Construction and Analysis of Dual Selectable Microcell Hybrids and their Use in the Identification of Suppressor Gene Loci

Marsha D. Speevak
Thesis submitted to the Department of Biochemistry in partial fulfilment of the requirements for the degree of Doctor of Philosophy

University of Ottawa
Ottawa, Ontario, Canada
August 13, 1996

ISBN 0-612-20019-1

Canada

Name Marsha SPEEVAK

Dissertation Abstracts International and *Masters Abstracts International* are arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation or thesis. Enter the corresponding four-digit code in the spaces provided.

GENETICS
SUBJECT TERM

0369
SUBJECT CODE

UMI

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences
Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences
Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Abstract

Cancer can be considered as a class of genetic diseases which arises as a result of mutations in oncogenes and tumour suppressor genes. These mutations alter the function, morphology, and behaviour of the affected cells, ultimately leading to tumour initiation, progression, and metastasis. These phenotypic changes also impact upon the ability of tumour cells to either respond to chemotherapy with cell death, or evade chemotherapeutic-induced cell death through drug-resistant pathways.

To identify cancer-related genes which contribute to cell survival during chemotherapy, hybrids between normal human fibroblasts and murine melanoma cells (B78) were generated and screened for the ability to survive short term exposure to the chemotherapeutic agent, PALA [*N*(phosphonoacetyl)-L- aspartate]. Whereas PALA induced growth arrest in normal fibroblasts, B78 responded to PALA with apoptosis (active cell death). It was hypothesized that the introduction of normal human chromosomes from the fibroblasts into B78 would result in an alteration in some of the hybrids' responses to the drug; in this instance, survival via growth arrest as opposed to apoptosis. Those human chromosomes which were associated with improved PALA survival were theorized to express genes responsible for cellular survival during chemotherapeutic treatment.

The hypothesis was first tested using whole cell hybrids constructed between human fibroblasts and B78. Whole cell hybrids showed improved survival during PALA exposure, as opposed to B78, and showed morphological features of growth arrest. To identify individual human chromosomes involved in this response, a panel of dual selectable microcell hybrids was constructed and screened. These hybrids contained one or more

normal human chromosomes in a B78 background. A drug selectable marker permitted the selective retention, or removal of the tagged human chromosome, as desired. The property of double selection was required to show that phenotypic changes were always due to the presence or removal of the tagged chromosome, rather than due to clonal variation. A pharmacological screen (using PALA) of the microcell hybrid panel resulted in the identification of human chromosome 3, which was consistently associated with improved survival and growth arrest during drug exposure. To attempt to identify genes which were involved in drug survival, a molecular strategy, known as differential display reverse transcriptase PCR (DDRT-PCR) was employed. This strategy was used to identify those cDNAs derived from differentially expressed mRNAs during PALA exposure in hybrids that showed improved survival during PALA exposure, as compared to B78 and the hybrids that died readily in the presence of PALA. Several candidate cDNAs were identified, cloned and sequenced. Characterization of these cDNA's may ultimately lead to the identity of new survival genes and an improved understanding of cancer cell drug responses.

Dedication

In memory of my mother, Beverly Abney and my mother-in-law, Deborah Speevak.

Acknowledgments

I would like to thank those responsible for encouraging, supporting, and helping me towards achieving this goal.

I wish to thank Dr. Hung-Shu Wang, who first took my interest in pursuing a Ph. D. seriously enough to encourage me to take the plunge, and who introduced me to my supervisor, Dr. Mario Chevrette. Thank-you for believing in me, Hung-Shu, through thick and thin. I would never have had the courage to do this without your support. I also thank Dr. Mario Chevrette, for taking a chance on me. Your enthusiasm for science never faltered, even on the bad days. You were open-minded, generous, helpful, and always available. Most astounding, you never criticized or lost patience, a true feat of endurance. I don't believe I could have thrived better under any other supervisor.

I would also like to thank Dr. Henriette Gourdeau, and Dr. Martin Tenniswood, whose expertise in the area of apoptosis research was generously offered on numerous occasions. Thank-you for the time you took to critique my manuscripts, I am truly grateful for your help. Also, I am grateful to the members of my advisory committee, Dr. John Bell, Dr. David Blakey, and Dr. Alex MacKenzie. Thank-you for your valuable advice, throughout the twists and turns that my topic took me.

I would also like to thank my lab mates, Nathalie Bérubé and Jean McGowan-Jordan. You were always tolerant of my ways, and generous with your solutions! Thanks for your ability to listen, and your supportiveness. Your friendship and companionship over the past five years have meant a lot to me, and will, I hope, continue. Also, thank-you Genny Giroux

and Patricia Tellis, for your great work, attention to detail, and positive attitudes. You both really made the lab tick.

At various times, throughout the last five years, I have received help from the students and staff of the 4th floor of Guindon Hall. These include Drs. Filion and Frank, Mr. Bob Gricken, and our loyal secretaries, Joanne and Julie. Also, to Sean, Jonathan and Anna, thank-you for your assistance. You may not remember the whens or wheres, but I do, and I appreciate it.

Finally, a short word of thanks to my husband David, and to my children, Jonathan and Leah. David, you have been the best! You are an incredible father, and terrific husband. Thanks from the bottom of my heart for believing in me. To my kids, thanks for bearing with me, I'm sorry it's taken so long, and I'm sorry there were so many nights and weekends that we lost together. You have been wonderful, and patient, and good, and I love you very much.

Table of Contents

List of Tables

List of Figures and Illustrations

Abbreviations

ALB	albumin gene
Alu-PCR FISH	competitive reverse chromosome painting
bp	base pair
BSA	bovine serum albumin
C	Centigrade
CAD	carbamyl phosphate synthetase-aspartate transcarbamylase-dihydroorotase
CAK	cdk-activating kinase
cdk	cyclin dependent kinase
cDNA	complementary DNA
con A	Concanavalin A
DAPI	4',6'-diamidino-2-phenylindole
DDX	arbitrary decamer
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dNTP	deoxynucleotide 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DDRT	differential display reverse transcriptase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothrietol
EDTA	ethylenediaminetetra-acetic acid
FITC	fluorescein isothiocyanate
FISH	fluorescence *in situ* hybridization
g	gram
HSF	human skin fibroblasts
hr	hour
kb	kilobase
kD	kiloDalton
mg	milligram
ml	millilitre
min	minute
mRNA	messenger RNA
oligo-(dT)	oligodeoxythimidylate

PAGE	polyacrylamide gel electrophoresis
PALA	[N(phosphonacetyl)-L-aspartate]
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	$-\log_{10}[H+]$
PHA	phytohaemagglutinin
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
SSC	Sodium Chloride/trisodium citrate
SDS	sodium dodecyl sulfate
TNHC	tagged normal human chromosome
TCA	Trichloracetic acid
THD	total human DNA
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TUNEL	Tdt-mediated dUTP nick end labelling
U	unit of activity
UV	ultraviolet
V	volts
WSC	cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluene-sulfonate
T11MN	anchored oligo-dT primer
µl	microlitre

Supplies

Ab-1	Oncogene Science
Acetic Acid	BDH Chemical
Acrylamide	Boehringer Mannheim
Agar Noble	Difco
Agarose	Gibco
Agarose, Nusieve	Mandel Scientific
Albumine, bovine	Sigma
Ammonium Acetate	BDH Chemical
Ammonium Persulfate	BDH Chemical
Ampicillin	Boehringer Mannheim
Amplitaq DNA polymerase	Applied Biosystem Canada
Amyl Alcohol	BDH Chemical
Anti-avidin antibody	Oncor
Anti-mouse antibody	Oncogene Science
Antifade	Oncor
Aprotinin	Boehringer Mannheim
Bis-acrylamide	Boehringer Mannheim
Biodyne Membrane	Gibco
Bioprime DNA Labelling System	Gibco
Bromophenol Blue	BDH Chemical
Butan-1-ol	BDH Chemical
Cesium Chloride	Toronto Research Chemical
Chemiluminescence kit	Amersham
Chloroform	BDH Chemical
Chromosome *in situ* kit	Oncor
Citric Acid	BDH Chemical
Colcemid	Gibco
Collagenase A	Boehringer Mannheim
Concanavalin A	Sigma
Cot-1 DNA, human	Gibco
Counterstain, propidium iodide	Oncor
Cytochalasin B	Sigma
DAPI	Boehringer Mannheim
dATP ^{35}S	Amersham
dCTP $\alpha^{32}P$	Amersham

DEPC	BDH Chemical
Dextran sulfate	Pharmacia
Dimethylformamide	BDH Chemical
Dimethlsulfoxide	BDH Chemical
Dithiothreitol	Boehringer Mannheim
DME/F-12 1:1	Gibco
DMEM	Gibco
DNA, salmon sperm	ICN
DNA staining kit	Coulter Electronics
DNA standard for fluorimetry	Canberra Packard Canada
DNA Lambda	Gibco
dNTP	Pharmacia
dNTP solution set	Boehringer Mannheim
EDTA Na_2	BDH Chemical
En3Hance	Dupont
Ethidium Bromide	BDH Chemical
Fetal bovine serum	Gibco
Fetal bovine serum, dialyzed	Gibco
Film, autoradiography XAR-5	Kodak
Film, autoradiography X-OMAT	Kodak
Film, Ektachrome 400	Kodak
Film, T Max	Kodak
FISH detection kit	Oncor
FITC-Avidin	Oncor
Formaldehyde	BDH Chemical
Formamide	Gibco
Ganciclovir	Syntex
Geniticin (G418)	Sigma
Giemsa stain Harleco	BDH Chemical
Glycerol	Gibco
Glycogen	Boehringer Mannheim
HEPES	Boehringer Mannheim
Hexanucleotide mixture	Boehringer Mannheim
Human placental RNAse inhibitor	Gibco
Hygromycin B	Sigma and Boehringer Mannheim
Immersion oil	Canlab
Kanamycin	Sigma

Klenow enzyme	Boehringer Mannheim
Leishman stain	BDH Chemical
Leupeptin	Boehringer Mannheim
Longranger solution	Johns Scientific
Magnesium acetate	BDH Chemical
MapPairs oligonucleotides	Research Genetics
MEM	Gibco
Mercaptoethanol	Sigma
Methanol	BDH Chemical
Methylene bisacrylamide	BDH Chemical
MOPS	USB
Nitrocellulose membrane	BioRad
Nylon membrane	Pall Biodyne
Ouabain	Sigma
PALA	National Cancer Institute
Penicillin Streptomycin	Gibco
Phenol	Toronto Research Chemical
Phenol red	BDH Chemical
Phenylmethylsulfonyl fluoride (PMSF)	Boehringer Mannheim
Phosphate Buffer	Fisher Scientific
Phytohemagglutinin	Difco
Poly A mRNA isolation kit	Qiagen
Polyethylene glycol 1540	NBS Biologicals
Potassium acetate	BDH Chemical
Potassium chloride	BDH Chemical
Prime It II random priming kit	Professional Diagnostics
Propan-2-ol	BDH Chemical
Protein Assay kit	Biorad
Proteinase K	Boehringer Mannheim
Random primers	Gibco
Ready safe cocktail	Beckman
Restriction enzymes	Boehringer Mannheim, Pharmacia, Gibco
RNAse A	Boehringer Mannheim
RNAse H	Pharmacia
Sequenase DNA sequencing kit	Amersham
Skim milk	Canlab
Sodium acetate	BDH Chemical

Sodium azide	BDH Chemical
Sodium chloride	BDH Chemical
Sodium citrate	BDH Chemical
Sodium deoxycholate	BHD Chemical
Sodium dodecyl sulfate (SDS)	Gibco
Sodium fluoride	BDH Chemical
Sodium hydrogen carbonate	BDH Chemical
Sodium hydrogen orthophosphate dodecahydrate	BDH Chemical
Sodium hydroxide	BDH Chemical
Sodium orthovanadate	Sigma
Spermine	Sigma
Spermidine	Sigma
Sucrose	Gibco
Superscript reverse transcriptase	Gibco
T4 DNA ligase	Invitrogen
T4 DNA polymerase	Invitrogen
TA cloning kit	Invitrogen
Trichloracetic acid	BDH Chemical
TEMED	BDH Chemical
Tetrasodium pyrophosphate	BDH Chemical
Tris	Gibco
Triton X-100	Boehringer Mannheim
tRNA, yeast	Boehringer Mannheim
Trypan blue	BDH Chemical
Trypsin EDTA	Gibco
Trypsin (bacto)	Gibco
Tween 20	BDH Chemical
Urea	BDH Chemical
WSC	Sigma
X-gal	Gibco
Xylene cyanol	BDH Chemical
Xylene	BDH Chemical

Chapter 1. General Introduction

1.1 Perspective

Basic cancer research encompasses a wide range of scientific endeavours, including molecular and cellular biology, immunology, microbiology and genetics. In addition, pharmacology and radiation biology provide the basis for the development of new cancer therapies. These diverse sciences contribute to the understanding of the etiology of cancer, and aid in the search for new anti-cancer therapies. The tools and strategies used in cancer research are thus varied, and are of necessity constantly changing. It is therefore significant that *in vitro* model systems play a common and important role in most, if not all of these research fields. *In vitro* mammalian model systems provide insight into the *in vivo* characteristics and behaviour of tumour cells. The cell lines used in these systems may be tumorigenic, or only partially transformed, and exhibit a variety of phenotypic differences as compared to the originating, normal cell types. These deviations include changes in morphology, function and behaviour, and are the basis of malignancy.

It is now clear that the cancer cell phenotype arises following activating mutations in growth promoting genes known as oncogenes, and inactivation of growth inhibiting genes known as tumour suppressor genes. These mutations impact upon the tumour cell's ability to survive outside the normal constraints imposed by the genetic program, and result in deregulated proliferation leading to invasion, and metastasis. Additionally, alterations in cancer-related genes can affect the ability of the cancer cell to withstand chemotherapy and radiation. The retention, or loss of the ability to die when challenged is therefore a significant attribute of the cancer cell, and is largely responsible for the ultimate success or failure of anticancer treatments. The identification of the molecular controls of cell survival

and death is therefore prerequisite to the development of more effective anti-cancer strategies. In this thesis, an *in vitro* mammalian model system was used to map and identify genes involved in cellular drug-induced survival responses.

1.2 Cancer as a Class of Genetic Diseases

Cancer is a multi-stepped genetic disease, arising from the mutation and inactivation of oncogenes and tumour suppressor genes (Knudson, 1971; Barrett and Ts'o, 1978; Bookstein and Allred, 1993; Nordenskjold and Cavanee, 1988; Mikkelsen and Cavenee, 1990). Historically, the concept of tumour cell fluidity as the basis of cancer progression arose prior to the establishment of modern genetics (Armitage and Doll, 1954). However, this concept eventually evolved into the theory of genomic instability as the basis of tumorigenesis (Nowell, 1976). In contrast to normal cells, which have relatively stable genomes, cancer cells undergo multiple genetic transformations during tumour initiation, progression, and metastasis (Holliday, 1989). The tumour cell's ever changing genotype is thus largely responsible for the morphological and behavioural changes which allow the tumour to grow and evolve (reviewed, Bevilacqua and Caligo, 1991; Bryant, 1993).

Cancer-related genes, their chromosomal loci, and their function continue to be the focus of intensive study by cancer researchers. However, following several decades of effort, a resolution to the problem of cancer remains elusive. This is due largely to the complexity of gene activity in higher organisms, which differs dramatically between organs and between specialized, differentiated cell types. It is this complexity which precludes the definition of a single set or sequence of mutational events that lead to oncogenesis.

1.3 The Cytogenetics of Cancer

During the 1960's and 1970's, investigators and clinicians began to establish a link between genomic integrity at the chromosomal level and tumorigenicity. The first evidence of association of a specific genetic alteration with cancer was the identification of the Philadelphia (Ph^1) chromosome (Nowell and Hungerford, 1960). This marker chromosome consistently appeared in the peripheral lymphocytes and bone marrow of chronic myelogenous leukemia (CML) patients, and was identified by clinical cytogeneticists as a chromosome 22 in translocation with chromosome 9 (Caspersson et al., 1970; Rowley, 1973). Over time, a variety of chromosome alterations were found to be consistently associated with haematological malignancies and solid tumours (Niebuhr, 1977; Kaiser-McCaw, 1977; Zech, 1975; reviewed, Weinberg, 1991). Many of these chromosomal abnormalities have since been shown to cause activation of oncogenes through alteration in transcriptional regulation or through gene fusion and inactivation of tumour suppressor genes, through deletion (reviewed, Rabbitts, 1994).

1.31 Somatic cell hybridization

The earliest identified oncogenes were discovered through viral DNA transfection experiments (Weinberg, 1981; Cooper, 1982). However, the genetic basis of malignancy, and the concept of tumour suppression were initially studied many years earlier, by means of the technique of somatic cell hybridization (Fig. 1.1a). The first somatic cell hybrid studies between mouse cell lines of different malignant potential suggested that the

malignant phenotype was dominant over the normal phenotype (Barski and Cornefert, 1962; Scaletta and Ephrussi, 1965). However, later studies showed that normal DNA could suppress the cancer phenotype (Harris, 1971; Stanbridge, 1976; Stanbridge, 1988). These investigators discovered that the fusion of non-tumorigenic mouse cells with tumorigenic mouse cells resulted in the transient suppression of the tumorigenic phenotype, as determined by the failure of the hybrid cells to produce tumours when injected into immuno-deficient mice. They noted however, that the hybrid cells were unstable, and chromosomes were lost during *in vitro* cell culture, eventually leading to the return of the tumorigenic phenotype. The suppression of the malignant phenotype by normal chromosomes was similarly shown in human x human hybrids. Intraspecific human hybrids were found to be more stable, and retained the suppressed phenotype over extended passaging (Stanbridge, 1976; Stanbridge et al., 1982). These findings suggested that normal chromosomes contain critical growth-regulating genes, now known as tumour suppressor genes, which become inactivated during tumorigenesis (Harris and Klein, 1969; Stanbridge and Cavanee, 1989).

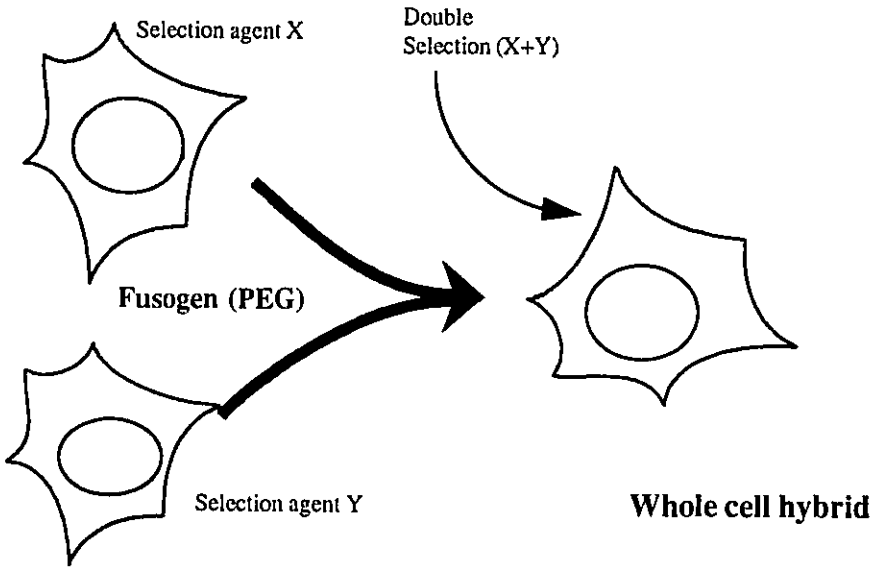
To attempt to identify the specific chromosomes involved in suppression of malignancy, interspecific hybrids between rodent and human cell lines were constructed (Klinger, 1982; Klinger and Shows, 1983; Craig and Sanger, 1985). The majority of these studies involved the use of transformed Chinese hamster cell lines, either embryonic fibroblast (CHEF) or ovarian (CHO) in origin. However, chromosomal instability was very pronounced in these cells, with rapid, non-random rejection of the human chromosomes occurring at early passage (Croce, 1984). The near-immediate loss of human chromosomes, accompanied by loss of the suppression of malignancy conspired to hamper the identification

of specific normal human chromosomes responsible for tumour suppression. However, a number of human chromosomes were eventually identified which were selectively lost from hybrids in conjunction with a reversion to tumorigenicity. The first of those identified included human chromosomes 2, 4, 9, 10, 11, 14 and 17 (Evans et al., 1982; Klinger, 1982; Klinger and Shows, 1983; Stoler and Bouck, 1985).

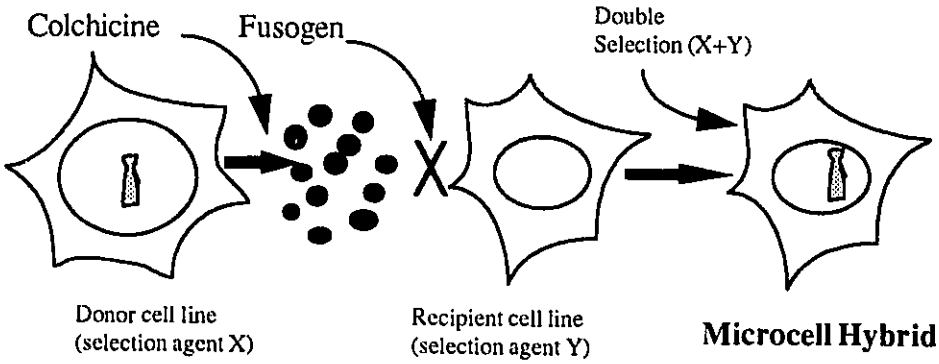
Figure 1.1 Fusion techniques
(a) Whole cell fusion. Two cell types, with different phenotypes eg. normal vs. tumorigenic are fused together usually by use of a fusogen, polyethylene glycol (PEG). The fused cells are called heterokaryons. Following fusion, hybrids are selected for survival, as opposed to the parental cells, by use of double selection: one selection method which is toxic to only one parental cell type (eg. ouabain for human cells) and a second selection method which is toxic to the other parental cell type (eg. HAT [hypoxanthine/aminopterin/thymidine] medium for some rodent cell lines). Drug resistant colonies contain chromosomes from both parental cell types
(b) Microcell fusion. The donor cell line is treated with the mitotic spindle inhibitor, colchicine. Prolonged treatment results in the formation of micronuclei which contain one or a few chromosomes. The micronuclei are stripped from the cells by centrifugal force, and are fused to the recipient cell line using PEG. Double drug selection is used to kill the parental cell types, allowing only the microcell hybrids to survive. To improve the variety of donor chromosomes retained in the recipient cells, a drug selectable marker is usually inserted (by electroporation or transfection) into the donor cell line chromosomes, prior to fusion.

Figure 1.1

(a) Whole cell fusion



(b) Microcell fusion



1.32 Microcell fusion

One impediment in the analysis of whole cell hybrids was the large amount of normal genomic material which tended to be introduced into the tumorigenic cells. The microcell fusion technique of chromosome transfer was developed as an improvement over whole cell fusion, since this method allowed introduction of small parcels of only one or a few chromosomes into the recipient cell line (Ege et al., 1977; Fournier and Ruddle, 1977) (Fig. 1.1b). However, selection for retention of the introduced chromosome was dependent upon complementation of genotypes through the presence of a dominant selectable marker on the introduced chromosome. This restricted microcell fusion to only a few human chromosomes, such as chromosome X and 17, which could complement a recessive rodent recipient cell phenotype. This disadvantage was eventually eliminated by the introduction of dominant selectable markers transfected into individual human chromosomes. Using this technique, any human chromosome, which was tagged with the genetic marker, could be introduced by microcell fusion into any genetic background (Saxon et al., 1985). A number of human chromosomes have now been stably introduced into tumorigenic human cell lines and have been shown to be associated with suppression of the malignant phenotype (Saxon et al., 1986; Weissman et al., 1987; Banerjee et. al., 1992; Speevak et al., 1995).

1.33 Cytogenetic breakpoints and cancer-related genes

In the 1980's, the advent of molecular biology led to the identification of viral and cellular oncogenes, the activation of which triggered tumorigenic growth. Some of these

were found to be associated with already known cancer-related chromosomal alterations. For example, it was discovered that the Ph^1 chromosome contained an activated oncogene, c-*abl*, fused to the *bcr* region of chromosome 22 (de Klein et al., 1982). Many other oncogenes important in human cancer have since been identified and mapped to cancer-related cytogenetic translocations (reviewed, Rabitts, 1994). Additionally, tumour suppressor genes have been mapped to sites of chromosomal deletions, both in paediatric tumours, such as Wilm's tumour and retinoblastoma, and in a variety of adult tumours, including renal, lung and colon cancers (Zbar, 1989; Haber and Housman, 1991; Lanfrancone et al., 1994). Accumulated evidence indicates that activation of growth promoting oncogenes, accompanied by inactivation of growth limiting tumour suppressor genes are required for progression of tumours to full malignancy (Stanbridge and Nowell, 1990; Weinberg, 1991).

1.4 Cell Cycle Regulation

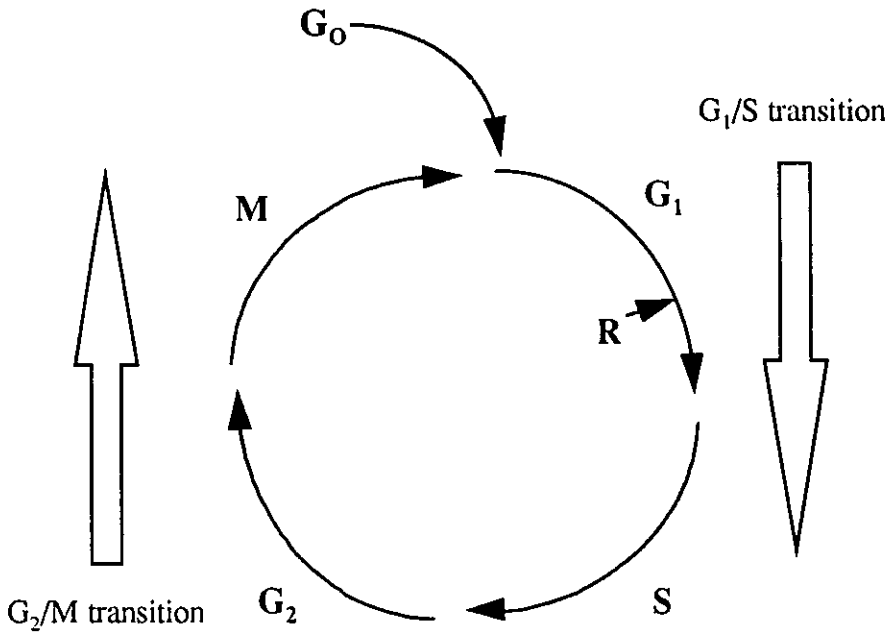
Deregulation of proliferation occurs during tumour progression following changes in the activity of cell cycle control genes, many of which are oncogenes and tumour suppressor genes. During the past 30 years, yeast has been used as an *in vitro* model organism for identifying eukaryotic genes involved in cell cycle control (Hartwell, 1967; Hartwell et al., 1970). Since the genes responsible for cell cycle regulation are highly conserved across species boundaries, advances in yeast genetics have led to a better understanding of mammalian cell cycle control. For example, the identification of yeast cyclins (proteins responsible for the advancement of the cell cycle) led to the isolation of

several human cyclins, identified by their ability to complement yeast cyclin defects (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991). In much the same manner, a number of mammalian cyclin dependent kinases (cdks) were cloned, all of which show regions of striking homology to their yeast counterparts (Lee and Nurse, 1987; Elledge and Spottswood, 1991; Meyerson et al., 1992; Okuda et al., 1992). Recent evidence now suggests that defects in genes involved in cell cycle progression, such as the cyclins and cdk's, as well as cdk-inhibitors may increase genetic instability, and accelerate the process of cancer progression (Hartwell and Kastan, 1994).

The cell cycle consists of a series of transitions that take place upon the prior completion of the preceding step (Hartwell and Weinert, 1989). The cell cycle concludes with the equal division of the cellular contents, into genetically identical daughter cells. This process is orchestrated by complex interactions between cell cycle regulatory proteins (Hartwell and Kastan, 1994). The major cell cycle transitions include: the first preparatory step (G1); DNA replication (S-phase); the second preparatory step (G2); and mitosis, the period of chromosomal condensation concluding with cell division (M-phase) (Howard and Pelc, 1953) (Fig. 1.2). The cell cycle is driven by cell cycle control genes which are negatively regulated by feedback mechanisms and checkpoints which ensure that the cell is ready for the next stage of the cycle (Hartwell and Weinert, 1989; Murray, 1992). Failure of negative feedback control, which occurs primarily during G1 and G2, can lead to mutation, transformation, or cell death (Peeper et al., 1994). Thus genes which are responsible for the negative regulation of the cell cycle are likely to be important in the prevention of tumorigenesis (Hartwell and Kastan, 1994).

Figure 1.2 The cell cycle.
The cell cycle consists of a series of transitions that depend upon the completion of the prior phase to proceed to the next step. The cell begins in G_0, or quiescence, and enters the first preparatory step, G1. If external signals indicate satisfactory conditions for proliferation, the cell passes through the restriction point R (START in yeast). R defines the point of no return, in that the cell is now committed to continue upon its course, and divide. However, cell division can be delayed or aborted, during any stage of the cell cycle. At S, the DNA is replicated, and the cell proceeds into G2, the second preparatory step. During this time, the cell checks the fidelity of DNA replication. Also, the centrosome organizes the microtubule machinery required to accurately segregate the chromosomes into the daughter cells. During the following step, M, the chromosomes separate into sister chromatids, and are drawn to opposite poles. Finally, at the end of M, the cytoplasm is divided, the cell membrane is cleaved, and two daughter cells emerge. The G_1/S and G_2/M transitions provide the cell with the opportunity to delay or abort the cell cycle, via checkpoints at G1 and G2.

Figure 1.2



1.41 G1 cell cycle control.

The restriction point (R) in mammalian cells defines the primary checkpoint, late in G1. Passage through R causes the cell to proceed irreversibly into the cell cycle (Pardee, 1989). G1 exit, and passage through each successive phase of the cell cycle is mediated, at least in part, by cyclin-cdk complexes (Pines, 1993; Sherr, 1993).

The following model describes the actions of cell cycle control proteins during G1 progression (Fig. 1.3). Following mitogenic stimulation, D-type cyclins are synthesized early in G1 and form complexes with cdks (cdk4 and cdk6) in a growth factor dependent manner. Later in G1, E type cyclins form complexes with cdk2 (Matsushime et al., 1991; Matsushime et al., 1992; Won et al., 1993; Bates et al., 1994; Meyerson and Harlow, 1994). Progression through the restriction point R requires activation of the cyclin/cdk complexes by cyclin activating kinase, CAK (Fisher and Morgan, 1994; Mäkalä et al., 1994). Once activated, the cyclin/cdk complexes phosphorylate pRB and other key substrates, resulting in the release of transcription factors required for the activation of S-phase genes (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993; Nevins, 1992; Hinds and Weinberg, 1994). Cyclin E also associates with an RB-related protein, p107, and E2F to initiate E2F-dependent transcription of genes required for S-phase transition (Lees et al., 1992; Dynlacht et al., 1994). Progress through G1 can be prevented by the INK4 proteins, $p15^{INK4b}$ and $p16^{INK4a}$, which compete with the D cyclins for cdk4 and cdk6 (Dulic et al., 1992; Koff et al., 1992; Harper et al., 1995; Sherr and Roberts, 1995). Also, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ can bind to cyclin D and cyclin E complexes thereby blocking their activation by CAK (Harper et al., 1993; El-Deiry et al., 1993; Xiong et al., 1993; Noda et al., 1994; Polyak et al., 1994b;

Toyoshima and Hunter, 1994; Zhang et al., 1994; Harper et al., 1995). Following passage into S-phase, the D cyclins are degraded via the ubiquitin pathway (Glotzer at al., 1991; Hershko et al., 1991).

Cyclin D1 appears to be oncogenic, probably due to its growth promoting abilities. Chromosomal translocations between cyclin D1 and tissue specific promoters have been found in parathyroid adenomas and B cell lymphomas (Motokura et al., 1991; Withers et al., 1991; Seto et al., 1992). Over expression of cyclin D1 due to gene amplification and deletions in the 3'-untranslated region has also been discovered in a variety of carcinomas (Lammie et al., 1991; Jiang et al., 1992; Buckley et al., 1993; Tsuruta et al., 1993; Lebwohl et al., 1994). Cyclins D2, D3 and cyclin E may also be oncogenic due to their similar roles in promoting proliferation; however, this has not been confirmed (Hunter and Pines, 1994).

The cdk-inhibitors $p16^{INK4a}$ and $p15^{INK4b}$ are candidate tumour suppressor genes, *MTS1* and *MTS2* respectively (Kamb et al., 1994; Nobori et al., 1994). $p16^{INK4a}$ is also a candidate for the familial melanoma gene, *MLM* (Hussussian et al, 1994). p15 is apparently induced by TGFβ, and may co-operate with p27 in mediating this pathway of growth arrest, by blocking cyclin D/cdk formation, and freeing p27 to bind and inhibit cyclin E/cdk complex activation (Hannon and Beach, 1994; Peters, 1994; Sherr and Roberts, 1995).

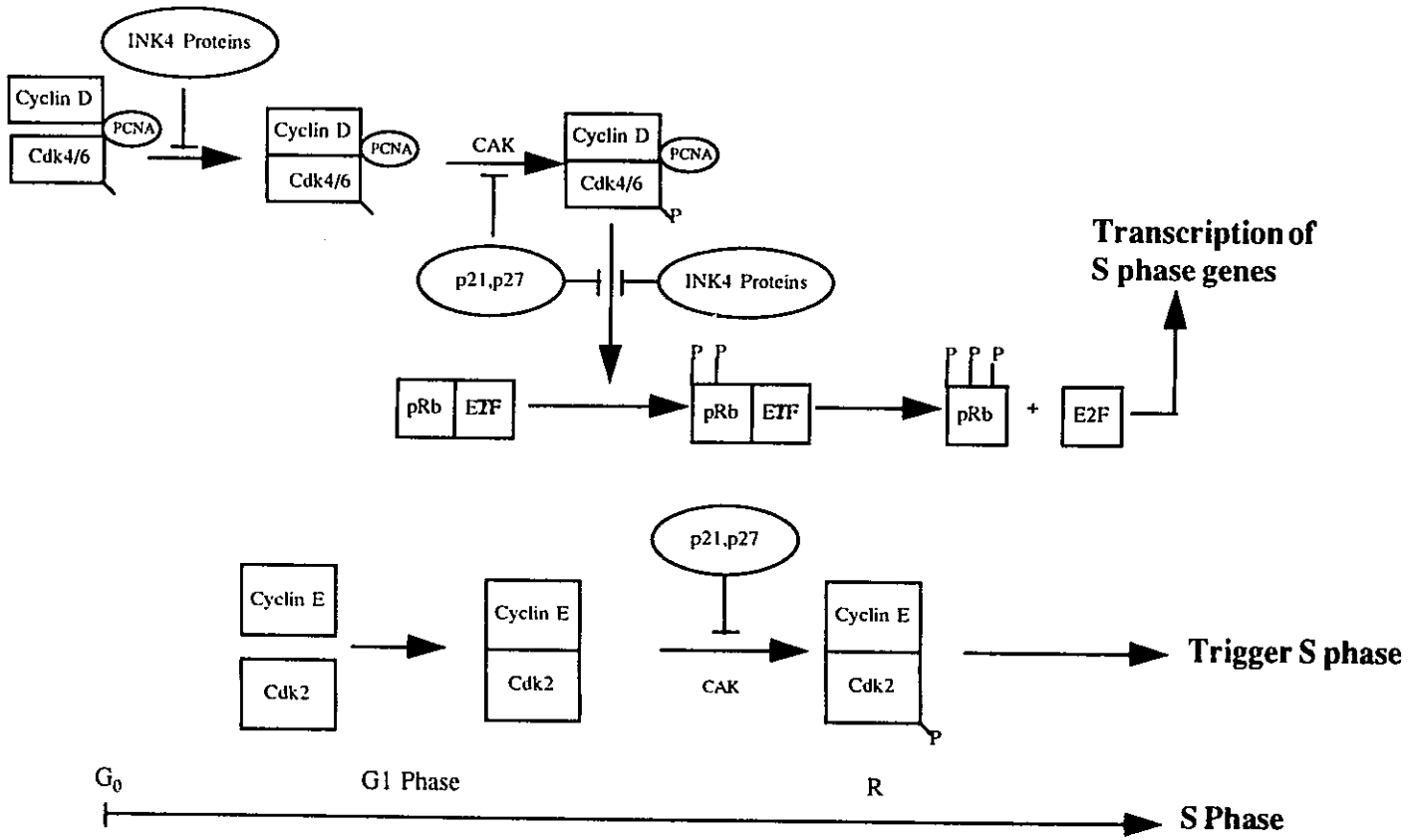
It is thought that, during G1 progression, the ratio of cyclin/cdk complexes begin to outnumber the available cdk-inhibitors, and become activated by CAK. p16 appears near the end of the cycle (possibly transcribed following E2F release by pRB) and displaces cyclin D, which is then ubiquinated (Parry et al., 1995).

$p21^{CIP1/WAF1}$ is upregulated by the tumour suppressor, p53, and is induced by conditions which produce increased p53 levels, such as DNA damage (Di Leonardo et al., 1994; Dulic et al., 1994; El-Deiry et al., 1994). However, p21 appears to be induced by other, p53-independent factors as well (Michieli et al., 1994). It is not presently clear if p21 is a tumour suppressor, or is merely a downstream component of the tumour suppressor activities of p53 (Hunter and Pines, 1994).

p21 contains homologies to $p27^{KIP1}$. This cdk inhibitor is activated by transforming growth factor beta (TGFβ), and may thus contribute to the TGFβ/RB growth arrest pathway (Polyak et al., 1994a; Slingerland et al., 1994). p27 may also mediate contact-inhibition in normal dividing cells (Polyak et al., 1994a).

Figure 1.3 Regulation of G1 progression
Following mitogenic stimulation, D-type cyclins are synthesized early in G1, and form complexes with cdk's (cdk4 and cdk6), in a growth factor dependent manner. Later in G1, Cyclin E forms complexes with cdk2. Progression through the restriction point R requires activation of the cyclin/cdk complexes, by CAK. Once activated, the cyclin/cdk complexes phosphorylate pRB, and other key substrates, resulting in the release of transcriptions factors required for the activation of S-phase genes (E2F shown here). Progress through G1 can be prevented by INK4 proteins, p15 and p16, which can compete with the D cyclins for cdk4 and cdk6. Also, p21 and p27 can bind to cyclin D and cyclin E complexes, thereby blocking their activation by CAK. Figure 1.3 was modified from Sherr and Roberts, 1995.

Figure 1.3


INK4 Proteins
Cyclin D
PCNA
Cdk4/6
Cyclin D
PCNA
Cdk4/6
CAK
Cyclin D
PCNA
Cdk4/6
P
p21,p27
INK4 Proteins
Transcription of
S phase genes
P P
P P P
pRb
E2F
pRb
E2F
pRb
+
E2F
p21,p27
Cyclin E
Cdk2
Cyclin E
Cdk2
CAK
Cyclin E
Cdk2
P
Trigger S phase
G0
G1 Phase
R
S Phase

1.42 S-phase and G2/M cell cycle control

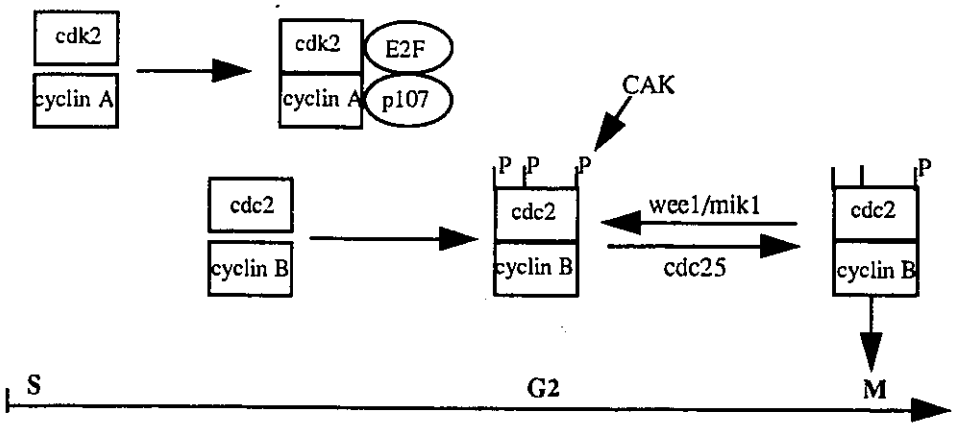
Following G1, E2F with its binding protein, DP-1, stimulates entry in S-phase by transactivation of S-phase genes (Dalton, 1992; Ogris E., et al., 1993; Pearson et al., 1991; Lam and Watson, 1993; Girling et al., 1993; Bandara et al., 1993). Cyclins A (S and G2/M specific) and cyclin B (G2/M specific) take over the cell cycle activities (Pagano et al., 1992; Ghiara et al., 1991) (Fig. 1.4). During S phase, cyclin A combines with cdk2 to form an activated complex, which interacts with E2F and p107 (Ewen et al., 1991; Devoto et al., 1992; Ewen et al., 1992; Faha et al., 1992). This complex guides the cell through S-phase, inactivating E2F-dependent transcription of immediate early genes; however, cyclin A is eventually destroyed at the beginning of G2 (Minshull et al., 1990; Pines and Hunter, 1990; Cao et al., 1992; Krek et al., 1994). Following S, cyclin B/cdc2 accumulates in the inactive form, via phosphorylation on tyrosine residue 15 (Tyr-15) and threonine residue 14 (Thr-14) by wee1/mik1 kinases (Parker et al., 1992; Lock and Wickramasinghe, 1994). At the end of G2, the complexes are activated by dephosphorylation on these sites by cdc25, and phosphorylation on Thr-161 by CAK, signalling the beginning of M (Nurse et al., 1976; Dunphy, 1994). Proteolysis of the A and B cyclins occur during late M.

During M phase, the cellular DNA condenses into compact, replicated chromosomes, held together by a duplicated centromere. Guided by the centrosome, the organizational centre for mitosis, mitotic spindles draw the chromosomes to the centre of the cell, the sister chromatids segregate and are pulled to opposite poles, and the cellular contents are divided equally between the two daughter cells (Bornens, 1992). Cyclin destruction, and other ubiquitin-dependent events are required for passage through the final stage of cell division

(Minshull et al., 1990; Pines and Hunter, 1990; Glotzer et al., 1991; Hunt et al., 1992; Surana et al., 1993; Amon et al., 1994). Failure of these events may contribute to cellular transformation, since abnormalities in spindle formation are frequently observed in tumour cell lines (Hunter and Pines, 1994). Also failure to dispose of cyclin A may be oncogenic, since it shows transforming ability when stabilized or overexpressed (Wang et al., 1992; Guadagno et al., 1993).

Figure 1.4 S and G2 transitions.
During S, cyclin A combines with cdk2 to form an activated complex, which interacts with E2F, and p107. Following S, cyclin B/cdc2 accumulates in the inactive form, via phosphorylation by wee1/mik1 kinases. At the end of G2, the complex is activated by dephosphorylation by cdc25, and phosphorylation by CAK, signalling the beginning of M. Proteolysis of the A and B cyclins occurs at anaphase. Figure 1.4 was modified from Lock and Wickramasinghe, 1994.

Figure 1.4


cdk2
cyclin A
cdk2
E2F
cyclin A
p107
CAK
P P P
cdc2
cyclin B
cdc2
cyclin B
wee1/mik1
cdc25
P
cdc2
cyclin B
S
G2
M

1.5 Cell cycle Checkpoints

When subjected to stressful conditions such as DNA damaging agents, most normal eukaryotic cells undergo cell cycle arrest (Hartwell and Weinert, 1989; Murray, 1992). This response allows the cell to evaluate and repair damage, and is mediated by specific gene products, known as checkpoint control proteins (Weinert and Hartwell, 1988; Enoch and Nurse, 1991; Weinert et al., 1994). The major properties of checkpoints are 1) they are signal transduction systems 2) most are non-essential to normal function 3) they ensure the fidelity of genome replication during cell division 4) they are adaptive and diminish in impact during prolonged stimulation, and 5) they induce different cellular responses, including growth arrest and apoptosis (Hartwell and Kastan, 1994). It is theorized that checkpoint controls, by maintaining the integrity of the genome, are candidate tumour suppressor genes, and that checkpoint defects are responsible for tumorigenesis, *in vivo* (Murray, 1992; Hartwell, 1992). The major checkpoints in eukaryotic cells occur at the G_1/S and the G_2/M transitions (reviewed, Peeper et al., 1994; Feilotter et al., 1992). The G_1/S checkpoint is best understood in mammalian cells, and appears to be orchestrated mainly by p53 and pRB (reviewed, Cox and Lane, 1995; Weinberg, 1995).

1.51 G1 checkpoints

p53 protein was originally discovered when it coprecipitated with large T antigen of the SV40 virus, and was at that time thought to be oncogenic (Linzer and Levine, 1979). It was later determined that only mutant forms of p53 had transforming ability, and that wild-

type *p53* ($p53^{+/+}$) was actually a tumour suppressor gene (Jenkins et al., 1984). p53 has since been shown to exhibit multiple biological functions, including induction of G1 arrest and apoptosis, inhibition of tumour cell growth, and preservation of genomic stability, as well as the biochemical functions of transcriptional activation and repression, inhibition of DNA replication, and interaction with a variety of nuclear proteins (reviewed, Pietenpol and Vogelstein, 1993).

The major role of *p53* appears to be that of tumour suppressor gene, since its mutation has been shown to be strongly associated with tumour formation in mice and humans, and yet it seems to be non-essential for normal development (Hollstein et al., 1991; Donehower et al., 1992; Malkin, 1994). p53 mediates cellular checkpoint responses to DNA damage, and it is theorized that this function defines its tumour suppressor qualities (reviewed, Sanchez and Elledge, 1995). p53 protein levels rise following DNA damage, leading to G1 arrest in most normal cell types (Maltzman and Czyzyk, 1984; Lu and Lane, 1993; Kastan et al., 1993). One of the targets of the p53 checkpoint appears to be *CIP1/WAF1*, the gene encoding the cdk-inhibitor, p21. Another important target of p53 during DNA damage appears to be *GADD45*, a member of the growth arrest and DNA damage family of genes which are up regulated following ultraviolet irradiation and other DNA damaging stimuli (Fornace et al., 1988; Kastan et al., 1992). The gadd45 protein was recently shown to associate with PCNA, which is required for excision repair following DNA damage. Gadd45 appears to enhance PCNA-dependent repair, and also may serve to promote G1 arrest by sequestration of PCNA from cyclin/cdk complexes (Shivji et al., 1992; Sanchez and Elledge, 1995). Another significant finding, implicating *p53* as a checkpoint

control gene, is that $p53^{+/+}$ is required for the maintenance of genomic stability, possibly through direct involvement in centrosome duplication during mitosis (reviewed, Hartwell, 1992; Livingstone et al., 1992; Yin et al., 1992; Fukasawa et al., 1996).

The tumour suppressor pRB is implicated, in addition to p53, as a G1 checkpoint protein. However, *RB-1*, the gene encoding pRB, differs from *p53* in that it appears to be essential for normal mammalian development, and is intimately involved in the negative feedback control of the normal cell cycle (Jacks et al., 1992; Clarke et al., 1992; reviewed, Weinberg, 1995). *RB-1* is named for retinoblastoma, a rare paediatric eye tumour, which was the basis of Knudson's "two hit hypothesis", now famous as the hypothesis that launched the multi-step theory of tumorigenesis into the genetic era (Knudson, 1971; Friend et al., 1986; Lee et al., 1987a; Fung et al., 1987). Eventually, *RB-1* was established as an important tumour suppressor gene, inactivated in a wide range of adult tumours (Weinberg, 1991).

It is believed that pRB, which is phosphorylated late in G1 and dephosphorylated upon the cell's emergence from M, plays an important regulatory role in the passage of the cell through the restriction point. (Lee et al., 1987b; Buchkovich et al., 1989; DeCaprio et al., 1989, 1992; Chen et al., 1989; Mihara et al., 1989). Experimental evidence supports the model that hypophosphorylated pRB is active in promoting G1 growth arrest, and that the hyperphosphorylated form of pRB is inactive throughout the cell cycle (Weinberg, 1995). Experimental evidence strongly implicates the G1 cyclins D and E, in conjunction with their cdk partners, in the inactivation of pRB by phosphorylation, late in G1 (Kato et al., 1993; Ewen et al., 1993; Hinds et al., 1992).

