INDUCTION OF GENOMIC INSTABILITY DURING TRANSFORMATION OF HUMAN CELLS WITH SV40 LARGE T ANTIGEN

INDUCTION OF GENOMIC INSTABILITY DURING TRANSFORMATION OF HUMAN CELLS WITH SV40 LARGE T ANTIGEN

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ABSTRACT

Human cells transformed by SV40 large T antigen achieve an extended lifespan and continue to grow and divide past the normal growth limit. This extended lifespan often ends at crisis when the cells die through fatal cell division. A few cells will survive this crisis and continue to proliferate indefinitely and are therefore considered immortal. Transformation of cells by SV40 large T antigen is associated with the induction of genomic instability at early times. This instability may contribute to a cells surviving crisis and becoming immortal through the chance disruption of genes involved in cell proliferation and regulation of cell death. Genetic instability is also observed in human tumours and the mechanisms by which it occurs both in tumour cells and SV40 transformed cells may be similar. In order to investigate these mechanisms, human and rodent cells were transfected with wild type and mutant forms of SV40 large T antigen and analyzed cytogenetically. The results of this study demonstrate that the amino terminal 147 amino acids of SV40 large T antigen are sufficient for the induction of genomic instability and at least three regions within this amino terminal fragment are

necessary. One between amino acids 17 and 27. A second being the retinoblastoma protein binding site, and the third between amino acids 130 and 147. Finally, binding of T antigen to p53 appears to not be required for the induction of genomic instability, but may be necessary for the survival of aberrant cells. There is an apparent correspondence between the ability of T antigen mutants to induce genomic instability, and their abilities to induce cellular DNA synthesis and to transform and immortalize cells.

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LIST OF ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
AT	Ataxia telangiectasia
ATP	adenosine triphosphate
bp	base pair
BRK	baby rat kidney
C	degrees celsius
cm	centimetre
DNA	deoxyribonucleic acid
dl	deletion mutant
FCS	fetal calf serum
G1	gap phase 1
G2	gap phase 2
µg, mg, g	micrograms, milligrams, grams
his	histidinol
HPV	human papillomavirus
H-CMV	human cytomegalovirus
KCl	potassium chloride
KD	kilodalton
LTR	long terminal repeat
µl, ml, l	microlitre, millilitre, litre
µM, mM, M	micromolar, millimolar, molar

M1, M2	mortality stage one, two
MAb	monoclonal antibody
N	amino
Na	sodium
NaCl	sodium chloride
NBCS	new born calf serum
neo	neomycin
PAb	polyclonal antibody
PBS	phosphate buffered saline
RB	retinoblastoma
RNA	ribonucleic acid
S	DNA synthesis phase
SSC	0.15M sodium chloride, 0.015M sodium citrate, pH 7.0
SV40	simian virus 40
T,t	large and small T antigen
TBS	Tris buffered saline
TRIS	Tris(hydroxymethyl)aminomethane
V	volts
v/v	volume/volume
wt	wild type
XP	Xeroderma pigmentosum
$ZnCl_2$	zinc chloride

INTRODUCTION

1.1 Regulation of cell cycle

The normal animal cell cycle consists of one round of DNA replication (S phase), followed by the segregation of the replicated chromosomes into two daughter cells (M phase), with each of these events being separated by a gap period (G1,G2) during which the cell increases in size and synthesizes the necessary proteins required for DNA synthesis and mitosis. The progression of the cell cycle is under genetic control, being regulated both positively and negatively by the organized expression of various genes. The major regulatory mechanisms controlling cell cycle progression appear to act in the G1 phase and act in response to external stimuli in the form of growth factors or mitogens. The type of mitogens present or recognized by specific receptors on the cell surface determines whether the cell will re-enter the cell cycle or enter a resting stage called quiescence, and leads to the induction or activation of a number of genes or gene products involved in cell cycle control. Tumour suppressor genes such as the retinoblastoma gene product (pRB), and p53 are among the proteins whose actions are responsive to mitogens and are important for the regulation of the cell cycle in actively

dividing cells, as well as in the control of cellular quiescence and senescence. In normal, cycling cells pRB controls the onset of S phase through inhibition of protein kinases and transcription factors in G1 and is itself regulated through phosphorylation (reviewed in Levine, 1993; Muller et al., 1993). p53 has an important role in monitoring the integrity of the genome. Cells showing DNA breaks are prevented from progressing from G1 through the cell cycle by p53 until the damage can be repaired. If the damage is too extensive p53 can also channel the cell into an apoptotic pathway (reviewed in Lane, 1992). Growth suppression by p53 is also operational in senescent cells (Bond et al., 1994; reviewed in Lane, 1992).

Normal human somatic cells are restricted in their proliferative capacity to a certain number of cell divisions called the Hayflick limit. Beyond this limit cells cease cycling, though still remain viable (Hayflick, 1965; reviewed in Goldstein, 1990 and Harley, 1991). Cellular senescence is characterized by irreversible growth arrest, a decrease in the response to mitogen stimulation, failure to phosphorylate the retinoblastoma gene product and to express or activate the p34cdc2 kinase and its associated cyclins (Stein et al., 1990, 1991; Hara et al., 1991; Dulic et al., 1993; reviewed in McCormick and Campisi, 1991). Senescence is a dominant state as determined by cell fusion of immortal cells with senescent

cells (reviewed in McCormick and Campisi, 1991) and is likely the result of expression of repressors of intracellular responses to growth signals rather than a breakdown of the response pathway itself. While, immortality appears to be recessive as hybrids created from SV40 immortalized cells and normal cells go through another crisis stage and only rarely produce other immortal clones, suggesting the presence of dominant factors in the normal cell that prevent cell growth (Pereira-Smith and Smith, 1987).

1.1.1 pRB

The retinoblastoma gene product (pRB) is a ubiquitously expressed protein which is found inactivated in a wide range of human tumours and in every case of retinoblastoma in humans. Retinoblastoma is a childhood cancer resulting from an inherited mutation in one or both RB alleles (reviewed in Levine, 1993). Normally pRB is expressed in both cycling and senescent cells and its activity is regulated by phosphorylation, likely by the protein kinase p34cdc2, in a cell cycle dependent manner (reviewed in Muller et al., 1993). This hypothesis is supported by the facts that the phosphorylation sites in pRB are in p34cdc2 consensus sequences, the two proteins form a complex in vivo, and p34cdc2 phosphorylates pRB in vitro (Lees et al., 1991; Hu et al., 1992). In its underphosphorylated form, which is common

in quiescent and G1 phase cells, pRB is thought to be active as a growth suppressor and restrict the G1-S transition of the cell cycle (DeCaprio et al., 1988; Ludlow et al., 1989). Introduction of the RB gene back into a tumorigenic retinoblastoma cell line, in which the gene was initially deleted, abrogates these cells ability to produce tumours in nude mice demonstrating pRB's growth suppressing function (Huang et al., 1988). Furthermore, heterozygous loss of the RB gene in transgenic mice increases predisposition to tumour formation while homozygous deletion results in lethal developmental defects (reviewed in Muller et al, 1993). In agreement with the finding that the underphosphorylated form is an active growth suppressor, phosphorylation of pRB is impaired in senescent cells (Stein et al, 1990).

pRB affects cell growth through its interaction with many cellular proteins, such as the family of E2F/DRTF transcription factors. This particular interaction is also dependent on the phosphorylation state of pRB. The underphosphorylated form of pRB associates with the E2F protein during the G1 phase of the cell cycle (reviewed in Muller et al., 1993). Upon phosphorylation of pRB, near the G1/S boundary, the pRB/E2F complex dissociates and the E2F transcription factor is free to stimulate the transcription of many genes whose products are involved in the synthesis of DNA precursors or are required for DNA replication (reviewed in

Muller et al., 1993). E2F also mediates the autoregulation of the RB gene expression. The RB gene promoter contains an E2F recognition site. Binding of E2F to this site transactivates RB gene expression which in turn results in overexpression of pRB which suppresses the E2F stimulation of the RB promoter. Therefore, pRB expression is negatively regulated through a feedback loop mediated by E2F (Shan et al., 1994).

pRB also forms complexes with the proteins of transforming DNA tumour viruses such as simian virus 40 (SV40) large T antigen, adenovirus E1A, and the human papillomavirus (HPV) E7 protein (DeCaprio et al., 1988; Dyson et al., 1989; Egan et al., 1989; Dyson et al., 1990). Since it is the active underphosphorylated form of pRB with which these viral proteins associate, the complex formation is likely a mechanism for the viruses to prevent the growth suppression activity of pRB and allow their own replication in growing cells (Ludlow et al., 1989; Munger et al., 1991; Dyson et al., 1992).

pRB belongs to a family of cellular proteins that also includes p107 and p130. These proteins share amino acid sequence homology and also have overlapping or complementary functions including interactions with some of the same cellular and viral proteins (Zhu et al., 1993).

1.1.2 p53

p53 is a nuclear phosphoprotein that is also a negative regulator of cell proliferation. 50% of all human cancers contain mutations in the p53 gene, an observation that has caused an intense search for the biochemical functions of this protein. The presence of an acidic domain near the amino terminus of the protein, that is similar to that of many transcription factors, along with the nuclear localization signal suggests that p53 may function as a transcription factor. In support of this, a chimeric protein, consisting of the acidic domain of p53 fused to the DNA binding domain of GAL4, could activate transcription from the GAL4 operon (reviewed in Vogelstein and Kinzler, 1992).

p53 has been found to inhibit cell proliferation in response to DNA damage at a G1 checkpoint (Kastan et al., 1991; reviewed in Lane, 1992 and Vogelstein and Kinzler, 1992). Knockouts of the p53 gene in nude mice are associated with instability of the genome (Livingstone et al., 1992), supporting the hypothesis that p53 in some way monitors the integrity of chromosomes and acts to prevent the cell from dividing until DNA repair can be completed (Kastan et al., 1991, 1992; Kuerbitz et al., 1992; reviewed in Hartwell, 1992 and Lane, 1992). Such instability could generate other genetic alterations which could lead to cancer as first suggested by Boveri in 1914 (Boveri, 1914; Nowell, 1976).

The mechanism by which p53 inhibits proliferation of cells exhibiting gaps or breaks in the DNA may be the stimulation of production or the activation of another protein which then inhibits factors needed to drive the cell through the cell cycle (reviewed in Marx, 1993; Slebos et al., 1994). The same inhibitory pathway appears to be active in senescent cells to stop their division (Bond et al., 1994; reviewed in Marx, 1993) (see Sdi 1 below). Recently, several labs working independently have discovered a protein that links p53 to the regulation of the cell cycle and to the pRB pathway and is one of a family of proteins with similar structure and likely function (Polyak et al., 1994). Cip1, or Cdi1 is a 21 kd protein found to interact both with cyclins and with protein kinases (Gyuris et al., 1993; Harper et al. 1993). For clarity this protein will be referred to as p21. Both the level of p21 mRNA and the interaction of the protein with cdks are regulated in a cell cycle dependent manner (Li et al., 1994). p21 is active at the G1/S transition and in the G2 phase of the cell cycle where it forms stable complexes with the cdk2 and cdc2 protein kinases respectively (Gyuris et al., 1993; Harper et al., 1993; Li et al., 1994) and inhibits their activity (Xiong et al., 1993). Through its inhibition of the cdk protein kinases, p21 prevents the phosphorylation and therefore the deactivation of pRB leading to G1 cell cycle arrest (Harper et al., 1993). In vitro, p21 has a phosphatase

activity specific for tyrosine residues (Gyuris et al., 1993). Overexpression of this protein delays progression through the cell cycle, dependent on its phosphatase activity (Gyuris et al., 1993; Xiong et al., 1993), implying the deactivation of cdk2 occurs through dephosphorylation by p21. In senescent cells, p21 has been identified as Sdi 1, a protein whose expression is upregulated in these cells (Noda et al., 1994). p21 was also identified as WAF1, the protein product of a gene whose expression is stimulated by wild type p53 expression (El-Deiry et al., 1993).

Other genes have been found to be regulated by p53 expression. One of these genes is the murine double minute (mdm-2) gene. Mdm-2 is an oncogene that is found amplified in human sarcomas and whose first intron contains a p53 response element (Leach et al., 1993). High level expression of p53 leads to high mdm-2 expression. The mdm-2 gene product forms a complex with p53 (Leach et al., 1993) and regulates p53 transcriptional activity (Oliner et al., 1992) therefore these two proteins are likely involved in a autoregulatory feedback loop (Wu et al., 1993). Another gene that may be regulated by p53 is GADD45. The GADD45 gene product is expressed in cells in response to ionizing radiation (Papathanasiou et al., 1991) and therefore may be part of or associated with the DNA repair system activated by the presence of the DNA breaks caused by irradiation or play a role in the cell cycle arrest observed

after DNA damage (Kastan et al., 1992; Zhan et al., 1994). Interestingly, GADD45 expression is not inducible in either Ataxia telangiectasia (AT) cells or in cells that have mutant p53 (Papathanasiou et al., 1991; Kastan et al., 1992). A p53 binding site is located in the promoter of the GADD45 gene implying that p53 directly interacts to control the expression of GADD45 (Kastan et al., 1992). Ataxia-telangiectasia, as will be explained in more detail later, is a genetic disease that is characterized by an increased susceptibility to cancer and a high frequency of chromosomal aberrations. AT cells may not induce the expression of p53 in response to irradiation (Kastan et al., 1992; Lu et al., 1993) implying that the AT gene products function upstream from p53 in a pathway involving the AT gene products, p53 and GADD45 (Kastan et al., 1992; reviewed in Hartwell, 1992).

p53, like pRB is a target of many DNA tumour virus early gene products. Wild type but not mutant forms of p53 associate with these viral proteins suggesting that this complex formation may be the viruses mechanism of preventing the growth suppressing activity of p53 in response to the DNA damage induced by these viruses (reviewed in Levine, 1990, 1993; Lin and Simmons, 1991; Shay et al., 1991 a,b; Fanning and Knippers, 1992; Peeper and Zantema, 1993). p53 is stabilized in a non functional complex by its association with viral proteins such as SV40 large T antigen and adenovirus E1B

55K (reviewed in Manfredi and Prives, 1994) or targeted to be degraded through the ubiquitin pathway by complexing with the polyomavirus E6 protein (Scheffner et al.,1990; Crook et al., 1991). The effect however, is the same: the repression or abolition of p53 functions, such as gene transactivation, required for cell cycle arrest in response to DNA damage (Meitz et al., 1992; Hoppe-Seyler and Butz, 1993; Kessis et al., 1993; Gu et al., 1994). Like the interaction with pRB, this repression of p53 function may allow growth of the virus.

1.2 Transformation and Immortalization

Transformation has been defined as the acquisition of one or more of the following properties by cells: altered morphology, focus formation, low serum requirement, anchorage independence, shortened doubling time, increased saturation density, extended or indefinite lifespan and tumorigenicity (reviewed in Chang, 1986). Many transformed cells acquire only a few of these properties and there appear to be several stages of transformation. For example, a cell can be transformed, in that it exhibits several of the traits mentioned above, but does not induce tumours in nude mice and therefore is not neoplastically or fully transformed. Minimal transformation is characterized by changes in morphology and an extended lifespan. Transformation is also often accompanied by a change from a diploid to an aneuploid karyotype as well

as by changes in chromosome structure. Chromosomal abnormalities are common in many human tumours and in tumour derived cell lines, therefore, genomic instability may be one of the few reliable markers of transformation in vitro.

Since there have been few reports of transformants arising spontaneously from normal human cells in culture (Takahashi et al., 1990), and none have been reproducible except for those involving p53 minus cells (Bischoff et al., 1990; Reddel, R., pers. comm.), it is assumed that treatment of cells with a carcinogen is necessary to establish human cell lines. Transformation of cells with viral oncogenes, physical agents, or chemical carcinogens overrides the normal growth control through functional inactivation of cellular proteins causing the cell to acquire an extended lifespan among other transformed phenotypes (reviewed in Strauss and Griffin, 1990). Most of these transformed human cells will continue to grow until a proliferative crisis when they will die. The survival of cells through crisis is a rare event which results in an cell line with an indefinite lifespan and therefore termed immortal (reviewed in Chang, 1986; Shay and Wright, 1989).

There appear to be multiple steps leading to the immortalization of cells. The first may be the deregulation of genes responsible for the control of cell growth, such as pRB, (reviewed in Strauss and Griffin, 1990; Shay et al., 1991a).

Loss of regulation of the G1/S transition may be a factor in transformation since normal cells arrest early in G1 in response to cell cycle inhibitors of protein kinases, while transformed cells are not affected by these inhibitors (Gadbois et al.,1992). In senescent cells, arrest at the G1/S interfase has been called by some mortality stage one (M1) and results in a loss of mitogen responsiveness (Wright et al., 1989; reviewed in Shay et al., 1991b). The cells then cease cycling but remain viable. This cell cycle arrest can be overcome in senescent cells and prevented from occurring in still cycling cells by stimulating cellular DNA synthesis with viral proteins such as the SV40 large T antigen (Wright et al., 1989; reviewed in Shay et al., 1991b). The M2 or mortality stage two, which is the same as the proliferative crisis transformed cells undergo at the end of their extended lifespan, is controlled by a mechanism independent of M1 that results in cell death instead of growth arrest. One possible hypothesis to explain this difference between cells at senescence and at crisis could may relate to the loss of telomeric DNA caused by the additional cell divisions during the extended lifespan past and the lack of active telomerase in somatic cells. By crisis the ends of the chromosomes may have become unstable and recombinogenic and cell death may result (Counter et al., 1992). The inactivation of M2 likely requires rare mutational events, such as the activation of

telomerase, (Counter et al., 1992) independent of the original transforming function (Wright et al., 1989). Since early occurrence of karyotypic instability is a common characteristic of both in vitro transformed cells and human tumours, the necessary mutagenic events required to immortalize a transformed cell may result from a chance disruption in the genes regulating cell death at the M2 or crisis stage as a result of this instability. Therefore, karyotypic instability may contribute to and be necessary for the immortalization of transformed cells. This mutational hypothesis accounts for the rarity with which transformed human cells become immortal since the required mutagenic events likely occur at random.

1.3 DNA Tumour Viruses

The DNA tumour viruses have become useful tools in the study of the role of genomic instability in transformation and immortalization of human cells in vitro. Viruses such as Simian Virus 40 (SV40), Polyomavirus, Adenovirus, and Human Papillomavirus (HPV) induce chromosomal aberrations and aneuploidy when infecting human cells in culture (Wolman et al., 1964; McDougall, 1971; Graham et al., 1977; Caporossi and Bacchetti, 1990; White et al., 1994). Each of these viruses encode proteins that are responsible for both the induction of genomic instability and the transformation of the cell. Cells

transformed by these viral proteins also undergo proliferative crisis but to different extents depending on the type of cell and the viral protein used to transform (Shay et al., 1993a; DeSilvia et al., 1994).

1.3.1 SV40

SV40 is a member of the papovaviridae subfamily called polyomavirus. Originally discovered as a viral contaminant in polio vaccine stocks in 1960, SV40's natural host range is restricted to rhesus monkeys (Topp et al., 1981; Norkin, 1982) where it produces a lytic infection. The virus can also infect green monkey kidney cell lines (Topp et al., 1981; Brady and Salzman, 1986). In human and rodent cells, which are semi and non-permissive for SV40, infection leads to the random integration of the viral DNA into the host genome. Expression of the early gene products from the integrated DNA changes the phenotype of the infected cell and is responsible for their transformation and the induction of genomic instability.

There are three proteins encoded by the early region of SV40: small t antigen, 17KT, and large T antigen. These proteins are created by the differential splicing of a single mRNA that is produced once the virion particle has reached the nucleus. Small t antigen is made by the removal of nucleotides 451-4638 of the mRNA. The resulting protein has 174 amino acids, the first 82 being identical to those of large T, and

is found in the cytoplasm of the infected cell. Small t does not seem to be required for lytic infection in vitro (Acheson, 1981; Brady and Salzman, 1986). Some of its functions include the activation of transcription of certain polII and polIII promoters (Loeken et al., 1988), and association with two cellular proteins which are the regulatory subunits (A and C) of protein phosphatase 2A (Rundell, 1987; Joshi and Rundell, 1990). This interaction is found to stimulate the Map kinase pathway and induce cell proliferation (Sontag et al., 1993). Through these functions and likely others, small t antigen has been found to enhance the transforming ability of limiting concentrations of large T antigen (Bikel et al., 1987).

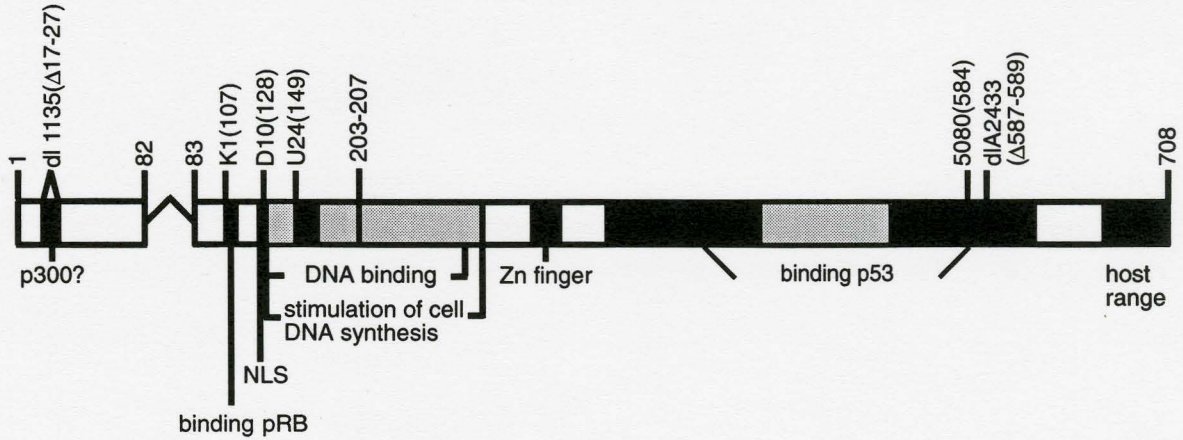
The 17kT protein consists of 135 amino acids, the first 131 of which are identical to large T (Zerrahn et al., 1993). This protein and the corresponding mRNA were found in all the SV40 transformed and infected cells analyzed. Transfection of the 17KT cDNA transformed rat fibroblasts (Zerrahn et al., 1993). The transforming functions of this protein are likely identical to those of large T and the expression of this amino terminal domain might be a way of regulating the activities of this domain independently from the carboxy terminal function of large T (Zerrahn et al., 1993).

