



Université d'Ottawa • University of Ottawa



Université d'Ottawa - University of Ottawa

FACULTÉ DE ÉTUDES SUPÉRIEURES
ET POSTDOCTORALES

FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES

Christine CARSON

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

Ph.D.(Biochemistry)

GRADE - DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Initiation of Nuclear Disassembly during Apoptosis

M. Sikorska

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

D. Gray

A. MacKenzie

H. McBride

Z. Zakeri

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES
SUPÉRIEURES ET POSTDOCTORALES

SIGNATURE

DEAN OF THE FACULTY OF GRADUATE
AND POSTDOCTORAL STUDIES

INITIATION OF NUCLEAR DISASSEMBLY DURING APOPTOSIS

by

CHRISTINE CARSON

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfilment of the requirements for
the PhD degree in the
Department of Biochemistry

© Copyright by Christine Carson 2003



National Library
of Canada

Bibliothèque nationale
du Canada

Acquisitions and
Bibliographic Services

Acquisitions et
services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 0-612-89991-8

Our file Notre référence

ISBN: 0-612-89991-8

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this dissertation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de ce manuscrit.

While these forms may be included in the document page count, their removal does not represent any loss of content from the dissertation.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Apoptosis has long been recognised by two hallmarks of the end stage of nuclear breakdown: collapse of the chromatin and oligonucleosomal cleavage of the DNA. Mitochondria are also recognised as playing several pivotal roles in the apoptotic response downstream of death receptor signalling and upstream of effector caspase activation in cells with a type II response. Understanding of the integration of nuclear and cytoplasmic events has been limited to the demonstration of mitochondrial control of several endonucleases capable of oligonucleosomal cleavage. Control of initial nuclear events, such as high molecular weight (HMW) DNA and specific nuclear matrix cleavage, and their relationship to the cytoplasmic steps of cell death has not been elucidated. Examination of the relationship between events in the cell death path is limited by the stochastic nature of the process in all known models, which forces investigators to deal with mixed populations, combined with the tight temporal relationship of events in single cells. To determine whether a mitochondria-based signal could be responsible for the initial nuclear events of apoptosis, the timecourse of cell death in both primary rat thymocytes treated with dexamethasone or VM-26 (teniposide) and human Jurkat T cells treated with anti-Fas was first established using several cytoplasmic and nuclear criteria of cell death. Initial nuclear events were characterised at the single cell level at early time points in the apoptotic response by staining of the chromatin, immunofluorescence labelling of nuclear matrix and in situ detection of DNA breaks by TUNEL. Reorganisation of the matrix attachment region (MAR)-binding protein SATB1 was uniquely detected prior to chromatin collapse but after the

appearance of initial DNA breaks. Early reorganisation of a portion of SATB1 was followed by increasing DNA cleavage, the reorganisation of the internal nuclear matrix, represented by NuMA, coincident with chromatin collapse and maximal DNA breaks. Peripheral nuclear proteins, represented by lamin B, lamin B receptor (LBR) and PI2, were not affected until after chromatin collapse, if at all. SATB1 reorganisation and initial DNA breaks were then compared with mitochondrial function detected in live Jurkat cells by uptake of DiOC6₍₃₎, using flow cytometry and cell sorting to isolate those cells with normal mitochondrial membrane potential, normal size and intact plasma membrane from the mixed population of anti-Fas treated Jurkats. At the single cell level, changes in nuclear morphology, initial DNA cleavage and early reorganisation of SATB1 were detected in a minority of the “apparently normal” cells that displayed a normal mitochondrial membrane potential. Specific cleavage of SATB1 in these cells was detected by Western blotting and accumulation of single strand breaks demonstrated as well, using two-dimensional electrophoresis of DNA. Microarray analysis using human 19K arrays demonstrated change in gene expression in anti-Fas treated Jurkats relative to controls, some of which was specific to the “apparently normal” cells. Clearly, initial nuclear events preceded loss of mitochondrial membrane potential in apoptotic Jurkats, and could not have been mediated by a signal resulting from loss of mitochondrial function. Interestingly, microarray analysis indicated that early changes in gene expression might actually impact on mitochondrial homeostasis, via changes in endoplasmic reticulum or heat shock proteins, and thus contribute to coordinated breakdown of the nuclear and cytoplasmic compartments during apoptosis.

ACKNOWLEDGEMENTS

The biggest thanks to Dave for living through this, and so many other parts of life, all over again with me.

I also wish to express my thanks to the many people at the Institute for Biological Sciences whom I have enjoyed working with and for during the course of this degree. First and foremost, I am grateful to Marianna for her contributions to my development as a student and a scientist. You set an outstanding standard for scientific and personal style. Thanks also to Roy - it has been a pleasure to work for you both. I have thoroughly enjoyed the chance to be part of the unique group of people in the Sikorska/Walker labs. Special mention should be made of Julie - the best experimental partner a gal could ever hope for and a nice person to boot, of all my office mates over the years - Henriette, Julie, Harvey, Caroline, Hung , and of the other “old girls” (and “old boys”) - Dao, Lucia, Marilena, Qing, Cathie, Brandon, Domenic and Pandey.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xiii
CHAPTER ONE: INTRODUCTION	1
1.1 CYTOPLASMIC CHANGES IN APOPTOTIC CELLS	1
1.1.1 Apoptotic signalling from death receptors	2
1.1.2 Mitochondrial roles in apoptosis	6
1.1.3 Activation of effector caspases	12
1.2 NUCLEAR REMODELLING DURING APOPTOSIS	14
1.2.1 DNA cleavage in apoptotic cells	14
1.2.2 Proteolysis of nuclear matrix and chromatin collapse	18
1.3 INTEGRATION OF NUCLEAR AND CYTOPLASMIC EVENTS IN APOPTOSIS	20
1.4 HYPOTHESIS	23
1.5 RESEARCH OBJECTIVES	24
1.6 EXPERIMENTAL STRATEGY	24
1.6.1 Define early nuclear events in representative lymphocytic models of apoptosis	24
1.6.2 Analyse the relationship between loss of mitochondrial function and early nuclear changes	24
1.6.3 Examine the effect of death receptor signalling on nuclear function	25
CHAPTER TWO: CHARACTERISATION OF EARLY NUCLEAR EVENTS IN LYMPHOID MODEL SYSTEMS	26
2.1 MATERIALS AND METHODS	30
2.1.1 Cell culture and treatments	30
2.1.2 Detection of apoptosis based on chromatin collapse	31
2.1.3 <i>In situ</i> detection of DNA breaks	31
2.1.4 Flow cytometric assessment of mitochondrial function	32
2.1.5 Detection of filamentous actin using flow cytometry	32
2.1.6 Pulsed field gel electrophoresis of DNA	33
2.1.7 Western blot analysis of proteins	34
2.1.8 Labelling of Nuclear Antigens and DNA	34

2.1.9 Antibodies	35
2.1.10 Microscopy	35
2.2 RESULTS	36
2.2.1 Kinetics of cell death in lymphocytic cells as measured by characteristic nuclear changes	36
2.2.2 Kinetics of cell death as determined by cytoplasmic changes	39
2.2.3 Biochemical changes in apoptotic lymphocytic cells	42
2.2.4 Morphology of nuclear matrix in lymphocytic cells	49
2.2.5 Morphological changes in the MAR-binding protein SAT B1 during apoptosis in lymphocytic cells	51
2.2.6 Apoptosis-associated morphological changes in the distribution of NuMA	54
2.2.7 Morphological change in components of the nuclear rim in apoptotic lymphocytic cells	57
2.2.8 Reorganisation of SATB1 precedes chromatin collapse	61
2.2.9 Apoptosis-related changes in nuclear morphology follow initial DNA cleavage	62
2.3 DISCUSSION	66
2.3.1 Apoptosis-associated cleavage does not immediately disrupt the nuclear matrix structure	67
2.3.2 Reorganisation of SATB1 is closely associated with initial changes in chromatin configuration and nuclear morphology	69
2.3.3 Roles of nuclear matrix breakdown in apoptosis	71
CHAPTER 3 : RELATIONSHIP BETWEEN EARLY NUCLEAR EVENTS AND MITOCHONDRIAL CHANGE IN APOPTOTIC JURKAT CELLS	75
3.1 MATERIALS AND METHODS	79
3.1.1 Cell culture and treatment	79
3.1.2 Flow cytometric assessment of mitochondrial function in early apoptotic cells	79
3.1.3 Sorting of subpopulations from Jurkat cultures	80
3.1.4 Examination of nuclear morphology	81
3.1.5 Microscopy of live, sorted DiOC6-stained cells	81
3.1.6 <i>In situ</i> labelling of DNA breaks	81
3.1.7 Two-dimensional gel electrophoresis of DNA	82
3.1.8 Western blotting for SATB1	82
3.1.9 Immunostaining of nuclear antigens	82
3.2 RESULTS	82
3.2.1 Identification and isolation of early apoptotic Jurkat cells	83
3.2.2 Apoptotic Jurkat cells display initial changes in nuclear morphology before loss of mitochondrial membrane potential	88
3.2.3 Detection and characterisation of DNA cleavage that precedes loss of mitochondrial function	97

3.2.4	Degradation and reorganisation of SATB1 in early apoptotic cells with normal mitochondrial function	104
3.3	DISCUSSION	107
3.3.1	Diverse signals for cell death may emanate from the Fas receptor	107
3.3.2	Transmission of the apoptotic signal from Fas receptor to the nucleus	109
3.3.3	Effector caspases do not mediate the initial nuclear events of apoptosis	111
3.3.4	Control and coordination of initial nuclear degradation events	112
CHAPTER 4	CHANGES IN GENE EXPRESSION IN EARLY APOPTOTIC JURKAT CELLS	116
4.1	MATERIALS AND METHODS	119
4.1.1	Cell culture and treatment	119
4.1.2	Flow cytometric assessment of mitochondrial function and cell sorting	119
4.1.3	Isolation of RNA for microarray analysis	119
4.1.4	Preparation of labelled cDNA, hybridisation and scanning of microarrays	120
4.1.5	Microarray data processing and analysis	121
4.1.6	QPCR analysis of gene expression	122
4.1.7	Detection of filamentous actin in sorted subpopulations	123
4.2	RESULTS	125
4.2.1	Changes in gene expression in anti-Fas treated Jurkat cells	125
4.2.2	Identification of genes with altered expression induced by anti-Fas treatment	132
4.2.3	Identification of candidate genes implicated in the initial steps of the apoptotic response	141
4.3	DISCUSSION	150
4.3.1	Impact of Fas-mediated apoptosis on gene expression	150
4.3.2	Cell death induced by the Fas receptor may impact on the TGF- β signalling path	152
4.3.3	Fas-mediated changes in gene expression may affect mitochondrial homeostasis	153
4.3.4	Impact of Fas-mediated change in gene expression on the actin cytoskeleton	155
5	CONCLUSION	158
6	REFERENCES	160
7	APPENDICES	185
7.1	APPENDIX A: SOURCE OF CHEMICALS	185

7.2 APPENDIX B: UPREGULATED SEQUENCES	187
7.3 APPENDIX C: DOWNREGULATED SEQUENCES	190
7.4 APPENDIX D: MANUSCRIPT	196
8 CURRICULUM VITAE	233
9 CONTRIBUTIONS OF COLLABORATORS	239

LIST OF TABLES

		Page
<u>Table 1</u>	Primers for QPCR	124
<u>Table 2</u>	Apoptosis-related genes affected by anti-Fas treatment of Jurkat cells	136
<u>Table 3</u>	Gene sequences for membrane proteins demonstrating change in expression induced by anti-Fas treatment of Jurkat cells	137
<u>Table 4</u>	Kinase and phosphatase genes affected by anti-Fas treatment of Jurkat cells	138
<u>Table 5</u>	Protease-associated genes demonstrate change in expression in anti-Fas treated Jurkat cells	139
<u>Table 6</u>	Gene sequences for G proteins demonstrate change in expression induced by anti-Fas treatment of Jurkat cells	140
<u>Table 7</u>	RNA transcription and processing-associated genes demonstrate change in expression in anti-Fas treated Jurkats	142
<u>Table 8</u>	Genes for mitochondrial proteins affected by anti-Fas treatment of Jurkat cells	143
<u>Table 9</u>	Gene sequences for ER and Golgi proteins demonstrate change in expression induced by anti-Fas treatment of Jurkat cells	144
<u>Table 10</u>	Cytoskeleton and ECM-associated genes demonstrate change in expression in anti-Fas treated Jurkat cells	145
<u>Table 11</u>	Identification of anti-Fas regulated genes in early apoptotic Jurkat cells	147

LIST OF FIGURES

		Page
<u>Figure 1</u>	Detection of apoptosis based on chromatin collapse in lymphocytic cells	37
<u>Figure 2</u>	Kinetics of apoptosis in dexamethasone-treated thymocytes measured by TUNEL labelling of DNA breaks	40
<u>Figure 3</u>	Detection of apoptosis in lymphocytic cells based on loss of mitochondrial function	41
<u>Figure 4</u>	Kinetics of apoptosis in dexamethasone-treated thymocytes measured by loss of f-actin	43
<u>Figure 5</u>	DNA degradation in apoptotic lymphocytic cells	44
<u>Figure 6</u>	Degradation of nuclear matrix in apoptotic lymphocytic cells	47
<u>Figure 7</u>	Nuclear matrix staining in lymphocytic cells	50
<u>Figure 8</u>	Reorganisation of SATB1 in VM-26 treated thymocytes	52
<u>Figure 9</u>	Reorganisation of SATB1 in dexamethasone treated thymocytes	53
<u>Figure 10</u>	Reorganisation of SATB1 in anti-Fas treated Jurkat cells	55
<u>Figure 11</u>	Reorganisation of NuMA in apoptotic thymocytes	56
<u>Figure 12</u>	Reorganisation of NuMA in anti-Fas treated Jurkat cells	58
<u>Figure 13</u>	Morphology of nuclear rim proteins in apoptotic thymocytes	59
<u>Figure 14</u>	Relationship of morphological changes in nuclear matrix to chromatin collapse in apoptotic lymphocytic cells	60
<u>Figure 15</u>	Relationship between SATB1 reorganisation and DNA cleavage in apoptotic thymocytes	63
<u>Figure 16</u>	Relationship between SATB1 reorganisation and DNA cleavage in anti-Fas treated Jurkat cells	64
<u>Figure 17</u>	Multiparameter flow cytometric detection of early apoptotic Jurkat cells	84
<u>Figure 18</u>	Identification of three subpopulations in cultures of anti-Fas treated Jurkat cells	85

<u>Figure 19</u>	Distribution of subpopulations in apoptotic Jurkat cell cultures	87
<u>Figure 20</u>	Sorting of “Apparently Normal” cells from apoptotic Jurkat populations selects for cells of normal size	89
<u>Figure 21</u>	Sorting of “Apparently Normal” cells from apoptotic Jurkat populations selects for cells with normal mitochondrial function and intact plasma membrane	90
<u>Figure 22</u>	Nuclear morphology of sorted “apparently normal” cells	92
<u>Figure 23</u>	Nuclear morphology of sorted “apoptotic” and “dead” cells	93
<u>Figure 24</u>	Accumulation of cells with altered nuclear morphology in the sorted “apparently normal” subpopulation of anti-Fas treated Jurkats	94
<u>Figure 25</u>	All cells in the sorted “apparently normal” subpopulation retain their mitochondrial membrane potential	96
<u>Figure 26</u>	The size profile of sorted “apparently normal” cells is not changed by treatment with anti-Fas	98
<u>Figure 27</u>	In situ detection of DNA breaks by TUNEL in sorted subpopulations of apoptotic Jurkat cells	99
<u>Figure 28</u>	Biochemical detection of DNA breaks in sorted subpopulations of anti-Fas treated Jurkat cells	101
<u>Figure 29</u>	Accumulation of single strand breaks in sorted “apparently normal” Jurkat cells after induction of apoptosis by anti-Fas	103
<u>Figure 30</u>	Degradation of SATB1 in sorted “apparently normal” Jurkat cells	105
<u>Figure 31</u>	Reorganisation of SATB1 in sorted “apparently normal” Jurkat cells displaying altered nuclear morphology	106
<u>Figure 32</u>	Analysis of change in gene expression using human 19K DNA microarrays	126
<u>Figure 33</u>	Processing of microarray data	128
<u>Figure 34</u>	Alterations in gene expression detected in anti-Fas treated Jurkats	131
<u>Figure 35</u>	Limited identification of genes with altered expression in anti-Fas treated Jurkats	133

<u>Figure 36</u>	Functional categories of identified genes affected by anti-Fas treatment	135
<u>Figure 37</u>	Changes in gene expression specific to sorted “apparently normal” Jurkat cells induced to die by anti-Fas treatment	148
<u>Figure 38</u>	Changes in gene expression detected only in unsorted, whole populations of Jurkat cells treated with anti-Fas	149
<u>Figure 39</u>	Preservation of the f-actin network in the “apparently normal” subpopulation of anti-Fas treated Jurkats	157

LIST OF ABBREVIATIONS

$\Delta\psi_m$: mitochondrial membrane potential
AICD: activation-induced cell death
AIF: apoptosis-inducing factor
ALPS: autoimmune lymphoproliferative syndrome
Apaf: apoptotic protease activating factor
BUR: base-unpairing region
CAD: caspase-activated DNase
CAGE: conventional agarose gel electrophoresis
CARD: caspase recruitment domain
CTL: cytotoxic T lymphocyte
CW: clockwise
DAP: death associated protein
DD: death domain
DED: death effector domain
DEDAF: death effector domain-associated factor
DEDD: death effector domain DNA-binding protein
DFF: DNA fragmentation factor
DIABLO: direct IAP binding protein with low pI
DISC: death-inducing signalling complex
dsDNA: double strand DNA
ECM: extracellular matrix
ER: endoplasmic reticulum
EST: expressed sequence tag
FADD: Fas-associated protein with death domain
FIGE: field-inverse gel electrophoresis
FLICE: FADD-like ICE protease
FLIP: FLICE inhibitory protein
FS: forward scatter
GFP: green fluorescent protein
GO: gene ontology
HIV: human immunodeficiency virus
HMW: high molecular weight
ICAD: inhibitor of caspase-activated DNase
ICE: interleukin- β converting enzyme
Ig: immunoglobulin
JNK: Jun kinase
LBR: lamin B receptor
LMPCR: ligation-mediated PCR
LMW: low molecular weight
MAR: matrix attachment region
NGF: nerve growth factor
NuMA: nuclear mitotic apparatus protein

OD: optical density
OMIM: online Mendelian inheritance in man
PAGE: polyacrylamide gel electrophoresis
PARP: poly-ADP ribose polymerase
PBS: phosphate-buffered saline
PCD: programmed cell death
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
PFGE: pulsed-field gel electrophoresis
PKC: protein kinase C
PML: promyelocytic leukemia
PP1: protein phosphatase 1
PT: permeability transition (of mitochondria)
QPCR: quantitative PCR
Rb: retinoblastoma protein
RFLP: restriction fragment length polymorphism
RT: reverse transcriptase
SATB1: special AT-rich sequence binding protein 1
SMAC: second mitochondrial activator of caspases
snRNP: small ribonucleoprotein
SSC: side scatter
ssDNA: single strand DNA
TGF: transforming growth factor
TNF: tumour necrosis factor
tRNA: transfer RNA
TUNEL: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling

CHAPTER ONE: INTRODUCTION

Apoptosis, or programmed cell death (PCD), has gained tremendous attention over the past decade as the common cellular mechanism underlying a host of normal and pathological states at the organismal level. It plays an integral role in the removal of unwanted cells during animal embryogenesis and participates in the normal turnover of regenerating systems, such as the epidermis and the hematopoietic system, in mature vertebrates. Repression of normal levels of apoptosis can be a contributing factor in cancer. In contrast, induction of apoptosis plays a role in neuronal damage subsequent to ischemia and in neurodegenerative disease. (For a general review see Vaux, 2002).

Apoptosis is a controlled process, in which a cell engages a specific program of changes leading to “suicide” in response to appropriate signals. It results in the breakdown of cellular components, including organelles, and their compartmentalisation into small, membrane-bound apoptotic bodies. These remnants are then absorbed by neighbouring cells or appropriate immune scavenger cells. Since there is no release of cellular contents the process does not elicit an immune response. Signal reception and transduction, alteration in gene expression, activation of the endonucleases and proteases responsible for degradation of cell components and alteration of normal cell structure to allow formation of apoptotic bodies must all play a part in the apoptotic response. The coordination of key events remains an active area of research, and the point(s) at which the process becomes irreversible is (are) not yet well understood.

1.1 CYTOPLASMIC CHANGES IN APOPTOTIC CELLS

The induction of apoptosis leads to a host of changes in the cytoplasm of a cell. The apoptotic response in a number of cell systems is known to involve cytoskeletal disruption or collapse, change in mitochondrial function, activation of the caspase family of proteases, increase in intracellular calcium, acidification of the intracellular environment and loss of the phospholipid asymmetry and integrity of the plasma membrane. For the purposes of exploring the relationship of early nuclear events to cytoplasmic changes, I will focus on three pivotal steps: transduction of the apoptotic signal from “death receptors”, loss of mitochondrial function and activation of caspases.

1.1.1 Apoptotic signalling from death receptors A number of cell surface receptors of the TNF/NGF superfamily are capable of initiating an apoptotic response. These “death receptors” are characterised by both the cysteine-rich extracellular domains that define the superfamily and an intracellular “death domain”. Each death receptor is activated by a corresponding, membrane-bound ligand. The best characterised example is CD95, the Fas receptor (recently reviewed in Sartorius et al., 2001; Wajant, 2002; Mundle and Raza, 2002). Both mouse and human mutations of the receptor or Fas ligand are known. Absence of functional Fas receptor or ligand leads to immune disorders featuring enlargement of the lymph nodes, impaired deletion of T cells during their development, and autoimmunity in *lpr* and *gld* mice, and in humans suffering from ALPS (Krammer et al., 1994; Nagata and Golstein, 1995; Jackson and Puck, 1999). In addition, Fas receptor-induced apoptosis contributes to elimination of peripheral lymphocytes after an immune response, during “activation induced cell death” and is one of the mechanisms used by CTL cells to kill their targets (Sartorius et al., 2001). Thus, *in vivo*, Fas-mediated

cell death appears to be primarily associated with lymphocytes, despite the fact that the receptor is widely expressed (Oehm et al., 1992). Fas receptor possesses no inherent enzyme activity, but functions via activation-dependant recruitment of signalling molecules. Oligomerization of the receptor is required for activation; the activated form is thought to be a trimer, in keeping with the other members of the TNF-R family (Dhein et al., 1992; Banner et al., 1993; Mongkolsapaya et al., 1999; Hymowitz et al., 1999; Mundle and Raza, 2002; Tibbetts et al., 2003). Formation of a death-inducing signalling complex (DISC) at the cytoplasmic face of the Fas receptor, modulation of Fas signalling at the level of the DISC and transmission of the death signal from the receptor to mitochondria or effector caspases have all been described.

The cytoplasmic death domain of the Fas receptor comprises 68 amino acid residues that form 6 alpha helices arranged in a novel fold, a structural motif that forms the basis for selection of binding partners (Huang et al., 1996). Several signalling proteins that associate specifically and rapidly with the Fas receptor following stimulation have been characterised, and together they form the DISC (Kischkel et al., 1995). The first of these is the adaptor molecule FADD (Fas-associating protein with death domain) (Kischkel et al., 1995). The association of FADD and Fas has been demonstrated using both yeast two hybrid assay and co-immunoprecipitation and is mediated by the death domain on both proteins (Chinnaiyan et al., 1995). A novel “death effector domain” (DED) on FADD was also identified, through which it recruited other signalling components that promote the apoptotic response (Chinnaiyan et al., 1995; Chinnaiyan et al., 1996). The apoptosis-promoting protein recruited by FADD was then identified using

peptide sequencing of gel-isolated proteins as caspase 8, originally called FLICE (FADD-like ICE protease). Caspase 8 contains two DEDs, through which it interacts with FADD (Muzio et al., 1996). It is recruited to the DISC as an inactive zymogen. Recent evidence suggests that initiator caspases are active in their dimerised form, although binding to the DISC has clearly been associated with caspase 8 cleavage and its prodomain remains bound to the receptor signalling complex (Muzio et al., 1996; Chen and Wang, 2002; Tibbetts et al., 2003). Caspase 10 may also be capable of initiating apoptosis from death receptors in humans (Wang et al., 2001). Caspase 8 processing is thought to be the result of autoproteolysis stimulated by close association of the molecules, according to the “induced proximity” model, and results in release of the active enzyme into the cytoplasm, where it mediates downstream transmission of the death inducing signal.

Modulation of Fas receptor signalling at the level of the DISC can occur by a number of mechanisms. Soluble Fas ligand, which is not capable of inducing apoptosis, can be generated by metalloproteases (Hohlbaum et al., 2000). “Decoy” receptor DcR-3 (TR6), which binds Fas ligand but does not transmit a death signal and soluble Fas receptor, from which the transmembrane domain has been removed via alternate splicing, may interrupt signals generated by presentation of Fas ligand (Yu et al., 1999; Papoff et al., 1999). Fas receptor is a glycoprotein and its death-promoting effects may also be modified by glycosylation (Keppler et al., 1999). The receptor is known to associate directly with a labile protein inhibitor, Fap-1 (Mundle and Raza, 2002). Finally, a family of FLICE-inhibitory proteins (FLIPs) have been identified from database searches for DED-containing sequences. The family comprises v-FLIPS, found in certain herpes

viruses and a corresponding human homologue, c-FLIP, described by a number of groups (reviewed in (Sartorius et al., 2001; Wajant, 2002). There are two known splice variants of c-FLIP. The short form containing two DEDs resembles the v-FLIPS, while the long form also contains an inactive caspase 8 enzymatic site (Scaffidi et al., 1999). All the FLIPs are capable of interacting with FADD via their DEDs, where they can affect the generation of active caspase 8 at the DISC (Chang et al., 2002a; Tibbetts et al., 2003).

Transmission of the apoptotic signal from the DISC via two cell-type specific pathways has been described. In type I cells, caspase 8 is efficiently activated at the DISC, resulting in conversion of the entire cytoplasmic pool of procaspase 8 into active enzyme (Medema et al., 1997; Scaffidi et al., 1998). Rapid activation of the effector protease caspase 3 follows, which mediates breakdown of cellular components during apoptosis (as discussed below) (Scaffidi et al., 1998). This path is active in thymocytes, peripheral T cells and some lymphoid cell lines (Scaffidi et al., 1998; Kuida et al., 1998; Hakem et al., 1998; Yoshida et al., 1998). In type II cells, less of the total Fas receptor is exposed on the cell surface, DISC formation is markedly less efficient and only limited amounts of active caspase 8 are generated at this step (Scaffidi et al., 1998; Mundle and Raza, 2002). This small pool of active caspase 8 is responsible for cleavage of the cytoplasmic protein Bid, a pro-apoptotic member of the Bcl-2 family of proteins. Truncated Bid translocates to mitochondria, where it interacts with the organelle-specific lipid cardiolipin and Bax or Bak, inducing both loss of the mitochondrial membrane potential and release of apoptogenic factors from the mitochondrial intermembrane space (Luo et al., 1998; Li et al., 1998; Lutter et al., 2000; Esposti, 2002; Zamzami and

Kroemer, 2003). Efficient activation of effector caspases, mediated by an “apoptosome” complex (as discussed in detail below) follows loss of mitochondrial function in this pathway. Type II signalling is found in Jurkat and CEM T lymphocytic cell lines, hepatocytes and fibroblasts (Scaffidi et al., 1998; Cecconi et al., 1998; Yin et al., 1999).

1.1.2 Mitochondrial roles in apoptosis Mitochondria have been identified as important elements in the apoptotic response, in part using studies of cell-free systems. A number of labs developed *in vitro* models for the study of apoptosis by combining the cytosolic fraction of a model cell type with exogenous, normal nuclei. Typically, the cytosolic fractions used are harvested from cells destined to die. The first report of such a system used chicken hepatoma cells subjected to sequential S phase/M phase synchronisation (Lazebnik et al., 1993). Others have used “old” *Xenopus* eggs laid several weeks after gonadotropin injection (Newmeyer et al., 1994) or the cytosol from either W4, Jurkat or CEM cells treated with anti-Fas, UV radiation or ceramide, all harvested after a short treatment period when few cells displayed the typical features of apoptosis (Enari et al., 1995; Martin et al., 1995). The first report of a cell-free system documented the apoptotic changes seen in the normal interphase nuclei added to the cytosolic extract, including condensation of the chromatin and generation of oligonucleosomal DNA fragments (Lazebnik et al., 1993). Further studies defined the biochemical constituents of the apoptosis promoting activity and determined how it was controlled (Newmeyer et al., 1994; Enari et al., 1995; Martin et al., 1995). Importantly, Newmeyer et al. (1994) demonstrated that induction of apoptosis depended on the presence of a heavy membrane fraction consisting predominantly of mitochondria. Since

then, loss of the transmembrane potential across the inner mitochondrial membrane and release of apoptosis-inducing factors from mitochondria have been identified as key events in the effector phase of programmed cell death. Mitochondria are also one of the sites of action of the Bcl-2 family of proteins.

Studies in numerous model systems have since demonstrated an interruption in mitochondrial function associated with apoptosis. Typically, mitochondrial function is assessed in living cells by their uptake of cationic lipophilic dyes such as DiOC6₍₃₎, JC-1 and Rhodamine 123. Staining with these dyes depends on the presence of a normal proton gradient across the inner mitochondrial membrane and the resulting mitochondrial transmembrane potential ($\Delta\psi_m$). Stained cells are examined by microscopy or flow cytometry, where those with functional mitochondria and high levels of staining can be easily distinguished from those with nonfunctional mitochondria and less intense staining. Use of this technique, most prominently by the research group of Guido Kroemer, has shown that loss of $\Delta\psi_m$ is characteristic of apoptosis in dexamethasone-induced thymocytes and lymphocytes (Petit et al., 1995; Zamzami et al., 1995b), in lymphocytes subject to negative selection or irradiation *in vivo* (Castedo et al., 1995) and in a number of model cell lines, irrespective of the inducer used to trigger programmed cell death (Zamzami et al., 1995a). Loss of $\Delta\psi_m$ in apoptotic cells is an irreversible step. Flow cytometry combined with cell sorting has been used to demonstrate that dexamethasone-treated lymphocytes which retain a high mitochondrial transmembrane potential can be rescued by culture in the presence of glucocorticoid receptor antagonist and cytokines, while those with decreased $\Delta\psi_m$ are not viable (Zamzami et al., 1995b).

Further, loss of mitochondrial function appears to be sufficient to induce apoptosis; a number of mitochondrial respiratory chain inhibitors induce apoptosis and the ability of anti-apoptotic compounds to prevent cell death correlates with their ability to prevent loss of $\Delta\psi_m$ (Zamzami et al., 1995b). It has been postulated that loss of $\Delta\psi_m$ could be caused by a phenomenon long studied in isolated mitochondria: the permeability transition (PT) (Zamzami et al., 1995a). PT involves the opening of megachannels or PT pores in the inner mitochondrial membrane at sites which abut the outer membrane, resulting in leakage of solutes from the organelle, collapse of the normal $\Delta\psi_m$ and cessation of ATP synthesis. It is a self-amplifying phenomenon which can spread between mitochondria in a population, as the calcium and glutathione lost from the mitochondrial matrix are capable of increasing the megachannel gating potential in other organelles. Using a cell-free system that mixed isolated mitochondria and normal nuclei, it has been demonstrated that induction of PT in the mitochondria resulted in apoptotic changes in the nuclei present, and that agents which prevented PT could prevent apoptosis in an inducer-independent manner in both the cell-free system and primary cell culture (Zamzami et al., 1996).

The release of apoptosis-inducing factors from mitochondria, beginning with cytochrome c and AIF, was first detected using cell-free models of apoptosis. The research group led by Xiaodong Wang developed a variant of the system which used the cytosolic fraction from normal cells prepared under conditions which caused breakage of the mitochondrial membranes, and demonstrated that an apoptotic response could be generated in interphase nuclei added to the extract in the presence of dATP (Liu et al.,

1996). Three proteins which promoted the activation of caspase 3 and an apoptotic response in exogenous nuclei were isolated from this preparation, and dubbed apoptotic protease activating factors (Apafs) 1-3. Apaf 2 was immediately identified as cytochrome c, released from the mitochondria during preparation of the cytosol (Liu et al., 1996). Immuno-depletion and add-back experiments showed that activation of caspase 3 was dependant on the presence of cytochrome c in the cytosol. Altering the preparation protocol to ensure that mitochondria remained intact resulted in the absence of cytochrome c and loss of apoptotic potential in the extract (Liu et al., 1996). Cytochrome c release from mitochondria during apoptosis has since been documented in other cell-free systems (Kluck et al., 1997; Yang et al., 1997a) and microinjected cytochrome c has been shown to induce apoptosis in a variety of cell lines (Zhivotovsky et al., 1998). Mitochondrial release of cytochrome c thus appears to be a common feature of apoptosis. Using a cell-free system which mixed mitochondria with normal nuclei, another soluble protein factor capable of inducing apoptotic changes, AIF, was found to be released from mitochondria via PT (Zamzami et al., 1996). AIF was shown to be a single 50 kDa polypeptide normally present in the mitochondrial intermembrane space. In cell-free conditions, AIF caused chromatin condensation and DNA cleavage in normal nuclei, but not proteolysis of nuclear matrix, and could be inhibited by the general caspase inhibitor Z-VAD.fmk, but not by more specific inhibitors of either caspase 1 or 3 (Susin et al., 1996). Further work has characterised AIF as a ubiquitously expressed oxidoreductase protein, targeted to mitochondria by an amino-terminal mitochondrial localisation sequence in healthy cells (Daugas et al., 2000a). Upon induction of apoptosis it

translocates to the nucleus. In cell free-systems, purified AIF can induce DNA fragmentation into 50Kb fragments in the absence of cytosol, indicating that AIF acts directly on nuclear target(s) (Susin et al., 1999; Daugas et al., 2000a). Other constituents of the apoptotic response released from mitochondria include endonuclease G, Omi/HtrA2 and Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000; Li et al., 2001; Suzuki et al., 2001; van Loo et al., 2002).

Mitochondria have also been identified as an important site of action for the bcl-2 family of apoptosis-modulating proteins. Using electron microscopy and cell fractionation, bcl-2 has been localised to the membranes of the nuclear envelope, the smooth endoplasmic reticulum and the outer mitochondrial membrane. In the mitochondria, bcl-2 is located in discrete patches reminiscent of pores, concentrated in areas where the inner and outer mitochondrial membranes are closely juxtaposed (Monaghan et al., 1992; Krajewski et al., 1993). In several cell-free systems the ability of bcl-2 to inhibit apoptotic changes has been shown to depend specifically on its interaction with the mitochondria in the extract (Newmeyer et al., 1994; Zamzami et al., 1996). The protective effect of bcl-2 in mitochondria correlates with preservation of normal $\Delta\psi_m$ in cells over-expressing the protein (Hennet et al., 1993; Susin et al., 1996; Shimizu et al., 1996) and prevention of the release of AIF and cytochrome c (Susin et al., 1996; Kluck et al., 1997; Yang et al., 1997a). Other pro- (bcl-xL) and anti- (Bax, Bid, Bim) apoptotic members of this protein family have also been shown to exert their effects via interaction with mitochondria (Adams and Cory, 2001). The mechanism(s) of action of Bcl proteins is the focus of much experimentation and controversy. Studies have shown that truncated

forms of bcl-2, bcl-x_L and Bax are able to form channels in synthetic liposomes and planar bilayers containing acidic lipids (Minn et al., 1997; Schendel et al., 1997). Formation of mitochondrial pores by Bax and the influence of other family members on the process, particularly Bid and Bcl-2, has been the subject of much study. Although pore formation by full length proteins under physiological conditions has yet to be demonstrated, the formation or alteration of pores is currently the most accepted hypothesis of Bcl-2 family protein function (Scorrano and Korsmeyer, 2003; Zamzami and Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003). Interaction of bcl-2 family members with the voltage-dependant anion channel in the outer mitochondrial membrane and the adenine nucleotide translocator in the inner mitochondrial membrane, both components of the PT pore, has also been described (Marzo et al., 1998; Shimizu et al., 1999) and models proposed in which pore formation by Bcl-2 proteins is initiated by transient PT, or in which effective release of cytochrome c depends on transient PT-associated reorganisation of the mitochondrial cristae (Scorrano and Korsmeyer, 2003; Zamzami and Kroemer, 2003). In addition to its role in mitochondria, bcl-2 has been shown to prolong cell survival after cytochrome c release or microinjection (Zhivotovsky et al., 1998; Rosse et al., 1998). This downstream protection was postulated to result from its function as an adaptor protein, binding to the caspase-activating apoptosome and blocking its subsequent association with caspases in a manner analogous to the interactions observed between homologous proteins in the *C. Elegans* model system (Adams and Cory, 2001). However, in mammals, no such complexes exist in healthy cells; the apoptosome adaptor Apaf-1 is a monomeric cytoplasmic protein which

oligomerises and binds to caspase 9 only after interacting with cytochrome c (Moriishi et al., 1999; Hausmann et al., 2000). Very recently, Bcl-2 regulation of effector caspase activation independent of the apoptosome has been documented in Apaf 1 $-/-$ and caspase 9 $-/-$ lymphocytes *in vivo*, associated with decreased activation of one or more initiator procaspases, suggesting that Bcl-2 may interact with alternate apoptotic scaffold proteins (Marsden et al., 2002).

1.1.3 Activation of effector caspases Activation of the effector caspases has emerged as a hallmark of the apoptotic response in recent years, with the most intense focus on the role of caspase 3 in apoptosis. The use of cell-free systems has been instrumental in demonstrating the pathways for control of caspase 3 activation.

Caspase 3 activation during apoptosis is seen in a variety of cell types following induction of apoptosis by divergent mechanisms and has been implicated in neuronal cell death *in vivo*. Cleavage of the peptide substrate DEVD by caspase 3 in the cytosolic fraction of mouse W4 cells incubated with apoptosis-inducing anti-Fas antibody was shown to increase steadily with time (Enari et al., 1996). In Jurkat cells, both Fas ligand activation and etoposide-induced DNA damage increase cytosolic caspase 3 activity (Eischen et al., 1997). Increase in active caspase 3 has also been seen after etoposide treatment of a number of other lymphoid cell lines (Martins et al., 1997; Faleiro et al., 1997). An *in vitro* study of the interaction of CTL granzyme B and procaspases revealed that the former cleaves procaspase 3, generating the active enzyme (Quan et al., 1996). Finally, caspase 3-knockout mice demonstrate failed neuronal apoptosis during development and resulting alterations in brain structure, indicating a vital role for this

enzyme in neurogenic apoptosis *in vivo* (Kuida et al., 1996). Thus, caspase 3 activation is an apoptotic event common to most dying cells. Caspases 6 and 7 also fit this profile; they are activated in response to many inducers (Martins et al., 1997; Faleiro et al., 1997; Eischen et al., 1997). The numerous substrates of these caspases include structural proteins, such as fodrin and lamins, signalling molecules, such as PKC δ and apoptosis-associated targets such as ICAD/DFF45 (Nicholson and Thornberry, 1997; Villa et al., 1997). Significantly, they do not include other procaspases. This profile indicates that they are effector caspases, responsible for cellular disassembly and disruption of normal signalling mechanisms.

An understanding of the molecular mechanisms which control effector caspase activation has emerged from studies of apoptosis in cell-free systems, in which the apoptotic response of exogenous nuclei depended on the presence of activated caspases, among other soluble cytosolic proteins (Enari et al., 1995; Martin et al., 1995). As described above, the research group of Xiaodong Wang identified three apoptotic protease activating factors in their cell-free system, and immediately identified Apaf-2 as cytochrome c. Apaf 3 was then shown to be caspase 9. Finally, Apaf 1 was found to be a human homologue of the death-promoting protein CED-4 of *C. elegans*. Like its counterpart, Apaf 1 acts as an adaptor, bringing together the elements needed for enzyme activation in an apoptosome complex. It first binds to cytochrome c, which stimulates binding to nucleotide. Oligomerisation of Apaf-1 is then triggered and exposes caspase recruitment domains (CARDs) on the protein. Multiple procaspase 9 molecules then bind to the complex and autoactivate. Active caspase 9 then mediates activation of caspase 3

(Zou et al., 1997; Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Zou et al., 1999; Jiang and Wang, 2000, reviewed in Adams and Cory, 2003). In type II apoptotic cells, caspase 3 activation is controlled principally by the apoptosome, whose formation depends on release of constituent factors from the mitochondria. Alternatively, caspase 3 can be activated directly by a receptor-initiated caspase cascade in type I cells. However, it is worth noting that loss of mitochondrial membrane potential, release of pro-apoptotic factors from mitochondria and formation of the apoptosome also occur in type I cells, in addition to the receptor-initiated caspase cascade (Scaffidi et al., 1998).

1.2 NUCLEAR REMODELLING DURING APOPTOSIS

Scientists have long used two indicators of nuclear change during apoptosis to detect and monitor the process: oligonucleosomal cleavage of the DNA and collapse of the chromatin. These hallmarks represent the end stage of nuclear degradation detectable using biochemical methods or microscopy. They are the culmination of an orderly process that our lab, among others, has partially elucidated. DNA cleavage and collapse of the nuclear structure during apoptosis are integrated events. Current research has defined some of the molecular mechanisms responsible for internucleosomal cleavage and proteolysis of nuclear matrix proteins, but important questions about the initial nuclear events of apoptosis remain unanswered.

1.2.1 DNA cleavage in apoptotic cells DNA cleavage is a common event in the apoptotic response of all systems studied to date. It proceeds in an orderly manner which

reflects chromatin structure, generating fragments of decreasing size. Using pulsed field gel electrophoresis (PFGE), a technique able to resolve fragments of 1 Mbp to 200 bp on a single gel, we have demonstrated that during apoptosis the first DNA fragments detectable are 50 Kbp- 1 Mbp in length, resulting from double-strand cleavage of the DNA at vulnerable sites in the chromatin structure. Cleavage between rosettes generates 300 Kbp fragments, while the 50 Kbp fragments arise from cleavage between DNA loops (Walker et al., 1994; Walker et al., 1995). Others have also detected large scale DNA fragmentation which precedes oligonucleosomal cleavage in apoptotic mammalian cells (Cohen et al., 1994; Lagarkova et al., 1995). Proteolysis of nuclear matrix proteins that interact with chromatin can be detected by Western blot concurrent with generation of these large fragments, and may play a role in mediating endonuclease access to the DNA (Weaver et al., 1996). In a number of model systems apoptotic DNA cleavage proceeds no further. However, in the majority of cases, cleavage in the linker DNA which joins nucleosomes follows, generating fragments that are multiples of the 200 bp nucleosome in length and which appear as a classic oligonucleosomal ladder when separated by conventional agarose gel electrophoresis (CAGE) (Walker et al., 1994; Walker et al., 1995).

More recently, work in our lab has demonstrated that apoptotic DNA cleavage may be initiated by single strand breaks. Single-strand breaks were previously detected, along with oligonucleosomal fragments in apoptotic mouse thymocytes subject to CAGE under denaturing conditions (Peitsch et al., 1993). We employed a two -dimensional electrophoresis technique to examine the relationship of the single strand breaks to the

process of double-strand DNA fragmentation during apoptosis. PFGE was used first, to separate the HMW and oligonucleosomal DNA fragments. The DNA was then subject to S1 treatment, cleaving the fragments opposite the sites of any hidden single-strand nicks in their DNA, and the samples examined by CAGE in the second dimension. We thus demonstrated that accumulation of single-strand breaks in apparently “intact” DNA preceded generation of smaller DNA fragments in apoptotic cells (Walker et al., 1997). Single-strand DNA (ssDNA) has also been detected by antibody binding in breast cancer cells treated with cisplatin, where labelling was seen coincident with chromatin margination and high molecular weight (HMW) DNA cleavage. Accumulation of single strand breaks preceded both complete collapse of the chromatin and loss of cell adherence (Frankfurt et al., 1996). As single strand breakage becomes more extensive it may itself lead to cleavage of the chromatin into discrete fragments. Due to chromatin configuration and association with nuclear matrix proteins, only a fraction of the genomic DNA would be vulnerable to the initial cleavage events of apoptosis. Among these rare sites, it is conceivable that enough single strand breaks may accumulate to generate a double strand break. (Walker et al., 1997; Sikorska and Walker, 1998; Robertson et al., 2000). Indeed, we have recently mapped the sites of the initial ssb of apoptosis in Jurkat cells, and found they accumulated in MAR sequences. Breaks in these regions were often offset by only 1-2 nucleotides on the two DNA strands and would be resolved as a dsb in PFGE (Liu et al., 2003).

Identification of the endonucleases responsible for DNA cleavage in apoptotic cells has long been an active area of research (for review see Sikorska and Walker, 1998;

Robertson et al., 2000). Endonuclease activities have been characterised biochemically and numerous candidate enzymes capable of internucleosomal DNA cleavage have been identified over the years (see, for example, (Pandey et al., 1997). Internucleosomal cleavage by several endonucleases following release of apoptogenic factors from mitochondria and activation of caspase 3 has been demonstrated, beginning with the cytoplasmic enzyme CAD/DFF40. In healthy cells, CAD/DFF40 is complexed to its inhibitor ICAD/DFF45. During apoptosis, caspase 3 cleaves the inhibitor, resulting in translocation of CAD/DFF40 into the nucleus by means of its nuclear localisation signal, and its activation (Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998; Tang and Kidd, 1998). Endonuclease G, an enzyme normally sequestered in the mitochondria, has also been implicated in oligonucleosomal cleavage. In this case, initiation of DNA cleavage is controlled solely by compartmentalization of the enzyme (Li et al., 2001). Release of AIF from mitochondria and its translocation to the nucleus has been linked to generation of 50 Kbp DNA fragments in apoptotic cells, although AIF does not display endonuclease activity itself. In *C. elegans*, AIF has been shown to cooperate with endonuclease G. Its partner(s) in mammalian cells have not been identified (Wang et al., 2002; Penninger and Kroemer, 2003). A constitutive endonuclease with topoisomerase II-like activity has also been recently been implicated in generation of 50 Kbp fragments in apoptotic neuroblastoma cells (Solovyan et al., 2002). The endonuclease(s) responsible for initial single strand and HMW DNA cleavage in apoptotic cells, apparently a nuclear protein(s), remain elusive (Robertson et al., 2000).

1.2.2 Proteolysis of nuclear matrix and chromatin collapse Apoptotic cells were first recognised by their nuclear morphology. Collapse of the chromatin to form pyknotic nuclei or multiple small, round apoptotic bodies remains a hallmark indicator of apoptosis to this day. We, among others, have shown that in thymocytes chromatin collapse is preceded by cleavage of nuclear matrix proteins and accompanied by rearrangement of the nuclear matrix.

Changes in the structure of apoptotic nuclei can be detected by biochemical assay, as numerous nuclear proteins are specifically cleaved in apoptotic cells, including U1 snRNP, PARP, and lamins (Kaufmann et al., 1993; Casciola-Rosen et al., 1994; Oberhammer et al., 1994). We have shown that cleavage of nuclear matrix is a very early event, detectable within half an hour of the induction of apoptosis by dexamethasone in thymocytes. Cleavage occurred in both the internal nuclear matrix, represented by the nuclear mitotic apparatus protein (NuMA), and in the peripheral nuclear lamina, represented by lamin B. However, nuclear envelope proteins, represented by the PI2 antigen, remained intact throughout the apoptotic response (Weaver et al., 1996). Immunofluorescence microscopy was also used to demonstrate that chromatin collapse and reorganisation of NuMA into the portion of the nucleus not occupied by the mass of DNA occurred simultaneously at the single cell level. Disappearance of the nuclear lamina followed closely, while staining for nuclear envelope protein remained intact throughout the apoptotic response (Weaver et al., 1996).

Several effector caspases have been implicated in the process of nuclear matrix breakdown (for reviews see Nicholson and Thornberry, 1997; Villa et al., 1997; Wolf and

Green, 1999): specific cleavage of the U1 70 kDa small ribonucleoprotein is carried out by a caspase (Casciola-Rosen et al., 1994); PARP has been identified as a caspase 3 substrate (Lazebnik et al., 1994) and cleavage of lamins, NuMA and the DNA-binding protein SATB1 may be due to caspase 6 (Zhivotovsky et al., 1997; Robertson et al., 2000; Galande et al., 2001). In all caspase studies, questions of nuclear matrix accessibility to cytoplasmic effector proteases remain. Caspase activity has been documented in the nuclear fraction of HL-60 cells induced to die by treatment with etoposide after a lag time of one hour (Martins et al., 1997), and caspase 3 is translocated to the nucleus in Fas-treated Jurkats after activation in the cytoplasm (Ramuz et al., 2003). Thus, in dying cells effector enzymes do gain access to the nuclear interior in due course. To date, only procaspase 1 and 2 have been detected in the nuclei of healthy cells (Mao et al., 1998; Zhivotovsky et al., 1999; Guo et al., 2002). Both are considered initiators of further caspase signalling, not effectors, based on their structure and their oligomerization-mediated activation (Baliga et al., 2003). Translocation of caspase 8 and procaspase 9 to the nucleus upon reception of a death signal has also been documented (Krajewski et al., 1999; Komoriya et al., 2000; Gajkowska and Wojewodzka, 2002). Caspase-dependant alteration of nuclear envelope permeability has been proposed, and would provide a convenient mechanism for nuclear transport of the downstream effector, caspase 3, but remains a controversial point (Ferrando-May et al., 2001; Faleiro and Lazebnik, 2000). The fact that proteolysis of nuclear matrix proteins begins concurrently with HMW damage in thymocytes, (Neamati et al., 1995; Weaver et al., 1996) suggests that initial proteolytic events are not mediated by active caspase 3.

1.3 INTEGRATION OF NUCLEAR AND CYTOPLASMIC EVENTS IN APOPTOSIS

Changes in gene expression are known to be an integral part of the apoptotic response in many model systems, contributing to both cytoplasmic and nuclear events in dying cells. In contrast, the physical process of nuclear destruction has generally been considered a late event. This is likely true for the end points of nuclear breakdown: collapse of the chromatin and generation of oligonucleosomal DNA fragments. However, evidence suggesting that initial nuclear events in apoptotic cells may accompany or precede cytoplasmic events has also been published.

Several transcription factor families known to play a role in cell growth have also been implicated in apoptotic signalling in a variety of systems, most notably myc, E2F and AP1. Myc-induced proapoptotic effects have been shown to impact the process of receptor-mediated cell death in some models and are mediated in part by p19ARF and the adaptor protein Bin1. Myc may also affect its targets by altering chromatin configuration to allow for effective transcription; it is known to bind complexes with histone acetylase activity (Prendergast, 1999; Luscher, 2001). Studies of knockout mice have implicated E2F1 in the induction of apoptosis during negative selection of thymocytes in vivo and in vitro studies have shown a p38-dependant activation of E2F1 during Fas-mediated apoptosis in both Jurkat cells and primary neuronal cultures. In these cases, p53 was found to be an important modulator of E2F1 proapoptotic effects (Wang et al., 1999; Garcia et al., 2000; Hou et al., 2002 and reviewed in Ginsberg, 2002). Amongst the members of the AP1 family, activity of both c-Jun and c-Fos has been associated with

apoptosis in neuronal model systems and a role for c-Jun has been described in DNA damage-mediated apoptosis in lymphocytic and fibroblast model systems. AP1 promotes p53-dependant events associated with cell death while attenuating cell cycle arrest responses (Shaulian and Karin, 2001). p53 is well known to impact the cytoplasmic events of cell death. Its effects include direct activation of genes coding for pro-apoptotic members of the bcl-2 protein family, which act on the outer mitochondrial membrane, where they promote permeability transition and hence activation of effector caspases (reviewed in Wu and Deng, 2002). These changes in gene expression affect the apoptotic response during the initiator phase, prior to loss of mitochondria function.

In contrast, the literature clearly identifies a number of cytoplasmic events which precede oligonucleosomal cleavage or chromatin collapse during apoptosis. In Jurkat cells induced to die by activation of the Fas ligand, a transient increase in caspase 1-like activity is seen within 30 minutes, before any increase in the proportion of cells with apoptotic nuclear morphology is detectable (Greidinger et al., 1996). In both dexamethasone-induced thymocytes and lymphocytes, loss of $\Delta\psi_m$ is a more sensitive measure of apoptosis in the population than accumulation of DNA breaks visualised by nick-end labelling or oligonucleosomal cleavage of DNA (Petit et al., 1995; Zamzami et al., 1995b). Finally, both endonuclease G and CAD, enzymes responsible for oligonucleosomal cleavage, are activated only following release of apoptogenic factors from mitochondria and, for the latter, caspase 3-mediated cleavage of its inhibitor ICAD (Sakahira et al., 1998; Li et al., 2001).

Despite these trends, evidence has occasionally surfaced which documents parallel, independent paths for nuclear and cytoplasmic breakdown. Dissociation of the loss of mitochondrial function, as assessed by MTT assay, and oligonucleosomal DNA fragmentation has been documented in several T-cell hybridomas. Both DNA fragmentation in the absence of mitochondrial change and mitochondrial failure in the absence of DNA cleavage were noted in these cells during apoptosis (Vukmanovic and Zamoyska, 1991). Similarly, it has been demonstrated that anucleate cytoplasts exposed to inducers of apoptosis exhibit loss of mitochondrial function and change in plasma membrane permeability. In this system, the cytoplasmic effector path in apoptosis can function independently from nuclear events. Whether the reverse is also true was not addressed (Jacobson et al., 1994). Finally, a microscopy study of thymocytes and several lymphocytic cell lines found that plasma membrane permeability to propidium iodide and collapse of the chromatin were parallel but independent events (Lizard et al., 1995).

In our studies of dexamethasone-treated thymocytes, we have found that nuclear matrix cleavage and HMW DNA fragmentation in the population as a whole was detectable within 30 minutes of the induction of apoptosis. In contrast, oligonucleosomal fragmentation was not detected for 2 hours, the majority of the cells did not display apoptotic nuclear morphology until 4 hours and cells did not become permeable to trypan blue dye until 12 hours after the apoptotic stimulus was applied (Weaver et al., 1996). Lamin B degradation has also been observed preceding oligonucleosomal DNA fragmentation in thymocytes by others (Oberhammer et al., 1994; Neamati et al., 1995).

The relative timing of these events suggests that initial nuclear events occur early in the apoptotic response in these cells.

Interpretations regarding the timing of apoptotic events are all limited by the fact that biochemical assays measure the trend in the population as a whole. They may not reflect accurately the relationship of rapid events at the single cell level in a population that is not completely synchronised in its response, as is the case in all known model systems for apoptosis. Studies of live cells at the single cell level and using flow cytometry have confirmed the stochastic nature of cell entry into the effector phase of apoptosis, detected by capsase activation, loss of plasma membrane integrity, release of cytochrome c and phosphatidyl serine exposure. In contrast, progress through the effector phase occurred with similar kinetics in all cells (Goldstein et al., 2000; Smolewski et al., 2002). A thorough examination of the relationship between nuclear and cytoplasmic events will require the use of methods capable of simultaneously examining several parameters at the single cell level. Our observations in apoptotic thymocytes suggest that cell death involves early transmission of the apoptotic stimulus to the nucleus, followed by parallel paths of nuclear and cytoplasmic destruction.

1.4 HYPOTHESIS

I propose that transduction of the apoptotic signal to the nucleus is a relatively early event in apoptosis, allowing for coordinated breakdown of the cytoplasmic and nuclear structures of a cell undergoing programmed cell death.

1.5 RESEARCH OBJECTIVES

- 1) Characterise the early stages of nuclear breakdown in different lymphocytic models of apoptosis in order to establish common events appropriate for further examination relative to cytoplasmic markers of apoptosis.
- 2) Define the relationship between loss of mitochondrial function and initial nuclear events at the single cell level in anti-Fas treated Jurkat T cells, a model in which activation of effector caspases is known to be mitochondria-dependant.
- 3) Characterise the impact of apoptotic signal transduction to the nucleus on nuclear function, as evidenced by changes in gene expression and identify potential mechanisms capable of transmitting the apoptotic signal to the nucleus in anti-Fas treated Jurkat cells by analysing changes in gene expression profiles.

1.6 EXPERIMENTAL STRATEGY

1.6.1 Define early nuclear events in representative lymphocytic models of apoptosis.

- Determine a suitable timeframe for analysis of early apoptotic changes based on both nuclear and cytoplasmic markers in several established lymphocytic model systems.
- Characterise common early nuclear changes at the single cell level using immunofluorescence staining of nuclear antigens and in situ labelling for DNA breaks.
- Order these events using double-labelling for the markers of interest.

1.6.2 Analyse the relationship between loss of mitochondrial function and early nuclear changes in anti-Fas treated Jurkat cells at the single cell level in order to

establish whether a mitochondria-based signal might be responsible for the initiation of nuclear disassembly during apoptosis.

- Isolate subpopulations of live cells treated with anti-Fas based on their mitochondrial membrane potential, using flow cytometry and cell sorting
- Examine them separately at the single cell level for early nuclear events, including changes in nuclear morphology, distribution of nuclear antigens of interest and in situ DNA breaks, with the main focus on the subpopulation(s) that maintain a normal mitochondrial membrane potential.
- Analyse DNA and nuclear matrix integrity using biochemical assays, especially in those cells which still appear normal.

1.6.3 Examine the effect of death receptor signalling on nuclear function

- Compare gene expression profiles of the whole population of anti-Fas treated Jurkat cells to that of control cultures using human 19.2K cDNA microarrays in order to ascertain whether gene expression is affected by death receptor-mediated signalling.
- Compare the expression profiles of whole Jurkat populations and the sorted cell population which maintains a normal mitochondrial membrane potential in order to identify candidate genes involved in the early steps of the apoptotic response.
- Using functional analysis of affected sequences, as determined by GO and other annotations in the publicly available databases, predict the impact of changes in gene expression induced by anti-Fas on the cytoplasmic and nuclear events of apoptotic breakdown.

CHAPTER TWO: CHARACTERISATION OF EARLY NUCLEAR EVENTS IN LYMPHOID MODEL SYSTEMS

Apoptotic cells were first identified based on their distinctive nuclear morphology and both collapse of the chromatin and DNA cleavage remain hallmarks of the process of programmed cell death to this day. Apoptotic changes in the nuclear matrix, the proteinaceous scaffold that provides structural support for the organelle and interacts with DNA to determine the 3D structure of the chromatin, have been extensively studied.

The interphase nucleus is surrounded by the nuclear envelope, a double membrane structure traversed by pores which control the permeability of the organelle (Suntharalingam and Wentz, 2003). The outer nuclear envelope is continuous with the endoplasmic reticulum. Inner nuclear envelope proteins diffuse to the compartment from the ER and are specifically retained in this portion of the membrane via their interactions with chromatin or the matrix (Worman and Courvalin, 2000). Beneath the nuclear envelope lies the nuclear lamina, a mesh of proteinaceous 10 nm filaments. The lamin proteins comprise a distinct sub class (type V) of the intermediate filament family of cytoskeletal proteins, with whom they share both sequence similarities and a common structure. Lamin interactions with a number of inner nuclear envelope proteins, nuclear pore complexes and chromatin have all been documented (see Goldberg et al., 1999; Gruenbaum et al., 2000). Within the nuclear interior or nucleoplasm reside proteins that function in transcription, replication and repair of the DNA, as well as those that maintain the structural integrity of the nucleus. Of the latter, NuMA is of particular note. It shares structural features with the intermediate filament family members, namely a central rod

domain containing heptad repeats of hydrophobic amino acids (Compton and Cleveland, 1994).

In the interphase nucleus, chromatin configuration is mediated via binding of particular DNA sequences, called matrix attachment regions (MARs) or scaffold attachment regions to the proteins of the nuclear matrix. MARs, sequences of 300-2000 bp, are typically found at the base of chromatin loops (deBelle et al., 1998) and are characterised by structural elements, rather than a strict consensus sequence. Most significantly, they contain a core unwinding element or base unpairing region (BUR) of up to 200 bp, which consists of ATC tracts; stretches of DNA that exclude guanine residues on one strand. BURs have a high probability of becoming single stranded under conditions of superhelical strain typical of duplex DNA (Kohwi-Shigematsu and Kohwi, 1990; Benham et al., 1997; Bode et al., 2000). These short stretches of single stranded DNA mediate the association of MARs with the proteinaceous scaffold of the nuclear matrix (Bode et al., 1992; Luderus et al., 1994). MAR-binding proteins also interact specifically with the minor groove of the DNA in these regions (Dickinson et al., 1992; Luderus et al., 1994), and can be isolated based on this property (Kohwi-Shigematsu et al., 1998). The list of MAR-binding proteins is ever-growing and includes both widely-expressed proteins such as PARP, the Ku subunit of DNA-PK and nucleolin (Dickinson and Kohwi-Shigematsu, 1995; Galande and Kohwi-Shigematsu, 1999) and tissue-specific proteins, such as the B cell-associated Brg1, thymus-associated SMAR1 or p114 and HMG-I(Y), which are both associated with breast carcinoma (Yanagisawa et al., 1996; Liu et al., 1999b; Chattopadhyay et al., 2000). Interaction of these proteins with MARs affects chromatin

configuration and accessibility and can influence gene expression. The T-cell specific MAR-binding protein SATB1 has been particularly well studied in this regard. SATB1 was identified in 1992 based on an in vitro binding assay to isolate proteins which interacted with a synthetic MAR sequence bound to a column, and shown to interact with the MAR site in the enhancer region of the IgH gene (Dickinson et al., 1992). SATB1 interaction with other MARs, including that flanking the major breakpoint region of BCL2, has also been demonstrated (Ramakrishnan et al., 2000). Binding of SATB1 to MARs has been shown to suppress transcription of reporter genes in vitro (Kohwi-Shigematsu et al., 1997). In vivo, gene expression profiles in knockout mice revealed that SATB1 acts primarily as a repressor of transcription (Alvarez et al., 2000). SATB1 acts by both tethering specific target sequences to the nuclear scaffold and recruiting chromatin remodelling complexes, resulting in gene regulation over regions of DNA up to 10 Kb long (Yasui et al., 2002; Cai et al., 2003).

Specific proteolysis of a subset of nuclear matrix proteins during apoptosis has been well documented (Gerner et al., 1998; Martelli et al., 1999). Proteins targeted for breakdown include PARP (Kaufmann et al., 1993) and U1 snRNP (Casciola-Rosen et al., 1994). Interestingly, many of the nuclear matrix proteins cleaved during apoptosis demonstrate MAR-binding activity, including lamin B, NuMA, PARP, SAT B1 and SAF-A (Luderus et al., 1994; Gotzmann et al., 2000; Kipp et al., 2000). Cleavage of many of these nuclear substrates by caspases has been documented in vitro and in vivo (Lazebnik et al., 1994; Casciola-Rosen et al., 1996; Slee et al., 2001; Wolf and Green, 1999). However, the potential complexity of cleavage events is well illustrated by studies of lamin

proteolysis. Lamins are both a caspase 6 substrate and subject to cleavage by an endogenous nuclear serine protease in rat thymocytes and Jurkat cells. Caspase 6-mediated cleavage of lamins was the predominant mechanism of lamin breakdown in apoptotic Jurkats (Zhivotovsky et al., 1997). In the case of CTL-mediated apoptosis, cleavage of lamin B by both granzyme B and caspases at the same site has been documented, while granzyme A and caspase cleave the A lamins at distinct sites, but generate fragments of identical size when analysed by Western blot (Zhang et al., 2001). This redundancy suggests that breakdown of certain nuclear matrix components may be essential to progression or completion of the apoptotic pathway.

We, among others, have demonstrated that cleavage of lamin B and NuMA can be detected by Western blot very early in the apoptotic response in dexamethasone-treated thymocytes (Oberhammer et al., 1994; Neamati et al., 1995; Weaver et al., 1996). Degradation of these proteins occurred concurrently with high molecular weight cleavage of the DNA and preceded both oligonucleosomal DNA cleavage and loss of plasma membrane integrity. In contrast, proteins of the nuclear envelope remained intact throughout the apoptotic response (Weaver et al., 1996).

