A MUTATIONAL ANALYSIS OF POLYOMA VIRUS LARGE T-ANTIGEN.

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ABSTRACT

Polyoma virus (PyV) is a murine papovavirus which induces neoplasia *via* the concerted action of three T- (tumour-) antigens. The 100kD PyV large T-antigen (LT) is a multifunctional protein involved in several aspects of the lytic or transforming behaviour of the virus. LT is absolutely required for viral DNA replication, and is also a transcriptional regulator, repressing its own transcription, and activating the PyV late transcription unit, as well as several other promoters of viral and cellular origin. LT is able to immortalise primary rodent fibroblasts, co-operates with the PyV middle and small T-antigens to induce full transformation, and reduces cellular growth factor requirements.

The aim of this thesis is to map the known biochemical and biological functions of LT to specific domains within the protein, and to assess the contribution of these functions to viral reproduction and to oncogenesis. To this end, a series of mutants spanning the LT coding region was constructed by in-phase linker-insertion. In addition, LT genes were isolated from a number of previously existing mutant viral genomes.

The ability of these mutant LT genes to promote DNA replication from the PyV origin in permissive cells was assessed. The capacity of the mutant LT proteins to activate transcription was investigated in another transient assay in which the mutant genes were introduced into mouse cells together with target plasmids containing the chloramphenicol acetyltransferase reporter gene linked to the PyV late promoter. The LT mutants were also assayed for their ability to immortalise early passage rat embryo fibroblasts, and to reduce cellular growth factor requirements.

The results of these investigations, in conjunction with published data, are used to produce a genetic map of PyV LT. The contribution of PyV LT to the viral life cycle, and the biochemical mechanisms underlying the biological activities of the protein, are discussed.

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ABBREVIATIONS

bp	base pair
C-terminus	Carboxy-terminus
CAT	Chloramphenicol Acetyltransferase
CKII	Casein Kinase II
CTF	CAAT-binding Transcription Factor
EC cells	Embryo Carcinoma cells
EGF	Epidermal Growth Factor
IGF-1	Insulin-Like Growth Factor-1
kb	kilobase pair
kD	kiloDalton
LT	Large T-Antigen
MHC	Major Histocompatibility Complex
MNBF	Mononucleotide Binding Fold
MT	Middle T-Antigen
NF-1	Nuclear Factor-1
N-terminus	Amino-terminus
ori	Origin of DNA Replication
PDGF	Platelet-Derived Growth Factor
PI	Phosphatidylinositol
PKC	Protein Kinase C
PPP	Platelet-Poor Plasma
PyV	Polyoma Virus
REF	Rat Embryo Fibroblast
SCE	Sister Chromatid Exchange
ST	Small T-Antigen
SV40	Simian Virus 40
T-Antigen	Tumour-Antigen
TGF	Transforming Growth Factor
TPA	12-*o*-Tetradecanoylphorbol-13-Acetate
ts	Temperature sensitive
TSTA	Tumour-Specific Transplantation Antigen
VP	Virion Protein

I: INTRODUCTION

A: DISCOVERY AND CHARACTERISATION OF POLYOMA VIRUS

Polyoma virus (PyV) was discovered by Gross, in 1953, as a contaminant of preparations of murine leukaemia virus, and is named after its ability to induce a wide range of tumours, of both mesenchymal and epithelial origin, in newborn mice. The pioneering work of Eddy, Stewart, Dulbecco, Stoker and others established conditions for the growth of the virus in tissue culture, and transformation *in vitro*. These early studies, together with the biological properties of PyV and its structural and functional organisation, are the subject of a number of review articles (Tooze, 1981; Griffin and Dilworth, 1983; Basilico, 1984) and many of the original references will not be cited here.

Cells from the mouse, the natural host of PyV, are permissive for viral growth, and infection with PyV leads to cell lysis and the release of viral particles, although rare transformed cells do arise. In contrast, rat or hamster cells are only semi-permissive for viral growth, little virus is produced, and foci of transformed cells arise at high frequency.

The phenotype of PyV-transformed cells generated *in vitro* is indistinguishable from that of cell lines derived from tumours generated by the virus. The cells have an indefinite lifespan in tissue culture, are not contact-inhibited, grow in soft agar, and exhibit a reduced requirement for serum growth factors.

Although inoculation of newborn mice, rats, or hamsters with high titres of PyV leads to the formation of tumours, immunocompetent adult rodents are generally resistant to the oncogenic effects of the virus. In fact, PyV is not normally associated with any disease of its natural host, and most wild populations of mice are found to be infected with the virus, with no obvious pathological effects.

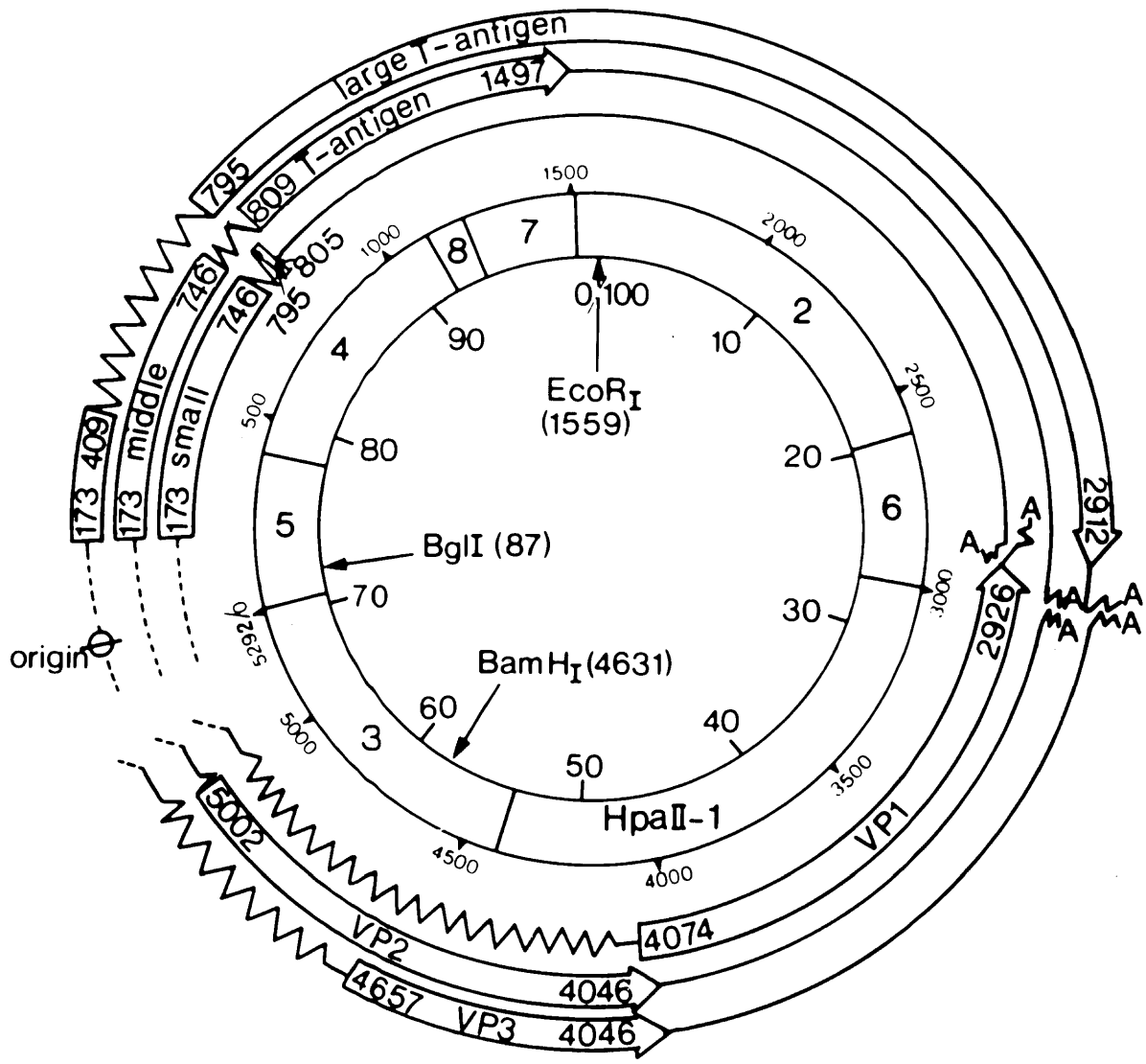
PyV has been classified as a member of the papovavirus family, which also contains the monkey virus SV40, and the human viruses BK and JC. SV40, which has considerable structural and functional similarity to PyV, replicates in human and monkey cells, but transforms cells of rodent origin, and is tumorigenic in newborn mice, rats, and hamsters.

Figure I.1: A Physical and Transcriptional Map of the Polyoma Virus Genome

The *Hpa*II restriction map of the PyV genome is shown, together with the transcription patterns of the early and late regions. Map units begin from the unique *Eco*RI restriction site, and nucleotide numberings, beginning at the viral origin of replication, are those of Soeda *et al.* (1980a).

Taken from Ito and Griffin (1987).

POSTULATED LANDMARKS ON POLYOMA VIRUS (A2 STRAIN).



5292 bp

B: ORGANISATION AND EXPRESSION OF THE PyV GENOME

1: Early and Late Transcription Units

Polyoma virus has an icosahedral capsid containing a supercoiled, circular DNA genome of approximately 5.3 kilobase-pairs, which is divided into two, functionally distinct, coding regions, located on either side of non-coding, regulatory sequences (see Figure I.1).

The early region, comprising nucleotides 1-2920 (nucleotide numbers throughout are for the A2 strain of PyV, sequenced by Soeda *et al.*, 1980a), encoding the three viral T- (or tumour-) antigens, is required for the replication of the viral genome, and is sufficient to transform rodent cells *in vitro*. Viral DNA replication is followed by expression of the late region (nucleotides 5292-2908), which encodes the capsid structural proteins, and is dispensible for transformation.

Transcription of the early region initiates at a number of sites (nucleotides 148-153, and a minor site at nucleotide 300), producing primary transcripts which undergo alternative splicing to generate three distinct families of mRNAs (see Figure I.1). Use of the donor and acceptor splice sites at nucleotides 409 and 795 respectively, produces messages encoding the 100kD large T-antigen (LT). The other two families of early messages employ the donor splice site at nucleotide 746, but utilise different acceptor sites. A 55kD protein, middle T-antigen (MT), is encoded by mRNAs spliced at nucleotide 809, and use of the acceptor site at nucleotide 805 produces messages encoding the 22kD small T-antigen (ST). The three T-antigens encoded by the PyV early region share the same N-terminal sequences (the so-called common region), but have unique C-termini, as they each utilise a different reading frame following the splice.

Polyadenylation of the early messages is usually directed by a signal located at nucleotide 2915, although a minority of lytic transcripts utilise an alternative processing signal at nucleotide 1476 (Kamen *et al.*, 1979). Further heterogeneity of early transcription is provided by the use of additional cap sites (nucleotides 5230-5265, 14, and 20) late in lytic infection (see Figure I.2).

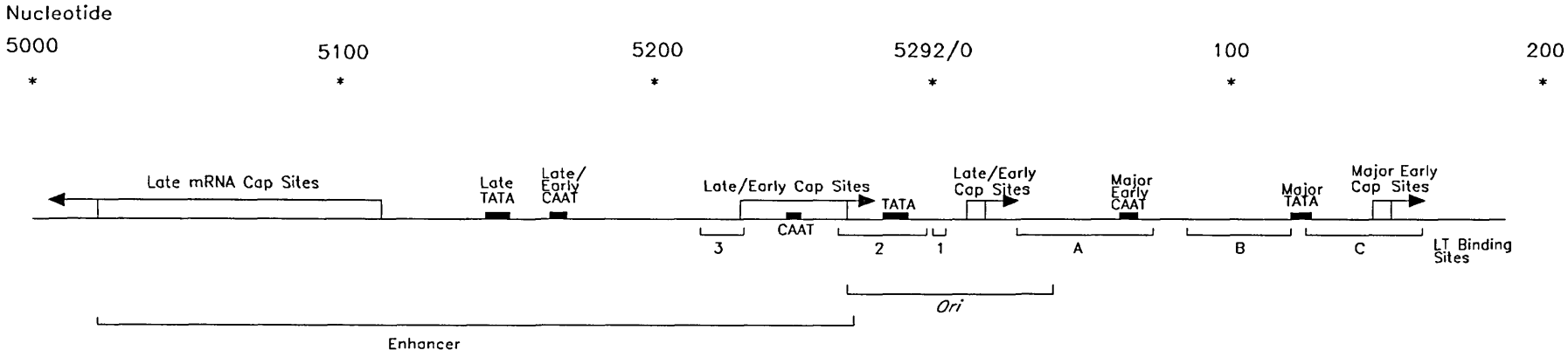
The late proteins, VP1, VP2, and VP3, are translated from three distinct families of messages, generated from a single family of precursor transcripts by a complex combination of splicing events. The late mRNAs, which are transcribed from the opposite DNA strand to the early messages, have extremely heterogeneous 5' ends, mapping to the region between nucleotides 5120 and 5021. All the late mRNAs contain multiple copies of nucleotides 5076-5020, generated by splicing of long precursor molecules comprising multimeric transcripts of the

Figure I.2: Functional Organisation of the PyV and SV40 Non-Coding Regions.

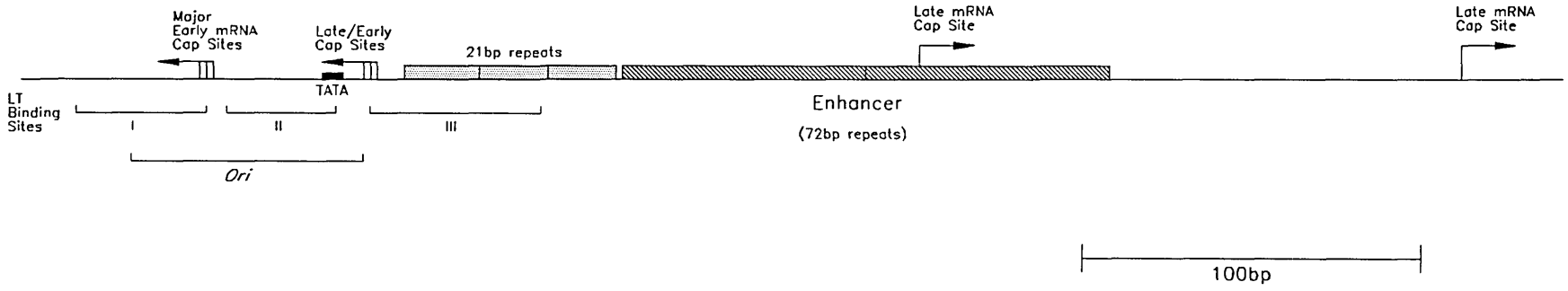
A: The locations of the PyV enhancer, origin of replication (*ori*), promoter elements (CAAT and TATA box homologies), mRNA cap sites, major LT-binding sites A, B, C, and minor sites 1, 2, and 3 (Cowie and Kamen, 1984) are shown. Late/Early refers to early region transcripts produced at later times of infection. Nucleotide numberings are those of Soeda *et al.* (1980a).

B: The locations of the SV40 enhancer (72bp repeats), *ori*, promoter elements (TATA box homology and 21bp repeats), mRNA cap sites, and LT-binding sites I, II, and III are shown. Late/Early refers to early region transcripts produced at later times of infection. Adapted from Das *et al.* (1985).

A


Nucleotide
5000
5100
5200
5292/0
100
200
Late mRNA Cap Sites
Late TATA
Late/ Early CAAT
Late/Early Cap Sites
TATA
Late/Early Cap Sites
Major Early CAAT
Major TATA
Major Early Cap Sites
CAAT
3
2
1
A
B
C
LT Binding Sites
Ori
Enhancer

B


Major Early mRNA Cap Sites
Late/Early Cap Sites
21bp repeats
Late mRNA Cap Site
Late mRNA Cap Site
TATA
LT Binding Sites
I
II
III
Enhancer
(72bp repeats)
Ori
100bp

entire late strand. This reiterated leader unit appears to fulfil an essential spacer function, affecting late mRNA splicing and stability, and can be replaced by unrelated DNA sequences (Adami and Carmichael, 1986; 1987). Another splice links the leader sequence to the body of the message, which has undergone one of the three possible splicing events shown in Figure I.1. The amino acid sequence of VP3 is identical to the C-terminal two thirds of VP2.

The SV40 genome is organised in a very similar manner to PyV, with a functional and structural division into early and late regions. Although SV40 encodes LT and ST proteins with considerable similarity to their PyV counterparts, it does not produce a middle T-antigen.

2: The Non-Coding Region

The PyV non-coding region governs the expression of the early and late transcription units, and contains the viral origin of replication (see Figure I.2).

i: Enhancer

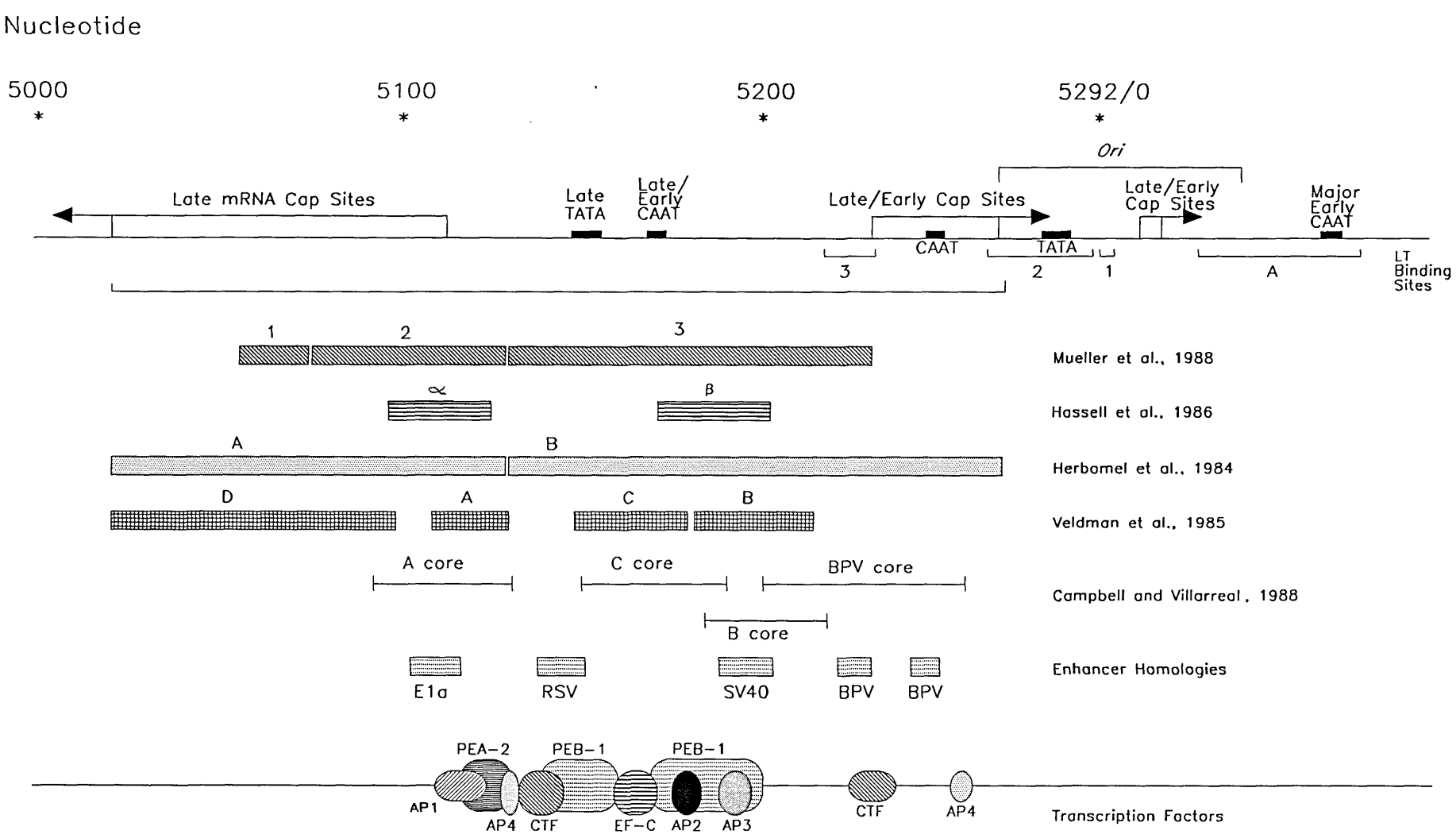
Enhancer elements are able to activate transcription when placed upstream or downstream of a gene, in either orientation, and at variable distances from the cap site (reviewed by Maniatis *et al.*, 1987). The *Bcl*I-*Pvu*II restriction fragment of PyV (nucleotides 5022-5267) possesses enhancer activity, stimulating transcription from the early and late promoters (de Villiers and Schaffner, 1981).

PyV, unlike SV40, requires a functional enhancer for viral replication (de Villiers *et al.*, 1984), although heterologous enhancers, of viral or cellular origin, can substitute for the endogenous sequences. Deletion analyses of the PyV enhancer have revealed a number of redundant sequence elements involved in the activation of gene expression and DNA replication (see Figure I.3). Herbomel *et al.* (1984) have defined two independent enhancer elements (A and B) separated by the PvuII site at nucleotide 5128. The less active B enhancer can be replaced by other viral or cellular enhancers, altering the tissue specificity of gene expression and replication (Campbell and Villarreal, 1986). However, the interaction between different enhancer elements is complex, exemplified by the unexpected pancreatic specificity exhibited by a recombinant polyoma virus with a hybrid PyV/murine leukaemia virus enhancer (Rochford *et al.*, 1987).

Hassell and colleagues (Hassell *et al.*, 1986; Mueller *et al.*, 1988) have identified three enhancer elements (1, 2, and 3) any two of which could activate gene expression, and also two replication elements (α and β) which overlap and may be identical to, elements 2 and 3. The replication elements were only active,

Figure I.3: Organisation of the PyV Enhancer

The location of the enhancer elements described in Section I.B.2.i are shown, together with regions of homology to the SV40, adenovirus E1a, bovine papillomavirus (BPV), and Rous Sarcoma Virus (RSV) enhancers (Ito and Griffin, 1987). The binding sites of transcription factors are also shown (Jones *et al.*, 1988; Wingender, 1988). Nucleotide numberings are those of Soeda *et al.* (1980a).


Nucleotide
5000
5100
5200
5292/0
Ori
Late mRNA Cap Sites
Late TATA
Late/ Early CAAT
Late/Early Cap Sites
Late/Early Cap Sites
Major Early CAAT
CAAT
TATA
3
2
1
A
LT Binding Sites
1
2
3
Mueller et al., 1988
α
β
Hassell et al., 1986
A
B
Herbomel et al., 1984
D
A
C
B
Veldman et al., 1985
A core
C core
BPV core
B core
Campbell and Villarreal, 1988
E1a
RSV
SV40
BPV
BPV
Enhancer Homologies
PEA-2
PEB-1
PEB-1
AP1
AP4
CTF
EF-C
AP2
AP3
CTF
AP4
Transcription Factors

however, if placed in juxtaposition to the origin of replication.

Veldman *et al.* (1985) defined four enhancer elements (A, B, C, and D) capable of activating gene expression and DNA replication. Again, these elements were found to be functionally redundant, with a combination of any two or three being as effective as the whole enhancer. Indeed, two copies of element A (corresponding to the A element of Herbomel and coworkers and element 2/α of Hassell and colleagues) could activate DNA replication, although three copies were needed to stimulate transcription.

Mutations of individual nucleotides in the PyV enhancer were found to alter DNA replication and gene expression simultaneously (Tang *et al.*, 1987), again suggesting that the enhancer fulfils the same function in the two processes. However, when synthetic oligonucleotides corresponding to different enhancer elements (see Figure I.3), were substituted for the entire B enhancer, an uncoupling of the effects on DNA replication and transcription was observed (Campbell and Villarreal, 1988). All the oligonucleotides were capable of stimulating gene expression to some extent, and could also, with the exception of the BPV core, activate DNA replication. These effects were cell-line specific, and no core sequence was able to mimic the behaviour of the wild-type enhancer. Activation of transcription but not DNA replication, and *vice versa*, was observed in some cell types, as were polar effects on replication but not transcription.

In conclusion, the PyV enhancer appears to be a complex mosaic of independent elements, which combine to activate gene expression and DNA replication in a tissue-specific manner. These enhancer elements interact with a number of cellular transcription factors (see Figure I.3). The rôles of the enhancer in replication and transcription appear to be very similar, but not identical. The SV40 enhancer, although not required for DNA replication, is also composed of a number of redundant sequence elements (reviewed by Ondek *et al.*, 1988).

ii: Promoters

The early and late promoters of PyV appear to be activated by the same enhancer sequences (Kern *et al.*, 1985), with the early promoter exhibiting the greater activity, in the absence of LT (Bourachot *et al.*, 1982). The major early promoter displays the "classical" organisation described by Maniatis *et al.* (1987), with the major early cap sites located approximately 31bp downstream (the terms "downstream" and "upstream" are used throughout to denote sequences located immediately to the 3' or 5', respectively, of a particular sequence element) of a

TATA box-like sequence, and 90bp away from a potential CAAT element (see Figure I.2). A functional rôle for these elements has not been established *in vitro*, as they can be deleted without major effects on the position or frequency of transcriptional initiation (Jat *et al.*, 1982a; Dailey and Basilico, 1985). However, mutant viruses lacking the TATA box and nearby upstream sequences, produced early mRNAs with extremely heterogeneous 5' ends (Farmerie and Folk, 1986).

The minor cap sites at nucleotides 14, 20, and 300 are also found to be located at the appropriate distances from TATA and CAAT box consensus sequences (Kamen *et al.*, 1982). However, the transcriptional start sites between nucleotides 5230 and 5265, utilised in the later stages of infection (Fenton and Basilico, 1982; Kamen *et al.*, 1982), lie downstream of sequences containing a potential CAAT box, but no TATA box. The site of transcriptional initiation from the SV40 early promoter (see figure I.2) is determined by a TATA box, located upstream from three 21bp repeats, not found in the PyV early promoter, which are required for maximal transcription (Das *et al.*, 1985).

The only consensus element apparent in the PyV late promoter is a weak TATA box homology at nucleotide 5155. Although some late transcripts do initiate approximately 30bp downstream from this sequence (Jat *et al.*, 1982b), the heterogenity of late mRNA cap sites is typical of transcription from a promoter lacking a functional TATA box. Sequences between nucleotide 5246 and the major early cap sites have also been observed to influence late gene expression (Kern *et al.*, 1985). Similarly, the SV40 late promoter (see Figure I.2) lacks a TATA box, resulting in late mRNAs with heterogeneous 5' ends, although there is a predominant cap site selected by a combination of three sequence elements, one of them located in protein coding sequences (Ayer and Dynan, 1988). The efficiency of transcription from the late promoter is reduced by mutations and deletions affecting the enhancer, 21bp repeats, and other sequences (Das *et al.*, 1985).

iii: Origin of Replication

Replication of the PyV genome requires, in *cis*, an enhancer and an origin (de Villiers *et al.*, 1984; DePamphilis, 1988). The minimal sequences capable of functioning as an origin of replication (the *ori* core) lie between nucleotides 5267 and 42 (Luthman *et al.*, 1982; Katinka and Yaniv, 1983), and are comprised of a 34bp sequence with dyad symmetry, flanked by an AT-rich region and a 14bp inverted repeat. Dailey and Basilico (1985) also include LT-binding site A in the minimal origin sequences. Although the PyV *ori* core is approximately 80% homologous to its SV40 counterpart, the organisation of the two viral non-coding

regions is different (see Figure I.2), as the PyV *ori* is separated from the early promoter while the two elements overlap in SV40 (Farmerie and Folk, 1984).

iv: LT-Binding Sites

PyV LT binds to a number of sites in the viral non-coding region. Cowie and Kamen (1984) employed DNAase I footprinting to localise three high-affinity LT-binding sites (A, B, and C) to the region between the *ori* core and the major early cap sites (see Figure I.2). In addition, three lower-affinity binding sites were found, two (1 and 2) within the 34bp dyad symmetry element in the *ori* core, and another (3) at the late side of the origin. All six binding sites were found to contain multiple copies of the consensus sequence G(A>G)GGC, which is also recognised by SV40 LT. Methylation interference experiments (Cowie and Kamen, 1986) confirmed this pentanucleotide sequence to be the site of contact between LT and the viral genome.

SV40 LT binds to three sites (I, II, and III) in the non-coding region (Das *et al.*, 1985). Binding to site II, located in the *ori* (see Figure I.2), is essential for viral DNA replication.

C: Biological and Biochemical Properties of the PyV T-Antigens.

The early region of PyV is essential for viral growth and is also responsible for cellular transformation. The biological and biochemical properties of the individual PyV T-antigens, their contribution to the lytic and transforming activities of the virus, and their functional similarity to their SV40 counterparts, are described below.

First, the properties of three classes of mutant viruses (see Figure I.4), defective in one or more of the T-antigens (reviewed by Griffin and Dilworth, 1983), will be described.

1: Mutant Polyoma Viruses

i: Tsa Mutants

The *tsa* mutants of PyV have a temperature-sensitive LT, with the defect mapping to the C-terminal region of the protein. The mutant viruses express normal MT and ST, and are defective for replication, and the initiation, but generally not the maintenance, of transformation.

ii: Mlt Mutants

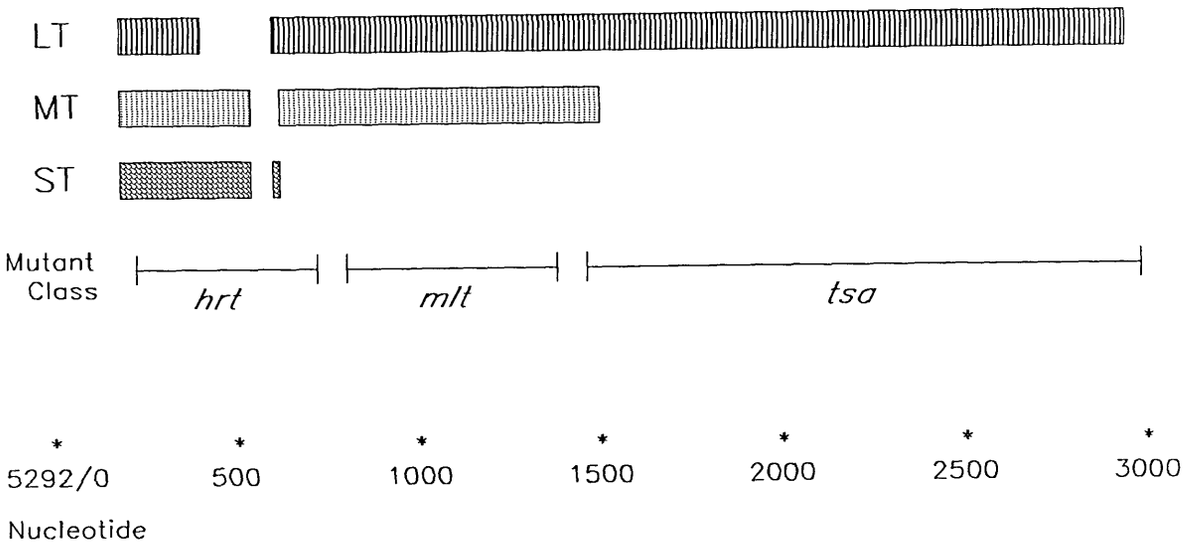
The *mlt* mutants of PyV express a normal ST protein, but have mutated MT- and LT-antigens, resulting in a wide variety of phenotypes, including wild-type. For example, the deletion mutant *dl*8 (Griffin and Maddock, 1979) replicates poorly, but transforms semi-permissive cells more effectively than the wild type virus. However, the *dl*23 (Griffin and Maddock, 1979) and *dl*2208 (Nilsson *et al.*, 1983) deletion mutants grow well, but transform inefficiently.

iii: Host-Range Transformation-Defective Mutants

The host-range transformation-defective (*hrt*) mutants of PyV were originally selected for their ability to grow on PyV-transformed, but not normal, mouse 3T3 cells (reviewed by Benjamin, 1982). Subsequently, *hrt* mutants, which lack functional MT and ST but express normal LT, as the mutations map to the LT intron, were found to grow on a restricted range of cell types, including primary mouse embryo cells. Cells supporting the growth of *hrt* mutants were hypothesised to express, constitutively, "permissivity factors" normally induced by MT and/or ST. Mutant viruses of the *hrt* class produce viral genomes to a level

Figure I.4: Map of the Three Classes of PyV Early Region Mutant.

The loci of the *tsa*, *hrt*, and *mlt* classes of PyV mutant are shown in relation to the viral genome and T-antigen coding sequences. Nucleotide numberings are those of Soeda *et al.* (1980a).


LT
MT
ST
Mutant Class
hrt
mlt
tsa
5292/0
500
1000
1500
2000
2500
3000
Nucleotide

30-40% of that achieved by the wild-type virus, but fail to encapsidate them into viral particles. This block in virion formation correlates with a defect in post-translational modification, particularly phosphorylation, on serine and threonine residues, of the major capsid structural protein, VP1 (Garcea and Benjamin, 1983; Garcea *et al.*, 1985). Viral DNA packaging and VP1 modification, in the form of phosphorylation and acetylation (Benjamin, 1984), is restored by coinfection of a wild-type virus. An additional defect in the acetylation of histones H3 and H4, derived from the viral chromatin, has also been observed for *hrt* viruses (Schaffhausen and Benjamin, 1976).

2: Large T-Antigen

PyV Large T-antigen has a predicted molecular weight of 88kD but migrates in polyacrylamide gels as a 100kD species. The protein, which has a 42% amino acid sequence homology to SV40 LT (Soeda *et al.*, 1980b), is found almost exclusively in the nucleus, and is associated with the nuclear cage (Humphrey and Pigiet, 1987a), but is excluded from the nucleolus (Dilworth *et al.*, 1986).

i: DNA Replication

Replication of the PyV genome is LT-dependent and proceeds bidirectionally from the viral origin, in a semi-discontinuous manner (reviewed by De Pamphilis and Wasserman, 1982; De Pamphilis, 1988). SV40 DNA replicates in a very similar fashion, differentiated mainly by its host cell-specificity (monkey or human cells for SV40 replication, mouse cells for PyV).

Murakami *et al.* (1986b) have defined conditions for the replication of PyV DNA *in vitro*. Multiple rounds of DNA synthesis can occur on a PyV *ori*-containing template incubated with a murine cell extract, purified PyV LT, and a supply of ATP. Replication does not occur in an extract of non-permissive cells, unless a purified murine DNA polymerase α-primase complex is added. A similar requirement for a human or simian polymerase α-primase complex has been demonstrated for the replication of SV40 DNA *in vitro* (Murakami *et al.*, 1986a), suggesting that a specific interaction between the replicase complex and LT-antigen may be a factor in determining viral host range. In fact, Smale and Tjian (1986) have demonstrated the binding of SV40 LT to DNA polymerase α, and suggest that this may target the polymerase to the viral origin of replication.

Analysis of the kinetics of SV40 DNA replication *in vitro* has revealed the existence of a lag period before the onset of rapid DNA synthesis. This delay can be prevented by pre-incubating the reaction mixture in the absence of

deoxyribonucleoside triphosphate precursors (Wold *et al.*, 1987). Preincubation of the SV40 DNA template together with SV40 LT and a purified fraction of cellular proteins produces, in the presence of topoisomerase I, a localised unwinding of the DNA helix, proceeding bidirectionally from the origin of replication (Wold *et al.*, 1987; Dodson *et al.*, 1987; Dean *et al.*, 1987a). Unwinding of the helix is ATP-dependent and specific for a functional *ori*. Although an *E. coli* single-stranded DNA binding protein, SSB, can substitute partially for the fraction of cellular proteins, it is likely that other cellular proteins are involved, as a significant reduction in the lag time is observed when pre-incubation is performed in the absence of template (Wold *et al.*, 1987). This so-called pre-synthesis reaction may represent the formation of a complex between cellular protein(s) and SV40 LT at the viral *ori*, leading to a localised melting of the DNA helix. A nucleoprotein complex of this type has been visualised by electron microscopy (Dean *et al.*, 1987b).

In addition to its *ori*-specific DNA-unwinding activity, SV40 LT possesses a general, sequence-independent DNA helicase activity (Stahl *et al.*, 1986). Processive, ATP-dependent unwinding of the DNA helix occurs in a 3'-5' direction when SV40 LT is presented with a DNA duplex flanked by a 3' single-stranded region (Goetz *et al.*, 1988; Wiekowski *et al.*, 1988). The presence of SV40 LT at the replication fork (Tack and Proctor, 1987) and the inhibitory effects of α-LT monoclonal antibodies on the elongation of SV40 DNA synthesis (Wiekowski *et al.*, 1987) suggests that LT may act as a processive helicase, separating the DNA strands at the replication fork, in a manner analogous to the dnaB protein of *E. coli*, although that protein unwinds the DNA helix in a 5'-3' direction (Kornberg, 1988). Indeed, α-SV40 LT monoclonal antibodies which inhibit the DNA helicase, but not ATPase, activity of the protein also prevent DNA replication (Wiekowski *et al.*, 1987), supporting a previous observation that the *ori*-binding and ATPase activities of SV40 LT were necessary, but not sufficient, for DNA replication (Stillman *et al.*, 1985).

Several of the cellular components of the SV40 *in vitro* DNA replication system have now been purified, including RF-A, a complex of three proteins which binds DNA, with a preference for single-stranded sequences. This complex is not required for the pre-synthesis reaction, unlike the more poorly-defined SSI protein(s) (Fairman and Stillman, 1988).

Formation of the pre-synthesis complex is followed by initiation of DNA replication, involving the DNA polymerase α-primase complex. However, elongation requires the proliferating cell nuclear antigen (PCNA) for co-ordinated synthesis of the leading and lagging strands (Prelich and Stillman, 1988). PCNA, or cyclin, is the auxiliary protein of DNA polymerase δ (Prelich *et*

al., 1987) and it appears to be this polymerase, rather than polymerase α, which is responsible for the elongation of DNA on the leading strand. DNA polymerase α seems to be required for replication on the lagging strand and for initiation (Prelich and Stillman, 1988), conclusions supported by the differential sensitivity to inhibitors of the initiation and elongation stages of SV40 replication *in vitro* (Decker *et al.*, 1987). Elongation also requires topoisomerase I or II to relieve the torsional strain induced by the accumulation of positive supercoils ahead of the replication fork, and topoisomerase II to separate catenated, fully-replicated daughter molecules (Yang *et al.*, 1987). However, replication of SV40 and PyV DNA *in vitro* has been reported to take place in a simple defined system containing only LT, the appropriate polymerase α-primase complex, topoisomerase I, a highly-purified HeLa cell extract containing a single-stranded DNA-binding protein (which may correspond to RF-A, and can be replaced by SSB from *E. coli*), and a source of ATP. Conversion of the reaction products to a covalently-closed, circular form requires an additional cellular extract, however (Wobbe *et al.*, 1987).

To summarise, the replication of the SV40 chromosome can be sub-divided into several distinct stages: a complex between LT and cellular protein(s) forms at the viral *ori*, resulting in the LT-catalysed melting of the DNA helix. LT, with its affinity for single-stranded DNA (Auborn *et al.*, 1988), can then enter to form a pre-synthesis complex analagous to the pre-initiation complex of *E. coli*, containing the dnaA, dnaB, and dnaC proteins (Kornberg, 1988; Bramhill and Kornberg, 1988). Unwinding of the DNA helix, stabilised by single-stranded DNA-binding proteins, allows the polymerase α-primase to synthesise an RNA primer and initiate DNA synthesis. Continued synthesis of the leading DNA strand is performed by DNA polymerase δ, while polymerase α copies the lagging strand. LT acts processively to unwind the helix at the replication fork, with cellular topoisomerases removing the resulting positive supercoils. DNA synthesis ceases when the two replication forks meet, RNA primers are removed by the combined action of RNaseH, DNA ligase, and DNA polymerases, and, finally, the two daughter molecules are segregated by topoisomerase II. In comparison, the rôle of PyV LT in DNA replication is poorly defined, although the protein is known to possess DNA-binding, ATPase, and DNA helicase activities (Clertant *et al*, 1984; Murakami *et al.*, 1986b). *Ori*-binding of PyV LT has been shown to be necessary, but not sufficient for DNA replication (Nilsson and Magnusson, 1984).

Both PyV and SV40 LTs bind nucleotides at a site distinct from the ATPase catalytic site (Clertant *et al.*, 1984), and it has been suggested that ATP bound to SV40 LT could act as a primer for DNA synthesis (Bradley *et al.*, 1984). The RNA molecule(s) linked to SV40 LT could also play a rôle in the initiation of

DNA synthesis (Carroll *et al.*, 1988). However, under conditions which support DNA replication *in vitro*, binding of SV40 LT to site II is enhanced by the presence of ATP, or one of its non-hydrolysable analogues, implying an allosteric rôle for nucleotide binding (Deb and Tegtmeyer, 1987; Dean *et al.*, 1987b; Borowiec and Hurwitz, 1988). The inhibitory effect of ATP on site II-binding observed by Vogt *et al.* (1986) may result from differences in experimental conditions.

The ability of SV40 LT to promote DNA replication may also be regulated by the phosphorylation state of the protein. Partial dephosphorylation of LT with alkaline phosphatase stimulates SV40 DNA replication *in vitro*, without affecting the ATPase or DNA helicase activities of the protein (Grässer *et al.*, 1987; Mohr *et al.*, 1987; Klausing *et al.*, 1988), although the possibility of a concomitant increase in site II-binding is a matter of some dispute. Binding of SV40 LT to sites I and II is affected by site-directed mutagenesis of the protein's ten phosphorylation sites, although a higher affinity for site II does not necessarily result in increased DNA replication (Schneider and Fanning, 1988). ATPase activity is again unaffected, but phosphorylation of tyr^{124} appears to be important for DNA binding and viral replication, confirming the results of other studies (Paucha *et al.*, 1986; Klausing *et al.*, 1988).

SV40 LT exists in multiple molecular forms, with distinct biophysical and biochemical characteristics. Only a subclass of LT, comprised mainly of newly-synthesised molecules which are monomeric and under-phosphorylated, exhibits a high degree of *ori*-binding activity. In contrast, "older" SV40 LT molecules are highly-phosphorylated, found predominantly in tetrameric complexes, and exhibit low DNA-binding activity (Scheidtmann *et al.*, 1984; Rünzler *et al.*, 1987). The DNA-binding subclass of LT can be further subdivided into populations with different affinities for site I and sites II and III, with the site II/III-binding population appearing immediately before the onset of SV40 DNA replication (Stetter *et al.*, 1988). In addition, only monomeric forms of SV40 LT exhibit DNA helicase activity (Deb and Partin, 1988).

Finally, a rôle for the oligomeric forms of SV40 LT in DNA replication is suggested by the observation that a mutant LT with a *ts* replication activity retains the ability to bind DNA at the non-permissive temperature, but no longer forms oligomers (Schürmann *et al.*, 1985). In addition, mutation of tyr^{124}, which abolishes site II-binding and DNA replication, also prevents oligomerisation (Montenarh and Müller, 1987). Oligomeric forms of LT have, in fact, been observed at the SV40 *ori* (Dean *et al.*, 1987b).

In conclusion, the contribution of SV40 LT to the initiation and elongation of viral DNA replication may be regulated by allosteric effectors, post-translational

modification, and oligomerisation. The phosphorylation sites of PyV and SV40 LT are generally not conserved (Hassauer *et al.*, 1986, Bockus and Schaffhausen, 1987b), and the functional significance of PyV LT phosphorylation is unknown, although *tsa* mutants of PyV LT are under-phosphorylated (Bockus and Schaffhausen, 1987a). Two subclasses of LT, exhibiting different phosphorylation states, also differ in their mode of association with the nucleus (Humphrey and Pigiet, 1987b). The effects of nucleotide binding and oligomerisation on the behaviour of PyV LT have also not been assessed.

ii: Transcriptional Activation

The infection of quiescent cells with PyV or SV40 produces a sequential stimulation of cellular RNA synthesis (Matter *et al.*, 1983). A virally-induced increase in hnRNA synthesis is observed 4-5 hours after the onset of T-antigen production, with an increase in the levels of 45S pre r-RNA, 5S rRNA, and tRNA following some 5-6 hours later. This stimulation of transcription is followed by the activation of the cellular DNA replication apparatus, the entry of the host cell into S-phase, and the production of viral progeny (Tooze, 1981), in a sequence of events very similar to that observed after the serum stimulation of quiescent fibroblasts (see Section I.C.2.iv).

Several cellular genes exhibit altered patterns of expression following transformation or lytic infection with PyV or SV40. For example, PyV- or SV40-transformed cells contain elevated levels of MHC class I gene transcripts (Majello *et al.*, 1985; Rigby *et al.*, 1985), and an increase in the expression of a MHC class I *H-2K* gene is observed following infection with PyV or SV40 (Rosenthal *et al.*, 1985).

A number of genes with elevated steady-state mRNA levels have been cloned by differential screening of cDNA libraries derived from PyV LT-expressing rat cell lines (Glaichenhaus *et al.*, 1985; 1986a; 1986b). Two genes, one, of mitochondrial origin, encoding subunit II of cytochrome oxidase, are also transcribed at increased rates in rat cells immortalised by the adenovirus E1a or rearranged c-*myc* genes. An increase in mitochondrial gene expression, which occurs co-ordinately for several mitochondrial transcripts in PyV LT-expressing cells, has also been observed in SV40-transformed mouse cells (Rigby *et al.*, 1985). Glaichenhaus and colleagues also found that mRNAs from four other genes accumulated in the G_1 phase of the cell cycle in PyV-transformed cells, but (to a lesser extent) in the G_2 phase of their non-transformed progenitors.

Other workers have attempted to identify PyV and SV40 LT-binding sites in cellular chromatin, in the hope that they may represent targets for transcriptional

regulation. Using this approach, Lane *et al.* (1985) have isolated SV40 LT-binding sequences which hybridise to mRNAs expressed at high levels in SV40-transformed, but not normal, cells. Similarly, a potential PyV LT-binding site has been isolated from mouse genomic DNA (Galup *et al.*, 1987). This sequence can be maintained in an autonomously replicating state in transgenic mouse strains, segregates stably through successive cell divisions, and is homologous to yeast centromeres and a human autonomous replication sequence (Léopold *et al.*, 1987).

The contributions of the individual T-antigens to the modulation of cellular and viral gene expression, and the functional significance of these effects, are not fully defined, but will be discussed below.

PyV late transcripts are rarely found in transformed cell lines, implying that the late promoter may not be active in non-permissive cells, and/or when contained in viral DNA integrated into host cell chromatin. However, selectable marker genes are expressed stably in non-permissive cells, when linked to the PyV late promoter (Bourachot *et al.*, 1982; Kern *et al.*, 1985). The restriction of late gene expression is therefore likely to occur post-transcriptionally, possibly at the level of message stability. Although transcription from the late promoter can occur in the absence of the PyV early antigens, it increases significantly in their presence (Kern *et al.*, 1986). Cotransfection of the LT gene with a non-replicating plasmid containing a truncated early region and the CAT reporter gene under PyV late promoter control, can result in a 60-fold increase in CAT activity. Transcriptional activation by LT requires a functional enhancer, but a significant level of stimulation is observed when LT-binding sites A, B, C, and 1 are deleted. Kern *et al.* (1986) also observed a low level of activation by a mutant LT-antigen defective in origin binding. Deletion of the truncated early region (encoding full length MT, ST, and an N-terminal LT fragment) was found to increase the basal level of CAT activity 10-fold, suggesting that the removal of the competing early transcription unit, either physically or by autoregulation (see next section), could be a major component in the increase of late gene expression observed during the later stages of lytic infection. Cotransfection of a LT gene with a late promoter/CAT plasmid lacking the early region produced a lower level of activation than observed in the presence of the truncated early region, and cotransfection of this reporter plasmid together with a complete PyV early region, or a combination of LT and MT genes, was found to be necessary for maximal stimulation.

The PyV early region has also been observed to stimulate a variety of cellular and viral promoters (Pannuti *et al.*, 1987a), including the rabbit β-globin, chicken 2(α)-collagen, and mouse *H-2K* promoters. This effect was attributed mainly to MT, although a small stimulation was observed in the presence of LT,

but not ST. Trans-activation was confined to transient assays, with the exception of the *H-2K* promoter, which could be activated even in an integrated state. PyV LT can also bind to, and activate, the human *hsp*70 promoter, although the extent of transactivation is unaffected by deletion of the LT-binding sites found within the promoter (Kingston *et al.*, 1986). Again, LT alone produces a smaller effect than the entire early region, implying a rôle for the other T-antigens.

Thus PyV LT may be able to activate its own late promoter by two mechanisms, one *via* binding to specific DNA sequences, and another, indirect, method which may involve protein-protein interactions. Similarly, LT can activate heterologous cellular and viral promoters by a mechanism which is independent of DNA binding. The other T-antigens, particularly MT, also appear to play a significant rôle in transcriptional activation. SV40 LT activates the SV40 late promoter *via* a complex interaction with a number of sequence elements in the viral control region (Keller and Alwine, 1985; Brady *et al.*, 1985). Elements within the origin and enhancer appear to act synergistically to bind cellular factors, which exhibit altered binding properties in the presence of LT (Gallo *et al.*, 1988). There is also evidence for negative regulation of the late promoter, which can be relieved by the introduction of specific competitor DNA (Brady and Khoury, 1985; Alwine and Picardi, 1986), although the two groups identify different regions of the promoter as the site of interaction with the putative repressor.

