A STUDY ON THE FUNCTIONS OF THE E1B MINOR PRODUCTS OF ADENOVIRUS TYPE 5

A STUDY ON THE FUNCTIONS OF THE E1B MINOR PRODUCTS OF ADENOVIRUS TYPE 5

by

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ABSTRACT

The E1b transforming region of Adenovirus type 5 encodes minor products of 93R and 156R in addition to the more abundant proteins 176R, 496R, and 84R. The goal of this study was to elucidate the function of 93R and 156R to gain a better understanding of their role in oncogenic transformation and productive infection. Mutant viruses were constructed, whose normal splicing pattern was disrupted by point mutations in the 3' acceptor sites for the 1.26 and 1.31Kb mRNAs, which code for the 156R and 93R products, respectively. In the construction of these mutations, it was necessary to ensure that they did not affect the coding region for 496R. These mutants produced transformed foci in primary rat kidney cells with wild type efficiencies in DNA-mediated transformation assays. In the mutant designed to eliminate 156R, although the two wild type 156R species were absent, two new species running slightly faster on SDS-PAGE were detected. These proteins were recognized by sera specific to both the N- and C-termini of 496R, suggesting the utilization of an in-frame cryptic splice acceptor site. Use of this site probably resulted in the production of a mRNA encoding a modified 156R. These mutant proteins also seemed to be produced at the expense of 496R. The mutant designed to eliminate 93R grew with titres equivalent to wild type dl309, yet it was not clear whether a modified protein was produced in this case as well.

TO MY PARENTS WHO SUPPORTED ME

WITH THEIR LOVE AND UNDERSTANDING

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Ad2, 5, 12, etc.	Adenovirus type 2, 5, 12, etc
NH_4Cl	ammonium chloride
bp	base pairs
BRK	baby rat kidney
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CASA	casamino acids
cDNA	copy DNA
CsCl	cesium chloride
cpm	counts per minute
dATP, dCTP, dGTP, dTTP	deoxynucleotide triphosphate
dCMP	deoxycytosine monophosphate
DBP	DNA binding protein
ddH_2O	double distilled water
°C	degrees celsius
dl	deletion
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	dithiothreitol
E1, E2, E3, E4	early regions 1, 2, 3, and 4
EDTA	ethylenediamine tetracetic acid
et al.	and co-workers
EtBr	ethidium bromide
FCS	fetal bovine serum
Fig	figure
g	gram
HA	hemagglutination
HCl	hydrochloric acid
HS	horse serum
KCl	potassium chloride
K_2HPO_4	di-potassium hydrogen orthophosphate
KH_2PO_4	potassium phosphate
Kd	kilodaltons
LiCl	lithium chloride
M	molar
$MgCl_2$	magnesium chloride
$MgSO_4$	magnisium sulfate
μCi	microcuries
μg	micrograms
μL	microlitres

MEM	minimal essential medium
mg	miligrams
mL	mililitre
mM	milimolar
mmol	milimoles
moi	multiplicity of infection
mRNA	messenger RNA
mu	map units
MW	molecular weight
N	normal
N_2	nitrogen
ng	nanograms
NaCl	sodium chloride
Na_2HPO_4	di-sodium hydrogen phosphate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCS	newborn calf serum
OD	optical density
ORF	open reading frame
PAGE	polyacryamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol 8000
Pen-Strep	penicillin-strepomycin
pfu	plaque forming units
pmol	picomoles
pol	polymerase
pi	post infection
p-TP	preterminal protein
R	amino acid residue
Rb	retinoblastoma
RNA	ribonucleic acid
rpm	rotations per minute
S	sedimentation coefficient
^{35}S	sulfur-35
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
ssDNA	single stranded DNA
SSPE	saline sodium phosphate EDTA
SV40	simian virus type 40
TBE	tris borate buffer
TE	tris-EDTA buffer
T_H	hybridization temperature
tk	thymidine kinase
Tris	tris (hydroxymethyl) aminomethane

UV	ultra violet
V	volts
wt	wild type
w/v	weight per volume
^{32}P	phosphorus-32

INTRODUCTION

1.1 Adenoviridae

1.1.1 Adenoviruses

Human adenoviruses were first identified and isolated by Rowe *et al.* (1953) as a cytopathogenic agent from adenoids undergoing spontaneous degeneration in tissue culture. Rowe *et al.* (1953) proposed that this agent be called the "adenoid degeneration agent" or "A. D. agent", with the current name of Adenovirus being adopted in 1956 (Enders *et al.*, 1956). Adenoviruses were subsequently found to be the causative agents for other diseases such as acute respiratory diseases (Dingle *et al.*,1968), epidemic keratoconjunctivitis (Hogan *et al.*, 1942; Jawetz 1959), acute haemorrhagic cystitis (Numazaki *et al.*, 1973), and gastroenteritis (Fox *et al.*, 1969), among others. Interest in adenoviruses was kindled by the discovery that human adenovirus type 12 (Ad12) could cause tumours in newborn hamsters (Trentin *et al.*, 1962). This was the first demonstration of a human pathogenic agent inducing tumours in animals and in the years following this discovery it was shown that oncogenicity was a property of other Ad serotypes as well, in a number of different rodent species (Heubner *et al.*, 1962, 1965; Girardi *et al.*, 1964; Larsson *et al.*, 1965; Pereira *et al.*, 1965; Rabson *et al.*, 1964; Trentin *et al.*, 1968). Not all human adenovirus

serotypes can induce tumours in rodents but all are able to transform primary rodent cells in culture (Freeman *et al.*, 1967; McAllister *et al.*, 1969a,b), with the resulting transformed cell able to induce tumours upon injection into rodents, especially if the serotype is oncogenic or if the animal is immunosupressed or immunoincompetent (Branton *et al.*, 1985; Freeman *et al.*, 1967; Gallimore, 1972; Gallimore *et al.*, 1977) . To date it seems that human Ads have not been associated with tumours or other cancers in humans (Green *et al.*, 1980), thereby serving as a safe model system to study oncogenesis and other cellular processes.

1.1.2 Classification of Adenoviruses

The family Adenoviridae is divided into two genera, Mastadenovirus (mammalian) and Aviadenovirus (avian) (Norrby *et al.*, 1976). This classification is based on immunological properties of antigenic determinants on surface proteins of the virion capsid, such as the hexon, fibre, and penton polypeptides (Norrby and Wadell, 1969; Wadell and Norrby, 1969; Philipson *et al.*,1975; reviewed in Ishibashi and Yasue, 1984).

There are currently 42 serotypes of human adenoviruses, which are separated into different groups based on certain biological and structural properties. Originally adenovirus serotypes were subclassified into 4 groups based on hemagglutination (HA, the determinant being the fibre polypeptide) of rat and rhesus monkey erythrocytes (Rosen, 1960), and updated by Heirholzer

(1973). Other classifications have been based on G+C content (Pina and Green, 1965), oncogenicity (Green, 1970), molecular weights of polypeptides V, VI, and VII (Wadell, 1978), and DNA homology (Green *et al.*, 1979). Table 1.1 shows the overall classification of human adenoviruses. Briefly, the adenoviruses have been classified into the highly oncogenic group A, the weakly oncogenic group B, and the non-oncogenic groups C, D, E, F, and G. Most of the research to date has involved Ad2 and Ad5 from group C and Ad12 from group A. The present study was centered on Ad5, thus the remainder of this discussion will be concerned with this group C virus and the closely related Ad2 serotype.

1.1.3 Structure of the Virion

Adenoviruses are non-enveloped and possess an icosahedral shape, with 20 triangular surfaces and 12 vertices (Horne *et al.*, 1959). The virion is made up of at least nine different polypeptides (Maizel *et al.*, 1968). Some of these proteins associate to form the capsid, which is made of 252 subunits called capsomeres, of which 240 are trimeric "hexons" that make up the 20 surfaces and 30 edges and 12 are "pentons" that form the 12 vertices (Ginsberg *et al.*, 1966). The central surfaces or " facets" are made up of groups-of-nine (GON) hexons, which are released if the capsid is dissociated under gentle conditions (Prage *et al.*, 1970). Protein IX, which is encoded partially within Early Region 1b (see below), is the "glue" that holds the GON's together and is found as a trimer

TABLE 1.1: CLASSIFICTION OF ADENOVIRUSES

Sub-genus	Species	DNA			Apparent molecular weight of the major internal polypeptides			Hemagglutination pattern[3]	Oncogenicity in newborn hamsters
		Homology (%)[1]	G+C %	# of SmaI fragments[2]	V	VI	VII		
A	12,18 31	43-69 (8-20)	48	4-5	51-51.5K 46.5-48.5K[4]	25.5-26K	18K	IV	High (tumours in most animals in 4 months
B[5]	3,7,11,14, 16,21,34, 35	89-94 (9-20)	51	8-10	53.5-54.5K	24K	18K	I	Weak (tumours in few animals in 4-8 months
C	1,2,5,6	99-100 (10-16)	58	10-12	48.5K	24K	18.5K	III	nil
D[5]	8,9,10,13, 15,17,19, 20,22,23, 24,25,26, 27,28,29, 30,32,33, 36,37,38,39	94-99 (4-17)	58	14-16	50-50.5K[6]	23.2K	18.2K	II	nil
E	4	(4-23)	58	16-19	48K	24.5K	18K	III	nil
F	40	n.d.	n.d.	9	46K	25.5K	17.2K	IV	nil
G	41	n.d.	n.d.	11-12	48.5K	25.5K	17.7K	IV	nil

not classified: Ad 42

1 Per cent homology within the group and in brackets: homology with members of other groups

2 DNA fragments were analysized on 0.8-12% agarose gels. DNA fragment smaller than 400bp not resolved

3 I, complete agglutination of monkey erythrocytes ; II complete agglutination of monkey erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV agglutination of rat erythrocytes only after addition of heterotypic antisera.

4 Polypeptide V of Ad 31 was a single band of 48K

5 Only DNA restriction and polypeptide analysis have been performed on Ad 32 - Ad 39

6 Polypeptides V and VI of Ad8 showed apparent molecular weights of 45K and 22K respectively. Polypeptide V of Ad30 showed an apparent molecular weight of 48.5K.

Modified from Wadell (1980)

between adjacent hexons (Furcinitti *et al.*, 1989). The penton consists of a pentameric penton base polypeptide, from which projects a trimeric fibre, and 5 hexon molecules, which surround the base (J. van Oostrum and R.M. Burnett,1985).

1.1.4 Lytic Infection

The early events of lytic infection, from the introduction of the virus to the cell to the presence of "naked" viral DNA in the cell's nucleus, are completed within 2 hours at 37°C (Lonberg-Holm and Philipson, 1969). Within 30 minutes upon infection, potentially thousands of virus particles are attached to the cell via an interaction between the fibre of the virus and a specific cell membrane receptor (Lonberg-Holm and Philipson, 1969; Philipson, 1967; Wohlfart *et al.*, 1985). Attachment is soon followed by penetration of the cell by viropexis, or the formation of endocytic membrane vacuoles containing the virus (Chardonnet and Dales, 1970). The endocytic vesicle then ruptures, releasing the virus, probably due to a lowering of the pH, that results in an alteration of the virion surface, causing rupture (Pastan *et al.*, 1987). Release of the virus also results in a partial uncoating of the viral capsid as seen by an increased sensitivity to DNase and by density measurements (Lawrence and Ginsberg, 1967; Sussenbach, 1967). The uncoating seems to involve the loss of the penton capsomeres and occurs largely at the perinuclear region (Chardonnet and Dales, 1970; Dales and Chardonnet,

1973; Morgan *et al.*,1969; Sussenbach, 1967), with the entire process of penetration, release, and uncoating occurring with a half life of 15 minutes (Lonberg-Holm and Philipson, 1969). Further studies showed that actual transport to the nucleus most probably occurs by means of microtubules (Dales and Chardonnet, 1973; Luftig and Weihing, 1975). The uncoated virion, which is attached at the nuclear pore complex, releases its nucleoprotein core into the nucleus through the nuclear pores (Chardonnet and Dales, 1970; Chardonnet and Dales, 1972). The viral DNA may then associate with cellular histones, following loss of the core proteins, with the final structure appearing very much like the cellular nucleosome arrangement (Tate and Philipson, 1979), although these results have been recently challenged (Wong and Hsu, 1988).

The replicative cycle for adenoviruses is separated into early and late phases. The early phase commences 45 minutes post-infection (p.i.) with the transcription of E1a, while the other early regions (see below) are transcribed by 2-3 hours p.i., and many continue to be transcribed well into the late phase, which starts with the onset of viral replication, at about 6 hours p.i. (Nevins *et al.*, 1979). One virus cycle is completed in 32-36 hours.

1.1.5 Structure of the Genome

The Adenovirus genome is a linear, double-stranded, DNA molecule, with a molecular weight of 20-25 X 10^6 (Green *et al.*, 1967) and an approximate

length of 36,000 base pairs (Ads 2 and 5) (Akusjarvi *et al.*, 1984; Alstrom *et al.*, 1984; Gingeras *et al.*, 1982; Roberts *et al.*, 1984). The 5' end of each DNA strand is covalently linked to a 55Kd terminal protein via a dCMP phosphodiester bond, that is thought to, along with its 80Kd precursor, play a role in replication (Rekosh *et al.*, 1977; Robinson *et al.*, 1973).

Adenovirus transcripts are made from a series of temporally organized regions, which are transcribed by RNA polymerase II . These regions include immediate-early (E1a), delayed early (E1b, E2a, E2b, E3, and E4), intermediate (IVa2, and IX), and late (L1-5) regions on both the l and r strands (see Figure 1.1).

1.1.5.1 Early Region 1

Early region 1 (E1) corresponds to the left-hand 11.2% of the adenovirus genome and is responsible for the control of many viral and cellular processes (Figure 1.2). The E1 region is comprised of two transcription units on the r strand, E1a and E1b. E1a and E1b are independently promoted, with E1a extending from 1.3 to 4.5 and E1b from 4.6 to 11.2 map units (Berk and Sharp, 1977, 1978; Chow *et al.*, 1979; Wilson *et al.*, 1979).

In determining the portion of the adenoviral genome required for transformation of primary rodent cells, Gallimore et al (1974) found that the E1 sequences were the only sequences consistently expressed in transformed cell

Figure 1.1: Transcription Map of Group C Adenoviruses. A schematic representation of the Ad 5 genome, with transcription units from the r and l strands represented above and below the central lines, which are divided into 100 units. The messages are shown with a capped end representing the initiation site, an arrow representing the polyadenylation site, and gaps representing splicing. The messages designated E represent the early regions, while those designated L represent late regions. All the late messages are promoted from the major late promoter at 16.3 and contain the triparted leader indicated 1, 2, and 3. Polypeptides encoded on the messages are listed above and below their corresponding mRNA and are shown in either number of amino acids (R) or kilodaltons (K, molecular weight) or with a Roman numeral in the case of some of the late proteins.

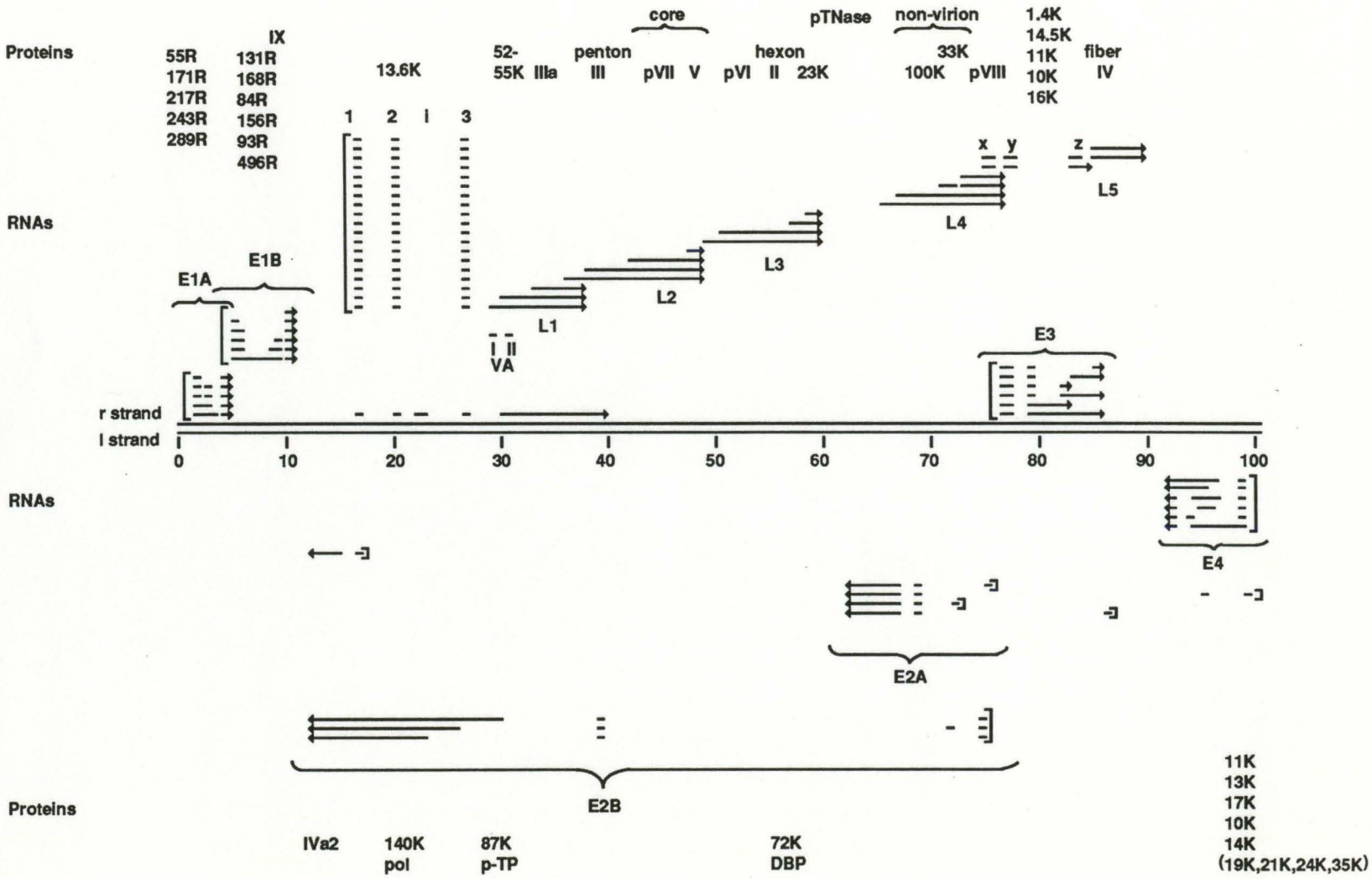
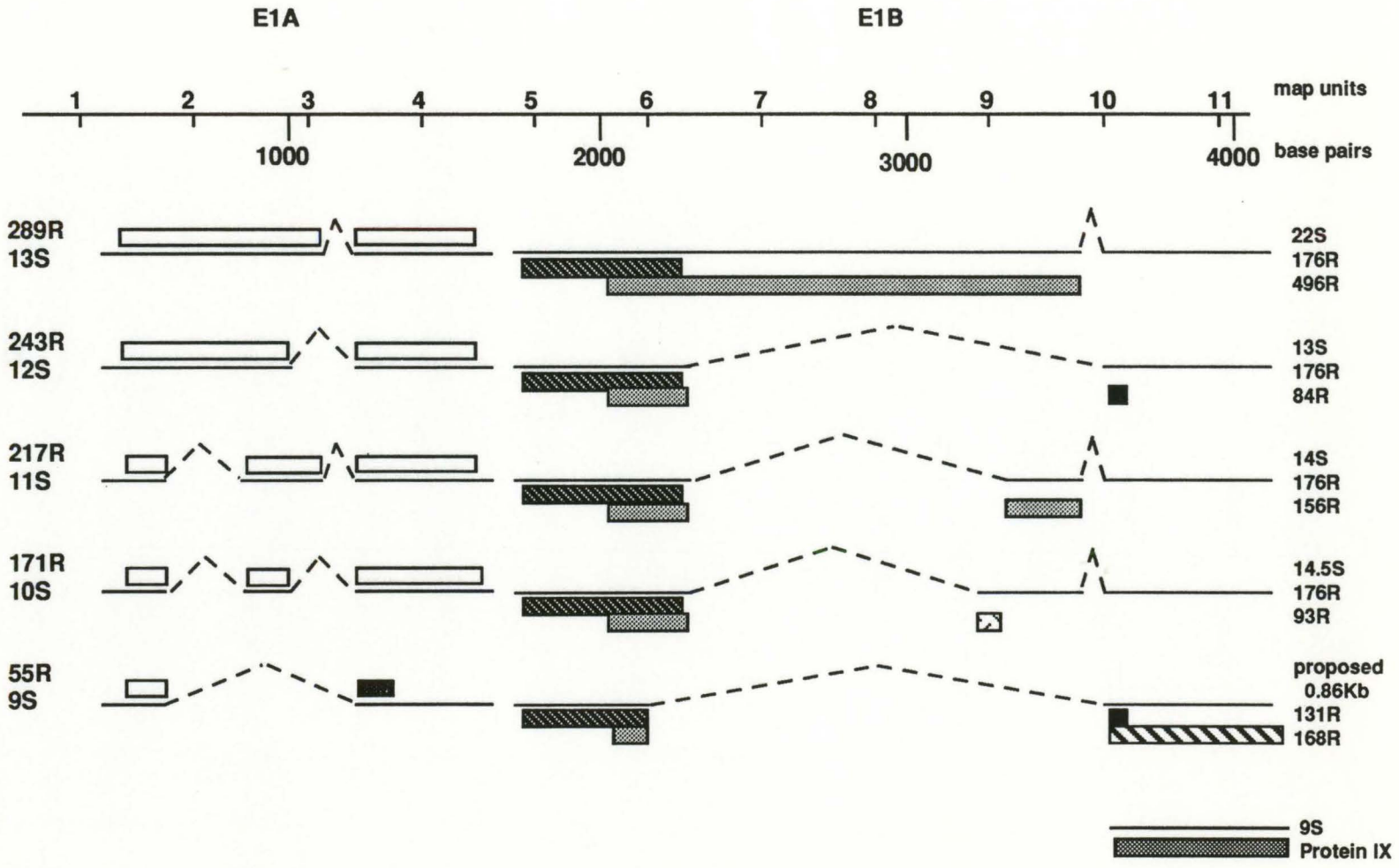

Proteins
IX
55R
131R
171R
168R
217R
84R
243R
156R
289R
93R
496R
13.6K
core
pTNase
non-virion
1.4K
14.5K
11K
10K
16K
52-
55K IIIa
penton
III
pVII V
hexon
pVI II 23K
33K
100K pVIII
fiber
IV
1 2 I 3
x y
z
L5
L4
RNAs
E1B
E1A
L3
L2
L1
I II
VA
E3
r strand
l strand
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A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

Figure 1.2: **The Adenovirus 5 E1 Transcription Unit. Shown are the independantly promoted E1a and E1b transcription units. The region on the genome is shown in both map units and base pairs from the left end. Open reading frames are shown by open or shaded boxes indicating different reading frames. The mRNAs are shown by solid lines, while spliced regions are indicated by the dashed lines. Beside the mRNAs and proteins are given the corresponding sedimentation values and residue numbers, respectively. For the E1b messages the splice donors and acceptors are given below: 22S (donor= 3510, acceptor= 3595), 13S (d= 2255, a= 3595), 14S (d1= 2255, a1= 3275, d2= 3510, a2= 3595), 14.5S (d1= 2255, a1= 3216, d2= 3510, a2= 3595),and 0.86Kb (d= 2090, a= 3595). Adapted from Ulfendahl et al., (1987) and Lewis and Anderson, (1987).**


E1A
E1B
1
2
3
4
5
6
7
8
9
10
11
map units
1000
2000
3000
4000
base pairs
289R
13S
22S
176R
496R
243R
12S
13S
176R
84R
217R
11S
14S
176R
156R
171R
10S
14.5S
176R
93R
55R
9S
proposed
0.86Kb
131R
168R
9S
Protein IX

lines. The E1 region was also determined to be the only sequences required for DNA mediated transformation (Graham *et al.*, 1974b) Specifically, it was found that the Hind III-G fragment, which encompasses E1a and part of E1b (approx. 8% of the genome), was sufficient for oncogenic transformation in DNA mediated assays (Graham *et al.*, 1974a; Schrier *et al.*, 1979; Shiroko *et al.*, 1977; Yano *et al.*, 1977). Because of the association of region E1 with oncogenic transformation, much research has been conducted on this region to elucidate the function(s) of proteins encoded by E1 and to determine their role in transformation.

