

TRANSFORMATION AND PLAQUE FORMING ABILITY
OF ADENOVIRUS TYPE 5 E1A INSERTION MUTANTS

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By

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ABSTRACT

The E1 region of the group C adenoviruses is able to induce oncogenic transformation of rodent cells in culture and is also necessary for the efficient transcription of other viral genes. The proteins of the E1a transcription unit have been shown to play a pivotal role in both of these activities. In order to better understand the regions of the E1a proteins required for transformation and viral growth a program of random insertion mutagenesis was undertaken by D. S. Bautista to help identify important domains. The 39bp linker insertion oligonucleotides were designed to encode a 13 amino acid in frame insertion in one orientation or a closed reading frame insertion in the opposite orientation. As well the insertion mutants could be collapsed by digestion with BamHI to generate a 2 amino acid in frame insertion. Using this method all three types of mutants were generated at 18 different sites within the E1a coding sequences. The purpose of this project was to assay these E1a mutants for the ability to cooperate with E1b in the transformation of primary baby rat kidney cells using DNA-mediated transfection and also to 'rescue' the mutants into infectious virus and study the ability of the mutant virus to replicate on HeLa cells.

Results showed that only closed reading frame mutations upstream of the unique region were completely negative for transformation. Conversely, 13aa or 2aa insertions outside of the unique region impaired but did not abolish transformation. However 8 of the 9 insertions in the unique region of the 289R protein of E1a were defective for

transformation of BRK cells in Ela plus Elb DNA-mediated transformation assays. To determine whether the unique region played a direct role in the transformation process or if it had an indirect role such as the transactivation of Elb the transformation assay was carried out using selective media that allows growth of foci transformed by Ela alone. Results from this assay showed that the unique region mutants combined with Elb were able to transform with about the same efficiency as Ela alone. The transformation assay was also performed using the unique region mutants in an Ela only background cotransfected with the EJ-ras oncogene. Results from these experiments showed that the unique region mutants in an Ela only background could cooperate with ras in transformation as well as wild-type Ela. From these results it was concluded that the unique region does not play a direct role in transformation by El but is required for the efficient expression of Elb which results in wild type transforming frequencies. The actual role of Elb in transformation is unknown.

The insertion mutants were also 'rescued' back into infectious virus to study their effect on the ability of the viruses to replicate. The results showed that only viruses in which the unique region was either eliminated or altered were defective for growth on HeLa cells. Transactivation assays carried out by D. Bautista showed results which were comparable to results of infectivity assays. Taken together the results suggest that only the unique region is required for transactivation and only the ability of Ela to transactivate is of importance for viral replication in HeLa cells.

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LIST OF ABBREVIATIONS

aa	amino acid
Ad 2, 5, 12	Adenovirus type 2, 5, 12
alk-SDS	alkaline-SDS
ATP	adenosine triphosphate
bp	base pairs
BRK	baby rat kidney
BRL	Bethesda Research Laboratories
CaCl ₂	calcium chloride
°C	degrees Celsius
cpe	cytopathic effect
CR 1, 2, 3	conserved region 1, 2, 3
CREF	cloned rat embryo fibroblasts
CRF	closed reading frame
CsCl	cesium chloride
DDW	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E1 - E4	early region 1-4 of adenovirus genome
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediamine tetracetic acid
et al	and co-workers
EtBr	ethidium bromide
EtOH	ethanol
F11	minimal essential media
FCS	fetal bovine serum
g	grams
GTE	glucose-tris-EDTA buffer
HCl	hydrochloric acid
hr	hour
HS	horse serum
Jok	Joklik's modified media
K	kilodalton
kb	kilobase
KCl	potassium chloride
KH ₂ PO ₄	potassium dihydrogen phosphate
KOAc ⁴	potassium acetate
l	litre
LB	Luria Bertani broth
L-Glu	L-glutamine
MEM	alpha minimal essential media
mg	milligram
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulfate
min	minute

ml	millilitre
MLP	major late promoter
mm	millimeter
mM	millimolar
M	molar
MnCl ₂	manganese chloride
mRNA	messenger ribonucleic acid
mu	map units
MW	molecular weight
NaCl	sodium chloride
Na ₂ HPO ₄	disodium hypophosphate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCS	newborn calf serum
nm	nanometer
nt(s)	nucleotide(s)
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
Pen-Strep	penicillin-streptomycin
PCNA	proliferating cell nuclear antigen
pfu	plaque forming units
R	residue
RbCl	rubidium chloride
rpm	revolutions per minute
S	sedimentation coefficient
SDS	sodium dodecyl sulfate
sec	seconds
SSC	sodium chloride sodium citrate solution
SV40	simian virus 40
TAE	tris-acetate-EDTA solution
TBE	tris-boric acid-EDTA solution
TE	tris-EDTA buffer
TFB	transformation buffer
TNE	tris-NaCl-EDTA solution
Tris	tris (hydroxymethyl) aminoethane
ug	microgram
ul	microlitre
UR	unique region
UV	ultraviolet
v/cm	volts per centimeter
vol	volume
wt	wild-type

Introduction

1.1 The Adenoviruses

A cytopathogenic agent identified by Rowe et al (1953) from cultured human adenoid cells undergoing "spontaneous" degeneration was in fact the first isolation of a human adenovirus. This cytopathogenic agent was able to cause the same degeneration in other human cell cultures and was subsequently found to be the infectious agent responsible for acute respiratory diseases, epidemic keratoconjunctivitis and gastroenteritis. One decade later Trentin et al (1962) found that the adenovirus serotype 12 (see section 1.2 for classification) was able to induce tumors when injected into newborn hamsters and this was soon proven a property of several of the adenovirus strains (Huebner et al, 1962; Rabson et al, 1964; Yabe et al, 1964; Huebner et al, 1965; Pereira et al, 1965). As the first human virus shown to induce tumors in animals the adenoviruses became the subject of intense research as a possible model, if not causative agent, for cancer in humans. At present it seems the adenoviruses play no role in human carcinomas but they continue to provide a useful and safe model for study of the molecular basis of oncogenic transformation. As well the adenoviruses stimulated research on mRNA processing and the regulation of gene expression.

