IDENTIFICATION OF THE SV40 GENE PRODUCT(S) REQUIRED FOR INDUCTION OF CHROMOSOMAL ABERRATIONS IN HUMAN CELLS

by

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ABSTRACT

Expression of the Simian Virus 40 (SV40) early region in human cells results in the induction of chromosomal aberrations and polyploidy, and in transformation. To understand how genetic damage occurs and what role it plays in transformation, human diploid fibroblasts and embryonic kidney cells were transfected with plasmids encoding wild type or mutant forms of the viral early region, and the neo gene. Clones selected for G418 resistance and expressing viral genes were initially analyzed within 20 cell divisions. The results of this study demonstrate that expression of the SV40 large T antigen is sufficient for the induction of chromosomal damage and ploidy changes, and that small t does not contribute to these processes. Mutant plasmids either lacking the SV40 origin of DNA replication, or encoding a large T mutant defective in its ability to bind the retinoblastoma gene product (Rb) were as proficient as wild type plasmids, indicating that both viral DNA replication and binding of T antigen to Rb are not required for cytogenetic damage. On the other hand, preliminary results with a plasmid encoding a T antigen mutant unable to bind the cellular p53 protein suggest that formation of this complex may be important for

cytogenetic damage. This study has also shown that chromosomal aberrations, but not necessarily polyploidy, increase in frequency and complexity upon subculturing of the clones regardless of whether such populations arrest at crisis or yield immortal lines. These results are compatible with the hypothesis that large T antigen destabilizes the cellular genome, and that specific mutations arising from this process may contribute to cell immortalization.

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AEC	3-amino-9-ethylcarbazole
ATP	adenosine triphosphate
bp	base pair
BCIP	bromochloroindolyl phosphate
CML	chronic myelogenous leukaemia
cpm	counts per minute
Ci	curie(s)
°C	degrees celsius
DNA	deoxyribonucleic acid
dl	deletion mutant
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
ug, mg, g	microgram, milligram, gram
HEK	human embryonic kidney
hr(s)	hour(s)
Ig	immunoglobulin
Kd	kilodalton
Kb	kilobase
ul, ml, l	microlitre, millilitre, litre
mu	map units

min(s)	minute(s)
uM, mM, M	micromolar, millimolar, molar
MW	molecular weight
nm	nanometres
NBCS	new born calf serum
NBT	nitroblue tetrazolium
nt(s)	nucleotide(s)
PBS	phosphate buffered saline
pm	point mutant
PPO	2,5-diphenyloxazole
Rb	retinoblastoma
rpm	rotations per minute
sec(s)	second(s)
SV40	simian virus 40
SSC	0.15M sodium chloride, 0.015M sodium citrate, pH 7.0
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TRIS	Tris(hydroxymethyl)aminomethane
V	volts
v/v	volume/volume
w/v	weight/volume
wt	wild type
XP	Xeroderma Pigmentosum

INTRODUCTION

1.1 Genetic Instability and Cancer

The progression of cells towards malignancy involves a number of stages, one of which is likely to be the accumulation of mutations in the genome (Bishop, 1987; Klein, 1987; Tomatis, 1989; Boyd and Barrett, 1990). In the genesis of cancer, one cell may acquire a mutation, either spontaneously or from exposure to carcinogens. This mutation by itself, or with additional ones acquired by the cell's descendants, could confer increased growth rate and extended life span, allowing the mutated cells to out compete normal cells. Aberrant cellular genomes have been observed in neoplasias, and can contain both gross alterations of the cell karyotype, such as changes in chromosome number, deletions, amplification, and translocations, and mutations in the cell DNA. Both types of events can lead to deregulation of gene expression or to functional changes of gene products, which may be essential for the initiation and the progression of cancer. A number of observations support this hypothesis.

Genomic instability and genetic alterations are common features of malignant cells derived from lymphomas or solid

tumours (Holiday, 1989; Boyd and Barrett, 1990; Evans, 1990). Analysis of the karyotype of these cells has further suggested that a causal relationship may exist between specific rearrangements and tumour development. This is exemplified by the alterations in karyotype observed in leukaemias and lymphomas, which commonly contain chromosomal duplications and deletions (Weinstein, 1988), as well as specific chromosomal markers. The classic example of the latter, originally described by Nowell and Hungerford (1960), is present in chronic myelogenous leukaemia (CML). 92% of CML patients carry a specific translocation between chromosome 22 (q11) and chromosome 9 (q34), which results in the formation of a chimeric chromosome termed the Philadelphia chromosome (Nowell and Croce, 1987; Evans, 1990; Testa, 1990). In the remaining 8% of patients, chromosome 22 recombines with a chromosome other than 9, or is involved in a more complex rearrangement with chromosome 9 and a third chromosome (LeBeau, 1990). Specific translocations, all of which in some fashion involve chromosome 8q24, are also observed in cells derived from Burkitt's lymphoma (Evans, 1990; LeBeau, 1990).

Genetic instability is also observed in solid tumours. As an example, changes in both chromosome number and structure are frequently observed in cells derived from human malignant melanoma. In addition, individual tumour lines can contain marker chromosomes which are not found in the normal tissue

from the donor, the most common of these being deletions in chromosome 3, or additional copies of chromosomes 7 and 22 (Liao et al., 1975; McCulloch et al., 1976).

Generally, malignant tumours tend to be more genetically unstable than benign tumours. This property is thought to generate more genetic variants, which may have acquired a selective growth advantage through genetic changes, and thus might expand and form a subpopulation within a tumour (German, 1983; Volpe, 1988). For example, cells entering the terminal acute phase of CML tend to exhibit new abnormalities, beside the translocation between chromosome 22 and 9 commonly observed at the early stages of the disorder (Nowell and Croce, 1987; LeBeau, 1990).

Many of the mutations and rearrangements found in cancers cells both at the early and late stages of carcinogenesis, appear to affect cellular proto-oncogenes and tumour suppressor genes, whose products are thought to be involved in the regulation of normal cell proliferation and differentiation (Weinberg, 1985; Bowden, 1990; Boyd and Barrett, 1990). Activation of the proto-oncogenes can be accomplished by two pathways: Structural and conformational changes in the protein by point mutations and/or deletions in the gene; and/or deregulation of gene expression, which could occur by translocation of the proto-oncogene to a region with a new series of regulatory elements (Bowden, 1990), or by

DNA amplification, as is observed in many human carcinomas (Barker and Hsu, 1979). Oncogenic activation by rearrangement is found in the previously mentioned Philadelphia chromosome, where the reciprocal translocation between chromosomes 22 and 9 results in the repositioning of the abl proto-oncogene from band 22q11 into a region of a gene (BCR) on chromosome 9 termed the breakpoint cluster region (bcr) (Nowell and Croce, 1987; LeBeau, 1990). This leads to the production of a fusion protein which contains the functional domain of the c-abl gene product, but has higher enzymatic activity than the normal protein (LeBeau, 1990). A similar example of proto-oncogene activation occurs in cells of Burkitt's lymphoma, which carry translocations between chromosome 8, at the c-myc proto-oncogene locus, and chromosomes 2, 14, or 22, at the loci of the heavy, kappa, or lambda chains of immunoglobin (Nowell and Croce, 1985; Evans, 1990; LeBeau, 1990). The transposition of c-myc to the Ig loci results in deregulation of c-myc expression, allowing it to be expressed constitutively (Nowell and Croce, 1985; Cesarman et al., 1987; Boyd and Barrett, 1990).

Mutations and deletions are the prevalent mechanisms involved in the loss of function of tumour suppressor genes (Klein and Klein, 1985; Bishop, 1987), a phenomenon which is thought to release cells from quiescence, thus allowing their unlimited proliferation. This hypothesis is supported by

several pieces of evidence. Hybrid cells, generated by fusion of tumour cells with primary human cells, lose their proliferative ability and enter a crisis stage, from which only some recover. This implies that the normal cell contributes some dominant tumour suppressor factor(s), which must be lost in order for the hybrid cell to regain proliferative ability and remain tumourigenic (Pereira-Smith and Smith, 1983; Pereira-Smith and Smith, 1987).

In addition, it has been noted in malignancies such as retinoblastoma, Wilm's tumour, osteosarcoma, and small cell lung carcinoma that inactivation or loss of specific chromosomal regions is required for their development (Bishop, 1987; Klein, 1987; Sager, 1989; Weinberg, 1990). For example, cells from Wilm's tumour usually contain deletions at band 11p13, and the high frequency of alterations at this locus suggests that they may be causal to the development of this cancer. This hypothesis is supported by the observation that reintroduction of a normal chromosome 11 into Wilm's tumour cells represses their ability to form tumours, while introduction of other chromosomes (X and 13) does not (Weissman et al., 1987). Patients suffering from retinoblastoma (Rb) commonly have deletions or point mutations in one or both copies of chromosome 13 (band q14) (Cavanee et al., 1983; Dryja et al., 1986; Lee et al., 1987; Horowitz et al., 1989), where a gene, termed the retinoblastoma, or Rb-

1 gene, has been mapped. This gene codes for a nuclear protein of 110kd, which is present in normal individuals, and absent in retinoblastoma patients carrying the chromosome 13 deletion (Friend et al., 1986; Lee et al., 1987). Alterations of the Rb gene have also been noted in a number of other carcinomas, notably osteosarcoma, soft tissue sarcomas, and small cell lung carcinomas (Friend et al., 1986; Harbour et al., 1988; Huang et al., 1988). These observations suggest that mutations to this locus are causal to the development of a variety of cancers. In support of this hypothesis, reintroduction of a normal Rb gene into retinoblastoma cells results in loss of the neoplastic phenotype (Huang et al., 1988).

A second tumour suppressor gene, p53, has been identified on the short arm of human chromosome 17. This gene is commonly mutated or deleted in lung cancer (Levine, 1990), and in over 75% of colorectal carcinomas (Vogelstein et al., 1988; Baker et al., 1989; Rodriques et al., 1990). p53 encodes a nuclear protein thought to be involved in cell cycle dependent regulation of DNA synthesis (Milner and Milner, 1981; Mercer, 1984; Reich and Levine, 1984). Wild type p53 protein has been shown to repress the neoplastic phenotype when reintroduced into cells transformed with ras and mutated p53, or E1A (Finlay et al., 1989).

Further evidence about the importance of mutations for

initiation and progression of cancer comes from the hereditary basis of numerous malignancies, e.g. colorectal cancer, breast cancer, osteosarcoma, retinoblastoma, and Wilm's tumour (Knudson, 1985; Den Otter et al., 1990; Muller, 1990a; Muller, 1990b). In all cases, a causal relationship appears to exist between congenital cytogenetic defects and development of the disease. In children, the classic example of inherited cancer is retinoblastoma, where individuals suffering from the hereditary form of the disease inherit an already mutated or deleted retinoblastoma gene, leaving only one allele to be lost or inactivated by subsequent mutational events (Den Otter et al., 1990; Muller, 1990a). Hereditary colorectal cancer provides an example of an inherited cancer in adults, where affected individual commonly inherit deletions in chromosomes 5q, 18q, and 17p (Muller, 1990a).

Treatment for cancer often involves agents, both physical and chemical, which in themselves may be mutagenic or clastogenic (Der Kinderen et al., 1988), and cases of treated patients becoming predisposed to secondary malignancies have been reported (Murphee and Benedict, 1984; Tucker et al., 1988; van den Berg-de Ruiter et al., 1990). This suggests that the cytogenetic damage induced by treatment for the primary disorder may contribute to the development of secondary malignancies.

The initiation of some cancers may also involve

deficiencies in DNA repair mechanisms. For example, Xeroderma pigmentosum (XP) is associated with a high frequency of chromosome breakage and reunion, and patients with this disease tend to be more prone to sunlight induced skin cancer, as well as to other malignancies such as breast cancer, adenoma or leukaemia (Cleaver et al., 1989; Muller, 1990b). Bloom's syndrome is also associated with a high rate of chromosomal instability, and patients with this disease have a high incidence of leukaemias, lymphomas and adenocarcinomas (Muller, 1990b).

Finally, studies on human populations and in vitro studies have shown that most carcinogens, both physical and chemical, induce mutations in the DNA and alterations in the number and structure of chromosomes (Barrett et al., 1978; Evans, 1984; Weinstein, 1988; Tomatis, 1989). Smoking is a most potent carcinogen, and is among the main causes of lung cancer. The results from numerous studies demonstrate a highly significant increase in aneuploidy, chromosome rearrangements, and loss of heterozygosity at specific markers (such as chromosome 17p13, the p53 locus) in blood cells from both light and heavy smokers (Vijayalaxmi and Evans, 1982; Chen et al., 1989; Harris et al., 1990).

1.1.1 Transformation

Similar to carcinogenesis, transformation of cells *in vitro* involves multiple steps. Transformation has been operationally defined as the acquisition by the cells of one or more properties, such as altered morphology, focus formation, low serum requirements, loss of anchorage dependence, shortened doubling time and increased saturation density, extended or indefinite lifespan, and tumourigenicity (Potter et al., 1970; Oshima, 1977; Sack, 1981; Chang, 1986; Christian et al., 1987). However, many transformed cells, particularly human cells, acquire only some of these properties. For example, some transformed human cell lines which are tumourigenic in nude mice remain anchorage dependent (Marshall et al., 1977). Most transformed cells acquire an extended lifespan, however the majority eventually reach a crisis stage where the transformed population ceases to proliferate. Cells that survive this stage gain an unlimited proliferative capacity and are termed immortal, a property which is frequently acquired by transformed rodent cells, but is a rare event in transformed human cells (Chang, 1986; Shay and Wright, 1989).

Genetic instability is a common feature of transformed cells, whether they originated following treatment with chemical, physical or viral agents (DiPaolo, 1983; Chang,

1986). Early cytogenetic studies indicated that genetic instability was not required for the occurrence of morphological changes in transformed cells (Moorehead and Saksela, 1965; Walen, 1981; Walen, 1987). Primary human epithelial cells infected with SV40, at early passages had altered morphology and had lost contact inhibition, but were found to possess normal diploid karyotypes. Cytogenetic analysis at later passages, however, revealed chromosomal instability. These observations suggest that morphological changes acquired by transformed cells may be independent of the induction of cytogenetic damage. Additionally, it has been observed that genetic instability in human cells infected with DNA viruses or transfected with viral DNA, occurs prior to the onset of crisis of the transformed population, and is associated with the expression of the viral oncogenes. This is the case for human 293 cells, transformed by Adenovirus type 5 DNA, and expressing the viral early region 1 (E1) (Graham et al., 1977; L. Wei and S. Bacchetti, unpublished), as well as for human fibroblasts transformed by simian virus 40 (SV40), or by the SV40 early region (Moorehead and Saksela, 1965; Chang et al., 1986; Walen, 1987; Shay and Wright, 1989). These results suggest that expression of the viral oncogenes might correlate with cytogenetic damage. This damage may give rise to populations of genetic variants amenable to selection for a number of phenotypes, including the ability to survive

crisis and form tumours. Thus, it appears possible that cytogenetic damage represents an initiating event in transformation, and might contribute as a mutational process, to the development of fully transformed cells.

1.1.2 SV40-Induced Transformation as a Model System for Cancer

For some time it has been known that DNA viruses can induce both cytogenetic changes and transformation in cultured cells (Shein and Enders, 1962; Koprowski et al., 1963; Wolman et al., 1964; Moorehead and Saksela, 1965; Chang, 1986). As mentioned, the induction of genetic damage appears to be important in the initiation and development of transformed cells *in vitro*. SV40 has been frequently used in studies on virus-induced cytogenetic damage, and is of particular interest in this respect as aberrations similar to those observed in human malignancies have been observed in human cells transformed *in vitro* by the virus. In addition, although, SV40 transformed human cells are seldom tumourigenic when injected into animals, and the virus does not appear to have a role in human cancer, SV40 continues to provide a good model for the study of oncogenic transformation. The present study was undertaken to investigate the mechanisms involved in the induction of chromosomal aberrations and of polyploidy

following expression of the SV40 early region in transfected human cells strains, and what contribution this damage makes towards immortalization.

1.2 Simian Virus 40

1.2.1 Classification and Structure

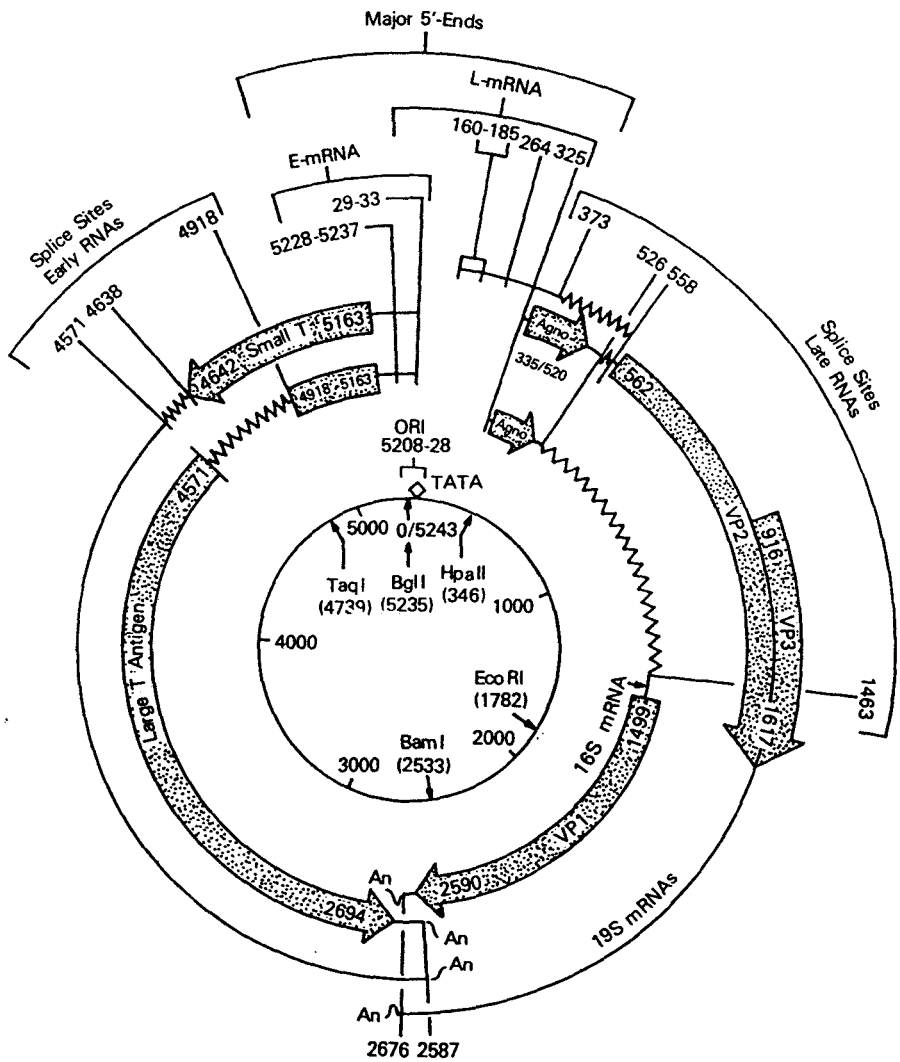
Simian Virus 40 (SV40) is a member of the papovaviridae subfamily known as the polyoma viruses. SV40 was discovered as a viral contaminant in polio vaccine stocks by Sweet and Hillerman in 1960. The natural host range of SV40 is restricted to rhesus monkeys (Topp et al., 1981; Norkin, 1982), however, productive infection of the virus can also occur in permissive green monkey kidney cell lines (Topp et al., 1981; Brady and Salzman, 1986). In addition, SV40 is capable of productive infection in semi-permissive human cells, although virion production occurs at a much lower (100x) efficiency than in permissive systems (Girardi et al., 1965). The virion particle consists of a double stranded, covalently closed circular DNA genome, usually in a superhelical form, enclosed in an icosahedral protein coat 45nm in diameter. SV40 viral DNA is normally associated with four of the cellular histones, H2A, H2B, H3, and H4, to form nucleoprotein structures similar to those found in cellular

chromatin (Cremisi et al., 1976; Griffith et al., 1975). The virion coat, or capsid, is composed of three viral proteins, VP1, VP2, and VP3. VP1 is the most prominent component, and constitutes 75% of the total virion protein, while VP2 and VP3 together constitute 5% (Brady and Salzman, 1986). The three viral structural proteins are arranged into 72 protein subunits referred to as capsomeres. Original analysis suggested the capsid consists of 12 pentameric and 60 hexameric capsomeres (Finch and Crawford, 1975; Griffin, 1981). However, analysis of the structurally similar polyoma virus capsid demonstrated that the hexavalent capsomeres were actually pentamers (Rayment et al., 1982). This suggests that the virion coat in SV40 may also be built solely of pentameric capsomeres.

1.2.2 Genome Organization and Products

As mentioned, the SV40 viral genome is a closed, circular molecule 5243 bp in length, whose entire sequence has been determined (Reddy et al., 1978; Fiers et al., 1978). The viral genes are grouped into two functional regions, early and late, defined by the onset of viral replication. A diagrammatic representation of the SV40 genome is given in Figure 1. The early region contains two overlapping genes, expressed before, and to a lesser extent after viral DNA

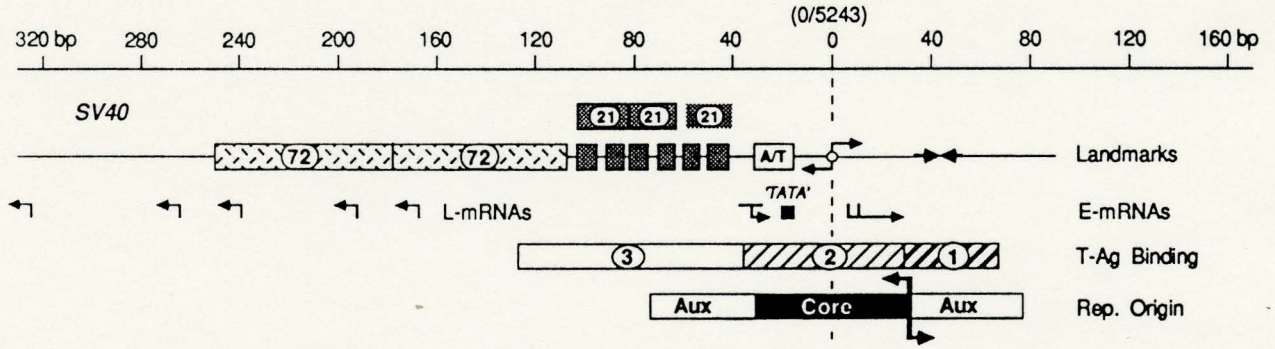
Figure 1: Structure of the SV40 genome.
(Adapted from DePamphilis and Bradley, 1986)


Major 5'-Ends
L-mRNA
160-185
264
325
E-mRNA
29-33
5228-5237
373
526
558
Splice Sites
Early RNAs
4918
4571
4638
Small T
5163
4642
4918-5163
Agno 3
335/520
562
Agno 5
Splice Sites
Late RNAs
ORI
5208-28
TATA
4571
5000
0/5243
TaqI
(4739)
BglI
(5235)
HpaII
(346)
1000
4000
EcoRI
(1782)
BamI
(2533)
3000
2000
16S mRNA
1499
VP2
916
VP3
VP1
1617
1463
Large T Antigen
An
2590
2694
An
An
19S mRNAs
An
2676
2587

replication, which encode the two non-structural proteins, large T antigen and small t antigen. The late region contains four overlapping genes expressed after viral DNA replication, which encode the structural proteins VP1, VP2, and VP3, plus an non-structural protein called LP-1 or agnoprotein (Brady and Salzman, 1986).