1.1.5.2 Early Region 1a

As outlined above, E1a proteins are the first viral gene products to be expressed. Through alternative splicing (see Figure 1.2), the E1a region produces five mRNAs, which sediment at 13S, 12S, 11S, 10S, and 9S (Berk and Sharp, 1978; Perricaudet *et al.*, 1979; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). The 13S and 12S messages predominate and produce two highly related gene products of 289 and 243 residues (R), respectively, differing only by the 46 amino acid unique region in the 13S product (Perricaudet *et al.*, 1979). These two proteins, located in the nucleus of infected cells (Feldman and Nevins, 1983; Schmitt *et al.*, 1987; Yee *et al.*, 1983), run on a SDS-polyacrylamide gel as a family of heterogeneous polypeptides due to differences in phosphorylation

(Richter *et al.*, 1988; Tremblay *et al.*, 1989, 1988; Yee *et al.*, 1983) These proteins have nominal molecular weights of 52 and 48.5Kd for the 13S product and 50 and 45Kd for the 12S product (Rowe *et al.*, 1983; Smart *et al.*, 1981; Yee and Branton, 1985a). The 11S and 10S messages encode proteins of 35 and 30Kd, respectively, which are produced later in infection (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). The 9S message splices into a different reading frame to produce a protein of 6.1Kd, with a novel C-terminus (Virtanen and Pettersson, 1983).

Proteins from the E1a region have a number of functions in lytic infection. One of the primary functions is the transactivation of other genes, mainly the other early viral genes, including E1a itself (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981). The transactivation region is located mainly in the unique region (conserved region 3) of the larger 13S product, which is both necessary (Culp *et al.*, 1988; Jelsma *et al.*, 1988) and sufficient for transactivation (Lillie and Green, 1989). E1a products are also able to repress transcription (Borelli *et al.*,1984), with the 12S product being the major protein involved (Lillie *et al.*, 1986).

The role of E1a in transformation has been extensively studied and although E1a on its own is incapable of fully transforming primary rodent cells, it possesses an immortalizing function (Houweling *et al.*, 1980). To obtain fully transformed cells, E1a must cooperate with another oncogene, such as E1b or Ha-ras, or other viral genes, such as polyoma middle-T (Land *et al.*, 1983; Ruley,

1983). If, however, E1a is expressed at high levels in the cell, growth properties and morphology of fully transformed cells have been reported in established cell lines (Senear and Lewis, 1986), although these cells were not tested for tumourgenicity.

E1a is also known to be associated with certain cellular proteins of 65, 68, 105, 107, and 300Kd (Egan *et al.*, 1987,1988; Yee and Branton, 1985b). The identities of these proteins are unknown, with the exception of the 105Kd protein, which was identified as the product of the recessive oncogene Rb-1 (Whyte *et al.*, 1988), whose binding to E1a products seems to be required for transformation by adenoviruses (Egan *et al.*, 1989;Jelsma *et al.*, 1989).

1.1.5.3 Early Region 1b

Products of the E1b transcription unit (Figure 1.2) are expressed from two overlapping open reading frames (ORFs). The nucleotide and residue numbers given below are for Ad5, although details on the minor messages were originally obtained from the Ad2 system. The first ORF, which starts at nucleotide 1714, encodes a protein 176 residues (R). The second ORF encodes a protein of 496R, which starts at nucleotide 2019, and which is encoded on a different reading frame (Bos *et al.*, 1981; Gingeras *et al.*, 1982). The 176R protein migrates on SDS-PAGE as a 19Kd doublet in SDS-polyacrylamide gels (Rowe

et al., 1983; McGlade *et al.*, 1987), while the 496R proteins runs at 58Kd. These species are often referred to as the 19K and 58K proteins. Two major E1b mRNA species of 22S (2.2 Kb) and 13S (1.0 Kb) exist, which share common 5' and 3' termini as a result of alternative splicing of a common precursor (Berk and Sharp, 1978; Chow *et al.*, 1979; Kitchingham and Westphal, 1980; Perricaudet *et al.*, 1980). The 22S and 13S messages are under temporal control, with the 22S message being the most abundant E1b message early in infection, with the 13S message becoming the predominant message at later times (Spector *et al.*, 1978). This temporal control seems to be regulated at the level of RNA splicing (Montell *et al.*, 1984). While the 496R protein is encoded only by the 22S mRNA, the 176R product is encoded by both of the major E1b messages (Bos *et al.*, 1981; Esche *et al.*, 1980). Other E1b messages that encode 176R include two minor mRNAs of 1.26 Kb and 1.31 Kb, which also encode proteins of 156R and 93R, respectively. The 156R protein shares both amino and carboxyl termini with the 496R, while the 93R protein possesses the amino terminus of 496R and a novel carboxyl terminus due to splicing into a different reading frame (Anderson et al.,1984; Lewis and Anderson, 1987). The 13S message also encodes an 84R protein that shares the same amino terminus as 496R, 156R and 93R, while splicing into a third reading frame, yielding a different carboxyl terminus from 496R (Green *et al.*, 1982; Lucher *et al.*,1984; Matsuo *et al.*, 1982). There may exist a fourth mRNA of 0.86 Kb, which, like the other messages, starts at nucleotide 1701 (Baker and Ziff, 1981), but uses a

different splice donor to produce possible 176R-and 496R-related products (Lewis and Anderson, 1987). Within the region encompassed by E1b is found the gene for protein IX, which is transcribed as a 9S mRNA from its own promoter and is expressed late in infection (Pettersson and Mathews, 1977).

The 176R protein has been found to be acylated (McGlade *et al.*, 1987) and phosphorylated (McGlade *et al.*, 1989). Immunofluorescence has shown 176R to be subcellularly localized to the nuclear membrane and perinunclear region (McGlade *et al.*,1987), as well as with intermediate filaments, which, along with the nuclear lamina, are disrupted upon transient expression of the 176R protein (White and Cipriani, 1989). The 176R product plays an important role in viral growth as was shown by complementation of E1b deletion viruses by fragments containing 176R but not 496R (Klessig *et al.*, 1982) and by a mutation in this region that is reduced for late polypeptide synthesis (Pilder *et al.*, 1984). Mutations in the coding region for 176R display interesting phenotypes. First of all, such mutants cause rapid cell lysis and a cytopathic effect (cyt phenotype) (Pilder *et al.*, 1984; Subramanian *et al.*, 1984; Takamori *et al.*, 1968; White *et al.*, 1984, 1987) that is associated with the formation of large plaques (Chinnadurai, 1983; Subramanian *et al.*, 1984). Secondly, mutants in 176R are associated with the degradation of viral and cellular DNA (deg phenotype) (Ezoe *et al.*, 1981; Lai Fatt *et al.*, 1982; Pilder *et al.*, 1984; White *et al.*, 1984). From these and other data, 176R has been implicated in the stabilization or protection of newly replicated viral DNA and thus directly or

indirectly prevents degradation by a cellular nuclease, allowing increased expression of viral genes (Herrmann and Mathews, 1989). Finally, it seems that 176R may have a transactivation function as well that may affect a number of cellular and viral promoters including E1a and E1b, itself (Herrmann *et al.*, 1987; Jochemsen *et al.*, 1987; Yoshida *et al.*, 1987).

The 496R protein is a phosphoprotein (Malette *et al.*, 1983) located in the nucleus and the perinuclear region (Rowe *et al.*, 1983) and has been implicated in a number of viral processes. Firstly, the 496R product appears to affect the transport to and accumulation of viral messages in the cytoplasm by facilitating transport of or by stabilizing late viral mRNAs, while having the opposite effect for cellular messages (Babiss et al., 1985; Pilder *et al.*, 1986). The 496R protein also plays a role, although poorly understood, in the shut-off of host protein synthesis (Babiss and Ginsberg, 1984). Finally, 496R is known to complex to a number of cellular and viral proteins, the most notable being the cellular protein p53 (Sarnow *et al.*, 1982) which has been suggested to be an anti-oncogene like Rb1 (Finlay *et al.*, 1988, Hinds *et al.*, 1989). The 496R protein from Ad 5 physically binds to p53 and concentrates in a "cytoplasmic body" consisting of a cluster of 8nm filaments (Sarnow *et al.*, 1982; Zantema *et al.*, 1985a,b), which localize as DNase sensitive structures in discrete areas of the nucleus and perinuclear structures that include centrosomes (Zajdel and Blair, 1988). Interestingly, the equivalent Ad 12 485R has not been shown to associate physically with p53 even though p53 is present at high levels in the nucleus of

transformed cells (Grand and Gallimore, 1984), suggesting some sort of stabilizing effect. The p53 protein is also known to bind to SV40 large T antigen (Sarnow *et al.*, 1982). In addition to p53, 496R also binds to a 34Kd product from the adenovirus E4 region (Sarnow *et al.*, 1984) and this complex may be important for the accumulation of late viral messages as well as host protein shut-off (Halbert *et al.*, 1985). The binding sites of these proteins on 496R are unknown, although the binding site of p53 on SV40 large T has been identified (Scmieg and Simmons, 1988).

Unlike E1a, E1b polypeptides cannot independently transform primary rodent cells or established rat cell lines, although 176R or 496R encoded on plasmids cooperate with E1a plasmids to transform primary cells (Logan *et al.*, 1984; Solnick and Anderson, 1982; van den Elsen *et al.*, 1983). In fact, not much is known concerning E1b products and transformation except that both 176R and 496R (or at least the amino terminus) are required (Babiss *et al.*, 1984; Barker and Berk, 1987; Bernards *et al.*, 1986; Chinnadurai, 1983; Edbauer *et al.*, 1988; Logan *et al.*, 1984; McLorie *et al.*, 1990, submitted for publication; Takemori *et al.*, 1984), although it has also been suggested that either 176R or 496R alone with E1a can transform cells at a reduced level (Bernards *et al.*, 1986; McLorie *et al.*, 1990, submitted). Some studies using host range II (hrII) mutants, however, suggested that the 496R protein of E1b may be dispensible in DNA mediated transformation (Rowe and Graham, 1983). The reason for this discrepancy remains unclear. From the above and other data, it has been

suggested that 176R and 496R function to transform cells by independent (McLorie *et al.*, 1990, submitted; White and Capriani, 1990) but cooperative pathways (McLorie *et al.*, 1990, submitted).

The role of the minor 496R-related products in lytic infection or oncogenic transformation remains unclear. Some indirect studies have suggested that these proteins play little or no role in the above processes. For example, Montell *et al.* (1984) mutated the splice donor site that creates the messages that produce the 84R, 93R, and 156R proteins and found that the mutant was not defective for productive infection of HeLa cells nor was it defective for transformation of primary rat cells or CREF cells. These data suggested that the minor products are of no functional importance . Montell *et al.* (1984) also noted, however, that cryptic splice donors were used and thus modified yet functional proteins may still have been produced. Thus the exact form of these proteins may not be important for their function (Lewis and Anderson, 1987). Also, the studies using the Hind III-G fragment suggest that at least the amino terminus is important in transformation. From the above data, it has been suggested that perhaps the minor E1b proteins function to vary the expression of the common amino terminal of 496R throughout the cell by differential localization (Lewis and Anderson, 1987).

Although the function of E1b products in transformation is unclear, it is thought that the products of E1a somehow are able to immortalize cells, while E1b functions are required to establish and maintain the fully transformed

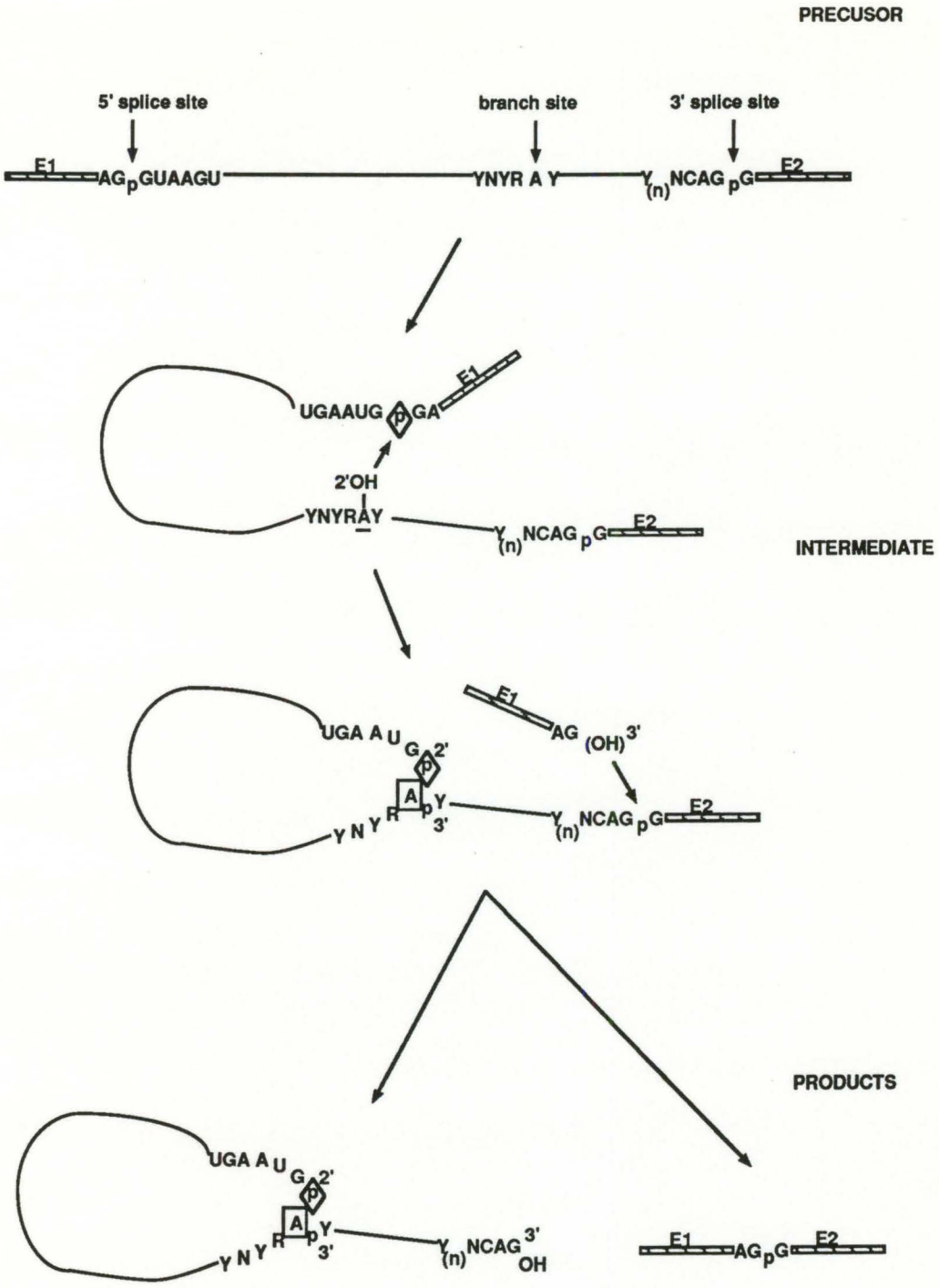
phenotype (Branton *et al.*, 1985). Also, it is unclear whether 496R (and perhaps the minor products) functions in transformation by simply binding to p53 or by more complex processes. At any rate much more study is required before the understanding of E1b functions reaches the same level as is found with E1a.

1.2 Eucaryotic mRNA Splicing: A Background

Splicing of eucaryotic messages was first discovered in the Adenovirus 2 system by RNA/DNA hybridization and electron microscopy (Berget *et al.*, 1977). Adenoviruses were used to study splicing as the genome was small enough to be easily worked with and it was known that during late stages of infection long viral RNA was synthesized, so it was thought that viral mRNA regulation may be similar to the cellular counterpart (Sharp, 1987). Soon after this discovery in the adenovirus system, it was revealed that cellular genes are indeed post-transcriptionally regulated by RNA splicing (Chambon, 1977). The descriptions of the adenovirus messages above reveal just how complex splicing of RNA can be (Sharp, 1987).

Splicing of RNA is thought to be carried out with the formation of an intermediate followed by excision of the intron and ligation of the exons (see Figure 1.3). All introns are said to have conserved sequences at their 5' and 3' termini or splice sites. These sequences will always have a Guanosine-Uracil (GU) at the 5' end and an Adenosine-Guanosine (AG) at the 3' terminus.

Figure 1.3: **Mechanism of mRNA splicing. The precusor is shown with the 5' splice site , 3' splice site, and branch site sequences labeled. On either side of the intron are the 5' and 3' exons (E1 and E2, respectively). Consensus sequences are shown at the various sites: A= adenosine, G= guanosine, C= cytosine, U= uracil, Y= pyrimidine, R= purine, and N= any nucleotide. Progression from step to step and as well as nucleophilic attacks on phosphodiester bonds by free hydroxyl groups (OH) are shown by arrows. The intermediate molecules are diagramed in the middle, while at the bottom are the two products of splicing: the discarded intron lariat and the spliced exons. Adapted from Sharp, (1987).**


PRECUSOR
5' splice site
branch site
3' splice site
E1
AGpGUAAGU
YNYR A Y
Y(n)NCAGpG
E2
UGAAUG
p
GA
2'OH
YNYRAY
INTERMEDIATE
UGA A U G
2'
A
R
pY
3'
Y N Y
AG
(OH)3'
PRODUCTS
OH

Splicing starts by the formation of a branch through a 2'-5' phosphodiester bond between the 5' terminal guanosine and an adenosine within the intron, 20-50 nucleotides upstream of the 3' splice site (Grabowski *et al.*, 1984; Reed and Maniatis; Ruskin *et al.*, 1984). This structure is generally called the lariat intermediate. Formation of the branch and excision of the exon are soon followed by cleavage at the 3' splice site and ligation of the exons and release of the lariat intron (Sharp 1987). Splicing occurs through the formation of the spliceosome or splicing body made up of a number of particles called small nuclear ribonucleoprotein (snRNP) particles named U1, U2, U4, U5, and U6 (Busch *et al.*, 1982; Lerner *et al.*, 1981). The presence of and recognition of sequences on the RNA, by the snRNPs, are critical for splicing (Sharp, 1987).

1.3 Rationale and Approach to Study

At the onset of this study, very little was known of the functions of the 496R related products 156R, 93R and 84R, since little or no studies had been centered on these proteins other than their identification. It was thought that two approaches could be used to attempt to elucidate the function of the minor products. The first included the construction of the appropriate cDNA virus to study to what extent the various minor proteins could substitute for 496R. This approach still remains to be pursued. The second approach was to attempt to

disrupt splicing of the minor messages by mutating the splice acceptor site in the appropriate message. This approach was adopted in order to answer the question whether eliminating these messages, and thus the proteins, had any effect on viral functions, particularly those functions normally attributed to 496R. This seemed the approach to take given the availability of good mutagenesis techniques and the development of efficient virus rescue protocols.

Materials and Methods

2.1. Bacterial Strains

Escherichia coli strains CJ236 (dut$^-$, ung$^-$, thi$^-$, rel A$^-$; pCJ105(Cm^r)) and MV1190 (delta(lac-pro AB), thi, sup E, delta(srt-rec A)306::Tn 10(tet^r)[F':tra D36, pro AB, lac I^{q}ZdeltaM15]) were obtained from Bio-Rad Laboratories and used in the M13 site-directed mutagenesis.

Escherichia coli strain LE392 (F$^-$ supE44, supF58, lacY1, galT22, metB1, trpR55, hsdR514($R_k^-M_k^+$)) was used to grow the mutagenic plasmids.

2.2. Bacterial Culture

2.2.1 Liquid Culture

All strains were grown in Luria-Bertani (LB) broth (10g tryptone, 5g yeast extract, 10g NaCl per litre distilled water, autoclaved) with the following exceptions or additions. For growth of CJ236 chloramphenicol (30 μg/ml final concentration) was added or for growth of LE392 containing pXC38 ampicillin (10-20 g/ L) was added. Overnight (preparatory) cultures of MV1190 were grown in glucose-minimal salts media (6g K_2HPO_4, 3g KH_2PO_4, 1g NH_4Cl, and 0.5g NaCl per litre distilled water, after autoclaving and cooling, addition of 20mL

20% CASA, 10 mL 20% glucose, 1mL 1M $MgSO_4$, 100μL 1M $CaCl_2$, 100μL 1% vitamin B1, and 10 μg/mL final volume tetracycline). All cultures were incubated at 37°C with shaking.

