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IDENTIFICATION OF NOVEL IN VITRO OLIGONUCLEOTIDE-PRIMED LABELLING SITES IN THE DNA OF TERMINALLY DIFFERENTIATED MYELOID CELLS

A thesis submitted to the
School of Graduate Studies
University of Ottawa

In partial fulfillment of the
requirements for the Degree of
Master of Science

Department of Microbiology and Immunology,
School of Medicine.

by

Yogarany Adcharamoorthy

Yogarany Adcharamoorthy, Ottawa, Canada, 1990

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UNIVERSITÉ D'OTTAWA
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ABSTRACT

The biochemical and genetic consequences of the double helical structure of DNA formulated by Watson and Crick (1953) continue to be under investigation for more than 40 years. DNA is not a conformationally homogeneous molecule; instead it has the capability of adopting several types of structures as dictated by its sequence. This double stranded DNA molecule get disrupted temporarily during processes such as replication, transcription and repair. It has also been demonstrated that mammalian DNA including human DNA contain single-stranded DNA regions in their genome.

Many methods have been used for quantifying single stranded breaks in cellular DNA, but no methods have been developed for analyzing DNA sequences at or near break sites. As part of this thesis project, I have developed a new approach to analyze DNA sequences at or near break sites. Cells are first embedded in agarose beads, a technique similar to that used in pulse-field-gel electrophoresis. Embedded cells are lysed <u>in situ</u>, subjected to mild alkali treatment and the single-stranded DNA used as template for labelling by the oligonucleotide-primer labelling method. The labelled DNA is then used to probe a battery of genes immobilized on a membrane. As a control, a similar membrane is probed with randomly labelled, completely denatured DNA.

Using this new method, I attempted to identify sequences at or near oxyradical-induced breaks in human granulocyte DNA. An unexpected but exciting new observation was made. In human granulocyte DNA, five

genes (FOS, CSF2, JUN ERB-B2 and TRF) were found to be 100-1000x more intensely labelled than other genes tested or than they were when probed with randomly labelled control DNA. These sites of labelling were named Selective Labelling Points (SLPs). Further experiments showed that SLPs were present in PMN DNA even in the absence of oxyradical-induced breaks. Characterization of SLPs showed that they are likely single-stranded regions found only in the DNA of terminally differentiated myeloid (granulocyte and monocyte) cells of human and mouse but not in the DNA of other blood cells (lymphocytes) or several cultured cell lines. The appearance of the SLPs was shown to be pH dependent. The precise sequences in the DNA in which they originate have not yet been identified.

Further characterization of these novel sites may give some insight into the nature of pre-existing single-stranded regions in mammalian cell DNA and their significance in the process of terminal differentiation of cells.

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DEDICATION

I dedicate this thesis to my parents

TABLE OF CONTENTS

ABSTRACT.. ii

ACKNOWLEDGEMENTS.. iv

DEDICATION.. v

TABLE OF CONTENT.. vi

LIST OF FIGURES... xiii

LIST OF TABLES.. xv

ABBREVIATIONS... xvi

I. INTRODUCTION... 1

1. OXYRADICAL MEDIATED DNA STRAND BREAK.......................... 1

2. NUCLEAR MATRIX.. 2

2.1. Proteins of the Nuclear Matrix.............................. 3

2.2. Nuclear Matrix and DNA Loop Domains......................... 4

2.3. DNA-Sequence Specific Attachment Sites...................... 5

2.4. DNA Loop Domains and A Model Chromosome Structure............ 6

3. UNUSUAL DNA STRUCTURE... 7

3.1. Left Handed DNA or Z-DNA.................................... 7

3.1.1. DNA Sequence that Favor Z-DNA Formation................... 7

3.1.2. Stability of Z-DNA.. 8

3.1.3. Identification of Z-DNA In Vitro.......................... 8

3.1.4. Biological Roles of Z-DNA................................. 9

(a) Z-DNA and transcriptional enhancers.......................... 9

(b) Z-DNA and chromatin structure...... 9

3.2. Cruciform Structure.......................... 10

3.3. Purine-Pyrimidine Sequences.................. 10

3.3.1. Natural Occurrence.................... 10

3.3.2. Triplexes at Pur.Pyr Sequences......... 11

3.3.3. Models of Pur.Pyr Sequences........... 11

3.3.4. H-DNA................................ 12

3.3.5. Pur.Pyr Sequences in Promotor Region................................ 12

3.3.6. Pur.Pyr Sequences in Transcriptional Active Genes......................... 13

3.3.7. Pur.Pyr Sequences near Splice Sites.... 13

4. **CHROMOSOMAL FRAGILE SITES**.......................... 14

4.1. History....................................... 14

4.2. Nomenclature.................................. 14

4.3. Major Groups.................................. 14

4.4. Expression of Fragile Sites.................. 17

4.4.1. Folate Sensitive...................... 17

4.4.2. Distamycin-A-inducible Sites.......... 17

4.4.3. Bromo deoxy uridine induced Sites...... 18

4.5. Mechanisms of Fragile Site Formation......... 18

4.6. Biologic Significance........................ 19

5. **MYELOID LINEAGE**.................................. 21

6. STATEMENT OF OBJECTIVES.......................... 21

II. MATERIALS AND METHODS.......................... 22

1. BLOOD CELLS.......................... 22

1.1. Isolation Procedure.......................... 22

1.1.1. Human Mononuclear Cells.......................... 22

1.1.2. Human T and B Lymphocytes.......................... 23

1.1.3. Human Granulocytes.......................... 23

1.1.4. Mouse Granulocytes.......................... 24

1.2. Conditions for Treating Blood Cells.......................... 25

2. CELL CULTURE.......................... 25

2.1. Maintenance and Passage of Human Cell Lines.......................... 25

2.1.1. Nonadherent Cells.......................... 25

2.1.2. Adherent Cells.......................... 25

2.2. Conditions for Treating Cultured Cells.......................... 26

2.2.1. Differentiation of U937 Cells.......................... 26

2.2.2. Differentiation of K562 Cells.......................... 26

2.3. Treatment of A431 cells to induce topisomerase I breaks........................ 27

3. **COUNTING CELLS**.................................... 27

3.1. Cytospinning and differential staining........ 27

3.2. Use of Coulter counter........................ 27

4. **ENCAPSULATION OF CELLS IN AGAROSE BEADS**............ 28

5. **QUANTITATION OF DNA**................................ 29

5.1. Embedded DNA.................................. 29

5.2. Non Embedded DNA.............................. 30

6. **LABELLING OF DNA**.................................. 30

6.1. Oligonucletide-Primer Method.................. 30

6.1.1. Embedded DNA.......................... 30

6.1.2. Non Embedded DNA...................... 30

(a) Plasmid DNA....................... 30

(b) Genomic DNA....................... 31

6.2. End-Labelling Method.......................... 31

6.3. Nick-Translation Method....................... 31

7. **MONITORING OF ^{32}P-dCTP INCORPORATION**............... 31

8. **PREHYBRIDIZATION AND HYBRIDIZATION CONDITIONS**...... 32

9. **FILTER WASHES AND FILM EXPOSURE**................... 32

10. **STRIPPING RADIOACTIVE PROBE FOR REUSE OF MEMBRANE**.. 33

11. **DNA EXTRACTION**.................................... 33

11.1. Plasmid Extraction........................... 33

11.1.1. Whole Plasmid........................ 33

11.1.2. DNA Fragment Isolation from Agarose gel.. 33
11.2. DNA Extraction from Human Granulocytes........ 33
12. RNA EXTRACTION..................................... 34
13. IMMOBILIZATION OF DNA ON MEMBRANES................ 35
13.1. Binding Study-Comparison of Binding Capacity and Retentiveness for DNA of Different Membranes.......................... 35
13.1.1. Types of Membranes.................... 35
13.1.2. Salt Conditions....................... 35
13.1.3. Application of DNA Samples............ 35
13.2. SLPs Study-Standard Conditions for Binding DNA to Membranes.................... 35

III. RESULTS... 37
1. BINDING STUDY...................................... 37
2. PRINCIPLE OF THE EMBEDDING METHOD................. 38
3. INITIAL POSITIVE FINDING.......................... 41
4. SLPs IN OTHER GENES............................... 43
5. PRESENCE OF SLPs IN INSERTS ISOLATED FROM THEIR PLASMID VECTORS.................................. 43
6. SLPs AND INTACT BEADS............................. 43
7. RELATIONSHIP BETWEEN SLPs AND TRANSCRIPTIONALLY ACTIVE GENES...................................... 47
8. SLPs PRE-EXIST IN HUMAN GRANULOCYTES AND ARE NOT INDUCED IN VITRO................................... 47

9. DO SLPs ARISE FROM NATURALLY OCCURRING SINGLE-STRANDED REGIONS IN DNA? 47
10. pH DEPENDENT APPEARANCE OF SLPs 50
11. SLPs ARE PRE-EXISTING SINGLE-STRANDED REGIONS IN THE DNA 50
12. SLPs AND TOPOISOMERASE I ACTIVITY 55
13. SLPs IN OTHER HUMAN CELL TYPES 55
13.1. Human Blood Cells 55
13.2. Other Human Cell Types 55
14. SLPs IN MOUSE BLOOD CELLS 59
15. SLPs IN DIFFERENTIATED CELLS 59
15.1. In U937 Cells 59
15.2. In K562 Cells 59

IV. DISCUSSION 69
1. INITIAL INTEREST IN STUDYING OXYRADICAL-INDUCED DNA BREAKS 69
2. APPROACH USED TO INVESTIGATE SEQUENCES NEAR DNA BREAKPOINTS WHICH LED TO THE DISCOVERY OF SLPs 70
3. GENE SELECTION 71
4. HANDLING OF EMBEDDED CELLS 72
5. TRANSCRIPTIONALLY ACTIVE GENES 72
5.1. Hypersensitivity to nucleases 72
5.2. Topisomerase activity 74
6. SINGLE-STRANDED REGIONS OF DNA 75

6.1. Pre-existing single-stranded regions.......... 75

6.2. Structure of SLPs.......................... 76

7. LOCATION OF CHROMOSOMAL FRAGILE SITES.............. 77

8. TERMINAL DIFFERENTIATION........................... 78

9 MODEL OF SLPs FORMATION............................ 79

10. CONCLUSIONS...................................... 80

REFERENCES... 83

APPENDIX... 93

LIST OF FIGURES

Fig. 1: Principle of the embedding method.................. 40

Fig. 2: SLPs are identified by some target genes in human granulocytes................................ 42

Fig. 3: SLPs are identified at another site (c-JUN)........ 42

Fig. 4: DNA fragment analysis of fos gene.................. 45

Fig. 5: SLPs disappear when the beads are broken........... 46

Fig. 6: Northern analysis of SLPs positive genes........... 48

Fig. 7: Oxyradical-induced breaks are not responsible for the appearance of SLPs in human granulocytes....... 49

Fig. 8: S1 endonuclease digestion of embedded DNA.......... 51

Fig. 9: The presence of SLPs is pH dependent............... 52

Fig. 10: SLPs disappear in the absence of Oligo-Primers..... 53

Fig. 11: SLPs are seen under non-denaturing conditions...... 54

Fig. 12: SLPs are absent in the presence of camptothecin.... 56

Fig. 13: SLPs in other blood cells.......................... 57

Fig. 14: SLPs are absent in MeWo cells...................... 58

Fig. 15: SLPs are absent in A431 cells...................... 60

Fig. 16: SLPs are absent in K562 cells...................... 61

Fig. 17: SLPs are absent in U937 cells...................... 62

Fig. 18: SLPs are slightly positive in HL60 cells........... 63

Fig. 19: SLPs are found in mouse mononuclear cells and granulocytes............................ 64

Fig. 20: SLPs are present in differentiated U937 cells...... 65

Fig. 21: SLPs disappear after 48 hr of U937 cells.......... 66

Fig. 22: SLPs are absent in differentiated K562 cells....... 68

LIST OF TABLES

TABLE 1: Binding of single-stranded DNA to Hybond and Immobilon as a function of fragment size....... 39

TABLE 2: Genes tested for SLPs and their chromosome location................................ 44

ABBREVIATIONS

BSA	bovine serum albumin
CDTA	trans-1,2-diaminocyclohexane-N,N',N'-tetra acetic acid.
CT	CDTA-Tris solution
DABA	3,5-diaminobenzoic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
GLB	glass loading buffer
H_2O_2	hydrogen peroxide
MNC	mononuclear cells
O_2^-	superoxide anion
PBS	phosphate buffered saline
PMNs	polymorphonuclear leukocytes
SDS	sodium dodecyl sulfate
SLP	selective labelling points
SSC	standared saline citrate
TPA	12-O-tetradecanoylphorbol-13-acetate

I. INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are cells which take part in the inflammatory and immune responses. Their role is to recognize, phagocytize and destroy foreign objects such as bacteria and other opsonized particles (complement-coated). PMNs respond to these stimuli with a respiratory burst (oxidative burst). The first observation about the respiratory burst was as early as 1933 by Baldridge et al. They observed that white blood cells engaged in phagocytosis undergo a striking increase in oxygen consumption. The burst is defined as cyanide-insensitive O_2 consumption which can be stimulated by soluble and particulate stimuli with the production of superoxide anion (O_2^-) (Babior et al., 1973). Superoxide anion can in turn give rise to H_2O_2 and other reactive species, collectively termed oxyradicals or reactive oxygen species.

1. OXYRADICAL-MEDIATED DNA DAMAGE IN POLYMORPHONUCLEAR CELLS

It has been well documented that these oxygen species and oxidizing species derived from them can cause extensive damage to proteins and lipids. However, not much is known about PMN-mediated intracellular DNA damage. The formation of DNA strand breaks in TPA stimulated PMNs was first demonstrated by Birnboim (1982). It was clear that hydrogen peroxide was involved, since some of the damage was abolished by the addition of catalase, which converts H_2O_2 to O_2 and H_2O (Birnboim, 1982). A three-fold variation in O_2^- produced only a small change in the number of strand breaks (Birnboim et al., 1988). The number of DNA strand breaks induced in TPA stimulated PMNs did not

exceed a maximum of 5000-6000 breaks/cell despite an increase in the extracellular O_2^- generated. This and other observations with metabolic inhibitors suggested that O_2^- could be acting at an extracellular target, implying that a transmembrane signal might be involved to trigger strand breaks in nuclear DNA (Birnboim 1988).

Therefore initially this project was designed to determine whether O_2^--induced DNA breaks occur at random or specific sites in the genome. During the course of my experiments, an unexpected still an interesting observation was made. In human granulocytes some genes were selectively labelled even without oxyradical production. Several evidences suggested that SLPs correspond to single-stranded regions of DNA in some cells (myeloid) and not in others. They may arise because of differences in chromatin structure or nuclear matrix attachment points. They may originate at so called unusual structures in DNA. I will review briefly some features of chromatin, nuclear matrix and unusual DNA structures which may be relavent to this study.

2. NUCLEAR MATRIX

The enclosure of DNA within its own compartment or organelle, the nucleus, was a major evolutionary event that separates eukaryotic from prokaryotic organisms. The development of the nucleus was likely the result of increased organizational and regulatory problems associated with the large genomes of more complex organisms. The organization of the nucleus solved these problems in several ways. The nuclear envelope separates DNA from cytoplasmic processes, while allowing selective transport through the nuclear pores to create a distinct biochemical

environment in the nucleus.

The importance of cytoskeletal elements in the regulation of biological function is becoming increasingly apparent (Shaper et al., 1979, Isaac et al., 1981, Porter and Tucker 1981). One of these structural components is the nuclear matrix, which is an insoluble skeletal frame work of the nucleus.

The nuclear matrix is a universal feature of eukaryotic nuclei. "Residual" nuclear structures have been isolated from a wide variety of mammalian and non-mammalian sources (Barrack and Coffey 1982). Different nomenclature has been used by different investigators to refer to the nuclear matrix: nuclear framework, nuclear skeleton, nuclear scaffold, nuclear ghost and nuclear cage. Studies of the nuclear matrix (Berezney and Coffey. 1974, 1976, 1977) actually preceded those of the cytoskeleton. The widespread acceptance of the presence of this structure has been slower because of the difficulties in developing specific fluorescent antibodies to these very insoluble proteins (Barrack et al., 1982).

2.1. Proteins of the Nuclear Matrix

The nuclear matrix represents about 10-20% of the total mass of nuclear protein mass and is composed of non-histone proteins. Three polypeptide fractions of 62-70 kDa appear to be associated with the pore complex-lamina component of the matrix (Aaronson and Blobel 1975, Dwyer and Blobel 1976). These three lamina proteins have been termed lamin A, lamin B and lamin C and appear to be phosphorylated at the time of disassembly of the nuclear envelope during mitosis (Gerace and Blobel 1980).

2.2. Nuclear Matrix and DNA Loop Domains

The higher-order structure of DNA within the nucleus and within the metaphase chromosome remains an important unsolved problem of molecular biology. At least three higher order levels of DNA organization have been identified in the past two decades: the nucleosome, the 30nm solenoid and the DNA loop domain.

Newly synthesized DNA is tightly associated with the matrix (Berezney and Coffey 1974, 1976). Pardoll et al. (1980) found that the nuclear matrix contains sites which support the replication of DNA loops; the latter were demonstrated to be equivalent to replicons-the basic lengths of DNA synthesized as discontinuous units (Hubermann and Riggs 1968). According to the model of Pardoll et al. (1980), multiple loops of DNA are attached at their base to the nuclear matrix. The two sites of attachment for each loop contain a fixed site for DNA replication. During replication, each DNA loop moves through each of two fixed replicating complexes. As the parental DNA loop passes through the fixed replication sites, two daughter loops are formed. It has been demonstrated that DNA loop domains are attached at their bases to the nuclear matrix (Vogelstein et al., 1980). Vogelstein et al (1980) also observed that these DNA loop structures, in the presence of ethidium bromide, formed a large fluorescent halo surrounding and extending beyond the periphery of the nuclear matrix. When the concentration of ethidium bromide was increased to high levels, a rewinding of the DNA halo back to the periphery of the matrix was seen. However, if the DNA was nicked, then the halo structures could not be rewound by elevating the concentration of ethidium bromide. These experiments suggested that the nuclear matrix organizes DNA so that it

has topological properties equivalent to those of covalently closed circular DNA. These loop-domains are maintained through mitosis attached to a residual chromosome core scaffold. Vogelstein et al (1980) directly measured the size of the fluorescent matrix-halo structures and determined, from the halo radius which was approximately 15μm (90,000 bp of DNA). Several investigators (Earnshaw and Laemmeli 1983, Hancock and Boulikas 1982) have estimated that the size of the DNA-loop domain ranges between 10 and 180 Kbp with an average of 63,000 (20μm of DNA double helix). Each loop would be large enough to contain 315 nucleosomes wound with six per turn into a solenoid structure such as that proposed by Finch and Klug (1976). This 30 nm solenoid would form the filament of a loop. Based on a total DNA content of $6x10^9$base pairs per diploid nucleus, there would be 95,000 such loops within a cell. Both in vivo and in vitro studies of DNA synthesis have shown that over 70% of the replicating sites are contained within the nuclear matrix (Smith et al., 1984).

2.3. DNA-Sequence Specific Attachment Sites

The organization of chromatin into loop domains was demonstrated by the finding that characteristic loops can be identified from generation to generation (Callan 1982). This finding suggested that the organization of chromatin into loop domains is determined by the interactions of specific DNA sequences with nuclear matrix proteins. It was shown that such sequences do exist (Mirkovitch et al., 1984, Gasser and Laemmli 1986). The key element in identification of these sequences was the development of a method for extracting proteins from interphase nuclei that maintains specific protein-DNA interactions. Using this

method, specific nuclear matrix binding sequences have been identified in the Drosophila histone gene product (Mirkovitch et al., 1984), hsp 70 gene (Mirkovitch et al., 1984), alcohol dehydrogenase (Gasser and Laemmli 1986), mouse kappa immunoglobulin gene (Cockerill and Gerrard 1986) and ribosomal RNA genes (Keppel et al., 1986). Different sites are not similar in sequence but all are about 200 bp long and A-T rich (70%). Because these sites remain bound to the nuclear matrix after protein extraction, they have been named either Scaffold Attachment Regions (SAR) or Matrix Attachment Regions (MAR).