SV40 LT can stimulate the SV40 late promoter in the absence of DNA binding sites (Keller and Alwine, 1985), and can also activate, but not bind to, the adenovirus E2 promoter, in a cell-specific manner (Loeken *et al.*, 1986).

iii: Transcriptional Repression

The ability of PyV LT to repress early region expression was first demonstrated by Cogen (1978), following the discovery of a similar phenomenon in SV40 (Tegtmeyer *et al.*, 1975; Reed *et al.*, 1976).

Autoregulation by SV40 LT is thought to be mediated through the binding of the protein to sites I and II (Figure I.2) which cover the early cap sites and TATA box (Rio and Tjian, 1983). The steric hindrance caused by the binding of LT at these sites could prevent transcriptional initiation by RNA polymerase II. In addition, a third LT-binding site (Site III) located in promoter sequences further upstream is required for maximal repression (Das and Salzman, 1985). SV40 LT bound at this upstream site could prevent the entry of RNA polymerase II or the binding of transcription factors.

PyV LT may down-regulate early transcription by a similar promoter-exclusion mechanism, but the rôle of the protein in repression has yet to be defined. Deletion of LT-binding site C (see Figure I.2), which covers the major early cap sites and TATA box, has a very limited effect on early transcription (Farmerie and Folk, 1984), an observation confirmed by the work of Dailey and Basilico (1985), who implicate binding sites A, 1, and 2 as the targets for repression by LT. However, LT-binding site C alone appears to be sufficient for maximal repression by LT (Kern *et al.*, 1986). The apparent contradictions in these data may result from the pleiotropic effects of deletions in the early promoter region, and it is probable that binding of LT to several sites will contribute to autoregulation. There is also evidence for an indirect mode of repression, as a mutant PyV LT-antigen, defective in DNA binding, can down-regulate the early promoter (Kern *et al.*, 1986).

iv: Effects on Host Cell DNA Synthesis

PyV infection of rodent cells reduces their requirement for serum growth factors, and induces cellular DNA replication. These phenomena are probably, although not necessarily, related, and will be described separately.

a: Competence/Progression

On reaching confluence, or in the absence of essential growth factors or nutrients, cells leave the cell cycle in G_1, and enter a quiescent state known as G_0. The addition of fresh serum to quiescent Balb c/3T3 cells stimulates them to re-enter the cell cycle and reinitiate DNA synthesis (see Figure I.5), following an ordered sequence of events (reviewed by Cochran, 1985; Pardee *et al.*, 1986; Denhardt *et al.*, 1986).

The addition of platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) to quiescent Balb c/3T3 cells induces a state of "competence", allowing them to traverse the cell cycle in response to the progression factors present in platelet-poor plasma (PPP). The competent state is reached 2-3 hours after treatment with PDGF, which is not required continuously, and is stable, with a half-life of 10-20 hours. Competence requires *de novo* transcription and can be transferred by cell fusion or the microinjection of purified mRNA. Cycling cells are rendered competent during the previous growth cycle. Competent cells enter S-phase at least 12 hours after the addition of PPP, which can be replaced by a combination of epidermal growth factor (EGF), and

Figure I.5: A Schematic Representation of the Mammalian Cell Cycle

The sequence of events following the serum stimulation of quiescent Balb c/3T3 cells (described in Section I.C.2.iv.a) are shown, together with the serum growth factors required for each stage of the cell cycle.

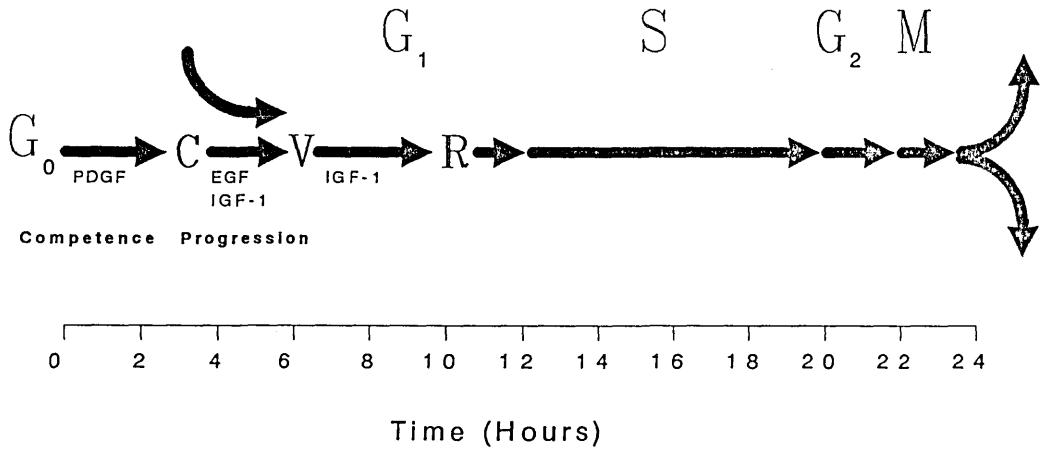
Adapted from Pardee *et al.* (1986).

Abbreviations

PDGF: Platelet-Derived Growth Factor

EGF: Epidermal Growth Factor

IGF-1: Insulin-Like Growth Factor-1


G1
S
G2
M
G0
PDGF
C
EGF
IGF-1
V
IGF-1
R
Competence
Progression
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (Hours)

insulin-like growth factor 1 (IGF-1), also known as somatomedin-C. Hyperphysiological concentrations of insulin can substitute for IGF-1.

Six hours after treatment with progression factors, competent cells reach a control point, V, at which they have restored their synthetic capacity, and are identical to cycling cells in G_1. Progression beyond V requires IGF-1 only, along with high levels of transcription and translation. Approximately four hours later, the cells reach a restriction point, R, at which they commit themselves to entry into S-phase. Progression beyond this point is independent of growth factors and transcription, although IGF-1 may be required for transition through a possible control point, W, located immediately before S-phase. Cells commence DNA synthesis two hours after passing through R, and proceed through S, G_2, M, and cytokinesis into G_1. Physiological regulation of the cell cycle is confined to G_1, during which the cells respond to the prevailing environmental conditions, and either continue to cycle or return to a quiescent state.

In an attempt to define the molecular basis of the competent state, a number of groups have identified genes which are induced in response to PDGF (reviewed by Denhardt *et al.*, 1986). Expression of the proto-oncogenes c-*myc* and c-*fos* is stimulated by treatment with PDGF (Kelly *et al.*, 1983; Kruijer *et al.*, 1984; Greenberg and Ziff, 1984; Cochran *et al.*, 1984). Induction occurs rapidly (within 30 seconds for c-*fos*) and is enhanced in the presence of cycloheximide, suggesting that the genes may be under the negative control of a labile protein. In addition, several PDGF-regulated genes have been identified by differential screening of cDNA libraries derived from Balb c/3T3 cells (Cochran *et al.*, 1983; Lau and Nathans, 1987). Expression of these genes is stimulated by competence, but not progression, factors, and is often superinducible in the presence of cycloheximide. There appear to be cell-cycle constraints on the regulation of "competence" genes by PDGF however, as c-*fos* and three other genes (JE, KC, and, JB) cloned by differential screening are only induced in quiescent cells, while c-*myc* is induced maximally in cycling cultures (Rollins *et al.*, 1987).

The c-*myc* gene does appear to fulfil a functional rôle in the production of the competent state, as microinjection of the purified *myc* protein, or gratuitous induction of the c-*myc* gene, allows cells to grow in PDGF-free medium, and renders them hypersensitive to EGF (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985). However, the reduced mitogenic effect, in comparison to the response to PDGF itself, indicates that c-*myc* is not the only mediator of PDGF action. For example, JE appears to encode a protein with cytokine-like properties, and could possibly act in an autocrine manner to stimulate cell growth (Rollins *et al.*, 1988). Finally, the growth of cells may also be regulated by inhibitory factors, for

example, mouse α/β interferon has been shown to inhibit the establishment of competence in Balb c/3T3 cells (Lin *et al.*, 1986).

Following the identification of c-*myc* as a competence gene, a number of attempts have been made to characterise the effects of other cellular and viral genes on growth factor requirements (Armelin *et al.*, 1985; Zhan and Goldfarb, 1986; Kelekar and Cole, 1987). The results obtained by different groups of workers are frequently in disagreement, possibly reflecting the different assays and cell lines employed. It should be noted that cell lines as similar as Balb c/3T3 (utilised for the competence/progression studies described above) and Swiss 3T3, have very different growth factor requirements, with PDGF acting as a complete mitogen for the latter (Rozengurt, 1986). A similar lack of consensus is apparent for the effects of the PyV early antigens on cellular growth factor requirements.

Binding of the PyV capsid to its cell-surface receptor transiently induces c-*myc*, c-*fos*, and JE expression, and allows Balb c/3T3 cells to respond to progression factors (Zullo *et al.*, 1987). This mitogenic effect is independent of PyV early gene expression, and can be induced by the purified VP1 capsid protein.

Expression of PyV LT, but not MT, or ST, allows the established FR3T3 rat cell line to form colonies in 0.5% newborn calf serum when seeded at low density (Rassoulzadegan *et al.*, 1982; Mougneau *et al.*, 1984). In addition, introduction of LT into a MT-expressing FR3T3 cell-line (MTT cells) allows focus formation and anchorage-independent growth at low serum concentrations. The introduction of ST into MTT cells leads to the formation of small, slow-growing colonies in soft agar containing 0.5% serum. Balb c/3T3 cells expressing LT or MT were capable of colony formation when seeded at low density in PPP (Armelin *et al.*, 1985). In contrast, the introduction of LT into NIH 3T3 cells by a retroviral vector had no effect on cellular growth factor requirements, but MT allowed growth in PPP, and abolished the need for EGF, but not IGF-1 (Cherington *et al.*, 1986). A number of clonal derivatives of the F111 rat cell line, transformed by defective PyV viruses encoding functional ST and MT only, exhibited a range of serum requirements (Priehs *et al.*, 1986). The extent of serum independence did not appear to correlate with the presence of stable fragments of LT-antigen.

In addition to the transient stimulation of competence gene function resulting from the binding of PyV capsids to the cell surface, a second, prolonged, phase of induction follows the expression of the early antigens (Zullo *et al.*, 1987). The use of mutant viruses indicated that MT and LT were dispensable for this second phase of mRNA accumulation. However, the expression of MT under the control of an inducible promoter in Balb c/3T3 cells was found to produce an increase in c-*myc* message levels. The actual effect of this second phase of mRNA

accumulation on cell growth was not assessed, however. Clearly, all three early antigens of PyV may be capable of altering cellular growth factor requirements, both in an assay- and cell line-specific manner.

SV40 infection has been shown to complement both competence and progression activities (Cochran, 1985), and SV40-transformed cells have been found to secrete PDGF-related molecules (Ross *et al.*, 1986).

b: Induction of Host Cell DNA Synthesis

PyV *tsa* mutants, which produce thermolabile LT-antigens, induce cellular DNA synthesis even at the non-permissive temperature (Fried, 1970; Eckhart, 1975). However, the *hrt* mutant NG59, which lacks functional MT and ST, can also induce host cell DNA replication (Schlegel and Benjamin, 1978). This mitogenic effect of NG59 is restricted to one round of cellular DNA synthesis, whereas wild-type virus can induce multiple rounds of replication. As Zullo *et al.* (1987) found that NG59 infection triggered the first phase of competence gene expression, but not the second, it is possible that the mitogenic action of NG59 may merely be the consequence of capsid binding. Prolonged stimulation of cellular DNA synthesis would therefore appear to involve the expression of MT and/or ST. However, LT may also play a rôle, as transfection of a LT gene (but not MT or ST) stimulates density-arrested FR3T3 cells to re-enter S-phase (Rassoulzadegan *et al.*, 1983a). Microinjection of purified SV40 LT is sufficient to induce one round of cellular DNA synthesis (Tjian *et al.*, 1978), but ST may be required for subsequent rounds of replication (Martin, 1981).

v: Immortalisation and Transformation

Transformation by PyV virus (reviewed by Griffin and Dilworth, 1983; Cuzin, 1984; Basilico, 1984) involves the co-ordinated action of the three viral early antigens. Although early studies utilising mutant viruses helped to define the contribution of the early proteins to the process of cellular transformation, a detailed analysis was facilitated by the construction of a set of modified viral genomes, each capable of expressing only one of the three T-antigens (Treisman *et al.*, 1981; Zhu *et al.*, 1984).

Transfection of a MT-only plasmid into Rat-1 cells induced them to form foci, grow in soft agar, and produce tumours in syngeneic rats (Treisman *et al.*, 1981), but the same plasmid could not transform primary rat cells (Rassoulzadegan *et al.*, 1982). Subsequently, the ability of LT to immortalise early passage rodent cells, allowing them to proliferate indefinitely in tissue

culture, was demonstrated (Rassoulzadegan *et al.*, 1983b). Cell lines established by LT could then be transformed by supertransfection of a MT gene, suggesting that a combination of LT and MT should transform primary rodent cells. In fact, a combination of the two genes immortalises, but does not transform, early passage rodent cells, and simultaneous expression of all three early antigens is required for transformation. Cell lines established by cotransfection of LT and MT progress spontaneously to a transformed phenotype on passaging in culture (Lemieux, 1986), and can be transformed by the introduction of a ST gene.

Although a truncated LT, in concert with MT and ST, can transform early passage rodent cells, a full-length protein is required when the genes are introduced by viral infection, presumably reflecting the role of C-terminal LT sequences in promoting the integration of viral DNA (Basilico, 1984).

A rôle for LT in the maintenance of the transformed state has been suggested by Cuzin and coworkers on the basis of the observation that FR3T3 cells transformed by the *tsa*A mutant virus exhibit a transformation-stable (A) or -unstable (N) phenotype at the restrictive temperature (Seif and Cuzin, 1977). A-type clones were found to contain a stable, truncated LT species, while the N clones expressed a thermolabile, full-length protein, and could be converted to the A phenotype by the introduction of a truncated LT gene (Rassoulzadegan *et al.*, 1981). These data are in apparent contradiction to the results of later experiments employing cloned DNA fragments, and may reflect the different numbers of genes that were introduced by infection or transfection.

Bastin and colleagues have produced data on PyV transformation which differ frequently from the results of Cuzin, Kamen and coworkers detailed above. Employing an *in vivo* transformation system, they have demonstrated that a combination of MT and LT or ST genes can substitute for wild-type DNA in producing tumours on inoculation of newborn rats or hamsters (Asselin *et al.*, 1983; Bouchard *et al.*, 1984). The fibrosarcomas and liposarcomas arising at the site of inoculation can also be induced by MT DNA alone, in newborn hamsters, but not rats, albeit with a latency period 5-10 times longer than found for wild-type DNA. *In vitro*, a combination of MT and LT or ST was found to produce a partial transformation of early passage rat embryo fibroblasts (REFs), with progression to a fully-transformed phenotype occurring at high frequency (Bouchard *et al.*, 1986). A similar partially-transformed phenotype resulted from the cotransfection of all three T-antigen genes, suggesting that cellular factors were contributing to transformation. The progression to a fully-transformed state was not accompanied by an increase in MT expression, as had been found previously for FR3T3 cells transfected with MT (Gélinas and Bastin, 1985).

Transgenic mice expressing MT develop endothelial tumours, but LT-expressing mice have no abnormal phenotype (Bautch *et al.*, 1987; Williams *et al.*, 1988).

The co-operation of the PyV T-antigens in the transformation of rodent cells is paralleled by the combined action of the adenovirus E1a and E1b proteins (reviewed by Bernards and van der Eb, 1984) and, more generally, by the co-operating oncogene model of transformation (Land *et al.*, 1983; Ruley, 1983). In this model, cellular and viral oncogenes are classified into two groups (reviewed by Weinberg, 1985). Group I oncogenes, including PyV LT, adenovirus E1a, and c-*myc*, exhibit immortalising activity, and co-operate with Group II oncogenes, including PyV MT, adenovirus E1b, and EJ-*ras*, to transform primary rodent cells. Group II oncogenes are also capable of transforming established cell lines.

Although it has proved useful, this classification of oncogenes is clearly an over-simplification, as the distinction between the two categories is far from rigid. For example, EJ-*ras* can immortalise primary rodent cells (Spandidos and Wilkie, 1984; Kelekar and Cole, 1987), and established cell lines expressing Group I oncogenes are found to be tumorigenic in nude mice (Keath *et al.*, 1984). Similarly, PyV MT has been found to immortalise REFs at low frequency (Asselin and Bastin, 1985), and can, like EJ-*ras*, establish and transform primary Chinese hamster cells in a single step, when linked to transcriptional enhancers (Spandidos and Wilkie, 1984; Spandidos and Riggio, 1986). An additional simplification in the co-operating oncogene model is indicated by the observation that not all combinations of Group I and II oncogenes lead to transformation, for example, PyV LT co-operates with EJ-*ras*, but not PyV MT, to transform non-established rodent cells.

SV40 LT has been shown to immortalise primary rodent cells and to transform established cell lines (reviewed by Livingston and Bradley, 1987). Retroviral vectors expressing SV40 LT immortalise early passage REFs at high frequency, but the resulting cell lines are rarely tumorigenic (Jat and Sharp, 1986). Similarly, infection of the F111 rat fibroblast cell line with an SV40 LT-expressing recombinant retrovirus produces only a small number of transformants, although a much greater effect is observed if the NIH 3T3 mouse fibroblast cell line is used (Brown *et al.*, 1986; Jat *et al.*, 1986). SV40 can transform primary and established cells in the absence of functional ST, but with an increased sensitivity as to the type and growth state of the host cell (Sleigh *et al.*, 1978; Martin *et al.*, 1979). Mutants of SV40 with an altered ST protein can grow normally, but have a reduced transformation range, failing to transform the Balb c/3T3 cell line, and quiescent cells, for example. Balb c/3T3 cells can be transformed by a LT-only virus, at high multiplicities of infection, but this requirement for high

concentrations of LT in transformation is greatly reduced by the presence of ST (Bikel *et al.*, 1987).

vi: Effects on Cellular Differentiation

The ob17 and 3T3-F442A mouse preadipocyte cell lines can be induced to differentiate *in vitro* to form fat cell clusters, but fail to do so if transformed by PyV (Grimaldi *et al.*, 1984). Differentiation of 3T3-F442A cells is also blocked by the introduction of the LT or MT genes *via* a retroviral vector, although the effects of MT are unstable (Cherington *et al.*, 1986). Ob17 cells can be induced to differentiate in serum concentrations greater than 1%, and cease to be viable in medium containing less than 0.3% serum. In contrast, MT-transformed derivatives of ob17 are viable, and differentiate, but do not proliferate, in serum-free medium (Grimaldi *et al.*, 1984). MT-expressing ob17 cells continue to differentiate in serum concentrations up to 3%, but adipocyte conversion is abolished in 10% serum, and the abilities of MT-transformed ob17 cells to differentiate and proliferate in low serum concentrations were found to be inversely related. Cells transformed by the complete PyV early region were incapable of differentiation at any serum concentration. MT-transformed ob17 cells secrete proteins of 28kD and 10kD which respectively stimulate the growth and differentiation of the parental ob17 cells (Czerucka *et al.*, 1986).

SV40 LT also blocks the differentiation of 3T3-F442A cells, producing a more pronounced effect than its PyV equivalent. SV40 LT can also, unlike PyV LT, block the differentiation of the M1 premonocyte cell line (Cherington *et al.*, 1987, 1988).

vii: Effects on Sister Chromatid Exchange

Rat fibroblasts expressing PyV LT, deregulated c-*myc*, or adenovirus E1a genes, exhibit an increased rate of sister chromatid exchange (SCE). The frequency of SCE, the exchange of two DNA strands at apparently homologous loci, reflects the level of recombination occurring within the cell. In contrast, PyV MT and the EJ-*ras* oncogene, despite transforming the cells, have no effect on the rate of SCE. Cell lines expressing LT, c-*myc*, or E1a, in combination with MT or EJ-*ras* continue to display the background rate of SCE. The genomic instability induced by LT is reflected in the abnormal karyotypes displayed by cell lines expressing the gene (Cerni *et al.*, 1986; 1987).

viii: Recombination

The integration of PyV DNA into the host cell chromatin, and its subsequent excision, is influenced by LT antigen (reviewed by Basilico, 1984). The viral genome usually integrates as a concatamer in the presence of functional LT, but as a single copy in its absence. Together with the observation that host-viral junctions had arisen by illegitimate recombination, this suggested that LT promoted rolling-circle replication of the viral genome, with the resulting oligomers integrating *via* a double cross-over event. Similarly, it has been proposed that LT promotes the excision of integrated genomes by inducing replication *in situ*, with free viral episomes arising by homologous recombination of duplicated sequences. Piché and Bourgaux (1987a,b) have suggested that PyV LT may, in fact, have a direct involvement in the excision of viral genomes. Mouse cells transformed by a *tsa* mutant generate a hybrid mouse/PyV replicon at the permissive temperature. This hybrid molecule then undergoes an internal recombination event, generating an infectious viral genome. The homogeneity of the products, and the frequency at which they are formed, suggest that they were generated by site-specific, rather than illegitimate or homologous, recombination. The requirement for LT in this process implicates the protein as a possible site-specific recombinase. The same authors have also proposed a similar activity for SV40 LT.

ix: Mutagenic Activity

The PyV early region is mutagenic, increasing the spontaneous mutation rate at the *hprt* locus (M. Strauss, personal communication). None of the individual early antigens were mutagenic, but transfection of ST, in combination with LT or MT, produced a mutagenic effect. LT alone was found to increase the mutation rate when overexpressed from a metallothionein promoter.

SV40 also possesses a mutagenic activity, dependent on a functional early region (Theile *et al.*, 1987).

x: Nuclear Localisation Signals

A sequence of predominantly basic amino acids (pro lys lys^{128} lys arg lys val) is required for the efficient nuclear localisation of SV40 LT (Lanford and Butel, 1984; Kalderon *et al.*, 1984). A similar sequence (pro lys lys^{282} ala arg glu asp) determines the nuclear location of PyV LT, in conjunction with a second sequence (val ser arg lys^{192} arg pro arg) which has no equivalent in SV40 LT, but

is found in a number of other nuclear proteins (Richardson *et al.*, 1986; Allday *et al.*, 1988).

xi: TSTA

PyV-induced tumours cannot be transplanted into syngeneic adult animals because of the presence of a tumour-specific transplantation antigen (TSTA) which promotes immunological rejection (Tooze, 1981). The contributions of the individual T-antigens to this effect have been investigated by the construction of vaccinia vectors expressing LT, MT, or ST (Lathe *et al.*, 1987). Vaccination with LT or MT prevents tumour formation when young rats are inoculated with PyV-transformed, syngeneic rat cells. Immunisation with LT or MT is also found to induce the rejection of existing tumours.

xii: LT-Protein Interactions

SV40 LT associates with a number of cellular proteins, including p53, which, in the absence of LT, is capable of immortalising primary rodent cells, transforming established cell lines, and increasing the tumorigenicity of transformed cells (reviewed by O'Reilly, 1986). There appears to be competition between p53 and the cellular DNA polymerase α for a binding site on SV40 LT (Smale and Tjian, 1986; Gannon and Lane, 1987). A complex between SV40 LT and the cellular transcription factor AP-2, which binds to the SV40 and PyV enhancers and a number of cellular promoters, has also been identified (Mitchell *et al.*, 1987), and LT also associates with the retinoblastoma anti-oncogene product, *Rb* (DeCaprio *et al.*, 1988). Finally, tyrosine and serine protein kinase activities, of cellular origin, are reported to associate with LT (Walser and Deppert, 1986). In contrast, little is known about the protein-protein interactions of PyV LT, although it does associate with the PyV capsid protein VP1 (Dilworth and Griffin, 1982), and can be cross-linked to cellular proteins of 70, 72, 129, and 150kD, which are believed to be components of the nuclear matrix (Humphrey and Pigiet, 1987b).

3: Middle T-Antigen

PyV middle T-antigen, which has no equivalent in SV40, has a predicted molecular weight of 49kD, but is observed as a 56/58kD doublet. The protein is associated with most cytoplasmic membranes, but is found mainly in the endoplasmic reticulum, with a small proportion in the plasma membrane (Dilworth *et al.*, 1986).

i: Interaction With $pp60^{c\text{-}src}$

PyV MT associates with the $pp60^{c\text{-}src}$ protein in vivo and in vitro (Courtneidge and Smith, 1983) resulting in a 5-10 fold increase in the total $pp60^{c\text{-}src}$-related tyrosine kinase activity (Bolen *et al.*, 1984). Only the $pp60^{c\text{-}src}$ molecules bound to MT, representing 5-10% of the detectable $pp60^{c\text{-}src}$ population (Bolen *et al.*, 1987), exhibit enhanced tyrosine kinase activity (Courtneidge, 1985). The tyrosine kinase activity of free, but not MT-associated, $pp60^{c\text{-}src}$ was found to be reduced in the presence of phosphatase inhibitors, implying that MT might act by disrupting the regulatory phosphorylation of $pp60^{c\text{-}src}$ activity (Courtneidge, 1985). In support of this hypothesis, $pp60^{c\text{-}src}$ molecules from non-transformed cells, or free molecules from PyV-transformed cells, were found to be phosphorylated on $tyrosine^{527}$. In contrast, MT-associated $pp60^{c\text{-}src}$ was phosphorylated on tyr^{416} but not tyr^{527} (Cartwright *et al.*, 1986). The contribution of the two phosphorylation sites to the regulation of $pp60^{c\text{-}src}$ activity was analysed by replacing tyr^{416} and/or tyr^{527} with other, non-phosphorylatable, residues (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Cartwright *et al.*, 1987). Mutation of tyr^{527} increased the transforming and tyrosine kinase activities of $pp60^{c\text{-}src}$, which was still found to be phosphorylated on tyr^{416}. Replacement of tyr^{416} alone had no obvious effect, but had a marked inhibitory effect on the tyrosine kinase and transforming activities of the tyr^{527} mutant.

These data suggest that phosphorylation of $pp60^{c\text{-}src}$ at tyr^{527} has a down-regulatory effect on the tyrosine kinase and transforming activities of the protein, and that binding of MT could activate $pp60^{c\text{-}src}$ by masking this phosphorylation site. A kinase-defective $pp60^{c\text{-}src}$ molecule is still phosphorylated on tyr^{527} when overexpressed in chicken cells (Jove *et al.*, 1987; Schuh and Brugge, 1988), although this may represent phosphorylation by endogenous $pp60^{c\text{-}src}$ (Cooper and MacAuley, 1988).

Only 10-15% of detectable MT molecules are stably complexed with $pp60^{c\text{-}src}$ (Dilworth, 1982; Bolen *et al.*, 1987), but it is the amount of MT involved in

complex formation, rather than the total concentration of MT, which correlates with degree of transformation (Raptis *et al.*, 1985). TPA, an activator of the serine kinase, protein kinase C (PKC), stimulates the formation and tyrosine kinase activity of MT/pp60$^{c\text{-}src}$ complexes (Matthews and Benjamin, 1986; Raptis *et al.*, 1986), and also potentiates the transforming activity of MT (Raptis and Whitfield, 1986). The addition of TPA leads to the phosphorylation (on serine residues) of pp60$^{c\text{-}src}$ (Gould *et al.*, 1985) and the 56kD form of MT, converting it to a 58kD species, which is preferentially incorporated into complexes with pp60$^{c\text{-}src}$ (Matthews and Benjamin, 1986). MT does in fact act as a substrate for purified PKC, *in vitro* (Hirata *et al.*, 1984).

Thus, PKC may promote the formation of transformation and kinase-active complexes by phosphorylating MT and/or pp60$^{c\text{-}src}$. A strong correlation between pp60$^{c\text{-}src}$-binding and activation and cellular transformation has been found for mutants of MT (reviewed by Markland and Smith, 1987). However, transformation-defective MT mutants capable of binding to, and activating, pp60$^{c\text{-}src}$ do exist, implying that this event may be necessary, but not sufficient, for transformation. Indeed, the tyr^{527} mutants of pp60$^{c\text{-}src}$ are less potent transforming agents than MT itself (Cartwright *et al.*, 1987), and it is estimated that 25-50% of MT-associated tyrosine kinase activity is provided by kinases other than pp60$^{c\text{-}src}$ (Bolen *et al.*, 1987).

ii: Interaction With PI Kinase

Cells infected with transformation-competent PyV virus have increased levels of phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-1,4-bisphosphate (PIP_2), and phosphatidylinositol-1,4,5-trisphosphate (IP_3) (Kaplan *et al.*, 1986). These three compounds are components of an intracellular signalling pathway (see Figure I.6) mediating the response to several growth factors, including PDGF (reviewed by Berridge, 1987). Binding of PDGF to its cell-surface receptor stimulates the phospholipase C-catalysed cleavage of PIP_2, generating the two secondary messengers diacylglycerol (DAG) and IP_3. DAG binds to, and activates, protein kinase C, a process mimicked by phorbol esters such as TPA, while IP_3 acts to release Ca^{2+} ions from intracellular stores. PIP_2 is generated from phosphatidylinositol (PI) *via* PIP, by the phosphorylating action of phosphatidylinositol kinases (PI kinases).

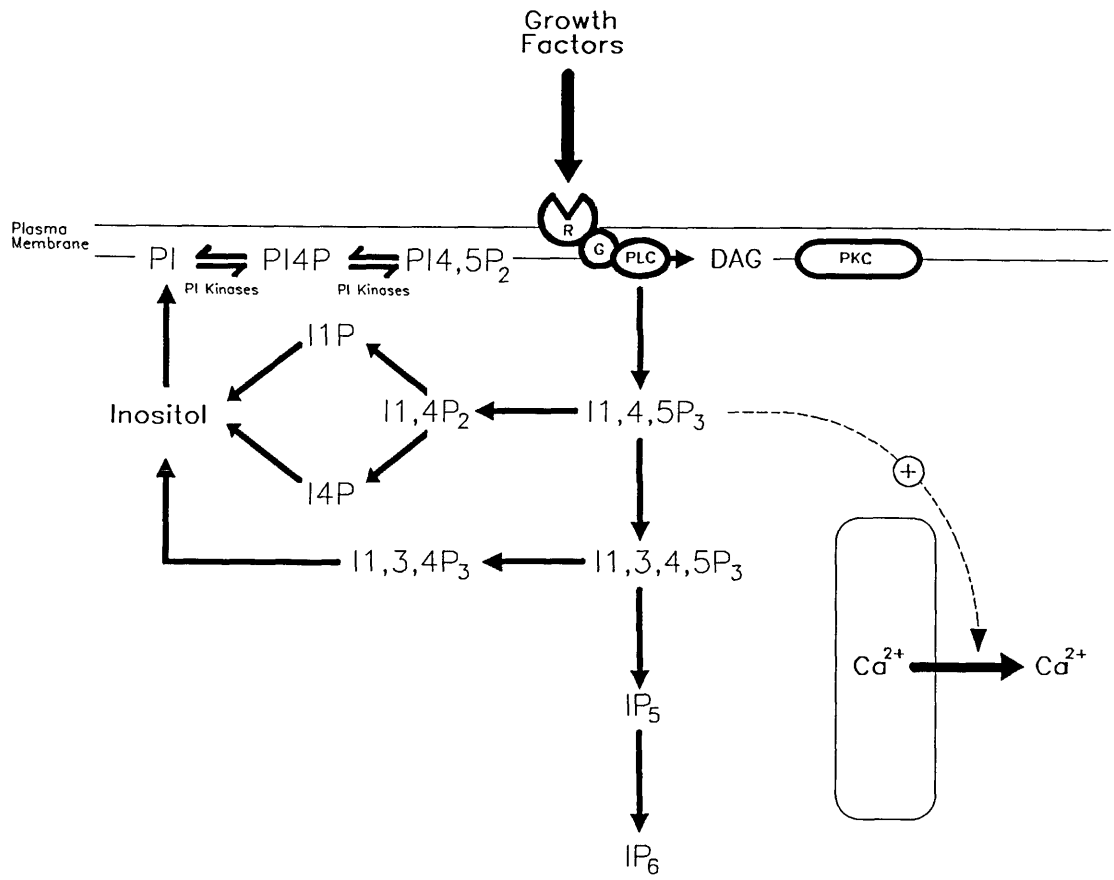
MT/pp60$^{c\text{-}src}$ complexes isolated from PyV-infected, or -transformed, cells have an associated PI kinase activity (Whitman *et al.*, 1985; Kaplan *et al.*, 1986) contributed by a third component, of molecular weight 80-85kD (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987). This PI kinase does not complex with pp60$^{c\text{-}src}$

Figure I.6: Schematic Representation of the Phosphatidylinositol Signalling Pathway.

The metabolism of phosphatidylinositol and its derivatives is shown. Following the binding of growth factors to cell surface receptors, phospholipase C (coupled to the receptor *via* a G protein) is stimulated to hydrolyse PIP_2, generating the secondary messengers DAG and $I1,4,5P_3$. DAG stimulates protein kinase C, while $I1,4,5P_3$ induces the release of Ca^{2+} from intracellular stores. Adapted from Berridge (1987).

Abbreviations

R : Receptor
PLC : Phospholipase C
G : G protein
PKC : Protein Kinase C
PI : Phosphatidylinositol
PI4P : Phosphatidylinositol-4-phosphate
$PI4,5P_2$: Phosphatidylinositol-4,5-bisphosphate
$I1,3,4P_3$: Inositol-1,3,4-trisphosphate
$I1,4,5P_3$: Inositol-1,4,5-trisphosphate
I1,3,4,5P : Inositol-1,3,4,5-tetrakisphosphate
I1P : Inositol-1-phosphate
I4P : Inositol-4-phosphate
IP_5 : Inositol pentaphosphate
IP_6 : Inositol hexaphosphate
I : Inositol
DAG : Diacylglycerol


Growth
Factors
R
G
PLC
DAG
PKC
Plasma
Membrane
PI
PI4P
PI4,5P2
PI Kinases
PI Kinases
I1P
Inositol
I1,4P2
I1,4,5P3
I4P
I1,3,4P3
I1,3,4,5P3
IP5
IP6
Ca2+
Ca2+

in the absence of MT, but associates instead with the PDGF receptor, which phosphorylates PI kinase in response to PDGF stimulation (Kaplan *et al.*, 1987).

The predicted molecular weight of a MT/pp60$^{c\text{-}src}$/PI kinase complex is approximately 200kD, in close agreement with the 220kD size observed by Courtneidge and Smith (1983). These three proteins may therefore represent the sole components of the complex.

iii: Interaction With p62$^{c\text{-}yes}$ and Other Cellular Proteins

Chick cells transformed by the MT gene, expressed in a retroviral vector, are found to contain complexes between MT and p62$^{c\text{-}yes}$, a pp60$^{c\text{-}src}$-related tyrosine kinase (Kornbluth *et al.*, 1987). These complexes are distinct from the pp60$^{c\text{-}src}$/MT association described above, and there is no evidence for the existence of ternary complexes containing all three proteins. No significant increase in the tyrosine kinase activity of p62$^{c\text{-}yes}$ was observed however, and the existence of a similar complex in mammalian cells has yet to be demonstrated.
PyV MT also associates with a number of other cellular proteins (Grussenmeyer *et al.*, 1985; 1987). MT is found with proteins of molecular weight 37kD and 61kD in a complex distinct from that formed by MT and pp60$^{c\text{-}src}$. The 37kD and 61kD proteins are complexed in uninfected cells, and also associate with SV40 ST. However, MT from the *hrt* mutant NG59 is instead found to be associated with a 73kD heat shock protein (Grussenmeyer *et al.*, 1985; Walter *et al.*, 1987), which is found, to a lesser extent, in all MT preparations. A MT-associated protein of 60kD was observed earlier by Dilworth and Griffin (1983). Finally, an 88kD protein has also been found to bind to MT, although this may correspond to the PI kinase described above.

vi: Cell-to-cell Communication

Intercellular communication, as measured by the migration of a fluorescent dye, is reduced in cells expressing MT (Azarnia and Loewenstein, 1987). This downregulation is probably mediated through pp60$^{c\text{-}src}$, as overexpression of that protein in NIH 3T3 cells reduces intercellular communication (Azarnia *et al.*, 1988). The effect is enhanced by mutating the tyr^{527} residue of pp60$^{c\text{-}src}$, but mutation of tyr^{416} restores junctional permeability to normal levels, even in combination with the tyr^{527} mutation.

The importance of intercellular junctional communication is demonstrated by the growth-inhibitory effects of normal cells on their transformed counterparts when cell-to-cell channels are induced by cAMP (Mehta *et al.*, 1986). This may

result from the transmission of growth-controlling molecules between the two cell types.

SV40 is also found to down-regulate intercellular communication, by a mechanism requiring both T-antigens (Azarnia and Loewenstein, 1984).

vii: Other Effects of MT

Cells transformed by polyoma virus have an altered microfilament staining pattern, with little bundling observed. This effect can be induced by transformation with a MT-only plasmid, but LT or ST have little or no effect on the formation of actin cables (Liang *et al.*, 1984; Ito *et al.*, 1986).

Cells transformed by PyV secrete transforming growth factors (TGFs) which induce indicator cell lines to grow in soft agar (Kaplan and Ozanne, 1982). The production of TGFs, which allow PyV-transformed cells to grow in the absence of all serum growth factors apart from insulin, correlates with the presence of MT. However, the cellular response to TGFs may be modulated by LT and/or ST, as cells expressing these T-antigens display an enhanced sensitivity to the effects of the factors.

SV40-transformed cells also express TGFs, under the control of the LT gene, although ST may modulate the cellular response to the factors (Kaplan *et al.*, 1981).

4: Small T-Antigen

The small T-Antigen of PyV shares a 30% amino acid sequence homology with its SV40 equivalent (Soeda *et al.*, 1980b), and is located mainly in the nucleus, but is excluded from the nucleolus (Noda *et al.*, 1986). All but the four C-terminal residues of ST, which are apparently translated from a different reading frame following the splice, are present in MT.

i: Contribution to the hrt Phenotype

A mutant virus expressing LT and ST, but not MT, resembles the wild-type virus in its ability to grow on 3T3 cells and to modify VP1 post-translationally. Similar results are obtained when an *hrt* mutant and a virus expressing ST as the only early antigen are coinfected (Benjamin, 1984). A defective virus expressing LT as the only early antigen, constructed by Templeton *et al.* (1986), behaves like an *hrt* mutant, failing to replicate in mouse 3T6 cells. However,

cotransfection of the LT-only viral genome with a ST gene restores the ability to form plaques on normal 3T6 cells. A truncated MT antigen was found to substitute for ST in this assay. Similar results were obtained by Berger and Wintersberger (1986), who found that the autonomous replication of a LT-encoding plasmid was enhanced by cotransfection with a ST gene. Therefore it appears that ST is capable of correcting, at least partially, the defects in viral DNA accumulation and encapsidation displayed by *hrt* mutants.

ii: Effects on Saturation Density and Growth in Soft Agar

Cell lines expressing PyV ST have proved difficult to isolate, possibly because high levels of the protein appear to induce the detachment of cells from the surface of the tissue culture dish (Lemieux, 1986). However, this problem has been circumvented by the construction of recombinant retroviral vectors expressing ST (Cherington *et al.*, 1986; Noda *et al.*, 1986). Cell lines resulting from retroviral infection grow to higher saturation densities than their parent cells, continuing to divide after reaching confluence to produce flat, dense foci. A small proportion of cells expressing ST form colonies in soft agar, and the presence of the protein enhances the anchorage-independent growth of cells transformed by the MT gene (Ito *et al.*, 1986; Noda *et al.*, 1987).

iii: Other Properties of ST

Cells expressing ST exhibit an enhanced lectin agglutinability, reflecting cell-surface changes which appear to be an important factor in the altered growth properties of transformed cells (Liang *et al.*, 1984, Cherington *et al.*, 1986). PyV ST associates with cellular proteins of molecular weight 32kD and 56kD (Rundell *et al.*, 1981; Ito *et al.*, 1986), but their relationship to the similar-sized proteins associated with PyV MT and SV40 ST has yet to be established.

The effects of SV40 ST on transformation and induction of cellular DNA synthesis have been described in an earlier section. The protein has also been reported to promote the dissolution of actin cables in rat cells (Bikel *et al.*, 1986), a property residing in PyV MT rather than ST. However, Phillips and Rundell (1988) have found that C310T1/2 mouse cells expressing ST retain a normal cytoskeletal organisation, suggesting that the protein does not have a direct effect on the formation of microfilament bundles. These cells exhibit a slightly increased saturation density (in low serum), unlike Balb c/3T3 cells expressing ST (Bikel *et al.*, 1987).

D: OBJECTIVES OF THE THESIS

Polyoma virus large T-antigen, acting alone, or in combination with the other PyV early antigens, has a profound influence on both viral reproduction and host cell metabolism. An understanding of the biochemical mechanisms underlying the biological effects of PyV LT is therefore likely to provide major insights into the mechanisms controlling DNA replication, gene expression, and cell growth. This task is complicated, however, by the multifunctional nature of LT, as described earlier in this chapter. The aim of this thesis is to simplify the problem by mapping individual functions to different domains of the protein. The approach taken is to construct a panel of mutant LT genes, and then assess their ability to promote DNA replication, immortalise primary cells, reduce cellular serum growth factor dependence, and trans-activate the PyV late promoter. The relationships between these four activities can then be assessed.

The results obtained during this study are elaborated in Chapter II, and discussed in detail in Chapter III. Chapter IV consists of the materials and methods used in this study.

II: RESULTS

A: MUTAGENESIS

In recent years, a number of methods for introducing mutations into cloned genes have been developed. One approach, which has become widely used, is in-phase, linker-insertion mutagenesis. Developed by Heffron *et al.* (1978), and refined by Stone *et al.* (1984), this is a semi-random technique, introducing a 6 base pair linker sequence into one of a number of defined sites. The reading frame is maintained, and a mutant protein with a two amino acid insertion is created.

A suitable target for LT mutagenesis was constructed by isolating the minimal sequences required for expression of polyoma virus LT from the plasmid pPyLT1 (Zhu *et al.*, 1984); pPyLT1 contains a complete viral genome, modified by removal of the LT intron sequences, and expresses LT only. The *Bcl*I-*Hinc*II fragment (nucleotides 5021-2962) of pPyLT1, containing the LT coding sequences, the viral enhancer, and the early region promoter and polyadenylation signals, was subcloned into the pEMBL8(-) vector, as shown in Figure II.1. The resulting plasmid, designated pELT, was then subjected to in-phase, linker-insertion mutagenesis.

The linker-insertion strategy employed here (shown in Figure II.1), exploits the ability of ethidium bromide to limit the restriction endonuclease digestion of plasmid DNA (Parker *et al.*, 1977). A restriction enzyme which cuts the gene of interest at frequent, evenly-spaced sites, leaving blunt ends, is selected and assessed for its sensitivity to inhibition by ethidium bromide. *Alu*I, the restriction enzyme employed in this study, was found by titration to produce maximal levels of linear pELT molecules in the presence of 20μg/ml ethidium bromide, and form III molecules were isolated from low melting temperature agarose gels. This mixed population of linearised plasmids, cleaved at one of the possible *Alu*I sites, was ligated to a 150-fold molar excess of a dodecanucleotide linker, comprising a dimer of the *Xho*I recognition site. (A 12-mer is easier to manipulate than a 6-mer). Following extensive digestion with *Xho*I, repurification of linear molecules, recircularisation, and transformation of competent *E. coli* cells, 140 ampicillin-resistant colonies were obtained.

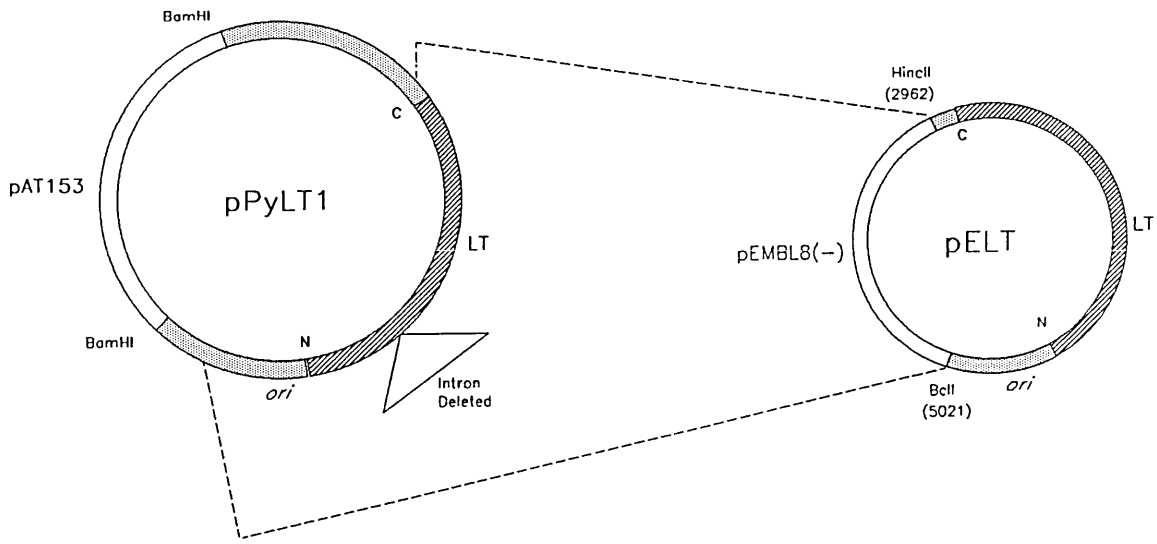
DNA was isolated from the resistant colonies and digested with *Bgl*I and *Xho*I - there are no *Xho*I sites in the native pELT plasmid - to reveal the presence of linker molecules in LT coding sequences (see Figure II.2). Of the 100 clones analysed in this way, 42 were found to have *Xho*I linkers in LT coding

Figure II.1: Cloning and Mutagenesis of pELT.

A: The *Bcl*I-*Hinc*II fragment of pPyLT1 was subcloned into pEMBL8(-), as described in Materials and Methods, to generate pELT. pPyLT1 (Zhu *et al.*, 1984) contains a complete viral genome, modified by removal of the LT intron sequences, and expresses LT as the only PyV early antigen. N and C represent the amino and carboxy termini, respectively, of LT. The functional organisation of the PyV sequences contained in pELT is shown in Figure II.3.

B: Schematic representation of the semi-random linker-insertion strategy (Stone *et al.*, 1984) employed to generate the AX series of mutants. *Alu*I-linearised pELT DNA was ligated to an excess of a 12bp *Xho*I linker. Following digestion with *Xho*I, the plasmid is recircularised by ligation, generating a 6bp in-phase, linker-insertion.