2.2.2. Solid Culture

LB plates were prepared by adding 15g Bacto agar to 1 litre LB broth (above). After autoclaving and allowing to cool to 42°C, the media was supplemented with chloramphenicol (for growth of CJ236) or ampicillin at the concentrations listed above. For growth and short term storage of MV1190, plates of glucose-minimal medium was prepared by preparing the media as above with the addition of 15g agar per litre prior to autoclaving. For both types of plates the agar medium was poured into Fischer plastic petri dishes and, after allowing the agar to solidify, were stored at 4°C. For short term storage, cultures were streaked on the appropriate medium, inverted and incubated overnight at 37°C, after which plates were placed at 4°C and colonies picked and grown up as needed.

2.2.3. Culture Storage

150μL of glycerol was added to 850μL of an overnight liquid culture in a 4 mL glass vial and stored at -70°C. When required, cultures were started by scraping the surface of the frozen stock with a sterile inoculating loop and transferring to a liquid culture.

2.3. M13 Mutagenesis (Taken from the Bio-rad Muta-Gene^{tm} in vitro Mutagenesis Kit Instruction Manual, 1987 as taken from Zoller and Smith (1983) and modified by Kunkel, 1985)

2.3.1. Preparation of Competent Cells

MV1190 was streaked on glucose minimal media plates and incubated at 37°C, until well defined colonies appeared. For inoculant for either competent cell production or transformation, the day before the procedure was to be done 10mL of glucose-minimal medium was inoculated with a single MV1190 colony and incubated overnight with shaking. 200-250mL of LB broth was inoculated with the overnight culture to give an initial absorbence reading (A_{590}) of 0.1 and incubated at 37°C with shaking. After the culture reached an absorbence (A_{590}) of 0.8-0.9, the culture was harvested at 0°C for 5 minutes at 5000 rpm. The pellet was resuspended in 50mL cold 100mM $CaCl_2$, using a pre-chilled pipet. The cells were again harvested by centrifugation and resuspended in 10mL 100mM $CaCl_2$, after which 100mL 100mM $CaCl_2$ was added. The resuspension was kept on ice for 30-90 minutes and spun down, drained and resuspended in 12.5mL 85mM $CaCl_2$, 15% glycerol. Aliquots of 0.3 mL were prepared and frozen in liquid N_2 and stored at -70°C where competence could be retained for 6-9 months. The aliquots were thawed on ice for use.

2.3.2. Transformation with M13

0.3mL of competent cells were thawed on ice. 1-10ng of a cloned M13 ligation reaction or 3-10μL of a synthesis reaction after dilution with TE (see below) was added. The mixture was gently mixed and held on ice for 30-90 minutes. The cells were then heat shocked at 42°C for 3 minutes and returned to ice. Transformation of LE392 by plasmids was performed in the same manner.

2.3.3. Plating of Transformants

Plating of transformants was done immediately following transformation. The transformed cells were mixed well and 10, 50, or 100μL was added to 0.3mL of an MV1190 overnight culture (prepared as described above) in a sterile 13 x 100 mm test tube. Next, 50uL of 2% X-gal which had been dissolved in dimethyl formamide and 20μL of 100mM Isopropyl-1-thio-ß-D-galactoside (IPTG) was added to 2.5mL of LB top agar (0.7% agar) that had been cooled to 50°C. This mixture was then added to the transformed cells, which were then mixed and immediately poured on LB plates. After the top agar cooled, the plates were inverted and incubated at 37°C overnight. The following morning the resulting plaques were picked by either inserting a sterile glass pipet into the plaque and blowing the plug into 150μL of TE buffer (10mM Tris-HCl, 1mM Ethylene-diamine Tetraacetic acid (EDTA), pH 8.0) or by touching the tip of a sterile wooden rod to the plaque and transferring the phage to the same amount of TE buffer by vigourous shaking. Clear plaques were picked over blue plaques as they

represented M13 phage with inserts as opposed to those without inserts. The phage was then titred and screened for the insert (see below).

2.3.4. Titering the M13 Phage

Serial dilutions, ranging from 10^{-2} to 10^{-10} in powers of 2, of the cloned M13 phage were prepared. 15μL of each dilution was added to 0.3mL competent cells and 2.5mL LB top agar (as described previously) and pour plated on LB plates. After the agar solidified, the plates were inverted and incubated at 37°C overnight. The following morning the plaques were counted and the titre calculated as pfu/mL.

2.3.5. Growth of Uracil-Containing Phage

50mL LB broth with 30μg/mL chloramphenicol (150μL 10mg/mL stock) in a 500mL Erlenmeyer flask was inoculated with 500μL overnight culture of CJ236 and incubated at 37°C with shaking. Approximately 6 hours later 50μL of cloned M13 phage was added and the infection was allowed to continue for no longer than 6 hours (usually 4-5 hours) at 37°C with shaking. After this time the culture was harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C in a Sorvall SS34 rotor. One quarter (1/4) volume 3.5M ammonium acetate, 20% Polyethylene glycol 8000 (PEG), was added to the supernatent and the mixture was added to a fresh centrifuge tube and held on ice for at least 30 minutes or overnight at 4°C. After the incubation the phage was pelleted by centrifugation

at 10,000 rpm for 15-20 minutes at 4°C (Sorvall). The supernatent was poured off carefully and the tube was drained thoroughly and the pellet resuspended in 100-200μL TE buffer. The stock is then titred on MV1190 and CJ236 and the efficiency of titre on each was compared. The efficiency of titre on MV1190 had to be at least 10^4 fold less than on CJ236.

2.3.6. Extraction of DNA

2.3.6.1 Large Scale Preparation of RF DNA

50mL LB broth was inoculated with 500μL MV1190 and 500μL (10μL per mL medium) stock cloned M13 phage and incubated at 37°C with shaking for no longer than 6 hours. After incubation the culture was harvested by centrifugation at 10,000 rpm for 10-15 minutes at 4°C. The supernatent was poured off and saved for single-stranded DNA (ssDNA). The pellet was resuspended in 1mL solution I (5mg/mL lysozyme (Boehringer Mannheim), 50 mM glucose, 10mM EDTA, and 25mM Tris-HCl pH 7.6). The resuspension was allowed to sit at room temperature for 5 minutes and transferred to a fresh corex tube. To the resuspension 2mL of freshly prepared solution II (0.2N NaOH, and 1% Sodium Dodecyl Sulfate (SDS)) was added and mixed gently. To the above 1.5mL solution III (3M Sodium Acetate (NaOAc) pH 4.8) was added, mixed gently, and placed on ice for 10 minutes. The mixture was centrifuged at 10,000 rpm for 20 minutes at 4°C (Sorvall) and the supernatant was transferred to a clean corex tube. To the

supernatent was added 0.6 volume cold isopropanol. The mixture was mixed and set at room temperature for 15 minutes. The DNA was recovered by centrifugation at 10,000 rpm for 25 minutes at room temperature (Sorvall) and the pellet was washed in cold 70% ethanol. The pellet was dried and resuspended in 200μL TE buffer and transferred to an Eppendorf tube. To the Eppendorf tube was added 400μL cold 5M LiCl, the contents were mixed, and allowed to stand on ice for 30 minutes. The supernatent was recovered and 0.1 volumes NH_4OAc, 3 volumes ethanol were added, and the DNA recovered by centrifugation in a microfuge (Microspin 24, Sorvall). The DNA pellet was resuspended in 80μL TE buffer and 20μL RNAse (10 mg/mL) and incubated at 37°C for 20 minutes. To this was added an equal volume 13% PEG, 1.6M NaCl and the contents held on ice for 30 minutes or overnight at 4°C. The DNA was then extracted with phenol twice and precipitated with 2 volumes cold ethanol. The pellet was resuspended in 50-200μL TE buffer. An aliquot of the resuspension was taken and analyzed with the appropriate enzymes and gel electrophoresis.

2.3.6.2. Small Scale Preparation of RF DNA

1.5-3.0mL of LB broth was inoculated with 15-30μL overnight culture of MV1190 and 15-30μL cloned M13 phage and incubated at 37°C with shaking for no longer than 6 hours. Cells were harvested by centrifugation and the pellet resuspended in 100μL solution I and incubated on ice for 30 minutes. At this point 200μL solution II was added and incubated on ice for 5 minutes at which

point 150μL solution III was added, after which the mixture was held on ice for an additional 60 minutes. The solution was then centrifuged at room temperature in a microfuge and 400μL of supernatent was removed and transferred to a new sterile Eppendorf tube. One mL cold ethanol was added and the tube placed at -70°C for 15 minutes, after which the contents were spun down in a microfuge at 4°C and the supernatent discarded. The pellet was resuspended in 150μL sterile double distilled water (ddH_2O) and phenol/chloroform extracted 3 times and ethanol precipitated (as above). The resulting pellet was dissolved in 500μL 0.1M NaOAc, 0.5M Tris-HCl pH 8.0 and 2 volumes ethanol were added and the mixture placed at -70°C. The DNA was pelleted by centrifugation 15 minutes at 4°C and the supernatant discarded. The ethanol precipitation was repeated and the resulting pellet was washed with 70% ethanol and dried by speed vac. This pellet was resuspended in 22.5μL ddH_2O and 2.5μL RNase was added and incubated at 37°C for 30 minutes. This sample was then stored at -20°C.

2.3.6.3. Large Scale Preparation of Single Stranded DNA

The supernatant from the large scale preparation of RF DNA (as described above) was centrifuged again at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a clean corex tube and 6.67mL 20% PEG, 2.5M NaCl was added to the supernatant and incubated on ice for at least 15 minutes or longer (about 1 hour). The phage was centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was removed by aspiration. The pellet was

resuspended in 300μL TE and transferred to an Eppendorf tube. The DNA was extracted 3 times with phenol/chloroform and ethanol precipitated. The DNA was pelleted, washed with 70% ethanol, dried, and resuspended in 50μL TE and stored at -20°C until used.

2.3.7. Synthesis of the Mutagenic Strand

2.3.7.1. Construction of the Mutagenic Oligonucleotides

The mutagenic oligonucleotides containing the appropriate base changes (see results) were prepared at the Biotechnology Institute at McMaster University. The absorbence (A_{260}) was measured and the oligonucleotide was lyophilized after synthesis. Upon delivery the oligonucleotides were resuspended in ddH_2O to a concentration of 50 pmol/mL.

2.3.7.2. Phosphorylation of the Oligonucleotide

To a 0.5mL microfuge tube was added 200 pmol oligonucleotide, 100mM Tris (pH 8), 10mM $MgCl_2$, 5mM DTT, 0.4mM ATP (neutralized), and ddH_2O to 30μL. The contents were mixed and 4.5 units of T4 polynucleotide kinase was added. The reaction mixture was incubated at 37°C for 45 minutes and the reaction stopped by heating at 65°C for 10 minutes.

2.3.7.3. Annealing of the Primer to the Template

The uracil-containing ssDNA was prepared by the extraction of ssDNA discussed above. To a 0.5mL microfuge tube was added 0.1 pmol uracil template, 2-3 pmol mutagenic oligonucleotide, 1uL 10X Annealing Buffer (200mM Tris-HCl pH 7.4, 20mM $MgCl_2$, 500mM NaCl), and ddH_2O to a final volume of 10μL. The molar ratio of primer to template was between 20:1 to 30:1. The reaction mixture was then placed in a 70°C water bath and the temperature was allowed to drop to 30°C over a 40 minute period (approx. 1°C/minute). The reaction mixture was then placed in an ice water bath and the synthesis of the complementary strand performed. A second reaction was run without primer in order to test for endogenous priming.

2.3.7.4. Synthesis of the Complementary DNA Strand

To the primed template in the ice water bath was added 1uL 10X Synthesis buffer (5mM each deoxynucleotide triphosphate, 10mM ATP, 100mM Tris pH 7.4, 50mM $MgCl_2$, 20mM dithiothreitol [DDT]), 2-4 units T4 DNA ligase (Pharmacia), 1 unit T4 DNA polymerase. The reaction was placed on ice for 5 minutes, followed by 5 minutes at 25°C, and then at 37°C for 60 minutes. After the final incubation period, 90μL of TE was added and the reaction stopped by freezing. The reaction could be stored at -20°C up to a month before use. DNA synthesis was monitored by agarose gel electrophoresis as the synthesised DNA ran slower than the ssDNA and could be differentiated from the double stranded

RF DNA. After determining that the synthesis reaction was successful, 3-10uL of the reaction was used to transform 300μL of competent MV1190, which were then plated on LB plates with a lawn of an overnight culture of MV1190.

2.3.8. Growth of Resultant Plaques

The following day, 10-20 plaques were picked and the phage were suspended in 150μL sterile TE. The phage isolates were then plaque purified twice by pour plating the phage on MV1190 as described previously and picking the resulting plaques which were then grown up as discussed above and screened as described below.

2.4. Cesium Chloride Banding of Large-Scale Plasmid DNA Preparations

A one litre culture of transformed LE392 was centrifuged at 6000 rpm for 15 minutes at 4°C and the pellet resuspended in 36mL of solution I (see above). After incubating for 10 minutes at room temperature, 80mL of solution II was added and incubated for 5 minutes on ice. After addition of 40mL of solution III, the contents were incubated on ice for an additional 15 minutes. The contents were then centrifuged for 10 minutes at 6000 rpm, the supernatent was filtered through cheese cloth, and 0.6 volumes of isopropanol were added. The contents were mixed and centrifuged for 15 minutes at 6000 rpm. The pellet was resuspended in 5mL 0.1X SSC (3M NaCl, 0.3M Sodium Citrate), centrifuged at 10,000 rpm for 10 minutes and adjusted to 5mL with 0.1 SSC. Then 2.0mL of

1mg/mL pronase 0.8% SDS was added and the mixture was incubated at 37°C for 30 minutes. Then 8.4g of CsCl was added, dissolved, and the contents were centrifuged at 10,000 rpm for 10 minutes. The supernatent was then transferred to Beckman polyallomer VTi 65.1 ultracentrifuge tubes, which were then capped with light paraffin oil and 250μL of 6 mg/mL ethidium bromide (EtBr). The tubes were sealed by heat and mixed. Centrifugation took place in a Beckman ultracentrifuge at 55,000 rpm for 15-18 hours using a Beckman Vti 65.1 rotor. Plasmid bands were collected from the tube using a 18G1/2 or 20G1 1/2 needle and allowing the band to drip into a 15mL polystyrene tubes (Corning). The plasmid was then cleaned up by 3 extractions of equal volumes of isopropanol saturated with 20X SSC and then dialysed in 1X TE overnight. The following morning the TE was changed and dialysis continued for another 6-8 hours. The DNA was ethanol precipitated, dried, and resuspended in 50-150μL TE.

2.5. Determination of DNA Concentration

Concentration of DNA preps was determined as outlined in Maniatis *et al.* (1982). Briefly, the DNA prep was diluted 1:500 in sterile ddH_2O and the absorbence at 280nm and 260nm was determined. An OD_{260} of 1 is roughly equivalent to 50 μg/mL double stranded DNA, 40μg/mL single stranded DNA, and 20μg/mL for oligonucleotides. A pure preparation of DNA has a OD_{260}/OD_{280} of 1.8 and any contamination would significantly reduce that value.

2.6. Enzyme Reactions

2.6.1. Restriction Endonuclease Digests

Restriction endonucleases were purchased from Pharmacia, Bethesda Research Laboratories (BRL), or New England Biolabs. Digests were done at 37°C or at 60°C in the case of BstEII for 4 hours, using 1 unit of enzyme for each mg of DNA. Digests using BstEII, Hind II, and PstI were carried out in a buffer of 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, and 50mM NaCl, while digests using BglII were carried out in a buffer of 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, and 100mM NaCl.

2.6.2. T4 DNA Ligase

The T4 DNA ligase was purchased from Pharmacia. The DNA fragments to be ligated were combined such that the smaller insert that was to be mutagenized was in 5 to 10 fold molar excess over the DNA it was to be cloned into. To this mixture was added 0.5mM ATP, 20mM Tris pH 7.6, 10mM DTT, 10mM $MgCl_2$, and one unit of T4 DNA ligase. The reaction was incubated at room temperature for a minimum of 4 hours. This mixture was then used directly to transform the appropriate competent cells.

2.7. DNA Gel Electrophoresis

2.7.1. Agarose Gel Electrophoresis

Agarose gels were generally made to 1% agarose (BRL) which was dissolved in Tris-Borate (TBE) buffer (0.089M Tris-borate, 0.089M boric acid, and 0.002M EDTA) by boiling, which once cooled to about 42°C started to solidify. Prior to this the gel was poured into a horizontal gel apparatus and then allowed to solidify. Loading buffer (20% glycerol, 2% SDS, and 0.5% bromophenol blue) was added to the DNA sample at a ratio of approximately 1:5. Between 10 to 30μL of DNA sample was loaded in each well, depending on the thickness of the gel, after the tank was filled with TBE. Once the gel had run it was emersed in water or TBE containing 0.5μg/mL EtBr and allowed to soak 15 to 45 minutes. After soaking the gel was photographed under UV light with a mounted Polaroid 107 camera and high speed Polaroid 667 film.

2.7.2. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels were made with 5% Acrylamide (Bio-Rad, 30% acrylamide, 1% N-N' Methylene-bis-acylamide). For polymeration of the gel 200μL of Ammonium persulfate (APS) and 90uL of TEMED (Bio-Rad, N,N,N',N'-Tetra-methylethylenediamine) was added. The gel upon polymerization was placed in a vertical gel apparatus and up to 30μL of sample was loaded per

well. DNA bands were visualized in the same manner as with agarose gels.

2.7.3. Extraction of DNA Fragments from Polyacrylamide Gels

Isolation of DNA from polyacrylamide gels was performed as in Maniatis *et al.*, 1982. Briefly, polyacrylamide gels were run and stained as described above. Using a long wavelength UV lamp the DNA band of interest was located and cut out with a sharp scalpel blade. The gel fragment containing the DNA was placed in a 1.5mL Eppendorf tube and was crushed with a teflon plunger. To the crushed gel was added 600μL Elution Buffer (0.5M ammonium acetate and 1mM EDTA pH8.0) and the mixture was vortexed. The tube was then wrapped in foil and placed on a rotator (Labindustries) at 37°C overnight. The next day,the tube was centrifuged for 10 minutes in a microfuge and the supernatent was removed and kept. To the "pellet" was added an additional 300μL of Elution Buffer, the tube was spun as before, and the supernatent was pooled with that previously collected. The supernatents were then filtered through a small column of glass wool and ethanol precipitated twice. The DNA was then washed with 70% ethanol, dried, and resuspended in 10μL TE.

2.8. DNA Sequencing

DNA sequencing was conducted as per instructions outlined in the ^{T7}Sequencing^{TM} Kit from Pharmacia.

2.8.1. Annealing of the Primer to ssDNA

Single-stranded DNA was prepared as outlined above. The concentration of the single-stranded template was then adjusted to 0.2μg/uL and the concentration of the primer was adjusted to 0.8μM. To a 1.5mL Eppendorf tube was added 10μL template, 2μL primer, and 2μL Annealing buffer. The reaction was then vortexed, centrifuged briefly, and incubated at 60°C for 10 minutes. The tube was placed at room temperature for 10 minutes before proceeding to the sequencing reactions.

2.8.2. Annealing of the Primer to dsDNA

Concentrations of DNA components were adjusted as before. The dsDNA was denatured by adding 2μL of 2M NaOH to the template and incubating at room temperature for 10 minutes. The DNA was ethanol precipitated, dried, and resuspended in ddH_2O and the annealing buffer and primer were added and incubated at 37°C for 20 minutes. The reaction was left at room temperature prior to proceeding to the sequencing reactions.

2.8.3. Sequencing Reactions

The T7 polymerase was diluted to 1.5 units/μL with cold enzyme dilution buffer. Four 1.5mL Eppendorf tubes were then labelled A, G, T, and C. To "read short" (up to 500 nucleotides) 2.5μL of the appropriate nucleotide **mix-Short** was

added to each tube while to read long (up to 1000 nucleotides) the appropriate **mix-long** was added to each tube. To the tube containing the annealed primer and template was added 3μL **Labelling mix** (dCTP, dGTP, and DTTP in solution), 10uCi [α-^{35}S]dATP, and 2μL of the diluted T7 DNA polymerase. The reaction was incubated at room temperature for 5 minutes. After the 5 minute labelling period 4.5μL of the Labelling reaction was added to each of the four sequencing mixes, which had been prewarmed at 37°C and allowed to incubate at 37°C for 5 minutes. After 5 minutes, 5μL of stop solution (95% deionized formamide, 20mM Na_2EDTA, 0.05% Xylene Cynol FF, and 0.05% Bromophenol Blue). The sample to be loaded was then heated at 80°C for 2 minutes and was kept at this temperature until loading of the gel was complete. Approximately 1-2μL of sample was loaded in each well and the gel was run with additional loading of sample if required.

2.8.4. Gel System

The gel used for sequencing was a 6% polyacrylamide (38:2 acrylamide:bis-acrylamide) in TBE with 42% w/v urea. The gel was run in a 2010 vertical Macrophor Electrophoresis Unit (LKB Bromma) and the temperature in the constant circulating plate kept at 55°C. The gel was prerun at 1800V for 30 minutes, the samples were loaded, and run at 3000V for 1.5 to 3 hours as required. The gel was placed in 10% acetic acid, 10% methanol for 20 minutes and then was dried in an oven at 80°C for approximately 2 hours.

2.9. Tissue Culture

2.9.1. Media and Equipment

Forma Scientific (Caltec Scientific) or National (Wernicke) incubators set at 37°C, 95% air, and 5% CO_2 were used for growth of all cell types and all protocols requiring incubation. All sterile work was done in SterilGARD (Baker Co., inc.) or Caltec Scientific hoods. Cells were cultured in 60mm or 100mm dishes (Corning) or 150mm dishes (Nunclon). Media used were either F11 minimal essential medium (F11 MEM), alpha minimal essential medium (α-MEM), or Joklik's modified medium. Sera used were fetal bovine serum (FCS), newborn calf serum (NCS), and horse serum (HS). Also used were Penicillin-Streptomycin (Pen-Strep, Gibco 100X solution contains 10,000μg/mL penicillin and 10,000μg/mL streptomycin, 1% L-glutamine, and fungizone (Gibco). Other reagents include 1X phosphate buffered saline double minus ($PBS^{=}$, 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, made to 1 litre with ddH_2O), PBS^{++} (PBS with 0.1mg/mL $CaCl_2$ and 0.1mg/mL $MgCl_2$), 1X trypsin-EDTA (dilution of 5.0g/100mL trypsin (Gibco) and 2.0g/100mL EDTA in 1X PBS), and 1X citric saline (made from 10X stock: 50g KCl, 22g Na-citrate in 500mL ddH_2O).