Hypophosphorylated pRB binds and inhibits the E2F transcription factor; however, upon inactivation, pRB releases E2F (Chellappan et al., 1991; Helin et al., 1993). It is probable that pRB thereby controls the expression of E2F dependent genes, which include c-*myc*, *cdc2*, *DHFR*, and others, all of which are involved in cell cycle progression (Nevins, 1992; Helin and Harlow, 1993). The active form of pRB has also been shown to bind and inhibit the proto-oncogene *c-abl* by inhibition of its kinase domain (Welch and Wang, 1993). Regulation of RB itself occurs for the most part indirectly by signal pathways such as TGFβ, cyclic AMP (cAMP), and by contact inhibition, through inhibition of the kinase activities of the cyclin/cdk complexes which would normally inactivate pRB, rather than through regulation at the transcriptional or translational levels (reviewed, Weinberg, 1995). DNA damage can also influence pRB activity via up regulation of p21 by p53, resulting in failure of G1 cyclin/cdk's phosphorylation of pRB (Kastan et al., 1991; Hall et al., 1993).

pRB appears also to play a positive role in the promotion of transcription of growth arrest genes. When over-expressed, pRB transactivates transcription of cell cycle arrest genes, *TGFβ1* and *TGFβ2*, by association with activating transcription factor-2 (ATF-2). (Kim et al., 1991; Kim et al., 1992). pRB also binds with the *brahma*-related gene, *BRG-1*, which is a transcriptional co-factor, and possibly together form an active complex which induces growth arrest (Khavari et al., 1993; Randazzo et al., 1994; Dunaief et al., 1994).

1.52 S and G2 checkpoints

The S and G_2/M transitions are best understood in yeast (reviewed, Feilotter et al., 1992). As discussed earlier, the latter stages of the cell cycle are positively regulated by

cdc25, which dephosphorylates, and thereby activates cdc2 kinase. Negative regulation is provided by wee1, which inactivates cdc2 (Parker et al., 1991, 1992). wee1 is itself regulated through phosphorylative inactivation by cdr1/nim1 and dephosphorylative activation by pyp1/pyp2 phosphatases (Russell and Nurse, 1987; Feilotter et al., 1991; Millar et al., 1992). Human *WEE1*, which was cloned by complementation of yeast mutant *wee1*, has been shown to play a role in the negative regulation of M, by preventing cytoplasmic cdc2 entry into the nucleus (Igarashi et al., 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Heald et al., 1993).

The control of S and G2 events are further guided by checkpoints which ensure that all S and G2 stage-related events have accurately and completely taken place prior to entry into the next stage of the cell cycle (Hartwell and Weinert, 1989). The sensing parameters used by the checkpoints are either related to completion of DNA synthesis (cdc25 pathway), or to cell size, nutritional requirements, and DNA damage (wee1 pathway) (Feilotter et al., 1992; Rowley et al., 1992a). Additionally, pathways appear to exist which respond to parameters such as chromosomal condensation, and centrosomal organisation of microtubules and spindle poles (Downes et al., 1994; Fukasawa et al., 1996; reviewed, Hartwell and Kastan, 1994).

Several yeast checkpoint mutants have been discovered which have mammalian counterparts, the best known being the *rad* mutants. These genes appear to be required for mitotic delay following radiation or chemically induced DNA damage (Rowley et al., 1992b; Al-Khodairy and Carr, 1992). For example, members of the *rad3* yeast mutant family are known to be involved in excision repair and are homologous to the human xeroderma

pigmentosa (*XP*) gene family, and the mammalian excision repair cross-complementing (*ERCC*) factors (reviewed, Aboussekhra and Wood, 1994). Mutations in these genes are associated with increased risk of skin cancer due to failure of DNA repair following UV exposure (sun hypersensitivity). Furthermore, the human homolog of another yeast G2 checkpoint gene, *mec1*, was recently cloned, and identified as *ATM*, the gene responsible for ataxia telangiectasia (AT) (Savitsky et al., 1995). Patients with AT have an increased cancer risk as well as hypersensitivity to ionizing radiation (Friedberg et al., 1995). Mutations in *ATM* and *mec1* result in failure of S and G2 checkpoint delays, following DNA damage, leading to transmission of unrepaired DNA (Paulovich and Hartwell, 1995).

The tremendous burden of oncogenic changes that can take place over a human lifetime illustrates the importance of fidelity of transmission of DNA during cell division. DNA damage is for the most part controlled and prevented by cell cycle checkpoints. However, if damage is too severe to repair, an alternative response is available. This alternative response, which causes the cell to "commit suicide", is known as apoptosis.

1.6 Apoptosis

Active cell death, or apoptosis is an essential physiological process that is required by multicellular organisms for normal development, tissue homeostasis, and cell-mediated cytotoxicity (Williams, 1991; Fesus, 1993). Apoptosis is thus an important process used by organisms to control and regulate cell numbers and growth. Furthermore, apoptosis is the mechanism whereby an organism can eliminate cells that are irreparably damaged by aging, or by external, unscheduled events, such as viral invasion, or DNA damage (reviewed,

Martin, 1993; Vaux et al., 1994). Apoptosis is thus the ultimate checkpoint response. Given the importance of apoptosis in maintaining homeostasis, it is perhaps not surprising that the apoptosis pathway is employed by the cell to combat oncogenesis, and is targeted for disruption during tumour progression (reviewed by Harrington et al., 1994; Hoffman and Liebermann, 1994). It is now widely accepted that cellular resistance to apoptosis can be oncogenic leading to inappropriate survival and proliferation of cells (Williams, 1991; Harrington et al., 1994; Hoffman and Liebermann, 1994).

1.61 Cellular changes during apoptosis

Experimental evidence suggests that apoptosis is a genetically controlled signalling pathway. For example, inhibitors of RNA and protein synthesis have been shown to block apoptosis under a wide variety of conditions, suggesting that the process of apoptosis requires transcription and translation of apoptosis-promoting factors (Martin, 1993). However, in some cases, for example, cell-mediated cytotoxicity, apoptosis can occur in the absence of these events (Doherty, 1993; Martin, 1993). Distinct morphological and biochemical features that identify death by apoptosis, as opposed to death by necrosis, have been observed and described on numerous occasions (reviewed, Peitsch et al., 1994; Bortner et al., 1995). Cells undergoing necrosis show no evidence of process-specific regulatory events and usually die following cytoplasmic swelling, and bursting of the membrane. In contrast, apoptotic cells, in addition to regulation by apoptosis-specific "death" genes, undergo morphological changes, such as cytoplasmic shrinkage and chromatin condensation. The cellular DNA becomes fragmented, followed by nuclear disintegration, and membrane

blebbing. Eventually, the cell breaks up into apoptotic bodies, which are phagocytosed by surrounding, healthy cells. A cardinal feature of apoptosis in most cell types, is the nucleosomal degradation of DNA into 180bp sized fragments, which, when electrophoresed, appear as a "DNA ladder" (Wyllie, 1980). However, other types of fragmentation have been described in apoptotic cells, and therefore failure to demonstrate a DNA ladder does not necessarily indicate the absence of apoptosis (Bortner et al., 1995).

Apoptosis has been identified during T cell maturation and immune response following cytokine-induced activation of membrane receptors, such as Fas/Apo-1(CD95) and tumour necrosis factor receptor-I (TNFR-1) (reviewed, Smith et al., 1994). The activated receptors transduce the apoptotic signal to receptor-associated effectors such as TRADD, FADD and RIP (Hsu et al., 1995; Chinnaiyan et al., 1995; Stanger et al., 1995). Other downstream components of the death signal include proteases of the interleukin-1β (ICE) family (Martin and Green, 1995; Tewari and Dixit, 1995; Enarl et al., 1995; Los et al., 1995). Apoptosis-induced DNA fragmentation is frequently Ca^{2+}-dependent, and occurs as following activation of DNA endonucleases, such as DNase I and II, and NUC-18, which attack the nuclear DNA (Pietsch et al., 1994; Martin et al., 1994; Martin and Green, 1995). The death signal can be blocked in the early stages (prior to proteolysis and endonucleolysis) by a number of cellular factors, the best characterized of which belong to the Bcl-2 family of apoptosis inhibitors and inducers (reviewed, Nuñez and Clarke, 1994).

1.62 Ced-9 and the Bcl-2 family

Genetic studies of the nematode, *Caenorhabditis elegans* (*C. elegans*), have revealed three important death genes: *ced-3*, *ced-4*, which are required for cell death, and *ced-9*, which protects from cell death, and which have since been shown to function similarly in mammals (reviewed, Hengartner and Horvitz, 1994b). The human proto-oncogene, *bcl-2*, is the mammalian homolog of *ced-9*, and has been shown to be so highly conserved, that it can complement *ced-9* mutants in *C. elegans* (Vaux et al., 1992). *bcl-2* was first identified following cloning of the B-cell lymphoma cytogenetic breakpoint t(14;18) and has since been shown to block cell death under a variety of conditions (Bakhshi et al., 1985; Cleary and Sklar, 1985; Vaux et al., 1988; Hockenbery et al., 1990; Nuñez et al., 1990). Analysis of sequences of a number of *bcl-2* and death related genes has led to the identification of highly conserved domains, known as the *bcl-2* homology domains 1 and 2 (BH1 and BH2). Some of the genes belonging to this family have functions similar to that of *ced-9* and *bcl-2*, in that they act to prevent cell death (Neilan et al., 1993). However, others have the opposite effect, and act to induce cell death (Table 1.1) (Oltvai et al., 1993). As well, some members of the family (such as *bcl-x*) produce messages that are alternatively spliced, with the result that one form of the transcript is anti-apoptotic, while the alternate form promotes apoptosis (Boise et al., 1993). Most of the *bcl-2*-related genes were identified by their ability to bind bcl-2, and by their BH1 and BH2 domains. It is probable that many of them interact *in vivo*, to either protect cells from apoptosis, or to allow apoptosis to occur. For example, Bax has been shown to dimerize with itself, heterodimerize with Bcl-2, and when in excess (in homodimer conformation), promote apoptosis (Oltvai et al., 1993).

Table 1.1 Bcl-2 family of genes.

Gene	Gene Product	Function	Expression
bcl-2	Bcl-2_{α}	Blocks apoptosis	Embryonic tissues; adult stem cells
bcl-x	Bcl-x_L	Blocks apoptosis	Similar to Bcl-2
	Bcl-x_s	Promotes apoptosis	Human thymus
	Bcl-x_{β}	Blocks apoptosis	Similar to Bcl-x_L
bax	Bax_{α}	Promotes apoptosis	Widespread
A1	A1	Blocks apoptosis	Haematopoietic tissues
mcl-1	Mcl-1	Blocks apoptosis	Myeloid cells
bad	Bad	Promotes apoptosis	?
bag-1	Bag-1	Blocks apoptosis	?
bak	Bak	Promotes apoptosis	Widespread

(Nuñez and Clarke, 1994;Yang et al., 1995; Takayama et al., 1995; Kiefer et al., 1995)

1.63 Ced-3 and ICE

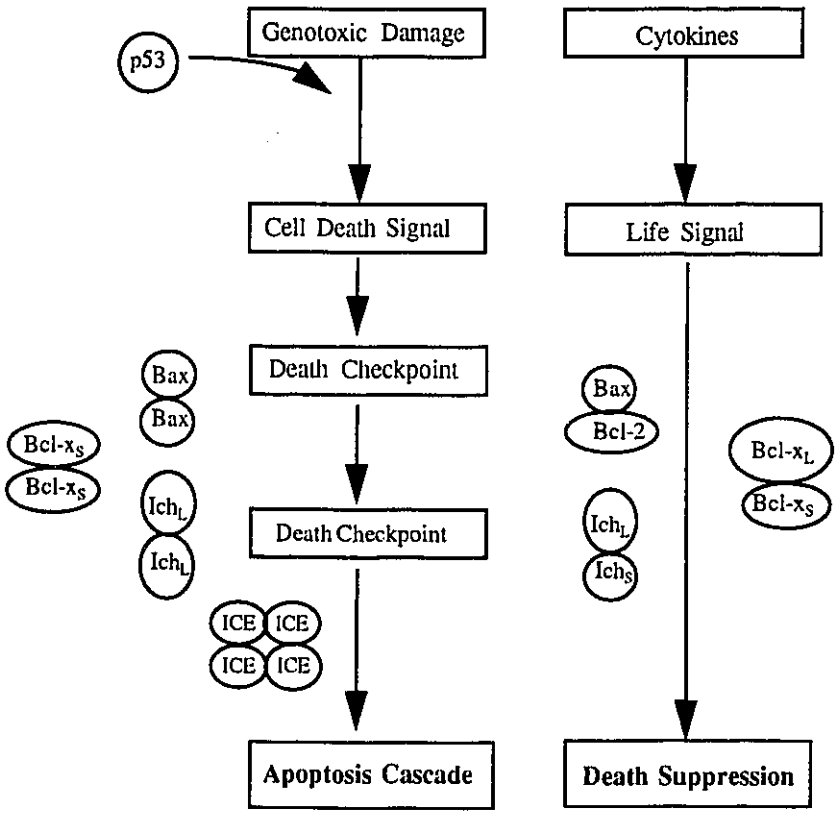
The mammalian homolog to the death promoting gene, *ced-3*, is the gene coding for interleukin-1β converting enzyme (*ICE*) (Yuan et al., 1993). ICE is a cysteine protease which is involved in the conversion of the inactive interleukin (IL)-1β precursor into the active form (Thornberry et al., 1992). Overproduction of ICE induces apoptosis in mammalian cells, probably through protease activity (Miura et al., 1993; Hengartner and Horvitz, 1994). Several additional mammalian *ced-3*-related genes have been cloned recently, including *ICH-1/NEDD2*, ICE_{rel}-*II*, ICE_{rel}-*III*, *CPP-32* and *MCH2* (Wang et al., 1994; Kumar et al., 1994; Fernandes-Munday et al., 1995; Alnemri et al., 1994, 1995). Some of the ced-3 family members show alternative splicing, in a manner similar to Bcl-x (Table 1.1). For example, Ich-1 is alternatively spliced into Ich-1_L and Ich-1_S. The short version, Ich-1_S, is anti-apoptotic, and counteracts Ich-1_L (Wang et al., 1994). As well, the apoptotic effects of at least some of the ICE family members appear to be negated by the viral anti-cell death gene, *crmA* (Ray et al., 1992; Wang et al., 1994). The biological substrate for CPP-32 (apopain) and MCH2 appears to be poly(ADP-ribose) polymerase (PARP), an enzyme required for DNA repair and fidelity, and which is inactivated by proteolytic cleavage at the onset of apoptosis (Nicholson et al., 1995; Tewari et al., 1995).

It has been proposed that all cells use a common apoptosis pathway, consisting of an amplifying protease signalling cascade (Fig. 1.5) (Martin and Green, 1995). This cascade involves the activation of proteases, such as apopain, which in turn process and activate additional proteases. These enzymatic events are thought to lead to the activation of other molecules, such as endonucleases, and inactivation of apoptosis inhibitors, cytoskeletal

proteins, and DNA repair enzymes. These events culminate with the characteristic physiological changes observed in apoptotic cells.

Figure 1.5 Model of cell death checkpoints.
The death signal, often originating via p53-dependent events, instigates a cascade of signals which proceed depending upon the relative amount of survival and death proteins, such as Bcl-2 and Bax, and Ich_L and Ich_S. Bcl-2 dimerizes with Bax, resulting in a cell "survival" condition, that prevents apoptosis. Changes in the ratio between Bcl-2 and Bax, that result in the ability of Bax to homodimerize enables apoptosis to occur. Similarly, heterodimers between Ich_L and Ich_S are anti-apoptotic, but Ich_L homodimers, when formed, allow apoptosis to proceed. The ICE protease is active when in tetrameric form. The exact order of the pathway is as yet unknown. Figure 1.5 was modified from Oltvai and Korsmeyer, 1994.

Figure 1.5


Genotoxic Damage
Cytokines
p53
Cell Death Signal
Life Signal
Death Checkpoint
Bax
Bax
Bcl-xS
Bcl-xS
IchL
IchL
Death Checkpoint
ICE
ICE
ICE
ICE
Bax
Bcl-2
IchL
IchS
Bcl-xL
Bcl-xS
Apoptosis Cascade
Death Suppression

1.64 Oncogenes, tumour suppressor genes, and apoptosis

Proliferation is strictly guarded in normal cells by negative feedback controls, which induce growth arrest, and prevent disorganized growth. However, under certain conditions, and depending upon cell type, cells may choose to undergo apoptosis to control growth. For example, *c-myc* is a powerful inducer of proliferation when over-expressed, however, when under the influence of activated *c-myc*, cells respond to poor growth conditions with apoptosis, as opposed to growth arrest (Wurm et al., 1986; Evan et al., 1992). The adenovirus *E1A* gene behaves in a similar fashion. E1A is oncogenic and stimulates proliferation, but also causes increased sensitivity to apoptosis-induction (White et al., 1991). The observation that promoters of proliferation can also sensitize cells to apoptotic stimuli, has led to the formulation of two models of oncogene-activated apoptosis (Harrington et al., 1994) (Fig. 1.6a and b). The first model, known as the conflict model, proposes that independent, opposing signals, one from a growth promoting oncogene, and the other from an external, cytostatic factor cause conflict in signalling pathways, resulting in apoptosis. The dual signal model proposes that the oncogene emits signals for both apoptosis and proliferation. Normal proliferation then requires the addition of survival factors to inactivate the apoptosis pathway. The *bcl-2* oncogene differs dramatically from oncogenes such as *c-myc*, which sensitize cells to apoptosis. In contrast, Bcl-2 reduces cell sensitivity to apoptosis induction. Overexpression of Bcl-2 can lead to increased resistance to apoptosis induced by radiation, cytokines, and chemotherapeutic drugs, and can negate the effects of apoptosis-sensitivity conferred by oncogenes such as *c-myc* and *E1A* (Fanidi et al., 1992; Mayashita and Reed, 1992; Rao et al., 1992). Thus the expression of survival genes like *bcl-*

2 may be favoured during tumour evolution, since their expression suppresses the negative effects of apoptosis-inducing oncogenes.

During oncogenesis, in addition to the over-expression of survival genes, "death" genes may be inactivated. For example, the tumour suppressor gene p53 has been identified as an apoptosis promoter that is inactivated in a large percentage of tumours. The association between p53 and apoptosis-induction was first noted following treatment of cells with a variety of agents that induce apoptosis, but which also activate the tumour suppressor p53 (Kastan et al., 1991; Kuerbitz et al., 1992; Lowe et al., 1993*a*; Lowe and Ruley, 1993). Furthermore, the introduction of $p53^{+/+}$ into $p53^{-/-}$ tumour cells induces apoptosis (Shaw et al., 1992; Yonish-Rouach et al., 1991). Stabilization of p53 protein is associated with viral oncoprotein E1A-induced apoptosis (Rao et al., 1992; Lowe and Ruley, 1993; Debbas and White, 1993). However, E1A-induced apoptosis is relieved by E1B 19K protein, which binds and inactivates p53 (Lows et al., 1993a). Mutation or inactivation of p53 results in decreased sensitivity of cells to induction of apoptosis by viral agents, radiation and chemotherapeutics (Lows et al., 1993a; McIlwrath et al., 1994). However, most normal cell types which have functional p53, show a preference for p53-dependent growth arrest, over apoptosis (Lows et al., 1993a; Fisher, 1994). A further connection between p53 and apoptosis during *in vivo* tumorigenesis was recently established by the observation that continued tumour growth is dependent upon the inactivation of p53 in early tumour cells, thus preventing hypoxia-induced apoptosis (Graeber et al., 1996). These findings explain the predominance of p53 mutations in human solid tumours and indicate an important functional association between p53 and induction of the apoptosis cascade, and further

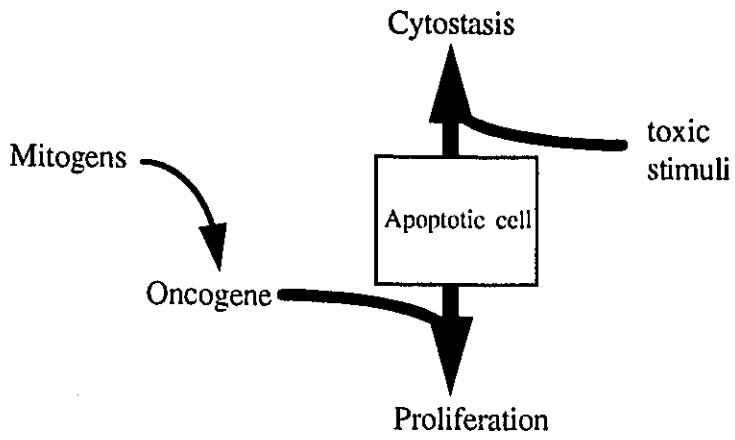
establish p53 in the role of "guardian of the genome"(Lane, 1992; Kinzler and Vogelstein, 1996).

In contrast to p53, pRB has been shown to inhibit apoptosis. For example, inactivation of *RB-1* during murine lens development results in failure of lens fibre cells to differentiate, and cause abnormal proliferation and extensive apoptosis (Pan and Griep, 1994; Morgenbesser et al., 1994). In addition, transfection of an $RB^{-/-}$ tumour cell line, SAOS-2, with wild-type *RB-1* resulted in decreased sensitivity to induction of apoptosis by irradiation (Haas-Kogan et al., 1995). Overexpression of pRB can protect tumour cells from p53-mediated apoptosis, suggesting that the decision making process between growth arrest and apoptosis may belong to pRB and related proteins (Haupt et al., 1995).

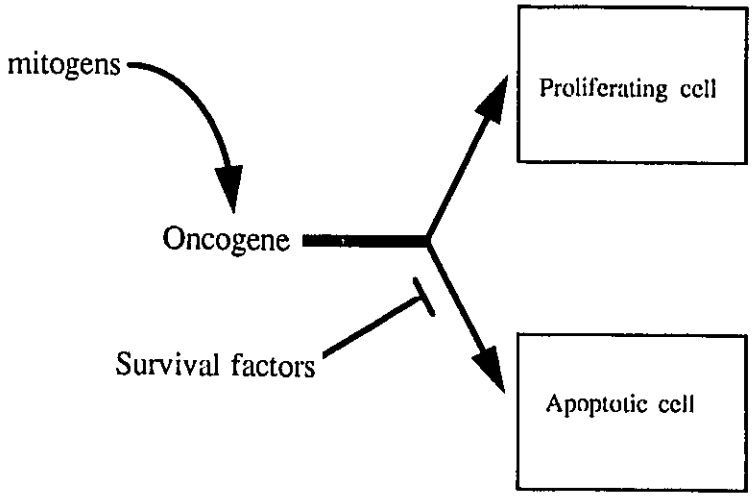
Figure 1.6 Models for oncogenic induction of apoptosis.
(a) Conflict model. The cell is subjected to two independent signals: one, leading to the cell cycle, is mediated by mitogens; the other, leading to growth arrest, is mediated by toxic stimuli. Conflict between these signals induces apoptosis.
(b) Dual signal model. The oncogene mandatorily emits signals for both pathways of growth and of apoptosis. The apoptosis pathway is blocked by survival factors which are removed by toxic stimuli.
Figure 1.6 was modified from Harrington et al., 1994.

Figure 1.6

(a) Conflict model



(b) Dual signal model



1.65 The role of apoptosis in cancer therapy

Antitumour drugs can be classified according to their mode of action and their intracellular targets (Table 1.2). The majority of these agents disrupt major cellular activities such as purine and pyrimidine biosynthesis, DNA synthesis and function, RNA transcription and protein translation (Traganos, 1990). Many of these drugs also have secondary effects, including changes in ion gradients, membrane composition and ultrastructure (Tritton and Hickman, 1990). For many years, it was assumed that anticancer therapies worked by targeting rapidly dividing cells; however, it is now apparent that most antineoplastic therapies accomplish tumour cell killing via induction of the apoptosis pathway. Thus, insensitivity to signals mediating this pathway is theoretically a mode of tumour cell drug resistance (Fisher, 1994).

Despite decades of research, drug resistance remains a serious impediment to a permanent cancer remission. Acquired drug resistance arises due to a variety of mechanisms that involve alterations in drug processing within the cell. These include the upregulation of transmembrane transporter molecules, such as P-glycoprotein (Pgp), the multidrug resistance associated protein (MRP), and the vault protein LRP (Endicott and Ling, 1989; Cole et al., 1992; Zaman et al., 1994; Scheffer et al., 1995). The molecular mechanisms of intrinsic drug resistance are only recently being unravelled, and may revolve around the process of apoptosis. For example, there is evidence that stress-induced cell death may be mediated by a sphingomyelin signalling pathway initiated at the cell membrane, which, if disrupted, leads to apoptosis insensitivity (Hannun and Obeid, 1995; Verheij et al., 1996). Furthermore, studies have shown that tumours with mutant p53 show increased drug

resistance, and a poorer response to therapy than tumours with intact and normally functioning p53 (reviewed, Fisher, 1994; Lows et al., 1994). In addition, expression of *bcl-2* has been shown to protect tumour cells from the effects of a variety of anticancer treatments (Collins et al., 1992; Miyashita et al., 1992, 1993; Fisher et al., 1993). Thus, the status of death and survival genes may play an important role in tumour cell responses to chemotherapy and radiation treatment (Fisher, 1994). The identification of genes that contribute to cellular survival, particularly in the absence of the frequently inactivated p53, should ultimately lead to improved methods of chemotherapeutic intervention.

Table 1.2 Antineoplastic drugs and their targets

Category	Class	Examples	Blocks
Alkylating agents	nitrogen mustards; nitrosoureas; trazenes; ethylenimines	methclorethamine, cyclophosphamide, chlorambucil	DNA function; S-phase sensitive
Antimetabolites	antifolates; antipurines; antipyrimidines	aminopterin; methotrexate; ara C; PALA	DNA, RNA, protein synthesis; cell cycle specific; G1/S sensitive
Antibiotics	DNA binding; intercalators;	actinomycin D; adriamycin; daunorubicin; mitomycin C	DNA; RNA; protein synthesis; S, G2/M sensitive (DNA) not cell cycle specific (RNA)
Antimitotics	vinca alkaloids	vincristine; vinblastine	mitotic spindle; G2/M sensitive
Miscellaneous	hormones; cytokines; enzymes	L-asparaginase; hydroxyurea-prednisolone	various targets: DNA synthesis; membrane receptors; G2 checkpoints

1.7 Strategy

Experimental findings indicate that during tumour progression, genes which normally act to "brake" the cell cycle are likely to be selected against, and genes which normally act to promote the cell cycle are likely to be retained, or upregulated. In addition, genes which cause or reduce apoptosis-sensitivity are likely to be important factors in tumour cell survival upon exposure to stressful conditions, such as hypoxia during tumour growth, and chemotherapeutic intervention. It is probable that these limiting conditions represent turning points in terms of the evolution of the tumour. It is theorized that rare tumour cells may survive stressful conditions via increased expression of survival genes, or reduced expression of death genes. These rare survivors are thought to be the founder cells responsible for the emergence of secondary cancers following initially successful chemotherapeutic treatment.

To map and clone new genes involved in the cellular survival response to chemotherapeutic agents, a strategy using somatic cell genetics, and an *in vitro* drug screening procedure was chosen. Hybrids containing individually tagged human chromosomes were screened for their ability to undergo growth arrest, and suppress apoptosis during short term *in vitro* chemotherapeutic treatment. Similar strategies have been used by others to map genes responsible for suppression of drug-induced responses. For example, suppressors of glucocorticoid-induced apoptosis were mapped in intraspecific hybrids to rat chromosomes 16 and 19, and Fanconi anaemia complementation group D was mapped to human chromosome 3, following analysis of drug-induced chromosome breakage in microcell hybrids (Gourdeau and Walker, 1994; Whitney et al., 1995). An alternative to the use of microcell hybrids, involves the introduction of cDNA into "sensitive" cell lines,

and the cloning of the exogenous cDNA in "resistant" cells (Teitz et al., 1990). However, this strategy may fail, particularly if the mRNA is unusually long or rare, since it is difficult to ensure that the cDNA library used consists of a complete representation of cDNAs which are full length and functionally intact.

Here, the experimental chemotherapeutic agent, *N*-(phosphonoacetyl)-L-aspartate (PALA) was chosen as the selection agent to map human chromosomes that carry genes responsible for growth arrest and suppression of apoptosis during chemotherapeutic treatment. PALA was chosen because 1) its mode of action is well known (inhibitor of the trifunctional CAD enzyme) (Swyryd et al., 1974), 2) the mechanism of acquired drug resistance is known (CAD gene amplification) and therefore could be monitored (Wahl et al., 1979), 3) it is capable of inducing p53-independent apoptosis (Livingstone et al., 1992; Yin et al., 1992), and 4) it is known to induce growth arrest in normal cells (Tlsty, 1990; Wright et al., 1990). The methods for monitoring the hybrid responses to the drug included qualitative assessment by microscopic examination of cell viability, and the appearance of apoptotic ladders, and by quantitative assessment, using trypan blue viability exclusion, and flow cytometry.

Chapter 2 of this thesis describes the construction and analysis of the microcell hybrids used in later experiments. Chapter 3 delineates preliminary experiments using whole cell hybrids, to evaluate the ability of normal human chromosomes to suppress the PALA-induced apoptosis phenotype of the murine cell line, B78. Chapter 4 describes the results of the drug screen used to identify the human chromosome responsible for cellular survival during PALA exposure. Finally, Chapter 5 describes the molecular techniques used to

identify candidate cDNAs which are potentially involved in the apparent chromosome 3-induced PALA survival pathway. In Chapter 6, conclusions are drawn regarding the experimental findings described in this thesis. It should also be noted that part of Chapter 2, and Chapters 3 and 4 were published in peer-reviewed journals (Speevak et al., 1995; Speevak and Chevrette, 1994; Speevak and Chevrette; 1996).

Chapter 2. Construction and Analysis of Microcell Hybrids

2.1 Abstract

A panel of human x murine microcell hybrids containing individual normal human chromosomes tagged with a dual selectable marker was constructed. Over 500 independent "B78MC" microcell hybrids were generated, and more than 200 individually isolated. The human chromosome content of a number of B78MC hybrids was identified. The hybrids were shown to possess the properties of positive and negative selection for the retention and removal of the human tagged chromosomes. Since its construction, this panel has proven to be useful in the mapping of tumour suppressor genes and genes involved in the suppression of apoptosis.

2.2 Introduction

Over the past 25 years, somatic cell hybrids have proven to be powerful tools in the study of cancer, cellular differentiation and gene mapping (Harris et al., 1969; Stanbridge, 1977). With the development of the microcell fusion technique, which permitted the transfer of only one or a few chromosomes into recipient cell lines, somatic cell hybrids became even more valuable as a means of genetic research (McNeill and Brown, 1980; Fournier, 1981; Leach et al., 1989). For example, microcell hybrids have been successfully used recently to map the location of tumour suppressor genes and to identify and clone tissue specific extinguishers by virtue of phenotypic alterations associated with the introduced chromosome

(Anderson and Stanbridge, 1993; Zarrilli et al., 1993; Bérubé et al., 1994; McGowan-Jordan et al., 1994; Jones et al., 1992).

Hybrid panels have been reported which were generated from human cell lines that were either abnormal or transformed, or relied upon complementation with the recipient cells for retention of the introduced chromosomes (Fournier and Ruddle, 1977; Warburton, 1990). Technical limitations are therefore a concern when attempting to use these panels for further chromosome transfer. Also, interpretation of phenotypic changes occurring after chromosome transfer can often be complicated by the fact that immortalized or tumorigenic recipient cell lines are usually genetically heterogeneous. For example, hybrids may be produced which show loss of tumorigenicity following introduction of a normal chromosome. However, the lack of tumorigenicity may be due to clonal variation, rather than due to the presence of the newly introduced chromosome. An appropriate test for association of tumour suppression with the introduced chromosome is to remove the introduced chromosome from the hybrid, and verify reversal of phenotype (Hansen and Cavanee, 1988).

Here, I report the generation of a new panel of microcell hybrids, which allows the transfer and removal of individual human chromosomes into and out of recipient cell lines. Early passage human skin fibroblasts were tagged with a dual selectable marker, tgCMVHy/TK, and microcells of these fibroblasts were fused with a murine melanoma cell line, B78. The panel, named B78MC, contains tagged normal human chromosomes which can be selectively retained or removed from hybrid cells using dominant positive or negative

selection (Speevak et al., 1995). This panel has already proven to be an extremely valuable tool in genetic complementation experiments.

2.3 Materials and Methods

2.31 Cell lines and culture conditions

The normal human fibroblasts (HSF-5) used in this study were isolated from neonatal foreskins. The foreskins were finely minced, rinsed with PBS, and treated with Hepes/collagenase buffer (4:1 Hepes [6.7mM KCl ;142mM NaCl; 10mM Hepes] : collagenase [5mg/ml collagenase; 50mM $CaCl_2 \cdot H_2O$; 120mM NaCl; 10mM Hepes; pH7.4]) to disaggregate the fibroblasts. The cells were plated in $75cm^2$ flasks and incubated at 37°C (5% CO_2) until confluence. The fibroblasts were passaged at least twice prior to storage in liquid nitrogen. HSF-5 was cultured in Hams-F12-Dulbecco's modified Eagle medium (F-DV) or in Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum. Cultures of hygromycin resistant human fibroblasts, HF-HT4, were further supplemented with 100µg/ml hygromycin B.

B78, the recipient cell line in microcell fusion experiments, is an immortalized and tumorigenic murine melanoma cell line (gift from M. Thayer, originating from murine melanoma cell line, B16), and was cultured in Dulbecco's Modified Eagle Medium, high glucose (DMEM) supplemented with 10% fetal bovine serum. The B78MC microcell hybrids were also cultured in DMEM, 10% fetal bovine serum, plus 400µg/ml hygromycin B.

2.32 Plasmid Preparations

E. coli containing plasmids were grown in selective medium (LB [1% tryptone; 0.5% yeast extract; 0.5% NaCl; 10^{-3}N NaOH] plus appropriate antibiotic), at 37°C in overnight cultures. Plasmid DNA was extracted from 2 and 250 ml cultures using standard protocols (Zhou et al., 1990; Birnboim and Doly, 1979).

2.33 Transfections

Exponentially growing fibroblasts were trypsinized, washed free of serum and resuspended in HEBS buffer (137mM NaCl; 21mM Hepes; 4.8mM KCl; 0.75mM Na_2HPO_4) at a concentration of 10^6 cells/ml. Supercoiled plasmid tgCMV/HyTK (Lupton et al., 1991) was added to 1ml aliquots of the suspension, at a final concentration of 4µg/ml. The mixture was electroporated using the Bio-Rad Gene Pulser (300V, 960µF).

The electroporated fibroblasts were allowed to recover at 37°C in non-selective complete medium, (1:1 F12 Hams: DMEM, plus 10% fetal bovine serum) overnight. Following this, the cells were fed with selective medium, containing 100µg/ml of hygromycin B, to select for incorporation of the plasmid into the human fibroblast genome. At this concentration of hygromycin B, fibroblasts which failed to incorporate the plasmid died, and those retaining the plasmid continued to grow and form colonies. The colonies (770) were pooled and named HF-HT4.

2.34 Ganciclovir sensitivity testing

To test HF-HT4 fibroblast sensitivity to ganciclovir, $2x10^5$ HSF-5 and HF-HT4 cells were exposed to a range of ganciclovir doses (0 to 1mM) during a 10 day culture period. Growth potential was assessed by comparison of the ability of HSF-5 and HF-HT4 to reach confluency in the presence of ganciclovir, as compared to controls (in the absence of ganciclovir).

2.35 Microcell fusions

Microcells from HyTK tagged fibroblasts were prepared and fused with the recipient cell line, B78, according to published procedures (McNeill and Brown, 1980; Fournier, 1981). To induce micronuclei formation, 12 large ($150cm^2$) flasks of 75% confluent fibroblasts were exposed to colchicine, at a final concentration of 10µg/ml, for 48 hr. The micronucleated population of HF-HT4 was trypsinized and redistributed onto sterile plastic ConA "bullets" in 150mm petri dishes, at a concentration of 10^6 cells/bullet. The bullets were incubated at 37°C for approximately 2 hours, allowing the fibroblasts to attach and flatten. The bullets were then suspended in sterile, capped centrifuge tubes containing serum-free enucleation medium (MEM plus 10µg/ml cytochalasin B). The cells were then centrifuged at high speed (15krpm), resulting in the shearing of the microcells from the cytoplasm, and pelleting to the bottom of the tubes. The bullets, which still retained the enucleated cell remnants were removed from the centrifuge tubes, and the medium was aspirated. The pelleted microcells were then resuspended in a small amount of serum-free medium, and filtered twice to remove debris. The microcells were again centrifuged and the

pellets were resuspended in serum free medium, to a volume of 0.5 ml for each 4-6 bullets used. The recipient cell line, B78, was grown to confluency in 25cm^2 flasks, one flask for each 4-6 bullets used, plus a control flask. In preparation for microcell fusion, the recipient flasks were rinsed, and 0.5ml of serum free medium containing 200µg/ml phytohemagglutinin-P (PHA) was added to each flask, followed by 0.5ml of microcell suspension, with the exception of the control flask, which received 0.5ml of medium alone. The flasks were then incubated for 15min prior to fusion, in order for the microcells to become agglutinated to the recipient cells, via the action of the PHA. To fuse the microcells with the recipient cells, the flasks were rinsed with serum-free medium, and exposed to 50% (w/w) polyethylene glycol 1540 (PEG) for 60sec. The flasks were immediately rinsed repeatedly with serum-free medium, to completely remove the PEG. The flasks were then fed with complete medium (DMEM plus 10% fetal bovine serum) and allowed to recover overnight, at 37°C. Each flask was then subdivided into four 100mm petri dishes, and fed with selective medium: 3mM ouabain, to kill any viable fibroblasts, and 400µg/ml hygromycin B, to kill recipient B78 cells which had failed to incorporate a tagged human chromosome from the HF-HT4 microcells.

2.36 Genomic DNA Isolation

Genomic DNA was isolated by a modified salt-extraction method (Miller et al., 1988). Cells were trypsinized, centrifuged (1100rpm) and pelleted. Pellets were resuspended in 0.9% NaCl (saline) and the suspensions were transferred to a 1.5ml eppendorf tube. The cells were pelleted, and the saline supernatant was aspirated and discarded. The

pellet was resuspended in 300µl TSM / NP40 (140µM NaCl; 10µM Tris-HCl [pH7.4]; 1.5mM $MgCl_2$ plus 0.5% NP40). The cells were vortexed lightly and allowed to sit on ice for 3 min to allow cell lysis to occur. The suspension was microfuged 10sec and the supernatant, containing cellular RNA was decanted to a separate tube for later use. The pellet, containing intact nuclei, was resuspended in 1ml nuclei dropping buffer (0.075M NaCl; 0.024M EDTA, pH 8.0), which was then transferred to a 15ml centrifuge tube containing 4ml nuclei dropping buffer, proteinase K and SDS to a final concentration of 0.2mg/ml and 0.5% respectively. The suspension was incubated overnight at 37°C on an orbital shaker (Labquake). Following this, 5ml H_2O and 5ml 6M NaCl were added to the lysate, and the suspension was mixed at room temperature for 1-2 hours on the orbital shaker. The suspension was centrifuged at 3000rpm (Beckman J-6B) for 20min to pellet the debris. The supernatant, containing the genomic DNA, was transferred to a 50ml tube containing 2 volumes of 99% ethanol. The tube was gently inverted several times to precipitate the DNA. The precipitated DNA was spooled out and resuspended in 3ml TE buffer (10mM Tris-HCl [pH 8.0]; 1mM EDTA [pH 8.0]) on an orbital shaker overnight. To further purify and concentrate the DNA, the DNA was reprecipitated with 1/10 volume 7.5M ammonium acetate and 2 volumes 99% ethanol. The precipitated DNA was spooled out and resuspended in 1ml low TE (10mM Tris-HCl [pH 8.0]; 0.1mM EDTA [pH 8.0]). The DNA was quantified by fluorometry (TKO 100 Mini Fluorometer), using the manufacturer's specifications (Hoefer Scientific Instruments).

2.37 Cytogenetic analysis of hybrids

Metaphase chromosomes were prepared according to standard cytogenetic techniques. Exponentially growing cells were incubated in medium containing 50ng/ml colchicine for 2 hours. The cells were harvested by trypsinization and centrifugation (1100 rpm) followed by a 20min hypotonic treatment (0.075M KCl) to swell the cells. A few drops of Carnoy's fixative (3:1 methanol:acetic acid) were added to the mixture, followed immediately by centrifugation. The cells were then fixed at least 3 times in Carnoy's fixative. Finally, cell suspensions were dropped onto iced, dry slides and air dried.

For G-banding (Seabright, 1971), 1-2 week old metaphase slides were digested with trypsin (0.25% in saline) for 10-20sec at room temperature. The slides were rinsed in saline and water, and stained for 1 min with Giemsa (1:3 Harleco:Fisher Giemsa diluted 1:10 in PBS). The slides were rinsed with water and allowed to air dry. The metaphase spreads were analyzed by light microscopy, with a 100X oil immersion Planapochromat objective, and photographed using a Zeiss Photomicroscope II and TMAX -100 (Kodak) black and white film.

To prepare the chromosome slides for fluorescence *in situ* hybridization (FISH), metaphase slides were dehydrated in an iced cold ethanol series (two minutes in each of 70%; 95%; 100% ethanol). Following air drying, the metaphase chromosomes were denatured in 70% formamide/2XSSC (300mM NaCl; 30mM trisodium citrate) at 70°C, for 2min. The slides were again dehydrated in an ethanol series, and air dried. Denatured, biotinylated probe was applied to the slide, coverslipped, and incubated at 37°C overnight. FISH using total human DNA (THD) probe (Oncor) was used to determine the total number

of human chromosomal elements in the hybrids. Biotinylated THD probe was denatured for 10min at 70°C, cooled briefly on ice and applied to denatured B78MC chromosome slides. The slides were coverslipped and incubated in a moist chamber at 37°C overnight. Probe hybridization was detected with a commercially available FISH detection kit, using FITC-conjugated avidin, anti-avidin antibodies, and a second layer of FITC-avidin to amplify the signal. The slides were counterstained with propidium iodide (PI, 0.25µg/ml), and the chromosome spreads were visualized on a Zeiss Axiophot fluorescence microscope (100X oil objective), and photographed with Ektachrome colour slide film, ASA 400 (8-15sec exposure).

DNA from individual B78MC hybrids was used to prepare *Alu*-PCR FISH probes. Briefly, unique sequences existing between *Alu* repeats within the human chromosomes in the hybrids were PCR amplified using *Alu* primers 153, 154, 450 and 451 (Dorin et al., 1992), biotinylated by random priming (BioPrime), and hybridized to normal human chromosome spreads (Bérubé et al. 1994; McGowan-Jordan et al. 1994; Speevak et al., 1995).

2.38 PCR analysis of hybrids

PCR amplification using oligonucleotide primers identifying human microsatellite sequences (sold as MapPairs by Research Genetics, Huntsville, Ala, USA) was used to verify the presence or absence of individual human chromosomes in the hybrids. High molecular weight DNA (50ng) from B78 (negative control), human cells (positive control), the B78MC hybrid or its derived subclones was added to PCR hybridization mixtures containing 10pmol

of each primer, 200mM deoxyribonucleotide triphosphates, PCR buffer (10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 50mM KCl, and 0.01% gelatin) and 0.75 U of AmpliTaq (Perkin-Elmer Cetus) in a volume of 25µl. Samples were incubated in a Perkin-Elmer DNA thermal cycler 480 with the following parameters: 94°C for 5 min followed by 35 cycles of successive denaturation (94°C for 30 sec), annealing (60°C or 55°C for 30 sec) and synthesis (72°C for 30 sec). A final cycle of 72°C for 5 min ended the amplification program. The amplified products were analyzed by gel electrophoresis on 2% agarose (Nu-Sieve 3:1, FMC, Rockland, ME, USA) gels stained with ethidium bromide. Primers used are as follows: D3S1210, chromosome 3; D4S415, chromosome 4; D5S107, chromosome 5; D6S254, chromosome 6; EGFR, chromosome 7; PENK, chromosome 8; D9S146, chromosome 9; D10S111, chromosome 10; Int2, chromosome 11; D12S43, chromosome 12; D13S131, chromosome 13; TCRD, chromosome 14; GABRA5, chromosome 15; SPN, chromosome 16; D17S957, chromosome 17; D18S44, chromosome 18; D19S49, chromosome 19; D20S17, chromosome 20; D21S270, chromosome 21; D22S274, chromosome 22. The presence of chromosomes 1, 2, X and Y in the hybrids was determined by *Alu*-PCR FISH alone.

2.4 Results

2.41 Isolation and tagging of human fibroblasts

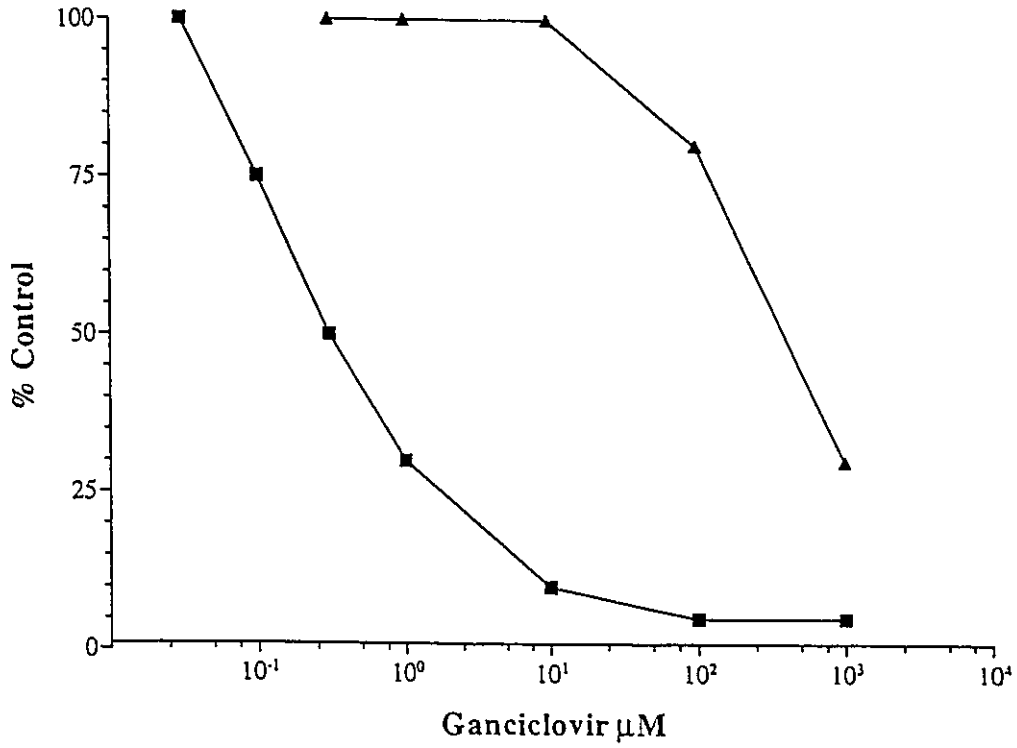
To establish a microcell donor cell line containing dual selectable tagged human chromosomes (THCs), normal human fibroblasts were isolated from a pool of 7 neonatal

foreskins. The fibroblasts were expanded, and passaged twice to stabilize their *in vitro* growth prior to use. Early passage fibroblasts (HSF-5), were then tagged by electroporation with the plasmid tgCMV/HyTK (Lupton et al., 1991). This plasmid contains the selectable fusion gene, HyTK, which consists of the hygromycin phosphotransferase gene (hph) fused in-frame with the herpes simplex virus type 1 thymidine kinase gene (HSV-1TK). HyTK confers hygromycin B resistance through the hph gene, and ganciclovir sensitivity through the HSV-1 TK gene. Approximately 3 x 10^7 fibroblasts were electroporated. Selection for stable integration of the plasmid into individual chromosomes was accomplished via resistance to hygromycin B. The majority of electroporated fibroblasts died within 4-6 days of selection in 100μg/ml hygromycin B medium, due to failure to incorporate the plasmid. However, rare, hygromycin resistant colonies began to appear following 2-3 weeks of hygromycin selection. Ultimately, a total of 770 individual, stably transfected fibroblast colonies were obtained (transfection efficiency of approximately $3x10^{-5}$). The hygromycin resistant colonies were pooled and named HF-HT4.

To verify that the TK portion of the HyTK gene was functioning, the HF-HT4 and the HSF-5 fibroblasts were exposed to a wide dosage range of ganciclovir (GCV) (Fig.2.1). A comparison of growth potential showed that HF-HT4 was approximately 2000 times more sensitive to GCV than HSF-5, suggesting that the TK portion of the gene was intact and fully operational. The HF-HT4 fibroblasts were considered therefore to be suitable as a microcell donor cell line.

Figure 2.1 Comparison of ganciclovir sensitivities.
Characterization of ganciclovir sensitivity of HSF-5,(▲) and HF-HT4(■) fibroblasts (day 9). The dose of GCV required to reduce the growth potential of HF-HT4 by 50% was 0.25μM. The dose required to produce a similar reduction in growth potential of HSF-5 was approximately 500μM.