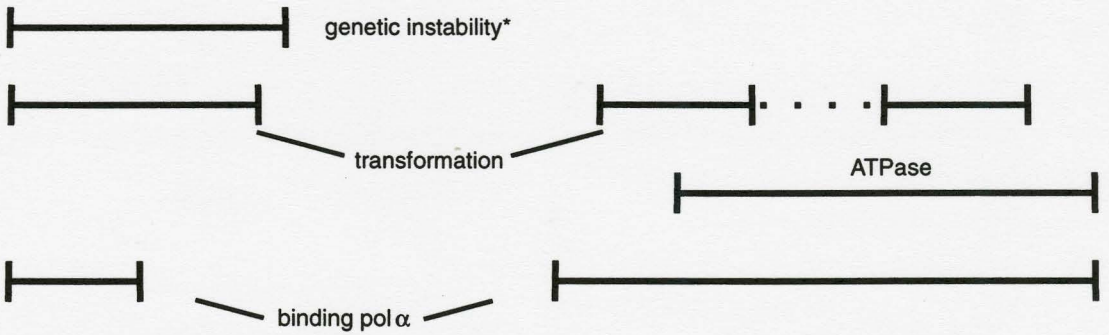
SV40 large T antigen is a large protein, consisting of 708 amino acids, and mainly localized to the nucleus of both infected and transformed cells (Staufenbiel and Deppert, 1983;

Covey et al., 1984). It is post-translationally modified through glycosylation, phosphorylation, and acylation (Peden et al., 1989) and also undergoes structural changes which generate multiple forms. The protein itself has multiple biochemical functions and appears to contain discreet functional domains (Figure 1). It is involved in viral replicative processes, in virus induced transformation of the host cell and is sufficient for the induction of genomic instability observed during transformation (Ray et al., 1990; Stewart and Bacchetti, 1991). For viral replication, large T has its own topoisomerase (Mann, 1993) DNA helicase (Clark et al., 1983) and ATPase activity (Bradley, 1990) essential for DNA unwinding. It also binds to the viral origin of replication and stimulates replication (Paucha et al., 1986). Since SV40 does not encode its own polymerase, large T appropriates DNA polymerase alpha from the host cell to synthesize both viral and host cell DNA (Smale and Tjian, 1986; Dornreiter et al., 1990). As well, large T induces transcription from the viral promoters. There are three T antigen binding sites in the viral origin of replication through which the expression of late and early genes of the viral genome can be autoregulated (Paucha et al., 1986; Arthur et al., 1988; Simmons et al., 1990). Large T antigen, also, modifies host cell gene expression directly through binding to non-specific and specific sites in the host cell genome

Figure 1: Partial Functional Map of Large T Antigen. The figure shows some of the identified functional domains and the areas disrupted by several of the mutants used in this investigation. Functional domains not included in this map are:

1) Transactivation of viral genes (aa 1-121)	Srinivasan et al., 1989.
2) Binding to Hsp 73 (aa 1-178)	Sawai and Butel, 1989.
3) Phosphorylation, Adenylation Sites (aa 106-124)	Scheidtmann et al., 1982.
(aa 639-701)	Van Roy et al., 1983. Simmons, 1984.
(aa 418-528)	Clertant et al., 1984. Bradley et al., 1987.
4) Binding to SV40 origin (aa 152-155 and 203-207)	Simmons et al., 1990.
5) DNA helicase activity (aa 152-155,182-187,203-207)	Simmons et al., 1990.
6) Binding to cellular DNA (aa 277-362)	Prives et al., 1982.
7) Stimulation of rRNA synthesis (aa 420-509)	Soprano et al., 1983.
8) Covalent linkage of RNA (aa 639-679)	Carroll et al., 1988.





* as found by this investigation

(Prives et al., 1982; Gruss et al., 1988) and indirectly through association with cellular factors that also mediate gene expression. The cellular proteins that SV40 large T antigen forms complexes with include the previously mentioned DNA polymerase alpha, the heat shock protein hsp 73, transcription factor AP-2, a 185 Kd protein (Kohrman et al., 1992) as well as cell cycle regulatory proteins such as p120, mdm-2, (Brown et al., 1993) p107, pRB (Yee and Branton, 1985; DeCaprio et al., 1988) and p53 (Peden et al., 1989). The region of T antigen necessary for the induction of cellular DNA synthesis, which overlaps with the DNA binding domain, appears to co-operate with both the pRB and p53 binding regions for this effect (Dobbelstein et al., 1992). The amino terminal 147 of SV40 large T antigen, sufficient for the transformation of rodent cells, (Chen and Paucha, 1990; Sompayrac and Danna, 1988, 1992), has also been found to be sufficient for the induction of host cell DNA synthesis (Sompayrac and Danna, 1994), implying a possible correlation between these two functions. The amino terminal 121 amino acids are capable of inducing tumours in transgenic mice but these tumours form at a much slower rate than those induced by full length large T antigen (Saenz-Robles et al., 1994). While it is not yet known if this fragment of large T antigen can induce DNA synthesis, it still retains the ability to bind to the pRB family of proteins (Saenz-Robles et al., 1994) and

contains the binding site for DNA polymerase alpha between amino acids 1 to 83 (Dornreiter et al., 1990). Therefore, it may only be impaired in the induction of DNA synthesis with respect to full length large T and T147. Large T antigen function is mirrored in proteins of other transforming DNA viruses indicating the importance of the specific host cell functions affected by all these viruses for the regulation of cell growth.

1.3.2 Other Viruses

Other DNA tumour viruses encode transforming proteins that have functional similarities to SV40 large T antigen. Three of these are polyomavirus, adenovirus, and human papillomavirus. These viruses are also commonly used to investigate transformation and immortalization of cells in vitro.

Polyomavirus encodes two gene products involved in cellular transformation and immortalization: the large T antigen and the middle T antigen. The large T antigen is immortalizing (reviewed in Strauss and Griffin, 1990), and like SV40 large T, binds to pRB and p107. However, unlike SV40 large T, it does not bind to p53 (Dyson et al., 1990). Polyomavirus large T antigen is also required for viral DNA replication, has DNA binding activity, ATPase and topoisomerase activity and stimulates host cell DNA synthesis

as well as activating transcription from cellular genes much like SV40 large T antigen (Topp, 1981). The immortalizing functions of polyomavirus large T resides in the amino terminal portion of the protein (Asselin and Bastin, 1985; Holman et al., 1994), and binding to pRB is necessary for this effect (Manfredi and Prives, 1990; LaRose et al., 1991). As with transformation and immortalization by SV40 large T antigen, karyotypic instability, chromosome aberrations and DNA amplifications are also a common feature of transformation and immortalization by polyomavirus large T antigen (St. Onge et al., 1990; 1993 a,b).

Middle T antigen of polyomavirus and small t antigen of SV40 also show similarity. Both of these proteins bind to two of the same cellular proteins, 61K and 37K, which are the A and C regulatory subunits of phosphatase 2A (Rundell, 1987; Walter et al., 1988; Joshi and Rundell, 1990). Middle T antigen is the principal transforming protein of polyomavirus and is required for the cells to become tumorigenic in nude mice (reviewed in Strauss and Griffin, 1990).

In adenovirus, the E1A region is sufficient to immortalize primary rodent cells (Graham et al., 1977) while both the E1A and E1B regions are required for neoplastic transformation (Houweling et al., 1980; White and Cipriani, 1990). The adenovirus E1A gene products interact with some of

the same cellular factors as SV40 large T antigen such as the pRB family of proteins (Yee and Branton, 1985; Harlow et al., 1986; Whyte et al., 1988). The adenovirus E1A proteins also bind to another cellular protein, p300 (Yee and Branton, 1985). While SV40 large T antigen apparently may not bind to p300, there is evidence that the amino terminus of large T has a complementary activity since a mutant of adenovirus E1A unable to bind p300, and therefore devoid of transforming activity, can be complemented by this region (Yaciuk et al., 1991). In addition, the adenovirus E1A proteins induce cellular DNA synthesis, cytogenetic damage and can be lethal in certain cells (Braithwaite et al., 1983; Caporossi and Bacchetti, 1990; Debbas and White, 1993). Adenovirus E1A gene products also bind to two additional proteins important for cell cycle regulation, cyclin A and p33cdk2 (Pines and Hunter, 1990).

The adenovirus E1B region encodes two proteins, 55K and 19K. The 55K protein product associates with p53 (Sarnow et al., 1982). Adenoviral mutants that do not express the E1B proteins while retaining E1A expression have been found to induce apoptosis during transformation of primary rodent cells (Rao et al., 1992). This effect is inhibited to a large extent by the presence of the 19K protein, and to a lesser extent by the 55K protein. Apoptosis is prevented by the presence of both proteins. This implies that, while binding of the 55K

protein to p53 is necessary for the long term survival of transformed cells, it is not sufficient and E1B 19K functions are needed (Rao et al., 1992). Therefore, in adenovirus, the E1A proteins bear a functional similarity to the amino terminus of SV40 large T antigen, while the E1B 55K protein, in binding to p53, mimics one of the functions of the carboxy terminus of large T.

Human papillomavirus (HPV) is a third transforming virus whose proteins affect the infected cell in ways similar to SV40 proteins. Oncogenic types of this virus can be found in 70 to 80% of cervical carcinomas as evidence of its possible role in the induction of human cancer. There are again two gene products important for the transformation and immortalization of cells, E6 and E7. The E7 gene product can immortalize and induce cell proliferation on its own but is more efficient in the presence of E6. The E6 protein specifically interacts with p53 (Werness et al., 1990) while E7 binds to the retinoblastoma gene product, p107, p130 and cyclin A (Dyson et al., 1989, 1992; Banks et al., 1990; Tommasino et al., 1993). As mentioned in section 1.1.2, p53 is quickly degraded by the ubiquitin pathway in the presence of E6 (Scheffner et al., 1990; Crook et al., 1991) resulting in the abolition of p53 functions (Mietz et al., 1992; Hoppe-Seyler and Butz, 1993; Kessis et al., 1993; Gu et al., 1994). The HPV E6 and E7 proteins may also be involved in the

accumulation of aberrations in the host cell genome that occurs upon infection with HPV. Infection of cells with HPV, like the other viruses mentioned here, results in the disruption of cellular activities that monitor the status of the genome (Gu et al., 1994, White et al., 1994).

All three of the viruses described above have independently evolved similar methods to induce cellular proliferation and attain control over an infected cell. The interaction of each virus' transforming proteins with cell cycle regulating proteins and tumour suppressors such as pRB and p53 is a common theme which indicates the vital importance these cellular proteins have in the regulation of cell growth and in the resistance to malignant transformation. All four of the DNA tumour viruses mentioned above are commonly used in studies of cellular transformation and immortalization in human cells. In this investigation SV40 is used due to its high transformation efficiency. It is an added convenience that the functions found in various studies to be involved in transformation and immortalization are located on a single protein, large T antigen.

1.4 Transformation by SV40

Although SV40 has never been linked to human cancer, it does cause tumours in rodents (Diamondopoulos, 1978; Lewis and Martin, 1979) making it useful as a model to study the

mechanisms of malignancy. Both rodent and human systems have been used to study the transforming properties of SV40. These two systems differ in that neoplastic transformation and immortalization of cells, not only by SV40, is common in rodent but not human cells (reviewed in Chan, 1986). In most cases, the phenotype of transformed human cells consists of altered morphology, growth properties and extended lifespan, however, the cells are not immortal or tumorigenic. In most cases in the following literature review cells referred to as transformed have acquired altered morphology and growth characteristics.

Most of the studies on SV40 have been done in rodent cells. These cells are non-permissive for SV40 so viral DNA replication and late gene expression do not occur though the early region genes are expressed (Salzman et al., 1986), indicating that the early region is sufficient for the transformation of rodent cells. Human cells are semi-permissive for SV40, so viral replication and production can occur, though at a lower level than in permissive cells (Girardi et al., 1965). Cells that survive infection usually contain an integrated viral genome and become transformed (Campo et al., 1978), though immortalization of human cells is a rare event (reviewed in Chang, 1986; Shay and Wright, 1989). Most of the transformed cells will attain an extended lifespan until they enter a proliferative crisis and die. A few cells

may survive this crisis and are therefore considered immortal. This suggests that viral functions are not sufficient to completely overcome the blocks that limit the lifespan of human cells and additional mutational events must occur to remove these blocks (reviewed in Sack, 1981; Shay and Wright, 1989).

In both rodent and human cell, large T antigen is sufficient for cell transformation (Zouzias et al., 1980; Mayne et al., 1986; Jat and Sharp, 1986, 1989; Shay et al., 1993b). Two regions of SV40 large T antigen have been found to be involved in viral induced transformation in both systems (Figure 1). For human cells, the first region, located at the amino terminus, includes the pRB binding site. The second is located at the carboxy terminus and includes the p53 binding site (reviewed in Manfredi and Prives, 1994). For rodent cells, the first region is located within the amino terminal 147 amino acids (Sompayrac and Danna, 1988, 1992; Chen and Paucha, 1990; Symonds et al., 1993; Thompson et al., 1990; Zhu et al., 1992) and may be within the first 121 amino acids (Saenz-Robles et al., 1994), the second is near the carboxy terminus (Asselin and Bastin, 1985; Sompayrac and Danna, 1988; Tevethia et al., 1988; Zhu et al., 1992). These regions may function independently, at least in rodents, because mouse C57Bl/6 cells can become immortal both with and without the first 127 amino acids (Thompson et al., 1990).

Evidence exists that some functions of T antigen are not required for transformation (Figure 1). These include replicative functions as mutants defective for viral replication can still transform (Prives et al., 1983; Gish and Bothchan, 1987; Manos and Gluzman, 1988). Therefore, ATPase activity, binding to the viral origin as well as viral host range functions are not required (Small et al., 1982; Paucha et al., 1986; Brown and Gallimore, 1987; Canaani et al., 1986; Gish and Botchan, 1987; Neufeld et al., 1987; Auborn et al., 1989; Radna et al., 1989).

T antigen proteins with mutations in the nuclear localization signal can also still transform rodent cells as assayed by focus formation. However, immortalization is impaired in some cases (Kalderon and Smith, 1984; Thompson et al., 1990), implying that immortalization may require nuclear functions of large T (Clark et al, 1983; Stahl and Knippers, 1987).

The regions required for transformation and immortalization of both human and rodent cells contain binding sites for host cell proteins, including the pRB family of proteins, p53 and DNA polymerase alpha (Figure 1). T antigen could function to transform cells through deactivation or, in the case of DNA polymerases alpha, utilization of these and other host cell proteins required for growth regulation and suppression. The pRB binding region of large T antigen,

between amino acids 105 to 114 (DeCaprio et al., 1988), is important for transformation in human systems. Mutant pRB proteins that are unable to suppress growth have been found in several human cancers and share the property that none of these mutants will bind to SV40 large T antigen. Therefore, it is likely that SV40 large T antigen binds to pRB through a domain of pRB that is important for its function and the same mutations that deactivate pRB prevent T antigen binding. This implies that binding to T antigen by wild type pRB to prevent its growth suppression function is important for the transformation of human cells (Horowitz et al., 1989; Huang et al., 1990). SV40 large T antigen's binding to pRB appears to be dispensable for transformation in rodent cells though in some cases the efficiency of mutants that do not bind pRB appears to be reduced as assayed by focus formation, anchorage independence, and growth in low serum (Kalderon and Smith, 1984; Sompayrac and Danna, 1984, 1988; Thompson et al., 1990). Large T antigen binding to pRB is also important for the induction of cellular DNA synthesis depending on the cell type (Sakamoto et al., 1993).

Binding of large T to p53 may not be required for morphological transformation (Sompayrac and Danna, 1983, 1989), but appears to be important for the extended lifespan and possible immortalization of rodent cells (Tevethia et al., 1984,1988; Zhu et al., 1991; Kierstead and Tevethia, 1993).

Mutants of T antigen that no longer bind p53 are also unable to transform or immortalize human cells (Lin and Simmons, 1991). These cells do not acquire an extended lifespan and are only partially transformed as assayed by efficiency of focus formation (Lin and Simmons, 1991), likely due to active growth suppression by the free p53. SV40 large T antigen binds to p53 and abrogates its transcriptional activity through stabilization of p53 in a non functional complex (Jiang et al., 1993) thus preventing it growth suppressing activity in both human and rodent cells.

It is not known whether binding to DNA polymerase alpha is important for T antigen ability to transform human or rodent cells, though it is likely important for the induction of DNA synthesis by SV40 large T antigen (see below). The transactivation of cellular genes by large T antigen is required for the morphological transformation of rodent cells (Alwine et al., 1987), but may not be required for immortalization as large T mutants defective for this function can still immortalize (Thompson et al., 1990). As yet no information is available on the role of transactivation of cellular genes in transformation by large T in human cells.

The ability to induce cellular DNA synthesis is a property of SV40 large T antigen that may be important for transformation. This property of the protein has been investigated so far only in rodent cells. The amino terminal

147 amino acids transform and induce cellular DNA synthesis at levels comparable to wild type SV40 large T (Sompayrac and Danna, 1988, 1990, 1994). Further deletions, of amino acids 131 to 147 may reduce both effects (Dobbelstein et al., 1992). As previously mentioned it is not known if the amino terminal 121 amino acids can still induce DNA synthesis, though they can transform (Saenz-Robles et al., 1994) and still contain a binding region for DNA polymerase alpha. It has been reported that large T antigen expression affects the duration of the cell cycle by increasing G2 and M and decreasing G1 (Sladek and Jacobberger, 1992). Since proteins necessary for DNA synthesis are often synthesized in G1, a shortened G1 could result in a depletion of these proteins and may affect the accuracy of cellular DNA synthesis.

SV40 large T antigen also induces genomic instability at early passages after transformation (Moorehead and Saksela, 1963; Todaro, 1963; Wolman; 1964) as do the viral proteins of other DNA tumour viruses such as polyomavirus, adenovirus and human papillomavirus as previously mentioned (Graham et al., 1977; Braithwaite et al., 1983; Caporossi and Bacchetti, 1990; St. Onge et al., 1990; 1993 a,b; Debbas and White, 1993; White et al., 1994). The presence of small t, viral replication and large T nuclear localization which are not required for transformation, are also not required for this effect (Ray et al, 1990; Stewart and Bacchetti, 1991; Woods et al., 1994).

The persistent presence of SV40 large T antigen is required in throughout the cell cycle and specifically in G2 in order to induce aneuploidy and possibly structural aberrations in human cells in support of the hypothesis that the viral protein is responsible for the induction of genomic instability (Friedrich et al., 1994). It is possible that mutations caused by this instability also aid in the development of immortalization and a neoplastically transformed phenotype in human cells. Human cells transformed with SV40 large T antigen, as well as by proteins from other DNA tumour viruses, gradually over time become tumorigenic without the need for additional treatment, implying that the mutagenic effect of these viral proteins may be sufficient (DeRonde et al., 1989; Ray and Kraemer, 1993).

The contribution of SV40 small t antigen in the process of transformation has not been studied in great detail, though the protein is required for the morphological transformation of human fibroblasts as assayed by focus formation (DeRonde et al., 1989). In human cells, small t interacts with protein phosphatase 2A to stimulate the map kinase pathway and induce cell proliferation (Sontag et al., 1993). This indicates that small t contributes to the proliferative phenotype of transformed cells. The role of small t antigen in the transformation of rodent cells is less clear but it may play an enhancing role when the concentration of large T antigen is

limiting (Bikel et al., 1987). The mechanism of this effect remains unknown.

1.5 Genetic Instability, Cancer and Cell Transformation

1.5.1 Genetic Instability and Cancer

For a cell to change from normal growth patterns to the rapid cell division common in tumours likely requires many mutations in the genome (Bishop, 1987; Klein, 1987; Tomatis, 1989; Boyd and Barrett, 1990). One cell may acquire a mutation, either spontaneously or through exposure to a carcinogen, that confers an increased growth rate and extended lifespan allowing this cell to out-compete normal cells. This deregulation of growth may result in the destabilization of the genome, as seen in many tumours. This destabilization may be caused by unregulated and inappropriately timed DNA synthesis and mitosis, and along with the rare occurrence of other growth supporting or cell death-avoiding mutations that may lead to the possible immortalization of the cell during the progression of the cancer. Furthermore, most carcinogens induce mutations in the DNA and other structural chromosome abnormalities reinforcing the importance of genomic instabilities in tumour progression.

Two types of genes are usually targeted in the evolution of most cancer: proto-oncogenes and tumour suppressor genes. The activation of proto-oncogenes results in

proliferative growth signals to the cell, while the tumour suppressor genes such as p53 and pRB are usually found deactivated resulting in the loss of growth restrictive signals. Activation of proto-oncogenes and deactivation of tumour suppressor genes can occur through two pathways: structural and conformational changes in the protein by point mutations and/or deletions in the gene; and/or deregulation of gene expression. Some examples of rearranged proto-oncogenes are c-abl and c-myc. Two of the most commonly affected tumour suppressor genes are RB and p53.

The c-abl proto-oncogene is activated in chronic myelogenous leukaemia through a translocation between chromosomes 22 and 9, resulting in the Philadelphia chromosome (Nowell and Croce, 1987; LeBeau, 1990). Burkitts lymphoma cells carry a similar translocation between chromosome 8, at the c-myc proto-oncogene locus and chromosomes 2, 14, or 22 at the loci for the heavy, kappa, or lambda chains of immunoglobin (Nowell and Croce, 1987; Evans, 1990; LeBeau, 1990) that results in the constitutive expression of c-myc.

The tumour suppressor gene retinoblastoma is inactivated by deletions and point mutations at the locus on chromosome 13 q14. As previously mentioned, alterations in this gene are present not only in retinoblastoma but also in other cancers such as osteosarcoma, soft tissue sarcomas and small cell lung carcinomas (Friend et al., 1986; Harbour et

al., 1988; Huang et al., 1988). Retinoblastoma is a common childhood tumour in which one defective RB allele is inherited and the other allele is subsequently altered, and is an example of the hereditary basis of cancer. Also previously mentioned, the p53 gene is also commonly affected in many cancers. This gene, located on the short arm of chromosome 17 in humans, has been found mutated in as many as 50% of human cancers (reviewed in Marx, 1993). As well, the genes of several proteins associated with or affected by p53 have also been found mutated in cancers and in genetic diseases which increase cancer susceptibility such as mdm-2 in sarcomas (Oliner et al., 1992; Leach et al., 1993), and genes regulating GADD45 expression in patients suffering from Ataxia-telangiectasia (Kastan et al., 1992), indicating that these proteins likely function in a pathway important for the regulation of cell growth.