Directly between the early and late regions is a 300 bp region containing a series of regulatory elements referred to as the SV40 control region (see Figure 2). This region includes the viral replication origin, as well as the early and late promoters and various enhancer elements (Salzman et al., 1986; Kelly 1988; Kelly et al., 1988; Nussinov, 1990). The minimal region ascribed to the origin of replication is 64-70 base pairs in size, within and around which are located three binding sites for large T antigen. Large T antigen sites I and II contain a consensus GAGGC recognition sequence, and a AT rich region which induces bending of the helix, important for large T antigen binding (DeLucia et al., 1983; Deb et al., 1986), plus the transcription start sites for early gene expression (Kelly, 1988; Nussinov, 1990). Immediately adjacent to this region is an AT rich block which acts as a TATA box for early transcription, followed by one imperfect and two perfect 21 base pair long direct repeats. Each 21 bp repeat consists of duplicate GGGCGG sequences, which are a binding site for the cellular transcription factor

Figure 2: Organization of the SV40 control region
(Adapted from DePamphilis and Bradley, 1986)


(0/5243)
320 bp
280
240
200
160
120
80
40
0
40
80
120
160 bp
SV40
21
21
21
72
72
A/T
Landmarks
'TATA'
L-mRNAs
E-mRNAs
3
2
1
T-Ag Binding
Aux
Core
Aux
Rep. Origin

Sp1 (Dynan and Tjian, 1983). These are followed by two 72 base pair repeat enhancer elements, the outer most of which contains the late promoter (Brady and Salzman, 1986; DePamphilis and Bradley, 1986; Nussinov, 1990). The 72 base pair enhancers can be further subdivided into smaller 15-29 bp long cooperative elements termed A, B, and C, which themselves consist of smaller subunits called enhansons (Ondek et al., 1988).

1.2.3 Infection by SV40: Absorption, Penetration, and Uncoating

Initial events in the productive infection process involve adsorption of the virion to the cell surface and endocytosis of the virion particle into the cytoplasm. Adsorption of the virion is mediated by one or more of the VP1 species on the virion capsid and by a specific receptor on the cell surface (Barbanti-Brodano et al., 1970; Hummler et al., 1970; Maul et al., 1978). The absorbed virion is internalized in an endocytotic vesicle, within which it is conveyed to the nucleus of the cell within one half to two hours after infection (Acheson, 1976; Maul et al., 1978; Clayson and Compans, 1988). Once it has reached the nucleus, the vesicle fuses with the nuclear membrane, and the intact virion is transported across the inner membrane into the nucleoplasm,

where removal of the membrane envelope and viral uncoating occur (Barbanti-Brodano et al., 1970; Maul et al., 1978).

1.2.4 Early Genes and Early Gene Expression

Early gene transcription commences once the virion particle reaches the nucleus and is uncoated. Large and small tumour antigen messages are produced by differential splicing of the same RNA transcript, which remove nucleotides 4918-4571 (the large T intron) for large T antigen, and 451-4638 for small t antigen messages. Both proteins therefore share the first 82 amino acids, and the same polyadenylation site (Acheson, 1981; Brady and Salzman, 1986).

The small t antigen sequence encodes a protein of 174 amino acids or 17 kd, found primarily in the cytoplasm of infected cells, which does not appear to be required for lytic infection *in vitro* of both permissive and semi-permissive systems (Acheson, 1981; Brady and Salzman, 1986). The biochemical functions of small t antigen are not well known, however some properties which have been ascribed to the protein include activation of transcription of certain polII and polIII promoters (Loeken et al., 1988), association with two cellular proteins (Yang et al., 1979; Rundell, 1987), and promotion of continued cell cycle progression in non-permissive cell systems (Hiscott and Defendi, 1981). Small t

antigen may also enhance the process of immortalization and morphological transformation in both human and rodent systems (Rubin et al., 1982,; Bikel et al., 1987; DeRonde et al., 1989; Jog et al., 1990).

The gene for large T antigen encodes a polypeptide 708 amino acids, or 94 kd. The majority of this protein is located within the nucleus of cells, either as free molecules in the nucleoplasm, or bound to chromatin or to an insoluble structure referred to as the nuclear matrix, both in SV40-transformed rodent cells (Staufenbiel and Deppert, 1983) and in SV40-infected or -transformed simian cells (Covey et al., 1984). A small amount (5-10%) of T antigen is located in the cytoplasm, and is associated with the plasma membrane (Michel and Schwyzer, 1982; Shirbeck and Deppert, 1989). Analysis of the protein extracted from SV40-infected or -transformed cells has revealed that large T antigen is post-translationally modified by glycosylation, phosphorylation, and acylation (Peden et al., 1989), and assumes several oligomeric states (Myers et al., 1981; Bradley et al., 1982). Changes in the structure of T antigen generates multiple forms of the protein, which in turn may be able to perform separate biochemical functions. For example, changes in the phosphorylation state of large T antigen can affect its replicative function (Prives et al., 1990), while dephosphorylation reduces the proteins ability to renature SS

DNA sequences (Schiedner et al., 1990). In addition, chromatin and nuclear matrix associated forms of the protein display different DNA binding and ATPase activities (Schrimbeck and Deppert, 1989).

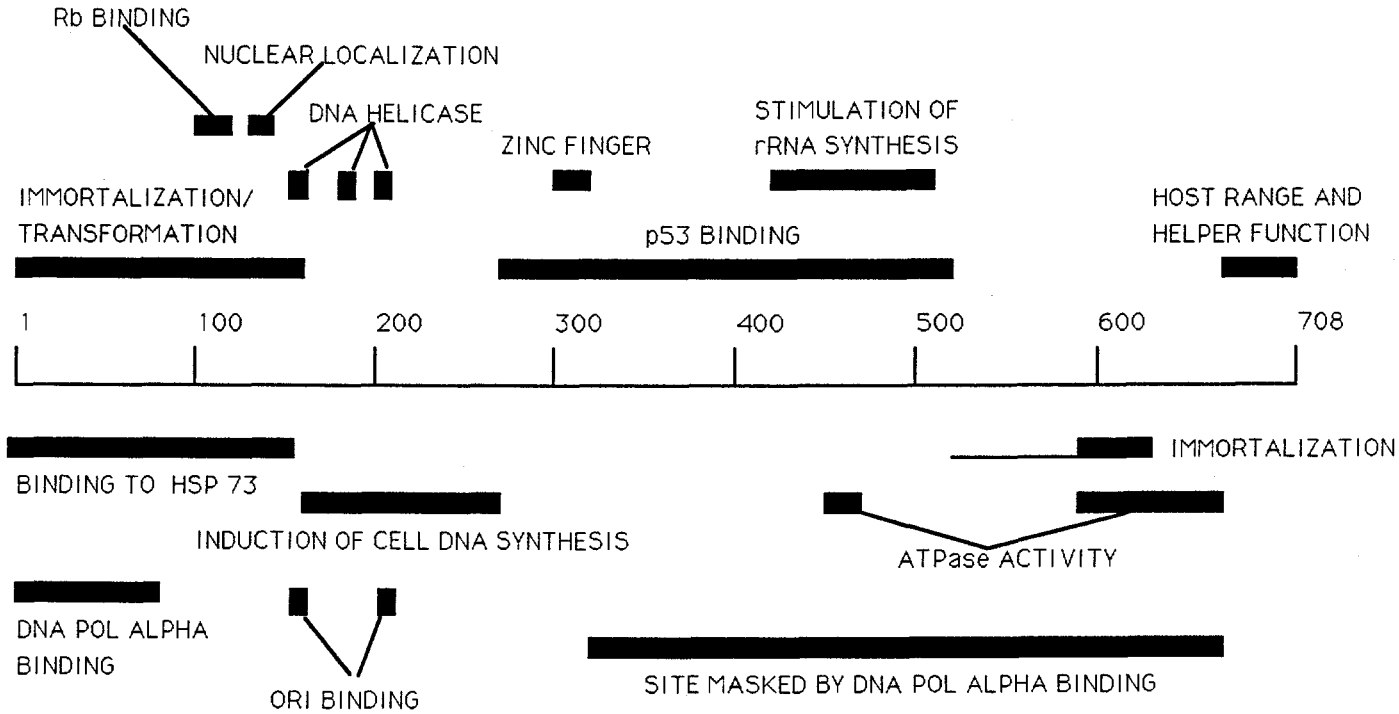
Large T antigen is a multifunctional protein, involved in both the viral replicative process and in virus-induced transformation (Livingston and Bradley, 1987; Stahl and Knippers, 1987). Mutational analysis of the SV40 genome has indicated that the T antigen polypeptide has distinct functional domains, as illustrated in Table 1 and Figure 2a. A number of independent biochemical functions have been ascribed to this protein. Large T antigen is required for viral DNA replication, which involves its ability to bind to the viral replication origin (Paucha et al, 1986; Arthur et al., 1988; Simmons et al., 1990), its ATPase and DNA helicase activities, essential for DNA unwinding activities (Clark et al., 1981; Clark et al., 1983; Stahl et al., 1986; Mole et al., 1987), and its binding to DNA polymerase alpha (Smale and Tjian, 1986; Dornreiter et al., 1990). Viral DNA replication may also involve a single sequence element, found in the protein, with an arrangement of cysteines and histidines characteristic of a zinc finger motif (Loeber et al., 1989), or the ability of the protein to renature homologous DNA strands (Schneider et al., 1990), and catalyse non-ATPase dependent RNA unwinding activity (Schneffner et al., 1989).

Table 1: Location of Functions on SV40 Large T Antigen*

Amino acids	Function	Ref
1-83	Binding to DNA polymerase alpha	Dornreiter et al., 1990
1-121	Transactivation of heterologous viral genes	Srinivasan et al., 1989
1-178	Binding to cellular hsp 73	Sawai and Butel 1989
101-118	Sequence sufficient for binding of retinoblastoma (Rb) anti-oncogene product	Moran 1988
105-114	Amino acid sequence that must remain intact for binding Rb or p115/118/120	DeCaprio et al., 1988; Ludlow et al., 1989; Ewen et al., 1989
106-124	Phosphorylated Ser and Thr residues	Scheidtmann et al., 1982; Van Roy et al., 1983; Simmons, 1984
126-132	Nuclear locatization	Kalderon et al., 1984a,b; Colledge et al., 1986
152-155 203-207	Binding to SV40 origin: most critical amino acids defined by mutagenesis	Simmons et al., 1990
160-272	Stimulation of cell DNA synthesis	Soprano et al., 1983
152-155 182-187 203-207	DNA helicase activity	Simmons et al., 1990
277-362	Nonspecific binding to cellular DNA	Prives et al., 1982
271-517	Binding to cellular p53	Mole et al., 1987; Schmeig and Simmons, 1988
302-320	Zinc finger motif	Loeber et al., 1989
320-670	Site masked by pol α binding	Smale and Tjian 1986; Dornreiter et al., 1990
590-669 453-468	ATPase activity	Clark et al., 1981, 1983; Mole 1987
418-528	Adenylation site	Clertant et al., 1984; Bradley et al., 1987
420-509	Stimulation of rRNA synthesis	Soprano et al., 1983
639-679	Covalent linkage of RNA	Carroll et al., 1988
639-701	Phosphorylated Ser and Thr residues	Scheidtmann et al., 1982; Van Roy et al., 1983; Simmons 1984
670-708	Post-transcriptional regulation of SV40 late mRNA; host range function; adenovirus helper function	Tornow and Cole 1983; Pipas et al., 1988; Tornow et al., 1985; Khalili et al., 1988
Functions of large T not located to domains:		
	renaturation of homologous DNA strands	Scheidner et al., 1990
	RNA unwinding non-ATPase dependent activity	Schneffner et al., 1989

* Adapted from Thompson et al., 1990

Figure 2a: Location of Functions on SV40 large T antigen. The numbers refer to amino acid numbers (Adapted from Table 1).


Rb BINDING
NUCLEAR LOCALIZATION
DNA HELICASE
ZINC FINGER
STIMULATION OF
rRNA SYNTHESIS
IMMORTALIZATION/
TRANSFORMATION
p53 BINDING
HOST RANGE AND
HELPER FUNCTION
1
100
200
300
400
500
600
708
IMMORTALIZATION
BINDING TO HSP 73
INDUCTION OF CELL DNA SYNTHESIS
ATPase ACTIVITY
DNA POL ALPHA
BINDING
ORI BINDING
SITE MASKED BY DNA POL ALPHA BINDING

Viral replication functions are also aided by post translational regulation and viral host range functions found on the carboxy end of the protein (Tornow and Cole, 1983; Pipas, 1985; Tornow et al., 1985; Khalili et al., 1988).

Regulation of both early and late viral gene expression is also mediated by large T antigen. Binding of the protein to the three T antigen sites in the viral origin, the domain for which has been mapped to amino acids between 131-259, serves to autoregulate early and late viral gene expression (Paucha et al, 1986; Arthur et al., 1988; Simmons et al., 1990). Activation of late gene expression by the protein is accomplished in part by T antigen-mediated viral DNA replication. However, the protein can also activate late gene expression in the absence of viral DNA replication (Brady et al., 1984). In this instance, promoter elements are acted upon by a cellular trans-activating factor which is either modified, activated, or induced by large T antigen (Alwine et al., 1987). This conclusion is supported indirectly by evidence that transactivation of both the late and early promoters is observed without nuclear location of T antigen (Wildeman, 1989). Viral gene transactivation functions have been mapped to the amino-terminal 120 amino acids of the protein (Srinivasan et al., 1989).

SV40 makes use of the hosts RNA polymerases and cellular factors for transcription, as it does not encode for its own

transcription factors (Salzman et al., 1986). The early promoter contains two transcriptional start sites, referred to as downstrean and upstream. Initial transcription is from the downstream initiation site, and requires at least one copy of the 21 bp repeat sequences, as well as an intact 72 bp enhancer sequences (Buchman et al., 1984). The second (upstream) transcription start site is switched to as the virus shifts into the late cycle of infection (Buchman et al., 1984; Buchman and Berg, 1984). GC rich regions are important for transcription from the downstream site. Transcription from the early promoter is regulated by binding of large T antigen to its three adjacent binding sites in the control region. Large T binds with highest affinity to site I, allowing repression of early transcription from the downstream start site later in infection by preventing RNA polymerase binding to the early initiation site (Reed, 1976; Tegtmayer, 1975; Buchman et al., 1984; Buchman and Berg, 1984; DePamphilis and Bradley, 1986). Subsequent to this, transcription from the early promoter shifts to the upstream start site. The switch of initiation sites further regulates early transcription as the upstream initiation site is only 10-20% as efficient as the downstream site (Buchman and Berg, 1984).

In addition, T antigen appears to be involved in modification of several host functions, including the

modulation of cellular gene expression by binding to both non-specific and high affinity sites in the cell genome (Prives et al., 1982; Gruss et al., 1988). Modification of cellular trans-acting factors by the protein may also assist in activation of cellular target promoters, which is known to occur in both lytic infection and transformation (Tack and Beard, 1985; Alwine et al., 1987). For example, T antigen has been reported to be involved in stimulation of cellular rRNA synthesis (Soprano et al., 1983). Large T antigen is known to bind to several cellular proteins, which include the Rb and p53 tumour suppressor gene products (Mole et al., 1987; DeCaprio et al., 1988; Moran, 1988, Schmeig and Simmons, 1988; Ludlow et al., 1989; Ewen et al., 1989), a cellular protein of 118-120 kd (Ewen et al., 1989), and the heat shock protein, hsp 73 (Sawai and Butel, 1989). Binding to large T antigen may affect the stability and/or function of these proteins, as is the case for p53 whose half-life is extended from 15-20 minutes to more than 24 hours when bound to large T antigen (Oren et al., 1981; Stahl and Knippers, 1987). p53 also competes with DNA pol α for binding to T antigen, which may interfere with SV40 DNA synthesis by potentially blocking formation of a T antigen-DNA pol α complex (Gannon and Lane, 1987). Mouse p53 (but not human p53) specifically inhibits SV40 origin-dependent DNA replication, which suggests that this competition between p53 and DNA pol α for T antigen may

be the basis for host permissiveness (Braithwaite et al., 1987; Wang et al., 1989). Additionally, T antigen induces cellular DNA synthesis, the domain for which has been mapped to amino acids 160-272 of the protein (Soprano et al., 1983).

1.2.5 Viral DNA Replication

Replication of SV40 DNA occurs approximately 12-20 hours after infection by the virion, and signifies the beginning of the late portion of the life cycle (Acheson, 1976; DePamphilis and Bradley, 1986). Large T antigen plays an essential role in viral DNA replication, by binding and interacting with the first and second T antigen sites in the control region. T antigen is capable of melting an imperfect palindrome located in the second site, allowing the protein to enter the DNA helix (Simmons et al., 1990). Once bound and into the helix, T antigen's ATP-dependent helicase activity catalyses unwinding of the two strands to establish two replication forks and allow replication (Kelly, 1988). This unwinding process is assisted by cellular accessory proteins, such as the replication protein A, which prevents reassociation of the single strands. Initiation and elongation also use cellular DNA polymerases alpha and delta, proliferating cell nuclear antigen (PCNA, or cyclin), topoisomerases I and II, protein phosphatase 2A ($PP2A_c$), replication factor C, and other as yet

undefined cellular components (Weinberg et al., 1990). Replication of the DNA is bidirectional and semi-discontinuous, with leading and lagging strands. Termination does not require specific nucleotide sequences, but occurs when the two replication forks meet approximately half way around the viral genome. The two intertwined daughter viral genomes are then separated by topoisomerase II. (DePamphilis and Bradley, 1986; Kelly, 1988; Kelly et al., 1988)

1.2.6 Late Gene Expression

Transcription from the late promoter occurs after replication of the viral genome. At this point there may be loss or removal of negative cellular factors which help to repress the late promoter during the early life cycle of the virus (Brady and Khoury, 1985). Late gene expression requires the 72 base pair enhancer sequences, one of which contains the late promoter. Binding of the large T antigen to the second binding site also appears to be important for late gene activation, as is the presence of uncharacterized positive cellular factors (Keller, 1984; Brady and Khoury, 1985). As mentioned, large T antigen can also induce late promoter expression by an indirect route, possibly activating or modifying cytoplasmic factors in the transcription machinery (Alwine et al., 1987; Wildeman, 1989).

Late gene expression, predominantly of the three viral structural proteins, accounts for up to 10% of RNA produced by the infected cell (Acheson, 1976). The open reading frame for the major virion protein, VP1, encompasses nucleotides 1488-2574. VP1 is separated into six distinct species, 1 major and 5 minor by post-translational modification (O'Farrell and Goodman, 1976). The open reading frames for VP1 and VP2/VP3 overlap for approximately 113 nucleotides. VP2 and VP3 are encoded by nucleotides 545-1601 and 899-1601 respectively, and as such share two thirds of their C-terminal end (Brady and Salzman, 1986; Wychowski et al., 1987). The agnoprotein is encoded in the late region leader sequence by nucleotides 335-520 (Jay et al., 1981; Brady and Salzman, 1986).

1.2.7 Virus Assembly and Release

Assembly of the viral structural proteins VP1-3 plus the DNA-histone complexes into mature virions, though not completely understood, occurs within the cell nucleus in a gradual fashion (Brady and Salzman, 1986). VP1 contains a nuclear migration signal (Wychowski et al., 1986), and VP2 and VP3 have a nuclear accumulation signal (Kasamatu and Nehorayan, 1979; Wychowski et al., 1987), which are required to transport the proteins to the nucleus after synthesis (Hummler et al., 1970). The role of the agnoprotein in virus

assembly is less certain, however there is evidence that it may aid efficient virion particle assembly by assisting the transport of VP1 to the nucleus (Ng et al., 1985; Carswell and Alwine, 1986; Stacey et al., 1990).

Virus DNA begins to be incorporated into virion structures within 20 minutes of replication, however the entire process requires eight hours to complete (Acheson, 1981). Virus assembly involves several intermediates, the first a 75S viral DNA chromatin structure consisting of viral DNA and all five of the cellular histones, then a 200S previrion form. This is followed by a 240S immature nuclear virion, where by some process the H1 histone is removed, and then by release of the mature virion (Acheson, 1981; Brady and Salzman, 1986). Release of the virus may be accomplished by alteration of the structure or the permeability of the cellular membranes (Norkin, 1977; Acheson, 1981), or, as it has been recently suggested, by a receptor-mediated vesicular transport process (Clayson et al., 1988).

1.3 Transformation by SV40

For many years, transformation of cells <u>in vitro</u> has been used as a model system to define the steps involved in malignancy. SV40 was one of the first viruses shown to possess transforming properties (Shein and Enders, 1962;

Koprowski et al., 1963). Although SV40 has never been linked to human cancer, the discovery that SV40 caused tumours in rodent systems (Diamondopoulos, 1978; Lewis and Martin, 1979) sparked interest in this agent as a model of molecular mechanisms in malignancy. Two main systems, rodent and human, have been used in studies of the transforming properties of SV40. Although similar in many of the properties of transformation, the two systems differ in that full transformation of cells to immortalization and tumourigenicity by SV40 although common in rodent, is a rare event in human cells (Chang, 1986). Cells transformed by SV40 acquire altered morphology and growth properties, including focus formation, low serum requirements, loss of anchorage dependence, shortened doubling time, and increased saturation density, as well as karyotypic alterations, and tumourigenicity (Potter et al., 1970; Oshima, 1977; Sack, 1981; Christian et al., 1987). As mentioned, SV40-transformed cells may also gain the ability to be passaged indefinitely (immortal) in culture. In the following review of the literature, cells which are referred to as being transformed most commonly have acquired only altered morphology and growth properties. Cases where transformants are also immortal and/or tumourigenic will be indicated.

Rodent cells have been used in the majority of the studies on transformation by SV40. These cells are non-

permissive for SV40, in the sense that they allow expression of the early viral functions, but not viral DNA replication or late gene expression (Salzman et al., 1986). Rodent cell cultures can spontaneously generate immortalized cell lines, but the frequency of this event is significantly increased by infection with SV40 (Barrett et al., 1978; Topp et al., 1981; Sack, 1981; Kraemer et al., 1983). In addition, SV40 transformed rodent cells are often tumourigenic when injected back into nude mice (Shin et al., 1975).