Figure 2.1



2.42 Microcell fusion of tagged fibroblasts with B78

Microcells were prepared from the HF-HT4 cells and fused with B78, a murine melanoma cell line. Hygromycin selection was used to kill those B78 cells which failed to retain a THC from HF-HT4. Ouabain was included in the selection medium as well, to ensure that no human fibroblasts survived the procedure. All the B78 cells in the control flask died within 5-7 days post-fusion. The majority of recipient cells in the microcell fusion flasks also died post-fusion. However, individual hygromycin and ouabain resistant colonies appeared during this time, and were ready to be picked within 2-3 weeks. These colonies were presumed to have survived hygromycin selection through retention of a THC within a murine ouabain resistant genetic background. Using cloning rings, the colonies were picked, numbered, and expanded into 25cm^2 flasks. A total of 208 individual colonies were picked in this manner, from three independent fusions. The HF-HT4 x B78 microcell hybrids were called B78MCn hybrids (n=1 to 208). The B78MC hybrids were stored individually in liquid nitrogen, and in pools of 10-14 hybrids (B78MCPn pools [n=1-18]). Additionally, several hundred unpicked colonies (MHP hybrids) were pooled, expanded and stored in liquid nitrogen for later use.

2.43 Human chromosome content of microcell hybrids

Analysis of the human chromosome content of the B78MC hybrids required a combination of methods (Table 2.1). Molecular cytogenetic methods, including FISH using total human DNA probe (THD FISH) and *Alu*-PCR FISH proved to be the most informative, as well as standard PCR amplification with primers specific for different human

chromosomal loci. In some cases, it was possible to identify human chromosomes present in hybrids by the classical cytogenetic method, G-banding. For example, a normal, non-rearranged human chromosome 12 was identified by G-banded analysis in B78MC30 (Fig.2.2a). However, due to the large number of mouse chromosomes (over 80 per spread), it was often difficult to find the human chromosome hidden amongst them. Also, human chromosomes were frequently unrecognizable, due to multiple rearrangements resulting from the microcell fusion procedure, and therefore could easily be missed. For example, the two human chromosomes in B78MC96 were difficult to recognize by G-banding, and could not be classified due to complex rearrangements (Fig. 2.2b). Using the additional techniques described below, the human chromosomes were later determined to be complex rearrangements of chromosomes 7, 10, 12 and 13.

Molecular cytogenetic methods were employed to characterize the human chromosome content of the B78MC hybrids with greater precision than that offered by G-banding. For example, THD-FISH analysis was used to establish with certainty the number of human chromosomes in each hybrid analyzed. Biotinylated THD probe was hybridized to B78MC hybrid chromosome spreads and detected using FITC and PI counterstain. The FITC hybridization signal on the human chromosomes appeared as green fluorescence on a background of red, PI stained mouse chromosomes. This method was an improvement over G-banding, in that the bright FITC signal easily permitted the identification of individual human chromosomes amidst the multitude of mouse chromosomes in each spread. For example, THD FISH on B78MC96 easily revealed two human chromosomes, one large and one small, amongst the many B78 chromosomes (Fig. 2.3a). However, although THD FISH

was a more accurate method of detection of the number of human chromosomes present in a hybrid, it did not allow the classification of individual chromosomes, and did not distinguish between intact chromosomes, and fragmented chromosomes. To further establish the identity of the human chromosomes present in the hybrids, *Alu*-PCR FISH was employed (Lichter et al., 1990; Dorin et al., 1992; Liu et al., 1993). For *Alu*-PCR FISH, *Alu* sequences were used as primers to PCR amplify the human DNA present in the hybrids. This resulted in preferential amplification of human DNA, since *Alu* sequences are common and regularly spread out through the human genome, but are rare in the murine genome. Following biotinylation, the *Alu*-PCR products were used as FISH chromosome painting probes on normal male human chromosome spreads. The human chromosomes which hybridized to the *Alu*-PCR probe were complementary to the human chromosomes contained in the hybrid from which the probe was derived, thus revealing the major human elements present in the hybrid being analyzed. This technique allowed the identification of the number, size, and integrity of human chromosomes present in the majority of cells of an individual B78MC hybrid. For example, *Alu*-PCR FISH using B78MC96-derived probe revealed at least 4 pairs of independent human chromosomal elements (Fig.2.3b). This finding indicated that the human chromosomes present in B78MC96 were highly rearranged and derived from at least 4 different human chromosomes.

To narrow down the search for specific human chromosomes present in the hybrid panel, a PCR screening approach was used. PCR primers, specific for the chromosome of interest were used to screen the 18 pools of hybrids (B78MCP1-18). Following identification of individual pools which showed strong amplification of the representative

sequence, DNA from the individual hybrids present in the positive pools was screened in the same manner. For example, the pools were screened for the presence of human chromosome 13 using D13S131. The PCR positive hybrids from the positive pools were then screened individually (hybrids from B78MCP1 shown, Fig.2.4). The positive hybrids were then analyzed by *Alu*-PCR FISH and PCR to determine their complete human chromosome content. Numerous B78MC hybrids were analyzed, using some or all of the techniques described above, resulting in a nearly complete microcell hybrid panel (Table 2.2). Characterization of a number of B78MC hybrids revealed that the majority contained one to several human chromosomal elements, many of which were rearranged, deleted, or translocated.

Retention of the THC in the B78MC hybrids was achieved through dominant positive selection with hygromycin B during routine cell culture. In order to verify that the hybrids were susceptible to dominant negative selection as well, their sensitivity to GCV was tested (Table 2.3). In the presence of GCV, most cells died rapidly, leading to the emergence of rare colonies of cells which were insensitive to GCV (GCV^R) . To determine if the resistant colonies had lost the THC or had simply resulted from the inactivation of the HyTK fusion gene, several colonies were picked from GCV treated cultures of B78MC96 and expanded (B78MC96-R). Complete removal of the tagged chromosome from the B78MC96-R subclones was confirmed by THD FISH (Fig.2.5). Prior FISH and PCR analysis of B78MC96 had shown the presence of human chromosomes 7, 10, 12 and 13 in this hybrid. PCR amplification of B78MC96-R hybrids using a chromosome 10 specific primer pair showed that this locus was no longer detectable, indicating that this locus was linked to the

tgCMV/HyTK tag (Fig.2.6). This procedure was repeated for a number of B78MC hybrids, and in most cases analyzed, GCV selection resulted in the specific loss of the THC from the hybrid cells.

While most B78MC hybrids were sensitive to GCV, some, like B78MC32 and B78MC108 were unexpectedly resistant (Table 2.3). Experimental observations suggest that phenotypic variability of the B78 cell line may be responsible for the GCV resistance seen in some of these hybrids. For example, human chromosome 4 from B78MC108 (insensitive to GCV) and the tagged portion of human chromosome 12 from B78MC9 (sensitive) were transferred into two human cell lines, PA-1 and DU-145 respectively. As expected, GCV^R segregants were easily obtained from the DU-145 cells that contained the extra fragment of chromosome 12 (Bérubé et al., 1994). However, interestingly, GCV^R segregants were also obtained from PA-1 hybrids containing the tagged chromosome 4 transferred from B78MC108 (McGowan-Jordan et al., 1994), which had been found to lack sensitivity to GCV (Table 2.3). This suggests that the lack of sensitivity to GCV of some B78MC hybrids was due to a B78 phenotypic variant, and not due to the inactivation of the HyTK gene. These results show that the B78MC hybrids can be successfully used as donors in microcell fusion experiments and that the property of dual selection is transferrable to other cell lines, in association with the tagged human chromosome.

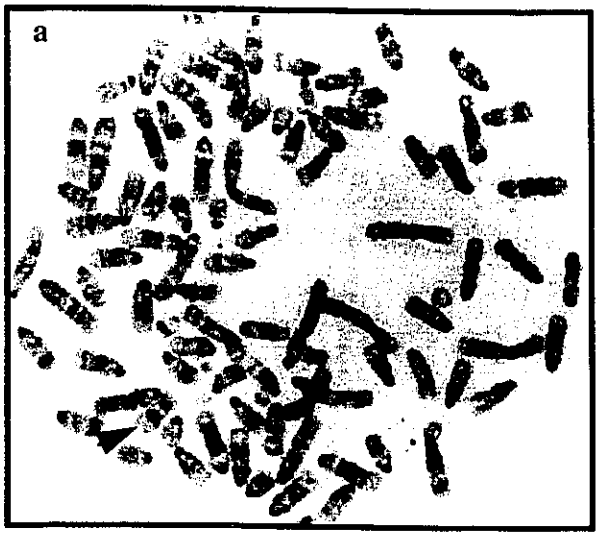
Table 2.1 Comparison of analysis techniques used to identify human chromosome content in the B78MC hybrids.

Analysis Technique	Advantages	Disadvantages
G-banding	Identifies and classifies human chromosomes; detects rearrangements and deletions.	Requires skill in chromosome identification; difficult to pick out rearranged chromosomes from mouse chromosomes; difficult and time-consuming to determine mosaicism in hybrid.
THD FISH	Identifies number and shape of human chromosomes in each cell; detects mouse; human translocations; allows rapid analysis of mosaicism	Does not allow classification of human chromosomes; does not detect rearrangements other than mouse; human translocations.
Alu-PCR FISH	Identifies all human elements in the hybrid; can infer deletions and rearrangements; verifies monochromosomal hybrids	Time consuming; identifies major human elements only; does not identify mosaicism; intrachromosomal rearrangements not detected
Standard PCR	Rapid screening tool to identify hybrids containing a desired chromosome; once content is known, allows easy tracking of changes following dilution cloning of GCV treatment.	Does not determine intactness of chromosome; many false positives due to presence of spurious fragments of human chromosomes in minority of hybrid cells.

Figure 2.2 G-banded analysis of the hybrids.
(a) G-banded chromosomes from B78MC30, revealed the presence of human chromosome 12 (arrow).
(b) G-banding of B78MC96, identified two rearranged human chromosomes of unknown origin (arrowheads). These two chromosomes were later determined to consist of parts of human chromosomes 7, 10, 12 and 13. The mouse chromosomes can be distinguished from the human chromosomes by the intensely dark staining around the mouse chromosome centromeres.

Figure 2.2

B78MC30



B78MC96

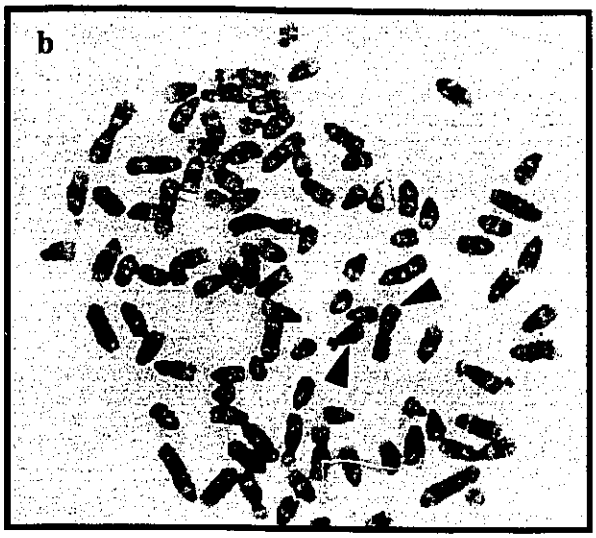


Figure 2.3 FISH analysis of B78MC hybrids.
(a) THD FISH identified with certainty, two human chromosomes, one large and one small, in B78MC96.
(b) *Alu*-PCR FISH using probe made from B78MC96 revealed hybridization to multiple human chromosomes, indicating that this hybrid contained complex rearrangements involving more than two human chromosomes. Further analysis, using additional techniques indicated that the human elements included chromosomes 7, 10, 12 and 13.

Figure 2.3

Figure 2.4 PCR screening of MCP pools.
Individual hybrids were screened using MapPair primer set D13S131 (Research Genetics) to detect the presence of human chromosome 13. M, 100bp ladder; Human DNA, positive control; B78, negative control; MCP1, B78MCP1 (pool 1); MC61 and MC134, B78MC hybrids containing chromosome 13 identified from a previous screen; MC109, a B78MC hybrid lacking chromosome 13. The remaining lanes are hybrids from B78MCP1. B78MC16, from pool 1 appears to be positive for human chromosome 13 at the D13S131 locus.

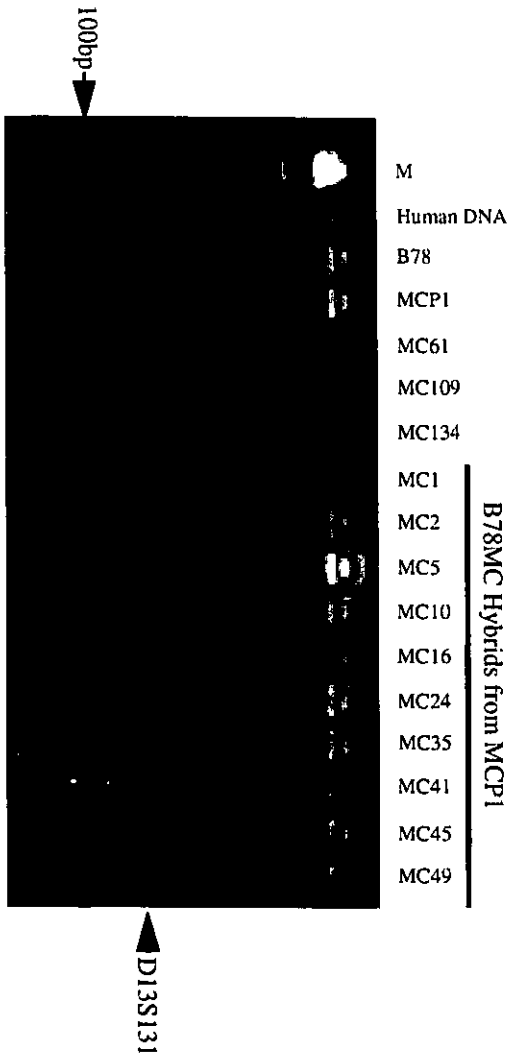

100bp
M
Human DNA
B78
MCP1
MC61
MC109
MC134
MC1
MC2
MC5
MC10
MC16
MC24
MC35
MC41
MC45
MC49
B78MC Hybrids from MCP1
D13S131

Figure 2.4

Figure 2.5 THD FISH of GCV^R segregant.
THD FISH performed on B78MC96-R3 chromosome spreads using total labelled human DNA. All cells analyzed contained a single large human chromosome, suggesting that the small chromosome present in B78MC96 (se Fig. 2.3) was tagged, since it was removed during GCV selection.

Figure 2.5

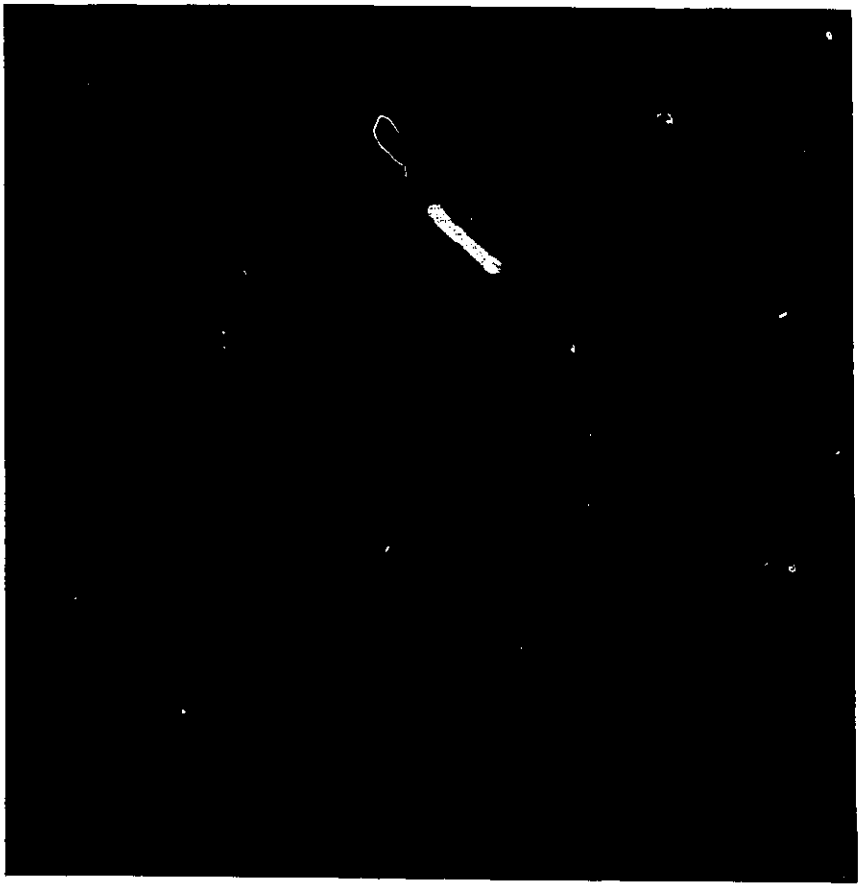


Figure 2.6 Removal of tagged chromosome by GCV selection.
PCR amplification shows removal of a human chromosome 10 locus (D10S111) upon GCV selection. M, 100bp ladder; Human DNA, positive control; B78, negative control. The arrowhead indicates the D10S111 amplicon which was present in the human DNA lane, and the B78MC96 lane, but absent in the three segregant lanes (-R1, -R2, and -R3). The lower fragments in the last 5 lanes represent amplicons derived from mouse DNA.

Figure 2.6

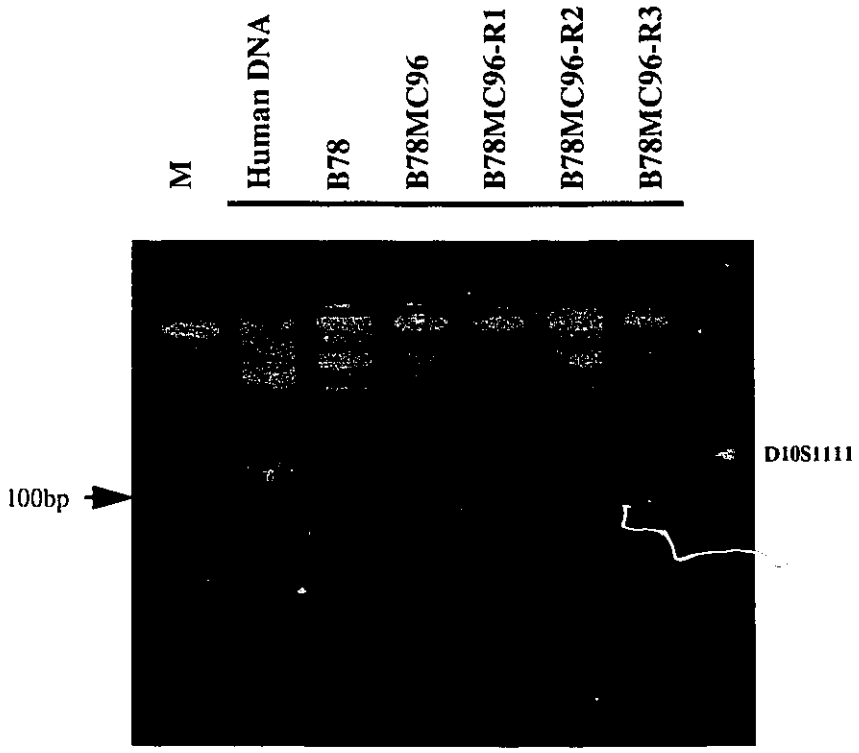

M
Human DNA
B78
B78MC96
B78MC96-R1
B78MC96-R2
B78MC96-R3
100bp
D10S1111

Table 2.2 Panel of B78MC hybrids.
Hybrids were analyzed by one or more of the following methods to determine the human chromosome content: G-banded analysis, THD FISH, *Alu*-PCR FISH, and PCR.

Table 2.2 Panel of B78MC hybrids

MC Hybrid	Human Chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
MC195	+						+				+													
MC32		+										+			+									
MC185			+																					
MC108				+																				
MC126					+										+									
?																								
MC1							+					+												
MC44								+		+														
MC173									+		+													
MC77										+					+									
MC190			+								+													
MC9												+												
MC16												+	+											
?																								
MC31								+				+			+									
MC197																+	+							
MC27							+										+					+		
MC89																		+						
MC192																	+		+					
MC63							+					+					+			+				
MC142		+																			+			
MC43																						+		
MC71							+																+	
?																								

Table 2.3 GCV response of B78MC hybrids.

Hybrid	Human Chromosome Content	GCV
B78MC32	[a] del(2), 12, **15**	resistant
B78MC166	[b] **rea(3)**	sensitive
B78MC185	**3**	resistant
B78MC108	**4**	resistant
B78MC63	**7**, 12, del(17)	sensitive
B78MC96	[c] cr(7), cr(10), cr(12), cr(13)	sensitive
B78MC77	**10**, del(15)	sensitive
B78MC9	**del(12)**, [d] f(12)	sensitive
B78MC9-5	**del(12)(q14)**	not tested
B78MC27	[e] **t(7;22)**; 17	sensitive
B78MC152	**12**, 17	sensitive

Note: Bold numeric denotes tagged chromosome.
[a] del indicates deletion; [b] rea indicates rearranged; [c] cr indicates complex rearrangement.
[d] f indicates fragment ; [e] t indicates translocation.

2.5 Discussion

A panel of microcell hybrids (B78MC) was constructed between normal human fibroblasts and the murine melanoma cell line, B78. This panel showed the unique trait of dual selectability of the human chromosomes. The retention or removal of the human chromosomes was accomplished via the presence of HyTK, a fusion gene introduced into the human chromosomes prior to microcell fusion. The ability to introduce and remove a human chromosome into any cell line, independent of its genetic makeup, is a valuable tool in the study of genetic suppressors. For example, cancer cell lines which have received a normal human chromosome by microcell fusion may show alterations in phenotype, such as loss of tumorigenicity, or morphological changes suggestive of differentiation. However, it is possible that the changes in phenotype could be due to clonal variation of the recipient cells, rather than due to the addition of the normal chromosome. The dual selectability of the B78MC panel allows the researcher to test for this, by choosing to select for removal of the human chromosome from the recipient cell line. If the resulting segregants, which lack the tagged chromosome, show reversion to the original phenotype, this provides strong evidence that the observed phenotypic changes were associated with the presence or absence of the normal human chromosome, and not due to genetic variability of the recipient cell line.

Selected hybrids from the B78MC panel have already been used successfully to show tumour suppression associated with human chromosome 4, in the human teratocarcinoma cell line, PA-1 (McGowan-Jordan et al., 1994) and chromosome 12, in the human prostate cell line, DU-145 (Bérubé et al., 1994). In these cases, two monochromosomal hybrids, B78MC108 (containing human chromosome 4) and B78MC9 (containing a centric fragment

of chromosome 12) were used. Microcells from each were fused with the human recipient cell lines, and individual clones were picked. Tumorigenicity was evaluated via subcutaneous injection into nude mice. In each case, tumour formation was shown to be delayed, or absent. Removal of the tagged chromosome from the human recipient cell lines resulted in the return of their original, tumorigenic phenotypes. Furthermore, in the case of PA-1, introduction and removal of chromosome 4 was associated with gain and loss of additional characteristics, including retinoic acid responsiveness and senescence (McGowan-Jordan and Chevrette, manuscript in preparation). The successful, selective transfer of human chromosomes from B78MC hybrids, into human tumorigenic cell lines, and their selectable removal provides ample proof of their usefulness in tumour suppressor gene research.

Since reporting the construction and analysis of the B78MC panel of hybrids, a number of independent researchers expressed interest in using this panel for their investigations, resulting in several interesting collaborations. Locally, Dr. D. Gray used B78MC185 to confirm the localization of a newly identified oncogene, *ump* to human chromosome 3 (Gupta et al., 1994). Ongoing collaborations include that of Dr. D. Littman (New York University, New York), who is using MHP (a pool of unpicked B78MC hybrids) to localize and identify the human accessory gene responsible for permissiveness to HIV infectibility in the presence of CD4. At the Montreal Neurological Institute, Dr. E. Shoubridge is similarly using MHP to localize and identify a human gene responsible for acute congenital lactic acidosis. The panel and individual hybrids are also currently being used by Dr S. Imreh (Karolinska Institute, Sweden), Dr. R.E.K. Fournier and M. Groudine

(Fred Hutchinson Cancer Research Center, Seattle), Dr. W. Cavanee (Ludwig Institute, California) and Dr. Y. Bao-Zhu (NIH, Maryland) for suppressor gene research. These collaborations reveal the value of the panel of B78MC hybrids to the international research community, and its continued use is expected to lead to advances in numerous fields of genetic research. It should be noted here that a similar panel of dual selectable hybrids has also been recently produced by a European group (Cuthbert et al., 1995).

2.6 Acknowledgments

I am grateful to Dr. J. Collins and to the Department of Obstetrics of the Ottawa Civic Hospital for the neonatal foreskins used in this work. I would also like to thank Dr. M. Thayer for the B78 cell line, Dr. S. Lupton for the marker tgCMV/HyTK, and Syntex for ganciclovir. I would like to thank Nathalie Bérubé, Céline Bisson, Kalpana Phansalker and Jean McGowan-Jordan for their individual assistance in the analysis of the B78MC hybrids.

Chapter 3. Suppression of Apoptosis in Whole Cell Hybrids

3.1 Abstract

In vitro exposure of tumorigenic cell lines to the chemotherapeutic agent PALA [*N*(phosphonoacetyl)-L-aspartate] usually results in cell death (shown here to be apoptosis), followed by clonal growth of rare survivors. On the other hand, normal diploid cells respond to PALA by arresting in G1 and G2 of the cell cycle. The tumour suppressor gene, *p53*, a G1 control gene, appears to mediate PALA-induced growth arrest in normal cells. In contrast, most tumorigenic and immortalized cell lines undergo p53-independent cell death in response to PALA. To determine if normal human chromosomes, such as chromosome 17 (the chromosomal locus for *p53*), can protect against PALA-induced apoptosis in a tumorigenic background, whole cell hybrids between normal human fibroblasts and B78, a murine melanoma cell line were generated. A whole cell hybrid (BHF12) responded to PALA with morphological features of growth arrest, while at later passage, the same hybrid showed evidence of apoptosis. In order to determine if loss of human chromosomes was associated with a reversion to PALA-induced apoptosis, subclones of BHF12 were isolated and tested for PALA-induced apoptosis. The subclones showed an increase in PALA-induced apoptosis as compared to early passage BHF12, but apoptosis was delayed as compared to the B78 parental cell type. *Alu*-PCR FISH and PCR amplification were used to identify and compare the human chromosome content of BHF12 at early passage and its subclones.

3.2 Introduction

Induction of apoptosis in tumour cells has recently been identified as a primary mechanism for the cytotoxic action of radiation and chemotherapeutic agents (Walker et al., 1991; Lennon et al., 1991; Dive and Hickman, 1991). Tumour cell sensitization to apoptosis induction appears to be related to oncogene activation, since overexpression of oncogenes such as E1A and *myc* has been shown to cause normal fibroblasts to undergo apoptosis when subjected to serum deprivation (Rao et al., 1992; Evan et al., 1992). These findings have led to the hypothesis that abnormal proliferative signalling results in activation of the cell death program (Symonds et al., 1994; Harrington et al., 1994). The apoptotic response to anti-cancer therapies seems to be a property specific to tumour cells, since normal cells, which retain strong negative growth controls, usually respond to these treatments with growth arrest (Lowe et al., 1993*a*). However, tumour cells may be insensitive to treatment, resulting in relapse and secondary malignancies. A number of mechanisms for tumour cell drug resistance and survival have been proposed and studied. For example, tumour cell dormancy, with re-entry into the proliferative state upon improvement in cell growth conditions, is a possible mechanism of drug resistance in metastatic cells (Israel, 1990). Alternatively, cancer cells may be able to bypass growth arrest and apoptosis pathways through avoidance of the toxic effects of treatment. Proposed drug survival mechanisms which minimize chemotherapeutic toxic effects, and permit continued proliferation include alterations in drug transport, and gene amplification (Wahl et al., 1979; Chabner and Myers, 1989; Ling, 1992; Scheffer et al., 1995; Roninson; 1992; Simon and Schindler, 1994). Gene amplification is also a marker for genomic instability, and is responsible for oncogene overexpression in some advanced tumours (De Vita, 1985; Nowell, 1976; Schimke, 1984; Stark

et al., 1990; Ferguson, 1991; Biedler, 1992). However, recent experimental evidence also suggests that tumour cell resistance may be due to loss of apoptosis activating genes, such as *p53*, and increased expression of survival genes, such as *bcl-2* (Williams, 1991; Lowe and Ruley, 1993; Fisher, 1994; Hickman et al., 1994). For these reasons, it is important to identify the genes which regulate growth and apoptosis and which may be targeted for mutation or inactivation during oncogenesis. This will ultimately lead to a more complete understanding of tumour cell survival following drug and radiotherapy.

Although *p53* has been shown to be involved in the promotion of drug- and radiation-induced apoptosis in oncogene-sensitized cells (Debbas and White, 1993; Lowe and Ruley, 1993), it has also been implicated in the promotion of growth arrest in normal cells, following exposure to the same anti-cancer treatments (Lowe et al., 1993*a*). This suggests a dual role for *p53* in the promotion of growth arrest and apoptosis. Loss of $p53^{+/+}$ alone is sufficient to cause the loss of the ability of normal fibroblasts to undergo growth arrest, and leads to genomic instability (Livingstone et al., 1992; Yin et al., 1992). However, other genes, in addition to *p53*, have been shown to be capable of promoting growth arrest, in addition to playing a role in the apoptosis pathway. For example, *bcl-2* has been shown to induce growth arrest, and suppress apoptosis in irradiated $p53^{-/-}$ lymphoblasts, and an *RB*-mediated, *p53*-independent G1 arrest has been shown in human leukemic cell lines, following exposure to anticancer drugs (Strasser et al., 1994; Qing et al., 1995).

Exposure of tumorigenic cells to the anti-cancer agent, PALA, has been used previously to reveal CAD (carbamyl phosphate synthase, aspartate transcarbamylase and dihydro-orotase) gene amplification in colonies of rare surviving transformed cells (Swyrd 1974; Wahl et al., 1979;

Otto et al., 1989; Tlsty et al., 1989; Tlsty et al., 1990; Wright et al., 1990). Using PALA, loss of *p53* activity was identified as a primary event leading to loss of growth arrest in normal cells, instigation of apoptosis, and resulting in the emergence of gene amplification potential. However, this occurred in conjunction with marked destabilization of the genome and/or immortalization (Livingstone et al., 1992; Yin et al., 1992), indicating that further genetic alterations could be involved in the PALA-induced responses in these cells. In another experiment, PALA was used to show that the gene amplification potential of tumorigenic cell lines was suppressed in somatic cell hybrids between human tumorigenic cell lines and normal fibroblasts (Tlsty et al., 1992), presumably through induction of a growth arrest pathway. More recently, somatic cell hybrids between rat and mouse cells have been used to identify rat chromosomes involved in trans repression of glucocorticoid-induced apoptosis (Gourdeau and Walker, 1994). A similar strategy has been used here, in order to identify human chromosomes harboring genes involved in the promotion of PALA-induced growth arrest and suppression of apoptosis. B78, a murine melanoma cell line, was fused to normal human fibroblasts (HSF-5), generating a series of mouse x human whole cell hybrids referred to as BHF hybrid clones. BHF clones were exposed to PALA, and the human chromosome content of clones which appeared to be growth arrested in the presence of PALA, was compared with that of clones which became apoptotic in the presence of the drug. Analysis of PALA-induced apoptosis in these whole cell hybrids suggested that several human chromosomes other than, or additional to chromosome 17, may be involved in the promotion of cell survival, and the suppression of apoptosis.

3.3 Materials and Methods

3.31 Cell lines

Whole cell hybrids were generated between B78, a pseudotetraploid murine melanoma cell line and HF-HT4, a population of human foreskin fibroblast cells (HSF-5) tagged with a hygromycin resistance marker, tgCMV/HyTK (Speevak et al., 1995). Culture conditions for the parental cell lines are described in Chapter 2, Materials and Methods. B78 (5×10^4 cells) was fused with HF-HT4 (5×10^5 cells) using 50% (w/w) PEG for 60 sec. One day after fusion, the cultures were split into 150cm^2 flasks and whole cell hybrids (BHF series) were selected with 400µg/ml hygromycin B and 3mM ouabain in DMEM-high glucose with 10% fetal bovine serum. Surviving colonies were picked and expanded, and hygromycin selection alone was used during all further culturing. Subclones of individual BHF hybrids were isolated by plating the hybrids at very low density (100 cells per 150cm^2 flask) and picking emerging colonies. The BHF hybrids were maintained in DMEM, supplemented with 10% fetal bovine serum and 400µg/ml hygromycin B. During PALA assays, the cells were cultured in the presence of 10% dialysed fetal bovine serum.

3.32 PALA assay

The anti-metabolite, PALA (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute), is a specific inhibitor of aspartate transcarbamylase activity of the tri-functional CAD enzyme. LD_{50} doses were determined for each cell line, hybrid clone and subclone by interpolation of dose

response curves ranging from 0 to 4 μg/ml. PALA exposure was 9 X LD_{50} in the DNA ladder assays, and 5 X LD_{50} in the flow cytometry assays. Cytotoxic delay during PALA exposure was determined by microscopic evaluation of the cells (shrinkage, rounding up, detachment). Apoptosis was evaluated by TUNEL assay and detergent soluble DNA analysis, and flow cytometry.

3.33 TUNEL analysis of PALA-induced cell death

B78 cells were plated onto tissue culture chamber slides, and incubated in the presence and absence of 9 X LD_{50} PALA for 4 days. To identify apoptotic cells, a TUNEL (TdT-mediated dUTP nick end labelling) assay was performed, using an *In situ* Cell Death Detection Kit (Boehringer Mannheim). Briefly, adherent cells on the chamber slides were fixed in 4% paraformaldehyde solution (in PBS, pH 7.4) for 30min, followed by permeabilization (0.1% Triton X-100; 0.1% sodium citrate) for 2min on ice. The slides were rinsed with PBS, and incubated with TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase (TdT) and fluorescein labelled nucleotides, for 1hr at 37°C in the dark. The slides were rinsed with PBS, counterstained with propidium iodide (Oncor) and analyzed by fluorescence microscopy.

3.34 DNA fragmentation analysis

Hybrids and parental cell lines were plated at a density of 3 x 10^6 cells per 75cm^2 flask, in the presence or absence of PALA. PALA-exposed B78 was harvested at onset of apoptosis (usually by day 3 of the experiment). Cell lines in which PALA induced growth arrest were

maintained in PALA until evidence of cytotoxicity, or to 21 days. Cells were trypsinized and combined with the medium containing cellular debris and apoptotic bodies. The suspension was centrifuged and detergent soluble DNA was isolated as described elsewhere (Walker et al., 1993). Samples were loaded on a 1% agarose gel containing ethidium bromide to detect DNA fragmentation. Residual degraded RNA (below 100 bp size) was detectable only in some controls and PALA-exposed growth arrested cell lines.

3.35 Molecular cytogenetic analysis of hybrids

Metaphase chromosomes were prepared according to standard cytogenetic techniques. FISH using total human DNA probe and an *in situ* hybridization kit (Oncor) were used to determine the total number of human chromosomal elements in the BHF hybrids. Using these techniques, BHF12 was selected for use in PALA-response assays since this hybrid was found to contain a large number of whole human chromosomes. *Alu*-PCR FISH was used as described elsewhere (Speevak et al., 1995) to identify the individual human chromosomal elements present in BHF12 and its subclones.

3.36 PCR analysis of hybrids

Under the conditions of *Alu*-PCR FISH it was possible to identify human chromosomes 1, 2, 3, 16, X, Y with certainty based on their morphology. To identify the other human chromosomes, PCR amplification using oligonucleotide primers identifying human microsatellite sequences was used to verify the presence or absence of individual human chromosomes in the hybrids (see Chapter 2, Materials and Methods).

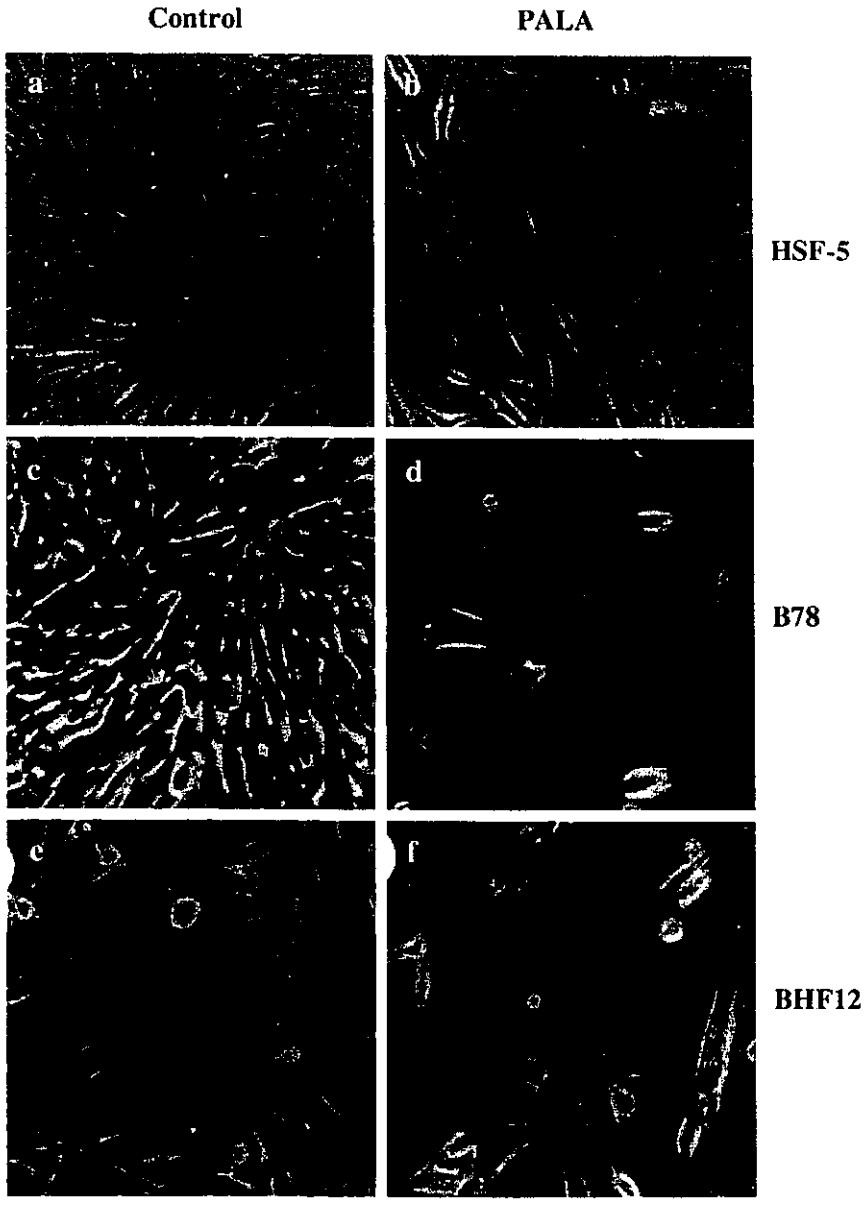
3.4 Results

3.41 Morphological changes in response to PALA

Normal fibroblasts, HSF-5 (Fig. 3.1a), responded to PALA with cytoplasmic and nuclear enlargement, suggestive of growth arrest (Fig. 3.1b), while B78 (Fig. 3.1c), responded to PALA with cell death in the majority of cells (Fig. 3.1d). To determine if the introduction of normal human chromosomes into B78 would result in suppression of the cytotoxic effect of PALA, whole cell hybrids were constructed between B78, and the HyTK tagged fibroblasts, HF-HT4. A whole cell hybrid, BHF12, was analyzed for PALA response and showed evidence of growth arrest during PALA exposure. The morphological changes, including nuclear and cytoplasmic enlargement, observed in the majority of BHF12 cells during PALA exposure, were suggestive of growth arrest (BHF12, passage 4 shown, Fig. 3.1e, control, 3.1f, PALA). Continued exposure to PALA failed to result in proliferation, and BHF12 continued to maintain a single cell, enlarged morphology for up to 21 days of PALA exposure (at earliest passage). These results suggested that the whole cell hybrids contained gene(s) from the fibroblast parental cells which controlled cell growth in response to toxic conditions. These genes were presumably inactivated (either lost or mutated) in the B78 mouse melanoma parent.

Figure 3.1 Morphology of cells in the presence or absence of the anti-metabolite, PALA.
(a, b) HSF5, normal human fibroblasts; (c, d) B78, murine melanoma; (e, f) BHF12, somatic cell hybrid between B78 and HSF5. (a, c, e), controls, no PALA. (b, d, f), 9 X LD_{50} PALA, 3 days exposure.

Figure 3.1



3.42 Analysis of PALA-induced cell death

Apoptosis was suspected to be the mechanism of PALA-induced cell death in B78. Typically, apoptosis is associated with cytoplasmic shrinkage, membrane blebbing and nuclear collapse into apoptotic bodies due to endonuclease cleavage (Durall and Wyllie, 1986; Compton, 1992). Endonucleolysis is known to result in cleavage of nuclear DNA into oligonucleosomal fragments, which can be detected by the presence of "DNA laddering" on agarose gels, and by enzymatic labelling of apoptosis-induced DNA strand breaks, using the TUNEL assay (Gavrieli et al., 1992; Portera-Cailliau, 1994). This assay labels the free 3'-OH termini of apoptosis-induced DNA strand breaks using fluoresceinated nucleotides and the enzyme, TdT, allowing the visualization of apoptotic nuclei, which stain a bright fluorescent green. Counterstaining with propidium iodide allows the visualization of the nuclei in the absence of fluorescein.

To confirm that apoptosis was responsible for PALA-induced cell death in B78 cells, a TUNEL assay was performed on PALA-exposed B78 (Fig. 3.2). Propidium iodide stained control cells showed large, interphase nuclei (Fig. 3.2a). In contrast, the PALA-exposed cells showed small, darkly staining nuclei suggestive of chromatin condensation (Fig.3.2b). Some of these nuclei were shown, by the TUNEL assay, to fluoresce brightly with FITC, indicating apoptosis-induced DNA fragmentation (Fig.3.2d). In contrast, control cells stained poorly with FITC, indicating a lack of incorporation of fluorescein-labelled nucleotides, and no DNA fragmentation (Fig.3.2c).

To verify that PALA was capable of inducing DNA laddering, and to determine if this was suppressed in BHF12, detergent soluble DNA was isolated from PALA exposed cell lines,

and electrophoresed on an agarose gel (Fig. 3.3). Each cell line was exposed to a normalized (9 X LD_{50}) dose of PALA. By day 3 of exposure, HSF-5 appeared to be growth arrested in the presence of PALA, and when PALA exposure was extended to 12 days or more, no DNA ladder was detectable from the cells and medium combined. In contrast, exposure of B78 to PALA resulted in extensive nucleosomal degradation within 3 days. Early passage BHF12 ($BHF12_E$, <passage 4) showed little cell death during PALA exposure. Detergent soluble DNA isolated from $BHF12_E$ following 12 days of PALA treatment failed to produce a DNA ladder (Fig. 3.3).

With passaging, it was noted that BHF12 became more sensitive to PALA-induced cytotoxicity. Whereas $BHF12_E$ withstood PALA exposure for up to 21 days without appreciable cell death, by passage 5, ($BHF12_M$, mid-passage) some cells showed shrinkage and detachment during PALA exposure. Whole cell human x rodent hybrids are known to rapidly, and non-randomly, reject the introduced human chromosomes. If the human chromosomes present in BHF12 were responsible for the suppression of PALA-induced apoptosis, their loss would be expected to lead to increased PALA-induced cell death. In an attempt to determine if chromosome loss was associated with an increase in PALA-induced cytotoxicity, subclones of BHF12 were isolated and their PALA responses were assayed (summarized in Table 3.1). The subclones showed a similar sensitivity to PALA-induced cell death, as compared to BHF12 at mid passage. BHF12/1 and BHF12/5 appeared to be the most resistant to PALA when first isolated ($BHF12/1_E$ and $BHF12/5_E$) and showed a delay in PALA-induced cytotoxicity of up to 12 days (Table 3.1). $BHF12/3_E$ appeared to be the most sensitive to PALA, showing the beginnings of PALA-induced cytotoxicity by day 8 of exposure. At later passage, the BHF12

subclones showed smearing and laddering following a short exposure to PALA when assayed by gel electrophoresis of detergent soluble DNA (BHF12/1_L and BHF12/3_L shown, Fig. 3.3).

Figure 3.2 TUNEL labelling of B78 in the presence and absence of PALA. (a,b), propidium iodide staining only. (c,d) FITC, (TUNEL). The arrowheads indicate apoptotic bodies associated with PALA exposure. (a, c), B78, control, after 4 days of normal growth. (b, d), B78, PALA (9 X LD_{50}), 4 days.

Figure 3.2

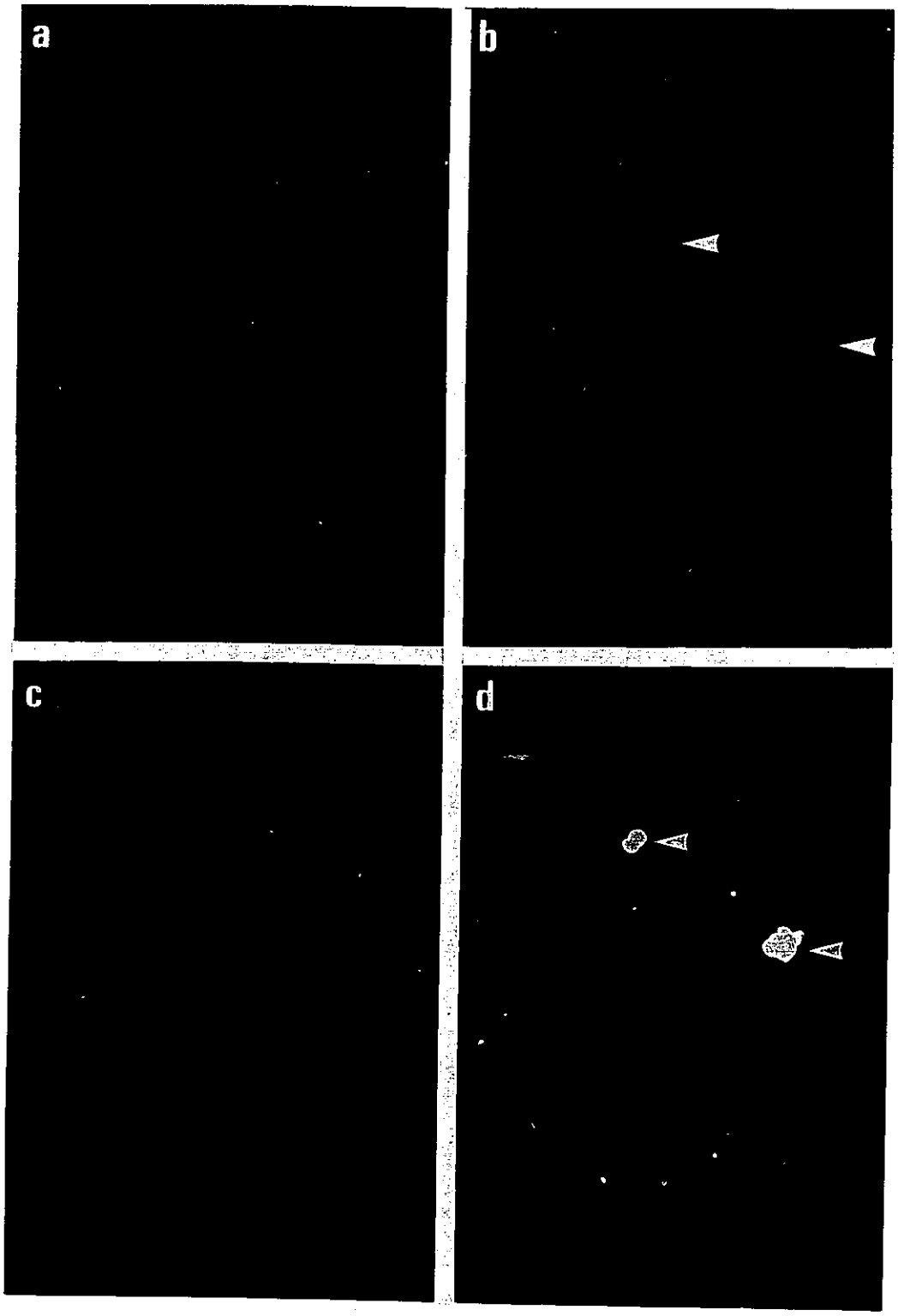


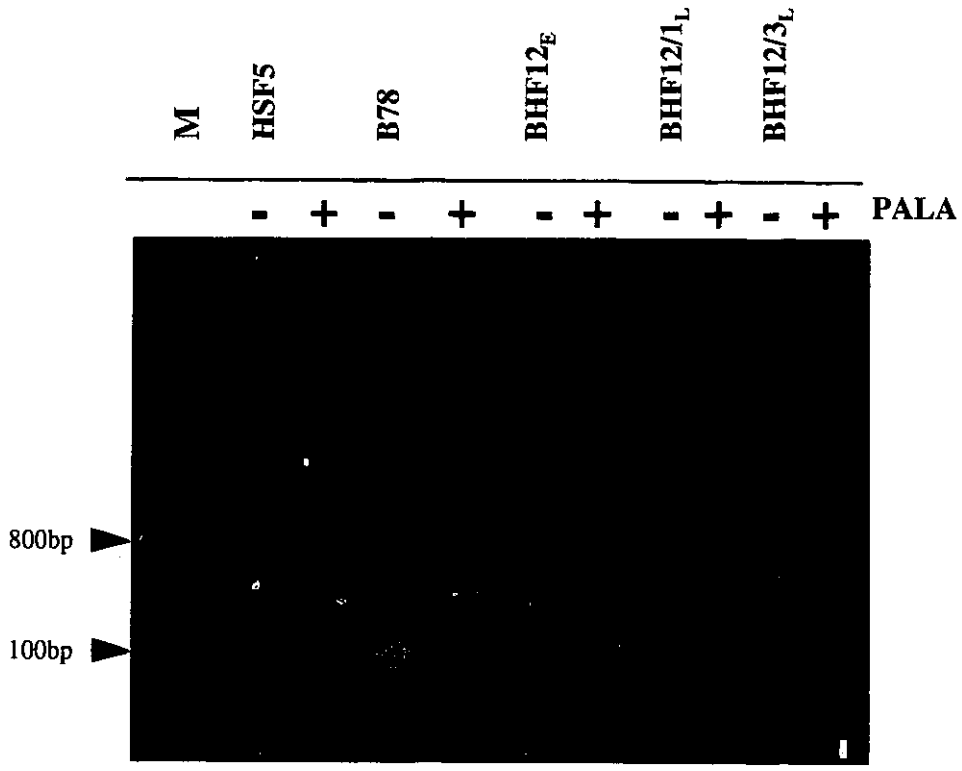
Table 3.1 Responses of BHF12 hybrids to PALA. PALA sensitivity (LD_{50}), delays before onset of cytotoxicity and DNA laddering were determined for individual cell lines, as described in Materials and Methods, this chapter.
[a] Cytotoxicity delay: number of days before microscopic evidence of cell death becomes apparent (cellular shrinkage and detachment), from early to later passage.
[b] DNA ladder was qualitatively assessed: (++++): extensive, easily defined nucleosomal ladder; (++): less intense nucleosomal ladder: (-): no detectable DNA degradation.