Other examples of the importance of genetic mutations in cancer progression come from a variety of inherited syndromes all of which are characterized by the patients increased susceptibility to cancer. Xeroderma pigmentosum is associated with a high frequency of chromosome breakage in affected cells and a sensitivity to sunlight-induced skin cancer as well as other malignancies resulting from a deficiency in DNA repair mechanisms (Cleaver et al., 1990; reviewed in Muller, 1990). Ataxia-telangiectasia is a human

autoimmune disorder that is characterized by hightened sensitivity to radiation and an increased incidence of cancer (Gatti et al., 1991). AT cells fail to arrest DNA synthesis following irradiation due to the previously mentioned (Section 1.1.2) mutation in the AT genes that normally stimulate both p53 and GADD45 production to induce growth arrest (Kastan et al., 1992). Patients suffering from the Li-Fraumeni Cancer Syndrome show a high frequency of multiple types of cancer, resulting from an apparent inherited predisposition toward the development of cancer (Li et al., 1988). Cells from Li-Fraumeni patients are either heterozygous or homozygous for mutations in the p53 gene in support of not only the genetic basis of cancer but also the importance of p53 for normal cell growth (Livingstone et al., 1992; Yin et al., 1992). Finally, Bloom syndrome, is a rare autosomal recessive disease in which the cells of the affected individual show a high degree of genomic instability (Groeden et al., 1990) and once again the patients are predisposed toward the development of cancer. The affected gene has yet to be identified but is localized to the q arm of chromosome 15 (German et al., 1994).

1.5.2 SV40 Induced Genetic Instability

Investigating the causes of cytogenetic damage is critical in the study of cancer since genomic instability is a characteristic of many tumours and may contribute to the

development of a malignant phenotype (German, 1983; Yunis et al., 1987). Transformation of human cells in vitro by SV40 large T antigen has been used as a model to study the induction of genomic instability since genomic instability is also a characteristic of these cells.

Genomic instability can be detected in human cells early after transformation with SV40 large T antigen or infection with the SV40 virus. At first chromosome gaps and breaks are detected, then more complex aberrations, with dicentrics being the most common (Todaro, 1963; Wolman et al., 1964). Translocations and chromosome rings also occur. Most cells become aneuploid at some point. As a cell approaches crisis, the number and complexity of the aberrations increase (Wolman et al., 1964; Chang et al., 1986).

Since not all cells transformed by SV40 large T antigen survive crisis to become immortal, clearly the viral protein functions are not sufficient for this effect. The observed genomic instability however, may result in mutations in the appropriate genes allowing the avoidance of death at crisis and the generation of an immortal cell line. The number and complexity of aberrations may also be related to the ability of SV40 transformed human cells to induce tumours in nude mice. Cells from an early passage of a SV40 transformed population could not induce tumours while those from a later passage of the same populations could (Brown and Gallimore,

1987). This implies that new mutational events were required for these cells to become malignant.

Transfection of cells with plasmids expressing only SV40 large T antigen established this protein as necessary and sufficient for the induction of genomic instability at levels similar to those found in cells transformed with the entire SV40 genome (Chang et al., 1986; Ray et al., 1990; Stewart and Bacchetti, 1991). In addition, at non-permissive temperatures an SV40 mutant with a temperature sensitive large T antigen was unable to induce genomic instability but regained the ability at permissive temperatures (Gorbunova et al., 1982). A recent report states that the origin of replication and the early control region are sufficient to induce instability at regions of plasmid integration and that SV40 large T antigen is not required (Hunter and Gurney, 1994). However, the instability could result simply from the integration of foreign DNA and therefore is not representative of the overall genomic instability observed during transformation with SV40 large T antigen.

There are a number of functions of large T antigen that could be involved in the induction of genomic instability as there are for transformation. The protein can bind to specific viral and cellular DNA sequences (Prives et al., 1982; Paucha et al., 1986; Arthur et al., 1988). It has been suggested that protein-protein interactions between separate

SV40 large T antigen molecules located on different DNA sites could result in the recombination between the DNA at these sites (Schiedner et al., 1990). T antigen can also induce cellular DNA synthesis (Chou and Martin, 1975; Thompson et al., 1990) which could also contribute to genomic instability through unregulated or inappropriate timing of chromosome replication.

1.6 Hypothesis and Project Goals

DNA viruses such as SV40 can induce genomic instability at the early stages of transformation of human cells in vitro. This is of interest because human malignancies also show similar structural aberrations to that of large T antigen transformed cells in vitro. One hypothesis is that mutations caused by genetic instability may contribute to the rare immortalization of large T transformed cells in vitro and to the malignant transformation of cells in vivo. As well, the mechanism by which the virus induces genomic instability in vitro may be similar to the mechanism that causes instability in cancer cells and involve the same cellular factors.

The present study was undertaken to answer questions about the mechanisms behind SV40 induced genetic instability in human cells: (1) What are the specific functions of large T involved in the induction of genomic instability ? (2) What is the role of binding to p53 in the transformation of human

cells? The approach was to transfect both primary and established cells of human and rodent origin, with plasmids bearing the early region of SV40 and mutants thereof. Several independent clones were isolated for each plasmid used and cytogenetic analysis was done within 28 cell divisions of the original cell.

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

MATERIALS AND METHODS

2.1 Cells

Human skin fibroblasts, strains 423 (Chang et al., 1986) and NFS-5A (C.B. Harley, unpubl.), primary cultures of baby rat kidney (BRK) cells, and an established rat embryo fibroblast line (Rat II) were grown in ∝-MEM (GIBCO) with 10% fetal calf serum (FCS), 1-glutamine and antibiotics. HT1080 (Rasheed et al., 1974), a tumour derived cell line, was grown in ∝-MEM with 7.5% fetal calf serum, 1-glutamine and antibiotics. SV40 transformed African green monkey kidney cells (COS-1) (Gluzman et al., 1981) were grown in ∝-MEM with 10% newborn calf serum (NBCS), 1-glutamine and antibiotics.

2.2 Plasmids

pSV2neo and pSV3neo (Southern and Berg, 1982) contain the neomycin resistance (neo) gene or the SV40 early region and the neo gene respectively. The neo gene encodes the bacterial phosphotransferase APH(3')II, which inactivates the aminoglycoside G418, a toxic compound used for selection of expressing cells (Davies and Smith, 1978; Southern and Berg, 1982). pSV2his (Hartman and Mulligan, 1988) contains the histidinol (hisD) resistance gene encoding the bacterial

enzymes that allow the breakdown of histidinol into histidine. Histidinol, an analogue of the amino acid histidine, cannot be metabolized by cells not expressing the histidinol resistance gene (Hartman and Mulligan, 1988), and is used as a substitute for histidine in the selection of expressing cells. pUCT147 and pRT147 (Sompayrac and Danna, 1988 and 1992) have a point mutation that changes the codon for amino acid 148 of SV40 large T to a stop codon and therefore express the N terminal 147 amino acids of large T (T147) under the control of the SV40 promoter or a viral LTR respectively. Table 1 provides a summary of the SV40 T antigen mutants used. pRT147 also encodes the neo gene. pMTΔT147Ddl (Sompayrac and Danna, 1994) contains the neo gene and the truncated T antigen under the control of two separate mouse metallothionin promoters, the one for the truncated T antigen being mutated to allow inducible expression. A 21 bp deletion in the promoter reduces the basal level of expression but still allows for induction with 0.1 mM $ZnCl_2$. An additional deletion of the first exon of large T prevents the expression of small t antigen by pMTΔT147Ddl while pRT147 still maintains small t expression. pSV131 (Arthur et al., 1988, Dobbelstein et al., 1989) has the codon for amino acid 131 of large T changed to a stop codon and therefore expresses the N terminal 130 amino acids of large T antigen as well as small t. pSV2neoSV24 (Asselin and Bastin, 1985) has a deletion from nucleotide 4407 to

Table 1: Properties of T antigen mutants used in this study[a]

Mutant	Mutation	T expressed	t expression	Selectable Marker
T147	Δ148-708aa	1-147	+	neo
T147D	Δ148-708aa	1-147	-	neo
T137	Δ138-708aa	1-137	-	neo
T130	Δ131-708aa	1-130	+	-
T130-K1	Δ131-708aa glu(107)-lys	1-130	+	-
dl1135	Δ17-27aa	1-708 (Δ17-27)	-	neo
K1	glu(107)-lys	1-708	+	-
D10	lys(128)-thr	1-708	+	-
y2Xmet128-70K	Δ1-127aa lys(128)-met	128-708	-	-
dlA2433	Δ587-589aa	1-708 (Δ587-589)	+	-
5080	ile(573)-phe	1-708	+	+
5081	pro(584)-leu	1-708	+	+

a) references in text

nucleotide 3180 of large T, that produces a reading frame shift and results in the expression of a protein containing the first 137 amino acids of large T antigen plus 6 additional residues not normally occurring in large T. This construct also contains the neo gene and does not express small t. dlA2433 contains a 9 base pair deletion in large T that prevents the protein from binding to p53 by removing amino acids 587-589 (Cole et al., 1986; Tevethia et al., 1988). p5080 and p5081 (Peden et al., 1989) contain mutations in the p53 binding domain of large T. The mutation in p5080 prevents large T antigen from binding to p53, while the p5081 mutation is silent. Recombinants encoding the neo resistance gene were constructed by Catherine LeFeuvre (McMaster) and used for transfection. The three previous constructs all express small t. py2xmet128-70k (Anderson et al., 1988) contains a deletion of the first 127 amino acids of T antigen, a substitution of a met codon for the lys^{128} codon in the nuclear localization signal (Kalderon et al., 1984 a,b) and does not express small t. K1 (Kalderon et al., 1984; DeCaprio et al., 1988) encodes full length large T antigen with a point mutation in the pRB binding domain. D10 (Kalderon et al., 1984) encodes a full length large T antigen that has a point mutation in the nuclear localization signal. Both the K1 and D10 constructs express small t. pSV131-K1 (Dobblestein et al., 1992) contains the same deletion as the pSV131 construct with the addition of

the same point mutation as the K1 construct. pRSVBneo1135 (Symonds et al., 1993) has an amino terminal deletion of the nucleotides encoding amino acids 17 to 27 of large T. Due to a deletion of the splice site acceptor for small t, this construct expresses only large T. pJL16 and pJL17 (Bacchetti and Graham, 1993) encode wild type and a dominant mutant form of human p53 respectively under the control of the H-CMV promoter. The mutant protein has two point mutations: the first at amino acid 135 affects function; the second at amino acid 72 is functionally silent but changes the protein conformation and electrophoretic mobility in some cell types. All the plasmids were grown in Escherichia coli (strain DH5 ∝ or LE392), and supercoiled DNA was purified by isopycnic centrifugation in cesium chloride-ethidium bromide gradients. Plasmids not encoding the neo or hisD genes were transfected along with pSV2neo or pSV2his respectively.

2.3 Transfections

2.3.1 Carrier DNA Preparation

Carrier DNA was prepared from isogenic cells by pronase digestion, followed by phenol-chloroform extraction. The DNA was then dialysed extensively, first against sterile water and then against 0.1 x SSC to remove the phenol, and harvested aseptically (Graham and Bacchetti, 1983).

2.3.2 Calcium phosphate transfection

Transfections were done using the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). HT1080 and Rat II are established cell lines whose lifespan is unlimited and were used at the age for each transfection. Strains 423 (Chang et al, 1986) and NFS5a were used at approximately 20 population doublings. BRK cells were transfected as primary cultures. All cells were seeded at approximately 2.5 X 10^5 cells per 10 cm dish and transfected 24 hours later with 5-10 µg of plasmid DNA and high molecular weight isogenic carrier DNA. Following an overnight incubation with the DNA precipitate, the cells were refed with growth medium and 24 hours later with medium containing G418 (400 µg/ml; Geneticin, GIBCO) or 0.3 mM histidinol (Sigma). Following selection, clones were isolated and transferred to 25 cm^2 Corning bottle. At confluence, the cells were subcultured for immunostaining and cytogenetic analysis. In all cases, immunostaining was done at approximately 23 divisions of the parental cell, while cytogenetic analysis was done between 25 and 27 divisions of the parental cell.

2.4 Immunolabelling of Cells

Immunolabelling was done on cells grown on coverslips to 50-70% confluence. The coverslips were washed in PBS and then immersed in acetone for 15 minutes at -20 C for fixation.

The coverslips were then mounted on to slides and stored at 4 C for 16 to 18 hours before staining. Antibodies were obtained from Oncogene Science (MAb 416, PAb 1801 and 240) or harvested from hybridomas (MAb 412 and 419). Mouse monoclonal antibodies MAb 412 (Gurney et al., 1980) and MAb 419 (Harlow et al., 1981) which react with large T, and large T plus small t respectively as well as MAb 416 (Harlow et al., 1981) which reacts with the amino terminus of large T but does not recognize small t were used as primary antibodies. PAb 1801 (Banks et al., 1986) and PAb 240 (Gannon et al., 1990) which recognize both mutant and wild type or only mutant p53 respectively were used for p53 staining. The cells were reacted for 1 hour with primary antibody at a 1:500 dilution in 1 x TBS (1 M Tris, 2 M NaCl) and then washed twice for 10 min with 1 x TBS. The cells were reacted for 1 hour with a goat anti-mouse antibody conjugated to horse radish peroxidase (BioRad) at a 1:25 dilution in 1 x TBS, then washed twice for 10 minutes in 1 x TBS followed by two 10 minute washes with acetate buffer (20 mM Na acetate, 50 mM acetic acid). The staining was done for 10 minutes in AEC substrate solution [5% AEC (4% 3-amino-9-ethylcarbazole in NN-dimethylformamide) in acetate buffer plus 1 drop of 30% hydrogen peroxide]. Counterstaining was done for 5-60 seconds in Mayer's haematoxylin (Drury and Wallington ed., 1967) with blueing in Scot's tap water (Drury and Wallington ed., 1967). The cells

were then analyzed by light microscopy. Cells positive for SV40 tumour antigens expression stained red while negative cells were blue. Unless otherwise stated, 100% of the cells analyzed showed positive expression. It should be noted that immunostaining is not a quantitative technique and therefore was not used to accurately determine the level of protein expression.

2.5 Cytogenetic Analysis

Chromosome spreads were prepared from cells seeded at approximately 1/8 confluence and arrested 24-48 hours later in metaphase by treatment with 0.1 µg/ml colcemid for 3-5 hours. The cells were then trypsinized from the plate, suspended in pre-warmed hypotonic 0.075 M KCl and incubated at 37 C for 23 minutes. The cells were fixed with cold methanol: acetic acid (3:1) overnight (15-18 hours). Chromosome spreads were then made by dropping 3-5 drops onto cleaned, wet slides. In some cases the cells were grown on coverslips. The coverslips were removed from the plates and hypotonic treatment and fixation were as above. Cytogenetic analysis was done by light microscopy on chromosomes stained with 5% Giemsa.

50 metaphases from each clone were scored for number of structural aberrations and of chromosomes. In all cases cells containing 1 or more structural aberrations were considered aberrant regardless of ploidy. The total number of

structural aberrations in 50 metaphases was also counted. Cells with more than 50 chromosomes were considered aneuploid. In the case of HT1080 cells, aberrations associated with the cell line, such as marker chromosomes, were discounted to make results comparable between cell types.

2.6 Western Immunoblot

Lysates were prepared from cells growing in 60 mm plates by washing the monolayer with PBS, adding 500 µl of NP40 lysis buffer, collecting the lysate into an eppendorf tube and incubating on ice for 30 minutes, then centrifuging to remove the cell pellet. The amount of protein in each sample was quantitated using the BioRad DC protein assay for detergent containing samples. 30 µg of total cellular protein was loaded on either a 9 or 12% mini gel (100 µg for a large gel). After electrophoresis, the gel was equilibrated for 30 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v)), then transferred to nitrocellulose using the BioRad transblot cell. The transfer was done at 70 V at 4 C for 1 1/2 hours for the mini gel and 5 hours for the large gel. The nitrocellulose membrane was then air dried and stored a 4 C. For the western blot, the membrane was rehydrated and blocked with 5% Carnation skim milk in PBS lacking calcium and magnesium. The blot was then incubated for 1 hour in the primary antibody [either MAb 412 (Gurney et al., 1980), MAb

416 (Harlow et al., 1981), or PAb 1801 (Banks et al., 1986)] diluted 1:500 in the milk solution. The blot was washed once for 15 minutes and 4 times for 5 minutes with PBS, then incubated for 1 hour with the secondary antibody (goat anti-mouse hydrogen peroxidase conjugated antibody (BioRad)) diluted 1:1000 in the milk solution. The blot was then washed again as described above, and developed for 1 minute using the DuPont NEN chemiluminescence reagents. Excess reagents were removed by blotting the membrane in Whatmann paper. The membrane was then exposed for 5 seconds to 1 minute on DuPont NEF reflections autoradiography or Kodak XAR-5 preflashed film. Attempts to do Western blots with MAb 419 were unsuccessful due to high background and non specific interactions, though, MAb419 was used successfully by Catherine LeFeuvre for the Western blot of the D10 expressing clones.

RESULTS

3.1 Cytogenetic Analysis of Clonal Cell Populations Expressing Carboxy-terminal Truncations of SV40 Large T Antigen

Previous work has established that the expression of SV40 large T antigen is sufficient for genome destabilization (Ray et al., 1990; Stewart and Bacchetti, 1991). In order, to correlate the induction of genomic instability with specific functions of the protein, a series of mutants of large T antigen were analyzed. Mutants encoding the amino terminal sequences of large T antigen: T147, T137, and T130, consisting of the first 147, 137, and 130 amino acids respectively, are known to transform and immortalize rodent cells with varying degrees of efficiency. (Sompayrac and Danna, 1988, 1991, 1992; Asselin and Bastin, 1985; Arthur et al, 1985). To investigate whether these mutants were also capable of inducing genomic instability, in support of the hypothesis that this mutagenic process contributes to immortalization (Ray et al., 1990; Stewart and Bacchetti, 1991), cells were transfected with these mutants and analyzed as described in Materials and Methods. As stated in the that section some of the plasmid constructs

Figure 2: Cytogenetic Analysis of HT1080 and BRK Cells Tranfected with the pRT147 Mutant. The mean number of aberrant (■), of aneuploid (▩) cells and of aberrations (▨) per 50 cells are indicated for each group of clones transfected with a given plasmid. Error bars above each column represent the standard error of the mean. The number of clones in each group is indicated in brackets below the plasmid name. pSV2neo and pSV3neo contain respectively the neomycin resistance gene or this gene plus the entire SV40 early region. pRT147 encodes a truncated T antigen (aa 1-147) under control of a retroviral LTR and also expresses small t. Results from clones derived from HT1080 and baby rat kidney cells are shown in panels A and B respectively.

used express small t while others do not. Since, it has been shown previously that small t is not required for induction of genomic instability (Ray et al., 1990; Stewart and Bacchetti, 1991), small t expression was not taken into consideration in the analysis of the results. pSV2neo was cotransfected with the plasmid constructs that do not encode the neo gene (Table 1).

Plasmids expressing T147 under the control of the SV40 (pUCT147) or an LTR (pRT147) promoter were transfected into human neonatal skin fibroblasts (strain NFS-5A), and adult human fibroblasts (strain 423) (Chang et al, 1986). The same cell types were also transfected with pSV3neo, which encodes the wild type SV40 early region linked to the neo gene, or with pSV2neo, which encodes only the neo gene. These controls were repeated for each transfection. Although, many clones were consistently obtained with the pSV2neo and pSV3neo plasmids, repeated attempts to obtain G418 clones expressing the truncated T antigen were unsuccessful. In most cases no clones were obtained following selection. When clones did arise, none were found to express the T147 protein or small t. Since previous work with these particular constructs had been done using rodent cells, to assess whether the problem was species related control transfections were carried out with the pRT147 construct in primary baby rat kidney cells (BRK) and the

established rat cell line, RatII. In addition, to assess whether the problem was specific to primary human cells, HT1080, an established human cell line, was also transfected. Clones expressing T147 were obtained in every cell type along with the appropriate control clones. Since BRK cells cannot be passaged in culture without being transformed no clones containing pSV2neo as negative control were obtained with these cells.

The results of immunostaining for T147 expression in HT1080, Rat II and BRK cells are given in Table 2, and compared to the immunostaining of pSV2neo and pSV3neo controls. As expected, cells transfected with pSV2neo were negative for expression of SV40 large T antigen, showing the blue colour of the counterstain and no reactivity to the MAb 419 antibody. Cells transfected with pSV3neo were positive for expression of the tumour antigen indicated by the red staining of the cells with an intensity slightly lower than that of SV40 transformed cos-1 cells and, in the case of HT1080 and BRK cells, variable between clones. Clones expressing T147 did so at a level that was somewhat lower than in pSV3neo controls, but also variable between clones and within each clonal population.

Cytogenetic analysis revealed that HT1080 or BRK cells expressing T147 were aberrant, in terms of both chromosome number and structure. As shown in Figure 2 and Tables 3 and

Table 2: Intensity of Immunostaining of HT1080, BRK and Rat II Cells Transfected with pSV2neo, pSV3neo or pRT1147

Cells	Clone	Plasmid Transfected	Intensity with 419[a]
HT1080	1	pSV2neo	-
	2	"	-
	3	"	-
	4	"	-
	1	pSV3neo	+++
	2	"	++
	3	"	+++
	1	pRT147	++
	2	"	++
	3	"	++
	4	"	++
	5	"	++
BRK	1	pSV3neo	++
	2	"	+++
	3	"	+++
	1	pRT147	+
	2	"	++
	3	"	++
	4	"	+
	5	"	+
Rat II	1	pSV2neo	-
	2	"	-
	3	"	-
	1	pSV3neo	+++
	2	"	+++
	3	"	+++
	1	pRT147	++
	2	"	++
	3	"	++

a) intensity with respect to SV40 transformed African Green Monkey cells (cos-1) (++++) with MAb 419

Figure 2: Cytogenetic Analysis of HT1080 and BRK Cells Tranfected with the pRT147 Mutant. The mean number of aberrant (■), of aneuploid (▩) cells and of aberrations (▨) per 50 cells are indicated for each group of clones transfected with a given plasmid. Error bars above each column represent the standard error of the mean. The number of clones in each group is indicated in brackets below the plasmid name. pSV2neo and pSV3neo contain respectively the neomycin resistance gene or this gene plus the entire SV40 early region. pRT147 encodes a truncated T antigen (aa 1-147) under control of a retroviral LTR and also expresses small t. Results from clones derived from HT1080 and baby rat kidney cells are shown in panels A and B respectively.

