# PHOSPHORYLATION OF THE AD5 E1B 55K PROTEIN

# IDENTIFICATION OF PHOSPHORYLATION SITES AT THE CARBOXY TERMINUS OF THE 55-K (496R) ADENOVIRUS TYPE 5 E1B PROTEIN

By

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## Abstract

The 55K product of early region 1B (E1B) of human adenoviruses is required for viral replication and participates in cell transformation. Both biochemical and genetic approaches have been used to show that this 496-residue (496R) protein of adenovirus (Ad5) is phosphorylated at both serine and threonine residues at sites near the carboxy terminus within sequences characteristic of a casein kinase II. Mutations which converted serines 490 and 491 to alanine residues decreased virus replication and reduced the efficiency of transformation of primary baby rat kidney cells, suggesting that phosphorylation at these sites is of importance in the regulation of 55K biological activity.

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| | |
|---|---|
| Ad | Adenovirus type |
| APS | Ammonium persulfate |
| bp | basepair(s) |
| BRK cells | baby rat kidney cells |
| cpe | cytopathic effect |
| CR | conserved region |
| cyt | cytopathic phenotype |
| dATP, dCTP, dGTP, dTTP | deoxynucleotides triphosphates |
| DBP | DNA binding protein |
| $ddH_2O$ | double distilled water |
| deg | degradation phenotype |
| $^0C$ | degrees celsuis |
| dl | deletion |
| DNA | deoxyribonucleic acid |
| E1, E2, E3, E4 | early region 1, 2, 3, 4 |
| EDTA | ethylenediamine tetracetic acid |
| et al | and co-workers |
| EtOH | ethanol |
| F11 | F11 media |
| FCS | fetal calf serum |
| Fig. | figure |
| g | gram |
| HPLC | high pressure liquid chromatography |
| hrs | hours |
| HS | horse serum |
| JOK | Joklik media |
| K | kiloDalton |
| kb | kilobase(s) |
| kvolts | kilovolts |
| l | litre |
| LB | Luria-Bertani broth |
| M | molar |
| ma | milliamps |
| mCi | millicuries |
| $Met^-$ | methionine minus |
| mg | milligram(s) |
| ml | millilitre(s) |
| MLP | major late promoter |
| min | minute(s) |
| mM | millimolar |
| moi | multiplicity of infection |
| mRNA | messenger RNA |
| m.u. | map units |
| μ | micro |
| N | Normal |
| NBCS | newborn calf serum |

| | |
|---|---|
| nm | nanometre |
| OD | optical density |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| pfu | plaque forming units |
| $Phos^-$ | phosphate minus |
| p.i. | post infection |
| pm | point mutation |
| pmoles | picomoles |
| P/S | penicillin-streptomycin |
| R | residue (amino acid number) |
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| sec | second(s) |
| SSC | saline sodium citrate |
| TBE | tris-borate-EDTA buffer |
| TLC | thin layer chromatography |
| UV | ultra violet |
| v | volts |
| v/v | volume to volume |
| w/v | weight to volume |
| wt | wild-type |

# I. Introduction

## I.I.1. Adenovirology

Adenoviruses were first isolated in 1953 from human adenoids (Rowe et al 1953). Several serotypes were quickly identified and in 1962 it was discovered that Adenovirus 12 (Ad12) could induce tumours when injected into rodents. This was the first reported case of a human virus with oncogenic potential (Trentin et al 1962; Huebner et al 1962). This discovery catapulted adenoviruses to a prominent position in the field of microbiology and resulted in many discoveries about both their replicating and transforming abilities. It should be noted that no link has been shown between adenoviruses and any human malignancies (Horwitz 1990).

In addition, adenoviruses have served as an excellent model for eukaryotic systems. The processes of mRNA splicing (Klessig 1977; Chow et al 1979), DNA replication (Challberg et al 1979) as well as protein transport and assembly (Horwitz et al 1969) have all been studied in adenovirus infected cells.

## I.I.2. Classification of Human Adenovirus

There have been many different methods of classification of adenoviruses since their discovery. These include: the ability of the viruses to haemagglutonate rat and rhesus monkey red blood cells (Rosen 1958; Rosen 1960 and Hierholzer 1973); oncogenicity (Huebner et al 1965; Trentin et al 1968); the separation of structural proteins by SDS polyacrylamide electrophoresis (Wadel 1979); and guanine and cytosine (G+C) composition (Green and Pina 1964; Pina and Green 1965; Green et al 1967).

The most widely accepted classification system is based on DNA sequence homology (Green et al 1979). Recently, this system was used to describe six subgroups containing 41 different adenovirus serotypes. The subgroups A-F share a high degree of homology within groups and low homology between groups.

Group A (Ad12; Ad18 and Ad31) adenoviruses share 48%-69% DNA homology, are highly oncogenic, and have G+C content of 48%-49%. The remainder of the groups have DNA homology greater than 85% within, and less then 20% between, the groups. Group B (Ad3; Ad7; Ad11; Ad14; Ad16; Ad21; Ad34 and Ad35) adenoviruses are moderately oncogenic and have G+C content of 50%-52%. Group C (Ad1; Ad2; Ad5 and Ad6) adenoviruses are referred to as weakly oncogenic or non-

oncogenic and have G+C content of 57%-59%. Group D and E adenoviruses, both contain weakly or non-oncogenic viruses and have a G+C content of 57%-61%. Group F contains a two viruses, Ad40 and Ad41, of which little is known (Horwitz 1990). The remainder of this discussion will deal with Ad5 and a highly related serotype Ad2, both of which are members of group C.

## I.II. The Structure of the Virion

Adenoviruses are non-enveloped viruses which have an icosahedral structure (20 triangular surfaces and 12 vertices). The virus particle is approximately 65-80nm in diameter (Horne et al 1959). The virus consists of an outer capsid coat and an inner core structure. Each of the twelve vertices of the capsid contains a "fibre" which projects from a penton base (Ginsberg et al 1966).

The virion consists of at least nine separate polypeptides (II-X) (Maizel et al 1968a and 1968b). The capsid is composed of 252 subunits called capsomeres, each of which consists of 240 hexons and 12 pentons. The hexon is a trimer of polypeptide II and is designated hexon because it has six neighbouring capsomeres (Horwitz et al 1970). Each penton is comprised of a penton base made of five molecules of polypeptide III (penton) and a fibre which comprises a trimer

of polypeptide IV, which projects from the base (Horwitz 1990).

The polypeptides VI, VIII and IX are found associated with hexon capsomeres after isolation procedures (Everitt et al 1973; Everitt et al 1975; Colby and Shenk 1981). Polypeptide IIIa is also known as the penton associated protein because it is associated with hexons which surround the pentons at each of the vertices (Everitt et al 1973).

The capsid can be removed in several different ways leaving the core structure (Brown et al 1975; Colby and Shenk 1981) which contains four additional proteins (Maizel et al 1968b; Hosakawa and Sung 1976), including two arginine-rich proteins (polypeptides VII and V) (Maizel et al 1968b; Russell et al 1968). Each virion contains 1070 copies of polypeptide VII which form nucleosome-like structures (Brown et al 1975).

Each nucleosome consists of six proteins which associate as dimers. There are 180 copies of polypeptide V which serve as linkers between nucleosomes (Mirza and Weber 1982; Corden et al 1976; Everitt et al 1975; Brown et al 1975). The average length of DNA contained around the nucleosome and in the spacer region is 200bp, however, electron microscopy has revealed that the nucleosomes are spaced at irregular distances (Corden et al 1976, Mirza and Weber 1982; Tate and Philipson 1979). Partial micrococcal nuclease digestion revealed that each nucleosome contains 150bp of DNA and has a

variable linker of about 50bp (Mirza and Weber 1982).

The core contains a third arginine-rich protein designated μ which may be involved in virus genome condensation and packaging (Hosakawa and Sung 1976; Anderson et al 1989).

The final protein found in the core is the 55K terminal protein (TP). The TP is covalently linked to the 5'ends of the viral genome and plays an essential role in priming viral DNA replication (Challberg and Kelly 1989; Challberg et al 1980; Kelly et al 1988; Rekosh et al 1977). The 55K TP is produced by cleavage of a 80K precursor (pTP) and is often found linked to DNA in cells as well as in *in vitro* systems (Challberg and Kelly 1981; Challberg et al 1982; Lichy et al 1981).

The viral genome is linear and double stranded, and has a length of approximately 36kb and a molecular weight of about 23 x $10^6$ Daltons (Green et al 1967b). The TP is covalently linked to both of the 5' ends of the adenovirus genome by a phosphodiester bond between the β-OH of a serine residue and the α-phosphoryl group of dCMP (Challberg and Kelly 1979; Tamanoi and Stillman 1982).

Another unique property of the viral genome is the presence of inverted terminal repeats. Ad5 has a 103bp perfect inverted terminal repeat (Steenberg et al 1977). These inverted repeats have been implicated in the formation

of ssDNA panhandle structures after denaturation and renaturation (Wolfson and Dressler 1972). These panhandle structures may play an important role in viral replication (Horwitz 1990).

## I.III. Adenovirus Replication

Adenovirus replication is divided into early and late phases to separate events before and after DNA replication (Horwitz 1990), although some functions are not strictly limited to these categories (Akusjarvi and Persson 1981; Lewis and Mathews 1980).

Each infected cell will produce approximately 10000 viruses before cell death occurs by lysis (Green and Daesch 1961). The infectivity of these particles is measured in plaque forming units/millilitre (pfu/ml). Depending on the serotype, each pfu comprises between 11 and 2300 virus particles because of the occurrence of defective particles (Green et al 1967a).

### I.III.2. Attachment, Penetration and Uncoating

There have been numerous studies on the entry of adenoviruses into HeLa and or KB cells. Viruses attach to the cell via an interaction between fibres and undefined receptors

on the cell surface (Chardonnet and Dales 1970b; Hennache and Boulanger 1977; Longberg-Holm and Philipson 1969), which number approximately 10000 per cell (Philipson et al 1968).

The virus particles appear to be internalized primarily by endocytosis (Chardonnet and Dales 1970a; Marsh and Helenius 1989). The internalized virus is initially located in endosomes until their release by a pH-dependent event (Marsh and Helenius 1989). After their release, virus particles have been shown to lack some of the penton capsomeres, as revealed by a increase in DNAse sensitivity and the results of radiolabelling experiments (Sussenbach 1967; Longer-Holm and Philipson 1969).

After release from the endosomes the viruses move quickly to the perinuclear region, possibly by a mechanism involving microtubules (Dales and Chardonnet 1973). The viral DNA and its associated proteins are then transported through the nuclear pores by an ATP-dependent process which leaves capsid shells with translucent centers (Chardonnet and Dales 1970a; Dales and Chardonnet 1973).

## I.IV. Early Transcription

Shortly after entering the nucleus, transcription from the four early regions begins. The two strands of the adenovirus genome are referred to as the r strand (which is

usually depicted as the upper strand and is transcribed left to right) and the l strand (normally depicted as the lower strand and is transcribed in a right to left direction)(Sharp et al 1974). Two of the early regions are transcribed from the r strand (E1 and E3), and two from the l strand (E2 and E4)(Sharp et al 1974) (Fig.1).

Each of these regions contains at least one promoter (Berk and Sharp 1977; Wilson et al 1979) which depend on the expression of the E1A protein early in infection (Berk et al 1979; Jones and Shenk 1979). The E1 and E2 regions contain multiple promoters. E1 region is subdivided into three separate regions E1A, E1B, and the gene encoding protein IX, and each has its own promoter (Wilson et al 1979).

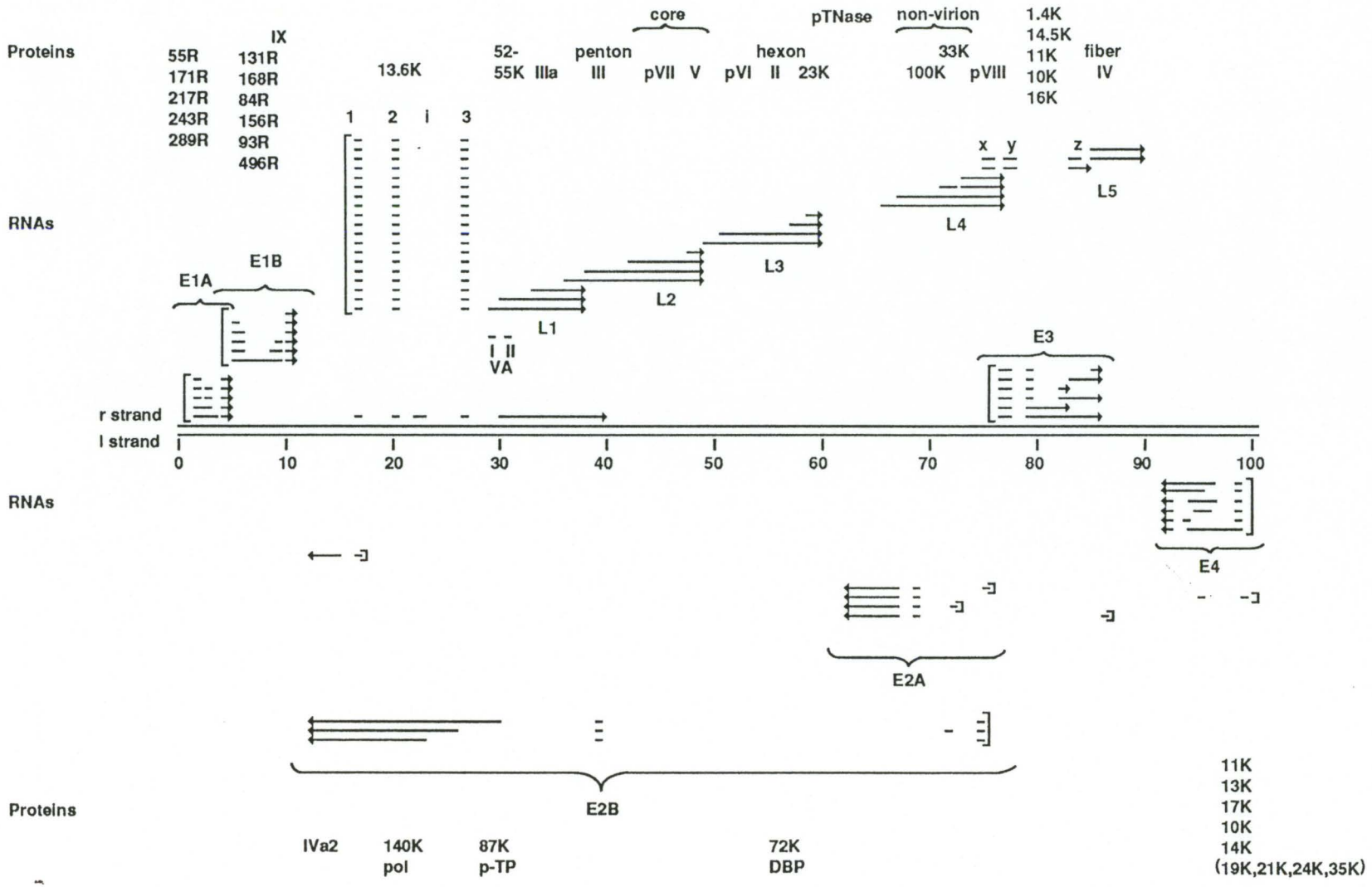
All of the early regions produce mRNA transcripts which are subject to differential splicing to produce families of proteins, many of which are coded for in overlapping regions (Berk and Sharp 1979; Chow et al 1979; Kitchingman and Westphal 1980).

### I.V.1. Early Proteins

#### I.V.1A. E1A Proteins

The E1A region is located between 1.3 and 4.5 map units (m.u.) and has been shown to produce six proteins (Harter and

Figure 1: Structure of the Adenovirus type 2 genome. (Adapted from Horwitz 1990)


Proteins
IX
55R
131R
171R
168R
217R
84R
243R
156R
289R
93R
496R
13.6K
core
pTNase
non-virion
1.4K
14.5K
11K
10K
16K
52-
55K
IIIa
penton
III
pVII
V
hexon
pVI
II
23K
33K
100K
pVIII
fiber
IV
RNAs
1
2
i
3
x
y
z
L5
L4
L3
L2
L1
E1B
E1A
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)

Lewis 1978). These polypeptides exist as multiple electrophoretically different species due to extensive post-translational modification (Yee et al 1983; Harlow et al 1985; Yee and Branton 1985a). The two major products are the 289 and the 243 residue peptides (Branton and Rowe 1985). These products differ only by the inclusion of a 46 amino acid (aa) unique sequence present in the 289 residue product (Jelsma et al 1988; Montell et al 1982). Two minor products of the E1A region encoded by a 10S and an 11S mRNA, are identical to those of the 12S and the 13S mRNAs except that because of a splicing event, 72aa, including CR1, are removed(Stephens and Harlow 1987). These minor products cannot substitute for the 289R and the 243R products in transformation (Stephens and Harlow 1987).

The two major E1A products produce 4 diffuse bands with molecular weights ranging between, approximately, 40K-50K. Each protein migrates on SDS-PAGE as two bands which differ due to phosphorylation at serine-89 and perhaps serine-96 (Dumont et al 1989 and 1993; Tremblay et al 1988 and 1989). Elimination of the ser-89 phosphorylation site was shown to cause a small effect on the ability of E1A to transform primary cells (Dumont et al 1993).

Comparison of various adenovirus serotypes has revealed that the E1A regions share three highly conserved domains termed CR1, CR2 and CR3 (Kimelman et al 1985; Schneider et al

1987). CR1 and CR2 are located near the N-terminus, and CR3 comprises the 46aa unique sequence (Zerler et al 1987). These regions have been shown to be important for a number of the functions attributed to E1A. In addition to transformation, which will be discussed shortly, E1A has been linked to a number of other functions.

### I.V.1B. E1A Functions

E1A has been shown to activate transcription from some cellular promoters (Zerler et al 1987; Horwitz 1990; Bagchi et al 1990; Bandara and La Thangue 1991; Raychuadhuri et al 1991) and to repress various cellular transcriptional enhancers (Borelli et al 1984). Other functions include the induction of differentiation of F9 stem cells (Montano and Lane 1987) and the stimulation of DNA synthesis (Zerler et al 1987; Howe et al 1990; Shepperd et al 1993).

The ability of E1A proteins to repress transcription driven by a variety of enhancers (Borelli et al 1984; Horwitz 1990) has been mapped to the amino terminus as well as CR1 (Borelli et al 1984; Lillie et al 1986; Barbeau et al 1992). The mechanism of repression is currently poorly understood.

E1A facilitates many of its functions through interactions with various cellular factors. E1A is found complexed with $p105^{RB}$ and a related p107 polypeptide, and at

least three other proteins ($p60^{cycA}$, p130, and p300). All of these associated proteins interact with E1A through the amino terminus and/or CR1 and CR2 (Yee and Branton 1985b; Egan et al 1988 and 1989; Barbeau et al 1992).

Transactivation of various promoters has long been linked to the 46aa unique region of ad5 E1A (Jelsma et al 1988), however, transactivation also appears to be the result of the ability of E1A proteins to free a transcription factor, E2F, from an inactive complex (Bagchi et al 1990; Bandara and La Thangue 1991; Raychuadhuri et al 1991). This may represent a distinct E1A transactivation function as it requires only the 243R E1A product, which lacks the 46aa unique region. E2F then associates with and activated by an E4-19K protein (Hardy et al 1989; Neill et al 1990). The discovery that $p105^{RB}$ was found in the inactive complex with E2F shed further light on E1A transactivation (Bagchi et al 1991). Recently, it was shown that $p60^{cycA}$, a regulatory subunit of the cdc2 cell cycle protein kinase family, and p107, a $p105^{RB}$-related protein, also interact with E2F during S-phase in uninfected cells (Cao et al 1992; Shirodkar et al 1992). Therefore, it appears that E1A proteins may induce cell poliferation by interfering with the regulation by $p105^{RB}$ and p107 of the transcriptional factor E2F which regulates expression of genes required for entry into S-phase.