2.9.2. Cell Lines

293 cells are a human embryonic kidney cell line that had been transformed by adenovirus 5 DNA (Graham *et al.*, 1977) and were maintained in F11 MEM supplemented with 10% FCS, 1% L-glutamine, 1.25% amino acids,1.25% vitamin supplement,1% fungizone, and 1% Pen-Strep. KB cells are an epidermoid carcinoma derived cell line (Eagle, 1955) and were maintained in α-MEM supplemented with 10% FCS, 1% L-glutamine,and 1% Pen-Strep. HeLa cells are an epitheloid carcinoma derived cell line (Gey *et al.*, 1952) and were maintained in α-MEM supplemented with 10% NCS, 1% L-glutamine, and 1% Pen-Strep.

2.9.3. Passaging of Cells

For passaging of 293, when the cells had reached confluency the medium was removed and the cells were washed with approximately 5mL of citric saline. After the citric saline was removed, an additional 2mL citric saline was added and removed and the cells were incubated for 3 minutes at 37°C. After incubation the cells were manually dislodged by striking the dish against the side of the hood and the cells were returned to the incubator for an additional 2 minutes. The cells were then "banged" again, resuspended in media, and plated in 60mm, 100mm or 150mm plates. KB and HeLa cells were passaged in a similar manner except that the cells were washed with PBS and Trypsin-EDTA was used instead of citric saline.

2.10. Transformation of Primary Baby Rat Kidney BRK Cells

2.10.1. Preparation of BRK Cells

Week old Wistar rats were obtained and sacrificed by cervical dislocation. The kidneys were removed and placed in a tissue culture dish containing $PBS^{=}$. Excess tissue and blood vessels were carefully removed from the kidneys using forceps and the cleaned kidney was placed in fresh $PBS^{=}$. All the cleaned kidneys were placed in a sterile 100mL bottle and were broken up with long handled scissors until the mixture became "soupy" in appearance. Ten (10) mL of 2X trypsin in PBS was added to the cells and the entire mixture transferred to a new bottle containing 30mL 2X trypsin. The cells were then stirred at a medium speed for 15-20 minutes. The mixture was allowed to settle for approximately 30 minutes and the supernatent was transferred to a fresh 100mL bottle containing ice cold FCS to stop the trypsin digestion. New trypsin (30mL) was added to the debris and the procedure repeated and the supernatents combined and centrifuged at 3000 rpm for 5 minutes in 50mL Falcon tubes (Corning). The cells were resuspended in α-MEM (10% FCS, 1% L-glutamine, and 1% Pen-Strep) and incubated at 37°C for 15 minutes. The cells were filtered through double thick cheesecloth and medium added (60mL per pair of kidneys). The cells were then plated in 60mm plates using 5mL cell suspension per plate and incubated at 37°C overnight. The following morning, the medium was changed to eliminate dead cells and other debris. The cells were allowed to grow to 70-80% before

transfection.

2.10.2. DNA-Mediated Transfection of BRK cells

The BRK cells were transfected by the calcium phosphate method of Graham and van der Eb (1973) as modified by Wigler *et al.* (1979). Generally 5μg plasmid/ 60mm dish BRK was added to a 15mL polystyrene centrifuge tube (Corning) along with 5μg salmon sperm DNA/60mm dish BRK, and ddH_2O to 0.9mL. Then 100ML 2.5M $CaCl_2$ was added and the mixture was added drop by drop to 0.25mL 2X HbS (8g NaCl, 0.37g KCl, 0.125g Na_2HPO_4, 5.0g Hepes [Calbiochem], 1.0g dextrose, pH 7.1) per dish BRK cells. While the $CaCl_2$ mixture was being added the 2X HbS was bubbled by passing air through the mixture. After the plasmid mixture had been added, the mixtures were allowed to incubate at room temperature for 30 minutes. Then 0.5mL of the precipitate was added to each of 4-8 60mm dishes of BRK with 5mL of α-MEM medium and were left overnight to allow uptake of the DNA. The next morning the media was changed with fresh α-MEM. Selection for transformed cells was done by changing the medium to Jokliks medium supplemented with 5% HS, 1% L-glutamine, and 1% Pen-Strep, 4 days post-transfection. The medium was changed every 3-4 days for approximately 2-3 weeks, when the cells were fixed with 3:1 methanol/acetic acid for 30 minutes, dried, and Giemsa stained (Fischer Scientific, diluted 1 in 20 with PBS). Transformed colonies were then counted and viewed under the microscope for confirmation if required.

2.11. Rescue of Mutations into Infectious Virus

Mutations were rescued into virus by the method of McGrory *et al.* (1988). Briefly, pJM17 (McGrory *et al.*, 1988) is a non-infectious plasmid, which is a derivative of pFG140 (Graham, 1984), a derivative of dl309 (Jones and Shenk, 1979) that contains a 4.3 Kb insert that disallows packaging of the genome into the viral capsid. All mutations were rescued into plasmid pXC38 (McKinnon *et al.*, 1982) that contains the leftmost 22 to 5788 base pairs of the Ad 5 genome. Cotransfection of 293 cells with these two plasmids resulted in recombination between the two plasmids that created an infectious virus that was packaged normally. The virus was then screened for the mutation(s) by restriction analysis.

2.11.1. Transfection of 293 Cells

293 cells were passaged as described above into 60mm dishes and were transfected when they reached a confluency of 70-80%. Transformation was performed as described above with the following differences. In addition to the 5μg/dish of the plasmid carrying the mutation, 5μg/dish of pJM17 (McGrory *et al.*, 1988) was used. The $CaCl_2$ cocktail when added to the 2X HbS was not bubbled but was instead merely added carefully drop by drop. The precipitate was added to the cells as described previously and the cells were incubated at 37°C for 4-5 hours to allow the uptake of the DNA. After incubation the medium was removed and the dishes were overlayed with 10mL F11 that was supplemented with 5% HS, 1% fungizone, 1% L-glutamine, 1% Pen-Strep, and 1% agarose. The

dishes were then returned to the incubator at 37°C and plaques were generally visible in just over a week. The cells were usually fed with fresh F11 and 1% agarose sometime midway during the incubation period.

2.12. Screening of Viral Recombinants

2.12.1. Harvest of Plaque Isolates

Plaques were picked using a sterile pasteur pipet and transferred to 250μL of PBS^{++} containing 10% glycerol. These isolates could then either be stored at -70°C or expanded immediately if 293 cells were ready. To expand the agarose plug, either an aliquot of the PBS suspension or the entire plug was used to infect 293 cells 80-90% confluent. After allowing the virus to absorb for 60 minutes, fresh F11 with 5% HS, 1% L-glutamine, and 1% Pen-Strep was added to the dish.

2.12.2. Harvest of Liquid Infections and Extraction of DNA

The harvest of liquid infections was performed in one of two methods depending on the virus. If only the recovery of the virus was desired then the medium was removed in a sterile hood and transferred to a 15mL Corning tube. Some medium was left on the cells which were carefully scraped using a rubber scraper and transferred to tube containing the medium. The cells were then pelleted at 1000 rpm and the supernatent sterilely transferred to a new Corning tube containing 100% glycerol. The pellet was resuspended in 1-2mL fresh

medium and freeze-thawed 3 times in liquid N_2 and in a 37°C water bath. The suspension was then centrifuged at 1000 rpm and the supernatent transferred to 4mL glass vials (containing 0.250mL glycerol) in 0.5mL aliquots, which were then stored at -70°C until required. On the other hand, if recovery of viral DNA was required a Hirt extraction (Hirt, 1967) was performed. The medium was again removed in a sterile hood and saved. To the dish was then added 0.8mL of 0.6% SDS in 10mM Tris-EDTA (pH 7.5) and left at room temperature for 20 minutes. The cells were then scraped gently into 1.5mL eppendorf tubes. After addition of 200μL of 5M NaCl, the mixture was left overnight at 4°C. The following morning the contents were centrifuged in a microfuge for 30 minutes at 4°C and the supernatent recovered. The DNA was then treated with RNase (40μg/mL final concentration) and then with Proteinase K (50μg/mL final concentration). The sample was then phenol/chloroform extracted twice, ethanol precipitated, washed in 70% ethanol, dried, and resuspended in 100μL ddH_2O. Of this resuspension 10-30 μL were used for restriction analysis depending on the DNA concentration. Other than digesting with the restriction enzymes characteristic of the mutations, viral DNA was digested with HindIII or PstII to test for gross rearrangements that may have arisen though undesired recombination events. Samples were run on 1% agarose gels and visualized by EtBr staining or by Southern blot analysis (see below).

2.12.3. Southern Blot Analysis

Southern transfer was carried out by the method of Southern (1975) as outlined in Maniatis *et al.* (1982). Briefly, after running the 1% agarose gel, the gel was treated with 0.5M NaOH, 1M NaCl for 1 hour, changing the solution after 30 minutes. The gel was rinsed with ddH_2O and neutralized with 3M NaCl, 0.5M Tris (pH 7.4-7.5) for 2X 30 minutes. The DNA was then transferred to nitrocellulose overnight by the procedure outlined in Maniatis *et al.* (1982) using 10X SSC. After transfer, the nitrocellulose was washed in 6X SSC for 5 minutes with shaking, air dried, and baked at 80°C in an oven for 1.5-2 hours. Prior to prehybridization, the nitrocellulose was soaked in 6X SSPE (3M NaCl, 200mM NaH_2PO_4:H_2O, 20mM EDTA pH7.4). Prehybrization took place in vacuum sealed (Decosonic) bags using 6X SSPE, 0.5% SDS, 5X Denhardt's solution (50X solution: 5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA, ddH_2O to 500mL, filtered, and stored at -20°C) all at T_H ([2°C/AorT of oligo + 4°C/Gor C of oligo]-5°C). Hybridization of the oligonucleotide took place overnight at T_H, using 200ng/mL tRNA and 1-10x10^6 cpm/mL of the oligo probe. The oligonucleotide was labelled by the procedure outlined above for phosphorylating an oligonucleotide of Zoller and Smith (1983) using 100μCi γ-^{32}PATP (New England Nuclear [NEN]). After hybridization, the nitrocellulose was washed in 2X SSPE/0.1% SDS 3 X 20 minutes at room temperature and 1 X 20 minutes at T_H. The blot was then airdried, put on 3MM paper, wrapped in clear plastic wrap, and exposed on Kodak XR-5 fast (X-ray) film at -70°C.

2.13. Plaque Purification of Viral Recombinants

After the viral recombinant had been identified by the procedures above, 293 cells in 60mm dishes were infected from the virus-containing medium from the expanded agarose plug,that had been serially diluted in F11. The dishes were then overlayed with F11 with 1% agarose as described above. After one week, a plaque was isolated and expanded into a liquid infection of 293 cells. The DNA was recovered and analyzed as outlined above, and if the virus proved to be correct the process was repeated using the medium from the first purification step. After the second purification, the virus was recovered by freeze-thawing and transferred to 4mL dram vial, which was then stored at -70°C and used as a stock for future work.

2.14. Determination of Virus Titres

HeLa and /or 293 cells were split into 60mm dishes and were infected when they reached 80-90% confluency. The viral stock was diluted serially from 10^{-3} to 10^{-10} and 0.5mL was used to infect the cells. After an absorption period of 60 minutes the dishes were overlayed with F11 supplemented with 5% HS, 1% fungizone, 1% L-glutamine, 1% Pen-Strep, and 1% agarose. Plaques were counted over a period of 7-9 days post-infection. The procedure was repeated with different cells and results were calculated as pfu/mL and these numbers were used for future infection where a certain multiplicity of infection (moi) was required. For comparing the titres on 293 and HeLa cells, the ratio between 293

and HeLa titres for the mutant was expressed relative to the ratio between 293 and HeLa cells for dl309 (the wild type virus).

2.15. Protein Analysis

2.15.1. Preparation and infection of Cells

KB cells were cultured in 100mm dishes (Corning) in α-MEM supplemented with 10% FCS, 1% L-glutamine, and 1% Pen-Strep and were maintained as described above. 293 cells were cultured in 100mm dishes in F11 supplemented with 10%FCS, 1.25% amino acids, 1.25% vitamins, 1% L-glutamine, 1% fungizone, and 1% Pen-Strep. Cells were infected at an moi of 35 pfu/cell (Rowe *et al.*, 1983).

2.15.2. Antisera and Immunoprecipitation

Antisera (peptide) to the amino termini of the 496R, 84R, 93R, and 156R proteins (termed 58N-2) and to the carboxy termini of 84R, 93R,and 496R proteins (termed 84-C2, 93R-C1, and 58-C1 respectively), were made available and described elsewhere (Brown *et al.*, in progress; McGlade *et al.*, 1987; and Yee *et al.*, 1983). Cells were labelled from 16-18 hours post-infection with 100-250μCi [^{35}S]methionine (Amersham Corp.; specific activity 1,300 Ci/mmol) and harvested at 18 hours post-infection. At this point the medium was removed by aspiration and the cells were gently scraped using a rubber scraper. The cells

were resuspended in 2mL cold PBS and transferred to a 15mL Falcon tube. The dish was washed with 2mL of fresh cold PBS and the suspension was added to the first. The cells were then centrifuged at 2000 rpm for approximately 5 minutes in a Chilspin 2 (MSE), the supernatant was removed, and the cell pellet resupended in cold PBS. The resuspension was centrifuged once more and the wash procedure repeated. The pellet was this time resuspended in cold RIPA buffer (50mM Tris, 150mM NaCl, 1% Na Dodecylcolate, 0.1% SDS, 1% Triton X, 100u Aprotinin, pH 7.2) at a quantity of 0.5mL per immunoprecipitation reaction. The resuspension was then vortexed briefly and centrifuged at 10,000 rpm for 10 minutes (Sorvall). The supernatant was removed and saved as cell extract. The immunoprecipitation reactions were set up by adding 100-200μL Protein A Sepharose CL4B Beads (Pharmacia), 0.5mL cell extract, 10-30μL antiserum, and peptide if a chase was desired. The reaction tubes were rotated end over end for at least 4 hours and usually overnight, before being centrifuged, aspirated, and washed 3X with RIPA and 2X 5M LiCl. The sample was then resuspended in sample buffer (10% Stacking buffer [see below], 1% SDS, 10% glycerol, 0.1% ß-mercaptoethanol, 50mM Tris-HCl pH 6.8, and 0.001% bromophenol blue) and boiled prior to approximately 30uL being loaded per well.

2.15.3. SDS Polyacryamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described previously (Yee *et al.*, 1983). The separating gel was usually 15% polyacrylamide, while the stacking gel was 5%.

The ratio of N-N'bismethylene acrylamide to acrylamide was 1:30. The separating buffer was 1.5M Tris-HCl (pH 8.8), while the stacking buffer was 0.5M Tris-HCl (pH 6.8). The gel was ran overnight at constant current such that the voltage varied from 50 to 150V over the 12-16 hours. The gel was then fixed with a fixing solution of isopropanol, water, acetic acid (25:65:10) and scintillated for 30 minutes in Amplify (Amersham). Following this the gel was dried over a steam dryer under vacuum and visualized by Kodak XR-5 film.

RESULTS

3.1 Characterization of the Minor E1b Products.

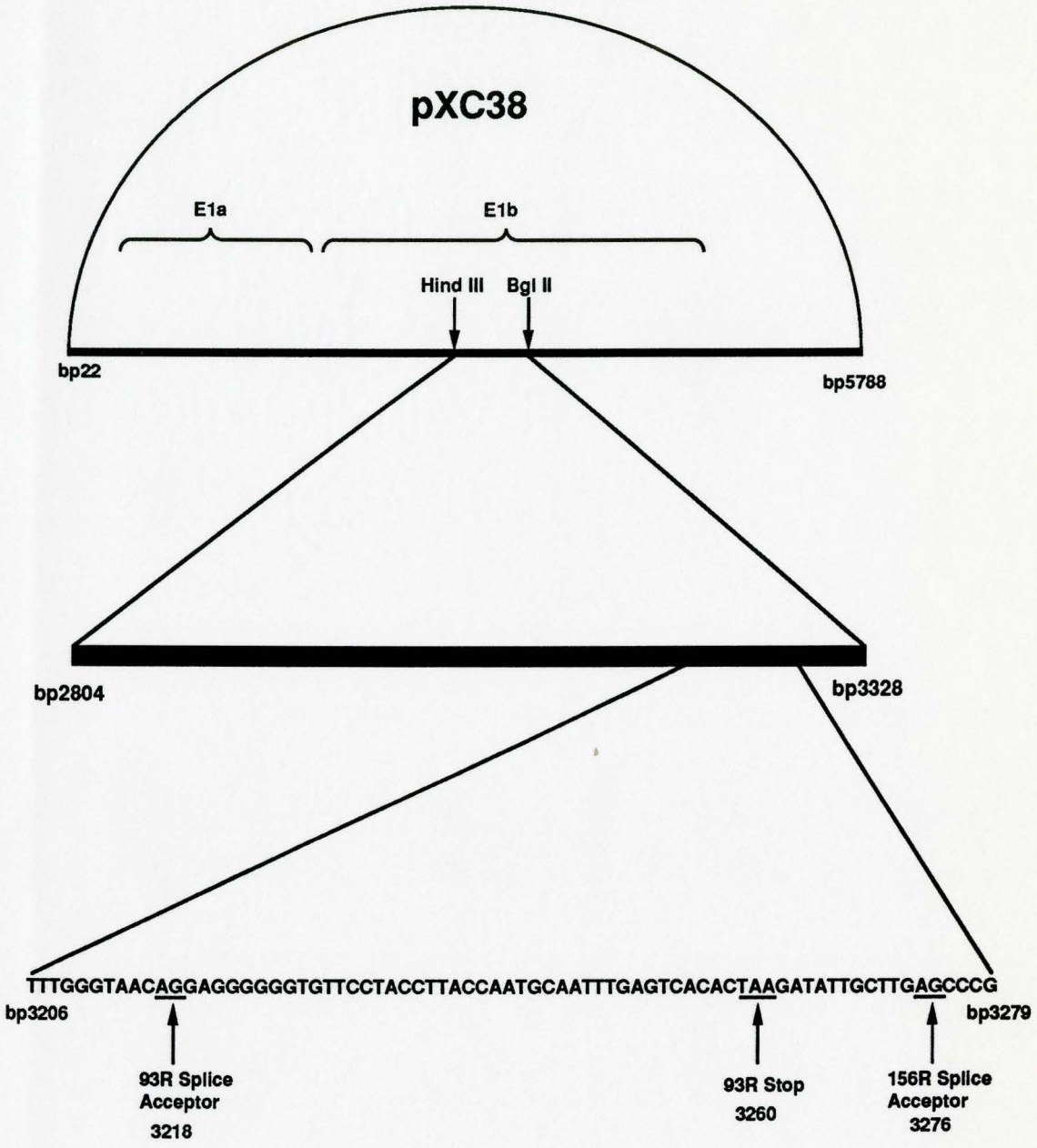
This study involved the analysis of the function of the E1b minor products 156R and 93R, through the generation of mutants that affect the individual acceptor sites of the 1.26 and 1.31Kb messages. The resulting mutants, which were hopefully deficient in the production of 93R or 156R, were tested for their ability to transform primary rat cells by DNA mediated assays and their ability to carry out productive infection in human cells.

3.2 Site Directed Mutagenesis of the 3' Splice Sites of the 1.26 and 1.31Kb messages.

3.2.1 Cloning into M13

Adenovirus type 5 sequences were obtained from the plasmid pXC38, which contains the adenoviral sequences from bases 22 to 5788 (the first 16%) and thus all of E1a and E1b (McKinnon et al., 1982). A working fragment of 524 base pairs was cloned from pXC38 that ranged from the restriction endonuclease HindIII site at 2804 to the BglII site at 3328 in the Ad sequences and which contained the splice acceptor sites for both the 1.26 and 1.31Kb mRNAs (Figure 3.1). The fragment was isolated, after digestion with restriction enzymes, by elution from a 5% polyacrylamide gel as described in section 2.7.3. This fragment

Figure 3.1 **Schematic Representation of the Plasmid pXC38. Also shown is the 524 base pair "working" fragment from the HindIII restriction site at 2804 to the BglII site at 3328 of the adenovirus genome. The sequence around the splice acceptor sites of the 1.26 and 1.31Kb mRNAs (labelled 156R and 93R splice acceptors, respectively) is also shown, with the 93R stop codon labelled as well. The nucleotide numbers given refer to the nucleotide numbers as found in the intact wild type virus and start with the letters bp, meaning base pair.**

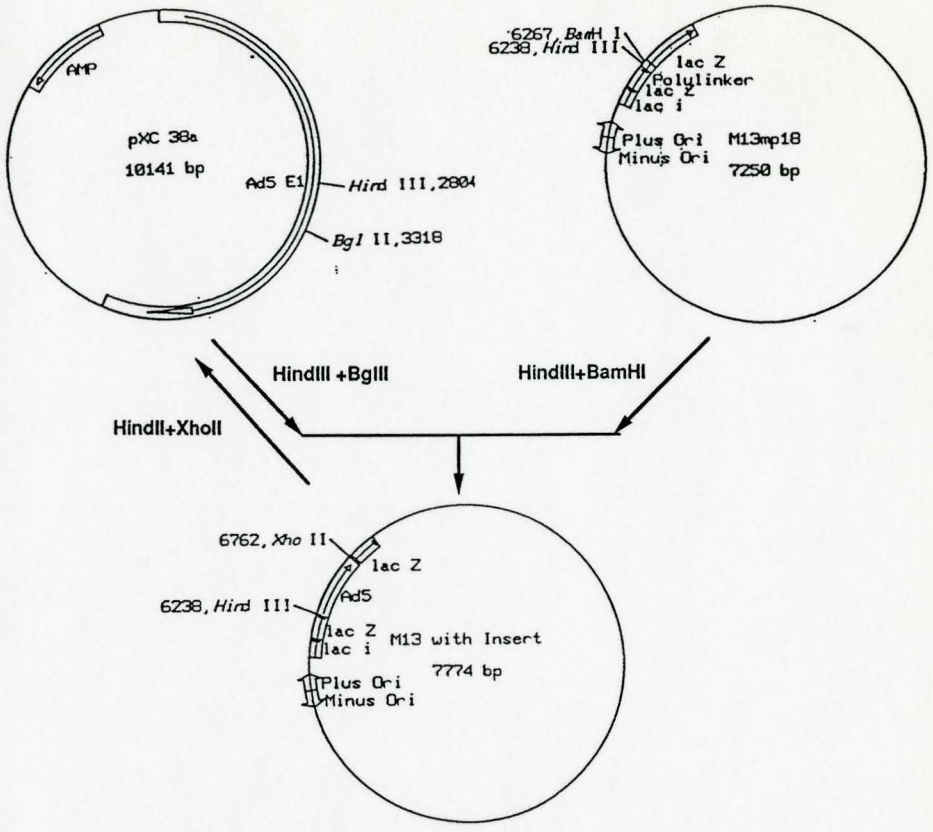

pXC38
E1a
E1b
Hind III
Bgl II
bp22
bp5788
bp2804
bp3328
TTTGGGTAACAGGAGGGGGGTGTTCCTACCTTACCAATGCAATTTGAGTCACACTAAGATATTGCTTGAGCCCG
bp3206
bp3279
93R Splice Acceptor 3218
93R Stop 3260
156R Splice Acceptor 3276

was subsequently cloned into the M13mp18 cloning vector in the multicloning polylinker at the HindIII and BamHI sites, which creates a new XhoII site at the BglII/BamHI site, as the overhanging sequence for all three enzymes are identical (Figure 3.2). The fragment could be recovered by digestion with the enzymes HindIII and XhoII, which would rescue the BglII site in the working fragment.