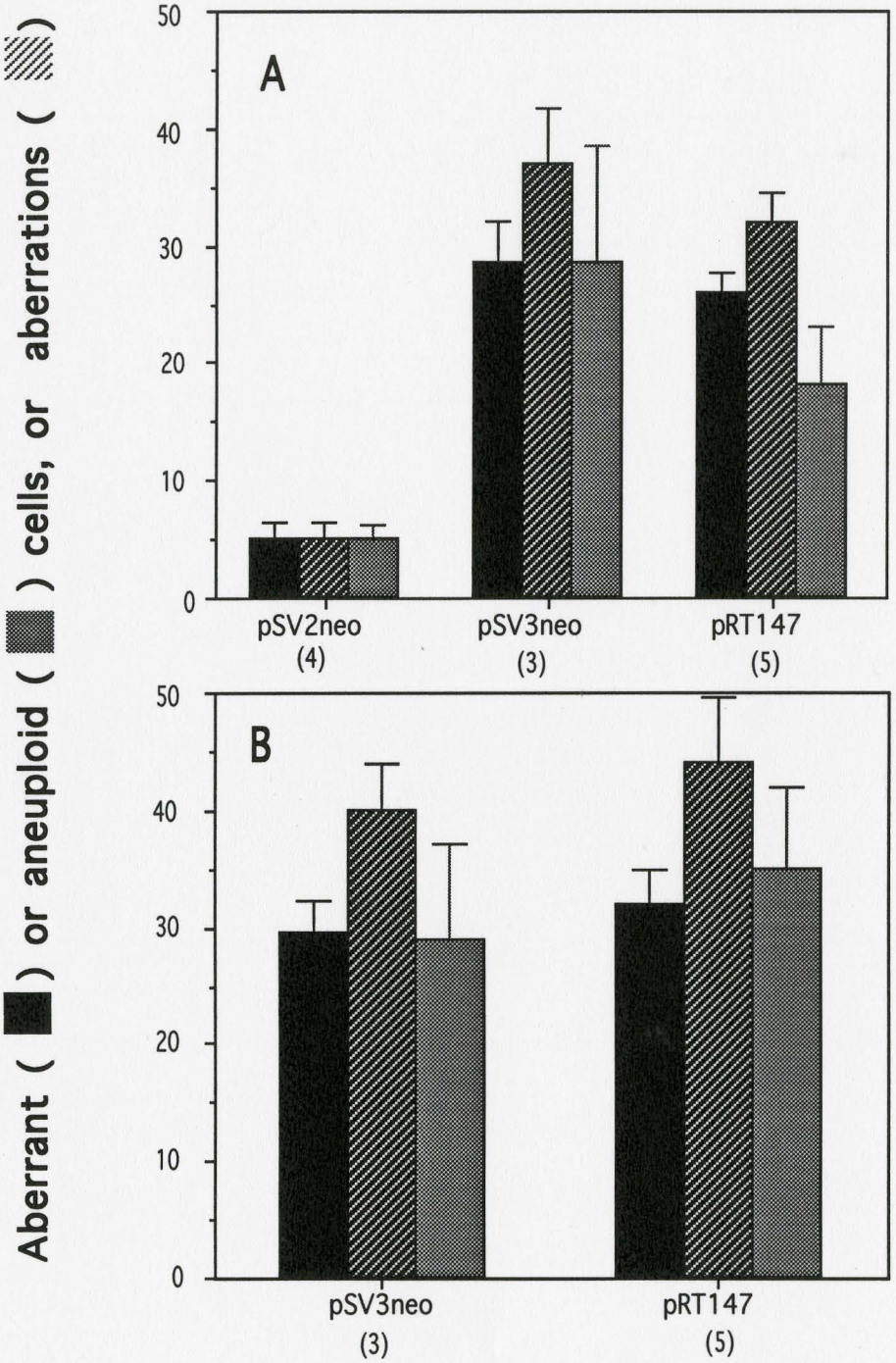

Aberrant ( ) or aneuploid ( ) cells, or aberrations ( )
A
B
50
40
30
20
10
0
pSV2neo
(4)
pSV3neo
(3)
pRT147
(5)
pSV3neo
(3)
pRT147
(5)

Table 3: Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo or pRT147

# OF ABERRANT METAPHASES IN 50 CELLS												
	pSV2neo				pSV3neo			pRT147				
Clone	1	2	3	4	1	2	3	1	2	3	4	5
Aneuploid	8	2	4	5	32	10	44	36	12	18	17	7
With Structural Aberrations	7	1	6	6	31	21	33	27	22	28	31	22
STRUCTURAL ABERRATIONS IN 50 CELLS												
Breaks	2		5	2	23	14	22	20	9	19	27	14
Fragments				2			3	2	2	10		
Double Minute								1		3		2
Di/tricentrics	5	1	1		20	11	15	15	14		11	11
Rings				1								1
Translocations				1								1
Duplications												
Total	7	1	6	6	43	28	41	38	25	31	38	29

50 metaphases were scored for each clone and the number of structurally aberrant and of aneuploid cells as well as the total number of structural aberrations in 50 cells were totaled

Table 4: Cytogenetic Properties of BRK Cells Transfected with pSV3neo or pRT147

# OF ABERRANT METAPHASES IN 50 CELLS								
	pSV3neo			pRT147				
Clone	1	2	3	1	2	3	4	5
Aneuploid	41	32	14	26	50	47	12	41
With Structural Aberrations	24	33	32	29	41	3	23	33
STRUCTURAL ABERRATIONS IN 50 CELLS								
Breaks	14	21	16	22	33	26	9	16
Fragments	9	6	8	10	9	1	2	
Double Minute		1	4	1	1	4		4
Di/tricentrics	9	17	14	7	15	21	12	23
Rings					1		2	1
Translocations			1				1	
Duplications					2			
Total	32	45	43	40	59	52	26	44

50 metaphases were scored for each clone and the number of stucturally aberrant and of aneuploid cells as well as the total number of structural aberrations in 50 cells were totaled.

Table 5: Statistical Analaysis of Cytogenetic Properties of HT1080 and BRK Cells Transfected with pSV2neo, pSV3neo or pRT147

Cell Type	Plasmid	Aberration	wrt[a] pSV2neo	wrt[a] pSV3neo
HT1080	pSV2neo	# of aneuploid cells	----	0.0765
		# of aberrant cells	----	0.0047[c]
		total # of structural aberrations	----	0.0030
	pRT147	# of aneuploid cells	0.1072	0.3176[b]
		# of aberrant cells	0.0002	0.5394
		total # of structural aberrations	0.0003	0.3296
BRK	pRT147	# of aneuploid cells	----	0.6602
		# of aberrant cells	----	0.5952
		total # of structural aberrations	----	0.6200

a) wrt = with respect to
b) values above 0.05 indicate there is no significant difference between the two data sets.
c) values below 0.05 indicate that the two data sets are significantly different.

4, the levels of structural aberrations were comparable to the pSV3neo positive controls and significantly higher than those of the pSV2neo negative controls. Statistical analyses, done in all cases by the T-test using the NCSS stats program at a 95% confidence interval, revealed no significant difference between the overall level of structural aberrations in clones expressing T147 and those expressing full length large T antigen (pSV3neo) (Table 5). However, in HT1080, pSV2neo clones were not significantly different from T147-expressing cells in terms of frequency of aneuploid cells. It should be noted that the statistical analysis is only as accurate as the data provided. The 95% confidence interval means that there is a 95% chance that the statistic is meaningful. The error bars on the graphs also represent the distribution of the data, but it should be considered that these are only accurate within a 70% confidence interval. In many of the sets of clones for the mutant T antigens described in this study there is often an outlier clone with values in terms of structural aberrations and more frequently aneuploidy extremely divergent from the rest of the population. The existence of this clone greatly widens the distribution of the data and increases the level of error in statistical comparison with other data sets. In order to rectify this problem a much larger number of clones would need to be analyzed to even out the distribution of

data points. As an example of this, the frequency of aneuploid cells varies greatly between clones for many of the constructs used. This may be due to a temporal difference in the first appearance of an aneuploid cell in the population and results in a wide range of values, making accurate statistical analysis difficult without a much larger sample size than used here.

The cytogenetic analyses are detailed in Tables 3 (HT1080), 4 (BRK) and 6 (Rat II). For HT1080, pSV3neo cells contained on average 28 structurally aberrant and aneuploid cells in a total of 50 cells; T147 transfectants contained 26 structurally aberrant and 18 aneuploid cells, whereas pSV2neo transfectants contained only 5 of each in 50 metaphases (Table 3). Structural aberrations consisted mostly of chromosome breaks and dicentrics with double minutes and translocations being less frequently observed. The total number of structural aberrations per aberrant cell was constant in clones expressing pSV2neo, pSV3neo, T147 and as shown later, other T antigen mutants, and likely limited by the extent of structural aberrations an individual cell can survive. Thus this parameter appears dependent upon the number of structurally aberrant cells in the population and may be redundant. Cytogenetic analysis of BRK cells (Table 4), revealed that while the level of both structural aberrations and aneuploidy detected in pSV3neo transfectants

Table 6: Cytogenetic Properties of Rat II Cells Transfected with pSV2neo, pSV3neo or pRT147

# OF ABERRANT METAPHASES IN 50 CELLS			
	pSV2neo	pSV3neo	pRT147
Aneuploid	34	41	18
With Structural Aberrations	9	30	21
STRUCTURAL ABERRATIONS IN 50 CELLS			
Breaks	12	12	7
Fragments		6	
Double Minute		6	14
Di/tricentrics		15	5
Rings			
Translocations			
Duplications	1		
Total	13	39	26

50 metaphases were scored for each clone and the number of structurally aberrant and of aneuploid cells as well as the total number of structural aberrations in 50 cells were totaled.

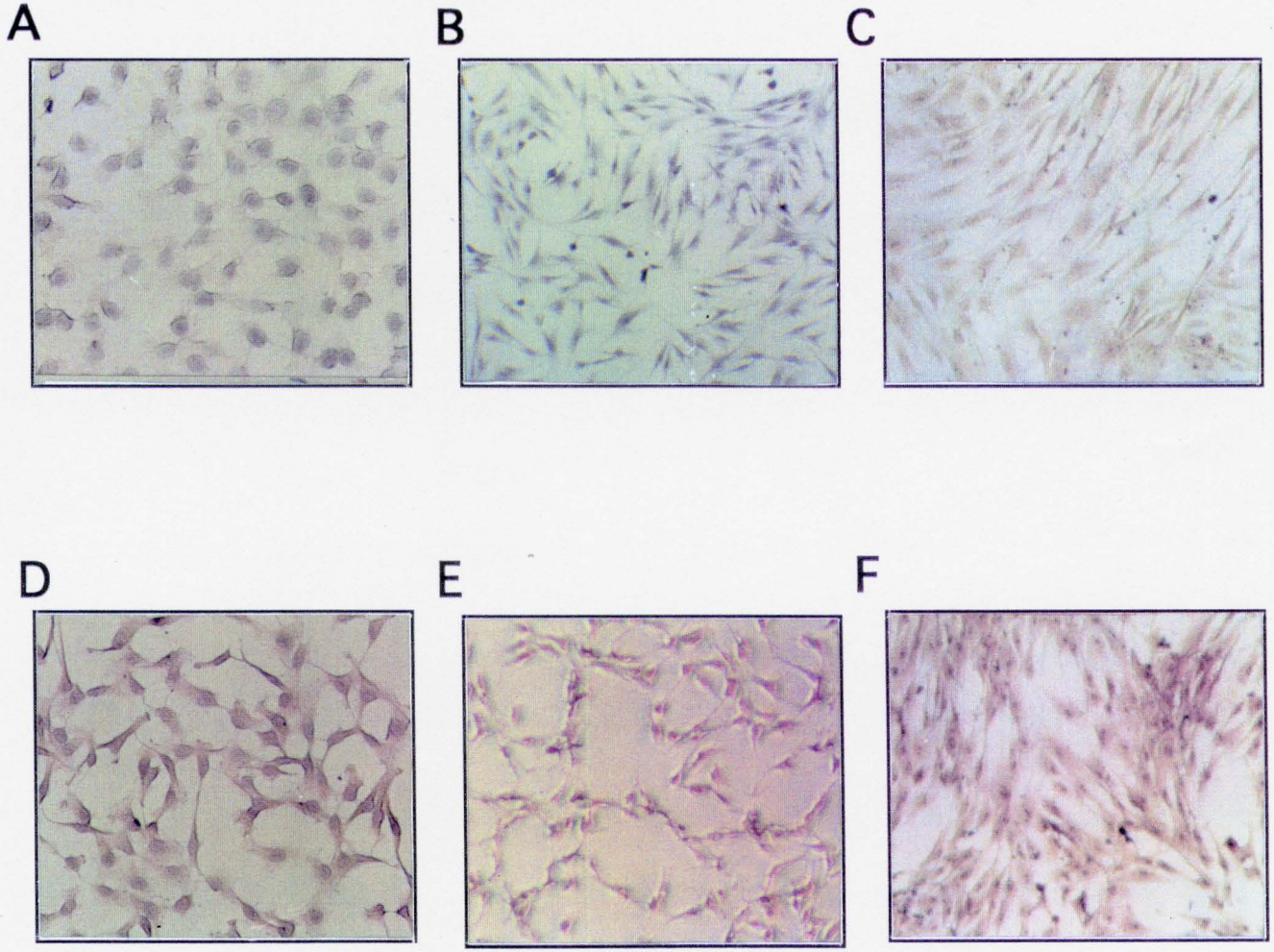
were similar to those of HT1080 and Rat II pSV3neo clones, a slightly higher level of structurally aberrant cells on average occurred in T147 transfectants when compared to HT1080 and Rat II. This could possibly indicate a difference in the degree of sensitivity to the T147 protein between cell types, although the levels of aberrations were similar between cell types when individual clones are considered. A much larger number of clones from both cell types would need to be analyzed to determine if this difference was significant. Cytogenetic analyses of one RATII clone for each plasmid type transfected gave results comparable with those of HT1080 and BRK with all constructs (Table 6) in terms of number of structural aberrations. The clone containing pSV2neo showed a high number of aneuploid cells even in comparison to the pSV3neo clone. Had a more extensive analysis of Rat II clones containing pSV2neo been carried out, it is probable that the number of aneuploid cells observed in this particular clonal population was the exception rather than the rule. However, since the results with HT1080 and BRK cells indicated that expression of T147 was not restricted to rodent cells and not dependent upon the established or primary status of the cells, no further analyses of Rat II was done.

These results described above indicate that amino acids 1-147 of SV40 large T antigen are sufficient for

genome destabilization in primary and in established rat cells and in established human cells. Combined with the inability to obtain clones from primary human cells following transfection with pRT147, these results also imply that the truncated T antigen may be growth inhibitory to primary human cells.

In order to further investigate the effect of T147 on primary human cells, human fibroblasts (strain NFS-5A), and HT1080 cells and Rat II cells as controls, were transfected with a T147 construct under the control of an inducible metallothionein promoter (pMTΔT147; Sompayrac and Danna, 1994). As described in the Materials and Methods, this promoter has a 21 bp deletion that prevents basal level expression while still allowing induction with $ZnCl_2$. Clones were isolated by G418 selection without inducing expression of the truncated T antigen, and then were expanded for analysis in the absence and presence of 0.1 mM $ZnCl_2$ for induction of expression. In the absence of induction the protein was undetectable by immunostaining with MAb 419 in both HT1080, and Rat II cells but, was detected at very low levels in fibroblasts (Figure 3A, 3B, 3C), suggesting that the promoter may be marginally leaky in the latter cells. Expression of the truncated T antigen increased in all three cell types after induction with $ZnCl_2$. HT1080 and Rat II transfectants were induced for 40 hours only. Human

Figure 3: Expression of T147 in HT1080, Rat II Cells and Human Fibroblasts (Strain NFS-5A) by Immunostaining. The panels show expression both before and after induction with 0.01 mM $ZnCl_2$ in cells transfected with pMTΔT147Ddl. Clonal populations were immunostained with MAb 419 before induction with $ZnCl_2$: A, HT1080; B, Rat II; C, human fibroblasts (strain NFS-5A), and after induction with $ZnCl_2$ D, HT1080 (40 hours in $ZnCl_2$); E, Rat II (40 hours in $ZnCl_2$); F, human fibroblasts (strain NFS-5A) (70 hours in $ZnCl_2$).


A
B
C
D
E
F

fibroblasts transfectants were induced for 40, 70 or 150 hours (Figure 3). For latter cell type, only the uninduced and 70 hour time point are shown in figure 3.

Since, as mentioned in the Materials and Methods, in my hands MAb 419 gave high background and non specific recognition in Western blot, MAb 416 was used to detect protein expression. However, attempts to look at T147 expression in HT1080 cells by Western blot were unsuccessful even with this antibody. Since T147 can be detected by immunostaining with both MAb 416 and 419, this failure to detect expression by Western remains unexplained.

Cytogenetic analysis showed that without induction, the karyotype of pMTΔT147 transformed HT1080 cells remained normal (Figure 4). Upon induction with $ZnCl_2$ for 40 hours the karyotype became significantly aberrant and was comparable to that of the pSV3neo control cells (Figure 4 and Table 7 and 8). The pMTΔT147 transformed fibroblasts without induction appeared slightly more aberrant karyotypically than pSV2neo negative controls, with 6 structurally aberrant and 8 aneuploid cells per 50 metaphases on average whereas pSV2neo transfectants had 1 structurally aberrant and 2 aneuploid metaphases (Figure 4, Table 9). Although, statistical analysis revealed these differences were not significant (Table 10), this result is in agreement with the immunostaining data suggesting

Figure 4: Cytogenetic Analysis of HT1080 Cells and Human Fibroblasts (Strain NFS-5A) Transfected with the pMTΔT147Dd1 Mutant. Symbols are as in Figure 2. pMTΔT147Dd1 encodes a truncated T antigen (T147) under control of an inducible metallothionin promoter. Results from clones derived from HT1080 and human fibroblasts (strain NFS-5A) are shown in panels A and B respectively. Two of the pSV2neo clones from human fibroblasts included here were analyzed by Catherine LeFeuvre.

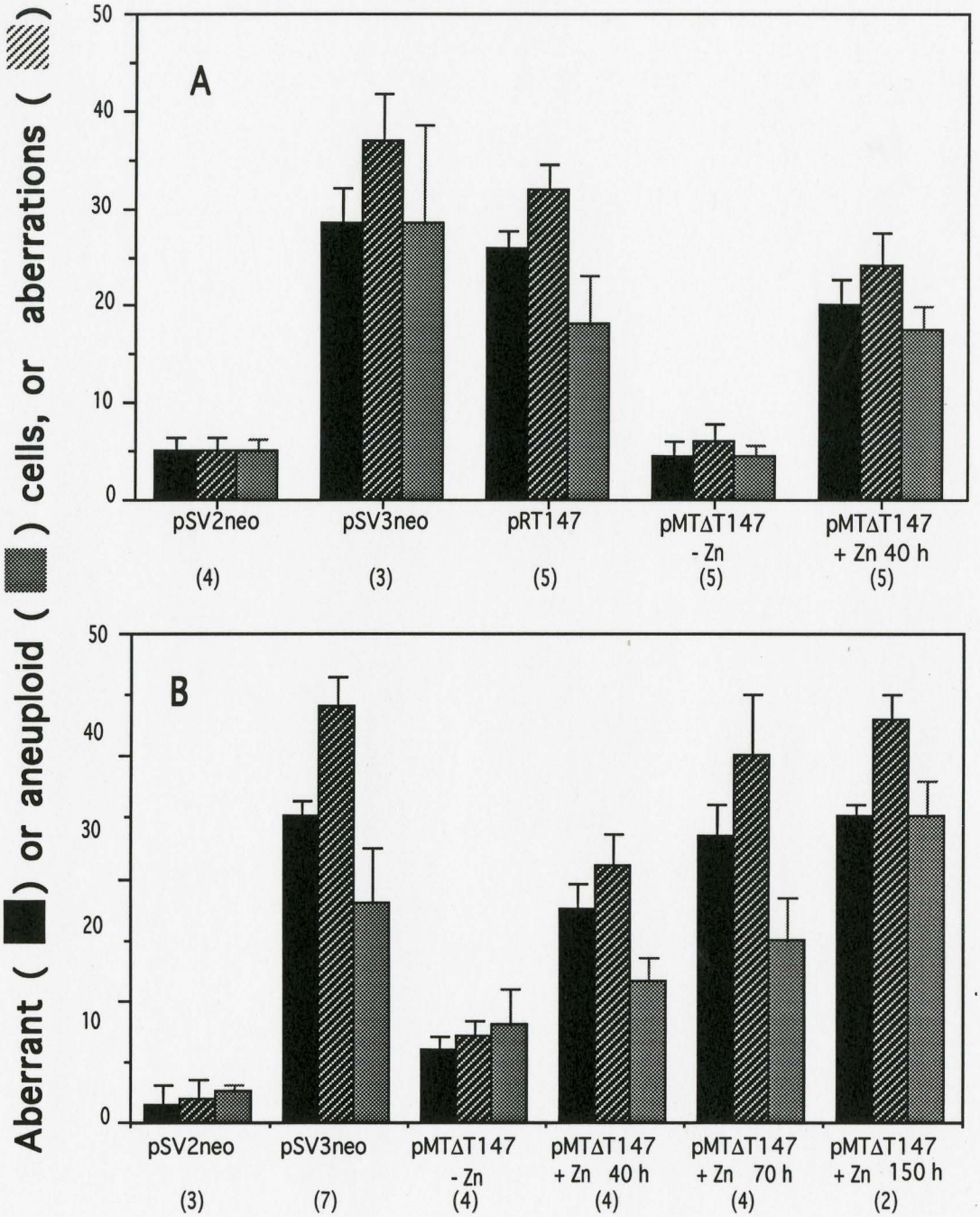

Aberrant (■) or aneuploid (▒) cells, or aberrations (▨)
A
50
40
30
20
10
0
pSV2neo
(4)
pSV3neo
(3)
pRT147
(5)
pMTΔT147
- Zn
(5)
pMTΔT147
+ Zn 40 h
(5)
B
50
40
30
20
10
0
pSV2neo
(3)
pSV3neo
(7)
pMTΔT147
- Zn
(4)
pMTΔT147
+ Zn 40 h
(4)
pMTΔT147
+ Zn 70 h
(4)
pMTΔT147
+ Zn 150 h
(2)

Table 7: Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo or pMT ΔT147Ddl

Clone	Aneuploid Cells	Aberrant Cells	Breaks	Fragments	Double Minute	Di/ Tricentrics	Rings	Translocations	Duplications	Total
pSV2neo										
1	8	7	2			5				7
2	2	1				1				1
3	4	6	5			1				6
4	5	6	2	2			1	1		6
pSV3neo										
1	32	31	23			20				42
2	10	21	14			11	1		1	28
3	44	33	22	3		15		1		41
no Zn										
1	6	7	4			3		1		8
2	1	3	2			4				6
3	7	9	7	1	1	2	1			12
4	4	1	1			1				2
5	5	3	2			1				3
+ Zn										
1	18	16	13		1	4		2		20
2	20	25	18	2		9		3		32
3	24	25	22			6		3		31
4	14	22	13		2	5		2		22
5	20	11	7	1	1	3		1		13

Cells were induced for 40 h by adding $ZnCl_2$ to a concentration of 0.1 mM along with fresh growth media every 24 hours. 50 metaphases of each clone were scored for structurally aberrant and aneuploid cells as well as the total number of structural aberrations in 50 cells.