1.2 Adenovirus Classification

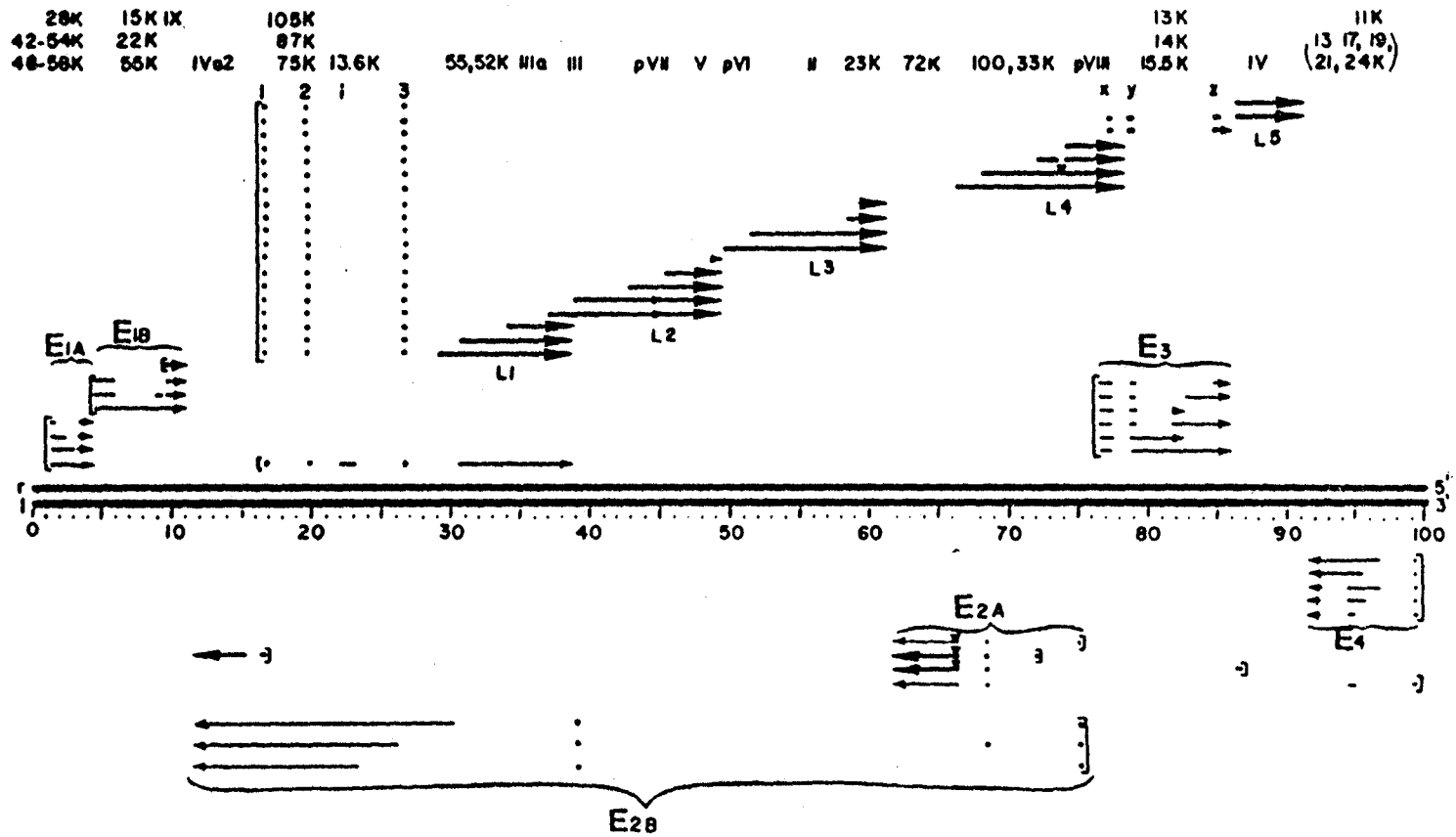
The most recent method of classification for the adenoviruses is based on the degree of homology between the genomes of the various serotypes (Green et al, 1979). Previous classification has been based upon properties such as the degree of oncogenicity (Trentin et al, 1968; Green, 1970), hemagglutination (Rosen, 1970), and the DNA guanosine-cytosine content (Pina and Green, 1965). DNA homology is likely the most valid measure of similarity and in most cases mirrors the similarities between serotypes based on the other properties mentioned. On this basis the adenoviruses have been classified as: the highly oncogenic class A (types 12, 18 and 31), moderately oncogenic class B (types 3, 7, 11, 14, 16 and 21), and the non-oncogenic classes C (types 1, 2, 5 and 6), D (types 8, 9, 10, 13, 15, 17, 19, 20, and 22-30) and E (type 4) (Green et al, 1979). In all cases there is less than 25% homology between classes and greater than 90% within a class except for class A viruses which share only 48-69% homology. It should be noted that oncogenicity in this context refers only to the ability of the virus to cause tumors when injected into animals and that all adenovirus types are able to induce the transformation of cells in tissue culture. This paper will deal predominately with the group C adenovirus type 5 (Ad5) and the highly related serotype adenovirus type 2 (Ad2).

1.3 Adenovirus Type 5

Adenovirus type 5 is a DNA virus with a linear double stranded genome of about 36,000 base pairs (bp) (Green et al, 1967) containing inverted terminal repeats of 103bp (Garon et al, 1972; Wolfson and Dressler, 1972) as well as a protein attached to each of the 5' ends through a phosphodiester linkage (Robinson et al, 1973; Sharp et al, 1976; Carusi, 1977; Rekosh et al, 1977). The viral genome is encapsidated in an icosahedral shell consisting of 240 hexons and 12 pentons (Valentine and Pereira, 1965). Each penton has a protruding fiber involved in the adsorption process to infected cells (Valentine and Pereira, 1965; Levine and Ginsberg, 1967; Philipson et al, 1968).

Within 30 minutes following adsorption and penetration of the cell membrane the virion capsid is removed and the viral genome enters the cell nucleus (Morgan et al, 1969). Transcription of the viral genome then begins in a temporal nature with expression of transcription units divided into either early (expressed predominately before DNA replication) or late (expressed predominately after DNA replication) (Figure 1). The early genes E1a, E1b, E2, E3 and E4 are transcribed within the first two hours of infection with E1a being the first detectable (Nevins et al, 1979; Rowe et al, 1984b) and plateau prior to DNA replication, about 3 to 7 hours, then begin to decline except for E1a and E1b which remain relatively constant (Nevins et al, 1979; Rowe

Figure 1. Composite transcription map of adenovirus type 2. Transcripts originating from the r and l strands are indicated above and below the genome, respectively. The genome is divided into map units (mu) where 100mu is equivalent to 36,000bp. Brackets indicate the position of promoters for the various transcripts and arrows represent the mRNAs produced, spaces in the arrows indicate introns spliced out of the mature transcripts. Arrow heads indicate the polyadenylation sites. The early transcription units are denoted as E1a, E1b, E2a, E2b, E3, and E4 while late transcription units are termed L1, L2, L3, L4 and L5. Proteins from each transcript are indicated above the map by molecular weight in kilodaltons (K) or by Roman numerals. The numbers 1, 2 and 3 show the three segments which are spliced to form the tripartite leader of the major late promoter (MLP). Taken from Chow et al (1979b) as modified by (Tooze, 1981).



et al, 1984b). Between 8 and 12 hours post-infection DNA replication begins (Green et al, 1970). Also during this period the transcription of some early genes increases probably as a reflection of the increase in number of DNA templates (Shaw and Ziff, 1982). At the same time the IX and IVa2 promoters reach their maximum activity (Wilson et al, 1979b) and transcription from the major late promoter (MLP) begins and peaks at about 18hr post-infection (Chow et al, 1979). The induction of DNA synthesis and late gene expression are regulated in part by products of the early genes (Sharp, 1984). Late products are predominately involved in the production and assembly of virion progeny (Sharp, 1984; Philipson, 1984).

1.4 Early Region 1

As mentioned, the ability of the human adenoviruses to induce tumors in vivo or to oncogenically transform mammalian cells in culture stimulated vast interest in these agents. Several independent studies led to the identification of early region 1 (E1) as the transforming region of the adenovirus genome. One line of investigation used either sheared or restricted fragments of viral DNA to transform cells (Graham et al, 1974; van der Eb et al, 1977) and this method allowed the transforming region to be accurately mapped to the left end 1 to 6% of the viral genome. Further studies showed that a 1.7×10^6 MW fragment of DNA corresponding to the left end 5.5% of Ad5 was sufficient for

"immortalization" of primary cells (van der Eb et al, 1977; van der Eb and Houweling, 1977).

Gallimore et al (1974) used the kinetics of renaturation between the DNA of nine Ad2-transformed cell lines and viral DNA to determine viral DNA content within the cells and found that although none of the cell lines contained the entire genome the left 14% was always present. Similar conclusions were reached by Sambrook et al (1974) and Flint et al (1975; 1976) in experiments with independent adenovirus transformed cell lines.