Several observations indicate that the region of SV40 DNA responsible for the transformation of rodent cells is limited to the early region of the viral genome and its two protein products, large and small tumour antigen. As mentioned, viral late functions and viral DNA replication do not occur in non-permissive systems. In addition, temperature sensitive mutant of large T antigen are unable to transform rodent cells at restrictive temperatures (Petit et al., 1983). Lastly, SV40 T antigen alone is capable of efficiently immortalizing rodent primary fibroblasts (Jat and Sharp, 1986; Jat and Sharp, 1989).

Two regions of large T antigen have been implicated as being essential for viral induced transformation in rodent systems. One is located at the amino terminal 157 amino acids of the protein (Sompayrac and Danna, 1988; Montano et al., 1990), whereas the second region is near the carboxy terminus

(Asselin and Bastin, 1985; Sompayrac and Danna, 1988; Tevethia et al., 1988). These two regions may function independently of one another, as mouse C57B1/6 cells can become immortal and tumourigenic both with and without the first 127 aa of large T antigen (Thompson et al., 1990).

SV40 T antigen mutants which are defective for viral DNA replication still transform at a wild type efficiency in rodent systems (Prives et al., 1983; Gish and Botchan, 1987; Manos et al., 1988). This suggests that T antigen replicative functions may not be required for transformation, an hypothesis supported by the observation that T antigen mutants defective for either ATPase activity or binding to the viral origin are transformation positive (Paucha et al., 1986; Gish and Botchan, 1987). Conversely, SV40 mutants defective for transformation function normally in viral replication in simian and rodent cells (Kalderon and Smith, 1984).

Evidence also exists to suggest that separate T antigen functions are required for morphological changes and immortalization. Studies with SV40 mutants containing alterations in the nuclear location signal of large T antigen, which prevented migration of the protein to the nucleus, demonstrated that T antigen was still able to morphologically transform Rat-1 cells but could not immortalize them (Kalderon and Smith, 1984). This is consistent with data suggesting that immortalization requires nuclear function(s) of large T

antigen (Clark et al., 1983; Stahl and Knippers, 1987), but is at variance with the fact that mutants of large T antigen which locate to the cytoplasm are still immortalization proficient (Vass-Marengo et al., 1986; Thompson et al., 1990).

Increasing evidence indicates that cellular proteins are involved in assisting T antigen in the development of both morphologically transformed and immortal phenotypes. As an example, the Rb binding region (aa 105-114) of large T antigen (DeCaprio et al., 1988) has been known for some time to be involved in transformation of rodent systems (Kalderon and Smith, 1984; Sompayrac and Danna, 1984; Sompayrac and Danna, 1988; Thompson et al., 1990). As mentioned, Rb is involved in suppression of neoplastic phenotypes, thus large T binding to Rb may remove this suppression. However, binding of the Rb protein may not be required for immortalization, as deletion or point mutants which lack the Rb binding site can still efficiently promote immortalization of primary rodent cells (Chen and Paucha, 1990; Thompson et al., 1990).

In contrast, binding to p53 may not be required for morphological transformation, as deletion mutants of large T which have lost the ability to bind to p53 could still morphologically transform these cells (Sompayrac et al., 1983; Sompayrac and Danna, 1989). Instead, p53 binding to large T antigen appears to be important for efficient immortalization. Mutants of T antigen which still bind to p53 can immortalize

primary mouse cells (Thompson et al., 1990), while mutants with alterations or deletions to the p53 binding site and therefore unable to bind p53, are unable to promote immortalization (Reddy, 1982; Tevethia et al., 1984; Tevethia et al., 1988). In addition, p53 is expressed at elevated levels, compared to normal tissues, in SV40 transfected cells and in SV40 induced tumours in hamsters (Marks et al., 1989), and is complexed with T antigen in transgenic mouse tumours (Butel et al., 1990).

One mechanism suggested for T antigen-induced transformation is the modification by T antigen of cellular components in the cytoplasm. As mentioned, large T antigen influences gene expression by trans-activating many cellular genes, a phenomenon known to occur during transformation (Tack and Beard, 1985; Alwine et al., 1987). Transactivation is required for the induction of morphological changes associated with transformation (Alwine et al., 1987). Additionally, T antigen interaction with the cellular chromatin is important for maintaining the morphological transformed phenotype in rodent cells (Richter and Deppert, 1990). However, transactivation function may not be required for immortalization, as SV40 mutants defective for this function of T antigen can still immortalize rodent cells (Thompson et al., 1990).

A number of other properties of large T antigen, such as DNA helicase and ATPase activity (Manos and Gluzman, 1985; Tevethia et al., 1988; Auborn et al., 1989) are not required for immortalization. In addition, the carboxy-terminal 82 amino acids of T antigen, which include the viral host range functions, is also dispensable for this process in rodent cells (Tevethia et al., 1988). Several other properties of the protein, including the induction of cell DNA synthesis, stimulation of rRNA synthesis, and binding to DNA pol α, remain potential candidates for assisting in immortalization, but have yet to be investigated.

The role of small t antigen in the transformation process in rodent systems is less clear. It has been suggested that small t may be of importance in morphological transformation when non-growing cells are used, and when concentrations of T antigen are limiting (Sleigh et al., 1978; Rubin et al., 1982; Bikel et al., 1987). Small t antigen may also cooperate with T antigen in inducing tumours in slowly dividing rodent cells (Choi et al., 1988). Recently, it has been demonstrated that the common region of large and small t antigens contains a domain required, along with the unique region of small t antigen, for anchorage-independent growth of T antigen-transformed BALB/c3T3 cells (Montano et al., 1990). However, the mechanism by which small t antigen can enhance morphological transformation and tumourigenicity by large T is

unknown. Jog et al. (1990) demonstrated that mutants of small t antigen, which were reduced in their ability to enhance transformation were able to transactivate polII promoters and to bind cellular proteins. This suggests that these activities cannot account for the enhancement of transformation by small t antigen.

SV40 induced transformation has also been characterized in human cells, although not as extensively as in rodent systems. Transformation of human cells by SV40 was first reported in the early 1960s (Koprowski, 1962; Shein and Enders, 1962). As previously mentioned, human cells are semi-permissive for SV40, meaning that viral replication and production can occur, though at a much lower efficiency than in permissive cells (Girardi et al., 1965). Survivors of infection with SV40 may contain integrated viral genomes (Campo et al., 1979) and become transformed, however they are still capable at a very low frequency (0.1%) of producing infectious virions (Girardi et al., 1965; Huebner et al., 1975; Fogh and Loveless, 1978).

As mentioned, immortalization of human cells by SV40 is a rare event (Chang, 1986; Shay and Wright, 1989). This suggests that expression of viral functions in human cells is not sufficient in itself to remove or avoid the block(s) which limits <u>in vitro</u> lifespan, and that at the least a second mutational event must occur to inactivate these blocks (Sack,

1981; Shay and Wright, 1989). SV40 morphologically transformed cells can gain an extended lifespan compared to untransformed cells (Girardi et al., 1965; Shay and Wright, 1989). However, most of them eventually reach a crisis stage at which point the transformed population ceases to proliferate. Cells in the crisis stage still continue to produce DNA but fail to successfully complete cell division (more cells dying off than those successfully dividing), with increasing cell detachment and the appearance of large and multinucleated cells within the population (Wolman, 1964; Girardi et al., 1965; Donahue and Stein, 1989). Cells surviving the crisis stage resume growth and have unlimited proliferative capacity, and thus are termed immortal. Immortality appears recessive, as hybrid cell populations created from SV40 immortalized cells and normal human fibroblasts still go through a crisis stage and give rise to new immortal cells. This suggests the normal cells contain dominant factor(s) which prevent cell proliferation (Pereira-Smith and Smith, 1987).

Full transformation of normal human diploid cells to tumourigenicity has proven to be more difficult than in rodent cell types. This indicates that the persistence of SV40 transforming proteins is not by itself sufficient to confer to human cells a malignant phenotype. For example, SV40 transfected human foetal kidney cells which have acquired

transformed morphology rarely produced tumours when injected into nude mice (Poirier et al., 1988). Human uroepithelial cells infected with SV40 acquired many of the altered phenotypic traits associated with morphological transformation, and an immortal lifespan, but remained nontumourigenic in nude mice (Christian et al., 1987; Meisner et al., 1988). Finally, SV40 established human keratinocyte cell lines will not form malignant tumours in nude mice without either extensive passaging in vitro (Brown and Gallimore, 1987), or the contribution of another transforming virus (Adenovirus 12), or of a chemical carcinogen (Rhim et al., 1985; Rhim et al., 1986).

Similar to the case in rodent cells, SV40 sequences responsible for morphological transformation and immortalization have been mapped to the early region of SV40. Both early gene products are found in SV40 transformed human cells (Sack, 1981; Chang, 1986; Mayne et al., 1986; Neufeld et al., 1987). Origin-defective SV40 DNA can transform human cells more efficiently than wild type DNA (Small et al., 1982; Neufeld et al., 1987). Further, experiments with primary human cells transfected with either a fragment of SV40 DNA containing the early region and the viral origin of replication, or the early region cloned into a plasmid demonstrated that the early region was both necessary and sufficient for morphological transformation and

immortalization (Zouzias et al., 1980; Mayne et al., 1986; Chang et al., 1986).

Also in agreement with studies on rodents, large T antigen appears to be the major protein responsible for SV40 induced transformation in human systems. It was recognised from early studies with SV40 that both the level of large T antigen in infected cells, and the number of T antigen producing cells correlated with the frequency of transformation (Aaronson and Todaro, 1968; Aaronson, 1970; Potter, 1970). In addition, human fibroblasts immortalized with SV40 mutant which encodes a temperature sensitive large T antigen, lost their proliferative ability at non-permissive temperatures (Radna et al., 1989).

Resolution of what functions of large T are involved in transformation of human cells has been limited, however morphological transformation and immortalization of these cells, similar to rodents, does not require viral replication. Origin minus or defective mutants of SV40 are capable of inducing both processes in human cells with greater efficiency than the wild type (Small et al., 1982; Brown and Gallimore, 1987; Canaani et al., 1987; Neufeld et al., 1987; Radna et al., 1989). In addition, large T mutants, defective for binding to Rb, which as discussed is required for morphological transformation, could still induce viral DNA

replication and transcriptionally activate early and late viral promoters (Trifillis et al., 1990).

One model suggested for T antigen's role in transformation is to out compete cellular proteins which bind to functional regions of the Rb protein (Huang et al., 1990). Mutant Rb proteins present in human cancers do not bind to large T, which suggests that binding of large T to functional regions of Rb is important for transformation of human cells in vitro (Horowitz et al., 1989; Huang et al., 1990). This does not rule out the possibility that interaction of other cellular proteins with T antigen is also important for transformation. The Rb binding region of Large T also binds to a cellular protein of 118-120 kd, which suggest these two proteins (Rb and p118/120) may share similar roles in transformation (Ewen et al., 1989). It is not yet known whether binding to p53 assists as well in large T induced transformation of human cells.

What contribution small t antigen makes towards transformation of human cells is less clear. It has been demonstrated that human cells transfected with plasmids which expressed only large T antigen did not acquire altered morphology (DeRonde et al., 1989, Ray et al., 1990). This indicates that small t contributes at least towards the development of the morphological transformed phenotype in human cells.

1.4 SV40 Induced Genetic Instability

As mentioned, cytogenetic damage has been of interest in the study of cancer, since genetic instability is often a feature of tumour cells, and may contribute to the development of the malignant phenotype (Yunis, 1987). More specifically, SV40 is of particular interest in the study of cytogenetic damage, as aberrations similar to those observed in human malignancies have been observed in human cells transformed *in vitro* by the virus.

SV40 induction of genetic damage appears to follow a basic format. In most cells infected or transfected by SV40, the presence of chromosomal breaks and gaps is detected first at early times after either method (Moorehead and Saksela, 1963; Todaro, 1963; Wolman, 1964). Subsequently, more complex rearrangements occur, including dicentrics, translocations and fragments, as well as heteroploidy and aneuploidy, all of which increase as the cells progress towards crisis (Moorehead and Saksela, 1963; Moorehead and Saksela, 1965; Wolman et al., 1964; Marshak et al., 1975; Chang et al., 1986; Walen, 1987). Cells infected with SV40 or transfected with the early region of SV40 also exhibit increased levels of sister chromatid exchanges and DNA amplification (Nichols, 1978; Nichols et al., 1985). The rate at which genetic damage develops can depend on the age of the cells. Infection of older cells (ie.

cells which have undergone a large number of cell divisions) gives rise to a more rapid development of genetic damage compared to younger cells (Todaro, 1963; Wolman et al., 1964).

Evolution of the aberrant karyotype has been observed in SV40 transfected cells which attain the immortalized state (Brown and Gallimore, 1987; Christian et al., 1987; Meisner et al., 1988; Poirier et al., 1988; Burholt et al., 1989; Shay and Wright, 1989). This suggests a causal relationship between continuous genetic alteration and immortalization by SV40. Continued karyotypic rearrangements could generate a population of genetic variants, some of which would allow continued cell proliferation and confer selective advantage over other cells in the transformed population. For example, human foetal keratinocytes transfected with an origin minus SV40 underwent an evolution of karyotype from polyploidy and low level of structural defects, to presence of dicentric, ring chromosomes, translocations and fragments between passages 3 to 20 (Poirier et al., 1988). A similar loss and gain of chromosomal material is observed in SV40 transformed cells from a pleural effusion of a large cell carcinoma (Burholt et al., 1989). Evolution of the karyotype also correlates with the progressive ability of SV40 transformed human cells to form tumours in nude mice, indicating the requirement of new mutational events for development of a malignant phenotype. Cells from an early passage of a SV40-

transformed population lacked the ability to form tumours, but acquired this trait in late passages as the karyotype became more aberrant (Brown and Gallimore, 1987).

Acquisition and maintenance of marker chromosomes is a common feature in SV40-transformed cell lines (Brown and Gallimore, 1987; Marlens et al., 1988; Meisner et al., 1988; Burholt et al., 1989). For example, SV40 immortalized human foetal keratinocytes acquired marker chromosomes, as well as monosomy for chromosomes 7, 11, 15, and 18 (Brown and Gallimore, 1987). This suggests that some feature(s) found in these markers are beneficial for the development and maintenance of the immortalized phenotype. Possession of marker chromosomes may, for example, provide a growth advantage over cells lacking the markers.

Experimental evidence indicates that the early region of SV40 and its proteins, large and small t antigen are responsible for the mutagenic activity of SV40. A SV40 mutant containing a temperature sensitive large T antigen was unable to induce chromosomal aberrations when the infected cells were grown at the non-permissive temperature (Gorbunova et al., 1982). These results were confirmed by transfection of human cells with plasmids bearing only the early region of SV40. The levels of chromosomal aberrations which resulted after the expression of the tumour antigens was similar to those found in human cells transformed with SV40 (Chang et al., 1986).

Large T antigen has a number of functions which may potentially be involved in the induction of genetic instability. As mentioned, the protein can bind to specific viral and cellular DNA sequences (Prives et al., 1982; Paucha et al., 1986; Arthur et al., 1988). In addition, it is capable of promoting recombination between homologous DNA segments, by reannealing of homologous SS DNA regions, either of inserted viral DNA or of cellular DNA sequences (St-Onge et al., 1990; Schiedner et al., 1990). Lastly, large T can induce host cellular DNA synthesis (Chou and Martin, 1975; Thompson et al., 1990), which could increase the chances of non-disjunction and replicative mutagenesis.

Many of the SV40-induced rearrangements observed in human cells involve large chromosomal segments including whole arms or apparently intact chromosomes, with telomeric fusions leading to stable dicentric or pseudo-dicentric chromosomes (Wolman, 1964; Walen et al., 1986; Walen, 1987; Meisner et al., 1988). For example, human amniocytes transformed by SV40 exhibit a preferential breakage resulting in dicentric chromosomes and translocations in telomeric and centromeric regions (Walen et al., 1986; Walen, 1987). It has been suggested that this reflects an affinity or preference of the mutagenic/clastogenic activity of SV40 to act upon centromeric and telomeric regions, although the mechanism(s) used are unknown.

Additionally, induction of genetic instability by SV40 may be related to virus induced DNA amplification. A common feature of SV40 immortalized cells is the amplification of cellular DNA sequences in double minutes or homogeneously staining regions (HSR) (Nichols et al., 1985). The human genome contains high affinity binding sites for large T antigen, many of which are found in the L1 family of repetitive elements. Accordingly, these elements are often amplified in SV40 newly transformed cells (Gruss, 1988), though appear to be lost in stably immortalized cells. Amplified genes near telomeric regions may be involved in formation of end-to-end chromosomal rearrangements (such as rings), by recombining and acting as a DNA bridge to hold chromosome ends together (Nichols et al., 1985). Free ended extrachromosomal amplified sequences may also be highly recombinogenic, and could interfere with large T induced sister chromatid exchanges, resulting in recombination between the amplified and chromosomal sequences to give rise to inversions and dicentrics (Schimke et al., 1985). The presence of unbanded DNA material resembling HSR within rearranged chromosomes has been detected in human cells transformed with a temperature sensitive mutant of large T antigen (Nichols et al., 1985).

1.5 Project Goals

The present study was undertaken to answer specific questions about SV40 induced genetic instability in human cells: (1) Are both large and small t antigens of SV40 required for the induction of chromosomal aberrations and polyploidy?; and (2) What oncogene functions are involved in the induction of the genetic damage? In addition, (3) since SV40-induced cytogenetic damage may play a role in immortalization, this study examined the progression of genetic changes during the growth of transfected cells prior to crisis. The approach taken involved transfection of diploid human cell (adult skin fibroblast or fetal kidney cells) with plasmids bearing the early region of SV40, or mutants thereof, either linked to, or cotransfected with a plasmid bearing the neomycin gene. To answer the first question, human fibroblasts were used in conjunction with plasmids bearing either the wild type early region of SV40, or SV40 mutants expressing only one of the two t antigen genes. To answer the second question, human fibroblasts were transfected with plasmids bearing an origin minus SV40 mutant, or mutants of large T antigen, defective for different functions of the protein, which were originally characterised in rodent systems. Human embryonic kidney (HEK) cells and the plasmid containing the wild type early region were used to

address the third question. In all cases, control transfections were performed using a plasmid encoding only the neo gene. Transfected cells were selected on the basis of G418 resistance, to ensure that any cytogenetic damage observed did not result from the selective pressure for transformed properties. Several independent colonies were expanded from each transfected population, and the cells analyzed cytogenetically within 20 cell divisions following transfection.

This study reports the results of these experiments and their interpretation.

MATERIALS AND METHODS

2.1 Cells

Human diploid skin fibroblasts (strain 423) (Chang et al, 1986) and human embryonic kidney cells (HEK) were cultured in 100 mm dishes (Corning) and maintained in α-MEM (GibCo) supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and 10% fetal calf serum. The fibroblasts were routinely subcultured at a 1:4 ratio and used for experiments between 6 and 10 passages. HEK cells were obtained by trypsinization of embryonal kidneys and used as secondary cultures and were not subcultured, except in the case of transfected clones which were passaged at a 1:4 ratio. Vero and COS-1 cells were cultured in 100 mm dishes (Corning), and maintained in α-MEM (Gibco) supplemented with 10% newborn calf serum. Both cell types were subcultured at a 1:10 ratio.

2.2 Plasmids

pSV2neo and pSV3neo (encoding respectively the neomycin gene or the neo gene plus the SV40 early region; Southern and Berg, 1982) were originally obtained from Dr. P. Berg.

Plasmid pX-8 contains an ori⁻ mutant of SV40 (Canaani et al., 1986), and was also obtained from Dr. P. Berg. Eight SV40 early region mutants were used (Table 1a): pdl2005, contains the neo gene and the SV40 early region with a 250 bp deletion in the large T intron. This plasmid expresses only the large T antigen (Sompayrac and Danna, 1983; J. Tevethia, personal communication). pdl536, contains the SV40 mutant pdl536 cloned into the BamH1 site of pSV2neo (J. Tevethia, personal communication). dl536 can only express small t antigen due to a deletion between nucleotides 4592-2666, which includes the large T splice acceptor site (Sompayrac and Danna, 1983). K1 is a large T mutant, cloned into pBR322, with a glu(107)→lys substitution which prevents large T antigen from binding to the Rb protein (DeCaprio et al., 1988). K1 is also transformation deficient and produces delayed foci, but has normal immortalization functions (Kalderon and Smith, 1984b). dl1138, cloned into pBR322, produces the first 160 amino acids of large T, due to a 29 bp out-of-phase deletion between nucleotides 4339-4311. This deletion also results in the addition of 15 nonsense amino acids before termination of the protein (Pipas et al., 1983; Soprano et al., 1983). dl1138 T antigen is mainly cytoplasmic, unable to induce cell DNA synthesis or activate silent rRNA genes, and is negative for viral replication and host range function. Further, this mutant lacks the ability to induce focus formation in rat

embryo fibroblasts. dlA2432, also cloned into pBR322, has a 12 bp in-phase deletion between nucleotides 3299-3286, which removes 4 amino acids from large T antigen. This mutant is non-viable, negative for viral DNA replication and lacks ATPase activity (Cole et al., 1986; Tevethia et al., 1988). dlA2433, cloned into pBR322, contains a 9 bp in-phase deletion in the carboxy-terminal end of the large T gene between nucleotides 3060-3050 (missing amino acids 587-589). This mutant is less stable than wild type (three hour half life versus 18 hours for wild type T antigen), is unable to form a stable complex with p53, is negative for viral DNA replication, has low ATPase activity, and is impared in its immortalization ability (Cole et al., 1986; Tevethia et al., 1988). pY2Xmet128-70K, cloned into pBR328, contains the TaqI-BamH1 fragment (which lacks the large T initiation codon) of a SV40 mutant with an in-phase deletion in the N-terminal region nucleotides 4501-4481. These deletions remove the first 127 amino acids of large T antigen. Y2Xmet128-70K also has a base change of A to T at nucleotide 4435 which changes the lysine codon at position 128 to a methionine (Anderson et al., 1988). This mutant is reported to have an enhanced immortalization ability (Anderson et al., 1988). Finally, pSV35-neo contains a SV40 mutant cloned into pSV2neo. This mutant has a large (approximately 800 bp) internal deletion of large T antigen between nucleotides 4459-3733, and is

unable to immortalize primary rat embryo fibroblasts (Asselin et al., 1985). The locations of all of the mutants within T antigen are indicated on Figure 3. Mutant plasmids were originally obtained from J. Tevethia (pdl2005, pdl536, dl1138, dlA2432, dlA2433, pY2Xmet128-70K), A. Smith (K1), and M. Bastin (pSV35-neo). All plasmids were propagated in E. coli (strain LE392 or DH5α), and supercoiled DNA was purified by isopycnic centrifugation in cesium chloride-ethidium bromide gradients, as described in Maniatis et al. (1982).