Table 3.1 Responses of BHF12 hybrids to PALA.

Cell Line	LD_{50} (μg/ml)	Cytotoxicity Delay[a]	DNA Ladder[b]
B78	1.4	3 days	++++
HSF-5	2.3	>21 days	-
$BHF12_E$	1.4	21 days	-
$BHF12_{M\text{-}L}$	1.4	12-6 days	++
$BHF12/1_{E\text{-}L}$	1.2	12-6 days	++
$BHF12/3_{E\text{-}L}$	1.3	8-4 days	++
$BHF12/5_{E\text{-}L}$	1.1	12-6 days	++

Figure 3.3 Nucleosomal degradation caused by PALA treatment.
Cells were grown in the absence (-) or presence (+) of PALA ($9XLD_{50}$). Note: B78 was harvested upon evidence of apoptosis, at 3 days of exposure. PALA exposure was extended to 12 days for HSF-5 and the $BHF12_E$ (early passage) which showed no sign of DNA laddering. $BHF12/1_L$ and $BHF12/3_L$ showed increased signs of apoptosis, with DNA laddering apparent after 3 days of PALA exposure. (M), 100bp ladder.

Figure 3.3



3.43 Human chromosomal content of BHF12 and its subclones

It was hypothesized that the absence of apoptosis in PALA-exposed $BHF12_E$ and the increase in PALA-induced cytotoxicity in late passage $BHF12_L$ and the subclones was due to the presence and subsequent loss of individual human chromosomes which suppress apoptosis. Theoretically, as the hybrid cells segregated the chromosome(s) responsible for suppression of apoptosis, they became more susceptible to PALA-induced apoptosis. FISH using total human DNA probe revealed that $BHF12_E$ contained multiple individual human chromosomes. In order to establish the human chromosome content of BHF12 and its subclones, *Alu*-PCR FISH and PCR amplification using human specific primers were performed. DNA was isolated from BHF12 and the subclones at early passage (passage 1-3), and PCR amplified using *Alu* specific primers. The PCR products were then biotinylated, and used as chromosome painting probes on normal human male chromosome spreads. Hybridization of the probe to specific chromosomes was representative of the human chromosome content of the hybrid. However, since only one of the chromosomes present in each hybrid would be expected to be tagged with the drug resistance marker, the human chromosome content of all the hybrids was considered to be unstable. Thus, the *Alu*-PCR FISH results were representative of the hybrids' overall human chromosome content, which might vary significantly from cell to cell and between passages.

Alu-PCR FISH revealed that chromosomes 4, 12, 13, 16 and 22 were present in BHF12 and all the subclones, at earliest passage. One or more of these chromosomes may have contributed to the delay in PALA-induced cytotoxicity observed in all the hybrids when first isolated. However, one of these chromosomes must be present in all hybrids, due to drug selection by hygromycin resistance. Chromosomes 1, 7, 9, X and Y were absent from all of the

BHF12 hybrids, and therefore were ruled out as contributing to the PALA survival phenotype. Each subclone of BHF12 lacked several different human chromosomes which were present in $BHF12_E$ (Table 3.2). $BHF12_E$ was nearly a complete whole cell hybrid, containing 18 of the 23 possible human chromosomes. Chromosomes 2, 10, 11, 19 and 20 were absent from all three subclones, but present in $BHF12_E$. The loss of these chromosomes is presumed to have contributed to the increased PALA-sensitivity in $BHF12_M$ and the subclones. However, their loss did not immediately cause a complete reversion to the parental B78 phenotype, since the subclones showed an intermediate response to PALA, with increased cytotoxic delays as compared to B78 (Table 3.1). Therefore, other chromosomes still present in the subclones may have been involved in the suppression of PALA-induced apoptosis. A comparison of the human chromosome content of the subclone $BHF12/3_E$ (Fig. 3.4 and Table 3.2), which showed the shortest delay in PALA-induced cytotoxicity (4-8 days), and $BHF12_E$, $BHF12/1_E$ and $BHF12/5_E$, all of which showed longer PALA-survival times, revealed specific human chromosomes which were absent in $BHF12/3_E$ but present in all or most of the other hybrids. Human chromosomes 5 and 8 were absent in $BHF12/3_E$ and present in the 3 remaining clones (Table 3.2). Also, human chromosome 3 was present in $BHF12/5_E$ and human chromosomes 14 and 17 were present in $BHF12/1_E$, but were absent in $BHF12/3_E$. This suggests that the presence of one or more of human chromosomes 3, 5, 8, 14 and 17 at early passage in BHF12, BHF12/1 and BHF12/5, and their absence in $BHF12/3_E$, may have contributed to improved cell survival during PALA exposure.

Figure 3.4 *Alu*-PCR FISH of BHF12 and BHF12/3
Comparison of human chromosome content by *Alu*-PCR FISH, using biotinylated *Alu*-PCR products from BHF12 (a) and one of its subclones, BHF12/3 (b) on normal human male chromosome spreads.

Figure 3.4

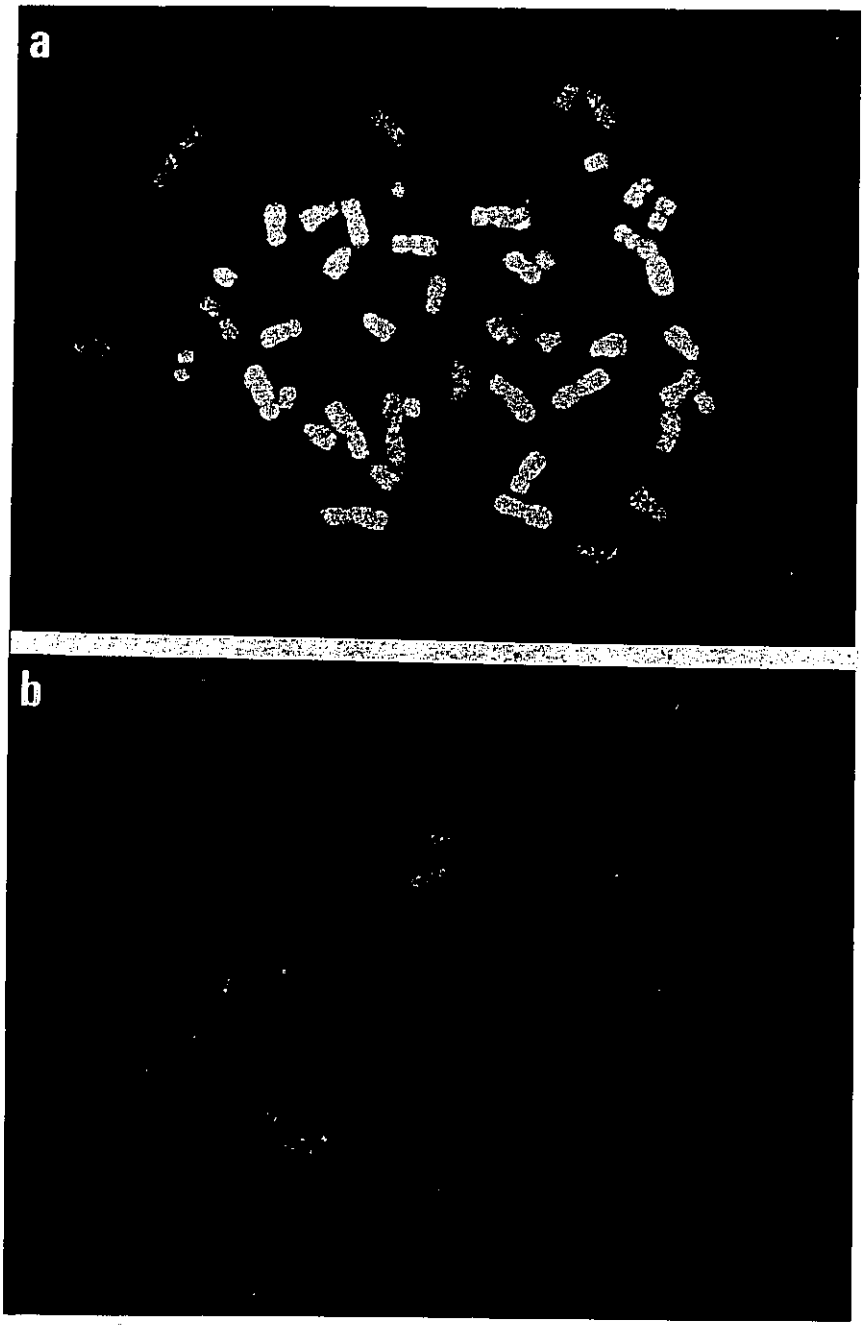


Table 3.2 BHF12 hybrid human chromosome content. Comparison of human chromosome content of BHF12 hybrids determined by *Alu*-PCR FISH and PCR amplification using human chromosome specific primers. (+) chromosome present, (-), chromosome absent. All results were obtained from early passage DNA.

Table 3.2 BHF12 hybrid human chromosome content.

Chromosomes	BHF12	BHF12/1	BHF12/5	BHF12/3
1	-	-	-	-
2	+	-	-	-
3	+	-	+	-
4	+	+	+	+
5	+	+	+	-
6	+	-	-	+
7	-	-	-	-
8	+	+	+	-
9	-	-	-	-
10	+	-	-	-
11	+	-	-	-
12	+	+	+	+
13	+	+	+	+
14	+	+	-	-
15	+	+	+	+
16	+	+	+	+
17	+	+	-	-
18	+	-	-	+
19	+	-	-	-
20	+	-	-	-
21	+	+	-	+
22	+	+	+	+
X	-	-	-	-
Y	-	-	-	-

3.5 Discussion

Somatic cell hybrids were generated between a murine melanoma cell line and normal human fibroblasts to identify human chromosomes potentially involved in PALA-induced growth arrest and suppression of apoptosis. The growth arrest phenotype conferred by the human fibroblast component of the hybrids appeared to dominate the PALA-induced apoptotic phenotype of the melanoma cell line, in $BHF12_E$ and its subclones. However, with passaging, the hybrids showed increased evidence of PALA-induced cytotoxicity and apoptosis, suggesting that loss of individual human chromosomes was responsible for the onset of cell death.

The analysis of the chromosome content of $BHF12_E$ and its subclones did not specifically pinpoint an individual human chromosome for involvement in growth arrest and apoptosis suppression. This was due to the fact that many human chromosomes were present in the hybrids, and that retention and loss of chromosomes was difficult to track, due to instability of the hybrids. As well, repetition of experiments was not feasible, due to the tendency of hybrid phenotypes to change with passaging. This is a common problem with whole cell hybrids, and limits the investigative possibilities. However, several chromosomes appeared to be associated with apoptosis, and its suppression. $BHF12/3_E$ showed the least delay in PALA-induced cell death of the subclones (4-8 days). This hybrid retained chromosomes 6 and 18, whereas $BHF12/1_E$ and $BHF12/5_E$ lacked these chromosomes. It is possible that retention of one or both of these chromosomes, in the absence of the chromosomes which were lost from $BHF12_E$ during subcloning, was detrimental and promoted cell death during PALA exposure. $BHF12/1_E$ and $BHF12/5_E$ showed longer delays in PALA-induced cytotoxicity, as compared to $BHF12/3_E$. Human chromosomes 5 and 8 were present in these two hybrids, but absent in BHF12/3. Also,

individually, chromosomes 3, 14, and 17 may have contributed to improved PALA survival in these two hybrids.

Chromosome 17 was present in $BHF12_E$ and $BHF12/1_E$, but absent in $BHF12/5_E$ and $BHF12/3_E$. Since $BHF12_E$ was very resistant to PALA-induced cell death (up to 21 days), it is possible that human *p53* contributed to growth arrest, perhaps in response to directions from genes on other introduced chromosomes, as opposed to the pro-apoptosis role it is expected to assume in transformed cell types. The fact that $BHF12/5_E$ showed a delay in PALA-induced cytotoxicity in the absence of chromosome 17 suggests that other genes, on other chromosomes have an anti-apoptotic, growth arrest function.

The molecular controls defining the conditions of choice between apoptosis and survival via growth arrest are still unknown. Tumour cells which express $p53^{+/+}$ may evoke p53-mediated apoptosis as opposed to growth arrest, as a result of conflicting growth control signals (Fisher, 1994). Apoptosis can also be induced in $p53^{-/-}$ cells (Lowe et al., 1993*b*; Clarke et al., 1993a); however, the threshold for induction is likely to be higher in the absence of $p53^{+/+}$(Kaufmann, 1989; Lennon et al., 1991; Sen and D'Incalci, 1992; Fisher, 1994). Recently, it was reported that the *WT1* gene product (located on human chromosome 11) was capable of inhibiting p53-mediated apoptosis, without affecting p53-mediated growth arrest (Maheswaran et al., 1995). Furthermore, *bcl-2* (chromosome 18) and *RB* (chromosome 13) have also been shown to suppress p53-mediated apoptosis (Chiou et al., 1994; Howes et al., 1994; Pan and Griep, 1994; Morgenbesser et al., 1994; White, 1994). Recent findings suggest that the apoptosis pathway, mediated by stress-induced protein kinases (SAPKs,) may be negatively regulated by p21, which is a downstream component of the p53 growth arrest pathway (Verheij et al., 1996; Shim et al.,

1996). These findings suggest that, under certain circumstances, these genes and perhaps others play a role in the modulation of p53 effects.

Recently, a new family of apoptosis inhibitors, unrelated to the *bcl*-2 family has been identified (Roy et al., 1995; Liston et al., 1996). These genes, known as *AIP* genes, were shown to suppress apoptosis induced by a variety of means. Chromosome 5, which was retained in $BHF12_E$, $BHF12/1_E$ and $BHF12/5_E$, is the locus for *NAIP*, one of these newly identified suppressors of apoptosis and which is transcriptionally active in human fibroblasts.

This study has shown that it is possible to suppress PALA-induced apoptosis in whole cell hybrids. This suggests that the survival response, presumably through induction of growth arrest is a dominant trait, and can overpower the apoptosis pathway. The sensitization of tumour cells to induction of apoptosis by chemotherapeutic agents, such as PALA, can be explained by the loss of activity of growth-regulating survival genes as well as by oncogene activation. This model of accumulated hits is similar to the theory of tumour suppressor gene action, as exemplified by multiple tumour suppressor gene losses observed in colon cancer (Stanbridge, 1990; Dunlop, 1992). It has recently been suggested that the ability of tumorigenic cell types to survive chemotherapeutic exposure may be due to resistance to apoptosis (Fisher, 1994). The findings described here support the view that chemotherapy success may be dependent upon the absence of genes which suppress apoptosis, and contribute to growth arrest. The cloning of growth-regulating survival genes should lead to improved cancer therapy strategies, as well as furthering the understanding of the regulation of cell growth and death.

3.6 Acknowledgments

I would like to thank the National Cancer Institute for the PALA, and Dr. Mario Chevrette, who was instrumental in the production of the BHF hybrids. Also, I would like to thank Haifa Al-Mazidi for performing the TUNEL assay.

Chapter 4: Human Chromosome 3 Mediates Growth Arrest and Suppression of Apoptosis in Microcell Hybrids

4.1 Abstract

Chemotherapeutic treatment of tumour cells leads to either tumour cell death (usually by apoptosis), or to the formation of drug resistant subpopulations. Known mechanisms of cancer cell drug resistance include gene amplification and increased expression of drug transporters. On the other hand, normal cells survive many forms of chemotherapy, with minimal damage, probably due to their capacity for growth arrest and stringent control of apoptosis. Microcell hybrids between B78 (murine melanoma) and HSF-5 (normal human fibroblasts) were analyzed to identify a new human chromosomal region involved in the promotion of drug-induced growth arrest and suppression of apoptosis. In these hybrids, the presence of human chromosome 3 was strongly associated with suppression of apoptosis via G1 and G2 growth arrest during exposure to the anti-metabolite *N*-(phosphonoacetyl)-L-aspartate (PALA) suggesting that gene(s) on chromosome 3 serve an anti-proliferative role in a drug-responsive growth arrest pathway.

4.2 Introduction

Negative regulation of the cell cycle is an important normal cellular response to environmental stress, such as depletion of growth factors or serum, increased crowding, or following administration of DNA damaging and chemotherapeutic agents (Del Sal et al., 1992., Kaufmann et al., 1991; Schneider et al., 1988; Schneider et al., 1991; Tolmach et al., 1977). Growth restriction is associated with delays in progression of the cell cycle, from G1 to S and G2 to M. These delays, or cell cycle checkpoints, reduce the probability of transmission of damaged or inaccurately replicated DNA to daughter cells (Al-Khodairy and Carr, 1992; Weinert et al.,

1988.). Upon improvement in the external environment, or when the damaged DNA is repaired, normal cells escape the transiently arrested state via positive signal transduction pathways (Almendral et al., 1988; Cochran et al., 1983; Lau et al., 1987). Tumour cells, on the other hand, often lack normal cell cycle checkpoints, and failing to respond appropriately to environmental stress, undergo apoptosis (Dive and Hickman, 1991; Eastman, 1990; Lennon et al., 1991). As a result, most tumour cells die readily following chemotherapeutic and radiation treatments. However, some cells may survive to later emerge as drug resistant tumour cell subpopulations. These resistant cells eventually become a major impediment to permanent remission following initially successful cancer therapy.

The tumour suppressor gene *p53* appears to play a central role in the activation of apoptosis in transformed cell types, while mediating growth arrest in normal cells (Kuerbitz et al., 1992; Livingstone et al., 1992; Lowe and Ruley, 1993; Lowe et al., 1993*a*, Yin et al., 1992). While $p53^{+/+}$ appears to be essential to the maintenance of genomic stability and efficient activation of growth arrest and apoptosis, additional genes can influence the cell's decision to arrest or die. For example, the retinoblastoma tumour suppressor protein, pRB, was recently linked with p53 in the control of cell growth and apoptosis, and was shown to be capable of protecting cells from p53-mediated apoptosis (Haupt et al., 1995). Furthermore, the ability of pRB to protect cells from stress-induced apoptosis was found to be independent of $p53^{+/+}$ expression (Haas-Kogan et al., 1995). This suggests that the inactivation of genes like *RB*, which have dual functions of both growth and apoptosis suppression, may not only result in decreased growth regulation, but also cause increased susceptibility to apoptosis, in a manner similar to oncogene activation (Evan et al., 1992; Haas-Kogan et al.; Shi et al., 1992).

To identify new loci implicated in the induction of drug-induced growth arrest and suppression of apoptosis, normal human chromosomes were transferred by microcell fusion, into the mouse melanoma cell line B78, and the resulting hybrids were exposed to the anti-metabolite and chemotherapeutic agent, *N*-phosphonoacetyl-L-aspartate (PALA).

As described previously (Chapter 3), B78 murine melanoma cell line was fused to normal human fibroblasts (HSF-5), generating a series of mouse/human whole cell hybrids referred to as BHF hybrid clones (Speevak and Chevrette, 1994). Analysis of PALA-induced growth arrest and apoptosis in these whole cell hybrids suggested that human chromosomes 3, 5, 8, 14 and 17 may be involved in the promotion of drug-induced growth arrest, and the suppression of apoptosis.

To further identify individual human chromosomes involved in this response, the B78MC panel of microcell hybrids was screened for the ability to undergo growth arrest during PALA exposure. Initially, the response of the microcell hybrids to treatment with PALA was measured by qualitative assessment of morphological changes indicative of growth arrest, followed by quantification of viability, growth and apoptosis. Flow cytometry was used to evaluate cell cycle responses to PALA. The analyses revealed that the presence of human chromosome 3 was specifically associated with reduced cell death and evidence of growth arrest. Removal of human chromosome 3 by dominant negative selection resulted in increased apoptosis, accompanied by a loss of growth arrest in the presence of PALA. It was concluded that a gene (or genes) on human chromosome 3 mediates drug-induced growth arrest and suppression of apoptosis in normal cells. The loss or inactivation of this gene may result in continued cell cycling during non-proliferative conditions, and sensitization of the cells to induction of apoptosis.

Alternatively, retention of this gene in cancerous cells may be implicated in tumour cell dormancy, and an increased apoptosis threshold.

4.3 Materials and Methods

4.31 Microcell hybrids

The generation of the B78MC hybrids has been described elsewhere (Chapter 2; Speevak et al., 1995). These hybrids contain different human chromosomes tagged with a dual selectable marker, tgCMV/HyTK (Lupton et al., 1991). Retention of the tagged chromosome was achieved through culture in medium supplemented with 400µg/ml hygromycin. The tagged chromosome was removed by culturing the cells in 100µM ganciclovir (provided by Syntex) until drug resistant colonies emerged. Subclones of B78MC166, which had lost the human chromosome 3 (B78MC166-R series), were thus obtained and were confirmed to lack human chromosome content by fluorescence in situ hybridization (FISH) using total human DNA probe, and by PCR amplification (see below). B78MC hybrids were grown in Dulbecco's Modified Eagle Medium-high glucose and the primary human fibroblast cells, HSF-5, were grown in Minimal Essential Medium. All cells were cultured in 10% fetal bovine serum, at 37°C and 5% CO_2. During PALA experiments, dialysed fetal bovine serum was used. All cell lines were free of mycoplasma, as determined by Hoechst 33258 staining (Chen, 1977).

4.32 PALA screen

The anti-metabolite PALA (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) is a specific inhibitor of aspartate transcarbamylase activity of the tri-functional CAD enzyme.

Eighteen pools of B78MC microcell hybrids (5 x 10^4 cells), each containing 10 - 14 individual clones, were initially exposed to 20μg/ml of PALA. Cell survival was qualitatively assessed by microscopic examination. Hybrid pools, showing evidence of growth arrest (reduced cytotoxicity, with enlarged, flattened single cell morphology), were selected for further screening. The individual hybrids represented in those pools were treated with PALA, and only hybrids showing better than 50% survival, accompanied by a growth arrest morphology, were selected for further study. B78MC hybrids selected in this manner were analyzed further, including identification of human chromosomal content and PALA-induced responses. Note: hybrids showing colonization during PALA exposure were not selected for further characterization, since these cells were at risk for containing amplified CAD genes.

4.33 PALA sensitivity determinations and gene amplification frequencies

LD_{50}'s and gene amplification frequencies were determined according to the established protocol (Otto et al., 1989). Briefly, the LD_{50} value (dose allowing 50% clonogenic survival) of each cell line was determined via interpolation of percent cell survival over a dose response range between 0 and 4μg/ml (0 and 11.5mM). For determination of CAD gene amplification frequencies, 10^6 cells were plated in triplicate in 100mm dishes and incubated in medium supplemented with PALA at a concentration of 9 x LD_{50}. Following 12 - 16 days of incubation,

with regular replacement of selection medium, the plates were rinsed, stained, and the number of colonies per dish were counted. In addition, for each experiment, cloning efficiencies were determined by plating 200 cells in duplicate 60mm dishes in non-selective medium. At 6 days, the dishes were stained, and colonies were counted. Cloning efficiency was calculated as the number of colonies counted per dish / 200. The gene amplification frequency was calculated as [the number of PALA resistant colonies counted per dish / the number of cells plated] x the plating efficiency.

4.34 Apoptosis and cell cycle analysis

2.5×10^5 cells were seeded in $25cm^2$ flasks and incubated with or without PALA (13 µg/ml) for up to 6 days. Cells were harvested at 24 hr or 48 hr intervals. The cells and medium were combined, an aliquot (0.5 ml) was taken for trypan blue viability testing and cell counting (by haemacytometer), and the remainder was centrifuged. The pellet was resuspended in 1ml PBS-EDTA, fixed with 3ml 95% ethanol, and stored at -20°C until analysis. For flow cytometric analysis, the fixed cells were centrifuged and resuspended in 0.25ml PBS-EDTA. The cell suspensions (0.25ml) were stained with 1ml Coulter DNA stain for 45 min at room temperature, and analyzed on an EPICS XL (Coulter) flow cytometer. The raw data were gated to remove doublets from the analyses. The cell cycles were determined using histograms gated to counts within the cell cycle range, and analyzed using Multicycle AV DNA Content cell cycle analysis software (Phoenix Flow Systems). The percent counts representative of apoptotic cells in the PALA treated samples were determined by quantifying the low fluorescence peak appearing in log scale histograms (Nicoletti et al., 1991).

Viable cell counts were determined from trypan blue negative counts, and total viable counts were determined by calculation of viable counts/ml x volume. The relative plating efficiencies (R.P.E.) were calculated as the viable growth in the presence of PALA /the viable growth in the absence of PALA, at a single time point after culture setup.

Fluorescence microscopy was used to examine nuclear changes in PALA-exposed cells following 2 days of growth. Chamber slide cultures of B78, grown in the presence or absence of PALA for 2 days were fixed with 70% ethanol for 30 minutes at -20°C. The slides were rinsed with water and stained with Hoechst 33258 (final concentration, 500 ng/ml) for 10 min . The slides were then rinsed with PBS and mounted. The samples were examined with a Zeiss Axiophot fluorescence microscope, and photographed with Ektachrome 400 colour slide film (exposure 1- 4 sec.).

DNA fragmentation analysis was performed as follows: each cell line was plated in two 150 cm^2 flasks. One flask was treated with 9 x LD_{50} PALA for 6 days, and the other flask served as a control, which was harvested at confluency. Cells were harvested, lysed, and detergent soluble DNA was extracted as described previously (Chapter 3). Samples were fractionated on a 1% agarose gel stained with ethidium bromide in order to detect nucleosomal degradation.

4.35 Fluorescence in situ hybridization analysis of hybrids

Human chromosomal content of the hybrids selected in the initial PALA screen was determined by FISH using total human DNA probe (Oncor) and by *Alu*-PCR FISH, as described previously (Chapter 2).

4.36 PCR analysis of human chromosome content

PCR amplification using oligonucleotide primers identifying human microsatellite sequences (sold as MapPairs by Research Genetics), was used to confirm the chromosome content, and to establish the presence or absence of individual chromosome 3 loci in the hybrids. To confirm the identity of the human chromosomes present in the hybrids, the following primers were used: chromosome 3, D3S1210 (3p) and D3S1215 (3q); chromosome 4, GABRB1; chromosome 7, D7S523; chromosome 9, D9S146; chromosome 11, D11S929; chromosome 12, D12S43; chromosome 13, D13S131; chromosome 17, D17S957; chromosome 22, D22S273. The PCR conditions used have been described in detail, Chapter 2.

4.37 Analysis of CAD gene copy number

Southern blots were prepared according to standard procedures. DNA was isolated from cells exposed to 13 μg/ml PALA for 0, 2 and 4 days (plus 2 day recovery), and digested with EcoR1 restriction enzyme (Boehringer Mannheim) using the manufacturer's recommended conditions. The digested DNA was ethanol precipitated, dissolved in TE buffer, and quantified by fluorometry (TKO 100 DNA Fluorometer, Hoefer Scientific Instruments). Equal amounts (6.5 μg) of DNA were loaded into each lane of a 0.8% agarose gel and fractionated by electrophoresis. The DNA was transferred to a nylon membrane by alkaline transfer (0.4N NaOH). The hamster CAD cDNA probe is a 6kb fragment derived from HindIII digested pCAD142 (Shigesada et al., 1985). The rat albumin cDNA probe is a 1.5 kb fragment derived from EcoR1 digested pHDQ835 (Turcotte et al., 1985). The probes were labelled via the random primer method, hybridized to the membrane overnight at 65°C (10% PEG/1.5XSSPE [225mM NaCl; 13mM

NaH_2PO_4-H_2O; 1.5mM Na_2EDTA-$2H_2O$]/7%SDS) and washed identically (2XSSC/0.1%SDS twice for 15 min at room temperature, and 0.2XSSC/0.1%SDS twice for 15 min at 42°C). Values for intensity of hybridization to a 2.3 kb murine CAD gene restriction fragment and a 5 kb albumin gene restriction fragment were determined by phosphorimaging (Molecular Dynamics ImageQuantNT, Molecular Dynamics).

4.38 Immunoblot analysis of cell lines

Total cellular protein extracts were prepared by lysing untreated cells with protein lysis buffer containing protease inhibitors (10mM Tris-HC [pH 7.4]; 150mM NaCl; 1%Triton X; 1% deoxycholate; 0.1% SDS; 0.5mM EDTA; 1mM PMSF; 2µg/ml aprotinin; 1 µg/ml leupeptin; 1µg/ml pepstatin) and quantified by Bio-Rad Protein Assay (Bio-Rad). After boiling, 50µg of cellular proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Following blocking with 0.5% milk-TBST (10mM Tris Cl [pH 8.0]; 150mM NaCl; 0.05% Tween 20), the blot was washed twice with TBST for 15 min and hybridized 1 h at room temperature with primary p53 antibody Ab-1, diluted to 10µg/ml in TBST. The blot was then washed, and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, diluted to 0.2µg/ml in TBST. The blot was washed again, and autoradiographed using chemiluminescence according to the manufacturer's instructions (Amersham Canada).

4.39 Irradiation of cell lines

Twenty-four hours after plating 250,000 cells/25cm^2 flask, cells were exposed to 0 or 5 grays of ^{137}Cs-gamma radiation (Gammacell-40 Self Contained Irradiator, Nordion International) at a rate of 1.08 grays/min. The cells were then incubated at 37°C for 24 and 96 hours. Flow cytometry was performed for cell cycle analysis as described above.

4.4 Results

4.41 PALA screening of the B78MC microcell hybrid series

Fusion between human tumorigenic cell lines and normal human fibroblasts results in the dominance of growth arrest and suppression of gene amplification in the presence of the anti-metabolite PALA (Tlsty et al., 1992). This finding suggests that recessive genes are responsible for the ability of cells to control the cell cycle, and that these genes are either deleted or mutated during the oncogenic process. The tumour suppressor gene *p53* has been shown to be involved in PALA-induced growth arrest in normal cells, and its loss is sufficient for growth arrest failure during PALA exposure (Livingstone et al., 1992; Yin et al., 1992). However, other genes may exist which, when mutated or inactivated, have a similar effect on the ability of the cell to respond to chemotoxic drugs with growth arrest.

Preliminary experiments with whole cell hybrids between murine melanoma cell line B78 and normal human skin fibroblasts, HSF-5, suggested that several chromosomes, in addition to human chromosome 17 (the locus for *p53*), were involved in PALA-induced growth arrest (Speevak and Chevrette, 1994). To further characterize the PALA-induced growth arrest

response conferred by individual human chromosomes, a panel of microcell hybrids constructed from B78 and HSF-5 (B78MC hybrids) was used. The panel was qualitatively screened for evidence of growth arrest and suppression of PALA-induced apoptosis.

To efficiently screen over two hundred newly generated B78MC hybrids, the hybrids were combined into eighteen pools. PALA (20μg/ml) was cytotoxic to B78 and to all of the B78MC pools tested, with the majority of cells dying by day 4 of drug exposure. However, in five of eighteen pools tested, nuclear and cytoplasmic enlargement, and a flattened morphology were observed in a minority of cells, suggesting the presence of a subpopulation capable of a PALA-induced growth arrest response. Individual hybrids from the five selected pools were subjected to PALA exposure and screened. Seven B78MC hybrids showed evidence of growth arrest and a reduction in the degree of PALA cytotoxicity (estimated at less than 50% cell death by day 6 of PALA exposure). The human chromosome content of these hybrids was then determined.

4.42 Alu-PCR FISH analysis of microcell hybrids

The human DNA present in the growth-arrested hybrids was PCR amplified using *Alu* primers, and the products were used as FISH probe on normal human male chromosome spreads (Dorin et al., 1992). Chromosomes painted by this method (*Alu*-PCR FISH) represent the highly retained human elements present in the hybrids. The *Alu*-PCR FISH positive findings were confirmed by PCR analysis using human chromosome specific primers. Overall, human chromosomes 3, 4, 9, 11, 12, and 13 were identified as highly retained in the seven hybrids; however, chromosome 3 and chromosome 12 were over-represented. Four of the seven hybrids

contained elements of chromosome 3, while three of the seven contained elements of chromosome 12. In order to rule out a bias in representation of chromosomes 3 and 12 in the B78MC panel, *Alu*-PCR FISH analysis of randomly selected B78MC hybrids was used to establish an estimate of the overall proportion of B78MC hybrids stably containing a chromosome 3 or 12. It was estimated that 30 - 50% of randomly selected B78MC hybrids contained human chromosome 12, whereas only 10 - 15% stably retained human chromosome 3. Thus, the increased representation amongst the PALA-selected hybrids of chromosome 12, but not chromosome 3, could be explained by a bias due to over-representation in the unselected B78MC hybrid panel. We therefore hypothesized that chromosome 3 had played a role in the PALA-induced growth arrest phenotype observed in four of the seven hybrids.

To further characterize the effect of human chromosome 3 on PALA-induced apoptosis, several hybrids with known human chromosome content were tested for their ability to undergo growth arrest and survive during PALA exposure. To represent all the chromosomes identified in the preliminary screen (3, 4, 9, 11, 12 and 13), the following hybrids were tested: B78MC166, B78MC56, B78MC108, B78MC173, B78MC16, and B78MC9. B78MC27, which contains a whole human chromosome 17, and chromosomes 7 and 22, was also evaluated.

4.43 Cell cycle analysis and apoptosis

The relative sensitivities of B78 and the hybrids to short term PALA exposure were compared using 13 µg/ml (standardized 9 x LD_{50} PALA dose for B78), delivered over an exposure period of 4 days. PALA has been used in the past to measure relative frequencies of gene amplification in a variety of cell lines (Livingstone et al., 1992; Otto et al., 1989; Schaefer

et al., 1993; Tlsty, 1990; Tlsty et al., 1992; Yin et al., 1992). CAD gene amplification allows clonogenic survival of immortalized and tumorigenic cells during long term PALA exposure and it is well established that this is a rare event (Otto et al., 1989; Tlsty et al., 1989). A 9 x LD_{50} PALA dose calibrated to each cell line was used in these studies, to compensate for differences in PALA sensitivities between cell lines. In contrast, the present study was designed to evaluate and compare the overall PALA-induced responses of growth arrest and apoptosis in the cell lines, following short term exposure, irrespective of CAD gene amplification potentials. For this reason, a calibrated 9 x LD_{50} PALA protocol was not required.

Preliminary analysis of B78 and the selected microcell hybrids indicated that there were no significant differences in their growth characteristics, or ploidy, which might potentially influence their responses to PALA. The ability of the cell lines to grow during short-term, continuous PALA exposure was evaluated by measuring the relative plating efficiencies (R.P.E.) of each of the cell lines at a single dose and time point. All of the cell lines tested showed low R.P.E., indicating growth failure in the presence of PALA (Table 4.1). PALA-induced growth failure can be due to either growth arrest, as seen in normal fibroblasts, or due to induction of apoptosis, as seen in B78 (Speevak and Chevrette, 1994). In order to differentiate these two processes in each of the hybrid cell lines, PALA-induced apoptosis was quantified by trypan blue staining and flow cytometric log scale histograms.

B78 showed a distinct intermediate peak on log scale histograms by day 4 of PALA exposure, which, when quantified, closely matched trypan blue positive counts taken from the same cell samples (Fig. 4.1 and Table 4.1). This peak corresponds to the population of apoptotic cells (Nicoletti et al., 1991). This peak was absent in HSF-5, which instead showed a depression

in the S-phase (between the G1 and G2 peaks), which was suggestive of growth arrest. However, trypan blue positive staining of PALA-exposed HSF-5 suggested a reduction in viability (Fig. 4.1 and Table 4.1). A limited degree of necrosis may therefore have occurred in the normal fibroblast population during PALA exposure.

As expected from the preliminary screening process, several hybrids showed a reduced level of apoptosis in comparison to B78 (Table 4.1). The most significant reduction in apoptosis was measured in two hybrids containing human chromosome 3. B78MC166, a monochromosomal hybrid containing a rearranged chromosome 3, and B78MC56, containing the short arm of chromosome 3 and the long arm of chromosome 17, showed significantly lower amounts of PALA-induced apoptosis as compared to B78 and the other hybrids tested (Table 4.1). These two hybrids showed a general increase in debris during PALA exposure, as determined by flow cytometry (Fig. 4.1), with a poorly distinguishable apoptotic peak. They also showed significantly increased viability in the presence of PALA, as compared to B78 and the other hybrids tested, while maintaining poor R.P.E. (Table 4.1). To determine if cell cycle checkpoints were responsible for loss of growth potential in B78MC166 and B78MC56, we compared the cell cycle responses of the cell lines in the presence of PALA, using single parameter flow cytometry to determine the relative numbers of cells in G1, S and G2/M.

A flow cytometric time course of the effect of PALA on the cell cycle is shown in Figure 4.2. During the first two days of PALA exposure, flow cytometric histograms of all the cell lines tested revealed accumulation of cells in S phase, a typical cellular response to anti-pyrimidine drugs, such as PALA (Fig. 4.2). The S phase delay was transient, and released spontaneously by

day 3 of exposure. Accumulation of dying cells began to occur at this time, resulting in a severe reduction in cycling cell counts by day 4.

Although cell death was not detectable, significant nuclear changes were already evident in some PALA exposed cells by day 2 of treatment (Fig. 4.3). At this time, some cells showed nuclear malformation and shrinkage, with chromatin condensation, suggestive of apoptosis. Many of the remaining cells showed nuclear enlargement, most likely due to partially completed DNA synthesis and G2 arrest. In spite of S-phase delay, the PALA-exposed cells proliferated slowly, and doubled in number at least once during the first two days of treatment, after which viable cell numbers either stabilized or declined (Fig. 4.4). Thus, the majority of cells passed through at least one cell cycle prior to undergoing either growth arrest or apoptosis in the presence of PALA.

Evaluation of flow cytometry data from day 4 of PALA exposure showed that B78MC166 and B78MC56 had an increase in G1 and G2 counts, accompanied by a decrease in S counts, as compared to B78 and the other hybrids. This response was similar to that of HSF-5, and resulted in G1:S and G2:S ratios which were significantly higher than B78 and the other hybrids (Table 4.1). To further analyse the effect of the presence of chromosome 3 on PALA-induced growth arrest, the G1:S ratios of B78MC166 and B78 were measured over a six day PALA exposure period. Both B78 and B78MC166 showed increasing G1:S ratios over most of the PALA exposure period. However, B78MC166 showed a more rapid increase, which, by day 4 of PALA exposure, was 3 fold higher than that of B78. The high G1:S ratio was maintained over the remaining exposure period (Fig.4.5). Furthermore, when subjected to increasing doses of PALA, B78MC166 underwent a dose responsive increase in the G1:S ratio, indicating a drug

responsive induction of G1 arrest. In contrast, B78 showed a depressed G1:S ratio over the same dosage range (Fig. 4.6). These findings indicate that human chromosome 3 contains a gene(s) which may play a role in growth arrest and apoptosis suppression.

Alu-PCR FISH and standard FISH using total human DNA probe revealed that B78MC166 contained a single rearranged human chromosome 3. B78MC56 contained the short arm of chromosome 3, and the long arm of chromosome 17. The PALA-induced growth arrest phenotype observed in B78MC56 was probably due to the presence of the short arm of human chromosome 3, since B78MC27, a hybrid containing chromosome 17, responded to PALA with growth arrest failure and a high percentage of apoptotic cells by day 4 of exposure (Table 4.1).

Figure 4.1 Comparison of log scale histograms of propidium iodide stained cells. Cells were exposed to 13μg/ml PALA for 4 days. The low fluorescence peak (arrow) observed in PALA-exposed cells represented a population of apoptotic cells. Controls were untreated cells in exponential phase.

Figure 4.1

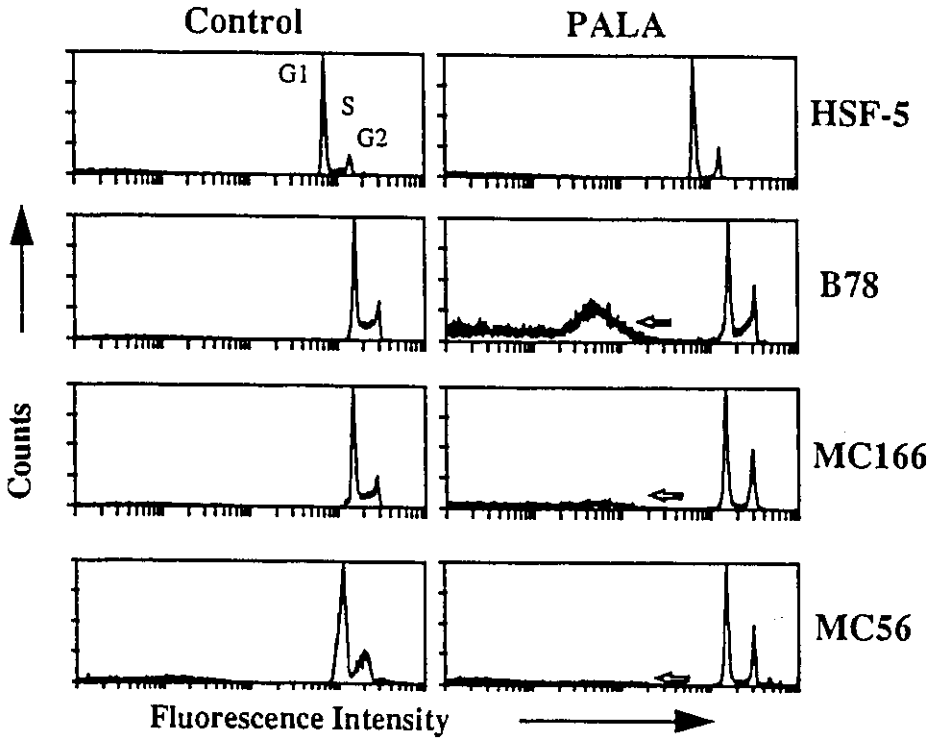

Control
PALA
G1
S
G2
HSF-5
B78
MC166
MC56
Counts
Fluorescence Intensity

Table 4.1 Cell line responses to short term PALA treatment. Cells were exposed to 13μg/ml PALA for 4 days and are means of 3 independent experiments.
[a] relative plating efficiency following 4 days of PALA exposure. Viable cells grown in the presence of 13μg/ml PALA/ viable cells in the absence of PALA.
[b] G1:S and G2:S ratios of cell lines determined from cell cycle analysis of flow cytometric histograms.
[c] Percent viability determined by trypan blue exclusion.
[d] Percent apoptosis determined by quantifying apoptotic peak from log scale flow cytometric histograms (counts in apoptotic range / counts in apoptotic range + counts in cell cycle range).
[e] μdel(3), abnormal human chromosome 3 containing several microdeletions.
* data significantly different from B78 ($P<0.01$) and all other hybrids tested ($P<0.05$). Statistical analysis method: One-way ANOVA, with Student-Newman-Keuls multiple comparisons post-test.
Note: B78MC hybrids are abbreviated to MC hybrids in this table.

Table 4.1 Cell line responses to short term PALA treatment.

Cell Line	Chromosome Content (human)	R.P.E.[a]	G1:S[b]	G2:S[b]	Viability (%)[c]	Apoptosis (%)[d]
HSF-5	46,XY	0.051	8.88	1.09	74.4	6.7
B78	none	0.016	1.63	0.52	48.9	61.6
MC166	3	0.033	5.02*	3.3*	75.7*	28.7*
MC166-R1	none	0.020	1.66	0.47	64.1	45.5
MC166-R2	none	0.017	2.38	2.0	42.2	53.9
MC166-R5	none	0.034	1.81	0.81	58.6	36.7
MC166-61	μdel(3)[e]	0.017	2.41	1.07	43.1	59.2
MC56	3p,17q	0.060	4.90*	1.68*	75.5*	24.2*
MC27	7q,22q,17	0.022	2.02	0.97	54.7	51.2
MC9	12	0.008	1.53	1.73	52.2	47.7
MC108	4	0.018	1.52	0.56	55.5	45.7
MC173	9,11	0.043	1.09	0.54	66.5	40.0
MC16	12,13	0.016	1.02	1.11	56.7	50.3

Figure 4.2 Linear scale flow cytometric histograms of cells continuously exposed to 13 μg/ml PALA over a 4 day period. Controls were exponential phase untreated cells. The relative positions of the cell cycle components are constant for all cell lines, and are indicated in the B78 Control panel.

Figure 4.2

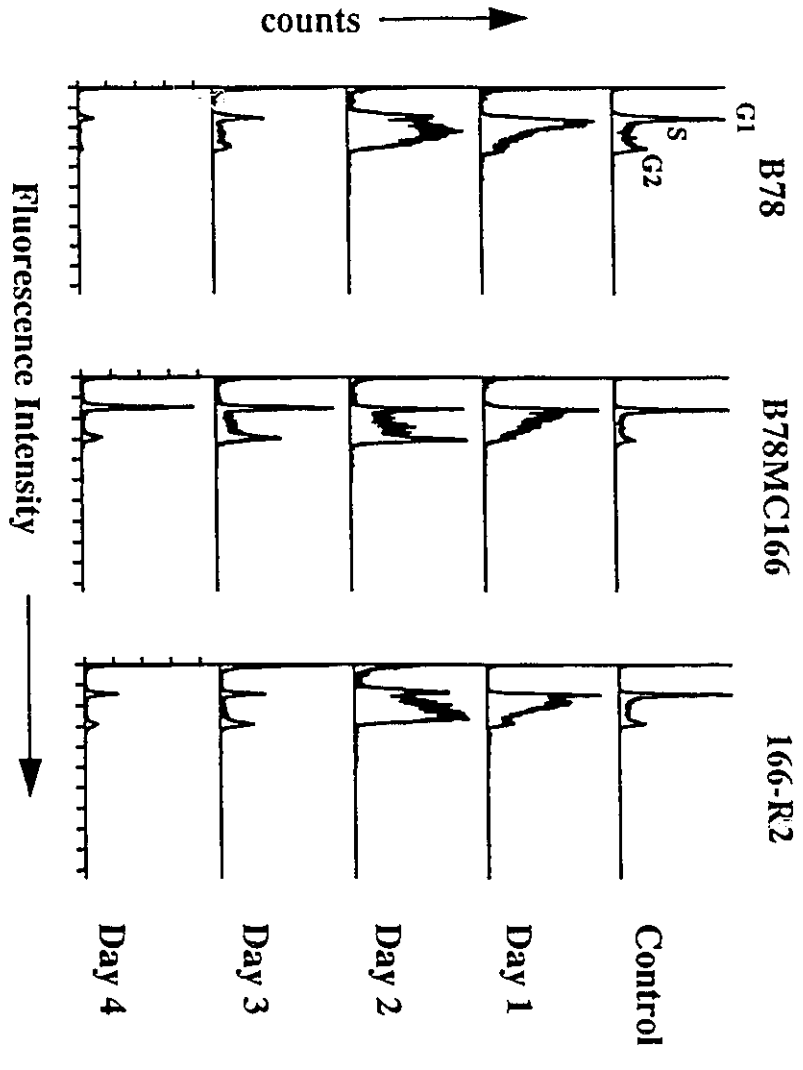

counts
B78
B78MC166
166-R2
G1
S
G2
Control
Day 1
Day 2
Day 3
Day 4
Fluorescence Intensity

Figure 4.3 *In situ* Hoechst 33258 stained cells.
(a) Untreated B78. (b) PALA-exposed B78, 13μg/ml, following 2 days of treatment. Arrowheads indicate nuclei with apoptotic changes. Malformed, enlarged nuclei are probably cells in S or G2 delay. m, mitotic figure.