Table 8: Statistical Analysis of Cytogentic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo or pMTΔT147Ddl

Plasmid	Aberration Type	wrt[a] pSV2neo	wrt[a] pSV3neo	wrt[a] no Zn
pSV2neo	aneuploid	----	0.0765	----
	aberrant	----	0.0047	----
	# struct. ab.	----	0.0030	----
pMTΔT147Ddl	aneuploid	0.9729[b]	0.0174[c]	----
	aberrant	0.9786	0.0004	----
	# struct. ab.	0.5987	0.0003	----
pMTΔT147Ddl 40h	aneuploid	0.0112	0.1994	0.0014
	aberrant	0.0082	0.1102	0.0012
	# struct. ab.	0.0086	0.5750	0.0024

a) wrt= with respect to
b) Values higher than 0.05 indicate there is no significant difference between the two data sets.
c) Values below 0.05 indicate that the two data sets are significantly different.

Table 9: Cytogenetic Properties of Human Fibroblasts (strain NFS-5A) Transfected with pSV2neo, pSV3neo or pMTΔT147Dd1

Plasmid	Clone	Aneuploid Cells	Aberrant Cells	Breaks	Fragments	Double Minute	Di/ Tricentrics	Rings	Translocations	Duplications	Total
pSV2neo	1	3	0								0
	2	0	0								0
	3	2	3	2			1				3
pSV3neo	1	17	23	10	4	5	8				27
	2	25	31	17		2	18		1		38
	3	29	27	12	1	2	21		1	1	38
	4	30	24	8	2		20		1		31
	5	28	26	15	3	1	13				32
pMTΔT147	1	1	4	1	1		1		1		4
no Zn	2	14	5		2		1			2	5
	3	6	7	1			2	1	2	2	8
	4	11	9				3			7	10
	1	16	20	7	2		8	1	5		23
	2	14	15	9	3	1	3		2		18
40 h	3	8	13	5	2		3		1	5	16
	4	9	22	8	5		9	4	2		28
	1	9	16	3	4		4		3	6	20
70 h	2	13	28	13	3	1	16	2	5	2	42
	3	12	19	5	2	1	13		2		42
	4	25	27	7	1		9		1	15	33
	1	28	26	10	3	1	18		2	1	35
150 h	3	22	24	5	2	2	15	1	4	2	31

Induction was by $ZnCl_2$ to 0.1 mM to the growth media every 24 hours for the noted time. 50 metaphases were scored for each clone the same as for table 4.

Table 10: Statistical Analysis of Cytogenetic Properties of Human Fibroblasts (Strain NFS-5A) Transfected with pSV2neo, pSV3neo or pMTΔT147Ddl

Plasmid	Abberation Type	wrt[a] pSV2neo	wrt[a] pSV3neo	wrt[a] no Zn	wrt[a] 40h Zn	wrt[a] 70h Zn
pSV2neo	aneuploid	----	0.1244[b]	----	----	----
	aberrant	----	0.0001[c]	----	----	----
	# struct. ab.	----	0.0003	----	----	----
pMTΔT147Ddl	aneuploid	0.2698	0.1560	----	----	----
	aberrant	0.0668	0.0000	----	----	----
	# struct. ab.	0.0809	0.0000	----	----	----
pMTΔT147Ddl 40h	aneuploid	0.0337	0.3454	0.3187	----	----
	aberrant	0.0082	0.0120	0.0032	----	----
	# struct. ab.	0.0088	0.0089	0.0030	----	----
pMTΔT147Ddl 70h	aneuploid	0.0815	0.6357	0.1872	0.4832	----
	aberrant	0.0046	0.4861	0.0007	0.1260	----
	# struct. ab.	0.0208	0.3945	0.0047	0.1969	----
pMTΔT147Ddl 150h	aneuploid	0.0026	0.8580	0.0219	0.0181	0.1407
	aberrant	0.0005	0.6369	0.0005	0.0798	0.6645
	# struct. ab.	0.0005	0.9592	0.0004	0.0494	0.6695

a) wrt = with respect to
b) Values above 0.05 indicate that there is no significant difference between the two data sets.
c) Values below 0.05 indicate that the two data sets are significantly different.

leakiness of the pMTΔT147 promoter in these cells. Upon induction of expression of the truncated T antigen, the karyotype of human fibroblasts transformed with pMTΔT147Dd1 became significantly aberrant (Figure 4 and Table 9 and 10) and the number of aberrant cells both in terms of aneuploidy and structural aberrations increased with the length of induction. Controls consisting of pSV3neo and pSV2neo transformed human fibroblasts exposed to $ZnCl_2$ for similar periods of time were not significantly different in terms of karyotype from untreated cells of the same clonal populations (not shown). Therefore, it is unlikely that exposure to $ZnCl_2$ had any effect on the karyotype of the cells, and the observed changes can be attributed to T147 expression.

After 40 hours induction, human fibroblasts exhibited a level of genomic instability intermediate between those of pSV2neo and pSV3neo controls and significantly different from both. By 70 hours induction, the cytogenetic properties of human fibroblasts were not significantly different from those of the pSV3neo controls (Table 9 and 10). The level of genomic instability continued to increase after 150 hours induction in human fibroblasts. Endoreduplications were common aberrations in both uninduced cells and after 40 hours of induction, the majority of aneuploid cells being perfectly tetraploid with sister

chromosomes still aligned. After 70 and 150 hours, clones showed an increased number of metaphases with structural aberrations such as gaps, breaks, and dicentrics as well as aneuploid cells including a number of endoreduplicated chromosome complements (Table 9).

Rat II cells transfected with pMTΔT147Dd1 showed karyotypic changes similar to those of HT1080 cells in both type and level of aberrations upon induction for 40 hours (Table 11), therefore further cytogenetic analysis was not continued. As noted for the pSV2neo control in these cells, aneuploidy was higher than in the negative controls for other cell types, indicating a general instability of the Rat II genome with respect to chromosome number, or simply, as stated earlier, a clonal variation.

Of note, is the fact that following induction of the T147, human fibroblasts also demonstrated a shortened lifespan, relative to both pSV2neo negative control transfectants and uninduced cells from the same clonal populations. Beyond 150 hours of induction or after about 6 population doublings (PD) the expression of T147 resulted in growth arrest (Table 12), while the uninduced cells from the same clonal population survived an average of 14 PD and pSV2neo clones grew for 20 PD.

These results established that the T147 protein is as proficient as full length large T antigen in the

Table 11: Cytogenetic Properties of Rat II Cells Transfected with pSV2neo, pSV3neo or pMTΔT147Ddl

# OF ABERRANT METAPHASES IN 50 CELLS				
Clone	pSV2neo	pSV3neo	pMTΔT147Ddl no Zn	pMTΔT147Ddl + Zn
Aneuploid	34	41	35	42
With Structural Aberrations	9	30	9	21
STRUCTURAL ABERRATIONS IN 50 CELLS				
Breaks	12	12	5	10
Fragments		6		3
Double Minute		6	3	2
Di/tricentrics		15	2	12
Rings				
Translocations				
Duplications	1			
Total	13	39	10	27

50 metaphases were scored for each clone and the number of structurally aberrant and of aneuploid cells were totaled as well as the total number of structural aberrations in 50 cells. The cells were induced for 40 hours with 0.1 mM $ZnCl_2$

Table 12: Lifespan of Human Fibroblasts (Strain NFS-5A) Transfected with pMTΔT147Ddl

Cell Type	Plasmid	# of Clones	Average Lifespan
NFS-5A	pSV2neo	3	20 PD
	pMTΔT147Ddl uninduced	4	14 PD
	pMTΔT147Ddl induced	4	6 PD

The lifespan is calculated from the time the clone is first isolated in the case of the pSV2neo clones and from the time of first induction with the pMTΔT147Ddl clones. The induced and uninduced cell populations were carried in parallel.

induction of genetic instability in primary as well as established human cells. Furthermore, they indicate that expression of T147 is growth inhibitory in primary human cells and support the hypothesis that the inability of rescuing clones of human fibroblasts transfected with pRT147 may be related to the growth inhibitory effect of the T147 protein.

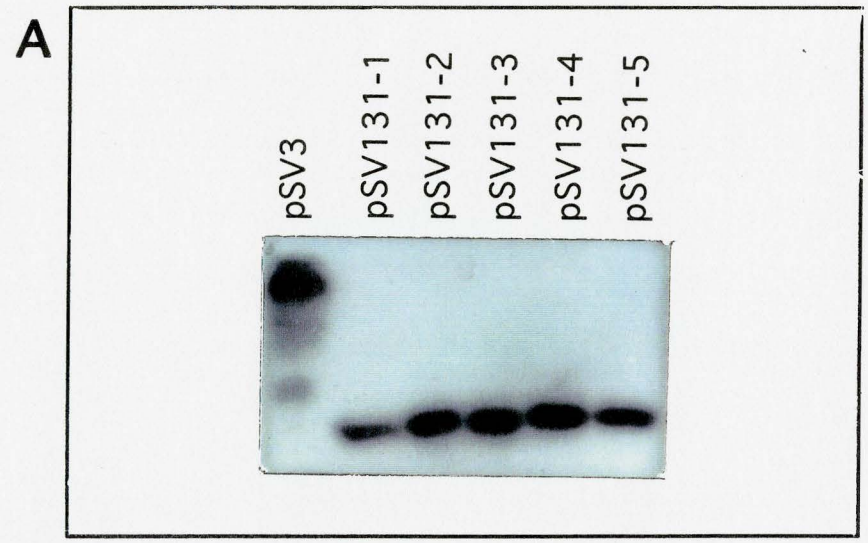
Among the functions of SV40 large T antigen that are deleted by the truncation to amino acid 147 is the ability to bind to p53. This in itself indicates that the T/p53 complex is not required for the induction of genomic instability. p53 is thought to play a role in monitoring the genome for aberrations in the DNA and to prevent aberrant cells from dividing until DNA repair is completed (reviewed in Lane, 1992). Since T147 does not bind p53, but still can induce aberrations, the inability of obtaining clones of primary human fibroblasts transfected with pRT147 and the limited growth of clones transfected with the inducible construct upon induction of expression of T147 may be explained by the presence of free p53 preventing aberrant cells from dividing. Therefore binding of p53 by T antigen appears to be required for the long term survival of aberrant cells as discussed further in section 3.3 of this report. A further implication from the data obtained with BRK cells is that rodent p53 may not be as stringent a

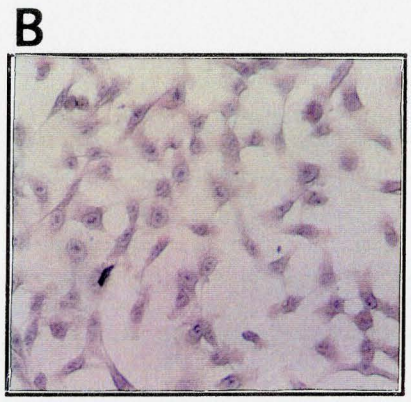
monitor of genome integrity as the human protein.

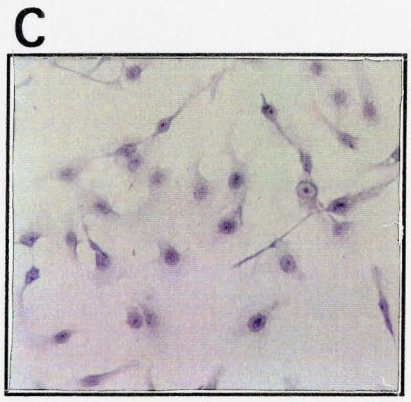
In order to further define the region of large T antigen required for the induction of genomic instability, plasmids pSV2neoSV24 and pSV131, expressing T137 and T130 under the control of the SV40 promoter, were transfected into HT1080 cells. These cells were used because the effect of full length T antigen in these cells was found to be comparable to that in human fibroblasts, and because these constructs, being deleted for the same functions as T147, are likely to be growth inhibitory in primary human cells.

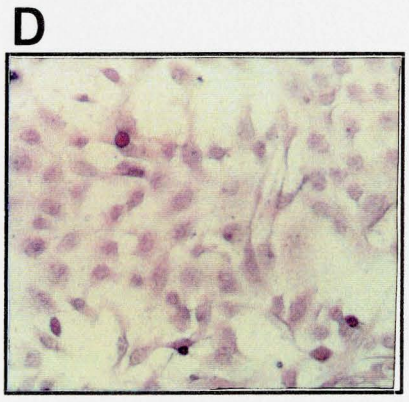
Clones were selected, isolated and subcultured in the same way as described earlier. Cells transfected with pSV131 stained red following immunostaining with MAb 419 (Figure 5D). The intensity of the stain varied across the population, with some cells showing a more intense red colour than others. Overall, however, it was comparable to that of a pSV3neo control (Figure 5B). A western blot using Mab 416 revealed that the pSV131 clones express T130 at varying levels, in most cases comparable to the expression of large T antigen in pSV3neo transfected cells (Figure 5A). Clones transfected with pSV2neoSV24 gave a much lower overall colour intensity after immunostaining with MAb 419 (Figure 5E). In some cases the red colour was almost undetectable indicating a low level of protein expression. This result was reproducible. Since pSV2neoSV24 does not

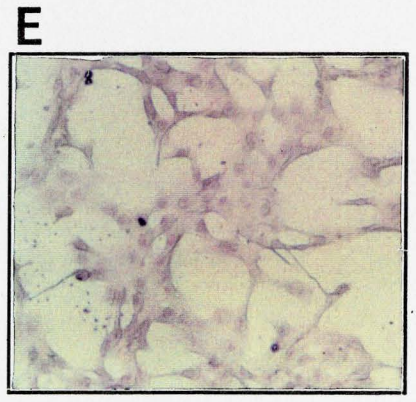
Figure 5: Expression of T130 and T137 in HT1080 Cells by Western Blot and Immunostaining. 5A, Cell lysates were prepared from pSV131 transformed populations and equal amounts of protein were separated by gel electrophoresis and transferred to nitrocellulose membranes. Following reaction with MAb 416, proteins were detected by chemiluminescence. Immunostaining with MAb 419. 5B, pSV3neo; 5C, pSV2neo; 5D, pSV131 (T130); 5E, pSV2neoSV24 (T137).


A
pSV3
pSV131-1
pSV131-2
pSV131-3
pSV131-4
pSV131-5


B


C


D


E

express small t, any positive signal must be due to expression of large T. As previously stated, Western blots using MAb 419 were unsuccessful, therefore MAb 416 was used. However, there was no detectable reaction of T137 with MAb 416, using both the immunostaining and western blot techniques, suggesting either very low protein expression or that the epitope for this antibody may be affected by the additional 6 amino acids tagged to the carboxy terminus of this protein.

Cytogenetic analysis of HT1080 cells expressing either the T137 or T130 large T antigen showed a reduced level of genomic instability compared to pSV3neo controls though still higher than the pSV2neo negative controls (Figure 6 and Table 13). Statistical analysis revealed that the data sets obtained for these two mutants are significantly different from pSV2neo and pSV3neo controls, again with the exception of the number of aneuploid cells, which was not significantly different from that of the pSV3neo controls for both mutants (Table 14). This indicates that the level of structural aberrations observed in HT1080 cells expressing T130 or T137 was intermediate between those of the negative control and those caused by full length large T antigen or by T147. As previously stated, the frequency of aneuploid cells may be dependent on the time of the first appearance of an aneuploid cell in the population.

Figure 6: Cytogenetic Analysis of HT1080 Cells Transfected with the pSV131 and pSV2neoSV24 (T137) Mutants. Symbols are as in Figure 2. pSV131 and pSV24 are plasmids expressing T130 and T137 respectively.

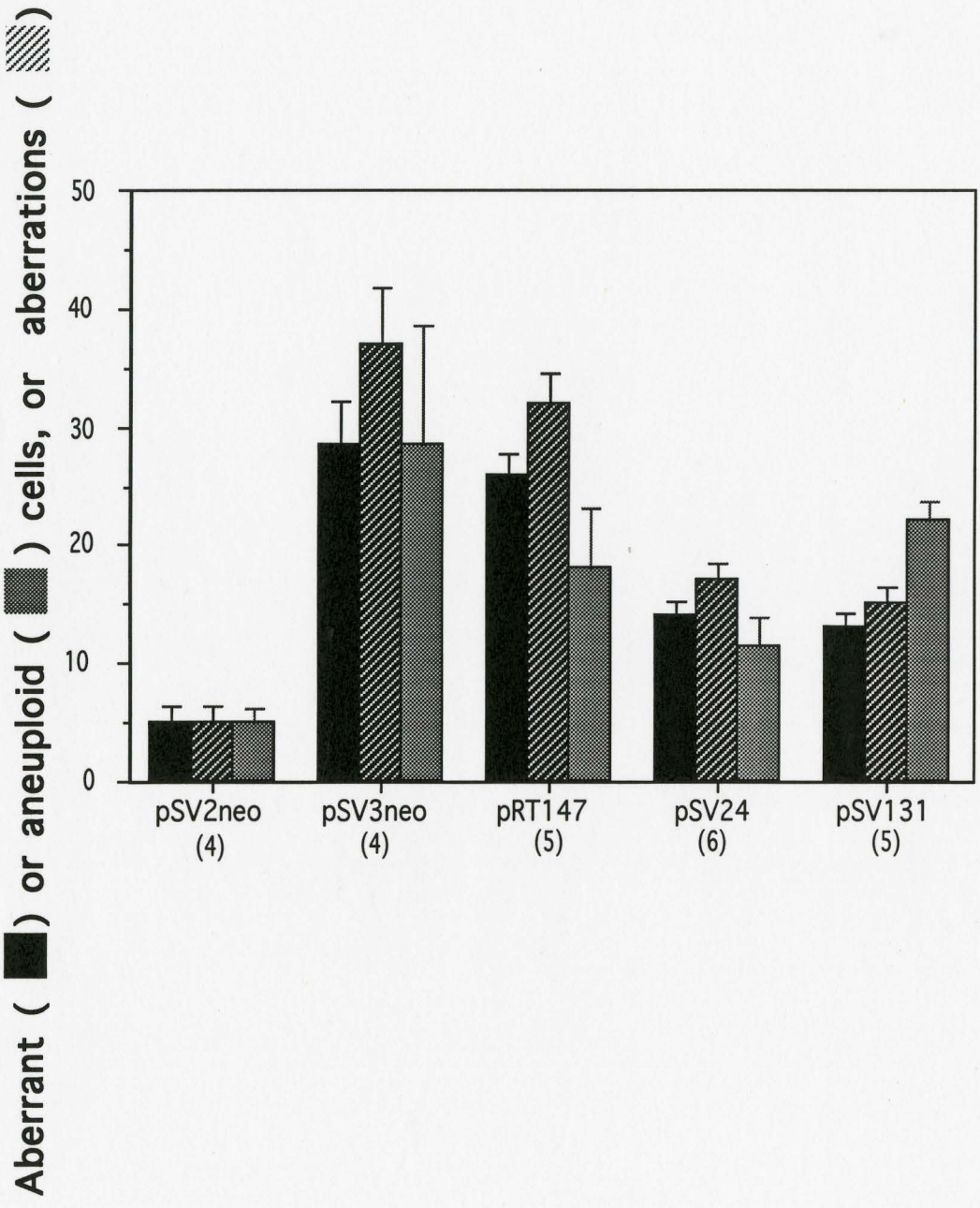

Aberrant ( ) or aneuploid ( ) cells, or aberrations ( )
50
40
30
20
10
0
pSV2neo
(4)
pSV3neo
(4)
pRT147
(5)
pSV24
(6)
pSV131
(5)

Table 13: Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo, pSV131 or pSV2neoSV24

TYPE OF ABERRANT METAPHASES IN 50 CELLS																		
	pSV2neo				pSV3neo				pSV131					pSV2neoSV24				
Clone	1	2	3	4	1	2	3	4	1	2	3	4	5	1	2	3	4	5	6
Aneuploid	8	2	4	5	32	10	44	27	25	28	31	8	15	5	17	19	8	6	14
With Structural Aberrations	7	1	6	6	31	21	33	29	15	11	14	13	13	14	13	15	9	18	16
TYPES OF STRUCTURAL ABERRATIONS IN 50 CELLS																			
Breaks	2		5	2	23	14	22	12	3	4	6	4	7	6	9	5	6	15	11
Fragments				2			3	6	2	1	1	2						1	1
Double Minute								1						1				1	1
Di/tricentric	5	1	1		20	11	15	13	8	6	7	5	5	4	3	6	2	2	2
Rings				1				2				1		1	1				1
Translocation				1				4	2	2	4	2	3	3	2	3	4	3	3
Duplications															2	4			
Total	7	1	6	6	43	28	41	38	15	13	18	14	15	15	17	18	12	22	19

50 metaphases were scored for each clone in terms of number of structurally aberrant and aneuploid cells and the total number of structural aberrations in 50 cells.