A


BamHI
C
pAT153
pPyLT1
LT
BamHI
N
ori
Intron
Deleted
HincII
(2962)
C
pEMBL8(-)
pELT
LT
N
BclI
(5021)
ori

B

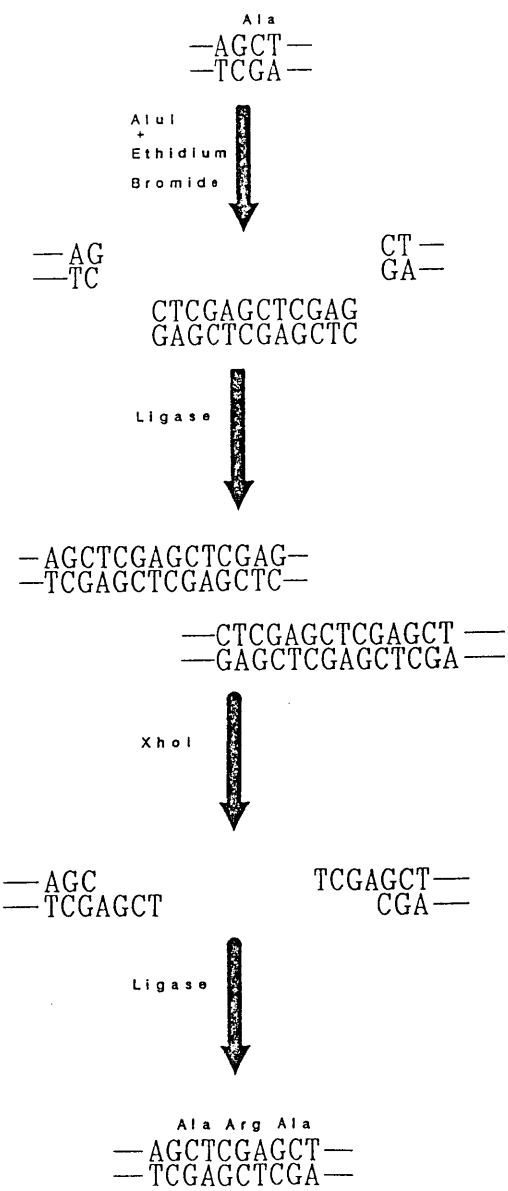
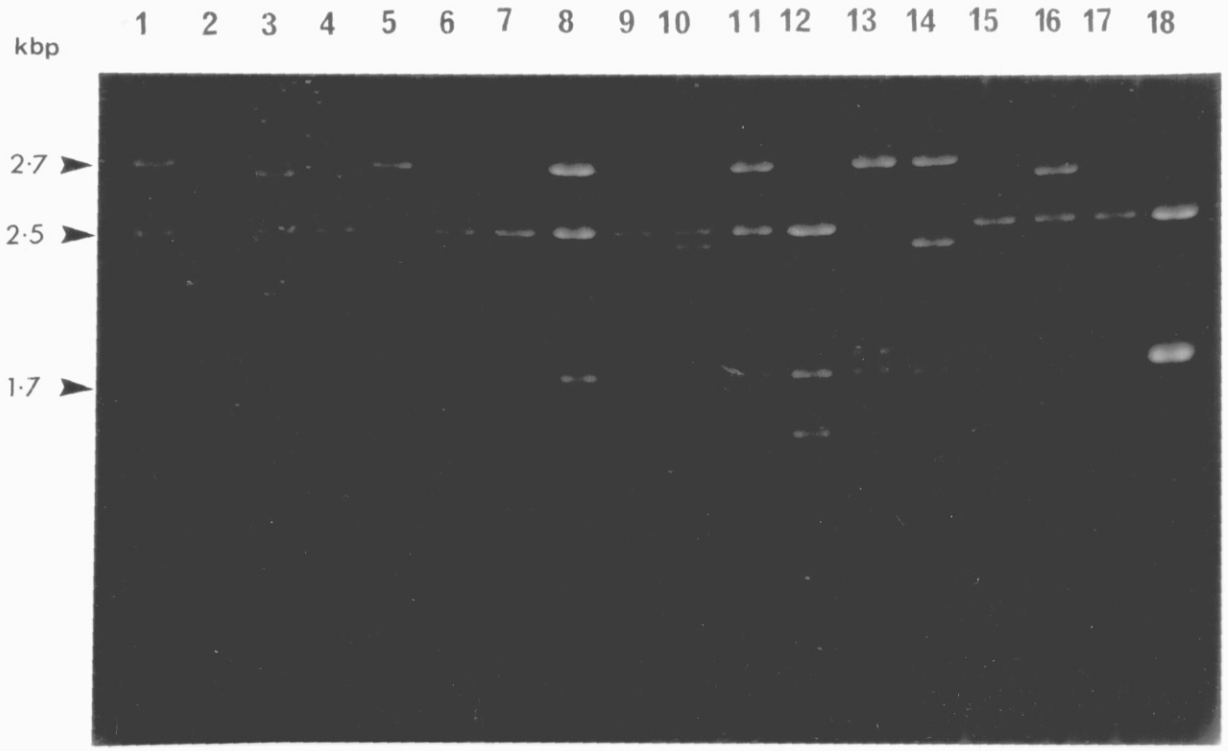

Ala
—AGCT—
—TCGA—
AluI
+
Ethidium
Bromide
—AG
—TC
CT—
GA—
CTCGAGCTCGAG
GAGCTCGAGCTC
Ligase
—AGCTCGAGCTCGAG—
—TCGAGCTCGAGCTC—
—CTCGAGCTCGAGCT —
—GAGCTCGAGCTCGA—
XhoI
—AGC
—TCGAGCT
TCGAGCT—
CGA—
Ligase
Ala Arg Ala
—AGCTCGAGCT—
—TCGAGCTCGA—

Figure II.2: Analysis of the Products of Linker-Insertion Mutagenesis

A: Plasmid DNA was extracted from the ampicillin-resistant colonies derived from the mutagenesis procedure shown in Figure II.1, digested with a combination of *Xho*I and *Bgl*I, and electrophoresed through a 1% agarose gel. A typical ethidium bromide-stained gel is shown. Lane 1 contains *Bgl*I-digested pELT DNA: fragments of 2.7, 2.5, and 1.7 kb (marked by arrows), containing LT coding sequences, vector sequences, and the PyV non-coding region respectively. Lanes 2-18 contain the *Bgl*I/*Xho*I-digested plasmid DNAs. The plasmids in Lanes 3, 4, 6, 7, 9, 10, 12, 15, 16, and 18 have an additional restriction site in the 2.7kb *Bgl*I fragment containing LT-coding sequences. Lanes 5 and 13 contain plasmids with *Xho*I sites in the 2.5kb vector band, Lanes 2 and 17 contain plasmids with deletions in PyV sequences, and Lanes 8, 11, and 14 contain plasmids with no linker insertions.

B: Plasmids containing potential linker insertions in LT-coding sequences (those in Lanes 3, 4, 6, 7, 9, 10, 12, 15, 16, and 18 of the gel shown in panel A) were digested with a combination of *Xho*I and *Hae*III, and end-labelled with α-[^{32}P] dCTP using the Klenow fragment of DNA polymerase I, as described in Chapter IV. Labelled fragments were separated by electrophoresis through an 8% polyacrylamide gel, and visualised by autoradiography, as shown. Radioactively-labelled, *Hae*III-digested pAT153 DNA fragments were used as markers, and the positions and sizes of these marker fragments are displayed on the left. The size of the labelled *Xho*I/*Hae*III fragments derived from the mutant plasmids, in conjunction with the size of the *Bgl*I/*Xho*I fragments, and the known positions of *Alu*I and *Hae*III restriction sites in the viral genome (Ito and Griffin, 1987), allowed the site of linker insertion to be defined. For example, Lane 1 contains labelled *Xho*I/*Hae*III fragments of approximately 340 and 260bp. *Bgl*I/*Xho*I digestion of this plasmid had previously cleaved the 2.7kb *Bgl*I fragment containing LT coding sequences into two fragments of approximately 2.0 and 0.7kb (Lane 6, panel A). These fragment sizes would be generated by digestion of a plasmid with a linker insertion at the *Alu*I site at nucleotide 1232.

A


kbp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
2·7
2·5
1·7

B


bp
1 2 3 4 5 6 7 8 9 10 11 12
587
458
339
267
234
213
192
184
124
104
89
64
57

sequences, 32 had insertions elsewhere, 9 were wild-type, and 17 were found to be deletions or rearrangements.

The 42 LT mutants were then subjected to a secondary screening procedure, to allow a more precise definition of the site of linker integration to be made. The clones were digested with a combination of *Hae*III and *Xho*I and the recessed 3' ends generated by *Xho*I cleavage of integrated linker sequences were repaired using the Klenow fragment of DNA polymerase I in the presence of α-[^{32}P]-dCTP. Resolution of the reaction products on acrylamide gels, followed by autoradiography, revealed a pair of radioactively-labelled bands for each clone, corresponding to the two halves of the pELT *Hae*III fragment interrupted by the *Xho*I linker (see Figure II.2). The length of these fragments, together with the size of the *Bgl*I/*Xho*I fragments obtained earlier, allowed the identification of the exact site of linker insertion.

Table 1 shows the distribution of the sites of insertion to be non-random, presumably reflecting the preference of *Alu*I for cleavage at certain sites, at least in the presence of ethidium bromide. Screening of the remaining 40 colonies, as well as the products of a second mutagenesis procedure, failed to yield molecules with insertions at the *Alu*I sites at nucleotides 933, 1656, or 1657. As mutants mapping to the *Alu*I sites at nucleotides 1350, 1362, and 1374, were considered likely to exhibit similar properties, only a mutant mapping to nucleotide 1374 was employed in this study (mutant AX1374). Mutations in the regions not covered by the *Alu*I series of mutants were created by the introduction of linkers at the *Acc*I, *Ava*I, *Eco*RI, *Hind*III, and *Xba*I restriction sites. All the mutants were then screened by digestion with *Sac*I or *Stu*I as additional sites for these enzymes would be created by multiple linker insertions. Mutants AVX1016, AX2006, AX2377, and AX2522 were found to contain multiple linker insertions, and were converted to a single-linker form by redigestion with *Xho*I followed by recircularisation.

In addition, the existing polyoma mutants *dl*8, *dl*23 (Griffin and Maddock, 1979), *dl*2208 (Nilsson *et al.*, 1983), RX14/*dl*2208, and RX33/*dl*2208 (Richardson *et al.*, 1986) were converted to a form compatible with the linker insertion mutants, as described in Section IV.2. Finally, mutant pELTRI, encoding the N-terminal 40% of LT, was created by deleting all LT-coding sequences downstream of the *Eco*RI site, located at nucleotide 1560 of the PyV genome. The precise locations of the mutations, and their effect on the amino acid sequence of PyV LT, are detailed in Table 2, and shown diagrammatically in Figure II.3.

TABLE 1: DISTRIBUTION OF LINKER-INSERTION SITES

AluI Site (nucleotide)	Number of Mutants
193	4
873	1
933	0
1145	1
1232	3
1350	1
1362	3
1374	2
1657	0
1668	0
2006	1
2033	1
2086	1
2227	2
2377	3

In addition, the sites of linker insertions in some mutants were not mapped precisely, but localised to a subset of possible *Alu*I sites:-

AluI Sites (nucleotides)	Number of Mutants
1145/1232	1
1350/1362/1374	11
2006/2033/2086	5

Table 2: Loci of the Linker Insertion, Deletion, and Deletion/Insertion Mutants.

The loci of the linker insertion, deletion, and insertion/deletion mutants used in this study, are shown, together with their effect on the amino acid sequence of PyV LT.

Mutants RX14 and RX33 were not used individually, but in combination with *dl*2208, as the double mutants RX14/*dl*2208 and RX33/*dl*2208, respectively.

A: LINKER INSERTION MUTANTS

MUTANT	LINKER INSERTED (base pairs)	LOCUS Modified Site	Nucleotide	Amino Acid	RESIDUE CHANGE From	To
AX193	*Xho*I (6)	*Alu*I	193	8	Ala	Ala Arg Ala
ACB368	*Bam*HI (10)	*Acc*I	368	66	Val	Val Gly Ser Gly
AX873	*Xho*I (6)	*Alu*I	873	106	Ser	Ser Ser Ser
AVX1016	*Xho*I (8)	*Ava*I	1016	154	Pro	Pro Thr Ser Arg Pro
AX1145	*Xho*I (6)	*Alu*I	1145	197	Ala	Ala Arg Ala
AX1232	*Xho*I (6)	*Alu*I	1232	226	Ala	Ala Arg Ala
AX1374	*Xho*I (6)	*Alu*I	1374	273	Ser	Ser Ser Ser
EX1560	*Xho*I (8)	*Eco*RI	1560	335	Glu	Glu Phe Leu Glu Glu
HX1656	*Xho*I (8)	*Hind*III	1656	367	Lys	Lys Leu Leu Glu Glu
ACB1809	*Bam*HI (10)	*Acc*I	1809	418	Val	Val Ser Gly Ser Gly
AX2006	*Xho*I (6)	*Alu*I	2006	484	Ala	Ala Arg Ala
AX2033	*Xho*I (6)	*Alu*I	2033	493	Ala	Ala Arg Ala
AX2086	*Xho*I (6)	*Alu*I	2086	510	Gln	Gln Leu Glu
AX2227	*Xho*I (6)	*Alu*I	2227	557	Lys	Lys Leu Gly
AX2377	*Xho*I (6)	*Alu*I	2377	607	Glu	Glu Leu Glu
XX2477	*Xho*I (8)	*Xba*I	2477	641	Leu	Leu Ala Ser Arg Leu
XX2522	*Xho*I (8)	*Xba*I	2522	656	Leu	Leu Ala Ser Arg Leu

B: DELETION AND INSERTION/DELETION MUTANTS

MUTANT	NUCLEOTIDES Deleted	AMINO ACIDS Deleted	Inserted	REFERENCE
dl8	990-1079	145-174		Smolar and Griffin, 1981.
dl23	1139-1240	195-228		Smolar and Griffin, 1981.
dl2208	1126-1182	191-209		Nilsson *et al.*, 1983.
RX14	1388-1407	278-284	Leu Glu Gly	Richardson *et al.*, 1986.
RX33	1417-1436	288-293	Ser Arg	Richardson *et al.*, 1986.
pELTRI	1560-2912	335-785		

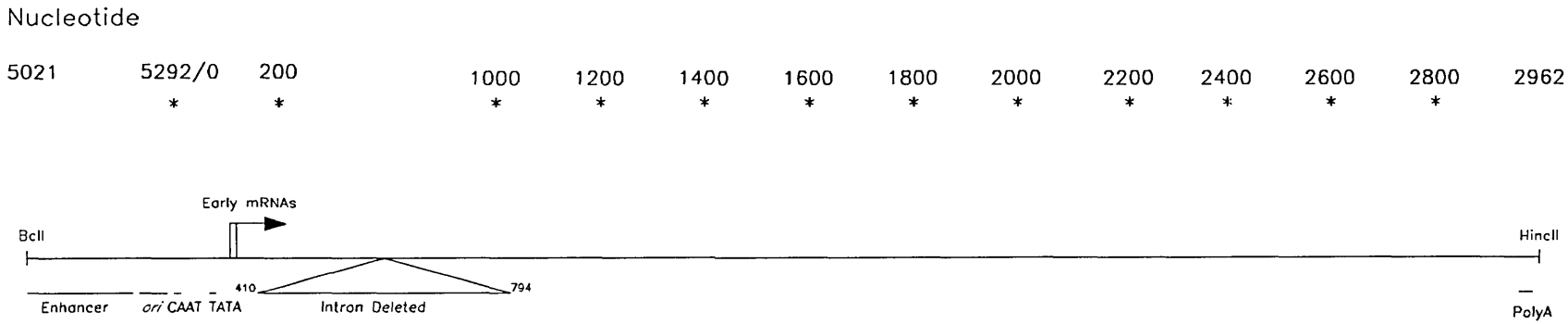
Figure II.3: Map of the Linker Insertion, Deletion, and Deletion/Insertion Mutants.

The functional organisation of the PyV sequence contained in pELT is shown, together with the loci of the mutations with respect to the amino acid sequence of LT and nucleotide sequence of PyV. The diagram shows:-

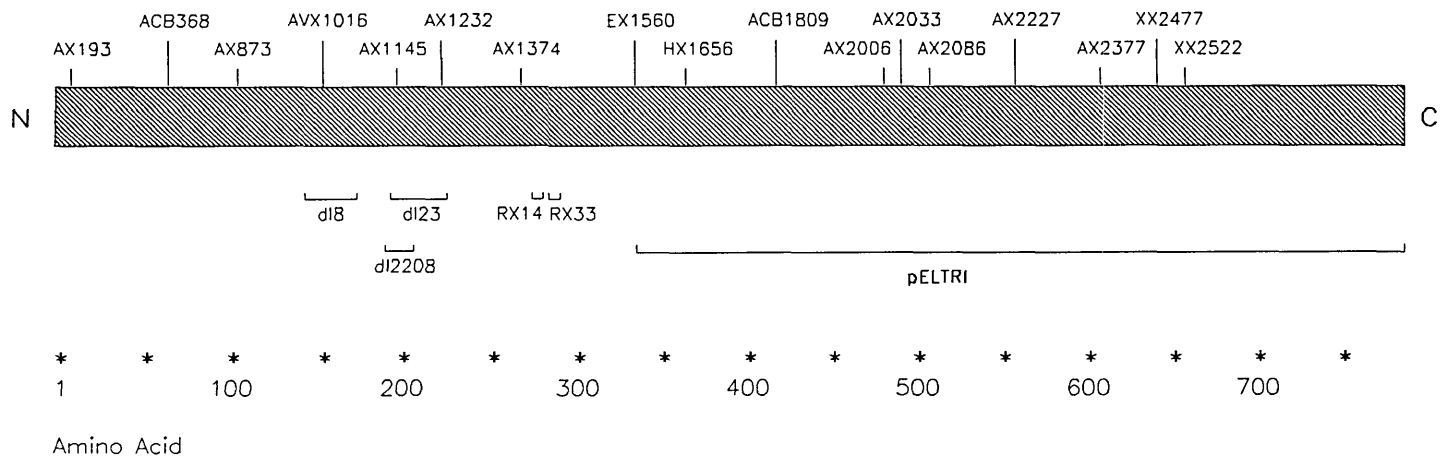
A: The functional organisation of the PyV sequence contained in pELT (see Figure II.1). pELT contains LT coding sequences, the PyV enhancer and origin of replication (*ori*), and the early region promoter and polyadenylation signal. The LT intron sequence (nucleotides 409-795) are deleted (Zhu *et al.*, 1984), ensuring that LT is the only PyV early antigen expressed. Nucleotide numberings are those of Soeda *et al.* (1980a).

B: The location of the linker-insertion mutants and the extent of the *dl*8, *dl*23, *dl*2208, RX14, RX33, and pELTRI deletions are shown with respect to the amino acid sequence of PyV LT. N and C represent the amino and carboxy termini, respectively, of LT.

A


Nucleotide
5021
5292/0
200
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
2962
Early mRNAs
BclI
HincII
410
794
Enhancer
ori
CAAT TATA
Intron Deleted
PolyA

B


ACB368
AVX1016
AX1232
EX1560
ACB1809
AX2033
AX2227
XX2477
AX193
AX873
AX1145
AX1374
HX1656
AX2006
AX2086
AX2377
XX2522
N
C
dl8
dl23
RX14 RX33
dl2208
pELTRI
1
100
200
300
400
500
600
700
Amino Acid

B: EXPRESSION IN BALB C/3T3 CELLS

To confirm the ability of the mutant plasmids to express LT, they were transfected into cells of the Balb c/3T3 mouse fibroblast line, along with the pSV2Neo selectable marker plasmid (Southern and Berg, 1982). Colonies of cells resistant to the cytotoxic effects of Geneticin (G418 sulphate) were isolated, expanded, and screened by immunofluorescence for the expression of LT. The majority of drug-resistant cells were found, on staining with α-LT monoclonal antibodies (Dilworth and Griffin, 1982), to display the nuclear fluorescence with nucleolar exclusion (Figure II.4) characteristic of PyV LT expression (Dilworth *et al.*, 1986). Immunoprecipitation, using the same antibodies, of [^{35}S]-methionine-labelled proteins from a Balb c/3T3 cell line expressing the AX2522 mutant revealed the presence of full-sized LT molecules (Figure II.5). In subsequent experiments, Balb c/3T3 cell lines transfected with pELT or AX1232 were also found to contain LT molecules of the expected size, but at extremely low concentrations, and attempts to identify LT-related proteins in cells expressing mutants AX193, AX873, AVX1016, and AX1145 proved unsuccessful (data not shown). LT could also not be detected, using Western blotting, in five independently-isolated Balb c/3T3 cell lines expressing mutant AX873, as detected by immunofluorescence (data not shown). The intensity of nuclear immunofluorescence displayed by the Balb c/3T3 cell lines was found to decrease with time, resulting in cells with weak, but detectable, LT staining. This suggests that the high levels of LT expression observed shortly after selection may have been produced by plasmids transiently associated with the cell, and that the stable lines which arise following the loss of these molecules exhibit a much lower level of LT expression. The AX2522-expressing cell line containing high levels of LT (Figure II.5) was metabolically labelled shortly after selection, when it would be expected to contain a high copy number of LT genes. In contrast, other cell lines which appear to contain little or no immunoprecipitable LT were labelled at much later times after selection, and may have contained rather lower numbers of LT genes. Indirect immunofluorescence would therefore appear to represent a more sensitive method than immunoprecipitation or Western blotting for the detection of PyV LT molecules in these cell lines. Another indication that the Balb c/3T3 cell lines do indeed express LT is that cells expressing AX193, AX1145, *dl*8 LT, or *dl*23 LT are capable of supporting the replication of a *tsa* mutant polyoma virus at the restrictive temperature (P. Bourgaux, personal communication).

All cell lines expressing mutant LT genes were found to exhibit the normal intracellular distribution of the protein, with the exception of cells expressing the

Figure II.4: Immunofluorescence Analysis of Balb c/3T3 Cell Lines Expressing Mutant PyV LT Genes.

Cells were fixed, permeabilised, and reacted with a combination of the LT-specific monoclonal antibodies α-PyLT-1 and -4 (Dilworth and Griffin, 1982). Photographs were taken using the x25 objective lens of a Zeiss Standard immunofluorescence microscope. The photomicrographs are:-

A: Balb c/3T3 cells infected with the A2 strain of PyV, fixed 24 hours post-infection. At this time point, the majority of cells express LT (Dilworth *et al.*, 1986).

B: A clone derived from a population of Balb c/3T3 cells transfected with a combination of pEMBL8(-) and pSV2Neo. These negative control cells display background immunofluorescence, but no specific nuclear staining.

C: A clone derived from a population of Balb c/3T3 cells transfected with a combination of AX193 and pSV2Neo.

D: A clone derived from a population of Balb c/3T3 cells transfected with a combination of AX873 and pSV2Neo.

E: A clone derived from a population of Balb c/3T3 cells transfected with a combination of AX1016 and pSV2Neo.

F: A clone derived from a population of Balb c/3T3 cells transfected with a combination of AX1145 and pSV2Neo.

The cells in panels A, C, D, E, and F display the nuclear fluorescence with nucleolar sparing characteristic of PyV LT expression (Dilworth *et al.*, 1986). Similar staining patterns were observed for all the other LT mutants, with the exception of RX14/*dl*2208 (see Figure II.6).

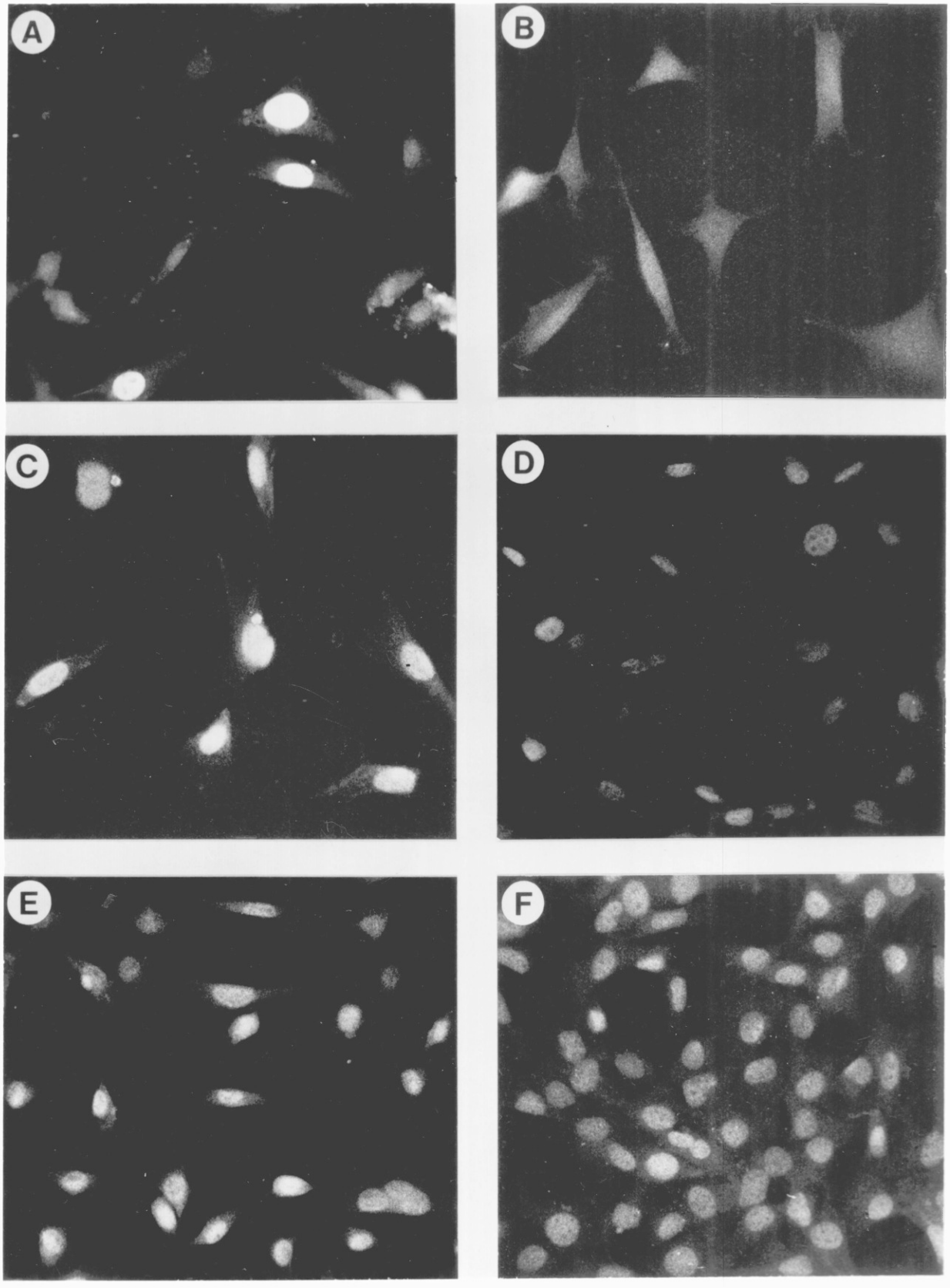
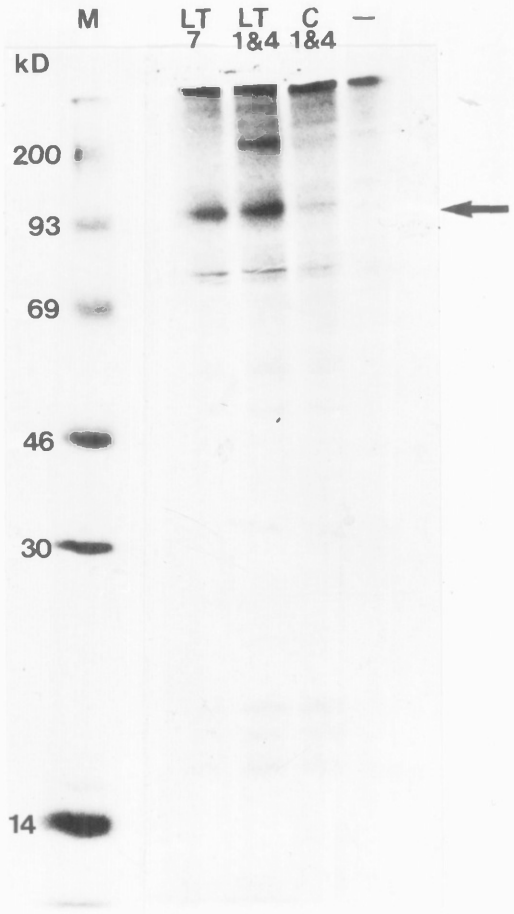

A
B
C
D
E
F

Figure II.5: Analysis of LT-related proteins in a Balb c/3T3 Cell Line Transfected With a Mutant LT Gene.

Cells from a Balb c/3T3-derived cell line transfected with a combination of LT mutant AX2522 and pSV2Neo were metabolically labelled with [^{35}S]-methionine, and PyV-related proteins were immunoprecipitated with monoclonal antibodies (Dilworth and Griffin, 1982) as indicated. Immunoprecipitated proteins were separated by electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, and visualised by autoradiography. The monoclonal antibodies used in each lane were:-

LT7:	α-PyLT-7 (LT-specific)
LT1+4:	A mixture of α-PyLT-1 and α-PyLT-4 (LT-specific)
C1+4:	A mixture of α-PyC-1 and α-PyC-4 (recogises the common region of LT, MT, and ST)
- :	Non-immune serum

The arrow marks the position of the 100kD LT-related protein. The 200kD protein immunoprecipitated by a combination of α-PyLT-1 and α-PyLT-4 is of cellular origin (Dilworth and Griffin, 1982). 100kD LT-related proteins were also identified in Balb c/3T3 cell lines expressing pELT or AX1232 (but not cell lines expressing AX193, AX873, AVX1016, and AX1145), although at much lower concentrations than in the AX2522-expressing cell line shown here.


M
LT 7
LT 1&4
C 1&4
—
kD
200
93
69
46
30
14

RX14/*dl*2208 double mutant. This mutant LT-antigen has deletions removing both of the protein's nuclear localisation signals, and was shown to be non-karyophilic when microinjected into Vero cells (Richardson *et al.*, 1986). However, Balb c/3T3 cell lines expressing RX14/*dl*2208 were found to contain mixtures of nuclear- and cytoplasmic- staining cells, with the proportion of each population varying greatly between independently-isolated clones (Figure II.6). Cell lines displaying almost exclusively nuclear staining were frequently isolated (Figure II.6, panels C and D), and, more rarely, clones with a predominantly cytoplasmic staining pattern were observed (Figure II.6, panel A). A number of cell lines exhibited intermediate distributions, with major populations of nuclear- and cytoplasmic-staining cells (Figure II.6, panel B). When cells from a mixed population, with equal proportions of nuclear and cytoplasmic LT staining members, were seeded at low density, and the resulting colonies recloned, all cells from the eight colonies examined were found to exhibit cytoplasmic fluorescence (Figure II.6, panels E and F).

C: DNA REPLICATION

A plasmid expressing PyV LT, and containing a functional PyV *ori*, is capable of autonomous replication in permissive cells. As pELT is a plasmid of this type, the ability of its mutant derivatives to replicate autonomously can be assessed.

The replication assay employed in this study exploits a difference between the DNA methylation mechanisms of eukaryotic and prokaryotic cells. Many strains of bacteria contain a *dam* methylase which methylates the adenine residue of the sequence GATC, rendering it resistant to cleavage by the *Mbo*I restriction endonuclease. Plasmids grown in dam^+ strains of *E. coli* will be resistant to *Mbo*I digestion, unless they have replicated subsequently in a dam^- environment. As eukaryotic cells do not contain an equivalent of the bacterial *dam* methylase, the acquisition of sensitivity to *Mbo*I digestion by transfected plasmid sequences indicates that DNA replication has occurred.

The mutant plasmids were therefore transfected into permissive mouse 3T6 cells, and low molecular weight DNA was harvested by Hirt extraction (see Chapter IV), 72 hours later. One fifth of the low molecular weight DNA isolated from the transfected cells was digested with *Mbo*I and electrophoresed through a 1% agarose gel before transfer to a nitrocellulose filter by Southern blotting. Following hybridisation of the filter with a nick-translated pELT probe, autoradiography revealed the input, *dam*-methylated plasmid DNA as forms I, II, and III plasmid DNAs, remaining undigested at the top of the gel, with the

Figure II.6: Immunofluorescence Analysis of Balb c/3T3 Cell Lines Expressing the PyV LT Nuclear Localisation Mutant RX14/*dl*2208.

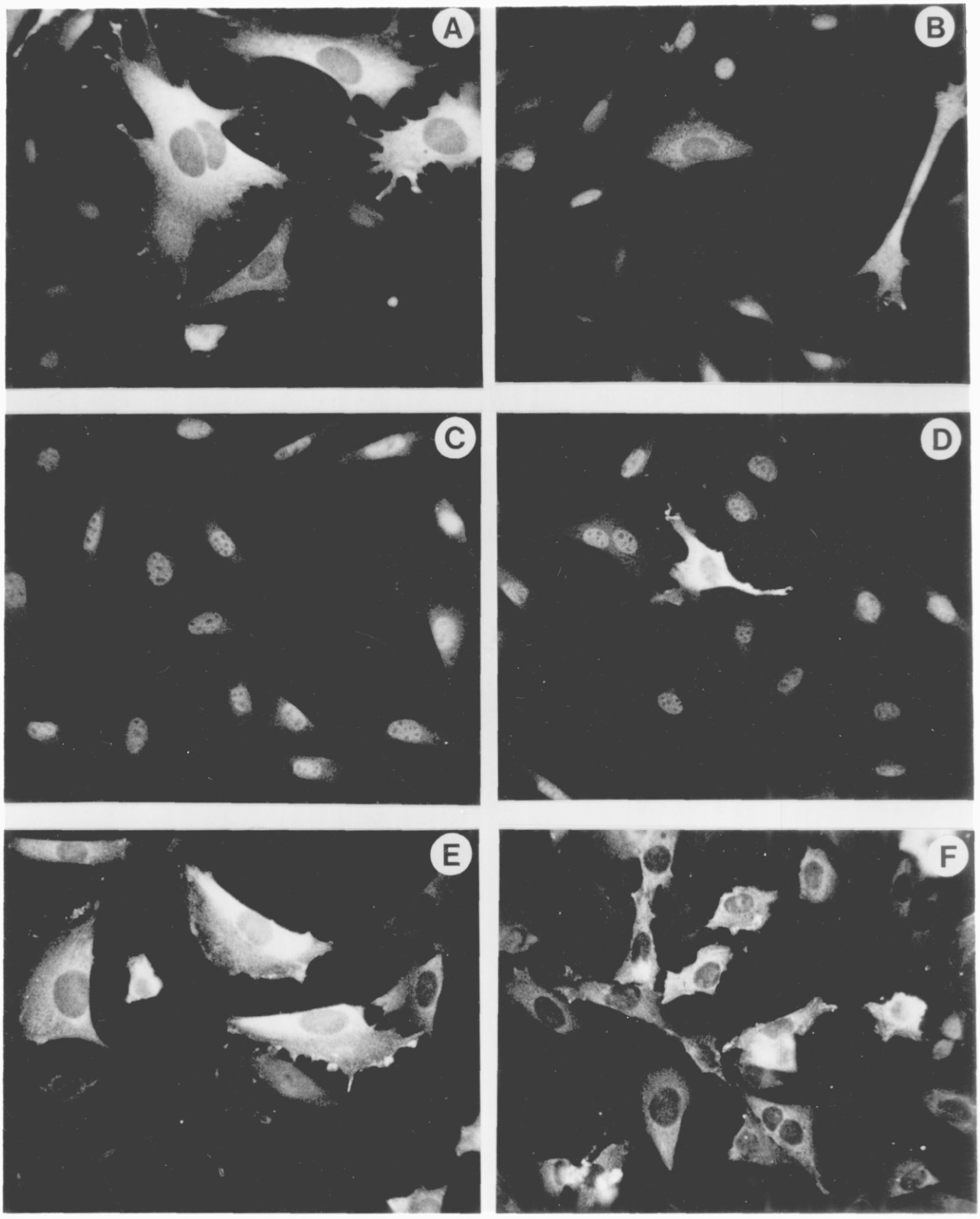
Cells were fixed, permeabilised, and reacted with a combination of the LT-specific monoclonal antibodies α-PyLT-1 and -4 (Dilworth and Griffin, 1982). Photographs were taken using the x25 objective lens of a Zeiss Standard immunofluorescence microscope. The photomicrographs are:-

A-D: Independently-isolated clones of Balb c/3T3 cells expressing the RX14/*dl*2208 LT mutant.

Panels A and B show heterogeneously-staining populations of cells with Panel A exhibiting a predominantly cytoplasmic distribution of LT, and Panel B containing a majority of nuclear-staining cells.

Panels C and D exhibit predominantly nuclear LT staining. The brightly staining cell in the centre of Panel D may represent a dying cell, rather than a genuine cytoplasmic variant, although cells of this type were not seen in populations of cells expressing the other LT mutants.

E-F: Independently-isolated subclones of a RX14/*dl*2208 cell line which exhibited heterogeneous nuclear and cytoplasmic immunofluorescence. The cell populations shown here (and cells from six other independently-isolated subclones) exhibit mainly cytoplasmic-staining, with little or no nuclear LT fluorescence.


A
B
C
D
E
F

*Mbo*I-digested, replicated DNA appearing as low molecular weight fragments (Figure II.7). The results of the replication assay (Table 3) are, however, incomplete, as a progressive decline in the level of pELT replication (the positive control) was observed in consecutive experiments. The use of alternative populations of 3T6 or Balb c/3T3 cells produced a temporary restoration of the replicative capacity of pELT, followed by a rapid decline suggesting that the permissivity of the murine cell lines for PyV DNA replication was lost on passaging *in vitro*. The 3T6 cells were still able to support viral growth, however (data not shown), implying that the defect in the DNA replication assay lay in inadequate transfection efficiencies or perhaps the absence of ST and/or MT. Neither the use of the DEAE-dextran transfection method or cotransfection with a ST-expressing plasmid was able to restore DNA replication of pELT, and several mutants (ACB368, ACB1809, *dl*8, *dl*23, *dl*2208, RX14/*dl*2208, and RX33/*dl*2208) could not tested for their ability to replicate autonomously. However, previous studies have shown that a plasmid expressing *dl*2208 LT, but not MT or ST, is capable of DNA replication (Nilsson and Magnusson, 1984), and Balb c/3T3 cells expressing LT genes from *dl*8 and *dl*23 (described in Section II.3) support the replication of a *tsa* PyV mutant at the non-permissive temperature (P. Bourgaux, personal communication). The use of *tsa* viral mutants to infect LT-expressing Balb c/3T3 cell lines, or transfection of PyV-transformed cell lines, or other cell lines which support the growth of *hrt* viruses (see Section I.C.1.c), might allow the ability of all the LT mutants to promote DNA replication to be assessed.

D: IMMORTALISATION

Rat embryo fibroblasts (REFs) have a limited lifespan *in vitro*, senescing after approximately 20 population doublings. The introduction of a PyV LT gene overcomes senescence, and allows the establishment of rat cell lines with an indefinite proliferative potential (Rassoulzadegan *et al.*, 1983b). Full-length LT molecules are often cytotoxic in the REF-immortalisation assay, however, presumably as a consequence of DNA replication (Land *et al.*, 1983), and a replication-defective clone encoding the N-terminal 40% of PyV LT immortalises efficiently (Rassoulzadegan *et al.*, 1983b; Land *et al.*, 1983). Based on these observations, only the derivatives of pELT with mutations mapping to this N-terminal region were assayed for their ability to promote establishment of rat cell lines. In addition, plasmids were digested with *Hind*III before transfection, thus

Figure II.7: Analysis of the Ability of the Mutant LT Genes to Promote DNA Replication.

Mouse 3T6 cells were transfected with 1μg pELT (positive control), its mutant derivatives, or pEMBL8(-) vector DNA (negative control). Low-molecular weight DNA was harvested by Hirt extraction 72 hours post-transfection, digested with *Mbo*I and electrophoresed through a 1% agarose gel. The DNA was then transferred to a nitrocellulose filter by Southern blotting and hybridised with a nick-translated pELT probe. The filter was washed and subjected to autoradiography.

The results of a typical DNA replication assay are shown. Arrows mark the positions of undigested input DNA (plasmid DNA forms I, II, and III), and lower molecular weight *Mbo*I-digested replicated DNA. DNA replication is observed for pELT, AX193, AVX1016, and AX1145, but not for pEMBL8(-), or AX1232, and AX1374, even on longer exposures.

Only a small amount of PyV-related low molecular weight DNA was extracted from cell populations transfected with AX873, AX1145, AX1232, and AX1374 in this experiment, although plasmid forms I, II, and III of these four mutants were clearly visible on longer exposures. As the lower molecular weight DNA fragments characteristic of DNA replication were readily detectable in the AX1145 Lane, the absence of similar fragments in Lanes AX873, AX1232, and AX1374 is likely to reflect the inability of these plasmids to replicate autonomously, rather than a failure to isolate adequate quantities of plasmid DNA. These observations were confirmed in subsequent experiments in which greater amounts of low molecular weight DNA were isolated.

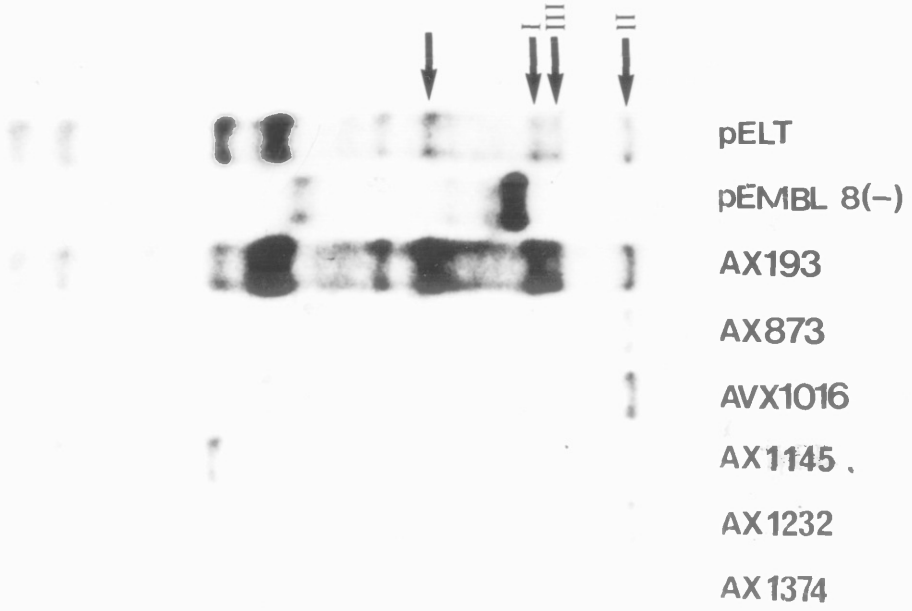

pELT
pEMBL 8(−)
AX193
AX873
AVX1016
AX1145
AX1232
AX1374

Table 3: Cellular Location and DNA Replication.

The intracellular locations of the mutant LT proteins, determined by immunofluorescence analysis of Balb c/3T3 cells transfected with the LT plasmids (see Figures II.4 and II.6), is shown.

The abilities of the mutant plasmids to replicate autonomously (see Figure II.7) are also shown.

MUTANT	LOCATION	REPLICATION
pELT	N	+
AX193	N	+
ACB368	N	#
AX873	N	-
AVX1016	N	+
AX1145	N	+
AX1232	N	-
AX1374	N	-
EX1560	N	-
HX1656	N	-
ACB1809	N	#
AX2006	N	-
AX2033	N	-
AX2086	N	-
AX2227	N	-
AX2377	N	-
XX2477	N	-
XX2522	N	-
*dl*8	N	(+)
*dl*23	N	(+)
*dl*2208	N	(+)
RX14/*dl*2208	N+C	#
RX33/*dl*2208	N	#
pELTRI	N	-

N= Nucleus
N+C= Nucleus and Cytoplasm
#= Ability to replicate could not assessed as positive control also failed to replicate (see text).
( )= Data from other laboratories

removing the coding sequences downstream of nucleotide 1656 (containing ATPase, and DNA helicase domains) and preventing DNA replication.

Immortalisation of secondary or tertiary REFs was performed essentially as described by Asselin and Bastin (1985). Cells were transfected with a combination of pSV2Neo and LT or vector DNA, and selected for resistance to the cytocidal effects of Geneticin. Very considerable cell death was observed after two weeks, but drug-resistant colonies became visible after approximately three weeks, and were isolated some 3-4 weeks post-transfection. Clones were then expanded and passaged at a 1:5 dilution on reaching confluence.

Cell populations cotransfected with pSV2Neo and the pEMBL8(-) vector (the negative control in these experiments) formed a limited number of drug-resistant colonies which frequently failed to survive transfer to 24-well dishes, and senesced shortly afterwards. None of these colonies could be expanded on to 5 cm dishes. In contrast, REFs transfected with a combination of pSV2Neo and pELTRI, which encodes the N-terminal 40% of LT (representing the positive control), produced a number of resistant colonies, many of which exhibited a distinctive cellular morphology not observed in the negative controls (see Figure II.8). These colonies were readily expanded into flat, contact-inhibited cell lines which could be maintained for more than three months in tissue culture. Similar cell lines were established by the majority of the mutant LT-antigens (see Table 4). Any clone which could be expanded on to three 5cm dishes was considered to be immortal, an assumption confirmed by passaging representative cell lines for at least ten subdivisions (1:5 dilution) without any noticeable loss of proliferative capacity.

The presence of LT in the established cell lines was confirmed both by immunofluorescence and by immunoprecipitation of metabolically-labelled cell lysates. The characteristic nuclear staining pattern of PyV LT was observed for all cell lines tested (Figure II.9), with the exception of a line established by the RX14/*dl*2208 nuclear localisation mutant, which exhibited nuclear LT fluorescence at early passages, but predominantly cytoplasmic staining following prolonged culture (Figure II.10). LT-related species of different molecular weights in the range of 35-42kD were found in 5 out of the 6 mutant-derived cell lines tested. Two independently cloned cell lines established by transfection with AX193 contained LT-related proteins of 39-40kD, and another two cell lines established by AX1374 contained LT species of approximately 42kD (Figure II.11). Cell lines established by transfection with AX1145 or RX14/*dl*2208 contained LT-related proteins of 35 and 42kD respectively (data not shown).

Some PyV-transformed rat cell lines containing a truncated early region employ the alternative polyadenylation signal at nucleotide 1476 (Kamen *et al.*,

Figure II.8: Morphology of Rat Fibroblast Cell Lines Established by Mutant LT Genes.

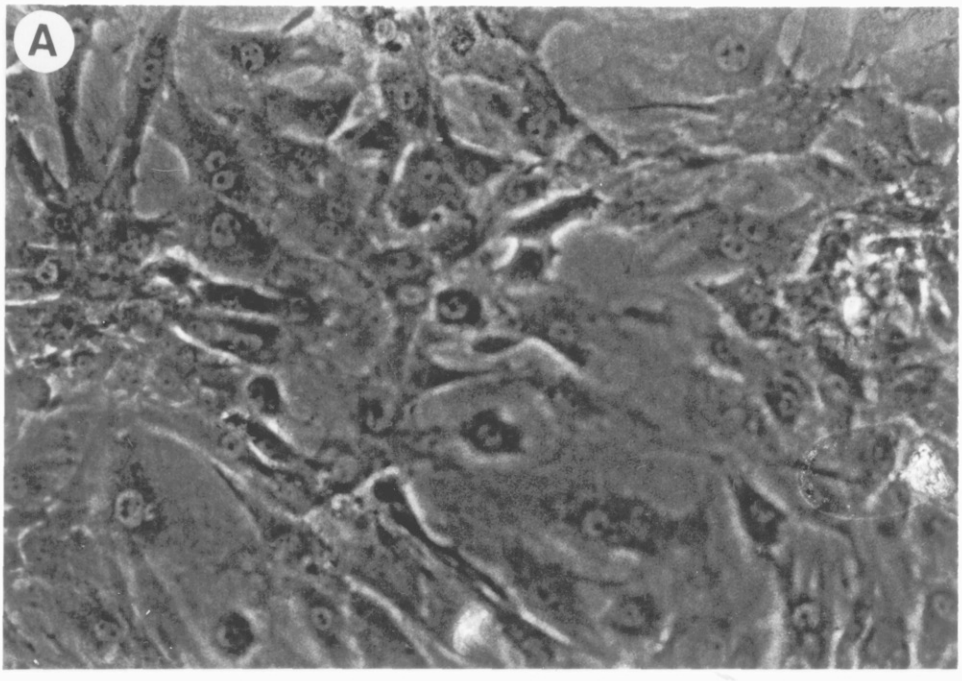
Cells, visualised using phase contrast, were photographed using the x10 objective lens of a Zeiss IM-35 microscope. The photomicrographs show:-

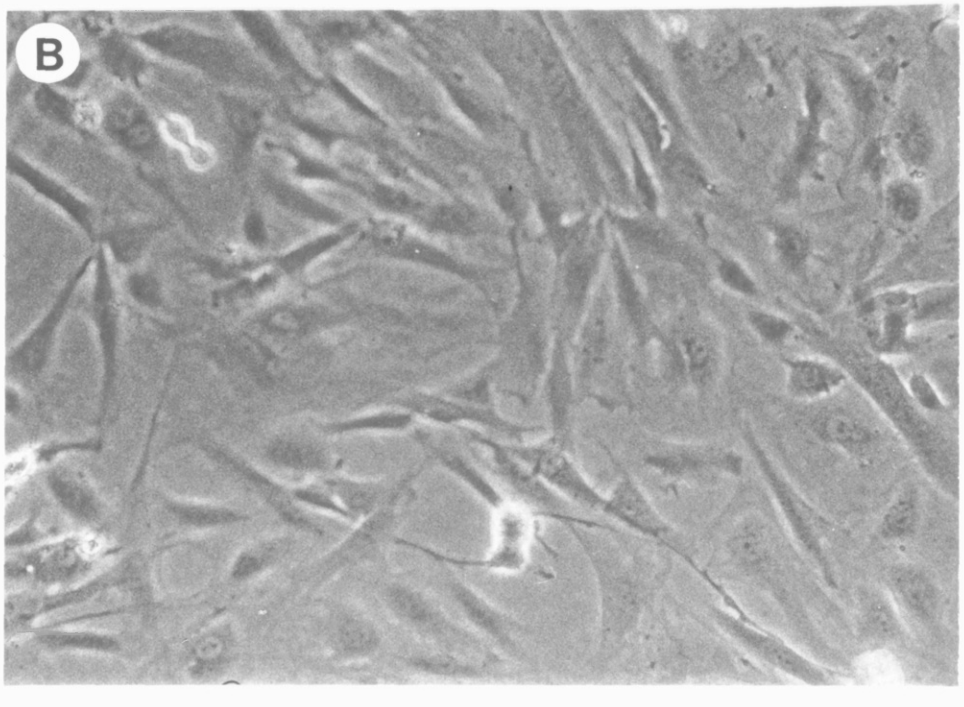
A: Tertiary rat embryo fibroblasts, transfected with salmon sperm DNA.

B: A rat cell line established by transfection with AX193.

C: A rat cell line established by transfection with AX1374.

The established cells in panels B and C exhibit a more elongated morphology than the flat, non-established cells in panel A. The morphology of the AX193 and AX1374-immortalised cell lines was typical of the other LT-established rat fibroblast lines.