Once the transforming region of the adenovirus genome had been identified it was of interest to define the products of this region in order to understand how they acted in the transformation process. This work began on several fronts and it soon became obvious that this relatively small region of DNA was able to generate many messages and even more protein species. Wilson et al (1979a) used UV irradiation transcription mapping to identify the active promoters within the E1 region and found three: one between 0-3mu (E1a), one between 4.4-8mu (E1b) and a third between 8-11.2mu which was active primarily late in infection. Messages initiated from these promoter elements were shown to consist of several related but differentially spliced mRNAs (Chow et al, 1979a; Kitchingman and Westphal, 1980; Berk and Sharp, 1978). Van der Eb et al (1979) isolated these mRNAs from infected cells, translated them in vitro, and showed the E1a region produced a 15K protein and unresolved proteins of 34-42K while the E1b region coded for proteins of

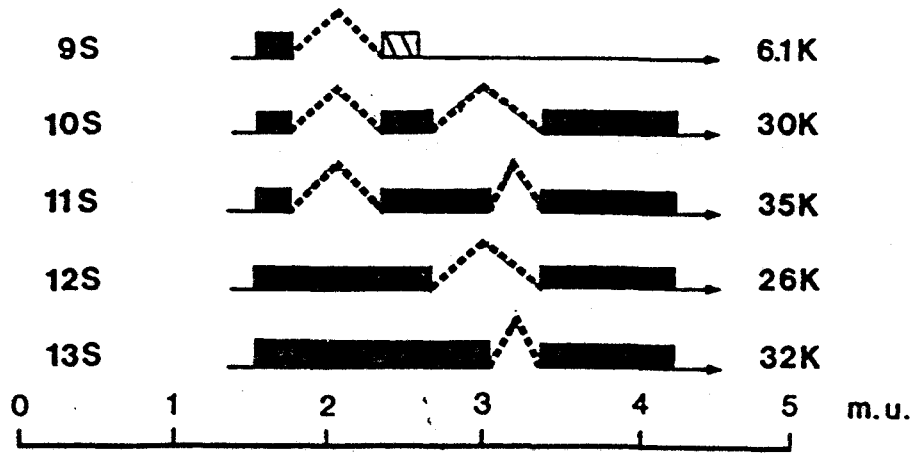
17, 18, 19, and 65K proteins. These protein products were mapped to their messages, again using an in vitro translation system, and it was shown that Ela produced a 28K protein from a 0.6kb mRNA late in infection, 42 and 54K proteins from a 0.9kb mRNA and 48 and 58K proteins from a 1.1kb mRNA, while E1b produced a 15K protein from a 0.9kb mRNA, an 18K protein from a 1.2kb message, a 57K protein from a 2.6kb message as well as a 22K protein produced late in infection (Esche et al, 1980). Similar results were found using the messages from transformed cells (Jochemsen et al, 1981; van den Elsen et al, 1983a; Lewis et al, 1976; Chow et al, 1979a; Kitchingman and Westphal, 1980; Halbert et al, 1979; Berk and Sharp, 1978) and immunoprecipitations of proteins with antisera from rodents containing Ad5-induced tumors (Schrier et al, 1979; Rowe et al, 1983b). Estimates of protein sizes differed due to the acidic and proline rich nature of the proteins as well as their extensive post-translational modifications (van der Eb et al, 1979).

1.4.1 Early Region 1a

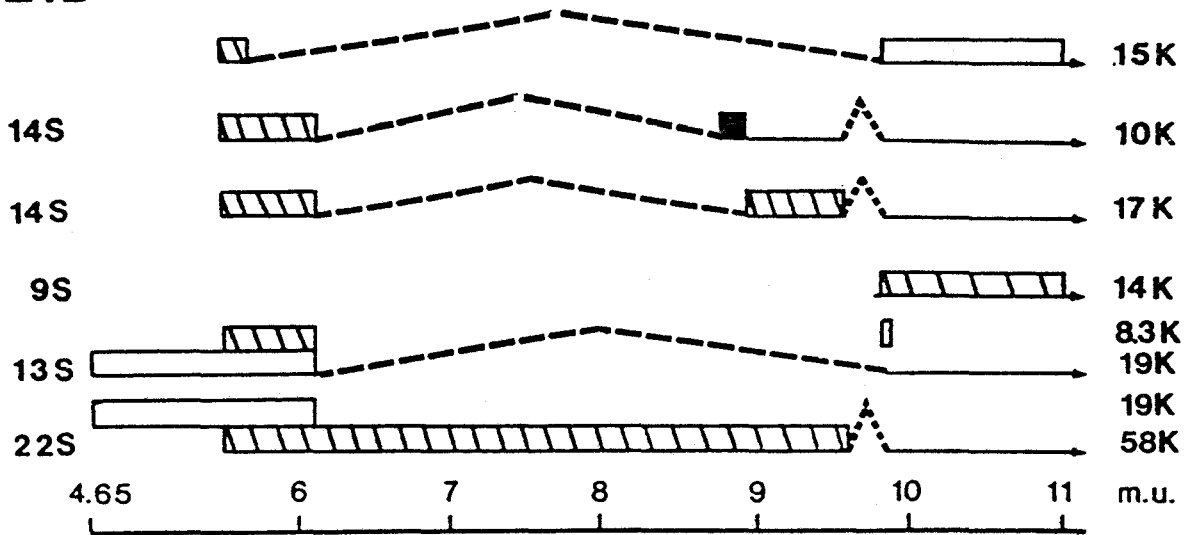
The Ela transcription unit has to date been shown to produce at least five distinct mRNA species (Chow et al, 1979a; Spector et al, 1978; Perricaudet et al, 1979; Baker and Ziff, 1981; Berk and Sharp, 1978; Harlow and Stephens, 1987) (Figure 2). One of these, the 9S mRNA, is produced mainly late during a productive viral infection (Spector et al, 1980; Chow et al, 1979b) and is undetectable in adenovirus

Figure 2. Map of the E1 transcription unit of adenovirus type 5. All E1 transcripts are synthesized from the r strand of the genome. a) The E1A region of the genome is divided into map units and the mRNA products are indicated above the genome by their sedimentation coefficients. Introns removed by splicing are indicated by carets, arrow heads indicate the polyadenylation sites, and the protein products are shown by molecular weight (K). All E1A proteins are translated from the same reading frame (solid bars) initiating at nt 560 except for the 9S protein which is spliced into a different reading frame as indicated by the hatched bar. b) The E1B unit is indicated in a similar manner. Different reading frames are indicated as either open, hatched or solid boxes.

E1A



E1B



transformed cells (Spector et al, 1980; Spector et al, 1978; Wilson et al, 1979b). The other species include the 13S, 12S, 11S and 10S mRNAs which share the same reading frame and translation initiation site, but due to differential splicing yield different protein products. The 13S and 11S mRNAs share the same splice acceptor/donor sites at nucleotides (nts) 1112 and 1229 of the Ad5 genome yet the 11S product has an additional splice site between nts 637 and 854 (Harlow and Stephens, 1987). The 12S and 10S mRNAs also share a splice site between nt 974 and 1229 while the 10S has the splice site between 637 and 854 (Harlow and Stephens, 1987). The role of the 11S and 10S messages in transformation and the virus growth cycle have not been clearly shown although they are known to be produced late in infection (Harlow and Stephens, 1987).