Table 14: Statistical Analysis of Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo, pSV131 or pSV2neoSV24

Plasmid	Aberration Type	wrt[a] pSV2neo	wrt[a] pSV3neo	wrt[a] pSV131
pSV2neo	# of aneuploid cells	----	0.0765	----
	# of aberrant cells	----	0.0047[c]	----
	total # of structural aberrations	----	0.0030	----
pSV131	# of aneuploid cells	0.0001	0.9506[b]	----
	# of aberrant cells	0.0070	0.0184	----
	total # of structural aberrations	0.0037	0.01111	----
pSV2neoSV24	# of aneuploid cells	0.0677	0.0541	0.6540
	# of aberrant cells	0.0013	0.0023	0.5368
	total # of structural aberrations	0.0003	0.0010	0.2401

a) wrt = with respect to
b) values higher than 0.05 indicate there is no significant difference between the two data sets.
c) values less than 0.05 indicate that the two data sets are significantly different.

This variable may therefore be a mere indicator of genomic instability rather than a measure of its actual extent. Statistical analysis also showed that the levels of aberrations in cells expressing T130 or T137 were comparable to each other in all respects (Table 14), ie. there was no significant difference between these two proteins in terms of their ability to induce genomic instability, though, it could be argued that the intermediate level of instability observed for cells expressing T137 could be due to the very low level of protein expression as shown in Figure 5E.

Based on these results, combined with those for the T147 mutants, the 17 amino acids between 130 and 147 appear to play a role in the induction of genomic instability. If the reduced level of aberrations observed in cells expressing T137 is due to the reduced activity of the mutant rather than low protein expression then this region could be narrowed to amino acids 137 to 147. On the other hand, if T137 induces genomic instability at levels comparable to those of T147 and full length large T antigen when expressed well, then the important amino acids are 130 to 137. In either case, the intermediate levels of genomic instability induced by the T130 and T137 proteins imply that other areas of T antigen must also be involved in this effect. These areas are likely to be found in the amino terminus since T147 is capable of inducing levels of genomic instability

comparable to those induced by full length large T antigen.

3.2 Analysis of Mutants Within the Amino Terminal Domain of T Antigen

In order to map the functions within the amino terminal 147 amino acids of large T antigen required for the induction of genomic instability, human fibroblasts (strain 423 and NFS-5A) were transfected with several T antigens (both full length and truncated) that contain mutations in the amino terminus. K1 (Kalderon and Smith, 1984) contains a point mutation at amino acid 107 that renders large T antigen unable to bind to pRB (DeCaprio et al., 1988), and presumably the associated p107 and p130 proteins (Dyson et al., 1989; Ewen et al., 1989; Whyte et al., 1989; P. Whyte, personal communication). In D10, a mutant studied by C. LeFeuvre, the mutation of residue 128 prevents the nuclear localization of the D10 antigen (Kalderon and Smith, 1984; Kalderon et al., 1984 a,b). The y2Xmet128-70K mutant contains a deletion of the first 127 amino acids of large T antigen and a substitution of lys^{128} with met. These mutations result in respectively, deletion of the pRB binding site and a mutation in the nuclear localization signal. The y2Xmet can still immortalize and oncogenically transform mouse embryo cells, though in some case with reduced efficiency (Thompson et al., 1990). Both the K1 and

D10 plasmids were transfected into 423 cells while y2Xmet128-70K was transfected into NFS-5A cells.

Mutant dl1135, expressed by the plasmid pRSVBneodl1135, was transfected into both HT1080 and primary human fibroblasts. Clones expressing dl1135 were easily obtained in HT1080 cells, but in three attempts with human fibroblasts, none were ever isolated. This mutant has a deletion of amino acids 17 to 27 of large T antigen and is unable to transform some cell types in culture (Symonds et al., 1993). No specific function has been assigned to this deleted region, but the sequence between amino acids 17 and 27 of SV40 large T antigen has been found to complement mutants of adenovirus E1A deficient in p300 binding (Yaciuk et al., 1991). As there is no known reason why this mutant should be lethal in primary cells, the lack of clones cannot be explained.

Plasmid pSV131-K1 encodes a double mutant T antigen that is a combination of the T130 truncation, and the K1 mutation above and was also transfected into HT1080 for the same reasons as previously listed for pSV131. As yet no information has been published on the transforming ability of this mutant.

Immunostaining was initially used to screen clones for expression of T antigen (Table 15). Cells expressing y2Xmet or K1 were found positive upon staining with MAb 412

and the reactivity was uniform within and between the clonal populations. T130-K1 transfectants were positive with MAb 419 (Figure 8) but negative with MAb 416. Since the construct expresses both large T and small t and MAb 419 recognizes both antigens whereas MAb 416 reacts only with large T, these results suggested expression of only small t. Mutant dl1135 showed a lower level of expression, also uniform in the population. No data was available from C. LeFeuvre on the immunostaining of cells expressing D10.

The level of protein expression of all of the mutants was analyzed by Western blots (Figure 7). Expression for K1, D10 (done by C. LeFeuvre) and y2Xmet varied from clone to clone independently of the level of genomic instability and for some clones in each set was comparable to the level of expression of T antigen in pSV3neo transformants. This suggests that beyond a threshold level, induction of aberrations is independent of the amount of T antigen. On the other hand, expression of dl1135, was always lower than that of wild type large T antigen (Figure 7). No expression of T130-K1 could be detected by Western blot with MAb 416 confirming the immunostaining results. Thus, it seems most likely that this mutant did not express large T in the transfected cells and therefore the cytogenetic results obtained with it must be interpreted with this possibility in mind.

Table 15: Intensity of Immunostaining in Cells Transfected with K1, py2Xmet128-70K, pRSVBneodl1135 or pSV131-K1

Cell Type	Clone #	Plasmid	Antibody	Intensity[a]
423	1	K1	412	++
	2	"	"	++
	3	"	"	++
	4	"	"	++
NFS-5A	1	y2Xmet	412	+++
	2	"	"	+++
	3	"	"	+++
	4	"	"	++
	5	"	"	+++
HT1080	1	dl1135	412	+
	2	"	"	+
	3	"	"	+
	4	"	"	+
	5	"	"	++
HT1080	1	pSV131-K1	419	+
	2	"	"	+
	3	"	"	+
	4	"	"	+
	5	"	"	+

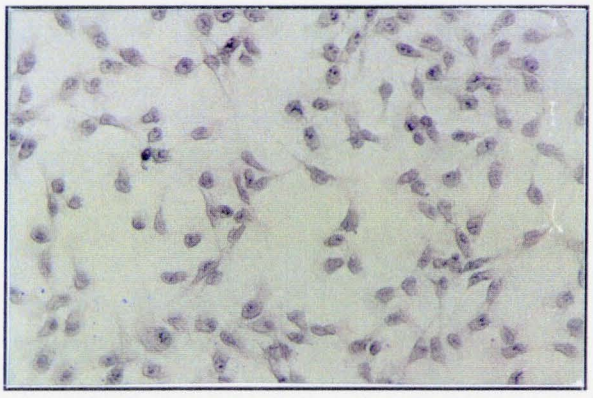
a) intensity with respect to cos-1 (++++)

Figure 7: Expression of Amino Terminal Mutants of SV40 Tumour Antigen by Western Blot. Cell lysates were prepared and treated as in Figure 5. Following reaction with mAb 412 (panels A,C,D), or mAb 419 (panel B) proteins were detected by chemiluminescence.

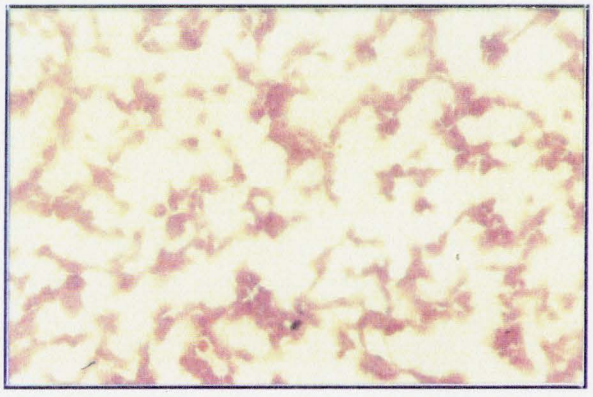
D
pSV3
dl1135-1
dl1135-2
dl1135-3
dl1135-4
dl1135-5
C
pSV3
y2Xmet-1
y2Xmet-2
y2Xmet-3
y2Xmet-4
y2Xmet-5
B
D10-1
D10-2
D10-3
D10-4
D10-5
cos-1
D10-6
A
pSV3-1
K1-1
K1-2
K1-3
K1-4

Figure 8: Expression of the T131-K1 Mutant in HT1080 Cells by Immunostaining. Immunostaining was done with MAb 419 A, pSV2neo; B, pSV3neo; and C, pSV131-K1

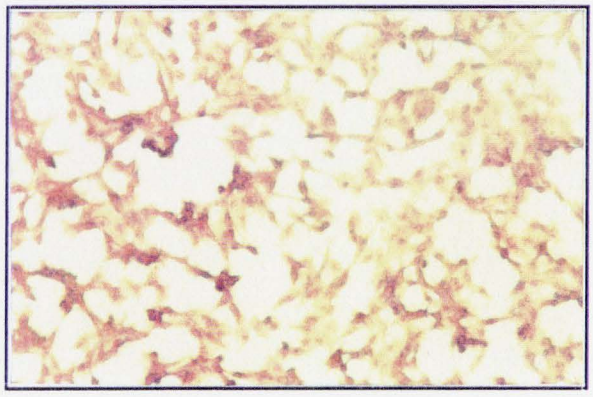
A



B



C



Cytogenetic analysis of cells expressing the K1 mutant revealed them to be abnormal in terms of structural aberrations and aneuploidy (Figure 9 and Table 16B). However, the levels were intermediate between the pSV2neo and pSV3neo controls (some of which were obtained and analyzed by N.Stewart and C. LeFeuvre) and significantly different from both (Table 16A,B and Table 17). These results indicate the binding of T antigen to and the resulting functional inactivation of the pRB family of proteins is required for the induction of genomic instability at wild type levels. Moreover, the residual instability detected in K1 expressing cells indicates that other T antigen functions participate.

The results with the D10 transfectants obtained by C. LeFeuvre were less easily explained. When averaged, these cells had significantly lower levels of structural aberrations than the pSV3neo controls but, did not differ with respect to aneuploid cells (Figure 9 and Table 17). Cells expressing D10 had 16 aberrant and 25 aneuploid on average in 50 metaphases compared to pSV3neo clones which had 28 aberrant and 27 aneuploid. When considered individually, however, some clones were as aberrant as pSV3neo controls (Table 16A,B). For example, one D10 clone had 32 structurally aberrant and 45 aneuploid metaphases (Table 16B) and therefore the level of genomic instability

Figure 9: Cytogenetic Analysis of Human Fibroblasts (Strain 423) Transfected with the K1 or D10 Mutants. Symbols are as shown in Figure 2. K1 and D10 (analyzed by C. LeFeuvre) are full length T antigen mutants with point mutations in the pRB binding domain or the nuclear localization signal respectively.

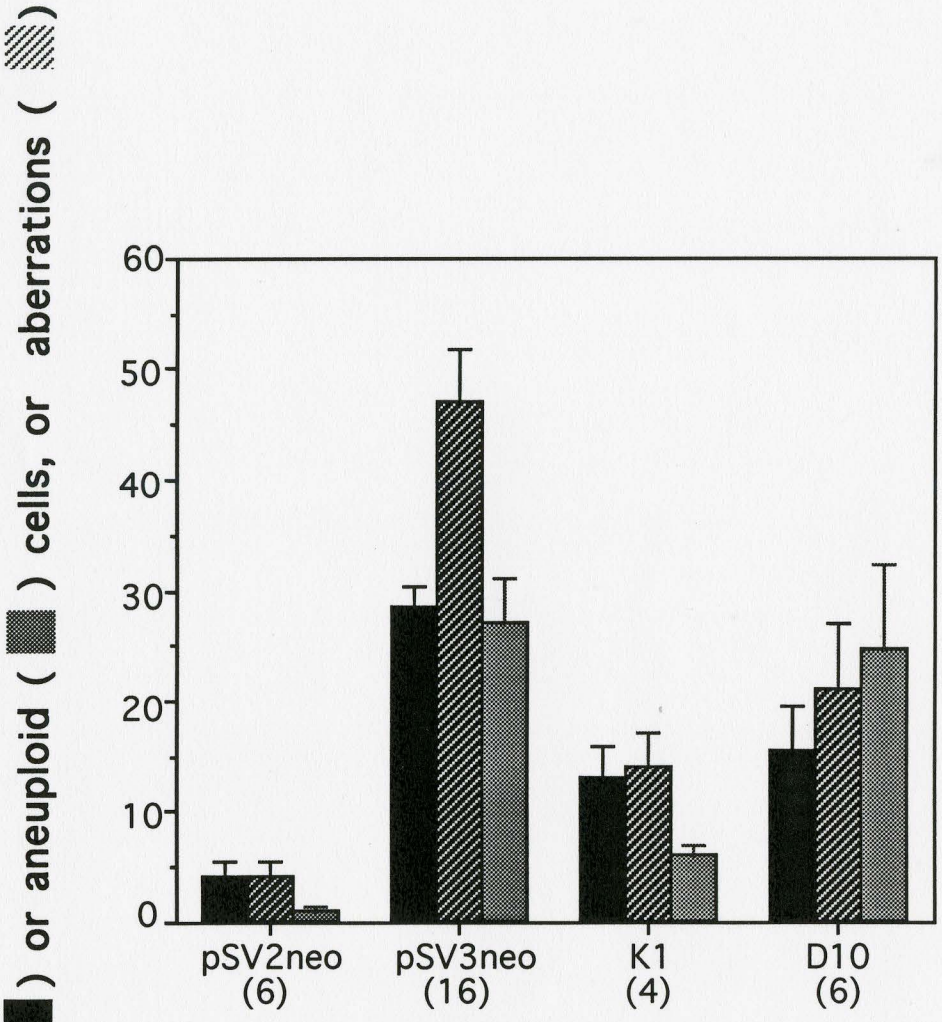

Aberrant ( ) or aneuploid ( ) cells, or aberrations ( )
60
50
40
30
20
10
0
pSV2neo
(6)
pSV3neo
(16)
K1
(4)
D10
(6)

Table 16A: Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with pSV2neo or pSV3neo

# OF ABERRANT METAPHASES IN 50 CELLS																						
	pSV2neo[a]						pSV3neo[a]															
Clone	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aneuploid	0	1	1	2	0	2	6	33	36	45	49	6	8	24	22	11	21	47	50	41	13	14
With Structural Aberrations	10	2	0	2	7	3	11	41	32	29	34	25	29	31	29	29	22	25	41	34	21	23
STRUCTURAL ABERRATIONS IN 50 CELLS																						
Breaks	8			2	4	1	3	22	8	15	13	27	22	18	5	4	6	1	2	4	10	8
Fragments	1				2	1	9	26	17	3	4	4	12	8	21	11	10	21	7	4	14	11
Double Minute	1							3		3	2	1	5	3	21	8	5	17	27	15	7	4
Di/tricentric		2			1	2		23	5	8	17	12	8	11	20	22	12	11	41	40	8	10
Rings								1		2	1		2	1							1	
Translocation								1			1	1			4			1	5	3		
Duplications							1															
Total	10	2	0	2	7	4	13	76	30	31	38	45	49	40	72	45	33	51	82	66	40	33

a) some of the pSV2neo and pSV3neo clones were analyzed by N. Stewart and C. LeFeuvre.
50 metaphases were scored for each clone in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations.

Table 16B: Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with K1 or D10

# OF ABERRANT METAPHASES IN 50 CELLS										
	K1				D10					
Clone	1	2	3	4	1	2	3	4	5	6
Aneuploid	10	4	7	3	20	49	9	12	4	45
With Structural Aberrations	6	17	11	18	18	11	6	20	6	32
STRUCTURAL ABERRATIONS IN 50 CELLS										
Breaks	2	11	2	5	8	8	1	10	1	13
Fragments			1	1	1	2		7	1	1
Double Minute		1			2	2			7	6
Di/tricentric	4	7	8	13	13	1	7	4	1	28
Rings							1		1	
Translocation										
Duplications	1	1						1		
Total	7	20	11	19	24	13	9	22	11	48

50 metaphases were scored for each clone in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations

Table 17: Statistical Analysis of Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with pSV2neo, pSV3neo, K1 or D10

Plasmid	Aberration Type	wrt[a] pSV2neo[d]	wrt[a] pSV3neo[d]
pSV2neo	# of aneuploid cells	----	0.0011
	# of aberrant cells	----	0.000
	total # of structural aberrations	----	0.000
K1	# of aneuploid cells	0.0016	0.0001
	# of aberrant cells	0.0007[c]	0.0090
	total # of structural aberrations	0.0115	0.0004
D10	# of aneuploid cells	0.0144	0.7824[b]
	# of aberrant cells	0.0248	0.0036
	total # of structural aberrations	0.0209	0.0079

a) wrt = with respect to
b) values higher than 0.05 indicate there is no significant difference between the two data sets.
c) values less than 0.05 indicate that the two data sets are significantly different.
d) some of the pSV2neo and pSV3neo clones were analyzed by N. Stewart and C. LeFeuvre

in this particular clone was comparable to and/or higher than that observed in pSV3neo transfectants. It could be argued that the some of the D10 mutant T antigen did localize to the nucleus, however none was detected by immunostaining, (Table 25). Therefore these results imply that cytoplasmically located T antigen can induce genomic instability as proficiently as the nuclear form.

The y2Xmet128-70K expressing clones also showed a reduced level of genomic instability with respect to structural aberrations and were comparable to K1 transfectants with 12 structurally aberrant on average in 50 scored (Figure 10 and Table 18). With respect to aneuploidy where they were comparable to the pSV3neo controls (Table 18 and 19). In mouse cells, the y2Xmet128-70K protein accumulates in the cytoplasm (Thompson et al., 1990) due to the substitution of a met codon for lys^{128} in the nuclear localization signal (Kalderon et al., 1984 a,b). However, in human fibroblasts, the protein was detected by immunostaining in both the cytoplasm and nucleus (Table 25). This suggests that the nuclear localization signal may still be partially functional or that other factors help transport the T antigen protein to the nucleus. The reduced induction of genomic instability by the y2Xmet128-70K protein is therefore likely due to the loss of pRB binding and other functions in the amino terminus.

Figure 10: Cytogenetic Analysis of Human Fibroblasts (Strain NFS-5A) Transfected with the py2Xmet128-70K Mutant. Symbols are as in Figure 2. y2Xmet is a truncation mutant of large T antigen missing the first 127 amino acids.

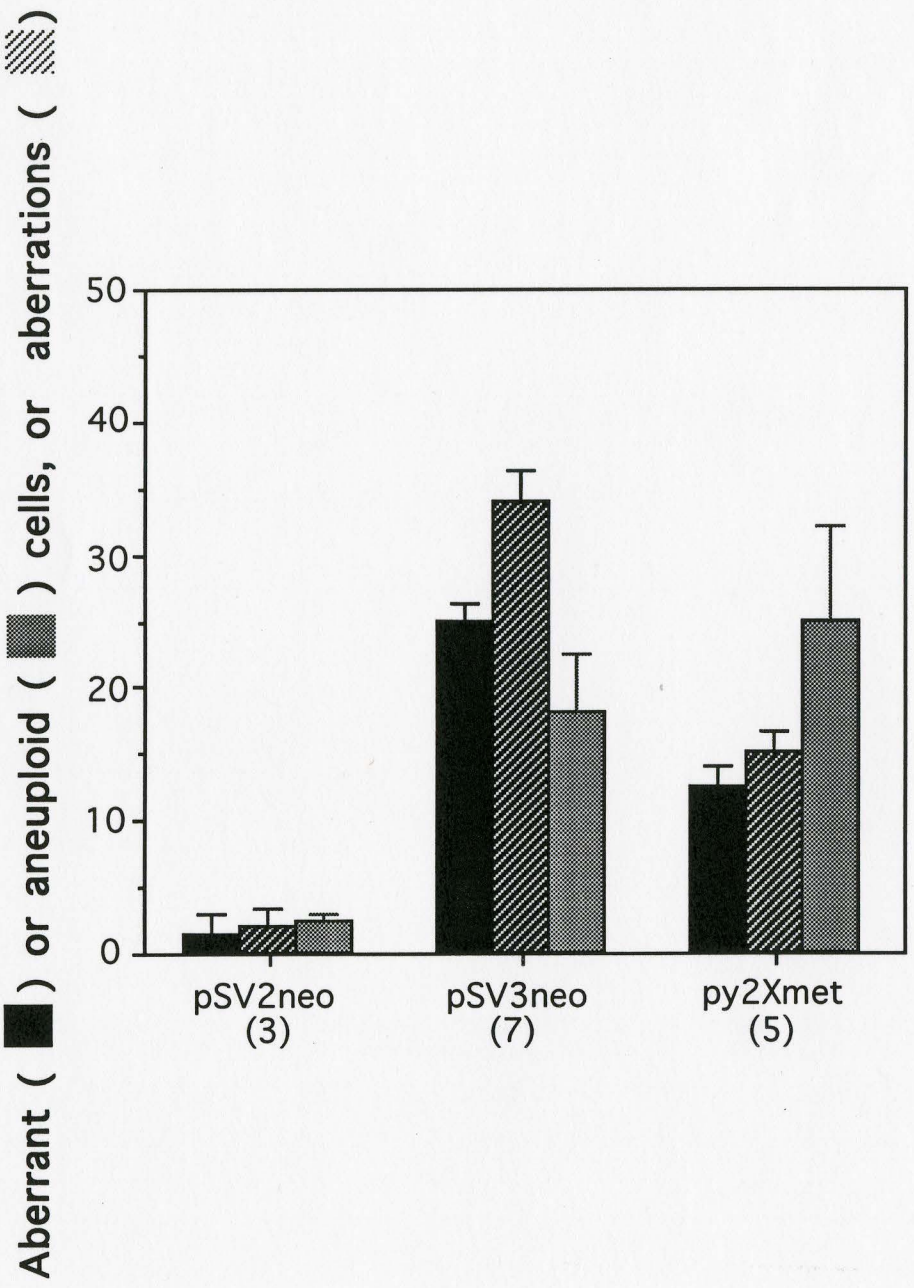

Aberrant (■) or aneuploid (▒) cells, or aberrations (▨)
50
40
30
20
10
0
pSV2neo
(3)
pSV3neo
(7)
py2Xmet
(5)

Table 18: Cytogenetic Properties of Human Fibroblasts (Strain NFS-5A) Transfected with pSV2neo, pSV3neo and py2Xmet128-70K

# OF ABERRANT METAPHASES IN 50 CELLS													
	pSV2neo			pSV3neo					py2Xmet				
Clone	1	2	3	1	2	3	4	5	1	2	3	4	5
Aneuploid	3	0	2	17	25	29	30	28	7	8	36	38	35
With Structural Aberrations	0	0	3	23	31	27	24	26	16	11	15	7	13
STRUCTURAL ABERRATIONS IN 50 CELLS													
Breaks			2	10	17	12	8	15	12	9	2	3	4
Fragments				4		1	2	3	1		4	1	6
Double Minute				5	2	2		1		2		1	3
Di/tricentric			1	8	18	21	20	23	5	2	7	1	2
Rings													
Translocation					1	1	1			2	2	1	
Duplications						1				1		1	
Total	0	0	3	27	38	38	31	32	18	16	15	8	15

50 metaphases were scored for each clones in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations

Table 19: Statistical Analysis of Cytogenetic Properties of Human Fibroblasts (Strain NFS-5A) Transfected with pSV2neo, pSV3neo or py2Xmet128-70K

Plasmid	Aberration Type	wrt[a] pSV2neo	wrt[a] pSV3neo
pSV2neo	# of aneuploid cells	----	0.1244
	# of aberrant cells	----	0.0001
	total # of structural aberrations	----	0.0003
py2Xmet	# of aneuploid cells	0.1186	0.4441[b]
	# of aberrant cells	0.0114	0.0001[c]
	total # of structural aberrations	0.0071	0.0001

a) wrt = with respect to
b) values higher than 0.05 indicate there is no significant difference between the two data sets.
c) values less than 0.05 indicate that the two data sets are significantly different.