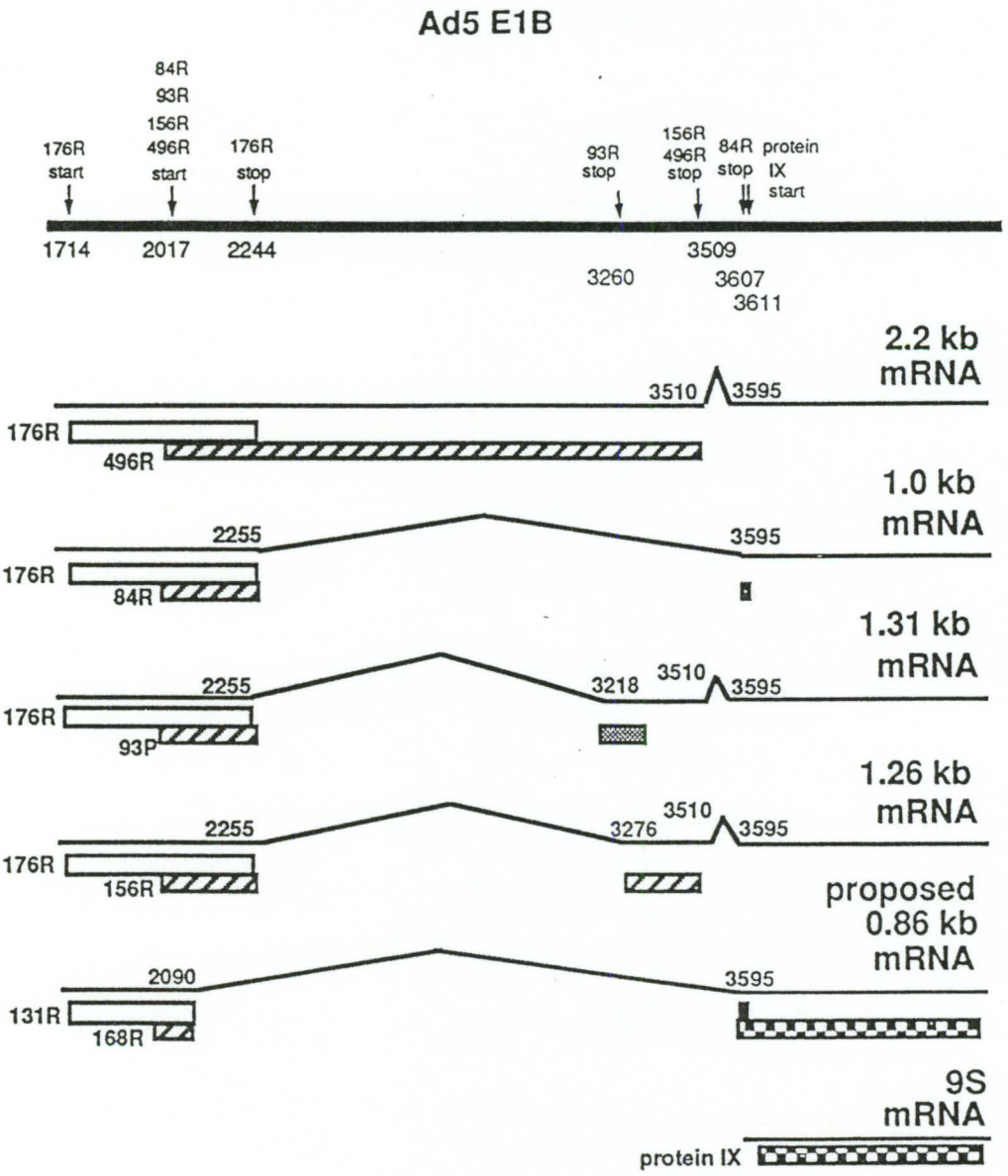
The identity and the function of the p300 protein remain unknown, however, some evidence suggests that it may represent another growth suppressing protein. E1A mutants which bind $p105^{RB}$ or p300 are capable of inducing DNA synthesis (Howe et al 1990), however, the binding of both $p105^{RB}$ and p300 is required for transformation suggesting that more than the initiation of DNA synthesis is required for transformation.

It appears that E1A uses several domains to interact with a variety of growth suppressor and cell cycle control proteins (DeCaprio et al 1989; Barbeau et al 1992). After infecting non-proliferating cells E1A seems to use these interactions to induce entry into S-phase and allow viral replication and transformation.

### I.V.2A. E1B Region

The E1B region which is located between 4.6 and 11.2 m.u., encodes at least six different spliced mRNA products during infection (Matsuo et al 1982; Lewis and Anderson 1987) (Fig.2). The two most studied proteins are 496R and 176R, also termed 55K and 19K. 19K is produced from both 22S and 13S mRNA transcripts, while only the 22S transcript is capable of producing the 55K product. The 19K and 55K coding sequences overlap but exist in different reading frames.

Figure 2: Transcription map of Ad5 E1B. (Adapted from Lewis and Anderson)


Ad5 E1B
84R
93R
156R
496R
start
176R
start
176R
stop
93R
stop
156R
496R
stop
84R
stop
protein
IX
start
1714
2017
2244
3260
3509
3607
3611
2.2 kb
mRNA
3510
3595
176R
496R
1.0 kb
mRNA
2255
3595
176R
84R
1.31 kb
mRNA
2255
3218
3510
3595
176R
93P
1.26 kb
mRNA
2255
3276
3510
3595
176R
156R
proposed
0.86 kb
mRNA
2090
3595
131R
168R
9S
mRNA
protein IX

Previous studies have revealed that the 55K protein is phosphorylated at two or more sites and contains phosphoserine and phosphothreonine at a ratio of about 2:1 (Mallette et al 1983). Three phosphopeptides were also identified, two major and one minor (Mallette et al 1983). The location and function of these sites are currently unknown.

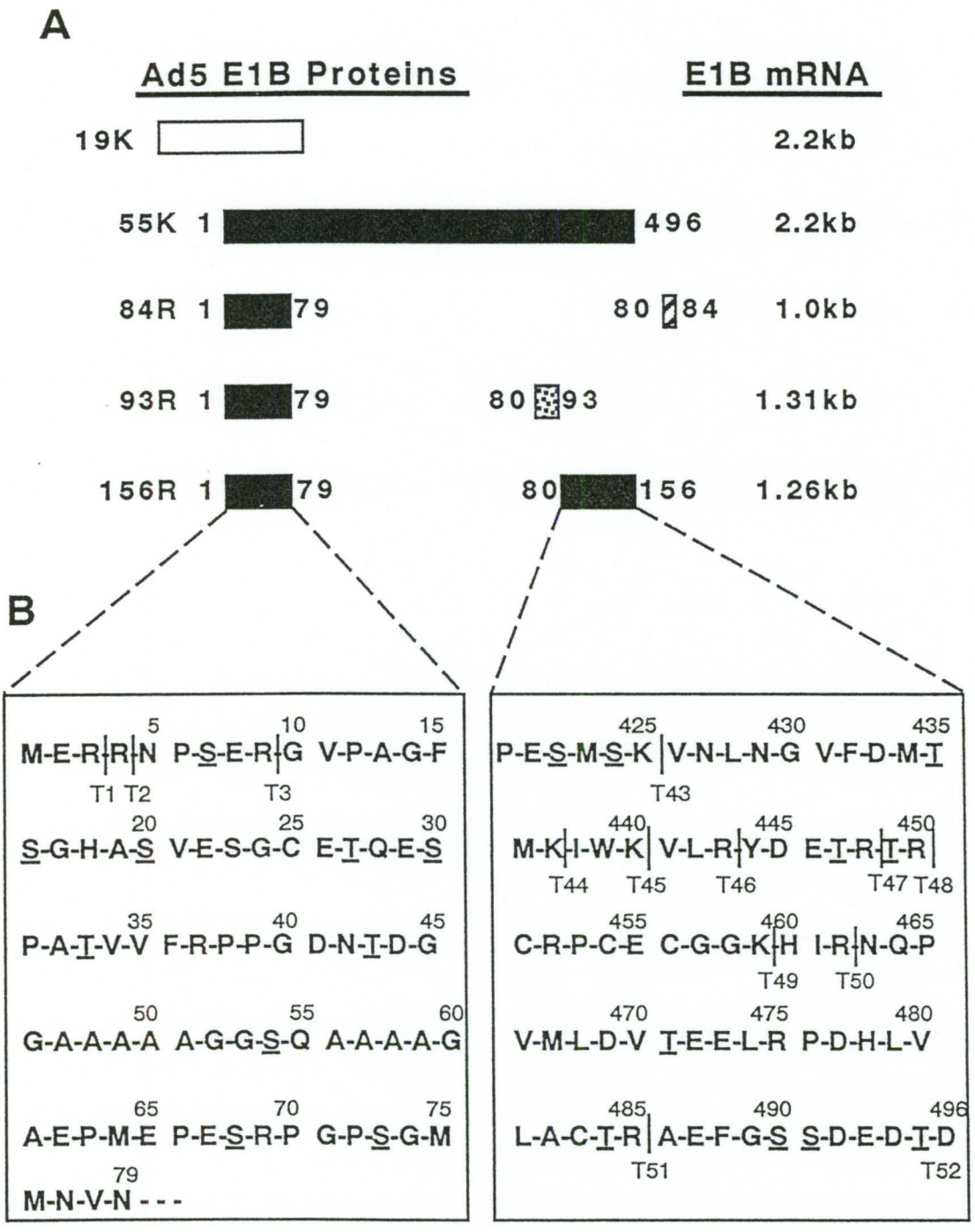
The 19K protein has been shown to be both phosphorylated (McGlade et al 1989) and acylated (McGlade et al 1987). Elimination of the phosphorylation site by point mutation had no major effect on transformation or other 19K functions. Bacterially produced 19K is found associated with the inner membrane of the bacteria and it has been suggested that an N-terminal alpha-helix may act as a translocation signal and be responsible for transport to the membrane (Dalie et al 1992). In infected cells the 19K protein is found localized in the nuclear envelope (White et al 1984a)

In addition to the major products, several minor products are produced which share homology with the 55K protein. A 156R peptide is produced and shares the first 79aa and the last 77aa with 55K. The 93R and the 84R peptides again share the amino terminal 79aa of the 55K peptide but are spliced into different reading frames and thus contain unique carboxy termini (Fig.3).

Figure 3: Ad5 E1B gene products, amino acids sequences.

A. Protein regions relative to the Ad5 E1B mRNAs that encode them and the corresponding number of residues have been illustrated. ▭ sequences unique to 19K; ▬ sequences present in 55K; ▨ sequences unique to 84R; ▩ sequences unique to 93R.

B. Amino acid sequences of 55K also present in 156R have been presented along with the predicted tryptic peptides (shown below the sequence) and positions of potential phosphorylation sites.


A
Ad5 E1B Proteins
E1B mRNA
19K
2.2kb
55K 1
496
2.2kb
84R 1
79
80
84
1.0kb
93R 1
79
80
93
1.31kb
156R 1
79
80
156
1.26kb
B
M-E-R-R-N P-S-E-R-G V-P-A-G-F
T1 T2 T3
S-G-H-A-S V-E-S-G-C E-T-Q-E-S
P-A-T-V-V F-R-P-P-G D-N-T-D-G
G-A-A-A-A A-G-G-S-Q A-A-A-A-G
A-E-P-M-E P-E-S-R-P G-P-S-G-M
M-N-V-N - - -
P-E-S-M-S-K V-N-L-N-G V-F-D-M-T
T43
M-K-I-W-K V-L-R-Y-D E-T-R-T-R
T44 T45 T46 T47 T48
C-R-P-C-E C-G-G-K-H I-R-N-Q-P
T49 T50
V-M-L-D-V T-E-E-L-R P-D-H-L-V
L-A-C-T-R A-E-F-G-S S-D-E-D-T-D
T51 T52

Previous studies in our lab had revealed that 55K and 156R share the same sites of phosphorylation (Takayesu et al unpublished). In addition, the 93R and 84R products appear not to be phosphorylated, or are phosphorylated at very low levels.

### I.V.2B. E1B Functions

### I.V.2C. The 19K Protein

The 19K protein has been shown to prevent degradation of cellular and viral DNA and to prevent the rapid onset of cytopathic effects (Pidler et al 1984; White et al 1984b). In cells infected by viruses which lack the 19K protein, large plaques are rapidly formed, a response referred to as the cytopathic (cyt) phenotype (Pidler et al 1984). These same cells show marked degradation of both cellular and viral DNA late in infection, referred to as the degradation (Deg) phenotype (Pidler et al 1984). It is now known that E1A induces apoptosis (cyt and deg phenotypes) and 19K functions to block this programmed cell death (White et al 1991; Rao et al 1992; White et al 1992).

The 19K protein has been shown to associate with and disrupt intermediate filaments and the nuclear lamina (White and Cipriani 1989), and it has been suggested that the

disruption of the intermediate filaments may be involved in some of the morphological changes associated with transformation as well as the ability of transformed cells to grow in an anchorage independent manner (White and Cipriani 1990).

Transcriptional regulation has also been attributed to the 19K protein. The 19K protein has been shown to transactivate early viral promoters as well as some cellular promoters (Hermmann et al 1987; Shiroki et al 1990). It has also been proposed as a transcriptional down regulator of the E1A proteins and therefore E1A transactivation (White et al 1988). Such studies , however remain controversial.

The major consequence of 19K expression appears to be to inhibit programmed cell death (apoptosis) induced as a consequence of the action of E1A proteins (White et al 1991; 1992; Gooding et al 1991;Rao et al 1992). The mechanism of inhibition is not known.

### I.V.2D. The 55K Protein

The E1B-55K protein has been shown to be important for the accumulation of late viral mRNA and host protein shutoff (Samulski and Shenk 1988; Weinburg and Ketner 1986; Babiss and Ginsberg 1984). Mutations affecting the 55K protein have been associated with an inability of adenovirus to block host cell

protein synthesis, as well as deficiencies in late gene expression and overall virus replication (Babiss and Ginsberg 1984; Bridge and Ketner 1990; Leppard and Shenk 1989; Logan et al 1984; Pidler et al 1986; Yew et al 1990). Complexes between 55K and the 34K viral E4 protein have been detected (Sarnow et al 1984) and are believed to be functionally important in late viral mRNA accumulation and host protein shutoff (Halbert et al 1985; Pilder et al 1986; Weinberg and Ketner 1986; Samuliski and Shenk 1988).

Currently, the 55K-34K complex is believed to act in parallel with another E4 protein (116R) to facilitate accumulation of unprocessed viral mRNA in the nucleus and possibly to play a role in DNA synthesis (Bridge and Ketner 1990) and transcriptional activation (Panning and Smiley 1993).

The 55K-34K complex has been found in nuclear viral inclusions which are the sites of viral transcription (Ornelles and Shenk 1991). It has been proposed that the complex interacts with a cellular protein required for cytoplasmic accumulation of mRNAs and localizes it to the viral inclusions. This would allow 55K and E4-34K to enhance late viral mRNA accumulation while inhibiting cellular mRNA accumulation (Ornelles and Shenk 1991). Recent studies have shown that different late mRNAs are not equally dependant on the 55K protein for accumulation in the cytoplasm. The

longest mRNA within each 3'-coterminal family displays the greatest dependence on 55K (Leppard 1993) supporting the hypothesis that the complex assists in the transport of matured mRNAs to the cytoplasm. A series of insertion mutants have been used to map a number of functional domains in the 55K protein (Yew et al 1990). Sequences around aa 180, between 262 to 326, and 442 to 474 have been shown to be crucial to viral replication. The central region of 55K, between aa 180 and 354 is important for host protein shutoff and late viral protein accumulation (Yew et al 1990).

The interaction between 55K and the antioncogene p53 (Sarnow et al 1982) has been the subject of much research and the function of this interaction will be discussed in the following sections.

### I.V.3. E2-E4 Proteins

The E2 region produces three proteins which are essential for viral replication. Expression of the E2 region begins approximately 2hrs after infection and reaches a maximum shortly before DNA synthesis begins (Nevins et al 1979). The E2 region has also been implicated in the negative regulation of transcription from the E4 region at late times in infection (Nevins and Winkler 1980).

The E2 region is divided into E2A and E2B. E2A encodes a 72K single-strand DNA binding protein (DBP) that functions during DNA replication to protect against nucleases (Neale and Kitchingman 1990). In the absence of the DBP, elongation is blocked at a maximum of approximately 700nt (Neale and Kitchingman 1990).

The E2B region encodes two proteins essential for viral replication. The first protein is the 120K DNA polymerase (Alestrom et al 1982). The other protein is the terminal protein precursor which is involved in the priming of DNA synthesis *in vivo* (Smart and Stillman 1982; Alestrom et al 1982).

The E3 and E4 regions are expressed at maximal levels three hours post infection and their expression is dependent on the prior expression of the E1A region (Nevins et al 1979). The E3 and E4 regions both code for multiple related proteins. E3 has been shown to be dispensable for growth in tissue culture (Lewis et al 1973) and seems to be involved in evasion of the host immune system (Kvist et al 1978; Burgert et al 1987 and Gooding et al 1988 and 1991). E4 is involved in shutoff of host protein synthesis, viral particle assembly, and accumulation and transport of late viral mRNA (Cutt et al 1987 and Falgout et al 1987).

The E3-19K protein has been shown to bind the human class I histocompatibility antigens, preventing terminal glycosylation and cell surface expression (Burget et al 1987). A 14.7K protein which is found in multiple serotypes of adenoviruses (Horton et al 1990) is responsible for inhibiting lysis of infected cells by tumour necrosis factor (Gooding et al 1988). A 10.4K protein has been implicated in the down regulation of the epidermal growth factor which may again modify the cell response to infection (Carlin et al 1989).

The E4 region has several functions which are important for efficient viral replication. The 34K product has been discussed above. Other E4 proteins have been implicated in viral assembly (Falgout and Ketner 1987) and in host protein synthesis shutoff (Babiss and Ginsberg 1984; Yew et al 1990).

## I.VI. DNA Synthesis

The replication of adenovirus DNA has been extensively studied in the hopes that it would serve as a model of eukaryotic DNA replication. The development of several *in vitro* replication systems have greatly aided in the discovery of factors which are required for efficient DNA replication (Challberg and Kelly 1989), including at least three viral products and several cellular polypeptides (Horwitz 1990).

The adenovirus genome has several unique features that are important for its replication. The E2 TP is covalently linked to the 5′ ends of the genome by a phosphodiester bond (Challberg and Kelly 1979; Tamanoi and Stillman 1982). In addition, it contains terminal inverted repeats. Both of these functions will be discussed below.

Adenovirus begins to replicate its DNA 8-12 hours post infection. The initiation of DNA synthesis requires the presence of an origin of replication, which is located in three highly conserved functional domains in the terminal repeats of the viral genome (Kelly and Wold 1988). Domain A contains nucleotides 1-18 and is the minimal essential origin (Wides et al 1987). The presence of domains B and C are not required but increase efficiency nearly 30 fold (Kelly and Wold 1988).

Initiation is enhanced by the presence of the cellular proteins nuclear factor I (NF1) and nuclear factor III (Wides et al 1987; van der Vilet et al 1988; Bosher et al 1992). These DNA-binding proteins have been shown to bind to the second conserved domain in the origin of replication (Rosenfeld et al 1987; van der Vilet et al 1988; Bosher et al 1992).

Adenovirus uses an unusual method of priming DNA synthesis which occurs via linkage of the 80K pTP to the first nucleotide (dCMP) Challberg and Kelly 1989; Kelly et al 1988).

*In vitro* studies and the use of temperature sensitive mutants have shown that the Ad DNA polymerase is required for this linkage to occur (Lichy et al 1982; Stillman et al 1982). The origin of initiation is also required for the covalent linkage of the pTP to the dCMP molecule (Tamanoi and Stillman 1982). Synthesis can occur from either end of the genome and proceeds from the 5′ to the 3′ end of the DNA (Arens and Yamashita 1978; Kelly et al 1988). The daughter strand, which is synthesized by the Ad DNA polymerase, displaces the parent strand of the same polarity as synthesis continues. The replication is semi-conservative and continuous, and it terminates when it reaches the opposite end of the genome (Horwitz 1971). The presence of the 72K DBP is essential for elongation of nascent DNA (Kaplan et al 1977; Horwitz 1978; Wides et al 1987), which also requires cellular topoisomeraseI (Wong and Hsu 1990).

It is not uncommon for replication to occur simultaneously from both ends of the genome or to have multiple replication forks originate from one end of the genome (Lechner and Kelly 1977). If single stranded parental DNA is released it may form a panhandled circular structure (Garon et al 1972). This panhandle structure mimics the double stranded DNA origin (Kedinger et al 1978; Lechner et al 1977). This structure has also been implicated in a rescue pathway for adenoviruses which contain small deletions in the

terminal repeats (Graham et al 1989).

## I.VII. Late Gene Expression

The commencement of DNA synthesis marks the beginning of the late phase. The production of many of the early proteins is greatly reduced at this time (Nevins et al 1979). There are five late families of genes that are processed from a single high molecular weight mRNA precursor (Bachenheimer and Darnell 1975; Goldberg et al 1977; Meissner et al 1977; Weber et al 1977). The synthesis of this precursor mRNA is driven by the major late promoter (MLP) and yields families of 3′ coterminal mRNAs (Shaw and Ziff 1980). The late mRNAs also share a 5′ region known as the tripartite leader. This region is about 160nt in length and represents the splicing together of three non-adjacent regions (Berget et al 1977; Chow et al 1977; Gelinas and Roberts 1977; Ziff and Evans 1978).

The precursor mRNA is processed in the nucleus to produce one of the many possible late mRNAs. Between 10% and 28% of the nuclear RNA is conserved after processing and polyadenlyation, and the remainder is subject to degradation (Nevins and Darnell 1978). The complete processing of the precursor mRNA is essential for the association of the mRNA with polyribosomes and the extension of the half-life of the mRNA (McGuire et al 1972; Zeevi et al 1982).

The late regions code for many of the structural components of the virion. These include hexon (polypeptide II), penton (polypeptide III), penton associated protein (polypeptide IIIa), fibre (polypeptide IV), core protein (polypeptide V), hexon associated protein (polypeptide VIII) (Ziff and Fraser 1978), non structural proteins 35K and 55K (D'Halluin et al 1978b; Persson et al 1979), and the viral associated RNAs VaI, VAII (Soderlund et al 1976).

## I.VIII. Early to Late Switch

The switch from early to late transcription is complex and not fully understood, however, several hypotheses have been presented. The expression of L1 55K and 52K proteins have been observed at early times (Akusjarvi and Persson 1981; Lewis and Mathews 1980) and served as focal point for examination of transcriptional control at early and late times. It seems that the MLP is under the influence of mRNA processing, poly (A) site selection, and attenuation at early times. At early times the MLP is active but the mRNA it produces is subject to differential splicing (Akusjarvi and Persson 1981; Lewis and Mathews 1980). This includes the addition of a leader sequence termed the "i" leader which may control expression at the translation level (Akusjarvi and Persson 1981). Differential splicing patterns at early and

late times have been observed for other proteins (Chow et al 1979) and may reflect changing conditions within the host cell (Nevins and Wilson 1981). The aberrant early expression of the MLP promoter also seems subject to attenuation preventing expression of L2-L5 regions at early times (Nevins and Wilson 1981).