To better define and order the process of apoptotic nuclear breakdown, and thus gain insight into the links between DNA cleavage and nuclear matrix breakdown, early nuclear events were characterised in both primary rat thymocytes and the human Jurkat T-cell line. Three separate inducers of apoptosis were employed in order to discriminate between path-specific and more general characteristics of nuclear breakdown, and to allow the selection of a model most suitable for the further study of the relationship between

cytoplasmic and nuclear events in apoptosis. Using biochemical assays, the timecourse of apoptotic cell death was readily established, but it proved difficult to separate closely related events in the mixed populations of dying cells using this approach.

Immunofluorescence and confocal microscopy were then employed to establish the early nuclear events of apoptosis detectable at the single cell level. In these experiments, internal nuclear matrix components were represented by NuMA and SAT B1, and peripheral nuclear proteins by lamin B, lamin B receptor (LBR) and the nuclear envelope protein recognised by the PI2 antibody. Counterstaining with the DNA dyes Hoechst 33258 or DAPI, and in situ labelling of DNA breaks by TUNEL were used to examine nuclear matrix changes relative to DNA collapse or cleavage, respectively.

2.1 MATERIALS AND METHODS

2.1.1 Cell culture and treatments Thymocytes were isolated from 150 - 200 g male Sprague-Dailey rats bred in the Institute using established protocols (Whitfield et al., 1968). Cells were suspended in RPMI 1640 tissue culture media supplemented with 5% fetal bovine serum at concentrations of $3-4 \times 10^7$ cells/ml, and suspensions were incubated on a rotator at 37°C in a 5% CO₂ atmosphere. Death was induced with either 1 µM dexamethasone or 10 µM of teniposide (VM-26). Jurkat cells were maintained in RPMI 1640 tissue culture media supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. Death was induced in growing cultures suspended at a density of 10^6 cells/ml by the addition of 100 ng/ml anti-Fas monoclonal antibody (clone CH-11) from a 0.5 mg/ml stock.

2.1.2 Detection of apoptosis based on chromatin collapse Live cells from treated and control cultures were stained for 10 minutes with 1 μ l/ml of 10 mM Hoechst 33342 and 7 μ l/ml of 1 mg/ml propidium iodide solution at 37 °C, while cells settled onto poly-l-lysine coated coverslips. Coverslips were then rinsed briefly in PBS, fixed for 5 minutes in freshly-made 3% EM grade paraformaldehyde, washed twice briefly in PBS and mounted onto slides cell side down, in liquid mounting medium containing anti-fade reagent. Cells were examined using fluorescence microscopy and scored as normal (Hoechst stain only, control nuclear morphology), early apoptotic (Hoechst stain only, collapsed chromatin), late apoptotic (Hoechst and propidium iodide stain, collapsed chromatin) and necrotic (Hoechst and propidium iodide stain, uncollapsed nuclear morphology). For each time point in an experiment, 400 cells in each of duplicate samples was scored.

2.1.3 *In situ* detection of DNA breaks DNA breaks were visualised by terminal deoxynucleotidyl transferase (TdT)-mediated nick end labelling (TUNEL) with biotinylated dUTP (Gavrieli et al., 1992), carried out as recently described (Walker et al., 2002). For flow cytometry, cells were fixed for 15 minutes on ice in 1% paraformaldehyde in PBS, resuspended in one part ice-cold PBS, then post-fixed with 5 parts ice-cold 70% ethanol. Fixed cells were rehydrated in PBS + 1% BSA for 15 minutes at room temperature, and DNA breaks labelled by incubating cells for 30 minutes at 37°C with 300 U/ μ l TdT in reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl pH=6.6, 2.5 mM CoCl₂, 0.25 mg/ml BSA) containing 10 μ M biotin-16-dUTP. The reaction was stopped with 2X TB buffer (600 mM NaCl, 60 mM sodium citrate), and the label detected by incubating for 1 hour at room temperature in the dark in 10 μ g/ml FITC-conjugated

streptavidin in staining buffer (2X TB buffer, 5% milk, 0.1% TX-100). Cells were then rinsed in 2X TB buffer containing 0.1% TX-100 and counterstained for 20 minutes at room temperature in the dark in PBS containing 1% BSA, 10 µg/ml DNase-free RNase and 2 µg/ml propidium iodide. TUNEL labelling and DNA content of 20,000 cells per sample were assessed simultaneously on a Coulter Epics Elite flow cytometer/sorter, using an excitation wavelength of 488 nm, and 525 (+/-5) nm and 610 (+/-5) nm bandpass filters. For microscopy, DNA breaks in cells fixed and single stained as described below were labelled by incubating cells for 1 hour at 37°C with 300 U/ml TdT in cacodylate buffer (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride) containing 10 µM biotin-16-dUTP. The reaction was stopped by immersion in 1X TB buffer, cells were blocked in 2% BSA in PBS, washed in PBS, and incorporated label detected by incubating for 30 minutes at room temperature in 10 µg/ml FITC- or CY3-conjugated streptavidin diluted in PBS. Coverslips were rinsed in PBS and mounted for microscopy.

2.1.4 Flow cytometric assessment of mitochondrial function Mitochondrial function was monitored by uptake of the potential-sensitive dye DiOC₆₍₃₎. 1-2 x 10⁶ live cells were incubated for 15 minutes at 37 °C in the dark in PBS containing 40 nM DiOC₆₍₃₎ (from a freshly-made 40 µM working solution and 4 mM stock, both in DMSO). Samples were then placed on ice and analysed in the dark within 1 hour on a Coulter Epics Elite flow cytometer, using a 488 nm excitation wavelength and 525 (+/-5) nm band pass filter. 10,000 events per sample were recorded.

2.1.5 Detection of filamentous actin using flow cytometry Staining of f-actin by phalloidin-FITC was carried out essentially as described (Endresen et al., 1995). Briefly,

cells were fixed for 60 minutes on ice in 1% paraformaldehyde in PBS, then permeabilised and free aldehyde groups reduced by incubating for 30 minutes at room temperature in PBS containing 1% BSA, 0.1% TX-100 and 0.1% NaBH₄. F-actin labelling was carried out for 1 hour at room temperature in the dark in PBS containing 2 U/ml FITC-phalloidin and 1% BSA. After rinsing in PBS + 1% BSA, 10,000 events per sample were analysed on a Coulter Epics Elite flow cytometer, using a 488 nm excitation wavelength and 525 (+/-5) nm band pass filter.

2.1.6 Pulsed field gel electrophoresis of DNA Protocols for PFGE analysis of DNA fragmentation have been described in detail (Walker et al., 1999). Briefly, cells were rinsed in nuclear buffer (15 mM Tris-HCl pH=7.4, 1 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 60 mM KCl, 15 mM NaCl), embedded in low melting point agarose plugs made up in nuclear buffer with 250 µg/ml proteinase K and proteins removed by incubation in TEEN buffer (10 mM NaCl, 10 mM Tris-HCl pH=9.5, 25 mM EDTA pH=9.5, 5 mM EGTA) containing 0.05% SDS and 125 µg/ml proteinase K for 1.5-3 hours at 37 °C. Plugs were then washed twice in TE buffer (10 mM Tris-HCl pH=8, 1 mM EDTA pH=8) and loaded onto 0.8% agarose gels made up and run in 0.5X TBE (45 mM Tris base, 45 mM boric acid, 6.25 mM EDTA). Yeast chromosome (225 kb-1.2 Mb), λ phage DNA (50 -1 Mb), PFGE low range (0.1-194 kb), HindIII digest of λ DNA (2kb-23kb) and 123bp ladder (100 - 5 kb) markers were used as size standards. Gels were run using a Bio-Rad FIGE mapper and established conditions (Walker et al., 1999). Gels were then stained with 1.5 µg/ml ethidium bromide for 30 minutes and destained in water.

Bands were visualised and gel images captured using an Ultra-Lum transilluminator (Paramount,CA) equipped with 300 nm excitation and 600 (+/-25) nm emission filters.

2.1.7 Western blot analysis of proteins Cellular proteins were extracted for 30 minutes on ice in RIPA buffer (50 mM Tris-HCl pH=7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% TX-100) and nuclear proteins extracted as described (Testolin et al., 1997). 40 µg aliquots of protein were separated by SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes using established conditions (Liu et al., 1999a). Blots were blocked with 5% milk in TBST (50 mM Tris-HCl, pH=7.5, 150 mM NaCl, 0.05% Tween-20) for 1 hour, then incubated at room temperature for 1 hour with primary antibody and 30 minutes with secondary antibody diluted in TBST + 5% milk. Blots were washed in TBST and bands visualised by colorimetric reaction.

2.1.8 Labelling of Nuclear Antigens and DNA For microscopic examination of nuclear antigens, cells were allowed to settle onto poly-l-lysine coated coverslips for 5 minutes then fixed for 5 minutes in 3% paraformaldehyde, washed twice briefly in PBS, permeabilised for 20 minutes in 0.2% Triton X-100 then washed three times for four minutes each time in PBS, in accordance with the method of Chaly et al (1984). Fixed cells were incubated for 1 hour in primary antibody, washed in PBS as above, then incubated in the dark for 45 minutes in the corresponding secondary antibody and washed again in PBS. For double staining, antibodies were applied sequentially with a 0.15% gelatin block used before application of the second primary. Finally, cells were counterstained either for 1 minute with 1 µg/ml of Hoechst 33258 or for five minutes in 0.2

µg/ml DAPI to label the DNA, rinsed twice briefly in PBS and coverslips were mounted onto slides.

2.1.9 Antibodies Primary antibodies used were: mouse monoclonal IgG anti-lamin B, diluted 1:25 for microscopy (clone 119D5-F1, (Weaver et al., 1996); mouse monoclonal IgG anti-NuMA, diluted 1:25 for microscopy or 1:1000 for Western blot; human polyclonal anti-lamin B receptor, diluted 1:500 for microscopy or 1:500 for Western blot (Ye and Worman, 1994); rabbit polyclonal anti-SATB1, diluted 1:500 for microscopy or 1:5000 for Western blot (Dickinson et al., 1992); rabbit polyclonal anti-lamin B, diluted 1:500 for microscopy or 1:2000 for Western blot (Chaudhary and Courvalin, 1993) or mouse monoclonal IgM anti-PI2, used at 1:100 (Chaly et al., 1984). Secondary antibodies used were: FITC- conjugated goat anti-mouse Ig G_(Fcγ), diluted 1:125; alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1:2000; FITC-conjugated goat anti-human IgG, diluted 1:100; CY3-conjugated donkey anti-rabbit IgG, diluted 1:600; alkaline phosphatase-conjugated donkey anti-rabbit IgG, diluted 1:5000 and FITC-conjugated goat anti-mouse IgM_(μ), diluted 1:150.

2.1.10 Microscopy Single-stained cells were examined using an Olympus BX50 fluorescence microscope and either photographed onto Ilford XP2 400 film or captured using Northern Exposure software. Double-labelled cells were photographed onto Kodak P1600 film and also examined on either a Leica-Wilde-Leitz or Zeiss LSM-410 inverted confocal laser scanning microscope equipped with an argon/krypton laser. Films were scanned and digital images were processed using the confocal system software and/or Adobe Photoshop.

2.2 RESULTS

2.2.1 Kinetics of cell death in lymphocytic cells as measured by characteristic nuclear changes

To define and order the events of nuclear breakdown, two lymphocytic cell models and three inducers of cell death were examined: rat primary thymocyte cultures treated with either 1 μ M dexamethasone or 10 μ M VM-26 (teniposide) and the human Jurkat T lymphocytic cell line treated with 100 ng/ml of death-inducing anti-Fas antibody (clone CH-11). In order to choose time points appropriate for the study of early changes in the nuclei of dying cells, the timecourse of cell death in the models was determined.

Cultures were first examined for changes in nuclear morphology. Figures 1A and 1B document the typical nuclear morphology of control and apoptotic thymocytes. Nuclei of controls featured crenellated edges, a central area of condensed chromatin and a thin rim of bright-staining DNA at the periphery. Apoptotic thymocyte nuclei seen after both dexamethasone and VM-26 treatment showed reorganisation of the chromatin into several large bodies at the nuclear periphery (top row of panel B) or a single, almost spherical chromatin mass (bottom row of panel B). The strong and uniform intensity of the DNA dye signal in these apoptotic bodies indicated that the chromatin had become highly concentrated; collapse of the normal chromatin structure had occurred. In Jurkat cells, control nuclei (Figure 1C) were slightly larger than thymocytes and characterised by both a smoother rim and a more uniform chromatin distribution throughout the nuclear interior, although a central area of slightly concentrated chromatin was also seen in these cells. Apoptotic Jurkat nuclei (Figure 1D) displayed both margination of several large chromatin masses (left hand column of the panel)

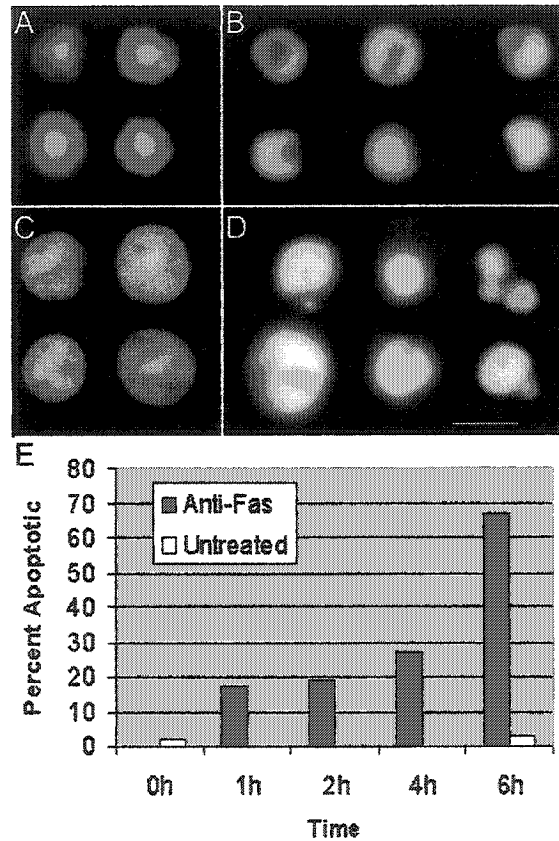


Figure 1 Detection of apoptosis based on chromatin collapse in lymphocytic cells

Primary cultures of rat thymocytes (A,B) and human Jurkat T cells (C,D) were stained with the DNA-binding dyes Hoechst 33248 and DAPI to reveal nuclear morphology. Typical samples of control nuclei from untreated cultures (A,C), apoptotic nuclei from dexamethasone or VM-treated thymocytes (B), and apoptotic nuclei from anti-Fas treated Jurkats (D) were assembled into panels. Bar = 10 μ m. Panel E: death of Jurkat cells in response to 100 ng/ml anti-Fas was monitored by cell counts following staining with Hoechst 33242 and propidium iodide to reveal nuclear morphology. Results of a typical experiment are shown.

and single, spherical collapsed chromatin bodies (middle column of the panel). In addition, formation of small, round archetypal “apoptotic bodies” was seen in a minority of nuclei in dying Jurkat cells (right hand column of the panel).

The kinetics of cell death in Jurkat cells treated with 100 ng/ml anti-Fas antibody were determined by monitoring chromatin collapse. Cells were incubated with the cell permeable DNA dye Hoechst 33342 to label all nuclei and propidium iodide to label the DNA in those cells which had lost their membrane integrity, then scored as intact (control nuclear morphology, Hoechst stain only), early apoptotic (collapsed nuclei, Hoechst stain only), late apoptotic (collapsed nuclei, Hoechst and propidium iodide labelling) or necrotic (uncollapsed nuclear morphology, Hoechst and propidium iodide labelling). The proportion of apoptotic nuclei (early + late) present at time points over a 6 hour time course was determined and the results presented in Figure 1E. Untreated Jurkat cultures contained 2-3% of nuclei with collapsed chromatin. After anti-Fas treatment, the percentage of apoptotic nuclei rose, increasing to 17-19% after 1-2 hours, almost 30% after 4 hours and constituting 2/3 of all nuclei by 6 hours of treatment. There was no accumulation of apoptotic nuclei in untreated control cultures over this same time frame. Anti-Fas treatment did not induce a necrotic phenotype in the cells (data not shown).

In addition to chromatin collapse, DNA cleavage has long been used as a biochemical hallmark of apoptosis. The response of thymocytes to 1 μ M dexamethasone was monitored by in situ labelling of DNA breaks using TUNEL and analysis by flow cytometry to determine the proportion of TUNEL positive nuclei over a 7 hour time course. Control cells were almost exclusively TUNEL-negative (Figure 2A, lower region, blue),

while treated cultures displayed a clearly distinguishable population of TUNEL-positive cells (Figure 2B, upper region, red). Comparison of the DNA content of these two subpopulations (X-axis) showed that accumulation of DNA breaks occurred in both the predominant G1 phase and the less common G2/M phase cells. Only a scant minority of TUNEL-positive cells displayed sub-G1 DNA content. Over the 7 hour time course, the proportion of TUNEL-positive cells rose from 2% in controls, to 20% after 2 hours treatment and 45% after 7 hours in the presence of dexamethasone (Figure 2C). VM-26-treated thymocytes showed a similar timecourse of response based on TUNEL labelling (data not shown).

2.2.2 Kinetics of cell death as determined by cytoplasmic changes The kinetics of cell death in thymocytes and Jurkat cells as detected by apoptotic changes to cytoplasmic components was also determined. Loss of mitochondrial function was monitored in both models by flow cytometric analysis of DiOC6₃ staining. Figures 3A and 3B show typical data from control and 2 hour anti-Fas treated cultures, respectively, presented as a two-parameter histograms showing both DiOC6 staining and side scatter of laser light (SSC). The latter is used to provide a clearer depiction of the shifted population, and does not depend on cell staining. Since uptake of DiOC6 depends on the presence of a normal membrane potential across the inner mitochondrial membrane, control cells display high levels of staining (right hand region, blue). After induction of apoptosis, an accumulation of cells with low levels of DiOC6 staining representing loss of mitochondrial membrane potential (left hand region, red) is seen in the culture. Over a 4 hour timecourse, the proportion of cells displaying loss of mitochondrial

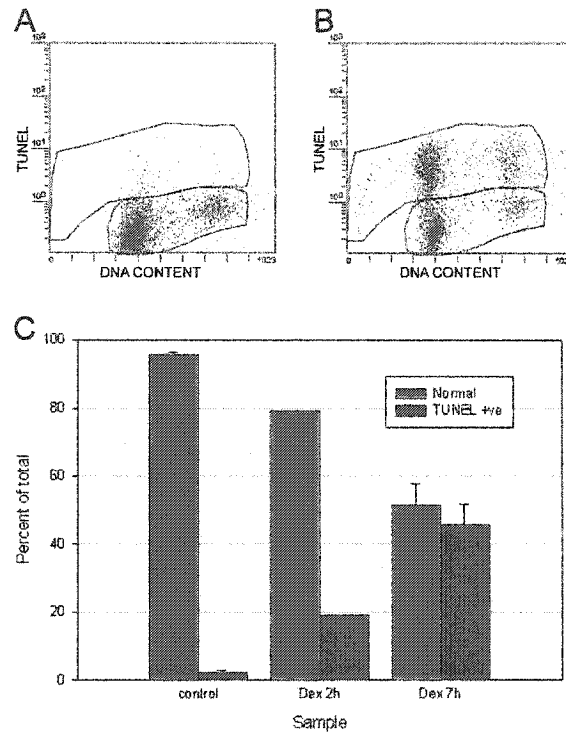


Figure 2 Kinetics of apoptosis in dexamethasone-treated thymocytes measured by TUNEL labelling of DNA breaks

Primary cultures of rat thymocytes were treated with $1\mu\text{M}$ dexamethasone for intervals up to 7 hours and induction of cell death as detected by TUNEL labelling of DNA breaks was assessed using flow cytometry. Sample flow histograms from 0 hours (A) and 7 hours of dexamethasone treatment (B) show the typical distribution of cells with background label (lower region, blue) and TUNEL-positive signal (upper region, red). Panel C: Kinetics of cell death in dexamethasone-treated thymocytes determined by flow cytometric analysis of TUNEL labelling. Results of triplicate experiments are shown.

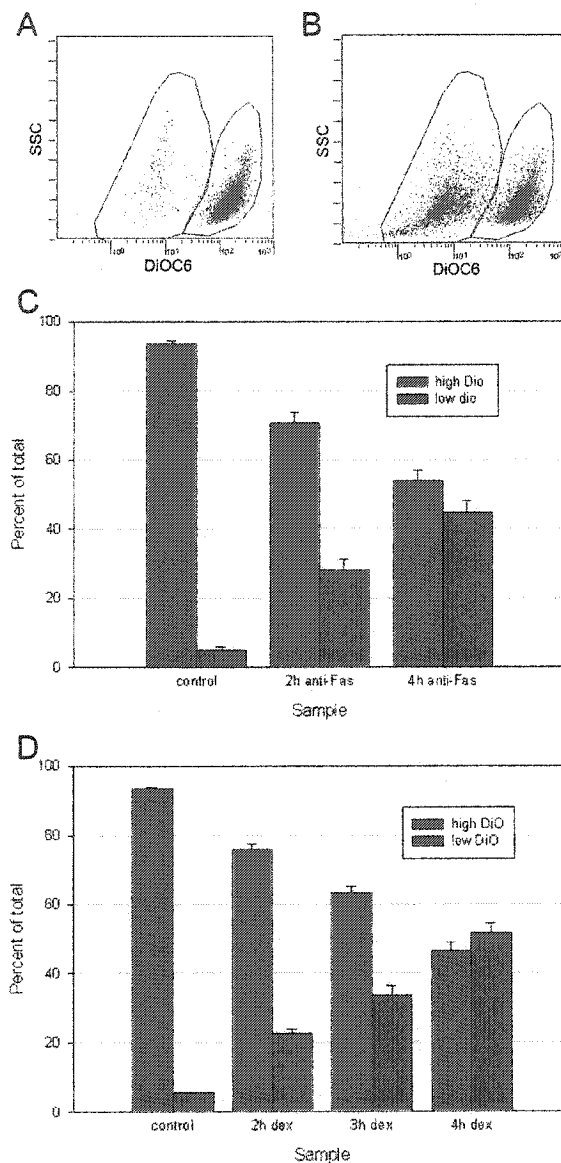


Figure 3 Detection of apoptosis in lymphocytic cells based on loss of mitochondrial function

Induction of cell death as detected by staining of functional mitochondria with the membrane potential-sensitive dye DiOC₆ was assessed using flow cytometry in human Jurkat T cell cultures treated with 100 ng/ml anti-Fas and primary cultures of rat thymocytes treated with 1 μ M dexamethasone for intervals up to 4 hours. Sample flow histograms from Jurkat cultures after 0 hours (A) and 2 hours (B) of anti-Fas treatment show the typical distribution of cells with constitutive label (right hand region, blue) and loss of DiOC₆ signal (left hand region, red). The kinetics of cell death in anti-Fas treated Jurkats (C) was monitored in quadruplicate experiments, and the response of dexamethasone treated thymocytes (D) was determined in triplicate.

function rose from 5% in controls to 28% at 2 hours of anti-Fas treatment and 45% after 4 hours in the presence of the inducer (Figure 3C). In thymocyte cultures treated with dexamethasone (Figure 3D) or VM-26 (data not shown), a similar timecourse was observed.

Cell death in thymocytes was also assessed by measuring the loss of filamentous actin. Cells were stained with FITC-conjugated phalloidin to detect F-actin, and assessed by flow cytometry. Figure 4A depicts the staining pattern of a typical control sample, which stained strongly with phalloidin (right hand region, blue). After 2 hours of dexamethasone treatment, a significant proportion of cells showed a marked drop in phalloidin staining (left hand region, red), indicating a loss of F-actin (Figure 4B). The proportion of cells which had lost their F-actin network increased from 1% in control cultures, to 18% after 2 hours of dexamethasone treatment and up to 47% after 4 hours in the presence of the drug.

Taken together, these results depict a consistent timecourse of response to inducers of cell death in the thymocyte and Jurkat cultures studied. In both cell types, loss of mitochondrial function proved to be the most sensitive marker of cell death studied by a small margin, particularly at the 2 hour time point. 20-25% of treated cells had undergone apoptosis by 2 hours according to both cytoplasmic and nuclear criteria, and this proportion increased to 45% by the 4 hour time point. Thus, further study of early nuclear events was confined to time points up to 4 hours.

2.2.3 Biochemical changes in apoptotic lymphocytic cells Using PFGE, DNA cleavage was assessed in thymocyte and Jurkat cell cultures, and the results presented in Figure 5. In primary thymocytes treated with 1 μ M dexamethasone, very high molecular weight

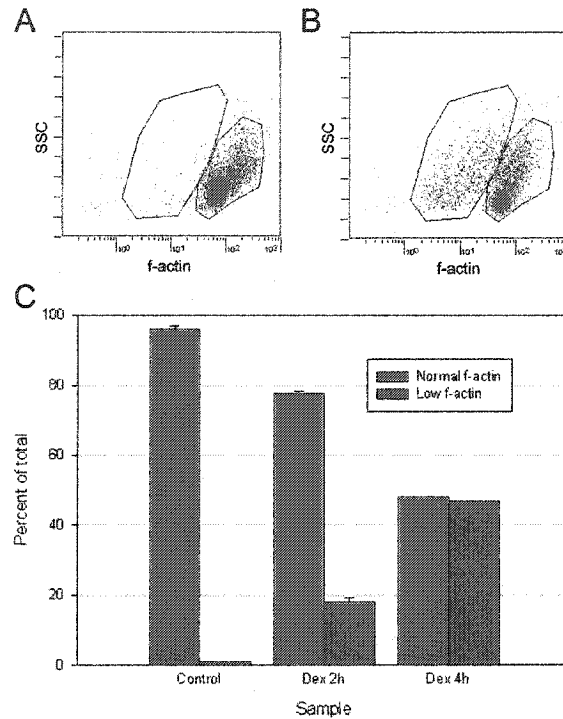


Figure 4 Kinetics of apoptosis in dexamethasone-treated thymocytes measured by loss of f-actin

Primary cultures of rat thymocytes were treated with $1\mu\text{M}$ dexamethasone for intervals up to 4 hours and induction of cell death as detected by FITC-phalloidin labelling of f-actin was assessed using flow cytometry. Sample flow histograms from 0 hours (A) and 2 hours (B) of dexamethasone treatment show the typical distribution of cells with background label (right hand region, blue) and f-actin-negative signal (left hand region, red). Panel C: Kinetics of cell death in dexamethasone-treated thymocytes determined by flow cytometric analysis of f-actin labelling. Results of duplicate experiments are shown.

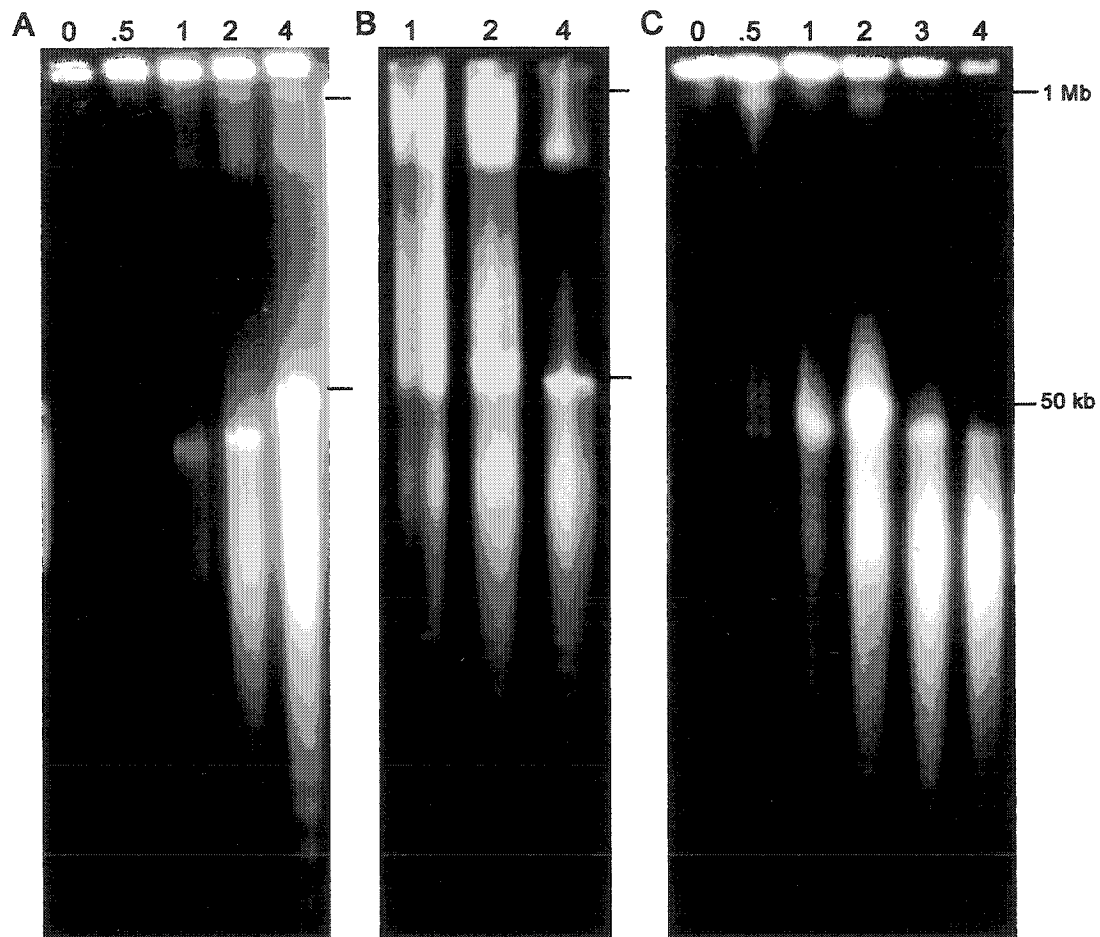


Figure 5 DNA degradation in apoptotic lymphocytic cells Chromatin breakdown during apoptosis in lymphocytic cells was characterised using pulsed field gel electrophoresis. The fragmentation of DNA from thymocytes treated with 1 μ M dexamethasone (A) or 10 μ M VM-26 (B) and Jurkat cells treated with 100 ng/ml anti-Fas (C) was assessed over a 4 hour time course. The positions of 50 kb and 1 Mb markers are indicated. Lanes are marked with incubation time (in hours).

DNA fragments (300Kb-1Mb) were first detected in the gel after only 30 minutes (Figure 5A, lane 0.5). Fragments of 50 kb and slightly smaller were first detected at 1 hour (Figure 5A, lane 1), became more pronounced by 2 hours of treatment and accounted for a large proportion of the DNA through to the end of the 4 hour incubation period (Figure 5A, lanes 2 and 4). In addition, a smear of LMW fragments was detected at 2 hours and became more pronounced at the 4 hour time point, with a light/dark banding pattern visible at its lower end, indicating the presence of an oligonucleosomal ladder. Thymocytes treated with 10 μ M VM-26 showed accelerated accumulation of DNA fragments. After 1 hour there was a significant accumulation of 50 kb and larger fragments (Figure 5B, lane 1) and by 2 hours, a smear of LMW fragments, including the oligonucleosomal ladder were seen (Figure 5B, lane 2). At 4 hours the 50 kb and LMW species accounted for the bulk of the DNA (Figure 5B, lane 4). In Jurkat cell cultures treated with 100 ng/ml anti-Fas, both very high molecular weight and 50 kb fragments were first visible after 30 minutes of treatment (Figure 5C, lane 0.5). From 30 minutes to 2 hours an increase in first the 50 kb size class, followed by a smear of LMW fragments was seen (Figure 5C, lanes 1-2). From 3 to 4 hours, the proportion of 50 kb fragments decreased and the LMW fragments accounted for the bulk of the DNA (Figure 5C, lanes 3-4). In Jurkats, an oligonucleosomal ladder was not clearly resolved. For pulsed field analysis, cells are embedded in low melting point agarose and the necessary protein digestion steps and washes are carried out on the agarose plug. Analysis of the accumulated plug wash showed that oligonucleosomal fragments of apoptotic Jurkat DNA were present in this fraction (data not shown). Accumulation of HMW fragments of 1 Mb-50 kb before generation of the classic LMW fragments and oligonucleosomal ladder was

seen for all the samples, although the timing and extent of DNA cleavage over the 4 hour time course varied. HMW fragments were the predominant species at the 30 minute time point in all cases, with a small amount of LMW fragmentation first detected in Jurkats and VM-treated thymocytes by 1 hour.

Specific cleavage of nuclear matrix proteins during apoptosis has been extensively described in lymphocytic and other model systems. The integrity of nuclear matrix proteins in apoptotic thymocytes and Jurkats was assessed by Western blotting. As presented in Figure 6, NuMA (panels A and D), SATB1 (panels B and E) and lamin B (panels C and F) were present as intact proteins of 240 kDa, 103 kDa and 67 kDa, respectively, in control thymocytes (A-C, lanes 0) and Jurkats (D-F, lanes 0). Upon induction of apoptosis, NuMA showed rapid degradation to the characteristic 220 kDa fragment (Hsu and Yeh, 1996) after 30 minutes in dex- or VM-treated thymocytes (Figure 6A, lane 0.5 and data not shown) and in anti-Fas treated Jurkats (Figure 6D, lane 0.5). The intact protein was no longer detectable in Jurkats after 1 hour of treatment (Figure 6D, lane 1) or in thymocytes after 2 hours (Figure 6A, lane 2). The MAR-binding protein SATB1 was also rapidly degraded in apoptotic cells; the 75 kDa cleavage product (Gotzmann et al., 2000) was detected in thymocytes after 30 minutes of VM-26 or dex treatment (Figure 6B, lane 0.5 and data not shown) and in Jurkat cells after 1 hour in the presence of anti-Fas (Figure 6E, lane 1). While a portion of the protein remained intact in thymocytes up to the 4 hour time point (Figure 6B, lanes 1-4), only the cleavage product was detected in Jurkat cells at later time points (Figure 6E, lanes 2-3). Cleavage of lamin B to the characteristic 46 kDa fragment

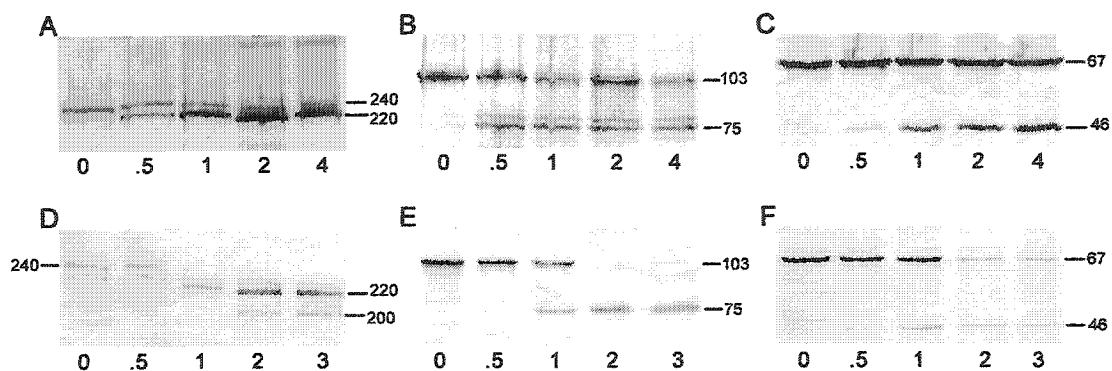


Figure 6 Degradation of nuclear matrix in apoptotic lymphocytic cells

The integrity of several nuclear matrix proteins during apoptosis in lymphocytic cells was assessed by Western blotting. NuMA was monitored in thymocytes treated with 1 μ M dexamethasone (A), while SAT B1 (B) and lamin B (C) were assessed in thymocytes treated with 10 μ M VM-26. NuMA (D), SAT B1 (E) and lamin B (F) were also monitored in Jurkat cells treated with 100 ng/ml anti-Fas. The size of intact proteins and fragments generated by apoptotic cleavage are indicated. Lanes are marked with incubation time (in hours).

(Oberhammer et al., 1994; Neamati et al., 1995; Weaver et al., 1996) was seen at the 1 hour time point in VM-26 treated thymocytes (Figure 6C, lane 1) and anti-Fas treated Jurkats (Figure 6F, lane 1). The intact protein also remained detectable throughout the 4 hour time course (Figure 6C, 6F, lanes 2-4). In general, cleavage was more extensive in Jurkat cells, which demonstrated earlier or more extensive loss of intact matrix proteins than did the thymocyte models. Lamin B receptor was not cleaved during the timecourse studied (data not shown), in accordance with previously published results showing that this nuclear envelope protein remains intact throughout the early stages of apoptosis (Duband-Goulet et al., 1998) and consistent with our previous findings for the nuclear envelope protein PI2, in dexamethasone-treated thymocytes (Weaver et al., 1996).

Taken together, the biochemistry data showed that cleavage of nuclear matrix proteins began concurrently with generation of HMW DNA fragments in my model systems, during the first 30 minutes to one hour of treatment. Drawing conclusions about the relationship of individual events at the single cell level was not possible based on the kinetic and biochemical data, since all the criteria studied show mixed populations throughout the timecourse. It was not possible to know, for example, that at 2 hours the 20% of cells which had lost their F-actin network or their mitochondrial function were the ones displaying the LMW DNA cleavage fragments detected by PFGE, or whether the HMW fragments detected at this same time point were present in all cells or only the minority that already appear dead. Given these limitations, the nuclear events of apoptosis were examined at the single cell level in an attempt to further characterise and order the process of nuclear breakdown.

2.2.4 Morphology of nuclear matrix in lymphocytic cells The nuclear matrix was examined at the single cell level by immunostaining for several representative proteins. SATB1 and NuMA were chosen as examples of internal nuclear matrix proteins, lamin B as a matrix protein confined to the nuclear periphery and lamin B receptor and PI2 as examples of nuclear envelope proteins. The distribution of these proteins in untreated control cells, as detected by confocal microscopy, is depicted in Figure 7. SATB1 was distributed throughout the nucleoplasm in both Jurkat cells (Figure 7A) and thymocytes (Figure 7B). The protein was excluded from nucleoli (as seen most clearly in the left hand nucleus in 7A), as well as the small marginal areas of condensed chromatin in thymocyte nuclei. Staining intensity varied only slightly throughout the nuclear interior; no organisation into nuclear bodies or speckles was seen. NuMA had a similar pan-nucleoplasmic distribution, and a remarkably uniform staining intensity (Figures 7C and 7D). As expected, lamin B stained the nuclear periphery in both cell types (Figures 7E and 7F). The signal was evenly distributed around the nuclear rim in Jurkats, while the intensity varied somewhat across the nucleus in thymocytes. A distinct lamin B-positive spot is visible in the interior of the right hand nucleus in panel E. Such internal foci of lamin protein have been described previously, and represent sites of post-translational processing of newly synthesized lamin proteins (Goldberg et al., 1999). Lamin B receptor staining was also confined to the nuclear rim (Figures 7G and 7H), and displayed a more even distribution in Jurkat cells than in thymocytes. Staining for the nuclear envelope protein PI2 was confined to the nuclear periphery in both cell types (data not shown).

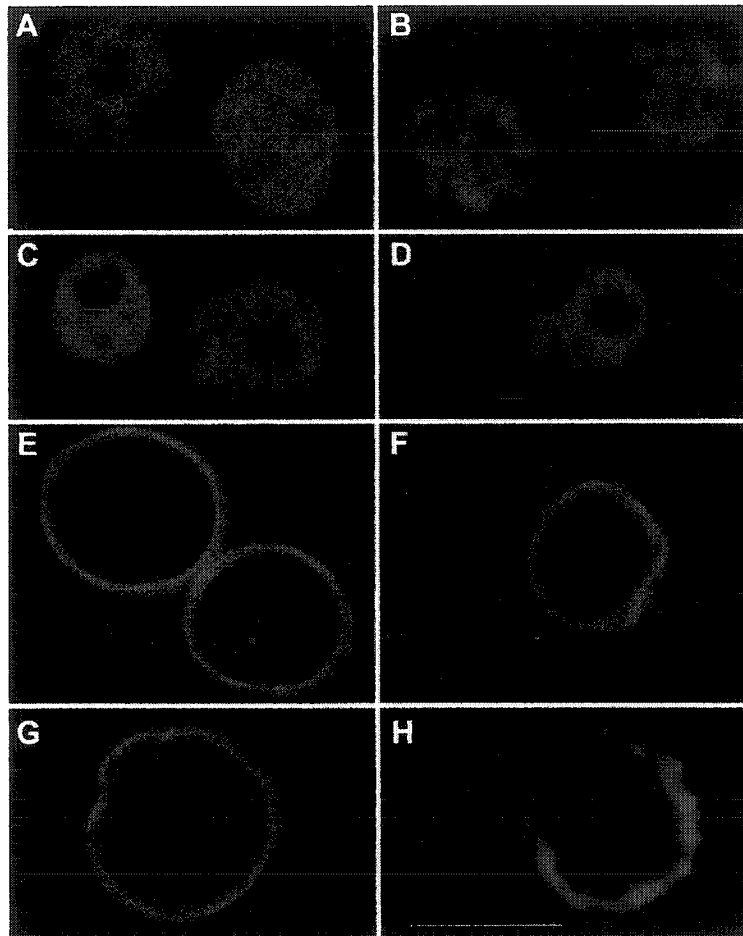


Figure 7 Nuclear matrix staining in lymphocytic cells

Control cultures of Jurkat cells (A,C,E,G) and thymocytes (B,D,F,H) were stained with antibodies to SATB1 (A,B), NuMA (C,D), lamin B (E,F) and lamin B receptor (G,H). Cells were examined using a confocal laser scanning microscope and the resulting digital images processed using the system software and Adobe Photoshop. Each panel depicts a representative slice through one or two nuclei. Bar = 10 µm.

2.2.5 Morphological changes in the MAR-binding protein SAT B1 during apoptosis in lymphocytic cells Changes in SATB1 distribution at the single cell level were extensively documented in both thymocytes and Jurkat cells, using both immunofluorescence and confocal microscopy, and results are presented in Figures 8-10. In freshly-isolated rat thymocytes, nuclei displayed typical morphology and SATB1 distribution (Figures 8A, 8A'). After 30 minutes incubation in either 10 μ M VM-26 or 1 μ M dexamethasone, some thymocyte nuclei featured increased areas of condensed chromatin relative to controls, although not apoptotic collapse (Figures 8B', 8C', 9B', arrowheads). These nuclei often showed dramatic relocation of a portion of the SAT B1 signal outside of the nuclear periphery as defined by Hoechst staining (Figures 8B, 8C, 9B, arrowheads). Relocation of a portion of the SATB1 signal was also seen in nuclei whose Hoechst morphology did not differ markedly from controls (Figures 9A, 9A'). The examples in Figures 8B and 9A clearly demonstrate that this reorganisation did not involve all of the SAT B1 present in the nucleus; some signal remained in the nucleoplasm. In all these cases, the staining pattern of SAT B1 was interpreted as showing relocation of a portion of the antigen to the cytoplasm. After 2 hours treatment with either VM-26 or dexamethasone, many nuclei displayed collapsed DNA typical of apoptotic cells (Figures 8D', 9C', arrows). Diffuse SATB1 staining of the remaining nucleoplasmic space to the side of or between the chromatin masses was present (Figures 8D, 9C, arrows). A similar distribution was detected through to 6 hours of drug treatment, but the staining intensity varied greatly from one nucleus to the next, and some nuclei appeared to have little remaining SAT B1 signal (Figures 8E, 8E', 9D, 9D' arrows). In Jurkat cells induced to die by activation of the Fas

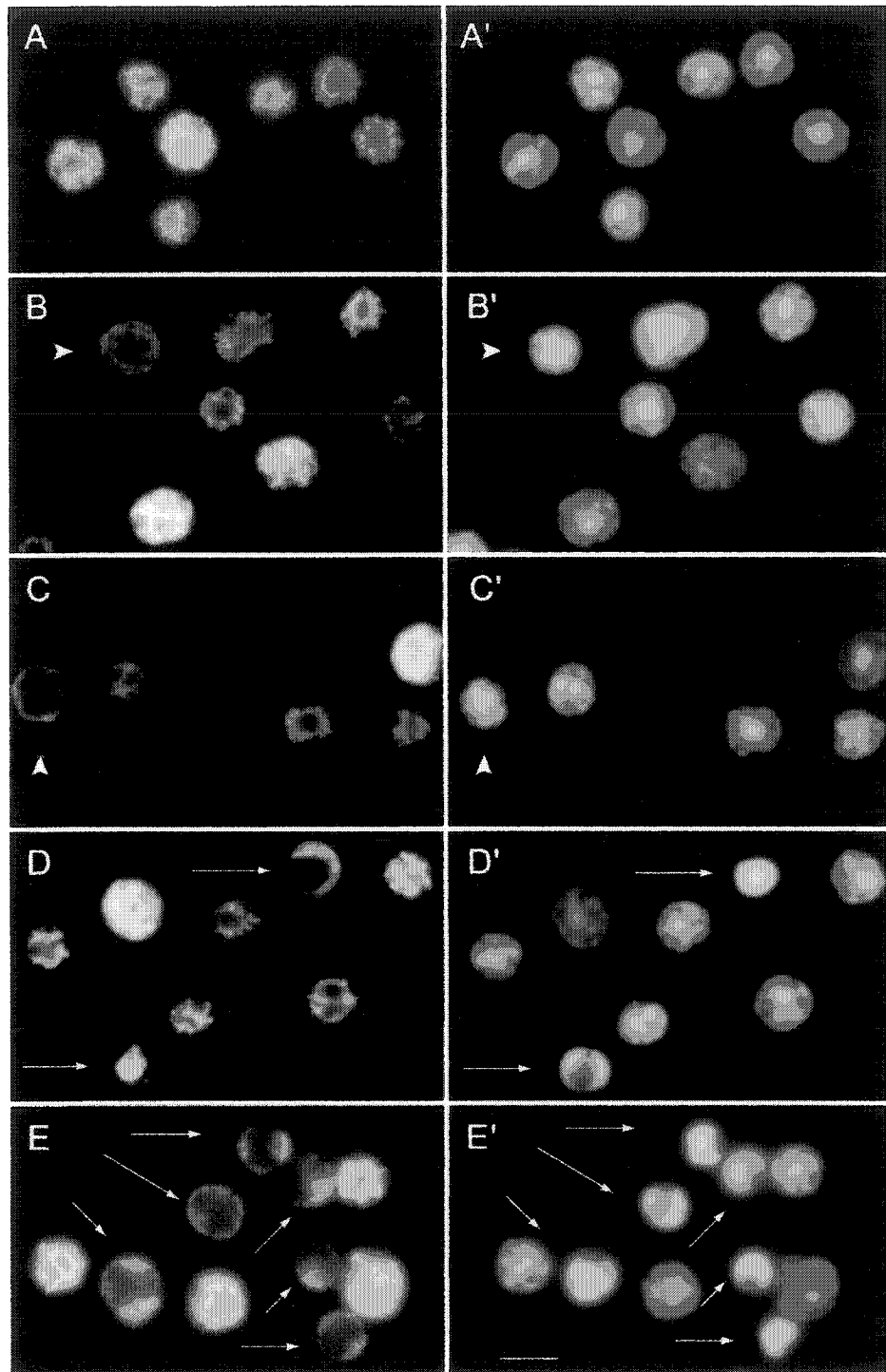


Figure 8 Reorganisation of SATB1 in VM-26 treated thymocytes

Primary cultures of rat thymocytes were immunostained to detect SATB1 (A-E) and counterstained with Hoechst 33258 to reveal nuclear morphology (A'-E') immediately after isolation (A, A') or after treatment with 10 μ M VM-26 for 30 minutes (B, B', C, C'), 2 hours (D, D') or 6 hours (E, E'). Arrowheads indicate nuclei which show reorganisation of SATB1 but not chromatin collapse, arrows indicate nuclei displaying apoptotic chromatin collapse. Bar = 10 μ m.

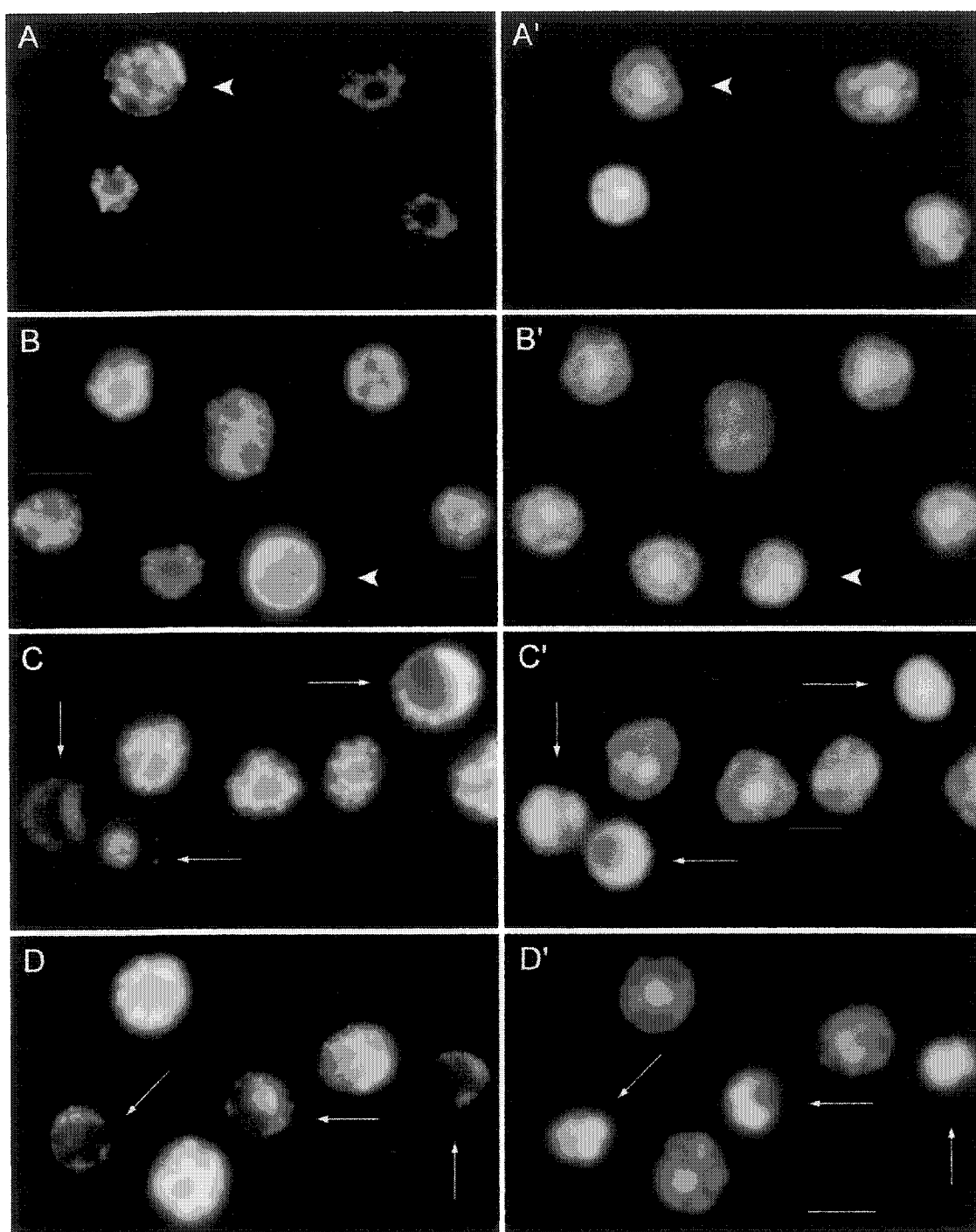


Figure 9 Reorganisation of SATB1 in dexamethasone treated thymocytes

Primary cultures of rat thymocytes were immunostained to detect SAT B1 (A-D) and counterstained with Hoechst 33258 to reveal nuclear morphology (A'-D') after treatment with 1 μ M dexamethasone for 30 minutes (A, A', B, B'), 2 hours (C, C') or 6 hours (D, D'). Arrowheads indicate nuclei which show reorganisation of SAT B1 but not chromatin collapse, arrows indicate nuclei displaying apoptotic chromatin collapse. Bar = 10 μ m.

receptor, SAT B1 displayed similar patterns of early and late reorganisation. Control cells displayed a pan-nucleoplasmic signal, excluded from the nucleolus (Figure 10A). In cells exposed to anti-Fas for 1 hour, a few nuclei showed apoptotic chromatin collapse, and a SAT B1 signal confined to the remaining nucleoplasmic space (Figure 10B). However, at time points up to and including 1 hour, early change in SATB1 distribution in the absence of chromatin collapse was also noted in a minority of cells. These displayed a marked decrease in the intensity of nucleoplasmic SATB1 signal, relative to both controls and neighbouring cells in the treated populations with normal nuclear morphology. Figure 10C shows a nucleus demonstrating low level SATB1 staining on the right and a nucleus displaying typical SATB1 staining on the left. This early change in SAT B1 did not involve all the protein; some remained in the nucleoplasmic space. However, unlike thymocytes, cytoplasmic SATB1 was not detected in these cells.

2.2.6 Apoptosis-associated morphological changes in the distribution of NuMA

Morphological changes in the distribution of NuMA during cell death were examined in order to document trends common to components of the internal nuclear matrix. In freshly-isolated thymocytes (Figures 11A, 11A') diffuse labelling of NuMA was seen in the nucleoplasm, a pattern indistinguishable from SAT B1. At early time points after 10 μ M VM-26 or 1 μ M dexamethasone (Figure 11B, 11B', 11D, 11D') nuclei with some increase in the central areas of condensed chromatin (but not collapse) stained slightly less intensely for NuMA, as a reflection of their decreased nucleoplasmic space, but relocation of a portion of the signal to the cytoplasm was never seen. Dramatic rearrangement of NuMA occurred at

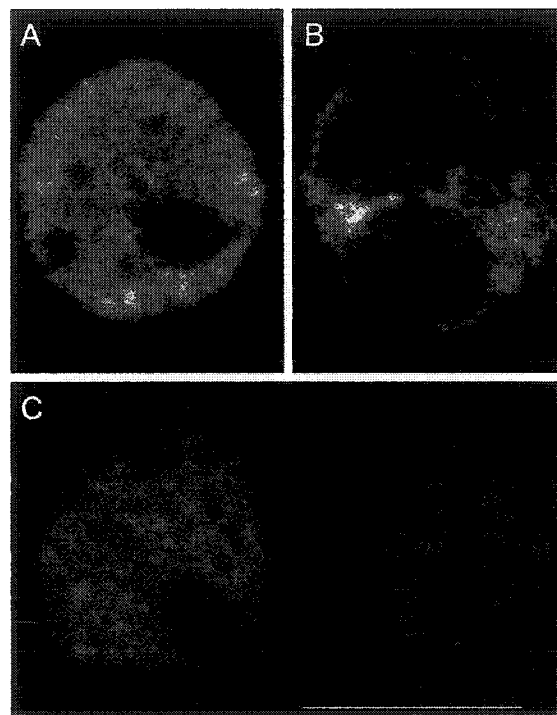


Figure 10 Reorganisation of SATB1 in anti-Fas treated Jurkat cells

Human Jurkat T cells were immunostained to detect SATB1 in untreated controls (A) and cells treated with anti-Fas for 1 hour (B, C). Cells were examined using confocal microscopy. Each panel depicts a representative slice through one or two nuclei. Bar = 10 μ m.

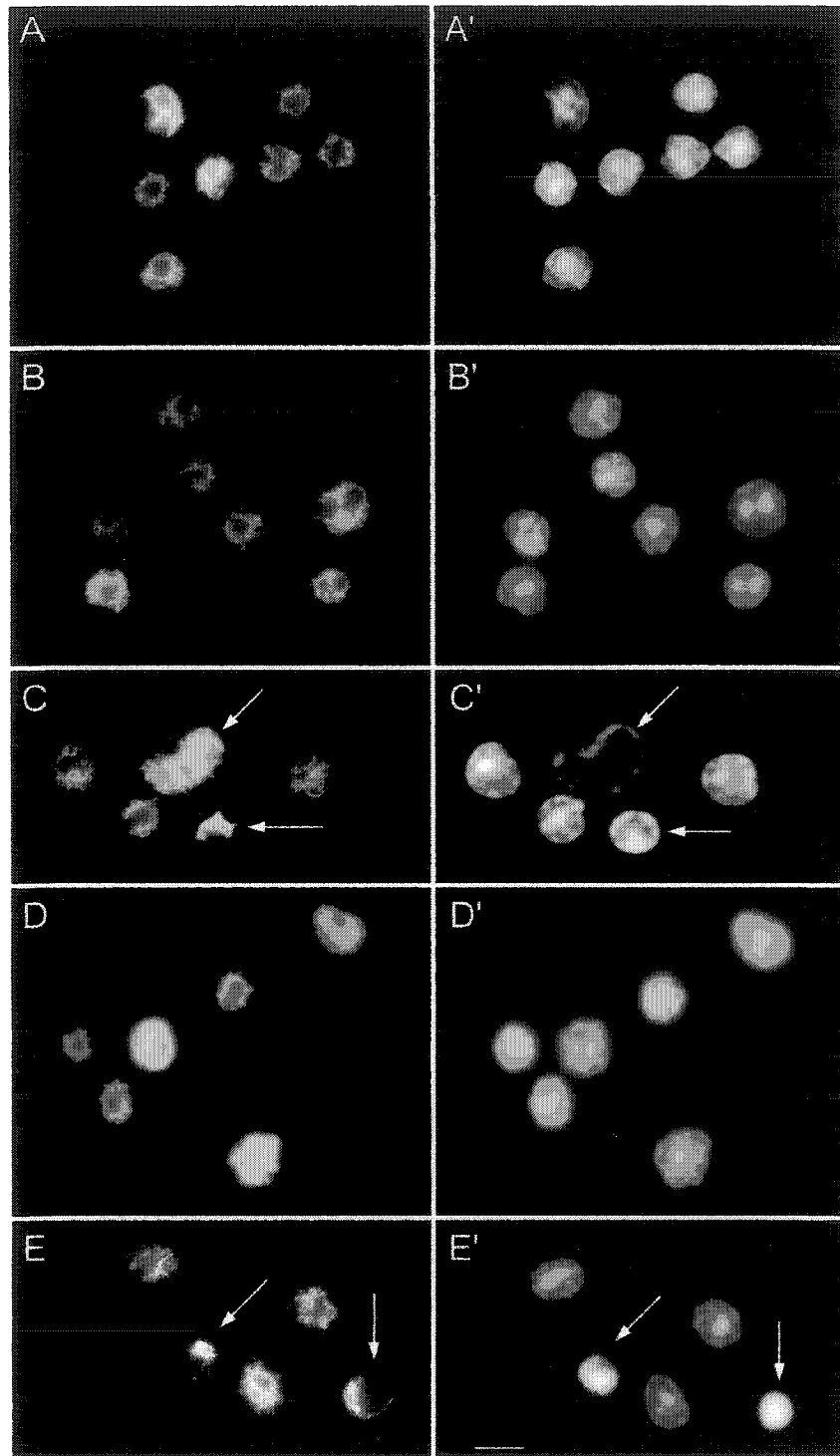


Figure 11 Reorganisation of NuMA in apoptotic thymocytes

Primary cultures of rat thymocytes were immunostained to detect NuMA (A-E) and counterstained with Hoechst 33258 to reveal nuclear morphology (A'-E') immediately after isolation (A, A') and after treatment with either 10 μ M VM-26 for 30 minutes (B, B') or 2 hours (C, C') or 1 μ M dexamethasone for 30 minutes (D, D') or 2 hours (E, E'). Arrows indicate nuclei displaying apoptotic chromatin collapse. Bar = 10 μ m.

later time points, coincident with the collapse of the DNA (Figures 11C, 11C', 11E, 11E'). In these nuclei NuMA staining was a bright, even signal in the remaining nucleoplasmic space, as seen for SAT B1. However, NuMA signal persisted in a smaller proportion of nuclei after 6 hours treatment (data not shown). Changes in the distribution of NuMA in apoptotic Jurkat cells were the same as those observed in thymocytes (Figure 12). Figure 12B depicts an apoptotic nucleus displaying a single mass of margined chromatin on the upper right hand side, and NuMA staining confined to the exterior of the mass, predominantly on the lower left hand side. Interestingly, remnants of the nucleolus persist and NuMA remains excluded from this compartment. Figure 12C depicts an apoptotic nucleus in which the collapsed DNA has formed two round apoptotic bodies. NuMA staining surrounds the chromatin masses.

2.2.7 Morphological change in components of the nuclear rim in apoptotic lymphocytic cells We have previously documented loss of lamin B signal in dexamethasone-treated thymocytes closely linked with chromatin collapse (Weaver et al., 1996). In contrast, I found that staining for lamin B in VM-26 treated thymocytes remained intact in the majority of nuclei at time points up to two hours, including those displaying apoptotic collapse of the chromatin (Figures 13A, 13A', 13B, 13B', arrows). However, some apoptotic nuclei did show a fainter, less even stain than those with normal nuclear morphology (horizontal arrow, 13B). This was interpreted as partial loss of the lamin signal, and this phenotype was seen more frequently after longer incubation times (data not shown). Jurkat cells induced to die with anti-Fas showed similar retention of the lamin B signal in apoptotic nuclei at time points up to 4 hours (Figure 14B and data not shown). Proteins of the nuclear envelope

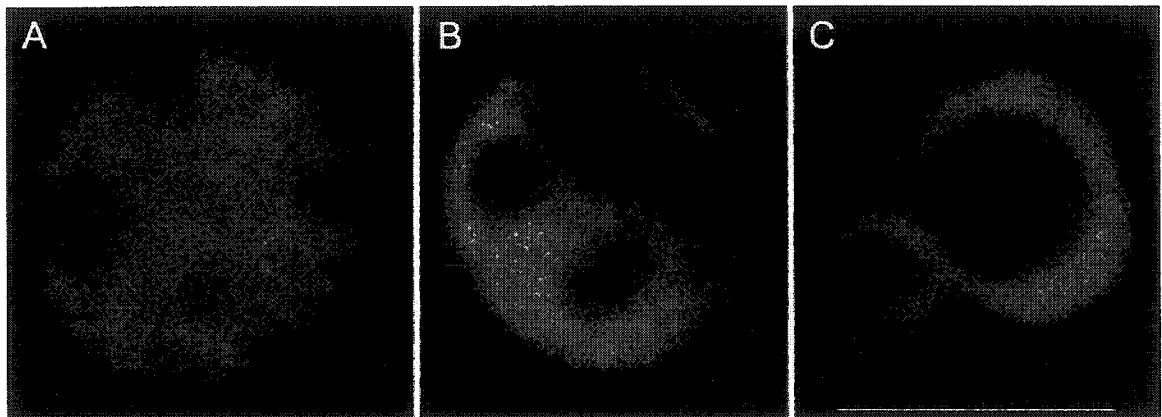


Figure 12 Reorganisation of NuMA in anti-Fas treated Jurkat cells

Human Jurkat T cells were immunostained to detect NuMA in untreated controls (A) and cells treated with anti-Fas for 1 hour (B,C). Cells were examined using confocal microscopy. Each panel depicts a representative slice through a single nucleus. Bar = 10 μm .