A


B

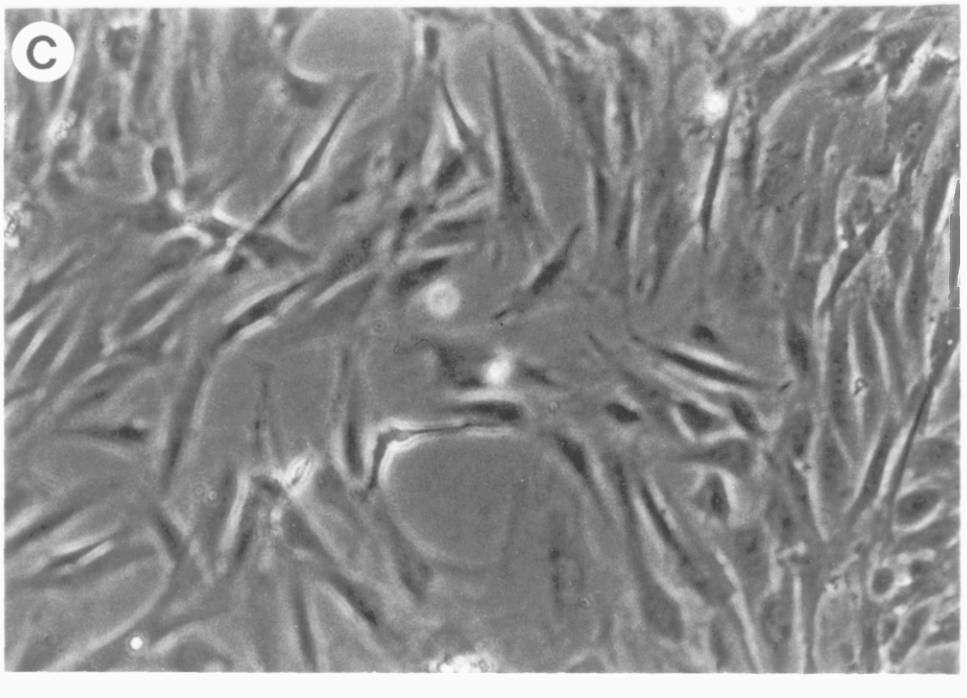

C

Figure II.9: Immunofluorescence Analysis of Rat Fibroblast Cell Lines Established by Mutant LT Genes.

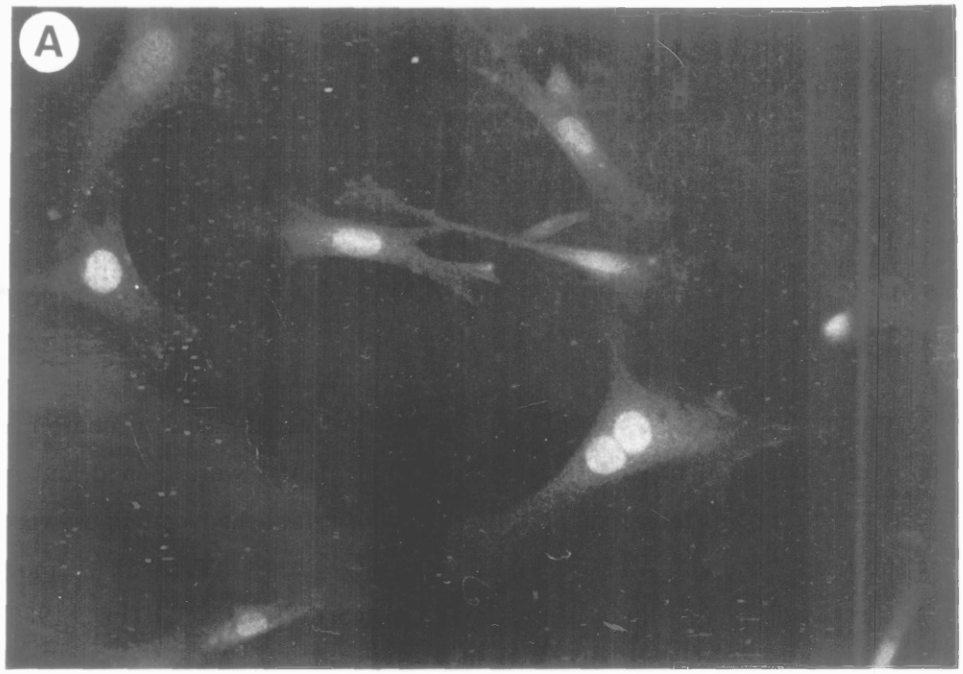
Secondary rat embryo fibroblasts were transfected with a combination of mutant LT genes and pSV2Neo. Drug resistant colonies were cloned and passaged to assess their proliferative capacity. Cells from established cell lines were fixed, permeabilised, stained with a combination of the LT-specific monoclonal antibodies α-PyLT-1 and α-PyLT-4 (Dilworth and Griffin, 1982), and photographed using the x25 objective lens of a Zeiss Standard immunofluorescence microscope. The photomicrographs are:-

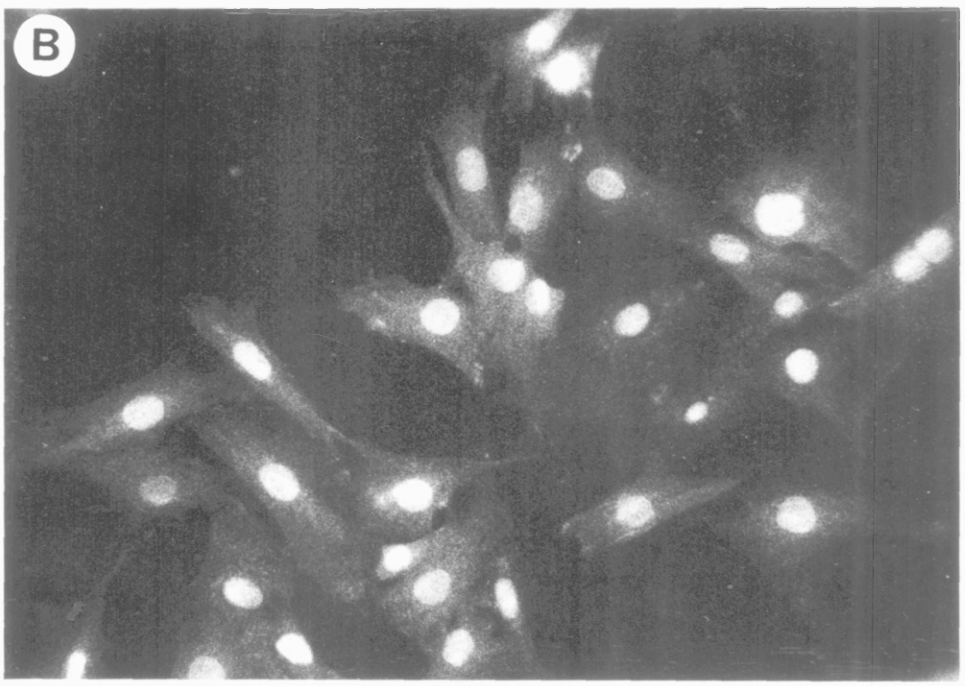
A: A rat fibroblast cell line established by transfection with mutant AX193.

B: A rat fibroblast cell line established by transfection with mutant AX1374.

C: A rat fibroblast cell line established by transfection with mutant RX33/*dl*2208.

The expected nuclear staining was observed, although the nucleolar exclusion characteristic of cells expressing PyV LT (Dilworth *et al.*, 1986) was less marked than in the LT-expressing Balb c/3T3 cell lines (see Figure II.4). This may reflect the smaller size of the truncated LT proteins expressed in the rat cell lines, which may enter the nucleolus more readily than the full-length proteins expressed in the Balb c/3T3 lines. Similar immunofluorescent staining was observed for cells immortalised by pELTRI, AX1145, ACB368, and AVX1016.


A


B

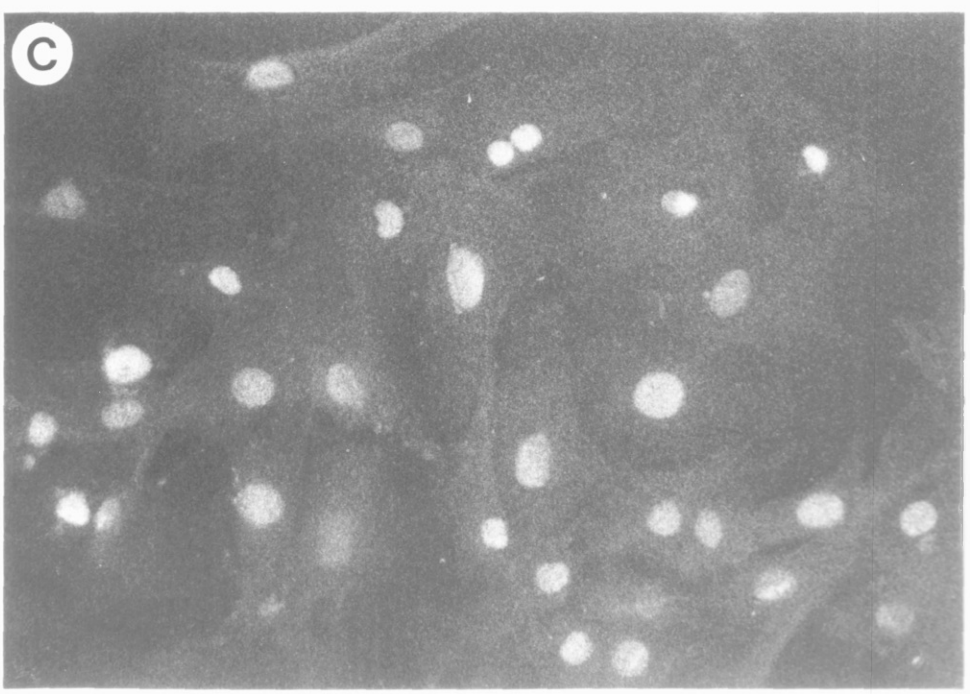

C

Figure II.10: Immunofluorescence Analysis of A Rat Fibroblast Cell Line Established by a Nuclear Transport Mutant of PyV LT.

Secondary rat embryo fibroblasts were transfected with a combination of RX14/*dl*2208 and pSV2Neo. Drug resistant colonies were cloned and an immortal cell line established. Cells from this cell line were fixed, permeabilised, stained with a combination of the LT-specific monoclonal antibodies α-PyLT-1 and α-PyLT-4 (Dilworth and Griffin, 1982), and photographed using the x25 objective lens of a Zeiss Standard immunofluorescence microscope. The photomicrographs are:-

A: An early passage of the RX14/*dl*2208 rat cell line. The cells exhibit the nuclear LT staining, with nucleolar exclusion, characteristic of wild-type PyV LT (Dilworth *et al.*, 1986).

B-D: Later passages (5 weeks later than the cells in panel A) of the RX14/*dl*2208 rat cell line. The cells exhibit predominantly cytoplasmic LT staining (panels B and C), with small patches of nuclear-staining cells (panel D). The staining pattern of these cells is similar to that shown in Figure II.6 for Balb c/3T3 cells expressing RX14/*dl*2208.

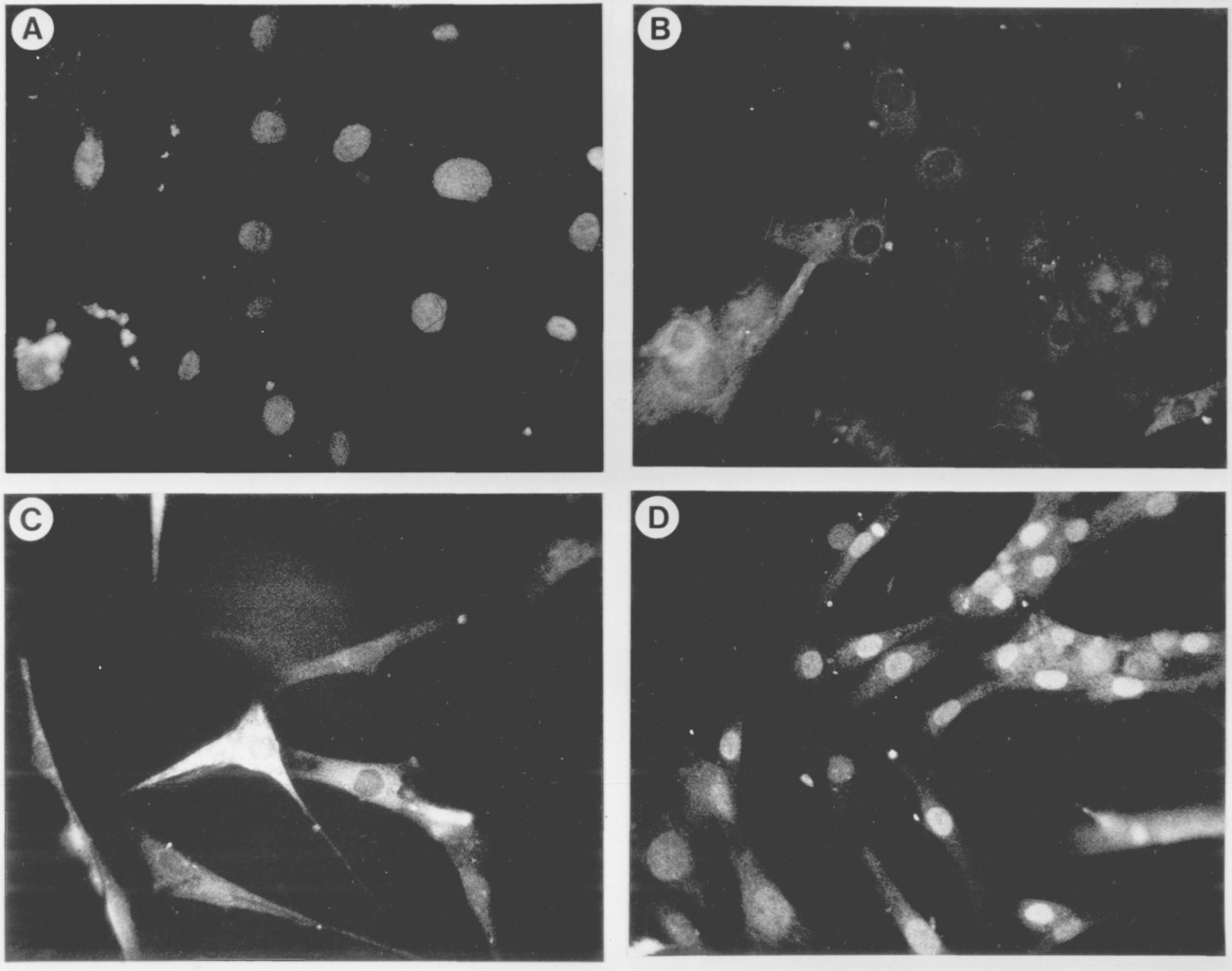

A
B
C
D

Figure II.11: Analysis of LT-Related Proteins in Rat Cell Lines Established by Mutant LT Genes.

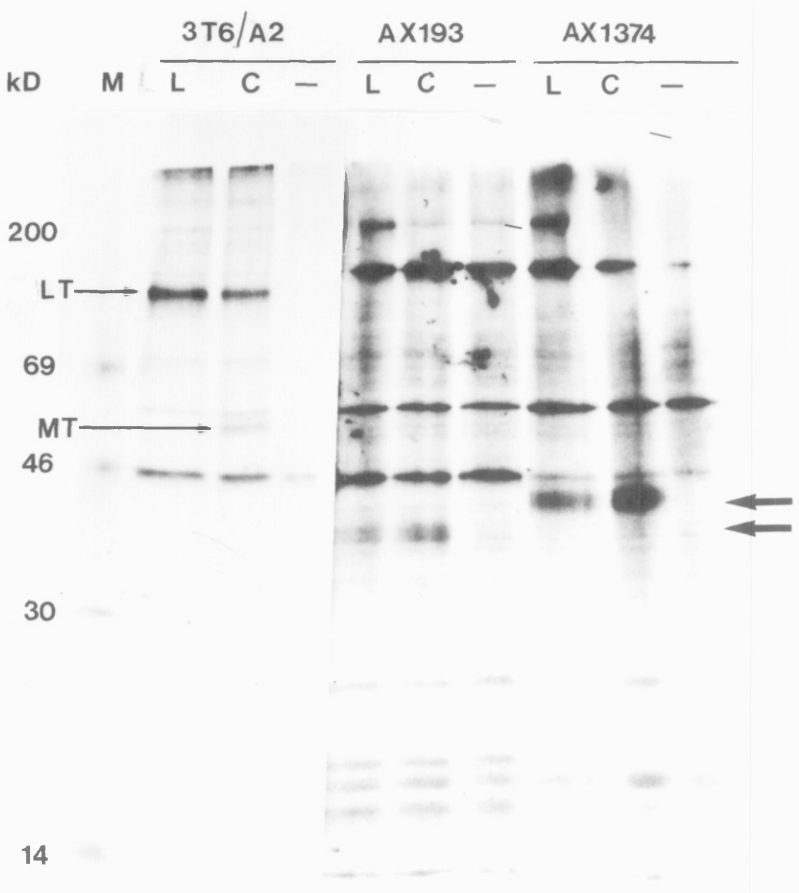
Cells from LT-immortalised cell lines were metabolically labelled with [^{35}S]-methionine, and PyV-related proteins were immunoprecipitated with the LT-specific monoclonal antibodies α-PyLT-1 and α-PyLT-4 (L), and the common region antibodies α-PyC-1 and α-PyC-4 (C) (Dilworth and Griffin, 1982), or non-immune serum (-). Immunoprecipitated proteins were separated by electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, along with [^{14}C]-labelled markers (M), and visualised by autoradiography. The cells used were:-

3T6/A2: Mouse 3T6 cells, infected with the A2 strain of PyV, and lysed 28 hours post-transfection. Arrows mark the positions of large (LT) and middle (MT) T-antigens.

AX193: Rat cells immortalised by transfection with the mutant AX193. LT-related proteins of a similar size (39-40kD) were detected in another, independently-isolated cell line established by transfection with AX193.

AX1374: Rat cells immortalised by transfection with the mutant AX1374. LT-related proteins of a similar size (42kD) were detected in another, independently-isolated cell line established by transfection with AX1374.

Arrows mark the positions of LT-related proteins of different molecular weights in the two rat cell lines. There appear to be a doublet of LT bands, particularly in the AX193-immortalised cell line, and this may represent distinct species of LT distinguished by post-translational modification. Full length wild-type LT can also be resolved into two distinct species by SDS-PAGE (Dilworth and Griffin, 1982).


3T6/A2
AX193
AX1374
kD
M
L
C
—
L
C
—
L
C
—
200
LT
69
MT
46
30
14

1980), generating a family of transcripts cleaved at one of three possible sites between nucleotides 1495 and 1514 (Norbury and Fried, 1987). Cleavage and polyadenylation at nucleotide 1506 generates a mRNA with a termination codon in the LT reading frame, which would encode a truncated LT of predicted molecular weight 37kD. A protein of 35-40kD was observed by Cuzin and coworkers (Rassoulzadegan *et al.*, 1983b; Guizani *et al.*, 1987) in cell lines established by LT genes truncated at the *Eco*RI restriction site at nucleotide 1560. In other cell lines however, a cellular polyadenylation signal is utilised in preference to the minor viral signal, and viral/cellular hybrid mRNAs are produced (Ruley *et al.*, 1988). The LT-related proteins translated from these messages are believed to terminate at the first in-frame termination codon encountered in the cellular sequences, resulting in LT molecules from different cell lines possessing varying numbers of unrelated C-terminal residues, and therefore different molecular weights. The variation in the sizes of the LT-related molecules immunoprecipitated from the immortalised cell lines in this study suggests that at least some of them may be hybrid viral/cellular proteins utilising a cellular termination codon. However, as integration of LT DNA into the host chromatin would presumably occur at random sites, producing hybrid proteins of different sizes, the observation that independently-isolated cell lines established by AX193 or AX1374 contain LT species of seemingly identical molecular weight implies that the same termination codon is utilised. The variation in the molecular weight of LT molecules found in cell lines established by different mutants (see Figure II.11) could reflect alterations in post-translational modification. For example the higher molecular weight species observed in AX1374-derived cell lines could be the result of further phosphorylation at the two additional serine residues introduced by the *Xho*I linker insertion (see Table 2).

The combined results of several immortalisation experiments are summarised in Table 4. Cell lines were established for mutants pELTRI, AX193, ACB368, AX873, AVX1016, AX1145, AX1374, *dl*2208, RX14/*dl*2208, and RX33/*dl*2208, but not for pELT, AX1232, or *dl*23, even though a number of colonies of the typical "immortalised" morphology (see Figure II.8) were obtained, in several experiments. These cells could be readily transferred to 24-well dishes, unlike colonies from negative control transfections, and some were expanded on to 6-well dishes. All these cell populations were ultimately lost through contamination of the tissue culture medium, however, precluding a definitive assessment of their proliferative potential. Nevertheless, in these experiments, cells transfected with pELT, AX1232, *dl*23 plasmids continued to proliferate at a time when colonies derived from negative control transfections had already senesced,

TABLE 4: IMMORTALISATION OF RAT EMBRYO FIBROBLASTS

MUTANT	COLONIES PICKED	CELL LINES ESTABLISHED*	NO. OF EXPERIMENTS
pELT	[+]		4
pEMBL8(-)	24	0	4
AX193	20	15	1
ACB368	15	4	1
AX873	11	2	4
AVX1016	16	3	2
AX1145	4	4	1
AX1232	[+]		4
AX1374	6	3	2
*dl*8	3	0	4
*dl*23	[+]		4
*dl*2208	15	1	3
RX14/*dl*2208	1	12	3
RX33/*dl*2208	13	4	3
pELTRI	8	6	1

* Colonies expanded on to three 5cm dishes were considered to be immortal.

[+] Colonies appeared to be immortal, but were lost due to tissue culture contamination, before they could be expanded on to three 5cm dishes.

suggesting that these LT genes had at least extended the lifespan of the rat cells. Data from M. Strauss (personal communication) show that a *dl*23 LT gene can immortalise human lung fibroblasts. In contrast, few colonies were obtained from cells transfected with *dl*8, and these invariably underwent senescence. In fact, in four successful immortalisation experiments, no drug-resistant colony derived from a population of REFs transfected with a combination of *dl*8 and pSV2Neo could be expanded on to a 6-well dish.

E: TRANSCRIPTIONAL ACTIVATION

The abilities of the LT mutants to trans-activate the PyV late promoter were assessed using two target plasmids, p48.19BECAT and pB27BECAT (Kern *et al.*, 1986), provided by Dr. C. Basilico. The p48.19BECAT plasmid contains the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the PyV late promoter. Processing of the CAT mRNA is directed by the SV40 ST splicing signals and the SV40 early region polyadenylation signal. The p48.19BECAT target plasmid also contains the PyV enhancer, origin of replication, and a truncated PyV early region expressing full-length MT and ST, and an N-terminal LT fragment (Figure II.12). An *Xho*I linker, inserted between viral nucleotides 35 and 37, abolishes the ability of the plasmid to replicate in permissive cells in the presence of PyV LT. The second target plasmid, pB27BECAT, is a derivative of p48.19BECAT generated by deletion of PyV nucleotides 5-242, containing LT binding-sites A, B, C, and 1, and does not bind PyV LT in an *in vitro* DNA-binding assay (Figure II.13).

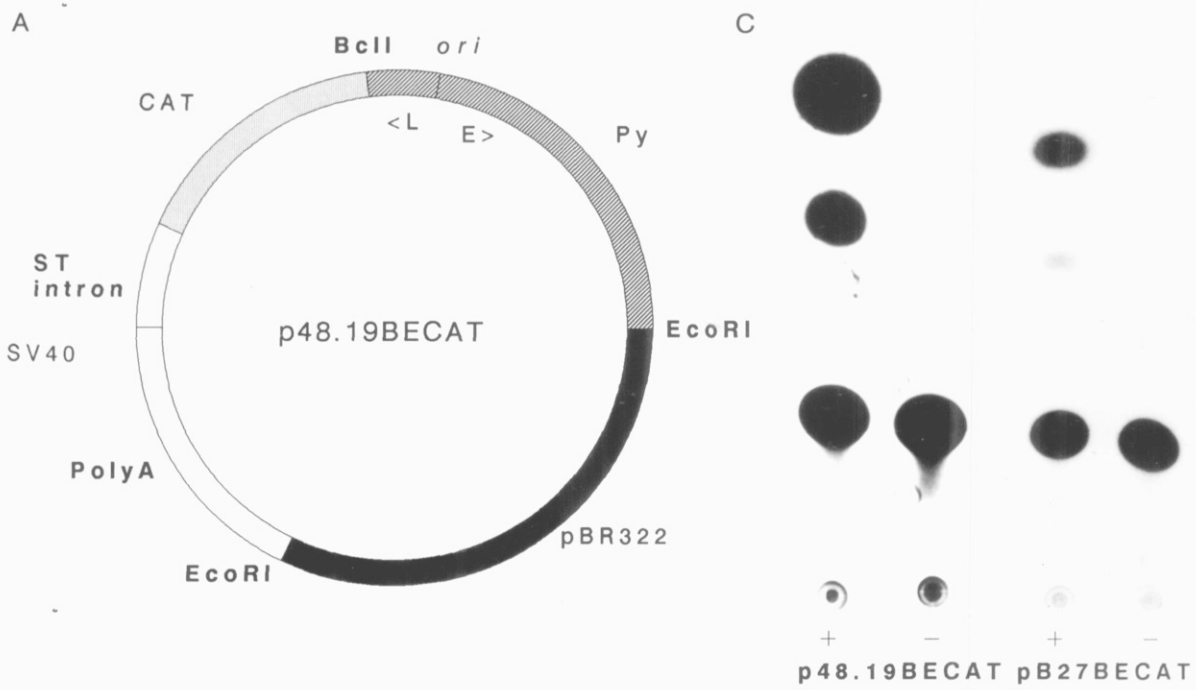
The mutant LT plasmids were co-transfected with p48.19BECAT into mouse NIH3T3 cells, which were harvested 70 hours later, and assayed for CAT activity. The combined results of a number of experiments, given in Table 5, reveal that the increases in CAT gene expression induced by the various LT mutants were inconsistent, varying greatly between experiments, a phenomenon also observed by Basilico and colleagues (Kern *et al.*, 1986). This variability may simply result from fluctuations in transfection efficiency, but could reflect genuine differences in the degree of gene activation achieved. In order to address these questions and to allow a more meaningful comparison of the trans-activating properties of the mutants, a third plasmid, pRSVβ-Gal (Gorman, 1985), was cotransfected into NIH3T3 cells with the LT mutant and p48.19BECAT. The pRSVβ-Gal plasmid contains the *E. coli lacZ* gene under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR), a promoter which is not significantly trans-activated by PyV LT (data not shown; Pannuti *et*

Figure II.12: Analysis of the Transcriptional Activation Functions of the LT Mutants.

A: A map of the target plasmid p48.19BECAT (Kern *et al.*, 1986). The plasmid contains the CAT reporter gene under the control of the PyV late promoter. Processing of the CAT mRNA is directed by the SV40 ST splicing signals, and the SV40 early region polyadenylation signal. The plasmid also contains the entire PyV non-coding region, and a truncated early region encoding full length MT and ST, and an N-terminal LT fragment.

B: A more detailed map of the PyV-derived sequences of p48.19BECAT, showing the functional organisation of the PyV non-coding sequences. The plasmid contains the PyV enhancer, early and late promoters, and the PyV *ori*, inactivated by the insertion of an *Xho*I linker sequence between PyV nucleotides 35 and 37. The locations of the LT DNA-binding sites (A, B, C, 1, 2, and 3), defined by Cowie and Kamen (1984), are shown. The target plasmid pB27BECAT was constructed by deleting PyV nucleotides 5-242 from p48.19BECAT, thus removing LT-binding sites A, B, C, and 1 (Kern *et al.*, 1986). Nucleotide numberings are those of Soeda *et al.* (1980a).

C: NIH3T3 cells were transfected with 2μg of p48.19BECAT or pB27BECAT and 2μg of pELT (+) or salmon sperm DNA (-). Cell lysates, prepared 68 hours post-transfection, were incubated with [^{14}C]-chloramphenicol and acetyl Co-A, the products separated by thin-layer chromotography, and visualised by autoradiography. Unmodified chloramphenicol, and its faster-migrating acetylated derivatives, are shown.


A
BclI
ori
CAT
<L
E>
Py
ST
intron
SV40
p48.19BECAT
EcoRI
PolyA
pBR322
EcoRI
C
+
−
+
−
p48.19BECAT pB27BECAT

B

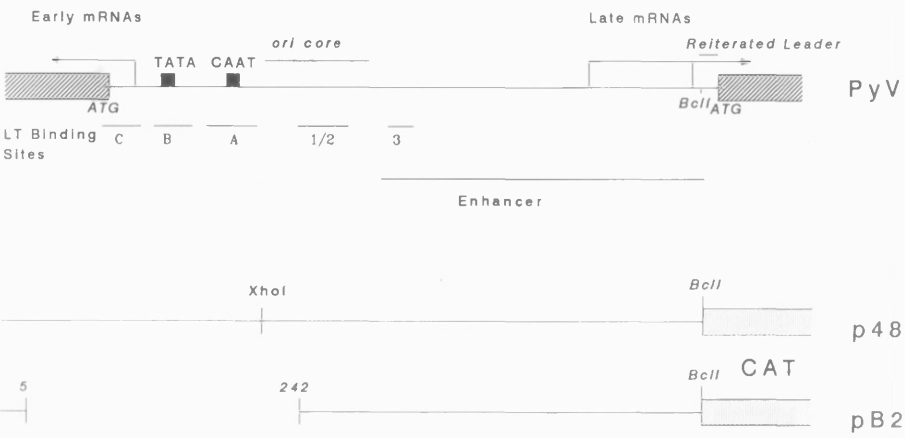
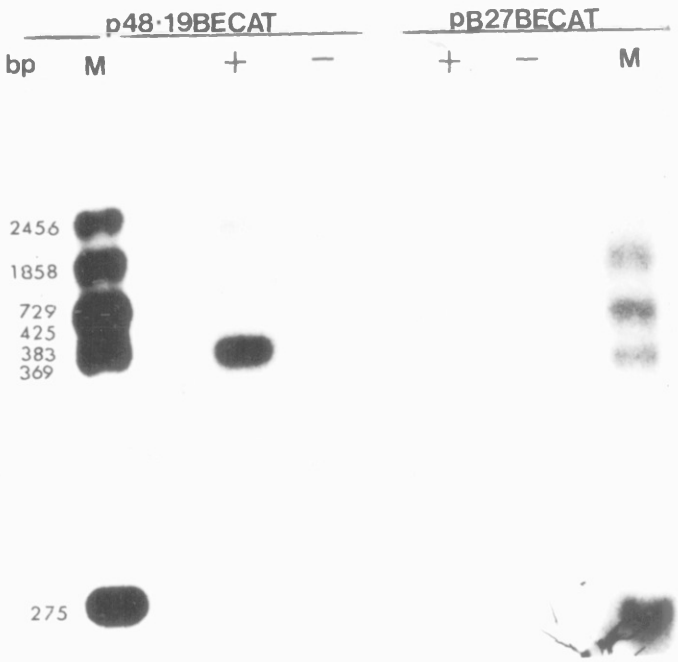

Early mRNAs
Late mRNAs
ori core
Reiterated Leader
TATA
CAAT
ATG
BclI
ATG
PyV
LT Binding Sites
C
B
A
1/2
3
Enhancer
XhoI
BclI
p48.19BECAT
5
242
BclI
CAT
pB27BECAT

Figure II.13: Analysis of the LT-Binding Capacity of p48.19BECAT and pB27BECAT.

Plasmids p48.19BECAT and pB27BECAT were digested with a combination of *Hinf*I and *Bgl*I, and end-labelled with α-[^{32}P]-dCTP using T4 DNA polymerase. Labelled DNAs were incubated with a protein lysate from A2-infected 3T6 cells, and LT-DNA complexes were immunoprecipitated using a combination of the LT-specific monoclonal antibodies (Dilworth and Griffin, 1982) α-PyLT-1 and α-PyLT-4 (+), or non-immune serum (-). Immunoprecipitated DNA fragments were separated by electrophoresis through a 1.5% agarose gel, using radioactively labelled, *Hinf*I/*Bgl*I-digested p48.19BECAT and pB27BECAT DNAs as markers (M), and detected by autoradiography. A 369bp DNA fragment from p48.19BECAT, containing LT-binding sites B and C, is immunoprecipitated by the α-LT monoclonal antibodies. LT does not bind to DNA fragments derived from pB27BECAT.


p48·19BECAT
pB27BECAT
bp
M
+
−
+
−
M
2456
1858
729
425
383
369
275

al., 1987a). The same cell extract was then assayed for CAT and β-galactosidase activities, allowing the extent of chloramphenicol acetylation to be corrected for transfection efficiency. All the LT mutant plasmids were assayed in the same experiment to minimise variability arising from different rates of cell growth *etc.*. A 10-fold variation in transfection efficiency, as shown by β-galactosidase activity, was observed in some experiments, suggesting that this may have been a major contributor to the lack of consistency in previous results. However, a 2- to 3-fold variation in the extent of trans-activation obtained in different experiments was still apparent. The average results from five experiments of this type are presented numerically in Table 5, and graphically in Figure II.14.

The results of similar experiments utilising pB27BECAT are detailed in Table 6, and presented graphically in Figure II.15. Again a high level of variability was obtained, but this was effectively eliminated by correction for transfection efficiency.

Confirming the earlier results of Kern *et al.* (1986), the PyV late promoter in pB27BECAT was found to be less susceptible to trans-activation by LT than its equivalent in p48.19BECAT. In the results presented here, wild-type LT produces a 3.3-fold activation of CAT expression from p48.19BECAT, and a 1.9-fold activation of pB27BECAT. This compares with activation in the range of 7.3- to 61.1-fold for p48.19BECAT and 1.9-fold for p27BECAT, observed by Kern *et al.* (1986). A comparison of the effects of the LT mutants on the two target plasmids is presented in Table 7 and shown graphically in Figure II.16. Clearly, these experiments must be repeated to allow the statistical significance of the results described above to be assessed.

Although the target plasmids themselves are replication-defective, this is not the case for all the LT mutant plasmids, some of which are capable of autonomous replication in the permissive environment provided by NIH3T3 cells. It is therefore possible that an increased intracellular concentration of LT, resulting from replication of the input plasmid, could arise. In order to assess the effect of such an increase in LT concentration on transactivation of the late promoter, the target plasmids were transfected into NIH3T3 cells along with differing concentrations of pELT. Figure II.17 shows the results of these experiments, revealing a linear increase in CAT activity, corrected for transfection efficiency, for lower amounts of pELT. At higher concentrations of pELT, the level of CAT expression from the two target plasmids was observed to plateau, and possibly become reduced, and this may reflect competition between the control regions of the two input plasmids for limiting cellular transcription factors. These results suggest that amplification of the LT plasmids by autonomous replication could influence the amount of trans-activation, and this

Table 5: Transcriptional Activation of p48.19BECAT.

NIH 3T3 cells were cotransfected with 2μg of p48.19BECAT and 2μg of pELT, or its mutant derivatives. Cells were harvested 70 hours post-transfection, and cell lysates were assayed for CAT activity. The mean activation (relative to CAT activity derived from cells transfected with a combination of p48.19BECAT and carrier DNA) is shown, together with the standard error of the mean.

The columns marked β-Gal Corrected contain the results of similar experiments in which NIH 3T3 cells were cotransfected with 2μg of p48.19BECAT, 2μg of pRSVβ-Gal, and 2μg of pELT, or its mutant derivatives. All the mutants were analysed in the same experiment. Cells were harvested 70 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The % chloramphenicol conversion, divided by β-galactosidase activity, is shown for each LT mutant. The degree of activation (relative to CAT activity derived from cells transfected with a combination of p48.19BECAT, pRSVβ-Gal, and carrier DNA) is shown.

n = number of transfections

- = no LT DNA

MUTANT	MEAN ACTIVATION	n	β-GAL CORRECTED*	
			% CONVERSION	ACTIVATION
pELT	2.6 ± 0.5	20	14.4	3.3
AX193	10.8 ± 3.4	8	15.9	3.6
ACB368	24.9 ± 9.9	8	14.2	3.2
AX873	11.1 ± 4.6	8	3.3	0.8
AVX1016	23.4 ± 9.3	8	12.9	2.9
AX1145	11.2 ± 3.9	8	13.0	3.0
AX1232	13.8 ± 7.7	8	4.1	0.9
AX1374	13.4 ± 4.9	6	4.7	1.1
EX1560	1.4 ± 0.3	9	4.0	0.9
HX1656	2.3 ± 0.3	4	4.2	1.0
ACB1809	6.9 ± 1.4	6	4.6	1.1
AX2006	41.3 ± 21.9	6	3.8	0.9
AX2033	3.1 ± 0.9	6	4.0	0.9
AX2086	1.8 ± 0.5	8	4.8	1.1
AX2227	4.3 ± 1.5	8	3.7	0.8
AX2377	2.2 ± 0.4	8	4.1	0.9
XX2477	1.7 ± 0.7	7	4.3	1.0
XX2522	5.1 ± 2.6	6	4.5	1.0
*dl*8	1.6 ± 0.6	6	5.5	1.3
*dl*23	1.0 ± 0.3	4	3.7	0.8
*dl*2208	5.7 ± 1.9	8	8.3	1.9
RX14/*dl*2208	20.1 ± 7.8	5	3.8	0.9
RX33/*dl*2208	7.1 ± 2.5	6	3.8	0.9
pELTRI	0.1 ± 0.1	4	4.4	1.0
-	1.0	18	4.4	1.0

* % chloramphenicol conversion divided by β-galactosidase activity (mean of 5 expts).

Figure II.14: A Graphical Representation of the Transactivation of p48.19BECAT by the LT Mutants.

NIH 3T3 cells were cotransfected with 2μg of p48.19BECAT, 2μg of pRSVβ-Gal, and 2μg of pELT, or its mutant derivatives. Cells were harvested 68 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The % chloramphenicol conversion, corrected for β-galactosidase activity, is shown for each LT mutant, plotted against amino acid position for the linker insertion mutants. The deletion and deletion/insertion mutants are shown separately.

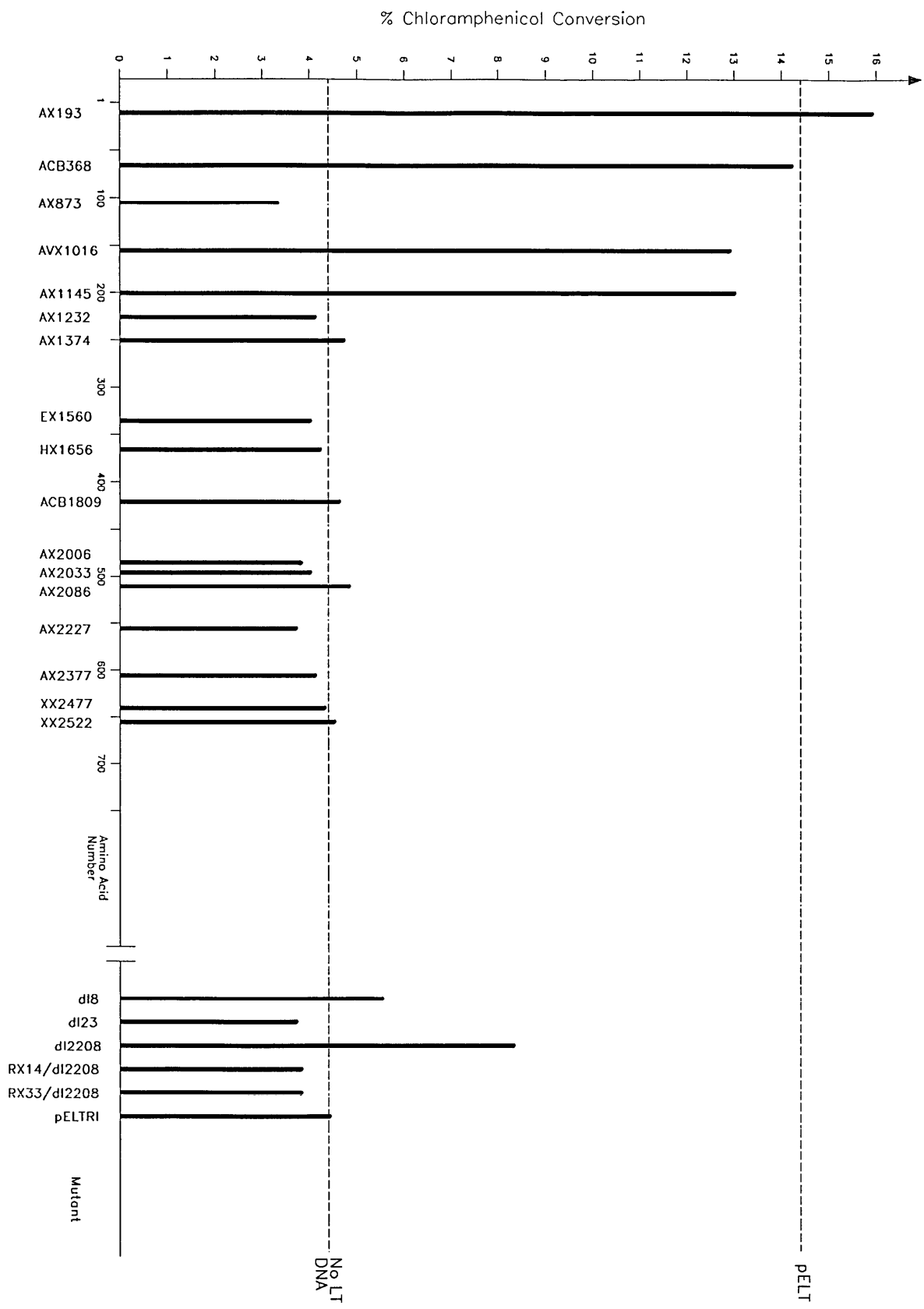

% Chloramphenicol Conversion
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
AX193
ACB368
AX873
AVX1016
AX1145
AX1232
AX1374
EX1560
HX1656
ACB1809
AX2006
AX2033
AX2086
AX2227
AX2377
XX2477
XX2522
dl8
dl23
dl2208
RX14/dl2208
RX33/dl2208
pELTRI
Mutant
1
100
200
300
400
500
600
700
Amino Acid Number
No LT DNA
pELT

Table 6: Transcriptional Activation of pB27BECAT.

NIH 3T3 cells were cotransfected with 2μg of pB27BECAT and 2μg of pELT, or its mutant derivatives. Cells were harvested 70 hours post-transfection, and cell lysates were assayed for CAT activity. The mean activation (relative to CAT activity derived from cells transfected with a combination of pB27BECAT, and carrier DNA) is shown for each mutant, together with the standard error of the mean.

The columns marked β-Gal Corrected, contain the results of similar experiments in which NIH 3T3 cells were cotransfected with 2μg of pB27BECAT, 2μg of pRSVβ-Gal, and 2μg of pELT, or its mutant derivatives. Cells were harvested 70 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The degree of activation (relative to CAT activity derived from cells transfected with a combination of pB27BECAT, pRSVβ-Gal, and carrier DNA) is shown for each mutant.

n = number of transfections

- = carrier DNA

MUTANT	MEAN ACTIVATION	n	β-GAL CORRECTED* ACTIVATION	n
pELT	4.4 ± 0.6	16	1.9	7
AX193	69.8 ± 10.4	12	2.5	7
ACB368	75.0 ± 22.9	12	1.9	7
AX873	6.1 ± 1.2	12	1.7	9
AVX1016	21.3 ± 7.3	12	1.7	7
AX1145	7.4 ± 2.0	17	3.1	10
AX1232	1.1 ± 0.9	22	1.5	6
AX1374	1.5 ± 0.2	20	1.0	6
EX1560	1.5 ± 0.3	25	0.9	4
HX1656	2.6 ± 0.7	23	1.0	7
ACB1809	13.3 ± 2.9	18	0.9	4
AX2006	9.6 ± 2.4	20	0.7	2
AX2033	7.0 ± 2.0	16	0.8	4
AX2086	1.4 ± 0.2	15	1.1	4
AX2227	3.4 ± 0.3	16	0.9	7
AX2377	1.2 ± 0.2	16	1.2	5
XX2477	3.6 ± 1.2	12	0.7	1
XX2522	5.8 ± 1.0	11	0.7	3
*dl*8	19.8 ± 5.5	12	1.2	7
*dl*23	15.7 ± 5.1	8	1.1	4
*dl*2208	93.1 ± 28.3	8	1.4	1
RX14/*dl*2208	7.6 ± 1.6	12	1.5	3
RX33/*dl*2208	3.0 ± 0.4	11	1.1	1
pELTRI	5.2 ± 1.0	10	0.8	5
-	1.0	64	1.0	14

* % chloramphenicol conversion divided by β-galactosidase activity.

Figure II.15: A Graphical Representation of the Transactivation of pB27BECAT by the LT Mutants.

NIH 3T3 cells were cotransfected with 2μg of pB27BECAT, 2μg of pRSVβ-Gal, and 2μg of pELT, or its mutant derivatives. Cells were harvested 68 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The transcriptional activation induced by each LT mutant (relative to CAT activity derived from cells transfected with a combination of pB27BECAT, pRSVβ-Gal, and carrier DNA), corrected for β-galactosidase activity, is plotted against amino acid position for the linker insertion mutants. The deletion and deletion/insertion mutants are shown separately.

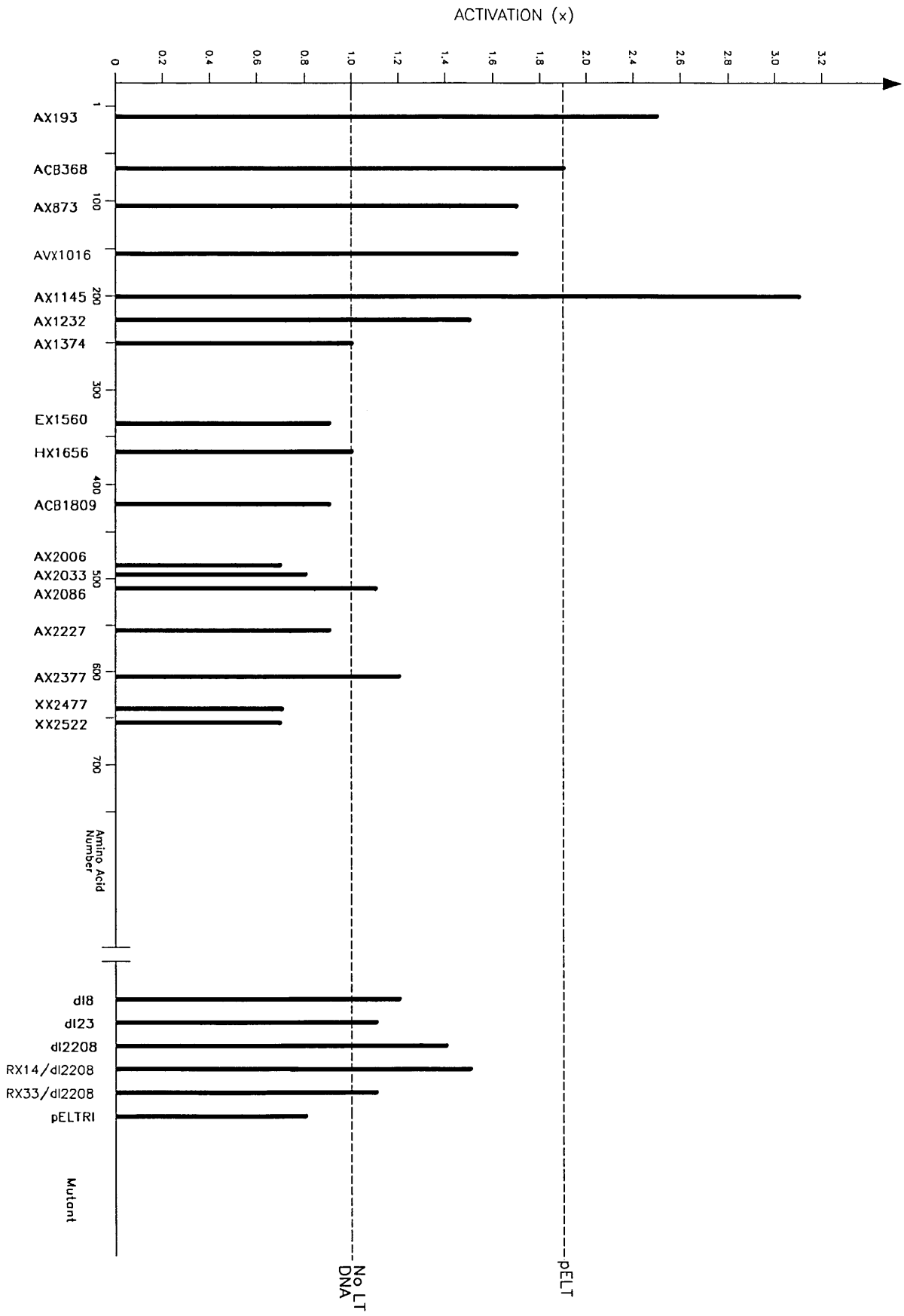

ACTIVATION (x)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.4
2.6
2.8
3.0
3.2
AX193
ACB368
AX873
AVX1016
AX1145
AX1232
AX1374
EX1560
HX1656
ACB1809
AX2006
AX2033
AX2086
AX2227
AX2377
XX2477
XX2522
dl8
dl23
dl2208
RX14/dl2208
RX33/dl2208
pELTRI
1
100
200
300
400
500
600
700
Amino Acid Number
Mutant
No LT DNA
pELT

TABLE 7: COMPARISON OF TRANSACTIVATION OF p48.19BECAT AND pB27BECAT

MUTANT	% ACTIVATION p48.19BECAT	pB27BECAT
pELT	100	100
AX193	112	160
ACB368	98	92
AX873	-11	80
AVX1016	85	80
AX1145	86	229
AX1232	-3	49
AX1374	3	2
EX1560	-4	-14
HX1656	-2	-2
ACB1809	2	-12
AX2006	-6	-28
AX2033	-4	-25
AX2086	4	11
AX2227	-7	-8
AX2377	-3	23
XX2477	-1	-34
XX2522	1	-33
*dl*8	11	16
*dl*23	-7	8
*dl*2208	39	38
RX14/*dl*2208	-6	58
RX33/*dl*2208	-6	13
pELTRI	18	-23

Figure II.16: A Graphical Comparison of the Abilities of the LT Mutants to Transactivate p48.19BECAT and pB27BECAT.