The use of a super-infection protocol has revealed that the viral genome itself plays an important role in the switch from early to late transcription (Thomas and Mathews 1980). The genome may require changes in the nucleosome structure which are only present after replication, or it may require compartmentalization for late transcription to occur (Thomas and Mathews 1980; Horwitz 1990).

## I.IX. Assembly of the Virion

Virus assembly begins with assembly of the capsomeres from the single peptides of hexon and penton (Horwitz 1990). The assembly of the hexon capsomeres occurs quickly while the penton capsomere assembly may take considerably longer (Horwitz et al 1969). The fibre protein has been shown to be nonessential for viral assembly (Falgout and Ketner 1987). The capsomeres assemble to form an empty shell or capsid (Horwitz 1990). Two scaffold proteins of 50K and 39K are involved in the formation of "light intermediate particles"

but must be removed before viral DNA entry may occur (D'Halluin et al 1978a and 1978b). The 50K protein has been identified as the viral protein $IVa_2$ which is encoded by the L strand (Persson et al 1979). Some of the internal virion precursor peptides, such as pVI and pVIII, also enter the capsid before the genome (Persson et al 1979; D'Halluin et al 1978a).

The viral DNA is packaged with the left end entering the capsid as directed by the encapsidation sequence (Hammarskjold and Winberg 1980; Hearing et al 1987). The small virion protein μ binds DNA and is believed to play an important role in the condensation of the viral genome for packaging (Anderson et al 1989). The entire viral genome enters the capsid before the core proteins, pVII and pV enter (D'Halluin et al 1978a). The maturation process is completed by the viral protease which cleaves the precursor proteins into their mature forms (Bhatti and Weber 1979).

## I.X.1. Transformation by adenovirus

The ability of adenovirus to cause tumors in rodents has long been known (Trentin et al 1962), and adenovirus serotypes have been classified by their ability to cause tumors (Huebner et al 1965; Trentin et al 1968). It was soon discovered, however, that even the "non-oncogenic" adenoviruses could

cause tumors in immunosuppressed rats (Gallimore 1972) or transform primary cells *in vitro* (McAllister et al 1969a and 1969b). The process of *in vitro* transformation of primary rat cells has allowed in-depth study of adenovirus transformation (Freeman et al 1967a and 1967b; Black et al 1967).

Studies of transformed cell lines have shown that the left end of the adenovirus genome is always integrated into the transformed cells (Galimore et al 1974; Graham et al 1974). The site of integration of the viral DNA appears to be random (Deuring et al 1981; Dorsch-Hasler et al 1980; Visser et al 1981). Small regions of homology between the viral and cellular genomes may assist in the integration (Deuring et al 1981) of a circular intermediate (Visser et al 1981).

Experiments with sheared DNA have shown that the leftmost 6% of the genome is sufficient for partial transformation (Graham et al 1974). This is the region which codes the E1A gene products, which are also essential for lytic infection.

### I.X.2A. E1A Transformation

*In vitro* studies have revealed that only the leftmost 4.5% of the viral genome is required for transformation of primary baby rat kidney (BRK) cells (Houwelling et al 1980). These partially transformed cells lack many of the characteristics of fully transformed cells such as a high

growth rate and the ability to reach high cell densities in monolayer cultures (Houwelling et al 1980). To obtain the fully transformed phenotype the presence of another transforming gene is required. E1A can cooperate with either the 19K or the 55K E1B products (McLorie et al 1991), but in addition, activated *ras* is also sufficient to produce fully transformed cells (Ruley 1983).

Human adenoviruses derive much of their oncogenic potential from their ability to form complexes with various cellular proteins (reviewed in Barbeau et al 1992). Many studies have shown that the amino terminus, CR1 and CR2 are important for transformation. As discussed earlier, E1A functions to affect the normal regulation of the cell cycle through complex formation with the normal regulatory proteins (DeCaprio et al 1989; Bagchi et al 1990; Cao et al 1992; Howe and Bayley 1992).

## I.X.3. The Role of E1B in Transformation

Initial studies of transformation of primary cells by adenoviruses revealed that both E1A and E1B were required for full transformation (Houwelling et al 1980). It was believed that the 19K protein in cooperation with E1A was sufficient for transformation, making 55K dispensable (Chinnaduria 1983; Babiss et al 1984). It was not until the two major E1B

products were studied independently that it was discovered that either the 55K or the 19K protein, in cooperation with E1A, could induce transformation by separate but additive pathways (White and Cipriani 1990; McLorie et al 1991).

The 19K protein, as discussed earlier, is believed to cooperate with E1A in transformation by preventing programmed cell death (apoptosis) induced by E1A (White et al 1992; Rao et al 1992; Subramanian et al 1993). The mechanism by which 19K blocks apoptosis is currently unknown.

The role of the 55K protein in transformation has become clearer in recent years and appears to involve complex formation with the cellular protein p53 (Sarnow et al 1982), which also has been shown to form complexes with products of other transforming viruses (Howley et al 1991; Lane and Benchimol 1990; Levine 1989; Schmieg and Simmons 1988). Mutants which fail to bind to p53 have been shown to be deficient for transformation (Yew et al 1990; Yew et al 1992).

p53 was initially identified as an oncogene by virtue of its ability to cooperate with E1A or *ras* in cellular transformation (Jenkins et al 1984). It was eventually realized that it actually is a tumour suppressor, following the discovery that mutations activate the oncogenic potential of p53 (Hinds et al 1989) and that wild type p53 could suppress transformation (Finlay et al 1989). The p53 gene is often found to be mutated in human malignancies (Chang et al

1993). p53 has been implicated in the induction of apoptosis, and thus complex formation with 55K may also be linked to E1A-induced programmed cell death (Debbas and White 1993; Lowe and Ruley 1993). The p53 protein has an increased half-life in infected and transformed cells (Zantema et al 1985), and it is believed that this increase in p53 levels in response to E1A may represent one aspect of E1A cytotoxicity (Lowe and Ruley 1993; Lane 1992). It has also been shown that the Ad5 55K protein sequesters p53 in discrete bodies in the nucleus and perinuclear region (Zantema et al 1985; Blair-Zajdel and Blair 1988). The p53 protein is normally found in the nucleus and it was suggested that 55K may inhibit its function at least partially by sequestering it from its normal targets. Thus E1B-55K and E1B-19K proteins may provide separate mechanisms to disable the apoptosis pathway (Debbas and White 1993).

In addition, p53 has been shown to transactivate transcription and it is believed to play a role in the regulation of the cell cycle through this transactivation ability (Diller et al 1990; Mercer et al 1990; Yew and Berk 1992). It has been shown that 55K binds to the N-terminus of p53 which contains the activation domain (Kao et al 1990; Braithwaite et al 1991; Yew and Berk 1992). 55K normally inactivates p53 transactivation activity (Yew and Berk 1992). Recently, however, a mutant was created which is able to bind

p53 but fails to inhibit p53 transactivation and is deficient for transformation (Yew et al 1990; Yew and Berk 1992). The molecular basis for this phenotype is not known.

## I.XI. Purpose of Study

It has been previously shown in this laboratory that 55K is phosphorylated on both serine and threonine residues. We wished to identify the major sites of phosphorylation of 55K and attempt to determine their importance in regulating the functions of 55K. We used both biochemical and genetic methods to map phosphorylation sites near the carboxy terminus of 55K and then, through the generation of mutants we have studied effects on replication, late protein synthesis, as well as, transformation efficiency.

## II. Materials and Methods

### II.I Bacterial Strains

The following *E.Coli* bacterial strains were used: LE392 and DH5.

### II.II Bacterial Culture Techniques

#### II.II.1. Liquid Cultures

LE392 and DH5 bacteria were grown in Luria-Bertani (LB) broth (10g Bacto-tyrptone, 5g Bacto-yeast extract and 5g NaCl per litre). LB broth was sterilized and prior to use ampicillin was added to a final concentration of 50μg/ml. Liquid cultures containing bacteria were incubated at 37°C with constant shaking.

#### II.II.2. Solid Cultures

Agar plates were prepared by adding 10g of Bacto-agar to 500ml of LB. After autoclaving, the agar-medium was cooled to 44°C and ampicillin was added to a final concentration of

50μg/ml. Approximately 20ml was dispensed into 100x15mm bacterial plates and allowed to cool. Bacteria were either spread or streaked out on plates, inverted and incubated at 37°C overnight.

### II.II.3. Culture Storage

Bacterial strains after being grown overnight were then stored by the addition of approximately 2ml of culture to 1ml of 50% sterile glycerol in a 4ml glass vial and stored at -70°C

## II.III. Preparation of Plasmid DNA

### II.III.1. Small Scale Plasmid Preparation

Clones were grown in 3ml LB and ampicillin overnight. The cells from approximately 1.5ml of culture were pelleted and resuspended in 100μl Lysozyme (2mg/ml Lysozyme in 50mM Glucose, 10mM EDTA and 25mM Tris pH 8.0). After a 30min incubation on ice 200μl of Alkaline SDS (0.2N NaOH and 1% SDS) was added and the sample was inverted to mix, producing a clear viscous suspension. Incubation on ice for 5min was followed by the addition of 150μl of 3M NaAc (pH 4.8) and mixing. After 60min on ice, the lysed cells were centrifuged

for 5min at 13000rpm, yielding a clear supernatant. The supernatant was transferred to a new Eppendorf tube and 1ml of 2-propanol was added to precipitate plasmid DNA. Plasmid DNA was pelleted by centrifugation for 10min at 13000rpm. The pellet was washed with 70% EtOH and centrifuged. The pellet was dried and resuspended in 50µl $ddH_2O$.

## II.III.2. Large Scale DNA Preparations

10ml overnight bacteria cultures, containing the plasmids of interest, would be scaled up to 1l the afternoon or evening before plasmid purification. The next day cells were pelleted in the Cryofuge 8000 in one 1000ml bottle for 10min at 5000rpm. The bacterial pellet was processed in the same manner as in the small scale protocol except that 40ml of lysozyme was used with 80ml alkaline SDS and 40 ml 5M KAc (60ml 5M KAc add 11.5ml glacial acetic acid and 28.5ml H2O.). The supernatant was then filtered through several layers of cheese cloth and the DNA precipitated by the addition of 100ml of isopropanol. After a 30min incubation at room temperature the DNA was pelleted for 10min at 5000rpm. The pellet was then dried for 15min and resuspended in 5ml of 0.1xSSC (20xSSC pH 7.0 stock: 175g NaCl, 88.2g sodium citrate in 1l $ddH_2O$) and 2ml of 50mM Tris and 10mM EDTA.

## II.III.3. Cesium Chloride Banding Procedure

After the addition of 8.6g of CsCl the DNA in solution was incubated on ice for 30min and then centrifuged in for 15min at 3800rpm. The supernatants were then loaded into 13ml Beckman Quick seal tubes along with 300µl of 10mg/ml Ethidium Bromide. The tube was then filled with light paraffin oil, balanced and sealed. The tubes were mixed, loaded, and spun in the Vti 65.1 rotor for 14-18hrs at 18°C at 55000rpm. The plasmid DNA was visualized with long wave length UV light and collected with a 18G-$^1/_2$ needle and a 3ml syringe through the side of the tube. The ethidium bromide was extracted with isopropanol which had been saturated with CsCl in 25mM Tris, 10mM EDTA. Approximately 3 volumes of Tris EDTA were added, and the DNA was precipitated with 8 volumes of 96% ethanol. The DNA was pelleted by centrifugation in a Beckman GPR at 3800, washed twice with 96% ethanol, and dried at 37°C. The DNA pellet was then resuspended in 0.5 to 1ml of 0.1xSSC.

## II.III.3.A. Determination of DNA Concentration

The concentration of DNA was determined by one of two methods. DNA concentration was determined by analysis at wavelengths of 260nm and 280nm followed by quantification by electrophoresis on an agarose gel with a standard of known

concentration. DNA concentration was also determined using the diphenylamine assay. Standard concentrations of salmon sperm DNA were prepared with the DNA of unknown concentration. The standards and unknowns were brought to a volume of 300µl using $ddH_2O$. One ml of the diphenylamine reagent was added to the samples followed by boiling for 10 min. The samples were allowed to cool to room temperature and then absorbance at 595nm was determined using a Beckman DU-7 spectrophotometer. The DNA standards were then plotted (absorbance 595nm versus DNA concentration) and unknown concentrations were determined by intrapolation.

## II.IV. Mutagenesis

Two basic types of mutagenesis were performed. The first type was insertional mutagenesis and was performed by the method of Bautista and Graham (1989). The second method involved point mutations created using the Polymerase Chain Reaction (PCR) method.

### II.IV.1. Insertional Mutagenesis

Oligonucleotides were provided by F.L.Graham and originally purchased from The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster

University. Oligonucleotides were resuspended to a final concentration of 50 pmoles/µl. Oligonucleotides were annealed by mixing equal molar amounts and heating to 65°C then gradually cooling to room temperature.

II.IV.2. Point Mutations created by PCR

PCR was performed according to the instructions provided by Perkin Elmer Cetus. Primer concentration and annealing temperatures varied for different primers and optimal conditions were determined through experimentation.

II.V.1. Restriction Endonuclease Digests

All restriction endonucleases were purchased from New England Biolabs, Bethesda Research Laboratories (BRL) or Pharmacia. The digestions contained $^{1}/_{10}$ volume of the appropriate reaction buffer provided by the supplier. The volume of enzyme used varied depending on the amount of DNA being digested. Digests were performed at 37°C (unless otherwise specified by the supplier) for 3-16hrs. Digests used for ligations were usually phenol-chloroform extracted after heat inactivation.

## II.V.2. Agarose Gel Electrophoresis

Agarose gels where made with 1% w/v agarose in TAE (0.4M Tris-Acetate, 2mM EDTA). The agarose TAE mixture was then boiled and allowed to cool to approximately 45°C, then poured into the gel mould after the addition of 0.5μg/ml of ethidium bromide. DNA samples were mixed with loading buffer (0.25%bromophenol blue, 0.25% xylene cyanol and 40% (w/v)sucrose in $H_2O$) at a 6:1 ratio. The DNA samples were usually run overnight at constant voltage of approximately 35volts with an appropriate marker (i.e. 1KB Ladder from BRL). The gels were visualized with UV light and photographed using a mounted Polaroid equipped with high speed Kodak 57 film.

## II.VI.1. Ligations

All ligations were performed using T4 DNA ligase. In a typical reaction 1μl of ligase would be added to a tube containing 1μl of 10x ligation buffer (from supplier) and DNA fragments. The volumes of the DNA fragments varied from one ligation to the next, but depended on the concentration of the fragments and the desired ratio of vector to insert. The ratio of vector to insert varied from 1:1 to 1:200 depending on the size of the vector and insert (Legerski and Robberson 1985). The final volume of a reaction was usually 10μl. The

ligation was incubated at room temperature for 1-16hrs depending on the complexity of the ligation. Before using the DNA for transformations an aliquot was removed for analysis on an agarose gel to check that ligation had worked.

## II.VII. Competent Cells

### II.VII.1. Calcium Chloride Technique

In the morning a 250ml culture was inoculated from an overnight culture of LE392. The culture was shaken at 37°C to allow appropriate aeration. The $OD_{660}$ was monitored and when the $OD_{660}$ reached 0.9 the culture was centrifuged at 6000rpm for 20 min. The bacterial pellet was resuspended in 50ml of ice cold 100mM $MgCl_2$ and then pelleted again. The pellet was resuspended in 110ml of 100mM $MgCl_2$ and incubated on ice for 30-90 min. The bacteria were then pelleted and resuspended in 12.5ml of 85mM $CaCl_2$ with 15% glycerol. Aliquots of 0.3ml were transferred to 1.5ml Eppendorf tubes and quick frozen in liquid $N_2$. The competent cells were stored at -70°C and tested with a known concentration of a plasmid for their ability to take up DNA.

### II.VII.2. Electroporation

DH5 cells were used for electroporation. The cells were grown overnight in a 10ml culture and in the morning they were used to inoculate a 1l flask of LB. The cells were grown in a shaker at 37°C until they reached an absorbance of between 0.5-1. The cells were chilled on ice for 15-30min, then centrifuged at 4000rpm for 15min at 4°C. The cells were resuspended in 0.5l of cold water and centrifuged again. Another 0.5l wash was performed as described above. The cells were then resuspended in 20ml of a 10% glycerol solution and centrifuged. The cells were resuspended in a volume of 3ml of the 10% glycerol solution and 100µl aliquots were quick frozen in liquid nitrogen. The cells were stored at -70°C until needed.

## II.VIII. Transformation of E. coli

### II.VIII.1. Calcium Chloride method

The competent LE392 cells were thawed on ice and 10-50µl of DNA from a ligation was added to the cells. The cells and DNA were incubated on ice for 30-90min and then heat shocked for exactly 2min, by agitation in water at 42°C. The cells were then incubated on ice for 5min, then 0.7ml of LB was

added and the cells were incubated with gentle shaking at 37°C for 0.5-1hrs to allow for expression of antibiotic resistance. The cells were then diluted in LB and 200µl were plated at $10^0$ and $10^{-1}$ concentrations on LB agar plates containing ampicillin. The plates were allowed to dry then inverted and incubated at 37°C overnight.

### II.VIII.2. Electroporation

The competent DH5 cells were thawed on ice, and then 1µl of the ligation DNA was added to 50µl of competent cells. The cells and DNA were then placed in a chilled electroporation cuvette and allowed to incubate for approximately 2min. The cuvette was then placed into the electroporation apparatus and electroporated at 2.25Kvolts 250ohms and 25µfaraday. The cells were then added to approximately 1ml of LB and incubated for 0.5-1hrs in a 37°C shaker. The cells were then diluted and plated as in the calcium chloride method.

### II.IX.1. Sequencing Reactions

Sequencing reactions were performed as instructed in the Sequenase Kit using the reagents provided and an appropriate primer.

## II.IX.2. Sequencing Gel Apparatus

The glass plates used for the sequencing apparatus were both cleaned with $ddH_2O$ and then with 70% ethanol one of the two plates was then treated with 5ml of 2% Repel Silane (in 1,1,1 Trichloroethane). 2l of 1xTBE were prepared (24.22 gm Tris, 10.27gm Boric Acid, 0.75 gm EDTA/2l) and allowed to dissolve completely. A 7% acrylamide gel was prepared by combining 6.65g acrylamide, 0.35g Bis-acrylamide and 42g urea in a 250ml flask and bringing the volume close to 100 ml using 1xTBE. The flask was incubated in the 65°C water bath until everything was completely dissolved then cooled on ice and filtered through 0.45 micron Nalgene filter. The volume was brought up to 100ml and the solution was placed on ice, while the plates were taped together with appropriate spacers. Immediately prior to the casting of the gel, 1 ml of fresh 10% ammonium persulfate was added to acrylamide/urea mixture and swirled to mix. 40µl of TEMED were added and the solution was again mixed. The gel was poured using 2 50ml syringes. The comb was inserted and the plates were clamped together from bottom up and across top. The gel was laid flat and allowed to polymerize for 60 min. The gel was then either used immediately or stored overnight for use the next day. Prior to loading, the gel was pre-run at 1800 v (100ma) for at least 1 hour. Approximately 2µl of each sample was loaded then run

at 1800volts for 1.5-2hrs before subsequent loadings were performed if necessary. The gel was dried on a gel dryer at 80°C for approximately 1hr then exposed to Kodak XAR-5 film.

## II.X. Plasmids

The plasmid pXC38 is a derivative of pXC1 (McKinnon et al 1982), containing the left 16% of the Ad5 genome with the Bam H1 site removed. The plasmid pUC19 was used for cloning PCR intermediates and the plasmid pJM17 was designed by J. McGrory et al (1988).

## II.XI. Isolation of DNA Fragments

### II.XI.1. DNA Fragment Elution

To isolate DNA fragment from acrylamide gels, large amounts of DNA were digested with the appropriate enzyme(s). The DNA digest was then loaded on a 5% acrylamide gel and the gel was then run an appropriate distance to separate the fragments of interest. The gel was then stained and the DNA fragment of interest, was excised using a scalpel, while being visualized with UV light. The gel fragment(s) was then transferred to an 1.5ml Eppendorf tube and crushed with a teflon plunger. The addition of 0.6ml of elution buffer (0.5M

ammonium acetate, 10mM magnesium acetate, 1mM EDTA, and 0.1% SDS) was followed by an overnight incubation in a 37°C shaker. The next day the acrylamide was pelleted by centrifugation at 13000rpm for 5min. The supernatant was removed and saved and the pellet was resuspended in 0.3ml of elution buffer by vortexing. The acrylamide was centrifuged again and the supernatants were pooled. The supernatant was filtered through siliconized glass wool and the DNA was precipitated by the addition of 2 volumes of 96% ethanol at -20°C. The DNA was pelleted by centrifugation at 13000rpm for 15min . The pellet was then washed once with 96% ethanol and once with 70% ethanol. After drying the pellet was resuspended in 20µl of $ddH_2O$.

### II.XI.2. Gene Clean Method

DNA fragments were isolated from agarose gels using the reagents and procedure provided in the Gene Clean Kit produced by Bio101 Inc.