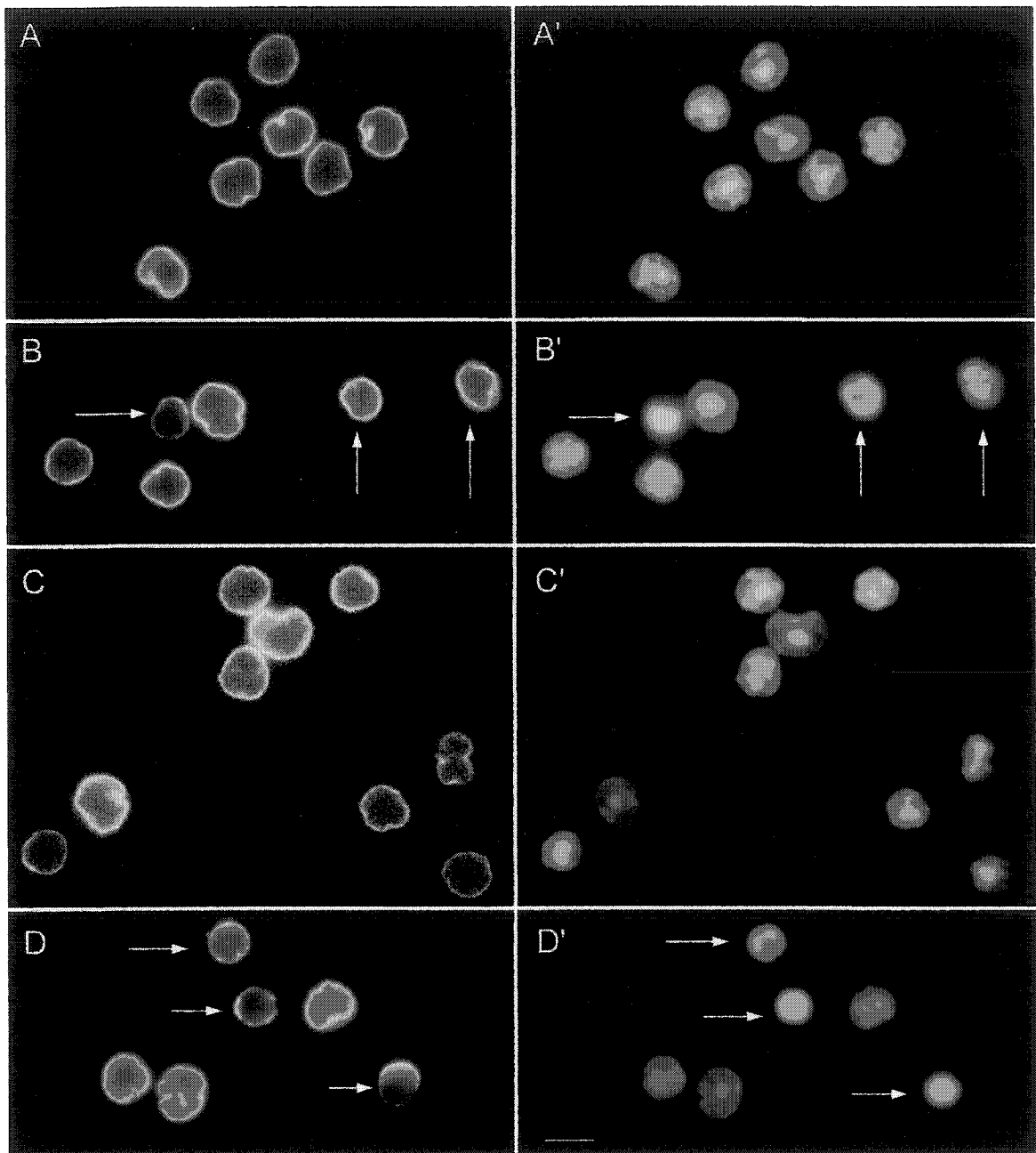


Figure 13 Morphology of nuclear rim proteins in apoptotic thymocytes

Primary cultures of rat thymocytes were immunostained to detect either lamin B (A,B) or the nuclear envelope protein F12 (C,D) and counterstained with Hoechst 33258 to reveal nuclear morphology (A'-D') after treatment with either 10 μ M VM-26 for 30 minutes (A,A') or 2 hours (B,B') or 1 μ M dexamethasone for 30 minutes (C,C') or 2 hours (D,D'). Arrows indicate nuclei displaying apoptotic chromatin collapse. Bar = 10 μ m.

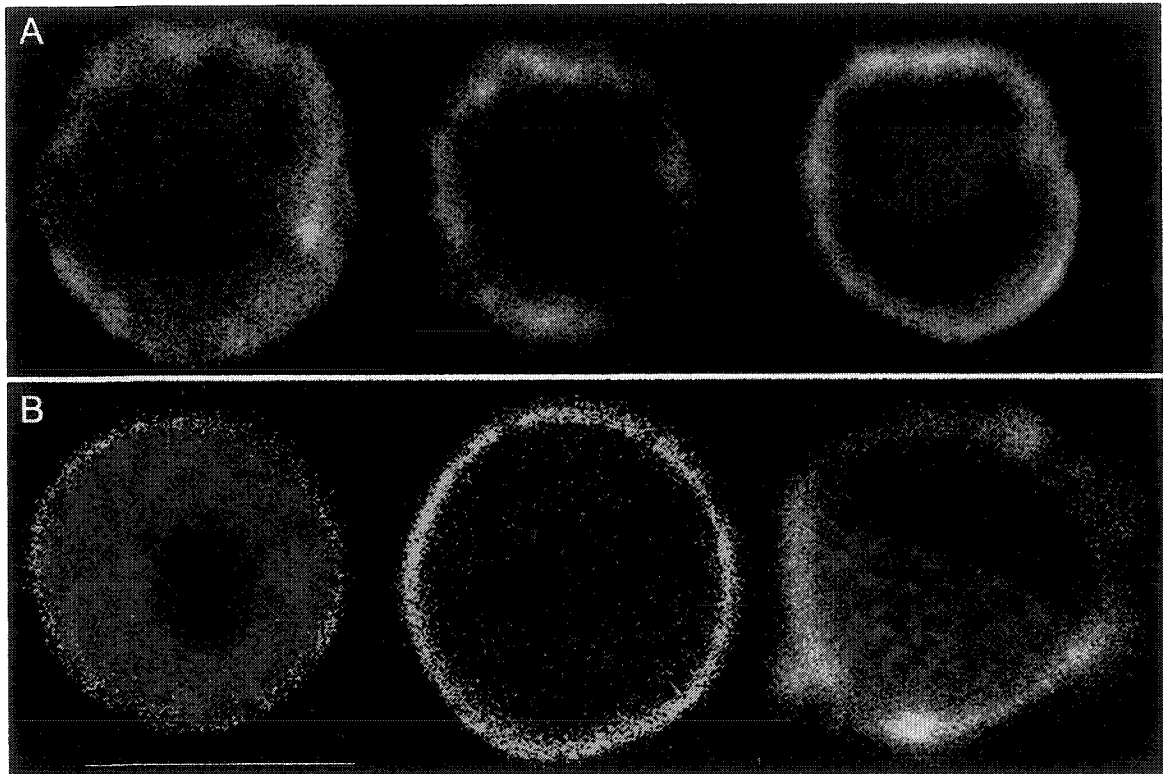


Figure 14 Relationship of morphological changes in nuclear matrix to chromatin collapse in apoptotic lymphocytic cells

Primary cultures of rat thymocytes treated with 1 μ M dexamethasone (A) and human Jurkat T cells treated with 100 ng/ml anti-Fas (B) were immunostained to detect SATB1 (red signal) and lamin B (green signal) or lamin B receptor (green signal, right hand nucleus in panel B) simultaneously. Cells were examined using confocal microscopy. Each panel depicts a representative slice through a single nucleus from untreated control cells (left hand nucleus), early apoptotic cells (middle nucleus) and cells displaying apoptotic collapse of the chromatin (right hand nucleus). Bar = 10 μ m.

demonstrated the most consistent morphological distribution throughout the apoptotic response. Staining for PI2 remained bright in thymocytes treated with dexamethasone (Figures 13C, 13C', 13D, 13D'), including those displaying chromatin collapse (indicated by arrows). The signal was often less evenly distributed around the nuclear rim in these dying cells, as seen best in the nucleus on the bottom right of panel D, but retained an intensity similar to those cells with normal nuclear morphology. In these cases, the sections of the nuclear rim which stained least brightly were often in closest contact with the collapsed chromatin. Thus, this variation in staining could be interpreted as decreased access of the epitope, or possibly redistribution of the epitope in the nuclear envelope similar to that seen for nuclear pores in dying cells (Worman and Courvalin, 2000). Likewise, the PI2 signal in VM-26 treated thymocytes and anti-Fas treated Jurkats remained intact (data not shown). Similar results were also observed for another nuclear envelope protein, lamin B receptor (Figure 14B and data not shown).

2.2.8 Reorganisation of SATB1 precedes chromatin collapse The results of single stains suggested that the reorganisation of SATB1 in apoptotic lymphocytic nuclei preceded chromatin collapse and alteration of other matrix components. Confocal microscopy of cells double stained for SAT B1 and either lamin B or nuclear envelope proteins demonstrated that this was indeed the case at the single cell level, as depicted in the representative nuclear sections in Figure 14. In control thymocytes (Figure 14A, left hand nucleus) and Jurkats (Figure 14B, left hand nucleus) SATB1 signal (red) was confined to the nuclear interior as defined by lamin B staining (green). Prior to collapse of the chromatin, loss of a portion of the SATB1 signal was detected in both thymocytes and Jurkats (middle nuclei in Figures

14A and 14B). Lamin B staining in these cells remained indistinguishable from controls. In thymocytes, relocation of SATB1 exterior to the nuclear rim as defined by lamin B was also seen. In those nuclei displaying collapse of the chromatin (right hand nucleus in both panels), the remaining SATB1 signal was confined to the nucleoplasmic space between or to the side of the chromatin masses, and within the nuclear boundary as defined by lamin B (Figure 14A), lamin B receptor (Figure 14B) or PI2 staining (data not shown).

2.2.9 Apoptosis-related changes in nuclear morphology follow initial DNA

fragmentation We have previously documented the accumulation of initial DNA breaks at the single cell level before chromatin collapse and loss of the nuclear lamin signal in dexamethasone-treated thymocytes (Weaver et al., 1996). Given that relocation of a portion of SAT B1 in apoptotic lymphocytic cells precedes collapse of the chromatin, I next examined the relationship between this early change and the accumulation of DNA breaks using confocal microscopy. In thymocytes treated with dexamethasone or VM-26 (Figure 15 and data not shown) and in anti-Fas-treated Jurkat cells (Figure 16) double stained to label SAT B1 (red signal) and DNA breaks (green signal), it was clear that initial DNA fragmentation preceded rearrangement of SAT B1. At early time points, nuclei displaying both a low level of DNA breaks and SAT B1 intensity and distribution indistinguishable from controls were present in the population (Figures 15A, 15B, 16A, 16B). In thymocytes, relocation of a portion of the SAT B1 signal was seen in nuclei with both this low intensity TUNEL signal (Figure 15C, upper nucleus) and those with a slightly stronger label, indicating more extensive DNA cleavage (Figure 15C, lower nucleus).

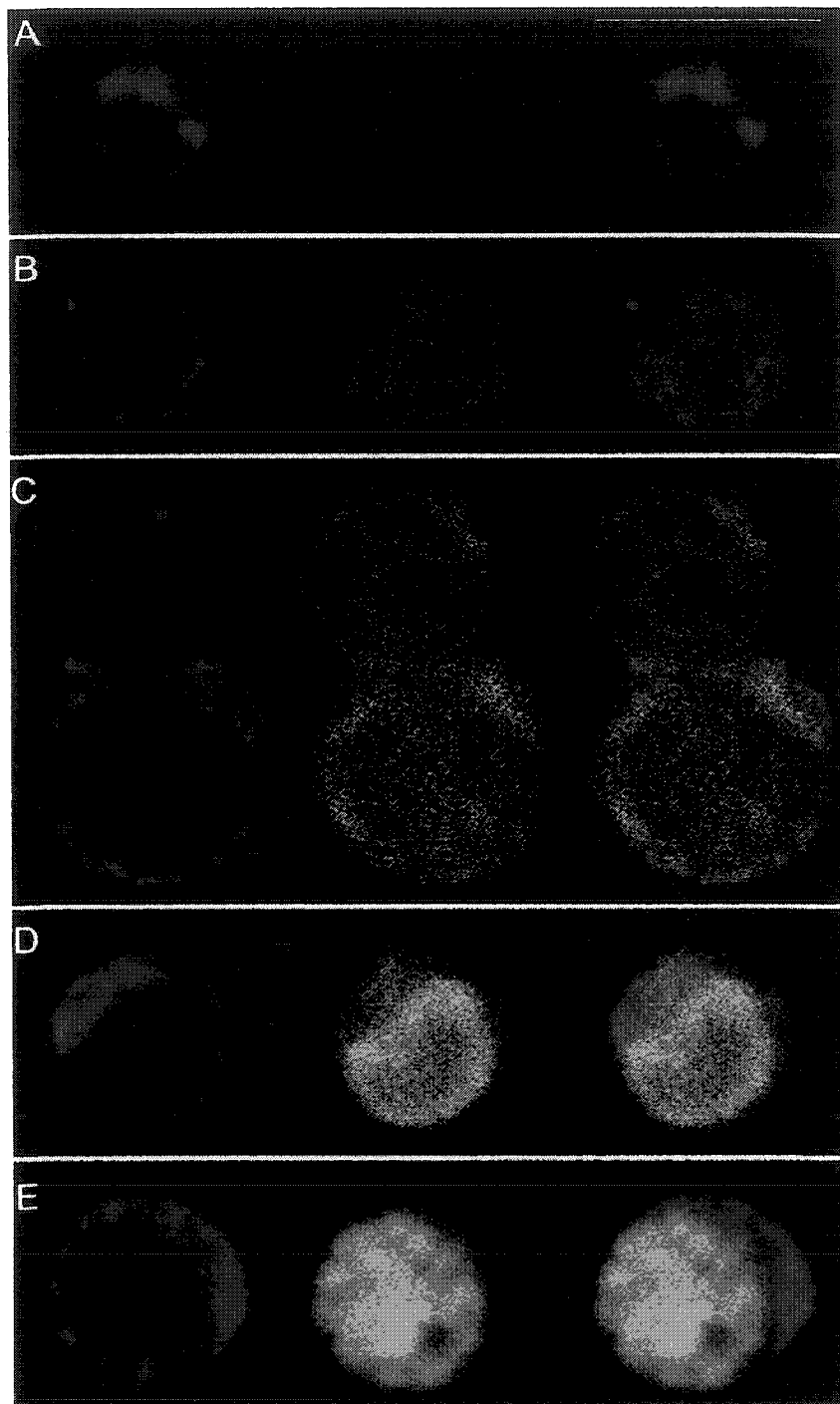


Figure 15 Relationship between SATB1 reorganisation and DNA cleavage in apoptotic thymocytes

Primary cultures of rat thymocytes were immunostained to detect SATB1 (A-E, red signal), then subjected to in situ labelling of DNA breaks by TUNEL (A-E, green signal) immediately after isolation (A) or after treatment with 1 μ M dexamethasone for 30 minutes (B,C) or 2 hours (D,E). Cells were examined using confocal microscopy. Each panel depicts a representative slice through one or two nuclei. The SATB1 signal is displayed on the left, TUNEL signal in the middle and the overlay of the two channels on the right. Bar = 10 μ m.

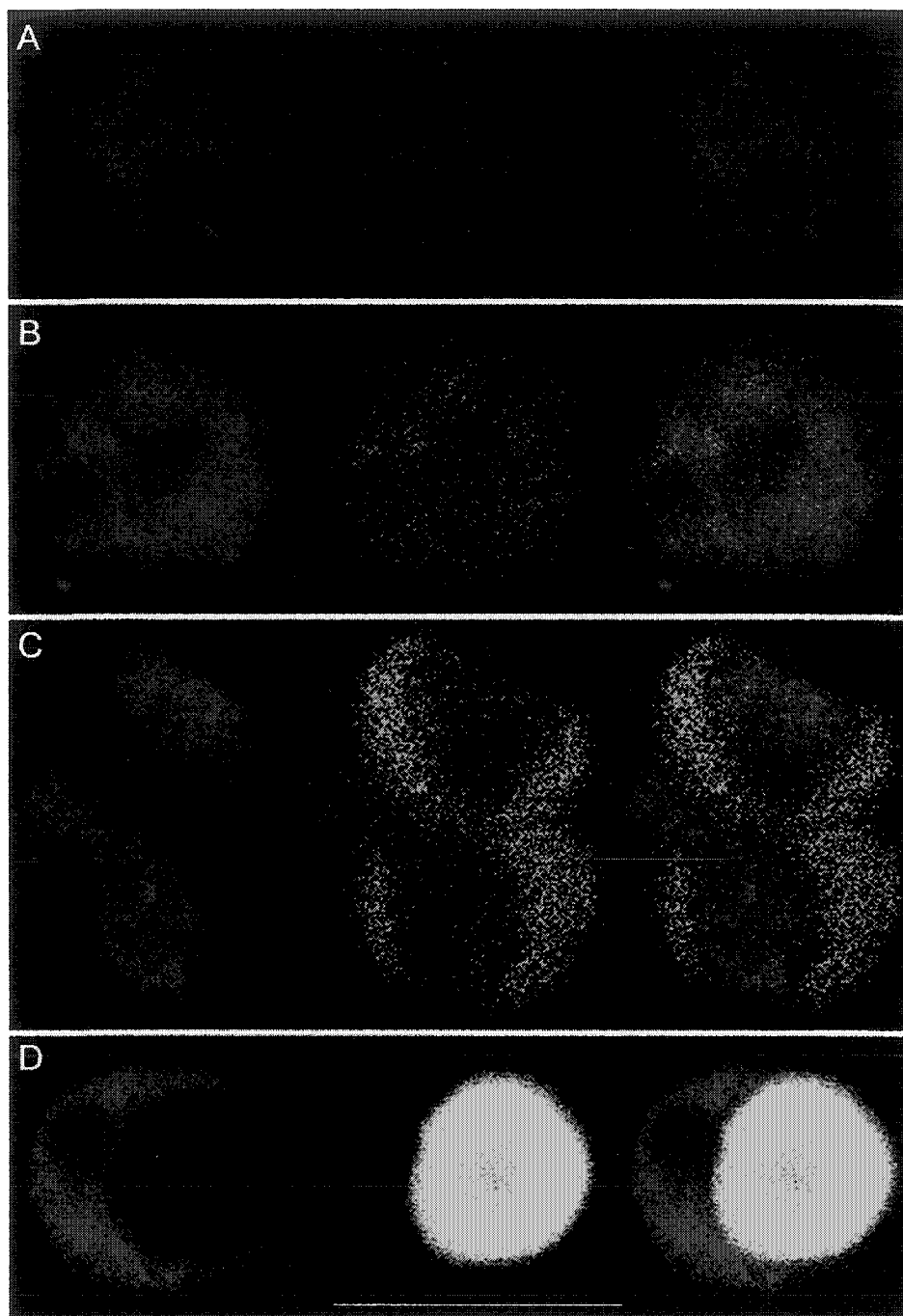


Figure 16 Relationship between SATB1 reorganisation and DNA cleavage in anti-Fas treated Jurkat cells

Human Jurkat T cells were immunostained to detect SATB1 (A-D, red signal), then subjected to in situ labelling of DNA breaks by TUNEL (A-D, green signal) immediately after isolation (A) or after treatment with 100ng/ml anti-Fas for 30 minutes (B) or 1 hour (C,D). Cells were examined using confocal microscopy. Each panel depicts a representative slice through a single nucleus. The SATB1 signal is displayed on the left, TUNEL signal in the middle and the overlay of the two channels on the right. Bar = 10 μm.

Loss of a portion of the SATB1 signal in Jurkat cells with low level TUNEL labelling was similarly noted (data not shown). Nuclei in which collapse of the chromatin had occurred featured relocation of SAT B1 to the remaining nuclear space and very bright to saturation-level labelling for DNA breaks (Figures 15D, 15E, 16C, 16D). Often, the mass of collapsed chromatin in these nuclei was most brightly labelled at its edges (Figure 15D, 16D), due to the limited ability of staining reagents such as streptavidin or antibodies to penetrate this structure completely (data not shown).

Considering together the results above, it is possible to define and order several of the steps involved in apoptotic nuclear collapse in thymocytes and Jurkat cells. While biochemical detection of the specific cleavage of nuclear matrix components NuMA, SAT B1 and lamin B accompanied accumulation of initial apoptosis-related DNA breaks detectable by agarose gel electrophoresis (Figures 5 and 6), morphological reorganisation of the nuclear matrix in apoptotic lymphocytes could not be detected in individual cells until after DNA cleavage had begun (Figures 15 and 16). Closely following initial DNA cleavage, a portion of the SAT B1 signal was lost from the nucleus, sometimes relocating to the cytoplasm (Figures 8, 9, 10, 14). More extensive cleavage, indicated by an increase in TUNEL labelling, was then observed (Figure 15). As cleavage progressed, collapse of the DNA, rearrangement of SAT B1 and NuMA into the remaining nucleoplasmic space and maximal TUNEL labelling for DNA breaks was detected concurrently (Figures 8-12, 14-16). Disappearance of the lamina followed this step, occurring almost simultaneously in the case of dexamethasone-treated thymocytes or as a distinctly later event in after VM-26 treatment

or in Jurkat cells. In contrast, the nuclear envelope proteins PI2 and lamin B receptor remained intact throughout the apoptotic response (Figures 13 and 14).

2.3 DISCUSSION

Apoptosis in lymphoid cell models, represented by both primary rat thymocytes and the human Jurkat T-cell line, was shown to involve rapid and consistent changes to both cytoplasmic structures (mitochondria and actin filaments, Figures 3 and 4) and the nucleus (DNA cleavage, nuclear matrix degradation and chromatin collapse, Figures 1,2,5,6). The time frame of the apoptotic response, as determined by each of these changes was remarkably consistent. A similar conserved time course of inducer-independent nuclear events in apoptotic lymphoid cells has previously been noted by others (Buendia et al., 1999) and my results concur with other studies of chromatin collapse in anti-Fas treated Jurkats (Taimen and Kallajoki, 2003). In my hands, the only exception to this pattern was seen in the Western blots for nuclear matrix proteins (Figure 6), where the intact form of NuMA in both thymocytes and Jurkats, and SATB1 protein in Jurkats was no longer detectable by the 2 hour time point, when only 20% of the cells were dead. This result suggested that biochemical cleavage of NuMA and SATB1 may precede chromatin collapse, LMW DNA cleavage, TUNEL labelling of DNA breaks, loss of filamentous actin and loss of mitochondrial function at the single cell level. The limitations posed by the temporal coincidence of events at the population level led to the examination of single cells, in order to better define the process of nuclear breakdown. Initial DNA cleavage preceded morphological reorganisation of the nuclear matrix in both thymocytes and Jurkats (Figures

15 and 16). It was not possible to correlate the initial low levels of TUNEL labelling seen with a particular phase of DNA cleavage, such as very HMW 300 kb - 1 Mb fragments, for example. However, it was clear that the technique labelled a class of break sites distributed throughout the nucleoplasm which were present in limited numbers, i.e. the abundance of these sites was well below saturation level labelling by TUNEL. Loss of a portion of the nuclear SATB1 signal, and its relocation to the cytoplasm in thymocytes preceded chromatin collapse (Figures 8,9,10,14), a novel observation, and was closely followed by an increase in TUNEL labelling (Figure 15). Reorganisation of NuMA, any remaining nucleoplasmic SATB1, chromatin collapse and saturation levels of TUNEL label were then detected simultaneously (Figures 8-12, 14-16), in accordance with others' results in Jurkat, BHK and HeLa cells and mouse spleenocytes (Sodja et al., 1997; Gueth-Hallonet et al., 1997; Gotzmann et al., 2000; Taimen and Kallajoki, 2003). Proteins of the nuclear rim showed more stable morphology (Figures 13 and 14), as observed by others in Jurkat, hepatoma and KE37 lymphoid cells (Duband-Goulet et al., 1998; Buendia et al., 1999; Taimen and Kallajoki, 2003).

2.3.1 Apoptosis-associated cleavage does not immediately disrupt the nuclear matrix structure Western blots clearly showed that cleavage of nuclear matrix proteins was detectable 30 minutes to 1 hour after the initiation of apoptosis, and that complete cleavage of NuMA and in some cases SATB1 was seen by 1-2 hours (Figure 6). In contrast, at the single cell level, many nuclei were identical to controls, with staining for NuMA, SATB1, lamin B and PI2 intact at the 2 hour time point. Reorganisation of NuMA was associated only with those cells that displayed chromatin collapse. Taken together, these observations

indicated that apoptosis-associated cleavage does not completely disrupt the interactions of nuclear matrix components with each other, in agreement with previous results from our lab and others (Weaver et al., 1996; Duband-Goulet et al., 1998; Buendia et al., 1999; Dynlacht et al., 2000). In the case of lamin B, apoptotic cleavage of the protein has been mapped to the hinge region upstream of the coil 2 domain, leaving a portion of the central rod domain intact (Rao et al., 1996). This coil 2 domain is necessary for formation of both lamin dimers and the lateral interactions that produce filaments (Goldberg et al., 1999). Thus, lamin polymerisation should not be disrupted by apoptosis-associated cleavage. Indeed, the lamin B 46kDA fragment remains insoluble in extracts prepared from apoptotic cells (Buendia et al., 1999). The cleavage site on NuMA lies at the junction of the central rod and tail domains, and also should not disrupt rod-mediated oligomerisation (Gueth-Hallonet et al., 1997).

The fact that both NuMA and lamin B are known to interact with MARs raises the possibility that it is the DNA binding capacity of the nuclear matrix that is affected by cell death-associated cleavage. Studies documenting the extent of nuclear matrix cleavage during apoptosis in lymphoid cells have shown that the proteins targeted for destruction, including NuMA, lamin B, LAP2, PARP, DNA topoisomerase I and II, and the Nup 153 protein of the nuclear filaments associated with pore complexes, all interact directly with the DNA. Many of those that remain intact, such as pore complex proteins p62 and gp210, Smith antigens and the nucleolar proteins B23 and fibrillarin do not contact DNA directly, although they may form part of complexes that interact with chromatin. This distinction is not absolute, however, as many DNA binding proteins are not targeted for proteolysis,

including PCNA, Ku, HP1, centromere proteins CENP A-C and histone H2B (Casiano et al., 1996; Buendia et al., 1999). Lamin B receptor shows another pattern, remaining intact until the very late stages of the apoptotic process, long after the cleavage of lamin B. In this case, it has been proposed that LBR may play a role in keeping the collapsed chromatin closely associated with the nuclear envelope while the cell disintegrates into small apoptotic bodies (Duband-Goulet et al., 1998).

2.3.2 Reorganisation of SATB1 is closely associated with initial changes in chromatin configuration and nuclear morphology The loss of a portion of the nuclear SATB1 signal and its relocation to the cytoplasm in thymocytes, prior to chromatin collapse and reorganisation of the other nuclear matrix antigens studied proved to be the most significant novel finding of this portion of the work. This observation in Jurkat cells has been published by our lab (Liu et al., 2003) and in thymocytes has been included in the manuscript of a collaborative study (Galande et al., 2001). Other studies of SATB1 in apoptosis have not examined early time points (before 2 hours) or nuclei that did not display chromatin collapse (Zweyer et al., 1997; Gotzmann et al., 2000), and so have not documented this phenomenon, which is most prominent at the 30 minute to 1 hour time points.

Early reorganisation of SATB1 was seen both in nuclei displaying a slight increase in the amount of condensed chromatin as well as those whose morphology was indistinguishable from controls (Figures 8A, 8B, 9A, 9B). Consequently, it could be postulated that this change promotes very early alterations in chromatin configuration and nuclear morphology in dying cells. Cleavage of SATB1 during apoptosis in Jurkat and HL-

60 cells has been described by others (Gotzmann et al., 2000) and a caspase 6 cleavage site mapped to the amino terminus, between the two known DNA binding domains and a PDZ domain involved in formation of dimers. The cleavage fragment of SATB1 is thus unable to dimerise. Surprisingly, although it still possesses its DNA binding domains, it is also incapable of binding DNA in vitro on a south western blot or in a gel shift assay (Galande et al., 2001). Thus, the SATB1 dimer represents the functional MAR-binding protein and cleavage of SATB1 will impact normal chromatin configuration, as its MAR-binding capacity is lost. Early morphological changes in apoptotic nuclei have also been described, although an appreciation of these subtle indicators of cell death has not entered into the dogma in this research area. Ultrastructural studies of U937 and glioma cells documented changes in nuclear morphology which preceded collapse of the chromatin. Nuclei were rounded and contained small accumulations of chromatin near their rim (Dini et al., 1996; Krajci et al., 2000). In the glioma cells, they often displayed a more distinct filamentous lamina and fibrous mesh associated with the nuclear pores than seen in controls, implying that the DNA normally associated with these structures no longer obscured their presence (Krajci et al., 2000). Altered nuclear morphology preceding chromatin collapse has also been noted in staurosporine-treated Jurkat cells, where it was most prominent at the 1 hour time point (Johnson et al., 2000).

These interpretations predict that other MAR-binding proteins should behave in an analogous manner to SATB1 during apoptosis, but the early reorganisation of SATB1 was distinct from the MAR binding proteins NuMA and lamin B also present in these cells. This difference may be due to the structural organisation of the proteins within the matrix. Lamin

B and NuMA are both incorporated into higher order structures, and exist as a meshwork of filaments in the interphase nucleus. MAR-binding proteins associated primarily with tissue-specific gene expression and not known to form filaments are more likely to behave in a SATB1-like manner. The ubiquitous protein SAF-A is another MAR-binding protein present in Jurkat cells. Caspase-mediated cleavage in its DNA binding domain during Fas-induced apoptosis produces a fragment which has no MAR-binding capacity, as is the case for the cleaved form of SATB1 (Gohring et al., 1997; Kipp et al., 2000). Its morphology early in the apoptotic response has not been studied. The fact that SAF-A forms filaments in the presence of DNA *in vitro* (Fackelmayer et al., 1994) indicates that its behaviour during apoptosis may resemble that of NuMA more than that of SATB1. Since apoptosis-associated cleavage disrupts the DNA binding activity of SATB1 (Galande et al., 2001), a portion of the protein may become soluble and display a change in morphology in the fixed and permeabilised cells used for microscopic examination. Early reorganisation of SATB1 did not involve all of the protein present in the nucleus. The portion remaining could represent intact protein, as has been detected in *in situ* matrix preparations from HL-60 cells (Martelli et al., 1999), and/or the remaining fragments of monomers that originally mediated interaction with the matrix, which have also been detected in nuclear matrices isolated from Jurkats (Gotzmann et al., 2000). I would also predict that blocking proteolysis of SATB1, for example by expression of a mutant lacking the cleavage site, should affect breakdown of the chromatin and interrupt the changes in nuclear morphology associated with cell death.

2.3.3 Roles of nuclear matrix breakdown in apoptosis The earliest events of nuclear breakdown detectable at the single cell level in my lymphocytic models were accumulation

of initial DNA breaks, reorganisation of a portion of SATB1 and increase in DNA cleavage detected by TUNEL (Figures 15 and 16). This sequence suggested that progressive DNA cleavage may depend on nuclear matrix degradation to provide access to the DNA, and that both proteolysis of nuclear matrix proteins and DNA cleavage are required for collapse of the chromatin and formation of apoptotic bodies. Indeed, a recent in vitro study demonstrated a requirement for intranuclear chromatin mobility for manifestation of DNA collapse in apoptotic nuclei (Widlak et al., 2002). The necessity of lamin cleavage in particular is also suggested by the redundant mechanisms for its proteolysis during apoptosis outlined in the introduction. A recent in vitro study showed that caspase 6-mediated cleavage of lamin A was required for proper chromatin condensation and apoptotic body formation in nuclei that express this isoform (Ruchaud et al., 2002). This hypothesis has also been directly tested in BRK cells, by expressing mutant lamins lacking a caspase cleavage site. The apoptotic response, as measured by membrane integrity and oligonucleosomal DNA cleavage, was delayed (but not prevented) in cells expressing the mutant lamins and collapse of the chromatin in these cells was impaired. At early time points, nuclei were convoluted, with DNA lining the periphery (Rao et al., 1996), morphology resembling early nuclear changes in apoptotic cells (Dini et al., 1996; Krajci et al., 2000). In contrast, lamin breakdown does not appear to be necessary for cell death in *C. elegans*. In this organism, lamin cleavage follows DNA fragmentation during apoptosis and was not mediated by CED-3, its sole caspase (Tzur et al., 2002).

Evidence also suggests that changes in the nuclear lamina can initiate an apoptotic response. In cycling fibroblasts and lymphocytic cells, reassembly of lamin B after mitosis

was specifically blocked by expression of a peptide containing a PP1 binding domain, which sequestered the phosphatase that mediates the dephosphorylation of B lamins necessary for their assembly at the nuclear envelope following cell division. In the absence of a normal lamina the cells died by apoptosis within 6 hours (Steen and Collas, 2001). The authors did not determine how the cell death signal was propagated, but did show that lamins A/C expression could be induced by the absence of Lamin B. Thus, it seems that a mechanism involving altered gene expression is likely (reviewed in (Burke, 2001). This result also provides a glimpse at the possible chain of events in disease states associated with mutant lamin genes, namely muscular dystrophies, lipidodystrophy and cardiomyopathies associated with lack of a functional lamin A/C gene. It has been proposed that loss of specific chromatin attachment sites on the lamina which are needed to establish or maintain patterns of gene expression in non-proliferating cells gives rise to the disease phenotype (reviewed in Worman and Courvalin, 2000; Cohen et al., 2001). The work of Steen and Collas shows that abnormalities in the lamina may indeed signal cell death.

Finally, it should be noted that cleavage of the DNA and collapse of the nucleus are of vital importance to the safe clearance of apoptotic cells in situ. Fragmentation of nuclei enables phagocytosis of apoptotic bodies by neighbouring cells, thus ensuring that their intracellular contents are not released. Less than optimal disposal of “cell corpses” can lead to autoantibody production, often associated with systemic autoimmune disease such as lupus erythematosus (reviewed in Martelli et al., 2001). A plethora of autoantibodies reactive with components of the nuclear matrix have been documented in the literature (Casiano et al., 1996 , for example) and serve as useful tools for this branch of cell biology

(including the anti-lamin B receptor antibody used for these studies). These autoantibodies typically recognise the cleaved fragment of proteins degraded during the apoptotic response, indicating that they likely arose from exposure to dying cells (Martelli et al., 2001). DNA cleavage, in turn, prevents the lateral transfer of genes to the phagocytosing cells. This is a very real danger; lateral transfer of both reporter genes and endogenous oncogenes from apoptotic cells to their phagocytes has been documented. In stable prostate cancer cell transfectants expressing distinct antibiotic resistance markers, coculture of cells was shown to give rise to colonies with dual resistance if one of the cell lines had been treated to induce apoptosis. Southern blot analysis of RFLPs showed that this “apoptotic conversion” involved transfer of genetic material from the dying cells into the healthy cells (which would have acted as phagocytes) only (de la Taille et al., 1999). Similarly, transfer of Epstein-Barr virus and HIV DNA via uptake of apoptotic bodies has been noted (Spetz et al., 1999; Holmgren et al., 1999). Lateral transfer of DNA may also play a role in the acquisition of multiple genetic alterations in tumours. Horizontal transfer of ras and myc oncogenes from apoptotic bodies which rendered the recipient cells capable of both focus formation in vitro and tumour formation in vivo has recently been described (Bergsmedh et al., 2001).

CHAPTER 3 : RELATIONSHIP BETWEEN EARLY NUCLEAR EVENTS AND MITOCHONDRIAL CHANGE IN APOPTOTIC JURKAT CELLS

In recent years, much research interest has focussed on cytoplasmic markers of apoptotic cell death. Transmission of the apoptotic signal from death receptors to mitochondria, and signalling from this organelle to downstream caspases have been prominent topics. Loss of mitochondria function appears to be an early event of the apoptotic response, preceding oligonucleosomal DNA fragmentation (Sartorius et al., 2001; Wang, 2001; Creagh and Martin, 2001).

Cell death receptors have been shown to signal through domain-based recruitment of both adaptor molecules and procaspases, which form the Death Inducing Signalling Complex (DISC) (Tartaglia et al., 1993; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). Autoproteolytic activation of the initiator procaspases in the DISC may result either in direct initiation of a proteolytic cascade of effector caspase activation, or transmission of the death signal to the mitochondria, via cleavage-based activation of Bid, a pro-apoptotic member of the Bcl-2 family (Medema et al., 1997; Luo et al., 1998; Scaffidi et al., 1998; Li et al., 1998; Gross et al., 1999; Chen and Wang, 2002).

Mitochondria have been shown to play several important roles in cell death. Loss of mitochondrial function, as detected by loss of the mitochondrial transmembrane potential across the inner mitochondria membrane, appears to be a common event in the cell death pathway (Castedo et al., 1995; Petit et al., 1995; Zamzami et al., 1995a; Zamzami et al., 1995b; Pastorino et al., 1996). Indeed, it has been proposed that this permeability transition (PT) of mitochondria serves as the initial event of the conserved

execution phase of apoptosis, and results from the role of mitochondria as central sensors of signals for cell death (Marchetti et al., 1996; Susin et al., 1997; Desagher and Martinou, 2000). However, it appears that mitochondrial permeability transition can also be a later step in some cell types, where it serves simply as an amplification loop of the death signal (Kluck et al., 1997; Yang et al., 1997a; Zhuang et al., 1998). Closely associated with mitochondrial PT is the release of a number of molecules implicated in the apoptotic response from the mitochondrial intermembrane space: AIF, SMAC/DIABLO, Omi/HtrA2, endonuclease G and cytochrome c are all sequestered in the mitochondria of healthy cells and function in the execution phase of apoptosis (Liu et al., 1996; Susin et al., 1999; Du et al., 2000; Verhagen et al., 2000; Li et al., 2001; Suzuki et al., 2001; Hedge et al., 2002). The mechanism responsible for release of these molecules remains to be established; both rupture of the outer mitochondrial membrane and pore-based mechanisms have been proposed, and the relationship between loss of mitochondrial function and the release of cytochrome c and AIF in particular has not been definitively established (Desagher and Martinou, 2000; Bernardi et al., 2001; Scorrano and Korsmeyer, 2003). Finally, mitochondria serve as important sites for the action of the bcl-2 family of proteins. The mechanism of action of bcl-2 family members and their role(s) in cytochrome c release are current topics of intense study in this field (Adams and Cory, 2001; Martinou and Green, 2001; Tsujimoto, 2003). Proapoptotic molecules released from the intermembrane space link mitochondria to effector caspase activation. Caspases 3, 6 and 7 have been quite clearly implicated as ubiquitous “executioners” (Martins et al., 1997; Faleiro et al., 1997), responsible for the proteolytic cleavage of many death-associated substrates in cell-free

assay systems (Slee et al., 2001), cultured and primary cell model systems (Greidinger et al., 1996; Casciola-Rosen et al., 1996; Hirata et al., 1998), and implicated in developmental programmed cell death in vivo in mice (Kuida et al., 1996). Cytochrome c, through its interactions with the Apaf-1 adaptor protein and nucleotide, is instrumental in formation of the “apoptosome” complex that recruits and activates procaspase 9, which in turn is responsible for the activation of caspase 3 and the other effector caspases (Zou et al., 1997).

Loss of mitochondrial function in apoptotic cells was found to be an early event of the apoptotic response, preceding accumulation of reactive oxygen species, loss of plasma membrane asymmetry and integrity and decrease in cell size (Petit et al., 1995; Zamzami et al., 1995a; Zamzami et al., 1995b; Castedo et al., 1996; Goldstein et al., 2000). In cells with type II signalling from death receptors, in programmed cell death necessary for normal neuronal development and during apoptosis induced by chemotherapeutic drugs, loss of $\Delta\psi_m$ preceded activation of effector caspases (Scaffidi et al., 1998; Yoshida et al., 1998; Cecconi et al., 1998). It also clearly preceded the final step of DNA breakdown. In both dexamethasone-induced thymocytes and lymphocytes, loss of $\Delta\psi_m$ was a more sensitive measure of apoptosis than accumulation of DNA breaks visualised by nick-end labelling or oligonucleosomal cleavage of DNA (Petit et al., 1995; Zamzami et al., 1995b).

Identification of the caspase 3-activated DFF/CAD and endonuclease G as enzymes capable of oligonucleosomal DNA degradation in apoptotic cells provided both caspase-dependant and -independent links (respectively) from the release of mitochondrial apoptogenic factors to the end stage of DNA degradation (Liu et al., 1997; Enari et al.,

1998; Li et al., 2001). In addition, characterisation of AIF revealed a caspase-independent link between the apoptotic increase in mitochondrial permeability and generation of 50 kb DNA fragments in the DNA of dying cells (Susin et al., 1999; Daugas et al., 2000b). However, none of these enzymes are responsible for the initial very HMW (300 kb-1Mb) and single strand DNA cleavage events of apoptosis.

To address the question of whether a mitochondria-based signal could initiate the nuclear events of apoptosis, I have made use of Jurkat T cells, a well-described model system in which the cytoplasmic events of cell death and the importance of mitochondrial PT in the effector phase of apoptosis have been documented (Scaffidi et al., 1998). Activation of effector caspases in these cells has been shown to follow loss of mitochondrial membrane potential in response to several inducers of apoptosis (Susin et al., 1997; Scaffidi et al., 1998; Gamen et al., 2000). The asynchronous nature of the apoptotic response in all known model systems, coupled with the tight temporal relationship of the events of cell death limit biochemical study of the process of apoptosis; many events appear to occur simultaneously when studied at the population level, and the relationships between them cannot be clearly discerned. Thus, it was important to examine apoptotic events in single cells. Since mitochondrial function is typically assayed by uptake of potential-sensitive dyes in live cells, and either in situ labelling of DNA breaks or immunostaining for nuclear antigens requires samples that are fixed and permeabilised, it was not possible to simply assay for these parameters in double-labelled cells. As an alternative, expression of fluorescent-tagged proteins implicated in early and later stages of nuclear reorganisation (i.e. SATB1 and NuMA, respectively) could be employed to

examine the relationship between alterations in protein distribution, nuclear morphology and $\Delta\Psi_m$ simultaneously at the single cell level. However, this approach does not allow biochemical detection of early nuclear events (such as ssDNA and nuclear matrix cleavage) or examination of DNA breaks relative to loss of mitochondrial function. Thus, flow cytometric analysis of live cells labelled to detect mitochondrial function was used to examine a population on a cell-to cell basis, and early apoptotic cells were isolated from the population as a whole by cell sorting. Nuclear events were then examined separately in those cells that still appeared normal. Using this approach, changes in nuclear morphology, initial DNA cleavage and reorganisation of the MAR-binding protein SATB1 were shown to precede loss of mitochondrial function, cell shrinkage and loss of plasma membrane integrity in anti-Fas treated Jurkat cells.

3.1 MATERIALS AND METHODS

3.1.1 Cell culture and treatment Jurkat cells were maintained and death induced as described in section 2.1.1.

3.1.2 Flow cytometric assessment of mitochondrial function in early apoptotic cells

Early apoptotic cells were defined as those displaying normal size and an intact plasma membrane capable of excluding the vital dye propidium iodide. Mitochondrial function was monitored by uptake of the potential-sensitive dye DiOC₆₍₃₎. 1-2 x 10⁶ live cells per sample were incubated for 15 minutes at 37 °C in the dark in PBS containing 1% BSA, 40 nM DiOC₆₍₃₎ (from a freshly-made 40 µM working solution and 4 mM stock, both in DMSO) and 5 µg/ml propidium iodide (from a 1 mg/ml stock). Samples were kept on ice

and analysed in the dark on a Coulter Epics Elite flow cytometer, using a 488 nm excitation wavelength and 525 (+/-5) nm and 610 (+/-5nm) band pass filters to detect DiOC6 and PI, respectively. Staining patterns were typically stable for 90-150 minutes, and samples were routinely analysed within 1 hour to avoid any deterioration of the signals. For colour compensation controls with no dye, DiOC6 only and propidium iodide only (the latter with 0.1% TX-100 added to ensure a strong positive signal) were used. Any signal at 610 nm in the DiOC6 control and any signal at 525 nm in the PI control were electronically reduced until they matched the background levels seen the unstained control sample. A minimum of 10,000 events per sample were recorded.

3.1.3 Sorting of subpopulations from Jurkat cultures For cell sorting, room temperature was equilibrated to 19°C overnight and the cell sorter allowed a minimum warm up time of 30 minutes to stabilise the frequency of the vibrations which generated the break off point of the sample stream. The system alignment and sort settings were adjusted using fluorescent beads, and the sorter programmed to generate the strictest conditions possible: one drop per sort event and rejection of any drops containing more than one particle. Sorting was tested using the fluorescent beads and conditions fine-tuned to produce a minimum sorting efficiency of 97%, stable over a two hour period. For sorting, samples containing 10^7 cells, stained with DiOC6 and PI as described above, were kept in the dark on ice and sorted subpopulations were collected in the dark in tubes containing ice-cold HANKS + 2% BSA. Sorted cells were kept on ice and processed within 1 hour. For each sample, the staining pattern at the beginning of the sort was recorded and monitored every 2-5 minutes throughout the sort period, along with the droplet breakoff

point. In response to any change in droplet breakoff or a shift of more than 2% in subpopulations detected by staining, the sort was aborted and collection tubes containing any suspect sorting events discarded.

3.1.4 Examination of nuclear morphology For microscopic examination of nuclei, sorted cells were collected by centrifugation for 5 minutes at 1200 RPM at 4 °C and allowed to settle onto poly-l-lysine coated coverslips for 5 minutes at 4 °C. Cells were rinsed briefly in ice-cold PBS, then fixed, permeabilised and the DNA stained as described in section 2.1.8. Cells were examined and recorded as per section 2.1.10. For each time point, 400 cells in each of duplicate samples was scored as displaying normal, “altered” (shrunken, with some increase in condensed chromatin) or collapsed nuclei (chromatin margined or in apoptotic bodies).

3.1.5 Microscopy of live, sorted DiOC6-stained cells Jurkat subpopulations isolated by sorting were collected by centrifugation at 4 °C, an aliquot of cells was placed on a microscope slide along with a drop of liquid mounting medium containing anti-fade reagent and a coverslip was lowered gently into place over the sample. Cells were examined on an Olympus BX50 fluorescence microscope for no more than 10 minutes and images captured using Northern Exposure software. No change in the staining pattern of the cells was detected during this time frame. Images were processed using Adobe Photoshop.

3.1.6 *In situ* labelling of DNA breaks Sorted cells were collected by centrifugation, attached to coverslips, fixed and permeabilised as described for examination of nuclear morphology above. DNA breaks were labelled as described in section 2.1.3.

3.1.7 Two-dimensional gel electrophoresis of DNA As for PFGE (section 2.1.6), cells were rinsed in nuclear buffer, embedded in low melting point agarose plugs and proteins digested. Plugs were then loaded onto 0.8% agarose gels. A maximum of two samples per gel were run along with λ phage DNA (50 -1 Mb) marker, using a Bio-Rad FIGE mapper and established conditions (Walker et al., 1999). Gels were stained and recorded as per 2.1.6, trimmed at the bottom, rotated 90 °CW in the tray, and DNA strands separated by soaking for 1 hour in alkaline buffer (50 mM NaOH, 1 mM EDTA) at 4°C while rocking gently. Conventional agarose gel electrophoresis was then carried out for 2 hours at 150V in cold alkaline buffer, gels were washed twice for 15 minutes each time in water and finally stained for 1 hour in ethidium bromide, before destaining and image recording.

3.1.8 Western blotting for SATB1 Cellular proteins from sorted cells were extracted and blots produced as described in 2.1.7. SATB1 was detected using rabbit polyclonal antibody 1:5000 (Dickinson et al., 1992) and alkaline phosphatase-conjugated donkey anti-rabbit IgG (1:5000).

3.1.9 Immunostaining of nuclear antigens Sorted cells were collected by centrifugation, attached to coverslips, fixed and permeabilised as for examination of nuclear morphology. Immunostaining was carried out as in 2.1.8. Antibodies to SATB1, lamin B and NuMA were used as described in 2.1.9.

3.2 RESULTS

For experiments probing the relationship between mitochondrial and nuclear events

in cell death, human Jurkat cells induced to undergo apoptosis by treatment with 100 ng/ml anti-Fas antibody were used as a model system. Flow cytometry and cell sorting was first employed to isolate early apoptotic cells, as defined by cytoplasmic criteria, from anti-Fas treated Jurkat cultures. Nuclear events, including changes in morphology, initial DNA cleavage and reorganisation of the nuclear matrix were then examined in the isolated subpopulations.

3.2.1 Identification and isolation of early apoptotic Jurkat cells Jurkat cells respond in a stochastic manner when induced to die by exposure to anti-Fas (Figures 1-4). In order to study nuclear changes in a uniform subpopulation, multiparameter flow cytometric analysis of cells labelled to detect cytoplasmic changes was employed to define early apoptotic cells within the population of dying Jurkats (Figure 17). Jurkats were incubated with both DiOC₆₍₃₎ to label cells with functional mitochondria, and propidium iodide to label cells which had lost their plasma membrane integrity. The staining patterns of normal size and small size cells were evaluated independently. Early apoptotic cells were defined as those that appeared intact by all three criteria: size, mitochondrial function and plasma membrane integrity. Figure 18 displays the subpopulations detected by this strategy in controls (A-A'') and in cultures exposed to 100 ng/ml anti-Fas for 1 hour (B-B''), 4 hours (C-C'') or 5 hours (D-D''). During the course of treatment, most of the cells retained a normal size profile, although a slight downward shift of the normal size population was noticed (Figures 18A-18D). A minute increase in the separate population of smaller cells was detectable at the 4 hour time point (Figure 18C) and became

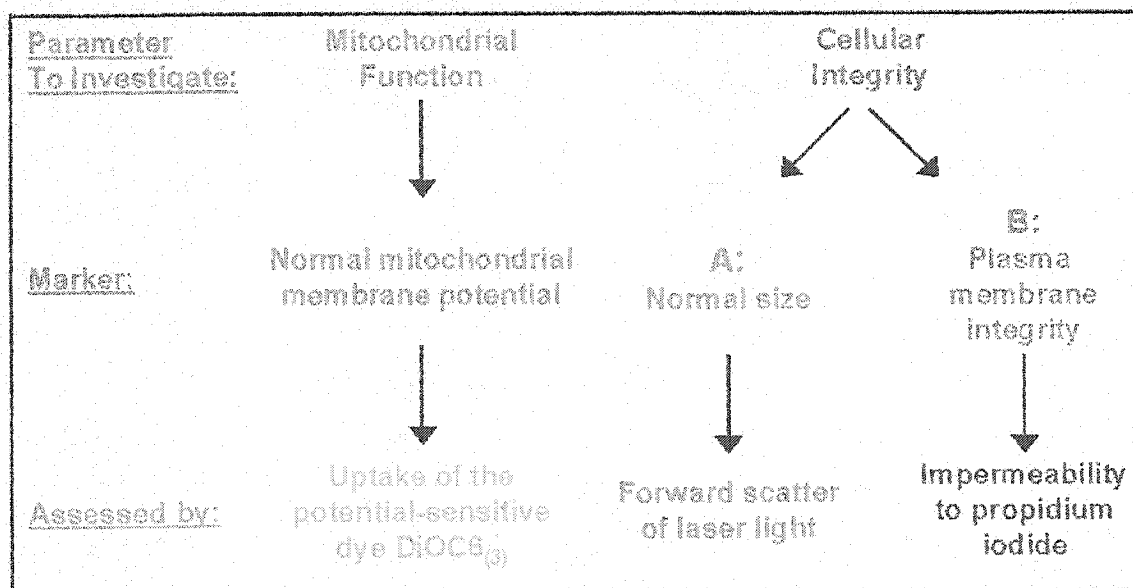


Figure 17 Multiparameter flow cytometric detection of early apoptotic Jurkat cells
 Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and at time points up to 4 hours early apoptotic cells within the induced population were identified based on the three parameter profile for live cell markers outlined above.

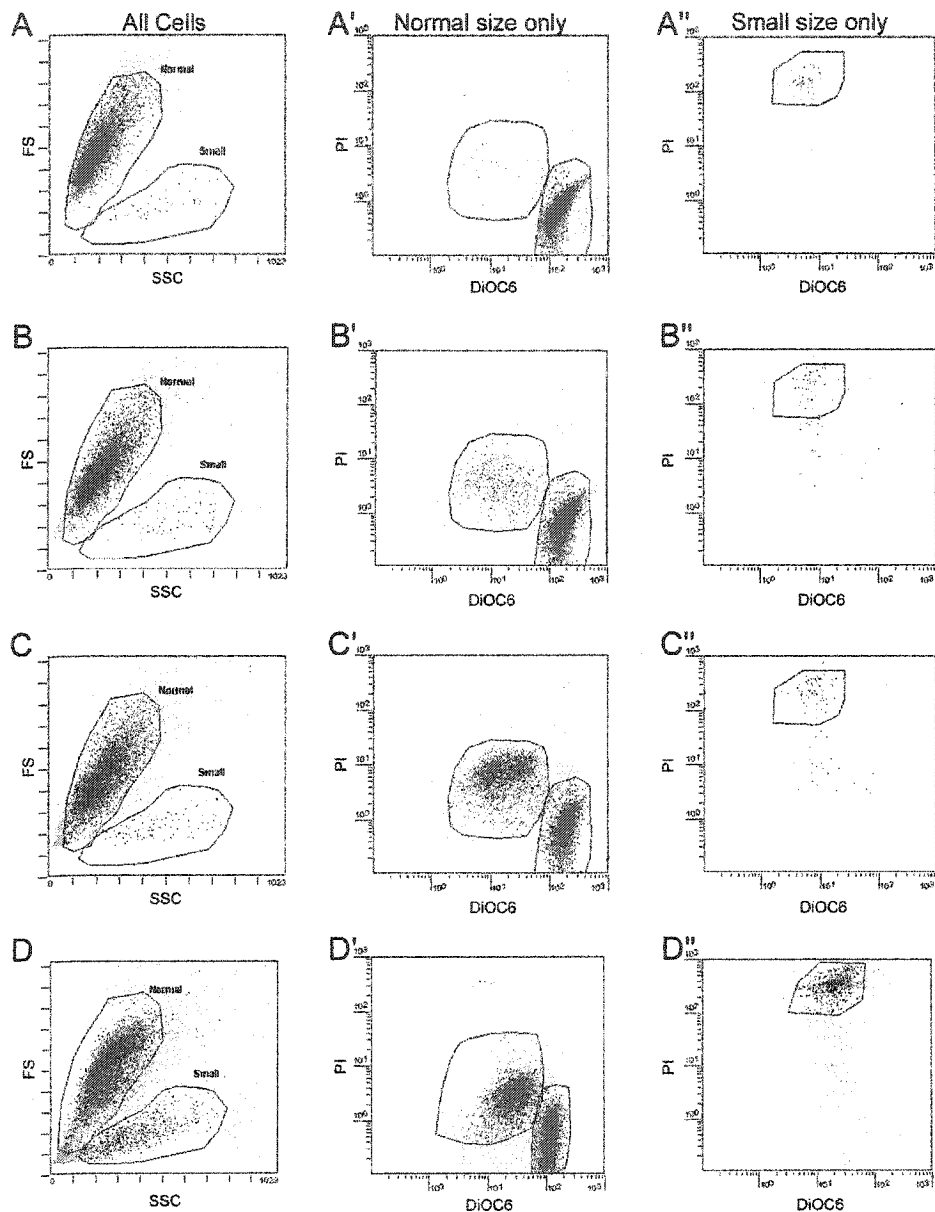


Figure 18 Identification of three subpopulations in cultures of anti-Fas treated Jurkat cells

Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and live cells from untreated controls (A,A',A''), or 1 hour (B,B',B''), 4 hour (C,C',C'') and 6 hour (D,D',D'') treated cultures were examined by flow cytometry. The distribution of cells based on size (as measured by forward scatter of laser light, FS) and granularity (as measured by side scatter of laser light, SSC) was determined for each sample (A-D), and then the mitochondrial membrane potential (as measured by uptake of the dye DiOC6) and plasma membrane integrity (as measured by exclusion of propidium iodide, PI) were assessed independently in the normal-sized (A'-D') and small-sized (A''-D'') cells. Based on these criteria, apparently normal cells (normal size, normal mitochondrial membrane potential and intact plasma membrane, indicated in blue) could be distinguished from both apoptotic (normal size, low mitochondrial membrane potential, compromised plasma membrane, indicated in red) and dead cells (small size, low mitochondrial membrane potential, permeable plasma membrane, indicated in black).

more pronounced if the treatment was extended to 5 hours (Figure 18D). The normal size cells contained two subpopulations (Figures 18A'-18D'). In controls, virtually all of them displayed low PI and high DiOC6 staining, indicating they had functional mitochondria and intact plasma membranes (right hand region, blue). With increasing time in the presence of anti-Fas, the proportion of these "apparently normal" cells decreased (Figure 19) and a second subpopulation appeared (left hand region, red). Although normal size, these cells showed a marked decrease in staining by DiOC6, indicating loss of mitochondrial membrane potential, accompanied by a small increase in propidium iodide staining. They were not completely permeable to PI; their uptake remained 100 fold less than that seen in the smaller, dead cells present in the population (Figures 18A''-18D'') and they did not have the bright PI staining typical of permeable cells when examined under the microscope (data not shown). These cells were considered "apoptotic". They accounted for a negligible proportion of control cultures, but just over 20% of the anti-Fas treated samples by 2 hours and close to 40% of the population at the 4 hour time point (Figure 19). The smaller size cells contained a third subpopulation that had lost mitochondrial membrane potential and were completely permeable to propidium iodide, designated "dead" cells. There was no significant accumulation of these cells during the first 4 hours of anti-Fas treatment (Figure 19); they became more frequent at later time points (Figure 18D'').

Having thus defined early apoptotic or "apparently normal" cells, I used cell sorting to separate this subpopulation from the other, overtly dead cells present in the treated cultures. Sorting effectively selected for the "apparently normal" cells in the mixed culture,

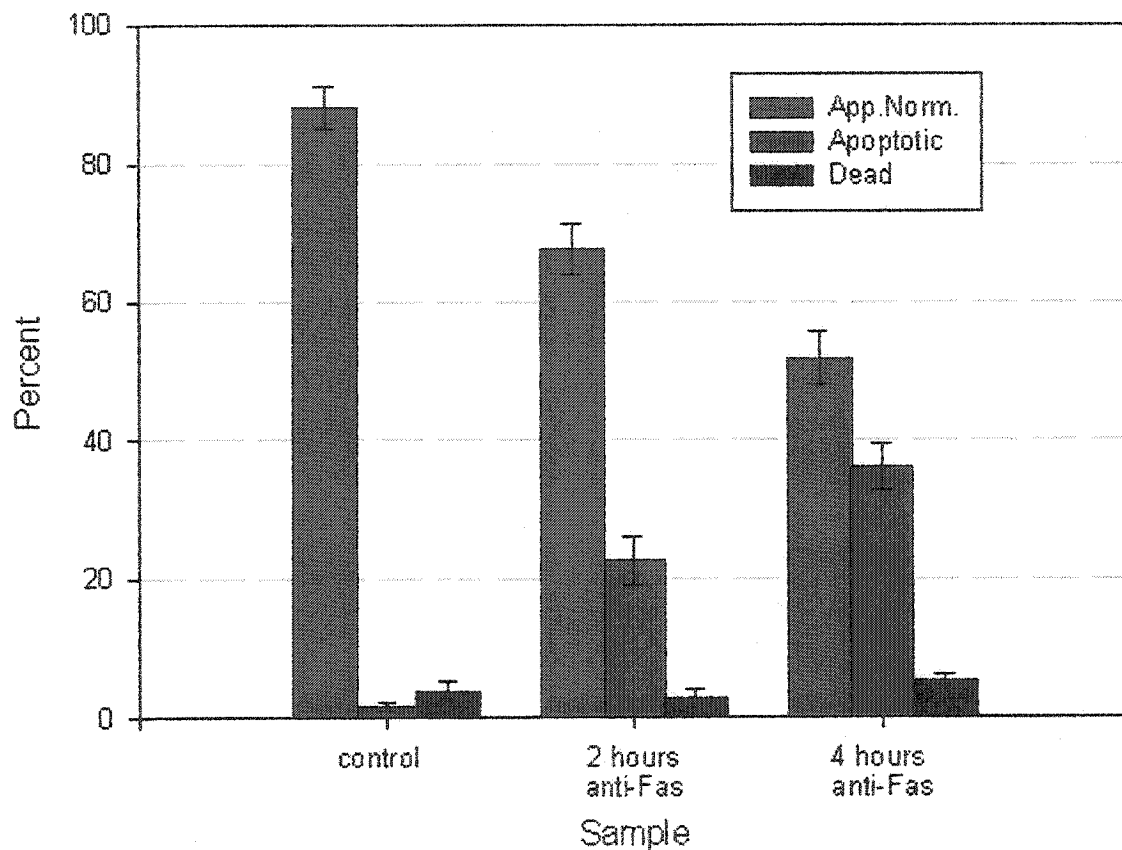


Figure 19 Distribution of subpopulations in apoptotic Jurkat cell cultures

Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and the proportion of apparently normal cells (normal size, normal mitochondrial membrane potential and intact plasma membrane, indicated in blue), apoptotic cells (normal size, low mitochondrial membrane potential, compromised plasma membrane, indicated in red) and dead cells (small size, low mitochondrial membrane potential, permeable plasma membrane, indicated in black) present in the population at time points up to 4 hours were monitored using flow cytometry.

as demonstrated in Figures 20 and 21. The size profile of the sorted “apparently normal” cells (Figures 20A’-20C’), showed that normal size cells were enriched from their already high proportion of the population as a whole (Figures 20A-20C). At the 1 hour time point normal size cells made up 96% of the whole population, while at 4 hours they accounted for 94% of cells. In “apparently normal” subpopulations sorted at these time points, normal size cells account for virtually the entire population. The proportion of small cells was reduced concurrently (Figures 20B, 20B’, 20C, 20C’). The “apparently normal” subpopulation displayed a tight size distribution identical to controls; sorting effectively removed those cells that accounted for the slight downward shift in normal population size seen in the whole population (compare Figure 20C’, 20A and 20C). In the whole population, an increasing proportion of the normal size cells became “apoptotic” after anti-Fas treatment, displaying loss of mitochondrial membrane potential (Figures 21A-21C). In the “apparently normal” subpopulations collected from these samples, almost all the cells had high DiOC6 staining, and low PI. Even with the region defining the “apoptotic” cells increased in size to maximize the estimation of error, less than 2.5% of cells in the sorted subpopulation fell into this category (Figures 21B’, 21C’). Taken together, Figures 20 and 21 also demonstrate that deterioration of the live cells in the sorted subpopulations did not occur under the strictly controlled conditions of temperature and collection time used for sorting.

3.2.2 Apoptotic Jurkat cells display initial changes in nuclear morphology before loss of mitochondrial membrane potential The nuclear morphology of sorted Jurkat cell subpopulations was examined in Hoechst- or DAPI-stained samples, and the distribution

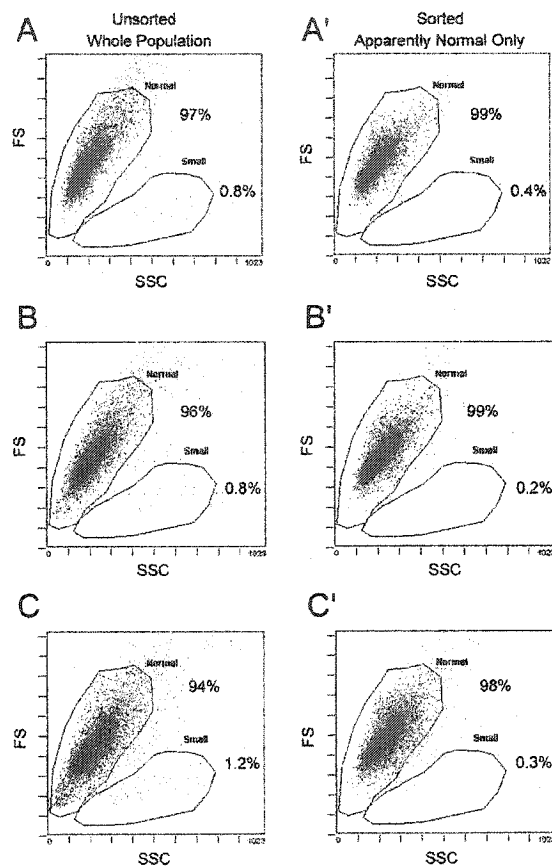


Figure 20 Sorting of "Apparently Normal" cells from a apoptotic Jurkat populations selects for cells of normal size

Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and live cells from untreated controls (A,A'), and both 1 hour (B,B') and 4 hour (C,C') treated cultures were examined by flow cytometry. The distribution of cells based on size (as measured by forward scatter of laser light, FS) and granularity (as measured by side scatter of laser light, SSC) was determined for both the entire population (A-C) and for the "apparently normal" cells separated by cell sorting (A'-C').