2.4. DNA Loop Domains and A Model Chromosome Structure

Pienta and Coffey (1984) constructed models of a single chromatid of human chromosome #4, focusing on the organization of a 30 nm filament into loop domains. First a 2 nm DNA double helix is wound twice around each histone octamer, forming nucleosomes each of which contain 160bp of DNA. Nucleosomes comprise the 10 nm beads-on-a-string fiber. This fiber of nucleosomes is then wound in solenoid fashion with 6 nucleosome particles/turn to form the 30 nm filament. A length of the 30 nm solenoid filament corresponding to 60 Kbp of DNA form a domain that loops out from attachment points in the nuclear matrix. In the chromosome these loops are radially organized 18 to each turn, forming the miniband. Finally 106 of these minibands are arranged along a central axis to form each chromatid.

3. UNUSUAL DNA STRUCTURES

For the past 40 years, scientists have been refining the double helical structure of DNA formulated by Watson and Crick in 1953. DNA is not a conformationally homogeneous molecule; rather, it has the capability of adopting several types of conformations as dictated by local sequences.

3.1. Left Handed DNA or Z-DNA

The left handed Z-DNA structure was not discovered through the investigation of left-handed double helical models; it emerged while solving the structure of a crystalline fragment of double-helical DNA. The structure of Z-DNA was solved from a crystal of a short double helix of a hexanucleoside pentaphosphate having the sequence $d(C_pG_pC_pG_pC_pG)$ (Wang et al., 1979). To convert a section of B-DNA to Z-DNA, the base pairs must flip so that they are now upside down relative to the orientation they had in B-DNA. This is brought about by rotation of purine residues about their glycosyl bond, from the anti to the syn conformation. In the case of the pyrimidines, both the bases as well as the sugars rotate. It is this rotation of the sugar that produces the zig-zag back bone conformation of Z-DNA. Both right handed B-DNA and left handed Z-DNA are double helical conformations with anti-parallel chains, held together by Watson-Crick hydrogen bonding between the bases.

3.1.1. DNA Sequences that Favor Z-DNA Formation

Since the syn conformation is more stable for purines than for pyrimidines, Z-DNA is favored in nucleotide sequences that have alterations of purines and pyrimidines. Not all sequences convert

equally readily to Z-DNA. Segments of DNA with repeating d(CG) sequences are the most favored for forming Z-DNA (Pohl and Jovin 1972). The next most effective sequences are $d(CA)_n$ or $d(TG)_n$ (Vorlickova et al., 1982). Long sequences of $d(CA/TG)_n$ represent a class of middle repetitive elements in eukaryotic genomes. These sequences form Z-DNA readily and their widespread distribution may indicate a significant biological function. Finally, d(AT) sequences favor Z-DNA least of all.

3.1.2. Stability of Z-DNA

In physiological solutions, Z-DNA is less stable than B-DNA largely owing to electrostatic repulsion between the negatively charged phosphates on opposite strand that are closer together in Z-DNA than in B-DNA. Z-DNA can be stabilized most effectively by negative supercoiling of DNA (Singleton et al., 1982).

3.1.3. Identification of Z-DNA in vitro

A biochemical assay used to identify Z-DNA _in vivo_ was based on the _in vitro_ observations that a target (recognition) site is not methylated by its specific methylase nor cleaved by its specific restriction endonuclease when the site is near (Singleton et al., 1983) or in a left handed Z helix (Zacharias et al., 1984). These enzymes do act on the same target sequences when they exist in a right handed B-structure. For example, when a Bam HI (GGATCC) or an EcoRI (GAATTC) site is located at the interface between a vector (right handed under all conditions) and an alternating $(C\text{-}G)_n$ insert (which may be either B or Z depending on the supercoil density of the plasmid), they are methylated by the respective Bam HI or EcoRI methylase only when the insert is right handed (Singleton et al., 1983).

3.1.4. Biological Roles of Z-DNA

(a) Z-DNA and Transcriptional Enhancers

Mapping of Z-DNA sequences in deproteinated, negatively supercoiled SV40 DNA revealed three segments of eight base pairs of alternating purines and pyrimidines that represent three major sites that form Z-DNA (Nordheim and Rich 1983). Also it was shown that Z-DNA segments are part of the transcriptional enhancer element of the viral early promotor (Gruss et al., 1981; Benoist and Chambon, 1981). Additionally, two segments of hypersensitivity to DNase I cleavage have been identified within this region, covering the origin of replication and the transcriptional enhancer. DNase I hypersensitivity is usually associated with transcriptionally active chromatin. Fine structure mapping of DNase I hypersensitive sites in the enhancer region of SV40 identified DNase I cleavage site approximately 25 bp on either side of the segments forming Z-DNA (Cereghini et al., 1983).

(b) Z-DNA and Chromatin Structure

There is at least one 50 bp or longer sequence of $d(CA/GT)_n$ per 50-100kb of eukaryote DNA (Hamada et al., 1982). Since these sequences can form Z-DNA readily at physiological negative superhelical densities (Nordheim and Rich 1983), they could play a role in domain activation (Rich 1983). The Z-forming segment is long enough for a B-to-Z (or Z-to-B) conversion which might change the superhelical density of the entire domain. A change in superhelical density might also regulate gene expression (Smith et al.,1981).

3.2. Cruciform Structure

Palindromic sequences in double-stranded DNA can rearrange to a tertiary structure called a cruciform (Gierrer 1966). In linear DNA, cruciforms are not favored thermodynamically and their formation requires activation and stabilization energy which may be provided by protein binding or supercoiling.

The first experimental evidence for the existence of cruciforms in purified supercoiled DNA was the observation that the single-strand specific endonuclease S1 could linearize circular double-stranded DNA by cleaving both strands in the middle of palindromic sequences (Panayotatos and Wells 1981, Lilley 1980). More recently a native cruciform structure has been shown in vivo (Panayotatos and Fontaine 1987). This paper also demonstrated that a cruciform structure can exist intracellularly and indicates that bacterial DNA has a substantial amount of single-stranded character in vivo.

3.3. Purine-Pyrimidine Sequences

3.3.1. Natural Occurrence

Stretches of DNA containing long sequences of purines on one strand and pyrimidines on the other (pur.pyr sequence) occur in both prokaryotes and eukaryotes. Their biological function is not known for certain but they are found at frequencies substantially higher than expected on a random basis in several human genes and eukaryotic viruses (Behe 1987, Beasty and Behe 1988). They are often associated with S1 nuclease hypersensitive sites (SHS) upstream of eukaryotic genes (Hentchel 1982, Larsen and Weintraub 1982). S1 hypersensitivity is only one of several attributes indicating that pur.pyr sequences have unusual

structures.

3.3.2. Triplexes at pur.pyr Sequences

Three stranded nucleic acids (triplexes) were widely studied in the 1960's with RNA and DNA polymers of repeating nucleotide sequences (Morgan and Wells 1968). It was discovered that only polymers that contained all purines in the complementary strand were suitable for formation of triplexes. For example, $(dA)_n.(dT)_n$ could form a triplex with another strand of $(dT)_n$ to generate $(dA)_n.2(dT)_n$, whereas the alternating $d(A\text{-}T)_n$ DNA could not adopt a triplex structure. Generally, but not always, two strands of pyrimidines were associated with one purine strand (eg., $(rA)_n.2(r(U))_n$). It is likely that the third (pyr) strand was associated with the duplex via Hoogsteen (or reversed Hoogsteen) pairs in the major groove. Although this pairing scheme presents no problem for A and T (or U) residues, it requires protonation of a cytosine in the third strand for stabilization in the case of G+C. The discovery (Hanvey et al., 1988) of extremely high pK (above 7) of dC residues in the polymeric form and the formation of triplexes at neutral pH with polymers containing 5-methyl cytosine (Lee et al., 1984) makes the occurrence of triplexes more plausible at GC sequences.

3.3.3. Models of pur.pyr Sequences

Recent interest in triplexes was generated by the enzymatic, chemical and physical properties of pur.pyr inserts in plasmids. At least four other models were proposed for the structure of short pur.pyr sequences containing A's and G's on one of the complementary strands. The most widely accepted model suggested that the reactivity of pur.pyr sequences would occur at major conformational adjustments caused by a buildup of strain resulting from different conformations of two strands.

This was the first model to account for the increased stability of the unusual structure of pur.pyr sequences at low pH as well as to propose the protonation of cytosines with the subsequent formation of Hoogsteen basepairs with guanine. It was suggested that the sequence $(AG)_n$ adopts a heteronomous structure, with each strand having a different back bone conformation possibly because of stacking differences of the two strands (Evans and Efstratiadis 1986).

3.3.4. H-DNA

The H-DNA model was based on the possibility that cytosine-protonation is involved in the unusual structure, the requirement for negative superhelical tension and the unwinding of the double helix on formation of the structure. An intra-molecular triplex is possible for short pur.pyr sequences which are a mirror repeat. Long sequences need two homologous segments that can interact to form the triplex is possible after the DNA is looped back upon itself. Models for a triplex in plasmids suggest the existence of two isomers of an intra-molecular triplex. In one isomer, the 5' half of the pyrimidine strand is the third strand, which interacts by Hoogsteen base pairing with the purine strand. In the other case, the 3' half of the pyrimidine strand is the third strand and the 5'half of the purine strand is unpaired.

3.3.5. Pur.Pyr Sequences in Promotor Regions

The promoters of mammalian ribosomal protein (rp) genes initiate transcription with high precision despite the fact that they do not contain well-defined TATA boxes (Hariharan et al., 1989). Initiation sites are generally situated within pur.pyr tracts flanked by regions of high G+C content.

3.3.6. Pur.Pyr Sequences in Transcriptionally Active Genes

Transcriptionally active chromatin is often, but not always, characterized by the presence of DNase I and/or S1 nuclease hypersensitive sites in the 5'or 3' end of genes. There are long (100bp or longer) poly (pur.pyr) tracts, containing multiple CT and CCCT repeats in the sequenced 5' end region of the two mammalian non-transcribed S1 sensitive sites (NTSS) (Yang-yen et al., 1985).

3.3.7. Pur.Pyr Sequences near Splice Sites

A long pur.pyr sequences in the -55 region of the chicken embryonic myosin chain imposes an altered structure on its 5' flanking DNA for at least 200 bp (McCarthy and Heywood 1987). In the human c-myc gene, a DNA sequence change as far away as 500 bp altered the S1 nuclease cleavage of a pur.pyr stretch (Boles and Hogan 1987). In all mammalian introns examined, so far the region proximal to the 3' splice site is rich in pyrimidines. This conservation implies an important role in splicing process (Reed and Maniatis 1985, Weiringa et al 1984). A point mutation changing the AG dinucleotide to GG in the second intron of the β-globin gene abolishes splicing at the normal site (Atweh et al., 1985).

Diagram 1 (a) shows a variety of possible unusual structures in DNA (Wells 1988) and 1 (b) model showing the triplex DNA formation at the poly purine-pyrimidine sequences (Wells et al., 1988).

4. CHROMOSOMAL FRAGILE SITES

4.1. History

The first report of a fragile site was published in 1965 by Dekaban who described a persisting clone of cell with an abnormal C group chromosome in a woman who had received radiation therapy (Dekaban 1965). Three years later, it was shown that these sites were heritable and used the term, a "fragile site". The chromosomal fragile site at Xq27, now known as fragile X, associated with mental retardation was described by Lubs in 1969 .

Chromosomal fragile sites, detected cytogenetically, are expressed, under appropriate in vitro conditions, as gaps and breaks involving one or both chromatids.

4.2. Nomenclature

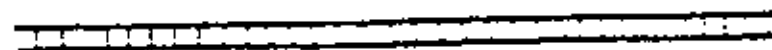
The cytogenetic nomenclature for fragile sites are now designated by "fra" followed by the chromosome number and the chromosome arm and band.

4.3. Major groups

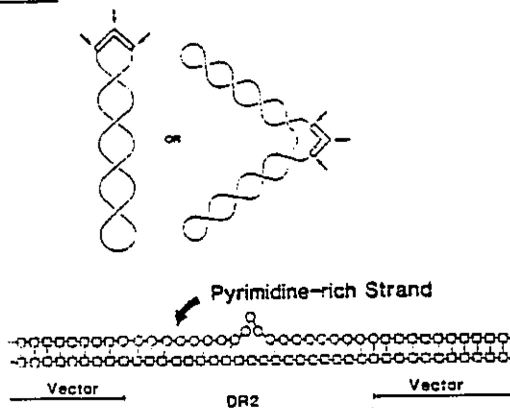
Classification depends on prevalence in the general population. There are two major groups of sites (Berger et al., 1985) known as : (a) Rare or Heritable site (b) Common or constitutive site <u>Heritable sites</u> are defined as specific points or loci that show the following features (LeBeau. 1986): (i) The chromosome has a non-staining gap of variable width that usually involves both chromatids. (ii) The gap involves the same locus in all individuals within a family or kindred and is

Left handed Z-DNA (therefore B-Z junctions)

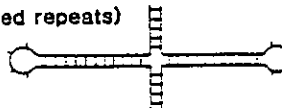
Pur · Pyr Structures



Anisomorphic DNA

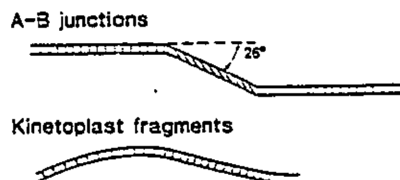


Cruciforms (at inverted repeats)



Bent (curved) DNA

A-B junctions



Slipped structures (at direct repeats)

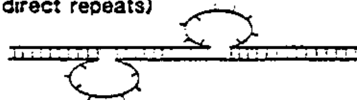


Diagram 1: (a) Unusual DNA structures (Wells 1980).

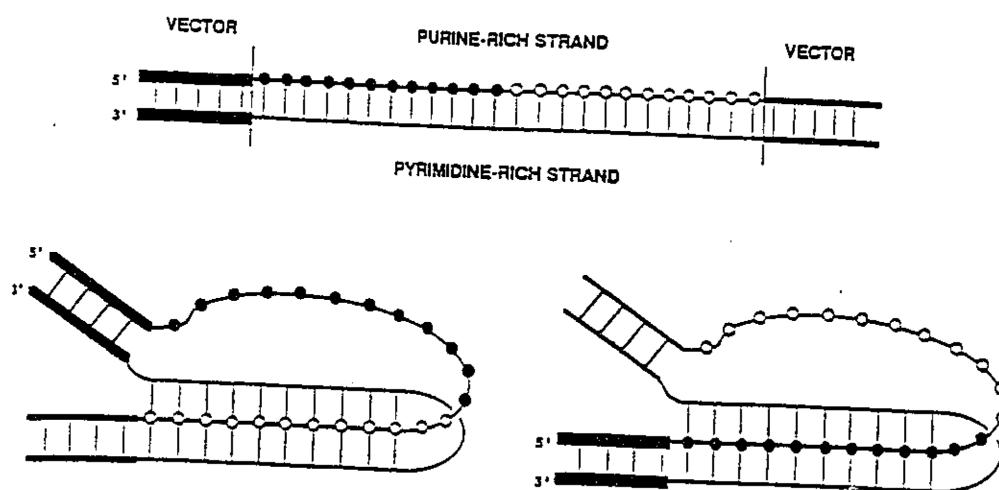


Diagram 1: (b) Model of triplex DNA at Purine/Pyrimidine sequences.

inherited in a Mendelian co-dominant fashion. (iii) The fragility expresses itself as the production of acentric fragments, deleted chromosomes or triradial figures. Common sites were defined as sites occurring frequently and caused by environmental factors such as chemicals, radiation and/or viruses. These may be observed in both chromatids.

4.4. Expression of Fragile sites

The key to the detection of these sites was the discovery that fragility may be expressed only under highly specific culture conditions and in some cases only in certain tissues (Sutherland 1977).

Heritable fragile sites can be grouped into three classes based upon the culture condition necessary for their expression.

4.4.1. Folate Sensitive sites

These sites are expressed under tissue culture conditions in which there is a relative deficiency of thymidine. This condition can be obtained by treatment with the antifolates methotrexate or aminopterin (Sutherland 1979) and by specific inhibition of thymidine synthetase with flurodeoxyuridine or fluorodeoxycytidine (Glover 1981, Jacky and Sutherland 1983).

4.4.2. Distamycin-A-inducible sites

These sites may be expressed spontaneously; however their expression is enhanced by the presence of distamycin A (Schmid et al., 1980), interferon (Threstrup et al., 1980), bromodeoxyuridine (Croci 1983) or Hoechst 33258 in culture medium.

4.4.3. Bromodeoxyuridine Induced Sites

These sites are expressed only if bromodeoxyuridine is present in culture medium for at least the final 8-24 hr of culture (Scheres and Hustinx 1980).

Common sites appear to occur in humans with a very high frequency. Expression of these are enhanced by chemicals such as aphidicolin and caffeine.

4.5. Mechanisms of Fragile Site Formation

The precise mechanism(s) of fragile site formation is unknown. Initially it was suggested that these lesions arose simply from despiralization of chromosomes that is mediated by alterations in DNA, histones, nonhistone proteins or divalent cations (Chaudhuri 1972). The nature of the inhibitors and inducers of the folate-sensitive sites suggests that the process is likely to occur during DNA synthesis. The common feature of these agents is that they interfere with the 5,10-methylenetetrahydrofolate-requiring conversion of deoxyuridylate to thymidylate. Sutherland (1977) proposed that the fragile site is created because a section of thymidine-rich DNA cannot complete its synthesis when the thymidine supply is restricted and that the site subsequently does not compact normally during mitosis. Goulion et al. (1980) have demonstrated that when the level of thymidine is low in culture medium, uracil is misincorporated into DNA during replication. This finding led these investigators to propose that the folate-sensitive sites appear as a result of heritable defects of DNA methylation along a chromosome region that normally binds a folding protein involved in chromosome condensation (Krumdieck and Howard-

Peebles 1983). The misincorporation of uracil in place of thymidine into DNA would produce a further loss of methyl groups, eventually precluding proper DNA-folding protein interactions.

Another mechanism is based upon the phenomenon of thymine-less death observed in bacteria (Hanawalt 1982). The misincorporated uracil is subject to recognition and removal by uracil-N-glycosylase, and the resulting apyrimidinic sites would be attacked by endonucleases. Each strand break would serve as a site for initiation of an excision-repair patch. In the absence of thymidine, dUMP would be incorporated and removed cyclically with lengthing of the patches during successive cycles. Ultimately double strand breakage would result in fragmentation cf the chromosome.

4.6. Biologic Significance

Generally, phenotypic consequences of the autosomal fra sites are unclear. Fra sites have been implicated in the pathogenesis of human tumors. Yunis (1983) found a correlation between 3 fragile sites and chromosomal rearrangements in malignant cells. He proposed that chromosomal rearrangements might be facilitated by the presence of heritable or constitutional sites. Le Beau and Rowley (1984) identified a remarkable concordance between the chromosomal location of fragile sites and the break points that occur in non-random abnormalities associated with leukemia and lymphomas.