### II.XII.1. Tissue Culture

Cell lines were maintained in 37°C incubators with 5% $CO_2$ and 95% humidity. Tissue culture dishes used were 60mm dishes from Corning, 100mm dishes from Nunclon and 150mm dishes from

Nunclon. Three types of media were used: minimum essential media (F11), alpha minimum essential media (MEM) and Joklik's modified media (JOK). Fetal calf serum (FCS), Newborn calf serum (NBCS) and Horse serum (HS) were all purchased from Gibco and heat inactivated at 56°C for 30min prior to use. Additional supplements used included Penicillin-streptomycin (P/S, purchased from Gibco), L-glutamine 1% v/v, fungizome, and Bacto-yeast extract.

## II.XII.2. Cell Lines

HeLa cells, a cell line derived from a cervical carcinoma, were maintained in MEM supplemented with 10%FCS, 1%P/S, 1%L-Glutamine and 1% Fungizome. KB cells are a cell line derived from nasopharangeal carcinoma tissue, were maintained in a similar manner. The human embryonic cell line 293 transformed by Ad5 DNA (Graham et al 1977) was maintained in F11 supplemented with 10% NBCS, 1%P/S, 1%L-Glutamine and 1% Fungizome.

## II.XII.3. Passaging Cell Lines

When a 150mm dish of HeLa or KB cells was nearly confluent the medium was removed and the dish was washed twice with sterile 1xPBS (8g NaCl, 0.2g KCl, 1.15g NaPHO in 1l

$ddH_2O$). The cells were then dislodged by incubation in 5ml of 1xTrypsin-EDTA for 5-10min. The cells were resuspended in media (25ml/plate) and plated. Passaging HeLa or KB cells 1/10 would produce plates ready for use in 3-4 days. A confluent plate of 293 cells would be split 1/3 to yield plates ready to be used in 3-4 days. The 293 cells were passaged by rinsing the cells twice with 5ml of 1x Citric Saline (10x Citric Saline Stock: 50g KCl, 22g Sodium Citrate in 500ml of $ddH_2O$) followed by incubation at 37°C for 5-10min. The cells were manually removed, resuspended in media and plated.

## II.XIII. Transformation of Primary Baby Rat Kidney (BRK) Cells

### II.XIII.1. Preparation of BRK Cells

Baby rats were used at the age of 6 days. The rats were sacrificed and the kidneys were removed and placed into 1xPBS with 2% P/S. The kidneys were cleaned by removing membranes and blood vessels. The kidneys were transferred to fresh PBS and P/S and further cleaned. The kidneys were rinsed in PBS and P/S and placed into a 50ml Corning tube. A long handled pair of scissors were used to mince the kidneys until a smooth suspension was produced. This suspension was placed into a

sterile 100ml Gibco bottle with 50ml of 2xTrypsin and spun using a stir bar for 15min. The particular matter was allowed to settle and the supernatant was transferred to 20ml of ice cold FCS. Additional 2xTrypsin was added to the Gibco bottle which was spun again for 15min. The particulate matter was allowed to settle and the supernatant was again transferred to the FCS. The cells in the FCS were transferred to 50ml Corning tubes and spun at 3000rpm for 10min at 4°C. The pelleted cells were resuspended in 100ml of MEM supplemented with 10%FCS, 1%P/S, 1% L-Glutamine and 1% Fungizome and incubated at 37°C for 30min. The cells were filtered through sterile cheese cloth and the final volume was brought up to 420ml using MEM (described above). The cells were then plated 5ml per 60mm dish.

### II.XIII.2. Transfection of BRK Cells

DNA mediated transfection of BRK cells was performed according to Graham et al. (1974) as modified by Wigler et al. (1979). In the morning following the preparation of the cells they were refed with MEM supplemented with 10%FCS, 1%P/S and 1%L-glutamine and 1% Fungizome. Later that day the BRK cells were 60-80% confluent and ready for transfection. To transfect the cells, an appropriate amount of experimental DNA was added to a 15ml Corning polystyrene tube. In addition,

200µl of 2.5M $CaCl_2$ (final concentration 125mM/ml) and 120µg of Salmon sperm DNA (final concentration of 30µg/ml) and brought to a final volume of 2ml using 0.1xTE. This solution was then added by single drops into 2ml of 2xHEPES (8g NaCl, 0.37g KCl, 0.125g $Na_2PO_4$, 5g HEPES, and 1g dextrose, brought to a final volume of 500ml using $ddH_2O$ and pH'ed to 7.1) which was sterilely aerated. The solution was then allowed to incubate at room temperature for at least 20min allowing the DNA precipitate to form. After incubation 0.5ml was added to each 60mm dish of BRK cells. The next day the cells were refed with MEM supplemented with 10% FCS, 1% P/S, 1% L-Glutamine and 1% Fungizome. After 48hrs the medium was removed and changed to the selection media, Joklik's supplemented with 5% HS, 1% P/S, 1% L-Glutamine and 1% Fungizome. The cells were refed with the selection media twice weekly for approximately two weeks. After which time the media was removed, the foci were fixed with 3:1 methanol:acetic acid, dried and stained with Giemsa stain diluted 1:20 in 0.1xPBS.

## II.XIV. Virus Rescue

Virus rescue was performed using the DNA-mediated transfection of 293 cells as described by Graham and Van der Eb (1973). 293 cells were split the day before the

transfection into 60mm dishes (one 150mm plate to 7-9 60mm plates), so that they would be 60-80 percent confluent at the time of use. All mutants were rescued into a dl309 background using the method of McGrory et al (1988). Briefly, to a 15ml polystyrene tube 2ml of 1x HEPES were added followed by 20-40μg of experimental DNA, an equal amount of pJM17 and finally 200μl of 2.5M CaCl. The mixture was mixed and incubated at room temperature for 20-30min. The DNA solution was then added to the cells (0.5ml/60mm dish) and incubated at 37°C in a $CO_2$ incubator for 4.5hr. The media was then removed and the cells were overlaid with 10ml of equal amounts of 2xF11 supplemented with 10% HS, 2% P/S, 2% L-glutamine, 2% fungizome, 0.4% yeast extract and 1% agarose. The overlay was allowed to solidify at room temperature for approximately 20min after which time the cells were incubated at 37°C until plaques or cytopathic effects were visible.

## II.XV. Screening Mutant Adenovirus Plaques

### II.XV.1. Harvest of plaque Isolates

All screening was performed utilizing 293 cells. Again 60mm dishes of 293 cells were set up to be 60-90% confluent at the time of use. Well isolated plaques were picked by punching out agar plugs using sterile Pasteur pipettes and

transferring the mashed agar to 0.5ml of $PBS^{++}$(PBS with 0.01g of $CaCl_2$ and 0.01g of $MgCl_2$ in 100ml of PBS) which was stored at -70°C until results of analysis were completed.

II.XV.2. Analysis of Viral DNA

The media from 293 dishes was removed and 0.2 ml of virus in $PBS^{++}$ was added. The virus was allowed to absorb at room temperature for 30min and then 5mls of F11 supplemented with 5% HS, 1% P/S, 1% L-glutamine, 1% fungizome and 0.2% yeast extract was added. The cells were incubated at 37°C until CPE was absolutely complete (all cells rounded and many floating) usually 3-4 days. A dish with complete CPE was placed in the laminar flow hoods for 20min to allow any floating cells to settle. The medium was gently removed with a pipette and about 3ml was saved for storage at -70°C in a sterile glass vial containing 0.5ml sterile glycerol. The remaining medium was removed by suction. To the 60mm dish 0.5 ml pronase-SDS (500μg/ml pronase, 10mM Tris pH 7.4, 10mM EDTA and 0.5% SDS) was added and allowed to digest at 37°C for 3-4 hrs. After the incubation the lysate was transferred to a 1.5ml Eppendorf tube and phenol extracted once with 0.5ml of phenol saturated with TNE (50mM Tris pH 8.0, 100mM NaCl and 10mM EDTA). The aqueous phase was collected with a Pasteur pipette and transferred to a fresh Eppendorf tube. The viral DNA was

precipitated with 1ml 96% ethanol and centrifugation at 13000rpm for 15min then washed 2x with 96% ethanol to remove any residual phenol. The DNA was dried completely in 37°C warm room and then redissolved in 50µl 0.1xSSC. The viral DNA was digested with an appropriate enzyme to confirm the presence of the mutations.

### II.XV.3. Plaque Purification of Viral Recombinants

All mutants once identified were plaque purified. In order to purify the plaques a serial dilution of the original plaque isolate was performed in $PBS^{++}$ and used to infect 60mm dishes of 293 cells. After absorbing for 0.5hrs the cells were overlaid with F11 and agarose (See Virus Rescue) and incubated at 37°C until plaques appeared. Again, well isolated plaques were picked and the viral DNA was re-analyzed as described in Analysis of Viral DNA.

### II.XV.4. Growing of Viral Stocks

To produce a viral stock several 150mm dishes of 293 cells were prepared to be 70-90% confluent when infected. Usually about 1ml of media, from the media saved from the last viral DNA analysis, was used to infect one 150mm dish. After the dish had reached complete CPE the cells were scraped from

the plate and resuspended in $PBS^{++}$plus 10% glycerol.

## II.XV.5. Titering of Viral Stocks

Appropriate cells (HeLa or 293's) were set to be 80-90% confluent when used. 293 cells were generally split, one 150mm dish into 7-9 60mm dishes, and HeLa cells were split one 150mm dish into 20-30 60mm dishes. The viral stocks were serially diluted ($10^{-4}$-$10^{-9}$) in $PBS^{++}$ and 200µl of each dilution was used to infect a duplicate series of plates. The virus was allowed to absorb for 30min at 37°C and then the plates were overlaid with F11 and agarose (see Virus Rescue). The overlay was allowed to solidify and the plates were then incubated at 37°C. On 293 cells plaques became visible within 4-5 days and were counted between 7-14 days. The HeLa plaques tended to take longer to appear and were counted between 7-21 days. HeLa plaques were visualized either by staining with a Neutral Red overlay or by removing the overlay fixing the cells and staining with Giemsa.

## II.XVI. Immunoprecipitations

### II.XVI.1. Infection of Cells

HeLa or KB cells were split to be 80% confluent at time of infection. The medium was removed from the dishes and saved, titred viral stocks were then diluted in $PBS^{++}$ to obtain a multiplicity of infection (moi) of 35 plaque forming units (pfu) per cell (unless otherwise stated). The virus was allowed to absorb at 37° for 30min and then 5 ml of the previously removed medium was added back. The infection was then allowed to progress for the desired period (usually 18hrs) at 37°C.

### II.XVI.2. Metabolic Labelling of Cells

Cells were incubated for 60min in prewarmed $methionine^-$ ($Met^-$) medium (3 ml per 60mm dish) or $phosphate^-$ ($Phos^-$) medium (5ml per 100mm dish). The medium was then removed and cells were labelled for 2hrs in medium contained 50-500uCi TranS-label per dish in $Met^-$ medium or 0.2mCi-1mCi $^{32}P$-orthophosphate in $phos^-$ medium.

## II.XVI.3. Cell Extracts

After the plates had been rinsed with ice cold $PBS^{++}$ approximately 0.5 ml RIPA (5ml 1M Tris-Cl pH 7.2, 3ml 5M NaCl, 0.5ml 20% SDS, 1ml Triton X100,and 1g Na Deoxycholate brought up to 100mls with $ddH_2O$ and sterilized by filtration) plus inhibitors (1:100 dilution of 3.5mg/ml PMSF in EtOH and 1:200 dilution of aprotinin 1.9mg/ml) were added to each 60mm dish. Cells were scraped and transferred to a 1.5ml Eppendorf tube and incubated on ice for 10-15min and then vortexed for 5-15sec. The crude extracts were clarified by centrifugation at 12000rpm at 4°C for 30min. The supernatant was carefully transferred to a fresh Eppendorf tube avoiding transfer of any pelleted matter. Cell extracts were either used to perform immunoprecipitations (IP's) immediately or frozen down at -70°C for use at a future date.

## II.XVI.4. Immunoprecipitation

To 0.5 ml of labelled extract 50µl of 50% protein A sepharose in RIPA was added. An appropriate amount of antibody (amount varies with the different serum or monoclonal) was added to the cell extract, which was then rotated on a mixer at 4°C for 2 to 4 hours. After incubation at 4°C the immunoprecipitations were washed 5X using 1ml of

RIPA. During the washing procedure samples were maintained on ice. After each wash the protein A Sepharose beads were pelleted by brief centrifugation and the supernatant was removed and discarded using a 1ml syringe and 26g needle. The protein A Sepharose beads were resuspended in 40µl of 2X SDS-PAGE loading buffer (2ml Tris-Cl pH 6.8, 1.6ml 20% SDS, 1.6ml glycerol, 0.8ml beta-mercaptoethanol, and 2ml $ddH_2O$) mixed, and boiled for 3-5min. Usually 20µl of the sample would be run on a 10% SDS-PAGE gel overnight and the remainder frozen down at -70°C until it was needed.

## II.XVII. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

### II.XVII.1. Casting SDS-PAGE gels

A running gel of the desired percentage (usually 10%) was prepared as follows: 10.1ml of $H_2O$, 6.3ml of 1.5ml Tris pH 8.8, 0.13ml of glycerol and 8.4 ml of 30% acrylamide-0.8% bisacrylamide mixture were mixed and then filtered. The glass gel plates were assembled as described in the Protean II manual. To the running gel 125µl of TEMED and 12µl of 10% APS were added and the gel was poured into the gel apparatus (leaving a space of approximately 1cm from the bottom of the comb). The running gel was overlaid with about 2 ml of 0.1% SDS ( or 70% ethanol) and allowed to polymerize for about

20min. While the running gel polymerized the 5% stacking gel was prepared (4.3ml $H_2O$, 1.8ml Tris pH 6.8, 0.04ml 20% SDS, and 1.3ml of 30% acrylamide-0.8% bisacrylamide) and filtered. The running gel was then washed thoroughly with $ddH_2O$ and drained well. After filtering 37µl of TEMED and 4µl of 10% APS were added to the stacking gel and it was poured into the apparatus and the comb was positioned. After polymerization was complete the comb was removed and the wells were rinsed thoroughly with $ddH_2O$.

## II.XVII.2. Electrophoresis

The gel apparatus was assembled as described in the manual and checked for leaks using 1X Tris-glycine running buffer (RB) (10X RB prepared with 30g Tris base, 144g glycine, 10g SDS, and brought to 1l with $ddH_2O$). The samples were loaded with a 50 µl Hamilton syringe and rinsed well between samples. $^{14}C$-labelled markers were run for reference along with the samples. The gel was run overnight at a constant current of 4 milli-amps.

## II.XVII.3. Fixing and Drying Gels

After electrophoresis was completed the gels disassembled and transferred into a plastic box. $^{35}S$ labelled protein gels were shaken for 10-15min in about 200ml DMSO. A second DMSO wash was completed and then the gel was shaken in a 22% PPO solution (in DMSO) for 1 hour. The gel was then rinsed in $ddH_2O$ and soaked for 15-30min in 500ml $ddH_2O$. The gel was transferred onto 3MM Whatman paper and dried for 1.5-2hr at 60°C. Gels containing $^{32}P$-labelled protein were fixed for 10min in 10% MeOH-10% HOAC. The gels were then rinsed for 10min in $ddH_2O$, then transferred to 3MM paper and dried for 1hr at 80°C. After gels were dried they were exposed to fast film (Kodak XAR) for an appropriate time and then developed.

## II.XVIII. Isolation of Proteins from SDS-PAGE Gels

For the purpose of isolation of proteins from SDS-PAGE gels immunoprecipitations were performed as outlined previously with the exception that several 150mm plates were usually labelled (3mCi/plate $^{32}P$ and 1mCi$^{35}S$/plate) to prepare the cell extracts. After performing the immunoprecipitations 300µl of 2X Laemlli sample buffer was added and the samples were boiled for 5-10min. The samples were loaded onto a SDS-

PAGE gel of the desired percentage and run as previously described.

The gel was then wrapped in saran wrap and marked with radioactive ink to allow alignment of the gel after exposure. A sheet of XAR5 film was exposed to the gel in a cardboard film holder for 0.5-2hrs and then developed. The film was then aligned with the gel and a heated probe was used to trace the outline of the desired bands onto the gel.

The bands were transferred into a 15ml polypropylene Corning centrifuge tube and crushed with a teflon plunger. 6ml of protein extraction buffer (100mM Ammonium Bicarbonate, 0.1% SDS, and 0.5% Beta-mercaptoethanol) were added and the tubes were boiled for 10min. The tubes were placed in the warm room (37°C) on the end over end rotator for 12-18 hours. The acrylamide was pelleted by centrifugation for 5min at 1000rpm, the supernatant was removed and placed in another 15ml polypropylene tube and placed on ice. The pellet was resuspended in an additional 6ml of protein extraction buffer and boiled for 10min, followed by rotation in the warm room for 2-4hrs. The tube was spun for 5min at 1000rpm and the supernatant was collected and pooled in a 50ml glass Corning centrifuge tube.

A 100μg of carrier protein (globlin) along with 20% TCA (stock solution 100% Trichloroacetic acid 100g Trichloroacetic acid brought to 100mls using $ddH_2O$) were added and the tube

was incubated on ice for 4 hours. The sample was centrifuged in a SS34 rotor at 4°C at 10000rpm for 30min. The pellet was washed 3X with 3ml of ice cold acetone and let dry at 4°C (on ice in the cold room). While the pellet dried fresh performic acid was made by mixing formic acid and hydrogen peroxide (95:5 vol/vol); the performic acid was incubated at room temperature for 1hr prior to use. The dried pellet was resuspended in 250µl of performic acid and transferred to a 1.5ml eppendorf tube, then incubated for 1hr. An additional 750µl of $ddH_2O$ was added, the sample was frozen in liquid nitrogen and lyophilized (or dried in the speedvac) overnight. The sample was then washed at least 2X with 1ml $ddH_2O$ and dried.

## II.XIX. Phosphoamino Acid Analysis by Thin Layer Chromatography (TLC)

Lyophilized proteins (6000cpm) were hydrolysed in 400µl of 6N HCl at 110°C for 2hrs. The sample was diluted to a final volume of 1ml and lyophilized overnight. The pellet was resuspended in 10µl of $ddH_2O$ and spotted onto a TLC plate, along with 3µg of each nonradioactive phosphoamino acid marker.

The samples were subjected to electrophoresis at pH 3.5 in a pyridine: acetic acid : 0.5M EDTA: $ddH_2O$ buffer

(5:50:2:943 by volume) on cellulose TLC plates (Polygram CEL300) at 1500volts for approximately 30min. The TLC plate was dried and the position of the phosphoamino acids was determined by staining with ninhydrin, followed by an incubation at 37°C for 30min. The TLC plate was exposed to XAR5 film overnight, then developed and aligned with TLC plate to allow identification of the labelled amino acids.

## II.XX. Trypsin Digestion of Proteins

After isolation from SDS-PAGE gels, proteins were resuspended in 500µl of 50mM $NH_4$-bicarbonate (pH 8.0) and 50µg of Worthington trypsin was added, then incubated at 37°C in the warm room on an end over end rotor for 6hrs. Another 50µg of trypsin was added followed by a 2hr incubation. The sample was dried by lyophilization overnight, washed 2X with $ddH_2O$ and dried in a speedvac for 3 hours after each wash. The tryptic peptides were either frozen down at -20°C or used for further analysis immediately.

## II.XXI. Thin Layer Chromatography (TLC)

The tryptic peptides were resuspended in 10µl of 1% ammonium carbonate (pH 8.9) and loaded onto the TLC plate. Electrophoresis in the first dimension was performed at 4°C at

1000volts with a formic acid:acetic acid:pyridine:dd$H_2O$ buffer (6:1.25:0.25:92.5) pH 1.9 for approximately 40min. The TLC plate was air dried then subjected to ascending chromatography using a n-butanol: pyridine: acetic acid: dd$H_2O$ buffer (50:40:10:40 by volume) for approximately 8hrs. The TLC was dried and exposed to XAR5 film for an appropriate amount of time.

## II.XXII. Analysis by High Performance Liquid Chromatography (HPLC)

Tryptic peptides were separated at room temperature on a Waters dual pump HPLC system with a 600E controller using a 4.6 X 250mm Ultrashpere ODS (C18) reverse phase column which had been pre-equilibrated with solution A (5% formic acid in water). Peptides were eluted from the column using either a linear 1 to 63% gradient of solution B (5% formic acid in ethanol) for 95min or using a stepwise protocol involving elution for 5min with solution B, followed by 65min with a 1 to 30% gradient with solution B, and terminated by 30min with a 30 to 60% with solution B. The flow rate was constant at 1ml/min and detection of labelled peptides was achieved using an on-line LB507A isotope detector (Berthold).

# III. RESULTS

## III.I.1. Production of 55K Insertion Mutants

Previous results (Takayesu and Branton, unpublished) suggested that one or more phosphorylation sites of E1B-55K were located towards the carboxyl terminus of the protein. In an attempt to map this site more precisely, two mutants were constructed using the method of Bautista and Graham (1989). A pair of synthetic 33bp oligonucleotides, with *BamHI* sticky ends(Fig.4A), was ligated into the *BglII* (3328bp) site in plasmid pXC38 (Fig.4B).

The oligonucleotides could insert such that in the open orientation an 11 amino acid insert containing Gln-Leu-Leu-Ser-Ala-His-Asn-Trp-Val-Arg-Ile was introduced immediately following amino acid 438 of the E1B-55K sequence ( Fig.4C). In the closed orientation the oligonucleotide yields 4 amino acids after residue 438 followed by a stop codon (Leu-Thr-Gln-Leu-End)(Fig.4D).