Figure 4.3

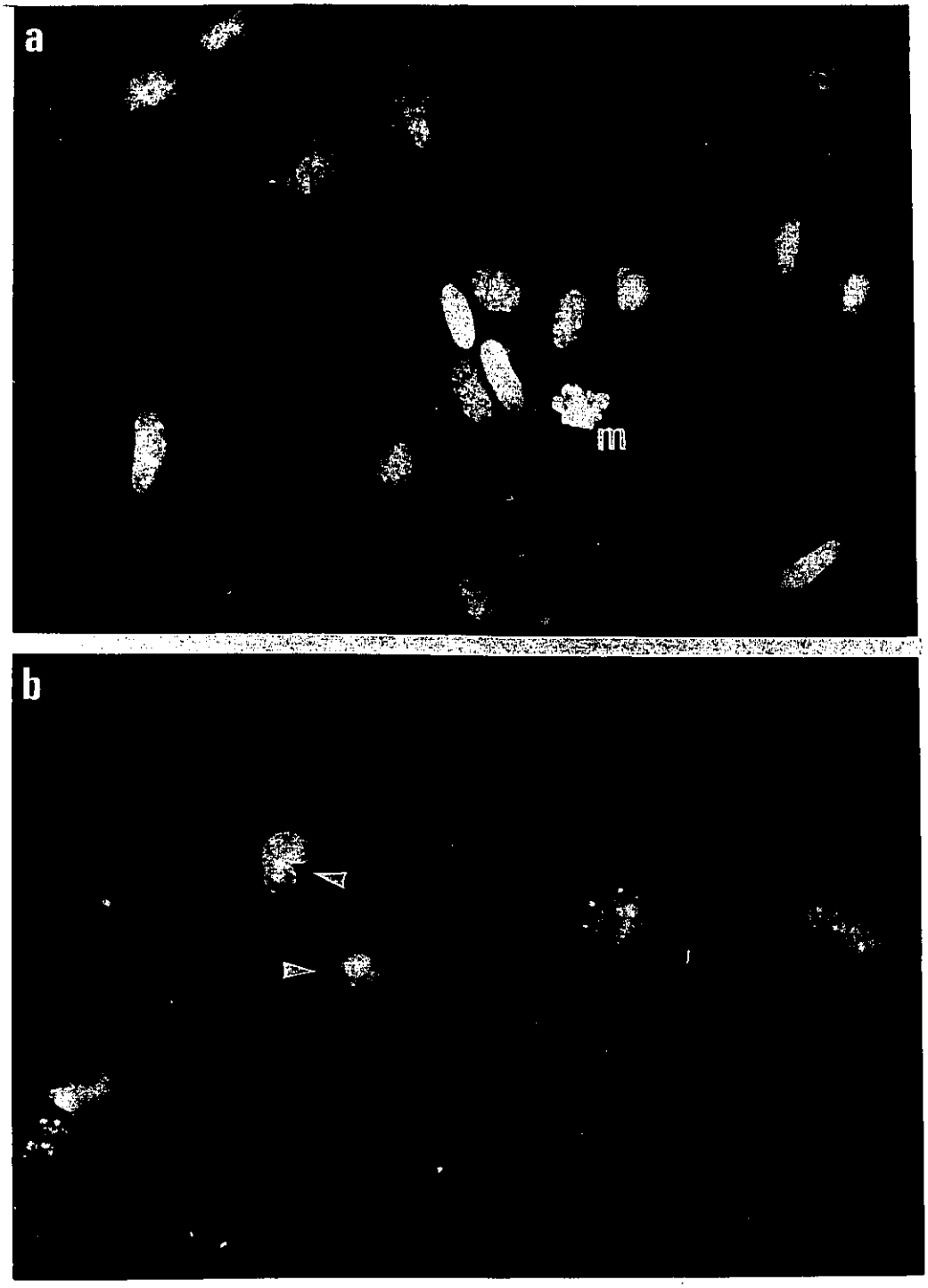


Figure 4.4 Viable growth during PALA exposure. Control was mean combined growth in the absence of treatment. B78,■ ; B78MC166,○; 166-R2,●; Control,▽

Figure 4.4

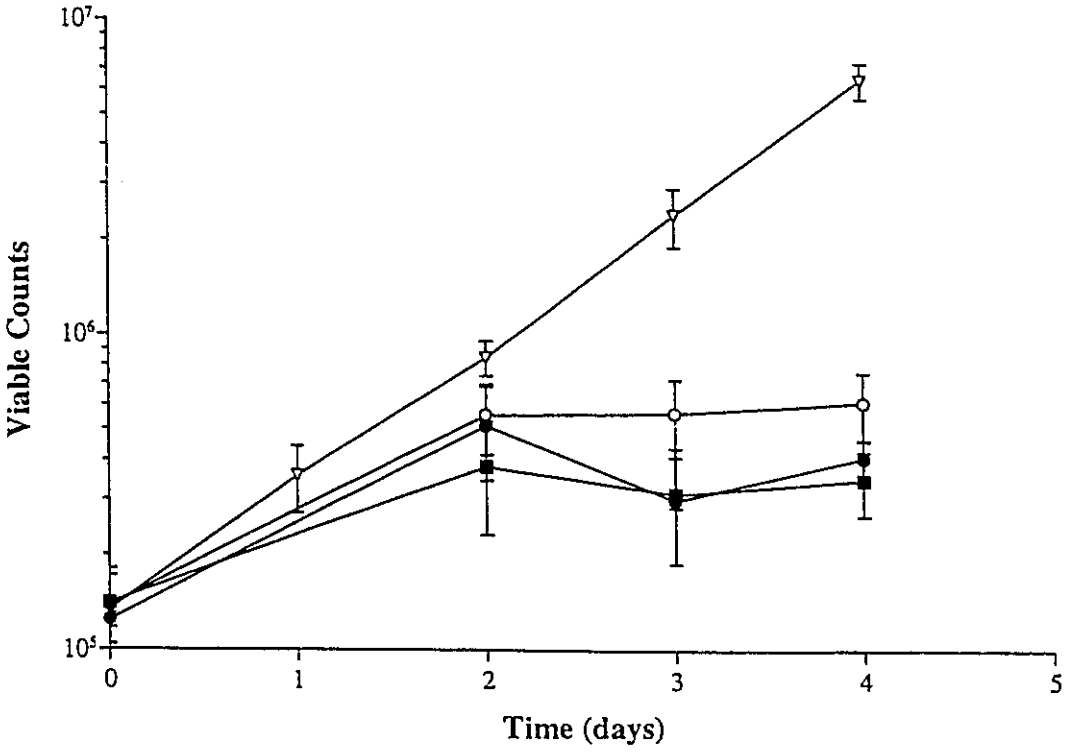


Figure 4.5 G1:S responses of cell lines during continuous PALA exposure (13μg/ml). The graph begins at day 2 of PALA exposure, following release from S phase delay. B78,■; B78MC166,○ 166-R2,●

Figure 4.5

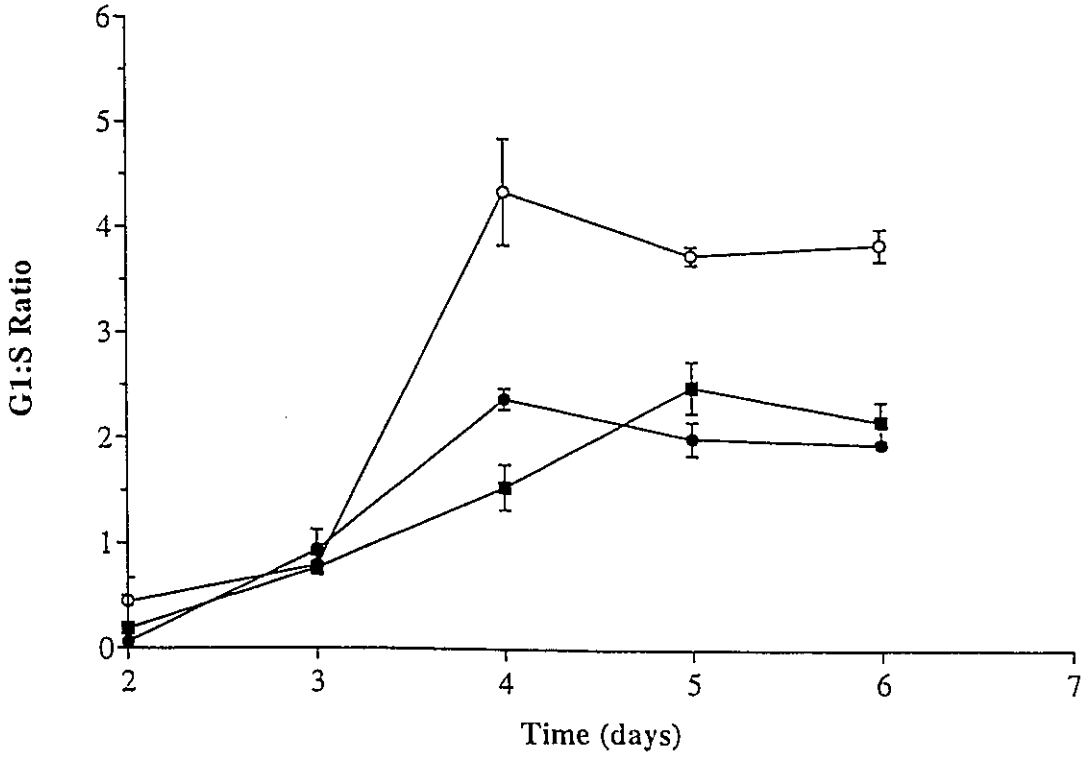
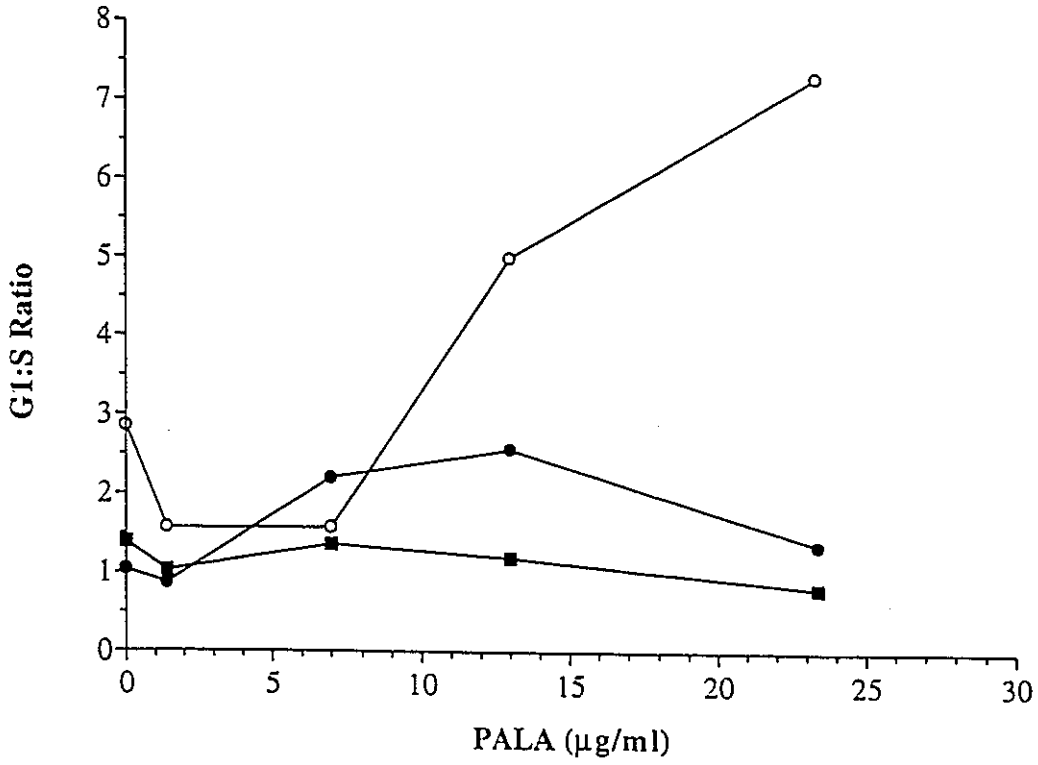


Figure 4.6 G1:S ratios at day 4 of PALA exposure, over a wide dosage range. The G1:S ratios of unexposed cells were obtained at confluency (day 4). B78,■; B78MC166,○; 166-R2,● .

Figure 4.6



4.44 Analysis of B78MC166 subclones

The B78MC panel of microcell hybrids is unique among human x rodent hybrids in that each hybrid in the panel contains an individually tagged human chromosome which can be retained, or removed via dominant positive or negative selection, as desired. This property can be used to test the hypothesis that a particular phenotype is associated with the presence of an introduced, tagged chromosome. If the phenotype in question is due to the presence of the tagged chromosome, as opposed to genetic heterogeneity between clones, one would expect removal of the tagged chromosome to result in loss of the phenotype. In this case, if a gene on chromosome 3 was responsible for the PALA-induced growth arrest phenotype and associated suppression of apoptosis, the subsequent loss of the gene, through removal of the tagged chromosome would then be expected to result in a return to the PALA-induced apoptotic phenotype and growth arrest failure. Using ganciclovir, we selected against the retention of human chromosome 3 in B78MC166, and isolated several ganciclovir-resistant subclones (166-R series). PCR amplification and FISH using total human DNA probe showed that these subclones lacked human chromosome 3 (not shown). We tested the PALA response of several ganciclovir selected subclones and determined that they showed reduced viability as compared to B78MC166, and an increase in apoptosis accompanied by low G1:S ratios during continuous PALA exposure (Table 4.1). We further evaluated the ability of one of the subclones, 166-R2, to respond to PALA with growth arrest. When subjected to PALA treatment, 166-R2 showed a B78-like response, with G1:S ratios failing to increase over time, and over a wide dosage range (Fig. 4.5 and Fig.4.6). These findings demonstrate that the presence of human chromosome 3, in

B78MC166, was indeed responsible for inducing growth arrest in response to PALA, and suppressing apoptosis.

At higher passages (> passage 8), B78MC166 became more sensitive to PALA-induced apoptosis as compared to early passage. We hypothesized that the reduction in apoptosis suppression in response to PALA in this hybrid at late passage was due to a lack of linkage between the chromosomal element responsible for the growth arrest phenotype and the selectable marker. This was supported by the fact that the chromosome 3 in B78MC166 was rearranged. As chromosome breakage is common during microcell fusion, we reasoned that chromosomal fragmentation occurring during the microcell fusion process could result in subsequent loss of acentric fragments during passaging. If an acentric fragment contained the gene providing the growth arrest phenotype, it could segregate from the tagged chromosome, and either be reincorporated into the hybrid genome, or lost from the cells. This would then explain the observed reduction in PALA-induced growth arrest phenotype of late passage B78MC166. To test this, we subcloned B78MC166 and analyzed this new set of hybrids (166-n series) for loss of the growth arrest phenotype. Subclone 166-61 showed an increase in PALA-induced apoptosis as compared to its B78MC166 parent (Fig. 4.7). This occurred in association with a low G1:S ratio at day 4 of exposure (Table 4.1). *Alu*-PCR FISH showed that while B78MC166 *Alu*-derived probe uniformly painted human chromosome 3, the *Alu*-probe from 166-61 revealed gaps in the fluorescence signal on both arms of chromosome 3 (Fig. 4.8). Some gaps appeared to localize to chromosome 3 fragile sites (chromosomal regions which can be chemically induced to break *in vitro*). PCR amplification using chromosome 3 specific primers identified some loci which were absent in 166-61, including D3S1276 at 3p13, D3S1302 at 3q12, and a large region

between 3q26 and 3q28 bracketed by D3S1564 and SST. These findings may explain the reversion of 166-61 to the B78 PALA-induced apoptotic phenotype, and suggest that the gene in question is located within one of the microdeleted regions of chromosome 3.

Figure 4.7 Nucleosomal degradation effect of PALA on cells. Cells were grown in the absence (-) or presence (+) of PALA (9 x LD_{50}) for 6 days. M, 100 bp ladder. 166 and -61 are abbreviations for B78MC166 and 166-61. The low molecular weight band visible in some lanes is digested remnant RNA.

Figure 4.7

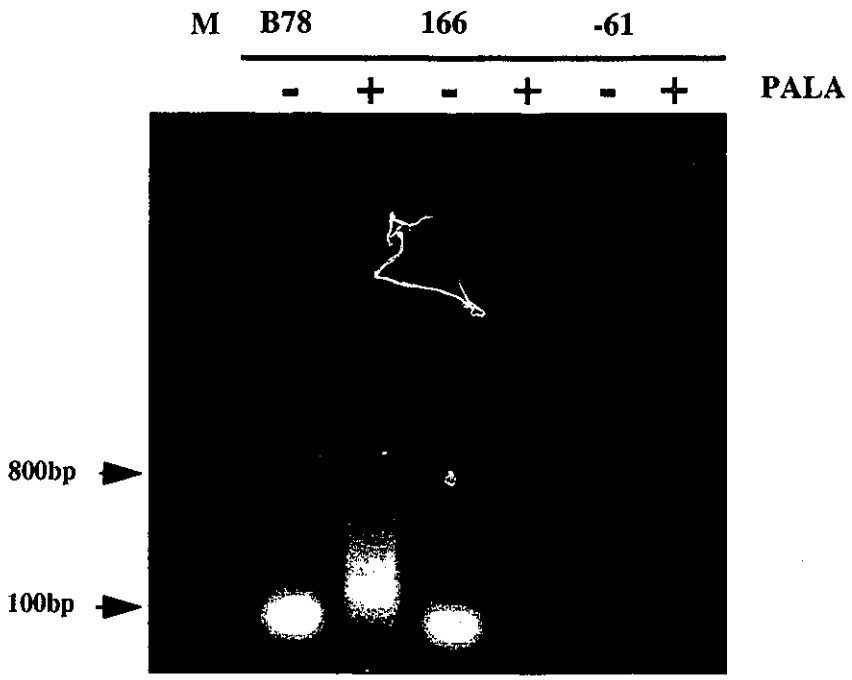
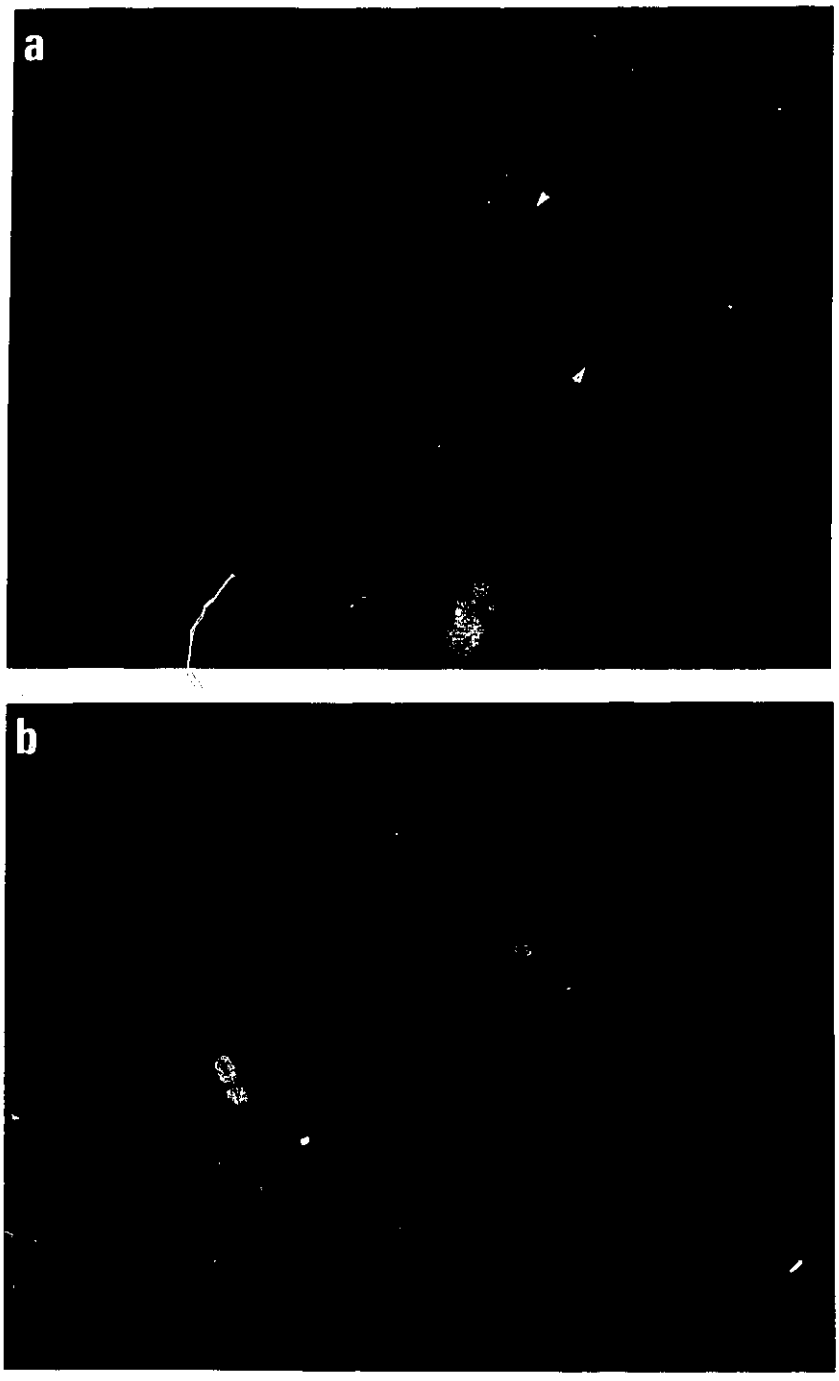


Figure 4.8 *Alu*-PCR FISH of chromosome 3 containing hybrids, performed on normal male human metaphase spreads. (a) Probe derived from 166-61, a revertant subclone of B78MC166. (b) Probe derived from B78MC166. The arrowheads in panel A indicate the largest deletion detected, at 3q26q28.

Figure 4.8



4.45 Evaluation of gene amplification frequencies

Long term exposure of immortalized and tumorigenic cells to high doses of PALA results in the emergence of drug resistant colonies containing amplified CAD genes (Yin et al., 1992). A calibrated PALA dose of 9 x LD_{50} is used to permit accurate comparisons of gene amplification potential between cell lines and types. To rule out the possibility that the hybrids had increased gene amplification potential, as compared to B78, PALA clonogenic assays were performed (Table 4.2). B78, HSF-5 and several hybrids were tested and showed PALA responsive LD_{50} measurements which were within the range reported in a variety of human and rodent cell types (Tlsty, 1990). B78MC166 and B78MC56, as well as HSF-5, were all found to have an LD_{50} roughly double that of B78. However, several B78MC166-derived cell lines which lacked the apoptosis suppression phenotype also showed similarly high LD_{50} measurements in comparison to B78 (Table 4.2). Thus, relative PALA sensitivities, by LD_{50} determinations, were not consistently predictive of the ability to suppress PALA-induced apoptosis. All of the hybrids tested showed gene amplification frequencies which were lower than that of B78, indicating that the CAD gene amplification potential of the hybrids was noncontributory to the growth arrest and apoptosis responses in the majority of cells.

Southern blot analysis was used to further confirm that the CAD gene was unamplified in the PALA-exposed cells of B78MC166 and B78MC56 as compared to B78 and the B78MC166-derived PALA-sensitive cell lines (Fig 4.9). The hybridization intensity of the CAD probe to each hybrid (0 days PALA treatment) was approximately equal to that of B78, when quantified by phosphorimaging, using hybridization to the albumin probe as a loading control. This indicated that the overall CAD gene copy number of each of the hybrids was similar to that

of B78, prior to PALA treatment. Quantification of the hybridization signals following 2 days and 4 days of treatment showed a trend for the CAD signal to increase slightly over the exposure period for all cell lines. However, B78MC166 and B78MC56 showed no evidence of an increased tendency to amplify the CAD gene during short term PALA exposure, as compared to B78 and the other hybrids tested.

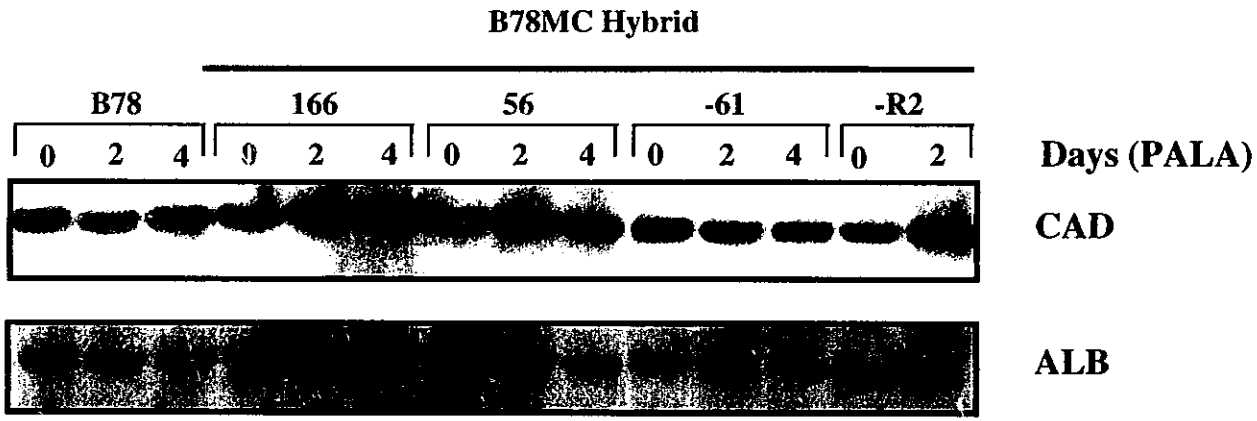
Table 4.2 Cell line characteristics and gene amplification frequencies.
[a] B78 and the hybrids were determined cytogenetically to be pseudotetraploid. HSF-5 is normal diploid.
[b] P.E., plating efficiency.
[c] C.G.T., cell generation time, determined according to Leibovitz et al., 1976.
[d] Frequency of colony formation determined by clonogenic assay (+/- SEM).
[e] N.D., not determined.

Table 4.2 Cell line characteristics and gene amplification frequencies.

Cell Line (ploidy)[a]	P.E.[b] (%)	C.G.T.[c] (hrs)	PALA LD_{50} (μg/ml)	Gene amplification[d]
B78(4n)	48.8±5.7	14.5±1.4	1.43±0.07	$8x10^{-5}\pm10^{-5}$
HSF-5(2n)	30.5±4.5	18.8±0.3	2.46±0.15	N.D.[e]
B78MC166(4n)	59.9±8.6	14.0±0.5	2.82±0.11	$5x10^{-6}\pm10^{-6}$
166-R1(4n)	64.7±4.5	14.1±1.0	2.73±0.03	$1x10^{-5}\pm10^{-5}$
166-R2(4n)	49.1±3.0	15.2±1.9	1.86±0.19	$1x10^{-5}\pm10^{-5}$
166-61(4n)	59.2±12.1	14.5±0.8	2.54±0.14	$2x10^{-6}\pm10^{-6}$
B78MC56(4n)	51.0±10.0	15.4±0.6	2.75±0.38	$1x10^{-5}\pm10^{-5}$

Figure 4.9 Southern analysis of PALA-exposed cell lines. DNA was isolated from cells exposed to 13 μg/ml PALA for 0, 2 or 4 days, EcoR1 digested, and hybridized with a hamster CAD probe (CAD). -61 and -R2 are abbreviations for 166-61 and 166-R2. Relative hybridization intensities were determined using a EcoR1 rat albumin probe (ALB) as a loading control. The locus for human albumin is chromosome 4q11-q13.

Figure 4.9


B78MC Hybrid
B78
166
56
-61
-R2
0 2 4 0 2 4 0 2 4 0 2 4 0 2
Days (PALA)
CAD
ALB

4.46 Determination of p53 protein status in B78 and hybrids

We suspected that B78 contained non-functional p53 protein. Tumorigenic cell lines with non-functional or mutant *p53* often show high cellular p53 protein levels, due to altered conformation and stabilization (Reihsaus et al., 1990; Vogelstein and Kinzler, 1992). This contrasts with non-transformed, $p53^{+/+}$ expressing cell lines, which, when analysed by immunoblotting, show very low, or undetectable levels of p53 protein, due to a very short half-life (Fritsche et al., 1993; Kastan et al., 1992; Lu and Lane,1993; Oren et al., 1981). To determine if p53 protein was stabilized in B78 and the hybrids, total cellular protein was extracted from the cell lines and analyzed by immunoblotting with a p53 monoclonal antibody, Ab-1, which reacts with both mutant and wild-type forms of p53 protein (Fig. 4.10). Untreated B78 and B78MC166-derived hybrids were found to contain high levels of p53 protein, as compared to normal human and murine cell lines (NH and NM, Fig. 4.10). This suggested that B78 derived cells contained inactivated p53 protein.

Tumorigenic and transformed cell lines which lack wild-type p53 protein activity are usually radioresistant and fail to undergo G1 arrest, due to failure of apoptosis (Kastan et al., 1992; Lee Bernstein, 1993; Lowe et al., 1993*a*; McIlwrath et al., 1994; Russell et al., 1995). Since radiation-induced G1 arrest and apoptosis are under the control of wild-type p53 protein, we tested the ability of B78 and B78MC166 to undergo either G1 arrest or apoptosis following ionizing radiation. The cells were exposed to 5 gray radiation, and following 24 hr or 96 hr of recovery, were harvested and analyzed by flow cytometry. Linear scale flow cytometric histogram profiles of both cell lines showed little perturbation following irradiation, and exhibited essentially no change in the percentage of cells in S phase, suggesting poor G1 and G2 checkpoint

activity (96 hr shown, Fig. 4.11). Ninety-six hours after irradiation, viability, as determined by trypan blue exclusion, was 95% and 92% for B78 and B78MC166 respectively. Furthermore, log-scale histograms of the irradiated cells showed little accumulation of debris and lacked a detectable apoptotic peak (Fig. 4.11). These findings are consistent with cells possessing mutant or non-functional p53 protein. These findings further suggest that the locus on human chromosome 3 is unable to mediate ionizing radiation-induced growth arrest in these cells.

Figure 4.10 Immunoblot of cell lines to detect stabilized p53 protein in B78 and B78MC166 hybrids. Controls: NH, normal human cells, HSF-5; TH, human tumorigenic cell line, DU-145; NM, normal murine cell line, NIH 3T3; TM, tumorigenic murine cell line, P19. 166, -61 and -R2 are abbreviations for B78MC166, 166-61 and 166-R2.

Figure 4.10

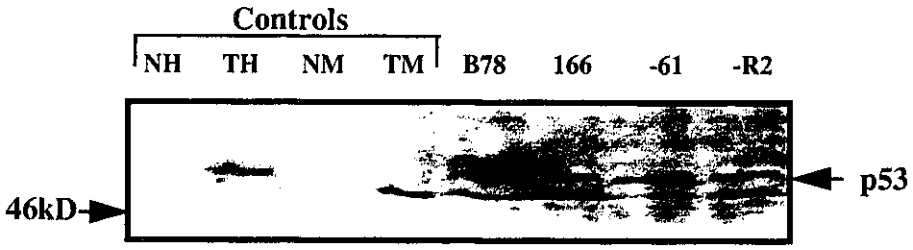
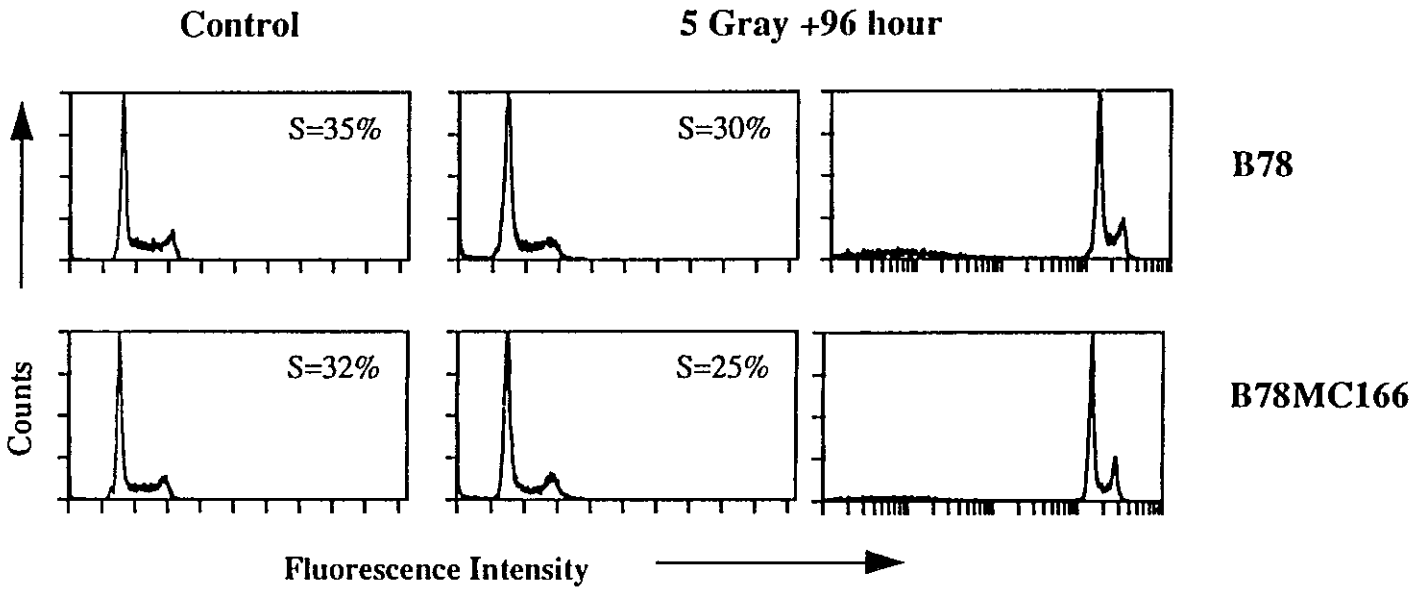


Figure 4.11 Linear and log-scale flow cytometric histograms of B78 and B78MC166, 96 hours after radiation (5 Gray). S is percent of cells in S-phase. Controls were growing untreated cells.

Figure 4.11


Control
5 Gray +96 hour
S=35%
S=30%
B78
S=32%
S=25%
B78MC166
Counts
Fluorescence Intensity

4.5 Discussion

In this study, microcell hybrids were used to show that the presence of human chromosome 3 is associated with a PALA-induced growth arrest phenotype accompanied by reduced apoptosis. Removal of chromosome 3, or microdeletions of specific regions of chromosome 3 from the hybrid, resulted in loss of the PALA-induced growth arrest and increased levels of apoptosis.

The G1:S ratios were significantly higher in PALA-exposed B78MC166 (which contained chromosome 3) and B78MC56 (which contained the short arm of chromosome 3) as opposed to B78 and the other hybrids. Increased G1:S ratios in the chromosome 3 containing hybrids were associated with significantly reduced levels of apoptosis. These findings suggest a G1 checkpoint linked to a gene on human chromosome 3. The G2:S ratio was also significantly higher in PALA-exposed B78MC166 and B78MC56 when compared with B78 ($P<0.01$), and removal of chromosome 3 from B78MC166 resulted in a significant reduction in the G2:S ratio (B78MC166-R hybrids, Table 4.1). However, because the G2:S ratio remained relatively high in 166-R2 as compared to B78 and the other hybrids, it is not clear if the G2 arrest appearing during PALA treatment was directly due to the presence of chromosome 3. Extended G2 arrest is thought to contribute to cell survival, following genotoxic exposure (Maity et al., 1994). In yeast, the failure of G2 arrest, due to checkpoint rad mutations, leads to cell death and genomic instability following irradiation damage (Al-Khodairy and Carr, 1992; Weinert and Hartwell. 1988). The presence of a strong G1:S ratio in B78MC166 and B78MC56 in response to PALA, may be due to the action of a similar mammalian checkpoint gene, acting to induce growth arrest in G1, with possible downstream effects in G2.

To explain the differences in the PALA-induced responses of B78 and the chromosome 3 containing hybrids, one can hypothesize that chromosome 3 contains a growth regulatory gene which also inhibits apoptosis. Due to cell to cell variations, only a proportion of cells in the chromosome 3-containing hybrids may actually synthesize the functional encoded protein in sufficient quantity. During PALA exposure, these cells may be able to avoid the apoptotic process by arresting in G1 following initial S phase delay. The ability to maintain a G1 arrest may result in increased survival as compared to B78 and the other hybrids. Cells which fail to observe the G1 checkpoint during PALA exposure may be destined to die via apoptosis. This would explain the observation of high day 4 G1:S ratios in the viable PALA-exposed chromosome 3 containing hybrid cells, since non-arrested cells would be selected for apoptosis. Some cycling cells may also survive via other drug resistance mechanisms, such as CAD gene amplification, or increased CAD gene copy number by chromosomal rearrangement (Schaefer et al., 1993). CAD gene amplification was shown to occur rarely in these hybrids, and their gene amplification potential was calculated to be less than that of B78 (Table 4.2). Gene amplification would not, therefore, be expected to emerge as a drug resistance mechanism in a significant proportion of the population of viable cells during the four day PALA exposure period. Southern blot analysis of DNA from treated and untreated cells confirmed that the CAD gene copy number was not appreciably different, or increased in B78MC166 and B78MC56 during short term PALA exposure, as compared to B78 and the other hybrids (Fig. 4.9).

Indirect evidence supports the view that CAD gene amplification was not responsible for the PALA-responsive phenotypes of B78MC166 and B78MC56. For example, it should be noted that *Alu*-PCR FISH failed to reveal any human chromosome 2 elements (the human locus

of the CAD gene) in the selected hybrids (B78MC166 shown, Fig.4. 8). Furthermore, if additional CAD genes were responsible for the reduced apoptosis observed in B78MC166, removal of human chromosome 3 would not have resulted in loss of the PALA-responsive phenotype of this hybrid. Reduced PALA-induced cell death was not accompanied by cellular proliferation, signalling classical drug resistance, in B78MC166 and B78MC56. Moreover, the differences in apoptosis and G1:S ratios could not be explained by differences in rates of proliferation, since the hybrids tested had cell generation times which were not significantly different from that of B78 (Table 4.2). This, and the high G1:S and G2:S ratios of B78MC166 and B78MC56 during PALA exposure support the view that continuous short term PALA exposure resulted in selection for survival of growth arrested cells in chromosome 3 containing hybrids, rather than survival through drug resistance by gene amplification.

Interestingly, *Alu*-PCR FISH revealed several human chromosome 3 breakpoints in 166-61 which appear to be in proximity of established cytogenetic markers for chemically-induced chromosome 3 breaks. One of these fragile sites, FRA3B, is closely linked to at least one candidate tumour suppressor gene locus, at 3p14.2 (Sutherland and D. H. Ledbetter, 1989.). Since the other growth arrested hybrid, B78MC56, contained only the p arm of chromosome 3, the microdeleted regions of the short arm of chromosome 3 are the candidate regions for the gene acting in PALA-induced growth arrest and suppression of apoptosis.

It was determined that B78 lacked wild-type p53 protein. This resulted in a poor G1 response to both PALA and ionizing radiation, but did not prevent PALA-induced apoptosis. Although the presence of $p53^{+/+}$ appears to be required for efficient activation of growth arrest and/or apoptosis, cells which lack functional p53 have been shown to be capable of undergoing

radiation- and drug-induced apoptosis, albeit at higher doses (Kaufmann, 1989; Lennon et al., 1991; Sen and D'Incalci, 1992; Strasser et al., 1994). This suggests that the apoptosis threshold of a particular cell is dependent upon its genetic makeup (Fisher, 1994; Fisher et al., 1993).

The presence of human chromosome 17, in hybrid B78MC27, failed to result in either increased sensitivity to PALA, or induction of G1 arrest (Table 4.1). It is possible that in B78MC27, human *p53*, under control of its natural promoter, mediated growth arrest inefficiently in the murine cells, or was inactivated either by mutation or by interaction with mutant murine p53 protein. Alternatively, p53 may be incapable of promoting drug-induced growth arrest in highly tumorigenic cells, or may only be effective in growth suppression in co-operation with other growth suppressor genes. These genes, acting as co-mediators of growth arrest during toxic conditions, may be required for efficient activation of growth suppression. It is likely that such growth regulators would be selected against during tumour progression. If some growth regulators are also survival genes, acting to suppress apoptosis, their inactivation may, as is the case with oncogene activation, result in both deregulated growth, and increased apoptosis. The chromosome 3-mediated growth arrest revealed in our experiments may represent part of a complex growth arrest pathway which operates in normal cells to suppress apoptosis and control the cell cycle during toxic conditions. Genes in this pathway, if intact in some tumour cells, may provide a mechanism for tumour cell drug resistance by raising the apoptosis threshold in the absence of p53. Alternatively, these genes could control proliferation during sub-optimal conditions, leading to dormancy, and thereby preventing apoptosis. An understanding of the molecular basis for differences in the responses of normal cells versus tumour cells to cancer therapy is of utmost importance. Identification of genes affecting tumour cell sensitization and

resistance to apoptosis will lead to new approaches to cancer therapy (Fisher, 1994). The eventual isolation of a survival gene implicated in suppression of drug-induced apoptosis, may thus help further the understanding of the mechanisms of cell death in anti-cancer therapy, as well as contribute to the study of cell cycle control in normal and neoplastic cells.

4.6 Acknowledgments

I would like to thank G.R. Stark for providing the hamster CAD probe; L. Filion for helpful advice; H. Gourdeau and M. Tenniswood for critical comments; J. Bell, D. Blakey and A. MacKenzie for valuable discussions.

Chapter 5. Molecular Analysis of Differentially Expressed Sequences Involved in Apoptosis Suppression

5.1 Abstract

Chromosome 3 containing microcell hybrids were shown to exhibit evidence of apoptosis suppression and growth arrest during short term exposure to the chemotherapeutic agent, PALA. However, a hybrid with microdeletions in the short and long arms of chromosome 3 showed a revertant phenotype, with loss of growth arrest potential, and increased induction of apoptosis during PALA exposure. To identify genes that influenced the PALA response in these hybrids, gene expression was analysed by differential display reverse transcriptase assay. Several novel transcripts were isolated and sequenced, which may play a role in the promotion of growth arrest and suppression of apoptosis in these hybrids. Also, murine TIS7, an interferon-related gene, was identified, suggesting its involvement in the early response to PALA exposure.

5.2 Introduction

Techniques which permit the isolation of differentially expressed, unknown genes, are limited to the classic molecular techniques of differential screening and subtractive hybridization, and the more recently developed technique of differential display reverse transcriptase-PCR (DDRT-PCR) (Hedrick et al., 1984; Lee et al., 1991; Liang and Pardee, 1992). The earlier methods have disadvantages, in that they are unidirectional, and pairwise, permitting the selection of genes which are expressed in one cell line as compared to another. They do not allow detection of quantitative differences between samples, and often require multiple attempts to obtain the differentially expressed genes. In contrast, DDRT-PCR allows the detection of

differences, both qualitative, and quantitative, in gene expression, between multiple cell lines, or multiple treatments at once.

The DDRT-PCR method is based on the use of arbitrarily selected primers to PCR amplify a subset of cDNAs from each cell line or treatment being analysed. The arbitrary primers anneal to the cDNA template at a low temperature. PCR products result from amplification of cDNA fragments which have sequences which match, or nearly match the arbitrary primer. Differences in gene expression can then be detected by electrophoresing the PCR products (radiolabelled during the amplification step) on a sequencing gel. Using this method, and with a sufficient variety of arbitrary primers, it is theoretically possible to amplify almost all expressed sequences.

Analysis of B78MC microcell hybrids' response to the chemotherapeutic agent PALA suggested that PALA-induced apoptosis was inhibited in hybrids which contained human chromosome 3. Comparison of B78MC166, a chromosome 3 hybrid; 166-61, a subclone of B78MC166 with microdeletions; and B78MC56, an independent chromosome 3 hybrid containing the short arm of chromosome 3, suggested that the gene involved in the apoptosis suppression pathway resided upon the short arm of chromosome 3. DDRT-PCR was undertaken to try to isolate the cDNA derived from this gene, as well as other cDNAs which were differentially expressed in the suppressed hybrids during PALA exposure, but not necessarily mapping to chromosome 3. A similar strategy has been used successfully by others to identify genes induced by a variety of treatments, including vitamin response, radiation induction, retinoic acid response, and regulation by growth factors (Kumar and Haugen, 1994; Jung et al., 1994;

Donohue et al., 1994; McClelland et al., 1994). Here, 15 differentially expressed fragments were isolated in a partial analysis of differential expression between PALA-exposed cell lines.

5.3 Materials and Methods

5.31 Cell Culture and PALA assay

B78MC hybrids, B78 and HSF-5 were cultured as described previously (Chapter 2, Materials and Methods). During the PALA assay, the cells were grown in non-selective medium, supplemented with 10% dialzyed fetal bovine serum. The cells were cultured in 150 cm^2 flasks (approximately 10^6 cells per flask) and exposed to 13µg/ml PALA (diluted in sterile water) for 2 days, after which they were harvested by trypsinization and centrifugation. Dead and dying cells were discarded in the supernatant. Controls were cells which were cultured in parallel, in non-selective medium, supplemented with 10% dialysed serum only.

5.32 RNA Isolation

Pelleted cells were rinsed in 0.9% saline, transferred to 1.5ml microfuge tubes, and briefly microfuged. On ice, total RNA was released from the cells by detergent lysis (300µl 10mM Tris-HCl [pH8.0]; 150mM NaCl; 2mM $MgCl_2$; 0.5% NP-40). The mixture was microfuged briefly, and the supernatant, containing total cellular RNA was transferred to fresh tubes. TSE+S buffer (300µl) was added to the mixture (10mM Tris-HCl [pH8.0]; 150mM NaCl; 5mM EDTA; 0.2% SDS) and the RNA was extracted twice using 50% phenol / 50% chloroform:isoamyl alcohol (24:1). Finally, the RNA was precipitated with a 1/20 volume 3M

NaCl and 2 volumes 99% ethanol. The samples were stored at -20°C in this form, or pelleted and resuspended in 100µl TE (10mM Tris-HCl [pH8.0]; 1mM EDTA) + 0.1%SDS, and stored at -80°C. Purified messenger RNA was isolated from total RNA samples using an mRNA isolation kit, according to the manufacturer's instructions (Oligotex, Qiagen). RNA was quantified by spectrophotometer (wavelength 260nm). Purity was determined by the ratio of 260/280 (pure samples had a ratio of approximately 2).

5.33 Differential Display by PCR

For DDRT-PCR, a modified method of the originally described technique was used (Liang and Pardee, 1992; Liang et al., 1993; Bauer et al., 1993; Mou et al., 1994). RNA from PALA exposed (2 days) B78, 166-61 and B78MC166 were selected to identify cDNAs which were differentially expressed in B78MC166 as compared to 166-61 and B78. DNA was eliminated from total RNA by incubation in DNAse I buffer for 30min at 37°C (50µg RNA; 10U RNAse Inhibitor; 0.1M Tris-HCl [pH 8.3]; 0.5M KCl; 15mM $MgCl_2$; 10U DNAse I). The RNA was phenol/chloroform extracted and diluted to 200ng/µl. The RNA was stored at -20°C until use.

A subset of cDNAs representative of the mRNA present in each cell line were made by reverse transcription, using different anchored polyT primers (T11MN where M=A,C or G and N = T,A,C or G) followed by PCR amplification using the same T11MN primer, and an arbitrary decamer, DDX (Table 5.1). Samples, consisting of: 1X RT buffer, 0.1M DTT; 20µM dNTPs; 1µM T11MN; 10U RNAse Inhibitor and 400ng DNAse treated RNA, in 19µl, were incubated at 65°C for 5min to denature secondary structure, followed by 10min at 37°C to allow the primer

to anneal to the RNA. To each sample, 200U of Superscript Reverse Transcriptase was added, and the samples were incubated for 50min at 37°C, followed by 5min at 95°C to inactivate the enzyme.

The cDNAs were then PCR amplified in a reaction buffer consisting of: 3µl of cDNA; 1X PCR buffer (10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 50mM KCl, and 0.01% gelatin); 2µM dNTPs; 1µM T11MN; 0.2µM DDX where DDX was an arbitrary decamer; 10µCu ^{35}S dATP; and 3U AmpliTaq (Perkin-Elmer Cetus) in a volume of 20µl with an oil overlay. An overnight PCR program was performed on a Pharmacia Gene ATAQ Controller Thermal Cycler as follows: 40 cycles of denaturation at 94°C, 30sec; followed by annealing at 40°C, 2min; and extension at 72°C 30sec. After a post-extension of 72°C for 5min, the samples were refrigerated until use the following day. To analyze the PCR products, 4-6µl of denatured sample (95°C, 5min and kept on ice), containing 1x formamide loading buffer (0.025 % bromophenol blue; 0.025% xylene cyanol; 10mM EDTA in formamide) was loaded onto a large 6% polyacrylamide / 8M urea denaturing sequencing gel. The gel was electrophoresed for approximately 4 hours, at 1800V; 60W; 30 amps. The gel was transferred to 3MM Whatmann paper, covered with plastic wrap, and dried at 80°C for 1hr . Radioactive (^{32}P-dCTP) ink was used to mark the corners of the gel-covered Whatmann. The gel was then exposed to XAR Kodak film overnight.