Although an association of fragile sites with cancer breakpoints has been recognized, the exact nature of their relationship at the DNA or gene level remains obscure. In many instances, the genes located at the breakpoints of specific rearrangements were previously recognized as

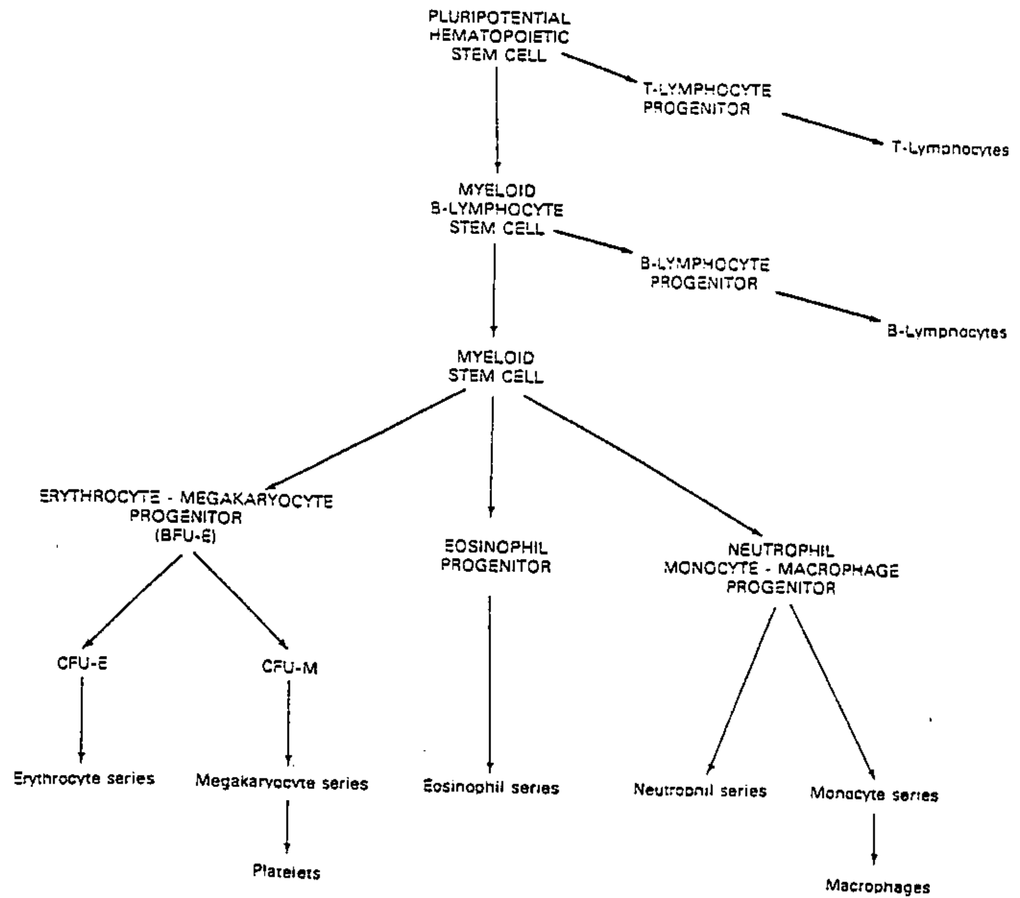


Diagram 2: Schematic representation of myeloid lineage.

proto-oncogenes.

5. MYELOID LINEAGE

Blood cell formation is commonly referred to as hematopoiesis. The tissue is which blood cells are produced is termed hematopoietic tissue. There are two kinds of hematopoietic tissues, myeloid and lymphatic. The term myeloid refers to the fact that erythocytes, granulocytes, monocytes and their precursors are formed in bone marrow. CSFs (colony stimulation factors) are a family of glycoproteins involved in the control of proliferation or differentiation of hematopoietic cells (Metcalf 1981). Blood cell lineages from pluripotent hematopoietic stem cells are shown in Diagram 2 (from Blood cell formation and the cellular basis of immune responses Ham et al., 1979, chp 12, pp306).

6. STATEMENT OF OBJECTIVES

The initial objective of this project was to determine whether DNA strand breaks in human PMNs (stimulated with TPA) occur at random or at specific sites. This was investigated by encapsulating freshly isolated human PMNs in agarose beads, denaturing with mild alkali treatment and labelling using oligo primer labelling. During the course of these experiments, it became evident that Selective Labelling Points (SLPs) were present at specific gene sites; however, they were not a result of oxyradical-mediated DNA breaks. Since this was a novel and interesting observation, I modified my original objective and carried out further experiments to investigate and characterize the nature of SLPs.

II. MATERIALS AND METHODS

1. BLOOD CELLS

1.1. Isolation Procedure

1.1.1. Human Mononuclear cells (MNC)

About 50-60 mL of blood was collected from normal volunteers in EDTA containing vacutainer (Beckton & Dickinson) and mixed carefully on a rocker platform (Bellco Biotechnology) to avoid small clots. Two volumes of B-Earles buffer (appendix, 5a) was added. Diluted blood was equally distributed to four 50 mL polypropylene centrifuge tubes. 12.5 mL of Ficoll-paque (density 1.077g/ mL; Pharmacia) was underlaid in each tube. The tubes were centrifuged in a swing-out rotor in a bench top centrifuge (International Equipment Company) at 450xg for 15 min at room temperature. The mononuclear cell layer formed between the top yellow plasma layer and the Ficoll-paque layer was removed with a transfer pipet and transferred into two 15 mL conical polypropylene tubes. The tubes were centrifuged at 600xg for 10 min at room temperature. The supernatant was removed and the pellets rinsed twice with B-Earles buffer. The recovered cells were counted in a Coulter counter. The mononuclear cell layer contained about 70-80% lymphocytes and 20-30% monocytes as estimated by morphology of cells stained with Differential Kwik after sedimentation onto a slide using a Cytospin. Distinguishing lymphocytes from monocytes by morpholoy and staining is recognized to be an unreliable, but was used as a rough guide to the cell types present. PMNs are readily distinguished and accounted for <2% of the nucleated cells in this layer.

1.1.2. Human T and B Lymphocytes

Purification of lymphocytes from mononuclear c lls were performed using Lymphokwik, a commercially available kit (One .ambda, Los Angeles, CA), which uses monoclonal antibodies and complement to lyse unwanted cell populations. In brief, the mononuclear cells (2.5-3x10^7), isolated as in section 1.1.1, were centrifuged in 15 mL conical glass tubes at 600xg for 10 min. The supernatant was removed by aspiration and the pellets were resuspended in 0.8 mL of Lymphokwik monoclonal antibody mixture. The cell/antibody suspension was then incubated at 37°C in a water bath for 30 min to allow lysis to occur. During the incubation, cell clumps were dispersed using a transfer pipet. When the incubation was over, 0.2 mL of phosphate buffered saline (PBS) was layered on top of the cell mixture. Tubes were centrifuged in a benchtop centrifuge at 600Xg for 12 min at room temperature. The supernatant was removed by aspiration and pellets were rinsed three times with PBS. Finally the cells were resuspended in B-Earles and counted using a Coulter counter. The purity of the cells (>90%) were estimated by morphology of cells stained with Differential Kwik staining on a Cytospin preparation.

1.1.3. Human Granulocytes

Whole blood was treated in the same manner as described up, to mononuclear cell isolation step (1.1.1). Granulocytes co-sediment with erythrocytes just above or within the bottom layer. The plasma, mononuclear cells and Ficoll-paque layers were removed by aspiration leaving the bottom layer undisturbed. The walls of the tubes were gently washed by adding 5 mL of B-Earles buffer down the side of the tubes and aspirating off the solution. Each packed cell layer was diluted with 10

mL of B-Earles and mixed with four volumes (40 mL) of NH_4Cl solution (appendix, 5a). The tubes were mixed by inversion and incubated in a water bath at 30^oC for about 10 min (until the color changed from bright red to purple indicating red cell lysis). The tubes were centrifuged at 600xg for 10 min at 2^oC. The combined pellets were suspended in 20 mL B-Earles buffer and the cell suspension was distributed into two 50mL polypropylene tubes. Then 20 mL of ice-cold water was added to each tube to lyse residual red cells by hypotonic shock. Exactly 60 sec after the addition of water, 2 mL of 1.7M NaCl was added to each tube to restore isotonicity. The tubes were centrifuged in a refrigerated centrifuge at 600xg for 10 min at 2^oC. Both pellets were resuspended in 20 mL of B-Earles. The cells were counted in a Coulter counter and the volume adjusted to $2x10^6$ cells/ mL. Purity of the cells were 98%, as estimated by morphology of cells stained on Cytospin preparation.

1.1.4. Mouse Granulocytes

About 4 mL of blood was collected from eleven C57 BL/6 male mice (41-47days old from Charles river). The animals were injected with 75 units of heparin about 5 min before killing. The isolation procedure was essentially the same as described for human granulocytes (1.1.3) but the volumes of the reagents were scaled down. Separation of lymphocytes, monocytes and granulocytes was not as clear-cut as for human blood but, because of the small volume of blood obtainable from mice, the technique was not repeated.

1.2. Conditions for Treating Blood Cells

5 mL of blood cells (MNC or granulocytes) at $2X10^6$cells/mL were incubated with 20μg/mL catalase and $2x10^{-8}$M TPA (from a 1mM stock solution in DMSO) together at 37°C for 40 min in 15 mL polypropylene tubes. The tubes were mixed by inversion every 10 min during incubation. The tubes were transferred to ice for 5 min and centrifuged at 600xg for 10 min at 2°C. The pellets were resuspended in 5 mL of B-Earles buffer.

2. CELL CULTURE

2.1. Maintenance and Passage of Human Cell Lines

2.1.1. Nonadherent Cells

HL60 (promyelocytic leukemia line), K562 (erythroleukemia line) and U937 (histiocytic lymphoma line) cells were maintained in culture medium (RPMI 1640) containing 10% fetal calf serum (Gibco Laboratories). The cells were allowed to grow to confluency and seeded at $10X10^5$ cells per 10 mL per 100mm polystyrene petri dishes. The plates were incubated at 37°C in a 5% CO_2 atmosphere incubator (GCA corporation).

2.1.2. Adherent Cells

A431 (epidermoid carcinoma line) and MeWo (melanoma line) were maintained in RPMI 1640 with 10% fetal calf serum. The cells were allowed to grow to confluency in 100mm polystyrene petri dishes. The monolayer cells to be passed were washed once with 5 mL of PBS and detached from the plastic surface with a mixture of 1x trypsin and EDTA (Gibco) for 5 min at 37°C. The cells were seeded at $10X10^5$ cells per 100 mm polystyrene dish.

2.2. Conditions for Inducing Differentiation Of Cells

2.2.1. Differentiation of U937 cells

U937 cells growing in culture medium as specified in 2.1.1 were induced to differentiate using TPA. U937 cells from five 100mm dishes were first pooled in a 50 mL polypropylene tube, then redistributed (10 mL of cells at $4X10^5$/ mL) to 100mm polystyrene petri dishes. TPA (0.2μM final concentration) was added to four dishes. Cells were harvested and analyzed at 10 and 20 or 24 and 48 hr after TPA addition in different experiments.

2.2.2. Differentiation of K562 Cells

K562 cells growing in culture medium as specified in 2.1.1 were induced to differentiate using hemin, sodium salt of n-butyric acid and cytosine β-D-arabinofuranoside. Four plates of K562 cells were pooled in a 50 mL polypropylene tube. About 10 mL of cell suspension at $2x10^6$cells/ mL was transferred to four fresh 100mm petri dishes. Three dishes of cells were treated individually with hemin ($1x10^{-4}$ in $1x10^{-3}$ NH_4OH), sodium salt of n-butyric acid ($2x10^{-3}$M;Sigma) or cytosine B-D-arabino furanoside ($5x10^{-7}$;Sigma). The fourth plate of cells was untreated (control). Induction of differentiation was allowed to proceed for 96 hr.

Induction of differentiation was scored by benzidine dihydrochloride staining (Rowley et al., 1981). Immediately before use, tetramethylbenzidine dihydrochloride (2mg/ mL in 0.5% acetic acid) was mixed with H_2O_2 (final 5μg/ mL) and added directly to an equal volume of cell suspension in a 24 multiwell plate. Hemoglobin-positive cells stained blue and hemoglobin negative cells were yellow, as determined

microscopically.

2.3. Treatment of A431 Cells to Induce topoisomerase I-Breaks

One mL of growing A431 cells at $2x10^6$cells/mL was transferred into a 2mL microfuge tube (Sarstedt). The cells were treated with 5μM A23187 at 37°C for 15 min to induce expression of fos gene and then 10μM camptothecin was added and incubation was continued for 5 min.

3. COUNTING CELLS

3.1. Cytospinning and Differential Staining

A cell suspension (blood cells) in B-Earles (0.2 mL containing $2x10^3$ cells) was loaded in the sample holder attached to a Shandon filter and microscope slide unit. The unit was spun at 600xg for 3 min. The cells were then fixed by dipping the slide into methanol for 10 sec, and stained with Differential Kwik staining solutions for 10 sec each. The slide was rinsed in water and then air dried. A drop of immersion oil was spotted on the slide and covered with a cover slip. The purity of each sample was then estimated by microscopically on the basis of morphology and staining characteristics.

3.2. Use of Coulter counter

A Coulter counter (Coulter Electronics Ltd) was used to estimate the cell number of both blood and cultured cells.

4. ENCAPSULATION OF CELLS IN AGAROSE BEADS

The method is adopted from Cook (1984). 1% (w/v) low melting agarose gel (BRL) in PBS was melted by heating in a microwave oven and cooled to 37°C in a water bath. 1 mL of prewarmed (37°C) blood cells or cultured cells at $2X10^6$/ mL in B-Earles was mixed with equal volume of molten agarose in a 15 mL siliconized Corex tube. The agarose/cell suspension was quickly poured into a 30 mL Corex centrifuge tube containing 4mL of prewarmed (37°C) paraffin oil (BDH; density 0.83g/mL). The tube was agitated at high speed on a vortex mixer for 30 sec at room temperature and then transferred to a 37°C water bath for 15 sec. The tube again was agitated for another 20 sec and then left on ice for 10 min. During chilling on ice, most of the oil formed a layer on top of the packed agarose beads. The oil layer was removed carefully using a plastic transfer pipette. The tube wall was washed with 4 mL of B-Earles buffer. Another 4 mL of B-Earles buffer was added and the tube was vortexed briefly to disperse beads. The agarose bead suspension was poured into a 15 mL conical glass tube. The tube was capped shaken on a wrist action shaker for 3 min. 1 mL aliquots of bead suspension were transferred to 1.5 mL conical screwcap microcentrifuge tubes. The tubes were centrifuged at room temperature in a microfuge at about 6000 rpm for 3 min. The beads were washed 3-5X with 0.5 mL of B-Earles. The beads were transferred to a fresh Sarstedt tube during the washes. After removing the supernatant, 0.2 mL of Deproteinizing Solution (Appendix, 5b) was added to the beads which were then incubated at 45°C for 45-60 min. The tubes were centrifuged in a microfuge and the deproteinizing solution was removed by washing repeatedly with B-Earles (0.5 mL buffer, 2 sec vortexing and 3 min incubation at 30°C). The

washing steps were repeated 5-6X. In alkali treatment experiments, 0.2 mL of Unwinding Solution (appendix, 5c) was added and the tubes were incubated at 30°C for 30 min. After alkali treatment, 0.1 mL of Neutralizing Solution (1M glucose) was added to drop the pH to about 11. In non-denaturing experiments, Unwinding Solution (2 vol) was premixed with Neutralizing Solution (1 vol) to drop the pH to 11 before addition to the tubes (see results).

5. QUANTITATION OF DNA

5.1. Embedded DNA

Agarose beads in pH 11 solutions were melted by heating at 62°C for 2-3 min. 20μl of molten agarose-DNA was then removed and transferred to 0.5 mL microcentrifuge tube. 20μl of 0.2N HCl and 100μL of 0.1 HCl were added to the tube. The tubes were left on ice for 15 min, to allow DNA to precipitate, then centrifuged at high speed in a microfuge at room temperature for 2 min. The supernatant was discarded and the pellet was suspended in 100μL of DABA (Aldrich, 0.4g/ mL in water). A standard curve was prepared by adding 0, 0.5, 1.0 and 1.5μg of human genomic DNA to tubes which were treated as described above.

Tubes were incubated at 60°C for 45 min. The contents of the tubes were transferred to glass tubes (16x100mm) containing 2.5 mL of 1N HCL. The tubes were read in the fluorescence spectrometer (Perkin-Elmer, model LS5) using excitation wavelength of 410nm and emission wavelength of 500nm.

5.2. Non-Embedded DNA

Pure plasmid or genomic DNA was quantitated in a Perkin-Elmer spectrophotometer by absorbance at 260nm. (A_M=6700; 50μg/ mL DNA gives A_{260} of 1.0).

6. LABELLING OF DNA

6.1. Oligonucleotide-primer method

This method was essentially the same as published earlier (Feinberg and Vogelstein 1983). Random deoxyhexanucleotides (Pharmacia) were used as primers (appendix, 3).

6.1.1. Embedded DNA

Embedded DNA in agarose (pH 11 solution) was melted at 62°C for 3 min. About 200ng was added to a labeling reaction mix as described in section 6.1.

6.1.2. Non-Embedded DNA

(a) Plasmid DNA

8μL of plasmid DNA (1.0μg) in CT buffer was nicked by boiling at 100°C for 5 min. The sample was chilled on ice and centrifuged in a microfuge for 5 sec. 2μL of 0.5N NaOH was added and the tube was incubated at 50°C for 30 min to complete the nicking and denaturation. The sample was centrifuged for 5 min and 40μL of sterile water was added to give a final DNA concentration of 20ng/μL. 2.5μL (50ng) of DNA was added into labelling reaction mix (appendix, 3).

(b) Genomic DNA

The labelling of this DNA was carried out by the two methods described in section 6.1.2 (a) and herein. 10μL of genomic DNA (1.0μg) in CT was nicked by boiling at 100°C for 5 min. The sample was chilled, on ice. 30μL of 35mM NaOH was added to the nicked DNA, and the tube incubated at 50°C for 40 min. 10μL of 1M glucose was added to give final DNA concentration of 20ng/μL and a final pH of about 11.0 (NaOH/glucose buffer).

6.2. End-Labelling Method

For studies of binding to membranes, plasmid DNA was digested with PstI and fragments were isolated from an agarose gel after electrophoresis (appendix, 1). Isolated fragments were end-labelled using terminal transferase enzyme (appendix, 2).

6.3. Nick Translation Method

This was performed following the procedure in BRL nick translation kit for binding study (appendix, 4).

7. MONITORING OF ^{32}P-dCTP INCORPORATION

The labelling reaction was terminated by addition of SDS to 0.4% and CDTA to 20mM. A small amount of the labelling mix (<1μL) was precipitated in the presence of 50 μg carrier RNA (50μL of 1mg/ mL ribosomal RNA in 0.5N NaCl) with 50μL of 1N HCl. After 15 min on ice the tubes were centrifuged. The acid soluble fraction of radiolabel was transferred to a scintillation vial and the acid precipitable fraction

was washed three times with cold 0.5N HCl. The supernatant and washes were combined in a scintillation vial containing 6 mL H_2O. The precipitate was dissolved in 100μL 1M NH_4OH and transferred to vial containing 6 mL of H_2O. The samples were counted in a liquid scintillation counter by Cerenkov radiation using a ^3H window.

8. PREHYBRIDIZATION AND HYBRIDIZATION CONDITIONS

The prehybridization (blocking) of filters was done in polypropylene bags (BRL). About 10 mL of prehybridization solution (appendix, 5a) was added to the bag which was sealed, avoiding air bubbles. Blocking of the membrane was allowed to proceed for 30 min at 65^oC in a shaking water bath. The prehybridization solution was removed and 10 mL of hybridization solution (appendix, 5b) and heat denatured radio-labelled DNA probes were added, and the bag was sealed. Hybridization was allowed to proceed at 65^oC for 12-18 hr.

9. FILTER WASHES AND FILM EXPOSURE

The radioactive probe was removed from the bag and the filter was washed twice with 250 mL Wash Solution I (2xSSC/ 0.1% SDS) for 5 min at room temperature. The filter was washed once with 150 mL Wash Solution II (0.16XSSC/ 0.1% SDS/ 10mM Na citrate pH 6.5) at room temperature for 3 min. A high stringency wash was carried by washing twice with prewarmed (65^oC) Washing Solution II at 65^oC for 20 min. The filters were dried at 80^oC for 20 min followed by exposure to preflashed Kodak (XAR-5) X-ray film at -20^oC for 48 hr with intensifying screens.

10. STRIPPING OF RADIOACTIVE PROBE FOR REUSE OF MEMBRANE

The filters were placed in a plastic box containing 250 mL Strip Solution (0.1% SDS/ 0.1XSSC) at >95°C. The box was shaken at room temperature for 15 min and the stripping was repeated once. After initial stripping, the filters were equilibrated with 0.1% SDS/2XSSC for 5 min. The effectiveness of the stripping step was determined by exposure of the membrane to X-ray film for 18 hr.

11. DNA EXTRACTION

11.1. Plasmid Extraction

11.1.1. Whole Plasmid

Plasmid DNA was extracted using the method described by Birnboim (1983), Marko et al., (1982) and Birnboim (unpublished), using glass powder.

11.1.2. DNA Fragment Isolation from Agarose gels

DNA fragments were isolated from agarose (0.8%) gel with sodium perchlorate and glass powder (appendix, 1).