The 13S and 12S mRNA products are the first detectable viral transcripts early in infection (Rowe et al, 1984b; Nevins et al, 1979; Spector et al, 1978) while the 9S product accumulates at only about 5% of these levels (Spector et al, 1978). The expression of E1a in cells transformed by adenovirus is similar to their expression early in infection (van den Elsen et al, 1983a; Sharp et al, 1974; Flint et al, 1975). The products of the 13S and 12S messages have been well characterized and encode two related proteins of predicted size 289 amino acids (289R) and 243 amino acids (243R) with the only difference being an additional 46 amino acids in the larger protein (Perricaudet et al, 1979). Both proteins are nuclear oriented in infected or

transformed cells (Feldman and Nevins, 1983) and both are phosphorylated (Yee et al, 1983) at multiple serine residues (Tremblay et al, 1988).

Activities attributed to the Ela proteins include the ability to transcriptionally activate other early viral genes during infection (Nevins, 1981; Bellett et al, 1985; Ricciardi et al, 1981; Jones and Shenk, 1979; Berk et al, 1979; Montell et al, 1982, Rowe et al, 1984b) or cotransfection (Leff et al, 1984; Svensson and Akusjarvi, 1984; Weeks and Jones, 1983; Imperiale et al, 1983) as well as transiently introduced genes in mammalian cells (Bos et al, 1983b; Imperiale et al, 1983; Weeks and Jones, 1983; Svensson and Akusjarvi, 1984; Green et al, 1983) and the stimulation of transcription of cellular genes such as the heat shock protein 70 (Nevins, 1982; Kao and Nevins, 1983) the B-tubulin gene (Stein and Ziff, 1984) and the cellular thymidylate synthase gene (Zerler et al, 1987). This activity has been mapped primarily to the 46 amino acids unique to the larger protein (Guilfoyle et al, 1985; Ricciardi et al, 1981; Montell et al, 1982; Lillie et al, 1987; Lillie et al, 1986), hence called the unique region (UR).

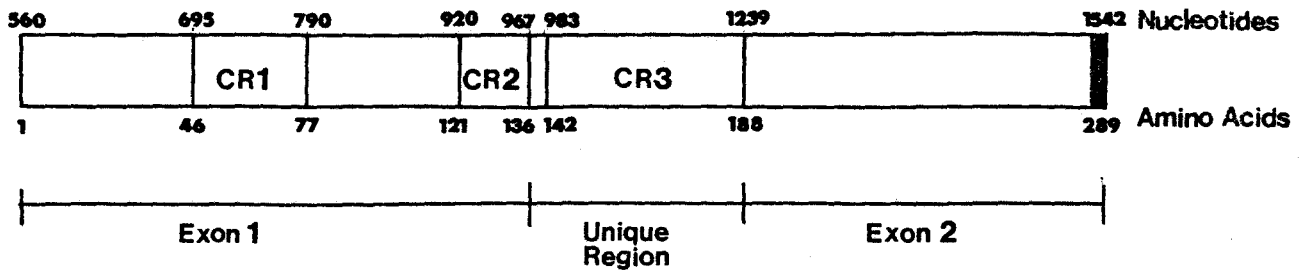
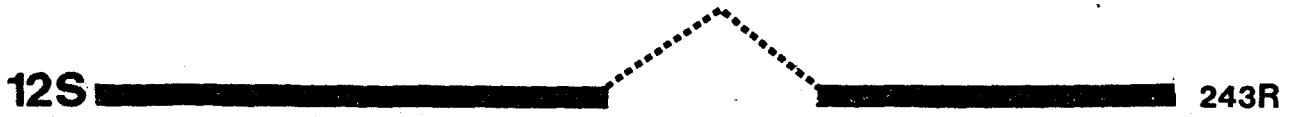
Other properties of Ela have been mapped to exon 1 of the 12S mRNA which is common to both the 289R and 243R proteins and these include: the ability to induce DNA synthesis in growth arrested rodent cells (Shimojo and Yamashita, 1968; Stabel et al, 1985; Braithwaite et al, 1981; Braithwaite et al, 1983; Lillie et al, 1987; Zerler et al, 1987; Kaczmarek et al, 1986; Shiroki et al, 1981; Youngusband et al, 1979; Rossini et al, 1979) although the 289R product seems to interfere with

this activity based on results with virus expressing only the 13S product such as pm975 (Montell et al, 1984; Spindler et al, 1985); the ability to induce the transcription of proliferating cell nuclear antigen (PCNA) (Zerler et al, 1987) a protein found primarily in rapidly replicating cells or cells stimulated to grow (Takasaki et al, 1984); and the ability to repress the enhancer-induced stimulation of transcription from the SV40 (Borelli et al, 1984; Velcich and Ziff, 1985), polyoma (Borelli et al, 1984; Hen et al, 1986) and Ela (Borelli et al, 1984) genes in a DNA-mediated transient assay or of enhancer-linked cellular genes following virus infection (Hen et al, 1985; Webster et al, 1988; Stein and Ziff, 1987). The Ela mediated repression of the enhancer element present in the Ela promoter (Hen et al, 1983) allows Ela to autoregulate its level of expression (Smith et al, 1985; Larsen and Tibbets, 1987; Borelli et al, 1984). Mutations that reduce the ability of the Ela products to repress transcription are often defective in transformation as well (Lillie et al, 1986; Schneider et al, 1987) but this correlation is not absolute in view of the fact that certain mutants are defective in repression but can co-operate with the ras oncogene in transformation (Velcich and Ziff, 1988; Kuppuswamy and Chinnadurai, 1987). Some of these Ela activities reflect the properties of cellular factors suggesting the presence of "Ela-like" proteins in some cell types (Hen et al, 1986; Montano and Lane, 1987; LaThangue and Rigby, 1987; Yoshida et al, 1987). All of these activities are carried out by unknown mechanisms although it seems the

proteins do not bind DNA directly (Ferguson et al, 1985,) in any specific manner (Chatterjee et al, 1988) but may exert their influence through binding to specific cellular proteins (Yee and Branton, 1985; Harlow et al, 1986). An example of such a phenomenon is a cellular 54K protein that is bound both by the 58K tumor antigen of E1b and the SV40 large T antigen in transformed cells (Sarnow et al, 1982).

Some of these functions have been tentatively mapped to regions highly conserved between adenovirus serotypes 5, 7, 12 and simian adenovirus 7 (Kimelman et al, 1985) termed conserved regions 1,2 and 3 (Figure 3). Conserved regions 1 and 2 (CR1 and CR2) seem to play roles in transformation (Moran et al, 1986a; Whyte et al, 1988; Lillie et al, 1986 and 1987), the induction of DNA synthesis (Zerler et al, 1987; Lillie et al, 1987) or mitosis (Zerler et al, 1987), repression (Velcich and Ziff, 1985; Lillie et al, 1986; Lillie et al, 1987) and activation of PCNA (Takasaki et al, 1984; Bravo and Macdonald-Bravo, 1985) while conserved region 3 (CR3 or UR) seems solely responsible for transactivation (Lillie et al, 1986 and 1987; Zerler et al, 1987). As yet exon 2 has not been associated with any of these activities except for a possible role in transactivation (Jelsma et al, 1988; Schneider et al, 1987). A fourth domain consisting of 5 amino acids at the carboxy terminus of the protein (Figure 3) has been shown to increase the rate of nuclear localization of the proteins (Krippel et al, 1985; Lyons et al, 1987).