NIH 3T3 cells were cotransfected with 2μg of p48.19BECAT or pB27BECAT, 2μg of pRSVβ-Gal, and 2μg of pELT, or its mutant derivatives. Cells were harvested 68 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The % transactivation (relative to pELT), corrected for β-galactosidase activity, is plotted against amino acid position for the linker-insertion mutants. The deletion mutants are shown separately.

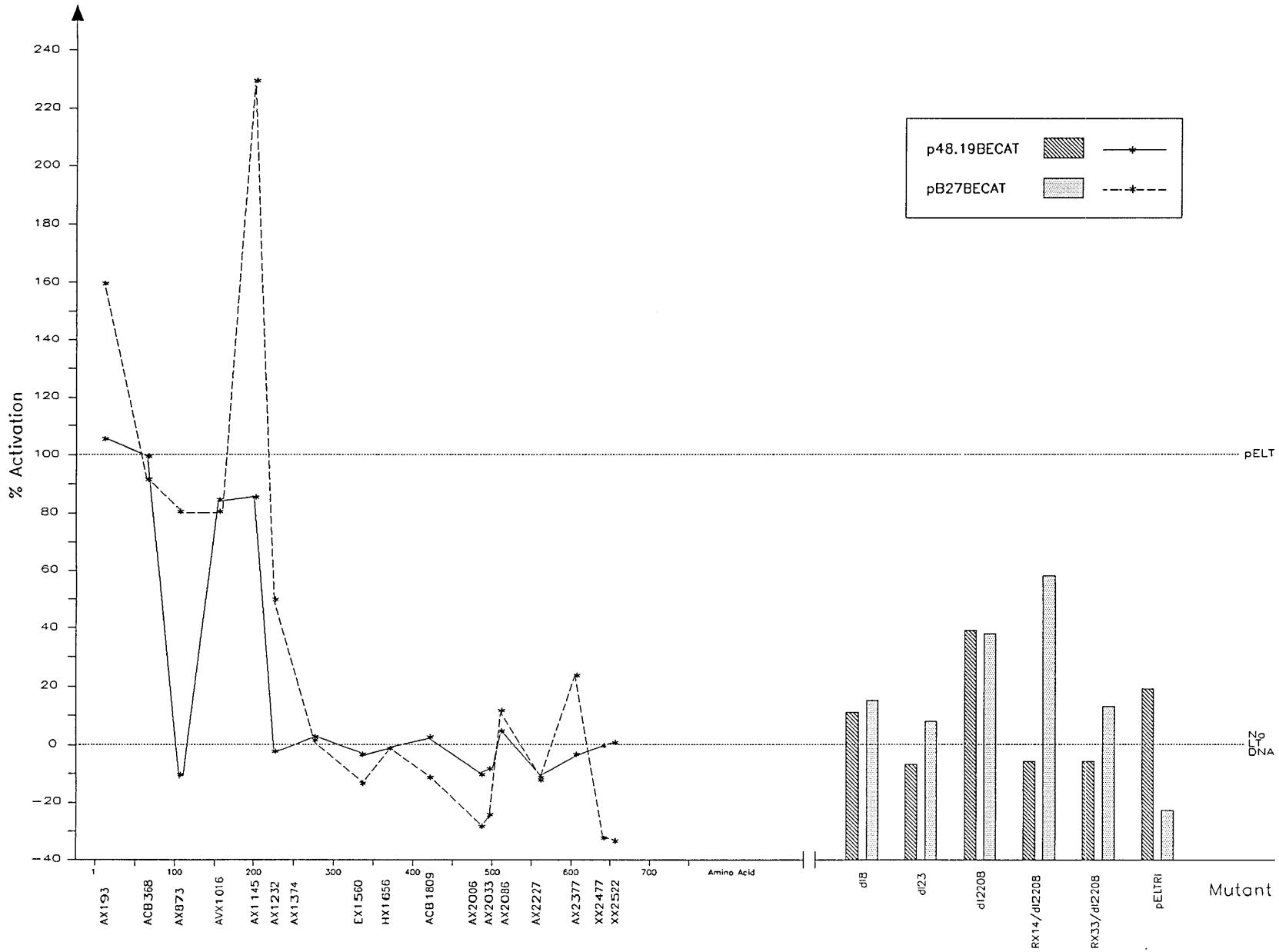
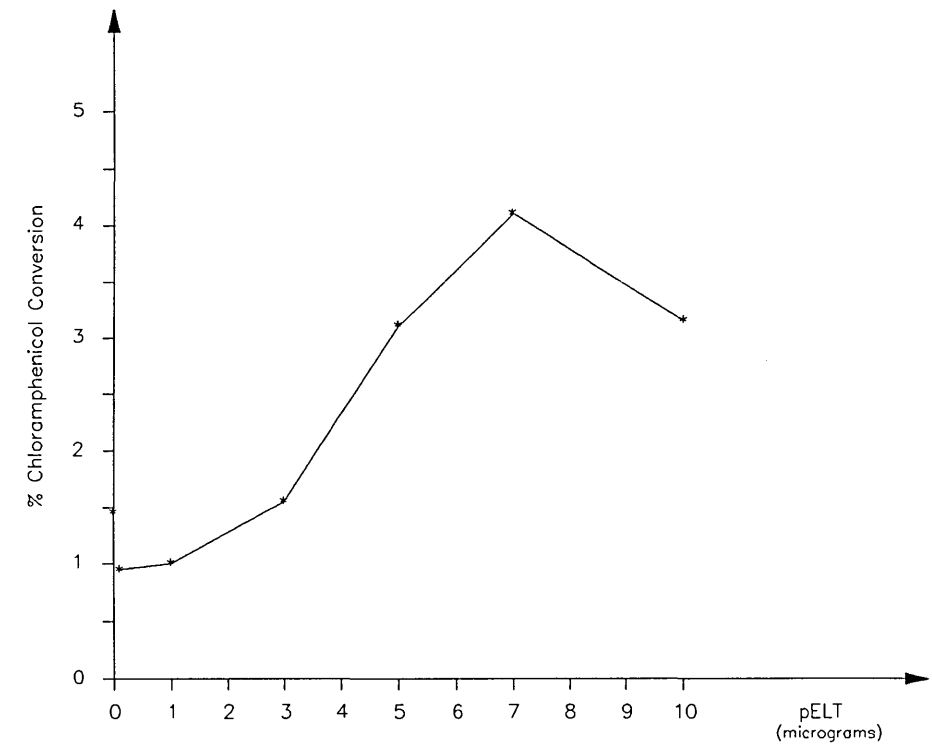

% Activation
240
220
200
180
160
140
120
100
80
60
40
20
0
−20
−40
p48.19BECAT
pB27BECAT
pELT
0 DNA
1
100
200
300
400
500
600
700
Amino Acid
AX193
ACB368
AX873
AVX1016
AX1145
AX1232
AX1374
EX1560
HX1656
ACB1809
AX2006
AX2033
AX2086
AX2227
AX2377
XX2477
XX2522
dl8
dl23
dl2208
RX14/dl2208
RX33/dl2208
pELTRI
Mutant

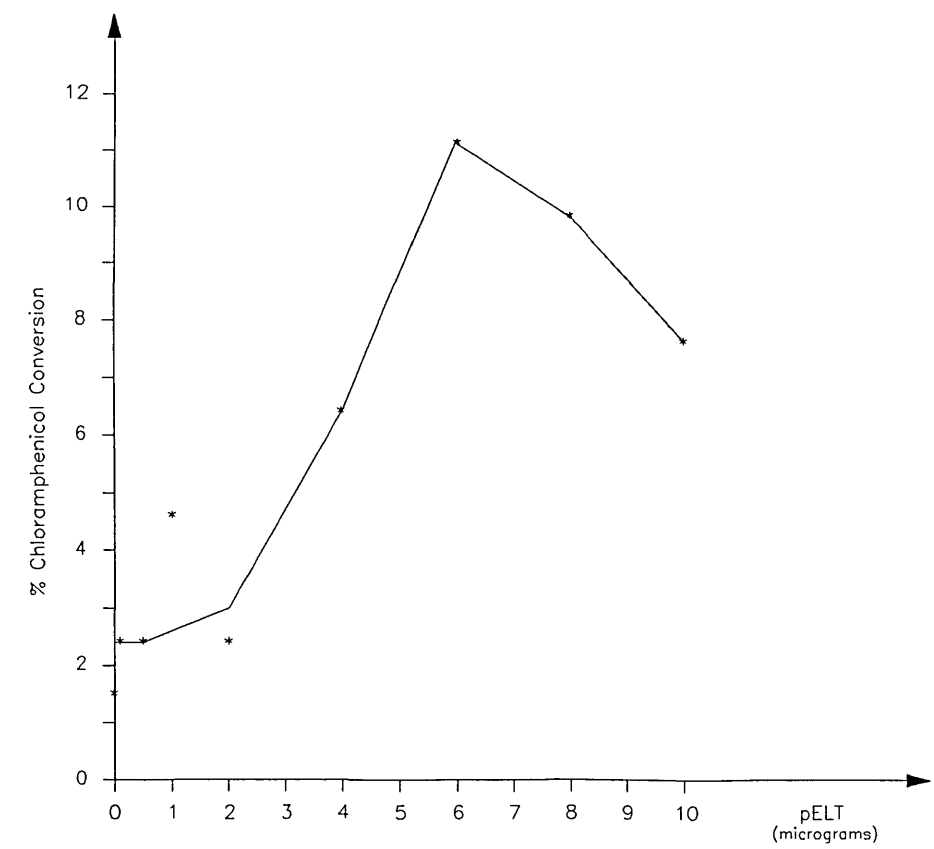
Figure II.17: A Graphical Representation of the Results of Titration Analyses on the Transactivation of p48.19BECAT and pB27BECAT by pELT.

2μg of p48.19BECAT (Graph A) or pB27BECAT (Graph B) were transfected, along with 2μg of pRSVβ-Gal and increasing concentrations of pELT, into NIH3T3 cells. Cells were harvested 68 hours post-transfection, and cell lysates were assayed for CAT and β-galactosidase activities. The % chloramphenicol conversion corrected for β-galactosidase activity, is shown for each concentration of pELT. In the transactivation experiments described in Tables 5 and 6, 2μg of each LT mutant were transfected into NIH3T3 cells.

A


% Chloramphenicol Conversion
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8 9 10
pELT
(micrograms)

B


% Chloramphenicol Conversion
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8 9 10
pELT
(micrograms)

must therefore be taken into consideration when interpreting the data presented here.

F: SERUM DEPENDENCE

The ability of the LT mutants to reduce the serum dependence of established cell lines was examined by employing the Balb c/3T3 cell lines described in section II.B. Identical numbers of cells, counted using a haemocytometer, were plated out in medium containing either 10% foetal calf serum or 10% ox plasma. Serum contains approximately ten times the level of PDGF found in plasma (20 ng/ml as opposed to 1-2 ng/ml), and the relative abilities of the cells to form colonies in the two media indicates the requirement of the cells for PDGF. As the ability to reduce the serum dependence of established cell lines has been localised to the N-terminus of the LT protein (Rassoulzadegan *et al.*, 1982), only cell lines expressing mutants which map to this region of LT were subjected to this assay. Balb c/3T3 cell lines transfected with a combination of pSV2Neo and pEMBL8(-) vector DNA were used as a negative control, and cell lines expressing pELT as a positive control. The results of one such experiment are shown in Figure II.18 and the overall results are detailed in Table 8. Cells expressing AX193, AX1232, RX14/*dl*2208 and *dl*23 were able to form colonies in ox plasma although at reduced efficiencies, compared to cells expressing wild-type LT, and not in all experiments. Mutants AX873, AVX1016, and AX1374 appeared to be totally defective in this assay, and although expression of *dl*8 LT allowed Balb c/3T3 cells to form colonies in ox plasma (in one experiment), cell growth appeared to be impaired (see Figure II.18).

As the level of LT expression in many of the Balb c/3T3 cell lines was extremely low (see Section II.B), and the behaviour of these cells in the serum dependence assay was inconsistent (see Table 9), parallel experiments were performed using rat cell lines established by the LT mutants, which appear to contain higher levels of LT (see Section II.E). In addition, clonal Balb c/3T3 cell lines were not available for ACB368 and pELTRI, and rat cell lines established by these mutants were employed as an alternative. As no suitable rat cell line was available, untransfected Balb c/3T3 cells were used as a negative control. The results of these experiments are summarised in Table 9. Cell lines established by mutants AX193, ACB368, AX1145, and AX1374 formed colonies in ox plasma at frequencies similar to those exhibited by cells immortalised by pELTRI (the positive control in these experiments). Mutants RX14/*dl*2208 and RX33/*dl*2208 also allowed rat cells to form colonies in ox plasma, although at reduced efficiencies.

Figure II.18: Analysis of the Ability of the Mutant LT Genes to Reduce the Serum Dependence of Balb c/3T3 Cells.

Balb c/3T3 cell lines, generated by transfection with a combination of pSV2Neo and either pELT (positive control), its mutant derivatives, or pEMBL8(-) vector DNA (negative control), were plated at a density of 500 cells/5cm dish in medium containing 10% foetal calf serum (Serum), or 10% ox plasma (Plasma). The medium was replaced every four days, and colonies were stained with Crystal Violet after 14 days.

In this experiment, cells expressing pELT, AX1232, and *dl*23 form colonies in medium containing ox plasma. The negative control cells, transfected with pEMBL8(-), fail to form colonies in medium containing ox plasma, as do cells expressing mutant AX873. Cells expressing *dl*8 grow poorly in ox plasma, forming small colonies.

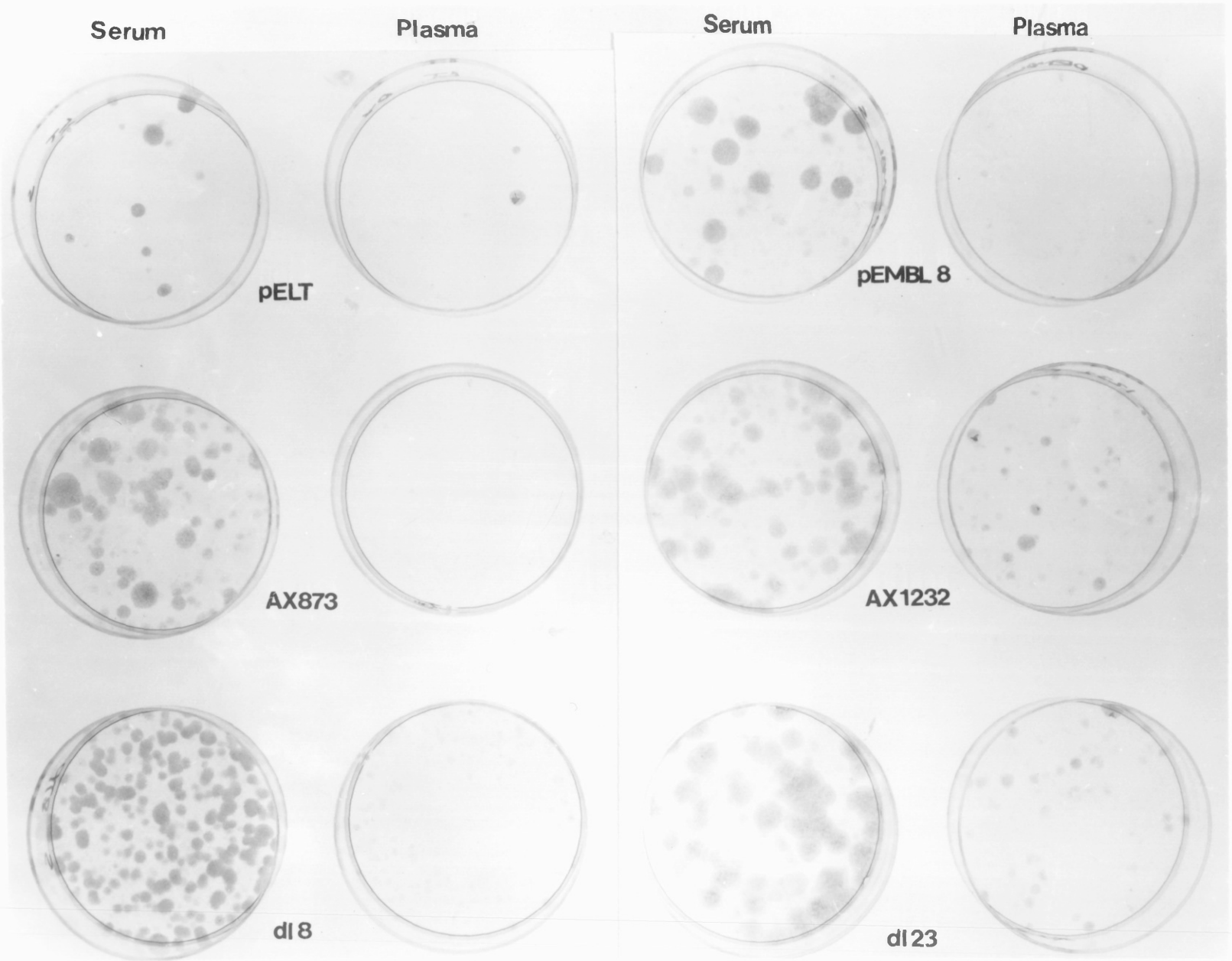

Serum
Plasma
pELT
Serum
Plasma
pEMBL 8
AX873
AX1232
dl 8
dl 23

TABLE 8: COLONY FORMATION OF LT-EXPRESSING BALB C/3T3 CELL LINES IN OX PLASMA

MUTANT	% COLONY FORMATION*		
	Expt. 1	**Expt. 2**	**Expt. 3**
pELT	100	1	71
pEMBL8(-)	2	0	0
AX193	16	10	-
AX873	0	0	6
AVX1016	1	1	-
AX1145	0	5	-
AX1232	0	1	28
AX1374	2	1	-
*dl*8	0	-	70
*dl*23	0	-	73
RX14/*dl*2208	6	-	-

* Ratio of colony formation in 10% serum to 10% plasma.

n = Number of Experiments

- = Not Done

TABLE 9: COLONY FORMATION OF LT-IMMORTALISED RAT CELL LINES IN OX PLASMA

MUTANT	% COLONY FORMATION*			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
pELTRI	#	-	-	100
Balb c/3T3@	5	#	2	3
AX193	90	-	30	94
ACB368	-	-	-	80
AVX1016	-	-	2	16
AX1145	-	-	-	100
AX1374	-	100	-	-
RX14/*dl*2208	-	40	-	-
RX33/*dl*2208	90	32	28	-

* Ratio of colony formation in 10% serum to 10% plasma

- = Not Done

= Cells lost due to tissue culture contamination

@ Used as a negative control

n = Number of Experiments

TABLE 10: COMBINED RESULTS OF EXPERIMENTS TO EXAMINE THE COLONY FORMATION OF LT-EXPRESSING CELL LINES IN OX PLASMA

MUTANT	BALB C/3T3 LINES		REF LINES	
	% COLONY FORMATION*	NUMBER OF EXPERIMENTS	% COLONY FORMATION*	NUMBER OF EXPERIMENTS
pELT#	57	3	-	-
pEMBL8(-)@	1	3	-	-
AX193	13	2	71	3
ACB368	-	-	80	1
AX873	2	3	-	-
AVX1016	1	2	9	2
AX1145	3	2	100	1
AX1232	10	3	-	-
AX1374	2	2	100	1
*dl*8	35	2	-	-
*dl*23	37	2	-	-
RX14/*dl*2208	6	1	40	1
RX33/*dl*2208	-	-	50	3
pELTRI	-	-	100	1
Balb c/3T3	-	-	5	3

- = Not Done

* Ratio (mean) of colony formation in 10% serum to 10% plasma

Positive Control

@ Negative Control

The combined results of these experiments are detailed in Table 10, and it is clear that different results were obtained for LT-expressing rat and mouse cells. Mutants AX1145 and AX1374 appear to promote the growth of rat cells, but not Balb c/3T3 cells, in ox plasma, and mutants AX193 and RX14/*dl*2208 also appear to have a greater influence on the growth of rat cells. By combining the results of the serum dependence assays it appears that pELT and mutants pELTRI, AX193, ACB368, AX1145, AX1232, AX1374, RX14/*dl*2208, RX33/*dl*2208, and *dl*23 retain at least some capacity to reduce cellular growth factor requirements. Mutants AX873 and, possibly, *dl*8 may be defective in the Balb c/3T3 assay, and AVX1016 appears to be unable to promote the growth of rat or mouse fibroblasts in medium containing low concentrations of PDGF.

Further experiments must be performed to confirm these preliminary results, and to assess the possibility that the LT mutants have differential effects on the growth of rat and mouse cells.

A summary of the results obtained for all the LT mutants with regard to their cellular localisation, ability to promote DNA replication, immortalise rat cells, trans-activate the PyV late promoter, and reduce the serum dependence of cells is presented in Table 11.

TABLE 11: SUMMARY

MUTANT	LOCATION	REPLICATION	% TRANS-ACTIVATION p48.19BECAT	% TRANS-ACTIVATION pB27BECAT	IMMORTALISATION	SERUM DEPENDENCE
pELT	N	+	100	100	[+]	+
AX193	N	+	112	160	+	+
ACB368	N	nd	98	92	+	+
AX873	N	-	-11	80	+	-
AVX1016	N	+	85	80	+	-
AX1145	N	+	86	229	+	+
AX1232	N	-	-3	49	[+]	+
AX1374	N	-	3	2	+	+
EX1560	N	-	-4	-14	nd	nd
HX1656	N	-	-2	-2	nd	nd
ACB1809	N	nd	2	-12	nd	nd
AX2006	N	-	-6	-28	nd	nd
AX2033	N	-	-4	-25	nd	nd
AX2086	N	-	4	11	nd	nd
AX2227	N	-	-7	-8	nd	nd
AX2377	N	-	-3	23	nd	nd
XX2477	N	-	-1	-34	nd	nd
XX2522	N	-	1	-33	nd	nd
*dl*8	N	(+)	11	16	-	+/-
*dl*23	N	(+)	-7	8	[+]	+
*dl*2208	N	(+)	39	38	+	nd
RX14/*dl*2208	N+C	nd	-6	58	+	+
RX33/*dl*2208	N	nd	-6	13	+	+
pELTRI	N	-	18	-23	+	+

N = Nucleus
N+C = Nucleus and Cytoplasm
( ) = Data from other laboratories
nd = not done
[ ] = Cell lines not established due to tissue culture contamination

III: DISCUSSION

This chapter is divided into three sections: the results presented in Chapter II are discussed in section A, and are related to the known properties of PyV LT, and other viral and cellular proteins, in section B. Conclusions are presented in section C.

A: Discussion of Results

1: Mutagenesis

A panel of PyV LT mutants has been constructed by a variety of methods: semi-random, linker-insertion mutagenesis (the AX series of mutants); linker-insertion at defined restriction sites (the ACB, AVX, EX, HX, and XX mutants); and deletion of C-terminal coding sequences to produce a truncated LT protein (pELTRI). In addition, LT genes from the existing PyV deletion mutants *dl*8, *dl*23 (Griffin and Maddock, 1979), and *dl*2208 (Nilsson *et al.*, 1983), and the double mutants RX14/*dl*2208 and RX33/*dl*2208 (Richardson *et al.*, 1986), have been isolated from the other PyV early genes, to produce LT-expressing plasmids in a format compatible with that of the linker insertion mutants.

The mutations are evenly distributed, occurring at 40-60 amino acid intervals throughout the entire LT coding sequence, with the exception of the C-terminal 120 residues (see Figure II.3 and Table 2, previous chapter). Although the mutants have been shown to contain single-linker insertions, the precise effect of the linkers on the coding sequence of PyV LT has not been confirmed by nucleotide sequence analysis, and the residue changes described in Table 2 (previous chapter) can therefore only be implied. Although the LT reading frame is predicted to be maintained following the linker insertion, LT-related proteins, of any size, were not detected in Balb c/3T3 cells transfected with the mutant genes. However, LT expression was detected in these cells by immunofluorescence (see Figures II.4 and II.6), using the LT-specific monoclonal antibodies α-PyLT-1 and α-PyLT-4, which recognise epitopes located between amino acid residues 150 and 250 of PyV LT (Griffin and Dilworth, 1982). Mutants AX193, ACB368, and AX873 contain linker insertions mapping to amino acids 8, 66, and 106 of PyV LT, respectively. Frame-shifts occurring at the site of insertion would therefore be expected to preclude translation, in the appropriate reading frame, of sequences encoding the epitopes recognised by the α-LT monoclonal antibodies. Mutants AX193, ACB368, and AX873 are therefore implicitly in-phase.

The insertion of 2-4 amino acids was expected to produce a localised effect on the secondary and tertiary structure of PyV LT, and this appears to have been a valid assumption, as neighbouring mutants are observed to exhibit distinct phenotypes (see Table 11, previous chapter). For example, mutant AX1145 (amino acid 197) is capable of promoting DNA replication, but mutant AX1232 (amino acid 226) is not. The internal deletions carried by mutants *dl*8, *dl*23, and *dl*2208, which remove 29, 33, and 18 amino acid residues, respectively, may have a more widespread effect on protein structure, and the phenotypes of these mutants are often inconsistent with those of the linker-insertion mutants mapping to the same region of LT (see Table 11, previous chapter). For example, mutant *dl*23 fails to transactivate the PyV late promoter in either of the target plasmids used in this study, while mutants AX1145 and AX1232, which map within the *dl*23 deletion, do induce transcriptional activation. The extent of the internal deletion appears to influence the phenotype of the mutant, as the *dl*23 deletion has a more dramatic effect on the ability of LT to activate the PyV late promoter than the smaller *dl*2208 deletion, which is almost entirely contained within the sequences removed by the *dl*23 deletion (see Figure II.3 and Table 2, previous chapter). This may reflect the greater effect of the *dl*23 lesion on the three-dimensional structure of LT. Combination of the *dl*2208 deletion with the RX14 or RX33 deletion/insertion mutations also produces a more dramatic effect on the ability of the protein to induce transcriptional activation of the intact PyV late promoter. However, in contrast to the neighbouring RX14 mutation, the RX33 deletion/insertion has a marked effect on the ability of *dl*2208 to stimulate CAT expression from pB27BECAT. This may reflect the greater effect on protein structure of the larger RX33 deletion.

The functional domains of LT identified in this study are shown in Fig III.1.

2: DNA Replication

The results of the replication assay in this study are incomplete, and no data are available for mutants ACB368 and ACB1809, or for *dl*8, *dl*23, *dl*2208, RX14/*dl*2208, and RX33/*dl*2208. Further experiments must be performed to assess the capacity of these mutants to undergo autonomous DNA replication. The majority of the linker-insertion mutants were tested satisfactorily in this assay, however, and only mutants AX193, AVX1016, and AX1145, mapping to the N-terminus of LT, retain the ability to promote DNA replication. Mutations in the C-terminal two thirds of LT (from mutant AX1232 at amino acid residue 226 to mutant XX2522 at amino acid 656) were found to abolish autonomous DNA replication. At least two distinct, non-contiguous, domains of LT appear to be

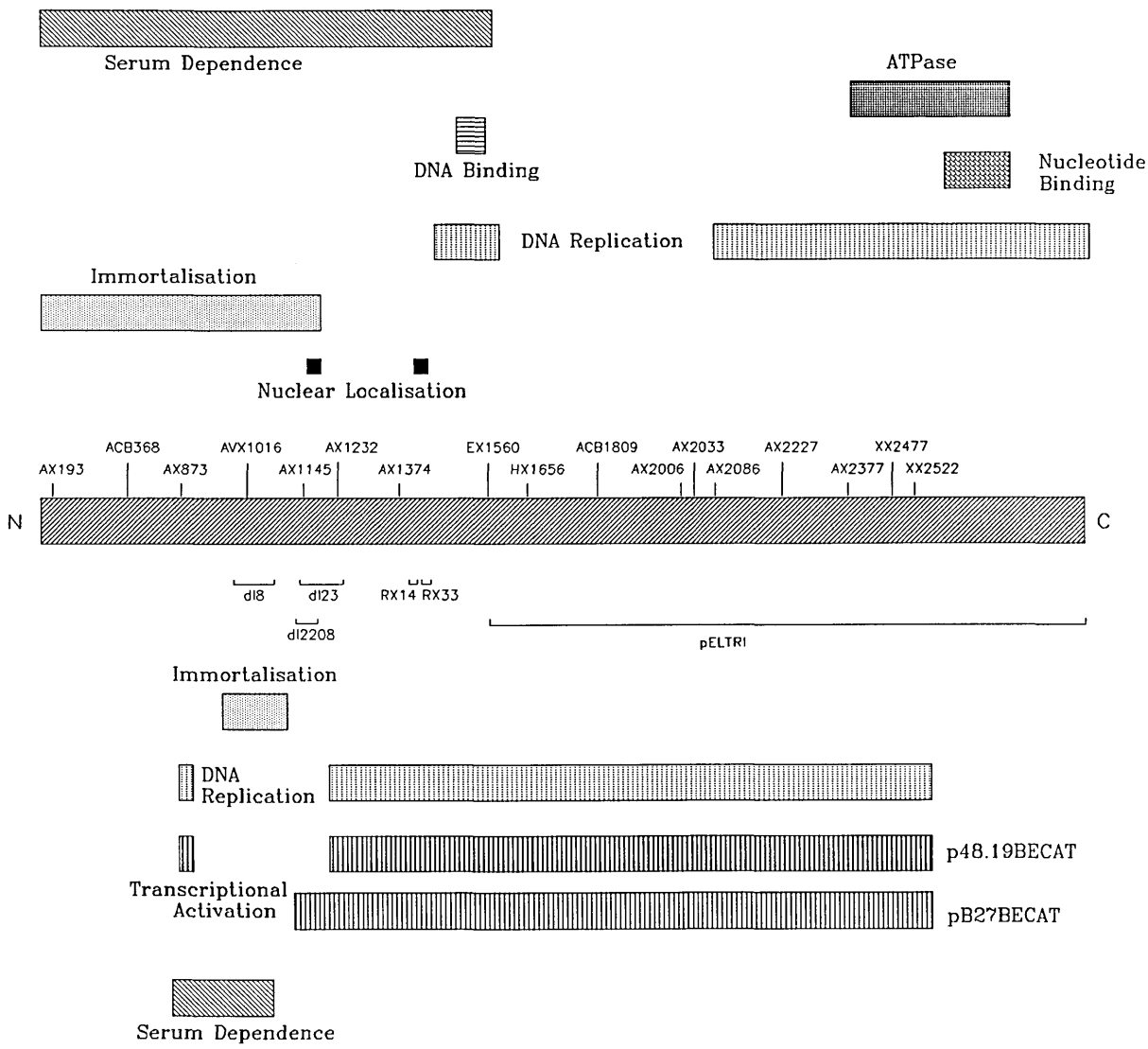
Figure III.1: Functional Domains of PyV LT.

The results presented in this study are shown diagrammatically, together with the functional domains identified in previous studies.

References

Immortalisation: Asselin and Bastin, 1985.
DNA Binding: Cowie *et al.*, 1986.
Reduction of Serum Dependence: Rassoulzadegan *et al.*, 1982.
ATPase: Bradley *et al.*, 1987.
Nucleotide Binding: Clertant *et al.*, 1984.
Nuclear Localisation Signals: Richardson *et al.*, 1986.

PUBLISHED DATA



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important for DNA replication: an N-terminal domain affected by a mutation sited at amino acid 106 (mutant AX873), and a domain located between mutant AX1145 (at amino acid 197) and the C-terminus (see Figure III.1). The importance of this C-terminal domain is supported by the observation that pELTRI, a mutant lacking the C-terminal 60% of PyV LT, is defective in the replication assay.

3: Transcriptional Activation

Experiments employing two target plasmids containing the CAT reporter gene have provided information on the ability of the LT mutants to transactivate the intact PyV late promoter (in p48.19BECAT), or a mutant promoter lacking the major LT-binding sites (pB27BECAT). However, additional studies are required to allow statistical confidence limits to be applied to the results presented in the previous chapter (Tables 5, 6, and 7). This should allow the significance of these results to be assessed, and help to address, for example, the possibility that some of the mutants (exemplified by AX873 and AX1374) are not entirely defective in these assays, but retain a residual ability to activate the PyV late promoter.

Only a minority of the LT mutants appear to activate the intact PyV late promoter contained in the target plasmid p48.19BECAT. Mutants AX193, ACB368, AVX1016, and AX1145 retain the ability of wild-type LT to transactivate the PyV late promoter, while *dl*2208 exhibits a reduced activity in this assay. With the possible exceptions of *dl*8 and pELTRI, which produce marginal and probably insignificant increases in CAT expression, all the other LT mutants appear to be defective in the ability to transactivate the PyV late promoter in p48.19BECAT.

As found earlier for DNA replication, two distinct, non-contiguous, domains of PyV LT appear to be important for transactivation of the intact late promoter: an N-terminal region affected by the AX873 mutation, and a C-terminal domain located between mutant AX1145 (at amino acid 197) and the C-terminus. The similarity of these localisations suggests that there may be a correlation between the ability of the LT mutants to induce transcriptional activation and to promote DNA replication (see Figure III.1 and Table 11, previous chapter). The only mutants which induce transcriptional activation are AX193, AVX1016, AX1145, ACB368 and *dl*2208, the first three of which have been demonstrated to promote DNA replication. The ability of the ACB368 and *dl*2208 plasmids to replicate autonomously could not be assessed in this study, although a *dl*2208 LT plasmid has previously been shown to replicate (Nilsson and Magnusson, 1984). However, mutants *dl*8 and *dl*23 are expected to promote DNA replication, but show little

or no ability to transactivate p48.19BECAT, and further experiments must be performed to confirm the correlation between the two activities. This apparent link between DNA replication and transcriptional activation could reflect the increased levels of LT resulting from the replication-induced amplification of a subset of the input plasmids. A titration experiment did suggest that a threshold level of LT was required for trans-activation of the PyV late promoter in p48.19BECAT and increases in the amount of input LT DNA were observed to produce an exponential increase in CAT activity (see Figure II.17). The problems caused by DNA replication of the LT plasmids could be circumvented in several ways, for example, by recloning the mutants into replication-defective vectors, or by employing an S1 nuclease protection assay to monitor the expression of LT and CAT genes simultaneously. However, the complete inability of the replication-defective mutants (exemplified by mutants AX873, AX1232, and AX1374), at least some of which would be expected to retain the capacity to bind to the PyV non-coding region, to increase CAT expression from p48.19BECAT, argues that the gene activation and DNA replication functions of LT may be genuinely related.

The results of similar experiments, utilising pB27BECAT, suggested that mutants ACB368, AX873, and AVX1016 retain the capacity of wild-type LT to stimulate a PyV late promoter lacking the major LT-binding sites, while mutants AX1232, *dl*2208, AX2377, and RX14/*dl*2208 exhibit reduced activities in this assay. Mutants AX193 and AX1145 appear to be considerably more active than pELT in stimulating the PyV late promoter in pB27BECAT, while *dl*8, AX2086, and RX33/*dl*2208 produce small and possibly insignificant stimulatory effects on CAT expression, and mutants AX1374, HX1656, AX2227, and *dl*23 appear to be completely defective in this assay. Finally, the remaining mutants, particularly AX2006, AX2033, XX2477, XX2522, and pELTRI, appear to exert an inhibitory effect on CAT expression. It appears, therefore, that LT residues between mutant AVX1016 (amino acid 154) and the C-terminus contribute to the activation of a PyV late promoter lacking LT-binding sites. Mutations mapping to this domain can abolish or enhance the ability of LT to activate gene expression, or can even induce repression of the PyV late promoter in p27BECAT. The regions of LT implicated in trans-activation of the mutant PyV late promoter differ from those involved in stimulation of the intact promoter, supporting the suggestion that LT employs two distinct mechanisms to stimulate gene expression (Kern *et al.*, 1986). The abilities of the LT mutants to stimulate CAT expression from pB27BECAT do not correlate with their ability to replicate autonomously (the replication-defective mutants AX873 and AX1232 are functional in this assay), but the involvement of the C-terminal two thirds of LT in both functions, again suggests

the existence of a mechanistic link between DNA replication and transcriptional activation. Even though CAT expression from pB27BECAT and p48.19BECAT is equally susceptible to increased concentrations of wild-type LT (see Figure II.17), some of the LT mutants (particularly AX873, AX1232, AX2377, and RX14/*dl*2208) have differential effects on the two target plasmids (see Table 11, previous chapter). For example, mutant AX873 fails to stimulate CAT expression from p48.19BECAT but is capable of activating the PyV late promoter in pB27BECAT. Similarly, mutant AX1232 stimulates CAT expression from pB27BECAT (although to a more limited extent than pELT), but not p48.19BECAT. As mutants AX873 and AX1232 trans-activate pB27BECAT they would therefore be expected to exert at least some effect on p48.19BECAT. Further experiments must be performed to address this point. The abilities of PyV LT to activate the intact PyV late promoter and a mutant derivative lacking LT-binding sites therefore seem to be genetically separable, providing additional evidence for the existence of alternative mechanisms of transcriptional activation.

The activation of pB27BECAT by the replication-defective mutants AX873 and AX1232 supports the suggestion that the inability of these mutants (and others) to increase CAT expression from p48.19BECAT derives from a genuine mechanistic defect rather than a failure to attain a threshold level of protein. It is still possible that amplification of the input plasmids could have distorted the data pertaining to the activation of pB27BECAT, and, again, future experiments must be designed to eliminate the effects of DNA replication.

4: Immortalisation

The ability of pELTRI to immortalise cells in this study confirms the observation (Rassoulzadegan *et al.*, 1983b; Land *et al.*, 1983) that the N-terminal 40% of LT is sufficient to induce cellular establishment. With the exception of *dl*8, all the LT mutants tested in this study, including AVX1016, which maps within the *dl*8 deletion (see Table 2, previous chapter), retain immortalising activity, although it is possible that some of them may have a reduced capacity to immortalise REFs, as no attempt was made to quantify the frequency of immortalisation in this study. Certainly, individual mutants were found to produce different numbers of drug resistant colonies with the characteristic "immortalised" morphology (see Figure II.8), although this could merely reflect fluctuations in transfection frequencies similar to those observed during the transactivation studies. A variation, from experiment to experiment, in the number of colonies produced by transfection of REFs with the same LT mutant was observed in this study, supporting this suggestion. The number of cell lines

established (see Table 4, previous chapter) is not necessarily an indication of the efficiency of immortalisation, as loss of cell populations to tissue culture contamination was a recurrent problem. For example, a number of potential cell lines established by transfection with RX14/*dl*2208 were lost in this way. The lack of immortalising activity exhibited in this study by *dl*8 LT, which has a deletion removing amino acids 145-174 (Smolar and Griffin, 1981) suggests that the essential PyV LT residues required for immortalisation lie in the vicinity of this region, with the usual caveat that internal deletions may have wide-ranging effects on the structure of the protein. The immortalising activity of LT appears to be less sensitive to the effects of linker insertion and internal deletion than the DNA replication, transcriptional activation, and serum dependence functions of the protein. Only one mutant, *dl*8, appears to be defective in this assay, and mutants AX873, AVX1016, and AX1145, mapping to the vicinity of this deletion (see Table 2, previous chapter), retain the ability to immortalise REFs. This could reflect the lack of a precise secondary structure requirement for the putative immortalising domain (see Figure III.4), and/or the small size of this domain.

5: Serum Dependence

The results of the serum dependence assays are extremely unsatisfactory, as there is a lack of consistency between the results of individual experiments employing Balb c/3T3 lines, and between the behaviour of rat and mouse cells expressing the same mutant LT gene (see Tables 8, 9, and 10, previous chapter). The inconsistency between the results of individual experiments using LT-expressing Balb c/3T3 cell lines may be the consequence of progressive decreases in LT expression (see Section II.B) resulting in cells containing insufficient quantities of LT to reduce cellular growth factor requirements. The LT-immortalised cell lines contain higher concentrations of LT, and appear to form colonies in ox plasma at higher frequencies than the equivalent murine cell lines. This may be because there is no direct selection pressure for LT expression in the Balb c/3T3 cells, while the rat cell lines may have a continued requirement for LT to prevent senescence. Serum dependence assays employing the LT-established rat cell lines may therefore provide more meaningful data on the ability of the mutants to reduce cellular growth factor requirements. The lack of an appropriate negative control cell line is a weakness of this assay, although spontaneously-immortalised cell lines derived from REFs, for example, REF52 cells (Franza *et al.*, 1986), may be suitable for this function. It is, however, possible that the LT mutants have differential effects on the growth of rat and

mouse fibroblasts in medium containing ox plasma. The extent of the nucleolar exclusion of LT in the rat cells is rather less marked than in the Balb c/3T3 cells (see Figures II.4 and II.9), and this alteration in cellular localisation may be a factor in determining the different behaviour of the two cell types in the serum dependence assay. Much more detailed serum dependence studies must now be performed, employing several, independently-isolated, clones of LT-expressing rat and mouse cells, to ensure that consistent and meaningful results are obtained.

The serum dependence assay employed here is unreliable, as Balb c/3T3 cells are capable of growth in medium containing ox plasma, although at a lower rate, presumably in response to the low concentrations of PDGF present in plasma. The time point at which staining is performed can therefore have a major influence on the extent of colony formation, complicating the interpretation of the results of the assay, as the distinction between "background" and genuine colonies is often unclear (see the cells expressing *dl*8 in Figure II.18). This problem was also encountered by Cuzin and colleagues (Rassoulzadegan *et al.*, 1982; Mougneau *et al.*, 1984) and Armelin *et al.* (1985). Alternative assays may therefore be more reliable, for example, cell proliferation in ox plasma or defined medium containing purified growth factors could be monitored by measuring the incorporation of [^{3}H]-thymidine into cellular DNA, a method used by Kelekar and Cole (1987), among others. This assay has the additional advantage that non-established REFs can be used as negative control cells, allowing a more meaningful assessment to be made of the effects of LT on the growth of rat cells. Preliminary attempts to employ this technique in the present study were unsuccessful, however, as the Balb c/3T3 cell lines detached from the tissue culture dishes during pre-incubation in serum-free medium. This assay measures the induction of cellular DNA synthesis, rather than cellular proliferation, and it is possible that these two activities may not always be linked (see Section III.B.6).

Although the results of the serum dependence assays are clearly only preliminary, and must be confirmed, mutants AX873, AVX1016, and *dl*8 appear to be unable to reduce cellular dependence on serum growth factors. This would imply that the serum dependence function of LT resides between amino acids 66 and 197, flanked by the ACB368 and AX1145 mutations.

6: Nuclear Localisation

The LT mutant RX14/*dl*2208 has deletions removing both of the wild-type nuclear localisation signals (see Section I.C.2.x, and Table 2, previous chapter), and had previously been demonstrated to localise exclusively to the cytoplasm of

Vero cells (Richardson *et al.*, 1986). In the same study, the RX33/*dl*2208 mutant was found to localise to both the nucleus and cytoplasm of Vero cells. In contrast, the results presented here demonstrate that the RX14/*dl*2208 protein can in fact enter the nucleus of rat and mouse fibroblasts (see Figures II.6 and II.10), and that RX33/*dl*2208 localises exclusively to the nucleus in Balb c/3T3 cells (data not shown) and rat fibroblasts (see Figure II.9). There are several possible explanations for the discrepancies between the results of these two studies. For example, Richardson and colleagues employed mutant plasmids expressing all three PyV early antigens, while the plasmids utilised in this study express LT alone, and it is possible that MT and ST could influence the entry of LT into the nucleus. Another difference is that Richardson and coworkers employed Vero cells, which are derived from the kidney of an African Green Monkey, while the cells used in this study are of rodent origin. Finally, these workers utilised microinjection to introduce PyV DNA into cells, and LT expression was assayed 16 hours later, while in the present study, the mutant LT genes were introduced to Balb c/3T3 or REF cells by calcium phosphate transfection, and LT expression was assayed at least 5 weeks later, following selection for drug-resistant clones of cells.

The results presented here demonstrate that PyV LT can enter the nucleus even in the absence of both wild-type nuclear localisation signals, implying that they merely influence the rate of nuclear accumulation. Individual cells, even in an apparently homogeneous Balb c/3T3 population, display different capacities to localise RX14/*dl*2208 to the nucleus, reflecting, perhaps, a variation in the number or properties of nuclear pores. Mixed populations of cytoplasmic- and nuclear-staining cells could have arisen from cross-contamination of homogeneously-staining clones. Single-cell clones derived from a heterogeneously-staining population were all found to exhibit exclusively cytoplasmic staining (see Figure II.6), suggesting that individual clones of cells may indeed localise LT either to the cytoplasm or the nucleus (as only eight clones were examined, the lack of nuclear-staining subclones could merely represent sampling errors). Alternatively, it is possible that cells with cytoplasmic staining become the dominant members of an initially heterogeneous population upon prolonged passaging in tissue culture. This could explain the apparent drift of the RX14/*dl*2208-immortalised cell line from a predominantly nuclear to a predominantly cytoplasmic-staining phenotype (see Figure II.10). Experiments to monitor progressive changes in the intracellular distribution of LT in the RX14/*dl*2208 cell lines, together with further single cell cloning experiments, must now be performed to address these points.

As expected, all the other LT mutants used in this study localised exclusively to the nucleus of Balb c/3T3 cells (Table 3, previous chapter). None of the linker insertions employed here interrupt either of the PyV LT nuclear localisation signals, although mutants AX1145 and AX1374 map in the vicinity of the signals centred on LT residues lys^{192} and lys^{282}, respectively (see Section I.C.2.x, Table2, and Figure III.1). It is possible that either of these mutations could have disrupted the function of the adjacent nuclear localisation sequences, but this would not have been detected in this experiment as the two PyV LT nuclear localisation signals function autonomously (Richardson *et al.*, 1986). Removal of the second nuclear localisation signal from these proteins by combining the AX1145 and AX1374 mutations with the RX14 and *dl*2208 mutations, respectively, to produce the double mutants AX1145/RX14 and AX1374/*dl*2208 could allow the effects of the linker insertions on the adjacent nuclear transport signals to be assessed. Mouse 3T6 cells infected with the *dl*8 or *dl*23 viruses exhibit exclusively nuclear staining (Richardson *et al.*, 1986), a result confirmed in this study by transfection of *dl*8 and *dl*23 LT genes into Balb c/3T3 cells.

The ability of the RX14/*dl*2208 mutant protein to enter the nucleus in a significant proportion of cells precludes any attempt to assess the importance of nuclear localisation to the biological and biochemical properties of PyV LT.

Immunofluorescence analysis of LT-expressing cell lines suggested that LT may have a greater capacity to enter the nucleolus in rat cells than in Balb c/3T3 cell lines (see Figures II.4 and II.9). As this appeared to apply to all the mutant LT proteins examined, this may be a non-specific effect, reflecting the smaller size of the truncated LT species in the rat cell lines, or differences between the nucleolar localisation mechanisms of rat and mouse cells.

6: Functional Domains of PyV LT

The functional domains of PyV LT identified in this study are shown diagrammatically in Figure III.1. In the case of SV40 LT, different sub-populations of the protein, varying in their post-translational modification or oligomeric state, appear to exhibit distinct properties. PyV LT could employ similar mechanisms to extend its functional diversity, and some of the defects described here could result from alterations in post-translational modification or oligomerisation, rather than lesions in functional domains. Equally, linker insertions could produce more widespread disruption of protein structure than was anticipated, affecting functions mapping to other regions of the protein.

The phenotypes of mutants AX193 and ACB368, which map to the common region of LT (amino acids 1-79), shared with the other PyV early antigens, are

similar, if not identical to that of the wild-type LT protein. This implies that the common region has little direct involvement in the activities assayed in this study, although mutants AX193 and ACB368 map to the N- and C-termini of the common region respectively, and functional domain(s) located between them may be unaffected. AX193 appears to exhibit an enhanced ability to stimulate CAT activity from pB27BECAT, suggesting that mutations in the common region may affect functions mapping to other parts of the protein. Alternatively the common region may play a more direct rôle in transcriptional activation.

The importance of the C-terminal 120 residues of PyV LT could not be assessed in this study, as none of the mutants map to this region of the protein. Although this region is presumably not involved in immortalisation or the reduction of serum dependence, it may be important for DNA replication and/or transcriptional activation. It would therefore be of interest to create mutants in this area and analyse their effects on the properties of LT.