11.2. DNA Extraction from Human Granulocytes

About 25x10^{6} human granulocytes were collected by centrifugation and suspended in 2 mL of B.Earles and 4 mL of DNA Extraction Buffer (appendix, 6b). The suspension was transferred to 15 mL glass tube and incubated with 50μg/ mL of proteinase K (Sigma) at 50°C overnight. An equal volume (6 mL) of phenol/chloroform (1g/mL) was added. The tube was shaken on a wrist action shaker at room temperature for 30 min and then centrifuged at 600xg for 5 min at room temperature. The aqueous layer

was removed carefully and the phenol/chloroform extraction was repeated 2-3 times until no interface material could be seen. Finally, one extraction with chloroform alone was performed. The aqueous layer was transferred to a 15 mL glass tube and clarified by centrifugation at 1000xg for 10 min. The supernatant was transferred to 10 mL beaker. An equal volume of ethanol at room temperature was slowly added. The DNA was collected by spooling on a glass rod. The spooled DNA was dissolved in CT buffer and stored over chloroform at room temperature. The concentration of DNA was determined as described in section 5.2. Note that leukocytes have very little RNA relative to fibroblasts so that a ribonuclease step was not necessary.

12. RNA EXTRACTION AND ELECTROPHORESIS

This was performed by the method of Birnboim (1988a). RNA for Northern analysis was denatured by combining 5μL formldehyde/phosphate (100μL HCHO, 6.6μL 1M sodium phosphate pH 6.8, 94μL formamide) and 5μL formamide to 10μL of RNA sample. Incubation was carried out at 55°C for 30 min. After chilling on ice for 1 min, samples were loaded onto a denaturing agarose gel (1.5mm thick vertical gel containing 0.2M formaldehyde, 1.2% agarose, 2mM CDTA and 20mM sodium phosphate (pH 6.8)) and electrophoresed for 90 min at 100 volts.

13. IMMOBILIZATION OF DNA ON MEMBRANES

13.1. Binding Study- Comparison of Binding Capacity and Retention of DNA of Different Membranes

13.1.1. Types of Membranes

Membranes used for this study were Immobilon (Millipore), Hybond-N (Amersham) and Zeta probe (Bio Rad).

13.1.2. Salt Conditions

The membranes were presoaked and applied in different salt concentrations: H_2O, 2xSSC, 6xSSC, 10xSSC and 50mM Na_2HPO_4/1M NaCl/1mM CDTA;pH adjusted to 11.0.

13.1.3. Application of DNA Samples

End-labelled DNA fragments were denatured by 0.1N NaOH at room temperature for 20 min. Denatured DNA fragments (20ng) was mixed with 2 μg of unlabelled, denatured carrier DNA. The carrier DNA (mouse) was denatured by boiling for 3 min and exposing to 0.1N NaOH at 50°C for 30-40 min. Samples were applied to different membranes using a "Slot Blot" (Biodot SF) apparatus (Bio Rad) under 40 cm water vacuum.

Nick translated human genomic DNA and oligoprimer labelled plasmid DNA was also denatured by boiling for 3 min at 100°C before applying to the membranes.

13.2. Standard Conditions for Binding DNA to Membranes for the SLPs Study

Hybond-N membrane was used for the majority of these studies. The membrane was pre-soaked in 10xSSC at room temperature for at least 15 min before DNA samples were applied using the Bio Rad "Slot Blot" (Bio SF) apparatus. The "target" plasmid DNA samples were prepared for application as follows. The plasmid containing the gene of interest was

nicked and depurinated by boiling for 3 min in CT and denatured in 0.1N NaOH at 50°C for 30 min. Serial dilutions of DNA were done in a solution with carrier RNA to avoid loss of DNA on the walls of tubes (10mM sodium phosphate/50 μg per mL yeast RNA in CT pH 6.8). Prior to applications, one volume (100μL) of diluted DNA was mixed with one volume (100μL) of 20xSSC. Samples were applied under 40 cm water vacuum. The membranes were dried at 80°C for 20 min and U.V irradiated for 3-4 min prior to the hybridization.

III. RESULTS

1. BINDING STUDY

Initial experiments were designed to study the ability of various membrane filters to bind DNA and to retain the fixed DNA during the various steps required for hybridization. This initially seemed important for my studies because interpretation of the results depended mainly upon the relative intensity of the signals. If bound DNA was not completely retained, then subtle differences might not readily be quantifiable. The retentiveness of three different membranes were compared under various conditions. The membranes were: (i) Hybond-N :- a hydrophilic nylon membrane with high physical strength (making it ideal for applications involving reprobing the blots); (ii) Zeta probe :- a charge-modified nylon membrane with DNA binding ability even at high pH condition; (iii) Immobilon :- a hydrophobic, polyvinylidene difluoride membrane said to bind DNA very tightly under some conditions.

Other experiments (data not shown) showed that binding capacity of Zeta-probe membrane was in the range of 1.0-1.5μg/5.25 mm^2(slot) and this to some extent limited the usefulness of this membrane in any experiment.

I focussed my attention on Hybond-N and Immobilon. The DNA samples were applied using a slot blot apparatus to have the area of application constant. Preliminary results with Immobilon indicated that DNA binding and retention is very high by using a phosphate buffer (50mM sodium phosphate/1mM CDTA/1M NaCl at pH 11.1) compared to 10xSSC (data not shown).

There were instances when it was necessary to use small size DNA

fragments. Therefore it was of interest to determine whether size influenced the ability of membranes to retain DNA. Although binding

affinity of this membrane for a 300 bp fragment was high initially (cycle I), it was lost in subsequent washes. Fragments from 970 to 4300 bp did not show any significant differences in their binding affinities (Table 1). Immobilon membrane bound even short DNA fragments with very high affinity. Binding of all sizes from 300-4300 bp was equally high (Table 1) with Immobilon membranes.

Although Immobilon membranes would appear to be useful for my experiments, when hybridization experiments with embedded and control DNA were performed, signals were not picked up. It seemed possible that DNA was bound so tightly that it was not accessible to the radioactive probe. Furthermore, it was a new product that was not readily available at the time some of my experiments were carried out. Therefore Hybond-N membrane were used in the experiments that follow for consistency, using the following conditions for binding: 10xSSC for presoaking and applying samples.

2. PRINCIPLE OF THE EMBEDDING METHOD

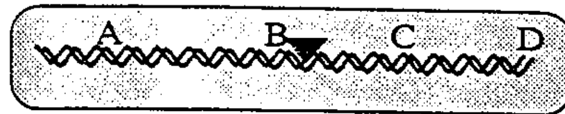
Cells to be analyzed were encapsulated in agarose beads (Cook. 1984). Encapsulated cells (Fig. 1; i) were lysed _in situ_ with deproteinizing solution and subsequently a short treatment with alkali (35mM) was performed; the rationale was to denature only a small region around the breakpoint (Fig. 1; ii). After "neutralization" to pH 11, the unwound single-stranded DNA was selectively labelled using oligo primer labelling method (Fig. 1; iii). The labelled DNA was used to

Table 1: BINDING OF SINGLE-STRANDED DNA TO HYBOND AND IMMOBILON MEMBRANE AS A FUNCTION OF FRAGMENT SIZE. About 20ng of ^{32}P-dCTP-end-labelled DNA fragments (300,750,970 and 1750 bp from human immunoglobulin kappa region gene insert 4300 bp fragment is from pBR322 plasmid) were mixed with 2.0μg of cold mouse DNA and applied using a slot blot apparatus. In cycle I membranes were incubated in prehybridization solution for 30 min and over night in cycle II. In cycle III, counts were taken after washed in solution (0.1%SDS/2xSSC) and high stringency wash solution (0.1%SDS/0.16xSSC/10mM sodium citrate). Drying was done at 80°C for 20 min. The DNA retained on the membrane was measured using liquid scintillation counter.

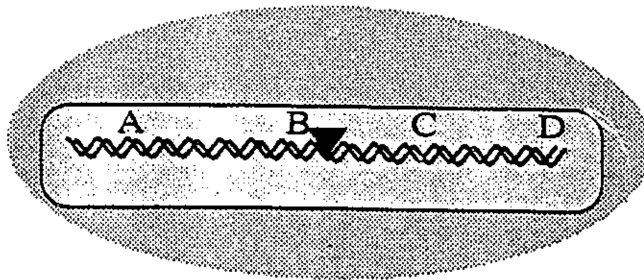
	Fragment size(bp)	CYCLE I	CYCLE II	CYCLE III
		% of loaded DNA retained		
1. Hybond-N 10xSSC	300	60.0	20.0	19.0
	750	72.0	38.0	40.0
	970	70.0	46.0	45.0
	1750	75.0	52.0	51.0
	4300	73.0	53.0	52.0
2. Immobilon phosphate	300	98.0	96.0	96.0
	750	97.0	97.0	97.0
	970	100.0	95.0	95.0
	1750	100.0	95.0	92.0
	4300	100.0	94.0	93.0

Fig. 1: PRINCIPLE OF THE EMBEDDING METHOD.
This is a diagramatic representation of the method developed to identify SLPs. Four genes A, B, C, D are shown to explain the rationale of this method.

LABELLING OF GENES AT DNA BREAKPOINTS
PRINCIPLE OF THE METHOD

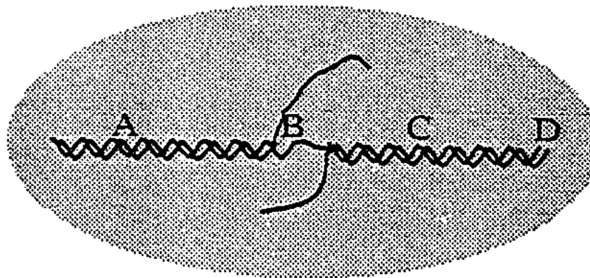


Granulocyte



Embed cell in agarose beads

(i)

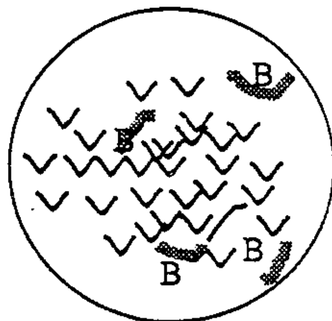


Treat with detergent and alkali

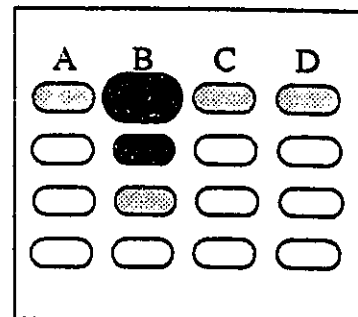
(ii)

Label single-stranded regions with ^{32}P

(iii)



Use labelled DNA to probe battery of genes on Slot Blot



(iv)

probe a battery of target genes immobilized on Hybond-N membrane (Fig. 1; iv). If the labelling points were specific, only some genes might give strong signals. As a control a similar membrane was probed with completely denatured, DNA in which every gene would be equally labelled.

3. INITIAL POSITIVE FINDING OF SLPs

The method developed to examine sequences near DNA breakpoints revealed an exciting new finding. Labelled embedded DNA from human granulocytes (stimulated with TPA) bound to some target genes 100-1000x more intensely than to other genes, compared to the control (Fig. 2). These target genes were FOS (c-fos), ERB-B2 (proto oncogenes), GM-CSF (CSF2) and transferrin receptor (TRF). Figure 3 shows that another gene JUN (c-jun) was also preferentially labelled, compared to the control. The preferential labelling sites were named Selective Labelling Points or "SLPs". Autoradiograms were scanned by laser densitometry. The absorbance are summarized in appendix (7,8).

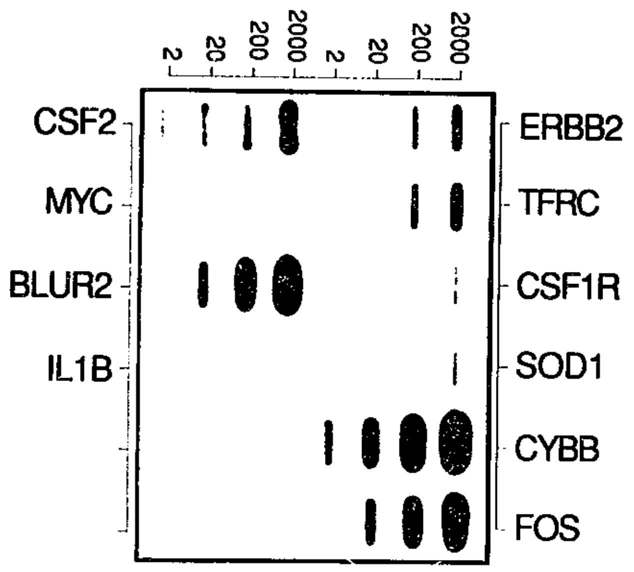
Two other genes, CYBB and Blur2, also showed strong labelling with both embedded and control DNA. This was because these are highly repeated sequences. Blur2 is a 302 bp DNA sequence which belongs to a family of Alu repeats (Deninger et al., 1981), present at about 100,000-300,000copies/genome. CYBB is a 5.4kb fragment of genomic DNA which contains a repeated sequence (ATCC 59404). Genes which were strongly positive in both control and embedded DNA (because of the presence of repeated sequences) could not be assessed for the presence of SLPs.

Fig. 2: SLPs ARE IDENTIFIED BY TARGET GENES IN HUMAN GRANULOCYTES. Human granulocytes were treated with TPA (10^{-8}) and catalase (20μg/mL), the cells were embedded, lysed (0.5% SDS, 20μg/mL proteinase K), alkali denatured in 35mM NaOH (unless otherwise stated), neutralized to (pH11) with glucose and labelled using oligoprimer labelling method. As control for all the experiments completely denatured human granulocyte (PMNs) DNA was labelled with the oligoprimer labelling. Whole plasmid containing genes of interest were immoblized on the membrane (unless otherwise stated) from 2000ng to 2ng. The percentage of incorporation of radio-label and the amount of DNA added to the labelling mix are shown below for each experiment.

Exp I: Control: Denatured human DNA
Incorporation (%) - 70
Amount of DNA (ng) - 50

Exp II: Embedded: Human granulocyte DNA
Incorporation (%) - 8
Amount of DNA (ng) - 200

II



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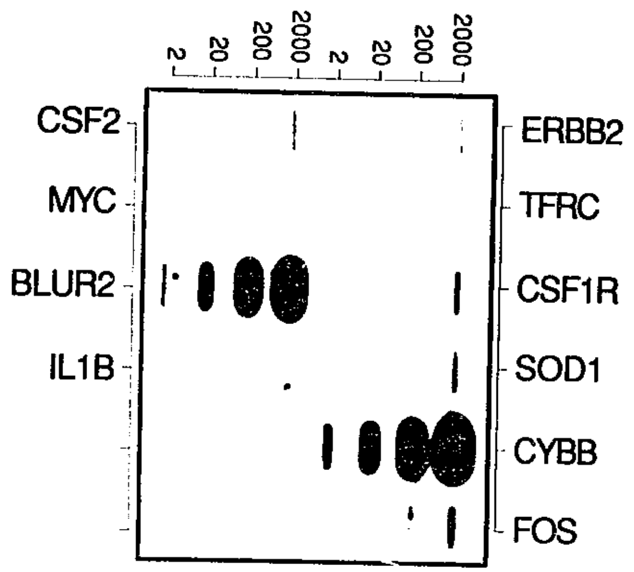
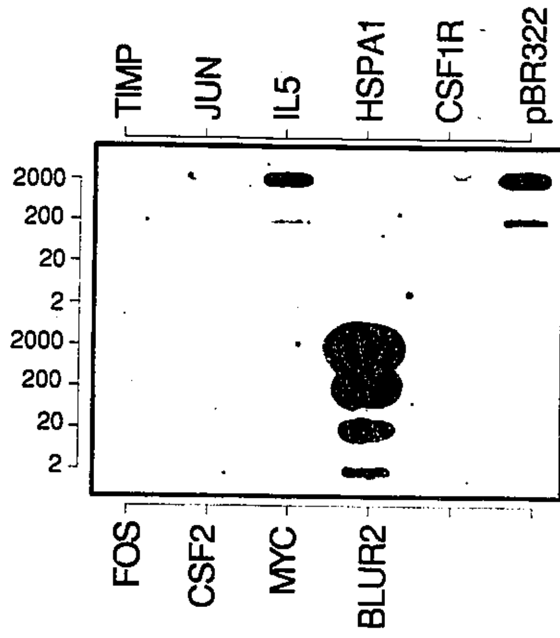


Fig 3: SLPs ARE IDENTIFIED AT ANOTHER GENE SITE (c-JUN).
Human granulocyte cell treatment, embedding, lysing, alkali treatment, neutralization and labelling were done as described in Fig. 2.

Exp I: Control: Denatured human DNA
Incorporation (%) - 69
Amount of DNA (ng) - 50

Exp II: Embedded: Human granulocyte DNA
Incorporation (%) - 8
Amount of DNA (ng) - 150

I



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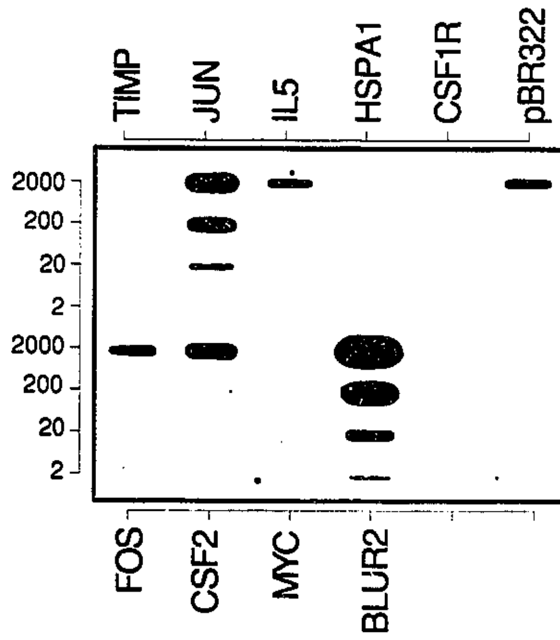

4. SLPs IN OTHER GENES

To investigate whether SLPs are present at or near other genes, a number of other target genes were immobilized and analyzed. The target genes tested are listed in Table 2 and ones positive for SLPs are identified by "***". The remainder are either negative or cannot be classified with certainty because of the presence of repeated sequences. Details about vectors and names of the plasmids of all target genes tested are shown in Table 2.

5. PRESENCE OF SLPs IN INSERTS ISOLATED FROM THEIR PLASMID VECTORS

For most experiments, plasmids containing an inserted gene were immobilized on the membranes. To confirm that SLPs were detecting the inserted gene and not the vector, isolated fragments from human fos genomic DNA were tested (Fig. 4). The absence of repeated sequences in these fragments were confirmed by Southern analysis (data not shown). Both the 4.3kb fragment which contains most of the coding sequences and the 1.0kb fragment from the downstream intron were positive (Fig. 4, I).

6. SLPs AND INTACT BEADS

SLPs are not an artifact related simply to encapsulation of cells in agarose beads. When the agarose beads were handled in a rough manner (pipetting vigorously), SLPs disappeared and an increase in percentage incorporation of radio-label was observed (Fig. 5). SLPs could be detected only when the beads were handled gently and maintained intact. If beads were broken causing some DNA spill out and unwinding in alkali, the SLP pattern disappeared and embedded DNA behaved as control DNA.

Table 2: GENES TESTED FOR SLPs AND THEIR CHROMOSOME LOCATION.
Names and symbols of genes immobilized and tested for presence of SLPs. The positive genes are marked (*).

