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THE INDIVIDUAL ROLES OF THE MAJOR E1B PROTEINS IN TRANSFORMATION AND THEIR FUNCTION IN THE LYTIC CYCLE OF ADENOVIRUS TYPE 5

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Abstract

Transformation by human adenovirus type 5 requires the cooperation of gene products from both the E1a and E1B early transcription units. Our major goal was to better understand the individual roles that the E1B proteins play in the transformation process. In order to determine the specific contribution made by the two major E1B proteins, 19K and 58K, mutants were constructed which were defective in the synthesis of each protein. Analysis with these mutants suggested that 58K appeared to be necessary for efficient plaque formation on human HeLa cells whereas 19K was not required. Mutants which failed to produce 19K or made a truncated 19K product displayed the cyt/deg phenotype characterized by production of large plaques and degradation of DNA. These properties were not apparent with point mutants at methionine 120 or serine 164 of 19K or with mutants defective for 58K production. All E1B mutants produce E1A at levels comparable to wild type adenovirus 5, suggesting that neither E1B protein affects the regulation of E1A expression. Of interest was the observation that in combination with E1A, both 19K and 58K were able to induce transformation of baby rat kidney cells. However, the efficiency of transformation was greatly increased if both these E1B products were present. It seems likely that the mechanism of transformation involving each of these E1B proteins utilizes different pathways, but these pathways appear to be additive.

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TO MY FAMILY
NEW AND OLD

Mike

Ria, Pat Anya, Elia
Maika, Jean Irene, Tim
Finn and
Sean

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LIST OF ABBREVIATIONS:

Ad2, 5, 12, etc.	adenovirus type 2, 5, 12 etc.
APS	ammonium persulfate
bp	base pairs
BRK cells	baby rat kidney cells
cpe	cytopathic effect
cyt	cytopathic phenotype
CREF	cloned rat embryo fibroblasts
CR1, 2, 3	conserved region 1, 2 and 3
dATP, dCTP, dGTP, dTTP	deoxynucleotide triphosphates
DBP	DNA binding protein
deg	degradation phenotype
dl	deletion
DNA	deoxyribonucleic acid
E1, E2, E3, E4	early regions 1, 2, 3 and 4
EDTA	ethylenediamine tetracetic acid
et al	and co-workers
FC	fetal calf serum
HS	horse serum
ITR	inverted terminal repeat
K	Kilodaltons
L	litre
L-glu	L-glutamine
M	molar
min	minute(s)
MEM	minimal essential medium
mg	milligram(s)
ml	millilitre(s)
mM	millimolar
moi	multiplicity of infection
mRNA	messenger RNA
mu	map unit(s)
μ	micro
NBC	new born calf serum
O/N	over night
ORF	open reading frame
PBS	phosphate-buffered saline
pfu	plaque forming units
pi	post infection
pm	point mutant
pol	polymerase
P/S	penicillin-streptomycin
R	residue (amino acid No.)
RF	replicative form
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
S	Svedberg

SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
TP	terminal protein
UV	ultraviolet radiation
wt	wild type

Chapter I.

I. Introduction.

I.I. Adenovirology.

I.I.1. Classification of human adenoviruses.

In 1953 an adenovirus was identified as the cytotoxic agent of cultured human adenoids (Rowe *et al.*, 1953). Adenoviruses infect a wide variety of species worldwide including human, simian, bovine, canine, murine, and avian (Pierara *et al.*, 1963). Over 41 human adenovirus serotypes been identified, and these primarily cause respiratory, ocular, and enteric diseases (Kelly *et al.*,1988). In 1962, Trentin *et al.* found that an adenovirus serotype was able to induce malignant tumours in newborn hamsters (Trentin *et al.*, 1962). The demonstration that this human virus was oncogenic initiated much interest in the study of adenoviruses. Adenoviruses have not been linked to any human malignancies but continue to provide a good and safe molecular model system to the study the basis of cancer.

Human adenoviruses (Ad) have been classified using several methods which seem to yield comparable subgroupings. These include hemagglutination (Rosen,

1968; Rosen *et al.*, 1969; Hiernolzer, 1973), antigen production (McAllister *et al.*, 1969), guanine plus cytosine (G + C) content (Green and Pina, 1964; Pina and Green, 1965), oncogenic potential (Huebner etal, 1965), and DNA sequence homology (Green *et al.*, 1979). Different adenoviruses within a given subgroup have 90% homology by DNA:DNA hybridization but have only approximately 23% homology with members of other subgroups (Green *et al.*, 1979). Horowitz (1990) has used the above criteria to describe six subgroups (A to F) of human adenovirus. Group A adenoviruses (which includes Ad12 and Ad31 serotypes) are highly oncogenic and have a G+C content of 48-49%. Group B viruses (including Ad3 and Ad31) are moderately oncogenic and have a G+C content of 50-52%. Group C adenoviruses (including Ad2 and Ad5) are weakly oncogenic or nononcogenic and have a G+C content of 57-59%. Groups D, E, and F (including Ad8, Ad4, and Ad40 respectively) viruses are all weakly oncogenic or nononcogenic with a G+C content of 57-59%. The following discussion will deal primarily with group C adenovirus type 5 (Ad5) and a highly related serotype adenovirus type 2 (Ad2).

I.I.2. The structure of the adenovirus virion.

Adenoviruses have a double stranded linear DNA genome of length of 35Kb. The adenovirus virion is made up of at least nine (II-IX) different polypeptides forming the capsid and core.

Adenoviruses are nonenveloped icosahedral viruses with a diameter of 700Å

(Horne *et al.*, 1959). There are three main polypeptides that form the capsid of the adenovirus virion, hexon (polypeptide II), penton base (polypeptide III), and a fiber protein (polypeptide IV) (Maizel *et al.*, 1968; Walter and Maizel 1974). The outer capsid consists of 252 morphological units termed capsomeres, which are composed of the major coat proteins (Horne *et al.*, 1959). The majority of these capsomeres, the 240 hexons, are formed of trimeric hexon polypeptides located on the 20 facets and along the 30 edges of the icosahedron (Horwitz *et al.*, 1970). The remaining 12 capsomeres, termed pentons, lie at the 12 vertices of the icosahedral capsid and are composed of penton base and penton fiber polypeptides (Ginsberg *et al.*, 1966; van Ostrum and Burnett, 1985). Pentons each consist of pentomeric penton base polypeptides with trimeric fiber polypeptides projecting outward (van Oostrum and Burnett, 1985). The conserved amino terminus of the fiber protein is attached to the penton base and the carboxyl end projects from the capsid to form a globular knob. There are also two minor proteins present in the capsid, polypeptides IIIa and IX. Polypeptide IIIa is present in 74 copies per virion and acts to connect to the icosahedral vertex regions to the core polypeptides (Everitt *et al.*, 1975; van Oostrum and Burnett, 1985). There are 247 copies of polypeptide IX per virion. These are associated with hexon and are involved in the stabilisation of hexon-hexon interactions (Colby and Shenk 1981; van Oostrum and Burnett, 1985).

The adenovirus core may be separated from the capsid proteins by differential degradation of the virion by heat, by pyridine, or by sarcosyl treatment. The core is a spherical virion subunit of diameter 21.6nm (Brown *et al.*, 1975). The

adenovirus core accounts for 20% of the virion protein (Russell *et al.*, 1968). It consists of 1070 copies of polypeptide VII, 180 copies of polypeptide V, and a minor μ protein (Walter and Maized, 1974; Anderson *et al.*, 1989). The major virion proteins VII and V are basic and resemble arginine rich histones but unlike histones they contain tryptophan and are immunoprecipitated with viral specific antisera (Hosokawa and Sung, 1976). Polypeptide VII is bound tightly to the viral DNA (Russell *et al.*, 1968;Brown *et al.*, 1975). Polypeptide V is found in close proximity to both capsid proteins (penton base and hexon) and the capsid polypeptide III (Everitt *et al.*, 1975). Freeze fracture techniques suggest that polypeptide V forms a shell around the virion core (Brown *et al.*, 1975). Staphylococcal nuclease digestion of adenovirus virion DNA generates 180 monomeric particles with a regular size of 150-200 base pairs (bp) (Corden *et al.*, 1976; Mirza and Weber, 1982). Based on stoichiometric analysis it has been calculated that each 150bp unit of core DNA is wrapped around 3 dimers of polypeptide VII. Another 30bp of linear DNA between the dimers of polypeptide VII are associated with 1 copy of polypeptide V (Corder *et al.*, 1976; Mirza and Weber, 1982).

The adenovirus genome is a linear duplex DNA molecule with a molecular weight of 23 x 10^6 daltons (Green *et al.*, 1967). There are number of distinctive features of the viral DNA. Firstly, when the genome is denatured and reannealed at low DNA concentration, single stranded circles with panhandle structures are observed (Wolfson and Dressler, 1972). This observation suggested that at the ends of the genome there are inverted terminal redundancies (Steenbergh *et al.*, 1977;

Challberg and Kelly, 1989). For Ad5 this terminal repeat consists of 103 base pairs. Secondly, the viral DNA isolated from adenovirus infected cells has a 80K precursor terminal protein (pTP) bound to each of 5′ ends of the DNA by a phosphodiester linkage (Rekosin *et al.*, 1977; Lichyetat, 1981; Tamonoi and Stillman, 1982; Kelly *et al.*, 1988). The pTP is involved in a novel mechanism of protein priming which allows the 5′ ends of the DNA to be replicated (Rekosin *et al.*, 1977).

I.I.3. Adenovirus replication.

The infectious cycle of adenovirus is divided into early (E) and late (L) phases, the latter commencing by definition with the onset of viral DNA replication. The adenovirus lytic cycle is efficient, resulting in 4000-10,000 plaque forming units (PFU) per cell, 36-48 hours post infection (pi) (Green and Daesch, 1961). A number of stages are involved in the adenovirus infectious cycle including attachment, penetration and uncoating, transcription of early genes, DNA replication, late gene transcription, early to late switch, and virion assembly.

I.I.3.a. Attachment, penetration and uncoating.

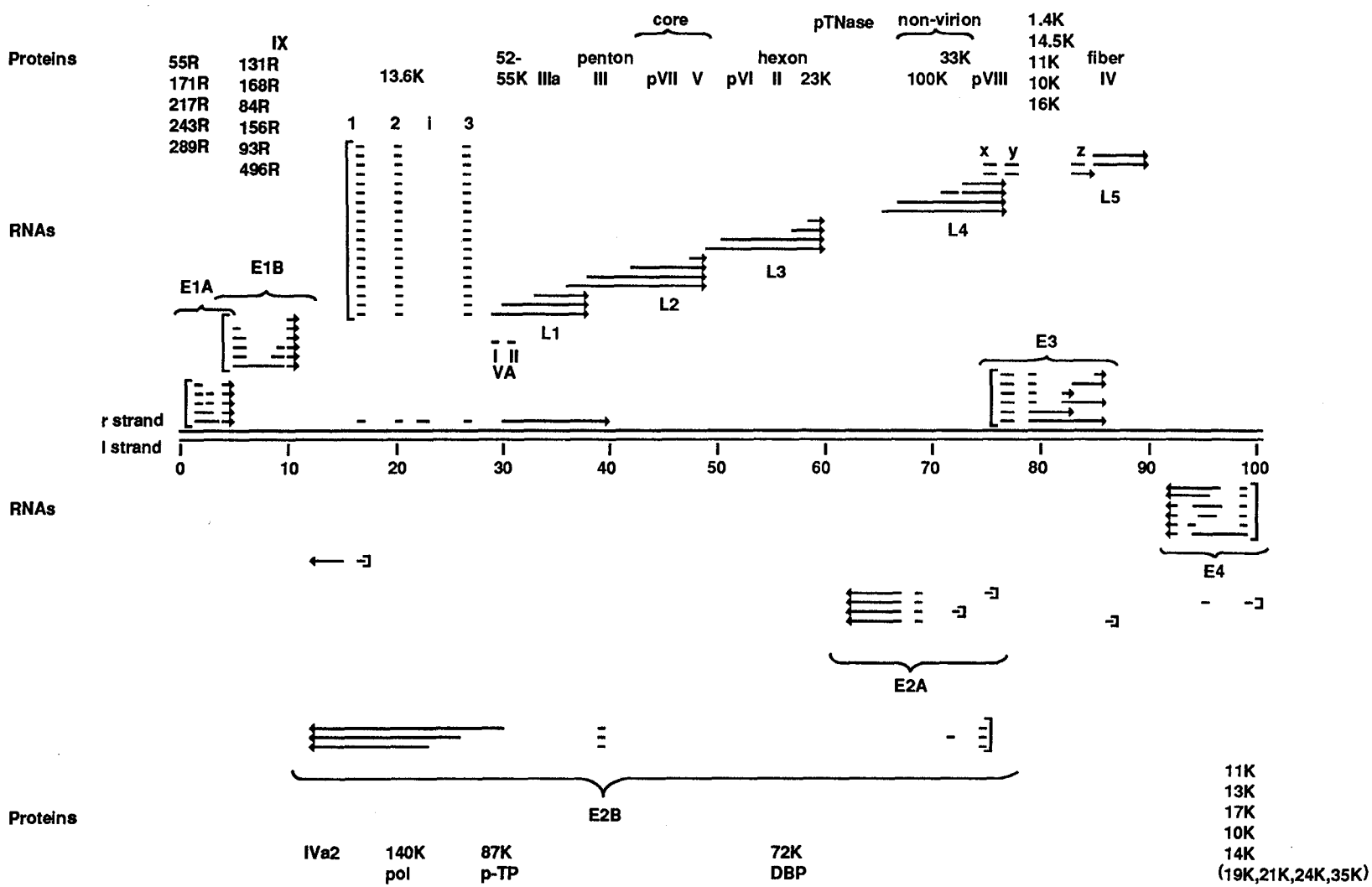
Adenoviruses attach to cell surface receptors through the fiber polypeptide located at each vertex projection of the icosahedron (Hannache and Boulanger, 1977). Once attached the virion is endocytosed, in what may be a low pH dependent process, into a coated vesicle formed from the preexisting cell membrane (Chardonnet and Dales, 1970; Marsh and Helenius 1989). Only minutes elapse before the adenovirus particles are transferred from the cell surface to the perinuclear site of uncoating. It has been suggested that the virion moves toward the nucleus along microtubule pathways (Dales and Chardonnet, 1973). Once in the proximity of the nuclear pore the virion is uncoated leaving a thin protein shell (polypeptide VII) enclosing the nucleoprotein (Morgan *et al.*, 1969; Chardonnet and Dales, 1970). The process of uncoating is mediated by ATPase (Dales and Chardonnet, 1973). Soon this thin protein shell ruptures and the nucleoprotein passes directly into the nuclear matrix (Morgan *et al.*, 1969).

I.I.3.b. Early transcription.

Early transcripts are complementary to both viral DNA strands, and are promoted in either the rightward (R) or leftward (L) direction (Figure 1). The early (E) cytoplasmic mRNAs are complementary to 4 defined regions (E1 to E4) on the viral genome (Berk and Sharp, 1978). Each E region gives rise to a family of RNA

Figure 1- Adenovirus type 5 genome.

The figure shows the transcription units of the Ad5 genome. Early region transcripts are designated E (E1A, E1B, E2A, E2B, E3 and E4), while the late region transcripts designated L (L1 to L5). The right and leftward coding strands are shown as r and l respectively. The numbers along the genome from 0 to 100 represent map units (mu or %). This diagram was a gift from S.A. Brown.


Proteins
IX
55R
171R
217R
243R
289R
131R
168R
84R
156R
93R
496R
13.6K
core
52-
55K
IIIa
penton
III
pVII
V
hexon
pVI
II
23K
pTNase
non-virion
33K
100K
pVIII
1.4K
14.5K
11K
10K
16K
fiber
IV
RNAs
1
2
I
3
E1B
E1A
L1
L2
L3
L4
L5
x
y
z
I
II
VA
E3
r strand
l strand
0
10
20
30
40
50
60
70
80
90
100
RNAs
E4
E2A
E2B
Proteins
IVa2
140K
pol
87K
p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

species resulting from alternative splicing patterns (Chow *et al.*, 1977; Chow *et al.*, 1979). The strand transcribed to the right contains E1 and E3. The E1 region is found within the leftmost 11% of the viral genome and can be broken down into three individually promoted regions termed E1A, E1B, and the gene for peptide IX (Wilson *et al.*, 1979). The L strand which is transcribed to the left codes for E4 and E2. The E2 region can be subdivided into two independently promoted regions E2A and E2B. Early transcripts can be further broken down into pre-early (E1A), delayed early (E1B and E4), and intermediate early transcripts (E2 and E3). The E1A gene products must first be expressed, for efficient transcription of E1B, E2A, E2B, E3, and E4 (Berk *et al.*, 1979; Jones and Shenk, 1979).

I.I.3.c. Early proteins.

The coding region for E1A resides at the left end of the adenovirus genome between 1.3 and 4.5 map units. E1A gene products are the first early region proteins produced and require no prior viral protein synthesis (Berk *et al.*, 1979; Nevins *et al.*, 1979; Glen and Ricciardi, 1988). ElA produces five transcripts of which the 13s and the 12s mRNAs are the major products. These messages encode proteins of 289 and 243 residues (R) that are identical except for the presence of 46 internal amino acids representing a unique region in the 289R protein. The unique region is responsible for the transcriptional transactivation of the other early regions (E2, E3, and E4) (Berk *et al.*, 1979; Jones and Shenk, 1979; Jelsma *et al.*, 1988).

The E1A gene products have a number of other functions including the induction of differentiation of F9 stem cells in culture (Montano *et al.*, 1987), stimulation of baby rat kidney (BRK) cell proliferation and DNA synthesis (Zerler *et al.*, 1987), transcriptional repression (Borrilli *et al.*, 1984; Velcich and Ziff, 1985), and cellular transformation (Houweling *et al.* 1980). These functions of E1A will be discussed in greater detail later.

The E1B region (4.6-11.2 map units) begins to be transcribed 60 to 90 minutes post infection and reaches maximal rate at 7 hours (Nevins *et al.*, 1979). Two major unrelated E1B protein, of 19 kilodaltons (K) and 58K are encoded by the E1b region. The 58K protein facilitates transport and accumulation of viral mRNA late after infection while blocking the same processes for cellular mRNA (host shut-off) (Pilder *et al.*, 1986; Samulski and Shenk, 1988). The 58K protein has also been shown to complex with both viral and cellular proteins. It is thought that E4-34K protein and the E1B-58K act along similar pathways to affect RNA metabolism (Weinburg and Ketner, 1986). The 58K protein has also been shown to bind to the cellular protein p53, which is the product of an anti-oncogene (Pilder *et al.*, 1984). The 19K protein plays a role in both protecting host and viral DNA from degradation and in both preventing enhanced cytotoxicity effect on host cells upon infection (Chinnadurai, 1983; White *et al.*, 1984). Proteins from both the E1A and E1B regions of adenovirus are involved in oncogenic transformation of rodent cells and will be discussed further in the following sections.

The E2 region is the last early region to be expressed, with initiation of

expression beginning 2 hours post infection (pi) (Nevins *et al.*, 1979). E2A codes for a 72K single stranded DNA binding protein (DBP) that protects nascent ssDNA from nuclease attack and separates it from the replication complex (Neale and Kitchingman, 1990). The DBP has also been implicated in the transactivational control of E4 region (Nevins and Winkler, 1980). The DBP has three highly conserved domains and has extensive charge heterogeneity as a result of post-translational phosphorylation (Linne *et al.*, 1979; Klein *et al.*, 1977; Neale and Kitchingman; 1990). The DBP is maximally expressed from 4-11 hours pi and synthesis is arrested with the onset of late gene transcription (Gilead *et al.*, 1976). The E2B region encodes the adenovirus terminal protein precursor which functions as a primer for the initiation of viral DNA replication (Smart and Stillman, 1987). The E2B region also codes for the 140K adenovirus DNA polymerase (Alestrom *et al.*, 1982).

The E3 and E4 regions are expressed following E1A and are transcribed maximally by three hours pi (Nevins *et al.*, 1979). The E3 transcription unit produces messages having a number of different open-reading frames and codes for at least three proteins involved in evading the host's immune responses. In infected host cells, the Ad2 E3 19K protein binds to human class I major histocompatibility (MHC) antigen and prevents its terminal glycosylation (Burgert *et al.*, 1985). In the Ad12 system the E1A gene product functions to down regulate transcription of the gene coding for MHC antigen in infected cells (Katon *et al.*, 1990). Another E3 protein of molecular weight 14.4K, inhibits the lysis of adenovirus infected cells by

tumour necrosis factor (TNF) (Gooding *et al.*, 1988; Horton *et al.*, 1990). An E3 10.5Kd protein induces an epidermal growth factor (EGF)-like endocytosis and degradation of the EGF receptor (Carling *et al.*, 1989; Tollefson *et al.*, 1990). Further study of the 10.4K protein may provide insight into the EGF signal transduction and antiviral host defenses (Tollefson *et al.*, 1990).

The E4 region codes for a number of polypeptides, including an 11K and a 34K protein. The 11K protein is found in two forms in the infected cell, one of which is associated with the nuclear matrix (Sarnow *et al.*, 1982). The 34K protein, encoded by E4 open reading frame (ORF) 6, plays a role in efficient viral growth during the lytic infection (Cutter *et al.*, 1987). The E4 34K protein has been found to be associated with the E1B 58K protein which is also necessary for viral growth (Sarnow, *et al.*, 1982). The E1B 58K - E4 34K complex mediates efficient transport of late viral messages from the nucleus to the cytoplasm and/or maintains stability of those messages on reaching the cytoplasm. Also a 116R protein encoded by E4 ORF-3 seems to act in parallel with the complex to permit normal viral DNA replication (Bridge and Ketner, 1990).

I.I.3.d. DNA synthesis.

In human cells productively infected with Ad2 and Ad5 the onset of viral DNA replication usually occurs between 8 and 12 hours after infection (Futtlerer and Winnacker, 1984). The development of an in vitro replication system for adenovirus

has been pivotal in the purification and analysis of the host and viral factors necessary for replication (Challberg and Kelly 1979).

There are at least three functional domains containing sequences (A, B, and C) that contribute to the overall efficiency of initiation of adenovirus DNA replication at either end of the adenovirus genome. Domain A contains nucleotides 1-18, which are the sequences sufficient for a functional origin (Wider *et al.*, 1987; Tamanoi and Stillman, 1983; Hay *et al.*, 1988). Within this 18bp are ten highly conserved nucleotides which are identical to all known adenovirus serotypes (Tamanoi and Stillman, 1983). The B and C domains which are located between 19 to 40bp and 41 to 50bp respectively, provide accessory sequences that significantly increase the ability of the minimal origin A to function as an origin (Wider *et al.*, 1987). Both viral and cellular proteins are required for initiation of replication.

The two viral proteins necessary are the 140K adenovirus DNA polymerase (pol) and the 80K precursor to the 55K terminal protein which serves as a primer (Stillman *et al.*, 1982; van der Vliet *et al.*, 1988). The adenovirus pol is tightly associated with the 80K pTP at the origin and may aid in catalyzing the transfer of dCMP to pTP (Challberg and Kelly, 1981; Thomas, Kelly and Wold, 1988). Initiation takes place at either terminus of the viral genome by the formation of a covalent bond between the pTP and the terminal dCMP of the viral genome. Initiation is strongly enhanced by the presence of three cellular DNA binding proteins NF1, ORPC, and ORPA (Wides *et al.*, 1987; van der Vliet *et al.*, 1988). The mechanism of action of their cellular proteins is unknown but their binding

sites have been mapped. ORPA binds domain A (Rosenfeld *et al.*, 1987), NF1 binds domain B and ORPC binds domain C (van der Vliet, *et al.*, 1988).

After initiation another viral protein is necessary for elongation of DNA synthesis . The DBP is dispensable for initiation but is required for elongation of nascent DNA chains (Wides *et al.*, 1987). The DBP is necessary to protect nascent single stranded DNA from nuclease attack and to separate it from the replication complex (Neale and Kitchingman, 1990). DNA synthesis continues until 25-30% of the adenovirus genome has been synthesized. For complete full length genomic synthesis another cellular protein factor NFII, or topoisomerase I is necessary (Nagata *et al.*, 1983; Wong and Hsu, 1990).

Adenovirus DNA replication is semiconservative (van der Vliet and Sassenbach, 1973). Once initiated the daughter strand is synthesized in the 5′ to 3′ direction, displacing the parental strand with the same polarity (Lechner and Kelly, 1977; van der Eb, 1973). At the end of the first round of replication the products consist of a double stranded DNA linear DNA molecule and a single stranded DNA molecule. With aid of the ITR this single stranded DNA may form a circular pan handle structure mimicking the double stranded DNA replication origin (Graham *et al.*, 1989). Thus both products of replication including the double stranded molecule and the single stranded panhandle structure, may continue to replicate as described above.

I.I.3.e. Late gene expression.

Coincident with the onset of viral DNA replication at 8 to 15 hours post infection, late protein synthesis increases 400-1000 fold (Shaw and Ziff, 1980). The major late promoter (MLP) initiates synthesis of a large nuclear precursor RNA that is spliced into mRNAs which fall into five 3′ co-terminal families, L1- L5 (Figure 1) (Flint 1977; Goldberg *et al.*, 1978). These mRNAs all share a common tripartite 5′ leader sequence with a capped terminus encoded at the RNA initiation site (Shaw and Ziff, 1980; Nevins and Darnell, 1978). The 15 to 20 mRNAs produced by the major late transcription unit code for virion structural proteins including hexon (polypeptide II), penton (polypeptide III), penton associated protein (polypeptide IIIa), fiber (polypeptide IV), core protein (polypeptide V), hexon associated protein (polypeptide VI), core protein (polypeptide VII), and hexon associated protein (polypeptide VIII) (Ziff and Fraser, 1978), non structural proteins 35K and 55K (D'Halluin *et al.*, 1978; Perssor *et al.*, 1979), and the viral associated RNAs VaI, VAII (Soderlund *et al.*, 1976).

I.I.3.f. Early to late switch.

The switch from early to late transcription is complex and involves many regulatory events including poly (A) addition, RNA splicing, promoter usage, and DNA replication. Proteins translated from the L1 family have been found early,

before E1A expression, as well as late in infected cells (Lewis and Mathews, 1980). The production of L1 mRNA early on in infection, may involve preferential poly(A) addition. The finding that selection of a poly (A) site varies between early and late times implies that the specificity does not reside in the DNA sequence but in a factor specific for a given poly(A) site (Nevins and Wilson, 1981). The early transcripts for the L1 genes also differ from those found late in transcription by the presence of an insertion of an additional leader sequence termed "i" within the tripartite leader. The presence of this insertion which results from differential splicing, prevents the expression of other late genes present on the large RNA transcript (Akusjari and Peersson, 1981). Differential splicing between early and late times has also been observed in other transcription units, including those from the E1 region (Chow *et al.*, 1979). Use of different promoters at early and late times has also been observed for the DBP (Chow *et al.*, 1979). Thomas and Mathews (1980) have shown that template replication is a requirement for late gene expression and that the accumulation of early gene products alone, does not suffice.

I.I.3.g. Virion assembly.

The first step in virion assembly is the formation of the major structural units of the capsid.namely the hexon, fiber and penton base, forom monomeric polypeptide chains as the newly synthesised molecules are being transported to the nucleus. This assembly occurs at the beginning of the late phase of viral infection

(Flint and Broker, 1981). The hexon capsomeres are incorporated into virions over a period of 24 hours but the penton base and fiber peptides assemble into penton capsomeres at a much slower rate, only 40% of the penton capsomeres are assembled by 45 minutes, the last 60% requiring a further 10 hours (Horwitz, 1969; Flint and Broker, 1981). The fiber protein has also been shown to be non-essential for adenovirus assembly (Falgout and Ketner, 1987). A 39K and 50K species are scaffolding proteins which are necessary during the formation of the capsid structure but are absent from the final product. These two scaffolding proteins are released from the capsid upon entry of the viral DNA (D'Halluin *et al.*, 1978). The 50K protein has been shown to be encoded by a gene located on the L strand, and is involved in the maturation process of the virus (Perssor *et al.*, 1979). A small 11K virion protein μ, bound tightly to the virion DNA, aids in condensation of the viral chromosome (Anderson *et al.*, 1989). Between 290 and 490bp from the left end of the viral genome, there exists a tandom repeat of a cis acting DNA encapsidation signal (Hammarskjold, 1980). One of these sequences is sufficient for DNA encapsidation as long as it is within 400bp of the ends of the viral genome (Hearing *et al.*, 1987). The left end of the viral genome enters the capsid first along with a viral encoded protease necessary for subsequent maturation of infectious adenovirus (Bhatti and Weber, 1979). In the last stages of viral morphogenesis all the precursor proteins pVI, pVII, pVIII, and pTP are cleaved and then the mature virion leaks from the nucleus into the cytoplasm (Horowitz, 1990). Adenovirus has no functions which induce cell lysis and so mature virions remain cell associated for long periods.

I.II. Transformation by adenovirus.