HT1080 cells expressing the dl1135 mutant, also showed a reduced level of aberrations (Figure 11 and Table 20) significantly different from both the pSV2neo and pSV3neo controls again with the exception of aneuploidy (Table 21). In this case, the level of aneuploidy was statistically comparable to that of both pSV2neo and pSV3neo controls due to the wide variation of the dl1135 clones for this particular variable. The averages over the five dl1135 clones obtained were 17 structurally aberrant and 17 aneuploid metaphases in 50 scored. The pSV2neo and pSV3neo values were: 5 aberrant, 5 aneuploid cells and 29 aberrant and 29 aneuploid cells respectively (Table 20). The reduced level of genomic instability observed with dl1135 may be due to low level protein expression as detected by Western blot (Figure 7) or the reported instability of the protein (J. Pipas, pers. comm.) However, amounts of dl1135 antigen were within the range detected for y2Xmet antigen, in different cell types, but using the same antibody. Since, the cytogenetic properties of y2Xmet clones were comparable regardless of expression levels, likley that the dl1135 protein was sufficiently expressed. Therefore, the region between a.a. 17 to 27 may contribute to the induction of genomic instability along with other functions of large T antigen. As stated earlier, no specific function is assigned to the region between a.a. 17 and 27 that is deleted in this

Figure 11: Cytogenetic Analysis of HT1080 Cells Transfected with the pSV131-K1 and pRSVBneodl1135 Mutants. Symbols are as in Figure 2. pSV131-K1 is a plasmid expressing a double mutant of the truncated T130 T antigen with the K1 mutation that prevents pRB binding. dl1135 contains a deletion of amino acids 17 to 27 in the amino terminus of T antigen.

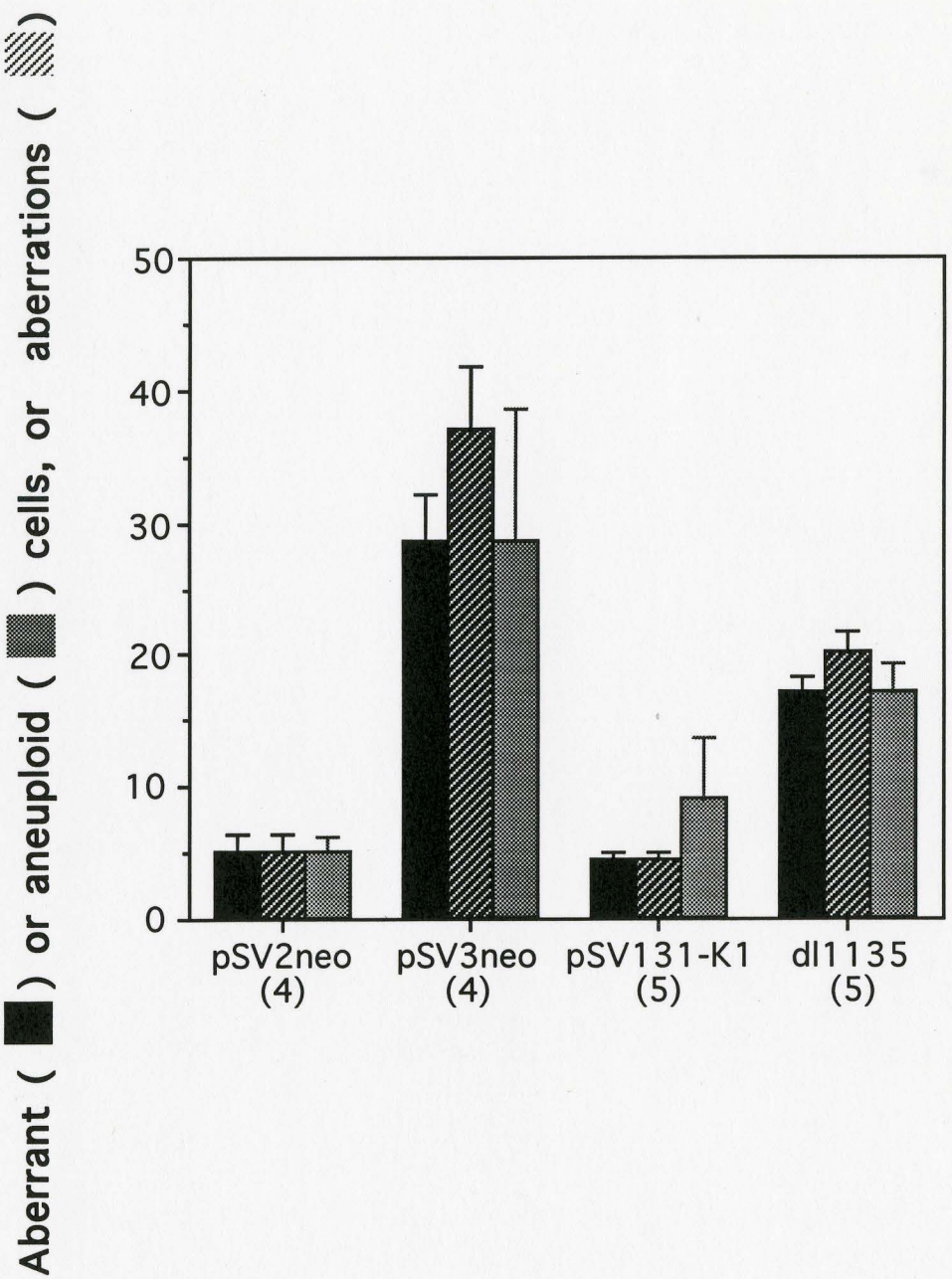

Aberrant (■) or aneuploid (▩) cells, or aberrations (▨)
50
40
30
20
10
0
pSV2neo (4)
pSV3neo (4)
pSV131-K1 (5)
dl1135 (5)

Table 20: Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo, pRSVBneodl1135 or pSV131-K1

# OF ABERRANT METAPHASES IN 50 CELLS																		
	pSV2neo				pSV3neo				dl1135					pSV131-K1				
Clone	1	2	3	4	1	2	3	4	1	2	3	4	5	1	2	3	4	5
Aneuploid	8	2	4	5	32	10	44	27	24	12	18	20	12	8	8	0	22	7
With Structural Aberrations	7	1	6	6	31	21	33	29	15	22	19	16	15	5	4	3	6	4
STRUCTURAL ABERRATIONS IN 50 CELLS																		
Breaks	2		5	2	23	14	22	12	6	11	10	10	5	2		1	1	1
Fragments				2			3	6	2	4	3		1	1		1		
Double Minute								1					3	1				
Di/tricentric	5	1	1		20	11	15	13	4	9	8	8	4		4	1	4	2
Rings				1				2		1	1		2					
Translocation				1				4	4		1	1	3	1			1	1
Duplications																		
Total	7	1	6	6	43	28	41	38	16	25	23	19	18	5	4	3	6	4

50 metaphases were scored for each clone in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations

Table 21: Statistical Analysis of Cytogenetic Properties of HT1080 Cells Transfected with pSV2neo, pSV3neo, pRSVBneodl1135 or pSV131-K1

Plasmid	Aberration Type	wrt[a] pSV2neo	wrt[a] pSV3neo	wrt[a] pSV131	wrt[a] K1
pSV2neo	# of aneuploid cells	----	0.0765[b]	----	----
	# of aberrant cells	----	0.0047[c]	----	----
	total # of structural aberrations	----	0.0030	----	----
dl1135	# of aneuploid cells	0.0038	0.1972	----	----
	# of aberrant cells	0.0004	0.0154	----	----
	total # of structural aberrations	0.0002	0.0057	----	----
pSV131-K1	# of aneuploid cells	0.3206	0.0000	0.0574	0.4723
	# of aberrant cells	0.4502	0.0001	0.0000	0.0114
	total # of structural aberrations	0.5002	0.0000	0.0000	0.0101

a) wrt = with respect to
b) values higher than 0.05 indicate there is no significant difference between the two data sets.
c) values less than 0.05 indicate that the two data sets are significantly different.

mutant. It is possible that the region either binds to p300 or has a comparable function due to its ability to complement adenovirus E1A mutants deficient in p300 binding (Yaciuk et al., 1991).

Cells containing the double mutant T130-K1, were not significantly different cytogenetically from the pSV2neo negative controls (Figure 10 and Table 20). When compared statistically to both the K1 and T130 clones, T130-K1 clones were significantly different (Table 21) indicating a possible reduction in this mutants ability to induce genomic instability. The averages were 9 aneuploid and 5 structurally aberrant metaphases in 50 metaphases scored (Table 20) with very little variation among the clones. However, as previously mentioned, expression of the T130-K1 protein could not be detected by Western blot or immunostaining with MAb 416 suggesting that the protein expression detected by immunostaining with MAb 419 was that of small t antigen. Therefore, due to the possibility that these clones do not express large T no conclusion can be derived from their cytogenetic analysis.

3.3 Analysis of Full Length T Mutants Defective in p53 Binding

In order to further investigate the role of p53 in the proliferation of T antigen transformed cells (see

Section 3.1) and in continuation of results obtained by C. LeFeuvre (unpubl.), human fibroblasts (strain 423) were transfected with 5080, a full length T antigen mutant that is defective for binding to p53 (Cole et al., 1986; Tevethia et al., 1988; Peden et al., 1989). A phenotypically wild type mutant 5081, was used along with pSV3neo as a second positive control. As usual, pSV2neo was used as a negative control.

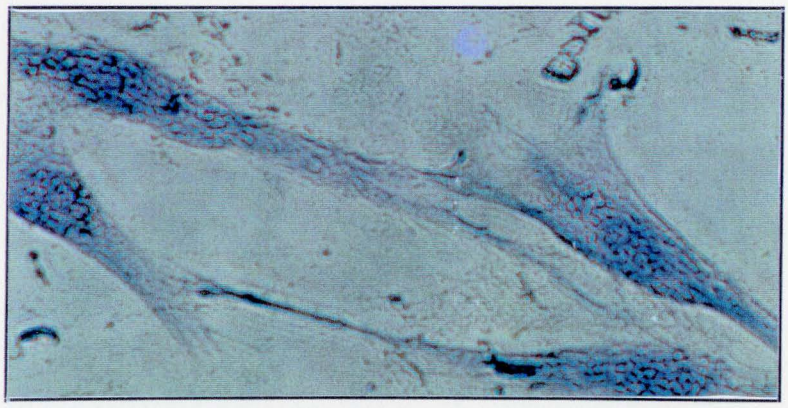
Expression of the mutant T antigen was severely reduced in cells expressing the 5080 mutant compared to cells transformed with pSV3neo and 5081 control plasmids as shown by immunostaining with MAb 419 (Figure 12).

Cytogenetic analysis revealed that clones expressing 5080 T antigen (Figure 13 and Table 22) had very low levels of structural aberrations and aneuploid cells. The level of genomic instability in these cells was not significantly different from that of the pSV2neo negative controls (Table 23). Cells expressing 5081 showed a high level of genomic instability that was not significantly different from that of the pSV3neo controls (Table 23). The results with the 5080 antigen are essentially identical to those obtained by N. Stewart with the mutant T antigen dlA2433, that also does not bind to p53.

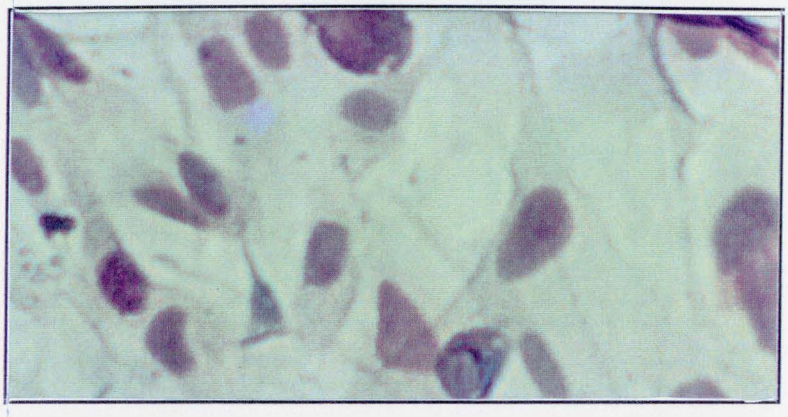
One possible explanation for this low level of aberrations is the low level of protein expression. The 5080

Figure 12: Expression of the 5080 and 5081 Mutants of SV40 Large Tumour Antigen in Human Fibroblasts (Strain 423) by Immunostaining. A, pSV2neo; B, pSV3neo; C, 5080; D, 5081 after immunostaining with MAb 419. Pictures were taken at a higher magnification due to the lower intensity of the 5080 staining.

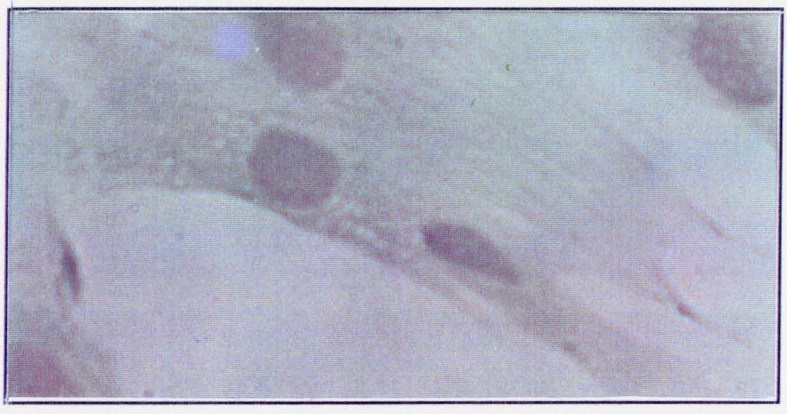
A



B



C



D

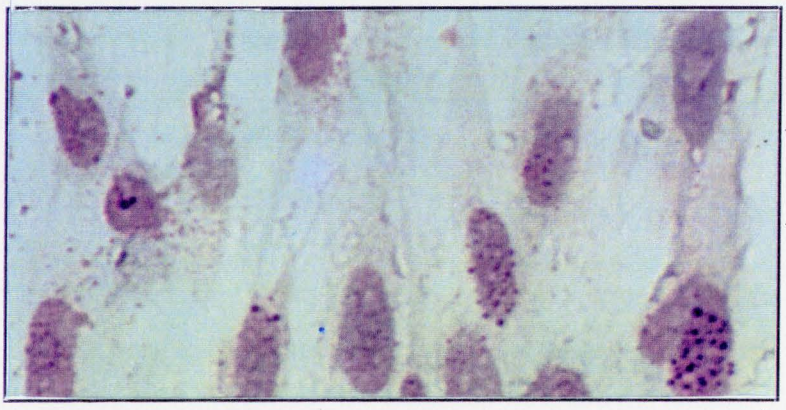


Figure 13: Cytogenetic Analysis of Human Fibroblasts (Strain 423) Transfected with the p5080neo, p5081neo and dlA2433 Mutants. Symbols are as in Figure 2. dlA2433 (analyzed by N. Stewart) and 5080 are full length T antigens with point mutations in the p53 binding domain. 5081 contains a silent mutation in the same region and is phenotypically wild type. Some of the 5080 and 5081 clones were analyzed by C. LeFeuvre.

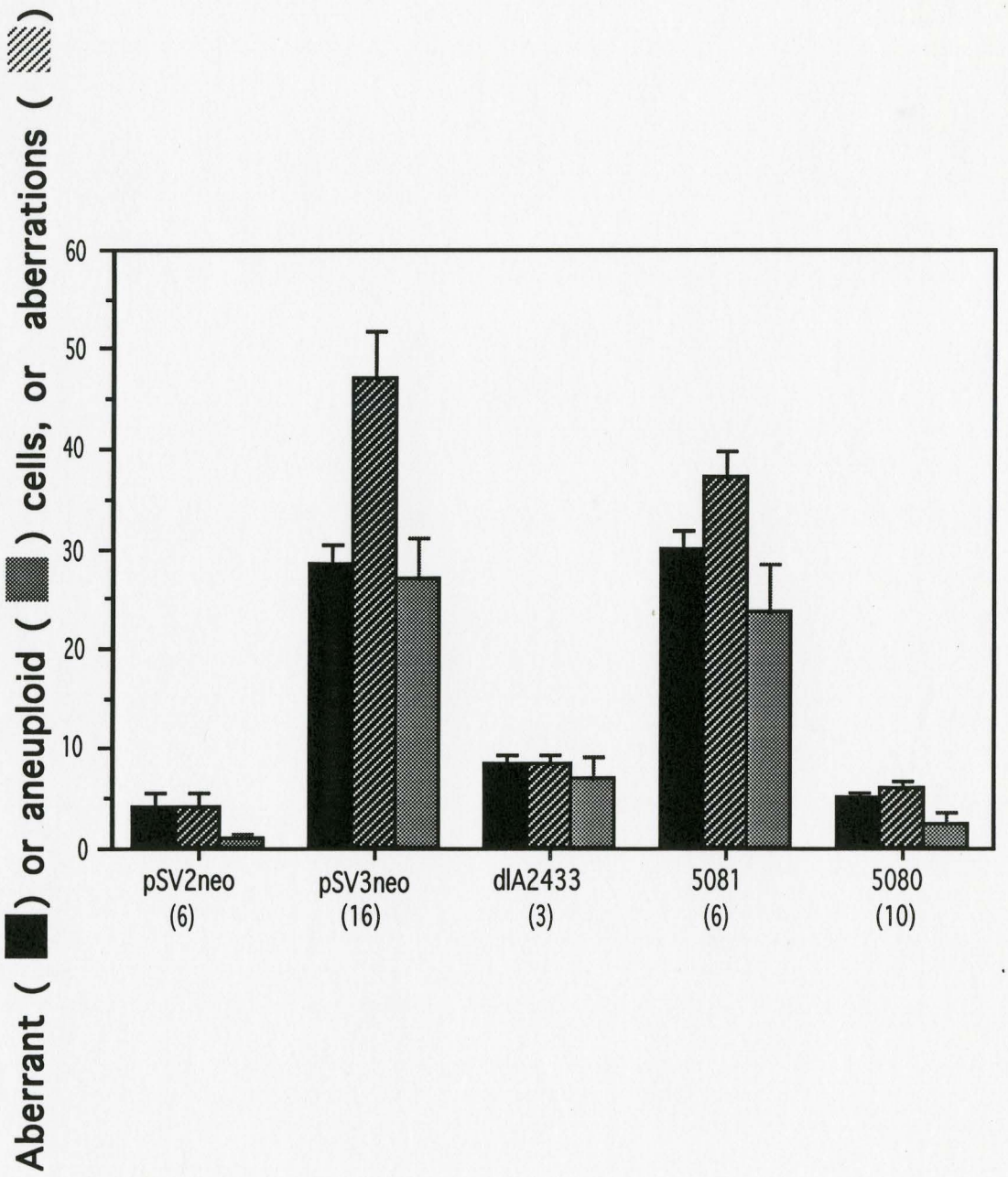

Aberrant ( ) or aneuploid ( ) cells, or aberrations ( )
60
50
40
30
20
10
0
pSV2neo
(6)
pSV3neo
(16)
dlA2433
(3)
5081
(6)
5080
(10)

Table 22: Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with p5080 and p5081

# OF ABERRANT METAPHASES IN 50 CELLS																
	p5080										p5081					
Clone	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6
Aneuploid	0	5	8	7	0	1	0	0	0	0	46	36	11	19	8	19
With Structural Aberrations	1	6	6	5	3	4	5	6	6	6	31	37	25	27	32	26
STRUCTURAL ABERRATIONS IN 50 CELLS																
Breaks		3	2	2	2	3	2	4	3	3	10	6	12	20	18	7
Fragments		3	3	2		1	2	1	3			16	6	7	7	7
Double Minute				1	1	1	1	2	1	2	2		5	5	13	6
Di/tricentric	1		1			2		1	2		15	22	7	2	5	9
Rings										1						1
Translocation													2	1	1	
Duplications		1									6	2				
Total	1	7	6	5	3	7	5	8	9	6	33	46	32	35	44	30

50 metaphases for were scored for each clone in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations. Cytogenetics for the pSV2neo and pSV3neo controls can be found in Table 16A.