Figure 3. Conserved Regions of the Ela proteins of adenoviruses 5, 7, 12 and simian adenovirus 7. The 12S and 13S messages of the Ela region are shown above the 289R protein product. Conserved regions are denoted as CR1, CR2 and CR3. Nucleotide positions are shown above the protein product and amino acid positions below. The degree of conservation between the different serotypes is 50%, 50% and 43%, respectively, for CR1, CR2 and CR3. The rapid nuclear localization signal consisting of the pentapeptide sequence Lys-Arg-Pro-Arg-Pro is indicated by the solid box at the carboxy-terminus of the protein. Modified from Moran and Mathews (1987).



1.4.2 Early Region 1b

The Elb transcription unit is also complex (Figure 2). All Elb messages are initiated at the same start site at nt 1699 (Bos et al, 1981; Gingeras et al, 1982) except for the 9S mRNA which has its own promoter and encodes the structural protein IX (Wilson et al, 1979b; Pettersson and Mathews, 1977; Chow et al, 1977). The Elb messages contain two overlapping open reading frames, the first of which starts at nt 1711 (Ad2) and encodes the 19K protein which can be made from either the 22S or 13S mRNAs (Bos et al, 1981). The second ORF starting at nt 2016 (Ad2) encodes the 58K protein which is produced only by the 22S mRNA (Esche et al, 1980). Other transcripts which include a 14S, 14.5S and 0.86kb mRNAs have been identified (Anderson et al, 1984; Lewis and Anderson, 1987; Virtanen and Pettersson, 1985) and share the same N-terminus as the 58K protein but as a result of differential splicing encode distinct polypeptides. The role of these proteins in viral replication and transformation is not yet as clearly understood as those of the 19K and 58K proteins.

The 19K protein is involved in viral replication since a mutation there results in about a 10 fold reduction in growth on HeLa cells (Pilder et al, 1984). Also mutants in this protein show a large-plaque phenotype (Chinnadurai, 1983; Takemori et al, 1984) and a cytotoxic, DNA degradation effect (Ezoe et al, 1981; Lai Fatt and Mak, 1982; Pilder et al, 1984; Barker and Berk, 1987; Takemori et al, 1984; White et al,

1984). Furthermore the 19K protein is able to enhance transcription of genes linked to various enhancer elements and relieve the repression activity of Ela on those same enhancer elements (Yoshida et al, 1987). Finally, the 19K protein is required for efficient transformation by both virus and DNA (Pilder et al, 1984; Chinnadurai, 1983; Babiss et al, 1984b; Bernardis et al, 1986; Barker and Berk, 1987; McKinnon et al, 1982; Takemori et al, 1984; Mak and Mak, 1983).

The 58K protein is also required for the efficient transformation of cells by virus (Babiss et al, 1984b; Bernardis, et al, 1986; Lassam et al, 1979) but its necessity in the DNA-mediated transformation of cells is less clear. Some mutants in the 58K are transformation defective in virus mediated assays but are near wild type in DNA transfection assays (Babiss et al, 1984b; Rowe and Graham, 1983a) while other mutants are equally defective in either assay (Barker and Berk, 1987). This anomaly may be due to the type of mutation present in view of the fact that a series of 58K mutants decreased in transforming ability as the size of the predicted truncated 58K protein decreased (Babiss et al, 1984b). The 58K protein also has a role in the shutoff of host cell protein synthesis (Babiss et al, 1985; Babiss and Ginsberg, 1984a) and the efficient transport of adenovirus RNA to the cytoplasm (Babiss et al, 1985; Pilder et al, 1986). Finally, the 58K protein has protein kinase activity in infected or transformed cells (Branton et al, 1979).

1.5 Transformation by Ad5 Early Region 1

To determine which of the Ela proteins is responsible in transformation, cDNA clones of the 12S and 13S messages were constructed in virus and assayed for transformation (Moran et al, 1986b). Although either message alone is able to transform cells both are required to induce the fully transformed phenotype: the ability to grow in low serum media, anchorage independent growth, and the ability to cause tumors when transformed cells are injected back into syngeneic animals. Also, BRK cells transformed with group 1 host-range mutants which express an intact protein only from the 12S mRNA are fibroblastic rather than epithelial, fail to grow in soft agar and are less tumorigenic than wild type transformed cells (Ruben et al, 1982). Other results with virus expressing a normal 12S product but an altered or absent 13S product showed either a defect in transformation (Carlock and Jones, 1981; Winberg and Shenk, 1984) or an abnormal transformed phenotype (Winberg and Shenk, 1984) that is cold sensitive (Babiss et al, 1986; Montell et al, 1984; Haley et al, 1984). A viral mutant with a wild-type 13S mRNA but defective 12S message transformed at reduced frequency (Montell et al, 1984) and the transformed cells were defective for anchorage-independent growth (Hurwitz and Chinnadurai, 1985).

The requirement of the Elb proteins in the transformation of rodent cells was revealed by studies showing that Ela alone is able to "immortalize" cells, that is to allow cells to grow indefinitely in

culture (Houweling et al, 1980; van der Eb et al, 1979), but Elb was needed to "co-operate" with Ela for the virus to produce the fully transformed phenotype. Results with complementation group II host range mutants, which synthesize the Elb 19K protein but not the Elb 58K protein (Lassam et al, 1979), showed that all were defective in transformation of rodent cells (Graham et al, 1978). In contrast, DNA extracted from the group II adenovirus mutants hr6 and hr50 are able to transform at wild-type levels even though no 58K protein is produced in the transformed cell lines (Rowe and Graham, 1983). Furthermore, Ela plus the N-terminus of Elb was shown to be sufficient to transform cells in a DNA-mediated transfection (Graham et al, 1974). This suggested that the 58K protein is the essential element for the initiation of transformation by virions but is dispensable for the maintenance of transformation (Rowe et al, 1984).

Several other groups have also shown that the Elb proteins are required for transformation (Barker and Berk, 1987; Chinnadurai, 1983; Bernardis et al, 1986; McKinnon et al, 1982; Mak and Mak, 1983) but it remains unclear whether they play an active role in the transformation process or an indirect role such as the efficient induction of expression of the Ela gene products. It is known that Ela products transactivate Elb (Bos and ten Wolde-Kraamwinkel, 1983; Berk et al, 1979; Nevins, 1981) and it has been shown that Ela mRNA and protein levels are higher when Elb is present (van den Elsen et al, 1982) so Elb may only increase the expression of Ela to allow the fully transformed

phenotype. Evidence supporting this has been provided by results showing that Ela alone under a strong promoter is able to induce the same transformed phenotype in NIH 3T3 cells as does Ela plus E1b (Senear and Lewis, 1986). However these results are for the established cell line NIH 3T3 which may be a poor model for the study of oncogenes (Land et al, 1983). It should be noted that E1b alone is not able to transform primary cells nor can it induce the transformed phenotype in previously immortalized cells (van den Elsen et al, 1983c). Furthermore, a virus defective in the production of Ela, but which still expresses E1b, is transformation negative, eliminating the possibility that Ela is only needed to activate E1b to produce the transformed phenotype (Solnick and Anderson, 1982).

It has also been shown that the Ela region can co-operate with other oncogenes such as the Ha-ras and polyoma middle T antigen genes to transform cells in culture leading to the hypothesis that certain oncogenes share similar pathways in transformation (Land et al, 1983; Ruley, 1983). For example, the immortalization of cells can be accomplished by Ela or the polyoma large T antigen but full transformation requires complementation by E1b, ras or the Py middle T antigen (Land et al, 1983; Ruley, 1983; Houweling et al, 1980).