	CHROM #	LOCUS SYMBOL	LOCUS NAME	SOURCE	PLASMID	VECTOR	cDNA/ GENOMIC DNA
***	1	JUN	v-jun oncogene homolog	Ref. 1	pCD10	pCD	C
	2	IGKC	immunoglobulin kappa constant region	ATCC 59172	HuCK	pBR322	G
		IL1B	interleukin 1, beta	ATCC 67024	YEpsec1-hIL1	YEpsec1	C
***	3	TFRC	transferrin receptor (p90)	ATCC 59324	pCDTR1	pCD	C
	4	KIT	v-kit oncogene homolog	ATCC 59492	phckit-171	pUC119	C
***	5	CSF2	colony stimulating factor 2 (GM-CSF)	ATCC 57594	pCD-hGM-CSF	pCD	C
		EGR	early growth response gene	Ref. 2	EGR #322	pUC13	C
		HSPA1	heat shock 70 kD protein 1	ATCC 57494	pH2.3	pAT153	G
		IL5	interleukin 5	ATCC 59394	phIL5-115.1	pBR322	C
		CSF1R	colony stimulating factor 1 receptor	ATCC 59292	pcfms104	pUC12	C
	6	ROS1	v-ros oncogene homolog	ATCC 59166	phroshi6	pUC19	G
		MYB	v-myb oncogene homolog	ATCC 57392	pHM2.6	pKH47	G
	8	SYR	src/yes related novel gene	ATCC 57588	pFYN	pUC19	C
		MYC	v-myc oncogene homolog	Ref. 3	pRyc7.4	pBR322	C
	9	ABL	v-abl oncogene homolog	ATCC 57102	pablK2	pBR322	G
		IFNB1	interferon, beta 1, fibroblast	ATCC 39517	pSY2501	pTrp3	C
	11	CAT	catalase	ATCC 57354	pCAT10	pSP65	C
		IGF2	insulin-like growth factor 2	ATCC 57482	pIGF2/8-1	pUC12	C
	12	KRAS2	v-Ki-ras2 oncogene homolog	ATCC 41027	pSW11-1	pUC13	C
		INT1	v-int-1 oncogene homolog	ATCC 57198	pAL.1	pBR322	G
***	14	FOS	v-fos oncogene homolog	Ref. 4	c-fos1	pBR322	C
	16	PRKCB	protein kinase C, beta polypeptide	ATCC 59288	phPKC-beta1-1	pUC12	C
		MT1	metallothiorein 1	Ref. 5	pmMT1	pBR322	C
	17	PRKCA	protein kinase C, alpha polypeptide	ATCC 59286	phPKC-alpha7	pUC12	C
***		ERBB2	v-erb-b2 oncogene homolog	ATCC 57584	pcER204	pCD	C
	18	YES1	v-yes1 oncogene homolog	ATCC 57582	pXEYes	pUC19	C
	19	APOC1	apolipoprotein CI	ATCC 57408	pucI	pUC	C
		APOC2	apolipoprotein CII	Ref. 6	apoCII	pSP64	C
		CGB	chorionic gonadotrophin, beta peptide	ATCC 57064	beta-hCG5	pXH3C	G
		CKMM	creatine kinase, muscle	ATCC 57678	pJN2CK-M	pUC12	C

	LDLR	low density lipoprotein receptor	ATCC 57004	pLDLR3	pCDV1	C
	LHB	luteinizing hormone beta polypeptide	ATCC 57016	Beta LH	pBR322	G
	PRKCG	protein kinase C, gamma polypeptide	ATCC 59290	phPKC gamma6	pUC13	C
	P1.4	anonymous probe	Ref. 7	p1.4	-	-
	P5.B	anonymous probe	Ref. 8	p alpha 5B	-	-
21	SOD1	superoxide dismutase 1, soluble	ATCC 39786	pSOD alpha2	pBRM	C
22	IGLC1	immunoglobulin lambda constant region 1	ATCC 57200	p1A5	pBR322	G
	PDGFB	v-sis oncogene homolog	ATCC 57050	pSM-1	pCDV1	C
X	CYBB	cytochrome b-245, beta polypeptide	ATCC 59404	379-10	pBR322	G
	TIMP	tissue inhibitor of metalloproteinase	Ref. 9	pTIMP	pGEM1	C
	pBLUR2	Alu family, highly repeated sequence	Ref. 10	pGEM3-Alu	pGEM3	
	pUC19	plasmid sequence				
	pBR322	plasmid sequence				

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3. Marcu et al. Proc.Natl.Acad.Sci.USA 80:519 (1983). 4. Curran et al. J.Virology 44:674 (1982).
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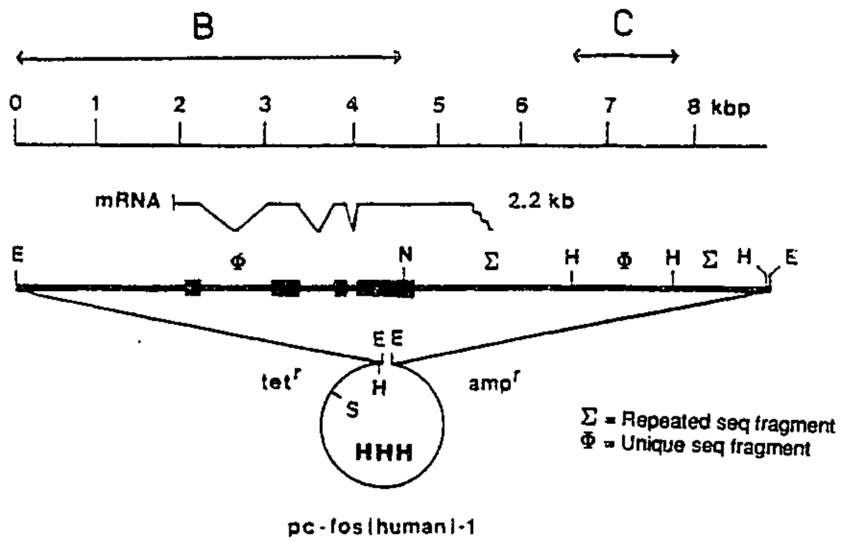
7. From T.Mckeithan, University of Chicago. 8. From T.McKeithan, University of Chicago.
9. Skup et al. J.Biol.Chem. 263:1439 (1988). 10. Deninger et al. J.Mol.Biol. 70:17 (1981).

Fig. 4: DNA FRAGMENT ANALYSIS OF FOS GENE.
Fragments from human fos genomic DNA were immobilized and tested for SLPs. The map of human genomic fos gene from Miller et al.(1984) is shown to indicate fragments free of repeated sequences were immobilized for the experiment. Cell treatment, embedding, lysing, alkali treatment, neutralizing and labelling were done as described in Fig. 2.

Exp I: Embedded : Human granulocyte DNA
Incorporation (%) - 5
Amount of DNA (ng) - 150

A: 13.3kb (whole plasmid)
B: 4.3kb (gene fragment)
C: 1.0kb (gene fragment)



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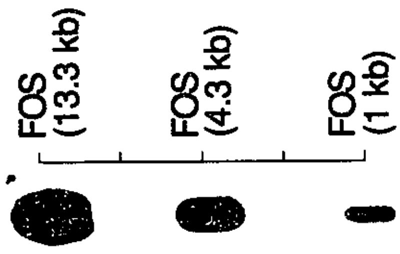
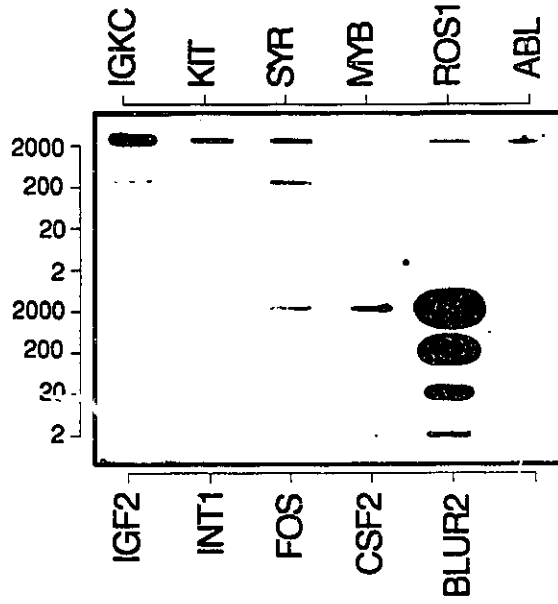


Fig. 5: SLPs DISAPPEAR WHEN THE BEADS ARE BROKEN.
Cells treatment, embedding, lysing, neutralizing and labelling were done as described in Fig. 2. The beads were broken before alkali treatment and then labelled.

Exp I: Control: Denatured human DNA
Incorporation (%) : 73
Amount of DNA (ng): 50

Exp II: Embedded: Human granulocyte DNA
Incorporation (%) : 40
Amount of DNA (ng): 150

I



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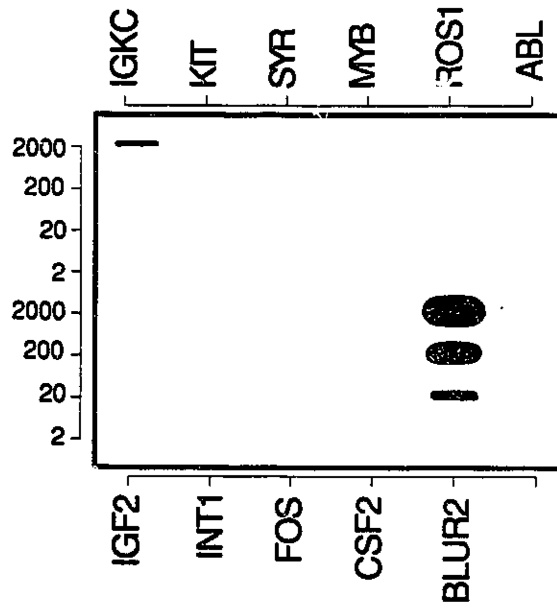


7. RELATIONSHIP BETWEEN SLPs AND TRANSCRIPTIONALLY ACTIVE GENES

Studies of eukaryotic DNA have revealed that the accessibility of chromatin to various nucleases in vivo differs greatly between transcriptionally active and inactive genes (Gross and Gerrard 1988). To determine whether SLPs occur when genes are transcriptionally active Northern analysis was performed with fos and CSF2. I was readily able to detect fos mRNA (Fig. 6, I) (as seen earlier, Sariban etal. 1988) but not CSF2 mRNA (Fig.6, II) in total cellular RNA. Other genes were not tested. If CSF2 mRNA is transcribed in granulocytes (PMNs), more sensitive methods may be needed to demonstrate this.

8. SLPs PRE-EXIST IN HUMAN GRANULOCYTES AND ARE NOT INDUCED IN VITRO

Human granulocytes stimulated with TPA liberate superoxide anion and hydrogen peroxide; these active oxygen species induce strand break in intracellular DNA of human granulocytes (Birnboim. 1982). To determine whether SLPs were due to oxyradical-induced strand breaks, TPA stimulated human granulocytes were compared with unstimulated cells. SLPs were observed equally in freshly isolated human granulocytes and TPA-stimulated granulocytes (Fig. 7, III). This result indicated that SLPs pre-exist in granulocytes and do not develop as a result of TPA treatment.

9. DO SLPs ARISE FROM NATURALLY OCCURRING STRANDED REGIONS IN DNA?

It has been known for several years that mammalian cells contain single stranded regions in the genome (Henson, 1978). Even though this has been speculated to be associated with DNA replication forks, its

Fig. 6: NORTHERN ANALYSIS OF SLP-POSITIVE GENES.
About 10ug of total RNA was extracted from human granulocytes and run on a formaldehyde denaturing agarose gel, blotted and probed with specified ^{32}P-labelled plasmid DNA.

Exp I: Control: Probing with Fos.
Incorporation (%) : 70
Amount of DNA (ng) : 50

Exp II: Test : Probing with CSF2
Incorporation (%) : 69
Amount of DNA (ng) : 50

The blot from the control was stripped and reprobed with CSF2 in Exp II. Position of 18s and 28s rRNA are shown by upper and lower dots respectively. These were detected by methylene blue staining of filters following exposure to X-ray film.

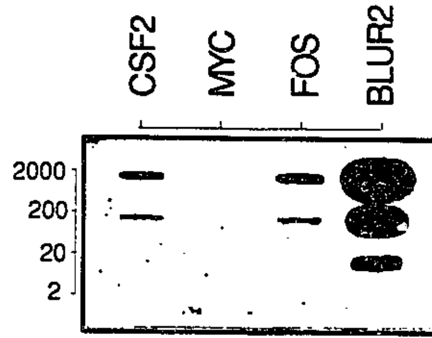
I

II

Fig. 7: OXYRADICAL-INDUCED BREAKS ARE NOT RESPONSIBLE FOR THE APPEARENCE OF SLPs IN HUMAN GRANULOCYTES.
The cell treatment, embedding, lysing,denaturing, neutralizing and labelling were done as described in Fig. 2 for Exp II. For Exp III, the cells were not treated (TPA) but followed the procedure as described in Fig. 2.

Exp I:	Control:	Denatured human DNA Incorporation (%) - 74 Amount of DNA (ng) - 50
Exp II:	Embedded:	Human granulocyte DNA (treated) Incorporation (%) - 10 Amount of DNA (ng) - 192
Exp III:	Embedded:	Human granulocyte DNA (untreated) Incorporation (%) - 11 Amount of DNA (ng) - 136

I



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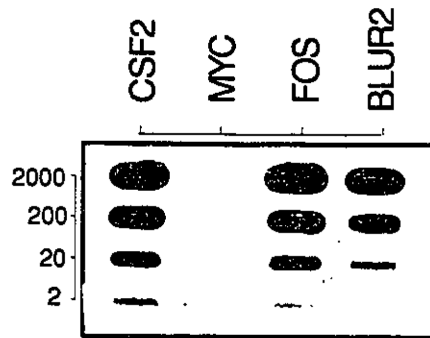

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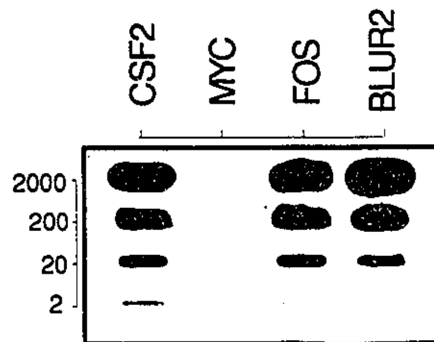


significance is not yet known. If SLPs are single stranded DNA, they could theoretically be made to disappear by digestion with a single-strand DNA specific enzyme such as S1 endonuclease from Aspergillus oryzae. However, when S1 nuclease digestion was performed, SLPs failed to appear even without S1 digestion (Fig. 8). That is, SLPs appear to be unstable at pH 4.5 in S1 nuclease buffer.

10. pH DEPENDENT APPEARANCE OF SLPs

The S1 nuclease results indicated that the appearance of SLPs is very pH dependent. Therefore, embedded DNA was exposed to three different pHs after deproteinizing and before labelling. SLPs were not seen at pH 4.5 (Fig. 9, I), were weakly positive at pH7.5 (Fig. 9, II) and strongly positive at pH11 (Fig. 9, III). This suggests that either they are unstable at low pH or are caused to appear at high pH.

11. SLPs ARE PRE-EXISTING SINGLE-STRANDED REGIONS IN THE DNA

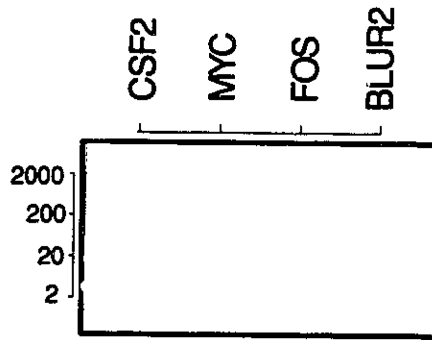
The requirement of oligo-nucleotide primers for labelling makes it likely that SLPs are regions of single stranded DNA, this is shown in Fig. 10. SLPs did not appear if the primers were omitted from the labelling mix. Further evidence supporting the pre-existence of single stranded regions in DNA came from experiments comparing the presence or absence of the alkali denaturation (Fig. 11). The presence or absence of alkali treatment (35mM NaOH) step did not affect specific labelling of Fos and CSF2 compared to myc. However, repeated Alu I sequences (Blur 2) gave a much stronger signal if alkali was used, indicating that denaturation occurs at random sites under these conditions.

Fig. 8: S1 ENDONUCLEASE DIGESTION OF EMBEDDED DNA.
Freshly isolated human granulocytes were embedded, lysed, alkali treated. In Exp I, beads were incubated in 1x S1 nuclease buffer (0.2M NaCl/0.05M sodium acetate/1mM $ZnCl_2$/0.5% glycerol pH 4.5) without S1. In Exp II, S1 nuclease was added and incubated in 1x buffer at 37°C and labelled in S1 nuclease buffer.

Exp I: Embedded: Human granulocyte DNA (no S1)
Incorporation (%) : 2
Amount of DNA (ng) : 150

Exp II: Embedded: Human granulocyte DNA (with S1)
Incorporation (%) : 2
Amount of DNA (ng) : 160

I



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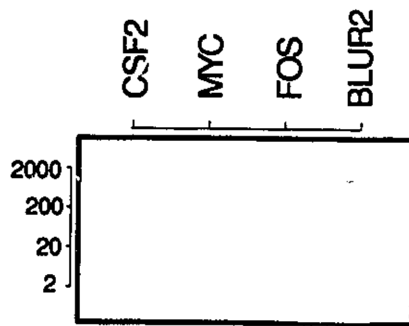


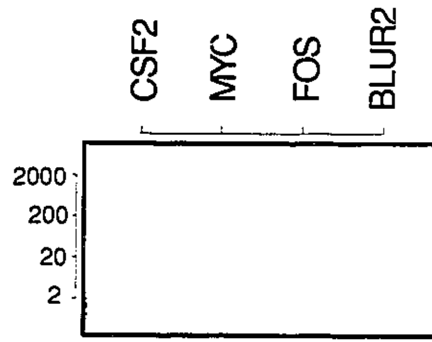
Fig. 9: THE PRESENCE OF SLPs IS pH DEPENDENT.
Freshly isolated human granulocytes were embedded, lysed, as described in Fig. 2. Then the embedded DNA was treated with pH 4.5, 7.5 and 11 buffers and labelled at these pH.

Exp I: Embedded: Human granulocyte DNA (pH 4.5)
Incorporation (%) : 1%
Amount of DNA (ng) : 210

Exp II: Embedded: Human granulocyte DNA (pH 7.5)
Incorporation (%) : 2%
Amount of DNA (ng) : 205

Exp III: Embedded: Human granulocyte DNA (pH 11)
Incorporation (%) : 2%
Amount of DNA (ng) : 198

I



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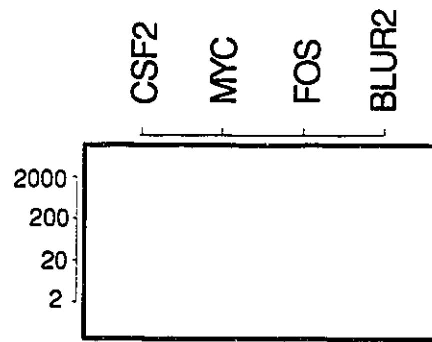

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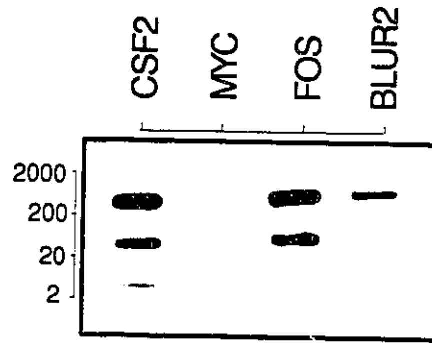


Fig. 10: SLPs DISAPPEAR IN THE ABSENCE OF OLIGO PRIMERS.
Freshly isolated human granulocytes were embedded, lysed, and labelled at pH 11. The oligo primers were removed from the reaction mix before labelling.

Exp I: Embedded: Human granulocytes DNA (no primers)
Incorporation (%) : 3
Amount of DNA (ng) : 200

I

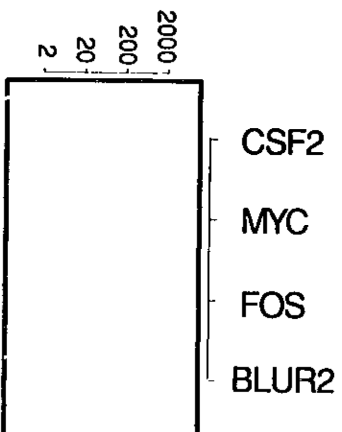


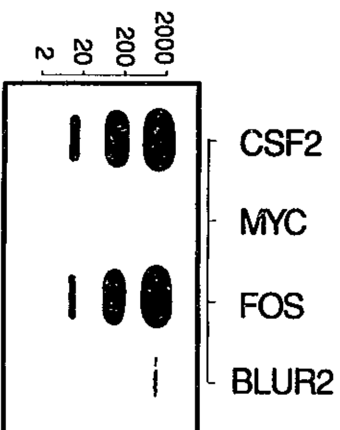
Fig. 11: SLPs ARE SEEN EVEN UNDER NON-DENATURING CONDITIONS.
Freshly isolated human granulocytes were embedded,lysed, alkali treated and labelled in pH 11 buffer as described in Fig. 2. In Exp II alkali treatment was not performed but pH 11 buffer (pre mixed, glucose/35mM NaOH) added before labelling.