In 1962, Trentin *et al.* showed that an adenovirus serotype (latter identified as Ad12), could induce tumour formation when injected into hamsters. Following this report, it was found that other adenovirus serotypes could induce tumours in immunosuppressed rats (Gallimore, 1972), and transform rodent cells in culture (Black *et al.*, 1967; Freeman *et al.*, 1967). Rodent cells in vitro are defined as being transformed by adenovirus according to a number of criteria, including uncontrolled cell growth coupled to a loss of contact inhibition, epithelioid morphology, sensitivity to calcium concentrations over 0.1mM, and expression of viral antigens (Freeman *et al.*, 1967a; Freeman *et al.*, 1967b). Gallimore (1974), showed that transformed rodent cells always retained at least the left 14% of the adenovirus genome (Gallimore *et al.*, 1974). These adenovirus sequences were integrated randomly into host chromosomal DNA (Sutter *et al.*, 1978; Dorsch-Hasler *et al.*, 1980; Vesser *et al.*, 1981). Patch homology between the adenovirus genome and host chromosomal DNA may play a role in this random integration event (Deuring *et al.*, 1981). There has also been evidence to suggest that the form of the genome which adenovirus integrates is circular (Vesser *et al.*, 1981). Graham (1984) transformed rodent cells with sheared adenovirus DNA, and found that the leftmost 1-6% of the adenovirus genome was required for partial transformation of BRK cells (Graham, 1974). The E1A coding region located within this left most 6% of the adenovirus genome plays

an important role in both the lytic and transfromation events of adenovirus.

I.III. E1A proteins.

The use of S1 nuclease (Berk and Sharp, 1978), DNA-RNA hybridization (Kitchingman and Westphal, 1980), and in vitro translation systems (Stephens and Harlow, 1987) have shown that the E1A region encodes at least 5 mRNA species having sedimentation coefficients of 13s, 12s, 11s, 10s, and 9s. The four largest mRNA, have common 5′ and 3′ termini but differ in their internal splicing patterns (Kitchingman and Westphal, 1980). The 9s mRNA has a 5′end which is identical to the other 4 mRNAs but differs at its 3′terminus as a result of having spliced into a different reading frame. The E1A mRNAs generate polypeptides of 289R, 243R, 217R, 171R and 55R respectively. The E1A proteins are relatively unstable, having a half-life of 30-90 minutes during a lytic infection (Branton and Rowe, 1985).

The two major E1A proteins of 289R and 243R are identical except for the presence of 46 internal amino acids which are unique to the 289R protein. Comparison of the amino acid sequences of the major E1A polypeptides from a variety of adenovirus serotypes reveal that the protein contains three highly conserved domains (Kimelaman *et al.*, 1985). Two of these conserved regions, termed CR1 and CR2, are in the first exon common to both the 289R and 243R products. The third domain, CR3, is essentially contained within the unique region present only in the 289R product. The protein products of the 13s and 12s mRNAs

when separated on a one-dimensional polyacrylamide gels generate 4 proteins migrating with relative weights of 30 to 60K (Yee *et al.*, 1983). Yee and Branton (1985a) determined that each of the major E1A mRNAs produce two major protein products; that is the 13s mRNA yields a 52 and a 48.5K protein whereas the 12s mRNA produces a 50 and a 45K protein (Yee and Branton, 1985a). These proteins can be further resolved using two-dimensional gel electrophoresis into as many as 60 different species (Harlow *et al.*, 1985). This heterogeneity has been attributed to both high proline content and post translational phosphorylation events (Yee *et al.*, 1983; Lucher *et al.*, 1985). Three phosphorylation sites on the major E1A products have been identified, located at ser-89, ser-96 and ser-219 (Tremblay *et al.*, 1988; Tremblay *et al.*, 1989). By mutating each of ser-89 and ser-96 to alanine it was shown that ser-89 was the principal phosphorylation site responsible for the major shift in molecular weight seen on SDS-polyacrylamide gels (Tremblay *et al.*, 1988; Dumont *et al.*, 1989). Extensive mutagenesis has been carried out on the E1A coding region resulting in the assignment of distinct functional domains along the E1A protein.

II.III.1. E1A function.

Viruses lacking E1A fail to produce normal levels of mRNA from the remaining viral early transcription units, indicating that E1A expression is necessary for transcriptional transactivation (Berk *et al.*, 1979; Jones and Shenk, 1979). The

transactivation domain of E1A has been mapped to the unique region of the 289R product (Kingston *et al.*, 1985; Lillie *et al.*, 1987; Jelsma *et al.*, 1988). Cellular proteins, such as the heat shock-70K species, have also been shown to be transactivated by E1A products (Nevins, 1982). E1A proteins do not bind directly to DNA and are thought to act by indirectly by increasing the concentration or activity of essential transcription factors such as E2F (Kovesdi *et al.*, 1986; Yoshinaga *et al.*, 1986).

In transient expression assays E1A has been shown to repress expression of ß-globin RNA and transcription from the SV40 enhancer and promoter (Borrelli *et al.*, 1984; Velcich and Ziff, 1985). Both 289 and 243R proteins are able to repress transcription, whereas only the 289 product can transactivate efficiently, suggesting that repression and transactivation of transcription are mediated differently by E1A proteins (Velcich and Ziff, 1985). CR1 and CR2 which are present in both major E1A products, have been shown to be essential for transcriptional repression (Lillie *et al.*, 1986 and 1987). The mechanism by which E1A proteins act to repress transcription is unknown but is thought to be mediated directly or indirectly by cellular proteins.

The N-terminal region of E1A has been shown to induce the differentiation of F9 stem cells in culture (Montano *et al.*, 1987). E1A has also been shown to stimulate baby rat kidney (BRK) cell proliferation and DNA synthesis (Zerler *et al.*, 1987; Howe et al., 1990).

I.III.2. E1A transformation.

BRK cells can be immortalized by E1A (4.5%) alone but such immortalized cell lines lack many of the characteristics of fully transformed cells such as anchorage independent cell growth and the ability to form tumours in rodents (Houweling *et al.*, 1980). Ruley (1983) showed that to obtain fully oncogenic transformed cell lines a second gene product was required. Co-transfection of primary rodent cells with E1A and either polyoma middle tumour antigen, activated Harvey ras, or E1B was found to be sufficient to both establish and maintain a fully oncogenic phenotype (Ruley, 1983). The 10s and 11s E1A messages do not play a role in oncogenic transformation by adenovirus (Stephens and Harlow, 1987). This suggests that CR1 which is spliced out in the 10s and 11s gene products is necessary for a fully transformed phenotype. Further studies suggested that CR1 and CR2 but not CR3 are required for E1A mediated transformation (Lillie *et al.*, 1986,1987; Schneider *et al.*, 1987). It has been suggested that cellular transformation may result in part from the transrepression by E1A of cellular mRNA synthesis (Schneider *et al.*, 1987).

The E1A proteins have been found to complex with a number of cellular proteins. Yee and Branton (1985b) identified at least five cellular polypeptides that associate with E1A products, including proteins of molecular weights 68K, 65K, 107K, 105K, and 300K (Yee and Branton, 1985b; Harlow *et al.*, 1986). Within the transforming domain of E1A are the binding sites for three cellular proteins, namely

107K, 105K, and 300K (Yee and Branton, 1985; Egan *et al.*, 1988; Egan *et al.*, 1989). The 105K protein has been identified as p105-Rb, the product of the retinoblastoma anti-oncogene (Whyte *et al.*, 1988; Egan *et al.*, 1989). Binding of p105-RB to E1A products is necessary for E1A-mediated transformation, but it is not sufficient. Association of E1A proteins with the 107K and 300K cellular proteins is also required for oncogenic transformation (Egan *et al.* 1988; Whyte *et al.*, 1989; Jelsma *et al.*, 1989).

I.IV. E1B coding region.

Relatively little is known about E1B functions, although the proteins produced by this region are required for productive infection of human cells and for complete transformation of rodent cells in co-operation with E1A gene products.

Transcription from the E1B gene results in the expression of at least 6 differently spliced mRNA species during a productive viral infection with the 22s and the 13s mRNA being the most prominent (Lewis and Anderson, 1987). The 22s mRNA codes for two unrelated proteins of 176 and 496 residues, commonly known as 19K and 58K respectively. The 13s mRNA encodes 19K and an 84R product which is composed of the first 79 amino acids of 58K and a proline rich tail. The 14.5s and 14s mRNAs code for other minor products related to 58k, termed 93R and 156R. Another 58K related mRNA has been proposed but has not yet been verified (see Figure 2) (Lewis and Anderson, 1987). In addition E1B region encodes the polypeptide pIX from its own promoter.

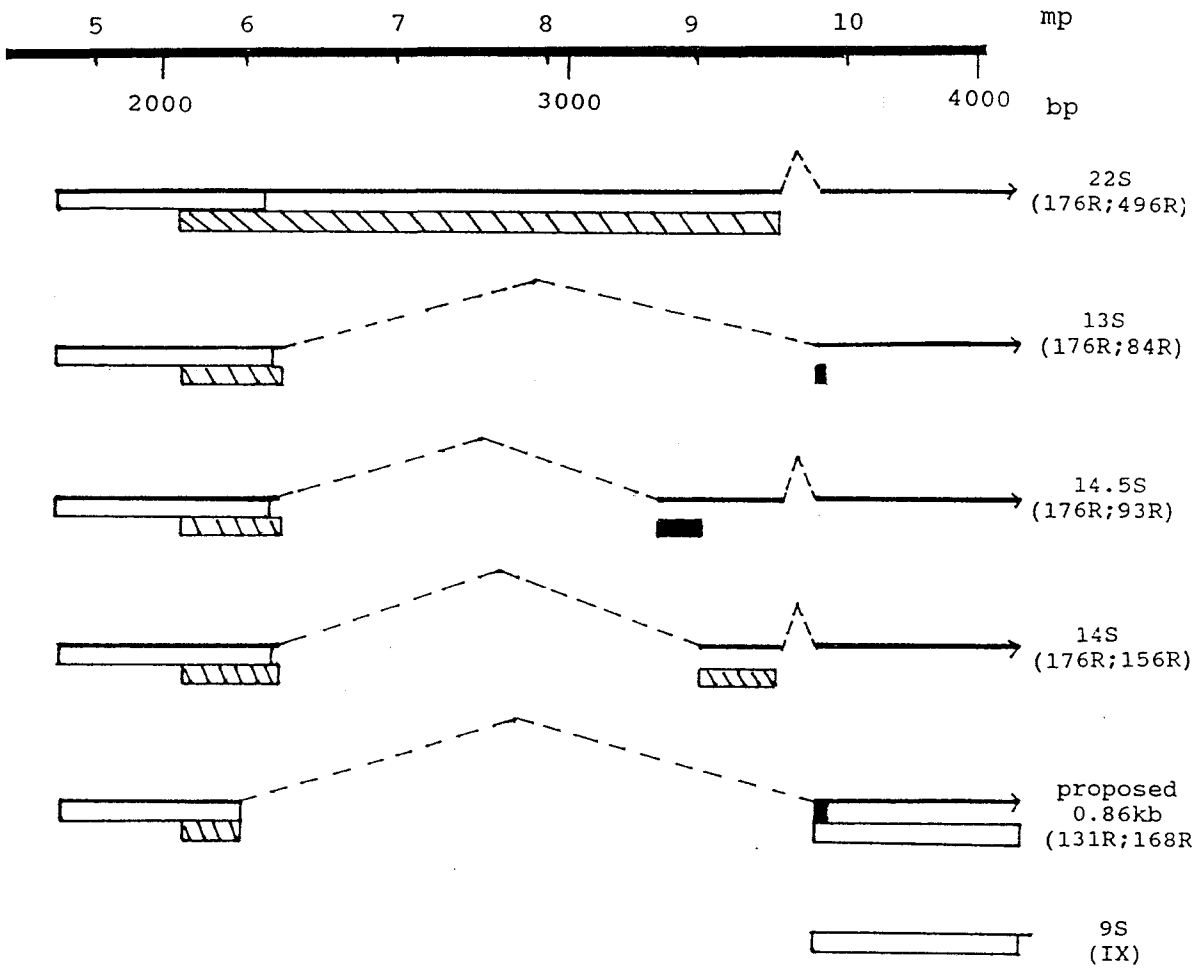
I.IV.1. The 58K E1B protein.

The 58K protein can be detected by immunoprecipitation as early as 5 hours post infection and the amounts increase until 20 hours post infection. The 58K protein is predominantly nuclear in infected cells but distinct cytoplasmic or perinuclear localization has also been shown (Rowe *et al.,* 1983). 58K is phosphorylated in infected and transformed cell lines on threonine and serine (Malette *et al.,* 1983; Schughart *et al.,* 1985).

Figure 2 -E1B map.

The figure shows the five products of transcription: 9s, 14.5s, 14s, 13s and a 22s mRNA plus a proposed sixth mRNA which would make a 168R protein.

E1B


5
6
7
8
9
10
mp
2000
3000
4000
bp
22S
(176R;496R)
13S
(176R;84R)
14.5S
(176R;93R)
14S
(176R;156R)
proposed
0.86kb
(131R;168R
9S
(IX)

Mutants affecting 58K fail to facilitate transport and accumulation of viral mRNA late after infection while blocking the same processes for cellular mRNA (host shut off) (Pilder *et al.,* 1986; Samulski and Shenk, 1988). Further research into late accumulation of viral mRNA by Leppard and Shenk suggested that 58K influences mRNA transport via an intranuclear effect on RNA metabolism (Leppard and Shenk, 1989). 58K has been shown to complex with both cellular and viral proteins. The 35K product of early region 4 of Ad5 associates with 58K in productively infected cells (Sarnow *et al.,* 1984). Similar to mutations in 58K, mutations in the coding region of E4 affect the viral growth cycle (DNA replication, late gene expression and host cell shut off) (Weinberg and Ketner, 1986). 58K has also been shown to complex with a cellular protein termed p53 (Sarnow *et al.,* 1984). Recently, p53 has been identified as an anti-oncogene which acts to block transformation (Finlay *et al.,* 1989).

I.IV.2. The 19K E1B protein.

Early in infection 19K is synthesised in low amounts but after DNA synthesis the 19K protein is produced in larger quantities. Immunofluorescence has shown that early in infection 19K is detected on cytoplasmic membranes and on the nuclear envelope (McGlade *et al.,* 1987; McGlade *et al.,* 1989). Late in infection it is exclusively in the nuclear envelope (White *et al.,* 1984). 19K is posttranslationally modified, phosphorylated at a low level on serine 164 and acylated near its amino terminus (McGlade *et al.,* 1988; McGlade *et al.,* 1989). The acylation of 19K perhaps plays a role in its membrane association. Recently White has shown that

19K associates with an intermediate filament component, vimenton (White and Cipriani, 1989; White and Cipriani 1990), which may suggest a role for 19K in the morphological changes observed during cellular infection and transformation.

Mutants in the coding region of 19K show a number of phenotypes. Such mutations cause an enhanced cytopathic effect, commonly called the cytocidal (cyt) phenotype. The cyt phenotype is associated with formation of large plaques (Chinnadurai, 1983; Subramanian *et al.,* 1984). HeLa and KB cells infected with 19K mutants show degradation of host and viral DNA (deg phenotype) (Chinnadurai, 1983; Subramanian *et al.,*1984; White *et al.,* 1984). The cyt and deg phenotypes have been shown to be functionally separable by White *et al.,* (White *et al.,* 1984). Using transient expression assays, White and others have further shown that 19K can activate all adenovirus early promoters (Herrmann *et al.,* 1987; White *et al.,* 1988). Others have suggested that E1B products modulate transcription from the E1A promoter and thus influence the effect of E1A gene products on transcription from other promoters (Natarajan, 1986). 19K has also been shown to transactivate enhancer-linked promoters and relieve enhancer repression (Yoshida *et al.,* 1987). More recently, Mathews *et al.,* has shown that 19K in transient assays acts to stabilize plasmid DNA, which allows for increased levels of transcriptional expression leading to high translation and accumulation of gene products (Herrmann and Mathews, 1989).

I.IV.3 The role of E1B in transformation.

The role of the individual E1B proteins in transformation has not yet been established. Earlier studies suggested that 19K and at least the amino terminal region of 58K are necessary for transformation (Graham and van der Ed, 1973a and 1973b). Further research has shown that the entire 58K gene is required for DNA and virion mediated transformation of cloned rat embryo fibroblasts (CREF) cells (Babiss *et al.,* 1984; Barker and Berk, 1987). Similar experiments with adenovirus type 12 showed that 58K is required to transform another established 3Y1 rat cell (Shiroki *et al.,* 1987). Transformation studies using primary baby rat kidney cells support the view that 58K is needed for transformation. The efficiency of transformation on BRK cells varies proportionally with the size of 58K truncation (Babiss *et al.,* 1984; Bernards *et al.,* 1986). No biochemical function of 58K has yet been linked to its role in transformation. However, one can speculate that the role of 58K in transformation is to bind p53, altering or blocking its biochemical function, thus disrupting cellular growth and regulation pathways.

It appears that adenovirus type 5 19K is required for virion and plasmid mediated transformation of CREF cells (Babiss *et al.,* 1984; Barker and Berk, 1987). However, in transformation assays with primary baby rat kidney cells, 19K is needed only for virion mediated transformation (Bernards *et al.,* 1986). This is similar to results obtained with adenovirus type 12 19K, indicating that it is required for transformation of established cell lines but not for primary rodent cells (Chinnadurai, 1983; Edbauer *et al.,* 1988). The role 19K plays in transformation is not yet well understood.

I.V. Purpose of Study.

Through analysis of mutants by several laboratories, distinct biological activities have been assigned to 'domains' within the E1A protein. No such extensive study has yet been carried out on the E1B products. The main impediment in the analysis of the individual E1B proteins (19K and 58K) is the overlap in their coding sequences. The initial objective of this study was to overcome the problem of overlapping coding sequences of 19K and 58K. This would enable subsequent mutagenic analysis of each E1B protein separately. Four individual mutants were constructed using the site directed M13 mutagenesis technique. Two of these mutagenic events, when combined eliminated the expression of 19K, while the other two when combined eliminated the production of 58K.

It is known that both E1A and E1B functions are required for full transformation by adenovirus. We wanted to investigate the individual roles of each E1B protein in co-operation with E1A in transformation assays. Our assay system involved analysis of the transformation efficiency of baby rat kidney cell by various E1B mutants.

The role of 19K and 58K in the lytic cycle of adenovirus was also investigated by a number of assays including plaquing efficiency on HeLa and 293 cells; DNA degradation in infected cells; cytotoxicity of viral infection and the effect on other viral protein synthesis by mutants in the E1B coding region.

Chapter II.

II. Materials and Methods.

II.I Bacterial strains.

E. coli bacterial strains used during this work included MV1190: delta (lac-ProAB), thi, supE, delta (srl-recA)::Tn10 (tet^r)[F':tra D36, Pro AB, lac I Z del tam15]. CJ236: dut-1, ung-1, thi-1, rel A-1, pCJ105 (cm^r) (Kunkel *et al.*, 1987). LE392: h5dr514 (rk-mk+) supE44, supF58, lacY, galK2, galT22, metB1, trp55, mcrA (Borck, *et al.*, 1976).

II.II. Bacterial culture techniques.

II.II.1. Liquid cultures.

Mv1190 culture medium consisted of M9 salts (6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCL, 1g NH_4Cl, and made up to 1L with distilled H_2O) sterilized, cooled, and supplemented with sterilized stocks to a final concentration of 1mM $MgSO_4$, 0.2% [wt/vol] glucose, 0.1mM $CaCl_2$, 0.4% [wt/vol] cas amino acids, and 0.0001%

[wt/vol] thiamine. Tetracycline was also added once the medium had cooled to a concentration of 10μg/ml. CJ236 bacteria were grown in 2xYT broth (16g Bacto-tryptone, 10g Bacto-yeast extract, 5g NaCl, and made to a final volume of 1 litre with distilled H_2O). The medium was sterilized, cooled, and chloramphenicol added to a final concentration of 30μg/ml.

LE392 bacteria were grown in Luria-Bertani (LB) broth (10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl) and resuspended in H_2O to a final volume of 1L. After sterilization and cooling ampicillin was added to a final concentration of 35-50μg/ml.

All liquid cultures were incubated at 37°C with constant shaking.

II.II.2. Solid cultures.

To prepare bottom agar, 20g of Bacto-agar was added to either 1L of LB, 2xYT, or M9, autoclaved, cooled to 44°C and then supplemented with the appropriate antibiotics (salts added in the case of M9). Approximately 20ml of agar-medium was then poured into 100x15mm bacterial plates (Phoenix Biomedical Products Inc.), allowed to solidify and then inverted and stored at 4°C. Plates containing tetramyocine, bing sensitive to light, were wrapped in tinfoil. To plate bacteria, cultures were either spread or streaked out on the plates, inverted, and then incubated overnight (O/N) at 37°C.

II.II.3. Culture storage.

From an O/N culture, of the above mentioned bacterial strains, 0.8ml of culture were mixed with 150μl of sterile glycerol in a 4ml glass vial and stored at -70°C.

II.III. Phage strains.

The bacterial phage strain used in the mutagenesis was M13mp18 obtained with the Muta-Gene: M13 *in vitro* Mutagenesis Kit (BIO-RAD).

II.IV. Phage culture techniques.

M13 phage were grown and manipulated according to Zoller and Smith (1984) as modified by Kunkel (1985).

II.IV.1. Preparation of single stranded-DNA.

II.IV.1.a. Small scale preparation of phage single stranded DNA.

For small scale preparations of M13, a single M13 plaque was added to 1.5ml of LB to which 15μl of O/N culture of MV1190 had been added in a 15ml corex tube (Corning). The culture was grown for a maximun of 8 hours at 37°C, after which it was transferred to a 1.5ml Eppendorf tube and centrifuged for 10 minutes (min) at 13000 rpm in a microcentrifuge at room temperature (RT). The supernatant was then transferred to a new 1.5ml Eppendorf and 50μl were removed as a stock. To the remaining supernatant 150μg of RNase A was added and incubated at 37°C for 10 min. Then 200μl of 2.5M NaCl/20% PEG 800 was added, mixed, and incubated at RT for 15 min. The phage were pelleted by a 10 min centrifugation at RT and the supernatant removed by aspiration. The phage pellet was then resuspended in 100μl of TE (10mM Tris-Cl pH 7.4, 1mM EDTA) and extracted three times with a mixture of phenol:chloroform:isoamylalcohol (50:34:12). Then 10μl of a 7.8M ammonium acetate solution was added along with 3 volumes of 100% ethanol to the phage lysate, mixed, and placed at -20°C for at least 1 hour. After centrifugation for 20 min at 13000 rpm the phage DNA was pelleted. The phage pellet was then washed with 70% ethanol and dried *in vaco* and resuspended in 50μl of TE.

II.IV.1.b. Large scale preparation of phage single stranded DNA.

A 1.5ml phage culture, as described above, was pelleted and the supernatant used to inoculate a larger culture. For every ml of LB in a large scale phage preparation, 10μl of an O/N MV1190 culture was added along with 10μl of phage stock. The scaled-up phage culture was grown for 7 to 8 hours at 37°C and single stranded DNA was isolated as described for small scale preparation, with volumes of reagents scaled up proportionally.

II.IV.2. Preparation of M13 replicative form (RF).

To a 5ml culture of M9 supplemented with salts, amino acids and glucose (M9+sup) 50μl of an O/N MV1190 was added along with 50μl of phage stock. The culture was grown at 37°C for 8 hours after which the cultures were centrifuged at 13000 rpm for 10 min and the supernatant retained. Subsequently, 2 hours after the phage culture was started, 2.5ml of MV1190 was added to 500ml of M9+sup and incubated at 37°C until an optical density (measured at a wavelength of 600nm (OD_{600})) read 0.7 to 0.8. The 5ml phage stock was then added to the MV1190 culture and grown for a further 2 hours. After this the cells were pelleted and the M13 RF was isolated by alkaline lysis plasmid preparation method (Birnboim and Doly, 1979).

II.IV.3. Alkaline lysis plasmid preparation method.

This procedure is according to Birnboim and Doly (1979) and can be scaled up or down using proportional reagent volumes.

The bacterial pellet from a 500ml culture was resuspended in 18ml of lysozyme solution (5mg/ml lysozyme, 50mM glucose, 25mM Tris-Cl (pH 8.0), and 10mM EDTA), mixed, and left at RT for 20 minutes. Then 40ml of alkalin-SDS solution (0.2N NaOH and 1% SDS) was added, gently mixed and left on ice for 5 to 10 min. To this mixture 20ml of ice cold 5M potassium acetate (pH 4.8) was then added, mixed well, and again left on ice for 1 hour or O/N. After this, centrifugation at 6000 rpm pelleted bacterial chromosomal DNA and the supernatant was transferred to a clean tube. For smaller scale preparations, an increase in the rpm may be necessary to pellet the DNA. The supernatent was extracted with a phenol mixture, precipitated with ethanol, dried, resuspended in TE and RNase to a final concentration of 20μg/ml was added or the samples were banded in a cesium chloride gradient.

II.IV.3.a. Cesium chloride banding procedure.

To the supernatant from potassium acetate precipitation 0.6 volumes of isopropanol was added and allowed to stand at RT for O/N. Centrifugation at RT was carried out at 6000 rpm for 30 min and the resulting pellet was

resuspended in 5ml of a 0.1xSSC solution (20x SSC pH 7 stock: 175g NaCl, 88.2g sodium citrate in 1L of H_2O). The resuspended pellet was transferred to a clean 15ml corex tube and 2ml of a solution containing 50mg/ml proteinase K, 140μl of 10% SDS solution, and 70μl of 0.5M EDTA stock solution, was added, mixed well, and centrifuged at 10,000 rpm for 15 min. The DNA preparation was transferred to a clean tube and the volume adjusted to exactly 7ml with 0.1xSSC. In this, 8.4g of CsCl was dissolved and incubated on ice for 15 min and then centrifuged again at 10,000 rpm for 10 min. The supernatant was then distributed in VTi65.1 tubes and balanced by weighing. A layer of 250μl of a 5mg/ml ethidium bromide (EtBr) stock was added and the remaining space in the quick-seal ultracentrifuge tube (Beckman Quick seal tubes) was filled with light paraffin oil saturated with H_2O. The tubes were sealed and spun at 55,000 rpm, 18°C, for 16-18 hours. The plasmid band was extracted from the tube under UV light with a 18G-½ needle and a 1ml syringe. The EtBr was extracted three times from the DNA using equal volumes of isopropanol saturated with 20xSSC in a 15ml corex tube. The DNA samples was then dialysed in 4L of 1XTE for 24 hours, with replacement of new TE solution at 12 hours. The samples were precipitated with three volumes of 100% ethanol and centrifuged at 10,000 rpm for 30 min, washed with 70% ethanol, and dried *in vacuo*. The DNA was then resuspended in 0.5 to 1ml of H_2O.

II.IV.3.b. Determination of DNA concentration.

The concentration of DNA was determined by analysis at wavelengths of 260 and 280nm. DNA was also quantified by electrophoresis on an agarose gel against a known concentration standard.

II.V. Mutagenesis.

Site directed oligonucleotide mutagenesis was carried out according to Zoller and Smith (1984) as modified by Kunkel (1985) using the mutagene kit supplied by BIO-RAD.

II.V.1. Uracil DNA template.

A 50ml culture of 2xYT plus chloramphenicol was inoculated with a single colony of CJ236 bacteria and incubated O/N at 37°C. One ml of this O/N culture was used to inoculate 50ml of 2xYT and grown to an OD_{600} of 0.4 at which point the culture was inoculated at a multiplicity of infection (MOI) of 0.2 or less with M13A or m13B. The culture was grown for a further 4 to 6 hours and the single stranded uracil containing phage template isolated as discussed in section **II.IV.1.b.** The phage obtained from this culture was titered on MV1190 and CJ236. Sufficient uracil was obtained if the phage titer was reduced by four

log when grown on MV1190. The mutagene kit recommended a uracil phage titre on MV1190 10^4 fold lower than that on CJ236.

II.V.2. Titre of M13 phage.

The M13 phage was titred by adding 0.2ml of O/N culture of MV1190 or CJ236 to 4 sterile 15ml corex tubes along with 100μl of a serial dilution of phage stock (10^2 fold, 10^4 fold, 10^6 fold, 10^8fold). To each tube was added 2.5ml of 50°C top agar (7g/L LB), mixed and poured onto a plate with bottom agar. The plates were allowed to cool and they were then inverted and incubated at 37°C O/N. The phage plaques were counted the next morning.

II.V.3. Phosphorylation of mutagenic oligonucleotides.

The four mutagenic oligonucleotides used in this study were all designed to alter the coding sequence by adding or deleting a diagnostic restriction enzyme site for easy verification of the mutants. The mutagenic oligonucloetides were obtained from the McMaster Institute for Biotechnology. 200 pmol of each mutagenic oligonucleotide was phosphorylated at its 5'end by combining in a 0.5ml Eppendorf tube 2μl of kinase buffer (final concentration of 100mM Tris (pH 7.0), 10mM $MgCl_2$, 5mM DTT, and 0.4mM ATP), 1μl of 10mM rATP and 4 units of T4 polynucleotide kinase in a total volume of 30μl. The reaction was

carried out for 1 hour at 37°C before being terminated by heating at 65°C for 10 min.

II.V.4. Annealing and synthesis of the mutagenic primer.

Uracil containing DNA (0.1 pmol) was added to 2 to 3 pmol of the phosphorylated mutagenic oligonucleotide and 1μl of annealing buffer (20mM Tris-HCL (pH 7.4) 2mM $MgCl_2$, and 50mM NaCl) and brought to a final volume of 10μl. The reaction was placed in a 70°C water bath for 5 min, transferred to a beaker with 70°C H_2O and allowed to cool over 40 to 50 min. After this, the annealing reaction was placed in an ice H_2O bath and the synthesis reaction was added. The synthesis reaction consisted of 1μl of synthesis buffer (0.4mM each dNTPs, 0.75mM ATP, 17.5mM Tris-HCl (pH 7.4), 3.75mM $MgCl_2$, and 21.5mM DTT), ligase (BRL), and T4 pol (Pharmacia). The reaction was incubated on ice at 25°C for 5 min, and then at 37°C for 90 min. After 90 min, 90μl of stop buffer (10mM Tris pH8.0, and 10mM EDTA) was added to the reaction and frozen at -20°C.