2.3 Generation of Recombinant Plasmids

Plasmids containing the SV40 mutants dl1138, dlA2432, and dlA2433 were digested with EcoR1 for three hours at 37°C to release the viral genome, and PvuI and SalI to destroy the pBR322 backbone. All restriction digests were performed according to standard proceedures recommended by the manufacturer. The cut vector were mixed with pSV2neo (linearized with EcoR1 and treated with alkaline phosphatase (Boehringer Mannheim GmbH) according to the proceedure recommended by the manufacturer), and ligated with T4 ligase ((Bethesda Research Laboratories), in 1 mM ATP, 10 mM DTT, 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$) overnight at 14°C. One half of the ligation mixtures were used to transform competent DH5α cells, prepared and transformed by the calcium chloride

Figure 3: Location of SV40 early region mutants. Deletions are indicated by black boxes. K1 carries a substitution at amino acid 107 (corresponding to nucleotides 4498-4496) in large T antigen. The deletion in dl2005 includes nucleotides 4876-4638, which removes most of the small t unique region, and the deletion in dl536 (nucleotides 4592-2666) removes the splice acceptor site for large T antigen. The deletion (black box) in dl1138 involves a 29 bp deletion which results in a truncated large T protein (deleted portion also shown as black box). The deletions in pSV35, dlA2432, and dlA2433 involve nts 4459-3733, 3299-3286, and 3060-3050, respectively.

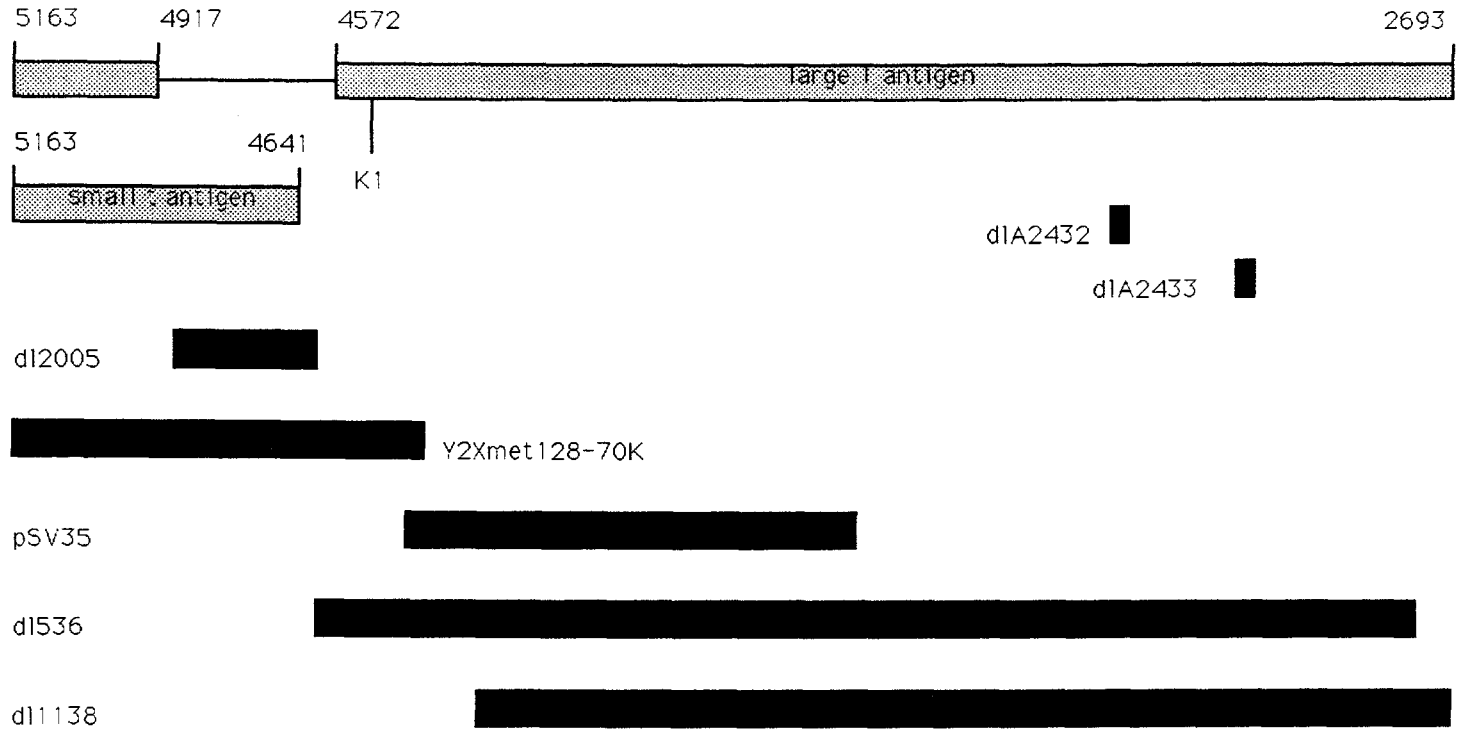

5163
4917
4572
2693
large T antigen
5163
4641
K1
small t antigen
dlA2432
dlA2433
dl2005
Y2Xmet128-70K
pSV35
dl536
dl1138

Table 1a: Map location and phenotype of SV40 early region mutants

SV40 Mutant	Defect	Phenotype**	Level of Expression*	Cytogenetic Damage*
pX-8	Mutated Origin of Replication	Negative for Viral DNA Replication	+	+
dl2005	In-phase Deletion of nts 4876-4638	Produces only large T antigen	+	+
dl536	In-phase Deletion nts 4592-2666	Produces only small t antigen	-	-
T Antigen Mutants				
K1	glu(107)→lys	Unable to Bind Rb Transformation Deficient Produces delayed foci	+	+
dlA2433	In-phase Deletion of aa 587-598	Unstable, Unable to Bind p53 Negative for Viral DNA Replication, Low ATPase Activity, Impaired Immortalization	+/-	-
dl1138	29 bp Out-of-phase Deletion removes aa 161-708	Cytoplasmic, Unable to induce Cellular and viral DNA synthesis or to Activate rRNA genes, Negative for Viral DNA Replication, host Range function, and focus formation	-	nd
dlA2432	In-phase Deletion aa 507-510	Non-Viable, Negative for Viral DNA Replication and ATPase Activity	-	nd
Y2Xmet128-70K	In-phase Deletion removes aa 1-127 lys(128)→met	Enhanced Immortalization Ability	-	nd
pSV35	In-phase Deletion aa 120-364	Unable to Immortalize primary Rat Embryo Fibroblasts	-	nd

- : Expression of viral antigens never detected
+ : Wild type expression viral antigens
nd : not determined
* : This study
** : References in text

technique, as described by Seidman (1989). Selection for transformed colonies was with Ampicillin or Kanamycin at a concentration of 40 ug/ml and 50 ug/ml respectively, in Luria agar plates. Several surviving colonies were grown up and used for small scale analytical preparations of plasmid DNA, as described by Birnboim and Doly (1979). All recombinants were characterized by digestion with restriction enzymes EcoR1, BglI and BglII, and electrophoresed in a 0.7% agarose gel. The appropriate clones were then grown up and used for large scale plasmid preparations, as described above.

2.4 Transfections

2.4.1 Preparation of Carrier DNA

Carrier DNA was prepared by pronase digestion of 423 cells, followed by chloroform-phenol extraction of the cell lysate. The DNA was then dialysed against 0.1 x SSC to remove any traces of phenol (Graham and Bacchetti, 1983).

2.4.2 Transfection

Strain 423 and HEK cells were transfected with plasmid DNA using the calcium phosphate coprecipitation technique of Graham and van der Eb (1973). For both cell types, cells were seeded at approximately 2.5 x 10^5 cells per 100 mm dish and

were transfected 24 hours later with 20-50 ug/dish of plasmid DNA and high molecular weight carrier DNA from strain 423 fibroblasts. The cells were incubated for either six hours or overnight in the presence of the DNA-calcium phosphate precipitate, then refed with fresh medium. 48 hours after transfection, they were subcultured at a 1:3 ratio in medium containing G418 (Geneticin, GibCo) at an effective dose of 400 ug/ml. The selective medium was changed at approximately weekly intervals until colonies could be picked with cloning cylinders and expanded. All clones analysed in the present study were independently generated.

2.5 Cytogenetic analysis

Chromosome spreads from human fibroblasts were prepared by seeding cells onto coverslips at a ratio of 1:4 or 1:8. After 24 or 48 hour growth, the cells were arrested in metaphase by treatment with colcemid (0.1 ug/ml) for 3-5 hours. The coverslips were then immersed in prewarmed hypotonic solution (0.075 M KCl) and incubated at 37°C for 15-20 minutes. Fixation was for twice 15 minute with cold (-20°C) methanol:acetic acid (Carnoy) (3:1 v/v). HEK cells were seeded in plates and harvested by trypsinization. Hypotonic treatment was for 15-20 minutes at 37°C, followed by fixation with methanol:acetic acid (3:1 v/v) for a minimum of

1 hour. Cells were given a quick rinse in methanol:acetic acid (1:1 v/v) and then placed back into Carnoy. Chromosomes preparations were by the drop method, onto dry slides washed in 100% ethanol. Chromosomes prepared by either method were stained with 5% Giemsa stain for 5 minutes at room temperature and analysed by light microscopy. For all cytogenetic analysis, frequency of chromosomal aberrations and ploidy were determined by scoring 50 metaphases from each clone. Chromosome spreads used for G-banding were prepared as above, and stained according to standard protocol.

2.6 Immunolabelling of Cells

For immunolabelling, cells were grown on coverslips to 50-70% confluency. In all experiments, COS-1 cells, which constitutively express both SV40 tumour antigens (Gluzman, 1981) were used as a positive control. Following a wash with PBS and fixation with acetone at -20°C, the coverslips were mounted onto slides. The cells were reacted for 1 hour with the mouse monoclonal antibody 419 which reacts within the first 82 amino acids of large T and small t antigens (Harlow et al, 1981), washed twice for 10 minutes in 1x TBS (1M Tris, 2M NaCl), then reacted for 1 hour with a rabbit anti-mouse antibody conjugated to horseradish peroxidase (Dimension Laboratories Inc. (DAKOPATTS a/s)). The cells were then

stained for 10 minutes in AEC substrate solution (5% AEC (4% 3-amino-9-ethylcarbazole in NN-dimethylformamide) in 20mM Na acetate, 50 mM acetic acid plus one drop of 30% hydrogen peroxide) and counterstained for 5 seconds in haematoxylin. A second coverslip was mounted with aquamount on top of the first to prevent the samples from drying, and the cells analyzed by light microscopy. In some experiments Mab 412 reacting with the carboxy terminal 26% of T antigen was used (Gurney et al., 1980).

2.7 Immunoprecipitation of cells

2.7.1 Labelling and Extraction of Proteins

For immunoprecipitations, cells were grown in 60 mm dishes to approximately 75% confluence before labelling. Cells were labelled for the indicated amount of time with 2.0 ml of 199-methionine free medium (Gibco) supplemented with 1% dialysed FCS, or if the label was overnight, 10% of the normal culture medium, plus 200 uCi of[^{35}S]-methionine (Amersham Corp., Arlington Heights, Ill; specific activity, 1300 Ci/mmol). After labelling, the radioactive medium was removed and the cells rinsed twice with cold PBS. Cell pellets were harvested by scraping into PBS with a plastic scraper, then pelleted by centrifugation (1000 rpm for 10 minutes), and the PBS aspirated off. Cell pellets were resuspended and lysed

for 30 minutes in 500 ul of RIPA buffer plus proteolytic inhibitors (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Na dodecyl sulfate (SDS), 1% Na deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (Sigma), and 0.11 trypsin inhibitor units of aprotinin (Sigma) per ml). The samples were then sonicated twice for 5 seconds each, and centrifuged at 15000 rpm for 30 minutes at 4°C. Following centrifugation, the supernatant was collected into another tube and the pellet discarded.

2.7.2 Immunoprecipitation of Proteins

500 ul aliquots of cell lysates were reacted with 5-10 ul of Mab 419 and/or Mab 412 (which binds to the carboxy terminus of T antigen (Gurney et al., 1980)), plus 500 ul of RIPA buffer plus inhibitors, in the presence of 200 ul of protein A-Sepharose beads (Pharmacia) (resuspended at a concentration of 10% in RIPA buffer), for 2 hours at 4°C. Immunocomplexes were collected by centrifugation and washed a minimum of three times in RIPA buffer minus proteolytic inhibitors. Samples for SDS-PAGE analysis were resuspended in 50 ul of 2x Laemmli buffer (100 mM Tris, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue), and boiled for 3 minutes. The samples were then centrifuged to pellet the

beads, and 5 ul of the supernatant removed for quantitation ofradioactivity in a Beckman liquid scintillation counter.

2.7.3 One Dimensional Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to Laemmli (1970), using 12% polyacrylamide separating gels, and stackings gel containing 5% polyacrylamide, both with a ratio of acrylamide to N-N'bismethylene acrylamide of 30 to 0.8. 45 ul of the prepared samples were loaded on the gels, which were run at 80V until the dye front was near the bottom (approximately 15-18 hours). The gel was trimmed to remove the dye front and washed in DMSO, then fluorographed using PPO-DMSO (Bonner and Laskey, 1974), washed and soaked in ddH_2O, and dried for 2 hours at 60°C. Autoradiography was at -70°C on Kodak XK-1 film.

2.8 Western Immunoblot

Preparation of the protein samples for western blot analysis was done by one of two methods. The first followed the preparation method described above, with cells harvested by trypsinization, and without radioactive labelling of the cells prior to harvesting. Samples from this method were

quantitated on the basis of cell number. For the second method, cell pellets were collected by trysinization into PBS and centrifuged for 10 minutes at 1000 rpm. The PBS was removed and the cell pellets resuspended in 200 ul of 0.1 M Tris and freeze-thawed 4 times, then centrifuged for 30 minutes at 4°C. Protein levels in the supernatant were quantitated by the Bio-Rad protein assay. 2x RIPA was added to the samples after quantitation. For both methods, SDS-PAGE electrophoresis was as described above.

After electrophoresis the gel was first equilibriated for 1 hour in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol [v/v]), then assembled into a Bio-Rad Transblot Cell with a nitrocellulose membrane (Bio-Rad) and 6 sheets of Whatmann 3 mm paper pre-soaked in transfer buffer. The transfer was at 4°C at 70V for a minimum of 4 hours. After transfer, the nitrocellose membrane was dried in air and stored at 4°C until required. The dried membrane was rehydrated and blocked in buffer A (0.05% Carnation instant skim milk [wt/v], 170 ul 30% Antifoam A [Sigma], 5 mls 5% Tween 20 [Polyoxyethylene Sorbitol Monolaurate, Sigma], and 0.5 ml 0.1% Trimersol [Sigma] made up to 500 ml with ddH20) for 45 minutes. After blocking, the blot was incubated for 90 minutes with Mab419 diluted 1:500 in buffer A, then washed 4 times 5 minutes with buffer A. The blot was then incubated for 1 hour with alkaline phosphatase conjugated goat anti-

mouse IgG (Bio Can Scientific Inc.), diluted 1:5000 in buffer A, then washed 2 times 5 minutes with buffer A, and 2 times 2 minutes with borate buffer (0.93 g/l, pH 9.5). Alkaline phosphatase colour reaction was performed by incubating the blot in developer mixture (45 ml borate buffer [pH 9.5], 5 ml 0.1% NBT in borate buffer, 0.5 ml 5 mg/ml BCIP in dimethylformamide, 100 ul 2M $MgCl_2$), for up to 2 hours, after which the colour reaction was stopped by washing the blot in ddH2O.

RESULTS

3.1 Cytogenetic Analysis of Clonal Cell Populations Expressing the SV40 Early Region.

Initial experiments were aimed at assessing the amount and type of cytogenetic damage in human cells expressing the wild type SV40 tumour antigens. To this end, human diploid skin fibroblasts (strain 423) (Chang et al., 1986), were cultured from frozen stocks as required, and used for transfections between passages six and ten. The cells were seeded and transfected, as described in Materials and Methods, with either 30 ug of the pSV3neo plasmid, which contains the wild type early region of SV40 (T^+, t^+, ori^+) linked to the neo gene, or 30 ug of the control pSV2neo plasmid, encoding only the neo gene. This gene codes for the bacterial phosphotransferase APH(3')II, which inactivates the aminoglycoside G418 (Davies and Smith, 1978; Southern and Berg, 1982). Following selection with G418 for expression of this marker, eight independent colonies from cultures transfected with pSV3neo and six independent colonies from cultures transfected with pSV2neo were isolated and expanded. For this, and all subsequent experiments, isolated colonies

were transferred to a 25 cm^2 Corning bottle and grown to confluence (designated passage 1). Immunostaining and cytogenetic analysis of transfected cells, as described in Materials and Methods, were performed after the cells had been subcultured once at a ratio of 1:4 (passage 2, corresponding to approximately 20 divisions of the parental cell). This was the earliest stage at which a sufficient number of cells were present to allow analysis of the colonies.

The results of the immunostaining of the 8 pSV3neo and 6 pSV2neo transfectants are given in Figure 4, and Table 2. As expected, pSV2neo transfected cells (Figure 4A) maintained their fibroblast morphology, and were negative for expression of the SV40 tumour antigens as determined by their lack of reactivity with the Mab 419 antibody (see also Table 2, clones FA1-6). These cells also retained the growth rate and pattern of the untransfected parental fibroblasts (data not shown). pSV3neo transfectants (Figure 4B), on the other hand, had altered (epithelioid) morphology and were all found positive for expression of the tumour antigens by immunostaining with Mab 419. All of the cells in each pSV3neo clone expressed these tumour antigens. Additionally, a fairly consistent intensity of stain was observed within each clonal population, and between different clones (Table 2, clones FB1-8). All of the pSV3neo clones grew at a faster rate and to higher

Figure 4: Expression of SV40 tumour antigens in transfected cells. Clonal populations selected after transfection of fibroblasts with: A, pSV2neo; B, pSV3neo (T^+, t^+); C, pdl2005 (T^+, t^-); or of HEK cells with pSV3neo (D), were immunostained with monoclonal antibody 419 which recognizes both large and small tumour antigen.

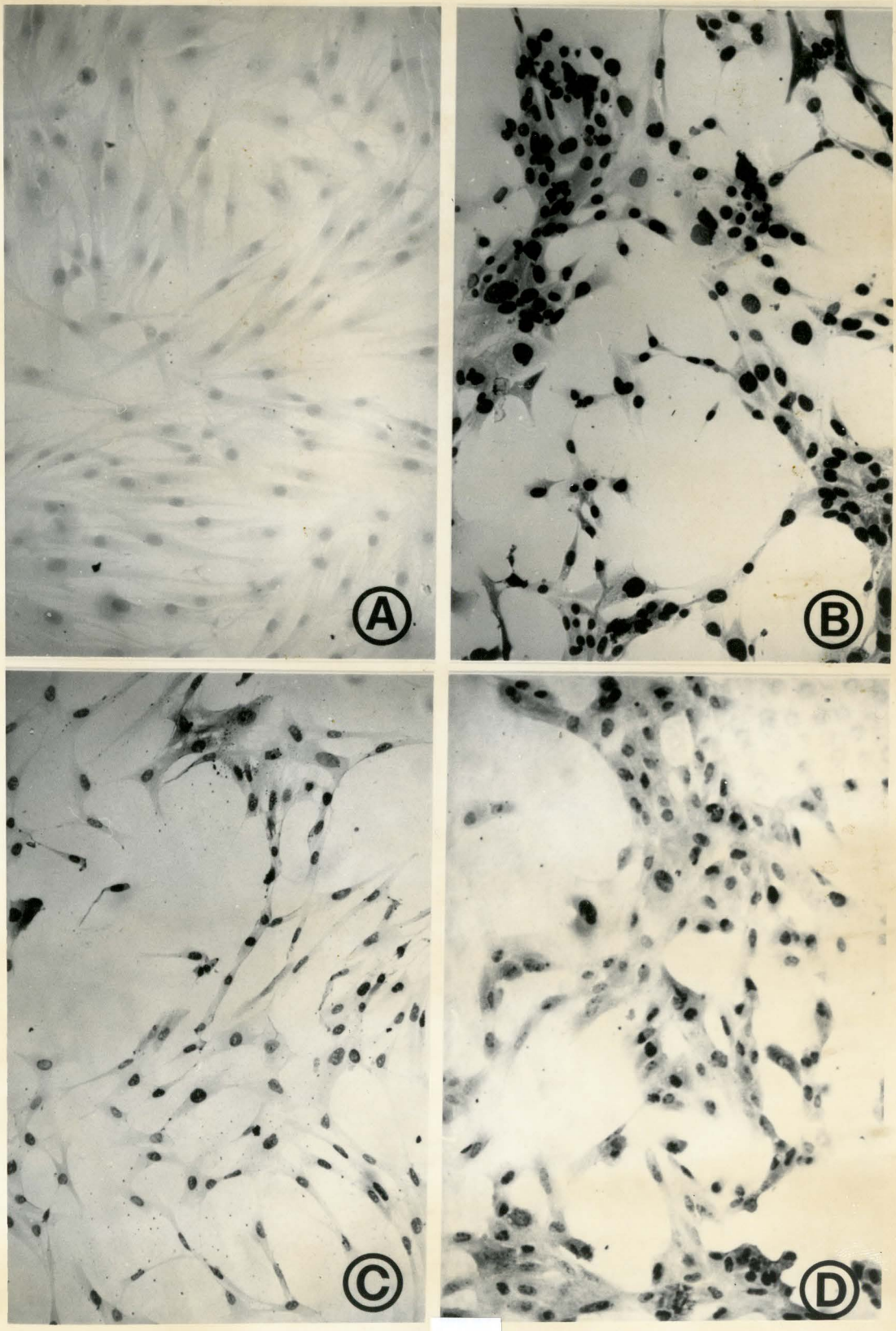

A
B
C
D

Table 2: Levels of Reactivity to Mab 419, as determined by immunostaining

Clone	Transfecting Plasmid	Intensity of stain *
FA1	pSV2neo	-
FA2	"	-
FA3	"	-
FA4	"	-
FA5	"	-
FA6	"	-
FB1	pSV3neo	++
FB2	"	++
FB3	"	++
FB4	"	++
FB5	"	++
FB6	"	++
FB7	"	++
FB8	"	++
FC1	pdl2005	++
FC2	"	++
FC3	"	++
FC4	"	++
FD1	pX-8	++
FD2	"	++
FD3	"	++
FD4	"	++
FD5	"	++
FE1	K1	++
FE2	"	++
FF1	pdlA2433	+
FF2	"	+
FF3	"	++
FF4	"	+
HA1	pSV3neo	++
HA2	"	++
HA3	"	++
HA4	"	++
HA5	"	++

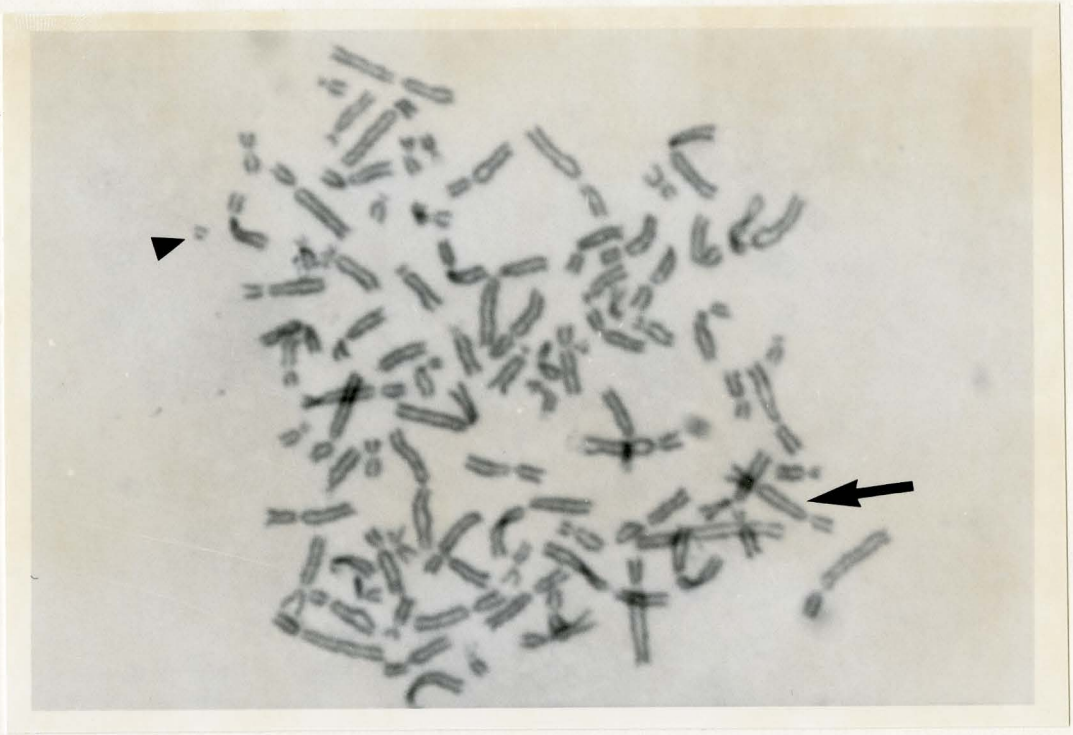
* evaluated by comparison with Cos-1 cells (++). Unless otherwise indicated in Results, 100 % of the cells in the population reacted with the Monoclonal antibody 419. (-) = undetectable

saturation densities than the untransfected parental cells (data not shown).