Figure 4: A. Oligonucleotides used to create the in3328 mutants.

B. Nucleotide and amino acid sequences of E1B-55K, showing the *BglII* site at 3328.

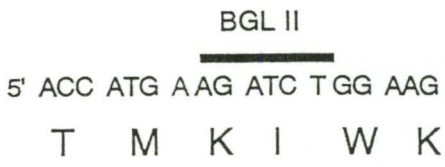
C. Nucleotide and amino acid sequences of in3328(+) showing the 11 amino acids inserted.

D. Nucleotide and amino acid sequences of in3328(-) showing the 4 amino acids inserted and end of the protein.

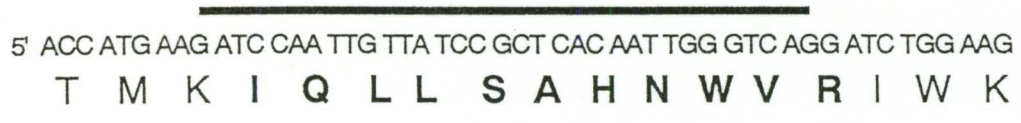
A

AB238 5' GATCCTGACCCAATTGTGAGCCGGATAACAATTG
AB239 GACTGGGTTAACACTCGCCTATTGTTAACCTAG 5'

B




C




D

## III.I.2. Mutagenesis by LOP Insertion

Digestion of plasmid pXC38 with *BglII* cleaves it at one site located at 3328bp (Fig.5A). This linearized plasmid was then ligated to the 33bp insert with *BamHI* cohesive ends (Fig.4A). The ligation produced plasmids containing one, two or three inserts as revealed by digestion with *StyI*. The insert caused a shift in the 289bp band to 333bp [which can be seen in Fig.5B lane A pXC38; lane B in3328(-) and lane C in3328(+)].

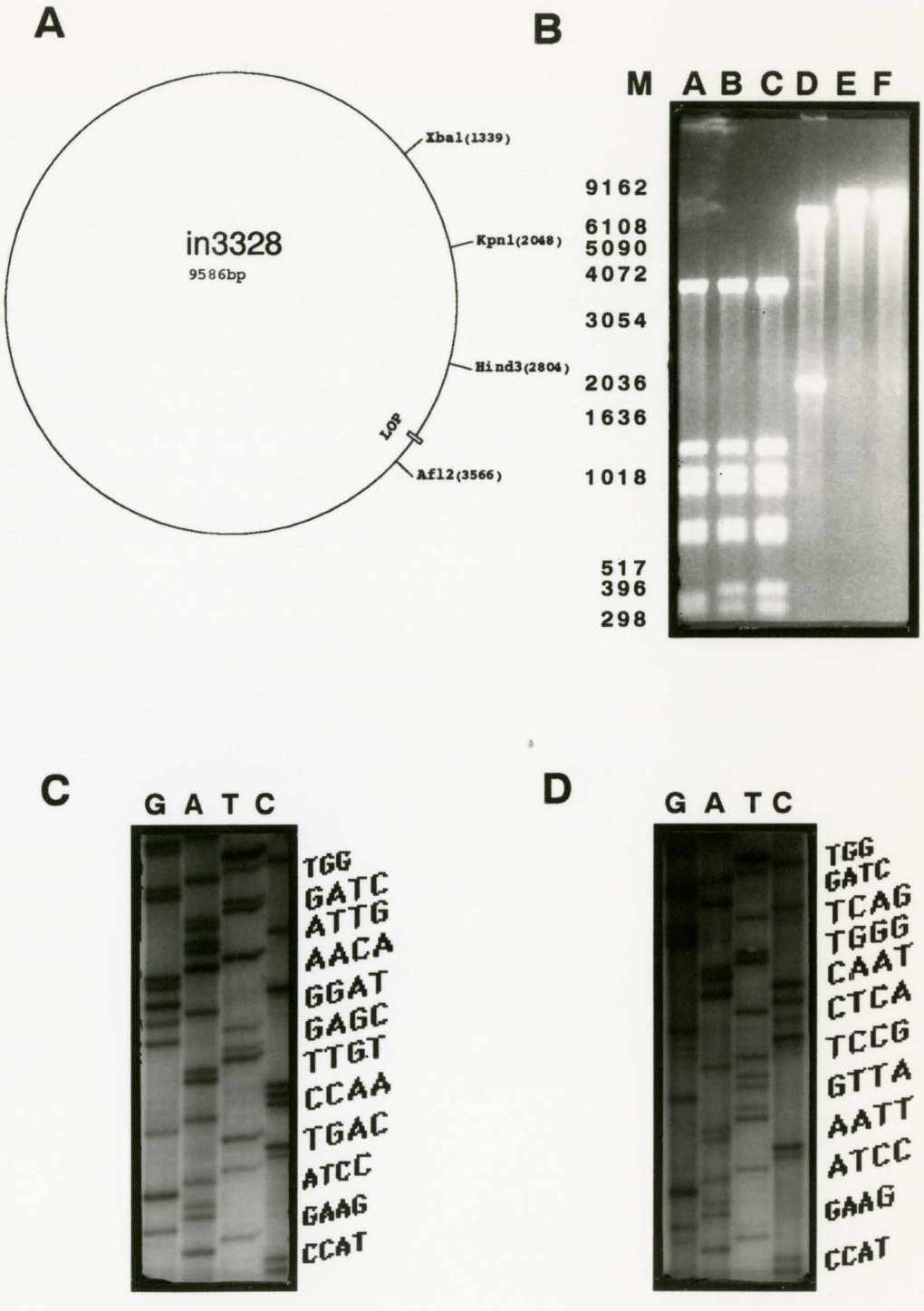
To confirm that the insert had been correctly ligated, plasmid DNA was digested by *BglII* and *XbaI*. pXC38 produces two fragments of 7542bp and 2011bp while in3328(-) or in3328(+) produces a single fragment of 9586bp as a result of the *XbaI* site at 1339bp (Fig.5B lanes D, E, and F). Plasmids containing single inserts were selected and sequenced to determine the orientation of the insert (Fig.5C and 5D). The plasmid with the insert in the forward orientation denoted in3328(+) inserted eleven amino acids ( Fig.5D). The plasmid containing the insert in the opposite orientation or closed orientation denoted in3328(-) inserted four amino acids and then coded for a stop codon truncating the protein (Fig.5C). These mutants were also rescued into plasmid pm1716/2072, which produces no E1B-19K protein (McLorie et al 1990), for use in transformation assays.

Figure 5: Characterization of the in3328 mutants.

A. A schematic of the plasmid in3328 showing the LOP insert at 3328.

B. *StyI* digest showing the shift of the 339bp fragment caused by the insertion of the LOP cassette. The lanes are pXC38 (lane A), in3328(-) (lane B) and in3328(+) (lane C). *BglII* and *XbaI* digest showing the elimination of the *BglII* site in the in3328 mutants. The lanes are pXC38 (lane A), in3328(-) (lane B) and in3328(+) (lane C). Position of the DNA markers are shown on the left in bp.

C. and D. are sequencing of the in3328 mutants inserts are denoted by the larger font. in3328(-) is in shown in C. and in3328(+) is shown in D.


A
in3328
9586bp
XbaI(1339)
KpnI(2048)
Hind3(2804)
LOP
Afl2(3566)
B
M A B C D E F
9162
6108
5090
4072
3054
2036
1636
1018
517
396
298
C
G A T C
TGG
GATC
ATTG
AACA
GGAT
GAGC
TTGT
CCAA
TGAC
ATCC
GAAG
CCAT
D
G A T C
TGG
GATC
TCAG
TGGG
CAAT
CTCA
TCCG
GTTA
AATT
ATCC
GAAG
CCAT

The plasmids, in3328(+) and in3328(-), were rescued into virus by cotransfection of 293 cells with pJM17. Well isolated plaques were picked and screened for the presence of the insert by digestion with *BglII*. Mutant virus was identified by the absence of the *BglII* site at bp 3328 thus eliminating a 3328bp and a 5586bp fragment and generating a new 8914bp species, in addition to the other fragments produced by the digestion (Fig.6B and 6C). Viruses containing the insert were plaque purified twice and then grown in large quantities on 293 cells and titred twice before use in experimentation.

To determine the levels of expression of the mutant viruses, KB cells were infected with dl309 and the insertion mutants at a moi of 35pfu. The cells were labelled with $^{35}$S-methionine and then immunoprecipitated using either 58-N1 serum or a 55K-specific monoclonal antibody 9C10.
In3328(-) produced no detectable product with either 58N-1 or 9C10 (Fig.7 lanes 3 and 4). In3328(+) produced a product but at greatly reduced levels compared to wild-type (Fig.7 lanes 5 and 6). Thus these mutants did not appear to be suitable for some of the studies to be carried out.

Figure 6: Characterization of the in3328 virus.

A. A schematic showing the distribution of *BglII* sites on the in3328 viral genome.

B. Expected fragments (in bp) for a *BglII* digest of wt (dl309) and the in3328 viruses.

C. *BglII* digest of the viruses. Lanes are: A wt; B in3328(-) and C in3328(+). Position of the DNA markers are shown on the left in bp.

**A**

in3328 Bgl II Digest

**B**

| Fragments (bp) | |
|---|---|
| Wt | in3328 |
| | 8914 |
| 6182 | 6182 |
| 5586 | |
| 5178 | 5178 |
| 3328 | |
| 2943 | 2943 |
| 2334 | 2334 |
| 2151 | 2151 |
| 1672 | 1672 |
| 1625 | 1625 |
| 1548 | 1548 |
| 1497 | 1497 |
| 1268 | 1268 |
| 351 | 351 |
| 272 | 272 |

**C**

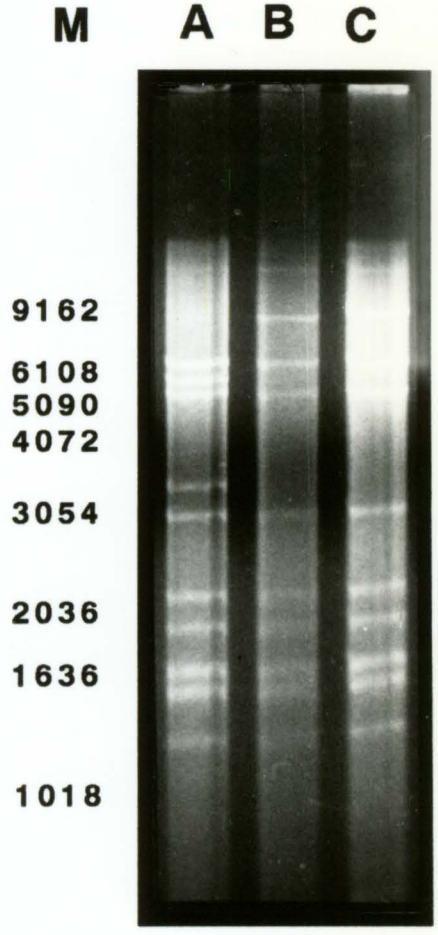
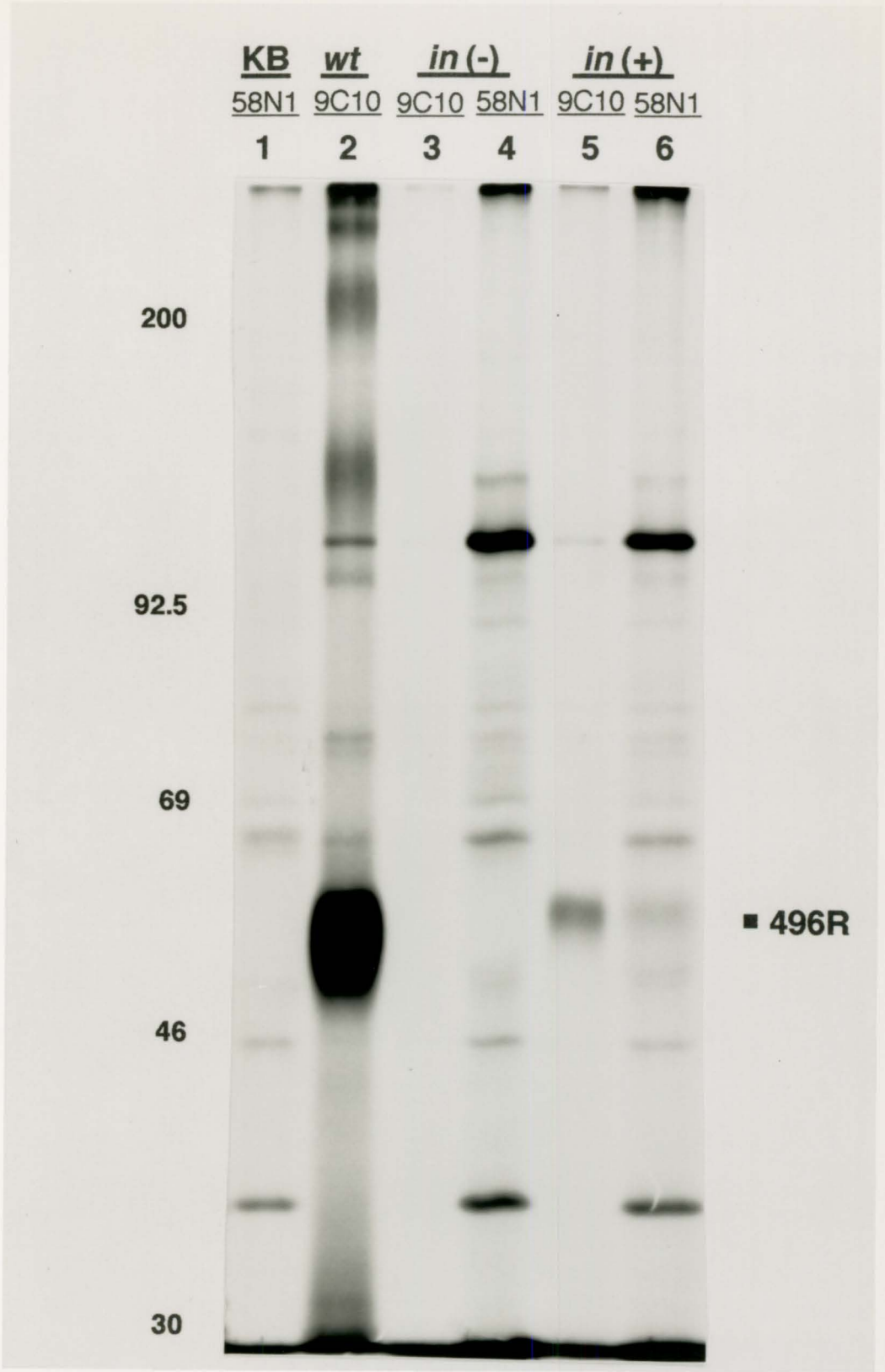


Figure 7: Analysis of 55K synthesized by in3328 mutants by SDS-PAGE. KB cells infected with wt ad5, mutants in3328(-) or in3328(+), or mock infected, were labelled with $^{35}$S-methionine, immunoprecipitated using 9C10 or 58-N1 antibodies and analyzed by SDS-PAGE. Lanes are indicated in the figure. The position of 496R (55K) has been indicated at the right and those of $^{14}$C-labelled markers ($x10^{-3}$) at the left.


KB
58N1
wt
9C10
in (-)
9C10
58N1
in (+)
9C10
58N1
1
2
3
4
5
6
200
92.5
69
46
30
496R

### III.II.1. Production of a Mutant Affecting the 55K Protein by PCR

As a result of the apparent instability of the insertion mutants an alternative approach was used to determine the importance of serines 490/491 which appeared to be the likely sites of phosphorylation near the carboxy terminus. PCR mutagenesis was used to change serines 490/491 to two alanine residues through the use of two mutagenic oligonucleotides which also generated a diagnostic *EagI* site (Fig.8A). In addition, an *XbaI* site was designed into AB1458 to assist in cloning of the PCR fragments. The oligonucleotides contained a total of four substitutions affecting only codons 490 and 491 (Fig.8C).

PCR was performed using AB1458 and AB1459 (Fig.8B) to yield a fragment of 224bp which was digested with *XbaI*, which cleaves at a site in AB1458 and *SacI* which cleaves after bp 3641 in the adenovirus sequence, and cloned into the pUC19. pUC19 was digested with *XbaI* and *SacI* and then alkaline phosphatase treated before being ligated (Fig.9). The resulting plasmid was called pTAH1 (Fig.9). The mutation was confirmed both by the presence of the newly created *EagI* site and by sequencing.

The PCR fragment produced by AB1457 and AB515 was approximately 750bp. However multiple products of similar

Figure 8: A. Oligonucleotides used in the construction of pm490A/491A.

B. A schematic of the PCR strategy using the oligonucleotides. The long lines represent the Ad genome and the arrows represent the various primers.

C. Nucleotide and amino acid sequences of pm490A/491A showing the change of serines 490/491 to alanines 490/491 and the creation of the *EagI* site (CGGCCG).

A

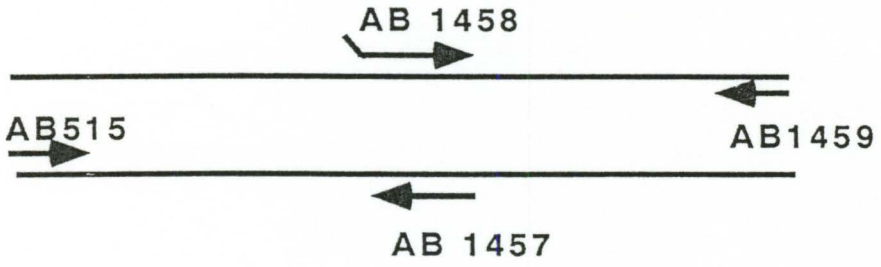
AB515 5' TTA GCG GTA CGG TTT

AB1457 5' TTC ATC GGC CGC GCC AAA CTC AGC

AB1458 5' GCT CTA GAG GCG CGG CCG ATG AAG ATA

AB1459 5' CCA TCA CAT TCT GAC GCA CC

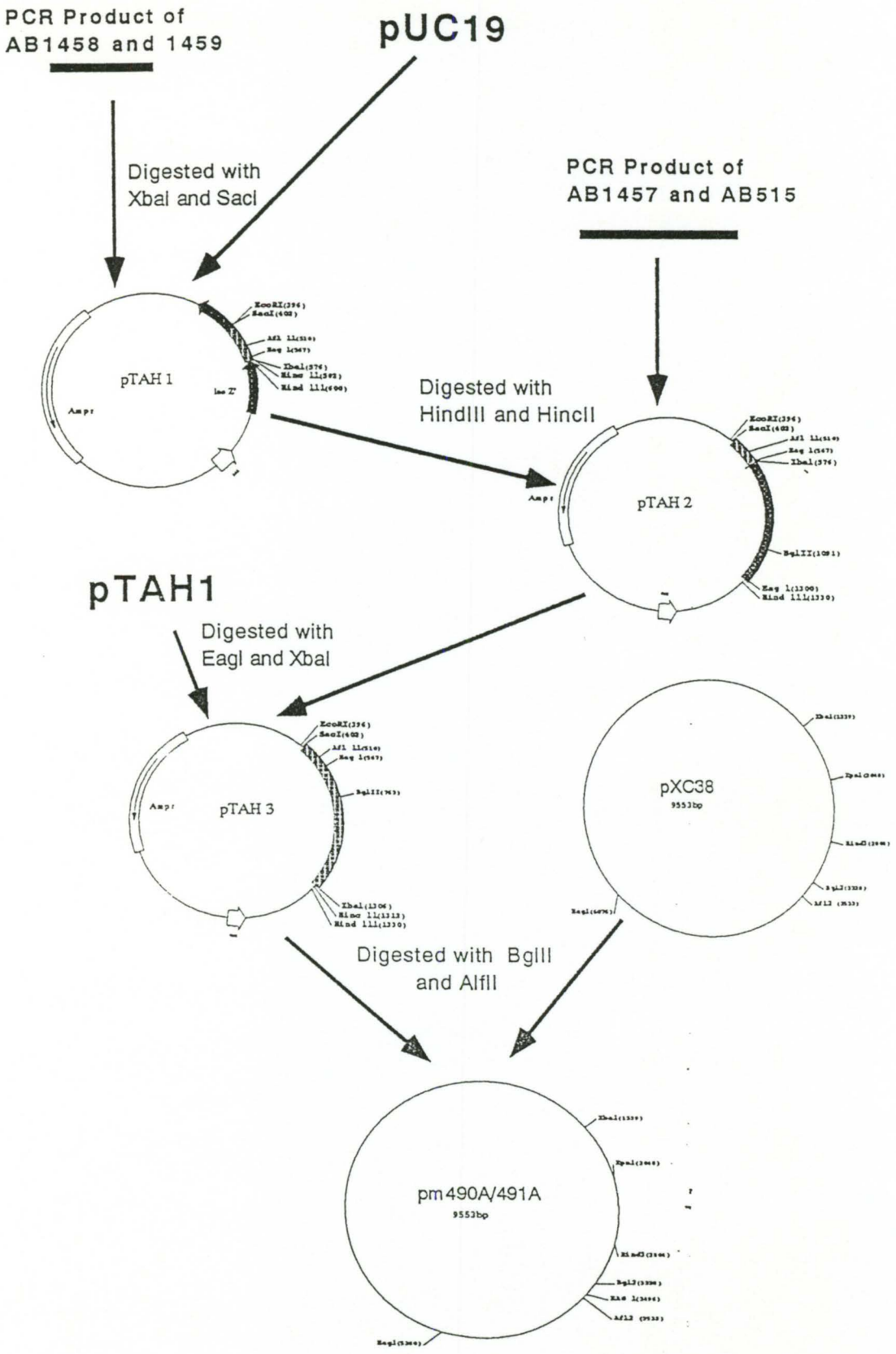
B




C

| | 489 | 490 | 491 | 492 |
|---|---|---|---|---|
| | GLY | SER | SER | ASP |
| WT | GGC | TCT | AGC | GAT |
| | | \| \| | \|\| | |
| PM490A/491A | GGC | GCG | GCC | GAT |
| | GLY | ALA | ALA | ASP |

Figure 9: A schematic of the construction of pm490A/491A. The cloning steps are depicted in the figure and discussed in the text of the results section.