II.VI. Competent cells.

The calcium chloride technique was used to make MV1190 and LE392 bacterial cells competent. Briefly a 200 to 250ml culture of appropriate medium

and antibiotics was inoculated to an OD_{600} of 0.1 from an O/N culture of either MV1190 or LE392. Once an OD_{600} of 0.9 was reached the cultures were centrifuged at 6000 rpm for 20 min and the cell pellet resuspended in 50ml ice cold 100mM $MgCl_2$. The cells were then immediately centrifuged again and the pellet resuspended in 110ml of 100mM $CaCl_2$ and left on ice for 30 to 90 min. Once again the cells were centrifuged and resuspended in 12.5ml of 85mM $CaCl_2$ plus 15% glycerol. Immediately after resuspension, 0.3ml aliquots of the cell suspension were transferred to 1.5ml Eppendorf tubes and quick frozen in liquid N_2. The competent cells were stored at -70°C.

II.VII. Transformation of bacteria.

A 0.3ml aliquot of competent cell was thawed on ice and 10 to 15μl of a synthesis reaction was added, mixed and held on ice for 30 to 90 min. The cells were then heat shocked by placing the tubes in a 42°C water bath for 3 min. The transformation reaction was then transferred to a 15ml corex tube with 0.7ml of LB and incubated at 37°C with constant shaking for 1/2 to 1 hour. Aliquots of 10, 50, 100, 200, and 250μl of the transformed cell cultures were added to 15ml tubes containing 2.5ml of 50°C top agar, mixed, and poured onto bottom agar containing appropriate antibiotics. Once the top agar solidified the plates were inverted and incubated at 37°C O/N.

II.VIII. Screening recombinant M13.

II.VIII.1. Restriction digest.

Restriction endonucleases were purchased from New England Biolabs, Bethesda Research Laboratories of Pharmacia. Digests were carried out on M13 phage RF or plasmid DNA (pXC38) at 37°C (except for *Taq 1* digests which were performed at 65°C) for 4 to 8 hours. After digestion 1/10 the digest volume of loading buffer (20% glycerol, 2% SDS, 0.5% bromophenol blue) was added to each digest. The digests were run on agarose gels (usually 1.2% agarose (BRL)) or on acrylamide gels (usually 5%). The gels were run approximately at 50 to 60 volts overnight and then stained with EtBr (1μg/ml). DNA was observed using a UV transilluminator and a mounted 107 polaroid camera using 667 polaroid film.

II.VIII.2. Sequencing single stranded M13 recombinant DNA.

II.VIII.2.a. T_7 sequencing reaction.

Sequencing was performed using the T_7 polymerase sequencing kit supplied by Pharmacia.

II.VIII.2.b. Sequencing gel system.

The LKB-Bromma 2010 Macrophor electrophoresis unit was used for all sequencing. The cooling plate was prepared by washing with H_2O, 70% ethanol, and acetone. This cleaning procedure was repeated and 5ml of Repulse Saline solution (LKB-Bromma) was rubbed onto the plate. The cooling plate was then washed twice with H_2O and 70% ethanol. The glass plate was similarly washed with H_2O and ethanol. Bind saline solution of 5ml of 100% ethanol, 15μl glacial acetic acid, and 15μl of Bind Saline (LKB-Bromma) was rubbed onto the glass plate. The glass plate was then washed with H_2O and 70% ethanol again before the second addition of the Bind Saline solution. The glass plate was washed once again with H_2O and 70% ethanol before the 1mm spacers were clamped into place between the glass plate and the cooling plate.

The sequencing gel was prepared using 10.5ml of a 19:1 solution of acrylamide to bis-acrylamide, 25.2g of urea, 14ml of 5xTBE (54g tris base, 27.5 borate, and 20ml 0.5M EDTA to a final volume of 1L), 0.7ml ammonium persulfate (APS), and brought to a final volume of 70ml with H_2O. The gel mixture was then filtered through a 115ml Nalgene filter, and 20μl of temed added. Immediately afterwards the sequencing gel was poured and a shark toothed comb was used to make the wells. The sequencing gel was pre-run at 1800 volts for ½ hour at a constant temperature of 55°C with 1xTBE as a buffer. After the gel was loaded, the samples were run at 3000 volts for 1 to 2½ hours at 55°C. At the end of the run the sequencing gel was soaked for ½ to 1 hour in 2L of a solution containing 10% of both acetic and methanol and then dried for 2

hours at 100°C. The gel was then exposed on Kodak XR-5 film for 12 to 48 hours at RT.

II.IX. Plasmids.

The plasmid pJm17 was designed by J. McGrory *et al.* (1988). The plasmid pXC38 is a derivative of pXC1 (McKinnon *et al.*, 1982), containing the left 16% of the Ad5 genome with a *Bam HI* site removed.

II.IX.1. Plasmid amplification.

The method used to amplify plasmids is based on Maniatis *et al.*, (1982) as modified by Frenkel and Bremer (1986). Briefly, 100μl of an overnight culture containing the appropriate antibiotic was used to inoculate a 25ml LB culture also containing the antibiotic. The culture was then incubated at 37°C with constant shaking until an OD_{600} of approximately 0.6 was reached. The entire 25ml LB culture was then used to inoculate a pre-warmed 500ml LB culture with antibiotic and incubated for a further 2.5 hours until an OD_{600} of 0.4 was obtained. Chloramphenical was then added to a final concentration of 10 to 20μg/ml and allowed to incubate O/N at 37°C with continual shaking.

II.X. Isolation of gel fragments.

To isolate restriction fragments for the purpose of recloning into another vector, 30μg of CsCl purified DNA was digested with appropriate restriction enzymes. After the digestion was complete the sample was split in three and run on a 5% acrylamide gel. The gel was to be stained with EtBr and using a hand held UV light the fragment of interest was visualized and cut out of the gel using a sterile scalpel. Each gel fragment was transferred into a sterile Eppendorf and mashed with a teflon plunger. To the Eppendorf was added 0.6ml of elution buffer (500mM ammonium acetate, 10mM magnesium acetate, 1mM EDTA, and 0.1% SDS) and elution took place in the dark at 37°C O/N. The next day centrifugation at 13,000 rpm pelleted the acrylamide. The supernatant was removed and saved, while 0.3ml more of elution buffer was added to the pelleted acrylamide, vortexed, and centrifuged again. The supernatents were pooled and filtered through siliconized glass wool. The DNA fragment was then precipitated with the addition of three volumes of 100% ethanol at -20°C. The DNA fragment was pellet by a 30 min 13,000 rpm centrifugation step, washed once with 100% ethanol and then with 70% ethanol and dried. The pellet was resuspended in 20 to 30μl of sterile H_2O.

II.XI. Ligation reaction.

All subcloning were forced clones involving gel purified vector and inserts. The ligation reactions were carried out according to King and Blakesley (1986), and Manaitis *et al.* (1982). In a 0.5ml Eppendorf tube 0.1μg of vector DNA was mixed with 0.01μg of fragment DNA, 6μl of 5x ligase buffer (0.25M Tris, 0.05M $MgCl_2$, 0.05M DDT, 5mM spermidine, 5mM ATP, and 0.5mg/ml BSA) (BRL), and 2 to 4 units of ligase (40,000 units/ml from Pharmacia). The ligation reaction was mixed, briefly centrifuged, and left at RT O/N. After this, the reaction mixture was diluted with 100μl of TE. A fraction of the ligation mixture, usually 20μl was run on an agarose gel in order to monitor the reaction. Then 10 to 20μl of the ligation mixture was used to transform 0.3ml of competent thawed cells.

II.XII. Screening for mutated plasmids.

The resulting bacterial colonies from a ligation and transformation reaction described above were picked and grown in 1.5ml minipreps. The plasmid DNA were extracted using the alkaline lysis protocol described earlier. Restriction digests using diagnostic enzymes were carried out in order to confirm the presence of the mutation in pXC38. Wild-type DNA were also digested with the diagnostic enzyme and loaded beside the potential mutant digest for comparison.

II.XIII. Transformation of primary baby rat kidney cells.

II.XIII.1. Preparation of baby rat kidney cells.

The method for the preparation of primary baby rat kidney (BRK) cells was based on that outlined by Graham and van der Eb (1973a). Briefly, one litter (usually twelve pups) of 6 day old Wistar rats were sacrificed by crushing the spinal chord at the neck, their kidneys removed and placed in 1xPBS= (1xPBS: 8g NaCl, 0.2g KCl, 1.15g NaHPO in 1L of H_2O with the addition of 1% penicillin-streptomycin (P/S)). After membranes and blood vessels were removed from the kidneys, they were transferred to 10ml of clean 1xPBS= in a 50ml Falcon tube (Becton, Dickinson). Using sterile scissors the kidneys were minced for 10 min. The kidneys were then resuspended in 30ml 2x Trypsin (Gibco) and stirred for 20 min with a sterile stir bar. After allowing the kidney debris to settle the supernatant was removed and added to 10ml cold fetal calf (FC) serum (Gibco). A fresh, 30ml aliquot of 2x trypsin was added to the remaining kidney tissue and the process repeated again. The combined trypsinized kidney cells in cold FC were centrifuged to pellet the cells at 1,000 rpm for 5 min. To the pelleted cells, 100ml of minimal essential medium (MEM) supplemented with 10% FC, 1% p/s,and 1% L-glutamine (L-glu), was added and incubated at 37°C for 15 min. The cells were then filtered through sterile cheese cloth into a 500ml Gibco bottle and the final volume brought up 500ml with MEM media

supplemented as above. While continually mixing the kidney cell suspension, 5ml aliquots were dispensed into 60mm tissue culture plates (Corning) and incubated O/N at 37°C with 5% CO_2 and 95% humidity. The following morning the kidney cells were refed with MEM supplemented as described above.

II.XIII.2. DNA mediated transformation of BRK cells.

DNA mediated transformation of BRK cells was performed according to Graham *et al.*, (1974) as modified by Wigler *et al.*, (1979). Twenty-four hours after plating the BRK cells were 70 to 80% confluent and ready for transfection using the calcium technique of Graham and van der Eb (1973b). To a 15ml corning polystyrene tube 40μg of plasmid(s) was added along with salmon sperm DNA to give a final 160μg of DNA per transfection cocktail, 200μl of 2.5M $CaCl_2$, and the final volume brought to 2ml with sterile distilled H_2O and mixed. The cocktail was then added dropwise to 2ml of 2x HEPES (8g NaCl, 0.37g KCl, 0.125g Na_2PO_4, 5.0g HEPES, 1g dextrose to a final volume of 500ml of sterile H_2O, pH 7.1) solution which has been sterilely aerated. The solution was then allowed to stand at RT for 30 min while the DNA precipitated. After this, 0.5ml of the solution was added to each of 8x60mm plates of BRK cell in 5ml of MEM medium. The next day the cells were refed with MEM medium and cultured for a further 48 hours and then switched to the selection medium Joklick supplemented with 5% horse, 1% P/S, and 1% L-glu. The cells were refed with selection medium every 3 to 4 days for the following 2 to 2½ weeks. After

removal of the medium the foci were fixed with 3:1 methanol:acetic acid, dried, and stained with Giemsa (from Harleco and diluted 1 in 20 with 0.1x PBS).

II.XIV. Tissue culture.

Confluent 100mm plates of KB cells (an established line derived from nasopharangeal carcinoma tissue) were diluted 1 in 10. A confluent plate of KB cells was first rinsed with 5ml of PBS and then 1ml of PBS was added along with 1 drop of trypsin-EDTA for 2 min and then removed. The cells were then dislodged and gently pipetted up and down to transfer the cells to 125ml of MEM supplemented with 10% FC, 1% p/s and 15 L-glu. The cells were then plated, 12ml per 100mm plate, and incubated at 37°C until used.

HeLa cells (a cell line derived from the cervical tumor of Helen Lane) were cultured in the same manner as the KB cells.

A confluent 150mm plate of 293 cells [derived from human embryonic kidney cells transformed by the leftmost 11% of the adenovirus type 5 genome (Graham *et al.*, 1977)] was split 1 in 3 by initially being washed with 10ml of PBS after which the PBS was removed and discarded. To the confluent plate 5ml of a 1x citric saline solution (10x citric saline stock: 50g KCl, 22g sodium citrate in 500ml of distilled H_2O) was added. The citric saline was immediately removed and the plate placed in the 37°C incubator for 5 to 7 min. The cells were then shaken loose and transferred to 60ml of F-11 medium (supplemented with 10% new born calf (NBC) serum, 1% P/S and 1% L-glu). The cells were then split 20ml per

plate and incubated until used (Graham *et al.*, 1977).

II.XV. Viruses.

The wild type adenovirus dl309 (Shenk *et al.*, 1977) used in these studies was grown and titered on 293 cells. Mutant adenoviruses were plaque purified at least three times and then titered on 293 cells.

II.XVI. Mutant virus rescue.

E1B mutants were rescued into dl309 wild type Ad5 by the technique of McGrory *et al.* (1988). This involved the DNA-mediated transfection of 293 cells using the calcium-phosphate technique (Graham and van der Eb, 1973a). Briefly, in individual 15ml corning polystyrene tube 20μg of each of the mutant plasmid was added to 20μg of pJM17, along with 100μl of 2.5M $CaCl_2$ and the final volume brought to 1ml with sterile H_2O and mixed. The plasmid pJM17 contained the entire adenovirus genome plus 43,000bp of pBR322 inserted at 3.7 mu. This solution was then added dropwise to 1ml of Hepes constantly being aerated with sterile air. The cocktail was mixed and allowed to stand at RT for 30 min to precipitate the DNA. To 80% confluent 293 cell on 4x 60mm dishes, 0.5ml of each cocktail was added dropwise. The cells were then allowed to incubate at 37°C for 4 hours and then the medium removed and replaced with an

1% agarose overlay 1:1 with 2xF-11 supplemented medium (2x F-11 supplemented with 10% amino acids, 10% vitamins, 2% P/S 2% L-glu, 1% sodium carbonate, 2% fungizone (Gibco)) and incubated for 10 days. The plaques were picked using a sterile Pasture pipet and stored in 0.5ml of TE at -20°C. In order to verify a viral mutant, 250μl of this virus stock was added to 80% confluent 293 cells and incubated for approximately 4 days or until CPE was observed. Upon CPE, the supernatant was transferred to a 15ml Falcon tube and stored at -20°C. The viral DNA was extracted for analysis using a Hirt extraction (Hirt, 1967).

II.XVI.1. Hirt extraction for isolating viral DNA.

The Hirt extraction was carried out according to Hirt (1967). The medium on infected 293 cells on a 60mm plate was removed and 0.4ml of a 0.6% SDS solution in 10mM TE (pH 7.0), was added for 20 min at RT. After this the cells were gently scraped from the plate into a 1.5ml Eppendorf tube. To this 150μl of 5M NaCl was added, mixed and left O/N at 4°C. The samples were then centrifuged at 13,000 rpm for 30 min and the supernatant transferred to a clean Eppendorf, extracted with 3 times with phenol:chloroform:isoamylalcohol (50:34:12), and then precipitated with 100% ethanol at -20°C. The DNA was pelleted by centrifugation, dried, and resuspended in 50μl of TE.

The presence of the mutation in the viral genomic background was confirmed by digestion with diagnostic enzymes and Southern blot analysis. The probe used in the Southern analysis was M13mp18 containing a pXC38 insert and labeled

with [^{35}P]dCTP using a nick translation kit (NEN).

II.XVII. DNA degradation assay.

Human KB cells grown on 60mm plates to 80% confluency were infected with wild type Ad5 or mutant virus (MOI of 30PFU) for 20 and 40 hours. After this the low molecular weight DNA was extracted using a modified Hirt technique (White and Stillman, 1984). Briefly, KB cells were harvested by gently scraping the monolayer into a 15ml corex tube. The cells were washed twice with PBS and centrifuged between each wash for 10 min at 1,000rpm. The cell pellet was then resuspended in 0.5ml of lysing solution (to a final concentration of 0.01M Tris pH 7.9, 0.005M EDTA, 0.1N NaCl) and transferred to a 1.5ml Eppendorf tube. Pronase was added to a concentration of 1mg/ml and then SDS was added to a final concentration of 0.5%. The samples were then incubated at 37°C for at least 2 hours. After incubation 150μl of NaCl was added to each tube, mixed, and left O/N at 4°C. The next day the Eppendorf tube(s) were spun for 30 min at 13,000rpm. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol and then ethanol precipitated with 2 volumes of 100% ethanol at -20°C. The DNA was pelleted, washed with 70% ethanol, dried *in vacuo* and resuspended in 40μl of TE with 10μg of RNase. The DNA was digested with Hind III for 4 to 6 hours and run on a 1% agarose gel alongside undigested DNA. The gel was then EtBr stained and a picture taken using a polaroid camera, as described earlier.

II.XVIII. Plaque formation assay.

293 and HeLa cells were grown on 60mm plates to confluency of 90 to 100% and then infected with serial dilutions of mutant and wild type virus. The serial dilution (10^{-3} to 10^{-11}) of wild type and mutant virus were performed by adding 0.2ml of virus stock two 1.8ml of 2xF11 medium. 0.5ml of this mixture was then added to a plate of HeLa or 293 cell plates and incubated for 1 hour at 37°C. The medium was removed and replaced with a 1:1 mixture of 2xF11 medium and 1% agarose. The plates were incubated for 2 weeks or until plaques could be analyzed for titre and large plaque phenotype.

II.XIX. Immunoprecipitation analysis.

II.XIX.1. Infection of human KB cells.

KB cells cultures were grown to a confluence of 80 to 90%, the medium was removed, saved, and replaced with 0.5ml of new medium containing 35 plaque forming units (PFU) per cell of wild type (dl309) or mutant adenovirus. The infection was incubated at 37°C for one hour after which the infection medium was removed and the 'saved' medium added back. The infection was allowed to proceed for a given time period usually 16 to 18 hours or as otherwise stated.

II.XIX.2. Labeling of KB cells.

Labeling of wild type, mutant or mock infected KB cells was performed at 14 to 16 hours post infection or as otherwise stated. Each 100mm dish of KB cells was washed once with methionine or cystine free medium and then incubated at 37°C with 2.5 ml of medium, lacking methionine or cystine, plus 100μCi of [^{35}S]methionine (specific activity of 1,300Ci/mmol) or 100μCi of [^{35}S]cystine (specific activity of 1,300Ci/mmol) respectively for 11/2 to 2 hours or as otherwise stated. In pulse-chase experiments, higher amounts of [^{35}S]methionine (usually 200μCi/100mm dish) were used and label was removed after 15 min.

II.XIX.3. Preparation of cell extracts.

Cell extracts were obtained as described by Yee *et al.*, (1983). Briefly, after removal of the labeling medium, cells were harvested by addition of 1ml cold PBS per plate and the cells were gently scraped from the plate into a 15ml falcon tube. Cells were washed three times with cold PBS, each time being centrifuged at 200rpm for 5 min at 4°C. The cell pellet was then resuspended in 1ml of Ripa buffer (50mM Tris pH 7.2, 150mM NaCl, 0.1% sodium deoxycholate, 1% Triton x-100, and 100,000 IU of aprotinin per ml). The amount of Ripa added depended on the number of immunoprecipitaions carried out on one plate of labeled cells, usually 1ml Ripa for one immunoprecipitation. After incubation on ice for 10 to

15 min, the mixture was centrifuged at 12,000 rpm for 20 min at 4°C. Then 1ml of this cell extract was added to 250μl of protein A-sepharose beads (Pharmacia) in a 1.5ml Eppendorf tube. The protein A-sepharose beads having been previously swelled were resuspended at a 1:10 dilution with Ripa buffer. Added to this was 25μl of a polyclonal serum or 10μl of a monoclonal serum. The immunoprecipitation reaction was then left O/N on an end-over-end rotator at 4°C. The next day the protein A beads were pelleted by centrifugation at 300 rpm for 20 seconds and washed three times with 1ml Ripa, spinning for 20 seconds between each wash. The beads were then similarly washed twice with a lithium chloride solution (100mM Tris pH 7.0, 200mM LiCl, and 0.1% 2-mercaptoethanol). To samples was then added 100μl of sample buffer (100mM Tris pH 6.5, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and boiled for 2 min and then let stand on ice.

II.XIX.4. SDS page gel preparation.

SDS page was performed as described by Yee *et al.* (1983). The stacking gel contained 5% polyacrylamide and the separating gel contained 15% (or as otherwise stated). The ratio of acrylamide to N-N'bismethylene acrylamide was 30 to 0.8. The gels were loaded with 25 to 50μl of the immunoprecipatated sample and run along with C^{14} labeled molecular weight markers (Amersham) at 60 volts until the dye front was through the stacking gel and then run at 80 to 90 volts O/N. Gels were fixed (40ml acetic acid, 100ml propanol, 360ml H_2O and

10ml of a 1% glycerol solution) and fluorographed in Enlightning (Dupont). Gels were dried and autoradiographed using Kodak XR-5 or XL-1 film.

Chapter III.

III. Results.

III.I. Mutagenesis of 19K and 58K

Unlike the study of E1A proteins, extensive mutagenesis of E1B is difficult due to the overlap in coding sequences of the major E1B products. To overcome this problem we constructed mutants which selectively eliminated the synthesis of either 19K or 58K.

Mutagenesis was carried out according to the technique of Zoller and Smith (1984) as modified by Kunkel (1985) using the Mutagene kit produced by BIO-RAD. The strategy of this type of mutagenesis was to clone the DNA to be mutated into the replicative form (RF) of M13mp18. The resulting recombinant was then used to transform CJ236 bacteria (an E.coli containing mutations in both the dUTPase and uracil N-glycosylase genes). This bacterial strain allowed the addition of uracils in to the single stranded phage template. The mutagenesis reaction was carried out on single stranded M13 uracil-containing DNA and the resulting products used to transform an *E. coli* strain with a functional uracil N-glycosylase, MV1190. Replication of the mutant M13 constructs in MV1190 selected against the paternal uracil-containing M13 DNA strand and enabled efficient selection of the mutant strand.

III.I.1. M13mp18 constructs.

To perform this type of mutagenesis it was necessary to obtain Ad5 E1B coding sequences in M13mp18 phage. Two such M13mp18 phage constructs were M13-A and M13-B, designed by S.W. Whalen and J.C. McGlade respectively (Figure 3). M13A contained the *Xba I* to *Kpn I* fragment from pXC38 (a plasmid containing the entire E1 region of Ad5 cloned into a pBR322 background) cloned into the *Xba I/ Kpn I* sites of the M13mp18 polylinker. The *Xba I/ Kpn I* fragment of pXC38 contained Ad5 sequences from nucleotides 1339 to 2048. M13-B contained the *Kpn I* to *Hind III* fragment from pXC38 cloned into the *Kpn I/Hind III* site of the M13mp18 polylinker. The *Kpn I* to *Hind III* fragment obtained from digestion of pXC38 contained Ad5 sequences from nucleotide 2048 to 2804. The combined coding sequences of these two m13mp18 constructs provided the entire coding sequences of 19K and 2/3 of the N-terminal coding sequences for 58K. These M13mp18 constructs allowed site directed mutagenesis of the E1B coding sequences.

III.I.2. Design of mutagenic oligonucleotides.

Four oligonucleotides for the purpose of site directed oligonucleotide mutagenesis were obtained from McMaster University Institute for Biotechnology (AB401, AB402, AB403, and AB404) (Figure 4). These oligonucleotides were designed to selectively eliminate the synthesis of 19K and 58K by disrupting the initiation codons for both 19K and 58K individually.

Oligonucleotide AB402 was designed to change the methionine initiation codon

Figure 3- The subcloning of E1B sequences into M13.

PXC38 fragments containing E1B sequences were subcloned into M13mp18. M13A was constructed by inserting the *Xba I* to *Kpn I* fragment of pXC38 (E1 sequences 1339 to 2048) into the *Xba I* to *Kpn I* polylinker sites of M13mp18 by S.G. Whalen. M13B similarly constructed by J.C. McGlade, subcloned the *Kpn I* to *Hind III* (E1 sequences 2048 to 2804) of pXC38 into the *Kpn I* to *Hind III* polylinker sites of M13mp18. These M13 constructs were used as templates for subsequent mutagenesis.

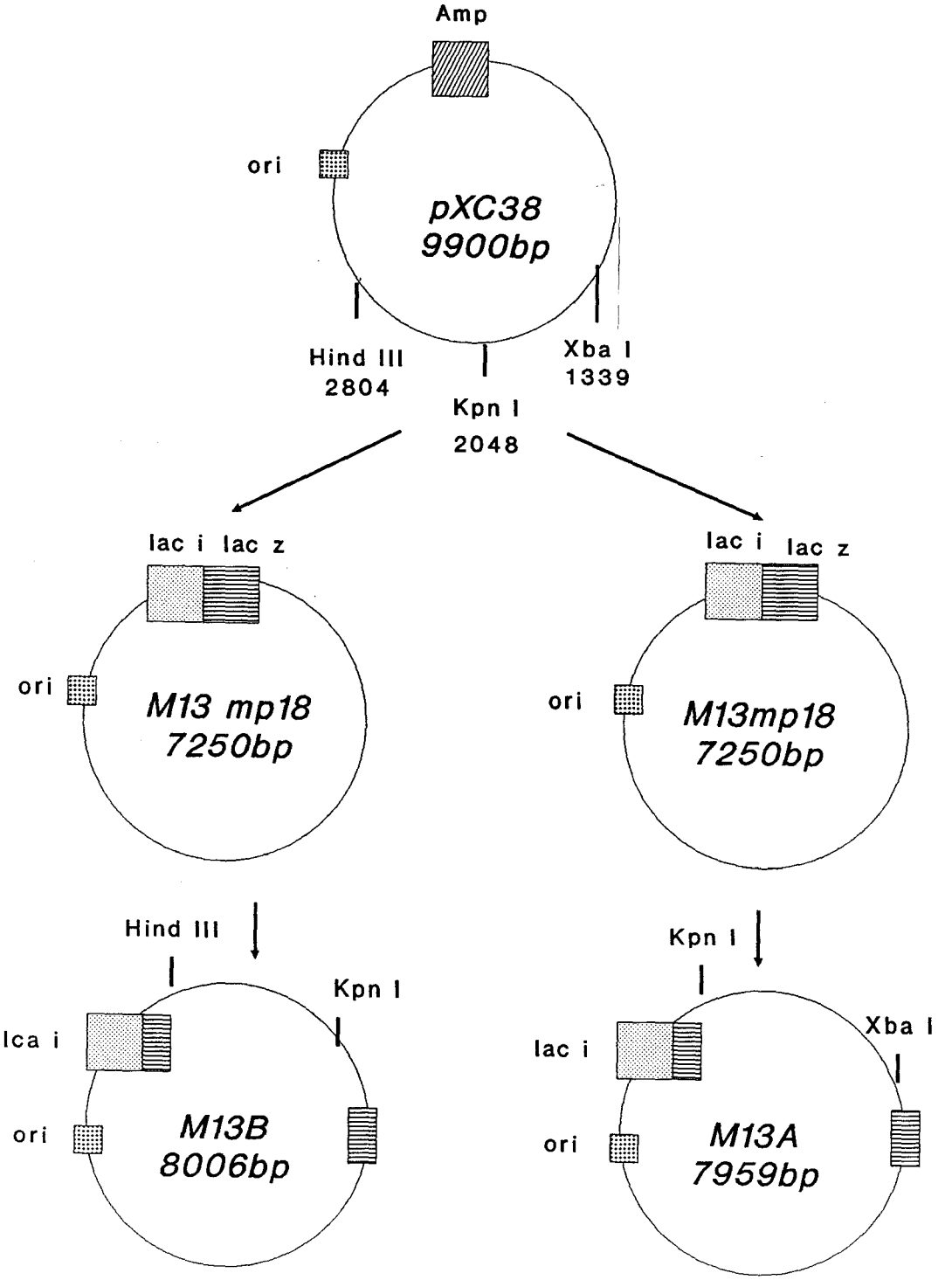

Amp
ori
pXC38
9900bp
Hind III
2804
Xba I
1339
Kpn I
2048
lac i lac z
ori
M13 mp18
7250bp
lac i lac z
ori
M13mp18
7250bp
Hind III
Kpn I
lca i
ori
M13B
8006bp
Kpn I
Xba I
lac i
ori
M13A
7959bp

Figure 4- Mutagenic oligonucleotides.

The figure illustrates the four mutagenic oligonucleotides obtained from McMaster Institute for Biotechnology. The nucleotides written below each sequence represent the wild type nucleotides. Letters represent: guanine (G), adenine (A), thymine (T), and cytosine (C).