For cytogenetic analysis ploidy and frequency and type of chromosomal aberrations were determined by scoring 50 metaphases from each clone. Examination of metaphases with aberrations are shown in Figure 4a and quantitative results for individual clones are presented in Table 3. pSV2neo transfectants retained an essentially normal diploid karyotype: depending upon the clone, polyploid (±4N) cells amounted to 0-4% of the total scored, and structural aberrations, consisting primarily of chromatid breaks and fragments, were observed in a maximum of 20% of the cells. On the other hand, pSV3neo transfectants contained a high fraction of polyploid cells (22-100%) and of structural aberrations (in 42-82% of the cells). Among the aneuploid cells the majority were ±4N but a significant percentage (0-10%) had ±8N complements. The largest proportion of structural aberrations detected consisted of dicentrics, fragments and double minutes; occasional chromosome and chromatid breaks, rings, and translocations or duplications were also observed. The average frequency of aberrations for each group of clones (obtained from the data presented in Table 3), is shown in Figure 5. Both the frequency of polyploid cells (open bars) and of cells with structural aberrations (shaded bars) in the pSV2neo clones were

Figure 4a: Metaphase spreads from FB (pSV3neo) fibroblast clones passage 2.

Large arrows: dicentric chromosome
small arrow: double minute
clear arrow: novel marker chromosome
triangle: fragments



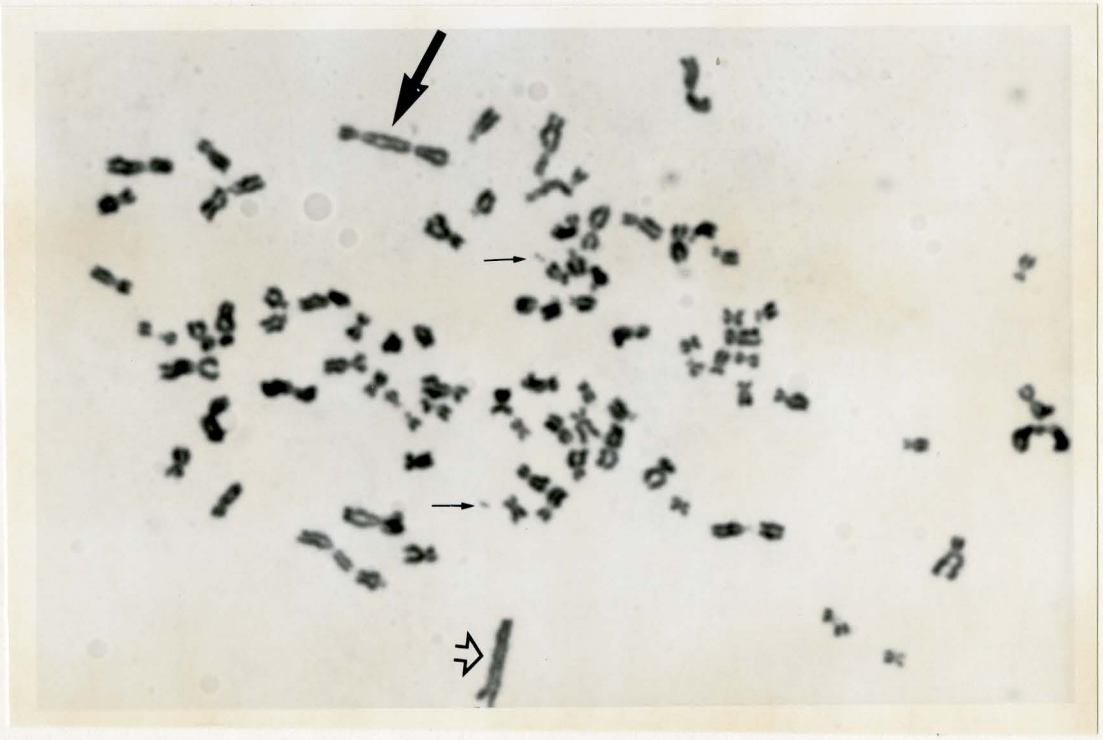
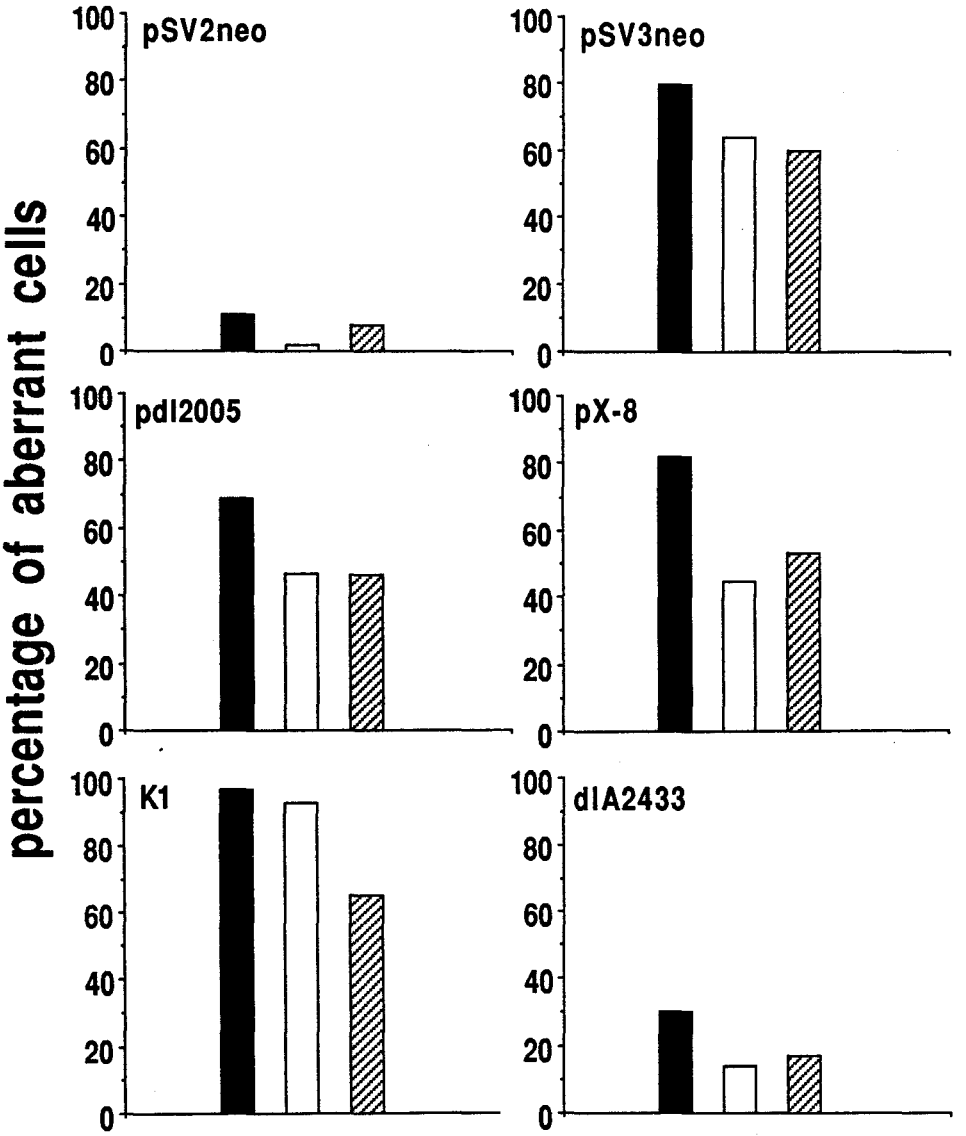


Table 3. Cytogenetic analysis of clonal populations generated by transfection of fibroblasts with pSV2neo and pSV3neo

	pSV2neo clones						pSV3neo clones							
	FA1	FA2	FA3	FA4	FA5	FA6	FB1	FB2	FB3	FB4	FB5	FB6	FB7	FB8
	Percentage of Cells *													
2N	100	98	98	96	100	96	56	78	58	6	0	18	74	72
±4N, ±8N	0	2	2	4	0	4	44	22	42	94	100	82	26	28
with structural aberrations*	20	4	0	4	14	6	58	58	44	50	82	68	42	46
	Number of Aberrations / 50 cells *													
breaks	8	0	0	2	4	1	5	4	6	1	2	4	10	8
fragments	1	0	0	0	2	1	21	11	10	21	7	4	14	11
double minutes	1	0	0	0	0	0	21	8	5	17	27	15	7	4
di/tricentrics	0	2	0	0	1	2	20	22	12	11	41	40	8	10
rings	0	0	0	0	0	0	1	0	0	0	0	0	1	0
translocations/ duplications	0	0	0	0	0	0	4	0	0	1	5	3	0	0
total	10	2	0	2	7	3	72	45	34	51	82	66	40	33

* = The percentage of cells containing structural aberrations or polyploidy, and the number of aberrations per 50 cells were determined by scoring up to 50 metaphases per clone.

Figure 5: Cytogenetic analysis of clonal populations generated by transfection of fibroblasts with SV40 plasmids. The average frequency of total aberrant cells (solid bars), of polyploid cells (open bars) and of cells with structural aberrations (shaded bars) for each type of clone was obtained from the data presented in Tables 3, 4, and 6.


percentage of aberrant cells
pSV2neo
pSV3neo
pdl2005
pX-8
K1
dlA2433
100
80
60
40
20
0

significantly (by a factor of at least 5) lower than that observed in the pSV3neo clones. These results confirm and extend those of others (Chang et al., 1986; Ray et al., 1990), and verify that the induction of cytogenetic damage in the cells correlates with the expression of the SV40 tumour antigens.

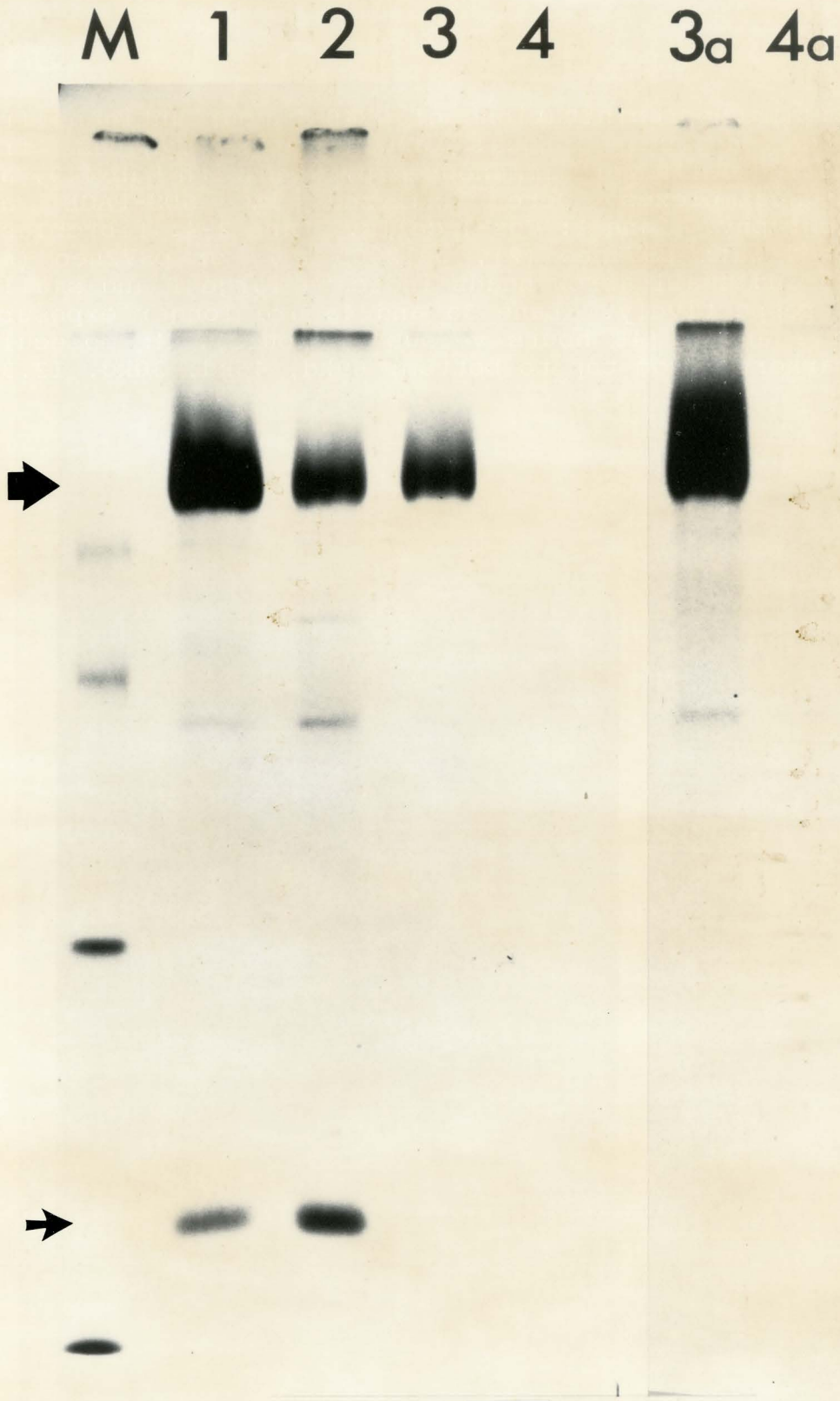
3.2 Role of Large T and Small t in the Induction of Cytogenetic Damage

At the onset of this study, it was not known whether the induction of cytogenetic damage required the expression of both SV40 tumour antigens, or if either antigen on its own was sufficient for this process. As mentioned in the Introduction, large T antigen is a multifunctional protein which is required for viral DNA replication, for both initiation and maintenance of SV40-induced immortalization of human cells, for complete cell transformation, and for the induction of cytogenetic damage (Sack, 1981; Canaani et al., 1986; Chang et al., 1986; Monier et al., 1986; Bikel et al., 1987; Radna et al., 1989). Recent evidence has indicated that small t antigen is required at least for morphological transformation of human cells by SV40 (DeRonde et al., 1989). It remains possible however, that small t antigen also has

multiple functions, and could be involved in the induction of aberrations.

To investigate the role of each tumour antigen in SV40-induced genetic damage, additional clones were generated by transfection of fibroblasts with 30 ug of plasmid pdl2005. This plasmid contains the neo gene and a mutated SV40 early region expressing only large T antigen due to a 250 bp deletion in the intron of the large T gene (Sleigh et al., 1978; Thompson et al., 1990). This experiment generated many colonies which, though surviving selection, were however not positive by immunostaining with Mab 419. Additional colonies contained only a low percentage of cells (10-20% within a clone) which reacted with the antibody. Nevertheless, four independent colonies in which 100% of the cells reacted with Mab 419 could be expanded and analyzed (Table 2, FC1-FC4). To certify that these cells expressed large T but not small t, cells from a representative clone (FC3) were labelled for 15 hours with [^{35}S]-methionine, and cell lysates immunoprecipitated and electrophoresed as indicated in Materials and Methods. The results, shown in Figure 6, indicate that FC3 cells (lane 3), expressed large T antigen in amounts comparable to those detected in pSV3neo transfectants (lane 2), but did not express small t antigen. Even upon longer exposure of the autoradiograph (lane 3a), small t antigen could not be detected. The pdl2005 clones appeared

Figure 6: Immunoprecipitations of SV40 tumour antigens from transfected cells. Equal amounts of lysates from radiolabelled cells were immunoprecipitated with Mab 419, and the immunocomplexes were analyzed by SDS-PAGE and autoradiography. Lane 1: Cos-1 cells. Lane 2-4: fibroblasts transfected with pSV3neo (2), pdl2005 (3), or pSV2neo (4). The large and small arrow heads indicate large T and small t antigen, respectively. Lane 3a and 4a are longer exposures of lanes 3 and 4 (48 hours versus 24 hours). M: molecular weight markers (from top to bottom) 69kd, 46kd, 30kd, 12.3kd


M
1
2
3
4
3a
4a

different from the parental 423 cells, in that they were more epithelial in nature and were arranged in a more disorganized pattern (Figure 4C). In both respects, however, they were not altered to the extent seen in the pSV3neo transfectants. They were nonetheless comparable to the latter clones in terms of their growth rate (data not shown).

Results from the cytogenetic analysis of the individual pdl2005 clones are shown in Table 4, and the average frequency of aberrations for each group of clones is shown in Figure 5. The pdl2005 clones were not significantly different from pSV3neo clones. Between 32 and 60% of the cells had 4N or 8N complements, and 30-60% also had structural aberrations, among which the most predominant were again dicentrics, fragments and double minutes. These results indicated that expression of large T antigen was sufficient for the induction of cytogenetic damage. Absence of small t antigen expression in these clones appeared to have little effect on the frequency of aberrations induced by large T antigen. This suggests that small t might not contribute to this process.

In an attempt to verify this hypothesis, fibroblasts were also transfected with pdl536-neo, a plasmid which contains the SV40 mutant pdl536 cloned into the BamH1 site of pSV2neo (J. Tevethia, personal communication). pdl536 can only express small t due to a deletion between nucleotides 4592-2666, which includes the large T splice acceptor site

Table 4: Cytogenetic analysis of clonal populations expressing early region mutants pd12005 or pd1536

	d12005 clones				pd1536 clones				
	FC1	FC2	FC3	FC4	FG1	FG2	FG3	FG4	FG5
	Percentage of Cells *								
2N	68	40	62	50	96	100	98	100	100
± 4N, ± 8N	38	60	38	50	4	0	2	0	0
with structural aberrations	30	60	42	52	6	2	3	2	5
	Number of aberrations / 50 cells *								
breaks	6	21	11	12	6	1	2	2	5
fragments	17	43	16	24	0	0	0	0	0
double minutes	1	5	10	6	0	0	0	0	0
di/tricentric	10	18	3	13	0	1	1	0	0
ring	0	0	0	0	0	0	0	0	0
translocation/ duplication	0	1	0	0	0	0	0	0	0
total	34	90	40	58	6	2	3	2	6

* = Determined by scoring up to 50 metaphases per clone

(Sompayrac and Danna, 1983). After selection with G418, five colonies were isolated and expanded. Their morphology and growth characteristics were similar to the parental fibroblasts, and they were found to retain normal diploid karyotypes in at least 84% of the cells (Table 4). However, screening of these clones for reactivity with Mab 419 by immunostaining, western blot or immunoprecipitation never detected the presence of small t (data not shown). Possibly the level of small t antigen was too low to be detected by these methods. Low levels of expression by this plasmid have been observed by others (L. Sompayrac, personal communication). In any case, the inability to detect small t antigen expression in these clones makes the cytogenetic data uninterpretable.

3.3 Effect of Viral DNA Replication on T Antigen-Dependent Cytogenetic Damage.

Having established that expression of large T antigen was sufficient for the induction of cytogenetic damage, the potential involvement of T antigen-dependent viral DNA replication was investigated. A previous study by Canaani et al. (1986) used SV40 DNA with a defective origin of replication to generate two immortal human cell lines. Karyotypic analysis of these cells revealed the existence of

Table 5: Cytogenetic analysis of clonal populations transfected with the origin defective pX-8 plasmid

	pX-8 clones				
	FD1	FD2	FD3	FD4	FD5
	percentage of cells *				
2N	76	62	70	24	46
±4N, ±8N	24	38	30	76	54
with structural aberrations	46	64	50	56	48
	Number of aberrations / 50 cells *				
breaks	4	19	11	23	15
fragments	11	32	15	32	14
double minutes	13	7	4	5	5
di/tricentrics	8	23	11	6	6
rings	0	0	0	0	2
translocations/ duplications	0	0	0	0	0
total	36	82	41	66	42

* = Determined by scoring up to 50 metaphases per clone

very few chromosomal aberrations. This suggested the possibility that lack of viral DNA replication (particularly of amplification and excision of integrated viral DNA, which can occur with wild type SV40 in semi-permissive human cells (Zouzias, 1980)), might abrogate induction of aberrations.

Experiments to test this possibility involved the use of a plasmid, pX-8, which bears an ori$^-$ mutant of SV40. Human fibroblasts were cotransfected with equal amounts (20 ug each) of the pX-8 ori$^-$ plasmid and pSV2neo. After selection with G418, 5 independent colonies were expanded and analyzed. All five colonies were found 100% positive for expression of the tumour antigens by immunostaining with Mab 419 (Table 2, FD1-FD5).

The results of the cytogenetic analysis for the individual clones are given in Table 4, and for the average frequency of aberrations for the group in Figure 5. The frequency and type of numerical and structural changes observed did not differ significantly from those seen in pSV3neo transfected cells. Thus, these results indicated that T-antigen-dependent viral DNA replication was not required for the induction of cytogenetic damage, and suggested the involvement of other properties of the viral protein.