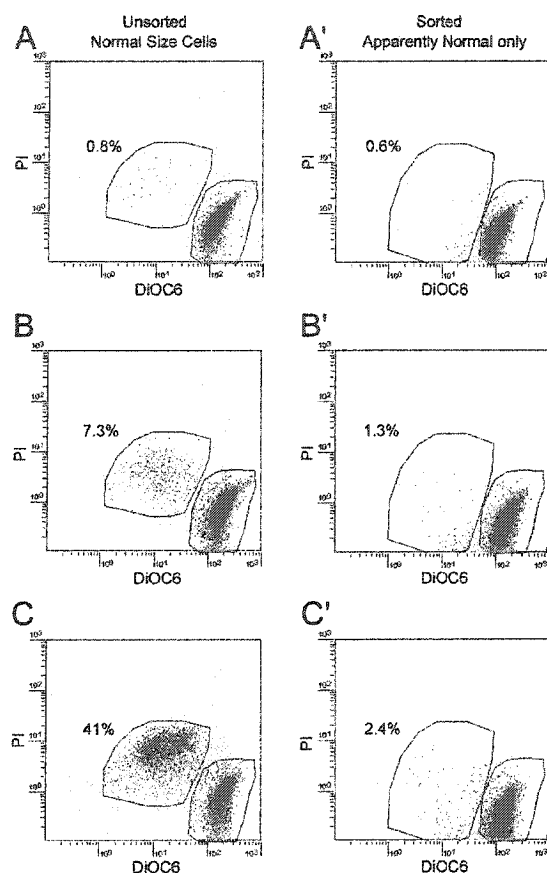


Figure 21 Sorting of "Apparently Normal" cells from a apoptotic Jurkat populations selects for cells with normal mitochondrial function and intact plasma membrane

Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and live cells from untreated controls (A,A'), and both 1 hour (B,B') and 4 hour (C,C') treated cultures were examined by flow cytometry. Mitochondrial membrane potential (as measured by uptake of the dye DiOC6) and plasma membrane integrity (as measured by exclusion of propidium iodide, PI) were assessed for both the normal-sized cells in whole population (A-C) and for the "apparently normal" cells separated by cell sorting (A'-C'). The left hand region which delineates the "apoptotic" subpopulation in A-C was expanded in A'-C' in order to maximise the estimation of sorting error.

of cells with distinct nuclear morphologies was assessed by cell counts (Figures 22-24). “Apparently normal” cells isolated from control cultures were round, with a limited cytoplasmic compartment in which small vacuoles were often visible (Figure 22A’, two upper cells; Figure 22B’, two middle cells). The bulk of the cell volume was occupied by the round nucleus (Figures 22A and 22B). The majority of the “apparently normal” cells isolated from anti-Fas treated Jurkat cultures were indistinguishable from healthy controls (Figures 22C’ and 22D’); there was no change in their nuclear morphology (Figures 22C, 22D). However, a minority of cells in this subpopulation had an “altered” nuclear morphology: small nuclei with increased areas of condensed chromatin (Figures 22C and 22D, arrows). This phenotype was distinct from both controls and apoptotic nuclei with collapsed chromatin. These “altered” cells often displayed higher contrast than their neighbours with normal nuclear morphology when viewed under phase contrast optics (Figures 22C’ and 22D’, arrows). In both the sorted “apoptotic” and “dead” subpopulations, chromatin collapse typical of apoptosis was detected in virtually all nuclei. “Apoptotic” nuclei featured marginated chromatin masses, large, single spheres of collapsed chromatin and classical apoptotic bodies (Figures 23A’ and 23B’). In the “dead” subpopulation, nuclei most frequently contained one or several small apoptotic bodies (Figure 23 C’), in keeping with their smaller size profile (Figures 18 and 23C). Both these subpopulations displayed very high contrast compared to healthy Jurkats when viewed using phase optics (Figures 23A-23C). Counts were used to monitor the distribution of cells with “altered” nuclear morphology and collapsed chromatin in the sorted subpopulations of Jurkats (Figure 24). In “apparently normal” cells isolated from control cultures, over 95% of nuclei

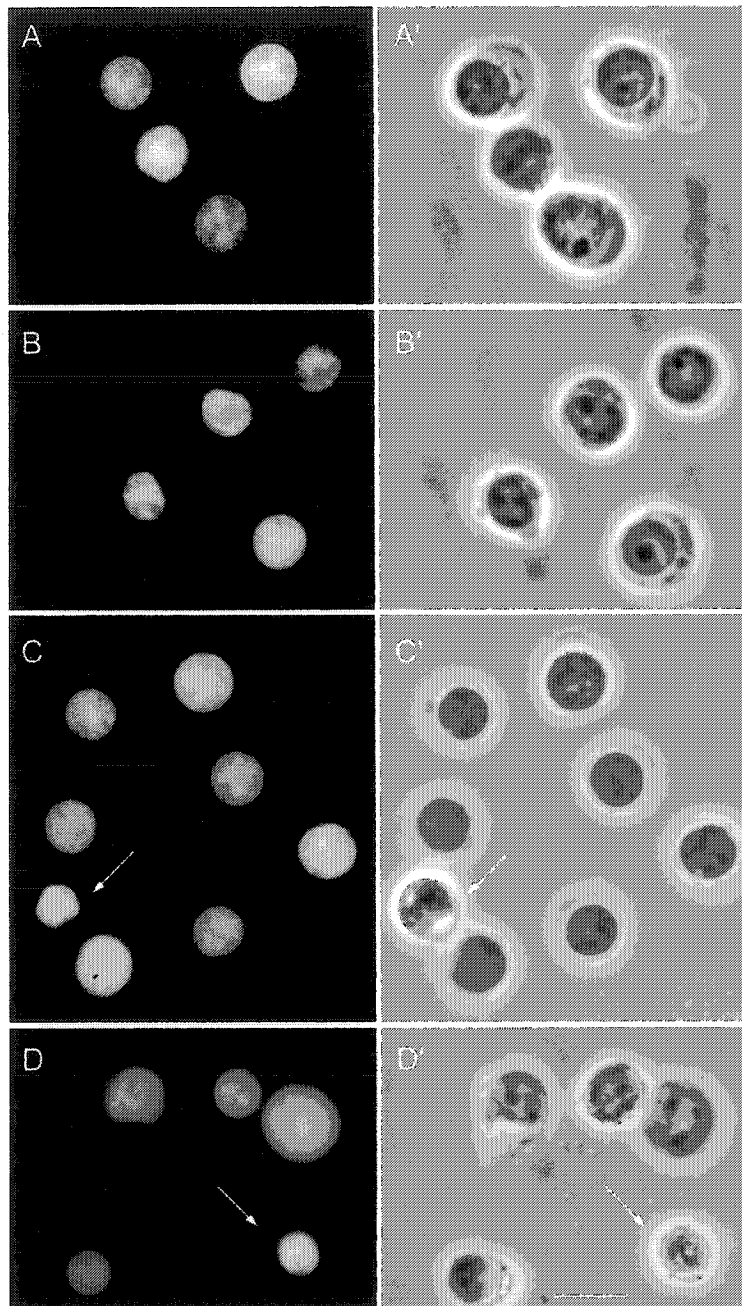


Figure 22 Nuclear morphology of sorted "apparently normal" cells
 Human Jurkat T cells were stained with DAPI to reveal nuclear morphology (A-D) and examined using phase contrast optics to detect cell size and shape (A'-D') of sorted "apparently normal" cells isolated from untreated control cultures (A,A',B,B') and from cultures induced to die by 2 hours exposure to 100 ng/ml anti-Fas (C,C',D,D'). Arrows indicate nuclei displaying decreased size and an increase in condensed chromatin. Bar = 10 μ m.

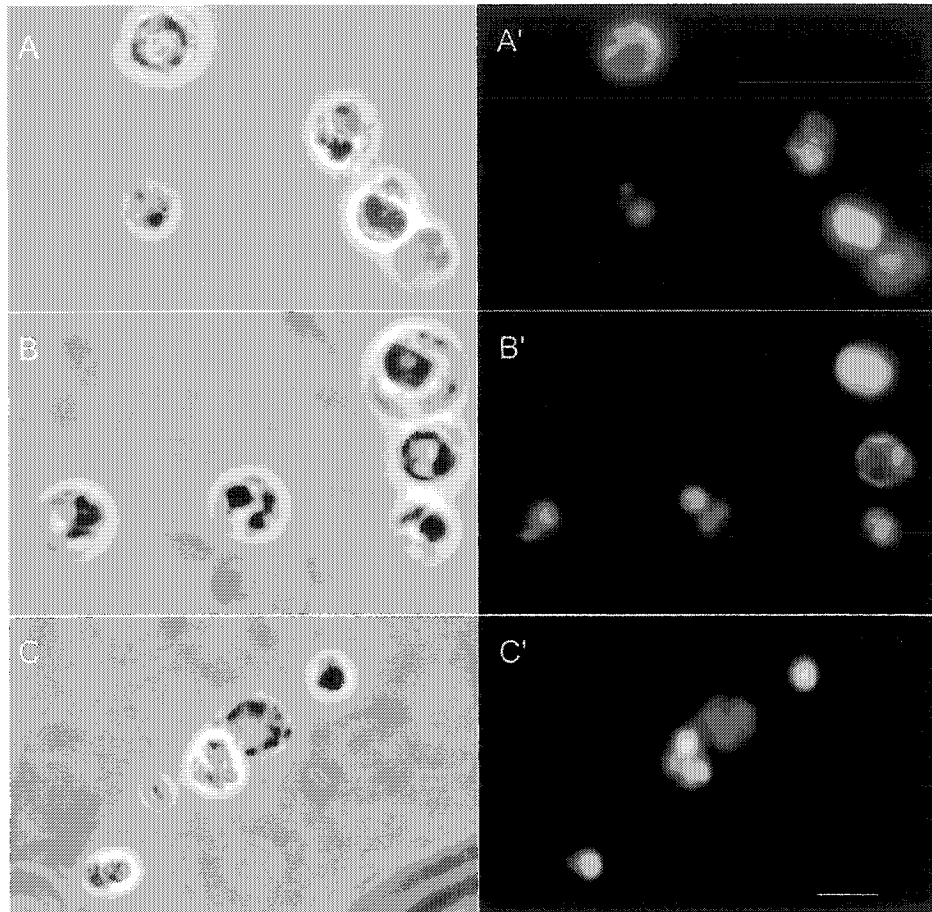


Figure 23 Nuclear morphology of sorted “apoptotic” and “dead” cells
 Human Jurkat T cells were examined using phase contrast optics to detect cell size and shape (A-C) and stained with DAPI to reveal nuclear morphology (A'-C') of sorted “apoptotic” (A,A',B,B') and “dead” cells (C,C') isolated from cultures induced to die by 4 hours exposure to 100 ng/ml anti-Fas. Bar = 10 μ m.

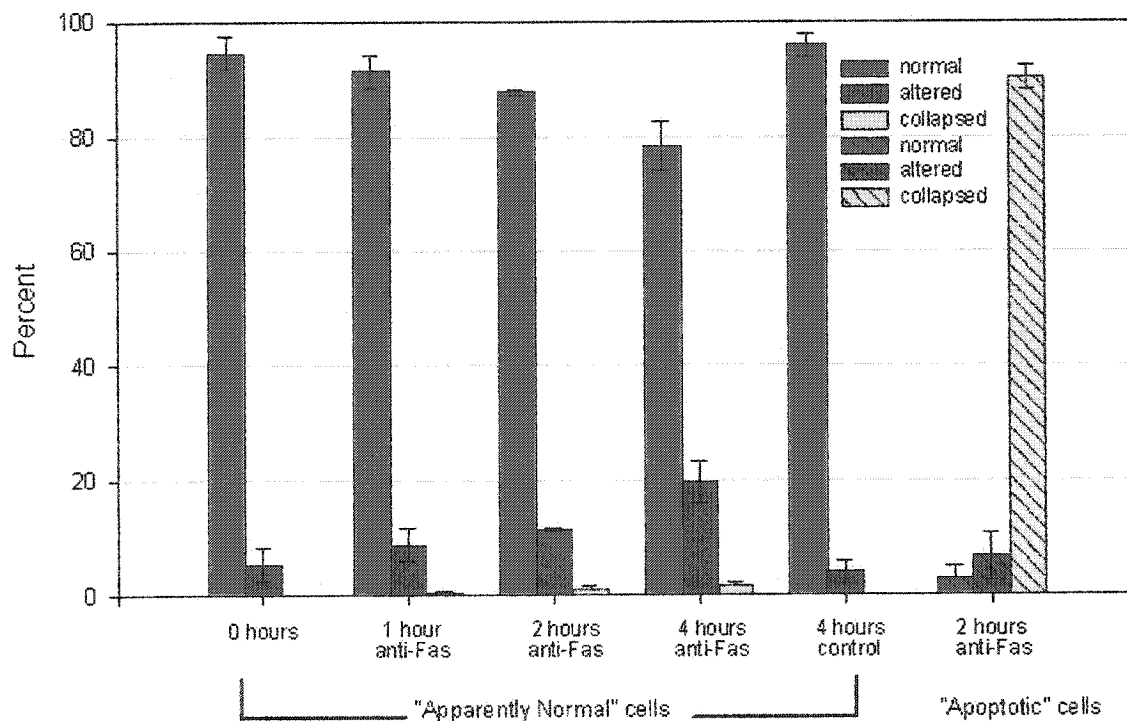


Figure 24 Accumulation of cells with altered nuclear morphology in the sorted "apparently normal" subpopulation of anti-Fas treated Jurkats

Human Jurkat T cells were treated with 100 ng/ml anti-Fas to induce apoptosis, and the proportion of sorted "apparently normal" cells displaying normal (indicated in blue), altered (shrunk, with some increase in condensed chromatin, indicated in red) and collapsed (chromatin margined or in apoptotic bodies, indicated in yellow) nuclei present in the population at time points up to 4 hours was monitored by cell counts following staining with DAPI.

displayed normal morphology. Approximately 4% of cells in these samples had “altered” nuclear morphology, and this fraction did not increase at time points up to 4 hours. Nuclei with collapsed chromatin were not seen. In contrast, “apparently normal” subpopulations isolated from anti-Fas treated cultures showed a steady decrease in the proportion of cells with normal nuclear morphology over the treatment period, to just over 90% after 1 hour, 88% at the 2 hour time point and down to 78% after 4 hours. Concomitantly, there was a rise in the proportion of cells containing “altered” nuclei to 8% after 1 hour, 11-12% by 2 hours and up to 20% at the 4 hour time point. Cells with collapsed chromatin did not make up a significant fraction of this subpopulation; less than 2% at the 4 hour time point, well within the margin of error due to sorting (Figure 21). Counts of the “apoptotic” subpopulation confirmed that over 90% of these cells did indeed display chromatin collapse, while approximately 6% had “altered” nuclear morphology and those with normal nuclei accounted for only 2.5%, a proportion equal to the sorting error rate (Figures 21 and 24, hatched bars).

The mitochondrial membrane potential and size profile of sorted “apparently normal” cells isolated from anti-Fas treated cultures were confirmed in live samples. “Apparently normal” cells from control cultures of Jurkats, when examined using phase contrast optics to discern cell size and fluorescence microscopy to detect DiOC6, featured bright, perinuclear labelling of the cytoplasm, a pattern consistent with the mitochondrial localisation of DiOC6 (Figure 25A’). “Apparently normal” cells isolated from cultures treated with anti-Fas for 4 hours showed a distribution and intensity of DiOC6 labelling indistinguishable from controls (Figures 25B’-25D’). Cells showing high contrast when viewed by phase

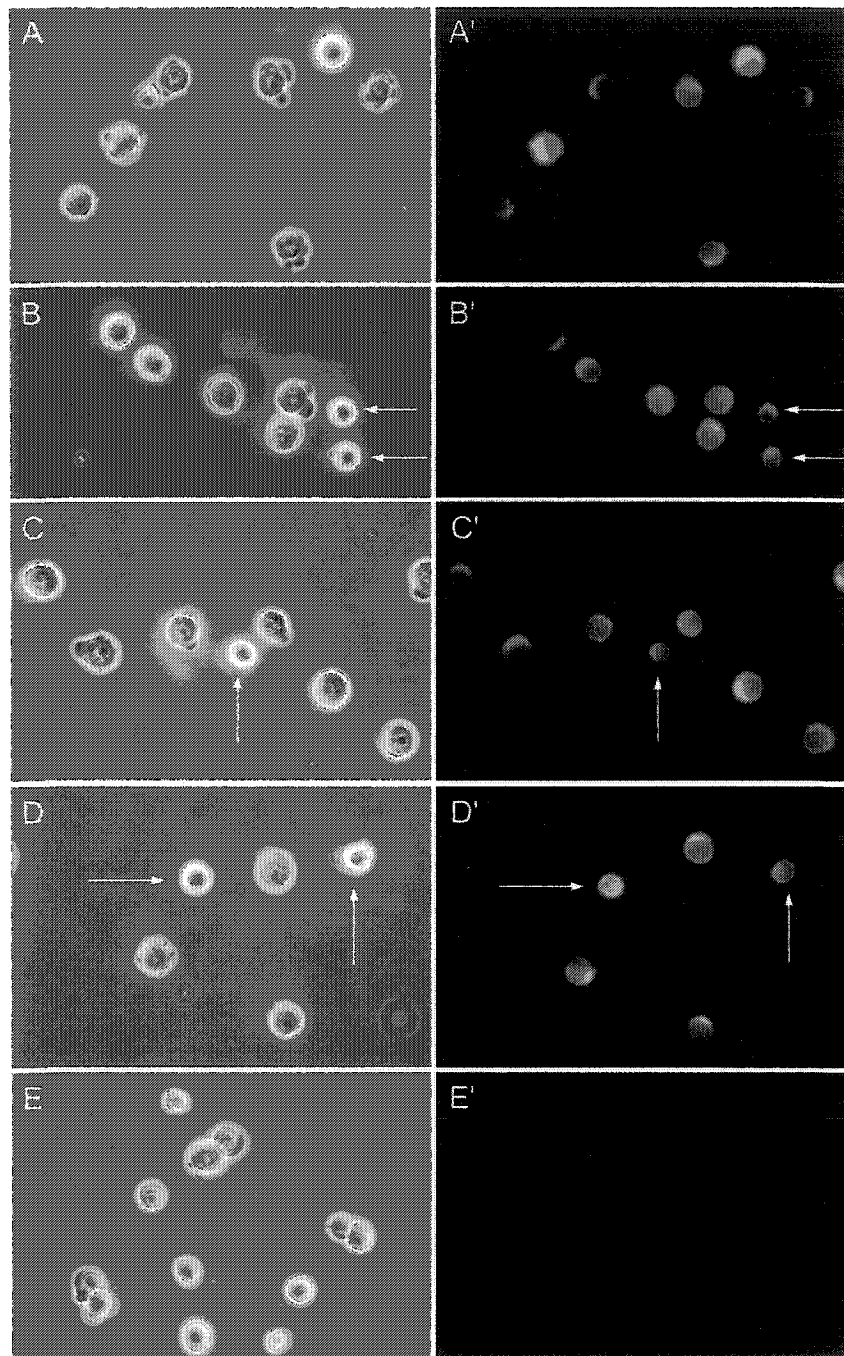


Figure 25 All cells in the sorted “apparently normal” subpopulation retain their mitochondrial membrane potential

Live samples of human Jurkat T cells were examined using phase contrast optics to detect cell size and shape (A-E) and fluorescence microscopy to reveal DiOC₆(3) staining (A'-E') in sorted “apparently normal” cells isolated from untreated controls (A,A') and both “apparently normal” (B,B',C,C',D,D') and “apoptotic” (E,E') cells isolated from cultures induced to die by 4 hours exposure to 100 ng/ml anti-Fas. Arrows indicate altered cells displaying shrunken nuclei.

(Figures 25 B-25D, arrows), along with a decrease in nuclear size, as outlined by the DiOC6 stain (Figures 25B'-25D', arrows) were frequently seen in these samples. They also displayed a robust DiOC6 signal, identical to both that of their neighbours with normal nuclear size and to controls. In contrast, cells of the "apoptotic" subpopulation retained no significant DiOC6 signal (Figure 25E'), even when the exposure time was tripled in an effort to pick up any residual staining. Figure 26 depicts an overlay of the size profiles of "apparently normal" cells isolated from untreated control cultures (blue) and 4 hour anti-Fas treated samples (red). Areas of overlap in the two profiles appear purple. The two samples share a similar forward scatter distribution (Y axis), indicative of cell size; the cells isolated from anti-Fas treated cultures are no smaller than those isolated from control cultures. Surprisingly, the distribution of Fas-induced cells extends slightly further out along both axes, indicating that a minority of these cells feature a slight increase in both "size" and side scatter of laser light, said to be indicative of cellular "granularity". The latter observation correlates well with the increased contrast of some of these cells when viewed under the microscope, and their slight increase in condensed chromatin.

3.2.3 Detection and characterisation of DNA cleavage that precedes loss of

mitochondrial function Samples were next analysed to detect DNA cleavage that might accompany the changes in nuclear morphology found in early apoptotic Jurkat cells. Sorted cells were first subject to *in situ* labelling of DNA breaks using TUNEL (Figure 27). In "apparently normal" cells isolated after 2 hours treatment with anti-Fas, nuclei with normal morphology did not show DNA cleavage detectable by TUNEL (Figures 27A, 27A', 27B,

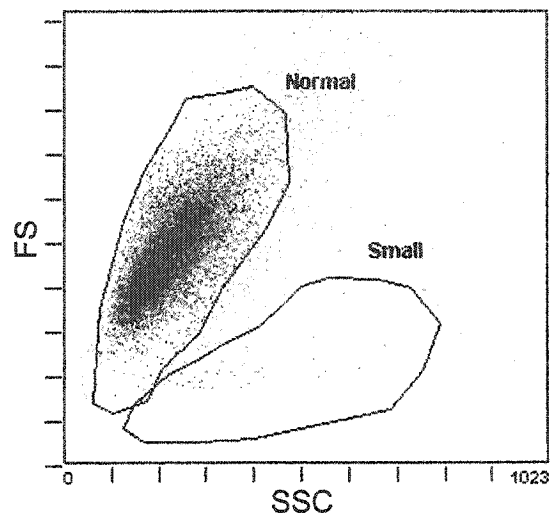


Figure 26 The size profile of sorted “apparently normal” cells is not changed by treatment with anti-Fas

The distribution of Jurkats based on size (as measured by forward scatter of laser light, FS) and granularity (as measured by side scatter of laser light, SSC) was determined by flow cytometry for “apparently normal” cells separated by cell sorting from both untreated control cultures (depicted in blue) and cultures treated for 4 hours with 100 ng/ml anti-Fas to induce apoptosis (depicted in red). Regions of overlap in the size profiles are depicted in purple.

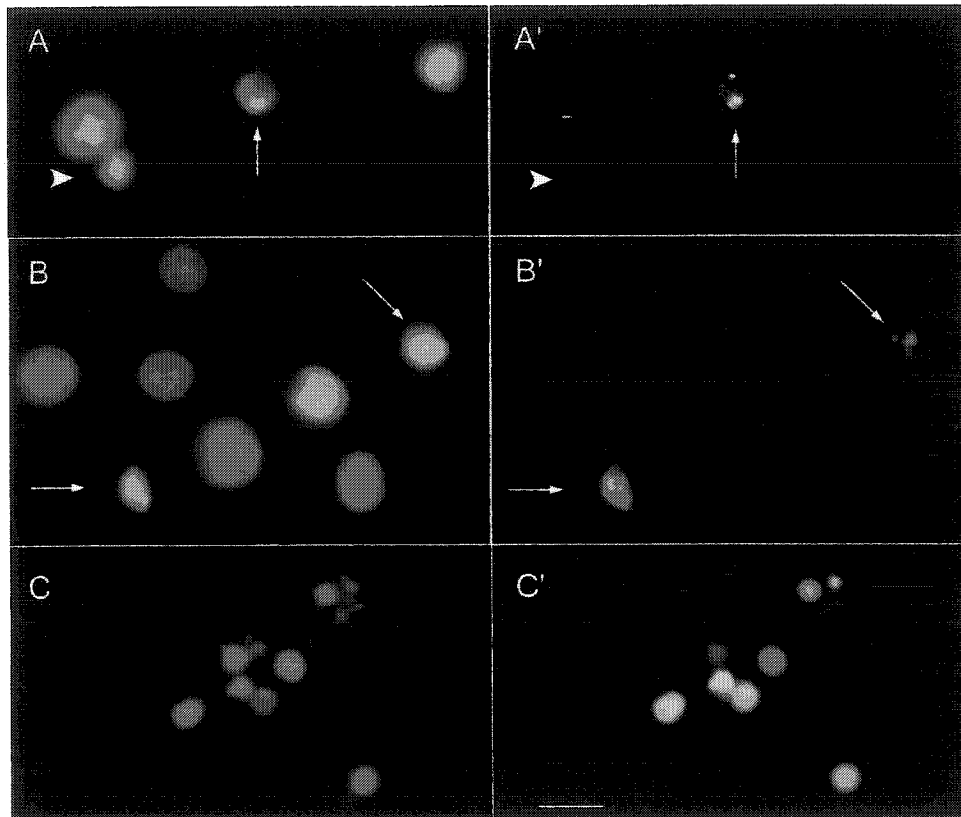


Figure 27 In situ detection of DNA breaks by TUNEL in sorted subpopulations of apoptotic Jurkat cells

Human Jurkat T cells treated with 100 ng/ml anti-Fas to induce apoptosis were stained with DAPI to reveal nuclear morphology (A-C) and subjected to in situ labelling by TUNEL to reveal DNA breaks (A'-C') present in both sorted "apparently normal" cells isolated after 2 hours of treatment (A,A',B,B') and in sorted "apoptotic" cells isolated after 4 hours of treatment (C,C'). Arrowhead indicates "apparently normal" cell displaying altered nuclear morphology but no detectable DNA damage. Arrows indicate "apparently normal" cells displaying DNA cleavage detectable by TUNEL. Bar = 10 μ m.

27B'). However, those that displayed "altered" nuclear morphology often did have low levels of TUNEL labelling (Figures 27A, 27A', 27B, 27B' , arrows). Interestingly, the TUNEL signal did not always cover the entire nucleus; it was often coincident with the areas of increased chromatin condensation present (Figures 27A', upper right hand nucleus in 27B'). Not all the nuclei with altered morphology were labelled by TUNEL (Figures 27A, 27A', arrowhead). The unlabelled nuclei with altered morphology may not have begun DNA cleavage, or the extent of cleavage may not have passed the threshold of damage detectable by TUNEL, which, judging by the low intensity of the label, lay close to the extent of DNA cleavage present in the "altered" nuclei. In sorted "apoptotic" cells isolated from anti-Fas treated Jurkat cultures, all the collapsed chromatin bodies present in the nuclei labelled with TUNEL, as expected (Figures 27C and 27C'). The intensity of label varied markedly, making it difficult to record a field of view accurately.

Biochemical analysis of sorted Jurkat subpopulations by two-dimensional gel electrophoresis of DNA was then employed to characterise the DNA cleavage detected at the single cell level in the "apparently normal" cells. Figure 28A outlines the technique, which is capable of resolving HMW fragments, LMW fragments and single strand damage on the same gel. PFGE is first used to separate fragments of 1 Mb-200 bp generated by double-strand breaks, then the entire gel is subject to an alkaline denaturing wash to separate the DNA strands of the fragments. The gel is then turned 90° and conventional agarose gel electrophoresis is carried out in the second dimension. If the fragments detected by PFGE were generated by double-strand breaks, only those less than 25 kb would travel in the

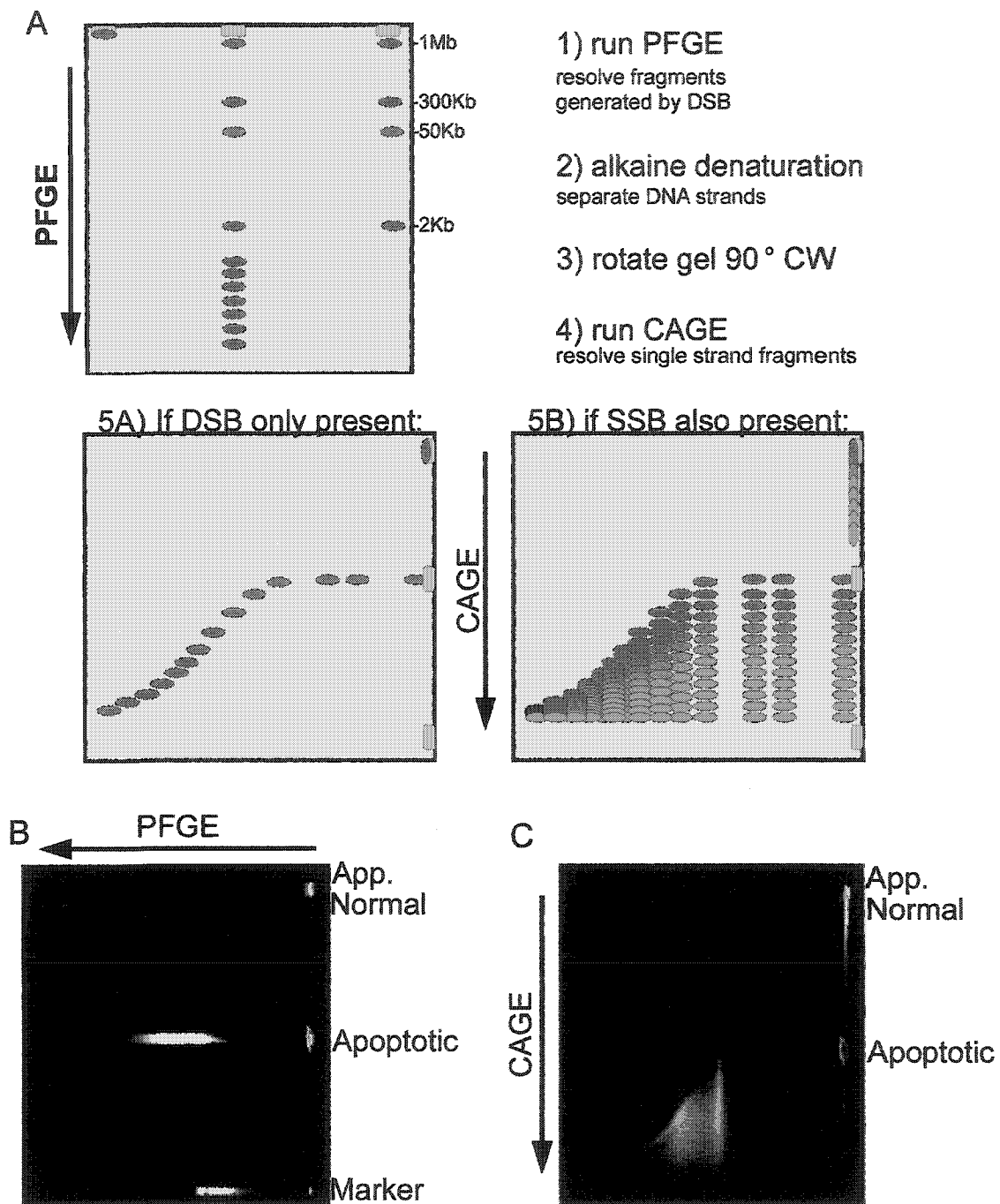


Figure 28 Biochemical detection of DNA breaks in sorted subpopulations of anti-Fas treated Jurkat cells

Chromatin breakdown during apoptosis in sorted "apparently normal" and "apoptotic" Jurkat cells isolated after 4 hours of treatment with 100 ng/ml anti-Fas was characterised using two dimensional gel electrophoresis (A). Images of a representative gel recorded after both PFGE in the first dimension to separate fragments generated by double strand breaks (B) and CAGE in the second dimension to reveal the accumulation of single strand breaks (C) are shown.

direction of the second dimension, and so only the oligonucleosomal fragments will shift, forming a diagonal line (depicted in section 5A). However, if the fragments detected by PFGE harboured single strand breaks along their lengths, separation of the strands will liberate single strand fragments of all sizes. The fragments less than 25 kb will be able to travel a distance proportional to their size in the second dimension, thus all the bands detected by PFGE will shift, generating a smear of DNA (as depicted in section 5B). When recorded after PFGE in the first dimension (Figure 28B), DNA from “apparently normal” anti-Fas treated cells appears intact, remaining in the well. In contrast, a smear of fragments of 50 kb and smaller are visible in the lane containing DNA from “apoptotic” cells. After denaturing of the strands and CAGE in the second dimension (Figure 28C), a smear of DNA was released from all the DNA bands from “apoptotic” cells. Significantly, release of fragments generated by single strand breaks was also detected in the supposedly intact DNA of the “apparently normal” cells that had remained in the well at the top of the gel following PFGE. Over the 4 hour timecourse of anti-Fas treatment single strand breaks progressively accumulated in the “apparently normal” cells; the DNA isolated after 2 hours induction contained fewer single strand breaks than that from cells which had been treated with the inducer for 4 hours (Figures 29A and 29B, lower lanes). “Apparently normal” cells isolated from untreated control cultures at the same time points showed no accumulation of DNA damage, although a minute, uniform smear of fragments was detected by the technique, indicating the presence of some single strand nicks under control conditions (Figures 29A and 29B, upper lanes). These nicks may represent normal sites of replication and/or repair (i.e. alkali-labile DNA lesions) or may result from normal handling of

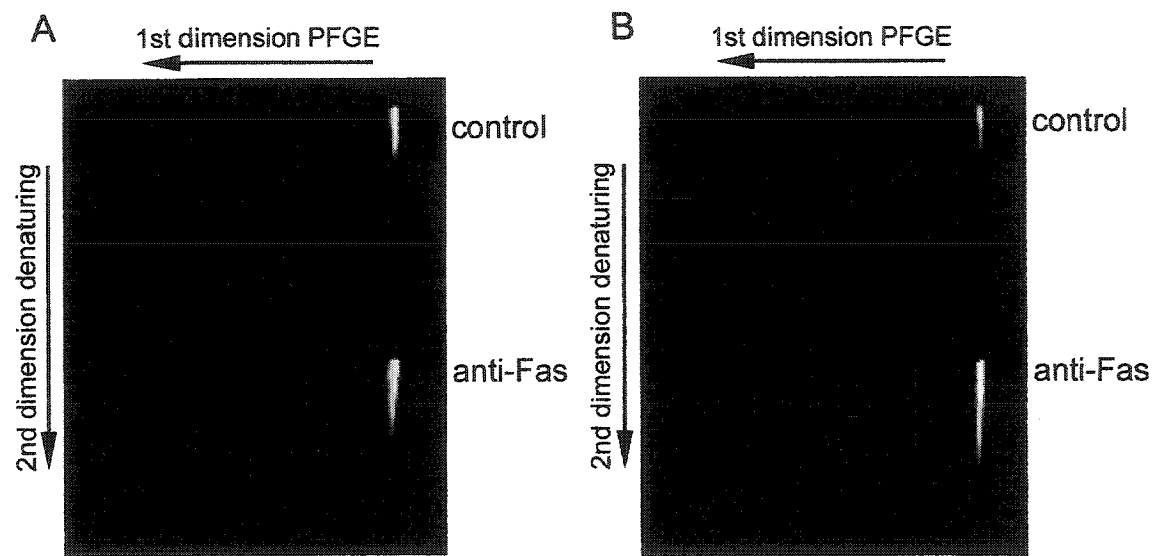


Figure 29 Accumulation of single strand breaks in sorted "apparently normal" Jurkat cells after induction of apoptosis by anti-Fas

Chromatin breakdown during the early phase of apoptosis in Jurkat cells was characterised using two dimensional gel electrophoresis of sorted "apparently normal" cells isolated from both untreated controls and cultures induced to die with 100 ng/ml anti-Fas after 2 hours (A) and 4 hours (B) of treatment.

the samples during processing. The level of “background” damage was limited, and easily distinguished from the induced increase in DNA fragments seen in the presence of anti-Fas.

3.2.4 Degradation and reorganisation of SATB1 in early apoptotic cells with normal

mitochondrial function In whole populations of thymocytes and Jurkat cells, early reorganisation of a portion of the MAR-binding protein SATB1 in apoptotic nuclei closely followed initial DNA cleavage events and preceded chromatin collapse (Figures 14-16).

Having detected initial DNA cleavage events in the “apparently normal” cells isolated from anti-Fas treated cultures of Jurkat cells, I then looked for the changes in SATB1 that would constitute the next steps in the process of nuclear breakdown in this subpopulation. The integrity of SATB1 was first examined by Western blotting (Figure 30). In extracts prepared from sorted “apparently normal” cells isolated from control cultures, antibody to SATB1 recognised a single band of 103 kDa, representing the intact protein (Figure 30, lane1).

However, if the “apparently normal” cells were isolated from cultures induced to die by anti-Fas, a band of 75 kDa was also detected (Figure 30, lane2). In sorted samples of “apoptotic” cells, only the cleavage product was detected (Figure 30, lane3). When examined in single cells, SATB1 was found evenly distributed throughout the nucleoplasm of “apparently normal” cells isolated from control cultures. Staining intensity did not vary across the subpopulation (Figure 31A-31A”). However, in “apparently normal” cells isolated from anti-Fas treated cultures, loss of a portion of the nucleoplasmic SATB1 signal in those cells that displayed altered nuclear morphology had occurred (Figure 31B-31B”, arrows). Staining for NuMA in “apparently normal” cells subject to anti-Fas treatment did not show any changes

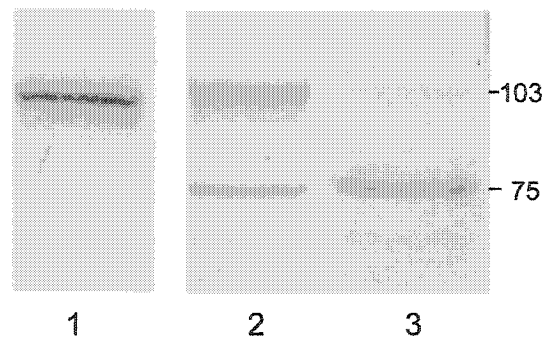


Figure 30 Degradation of SATB1 in sorted “apparently normal” Jurkat cells

The integrity of SATB1 was assessed by Western blotting in sorted “apparently normal” cells isolated from untreated control cultures (lane 1) and both “apparently normal” (lane 2) and “apoptotic” cells (lane 3) isolated from cultures treated with 100 ng/ml anti-Fas to induce apoptosis. The size of the intact protein and the fragment generated by apoptotic cleavage are indicated.

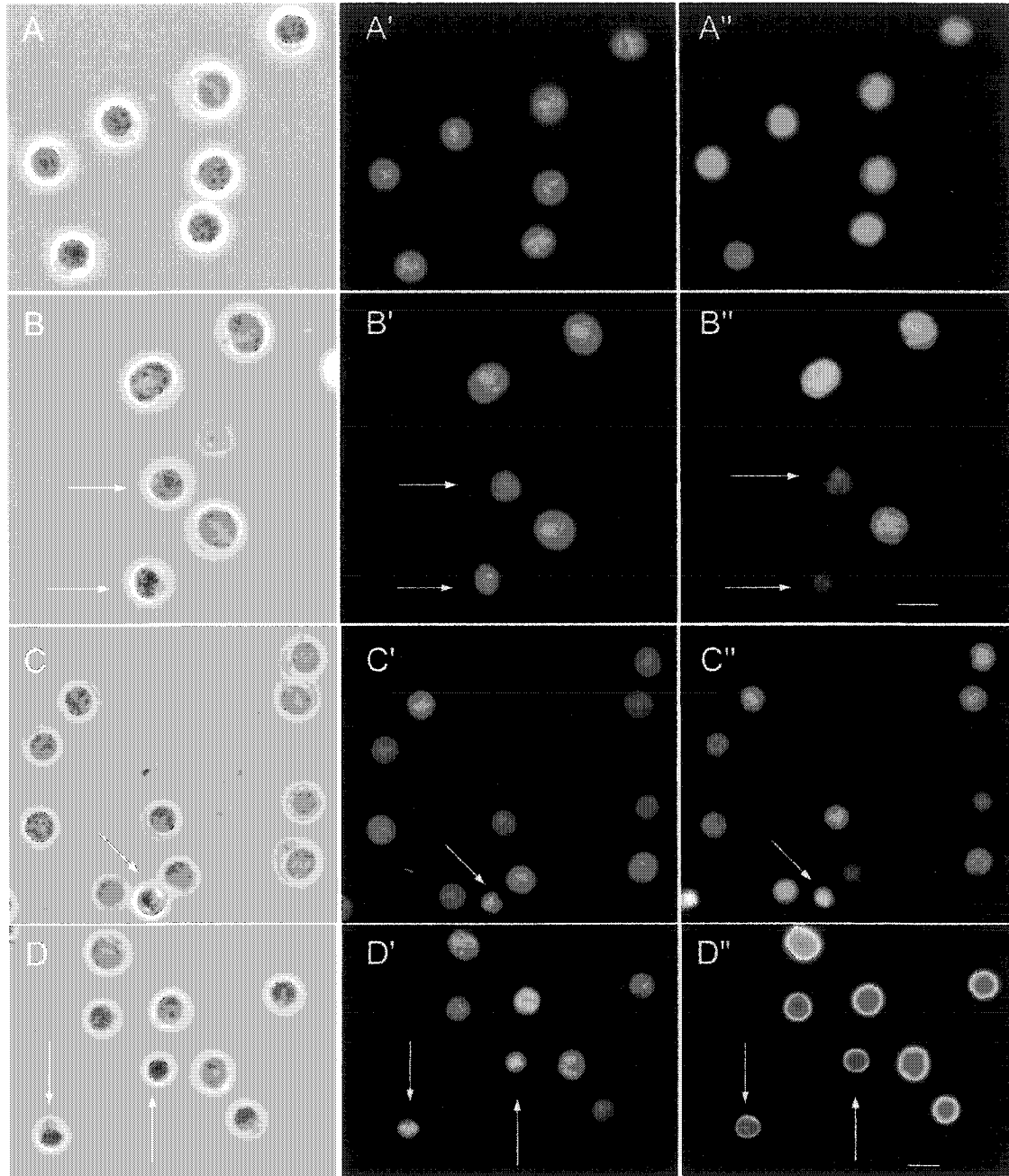


Figure 31. Reorganisation of SATB1 in sorted "apparently normal" Jurkat cells displaying altered nuclear morphology

Sorted "apparently normal" cells isolated from both untreated control cultures (A-A'') and Jurkats treated with 100 ng/ml anti-Fas for 4 hours to induce apoptosis (B-B'', C-C'', D-D'') were examined using phase contrast optics to detect cell size and shape (A-D), counterstained with DAPI to reveal nuclear morphology (A'-D') and immunostained to detect either SATB1 (A'', B''), NuMA (C'') or laminin B (D''). Arrows indicate "apparently normal" cells displaying altered nuclear morphology. Bars = 10 μ m.

specifically in those with “altered” nuclear morphology (Figures 31C-31C”, arrow), although the intensity of NuMA staining did vary somewhat cell-to-cell. Similarly, “apparently normal” cells with “altered” nuclear morphology showed no decrease in or redistribution of lamin B (Figures 31D-31D”, arrows). Taken together, these results indicate that early reorganisation of a portion of the SATB1 present in anti-Fas treated Jurkats preceded loss of mitochondrial function.

3.3 DISCUSSION

Using flow cytometry and cell sorting, a subpopulation of early apoptotic or “apparently normal” cells was identified in cultures of anti-Fas-treated Jurkats, and isolated from the overtly dead cells present in the culture. These cells appeared normal by three cytoplasmic criteria: cell size, mitochondrial membrane potential and plasma membrane integrity (Figure 18). However, changes in nuclear morphology (Figures 22 and 24), initial DNA cleavage in the form of single strand breaks (Figures 27 and 29) and specific cleavage and reorganisation of SATB1, which typically precedes collapse of the chromatin (Figures 30 and 31) were all observed in these cells. Clearly, the initial nuclear events of apoptosis preceded loss of both mitochondrial function and plasma membrane integrity during Fas-mediated cell death in Jurkat cells. These novel findings raised important questions about the signalling pathway(s) from the death receptor to the nucleus.

3.3.1 Diverse signals for cell death may emanate from the Fas receptor Induction of cell death through death domain-based recruitment of adaptor molecules and procaspases has been well documented for the Fas receptor and other members of the TNF- α

superfamily, as described in the introduction. However, other signal cascade(s) are activated in parallel to the caspase-mediated signalling, in particular Ras-mediated activation of JNK. Fas-mediated activation of both a plasma membrane-associated neutral sphingomyelinase and an acidic sphingomyelinase downstream of DAG has been documented in several lymphoid cell lines, including Jurkats. Selection for Fas-resistant mutants implicated the acid sphingomyelinase only in transmission of an apoptotic signal. This signal is transduced through a path that includes ceramide, ras and JNK (Cifone et al., 1995; Gulbins et al., 1995). Activation of JNK in response to the Fas receptor requires receptor aggregation, assembly of the DISC, FADD, caspase 8 and the ASK1 protein (Villunger et al., 2000; Hofmann et al., 2001). Fas-mediated activation of JNK was first described as necessary, but not sufficient for initiation of cell death (Gulbins et al., 1995; Brenner et al., 1997) and persistent Fas-independent activation of JNK in Jurkats has been shown to trigger FADD-dependent apoptosis (Chen and Lai, 2001). However, conflicting evidence suggesting that JNK activation does not contribute to the apoptotic response, but instead reflects activation of cellular stress defence mechanisms during the early phases of apoptosis, has also been published (Lenczowski et al., 1997; Wilson et al., 1999; Hofmann et al., 2001).

Induction of p38 kinase has also been noted in response to Fas treatment of Jurkats, and shown to mediate phosphorylation-based inactivation of Rb protein and hence activation of the E2F1 transcription factor (Wang et al., 1999). Use of specific inhibitors has revealed that p38 activation enhances the apoptotic response, perhaps through E2F1-

mediated effects, as is the case in Fas-treated primary neuron cultures (Brenner et al., 1997; Hou et al., 2002).

3.3.2 Transmission of the apoptotic signal from Fas receptor to the nucleus The identification of early nuclear events that precede loss of mitochondrial membrane potential raises the question of what mechanisms are responsible for transmission of the death signal from the receptor to the nucleus. Translocation of a second messenger is suggested by the requirement of active nuclear transport for effective manifestation of the nuclear events of apoptosis in Fas-mediated cell death (Yasuhara et al., 1997). Daxx, DEDAF and caspase 8 are known Fas-interacting proteins which may be capable of playing this role, and DEDD is a nuclear protein which may serve as a scaffold for death messenger signals.

Daxx was originally identified as an adaptor protein capable of binding to the Fas receptor independently of the FADD adaptor in yeast two-hybrid and GST pull down screens (Yang et al., 1997b). Daxx itself seemed capable of direct signal transmission to the nucleus; when examined by microscopy, it was found to be a nuclear protein associated with PML domains, known sites of transcription (Pluta et al., 1998; Michaelson et al., 1999; Ishov et al., 1999). Evidence showed that Daxx could affect Fas-mediated apoptosis from PML domains (Torii et al., 1999) and that Daxx enhances Fas-mediated apoptosis in B cells (Muromoto et al., 2003). However, Daxx translocation out of the nucleus upon induction of Fas-mediated apoptosis has not been observed, and coimmunoprecipitation did not confirm an association with Fas (Torii et al., 1999; Ishov et al., 1999), raising questions about its interaction with the receptor in vivo. In addition, mice bearing a

targeted disruption of Daxx demonstrated early embryonic death characterised by massive apoptosis, suggesting that Daxx normally acts as an anti-apoptotic molecule during development, not as a purveyor of pro-apoptotic signals (Michaelson et al., 1999). However, the animals do express a carboxy-terminal Daxx fragment (Michaelson et al., 1999) and it is this domain that has been implicated in both Fas-mediated signalling and Daxx effects on transcription when associated with PML domains (Yang et al., 1997b; Torii et al., 1999), making interpretation of the mouse data problematic. A recent RNAi study has provided further evidence that Daxx may play an anti-apoptotic role (Michaelson and Leder, 2003), making it at best a controversial candidate.

The more recently identified Death Effector Domain-Associated Factor (DEDAF) may be an alternate candidate for nuclear transmission of Fas death signals. This protein is capable of interacting with both cytosolic and nuclear DED-containing proteins (procaspase -8, procaspase-10, FADD and DEDD), although it does not itself contain a death effector domain. DEDAF promotes DISC formation and enhances death receptor-mediated apoptosis (Zheng et al., 2001). In the nucleus, DEDAF can interact with DEDD, a DNA-binding protein implicated in apoptosis-associated repression of transcription (Stegh et al., 1998; Schickling et al., 2001).

Finally, caspase 8 itself may be capable of nuclear signal transduction. Apoptosis-dependant translocation of caspase 8 to the nuclear envelope has been detected in ultrastructural and confocal microscope studies of colon cancer cells, neurons and Jurkats (Komoriya et al., 2000; Gajkowska and Wojewodzka, 2002; Benchoua et al., 2002). In the nucleus, caspase 8 can also associate with DEDD proteins (Alcivar et al., 2003).

DEDD proteins may well act as scaffolds for molecular integration of the initial nuclear events of apoptosis. DEDD is typically sequestered in nucleoli, but relocalises to the nucleoplasm upon interaction with DEDAF or caspase 8, implying that this interaction affects its apoptosis-associated nuclear function (Zheng et al., 2001).

3.3.3 Effector caspases do not mediate the initial nuclear events of apoptosis

Effector caspase activation in Fas-treated Jurkats is known to follow the permeability transition and release of proapoptotic molecules from the mitochondrial intermembrane space. Thus, the changes in nuclear morphology, SATB1 integrity and the initial DNA cleavage detected in the sorted “apparently normal” cells which display normal mitochondrial membrane potential would not be mediated by effector caspases. Although the release of cytochrome c necessary for apoptosome activation has been detected just prior to loss of mitochondrial membrane potential in Fas-treated Jurkats (Hearps et al., 2002), others in the lab have found no active caspase 3 present in sorted “apparently normal” cells after anti-Fas treatment using standard assays that measure the cleavage of fluorogenic substrate (data not shown), confirming that the initial nuclear events of apoptosis precede the biological effect of liberated cytochrome c in these cells.

A recent report has called into question the relationship between effector caspase activation and apoptosome formation in type II apoptosis. An in vivo study of Apaf^{-/-} and caspase 9^{-/-} lymphocytes demonstrated activation of caspase 7 in the absence of the apoptosome (Marsden et al., 2002), albeit with delayed kinetics relative to cell death in controls. This delayed onset raises doubts that the same mechanism could contribute to Fas-mediated events at early time points in Jurkats. Caspase 7 is expressed in Jurkat cells.

Although GFP-caspase 7 constructs have been detected predominantly in the cytoplasm in this cell type (Shikama et al., 2001), cleavage-mediated activation of the protease is known to unmask a cryptic nuclear localisation sequence in the catalytic domain and permit nuclear translocation (Yaoita, 2002). However, the apoptosome-independent caspase 7 activity detected by Marsden et al. was manifest in a DEVD cleavage assay, the same assay employed by my colleagues to detect effector caspases in our system, ruling out caspase 7 involvement in initial nuclear events in early apoptotic Jurkat cells.

These conclusions concur well with a study of initial nuclear events in staurosporine-treated Jurkat cells. Appearance of a minority of cells displaying initial nuclear changes, but not collapse of the chromatin, was noted at early time points in the presence of staurosporine. Addition of the drug in the presence of various caspase inhibitors demonstrated that this stage of nuclear reorganisation was not caspase-dependant. Similar caspase-independent early nuclear changes were noted in MCF-7, THP-1 and 3T3 cells induced to die with staurosporine as well as Jurkats treated with etoposide or TPEN, a heavy metal chelator (Johnson et al., 2000). Likewise, caspase 3-independent early changes in nuclear morphology have been noted in Fas-treated HeLa cells (Taimen and Kallajoki, 2003). My results also concur with previous reports showing that caspase 3 and 6 activation in anti-Fas treated Jurkat cells correlate with cleavage of lamins, and that initial changes in the morphology of apoptotic cells do not depend on lamin cleavage (Rao et al., 1996; Kawahara et al., 1998).

3.3.4 Control and coordination of initial nuclear degradation events DNA cleavage in apoptotic cells begins with accumulation of single strand breaks, as described in chapter

1, and then generates HMW fragments whose sizes (300kbp and 50 kbp) reflect cleavage of the chromatin at the base of rosette and loop structures, respectively, areas characterised by the presence of MAR sequences (deBelle et al., 1998). This pattern suggests that MARs are particularly susceptible sites for the initiation of DNA cleavage. Direct evidence for this hypothesis was first provided by a study of HMW DNA fragments produced during radiation-induced apoptosis, which used directional cloning to demonstrate the presence of AT-rich MAR-like sequences at the fragment ends (Khodarev et al., 2000). Our lab has also published evidence in support of this model. Initial DNA cleavage events in sequences comprising six matrix attachment regions (MARs) in anti-Fas treated Jurkat cells were mapped using ligation-mediated PCR. We detected an accumulation of single strand breaks concentrated in the short base unwinding regions contained within the MARs, relative to adjacent stretches of DNA. Further, breaks on the two strands of DNA were often offset by only one or two nucleotides, and hence would be resolved as a double-strand break by electrophoresis techniques (Liu et al, 2003). The endonuclease responsible for these initial DNA cleavage events, apparently a nuclear protein, has not yet been conclusively identified (Robertson et al., 2000). However, our lab among others has identified a candidate novel DNase I-like endonuclease. Unlike the other members of the DNase family, this "DNase Y" or "DNase γ " is located throughout the nucleus where it is tightly bound to the chromatin. It can mediate both single and double-stranded DNA cleavage and produces DNA fragments with 3'-OH ends typical of apoptotic cells (Liu et al., 1998; Shiokawa and Tanuma, 1998). Overexpression of this endonuclease has recently been shown to induce apoptosis (Saito et al., 2003).

The vulnerability of MARs to the initial DNA cleavage events of apoptosis may be mediated by proteolysis of nuclear matrix proteins, which is first detected by biochemical assay in a similar time frame (Figures 5 and 6). As discussed in Chapter 2, apoptotic cleavage of MAR-binding proteins can disrupt their ability to interact with the DNA. Several studies of oligonucleosomal cleavage products have shown that DNA-protein interactions protect the nucleotide strand. The frequency of DNA cleavage events observed in areas wrapped around core histones was less than that in linker regions, and transcription factor binding has also been shown to moderate DNA damage (Peitsch et al., 1993; Cairns and Murray, 1998). Analysis of the cleavage sites we mapped using LMPCR also supports this model (Liu et al, 2003). The endonuclease which produced the breaks showed no sequence specificity and distribution of the cuts followed no set pattern, indicating that the base composition in these regions did not determine the cutting sites. Rather, loss of protective interaction with MAR-binding protein rendered the areas vulnerable to endonuclease. The protease(s) responsible for early cleavage of MAR-binding proteins has (have) not been established. A number of these proteins contain sites for effector caspase-mediated cleavage, as outlined in Chapter 2, but their breakdown is initiated very early in the apoptotic response, and in the case of SATB1, before mitochondria-based activation of the effector phase of apoptosis (Figure 30). Initial cleavage of these proteins may be mediated by an alternate caspase. One candidate for this role is caspase 2, which has been shown to be present in the nucleus (Zhivotovsky et al., 1999). Caspase 2 is activated by oligomerisation (Baliga et al., 2003), acts on Bid and mitochondrial targets, rather than other caspases (Guo et al., 2002) and can induce the

mitochondrial events of apoptosis from the nucleus (Paroni et al., 2002). However, cleavage of nuclear matrix substrates was not examined in these studies, and thus a direct link between caspase 2 activation and the initiation of nuclear breakdown remains to be established. Alternatively, caspase 8 may play a role following translocation to the nucleus. Finally, caspase 6 may be activated by Fas signalling to nuclei; DEDD has been shown to activate caspase 6 (Schickling et al., 2001). While typically caspase 6 activation is thought to lie downstream of caspase 9 and 3, activation of caspase 6 in the absence of caspase 3 has been detected in Fas-treated Jurkat cell extracts (Kawahara et al., 1998) and in intact thymocytes treated with dexamethsone (Komoriya et al., 2000).

The nuclear DEDD proteins could provide an efficient link between Fas-mediated messengers and activation of caspase 6, the protease implicated in apoptotic degradation of SATB1 (Schickling et al., 2001). DEDD is also known to associate with several transcription factors and act as a repressor of transcription following its interaction with either DEDAF or caspase 8, and its associated relocation from the nucleolus to the nucleoplasm (Stegh et al., 1998; Alcivar et al., 2003). The non-caspase signalling paths activated by anti-Fas (JNK, p38) also impact gene expression. Thus, engagement of Fas paths could be predicted to affect nuclear function. An examination of changes in gene expression specific to early apoptotic Jurkat cells was therefore pursued, to demonstrate the effects of these signalling paths on gene expression and examine their potential contributions to the initial steps of nuclear breakdown.

CHAPTER 4 CHANGES IN GENE EXPRESSION IN EARLY APOPTOTIC JURKAT CELLS

Changes in gene expression during apoptosis, mediated by several transcription factor families, including myc, AP1 and E2F, have been described in a number of models systems. Their potential to affect the progression of cell death is exemplified by p53-mediated upregulation of proapoptotic members of the bcl-2 family known to compromise mitochondrial membrane integrity and promote PT (Prendergast, 1999; Shaulian and Karin, 2001; Ginsberg, 2002). However, death induced by the Fas receptor is commonly considered an exception to this trend, and presented as a process that proceeds independently of gene expression and de novo protein synthesis (Sartorius et al., 2001; Wajant, 2002). Activation of the caspase cascade instrumental to dismantling of cellular structure in response to death receptors, as described in the introduction, relies on constitutively expressed components. Fas-mediated apoptosis is also known to proceed in the presence of either actinomycin D, which represses transcription, or cyclohexamide, which represses translation. These experiments have been routinely interpreted as indicators that activation of caspases is both necessary and sufficient for Fas-mediated apoptosis to proceed (Sartorius et al., 2001).

However, enhancement of the apoptotic response by changes in JNK and p38 signalling has also been described in Fas-treated Jurkats, as detailed in section 3.3.1, although the impact of these signalling mechanisms on the process of cell breakdown has been the topic of some debate. A survey of known downstream targets of these paths shows potential for significant impact on the process of apoptotic cell breakdown. The

activation of JNK in Fas-treated Jurkats has been linked to activation of AP1 transcription factor composed of cJun and ATF2 subunits (Faris et al., 1998b) and increased expression of c-Fos (Siegmund et al., 2001) and Fas ligand (Faris et al., 1998a; Faris et al., 1998b). In addition, AP1 may activate a p53 apoptotic response. p53 also lies downstream of the E2F1 transcription factor, which is activated in Fas-treated Jurkats via p38-mediated inactivation of its inhibitor Rb (Wang et al., 1999). The apoptotic response mediated by p53 involves upregulation of sequences for Fas receptor, H-Ras, the proapoptotic bcl-2 family members Bax, PUMA and Noxa, and the apoptosome protein Apaf-1 (Kannan et al., 2001a; Kannan et al., 2001b; Wu and Deng, 2002). Both h-Ras and Apaf-1 upregulation by p53 involves a self-amplifying loop, through JNK and AP1 for ras and through the transcription factor Zac-1 for the apaf-1 gene (Shaulian and Karin, 2001; Rozenfeld-Granot et al., 2002).

Additionally, activation of cell proliferation-associated pathways by the Fas receptor has been documented recently (Budd, 2002). This response may depend on association of the adaptor FLIP with the DISC and results in upregulation of c-Fos and NFK β (Siegmund et al., 2001; Budd, 2002; Tibbetts et al., 2003). Like JNK and p38, activation of this alternate signalling path by the Fas receptor in Jurkats should have an impact on gene expression.

Interpretation of continued cell death in the presence of routinely-used inhibitors of transcription and translation is complicated by their own toxicity; Actinomycin D can act as a death stimulus in Jurkat cells (Kasai et al., 1998) and careful study has shown that cyclohexamide enhances the kinetics of apoptosis due to Fas treatment in this system

(Kim et al., 2000). Toxicity of these inhibitors may reflect their interruption of modulatory elements in the cell death response, which require gene expression and lie upstream of the effector phase of apoptosis (Chang et al., 2002b). Taken together, the limitations of inhibitor-based studies and documented activation of alternate signalling paths by Fas suggest that efficient execution of apoptosis in response to this inducer may involve expression of novel death-mediating sequences.

Results presented in Chapter 3 suggest that pre-mitochondrial transduction of caspase 8-mediated signals from the Fas receptor to the nucleus control the initial nuclear events of apoptosis. Caspase 8 or DISC-associated second messengers are candidate members of this signalling path. Interestingly, interaction of the nucleolar protein DEDD with either caspase 8 or the second messenger DEDAF results in reorganisation of the nuclear matrix associated with caspase 6 activation and altered transcription (Stegh et al., 1998; Schickling et al., 2001). A role for transcription in the process of Fas-induced apoptosis is also supported by evidence that PML, another nuclear protein known to form domains associated with transcription, is required for cell death in response to this signal and functions at an early point upstream of effector caspase activation (Wang et al., 1998). At the protein level, de novo synthesis of several proteins in response to Fas treatment of Jurkats has recently been detected in a proteomic study of these cells (Gerner et al., 2000).

Thus, Fas-mediated death, particularly early events, may be influenced by altered gene expression. To identify genes affected by anti-Fas signalling and derive some insight into genetic contribution to the apoptotic response in Jurkats, microarray analysis

was used to compare control and apoptotic populations. Separate analysis of “apparently normal” cells isolated from the whole populations by sorting was also carried out, to identify changes that may specifically accompany and mediate early nuclear events. Functional analysis of the genes displaying altered expression predicted important effects on mitochondria homeostasis and the actin cytoskeleton in apoptotic cells.

4.1 MATERIALS AND METHODS

4.1.1 Cell culture and treatment Jurkat cells were maintained as described in section 2.1.1. To ensure healthy, growing cultures for treatment, cells were plated 24 hours prior to experimentation at a density of 0.4×10^6 cells/ml. Before use, cell density was adjusted to 10^6 cells/ml, which typically represented a change of no more than 10%, as cultures had grown to $0.85 - 1.1 \times 10^6$ cells/ml overnight. Cell death was induced by the addition of 100 ng/ml anti-Fas monoclonal antibody (clone CH-11) from a 0.5 mg/ml stock.

4.1.2 Flow cytometric assessment of mitochondrial function and cell sorting Early apoptotic cells displaying normal size, intact plasma membrane and normal mitochondrial function were identified and isolated as described in sections 3.1.2 and 3.1.3.

4.1.3 Isolation of RNA for microarray analysis Total RNA was extracted from Jurkat cells and sorted subpopulations using TriReagent, in accordance with the manufacturer’s instructions. Briefly, cells were suspended in TriReagent at a density of 10^7 /ml for 5 minutes, and phases separated by the addition of 0.1V BCP for 15 minutes at room temperature. The aqueous phase was collected and 0.5V isopropanol added to extract the

RNA. After precipitation in 75% ethanol, RNA was resuspended in DEPC-treated water, its concentration and purity assessed by recording the OD at 260 and 280 nm, and its integrity confirmed by non-denaturing agarose gel electrophoresis. Samples from sorted subpopulations were kept at -20 °C for up to two weeks in TriReagent, then pooled to make up 1 ml aliquots for processing. RNA isolated from all samples and pooled according to treatment and time was stored at -80°C in 75% ethanol until sufficient quantity for microarray analysis had been accumulated. Control and treated samples were prepared in equal quantity on a daily basis over the duration of the collection period, to ensure that any variation in culture conditions would be reflected equally in both pools of RNA.