AB401

5'-TAAAGGATAAGTGGTCCGAAGAAACC-3'
G AG

AB402

5'-CTGACCTCATCGAGGCTTGGG-3'
G

AB403

5'-CCACAGATGCGTGGCCAGAAA-3'
A

AB404

5'-CAGCCACCTGTCAAACATTCATT-3'
AC

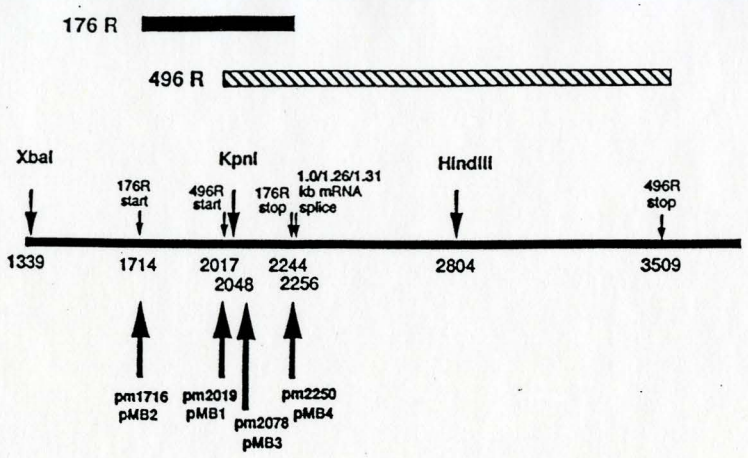
of 19K to an isoleucine codon. This mutation termed pm1716 (named after the nucleotide altered by the mutagenesis) would prevent the initiation of 19K and created a new *Taq I* site (Figure 5). Mutagenic oligonucleotide AB401 was designed to alter the initiation codon of 58K without disrupting the 19K coding sequences. This mutation termed for its nucleotide at 2019 altered the 58K coding sequence by changing the first two codons for methionine and glutamine to valine and valine (Figure 5). Mutant pm2019 also added a *Sau96 I* restriction enzyme site. Because of the previous observation by Barker and Berk, that 58K may reinitiate at an internal methionine we designed oligonucleotide AB404 which changed two nucleotide at 2250 and 2251 (Figure 5). So named for its nucleotide location, pm2250 mutated valine 78 to a stop signal just downstream from the third methionine of 58K. Mutant pm2250 also destroyed an Rsa I restriction enzyme site at this location. In order to ensure that reinitiation of 19K could not occur at an internal methionine AB403 was designed to mutate the only other methionine of 19K, methionine-120, to an alanine (Figure 5). The resulting mutation termed pm2072 destroyed a *Nsi I* restriction enzyme site but did not alter the 58K overlapping sequences.

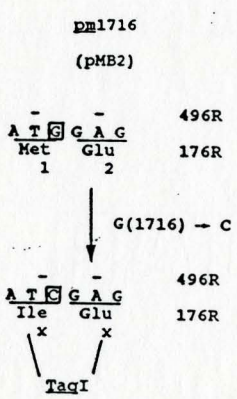
III.I.3. Mutagenesis.

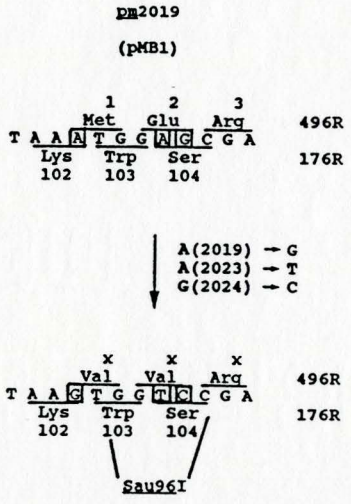
Uracil-containing M13A and M13B constructs were obtained as outlined in the Materials and Methods. Two individual annealing reactions were set up involving M13A single stranded DNA and one of the phosphorylated oligonucleotide AB 401 or 402. A further two annealing reactions were also set up involving M13B single

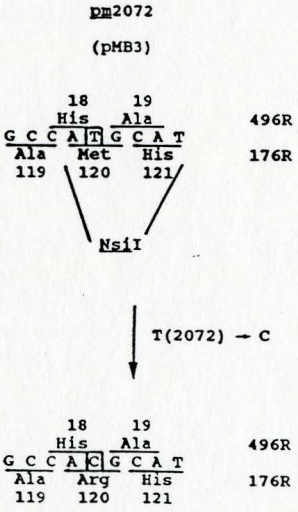
Figure 5 -E1B Mutants Design.

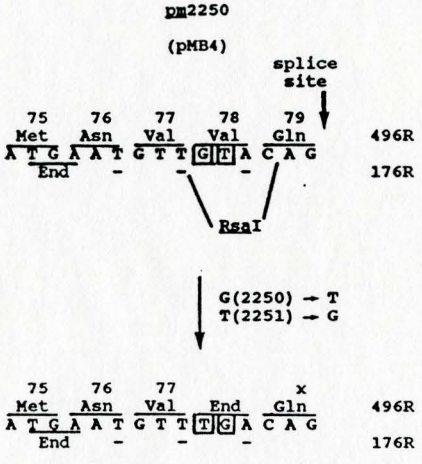
Site directed mutagenesis was performed on two M13mp18 constructs containing *Xba* I to *Kpn I* or *Kpn I* to *Hind III* inserts of the E1 region of adenovirus. Mutations were designed to eliminate the expression of either 19k or 58K without disrupting the other's coding sequence. Also for screening, mutations were designed to change the restriction pattern of diagnostic enzymes. For example, pm2019, eliminated the ATG initiation codon of 58K (496R) without changing the codon for 19K and created a new *Sau 96* restriction site (see text for greater detail).


176 R
496 R
XbaI
KpnI
HindIII
176R start
496R start
176R stop
1.0/1.26/1.31 kb mRNA splice
496R stop
1339
1714
2017
2048
2244
2256
2804
3509
pm1716 pMB2
pm2019 pMB1
pm2078 pMB3
pm2250 pMB4


pm1716
(pMB2)
496R
176R
Met Glu
1 2
G(1716) → C
Ile Glu
TaqI


pm2019
(pMB1)
1 2 3
Met Glu Arg
Lys Trp Ser
102 103 104
496R
176R
A(2019) → G
A(2023) → T
G(2024) → C
Val Val Arg
Sau96I


pm2072
(pMB3)
18 19
His Ala
Ala Met His
119 120 121
496R
176R
NsiI
T(2072) → C
Ala Arg His


pm2250
(pMB4)
splice site
75 76 77 78 79
Met Asn Val Val Gln
End
496R
176R
RsaI
G(2250) → T
T(2251) → G
End Gln

stranded DNA and one of the phosphorylated oligonucleotide AB403 or AB404. After annealing the oligonucleotide primers to their respective single stranded M13 templates, the complementary M13 DNA was synthesised. Since the mutant template contained thymidine, unlike the parental wild type strand containing uracil, transformation into MV1190 bacteria permitted selection for the mutagenic template. Mutant plaques were picked and both RF and single stranded M13 DNA were isolated and analyzed by diagnostic restriction analysis and sequencing, respectively.

Using the appropriate diagnostic enzyme, each of the mutant and wild type RF DNAs was digested and analyzed by agarose or acrylamide gels. The presence of the mutant pm1716 in M13-A RF sequences was determined by digestion with *Taq I* (Figure 6A and B). The addition of a *Taq I* site in pm1716 digested a 577bp fragment present in wild type M13 RF sequences into a 347 and a 230 bp fragment (Figure 6A and B). Digestion of wild type M13B and M13B-pm2072 RF DNAs with *Nsi I* and *Bgl II* showed the loss of an *Nsi I* site in the mutant sequences. Digestion of wild type M13 RF DNA with *Nsi I* produced two fragments of 1,300 and 5,950bp that in the mutant combined to form a single 7,250bp fragment (Figure 7A and B). The mutant pm2019 present in M13A sequences was diagnosed through the presence of a new *Sau96 I* site which converts a 943bp fragment present in wild type M13A RF into a 615 and a 340bp fragment (Figure 8A and B). Mutant pm2250 present in the M13B RF background was determined by the loss of a *Rsa I* site which converted the wild type M13B *Rsa I* fragments of 201 and 245bp into a single 455bp fragment (Figure 9A and B).

Upon restriction digest confirmation of the mutants, each of the four mutant phage was plaque purified twice. Small scale preparations of single stranded M13

Figure 6- Mutant pm1716 in M13A.

Mutant pm1716 disrupts the initiation codon for the 19K protein.

A. A cartoon of pm1716 depicts the *Taq I* sites within the pXC38 *Xba I/Kpn I* insert in M13A. The starred nucleotide represents the gain of a *Taq I* site at position 1711 in the mutant pm1716.

B. *Taq I* restriction digestion of M13 RF DNA. Lanes: Wild type M13A (A), two isolates of pm1716 (B and C), and molecular weight marker with base pair numbers to the right of the gel (D). To the left of the gel are fragment base pair numbers of importance. The mutant pm1716 is characterized by the gain of a *Taq I* site at nucleotide 1711 such that a 577bp fragment is digested into a 230 and a 347bp fragment. (see text for more detail).

C. Sequencing analysis of pm1716 confirmed the presence of the mutation at nucleotide 1716 as illustrated by the starred cytosine (C) to the right of the figure. The wild type nucleotide at this position would have been guanine (G). The lettering above the gel represents sequencing reactions for nucleotides guanine (G), adenine (A), thymine (T) and cytosine (C).

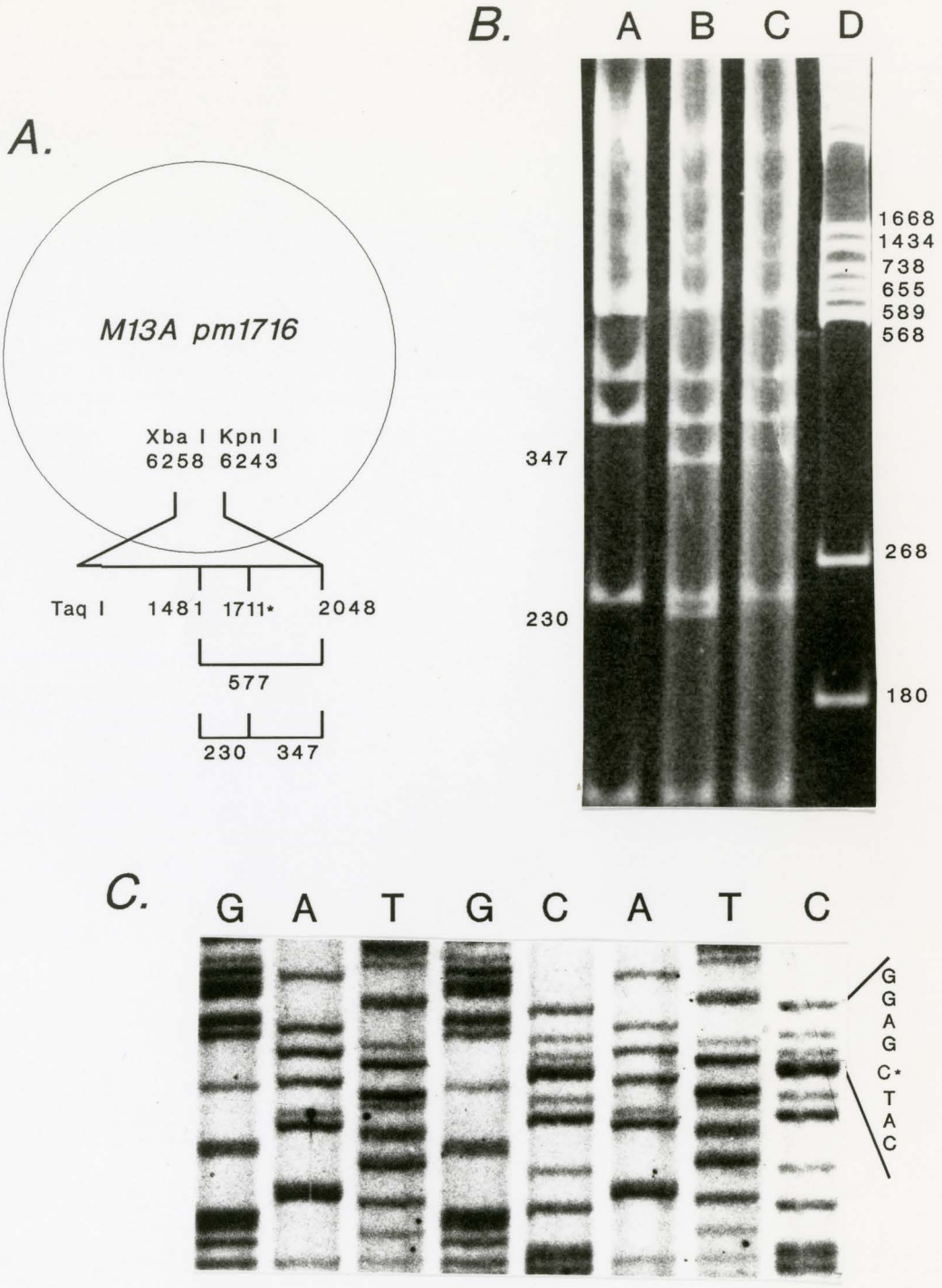

A.
M13A pm1716
Xba I Kpn I
6258 6243
Taq I
1481
1711*
2048
577
230
347
B.
A
B
C
D
1668
1434
738
655
589
568
347
268
230
180
C.
G
A
T
G
C
A
T
C
G
G
A
G
C*
T
A
C

Figure 7- Mutant pm2072 in M13B.

Mutant pm2072 prevents the potential initiation of 19K at an internal methionine.

A. A cartoon of the M13B pm2072 mutant illustrating the *Nsi I* sites within the *Hind III/Kpn I* pXC38 insert. The mutant pm2072 is characterized by the loss of a *Nsi I* site at the starred nucleotide 2071.

B. A diagnostic *Nsi I/ Bgl II* restriction enzyme digest of mutant and wild type M13B RF DNA. Lanes: molecular weight marker with base pair numbers to the Left of the gel (A), pm2072 (B), and wild type M13B (C). The pm2072 mutant is characterized by loss of a 1300 and 5950bp fragment, due to the mutation of a *Nsi I* restriction site, and the gain of a 7250bp band. The numbers to the right of the gel represent base pair numbers. (see the text for more detail).

C. Sequencing analysis of pm2072 confirms the presence of the mutation as illustrated by the starred guanine (G) to the right of the figure. The wild type nucleotide at this position would have been adenine (A) in place of the guanine (G). The lettering above the gel represent sequencing reactions for nucleotide: guanine (G), adenine (A), thymine (T), and cytosine (C).

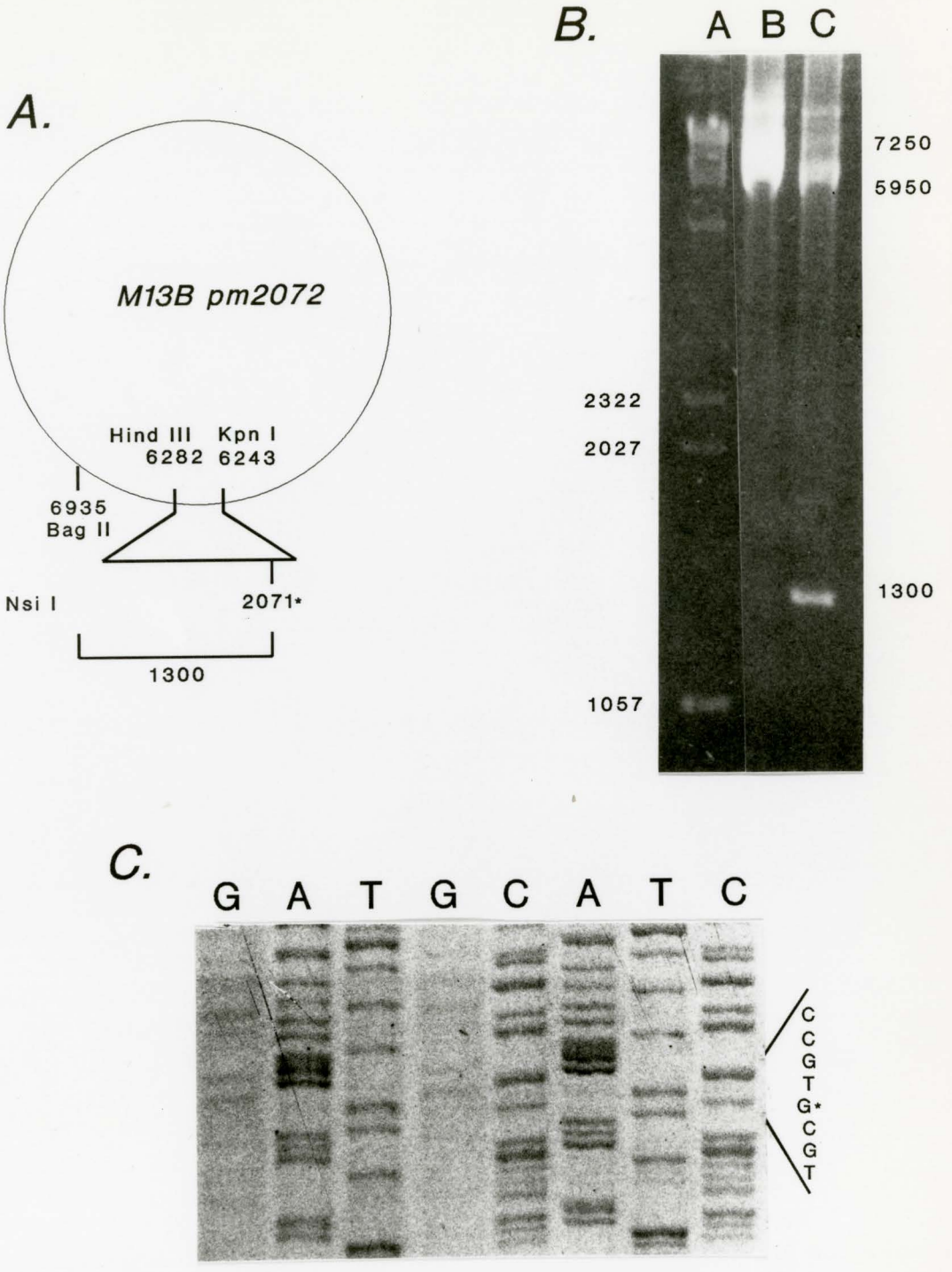

A.
M13B pm2072
Hind III
6282
Kpn I
6243
6935
Bag II
Nsi I
2071*
1300
B.
A B C
7250
5950
2322
2027
1300
1057
C.
G A T G C A T C
C C G T G* C G T

Figure 8- Mutant pm2019 in M13A.

Mutant pm2019 alters the initiation codon for 58K.

A. A cartoon of the *Sau96 I* restriction sites within the E1B sequences cloned in M13A. The diagram illustrates the gain of a Sau96 I site at the starred nucleotide 2019.

B. The addition of a *Sau96 I* diagnostic restriction enzyme site at nucleotide 2019 in pm2019 RF DNA cuts the wild type 943bp fragment into a 606 and a 337bp fragment. Lanes: molecular weight marker with number of base pairs to the left of the gel (A), wild type M13A (B), and pm2019 (C and D). The numbers to the right of the gel represent base pairs of significance.

C. Sequencing analysis of pm2019 confirms the presence of the mutation as illustrated by the starred cytosine (C), thymine (T), and guanosine (G) to the right of the gel. The wild type sequences at these positions would have been guanine (G), adenine (A) and adenine (A) respectively. The lettering above the gel represents the sequencing reactions for nucleotide: guanine (G), adenine (A), thymine (T), and cytosine (C).

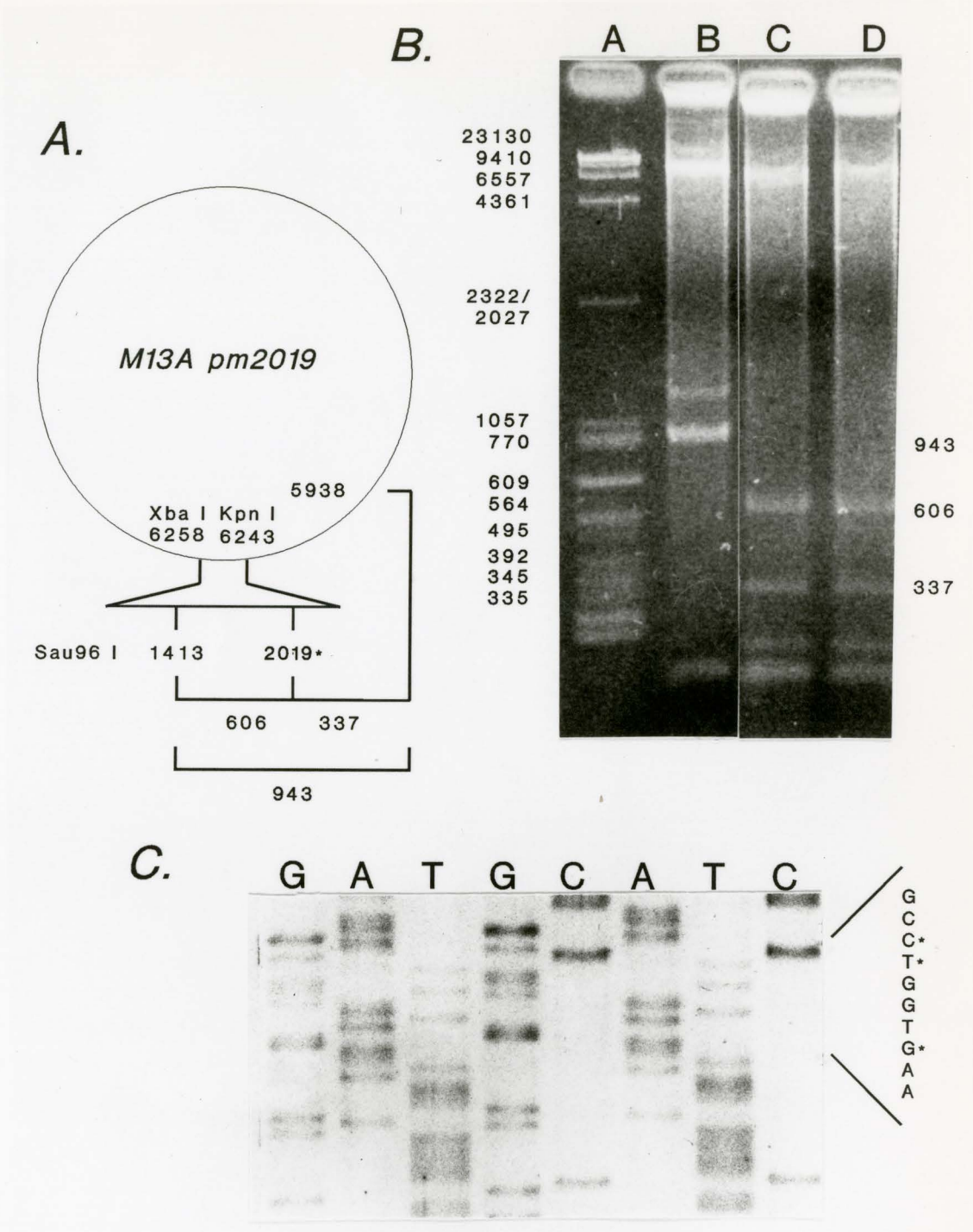

A.
M13A pm2019
5938
Xba I Kpn I
6258 6243
Sau96 I
1413
2019*
606
337
943
B.
A B C D
23130
9410
6557
4361
2322/
2027
1057
770
609
564
495
392
345
335
943
606
337
C.
G A T G C A T C
G C C* T* G G T G* A A

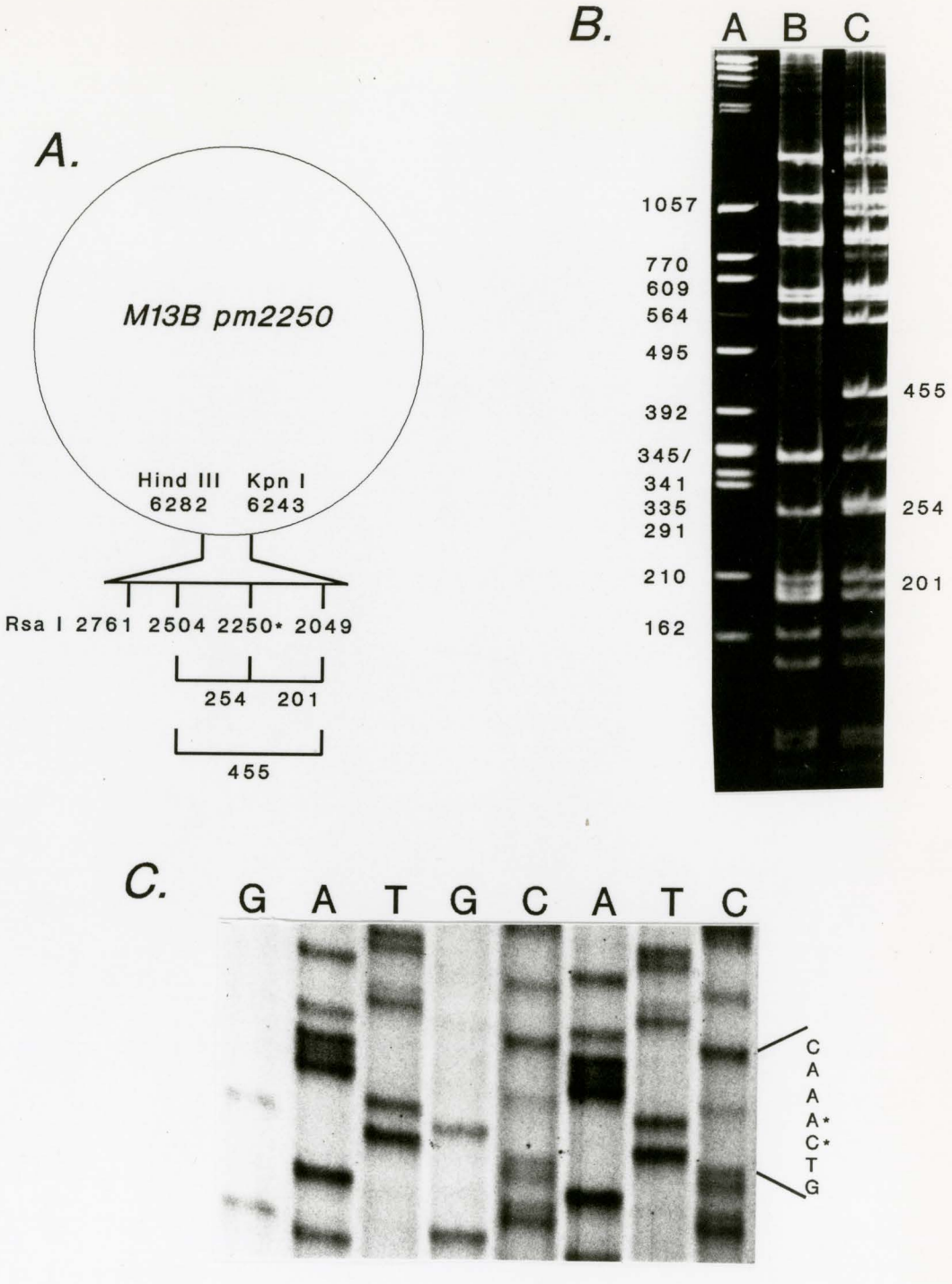
Figure 9- Mutant pm2250 in M13B.

Mutant pm2250 adds a stop codon three codons down-stream of the third methionine of 58K at nucleotide pm2250.

A. A cartoon of the M13B pm2250 mutant shows the loss of an *Rsa I* site at the starred nucleotide 2250.

B. The diagnostic *Rsa I* digest shows the loss of a 201 and a 254bp band in the wild type M13B, as illustrated by the base pair number at the right of the gel, while a 455bp band appears in pm2250 digest. Lanes: Molecular weight marker (A), wild type M13B construct (B), and pm2250 (C). The numbers to the left of the gel represent the base pair numbers of the molecular weight marker. (See text for more details).

C. Sequencing analysis of pm2250 confirmed the presence of the mutation as illustrated by the two starred nucleotides adenine (A) and cytosine (C) to the right of the gel. The wild type nucleotide at this position would have been cytosine (C) and adenine (A) respectively. The lettering above the gel represent the sequencing reactions for nucleotide: guanosine (G), adenine (A), thymidine (T), and cytosine (C).


A.
M13B pm2250
Hind III 6282
Kpn I 6243
Rsa I 2761 2504 2250* 2049
254 201
455
B.
A B C
1057
770
609
564
495
392
345/
341
335
291
210
162
455
254
201
C.
G A T G C A T C
C A A A* C* T G

DNA were carried out and sequenced using the Pharmacia T_7 sequencing kit (Figure 7C, 8C, 9C, and 10C).

III.II. Viral rescue.

III.II.1. Gel fragment purification.

Once the four mutations had been confirmed by DNA sequencing, large RF preparations of each mutant were grown. The RF DNA was extracted by the alkaline lysis method according to Birnboim and Doly (1979), and CsCl banded. The DNA concentration for each RF preparation was determined by spectrophotometric analysis and 30μg of each DNA was digested. Mutants pm1716 and pm2019 present in M13A constructs were digested with *Kpn I* and *Xba I* to yield a 709bp fragment, while mutants pm2072 and pm2250 present in M13B constructs were digested with *Kpn I* and *Hind III* to liberate a 756bp fragment. Each digest was run on a 5% acrylamide gel and these fragments were cut out of the gel and the DNA eluted.

Meanwhile large scale plasmid preparations of pXC38 were also produced. After CsCl banding the pXC38 DNA was digested with either *Kpn I* and *Xba I* or *Kpn I* and *Hind III*. The digests were run on an 5% acrylamide gel and fragments of 9431bp or 9384bp respectively were isolated and eluted from the gel.