1.6 Mutational Analysis of Ela

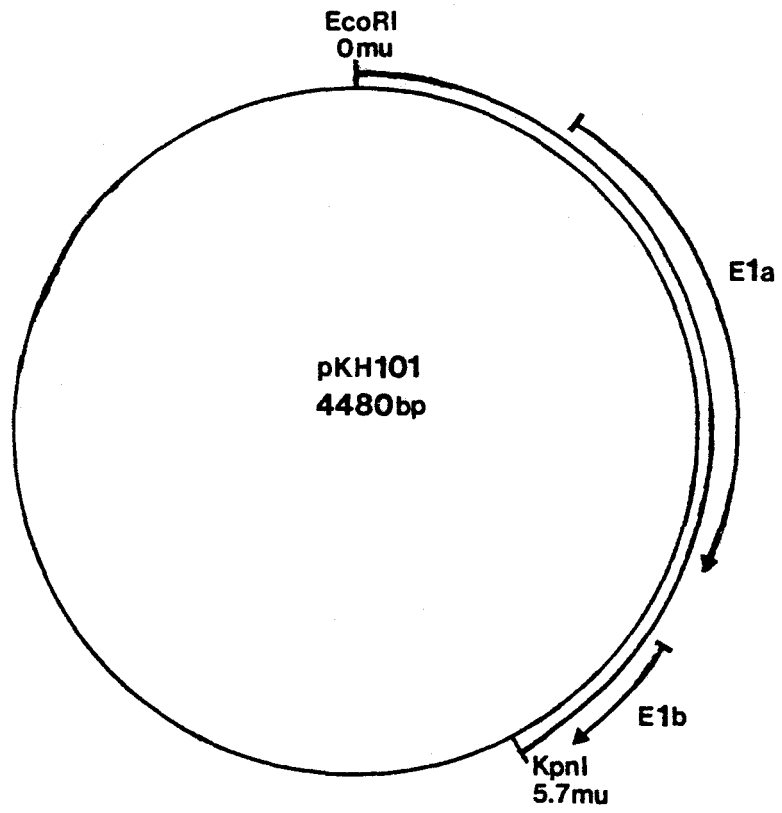
Because of the numerous biological activities attributed to Ela a great deal of interest has been focused on it in the hope of better

understanding the molecular basis for its actions. The purpose of this project was to employ the linker insertion mutants constructed in Ela by D.S. Bautista to study the role of the Ela proteins in the transformation of rodent cells and viral replication. This involved the construction of plasmids which contained the insertion mutants in an El background and assaying them for the ability to transform primary baby rat kidney cells in a DNA-mediated transfection. The mutants were also rescued back into virus and studied for their ability to replicate in HeLa cells.

1.6.1 Construction of Ela Insertion Mutants

Our laboratory initiated a programme of random insertion mutagenesis in the hope of identifying important regions in the Ela proteins required for specific activities or structural requirements. To generate these mutants the plasmid pKH101 (Figure 4) containing the Ela region cloned into the bacterial plasmid pUC19 was employed. Linker insertion mutants were generated by restricting the Ela region in pKH101 with a multicut endonuclease in the presence of a concentration of ethidium bromide that theoretically allows the enzyme to cut only once (D.S. Bautista, personal communication). Following digestion the plasmid was ligated to an excess of a 39bp oligonucleotide linker and used to transform bacteria. The linker was designed with the following properties. 1) As a convenient marker it contained the 21 base pair Escherichia coli (E. coli) lac operator sequence which when present in a

Figure 4. Map of the Ela plasmid pKH101. The Ela sequences of Ad5 are indicated by the open bar. The sequence extends from 0mu (EcoRI) to 5.7mu (KpnI). The arrows indicate the coding sequences of Ela and the partial coding sequences of Elb.



high copy number plasmid titres out the lac repressor molecules allowing expression of the beta-galactosidase gene. This results in the production of blue colonies in the presence of X-gal. ii) One orientation of the linker would generate a 39bp (13 amino acid) in-frame insertion while the opposite orientation would result in an out-of-frame insertion resulting in the premature termination of translation. iii) The linker is flanked by BamHI restriction enzyme sites so that digestion of the mutant plasmid with this enzyme and re-ligation results in a mutant with a 6bp (2 amino acid) in-frame insert. Using this method D.S. Bautista has generated mutants at eighteen sites in the Ela genome with each site having either a 13aa insert, a 2aa insert, or a 39bp insert causing premature truncation of the protein product (Figure 5).

Materials and Methods

2.1 Bacterial Strains

Escherichia coli strain HMS 174 (Rk12-, Mkl2+, Su-, recA-, rif^r) was used for all bacterial work.

2.2 Bacterial Culture Techniques

2.2.1 Liquid Culture

Luria-Bertani (LB) broth was used for all liquid culture (10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, 1g glucose made up to 1 litre and pH adjusted to 7.5 with NaOH). If required, antibiotics were added after sterilization to the final concentrations of: 40ug/ml ampicillin, 50ug/ml kanamycin, and 10ug/ml tetracycline. Cultures were incubated at 37°C with constant shaking.

2.2.2 Solid Culture

LB broth plus 1.9% agar (Bacto) was prepared and autoclaved, allowed to cool to 44°C, then supplemented with the appropriate antibiotic(s) at the concentrations listed above. The agar-media was then poured into plastic petri dishes (Fischer), allowed to solidify and then placed at 37°C overnight to dry. Plates were stored at 4°C until

used. To plate cells, cultures were either spread or streaked out on the plates, inverted, and then incubated overnight at 37°C.

2.2.3 Culture Storage

2ml of an overnight liquid culture plus 2ml of 40% glycerol were mixed in 4 dram glass vials and stored at -70°C. Cultures could be started from frozen stocks by scraping the surface with the tip of a sterile 1ml pipet and transferring the inoculum to a liquid culture.

2.3 Preparation and Transformation of E. Coli HMS 174 Cells

2.3.1 Calcium Chloride Technique

For transformation of cells with plasmids less than 10kb in size the calcium chloride technique of Mandel and Higa (1970) was used. A 0.5ml aliquot of an overnight culture was used to inoculate a 50ml culture of LB broth and grown at 37°C with constant agitation until an OD₆₆₀ of 0.4 to 0.6 was reached. The cells were then precipitated by centrifugation at 3000 rpm for 5 min and resuspended in 40 ml of ice cold CaCl₂-Tris solution (75mM CaCl₂, 5mM Tris pH 7.6) and allowed to sit on ice for a minimum of 40 minutes. The cells were then precipitated and resuspended in 2-3 ml of the transformation buffer. Aliquots of 200ul were placed in chilled 4 dram glass vials to which the DNA ligation mixture was added. The mixture then sat on ice for a minimum of 40 min with periodic shaking. After a 2 minute 42°C heat

shock a 0.8ml aliquot of LB broth was then added to each vial and incubated with agitation at 37°C for 30 min. The vial was then used to make serial dilutions in LB broth. From each dilution 200ul was plated out on LB agar plates containing the appropriate antibiotic(s). The plates were then inverted and incubated for 16-20 hours at 37°C.