3.4 T Antigen Functions Involved in Cytogenetic Damage

In order to address the question of which of the functions of large T antigen are involved in the cytogenetic effect, several plasmids bearing SV40 mutants with either deletion or point mutations in the large T gene were used in this study. The phenotypes of these mutants were as follows (see also Materials and Methods): dl1138 produces only the first 160 amino acids of large T. This mutant is mainly cytoplasmic, unable to induce cell DNA synthesis or activate silent rRNA genes, and is negative for viral replication and host range function. Further, it lacks the ability to induce focus formation in rat embryo fibroblasts (Pipas et al., 1983; Soprano et al., 1983).; dlA2432 is a non-viable mutant, negative for viral DNA replication and lacking ATPase activity (Cole et al., 1986; Tevethia et al., 1988); dlA2433 lacks the ability to complex with p53, has low ATPase activity, and is negative for viral DNA replication and for the ability to immortalize rodent cells (Cole et al., 1986; Tevethia et al., 1988); pY2Xmet128-70K, is reported to have an enhanced immortalization ability (Anderson et al., 1988); finally, K1 is unable to bind both the retinoblastoma protein and a 118/120 kd cellular protein, has reduced transformation

ability, but can still immortalize rodent cells (Kalderon and Smith, 1984; Ewen et al., 1989).

Initially, in an effort to improve the likelihood of both neo and mutant SV40 sequences being transfected into the fibroblasts, an attempt was made to link the sequences from some of the mutants to the neo gene. The DNA sequences for three mutants, dl1138, dlA2432, and dlA2433 were cut out of their original plasmids using EcoR1, and ligated into the EcoR1 site of pSV2neo, as indicated in Materials and Methods (data not shown). The new constructs, designated pSV2neo-dl1138, pSV2neo-dlA2432, and pSV2neo-dlA2433 were used to transfect fibroblast cells as above. Unfortunately, colonies which survived selection with G418 did not appear to be expressing the viral oncogenes, as determined by both immunostaining with Mab 419, and for pSV2neo-dl1138 and pSV2neo-dlA2432, immunoprecipitation with Mab 419 and Mab 412 (data not shown). One pSV2neo-dlA2432 colony appeared to be expressing small t antigen, but not large T.

Although it seemed unlikely for all three recombinants, these results may have reflected a problem with the construction of the recombinant plasmids. Rather than exploring this possibility, however, subsequent experiments attempted to achieve expression of the mutants in fibroblasts by cotransfection of either dl1138, dlA2432, or Y2Xmet-70K with pSV2neo. Mutant plasmids and pSV2neo were set up in

either a 2 : 1 ratio or equal molar amounts. Again, however, none of the colonies which survived selection reacted with either Mab 419 or Mab 412. Only one of the mutants (dlA2433) has been reported as being unstable, thus the reason the mutant proteins were not expressed is unknown.

At the same time, transfections were also done with pSV35, which already contained the neo gene. pSV35 has a large (approximately 800 bp) deletion in the interior of large T antigen between nucleotides 4459-3733, and is unable to immortalize primary rat embryo fibroblasts (Asselin et al., 1985). This mutant, in the present study, rarely produced colonies which survived the selection process. The few colonies which did, grew extremely poorly and failed to express the viral oncogenes, as determined by immunostaining with both Mab 419 and 412. Cotransfections with pSV2neo also failed to produce any colonies. The repeated failure of pSV35 to produce colonies may indicate that either the plasmid construct, or the mutant protein itself may be toxic to human cells.

Further experiments with K1 and dlA2433 proved to be more successful. Cotransfections with the K1 or dlA2433 plasmids and pSV2neo were set up as previously described. Two K1 colonies, after selection and expansion, tested 100% positive for tumour antigen expression when examined by immunostaining with Mab 419 (Table 2, FE1-FE2). These cells were similar to

the pdl2005 transfectants, being altered in morphology and growth characteristics relative to the parental cells, but not as much as the pSV3neo transfectants (data not shown). Four colonies arose from cotransfection of dlA2433 with pSV2neo, which after selection tested 80-90% positive in the immunostaining assay (Table 2, FF1-FF4). One of the four (FF4) grew extremely poorly and did not yield sufficient metaphases for cytogenetic analysis. Of the remaining three, two grew at a slightly slower rate than the pSV2neo transfectants, while one grew at a rate comparable to them. All exhibited morphology similar to the pSV2neo transfectants (Figure 9B, see also Figure 4A).

The results of the cytogenetic analysis for individual K1 clones are given in Table 6, and the average frequency of aberration in Figure 5. Both K1 clones exhibited aneuploidy and chromosomal aberrations at levels similar to those observed in the pSV3neo fibroblasts (cf. Table 3). As mentioned, the mutation in K1 prevents binding of large T antigen to the retinoblastoma and 118/120 kd proteins. Thus, these results indicated that this binding is not required for induction of genetic instability by large T antigen in human cells. Additionally, while both K1 and dl2005 transfectants exhibited aberrant karyotypes, neither exhibited morphological changes to the same extent as observed with the pSV3neo transfectants (data not shown). This suggests that

Table 6: Cytogenetic analysis of clonal populations expressing large T mutants K1 or dlA2433

	K1 clones		dlA2433 clones		
	FE1	FE2	FF1	FF2	FF3
	percentage of cells *				
2N	10	4	80	84	94
±4N, ±8N	90	96	20	16	6
with structural aberrations	58	72	18	20	14
	Number of aberrations / 50 cells *				
breaks	15	19	3	6	6
fragments	27	27	3	2	1
double minutes	2	12	1	0	0
di/tricentrics	5	13	0	1	0
rings	1	0	0	0	0
translocations/ duplications	2	1	0	0	0
total	58	84	9	10	7

* = Determined by scoring up to 50 metaphases per clone

alterations in morphology might be independent of the induction of cytogenetic damage by large T antigen.

In contrast to the K1 mutant, dlA2433 was impaired in its ability to induce cytogenetic damage (Table 6 and Figure 4). dlA2433 transfectants exhibited 6-20% polyploidy (0-2% ±8N), a level slightly higher than that detected in the pSV2neo clones (Table 3). The levels of structural aberrations in the dlA2433 transfectants, ranging from 14-20%, however, was comparable to the latter clones. These aberrations were mainly chromosome and chromatid breaks and gaps. The decreased ability of dlA2433 to induce cytogenetic damage suggested that one, or more of the functions affected by the dlA2433 mutation in T antigen might be important for the induction of genetic instability. Additionally, these functions of large T antigen affected by the mutation may also be important for induction of cell proliferation, since the dlA2433 mutant had a limited ability to enhance the growth properties of the fibroblasts.

3.5 Expression of Tumour Antigens by K1 and pdlA2433

To verify that the amount of large T antigen in the K1 clones was similar to that in pSV3neo transfectants, and to discount the possibility that the lack of cytogenetic damage in the dlA2433 clones was due to decreased levels of this

protein, it seemed necessary to determine the level of large T antigen expression in these transfectants. A comparison was made between the results from the immunostaining analysis done on the K1 and dlA2433 clones, and the equivalent analysis done on the pSV3neo clones. This was possible as the positive controls (COS-1 cells) in all three experiments exhibited comparable reactivity to Mab 419. The intensity of staining with Mab 419 in the K1 transfectants was comparable to that observed with the pSV3neo transfectants (Table 2). This suggested that both sets of clones expressed similar levels of the viral oncogenes. To confirm this, cell pellets from the K1 clones and a representative pSV3neo clone were collected and quantitated for amount of total protein by the Bio-Rad assay, and used for quantitative western analysis, as described in Materials and Methods. Relative levels of T antigen expression were determined by visual inspection of the stained gel.

The results of this analysis are shown in Figure 7. Both K1 clones (lanes 1 and 2) appear to express levels of large and small t antigen, comparable to or higher than the wild type pSV3neo transfectant (lane 3). These clones were further analyzed by immunoprecipitation with Mab 419, following a three hour label of the cells with [^{35}S]-methionine (Figure 8A). Again, both clones (FE1 and FE2) appear to express levels of both large and small t antigen comparable to the

Figure 7: Western blot analysis of SV40 tumour antigens in K1 and dlA2433 clones. Equal amounts of protein from fibroblasts transfected with either K1 or dlA2433 were electrophoresed and the protein electroblotted to a nitrocellulose membrane. The proteins were immunostained with the monoclonal antibody 419. The large and small arrow heads indicate large T and small t, respectively. M: molecular weight markers (from top to bottom) 180 kd, 116 kd, 84 kd, 58 kd, 48.5 kd, 36.5 kd, 26.6 kd

		clone	Transfecting Plasmid
Lane	1	FE1	K1
	2	FE2	K1
	3	FB7 (wt)	pSV3neo
	4	FF2	pdlA2433
	5	FF3	pdlA2433

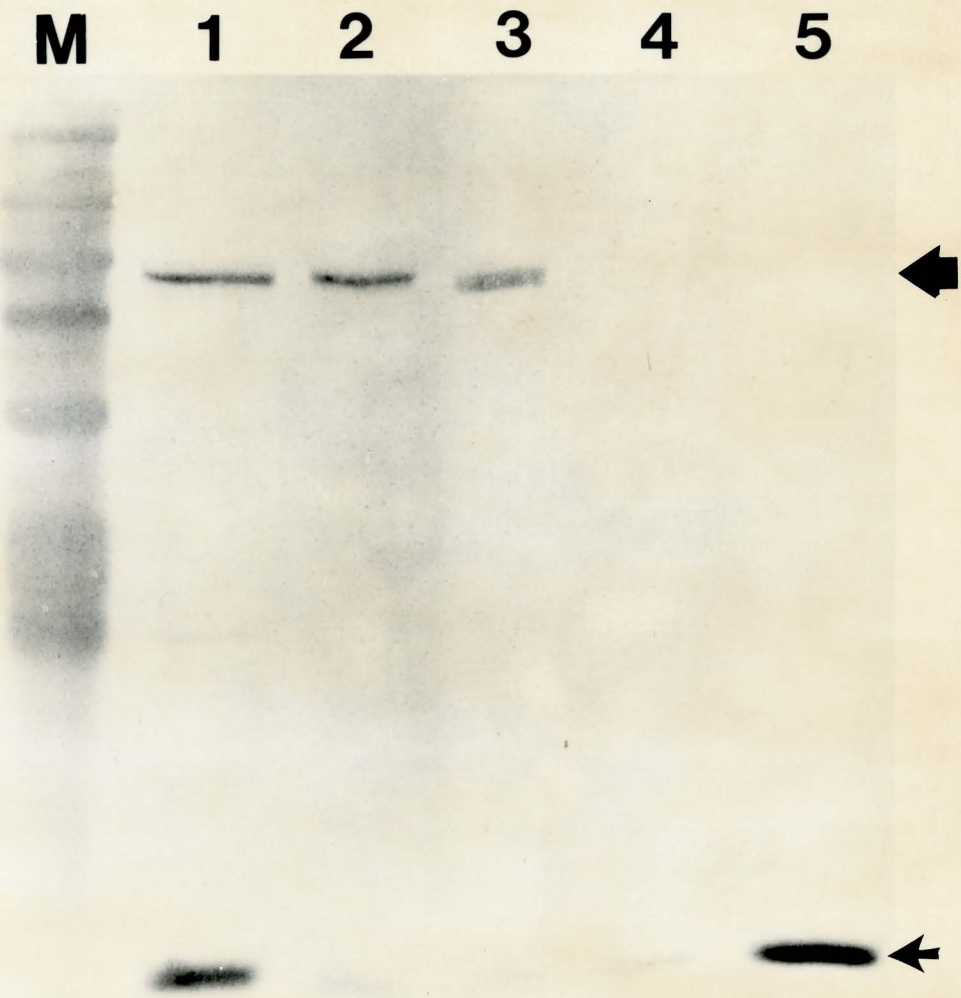
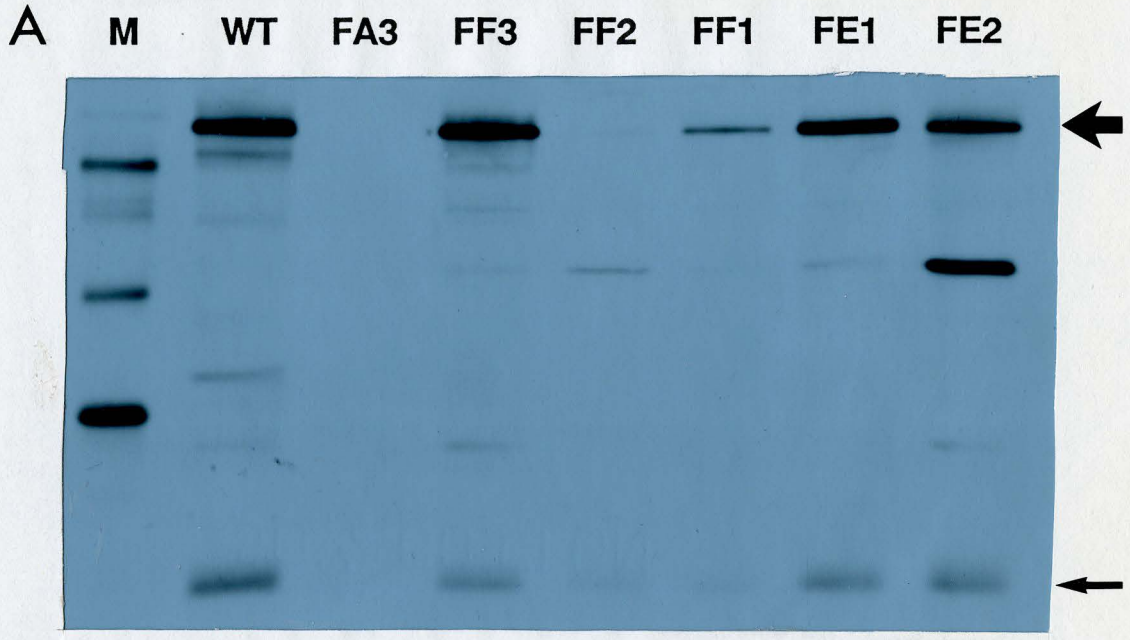

M
1
2
3
4
5

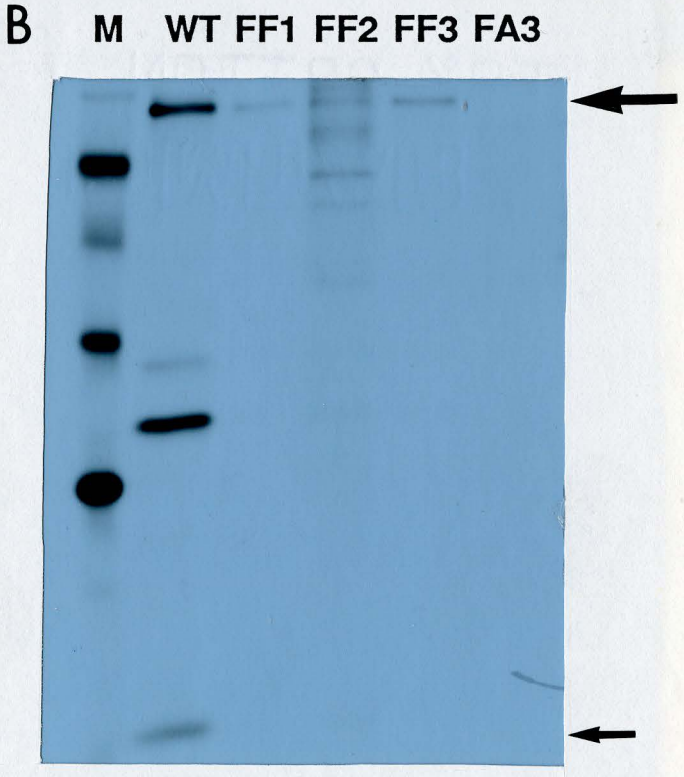
Figure 8: Immunoprecipitations of SV40 tumour antigens from K1 and dlA2433 transfected cells.
(A) Analysis at passage 2 of clonal populations of fibroblasts transfected with either K1 or dlA2433. Equal amounts of lysates from cells radiolabelled with [^{35}S]-methionine for three hours, were immunoprecipitated using Mab 419. Following electrophoresis of cell lysates, the gel was dried and exposed to Kodak XR-1 film for three days at -70°C. Large and small arrow heads indicate large T and small t antigen, respectively. M: molecular weight markers (from top to bottom) 69 kd, 46 kd, 30 kd (dark bands)

		clone	Transfecting plasmid
Lane	1	FB7 (wt)	pSV3neo
	2	FA3	pSV2neo
	3	FF3	pdlA2433
	4	FF2	pdlA2433
	5	FF1	pdlA2433
	6	FE1	K1
	7	FE2	K1

(B) Analysis at passage 4 of clonal populations of fibroblasts following transfection with dlA2433. Cells were labelled with [^{35}S]-methionine for one hour. Preparation of cell lysate, immunoprecipitation and electrophoresis were as above. The dried gel was exposed to XR-1 film for five days at -70°C. Large and small arrow heads indicate small and large T antigen, respectively. M: molecular weight markers (as above)

		clone	Transfecting plasmid
Lane	1	FB7 (wt)	pSV3neo
	2	FF1	pdlA2433
	3	FF2	pdlA2433
	4	FF3	pdlA2433
	5	FA3	pSV2neo

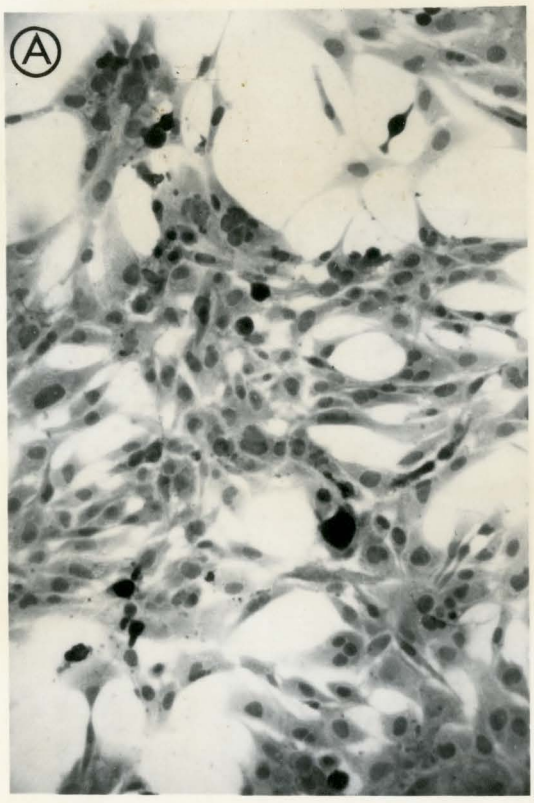

A
M
WT
FA3
FF3
FF2
FF1
FE1
FE2

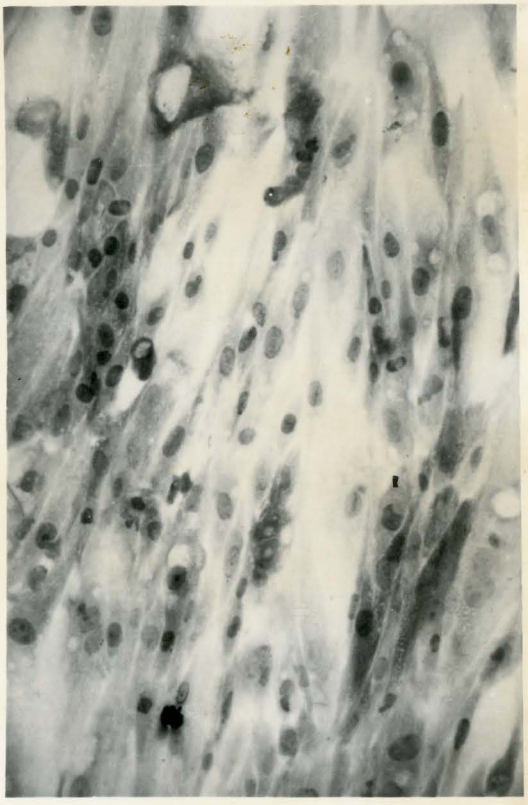

B
M
WT
FF1
FF2
FF3
FA3

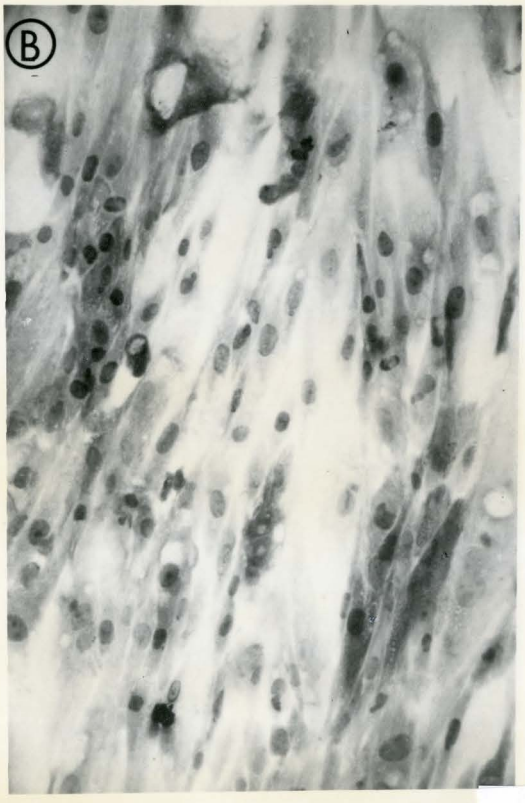
pSV3neo transfectant (wt). These results indicate that the amount of cytogenetic damage in the K1 transfectants is not due to elevated levels of large T protein, and confirm that the mutation in K1 does not affect the functions of large T antigen involved in the induction of cytogenetic damage.

The results of immunostaining of the dlA2433 transfectants showed that although all three clones exhibited primarily nuclear staining, two of them (FF1 and FF2) stained less intensely than pSV3neo clones (Table 2). The third clone (FF3) however was comparable to pSV3neo transfectants (Table 2, see also Figure 9A). This suggested that at least FF1 and FF2 might be expressing reduced levels of the tumour antigens. Cellular extracts from FF2 and FF3 at passage 2.0, were used in quantitative western analysis, as described above (see lanes 4 and 5 of Figure 7). Small t antigen was detected in both clones, in amounts comparable to or higher than the pSV3neo clone (lane 3). Somewhat unexpectedly, however, large T antigen was not detected in either FF2 or FF3. To investigate this further, all three dlA2433 transfectants were analyzed, again at passage 2, by immunoprecipitation with Mab 419 following a three hour label with [^{35}S]-methionine (see Figure 8A). The length of label used in this and a subsequent immunoprecipition (Figure 8B) was chosen due to the shorter halflife (three hours, versus 18 hours for wild type T antigen) of the dlA2433 mutant. In agreement with the results

Figure 9: Expression of SV40 tumour antigens in cells transfected with dlA2433. Clonal populations selected after transfection of fibroblasts with: (A) left panel -pSV3neo (control) (at passage 2); right panel - dlA2433 clone FF3 (at passage 2); (B) left panel - FF3 (at passage 2); right panel - FF3 (at passage 4), were immunostained with monoclonal antibody 419.