III.II.2. Ligation into pXC38.

Ligation reactions were performed in order to subclone mutated E1B sequences back into pXC38. The *Xba I* to *Kpn I* inserts containing mutant pm1716 and pm2019 were cloned individually into the *Xba I/Kpn I* digested pXC38 sequences. Similarly the *Kpn I* to *Hind III* inserts containing mutants pm2072 and pm2250 were cloned into the *Kpn I/Hind III* digested pXC38 sequences. Each ligation reaction was used to transform LE392 bacteria. Ampicillin-resistant colonies for each mutant were analyzed by diagnostic restriction enzyme digest to confirm the E1B mutations.

To obtain both 19K mutants pm1716/2072 in the same pXC38 construct, the *Kpn I* to *Hind III* insert containing the pm2072 mutant was cloned into the *Kpn I/Hind III* vector of pm1716 (Figure 10). Diagnostic restriction enzyme digestion of pm1716/2072 showed that the mutant pm1716 is present in the double mutant by the disappearance of a 3726bp in the wild type pXC38 sequence and the appearance of a 3481 and a 235 bp band in the mutant. It is difficult to note the disappearance of the 3726bp band and the appearance of the 3481 band but the appearance of the 235bp band is readily detectable. Digestion of pm1716/2072 with *Nsi I* and *Bgl II* for the detection of pm2072 mutant showed that in the mutant a 1247bp and a 7629bp fragment combine to form a 8876 band (Figure 10).

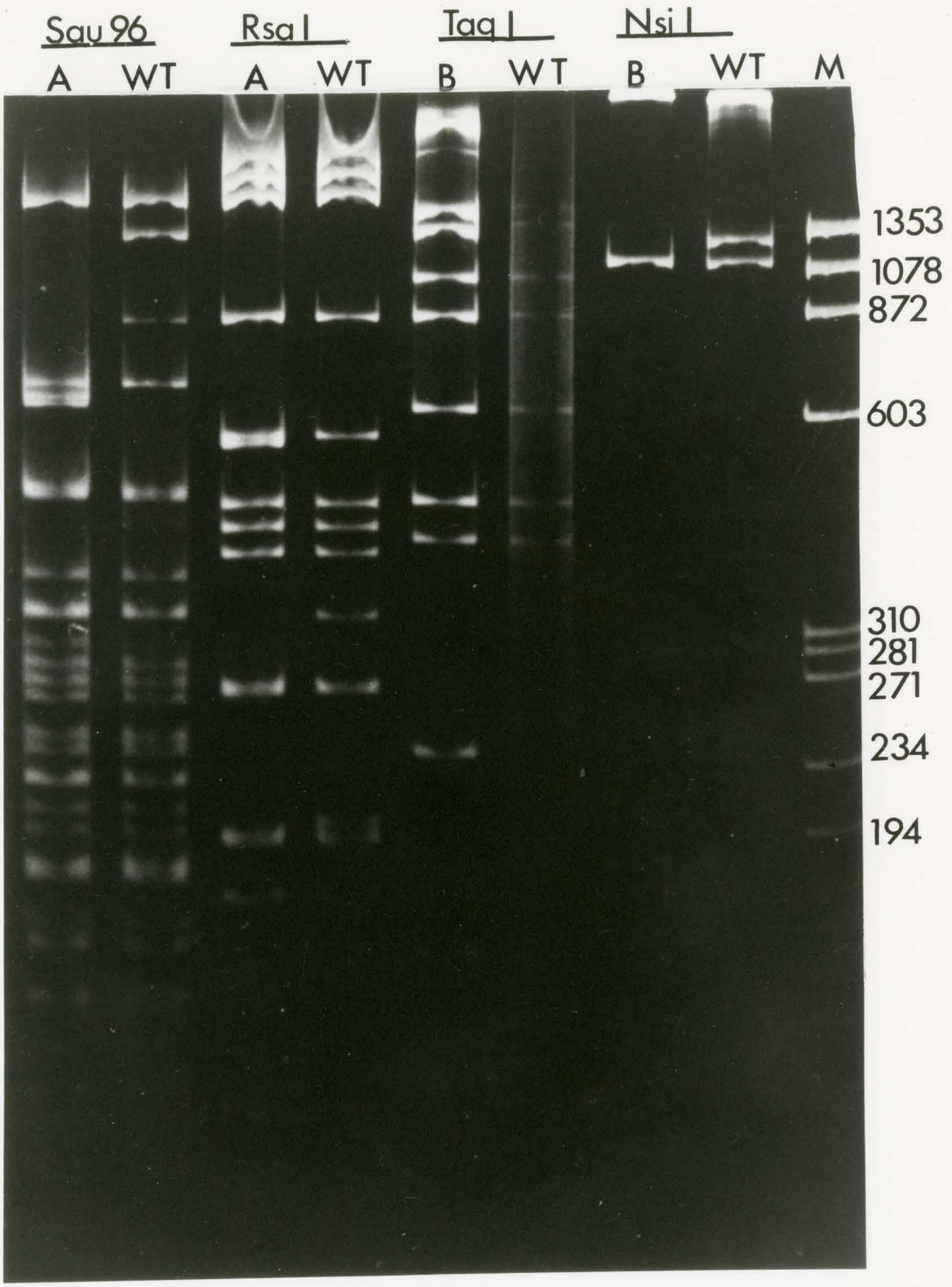
To obtain both 58K mutants pm2019/2250 in the same pXC38 construct, the *Kpn I* to *Xba I* insert containing mutant pm2019 was cloned into the *Kpn I/Xba I* vector of pm2250. Diagnostic *Sau96 I* digestion of pm2019/2250 identified the presence of pm2019 mutation (Figure 10). *Sau96I* is sensitive to DCM-methylation and it cuts 47 times within PXC38 sequences, making it difficult to interpret the

Figure 10- Diagnostic enzyme digest of mutant pXC38 constructs.

The positive M13 mutants were subcloned into pXC38 (containing the E1 region of adenovirus type 5). Diagnostic enzyme for each mutant (as described in Figure 2) were used to identify mutant pXC38 constructs.

The mutant pm2029/2250 contains two mutations which eliminate the synthesis of 58K. The restriction digests represent DNA from pm2019/2250 (A), wild type pXC38 (wt) and pm1716/2072.(B)
1. *Sau96 I* digest confirms mutation pm2019. Because of DAM methylation the Sau96 digest in the mutant shows a less intense 816bp band and the appearance of a 604 and 212bp band.
2. *Rsa I* confirms mutant pm2250 by the disappearance of a 201 and a 323bp band in the wild type and the appearance of a 524bp band in the mutant.

The mutant pm1716/2072 contains two mutations which eliminate the synthesis of 19K.
1. *Taq I* confirms pm1716 by the digestion of a 3726bp fragment into a 3481 and a 235bp fragment.
2. *Nsi I* digest is actually a *Nsi I/Bag II* digest confirming the presence of pm2072 by the elimination of a 1257 and a 7618bp fragment to produce in the mutant a 8875bp fragment.


Sau 96
Rsa I
Taq I
Nsi I
A
WT
A
WT
B
WT
B
WT
M
1353
1078
872
603
310
281
271
234
194

products of the digest. However, the mutant pm2019 was diagnosed by the loss of a 816bp band and the appearance of 604 and a 212bp fragment. Methylation sensitivity of the *Sau96 I* enzyme prevents efficient digestion by this enzyme at certain DNA sites. *Sau96 I* digestion of wild type DNA yields among other fragments, a fragment of approximatley 10,00bp due to the incomplete digestion of this fragment into a 816bp and a fragment of approximatly 200bp. The characteristic *Sau96 I* fragments which identified the mutant pm2019 were the loss of the partially digested 10,000bp fragment along with the 816bp fragment and the gain of a 604 and a 212bp band. To confirm the mutation pm2250 in the double mutant pm2019/2250, a diagnostic *Rsa I* digest was performed (Figure 10). The presence of pm2250 was determined by the loss of both a 201 and a 323bp band and the gain of a 523bp band.

III.II.3. Rescue of E1B mutants into Ad5 virus.

In order to rescue the E1B mutants from the E1 containing plasmid (pXC38), the homologous recombination technique of virus rescue according to McGrory et al. (1988) was used. Subconfluent monolayers of 293 cells were co-transfected with pJM17 (containing the entire Ad5 genome plus an insert in the E1 region) and with each mutant plasmid pm1716/2072, pm2019/2250, pm2072, pm2019, and pm2250. Within 7 to 10 days viral plaques appeared and were picked with sterile Pasture pipettes and either stored or used to infect a monolayer of 293 cells. Once infected 293 showed CPE, the monolayer was harvested and the viral DNA extracted for diagnostic digestion.

Figure 11- Rescue of pm1716/2072 and pm2072 into virus.

After 20hr pi, 293 cells were harvested for viral DNA employing the Hirt extraction method. The viral DNA was digested using the diagnostic *Nsi I* and Bag II restriction enzymes to show the presence of the mutation at nucleotide 2072. Lanes: an isolate of pm1716/2072 (A and B), wild type dl309 (C), and pm2072 (D). The wild type fragment of 2344 and 2071 shifted to a 4415 fragment, due to a loss of a *Nsi I* site, seen in both isolates of pm1716/2072 and pm2072. The numbers to the right of the gel represent base pair numbers of interest.

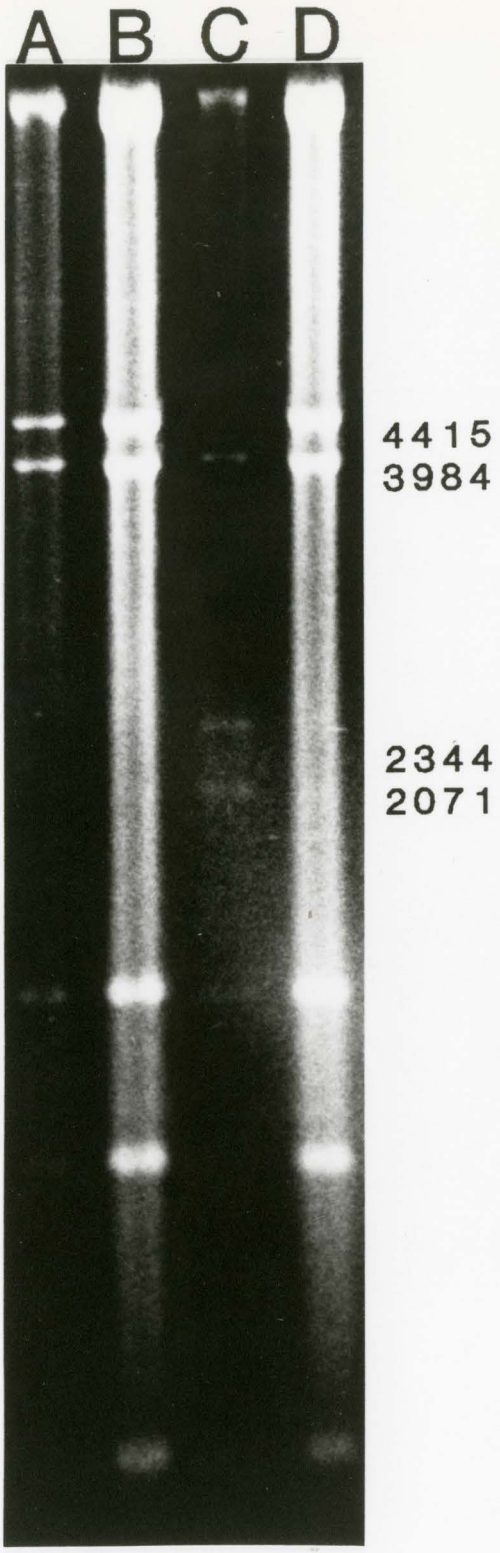
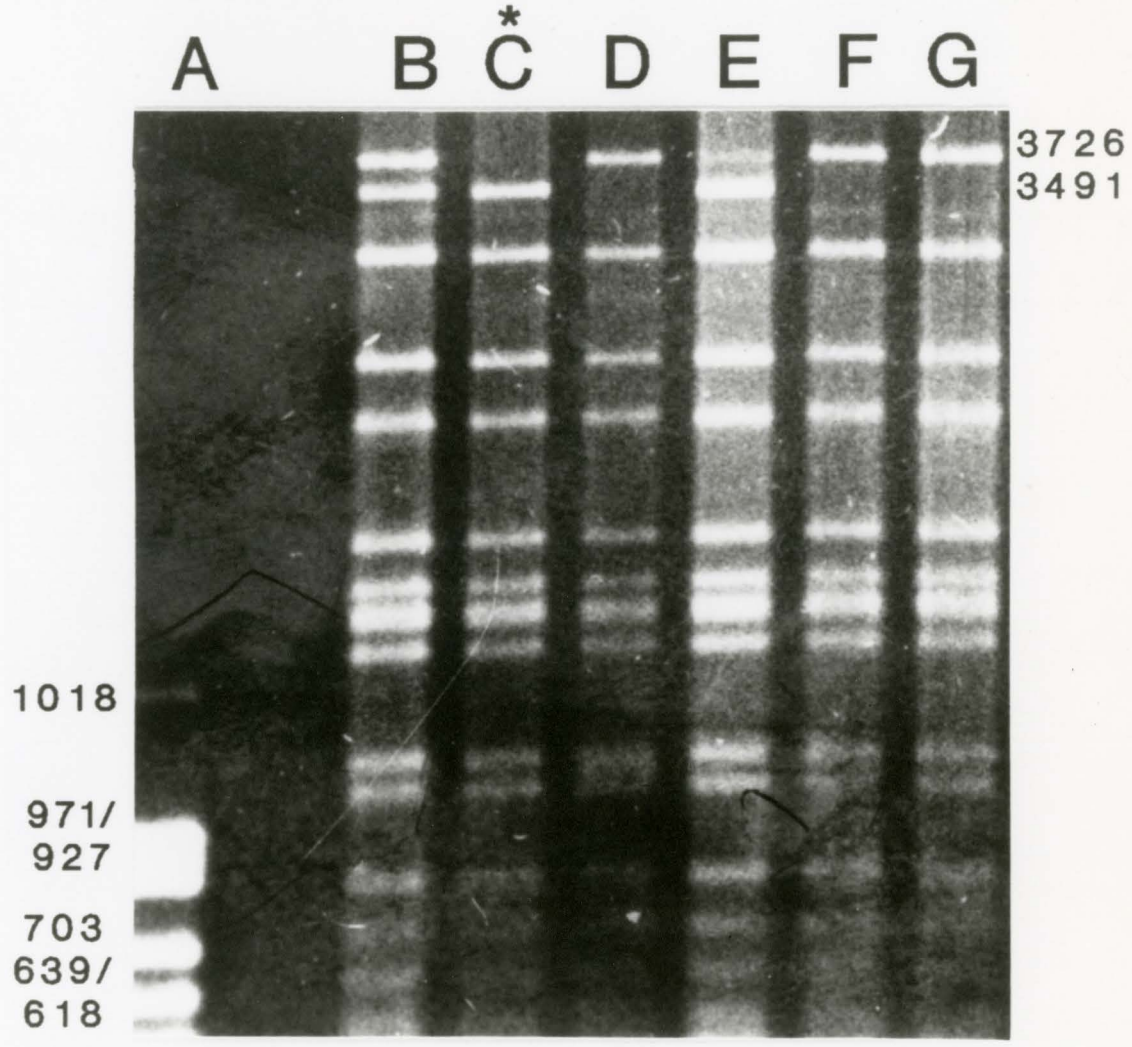

A B C D
4415
3984
2344
2071

Figure 12- Rescue of pm1716/2072 into virus.

293 cells were infected with wild type and mutant virus, 20hr pi Hirt extraction of the viral DNA was performed and digested with *Taq I* restriction enzyme. The mutation at nucleotide 1716 was characterized by a 3491bp fragment, while the wild type was characterized by a 3726bp fragment. The lanes represent digested viral DNA extracted from 293 cells infected with isolates of pm1716/2072 contaminated with wild type virus (B) and (E), wild type virus (D) and (F) and pm1716/2072 (C). Lane (A) represents a molecular weight marker with bp numbers to the left of the gel. The starred lane (C) represents a mutation at nucleotide 1716; this virus also has a mutation at nucleotide 2072 (see figure 11). The numbers to the right of the gel represent bp numbers of importance.


A
B
C*
D
E
F
G
3726
3491
1018
971/
927
703
639/
618

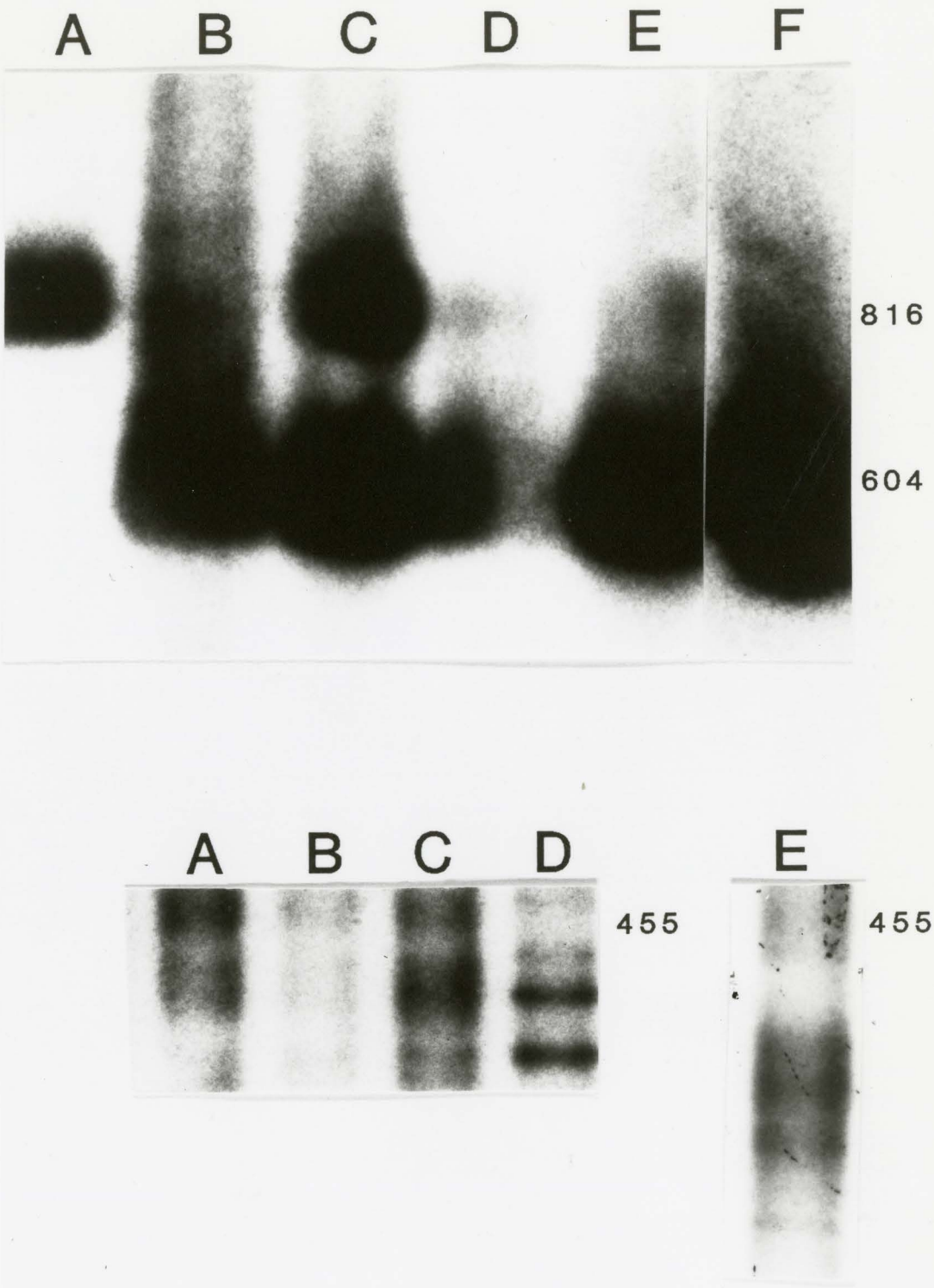
The presence of the mutation at nucleotide 2072 in the viruses pm1716/2072 and pm2072 was confirmed by Nsi I restriction digestion of Hirt extracted viral DNA (Figure 11). The wild type DNA (Lane C) was characterized by the presence of 2344 and 2971bp bands, that combined to form a 4415bp fragment in the mutant viruses pm1716/2072 (Lanes A and B) and pm2072 (Lane D). The presence of the pm1716 mutation in mutant virus was confirmed by a *Taq I* digest (Figure 12). Wild type (Lane G) was characterized by a fragment of 3726bp, whereas the mutation at nucleotide 1716 (Lane C) resulted in the loss of the 3726bp fragment and the gain of a 3491bp fragment.

The diagnostic enzyme digestion of the 58K mutant viruses cut the viral DNA to such an extent that individual fragments were impossible to interpret. Southern blot analysis using a [P^{32}]dCTP labeled nick translated M13A or M13B probe was used to detect these mutations. Figure 13 illustrates a *Sau96 I* digest of viral DNA from dl309 (lane A), pm2019/2250 (lane B to E), and pm2019 DNA (lane F). The presence of the mutant at 2019 is confirmed by the loss of a 816bp band and the gain of 604 and 212bp fragments. [The 212 fragment had run off the gel in this experiment]. Lane C represented an isolate of pm2019/2250 that was contaminated with wild type virus. Southern analysis was also used to confirm the existence of the mutation at nucleotide 2250 in both mutant viruses pm2019/2250 and pm2250. Upon digestion with the diagnostic Rsa I restriction enzyme, mutants pm2019/2250 and pm2250 were confirmed by the detection of a 455bp band (Figure 13).

Figure 13- Southern analysis of pm2019/2250, pm2019 and pm2250.

A. *Sau 96 I* digests were performed on Hirt extracted viral DNA from 293 cells infected with : wild type dl309 (lane A), isolates of pm2019/2250 (B-E) and pm2019 (F). The digested DNA was transferred to nitrocellulose and blotted with a [P^{32}] dCTP nick translated M13A probe. The wild type dl309 virus DNA is characterized by a band at 816bp, which because of the addition of a *Sau96 I* site in the mutant, is digested into a 604 and a 212bp band. Unfortunately the 212bp fragment had run off the gel in this experiment. The mutant pm2019/2250 in lane (C) represents an isolate contaminated with wild type virus. The virus pm2019/2250 in lane (B) and pm2019 in lane (F) were further plaque purified and used in subsequent studies.

B. 293 cells were infected with: Isolates of pm2019/2250 (lanes A to C), wild type dl309 (D), and pm2250 (E). The viral DNA was Hirt extracted and digested with *Rsa I* for Southern blot analysis with a [P^{32}] dCTP nick translated M13B probe. The presence of the mutation at nucleotide 2250 was shown by the presence of a band at 455bp fragment.


A
B
C
D
E
F
816
604
A
B
C
D
455
E
455

III.III. Analysis of mutant E1B protein synthesis.

III.III.1. 19K mutant protein pattern.

Two mutant 19K viruses were rescued, one containing mutations in both the first and second methionine, pm1716/2072, and the second with single mutation changing Met 120 to an arginine, pm2072. In order to determine the Ad5 proteins produced, cell extracts were immunoprecipitated using 19C and 58N2 antipeptide sera. Infected human KB cells were labeled 16-18 hours post infection with either [^{35}S]methionine or [^{35}S]cysteine (the latter is to label a potential 19K product produced by pm1714/2072, which should lack methionine). Figure 14 shows the results obtained with the 19K mutants. The double 19K mutant (lane C) synthesized no protein recognized by the 19C1 serum, while pm2072 synthesized a mutated 19K at levels comparable to wild type adenovirus dl309 (compare lanes A and D). Using 58N2 serum, wild type production of 58K and related products were produced by the double mutant construct (pm 1716/2072) which failed to synthesise 19K.

III.III.2. 58K mutant protein pattern.

Three 58K mutants were rescued into virus, two single mutants, pm2019 and pm2250, and a double, pm2019/2250. Unlike Barker and Berk with their mutant, we did not detect any reinitiated 58K product with pm2019 (Figure 15 lanes E and K). Experiments using 300μCi [^{35}S]methionine per dish of KB cells to label viral proteins, pulse labelling experiments with [^{35}S]methionine, [^{32}P]orthophosphate

Figure 14 -Protein pattern of mutations in 19K coding sequences.

This figure is composed of two exposures of the same gel. Human KB cells were infected with wild type and mutant virus for 16 to 18 hours and labelled for 2 hours with [^{35}S]cystine. Lanes A-E represent immunoprecipitations, with 19-C1 serum, of extracts from KB cells infected with: dl309 (A), dl309 plus 10μg 19C1 peptide (B), pm1716/2072 (C), pm2072 (D), Mock (E). Lanes F-J represent immunoprecipitaions, with 58N2 serum, of KB cells infected with: dl309 (F), dl309 plus 10μg 58N2 peptide (G), pm1716/2072 (H), pm2072 (I), and Mock (J). The position of molecular weight markers are to the left and those of E1B proteins are to the right. (m)- ^{14}C-labelled molecular weight markers.

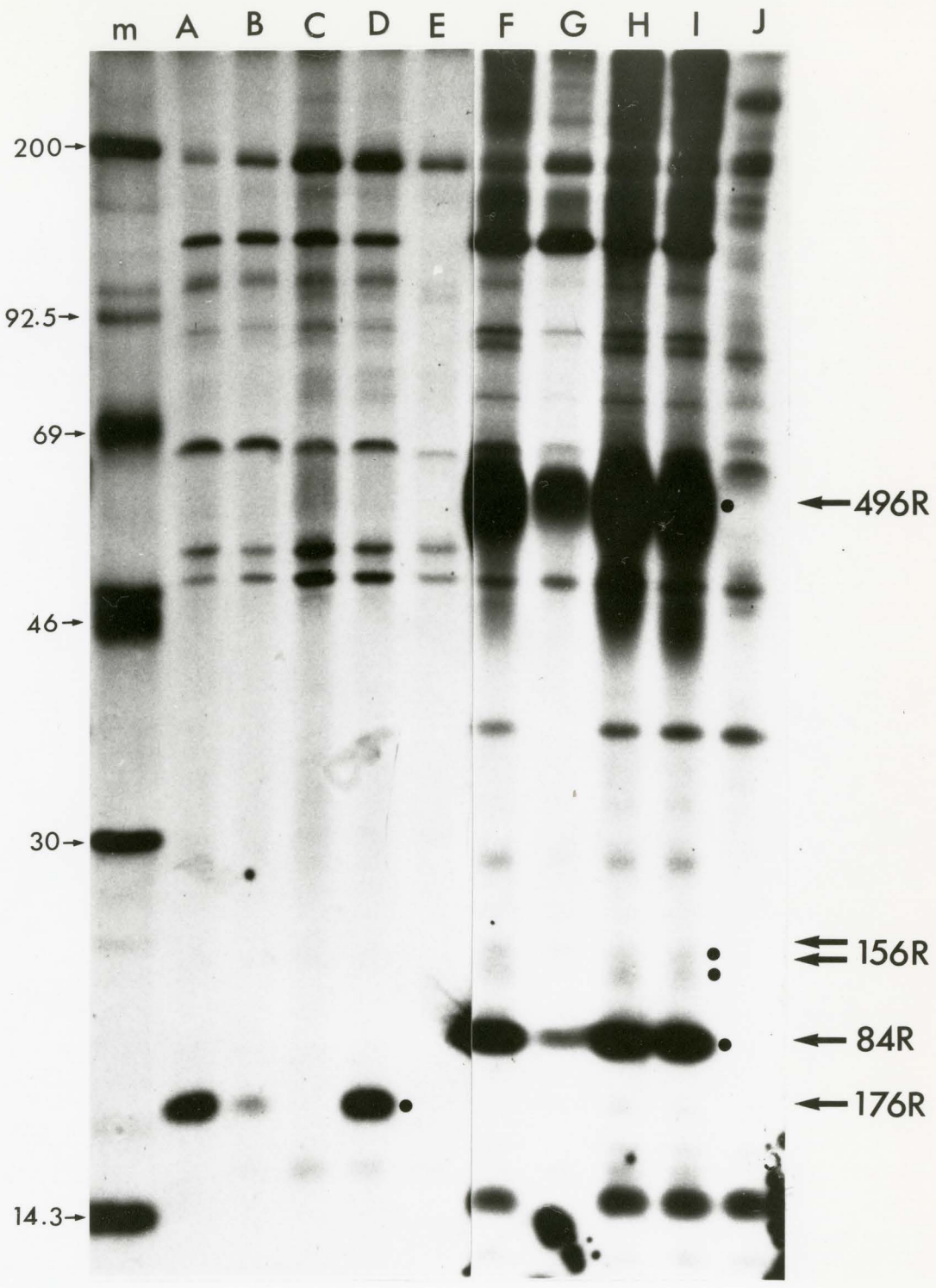

m
A
B
C
D
E
F
G
H
I
J
200
92.5
69
46
30
14.3
496R
156R
84R
176R

Figure 15 -Protein pattern of mutations in the 58K coding sequence.