4.1.4 Preparation of labelled cDNA, hybridisation and scanning of microarrays

For each sample, a 20 µg aliquot of RNA was removed from the pool, precipitated with 0.1V 3M NaCl, the pellet washed in 80% ethanol and dried. A fluorescently-labelled cDNA copy was generated in a 40 µl reaction mix (1X superscript II first strand buffer (from 5X stock) containing 150 pM AncT mRNA primer (5' T₂₀ VN 3'), 20 mM each of dATP, dGTP and dTTP, 2 mM dCTP, 1 mM Cy3- or Cy5-conjugated dCTP, 0.4M DTT and the RNA aliquot). The mix was first heated to 65°C for two minutes, then 42 °C for 5 minutes to denature the RNA and anneal the AncT primer. Reverse transcription was carried out for 2 hours, 45 minutes at 42°C in the dark, following the addition of 2 µl RT Superscript II and 1 µl RNasin. The reaction was stopped by the addition of 5 µl of 50 mM EDTA (pH=8), and RNA templates hydrolysed by the addition of 2 µl 10 N NaOH and heating to 65 °C for 20 minutes. The reaction was then neutralised by adding 4 µl

5M acetic acid, the pH verified, and incorporation of the labelled probe confirmed using agarose gel electrophoresis. Before hybridisation, the probes from control and treated samples to be directly compared were combined, precipitated and resuspended in 85 μ l of DIG Easy Hyb buffer containing 0.5 μ g/ μ l yeast tRNA and 0.5 μ g/ μ l single stranded sperm DNA, heated to 65 °C for 2 minutes and cooled to room temperature. Probes were hybridised for 18 hours at 37 °C in a dark humid chamber to a pair of human 19K microarray slides placed face to face, by filling the space between the slides. Slides were then washed three times for 10 minutes each time in 1X SSC containing 10% SDS at 50 °C, rinsed once in 1X SSC and dried by gentle centrifugation. Arrays were kept in the dark and scanned within 24 hours. Cy3 and Cy5-conjugated probe binding was detected by sequential scans using a Packard ScanArray 5000. Fluorescence intensity data was extracted from the scanned chip image using the system's QuantArray microarray analysis software. The location of spots as determined by the Quant Array program was verified manually for each of the 32 subarrays on every microarray chip, and adjustments made on a row-by-row basis as required. Fluorescence signal due to dirt or scratches on the slide was flagged manually for removal from further analysis. For each sample triplicate experiments, including reciprocal labelling of cDNAs, were carried out.

4.1.5 Microarray data processing and analysis Raw intensity data from Quant Array was subject to processing in Microsoft Excel. Using a macro written for the purpose (Normaliser2, by Brandon Smith, © NRC 2001) median background fluorescence was assessed on each subarray and subtracted from the spot intensities, the data was normalised using a linear regression correction method and suspect data spots were

flagged and removed from both the equation for normalisation and further analysis. Four data flags were employed: an inadequate signal/background ratio (set at 2.5X), signal intensity too low for analysis (set to remove the lowest 5% of spots), signal intensity too high for a legitimate spot (set to remove the top 2% of spots) and unacceptable signal variation across a spot (set for standard deviation of spot intensity more than 500% of mean spot intensity). The ratio of treated to control signals was then determined for each spot and reported on a log₂ scale. Data was analysed in Excel to select for spots with a mean log ratio of at least ± 0.6 , for which all three replicates were present and unflagged. A p-value threshold of ≤ 0.05 was then assessed by single sample t-test of the log₂ ratio vs. 0 to select for sequences with consistently altered gene expression.

Annotation for spots of interest, identified by their clone ID, was obtained first from SOURCE (Stanford University), then through publically-accessible databases both at NCBI and the Weizmann Institute of Science, principally Unigene, Locus Link, OMIM, PubMed and Gene Cards.

4.1.6 QPCR analysis of gene expression For QPCR, trace DNA contamination was removed from the RNA using the DNA-free kit (Ambion Inc., Austin, TX) with several modifications: 4U DNaseI/100 μ l was incubated with 0.4 μ g/ μ l RNA in the buffer supplied for 2 hours at 37 °C, and the reaction stopped with 0.2 V of DNase inactivation reagent. RNA in the supernatant was precipitated with 0.5V lithium chloride (Ambion) overnight at -20 °C, washed in 75% ethanol, dried and resuspended in 0.1V water. RNA quality was re-evaluated by agarose gel electrophoresis and OD 260/280, and absence of trace DNA contamination confirmed by QPCR amplification of beta-actin on a 5 μ g

aliquot. cDNA was generated in a volume of 40 µl per 20 µg RNA aliquot, in a reaction mix containing 0.5 n moles/µl dNTP (Invitrogen) instead of the nucleotide mixture for microarray. Reverse transcription was carried out for 2 hours at 42 °C, reaction stopped by adding 5 µl of 500 mM EDTA, then RNA hydrolysed and the mix neutralised as described (Walker et al., 2003). cDNA was purified and quantitated using the Qiaquick PCR kit (Qiagen) and Oligreen dye (Molecular Probes), as instructed. For QPCR, 1.2-2 ng cDNA was amplified in 25 µl of SYBRgreen PCR master mix (Applied Biosystems) containing 0.15 pM each of gene-specific forward and reverse primers. Fluorescent product was detected using an ABI Prism 7000 system, set for 1 cycle of 2 minutes at 50 °C, 1 cycle of 10 minutes at 95 °C and 40-43 cycles of 15 seconds at 95 °C, 1 minute at 60 °C. Product dissociation was monitored to confirm specificity of the primers. Forward and reverse primers for sequences of interest (Qiagen) were designed using Primer Express (Applied Biosystems) and are listed in Table 1.

4.1.7 Detection of filamentous actin in sorted subpopulations Staining of f-actin by phalloidin-FITC was carried out as described in section 2.1.5 on “apparently normal” and “apoptotic” subpopulations isolated by sorting, and analysed by flow cytometry.

Oligo	Sequence from 5'-3'	Position	PCR product
APOD-f	TGGCCACCGACTATGAGAACTA	423-444	81bp
APOD-r	TCCAAGCAAAATCCACGTGA	484-503	
ARHGEF6-f	AACCAGAAGCAACGGCCAG	2093-2111	81 bp
ARHGEF6-r	TCTCTGACCTCGTCCTTCAAGG	2152-2173	
BTF-f	GCTGCTTGCTAGTACACTTGTCCA	1960-1983	84 bp
BTF-r	GCCTGTGGCAACTTAATGTGGT	2022-2043	
Caspase8-f	TCTCCAAGAGAACAGGATAGTGAATC	922-947	83 bp
Caspase8-r	ATCAG ACAGTATCCCCGAGGTTT	982-1004	
Cdk5-f	CGTCGTGCCCAAACTCAAT	774-792	82 bp
Cdk5-r	TGCTGAGATACGCTGGACAGG	835-855	
CLN8-f	GTTCTTGTGCGGACTGGCTC	933-952	81 bp
CLN8-r	GGATTGAGAAGCTG CTGAGTCTTC	990-1013	
Cox6-f	CAGGCGTCTGCGAAATCATA	108-127	83 bp
Cox6-r	GATCAGCCACACGAACTTATACAA	166-190	
E2F4-f	GGGCAGAAGAAGTACCAGATTAC	585-608	81 bp
E2F4-r	GCTCCATGCCTCCTTGTTC	646-665	
ECM2-f	GCTCTATCACGTCCTGCTCTAT	1711-1732	81 bp
ECM2-r	CCAGGGATCCGTTCAATCTG	1772-1791	
EIF3S6-f	GGTG GCTTGTCTTGAGGATTTT	997-1018	81 bp
EIF3S6-r	CTGATACACTGGTGGATGCGA	1057-1077	
GNG11-f	CTTCACATCGAAGATTGCCAG	361-382	84 bp
GNG11-r	TTGTCTCTGCAACTTCACCTCTTTG	420-444	
GOLGA1-f	GACCCGATAATGAGCTCTTCGA	2307-2328	82 bp
GOLGA1-r	GTGTTATTCTGTGACGGAAGGC	2368-2388	
HSPA1B-f	TGACAACAGGCTGGTGAACC	903-922	81 bp
HSPA1B-r	ACGGCTCGCTTGTCTGG	966-983	
HSPA8-f	TCCAGGCAGCCATCTTGTCT	1202-1221	79 bp
HSPA8-r	AGGGAAAGAGGAGTGACATCCA	1259-1280	
ILX3-f	CCTTCCAAGATGCCCAAGAA	1284-1303	81 bp
ILX3-r	GGCATAGGTGGTGGCTTGGTG	1345-1364	
JAK1-f	GTGCCACCTAACTGTCCAGA	3384-3404	81 bp
JAK1-r	AAGCTTGTCCGATTGGATGG	3445-3464	
KCNJ13-f	ATCAAAGTCTCTGGCTACTCTGCT	889-912	81 bp
KCNJ13-r	TCCTGCATTGCTGAAAGGAAT	949-969	
NDST3-f	CTCCAGTACAACCTGGCCACA	2061-2081	81 bp
NDST3-r	TGTCATCGCAAGGATTCTGC	2122-2141	
PPP2CB-f	CTGTTATCGTTGTGGGAACCAG	816-837	81 bp
PPP2CB-r	GCCGGGTCAAATTGAAGGA	878-896	
Serpinf2-f	CACTTCCAGGGTTTCTGGAGG	730-750	81 bp
Serpinf2-r	CGTGAAGTCTCGTCCAGGT	791-810	
SMARCA2-f	AGCCAAACCTGTAGTGAGCGA	4773-4793	81 bp
SMARCA2-r	TCATCCGTCCCACTTCTTCT	4833-4853	
SMURF2-f	ATGATCCAAGAGTGCCAGG	1027-1046	81 bp
SMURF2-r	TACGGATCTCCCATCCAGGA	1088-1107	

Table 1: QPCR primers

Primers were designed for amplification of sequences whose expression changed when analysed by microarray in anti-Fas treated Jurkats. Sequences represent known genes altered specifically in early apoptotic cells, as well as genes present in a number of functional categories identified by microarray analysis.

4.2 RESULTS

Microarray analysis of changes in gene expression in anti-Fas treated cells relative to controls was carried out, both to demonstrate that treatment with anti-Fas had an impact on gene expression and to generate insight into events of the initiation phase of apoptosis in Jurkat cells by examining transcriptional changes specific to early apoptotic cells. Human 19K microarrays purchased through the University Health Network in Toronto were employed for analysis of gene expression. These arrays offered the advantage of representing a significant portion of the human genome; the 19,200 cDNA spots on the arrays represent roughly 15,000 unique sequences, or up to 50% of the predicted total of human genes.

4.2.1 Changes in gene expression in anti-Fas treated Jurkat cells For microarray analysis, RNA isolated from control and anti-Fas treated Jurkat cells, both whole populations and sorted, “apparently normal” subpopulations, was reverse-transcribed using a direct-labeling approach. After cDNA hybridisation, problems with the quality of some of the arrays became evident (Figure 32). Figures 32A-32A” depict an array of good quality. The chip is organised in subarrays of 600 spots (24 x 25), laid out in a grid of 4 columns and 8 rows. Following simultaneous hybridisation with the cDNA of sorted, apparently normal cells isolated from control cultures (coloured red, Figure 32A), and anti-Fas treated cultures (coloured green, Figure 32A’), an overlay of the two signals clearly shows that expression of most of the sequences represented on the array does not change (spots appear yellow, Figure 32A”) . The signals displayed good balance across a range of intensities. In contrast, some arrays displayed

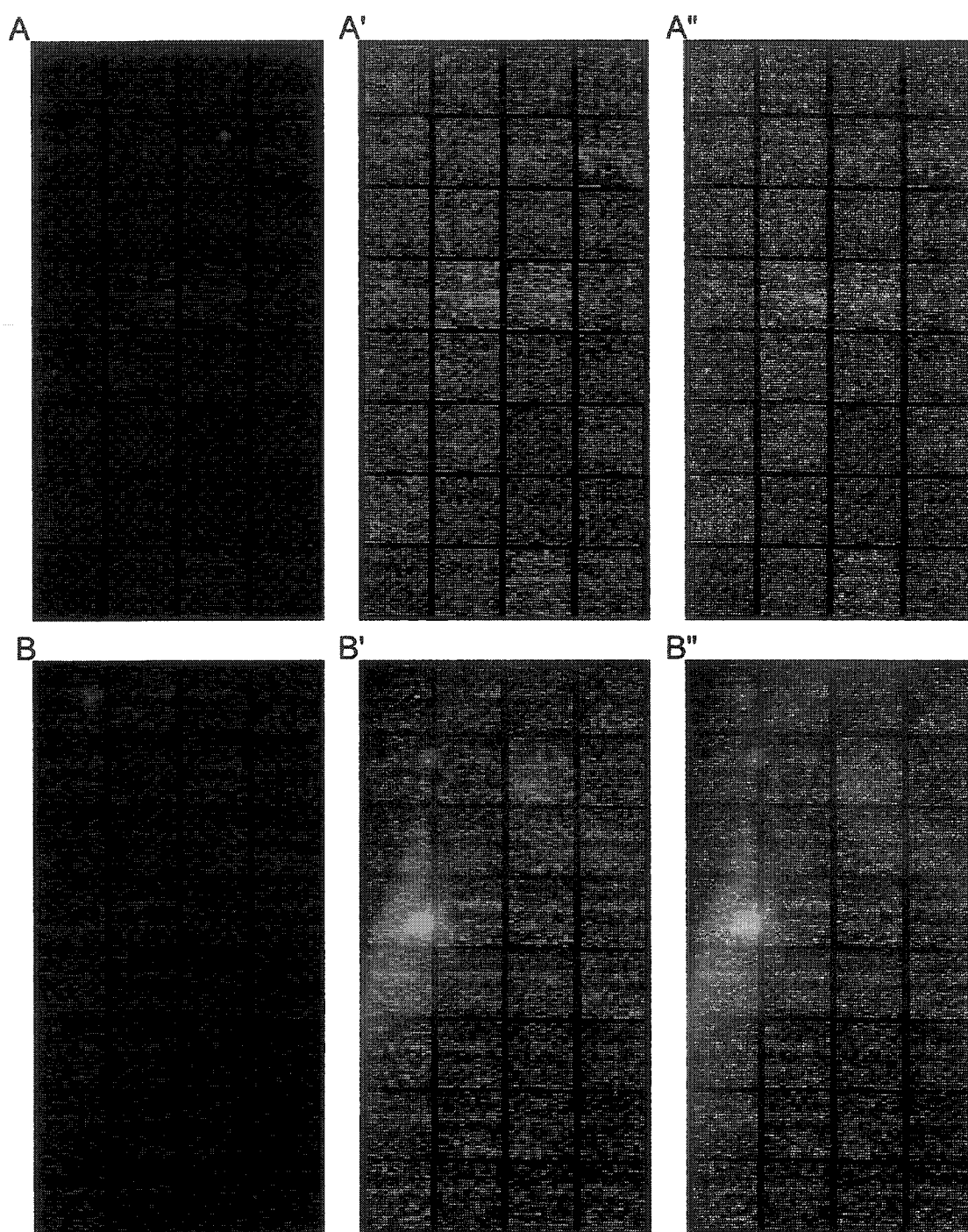


Figure 32 Analysis of change in gene expression using human 19K DNA microarrays
 Gene expression in anti-Fas treated Jurkats (A', B') relative to controls (A, B) was assessed by microarray analysis of RNA isolated from both sorted "apparently normal" cells (A-A'') and whole populations (B, B''). The overlay of the two signals is depicted in A'' and B''; spots which hybridised to both treated and control RNA appear yellow. Representative arrays of good quality (A-A'') and poor quality (B-B'') are depicted.

problems in hybridisation and background (Figures 32B-32B'). Large areas of non-specific binding of one of the labelled cDNAs are visible; there is a background spot due to the signal from the anti-Fas treated sample in column 1, row 4 (Figure 32B') and problem areas due to the signal from the control over most of row 1, column 1 and rows 2 and 3, column 3 (Figure 32B). In addition, the control sample also shows a non-specific reduction in hybridisation over the bottom right hand corner of the array. When the two signals were overlaid (Figure 32B''), this area appeared green, giving the false impression of massive increase in gene expression in the treated sample. These problems in array quality result from interaction of the fluorochromes with the chemical coating and masking agents used to treat the glass slides during manufacture. Error due to these imperfections was eliminated by the use of data processing and analysis strategies.

Three data processing steps were employed. The data distribution of representative good and poor quality microarrays after each of the steps is depicted in Figure 33. Background subtraction was first carried out separately on each subarray, using the median value, to produce a more accurate intensity reading for each spot. Figure 33A shows that after background subtraction, a large proportion of the data from poor quality microarrays fell outside the limitation of 2-fold change considered significant (indicated by red lines). In contrast, the data cluster from a good quality microarray was typically distributed within the limitation of 2-fold change, although the distribution of low intensity spots in the dataset came very close to this limit (Figure 33B). Data was then normalised using linear regression, to correct for the effects of any imbalance in scanning the two signals and differences in

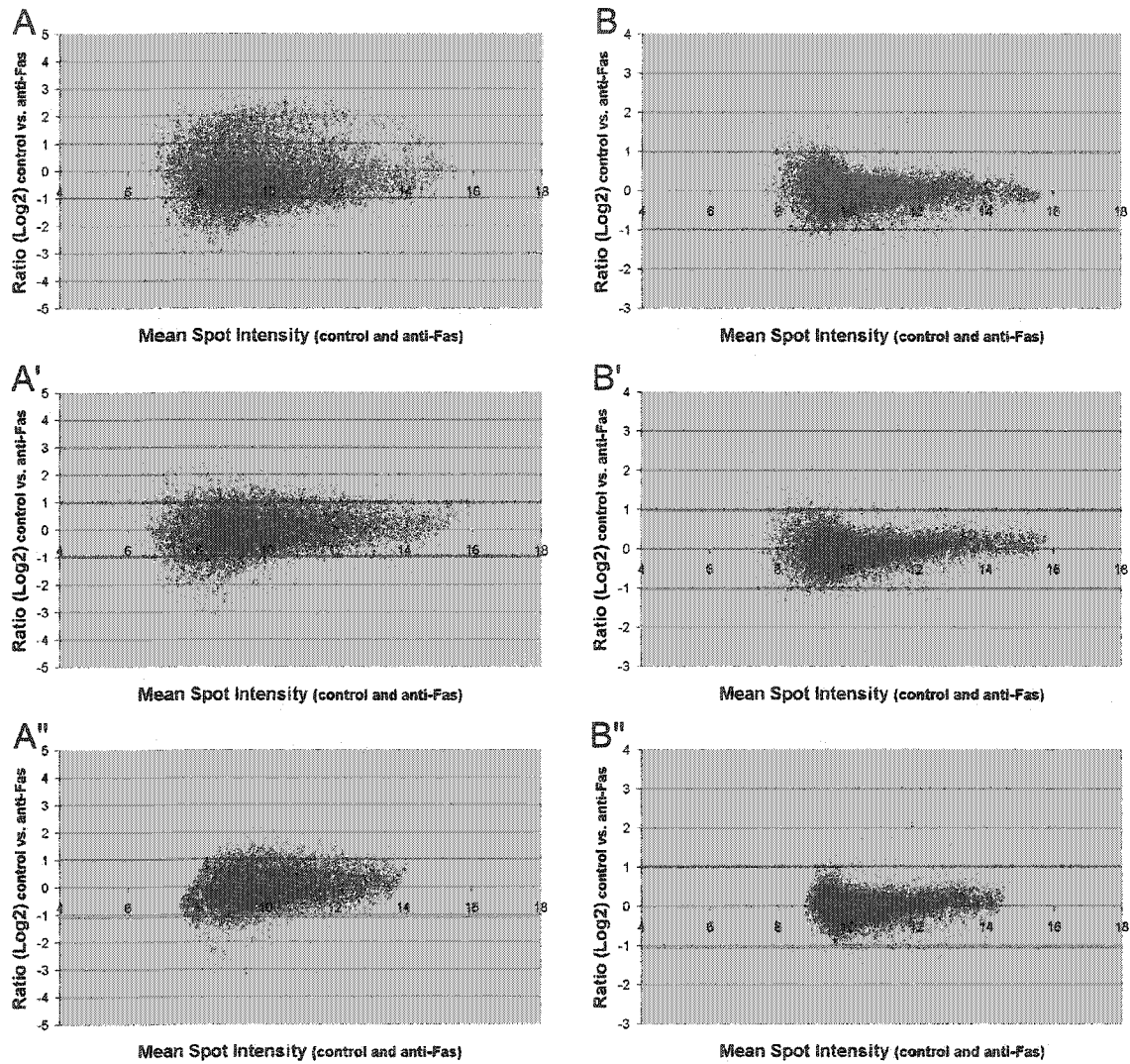


Figure 33 Processing of microarray data

Microarray data from both whole populations (A-A'') and sorted "apparently normal" cells (B-B'') was subject to data processing to remove background signal (A, B), normalise the data using linear regression (A', B') and remove flagged data spots (A'', B''). Data from representative arrays of poor quality (A-A'') and good quality (B-B'') are depicted.

incorporation and fluorescence efficiency of the dyes used to label the two pools of cDNA. Normalisation of the data resulted in a tighter distribution of all data sets and corrected for a significant portion of the error introduced by the poor quality of some microarrays (Figures 33A' and 33B'). Finally, data spots of questionable quality were flagged and removed. Signals due to obvious spots of dirt or scratches on the array, spots with an inadequate signal/background ratio, spots whose intensity was either too low or too high to generate an accurate measurement and spots which showed obvious errors in printing were flagged. Removal of flagged data further reduced the error in datasets, particularly that due to low intensity spots (Figures 33A'' and 33B''). After data processing, the datasets from good quality arrays typically fell in a tight distribution, with only a few spots representative of specific changes exceeding the 2-fold limitation (Figure 33B''). The datasets from poor quality arrays, although much improved, still had a distribution that exceeded the 2-fold limitation slightly, obscuring some of the specific, significant changes in gene expression present in these samples (Figure 33A''). This remaining error was addressed in the data analysis strategy. Data was filtered in Excel to select for spots which demonstrated a mean change in expression of more than 1.5-fold, were not flagged in any of the replicates and which demonstrated consistent up or down-regulation, as assessed by paired t-test. This strategy minimised the detection of changes in gene expression in the anti-Fas treated Jurkat cells, as spots showing legitimate change in expression which happened to fall in an area of poor quality on any of the triplicates would be eliminated due to their lack of reproducibility.

In addition, QPCR verification of select sequences which demonstrated altered expression in the microarray analysis was carried out. Expression of 20 sequences representing the various functional groups of identified genes, but focused particularly on those found to change specifically in early apoptotic cells, were examined using QPCR. 65% of the microarray results were validated by the QPCR analysis, the results of which are reported in Tables 2-11 and Figures 37-38. The validation rate was similar for both the whole population (7/10 sequences) and the sorted “apparently normal” cells (6/10).

Following microarray analysis, change in the expression of 867 sequences, representing 4.5% of the total number of spots interrogated, was identified in anti-Fas treated Jurkats (Figure 34). Very few of these changes were consistently observed in the sorted, “apparently normal” cells; sequences with altered expression in this subpopulation accounted for only 0.2% of total spots (Figure 34A). As expected, the vast majority of the sequences showed no significant change, illustrating the powerful ability of microarray analysis to define limited, specific changes in a large sample. In the population of anti-Fas-treated Jurkats as a whole, gene increases and decreases accounted for almost equal proportions of the changes observed. Two hours appeared to be an early time point for measuring change in gene expression; a greater proportion of sequences were affected at the 4 hour time point, where the majority of the decreased genes were noted (Figure 34B). In the sorted, “apparently normal” cells, decreases accounted for 2/3 of the changes in gene expression observed, but the proportion of decreased genes was more evenly distributed between the two time points (Figure 34A). This distinct accumulation of sequences with decreased expression in the

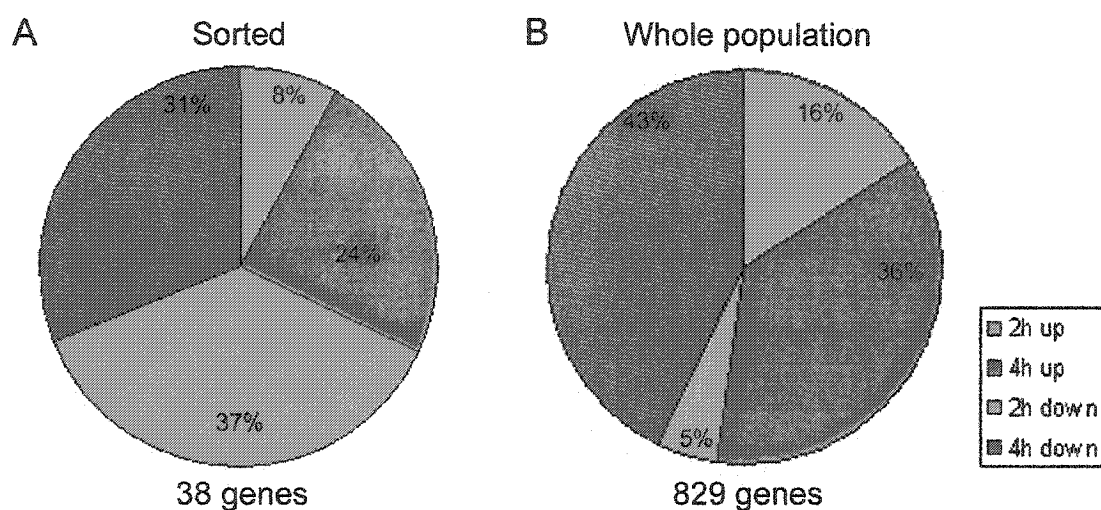


Figure 34 Alterations in gene expression detected in anti-Fas treated Jurkats

Consistent, reproducible gene upregulation (orange) and downregulation (blue) was detected in both sorted "apparently normal" cells (A) and unsorted Jurkats (B) treated with 100 ng/ml anti-Fas using microarray analysis.

“apparently normal” cells at 2 hours suggests that these changes may represent unique events associated with the earliest steps of the apoptotic response.

4.2.2 Identification of genes with altered expression induced by anti-Fas treatment

Sequences with altered expression were identified on the microarrays by a clone ID number. The associated Unigene ID, gene name, symbol, GO and other annotations, description and publication records for each differentially expressed sequence were extracted from the public databases at Stanford, NCBI and the Weizmann Institute. The results of these searches are summarised in Figure 35. For both the sorted “apparently normal cells (Figures 35A, 35A’) and the whole population (Figures 35B, 35B’), useful information was available for only one third of the sequences. The rest were either completely unknown, having no Unigene ID, or uncharacterised, having a Unigene ID number, but for which no further information beyond “EST”, “cDNA” or a numbered protein identifier were available. Thus, the dataset retains an enormous potential for association of novel sequences with the initial events of cell death in Jurkats.

For 274 sequences whose expression in Jurkat cells was altered by anti-Fas treatment, information was available in the public databases. A complete list of identified sequences with increased expression is presented in Appendix B, while those with decreased expression are presented in Appendix C. Based on GO and other annotation supplied by the SOURCE database at Stanford University, these sequences were classified according to function. Three major categories of genes were affected by the induction of apoptosis: those whose products are involved in intracellular signalling, genes which code for proteins associated with RNA

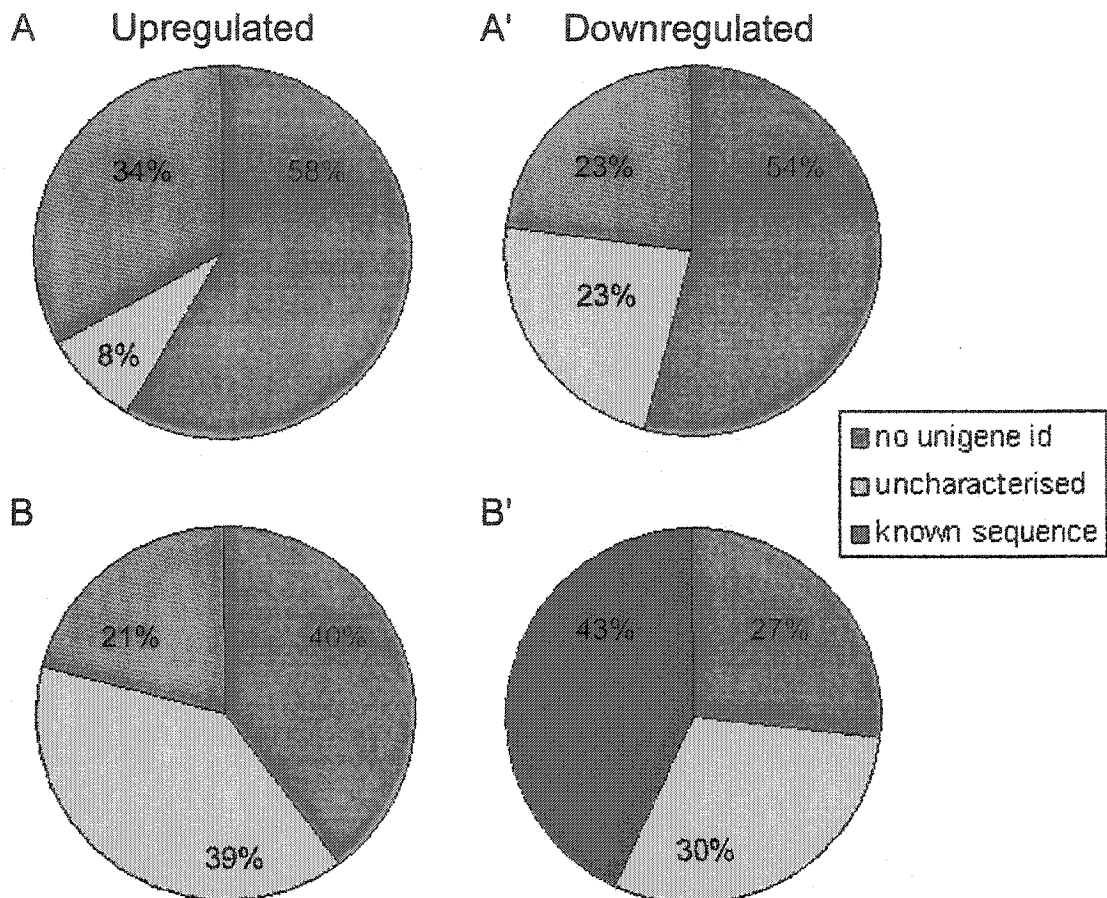


Figure 35 Limited identification of genes with altered expression in anti-Fas treated Jurkats

Sequences in both sorted "apparently normal" (A, A') and whole populations (B, B') of anti-Fas treated Jurkat cells which were upregulated (A, B) or downregulated (A', B') were annotated using public database information. Sequences were classified in three categories: those having no basic expression and sequence record at all in Unigene (NCBI), those assigned a Unigene number but about which no further information existed, and those for which information could be found.

transcription and translation, and those that code for organelle-specific proteins (Figure 36). 12% of the genes were not annotated, or did not fit these broad categories (“other”, Figure 36).

Over half of the identified gene changes observed in anti-Fas-treated Jurkat cells impacted on intracellular signalling pathways (Figure 36), with the vast majority being decreased. Cell signalling sequences were further subdivided into 5 partially overlapping categories: signals associated specifically with apoptosis or cell stress (Table 2), receptors and other membrane proteins (Table 3), kinases/phosphatases (Table 4), proteases (Table 5), and G-protein second messengers (Table 6). Known apoptosis-associated sequences accounted for 5 % of the changes in identified genes (Table 2) and included caspase and bcl-2 -related sequences as well as heat shock proteins. The latter have been implicated in mediation of the apoptotic response through their interactions with Bag-1 chaperones and CXCR4, among other proteins. Among receptor and other membrane protein sequences (Table 3), decreased expression of a number of leukocyte-specific cell surface markers was detected, along with a mixed impact on membrane transporters. Anti-Fas induced equal amounts of increases and decreases in phosphorylation-mediated second messengers (Table 4). Increase of several nuclear proteins along with mixed impact on cell cycle-related sequences were noted. Protease-related molecules affected by anti-Fas treatment (Table 5) included several members of ubiquitin/proteasome-mediated degradation pathways along with molecules implicated in serine protease activity. A substantial number of G-protein signal pathway constituents were affected by anti-Fas treatment of Jurkat cells (Table 6). Most noticeable in this category were

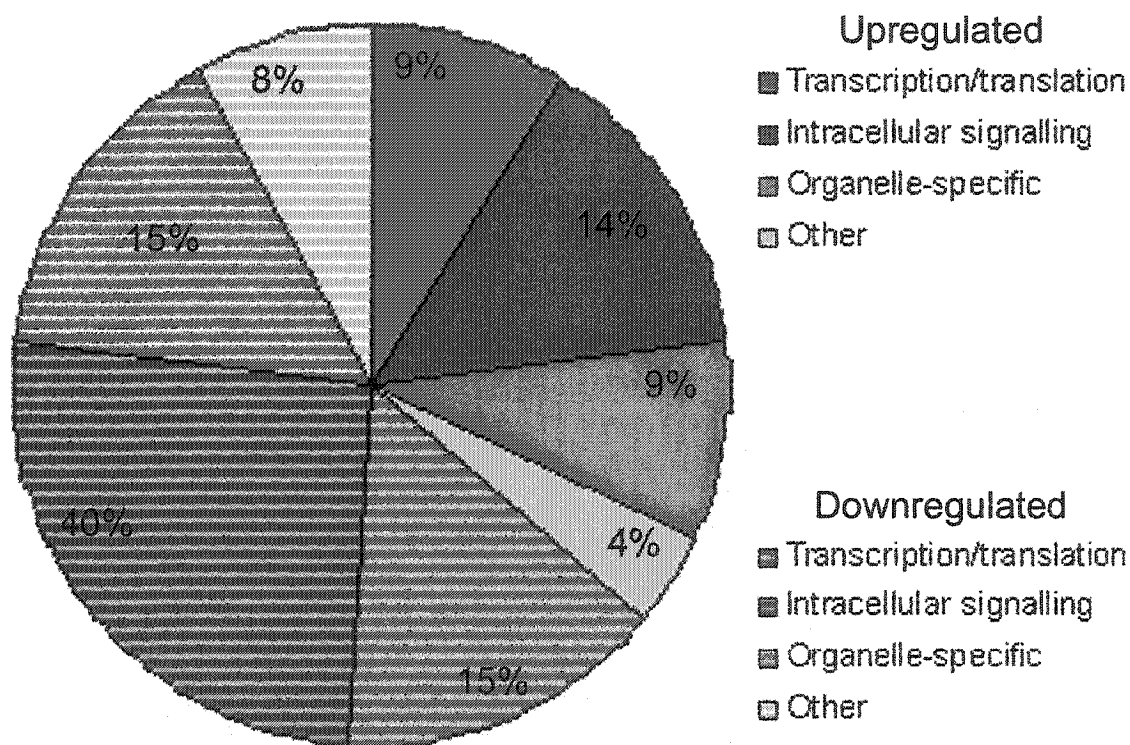


Figure 36 Functional categories of identified genes affected by anti-Fas treatment
 Genes affected by anti-Fas treatment of Jurkat cells, for which information was available, were classified by function, based initially on their GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.168159	bifunctional apoptosis regulator	BFAR	2.77	
Hs.381231	caspase 8, apoptosis-related cysteine protease	CASP8	2.21	1.3
Hs.70582	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	DDX54	1.52	
Hs.180383	dual specificity phosphatase 6	DUSP6	2.46	
Hs.132898	fatty acid desaturase 1	FADS1	1.55	
Hs.169476	glyceraldehyde-3-phosphate dehydrogenase	GAPD	2.32	
Hs.180414	heat shock 70kDa protein 8	HSPA8	1.63	2.25
Hs.15259	BCL2-associated athanogene 3	BAG3	-2.7	
Hs.80338	Bcl-2-associated transcription factor	BTF	-2.4	-1.1
Hs.75189	death-associated protein	DAP	-1.8	
Hs.75652	glutathione S-transferase M5	GSTM5	-1.6	
Hs.75452	heat shock 70kDa protein 1A	HSPA1A	-2.3	-2.1
Hs.284279	heme oxygenase (decycling) 2	HMOX2	-1.7	
Hs.406455	prosaposin	PSAP	-1.5	
Hs.115721	protease, serine, 25	PRSS25	-1.6	

Table 2 Apoptosis-related genes affected by anti-Fas treatment of Jurkat cells

Fourteen gene sequences affected by anti-Fas treatment of Jurkat cells were identified as playing a role in apoptosis, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.90107	adhesion regulating molecule 1	ADRM1	3.08	
Hs.84999	ATPase, Cu++ transporting, beta polypeptide (Wilson disease)	ATP7B	2.71	
Hs.168159	bifunctional apoptosis regulator	BFAR	2.77	
Hs.454442	chromosome 20 open reading frame 142	C20orf142	1.52	
Hs.198612	G protein-coupled receptor 51	GPR51	2.32	
Hs.355851	gamma-aminobutyric acid (GABA) A receptor, gamma 2	GABRG2	1.69	
Hs.76828	glypican 5	GPC5	2.24	
Hs.8088	Sec 6 (S. cerevisiae) homolog	SEC6	1.57	
Hs.113602	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6	SLC1A6	1.68	
Hs.1074	surfactant, pulmonary-associated protein C	SFTPC	1.52	
Hs.9572	adenylate cyclase 5	ADCY5	-1.7	
Hs.75741	amiloride binding protein 1 (amine oxidase (copper-containing))	ABP1	-1.7	
Hs.75736	apolipoprotein D	APOD	-1.7	5.5
Hs.75478	ATPase, Class VI, type 11B	ATP11B	-1.7	
Hs.134514	ATP-binding cassette, sub-family A (ABC1), member 7	ABCA7	-1.7	
Hs.55879	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	ABCC10	-1.6	
Hs.75929	cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	-1.7	
Hs.79630	CD79A antigen (immunoglobulin-associated alpha)	CD79A	-1.8	
Hs.5298	CGI-45 protein	CGI-45	-1.7	
Hs.89414	chemokine (C-X-C motif) receptor 4	CXCR4	-2.4	
Hs.132834	hematopoietic protein 1	HEM1	-1.5	
Hs.424053	homer homolog 3 (Drosophila)	HOMER3	-1.8	
Hs.101257	hypothetical protein MGC3295	MGC3295	-1.6	
Hs.79356	Lysosomal-associated multispinning membrane protein-5	LAPTM5	-1.5	
Hs.77961	major histocompatibility complex, class I, B	HLA-B	-3.4	
Hs.271954	pan-hematopoietic expression	PHEMX	-1.9	
Hs.296361	potassium inwardly-rectifying channel, subfamily J, member 13	KCNJ13	-1.7	1.29
Hs.298184	potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2	-1.7	
Hs.406455	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	-1.5	
Hs.27621	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	SEMA5A	-1.9	
Hs.301871	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	SLC37A1	-1.6	
Hs.406590	T-cell activation protein	PGR1	-2.2	
Hs.125359	Thy-1 cell surface antigen	THY1	-1.6	

Table 3 Gene sequences for membrane proteins demonstrate change in expression induced by anti-Fas treatment of Jurkat cells

Thirty-three gene sequences affected by anti-Fas treatment of Jurkat cells were identified as coding for receptors and other membrane proteins, based on GO and other annotation provided by the SOURCE, NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.18268	adenylate kinase 5	AK5	3.97	
Hs.194382	ataxia telangiectasia mutated (includes complementation groups A, C and D)	ATM	1.51	
Hs.18586	CDC42 binding protein kinase alpha (DMPK-like)	CDC42BPA	1.92	
Hs.82932	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	1.73	
Hs.180383	dual specificity phosphatase 6	DUSP6	2.46	
Hs.321045	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	1.91	
Hs.50651	Janus kinase 1 (a protein tyrosine kinase)	JAK1	2.39	2.6
Hs.211586	phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	PIK3R2	1.69	
Hs.103081	ribosomal protein S6 kinase, 70kDa, polypeptide 2	RPS6KB2	1.60	
Hs.23348	S-phase kinase-associated protein 2 (p45)	SKP2	1.51	
Hs.75393	acid phosphatase 1, soluble	ACP1	-1.6	
Hs.8417	calcium/calmodulin-dependent protein kinase kinase 1, alpha	CAMKK1	-1.6	
Hs.166071	cyclin-dependent kinase 5	CDK5	-1.9	- 3.9
Hs.695	cystatin B (stefin B)	CSTB	-1.6	
Hs.7835	mannose receptor, C type 2	MRC2	-2.2	
Hs.23643	Mst3 and SOK1-related kinase	MST4	-1.6	
Hs.48802	myotubularin related protein 9	MTMR9	-2.6	
Hs.315366	protein kinase C, eta	PRKCH	-1.8	
Hs.80350	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	PPP2CB	-2.8	- 2.7
Hs.10037	Sac domain-containing inositol phosphatase 3	SAC3	-1.5	
Hs.26054	SH2 domain containing 3C	SH2D3C	-1.6	
Hs.146355	v-abl Abelson murine leukemia viral oncogene homolog 1	ABL1	-1.7	

Table 4 Kinase and phosphatase genes affected by anti-Fas treatment of Jurkat cells

Twenty-two kinase or phosphatase gene sequences affected by anti-Fas treatment of Jurkat cells were identified, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.23348	S-phase kinase-associated protein 2 (p45)	SKP2	1.51	
Hs.423615	cell division cycle 34	CDC34	-1.6	
Hs.695	cystatin B (stefin B)	CSTB	-1.6	
Hs.108327	damage-specific DNA binding protein 1, 127kDa	DDB1	-2.0	
Hs.194477	E3 ubiquitin ligase SMURF2	SMURF2	-1.6	-3.2
Hs.59242	furin (paired basic amino acid cleaving enzyme)	FURIN	-1.7	
Hs.14456	neural precursor cell expressed, developmentally down-regulated 1	NEDD1	-1.6	
Hs.75512	neural precursor cell expressed, developmentally down-regulated 8	NEDD8	-2.0	
Hs.419094	protease, serine, 1 (trypsin 1)	PRSS1	-1.6	
Hs.115721	protease, serine, 25	PRSS25	-1.6	
Hs.79387	proteasome (prosome, macropain) 26S subunit, ATPase, 5	PSMC5	-1.6	
Hs.76913	proteasome (prosome, macropain) subunit, alpha type, 5	PSMA5	-2.1	
Hs.76913	proteasome (prosome, macropain) subunit, alpha type, 5	PSMA5	-2.8	
Hs.118926	septrin/SUMO-specific protease 3	SEN3	-1.8	
Hs.234726	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	SERPINA3	-1.6	
Hs.159509	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	SERPINF2	-1.9	-1.9
Hs.27258	Siah-interacting protein	SIP	-2.2	
Hs.75355	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	UBE2N	-2.2	
Hs.11184	ubiquitin-conjugating enzyme E2R 2	UBE2R2	-1.7	

Table 5 Protease-associated genes demonstrate change in expression in anti-Fas treated Jurkat cells

Eighteen protease-associated gene sequences affected by anti-Fas treatment of Jurkat cells were identified, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.24340	centaurin, beta 2	CENTB2	1.56	
Hs.302161	endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	EDG8	1.52	
Hs.198612	G protein-coupled receptor 51	GPR51	2.32	
Hs.340636	GTP binding protein 5 (putative)	GTPBP5	2.00	
Hs.79307	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	ARHGEF6	2.97	2.3
Hs.9347	regulator of G-protein signalling 14	RGS14	1.64	
Hs.25951	Rho guanine nucleotide exchange factor (GEF) 3	ARHGEF3	1.65	
Hs.172813	Rho guanine nucleotide exchange factor (GEF) 7	ARHGEF7	1.64	
Hs.8088	Sec 6 (S. cerevisiae) homolog	SEC6	1.57	
Hs.241503	spastic paraplegia 3A (autosomal dominant)	SPG3A	1.69	
Hs.348024	v-ras simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	RALB	2.52	
Hs.41654	CAMP-binding guanine nucleotide exchange factor IV (cAMP-GEFIV) mRNA, clone W15, partial sequence		-1.8	
Hs.6522	elongation factor for selenoprotein translation	SELB	-1.8	
Hs.56845	GDP dissociation inhibitor 2	GDI2	-2.2	
Hs.51147	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	GNAT1	-1.7	
Hs.155090	guanine nucleotide binding protein (G protein), beta 5	GNB5	-1.5	
Hs.78993	IQ motif containing GTPase activating protein 2	IQGAP2	-1.6	
Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-1.6	
Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-1.8	
Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-2.1	
Hs.16258	RAB24, member RAS oncogene family	RAB24	-1.8	
Hs.77273	ras homolog gene family, member A	ARHA	-2.9	
Hs.75082	ras homolog gene family, member G (rho G)	ARHG	-1.7	
Hs.194695	ras homolog gene family, member I	ARHI	-1.7	

Table 6 Gene sequences for G proteins demonstrate change in expression induced by anti-Fas treatment of Jurkat cells

Twenty-four gene sequences affected by anti-Fas treatment of Jurkat cells were identified as coding for G-protein signalling components, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

components known to impact on JNK signalling and the cytoskeleton.

Changes in genes involved in chromatin configuration, transcription, RNA processing and translation accounted for 24% of the identified sequences affected by anti-Fas treatment of Jurkat cells (Figure 36). The predominant trend in this category was decrease in mRNA, as typified by the example of genes involved in transcription and RNA processing (Table 7).

Organelle-specific genes accounted for a further 24% of the identified sequences affected by anti-Fas treatment of Jurkat cells (Figure 36). While mitochondria and ER/Golgi-associated sequences showed equal amounts of decrease and increase (Tables 8 and 9), genes for cytoskeletal and extracellular matrix components (Table 10) showed a distinct trend of decreased expression. Amongst the mitochondrial genes (Table 8), sequences related to both energy generation and mitochondrial translation were affected. ER/Golgi sequences affected by anti-Fas treatment (Table 9) included a number of vesicle coat proteins involved in endocytotic trafficking (AP3B2, AP3D1, COPA, COPB, SEC6, STX10). Sequences coding for cytoskeletal and ECM proteins (Table 10) with decreased expression included genes for proteins that mediate cytoskeletal interaction with the cell periphery (FBLN2, ECM2, NDST3, PSG11, ITGA4, LAD1). The less common increased sequences included several actin-interacting proteins.

4.2.3 Identification of candidate genes implicated in the initial steps of the apoptotic response A very limited subset of the spots interrogated by microarray showed change specific to sorted cells, and amongst this select group a unique trend of decrease at 2h was observed (Figure 34A). Consistent with this result, a number of identified genes uniquely

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.380969	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	COPS5	3.65	
Hs.31551	core-binding factor, runt domain, alpha subunit 2; translocated to, 1; cyclin D-related	CBFA2T1	1.63	
Hs.406404	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	HNRPD	1.51	
Hs.301005	histone H2A.F/Z variant	H2AV	2.03	
Hs.314169	KIAA1618 protein	KIAA1618	1.56	
Hs.405961	old astrocyte specifically induced substance	OASIS	1.71	
Hs.101742	ribosomal large subunit pseudouridine synthase C like	RLUCL	4.28	
Hs.195614	splicing factor 3b, subunit 3, 130kDa	SF3B3	2.05	
Hs.193989	TAR DNA binding protein	TARDBP	4.49	
Hs.142157	transcription termination factor, RNA polymerase II	TTF2	1.63	
Hs.343586	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	2.62	
Hs.32511	zinc finger protein 450	ZNF450	1.66	
Hs.80338	Bcl-2-associated transcription factor	BTF	-2.4	-1.1
Hs.183085	CCR4-NOT transcription complex, subunit 6-like	CNOT6L	-1.7	
Hs.108371	E2F transcription factor 4, p107/p130-binding	E2F4	-2.0	-2.9
Hs.154365	E74-like factor 1 (ets domain transcription factor)	ELF1	-1.5	
Hs.48576	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	ERCC5	-1.6	
Hs.256583	interleukin enhancer binding factor 3, 90kDa	ILF3	-2.5	-2.0
Hs.373499	NS1-associated protein 1	NSAP1	-1.6	
Hs.100221	nuclear receptor subfamily 1, group H, member 2	NR1H2	-1.7	
Hs.84928	nuclear transcription factor Y, beta	NFYB	-1.6	
Hs.296585	nucleolar protein 5A (56kDa with KKE/D repeat)	NOL5A	-1.8	
Hs.154104	pleiomorphic adenoma gene-like 2	PLAGL2	-1.5	
Hs.171880	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	POLR2A	-1.6	
Hs.421667	POP4 (processing of precursor, S. cerevisiae) homolog	POP4	-2.0	
Hs.361071	retinoic acid receptor, alpha	RARA	-1.6	
Hs.202430	ring finger protein 1	RING1	-1.6	
Hs.83715	Sjogren syndrome antigen B (autoantigen La)	SSB	-1.6	
Hs.334826	splicing factor 3b, subunit 1, 155kDa	SF3B1	-1.6	
Hs.406186	splicing factor 3b, subunit 4, 49kDa	SF3B4	-1.6	
Hs.406186	splicing factor 3b, subunit 4, 49kDa	SF3B4	-1.7	
Hs.433581	splicing factor, arginine/serine-rich 11	SFRS11	-1.7	
Hs.146355	v-abl Abelson murine leukemia viral oncogene homolog 1	ABL1	-1.7	
Hs.149923	X-box binding protein 1	XBP1	-1.7	
Hs.78434	zinc finger protein 133 (clone pHZ-13)	ZNF133	-1.7	
Hs.145956	zinc finger protein 226	ZNF226	-1.5	

Table 7 RNA transcription and processing-associated genes demonstrate change in expression in anti-Fas treated Jurkat cells Thirty-six transcription-associated gene sequences affected by anti-Fas treatment of Jurkat cells were identified, based on GO and other annotation provided by the SOURCE database at Stanford University, and information contained in the NCBI and Weizmann Institute databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change
Hs.193974	glutathione reductase	GSR	1.66
Hs.380929	lactate dehydrogenase D	LDHD	1.74
Hs.109059	mitochondrial ribosomal protein L12	MRPL12	1.92
Hs.211914	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	NDUFS7	1.72
Hs.75438	quinoid dihydropteridine reductase	QDPR	5.28
Hs.8372	ubiquinol-cytochrome c reductase (6.4kD) subunit	UQCR	1.54
Hs.81634	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit b, isoform 1	ATP5F1	-1.7
Hs.155433	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP5C1	-2.4
Hs.433307	branched chain keto acid dehydrogenase E1, alpha polypeptide (maple syrup urine disease)	BCKDHA	-1.6
Hs.29331	carnitine palmitoyltransferase 1B (muscle)	CPT1B	-1.7
Hs.149894	mitochondrial translational initiation factor 2	MTIF2	-1.5
Hs.51299	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	NDUFV2	-1.5
Hs.184877	solute carrier family 25 (mitochondrial carrier, oxoglutarate carrier), member 11	SLC25A11	-2.1

Table 8 Genes for mitochondrial proteins affected by anti-Fas treatment of Jurkat cells

Thirteen gene sequences associated with mitochondria or energy generation which were affected by anti-Fas treatment of Jurkat cells were identified, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.21022	adaptor-related protein complex 3, beta 2 subunit	AP3B2	1.71	
Hs.127675	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	CLN8	1.72	1.66
Hs.79946	cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	1.62	
Hs.104117	cytochrome P450, family 3, subfamily A, polypeptide 5	CYP3A5	1.86	
Hs.104117	cytochrome P450, family 3, subfamily A, polypeptide 5	CYP3A5	1.63	
Hs.79222	galactosidase, beta 1	GLB1	1.52	
Hs.182982	golgin-67	GOLGIN-67	1.76	
Hs.1480	histidine rich calcium binding protein	HRC	1.85	
Hs.77665	KIAA0102 gene product	KIAA0102	2.79	
Hs.25253	mannosidase, alpha, class 1A, member 1	MAN1A1	1.81	
Hs.298229	prefoldin 2	PFDN2	3.04	
Hs.8088	Sec 6 (S. cerevisiae) homolog	SEC6	1.57	
Hs.334725	similar to putative transmembrane protein; homolog of yeast Golgi membrane protein Yif1p (Yip1p-interacting factor)	LOC90522	2.17	
Hs.458032	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	TGM2	2.13	
Hs.75616	24-dehydrocholesterol reductase	DHCR24	-2.1	
Hs.379781	adaptor-related protein complex 3, delta 1 subunit	AP3D1	-1.6	
Hs.75887	coatamer protein complex, subunit alpha	COPA	-1.6	
Hs.3059	coatamer protein complex, subunit beta	COPB	-1.7	
Hs.274464	diaphorase (NADH) (cytochrome b-5 reductase)	DIA1	-2.1	
Hs.154332	ER degradation enhancing alpha mannosidase-like	EDEM	-1.8	
Hs.59242	furin (paired basic amino acid cleaving enzyme)	FURIN	-1.7	
Hs.172647	golgi autoantigen, golgin subfamily a, 1	GOLGA1	-2.7	- 1.3
Hs.77665	KIAA0102 gene product	KIAA0102	-1.5	
Hs.410578	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	P4HB	-1.5	
Hs.16258	RAB24, member RAS oncogene family	RAB24	-1.8	
Hs.75268	sialyltransferase 4C (beta-galactoside alpha-2,3-sialyltransferase)	SIAT4C	-1.7	
Hs.43812	syntaxin 10	STX10	-1.5	
Hs.2022	transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)	TGM3	-1.7	

Table 9 Gene sequences for ER and Golgi proteins demonstrate change in expression induced by anti-Fas treatment of Jurkat cells

Twenty-eight sequences affected by anti-Fas treatment of Jurkat cells were identified as coding for ER or Golgi components, based on GO and other annotation provided by the SOURCE, NCBI and Weizmann Institute public databases.

Unigene ID	Gene Name	Gene Symbol	Microarray Fold-change	QPCR Fold-change
Hs.333257	advillin	AVIL	2.32	
Hs.433989	decorin	DCN	1.65	
Hs.298229	prefoldin 2	PFDN2	3.04	
Hs.352054	pregnancy specific beta-1-glycoprotein 9	PSG9	1.56	
Hs.8088	Sec 6 (S. cerevisiae) homolog	SEC6	1.57	
Hs.75741	amiloride binding protein 1 (amine oxidase (copper-containing))	ABP1	-1.7	
Hs.9613	angiopoietin-like 4	ANGPTL4	-2.3	
Hs.75929	cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	-1.7	
Hs.82916	chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A	-1.8	
Hs.146428	collagen, type V, alpha 1	COL5A1	-1.7	
Hs.235368	collagen, type V, alpha 3	COL5A3	-1.8	
Hs.108885	collagen, type VI, alpha 1	COL6A1	-1.6	
Hs.2441	C-type lectin BIMLEC precursor	BIMLEC	-1.6	
Hs.35094	extracellular matrix protein 2, female organ and adipocyte specific	ECM2	-1.5	-1.8
Hs.198862	fibulin 2	FBLN2	-1.5	
Hs.29283	hyaluronan and proteoglycan link protein 3	HAPLN3	-1.7	
Hs.40034	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	ITGA4	-1.9	
Hs.166184	intersectin 2	ITSN2	-1.7	
Hs.18141	ladinin 1	LAD1	-1.6	
Hs.20028	N-deacetylase/N-sulfotransferase (heparan glucosaminy) 3	NDST3	-1.9	-4.2
Hs.73947	peptidase D	PEPD	-1.7	
Hs.75737	pericentriolar material 1	PCM1	-1.7	
Hs.398049	pregnancy specific beta-1-glycoprotein 11	PSG11	-1.8	
Hs.77273	ras homolog gene family, member A	ARHA	-2.9	

Table 10 Cytoskeleton and ECM-associated genes demonstrate change in expression in anti-Fas treated Jurkat cells

Twenty-four gene sequences coding for cytoskeletal or extracellular matrix proteins affected by anti-Fas treatment of Jurkat cells were identified, based on GO and other annotation provided by the SOURCE database at Stanford University, and supplemented by information contained in the NCBI and Weizmann Institute public databases.

decreased at 2 hours in the sorted “apparently normal” cells were noted (Table 11). They included SERPIN F2 (Figure 37A) and SMURF2, which codes for an E3 ligase, as well as NDST3 and ECM2, sequences for proteins involved in cell/matrix interactions. Sequences with this expression pattern could arguably represent genes involved in the initial response to the apoptotic insult. Sequences which changed uniquely in the “apparently normal” cells at the 4 hour time point coded for the cognate heat shock 70 kDa protein (Hsc70) and the ER membrane protein CLN8 (Figure 37B) and are also excellent candidates for genes involved in the initial steps of apoptosis. However, studies of apoptosis in the presence of inhibitors of transcription and translation have revealed that gene-dependant modulation of the death response can occur in these cells (Kim et al., 2000; Chang et al., 2002b). Thus, the potential contribution of these genes to mitigation, rather than promotion of the cell death signal cannot be ruled out. Clarification of their roles will depend on further testing.

Some of the identified sequences interrogated by microarray analysis demonstrated changes in both the unsorted and sorted subpopulations, providing a strong indicator of their involvement in the apoptotic response, although the proteins that they code for may be involved in events of the effector stages of apoptosis, rather than its initiation. These sequences included AP3B2, which codes for an ER adaptor complex protein and ADRM1, whose protein product is involved in cell adhesion (Figure 38A).

In contrast to the above select groups, most of the identified sequences altered in

<u>Name</u>	<u>Clone ID</u>	<u>UGCluster</u>	<u>Microarray mean fold change ($\pm$SEM)</u>	<u>QPCR fold change</u>
<u>Upregulated</u>				
CASP8: caspase 8, apoptosis-related cysteine protease	125581	Hs.381231	2.21 $\pm$ 0.31	1.28
hypothetical protein	308830	Hs.349344 or Hs.293615	1.74 $\pm$ 0.2	nd
CLN8: ceroid-lipofuscinosis, neuronal 8	272612	Hs.127675	1.72 $\pm$ 0.21	1.66
HSPA8: heat shock 70kDa protein 8	208059	Hs.180414	1.63 $\pm$ 0.14	2.25
EIF3S6: eukaryotic translation initiation factor 3, subunit 6 48kDa	5548410	Hs.106673	1.62 $\pm$ 0.08	-3.54
<u>Downregulated</u>				
NDST3: N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	125806	Hs.20028	-1.9 $\pm$ 0.09	-4.21
SERPINF2: serine (or cysteine) proteinase inhibitor (alpha-2 antiplasmin), member 2	212807	Hs.159509	-1.9 $\pm$ 0.26	-1.92
HOMER3: homer homolog 3 (Drosophila)	5847747	Hs.424053	-1.8 $\pm$ 0.25	nd
APOD: apolipoprotein D	4042897	Hs.75736	-1.7 $\pm$ 0.17	5.54
Homo sapiens cDNA FLJ35131 fis, clone PLACE6008824.	291575	Hs.43744	-1.7 $\pm$ 0.23	nd
KCNJ13: potassium inwardly-rectifying channel, subfamily J, member 13	4814905	Hs.296361	-1.7 $\pm$ 0.06	1.29
SMURF2: E3 ubiquitin ligase	35711	Hs.194477	-1.6 $\pm$ 0.03	-3.23
Homo sapiens, clone IMAGE:3937520, mRNA, partial cds	179266	Hs.250879	-1.6 $\pm$ 0.06	nd
FLJ12085: hypothetical protein FLJ12085	377586	Hs.48827	-1.6 $\pm$ 0.12	nd
ESTs	34598	Hs.356349	-1.5 $\pm$ 0.05	nd
ECM2: extracellular matrix protein 2, female organ and adipocyte specific	124065	Hs.35094	-1.5 $\pm$ 0.07	-1.79
FLJ21615: hypothetical protein FLJ21615	258763	Hs.44159	-1.5 $\pm$ 0.13	nd

Table 11 Changes in gene expression specific to early apoptotic Jurkat cells
Sequences affected by anti-Fas treatment of Jurkat cells specifically in the sorted “apparently normal” subpopulation, and for which information was available in the NCBI and Weizmann Institute public databases, were first identified using microarray analysis, then confirmed using QPCR.

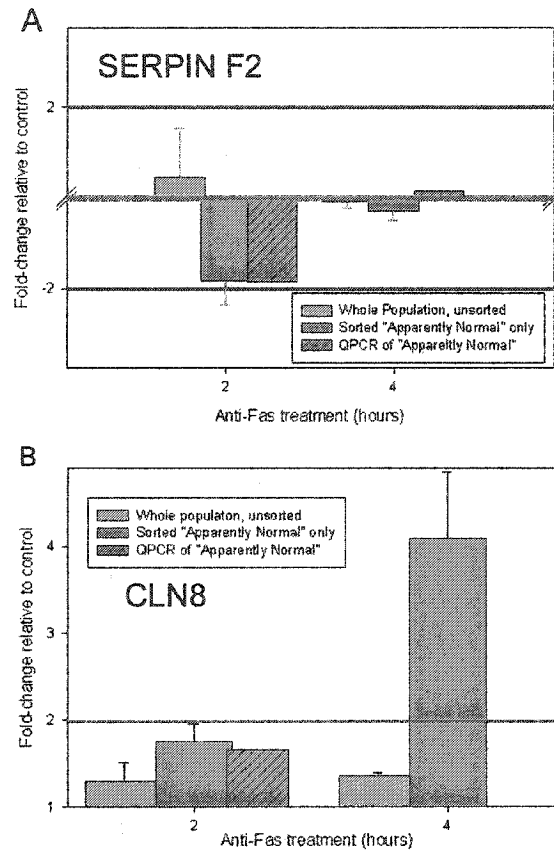


Figure 37 Changes in gene expression specific to sorted "apparently normal" Jurkat cells induced to die by anti-Fas treatment

Alterations in gene expression relative to controls and specific to the sorted "apparently normal" cell subpopulation were detected in anti-Fas treated Jurkat cultures at both 2 hours, as depicted for the representative sequence SERPINF2 (A), and 4 hours, represented by the sequence for the ER protein CLN8 (B).

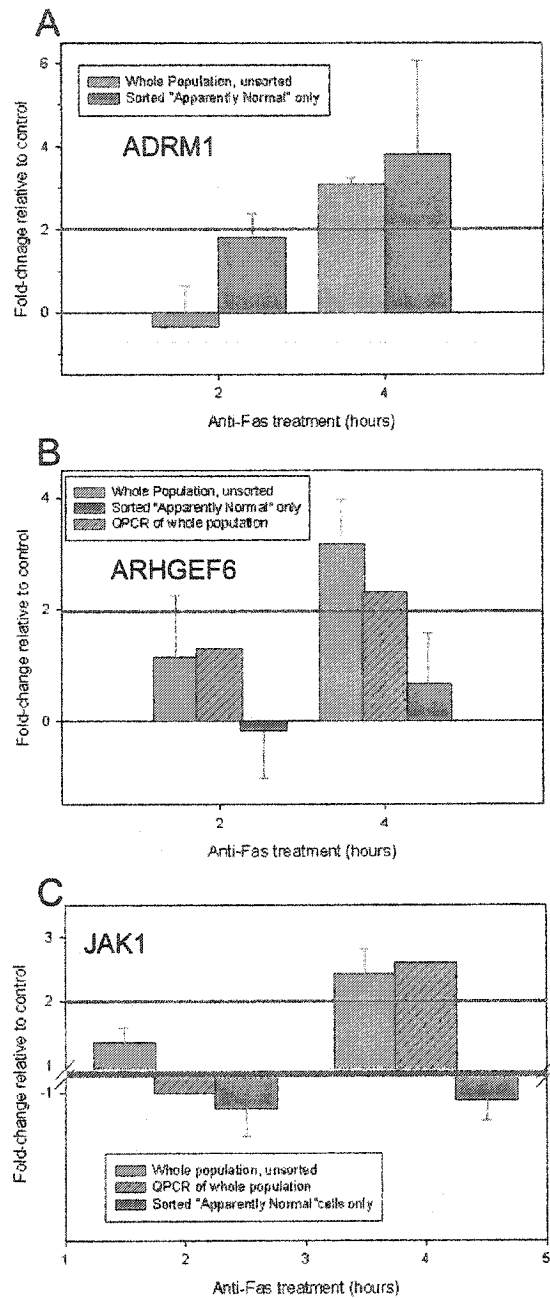


Figure 38 Changes in gene expression in unsorted Jurkat cells induced to die by anti-Fas treatment
Alterations in gene expression relative to controls were detected in anti-Fas treated Jurkat cultures, as depicted for the representative sequences for adhesion regulating molecule 1, ADRM1 (A), the Rac/cdc42 GEF, ARHGEF6 (B), and Janus kinase 1, JAK1 (C).

anti-Fas treated Jurkat cells changed significantly in the unsorted population only. Their involvement in the apoptotic response would thus be limited to the effector phase of the cell death process. The genes coding for Rac/cdc42 guanine nucleotide exchange factor (ARHGEF6, Figure 38B) and Janus kinase 1(JAK1, Figure 38C) are typical examples.