It appears that the DNA replication, transcriptional activation, immortalisation, and serum dependence functions of LT all map to different regions of the protein. For example, the C-terminal two thirds of LT appear to be important for transcriptional activation and DNA replication, but not for the immortalisation or serum dependence functions of PyV LT, which map to the N-terminal third of the protein. These functional domains overlap, however, and there may be mechanistic links between these activities of PyV LT, particularly the activation of the PyV late promoter and DNA replication. The ability of LT to immortalise cells seems to be dissociable from all the other functions of LT tested in this study, although there is some overlap between the putative immortalisation and serum dependence domains.

B: General Discussion

1: Mutagenesis

Of the numerous methods available for mutagenising cloned genes (reviewed by Smith, 1985; Botstein and Shortle, 1985), in-phase, linker-insertion mutagenesis is perhaps the most appropriate for the initial identification of functional domains within a protein. The mutagenesis procedure is technically straight-forward, yielding products which are easily characterised and can be used directly without reconstruction. The mutants generated here and elsewhere have a 2-4 amino acid insertion which is expected to produce a localised disruption of the three-dimensional protein structure, confining the effect to a single functional domain. Although deletion mutagenesis can also provide a great

deal of information, the removal of as many as 20-30 amino acids is likely to produce rather more wide-ranging effects on the conformation of the protein. The linker-insertion strategy does have the disadvantage that the loci of the mutants are determined by the presence of suitable restriction sites, not all of which are equally susceptible to mutation (see Table 1, previous chapter). Nevertheless, in-phase, linker-insertion mutagenesis has been employed successfully to define functional domains in a number of viral and cellular proteins, including the human glucocorticoid receptor (Giguère *et al.*, 1986), the human oestrogen receptor (Kumar *et al.*, 1987), and v-*erbB* (Ng and Privalsky, 1986). The functional domains of PyV LT identified in this study by linker-insertion mutagenesis are shown diagrammatically in Figure III.1, along with the results of earlier attempts to map the protein. Figure III.2 shows a comparison of the amino acid sequences and functional organisation of PyV LT and SV40 LT.

2: DNA Replication

The conservation of the DNA-binding, ATPase, DNA helicase, and nucleotide-binding activities of PyV LT and SV40 LT, together with the similarity of PyV and SV40 replication, *in vivo* and *in vitro*, suggests that the two LT proteins may play similar rôles in DNA replication. If PyV LT does indeed conform to the SV40 LT paradigm (described in Section I.C.2.i), the following model for replication of the PyV chromosome can be proposed. PyV LT, in conjunction with cellular proteins, binds to the viral *ori*, and promotes a localised melting of the DNA duplex. LT can then use its non-specific DNA helicase activity, in the presence of single-stranded DNA-binding proteins, to separate the DNA strands, allowing the cellular replicase to initiate DNA synthesis. Elongation, with LT acting as a processive helicase at the replication fork, can then occur. Confirmation of the involvement of DNA polymerase δ, PCNA, and other cellular proteins in PyV replication must await the further characterisation of the components of *in vitro* PyV replication systems.

Several functions of PyV LT which are required for viral DNA replication have been localised to functional domains within the protein (see Figure III.1). The DNA-binding domain of PyV LT has been identified by virtue of its structural homology to its functional counterpart in SV40 LT. Site-directed mutagenesis of this region (amino acids 277-234 of PyV LT) has generated LT proteins which lack both *ori*-specific and non-specific DNA-binding activities, and are defective in a DNA replication assay (Cowie *et al.*, 1986). Detailed knowledge of the structures of several nucleotide-binding proteins has allowed a consensus domain known as a "mononucleotide binding fold" (MNBF) to be

Figure III.2: A Structural and Functional Comparison Between PyV LT and SV40 LT.

A comparison between the amino acid sequences of PyV LT and SV40 LT is shown. Vertical lines represent identical residues identified by computer sequence comparisons performed by M. Ginsburg and M. D. Jones.

The functional domains of the two proteins are also shown.

References

PyV LT

Immortalisation: Asselin and Bastin, 1985.

DNA Binding: Cowie *et al.*, 1986.

Reduction of Serum Dependence: Rassoulzadegan *et al.*, 1982.

ATPase: Bradley *et al.*, 1987.

Nucleotide Binding: Clertant *et al.*, 1984.

Nuclear Localisation Signals: Richardson *et al.*, 1986.

SV40 LT

Livingston and Bradley, 1987.

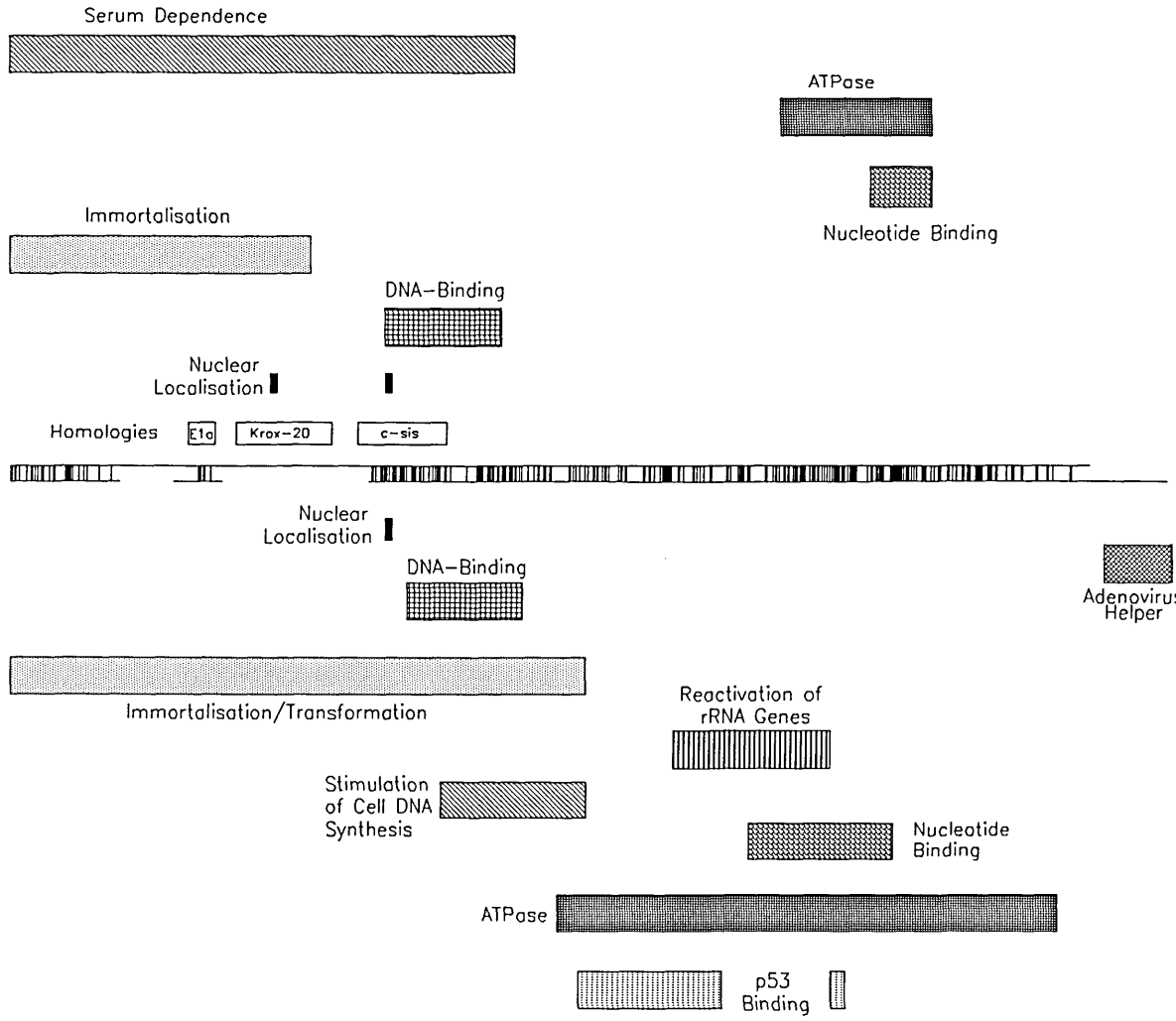
Homologies

E1a: Moran, 1988.

Krox-20: Chavrier *et al.*, 1988.

c-*sis*: Stabel *et al.*, 1985.

PyV LT



SV40 LT

identified. Computer analysis of the primary and secondary structures of PyV LT has located a nucleotide-binding domain of this type between amino acids 565 and 675 (Bradley *et al.*, 1987). The corresponding MNBF in SV40 LT appears to be important for ATPase activity, suggesting that it could represent the enzymatic active site. However, this region of SV40 LT also contains a nucleotide-binding site which is distinguishable from the ATPase active site. PyV LT also possesses a distinct nucleotide-binding site, (located between amino acids 636 and 675), and a truncated PyV LT species lacking the majority of the MNBF domain fails to bind nucleotides, yet retains ATPase activity, suggesting that the ATPase catalytic domain is located further towards the N-terminus (Clertant *et al.*, 1984). This conclusion is supported by a deletion analysis of SV40 LT which revealed the importance of the protein sequences between the *ori*-binding domain and the MNBF for ATPase activity (Cole *et al.*, 1986). The MNBF may therefore constitute a binding site for allosteric effectors. Nevertheless, point and in-phase deletion mutations mapping between amino acids 641 and 686 of PyV LT produce defects in DNA replication, and the mutant proteins can inhibit the replicative activity of wild-type LT (Nilsson and Magnusson, 1984). The DNA helicase activity has not been mapped precisely in PyV or SV40 LT, but is inhibited by monoclonal antibodies which bind to the C-terminal two thirds of SV40 LT, and requires ATPase activity (Wiekowski *et al.*, 1986). Finally, the *tsa* mutants of PyV LT, which are temperature-sensitive for DNA replication, map between amino acid 360 and the C-terminus (Ito and Griffin, 1987).

Mutations mapping to the N-terminal third of PyV LT do not normally affect viral viability, but can reduce the extent of DNA replication (Griffin and Maddock, 1979). This is exemplified by the LT genes of the *mlt* mutants *dl*1042 and *dl*2208, which exhibit a reduced capacity for DNA replication, but only in the absence of wild-type MT and ST (Nilsson and Magnusson, 1984). Another *mlt* mutant, the *dl*8 virus, grows poorly, although this could result, at least partially, from some defect in MT (Griffin and Maddock, 1979). It would be of interest to confirm and extend these observations by subjecting the panel of LT mutants generated in this study to a quantitative DNA replication assay, in the presence or absence of wild-type MT and ST. This approach could identify a domain of LT which can be complemented by MT and/or ST. Cotransfection of mutant and wild-type LT genes could also identify trans-dominant replication mutants of the type described by Nilsson and Magnusson (1984). XX2477 and XX2522, mutants generated in this study, are potential members of this category, as they map to the proposed nucleotide-binding region (Figure III.1).

The results of the replication assays in this study (Table 3, previous chapter), although incomplete, are consistent with the above data. On the basis of the

model for PyV replication presented above, and by analogy with SV40 LT, mutations affecting a number of properties of PyV LT (namely, DNA-binding; DNA helicase; ATPase; binding of, and response to, allosteric effectors; post-translational modification; and interaction with components of the cellular replication apparatus) can be predicted to affect viral DNA replication. In this study, only mutants AX193, AVX1016, and AX1232, mapping to the N-terminus of LT, were shown to retain the ability to promote DNA replication. Mutations in the C-terminal two thirds of LT (from mutant AX1374 at amino acid residue 273 to the C-terminus) were found to abolish autonomous replication, and may affect the ATPase, DNA-binding, nucleotide-binding, and, DNA helicase activities of the protein, which are believed to be located in this region (see Figure III.1). Mutant AX1374, for example, may be defective in *ori*-binding, as it contains a linker insertion in a position adjacent to the LT DNA-binding domain. However, two mutants, AX873 and AX1232, mapping outside this C-terminal region are also replication-defective. These mutations may disrupt C-terminal functions, as monoclonal antibodies binding to the N-terminal third of LT inhibit ATPase activity (Clertant *et al.*, 1984), or could affect another domain of the protein, involved perhaps in oligomerisation or interaction with cellular components of the DNA replication apparatus. Inappropriate post-translational modification of LT, disrupting normal regulation, could also affect DNA replication. For example, phosphorylation of one or both of the additional serine residues carried by mutant AX873 (see Table 2, previous chapter) could affect the replicative activity of LT. The PyV LT phosphorylation sites are normally clustered between amino acids 71 and 191 (Hassauer *et al.*, 1986), but several of the linker insertions (AX1374, ACB1809, XX2477, and XX2522) create potential sites outside this region (see Table 2, previous chapter).

The DNA-binding, ATPase, DNA helicase, and nucleotide-binding activities of LT can all be assayed *in vitro* given sufficient quantities of the protein. The level of LT expression in the Balb c/3T3 cell lines created in this study is too low to allow DNA-binding activities of the mutants to be assessed using the method of Cowie and Kamen (1984), described in Section IV.B.10 (data not shown), and is presumably also inadequate for the other assays. Although higher concentrations of protein could be obtained by expressing the mutant LT genes in bacteria, prokaryotically-expressed SV40 LT exhibits altered DNA-binding properties, presumably as a consequence of inappropriate or incorrect post-translational modification (Strauss *et al.*, 1987). Therefore, high-level expression of the mutant genes in eukaryotic cells, employing the adenovirus major late promoter (Cowie *et al.*, 1986), or a baculovirus vector (Rice *et al.*, 1988), for

example, might provide a more appropriate method of examining the nature of the biochemical lesions underlying defects in replication.

Although the replication of PyV and SV40 occurs by similar mechanisms there are some differences between the two processes, exemplified by the requirement for a transcriptional enhancer in replication of the PyV chromosome. The constraints on the proximity and orientation of the enhancer with respect to the *ori* (see Section I.B.2.i) suggests that there may be a specific interaction between the two sequences, possibly *via* the association of *ori*-bound LT and an enhancer-bound cellular protein (see Figure I.3). However, the ability of several sub-enhancer elements, and even heterologous enhancers, to substitute for the PyV enhancer implies an alternative mechanism, unless LT can interact with a number of cellular proteins. Another difference between SV40 and PyV replication is their requirement for the replicase complex from the appropriate permissive cells. As SV40 LT has been demonstrated to bind simian DNA polymerase-α (Smale and Tjian, 1986), PyV LT can be predicted to bind another, presumably non-conserved, domain of the murine replicase complex. This difference in binding specificity could explain the apparent failure of PyV LT to bind p53, a conserved cellular protein which competes with DNA polymerase-α for the same binding site on SV40 LT, and may regulate SV40 DNA replication (Gannon and Lane, 1987; Braithwaite *et al.*, 1987).

3: <u>Transcriptional Activation</u>

LT could employ a number of possible mechanisms to activate the PyV late promoter; for example, down-regulation of the competing early transcription unit appears to increase late transcription (Kern *et al.*, 1986). Binding of LT to the PyV non-coding region could alter the conformation of the chromatin, facilitating binding of the many transcription factors which interact with DNA sequences in the PyV non-coding region (see Figure I.3). The extent of transcriptional activation induced by these mechanisms would presumably increase in parallel with the intracellular concentration of LT, until the LT-binding sites in the viral non-coding region become saturated. In this study, there appears to be a direct correlation between the abilities of the LT mutants to promote DNA replication and to activate the PyV late promoter in p48.19BECAT (see Table 11, previous chapter). It is conceivable, for example, that changes in DNA conformation accompanying the binding of LT to the viral *ori* and the localised melting of the DNA helix, could potentiate the binding of transcription factors. Trans-activation could therefore be a by-product of the formation of a DNA replication pre-synthesis complex, and would be affected by the same

lesions which prevent DNA replication. Alternatively, LT-induced binding of cellular factors to the enhancer or *ori* could be required for replication of the viral chromosome, and it is possible that defects in transcriptional activation could affect DNA replication, rather than *vice versa.* For example, the cellular transcription factor NF-1/CTF, which binds to the PyV non-coding region, is also required for the replication of the adenovirus chromosome (Jones *et al.*, 1987). The fact that p48.19BECAT carries a non-functional PyV *ori* is likely to preclude widespread unwinding of the DNA helix, but the strand separation normally induced by LT could allow access for sequence-specific, single-stranded DNA-binding proteins, one of which binds to the 21bp repeats of SV40 (Gaillard *et al.*, 1988).

Although LT is able to stimulate gene expression by binding to specific DNA sequences, a binding-defective LT mutant can still activate the PyV late promoter (Kern *et al.*, 1986). Similarly, LT induces a small, but reproducible, increase in CAT expression from pB27BECAT (see Table 6, previous chapter), a target plasmid lacking all three major LT-binding sites, and one minor site (Figure II.12). Minor sites 2 and 3 are retained in this construction, but LT appears to employ an alternative mechanism, independent of DNA-binding, to activate the PyV late promoter, as well as heterologous promoters lacking LT-binding sequences. A number of transcriptional-activating proteins, including adenovirus E1a, SV40 LT, and c-*myc*, are capable of operating in the absence of sequence-specific DNA-binding. Deletion analysis of E1a-inducible promoters has indicated that trans-activation is mediated through the binding of cellular transcription factors (Berk, 1986; Nevins, 1986; 1987), and E1a has been proposed to act, *via* chemical modification or direct physical interaction, by stimulating the incorporation of these factors into transcription complexes (Reichel *et al.*, 1988). Similarly, SV40 LT appears to activate its late promoter by enhancing the binding of cellular factors (Gallo *et al.*, 1988), and although equivalent experiments have not been performed for PyV LT-inducible promoters, similar mechanisms of action could be proposed. It is interesting to note that amino acids 160-234 of PyV LT display a marked similarity to part of a putative murine transcription factor, Krox-20 (see Figure III.3), which is induced during G_0/G_1 transition (Chavrier *et al.*, 1988). Krox-20 is one of a number of murine genes identified by virtue of their homology to the zinc-finger domain of the Drosophila melanogaster "gap" gene, Krüppel. This homology is confined to the zinc-finger motif, a conserved DNA-binding domain found in a number of prokaryotic and eukaryotic proteins (Klug and Rhodes, 1987; Evans and Hollenberg, 1988), including the transcription factor Sp1, which binds to the 21bp repeats of SV40 (Kadonaga *et al.*, 1988). The section of

Figure III.3: Structural Relationship Between PyV LT and Krox-20 Polypeptides.

A: The amino acid sequences of PyV LT and the putative murine transcription factor Krox-20 are compared. An asterisk denotes identical residues or conservative changes. The position of LT mutants mapping to this region are also shown. Adapted from Chavrier *et al.* (1988).

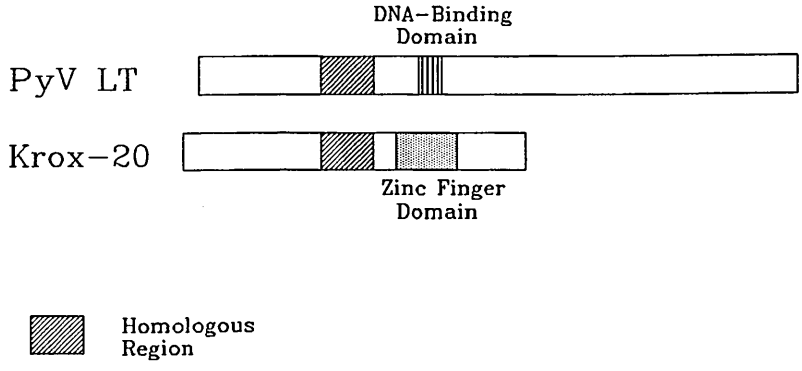
B: The relationship between the homologous region and the respective DNA-binding domains of the two proteins is shown.

A

```
180 TVPAGPDRKPFPC---PLDSLRVPPPLTPLSTIRNFTLGGPGAGVTG Krox-20
    * *  ****  **   * ***   ** **** *    ** ***  *
160 TPAHTAGRRRNPCVAEPDDSISPDPPRTPVSRKRPRPAGATGGGGGG PyV LT
                                        -
                                      AX1145
                                      ----------
                                         dl23

224 PGASGGGEGPRLPGSGSAAVTATPYNPH Krox-20
      * ***   *  ** * *  * *** *
207 VHANGGSVFGHPTGGTSTPAHPPPYHSQ PyV LT
                      -
                    AX1232
    ----------------------
        dl23
```

B

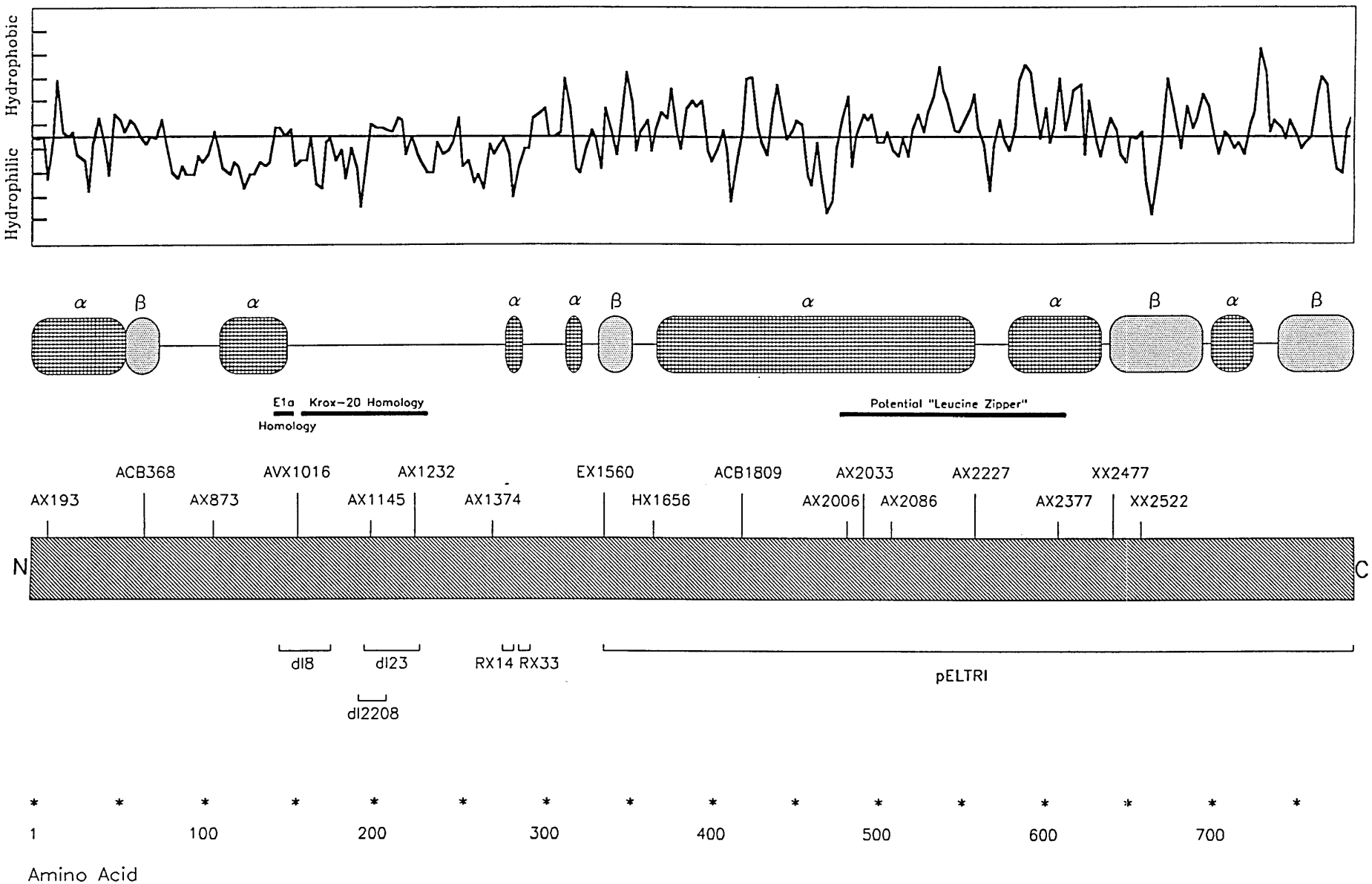


Krox-20 bearing homology to PyV LT lies outside the zinc-finger domain, but the spatial relationship between this conserved region and the DNA-binding domain is maintained in PyV LT (see Figure III.3). It is tempting to speculate that PyV LT has acquired a functional domain from Krox-20, allowing it to mimic the activity of a cellular transcription factor, possibly by interacting with other components of the transcription apparatus. The replacement of the zinc-finger region of Krox-20 with the DNA-binding domain of PyV LT would presumably alter the spectrum of promoters interacting with this factor. The increased abilities (in comparison to pELT) of AX193 and AX1145 to stimulate CAT expression from pB27BECAT could therefore reflect an enhanced affinity of these LT molecules for cellular proteins. The AX1145 linker insertion maps within the Krox-20 homology (see Figure III.3), introducing an additional charged arginine residue (see Table 2) into an already hydrophilic region (see Figure III.4), and this may alter the conformation of LT, perhaps making the Krox-20 region more accessible to cellular proteins. The AX193 mutation, produces a smaller, and possibly less significant effect, and this could either reflect long-range effects on the conformation of the Krox-20 region, or, perhaps, the existence of an additional site of interaction with cellular proteins. Although the region of PyV homologous to Krox-20 contains a number of acidic residues it does not appear to constitute an "acid blob", a transcriptional activator domain, originally identified in the yeast protein GCN4, which is believed to mediate the interaction between individual components of a transcription complex (Hope *et al.*, 1988). Nevertheless, the mutants AX1232, *dl*8, and *dl*23, and *dl*2208 which map in the vicinity of this conserved region (see Figures III.1 and III.3), are all at least partially impaired in their ability to trans-activate the PyV late promoter in pB27BECAT (see Table 7, previous chapter), implying that LT sequences bearing homology to Krox-20 are indeed involved in transcriptional activation. Mutant *dl*2208, which has a deletion removing amino acids 191-209 (Nilsson *et al.*, 1983), is capable of stimulating CAT expression from pB27BECAT, although at a reduced level compared to pELT, suggesting that amino acid residues N-terminal to amino acid 191 may represent the putative site of interaction with cellular proteins. As mutations mapping to the C-terminus of LT also affect the ability of the protein to increase CAT expression from pB27BECAT (see Tables 6 and 7, previous chapter), it is possible that this alternative mode of trans-activation may require ATPase and/or nucleotide-binding activities. Dissociation of a LT/transcription factor complex is likely to be an energy-dependent process, and lesions affecting ATP hydrolysis could therefore prevent complex turnover, leading to the sequestration of transcription factors by LT. This could explain the inhibition of late promoter

Figure III.4: Hydropathicity Plot and Predicted Secondary Structure of PyV LT.

The hydropathicity profile of PyV LT, and the predicted secondary structure of the protein (using the rules of Chou and Fasman, 1978) are shown. The predicted α-helix (α) and β-pleated sheet (β) structures are indicated, together with the loci of the LT mutants (Table II.2), with respect to the amino acid sequence of the protein. The locations of the E1a homology (Stabel *et al.*, 1985; Moran, 1988), the Krox-20 homology (Chavrier *et al.*, 1988), and the putative "leucine zipper" are shown.

Computer analysis (employing the programs of R. Staden) was performed by M. Ginsburg.


Hydrophobic
Hydrophilic
α β α α α β α α β α β
E1a Homology
Krox-20 Homology
Potential "Leucine Zipper"
ACB368
AVX1016
AX1232
EX1560
ACB1809
AX2033
AX2227
XX2477
AX193
AX873
AX1145
AX1374
HX1656
AX2006
AX2086
AX2377
XX2522
N
C
dl8
dl23
RX14 RX33
pELTRI
dl2208
1
100
200
300
400
500
600
700
Amino Acid

activity induced by several of the LT mutants (AX2006, AX2033, XX2477, and XX2522). An alternative explanation is suggested by the observation that amino acids 480-610 of PyV LT contain a number of leucine residues spaced at 7 or 14 residue intervals, and are predicted to adopt an α-helical conformation (see Figure III.4). This region may therefore constitute a "leucine zipper", a hypothetical structural motif which is proposed to allow the association of proteins to form homo- or hetero-oligomers (Landschultz *et al.*, 1988). Mutations in the C-terminal region of LT could disrupt the leucine zipper, impairing the formation of complexes, thus abolishing transcriptional activation (mutants AX2086 and AX2227), or preventing their dissociation, which could again lead to the inhibition of late promoter activity (mutants AX2006 and AX2033). A transactivation-defective E1a mutant has, in fact, been shown to block the stimulatory effects of the wild-type protein on adenovirus gene expression (Glenn and Ricciardi, 1987), and this could represent another example of transcription factor sequestration. Although the Krox-20 homology is not exhibited by SV40 LT (see Figure III.1), the latter protein has been shown to bind to the AP-2 (activator protein-2) transcription factor (Mitchell *et al.*, 1987).

MT also exerts a significant stimulatory effect on the PyV late promoter, although this must involve an indirect mechanism, given the extranuclear location of the protein. Another membrane-bound oncogene, EJ-*ras*, activates the PyV enhancer *via* increased binding of the AP-1 (activator protein-1) transcription factor complex (Wasylyk *et al.*, 1987; Imler *et al.*, 1988). The p21 *ras* gene product is a putative transducer protein (Hanley and Jackson, 1987) which may couple growth factor receptors to components of the phosphatidylinositol (PI) intracellular signalling pathway (see Figure I.6), and *ras*-induced activation of AP-1 appears to be mediated through protein kinase C (PKC). This represents another manifestation of the complex interplay between PyV and PI metabolism (see Sections I.C.3.i and ii). The association of MT with a cellular PI kinase suggests that PI metabolism may be stimulated or deregulated in some, currently undefined, way in PyV-infected or transformed cells. If MT does, in fact, induce an increase in mitogenic flux through the PI pathway the resultant stimulation of PKC could phosphorylate and activate MT (a substrate of PKC, see Section I.C.3.i), potentially creating a positive feedback loop. The autocrine or paracrine effects of TGFs secreted by PyV-transformed cells could produce an even greater stimulation of PI metabolism. In fact, several of the genes activated by PyV early antigens, including *hsp*70, metallothionein, and the PyV transcription units, are also induced by serum growth factors and activators of protein kinase C (Wu *et al.*, 1986; Imbra and Karin, 1987; Imler *et al.*, 1988). It is therefore possible that at least some of the effects of PyV on gene expression are a

secondary effect of TGF production. For example, transcription of both c-*fos* and c-*jun*, which encode components of the AP-1 transcription complex, is induced by serum (Rauscher *et al.*, 1988). The promiscuous transactivation of gene expression by PyV MT could therefore reflect the ability of that protein to induce growth factors, which stimulate the growth rate of the cell, and increase the production of transcription factors. These factors may correspond to the "permissivity factors" induced by MT and/or ST, postulated by Benjamin (1982), to account for the restricted growth range of *hrt* mutant viruses (see Section I.C.1.iii). Transcription factors may then be subjected to post-translational activation by MT, possibly *via* a protein phosphorylation mechanism, and may also interact with LT. A mechanism of this type, would, in addition to stimulating PyV gene expression, result in a generalised up-regulation of cellular transcription, with potentially dramatic effects on cell growth and behaviour. It should be noted that TPA, an analogue of diacylglycerol, the physiological activator of PKC (see Figure I.6), is able to substitute for MT by co-operating with PyV LT in a rat embryo fibroblast transformation assay (Connan *et al.*, 1985). Variations in the degree of transcriptional activation induced by the LT mutants when p48.19BECAT was used as the target plasmid (see Table 5, previous chapter) may reflect differences in cellular growth rates between individual experiments, producing fluctuations in the availability of transcription factors. The comparative reproducibility of the results when pB27BECAT is used as the target plasmid (see Table 6, previous chapter) may either reflect the absence of MT (pB27BECAT lacks the PyV early region promoter and N-terminal T-antigen coding sequences, see Figure II.12), or the deletion of binding sites for growth-regulated transcription factors. Variations in the growth rates of different populations of NIH3T3 cells could also explain the reduced effect of wild-type LT, in comparison to the results of Kern *et al.* (1986), on the PyV late promoter in p48.19BECAT, but not pB27BECAT, observed in this study.

As stimulation of the mouse α2(I)-collagen promoter by TGF-β is directed through an NF-1/CTF binding sequence (Rossi *et al.*, 1988), it may be significant that initiation of early region transcription at later times in PyV infection, when the infected cells are producing TGFs, occurs downstream from NF-1 binding sites (see Figures I.2 and I.3). Although TGFs can stimulate the growth of fibroblasts they also have potent growth-inhibiting effects on other cell types, and can inhibit fibroblast growth, in combination with other factors (Massagué, 1987; Sporn and Roberts, 1988). The effect of TGFs on the transcription of PyV genes may therefore depend on cell type as well as the variety of factors acting on the cell, and this may be a factor in determining the

persistence of PyV. Clearly the activation of the PyV late promoter is a complex phenomenon involving the direct and indirect effects of LT and MT.

4: Immortalisation

The ability of pELTRI to immortalise early passage rat embryo fibroblasts (Table 4, previous chapter) confirms the earlier observation that the immortalising activity of PyV LT resides in the N-terminal 40% of the protein (Rassoulzadegan *et al.*, 1983b; Land *et al.*, 1983). In fact a truncated gene encoding the first 219 amino acids of LT has been reported to possess immortalising activity, albeit of reduced efficiency (Asselin and Bastin, 1985). The results of the present study support this conclusion, as mutants AX1232 and AX1374, mapping outside this region, appear to retain immortalising activity. In fact, only the N-terminal 190 amino acids may be required for immortalisation, as mutants *dl*23, lacking amino acids 195-228 (Smolar and Griffin, 1981), and mutants *dl*2208, RX14/*dl*2208, and RX33/*dl*2208, lacking amino acids 191-209 (Nilsson *et al.*, 1983) are also able to promote cellular establishment. The lack of immortalising activity exhibited in this study by *dl*8 LT, which has a deletion removing amino acids 145-174 (Smolar and Griffin, 1981), is consistent with this localisation, although the deletion may have affected the structure of another region of the protein. Even though *dl*8 LT appears to lack immortalising activity, the *dl*8 virus is at least as tumorigenic in nude mice as the prototypic A2 strain of PyV (Griffin and Maddock, 1979). Although there is an inherent difficulty associated with the demonstration of a negative result, the implication is that an immortalisation-defective LT can complement the other PyV early antigens in tumorigenesis. As the deletion in the genome of the *dl*8 virus also affects MT (*dl*8 is a *mlt* mutant - see Figure I.4), it is possible that *dl*8 MT has altered properties, and could compensate for the immortalising defect in LT. Certainly, over-expression of wild-type MT can induce cellular transformation in the absence of the other PyV early antigens (Spandidos and Riggio, 1986). Therefore *dl*8 LT, and the other LT mutants used in this study, must be tested in an oncogene co-operation transformation assay along with wild-type MT and ST, or an activated *ras* gene, to allow a more controlled assessment of their oncogenic potential. Interestingly, mutants of E1a display differential abilities to co-operate with MT or an activated *ras* gene in this assay (Zerler *et al.*, 1986).

If *dl*8 LT is indeed able to contribute to full transformation without displaying immortalising activity, this would represent an additional contribution to the growing body of evidence disputing the rôle of immortalisation in the development of cancer. For example, the REF52 cell line, and another rat line

established by a mutant p53 gene, are both refractory to transformation by EJ-*ras* (Jenkins *et al.*, 1985; Franza *et al.*, 1986). REF52 cells, although an established line, behave like primary REFs in an oncogene co-operation assay, and can only be transformed by a combination of EJ-*ras* and an "immortalising" gene such as adenovirus E1a. Conversely, mutants of E1a are able to co-operate with activated *ras* genes in an *in vitro* transformation assay, yet fail to immortalise primary rodent cells (Schneider *et al.*, 1987; Kuppuswamy *et al.*, 1988). These data suggest that oncogene co-operation and immortalisation are separable activities, and that *in vitro* establishment is not necessarily a prerequisite for transformation.

Immortalisation and senescence (reviewed by Pontén, 1985) are terms, defined *in vitro*, which are used to describe the ability of cells to survive in tissue culture when removed from their normal environment. It is not clear that the proliferative potential of cells is necessarily limited *in vivo*, although many cells do undergo terminal differentiation and cease to divide. Spontaneous immortalisation, particularly of cells from higher mammals, is a rare event, and it is generally observed that only a minor population of cells from a subset of tumours can be propagated indefinitely *in vitro*. It is possible that senescence could merely reflect the failure of cells to adapt to the alien conditions employed for tissue culture, rather than any intrinsic lack of proliferative potential. Immortal cells could represent rare variants with the ability to remain viable and divide in this environment, and the so-called immortalising genes may simply allow the cells expressing them to adapt to the prevailing conditions, perhaps by mediating the cellular response to positive factors present in foetal calf serum or by preventing the effects of growth-inhibitory factors. Although cellular immortalisation is a fascinating problem in itself, the activity of the mutant LT genes in an oncogene co-operation assay, although clearly another artificial and over-simplified system, may provide a more meaningful insight into the contribution of LT to oncogenesis.

5: Serum Dependence

The results of the serum dependence assays performed in this study are extremely inconsistent, and must be confirmed by additional experiments before any firm conclusions can be formed. Nevertheless, the preliminary results presented here did suggest that mutants AX873, AVX1016, and, possibly, *dl*8, which map within a 70 amino acid region of LT (see Table 2, previous chapter, and Figure II.3) may be unable to reduce cellular dependence on serum growth factors, and future studies should address this possibility. Although the data

presented here are far from convincing, the possible implications of this potential localisation will be discussed below, and in the following section.

Expression of a deregulated c-*myc* gene allows indicator cell lines to respond to low concentrations of a variety of growth factors (Sorrentino *et al.*, 1986; Stern *et al.*, 1986; Leof *et al.*, 1987). PyV LT displays a similar ability to sensitize PyV-transformed cells to the stimulatory effects of MT-induced transforming growth factors (TGFs) (Kaplan and Ozanne, 1982). Although these factors have not been characterised, the mitogenic effects of a combination of TGF-α and -β could account for the ability of PyV-transformed cells to grow in the absence of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). By proposing that LT amplifies the intracellular response to TGFs induced by MT, it is possible to reconcile the apparently contradictory data (described in Section I.C.2.iv.a) pertaining to the effects of LT and MT on the serum dependence of established cells. Thus, in the study of Armelin *et al.* (1985), LT expression could allow Balb c/3T3 cells to grow in medium containing platelet-poor plasma (PPP) by amplifying the response to the low concentrations of PDGF that it contains. In contrast, MT could produce the same effect by inducing TGF-β, which in turn activates expression of c-*sis*, encoding PDGF (Leof *et al.*, 1986). The growth of FR3T3 cells cultured in 0.5% serum is limited by the availability of PDGF, EGF, and insulin-like growth factor-1 (IGF-1), but expression of LT could promote cell division (Rassoulzadegan *et al.*, 1982) by sensitizing the cells to all three growth factors. In the same study, MT had no effect, as induction of TGFs by MT can only circumvent the requirement for EGF (TGF-α mimics the action of EGF) and PDGF, but not IGF-1. However, when IGF-1 is provided as the only serum growth factor, MT is able to promote the growth of NIH3T3 cells (Kaplan and Ozanne, 1982). Although LT does not allow NIH3T3 cells to grow in platelet-poor plasma (Cherington *et al.*, 1986), this cell line displays an unusually low requirement for PDGF, and behaves anomalously in PPP (Armelin *et al.*, 1985).

This hypothesis is also consistent with the mitogenic effects of PyV infection on Balb c/3T3 cells (Zullo *et al.*, 1987). The second phase of "competence" gene expression following the initial response to capsid binding is dependent on MT and ST, but not LT expression (see Section I.C.2.iv.a). The effects of MT could again be mediated through the induction of c-*sis* expression by TGF-β, and LT would be expected to operate in parallel to, or downstream from, the "competence" genes. The combined effects of the T-antigens and VP1 induce quiescent cells to re-enter the cell cycle and reassemble their transcriptional and DNA replication machinery, providing the optimal environment for viral reproduction. This represents a common strategy employed by several tumour

viruses, including SV40, adenovirus, and Epstein-Barr Virus (Spindler *et al.*, 1985; Gordon *et al.*, 1986).

The ability of c-*myc* to sensitize cells to the effects of a variety of growth factors has led to the suggestion that *myc* may be a component of a number of intracellular signalling pathways. The protein could possibly act as a transcription factor, modulating gene expression in response to growth factor stimulation, and it is conceivable that LT could act in a similar manner, mediating the nuclear response to TGFs induced by MT. This hypothesis is particularly attractive in view of the homology between LT and Krox-20 (see Figure III.3), one of a number of putative cellular transcription factors induced by serum stimulation (Sukhatme *et al.*, 1988; Ryder *et al.*, 1988). There is, however, no obvious correlation between the ability of the LT mutants to activate CAT expression from pB27BECAT or p48.19BECAT and to reduce the serum dependence of Balb c/3T3 cells (see Table 11, previous chapter). For example, mutant pELTRI, or an equivalent fragment of LT (Rassoulzadegan *et al.*, 1982; Mougneau *et al.*, 1984), reduces the serum requirement of cells, but pELTRI has an inhibitory effect on CAT expression from pB27BECAT. Intriguingly, mutant AX873 may be defective both in the ability to reduce serum dependence and to promote viral DNA replication, suggesting the existence of a mechanistic link between the two processes. This possibility is reinforced by the observation that cells expressing high levels of the c-*myc* protein can support limited SV40 DNA replication in the absence of SV40 LT (Iguchi-Ariga *et al.*, 1987a; Classon *et al.*, 1988). Similarly, *myc* is able to substitute for PyV LT in promoting viral replication (M. Strauss, personal communication) and has also been proposed to participate in cellular DNA synthesis, although this remains a matter of some controversy. Reduction of endogenous c-*myc* expression by antisense RNA or antisense oligonucleotides has been reported to inhibit the growth of premonocyte cell lines, and to prevent G_1/S, but not G_0/G_1, transition in T-cells (Yokoyama and Imamoto, 1987; Heikkila *et al.*, 1987; Wickstrom *et al.*, 1988). However, inhibitory effects of α-*myc* monoclonal antibodies on DNA replication in whole cells, isolated nuclei, and cellular extracts (Studzinski *et al.*, 1986; Iguchi-Ariga *et al.*, 1987b) are not observed in all laboratories, and inhibition may be the result of contamination by inhibitors of DNA polymerases (Kaczmarek *et al.*, 1986; Gutierrez *et al.*, 1987; 1988). *Myc* has also been reported to bind to putative cellular *ori* sequences (Iguchi-Ariga *et al.*, 1987b).

The adenovirus E1a genes share the ability of PyV LT, SV40 LT and c-*myc*, to reduce the requirement of established cells for PDGF (Braithwaite *et al.*, 1983; Stabel *et al.*, 1985; Spindler *et al.*, 1985; Oda *et al.*, 1986). Transformation by the adenovirus E1 region allows human embryonic kidney cells to support replication

of an SV40 plasmid, even though LT expression is extremely low, and appears to be antagonistic to DNA replication (Lebkowski *et al.*, 1985; Lewis and Manley, 1985). The limited expression of SV40 early genes in these cells may result from the ability of E1a to repress expression from SV40 and PyV promoters (Borrelli *et al.*, 1984; Velcich and Ziff, 1985; Velcich *et al.*, 1986). E1a also stimulates the expression of adenovirus early genes, and defects affecting this function severely limit viral growth, although E1a-defective adenoviruses do express their early genes in murine teratocarcinoma cell lines (EC cells), implying that these cells contain an E1a-like activity (Imperiale *et al.*, 1984). When induced to differentiate, EC cells fail to support early gene expression by E1a-defective adenoviruses. Differentiation of EC cells is accompanied by the loss of constitutive expression of *hsp*70, a gene activated by E1a (Nevins, 1982), and the disappearance of cellular transcription factors which bind to E1a-inducible adenovirus early promoters (Reichel *et al.*, 1987; La Thangue and Rigby, 1987).

EC cells, unlike their differentiated derivatives, are not permissive for PyV infection, as a result of a transcriptional block to PyV early gene expression (Amati, 1985). PyV mutants which are capable of growth in undifferentiated EC cells have mutations or rearrangements in the non-coding region rendering them resistant to transcriptional repression by adenovirus E1a (Hen *et al.*, 1986), suggesting that the failure of wild-type viruses to reproduce in EC cells could result from the repressive effects of a cellular E1a-like activity. However, some of these non-coding region mutations also create an additional binding site for the positively-acting transcription/replication factor, NF-1/CTF (Kovesdi *et al.*, 1987; Satake *et al.*, 1988; Tseng *et al.*, 1988). Although viral growth is prevented, PyV DNA is, in fact, capable of replication in undifferentiated EC cells, but this ability is lost on differentiation (Dandolo *et al.*, 1984).

Therefore c-*myc*, E1a, and a putative cellular E1a equivalent may be able to substitute, at least partially, for PyV LT and SV40 LT in the promotion of viral DNA replication. Although further studies are necessary, the phenotype of mutant AX873 (and possibly *dl*8, which may have at least partial defects in DNA replication and the reduction of serum dependence) suggests that the shared ability of *myc*, E1a, SV40 LT, and PyV LT to reduce cellular growth factor requirements may underly their common participation in viral DNA replication. This effect is likely to be non-specific, the consequence of a generalised up-regulation of the capacity of the cell to replicate DNA. However, mutant AVX1016, which may also be defective in the serum dependence assay is still capable of promoting viral DNA replication, although the possible inabilities of mutants AX873 and AVX1016 to promote cell growth in ox plasma could result from different lesions (see next section). The reduction of cellular serum

dependence by E1a can be dissociated from the ability of the protein to activate gene expression (Zerler *et al.*, 1987), and this may also be the case for PyV LT, as mutant AX1374, for example, fails to activate the PyV late promoter, but appears to allow rat cells to proliferate in medium containing ox plasma. PyV LT and E1a may therefore act by protein-protein interaction, perhaps facilitating the association of DNA with the replicative machinery. Clearly, PyV and SV40 LT have acquired additional functions, including *ori*-binding and DNA helicase activities, which allow them to participate directly and specifically in the replication of their respective viral chromosomes. This is illustrated by the observation that replication of PyV DNA is subject to cell-cycle constraints in the absence of LT, but not in its presence (Dandolo *et al.*, 1984).

6: A Possible Conserved Domain In PyV LT, SV40 LT, Myc, and E1a.

A structural basis for the common ability of PyV LT, SV40 LT, E1a, and *myc* to promote viral DNA replication and reduce cellular dependence on serum growth factors is suggested by the observation that these four proteins, along with other papovavirus LTs, share the short amino acid sequence shown in Figure III.5 (Ralston and Bishop, 1983; Stabel *et al.*, 1985). In the case of E1a, this homologous sequence is located within domain 2 (see Figure III.5), one of three regions conserved between the E1a proteins of different adenovirus serotypes (Moran and Matthews, 1987). Domains 1 and 2 are both required for co-operation with an activated *ras* gene in transformation of BRK cells, but act autonomously, and function in this assay even when expressed from separate vectors (Moran and Zerler, 1988; Whyte *et al.*, 1988a). The induction of cellular DNA synthesis by E1a requires domain 1, although mutations and deletions affecting domain 2 delay the onset of S-phase. Lesions in domain 2 also prevent adenovirus-induced stimulation of mitosis and cellular proliferation, and affect the ability of E1a to immortalise primary rodent cells and repress gene expression (Lillie *et al.*, 1986; Zerler *et al.*, 1987; Moran and Zerler, 1988). Domain 2, and its homologues, is a potential target for casein kinase II (CKII), a cellular protein kinase activated by hormones and growth factors (Kuenzel *et al.*, 1987; Sommercorn *et al.*, 1987). Purified CKII does in fact phosphorylate SV40 LT, on ser^{106} and ser^{112}, *in vitro* (Grässer *et al.*, 1988). Domain 3, unique to the 289aa E1a protein, is responsible for transcriptional activation, and a synthetic peptide representing this region is capable of activating the E1a-inducible adenovirus E3 promoter (Lillie *et al.*, 1987).

When amino acids 101-118 of SV40 LT are substituted for domain 2 of E1a the resulting chimeric protein is active in immortalisation and *ras* co-operation,

Figure III.5: Structural Relationships Between SV40 LT, PyV LT, E1a and c-*myc* Polypeptides.

A: The amino acid sequences of SV40 LT, PyV LT, c-*myc* (murine) and adenovirus E1a (from adenovirus serotypes 2 and 5) are compared. Serine residues representing potential phosphorylation sites for casein kinase II are underlined. Asterisks mark identical residues. The location of the PyV LT mutants AVX1016 and *dl*8 are also shown.
Adapted from Moran (1988) and Figge *et al.* (1988).

B: The combined primary and secondary structure consensus for the polypeptides shown in panel A are shown. X represents a spacer of 1-8 amino acids, often containing prolines and serines. Adapted from Figge and Smith (1988).

C: Domains of E1a conserved between the E1a proteins of different serotypes of adenovirus are shown. The E1a sequence shown in panel A is that of domain 2. Adapted from Moran and Mathews, (1987).