A




B

of immunostaining (Table 2, and Figure 9A), FF3 (lane 3) was found to express levels of large T and small t antigen comparable to the wild type control (lane 1). Expression of both SV40 antigens in FF1 and FF2 (lanes 4 and 5) was however lower than in wild type. The reasons why large T antigen was not detected in the western blot remain unknown, but seem likely related to the instability of the protein or unknown technical problems.

Both the small sample size (three clones of which only one clearly positive), and the problems encountered in detecting T antigen expression make any conclusions drawn from the results with the dlA2433 mutant somewhat preliminary. However, the comparable intensity (relative to the pSV3neo transfectants), the nuclear location of the immunostain in FF3, and the results of immunoprecipitation suggest that, for this clone, the inability of dlA2433 to induce cytogenetic damage can not be attributed to a decrease in expression of large T antigen. Thus, dlA2433 may lack the functions involved in this process. The results with FF1 and FF2 can not be similarly interpreted, due to their intermediate levels of expression of large T antigen.

Upon culturing of all of the dlA2433 clones, the level of T antigen expression was found to decrease. Each clone was reanalysed at passage 4 (i.e. at four population doublings past the previous analysis) by both immunoprecipitation

(following a one hour label with [^{35}S]-methionine, the length of which again due to the short halflife of dlA2433) and immunolabelling with Mab 419. Results from the immunoprecipitation are shown in Figure 8B. FF1 and FF2 (lanes 2 and 3) still expressed less T antigen than FF3 (lane 4). However, even the latter clone appeared to express substantially lower levels of this protein than the pSV3neo control (lane 1). Figure 9B illustrates the analysis of FF3 cells by immunostaining with Mab 419. In the left panel are cells stained at passage 2, and in the right cells stained at passage 4. As can clearly be seen, at passage 2 all cells were positive, whereas by passage 4 both the number of cells expressing the tumour antigens, and the intensity of the immunostain had dropped dramatically. A similar phenomenon was observed with FF1 and FF2 (data not shown). The reason for the loss of expression of the tumour antigens were not investigated. Although this phenomenon prevented further cytogenetic analysis of the clones, the results obtained with FF3 cells at passage 2, when expression of the tumour antigens was similar to pSV3neo clones suggest that the dlA2433 mutation abrogates the induction of cytogenetic damage.

3.6 Accumulation and Evolution of Cytogenetic Damage Prior to Crisis

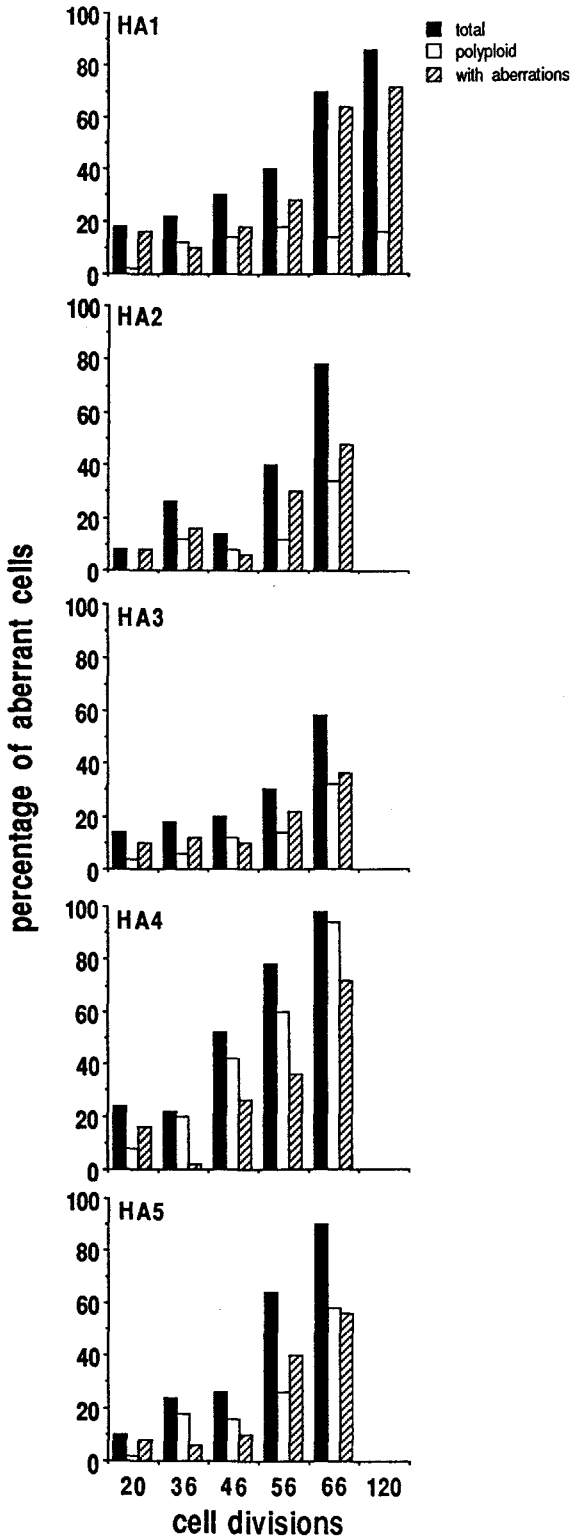
The third main objective of this study was to assess the role of cytogenetic damage in cell immortalization, and identify early genetic events which might contribute to this process. As shown above, the fibroblasts were very susceptible to T antigen induced damage at early times after transfection (passage 2), which made them unsuitable for the detection of initial aberrations and their subsequent evolution. Thus, a second human cell type, human embryonic kidney (HEK), originally obtained from Irene Mak (McMaster University), was used in experiments to address this objective. In infection with adenoviruses, these cells appeared relatively resistant to genetic damage (Caporossi and Bacchetti, 1990; Schramayr et al. 1990). In addition, HEK cells can be subcultured for only a limited number of passages, compared to fibroblasts (Poirier et al., 1988; Shay and Wright, 1989), which may allow more rapid identification of clones with an increased lifespan.

HEK cells were seeded and transfected with pSV3neo, in the same manner as the fibroblasts, with the exception that cells were incubated with the calcium-phosphate DNA-precipitate for six hours instead of overnight. Following

selection with G418, five colonies were isolated and expanded. Analysis of these colonies was carried out after the same number of cell divisions (approximately 20) as for the fibroblasts. At this stage HEK cells had acquired changes in morphology and growth properties (Figure 4D), one of the most striking being their vigourous and continued proliferation in culture. 100% of the cells in every clone could be immunostained with Mab 419, as intensely as the pSV3neo fibroblast clones (Table 2, see also Figure 4D). This suggested that both sets of clones expressed similar levels of the viral oncogenes.

Results of the cytogenetic analysis of the HEK clones at passage 2 are shown in Figure 10, and are expressed as polyploidy and frequencies of structural aberrations present in the individual clones. A substantially lower amount of cytogenetic damage was detected at this passage in the HEK clones compared to the fibroblasts. Up to 92% of the HEK cells had retained a diploid and normal karyotype: the maximum frequency of polyploid was 8% and the maximum frequency of cells with structural aberrations (mainly breaks and gaps) amounted to 16%. This suggested possible differences in the cytogenetic response of different cell types (fibroblast versus kidney) to the SV40 oncogenes. Alternatively, differences in the age of the cells (adult as opposed to embryonic) might affect the cytogenetic response. In

Figure 10: Cytogenetic analysis of HEK clones. Clonal populations of HEK cells transfected with pSV3neo and expressing SV40 antigens were analyzed at the indicated number of cell divisions after transfection. The frequency of total aberrant cells (solid bars), of polyploid cells (open bars) and of cells with structural aberrations (shaded bars) are plotted versus the *in vitro* age of the populations.


HA1
HA2
HA3
HA4
HA5
total
polyploid
with aberrations
percentage of aberrant cells
0
20
40
60
80
100
20 36 46 56 66 120
cell divisions

agreement with findings with the dl2005 and K1 transfectants, no correlation was observed between the induction of morphological and growth changes and extent of cytogenetic damage in the HEK clones, which suggested these processes might be independent of each other.

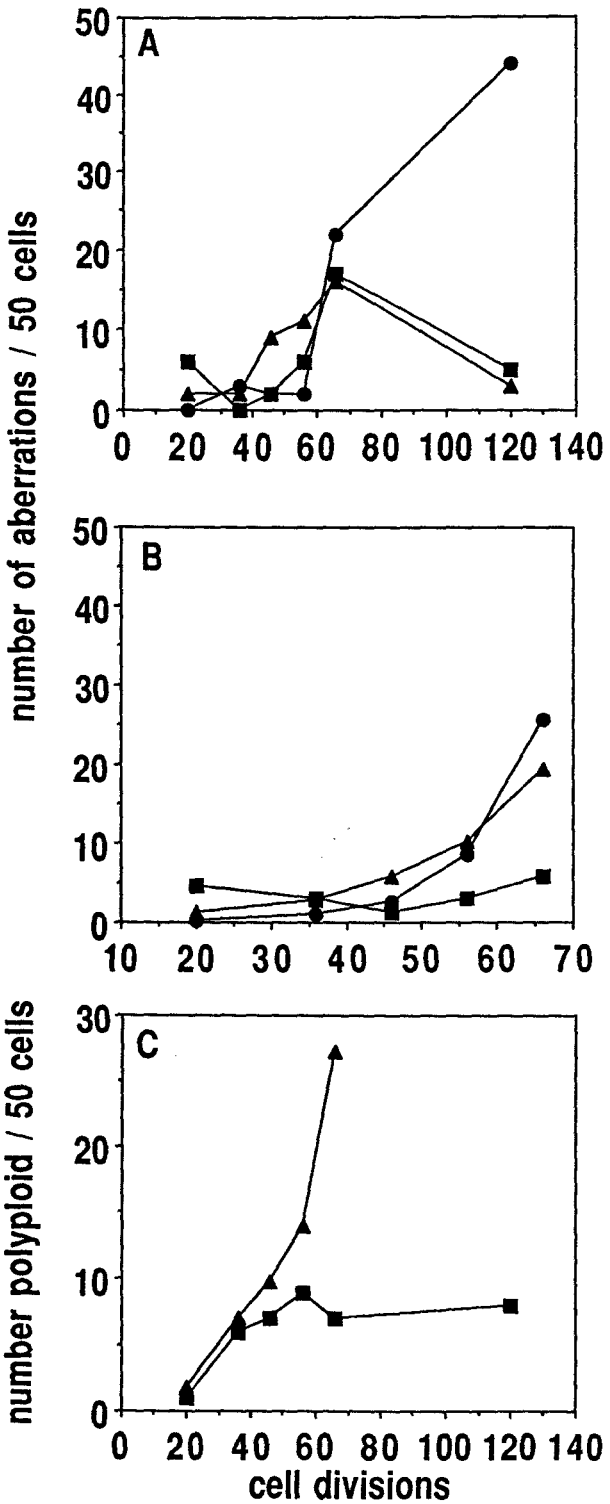
Attempts to generate control HEK pSV2neo clones were not as successful. These clones did not undergo sufficient divisions or generate a sufficient number of metaphases for analysis. However, of those metaphases scored, all retained the normal diploid karyotype (data not shown). As well, polyploidy and/or aberrations were rarely detected in untransfected HEK cells (data not shown; Caporossi and Bacchetti, 1990).

To investigate the evolution of cytogenetic damage in the pSV3neo HEK transfectants, all clones were kept in culture and screened for karyotypic aberrations at passages 10, 15, 20, and 25 (corresponding to approximately 36, 46, 56, and 66 cell divisions). The results of this analysis are also shown in Figure 10. As the cells were subcultured, complex structural aberrations, predominantly dicentrics and fragments, developed and increased in number. By passage 20-25, all five transfectants contained levels of structural aberrations comparable to those observed in the pSV3neo fibroblast transfectants at passage 2. The fraction of polyploid cells also increased upon passage, except for one clone (HA1).

Figure 11 outlines the change in frequency of various structural aberrations (A and B) and polyploidy (C) as the cells are subcultured. There was a gradual increase in both the frequency of breaks and gaps (■) and fragments (▲) in all five clones up to passage 25 (66 cell divisions). On the other hand, the frequency of complex rearrangements (•) (e.g. dicentrics) remained at a low level for the first 15 passages, then increased sharply around passage 20-25.

The growth rate of four of the five HEK clones declined progressively after passage 25, but no sign of cell death was detected. By passage 30, all four had ceased to grow, and no immortal lines could be derived from any of them. One clone, HA1, however continued to grow vigorously without any apparent sign of crisis; HA1 cells have been passaged over 80 times in culture and can be considered immortal. Additional cytogenetic analysis on the HA1 clone was performed at passage 51 (±120 divisions). As shown in Figure 10, the overall frequency of cells containing structural aberrations had continued to increase. Breaks and fragments had dropped however in number by this passage, in favour of more complex rearrangements (Figure 11A). The frequency of polyploid cells (■) remained essentially constant, at levels significantly lower than those observed during the later passages for the other clones (▲). This may indicate that polyploidy may not be essential for, or may even interfere with, immortalization,

Figure 11: Frequency of structural aberrations and polyploidy in HEK clones. Clonal populations of HEK cells transfected with pSV3neo and expressing SV40 antigens were analyzed at the indicated number of cell divisions after transfection. The frequency of breaks and gaps (■), fragments (▲), and complex rearrangements (dicentrics, rings, and translocation/duplications) (•), are plotted versus the <u>in vitro</u> age of the population. The frequency of aberrations was determined by scoring 50 metaphases for each clone. (A) Frequency of aberrations observed in HA1 (immortal clone), at cell divisions 20-120. (B) Average frequency of aberrations for limited lifespan clones (HA2-HA5), at cell divisions 20-66. (C) Frequency of polyploidy in HA1 (■), and HA2-HA5 clones (▲).


A
B
C
number of aberrations / 50 cells
number polyploid / 50 cells
cell divisions
0
10
20
30
40
50
60
70
100
120
140

though it is difficult to confirm this hypothesis with such a small sample size.

3.6.1 Detection of a Novel Marker Chromosome in Transfected Cell Populations

Among the commonly seen structural aberrations (dicentrics, fragments, and double minutes) observed in the immortalized HA1 clone, the most frequent rearrangement (66% of total aberrations) in 64% of the cells consisted of a marker chromosome, an example of which is shown in Figure 12. Several attempts were made to identify the origin of this marker by G-banding (not shown), however the banding pattern obtained proved to be very difficult to decipher. The top one third of the marker may be from a member of group C with a deletion removing part of the p (short) arm. A common feature of this chromosome is an unbanded light staining region, roughly in the middle of the q arm (small arrow in Figure 12), which may reflect uncoiling of the DNA. Retrospective analysis of all the transfectants revealed the presence of the same marker in two of the HEK clones with limited life span, and in eight of the fibroblast clones (4 pSV3neo, 2 pdl2005, and both K1 clones) (Table 7). This marker was never detected in the clones generated by transfection with the pX-8 plasmid. However, since the frequency of this marker is quite low in

Figure 12: Metaphase spread from HEK clone HA1 passage 51.
Large arrow - Marker chromosome
small arrow - region of lighter staining within marker

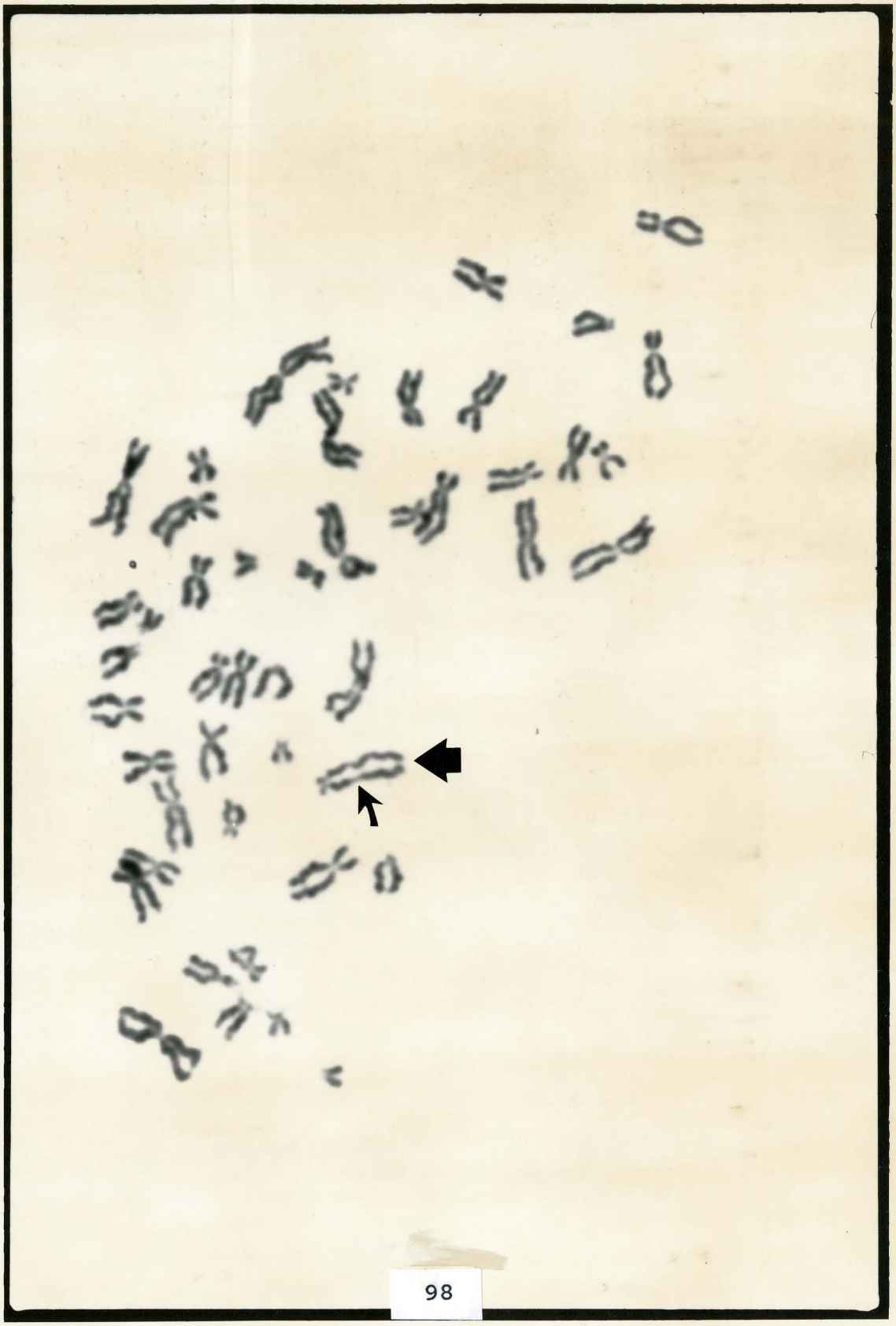


Table 7: Presence of Marker Chromosome in individual clones

Clone	Transfecting Plasmid	Number of marker*	passage number**
FA1	pSV2neo	0	2
FA2	"	0	2
FA3	"	0	2
FA4	"	0	2
FA5	"	0	2
FA6	"	0	2
FB1	pSV3neo	4	2
FB2	"	0	2
FB3	"	0	2
FB4	"	1	2
FB5	"	5	2
FB6	"	3	2
FB7	"	0	2
FB8	"	0	2
FC1	dl2005	0	2
FC2	"	1	2
FC3	"	0	2
FC4	"	3	2
FD1	pX-8	0	2
FD2	"	0	2
FD3	"	0	2
FD4	"	0	2
FD5	"	0	2
FE1	K1	2	2
FE2	"	1	2
FF1	dlA2433	0	2
FF2	"	0	2
FF3	"	0	2
HA1	pSV3neo	1/39	25/51
HA2	"	0	all passages
HA3	"	0	all passages
HA4	"	1/1/3	15/20/25
HA5	"	1/1	20/25

* - per 50 metaphases
** - passage number of cytogenetic analysis

most of the transfectants (occurring generally once or twice in 50 metaphases), it is possible that this marker does exist in the pX-8 transfectants and was not detected due to the size of the sample.

3.7 Analysis of HEK Transfectants

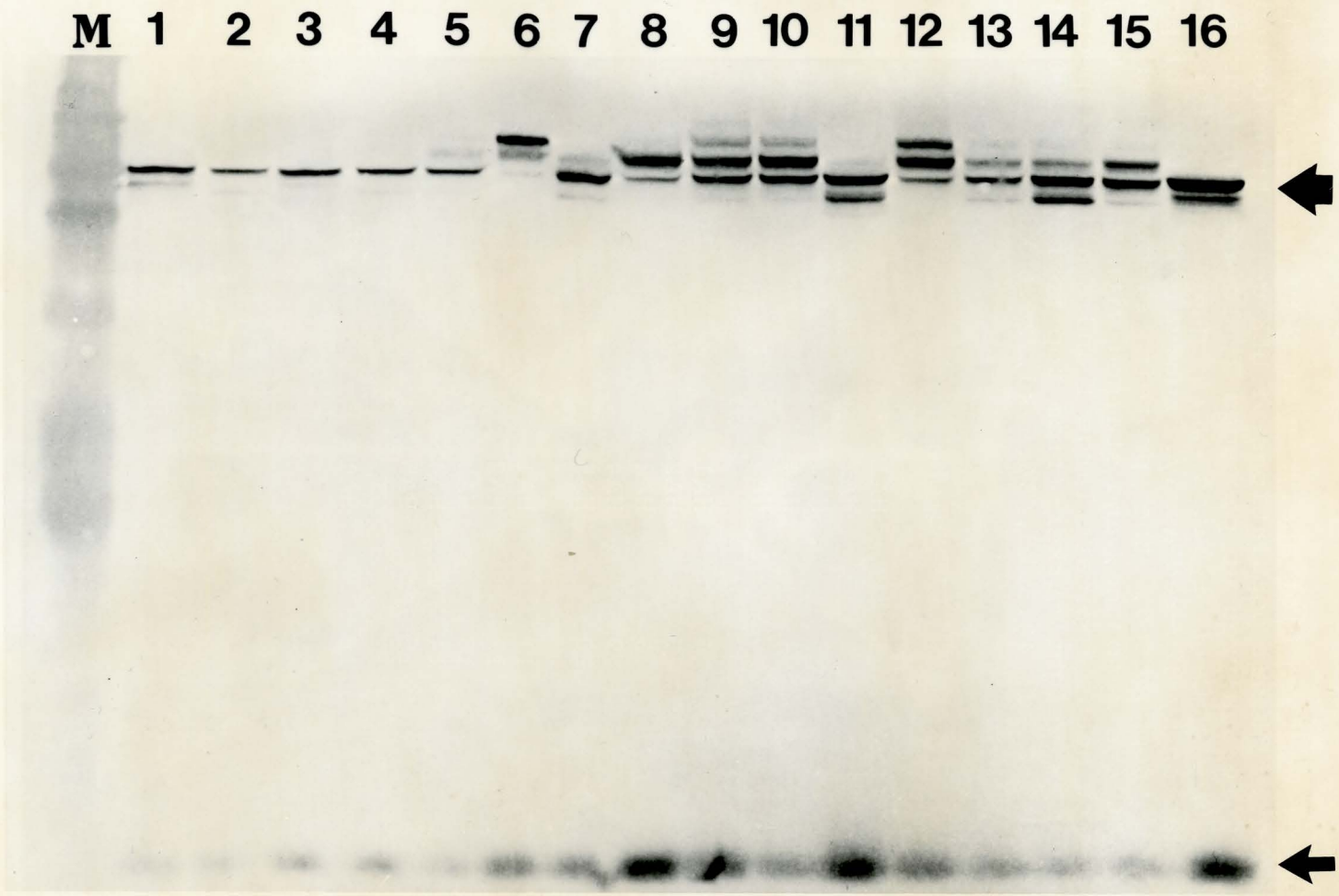
To address the possibility that the limited lifespan of four of the HEK clones, and the survival past crisis of the HA1 clone were due to differences in the level of expression of large T antigen, this latter aspect was quantitated by western blot. The results of this analysis are given in the next section.