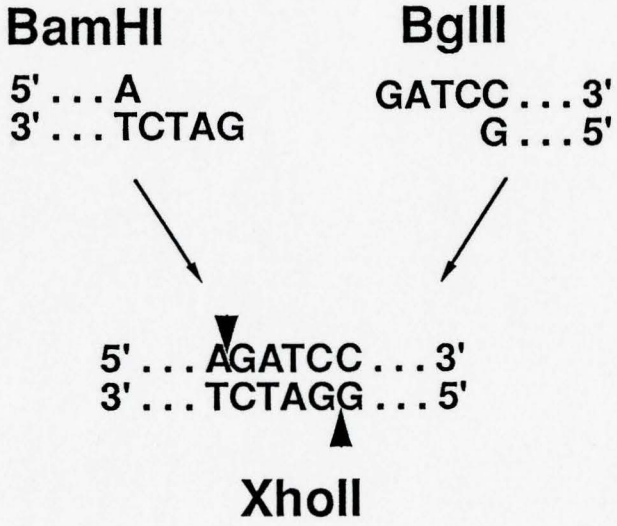
After ligation of the adenoviral sequence into M13, the cloned M13 was used for the transformation of E. coli MV1190 cells. The polylinker sequences in M13 are located near the start of the lac DNA of the ß-galactosidase gene, which is found in the intergenic region of the bacteriophage between phage genes IV and II. Any inserted DNA in the polylinker was expected to disrupt the ß-galactosidase gene, resulting in the formation of clear plaques. Following transformation and plating, clear plaques were picked and the cloned phage was expanded. For confirmation of the presence of the insert, DNA was isolated and screened by digesting the cloned M13 with HindIII and XhoII, followed by agarose gel electrophoresis. The expected restriction fragments are shown in Figure 3.5 and include a 700 base XhoII-XhoII fragment, the 524 base HindIII-XhoII fragment, and a third XhoII-HindIII fragment that consists of the remainder of the M13 vector. The pattern of DNA fragments resulting from digestion with XhoII and HindIII are shown in Figure 3.6, lanes A-D.

Figure 3.2 **Cloning into and out of M13. A: Shown in the upper portion are the parental plasmid pXC38 and the bacteriophage M13mp18, with the restriction sites used in the cloning labelled in the appropriate places. Arrows pointing down indicate digestion with the enzymes listed next to the arrows and subsequent ligation of the pXC38 fragment into the M13 vector, with the HindIII and the newly created XhoII site labelled (below). The arrow pointing up indicates digestion with the enzymes listed to the left of the arrow, used in the rescue of the mutated fragment back into pXC38. B: Shown here are the 5' overhanging sequences of the BamHI and BglII sites and the subsequent ligation and formation of the new XhoII site.**

A


AMP
pXC 38a
10141 bp
Ad5 E1
Hind III,2804
Bgl II,3318
6267, BamH I
6238, Hind III
lac Z
Polylinker
lac Z
lac i
Plus Ori
Minus Ori
M13mp18
7250 bp
HindIII +BglII
HindIII+BamHI
HindII+XhoII
6762, Xho II
lac Z
Ad5
6238, Hind III
lac Z
lac i
M13 with Insert
7774 bp
Plus Ori
Minus Ori

B


BamHI
5' . . . A
3' . . . TCTAG
BglII
GATCC . . . 3'
G . . . 5'
5' . . . AGATCC . . . 3'
3' . . . TCTAGG . . . 5'
XhoII

3.2.2 Oligonucleotide-directed Mutagenesis.

The mutagenic oligonucleotides used were 21mers, with 10 nucleotides on either side of the mutated nucleotide (Figure 3.3). This size was used in order to achieve as high a degree of stringency and efficiency of priming as possible. Figure 3.3 shows the steps in M13 mutagenesis. Briefly, the method involves the hybridization of the mutagenic primers to the M13-adenoviral clone followed by extension of the primer by T4-polymerase. The clone template used has uracil incorporated into the DNA rather than thymidine (due to growth of the phage clone in CJ236, which lacks the enzymes dUTPase and uracil N-glycosylase), while the newly synthesized DNA strand is normal. This situation allows for selection of the new mutated strand on transformation into MV1190 (see 2.3.7), with the uracil-incorporated strand being inactivated at high efficiencies by uracil N-glycosylase.

The actual mutations introduced into the DNA were planned in such a way as to meet several criteria. First of all the mutations must involve either the terminal A or G of the intron, or any of the other nucleotides of the splice site consensus sequence known to disrupt splicing (Sharp, 1987; see Discussion). Secondly, the mutations must be conservative changes and not change the coding sequence for the 496R protein to ensure that any resulting phenotype is due to the loss of the minor protein and not to any potential change in function of 496R. This can be achieved by taking advantage of the degeneracy of the genetic code (Table 3.1). Finally, for screening purposes the mutations were designed to

Figure 3.3 M13 Mutagenesis. A: The sequence of the 21mer oligonucleotide mutagenic primers, with the respective mutated nucleotide indicated by an underline. B: A schematic representation of the steps in mutagenesis. The cloned M13-Ad5 template is shown with uracils incorporated into the DNA sequence as well as two of the complementary nucleotides of the binding site of the mutagenic primer. The next steps involving the hybridization of the primer (in the case shown oligo #1-93) to the template, followed by elongation by T4 DNA Polymerase, and ligation are also shown. These steps are followed by transformation of E. coli MV1190 cells, which leads to inactivation of the uracil incorporated strand and selection of the mutated strand.

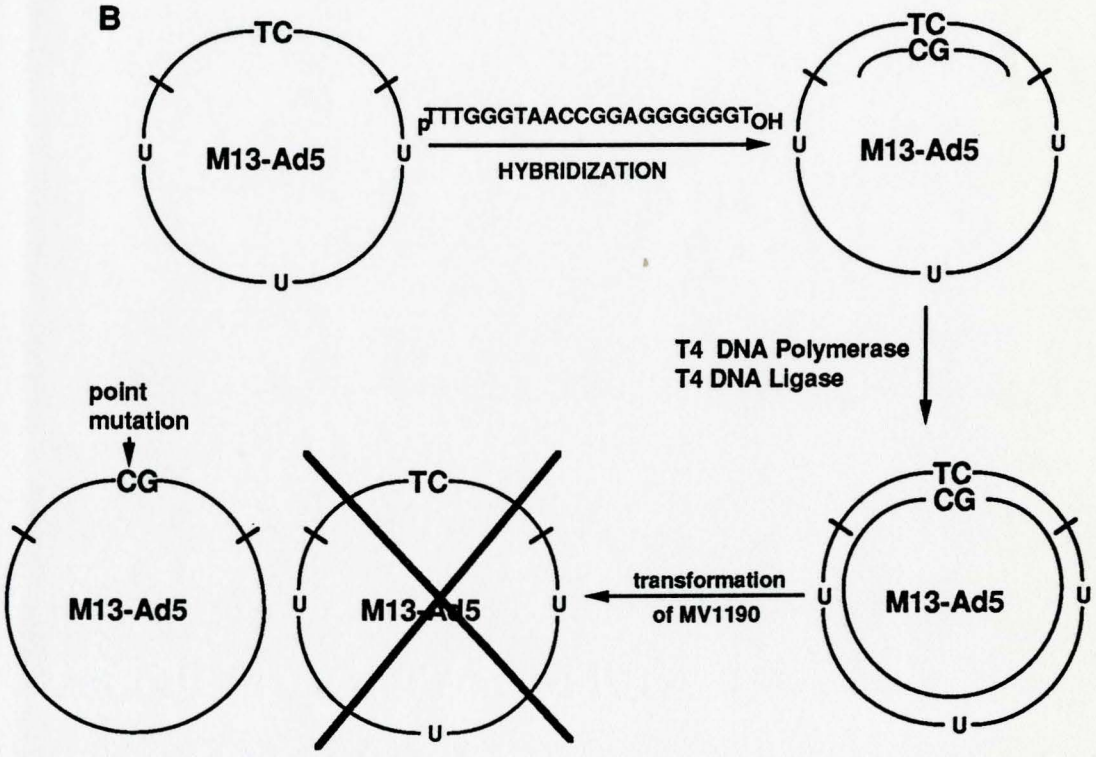
A

Oligonucleotide # 1-93

TTTGGGTAACCGGAGGGGGGT

Oligonucleotide # 2-156

TATTGCTTGAACCCGAGAGCA



introduce a change in the restriction endonuclease digestion pattern by adding or removing a site.

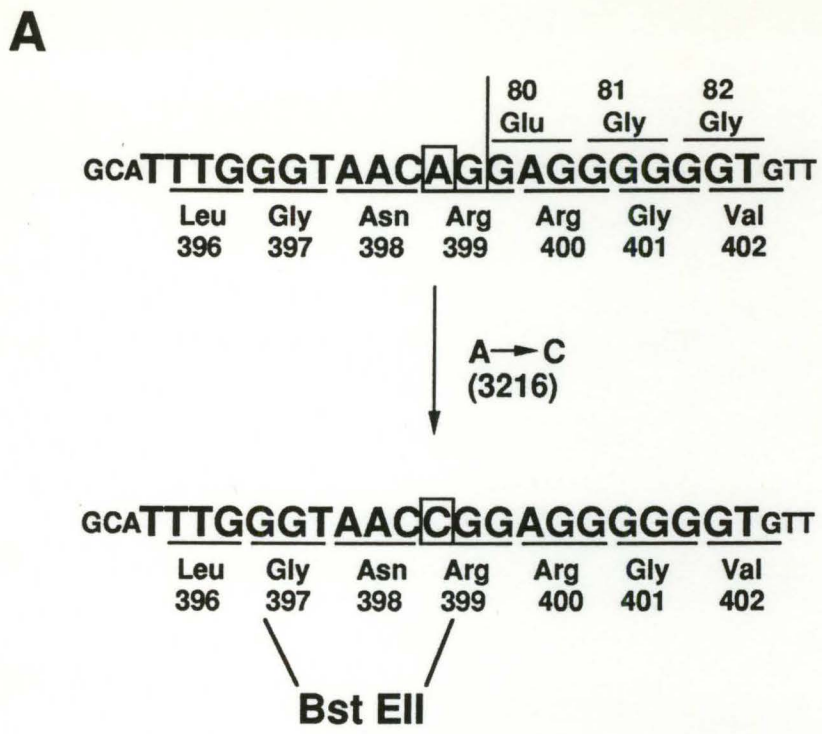
Observance of the above criteria is outlined in Figure 3.4. In order to disrupt the splicing of the 1.31Kb message that produces the 93R protein, the adenosine (A) of the AG 3' splice acceptor was converted to a cytosine (C). Changing this nucleotide has no effect on the amino acid coding sequence for 496R (Table 3.1). The conversion of A to C also created a new BstEII site, whose recognition sequence is GGTNAACC. In the case of the mutation disrupting the 1.26Kb mRNA and subsequently the production of 156R, the G of the AG consensus sequence was mutated to an A. The resulting codon change in the 496R coding sequence was a GAG to a GAA, which conserved the glutamic acid codon at that site (Table 3.1). The nucleotide change also eliminated a BanII restriction endonuclease site, which has a recognition sequence of G(Pu)GC(Py)C. The gain of a restriction site in the one case and the loss of a site in the other allowed for a rapid screening procedure for the presence of the mutations. Figure 3.5 outlines the expected DNA fragments produced from a digestion of the M13-93R and M13-156R mutants by digestion with HindIII/BstEII and HindIII/BanII, respectively. The predicted restriction pattern for the M13-93R mutant, digested with HindIII and BstEII, would be to create a single 409 base fragment due to the addition of the BstEII site, while the wild-type (the M13-adenoviral clone) would yield only the linearized genome, cut at the HindIII site. The elimination of the BanII site in the M13-156R mutant would yield a 469 base fragment that would shift to a 538 base fragment, which corresponds to a fragment cut at one end at

Table 3.1 The Genetic Code, Displaying Its Degeneracy

FIRST POSITION (5' END)	SECOND POSITION U	SECOND POSITION C	SECOND POSITION A	SECOND POSITION G	THIRD POSITION (3' END)
U	phe	ser	tyr	cys	U
	phe	ser	tyr	cys	C
	leu	ser	stop	stop	A
	leu	ser	stop	trp	G
C	leu	pro	his	arg	U
	leu	pro	his	arg	C
	leu	pro	gln	arg	A
	leu	pro	gln	arg	G
A	ileu	thr	asn	ser	U
	ileu	thr	asn	ser	C
	ileu	thr	lys	arg	A
	met	thr	lys	arg	G
G	val	ala	asp	gly	U
	val	ala	asp	gly	C
	val	ala	glu	gly	A
	val	ala	glu	gly	G

phe: phenylalanine
leu : leucine
ileu: isoleucine
met: methionine
val : valine
ser : serine
pro: proline
thr : threonine
ala : alanine
tyr : tyrosine
his : histidine
gln : glutamine
asn: asparagine
lys : lysine
asp: aspartic acid
glu : glutamic acid
cys: cysteine
trp : tryptophan
arg: arginine
gly : glycine

Figure 3.4 The Introduction of the Mutations into the Adenovirus Sequence. A: The sequence around the splice acceptor site for the 1.31Kb mRNA coding for the 93R protein, showing the nucleotide sequence, the splice acceptor site (vertical line), the codons for the 496R protein (underlined), the codons for the 93R protein (overlined), and the amino acid sequence and number for both the 496R and 93R proteins under and over the nucleotide sequence, respectively. The mutated nucleotide is shown in a box, with the actual change shown next to the arrow, which indicates the point mutagenesis. Also shown is the change in the restriction enzyme pattern, which in this case is the creation of a BstEII site. Note that in mutating the site there is no change in the amino acid sequence of 496R (Table 3.1). B: The sequence surrounding the 1.26Kb mRNA is shown as in A, with the amino acid sequence of 156R shown, as well as the elimination of the BanII site. Note again that the amino acid sequence of 496R is unaltered by the mutation.


A
80 81 82
Glu Gly Gly
GCATTTGGGTAACAGGAGGGGGGTGTT
Leu Gly Asn Arg Arg Gly Val
396 397 398 399 400 401 402
A→C
(3216)
GCATTTGGGTAACCGGAGGGGGGTGTT
Leu Gly Asn Arg Arg Gly Val
396 397 398 399 400 401 402
Bst EII

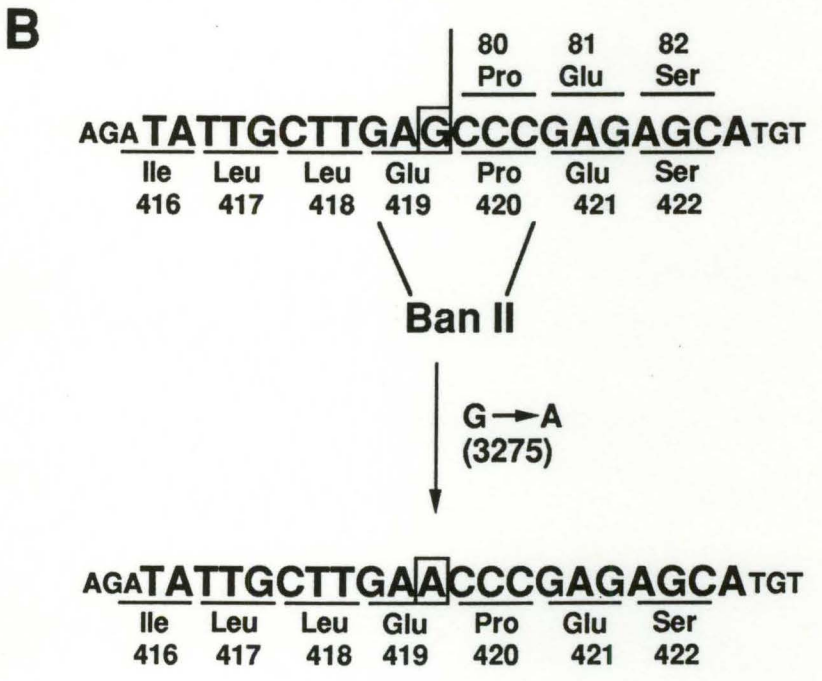
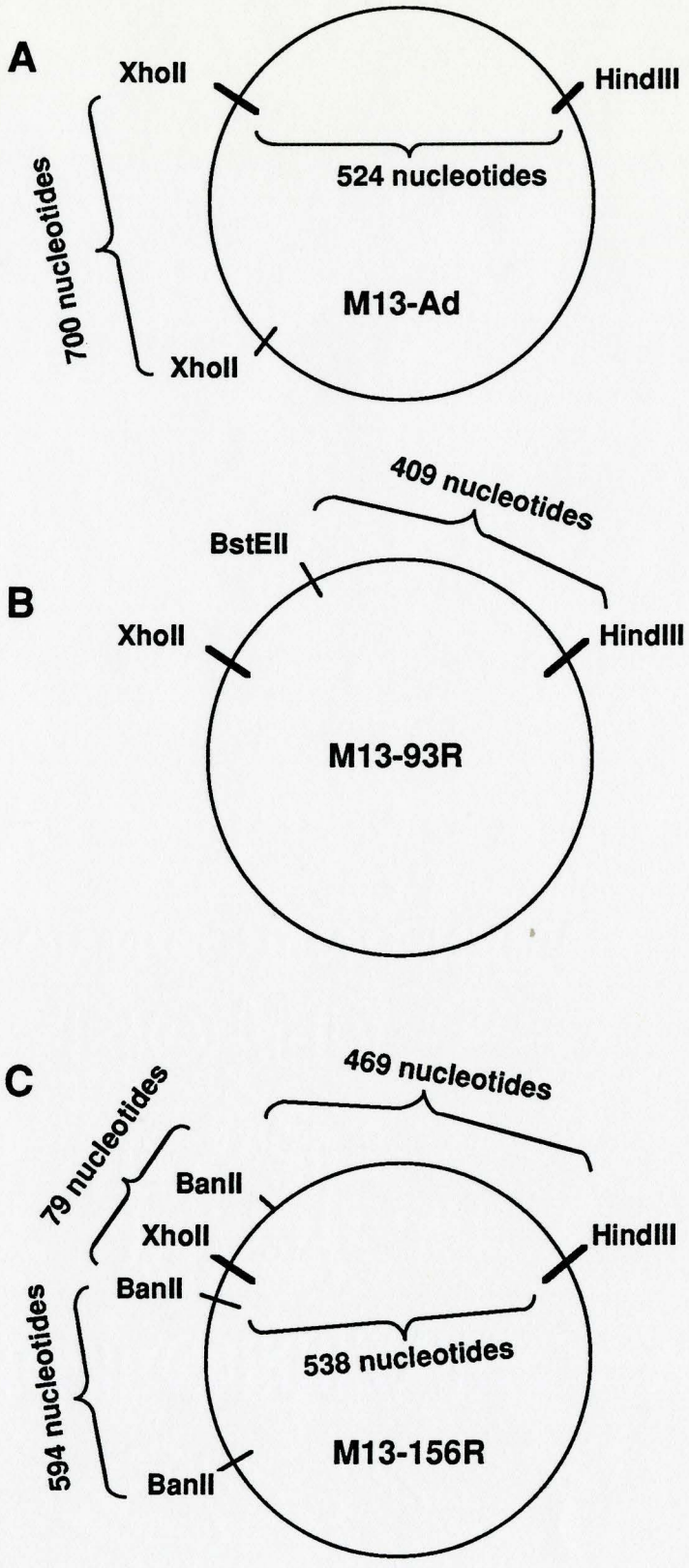

B
80 81 82
Pro Glu Ser
AGATATTGCTTGAGCCCGAGAGCATGT
Ile Leu Leu Glu Pro Glu Ser
416 417 418 419 420 421 422
Ban II
G→A
(3275)
AGATATTGCTTGAACCCGAGAGCATGT
Ile Leu Leu Glu Pro Glu Ser
416 417 418 419 420 421 422

Figure 3.5 **The Expected Restriction Fragments of the M13 Vectors. A: Shown are the expected fragments produced by digesting the cloned M13-Ad (wild type) vector with HindIII and XhoII. Expected are the 524 base pair "working" adenovirus fragment, a 700 base pair M13 fragment, and the remainder of the M13 sequences. B: Shown are the expected fragments produced by digesting the M13-93R vector containing the 93R splice acceptor mutation, which creates a BstEII site, with HindIII and BstEII. Expected in the mutant are a 409 base pair fragment and the remainder of the vector sequences, while in the wild type vector only the opened vector is expected. C: Shown are the expected fragments produced by digesting the M13-156R vector containing the 156R splice acceptor mutation, which leads to a loss of a BanII site, with HindIII and BanII. Expected in the wild type vector are a 469 base pair fragment, a 79 base pair fragment, a 594 base pair fragment, and the remainder of the vector, while in the mutant vector a 538 base pair fragment is expected instead of the 469 and 79 base pair fragments.**


A
XhoII
HindIII
524 nucleotides
700 nucleotides
M13-Ad
XhoII
B
409 nucleotides
BstEII
XhoII
HindIII
M13-93R
C
469 nucleotides
79 nucleotides
BanII
XhoII
HindIII
BanII
538 nucleotides
594 nucleotides
M13-156R
BanII

the HindIII site and at the other end at a BanII site just outside the polylinker region in the M13 sequences. Also produced was a 594 base fragment of M13 sequences (see Figure 3.5) and in the "wild type" phage a 79 base fragment, which was not resolved on the agarose gel. These fragments are seen as the appropriate bands on an ethidium bromide stained 1% agarose gel (Figure 3.6).