4.3 DISCUSSION

Microarray analysis revealed that Fas-mediated apoptosis resulted in change in gene expression. Changes in 4.7% of interrogated sequences were detected, a result comparable with proteomic analysis of the same system, which found alterations in 5.3% of the 1000 proteins surveyed (Gerner et al., 2000). The magnitude of the changes I detected was modest, in keeping with the short experimental time frame. Altered gene expression was confirmed using QPCR in a selected set of genes. 65% of gene changes observed by microarray were confirmed by QPCR in the sample set, a reasonable rate given that most changes lay close to the cut off for non-specific noise in the microarray system. Gene changes specific to early apoptotic cells were detected, which could be considered candidate initiators of the apoptotic response in receptor-mediated death of Jurkats. Functional analysis of altered sequences predicts some impact of Fas-mediated signalling on TGF- β pathway components, mitochondrial homeostasis and the actin cytoskeleton.

4.3.1 Impact of Fas-mediated apoptosis on gene expression Changes in expression of a select set of genes were manifest particularly in the sorted “apparently normal” subpopulation, indicating that the products of these sequences could participate in

initiation of the cell death pathway (Table 11). Would alterations in gene expression be manifest at the protein level in this subpopulation? Early apoptotic cells do not display cytoplasmic changes associated with apoptosis, and may well contain intact protein translation machinery in their cytoplasm. In addition, a mechanism of differential translation of RNA in apoptotic cells has been described. Sequences whose mRNAs form IRES structures, including XIAP, have been shown to be preferentially preserved and translated in dying cells (Holcik et al., 2000a; Holcik et al., 2000b). This mechanism may allow for the alterations in specific mRNA sequences detected in early apoptotic cells to be manifested at the protein level during later stages of the cell death path. Indeed, changes at the protein level are detected 5-12 hours after initiation of apoptosis by anti-Fas in this system (Gerner et al., 2000).

Although microarray analysis demonstrated that the impact of anti-Fas mediated apoptosis on gene expression involved both up and down-regulation in almost equal measure, particularly in the unsorted cells (Figure 34), analysis of identified sequences by function predicts profound differences in the effect of Fas signalling on particular cellular processes or compartments (Figure 36, Tables 2-10). Results predict a shutdown of several key cellular systems. Generation of RNA and proteolytic degradation of proteins would be compromised (Tables 5 and 7), and an interruption in normal vesicular trafficking is predicted by some of the changes in both ER and cytoskeleton-related sequences (Tables 9 and 10). In contrast, kinase/phosphatase and G protein second messenger sequences demonstrated changes more representative of diverse, pathway-specific effects (Tables 4 and 6). Fas treatment also produced a mixed effect on

mitochondria and known apoptosis-related sequences (Tables 2 and 8).

4.3.2 Cell death induced by the Fas receptor may impact on the TGF- β signalling path

The microarray analysis carried out for this thesis revealed possible cross-talk between the Fas receptor and the TGF- β signaling path. TGF- β signaling has been associated with growth arrest, differentiation and cell death in different contexts (Moustakas et al., 2001; Shi and Massague, 2003). The known effects of TGF- β on T cells are complex. In developing and peripheral T cells in TGF- β knockout mice, as well as during AICD of T cells in vitro, this pathway has an anti-apoptotic effect (Genestier et al., 1999; Chen et al., 2001). However, during the post-activation phase of wound healing, TGF- β promotes apoptosis of activated T cells, through cytokine-dependance pathways (Sillett et al., 2001).

Decreased mRNA for SMURF2 (Table 5), the E3 ubiquitin ligase which mediates the degradation of three of the transcription factors (SMADs 2, 3 and 7) responsible for TGF- β -driven events was detected specifically in early apoptotic Jurkat cells (Table 11). Crosstalk from the Fas receptor to elements of the TGF- β signaling pathway has been described only recently in lung epithelial cells and mouse liver (Hagimoto et al., 2002; Leu et al., 2003). My results indicate that decreased degradation of SMADs, due to more limited amounts of SMURF2, may contribute to this positive cross-talk.

The downstream targets of SMADs 3, 2 and 7 are so diverse and context-specific that no easy prediction of outcome can be made (Attisano and Wrana, 2003). One of the important known effects of TGF- β signaling is the repression of myc, mediated by a complex containing SMAD 3, SMAD 4, E2F4 and E2F5 (Chen et al., 2002). However,

decreased amounts of E2F4 mRNA was detected by microarray analysis (Table 7); thus, in anti-Fas treated Jurkats, repression of myc is not likely. The most suitable SMAD-mediated effect to examine in Fas-treated Jurkats cells may be expression of DAP kinase, a known death-associated protein first identified in HeLa cells and recently reported to be induced by SMADs 2, 3 and 4 as part of the TGF- β apoptotic response (Deiss et al., 1995; Jang et al., 2002). It is known to participate in Fas-induced apoptosis, acting upstream of cytochrome c release, loss of mitochondrial membrane potential and activation of effector caspases (Cohen et al., 1999; Jang et al., 2002). DAP kinase is associated with the cytoskeleton and was recently shown to regulate membrane blebbing during receptor-mediated apoptosis (Cohen et al., 1997; Inbal et al., 2002). Consistent with my data predicting that increased DAP kinase expression would occur following the initial nuclear events of Fas-mediated apoptosis, nuclear fragmentation was not dependant on DAPK (Inbal et al., 2002).

4.3.3 Fas-mediated changes in gene expression may affect mitochondrial

homeostasis The changes in gene expression detected in anti-Fas treated Jurkats may impact on mitochondrial homeostasis through heat shock protein, the ER protein CLN8 and JNK expression.

Expression of the cognate isoform of heat shock 70 kDa protein (Hsc70) was increased in early apoptotic cells (Table 11). Hsc70, along with co-chaperones Hsp40 and Bag-1, has been implicated in import and refolding of mitochondrial proteins as well as cytoplasmic sequestration of p53 (Terada et al., 1997; Terada and Mori, 2000; Zyllicz et al., 2001). In Jurkat cells, conflicting reports testify to both inhibition and

enhancement of apoptosis mediated by Hsp70, including a role early in the apoptotic response, before loss of mitochondrial membrane potential (Liossis et al., 1997; Creagh et al., 2000; Ricci et al., 2001). This conflicting evidence may reflect the existence of over 11 human Hsp70 isoforms, and the lack of precision in defining isoform-specific effects in the reports published. Hsc70 in particular may impact on mitochondria via several mechanisms. Provocatively, recent reports suggest that p53 can exert a transcription-independent proapoptotic effect directly on mitochondria, in part through binding of partners such as WOX1 and heat shock 70 Kda proteins (Marchenko et al., 2000; Chang, 2002).

The ER transmembrane protein CLN8 was also increased specifically in early apoptotic cells (Tables 9 and 11). Recently, destabilisation of mitochondrial homeostasis and increased sensitivity to apoptosis following transfer of intracellular Ca^{++} from the endoplasmic reticulum to mitochondria have been described. These changes precede loss of mitochondrial membrane potential and are mediated in part by Bcl-2 family members (Hajnóczky et al., 2003; Demareaux and Distelhorst, 2003). My microarray results suggest that CLN8 may contribute to this internal ion flux in early apoptotic Jurkats.

Finally, activation of the JNK path by Fas receptor may contribute to the catastrophic mitochondrial events that mark the transition to the effector phase of apoptosis in this system. In stress or DNA damage-induced apoptosis, JNK has been shown to colocalise with mitochondria via specific protein interactions (Kharbanda et al., 2000; Wiltshire et al., 2002; Aoki et al., 2002). Here, JNK is capable of direct induction of the mitochondrial events of apoptosis, through interaction with pro-apoptotic members

of the Bcl-2 family, including Bim (Aoki et al., 2002; Lei et al., 2002; Putcha et al., 2003). JNK may also impact on mitochondrial function through AP1 transcription-mediated effects on p53. Increased expression of p53 and the apoptosis-specific, mitochondria-associated genes it induces, such as Bax, Puma and Noxa was not detected in the microarray results, arguing against substantial engagement of either the JNK or p38 transcription paths in Fas treated Jurkats in my hands. However, the synthesis of h-Ras predicted by engagement of these pathways has been detected at later time points by others (Gerner et al., 2000).

4.3.4 Impact of Fas-mediated change in gene expression on the actin cytoskeleton

Expression of numerous sequences coding for actin-related proteins were altered in anti-Fas treated Jurkats, strongly implicating the actin cytoskeleton as a target of apoptosis-specific gene changes. In the nucleus, changes in the actin cytoskeleton may contribute specifically to the early events of apoptosis.

Decreased expression of several Rho isoforms (AHRA, AHRG, AHRI, Table 6) known to mediate stress fibre formation in fibroblasts (Lim et al., 1996) and proteins which connect the actin cytoskeleton and the plasma membrane (CXCR4, ECM2, Table 10) predict a decrease in the physical connections between the cells and their environment, likely a component of the fragmentation of dying cells into apoptotic bodies. In contrast, sorted “apparently normal” cells from anti-Fas treated Jurkat cultures displayed an f-actin signal indistinguishable from controls when assessed by flow cytometric analysis of FITC-phalloidin staining. Decrease in f-actin was observed only in sorted “apoptotic” cells (Figure 39). F-actin is necessary for effective budding of

collapsed chromatin into apoptotic bodies (Dini et al., 1996; Krajci et al., 2000).

Consistent with a role in the effector phase of cell death, microarray analysis detected changes in actin-related sequences predominantly in the whole, unsorted population.

Only ECM2, an integrin ligand, showed change in expression specifically in sorted early apoptotic cells.

In the nucleus, changes in the actin-based cytoskeleton may contribute to initiation of DNA cleavage. Hsc70, the heat shock isoform which demonstrated increased expression specific to early apoptotic cells, can translocate to nuclear aggregates during apoptosis (Dastoor and Dreyer, 2000) and has been proposed to play a role in actin-based remodelling of chromatin (Boyer and Peterson, 2000). Intriguingly, others in the lab have found that the activity of DNaseY, the candidate nuclease for initiation of apoptotic DNA cleavage, is modulated by interaction with actinin (manuscript in preparation). Thus, changes in the nuclear matrix which affect actin and related structural proteins could link the increase in Hsc70 in early apoptotic Jurkats to initial DNA cleavage events.

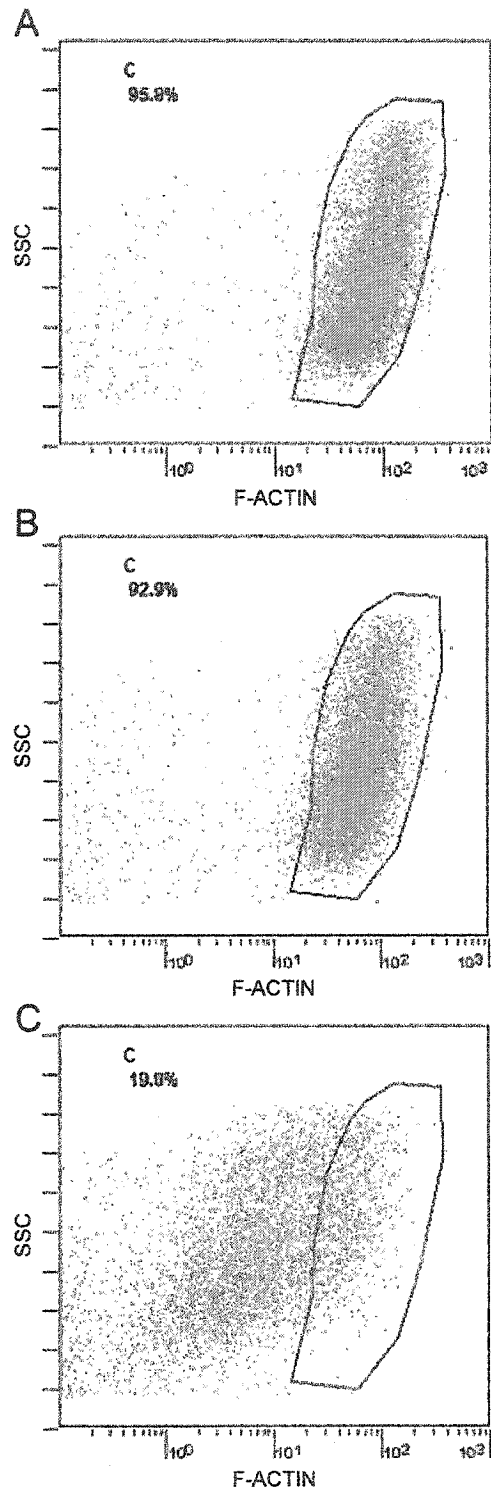


Figure 39 Preservation of the f-actin network in the “apparently normal” subpopulation of anti-Fas treated Jurkats

F-actin integrity was assessed in sorted “apparently normal” cells isolated from untreated control cultures (A) and both “apparently normal” (B) and “apoptotic” cells (C) isolated from cultures treated for 4 hours with 100 ng/ml anti-Fas to induce apoptosis, using flow cytometric analysis of cells labelled with FITC-phalloidin.

5 CONCLUSION

Apoptosis, or programmed cell death is a fundamental process which contributes to a host of normal and pathological phenomena. Although first identified by the hallmark nuclear events of the process, increasingly attention has focussed on the cytoplasmic events in dying cells and their integration, particularly the relationships between death receptor signalling, loss of mitochondrial function and integrity, and activation of caspases. Understanding of the integration of nuclear and cytoplasmic events has lagged, progress being limited to identifying mitochondrial control of several endonucleases capable of oligonucleosomal cleavage. Control of initial nuclear events, such as HMW DNA and specific nuclear matrix cleavage, and their relationship to the cytoplasmic steps of cell death is not understood. Examining the relationship between events in the cell death path is limited by the stochastic nature of the process in all known models, which forces investigators to deal with mixed populations, combined with the tight temporal relationship of events in single cells. My survey of cell death markers and assays for DNA and matrix cleavage in several lymphocytic cell lines illustrated this limitation; all showed a similar timecourse of change and it was not possible to discern the relationship between individual events of the cell death response. A microscopy-based study of nuclear events at the single cell level proved more fruitful, identifying accumulation of initial DNA breaks as the first detectable event in both thymocytes and Jurkat cells, which was closely followed by reorganisation of a portion of the MAR-binding protein SATB1. Comparison of these events to loss of mitochondrial function, a relatively early cytoplasmic event assessed by uptake of potential-sensitive dye in live

cells, was achieved by cell sorting to remove the early apoptotic cells which still retained a normal mitochondrial membrane potential from the mixed population present after anti-Fas treatment of Jurkat cells. Change in nuclear morphology, initial DNA cleavage and reorganisation of SATB1 were detected in a minority of these cells. Biochemical assays and microarray analysis showed that these changes involved accumulation of single strand breaks, specific cleavage of SATB1 and alterations in gene expression. Clearly, a mitochondria-based signal was not responsible for transmission of the apoptotic stimulus to the nucleus. Signal transmission to the nucleus early in apoptosis may involve caspase 8 itself, or adaptor proteins such as DEDAF and cause reorganisation of the nuclear matrix adaptor protein DEDD with which they are known to interact. DEDD reorganisation is associated with activation of caspase 6, the protease responsible for cleavage of SATB1. It also results in altered transcription. Accordingly, changes in gene expression specific to early apoptotic cells could be detected after anti-Fas treatment of Jurkat cultures using microarray analysis and QPCR. Gene changes confirmed in early apoptotic cells could contribute to alterations in mitochondrial homeostasis, and hence the loss of mitochondrial function associated with apoptosis. Key candidates for nuclear-triggered mitochondrial effectors were the cognate heat shock 70 KDa protein isoform and the ER membrane protein CLN8. Hsc70 may also be involved in the initiation of DNA cleavage, through reorganisation of the nuclear actin network. Overall, results suggest that signalling from the nucleus to the mitochondria and the cytoskeleton could play a significant role in coordinating the breakdown of nuclear and cytoplasmic structures during apoptosis.

6 REFERENCES

- Adams,J.M. and Cory,S. (2001). Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* 26, 61-66.
- Adams,J.M. and Cory,S. (2003). Apoptosomes: engines for caspase activation. *Curr. Opin. Cell Biol.* 14, 715-720.
- Alcivar,A., Hu,S., Tang,J., and Yang,X. (2003). DEDD and DEDD2 associate with caspase-8/10 and signal cell death. *Oncogene* 22, 291-297.
- Alvarez,J.D., Yasui,D.H., Niida,H., Joh,T., Loh,D.Y., and Kohwi-Shigematsu,T. (2000). The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes Dev.* 14, 521-535.
- Aoki,H., Kang,P.M., Hampe,J., Yoshimura,K., Noma,T., Matsuzaki,M., and Izumo,S. (2002). Direct activation of mitochondrial apoptosis machinery by c-Jun N- terminal kinase in adult cardiac myocytes. *J. Biol. Chem.* 277, 10244-10250.
- Attisano,L. and Wrana,J.L. (2003). Signal Transduction by the TGF-b superfamily. *Science* 296, 1646-1647.
- Baliga,B.C., Colussi,P.A., Read,S.H., Dias,M.M., Jans,D.A., and Kumar,S. (2003). Role of prodomain in importin-mediated nuclear localization and activation of caspase-2. *J. Biol. Chem.* 278, 4899-4905.
- Banner,D.W., D'Arcy,A., Janes,W., Gentz,R., Schoenfeld,H.J., Broger,C., Loetscher,H., and Lesslauer,W. (1993). Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* 73, 431-445.
- Benchoua,A., Couriaud,C., Guegan,C., Tartier,L., Couvert,P., Friocourt,G., Chelly,J., Menissier-de Murcia,J., and Onteniente,B. (2002). Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2. *J. Biol. Chem.* 277, 34217-34222.
- Benham,C., Kohwi-Shigematsu,T., and Bode,J. (1997). Stress-induced duplex DNA destabilization in scaffold/matrix attachment regions. *J. Mol. Biol.* 274, 181-196.
- Bergsmedh,A., Szeles,A., Henriksson,M., Bratt,A., Folkman,M.J., Spetz,A.L., and Holmgren,L. (2001). Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. U. S. A* 98, 6407-6411.
- Bernardi,P., Petronilli,V., Di Lisa,F., and Forte,M. (2001). A mitochondrial perspective on cell death. *Trends Biochem. Sci.* 26, 112-117.

- Bode,J., Benham,C., Ernst,E., Knopp,A., Marschalek,R., Strick,R., and Strissel,P. (2000). Fatal connections: when DNA ends meet on the nuclear matrix. *J. Cell Biochem. Suppl* 35, 3-22.
- Bode,J., Kohwi,Y., Dickinson,L., Joh,T., Klehr,D., Mielke,C., and Kohwi-Shigematsu,T. (1992). Biological significance of unwinding capability of nuclear matrix- associating DNAs. *Science* 255, 195-197.
- Boyer,L.A. and Peterson,C.L. (2000). Actin-related proteins (Arps): conformational switches for chromatin-remodelling machines? *Bioessays* 22, 666-672.
- Brenner,B., Koppenhoefer,U., Weinstock,C., Linderkamp,O., Lang,F., and Gulbins,E. (1997). Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J. Biol. Chem.* 272, 22173-22181.
- Budd,R.C. (2002). Death receptors couple to both cell proliferation and apoptosis. *J. Clin. Invest* 109, 437-441.
- Buendia,B., Santa-Maria,A., and Courvalin,J.C. (1999). Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J. Cell Sci.* 112 (Pt 11), 1743-1753.
- Burke,B. (2001). Lamins and apoptosis: a two-way street? *J. Cell Biol.* 153, F5-F7.
- Cai,S., Han,H., and Kohwi-Shigematsu,T. (2003). Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat. Genet.* 34, 42-51.
- Cairns,M.J. and Murray,V. (1998). Detection of protein-DNA interactions at beta-globin gene cluster in intact human cells utilizing hedamycin as DNA-damaging agent. *DNA Cell Biol.* 17, 325-333.
- Casciola-Rosen,L., Nicholson,D.W., Chong,T., Rowan,K.R., Thornberry,N.A., Miller,D.K., and Rosen,A. (1996). Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J. Exp. Med.* 183, 1957-1964.
- Casciola-Rosen,L.A., Miller,D.K., Anhalt,G.J., and Rosen,A. (1994). Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* 269, 30757-30760.
- Casiano,C.A., Martin,S.J., Green,D.R., and Tan,E.M. (1996). Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)- mediated T cell apoptosis. *J. Exp. Med.* 184, 765-770.
- Castedo,M., Hirsch,T., Susin,S.A., Zamzami,N., Marchetti,P., Macho,A., and Kroemer,G. (1996). Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J. Immunol.* 157, 512-521.

- Castedo,M., Macho,A., Zamzami,N., Hirsch,T., Marchetti,P., Uriel,J., and Kroemer,G. (1995). Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo. *Eur. J. Immunol.* 25, 3277-3284.
- Cecconi,F., Alvarez-Bolado,G., Meyer,B.I., Roth,K.A., and Gruss,P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 94, 727-737.
- Chaly,N., Bladon,T., Setterfield,G., Little,J.E., Kaplan,J.G., and Brown,D.L. (1984). Changes in distribution of nuclear matrix antigens during the mitotic cell cycle. *J. Cell Biol.* 99, 661-671.
- Chang,D.W., Xing,Z., Pan,Y., Algeciras-Schimmich,A., Barnhart,B.C., Yaish-Ohad,S., Peter,M.E., and Yang,X. (2002a). c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J.* 21, 3704-3714.
- Chang,N.S. (2002). A potential role of p53 and WOX1 in mitochondrial apoptosis (review). *Int. J. Mol. Med.* 9, 19-24.
- Chang,S.H., Cvetanovic,M., Harvey,K.J., Komoriya,A., Packard,B.Z., and Ucker,D.S. (2002b). The effector phase of physiological cell death relies exclusively on the posttranslational activation of resident components. *Experimental Cell Research* 277, 15-30.
- Chattopadhyay,S., Kaul,R., Charest,A., Housman,D., and Chen,J. (2000). SMAR1, a novel, alternatively spliced gene product, binds the Scaffold/Matrix-associated region at the T cell receptor beta locus. *Genomics* 68, 93-96.
- Chaudhary,N. and Courvalin,J.C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* 122, 295-306.
- Chen,C.R., Kang,Y., Siegel,P.M., and Massague,J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* 110, 19-32.
- Chen,M. and Wang,J. (2002). Initiator caspases in apoptosis signalling pathways. *Apoptosis*. 7, 313-319.
- Chen,W., Jin,W., Tian,H., Sicurello,P., Frank,M., Orenstein,J.M., and Wahl,S.M. (2001). Requirement for transforming growth factor beta1 in controlling T cell apoptosis. *J. Exp. Med.* 194, 439-453.
- Chen,Y. and Lai,M.Z. (2001). c-Jun NH2-terminal kinase activation leads to a FADD-dependent but Fas ligand-independent cell death in Jurkat T cells. *J. Biol. Chem.* 276, 8350-8357.

- Chinnaiyan,A.M., O'Rourke,K., Lane,B.R., and Dixit,V.M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275, 1122-1126.
- Chinnaiyan,A.M., O'Rourke,K., Tewari,M., and Dixit,V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81, 505-512.
- Chinnaiyan,A.M., Tepper,C.G., Seldin,M.F., O'Rourke,K., Kischkel,F.C., Hellbardt,S., Krammer,P.H., Peter,M.E., and Dixit,V.M. (1996). FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.* 271, 4961-4965.
- Cifone,M.G., Roncaioli,P., De Maria,R., Camarda,G., Santoni,A., Ruberti,G., and Testi,R. (1995). Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. *EMBO J.* 14, 5859-5868.
- Cohen,G.M., Sun,X.M., Fearnhead,H., MacFarlane,M., Brown,D.G., Snowden,R.T., and Dinsdale,D. (1994). Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. *J. Immunol.* 153, 507-516.
- Cohen,M., Lee,K.K., Wilson,K.L., and Gruenbaum,Y. (2001). Transcriptional repression, apoptosis, human disease and the functional evolution of the nuclear lamina. *Trends Biochem. Sci.* 26, 41-47.
- Cohen,O., Feinstein,E., and Kimchi,A. (1997). DAP-kinase is a Ca²⁺/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J.* 16, 998-1008.
- Cohen,O., Inbal,B., Kissil,J.L., Raveh,T., Berissi,H., Spivak-Kroizaman,T., Feinstein,E., and Kimchi,A. (1999). DAP-kinase participates in TNF- α . *J. Cell Biol.* 146, 141-148.
- Compton,D.A. and Cleveland,D.W. (1994). NuMA, a nuclear protein involved in mitosis and nuclear reformation. *Curr. Opin. Cell Biol.* 6, 343-346.
- Creagh,E.M., Carmody,R.J., and Cotter,T.G. (2000). Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. *Exp. Cell Res.* 257, 58-66.
- Creagh,E.M. and Martin,S.J. (2001). Caspases: cellular demolition experts. *Biochem. Soc. Trans.* 29, 696-702.
- Dastoor,Z. and Dreyer,J. (2000). Nuclear translocation and aggregate formation of heat shock cognate protein 70 (Hsc70) in oxidative stress and apoptosis. *J. Cell Sci.* 113, 2845-2854.

- Daugas,E., Nochy,D., Ravagnan,L., Loeffler,M., Susin,S.A., Zamzami,N., and Kroemer,G. (2000a). Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett.* *476*, 118-123.
- Daugas,E., Susin,S.A., Zamzami,N., Ferri,K.F., Irinopoulou,T., Larochette,N., Prevost,M.C., Leber,B., Andrews,D., Penninger,J., and Kroemer,G. (2000b). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J.* *14*, 729-739.
- de la,Taille.A., Chen,M.W., Burchardt,M., Chopin,D.K., and Buttyan,R. (1999). Apoptotic conversion: evidence for exchange of genetic information between prostate cancer cells mediated by apoptosis. *Cancer Res.* *59*, 5461-5463.
- deBelle,I., Cai,S., and Kohwi-Shigematsu,T. (1998). The genomic sequences bound to special AT-rich sequence-binding protein 1 (SATB1) in vivo in Jurkat T cells are tightly associated with the nuclear matrix at the bases of the chromatin loops. *J. Cell Biol.* *141*, 335-348.
- Deiss,L.P., Feinstein,E., Berissi,H., Cohen,O., and Kimchi,A. (1995). Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* *9*, 15-30.
- Demareaux,N. and Distelhorst,C. (2003). Apoptosis - the calcium connection. *Science* *300*, 65-66.
- Desagher,S. and Martinou,J.C. (2000). Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* *10*, 369-377.
- Dhein,J., Daniel,P.T., Trauth,B.C., Oehm,A., Moller,P., and Krammer,P.H. (1992). Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J. Immunol.* *149*, 3166-3173.
- Dickinson,L.A., Joh,T., Kohwi,Y., and Kohwi-Shigematsu,T. (1992). A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell* *70*, 631-645.
- Dickinson,L.A. and Kohwi-Shigematsu,T. (1995). Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. *Mol. Cell Biol.* *15*, 456-465.
- Dini,L., Coppola,S., Ruzittu,M.T., and Ghibelli,L. (1996). Multiple pathways for apoptotic nuclear fragmentation. *Exp. Cell Res.* *223*, 340-347.
- Du,C., Fang,M., Li,Y., Li,L., and Wang,X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* *102*, 33-42.

- Duband-Goulet, I., Courvalin, J.C., and Buendia, B. (1998). LBR, a chromatin and lamin binding protein from the inner nuclear membrane, is proteolyzed at late stages of apoptosis. *J. Cell Sci.* *111* (Pt 10), 1441-1451.
- Dynlacht, J.R., Earles, M., Henthorn, J., and Seno, J.D. (2000). Different patterns of DNA fragmentation and degradation of nuclear matrix proteins during apoptosis induced by radiation, hyperthermia or etoposide. *Radiat. Res.* *154*, 515-530.
- Eischen, C.M., Kottke, T.J., Martins, L.M., Basi, G.S., Tung, J.S., Earnshaw, W.C., Leibson, P.J., and Kaufmann, S.H. (1997). Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. *Blood* *90*, 935-943.
- Enari, M., Hase, A., and Nagata, S. (1995). Apoptosis by a cytosolic extract from Fas-activated cells. *EMBO J.* *14*, 5201-5208.
- Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* *391*, 43-50.
- Enari, M., Talanian, R.V., Wong, W.W., and Nagata, S. (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* *380*, 723-726.
- Endresen, P.C., Prytz, P.S., and Aarbakke, J. (1995). A new flow cytometric method for discrimination of apoptotic cells and detection of their cell cycle specificity through staining of F-actin and DNA. *Cytometry* *20*, 162-171.
- Esposti, M.D. (2002). The roles of Bid. *Apoptosis* *7*, 433-440.
- Fackelmayer, F.O., Dahm, K., Renz, A., Ramsperger, U., and Richter, A. (1994). Nucleic-acid-binding properties of hnRNP-U/SAF-A, a nuclear-matrix protein which binds DNA and RNA in vivo and in vitro. *Eur. J. Biochem.* *221*, 749-757.
- Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997). Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* *16*, 2271-2281.
- Faleiro, L. and Lazebnik, Y. (2000). Caspases disrupt the nuclear-cytoplasmic barrier. *J. Cell Biol.* *151*, 951-959.
- Faris, M., Kokot, N., Latinis, K., Kasibhatla, S., Green, D.R., Koretzky, G.A., and Nel, A. (1998a). The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. *J. Immunol.* *160*, 134-144.

- Faris,M., Latinis,K.M., Kempiak,S.J., Koretzky,G.A., and Nel,A. (1998b). Stress-induced Fas ligand expression in T cells is mediated through a MEK kinase 1-regulated response element in the Fas ligand promoter. *Mol. Cell Biol.* *18*, 5414-5424.
- Frankfurt,O.S., Robb,J.A., Sugarbaker,E.V., and Villa,L. (1996). Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. *Exp. Cell Res.* *226*, 387-397.
- Gajkowska,B. and Wojewodzka,U. (2002). A novel embedment-free immunoelectron microscopy technique reveals association of apoptosis-regulating proteins with subcellular structures. *Histochem. J.* *34*, 441-446.
- Galande,S., Dickinson,L.A., Mian,I.S., Sikorska,M., and Kohwi-Shigematsu,T. (2001). SATB1 cleavage by caspase 6 disrupts PDZ domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Mol. Cell Biol.* *21*, 5591-5604.
- Galande,S. and Kohwi-Shigematsu,T. (1999). Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. *J. Biol. Chem.* *274*, 20521-20528.
- Gamen,S., Anel,A., Perez-Galan,P., Lasier,P., Johnson,D., Pineiro,A., and Naval,J. (2000). Doxorubicin treatment activates a Z-VAD-sensitive caspase, which causes deltapim loss, caspase-9 activity, and apoptosis in Jurkat cells. *Exp. Cell Res.* *258*, 223-235.
- Garcia,I., Murga,M., Vicario,A., Field,S.J., and Zubiaga,A.M. (2000). A role for E2F1 in the induction of apoptosis during thymic negative selection. *Cell Growth Differ.* *11*, 91-98.
- Gavrieli,Y., Sherman,Y., and Ben Sasson,S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* *119*, 493-501.
- Genestier,L., Kasibhatla,S., Brunner,T., and Green,D.R. (1999). Transforming growth factor beta1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via downregulation of c-Myc. *J. Exp. Med.* *189*, 231-239.
- Gerner,C., Frohwein,U., Gotzmann,J., Bayer,E., Gelbmann,D., Bursch,W., and Schulte-Hermann,R. (2000). The Fas-induced apoptosis analyzed by high throughput proteome analysis. *J. Biol. Chem.* *275*, 39018-39026.
- Gerner,C., Seelos,C., and Sauermann,G. (1998). Alteration of nuclear matrix protein composition during apoptosis in rat embryo cells. *Exp. Cell Res.* *238*, 472-480.
- Ginsberg,D. (2002). E2F1 pathways to apoptosis. *FEBS Lett.* *529*, 122-125.

- Gohring,F., Schwab,B.L., Nicotera,P., Leist,M., and Fackelmayer,F.O. (1997). The novel SAR-binding domain of scaffold attachment factor A (SAF-A) is a target in apoptotic nuclear breakdown. *EMBO J.* 16, 7361-7371.
- Goldberg,M., Harel,A., and Gruenbaum,Y. (1999). The nuclear lamina: molecular organization and interaction with chromatin. *Crit Rev. Eukaryot. Gene Expr.* 9, 285-293.
- Goldstein,J.C., Kluck,R.M., and Green,D.R. (2000). A single cell analysis of apoptosis Ordering the apoptotic phenotype. *Ann. N. Y. Acad. Sci.* 926, 132-141.
- Gotzmann,J., Meissner,M., and Gerner,C. (2000). The fate of the nuclear matrix-associated-region-binding protein SATB1 during apoptosis. *Cell Death. Differ.* 7, 425-438.
- Greidinger,E.L., Miller,D.K., Yamin,T.T., Casciola-Rosen,L., and Rosen,A. (1996). Sequential activation of three distinct ICE-like activities in Fas- ligated Jurkat cells. *FEBS Lett.* 390, 299-303.
- Gross,A., Yin,X.M., Wang,K., Wei,M.C., Jockel,J., Milliman,C., Erdjument-Bromage,H., Tempst,P., and Korsmeyer,S.J. (1999). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* 274, 1156-1163.
- Gruenbaum,Y., Wilson,K.L., Harel,A., Goldberg,M., and Cohen,M. (2000). Review: nuclear lamins--structural proteins with fundamental functions. *J. Struct. Biol.* 129, 313-323.
- Gueth-Hallonet,C., Weber,K., and Osborn,M. (1997). Cleavage of the nuclear matrix protein NuMA during apoptosis. *Exp. Cell Res.* 233, 21-24.
- Gulbins,E., Bissonnette,R., Mahboubi,A., Martin,S., Nishioka,W., Brunner,T., Baier,G., Baier-Bitterlich,G., Byrd,C., Lang,F., and . (1995). FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. *Immunity.* 2, 341-351.
- Guo,Y., Srinivasula,S.M., Druilhe,A., Fernandes-Alnemri,T., and Alnemri,E.S. (2002). Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J. Biol. Chem.* 277, 13430-13437.
- Hagimoto,N., Kuwano,K., Inoshima,I., Yoshimi,M., Nakamura,N., Fujita,M., Maeyama,T., and Hara,N. (2002). TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. *J. Immunol.* 168, 6470-6478.
- Hajnoczky,G., Davies,E., and Madesh,M. (2003). Calcium signaling and apoptosis. *Biochem. Biophys. Res. Commun.* 304, 445-454.
- Hakem,R., Hakem,A., Duncan,G.S., Henderson,J.T., Woo,M., Soengas,M.S., Elia,A., de la Pompa,J.L., Kagi,D., Khoo,W., Potter,J., Yoshida,R., Kaufman,S.A., Lowe,S.W.,

- Penninger, J.M., and Mak, T.W. (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* 94, 339-352.
- Hausmann, G., O'Reilly, L.A., van Driel, R., Beaumont, J.G., Strasser, A., Adams, J.M., and Huang, D.C. (2000). Pro-apoptotic apoptosis protease-activating factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-x(L). *J. Cell Biol.* 149, 623-634.
- Hearps, A.C., Burrows, J., Connor, C.E., Woods, G.M., Lowenthal, R.M., and Ragg, S.J. (2002). Mitochondrial cytochrome c release precedes transmembrane depolarisation and caspase-3 activation during ceramide-induced apoptosis of Jurkat T cells. *Apoptosis*. 7, 387-394.
- Hedge, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T., and Alnemri, E.S. (2002). Identification of Omi/HtrA2 as a mitochondrial serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J. Biol. Chem.* 277, 432-438.
- Hennet, T., Bertonni, G., Richter, C., and Peterhans, E. (1993). Expression of BCL-2 protein enhances the survival of mouse fibrosarcoma cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res.* 53, 1456-1460.
- Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998). Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J. Exp. Med.* 187, 587-600.
- Hofmann, T.G., Moller, A., Hehner, S.P., Welsch, D., Droge, W., and Schmitz, M.L. (2001). CD95-induced JNK activation signals are transmitted by the death-inducing signaling complex (DISC), but not by Daxx. *Int. J. Cancer* 93, 185-191.
- Hohlbaum, A.M., Moe, S., and Marshak-Rothstein, A. (2000). Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. *J. Exp. Med.* 191, 1209-1220.
- Holcik, M., Sonenberg, N., and Korneluk, R.G. (2000a). Internal ribosome initiation of translation and the control of cell death. *Trends Genet.* 16, 469-473.
- Holcik, M., Yeh, C., Korneluk, R.G., and Chow, T. (2000b). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene* 19, 4174-4177.
- Holmgren, L., Szeles, A., Rajnavolgyi, E., Folkman, J., Klein, G., Ernberg, I., and Falk, K.I. (1999). Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood* 93, 3956-3963.

- Hou, S.T., Xie, X., Baggle, A., Park, D.S., Chen, G., and Walker, T. (2002). Activation of the Rb/E2F1 Pathway by the Nonproliferative p38 MAPK during Fas (APO1/CD95)-mediated Neuronal Apoptosis. *J. Biol. Chem.* 277, 48764-48770.
- Hsu, H.L. and Yeh, N.H. (1996). Dynamic changes of NuMA during the cell cycle and possible appearance of a truncated form of NuMA during apoptosis. *J. Cell Sci.* 109 (Pt 2), 277-288.
- Huang, B., Eberstadt, M., Olejniczak, E.T., Meadows, R.P., and Fesik, S.W. (1996). NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. *Nature* 384, 638-641.
- Hymowitz, S.G., Christinger, H.W., Fuh, G., Ultsch, M., O'Connell, M., Kelley, R.F., Ashkenazi, A., and de Vos, A.M. (1999). Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol. Cell* 4, 563-571.
- Inbal, B., Bialik, S., Sabanay, I., Shani, G., and Kimchi, A. (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J. Cell Biol.* 157, 455-468.
- Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F., III, and Maul, G.G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J. Cell Biol.* 147, 221-234.
- Jackson, C.E. and Puck, J.M. (1999). Autoimmune lymphoproliferative syndrome, a disorder of apoptosis. *Curr. Opin. Pediatr.* 11, 521-527.
- Jacobson, M.D., Burne, J.F., and Raff, M.C. (1994). Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* 13, 1899-1910.
- Jang, C.W., Chen, C.H., Chen, C.C., Chen, J.Y., Su, Y.H., and Chen, R.H. (2002). TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat. Cell Biol.* 4, 51-58.
- Jiang, X. and Wang, X. (2000). Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J. Biol. Chem.* 275, 31199-31203.
- Johnson, V.L., Ko, S.C., Holmstrom, T.H., Eriksson, J.E., and Chow, S.C. (2000). Effector caspases are dispensable for the early nuclear morphological changes during chemical-induced apoptosis. *J. Cell Sci.* 113 (Pt 17), 2941-2953.
- Kannan, K., Amariglio, N., Rechavi, G., Jakob-Hirsch, J., Kela, I., Kaminski, N., Getz, G., Domany, E., and Givol, D. (2001a). DNA microarrays identification of primary and secondary target genes regulated by p53. *Oncogene* 20, 2225-2234.

- Kannan,K., Kaminski,N., Rechavi,G., Jakob-Hirsch,J., Amariglio,N., and Givol,D. (2001b). DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1. *Oncogene* 20, 3449-3455.
- Kasai,T., Ohguchi,K., Nakashima,S., Ito,Y., Naganawa,T., Kondo,N., and Nozawa,Y. (1998). Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jurkat T cells. *J. Immunol.* 161, 6469-6474.
- Kaufmann,S.H., Desnoyers,S., Ottaviano,Y., Davidson,N.E., and Poirier,G.G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* 53, 3976-3985.
- Kawahara,A., Enari,M., Talanian,R.V., Wong,W.W., and Nagata,S. (1998). Fas-induced DNA fragmentation and proteolysis of nuclear proteins. *Genes Cells* 3, 297-306.
- Keppler,O.T., Peter,M.E., Hinderlich,S., Moldenhauer,G., Stehling,P., Schmitz,I., Schwartz-Albiez,R., Reutter,W., and Pawlita,M. (1999). Differential sialylation of cell surface glycoconjugates in a human B lymphoma cell line regulates susceptibility for CD95 (APO-1/Fas)- mediated apoptosis and for infection by a lymphotropic virus. *Glycobiology* 9, 557-569.
- Kharbanda,S., Saxena,S., Yoshida,K., Pandey,P., Kaneki,M., Wang,Q., Cheng,K., Chen,Y.N., Campbell,A., Sudha,T., Yuan,Z.M., Narula,J., Weichselbaum,R., Nalin,C., and Kufe,D. (2000). Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. *J. Biol. Chem.* 275, 322-327.
- Khodarev,N.N., Bennett,T., Shearing,N., Sokolova,I., Koudelik,J., Walter,S., Villalobos,M., and Vaughan,A.T. (2000). LINE L1 retrotransposable element is targeted during the initial stages of apoptotic DNA fragmentation. *J. Cell Biochem.* 79, 486-495.
- Kim,K.M., Lee,K., Hong,Y.S., and Park,H.Y. (2000). Fas-mediated apoptosis and expression of related genes in human malignant hematopoietic cells. *Exp. Mol. Med.* 32, 246-254.
- Kipp,M., Schwab,B.L., Przybylski,M., Nicotera,P., and Fackelmayer,F.O. (2000). Apoptotic cleavage of scaffold attachment factor A (SAF-A) by caspase-3 occurs at a noncanonical cleavage site. *J. Biol. Chem.* 275, 5031-5036.
- Kischkel,F.C., Hellbardt,S., Behrmann,I., Germer,M., Pawlita,M., Krammer,P.H., and Peter,M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* 14, 5579-5588.
- Kluck,R.M., Bossy-Wetzel,E., Green,D.R., and Newmeyer,D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275, 1132-1136.

- Kohwi-Shigematsu, T., deBelle, I., Dickinson, L.A., Galande, S., and Kohwi, Y. (1998). Identification of base-unpairing region-binding proteins and characterization of their in vivo binding sequences. *Methods Cell Biol.* 53, 323-354.
- Kohwi-Shigematsu, T. and Kohwi, Y. (1990). Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. *Biochemistry* 29, 9551-9560.
- Kohwi-Shigematsu, T., Maass, K., and Bode, J. (1997). A thymocyte factor SATB1 suppresses transcription of stably integrated matrix-attachment region-linked reporter genes. *Biochemistry* 36, 12005-12010.
- Komoriya, A., Packard, B.Z., Brown, M.J., Wu, M., and Henkart, P.A. (2000). Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable fluorogenic caspase substrates. *J. Exp. Med.* 191, 1819-1828.
- Krajci, D., Mares, V., Lisa, V., Spanova, A., and Vorlicek, J. (2000). Ultrastructure of nuclei of cisplatin-treated C6 glioma cells undergoing apoptosis. *Eur. J. Cell Biol.* 79, 365-376.
- Krajewski, S., Krajewska, M., Ellerby, L.M., Welsh, K., Xie, Z., Deveraux, Q.L., Salvesen, G.S., Bredesen, D.E., Rosenthal, R.E., Fiskum, G., and Reed, J.C. (1999). Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proc. Natl. Acad. Sci. U. S. A* 96, 5752-5757.
- Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res.* 53, 4701-4714.
- Krammer, P.H., Dhein, J., Walczak, H., Behrmann, I., Mariani, S., Matiba, B., Fath, M., Daniel, P.T., Knipping, E., Westendorp, M.O., and . (1994). The role of APO-1-mediated apoptosis in the immune system. *Immunol. Rev.* 142, 175-191.
- Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., and Flavell, R.A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94, 325-337.
- Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R.A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384, 368-372.
- Lagarkova, M.A., Iarovaia, O.V., and Razin, S.V. (1995). Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers. *J. Biol. Chem.* 270, 20239-20241.

- Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G., and Earnshaw, W.C. (1993). Nuclear events of apoptosis in vitro in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. *J. Cell Biol.* 123, 7-22.
- Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371, 346-347.
- Lei, K., Nimnual, A., Zong, W.X., Kennedy, N.J., Flavell, R.A., Thompson, C.B., Bar-Sagi, D., and Davis, R.J. (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. *Mol. Cell Biol.* 22, 4929-4942.
- Lenczowski, J.M., Dominguez, L., Eder, A.M., King, L.B., Zacharchuk, C.M., and Ashwell, J.D. (1997). Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. *Mol. Cell Biol.* 17, 170-181.
- Leu, J.I., Crissey, M.A., and Traub, R. (2003). Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. *J. Clin. Invest* 111, 129-139.
- Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94, 491-501.
- Li, L.Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412, 95-99.
- Lim, L., Manser, E., Leung, T., and Hall, C. (1996). Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.* 242, 171-185.
- Liossis, S.N., Ding, X.Z., Kiang, J.G., and Tsokos, G.C. (1997). Overexpression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat T cells. *J. Immunol.* 158, 5668-5675.
- Liu, Q.Y., Pandey, S., Singh, R.K., Lin, W., Ribocco, M., Borowy-Borowski, H., Smith, B., Leblanc, J., Walker, P.R., and Sikorska, M. (1998). DNaseY: a rat DNaseI-like gene coding for a constitutively expressed chromatin-bound endonuclease. *Biochemistry* 37, 10134-10143.
- Liu, Q.Y., Ribocco-Lutkiewicz, M., Carson, C., Testolin, L., Bergeron, D., Kohwi-Shigematsu, T., Walker, P.R., and Sikorska, M. (2003). Mapping the initial DNA breaks in apoptotic Jurkat cells using ligation-mediated PCR. *Cell Death Diff.* 10, 278-289.
- Liu, Q.Y., Ribocco, M., Pandey, S., Walker, P.R., and Sikorska, M. (1999a). Apoptosis-related functional features of the DNaseI-like family of nucleases. *Ann. N. Y. Acad. Sci.* 887, 60-76.

- Liu, W.M., Guerra-Vladusic, F.K., Kurakata, S., Lupu, R., and Kohwi-Shigematsu, T. (1999b). HMG-I(Y) recognizes base-unpairing regions of matrix attachment sequences and its increased expression is directly linked to metastatic breast cancer phenotype. *Cancer Res.* 59, 5695-5703.
- Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86, 147-157.
- Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89, 175-184.
- Lizard, G., Fournel, S., Genestier, L., Dhedin, N., Chaput, C., Flacher, M., Mutin, M., Panaye, G., and Revillard, J.P. (1995). Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* 21, 275-283.
- Luderus, M.E., den Blaauwen, J.L., de Smit, O.J., Compton, D.A., and van Driel, R. (1994). Binding of matrix attachment regions to lamin polymers involves single-stranded regions and the minor groove. *Mol. Cell Biol.* 14, 6297-6305.
- Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94, 481-490.
- Luscher, B. (2001). Function and regulation of the transcription factors of the Myc/Max/Mad network. *Gene* 277, 1-14.
- Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., and Wang, X. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat. Cell Biol.* 2, 754-761.
- Mao, P.L., Jiang, Y., Wee, B.Y., and Porter, A.G. (1998). Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. *J. Biol. Chem.* 273, 23621-23624.
- Marchenko, N.D., Zaika, A., and Moll, U.M. (2000). Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J. Biol. Chem.* 275, 16202-16212.
- Marchetti, P., Castedo, M., Susin, S.A., Zamzami, N., Hirsch, T., Macho, A., Haeflner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996). Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med.* 184, 1155-1160.
- Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, P.G., Huang, D.C., Cecconi, F., Kuida, K., Tomaselli, K.J., Roy, S., Nicholson, D.W., Vaux, D.L., Bouillet, P., Adams, J.M., and Strasser, A. (2002). Apoptosis initiated by Bcl-2-regulated caspase

activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* 419, 634-637.

Martelli,A.M., Bortul,R., Fackelmayer,F.O., Tazzari,P.L., Bareggi,R., Narducci,P., and Zweyer,M. (1999). Biochemical and morphological characterization of the nuclear matrix from apoptotic HL-60 cells. *J. Cell Biochem.* 72, 35-46.

Martelli,A.M., Zweyer,M., Ochs,R.L., Tazzari,P.L., Tabellini,G., Narducci,P., and Bortul,R. (2001). Nuclear apoptotic changes: an overview. *J. Cell Biochem.* 82, 634-646.

Martin,S.J., Newmeyer,D.D., Mathias,S., Farschon,D.M., Wang,H.G., Reed,J.C., Kolesnick,R.N., and Green,D.R. (1995). Cell-free reconstitution of Fas-, UV radia. *EMBO J.* 14, 5191-5200.

Martinou,J.C. and Green,D.R. (2001). Breaking the mitochondrial barrier. *Nat. Rev. Mol. Cell Biol.* 2, 63-67.

Martins,L.M., Kottke,T., Mesner,P.W., Basi,G.S., Sinha,S., Frigon,N., Jr., Tatar,E., Tung,J.S., Bryant,K., Takahashi,A., Svingen,P.A., Madden,B.J., McCormick,D.J., Earnshaw,W.C., and Kaufmann,S.H. (1997). Activation of multiple interleukin-1beta converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. *J. Biol. Chem.* 272, 7421-7430.

Marzo,I., Brenner,C., Zamzami,N., Jurgensmeier,J.M., Susin,S.A., Vieira,H.L., Prevost,M.C., Xie,Z., Matsuyama,S., Reed,J.C., and Kroemer,G. (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 281, 2027-2031.

Medema,J.P., Scaffidi,C., Kischkel,F.C., Shevchenko,A., Mann,M., Krammer,P.H., and Peter,M.E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.* 16, 2794-2804.

Michaelson,J.S., Bader,D., Kuo,F., Kozak,C., and Leder,P. (1999). Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev.* 13, 1918-1923.

Michaelson,J.S. and Leder,P. (2003). RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. *J. Cell Sci.* 116, 345-352.

Minn,A.J., Velez,P., Schendel,S.L., Liang,H., Muchmore,S.W., Fesik,S.W., Fill,M., and Thompson,C.B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 385, 353-357.

Monaghan,P., Robertson,D., Amos,T.A., Dyer,M.J., Mason,D.Y., and Greaves,M.F. (1992). Ultrastructural localization of bcl-2 protein. *J. Histochem. Cytochem.* 40, 1819-1825.

- Mongkolsapaya,J., Grimes,J.M., Chen,N., Xu,X.N., Stuart,D.I., Jones,E.Y., and Screaton,G.R. (1999). Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. *Nat. Struct. Biol.* 6, 1048-1053.
- Moriishi,K., Huang,D.C., Cory,S., and Adams,J.M. (1999). Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc. Natl. Acad. Sci. U. S. A* 96, 9683-9688.
- Moustakas,A., Souchelnytskyi,S., and Heldin,C.H. (2001). Smad regulation in TGF-beta signal transduction. *J. Cell Sci.* 114, 4359-4369.
- Mundle,S. and Raza,A. (2002). Defining the dynamics of self-assembled Fas-receptor activation. *Trends in Immunology* 23, 187-194.
- Muromoto,R., Yamamoto,T., Yumioka,T., Sekine,Y., Sujiyama,K., Shimoda,K., Oritani,K., and Matsuda,T. (2003). Daxx enhances Fas-mediated apoptosis in a murine pro-B cell line, BAF3. *FEBS Lett.* 540, 223-228.
- Muzio,M., Chinnaiyan,A.M., Kischkel,F.C., O'Rourke,K., Shevchenko,A., Ni,J., Scaffidi,C., Bretz,J.D., Zhang,M., Gentz,R., Mann,M., Krammer,P.H., Peter,M.E., and Dixit,V.M. (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* 85, 817-827.
- Nagata,S. and Golstein,P. (1995). The Fas death factor. *Science* 267, 1449-1456.
- Neamati,N., Fernandez,A., Wright,S., Kiefer,J., and McConkey,D.J. (1995). Degradation of lamin B1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. *J. Immunol.* 154, 3788-3795.
- Newmeyer,D.D., Farschon,D.M., and Reed,J.C. (1994). Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79, 353-364.
- Newmeyer,D.D. and Ferguson-Miller,S. (2003). Mitochondria: Releasing power for life and unleashing the machineris of death. *Cell* 112, 481-490.
- Nicholson,D.W. and Thornberry,N.A. (1997). Caspases: killer proteases. *Trends Biochem. Sci.* 22, 299-306.
- Oberhammer,F.A., Hochegger,K., Froschl,G., Tiefenbacher,R., and Pavelka,M. (1994). Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* 126, 827-837.
- Oehm,A., Behrmann,I., Falk,W., Pawlita,M., Maier,G., Klas,C., Li-Weber,M., Richards,S., Dhein,J., Trauth,B.C., and . (1992). Purification and molecular cloning of the APO-1 cell

surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.* 267, 10709-10715.

Pandey,S., Walker,P.R., and Sikorska,M. (1997). Identification of a novel 97 kDa endonuclease capable of internucleosomal DNA cleavage. *Biochemistry* 36, 711-720.

Papoff,G., Hausler,P., Eramo,A., Pagano,M.G., Di Leve,G., Signore,A., and Ruberti,G. (1999). Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. *J. Biol. Chem.* 274, 38241-38250.

Paroni,G., Henderson,C., Schneider,C., and Brancolini,C. (2002). Caspase-2 can trigger cytochrome c release and apoptosis from the nucleus. *J. Biol. Chem.* 277, 15147-15161.

Pastorino,J.G., Simbula,G., Yamamoto,K., Glascott,P.A., Jr., Rothman,R.J., and Farber,J.L. (1996). The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. *J. Biol. Chem.* 271, 29792-29798.

Peitsch,M.C., Muller,C., and Tschopp,J. (1993). DNA fragmentation during apoptosis is caused by frequent single-strand cuts. *Nucleic Acids Res.* 21, 4206-4209.

Penninger,J. and Kroemer,G. (2003). Mitochondria, AIF and caspases- rivaling for celdeath execution. *Nat. Cell Biol.* 5, 97-99.

Petit,P.X., Lecoœur,H., Zorn,E., Dauguet,C., Mignotte,B., and Gougeon,M.L. (1995). Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J. Cell Biol.* 130, 157-167.

Pluta,A.F., Earnshaw,W.C., and Goldberg,I.G. (1998). Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. *J. Cell Sci.* 111 (Pt 14), 2029-2041.

Prendergast,G.C. (1999). Mechanisms of apoptosis by c-Myc. *Oncogene* 18, 2967-2987.

Putchu,G.V., Le,S., Frank,S., Besirli,C.G., Clark,K., Chu,B., Alix,S., Youle,R.J., LaMarche,A., Maroney,A.C., and Johnson,E.M. (2003). JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* 38, 899-914.

Quan,L.T., Tewari,M., O'Rourke,K., Dixit,V., Snipas,S.J., Poirier,G.G., Ray,C., Pickup,D.J., and Salvesen,G.S. (1996). Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. *Proc. Natl. Acad. Sci. U. S. A* 93, 1972-1976.

Ramakrishnan,M., Liu,W.M., DiCroce,P.A., Posner,A., Zheng,J., Kohwi-Shigematsu,T., and Krontiris,T.G. (2000). Modulated binding of SATB1, a matrix attachment region protein, to the AT-rich sequence flanking the major breakpoint region of BCL2. *Mol. Cell Biol.* 20, 868-877.

- Ramuz,O., Isnardon,D., Devilard,E., Charafe-Jauffret,E., Hassoun,J., Birg,F., and Xerri,L. (2003). Constitutive nuclear localization and initial cytoplasmic apoptotic activation of endogenous caspase-3 evidenced by confocal microscopy. *Int. J. Exp. Path.* 84, 75-81.
- Rao,L., Perez,D., and White,E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. *J. Cell Biol.* 135, 1441-1455.
- Ricci,J.E., Maulon,L., Battaglione-Hofman,V., Bertolotto,C., Luciano,F., Mari,B., Hofman,P., and Auberger,P. (2001). A Jurkat T cell variant resistant to death receptor-induced apoptosis. Correlation with heat shock protein (Hsp) 27 and 70 levels. *Eur. Cytokine Netw.* 12, 126-134.
- Robertson,J.D., Orrenius,S., and Zhivotovsky,B. (2000). Review: nuclear events in apoptosis. *J. Struct. Biol.* 129, 346-358.
- Rosse,T., Olivier,R., Monney,L., Rager,M., Conus,S., Fellay,I., Jansen,B., and Borner,C. (1998). Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* 391, 496-499.
- Rozenfeld-Granot,G., Krishnamurthy,J., Kannan,K., Toren,A., Amariglio,N., Givol,D., and Rechavi,G. (2002). A positive feedback mechanism in the transcriptional activation of Apaf- 1 by p53 and the coactivator Zac-1. *Oncogene* 21, 1469-1476.
- Ruchaud,S., Korfali,N., Villa,P., Kottke,T.J., Dingwall,C., Kaufmann,S.H., and Earnshaw,W.C. (2002). Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. *EMBO J.* 21, 1967-1977.
- Saito,R., Mizuno,M., Kumabe,T., Yoshimoto,T., Tanuma,S., and Yoshida,J. (2003). Apoptotic DNA endonuclease (DNase-gamma) gene transfer induces cell death accompanying DNA fragmentation in human glioma cells. *Journal of Neurooncology* 63, 25-31.
- Sakahira,H., Enari,M., and Nagata,S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391, 96-99.
- Sartorius,U., Schmitz,I., and Krammer,P.H. (2001). Molecular mechanisms of death-receptor-mediated apoptosis. *Chembiochem.* 2, 20-29.
- Scaffidi,C., Fulda,S., Srinivasan,A., Friesen,C., Li,F., Tomaselli,K.J., Debatin,K.M., Krammer,P.H., and Peter,M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17, 1675-1687.
- Scaffidi,C., Schmitz,I., Krammer,P.H., and Peter,M.E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* 274, 1541-1548.

- Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S., Montal, M., and Reed, J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. *Proc. Natl. Acad. Sci. U. S. A* 94, 5113-5118.
- Schickling, O., Stegh, A.H., Byrd, J., and Peter, M.E. (2001). Nuclear localization of DEDD leads to caspase-6 activation through its death effector domain and inhibition of RNA polymerase I dependent transcription. *Cell Death. Differ.* 8, 1157-1168.
- Scorrano, L. and Korsmeyer, S.J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem. Biophys. Res. Commun.* 304, 437-444.
- Seshagiri, S. and Miller, L.K. (1997). *Caenorhabditis elegans* CED-4 stimulates CED-3 processing and CED-3- induced apoptosis. *Curr. Biol.* 7, 455-460.
- Shaulian, E. and Karin, M. (2001). AP-1 in cell proliferation and survival. *Oncogene* 20, 2390-2400.
- Shi, Y. and Massague, J. (2003). Mechanisms of TGF- β signalling from cell membrane to the nucleus. *Cell* 113, 685-700.
- Shikama, Y., U, M., Miyashita, T., and Yamada, M. (2001). Comprehensive studies on subcellular localizations and cell death-inducing activities of eight GFP-tagged apoptosis-related caspases. *Experimental Cell Research* 264, 315-325.
- Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996). Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* 13, 21-29.
- Shimizu, S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399, 483-487.
- Shiokawa, D. and Tanuma, S. (1998). Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase gamma. *Biochem. J.* 332, 713-720.
- Siegmund, D., Mauri, D., Peters, N., Juo, P., Thome, M., Reichwein, M., Blenis, J., Scheurich, P., Tschopp, J., and Wajant, H. (2001). Fas-associated death domain protein (FADD) and caspase-8 mediate up-regulation of c-Fos by Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) via a FLICE inhibitory protein (FLIP)-regulated pathway. *J. Biol. Chem.* 276, 32585-32590.
- Sikorska, M. and Walker, P.R. (1998). Endonuclease activities in apoptosis. In *When Cells Die*, R.A. Locks, Z. Zakeri, and J.L. Tilly, eds. Wiley-Liss, pp. 211-242.

- Sillett,H.K., Cruickshank,S.M., Southgate,J., and Trejdosiewicz,L.K. (2001). Transforming growth factor-beta promotes 'death by neglect' in post- activated human T cells. *Immunology* 102, 310-316.
- Slee,E.A., Adrain,C., and Martin,S.J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J. Biol. Chem.* 276, 7320-7326.
- Smolewski,P., Graberek,J., Halicka,H.D., and Darzynkiewicz,Z. (2002). Assay of caspase activation in situ combined with probing plasma membrane integrity to detect three distinct stages of apoptosis. *J. Immunol. Methods* 265, 111-121.
- Sodja,C., Walker,P.R., Brown,D.L., and Chaly,N. (1997). Unique behaviour of NuMA during heat-induced apoptosis of lymphocytes. *Biochem. Cell Biol.* 75, 399-414.
- Solovyan,V.T., Bezvenyuk,Z.A., Salminen,A., Austin,C.A., and Courtney,M.J. (2002). The role of topoisomerase II in the excision of DNA loop domains during apoptosis. *J. Biol. Chem.* 277, 21458-21467.
- Spetz,A.L., Patterson,B.K., Lore,K., Andersson,J., and Holmgren,L. (1999). Functional gene transfer of HIV DNA by an HIV receptor-independent mechanism. *J. Immunol.* 163, 736-742.
- Steen,R.L. and Collas,P. (2001). Mistargeting of B-type lamins at the end of mitosis: implications on cell survival and regulation of lamins A/C expression. *J. Cell Biol.* 153, 621-626.
- Stegh,A.H., Schickling,O., Ehret,A., Scaffidi,C., Peterhansel,C., Hofmann,T.G., Grummt,I., Krammer,P.H., and Peter,M.E. (1998). DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. *EMBO J.* 17, 5974-5986.
- Suntharalingam,M. and Wente,S.R. (2003). Peering through the pore: nuclear pore complex structure, assembly and function. *Dev. Cell* 4, 775-789.
- Susin,S.A., Lorenzo,H.K., Zamzami,N., Marzo,I., Snow,B.E., Brothers,G.M., Mangion,J., Jacotot,E., Costantini,P., Loeffler,M., Larochette,N., Goodlett,D.R., Aebersold,R., Siderovski,D.P., Penninger,J.M., and Kroemer,G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397, 441-446.
- Susin,S.A., Zamzami,N., Castedo,M., Daugas,E., Wang,H.G., Geley,S., Fassy,F., Reed,J.C., and Kroemer,G. (1997). The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/. *J. Exp. Med.* 186, 25-37.

- Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med.* 184, 1331-1341.
- Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell* 8, 613-621.
- Taimen, P. and Kallajoki, M. (2003). NuMA and nuclear lamins behave differently in Fas-mediated apoptosis. *J. Cell Sci.* 116, 571-583.
- Tang, D. and Kidd, V.J. (1998). Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. *J. Biol. Chem.* 273, 28549-28552.
- Tartaglia, L.A., Ayres, T.M., Wong, G.H., and Goeddel, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 74, 845-853.
- Terada, K., Kanazawa, M., Bukau, B., and Mori, M. (1997). The human DnaJ homologue dj2 facilitates mitochondrial protein import and luciferase refolding. *J. Cell Biol.* 139, 1089-1095.
- Terada, K. and Mori, M. (2000). Human DnaJ homologs dj2 and dj3, and bag-1 are positive cochaperones of hsc70. *J. Biol. Chem.* 275, 24728-24734.
- Testolin, L., Carson, C., Wang, Y., Walker, P.R., Armato, U., and Sikorska, M. (1997). Jun and JNK kinase are activated in thymocytes in response to VM26 and radiation but not glucocorticoids. *Exp. Cell Res.* 230, 220-232.
- Tibbetts, M.D., Zheng, L., and Lenardo, M.J. (2003). The death effector domain protein family: regulators of cellular homeostasis. *Nat. Immunol.* 4, 404-409.
- Torii, S., Egan, D.A., Evans, R.A., and Reed, J.C. (1999). Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO J.* 18, 6037-6049.
- Tsujimoto, Y. (2003). Cell death regulation by the Bcl-2 protein family in the mitochondria. *Journal of Cellular Physiology* 195, 158-167.
- Tzur, Y.B., Hersh, B.M., Horvitz, H.R., and Gruenbaum, Y. (2002). Fate of the Nuclear Lamina during *Caenorhabditis elegans* Apoptosis. *J. Struct. Biol.* 137, 146-153.
- van Loo, G., Saelens, X., van Gurp, M., MacFarlane, M., Martin, S.J., and Vandenabeele, P. (2002). The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Differ.* 9, 1031-1042.
- Vaux, D.L. (2002). Apoptosis timeline. *Cell Death Differ.* 9, 349-354.

- Verhagen,A.M., Ekert,P.G., Pakusch,M., Silke,J., Connolly,L.M., Reid,G.E., Moritz,R.L., Simpson,R.J., and Vaux,D.L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102, 43-53.
- Villa,P., Kaufmann,S.H., and Earnshaw,W.C. (1997). Caspases and caspase inhibitors. *Trends Biochem. Sci.* 22, 388-393.
- Villunger,A., Huang,D.C., Holler,N., Tschopp,J., and Strasser,A. (2000). Fas ligand-induced c-Jun kinase activation in lymphoid cells requires extensive receptor aggregation but is independent of DAXX, and Fas-mediated cell death does not involve DAXX, RIP, or RAIDD. *J. Immunol.* 165, 1337-1343.
- Vukmanovic,S. and Zamoyska,R. (1991). Anti-CD3-induced cell death in T cell hybridomas: mitochondrial failure and DNA fragmentation are distinct events. *Eur. J. Immunol.* 21, 419-424.
- Wajant,H. (2002). The Fas signalling pathway: more than a paradigm. *Science* 296, 1635-1636.
- Walker,P.R., Carson,C., Leblanc,J., and Sikorska,M. (2002). Labeling DNA damage with terminal transferase. Applicability, specificity, and limitations. *Methods Mol. Biol.* 203, 3-19.
- Walker,P.R., Leblanc,J., and Sikorska,M. (1997). Evidence that DNA fragmentation in apoptosis is initiated and propagated by single-strand breaks. *Cell Death Diff.* 4, 506-515.
- Walker,P.R., Leblanc,J., Smith,B., Pandey,S., and Sikorska,M. (1999). Detection of DNA fragmentation and endonucleases in apoptosis. *Methods* 17, 329-338.
- Walker,P.R., Pandey,S., and Sikorska,M. (1995). Degradation of chromatin in apoptotic cells. *Cell Death Diff.* 4, 97-104.
- Walker,P.R., Weaver,V.M., Lach,B., Leblanc,J., and Sikorska,M. (1994). Endonuclease activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. *Exp. Cell Res.* 213, 100-106.
- Wang,J., Chun,H.J., Wong,W., Spencer,D.M., and Lenardo,M.J. (2001). Caspase-10 is an initiator caspase in death receptor signaling. *Proc. Natl. Acad. Sci. U. S. A* 98, 13884-13888.
- Wang,S., Nath,N., Minden,A., and Chellappan,S. (1999). Regulation of Rb and E2F by signal transduction cascades: divergent effects of JNK1 and p38 kinases. *EMBO J.* 18, 1559-1570.
- Wang,X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev.* 15, 2922-2933.

- Wang,X., Yang,C., Chai,J., Shi,Y., and Xue,D. (2002). Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. *Science* 298, 1587-1592.
- Wang,Z.G., Ruggero,D., Ronchetti,S., Zhong,S., Gaboli,M., Rivi,R., and Pandolfi,P.P. (1998). PML is essential for multiple apoptotic pathways. *Nat. Genet.* 20, 266-272.
- Weaver,V.M., Carson,C.E., Walker,P.R., Chaly,N., Lach,B., Raymond,Y., Brown,D.L., and Sikorska,M. (1996). Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. *J. Cell Sci.* 109 (Pt 1), 45-56.
- Whitfield,J.F., Perris,A.D., and Youdale,T. (1968). Destruction of the nuclear morphology of thymic lymphocytes by the corticosteroid cortisol. *Exp. Cell Res.* 52, 349-362.
- Widlak,P., Palyvoda,O., Kumala,S., and Garrard,W.T. (2002). Modeling apoptotic chromatin condensation in normal cell nuclei. *J. Biol. Chem.* 277, 21683-21690.
- Wilson,D.J., Alessandrini,A., and Budd,R.C. (1999). MEK1 activation rescues Jurkat T cells from Fas-induced apoptosis. *Cell Immunol.* 194, 67-77.
- Wiltshire,C., Matsushita,M., Tsukada,S., Gillespie,D.A., and May,G.H. (2002). A new c-Jun N-terminal kinase (JNK)-interacting protein, Sab (SH3BP5), associates with mitochondria. *Biochem. J.* 367, 577-585.
- Wolf,B.B. and Green,D.R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J. Biol. Chem.* 274 , 20049-20052.
- Worman,H.J. and Courvalin,J.C. (2000). The inner nuclear membrane. *J. Membr. Biol.* 177, 1-11.
- Wu,X. and Deng,Y. (2002). Bax and BH3-domain-only proteins in p53-mediated apoptosis. *Front Biosci.* 7, d151-d156.
- Yanagisawa,J., Ando,J., Nakayama,J., Kohwi,Y., and Kohwi-Shigematsu,T. (1996). A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast carcinomas and not in normal and benign breast disease tissues. *Cancer Res.* 56, 457-462.
- Yang,J., Liu,X., Bhalla,K., Kim,C.N., Ibrado,A.M., Cai,J., Peng,T.I., Jones,D.P., and Wang,X. (1997a). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275, 1129-1132.
- Yang,X., Khosravi-Far,R., Chang,H.Y., and Baltimore,D. (1997b). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* 89, 1067-1076.
- Yaoita,Y. (2002). Inhibition of nuclear transport of caspase-7 by its prodomain. *Biochem. Biophys. Res. Commun.* 291, 79-84.

- Yasuhara,N., Eguchi,Y., Tachibana,T., Imamoto,N., Yoneda,Y., and Tsujimoto,Y. (1997). Essential role of active nuclear transport in apoptosis. *Genes Cells* 2, 55-64.
- Yasui,D., Miyano,M., Cai,S., Varga-Weisz,P., and Kohwi-Shigematsu,T. (2002). SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* 419, 641-645.
- Ye,Q. and Worman,H.J. (1994). Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane. *J. Biol. Chem.* 269, 11306-11311.
- Yin,X.M., Wang,K., Gross,A., Zhao,Y., Zinkel,S., Klocke,B., Roth,K.A., and Korsmeyer,S.J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400, 886-891.
- Yoshida,H., Kong,Y.Y., Yoshida,R., Elia,A.J., Hakem,A., Hakem,R., Penninger,J.M., and Mak,T.W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 94, 739-750.
- Yu,K.Y., Kwon,B., Ni,J., Zhai,Y., Ebner,R., and Kwon,B.S. (1999). A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J. Biol. Chem.* 274, 13733-13736.
- Zamzami,N. and Kroemer,G. (2003). Apoptosis: Mitochondrial Membrane Permeabilization- the (W)hole Story? *Curr. Biol.* 13, R71-R73.
- Zamzami,N., Marchetti,P., Castedo,M., Decaudin,D., Macho,A., Hirsch,T., Susin,S.A., Petit,P.X., Mignotte,B., and Kroemer,G. (1995a). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med.* 182, 367-377.
- Zamzami,N., Marchetti,P., Castedo,M., Zanin,C., Vayssiere,J.L., Petit,P.X., and Kroemer,G. (1995b). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181, 1661-1672.
- Zamzami,N., Susin,S.A., Marchetti,P., Hirsch,T., Gomez-Monterrey,I., Castedo,M., and Kroemer,G. (1996). Mitochondrial control of nuclear apoptosis. *J. Exp. Med.* 183, 1533-1544.
- Zhang,D., Beresford,P.J., Greenberg,A.H., and Lieberman,J. (2001). Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytotoxicity. *Proc. Natl. Acad. Sci. U. S. A* 98, 5746-5751.
- Zheng,L., Schickling,O., Peter,M.E., and Lenardo,M.J. (2001). The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. *J. Biol. Chem.* 276, 31945-31952.

- Zhivotovsky,B., Gahm,A., and Orrenius,S. (1997). Two different proteases are involved in the proteolysis of lamin during apoptosis. *Biochem. Biophys. Res. Commun.* 233, 96-101.
- Zhivotovsky,B., Orrenius,S., Brustugun,O.T., and Doskeland,S.O. (1998). Injected cytochrome c induces apoptosis. *Nature* 391, 449-450.
- Zhivotovsky,B., Samali,A., Gahm,A., and Orrenius,S. (1999). Caspases: their intracellular localization and translocation during apoptosis. *Cell Death Differ.* 6, 644-651.
- Zhuang,J., Dinsdale,D., and Cohen,G.M. (1998). Apoptosis, in human monocytic THP.1 cells, results in the release of cytochrome c from mitochondria prior to their ultracondensation, formation of outer membrane discontinuities and reduction in inner membrane potential. *Cell Death. Differ.* 5, 953-962.
- Zou,H., Henzel,W.J., Liu,X., Lutschg,A., and Wang,X. (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90, 405-413.
- Zou,H., Li,Y., Liu,X., and Wang,X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* 274, 11549-11556.
- Zweyer,M., Riederer,B.M., Ochs,R.L., Fackelmayer,F.O., Kohwi-Shigematsu,T., Bareggi,R., Narducci,P., and Martelli,A.M. (1997). Association of nuclear matrix proteins with granular and threaded nuclear bodies in cell lines undergoing apoptosis. *Exp. Cell Res.* 230, 325-336.
- Zylicz,M., King,F.W., and Wawrzynow,A. (2001). Hsp70 interactions with the p53 tumour suppressor protein. *EMBO J.* 20, 4634-4638.

7 APPENDICIES

7.1 APPENDIX A: SOURCE OF CHEMICALS

123 bp DNA ladder (Gibco BRL, Burlington, ON)
5'-T₂₀VN-3' (Cortec, Queen's Universtiy, Kingston, ON)
5x First Strand Reaction Buffer, Superscript II (Invitrogen Life Technologies, Burlington, ON)
Acetic acid (BDH, Toronto, ON)
Agarose (Gibco BRL, Burlington, ON)
alkaline phosphatase goat anti-mouse IgG (Promega, Madison, WI)
alkaline-phosphatase donkey anti-rabbit IgG (Promega, Madison, WI)
Anti-Fas monoclonal antibody, Clone CH-11 (Upstate Biotech Inc., Lake Placid, NY)
Anti-NuMA, A-204 (Matritech, Cambridge, MA)
Arabidopsis RNA (Promega, Madison, WI)
BCIP/NBT substrate for Western blotting (KPL, Gaithersburg, MA)
BCP (MRC Inc., Cincinnati, OH)
Biotin-16-dUTP (Boehringer Mannheim, Laval, PQ)
Boric acid (BDH, Toronto, ON)
BSA, A-7030 (Sigma Chemical Co., St. Louis, MO)
Cacodylate buffer (Gibco BRL, Burlington, ON)
CoCl₂ (Sigma Chemical Co., St. Louis, MO)
CY3 -anti-rabbit IgG (Jackson ImmunoResearch/BioCan Scientific, Mississauga, ON)
CY3 -streptavidin (Jackson ImmunoResearch/BioCan Scientific, Mississauga, ON)
CY3-dCTP (Amersham Biosciences, Baie d'Urfe, QC)
CY5-dCTP (Amersham Biosciences, Baie d'Urfe, QC)
DAPI (Sigma Chemical Co., St. Louis, MO)
DEPC (Sigma Chemical Co., St. Louis, MO)
DTT (Gibco BRL, Burlington, ON)
dATP (Amersham Biosciences, Baie d'Urfe, QC)
dCTP (Amersham Biosciences, Baie d'Urfe, QC)
Deoxycholate (Sigma Chemical Co., St. Louis, MO)
Dexamethasone (Sigma Chemical Co., St. Louis, MO)
dGTP (Amersham Biosciences, Baie d'Urfe, QC)
DIG Easy Hyb buffer (Boehringer Mannheim, Laval, PQ)
DiOC₆₍₃₎ (Molecular Probes, Eugene, OR)
DMSO (Sigma Chemical Co., St. Louis, MO)
DNase-free Rnase (Sigma Chemical Co., St. Louis, MO)
dTTP (Amersham Biosciences, Baie d'Urfe, QC)
EDTA (Sigma Chemical Co., St. Louis, MO)
EDTA (Sigma Chemical Co., St. Louis, MO)
EGTA (Sigma Chemical Co., St. Louis, MO)
Ethidium bromide (Sigma Chemical Co., St. Louis, MO)
Fetal bovine serum (Gibco BRL, Burlington, ON)

FITC- anti-human IgG (Jackson ImmunoResearch/BioCan Scientific, Mississauga, ON)
 FITC-anti mouse IgM (Cappel/Organon Teknika, Scarborough, ON)
 FITC-anti-mouse IgG (Jackson ImmunoResearch/BioCan Scientific, Mississauga, ON)
 FITC-phalloidin (Molecular Probes, Eugene, OR)
 FITC-streptavidin (Jackson ImmunoResearch/BioCan Scientific, Mississauga, ON)
 Gelatin (Bio-Rad, Mississauga, ON)
 HANKS balanced salt solution (Gibco BRL, Burlington, ON)
 HindIII digest of λ DNA (Gibco BRL, Burlington, ON)
 Hoechst 33342 (Sigma Chemical Co., St. Louis, MO)
 Hoechst 33258 (Sigma Chemical Co., St. Louis, MO)
 Immuno-Check fluorescent beads (Beckman Coulter, Burlington, ON))
 Isopropanol (BDH, Toronto, ON)
 KCl (Anachemia, Lachine, QC)
 Lambda phage DNA (New England Biolabs, Inc., Mississauga, ON)
 Low melting point agarose (Gibco BRL, Burlington, ON)
 NaBH₄ (BDH, Toronto, ON)
 NaCl (Sigma Chemical Co., St. Louis, MO)
 NaOH (BDH, Toronto, ON)
 Paraformaldehyde (J.B. EM Services Inc., Pointe Claire, Dorval, PQ)
 PFGE low range markers (New England Biolabs, Inc., Mississauga, ON)
 Poly-l-lysine (Sigma Chemical Co., St. Louis, MO)
 Potassium cacodylate (Sigma Chemical Co., St. Louis, MO)
 Propidium iodide (Molecular Probes, Eugene, OR)
 Proteinase K (Gibco BRL, Burlington, ON)
 RNasin (Promega, Madison, WI)
 RPMI 1640 tissue culture media (Gibco BRL, Burlington, ON)
 Salmon sperm DNA (Sigma Chemical Co., St. Louis, MO)
 SDS (BDH, Toronto, ON)
 Sodium citrate (BDH, Toronto, ON)
 Spermidine (Sigma Chemical Co., St. Louis, MO)
 Spermine (Sigma Chemical Co., St. Louis, MO)
 Superscript II (Invitrogen Life Technologies, Burlington, ON)
 TdT (Gibco BRL, Burlington, ON)
 Tri-Reagent (MRC Inc., Cincinnati, OH)
 Tris base (BDH, Toronto, ON)
 Tris-HCl (Fisher, Fair Lawn, NJ)
 Triton X-100 (Pierce/ Chromatographic Specialties Inc., Nepean, ON)
 Tween-20 (Sigma Chemical Co., St. Louis, MO)
 Vectasheild mounting medium (Vector/ Novocastra Laboratories Ltd., Burlington, ON)
 Yeast chromosomes (New England Biolabs, Inc., Mississauga, ON)
 Yeast tRNA (Gibco BRL, Burlington, ON)

7.2 APPENDIX B: UPREGULATED SEQUENCES

Clone ID	UG Cluster Name	Symbol	Mean fold change
47510	Hs.21022	adaptor-related protein complex 3, beta 2 subunit	AP3B2 1.71
46408	Hs.18268	adenylate kinase 5	AK5 3.97
5475349	Hs.90107	adhesion regulating molecule 1	ADRM1 3.08
430028	Hs.333257	advillin	AVIL 2.32
149080	Hs.128749	alpha-methylacyl-CoA racemase	AMACR 1.69
270248	Hs.334707	aminoacylase 1	ACY1 1.60
116771	Hs.268571	apolipoprotein C-I	APOC1 1.94
487938	Hs.356559	ARF GTPase-activating protein	MRIP2 1.80
188230	Hs.194382	ataxia telangiectasia mutated (includes complementation groups A, C and D)	ATM 1.51
30144	Hs.84999	ATPase, Cu ⁺⁺ transporting, beta polypeptide (Wilson disease)	ATP7B 2.71
5578702	Hs.168159	bifunctional apoptosis regulator	BFAR 2.77
125581	Hs.381231	caspase 8, apoptosis-related cysteine protease	CASP8 2.21
41975	Hs.18586	CDC42 binding protein kinase alpha (DMPK-like)	CDC42BPA 1.92
261838	Hs.24340	centaurin, beta 2	CENTB2 1.56
272612	Hs.127675	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	CLN8 1.72
273069	Hs.9825	CGI-128 protein	CGI-128 6.07
142588	Hs.125849	chromosome 8 open reading frame 2	C8orf2 1.63
4716125	Hs.99886	complement component 4 binding protein, beta	C4BPB 2.17
240712	Hs.99886	complement component 4 binding protein, beta	C4BPB 1.83
25386	Hs.380969	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	COP5 3.65
5842306	Hs.82932	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1 1.73
51398	Hs.271221	cyclin M2	CNNM2 2.49
51398	Hs.271221	cyclin M2	CNNM2 2.84
338479	Hs.426266	cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (sub-family C, member 7)	CFTR 1.68
258870	Hs.79946	cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1 1.62
248397	Hs.104117	cytochrome P450, family 3, subfamily A, polypeptide 5	CYP3A5 1.86
248397	Hs.104117	cytochrome P450, family 3, subfamily A, polypeptide 5	CYP3A5 1.63
470966	Hs.70582	DEAD box helicase 97 KDa	MGC2835 1.52
5851583	Hs.433989	decorin	DCN 1.65
429954	Hs.11779	dehydrogenase/reductase (SDR family) X chromosome	DHR5X 1.55
4450636	Hs.279696	DKFZP566I1024 protein	DKFZP566I1024 3.14
21815	Hs.180383	dual specificity phosphatase 6	DUSP6 2.46

35818	Hs.97925	elongation protein 4 homolog (<i>S. cerevisiae</i>)	ELP4	2.04
280327	Hs.302161	endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	EDG8	1.52
5548410	Hs.106673	eukaryotic translation initiation factor 3, subunit 6 48kDa	EIF3S6	1.62
111168	Hs.57783	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa	EIF3S9	1.55
5314001	Hs.132898	fatty acid desaturase 1	FADS1	1.55
4702445	Hs.418650	ferritin, heavy polypeptide 1	FTH1	2.39
35715	Hs.198612	G protein-coupled receptor 51	GPR51	2.32
3445214	Hs.79222	galactosidase, beta 1	GLB1	1.52
270088	Hs.76686	glutathione peroxidase 1	GPX1	1.91
261910	Hs.193974	glutathione reductase	GSR	1.66
5755043	Hs.169476	glyceraldehyde-3-phosphate dehydrogenase	GAPD	2.32
52759	Hs.76828	glypican 5	GPC5	2.24
123257	Hs.182982	golgin-67	GOLGIN-67	1.76
5474468	Hs.340636	GTP binding protein 5 (putative)	GTPBP5	2.00
208059	Hs.180414	heat shock 70kDa protein 8	HSPA8	1.63
471910	Hs.406404	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	HNRPD	1.51
116772	Hs.58169	highly expressed in cancer, rich in leucine heptad repeats	HEC	2.31
252928	Hs.1480	histidine rich calcium binding protein	HRC	1.85
121452	Hs.301005	histone H2A.F/Z variant	H2AV	2.03
5424961	Hs.321045	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	1.91
171569	Hs.50651	Janus kinase 1 (a protein tyrosine kinase)	JAK1	2.39
5405608	Hs.301206	kinesin family member 3B	KIF3B	1.64
446605	Hs.380929	lactate dehydrogenase D	LDHD	1.74
5725681	Hs.166254	likely ortholog of rat vacuole membrane protein 1	VMP1	2.38
248874	Hs.180398	LIM domain containing preferred translocation partner in lipoma	LPP	1.58
154190	Hs.25253	mannosidase, alpha, class 1A, member 1	MAN1A1	1.81
5784795	Hs.118786	metallothionein 2A	MT2A	1.55
128544	Hs.109059	mitochondrial ribosomal protein L12	MRPL12	1.92
172504	Hs.211914	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	NDUFS7	1.72
342879	Hs.81469	nucleotide binding protein 1 (MinD homolog, <i>E. coli</i>)	NUBP1	1.99
24747	Hs.405961	old astrocyte specifically induced substance	OASIS	1.71
110771	Hs.49760	origin recognition complex, subunit 6 homolog-like (yeast)	ORC6L	1.55
110771	Hs.49760	origin recognition complex, subunit 6 homolog-like (yeast)	ORC6L	1.90
174837	Hs.211586	phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	PIK3R2	1.69
48265	Hs.82285	phosphoribosylglycinamide formyltransferase,	GART	1.86

		phosphoribosylglycinamide synthetase,		
		phosphoribosylaminoimidazole synthetase		
257510	Hs.298229	prefoldin 2	PFDN2	3.04
4720030	Hs.352054	pregnancy specific beta-1-glycoprotein 9	PSG9	1.56
23059	Hs.74519	primase, polypeptide 2A, 58kDa	PRIM2A	1.79
5809010	Hs.150580	putative translation initiation factor	SUI1	1.62
488208	Hs.75438	quinoid dihydropteridine reductase	QDPR	5.28
42762	Hs.79307	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	ARHGEF6	2.97
5590426	Hs.9347	regulator of G-protein signalling 14	RGS14	1.64
3266934	Hs.25951	Rho guanine nucleotide exchange factor (GEF) 3	ARHGEF3	1.65
277041	Hs.172813	Rho guanine nucleotide exchange factor (GEF) 7	ARHGEF7	1.64
51604	Hs.101742	ribosomal large subunit pseudouridine synthase C like	RLUCL	4.28
5558091	Hs.356795	ribosomal protein L41	RPL41	1.89
4248813	Hs.409045	ribosomal protein L6	RPL6	1.81
4373635	Hs.275865	ribosomal protein S18	RPS18	1.65
324712	Hs.103081	ribosomal protein S6 kinase, 70kDa, polypeptide 2	RPS6KB2	1.60
232533	Hs.334725	similar to putative transmembrane protein; homolog of yeast Golgi membrane protein Yiflp (Yip1p-interacting factor)	LOC90522	2.17
194357	Hs.8088	similar to <i>S. cerevisiae</i> Sec6p and <i>R. norvegicus</i> rsec6	SEC6	1.57
52422	Hs.113602	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6	SLC1A6	1.68
38747	Hs.241503	spastic paraplegia 3A (autosomal dominant)	SPG3A	1.69
43059	Hs.177011	spermatogenesis associated 7	SPATA7	1.54
4499521	Hs.195614	splicing factor 3b, subunit 3, 130kDa	SF3B3	2.05
301084	Hs.1074	surfactant, pulmonary-associated protein C	SFTPC	1.52
504179	Hs.334791	tetraspanin similar to uroplakin 1	LOC90139	2.33
239918	Hs.142157	transcription termination factor, RNA polymerase II	TTF2	1.63
52027	Hs.75307	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	TGM2	2.13
4849718	Hs.8372	ubiquinol-cytochrome c reductase (6.4kD) subunit	UQCR	1.54
154663	Hs.348024	v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	RALB	2.52
248849	Hs.380730	zinc finger protein	FLJ10697	1.54
5838665	Hs.343586	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	2.62

7.3 APPENDIX C: DOWNREGULATED SEQUENCES

Clone ID	UGCluster	Name	Symbol	Mean fold change
579768 7	Hs.75616	24-dehydrocholesterol reductase	DHCR24	-2.1
231663	Hs.195471	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	PFKFB3	-1.6
179357	Hs.75393	acid phosphatase 1, soluble	ACP1	-1.6
129349	Hs.421956	AD024 protein	AD024	-1.5
486852	Hs.9572	adenylate cyclase 5	ADCY5	-1.7
42284	Hs.75102	alanyl-tRNA synthetase	AARS	-1.7
470837 5	Hs.76847	alpha glucosidase II alpha subunit	G2AN	-1.6
488263	Hs.75741	amiloride binding protein 1 (amine oxidase (copper-containing))	ABP1	-1.7
324710	Hs.27669	amyotrophic lateral sclerosis 2 (juvenile)	ALS2	-1.7
252336	Hs.9613	angiopoietin-like 4	ANGPTL4	-2.3
404289 7	Hs.75736	apolipoprotein D	APOD	-1.7
415278	Hs.81634	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit b, isoform 1	ATP5F1	-1.6
592762 3	Hs.81634	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit b, isoform 1	ATP5F1	-1.7
484765	Hs.155433	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP5C1	-2.4
265701	Hs.75478	ATPase, Class VI, type 11B	ATP11B	-1.7
30144	Hs.84999	ATPase, Cu ⁺⁺ transporting, beta polypeptide (Wilson disease)	ATP7B	-1.8
182933	Hs.134514	ATP-binding cassette, sub-family A (ABC1), member 7	ABCA7	-1.7
322866	Hs.55879	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	ABCC10	-1.6
554129 3	Hs.15259	BCL2-associated athanogene 3	BAG3	-2.7
172475	Hs.80338	Bcl-2-associated transcription factor	BTF	-2.4
206781	Hs.172382	betaine-homocysteine methyltransferase 2	BHMT2	-1.8
165971	Hs.433307	branched chain keto acid dehydrogenase E1, alpha polypeptide (maple syrup urine disease)	BCKDHA	-1.6
491240	Hs.44685	C3HC4-like zinc finger protein	ZFP26	-1.6
151620	Hs.424931	CAC-1	CAC-1	-1.7
146832	Hs.75929	cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	-1.7
489176 8	Hs.8417	calcium/calmodulin-dependent protein kinase kinase 1, alpha	CAMKK1	-1.6
494144 2	Hs.29331	carnitine palmitoyltransferase 1B (muscle)	CPT1B	-1.7
434621	Hs.183085	CCR4-NOT transcription complex, subunit 6-	CNOT6L	-1.7

3		like		
115281	Hs.79630	CD79A antigen (immunoglobulin-associated alpha)	CD79A	-1.8
200227	Hs.423615	cell division cycle 34	CDC34	-1.6
171672	Hs.146409	cell division cycle 42 (GTP binding protein, 25kDa)	CDC42	-1.8
158463	Hs.271614	CGI-112 protein	CGI-112	-2.6
260007	Hs.182986	CGI-36 protein	CGI-36	-1.6
143403	Hs.5298	CGI-45 protein	CGI-45	-1.7
553590				-1.8
7	Hs.82916	chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A	
144944	Hs.89414	chemokine (C-X-C motif) receptor 4	CXCR4	-2.4
213740	Hs.75470	chromosome 1 open reading frame 29	C1orf29	-1.8
232727	Hs.90918	chromosome 11 open reading frame 10	C11orf10	-1.7
271377	Hs.196437	chromosome 2 open reading frame 6	C2orf6	-1.6
417644	Hs.3569	chromosome 20 open reading frame 155	C20orf155	-2.2
154969	Hs.26213	chromosome 20 open reading frame 167	C20orf167	-1.6
491630	Hs.282882	chromosome 21 open reading frame 109	C21orf109	-1.9
486047	Hs.84162	chromosome 3p21.1 gene sequence	HUMAGCG B	-1.7
326032	Hs.425456	cisplatin resistance related protein CRR9p	CRR9	-1.9
342613	Hs.75887	coatamer protein complex, subunit alpha	COPA	-1.6
299740	Hs.3059	coatamer protein complex, subunit beta	COPB	-1.7
326223	Hs.146428	collagen, type V, alpha 1	COL5A1	-1.7
154173	Hs.235368	collagen, type V, alpha 3	COL5A3	-1.8
110331	Hs.108885	collagen, type VI, alpha 1	COL6A1	-1.6
207989	Hs.2441	C-type lectin BIMLEC precursor	BIMLEC	-1.6
220505	Hs.166071	cyclin-dependent kinase 5	CDK5	-1.9
299183	Hs.695	cystatin B (stefin B)	CSTB	-1.6
207669	Hs.38039	D11lgp1e-like	LGP1	-1.7
592333				-2.0
1	Hs.108327	damage-specific DNA binding protein 1, 127kDa	DDB1	
578742				-2.3
8	Hs.103180	DC2 protein	DC2	
239801	Hs.75189	death-associated protein	DAP	-1.8
126416	Hs.89135	deltex homolog 2 (Drosophila)	DTX2	-1.6
266114	Hs.274464	diaphorase (NADH) (cytochrome b-5 reductase)	DIA1	-2.1
272658	Hs.234265	DKFZP586G011 protein	LAP1B	-1.6
247551	Hs.108371	E2F transcription factor 4, p107/p130-binding	E2F4	-2.0
486340	Hs.2331	E2F transcription factor 5, p130-binding	E2F5	-1.7
35711	Hs.194477	E3 ubiquitin ligase SMURF2	SMURF2	-1.6
299500	Hs.154365	E74-like factor 1 (ets domain transcription factor)	ELF1	-1.5
40163	Hs.6522	elongation factor for selenoprotein translation	SELB	-1.8
376315	Hs.434075	endothelial-derived gene 1	EG1	-1.6
580607				-1.7
0	Hs.82193	esterase D/formylglutathione hydrolase	ESD	
305122	Hs.155103	eukaryotic translation initiation factor 1A, Y	EIF1AY	-1.7

		chromosome		
		excision repair cross-complementing rodent		-1.6
470196	Hs.48576	repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	ERCC5	
124065	Hs.35094	extracellular matrix protein 2, female organ and adipocyte specific	ECM2	-1.5
485648	Hs.198862	fibulin 2	FBLN2	-1.5
209255	Hs.172694	fring	LOC117584	-1.8
340861	Hs.59242	furin (paired basic amino acid cleaving enzyme)	FURIN	-1.7
299692	Hs.56845	GDP dissociation inhibitor 2	GDI2	-2.2
151611	Hs.1674	glutamine-fructose-6-phosphate transaminase 1	GFPT1	-1.5
28505	Hs.75652	glutathione S-transferase M5	GSTM5	-1.6
233182	Hs.172647	golgi autoantigen, golgin subfamily a, 1	GOLGA1	-2.7
282232	Hs.425623	growth differentiation factor 11	GDF11	-2.2
221208	Hs.51147	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	GNAT1	-1.7
197641	Hs.155090	guanine nucleotide binding protein (G protein), beta 5	GNB5	-1.5
22658	Hs.75452	heat shock 70kDa protein 1A	HSPA1A	-2.3
321792	Hs.132834	hematopoietic protein 1	HEM1	-1.5
304843	Hs.284279	heme oxygenase (decycling) 2	HMOX2	-1.7
219656	Hs.20993	high-glucose-regulated protein 8	HGRG8	-1.6
584774				-1.8
7	Hs.424053	homer homolog 3 (Drosophila)	HOMER3	
111571	Hs.142442	HP1-BP74	HP1-BP74	-1.7
163221	Hs.102950	immunoglobulin lambda joining 3	IGLJ3	-1.6
489326	Hs.40034	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	ITGA4	-1.9
194088	Hs.256583	interleukin enhancer binding factor 3, 90kDa	ILF3	-2.5
118365	Hs.166184	intersectin 2	ITSN2	-1.7
149156	Hs.78993	IQ motif containing GTPase activating protein 2	IQGAP2	-1.6
196727	Hs.300670	KIAA1204 protein	CDGAP	-1.6
121551	Hs.18141	ladinin 1	LAD1	-1.6
240325	Hs.93199	lanosterol synthase (2,3-oxidosqualene- lanosterol cyclase)	LSS	-1.9
472327				-1.6
1	Hs.7917	likely ortholog of mouse hypoxia induced gene 1	HIG1	
480337				-1.9
0	Hs.166254	likely ortholog of rat vacuole membrane protein 1	VMP1	
503209	Hs.79356	Lysosomal-associated multispinning membrane protein-5	LAPTM5	-1.5
129140	Hs.79078	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	-2.0
300080	Hs.7457	MAGE-E1 protein	MAGE-E1	-1.7
207215	Hs.77961	major histocompatibility complex, class I, B	HLA-B	-3.4
342581	Hs.7835	mannose receptor, C type 2	MRC2	-2.2
120559	Hs.381097	metallothionein 1F (functional)	MT1F	-2.1
511370	Hs.130607	mevalonate kinase (mevalonic aciduria)	MVK	-1.9

8				
289588	Hs.149894	mitochondrial translational initiation factor 2	MTIF2	-1.5
111377	Hs.23643	Mst3 and SOK1-related kinase	MST4	-1.6
291608	Hs.21321	myotrophin	MTPN	-1.9
382175	Hs.48802	myotubularin related protein 9	MTMR9	-2.6
428542	Hs.51299	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	NDUFV2	-1.5
125806	Hs.20028	N-deacetylase/N-sulfotransferase (heparan glucosaminy) 3	NDST3	-1.9
110756	Hs.14456	neural precursor cell expressed, developmentally down-regulated 1	NEDD1	-1.6
204695	Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-1.8
204695	Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-1.6
582990	Hs.155595	neural precursor cell expressed, developmentally down-regulated 5	NEDD5	-2.1
8				
220392	Hs.75512	neural precursor cell expressed, developmentally down-regulated 8	NEDD8	-2.0
309825	Hs.55069	neurexophilin 3	NXPH3	-1.8
159442	Hs.433222	Niemann-Pick disease, type C2	NPC2	-1.8
504661	Hs.373499	NS1-associated protein 1	NSAP1	-1.6
142899	Hs.179909	nuclear receptor coactivator 6 interacting protein	NCOA6IP	-2.0
181113	Hs.100221	nuclear receptor subfamily 1, group H, member 2	NR1H2	-1.7
305947	Hs.84928	nuclear transcription factor Y, beta	NFYB	-1.6
522634	Hs.296585	nucleolar protein 5A (56kDa with KKE/D repeat)	NOL5A	-1.8
5				
51221	Hs.106635	ortholog of rat pippin	PIPPIN	-1.6
210873	Hs.20588	pancreatic polypeptide 2	PPY2	-1.6
235072	Hs.271954	pan-hematopoietic expression	PHEMX	-1.9
154550	Hs.73947	peptidase D	PEPD	-1.7
186842	Hs.75737	pericentriolar material 1	PCM1	-1.7
342212	Hs.3343	phosphoglycerate dehydrogenase	PHGDH	-1.5
308139	Hs.154104	pleiomorphic adenoma gene-like 2	PLAGL2	-1.5
264589	Hs.171880	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	POLR2A	-1.6
481490	Hs.296361	potassium inwardly-rectifying channel, subfamily J, member 13	KCNJ13	-1.7
5				
271599	Hs.298184	potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2	-1.7
		procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	P4HB	-1.5
203560	Hs.410578			
584108	Hs.406455	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	-1.5
1				
328493	Hs.419094	protease, serine, 1 (trypsin 1)	PRSS1	-1.6

109111	Hs.115721	protease, serine, 25	PRSS25	-1.6
5442460	Hs.79387	proteasome (prosome, macropain) 26S subunit, ATPase, 5	PSMC5	-1.6
5809140	Hs.76913	proteasome (prosome, macropain) subunit, alpha type, 5	PSMA5	-2.1
5933524	Hs.76913	proteasome (prosome, macropain) subunit, alpha type, 5	PSMA5	-2.8
502910	Hs.315366	protein kinase C, eta	PRKCH	-1.8
469697	Hs.80350	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	PPP2CB	-2.8
486400	Hs.54649	putative nucleic acid binding protein RY-1	RY1	-1.5
5788140	Hs.77273	ras homolog gene family, member A	ARHA	-2.9
158086	Hs.75082	ras homolog gene family, member G (rho G)	ARHG	-1.7
46531	Hs.194695	ras homolog gene family, member I	ARHI	-1.7
41657	Hs.252831	reticulon 3	RTN3	-1.7
155460	Hs.361071	retinoic acid receptor, alpha	RARA	-1.6
198546	Hs.202430	ring finger protein 1	RING1	-1.6
261760	Hs.10037	Sac domain-containing inositol phosphatase 3	SAC3	-1.5
665234	Hs.27621	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	SEMA5A	-1.9
128506	Hs.118926	sentrin/SUMO-specific protease 3	SENP3	-1.8
248143	Hs.234726	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	SERPINA3	-1.6
4335316	Hs.26054	SH2 domain containing 3C	SH2D3C	-1.6
5932330	Hs.109051	SH3 domain binding glutamic acid-rich protein like 3	SH3BGRL3	-1.7
248446	Hs.27258	Siah-interacting protein	SIP	-2.2
263152	Hs.75268	sialyltransferase 4C (beta-galactoside alpha-2,3-sialyltransferase)	SIAT4C	-1.7
418103	Hs.83715	Sjogren syndrome antigen B (autoantigen La)	SSB	-1.6
49181	Hs.184877	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	SLC25A11	-2.1
151634	Hs.301871	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	SLC37A1	-1.6
114074	Hs.334826	splicing factor 3b, subunit 1, 155kDa	SF3B1	-1.6
44947	Hs.406186	splicing factor 3b, subunit 4, 49kDa	SF3B4	-1.7
5806153	Hs.406186	splicing factor 3b, subunit 4, 49kDa	SF3B4	-1.6
294465	Hs.433581	splicing factor, arginine/serine-rich 11	SFRS11	-1.7
308013	Hs.284161	SPPL2b	SPPL2B	-1.5
324920	Hs.56045	src homology three (SH3) and cysteine rich domain	STAC	-1.6
161443	Hs.180403	STRIN protein	STRIN	-1.7
307040	Hs.43812	syntaxin 10	STX10	-1.5

136054	Hs.406590	T-cell activation protein	PGR1	-2.2
324303	Hs.125359	Thy-1 cell surface antigen	THY1	-1.6
301735	Hs.2022	transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)	TGM3	-1.7
48175	Hs.341203	transmembrane 6 superfamily member 1	TM6SF1	-1.6
44740	Hs.86228	TRIAD3 protein	TRIAD3	-1.9
592807	Hs.75355	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	UBE2N	-2.2
165966	Hs.11184	ubiquitin-conjugating enzyme E2R 2	UBE2R2	-1.7
546872	Hs.146355	v-abl Abelson murine leukemia viral oncogene homolog 1	ABL1	-1.7
145941	Hs.288283	WD-repeat protein	HAN11	-2.0
592001	Hs.180900	Williams-Beuren syndrome chromosome region 1	WBSCR1	-2.1
213933	Hs.149923	X-box binding protein 1	XBP1	-1.7
50794	Hs.78434	zinc finger protein 133 (clone pHZ-13)	ZNF133	-1.7
488025	Hs.145956	zinc finger protein 226	ZNF226	-1.5

7.4 APPENDIX D: MANUSCRIPT

INITIAL NUCLEAR EVENTS OF APOPTOSIS PRECEDE LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL IN ANTI-FAS TREATED JURKAT CELLS

**RUNNING TITLE: NUCLEAR EVENTS PRECEDE MITOCHONDRIAL EVENTS
IN JURKAT DEATH**

Christine Carson, Julie LeBlanc, P. Roy Walker and Marianna Sikorska*
Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario,
Canada K1A 0R6

*Corresponding author: Institute for Biological Sciences, NRC, 1200 Montreal Rd.,
Bldg. M-54, Ottawa, Ontario, Canada, K1A 0R6. Phone 613-993-5916. Fax 613-990-
7963. E-mail: Marianna.Sikorska@nrc-cnrc.gc.ca.

ABSTRACT

During anti-Fas mediated apoptosis in Jurkat cells, oligonucleosomal cleavage of DNA and activation of cytoplasmic effector caspases follow release of apoptogenic factors from mitochondria, an event closely associated with loss of membrane potential. In this report we document that initiation of nuclear endonucleolysis and proteolysis occurs upstream of mitochondria-mediated events. Early apoptotic cells, defined by their normal mitochondrial membrane potential, size and plasma membrane integrity, were isolated by cell sorting. These cells demonstrated altered nuclear morphology associated with initial DNA cleavage in the form of single strand breaks, cleavage and loss of the matrix attachment region (MAR)-binding protein SATB1 and altered gene expression. These results are consistent with a model in which transduction of the apoptotic stimulus from the death-inducing signaling complex (DISC) to the nucleus initiates nuclear events via interaction with, and reorganization of, existing subnuclear domains. Such domains are known to be involved in both caspase activation and transcription.

KEYWORDS: DNA cleavage, SATB1, proteolysis, microarray, caspase 3

ABBREVIATIONS: MAR, matrix attachment region of DNA; SATB1, special AT-rich sequence binding protein 1; DISC, death-inducing signaling complex; SMAC, second mitochondrial activator of caspases; DIABLO, direct IAP binding protein with low pI; AIF, apoptosis inducing factor; DFF, DNA fragmentation factor; CAD, caspase-activated Dnase; HMW, high molecular weight; LMPCR, ligation-mediated PCR; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PFGE, pulsed-field gel electrophoresis; LMW, low molecular weight; PI, propidium iodide; CAGE, conventional agarose gel electrophoresis; QPCR, quantitative PCR; HSP, heat shock protein; ER, endoplasmic reticulum; CLN8, gene contributing to ceroid-lipofuscinosis, neuronal 8; NDST3, N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3 gene; ECM2: extracellular matrix protein 2 gene; SMURF2, SMAD ubiquitination regulatory factor 2 gene; SERPINF2, serine proteinase inhibitor, member 2 gene; DED, death effector domain; DEDD, death effector domain DNA-binding protein; DEDAF, death effector domain-associated factor; TGF, transforming growth factor

INTRODUCTION

Apoptosis is commonly detected by monitoring nuclear collapse, DNA cleavage or loss of mitochondrial membrane potential, all hallmark processes of programmed cell death. Mechanisms linking loss of potential across the inner mitochondrial membrane to upstream signaling by death receptors on one hand, and to downstream DNA cleavage, on the other, have been partially elucidated. Cell death receptors, exemplified by the Fas receptor, CD95, have been shown to signal through domain-based recruitment of adaptor molecules and procaspases, which together form the DISC¹⁻⁵. Activation of initiator procaspases in the DISC⁶⁻⁸ results in direct initiation of a proteolytic cascade of effector caspase activation in cells which display a type I or extrinsic pathway response^{9,10}. Alternatively, in cells which employ the intrinsic death pathway, or type II signalling from death receptors, signals are transmitted from initiator caspases to the mitochondria⁹. This occurs¹¹ via cleavage and activation of Bid, a pro-apoptotic member of the Bcl-2 family^{12,13}. In these cells, efficient activation of effector caspases is controlled by the apoptosome¹⁴⁻¹⁶. Apoptosome formation depends on release of mitochondrial factors^{17,18}, an event closely associated with pore formation and loss of mitochondrial membrane potential¹⁹⁻²¹. Numerous molecules found in the mitochondrial intermembrane space have been implicated in the apoptotic response^{22,23}. They contribute either to proteolytic degradation of cellular components (e.g. cytochrome c²⁴, SMAC/DAIBLO^{25,26} and Omi/HtrA2^{23,27,28}) or to cleavage of the DNA into characteristic 50 Kbp and oligonucleosomal fragments (e.g. AIF²⁹ and endonuclease G³⁰).

Loss of mitochondrial function has proven to be an early and pivotal cytoplasmic event in the intrinsic apoptotic response. It precedes accumulation of reactive oxygen species³¹, loss of plasma membrane asymmetry and integrity³² and decrease in cell size³³. It also clearly precedes the final steps of DNA breakdown^{34,35}. Enzymes capable of oligonucleosomal DNA degradation in apoptotic cells provide both caspase-dependent (caspase 3-activated DFF/CAD³⁶⁻³⁸) and independent links (endonuclease G³⁰) from the release of mitochondrial apoptogenic factors to the end stage of DNA degradation. In addition, AIF links the apoptotic increase in mitochondrial permeability and generation of 50 Kbp DNA fragments in the DNA of dying cells^{29,39}.

We, among others, have shown that apoptotic DNA cleavage is hierarchical (reviewed in⁴⁰). Cleavage begins with accumulation of single strand breaks at vulnerable points in the DNA⁴¹⁻⁴⁴ and proceeds to generation of very high molecular weight (HMW) fragments (300 Kbp-1Mbp) before the appearance of the 50 Kbp and oligonucleosomal fragments more widely used as assays to detect apoptosis⁴⁵⁻⁴⁹. In apoptotic thymocytes and Jurkat cells initial DNA damage can be detected very early using pulsed-field and two-dimensional gel electrophoresis. This damage is accompanied, and perhaps mediated by, specific cleavage of a subset of nuclear matrix proteins^{44,50}. To further examine this proposed relationship we have recently mapped initial DNA cleavage events in Fas-treated Jurkats using ligation-mediated PCR (LMPCR)⁴⁴. Accumulation of single strand breaks could be detected preferentially in the base-unpairing region of several matrix attachment sequences, which are the stretches of DNA mediating interaction with the proteins of the nuclear matrix. Intriguingly, we noted that biochemical detection of early nuclear cleavage events preceded measurable change in apoptotic index as monitored by reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), an assay for mitochondrial enzymatic function.

In this report, we assess whether a signal released from mitochondria upon loss of membrane potential and integrity could be responsible for the early nuclear events of

apoptosis in Jurkat cells. Anti-Fas mediated apoptosis in this cell line is known to evoke a type II response, wherein loss of mitochondrial membrane potential and associated release of pro-apoptotic molecules from the mitochondrial intermembrane space constitute the first steps of the effector phase of cell death⁹. Since kinetic studies of loss of mitochondrial function and initial DNA and nuclear matrix cleavage produced overlapping profiles for these events, we pursued methods capable of simultaneously examining several parameters at the single cell level. Flow cytometric analysis was used to examine anti-Fas treated Jurkat cell populations on a cell-to cell basis, and early apoptotic cells with normal mitochondrial function were isolated by cell sorting. Nuclear events were then examined specifically in those cells that still appeared normal. Using this approach initial DNA cleavage, along with changes in nuclear morphology, integrity and organisation of the MAR-binding protein SATB1, and gene expression were shown to precede loss of mitochondrial function, cell shrinkage, and loss of plasma membrane integrity.

RESULTS

Kinetics of apoptosis in anti-Fas treated Jurkat cells Loss of mitochondrial function, DNA cleavage, and breakdown of nuclear matrix protein were first monitored in Jurkat cells treated with 100 ng/ml anti-Fas over a 4 hour timecourse. The proportion of cells displaying loss of mitochondrial function, detected by decreased incorporation of the potential-sensitive dye DiOC₆₍₃₎, rose from 5% in controls to 28% at 2 hours of anti-Fas treatment and 45% after 4 hours in the presence of the inducer (Figure 1A). Apoptosis-associated DNA cleavage was assessed using pulsed field gel electrophoresis (PFGE). Both very high molecular weight and 50 Kbp fragments were first detected after 30 minutes of treatment (Figure 1B, lane 0.5). From 30 minutes to 2 hours, an increase in the 50 Kbp size class, followed by a smear of low molecular weight (LMW) fragments, was seen (Figure 1B, lanes 1-2). From 3 to 4 hours, the proportion of 50 Kbp fragments decreased and the LMW fragments accounted for the bulk of the DNA (Figure 1B, lanes 3-4). The MAR-binding protein SATB1 was rapidly degraded in treated Jurkats, as expected⁴⁴. The 75 kDa apoptosis-specific cleavage product⁵¹ was detected after 1 hour in the presence of anti-Fas (Figure 1C, lane 1) and accounted for the vast majority of the protein at later time points (Figure 1C, lanes 2-3). The relationship between these apoptotic events could not be elucidated based on the kinetic and biochemical data. It was not possible to know, for example, that at 2 hours the cells which had lost their mitochondrial function were the ones displaying the LMW DNA cleavage fragments detected by PFGE, or whether the HMW fragments detected at this same time point were present in all cells or only the minority that already appear dead. Thus, loss of mitochondria function was examined further at the single cell level, where we have recently defined accumulation of initial DNA breaks and loss of a portion of the nuclear matrix protein SAT B1 as the earliest nuclear events⁴⁴.

Identification and isolation of early apoptotic Jurkat cells Since mitochondrial function is measured in live cells by the uptake of potential-sensitive dye while labelling of nuclear antigens or DNA breaks requires fixed and permeabilised cells, it was not possible to compare dye uptake with nuclear events directly. Instead, flow cytometry and cell sorting were first employed to isolate early apoptotic cells, as defined by cytoplasmic criteria, from anti-Fas treated Jurkat cultures. Nuclear events were then examined in the isolated subpopulations.

The staining patterns of normal size and small size cells were evaluated independently in cultures stained with DiOC₆₍₃₎ to label functional mitochondria and propidium iodide to assess plasma membrane integrity (Figure 2). The normal size cells contained two subpopulations (Figures 2A', 2B'): "apparently normal" cells which displayed functional mitochondria and intact plasma membranes (right hand region, blue) and "apoptotic" cells (left hand region, red), which showed loss of mitochondrial membrane potential, accompanied by a small increase in propidium iodide (PI) staining. Their uptake of PI remained 100 fold less than that of the smaller, dead cells present in the population (Figures 2A'', 2B'') and they did not have the bright PI staining typical of permeable cells when examined under the microscope (data not shown). The apoptotic subpopulation accounted for a negligible proportion of control cultures, but increased to just over 20% of the anti-Fas treated samples by 2 hours, and 40% at the 4-hour time point (data not shown). The smaller size cells contained a third subpopulation that had lost mitochondrial membrane potential and were completely permeable to propidium iodide, designated "dead" cells. There was no significant accumulation of "dead" cells

during the first 4 hours of anti-Fas treatment, but they became more frequent at 5 hours and later time points (Figure 2B", and data not shown).

The early apoptotic or "apparently normal" subpopulation was efficiently separated from the other cells present in Jurkat cultures by sorting (Figure 3). In the whole culture, an increasing proportion of the normal size cells became apoptotic after anti-Fas treatment (Figures 2A', 2B', 3A, 3B). In the "apparently normal" subpopulations collected from these samples, 1% or less of the cells expressed an apoptotic phenotype (Figures 3A', 3B', 4D', 4E'). Taken together, Figures 3, 4D' and 4E' also demonstrate that deterioration of the live cells in the sorted subpopulations did not occur under the strictly controlled conditions of temperature and collection time used for these experiments.

Apoptotic Jurkat cells display initial changes in nuclear morphology before loss of mitochondrial membrane potential The nuclear morphology of sorted Jurkat subpopulations was examined in DAPI-stained samples, and the distribution of cells with distinct profiles assessed by cell counts (Figure 4). The majority of the "apparently normal" cells isolated from anti-Fas treated cultures were indistinguishable from healthy controls (Figures 4A, 4A', 4B, 4B'). However, a minority displayed "altered" morphology: small nuclei with increased areas of condensed chromatin, and higher contrast than their neighbours when viewed under phase optics (Figure 4B, 4B', arrow). This phenotype was distinct from both controls and apoptotic nuclei with collapsed chromatin. Cells with "altered" morphology accounted for less than 4% of the "apparently normal" subpopulation from controls, rising to 20% of "apparently normal" cells after 4h anti-Fas treatment. Both the sorted apoptotic and dead subpopulations featured chromatin collapse in virtually all nuclei and very high contrast when viewed using phase optics (Figure 4C, 4C' and data not shown).

The nuclear size profile of early apoptotic cells was confirmed in live samples. "Apparently normal" cells from control cultures featured bright, perinuclear DiOC6 labelling, a pattern consistent with mitochondrial localization (Figure 4D'). In the "apparently normal" subpopulation from treated cultures, the distribution and intensity of DiOC6 was indistinguishable from controls, as demonstrated by images captured using a fixed exposure time, including in those cells displaying high phase contrast and decreased nuclear size, as outlined by the fluorescent stain (Figures 4E, 4E', arrows). In contrast, cells of the apoptotic subpopulation had no significant DiOC6 signal (Figure 4F'), even when the exposure time was tripled in an effort to pick up any residual staining.

Detection and characterization of DNA cleavage that precedes loss of mitochondrial function We next examined the early apoptotic Jurkat subpopulation for initial DNA cleavage, the earliest morphological change in anti-Fas treated Jurkats detectable in single cells⁴⁴. In "apparently normal" cells isolated after 2 hours treatment, nuclei with normal morphology did not show any detectable DNA cleavage, but those displaying altered morphology often had low levels of TUNEL labelling (Figures 5A, 5A', 5B, 5B', arrows). Interestingly, the TUNEL signal was often coincident with the areas of increased chromatin condensation. Not all the nuclei with altered morphology were labelled by TUNEL (Figures 5A, 5A', arrowhead). The latter may not have begun DNA cleavage, or the extent of cleavage may not have passed the threshold detectable by TUNEL. In sorted apoptotic cells from treated cultures, all the collapsed chromatin bodies present in the nuclei labelled with TUNEL, as expected (Figures 5C, 5C'), although the intensity of label varied markedly cell-to cell.

Biochemical analysis of sorted Jurkat subpopulations by two-dimensional gel electrophoresis of DNA, which is capable of resolving HMW, LMW and single strand damage, was then employed to characterize the DNA cleavage detected at the single cell level. When recorded after PFGE in the first dimension (Figure 6A), DNA from “apparently normal” anti-Fas treated cells remained in the well, indicating that no significant accumulation of double strand breaks had occurred. In contrast, fragments of 50 Kbp and smaller were detected in the DNA from sorted “apoptotic” cells. After denaturing of the strands and conventional agarose gel electrophoresis (CAGE) in the second dimension (Figure 6B), a smear of fragments, indicative of single strand breaks present in the DNA, was released from both the bands detected in “apoptotic” cells and, significantly, from the DNA of the “apparently normal” cells that had remained in the well at the top of the gel following PFGE (Figure 6B, arrow). Following exposure to anti-Fas, single strand breaks progressively accumulated in the “apparently normal” subpopulation. The DNA from cells isolated after 2 hours treatment contained fewer fragments generated due to single strand breaks than that from cells which had been treated for 4 hours (Figures 7A, 7B, arrows). “Apparently normal” cells isolated from control cultures at the same time points showed no accumulation of DNA damage (Figures 7A, 7B, upper lanes).

Degradation and reorganization of SATB1 in early apoptotic cells with normal mitochondrial function In individual Jurkat cells, we have shown that loss of a portion of the MAR-binding protein SATB1 in the nuclei of Fas-treated cells closely followed initial DNA cleavage events and preceded chromatin collapse⁴⁴. SATB1 was found throughout the nucleoplasm of “apparently normal” cells isolated from control cultures. Staining intensity did not vary across the subpopulation (Figures 8A-8A’). In contrast, loss of a portion of the nucleoplasmic SATB1 signal in cells with altered nuclear morphology was detected in “apparently normal” cells from treated cultures (Figure 8B-8B’, arrows). The integrity of SATB1 was then examined by Western blotting (Figure 8C). In control “apparently normal” cell proteins, antibody to SATB1 recognised the intact protein, a single band of 103 kDa (lane1). In “apparently normal” cell protein isolated from cultures induced to die by anti-Fas, the 75 kDa cleavage product was also detected (lane2). In samples of sorted “apoptotic” cell protein, only the cleavage product was seen (lane3).

Caspase 3 is not activated in early apoptotic cells Caspase 3 activation in anti-Fas treated Jurkats is known to depend on the release of mitochondrial factors; these cells employ a type II response to receptor-mediated death^{9,11}. Caspase 3 activity was examined in sorted subpopulations to determine whether post-mitochondrial activation of this effector protease had begun in the “apparently normal” cells. Figure 9 shows that a standard fluorogenic assay detected no significant activation of caspase 3 relative to controls in the “apparently normal” cells from treated cultures at times up to 4 hours. In contrast, the apoptotic subpopulation displayed robust caspase 3 activity that increased with time in the presence of the death inducer.

Change in gene expression in “apparently normal” Jurkats induced by anti-Fas treatment We next addressed whether nuclear function was also altered in the “apparently normal” subpopulations from treated cultures. Microarray analysis of changes in gene expression in anti-Fas treated cells relative to controls was carried out using human 19K microarrays purchased through the University Health Network in Toronto. Reproducible change in the expression of 829 sequences, representing 4.5% of the total number of spots interrogated, was identified in whole cultures of anti-Fas treated

Jurkats (Figure 10). Very few of these changes were consistently observed in the sorted, “apparently normal” cells; sequences with altered expression in this subpopulation accounted for only 0.2% of total spots (Figure 10). In the population of anti-Fas-treated Jurkats as a whole, upregulation and downregulation accounted for almost equal proportions of the changes observed, with almost all the downregulated genes clustered at the 4-hour time point. In the sorted, “apparently normal” cells, downregulation accounted for 2/3 of the changes in gene expression observed, evenly distributed between the two time points. This distinct pattern of decreased gene expression in the “apparently normal” cells at 2 hours suggests that these changes may represent unique events associated with the earliest steps of the apoptotic response.

Confirmation of change in gene expression was obtained using quantitative PCR (QPCR) analysis of sequences coding for characterized proteins. For both the sorted “apparently normal” cells and the whole population, useful annotation was available in the public databases for only one third of the sequences. The rest were either completely unknown, having no Unigene ID, or uncharacterized, having a Unigene ID for which no information beyond “EST”, “cDNA” or a numbered protein identifier were available. 60% of the gene changes observed by microarray were validated in both unsorted Jurkats (data not shown) and the sorted “apparently normal” subpopulation by QPCR (Table 1, results in bold). Based on this dual analysis, anti-Fas treatment was shown to increase expression of the sequences coding for cognate 70 kDa heat shock protein (HSPA8, ⁵²) and the endoplasmic reticulum (ER) transmembrane protein CLN8 ⁵³ early in the apoptotic response. Decreased expression of NDST3 and ECM2, genes coding for components of the cell/extracellular matrix interaction machinery ^{54,55} as well as the E3 ligase SMURF2 and SERPINF2 sequences, whose proteins are involved in directed proteolysis ^{56,57} was confirmed in early apoptotic cells. This profile implicates interorganelle communication, altered cellular interaction with the external environment and protein turnover in the initial steps of the apoptotic response in Jurkat cells.

DISCUSSION

Using flow cytometry and cell sorting, we have isolated a subpopulation of early apoptotic or "apparently normal" cells from cultures of anti-Fas-treated Jurkats. These cells appeared normal by three cytoplasmic criteria: cell size, mitochondrial membrane potential and plasma membrane integrity (Figures 2, 4E, 4E') and demonstrated no increase in caspase 3 activation (Figure 9). However, changes in nuclear morphology (Figures 4, 5A, 5B), initial DNA cleavage in the form of single strand breaks (Figures 5, 6, 7) and cleavage and reorganization of the nuclear matrix protein SATB1 (Figure 8) were all observed in these cells. Clearly, the initial nuclear events of apoptosis preceded loss of both mitochondrial function and effector caspase activation during Fas-mediated cell death in Jurkats. Further, induction of the Fas pathway resulted in changes in gene expression detectable by microarray, a small subset of which were specific to the early apoptotic cells. These novel findings raise important questions about the possible signalling pathway(s) from the death receptor to the nucleus.

Early morphological changes in apoptotic nuclei of several cell types have been described by several other groups, although an appreciation of these subtle indicators of cell death has not entered into the dogma. Nuclei were rounded and contained small accumulations of chromatin near their rim⁵⁸⁻⁶⁰, as we observed in sorted "early apoptotic" cells. In glioma cells, ultrastructural studies revealed a more distinct filamentous lamina and fibrous mesh associated with the nuclear pores than seen in controls, implying that the DNA normally associated with these structures no longer obscured their presence⁵⁹. Consistent with this prediction, we detected cleavage of the MAR-binding protein SAT B1 in sorted early apoptotic cells, and loss of the SATB1 signal was seen specifically in those which displaying altered nuclear morphology (Figure 8). SATB1 is known to lose its DNA-binding capacity upon apoptotic cleavage⁶¹. Thus, cleavage of SATB1, or analogous proteins in other cell types, would indeed release DNA from areas of the nuclear matrix to which it was normally bound early in the apoptotic response.

The early nuclear events of apoptosis are not effector caspase-mediated. Jurkat cells are a well-characterized model system for the study of cell death in which perturbation of mitochondrial membrane integrity initiates the effector phase⁹⁻¹¹. Although AIF and endonuclease G both play roles in apoptotic DNA cleavage^{29,30,62}, our data clearly demonstrate that transmission of the apoptotic stimulus to the nucleus occurs prior to loss of mitochondrial membrane potential, which is linked to the release of these proapoptotic molecules. The release of cytochrome c from mitochondria, required for caspase 3 activation, may occur immediately prior to general increase in mitochondrial membrane permeability⁶³⁻⁶⁵. However, we show that initial nuclear events also precede activation of caspase 3 (Figure 9), and hence any biological effect of liberated cytochrome c in these cells. Finally, recent examination of Apaf-1 knockout lymphocytes in vivo has demonstrated that direct activation of effector caspase 7 by apical caspases may also occur in a type II apoptotic response, although the kinetics of cell death were delayed in the absence of the mitochondria-dependant apoptosome⁶⁶. However, this caspase 7 activity was manifest as DEVDase-specific cleavage of fluorogenic substrate, the exact assay used to monitor effector caspase activation in our experiments. Thus, the early nuclear events described cannot be attributed to direct activation of the effector caspase 7. Our results concur well with studies of early nuclear morphological changes in staurosporine-treated Jurkat and HeLa cells, which showed that

manifestation of “altered” nuclear morphology akin to that depicted in Figures 4 and 5 was effector caspase-independent^{60,67}.

The protease responsible for the SATB1 cleavage in early apoptotic cells may well be an alternate caspase. Caspase 6 activation can precede caspase 3 cleavage in some cells⁶⁸, and caspase 2 has been shown to initiate the mitochondrial events of apoptosis from within the nucleus⁶⁹. Caspase 6-mediated cleavage of SATB1 during apoptosis has been documented^{51,61}, making it the more likely candidate. The endonuclease responsible for initial single strand DNA cleavage events has not yet been conclusively identified⁴⁰. However, our lab among others has identified a candidate DNase I-like endonuclease. Unlike the other members of the DNase family, this “DNase Y” or “DNase γ ” is located throughout the nucleus where it is tightly bound to the chromatin. It can mediate both single and double-stranded DNA cleavage and produces DNA fragments with 3'-OH ends typical of apoptotic cells⁷⁰⁻⁷³. Overexpression of the protein can result in induction of DNA cleavage typical of apoptosis in the absence of a death signal⁷⁴.