3.7.1 Expression of Large T in HEK Transfectants

Cellular extracts were prepared in Tris buffer as described in Materials and Methods, from each of the HEK clones at passages two, ten and twenty, as well as from HA1 at passage 74. Protein concentration was determined by Bio-rad assay, and equivalent amounts of protein from each sample were used in western blot analysis. Unexpectedly, multiple bands reacting with Mab 419 and presumably corresponding to large T antigen were detected in many of the samples (Figure 13). To determine if this was due to incomplete solubilization of

Figure 13: Western Blot analysis of SV40 tumour antigens from HEK clones. Clonal populations of HEK cells transfected with pSV3neo were analyzed at passages 2, 10 and 20, plus passage 74 for HA1. Cell lysates were quantitated for protein concentration by Bio-rad assay. Equivalent amounts of protein were electrophoresed, the protein electroblotted onto a nitrocellulose membrane, and stained with Mab 419. Large and small arrow heads indicate large T and small t, respectively. M: molecular weight marker (from top to bottom) 180 kd, 116 kd, 84 kd, 58 kd, 48.5 kd, 36.5 kd, 26.6 kd

		clone	passage
Lane	1	HA1	2
	2	HA2	
	3	HA3	
	4	HA4	
	5	HA5	
	6	HA1	10
	7	HA2	
	8	HA3	
	9	HA4	
	10	HA5	
	11	HA1	20
	12	HA2	
	13	HA3	
	14	HA4	
	15	HA5	
	16	HA1	74


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

oligomers of T antigen, the experiment was repeated using two methods. The original cell extracts were sonicated 3 times 5 seconds, while new cell extracts were obtained from the HEK clones using RIPA solubilization and sonication as outlined in Materials and Methods. Both sets of extracts were used in separate western blot analysis, and again multiple bands for large T antigen, comparable to those in Figure 13 were observed (not shown). This pattern has been reported by other researchers (e.g. see Thompson, 1990), and attributed to changes in the phosphorylation state of large T. Alternatively, this pattern might be the result of tandem duplications of the viral early region following integration of viral DNA into the cellular genome. Duplication of the T-antigen-coding sequences can result in the expression of higher molecular weight forms of T antigen, termed super-T-antigen (May et al., 1981; May et al., 1983; Levitt et al., 1985; Monier, 1986; Blanck et al., 1988).

Protein levels were compared by visual inspection of the stained gel, taking into account all of the bands. Overall, there did not appear to be any significant differences in the level of expression of T antigen among the HEK clones (whether immortal or with limited lifespan) at the different passages (cf. 2-5, 7-10, 11-14). Within each clone however, expression increased with passage in culture (eg HA2, lanes 2, 7, 12, or HA1, lanes 1, 6, 11, 16). This could be due to increased copy

number of the viral genome, although this possibility remains to be assayed. All of the limited lifespan clones expressed at least two major, plus several minor forms of large T antigen, except at passage 2. HA1 on the other hand always expressed a single major form of large T antigen. Whether any of these factors had an influence on the survival of HA1 post crisis, compared to the other clones, is difficult to determine on the basis of these results. Nevertheless, differences in the level of T antigen expression do not appear to be the reason for HA1 immortalization.

DISCUSSION

Transformation of mammalian cells appears to be a multifactorial process, which can be induced by treatment with chemical or physical agents, or with viruses (DiPaolo, 1983; Chang, 1986). Thus transformation can result from the addition of new genetic information (e.g. viral oncogenes), or more commonly from the induction of genetic changes within cells (Chang, 1986). In the case of virally transformed cells, the presence of the viral oncogene(s) appears to be sufficient to alter cell growth and morphology (Wolman et al., 1964; Walen, 1981; Walen, 1987). However, at least in human cells these alterations are rarely accompanied by acquisition of an immortal state. This suggests that expression of the viral oncogene(s) is not in itself sufficient to ensure unlimited lifespan in vitro (Sack, 1981; Chang, 1986; Shay and Wright, 1989). Induction of genetic instability in transformed cells has been shown to occur as an early event following the expression of the viral oncogenes, and to precede the onset of crisis and the establishment of the transformed population (Moorehead and Saksela, 1965; Graham et al., 1977; Chang et al., 1986). Moreover, in essentially all cases immortal cells are genetically abnormal. Both of these

findings are compatible with the hypothesis that genetic damage may be important for both the initiation and development of the immortal phenotype.

In the case of SV40, studies with human cells have shown that this virus induces a variety of genetic damage (e.g. chromosome breaks and gaps, more complex rearrangements, and heteroploidy), all of which increase as the cells progress towards crisis and become immortal (Wolman et al., 1964; Moorehead and Saksela, 1965; Marshak et al., 1975; Nichols et al., 1985; Walen, 1987). Several investigators have further reported that the damage induced by SV40 involves preferential breakage in telomeric and centromeric regions of chromosomes (Wolman et al., 1964; Walen et al., 1986; Walen, 1987; Meisner et al., 1988), although the significance of these observations is not clear at present.

Previous work by Chang et al. (1986) has described the chromosome damage induced in human fibroblasts by a plasmid bearing only the early region of SV40. Levels of cytogenetic damage detected after the expression of the viral genes were comparable to those reported for virally transformed cells, which suggested that the early region of SV40 was sufficient for the induction of aberrations. The present study ensued from these observations, and was undertaken to determine which of the SV40 early proteins was required for the induction of chromosomal aberrations in human diploid cells, and to begin

to define oncogene functions involved in this process. In addition, since we postulated that SV40-induced cytogenetic damage may play a role in immortalization, this study examined the progression of genetic changes during growth of the transfected cells prior to and after crisis.

The results obtained in this study indicate that the expression of large T antigen is both necessary and sufficient for the induction of chromosomal damage, based on the wild type karyotype of cells transfected with the dl2005 mutant, which does not express small t. In addition, small t antigen may not contribute to the clastogenic process, since levels of damage in dl2005 transfectants were not significantly different from those in cells expressing the wild type SV40 early region (pSV3neo clones), and since a transfectant (FF3), which expressed small t antigen at normal levels in the presence of a mutated large T antigen, displayed a near normal karyotype. These results are in agreement with a concomitant study by Ray et al. (1990), and also parallel those from previous studies with adenovirus, which reported the requirement for only one of the viral oncogenes in the induction of genetic damage in human cells (Caporossi and Bacchetti, 1990; Schramayr et al., 1990).

Data presented in this study (Section 3.2) also indicate that the acquisition of morphological characteristics associated with transformation of cells by wild type SV40

requires the presence of small t antigen, since the morphology of the dl2005 clones was less altered than that of the pSV3neo clones. The observation that a second set of transfectants (K1) expression a mutant large T and a wild type small t was also morphologically less abnormal than the pSV3neo clones suggests, however, that large T plays a role of its own in the alteration of cell morphology. The K1 large T mutant is unable to bind the Rb protein. A role for the Rb protein in the alteration of cell morphology is therefore suggested by these results, in agreement with findings of previous studies in rodent cells (Chen and Paucha, 1990; Thompson et al., 1990). As already suggested by others, small t antigen may therefore contribute towards alterations of cell morphology, whereas large T antigen alone appears capable of altering cell growth (Sleigh et al., 1978; Bikel et al., 1987; DeRonde et al., 1989; Donahue and Stein, 1989; Jat and Sharp, 1989; Montano et al., 1990).

Lastly, the results presented in this report suggested that morphological and growth changes associated with expression of the viral oncogenes may be independent from the occurrence of cytogenetic damage. This conclusion is based on the facts that the K1 and dl2005 transfectants have a less altered morphology and high levels of aberrations, and conversely, HEK clones at early passages are significantly

altered in morphology and growth rate, but have low levels of cytogenetic damage.

The mechanism, either direct or indirect, by which large T antigen induces aberrations, is presently unknown. At the onset of this study, the ability of the protein to initiate viral DNA replication by binding to the replicative origin was considered among the most likely (DePamphilis and Bradley, 1986). Autonomous replication, as well as integration, amplification, and excision of viral DNA can all occur in semi-permissive human cells infected or transfected with wt SV40 (Zouzias et al., 1980), and the latter three processes in particular might induce considerable damage in the cell chromosomes (Gish and Botchan, 1987; Ruiz and Wahl, 1990). However, as determined in this study (Section 3.3), and as indicated in the parallel study by Ray et al. (1990), plasmids bearing an SV40 mutant lacking a functional origin of replication were as efficient at inducing cytogenetic damage as wild type SV40. Thus, T antigen mediated viral DNA replication does not appear to be necessary for damage induction.

These results contradict a previous study by Canaani et al. (1986), which suggested that an ori$^-$ mutant of SV40, unable to replicate and excise, was less efficient in the induction of aberrations, a contradiction which remains difficult to explain. Canaani's results were based on two

immortal fibroblast clones derived from cells of a Xeroderma pigmentosum patient. However, since XP cells possess impaired DNA repair, one might have expected an increased, rather than decreased level of cytogenetic alterations compared to normal fibroblasts. Also of note is the fact that both immortal clones exhibited identical karyotypes. Most immortal SV40-transformed lines contain a wide spectrum of chromosomal defects, although certain clonal cell lines can carry common marker chromosomes (Sack, 1981; Brown and Gallimore, 1987; Poirier et al., 1988; Burholt et al., 1989). This suggests that both of Canaani's rare immortal lines were derived from the same parental cell. To this date, Canaani's study remains an isolated incident in the literature. The induction of aberrations at wild type levels by the origin defective mutant reported in this study and by Ray et al. (1990), indicates that damage is caused by processes other than viral DNA replication.

Of the two T antigen mutants which could be assayed in this study, dlA2433 was found unable to induce chromosomal damage. The mutation in dlA2433 affects among other functions the ability of T antigen to bind the cellular p53 protein, and thus results with this mutant suggest a potential role for the T antigen-p53 complex in the induction of aberrations. However, although all three dlA2433 transfectants were positive for T antigen expression, only in one (FF3) was the

amount of the protein comparable to that in the pSV3neo clones. Moreover, T antigen expression in all three dlA2433 clones was found to be unstable. Although no data are available relating T antigen expression levels to amount of damage, the properties of the dlA2433 clones temper any conclusions which can be drawn on the role of p53 binding in induction of damage.

Further studies are therefore required to clarify the relationship between binding of p53 to T antigen and the induction of cytogenetic damage by the latter protein. Nonetheless, the results with the dlA2433 mutants raise questions on how the interaction between T antigen and p53 could induce genetic damage. As mentioned, p53 appears to be involved in cell cycle dependent regulation of DNA synthesis (Milner and Milner, 1981; Mercer, 1984; Reich and Levine, 1984). Binding of p53 to T antigen is known to stabilize the former, to induce in the latter a kinase activity which phosphorylates bound p53, and to be required for efficient transformation of cells by large T antigen (Scheidtmann and Huber, 1990). p53 may be prevented, by the binding of T antigen to the same site(s) in p53 required for interaction with cellular protein(s), from participating in pathways involved in cell cycle regulation. Alternatively, the T antigen-p53 complex could prevent p53/cellular protein interaction by inducing conformational changes in p53.

Phosphorylation might also induce conformational changes in p53, as this protein in normal cells is underphosphorylated, and displays a different electrophoretic ability compared to the form found in SV40-transformed cells (Scheidtmann and Huber, 1990). Alteration of p53 participation with cell cycle pathways might potentially affect the normal rate of cellular DNA synthesis. Uncontrolled cellular DNA synthesis, without corresponding mitosis, could then result in both polyploidy and gene amplification. This may in turn alter the copy number of gene(s) which are required for the maintenance of a normal diploid karyotype, and thus lead to further destabilization of the genome (Holliday, 1989).

Another possibility, not necessarily independent of the previous, is the combined interaction of T antigen, p53, and a second cellular protein to achieve the mutagenic effect. Such a mechanism could stabilize or alter the cellular protein, which in its new form could be able to induce chromosomal damage. The hsp 70 family of heat shock proteins has been reported to bind p53 in transformed cells which overexpress the latter (Sturzbecker et al., 1987). Further, a member of this family, hsp 73 has been identified as binding to large T antigen (Sawai and Butel, 1989). However, interaction of both p53 and hsp 73 with T antigen are independent of each other (Sawai and Butel, 1989), making it unlikely they are involved in this type of mechanism.

Altered binding to p53 could also disrupt other functions of T antigen involved in the induction of cytogenetic damage. Previous studies have indicated that DNA pol α and p53 compete for binding to T antigen (Braithwaite et al., 1987; Gannon and Lane, 1987), possibly due to steric hindrance by p53 of DNA pol α binding (Dornreiter et al., 1990). Disruption of this competition by abrogation of p53 binding might alter T antigen's stimulation of cell DNA synthesis, and thus its ability to generate polyploidy to destabilize the genome. DNA pol α, p53, and T antigen are also known to form a complex at certain concentrations in vitro (Gannon and Lane, 1987), which may also be required for the induction of cytogenetic damage, via the stimulation of cell DNA synthesis. However, whether this complex exists in vivo has yet to be determined.

The mutation in dlA2433 also affects the ATPase (helicase) abilities of the mutant T antigen, which prevents it from replicating viral DNA. Thus, the results obtained with the dlA2433 clones might also indicate that the lack of ATPase function was responsible for loss of the genotoxic properties of T antigen. However, as indicated in this study, viral DNA replication is not necessary for the mutagenic effect, and this suggests that these defects in dlA2433 are not likely to be involved.

The other mutant assayed in the study was K1. Data obtained from the K1 transfectants indicates that binding to

either Rb or p118/120 to T antigen is not required for the induction of aberrations by the latter protein. This interpretation is consistent with the previous observations that Rb may be required for morphological transformation of cells by large T antigen, and that morphological and mutagenic functions of T antigen appear to be separate. Rb also binds to sequences, in the Ad5 E1A products and in the human papillomavirus type 16 E7 proteins, which are structurally and functionally similar to the large T antigen Rb binding domain (Figge et al, 1988; Moran, 1988; Whyte et al., 1989; Vousden and Jat, 1989). Interestingly, a previous study with Ad5 indicates that the virus ability to induce chromosomal damage does also not correlate with E1A binding to Rb (Caporossi and Bacchetti, 1990).

Several other activities of T antigen not assayed in this study remain potential candidates for having a role in the induction of cytogenetic damage. T antigen is responsible for several alterations of the growth cycle, in particular for the induction of host cellular DNA synthesis (Chou and Martin, 1975; Soprano et al., 1981), which, as described above in relation to p53 could result in polyploidy and destabilization of the cellular genome. The generation of more complicated rearrangements, such as inversions and dicentrics, may further be directly aided by the single stranded recombinational activities of T antigen (Schneider et al., 1990). Of note is

the recent discovery by Gruss et al. (1988) that the human L1 family of repetitive DNA elements contains specific binding sites for T antigen. L1 sequences tend to appear as extrachromosomal elements in SV40-transformed human cell lines, which suggests that T antigen either directly or indirectly induces the mobilization of L1 elements (Gruss et al., 1988). Excision of L1 elements may result in chromosomal breakage, while recombination between intrachromosomal amplified sequences could give rise to the more complicated rearrangements commonly observed in cells transformed by SV40, such as dicentrics and translocations. Finally, one other candidate property for induction of cytogenetic alterations is the interaction between T antigen and cellular transcription factors, which assists in activation of normal cellular target promoters (Alwine et al., 1987), such as cellular rRNA (Soprano et al., 1983). This interaction could disrupt the balance of proteins involved in maintaining stability of the genome. For example, T antigen-induced transcription might increase the production of nucleases (e.g. endo- or exonuclease), ligases, or recombinases similar to the bacterial RecA protein, which could in turn act upon cellular DNA to produce chromosomal damage.

The lower amount and complexity of genetic damage observed in the HEK pSV3neo clones, relative to the fibroblasts at the age in culture, along with the former cells

altered morphology and growth rate, suggests that there are different responses to the genotoxic effect of T antigen between different cell types. It is significant that the frequency of immortalization, as has been noted elsewhere, also varies between different cell types, ie. mesenchymal versus epithelial (Girardi et al., 1965; Defendi et al., 1982; Christian et al., 1987). Together, these observations suggest that some factor(s) intrinsic to a given cell type may influence both processes, although what these factors are has yet to be determined.

Alternatively, differences in cell susceptibility may lie in the relative age of the two cell types used in this study. The HEK cells, although of similar age in culture as the fibroblasts, were younger in terms of the number of cell divisions they had undergone since fertilization. Older human fibroblasts, i.e. cells which have undergone a large number of cell divisions and are in the declining phase of growth, have been reported by others to acquire chromosomal alterations after infection with SV40 more rapidly than younger fibroblasts (Wolman et al., 1964). One reason for age dependent susceptibility could be that older cells have already acquired some of the critical mutations leading towards the development of genetic instability. For example, there could be a loss in the efficiency of DNA repair mechanisms as the cells age, which would inhibit the repair of

genetic damage induced by the virus.

One aspect of T antigen-induced cytogenetic damage in the HEK clones described here, which has also been noted in other cell types (Wolman et al., 1964; Moorehead and Saksela, 1965; Poirier et al., 1988), is the evolution of the structural abnormalities from breaks and gaps to more complex forms like dicentrics, rings and fragments. This suggests that the former aberrations may serve as a substrate for the latter. Whether continued T antigen expression is required for the evolution of the aberrant karyotype, or whether the protein is responsible only for the initial damage and/or destabilization of the genome could not be determined by the experimental protocol used. Findings with both fibroblasts and HEK clones are compatible with either possibility.

Although accumulation of substantial structural alteration in the karyotype occurred both in the immortal HA1 clone and in the limited lifespan clones, the level of polyploidy in HA1 remained consistently at a lower level, relative to latter clones. Others have also reported that many SV40-induced immortal cell lines tend to maintain near diploid karyotypes (Marlhens et al., 1988; Meisner et al., 1988; Poirier et al., 1988). These observations suggest the possibility that high levels of polyploidy might interfere with immortalization: a polyploid karyotype may for example be simply too cumbersome to allow normal cell division.

Duplication of the entire chromosome complement might also increase the gene dosage of factors involved in regulating or repressing cellular proliferation (eg. tumour suppressor genes such as p53 and Rb), which would have to be either lost or mutated to permit cell growth. However, the small sample size analyzed in the present study does not permit a definite conclusion on the role of polyploidy in cell survival.

A common feature in the karyotype of the HA1 immortal cell line was the presence of a marker chromosome. The high frequency (64% of cells) of this marker in the HA1 cells implies that this rearrangement may have contributed towards immortalization, possibly by conferring an initial growth advantage. Although the mere acquisition of this marker does not appear sufficient to ensure an unlimited lifespan, as two other HEK clones possessed the marker at a low frequency but did not yield immortal cell lines, its presence in both HEK and fibroblast clones suggests that a common, and possibly specific mechanism, may exist for inducing this arrangement. It would be interesting to see if all HEK and fibroblast SV40-transfected clones carried this marker. This might be achieved by expanding the cytogenetic analysis of the clones. Since the marker may be composed of a number of chromosomes, it would also be of interest to identify the origin of the

chromosomes involved, and the specific sites at which they are joined.

Although not directly investigated (by inspection of the integrated viral DNA sequences), the expression of unusual T antigen proteins resembling super-T-antigens in all five HEK clones might be the result of tandem duplications of the viral early region. This suggests that there may be specific rearrangements within SV40 sequences and/or at their site of integration as the cells are passaged.

Control HEK clones, generated by transfection with pSV2neo, grew for only a very limited period, as expected, whereas all T antigen positive clones acquired an extended lifespan. However, the majority of these clones (4/5) ceased growing after about 60 divisions, despite the presence of both large T antigen (Section 3.8) and substantial karyotypic abnormalities, and only one continued to grow into an immortal line. This observed low frequency of immortalization is in agreement with results reported by others for human cells (Chang et al., 1986; Shay and Wright, 1989). Thus, the occurrence of cytogenetic damage, the expression of T antigen, or both do not appear to ensure the acquisition of immortality by all cells. This is consistent with a mutagenic theory of transformation which would predict that if genetic damage generates random rearrangements, only some cells would acquire the necessary combination of alterations in critical genes to

allow survival past crisis. However, genetic damage is very likely required to achieve the immortal state, as to this date no established cell line has been found which has a completely normal karyotype.

Several issues remain to be addressed beyond this study, one of which is whether p53 binding is the only property of T antigen required for induction of cytogenetic damage. To answer this question, the number of transfectants which stably express the dlA2433 mutant could be expanded. Additionally, other T antigen mutants deficient for p53 binding could be assayed to confirm the results obtained with dlA2433. Both approaches are currently being followed in our laboratory. Another question worth pursuing is whether the continued expression of large T antigen is required for the evolution of the aberrant karyotype. This might be approached by generating fibroblast transfectants which express a temperature sensitive mutant of large T antigen, which could be turned off and on as desired. Alternatively, as the fibroblasts have a limited lifespan in culture, an established cell line with a stable karyotype (e.g. HT-1080, Rasheed et al., 1974) could be used instead for more long term studies.

It would also be of interest to determine whether age in culture is a factor in the susceptibility of cell strains to cytogenetic damage. This could be accomplished by generating pSV3neo clones from a older population (in culture) of HEK

cells. However, the limited lifespan in culture of this cell strain may not allow to assay cells with a large enough difference in the number of cell divisions to see an effect. Alternatively, a population of embryonic human fibroblasts could be used to generate clones to see if a corresponding decrease in T antigen-induced cytogenetic damage occurs. Lastly, it might also be useful to expand the number of immortal clones (both HEK and fibroblast), to assist in identifying common cytogenetic traits, such as the marker chromosome, which may influence survival past crisis.

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