2.3.2 Hanahan Technique

For transformation of HMS 174 cells with plasmids greater than 36kb in length the technique of Hanahan (1983) was used. A 0.5ml aliquot of an overnight culture was transferred to a 50ml flask of SOB medium (20.0g tryptone, 5g yeast extract, 0.59g NaCl, and 0.19g KCl per litre, to which 10ml of sterile 1M MgCl₂, 1M MgSO₄ was added after autoclaving). The cells were grown to an O.D.₅₅₀ of 0.45 to 0.55 then chilled on ice for 10-15 minutes and then pelleted. Cells were then resuspended in 20ml of transformation buffer (TFB: 10mM K-MES pH 6.2, 100mM RbCl, 45mM MnCl₂, 10mM CaCl₂, and 3mM Hexamine cobalt (III) Cl₃; filter sterilized) and left on ice for 30 minutes. The cells were then pelleted and resuspended in 2ml of TFB. To this was added 35ul/ml of fresh DMSO, chilled on ice for 5 minutes, then 35ul/ml of dithiothreitol (DTT) was added (2.25M in 40mM KOAc pH 6.0), and chilled for 10 more minutes. Again DMSO was added, 35ul/ml, and chilled for 5 minutes. Aliquots of 200ul were transferred to chilled 4 dram glass vials to which the DNA was added in a 10ul volume or less. The mixture was chilled on ice for 30-60 minutes and then heat shocked at 42°C for 90

seconds. To each vial 800ul of SOB plus 20mM glucose was added and then incubated at 37°C with gentle agitation for one to two hours. Appropriate dilutions were then plated on agar plates containing the desired antibiotic(s).

2.4 Screening of Recombinant DNA Colonies

2.4.1 Small Scale DNA Preparation

Screening of recombinant DNA colonies was by the method of Birnboim and Doly (1979). Briefly, well isolated colonies were picked with a wooden stick and used to inoculate a 2.5ml LB broth culture containing the desired antibiotic. The cultures were then grown for a minimum of 4 hours. Cells were then pelleted in 1.5ml Eppendorf tubes and resuspended in 100ul GTE (50mM glucose, 25mM Tris-Cl pH8.0, 10mM EDTA) with 2ug/ul lysozyme (Boeringer-Mannheim) and incubated at room temperature for 20 min. 200ul of alkaline-SDS (0.2N NaOH, 1% SDS) was then added, mixed well and incubated on ice for 5 min. Then 150ul of 3M NaOAc pH4.8 was added, mixed by vortexing briefly and allowed to sit on ice for 30-60min. The tubes were then spun at 13000 rpm for 5min and the supernatant was ethanol precipitated twice. Ethanol precipitation simply involves adding 2 vol of ice cold ethanol to the DNA solution, mixing well and centrifuging at 13000 rpm for 5min. The dried pellet was resuspended in 100ul of 0.1xSSC (made from 20x stock: 175.3g NaCl and 88.2g sodium citrate dissolved in water, pH adjusted to 7.0 with NaOH,

and made up to 1 litre volume) and a 10-20ul aliquot analyzed by digestion with the appropriate enzymes and gel electrophoresis. Desired clones were streaked out on agar plates to obtain a well isolated colony which was grown up to be reanalyzed and frozen down at -70° C.

2.4.2 Large-Scale DNA Preparation

In order to obtain large amounts of plasmid DNA a 750ml LB broth culture, containing the desired antibiotic(s), of the E. coli clone was grown overnight and then centrifuged at 5000 rpm for 5min in a Sorvall RC-5B refrigerated centrifuge. The bacterial pellet was then processed according to the Birnboim and Doly technique essentially as described previously except that 100x more of the GTE-lysozyme, alk-SDS, and 3M NaOAc were used. The DNA was then pelleted by adding 0.6 vol of isopropanol, allowed to sit at room temperature for 30min and then spun at 8000rpm for 12min (Sorvall). The DNA pellet was resuspended in 10ml of double distilled water (DDW) and precipitated once more with 2 vol of ice cold ethanol. The pellet was resuspended in 5ml 0.1x SSC and 2ml TE (50mM Tris pH 8.0, 10mM EDTA).

2.4.3 CsCl Banding of Large-Scale DNA Preparation

To further purify the DNA preparation 8.8g of CsCl was added and the prep transferred to a Beckman polyallomer ultracentrifuge tube. Light paraffin oil and 200ul of ethidium bromide (EtBr) (4mg/ml) were

layered over the prep and the tube sealed by heat and inverted to mix. Banding was done by centrifugation in a Beckman Vti 65.1 rotor at 55000 rpm in a Beckman ultracentrifuge for 16-20 hours. The plasmid band was then extracted from the tube under UV light using a 18G1/2 needle and 1ml syringe. Three extractions with equal volumes of CsCl saturated, TE buffered propanol were done to remove EtBr from the prep. The DNA was then ethanol precipitated twice, dried at 37°C and resuspended in 0.5ml of 0.1x SSC.

2.5 Determination of DNA Concentration

Concentration of DNA preps was determined by the diphenylamine assay of Giles and Myers (1965). Standard concentrations of salmon sperm DNA were prepared along with DNA samples in a final volume of 250ul. To these were added 250ul of 20% perchloric acid, 500ul of 0.4% diphenylamine in glacial acetic acid, and 50ul of 0.16% acetaldehyde. Tubes were incubated at 37°C for 16-20 hours and then absorbance was determined by a Beckman DU-7 spectrophotometer scan from 700nm to 595nm. Absorbance at 595nm was recorded and DNA concentrations of the samples were calculated by linear regression analysis of the standards.

2.6 Enzyme Reactions

2.6.1 Restriction Endonuclease Digests

All restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) or New England Biolabs. Digests were carried out at 37°C for 2-4 hours using one unit of enzyme for each microgram of DNA in a buffer of 10mM Tris-HCl pH7.5, 50mM KCl, 10mM MgCl₂. Digests that were to be used for ligations were heat inactivated at 65°C for 30 min. Partial digests were carried out at room temperature for short periods of time, usually less than 2 min, and then heat inactivated.

2.6.2 T4 DNA Ligase

T4 DNA ligase was purchased from BRL. Appropriate amounts of the DNA molecules to be ligated were combined with 0.5mM ATP, 20mM Tris pH 7.6, 10mM DTT, 10mM MgCl₂ and one unit of T4 DNA ligase. The ligation mixture was incubated a minimum of 4 hours at room temperature or overnight at 15°C and then used for transformation of competent bacterial cells.

2.7 Agarose Gel Electrophoresis

Agarose gels were made with 1% w/v agarose (BRL) in TAE solution (0.4M Tris-Acetate, 2mM EDTA) which was boiled to dissolve the agarose

then allowed to cool to approximately 45°C. 0.1 ug EtBr per ml of TAE-agarose was then added prior to pouring the gel on a horizontal gel apparatus. DNA samples of 30ul were mixed with 5ul loading buffer (20% glycerol, 2% SDS, 0.5% bromophenol blue) then added to the wells of the gel. Adenovirus 5 DNA digested with HindIII was used as marker in all cases. Gels were run overnight at about 1V/cm and then photographed under UV light with a mounted Polaroid camera using high speed Kodak 57 film.

2.7.1 Isolation of DNA Fragments from Agarose Gels

To isolate DNA fragments, agarose gels were prepared in the same manner except the electrophoresis buffer used was TBE (12.1g Tris-Cl, 5.135g boric acid, 0.372g Na₂-EDTA per litre) and the gels were run in the dark. The DNA fragment to be isolated was visualized with UV light and the gel was cut with a scalpel just below the migrating band. A strip of boiled dialysis membrane was then inserted in the slice and the gel was allowed to continue running until all of the fragment had been trapped by the membrane. The DNA was then eluted from the membrane with 500ul lysing buffer (10mM Tris pH 7.4, 10mM EDTA, and 0.4% SDS), extracted with phenol (80ml double distilled phenol plus 20ml TNE: 0.05M Tris pH8.0, 0.1M NaCl, 0.01M EDTA, plus a few crystals of hydroxyquinoline to prevent oxidation) to remove any traces of agarose and EtBr, then ethanol precipitated twice and resuspended in 0.1x SSC at a final concentration of about 1ug/ul.