To isolate differential bands, the autoradiograph and gel-covered Whatmann were positioned, using the radioactive ink spots as guides. Differential bands were cut out using a clean razor blade. The polyacrylamide gel fragment was then transferred to a sterile 1.5ml microfuge tube. The PCR product present in the differential band was eluted by boiling in 100µl sterile water for 15min. The sample was microfuged for 2min, and the supernatant,

containing the selected PCR product, was transferred to a clean tube. The sample was then precipitated (1/10 volume 3M sodium acetate; 5µl glycogen(20µg/µl); 2 volumes 99% ethanol; -80°C for 30 min), and microfuged at 4°C for 15min. The supernatant was discarded, and the pellet was rinsed once with 500µl cold 85% ethanol. The pellet was allowed to dry, and the PCR product was resuspended in 10µl H_2O.

To re-amplify the excised band, the PCR product was amplified in a reaction buffer consisting of: 4µl from the excised band; 1x PCR buffer; 20µM dNTPs, 1µM T11MN, 0.2µM DDX and 2.5U AmpliTaq in 40µl. The samples were overlaid with oil, and PCR amplified using the PCR program as before. To verify that the re-amplification was successful, and to determine the size of the fragment, 10µl of the sample was electrophoresed on 2% Nusieve GTG (3:1) agarose gel containing ethidium bromide. One µl of the remaining 30µl was used to quantify DNA concentration of the sample (TK-100 fluorometer).

Table 5.1 List of 5' arbitrary decamer primers

Decamer	Sequence (5' to 3')									
DD21	T	A	C	A	A	C	G	A	G	G
DD22	T	G	G	A	T	T	G	G	T	C
DD25	G	G	A	A	C	C	A	A	T	C
DD27	T	C	G	A	T	A	C	A	G	G
DD29	T	C	G	G	T	C	A	T	A	G
DD30	G	G	T	A	C	T	A	A	G	G
DD32	C	T	G	C	T	T	G	A	T	G
DD42	G	A	T	C	G	C	A	T	T	G
DD43	G	A	T	C	T	G	A	C	T	G
DD44	G	A	T	C	A	T	G	G	T	C

Note: Decamers are 50% G/C and 50% A/T, and are non-selfcomplementary.

5.34 Subcloning of DNA from excised bands

The re-amplified DNA from the excised bands was subcloned into pCRII vector (TA Cloning Kit, Invitrogen). A 1:1 molar ratio of the PCR product with the pCRII cloning vector was used, based on the length of the PCR product, which ranged between 125 bp to 800 bp. An equimolar mixture of fresh PCR product was ligated to 50ng pCRII vector in 1x Ligation Buffer and 4U T4 DNA ligase, in a 10µl volume, at 14°C overnight. Following this, a 2µl aliquot of the ligation mixture was added to 50µl of competent cells in the presence of 20mM β-mercaptoethanol. The cells were incubated on ice for 30min, and heat shocked for 30sec at 42°C. The cells were then placed on ice for 2min. 450µl SOC (2% Tryptone; 0.5% Yeast Extract; 10mM NaCl; 2.5mM KCl; 10mM $MgSO_4$; 20mM glucose) medium was added to the cells, which were then incubated for 1hr at 37°C. For each transformation, 50µl and 200µl of cells were spread onto separate labelled LB agar plates containing 50µg/ml ampicillin and overlaid with 40µl of 40mg/ml X-Gal in dimethylformamide. Controls were self-ligation reactions, with pCRII vector only, to determine the rate of false white colony formation and transformation efficiency controls, to test the competency of the cells to be transformed by a control plasmid (pUC18). The plates were inverted and placed at 37° C overnight to allow colony growth. White colonies (recombinants) were picked and expanded overnight in 2ml LB (1% Tryptone; 0.5% Yeast Extract; 1% NaCl [pH7.0]) with ampicillin. Recombinants were labelled according to the originating primer sets used, and given an alphabetic name (eg. four MA21f1 clones were picked and labelled MA21f1a, f1b, f1c, and f1d).

Restriction analysis was performed on the recombinants to ensure that the inserts were the expected size. The insert was released from the vector by an EcoRI digest (10µl of plasmid DNA incubated for 2 hours at 37° C, in a digestion buffer consisting of 10U RNAse, 1x H buffer [Boehringer Mannheim] , 10U EcoR1, in a volume of 15µl). The digest was then electrophoresed on a 1% ethidium bromide agarose gel, with a 100bp ladder to determine the fragment size. Alternatively, the plasmid was PCR amplified as described in the PCR protocol above, and the PCR product was electrophoresed, and compared with a 100bp ladder marker.

5.35 Northern Blots

Each cell line was either untreated, and in exponential growth, or treated with PALA (13µg/ml) for 2 days prior to RNA isolation. Total RNA (15µg) or mRNA (0.5 or 1 µg) from each cell line was loaded onto a formaldehyde gel (1.2% agarose; 1x MOPS [20mM MOPS; 5mM sodium acetate; 0.5mM disodium EDTA]; 1.0M formaldehyde), and electrophoresed at approximately 56 V for 3 hours. Marker lanes were 1µg total RNA from B78 with 100µg/ml ethidium bromide added). For Northern transfer, the gel was placed on a Whatman paper covered sponge soaked in 10X SSC (20XSSC=3M NaCl; 0.3M trisodium citrate). A nylon membrane (Pall Biodyne) was cut to the same dimensions as the gel, and placed on top of the gel. A Whatman paper was placed over the membrane, and approximately 3 inches of blotting pads were placed on top. The RNA transfer was allowed to take place overnight. Following transfer, the lanes were marked, and the RNA was crosslinked to the membrane using a UV source (Stratalinker, Stratagene) set at

Autocrosslink. Membranes were stored in prehybridization solution (50% formamide; 0.5M $NaHPO_4$ [pH7.2]; 1mM EDTA; 5%SDS) at 4°C until use.

5.36 Dot Blots

Denatured aliquots (2ng in 2µl 0.4M NaOH/10mMEDTA, boiled 2min) of each cloned fragment were dotted onto a damp nylon membrane equilibrated in 0.4M NaOH. The dots were allowed to dry, the membrane was rinsed briefly in 2XSSC, and stored in PEG/SSPE hybridization buffer (see Materials and Methods, 4.37).

5.37 Preparation and labelling of probes

Probes were labelled with ^{32}P-dCTP by PCR-labelling, or by random priming, using Prime-It II Random Primer Labelling Kit (Stratagene) according to the manufacturer's instructions. For random priming, 25ng of DNA was denatured (100° C, 5min) in the presence of oligonucleotide primers, in a volume of 34µl. The denatured DNA was briefly centrifuged, and primer buffer (5X), 5Ci ^{32}P-dCTP (3000 Ci/mmol), and 5U Klenow were added to a final volume of 50µl. The mixture was incubated for 10min at 37° C, and stopped with 2µl stop mix. The mixture was then diluted to a volume of 220µl in low TE (10mM Tris-HCl [pH8.0]; 0.1mM EDTA). Probe specific activity was determined by TCA precipitation (1µl probe mixture in 2ml 5% TCA with 0.04% BSA) and quantified by scintillation counting. Unincorporated nucleotides were eliminated by precipitation of the probe with 40µg salmon sperm DNA and 2mM spermine. The probe was resuspended in 135µl low TE. A 1/10 volume of 4N NaOH and 5M NaCl were added to dissolve the pellet.

Prior to use, the probe was denatured by heating to 65°C for 5min, placed on ice for 2min, and added to 10ml hybridization solution.

Alternatively, PCR probes were made from re-amplified, excised cDNA PCR products for use on Nothern blots. The reaction mixture consisted of 25-50ng of re-amplified PCR product, 1x PCR buffer, 2.5µM dNTP-dCTP, 1µM T11MN, 0.2 µM DDX, 2.5U Amplitaq and 100µCi ^{32}P-dCTP (3000 Ci/mmol) in a volume of 20µl. Oil was overlaid onto the sample, and the previously described PCR program was run overnight. The ^{32}P-labelled PCR products were then removed from the oil by dotting onto parafilm, and transferred to a clean microfuge tube. The probe was diluted in 200µl low TE and was purified and quantified as described above for the random-primed probes.

5.38 Hybridization of Blots

Northern blots were placed face up in roller bottles and equilibrated at 42°C in 10ml prehybridization (50% formamide; 0.5M $NaHPO_4$ [pH7.2]; 1mM EDTA; 5%SDS) solution, in a hybridization chamber (Robbins Model 400) for at least 15min. Denatured probe was added to the solution and hybridization was allowed to take place at 42°C overnight. The hybridized blots were rinsed in 2XSSC/0.1% SDS (2x15min, 42°C) and 0.1XSSC/0.1%SDS (30min, 65°C). The blots were then wrapped and exposed appropriately on Kodak XAR-5 film with intensifying screens, or on Molecular Dynamics phosphorimaging screens. The intensity of signals was analysed by phosphorimaging (Molecular Dynamics) or by densitometry. Signals were calibrated to control hybridizations (tubulin or GAPDH).

Dot blots were equilibrated at 65°C in prehybridization solution (PEG/SSPE) for 2 hours. Denatured probe was added to the solution and hybridization took place at 65°C overnight. The blots were washed at 65°C in 2XSSC/0.1% SDS (2x15min) and 0.1XSSC/0.1%SDS (30min). The dot blots were exposed on Kodak XAR-5 film with intensifying screens, or on phosphorimaging screens (Molecular Dynamics).

5.4 Results

5.41 Differential display of cDNAs

Analysis of the PALA responses of B78 and the microcell hybrids, suggested that the turning point for survival via growth arrest as opposed to cell death via apoptosis occurred during day 2 of PALA exposure. Removal of PALA following one day of treatment, but not 2 days of treatment, partially rescued B78 from PALA-induced cell death (Fig. 5.1). Also, cell proliferation levelled off for all the cell lines at day 2 (Fig. 4.4), in spite of the fact that viability was still almost 100%. Also at day 2, all the cell lines showed similar flow cytometric histograms, and were still primarily blocked in S phase of the cell cycle (see Chapter 4, Fig. 4.2, and Fig. 4.4). Therefore, it was assumed that each cell line would have similar patterns of gene expression, except for those genes which affected the decision to undergo apoptosis, or survive via growth arrest. For these reasons, day 2 of PALA exposure was chosen as an appropriate starting point for identifying differentially expressed sequences.

PALA-exposed B78 and 166-61 were used in the DDRT-PCR screen, to represent cells that responded to PALA with apoptosis. PALA-exposed B78MC166 was chosen to

represent cell types that responded to PALA with growth arrest and improved survival. It was assumed that B78 and 166-61 would have matching displays during PALA exposure, except for cDNAs derived from human chromosome 3 that were non-conserved between the two species. It was also assumed that B78MC166 would have matching displays with B78 (the murine background), and 166-61 (micro-deleted human chromosome 3), but differ in genes expressed which were either linked to the microdeleted regions of human chromosome 3 in 166-61, or which were growth arrest specific (human or down-stream murine).

Ten different arbitrary decamers were selected (Table 5.1), in combination with 4 different T11MN primers: T11MA; T11MC; T11MG; and T11MT. DDRT-PCR was performed on RNA extracted from 2 day PALA-exposed cells, and the products were analysed for differential expression. The reactions were repeated, using cDNAs which were reverse transcribed on different days, and duplicate experiments were electrophoresed together, on sequencing gels (Fig. 5.2).

Forty independent subsets of mRNA species (4 anchored primers x 10 DDX primers) revealing approximately 120-150 bands per experiment were differentially displayed, in duplicate. Fifteen individual fragments were found which were differentially displayed, ie. present in greater intensity in the B78MC166 lanes as opposed to 166-61 and B78. The fragments were named for the primer sets used, and numbered sequentially (eg. MA32f1). The fragments were cut from the gel, extracted and reamplified. One of the fifteen fragments failed to reamplify, and was discarded (MG43f10). Fourteen fragments were re-amplifiable, and these were subcloned for further analysis.

Figure 5.1 Rescue of PALA-exposed B78.
B78 was exposed to PALA for 0, 1, 2 and 4 days prior to removal of PALA. The cells were washed and allowed to continue to grow in regular medium until cultured for a total of 4 days. Cell viability was measured by trypan blue. There was no significant difference in cell survival between 2 days and 4 days of PALA exposure ($P>0.05$). Cell survival following 1 day of PALA treatment was significantly higher than survival following 2 or 4 days of PALA treatment ($P<0.001$) (Bonferroni multiple comparisons test).

Figure 5.1

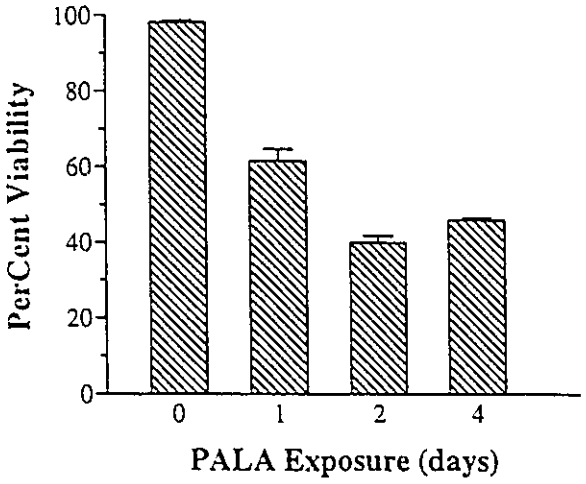
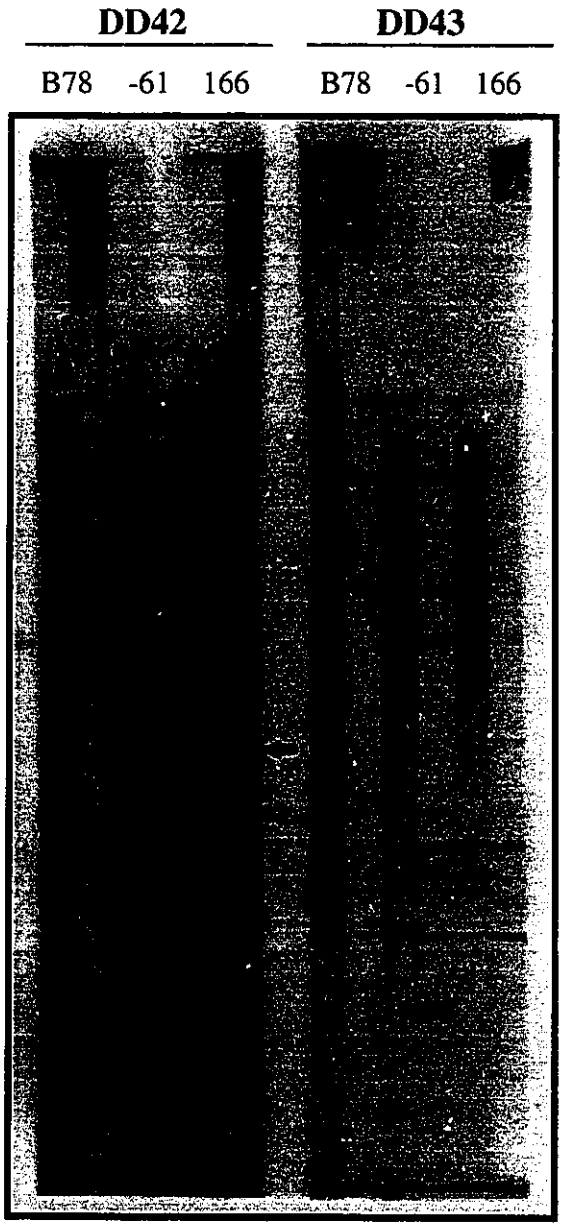


Figure 5.2 Example of DDRT-PCR sequencing gel, differential bands. cDNA was made from RNA from PALA exposed B78, 166-61 (-61), and B78MC166 (166), using, in this case, T11MA anchored primer. The cDNAs were used in PCR reactions using T11MA and different arbitrary decamers (shown here, DD42 and DD43). Each experiment was repeated once and the PCR products from the replicate experiments were electrophoresed in duplicate lanes. A differential fragment (MA42f5) is shown (arrow).

Figure 5.2



T11MA

5.42 Northern blot analyses

Northern blots were performed, using PCR-radiolabelled fragments as probes, and quantified by phosphorimaging to measure the relative level of expression of the corresponding RNA in the presence and absence of PALA. RNA was isolated from untreated, and PALA-treated (13 µg/ml, 2 days) cells from the three cell lines used for the DDRT-PCR experiments, and other cell lines with known PALA responses. Differential increased expression was expected to be detectable between cell lines which showed reduced PALA-induced apoptosis and growth arrest (growth arrested, GA cell lines: B78MC166 and B78MC56) as opposed to those that showed higher levels of PALA-induced apoptosis and no growth arrest (apoptotic, AP cell lines: B78, 166-61 and 166-R2). Differences in gene expression between the cell lines were detected using several of the DDRT fragment probes; however, they were weak and difficult to reproduce (Table 5.2). Probes MA21f1, MA29f2 and MG32f9 failed to reveal any differences in expression between cell lines. Several probes revealed a differential response to PALA in one or more lanes, but they did not show a consistent difference between GA and AP cell lines (probes MA42f5, MC25f6, MG44f11; MG44f12). The failure of these probes to detect differences in expression between the cell lines and their responses to PALA may be attributed either to spurious differences in the differential display, or due to mosaicism in the cell lines, which allowed the detection of differential expression by the DDRT-PCR technique, but diluted the detection of differences in expression by Northern blotting.

The MA32f4 probe detected two messages, one at 1.9kb, and the other, at 1.3kb, indicating that either at least 2 cDNA fragments were present in the original gel slice, or the

probe revealed an alternatively spliced mRNA (Table 5.2). Whereas the 1.3kb band showed no consistent differences between cell type, the 1.9kb band showed differential expression between the GA cell lines as compared to the AP cell lines (Fig. 5.3). This band showed up regulation or little effect in the GA lanes, and relatively strong down regulation in the AP lanes.

The MC44f13 and MC44f14 probes also indicated consistent differential expression pattern between AP and GA cell lines (Table 5.2). The 1.9kb message revealed by the MC44f14 probe did not appear to be strongly affected by PALA in the AP cell lines, but was relatively upregulated in the GA cell lines (Fig. 5.4). The MC44f13 probe revealed a 1.5kb message that was either weakly expressed (B78) or strongly down regulated in the presence of PALA, for all remaining AP cell lines. It was weakly down regulated in GA cell line B78MC166, but showed higher residual expression relative to the AP cell lines. The message also appeared to be up regulated in B78MC56 during PALA exposure (Fig. 5.5).

Table 5.2 Northern analysis of differentially displayed fragments.

Probe	mRNA Size	PALA Response	Expression
MA21f1	1.6kb	No change	Not differential
	1.5kb	No change	Not differential
	1.0kb	Induced in all	Not differential
MA29f2	0.9kb	Reduced in all	Not differential
MA32f3	N.D.		
MA32f4	1.9kb	Differential	MC166/MC56: up; others: down.
	1.3kb	Differential	B78: down; others: no change
MA42f5	1.5kb	Differential	MC56: up; others: unchanged, or down
MC25f6	2.9kb	Differential	R2: down; others: up
MC32f7	N.D.		homologous to f4
MG32f8	N.D.		homologous to f14
MG32f9	1.8kb	Reduced in all	No differential
MG44f11	1.6kb	Differential	MC166/56/166-R2: up; B78, MC166-61: down
MG44f12	2.1kb	Differential	MC166/MC56/R2:up; B78, 166-61: down
MC44f13	1.5kb	Differential	MC166/MC56: remains high; others: down
MC44f14	1.9kb	Differential	166/56: up; B78/166-61: unchanged
MC44f15	N.D.		

N.D., not determined

Figure 5.3 Northern blot analysis of MA32f4.
(a) Northern blot probed with labelled MA32f4. Cells were harvested and RNA was isolated following 2 days of growth in the presence (+) or in the absence (-) of PALA. Approximately 1μg polyA$^+$ RNA was loaded in each lane. GAPDH was the loading control.
(b) Quantification of each lane. Band intensities were determined by phosphorimaging, corrected relative to GAPDH and standardized to the B78 (-) lane. Light hatch, controls; dark hatch, PALA exposed.

Figure 5.3

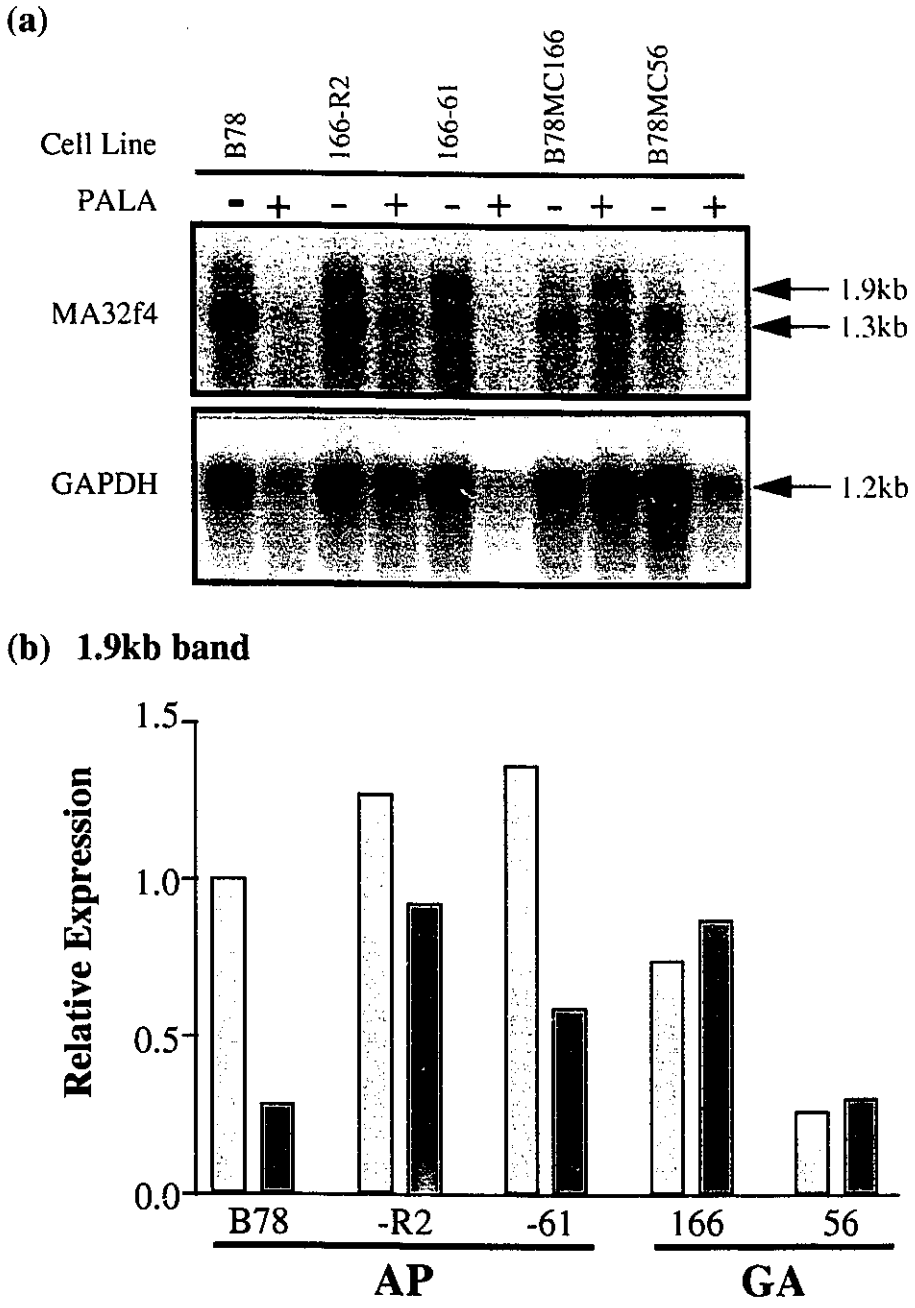
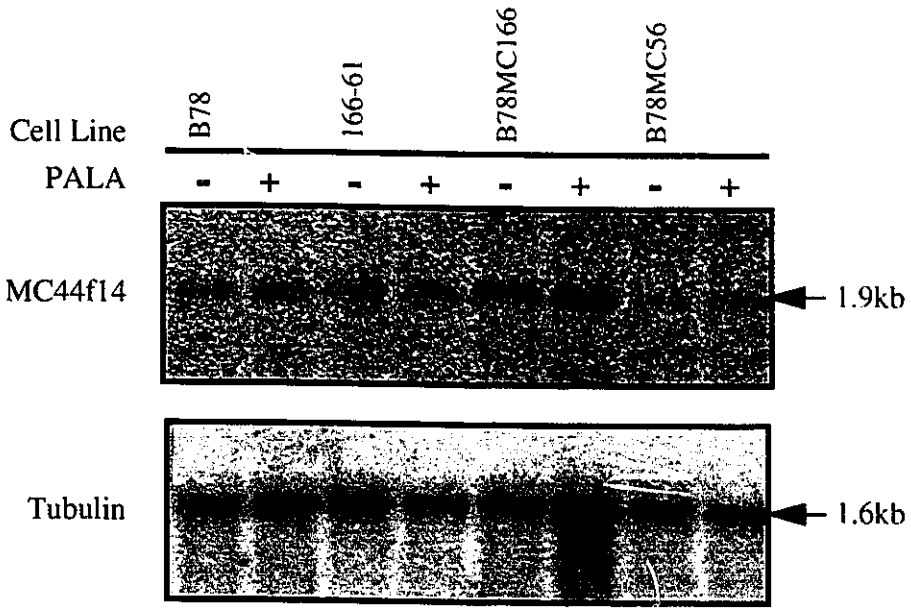

(a)
Cell Line
B78
166-R2
166-61
B78MC166
B78MC56
PALA
- + - + - + - + - +
MA32f4
1.9kb
1.3kb
GAPDH
1.2kb
(b) 1.9kb band
Relative Expression
1.5
1.0
0.5
0.0
B78
-R2
-61
166
56
AP
GA

Figure 5.4 Northern blot analysis of MC44f14.
(a) Northern blot probed with labelled MC44f14. Cells were harvested and RNA was isolated following 2 days of growth in the presence (+) or in the absence (-) of PALA. Approximately 15μg total RNA was loaded in each lane. Tubulin was the loading control.
(b) Quantification of each lane. Band intensities were determined by phosphorimaging, corrected relative to Tubulin and standardized to the B78 (-) lane. Light hatch, controls, dark hatch, PALA exposed.

Figure 5.4

(a)


Cell Line
B78
166-61
B78MC166
B78MC56
PALA
- + - + - + - +
MC44f14
1.9kb
Tubulin
1.6kb

(b)

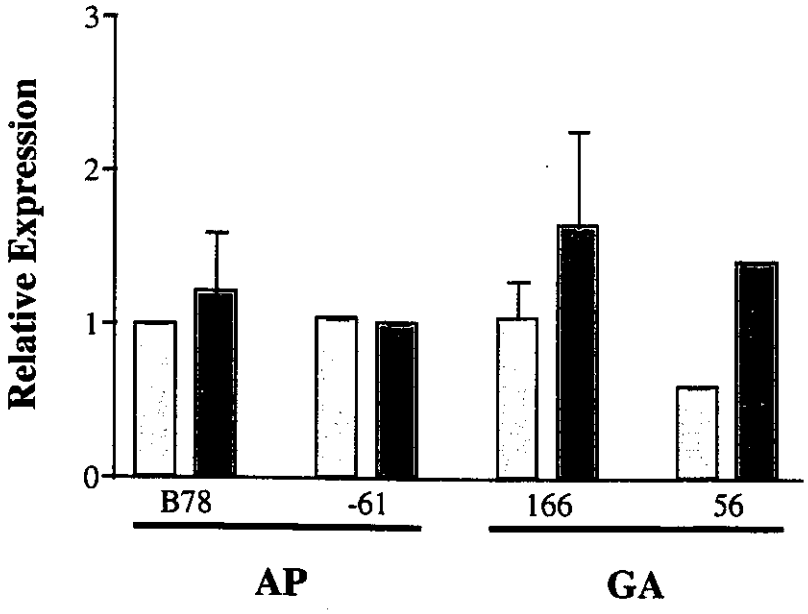
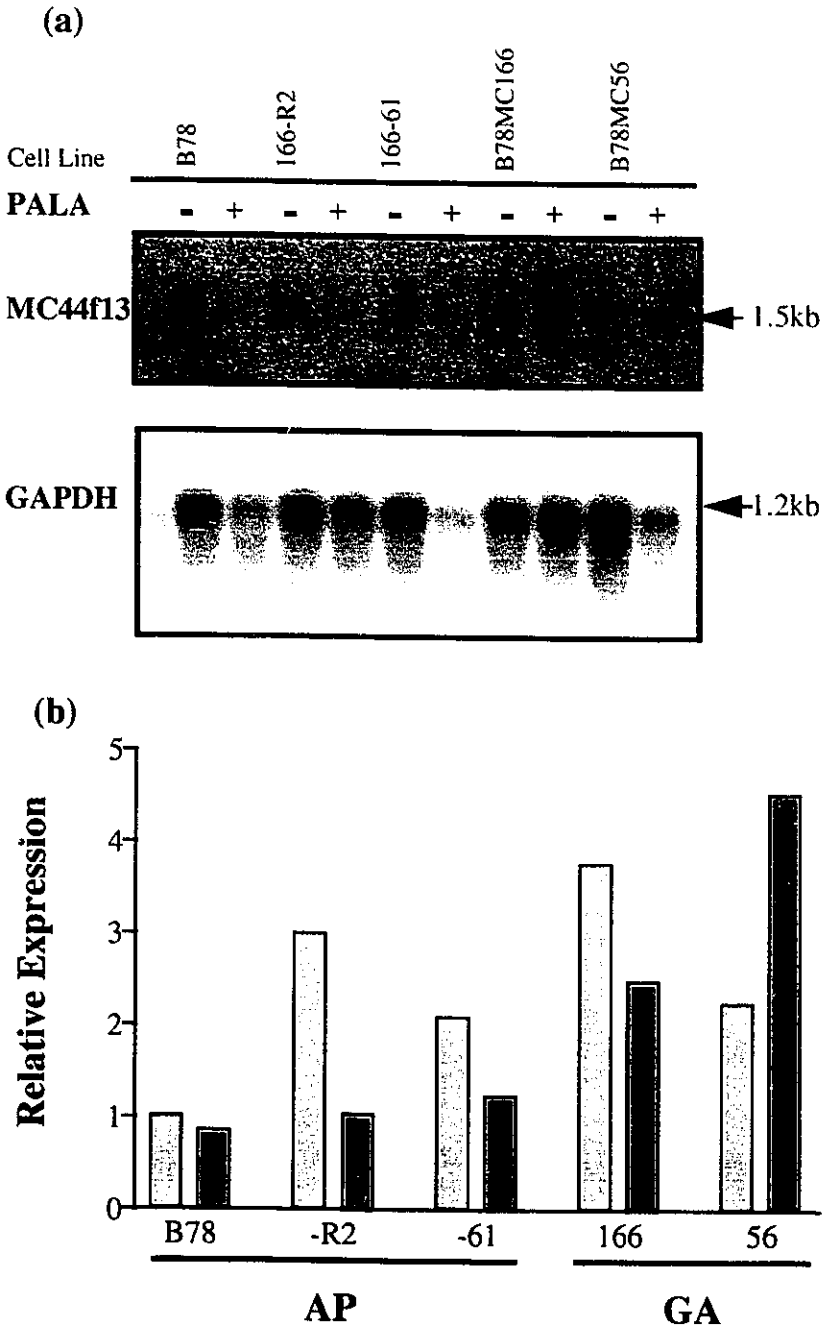

Relative Expression
3
2
1
0
B78
-61
166
56
AP
GA

Figure 5.5 Northern blot analysis of MC44f13.
(a) Cells were harvested and RNA was isolated following 2 days of growth in the presence (+) or in the absence (-) of PALA. Approximately 1μg polyA$^+$ RNA was loaded in each lane. The blot was probed with labelled MA32f4 probe, and GAPDH.
(b) Relative expression was quantified from the ratios of signal intensities between MC44f13 and GAPDH, and standardized to the B78 (-) lane. Light hatch, controls, dark hatch, PALA exposed.

Figure 5.5


(a)
Cell Line
B78
166-R2
166-61
B78MC166
B78MC56
PALA
- + - + - + - + - +
MC44f13
1.5kb
GAPDH
1.2kb
(b)
Relative Expression
0
1
2
3
4
5
B78
-R2
-61
166
56
AP
GA

5.43 Cloning and sequencing

To allow further analyses of the DDRT-PCR fragments, it was necessary to PCR-amplify them using the appropriate primers and subclone them into a cloning vector (pCRII, Stratagene). To ensure that the correct fragment was cloned, multiple colonies were picked and expanded, and plasmid DNA was extracted for analysis. The sizes of the cloned fragments were tested against the known size of the uncloned fragments, by PCR amplification, and by plasmid digests. To identify clones which corresponded to the desired amplifiable fragment, uncloned fragments were PCR-labelled and used as probes on dot blots containing DNA from each cloned fragment (Fig. 5.6). Those clones which were of similar size, and hybridized to the fragment probes from which they were derived were then sequenced (Table 5.3).

The T11 primers, which were complimentary to the poly A tail of mRNAs, also targetted the 3'-UTRs which are known to be high in A and T content (Proudfoot and Brownlee, 1976). Sequencing of the DDRT fragments revealed that most were indeed rich in A and T content A database search (BLASTN and BLASTX) revealed that the majority of fragments isolated were novel; most likely due to a paucity of 3'UTR sequences registered in the gene databases.

Sequencing of MA32f4-2c revealed no extensive homologies at the protein level. However, interestingly, a short region of this clone was homologous to part of exon 2 of murine *bcl-2*, a gene already known to be involved in the suppression of apoptosis (Fig. 5.7b). Sequencing of MC32f7a, an unrelated fragment, revealed identity between f4 and f7 (Fig. 5.7a). Both fragments were derived from amplification of a unique sequence flanked

by DD32 at both the 5' and 3' ends, and therefore the f4/f7 fragments probably originated from internal sequences. The fact that the same cDNA fragment was subcloned independently twice, in combination with the Northern analysis (Fig. 5.3) suggested that it was truly differentially expressed.

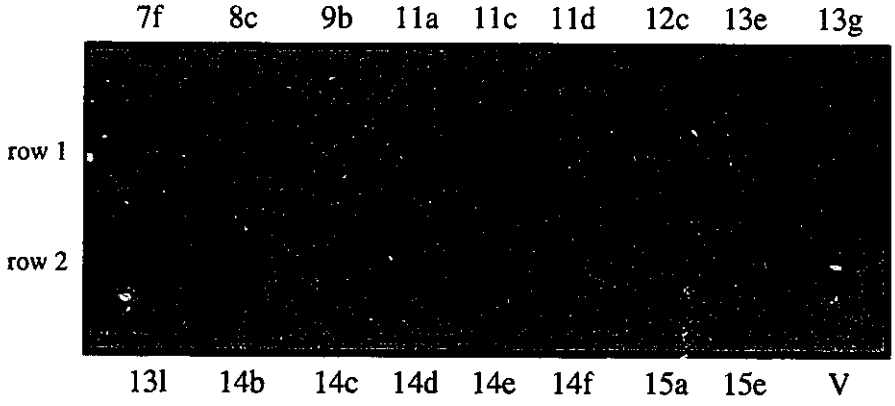
The MC44f14f sequence corresponded to murine *TIS7*, a β-interferon-related gene, possibly suggesting the involvement of this gene in growth arrest and cell survival in this system. The sequence of MC44f14f overlapped with an independently isolated fragment, MG32f8c (Fig. 5.8a and b). This occurred in spite of having been derived from cDNAs made from different anchored polyT primers (T11MC vs. T11MG) and different decamers (DD44 vs. DD32). Sequence analysis of MG32f8c and MC44f14f revealed that these fragments, which were amplified using both different decamers and anchored polyT primers, were nested fragments of *TIS7*, a murine β-interferon related gene (Fig. 5.8c). The longer fragment, MG32f8c, showed homology at both the protein level, and the nucleotide level (Fig 5.8a and 5.8d). MC44f14f was homologous to the 3'UTR of *TIS7*. Furthermore, the size of the RNA revealed by the MC44f14 probe was similar in size to that reported for *TIS7* (between 1.8 and 2.0kb). All of these findings suggest that *TIS7* was up regulated in GA cell lines during PALA exposure.

In contrast to the above finding, the majority of the DDRT fragments were found to be novel, and lacked agreement in the nucleotide and the predicted protein sequences. This was most likely due to the fact that the DDRT methodology was biased in favour of cloning fragments complementary to the 3' UTR of mRNAs. An example is MC44f13, a 290 bp fragment which revealed differential expression in the GA cell lines as compared to the AP

cell lines (Fig. 5.5), and showed no homologies to any known genes (Fig. 5.9). An analysis of its GC and AT content showed that, from the decamer-primed start of the fragment to nucleotide 113, the percentage of A+T bases was approximately equal to the percentage of C+G bases (52.2%). However, as the sequence approached the T11 primer, it became more A+T content rich. The percentage of A+T bases within the final 180 nucleotides leading to the T11 primer rose to 68.9%. As well, an analysis of the possible reading frames of the MC44f13 fragment (Clone Manager 3.11) revealed an open reading frame beginning at the first base of the decamer primer, and ending with the TAA stop codon at nucleotide 128 (shown underlined in Fig. 5.9). This coincides approximately with the change in base composition and may represent the end of the coding region of the message.

Figure 5.6 Dot blot of cloned fragments. Uncloned, labelled MC44f14 was used to probe a dot blot of the subcloned fragments (two rows shown, opposite). The probe hybridized strongly to plasmids MC44f14b, d, e, and f. The probe also hybridized faintly to MG32f8c, which contains a homologous sequence to f14 fragments. MC44f14c, which failed to hybridize was found to be a rearrangement of the vector. The labels refer to the fragment clone (i.e. 8c represents MG32f8, subclone c; please see Table 5.2). V is for vector, negative control.

Figure 5.6



Probe: MC44f14

Table 5.3 Characterization of DDRT-PCR fragments.

Fragment	Size (bp)[a]	Plas mid	Size (bp)[a]	Sequence Homology
MA21f1	500	d	500	N.D.
MA29f2	190/210	b	210	Novel sequence
MA32f3	800	c	800	N.D.
MA32f4	350	2c	320	Novel sequence; same as f7
MA42f5	600	d	400	Plasmid rearranged with vector-novel sequence
MC25f6	200/210	i	255	Novel sequence
MC32f7	300	a	340	Novel sequence; same as f4
MG32f8	400	c	400	TIS7; overlaps f14
MG32f9	150	b	150	Novel sequence
MG44f11	400	c	400	N.D.
MC44f12	500	c	500	N.D.
MC44f13	320	e	290	Novel sequence
MC44f14	150/250	f	280	TIS7; overlaps f8
MC44f15	125/225	a	190	Novel sequence

N.D., not determined

[a] bp size was estimated by gel electrophoresis and comparison with a 100bp ladder, or if sequenced, by counting nucleotides between and including the primers.

Figure 5.7 Sequences of identical cloned fragments MA32f4-2c and MC32f7a.
(a) Sequence of MC32f7a is shown. Lower case letters correspond to primer sequences. MA32f4-2c is shorter, ending at the internal (lower case italicized) primer site.
(b) Murine *bcl-2* exon 2 region (3443-3489) showing homology with f4/f7 clones. Superscript letters represent non-matching *bcl-2* bases.

Figure 5.7

a. MC32f7a

ctgcttgatg GAGGAGAAAT TTGGAAAAAA GAACCTAAAA TTTTAGGTGC AGCAAACACG ATTGTGGAAA GTAATGGAAA ATCTATTGTG G TGTGTCTAA AGCCAATCTG AAATATGAAC TTAACACATC TCGCTCTC CT AGTAGTACTT CTTCAAACTA TCTTGATCAT TTCTGGAGTA TTGTAACTCA GGAAGAAACA ATTTTCTATT TCGATGAAAA TAAAGAAGAA TGGATAAGTT GTGGAAATGG CATAAGTACT CAGTAATCGC CAACATAAAT AGACATTCAG TACTGCATNA AACATCGCCA ACCAAGCAAT TACACA *catcaagcag*ACCA TCAAGCAGTC catcaagcag

b. f4/f7 region of murine bcl-2 exon 2 homology (3' regulatory region)

TG^{G} GT^{G} T^{G} AAGCC^{CT} TCTGAAATATGAACT^{AC} A^{TG} CA^{GA} T^{G} GCTCTC

Figure 5.8 Sequences of *TIS7* related cloned fragments
(a) and (b) are representative sequences of *TIS7* related fragments, MG32f8 and MC44f14 respectively. The italicized lower case letters indicate the f14 priming sites The // in (a) represents an unsequenced region. The region of homology with *TIS7* is underlined.
(c) The extent of homology between f8/f14 and *TIS7* is shown. Superscript letters represent *TIS7* non-matching bases. -//- represents the unsequenced region in f8 and a region of non-homology presumed to be artifactual. The asterixes encompass the region of f8 which is homologous to a part of the coding region of *TIS7*.
(d) Homology between f8 and TIS7 at the protein level is shown. The final amino acid residue is followed by the stop codon, ATC. Note: the nucleotide sequences are shown in anti-sense conformation.

Figure 5.8

a. MG32f8c

gac*gaactac GACGTTGTGA ATTTTGGTAC TTCCAAAGAG CAAAACTTTC CGTAAATATA TTGAGACGTC GAAAGTTTCG AGCTTGTTTT CGAGCTTCGT TTACGGCTCT ATTCTCTCGT CTACAACCTC TTAAGAAC*AT CACAGACTT AAA//CGATG AGATTCATAT ATTCGA AATCTCTGTC AAAA*atagaaccag* TTGAATTTAT TAAAAACTAC ATCCCCACCC AACATAAAAT TAAATTACAT GTCACAATGT TTAATTACTC AAGAAATGAG AGACATTTTT ATTGACCATT GGTGTTTATT TCACAAACAC TA CAAACCA gctttttttttt

b. MC44f14f

ctagtaccag TTGAATTTAT TAAAAACTAC ATCCCCACCC AACATAAAAT TAAATTACAT GTCACAATGT TTAATTACTC AAGAAATAAT ATACATTTTT ATTGACCATT GGTGTTTATT TCACAAACAC TAGTACCAG Gcgttttttt tttt TTTTTT TAGTCTTTAA CCTCTTTAGG AATCGTGTTA AAAGATTTTT TATTGTCTGT AAAAACAATA TGTAATCTGA TAGTCTGTGA CCTGGATGGA ATTACCAATC TGTGAAATA *cgttttttttt*

c. f8/f14 region of homology with TIS7(273-439)

GAACTACGAC GTTGTGAATT TTGGTACTTC CAAAGAGCAA AACTTTCCG TAAATATATT GAGACGTCGA AAGTTTCGAG CTTGTTTTCG AGCTTCGTTT ACGGCTCTAT TCTCTCGTCT ACAACCTCTT AAGAACATC-//-AATCTCTGTC AAAAA^{A} *taga accag*TTGAA TTTATTAAAA ACTACATCCC CACCCAACAT AAAATTAAAT TACATGTCAC AATGTTTAAT TACTCAAGAA AT$^{\wedge}$ AGAGACA TTTTTATTGA CCATTGGTGT TTATTTCACA AACACTACAA ACCAgc$^{\wedge}$ tttt tttttt

d. f8/*TIS7* coding region

```
f8:          * TLKTMKVSRFERHLYNSAAFKARTKARSKCRDKRADVGEFL *
               TLKTMK+SRFERHLYNSAAFKARTKARSKCRDKRADVGEFL
TIS7:     409 TLKTMKISRFERHLYNSAAFKARTKARSKCRDKRADVGEFL 449
```

Figure 5.9 Sequence of novel cloned fragment MC44f13e.
The non-capitalized letters represent primer sites. The 3 underlined bases represent the first stop codon in an open reading frame beginning with the first nucleotide of the sequence. A second clone, MC44f13l (not shown) was identical in sequence to MC44f13e.

Figure 5.9

MC44f13e

ctagtaccag GTTTGTCCAA AACCAAGTCA GAACAAAACT TTTACTTACT GATTACCCAC TGGCGGGAGA GACAGCACAC AGAAACGTTT GCCTCCGGGA CCCTACATGA GACAAAGTAC TGTACTAAAC TAAACTAAAA ACCTTTTTTT TATTACTAAA AATTCGAGAA ACATAGAACC TAAAAAAAGG GGAATTTTAC AAGAATTTCT ATCGTTACCT TGGGTTGAGG TCCCGAMCGA AAAATAGTTG TCAATAAATA TTTAAATTTT AGAGGGACAC cgttttttttt

5.5 Discussion

The recently developed DDRT-PCR technique, was used to screen for differentially expressed cDNAs from cell lines having a differential response to short term PALA treatment. Four different anchored primers were used with 10 different arbitrary decamers, resulting in a cummulative display of approximately 6,000 fragments (about 150 bands/display track). It has been estimated that most cells express approximately 15,000 genes, and therefore, if each cDNA is represented by differential display as an independent band, at least 25 different arbitrary decamers should be used (25 decamers x 4 T11MN X 150 bands). However, the results of this partial screen suggest that two, and possibly more independently primed fragments can be displayed from the same cDNA, and therefore, fewer mRNAs may have been represented on the displays than expected. For example, on two separate occasions, the same cDNA fragment was isolated from independent differential screens (MA32f4/MC32f7 and MG32f8/MC44f14), using different combinations of primers. Thus, different fragments of a particular cDNA may be represented by differential display, several times. For this reason, it is not possible to estimate accurately what percentage of the active genome was screened here.

Fourteen differential fragments were isolated and cloned. Five of the fragments (MA32f4/MC32f7; MG32f8/MC44f14; and MC44f13) were candidates for further analysis, since they showed evidence of differential expression by Northern blot analysis (Table 5.2).

Candidate fragments, MA32f4/MC32f7 and MC44f13 fragment sequences showed no significant homologies to known genes. These fragments can be used however, to screen cDNA libraries and to isolate the full length cDNAs. Also, quantitative PCR could be used

to try to improve the detection of differences in expression of the candidates, in the presence or absence of PALA, and between cell lines.

The MG32f8/MC44f14 fragment was most interesting, due to its homology with the *TIS7* murine β-interferon-related cDNA. *TIS7* was identified as an immediate early gene (IEG) with antiviral properties that was inducible by a variety of treatments, and which showed homologies to human interferon-β1 (Skup et al., 1982; Kujubu et al., 1987; Varnum et al., 1989). IEGs, which include *c-fos* and *c-jun*, are believed to initiate genetic responses to changes in the cellular environment (Gubits et al., 1993). It is also interesting to note that human interferon-β1 (also known as human fibroblast interferon) has an antiproliferative effect on human melanoma cells (B78 is a murine melanoma cell line) and members of the interferon family have been shown to produce this affect via suppression of apoptosis in association with growth arrest (Ida et al., 1982; Graham et al., 1991; Krasagakis et al., 1993; Lotem and Sachs, 1995; Milner et al., 1995). The interferon-mediated growth arrest pathway appears to involve pRB, since suppression of phosphorylation of pRB can be triggered by interferons, possibly via a TGFβ-mediated pathway (Kerr et al., 1989; Resnitzky et al., 1992; Satterwhite and Moses, 1994). Furthermore, the interferon-β1 locus (9p21) is deleted in a number of human cancers, including familial melanomas (Diaz et al., 1988; Cannon-Albright et al., 1992). These findings lend credence to the possibility that murine *TIS7* may be involved in the growth arrest and suppression of apoptosis seen in PALA-exposed B78MC166 and B78MC56, and may be downstream from a chromosome 3 linked signalling gene. A simple test of this hypothesis, would be to observe the response of B78 to recombinant human interferon-β1 (playing the role of *TIS7* induction) in the presence and

absence of PALA. A good candidate for the role of signalling gene may be the TGFβ receptor II (TGFβRII) located on 3p22.

In summary, DDRT-PCR has provided with relative ease, a manageable number of candidate cDNAs, one of which is an interferon-related gene product, that may be involved in the promotion of cellular survival via growth arrest during chemotherapeutic treatment. Further screening, using new arbitrary decamers will lead to the cloning of more candidate genes. It is hoped that eventually, these investigations will lead to an improved understanding of the cellular decisions of life vs. death, as relating to chemotherapy and drug resistance.

5.6 Acknowledgments

I would like to thank the Ottawa Cancer Research Group for access to the phosphorimaging equipment used in this study. I would also like to thank Nathalie Bérubé for her work in adapting the DDRT-PCR methodology in our laboratory, and Dr. Mario Chevrette and Jean McGowan-Jordan for valuable discussions.