PCR Product of
AB1458 and 1459
pUC19
Digested with
XbaI and SacI
PCR Product of
AB1457 and AB515
pTAH 1
Digested with
HindIII and HincII
pTAH 2
pTAH1
Digested with
EagI and XbaI
pTAH 3
pXC38
9553bp
Digested with BglII
and AlfII
pm490A/491A
9553bp

size were produced by the PCR reaction. The full length fragment was to contain an internal *HindIII* and an *EagI* site (located 6bp from the end of the fragment). The 750bp PCR product was purified from a 5% acrylamide gel and then treated with the Klenow fragment to create a blunt-ended product, and then digested with *HindIII*. This fragment was then cloned into pTAH1 which had been digested with *HindIII* and *HincII*. After identifying appropriate clones, an *EagI* digest would collapse out the unwanted sequences between the two PCR products.

Potential clones were identified by the presence of a unique *BglII* site introduced through the adenovirus sequences. Two clones were identified by this method, however, after further digestion it was discovered that these clones both contained the fragment in reverse orientation. Further digestions showed that PCR fragments were incorrectly initiated and lacked the *HindIII* site. An incomplete product had been blunt ended into the *HincII* site in the incorrect orientation (Fig.9). This plasmid was called pTAH2.

Since a full length product was not required for future cloning pTAH2 was digested with *EagI* and *XbaI* and ligated to pTAH1 which had also been digested with *EagI* and *XbaI* (Fig.9). DNA fragments were purified from a 5% acrylamide gel and ligated, resulting in a plasmid, termed pTAH3 (Fig.9). This plasmid contained adenovirus sequences from approximately

2900bp (beginning shortly after the *HindIII*) through to the *SacI* site at 3641bp in a pUC19 background, including the newly created *EagI* site at 3486bp.

The plasmid pTAH3 was cleaved with *BglII* and *AflII* and run on a 1% agarose gel. The 184bp fragment was purified from the agarose gel using the Gene-Clean Kit produced by Bio101 Inc and ligated into pXC38 which had been digested with *BglII* and *AflII*(Fig.9). The plasmid was named pm490A/491A.

The mutations were confirmed by digestion with *EagI* (Fig.10B lanes A, B and C). The plasmid pXC38 produces a single fragment of 9553bp while pm490A/491A produces two fragments of 6963bp and 2590bp (Fig.10B lanes B and C). The plasmid was also sequenced from the *BglII* site to the *AflII* site to confirm the integrity of the PCR products (Fig.10C). Although the sequence presented in Fig.10 is some what ambiguous, it has also been sequenced by Branton and coworkers. The presence of the *EagI* restriction site also supports this sequence.

The mutant pm490A/491A was also rescued into pm1716/2072 for use in transformation assays. The presence of the *EagI* site was confirmed by digestion as seen in Fig.10 lane C.

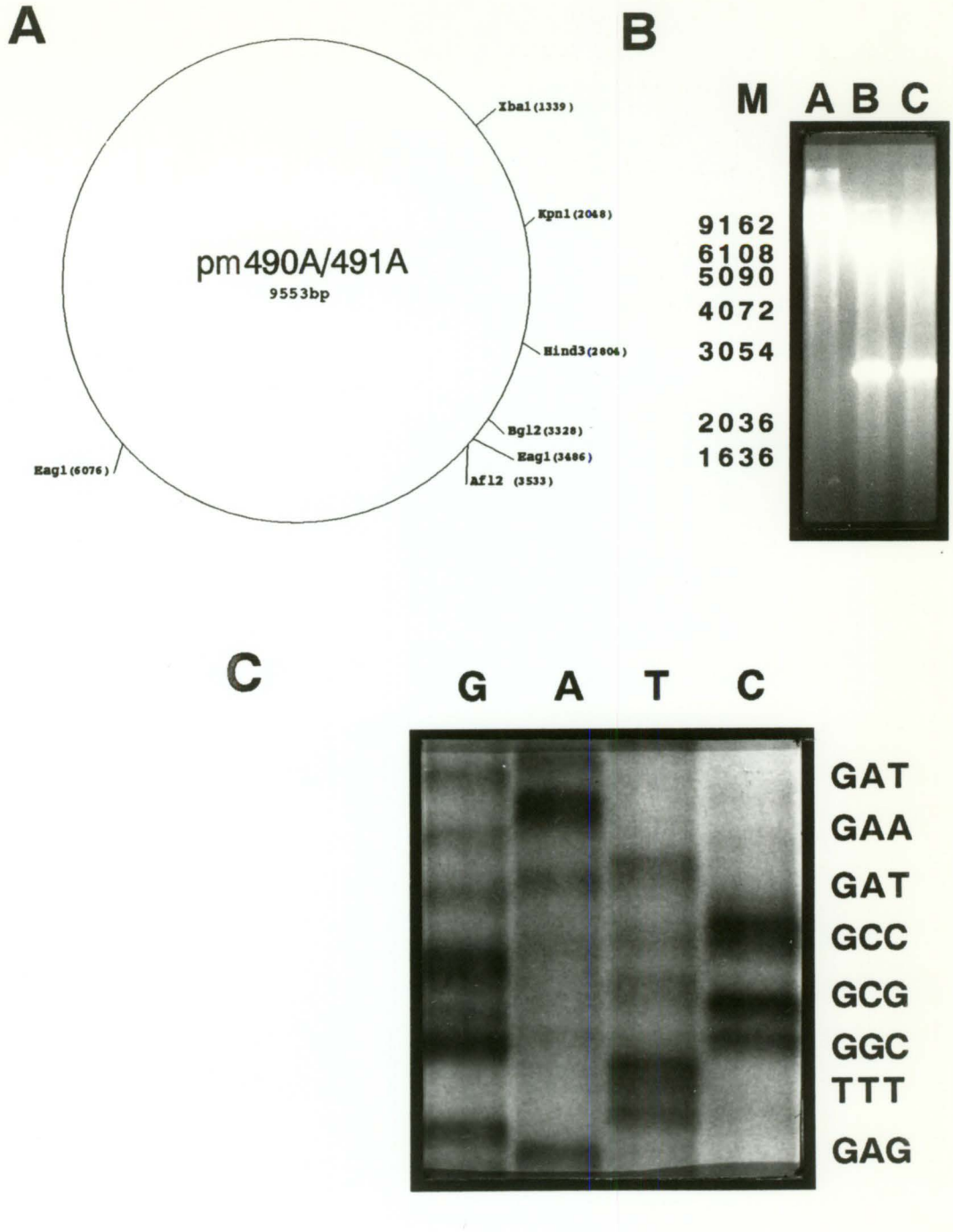
The plasmid pm490A/491A and pm490A/491A(19K$^-$) were rescued into virus by cotransfection with pJM17 of 293 cells. Plaques appeared after approximately 12 days and were picked after attaining a size of 2-3mm. The plaque isolates were

Figure 10: Characterization of the pm490A/491A and pm490A/491A(19K$^-$) mutants.

A. A schematic of the plasmid pm490A/491A showing the new *EagI* site at 3486.

B. *EagI* digest of plasmids confirming the second *EagI* site. Lanes are: A pXC38; B pm490A/491A and C pm490A/491A(19K$^-$). Position of the DNA markers are shown on the left in bp.

C. Sequencing of the pm490A/491A mutant. Nucleotide sequences are located on the figure.


A
pm490A/491A
9553bp
XbaI(1339)
KpnI(2048)
HindIII(2806)
Bgl2(3328)
EagI(3486)
Afl2 (3533)
EagI(6076)
B
M A B C
9162
6108
5090
4072
3054
2036
1636
C
G A T C
GAT
GAA
GAT
GCC
GCG
GGC
TTT
GAG

grown in 293 cells in 60mm dishes for 3-4 days after which time the viral DNA was harvested and digested with *EagI* to verify the presence of the mutation (Fig.11C and 11D). The addition of the *EagI* site at 3486bp resulted in the disappearance of a 6503bp fragment and the appearance of a 3486bp and a 3017bp fragment (Fig.11B). For the purposes of experimentation in the E1 region, dl309 is considered as wild-type, however, dl309 contains deletions and insertions in the E3 region (Jones and Shenk 1979). This procedure affected one of the *EagI* sites normally found in the wt virus resulting in the combination of a 5409bp fragment and a 1016bp fragment to produce a fragment approximately 6400bp (Fig.11B). The mutation in pm490A/491A creates an additional site at bp 3486, cleaving the 6503bp fragment into 3486bp and 3017bp fragments (Fig.11B). Unfortunately, because of the 6400bp fragment produced by the construction of dl309 and the previous existence of a 3017bp fragment, only the 3486bp fragment was readily visible in the mutant (Fig.11C lanes B and C). The 6503bp and the 6400bp fragments, however, can be verified in Fig.11D lanes B and C.

Virus which produced the expected patterns were plaque purified twice and the presence of the mutation was verified after each purification. Viral stocks were then grown on 150mm dishes of 293 cells and titred twice before being used for experimentation.

Figure 11: Characterization of the pm490A/491A virus.

A. A schematic showing the distribution of *EagI* sites on the pm490A/491A viral genome.

B. Expected fragments (in bp) for a *EagI* digest of wt, dl309 and pm490A/491A virus. The differences in expected fragments is discussed in the text.

C. *EagI* digest of the viruses. Lanes are: A dl309; B pm490A/491A, C pm490A/491A(19K$^-$) and D wt ad5. Position of the DNA markers are shown on the left in bp.

D. *EagI* digest of the viruses run to separate the upper fragments. Lanes are: A dl309; B pm490A/491A, C pm490A/491A(19K$^-$) and D wt ad5. Position of the DNA markers are shown on the left in bp.

A

pm490A/491A

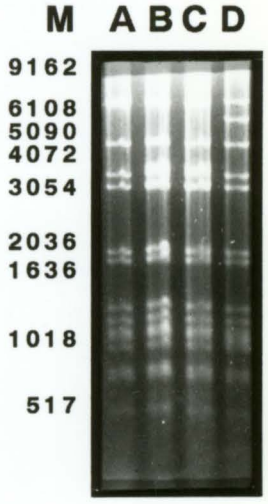
Eag III Digest

B

Fragments (bp)

| Wt | dl309 | pm490A/491A |
|---|---|---|
| 6503 | 6503 | |
| | 6400 | 6400 |
| 5409 | | |
| 4263 | 4263 | 4263 |
| | | 3486 |
| 3274 | 3274 | 3274 |
| 3017 | 3017 | 3017 |
| | | 3017 |
| 1891 | 1891 | 1891 |
| 1793 | 1793 | 1793 |
| 1293 | 1293 | 1293 |
| 1175 | 1175 | 1175 |
| 1104 | 1104 | 1104 |
| 1045 | 1045 | 1045 |
| 1016 | | |
| 960 | 960 | 960 |
| 796 | 796 | 796 |
| 762 | 762 | 762 |
| 556 | 556 | 556 |
| 253 | 253 | 253 |
| 246 | 246 | 246 |
| 222 | 222 | 222 |
| 188 | 188 | 188 |
| 80 | 80 | 80 |
| 49 | 49 | 49 |
| 40 | 40 | 40 |

C



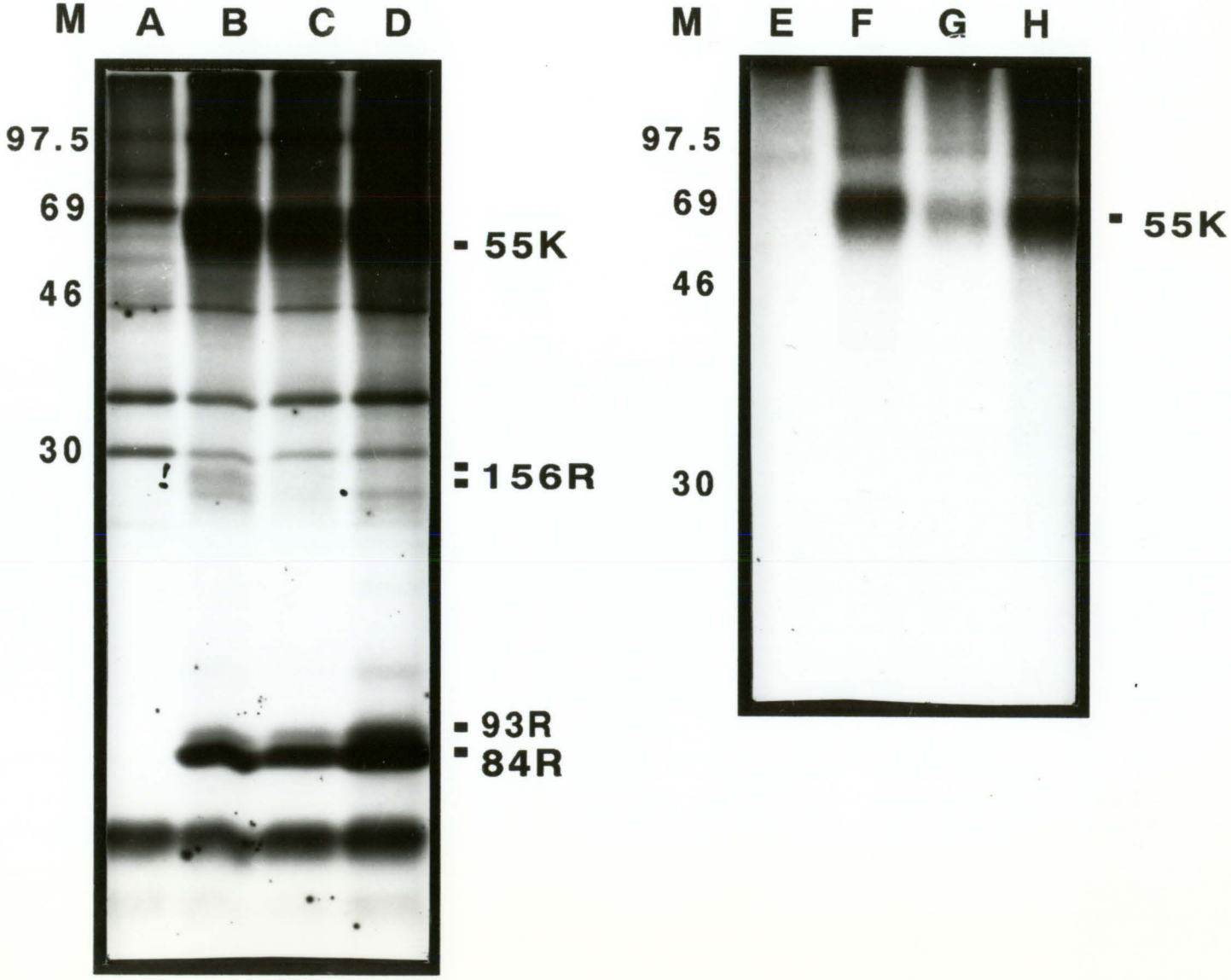

D

To examine the synthesis of 55K by pm490A/491A and to assess its level of phosphorylation, HeLa cells were infected at a moi of 35pfu/cell with dl309 or pm490A/491A. At 16hr p.i. the plates were labelled with $^{35}$S-methionine or $^{32}$P-orthophosphate for two hours and then the cells were then harvested and lysed. Immunoprecipitations were carried out using a 55K-specific monoclonal antibody 9C10, 58N serum, 19C serum and M73 (an E1A-specific monoclonal antibody), and precipitates were analyzed on 15% SDS-PAGE gels.

Fig.12 shows that in the case of dl309 and pm490A/491A the levels of $^{35}$S-methionine labelled protein, were similar (lanes B and C). However, with samples labelled with $^{32}$P-orthophosphate, 55K from pm490A/491A appeared less highly labelled compared to those obtained with dl309 (lanes F and G). With pm490A/491A(19K$^-$) the levels of labelling of 55K with both $^{35}$S and $^{32}$P were elevated (lane D and H), however, other experiments yielded similar results to pm490A/491A (Teodoro and Branton unpublished).

Also of interest was the 55K-related protein 156R. Previously it had been reported to migrate on SDS-PAGE as two species which differed by degree of phosphorylation (Takayesu et al Submitted for publication) which were clearly apparent with dl309 (Fig.12 lane B). However, with pm490A/491A (lane C) and pm490A/491A(19K$^-$)(lane D) the upper band appears to be absent.

Figure 12: Analyzes of 55K produced by pm490A/491A mutants by SDS-PAGE. HeLa cells were infected with wt ad5, mutants pm490A/491A, pm490A/491A(19K$^-$) or mock infected, were labelled with $^{35}$S-methionine(lanes A-D) or $^{32}$P-orthophosphate, immunoprecipitated using 58-N1 (lanes A-D) or 9C10 (lanes E-H) antibodies and analyzed on a 15% gel. Lanes are: A and E mock; B and F wt ad5; C and G pm490A/491A and D and H pm490A/491A(19K$^-$). The positions of 55K, 156R, 93R and 84R have been indicated at the right and those of $^{14}$C-labelled markers (x10$^{-3}$) at the left.


M
A
B
C
D
97.5
69
46
30
55K
156R
93R
84R
M
E
F
G
H
97.5
69
46
30
55K

To further characterize these mutant viruses, the levels of E1A proteins and 19K products produced by each were examined (Fig.13). The wt virus and pm490A/491A produced virtually identical levels of both E1A products and the E1B-19K product (Fig.13 lanes B,C and F,G respectively), while pm490A/491A(19K$^-$) appears to produce slightly less E1A protein (Fig.13 lane D). As expected this mutant did not express the E1B-19K product (Fig.13 lane H).

As additional evidence that serines 490/491 were indeed replaced by two alanine residues, the 58C serum was used to attempt to immunoprecipitate the 55K protein produced by pm490A/491A. The 58C serum is an anti-peptide sera produced against a peptide composed of the last 11 amino acids of the 55K protein (amino acids 486-496). The replacement of serines 490/491 with two alanine residues could result in a modification of the recognition site on the protein. As expected the 58C serum showed reduced affinity for the pm490A/491A 55K product (Fig 14).

Figure 13: Analysis of E1A and E1B-19K synthesized by pm490A/491A mutants by SDS-PAGE. HeLa cells infected with wt ad5, mutants pm490A/491A, pm490A/491A(19K$^-$) or mock infected, were labelled with $^{35}$S-methionine immunoprecipitated using M73 (lanes A-D) or 19C sera (lanes E-H) antibodies and analyzed on a 15% gel. Lanes are: A and E mock; B and F wt ad5; C and G pm490A/491A and D and H pm490A/491A(19K$^-$). The positions of the E1A proteins and the E1B-19K protein have been indicated at the right and those of $^{14}$C-labelled markers (x10$^{-3}$) at the left.