Exp I: Embedded: Human granulocyte DNA
Incorporation (%) : 11
Amount of DNA (ng) : 136

Exp II: Embedded: Human granulocyte DNA (no alkali)
Incorporation (%) : 3
Amount of DNA (ng) : 160

(Exp I & II are from two separate experiments)

II



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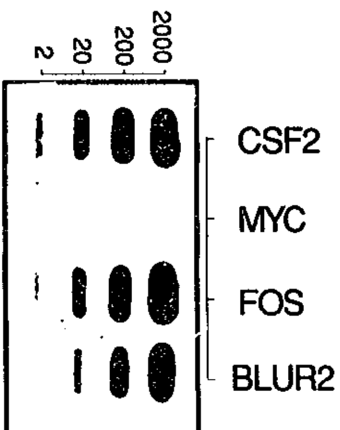


12. SLPs AND TOPOISOMERASE I ACTIVITY

The localization of topoisomerase I to actively transcribed chromatin by several studies (Gilmour and Elgins 1987; Stewart and Schutz 1987; Zhang et al., 1988) suggests that the torsional consequences of transcriptional elongation are relieved by this enzyme. Camptothecin, a cytotoxic alkaloid with anti- neoplastic properties (Horwitz, 1975) targets topoisomerase I with high specificity in vivo. Recently studies have shown that topoisomerase I activity in intact cells could be assessed by mapping camptothecin-trapped single stranded DNA cleavages of genomic DNA (Stewart et al., 1990). When camptothecin was used in the experiment with A431 cells, no significant differences were evident. SLPs therefore donot appear to be due to topisomease I (Fig. 12).

13. SLPs IN OTHER HUMAN CELLS

I have searched for the presence of SLPs in other human cell types.

13.1. Human Blood Cells

To see whether SLPs occur in other blood cells, human T+B lymphocytes were tested and shown to be negative (Fig. 13, II). However, SLPs were seen in a human total mononuclear cell fraction which contained approximately 20% monocytes (Fig. 13, I).

13.2. Other Human Cell Types

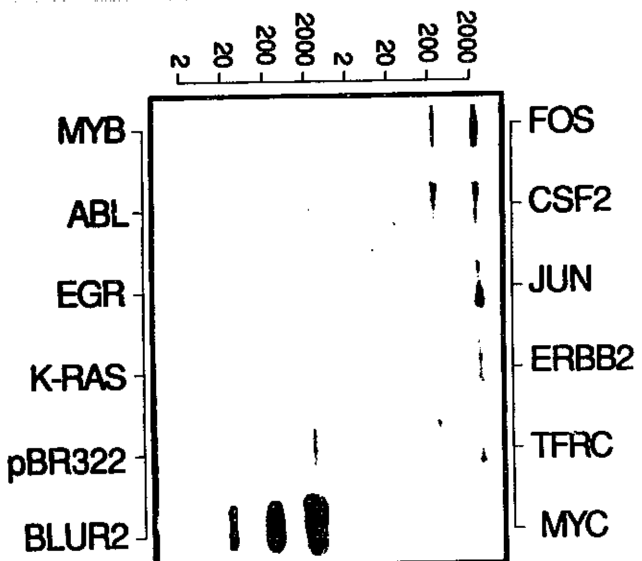
Other cell types tested were human primary fibroblasts, growing U937 cells (Sunstrom and Nilsson 1976), HL60 cells (Gallagher et al., 1979), MeWo cells (Kodera and Bean 1975), K562 cells (Lozzio et al.,

Fig. 12: SLPs ARE ABSENT IN THE PRESENCE OF CAMPTOTHECIN
A431 cells were treated with 5μM A23187 and 10μM camptothecin. The treated cells were embedded, lysed, alkali treated and neutralized and labelled as described in Fig. 2..

Exp I : Embedded: A431 cell DNA (camptothecin)
Incorporation (%) : 10
Amount of DNA (ng) : 170

Exp II: Embedded: A431 cell DNA (no camptothecin)
Incorporation (%) : 5
Amount of DNA (ng) : 150

II



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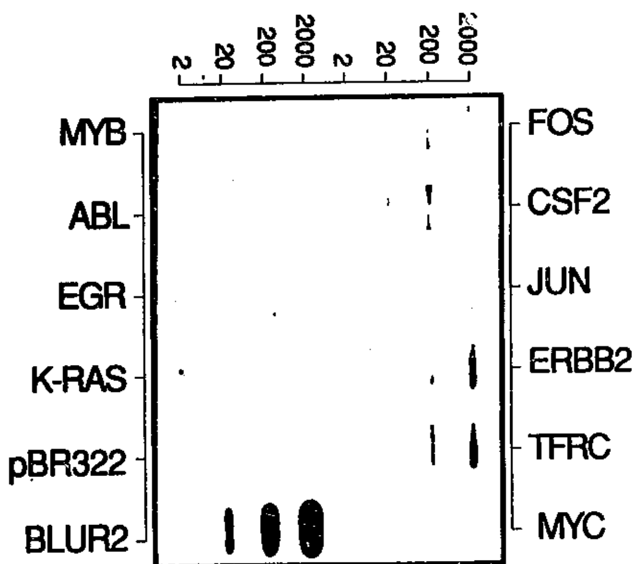
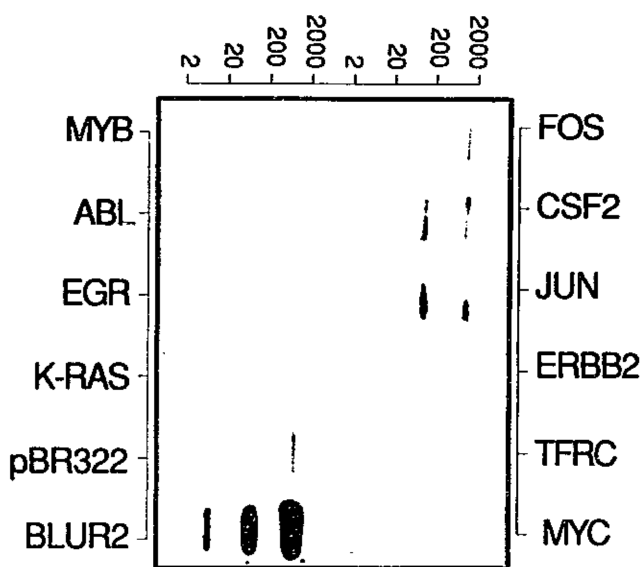


Fig. 13: SLPs IN OTHER BLOOD CELLS.
Human mononuclear cells (80% lymphocytes+ 20% Monocytes) were embedded, lysed and labelled in pH 11 buffer(Exp. I). Human lymphocytes were embedded, lysed, alkali treated, neutralized and labelled as described in Fig. 2 (Exp II). As a control a similar memebrane was labelled with completely denatured labelled DNA (data not shown).

Exp I: Embedded: Human mononuclear cell DNA
Incorporation (%) : 4
Amount of DNA (ng) : 200

Exp II: Embedded: Human lymphocyte DNA
Incorporation (%) : 7
Amount of DNA (ng) : 220

II



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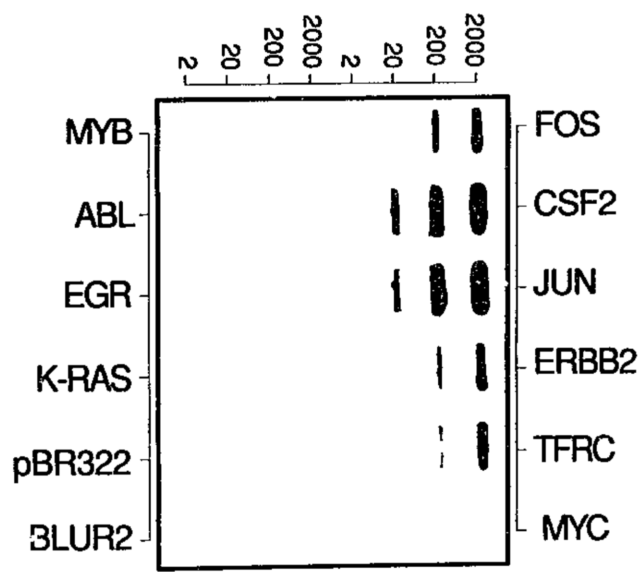
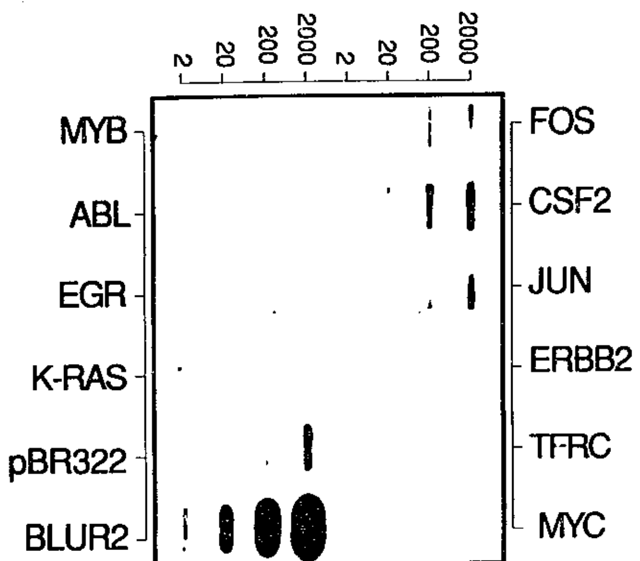


Fig. 14: SLPs ARE ABSENT IN MeWo CELLS.
Growing MeWo cells were embedded and lysed as described in Fig. 2. pH 11 buffer was added before labelling. Alkali treatment was not performed. As a positive control human granulocytes were embedded and labelled (data not shown).

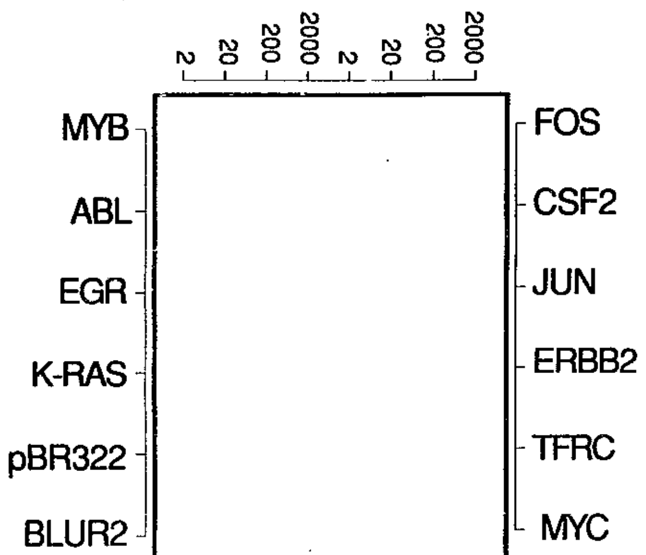
Exp I: Control : Denatured human DNA
Incorporation (%) : 60
Amount of DNA (ng) : 250

Exp II: Embedded: Growing MeWo DNA
Incorporation (%) : 2
Amount of DNA (ng) : 200

I



II



1975), A431 cells (Haigler et al., 1978). Growing MeWo cells (Fig. 14, II), A431 cells (Fig. 15), unstimulated K562 cells (Fig. 16) and unstimulated U937 cells (Fig. 17) were negative for SLPs. Growing HL60 cells (Fig. 18) looked weakly positive.

14. SLPs IN MOUSE BLOOD CELLS

Experiments were done with mouse granulocytes and mononuclear cells to compare SLPs in other species. In both mouse granulocytes (Fig. 19, II) and mononuclear cells (Fig. 19, I) some SLPs were strongly positive (fos, jun and CSF2).

15. SLPs IN DIFFERENTIATED CELLS

15.1. In U937 Cells

AS SLPs were found in granulocyte and monocytes, it was speculated that SLPs are a property of terminally differentiated myeloid cells. A time course experiment on differentiation of U937 cells to granulocyte-like cells was done. Growing U937 cells were induced to differentiate by TPA (Balsinde and Mollinedo 1988).

Unstimulated U937 cells were negative (Fig. 20, I). In TPA treated cells, SLPs started to appear at 10 hrs (Fig. 20, II) and became strongly positive by 20 hrs (Fig. 20, III). In a separate experiment 24, 48 and 72 hr time course was done. SLPs reached maximum intensity by 24 hr (Fig. 21, I) and remained the same for 48 hr (Fig. 21, II). By 72 hrs SLPs partially disappeared (Fig. 21, III).

15.2. In K562 Cells

In an attempt to find out whether SLPs occur in other differentiated cells, K562 cells were differentiated by hemin, sodium

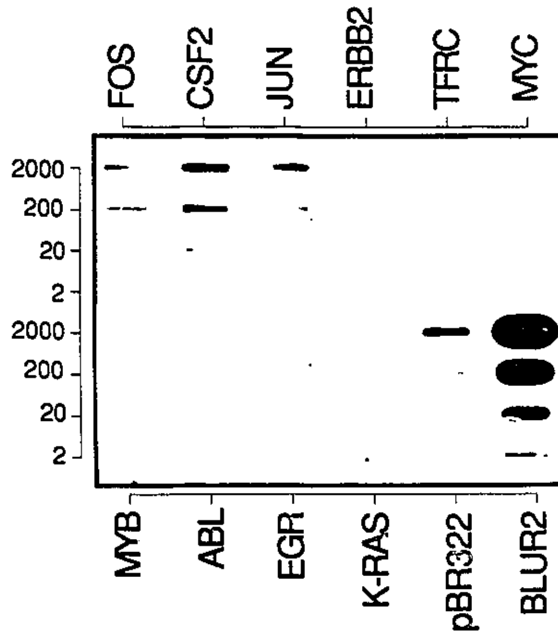
Fig. 15: SLPs ARE ABSENT IN A431 CELLS.
Growing A431 cells were embedded, lysed, alkali treated, neutralized and labelled as described in Fig. 2.

Exp I: Control : Denatured human DNA
Incorporation (%) : 60
Amount of DNA (ng) : 250

Exp II: Embedded : Growing A431 DNA
Incorporation (%) : 5
Amount of DNA (ng) : 150

(Exp I & II are from two separate experiments)

I



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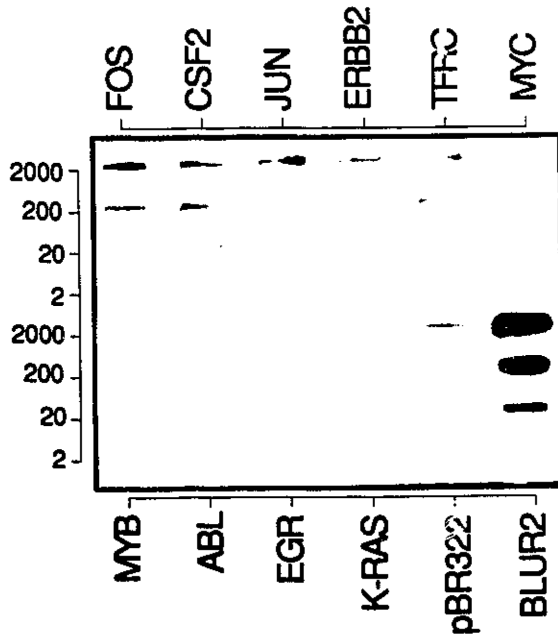


Fig. 16: SLPs ARE ABSENT IN K562 CELLS.
Growing K562 cells were embedded and lysed as described in Fig. 2. pH 11 buffer was added before labelling without alkali treatment. A similar membrane was probed with completely denatured DNA as a control (Fig. 14, Exp I).

Exp I: Embedded: Growing K562 DNA
Incorporation (%) : 3.4
Amount of DNA (ng) : 130

I

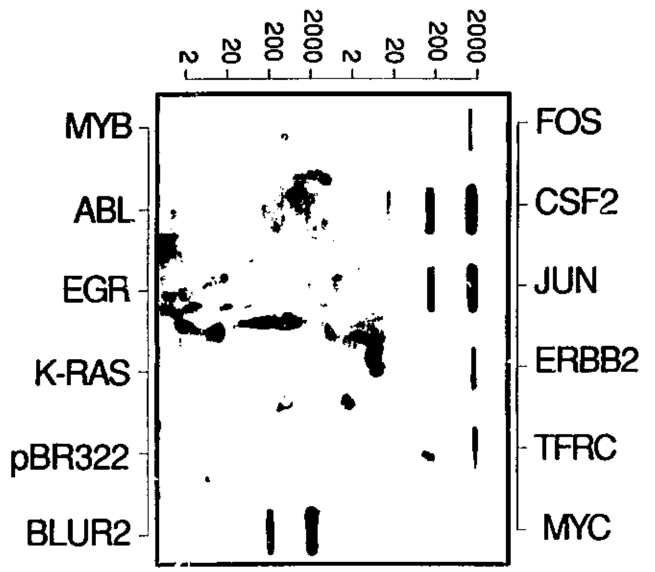


Fig. 17: SLPs ARE ABSENT IN U937 CELLS.
Growing U937 cells were embedded, lysed, alkali treated, neutralized and labelled as described in Fig. 2.

Exp I: Embedded: Growing U937 DNA
Incorporation (%) : 4
Amount of DNA (ng) : 170

I

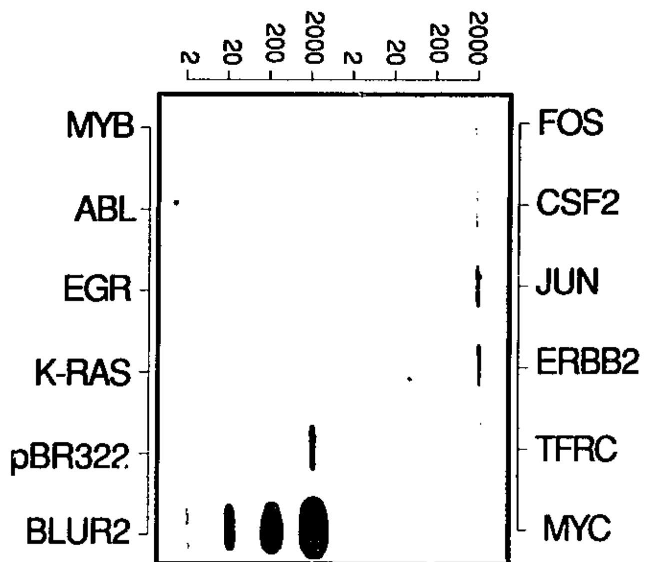


Fig. 18: SLPs ARE SLIGHTLY POSITIVE IN HL60 CELLS.
Growing HL60 cells were embedded, lysed, alkali treated, neutralized and labelled as described in Fig. 2. Completely denatured and labelled DNA was used as a control (Fig. 15, Exp I).