Chapter 6. General Conclusions

In this thesis, I have described the construction and analysis of a novel panel of microcell hybrids. This panel is unique, in that a dual selectable marker permits the selective retention or removal of the tagged chromosome as desired. When hybrids from the panel are used as donors in further microcell fusion experiments, reversal of the chromosome-mediated phenotypic change can be confirmed via dominant negative selective removal of the tagged chromosome. This solves the problem of heterogeneity within the genetic background of the recipient cells which could otherwise affect the interpretation of the results.

Although the B78MC panel of microcell hybrids was originally created as an intermediary tool for the transfer of human chromosomes into human tumorigenic cell lines to study and map new human tumour suppressor genes, we believed also that the panel could be used directly to screen for cancer-related genes, affecting a variety of phenotypes. In preliminary studies, not discussed in this thesis, the panel was used to screen for human chromosomes that could directly suppress the tumorigenic phenotype of B78. Tumour formation by subcutaneous injection in nude mice, as well as growth in soft agar were tests used to screen for new tumour suppressor loci. For example, human chromosome 10 appeared to be associated with reduced anchorage dependent growth by soft agar assay, and chromosome 12 was implicated in producing delays in tumour formation in nude mice. However, only a small fraction of the panel could be screened at a time using these techniques. Also, because these procedures were time consuming, extensive passaging occurred, during which time the phenotypes altered, most likely due to loss of untagged chromosomes associated with phenotypic changes. This resulted in difficulty in verifying the influence of a particular human chromosome. One alternative would have been to clone

purify the entire panel, and identify the individual tagged human chromosomes in each hybrid, to eventually obtain a complete panel of tagged human chromosomes from 1 to Y: a very long, and unimaginative preliminary step. The alternative chosen here, and described in this thesis, was to rapidly screen the entire panel at early passage for alterations in chemotherapeutic drug responses, blindly select the hybrids showing the desired response, and identify the specific human chromosomes involved in the response.

The ability of cancer cells to undergo apoptosis during chemotherapeutic treatments contrasts dramatically with the tendency of most normal cells to respond to the same treatments with growth arrest. The molecular mechanisms which influence these alternative responses are therefore important to determine, in order to improve the efficacy of cancer treatments, and to safeguard against tumour cell resistance to apoptosis. The tumour suppressor gene, *p53* has been pinpointed as a strong regulator of growth during stressful conditions in most normal cell types, and as an inducer of apoptosis in most transformed cell types. It has been suggested that p53 may simultaneously upregulate the growth arrest pathway (via induction of p21) and the apoptosis pathway (via induction of Bax), and depending upon the presence or absence of additional factors, one pathway may take precedence over the other (Han et al., 1996). However, the actual molecular conditions required by the cell (in the presence of $p53^{+/+}$) for selection of one course of action over the other have not yet been clarified.

p53-directed apoptosis is not the only line of defence against DNA damage. For example, following anticancer drug treatment, cells deficient in p21, a component of the p53-dependent growth arrest and apoptosis pathway, arrest briefly, but then continue to undergo

DNA replication resulting in malformed polyploid nuclei that eventually die via apoptosis (Waldman et al., 1996). This suggests a model whereby p53 and its downstream components direct a first tier of defence against DNA damage by prevention of DNA replication through G1 arrest or apoptosis, depending upon the cellular context. A second tier of damage control, which is p53-independent, may however be activated following transmission of damage during replication, thus eliminating transmitted DNA damage by apoptosis. However, other modes of survival exist, since cancer cells often manage to survive DNA-damaging chemotherapeutic and radiation treatments. Cancer cell survival has been attributed to increased expression of drug resistance genes which limit the ability of the treatment to induce damage. An alternate form of cancer cell survival may be via dormancy - the ability to lie low during chemotherapeutic treatment. However, the molecular basis of *p53*-independent dormancy is difficult to ascertain, since cancer cells display myriad mutations affecting the ability of the cell to survive under the diverse conditions of tumour initiation, progression, metastasis and finally, therapeutic interventions.

In this study, whole cell hybrids between B78, a tumorigenic murine cell line which responded to the chemotherapeutic agent PALA with apoptosis, and human fibroblasts which responded with growth arrest, were shown to be suppressed for ability to undergo apoptosis in response to PALA. To attempt to identify individual human chromosomes which affected the cellular decision to undergo apoptosis or growth arrest during PALA exposure, the B78MC panel of microcell hybrids was screened for evidence of differential response to PALA exposure. Using this technique, the short arm of human chromosome 3 was shown to be associated with increased resistance to induction of apoptosis via G1 arrest.

The primary goal following this finding was to attempt to clone the gene or cDNA located on human chromosome 3 and responsible for the growth arrest and apoptosis suppression phenotype. Due to the availability of two hybrids which were almost identical, except for small, microdeleted regions of human chromosome 3 (B78MC166 and B78MC166-61), it was possible to attempt to clone the desired cDNA immediately, via isolation of differentially expressed sequences. The differential display reverse transcriptase method was used in a partial analysis of differential sequences between B78, B78MC166-61 and B78MC166, and resulted in the isolation of 15 differentially displayed cDNAs. Five of these cDNAs were shown here to be candidates for the growth arrest pathway instigated by an as yet identified chromosome 3-linked gene.

One of the candidate cDNAs identified here was homologous to a portion of the murine interferonβ-related gene, *TIS7*. This is an interesting finding, given that interferons are known to be cytostatic, and are particularly effective against melanoma, which is the genetic background of the hybrids (Krasagakis et al., 1993). The differential upregulation of this murine gene in the growth arrested cells may be due to upstream influence by a gene residing on human chromosome 3.

The short arm of human chromosome 3 is a hot spot for tumour suppressor research. A number of candidate tumour suppressor genes have been mapped to chromosome 3, including *VHL*, at 3p25.2, a small cell lung and renal cell carcinoma gene at 3p21; and an ovarian cancer gene at 3p25, 3p23 and 3p21 (Rimessi et al., 1994; Smith et al., 1995). Also, there are several interesting candidate genes which may be involved in the chromosome 3-mediated response described in this thesis. For example, the xeroderma pigmentosa (XP)

complementation group C (*XPCC*) gene maps to 3p25 (Legerski et al., 1994). The XP genes are thought to be responsible for nucleotide excision repair, which is a G2 checkpoint process (Chapter 1). XP is a sun hypersensitivity syndrome, resulting in skin cancers, and patients with the *XPCC* mutation are particularly prone to development of malignant melanoma (Lynch et al., 1984). Since the B78 cell line is a melanoma cell line, it is possible that a mutation on the murine homolog of *XPCC* was complemented by the human *XPCC* on chromosome 3 and contributed in some way to promoting drug-induced growth arrest.

Another interesting candidate gene on chromosome 3p21-p14, is the protein-tyrosine phosphatase gamma (*PTPG*) gene. This gene has been shown to be deleted in several renal and lung carcinoma cell lines (LaFogia et al., 1991). The PTPG protein is a receptor type protein phosphatase, and it is interesting to speculate that such a protein may be involved in opposing the activities of kinases involved in transducing proliferative signalling in cancer cells. The introduction of this locus into B78 via chromosome 3 may provide the opposition to growth necessary to promote G1 arrest, and thereby avoid apoptosis induction. It is also interesting to note that *PTPG* maps to one of the microdeleted regions in 166-61.

Finally, a third possible candidate gene on chromosome 3 is transforming growth factor-beta receptor type II (*TGFβRII*). *TGFβRII*, which maps to 3p22, mediates growth inhibition via the TGFβ signalling pathway resulting in the hypophosphorylation of pRB (Chen et al., 1993; Mathew et al., 1994 and Fig. 6.1). This pathway may be involved in the PALA-responsive growth arrest pathway in the chromosome 3 hybrids, since functional pRB has previously been shown to suppress apoptosis in addition to inhibiting proliferation. It is possible that the mouse *TGFβRII*, which has been found to be inactivated in a subset of

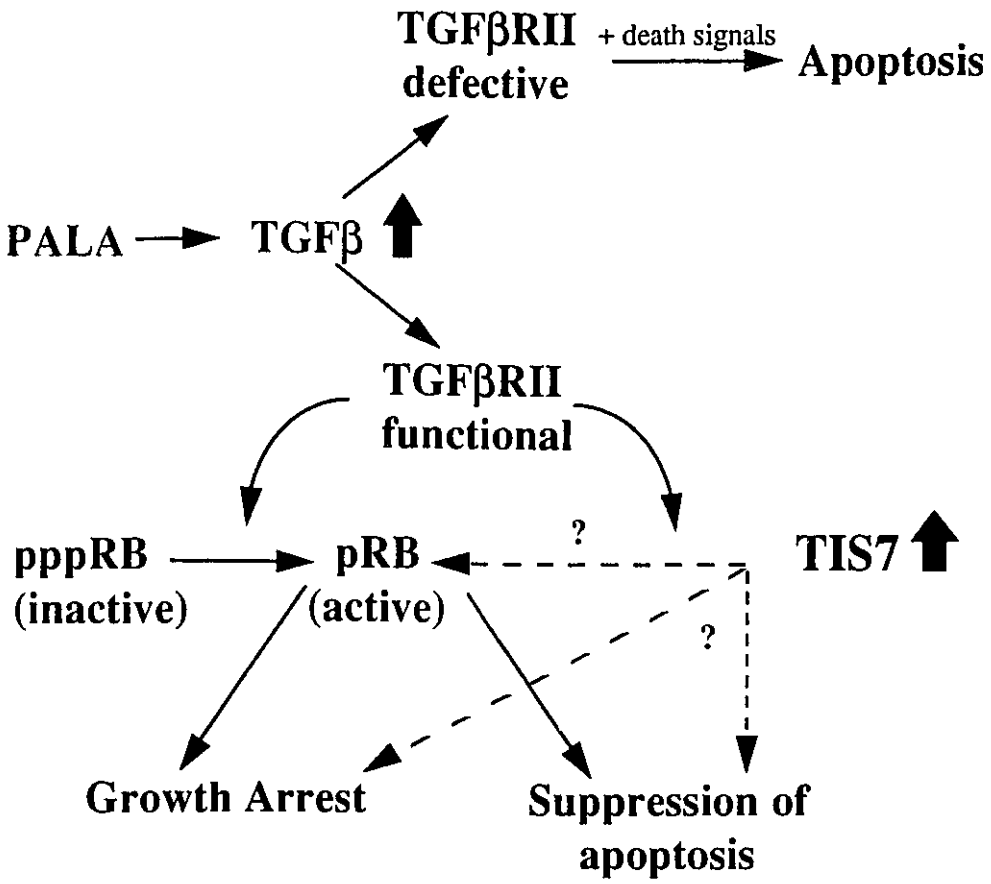
cancers, is down regulated in B78, and the re-introduction of its human counterpart resulted in re-establishing the TGFβ/pRB link in these cells (Markowitz et al., 1995). It is interesting to note that TGFβ activity has been previously shown to mediate the cytostatic effects of human interferons (Kerr et al., 1989). Furthermore, TGFβ has been shown to be a potent inhibitor of melanoma cell proliferation, although it has also been shown to stimulate some melanoma cells to grow (Newman, 1990; Rodeck et al., 1994; Krasagakis et al., 1995). It has also been suggested that insensitivity to TGFβ growth regulation may be involved in melanoma oncogenesis (Krasagakis et al., 1994). Since the murine interferon-related gene *TIS7* was upregulated during PALA-induced growth arrest and apoptosis suppression (Chapter 5), one wonders if there is a relationship between the introduction of *TGFβRII* on chromosome 3, upregulation of *TIS7*, and the ability of the cell to undergo growth arrest during PALA exposure. As *TIS7* was originally cloned due to its ability to be induced by the tumour promoter TPA (Varnum et al., 1989), and *TGFβ* has been shown to induce immediate early genes via the TPA responsive element (TRE) (de Groot and Kruijer, 1990), one can envision a pathway whereby PALA exposure induces a *TGFβ* growth arrest pathway in cells with intact *TGFβRII*, possibly mediated by *TIS7* (Fig. 6.1). The evaluation of the status of *TGFβRII* in B78MC166, and its revertant subclone 166-61 should provide an answer to this question. As well, since human *TIS7* (*hTIS7*) has recently been cloned and mapped to human chromosome 3, determining the expression pattern of *hTIS7* (using a human specific probe) could further clarify the model presented in Figure 6.1.

Future considerations for the continuation of this project will be to test the ability of some of these already cloned candidate genes to modulate the responses of B78 to

chemotherapeutic agents, such as PALA. Also, further analysis of the novel differentially expressed sequences identified by DDRT-PCR, such as f4/f7, and MC44f13 and continued screening with more arbitrary decamers will hopefully lead to the identification of the human chromosome 3 gene responsible for growth arrest and apoptosis suppression in these hybrids.

Figure 6.1 Hypothetical model of PALA-induced *TGFβ* growth arrest pathway. According to this model, PALA exposure triggers an autocrine production of TGFβ. Cells which lack functional *TGFβRII* (B78?), fail to undergo growth arrest, and follow an alternate apoptosis pathway. Cells containing functional *TGFβRII* (B78MC166?) are capable of transducing the *TGFβ* signal, possibly via induction of *TIS7*. Growth arrest and apoptosis suppression follows, most likely via activation of pRB.

Figure 6.1


TGFβRII
defective
+ death signals
Apoptosis
PALA
TGFβ
TGFβRII
functional
pppRB
(inactive)
pRB
(active)
?
TIS7
?
Growth Arrest
Suppression of
apoptosis

References

Aboussekhra, A., and R. D. Wood (1994). Repair of UV-damaged DNA by mammalian cells and *Saccharomyces cerevisiae*. Curr. Op. Genet. Dev. **4**:212-220.

Al-Khodairy, F., and A. M. Carr (1992). DNA repair mutants defining G_2 checkpoint pathways in *Schizosaccharomyces pombe*. EMBO **11**:1343-1350.

Amon, A., S. Irniger, and K. Nasmyth (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell **77**:1037-1050.

Almendral, J. M., D. Sommer, J. Perera, J. Burckhardt, H. Macdonald-Bravo, and R. Bravo (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140-2148.

Anderson, M. J., and E. J. Stanbridge (1993). Tumor suppressor genes studied by cell hybridization and chromosome transfer. Faseb J. **7**:826-883.

Armitage, P., and R. Doll (1954). The age distribution of cancer and a multistage theory of carcinogenesis. Br. J. Cancer **8**:1-12.

Bakhshi, A., J. P. Jenson, P. Goldman, J. J. Wright, O. W. McBride, A. L. Epstein, and S. J. Korsmeyer (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around J_H on chromosome 14 and near a transcriptional unit on 18. Cell **41**:899-906.

Bandara, L. R., V. M. Buck, M. Zamanian, L. H. Johnston, and N. B. La Thangue (1993). Functional synergy between DP-1 and E2F-1 in the cell cycle regulating transcription factor DRTF1/E2F. EMBO J. **12**:4317-4324.

Banerjee, A., H. -J. Xu, S. -X. Hu, D. Araujo, R. Takahashi, E. J. Stanbridge, and W. F. Benedict (1992). Changes in growth and tumorigenicity following reconstitution of retinoblastoma gene function in various human cancer cell types by microcell transfer of chromosome 13. Cancer Res. **52**:6297-6304.

Barrett, J. C., and P. O. Ts'o (1978). Evidence for the progressive nature of neoplastic transformation in vitro. Proc. Natl. Acad. Sci. U.S.A. **75**:3761-3765.

Barski, G. and F. Cornefert (1962). Characteristics of "hybrid"-type clonal cell lines obtained from normal and neoplastic cell lines obtained from mixed culture *in vitro*. J. Natl. Cancer Inst. **28**:801-821.

Bates, S., L. Bonetta, D. Macallan, D. Pary, A. Holder, C. Dickson, and G. Peters. (1994). CDK6 (PLSTIRE) and CDK4 (PSKJ3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. Oncogene **9**:71-79.

Bauer, D., H. Müller, J. Reich, H. Riedel, V. Ahrenkiel, P. Warhoe, and M. Strauss (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucleic Acids Res. **21**:4272-4280.

Bérubé, N. G., M. D. Speevak, and M. Chevrette (1994). Suppression of tumorigenicity of human prostate cancer cells by introduction of human del(12)(q14). Cancer Res. **54**:3077-3081.

Bevilacqua, G., and M. A. Caligo (1991). Suppressor genes in tumor progression. *In:* Hereditary Tumors, Eds.: M. L. Brandi, R. White, pp 199-205.

Biedler, J. L (1992). Genetic aspects of multidrug resistance. Cancer **70:**1799-1809.

Bishop, J. (1987). The molecular genetics of cancer. Science **235:**305-11.

Birnboim, H. C. and J. Doly (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

Boise, L. H., M. Gonzalez-Garcia, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nunez, and C. B. Thompson (1993). blc-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell **74:** 597-608.

Bookstein, R., and D. C. Allred (1993). Recessive oncogenes. Cancer **71:**1179-1186.

Bornens, M. (1992). *In:* The Centrosome, ed.:V. I Kalnins (Academic Press, New York) pp1-43.

Bortner, C. D., N. B. E. Oldenburg, and J. A. Cidlowski (1995). The role of DNA fragmentation in apotosis. Trends Cell Biol. **5:**21-26.

Bryant, P. J. (1993). Towards the cellular functions of tumour suppressors. Trends in Cell Biol. **3:**31-35.

Buchkovich, K., L. A. Duffy, and E. Harlow (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097-1105.

Buckley, M. F., K. J. Sweeney, J. A. Hamilton, R. L. Sini, D. L. Manning, R. I. Nicholson, A. deFazio, C. K. Watts, E. A. Musgrove, and R. L. Sutherland (1993). Expression and amplification of cyclin genes in human breast cancer. Oncogene **8:**2127-2133.

Cannon-Albright, L. A., D. E. Goldgar, L. J. Meyer, C. M. Lewis, D. E. Anderson, J. W. Fountain, M. E. Hegi, R. W. Wiseman, E. M. Petty, A. E. Bale, O. I. Olopade, M. O. Diaz, D. J. Kwiatkowski, M. W. Piepkorn, J. J. Zone, and M. H. Skolnick (1992). Assignment of a locus for familial melanoma, MLM to chromosome 9p13-p22. Science **258:**1148-1152.

Cao, L., B. Faha, M. Dembski, L.-H. Tsai, E. Harlow, and N. Dyson (1992). Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature **355:**176-179.

Caspersson, T., M. Hulten, J. Lindsten, and L. Zech (1970). Distinction between extra G like chromosomes by quinacrine mustard fluorescence analysis. Exp. Cell Res. **63:**240-243.

Chabner, B. A., and C. E. Myers (1989). Clinical pharmacology of cancer chemotherapy, p. 349-395. *In:* Cancer: Principles and Practice of Oncology, Eds.: V. T. DeVita, S. Hellman, and S. A. Rosenberg (J. B. Lippincott, Philadelphia).

Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins (1991). The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053-1061.

Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. Y. Wang, and W.-H. Lee (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1193-1198.

Chen, R.-H., R. Ebner, and R. Derynck (1993). Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. Science **260:**1335-1338.

Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell **81:**505-512.

Chiou, S.-K, L. Rao, and E. White (1994). Bcl-2 blocks p53-dependent apoptosis. Mol. Cell. Biol. **14:**2556-2563.

Clarke, A. R., E. R. Maandag, M. van Roon, N. M. T. van der Lugt, M. van der Valk, M. L. Hooper, A. Berns, and H. T. Riele (1992). Requirement for a functional Rb-1 gene in murine development. Nature **359:**328-330.

Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper, and A. H. Wyllie (1993). Thymocyte apoptosis induced by p-53-dependent and independent pathways. Nature **362:** 849-852.

Cleary, M. L., and J. Sklar (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. USA **82:**7439-7443.

Cochran, B. H., A. C. Reffel, and C. D. Stiles (1983). Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33:**939-947.

Cole, S. P., G. Bhardwaj, J. H. Gerlach, J. E. Mackie, C. E. Grant, K. C. Almquist, A. J. Stewart, E. U. Kurz, A. M. Duncan and R. G. Deeley (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science **258:**1650-1654.

Collins, M. K. L., J. Marvel, P. Malde, and A. Lopez-Rivas (1992). Interleukin 3 protects bone marrow cells from apoptosis induced by DNA damaging agents. J. Exp. Med. **176:**1043-1051.

Compton, M. M. (1992). A biochemical hallmark of apoptosis: Internucleosomal degradation of the genome. Cancer Metastasis Rev. **11:**105-119.

Cooper, G. M. (1982). Cellular transforming genes. Science **217:**801-806.

Cox, L. S., and D. P. Lane (1995). Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. BioEssays **17:**501-508.

Craig, R. W., and R. Sanger (1985). Suppression of tumorigenicity in hybrids of normal and oncogene-transformed CHEF cells. Proc. Natl. Acad. Sci. U.S.A. **82:**2062-2066.

Croce, C. M. (1984). Gene regulation in the expression of malignancy. Cancer Surveys **3:**287-298.

Cuthbert, A. P., D. A. Trott, R. M. Ekong, S. Jezzard, N. L. England, M. Themis, C. M. Todd and R. F. Newbold (1995). Construction and characterization of a highly stable human: rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenet. Cell Genet. **71:** 68-76

Dalton, S. (1992). Cell cycle regulation of the human cdc2 gene. EMBO J. **11:**1797-804.

Debbas, M., and E. White (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:** 546-554.

DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms,C.-M. Huang, and D. M. Linvingston (1989). The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085-1095.

DeCaprio, J. A., Y. Furukawa, F. Ajchenbaum, J. D. Griffin, and D. M. Livingston (1992). The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. Proc. Natl. Acad. Sci. USA **89:**1795-1798.

de Groot, R.P., and W. Kruijer (1990). Transcriptional activation by TGF beta 1 mediated by the dyad symmetry element (DSE) and the TPA responsive element (TRE). Biochem. Biophys. Res. Commun. **168:**1074-1081.

de Klein, A., A. G. van Kessel, G. Grosveld, C. R. Bartram, A. Hagemeijer, D. Bootsma, N. K. Spurr, N. Heisterkamp, J. Groffen, and J. R. Stephenson (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukemia. Nature **300:**765-767.

Del Sal, G. L., M. E. Ruaro, L. Philipson, and C. Schneider (1992). The growth arrest-specific gene, gas1, is involved in growth suppression. Cell **70:**595-607.

De Vita, V. T. 1985. Principles of chemotherapy, *In:* Cancer: Principles and Practice of Oncology, Eds., V.T. De Vita, S. Hellman, and S. A. Rosenberg (J.B. Lippincott, Philadelphia) pp 257-285.

Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins (1992). A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: $p33^{cdk2}$ is a component of the E2F-cyclin A complex. Cell **68:**167-176.

Diaz, M. O., S. Ziemin, M. M. Le Beau, P. Pitha, S. D. Smith, R. R. Chilcote, J. D. Rowley (1988). Homozygous deletion of the alpha- and beta(1)-interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. **85:**5259-5263.

Dive, C., and J. A. Hickman (1991). Drug-target interactions only the first step in the commitment to a programmed cell death? Br. J. Cancer **64:** 192-196.

Doherty, P. C. (1993). Cell-mediated cytotoxicity. Cell **75:**607-612.

Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. J. Montgomery, J. S. Butel, and A. Bradley (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature **356:**215-221.

Donohue, P. J., G. F. Alberts, B. S. Hampton, and J. A. Winkles (1994). A delayed-early gene activated by fibroblast growth factor-1 encodes a protein related to aldose reductase. J. Biol. Chem. **269:**8604-8609.

Dorin, J. R., E. Emslie, D. Hanratty, M. Farrall, J. Gosden, and D. J. Porteous (1992). Gene targeting for somatic cell manipulation: rapid analysis of reduced chromosome hybrids by *Alu*-PCR fingerprinting and chromosome painting. Hum. Molec. Genet. **1:**53-59.

Dowdy, S. F., P. W. Hinds, K. Louis, S. I. Reed, A. Arnold, and R. A. Weinberg (1993). Physical interactions of the retinoblastoma protein with human cyclins. Cell **73:**499-511.

Downes, C. S., D. J. Clarke, A. M. Mullinger, J. F. Giménez-Abián, A. M. Creighton, and R. T. Johnson (1994). A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells. Nature **372:**467-470.

Dulic, V., E. Lees, and S. I. Reed (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. Science **257:**1958-1961.

Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge, and S. I. Reed (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell **76:**1013-1023.

Dunaief, J. L., B. E. Strober, S. Guha, P. A. Khavari, K. Ålin, J. Luban, M. Begemann, G. R. Crabtree, and S. P. Goff (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell **79:**119-130.

Dunlop, M. G. (1992). Colorectal cancer genetics. Sem. Cancer Biol. **3:**131-140.

Dunphy, W.G. (1994). The decision to enter mitosis. Trends Cell Biol **4:**202-207.

Dynlacht, B. D., O. Flores, J. A. Lees, and E. Harlow (1994). Differential regulation of E2F trans-activation by cyclin/cdk2 complexes. Genes Dev. **8:**1772-1786.

Eastman, A. 1990. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells **2:**275-279.

Ege, T., N. R. Ringertz, H. Amberg, and E. Sidebottom (1977). Preparation of microcells. Methods Cell Biol. **15:**339-357.

El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, B. Vogelstein (1993). WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817-825.

El-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, K. G. Wiman, W. E. Mercer, M. B. Kastan, K. W. Kohn, S. J. Elledge, K. W. Kinzler, and B. Vogelstein (1994). WAF1/Cip1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. **54:**1169-1174.

Elledge, S. J., and M. R. Spottswood (1991). A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1. EMBO J. **10:**2653-2659.

Enarl, M., H. Hug, and S. Nagata (1995). Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature **375:** 78-81.

Endicott, J. A., and V. Ling. (1989). The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. **58:**137-171.

Enoch, T., and P. Nurse (1991). Coupling M phase and S phase: Controls maintaining the dependence of mitosis on chromosome replication. Cell **65:**921-923

Evan G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, S. Z. Penn, and D. C. Hancock (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:** 119-128.

Evans, E. P., M. D. Burtenshaw, B. B. Brown, R. Hennion, and H. Harris (1982). The analysis of malignancy by cell fusion. IX. Reexamination and clarification of the cytogenetic problem. J. Cell Sci. **56:**113-130.

Ewen, M. E., Y. Xing, J. Bentley-Lauwrence, and D. M. Livingston (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155-1164.

Ewen, M. E., B. Faha, E. Harlow, and D. M. Livingstone (1992). Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. Science **255:**85-90

Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Linvingston (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**487-497.

Faha, B., M. E. Ewen, L. H. Tsai, D. M. Livingstone, and E. Harlow (1992). Interaction between human cyclin A and adenovirus E1A-associated p107 protein. Science **255:**87-90.

Fanidi, A., E. A. Harrington, and G. I. Evan (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature **359:**554-556.

Fath, I., F. Schweighoffer, I. Rey, M.-C. Multon, J. Boiziau, M. Duchesne, and B. Tocque (1994). Cloning of a Grb2 isoform with apoptotic properties. Science **264:**971-974.

Feilotter, H., C. Lingner, R. Rowley, and P. G. Young (1992). Regulation of the G_2-mitosis transition. Biochem. Cell Biol. **70:**954-971.

Feilotter, H., P. Nurse, and P. G. Young (1991). Genetic and molecular analysis of cdr/nim1 in *Schizosaccharomyces pombe*. Genetics **127:**309-318.

Ferguson, P. J. (1991). The role of gene amplification in clinical resistance to chemotherapy: a review. J. Otolaryngol. **20:**130-136.

Fernandes-Alnemri, T., G. Litwack and E. S. Alnemri (1994). CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J. Biol. Chem. **269:**30761-30764.

Fernandes-Alnemri, T., G. Litwack and E. S. Alnemri (1995). Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res **55:** 2737-2742

Fesus, L. (1993). Biochemical events in naturally occurring forms of cell death. FEBS Lett. **328:**1-5.

Fisher, D. E. (1994). Apoptosis in cancer therapy: crossing the threshold. Cell **78:**539-542.

Fisher, R. P., and D. O. Morgan (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. Cell **78:**713-724.

Fisher, T. C., A. E. Milner, C. D. Gregory, A. L. Jackman, W. Aherne, J. A. Hartley, C. Dive, and J. A. Hickman (1993). bcl-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. Cancer Res. **53:**3321-3326.

Flores-Rozas, H., Z. Kelman, F. B. Dean, Z. Pan, W. J. Harper, S. J. Elledge, M. O'Donnell, and J. Hurwitz (1994). Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase and holoenzyme. Proc. Natl. Acad. Sci. **91:**8655-8659.

Fornace, Jr., A. J., I. Alamo, Jr., and M. C. Hollander (1988). DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA **85:**8800-8804.

Fournier, R. E. K., and F. H. Ruddle (1977). Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. U.S.A. **74:**319-323.

Fournier, R. E. K. (1981). A general high-efficiency procedure for production of microcell hybrids. Proc. Natl. Acad. Sci. USA **78:**6349-6353.

Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert, T. P. Dryja (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature **323:**643-646.

Friedberg, E. C., G. C. Walker, and W. Siede (1995). DNA repair and mutagenesis (Washington D.C.: American Society for Microbiology) pp662-669.

Fritsche, M., C. Haessler, and G. Brandner (1993). Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene **8:**307-318.

Fukasawa, K., T. Choi, R. Kuriyama, S. Rulong, and G. F. Vande Woude (1996). Abnormal centrosome amplification in the absence of p53. Science **271:**1744-1747.

Fung, Y.-K. T., A. L. Murphree, A. T'Ang, J. Qian, S. H. Hinrichs, and W. F. Benedict (1987). Structural evidence for the authenticity of the human retinoblastoma gene. Science **236:**1657-1661.

Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. **119:**493-501.

Gheira, J. B., H. E. Richardson, K. Suzimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed (1991). A cyclin B homolog in *S. cerevisiae*: chronic activation of the cdc28 protein kinase by cyclin prevents exit from mitosis. Cell **65:**163-174.

Girling, R., J. F. Partridge, L. R. Bandara, N. Burden, N. F. Totty, J. T. Hsuan, N. B. La Thangue (1993). A new component of the transcription factor DRTF1/E2F. Nature **362:**83-87.

Glotzer, M., A. W. Murray, and M. W. Kirschner (1991). Cyclin is degraded by the ubiquitin pathway. Nature **349:**132-138.

Gourdeau, H., and P. R. Walker (1994). Evidence for trans regulation of apoptosis in intertypic somatic cell hybrids. Mol. Cell. Biol. **14:** 6125-6134.

Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe and A. J. Giaccia (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature **379**:88-91.

Graham, G. M., L. Guarini, T. A. Moulton, S. Datta, S. Ferrone, P. Giacomini, R. S. Kerbel, and P. B. Fisher (1991). Potentiation of growth suppression and modulation of the antigenic phenotype in human melanoma cells by the combination of recombinant human fibroblast and immune interferons. Cancer Immunol. Immunother. **32**:382-390.

Guadagno, T. M., M. Ohtsubo, J. M. Roberts, and R. K. Assoian (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. Science **262**:1572-1575.

Gubits, R. M., C. R. Geard, and P. B. Schiff (1993). Expression of immediate early genes after treatment of human astrocytoma cells with radiation and taxol. Int. J. Radiat. Oncol. Biol. Phys. **27**:637-642.

Gupta, K., M. Chevrette, and D. A. Gray (1994). The Unp proto-oncogene encodes a nuclear protein. Oncogene **9**:1729-1731.

Haas-Kogan, D. A., S.C. Kogan, D. Levi, P. Dazin, A. T'Ang, Y-K. T. Fung, and M. A. Israel (1995). Inhibition of apoptosis by the retinoblastoma gene product. EMBO J. **14**:461-472.

Haber, D. A., and D. E. Housman (1991). Rate-limiting steps: the genetics of pediatric cancers. Cell **64**:5-8.

Hall, P. A., P. H. McKee, H. P. Menage, R. Dover, and D. Lane (1993). High levels of p53 protein in UV-irradiated normal human skin. Oncogene **8**:203-207.

Han, J., P. Sabbatini, D. Perez, L. Rao, D. Modha, and E. White (1996). The E1B 19K protein blocks apoptosis by intereacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev. **10**:461-477.

Hannon, G. J., and D. Beach (1994). $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. Nature **371**:257-261.

Hannun, Y. A., and L. M. Obeid (1995). Ceramide: an intracellular signal for apoptosis. TIBS **20**:73-77.

Hansen, M. F., and W. K. Cavanee (1988). Tumor suppressors: recessive mutations that lead to cancer. Cell **53**:172-173.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, S. J. Elledge (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75**:805-806.

Harper, J. W., S. J. Elledge, K. Keyomarsi, B. Dynlacht, L. H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, L. Connell-Crowley, E. Swindell, M. P. Fox, and N. Wei (1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell **6**:387-400.

Harrington, E. A., A. Fanidi, and G. I. Evan (1994). Oncogenes and cell death. Curr. Opin. Genet. Dev. **4**:120-129.

Harris, H., and G. Klein (1969). Malignancy of somatic cell hybrids. Nature **224**:1314-1316.

Harris, H., O. J. Miller, G. Klein, P. Worst, and T. Tachibana (1969). Suppression of malignancy by cell fusion. Nature **223:**363-368.

Harris, H. (1971). Cell fusion and the analysis of malignancy: The Croonian lecture. Proc. Roy. Soc. B **179:**1-20.

Hartwell, L. H. (1967). Macromolecular synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93:**462-466.

Hartwell, L. H., J. Culotti, and B. J. Reid (1970). Genetic control of the cell division cycle in yeast. I. Detection of mutants. Proc. Nat. Acad. Sci. U.S.A. **66:**352-359.

Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid (1974). Genetic control of the cell division cycle in yeast. Science **183:**46-51.

Hartwell, L. H., and T. A. Weinert (1989). Checkpoints: controls that ensure the order of cell cycle events. Science **246:**629-634.

Hartwell, L. H. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell **71:**543-546.

Hartwell, L. H., and M. B. Kastan (1994). Cell cycle control and cancer. Science **266:**1821-1828.

Haupt, Y., S. Rowan and M. Oren (1995). p53-mediated apoptosis in HeLa cells can be overcome by excess pRB. Oncogene **10:**1563-1571.

Heald, R., M. McLoughlin, and F. McKeon (1993). Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. Cell **74:**463-474.

Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis (1984). Isolation of cDNA clones encoding T cell specific membrane-associated proteins. Nature **308:**149-153.

Helin, K., and E. Harlow (1993). The retinoblastoma protein as a transcriptional repressor. Trends Cell Biol. **3:**43-46.

Helin, K., E. Harlow, and A. Fattaey (1993). Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol. Cell. Biol. **13:**6501-6508.

Hengartner, M. O., and H. R. Horvitz (1994). Programmed cell death in *Caenorhabditis elegans*. Curr. Opin. Genet. Dev. **4:**581-586.

Hershko, A., D. Ganoth, J. Pehrson, R. E. Palazzo, and L. H. Cohen (1991). Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. J. Cell. Biol. **266:**16376-16379.

Hickman, J. A., C. S. Potten, A. J. Merritt, and T. C. Fisher (1994). Apoptosis and cancer chemotherapy. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **345:**319-325.

Hinds, p. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70:**993-1006.

Hinds, P. W. and R. A. Weinberg (1994). Tumor suppressor genes. Curr. Opin. Genet. Dev. **4:**135-141.

Hockenbery D. G., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature **348:** 334-336.

Hoffman, B., and D. A. Liebermann (1994). Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive and negative modulators. Oncogene **9:**1807-1812.

Holliday, R. (1989). Chromosome error propagation and cancer. Trends in Genet. **5:**42-45.

Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris (1991). p53 mutations in human cancers. Science **253:**49-53.Howard, A. and S. R. Pelc (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relationship to chromosome breakage. Heredity (Suppl.) **6:**261-273.

Howes, K. A., N. Ransom, D. S. Papermaster, J. G. H. Lasudry, D. M. Albert, and J. J. Windle (1994). Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes Dev. **8:**1300-1310.

Hsu, H., J. Xiong, and D. V. Goeddel (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κ activation. Cell **81:** 495-504.

Hunt, T., F. C. Luca, and J. V. Ruderman (1992). The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. J. Cell Biol. **116:**707-724.

Hunter, T., and J. Pines (1994). Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell **79:**573-582.

Hussussian, C. J., J. P. Struewing, A. M. Goldstein, P. A. T. Higgins, D. S. Ally, D. Sheahan, W. H. J. Clark, M. A. Tucker, and N. C. Dracopoli (1994). Germline p16 mutations in familial melanoma. Nature Genet. **8:**15-21.

Ida, N., N. Uenishi, A. Kajita, and Y. Satoh (1982). Antitumor effect of human fibroblast interferon on the growth of human melanoma cells implanted in nude mice. Gann. **73:**952-960.

Igarashi, M., A. Nagata, S. Jinno, K. Suto, and H. Okayama (1991). Wee1(+)-like gene in human cells. Nature **353:**80-83.

Imai, Y., Y. Matsushima, T. Sugimura, and M. Terada (1991). Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of the retinoblastoma gene product. J. Virol. **65:**4966-4972,

Israel, L. (1990). Accelerated genetic destabilization and dormancy: two distinct causes of resistance in metastatic cells; clinical magnitude, therapeutic approaches. Clin. Exp. Metastases **8:**1-11.

Jacks, T., A. Fazeli, E. Schmitt, R. T. Bronson, M. Goodell, and R. A. Weinberg (1992). Effects of an RB mutation in the mouse. Nature **359:**295-300.

Jenkins, J. R., K. Rudge, and G. A. Currie (1984). Cellular immortalization by a cDNA clone encoding the transformation associated phosphoprotein p53. Nature **312:**651-653.

Jiang, W., S. M. Kahn, N. Tomita, Y. J. Zhang, S. H. Lu, and I. B. Weinstein (1992). Amplification and expression of the human cyclin D gene in esophageal cancer. Cancer Res. **52:** 2980-2983.

Jones, K. W., M. H. Shapero, M. Chevrette, and R. E. K. Fournier (1992). Subtractive hybridization cloning of a tissue-specific extinguisher; TSE1 encodes a regulatory subunit of protein kinase A. Cell **66:**861-872.

Jung, M., A. D. Kondratyev, and A. Dritschilo (1994). Elongation factor 1 delta is enhanced following exposure to ionizing radiation. Cancer Res. **54:**2541-2543.

Kaiser-McCaw, B., A. L. Epstein, H. S. Kaplan, and F. Hecht (1977). Chromosome 14 translocation in African and North American Burkitt's lymphoma. Int. J. Cancer **19:**482-486.

Kamb, A., N. A. Gruis, J. Weaver-Feldhaus, Q. Liu, K. Harshman, S. V. Tavtigian, E. Stockert, R. S. I. Day, B. E. Johnson, and M. H. Skolnick (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science **264:**436-440.

Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51:**6304-6311.

Kastan, M. B., Q. Zhan, W. S. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace, Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell **71:**587-597.

Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase, CDK4. Genes and Dev. **7:**331-342.

Kaufmann, S. H. (1989). Induction of endonucleotytic DNA cleavage in human acute myelogenous leukemia cells by etopiside, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. **49:**5870-5878.

Kaufmann, W. K., J. C. Boyer, L. L. Estabrooks, and S. J. Wilson (1991). Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes. Mol. Cell. Biol. **11:** 3711-3718.

Kerr, D. J., I. B. Pragnell, A. Sproul, S. Cowan, T. Murray, D. George, and R. Leake (1989). The cytostatic effects of alpha-interferon may be mediated by transforming growth factor-beta. J. Mol. Endocrinol. **2:**131-136.

Khavari, P. A., C. L. Peterson, J. W. Tamkun, D. B. Mendel, and G. R. Crabtree (1993). BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mititic growth and transcription. Nature **366:**170-174.

Kiefer, M. C., M. J. Brauer, V. C. Powers, J. J. Wu, S. R. Umansky, L. D. Tomei, and P. J. Barr (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature **374:**736-739.

Kim, S.-J., H.-D. Lee, P. D. Robbins, K. Busam, M. B. Sporn, and A. B. Roberts (1991). Regulation of transforming frowth factor β1 gene expression by the product of the retinoblastoma-susceptibility gene. Proc. Natl. Acad. Sci. USA **88:**3052-3056.

Kim, S.-J., S. Wagner, F. Liu, M. A. O'Reilly, P. D. Robbins, and M. R. Green (1992). Retinoblastoma gene product activates expression of the human TGF-β2 gene through transcription factor ATF-2. Nature **358:**331-334.

Kinzler, K. W., and B. Vogelstein (1996). Life (and death) in a malignant tumour. Nature **379:**19-20.

Klinger, H. P. (1982). Suppression of tumorigenicity. Cytogenet. Cell Genet. **32:**68-84.

Klinger, H. P., and T. B. Shows (1983). Suppression of tumorigenicity in somatic cell hybrids. II. Human chromosomes implicated as suppressors of tumorigenicity in hybrids with Chinese hamster ovary cells. J. Natl. Cancer Inst. **71:**559-569.

Knudson, A. G. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. U.S.A. **68:**820-3.

Knudson, A. (1977). Genetics and the etiology of human cancer. Adv. Hum. Genet. **8:**1-66.

Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. M. Roberts (1991). Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell **66:**1217-1228.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza, and J. M. Roberts (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689-1694.

Krasagakis, K., C. Garbe, and D. E. Orfanos (1993). Cytokines in human melanoma cells: synthesis, autocrine stimulation and regulatory functions- an overview. Melanoma Res. **3:**425-433.

Krasagakis, K., C. Garbe, P. I. Schrier, and C. E. Orfanos (1994). Paracrine and autocrine regulation of human melanocyte and melanoma cell growth by transforming growth factor beta in vitro. Anticancer Res. **14:**2565-2571.

Krasagakis, K., C. Garbe, C.C. Zouboulis, and C. E. Orfanos (1995). Growth control of melanoma cells and melanocytes by cytokines. Recent Results Cancer Res. **139:**169-182.

Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA **89:**7491-7495.

Kujubu, D. A., R. W. Lim, B. C. Varnum and H. R. Herschman (1987). Induction of transiently expressed genes in PC-12 pheochromocytoma cells. Oncogene **1:**257-262

Kumar, R., and Haugen, J. D. (1994). Human and rat osteoblast-like cells express stathmin, a growth-regulatory protein. Biochem. Biophys. Res. Commun. **201:**861-865.

Kumar, S., M. Kinoshita, M. Noda, N. G. Copeland, and N. A. Jenkins (1994). Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the Caenorhabditis elegans cell death gene ced-3 and the mammalian IL-1beta-converting enzyme. Genes Dev. **8:**1613-1626.

LaForgia, S., B. Morse, J. Levy, G. Barnea, L. A. Cannizzaro, F. Li, P. C. Nowell, L. Boghosian-Sell, J. Glick, A. Weston, C. C. Harris, H. Drabkin, D. Patterson, C. M. Croce, J. Schlessinger, and K. Huebner (1991). Receptor protein-trypsin phosphatase gamma is a candidate tumor suppressor gene at human chromosome region 3p21. Proc. Natl. Acad. Sci. USA **88:**5036-5040.

Lam, E. W., and R. J. Watson (1993). An E2F-binding site mediates cell cycle regulated repression of mouse B-myb transcription. EMBO J. **12:**2705-2713.

Lammie, G. A., V. Fantl, R. Smith, E. Schuuring, S. Brookes, R. Michalides, C. Dickson, A. Arnold, and G. Peters (1991). D11S128, a putative oncogene on chromosome 11q13 is amplified and expressed in squamous cell and mammary carcinomas and lined to BCL-1. Oncogene **6:**439-444.

Lane, D. P. (1992). Cancer: p53, guardian of the genome. Nature **358:**15-16.

Lanfrancone, L., G. Pelicci, and P. G. Pelicci (1994). Cancer genetics. Curr. Opin. Genet. Dev. **4:**109-119.

Lau, L. F., and D. Nathans (1987). Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. **4:**3145-3151.

Leach, R. J., M. J. Thayer, A. J. Schafer, and R. E. K. Fournier (1989). Physical mapping of human chromosome 17 using fragment containing microcell hybrids. Genomics **5:**167-176.

Lebwohl, D. E., R. Muise-Helmericks, L. Sepp-Lorenzino, S. Serve, M. Timaul, R. Bol, P. Borgen, and N. Rosen (1994). A truncated cyclin D1 gene encodes a stable mRNA in a human breast cancer cell line. Oncogene **9:**1925-1929.

Lee, M. G., and P. Nurse (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature **327:**31-35.

Lee, J. M., and A. Bernstein (1993). p53 mutations increase resistance to ionizing radiation. Proc. Natl. Acad. Sci. USA **90:**5742-5746.

Lee, S. W., C. Tomasetto, and R. Sager. (1991). Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc. Natl. Acad. Sci. USA **88:**2825-2829.

Lee, W. -H., R. Bookstein, F. Hong, L. J. Young, J. Y. Shew, and E. Y.-H. P. Lee (1987a). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science **235:**1395-1399.

Lee, W. -H., J.-Y. Shew, F. D. Hong, T. W. Sery, L. A. Donoso, L. -J. Young, R. Bookstein, and E. Y.-H. P. Lee (1987b). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature **329:**642-645.

Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow (1992). Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes Dev. **6:**1874-1885.

Legerski, R., and C. Peterson (1992). Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature **359:**70-73.

Leibovitz, A., J. C. Stinson, W. B. McCombes, C. E. McCoy, K. C. Mazur, and N.D. Mabry (1976). Classification of human colorectal adenocarcinoma cell lines. Cancer Res. **36:**4562-4569.

Lennon, S. V., S. J. Martin, and T. G. Cotter (1991). Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. Cell Prolif. **24:**203-214.

Levan, G., and F. Mitelman (1975). Clustering of aberrations to specific chromosomes in human neoplasias. Hereditas **79:**156-160.

Lew, D. J., V. Dulic, and S. I. Reed (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell **66:**1197-1206.

Liang, P. and A. B. Pardee (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:**967-971.

Liang, P., L. Averboukh, and A. B. Pardee (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: Refinements and optimization. Nucleic Acids Res. **21:**3269-3275.

Lichter, P., S. A. Ledbetter, D. H. Ledbetter, and D. C. Ward (1990). Fluorescence in situ hybridization with *Alu* and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines. Proc. Natl. Acad. Sci. USA **87:**6634-6638.

Ling, V. 1992. P-glycoprotein and resistance to anticancer drugs. Cancer **69:**2603-2609.

Liston, P., N. Roy, K. Tamal, C. Lefebvre, S. Baird, G. Cherton-Horvat, R. Farahani, M. McLean, J.-E. Ikeda, A. MacKenzie, and R. G. Korneluk (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature **379:**349-353.

Liu, P., J. Siciliano, D. Seong, J. Craig, U. Zhao, P.J. de Jong, and M. J. Siciliano (1993) Dual *Alu*-PCR primers and conditions for isolation of human chromosome painting probes from hybrid cells. Cancer. Genet. Cytogenet. **65:**93-99.

Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70:**923-935.

Linzer, D. I., and A. J. Levine (1979). Characterization of a 54kDalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17:**43-52.

Lock, L. F., and D. Wickramasinghe (1994). Cycling with CDKs. Trends Cell Biol. **4:**404-405.

Los, M., M. Van de Craen, L. C. Penning, H. Schenk, M. Westendorp, P. Baeuerle, W. Droge, P. H. Krammer, W. Fiers, and K. Schulze-Osthoff (1995). Requirement of an ICE/CED-3 protease for Fas/Apo-1-mediated apoptosis. Nature **375:**81-83.

Lotem, J., and L. Sachs (1995). Interferon-gamma inhibits apoptosis induced by wild-type p53, cytotoxic anti-cancer agents, and viability factor deprivation in myeloid cells. Leukemia **9:**685-692.

Lowe, S. W., and H. E. Ruley (1993). Stabilization of the p53 tumor suppressor gene is induced by adenovirus 5 E1A and accompanies apoptosis. Genes and Dev. **7:**535-545.

Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957-967.

Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature **362:** 847-848.

Lowe, S. W., S. Bodis, A. McClatchey, L. Remington, H. E. Ruley, D. E. Fisher, D. E. Housman, and T. Jacks (1994). *p53* status and the efficacy of cancer therapy *in vivo* Science **266:**807-810.

Lu, X., and D.P. Lane (1993). Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell **75:**765-778.

Ludlow, J. W., J. A. DeCaprio, C.-M. Huang, W.-H. Lee, E. Paucha, and D. M. Livingston (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell **56:**57-65.

Luethy, J. D., and N. J. Hobrook (1992). Activation of the gadd153 promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res. **52:** 5-10.

Lupton, S. D., L. L. Brunton, V. A. Kalberg, and R. W. Overell (1991). Dominant positive and negative selection using a hygromycin phosphotransferase-thymidine kinase fusion gene. Mol. Cell. Biol. **11:**3374-3378.

Lynch, H. T., R. M. Fusaro, and J. A. Johnson (1984). Xeroderma pigmentosum: complementation group C and malignant melanoma. Arch. Derm. **120:**175-179.

Maheswaran, S., C. Englert, P. Bennett, G. Heinrich, and D. A. Haber (1995). The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. Genes Dev. **9:**2143-2156.

Maity, A., W.G. McKenna, and R.J. Muschel (1994). The molecular basis for cell cycle delays following ionizing radiation: a review. Radiother. Onc. **31:**1-13.