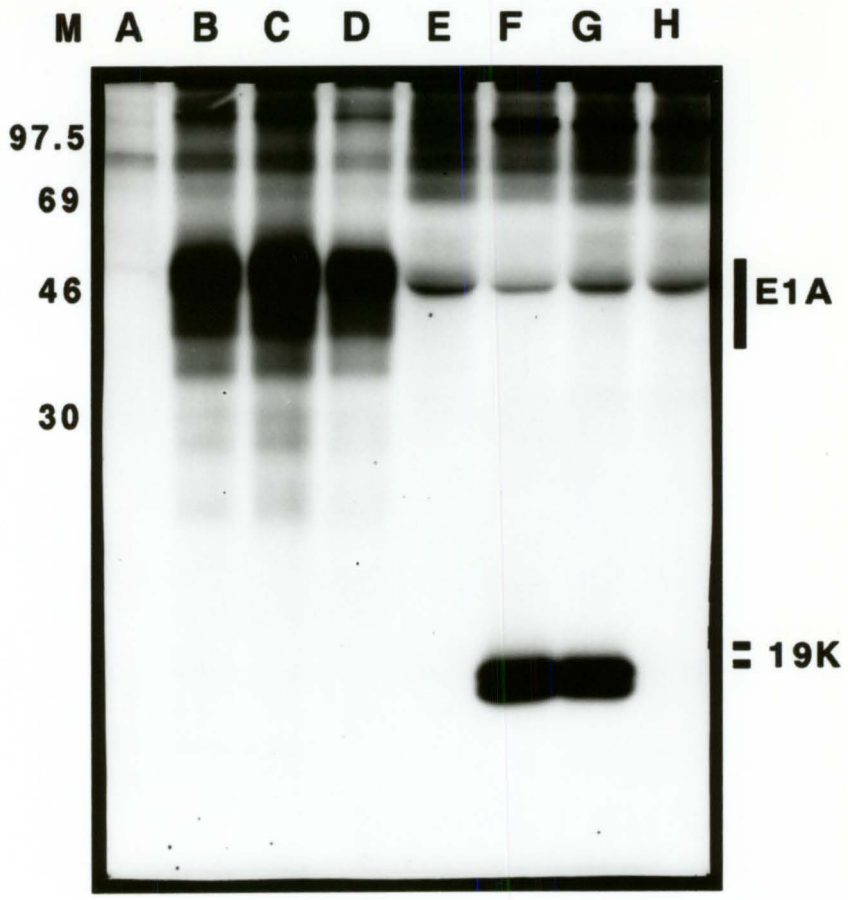
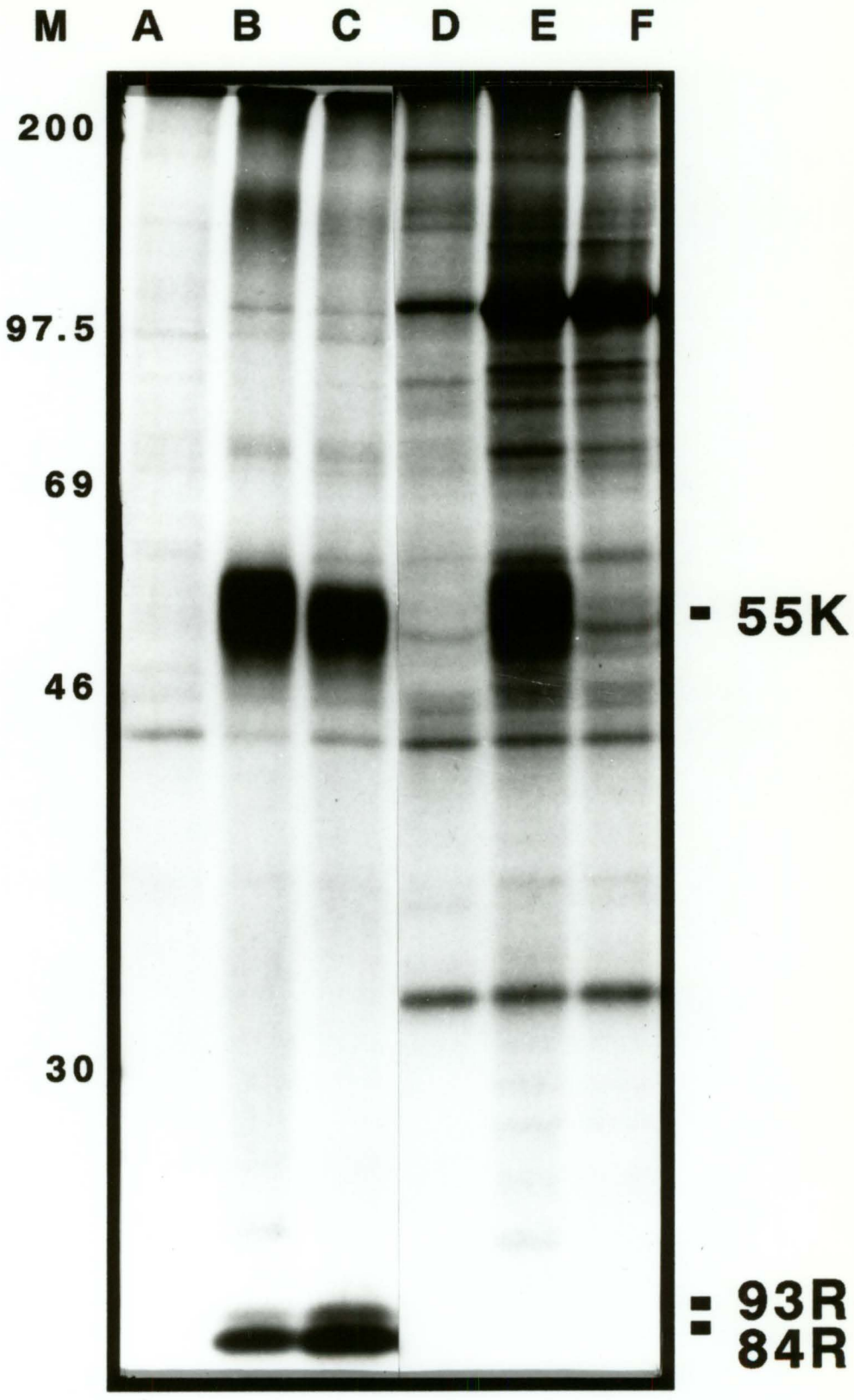

M A B C D E F G H
97.5
69
46
30
E1A
19K

Figure 14: Analyzes of 55K produced by pm490A/491A mutants by SDS-PAGE using 58C serum. HeLa cells were infected with wt ad5, mutant pm490A/491A or mock infected, were labelled with $^{35}$S-methionine, immunoprecipitated using 9C10 (lanes A-C) or 58-C1 (lanes D-F) antibodies and analyzed on a 10% gel. Lanes are: A and D mock; B and E wt ad5 and C and F pm490A/491A. The positions of 55K, 93R and 84R have been indicated at the right and those of $^{14}$C-labelled markers ($x10^{-3}$) at the left.


M
A
B
C
D
E
F
200
97.5
69
46
30
55K
93R
84R

## III.III. HPLC Analysis of Tryptic Digest of pm490A/491A[1]

Further analysis of the phosphorylation of 55K was performed through analysis of tryptic peptides of pm490A/491A 55K by HPLC. The 55K and 156R proteins from $^{35}$S-methionine and $^{32}$P-orthophosphate-labelled cells were isolated and subjected to tryptic digestion. The resulting peptides were analyzed by HPLC (Fig.15). Complete digestion of 55K yields 10 methionine containing peptides labelled B through K (Fig.15A). Two major $^{32}$P labelled peptides were evident (labelled II and III) and a third minor species was found eluting early from the gradient (peak I)(Fig.15C). The minor peak probably represented free phosphate which has previously been found to migrate at that position. Of the two major peaks, it appeared that neither co-migrated with a methionine-containing peptide.

Digestion of 156R yielded four methionine-containing peptides, three of which were identical to tryptic peptides produced from the 55K product (peaks B, G and I)(Fig.15B). A novel peptide (peak D1) was also present which probably represented the junction of tryptic peptides T4 and T43 which occurs in 156R. Analysis of $^{32}$P-labelled peptides indicated that both 55K and 156R yielded two identical phosphopeptides.

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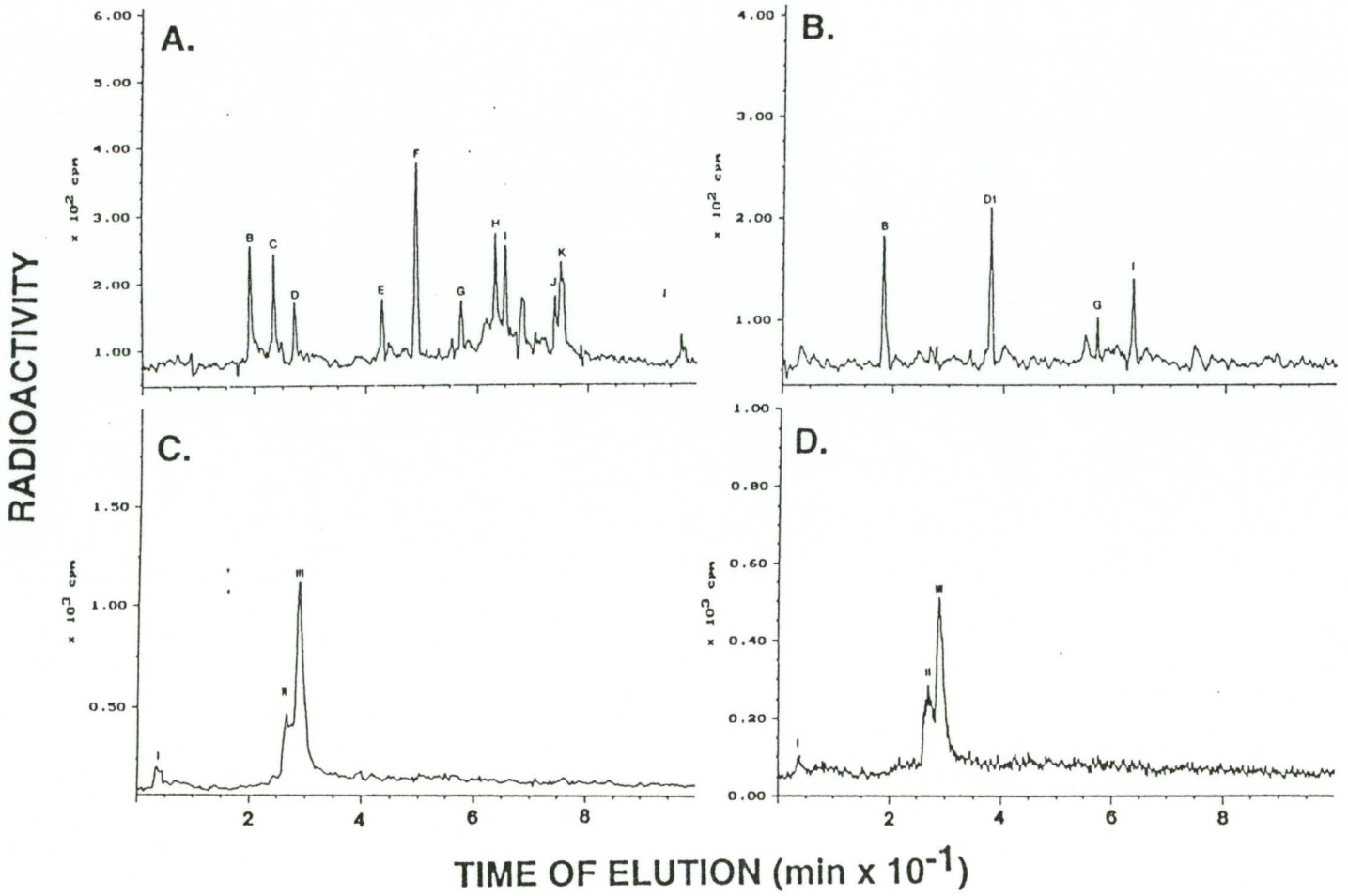
[1] HPLC analysis were performed at McGill university with the technical assistance of D. Takayesu

Figure 15: Analysis of tryptic peptides from 55K (496R) and 156R by HPLC. 55K and 156R labelled with $^{35}$S-methionine or $^{32}$P-orthophosphate were isolated by immunoprecipitation and SDS-PAGE and tryptic peptides were analyzed by HPLC as described in materials and methods. The amount of radioactivity has been shown in the ordinate and the time of elution on the abscissa. $^{35}$S-labelled and $^{32}$P-labelled 55K (A and C respectively). $^{35}$S-labelled and $^{32}$P-labelled 156R (B and D respectively).


A.
B.
C.
D.
RADIOACTIVITY
TIME OF ELUTION (min x 10-1)
x 10-2 cpm
x 10-3 cpm
B
C
D
E
F
G
H
I
J
K
D1
II
III

Analysis of peptides from pm490A/491A revealed that both $^{32}$P-labelled peptides present in 55K were absent (peak II and III) but that a third unique peptide (peptide IV) was apparent (Fig 16A and 16B). The 156R protein isolated from pm490A/491A-infected cells exhibited the same novel peptide and absence of peaks II and III (Fig 16C and 16D).

## III.IV. Phosphoamino Acid Analysis of pm490A/491A[2]

Previous studies had revealed that wild-type 55K is phosphorylated at both serine and threonine residues (Malette et al 1983). Phosphoamino acid analyses were performed to examine the amino acids which were phosphorylated in 55K from wt and pm490A/491A. The analyses revealed that while wt 55K contained predominantly phosphoserine and smaller amounts of phosphothreonine, pm490A/491A contained phosphothreonine and some phosphoserine.

Further analysis revealed that peak II of wt was composed of both phosphoserine and phosphothreonine, while peak III was due almost exclusively to phosphoserine. Analysis of peak IV of the pm490A/491A revealed the presence of phosphothreonine alone.

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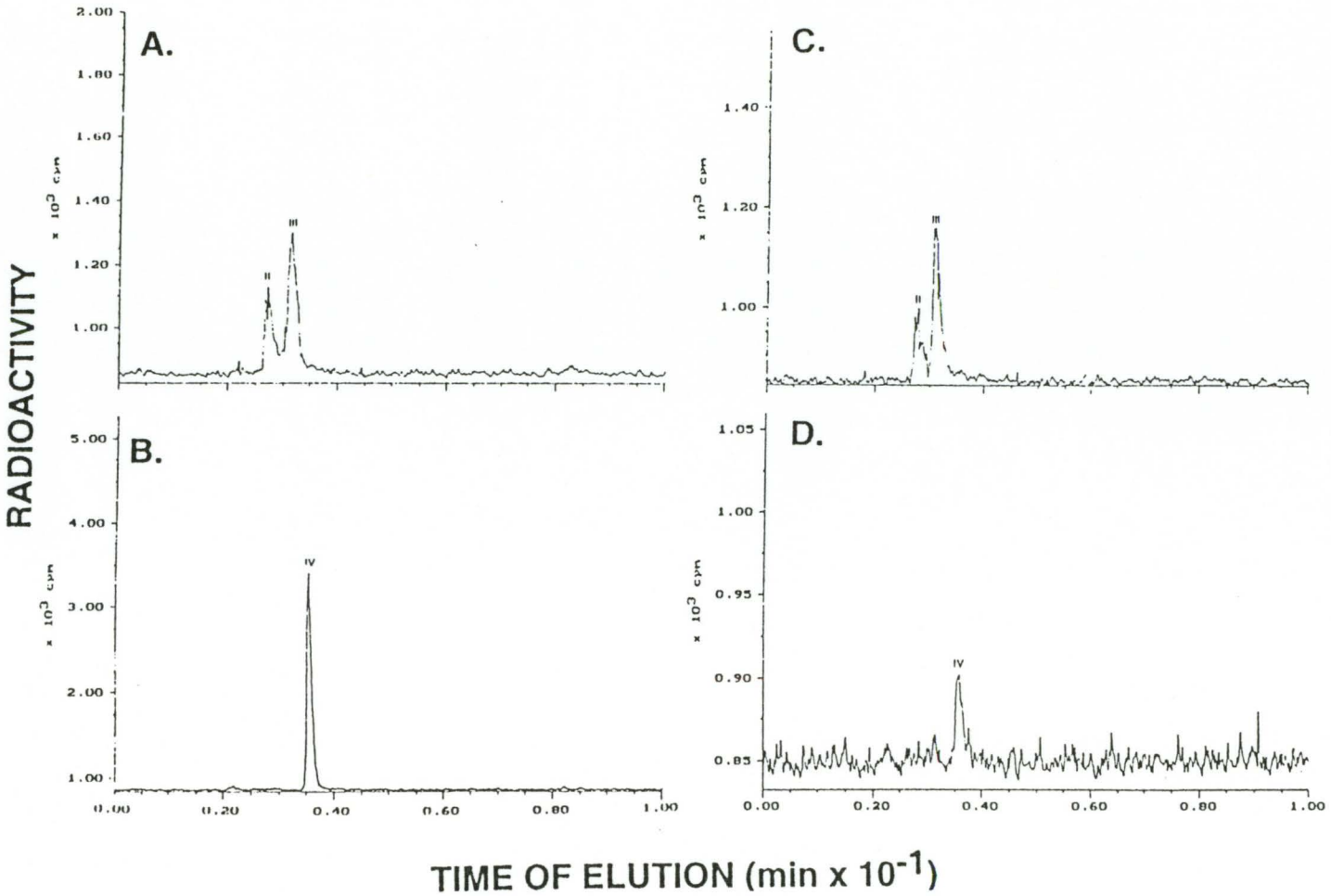
[2] Phosphoamino acid analysis was preformed by S. Whalen at McGill University and the results are found in Halliday et al.

Figure 16: Analysis of tryptic phosphopeptides of 55K and 156R synthesized by wt or mutant pm490A/491A by HPLC. Tryptic peptides from $^{32}$P-labelled 55K or 156R produced by wt ad5 or mutant pm490A/491A were analyzed by HPLC as described in the legend to figure 13. 55K from wt ad5 (A) and pm490A/491A (B). 156R from wt ad5 (C) and pm490A/491A (D).


A.
B.
C.
D.
II
III
IV
RADIOACTIVITY
x 10³ cpm
TIME OF ELUTION (min x 10⁻¹)

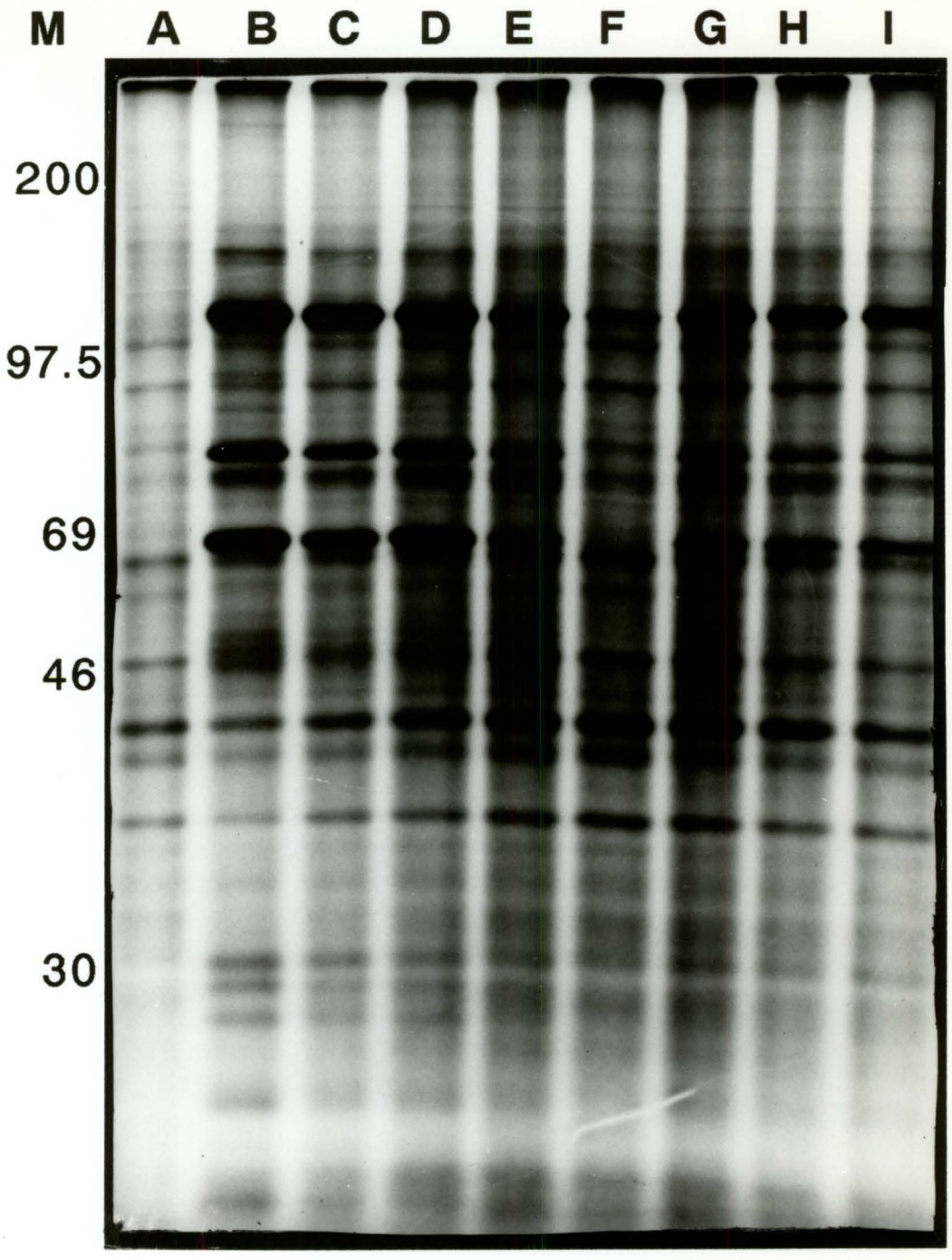
## III.V. Mutant Analysis

### III.V.1. Late Protein Synthesis

One of the functions attributed to the 55K protein is the ability to trigger host protein shutoff and assist in the accumulation of late viral mRNA in the cytoplasm. Mutants in the 55K protein which lack these abilities show reduced levels of late viral proteins and high levels of cellular proteins even at late times. To examine the abilities of the mutants to perform these functions HeLa cells were infected with wt or mutant Ad5 (including dl355 an E4 mutant provided by B. Panning) and the infection was allowed to continue for 36hrs.

Due to the apparent poor labelling of the mock control host protein shutoff is not evident, however the accumulation of viral late proteins can be evaluated. The wt virus (dl309) exhibited the greatest accumulation of late viral proteins (Fig.17 lane B). The 55K minus mutant (pm2019/2250) (McLorie et al 1990) seemed most severely impaired for viral late protein accumulation, even more severely than dl355 (Fig.17 lanes E and F). The three insertion mutants in3328(+), in3328(-) and in3328(-)(19K$^-$) seem equally impaired when compared to wt (Fig.17 lanes G, H, and I). Finally both pm490A/491A and pm490A/491(19K$^-$) seem to facilitate viral late proteins at almost wt levels (Fig.17 lanes C and D).

Figure 17: Analysis of viral late protein accumulation. HeLa cells were mock infected or infected with wt ad5 or a mutant adenovirus at a moi of 35pfu. After 35hrs the cells were labelled with $^{35}$S-methionine for one hour and harvested. Cellular extracts were then analyzed by SDS-PAGE. The lanes are: A mock; B wt ad5; C pm490A/491A; D pm490A/491A(19K$^-$); E dl355; F pm2019/2250; G in3328(-); H in3328(-)(19K$^-$) and I in3328(+).