The restriction patterns on agarose gel electrophoresis showed clearly that the mutations were present in the M13 clones. Yet, in order to ensure that no other mutations were introduced into the adenoviral sequence, which could affect the coding region of 496R, the entire mutated fragment was sequenced while still in the M13 construct. Particular attention was given to the area surrounding the mutated site as problems occasionally arise due to difficulties in initiation from the primer by the polymerase. The actual mutations were confirmed by this procedure, as is shown in Figures 3.7 and 3.8, for the 93R and 156R mutations, respectively. Single stranded and double stranded sequencing also confirmed that the remaining adenoviral sequences were free of undesired mutations. Double stranded sequencing was also performed on the adenoviral sequences upon rescue back into pXC38 to ensure that no mishaps occurred during this procedure (data not shown).

3.2.3 Rescue of Mutated Sequences into pXC38

Wild type pXC38 was digested with HindIII and BglII and the larger band containing the majority of the pXC38 sequences was purified from a 5%

Figure 3.6 Restriction Patterns of the Wild Type and Mutant M13 Vectors as run on a 1% Agarose Gel. Lane A is the lambda-ϕ174 phages digested with HindIII and HincIII respectively, used as markers with the sizes in base pairs shown. Lanes B,C,and D are the wild type, M13-93R mutant, and M13-156R mutant, respectively, all digested with HindIII and XhoII, which is indicative of the presence of the 524 base pair adenovirus insert. Lanes E and F are the M13-93R mutant and wild type vector, respectively, digested with HindIII and BstEII, showing the appearance of the 409 base pair fragment in the mutant. Lanes G and H are the M13-156R mutant and the wild type vector, respectively, digested with HindIII and BanII. Shown, as expected, is a shift in the mutant to a 538 base fragment from a 469 base pair fragment in the wild type.

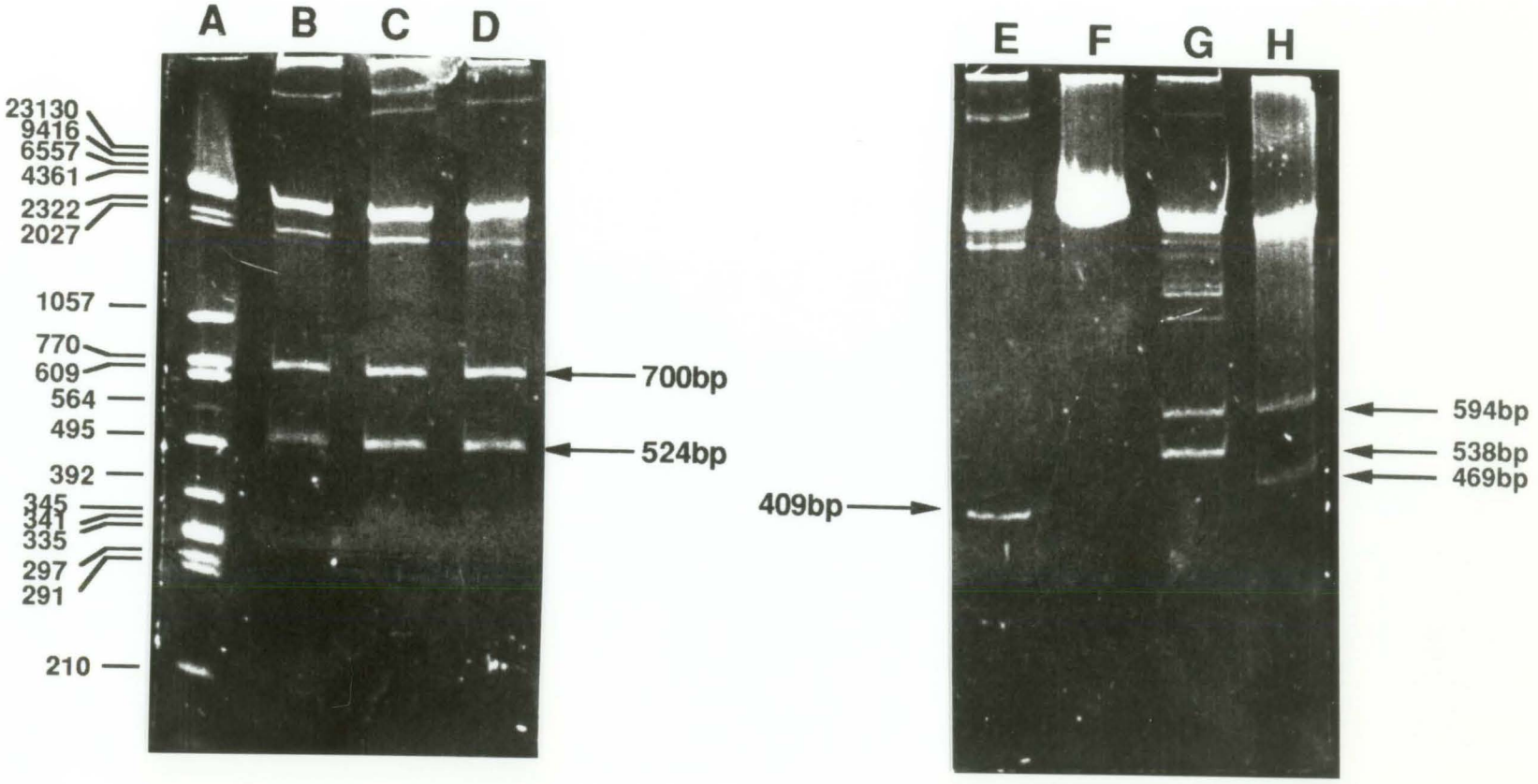

A
B
C
D
E
F
G
H
23130
9416
6557
4361
2322
2027
1057
770
609
564
495
392
345
341
335
297
291
210
700bp
524bp
409bp
594bp
538bp
469bp

Figure 3.7 Dideoxyribonucleic acid Sequencing of the Region Surrounding the 93R Splice Acceptor Mutation. Shown to the left of the sequencing gel is the sequence read from the region indicated. A indicates an Adenosine, G indicates a Guanosine, T indicates a Thymi dine, and C indicates a Cytosine at that position in the sequence. The letters along the top refer to the particular dideoxyribonucleic acid sequencing mix added to the reaction mixture loaded into each lane. The lanes were loaded in such a way as to ensure that every reaction mixture was loaded next to every other different reaction mixture in the series.

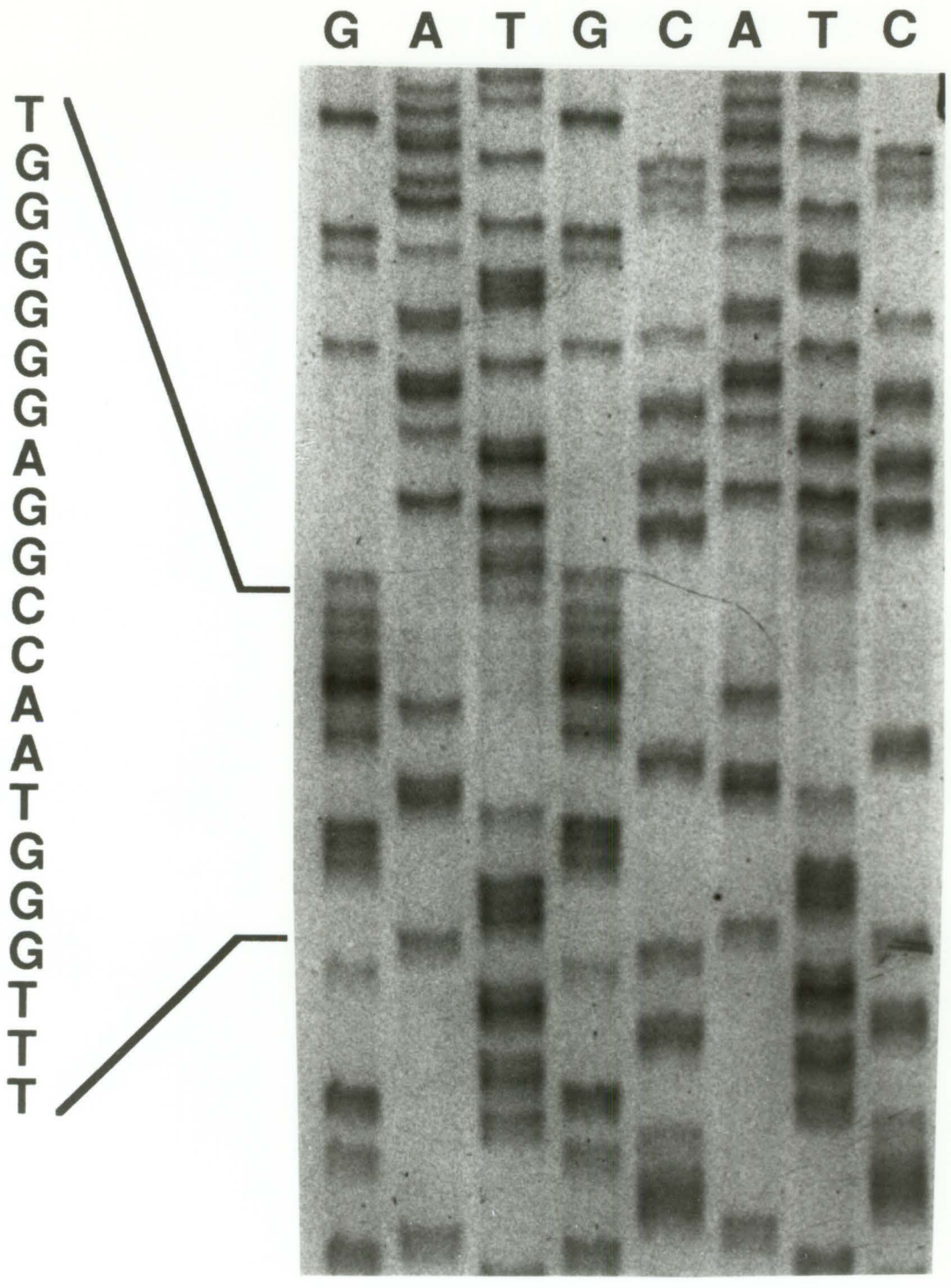
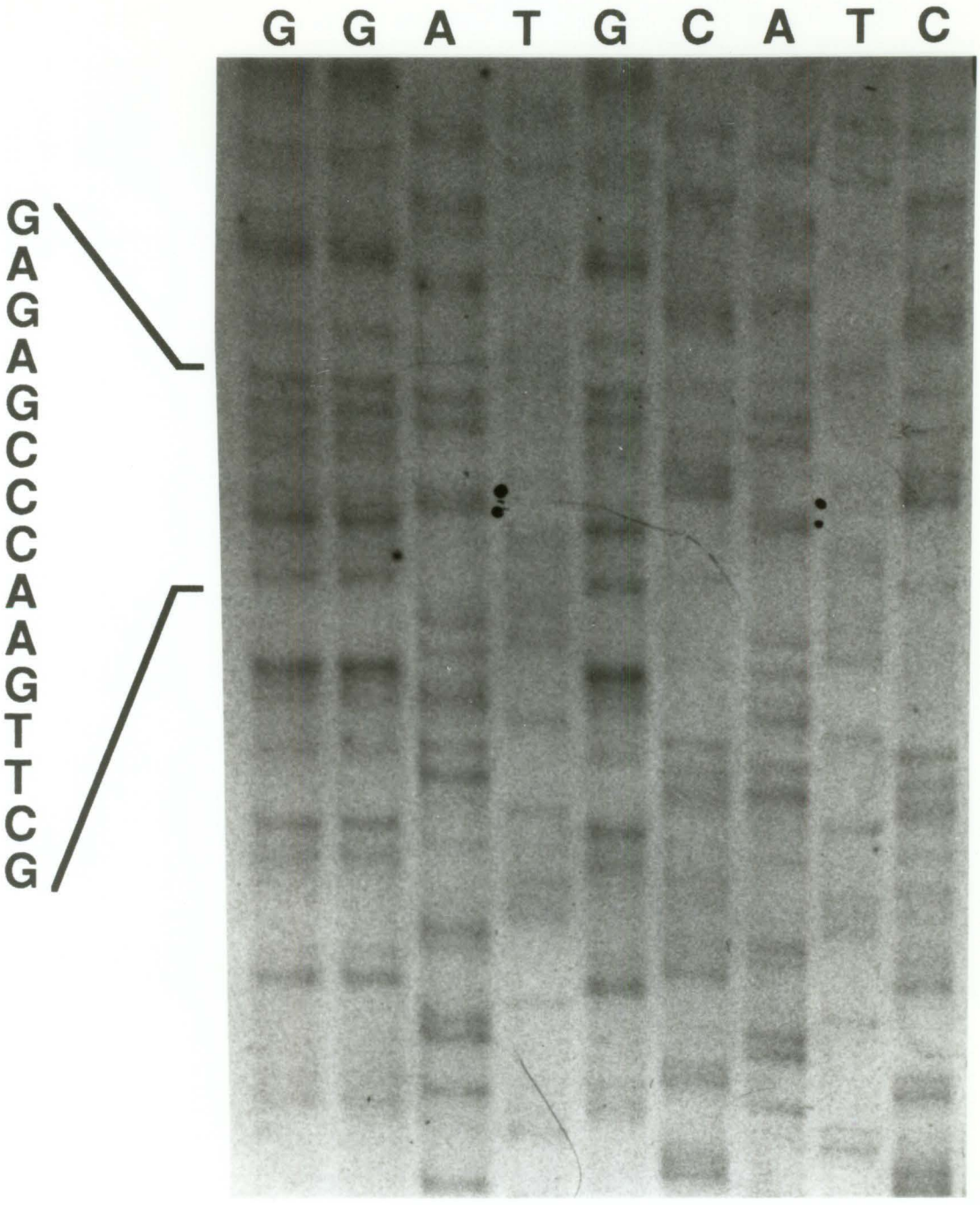

G A T G C A T C
T G G G G G A G G C C A A T G G G T T T

Figure 3.8 Dideoxyribonucleic acid Sequencing of the Region Surrounding the 156R Splice Acceptor Mutation. Shown to the left of the sequencing gel is the sequence read from the region indicated. A indicates an Adenosine, G indicates a Guanosine, T indicates a Thymidine, and C indicates a Cytosine at that position in the sequence. The letters along the top refer to the particular dideoxyribonucleic acid sequencing mix added to the reaction mixture loaded into each lane. The lanes were loaded in such a way as to ensure that every reaction mixture was loaded next to every other different reaction mixture in the series.

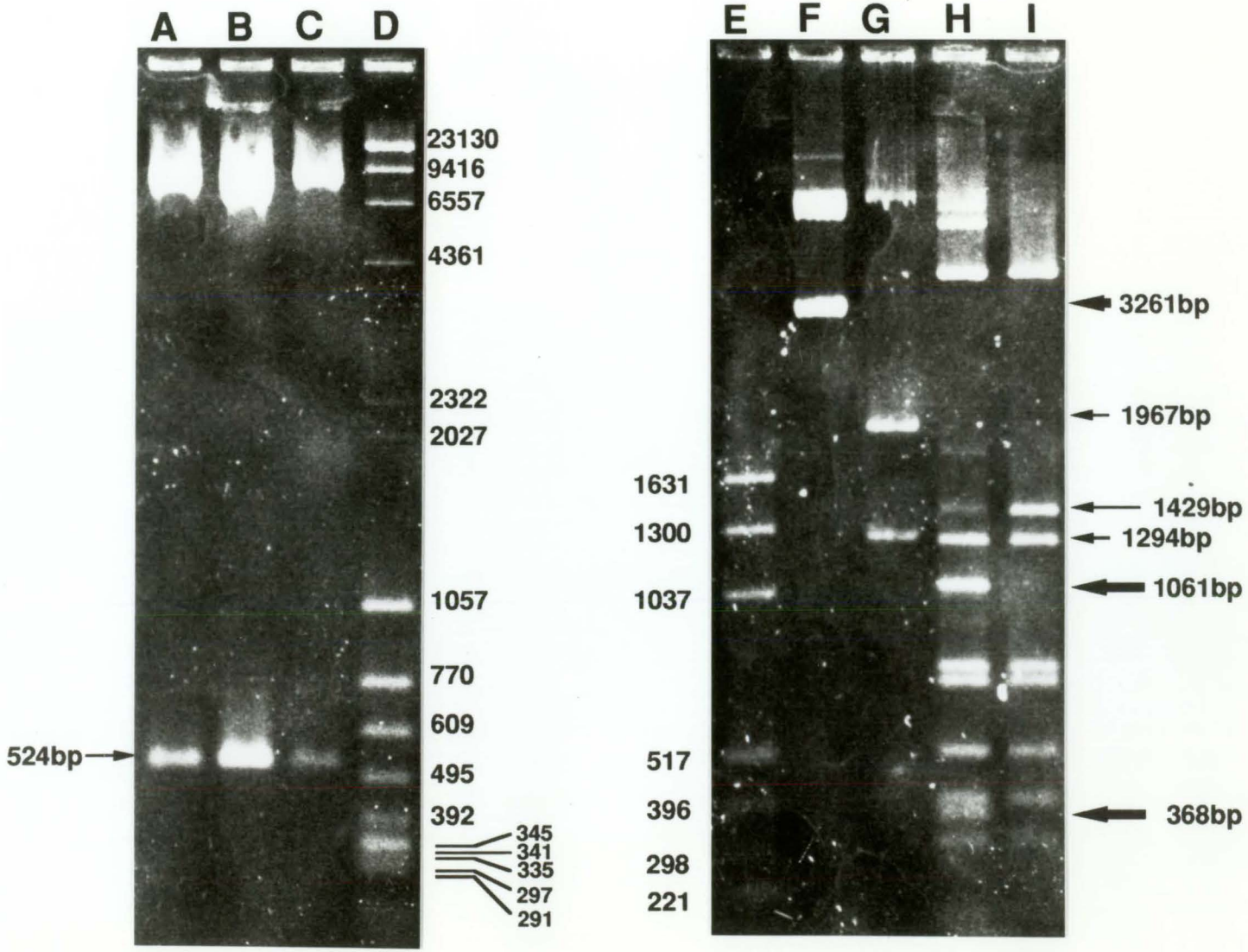

G G A T G C A T C
G
A
G
A
G
C
C
C
C
A
A
G
T
T
C
G

polyacrylamide gel. The mutant M13 clones were likewise digested with HindIII and XhoII and the 524 base fragment similarly isolated. The two DNA fragments were ligated as in Section 2.6.2 and used to transform competent E. coli LE392 cells. Once the cells were grown up, the DNA was isolated by the procedures outlined in Section 2.3.6 and screened by restriction analysis. The 93R mutant, now called pXC38/93-3216, was screened by digestion with BstEII. The mutant, differing from the wild type plasmid by the addition of a BstEII site, created two fragments of 1967 and 1294 base pairs from a 3261 base pair fragment seen in the wild type pXC38 (Figure 3.9). Also seen in Figure 3.9 is the restriction pattern observed for the 156R mutant (pXC38/156R-3275), which, due to the elimination of a BanII site, resulted in the combination of two smaller fragments of 368 base pairs and 1061 base pairs into a larger fragment of 1429 base pairs. The oberved restriction patterns for both mutants followed the predicted pattern based on the Ad5 genomic sequences. The two mutant plasmids were then used to test for DNA-mediated transformation in primary rodent cells and to rescue the mutations into virus by the method of McGrory and Graham (1988).

3.3 DNA-Mediated Transformation of Primary Rodent Cells

Plasmid DNA containing the appropriately altered sequences was used in transformation, which employed primary rat kidney cells. Due to the variability of the assay, it was necessary to repeat this experiment several times, using different DNA preparations and different preparations of baby rat kidney (BRK)

Figure 3.9 **Rescue of the Mutated Sequences into pXC38. Lanes A, B,and C are the wild type pXC38, pXC38/93-3216, and pXC38/156-3275, respectively, digested with HindIII and BglII, showing, in the case of the mutants, the presence of the mutated 524 base pair "working" fragment. Lane D is the lambda-ϕ174 molecular weight marker seen previously and lane E is pTK173 digested with HinfI used as a marker with the sizes in base pairs shown. Lanes F and G are pXC38 and pXC38/93-3216, respectively, digested with BstEII, showing the cleaving of the wild type 3261 base pair fragment to 1967 and 1294 base pair fragments in the mutant. Lanes H and I are pXC38 and pXC38/156-3275, respectively, digested with BanII, showing the formation of a 1429 base pair fragment in the mutant from a 1061 and a 368 base pair fragments in the wild type. The numbers and arrows to the right of the gel indicate the fragments mentioned above, with the thick arrows indicating the wild type fragments, the thin arrows indicating the mutant fragments, the short arrows representing the BstEII digested lanes, and the longer arrows representing the BanII digested lanes.**


A
B
C
D
23130
9416
6557
4361
2322
2027
1057
770
609
524bp
495
392
345
341
335
297
291
E
F
G
H
I
3261bp
1967bp
1631
1429bp
1300
1294bp
1061bp
1037
517
396
368bp
298
221

cells. It had been found previously that the condition of the BRK cells made it difficult at best to standardize this assay and it was necessary to repeat the assay at least three times (see McGrory thesis, 1988). It had also been determined previously that the optimum concentration of DNA used in the assay was 5μg (see McGrory thesis, 1988). The transformation assay was repeated three times for each plasmid and the number of transformed foci was determined (Table 3.2). It can be seen from Table 3.2 that for both mutant plasmids transformed foci were obtained at wild type efficiencies, suggesting that neither the 93R nor the 156R protein was involved in plasmid mediated transformation.

3.4 Rescue of the Mutations into Virus

Both mutations were rescued into virus by the method of McGrory et al. (1988) (see 2.11). As the plasmid pJM17 was used, all viruses were in a dl309 background, making dl309 the wild type virus used in all cases. Again, as determined by McGrory et al. (1988), 5μg of each plasmid and 5μg of pJM17 were used as the optimum DNA concentration to transfect 293 cells. Plaques were visible between 7 to 10 days post-transfection and were picked and expanded on 293 cells. DNA was harvested by Hirt extraction (2.12.2) and the DNA screened by restriction analysis.

The DNA from the 93R viral mutant (now called sa93R-3216) was digested with the restriction enzyme BstEII and run out on a 1% agarose gel and viewed by ethidiun bromide staining. Figure 3.10 shows the resulting restriction pattern

TABLE 3.2 Transformation of BRK Cells by Mutant and Wild Type Ad5 E1b Genes in E1 Plasmids.