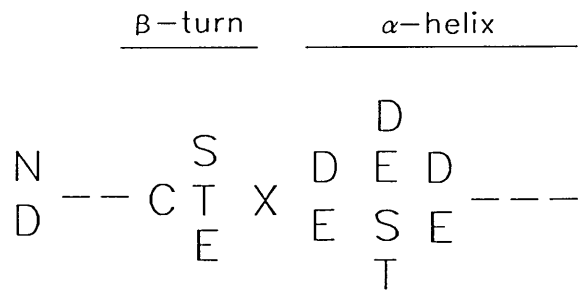
A

dl8

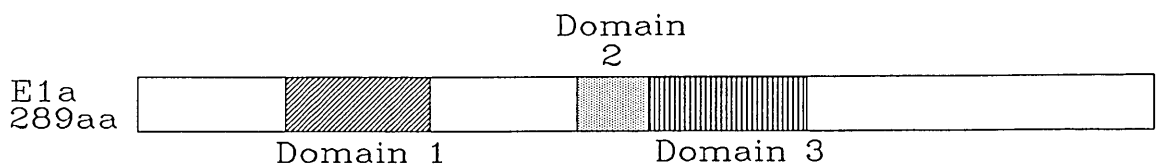
AVX1016

PyV LT	141	**DLFCYEEPLLSPNPSSPT** 158
Ad 2/5 E1a	121	**DLTCHEAGFPPSDDEDEEG** 139
SV40 LT	101	**ENLFCSEEM-PSSDDEATA** 118
c-*myc*	342	**CSSPRSSDTEEND** 354

B



C



and is also able to induce cellular DNA synthesis and proliferation, indicating functional as well as structural conservation (Moran, 1988). This E1a/SV40 LT chimera also associates with two cellular proteins, of molecular weight 105 and 107kD, whose binding to native E1a is disrupted by mutations or deletions affecting domain 2. The smaller of these proteins, which also associates with wild-type SV40 LT (DeCaprio *et al.*, 1988), has been identified as *Rb*, a nuclear phosphoprotein with potential DNA-binding activity, which is the product of the putative retinoblastoma anti-oncogene (Lee *et al.*, 1987; Whyte *et al.*, 1988b). The retinoblastoma anti-oncogene is one of a number of possible tumour-suppressor genes which have been proposed to limit the proliferation of cells (reviewed by Sager, 1986; Klein, 1987). Inactivation of members of this class of genes, by mutation or deletion, may lead to uncontrolled cell growth, and titration of *Rb* by E1a or SV40 LT could produce a similar effect. Although the mode of action of *Rb* has not been defined, it may be significant that retinoblastoma cells lack receptors for TGF-β and are not susceptible to the growth-inhibitory effects of that factor (Kimchi *et al.*, 1988).

Computer comparisons using a combination of the primary and predicted secondary structures of the conserved region shown in Figure III.5 have identified a similar structural motif in the cdc25 cell cycle control protein of the fission yeast *Schizosaccharomyces pombe* (Figge *et al.*, 1988; Figge and Smith, 1988). The cdc25 protein induces mitosis in *S. pombe* by activating cdc2, a protein kinase controlling G_2/M transition, which is conserved in higher eukaryotes as maturation-promoting factor (MPF), a mitotic inducer (Hayles and Nurse, 1986; Lee and Nurse, 1987; Robertson, 1988). Although their structural link to cdc25 is tenuous, there is some evidence to suggest that E1a, SV40 LT, and c-*myc* do indeed stimulate entry into mitosis. The control of mammalian fibroblast growth by serum factors is normally confined to the G_0/G_1 phase of the cell cycle, but a *ts* mutant rat fibroblast cell line becomes arrested in G_2 at the restrictive temperature in the absence of serum (Zaitsu and Kimura, 1988). This defect can be complemented by the addition of serum, or a combination of EGF and PDGF, or by infection with wild-type SV40, or a mutant derivative which fails to express ST. Another rat fibroblast cell line, REF52, becomes arrested in late S-phase or G_2 when transfected with an activated *ras* gene, but continues to cycle when expressing a combination of *ras* and SV40 LT (Hirakawa and Ruley, 1988). Similarly *ras* induces rat Schwann cells to become arrested in G_1 or G_2, but cointroduction of *ras* with SV40 LT, c-*myc*, or E1a allows the cells to continue to proliferate (Ridley *et al.*, 1988). Although these studies did not attempt to define the regions of SV40 LT, c-*myc*, and E1a required for

complementation of the cell-cycle defects, domain 2 of E1a has, as described above, been shown to stimulate entry into mitosis.

In *S. pombe*, cdc25 is one of at least three proteins modulating the activity of cdc2 in a dosage-dependent manner, employing a mechanism which may involve protein-protein interaction (Russell and Nurse, 1986; 1987). Thus E1a and SV40 LT could potentially induce G_2/M transition by mimicking the behaviour of a mammalian equivalent of cdc25 and titrating a growth-inhibitory protein, conceivably the retinoblastoma anti-oncogene product. This interaction could possibly be regulated by phosphorylation by CKII. In fission yeast, cdc2 also controls entry into S-phase at "start", a point in G_1 which may be equivalent to the restriction point, R, in the mammalian cell cycle (see Figure I.4). Although domain 2 of E1a, and its homologues, also stimulates cellular DNA synthesis, cdc25 does not appear to regulate the activity of cdc2 in G_1, at least in yeast.

Although amino acids 141-158 of PyV LT are homologous to domain 2 of E1a and contain potential CKII phosphorylation sites (see Figure III.3), the predicted secondary structure of this region deviates from the consensus described , as an α-helix is not encountered until some distance toward the C-terminus of the protein (Figure III.4), although the significance of this secondary structure has not been assessed. However, PyV LT mutants AX873, AVX1016 and *dl*8, which map within, or close to, this conserved region (see Figure III.1), may be impaired in the ability to promote the growth of Balb c/3T3 cells in medium containing ox plasma, suggesting that these sequences do in fact alter cellular growth factor dependence. Experiments to assess the ability of amino acids 141-158 of PyV LT to substitute for domain 2 of E1a, to bind *Rb*, and to stimulate G_2/M transition must now be performed.

Mutants AX873, AVX1016, and *dl*8 exhibit distinct phenotypes, with different abilities to promote DNA replication, immortalise cells, and transactivate the PyV late promoter (see Table 11, previous chapter), implying that this small region of PyV LT, in common with its E1a equivalent, may be important for a number of independent functions, including immortalisation and the reduction of serum dependence. This could reflect the existence of multiple species of LT, perhaps phosphorylated at different residues within this region, or, possibly, interactions with different cellular proteins. Site-directed mutagenesis of this region of PyV LT could lead to the dissociation of these different functions. By analogy with E1a, the ability of LT to induce cellular proliferation in ox plasma could involve a combination of the stimulatory effects on G_1/S and G_2/M transition. The apparent inabilities of mutants AX873 and AVX1016 to promote cell growth in ox plasma could therefore result from defects in the induction of G_1/S and G_2/M transition, respectively, explaining

their different abilities to promote viral DNA replication. If this was indeed the case, the two mutants would be expected to complement each other and promote cell growth in ox plasma.

A deletion analysis of SV40 LT (Soprano *et al.*, 1983) identified amino acids 160-272 of that protein as important for the induction of cellular DNA synthesis (see Figure III.2). Although it is possible that the deletions affected the structure adopted by amino acids 101-118 (the E1a domain 2 homology), Stabel *et al.* (1985) noted an homology between amino acids 224-269 of SV40 LT and a region of PDGF. This homology is also exhibited by amino acids 376-419 of PyV LT (see Figure III.6), and it would be of interest to analyse the effects of mutations mapping to this region (EX1560, HX1656, and ACB1809) on the ability of LT to reduce serum dependence.

7: Nuclear Localisation

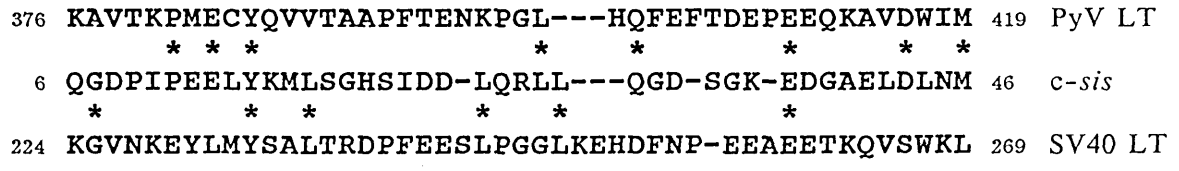
As described earlier in this chapter, the intracellular localisation displayed by LT mutants RX14/*dl*2208 and RX33/*dl*2208 in this study differs from that observed by Richardson *et al.* (1986). This could reflect the use of different experimental techniques, as the cell type and/or gene transfer method employed can clearly influence the intracellular distribution of PyV LT. The *dl*2208 LT protein is found in the nucleus and cytoplasm of mouse 3T6 cells infected by the *dl*2208 virus (Nilsson and Magnusson, 1984), but is localised exclusively to the nucleus of Vero cells microinjected with *dl*2208 early region DNA (Richardson *et al.*, 1986), or Balb c/3T3 cells transfected with a *dl*2208 LT gene (this study). A similar heterogeneity in cellular location has been observed for a nuclear transport mutant of SV40 LT which is found exclusively in the cytoplasm of rat cells but localises to both the nucleus and cytoplasm of mouse embryo cells (Lanford *et al.*, 1985a).

Nuclear localisation has been subdivided into two phases: (i) binding to the nuclear pore, possibly *via* associated fibrils, and (ii) a subsequent, energy-dependent translocation to the nucleus (Richardson *et al.*, 1988; Newmeyer and Forbes, 1988). RX14/*dl*2208 could therefore still enter the nucleus by binding to the opening of the nuclear pore, either randomly or *via* a vestigial binding activity.

Non-karyophilic mutants of SV40 LT transform established cells efficiently, but are impaired in the ability to transform primary cells and induce tumours in newborn hamsters (Lanford *et al.*, 1985a). However, mutants of this type retain the ability to immortalise primary rat cells, and co-operate with the PyV MT gene in a transformation assay (Vass-Marengo *et al.*, 1986). In addition,

Figure III.6: Structural Relationships Between SV40 LT, PyV LT, and c-*sis* Polypeptides.

The amino acid sequences of SV40 LT, PyV LT, and the c-*sis* gene product, PDGF, are compared. Asterisks mark identical residues. Adapted from Stabel *et al.* (1985).


376 KAVTKPMECYQVVTAAPFTENKPGL---HQFEFTDEPEEQKAVDWIM 419 PyV LT
6 QGDPIPEELYKMLSGHSIDD-LQRLL---QGD-SGK-EDGAELDLNM 46 c-sis
224 KGVNKEYLMYSALTRDPFEESLPGGLKEHDFNP-EEAEETKQVSWKL 269 SV40 LT

transgenic mice expressing a cytoplasmic variant of SV40 LT develop a variety of tumours (Pinkert *et al.*, 1987). Nuclear transport mutants of SV40 LT also trans-activate a variety of cellular and viral promoters (Pannuti *et al.*, 1987b), induce cellular DNA synthesis (Lanford *et al.*, 1985b), and support limited replication of an SV40 *ori*-containing plasmid, but not viral DNA replication (Colledge *et al.*, 1986). Interpretation of these data is complicated by the possibility that the observed effects could be induced by undetectable amounts of nuclear SV40 LT. The ability of the RX14/*dl*2208 PyV LT nuclear transport mutant to activate gene expression, immortalise primary cells and, possibly, reduce the serum dependence of cells is consistent with the results described above, but the ability of this mutant protein to enter the nucleus in a significant proportion of cells precludes any attempt to assess the importance of nuclear localisation to the behaviour of PyV LT.

8: Mechanism of Immortalisation

One of the primary aims of this study was to gain an insight into the biochemical mechanisms underlying the immortalisation of rodent cells by PyV LT. Previous work (confirmed in this study by the abili ty of pELTRI to immortalise REFs) has demonstrated that the C-terminal 60% of the protein, containing the ATPase, nucleotide-binding, and, possibly, the DNA helicase domains is dispensable for immortalisation (Rassoulzadegan *et al.*, 1983b), as is the ability to bind DNA (Cowie *et al.*, 1986). A number of oncogenes are also transcriptional regulators, suggesting that immortalisation and transformation could be triggered by modulation of the expression of cellular growth control genes (Kingston *et al.*, 1985; Nevins, 1986; Kaddurah-Daouk *et al*, 1987). Although the processes of immortalisation and transformation are undoubtedly accompanied by alterations in the cellular transcription pattern, these changes may be the secondary or tertiary effects of oncogene expression. This may apply, in particular, to putative target genes identified in differential screening studies using stable cell lines (Rigby *et al.*, 1985), or in transient assays performed 48-72 hours post-transfection (Kaddurah-Daouk *et al.*, 1987; Pannuti *et al.*, 1987a). Even genes which are the direct target of oncogene regulation may be unimportant for the control of cell growth. In this study there is no apparent correlation between the abilities of the mutant LT proteins to activate gene expression and to immortalise REFs (see Table 11, previous chapter). For example, AX1374, a mutant effectively devoid of any ability to stimulate the PyV late promoter, retains immortalising activity. Activation of the PyV late promoter represents a model system for the study of transcriptional regulation of

cellular genes by PyV LT, and it is conceivable that important cellular target genes are still activated by AX1374. It would be of interest to investigate the effect of this mutant on the expression of some of the putative cellular target genes, for example, *hsp*70, or metallothionein, although there is little evidence to suggest that these genes have any significance in growth control. The *hsp*70 gene is, however, activated by a number of other oncogenes, including c-*myc*, E1a, and SV40 LT (Kingston *et al.*, 1985), and the related dnaK protein of *E. coli* is important for cell growth and the replication of bacteriophage λ DNA (Kornberg, 1988). The *hsp*70 gene, in addition to its involvement in stress response, plays a rôle in the translocation of proteins into organelles (Deshaies *et al.*, 1988), a function which may help to explain the activation of the mitochondrial cytochrome oxidase subunit II gene by PyV LT (Glaichenhaus *et al.*, 1986a,b).

A similar lack of correlation between gene-activation and immortalising ability has been observed for mutants of adenovirus E1a (Moran *et al.*, 1986; Schneider *et al.*, 1987). In this case, a possible connection between immortalisation and transcriptional repression was identified, however, and it may therefore be of interest to examine the ability of the LT mutants to repress the PyV early promoter. A suitable target plasmid containing the CAT reporter gene linked to the PyV early promoter is available for this purpose (Kern *et al.*, 1986). Similarly, neither the transcriptional activation or repression functions of E1a are required for co-operation with activated *ras* genes to produce full transformation (Kuppuswamy and Chinnadurai, 1987; Velcich and Ziff, 1988). Again it is possible that undetected, vestigial trans-regulatory activity could suffice to modulate the expression of crucial cellular target genes, but it does appear that immortalisation and oncogene co-operation do not involve the direct effects of E1a on gene expression.

The results presented here suggest that, in addition to the activation of gene expression by two different mechanisms, the ability to reduce serum growth factor requirements can also be dissociated from the promotion of cellular establishment by PyV LT, as mutants AX873 and AVX1016 immortalise REFs, yet apparently fail to allow Balb c/3T3 cells to grow in medium containing ox plasma. This conclusion can only be tentative, however, given the unconvincing nature of the results of the serum dependence assays. If the AX873 and AVX1016 mutants are indeed unable to reduce cellular serum growth factor requirements, yet retain the ability to immortalise cells, this would contradict the previous suggestion that the two activities of PyV LT are directly linked (Mougneau *et al.*, 1984).

It must be kept in mind that the assays employed to reach the above conclusions may not accurately reflect the true behaviour of LT *in vivo*.

Alternatively, other, possibly undiscovered, properties of the protein may be important for immortalisation. For example, a block in differentiation of a mouse preadipocyte cell line, induced by c-*myc*, appears to result from the inability of these cells to leave the cell cycle and enter a distinct pre-differentiation state in G_0/G_1 (Freytag, 1988). LT could act in a similar way, preventing cells from entering a terminal differentiation program, with the result that they do not senesce, but continue to divide indefinitely. If this is indeed the case, the *dl*8 LT protein would be expected to be incapable of blocking differentiation. TGF-β also prevents preadipocyte differentiation (Ignotz and Massagué, 1985), and induction of this factor by MT could explain the retention of oncogenic ability by the *dl*8 virus. Since immortality is a recessive phenotype (Pereira-Smith and Smith, 1983) it could conceivably be induced by the mutational inactivation of cellular genes. At least one pair of alleles would have to be inactivated by LT, however, implying that mutagenesis would need to be targeted to produce the observed immortalisation frequencies. As mutations could arise from errors in LT-directed recombination, the ability of the LT mutants generated in this study to promote site-specific recombination is being investigated in the laboratory of Dr. P. Bourgaux (University of Sherbrooke, Canada). Interestingly, the ability of adenovirus E1a to induce chromosomal abnormalities appears to be related to its effect on the G_2 phase of the cell cycle (Braithwaite *et al.*, 1983), and it is therefore conceivable that the region of PyV LT exhibiting homology to E1a domain 2 may also be involved in mutagenesis.

The possibility remains, however, that no activity of PyV LT is uniquely responsible for cellular immortalisation, and that more than one biochemical function of the protein could promote establishment. Indeed, complementation experiments employing cell fusion techniques have identified at least two different cellular lesions underlying immortality, implying that cells can become established by alternative routes (Pereira-Smith and Smith, 1983). The immortalising activity of LT appears to be less sensitive to the effects of linker insertion and internal deletion than the DNA replication, transcriptional activation, and serum dependence functions of the protein. This could reflect the lack of a precise secondary structure requirement for the putative immortalising domain (see Figure III.4), or, alternatively, the possibility that LT can immortalise cells by more than one route. It may therefore be significant that the non-immortalising *dl*8 LT is the only mutant studied here to exhibit at least partial defects in both modes of gene activation, reduction in serum dependence, and, by inference, DNA replication.

9: Comparison With SV40 LT

The comparison of the amino acid sequences and functional domains of SV40 LT and PyV LT shown in Figure III.2 indicates that the two proteins are highly related throughout their sequences. However, a large section of PyV LT (amino acids 90-270) is unrelated to SV40 LT, apart from the short sequence shared with domain 2 of E1a, and contains sequences which may be derived from the cellular Krox-20 protein (see Figure III.3). On the other hand, SV40 LT contains a C-terminal domain, with no equivalent in PyV LT, which confers an extended host range on adenovirus, and also influences the growth of SV40 and the efficiency of capsid production (Cole and Stacy, 1988). SV40 LT also induces cells to overproduce rRNA, and can reactivate silent mouse rRNA genes in human/mouse hybrid cells (Soprano *et al.*, 1983). Although PyV infection also stimulates rRNA synthesis (Section I.C.2.ii) the viral proteins involved in this process have not been identified.

The example provided by SV40 LT (reviewed by Livingston and Bradley, 1987) has proved extremely useful in the study of PyV LT, but a rigid adherence to the SV40 paradigm is probably inadvisable, given the different biological properties of the two proteins, particularly the ability of SV40 LT to fully-transform cells. The SV40 LT sequences required for oncogenesis have not been defined unequivocally, probably reflecting the variety of transformation and immortalisation assays employed in different studies. For example, the N-terminal 35% of SV40 LT is able to immortalise rat cells and induce limited transformation, although lesions mapping outside this region affect the extent and efficiency of transformation (Colby and Shenk, 1982; Tevethia, 1984; Sompayrac and Danna, 1985; Asselin and Bastin, 1985). However, a similar N-terminal fragment fails to immortalise primary mouse cells, with only the C-terminal 81 amino acids of SV40 LT being absolutely dispensable for this activity (Tevethia *et al.*, 1988). Tevethia and colleagues also demonstrated that the transforming and immortalising activities of SV40 LT are genetically separable. It is possible that the transforming activity of SV40 LT may reside in a sub-population (<5%) of molecules which localise to the plasma membrane (Butel and Jarvis, 1986). There appears to be no equivalent membrane-bound population of PyV LT molecules.

C: CONCLUSIONS

The study of the properties of a panel of mutants, created mainly by in-phase linker-insertion, has allowed the genetic map of PyV LT to be extended and refined, revealing the protein as a complex mosaic of functional domains (see Figure III.1).

A number of LT mutants exhibit defects in the ability to promote DNA replication from the PyV *ori*, and may have lesions affecting the DNA-binding, ATPase, DNA helicase and nucleotide-binding activities of the protein, or its ability to interact with components of the cellular DNA replication apparatus. A possible correlation between the ability of the LT mutants to promote DNA replication and activate the PyV late promoter has also been observed. Several mutants retain the ability to activate a mutant PyV late promoter lacking the major LT DNA-binding sites, and at least one mutant (AX1145) appears to have an enhanced activity in this assay. The phenotype of AX1145 suggests that a region of PyV LT, bearing homology to the putative murine transcription factor, Krox-20, may be important for transcriptional activation in the absence of DNA binding. The abilities of LT to activate the intact PyV late promoter and a mutant derivative lacking LT-binding sites appear to be genetically separable, suggesting that LT activates gene expression by two alternative, but not necessarily independent, mechanisms.

It is the LT mutants mapping to the region located between amino acids 90-230 (AX873, AVX1016, AX1145, AX1232, *dl*8, and *dl*23) which exhibit the most intriguing phenotypes in this study, as they have defects in DNA replication, immortalisation, transcriptional activation, and the reduction of cellular serum growth factor requirements. Further studies should concentrate on this subset of mutants.

One mutant in this region, *dl*8, appears to be unable to immortalise rat embryo fibroblasts, and immortalisation appears to be genetically separable from all the other properties of LT assayed here, and, by implication, may not be required for full transformation. Preliminary, unconfirmed, results suggest that a small region of PyV LT, deleted in the *dl*8 mutant, with homologues in other viral and cellular proteins, may induce a reduction in cellular growth factor requirements and be also involved in immortalisation.

PyV LT contributes directly to the viral life cycle by promoting DNA replication, and regulating the expression of the late and early transcription units. In combination with MT and ST it also influences viral reproduction indirectly by stimulating cell growth and DNA replication, thus ensuring that the synthetic machinery of the host cell is primed to produce the maximal yield of viral

particles. The deregulation of normal growth controls following viral infection has profound effects on the cell, but these are normally limited by cell death that accompanies the release of viral particles. However, when viral production is prevented by mutations in replicative functions of LT, or, in a non-permissive environment, when appropriate cellular factors are not available, the effects on the growth of the infected cell are no longer self-limiting, and cellular transformation ensues. The properties of the mutants described here may help to provide information on the mechanisms of viral and cellular DNA replication and transcriptional regulation, as well as cellular growth control. Additional experiments to confirm the observations described in this thesis, and extend them to include oncogene co-operation, recombination, and the blocking of differentiation must now be performed. However, further insights into the contribution of PyV LT to oncogenesis and viral reproduction will probably require the identification and characterisation of the cellular proteins associated with PyV LT.

IV: MATERIALS AND METHODS

A: MATERIALS

1: Chemical Abbreviations

(d)ATP	(deoxy) Adenosine triphosphate
BSA	Bovine Serum Albumin
(d)CTP	(deoxy) Cytosine triphosphate
DMEM	Dulbecco's modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal Calf Serum
(d)GTP	(deoxy) Guanosine triphosphate
HEPES	*N*-2,hydroxyethylpiperazine-*N*'-2,ethane sulphonic acid
MOPS	3-(*N*-morpholino)propane sulphonic acid
OD	Optical Density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
NP-40	Nonidet P-40
PAGE	Polyacrylamide Gel Electrophoresis
pfu	plaque-forming unit
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
RT	Room Temperature
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TEMED	*N,N,N',N'*,tetramethyl ethylenediamine
TLC	Thin Layer Chromatography
Tris	Tris(hydroxymethyl)aminomethane
TTP	Thymidine triphosphate
U	Unit

2: Cells

Cell Line	Cell type	ATCC Number
Balb c/3T3 (clone S24)	Mouse fibroblast	CCL 163
NIH/3T3	Mouse fibroblast	CRL 1658
3T6	Mouse fibroblast	CCL 96

NIH3T3 and 3T6 cell lines, and primary rat and mouse embryo fibroblasts were provided by ICRF Cell Production Unit. Balb c/3T3 cells were supplied by Dr. D. Critchley, Leicester University.

3: Plasmids

Plasmid	Reference
pPyLT1	Zhu *et al.*, 1984
pEMBL8(-)	Dente *et al.*, 1983
pELT*dl*8, pELT*dl*23	Strauss *et al.*, 1986
p*dl*2208, pRX14/*dl*2208, pRX33/*dl*2208	Richardson *et al.*, 1986
p48.19BECAT, pB27BECAT	Kern *et al.*, 1986
pRSVβ-Gal	Gorman, 1985
pSV2Neo	Southern and Berg, 1982

Plasmids pELT*dl*8, pELT*dl*23, and pEMBL8(-) were provided by Dr M. Strauss (Academy of Sciences of the GDR, Berlin), p*dl*2208, pRX14/*dl*2208, and RX33/*dl*2208 were supplied by Dr. W. Richardson (University College London), p48.19BECAT and pB27BECAT were provided by Dr. C. Basilico (New York University), and pRSVβ-Gal was supplied by Dr. J. Burke (University of Sussex).

4: Reagents and Equipment

Chemicals were of Analar quality, and supplied by BDH unless otherwise stated.

Acrylamide	BDH
Agarose	Seakem
Antibiotics	Bacterial culture: Sigma Tissue culture: Gibco
Aprotinin	Sigma
BSA	BRL
Caesium chloride	Koch-Light
DNA Polymerase I	Kornberg: Boehringer Klenow fragment: Amersham
Foetal Calf Serum	Gibco
Nucleoside triphosphates	Sigma
Ox Plasma	Tissue Culture Services
Phenol	Fisons. Redistilled and buffered twice with 500mM Tris-HCl [pH8.0], 50mM EDTA, and twice with distilled water.
Proteinase K	BDH
Restriction enzymes	Biolabs
T4 DNA ligase	Biolabs
T4 Polynucleotide kinase	Pharmacia
T4 DNA Polymerase	Pharmacia

Tissue culture dishes	5cm, 9cm, and bioassay dishes: Nunc 6-well and 24-well dishes: Falcon
3MM Paper	Whatman
X-ray film	Kodak XAR-5

Fixed preparations of *Staphylococcus aureus* were provided by F. Fitzjohn, ICRF.

5: Solutions

PBS

1% (w/v) NaCl
0.025% (w/v) KCl
0.14% (w/v) NaH_2PO_4
0.025% (w/v) KH_2PO_4
10mM $CaCl_2$
6mM $MgCl_2$

SSC

150mM NaCl
150mM sodium citrate

Denhardt's solution

0.02% (w/v) BSA
0.02% (w/v) polyvinylpyrrolidone
0.02% (w/v) Ficoll 400

Phenol/Chloroform

49.5% (v/v) buffered phenol
49.5% (v/v) chloroform
1% (v/v) isoamyl alcohol
0.1% (w/v) 8-hydroxyquinoline

L-Broth

1% (w/v) bacto-tryptone
0.5% (w/v) yeast extract
1% (w/v) NaCl

Psi-Broth

2% (w/v) bacto-tryptone
0.5% (w/v) yeast extract
10mM NaCl
20mM $MgSO_4$
5mM KCl

TE

10mM Tris
1mM EDTA
pH7.6 (adjusted with HCl)

Versene

0.02% EDTA (w/v) in PBS
pH7.2

B: METHODS

1: Tissue Culture Techniques

i: Cell Culture

All established and non-established cells were grown, on tissue culture dishes, in DMEM supplemented with 10% FCS (with the exception of 3T6 cells which were cultured in DMEM supplemented with 5% FCS) in a 10% CO_2 : 90% air-humidified incubator at 37°C. On reaching confluence the cells were washed with versene, freed from the surface of the dish by the addition of a small volume of versene containing 0.25% trypsin (1min, 37°C), and replated, at an appropriate dilution, on to new dishes containing fresh medium.

ii: Storage of Cells

Sub-confluent dishes of cells were trypsinized as described above, disaggregated in 5ml of DMEM, 10% FCS and pelleted in a sterile 30ml universal (5 min, 1,100 rpm, bench centrifuge, RT). The cells were resuspended in 1ml of DMEM, 20% FCS, 10% DMSO and transferred to a 1.6ml cryotube before freezing at -70°C (4 hours) and storage in liquid nitrogen.

iii: DNA-Mediated Gene-Transfer

This was performed by an adaptation of the calcium phosphate co-precipitation method of van der Eb and Graham (1980). The cells were trypsinized and replated at a density of 500,000 cells/5cm dish 24 hours before transfection, and then refed with 4.5ml of fresh medium 4 hours pre-transfection.

A total of 10μg of DNA (a combination of plasmid and sonicated salmon sperm carrier DNAs) was added to 465μl of HeBS (70mM NaCl, 5mM KCl, 1mM NaH_2PO_4, 5mM glucose, 20mM HEPES, pH7.05) in a sterile bijou. Following the addition of 25μl of a sterile 2.5M $CaCl_2$ solution (20min, RT) a fine white precipitate was produced, and this was added to the tissue culture medium. The cells were then incubated under standard tissue culture conditions for 16 hours before the medium was removed, the cells washed with versene, and the addition of 5ml of fresh medium.

iv: Generation of Drug-Resistant Balb c/3T3 Cell Lines

Balb c/3T3 cells were transfected, as described above, with 5μg of LT plasmid and 1μg of pSV2Neo. The cells were trypsinized 72 hours post-transfection, and replated, at a density of 500,000 cells/5cm dish, in medium containing Geneticin (400μg/ml). The medium was replaced every four days, and widespread cell death was observed after 7-10 days of selection. Drug-resistant colonies, visible after 14 days, were isolated using cloning rings after 3-4 weeks and expanded into cell lines in medium containing 100μg/ml Geneticin.

v: Immortalisation of Early Passage Rat Embryo Fibroblasts.

Secondary REFs were plated on to 5cm tissue culture dishes at a density of 500,000 cells/dish. The following day, the cells were transfected with a combination of LT or pEMBL 8(-) DNA (5μg/dish) and pSV2Neo (1μg/dish). LT and vector plasmids were digested with *Hind*III prior to transfection to prevent autonomous replication. Digested DNAs were phenol-extracted and ethanol-precipitated before use. Seventy-two hours post-transfection, the cells were trypsinized and replated at a density of 500,000 cells/5cm dish, in medium containing 200μg/ml of Geneticin (Gibco). The medium was then changed every four days, and drug-resistant colonies were cloned 3-4 weeks later, and expanded where possible.

vi: Serum Dependence Assay (Armelin *et al.*, 1985).

Balb c/3T3 cells expressing the LT mutants (Section II.B), or rat cell lines established by the LT mutants (Section II.D) were trypsinized, suspended in serum-free DMEM, and counted, using a haemocytometer. Following serial dilution in serum-free DMEM, the cells were plated, at a density of 100 or 500 cells/5cm dish, into medium containing either 10% FCS or 10% ox plasma. The medium was changed every four days, and the colonies were visualised by crystal violet staining after two weeks. Staining was performed by removing the tissue culture medium and adding 5 ml of PBS containing 3.8% formaldehyde, 300mM sucrose, 0.1% crystal violet (20 mins, RT). The stain was then removed, and the plates rinsed with water and air-dried.

2: Virus Production and Infection

i: Production of Virus Stock

Polyoma virus was grown by infecting a bioassay dish of primary whole mouse embryo cells with plaque-purified virus from the A2 stock, at a multiplicity of infection of approximately 0.1 pfu/cell. Following incubation for 10 days at 37°C, an extensive cytopathic effect was observed, and the cells and media were freeze-thawed twice and stored at -20°C. The virus was sonicated briefly prior to infection.

ii: Infection of Cells

The medium covering a sub-confluent monolayer of cells was removed, the cells washed with serum-free DMEM, and a small volume of virus stock (1ml/9cm dish) was added. Following incubation at 37°C for 90 minutes, with frequent rocking, the monolayer was overlaid with fresh medium containing FCS, and incubation at 37°C was continued. This time-point was taken as the start of infection.

3: Molecular Cloning Techniques

i: Restriction Endonuclease Digestion of DNA

DNA was digested under the conditions described by their commercial suppliers. Routinely, 1μg of plasmid DNA was digested with an excess (5 units) of enzyme for 2-3 hours at 37°C, to ensure complete digestion.

ii: Removal of 5'-Terminal Phosphate Groups From DNA

Restriction endonuclease-digested DNA was dephosphorylated by incubation (37°C, 30min) with calf intestinal alkaline phosphatase (Boehringer, 0.5U/50pmol ends) in a buffer containing 50mM Tris-HCl, 0.1mM EDTA, [pH 8.0]. The enzyme was then inactivated by incubation (68°C, 45 mins) with nitriloacetic acid (final concentration of 10mM), followed by two phenol extractions and ethanol precipitation.

iii: Ligation of DNA Fragments

DNA fragments were ligated in a 30μl reaction mixture containing 50mM Tris.HCl [pH7.6], 10mM $MgCl_2$, 5% (w/v) polyethylene glycol 8,000, 1mM ATP, 1mM DTT and 1U T4 DNA ligase (14°C, 20 hours). Routinely a vector:insert ratio of 1:3 was used, with the total amount of DNA in the range of 200-300ng. The ligation mixture was diluted 3-fold with TE prior to bacterial transformation.

iv: Culture of Bacteria

Bacteria from the DH5 strain of *E. coli* were grown in L-Broth containing the appropriate antibiotics. Bacteria from the GM113 strain of *E. coli* (supplied by Dr. B. Sedgwick) were grown in L-Broth supplemented with methionine and thymidine. Antibiotics were used, where appropriate, at the following concentrations: ampicillin, 100μg/ml; chloramphenicol, 200μg/ml, tetracycline, 17μg/ml.

LBA plates were prepared from L-Broth containing 1.2% bacto-agar (Difco), and ampicillin (100μg/ml), and poured in 9cm Petri dishes (Sterilin).

v: Preparation of Competent Bacteria (Hanahan, 1985).

A single bacterial colony was transferred into 5ml of Psi-Broth, and shaken overnight at 37°C, before being used to inoculate a further 100ml of Psi-Broth, prewarmed in a 1l Erlenmeyer flask. The culture was then incubated at 37°C, with vigorous shaking until an OD_{550} of approximately 0.48 was achieved (2-2.5 hours). The cells were then transferred to a sterile 50ml tube (Falcon 2070), pelleted (2500rpm, bench centrifuge, 0°C), and resuspended in 30ml of ice-cold RF-1 (100mM $RbCl_2$, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 15% w/v glycerol, pH5.8, filter sterilised). Following incubation at 4°C for 20 minutes, the cells were pelleted (2,500rpm, bench centrifuge, 0°C) and resuspended in 8ml of RF-2 (10mM MOPS, 10mM $RbCl_2$, 75mM $CaCl_2$, 15% w/v glycerol, pH6.8, filter-sterilised). The bacteria were then aliquoted into 1.8ml cryotubes (Nunc 3-63401), and maintained at 4°C for 20 minutes, before flash-freezing in a dry-ice/ethanol bath and storage at -70°C.

vi: Transformation of Competent Bacteria (Hanahan, 1985).

Competent cells were partially thawed at RT, before incubation at 4°C (5 minutes). A 200μl aliquot of competent cells was mixed with DNA (<100ng, in a volume <50μl) in a cryotube (Nunc 3-63401), and maintained at 4°C for 20 minutes. The bacteria were then heat-shocked by incubation at 42°C for 2 minutes, followed by chilling on ice for a further 2 minutes. Four volumes of Psi-Broth were added, and the culture was incubated at 37°C, with gentle shaking, for 60 minutes. Aliquots of the transformation mixture (100-400μl) were spread on to 9cm LBA plates, which were inverted and incubated overnight at 37°C.

vii: Small Scale Preparation of Plasmid DNA (Wilimzig, 1985).

Cells from individual bacterial colonies were transferred, using a sterile toothpick, to a sterile 1.5ml microcentrifuge tube containing 1ml of LBA. After overnight incubation at 37°C on a shaking platform, the bacteria were pelleted (30 secs., microcentrifuge, RT), and resuspended in 100μl of TELT buffer (50mM Tris-HCl, 62.5mM EDTA, 0.4% Triton X-100, 2.5M LiCl, pH7.5). Following the addition of 10μl of a freshly-prepared solution of lysozyme (10mg/ml in H_2O), the bacterial suspension was placed in a boiling water bath for 1 minute, and then chilled on ice for 5 minutes. The precipitated protein, RNA, and chromosomal DNA was removed by centrifugation (8 minutes, microcentrifuge, RT) and the plasmid DNA in the supernatant was precipitated by the addition of an equal volume of isopropanol. Precipitated DNA was recovered by centrifugation (10 minutes, microcentrifuge, RT), redissolved in 40μl of TE, and used directly for restriction endonuclease digestion.

viii: Large Scale Preparation of Plasmid DNA (Birnboim and Doly, 1979).

A single bacterial colony was transferred, using a sterile inoculating loop, into a sterile universal (Sterilin), containing 10ml of LBA. Following overnight incubation at 37°C on a shaking platform, the culture was used to inoculate a 2l flask containing 1l of LBA, which was then shaken for a further 16 hours at 37°C. The bacteria were pelleted (Sorvall GS-3, 6,000rpm, 5min, 4°C), resuspended in 40ml of 50mM glucose, 25mM Tris-HCl [pH8.0], 10mM EDTA, 5mg/ml freshly-prepared lysozyme, and incubated at RT for 10 mins. Following the sequential addition of 80ml of 0.2M NaOH, 1% SDS (5min, 4°C), and 40ml of

3M sodium acetate [pH4.8] (10min, 4°C), the precipitated cellular debris was removed by centrifugation (Sorvall GS-3, 6,000rpm, 5min, 4°C). The DNA in the supernatant was then precipitated by the addition of 0.6 volumes of propan-2-ol (30 mins, -20°C), and pelleted by centrifugation (Sorvall GS-3, 7,000rpm, 15min, 4°C), before resuspension in 10ml of TE. The solution was phenol-extracted once, and the DNA reprecipitated by the addition of 2.5 volumes of ethanol (3 hours, -20°C). The DNA was then pelleted (Sorvall HB-4, 10,000rpm, 30mins, 4°C), and resuspended in 10ml of TE, before the addition of 10g of CsCl, and 1ml of ethidium bromide (5mg/ml in dH_2O). This solution was loaded into a Quick-Seal ultracentrifuge tube (Beckman), and centrifuged (Beckman Ti 70.1, 47,000rpm, 48 hours, 18°C) to separate form I plasmid DNA from other plasmid forms and contaminating high-molecular weight cellular DNA. The form I DNA was removed using a hypodermic syringe, extracted five times with CsCl-saturated propan-2-ol, diluted with two volumes of dH_2O, and precipitated by the addition of 2.5 volumes of ethanol (3 hours, -20°C). The plasmid DNA was then recovered by centrifugation (Sorvall HB-4, 10,000rpm, 30mins, 4°C), resuspended in TE, and adjusted to a concentration of 1mg/ml following quantitation by measurement of OD_{260}.

ix: Purification of DNA Fragments

Restriction endonuclease digested DNA was electrophoresed through a 1.1% low melting temperature agarose gel, and the appropriate band was excised from the gel and placed in a 15ml Falcon tube containing 5ml of Low Salt Buffer (0.2M NaCl, 20mM Tris-HCl [pH7.5], 1mM EDTA). The gel slice was then melted by transferring the tube to a 68°C water bath for 30 minutes, followed by equilibration in a 37°C water bath for 15 minutes. The agarose solution was then passed over an Elutip-D column (Schleicher and Schuell) according to the manufacturer's instructions, and the DNA was subsequently eluted using 0.4 ml of High Salt Buffer (1M NaCl, 20mM Tris-HCl [pH 7.5], 1mM EDTA). The DNA was then precipitated by the addition of 2.5 volumes of ethanol (3 hours, -20°C), pelleted by microcentrifugation (10 mins, RT), and redissolved in 10μl of TE.

x: Agarose Gel Electrophoresis

Agarose gels (0.8-1.1%) were prepared by adding agarose powder to 200ml of E buffer (40mM Tris-acetate [pH7.5], 2mM EDTA) and heating to 100°C (microwave, 5min). After cooling to 50°C, and the addition of ethidium bromide to a final concentration of 0.5μg/ml, the gel was cast on a horizontal glass plate (20cm x 15cm). Once set, the gel was transferred to a flat bed electrophoresis chamber containing 1800ml of E buffer with 0.5μg/ml ethidium bromide. DNA samples were mixed with 0.1 volumes of loading buffer (1.25% SDS, 2.5mM EDTA, 37.5% [w/v] sucrose, 0.15% bromophenol blue) and loaded on to the gel. The DNA fragments were then electrophoresed towards the anode (40mA, 16 hours, RT), and their migration was visualised under longwave UV light, and photographed using Polaroid Land 57 film.

Rapid analysis of DNA samples was performed using minigels (10cm x 8cm) formed by dissolving the appropriate amount of agarose in 50ml of TBE buffer (90mM Tris-borate, 90mM boric acid). Electrophoresis was performed in TBE buffer (60mA, 1 hour).

4: Mutagenesis

i: Construction of pELT and pELTRI

Following digestion of the plasmid pPyLT1 with *Bam*HI, the 4.9kb fragment containing the PyV coding sequences was purified from low melting temperature agarose and ligated to *Bam*HI-digested, dephosphorylated pEMBL8(-). The resulting plasmid (designated pEPyLT1) was then digested with *Hinc*II, diluted and recircularised by ligation. This plasmid was then grown in the GM113 strain *of E.coli* to prevent *dam* methylation, digested with *Bam*HI and *Bcl*I, and recircularised by ligation to produce pELT.

The mutant pELTRI was constructed by digesting the plasmid pEPyLT1A (the same insert as pEPyLT1, but in the reverse orientation) with *Eco*RI, recircularising the plasmid by ligation, and transfecting into GM113 cells. The resulting plasmid was then digested with *Hinc*II, recircularised by ligation, and used to transform competent DH5 bacteria. pELTRI contains PyV nucleotides 5021-1560.

ii: Ethidium Bromide Titration

Restriction endonuclease digestion reactions (40μl volume) containing 1μg of pELT, 2 units of enzyme, and ethidium bromide at different concentrations in the range 0-50μg/ml were incubated for 30 mins at 37°C, and terminated by the addition of electrophoresis loading buffer. The reaction products were then electrophoresed through a 1.1% agarose gel, with undigested and *Cla*I-linearised pELT as markers, to identify the concentration of ethidium bromide producing the optimum yield of linear form III molecules. The products of 12 similar reactions containing this concentration of ethidium bromide were then pooled, electrophoresed through low-melting temperature agarose, and the linear molecules purified.

The concentrations of ethidium bromide employed for different restriction enzymes were: *Acc*I, 1μg/ml; *Alu*I, 20μg/ml; *Ava*I, 25μg/ml, *Eco*RI, 30μg/ml; *Hind*III, 20μg/ml; *Xba*I, 20μg/ml.

iii: Construction of the AX Insertion Mutants (Stone *et al.*, 1984).

An oligonucleotide of the sequence 5'-d(CTCGAGCTCGAG)-3' (synthesised by Genetic Research Instrumentation Ltd.) was converted to a double-stranded linker by dissolving in TE (1mg DNA/ml), heating to 68°C for 15 minutes, cooling slowly for 20 minutes at RT, and then chilling on ice for 20 mins. 4μg of the linker was phosphorylated by incubation (37°C, 30mins) with 30U of T4 polynucleotide kinase in 50μl of kinase buffer (60mM Tris-HCl [pH 7.8], 10mM $MgCl_2$, 15mM β-mercaptoethanol, 2mM ATP).

*Alu*I-linearised pELT (approximately 1μg) was ligated to 1.5μg of the kinased *Xho*I linker. Following heat inactivation of T4 DNA ligase (68°C, 10 mins), the DNA was digested with an excess (150 units) of *Xho*I, and electrophoresed on a 1.1% low-melting temperature agarose gel to separate forms I, II, and III. The form III band was excised and the DNA was purified and ligated in a reaction volume of 40μl to promote recircularisation. The DNA was then transformed into competent bacteria (DH5), as described above.

iv: Construction of the ACB, AVX, EX, HX, and XX Mutants

Following ethidium bromide-limited digestion of pELT with the appropriate restriction enzyme, the overhanging 5' ends of the linearised plasmids were filled in using the Klenow fragment of DNA polymerase I. 1μg of linearised DNA was incubated (30 mins, RT) with 5U of Klenow enzyme in a buffer containing

50mM Tris-HCl [pH7.5] 5mM $MgCl_2$, 1mM β-mercaptoethanol, 100μM dATP, 100μM dCTP, 100μM dGTP, 100μM TTP. The reaction product was then used directly in a ligation reaction with a 10-fold molar excess of linker, digested with an excess of the appropriate restriction enzyme, recircularised by ligation, and used to transform competent *E. coli* of the DH5 strain.

The following linkers were used for mutagenesis. ACB368 and ACB1809: *Bam*HI 10-mer, 5'-Pd(CGGGATCCCG)-3' (Boehringer Mannheim); AVX1016, EX1560, HX1656, XX2477, XX2522: *Xho*I 8-mer, 5'-Pd(CCTCGAGG)-3' (Pharmacia).

v: Construction of dl8 and dl23

The plasmids pELT*dl*8 and pELT*dl*23 (Strauss *et al.*, 1986), containing truncated LT, were converted to full-length by linearisation with *Eco*RI and ligation to a purified *Eco*RI fragment from pEPyLT1A, containing the C-terminal LT sequences. The resulting plasmids are of the same format as pELT, with the exception that PyV sequences terminate at nucleotide 4632, rather than nucleotide 2962.

vi: Construction of dl2208, RX14/dl2208, and RX33/dl2208

The plasmids p*dl*2208, pRX14/*dl*2208, and pRX33/*dl*2208, comprised of viral genomes cloned at the *Bam*HI site of pAT153, were digested with *Bam*HI, and the PyV sequences were purified and ligated to *Bam*HI-digested, dephosphorylated pEMBL8(-). The resulting plasmids were digested with *Sca*I and ligated to a purified *Sca*I fragment from pELT containing nucleotides 886-2962 of PyV and sequences from pEMBL8(-). This plasmid was then digested with *Hinc*II and recircularised by ligation, yielding mutants with the same format as pELT.

vii: Screening for Linker Insertion Mutations

Plasmid DNA was purified from ampicillin-resistant colonies and digested with a combination of *Bgl*I and *Xho*I (or *Bam*HI in the case of ACB368 and ACB 1809). Digestion of pELT with *Bgl*I produces three fragments of length 2.7, 2.5, and 1.7 kb. Cleavage of the 2.7kb fragment indicated the presence of a linker, and the size of the fragments generated by *Xho*I or *Bam*HI digestion gave an approximate indication of the site of insertion, which was sufficient to identify all but the AX series of mutants. The exact site of insertion was identified for

the AX mutants using a secondary screening procedure. Plasmid DNA was digested with *Xho*I and *Hae*III and end-labelled by incubation with 20μM dATP, 20μM dGTP, 20μM TTP, 1μl of α-[^{32}P]-dCTP, and 1 unit of the Klenow fragment of DNA polymerase I (15mins, RT). Unincorporated nucleotides were removed by the addition of an equal volume of 5M ammonium acetate and three volumes of ethanol (-20°C, 3 hours). Labelled DNA was then pelleted by microcentrifugation (10min, RT), redissolved in TE, mixed with 0.1 volumes of loading buffer, and applied to an 8% polyacrylamide gel, along with *Hae*III-digested pAT153, end-labelled using T4 DNA polymerase (as described in Section IV.B.10.ii). The gel, containing 8% (w/v) acrylamide, 0.2% (w/v) *N,N'* methylene bis-acrylamide, 0.1% (w/v) ammonium persulphate, dissolved in TBE, was induced to polymerise by the addition of a 1/1000 volume of TEMED, and cast between gel plates separated by Plasticard spacers. Following elecrophoresis (20mA, 30mins) in TBE the gel was fixed in 10% acetic acid (15min, RT), dried under vacuum, and exposed to X-ray film. The size of the labelled *Xho*I/*Hae*III fragments together with the size of the *Bgl*II/*Xho*I fragments obtained previously, and knowledge of the nucleotide sequence of PyV allowed the location of the linkers to be identified.

5: DNA Replication Assay

i: Transfection of Plasmid DNA

3T6 cells were transfected with 1μg of LT plasmid or pEMBL8(-), as described in Section IV.B.1.iii.

ii: Extraction of Low Molecular Weight DNA (a modification of Hirt, 1967).

Cells were lysed, 72 hours post-transfection, by washing with PBS and adding 0.4ml of 0.6% SDS, 10mM EDTA, 10mM Tris-HCl [pH7.5] per 5cm dish tissue culture dish. Following incubation on an orbital shaker for 20mins at room temperature, 100μl of 5M NaCl was added, the lysate was transferred to a 1.5ml microcentrifuge tube, and incubated overnight at 4°C. The cellular debris was pelleted by microcentrifugation (30min, 4°C) and the supernatant was digested with 100μg of Proteinase K (60min, 37°C). Following extraction with an equal volume of buffered phenol, the DNA was precipitated with 2.5 volumes of ethanol (1 hour, -20°C), recovered by microcentrifugation (10mins, RT), and resuspended in 40μl of TE.