Human KB cells were infected with wild type and mutant virus for 16 to 18 hours and labelled with [^{35}S] methionine. (m)- ^{14}C-labelled molecular weight marker. Lanes A-G represent immunoprecipitations with 58N2 serum from extracts of KB cells infected with: dl309 (A), dl309 plus 10μg of 58-N2 peptide (B), PM2019/2250 (C), pm2250 (D), pm2019 (E), mock (F). Lanes G-L represent immunoprecipitations with 58C1 serum from extracts of KB cells infected with: dl309 (G), dl309 plus 10μg of 58C1 peptide (H), pm2019/2250 (I), pm2250 (J), pm 2019 (K), mock (L). Lanes M-R represent immunoprecipitations with 19C1 serum from extracts of KB cells infected with: dl309 (M), dl309 plus 10μg of 19C1 peptide (N), pm2019/2250 (O), pm2250 (P), pm2019 (Q), mock (R). Molecular weights of the marker are to the left of the gel and the E1B protein residue number to the right of the gel.

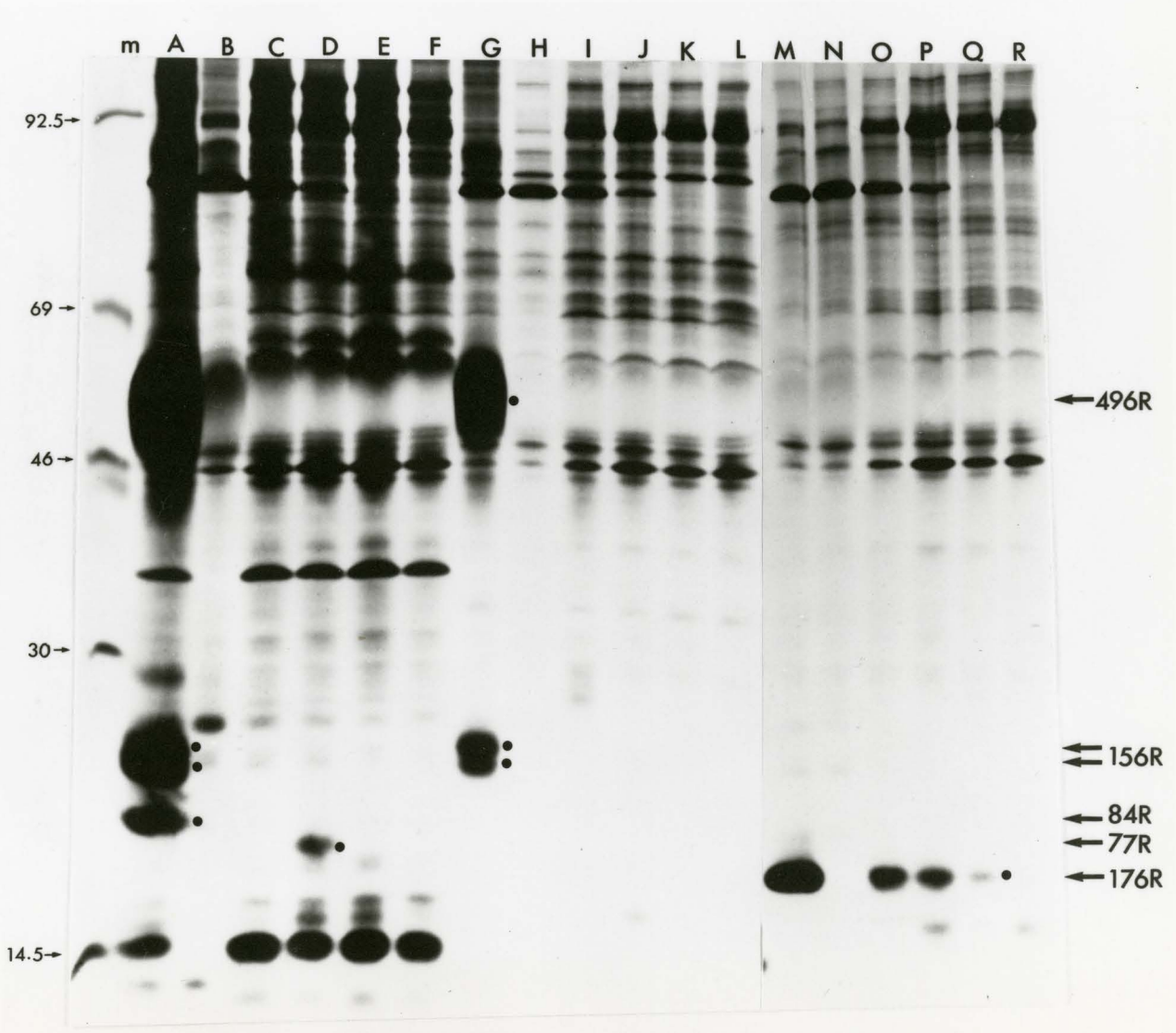
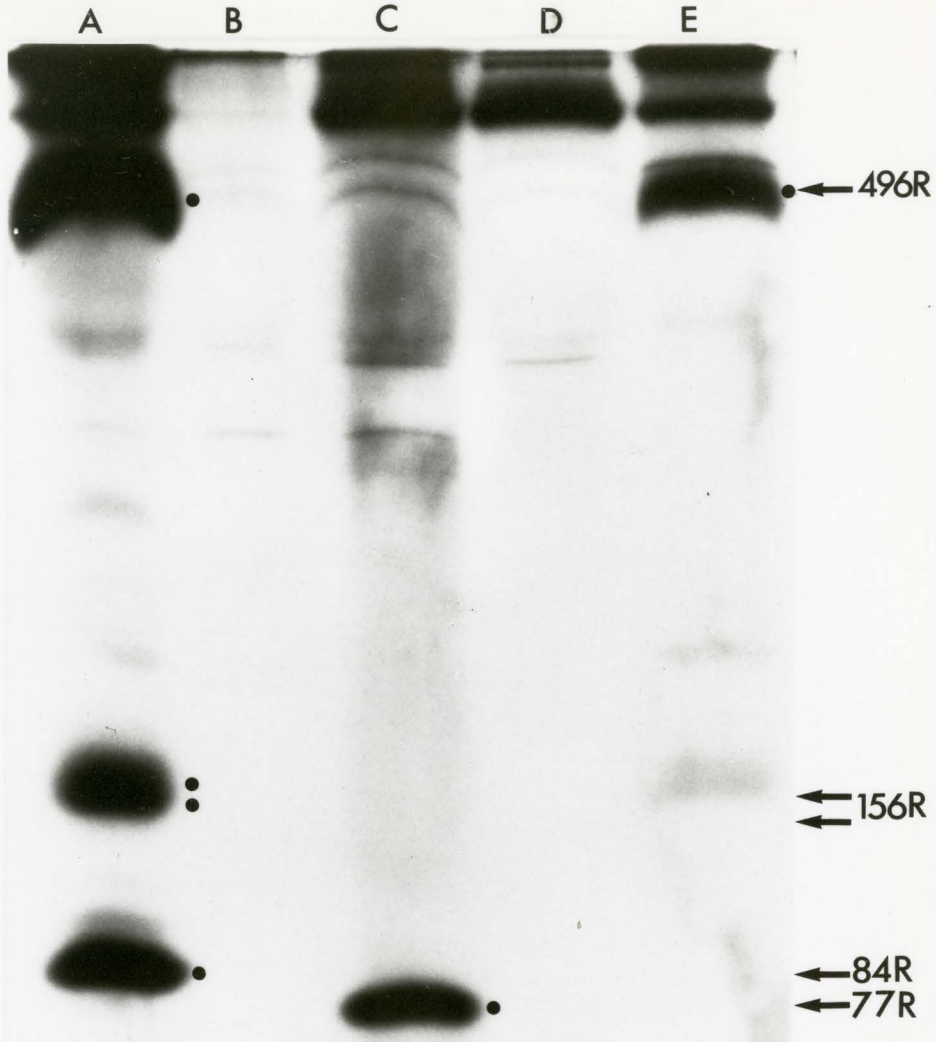

m
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
92.5
69
46
30
14.5
496R
156R
84R
77R
176R

Figure 16 -The truncated product of 58K.

Human KB cells were infected with dl309 (wt) or pm2250 for 16-18 hours and then labelled with [^{35}S]methionine for 2 hours. The extracts were combined with either the 58N2 or 58C1 serum and immunoprecipitates were analyzed by SDS-page. Lanes A-D 58N2 serum: dl309 (A), mock (B), pm2250 (C), pm2250 and 10μg of 58N peptide (D). Lane E represents an imunoprecipitation using the 58C1 serum of KB cells infected with dl309. The E1B residue number is shown to the right of the gel.


A
B
C
D
E
496R
156R
84R
77R

labelling, and the use of monoclonal antibodies to 58K, all failed to detect any reinitiated 58K product from pm2019 (data not shown). Mutant pm2250 synthesized a truncated form of 58K consisting of the first 75 amino acids (Figure 15 lane D, Figure 16 lanes C and D). The double mutant (pm2019/2250) did not synthesize detectable 58K or related products (lanes C and I) (Figure 15). To confirm that the mutations in the 58K coding sequence did not change the pattern of 19K synthesis, immunoprecipitation using 19C1 serum was carried out (lanes M to R) (Figure 15). Figure 15 shows that with all three 58K mutants, 19K was synthesized. The low level of 19K seen in lane Q (mutant pm2017) was due largely to the low moi used in this particular experiment.

III.IV. Replication of E1B mutant viruses.

To study the replication of 19K and 58K mutants, plaque formation on HeLa cells was compared to that on 293 cells which express E1A and E1B constitutively. Table 1 shows plaque ratios of mutant virus grown on HeLa and 293 cells. Mutants with alterations in 19K coding sequences displayed a HeLa/ 293 plaque ratio similar to that of wild type dl309 virus. These results confirmed previous observations that 19K is not essential for replication on human HeLa cells (Pilder *et al.*, 1984; White *et al.*, 1984; Bernards *et al.*, 1986; Barker and Berk, 1987). Figure 17 shows the large plaques morphology of pm1716/2072 and pm1969 as compared with wild type (dl309) plaque size. Mutant pm1969, a gift from Dr. F. Graham, makes reduced levels of a 85 amino acid truncated product of 19K. Mutant pm2204 changes the site of phosphorylation at serine 164 of 19K to a alanine (McGlade *et al.*,1989).

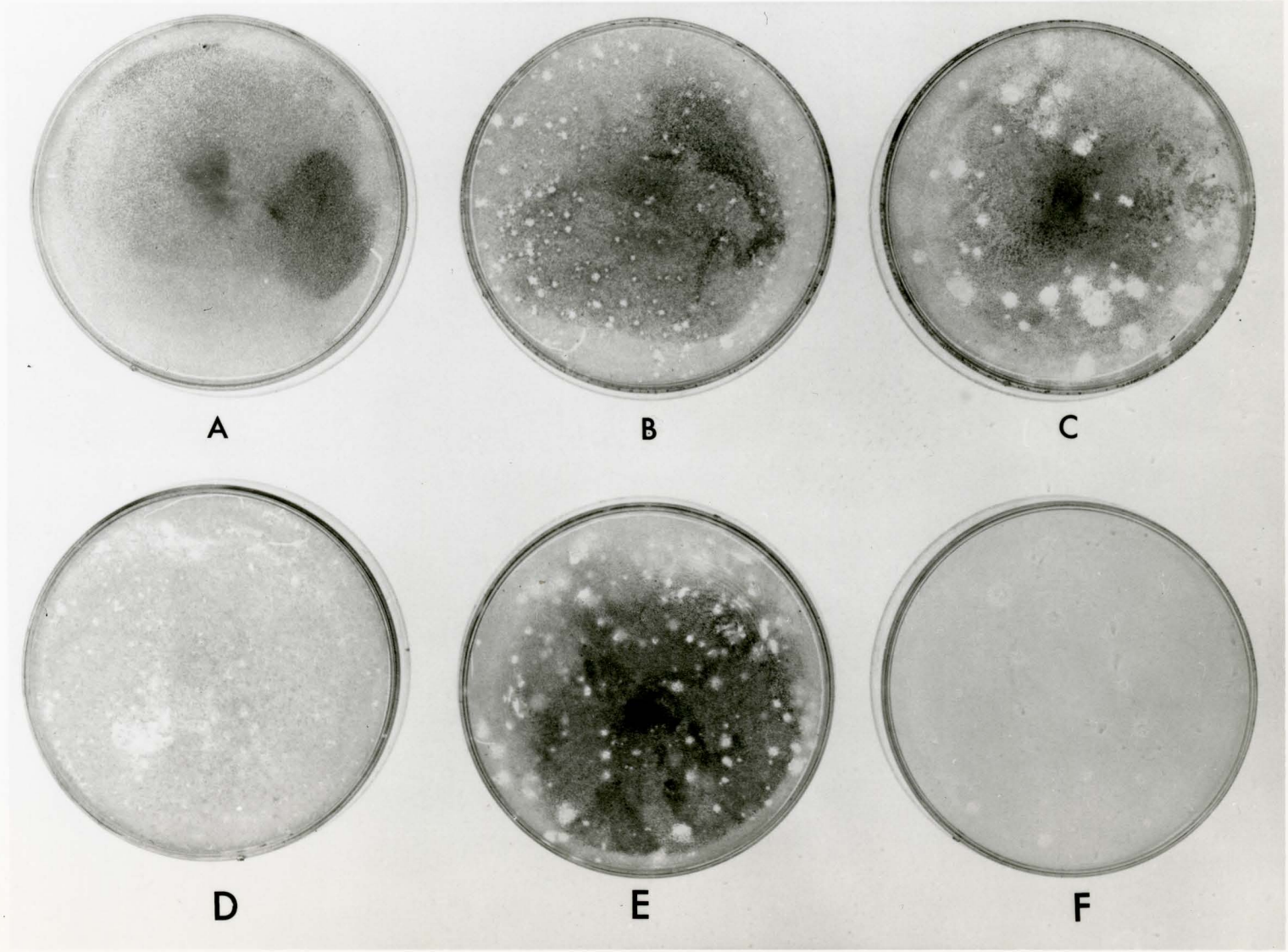
Table 1: **PLAQUE FORMATION BY MUTANT AND WT AD5 VIRIONS**

Virus	Plaque Titre (pfu/ml) on HeLa cells	293 cells	Plaque Ratio HeLa/293	Plaque Size
wt (dl309)	$1.2x10^{12}$	$2.6x10^{12}$	0.46	normal
pm1716/2072	$1.9x10^{10}$	$4.9x10^{10}$	0.36	large
pm2072	$6.0x10^{11}$	$4.0x10^{11}$	1.50	normal
pm2019/2250	$1.7x10^{9}$	$3.8x10^{11}$	0.004	normal
pm2019	$3.2x10^{8}$	$6.9x10^{10}$	0.0045	normal
pm2250	$2.89x10^{7}$	$1.5x10^{11}$	0.0002	normal

Assays were carried out 2-3 times with equal dilution of each virus on HeLa and 293 cells. The number of plaques was determined after 15 days. Each value represents the average plaque titre obtained.

Figure 17 -Cytocidal phenotype of 19K mutants.

The figure shows the results of a two week overlay of HeLa cells infected with: mock (A), dl309 (wt) (B), pm1716/2072 (C),pm2072 (D), pm1969 (E), and pm2204 (F). Cells were fixed, the agarose overlay removed and the cells were stained with Geimsa to visualize the plaques.


A
B
C
D
E
F

These results suggested that pm1716/2072 and pm1969 (and perhaps pm2204) displayed a cyt phenotype. However large plaque morphology was only an indirect method of determining the cytocidal phenotype of 19K mutants. Table 1 also shows that the plaque ratios for all three of the 58K mutants (pm2019/2250, pm2250 and pm2019), in particular pm2250, were greatly reduced. Thus, as suggested previously, 58K and related products appeared to be necessary for viral replication on HeLa cells but not for viral DNA synthesis (Figure 18 and 19)(Harrison *et al.*, 1977; Bernards *et al.*, 1986; Barker and Berk, 1987).

III.V. Degradation of cellular and viral DNA.

One function of 19K involves its direct or indirect protection of host cell DNA against degradation during a viral infection. To assess the effects of E1B mutants on host DNA degradation, undigested and *Hind III* digested DNA from infected KB cells were analyzed on a 1% agarose gel. In the absence of 19K (pm1716/2072) or in the presence of a severely truncated form of 19K (pm1969), viral and host cell DNA were degraded (see Figure 18 and 19). However, with point mutants at methionine 120 (pm2072) or at the phosphorylation site at serine 164 (pm2204), the deg phenotype was not observed (see Figure 18). Similarly, in the absence of 58K and related products (pm2019/2250) or mutated forms of 58K (pm2019 and pm2250), host cell DNA was not degraded, (see Figure 18 and 19). These results confirmed that the deg phenotype was induced by failure to express functional 19K.

Figure 18 -Analysis of DNA Degradation.

KB cells were infected with dl309 and mutant viruses. Cells were harvested 20 hours post infection and the DNA was extracted using a modifies Hirt technique (White *et al.*, 1984). *Hind III* (H) and undigested (U) DNA was electrophoresed on a 1% agarose gel. DNA was then visualized by ethidium bromide staining. A marker (m) to the left of the gel indicates the base pair number.

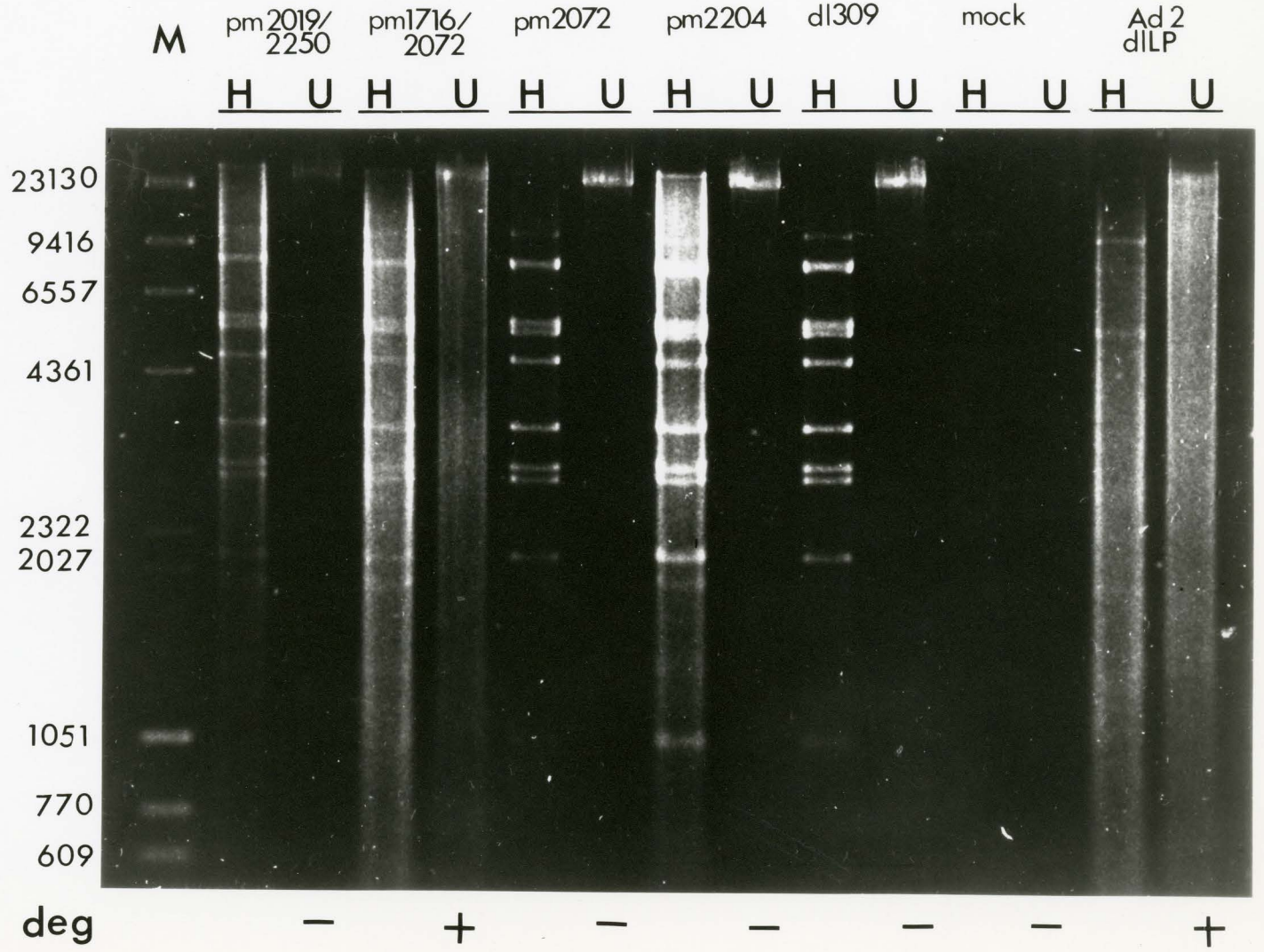
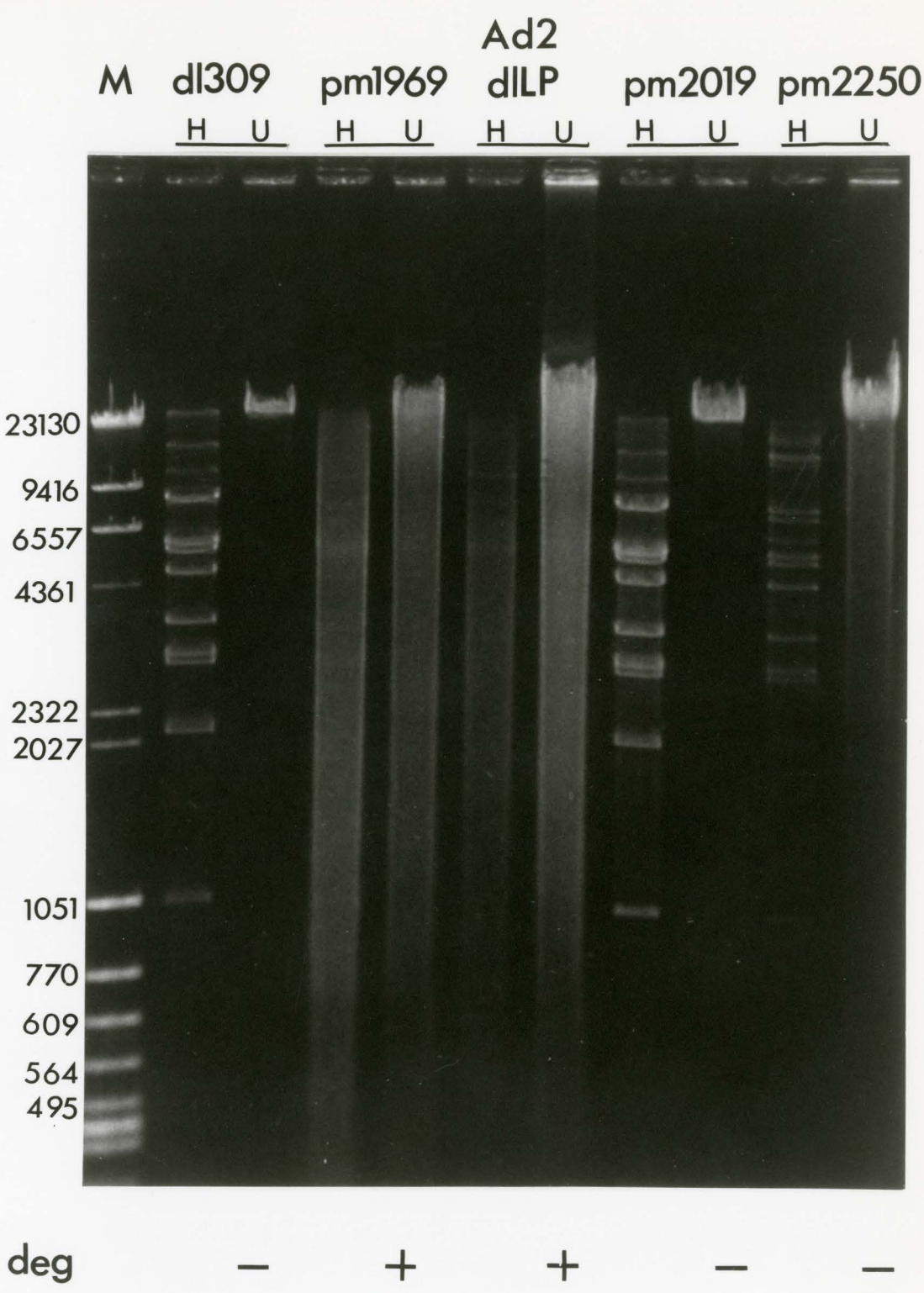

M
pm2019/ 2250
pm1716/ 2072
pm2072
pm2204
dl309
mock
Ad 2 dlLP
H U H U H U H U H U H U H U
23130
9416
6557
4361
2322
2027
1051
770
609
deg
−
+
−
−
−
−
+

Figure 19 -DNA Degradation.

KB cells were infected with dl309 or various mutants. The cells were harvested at 20 hours post infection and DNA was extracted using a modified Hirt technique (White *et al.*, 1984). *Hind III* digested (H) or undigested (U) DNA was loaded on a 1% agarose gel. After electrophoresis the gel was stained by ethidium bromide. A marker (m) to the left of the gel indicates the number of bases.


M
dl309
pm1969
Ad2 dlLP
pm2019
pm2250
H U
H U
H U
H U
H U
23130
9416
6557
4361
2322
2027
1051
770
609
564
495
deg
–
+
+
–
–

III.VI. Effects of E1B mutants on protein synthesis.

Several studies have suggested that E1B products play a role in the regulation of other adenovirus genes. In particular, it has been suggested that 19K may regulate E1A expression. To examine this possibility using our mutants, we compared the expression of both E1A products and the E2A-72K protein production in wild type- and mutant virus-infected KB cells. Figures 20 and 21 show preliminary results which illustrate that neither the 19K or 58K mutants greatly influenced the levels of E1a nor 72K protein synthesis. Early in infection there is no effect on the production of E1A and 72K proteins by the 58K mutant viruses. However, at late times during infection with 58K mutant viruses the level of E1A and 72K proteins was reduced (Data not shown). Further experimentation must be carried out to make any firm conclusions.

III.VII. DNA-mediated transformation.

The major interest in making mutations in the E1B coding sequences, aside from establishing constructs for future structure/function analysis, was to examine the role of individual E1B products in cellular transformation. To do this, baby rat kidney cells were transfected with plasmids expressing E1A plus wild type or mutant E1B protein. The results of several experiments using different DNA preparations are shown in Table 2. The numbers of transformed foci produced by mutant E1B plasmids were counted and expressed as a per cent of the number of foci observed

Figure 20 -Synthesis of E1A proteins by E1B mutants viruses.

KB cells were infected with various E1B mutants for 16-18 hours and labelled for 2 hours with [^{35}S]methionine. KB extracts were immunoprecipitated with monoclonal M73 (Oncogene Science). To the left are molecular weight ^{14}C-markers. Lane: dl309 (A), pm1716/2072 (B), pm2072 (C), pm2019/2250 (D), pm2250 (E), pm2019 (F).

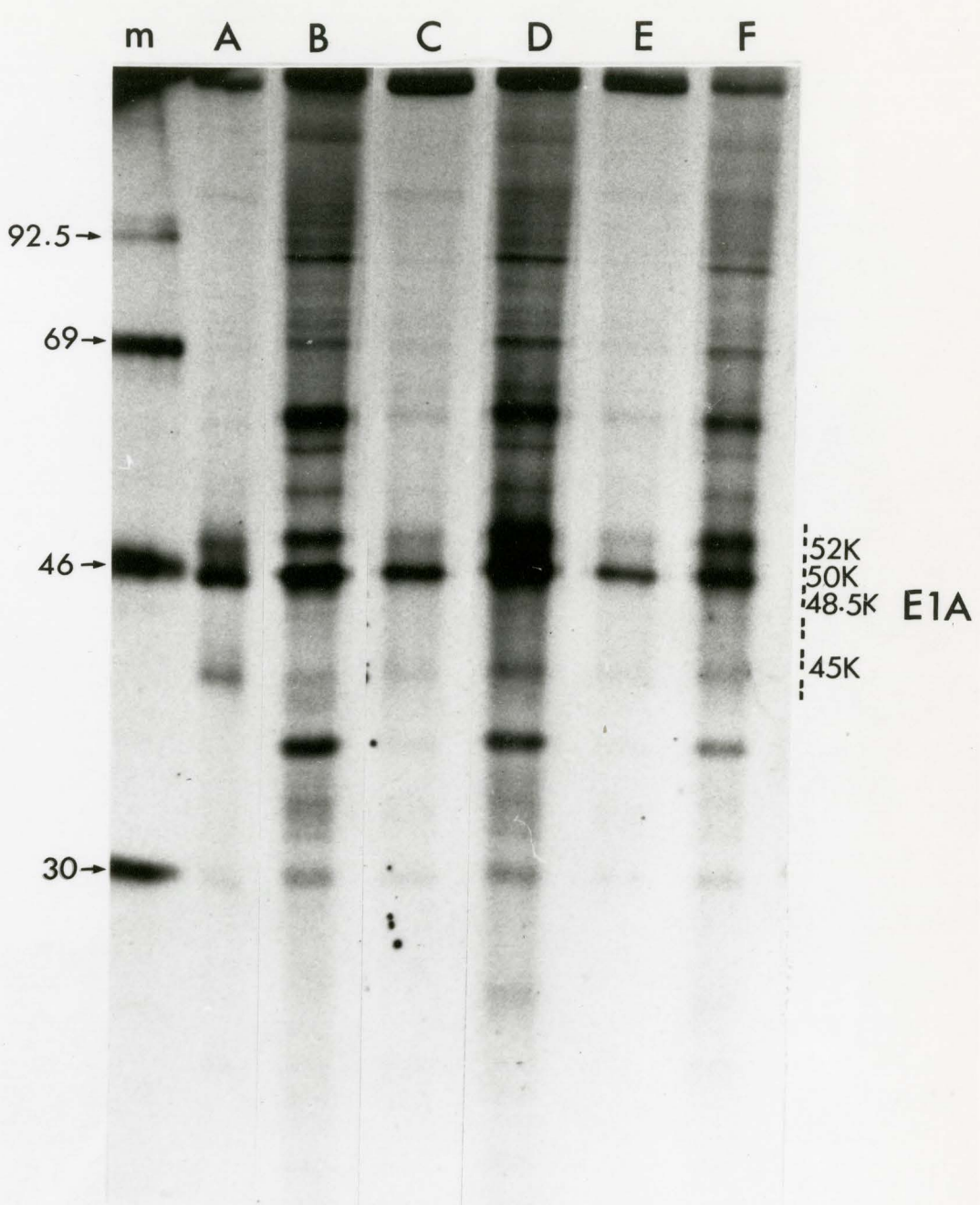

m
A
B
C
D
E
F
92.5
69
46
30
52K
50K
48.5K
45K
E1A

Figure 21 -Synthesis of 72K DNA binding protein by E1B mutants viruses.