Plasmid	Number of Foci			Average Number of Foci per Plate /standard deviation			%Wild Type
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3	
pXC38	15,17	3,2,2, 2,1,1	7,6,5,5, 4,4,2,1	16.00/ ±1.41	1.83/ ±0.75	4.25/ ±1.98	100
pXC38/93R-3216	nd	5,4,4,3, 3,2,1,0	9,5,5,4, 3,2,2,1	na	2.75/ ±1.67	3.88/ ±2.53	121
pXC38/156R-3275	14,13, 20,11	3,2,2, 2,1,1,	9,9,8,6, 5,4,4,2	14.50/ ±3.87	1.83/ ±0.75	5.88/ ±2.59	110
E1a Alone		0,0,0, 0,0,0			0		0

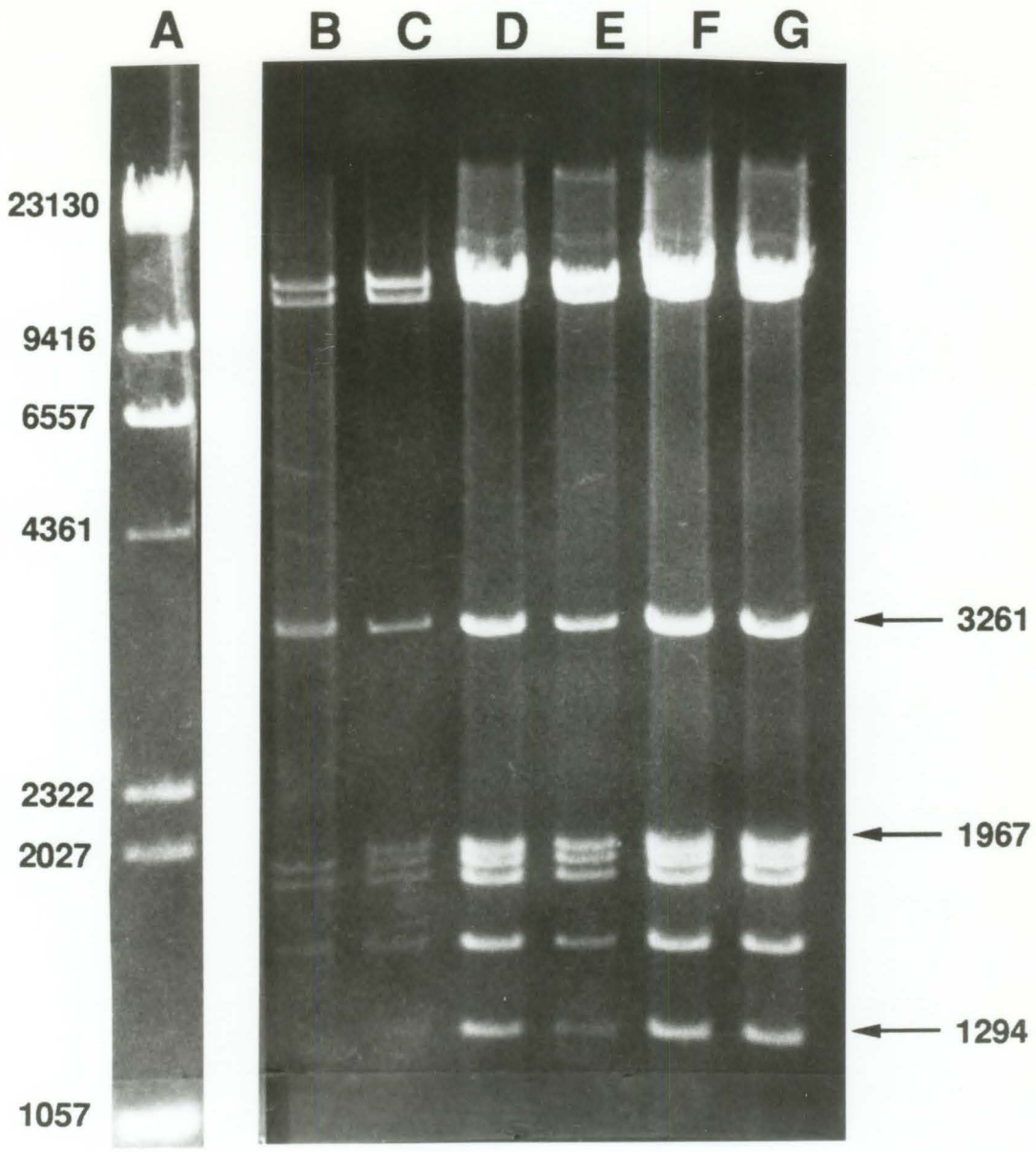
Baby Rat Kidney cells were prepared and transfected as described in the Materials and Methods. Shown here are the results of three experiments using pXC38/156R-3275 and wt ,and two experiments using the pXC3893R-3216 mutant and wt. The number of foci, the average number of foci per plate and the total percentage of wild type are given. Note: for the pXC38/93R-2316 mutant the % wild type is given compared to the total wild type foci for experiments 2 and 3 only.

The % wt was calculated as an average of the sum of the percentage of wt for each individual experiment as calculated by the following equation:

$$\%wt = 100\% \times \frac{1}{n} \sum_{i=1}^{n} \left(\frac{\bar{x}_i}{\bar{x}_{wt}} \right)$$

T-tests were conducted on all three experiments. The tests showed that differences in the data were not significant.

Figure 3.10 Rescue of the 93R Mutation into Virus. Lane A is the lambda-ϕ174 HincIII digest used as a marker. Lane B is the wild type dl309, digested with BstEII, showing the wild restriction pattern. Lanes C to G are different plaque isolates of the sa93R-3216 mutant. Shown to the right of the gel are the fragments of interest, showing the 3261 base pair fragment of the wild type virus, that is cleaved in the mutant to form the 1967 and 1294 base pair fragments. The number of mutants obtained is indicative of the efficiency of the rescue procedure.


A
B
C
D
E
F
G
23130
9416
6557
4361
2322
2027
1057
3261
1967
1294

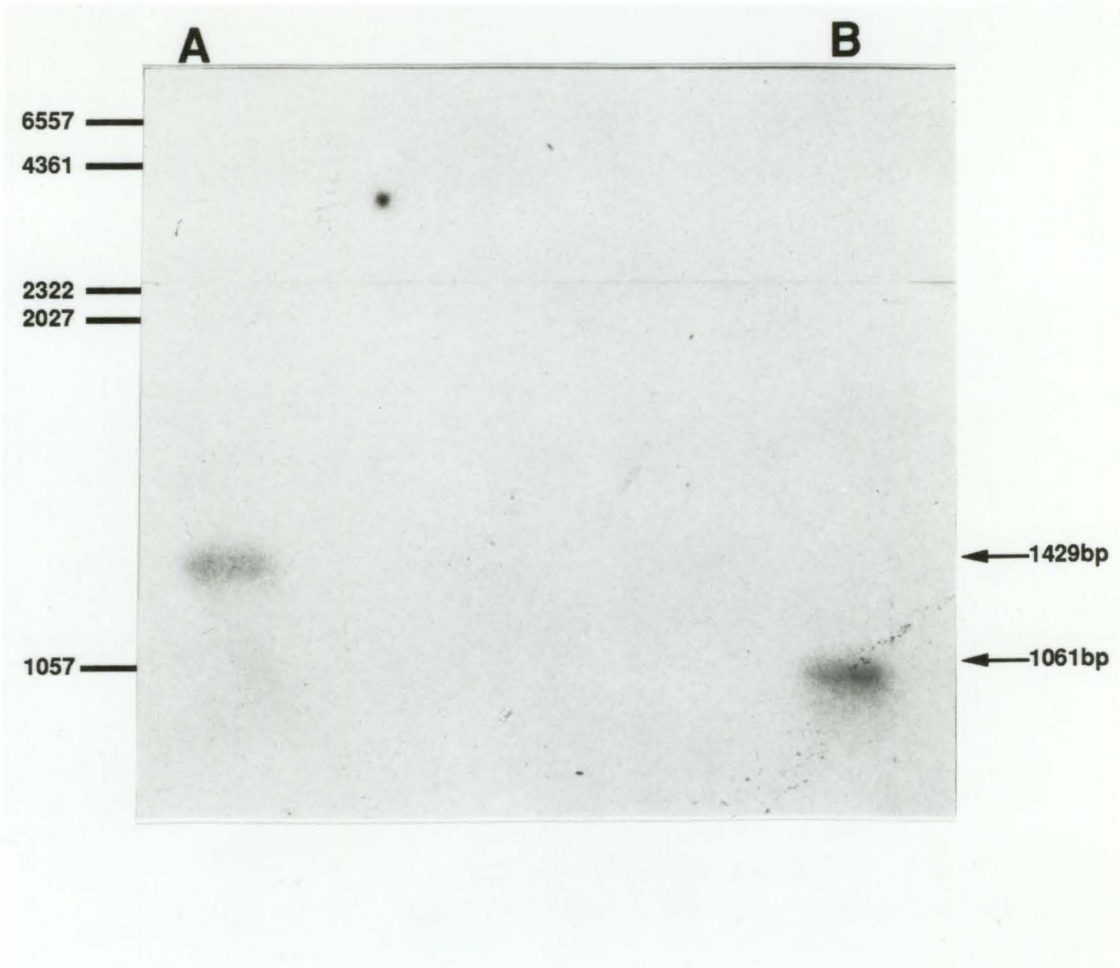
and, as expected from the plasmid data, a 3261 base pair fragment, seen in the wild type dl309 (lane B), was cleaved by the addition of the site to form 1967 and 1294 base pair fragments (lanes C-G). DNA from the 156R viral mutant (sa156R-3275) was similarly digested with BanII, but because of the large number of fragments produced, was screened by Southern blot analysis (see Section 2.12.3). The probe used was a sequencing oligonucleotide labelled 5' with ^{32}P. The oligonucleotide sequence was located upstream of the site of the mutation and should bind to the 1061 and 1429 base pair fragments seen in the plasmid data (Figure 3.9). Figure 3.11 is one such Soutern blot, showing the 1061 base pair fragment of the wild type (lane B) and the 1429 base pair of the mutant (lane A). This shift was, of course, due the loss of the BanII site at 3275. With both mutations rescued into virus, production of the proteins in question could be assessed and any resulting phenotype explored.

3.5 Analysis of the Proteins Produced by the Mutant Viruses

3.5.1 An Overview of the Anti-Peptide Sera Used

Antisera had been made previously by Dennis Takayesu to peptides based on the predicted sequence of 496R and the 496R-related proteins. The antisera were prepared in rabbits as described in Yee et al. (1983) against peptides corresponding to the amino termini of 496R, 156R, 93R, and 84R (all common, see Figure 1.2), which was called 58-N2,and to the carboxy termini of 93R and

Figure 3.11 Southern Blot Analysis of the 156R Mutation After rescue into Virus. Shown is an autoradiograph of a Southern blot, with lane a being a plaque isolate of the sa156R-3275 mutant and lane B being the wild type dl309, both digested with BanII and probed with a ^{32}P-labelled oligonucleotide. The numbers to the left of the gel refer to the fragments of the lambda-ϕ174 HincIII marker. Shown to the right of the gel are the sizes of the fragments to which the probe hybridized, showing the shift from the 1061 base pair fragment of the wild type to the 1429 base pair fragment of the mutant.


A
B
6557
4361
2322
2027
1057
1429bp
1061bp

84R, called 93-C1 and 84-C1, respectively. Also used was an antiserum which recognizes the carboxy terminus of 496R (and hence 156R as well) called 58-C1, which has been described previously (Yee et al., 1983). A summary of the peptides used can be found in Table 3.3.

3.5.2 Determination of the Proteins Produced

Proteins were immunoprecipitated by the various antisera by the method outlined in Section 2.15.2. The 496R major E1b protein has been found previously to migrate on an SDS-polyacrylamide gels as a single broad band with an apparent molecular weight of 58,000 (Lewis and Anderson, 1987; Yee et al., 1983; Brown et al., 1990, in preparation). The 155R and 82R species of Ad2 had also been identified, using antisera specific for the amino terminus of 495R and which ran with apparent molecular masses of 18,000 and 15,000 daltons, respectively (Anderson et al., 1984; Lewis and Anderson, 1987). Using the 58-N2 serum described above and in Table 3.3, the 496R protein was identified on SDS-PAGE at 58,000 daltons as expected. Also brought down by this antiserum was a major protein with an apparent molecular weight of 20,000 daltons that corresponded to the 84R protein, as it co-migrated with a similar major species brought down by the 84-C1 antisera specific to the carboxy terminus of 84R (D. Takayesu, unpublished). Immediately visible also were two species that migrated at around 25,000 daltons and which probably corresponded to the two 156R that differ only by the phosphorylation of the slower migrating species (D. Takayesu,

TABLE 3.3 SUMMARY OF ANTI-PEPTIDE SERA USED

Protein	Terminus	Name of Serum	Peptide Used
496R	N-terminus	58-N2	NH_2-M(Ac)ERRNPSERGY-COOH
496R	C-terminus	58-C1*	NH_2-YSDEDTD-COOH
93R	N-terminus	58-N2	see above
93R	C-terminus	93-C1	NH_2-YPTLPMQFESH-COOH
156R	N-terminus	58-N2	see above
156R	C-terminus	58-C1	see above

Peptides were purchased from Biosearch (San Rapael, California) or Bachem (Torrance, California) and treated as outlined in Yee et al. (1983).

New Zealand rabbits were used as the source of the antisera after being injected with the various peptides.

* Described previously in Yee et al. (1983).

unpublished). These two proteins were also precipitated by the 58-C1 antiserum, indicating that they do share both amino and carboxy termini with 496R (Figure 3.12, lane G). Figure 3.12 also shows wild type dl309 polypeptides immunoprecipitated with 58-N2 serum in either the presence or absence of the 58-N2 peptide to which the antibody was raised (lanes B and C, respectively). The 93R protein, on the other hand, was a very minor product that in previous studies have been found to migrate just above the 84R and not clearly visible without extensive exposure of the gel to the film (D. Takayesu, unpublished). Thus the 93R protein was only seen in these other studies using the antiserum specific to the carboxy terminus of 93R (93-C1), although even then it was often found nestled in among 4 or 5 as yet unidentified proteins. In addition, the antiserum to 93-C1 was of low affinity adding to the difficulties in identification of 93R.

When the proteins produced by the mutant virus sa156R-3275 were immunoprecipitated with 58-N2 the most surprising discovery was the existence of two species which migrated somewhat faster than the 156R protein produced by the wild type (Figure 3.12, lane D). These species were also evident when the 58-C1 serum was used (Figure 3.12, lane H) indicating that these proteins, like the 156R species, possessed both the amino and carboxy termini of 496R. Also like the 156R proteins, precipitation of these two new species could be blocked when the 58-N2 serum was combined with the 58-N2 peptide (Figure 3.13, lanes E and F). Similar competition was observed between the 58-C1 serum and its peptide (Figure 3.13, lanes S and T). It then seemed from these data that these new products were probably related to the 156R species and were produced from

Figure 3.12 Immunoprecipitation of Infected Cell Extracts Labelled with ^{35}S-Methionine and run on a 15% SDS-Polyacryamde Gel. Cells were infected with wild type or mutant viruses and harvested 16-18 hours post-infection. Lane A is a molecular weight marker with the weights of the components listed on the left in kilodaltons. Lanes B-F were immunoprecipitated with the 58-N2 serum and exposed on film 5 hours, with (B) wild type dl309 with peptide, (C) wild type dl309, (D) sa156-3275, (E) sa93-3216, and (F) mock-infected. Lanes G-J were immunoprecipitated with 58-C1 serum and exposed on film overnight, with (G) wild type dl309, (H) sa156-3275, (I) sa93-3216, and (J) mock-infected. Lanes K-N were immunoprecipitated with 93-C1 and exposed on film 4 weeks, with (K) wild type dl309, (L) sa93-3216, (M) sa156-3275, and (N) mock-infected. The arrow labelled 93R shows the region where the 93R protein has been found previously to run on SDS-PAGE. Note that 93R was not detected in this study.

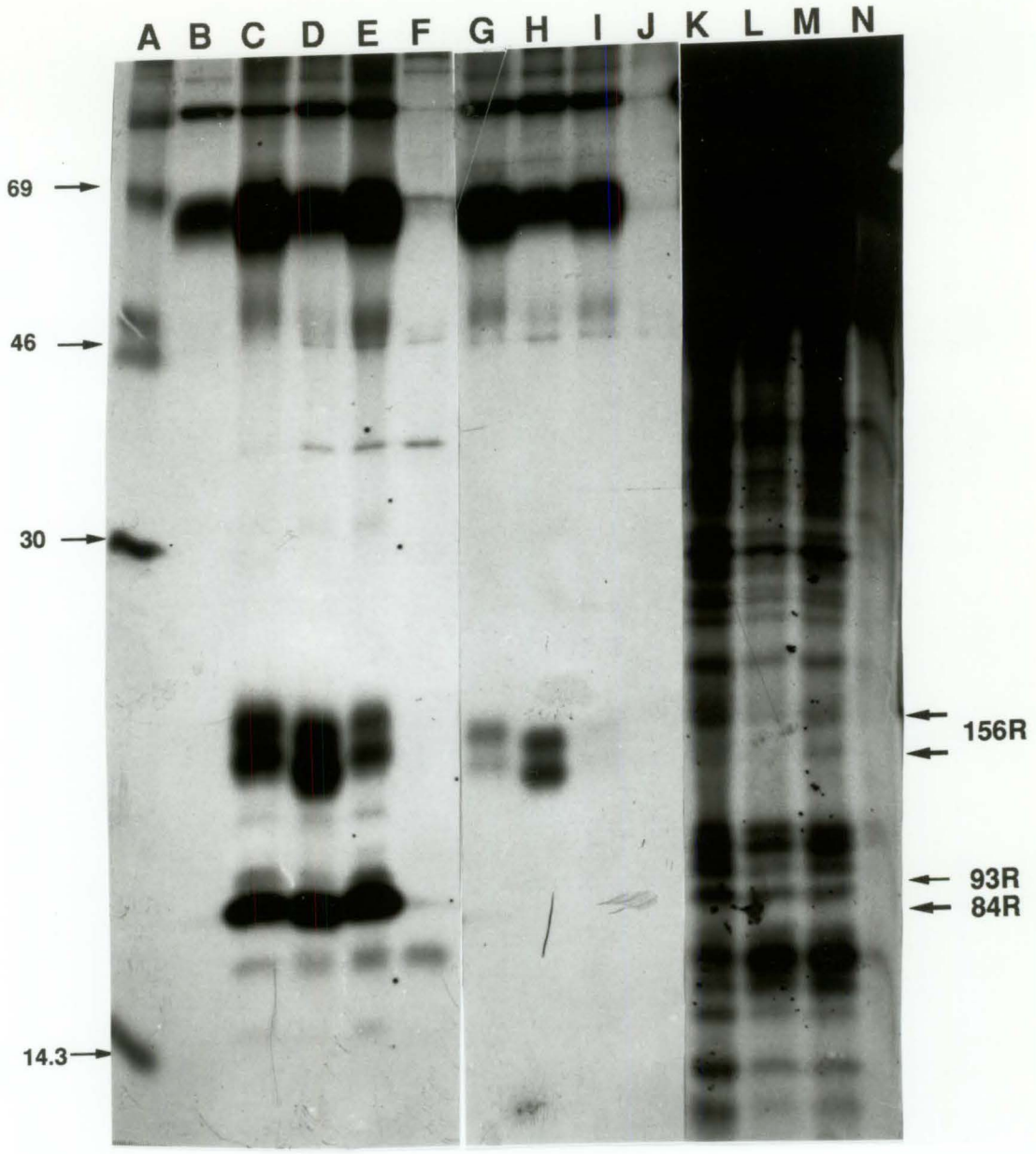
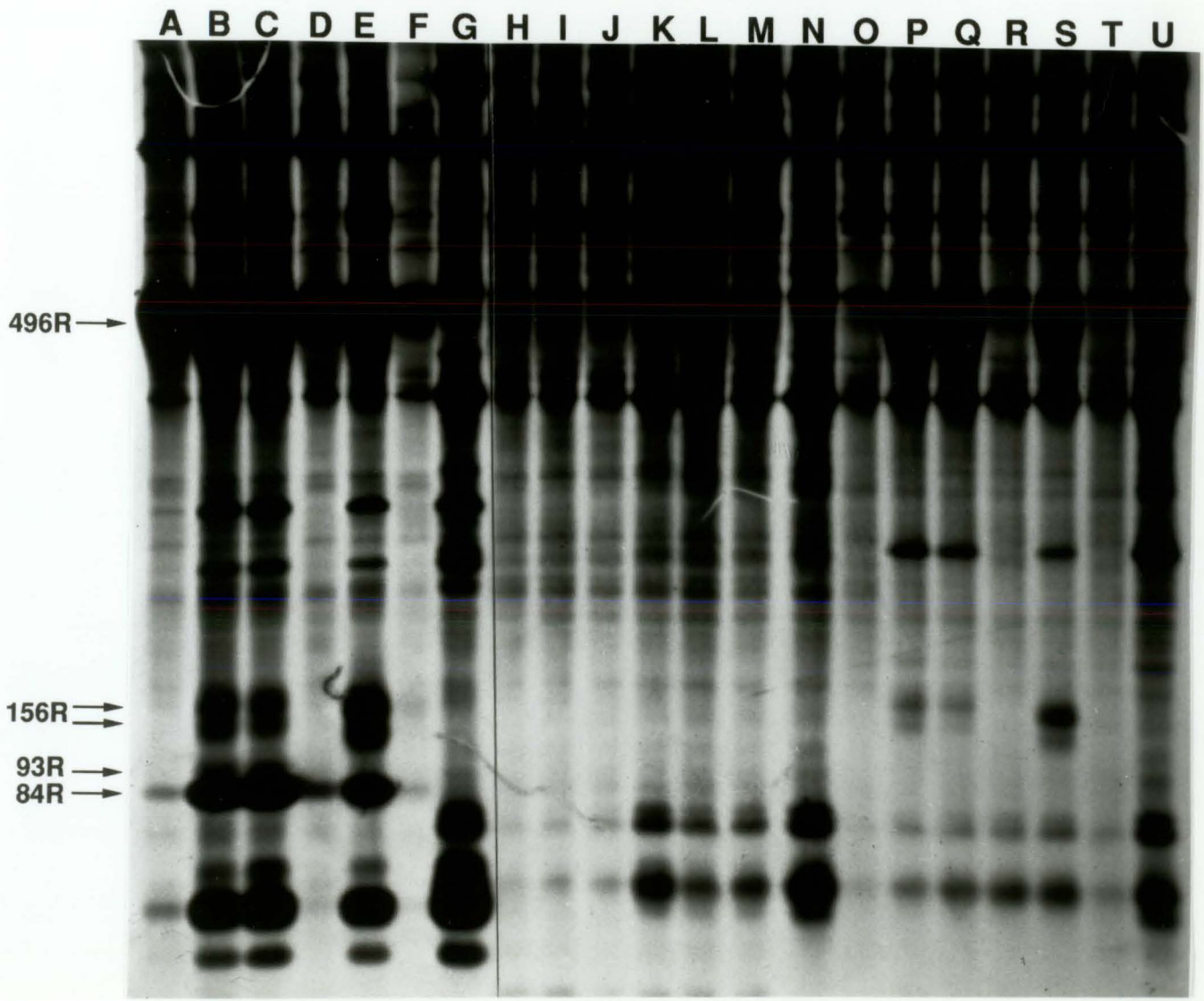