6: Blotting and Hybridisation

i: Preparation of Radioactively-Labelled DNA Probes : Nick Translation

Nick translation reactions (Rigby *et al*, 1979) were routinely performed using 50ng of DNA in a reaction mixture containing 50mM Tris-HCl [pH7.8], 5mM $MgCl_2$, 10mM β-mercaptoethanol, 50μg/ml BSA, 2μM dATP, 2μM dGTP, 2μM TTP, and 50μCi of α-[^{32}P]-dCTP (Amersham International, 2-3,000 Ci/mmol), and 20U of DNA polymerase I (Boehringer) in a total volume of 50μl. Following incubation at 14°C for 3 hours, labelled DNA was separated from unincorporated radionucleotides by passing the reaction mixture over a 3ml Dispo-column containing Sephadex-G75 (Pharmacia). The labelled DNA was eluted with TE, 0.1% SDS, and 0.4ml fractions were collected and monitored for radioactivity using a Geiger counter. The two fractions with the highest activity were used for hybridisation.

ii: Transfer of DNA to Nitrocellulose (Southern, 1975)

Following electrophoresis through agarose gels, DNA was denatured by immersing the gel in 0.5M NaCl, 0.5M NaOH for 60 min at RT, with gentle agitation. The gel was then neutralised in 0.5M NaCl, 1M Tris-HCl [pH7.6] (3hours, RT, with gentle agitation), and transferred to a glass plate, covered with 3MM paper soaked in 20xSSC, dipping into a reservoir of 20xSSC. The gel was surrounded with Saran Wrap, and covered with nitrocellulose (Schleicher and Schuell), pre-soaked in deionised H_2O, followed by several layers of 3MM paper. Finally, a stack of paper towels was placed on top of the 3MM paper, and weighted down with a 1kg lead weight. The DNA was allowed to transfer to the nitrocellulose overnight, the towels and 3MM paper were removed, the nitrocellulose washed in 2xSSC (5min, RT), air-dried, and baked, under vacuum, at 80°C for 2 hours.

iii: Hybridisation

Baked nitrocellulose filters were sealed into plastic bags containing 10ml of prehybridisation buffer (5xDenhardt's solution, 6xSSC, 0.1% [w/v] SDS, 1.5mM sodium pyrophosphate, 25mM NaH_2PO_4) and incubated at 68°C for 4 hours. The buffer was then replaced with hybridisation buffer (pre-hybridisation buffer, containing nick-translated DNA and 2mg of sonicated calf thymus DNA, denatured by placing in a boiling water bath for 8 minutes). Following

incubation at 68°C for 16 hours, with constant agitation, the filter was transferred to a plastic sandwich box containing 500ml of 0.3xSSC, 0.1% SDS and incubated at 68°C for 1 hour, with constant agitation. This procedure was then repeated with two changes of 0.1xSSC, 0.1% SDS. The filter was then air-dried, covered in Saran Wrap, and exposed to X-ray film.

7: Transcriptional Activation

i: Chloramphenicol Acetyltransferase Assay (Gorman, 1985).

The target plasmids p48.19BECAT and pB27BECAT (2μg) were co-transfected with the LT plasmids (2μg) into NIH3T3 cells, as described in Section IV.B.2.iii. Seventy hours post-transfection, the cells were washed with ice-cold PBS, transferred, using a rubber policemen, into a 1.5 ml microcentrifuge tube, pelleted (30 secs., microcentrifuge, 0°C) and resuspended in 100μl of Tris-HCl [pH7.8]. The cells were disrupted by three freeze-thaw cycles (5 minutes in a dry-ice/ethanol bath followed by 90 seconds at 37°C), and the debris removed by centrifugation (5 minutes, microcentrifuge, 4°C). A 30μl aliquot of the supernatant was then incubated for 60 minutes at 37°C in a reaction mixture containing 70μl 0.25M Tris-HCl [pH7.8], 42μl water, 8μl 10mM acetyl Co-A (Pharmacia), and 1μl [^{14}C]-chloramphenicol (40-50 Ci/mmol, NEN). Chloramphenicol and its derivatives were then extracted by vortexing the reaction mixture with 600μl of ethyl acetate. Following centrifugation (2 mins, microcentrifuge, RT) the upper, organic, phase was removed, and dried down under vacuum. Samples were then redissolved in 20μl of ethyl acetate and spotted on to a 20cm x 20cm thin-layer chromotography plate (Polygram SIL G/UV$_{254}$). Following ascending chromotography with a 95:5 mixture of chloroform:methanol the plates were coated with 2-methylnaphthalene containing 10% toluene and 0.4% (w/v) PPO, air-dried, and exposed to X-ray film. The extent of chloramphenicol conversion was quantitated by excising the regions of the TLC plate corresponding to the unconverted and monoacetylated forms of chloramphenicol and measuring radioactive emission in a scintillation counter.

ii: Measurement of β-Galactosidase Activity (Gorman, 1985).

Transfections of p48.19BECAT or pB27BECAT with LT plasmids were performed as described above, with the addition of 2μg of pRSVβ-Gal to the transfection mixture. Cells were harvested as described above, resuspended in 100μl of Tris-HCl [pH7.8] containing 5mM DTT, and disrupted by three freeze-

thaw cycles. A 50μl aliquot of the cell lysate was incubated together with 200μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside, Sigma), and 1μl of a solution containing 60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol at 37°C until a visible yellow colour was achieved. The reaction was terminated by the addition of 500μl of 1M Na_2CO_3, and the extent of ONPG hydrolysis was quantitated by measuring the OD_{420} of the solution.

8: Analysis of Proteins

i: Metabolic Labelling of Proteins With [^{35}S]-Methionine

The tissue culture medium of exponentially-growing cells was removed by aspiration, the cells were washed twice with methionine-free DMEM (at 37°C) and overlaid with methionine-free DMEM (Gibco) containing 5% FCS (dialysed at 4°C against PBS, and then filter sterilised). After incubation for 1 hour at 37°C the medium was replaced with methionine-free DMEM containing [^{35}S]-methionine (Amersham, >600 Ci/mmol). 0.3mCi of [^{35}S]-methionine, in a volume of 1ml was used to label a 5cm dish of cells, and labelling was performed for 4 hours, with regular rocking of the dish.

Labelled cells were washed twice with ice-cold PBS, transferred to a 1.5ml microcentrifuge tube using a rubber policeman, and lysed with 300μl of a buffer containing 100mM NaCl, 100mM Tris-HCl [pH 8.8], 0.5% NP-40, 1% Aprotinin (4°C, 15 min, with occasional vortexing). Cellular debris was removed by microcentrifugation (5min, 4°C), and the supernatant was pre-cleared by the addition of 0.5 volumes of a fixed preparation of *Staphylococcus aureus* (30mins, 4°C). Following microcentrifugation (2min, 4°C), supernatants were stored in liquid nitrogen.

ii: Immunoprecipitation of LT

Metabolically-labelled cell lysates were incubated (4°C, 60 mins) with 5μl each of α-PyV LT-1 and -4 (Dilworth and Griffin, 1982) and 400μl of NET (150mM NaCl, 5mM EDTA, 50mM Tris-HCl[pH7.4], 0.05% NP-40, 1% Aprotinin) containing 0.2% gelatin, in a 15ml centrifuge tube (Falcon 2095). Following incubation with 25μl of a fixed preparation of *Staphylococcus aureus* (30mins, 4°C, with occasional vortexing), 10ml of NET were added, and the adsorbed immune complexes pelleted by centrifugation (bench centrifuge, 2,500rpm, 15mins, 4°C). The complexes were resuspended in 700μl of RIPA (NET containing 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), transferred to

a 1.5ml microcentrifuge tube and pelleted (microcentrifuge, 15secs, RT). Following a further wash with RIPA, the pellet was resuspended in 20μl of sample buffer (100mM DTT, 0.05% SDS, 20% glycerol, 3mM Tris-HCl [pH6.8]), and heated in a boiling water bath for 3mins. The *Staphylococcus aureus* cells were removed by centrifugation (microcentrifuge, 5min, RT), and the proteins were loaded on to a 10% polyacrylamide gel, using a Hamilton microsyringe. Gels, cast in plates separated by 1mm spacers, were made from a stock solution containing 30% (w/v) acrylamide, 0.8% N,N' methylene bis-acrylamide, and polymerised by the addition of 0.1% (w/v) ammonium persulphate, and 1/1000 volumes of TEMED. Running gels were composed of 10% acrylamide (1:3 dilution of stock solution), 0.1% (w/v) SDS, 375mM Tris-HCl [pH8.8], and stacking gels of 5% acrylamide, 0.1% (w/v) SDS, 125mM Tris-HCl [pH 6.8]. The proteins were electrophoresed for 2 hours at 30mA, in buffer containing 25mM Tris-HCl, 192mM glycine, 0.1% (w/v) SDS, pH 8.3, the gel was fixed and stained in 50% methanol, 10% glacial acetic acid, 0.1% PAGE Blue G90 (BDH), destained in 10% methanol, 7% glacial acetic acid, dried under vacuum, and exposed to X-ray film.

9: Indirect Immunofluorescence (Dilworth *et al.*, 1986).

Cells were grown on Multitest slides (Flow) or Lab-Tek chamber slides (Miles), washed with PBS (37°C), and fixed in PBS containing 3.8% formaldehyde and 300mM sucrose (20min, initially at 37°C). The cells were then washed with PBS, permeabilised with PBS containing 1% Triton X-100 (5 min, RT), and washed several more times with PBS. Non-specific antibody binding sites were blocked with PBS containing 0.25% BSA and 0.25% gelatin (60 min, RT), before sequential incubation (30 min, RT) with tissue culture fluid containing the monoclonal antibodies α-Py LT1 and -4 (Dilworth and Griffin, 1982), a 1/50 dilution of rabbit α-rat IgG (Dako Immunochemicals), and a 1/50 dilution of fluorescein-conjugated goat α-rabbit IgG (Miles Biochemicals). Slides were washed extensively with several changes of PBS (>30 min) between incubation with each antibody. The rabbit and rat monoclonal antibodies were pre-absorbed on a methanol:acetone (1:1)-fixed monolayer of Balb c/3T3 or REF cells (30 min, RT), and then diluted in PBS containing 0.25% BSA and 0.25% gelatin, prior to use. Finally, the slides were mounted in PBS containing 90% glycerol and 1mg/ml *p*-phenylenediamine, and viewed using a Zeiss standard 16 microscope with a Neofluor x25 objective lens, and photographed using Ilford HP5 film.

10: Binding of PyV LT to DNA (Cowie and Kamen, 1984).

i: Preparation of a Cold LT lysate

A 9cm tissue culture dish of mouse 3T6 cells, infected with PyV 48 hours previously, was washed twice with ice-cold PBS and incubated for 30 minutes at 4°C following the addition of 1ml of lysis buffer (50mM Tris-HCl [pH8], 150mM NaCl, 2% NP-40, 1% Aprotinin). The cell lysate was then transferred to a 1.5ml microcentrifuge tube, and debris was pelleted (5 mins, microcentrifuge, 0°C). Supernatants were aliquoted and stored in liquid nitrogen.

ii: End-Labelling of DNA Fragments

Plasmids containing the LT DNA-binding sites were digested with a combination of *Bgl*I and *Hinf*I. Following heat inactivation of the restriction enzymes (68°C, 10min), 100ng of digested DNA was end-labelled by incubation with T4 DNA polymerase (5min, 37°C) in a buffer containing 33mM Tris acetate [pH 7.9], 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, and 100μg/ml BSA. dATP, dGTP, and TTP were then added to a final concentration of 100μM along with 1μl of α-[^{32}P]-dCTP (5min, 37°C). Finally, cold dCTP was added, to a final concentration of 100μM (10min, 37°C), the reaction mixture was diluted with TE, and, following heat inactivation (68°C, 10min) unincorporated radio-labelled nucleotides were removed by the addition of an equal volume of ammonium acetate and three volumes of ethanol (3hours, -20°C). Labelled DNA was recovered by microcentrifugation (10min, RT), redissolved in TE, and stored at -20°C.

iii: DNA-Binding

100μl of cell lysate was incubated with 5ng of end-labelled DNA fragments and 1ml of MB buffer (20mM sodium phosphate [pH7.0], 2mM DTT, 0.01% BSA, 1mM EDTA, 0.05% NP-40, 3% DMSO, 0.1M NaCl, 5μg/ml sheared calf thymus DNA) for one hour at 4°C. Following the addition of 5μl each of the monoclonal antibodies α-PyLT1 and α-PyLT4 (Dilworth and Griffin, 1982) (60 mins, 4°C), 30μl of a washed preparation of fixed *Staphylococcus aureus* cells were added to the samples (30 mins, 4°C). The cells were then pelleted (30 secs, RT), and washed three times with MW buffer (20mM Tris-HCl [pH8.0], 1mM EDTA, 2mM DTT, 0.01% BSA, 0.05% NP-40, 0.1M NaCl, 10μg/ml sheared calf

thymus DNA). DNA was then eluted from the immune complex by incubating the cells (10 min, RT) in 100μl of elution buffer (10mm Tris-HCl [pH7.5], 1mM EDTA, 2% SDS). The cells were then pelleted (5mins, RT) and the DNA in the supernatant was precipitated by the addition of 200 μg of carrier tRNA and 2.5 volumes of ethanol. The immmunoprecipitated DNA was pelleted, resuspended in 10μl of TE, and electrophoresed through a 1% agarose gel, which was dried under vacuum and exposed to X-ray film.

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APPENDIX

Immortalising Gene(s) Encoded by Epstein-Barr Virus

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IMMORTALISING GENE(S) ENCODED BY EPSTEIN–BARR VIRUS

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The causal role of Epstein–Barr virus (EBV) in infectious mononucleosis and its association with two human malignancies, Burkitt's lymphoma and nasopharyngeal carcinoma, are well-documented (Henle, Henle and Diehl, 1968; Klein *et al.*, 1974; de Thé *et al.*, 1978; Epstein and Achong, 1979; Klein, 1983). Less well-defined and of more recent interest is the recognition of diffuse, polyclonal B-cell lymphomas also associated with EBV. This type of disease, originally noted in studies with the cotton-top marmoset, has now been observed in a number of cases in man, most notably in patients with genetic disorders or receiving immunosuppressive treatment (Miller, 1984). To date, expression of a viral gene product has not been definitively linked to any of the diseases, although an EBV nuclear antigen(s), EBNA, is ubiquitous in EBV-infected cells and is tacitly assumed to be intimately involved in cellular transformation (Reedman and Klein, 1973; Klein and Klein, 1984). It is not clear in every case that viral gene expression need be a prerequisite for the transformation event, however, in that the chromosomal translocations detected in Burkitt's lymphoma cell lines at least allow the possibility of some sort of 'hit and run' mechanism mediated by the virus (Galloway and McDougall, 1983; Klein, 1983). Moreover, for a virus as large as EBV, more than one pathway to cellular immortalisation and transformation could exist. For example, alternative modes of transformation may be involved in the case of B-lymphocytes and epithelial cells.

This paper describes an attempt to resolve some of the questions about EBV transformation and in particular to answer the question of whether cellular alterations can be correlated with any particular region of the EBV genome. Studies focussing on the interaction of EBV with epithelial cells suggest that they can, and reinforce the

notion that B-lymphocytes and epithelial cells may be immortalised by different routes.

B-LYMPHOCYTE IMMORTALISATION

Before proceeding to our experimental results on epithelial cells, it seems pertinent to review briefly the literature on EBV-mediated B-cell proliferation. This is a highly efficient process. B-lymphocytes contain receptors for the virus, and infection of cells derived from human peripheral or umbilical cord blood, or from several species of non-human primates, leads to immortalisation (Falk *et al.*, 1974; Frank, Andiman and Miller, 1976). Recently, Katamine *et al.* (1984) have shown that cells can be immortalised by EBV at different stages of the B-cell differentiation pathway, even before the immunoglobulin gene rearrangements occur. Cells immortalised *in vitro* contain multiple copies of an episomal form of the complete viral genome, the precise copy number being characteristic of an individual cell line. The processes that control viral genome amplification are not known. Nor has the question of integration into the host chromosome been experimentally resolved, although it is assumed that in some cell lines, at least, integration must occur (Anderson and Lindahl, 1976). EBNA can be detected as early as 6–8 h post-infection, reaching a peak at 36 h if high multiplicities of infection are used (Robinson and Smith, 1981).

In attempting to understand the role of EBV in B-cell immortalisation much attention has been focussed on the non-transforming P3HR1 strain of the virus. This variant induces EBNA but is not a mitogen for normal B-lymphocytes and does not immortalise them (Miller *et al.*, 1974; Menezes, Leibold and Klein, 1975). DNA isolated from P3HR1 has been found to have a large deletion (in the *Bam*HI Y and H fragments, see Fig. 1) relative to transforming strains of EBV (Bornkamm *et al.*, 1980; Heller, Dambaugh and Kieff, 1981). Naturally, sequences removed by this deletion have been assumed to play a key role in the process(es) leading to immortalisation and there are data which support this (Stoerker and Glaser, 1983). Unfortunately, studies on P3HR1 have not turned out to be straightforward, since the virus produced by this cell line is extremely heterogeneous and numerous alterations, in addition to that cited above, can be detected by restriction enzyme analysis alone (Rabson *et al.*, 1982). Use of cloned cell lines (Heston *et al.*, 1982) should in

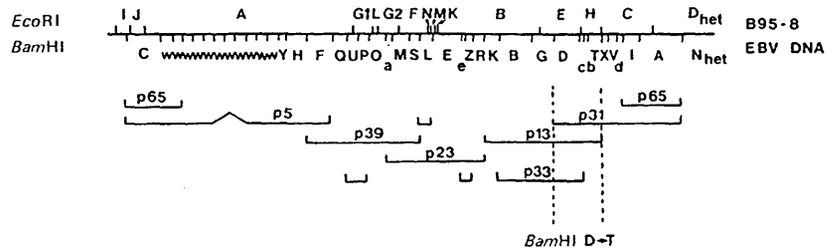


Fig. 1. A cosmid library of EBV DNA. EB virion DNA from B95-8 cells was partially digested with *Bam*HI, fragments about 40 kb in size ligated to *Bam*HI cleaved cosmid pHC79 DNA (from which the 5'-phosphates had been removed), and the resulting hybrid DNAs cloned according to the procedure of Hohn and Collins (1980). Individual recombinant DNAs were isolated and analysed as previously described (Griffin and Karran, 1984). Those that yielded results consistent with the known physical maps of EBV DNA (as shown) were selected to make up a library of largely overlapping fragments which consisted of six clones designated p65, p5, p39, p23, p13 and p31, as indicated. (p33 was selected originally as part of the library because 'miniprep' analysis showed it to contain the *Bam*HI R fragment, necessary to provide an overlap of p23 and p13. Further characterisation failed to confirm this. The heterogeneous terminal fragments, N_{het}, were presumably not represented within this library.)

time produce meaningful results with this strain of virus. In support of some role in immortalisation for the region of the genome deleted in P3HR1, the well-characterised Burkitt's lymphoma line, Daudi, also has a deletion that overlaps that of P3HR1; Daudi cells can be induced to produce virus which also appears to be non-transforming (Jones *et al.*, 1984; D. H. Crawford, personal communication). As Daudi is a primary tumour isolate, it is therefore important to study it further in this context.

In spite of the very considerable amount of data that has accumulated from studies on the immortalisation of B-lymphocytes with EBV, only a small part of which has been described above, there is still very little of significance that can be said regarding the role of the viral genome, except that (*a*) it seems to be highly relevant to the process, (*b*) it is retained, and (*c*) viral gene products are expressed to various degrees in immortalised cells. In a recent review, Miller (1984) has focussed on five interesting and potentially important regions of the genome including that covered by the P3HR1 deletion and that coding for EBNA. The others encompass the large internal repeat (*Bam*HI W), the termini, and a region (*Eco*RI J) that encodes two small, abundant RNA molecules. Whether any, or all, of these regions are relevant to the induction of continuous cell growth remains to be seen.

It is important to stress the difference between immortalisation *in*

vitro and tumourigenesis *in vivo*, since the first process need not, and frequently does not, lead to the other. This point has been recently emphasised elsewhere (Klein, 1981, 1983). Thus, EBV-immortalised lymphoblastoid cell lines are normally diploid, are not clonable in agarose and *do not* produce tumours in nude mice when injected subcutaneously (Nilsson *et al.*, 1977). On the other hand, cells derived from Burkitt's lymphomas are chromosomally abnormal, grow in agarose and *do* produce tumours *in vivo* in nude mice. The principle role of EBV appears to be to convert the normal mortal cells to continuously proliferating (immortalised) cells; it has been argued by Klein and others that the tumourigenic phenotype of the Burkitt's lymphoma cell is a consequence of the specific chromosomal translocations in these cells (Klein, 1979) and may be related to the altered location and expression of cellular oncogenes, especially c-*myc*, normally found on chromosome 8 (for instance, see Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982). If it is independent of viral gene expression, the nature of the co-factor that results in chromosomal rearrangement has not been elucidated. It may be a consequence of a more or less specific recombinational event (a genetic accident) that accompanies the continuously dividing immortalised B-cell, or possibly a specific external stimulus. It is perhaps noteworthy that *in vitro* such chromosomal translocations rarely occur spontaneously, so that the concept of an active co-factor in the B-cell malignancy must continue to receive serious consideration.

IMMORTALISATION OF EPITHELIAL CELLS

The undifferentiated, or poorly differentiated, carcinomas of the nasopharynx (so-called nasopharyngeal carcinoma, NPC) associated with EBV are a major health problem in certain areas of Southern China and to a lesser extent in other parts of the world (de Thé *et al.*, 1975; Zeng *et al.*, 1980). One of the uncertainties regarding this disease is the mode of entry by the virus, since no epithelial cells with receptors for EBV have yet been found. Whilst it cannot at this stage be concluded that such cells do not exist, an alternative mode of entry might involve a fusion event between an EBV-bearing B-cell and an epithelial cell of the nasopharynx (Sixbey *et al.*, 1983; Li *et al.*, 1984). The studies we have carried out on epithelial cells (Griffin and Karran, 1984, see below) have been designed to investigate the existence of a possible immortalising function(s) within the viral genome and to set up a model system *in vitro* for NPC.

To this end, virion EBV DNA isolated from a lymphoblastoid marmoset line, B95-8, was subjected to partial digestion with the endonuclease *Bam*HI; fragments about 40 kb in size were selected and cloned in the cosmid vector pHC79. In all, 780 clones (out of a total of about 40 000 EBV-positive clones produced) were analysed, from which the EBV library shown in Fig. 1 was derived. Individual recombinant EBV DNAs from this library were used to transfect a mixed population of epithelial and fibroblast cells derived from African green monkey (*Cercopithecus aethiops*) kidneys (AGMK) using the calcium phosphate co-precipitation method, essentially as described by van der Eb and Graham (1980), in the presence of carrier calf thymus DNA. Untreated (control) AGMK cells could be passaged for five or six generations in culture before reaching senescence. After several months in culture, following treatment with specific fragments of EBV DNA, small 'foci' were observed in a number of experiments, notably those in which cells had been transfected with DNA from the clone p65, with a mixture of all the cloned DNAs, or with control calf thymus DNA; large distinct 'foci' were observed in the AGMK cells transfected with DNA from clones p13 plus p33, and p31 (Fig. 2). Although none of the other foci could be propagated in culture, a number of cell lines were established from foci selected from the p13/p33 and p31 dishes. The following questions were asked with regard to cells that were propagated from foci: (1) Were they immortalised? That is, would they grow continuously in culture? (2) What type of cells were they? (3) Would they grow in the presence of low serum? (4) Would they grow in soft agar or produce tumours in susceptible animals, such as nude mice? (5) Did they contain integrated EBV DNA? (6) Did they express EBNA? On the basis of what is already known about the interaction of EBV with its natural hosts, the answers obtained from studies on these cells were perhaps not wholly surprising. That is, lines established from AGMK cells transfected with either of the overlapping fragments, p13/p33 and p31, could be subcultured weekly at a 1:10 dilution and grown continuously in culture for over $1\frac{1}{2}$ years. (At this stage, it was assumed that the cells were immortalised and they were not further propagated.) Morphologically, they appeared to be more like epithelial than fibroblast cells (Fig. 3*a*), and this was confirmed by immunofluorescent staining using a monoclonal antibody, LE61 (Lane, 1982), specific for epithelial cells (Fig. 3*b*). Further studies suggested that although the cells had properties, such as the ability to grow in low serum, characteristic of transformed or potentially

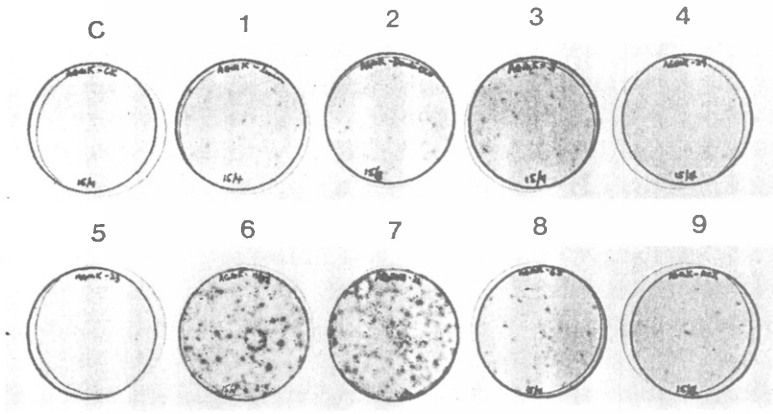


Fig. 2. Transfection studies. Dense focus assays of AGMK cells transfected with different fragments of EBV DNA. Sub-confluent dishes (50 mm) of early passage AGMK cells were transfected with EBV DNA in the presence of calf thymus carrier DNA and calcium phosphate for 6 h at 37°C (van der Eb and Graham, 1980), then media (3% DMEM/3% glutamine, 5% foetal calf serum) removed and fresh media added. After three days in culture, cells were trypsinised and replated at 1:5 dilution. Thereafter, they were grown at 37°C with weekly changes of media. Individual dishes contain AGMK cells transfected with calf thymus DNA only (C), or with linear virion DNA (1); episomal DNA from Daudi cells (2); recombinant DNA from clones p5 (3); p39 (4); p23 (5); p13 + p33 (6); p31 (7); p65 (8); and a mixture of recombinant DNAs (9), as noted. Foci were visualised using Leishman's stain and the data obtained with dishes that had been in culture for about two months are shown. (From Griffin and Karran, 1984.)

tumourigenic cells, they lacked others. Notably lacking was the ability to form colonies in semi-solid media. That is, in soft agar the immortalised cells were found to proliferate for several weeks, after which time no further growth was observed. Neither did the cells die since, even a month after growth had ceased, cells could be re-established in tissue culture on plastic dishes and proceeded to grow normally. Cells injected subcutaneously into nude mice failed to produce tumours (Griffin and Karran, 1984). The conclusions from studies at this juncture were that a minor population of kidney cells that resembled simple cuboidal epithelium had been induced to grow continuously in culture by sub-genomic fragments of EBV, but that the cells *per se* were not capable of inducing tumours *in vivo*. These findings were reminiscent of those obtained following infection of B-lymphocytes with EB virions (see Nilsson *et al.*, 1977). In marked contrast to studies *in vitro* on cellular transformation with *Herpes simplex* virus (Galloway and McDougall, 1983), EBV DNA 'footprints' were found in the chromosomal DNA of the immortalised AGMK cells, as exemplified in Fig. 4. At early passage (Fig. 4A) the pattern of exogenous DNA in the cells was complex and not only

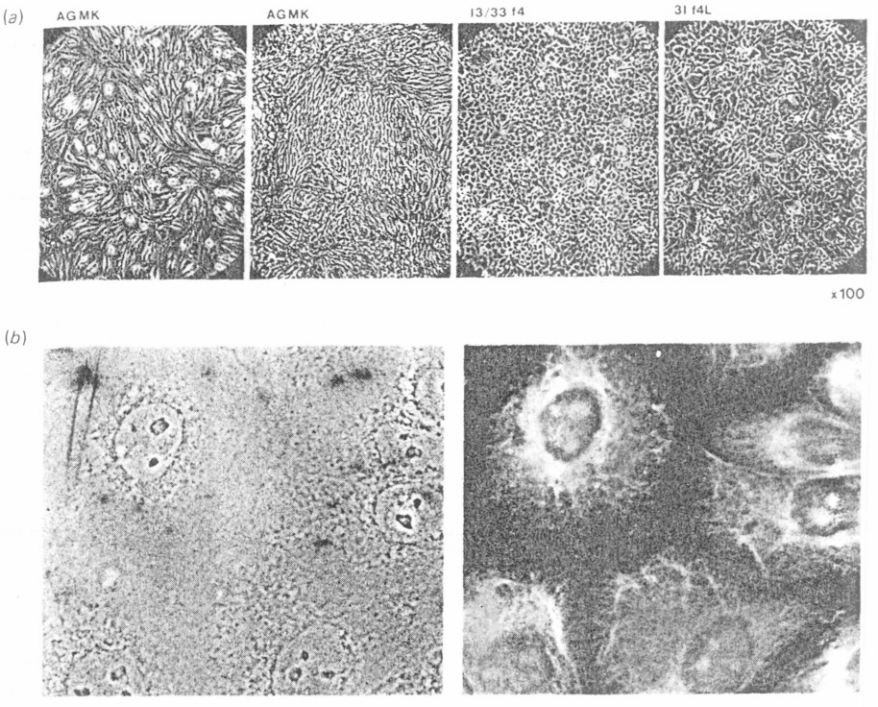


Fig. 3. Morphology of immortalised cells. (*a*) Light micrographs of the heterogeneous population of cells observed in (*left to right*) a sparse and a dense early culture of AGMK cells, and homogeneous cuboidal epithelial cells present in dense cultures of the cell lines 13/33f4 and 31f4L after 20 weeks in culture. 13/33f4 or 31f4L cells examined at earlier (4 weeks) or later (34–76 weeks) periods showed no obvious morphological differences from the 20-week culture. Living cells shown by phase-contrast optics.

(*b*) Sub-confluent 31f4L cells fixed and stained with the monoclonal antibody LE61 that specifically recognises kidney epithelium (Lane, 1982). Cells observed (*left*) by phase-contrast optics and (*right*) by immunofluorescence to show the tonofilament organisation.

were sequences characteristic of EBV DNA observed but those derived from the cosmid vector used in DNA cloning were also present. At later passage (Fig. 4B, C) the latter were lost, and the DNA carried by the cells was largely to be found in a single band that hybridised to EBV DNA. Although exhaustive analyses have not been carried out, the size of this fragment differed among cell lines (Fig. 4D) and the pattern on further passaging of any particular line appeared to remain constant. The single aspect of our data that was surprising is that in none of the cell lines was EBNA expression observed.

At this stage, it seemed important for a number of reasons to ascertain whether experiments such as those described above could be repeated in other systems. First and foremost was the need to

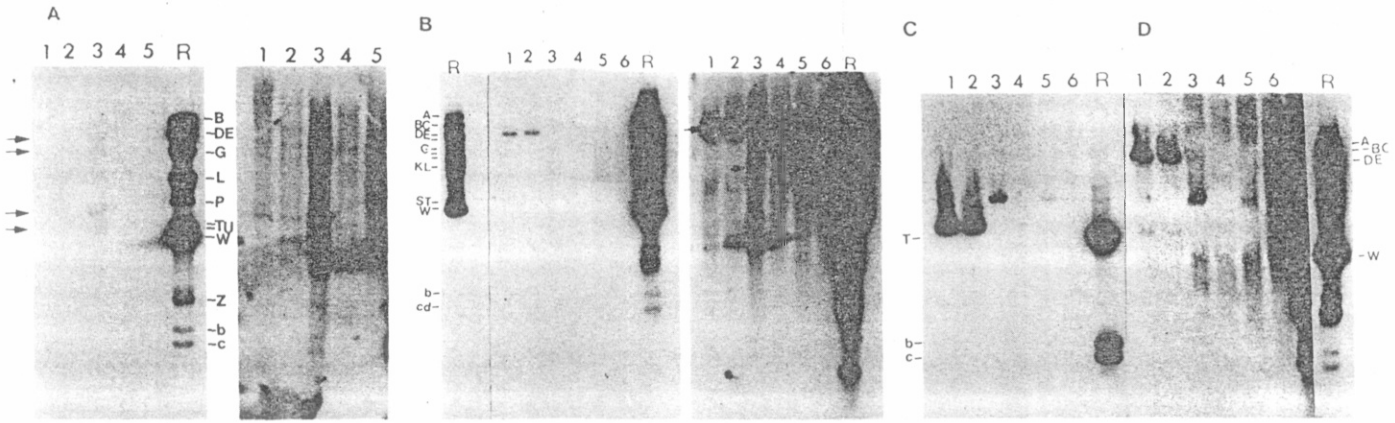


Fig. 4. Autoradiograms of *Bam*HI restriction enzyme-cleaved chromosomal DNA from immortalised AGMK cells hybridised against ^{32}P-labelled nick-translated EBV DNA.

Panel A, two exposures of results obtained with DNA isolated from cells passaged for only four weeks in culture following selection and establishment from individual foci: Panel B, two exposures of a similar analysis of DNA from cells passaged continuously for six months in culture. Whole cell DNA was cleaved with *Bam*HI, fragments separated by electrophoresis on 0.8% agarose slab gels, transferred to nitrocellulose filters and probed with ^{32}P-labelled DNA from a mixture of clones p13 and p33 under standard conditions. Tracks 1–5 contain DNA fragments from cell lines 13/33f2, f3, f4, f4L and f5, respectively, and track R contains EB virion DNA. Prominently hybridising bands are denoted by arrows.

Panel C, illustrates the patterns obtained when the probe was a nick-translated recombinant DNA containing the EBV fragment *Eco*RI H (which hybridises to EBV DNA *Bam*HI fragments T, b and c, as shown) or, panel D, EBV DNA from virions. (Data adapted from Griffin and Karran, 1984.)

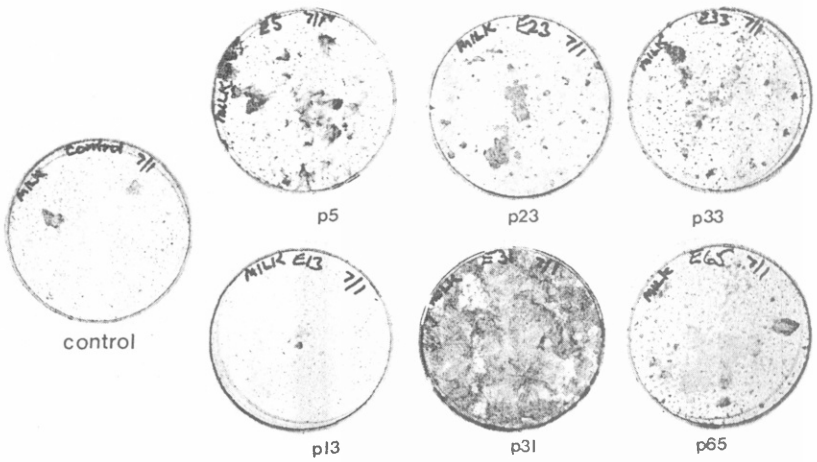


Fig. 5. Transfection studies on human cells. Cells from human breast milk epithelium (Chang *et al.*, 1982) were transfected with recombinant DNA from the EBV cosmid library (see Fig. 1), as illustrated. These dishes have been maintained for about four months with occasional subculturing and are visualised with Leishman's stain.

determine to what extent the immortalisation was a property of the AGMK cells themselves and/or how important was the contribution of EBV DNA to the cell phenotype. Secondly, there was the need to monitor chromosomal changes and alterations in the expression of genetic information so as to assess the role of co-factors in generating a fully malignant phenotype. If chromosomal alterations such as translocations can be used as a marker for the latter (Klein, 1981), the fact that AGMK cells have a diploid number of 60 could complicate any analysis of marker chromosomes in these cells.

Two different studies were set up to answer these questions. One involved transfection of cells from human milk with individual fragments from the recombinant EBV DNA library and the other involved similar types of experiments using primary kidney cells from common marmosets (*Callithrix jacchus*). (In contrast to AGMK cells, common marmoset cells, like human cells, have a diploid chromosome number of 46; moreover, the ready availability of marmoset colonies at the Royal College of Surgeons (Wedderburn *et al.*, 1984) will in the long run allow studies to be carried out *in vivo*). In both experiments, the fragment of EBV DNA found in the recombinant p31 was found to have a marked effect on cell growth. For a finite time (that is, over a six-month period) the human cells, as illustrated (four months) in Fig. 5, were stimulated to grow. Unlike experiments reported earlier with SV40 (Chang *et al.*, 1982), we have

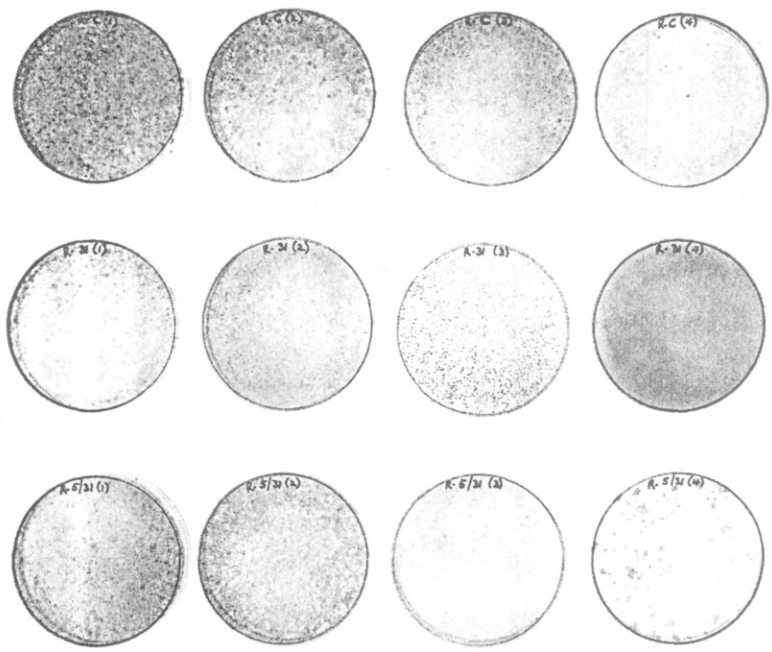


Fig. 6. Transfection studies on common marmoset kidney cells. Cells from primary cultures of common marmoset (*Callithrix jacchus*) kidneys were transfected with recombinant DNA from the EBV cosmid library (see Fig. 1) and sub-cultured at three-weekly intervals. Illustrated here (cells visualised with Leishman's stain) are: row A, control cells at passages 1 to 4 (*left to right*); row B, cells transfected with p31 recombinant DNA, passages 1 to 4, and similarly, row C, cells co-transfected with a mixture of p31 and p5 recombinant DNAs. By the fifth passage, the latter were completely confluent, resembling the fourth passage of cells seen in row B. The prefix 'R' on each dish means that cells were transfected again, about a week after the initial transfection, and before the sub-culture protocol was adopted. Cells transfected only a single time (not shown) were immortalised much more slowly, supporting the notion that gene dosage may be important in this process.

not, however, yet been successful in establishing a continuously growing line. More success has accompanied the studies with marmoset cells (D. King, L. Karran and B. E. Griffin, unpublished). Again, p31 DNA proved to be a growth stimulant. Although no densely growing foci were ever observed (Fig. 6), after about three months (or five passages) in culture, the epithelial population of cells had outgrown the fibroblasts and, by the tenth passage, a homogeneous population of epithelial cells (see Fig. 7A) was evident. From this time on, cells were sub-cultured weekly and have now been continuously in culture for nearly a year. As observed in the case of AGMK cells, these marmoset cells have only a finite capacity to grow in soft agar, which is nonetheless superior to that observed with

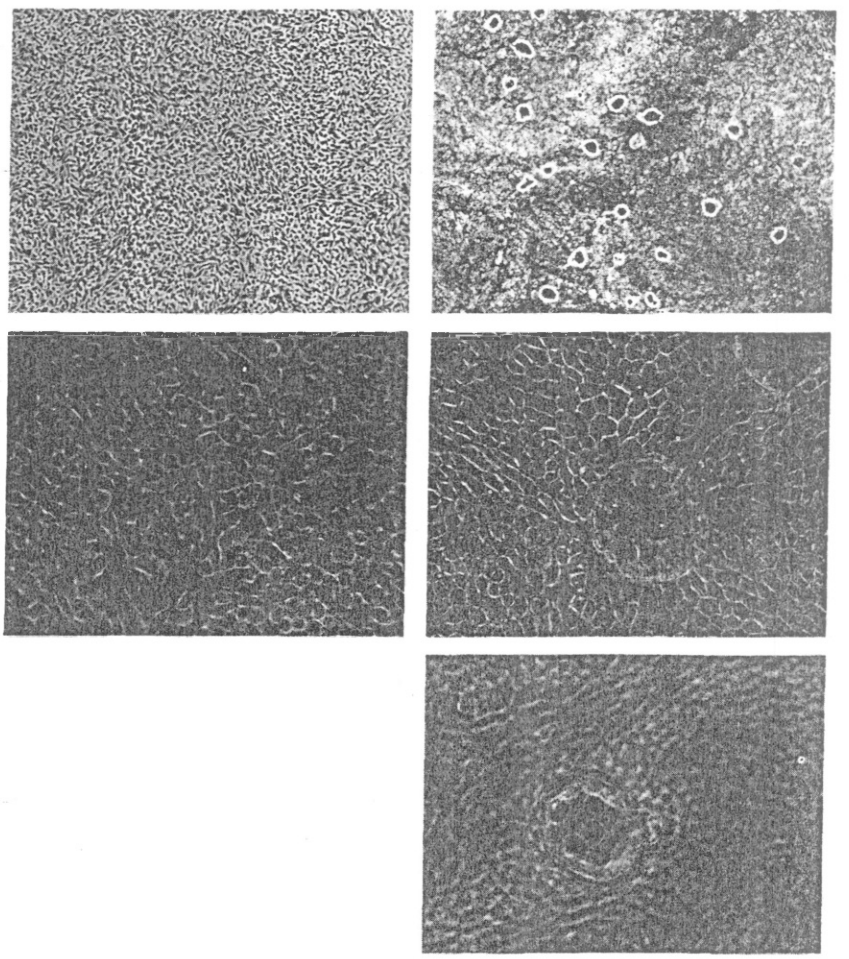


Fig. 7. Common marmoset kidney cells growth stimulated by sub-genomic fragments of EBV DNA. Cells at the tenth passage subsequent to transfection with recombinant DNA from the cosmid clone p31, or p31 and p5, examined by light microscopy. Panel A: Dense culture of cells designated R-31 (see Fig. 6) at two magnifications. Panel B: Dense cultures of cells designated R-5/31 (see Fig. 6). The presence of 'domes' or hemicysts is clearly evident in R-5/31 cultures. In bottom figure of B only cells comprising the dome are in focus.

Immunofluorescent staining of these cells with the monoclonal antibody LE61 (see Fig. 3*b*) showed the epithelial nature of all cells in both Panel A and Panel B (data not given).

control cells. Moreover, whereas the latter had completely senesced after two months in the semi-solid support, the p31 DNA–treated cells could be removed from soft agar and re-established on plastic. Finally, 'footprints' of the complete EBV DNA used in the transfection procedure were still evident in the marmoset cells after ten

passages, as had previously been observed in the AGMK cell experiments. Although these became simpler in late passage cells, no alterations in chromosomes were observed.

A very interesting phenotypic alteration of marmoset kidney cells was observed when they were transfected simultaneously with p31 recombinant DNA and a region of EBV DNA encompassed in another cloned fragment, p5 (see Fig. 1). Instead of a flat homogeneous population of epithelial cells becoming established, a much more mobile, apparently more highly differentiated population was observed. These have now also been in culture nearly a year but they grow slowly and, unlike cells transfected with p31 DNA alone, cannot be passaged weekly. Moreover, when individual dishes of cells are kept in culture past confluence, they develop 'domes' or hemicysts characteristic of actively transporting renal epithelium (Fig. 7B), similar to those observed, for example, in Madin–Darby canine kidney (MDCK) cells, a continuously growing line established from an apparently normal dog kidney (Lever, 1979; Jefferson *et al.*, 1980; Cereijido, 1984). Cells with the potential to form domes can be actively stimulated by certain solvents, such as DMSO, that are potent inducers of cell differentiation; conversely, dome formation can be abolished by ouabain, which inhibits transepithelial ion transport (Birek *et al.*, 1982). Similar effects in the presence of these agents were observed with the marmoset cells that had been co-transfected with p5 and p31 DNAs, suggesting that information obtained from the second fragment of EBV (contained in p5) might have relevance to active ion transport and/or cell differentiation. It may be noteworthy that the p5 recombinant DNA encodes three of the five regions of the EBV genome that Miller (1984) has suggested to be of especial importance to the biology of EBV.

These data taken together strongly support the notion of a function(s) within the EBV genome that, directly or indirectly, stimulates cell growth and at least in non-human primates can result in the immortalisation of sub-classes of epithelial cells. Based on the studies with the mixture of fragments (p31 and p5), it would appear that, in the presence of the immortalising function(s), other consequences of virus–cell interactions can be observed. Very preliminary data on some of the immortalised cells suggest that mRNAs with homology to EBV are being expressed. If this can be confirmed, it will stimulate us in our search for EBV-coded proteins that play important roles in the overall process of cellular immortalisation. Further experiments aimed at defining the minimum size of fragment which, when

transfected into a suitable cell, will produce continuously growing lines appear promising. Cleavage of the recombinant DNA from p31 with the restriction enzymes *Eco*RI, *Bgl*II and *Hin*dIII appear to abolish this activity, but cleavage with certain other enzymes apparently does not. These types of studies thus suggest that a minimum immortalising region may in time be identified and, with the aid of the total DNA sequence (Baer *et al.*, 1984), a viral gene localised.

CONCLUSIONS AND UNSOLVED PROBLEMS

Our data on immortalisation of primate epithelial cells in culture by specific sub-genomic fragments of EBV DNA are consistent with the large volume of information that has accumulated over a number of years from studies on the diseases associated with this virus. The latter support the notion that EBV can induce continuous proliferation of cells but that other stimuli (such as tumour promoters, chemical carcinogens, or cellular 'oncogenes') may be required for full expression of malignancy. By introducing viral DNA directly into the cell we have avoided the question of whether epithelial cells with receptors for EBV exist. Thus, at this juncture, we would suggest that, using genetic information initially supplied by the virus, we have obtained model epithelial cell lines that can be used for the investigation of agents needed in subsequent stages along the pathway to a malignant phenotype; that is, we are part of the way towards setting up a model system *in vitro* for nasopharyngeal carcinoma in non-human primates. Whether chromosomal alterations are involved in this process, as is the case with Burkitt's lymphoma, is not clear, although Mittelman *et al.* (1983) have observed a haploid change in chromosome 3 in two cases of NPC tumours. So far, our attempts to produce phenotypic alterations in immortalised monkey kidney cells by continuous passage in culture have not been successful. This essentially passive approach, which presupposes a 'genetic accident', may be an important step *in vivo* but is probably too slow to yield meaningful results *in vitro*. For example, passaging immortalised AGMK epithelial cells for nearly two years in culture failed to produce any marked alteration in the cells. Therefore, to accelerate the process, we have begun studies designed to determine if gene products from other viruses or cellular oncogenes can convert immortalised cells to potentially malignant ones; alternatively, we are investigating whether addition of chemicals observed to be active in

tumour promotion or cell differentiation can accomplish this effect. In this regard, two recently published papers are of considerable interest in that they study environmental factors that, together with EBV, may be involved in the development of NPC. Using the induction of viral early antigens in Raji cells as an assay, Ito *et al.* (1983) found that diterpene esters from plants of the Euphorbiacae family were highly effective in their ability to initiate expression of some EBV functions. Similarly, a group of scientists in Beijing (Zeng *et al.*, 1983) have investigated a large number of commonly used medicinal herbs to determine whether factors that can alter EBV gene expression, and presumably host gene expression, are present in Chinese medicines. Five species of plants from the Thymelacacae family were strongly positive in this respect, especially if used together with sodium butyrate, a chemical that stimulates cells to differentiate (Leder and Leder, 1975).

Our immortalisation studies focus attention on a portion of the viral genome that contains none of the 'five interesting and potentially important regions' of EBV discussed in a recent review (Miller, 1984). Thus, we are forced to conclude that although we find 'footprints' of EBV DNA in the continually growing monkey-epithelial cells, viral gene expression may not be an essential feature of the cellular immortalisation process. Alternatively, in the absence of good tissue culture systems for the virus, and considering its large size, it seems more likely that a very important viral function has up to now escaped detection.

Further, it is difficult to correlate our data with those of Stoerker and Glaser (1983), who essentially rescued transforming virus from a hybrid cell line that contained the DNA of the non-immortalising P3HR1 strain of EBV using a portion of the viral genome encompassed within *Bam*HI fragments W, Y and H (see Fig. 1). Theirs is a very complex experiment, and the structure of the DNA within their hybrid cell has not been characterised and may be very heterogeneous; also, their assay is immortalisation of cord blood lymphocytes. Our experiments, on the other hand, are more direct. Nonetheless, this apparent conflict of data exists. Among several projected explanations, the possibility that the mechanism by which EBV transforms B-lymphocytes and epithelial cells differs must receive strong consideration; that is, this large virus may produce the same phenotype in different types of cells by alternative routes. This is an important concept and needs further investigation. In the long run, the situation will undoubtedly prove even more complex than this; for example,

Åman, Ehlin-Henriksson and Klein (1984) have recently shown that among B-lymphocytes, only resting cells could apparently be infected and immortalised by EBV. Thus, not only the type of cell but the state of the cell may play an important role in determining whether it responds to the viral information in such a way as to become immortalised and ultimately malignant. EBV has set up an effective symbiotic relationship with its host(s) and it will obviously require a great deal of effort and insight to understand the relationship (see discussion in Rickinson, 1984). Our experiments should allow us to explore an important aspect (immortalisation) of this relationship.

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