Exp I: Embedded: Growing HL60 DNA
Incorporation (%) : 10
Amount of DNA (ng) : 190

I

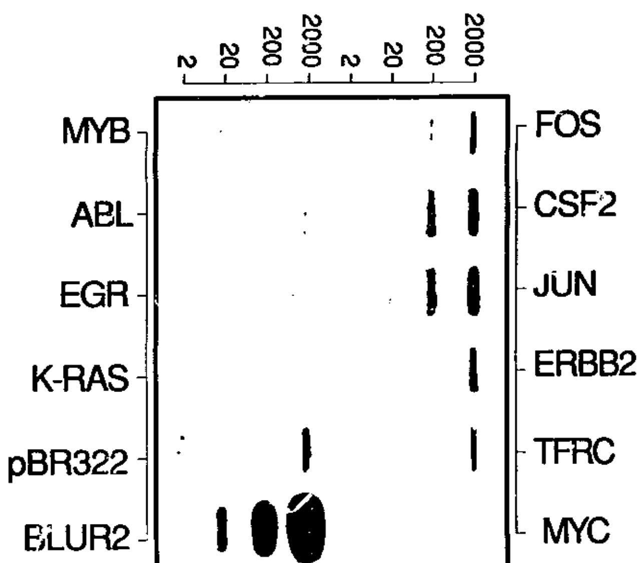
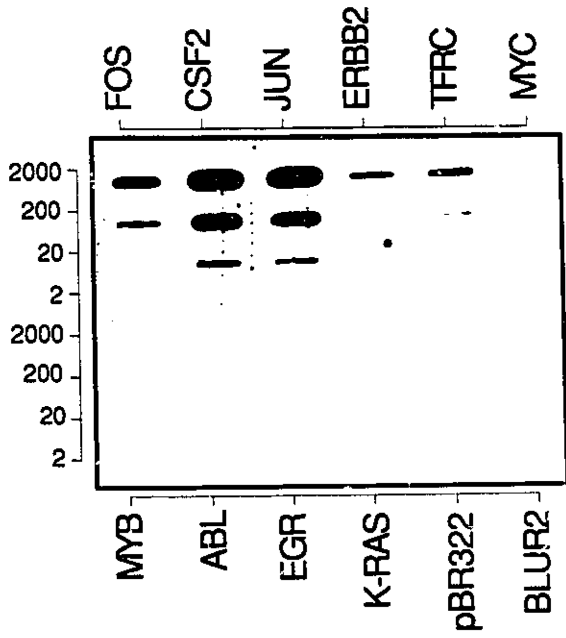


Fig. 19: SLPs ARE FOUND IN MOUSE MONONUCLEAR CELLS AND GRANULOCYTES. Freshly isolated mouse mononuclear cells and granulocytes were embedded and lysed as described on Fig. 2. pH 11 buffer was added before labelling , without alkali treatment.

Exp I: Embedded : Mouse mononuclear DNA
Incorporation (%) : 3.3
Amount of DNA (ng) : 170

Exp II: Embedded: Mouse granulocyte DNA
Incorporation (%) : 3.7
Amount of DNA (ng) : 150

I



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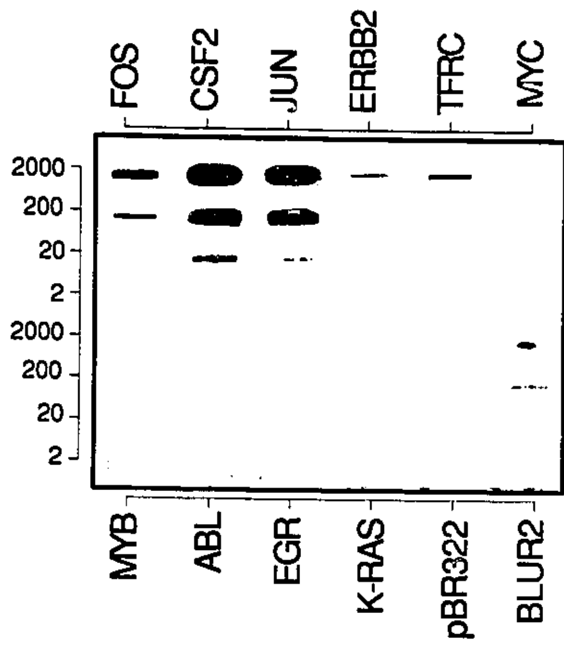


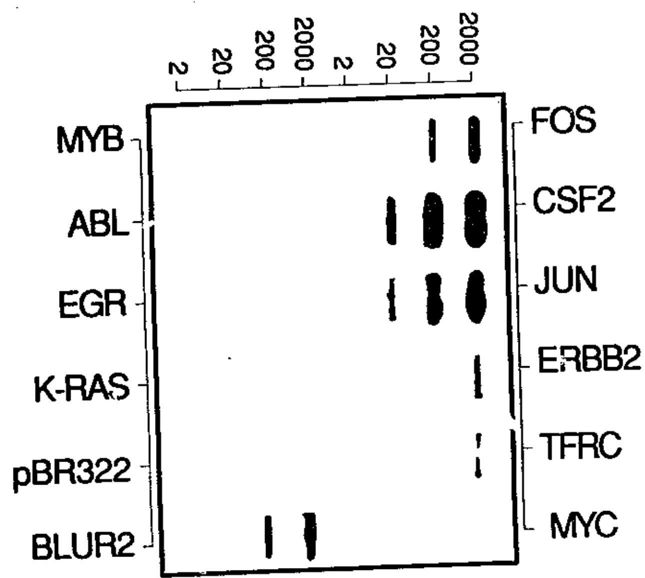
Fig. 20: SLPs ARE PRESENT IN DIFFERENTIATED U937 CELLS.
U937 cells were induced to differentiate by TPA (0.2μM) and embedded and lysed as described in Fig. 2. Labelled at 10 hr and 20 hr at pH 11 buffer without alkali treatment. As a control untreated cells were embedded and tested at 20 hr (Exp I).

Exp I: Embedded : U937 cells DNA at 20hrs (no TPA)
Incorporation (%) : 3.5
Amount of DNA (ng): 220

Exp II: Embedded : U937 cells DNA at 10hrs (+TPA)
Incorporation (%) : 4
Amount of DNA (ng) : 200

Exp III: Embedded : U937 cells DNA at 20hrs (+TPA)
Incorporation (%) : 5
Amount of DNA (ng) : 225

III



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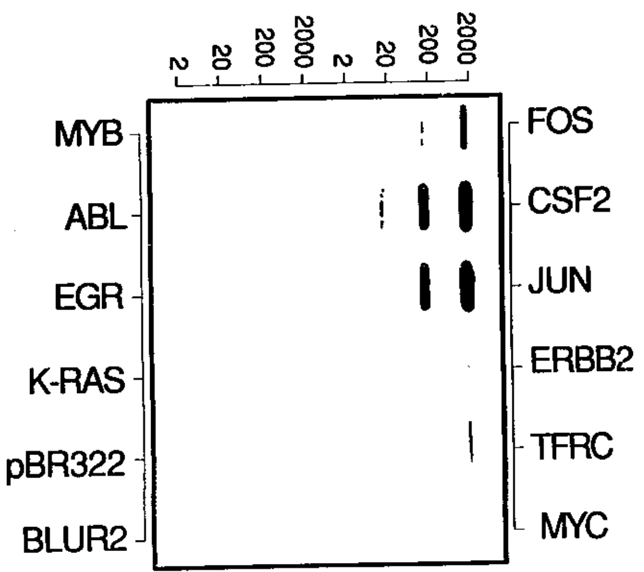

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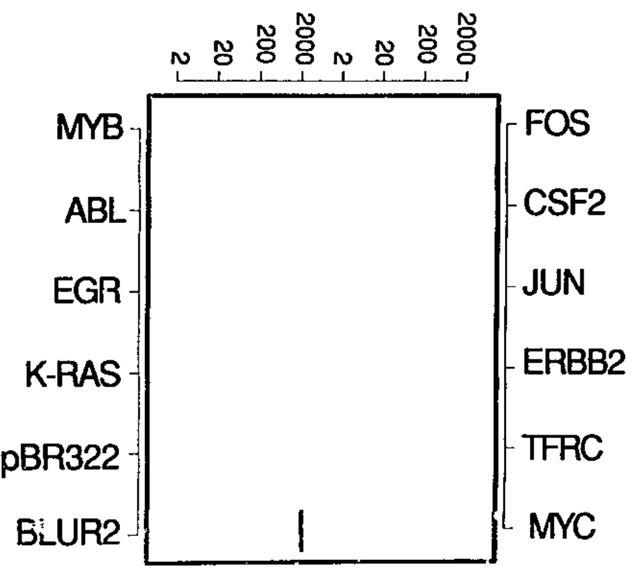


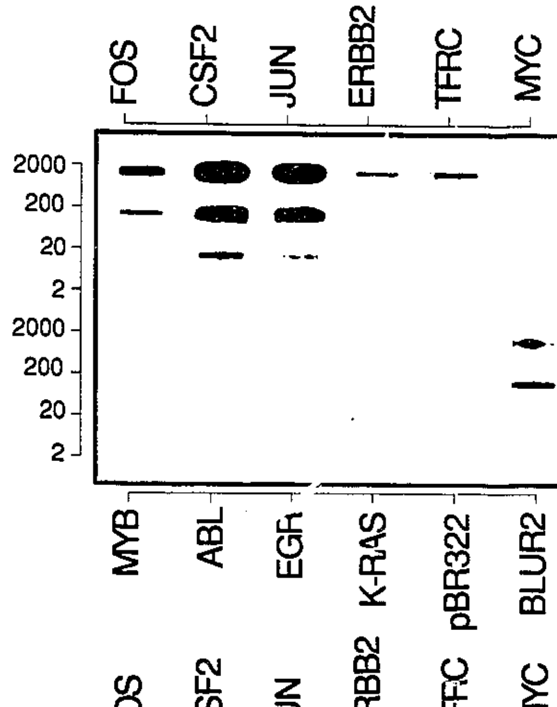
Fig. 21: SLPs DISAPPEAR AFTER 48 hr OF U937 CELL DIFFERENTIATION. In another experiment time course of U937 differentiation was followed at 24, 48 and 72 hr. The cells were embedded, lysed and labelled as described in Fig. 20.

Exp I: Embedded : U937 cell DNA at 24 hr (+TPA)
Incorporation (%) : 3.7
Amount of DNA (ng) : 170

Exp II: Embedded : U937 cell DNA at 48 hr (+TPA)
Incorporation (%) : 3
Amount of DNA (ng) : 250

Exp III: Embedded : U937 cell DNA at 72 hr (+TPA)
Incorporation (%) : 6
Amount of DNA (ng) : 150

I



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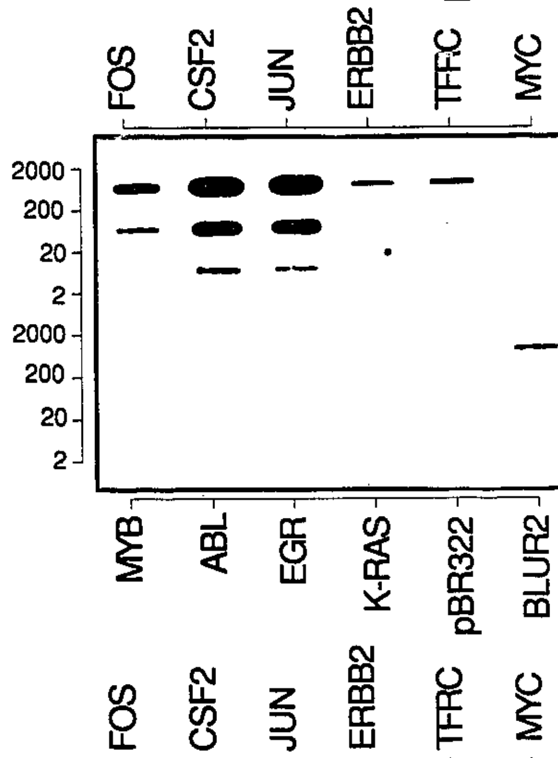

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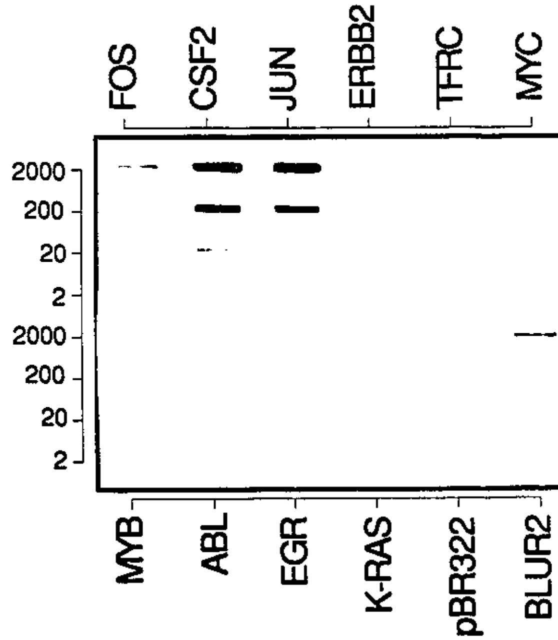
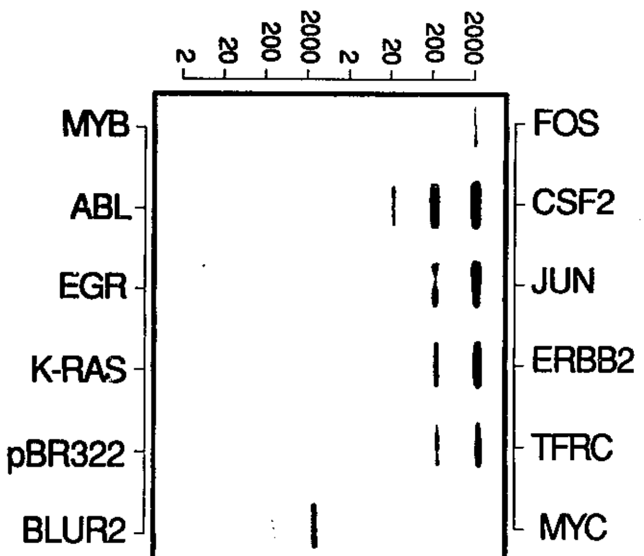


salt of n-butyric acid and cytosine β-D-arabinofuranoside. Induction of differentiation was allowed to proceed for 96 hr. Differentiated K562 cells did not give strong signals as U937 cells at positive SLPs. Fig. 22 shows the results of sodium salt of n-butyric acid-differentiated K562 cells. This experiment was done only once and has to be repeated.

Fig. 22: SLPs ARE ABSENT IN DIFFERENTIATED K562 CELLS.
K562 cells were induced to differentiate by sodium dibutyrate. Cells were embedded ,lysed and labelled as described in Fig. 20.
Exp I: Embedded : K562 cell DNA at 96 hr (sodium dibutyrate)
Incorporation (%) : 2.5
Amount of DNA (ng) : 210

I



IV. DISCUSSION

1. INITIAL INTEREST IN STUDYING OXYRADICAL-INDUCED DNA BREAK SITES

Active oxygen species such as superoxide anion and hydrogen peroxide occur widely in humans and animals during the course of normal cellular functions as well as after treatment with xenobiotics and ionizing radiation (Birnboim. 1988). Stimulation of polymorphonuclear leukocytes (PMNs) and activated macrophages by inflammatory compounds and tumor promotors results in activation of a "respiratory burst" in which a variety of reactive oxygen species (ROS) are released extracellularly (Badwey and Karnovsky 1980). These reactive oxygen species secreted by activated phagocytes have been shown to promote neoplastic transformation in C3H/10T1/2/ C18 fibroblast in vitro (Zimmerman and Cerutti 1984, Weitzman et al., 1985). Also, they were found to induce DNA base changes and sister chromatid exchanges (Weitberg et al., 1983) in neighboring cells. Furthermore Birnboim (1982) showed that oxyradicals liberated by stimulated human granulocytes introduce breaks in intracellular DNA of human granulocytes. It was also shown that the number of breaks did not exceed a maximum of 6000/genome (Birnboim 1988). This finding and others created an interest in analyzing sequences at the break sites in human granulocytes. My study was initially expected to give some insight into the mechanism of tumorigenesis, by free radicals, at the DNA level. As a consequence of my development of a technique to permit analysis of sequences at DNA break points, I discovered the presence of "Selective Labelling Points" in the DNA of myeloid cells (Fig. 3, 4, 13 I). Their presence in freshly isolated human granulocytes (i.e, cells

not treated with TPA)(Fig. 7) indicates that they pre-exist in these cells. Therefore, I continued my project concentrating on other blood cells and cultured cells in an attempt to characterize the nature and occurrence of these sites.

2. APPROACH USED TO INVESTIGATE SEQUENCES NEAR DNA BREAKPOINTS WHICH LED TO THE DISCOVERY OF SLPs

In order to achieve my goal of distinguishing specific from random break sites, it was necessary to analyze DNA sequences at or near DNA break points. If breaks occur randomly, each gene would have an equal probability of being found at or near a break point. On the other hand, if breaks occur at specific points, only some genes would be found at or near break points. To identify DNA sequences near break points, first DNA had to be isolated in a very high molecular weight form. Conventional methods of DNA purification introduce breaks randomly which could mask the presence of specific breaks. Even if isolated in a high molecular weight form, such naked DNA molecules are so long and fragile that they cannot be pipetted without breaking them. It was necessary for me to use a method that could isolate DNA in a high molecular weight form and at the same time allow analysis of DNA break sites in situ.

Pulse-field gel electrophoresis which was first described by Schwartz et al. (1984) is used extensively by many investigators to separate fragments of DNA in the size range of 50-300kb. Because this technique requires the manipulation of very high molecular weight DNA, embedding cells in agarose plugs has been used to prevent shearing of the DNA which would occur in solution during handling. I also used the

technique of embedding cells in agarose. Due to the large size and hence limited surface area of agarose plugs, diffusion time into the agarose would be expected to be very long for large molecules. Therefore, I adopted a method for embedding cells in agarose beads using modifications of the procedure described by Cook (1984). Initial experiments were done to establish conditions for embedding and labelling of single stranded regions of human granulocyte DNA. The labelled DNA was used to probe a battery of target genes immobilized on a membrane. Thereby I developed a new technique to analyze DNA sequences at or near break site in vivo or in vitro.

3. SELECTION OF TARGET GENES

It is now well established that human tumors, particularly leukemias and lymphomas, are characterized by non-random chromosomal abnormalities (Le Beau 1984). Moreover, the number of fragile sites that coincide with chromosomal breakpoints in cancer cells were found to be greater than expected by chance (Glover et al., 1984, Yunis and Soreng 1984). In many instances, genes located near chromosomal breakpoints were previously recognized as proto-oncogenes. In other cases, however previously unknown transforming sequences have been identified near fragile sites (Le Beau 1986). Many genes have been mapped in the region of fragile sites by cytogenetic studies, but no single gene had been identified at a precise location. Even though an association of fragile sites and cancer break points has been recognized, the exact nature of relationship at the DNA or gene level remains obscure.

At the time of selecting genes for immobilization, we hypothesized that the oxyradical-induced break sites in human granulocytes occur at

or near cytogenetically observed chromosomal fragile sites. Therefore, about 50 target genes were selected on the basis of their being near known chromosomal fragile sites .

4. HANDLING OF EMBEDDED CELLS

SLPs were detected by five target genes of fifty tested in human granulocytes (Fig. 2,3). SLPs appear not to be an artifact of the embedding procedure in agarose per se; rough handling of beads by vigorous pipetting caused the disappearance of SLPs (Fig. 5 II) along with an increase in percentage incorporation of radioactive label from 2-3% to 40%. A plausible reason for this increase in incorporation of label is that DNA spilled out from the broken beads and was denatured completely, during the 35 mM step; thereby increasing the a large amount of single-stranded DNA template for the labelling reaction. It is possible that SLPs require certain primers in the reaction mix. There are 4096 (4^6) different combinations of hexanucleotides in the mixture. Utilization of these certain specific primers by other non-"SLPs" sites on the single-stranded DNA might deplete the specific primers and may be responsible for the disappearance of the SLPs in roughly handled beads.

5. TRANSCRIPTIONALLY ACTIVE GENES

5.1. Hypersensitivity to nuclease

Transcription is associated with perturbation of higher order structure of chromatin, both within the transcribed unit and throughout

the chromosomal domain (Patient and Allan 1989). Some of these alterations are a consequences of transcription, which creates a nuclease hypersensitive sites (HS)(Gross and Gerrard 1988). Some HS have been shown to be nucleosome free. They also disrupt the continuity of the chromatin higher order fibre in which they are embedded (Ptashne et al., 1988) and tend to flank coding sequences of genes that are actively transcribing or poised for transcription. Transcriptional promotors and enhancers are found within HS. The most significant set of HS to be identified and characterized are those which delineate the dominant control region (DCR) of the human β-globin locus (Grosveld et al., 1987, Forrester et al., 1987). This group of five "super" HS are spread over 12kb of DNA and contain sequence elements capable of specifying both the tissue specificity and affecting the level of transcription of the locus. Jarman and Higgs (1988) have hypothesized that these sequences function as nuclear matrix attachment sites.