The initiation of nuclear events prior to reception of a mitochondria-based signal suggests early transmission of the death stimulus from the DISC to this organelle. Translocation of active caspase 8 itself may occur. Active caspase 8 has been detected in the nuclei of HeLa cells following a Fas receptor-mediated death signal, where it engaged in death effector domain (DED)-mediated interaction with the death effector domain DNA-binding protein (DEDD), a nuclear protein associated with nucleoli⁷⁵. Alternatively, a second messenger, such as the death effector domain-associated factor (DEDAF) may play a role. DEDAF has been shown to promote death receptor-mediated apoptosis, and to associate with DED-containing proteins, including procaspase-8 and DEDD⁷⁶. Caspase or second-messenger signalling that impacts on DEDD may well link to both changes in nuclear matrix organisation and gene transcription. Interaction of DEDD with either of these messengers causes its release from intranuclear structures and dispersal throughout the nucleus where it is associated with repression of transcription⁷⁶⁻⁷⁸. Accordingly, we detected altered gene expression specific to early apoptotic Jurkat cells, with notable down regulation of sequences at 2 hours (Figure 10) consistent with DEDD-mediated gene repression. DEDD reorganization may also impact on the initial cleavage of nuclear proteins and DNA. Activation of nuclear caspase 6 by DEDD has already been documented⁷⁸. The activity of DNase Y, the candidate nuclease for initial apoptotic cleavage events, is modulated by its interaction with actinin and so could be altered by changes in the nuclear matrix that affect actin and associated components (manuscript in preparation).

Our microarray analysis strategy surveyed a large number of unique sequences (approx. 15 000) for strictly consistent changes. This was achieved by rigorous rejection of suspect data spots combined with statistical analysis to confidently identify reproducible changes. This strategy minimised the detection of alterations in gene expression, as sequences showing legitimate changes which happened to fall in an area of poor quality on any of the triplicates would be eliminated. The magnitude of changes detected by microarray was modest, in keeping with the short experimental time frame. QPCR analysis validated 60% of the changes observed by microarray, a reasonable proportion given that many results lay close to the cut off for non-specific signals in the microarray system.

The functional significance of the changes in gene expression detected in early apoptotic Jurkat cells remains to be demonstrated. Inhibitor studies have indicated that the effector phase of mitochondria-dependant apoptosis and anti-Fas mediated death in Jurkats, in particular, does not absolutely require gene expression or de novo protein synthesis to proceed^{5,79}. However, our results are consistent with a proteomic study that detected synthesis of several proteins in this model system⁸⁰, and recent evidence showing that synthesis of the anti-apoptotic protein Mcl-1 must be blocked in order for the mitochondrial events of the intrinsic pathway to be engaged in at least some systems⁸¹. An impact on cell death can be predicted from the changes in known genes detected in our study. Decreased contact with the extracellular milieu via downregulation of NDST3 and ECM2 could be part of the mechanism of Fas receptor capping, an organisational change associated with its maximal activation¹¹, and later contribute to cell shrinkage and packaging into apoptotic bodies. Selectively altered proteolysis is predicted by the downregulation of SERPINF2 and SMURF2. The latter is an E3 ligase involved in attenuation of transforming growth factor (TGF)-beta mediated responses by targeting its associated transcription factors, SMADs, for proteosomal degradation^{82,83}. Intriguingly, TGF-β signalling has recently been shown to augment Fas-mediated apoptosis in lung epithelial cells⁸⁴ and TGF-β is activated quickly following Fas ligand treatment in both this system and in mouse liver in vivo^{84,85}. Our results indicate that decreased degradation of SMAD transcription factors mediated by Fas may contribute to positive cross talk between the two pathways. Finally, upregulation of several genes specific to early apoptotic Jurkat cells was detected. The cognate heat shock protein 70 (Hsc70) is constitutively expressed and normally functions in both protein folding and as an ATPase⁸⁶. Conflicting reports in the literature testify to the effects of poorly-identified HSP70 isoforms on apoptosis in Jurkats⁸⁷⁻⁸⁹. Intriguingly, the cognate isoform shows translocation to nuclear aggregates during apoptosis⁹⁰, is known to associate with p53⁹¹, and is proposed to be involved in actin-based remodelling of chromatin⁸⁶. Taken together with our microarray results, these observations suggest that Hsc70 may participate in the early nuclear events of apoptosis. Finally, upregulation of the ER transmembrane protein CLN8⁵³ was detected. The potentiation of mitochondria-mediated apoptosis by transfer of intracellular Ca²⁺ from the ER prior to loss of mitochondrial function has recently been described, and has been shown to involve Bcl-2 family members^{92,93}. Our results predict that CLN8 may contribute to this internal ion flux in early apoptotic Jurkats.

In summary, we demonstrate that the initial nuclear events in early apoptotic Jurkat cells precede mitochondrial dysfunction, plasma membrane perturbation, and effector caspase activation. Pre-mitochondrial transduction of the apoptotic signal to the nucleus by caspase 8 or a DISC-associated second messenger should impact on DEDD protein, and contribute to both matrix reorganization and alternations in transcription. Our results suggest that early transmission of the apoptotic signal to the nucleus causes A: DEDD-mediated activation of SATB1-cleaving caspase 6; B: actin-based chromatin reorganization influenced by increased Hsc70 expression; and C: nuclear matrix-based modulation of the endonuclease activity responsible for initial single strand DNA breaks. Taken together, these events would result in the observed nuclear changes in early apoptotic Jurkats.

MATERIALS AND METHODS

Cell culture and treatment Jurkat cells were maintained in RPMI 1640 (Gibco/Invitrogen Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a 5% CO₂ atmosphere. Cell death was induced in cultures suspended at 10⁶ cells/ml with 100 ng/ml anti-Fas monoclonal antibody (clone CH-11, Upstate Biotech Inc., Lake Placid, NY).

Flow cytometry and cell sorting To examine mitochondrial function, 1-2 x 10⁶ live cells were stained for 15 minutes at 37 °C in the dark in PBS containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and 40 nM DiOC₆(3) (Molecular Probes, Eugene, OR), from a freshly-made 40 µM working solution and 4 mM stock, both in DMSO (Sigma) and apoptotic index was measured in quadruplicate experiments. For identification of subpopulations, 5 µg/ml propidium iodide (PI, Molecular Probes) was added to the assay. Samples were kept on ice and a minimum of 10,000 events analysed in the dark on a Coulter Epics Elite flow cytometer, using 488 nm excitation and 525 (+/-5) nm and 610 (+/-5nm) band pass filters. Staining was stable for 90-150 minutes, and samples routinely analysed within 1 hour in numerous (i.e.>20) replicates. For cell sorting, conditions were set for one drop per sort event and rejection of drops containing more than one particle. Subpopulations from samples containing 10⁷ cells were collected in the dark in ice-cold HANKS (Gibco) + 2% BSA, kept on ice and processed within 1 hour. The stability of droplet breakoff and subpopulation distribution was confirmed throughout the sort.

Gel electrophoresis of DNA Analysis of DNA fragmentation by pulsed-field and two-dimensional gel electrophoresis was carried out in triplicate experiments using established protocols⁹⁴.

Western blot analysis of SAT B1 Cellular proteins were extracted and Western blotting for SATB1 carried out in triplicate experiments as recently described⁴⁴, using 40 µg protein aliquots.

Cell staining and imaging Sorted Jurkat subpopulations were collected by centrifugation at 900 g for 10 minutes at 4 °C. To assess DiOC₆(3) signal in live cells, an aliquot was mounted on a slide in a drop of liquid anti-fade mounting medium (Vectasheild, Vector/ Novocastra Laboratories Ltd., Burlington, ON), then examined on an Olympus BX50 microscope for no more than 10 minutes, during which time staining patterns remained stable. Images from duplicate experiments were captured using Northern Exposure software and a fixed exposure time. For examination of nuclear morphology, sorted cells from triplicate experiments were settled onto poly-l-lysine coated coverslips for 5 minutes at 4 °C, rinsed briefly in ice-cold PBS, fixed and permeabilised as previously described⁵⁰, then stained for five minutes in 0.2 µg/ml DAPI (Sigma), rinsed twice briefly in PBS and mounted in Vectasheild. Labelling of SAT B1 and detection of DNA breaks *in situ* by TUNEL were both carried out as previously detailed (^{44,95}, respectively) in triplicate. Images of fixed cells were photographed onto Ilford XP2 400 film and scanned. All images were processed using Adobe Photoshop.

Caspase 3 activity assay Caspase-3 activity was measured in subpopulations isolated from control and anti-Fas treated Jurkat cultures, as well as inhibitor-pretreated samples, using the ApoAlert fluorometric assay kit (Clontech Laboratories, Inc., Palo Alto, CA) in accordance with manufacturer's instructions. Fluorogenic product was detected with a Millipore CytoFluor 2350 (excitation 360 ± 40 nm; emission 460 ± 40 nm). Caspase activity from three independent experiments was calculated as fluorescence units/10⁶

cells/ minute and expressed as the percentage of activity observed in control subpopulations.

Isolation of RNA for gene expression analysis Cells were plated 24 hours prior to use at 0.4×10^6 cells/ml and density adjusted to 10^6 cells/ml at 0h, typically a change of <10%. Total RNA was extracted from whole Jurkat cultures in three independent experiments and sorted subpopulations in numerous (i.e. >20) replicates using TriReagent (MRC Inc., Cincinnati, OH), in accordance with the manufacturer's instructions.

Samples from sorted subpopulations were pooled in Tri-Reagent at -20 °C for up to two weeks to make up 1 ml aliquots for processing. RNA quality was assessed by agarose gel electrophoresis and OD 260/280. RNA from all samples was pooled according to treatment and time and stored at -80°C in 75% ethanol until use. Control and treated samples were prepared in equal quantity throughout the collection period, to ensure that culture variations would be reflected equally in both pools.

Microarray analysis For each microarray, 20 µg of total RNA was precipitated with 0.1V 3M NaCl, and washed in 80% ethanol, then a fluorescently-labelled cDNA copy generated in 40 µl of reaction mix containing 1X superscript II first strand buffer (Invitrogen), 3.75 pmoles/µl AncT mRNA primer (5' T₂₀VN 3', Cortec, Queen's University, Kingston, ON), 0.5 nmoles/µl each dATP, dGTP and dTTP, 50 pmoles/µl dCTP, 25 pmoles/µl Cy3- or Cy5-conjugated dCTP (all Amersham Biosciences, Baie d'Urfe, QC) and 10 nmoles/µl DTT (Invitrogen). Reverse transcription was carried out as detailed⁹⁶ after addition of 10 U/µl RT Superscript II (Invitrogen) and 1U/µl Rnasin (Promega). Samples were hybridised in buffer containing 0.5 µg/µl yeast tRNA (Invitrogen) and 0.5 µg/µl single stranded sperm DNA (Sigma), to human 19K microarray slides (University Health Network, Toronto, ON), and scanned as described⁹⁶. Triplicate experiments, including reciprocal labelling of cDNAs, were performed for each RNA sample. Spots were verified manually to flag imperfections for removal from further analysis. Raw intensity data was then pre-processed in Microsoft Excel, using established strategies⁹⁶: median background fluorescence was assessed per subarray, data normalised using linear regression correction and spots flagged and removed if they showed an inadequate signal/background ratio (set at 2.5X), low signal intensity (set to the 5th percentile), high signal intensity (set to the 98th percentile) or unacceptable spot quality (set for standard deviation of spot intensity >5x mean spot intensity). The log₂ ratio of treated to control signals was determined, and data analysed in Excel to select for spots with a mean log ratio of at least +/- 0.6 for which all three replicates were present (unflagged). A p-value threshold of ≤ 0.05 assessed by single-sample t-test of the log₂ ratio vs.0 was then applied to generate a list of sequences with consistently altered gene expression. Annotation for sequences identified by clone ID was obtained first from the SOURCE database (Stanford University) then through publicly accessible databases at NCBI and the Weizmann Institute of Science.

QPCR analysis For QPCR, trace DNA contamination was removed from the RNA using the DNA-free kit (Ambion Inc., Austin, TX) with several modifications: 4U DNase1/100 µl was incubated with 0.4 µg/µl RNA in the buffer supplied for 2 hours at 37 °C, and the reaction stopped with 0.2 V of DNase inactivation reagent. RNA in the supernatant was precipitated with 0.5V lithium chloride (Ambion) overnight at -20 °C, washed in 75% ethanol, dried and resuspended in 0.1V water. RNA quality was re-evaluated by agarose gel electrophoresis and OD 260/280, and absence of trace DNA

contamination confirmed by QPCR amplification of β -actin on a 5 μ g aliquot. cDNA was generated in a total volume of 40 μ l per 20 μ g RNA aliquot, in a reaction mix containing 0.5 n moles/ μ l dNTP (Invitrogen) instead of the nucleotide mixture for microarray. Reverse transcription was carried out for 2 hours at 42 °C, reaction stopped by adding 5 μ l of 500 mM EDTA, then RNA hydrolysed and the mix neutralised as described ⁹⁶. cDNA was purified and quantitated using the Qiaquick PCR kit (Qiagen) and Oligreen dye (Molecular Probes), as instructed. For QPCR, 1.2-2 ng cDNA was amplified in 25 μ l of SYBRgreen PCR master mix (Applied Biosystems) containing 0.15 pM each of gene-specific forward and reverse primers. Fluorescent product in triplicate samples was detected using an ABI Prism 7000 system, set for 1 cycle of 2 minutes at 50 °C, 1 cycle of 10 minutes at 95 °C and 40-43 cycles of 15 seconds at 95 °C, 1 minute at 60 °C. Product dissociation was monitored to confirm specificity of the primers. Forward and reverse primers for sequences of interest (Qiagen) were designed using Primer Express (Applied Biosystems) and are listed in Table 2.

REFERENCES

1. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 74:845-853
2. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81:505-512
3. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 14:5579-5588
4. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 85:817-827
5. Sartorius U, Schmitz I, Krammer PH (2001) Molecular mechanisms of death-receptor-mediated apoptosis. *Chembiochem* 2:20-29
6. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 16:2794-2804
7. Barnhart BC, Peter ME (2002) Two faces of caspase-8. *Nat Immunol* 3:896-898
8. Chen M, Wang J (2002) Initiator caspases in apoptosis signalling pathways. *Apoptosis* 7:313-319
9. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 17:1675-1687
10. Wajant H (2002) The Fas signalling pathway: more than a paradigm. *Science* 296:1635-1636
11. Mundle S, Raza A (2002) Defining the dynamics of self-assembled Fas-receptor activation. *Trends Immunol* 23:187-194
12. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94:481-490
13. Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94:491-501

14. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90:405-413
15. Zou H, Li Y, Liu X, Wang X (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 274:11549-11556
16. Adams JM, Cory S (2001) Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci* 26:61-66
17. Jiang X, Wang X (2000) Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem* 275:31199-31203
18. Tafani M, Karpinich NO, Hurster KA, Pastorino JG, Schneider T, Russo MA, Farber JL (2002) Cytochrome c release upon Fas receptor activation depends on translocation of full-length bid and the induction of the mitochondrial permeability transition. *J Biol Chem* 277:10073-10082
19. Reed JC, Green DR (2002) Remodeling for demolition: changes in mitochondrial ultrastructure during apoptosis. *Mol Cell* 9:1-4
20. Scorrano L, Korsmeyer SJ (2003) Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Comm* 304:437-444
21. Tsujimoto Y (2003) Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol* 195:158-167
22. van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P (2003) Mitochondrial intermembrane proteins in cell death. *Biochem Biophys Res Comm* 304:487-497
23. van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P (2002) The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Diff* 9:1031-1042
24. Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147-157
25. Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33-42
26. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53

27. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 8:613-621
28. Hedge R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES (2002) Identification of Omi/HtrA2 as a mitochondrial serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* 277:432-438
29. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397:441-446
30. Li LY, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412:95-99
31. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 182:367-377
32. Goldstein JC, Kluck RM, Green DR (2000) A single cell analysis of apoptosis Ordering the apoptotic phenotype. *Ann N Y Acad Sci* 926:132-141
33. Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, Kroemer G (1996) Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol* 157:512-521
34. Petit PX, Lecoq H, Zorn E, Dauguet C, Mignotte B, Gougeon ML (1995) Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* 130:157-167
35. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, Kroemer G (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 181:1661-1672
36. Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89:175-184
37. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391:43-50
38. Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391:96-99

39. Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* 476:118-123
40. Robertson JD, Orrenius S, Zhivotovsky B (2000) Review: nuclear events in apoptosis. *J Struct Biol* 129:346-358
41. Frankfurt OS, Robb JA, Sugarbaker EV, Villa L (1996) Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. *Exp Cell Res* 226:387-397
42. Walker PR, Leblanc J, Sikorska M (1997) Evidence that DNA fragmentation in apoptosis is initiated and propagated by single-strand breaks. *Cell Death Diff* 4:506-515
43. Peitsch MC, Muller C, Tschopp J (1993) DNA fragmentation during apoptosis is caused by frequent single-strand cuts. *Nucleic Acids Res* 21:4206-4209
44. Liu QY, Ribocco-Lutkiewicz M, Carson C, Testolin L, Bergeron D, Kohwi-Shigematsu T, Walker PR, Sikorska M (2003) Mapping the initial DNA breaks in apoptotic Jurkat cells using ligation-mediated PCR. *Cell Death Diff* 10:278-289
45. Walker PR, Weaver VM, Lach B, Leblanc J, Sikorska M (1994) Endonuclease activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. *Exp Cell Res* 213:100-106
46. Walker PR, Sikorska M (1997) New aspects of the mechanism of DNA fragmentation in apoptosis. *Biochem Cell Biol* 75:287-299
47. Walker PR, Pandey S, Sikorska M (1995) Degradation of chromatin in apoptotic cells. *Cell Death Diff* 4:97-104
48. Cohen GM, Sun XM, Fearnhead H, MacFarlane M, Brown DG, Snowden RT, Dinsdale D (1994) Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. *J Immunol* 153:507-516
49. Lagarkova MA, Iarovaia OV, Razin SV (1995) Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers. *J Biol Chem* 270:20239-20241
50. Weaver VM, Carson CE, Walker PR, Chaly N, Lach B, Raymond Y, Brown DL, Sikorska M (1996) Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. *J Cell Sci* 109 (Pt 1):45-56
51. Gotzmann J, Meissner M, Gerner C (2000) The fate of the nuclear matrix-associated-region-binding protein SATB1 during apoptosis. *Cell Death Differ* 7:425-438

52. Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C (2003) Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Comm* 304:505-512
53. Lonka L, Kytälä A, Ranta S, Jalanko A, Lehesjoki A (2000) The neuronal ceroid lipofuscinosis CLN8 membrane protein is a resident of the endoplasmic reticulum. *Human Mol Gen* 9:1691-1697
54. Grobe K, Ledin J, Ringvall M, Holmborn K, Forsberg E, Esko JD, Kjellen L (2002) Heparan sulfate and development: differential roles of the N-acetylglucosamine N-deacetylase/N-sulfotransferase isozymes. *Biochim Biophys Acta* 1573:209-215
55. Oritani K, Kincade PW (1998) Lymphopoiesis and matrix glycoprotein SC1/ECM2. *Leukemia and Lymphoma* 32:1-7
56. Lin X, Liang M, Feng X (2000) Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor- β signaling. *J Biol Chem* 275:36818-36822
57. van Gent D, Sharp P, Morgan K, Kalsheker N (2003) Serpins: structure, function and molecular evolution. *Intl J Biochem Cell Biol* 35:1536-1547
58. Dini L, Coppola S, Ruzittu MT, Ghibelli L (1996) Multiple pathways for apoptotic nuclear fragmentation. *Exp Cell Res* 223:340-347
59. Krajci D, Mares V, Lisa V, Spanova A, Vorlicek J (2000) Ultrastructure of nuclei of cisplatin-treated C6 glioma cells undergoing apoptosis. *Eur J Cell Biol* 79:365-376
60. Johnson VL, Ko SC, Holmstrom TH, Eriksson JE, Chow SC (2000) Effector caspases are dispensable for the early nuclear morphological changes during chemical-induced apoptosis. *J Cell Sci* 113 (Pt 17):2941-2953
61. Galande S, Dickinson LA, Mian IS, Sikorska M, Kohwi-Shigematsu T (2001) SATB1 cleavage by caspase 6 disrupts PDZ domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Mol Cell Biol* 21:5591-5604
62. Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G (2000) Mitochondria-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J* 14:729-739
63. Zhuang J, Dinsdale D, Cohen GM (1998) Apoptosis, in human monocytic THP.1 cells, results in the release of cytochrome c from mitochondria prior to their ultracondensation, formation of outer membrane discontinuities and reduction in inner membrane potential. *Cell Death Differ* 5:953-962
64. Hearps AC, Burrows J, Connor CE, Woods GM, Lowenthal RM, Ragg SJ (2002) Mitochondrial cytochrome c release precedes transmembrane depolarisation and

caspase-3 activation during ceramide-induced apoptosis of Jurkat T cells. *Apoptosis* 7:387-394

65. Waternouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, Green DR (2001) Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J Cell Biol* 153:319-328
66. Marsden VS, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, Roy S, Nicholson DW, Vaux DL, Bouillet P, Adams JM, Strasser A (2002) Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* 419:634-637
67. Taimen P, Kallajoki M (2003) NuMA and nuclear lamins behave differently in Fas-mediated apoptosis. *J Cell Sci* 116:571-583
68. Komoriya A, Packard BZ, Brown MJ, Wu M, Henkart PA (2000) Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable fluorogenic caspase substrates. *J Exp Med* 191:1819-1828
69. Paroni G, Henderson C, Schneider C, Brancolini C (2002) Caspase-2 can trigger cytochrome c release and apoptosis from the nucleus. *J Biol Chem* 277:15147-15161
70. Liu QY, Pandey S, Singh RK, Lin W, Ribocco M, Borowy-Borowski H, Smith B, Leblanc J, Walker PR, Sikorska M (1998) DNaseY: a rat DNaseI-like gene coding for a constitutively expressed chromatin-bound endonuclease. *Biochemistry* 37:10134-10143
71. Shiokawa D, Tanuma S (1998) Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase gamma. *Biochem J* 332:713-720
72. Baron WF, Pan CQ, Spencer SA, Ryan AM, Lazarus RA, Baker KP (1998) Cloning and characterisation of and actin-resistant DNase I-like endonuclease secreted by macrophages. *Gene* 215:291-301
73. Yakovlev AG, Wang G, Stoica BA, Simbulan-Rosenthal CM, Yoshihara K, Smulson ME (1999) Role of DNAS1L3 in Ca^{++} and Mg^{++} -dependent cleavage of DNA into oligonucleosomal and high molecular mass fragments. *Nucleic Acids Res* 1999-2005
74. Saito R, Mizuno M, Kumabe T, Yoshimoto T, Tanuma S, Yoshida J (2003) Apoptotic DNA endonuclease (DNase-gamma) gene transfer induces cell death accompanying DNA fragmentation in human glioma cells. *Journal of Neurooncology* 63:25-31
75. Alcivar A, Hu S, Tang J, Yang X (2003) DEDD and DEDD2 associate with caspase-8/10 and signal cell death. *Oncogene* 22:291-297

76. Zheng L, Schickling O, Peter ME, Lenardo MJ (2001) The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. *J Biol Chem* 276:31945-31952
77. Stegh AH, Schickling O, Ehret A, Scaffidi C, Peterhansel C, Hofmann TG, Grummt I, Krammer PH, Peter ME (1998) DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. *EMBO J* 17:5974-5986
78. Schickling O, Stegh AH, Byrd J, Peter ME (2001) Nuclear localization of DEDD leads to caspase-6 activation through its death effector domain and inhibition of RNA polymerase I dependent transcription. *Cell Death Differ* 8:1157-1168
79. Chang SH, Cvetanovic M, Harvey KJ, Komoriya A, Packard BZ, Ucker DS (2002) The effector phase of physiological cell death relies exclusively on the posttranslational activation of resident components. *Exp Cell Res* 277:15-30
80. Gerner C, Frohwein U, Gotzmann J, Bayer E, Gelbmann D, Bursch W, Schulte-Hermann R (2000) The Fas-induced apoptosis analyzed by high throughput proteome analysis. *J Biol Chem* 275:39018-39026
81. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X (2003) Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 17:1475-1486
82. Shi Y, Massague J (2003) Mechanisms of TGF- β signalling from cell membrane to the nucleus. *Cell* 113:685-700
83. Attisano L, Wrana JL (2003) Signal Transduction by the TGF- β superfamily. *Science* 296:1646-1647
84. Hagimoto N, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, Maeyama T, Hara N (2002) TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. *J Immunol* 168:6470-6478
85. Leu JI, Crissey MA, Traub R (2003) Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. *J Clin Invest* 111:129-139
86. Boyer LA, Peterson CL (2000) Actin-related proteins (Arps): conformational switches for chromatin-remodelling machines? *Bioessays* 22:666-672
87. Liossis SN, Ding XZ, Kiang JG, Tsokos GC (1997) Overexpression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat T cells. *J Immunol* 158:5668-5675
88. Creagh EM, Carmody RJ, Cotter TG (2000) Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. *Exp Cell Res* 257:58-66

89. Ricci JE, Maulon L, Battaglione-Hofman V, Bertolotto C, Luciano F, Mari B, Hofman P, Auburger P (2001) A Jurkat T cell variant resistant to death receptor-induced apoptosis. Correlation with heat shock protein (Hsp) 27 and 70 levels. *Eur Cytokine Netw* 12:126-134
90. Dastoor Z, Dreyer J (2000) Nuclear translocation and aggregate formation of heat shock cognate protein 70 (Hsc70) in oxidative stress and apoptosis. *J Cell Sci* 113:2845-2854
91. King FW, Wawrzynow A, Hohfeld J, Zylicz M (2001) Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70 and Hsp90 interactions with wild-type or mutant p53. *EMBO J* 20:6297-6305
92. Demareaux N, Distelhorst C (2003) Apoptosis - the calcium connection. *Science* 300:65-66
93. Hajnoczky G, Davies E, Madesh M (2003) Calcium signaling and apoptosis. *Biochem Biophys Res Commun* 304:445-454
94. Walker PR, Leblanc J, Smith B, Pandey S, Sikorska M (1999) Detection of DNA fragmentation and endonucleases in apoptosis. *Methods* 17:329-338
95. Walker PR, Carson C, Leblanc J, Sikorska M (2002) Labeling DNA damage with terminal transferase. Applicability, specificity, and limitations. *Methods Mol Biol* 203:3-19
96. Walker PR, Smith B, Liu QY, Famili AF, Valdes JJ, Liu Z, Lach B (2003) Data Mining of Gene Expression Changes in Alzheimer Brain. *Artificial Intelligence in Medicine* (in press)

Table 1 Identification of anti-Fas regulated genes in early apoptotic Jurkat cells

<u>Name</u>	<u>Clone ID</u>	<u>UGCluster</u>	<u>Microarray mean fold</u>	<u>QPCR fold</u>
<u>Upregulated</u>				
CASP8: caspase 8, apoptosis-related cysteine protease	125581	Hs.381231	2.21 ± 0.31	1.28
hypothetical protein	308830	Hs.349344 or Hs.293615	1.74 ± 0.2	nd
CLN8: ceroid-lipofuscinosis, neuronal 8	272612	Hs.127675	1.72 ± 0.21	1.66
HSPA8: heat shock 70kDa protein 8	208059	Hs.180414	1.63 ± 0.14	2.25
EIF3S6: eukaryotic translation initiation factor 3, subunit 6 48kDa	5548410	Hs.106673	1.62 ± 0.08	-3.54
<u>Downregulated</u>				
NDST3: N-deacetylase/N- sulfotransferase (heparan glucosaminyl) 3	125806	Hs.20028	-1.9 ± 0.09	-4.21
SERPINF2: serine (or cysteine) proteinase inhibitor (alpha-2 antiplasmin), member 2	212807	Hs.159509	-1.9 ± 0.26	-1.92
HOMER3: homer homolog 3 (Drosophila)	5847747	Hs.424053	-1.8 ± 0.25	nd
APOD: apolipoprotein D	4042897	Hs.75736	-1.7 ± 0.17	5.54
Homo sapiens cDNA FLJ35131 fis, clone PLACE6008824.	291575	Hs.43744	-1.7 ± 0.23	nd
KCNJ13: potassium inwardly-rectifying channel, subfamily J, member 13	4814905	Hs.296361	-1.7 ± 0.06	1.29
SMURF2: E3 ubiquitin ligase	35711	Hs.194477	-1.6 ± 0.03	-3.23
Homo sapiens, clone IMAGE:3937520, mRNA, partial cds	179266	Hs.250879	-1.6 ± 0.06	nd
FLJ12085: hypothetical protein FLJ12085	377586	Hs.48827	-1.6 ± 0.12	nd
ESTs	34598	Hs.356349	-1.5 ± 0.05	nd
ECM2: extracellular matrix protein 2, female organ and adipocyte specific	124065	Hs.35094	-1.5 ± 0.07	-1.79
FLJ21615: hypothetical protein FLJ21615	258763	Hs.44159	-1.5 ± 0.13	nd

Table 2: QPCR primers

Oligo	Sequence from 5'-3'	Position	PCR product
APOD-f	TGGCCACCGACTATGAGAACTA	423-444	81bp
APOD-r	TCCAAGCAAAATCCACGTGA	484-503	
ARHGEF6-f	AACCAGAAGCAACGGCCAG	2093-2111	81 bp
ARHGEF6-r	TCTCTGACCTCGTCCTTCAAGG	2152-2173	
BTF-f	GCTGCTTGCTAGTACACTTGTCCA	1960-1983	84 bp
BTF-r	GCCTGTGGCAACTTAATGTGGT	2022-2043	
Caspase8-f	TCTCCAAGAGAACAGGATAGTGAATC	922-947	83 bp
Caspase8-r	ATCAG ACAGTATCCCCGAGGTTT	982-1004	
Cdk5-f	CGTCGTGCCCAAACCTCAAT	774-792	82 bp
Cdk5-r	TGCTGAGATACGCTGGACAGG	835-855	
CLN8-f	GTTCCCTTGTCGGACTGGCTC	933-952	81 bp
CLN8-r	GGATTGAGAAGCTG CTGAGTCTTC	990-1013	
Cox6-f	CAGGCGTCTGCGAAATCATA	108-127	83 bp
Cox6-r	GATCAGCCACACGAAACTTATACAA	166-190	
E2F4-f	GGGCAGAAGAAGTACCAGATTCAC	585-608	81 bp
E2F4-r	GCTCCATGCCCTCCTTGTTCA	646-665	
ECM2-f	GCTCTATCACGTCCCGTCTAT	1711-1732	81 bp
ECM2-r	CCAGGGATCCGTTCAATCTG	1772-1791	
EIF3S6-f	GGTG GCTTGTCTTGAGGATTTTC	997-1018	81 bp
EIF3S6-r	CTGATACACTGGTGGATGCGA	1057-1077	
GNG11-f	CTTCACATCGAAGATTTGCCAG	361-382	84 bp
GNG11-r	TTGTCTCTGCAACTTCACCTCTTTG	420-444	
GOLGA1-f	GACCCGATAATGAGCTCTTCGA	2307-2328	82 bp
GOLGA1-r	GTGTTATTCGTGACGGAAGGC	2368-2388	
HSPA1B-f	TGACAACAGGCTGGTGAACC	903-922	81 bp
HSPA1B-r	ACGGCTCGCTTGTCTGG	966-983	
HSPA8-f	TCCAGGCAGCCATCTTGCT	1202-1221	79 bp
HSPA8-r	AGGGAAAGAGGAGTGACATCCA	1259-1280	
ILX3-f	CCTTCCAAGATGCCCAAGAA	1284-1303	81 bp
ILX3-r	GGCATAGGTGGTGGCTTGGTG	1345-1364	
JAK1-f	GTGCCACCTAACTGTCCAGA	3384-3404	81 bp
JAK1-r	AAGCTTGTCCGATTGGATGG	3445-3464	
KCNJ13-f	ATCAAGTCCTCTGGCTACTCTGCT	889-912	81 bp
KCNJ13-r	TCCTGCATTGCTGAAAGGAAT	949-969	
NDST3-f	CTCCAGTACAACCTGGCCCACA	2061-2081	81 bp
NDST3-r	TGTCATCGCAAGGATTCTGC	2122-2141	
PPP2CB-f	CTGTTATCGTTGTGGGAACCAG	816-837	81 bp
PPP2CB-r	GCCGGGTCAAATTGAAGGA	878-896	
Serpinf2-f	CACTTCCAGGGTTTCTGGAGG	730-750	81 bp
Serpinf2-r	CGTGAACTGCTCGTCCAGGT	791-810	
SMARCA2-f	AGCCAAACCTGTAGTGAGCGA	4773-4793	81 bp
SMARCA2-r	TCATCCGTCCCACTTCTTCT	4833-4853	
SMURF2-f	ATGATCCAAGAGTGCCCAGG	1027-1046	81 bp
SMURF2-r	TACGGATCTCCCATCCAGGA	1088-1107	

FIGURE LEGENDS

Figure 1 Kinetics of apoptotic changes in anti-Fas treated Jurkat cells

Jurkat cells were treated with 100 ng/ml anti-Fas for intervals up to 4 hours and analysed for loss of mitochondrial function using flow cytometry and the membrane potential-sensitive dye DiOC₆(₃) (A, error bars represent S.E.M.); chromatin breakdown, using PFGE (B); and cleavage of the nuclear matrix protein SAT B1 by Western blotting (C). In B, the positions of 50 Kbp and 1 Mb markers are indicated; in C, the size of both the intact protein and the apoptotic cleavage fragment are indicated

Figure 2 Identification of three cell subpopulations in anti-Fas treated Jurkat cultures Controls (A,A',A"), and 5 hour treated cultures (B,B',B") were examined by flow cytometry. The distribution of cells based on size (forward scatter, FS) and granularity (side scatter, SSC) was determined for each sample (A,B), and then the mitochondrial membrane potential (DiOC₆) and plasma membrane integrity (measured by exclusion of propidium iodide, PI) were assessed independently in the normal-sized (A'-B') and small-sized (A"-B") cells. Subpopulations of "apparently normal" cells (normal size, normal mitochondrial membrane potential and intact plasma membrane) are indicated in blue; apoptotic cells (normal size, low mitochondrial membrane potential, compromised plasma membrane) are indicated in red and dead cells (small size, low mitochondrial membrane potential, permeable plasma membrane) are indicated in black.

Figure 3 Isolation of the "apparently normal" subpopulation from Jurkat cultures Mitochondrial membrane potential (DiOC₆) and plasma membrane integrity (PI) were assessed using flow cytometry for both normal-sized cells in the whole population (A,B) and for "apparently normal" cells separated by cell sorting (A'-B') from untreated controls (A,A') and 4 hour treated cultures (B,B') of Jurkats.

Figure 4 Alteration of nuclear morphology in the "apparently normal" subpopulation of anti-Fas treated Jurkats Jurkat cells (A-C, phase contrast) were stained with DAPI to reveal the nuclear morphology (A'-C') of sorted "apparently normal" cells isolated from untreated controls (A,A'), and 2 hour treated "apparently normal" (B,B') and apoptotic (C,C') subpopulations. DiOC₆(₃) staining (D'-F') of live samples (D-F, phase contrast) was also assessed in control "apparently normal" cells (D,D') and 4 hour treated "apparently normal" (E,E') and apoptotic (F,F') subpopulations. Arrows indicate cells with altered nuclei displaying decreased size and increased condensed chromatin. Bar = 10 μ m.

Figure 5 Detection of DNA breaks in sorted anti-Fas treated Jurkat subpopulations

Treated Jurkat cells were stained with DAPI (A-C) and labeled by TUNEL to reveal DNA breaks (A'-C') present in "apparently normal" cells after 2 hours (A,A',B,B') and apoptotic cells at 4 hours (C,C'). Arrowhead indicates cell displaying altered nuclear morphology but no detectable DNA damage; arrows indicate cells with altered nuclear morphology and DNA breaks labelled by TUNEL. Bar = 10 μ m.

Figure 6 Characterisation of DNA breaks in sorted "apparently normal" Jurkats

DNA cleavage in “apparently normal” and apoptotic Jurkat cells isolated after 4 hours anti-Fas treatment was characterised using two dimensional gel electrophoresis. Images were recorded after both PFGE in the first dimension, to separate fragments generated by double strand breaks (A) and denaturing CAGE in the second dimension to reveal the accumulation of single strand breaks (B). Arrow indicates fragments generated by single strand breaks in the “apparently normal” subpopulation.

Figure 7 Single strand breaks accumulate in the “apparently normal” subpopulation in response to anti-Fas treatment

The integrity of DNA from the sorted “apparently normal” subpopulation of controls and treated Jurkat cultures after 2 hours (A) and 4 hours (B) was assessed using two dimensional gel electrophoresis. Arrows indicate accumulation of single strand breaks specific to the anti-Fas treated cells.

Figure 8 Reorganisation and cleavage of SATB1 in “apparently normal” Jurkat cells

Sorted “apparently normal” cells from control (A-A’) and 4 hour anti-Fas treated cultures (B-B’) were stained with DAPI (A-B) and immunostained to detect SATB1 (A’,B’). Arrows indicate cells displaying altered nuclear morphology. Bar = 10 μ m. The integrity of SATB1 was assessed by Western blotting (C) in sorted “apparently normal” cells from controls (lane 1) and “apparently normal” (lane 2) and apoptotic cells (lane 3) from treated cultures. The sizes of the intact protein and the apoptotic cleavage fragment are indicated.

Figure 9 Caspase 3 is not activated in the “apparently normal” Jurkat subpopulation

Caspase 3 activation was assessed by monitoring cleavage of the fluorogenic substrate DEVD-AMC in “apparently normal” and apoptotic cells isolated from control and anti-Fas treated Jurkat cultures. Specificity of the assay was demonstrated by pre-incubation of the samples with the inhibitor DEVD-FMK. Results depicted are typical of triplicate experiments.

Figure 10 Alterations in gene expression detected in anti-Fas treated Jurkats

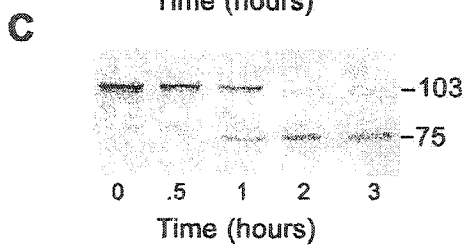
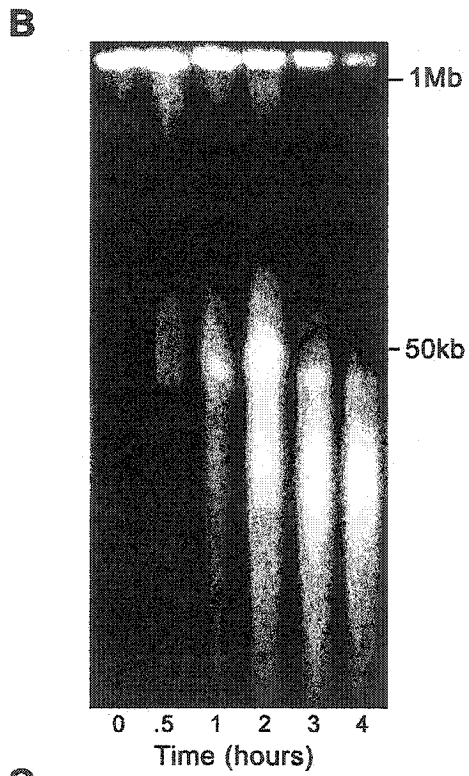
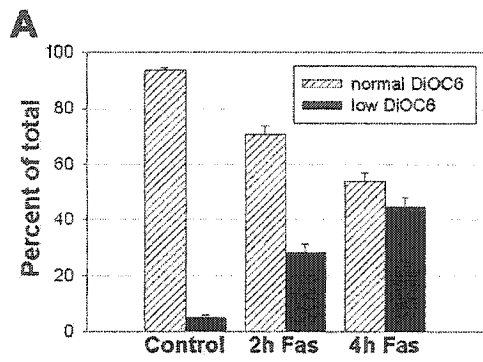
Consistent, reproducible gene upregulation (orange) and downregulation (blue) relative to controls was detected using microarray analysis in both sorted “apparently normal” cells and unsorted Jurkats.

Table 1 Identification of anti-Fas regulated genes in early apoptotic Jurkat cells

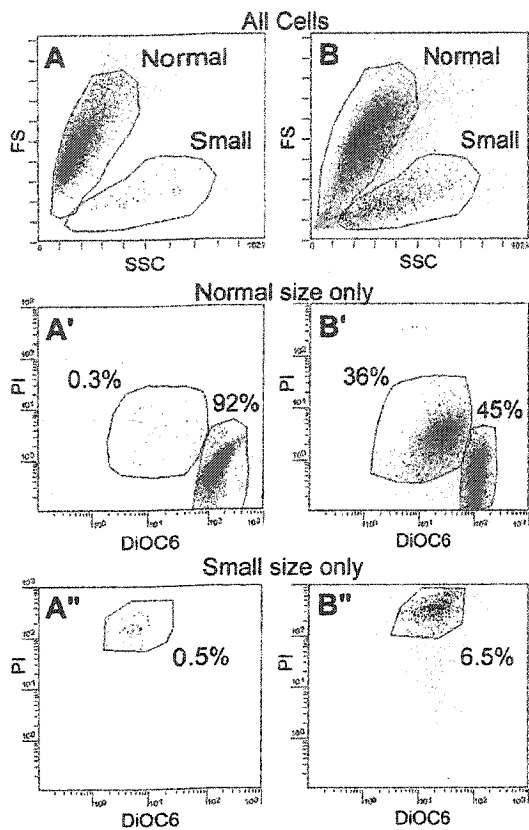
Sequences identified by microarray which coded for characterised genes and which were affected by anti-Fas treatment of Jurkat cells specifically in the sorted “apparently normal” subpopulation, were confirmed using QPCR. Results for validated sequences are indicated in bold.

Table 2 QPCR primers

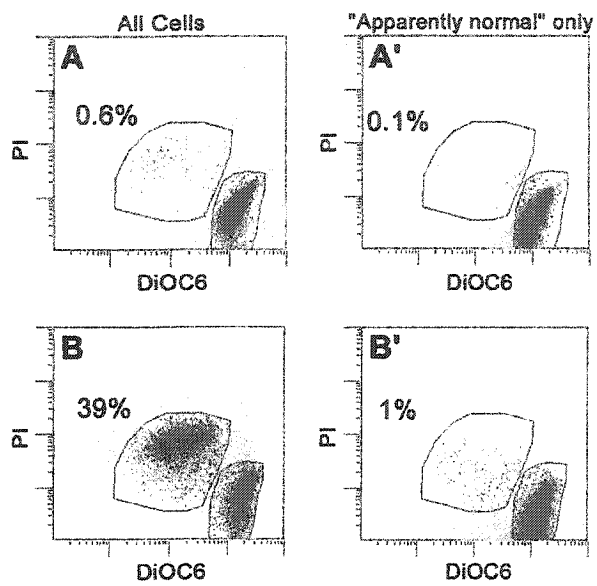
Primers were designed to amplify select sequences coding for known proteins, first identified as having changed expression in either whole cultures of anti-Fas treated Jurkats, or specifically in the sorted early apoptotic cells, using microarray analysis.



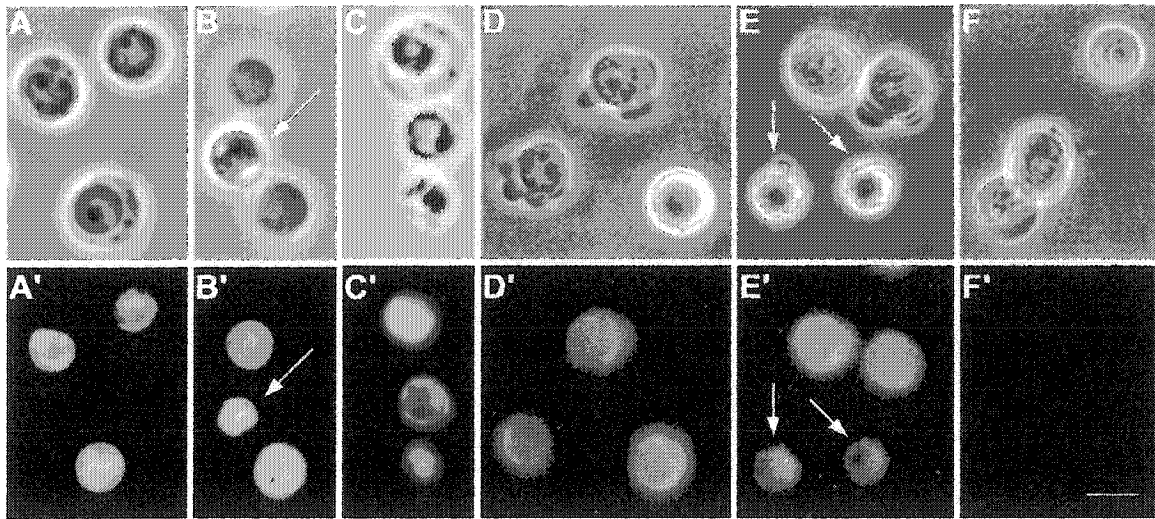
Carson et al., Figure 1



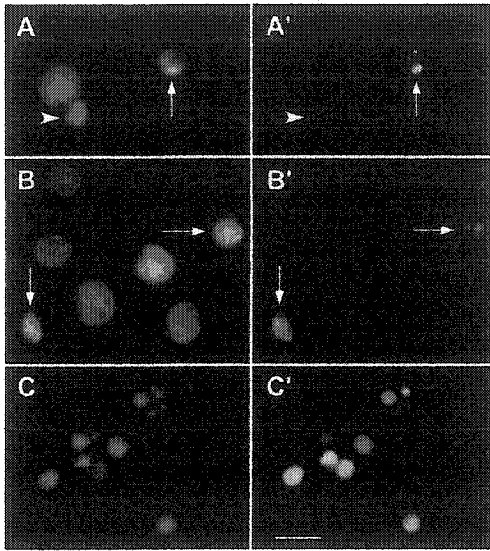
Carson et al., Figure 2



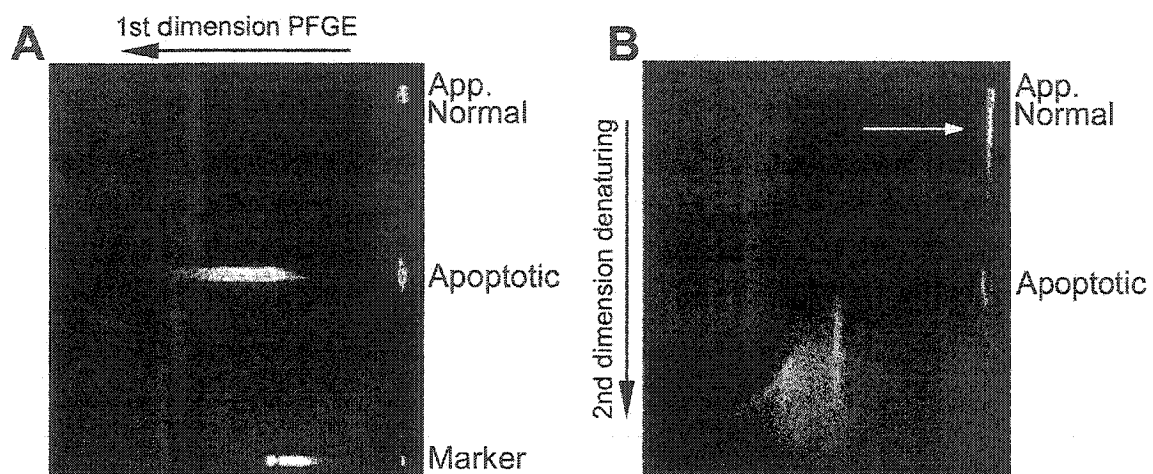
Carson et al., Figure 3



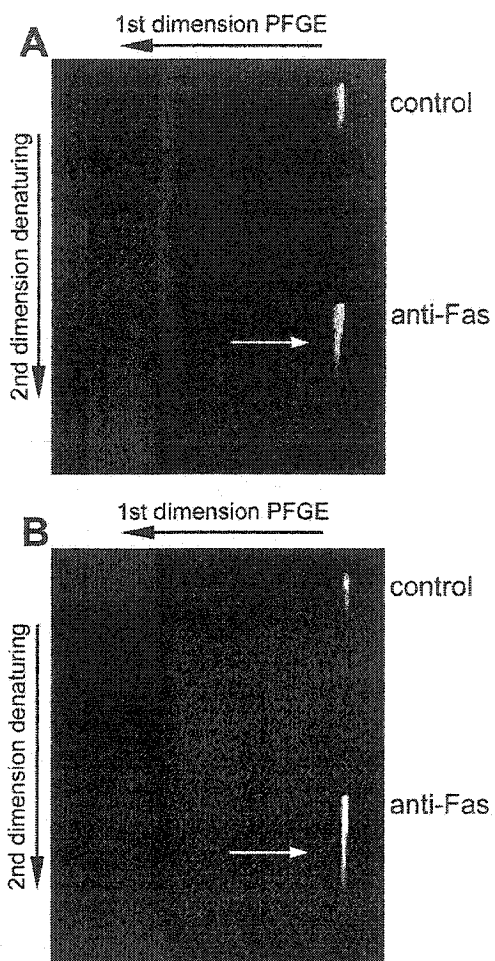
Carson et al., Figure 4



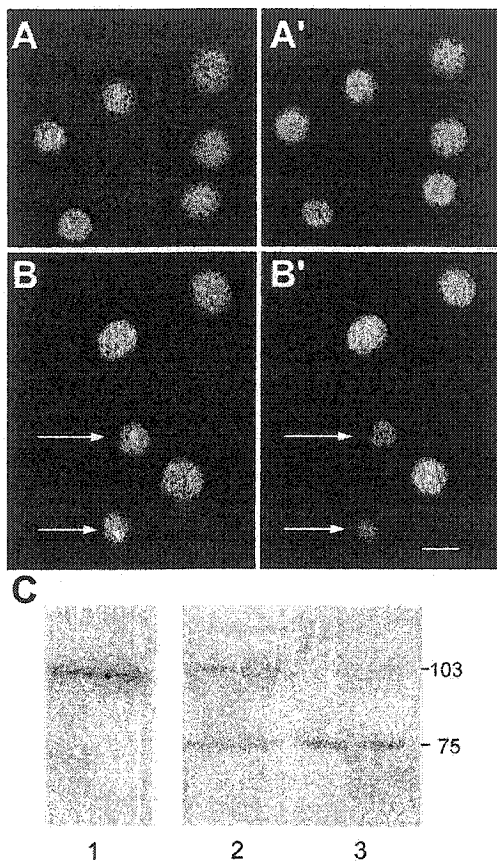
Carson et al., Figure 5



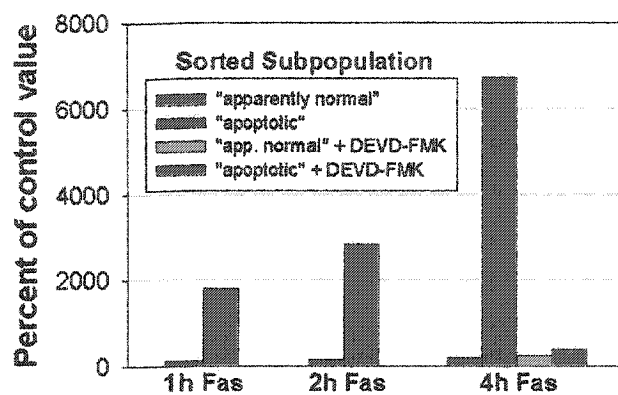
Carson et al., Figure 6



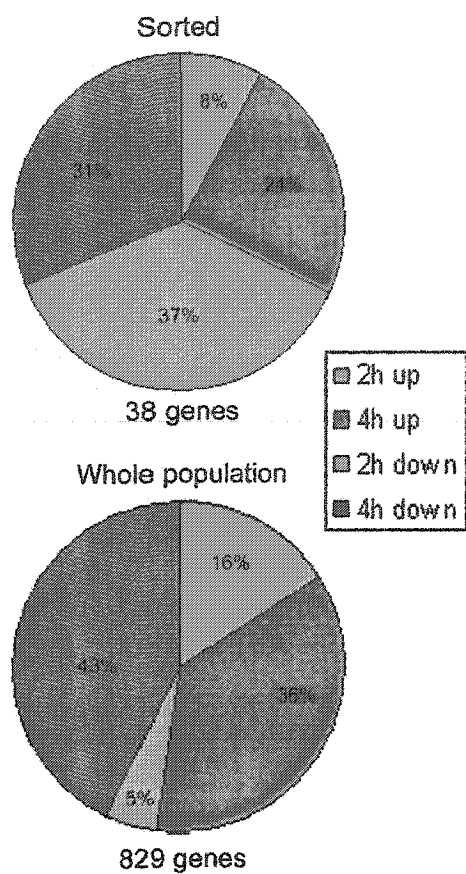
Carson et al., Figure 7



Carson et al., Figure 8



Carson et al., Figure 9



Carson et al., Figure 10

8 CURRICULUM VITAE

Chrsitine E. Carson

Education:

Doctoral Studies

- *University of Ottawa, Ottawa, Ontario September 1996 -
- *Time elapsed includes maternity leave from September 1999-August 2000 and December 2001-January 2002.
- *Supervisor: Dr. Marianna Sikorska, adjunct professor, Department of Biochemistry
- *Courses included Advanced Topics in Cell Regulation, Advanced Topics in Gene Expression and Protein Synthesis
- *Thesis project: Initiation of Nuclear Disassembly During Apoptosis. Examined the relationship between cytoplasmic and nuclear events during apoptosis at the single cell level, in order to identify those changes which preceded the initial steps of nuclear breakdown.
- *Techniques employed: A) Cell biology: immunocytochemical staining, metabolic labeling of live cells, *in situ* labeling of DNA breaks (TUNEL), flow cytometry, cell sorting and epifluorescence and confocal microscopy; B) DNA microarray analysis: isolation of RNA, reverse transcription to cDNA, microarray hybridization and scanning, data processing and cluster analysis; C) DNA cleavage analysis: conventional, pulsed field and two-dimensional gel electrophoresis of DNA; D) Cell culture: routine culture of hybridomas, hematopoietic, epithelial, fibroblastic and neuronal cell lines and neuronal differentiation of human NT2 cells.

Master of Science

- *University of Ottawa, Ottawa, Ontario 1990 - 1993
- *Degree granted 1995
- *Supervisor: Dr. Micheline Paulin-Levasseur, associate professor, Department of Biology
- *Courses included Techniques in Light and Electron Microscopy, Cell Biology.
- *Thesis in cell biology, entitled "Characterization of Lamins A and C Expression During Differentiation Of Human Leukemia Cells *in Vitro*".
- *Techniques employed: Immunocytochemical staining, epifluorescence microscopy, cell cycle analysis using both flow cytometry and pulse-labeling of cells, SDS-PAGE, Western blotting and ELISA.

Special Student

*University of Victoria, Victoria, B.C. 1988 - 1990

*Courses included Cellular Physiology, Applications of Genetic Principles.

Bachelor of Science 1983 - 1987

*McGill University, Montreal, Quebec

*Major in Human Genetics

*Graduated with Distinction

Experience:**Technical Officer TO-3 September 1996 – November 2001**

*Apoptosis Research Group, Institute for Biological Sciences, NRC, Ottawa, Ontario

*On a part-time basis, provided flow cytometry, cell sorting, immunofluorescence and confocal microscopy expertise to projects ongoing in the institute as needed.

Technical Officer TO-3 June 1994 - August 1996

*Apoptosis Research Group, Institute for Biological Sciences, NRC, Ottawa, Ontario

*Under the supervision of Dr. M. Sikorska and Dr. P. R. Walker, and as part of an industry-funded team studying the induction of apoptosis via depletion of vitamin B₁₂, performed microscopic analysis of nuclear morphology, flow cytometric analysis of both DNA cleavage and the expression of cell surface phosphatidyl serine and biological testing of putative peptide inducers of apoptosis, using MTT assay and microscopy.

Technical Officer TO-2 July 1993 - May 1994

*Apoptosis Research Group, Institute for Biological Sciences, NRC, Ottawa, Ontario

*Under the supervision of Dr. M. Sikorska, characterized changes in nuclear morphology occurring during programmed cell death in thymocytes using epifluorescence and confocal microscopy.

Teaching Experience:**Flow Cytometry Workshop**

*Developed and taught a workshop entitled "Using the flow cytometer to detect apoptosis".

*Lectured on the design and use of the flow cytometer and demonstrated four techniques: propidium iodide staining for DNA content, *in situ* labeling of DNA breaks (TUNEL), labeling of the surface marker Annexin V and staining for f-actin.

*Presented to Carleton University, 3rd year cell biology lab course at the request of Dr. N. Chaly, October 1996

*Presented to the Coulter Epics Elite Ontario Users Group, March 1996

Laboratory Demonstrator September 1990 - April 1993

*University of Ottawa Biology Department, Ottawa, Ontario

*Taught advanced cell biology and biochemistry techniques for the third year biology lab course, intermediate level embryology and genetics for second year lab and basic concepts of cell biology, genetics and cellular physiology for first year lab.

Training: Flow Cytometry

*Coulter Elite Operators Course, May 1994

Computer Skills:

*Experience with IBM, Macintosh

*Skilled operator of Elite and EXPO software for analysis of flow cytometry listmode data, Multicycle cell cycle analysis software, Zeiss LSM software for confocal microscope operation and ScanArray and QuantArray software for recording and processing of DNA microarray data.

*Excellent knowledge of Adobe Photoshop, CorelDraw, MSWord, Word Perfect, Excel and Sigma Plot.

Languages:

*My mother tongue is English.

*I speak and read fluently and write at a highly functional level in French.

*I understand, speak and read at a moderate level in German.

Awards:

*University of Ottawa Graduate Research Entrance Scholarship, 1990

*Student Poster Awards

Ottawa Life Sciences National Conference, Oct. 1996

CSBMCB 7th Annual Winternational Symposium, Quebec City, Quebec, February 1997

*from NRC: Merit Increase, 1996, 1998, 1999

IBS Award for group achievement/teamwork 1998

Invited Talks:

*Initial DNA cleavage precedes loss of mitochondrial function in apoptotic Jurkat cells

Speaker in symposium entitled "Cell death- the ultimate in development"

International Congress on Differentiation and Cell Biology

Gold Coast, Australia September 28, 2000

*Apoptosis: examining cell death at the single cell level

Seminar presented to the National Wildlife Research Center, Hull, QC December 21, 1998

Publications: Refereed Papers

- *Initial nuclear events of apoptosis precede loss of mitochondrial function in anti-Fas treated Jurkat cells**
C. Carson, J. Leblanc, P.R. Walker and M. Sikorska
manuscript in preparation
- *Mapping the initial DNA breaks in apoptotic Jurkat cells using ligation mediated PCR**
Q. Liu, M. Ribocco-Lutkiewicz, C. Carson, L. Testolin, D. Bergeron, J. Leblanc, T. Kohwi-Shigematsu, P.R. Walker and M. Sikorska
Cell Death and Differentiation **10**: 278-289 (2003)
- *Effects of neoplastic transformation and teniposide (VM26) on protein kinase C isoform expression in rodent fibroblasts**
Q.Y. Liu, C. Carson, M. Ribocco, L. Testolin, L. Raptis, P.R. Walker and M. Sikorska
Cancer Letters **153**: 13-23 (2000)
- *Neither caspase-3 nor DNA fragmentation factor is required for high molecular weight DNA degradation in apoptosis**
P.R. Walker, J. Leblanc, C. Carson, M. Ribocco and M. Sikorska
Annals of the New York Academy of Sciences, **887**: 48-59 (1999)
- *JUN and JNK kinase are activated in thymocytes in response to VM26 and radiation but not glucocorticoids**
L. Testolin, C. Carson, Y. Wang, P.R. Walker, U. Armato and M. Sikorska
Experimental Cell Research **230**: 220-232 (1997)
- *Induction of apoptosis in neoplastic cells by depletion of vitamin B₁₂**
P. R. Walker, B. Smith, C. Carson, J. LeBlanc, M. Sikorska, C. S. Woodhouse and A.C. Morgan
Cell Death and Differentiation **4**: 233-241 (1997)
- *Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes**
V. Weaver, C. Carson, P.R. Walker, N. Chaly, B. Lach, Y. Raymond, D.L. Brown and M. Sikorska
Journal of Cell Science **109**: 45-56 (1996)
- *Degradation of constitutive transcription factors during apoptosis in rat thymocytes**
I. de Belle, L. Testolin, S. Pandey, C. Carson, P.R. Walker, U. Armato and M. Sikorska
Biochemistry and Cell Biology **72**: 639-648 (1994)

Book Chapters

- *Labeling DNA damage with terminal transferase: applicability, specificity and limitations
P.R. Walker, C. Carson, J. Leblanc and M. Sikorska
Methods in Molecular Biology 203: In situ detection of DNA damage: methods and protocols ed: V.V. Didenko, pub: Humana Press Inc., Totowa, NJ (2002)

Abstracts

- *Initial nuclear events of apoptosis precede loss of mitochondrial function in anti-Fas treated Jurkat cells
C. Carson, J. Leblanc, P.R. Walker and M. Sikorska
Canadian Federation of Biological Societies 44th Annual Meeting
Ottawa, Ontario June 2001
- *Multiple mechanisms contribute to drug resistance in rat liver epithelial cells expressing polyoma virus middle T antigen
C. Carson, L. Testolin, K.N. Dhanraj, P.R. Walker and M. Sikorska
Biochemistry and Molecular Biology 99
San Francisco, California May 1999
- *Initiation of DNA fragmentation and early changes in nuclear morphology precede loss of mitochondrial membrane potential and activation of caspase-3 in apoptotic Jurkat cells
P.R. Walker, C. Carson, J. Leblanc, S. Pandey, M. Sikorska
Molecular Mechanisms of Apoptosis Regulation, an AACR special conference
California, January 1998
- *Interruption of PKC function increases sensitivity of rodent fibroblasts to the chemotherapeutic drug teniposide VM26
Q. Liu, M. Ribocco, C. Carson, L. Raptis, P.R. Walker and M. Sikorska
2nd Gordon Research Conference on Cell Death
New London, NH June 1997
- *Remodelling of the nuclear scaffold during apoptosis in rat thymocytes
C. Carson, B. Zurakowski, P.R. Walker, N. Chaly, D. Brown and M. Sikorska
Canadian Society of Biochemistry and Molecular and Cellular Biology 7th Annual Winter International Symposium
Quebec City, Quebec February 1997
- *Remodelling of the nuclear scaffold during apoptosis in rat thymocytes
C. Carson and M. Sikorska
Ottawa Life Sciences National Conference and Exhibition
Ottawa, Ontario October 1996

- *Site-specific cleavage of MAR-binding protein SATB1 and its disassociation from DNA occur early in apoptosis
M. Sikorska, S. Pandey, C. Carson, D. Bergeron, L. Dickinson, P.R. Walker and T. Kohwi-Shigematsu
Ottawa Life Sciences National Conference and Exhibition
Ottawa, Ontario October 1996

- *Instability of nuclear scaffold and chromatin destruction during apoptosis
M. Sikorska, C. Carson, V. Weaver, N. Chaly, D.L. Brown and P.R. Walker
Commercial Prospects for Apoptosis Research
Boston, MA May 1995

- *Correlations between DNA fragmentation and changes in nuclear morphology during dexamethasone-induced apoptosis in immature thymocytes
M. Sikorska, C. Carson, V. Weaver, N. Chaly, D.L. Brown and P.R. Walker
Canadian Federation of Biological Societies 37th Annual Meeting,
Montreal, Quebec June 1994

- *Instability of nuclear scaffold and chromatin destruction during apoptosis in thymocytes
M. Sikorska, V. Weaver, I. deBelle, C. Carson, and P.R. Walker
Cell Death in Cancer and Development
Chatham, MA October, 1993

9 CONTRIBUTIONS OF COLLABORATORS

Western blots: Western blots of thymocyte and Jurkat nuclear matrix proteins were produced by Bogdan Zurakowski and Dr. Domenic Bergeron.

PFGE: Gels of thymocyte and Jurkat DNA were produced by Julie Leblanc

Cell Sorting: for those experiments requiring more than one pair of hands, in particular the collection of RNA for microarray analysis, Julie Leblanc and I shared cell preparation, sorting and processing duties. While in general, Julie handled cell culture, I did the sorting and we shared the processing of the sorted samples, we did rotate jobs in order to ensure that results were consistent in both our hands.

Microarray analysis: Julie Leblanc prepared the cDNA and hybridised the arrays for one replicate of the unsorted, whole population.