Table 23: Statistical Analysis of Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with pSV2neo, pSV3neo, p5080 and p5081

Plasmid	Aberration Type	wrt[a] pSV2neo	wrt[a] pSV3neo	wrt[a] p5080
pSV2neo	# of aneuploid cells	----	0.0011	----
	# of aberrant cells	----	0.0000	----
	total # of structural aberrations	----	0.0000	----
p5080	# of aneuploid cells	0.3389[b]	0.0010[c]	----
	# of aberrant cells	0.5627	0.0000	----
	total # of structural aberrations	0.3800	0.0000	----
p5081	# of aneuploid cells	0.0035	0.6849	0.0005
	# of aberrant cells	0.0000	0.5795	0.0000
	total # of structural aberrations	0.0000	0.2418	0.0000

a) wrt = with respect to
b) values higher than 0.05 indicate there is no significant difference between the two data sets.
c) values less than 0.05 indicate that the two data sets are significantly different.

protein is abnormal in conformation and this impairs binding to pRB (Tack et al., 1989; Ludlow et al., 1990). As previously mentioned, (Section 3.2) binding to the pRB family may play a role in the induction of genomic instability. Therefore, the reduced binding of pRB by the 5080 protein could have impaired its functioning in our assay. The third possible explanation for the low level of aberrations induced by 5080 comes from the observation that the growth of 5080 and dlA2433 cells was slower and their lifespan shortened compared to pSV2neo transfectants (8 PD versus 20 PD) much like that of the pMTΔT147 transformed cells. As mentioned in the introduction, p53 is a growth suppressor gene that appears to monitor genomic integrity and stability and will prevent a cell from dividing until DNA repair is completed (Kastan et al., 1991; Kuerbitz et al., 1992). Binding to large T antigen may prevent p53's growth suppressing activity, and allow aberrant cells to continue to grow and divide. With a mutant large T antigen that does not bind p53, cells with aberrations induced by the presence of large T may be prevented from dividing by the presence of free p53. A few cells that have a lower level of aberrations, possibly due to low level protein expression, may escape this suppression for a time and be analyzable as metaphases. The damage surveillance pathway could also explain the growth inhibitory effect of T147

expression in human fibroblasts, whereas HT1080 cells, which express a mutant p53 (Anderson et al., 1994) are not affected. A shortened lifespan for human cells transformed by T antigen unable to bind p53 has been reported by others (Hara et al., 1991; Lin and Simmons, 1991; Maclean et al., 1994).

In order to verify that the effect of dlA2433 and 5080 mutant T antigens was due to the lack of binding to p53, an attempt to complement the 5080 mutant with a plasmid (pJL17) that expresses a dominant negative mutant p53 was made. This plasmid was transfected into cells already containing the 5080 protein as well as cotransfected with p5080 in human fibroblasts (strain 423). As a control, a plasmid expressing wild type p53 (pJL16) was also used. To investigate whether the effect of large T could be competed out by the overexpression of wild type p53, pJL16 was also cotransfected with the 5081 mutant T antigen or transfected into cells already containing the 5081 mutant. As a control, the mutant p53 was also used.

No expression of the 5080 protein could be detected by Western blot, likely due to the low level of expression of the protein combined with the poor growth of the cells. Figure 14 shows the expression of the 5081 mutant T antigen in 423 cells with respect to the level of expression of either wild type or mutant p53. The endogenous expression of

p53 occurred at a much lower level than that of large T antigen, while that of the transfected p53, mutant or wild type, was comparable to or higher than that of large T antigen.

The presence of the strong dominant mutant of p53 in cells expressing the 5080 mutant did not change the growth phenotype of these cells. In fact, they grew so poorly that it was impossible to obtain cytogenetic information on the clones. The control cells expressing 5080 with normal p53 also maintained the same phenotype. There was no difference between the cotransfected cells and the cells expressing the 5080 mutant prior to transfection with the p53 plasmids.

Cells expressing the 5081 mutant in the presence of high levels of normal or mutant p53 maintained the same phenotype of cells expressing only 5081, both in terms of rapid growth and being cytogenetically abnormal (Table 24). Again there was no difference between the cotransfected cells and the cells expressing the 5081 mutant prior to transfection with the p53 plasmids. These results are therefore inconclusive. It may be that the expression of the wild type or mutant p53 was not sufficient to override or affect the action of the T antigen in these cells. Two other possibilities are: (1) the timing of p53 expression with respect to T antigen did not allow for p53 to exhibit any effect. p53 may need to be expressed prior to T antigen to

act. (2) the hypothesis of p53's role in preventing the growth of aberrant cells is incorrect.

Figure 14: Expression of the 5081 Mutant and Wild Type or Mutant p53 in Human Fibroblasts (Strain 423) by Western Blot. Cell lysates were prepared and treated as in Figure 5. Reacted with mAb 412 and p53-2 and detected by chemiluminescence.

T-Ag
423
5081+JL16-1
5081+JL16-2
5081+JL16-3
5081+JL16-4
5081+JL17-1
5081+JL17-2
5081+JL17-3
50801+JL17-4

p53

Table 24: Cytogenetic Properties of Human Fibroblasts (Strain 423) Transfected with p5081 and pJL16 or pJL17

# OF ABERRANT METAPHASES IN 50 CELLS										
	p5081		p5081 + pJL16				p5081 + pJL17			
Clone	1	2	1[a]	2	3	4	1[a]	2	3	4
Aneuploid	45	36	40	22	28	38	16	37	24	48
With Structural Aberrations	31	37	37	31	14	37	32	29	17	28
STRUCTURAL ABERRATIONS IN 50 CELLS										
Breaks	10	6	15	18	5	20	23	12	3	18
Fragments		16	9	6	2	10	2		2	5
Double Minute	2		1			2		4	2	
Di/tricentrics	15	22	20	11	6	23	15	23	9	8
Rings										
Translocations					3				1	
Duplications	6	2		1						2
Total	33	46	46	36	16	55	41	39	17	33

a) the p53 plasmids were transfected into cells already expressing the 5081 mutant for clone #1 of both sets. All other clones were cotransfected.
50 metaphases were scored for each clone in terms of the number of structurally aberrant and aneuploid cells and the total number of structural aberrations

DISCUSSION

Multiple steps seem to be required in order for a cell to change from the normal growth pattern to the rapid unregulated growth associated with the development of tumours. These step are thought to be based on the mutation of cellular genes involved in growth regulation and cell cycle control. Multiple and possibly similar steps are also likely required for the development of a partially transformed phenotype (altered morphology and an extended lifespan) and of tumorigenicity or immortalization of human cells in vitro (reviewed in Strauss and Griffin, 1990; Shay et al., 1991a). Treatment of cells with viruses may mimic some of the steps as a direct consequence of the oncogene expression and while other events may occur as a result of the genetic instability induced by the presence of the oncogene. Mutagenic agents such as chemicals or radiation may also initiate the same process through the induction of genetic damage. Therefore, cells can become transformed and tumours can develop from the induction of multiple genetic changes within the cell, or in the case of in vitro transformation, by viruses through the addition of new genetic information in the form of the viral oncogenes followed by oncogene induced genetic instability (Chang, 1986).

The presence of viral oncogenes, such as SV40 large T antigen, in human cells appears to be sufficient for transformation with respect to altered cell growth and morphology (Wolman et al., 1964). However, since most virally transformed cells die at crisis, simply the presence of the viral oncogene is not sufficient for immortalization.

Genetic instability is common in human tumours and additionally in almost all instances immortal cells are genetically aberrant. An early event in the transformation of human cells by viral oncogenes is the induction of genetic instability, which precedes crisis and the establishment of an immortal population (Moorehead and Saksela, 1965; Graham et al., 1977; Chang et al., 1986). This apparent correlation between genomic instability and the development of a partially transformed phenotype (i.e. pre-immortal) supports the hypothesis that genomic instability through the disruption of genes involved in growth control and cell death may be causal in the development of an immortal cell line and in tumorigenesis in vitro.

Studies have shown that SV40 induces chromosome gaps and breaks as well as changes in chromosome number (Wolman et al., 1964; Moorehead and Saksela, 1965). The frequency and in some cases the complexity of these aberrations increase as the cells move toward crisis and become immortal (Wolman et al., 1964; Moorehead and Saksela, 1965) indicating destabilization

of the entire genome. Previous studies have shown that the presence of only SV40 large T antigen is required for the induction of genomic instability (Ray et al., 1990; Stewart and Bacchetti, 1991) and that a viral origin of replication is not required (Stewart and Bacchetti, 1991). Preliminary data have suggested a role for the binding of p53 by large T antigen in the induction of genomic instability. The present study followed on these observations and was undertaken to determine which specific functions of large T antigen were required for the induction of genomic instability, and to further clarify the role of p53 in this process.

The results obtained in this study indicate that the amino terminal 147 amino acids of SV40 large T antigen are sufficient for the induction of genomic instability. Within these 147 amino acids 3 specific regions have been identified as possibly being involved in this process: amino acids 17 to 27, the pRB binding site, and amino acids 130 to 147 (Figure 15). Another region, the p53 binding site, near the carboxy terminus, appears to be involved, not in the induction of genomic instability, but in the survival of aberrant cells.

Human and rodent cells expressing the truncated T147 protein showed levels of structurally aberrant and aneuploid cells comparable to those induced by full length large T antigen indicating that this amino terminal region was sufficient for the induction of genomic instability. An

Figure 15: Partial Functional Map of T147. The figure shows the known functions remaining in the amino terminal 147 amino acids of SV40 large T antigen as well as the location of the regions found by this study to be involved in the induction of genomic instability

147
T137
T130
D10(128)
K1(107)
d11135 (Δ17-27)
p300 ?
y2Xmet
(Δ1-128)
Binding polα
Transactivation
of genes

additional deletion to amino acid 130 reduced the observed level of aberrations (structural + aneuploid) indicating the importance of the 17 amino acids between 130 and 147 in the induction of genomic instability. This region may be reduced further since the mutant T137, which expresses only the first 137 amino acids of SV40 large T antigen also shows a reduced level of aberrations comparable to that of cells expressing the T130 protein. However, since the T137 mutant protein was expressed at low levels and was not detectable by Western blot, it is difficult to ascertain whether the reduced level of genomic instability was due to the inability of the protein to induce aberrations or to its insufficient expression. If T137 is reduced in its ability to induce aberrations, then deletion to amino acid 130 had no additional affect and the relevant region can be narrowed to amino acids 137 and 147. On the other hand, if T137 is capable of inducing genomic instability at levels comparable to those of T147 and the reduced levels observed were due to low protein expression, the critical region is localized between amino acids 130 to 137.

Since none of the previously mentioned mutants were negative for the induction of genomic instability clearly other functions within the amino terminus are also involved. Several amino terminal mutants were analyzed to explore this possibility.

Two specific functions contained in the amino terminus were tested with the K1 and dl1135 mutants (Figure 15), which are defective in pRB binding and in the activity complementary to p300 binding by E1A, respectively. Cells expressing these mutant proteins showed a reduced level of aberrations in comparison to cells expressing wild type T antigen indicating that both the region between amino acids 17 and 27 and the pRB binding site may be involved in the induction of genomic instability.

Deletion of the first 128 amino acids in the y2Xmet mutant also reduces but does not abrogate the induction of genomic instability. This deletion removes both amino acids 17 to 27 and the pRB binding site (Figure 15), making y2Xmet a double mutant, though not a precise one, of the K1 and dl1135 mutations. The level of aberrations induced by y2Xmet remains comparable to that induced by both single mutants indicating that deletion of both sites does not have an additive effect. This implies that the pRB binding site and the region between amino acids 17 and 27, may have overlapping or redundant functions with respect to the induction of genomic instability and are likely not independent of each other. The y2Xmet protein, although mutated in the nuclear localization signal, is located in both the nucleus and cytoplasm at least in human cells (Table 25). However, as the results for the D10 mutant show, nuclear localization is not required for the induction

Table 25: Additional Properties of T antigen mutants used in this study[a]

Mutant	Mutation	Binding to Cell Proteins	Localization[b]	Host DNA Synthesis	Transformation/ Immortalization[c]	Genetic Instability[c]
T147[d]	Δ148-708aa	$p53^-$	N	+	+	+
T137	Δ138-708aa	$p53^-$	N	ND	+	±
T131	Δ131-708aa	$p53^-$	N	±	+	±
dl1135	Δ17-27aa	$p300^{-?}$	N	ND	±	±
K1	glu(107)-lys	pRB^-	N	+	±	±
D10	lys(128)-thr	+	C	ND[e]	+	±,+
y2Xmet128-70K	Δ1-127aa lys(128)-met	pRB^-	N/C	ND	±	±
dlA2433	Δ587-589aa	$p53^-$	N	ND	±	-
5080	ile(573)-phe	$p53^-/pRB^{\pm}$	N	ND	-	-
5081	pro(584)-leu	wt	N	+	+	+

a) references in text
b) N = nuclear, C = cytoplasmic
c) + = wild type levels, - = undetectable, ± = intermediate levels
d) pRT147 and pUCT147, but not pMTΔT147Ddl , also encode small t antigen
e) ND = not done

of aberrations (see below).

Since none of the mutants mentioned above completely abrogate the ability to induce genomic instability, it could be hypothesized that functional interactions or redundancy exist between these three sites and deletions or mutations in more than one or all would be required to abrogate the induction of genomic instability or that there are additional regions in the amino terminus that are also involved. The possibility of functional redundancy is supported by the results with the y2Xmet mutant with respect to the region between amino acids 17 to 27 and the pRB binding site. Results with the double mutant T130-K1, where there is no induction of genomic instability, were unfortunately inconclusive since expression of large T was undetectable by Western blot and could not be ascertained by immunostaining.

Two functions that map to the region between amino acids 130 and 147 of SV40 large T antigen are DNA binding and the stimulation of host cell DNA synthesis (Tjian et al., 1978; Baumann et al., 1985; Dobbelstein et al., 1992; Sompayrac and Danna, 1994). Hypotheses can be formulated on how both of these functions could play a role in the induction of genomic instability. Since SV40 large T antigen binds to the host cell DNA and also forms multimeric complexes with itself, it is possible that molecules of SV40 large T antigen could crosslink the genomic DNA of the host cell through these

associations. These crosslinks could affect both replication and transcription of DNA and result in the gaps and breaks observed (Schneider et al., 1990). Evidence against this hypothesis comes from C. LeFeuvre's analysis of the D10 mutant which is impaired for nuclear localization. If DNA binding was required for the induction of genomic instability this mutant should be impaired to a noticeable extent. However, some D10 expressing clones exhibited levels of genomic instability comparable to those pSV3neo positive control clones. While it could be argued that a basal level of T antigen could be present in the nucleus, none was detected by immunostaining (Table 25). Furthermore, if the level of expression was undetectable, a reduced level of genomic instability may still be expected in all the clones.

Stimulation of host cell DNA synthesis could also result in structural aberrations within the genome as well as aneuploidy. DNA synthesis in a cell without the additional mitosis would result in a tetraploid cell. In fact, perfect tetraploid cells, with the sister chromatids still aligned, were frequently detected in human fibroblasts expressing the T147 mutant protein. The presence of an additional complement of chromosomes when mitosis is reached could result in mistakes in the segregation of the chromosomes resulting in aneuploidy, contribute to the formation of structural aberrations such as the observed gaps and breaks when a

chromosome segregates incorrectly and is broken or alter the copy number of genes required for the maintenance of the normal karyotype and lead to further destabilization (Holliday, 1989). The hypothesis that the stimulation of host cell DNA synthesis is linked to the induction of genomic instability is supported by the observation that T130 is reduced in its ability to stimulate DNA synthesis (Dickmanns et al., 1994) in correlation with its reduced ability to induce genomic instability, while T147 is capable of inducing both DNA synthesis (Sompayrac and Danna, 1994) and genomic instability at levels comparable to those of full length large T antigen. It is yet to be investigated whether T137 and y2Xmet can stimulate DNA synthesis though the later still contains the region implicated in this function (Tjian et al., 1978; Baumann et al., 1985; Dobbelstein et al., 1992; Sompayrac and Danna, 1994). Furthermore, both the region between amino acid 17 and 27 and the pRB binding site, implicated by this study to be involved in the induction of genomic instability, are also related to one specific function of SV40 large T antigen; the ability to induce cellular DNA synthesis.

Binding to pRB by E1A of adenovirus, E7 of human papillomavirus and by SV40 of large T antigen has been shown to be required for the transformation and immortalization of cells (Egan et al., 1989; Manfredi and Prives, 1990; LaRose et

al., 1991; Quartin et al., 1994; Slebos et al., 1994) Binding of pRB by the transforming proteins of various viruses has also been implicated in the induction of DNA synthesis (Howe et al., 1990, Dickmanns et al., 1994), thereby providing a correlation between induction of DNA synthesis and transformation. As stated earlier, binding of SV40 large T antigen to p300 has not yet been confirmed, though the area between amino acids 17 to 27 of SV40 large T antigen can functionally complement E1A mutants defective in p300 binding (Yaciuk et al., 1991). Transformation of cells and induction of DNA synthesis by adenovirus E1A requires binding to p300 (Egan et al., 1989; Howe et al., 1990) and the complementary region of SV40 large T antigen has also been implicated as necessary for transformation in some cell contexts (Symonds et al., 1993) and to overcome p53 induced growth arrest (Quartin et al., 1994). Furthermore, the extreme amino terminal region, between amino acids 19 and 28, of SV40 large T antigen has been found to have DNA synthesis stimulatory activity in the context of full length large T antigen (Dickmanns et al., 1994). The results described here and in the literature implicate the pRB binding site and the region between amino acids 17 and 27 of SV40 large T antigen, in the transformation and immortalization of human cells, in the induction of cellular DNA synthesis and in the induction of genomic instability in transformed cells and provide a correlation

between all four of these phenomena. Therefore, it could be hypothesized that if the ability of large T antigen to induce DNA synthesis could be abrogated entirely the induction of genomic instability would be arrested as well.

Future work should address this hypothesis by investigating mutants of large T that combine mutations in the three areas implicated by this study in terms of the ability to induce genomic instability and cellular DNA synthesis. A mutant with the dl1135 and K1 mutations in combination with a truncated T antigen (T130) would be deleted for all the regions implicated by this study and the results obtained would indicate if there are other functions within the amino terminus that are involved in the induction of genomic instability. Also it would be interesting to see the effect a full length T antigen mutant combining both the dl1135, and K1 mutations with a deletion of amino acids 130 to 147 as this would preserve any carboxy terminal functions that may also be involved. It can be hypothesized that both the pRB and putative p300 binding site may have some sort of regulatory influence on the region between amino acids 130 and 147 responsible for the induction of DNA synthesis, either through a direct interaction with the region between amino acids 130 to 147 or more likely indirectly by influencing the cell cycle through the interaction with pRB and possibly p300. In adenovirus, pRB is bound and functionally deactivated by its

association with the CR2 region of E1A while p300 is bound by both the CR1 and the CR2 regions (Whyte et al., 1989). pRB binding may also be affected or modulated by sequences in the CR1 (Whyte et al., 1989). Also, binding of both p300 and pRB is required in adenovirus for the induction of cellular DNA synthesis (Howe et al., 1990). Loss of binding of one or the other results in a reduced synthesis (Shepherd et al., 1993). Therefore, it is possible that in SV40 large T antigen the region between amino acids 17 to 27 while not actually binding to p300 may have a regulatory effect on the pRB binding site and vice versa. T147 and exon 1 of adenovirus 5 E1A are very similar in that both proteins interact with the same cellular proteins and have similar effects on transformed cells. Therefore, pRB binding and deactivation may be regulated similarly in both viral proteins and the deletions of either amino acids 17 to 27 or the pRB binding site in SV40 large T antigen would both result in the same effect.

Two additional functions also map to the amino terminus of large T antigen: the ability to bind to DNA polymerase α and the ability to transactivate cellular genes. Neither were investigated in the current study and future work should address them. Transactivation of cellular genes is not required for the immortalization of cells (Thompson et al., 1990) but is required for morphological transformation (Alwine et al., 1987). It could be hypothesized that the

transactivation of cellular genes could deregulate protein expression in the cell, including proteins involved in maintaining genome integrity, resulting in destabilization of the genome. The effect of binding to DNA polymerase ∝ has yet to be investigated in either transformation or induction of genomic instability. However, it is of interest to note that the two sites involved in binding this protein, one in the amino terminus (binding site), the other in the carboxy terminus (regulatory site), overlap with the putative p300 binding site and the p53 binding site respectively. Therefore, another hypothesis to explain the behaviour of the dl1135 mutant could be defective binding of DNA polymerase ∝ resulting in reduced induction of host cell DNA synthesis and consequentially, of genomic instability. Both of the above mentioned regions are required for transformation and, as found by this study, are related to the induction of genomic instability either directly in the case of the region between amino acids 17 and 27 or through allowing the survival of aberrant cells in the case of the p53 binding site. It is unknown, however, whether the mutants used in this study are affected in DNA polymerase ∝ binding.

The role of p53 in the induction of genomic instability was also addressed by this investigation. The results obtained with both the constitutively expressed and the inducible constructs of T147 indicate that binding to p53 is not

required for the induction of genomic instability, since p53 binding is one of the functions deleted in the truncated protein (Figure 15, Table 25). However, the growth phenotype of primary human fibroblasts expressing T147 implied that this mutant protein had a growth inhibitory effect. Since p53 is thought to play a role in monitoring the genome for DNA damage and preventing aberrant cells from dividing until DNA repair is completed (Kastan et al., 1991; reviewed in Lane, 1992 and Vogelstein and Kinzler, 1992), and the T/p53 complex is required for the ongoing proliferation of transformed cells (Tevethia et al., 1988; Peden et al., 1989; Hara et al., 1991; Lin and Simmons, 1991; Zhu et al., 1991, 1992; Kierstead and Tevethia, 1993; Maclean et al., 1994) it is possible that the presence of free p53 in cells expressing T147 was resulting in the growth arrest of aberrant cells. This hypothesis is supported by the behaviour of cells expressing the 5080 and dlA2433 mutant T antigen, which are full length protein, but also do not bind p53. Growth of cells expressing these mutants was also impaired and the cultures usually stopped growing about 5 population doublings after isolation of the clones. The low level of aberrations observed in these cells could be explained by the active selection against highly aberrant cells by p53 leaving only those cells with low expression of T antigen and a low level of aberrations to grow and divide and therefore be available for cytogenetic analysis. A further

implication from the results with the BRK cells which still express wild type p53 but did not appear to be growth restricted when expressing T147, is that rodent p53 is not as stringent a monitor of genome integrity as human p53.

In the presence of free p53, entry of SV40 large T antigen transformed cells into S phase is delayed, but the level of DNA synthesis eventually becomes comparable to that induced when p53 is bound (Dobbelstein et al., 1992; Sompayrac and Danna, 1994). This suggests that p53 plays a role in regulating the cell cycle rather than directly regulating DNA synthesis. p53 also competes with DNA polymerase α for binding to large T antigen through the carboxy terminal regulatory site (Braithwaite et al., 1987) and mutations that affect p53 binding may also affect DNA polymerase α binding and in this way influence DNA synthesis. If this is the case then perhaps the reason for the failure of our complementation studies using both wild type and mutant p53 combined with the 5080 and 5081 mutant T antigens was the timing of p53 expression with respect to the period of the cell cycle and with respect to T antigen expression. It would be interesting to repeat those experiments with transfecting p53 in first and thereby priming the cells before of T antigen is expressed.

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