KB cells were infected by wild type and mutant E1B viruses for 16 to 18 hours and labelled with [^{35}S]methionine for 2 hours. KB extracts were immunoprecipitated with an antipeptide 72K antibody. To the far left the molecular weight ^{14}C marker (m). KB cells infected with dl309 (A), mock (B), pm1716/2072 (C), pm2072 (D), pm1969 (E), pm2019/2250 (F), pm2250 (G), pm2019 (H).

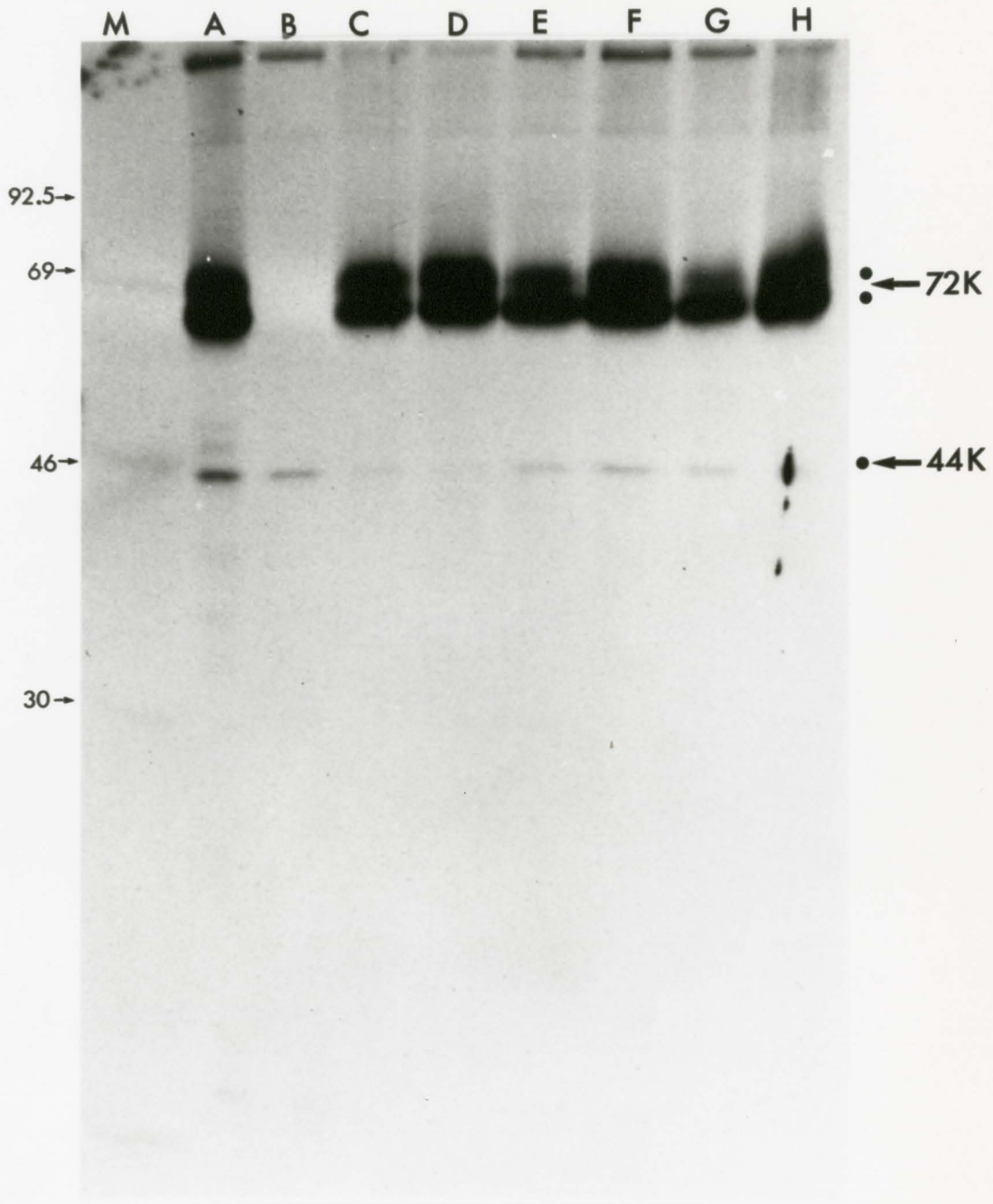

M
A
B
C
D
E
F
G
H
92.5
69
46
30
72K
44K

TABLE 2: TRANSFORMATION OF BABY RAT KIDNEY CELLS*

Plasmid	E1B Defect	Transformation %wt ± S.D.	No. of Experiments
pLE2 (E1A alone)	no E1B	0	1
pXC38 (wt)	none	100	8
pm1716	19K$^-$	37 ± 11	3
pm1716/2072	19K$^-$	24 ± 16	2
pm2072	19K (M-120 to R)	78 ± 28	3
pm2019	58K (dl 1 to 63)	77 ± 16	3
pm2250	58K (dl after 78)	46 ± 11	4
pm2019/2250	58K-	26 ± 10	3
pm1716/2072 + pm2019/2250	none	73 ± 7	2

* Cells were transfected with equal concentrations of plasmid DNA as described in Materials and Methods. Plasmid pLE2 which contains only the E1A coding region (Jelsma *et al.*, 1988) was used to determine the effects of E1A in the absence of the entire E1B region. The results of several separate experiments using independent plasmid DNA preparations have been presented. The average number of foci per dish in individual experiments with pXC38 varied from 29 to 45. The % transforming efficiency relative to pXC38 and the standard error of means have been presented. Analysis of variance at the 95% confidence level of the number of foci per dish in individual experiments using the Student T test indicated that transformation by pm1716, pm1716/2072, pm2250, and pm2019/2250 was significantly reduced relative to pXC38, that by pm1716/2072 + pm2019/2250 was not significantly reduced, and that by pm2072 and pm2019 was reduced at borderline significance.

using wild type E1B. Plasmids which contained both E1A and wild type E1B induced transformation at high efficiency. However E1A in the absence of E1B products was not capable of transformation under these assay conditions. Co-transfection of plasmids expressing only the 58K E1B protein, pm1716/2072, and plasmids only expressing the 19K E1B protein pm2019/2250 yielded transformants at levels approaching wild type. With the mutants that failed to express 19K (pm1716 and pm1716/2072), transformation occurred, but at a greatly reduced efficiency. Alteration of only methionine 120 had no effect on transformation. The double mutant, pm2019/2250, which failed to express any 58K or related products, also induced transformation, but at a greatly reduced efficiency. Truncation of 58K after the third methionine (pm2250), somewhat lowered the efficiency but simple elimination of the first methionine (pm2019) had little effect. These results were similar to those of Barker and Berk who suggested that internal reinitiation of 58K can yield a functional 58K-related product (Barker and Berk, 1987).

Chapter IV.

IV. Discussion.

Our major goal was to better understand the individual roles that the adenovirus E1B proteins play in the transformation of baby rat kidney cells. The overlap in the coding sequences of the two major E1B proteins, 19K and 58K, has made extensive mutational analysis impossible, hindering elucidation of their roles in the transformation process. To separate the coding sequences of 19K and 58K, four individual mutants were prepared. Constructs which failed to express either 19K or 58K were designed by mutating the initiation codons of each protein, thus preventing normal initiation of translation. Internal reinitiation of 19K at the only other methionine (residue 120) was prevented by altering this methionine to an arginine. An in-frame stop was created downstream from the third methionine of 58K because of the observation by Barker and Berk that 58K may reinitiate at an internal methionine. Each mutation was confirmed by sequencing and digestion with diagnostic restriction enzymes.

IV.I. Viral protein synthesis.

IV.I.1. Mutant 58K virus protein synthesis.

Immunoprecipitation experiments confirmed that no 58K or related products were synthesized by the double mutant pm2019/2250. With the single 58K mutant pm2250, a truncated product of 77R was produced. No reinitiated 58K product was detected with pm2019 in infected KB cells using a variety of labelling experiments with [^{35}S]methionine or by [^{32}P]orthophosphate labeling. These results differed from those obtained by Barker and Berk using a mutant (pm2022) which contained a stop codon after the second codon of Ad2 58K (Barker and Berk, 1987). Translation in mammalian cells has been shown to reinitiate at an internal AUG codon after previously initiating and terminating at an upstream site (Liu *et al.*, 1984). Because the initiation sequences of 58K were intact in the pm2022 mutant, ribosomes may have reinitiated at an internal site. The initiation codon of 58K in our pm2019 mutant, however, has been disrupted and thus initiation may have been prevented in the first place.

To ensure the mutations affecting the 58K coding region did not disturb synthesis of the overlapping 19K protein, immunoprecipitations using the 19C antipeptide serum were carried out. The immunoprecipitations were performed early (6 to 8 hours pi), and late (16 to 18 hours pi). At early times all three 58K mutants (pm2019, pm2250, and pm2019/2250) made wild type levels of 19K. However, late during infection there was a reduction in the amount of 19K produced by the mutant 58K viruses (data not shown). A similar phenomen for both E1A

products and the 72K-DBP was also observed with the 58K mutants (data not shown). It is possible therefore that the defect in 19K protein production was not specific but rather reflected a more general effect on intermediate and late viral protein production. This was not surprising since 58K plays a role in late viral mRNA transport (Leppard and Shenk, 1989). Further experimentation is however necessary in order to determine the effect of mutations in 58K on viral protein synthesis.

IV.I.2. Mutant 19K virus protein expression.

Using [^{35}S]cysteine-labelled cells and immunoprecipitation with either 19N or 19C antipeptide polyclonal serum, we did not detect synthesis of any 19K by pm1716/2072. With the point mutant pm2072, in which methionine 120 had been changed to arginine, wild type levels of 19K were produced. The truncation mutant pm1969 produced greatly reduced levels of 19K protein (data not shown). In all cases, 58K expression with the 19K mutants was similar to wild type levels. Similarly, mutations in the 19K coding sequence had no effect on either E1A or 72K protein expression, as determined by immunoprecipitation experiments.

IV.II. Plaquing efficiency.

IV.II.1. Plaquing efficiency of 58K mutant virus.

Table 1 illustrates that the 58K mutants were greatly impaired for their ability

to form plaques on HeLa cells, especially pm2250. Such reduced plaquing efficiency by 58K mutants has also been reported by a number of other groups (Harrison *et al.*, 1977; Benards *et al.*, 1986; Barker and Berk, 1987). The presence of the truncated 58K product expressed by pm2250 appeared to affect viral replication to a greater extent than did complete absence of 58K. The 58K appears to be necessary for Ad5 viral replication. The role of 58K in replication has been investigated by Leppard and Shenk (1989), who showed that 58K mutants fail to efficiently transport late viral mRNA from the nucleus to the cytoplasm, which in turn decreases protein production and slows viral growth. Perhaps the presence of a truncated 58K product further hinders the transport of late viral messages, thus leading to an even greater reduction in viral plaquing efficiency.

IV.II.2. Plaquing efficiency of 19K mutant viruses.

All of the 19K mutants behaved like wild type dl309 virus in that they were able to produce a productive viral infection in HeLa cells. Thus 19K appears not to be essential for viral replication. Unlike wild type, the double mutant pm1716/2072 and pm1969 created large plaques on HeLa cells. Infection by adenoviruses which express no 19K (eg. pm1716/2072), have generally been found to be more cytotoxic to cells, resulting in the production of large and morphologically altered plaques (Chinuadurai 1983, 1984).

The 19K protein has been shown to colocalize in the cytoplasm with the intermediate filament (IF) component vimentin (White and Cipriani, 1989). The 19K protein appears to alter the organization of these IF in the cytoplasm (White

and Cipriani, 1989; 1990). This disruption of IF by 19K during infection may be necessary to protect host cells from extensive cytotoxicity. Thus in the absence of 19K, pm1716/2072, or in the presence of a low amount of a truncated 19K protein, pm1969, the IF are not modified in such a way as to protect the cell against the cyt phenotype.

IV.III. DNA degradation caused by 19K mutant viruses.

The presence of 19K has been shown to protect host and viral DNA from degradation during infection (White *et al.*, 1984). None of the 58K mutant viruses were deficient for protection of the degradation of host or viral DNA. The mutant viruses which either did not produce 19K (pm1716/2072) or synthesised reduced amounts of a truncated product (pm1969), failed to protect DNA from degradation. As observed with other deg mutants, these mutants were also cytopathic (Chinnadurai, 1983; White *et al.*, 1984). White has shown that these two phenotypes may be separable (White *et al.*, 1984).

The mechanism by which 19K functions to protect DNA from degradation is unknown. White *et al.* (1987) have suggested that 19K acts on E1A products, which in turn mediate the deg phenotype. At the protein level, our results did not suggest a great alteration in the amount of E1A protein produced by the 19K deg mutants (pm1716/2072 or pm1969). Thus 19K acts not to increase E1A protein synthesis but rather to modify E1A function. White *et al.* (1984) have also suggested that localization of 19K protein to the nuclear envelope correlates with its ability to block the appearance of the deg phenotype. Vimentin shares extensive amino acid

homology with the nuclear lamin proteins. Thus it is not surprising that 19K also disrupts lamin structures as well (White and Cipriani, 1989). It is possible that 19K functions to protect against host and viral DNA degradation by altering the organization of the lamins.

The phosphorylation of the nuclear lamins by the cell cycle protein cdc2 at mitosis causes them to disassemble (Heald and McKeon, 1990; Moreno and Nurse 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). It is interesting to note that Ad5-19K, a phosphoprotein, has a consensus sequence for cdc2 kinase. Preliminary results show that 19K is phophorylated predominantly at the G_2/mitosis boundary (data not shown). If the deg phenotype is a direct result of the inability of 19K to alter lamin organization, then the phosphorylation of 19K may not play an extensive role in this process as pm2204, which lacks the serine 164 phosphorylation site, is wild type for both the deg and cyt phenotypes (McGlade, 1990).

IV.IV. The role of E1B in transformation.

Our transformation results suggested that, in combination with E1A, both 19K and 58K are able to induce transformation of baby rat kidney cells. Nevertheless, the efficiency of transformation was greatly increased if both E1B products were present. These results were similar to those of Bernards *et al.*, (1986) and White and Cipriani (1990), who also looked at transformation in baby rat kidney cells. The transformation results with mutants in both 19K and 58K coding sequences in established cell lines, as CREF and 3Y1 cells, suggested that both 19K and 58K are required for DNA-mediated transformation (Babiss *et al.*, 1984; Barker and Berk,

1987). These results indicate, perhaps, that primary rat cells are more sensitive to the biological effects of 19K and 58K than established cell lines. Since either 19K or 58K alone can transform BRK cells in cooperation with E1A, and their effects are additive when introduced together, the mechanisms of transformation utilized by each of these E1B proteins likely involve different pathways or at least separate steps of the same pathway. In further support of this hypothesis, 19K and 58K are unrelated in structure and they do not colocalize within the cell.

IV.IV.1. The contribution of 58K to transformation.

Sarnow *et al.* (1984) showed that 58K could be found associated with p53, a cellular phosphoprotein of 53K. The p53 gene has been identified as an anti-oncogene due to its ability to reduce transformation potential in cotransfection assays with activated ras (Finlay *et al.,* 1989). Mutant p53 gene products found in human cancers such as colorectal carcinomas can induce immortalization of early passage rat chondrocytes (Jenkins *et al.,* 1984; Baker *et al.,* 1989). It is possible that 58K may act in transformation by binding to the anti-oncogene product p53 in much the same manner as E1A binds to the retinoblastoma gene product (Egan *et al.,* 1988; Whyte *et al.,* 1988), thus preventing p53 from performing its normal function. The p53-58K complex has not been observed during lytic infection, but rather only in adenovirus-transformed cell lines (Sarnow et al., 1982; Braithwaite and Jenkins, 1989). The p53 in transformed cell lines is said to be competent, that is, competent p53 binds 58K. This suggests that transformation by adenovirus might in some way upregulate the (normally rare) competent form of p53, or select for a subpopulation

of cells expressing more of the p53 to which 58K may bind. As yet the function of p53 in cells remains unclear. Similarly, binding of p53 by 58K has yet to be shown to be a prerequisite for adenovirus transformation.

We have shown that a 77R-truncated product of 58K is unable to transform BRK cell efficiently. It is possible that this amino-terminal region of 58K does not contain sufficient information or that a region further to the carboxy terminus of 58K is involved in transformation. An argument for a carboxy-terminal region of 58K being important in transformation is supported by the results with pm2019 which is able to transform at wild type levels. However, pm2019 does not produce detectable levels of a reinitiated 58K product, suggesting that perhaps minute amounts of a reinitiated 58K protein is sufficient for significant transformation activity but not for wild type plaquing efficiency.

IV.IV.2. The contribution of 19K to transformation.

White and Cipriani (1989, 1990) have suggested that 19K plays a morphological role in transformation by interacting with IF protein and the nuclear lamins. The association with the IF may alter the cell morphology leading to anchorage independent growth and tumorigenicity. Disruption of the nuclear lamins by 19K may interfere with gene regulation leading to the transformed state (White and Cipriani, 1990). The 19K protein has also been shown to stabilize plasmid DNA in transient assays (Herrmann and Mathews, 1989). Thus in co-transformation assays with E1A, 19K may induce higher copy number of E1A plasmid and subsequent E1A protein levels which in turn may increase the efficiency of transformation. It would

be of interest to analyse the 19K mutants described above, as well as the 19K insertion mutants (Appendix), for IF disruption.

Our results also suggest that point mutations and small insertion in the coding sequences of 19K, such as pm2072, pm1772, pm1893, pm1969 (see Appendix) are able to transform at wild type levels. Thus these regions of 19K may not be important for 19K function in transformation.

IV.V. Conclusion and future considerations.

Thus it appears that individually, both 19K and 58K can co-operate with E1A to induce transformation of BRK cells, and that the individual roles of 19K and 58K in transformation are different. This is supported by the observation that when expressed together, they allow for increased efficiency of transformation. It should be noted, however, that in the present study, no attempt was made to characterize the biological properties of cells transformed by the individual E1B proteins. Thus it remains possible that to generate the whole range of transformed cell properties, both E1B products may be required.

The constructs, pm1716/2072 and pm2019/2250 also provide important tools for the mutational analysis of structure and function of both the 19K and 58K proteins, as they provide a means of overcoming problems caused by the overlap in the protein coding sequences. The individual mutagenesis of 19K or 58K may shed further light on the roles of these proteins in transformation by adenovirus.

Ultimately, the study of these two adenovirus co-oncogenes, in addition to E1A, may aid in the further general understanding of the molecular mechanisms of oncogenesis.

IV.VI. Appendix

Three insertion mutants in the 19K coding sequence made by D. Batista in Dr. F. Graham's lab were gifts. The mutants inserted two codons into the 19K coding sequences at nucleotide numbers 1772, 1893 and 1969. Specifically, pm1772 has a leucine and a glycine inserted after amino acid 20 of 19K; pm1893 has a proline and an arginine after amino acid 60; pm1969 has a leucine and a glycine inserted after amino acid 86 of 19K. These mutants in the 19K coding sequence were subcloned from pKP1772, pKM1893 and pKM1969 into the *Kpn I/Xba I* polylinker sites of pXC38. Double stranded sequencing was done to confirm the presence of these mutations. DNA-mediated transformations were carried out on BRK cells. The results from one transformation assay are reported in table 3.

TABLE 3. Transformation by 19K insertion mutants

Plasmid	foci/plate	average # foci/ plate	% transformation of wt
pXC38	239, 115, 177, 175	176	--
pm1772	63, 129, 162, 123, 53, 166, 167	123	70
pm1893	171, 158, 195, 176, 147	169	96
pm1969	109, 125, 130, 147, 163, 129, 158, 174	141	80

Further experimentation is required in order to perform statistical analysis of this data. However, it appears that all three of these 19K insertion mutants are wild type for transformation.

Chapter V.

V. References.

Akusjarvi, G. and H. Persson. 1981. Control of RNA splicing and termination in the major late adenovirus transcription unit. *Nature.* 292: 420-426.

Alestrom, P., G. Akusjavi, M. Pettersson and U. Pettersson. 1982. DNA sequence analysis of the region encoding the terminal protein and the hypothetical N-Gene product of adenovirus type 2. *J. Biol. Chem.* 257(22): 13492-13498.

Anderson, C.W., M.E. Young and S.J. Flint. 1989. Characterization of adenovirus 2 virion protein in Mu. *Virology.* 172: 506-512.

Babiss, L.E., P.B. Ficher and H.S. Ginsberg. 1984. Effect on transformation of mutations in the early region 1b-encoded 21- and 55-Kilodalton protein of adenovirus type 5. J. Virology. 52(2): 389-385.

Baker, S.J., E.R. Fearon, J.M. Nigro, S.R. Hamilton, A.C. Preisinger, J.M. Jessup, P. vanTuinen, D.H. LedBetter, D.F. Barker, Y. Namura, R. White and B. Vogelstein. 1989. Chromosome 17 deletion and p53 gene mutation in colorectal carcinomas. *Science.* 244: 217-221.

Barker, D.D. and A.J. Berk. 1987. Adenovirus proteins from both E1b reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology.* 156: 107-121.

Borelli, E., R. Hen and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature.* 312: 608-612.

Bernards, R., M.G.W. De Leeuw, A. Houweling and A.J. van der Eb.1986. Role of the adenovirus early region 1B tumor antigen in transformation and lytic infection. *Virology.* 150: 126-139.

Berk, A.J. and P.A. Sharp. 1978. Structure of the Adenovirus 2 early mRNA.*Cell.* 14: 695-711.

Berk, A.J. 1986. Adenovirus promoters and E1A transactivation. *Ann. Rev. Genet.* 20: 45-79.

Berk, A.J., F. Lee, T. Harrisio, J. William and P.A. Sharp. 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. *Cell.* 17: 935-944.

Bhatti, A.R. and J. Weber. 1979. Protease of adenovirus type 2: subcellular localization. *J. Biol. Chem.* 254 (24): 12265-12268.

Black, P.H., H.C. Turner and R.J. Huebner. 1967. Transformation of primary by adenovirus-type 3. *Nature.* 216: 171-173.

Borck, J., J.D. Beggs, W.J. Brammer, A.S. Hopkins and N.E. Murray. 1976. The construction *in vito* of transducing derivatives of phage lambda. *Mol.Gen. Genet.* 146: 199-207.

Braithwaite, A.W. and J.R. Jenkins. 1989. Ability of p53 and the adenovirus E1b 5 kilodalton protein to form a complex is determined by p53*J. Virology.* 63(4): 1792-1799.

Branton, P.E. and D.T. Rowe. 1985. Stabilities and interrelations of multiple species of human adenovirus type 5 early region 1 proteins in infected and transformed cells. *J. Virology.* 56(2): 633-638.

Bridge, E. and G. Ketner. 1990. Interaction of adenoviral E4 and E1b products in late gene expression. *Virology.* 174: 345-353.

Brown, D.T., M. Westphal, B.T. Burlingham, U. Winterhoff and W. Doerfler. 1975. Structure and composition of adenovirus type 2 core. *J. Virology.* 16(2):366-387.

Burgert, H.G., J.L. Maryanski and S. Kvist. 1987. 'E3/19K' protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-class I antigens. *Proc. Natl. Acad. Sci. USA.* 84: 1356-1360.

Carlin, C.R., A.E. Tollefson, H.A. Brady, B.L. Hoffman and W.S.M. Wold. 1989. Epidermal growth factor receptor is down-regulated by a 10,400 MW protein encoded by the E3 region of adenovirus. *Cell.* 57: 135-144.

Challberg, M.D. and T.J. Kelly (Jr). 1979. Adenovirus DNA replication *in vitro.Proc. Natl. Acad. Sci. USA.* 76(2): 655-659.

Challberg, M.D. and T.J. Kelly. 1989. Animal viruses: DNA replication. *Ann. Rev. Biochem.* 58: 671-717.

Chardonnet, Y. and S. Dales. 1970. Early events in interaction of adenovirus with HeLa cells: I. Release of the DNA genome. *Virology.* 40: 462- 477.

Chinnadurai, G. 1983. Adenovirus 2 lp^{+} locus codes for a 19Kd tumor antigen that plays an essential role in cell transformation. *Cell.* 33: 759-766.

Chow, L.T., T.B. Broker and J.B. Lewis. 1979. Complex splicing patterns of RNAs from the early region of adenovirus 2. *J. Mol. Biol.* 134: 265-303.

Chow, L.T., R.E. Gelinas, T.R. Broker and R.J. Roberts. 1977. An amazing sequence arrangement at the 5' ends of adenovirus 2 messager RNA.*Cell.* 12: 1-8.

Colby, W.W. and T. Shenk. 1981. Adenovirus type 5 virion can be assembled *in vitro* in the absence of detectable polypeptide IX. *J. Virology.* 39(3): 977-980.

Corden, J., H.M. Engelking and G.D. Pearson. 1976. Chromatin-like organization of the adenovirus chromosome. *Proc. Natl. Acad. Sci. USA.* 73(2):401-404.

Cutt, J.R., T. Shenk and P. Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptide synthesized in productively infected cells. *J. Virology.* 61(2): 543-552.

Dales, S. and Y. Chardonnet. 1973. Early events in the interaction of adenoviruses with HeLa cells IV. Association with microtubles and the nuclear pore complex during vectorial movement of the inoculam. *Virology.* 56: 465-483.

Deuring, R., U. Winterhoff, F. Tamanoi, S. Stabel and W. Doerfler. 1981. Site of linkage between adenovirus type 12 and cell DNAs in hamster transformed line CLAC3. *Nature.* 293: 81-84.

D'Halluin, J.C., G.R. Martin, M. Toppler and P.A. Boulanger. 1978. Adenovirus type 2 assembly analysed by reversible cross linking of labile intermediates. *J. Virology.* 26(2): 357-363.

D'Halluin, J.C., M. Milleville, P.A. Boulanger and G.R. Martin. 1978. Temperature-sensitive mutant of adenovirus type 2 blocked in virion assembly: accumulation of light intermediate particles. *J. Virology.* 26(2): 344-356.

Dorsch-Hasler, K., P.B. Fisher, I.B. Weinsterin and H.S. Ginsberg. 1980. Patterns of viral DNA integration in cells transformed by wild type or DNA-binding protein mutants of adenovirus type 5 and effect of chemical carcinogens on integration. *J. Virology.* 34(2): 305-314.

Dumont, D.J., M.L. Tremblay and P.E. Branton. 1989. Phosphorylation at serine 89 induces a shift in gel mobility but has little effect on the function of adenovirus type 5 E1A protein. *J. Virology.* 63(2): 987-991.

Edbauer, C., C. Lamberti, J. Tong and J. Williams. 1988. Adenovirus type 12 E1B 19-Kilodalton protein is not required for oncogenic transformation in rats. *J. Virology.* 62(9): 3265-3273.

Egan, C., S.T. Bayley and P.E. Branton. 1989. Binding of the Rb1 protein to E1A products is required for adenovirus transformation. *Oncogene.* 4: 383-388.

Egan, C., T.N. Jelsma, J.A. Howe, S.T. Bayley and P.E. Branton. 1989. Mapping of cellular protein-binding sites on the products of early region 1A of human adenovirus type 5. *Mol. Cell. Biol.*8: 3955-3959.

Everitt, E., L. Lutter and L. Philipson. 1975. Structural proteins of adenoviruses: XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology.* 67: 197-208.

Falgout, B. and G. Detner. 1987. Adenovirus early region 4 is required for efficient virus particle assembly. *J. Virology.* 61:3759-3768.

Finlay, C.A., P.W. Hinds and A.J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell.* 57:1083-1093.

Flint, J. 1977. The topography and transcription of adenovirus genome. *Cell.* 10:153-166.

Flint, S.J. and T.R. Broker. 1981. Lytic infection by adenovirus. in *DNA Tumor Viruses.* ed. J. Tooze. Cold Spring Harbour Laboratory. New York. pg. 443.

Freeman, A.E., P.H. Black, E.A. Vanderpool, P.H. Henry, J.B. Austin and R.J. Huebner. 1967. Transformation of primary rat embryo cells by adenovirus type 2. *Proc. Natl. Acad. Sci. USA.* 58:1205-1212.