A B C D E F G H I J K L M N
69
46
30
14.3
156R
93R
84R

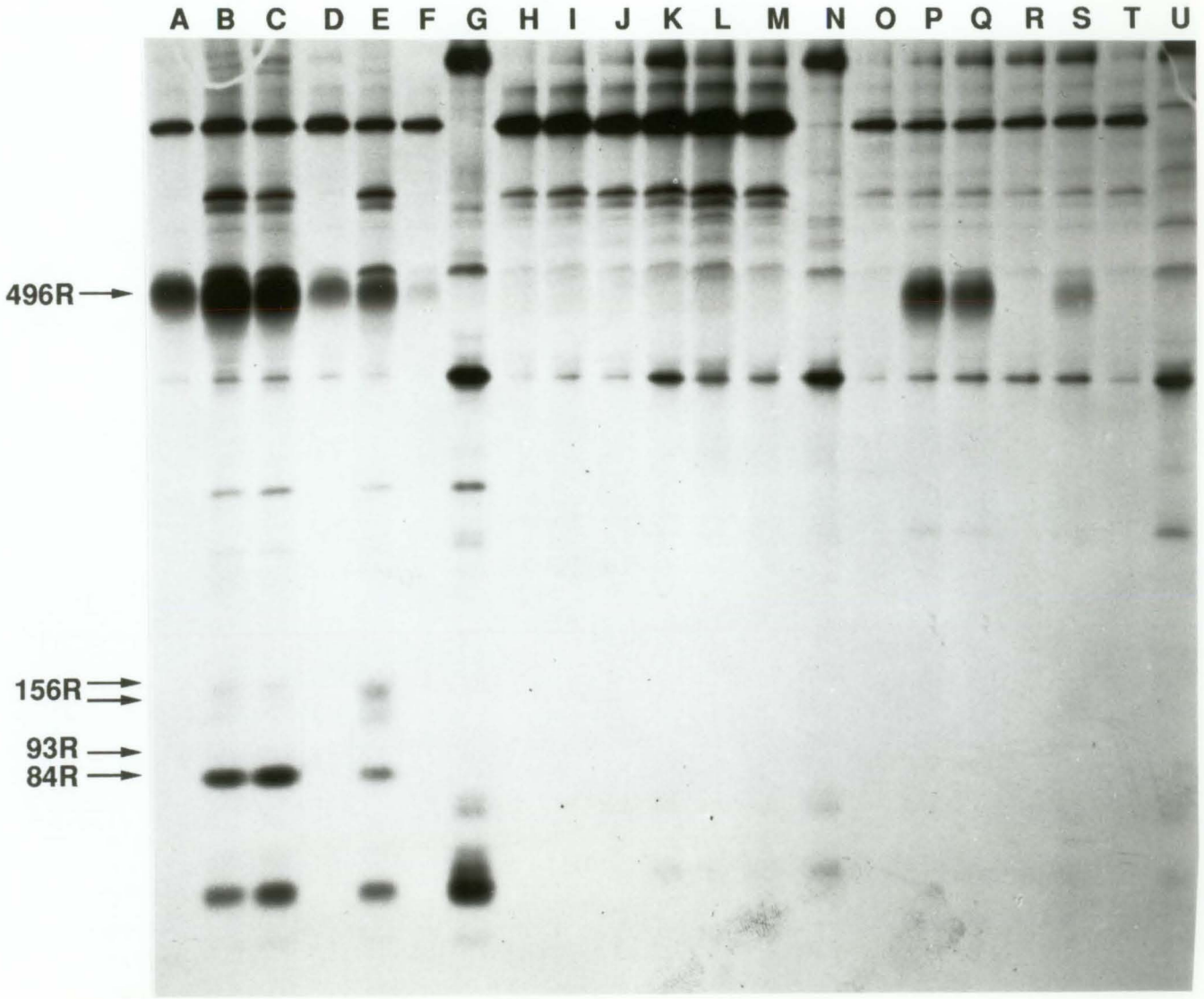
Figure 3.13 Immunoprecipitation of Infected Cell Extracts Labelled with ^{35}S-Methionine, showing with and without peptide chases and run on a 15% SDS-Polyacryamde Gel. Cells were infected with wild type or mutant viruses and harvested 16-18 hours post-infection. This is a 4 week exposure of the gel on film. Lanes A-G were immunprecipitated with the 58-N2 serum, with (A) wild type dl309 (wt) with peptide, (B) wt, (C) sa93-3216, (D) sa93-3216 with peptide, (E) sa156-3275, (F) sa156-3275 with peptide, and (G) mock-infected. Lanes H-N were immunoprecipitated with the 93-C1 serum, with (H) wt with peptide, (I) wt, (J) sa93-3216, (K) sa93-3216 with peptide, (L) sa156-3275, (M) sa156-3275 with peptide, and (N) mock-infected. Lanes O-U were immunoprecipitated with 58-C1 serum, with (O) wt with peptide, (P) wt, (Q) sa93-3216, (R) sa93-3216 with peptide, (S) sa156-3275, (T) sa156-3275 with peptide, and (U) mock-infected. The relative proteins are indicated on the left. The arrow labelled 93R shows the region where 93R has been known to run on SDS-PAGE in previous studies. Note that 93R was not detected in this study.


A B C D E F G H I J K L M N O P Q R S T U
496R
156R
93R
84R

an mRNA which used a new downstream splice acceptor (see Discussion). Another very interesting observation was the relative intensity of the bands for the mutant proteins when compared to the wild type 156R species. Figure 3.12, lanes C and D show the wild type and mutant viruses immunoprecipitated with 58-N2. It seems evident that the two new species seem more intense than the wild type 156R, but more interestingly the new species are far more intense compared with the mutant 496R (Figure 3.12, lane D) than the wild type 156R is to the wild type 496R (Figure 3.12, lane C). This increased relative intensity of the bands was also seen in Figures 3.13 and 3.14, lanes B and E (note that these figures are different exposures of the same gel). This result was consistent in subsequent gels. So not only are these species smaller, but they seem to be made made in increased amounts at the expense of 496R.

The results from the 93R mutant sa93R-3218 were more ambiguous. Due to the difficulties mentioned above, the 93R protein was never observed in the present experiments and thus it was not possible to determine if the mutant failed to produce 93R. Even though the proteins were extracted many times using the 93-C1 serum no change was observed in the migration pattern on SDS-PAGE. Figure 3.12, lanes K-N, shows a typical SDS-polyacryamide gel after a 4-6 week exposure. No apparent differences were observed between the wild type (lane K) and the 93R mutant (lane L). A number of explanations seem possible. Perhaps the 93-C1 antiserum was not avid enough to routinely yield detectable levels of 93R. In addition, the large number of other proteins were brought down non-specifically in the expected region of the gel and thus any changes in 93R would

Figure 3.14 Immunoprecipitation of Infected Cell Extracts Labelled with ^{35}S-Methionine, showing with and without peptide chases and run on a 15% SDS-Polyacryamde Gel. Cells were infected with wild type or mutant viruses and harvested 16-18 hours post-infection. This is an overnight exposure of the gel on film. Lanes A-G were immunprecipitated with the 58-N2 serum, with (A) wild type dl309 (wt) with peptide, (B) wt, (C) sa93-3216, (D) sa93-3216 with peptide, (E) sa156-3275, (F) sa156-3275 with peptide, and (G) mock-infected. Lanes H-N were immunoprecipitated with the 93-C1 serum, with (H) wt with peptide, (I) wt, (J) sa93-3216, (K) sa93-3216 with peptide, (L) sa156-3275, (M) sa156-3275 with peptide, and (N) mock-infected. Lanes O-U were immunoprecipitated with 58-C1 serum, with (O) wt with peptide, (P) wt, (Q) sa93-3216, (R) sa93-3216 with peptide, (S) sa156-3275, (T) sa156-3275 with peptide, and (U) mock-infected. The relative proteins are indicated on the left. The arrow labelled 93R shows the region where 93R has been known to run on SDS-PAGE in previous studies. Note that 93R was not detected in this study.


A B C D E F G H I J K L M N O P Q R S T U
496R
156R
93R
84R

be difficult to observe, or given the result with 156R it is possible that any change to the amino acid sequence of 93R due to possible alternative splicing may be slight and thus undetectable by SDS-PAGE (see Discussion).

In summary, it seemed that the mutant virus sa156R-3275 produced the 156R protein (although probably modified) that was intended to be eliminated by interfering with the splice acceptor site of the respective mRNA. A similar conclusion could not be reached concerning the mutant sa93R-3216, given that the protein could not be detected on SDS-PAGE.

3.6 Effects of the Mutations on Productive Infection

To study whether these mutants had any effect on viral replication, plaque formation on HeLa cells was compared to that on 293 cells (see Sections 2.9.3 and 2.14). Overall it was observed that the HeLa/293 plaque ratios were not significantly lower for the mutants than for the wild type dl309 (Table 3.4). The ratio of HeLa/293 cells for the mutant sa93R-3216 showed a 10 fold decrease from wild type. Given the rarity of 93R in infected cells this may be a significant result, yet generally in this assay there is little confidence in any result less than a thousand fold decrease. Thus, these data suggested that either the mutations introduced were not significant enough to inhibit viral growth or that mutation of these proteins did not affect growth in the presence of an intact 496R.

TABLE 3.4 PLAQUE TITRES ON HeLa AND 293 CELLS BY MUTANT AND WILD TYPE VIRUSES.

Virus	Plaque Titre (pfu/mL) on HeLa	293	Plaque Ratio HeLa/293
dl309 (wt)	$1.8X10^{10}$	$2.3X10^{10}$	0.78
sa93-3216	$2.0X10^{9}$	$2.7X10^{10}$	0.074
sa156-3275	$6.4X10^{9}$	$2.13X10^{10}$	0.30

Figures shown are the averages from two plaque titres. Viruses were plaqued in duplicate at several different dilutions on the 293 and HeLa cells. Plaques were counted after 10 to 15 days incubation at 37°C.

DISCUSSION

4.1 The Effects of Mutations in Splice Sites

The effects of mutations in 5' and 3' intron splice sites have been extensively studied in other systems and it has been determined that most changes in the consensus sequences will reduce or completely block the formation of the fully processed mRNA (Padgett *et al.*, 1986). In the case of the 5' splice donor any change in either the G or the U of consensus sequence AG:**GUAAGU** will completely disrupt splicing, most likely at the second stage of the intermediate and not at the level of cleavage (Sharp, 1987). Changes in the sequences flanking the GU can also inhibit the splicing process (Solnick, 1985). Mutations in the 3' consensus sequence can similarly reduce or eliminate splicing. Changes in the polypyrimidine tract can prevent formation of the spliceosome, while mutations in the terminal A or G can prevent cleavage at that point and thus cause the accumulation of the intermediate lariat (Sharp, 1987).

Mutations of splice sites have been very useful in the Adenoviral system for the functional analysis of both E1a and E1b gene products. Mutations of splice sites were used in E1a to separate the functions of the 289R 13S product from the 243R 12S product. Montell *et al.* (1982) mutated the 5' splice donor for the 12S message at nucleotide 973, such that the consensus GT was converted to a GG. This mutation prevented splicing at that point, creating a mutant that did not produce the 243R protein. Similarly, Montell and Berk (1984) mutated the GTA

of the consensus sequence at nucleotide 1114 to a GTT in order to disrupt the 5' splice donor for the 13S message, creating a mutant that did not produce the 289R product. Both these studies found that the mutations introduced to disrupt the splice site did not lead to the use of cryptic sites (see below) but instead the mutant viruses seemed to use the existing splice sites lying nearby (Montell and Berk, 1984). These existing splice sites were of course the 5' splice site for the other message thus ensuring that the desired message was eliminated and the other produced. These studies were special cases given the availability of nearby alternative splice sites. This was not the case with the E1b splice donor for the minor E1b products. Here Montell *et al.* (1984) converted the G of the consensus GT to a C and observed no obvious phenotype (see Introduction). Yet in this study, it was discovered that a downstream in-frame cryptic splice donor was used that would have added an additional 22 amino acids to the minor proteins, yet keeping the existing regions the same as the wild type sequence.

The observation of cryptic sites being used in lieu of mutated splice sites has been found in recent studies involving cellular genes as well, particularly at the 5' donor site. It seems that in the absence of the naturally occurring 5' splice site, a nearby splice site "of best fit" is used instead (Robberson *et al.*, 1990). These sites were located in the immediate vicinity of the mutated wild type site, with exon skipping occurring only when no cryptic sites were available (Robberson *et al.*, 1990). The best example of the use of cryptic sites is found in the ß-globin gene of humans and rabbits. In these genes, naturally occurring mutations in splice sites encourage the use of cryptic sites that cause the genetic abnormality

of ß-thalassaemia, such as hereditary persistence of foetal haemoglobin (HPFH) (Treisman *et al.*, 1983; Wieringa *et al.*, 1983). Treisman *et al.* (1983) also located a mutation in the second intron of human ß-globin that created a novel 5' splice site. In the latter case this mutation led to the activation of a normally silent 3' splice site upstream of the new donor that introduced an extra exon that could be eliminated by reverting the mutation that created the novel 5' donor (Treisman *et al.*, 1983). Cryptic sites are also known to be used efficiently *in vitro* when a normal 3' site in the human ß-globin gene is mutated (Krainer *et al.*, 1985). This result probably could have been expected given the lack of sequence specificity of 3' acceptor sites. This lack of specificity could account for many cases of the utilization of alternative splice sites (Reed, 1990).

Taking all the above into consideration, the current study set out to mutate the 3' acceptor sites of the 1.31 and 1.26Kb E1b messages that produce the 93R and 156R products, respectively. Although the concern for the potential use of cryptic 3' acceptor sites was present during the study, it was thought at the outset that, given the availability of nearby naturally utilized acceptor sites, the result would be the elimination of the desired message, and thus the protein, as was the case for the E1a splice mutants. Specifically, the nearby sites are the acceptor site for the other message in question (the 1.26 or 1.31 Kb message) and the acceptor for the 1.0Kb message that produces the 84R protein (see Introduction). Alternatively, perhaps cryptic sites would be used that could potentially destabilize the mRNA or protein resulting in their elimination or change the protein in such a way as to interfere with its function. Either result might have led to a better

understanding of the function of these minor products. As the case turned out neither result appeared to occur.

4.2 The 156R Splice Mutant

4.2.1 The Potential Existence of a Cryptic Site for the 156R Splice Mutant

The mutant plasmid that possessed the mutation at 3275 corresponding to the splice acceptor for the 1.26Kb mRNA, which produces the 156R protein, produced transformed foci on BRK cells with wild type efficiency. The corresponding mutant virus displayed plaque titres similar to those seen by the wild type dl309. These data, which initially suggested that 156R played little or no role in either transformation by DNA or viral growth, could be later accounted for by the production of the two new species running slightly faster on SDS-PAGE. These novel species possessed the same N- and C-termini as the wild type 156R species, differing only by their smaller size. These data were consistent with the explanation that these new species possessed an in-frame deletion caused by the use of an in-frame cryptic splice acceptor.

Although the studies to identify the potential new splice acceptor were not performed, a number of possible sites exist. The number of sites could be limited by the size of the new proteins as they ran only slightly faster than the equivalent wild type proteins on SDS-PAGE. Possible cryptic sites at 3280, 3292, 3328, and 3355 would give proteins of 154, 150, 138, 135, and 129 amino acids, respectively.

The actual site used could be determined by using appropriate S1 hybridization or PCR-mediated technique.

4.2.2 The Effects of the Use of the Cryptic Site on Protein Production

The most provocative effect the use of this cryptic site had on protein production was the accumulation of amounts of these mutant proteins greater than wild type proteins at the apparent expense of 496R. It could be that the new splice acceptor was a better 3' splice site than that currently used by the wild type virus. If this novel site was utilized more efficiently, the question arises as to why it is not used preferentially over the wild type site in the wild type virus? It is possible that the actual point mutation incorporated makes the upstream region a better binding site for the snRNP complex, which initiates splicing at this point, and making this site more prone to cleavage than the wild type site. Yet it is more likely that the wild type site is used over the new site due to its proximity to the branch point sequence. The importance of the distance between the AG of the splice site and the branch point is not clear, but it has been observed that in some cases the first AG downstream of the branch point is used as the splice acceptor (Krainer *et al.*, 1985; Reed and Maniatis, 1985). In the case of the 156R splice mutant, perhaps the cryptic site is a better site, yet is not used as the wild type site is physically closer to the branch point sequence. It is also provocative to postulate that, during evolution of the virus, the use of a weaker site at this point could have developed as some sort of regulatory mechanism to keep the

amounts of 156R to 496R at a certain ratio. The purpose of maintaining this ratio is unclear, since skewing this ratio, as was observed in the 156R splice mutant, seemed to have little or no effect on virus replication in HeLa cells. Perhaps, however, replication in epithelial cells, the normal targets of viral infection *in vivo*, could be affected.

The other interesting point to raise is the fact that the cryptic site is in-frame, thus preserving the N- and C-termini of the wild type 156R proteins. The questions that arise here are is this some kind of "safety" mechanism on the part of the virus; if so why did it develop; and what does this say about the importance of these proteins? If the use of the cryptic site is a safety mechanism to preserve the production of the proteins why would it have developed, given that only the wild type site is used normally. The explanation that is readily available is that perhaps the wild type site postdates the cryptic site, through the inclusion of a mutation, and was subsequently used due to its proximity to the branch point. The problem with this theory is that the use of the cryptic site seems to have no obvious detrimental effects on the virus and in itself may possess no selective disadvantage unless creation of the wild type site coincided with another event elsewhere in the virus. It also could be just coincidence that several in-frame AG dinucleotides are found downstream of an active splice site, which became available in the absence of the normal splice site. It is hard to say at this point which is the correct scenario.

Yet regardless of the origin of the splice site, the fact remains that the 156R proteins are largely preserved after mutation of the 3' splice acceptor. It would

be tempting to propose that the 156R product is required for Ad 5 functions, given the fact that it was still produced (at least in part) after the normal splice site was eliminated. Of course this statement cannot be put forward with certainty until mutants are constructed that completely eliminate these proteins.

In addition, it is not clear whether or not 156R is produced in 293 cells as it was not detectable using 58N-2 or 58C antisera (D. Takayesu, unpublished results; data not shown). If 156R is required for virus replication, it may not be possible to obtain a 156R$^-$ virus, given the current system of virus rescue and maintenance. Perhaps if 156R is not produced in 293 cells then only viruses with a functional 156R would be viable. At any rate this will not be made clear until the appropriate studies are undertaken.

4.3 The 93R Splice Mutant

It remains unclear as to what event(s) are occurring with the 93R splice mutant. Although the 93R product was never accurately identified in the current studies, neither was any change noted in the protein mobility pattern on SDS-PAGE for the mutant. This could mean that either the mutation was not present in the virus (which is unlikely given the expected DNA restriction pattern), the protein could not be identified given the poor affinity of the serum and the low levels of production of 93R or that any mutation that affected the amino acid sequence of the protein was so slight as to not be noticeable on SDS-PAGE. The problem in identifying 93R in these studies was probably a combination of the

latter two points above. Given the possibility that a cryptic site may be used in the case of the 156R splice mutant, perhaps a cryptic site is used in the case of the 93R splice mutant as well. If this is true the only available alternative AG lies at 3219, three nucleotides downstream from the wild type site, which would eliminate a single codon coding for a glutamic acid residue. Probably the elimination of this residue would be insufficient to be noticeable given the obscurities of the gel system used. Again the situation could be clarified by S1 hybridization or PCR techniques.

4.4 Conclusions, Criticisms, and Future Considerations

In summary, although some of the results presented here may be interesting, they were inconclusive. This study set out to characterize the minor E1b proteins by eliminating the 156R and 93R products through disruption of the splice acceptor sites for the 1.26 and 1.31Kb mRNAs, repectively. It did not succeed in doing so due to the probable utilization of an in-frame cryptic splice acceptor at least in the case of the 156R protein.

More questions than answers were raised in the course of this study. For example, what does the use of in-frame cryptic 3' splice acceptors really mean if that is indeed what is happening? Does it mean that these proteins are required for productive infection by the virus and certain built-in safety mechanisms are in place to ensure that they are produced? Or is the use of cryptic sites simply a product of the splicing mechanism and the fact that they may be in-frame merely

a coincidence? Thus the precise role of the 496R related proteins 156R and 93R as well as the other known E1b products remains to be discovered.

This then leads to a discussion of what may be done in the future to clarify the points raised in this thesis. The first step to continue this project would be the identification of the cryptic splice acceptor(s) by the appropriate S1 hybridization or PCR techniques. These results could lead to a clarification of what events are occurring in both the 156R and 93R mutants. These studies could naturally proceed into a mutational analysis of these cryptic sites to eliminate them in a continued attempt to eliminate the messages. For the 156R and 93R, there are a number of potential cryptic sites which could be mutated. This, however, may lead to the utilization of further cryptic site increasing the number of mutations that are required to be made in order to eliminate the messages. The effects of in-frame cryptic sites could be interesting as the protein is internally deleted, but this does not answer the original question posed at the onset of this study, which is how does the elimination of these proteins affect viral replication and transformation. The modified proteins may have a deleterious effect upon viral infection and thus confuse the results. The overall question could then be modified from asking what the effect the absence of these proteins has on productive infection and transformation to what can these proteins do on their own in the absence of 496R and the other related products. This can be acheived by creating a cDNA virus for each of these proteins. In short there exist further possible studies in order to elucidate the function of the E1b 496R related minor products.

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