M
A
B
C
D
E
F
G
H
I
200
97.5
69
46
30

III.V.2. Replication of Mutant Virus on HeLa and 293 Cells

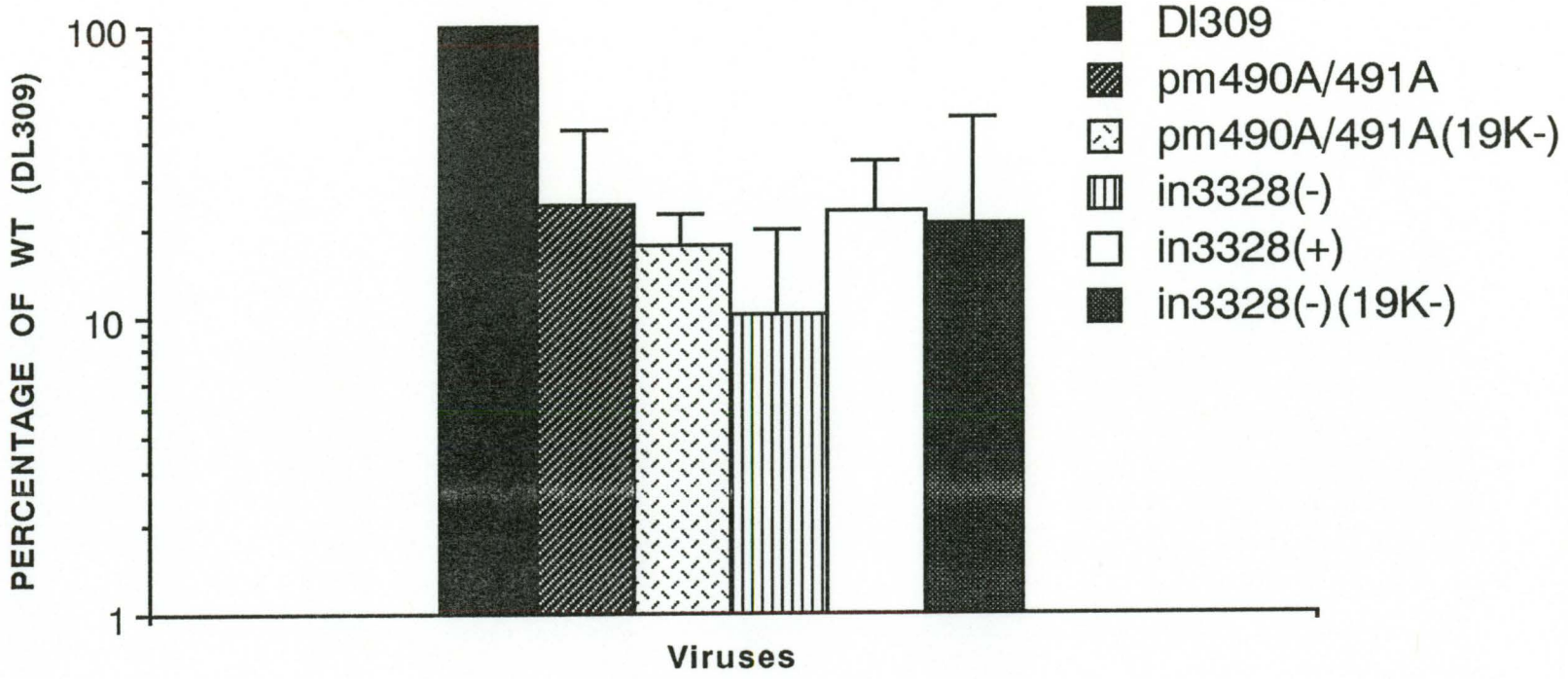
The 55K protein has been shown to be important for viral replication and plaquing efficiency. To examine the ability of pm490A/491A to replicate, viral stocks were titred on both HeLa cells and 293 cells which express the Ad5 E1 region constitutively and the ratio of the titre on Hela cells to that on 293 cells was measured. The ratio for the dl309 virus (wt) was taken as 100% and those of the other viruses were recorded as percentages of wt (Fig 18). The ratios for all the mutant viruses was 5-10 fold lower than wt. The in3328(-) virus was most severely reduced (10 fold) while pm490A/491A was the least impaired (approximately 5 fold) (Fig.18). The remaining viruses in3328(+), in3328(-)(19K$^-$) and pm490A/491A(19K$^-$) had similar reductions in their plaque forming abilities (Fig.18). These results suggested that the 55K protein produced by all these mutants were somewhat defective.

III.V.4. DNA Mediated Transformation

The ability of the 55K mutants to cooperate with E1A in DNA mediated transformation was also examined. For the purpose of these studies the plasmid pXC38 was used as a wild-type control. Transforming efficiencies of the various

Figure 18: Plaque formation by mutant and wt ad5. Assays were carried out 2-4 times and values presented represent averages of these experiments. Plaque formation on HeLa or 293 cells was determined in duplicate assays of several dilutions of virus. The standard deviation is shown by the error bars. The viruses are identified in the figure.

# Plaquing Efficiencies of Mutant Viruses



mutants were expressed as percentages of the level obtained with wild-type. Typical results of a transformation assay are depicted in Fig.19. The pXC38 plasmid yielded the highest number of colonies per μg of DNA. The plasmids pm490A/491A, in3328(-) and in3328(+) yielded intermediate results. The $19K^-$ mutant, pm1716/2072 yielded lower numbers of colonies while the pm490A/491A($19K^-$), in3328(-)($19K^-$) and in3328(+) ($19K^-$) plasmids produced virtually no colonies (Fig.19).

The data presented in Fig.20 represent the results of three experiments. The mutants pm490A/491A, in3328(-) and in3328(+) all yielded transformation at a level of 30-50% that obtained with wt pXC38 (Fig.20 top). As was shown previously (McLorie et al 1991) the plasmid pm1716/2072 which expresses 55K but no 19K consistently transformed at about 20% the level of wild-type pXC38 (Fig.20 top). The mutants in3328(-)($19K^-$), in3328(+)($19K^-$) and pm490A/491A($19K^-$) all transformed at very low levels, approximately 2-3% of the plasmid pm1716/2072 ($19K^-$ and wt 58K (Fig.20 bottom). These results indicated that mutation of serine 490/491 to alanine residues severely impaired the transforming ability of 55K.

Figure 19: Average numbers of colonies per dish is plotted versus μg of DNA for pXC38 (wt) and the various mutants in a DNA-mediated transformation assay. These are the results from one experiment and are typical of transformation assays. The plasmids are identified in the figure.

# Transformation of BRK Cells by the E1B Mutants

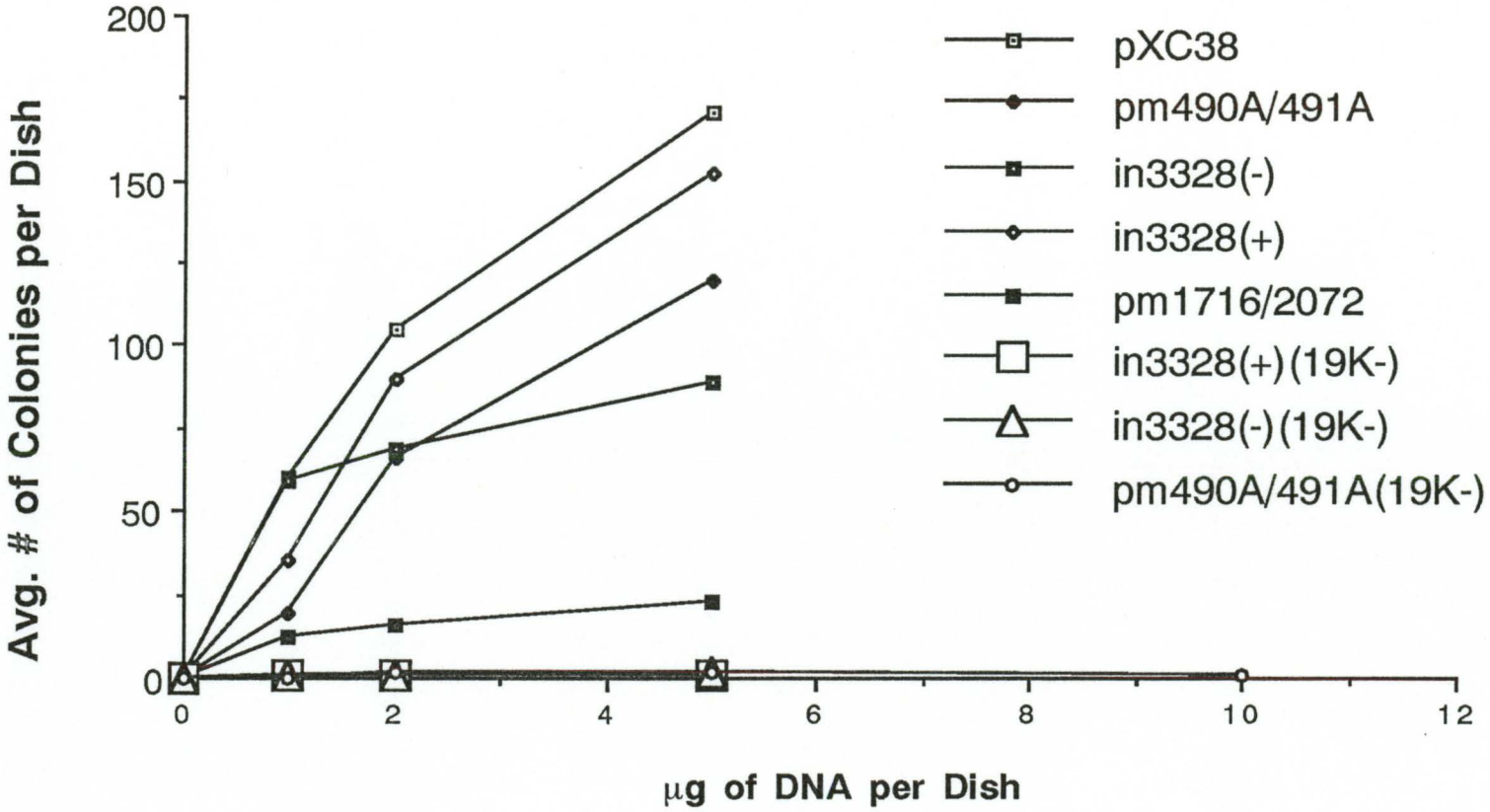
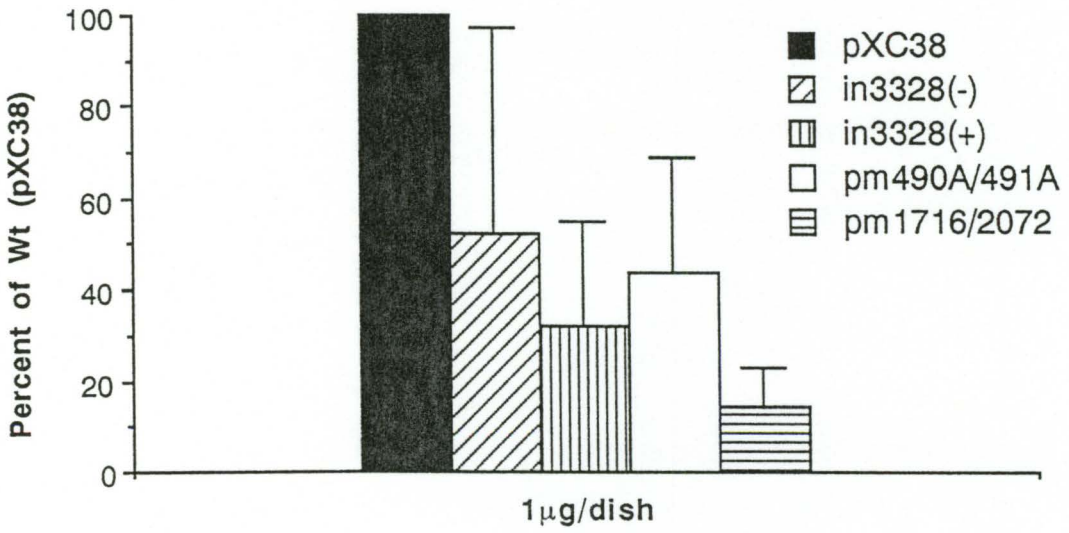
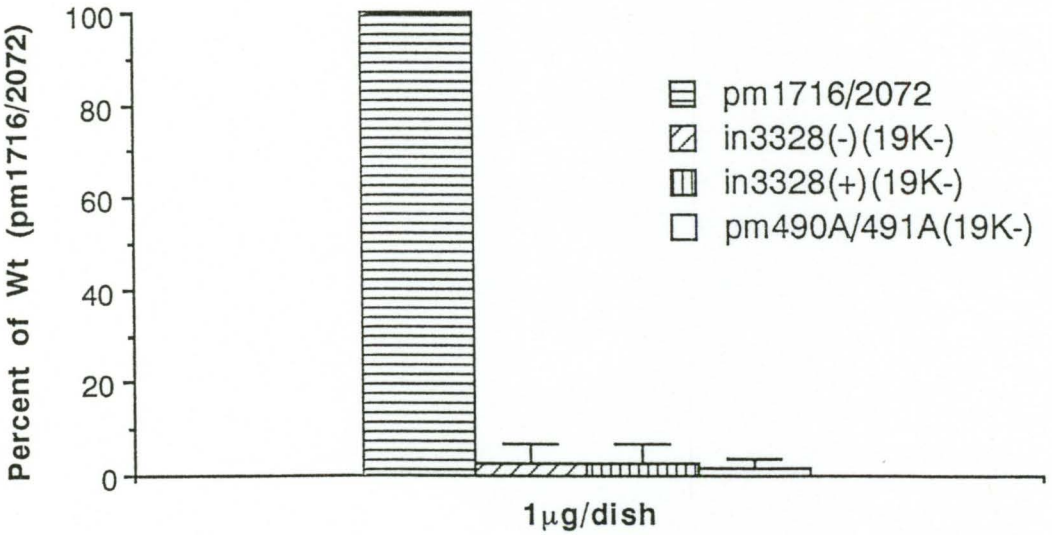


Figure 20: Transformation by wt (pXC38) and mutant plasmids. Results from a minimum of three experiments are expressed as a percent of wt (pXC38) in the upper figure. Values are averages and the standard deviation is shown by the error bars. In the lower graph the 19K$^-$ mutants are plotted as an average of pm1716/2019 (19K$^-$ background). Values are averages of a minimum of three experiments and the standard deviation is shown by the error bars.

## Transformation of BRK Cells by E1B Mutants




## Transformation of BRK Cells by E1B Mutants in 19K- Background



## IV. DISCUSSION

The purpose of this study was to elucidate the major sites of phosphorylation of the Ad5 E1B-55K protein and the significance of these sites in transformation and viral replication. Towards these ends two insertion mutants were produced [in3328(-) and in3328(+)] in an attempt to confirm previous data which suggested that the major sites of phosphorylation are located in the carboxy terminus (Takayesu et al). Specific point mutations which eliminated two putative sites of phosphorylation at serine 490/491 were also generated.

Previous studies had revealed that the 55K protein was phosphorylated at serine and threonine residues (Malette et al 1983). In addition it had been suggested that the 55K protein and the 156R protein share the same sites of phosphorylation (Takayesu et al). Since the 84R and the 93R proteins appeared not to be highly phosphorylated and were known to contain the amino terminal 79 amino residues of 55K and 156R, the potential location for the major 55K phosphorylation sites appeared to be in the last 77 amino acids. Within this region serine is present only in one tryptic peptide, T52 which contains two serines at residues 490 and 491, and a threonine

at residue 495.

In an attempt to confirm that T52 was indeed the major site of phosphorylation, two insertion mutants were created. Mutant in3328(-) encoded a 55K which was truncated after amino acid 438. This mutant proved unstable and could not be detected (Fig.7). The second insertion mutant in3328(+) inserted 11 residues between amino acids 438 and 439 (Fig.4). This mutant produced a detectable 55K product, however, levels were greatly reduced compared to wt (Fig.7). Thus these mutants were not suitable for future studies. Previous studies have shown that other insertions in 55K also produced unstable proteins (Yew et al 1990).

As discussed previously mutant pm490A/491A contains alanines in place of serines 490 and 491. It was necessary to eliminate both serine residues because studies of a similar site in the E7 protein of human papilloma virus showed that both were potential sites for phosphorylation by casein kinase II (Barbosa et al 1990). pm490A/491A produced levels of 55K similar to wt Ad5 but analysis of $^{32}$P-labelled 55K indicated that the levels of phosphorylation were lower in 55K from pm490A/491A, and the 55K migrated slightly faster on SDS-PAGE. Although phosphorylation of pm490A/491A 55K appeared reduced it was not eliminated and so additional sites clearly exist.

Previous studies had revealed that wt 55K produces two major $^{32}P$ tryptic peptides (Fig.15). When 55K from pm490A/491A was analyzed by HPLC both of these major peaks had been eliminated and a novel peak with different elution properties was present (Fig.16). There are several possible explanations for the origin of this new phosphopeptide. It may represent phosphorylation at another site on another tryptic peptide. However if this were the case, phosphorylation at serine 490/491 must regulate phosphorylation at this site as it was never detected in 55K synthesized by wt Ad5. It is also possible that this new phosphopetide is actually T52 phosphorylated at threonine-495. The replacement of serines 490/491 with two alanines could cause a shift in the elution of the peptide and explain its novel position. Analysis of wt 55K had shown that it contains both phosphoserine and phosphothreonine. With pm490A/491A, however, the 55K produced showed a predominance of phosphothreonine with some phosphoserine. Furthermore, the major phosphopeptide peaks produced by wt 55K contained both phosphoserine and phosphothreonine whereas the new peak found with pm490A/491A contained only phosphothreonine (Halliday et al). This suggested that the novel phosphopeptide of pm490A/491A may represent T52 phosphorylated at threonine-495.

Several functions associated with the 55K were examined to determine the importance of phosphorylation at serine 490/491. The insertion mutants created in this work were also tested for their functional abilities. These included the ability of 55K to facilitate host protein shutoff and viral late protein accumulation, to function in viral replication, and to cooperate with E1A in the transformation of BRK cells.

## IV.I. Viral Late Protein Accumulation

The mutants were examined after a 36hr infection for their ability to expedite viral late protein accumulation. It appears that phosphorylation at serines 490/491 plays a minor role, if any, in these functions as seen in Fig.17. Both pm490A/491A and pm490A/491A(19K$^-$) appear to have late viral protein accumulation comparable to wt ad5.

## IV.II. Viral Replication

The plaquing efficiencies of the mutant viruses were used as a measure of viral growth. The ratio of titre on HeLa cells to that on 293 cells with pm490A/491A was reduced to 25% of wt (dl309), and that of in3328(-) to approximately 10%. The remaining viruses had intermediate values. Others have also shown that viruses lacking a functional 55K protein have

reduced plaquing efficiencies (Yew et al 1990). It has been suggested that viruses with defective 55K are deficient for viral protein accumulation and accordingly viral growth is inhibited (Leppard and Shenk 1989). The reductions seen in Fig.18 may be a result of deficiencies in the host protein shutoff function or another related 55K function.

## IV.III. Transformation by pm490A/491A

One of the functions attributed to 55K that has received a great deal of study, has been its ability to cooperate with E1A to transform primary cells. Initial studies with pm490A/491A, in3328(-) and in3328(+) revealed that the mutants had reduced transforming ability compared to wt (pXC38) in a DNA mediated transformation assay. Recent studies by others, however, had shown that E1A is able to transform in cooperation with either 55K or the E1B-19K protein (McLorie et al 1991). In order to determine more accurately the effect of the serine 490/491 mutations, the mutants were rescued into constructs which fail to produce 19K. Thus transforming activity is entirely dependent upon 55K. pm1716/2072 which produces wt 55K transformed at levels of about 20% of wt. However, mutants pm490A/491A, in3328(-) and in3328(+) in the $19K^-$ background transformed at 2-4% of pm1716/2072 (Fig.20 bottom). The in3328(-) mutant was expected to transform at

reduced levels because it produces no detectable stable 55K product. The mutant in3328(+) produced very low levels of the 55K protein which may explain its reduced transforming ability. In addition others have shown that insertions in this region can affect transformation dependant upon 55K (Yew et al 1990). The pm490A/491A in the 19K$^-$ background, while producing almost the same level of 55K as wt, was severely deficient for transformation. These data suggested that phosphorylation of 55K at serine 490/491 may regulate its ability to cooperate with E1A in the transformation of primary cells.

The mechanism of transformation by 55K appears to involve interactions with the cellular p53 tumour suppressor (Kao et al 1990; Yew and Berk 1992). Correlations have been found between abilities of mutant 55K proteins to form complexes with p53 and their ability to transform cells. It has been shown that the central region of 55K is largely responsible for interaction with p53 (Kao et al 1990). Furthermore, a transformation defective mutant containing an insertion following Arg-443 of 55K, was found to complex with p53 but failed to inhibit p53 transcriptional activity. This result suggested that pm490A/491A and perhaps in3328(+) may both possibly be defective for transformation because of a deficit in the inactivation of p53 transcriptional activity.

## IV.IV. Conclusions and Future Considerations

In the future, experiments to determine whether pm490A/491A binds p53 and if so whether p53 transcriptional activity is impaired, may shed light on any regulatory roles that phosphorylation at serines 490/491 may have on 55K function. In addition, identification of the site of phosphorylation of the new phosphopeptide and potential minor sites towards the amino terminus should be pursued.

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