Freeman, A.E., P.H. Black, R. Wolford and R.J. Huebener. 1967. Adenovirus type 12-rat embryo transformation system. *J. Virology.* 1(2): 362-367.

Frenkel, L. and H. Bremer. 1986. Laboratory methods: Increased amplification of plasmids pBR322 and pBr327 by low concentration of chloramphenicol. *DNA.*5(6): 539-544.

Friefeld, B.R., M.O. Krevolin and M.S. Horwitz.1983. Effects of the adenovirus H5ts125 and H5ts107 DNA binding protein on DNA replication *in vitro*. *Virology.* 124: 380-389.

Futterer, J. and E.L. Winnacker. 1984. Adenovirus DNA replication. in: *Current topics in microbiology III: The molecular biology of adenovirus 3.* eds: W. Doerfler. Springer Verlag. Berlin.

Gallimore, P.H. 1972. Tumor production in immunosuppressed rats with cells transformed *in vitro* by adenovirus type 2. *J. Gen. Virol.* 16: 99-102.

Gallimore, P.H., P.A. Sharp and J. Sambrook. 1974. Viral DNA in transformed cells: II. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. *J. Mol. Biol.* 89: 49-72.

Gilden, R.V., J. Kern, A.E. Freeman, C.E. Martin, R.C. McAllister, H.C. Turner and R.J. Huebner. 1968. T and tumor antigens of adenovirus group C-infected and transformed cells. *Nature.* 219: 517-518.

Ginsberg, H.S., K. Sugawara, G. Shanmugam and M. Green. 1976. Synthesis of the adenovirus-coded DNA binding protein in infected cells. *J. Virology.* 18(2): 454-460.

Ginsberg, H.S., H.G. Pereina, R.C. Valentine and W.C. Wilcox. 1966. A proposed terminology for the adeonvirus antigens and virion morphological subunits. *Virology.* 288: 782-783.

Glen, G.M. and R. Ricciardi. 1988. Detailed kinetics of adenovirus type-5 steady-state transcription during early infection. *Virus Research* 9(1): 73-91.

Goldberg, S., J. Nevins and J.E. Darnell. 1978. Evidence from UV transcription mapping that late adenovirus type 2 mRNA is derived from a large precursor molecule. *J. Virology.* 25(3): 806-816.

Gooding, L.R., L.W. Elmore, A.E. Tollefson, H.A. Brady and W.S.M. Wold. 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell.* 53:341-346.

Graham, F.L., T. Harrison and J. Williams. 1978. Defective transforming capacity of adenovirus type 5 host range mutants. *Virology.* 86: 10-21.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn. 1977. Characterization of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 39: 59-77.

Graham, F.L. and A.J. van der Eb. 1973a. A new technique for the assay of infectivity of human adenovirus type 5 DNA. *Virology.* 52: 456-467.

Graham, F.L. and A.J. van der Eb. 1973b. Size and location of the transforming region in adenovirus type 5 DNA. *Virology.* 54: 536-539.

Graham, F.L., A.J. van der Eb and H.L. Heijneker. 1979. Size and location of the transforming region in human adenovirus type 5 DNA. *Nature.* 251:687-690.

Graham, F.L., J. Rudy and P. Brinkley. 1989. Infectious circular DNA of human adenovirus type 5: Regeneration of viral DNA termini from molecules lacking terminal sequences. *EMBO. J.* 8(7): 2077-2085.

Green, M. 1970. Oncogenic viruses. *Ann. Rev. Biochem.* 39: 701-756.

Green, M. and G. Daesch. 1961. Biochemical studies on adenovirus multiplication: II. Kinetics of nucleic acid and protein synthesis in suspension cultures. *Virology.* 13: 169-176.

Green, M., J.K. Mackey, W.M. Wold and P. Regden. 1979. Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based upon DNA genome homologies. *Virology.* 93: 481-492.

Green, M. and M. Pina. 1964. Biochemical studies on adenovirus multiplication: VI. Properties of highly purified tumorigenic human adenoviruses and their DNAs. *Proc. Natl. Acad. Sci. USA.* 51: 1251-1259.

Green, M., M. Pina, R. Kimes, P.C. Wesink, L.A. MacHattie and C.A. (Jr) Thomas. 1967. Adenovirus DNA: I. Molecular weight and conformation. *Proc. Natl. Acad. Sci. USA.* 57: 1302-1309.

Hammarskjold, M.L. and G. Winberg. 1980. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. *Cell.* 20: 787-795.

Harlow, E., B.R. Franza (Jr) and C. Schley. 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: Extensive heterogeneity in early region 1A products. *J. Virology.* 55(3): 533-546.

Harlow, E., P. Whyte, B.R. Franza and C. Schley. 1986. Association of adenovirus early region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* 6: 1579-1589.

Harrison, T., F.L. Graham and J. Williams. 1977. Host range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology.* 77: 319-329.

Hay, R.T., L. Clark, P.H. Cleat, M.P.G. Harris, E.C. Robinson and C.J. Witson. 1988. Requirements for the initiation of adenovirus type 2 and type 4 DNA replication. In: *Cancer cell (volume 6): Eukaryotic DNA replication.* ed. T. Kelly and B. Stillmanm. Cold Spring Harbour. New York, pg. 71-75.

Heald, R. and F. McKeon. 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell.* 61: 579-589.

Hearing, P., R.J. Samulski, W.L. Wishart and T. Shenk. 1987. Identification of a repeated sequence element required for efficient encapsidation of adenovirus type 5 chromosome. *J. Virology.* 61(8): 2555-2558.

Hennache, B. and P. Boulanger (1977). Biochemical study of KB-cell receptor for adenovirus. *Biochem. J.* 166: 237-241.

Hermmann, C., C.V. Dery and M.B. Mathews. 1987. Transactivation of host and viral genes by the adenovirus E1B 19K tumor antigen. *Oncogene.* 2: 25-35.

Herrman, C.H. and M.B. Mathews. 1989. The adenovirus E1B 19-Kilodalton protein stimulates gene expression by increasing DNA levels. *Mol. Cel. Biol.* 9(12): 5412-5423.

Hierholzer, J.C. 1973. Further sugrouping of human adenoviruses by differential hemagglutination. *J. Inf. Dis.* 128(4): 541-550.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26: 365-369.

Horne, R.W., S. Bonner, A.P. Waterson and P. Wildy. 1959. The icosahedral form of an adenovirus. *J. Mol. Biol.* 1: 84-86.

Horton, T.H., A.E. Tollefson, W.S.M. Wold and L.R. Gooding. 1990. A protein serologically and functionally related to the Group C E3 14,700 Kiladalton protein is found in multiple adenovirus serotypes. *J. Virology.* 64(3): 1250-1255.

Horwitz, M.S. 1990. Adenoviridae and their replication. In *Fields Virology* vol 2. eds. Fields, B.N., D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick and T.P. Monath. Raven Press. New York. pg: 1679-1721.

Horwitz, M.S., J.V. Maizel and M.D. Schaff. 1970. Molecular weight of adenovirus type 2 hexon polypeptide. *J. Virology.* 6(4): 569-571.

Horwitz, M.S., M.D. Scharff and J.V. Maizel. 1969. Synthesis and assembly of adenovirus 2. I. Polypeptide synthesis, assembly of capsomeres, and morphogenesis of the virion. *Virology.* 39: 682-694.

Hosokaqa, K. and M.T. Sung. 1976. Isolation and characterization of an extremely basic protein from adenovirus type 5. *J. Virology.* 17(3):924-934.

Houweling, A., P.J. van der Elsen and A.J. van der Eb. 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology.* 105: 537-550.

Huebner, R.J., M.J. Casey, R.M. Chanock and K. Schell. 1965. Tumor induced in hamsters by a strain of adenoviruses type 3: Sharing of tumor antigens and "neoantigens" with those produced by adenovirus type 7 tumors. *Proc. Natl. Acad. Sci. USA.* 54: 381-388.

Hurwitz, D.R. and G. Chinnadurai. 1985. Evidence that a second tumor antigen coded by adenovirus early gene region E1a is required for efficient cell transformation. *Proc. Natl. Acad. Sci. USA.* 82: 163-167.

Jelsma, T.N., J.A. Howe, C.M. Evelegh, N.F. Cunniff, M.H. Skiadopoulos, M.R. Floroff, J.E. Denman, and S.T. Bayley. 1988. Use of deletion and point mutations spanning the coding region of the Ad5 E1A gene to define a domain that is essential for transcriptional activation. *Virology.* 163: 494-502.

Jenkins, J.R., K. Rudge and G.A. Currie. 1984. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature.* 312: 651-654.

Jones, N. and T. Shenk. 1979. An adenovirus type5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA.* 76(8): 3665-3669.

Katon, S., K. Ozawa, S. Kondon, E. Soeda, A. Isreal, K. Shiroki, K. Fujinaga, K. Itakura, G.Gachelin and K. Yokayama. 1990. Identification of sequences responsible for positive and negative regulation by E1A in the promoter of H-2K^{bm1} class I MHC gene. *EMBO.* 9(1): 127-135.

Kelly, T.J., M.S. Wold, J. Li. 1988. Initiation of viral DNA replication. *Advances in Virus Research.* 1-34.

Kenny, M.K., L.A. Balogh and J. Hurwitz. 1988. Initiation of adenovirus DNA replication. *Virology.* 263(20):9801-9808.

Kimelman, D., J.S. Miller, D. Porter and B.E. Roberts. 1985. E1a region of the human adenoviruses and of the highly oncogenic adenovirus 7 are closely related. *J. Virology.* 53(2): 399-409.

King, P.V. and R.W. Blakesley. 1986. Optimizing DNA ligation for transformation. *Focus (BRL).* 8(1): 1-2.

Kingston, R.E., A.S. Baldwin and P.A. Sharp. 1985. Transcription control by oncogens. *Cell.* 41: 3-5.

Kitchingman, G.R. and H. Westphal. 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. *J. Mol. Biol.* 137: 23-48.

Klein, H., W. Maltzman and A.J. Levine. 1979. Structure-function relationship of adenovirus DNA-binding protein. *J. Biol. Chem.* 254(21): 11051-11060.

Kovesdi, I., R. Reichel and J.R. Nevins. 1986. Identification of a cellular transcription factor involved in E1A trans-activatiion. *Cell.* 45: 219-228.

Kunkel, T.A. 1985. Rapid efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82: 499-492.

Leppard, K.N. and T. Shenk. 1989. The adenovirus E1B 55k protein influences mRNA transport via an intranuclear effect on RNA metabolism. *EMBO.* 8(8):2329-2336.

Lewis, J.B. and C.W. Anderson. 1987. Identification of adenovirus type 2 early region 1B products that share the same amino terminus as do the 496R and 155R proteins. *J. Virology.* 61(12): 3879-3888.

Lewis, J.B. and M.B. Mathews. 1980. Control of adenovirus early gene expression: a class of immediate early products. *Cell.* 21: 303-313.

Lechner, R.L. and T.J. Kelly. 1977. The structure of replicating adenovirus 2 DNA molecules. *Cell.* 12: 1007-1020.

Lichy, J.H., M.S. Horwitz and J. Hurwitz. 1981. Formation of a covalent complex between the 800,000d adenovirus TP and 5'-dCMP *in vitro*. *Proc. Natl. Acad. Sci. USA.* 78(5): 2678-2682.

Lillie, J.W., M. Green and M.R. Green.1986. An adenovirus E1a protein region required for transformation and transcriptional repression. *Cell.* 46: 1043-1051.

Linne, T., H. Jornvall and L. Philipson. 1977. Purification and characterization of the phosphorylated DNA binding protein from adenovirus-type2-infected cells. *Eur. J. Biochem.* 78: 481-490.

Lillie, J.W., P.M. Loewenstein, M.K. Green and M. Green 1987 Functional domains of adenovirus type5 E1a proteins. *Cell* 50: 1091-1100.

Liu, C.C, C.C. Simonsen and A.D. Levinson. 1984. Initiation of translation at internal AUG codons in mammalian cells. *Nature.* 309: 82-85.

Lonberg-Holm, R. and L. Philipson. 1969. Early events of viral-cell interaction in an adenovirus system. *J. Virology.* 4(4): 323-338.

Lucher, L.A., P.M. Loewenstein and M. Green. 1985. Phosphorylation *in vitro* of *Escherichia coli* - produced 235R and 266R tumor antigens encoded by human adenovirus type 12 early transformation region 1A. *J. Virology.* 56(1): 183-193.

Maizel, J.V.(Jr), D.O. White and M.D. Schaff. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7, 12. *Virology.* 36: 115-125.

Malette, P., S.P. Yee and P.E. Branton. 1983. Studies on the phosphorylation of the 58,000 dalton early region 1B protein of human adenovirus type 5. *J. Gen. Virology.* 64: 1069-1078.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. *Molecular Cloning: A laboratory manual*. Cold Spring Harbour Laboratory.

Marsh, M. and A. Helenius. 1989. Virus entry into animal cells. *Advances in Virus Research.* 36: 107-139.

McAllister, R.M., M.O. Nicolson, A.M. Lewis, J.I. Macpherson and R.T. Huebner. 1969. Transformation of rat embryo cells by adenovirus type 1. *J. Gen. Virol.* 4: 29-36.

McAllister, R.M., M.O. Nicolson, G. Reed, J. Kern, R.V. Gilden and R.J. Huebner. 1969. Transformation of rodent cells by adenovirus 19 and other group D adenoviruses. *J. Nat. Cancer. Inst.* 43: 917-922.

McGlade, C.J. 1990. PhD thesis. McMaster University. Dept. Medical Sciences.

McGlade, C.J., M.L. Tremblay and P.E. Branton. 1989. Mapping of a phosphorylation site in the 176R (19KDa) early region of human adenovirus type 5. *Virology.* 168: 119-127.

McGlade, C.J., M.L. Tremblay, S.P. Yee, R. Ross and P.E. Branton. 1987. Acylation of the 176R (19-Kilodalton) early region 1b protein of human adenovirus type 5. *J. Virology.* 61(10):3227-3234.

McGrory, W.J., D.S. Bautista and F.L. Graham. 1988. A simple technique for the rescue of early region 1 into infectious human adenovirus type 5. *Virology.* 163: 614-617.

Mirza, M.A. and J. Weber. 1982. Structure of adenovirus chromatin. *Bioch. Biophys. Acta.* 696: 76-86.

Montano, X. and D.D. Lane. 1987. The adenovirus E1a gene induces differentiation of F_9 teratocarcinoma cells. *Mol. Cellul. Biol. 7(5): 1782-1790.*

Moreno, S. and P. Nurse. 1990. Substrates for $p34^{cdc2}$ *in vivo* veritas? *Cell.* 61: 549-551.

Morgan, C., H.S. Rosenkranz and B. Mednis. 1969. Structure and development of viruses as observed in the electron microscope: X. Entry and uncoating of adenovirus. *J. Virology.* 4(5): 777-796.

Nagata, K., R.A Guggenheimer and J.Hurwitz. 1983. Adenovirus DNA replication *in vitro*: Synthesis of full length DNA with purified protein. *Proc. Natl. Acad Sci. USA* 80: 4266-4270.

Natarajan, V. 1986. Adenovirus-2 E1a and E1b gene products regulate enhancer mediated transcription. *Nuc. Acid Res.* 14(23): 9445-9455.

Neale, G.A.M. and G.R. Kitchingman. 1990. Conserved region 3 of adenovirus type 5 DNA-binding protein is important for interaction with single stranded DNA. *J. Virology.* 64(2): 630-638.

Nevins, J.R. 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell.* 29: 913-919.

Nevins, J.R. and J.E. Darnell (Jr). 1978. Steps in the processing of Ad2 mRNA: poly $(A)^+$ nuclear sequences are conserved and poly (A) addition preceedes splicing. *Cell.* 15: 1477-1493.

Nevins, J.R., H.S. Ginsberg, J.M. Blanchard, M.C. Wilson and J.E. (Jr). Darnell. 1979. Regulation of the primary expression of the early adenovirus transcription unit. *J. Virology.* 32(3): 727-733.

Nevins, J.R. and J.J. Winkler. 1980. Regulation of early adenovirus transcription: A protein product of early region 2 specifically represses region 4 transcription. *Proc. Natl. Acad. Sci. USA.* 77(4): 1893-1897.

Nevins, J.R. and M.C. Wilson. 1981. Regulation of adenovirus 2 gene expression at the level of transcriptional termination and RNA processing. *Nature.* 290: 113-118.

Pastan, I., P. Seth, D. Fitzgerald and M. Willingham. 1986. Adenovirus entry into cells: New observation on an old problem. in: *Concepts in viral pathogenesis II.* eds: A.L. Natlans and M.B.A. Oldstone. Springer-Verlag, New York. pg. 141-146.

Persson, H., B. Mathisen, L. Philipson and U. Pattersson. 1979. A maturation protein in adenovirus morphogenesis. *Virology.* 93: 198-208.

Peter, M., J. Nakagawa, M. Doree, J.C. Labbe and E.A. Nigg. 1990. *In vitro* disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell.* 61: 591-502.

Pilder, S., M. Moore and S. Logan. 1986. The adenovirus E1B-55Kd transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* 6(2): 470-476.

Pilder, S., J. Logan and T. Shenk. 1984. Deletion of the gene encoding the adenovirus 5 early region 1b 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. *J. Virology.* 52(2): 664-671.

Pina, M. and M. Green. 1965. Biochemical studies on adenovirus multiplication, IX: Chemical and base composition analysis of 28 human adenoviruses. *Proc. Natl. Acad. Sci. USA.* 54: 547-551.

Rekosh, D.M.K., W.C. Russel and A.J.D. Bellet. 1977. Identification of a protein linked to the ends of adenovirus DNA. *Cell.* 11: 283-295.

Robinson, A.J., H.B. Younghusband and A.J.D. Bellett. 1973. A circular DNA-protein complex from adenoviruses. *Virology.* 56: 54-69.

Rosen, L. 1958. Hemagglutination by adenoviruses. *Virology.* 5: 574-577.

Rosen, L., J.F. Hovis and J.A. Bell. 1962. Further observation on typing adenovirus and a description of two possible additional serotypes. *Proc. Soc. Exp. Biol. Med.* 110: 710-715.

Rosenfeld, P.J., E.A. O'Neill, R.J. Wides and T.J. Kelly. 1987. Sequence-specific interaction between cellular DNA-binding proteins and the adenovirus origin of DNA replication. *Mol. Cellul. Biol.* 7(2): 875-886.

Rowe, D.T., P.E. Branton, R.L. Graham. 1983. Intracellular localization of adenovirus type 5 tumor antigen in productively infected cells. *J. Vriology.* 129: 456-468.

Rowe, W.P., R.J. Huebner, L.K. Gillmore, R.H. Parrott and T.G. Ward. 1959. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Expl. Biol. Med.* 84: 570-573.

Ruley, H.E. 1983. Adenovirus early region 1A encodes viral and cellular transforming genes to transform primary cells in culture. *Nature.* 304: 602-606.

Russell, W.C., W.G. Laver and P.J. Sanderson. 1968. Internal components of adenovirus. *Nature.* 219: 1127-1130.

Samulski, R.J. and T. Shenk. 1988. Adenovirus E1b 55-Mr polypeptide facilitates timely cytoplamic accumulation of adeno-associated virus mRNAs. *J. Virology.* 62(1): 206-210.

Sarnow, P., P. Hearing, C.D. Anderson, D.N. Halbert, T. Shenk and A.J. Levine. 1984. Adenovirus early region 1B 58,000-dalton tumorantigen is physically associated with an early region 4 35,000-dalton protein in productively infected cells. *J. Virology.* 49: 692-700.

Sarnow, P., P. Hearing, C.W. Anderson, N. Reich and A.J. Levine. 1982. Identification and characterization of an immunologically conserved adenovirus early region 11,000 Mr protein and its association with the nuclear matrix. *J. Mol. Biol.* 162: 565-583.

Schneider, J.F., F. Fisher, C.R. Golding and N.C. Jone. 1987. Mutational analysis of the adenovirus E1a gene: the role of transcriptional regulation in transformation. *EMBO. J.* 6(7): 2053-2060.

Schughart, K., E. Bause and H. Esche. 1985. Structure and expression of adenovirus type 12 E1B 58K protein in infected and transformed cells: Studies using antibodies directed against a synthetic peptide. *V. Res.* 3: 41-56.

Shaw, A.R. and E.B. Ziff. 1980. Transcripts from the adenovirus-2 major late promoter yield a single early family of 3′ coterminal mRNAs and five late families. *Cell.* 22: 905-916.

Shenk, T., N. Jones, W. Colby and D. Fowlker.1979. Functional analysis of adenovirus 5 host-range deletion mutants defective for transformation of rat embryo cells. *Cold Spring Harbour Sympo. Quant. Biol.* 44: 367-375.

Shiroki, K., K. Oltshima, Y. Fukui and H. Ariga. 1986. The adenovirus type 12 early-region 1B 58,000Mr gene product is required for viral DNA synthesis and for initiation of cell transformation. *J. Virology.* 57(3): 792-801.

Smart, J.E. and B.W. Stillman. 1982. Adenovirus terminal protein precursor: partial amino acid sequence and the site of covalent linkage to virus DNA. *J. Biol. Chem.* 257(22): 13499-13506.

Soderlund, H., U. Pettersson, B. Vennstrom and L. Philipson. 1976. A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. *Cell.* 7: 585-593.

Steenbergh, P.H., J. Matt, H.V. van Ormondt and J.S. Sussenbach. 1977. The nucleotide sequence at the termini of adenovirus type 5 DNA. *Nucleic Acids Research.* 4(12): 4371-4389.

Stephens, C. and E. Harlow. 1987. Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30Kd and 35Kd proteins. *EMBO. J.* 6(7): 2027-2035.

Stillman, B.W., F. Tamanoi and M.B. Mathews. 1982. Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. *Cell.* 31: 613-623.

Subramanian, T., M. Kuppuswamy, M. Mack and G. Chinnadurai. 1984. Adenovirus Cyt^+ locus, which controls cell transformation and tumorigenicity, is an allele of lp^+ locus, which codes for a 19-Kilodalton tumor antigen. *J. Virology.* J.V. 52:336-343.

Sutter, D., M. Westphal and W. Doefler. 1978. Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. *Cell.* 14: 568-585.

Tamanoi, F. and B.W. Stillman. 1982. Function of adenovirus terminal protein in the initiation of DNA replication. *Proc. Natl. Acad. Sci. USA.* 79: 2221-2225.

Tamanoi, F. and B.W. Stillman. 1983. Initiation of adenovirus DNA replication *in vitro* requires a specific DNA sequencec. *Proc. Natl. Acad. Sci. USA.* 80: 6446-6450.

Tate, V.E. and L. Philipson. 1979. Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. *Nucleic Acids Research.* 6(8): 2769-2785.

Thomas, G.P. and M.B. Mathews. 1980. DNA replication and the early to late transition in adenovirus infection. *Cell.* 22: 523-533.

Tollefson, A.E., P. Krajcsi, S. Yei, C.R. Carlin and W.S.M. Wold. 1990. A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. *J. Virology.* 64(2): 794-801.

Tremblay, M.L., D.J. Dumont and P.E. Branton. 1989. Analysis of phosphorylation sites in the exon 1 region of E1a proteins of human adenovirus type 5. *Virology.* 169: 397-407.

Tremblay, M.L., C.J. McGlade, G.E. Gerber and P.E. Branton. 1988. Identification of the phosphorylation sites in early region 1A proteins of adenovirus type 5 by amino acid sequencing of peptide fragments. *J. Bio. Chem.* 63(13):6375-6383.

Van der Eb, A.J. 1973. Intermediates in type 5 adenovirus DNA replication. *Virology.* 51: 11-23.

Van der Eb, A.J., C. Mulder, F.L. Graham and A. Houweling. 1977. Transformation with specific fragments of adenovirus DNAs: I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. *Gene.* 2: 115-132.

Van der Eb, A.J. and A. Houwelling .1977. Transformation with specific fragments of adenovirus DNA. II. Analysis of the viral DNA sequences present in cell transformed with a 7% fragment of adenovirus 5 DNA. *Gene.* 2:133-146.

Van der Vliet, P.C., J. Claessens, E. deVries, P.A.J. Leegwater, G.J.M. Puijn, W. van Driel and R.T. van Miltenberg. 1988. Interaction of cellular proteins with the adenovirus origin of DNA replicatiion. In: *Cancer Cell (volume 6) Eukaryotic DNA Replication.* eds: T. Kelly and B. Stillman. Cold Spring Harbour. New York. pg. 61-71.

Van der Vliet, P.C. and J.S. Sussenbach. 1973. The mechanism of adenovirus DNA synthesis in isolated nuclei. *Eur. J. Biochem.* 30: 584-592.

Van der Vliet, P.C. and J.S. Sussenbach. 1975. An adenovirus type 5 gene function required for initiation of viral DNA replication. *Virology.* 67: 415-426.

Van Oostrum, J. and R.M. Burnett. 1985. The molecular composition of adenovirus type 2 virion. *J. Virology.* 56: 439-448.

Velcich, A. and E. Ziff. 1985. Adenovirus E1a protein repres transcription from the SV_{40} early promoter. *Cell.* 40: 705-716.

Visser, L., D.C. Wassenaar, M.W. van Maarschalkerweerd and T.H. Rozijn. 1981. Arrangement of integrated viral DNA sequences in cells transformed by adenovirus type 2 and 5. *J. Virology.* 39(3): 684-693.

Walter, G. and J.V. Maizel (Jr). 1974. The polypeptides of adenovirus. IV. Detection of early and late virus-induced polypeptides and their distribution in subcellular fractions. *Virology.* 57: 402-408.

Ward, G.E. and M.W. Kirschner. 1990. Identifiction of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell.* 61: 561-577.

Weinburg, D.H. and G. Ketner. 1986. Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. *J. Virology.* 57(3): 833-838.

White, E., H.S. Blose and B.W. Stillman. 1984. Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. *Mol. Cell. Biol.* 4(12): 2865-2875.

White, E. and R. Cipriani. 1989. Specific disruption of intermediate filaments and the nuclear lamina by the 19-kDa product of the adenovirus E1B oncogene. *Proc. Natl. Acad. Sci. USA.* 86: 9886-9890.

White, E. and R. Cipriani. 1990. Role of adenovirus E1B proteins in transformation: Altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. *Molec. Cell. Biol.* 10(1): 120-130.

White, E., A. Denton and B. Stillman. 1988. Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. *J. Virology.* 62(9): 3445-3454.

White, E., T. Godzicher and B.W. Stillman. 1984. Mutations in the gene encoding the adenovirus early region 1B 19,000-molecular-weight tumor antigen cause the degradation of chromosomal DNA. *J. Virology.* 52(2): 410-419.

White, E. and B. Stillman. 1987. Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. *J. Virology.* 61(2): 426-435.

Whyte, P., J.J. Buchkovich, J.M. Herowitz, S. H. Friend, M. Raybuck, R.T. Weinberg and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: The adenovirus E1A protein binds to retinoblastoma gene product. *Nature.* 334: 124-129.

Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub and L. Chasin. 1979. DNA-mediated transfer of the adinine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 73(3): 1373-1376.

Wilson, M.C., N.W. Fraser and J.E. Darnell. 1979. Mapping of RNA initiation site by high doses of UV irradiation evidence for 3 independent promoters within the left 11% of adenovirus 2 genome. *Virology.* 96: 175-184.

Wider, R.J., M.D. Challberg, D.R. Rawlins and T.J. Kelly. 1987. Adenoviruses origin of DNA replication: sequence requirements for replication *in vitro*. *Mol. Cell. Biol.* 7(2): 864-874.

Wolfson, J. and D. Dressler. 1972. Adenovirus 2 DNA contains an inverted terminal repetition. *Proc. Natl. Acad. Sci. USA.* 69(10): 3054: 3057.

Wong, M. and M. Hsu. 1990. Involvement of topoisomerases in replication, transcription, and packaging of the linear adenovirus genome. *J. Virology.* 64(2): 691-699.

Yee, S.P. and P.E. Branton. 1985a. Analysis of multiple forms of human adenovirus type 5 E1A polypeptides using an anti-peptide antiserum specific for the amino terminus. *Virology.* 146: 315-322.

Yee, S.P. and P.E. Branton. 1985b. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology.* 147: 142-153.

Yee, S.P., D.T. Rowe, M.L. Tremblay, M. McDermott and P.E. Branton. 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. *J. Virology.* 46(3): 1003-1013.

Yoshida, K., L. Venkatesh, M. Kuppuswany and G. Chinnadurai. 1987. Adenovirus transforming 19-Kd T antigen has an enhancer-dependent trans-activation function and relieves enhancer repression mediated by viral and cellular genes. *Gene and Dev.* 1: 645-658.

Yoshinaga, S., N. Dean, M. Han and A. Berk. 1986. Adenovirus stimulation of transcription by RNA polymerase III: Evidence for an E1A dependent increase in transcription factor IIIC concentration. *EMBO. J.* 5(2): 343-354.

Zerler, B., R.J. Roberts, M.B. Mathews and E. Moran. 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. *Mol. Cell. Biol.* 7(2): 821-829.

Ziff, E.B. 1980. Transcription and RNA processing by the DNA tumor viruses. *Nature.* 287: 491-498.

Ziff, E. and N. Fraser. 1978. Adenovirus type 2 late mRNA's: structural evidence for 3'-coterminal species. *J. Virology.* 25(3): 897-904.

Zoller, M.J. and M. Smith. 1984. Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single stranded DNA template. *DNA.* 3(6): 479-488.