Furthermore, studies of eukaryotic DNA have revealed that the accessibility of chromatin to various nucleases in vivo differs greatly between transcriptionally active and inactive genes (Gross and Gerrard 1988). Some models suggests that single stranded nick (Meselson and Radding 1975) or a double strand break (Szostak et al., 1983) is the initiating lesion for genetic recombination.

In order to investigate whether the SLPs occur at or near transcriptionally active genes, Northern analysis was performed on two SLP- positive genes. Fos was confirmed to be transcriptionally active (Fig. 6 I) but no mRNA was seen for CSF2 (Fig. 6,II). This indicates that SLPs are likely not involved in transcription, although this cannot yet be firmly ruled out. Jun has recently been shown to be

transcriptionally active in granulocytes. Fos and Jun are known to be part of AP1-a transacting complex.

5.2. Topoisomerase Activity

Transcriptional elongation necessitates the movement of RNA polymerase along or through a double helical DNA template. If the polymerase rotates to follow the helical path, then the growing nascent RNA and accumulating protein complexes must also rotate (Stewart et al., 1990). If the polymerase does not rotate, it will generate and partition positive supercoils ahead of its movement and negative ones behind (Liu and Wang 1987). Evidence in favor of the latter case has been acquired from in vivo studies using yeast mutants (Brill and Sternglaz 1988). Supercoiling has been shown by Tsoa et al., (1989) to be dependent upon the presence of growing RNA, suggesting that the RNA tail prevents the polymerase from rotating and that each transcribing polymerase will therefore, serve to partition supercoils. Many studies have localized another enzyme called topoisomerase I to actively transcribed chromatin. Their studies also suggest that the torsional consequences of transcriptional elongation are relieved by this enzyme (Tsoa et al., 1989).

Topoisomerase I forms a transient covalent intermediate with double stranded DNA by transferring a phosphodiester bond from the phosphate backbone to a tyrosine hydroxyl group, thereby breaking one DNA strand (Vosberg 1985). Camptothecin, an alkaloid inhibitor, stabilizes this covalent intermediate in vitro (Stewart and Schutz 1987). Consequently, topoisomerase I activity in intact cells can be assessed by mapping camptothecin-trapped, single stranded cleavages of

genomic DNA (Stewart et al., 1990). A431 cells were incubated with A23187 to induce the expression of the fos gene in this cell type and subsequently treated with camptothecin to trap topoisomerase I intermediates. My experiment indicated (Fig. 12) that fos-SLP was not affected by either fos gene activation or inhibition of topoisomerase I activity. This supports the previous results suggesting that SLPs are not necessarily found at active genes.

6. SINGLE STRANDED REGIONS OF DNA

6.1. Pre-existing single-stranded regions

Although DNA is basically a double-stranded molecule, various metabolic processes such as replication, transcription and repair temporarily disrupt this structure. It has been suggested that DNA might contain permanent structural features acting as signals (Crick 1971). Henson (1978) demonstrated that the presence of single stranded regions in mammalian DNA. The presence of long regions of single-stranded DNA in human cells has been reported by Bjursell et al., (1979) as well. Other "unusual" DNA structures also contain single stranded DNA (Panayotatos et al., 1987). In agreement with the above findings, we report that human granulocytes (Fig. 2,3) and monocytes (Fig. 13 I) contain pre-existing SLPs (which may be single-stranded regions of DNA). This is supported by the presence of SLPs in freshly isolated cells (Fig. 7 III). The absence of SLPs if primers were omitted from a labelling mix (Fig. 10) suggest that gaps with 3'-OH ends are not present at SLPs sites. It is uncertain whether these single-stranded regions exist as such in situ or whether they are created selectively

during manipulation of the embedded cells. Even though a definitive conclusion was not drawn from the S1 nuclease experiment (Fig. 8), it led us to investigate the pH dependency of SLP structure.

6.2. Structure of SLPs

Although there are many reports of single- stranded DNA in vivo, their exact nature is not known. Many models support that single stranded DNA occur during DNA synthesis as replicating forks (Collins 1977). In sea urchins embryos two classes of single stranded DNA had been identified (Wortzman and Baker 1981): one is rich in replication forks regions and the other in histone gene DNA sequences.

The presence of single-stranded DNA has also been associated with presence of polypurine/polypyrimidine stretches in eukaryotic cells. Birnboim et al., (1973) was the first to report that eukaryotic cell DNA contained regions of asymmetric base composition. Even though the significance and the function of these sequences was unknown at that time, studies have since demonstrated that these may function as important regulatory sequences (Hariharan and Perry 1990). One of the models for the structure of polypurine/polypyrimidine sequences is "H-DNA", a triple-strand/single strand form. Stabilization of this structure has been shown to be pH dependent (Hanvey et al., 1988). Our results also showed that the structure adopted by SLPs may be pH dependent (Fig. 9). Contrary to what would be expected for the triple-strand DNA model, SLPs were stronger at higher pH (pH 11) but not seen at acidic pH (pH 4.5) where protonation of cytosine can stabilize H-DNA loops. This indicates that either SLPs are unstable at low pH or are made to appear at pH 11. Use or lack of use of 35 mM NaOH (pH 12.4;

denaturing condition) did not affect labelling of SLPs (Fig. 11), while a change in the "background" of Blur2 sequences was observed. The increase in labelling of repeated sequences suggests that some degree of unwinding of chromosomal DNA at random breakpoints can occur within the gel network. This result further supports the notion that SLPs are single-stranded in nature and do not require exposure to pH>12 to be detected. It appears that SLPs represent a bubble or loop structure with an exposed single-strand which can be labelled under the in vitro conditions.

7. LOCATION OF CHROMOSOMAL FRAGILE SITES

Sutherland et al., (1985) hypothesized that there exist a set of similar but not identical DNA sequences at a limited number of sites in the genome. If these become amplified, they result in fragile sites. Four out of five SLPs genes were located within the region of chromosomal fragile sites (CSF2, Jun, ERB-B2 and TRF). We cannot rule out the possibility of the fifth gene (Fos) may be located at or near a as yet undetected fragile site.

Association of poly purine/pyrimidine stretches with fragile site has been proposed (Sutherland and Baker 1986). Naturally occurring sequences of pur/pyr can be of the form $[(AG)_n(CT)_n]$. Amplification of such a sequence could result in a repeated structure. Such a structure would led to a single strand gap when replicated under conditions favoring fragile site formation.

8. **TERMINAL DIFFERENTIATION**

The fact that SLPs occur in granulocytes and monocytes and either not at all or weakly in other cell types (Fig. 14, 15, 16) shows that they might be a property of the granulocyte/monocyte/macrophages cell lineage. The presence of SLPs in myeloid cells of other species (Fig. 19) further supports the lineage specific nature of SLPs. SLPs are weakly positive in HL60 cells (Fig. 17). However, this may be due to presence of some granulocyte type cells in the population. The absence of SLPs in lymphocytes, where about 50% of cells can be stimulated to differentiate by a mitogen such as concavalin A (Setterfield et al., 1983) is consistent with the notion that they are a property of terminally differentiated cells. Differentiation experiments were performed on U937 cells (which resemble blast cells in lineage and were originally isolated from the pleural fluid of a patient with diffuse histiocytic lymphoma) (Balsinde et al., 1988) using TPA, which results in a mixed cell population of granulocytes and monocytes, SLPs started to appear as the cells started the process of differentiation (Fig. 20 II, III) ie., at 10 and 20 hr. SLPs were not detected in unstimulated U937 cells (Fig. 20 I). In a separate experiment, SLPs were positive up to 48 hr after TPA induction and diminished thereafter (Fig. 21). Disappearance may have been due to cell death as observed by trypan blue exclusion assay.

When a similar differentiation experiment was performed on K562 cells (erythroleukemia line) SLPs were not as strong as for U937 cells (Fig. 22). Further experiments have to be done to confirm this result.

Many changes at the level of chromatin and at the transcription stage take place during differentiation of cells. Cellular

differentiation serves a critical role in cell biology and is integrally involved with the control of cell proliferation (Tzen et al., 1988). The nuclear enzyme, ADP-ribosyl transferase (ADPRT), catalyses the formation of poly (ADP-ribose)-modified chromatin proteins from NAD^+ (Hayaishi and Ueda 1977) and is entirely dependent on DNA containing nicks (Halldorssan et al. 1978). Nuclear ADPRT activity is required for efficient DNA excision repair (Durkacz et al., 1980). It has been reported that ADPRT activity is increased during differentiation of primary chick myoloblast (Farzaneh et al., 1982). Farzaneh et al.(1982) had also observed that the appearance of single strand DNA breaks during differentiation which are not due to a general deficiency in DNA repair. Furthermore, ADP-ribosylation has been shown to be an integral component of mesenchymal cell differentiation (Midura et al., 1985). An increase in this enzyme indirectly indicates that DNA breaks occur during differentiation. Endogenous breaks in the DNA of differentiated cells may be a special character occurring at specific nucleotide sequences. The involvement of DNA strand breaks in some categories of cellular differentiation is assumed to be the molecular basis for the requirement of ADPRT in differentiation.

I have postulated that SLPs arise as a result from differences in chromatin structure in specific regions of the DNA in different cell types. The appearance of SLPs early in U937 cell differentiation suggests a function associated with myeloid differentiation, possibly terminal differentiation.

9. MODEL OF SLPs FORMATION

Since the SLPs are detected during terminal differentiation it is

very tempting to speculate that these occur as a result of changes in chromatin structure at the level of loop domain or matrix attachment points. Based on the evidence, I have proposed a model for SLPs formation (Diagram 3). In Dig.3, (a) represents a loop domain in SLP-negative cells such as lymphocytes, (b) and (c), represent loop domain in SLP-positive cells such as granulocytes. In (b) and (c) the DNA loop is under torsional strain due to their superhelical density. Genes along the loop domain is identified as A-I and assume that gene E is a SLP positive gene. In (c), when proteins are removed and the DNA is exposed to pH 11, the DNA strand at or near gene E separate to expose a denaturation bubble. On the other hand in (e), DNA at gene E may pre-exist as a single-stranded region in the cells which is stabilized by bound proteins. This bubble structure may be unstable at lower pHs.

10. CONCLUSIONS

Novel in vitro labelling sites have been identified. Their discovery has opened up new avenues for investigation. Evidence suggests that SLPs are single-stranded in nature and occur in certain cell types and not in others. Single-stranded regions in DNA can arise from different structures, such as H-DNA loops, Z-DNA structure, cruciform structures and other anisomorphic structures. The DNA structure which gives rise to SLPs has not yet been identified. Further more occurrence of SLPs in terminally differentiated myeloid specific cells suggests that SLPs may be involved in terminal differentiation.

Further experiments such as gene mapping of genomic SLP positive genes to identify sites at which SLPs originate will be necessary to characterize the nature of SLPs and other differentiating cell systems

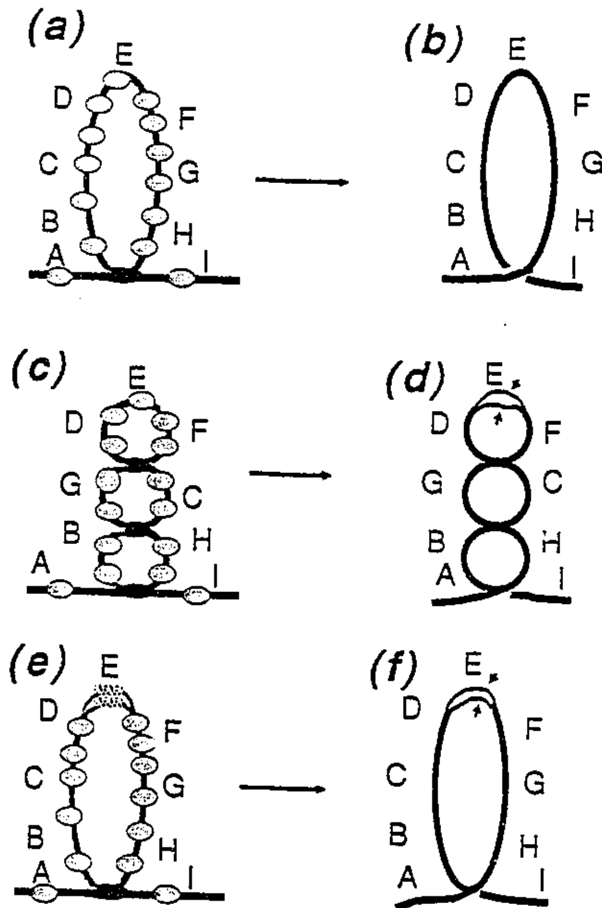


Diagram 3: A model for SLP formation.

will need to be examined to establish their relationship to differentiation of cells.

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APPENDIX

1. RECOVERY OF DNA FRAGMENTS FROM AGAROSE GEL

DNA fragments of interest were visualized by staining lightly with ethidium bromide (0.1μg/mL). An agarose plug containing the DNA fragment was transferred to a tared 10mL polypropylene tube. 2mL of Agarose Solubilizer (9M $NaClO_4$,15mM CDTA and 15mM Tris-cl, pH 7.5) was added per gram of agarose. The tube was vortexed vigorously and then heated at 45°C for 5min. About 1mg of glass powder in Glass Loading Buffer (GLB; 6M $NaClO_4$,10mM CDTA and 10mM Tris-Cl, pH 7.5) was added per μg of DNA expected to be recovered. The tube was rocked for 20 min at room temperature and then centrifuged for 5min at 400xg. The supernatant was discarded. The pellet of glass powder was suspended in 100μL of GLB and the suspension was transferred to 0.22μM (Millipore, Milex filter) unit set in a 0.5mL microfuge tube with the bottom cut open which was supported on a 1.5mL microfuge tube. The filter unit was centrifuged in a microcentrifuge for 2min and the glass bed was rinsed four times with 100ul of GLB. Finally the bed was washed two times with 70% ethanol. The filter unit was transferred to fresh microfuge tube and the DNA fragment bound to the glass was eluted with 50-75μL of CT buffer (not less than 5μL of CT per mg of glass powder). The elution was allowed to proceed overnight and finally the eluate was recovered by centrifugation in a microfuge for 2 min. The purity of DNA was assessed by agarose gel electrophoresis and recovery was estimated spectrophotometrically.

2. END LABELLING OR HOMO POLYMER TAILING

DNA fragments were isolated using the procedure of Appendix 1. End-labelled by procedure as described by Eschenfeldt et al(1987).

DNA fragment in CT buffer	- 1 pmol
tailing buffer	- 1x
BSA (10mg/mL from Sigma)	- 40μg/mL
Terminal transferase (16units/ul from Pharmacia)	- 128units/mL
^{32}P-dCTP(Amersham 10μci/μl)	- 20μci

Sterile H_2O to final volume of 25μl

reaction was allowed to proceed at 37°c for 30min.

(a) 2x Tailing buffer

Sodium cacodylate pH 7.0 (chelex purified)	- 0.2N
$CoCl_2$	- 4.0mM
Dithithrethiol	- 0.4mM

(b) CT

1.0mM CDTA

10.0mM Tris.HCL pH 7.5

3. OLIGO PRIMER LABELLING METHOD

Stock reagents

1.8M HEPES (pH 6.5 with NaOH)

Hexanucleotide mix (Pharmacia pd(N)6; 50 A_{260} U/mL in salt solution)

dNTPs, were prepared as follows:

10mg each of dNTPs from Pharmacia was dissolved in 160-180μl of 10mM CDTA (pH 7.0), calculated to give 100mM solution.

Working reagents

BSA (BRL) 10mg/mL in H_2O

Solution OLB: mix 200μl HEPES + 200μl hexanucleotide mix

Polymerase I: klenow fragment (Pharmacia, 1Unit/μl)

^{32}P-dCTP: 5'a-32P (Amersham)

Carrier RNA (1mg/mL ribosomal RNA in 0.5M Nacl)

Mercaptoethanol: 140mM

Reagents for labelling reaction

dATP, dGTP, dTTP	20μM of each
OLB solution	1/5 vol
BSA	20μg/mL
Mercaptoethanol	14mM
Nicked denatured DNA	20μg/mL

Sterile water for a final volume of 50μL

mixed gently and centrifuged

^{32}P-dCTP	50μci
Polymerase I	40units/mL

mixed and centrifuged again.

Reaction was allowed to proceed at 20°C for 3 hr.

4. S1 NUCLEASE DIGESTION

S1 nuclease - 45units

S1 nuclease buffer - 1x

Bead volume - 25μL

10x S1 nuclease buffer

NaCl - 2M

Sodium acetate pH4.5 - 0.5M

$ZnCl_2$ - 10mM

Glycerol - 5%

5. SOLUTIONS FOR HYBRIDIZATION

(a) Prehybridization Solution

*Denhardt's solution - 5x

yeast RNA - 1mg/mL

SSC - 6x

SDS - 0.5%

Sodium phosphate pH6.8 - 50mM

final volume to 10mL with H_2O

*See Molecular Cloning, A Laboratory Manual (1982). eds. T. Maniatis, E.F. Fritsch, J. Sambrook. Cold Spring Harbor Laboratory. p. 448.

(b) Hybridization Solution

Denhardt's solution - 5x

yeast RNA - 1mg/mL

SSC - 6x

SDS - 0.5%

Sodium phosphate pH6.8 - 50mM

dextran sulphate - 5%

final volume to 10mL with H_2O

5. SOLUTIONS FOR EMBEDDING

(a) B-Earles Buffer

NaCl	- 35mM
KCl	- 5mM
$MgSO_4$	- 0.8mM
Hepes	- 10mM

final pH was adjusted to 7.4

(b) Deproteinizing Solution

SDS	- 0.5%
Proteinase K (Sigma)	- 20μg/mL
Tris-HCl pH 7.5	- 50mM

final volume to 5mL with 2mM CDTA

(c) Unwinding Solution

NaOH	- 35mM

final volume to 5mL with 2mM CDTA

(d) Neutralizing Solution

Glucose	- 1M

6. OTHER REAGENTS

(a) 20xSSC

3.0M NaCl

0.3M sodium citrate; pH6.0

(b) DNA extraction solution

1.0M LiCl

1.0M Urea

5.0mM CDTA

50.0mM Tris-Cl; pH8.0

0.2% SDS

(c) NH_4Cl Solution

NH_4Cl	- 0.87%
Tris-HCL pH 7.2	- 10mM
$NaHCO_3$	- 10mM

(d) PBS

KCl	- 2.6mM
KH_2PO_4	- 1.1mM
NaCl	- 136mM
Na_2HPO_4	- 8.0mM

adjust pH to 7.4.

7. HUMAN GRANULOCYTES

GENE	CONTROL	EMBEDDED	T-VALUES
	X±SEM	X±SEM	
FOS	0.114±0.006	2.864±0.029	102, p<0.01
JUN	0.024±0.003	2.714±0.011	266, p<0.01
CSF	0.042±0.004	2.450±0.041	65, p<0.01
ERBB2	0.066±0.003	1.838±0.026	75, p<0.01
TFR	0.042±0.004	2.126±0.008	270, p<0.01
MYC	0.124±0.021	0.136±0.012	0.55, p>0.1*

*Statistically not significant. Values shown are densitometer (LKB, Ultrascan XL) reading of autoradiograms from replicate experiments such as shown in Fig.2 & 3. The absorbance readings (average±SEM) of 2000ng spots. The T values (Student's T-Test) and p values for differences for each gene using control (total DNA) and embedded DNA are shown (n=5).

8\. U937 CELLS (HISTIOCYTIC LYMPHOMA LINE)

GENE	Control	+TPA	T-value
	X±SEM	X±SEM	
FOS	0.093±0.007	1.900±0.047	43, $p<0.01$
JUN	0.028±0.017	2.600±0.082	35, $p<0.01$
CSF	0.042±0.006	2.230±0.047	52, $p<0.01$
ERBB2	0.040±0.014	1.500±0.047	34, $p<0.01$
TFR	0.060±0.014	1.370±0.029	47, $p<0.01$
MYC	0.108±0.037	0.143±0.029	0.55, $p>0.1$*

*Statistically not significant. Values shown are densitometer (LKB, Ultrascan XL) reading of autoradiograms from replicate experiments such as shown in Fig.21. The absorbance readings (average±SEM) of 2000ng spots. The T values (Student's T-Test) and p values for differences for each gene using control (-TPA) and embedded DNA (+TPA) are shown (n=4).