Mäkalä, T. P., J.-P. Tassan, E. A. Nigg, S. Frutiger, G. J. Hughes, and R. A. Weinberg (1994). A cyclin associated with the CDK-activating kinase MO15. Nature **371:**254-257.

Malkin, D. (1994). *p53* and the Li-Fraumeni syndrome. Bioch. Biophys. Acta **1198:**197-210.

Maltzman, W., and L. Czyzyk (1984). UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol. **4:**1689-1694.

Markowitz, S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, B. Vogelstein, M. Brattain, and J. K. V. Willson (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science **268:**1336-1338.

Martin, S. J. (1993). Apoptosis:suicide, execution or murder? Trends Cell Biol. **3:**141-144.

Martin, S. J., D. R. Green, and T. G. Cotter (1994). Dicing with death: dissecting the components of the apoptosis machinery. Trends Biochem. Sci. **19:**26-30.

Martin, S. J., and D. R. Green (1995). Protease activation during apoptosis: Death by a thousand cuts? Cell **82:**349-352.

Mathew, S., V. Murty, S. Cheifetz, D. George, J. Massague, and Chaganti, R. S. K. (1994). Transforming growth factor receptor gene TGFBR2 maps to human chromosome band 3p22. Genomics **20:**114-115.

Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:**701-713.

Matsushime, H., M. E. Ewen, D. K. Strom, J. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr (1992). Identification and properties of an atypical catalytic subunit ($p34^{PSKJ3}$/CDK4) for mammalian D-type G1 cyclins. Cell **71:**323-334.

McGowan, C. M., and P. Russell (1993). Human wee1 kinase inhibits cell division by phosphorylating $p34^{cdc2}$ exclusively on Tyr15. EMBO J. **12:**75-85.

McGowan-Jordan, I. J., M. D. Speevak, D. Blakey, and M. Chevrette (1994). Suppression of tumorigenicity in human teratocarcinoma cell line PA-1 by introduction of human chromosome 4. Cancer Res. **54:**2568-2572.

McIlwrath, A. J., P. A. Vasey, G. M. Ross, and R. Brown (1994). Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res. **54:**3718-3722.

McClelland, M., D. Ralph, R. Cheng, and J. Welsh (1994). Interactions among regulators of RNA abundance characterized using RNA fingerprinting by arbitrarily primed PCR. Nucleic Acids Res. **22:**4419-4420.

McNeill, C. A., and R. L. Brown (1980). Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc Nat. Acad. Sci. USA **77:**5394-5398.

Meyerson, M., G. H. Enders, C.-L. Wu, L.-K. Su, C. Gorka, C. Nelson, E. Harlow, L.-H. Tsai (1992). A family of human cdc2-related protein kinases. EMBO J. **11:**2909-2917.

Meyerson, M., and E. Harlow (1994). Identification of a G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077-2086.

Michieli, P., M. Chedid, D. Lin, J. H. Pierce, W. E. Mercer, and D. Givol (1994). Inhibition of WAF1/Cip1 by a p53-independent pathway. Cancer Res. **54:**3391-3395.

Mihara, K., X. R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y. K. Fung (1989). Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science **246:**1300-1303.

Mikkelsen, T., and W. K. Cavenee (1990). Suppressors of the malignant phenotype. Cell Growth Diff. **1:** 201-207.

Millar, J. B. A., P. Russell, J. E. Dixon, and K. L. Guan (1992). Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. EMBO J. **11:**4943-4952.

Miller, S. A., D. D. Dykes, and H. F. Polesky (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res. **16:**1215.

Milner, A. E., R. J. Grand, and C. D. Gregory (1995). Effects of interferon-alpha on human B cells: repression of apoptosis and prevention of cell growth are independent responses of Burkitt lymphoma lines. Int. J. Cancer **61:**348-354.

Minshull, J., R. Golsteyn, C. S. Hill, and T. Hunt (1990). The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. EMBO J. **9:**2865-2875.

Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan (1993). Induction of apoptosis in fibroblasts by IL-1b-converting enzyme, a mammalian homologue of the C. elegans cell death gene ced-3. Cell **75:**653-660.

Mittnacht, S., P. W. Hinds, S. F. Dowdy, and R. A. Weinberg (1991). Modulation of retinoblastoma protein activity during the cell cycle. Cold Spring Harbor Symp. Quant. Biol. **56:**197-209.

Morgenbesser, S. D., B. O. Williams, T. Jacks, and R. A. DePinho (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature **371:** 72-74.

Motokura, T., T. Bloom, H. G. Kim, H. Jüppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold (1991). A novel cyclin encoded by a *bcl*-linked candidate oncogene. Nature **350:**512-515.

Mou, L., H. Miller, J. Li, E. Wang, and L. Chalifour (1994). Improvements to the differential display method for gene analysis. Biochem. Biophys. Res. Comm. **199:**564-569.

Munday, N. A., J. P. Vaillancourt, A. Ali, F. J. Casano, D. K. Miller, S. M. Molineaux, T.T. Yamin, V. L. Yu and D. W. Nicholson (1995). Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. J. Biol Chem. **270:**15870-15876.

Murray, A. W. (1992). Creative blocks: cell-cycle checkpoints and feedback controls.Nature **359:**599-604.

Myashita, T., and J. C. Reed (1992). *Bcl-2* gene transfer increases relative resistnace of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. Cancer Res. **52:**5407-5411.

Myashita, T., and J. C. Reed (1993). *Bcl-2* oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood **81:**151-157.

Neilan, J. G., Z. Lu, C. L. Alfonso, G. F. Kutish, M. D. Sussman, and D. L. Rock (1993). An African Swine Fever virus gene with similarity to the proto-oncogene blc-2 and the Epstein-Barr virus BHRF1. J. Virol. **67:**4391-4394.

Nevins, J. R. (1992). E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424-429.

Newman, M. J. (1990). Inhibition of carcinoma and melanoma cell growth by type 1 transforming growth factor bet is dependent on the presence of polyunsaturated fatty acids. Proc. Natl. Acad. Sci. USA **87:**5543-5547.

Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, N. A. Munday, S. M. Raju, M. E. Smulson, T.-T.

Yamin, V. L. Yu, and D. K. Miller (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature **376:**37-43.

Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccard (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods **139:**271-279.

Niebuhr, E. (1977). Partial trisomies and deletions of chromosome 13. *In:* New Chromosomal Syndromes. Chromosomes in Biology and Medicine Monograph Series, ed.: J. J.Yunis (New York Academic Press, New York) pp273-299.

Ning, Y., J. W. Shay, M. Lovell, L. Taylor, D. H. Ledbetter, and O. M. Pereira-Smith (1991). Tumor suppression by chromosome 11 is not due to cellular senescence. Exp. Cell Res. **192:**220-226.

Nobori, T., K. Miura, D. J. Wu, A. Lois, K. Takabayashi, and D. A. Carson (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature **368:**753-756.

Noda, A., Y. Ning, S. F. Venable, O. M. Pereira-Smith, and J. R. Smith (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis unsing an expression screen. Exp. Cell Res. **211:**90-98.

Nordenskjold, M., and W. K. Cavenee (1988). Genetics and the etiology of solid tumors. [review]. *In:* Important Advances in Oncology, Eds.: V. T. DeVita, S. Hellman, and S. A. Rosenberg (J.B. Lippincott, Philadelphia) pp83-101.

Nowell, P. C., and D. A. Hungerford (1960). A minute chromosome in human chronic granulocytic leukemia. Science **132:**1497.

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. Science **194:**23-28.

Nuñez, G., and M. F. Clarke (1994). The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol. **4:**399-403.

Nurse, P., P. Thuriaux, and K. Nasmyth (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccaromyces pombe*. Mol. Gen. Genet. **146:**167-178.

Ogris E., H. Rotheneder, I. Mudrak, A. Pichler, and E. Wintersberger (1993). A binding site for transcription factor E2F is a target for trans activation of murine thymidine kinase by polyomavirus large T antigen and plays an important role in growth regulation of the gene. J. Virol. **67:**1765-1771.

Okuda, T., J. L. Cleveland, and J. R. Downing (1992). PCTAIRE-1 and PCTAIRE-3, two members of a novel cdc2/CDC28-related protein kinase gene family. Oncogene **7:**2249-2258.

Oltvai Z. N., C. L. Milliman, and S. J. Korsmeyer (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell **74:** 609-619.

Oltvai, Z. N., and S. J. Korsmeyer (1994). Checkpoints of dueling dimers foil death wishes. Cell **79:**189-192.

Oren, M., W. Maltzman, and A. J. Levine (1981). Posttranslational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol. Cell. Biol. **1**:101-110.

Otto, E., S. McCord, and T. D. Tlsty (1989). Increased incidence in CAD gene amplification in tumourigenic rat lines as an indicator of genomic instability of neoplastic cells. J. Biol. Chem. **264:** 3390-3396.

Padmanabhan, R., T. H. Howard, and B. H. Howard (1987). Specific growth inhibitory sequences in genomic DNA from quiescent human embryo fibroblasts. Mol. Cell. Biol. **7**:1894-1899.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. **11**:961-971.

Pan, H., and A. E. Griep (1994). Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: Implications for tumour suppressor gene function in development. Genes Dev. **8:** 1285-1299.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. Science **246**:603-608.

Parker, L. L., S. Atherton-Fessler, M. S. Lee, S. Ogg, J. S. Falk, K. I. Swenson, and H. Piwnica-Worms (1991). Cyclin promotes the tyrosine phosphorylation of $p34^{cdc2}$ in a wee1+ dependent manner. EMBO J. **10:**1255-1263.

Parker, L. L., S. Atherton-Fessler, and H. Piwnica-Worms (1992). $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. Proc. Natl. Acad. Sci. U.S.A. **89:**2917-2921.

Parker, L. L., and H. Piwnica-Worms (1992). Inactivation of the $p34^{cdc2}$-cyclin B complex by the human WEE1 tyrosine kinase. Science **257:**1955-1957.

Parry D., S. Bates, D. J. Mann, and G. Peters (1995). Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. EMBO J. **14:**503-511.

Paulovich, A. G., and L. H. Hartwell (1995). A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell **82:**841-847.

Pearson, B. E., H. P. Nasheuer, and T. S. Wang (1991). Human DNA polymerase alpha gene: sequences controlling expression in cycling and serum-stimulated cells. Mol. Cell Biol. **11:**2081-2095.

Peeper, D. S., A. J. van der Eb, and A. Zantema (1994). The G1/S cell-cycle checkpoint in eukaryotic cells. Biochim. Biophys. Acta **1198:** 215-230.

Peitsch, M. C., H. G. Mannherz, and J. Tschopp (1994). The apoptosis endonucleases: cleaning up after cell death? Trends Cell Biol. **4:**37-41.

Peters, G. (1994). Cell cycle: Stifled by inhibitions. Nature **371:**204-205.

Pietenpol, J. A., and B. Vogelstein (1993). No room at the p53 inn. Nature **365:**17-18.

Pines, J. (1993). Cyclins and cyclin-dependent kinases: Take your partners. Trends Biochem. Sci. **18:**195-197.

Pines, J., and Hunter, T. (1990). Human cyclin A is adenovirus E1-A associated protein p60 and behaves differently from cyclin B. Nature **346**:760-763.

Polyak, K., J. Y. Kato, M. J. Solomon, C. J. Sherr, J. Massagué, J. M. Roberts, and A. Koff (1994a). $p27^{Kip1}$, a cyclin-Cdk inhibitor links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev. **8**:9-22.

Polyak, K., M. -H. Lee, H. Erdjument-Bromage, P. Tempst, and J. Massagué (1994b). Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell **78**:59-66.

Portera-Cailliau, C., C. H. Sung, J. Nathans, and R. Adler (1994). Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. Proc. Natl. Acad. Sci. USA **91**:974-978.

Proudfoot, N. J., and G. G. Brownlee (1976). 3' non-coding region sequences in eukaryotic messenger RNA. Nature **263:** 211-214

Qing, P. D., A. Bing, and P. L. Will (1995). Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G_1 arrest and apoptosis. Proc. Natl. Acad. Sci. USA **92**:9019-9023.

Rabbitts, T. H. (1994). Chromosomal translocations in human cancer. Nature **372**:143-149.

Randazzo, F., M., P. Khavari, G. Crabtree, J. Tamkun, and J. Rossant (1994). *brg1*: a putative murine homologue of the *Drosophila brahma* gene, a homeotic gene regulator. Dev. Biol. **161**:229-242.

Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White (1992). The adenovirus E1A proteins induce apoptosis which is inhibited by the E1B and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:** 7742-7746.

Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup (1992). Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell **69**:597-604.

Reihsaus, E., M. Kohler, S. Kraiss, M. Oren, and M. Montenarh (1990). Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. Oncogene **5**:137-145.

Resnitzky, D., N. Tiefenbrun, H. Berissi, and A. Kimchi (1992). Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietic cells. Proc. Natl. Acad. Sci. USA **89**:402-406.

Rimessi, P., F. Gualandi, C. Morelli, C. Trabanelli, Q. Wu, L. Possati, M. Montesi, J. C. Barrett, and G. Barbanti-Brodano (1994). Transfer of human chromosome 3 to an ovarian carcinoma cell line identifies three regions on 3p involved in ovarian cancer. Oncogene **9**:3767-3474.

Rodeck, U., A. Bossler, U. Graeven, F. E. Fox, P. C. Nowell, C. Knabbe, and C. Kari (1994). Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. Cancer Res. **54**:575-581.

Roninson, I. B. (1992). From amplification to function:the case of the MDR1 gene. Mutat. Res. **276**:151-61.

Rowley, J. D. (1973). A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature **231**:503-506.

Rowley, R., J. Hudson, and P. G. Young (1992a). The wee1 protein kinase is required for radiation-induced mitotic delay. Nature **356**:353-355.

Rowley, R., S. Subramani, and P. G. Young (1992b). Checkpoint controls in *Schizosaccharomyces pombe:rad1*. EMBO J. **11**:1335-1342.

Roy, N., M. S. Mahadevan, M. McLean, G. Shutler, Z. Yaraghi, R. Farahani, S. Baird, A. Besner-Johnston, C. Lefebvre, X. Kang, M. Salih, H. Aubry, K. Tamai, X. Guan, P. Ioannou, T. O. Crawford, P. J. de Jong, L. Surh, J.-E. Ikeda, R. G. Korneluk, and A. MacKenzie (1996). The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell **80:** 167-178.

Russell, P., and P. Nurse (1987). The mitotic inducer *nim1+* functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. Cell **49**:569-576.

Russell, K. J., L. W. Wiens, G. W. Demers, D. A. Galloway, S. E. Plon, and M. Groudine (1995). Abrogation of the G2 checkpoint results from differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. Cancer Res. **55**:1639-1642.

Sanchez, Y., and S. J. Elledge (1995). Stopped for repairs. BioEssays **17**:545-548.

Satterwhite, D. J., and H. L. Moses (1994). Mechanisms of transforming growth factor-beta 1-induced cell cycle arrest. Invasion Metastasis **14**:309-318.

Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D. A. Tagle, S. Smith, T. Uziel, S. Sfez, M. Ashkenazi, I. Pecker, M. Frydman, R. Harnik, S. R. Patanjali, A. Simmons, G. A. Clines, A. Sartiel, R. A. Gatti, L. Chessa, O. Sanal, M. F. Lavin, N. G. J. Jaspers, A. M. R. Taylor, C. F. Arlett, T. Miki, S. M. Weissman, M. Lovett, F. S. Collins, and Y. Shiloh (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science **268**:1749-1753.

Saxon, P. J., E. S. Srivatsan, G. V. Leipzig, J. H. Sameshima, and E. J. Stanbridge (1985). Selective transfer of individual human chromosomes to recipient cells. Mol. Cell. Biol. **5**:140-146.

Saxon, P. J., E. S. Srivatsan, and E. J. Stanbridge (1986). Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. EMBO J. **5**:3461-3466.

Scaletta, L. J. and B. Ephrussi (1965). Hybridization of normal and neoplastic cells *in vitro*. Nature **205**:1165-1171.

Schaefer, D. I., E. M. Livanos, A. E. White, and T. D. Tlsty (1993). Multiple mechanisms of N-(phosphonoacetyl)-L-aspartate drug resistance in SV40-infected precrisis human fibroblasts. Cancer Res. **53**:4946-4951.

Scheffer, G.L., P.L.J. Wijngaard, M.J. Flens, M.A. Izquierdo, M.L. Slovak, H.M. Pinedo, C.J.L.M. Meijer, H.C. Clevers, and R.J. Scheper (1995). The drug resistance-related protein LRP is the human major vault protein. Nature Med. **1**:578-582.

Schimke, R. T. (1984). Gene amplification, drug resistance and cancer. Cancer Res. **44**:1735-1742.

Schneider, C., R. M. King, and L. Philipson (1988). Genes specifically expressed at growth arrest of mammalian cells. Cell **54**:787-793.

Schneider, C., S. Gustincich, and G. Del Sal (1991). The complexity of cell proliferation control in mammalian cells. Curr. Opin. Cell Biol. **3**:276-281.

Seabright, M (1971). A rapid banding technique for human chromosomes. Lancet **2**:971-972.

Sen, S., and M. D'Incalci (1992). Apoptosis. Biochemical events and relevance to cancer chemotherapy. FEBS Lett. **307**:122-127.

Serrano, M., G. J. Hannon, and D. Beach (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/Cdk4. .Nature 366, 704-707.

Seto, M., K. Yamamoto, S. Iida, Y. Akao, K. R. Utsumi, I. Kubonishi, I. Miyoshi, T. Ohtsuki, Y. Yawata, M. Namba, T. Motokura, A. Arnold, T. Takahashi, and R. Ueba (1992). Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. Oncogene **7**:1401-1406.

Shaw, P., R. Bovey, S. Tardy, R. Sahli, B. Sordat, and J. Costa (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA **89**:4495-4499.

Sherr, C. J. (1993). Mammalian G1 cyclins. Cell **73**:1059-1065.

Sherr, C. J., and J. M. Roberts (1995). Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev. **9**:1149-1163.

Shi, Y., J. M. Glynn, L. J. Guilbert, T. G. Cotter, R. P. Bissonette, and D. R. Green (1992). Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science **257**:212-214.

Shigesada, K., G.R. Stark, J.A. Maley, L.A. Niswander, and J.N. Davidson (1985). Construction of a cDNA to the hamster CAD gene and its application toward defining the domain for aspartate transcarbamylase. Mol. Cell Biol. **9**:1735-1742.

Shim, J., H. Lee, J. Park, H. Kim, and E.-J. Choi (1996). A non-enzymatic p21 inhibitor of stress-activated protein kinases. Nature **381**:804-807.

Shivji, M. K. K., M. K. Kenny, and R. D. Wood (1992). Proliferating cell nuclear antigen is required for DNA excision repair. Cell **69**:367-374.

Shivji, M. K. K., S. J. Grey, U. P. Strausfeld, R. D. Wood, and J. J. Blow (1994). Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr. Biol. **4**:1062-1068.

Simon, S. M., and M. Schindler (1994). Cell biological mechanisms of multidrug resistance in tumors. Proc. Natl. Acad. Sci. USA **91**:3497-3504.

Skup, D., J. D. Windass, F. Sor, H. George, B. R. G. Williams, H. Fukuhara, J. De Maeyer-Guignard, and E. De Maeyer (1982). Molecular cloning of partial cDNA copies of two distinct mouse IFN-β mRNAs. Nucleic Acids Res. **10**:3069-3084.

Slingerland, J. M., L. Hengst, C.-H. Pan, D. Alexander, M. Stampfer, and S. I. Reed (1994). A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. Mol. Cell. Biol. **14:**3683-3694.

Smith, C. A., T. Farrah, and R. G. Goodwin (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell **76:**959-962.

Smith, D. I., T. W. Glover, R. Gemmill, H. Drabkin, P. O'Connell, and S. L. Naylor (1995). Report of the fifth international workshop on human chromosome 3 mapping 1994. Cytogent. Cell Genet. **68:**126-146.

Speevak, M. D., and M. Chevrette (1994). Identification of chromosomes implicated in suppression of apoptosis in somatic cell hybrids. Biochem. Cell Biol. **72:**655-662.

Speevak, M. D., N. G. Bérubé, I. J. McGowan-Jordan, C. Bisson, S. D. Lupton, and M. Chevrette (1995). Construction and analysis of microcell hybrids containing dual selectable taged human chromosomes. Cytogenet. Cell Genet., **69:**63-65.

Speevak, M. D., and M. Chevrette (1996) Human chromosome 3 mediates growth arrest and suppression of apoptosis in microcell hybrids. Mol. Cell. Biol. **16:**2214-2225.

Stanbridge, E. J. (1976). Suppression of malignancy in human cells. Nature **260:**17-20.

Stanbridge, E. J. (1988). A brief review of the evidence of the genetic regulation of tumorigenic expression of somatic cell hybrids. I.A.R.C. Sci. Publ. **92:**23-31.

Stanbridge, E.J. (1990). Identifying tumour suppressor genes in human colorectal cancer. Science **247:**12-13.

Stanbridge, E. J., C. J. Der, C. J. Doersen, R. Y. Nishimi, D. M. Peehl, B. E. Weissman, and J. Wilkinson (1982). Human cell hybrids: Analysis of transformation and tumorigenicity. Science **215:**252-259.

Stanbridge, E. J., and W. K. Cavenee (1989). Heritable cancer and tumor suppressor genes: a tentative connection. *In*: Oncogenes and the Molecular Origins of Cancer. (Cold Spring Harbor Laboratory Press) pp281-305

Stanbridge, E. J., and P. C. Nowell (1990). Origins of human cancer revisited. Cell **63:**867-874.

Stanger, B. Z., P. Leder, T.-H. Lee, E. Kim, and B. Seed (1995). RIP: A novel protein containing a death domain that interacts with Fas/Apo-1 (CD95) in yeast and causes death. Cell **81:** 513-523.

Stark, G. R., M. Debatisse, G. M. Wahl, and D. M. Glover (1990). Gene rearrangement *In*: Frontiers in Molecular Biology, Eds.: B.D. Hames and D. M. Glover (IRL, Oxford) pp 99-149.

Stoler, A., and N. Bouck (1985). Identification of a single chromosome in the normal human genome essential for suppression of hamster cell transformation. Proc. Natl. Acad. Sci. U.S.A. **82:**570-574.

Strasser, A., A. W. Harris, T. Jacks, and S. Cory (1994). DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by bcl-2. Cell **79:**329-339.

Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J. **12:**1969-1978.

Sutherland, G. R., and D. H. Ledbetter (1989). Report of the committee on cytogenetic markers (HGM10). Cytogenet. Cell Genet. **51:**452-458.

Swyryd, E. A., S. S. Seaver, and G. R. Stark (1974). N-(phosphonoacetyl)-L-aspartate, a potent transition state analog inhibitor of aspartate transcarbamylase, blocks proliferation of mammalian cells in culture. J. Biol. Chem. **249:** 6945-6950.

Symonds, H., L. Krall, L. Remington, M. Saenz-Robles, S. Lowe, T. Jacks, and T. Van Dyke (1994). p53-dependent apoptosis suppresses tumour growth and progression in vivo. Cell **78:**703-711.

Takayama, S., T. Sato, S. Krajewski, K. Kochel, S. Irie, J. A. Millan, and J. C. Reed (1995). Cloning and functional analysis of BAG-1: A novel Bcl-2-binding protein with anti-cell death activity. Cell **80:**279-284.

Tanaka, N., M. Ishihara, M. Kitagawa, H. Harada, T. Kimura, T. Matsuyama, M. S. Lamphier, S. Aizawa, T. W. Mak, and T. Taniguchi (1994). Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell **77:**829-839.

Teitz, T., D. Eli, M. Penner, M. Bakhanashvili, T. Naiman, T. L. Timme, C. M. Wood, R. E. Moses, and D. Canaani (1990). Expression of the cDNA for the beta subunit of human casein kinase II confers partial UV resistance on xeroderma pigmentosum cells. Mutat. Res. **236:**85-97.

Templeton, D. J., S.-H. Park, L. Lanier, and R. A. Weinberg (1991). Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. USA **88:**3033-3037.

Tewari, M., and V. M. Dixit (1995). Fas- and TNF-induced apoptosis is inhibited by the poxvirus crmA gene product. J. Biol. Chem. **270:** 3255-3260.

Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen and V. M. Dixit (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is aCrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:** 801-809

Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard, M. J. Kostura, D. K. Miller, S. M. Molineaux, J. R. Weidner, and J. Aunins (1992). A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature **356:**768-774.

Tlsty, T. D., B. Margolin, and K. Lum (1989). Differences in the rates of gene amplification in nontumourigenic and tumourigenic cell lines as measured by Luria-Delbruck fluctuation analysis. Proc. Natl. Acad. Sci. USA **86:** 9441-9445.

Tlsty, T. D. (1990). Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc. Natl. Acad. Sci. USA **87:** 3132-3136.

Tlsty, T. D., A. White, and J. Sanchez (1992). Suppression of gene amplification in human cell hybrids. Science **255:** 1425-1427.

Tolmach, L. J., R. W. Jones, and P. M. Busse (1977). The action of caffeine on X-irradiated HeLa cells. I. Delayed inhibition of DNA synthesis. Radiat. Res. **71:**653-665.

Toyoshima, H., and T. Hunter (1994). p27, a novel inhibitor of G1-cyclin-Cdk protein kinase activity, is related to p21. Cell **78:**67-74.

Traganos, F. (1990). Single- and multiparameter analysis of the effects of chemotherapeutic agents on cell proliferation *in vitro*. In: Flow Cytometry and Sorting, Eds.: (Wiley-Liss) pp773-801.

Tritton, T. R., and H. A. Hickman (1990). How to kill cancer cells: Membranes and cell signaling as targets in cancer chemotherapy. Cancer Cells **2:**95-105.

Tsuruta, H., H. Sakamoto, M. Onda, and M. Terada (1993). Amplification and overexpression of EXP1 and EXP2/Cyclin D1 genes in human espophageal carcinomas. Biochem. Biophys. Res. Commun. **196:**1529-1536.

Turcotte, B., M. Guertin, M. Chevrette, and L. Bélanger (1985). Rat alpha$_1$-fetoprotein messenger RNA: 5'-end sequence and glucocorticoid-suppressed liver transcription in an improved nuclear run-off assay. Nucleic. Acids Res. **13:** 2387-2398

Varnum, B. C., R. W. Lim, and H. R. Herschman (1989). Characterization of TIS7, a gene induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. Oncogene **4:**1263-1265.

Vaux, D. L., S. Cory, and J. M. Adams (1988). Bcl-2 promotes the survival of haemopoietic cells and cooperates with c-myc to immortalize pre-B cells. Nature **335:**440-442.

Vaux, D. L., I. L. Weissman, and S. K. Kim (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. Science **258:**1955-1957.

Vaux, D. L., G. Haecker, and A. Strasser (1994). An evolutionary perspective on apoptosis. Cell **76:**777-779.

Vaux, D. L., S. Cory, and J. M. Adams (1988). bcl-2 gene promotes hemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature **335:**440-442.

Verheij, M., R. Bose, X. H. Lin, B. Yao, W. D. Jarvis, S. Grant, M. J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, and R. N. Kolesnick (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature **380:**75-79.

Vogelstein, B., and K.W. Kinzler (1992). p53 function and dysfunction. Cell **70:**523-526.

Waga, S., G. J. Hannon, D. Beach, and B. Stillman (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature **369:**574-578.

Wahl, G. M., R. A. Pudgett, and G. R. Stark (1979). Gene amplification causes overproduction of the first three exnzymes of UMP synthesis in N-(phosphonoacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254:** 8679-8689.

Waldman, T., C. Lengauer, K. W. Kinzler, and B. Vogelstein (1996). Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature **381:**713-716.

Walker, P. R., C. Smith, T. Youkale, J. Leblanc, J. Whitfield, and M. Sikorska (1991). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. Cancer Res. **51:** 528-535.

Walker, P. R., J. Kwast-Welfeld, H. Gourdeau, J. Leblanc, W. Neugebauer, and M. Sikorska (1993). Relationship between apoptosis and the cell cycle in lymphocytes: roles of protein kinase C, tyrosine phosphorylation, and AP1. Exp. Cell Res. **207:** 317-322.

Wang, J., F. Zindy, X. Chenivesse, E. Lamas, B. Henglein, and C. Brechot (1992). Modification of cyclin A expression by hepatitis B virus DNA integration in a hepatocellular carcinoma. Oncogene **7:**1653-1656.

Wang, L., M. Miura, L. Bergeron, H. Zhu, and H. Yuan (1994). Ich-1, an Ice/ced3-related gene, encodes both positive and negative regulators of programmed cell death. Cell **78:**739-750.

Warburton, D., S. Gersen, M. Yu, C. Jackson, B. Handelin, and D. Housman (1990). Monochromosomal rodent-human hybrids from microcell fusion of human lymphoblastoid cells containing an inserted dominant selectable marker. Genomics **6:**358-366.

Weinberg, R. A. (1981). Use of transfection to analyze genetic information and malignant transformation. Biochim. Biophys. Acta **651:** 25-35.

Weinberg, R. A. (1991). Tumor suppressor genes. Science **254:**1138-1146.

Weinberg, R. A. (1995). The retinoblastoma gene and cell cycle control. Cell **81:**323-330.

Weinert, T. A., and L. H. Hartwell (1988). The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science **241:**317-322.

Weinert, T. A., G. L. Kiser, L. H. Hartwell (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. **8:**652-665.

Weissman, B. E., P. J. Saxon, S. R. Pasquale, G. R. Jones, A. G. Geiser, E. J. Stanbridge (1987) Introduction of a normal human chromosome 11 into a Wilm's tumor cell line controls its tumorigenic expression. Science **236:**175-180.

Welch, P. J., and J. Y. J. Wang (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell **75:**779-790.

White, E., R. Cipriani, P. Sabbatini, and A. Denton (1991). Adenovirus E1B 19-kiloDalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. **65:**2968-2978.

White, E. (1994). p53, guardian of Rb. Nature **371:**21-22.

Whitney, M., M. Thayer, C. Reifsteck, S. Olson, L. Smith, P. M. Jakobs, R. Leach, S. Naylor, H. Joenje, and M. Grompe (1995). Microcell mediated chromosome transfer maps the Fanconi anaemia group D to chromosome 3p. Nature Genet. **11:**341-343.

Williams, G. T. (1991). Programmed cell death: Apoptosis and oncogenesis. Cell **65:**1097-1098.

Withers, D. A., R. C. Harvey, J. B. Faust, O. Melnyk, K. Carey, and T. C. Meeker (1991). Characterization of a candidate *bcl-1* gene Mol. Cell. Biol. **11:**4846-4853.

Won, K. A., Y. Xiong, D. Beach, and M. Z. Gilman (1992). Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89:**9910-9914.

Wright, J. A., H. S. Smith, F. M. Watt, M. C. Hancock, D. L. Hudson, and G. R. Stark (1990). DNA amplification is rare in normal human cells. Proc. Natl. Acad. Sci. USA, **87:** 1791-1795.

Wurm, F., K. Gwinn, R. Kingston (1986). Inducible over-expression of the mouse *c-myc* protein in mammalian cells. Proc. Natl. Acad. Sci. USA **83:**5414-5418.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature **284:**555-556.

Wyllie, A. H., M. J. Arends, R. G. Morris, S. W. Walker, and G. Evan (1992). The apoptosis endonuclease and its regulation. Semin. Immunol. **4:**389-397.

Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach (1993). p21 is a universal inhibitor of cyclin kinases. Nature **366:**701-704.

Xiong, Y., T. Connolly, B. Futcher, and D. Beach (1991). Human D-type cyclin. Cell **65:**691-699.

Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson, and S. J. Korsmeyer (1995). Bad, a heterodimeric partner for Bcl-x_L and Bcl-2 displaces Bax and promotes cell death. Cell **80:**285-291.

Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell **70:** 937-948.

Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature **352:**345-347.

Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1b-converting enzyme. Cell **75:**641-652.

Zaman, G. J., M. J. Flens, M. R. van Leusden, M. de Haas, H. S. Mulder, J. Lankelma, H. M. Pinedo, R. J. Scheper, F. Baas, and H. J. Broxterman (1994).The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc. Natl. Acad. Sci. USA **91:**8822-8826.

Zarrilli, R., S. Casola, A. Conti, C. B. Bruni, and B. Colantuoni (1993). Extinction of insulin-like growth factor II in intratypic hybrids of rat liver cells. Mol. Endocrin. **7:**131-141.

Zbar, B. (1989). Chromosomal deletions in lung cancer and renal cancer. *In*: Important Advances in Oncology, Eds.: V. T. De Vita Jr., S. Hellman, S. A. Rosenberg (J.B. Lippincott, Philadelphia) pp 41-59.

Zhang, H., G. J. Hannon, and D. Beach (1994). p21-containing cyclin kinases exist in both active and inactive states. Genes Dev. **8:**1750-1758.

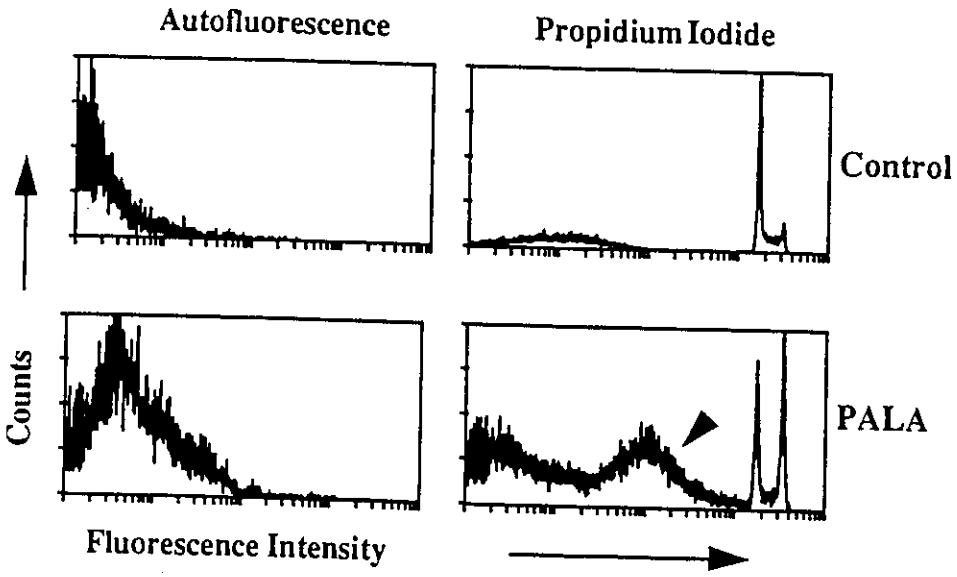
Zhou, C., Y. Yang, and A.Y. Jong (1990). Mini-Prep in ten minutes. Biotech **8:**172-173.

Appendices

A.1 Autofluorescence.

To determine the effect of PALA on autofluorescence of B78 cells, flow cytometry was performed on B78, untreated (Control), or exposed to PALA (13μg/ml) for 4 days. The cells were fixed, and were either stained with propidium iodide as per the protocol described in Chapters 3 and 4 (Propidium Iodide), or they were unstained (Autofluorescence). Flow cytometric log scale histograms show an increase in autofluorescence associated with PALA exposure. However, the autofluorescent peak did not overlap that of the "apoptosis" peak seen in the PALA-exposed, propidium iodide cells (arrow). This indicates that changes in the autofluorescence of the PALA-treated cells did not influence the formation of the "apoptosis" peak detected in the propidium iodide stained cells.

Figure A.1


Autofluorescence
Propidium Iodide
Control
PALA
Counts
Fluorescence Intensity

A.2 Sequences of Cloned fragments (not discussed in text)

MA29f2b

agccagtatc GTAGATTTGG TTCGAACGAA TGTTAGTGAT TTATTATACT ATAATATTCA TCGAATGTGT ACCGAACATT ATAGAGAAAT CTTAAACATC TAGAAAGAAC TTTGTAGATG AGATCAAGGA AAGATAGCAC TATGGACAAG AGTCACTTCA TAGAGGAGAC CTCGTCATTC CACCGTCGTA GGTAAATAGA C agtttttttt

MA32f5d

/TTCCAGCACA CTGGCGTAAC CTTAGTGGAT CCGAGCTCGG TACCAAGCTT GATGCATAGC TTGGTATTCT ATAGTGTCAC CTAAATAGCT TGGCGTAATC ATGGTATAGC TGTTTCCTGT GTGAAATTGT TAT/
(/ direct insertion into vector)

MC25f6i

ccttggttag GTTACTCCTT CTACCCCCAC CGATACCTCA CTGACCCTTC AATCTTATTT TGTAAAGACG AACTTGGGAA TTTCCATATA GGATTCATTT TAATCTAAAT GAAAATTATG TAATTCACAA AAGTAGTGGT CTTCCTACGA CCTTAAAGAT GAGTAAAATC TGGTCCCTCA GTCCAAACCC ACCTTTTTTT TTTTTTATTT ACGCTTACAC AAACCACGTA AGAAAGTACT CACC ccttttttttt

MG32f9b

ctgcttgatg TTAANNGGCA GCATCCAGAA GAACATGAAN CCGAATNCTN CAGATATNCA TCACACNNNN NNCCNNCGAG CATGCATCTA GAGGNCCAAT NNCCTATAGT GAG//CTATAG ATACTCAAGC TATGCATCAA GCTTGGNACC GAGCTNGGTC CACTAGTACG GCNGNNAGTG TGCTGGAATT CGGNTTCTGC TTGATGTTAA CCGGCAGCAT CCAGAAGAAC ATG/
(// unsequenced region; / direct insertion into cloning vector)

MC44f15a

//AAGCCGACTA GTACCAGACC TCGTCCCCGT CGACCCGAAA CGATAAGACC TCCTGTACCG GTTCAGTACC TGAAAGTCAA GCCTCCTGAA ACACCNCGTG ATCTACCACT ACCCNNTTTT TTTTTTCTTC TTNTTTTTGT CTTTAGTAAA AAAGGGATTA ACGAAGACCG GTACAACAAA TAGTGT cnttttttttt
(//unsequenced region)

MC44f15e

ctagtaccag TCTCTCTACA CNNNGACTCT TTAACCTTTG NCACTACTTT AAAAGAGTAA AACACGTTAT ATTAATTTAT TTGTCTGGTA ACGTATAAAT CATCGTTTTT TTTTTTTTGA TTTTGTAGAG ACCCTGAAAG ACCCCTGAAG TACCTCCCTA CAGTGGTTGG GGTGT cgttttttttt
(this fragment failed to hybridize with MC44f15 probe on dot blot)

A.3 Table of cloned differential fragments

Differential fragments were subcloned and analyzed by dot blot. The shaded boxes in the "Clone" column represent those clones which hybridized to the respective probe on the dot blot. Those clones which hybridized to the probe were presumed to be representative of the differential fragment and were sequenced. Some clones which did not hybridize to the dot blot were also sequenced, to confirm the validity of selecting clones by this technique.

A.3 Cloned differential fragments

FRAG	SIZE (bp)	DDRT	EXPRESSION	CLONED FRAGMENTS		
				PL	BP	SEQUENCE
MA21f1	500	+	detected 3 bands	a	500	
				b	500	
				d	500	hyb to f6
MA29f2	190/210	**	detected 1 band	a	400	novel
				b	190	same as f2a
				c	190	
				f	190	L17 rib protein
				d	210	
MA32f3	800	++	too weak	b	800	
				c	800	
				d	800	
MA32f4	300	+++	single band	1a	300	
				1b	300	novel same as f7a,c
				1c	300	
				2a	300	
				2b	300	
				2c	300	novel, same as 1b
				2d	300	
MA42f5	600/300/200	+	weak	c	600	
				d	600	Vector + novel
MC25f6	200/210	+	weak	a	200	
				g	200	
				j	200	
				e	210	Novel
				f	210	
				h	210	

A.3 continued

FRAG	SIZE (bp)	DDRT	NORTH	CLONED FRAGMENTS		
				PL	BP	SEQUENCE
MC25f6	200/210		weak	i	210	same as 6e
				l	210	
MC32f7	300	**	see f4	a	300	novel (same as f4)
				d	300	
				e	300	
				f	300	
				b	320	
				c	320	novel (same as f4)
MG32f8	400	++	see f14	c	400	IFN β (TIS7)
MG32f9	150	++	weak	b	150	novel
MG44f11	400	++	weak	a	400	
				c	400	
				d	400	
MG44f12	500	+	weak	c	500	
MC44f13	320	++	weak	e	320	novel
				g	320	
				i	320	
				k	320	
				l	320	same as f13e
MC44f14	150	++	weak	b	150	
				c	300	Vector
				e	150	
				f	150	IFN β (TIS7)
				d	200	IFN β plus rea
MC44f15	125	+	too weak	a	125	Novel
				e	125	Novel

Curriculum Vitae

NAME: Marsha D. Speevak

ADDRESS: 1669 Taché Way
Orléans, Ontario
K4A 2T7

TELEPHONE: 613-824-4467 (home)
613-562-5800 ext 8403 (work)
613-562-5440 (Fax)

EMAIL: mdspeevak@cyberus.ca

PERSONAL

1) **Date of Birth:** June 7, 1953

Nationality: American Citizen
Landed Immigrant in Canada, 1959

Marital Status: Married, with two children

EDUCATION

1) **Academic Degrees**

McGill University:
Montréal, Québec — B. Sc. Biology, 1975

University of Ottawa — Ph.D., Biochemistry (Winter, 1996)

2) **Technical Certifications**

C.S.L.T. — R.T. (C.G.) Registered Technologist, Specialty, Cytogenetics (1981)

A.R.T. (C.G.) Advanced Registered Technologist,
Specialty, Cytogenetics (1991)

SCHOLARSHIPS AND AWARDS

1) Ontario Graduate Studies Scholarship, 11,859.00 $
Sponsored by the Ontario Ministry of Education
University of Ottawa, Ottawa, Ontario
May, 1992-April, 1993.

2) Ontario Graduate Studies Scholarship, $11,859.00
Sponsored by the Ontario Ministry of Education
University of Ottawa, Ottawa, Ontario
May, 1993-April, 1994.

3) Ontario Graduate Studies Scholarship, $11,859.00
Sponsored by the Ontario Ministry of Education
University of Ottawa, Ottawa, Ontario
May, 1994-April, 1995.

4) C.R.S. Studentship, $15,050.00
Sponsored by the Cancer Research Society
1 Place Ville Marie, Suite 2332
Montreal, Quebec
July, 1995-July, 1996

5) University of Ottawa Tuition Scholarship, $3,400.00 per year
Graduate Studies Department
University of Ottawa, Ottawa, Ontario
May, 1992-May 1996

EXPERIENCE AND EMPLOYMENT HISTORY

Employment History

1) McGill University
Montréal, Québec

1975-1976	Research assistant, Polypeptide Laboratory, Biochemistry Deptartment

2) Fisher Scientific Company
Ottawa, Ontario

1976-1978	Inside sales representative

3) Children's Hospital of Eastern Ontario
Ottawa, Ontario

1978-1980	Cytogenetic technologist Cytogenetics Laboratory Genetics Division

4) Montreal Children's Hospital
Montréal, Québec

1980	Cytogenetic technologist Cytogenetics Laboratory Genetics Division

EXPERIENCE AND EMPLOYMENT HISTORY (continued)

5) Children's Hospital of Eastern Ontario
Ottawa, Ontario

1981-1982	Cytogenetic technologist Cytogenetics Laboratory Genetics Division
1982-1986	Prenatal counsellor Genetics Division
1986-1991	Cytogenetic technologist Promoted to Chief technologist, 1988 Cytogenetics Laboratory Genetics Division

A. REFERENCED PUBLICATIONS

1) Speevak, M. D., and M. Chevrette (1996). Human chromosome 3 mediates growth arrest and suppression of apoptosis in microcell hybrids. Mol. Cell. Biol. **16:**2214-2225.

2) Speevak, M. D., N. G. Bérubé, I. J. McGowan-Jordan, S. D. Lupton, and M. Chevrette (1995). Construction and analysis of microcell hybrids containing dual selectable tagged human chromosomes. Cytogenet. Cell Genet. **69**:63-65.

3) Speevak, M.D., and M. Chevrette (1994). Identification of chromosomes implicated in suppression of apoptosis in somatic cell hybrids. Biochem. Cell Biol. **72:**655-662.

4) McGowan-Jordan, I. J., M. D. Speevak, D. Blakey, and M. Chevrette. (1994). Suppression of tumorigenicity in human teratocarcinoma cell line PA-1 by introduction of human chromosome 4. Cancer Res. **54**: 2568-2572.

5) Bérubé, N.G., M. D. Speevak, and M. Chevrette. (1994). Suppression of tumorigenicity of human prostate cancer cells by introduction of human del(12)(q14). Cancer Res. **54**:3077-3081.

B. OTHER PUBLICATIONS

6) Ekker, M., M. D. Speevak, C. C. Martin, L. Joly, G. Giroux, and M. Chevrette (1996). Stable transfer of zebrafish chromosome segments to mouse cells. Genomics **33:**57-64.

7) Rajcan-Separovic, E., H.-S. Wang, M. D. Speevak, L. Janes, R. G. Korneluk, K. Wakasa, and J.- E.Ikeda (1995). Identification of the origin of double minutes in normal human cells by laser-based chromosome microdissection approach. Hum. Genet. **96:**39-43.

8) Goldsmith, C.L., M. D. Speevak, H.-S. Wang, B. Carpenter, and A. G.W. Hunter (1993). Mosaic r(13) in an infant with aprosencephaly. Am. J. Med. Genet. **47**: 531-533.

9) Speevak, M. D., C. Goldsmith, and H. -S. Wang (1992). A familial non-Robertsonian translocation resulting in a child with Down syndrome. Applied Cytogenet. **18**: 93-95.

10) Speevak, M. D., A. G. W. Hunter, D. M. Cox, and H.-S. Wang (1989). The use of computer database for storage and retrieval of cytogenetic data. Karyogram **15**: 51-53.

11) Speevak, M. D., A. G. W. Hunter, H. Hughes, and D. M. Cox (1985). A familial paracentric inv(1)(q42q44) resulting in a child with a del(1)(q42). Ann. Genet. **28**: 177-180.

12) Speevak, M.D., B. Clifford, D. M. Cox, and A. G. W. Hunter (1985). Detection at amniocentesis of a maternally inherited X;Y translocation. Clin. Genet. **27**: 595-599.

13) Hunter, A.G.W., B. Clifford, M. D. Speevak, and S. B. MacMurray (1985). Mosaic tetrasomy 21 in a liveborn male infant. Clin. Genet. **21**: 228-232.

C. ABSTRACTS

1) Speevak, M.D., and Chevrette, M. Microcell hybrids and their use in phenotype/genotype identification. Great Lakes Chromosome Conference, Toronto, Ontario, Canada, May 24-26, 1995.

2) Chevrette, M., Speevak, M.D., Joly, L., and Ekker, M. Mapping the zebrafish genome with a collection of zebrafish/mouse hybrids. EMBO Genomes and Chromosomes Symposium, Heidelberg, Germany, October 3-6, 1994.

3) Speevak, M.D., and Chevrette, M. Identification of chromosomes implicated in suppression of apoptosis in somatic cell hybrids following exposure to *N*(phosphonoacetyl)-L-aspartate. Molecular and Cell Biology of Apoptosis in Development, Disease and Cancer Symposium, Lake Placid, New York, U.S.A., September 29 - October 2, 1994.

4) Rajcan-Separovic, E., Wang, H.-S., Speevak, M.D., Janes, L., Korneluk, R.G., Wakasa, K., and Ideda, J.-E. Application of laser-based chromosome microdissection to clinical cytogenetics. Genosphere Symposium, Tokyo, Japan, Sepetember 21-22, 1994.

5) Speevak, M.D., Bérubé, N.G., McGowan-Jordan, I.J., Ekker, M., and Chevrette, M. Fluorescence *in situ* hybridization of microcell hybrids and their derivatives. Great Lakes Chromosome Conference, Toronto, Ontario, Canada, May 25-27, 1994.

6) Rajcan-Separovic, E., Speevak, M.D., Wang, H.-S., Janes, L., Korneluk, R.G., and Ikeda, J.-E. Application of laser microdissection for characterization of chromosomal material of unknown origin: a case of a marker and a 4p+

chromosome. Great Lakes Chromosome Conference, Toronto, Ontario, Canada, May 25-27, 1994.

7) Ekker, M., Joly, L., Speevak, M.D., and Chevrette, M. Stable transfer of zebrafish chromosome fragments into mouse cells. Cold Spring Harbor Laboratory Meeting on Zebrafish: Development and Genetics, U.S.A., April 27-May 1, 1994.

8) Speevak, M.D., Bérubé, N.G., and Chevrette, M. Analysis of tumor suppression in mouse melanoma microcell hybrids. American Association for Cancer Research, Genetics of Cancer meeting, Hilton Head, South Carolina, U.S.A., November 4-7, 1992.

9) Bérubé, N.G., Speevak, M.D., and Chevrette, M. Analysis of tumor suppression in microcell hybrids derived from prostate cell line DU 145. American Association for Cancer Research, Genetics of Cancer meeting, Hilton Head, South Carolina, U.S.A., November 4-7, 1992.