2.8 Tissue Culture Techniques

In all cases cells were maintained in Forma Scientific Steri-Cult incubators at 37°C, 5% CO₂, and 95% humidity and all work was carried out in Forma Scientific contamination control flow hoods. Tissue culture dishes were either 60mm from Corning or 150mm from Nunclon. Media used was minimum essential media (Fl1), alpha-minimum essential media (MEM), or Joklik's modified media (Jok). Serum was from Gibco: Fetal bovine serum (FCS), horse serum (HS), or newborn calf serum (NCS), all heat inactivated for complement at 56°C for 30 minutes. Additional supplements used were Penicillin-streptomycin (Pen-Strep, Gibco 100x solution contains 10000 u/ml penicillin and 10000 ug/ml streptomycin), L-glutamine (L-Glu) 1%v/v, fungizone (Squibb Canada, 100x solution contains 0.25mg/ml), and Bacto yeast extract (5% solution served as 25x stock). Other reagents used in tissue culture were: 1x PBS⁻ (phosphate buffered saline: 8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄, 0.2g KH₂PO₄ in one litre DDW supplemented with P-S), 1x PBS⁺⁺ (PBS supplemented with 0.1mg/ml CaCl₂ and 0.1mg/ml MgCl₂), pronase-SDS (0.5mg/ml pronase in lysing buffer: 10mM Tris pH7.4, 10mM EDTA diNa, 0.4% SDS), 2x trypsin-EDTA and 1x trypsin-EDTA (made by diluting Gibco 10x stock containing 5.0g/100ml trypsin and 2.0g/100ml EDTA in 1x PBS) and 1x saline-EDTA (made from 10x stock: 2g glucose, 2g di-Na-EDTA, 80g NaCl, 2g KCl, 11.5g Na₂HPO₄, 2g KH₂PO₄ in one litre DDW).

2.8.1 Cell Lines

293 cells (Graham et al, 1977) a human embryonic kidney cell line transformed by adenovirus 5 DNA, were maintained in F11 supplemented with 10% NCS, Pen-Strep, and L-Glu. HeLa cells were maintained in MEM supplemented with 10% NCS, Pen-Strep, and L-Glu.

2.8.2 Passaging of Cells

When 150mm dishes of 293 cells were near confluency they were passaged by removing the media and washing the cell monolayer once with 4ml of 1x saline-EDTA. The cells were then incubated with 1ml of saline-EDTA for 5-10 minutes at 37°C and then manually dislodged by banging the dish. The cells were then resuspended in media and plated either in 60mm dishes or 150mm dishes. Passaging of HeLa cells was performed in the same manner except that 1x trypsin-EDTA was used instead of saline-EDTA.

2.9 Transformation of Primary Baby Rat Kidney (BRK) Cells

2.9.1 Preparation of BRK cells

6-7 day old Wistar rats were obtained from the Charles River Company (Montreal, P.Q). The rats were sacrificed by cervical dislocation and the kidneys removed and placed in 1xPBS⁺. The kidneys

were then cleaned of excess membranes and blood vessels and transferred to a fresh solution of 1xPBS⁻. Using long handled scissors the kidneys were manually minced until the mixture had a soupy texture (no tissue fragments larger than about 1mm), usually about 10min. One vol of 2x trypsin-EDTA was added and the mixture stirred at medium speed for 20 min. The solution was allowed to settle and the supernatant was pipetted off into 10ml of ice cold FCS to stop the trypsin digest. Fresh 2x trypsin-EDTA was added to the remaining kidney tissue and the procedure repeated once more.

The kidney cells were then pelleted in 50ml Corning centrifuge tubes at 3000 rpm for 5min. The supernatant was discarded and the cells resuspended in 40ml of MEM plus 10% FCS, Pen-Strep and L-glu and allowed to incubate at 37°C for 15 min. The cells were then filtered through two layers of cheese cloth and brought up to a final volume of 50 to 60ml per pair of kidneys. The cells were then plated onto Corning 60mm tissue culture dishes at a volume of 5ml per dish. The following day cells were refed with MEM plus 10% FCS, Pen-Strep, and L-Glu. Generally, it was possible to seed 10-12 60mm dishes for each pair of kidneys.

2.9.2 DNA-Mediated Transfection of Primary BRK cells

When the BRK cells reached 70-80% confluence they were transfected via the calcium technique (Graham and van der Eb, 1973) as modified by Wigler et al (1979). The desired amount of plasmid DNA was added to a

15ml Corning polystyrene tube followed by 30ug of salmon sperm carrier DNA, and DDW up to a volume of 0.9ml. 100ul of 2.5M CaCl_2 was added to give a final volume of 1ml. This mixture was then added dropwise slowly to 1.0ml of 2xHepes (8g NaCl, 0.37g KCl, 0.125g Na_2HPO_4 , 5.0g Hepes [Calbiochem], 1.0g dextrose, pH adjusted to 7.1 and then brought up to a final volume of 500ml) whilst bubbling sterile air through the latter. The bubbling was allowed to continue 20 seconds after the last drop was added and then the samples were incubated for 30 minutes at room temperature to allow the DNA precipitate to form. After this time 0.5ml of the solution was added to each of 4x60mm dishes containing 5ml of culture medium and the DNA precipitate was allowed to be taken up overnight. The dishes were then refed the next day with MEM plus 10% FCS, Pen-Strep and L-glu. Selection for transformed cells began 4 days post-transfection by switching the media to either Jok plus 5% HS, Pen-Strep and L-Glu; MEM plus 5% FCS, Pen-Strep and L-Glu or F11 plus 5% FCS, Pen-Strep and L-Glu. Dishes were refed twice weekly until 14 days post-transfection in the case of transformation with Ela plus Elb or 21 days for transformation with Ela plus ras. At this point the medium was removed and the cells fixed with 3:1 Methanol:Acetic acid for 30min, dried, and stained with Giemsa (Fischer Scientific, diluted 1 in 20 with 0.1x PBS). Colonies of transformed cells were counted first visually and then confirmed under the microscope.

2.10 Rescue of Insertion Mutants into Infectious Virus

2.10.1 Preparation of 293 Cells for Transfection

293 cells used for DNA transfection were split as described in Section 2.8.2 and seeded in 60mm dishes. Generally, 6-8 60mm dishes could be seeded from one 150mm dish depending on the confluency of the cells and their rate of growth. As a rule the older the cell's passage number the faster would be their growth rate, taking this into consideration the cells would be from 70-80% confluent the next day and ready for transfection.

2.10.2 Transfection of 293 cells

DNA-mediated transfection of 293 cells was by the calcium technique (Graham and van der Eb, 1973). To 15ml polystyrene tubes the following were added in order: 0.5ml 2x HEPES, 10ug pJM17 (McGrory *et al*, 1988), 10ug of the plasmid containing the insertion mutation, DDW up to a volume of 0.9ml, then 100ul of 2.5M CaCl_2 . The cocktail was mixed and allowed to stand at room temperature for 30 minutes. 0.5ml was added to each of two 60mm dishes of 293 cells prepared as described above. The plates were gently swirled to distribute the DNA precipitate and then allowed to incubate at 37°C for 4 to 5 hours to allow the uptake of the DNA. After this time the media was removed and the dishes were overlaid with F11 supplemented with 5% HS, P-S, fungizone, L-glutamine,

yeast extract, and 0.5% agarose and returned to the incubator. Plaques were normally visible within 8-9 days.

2.10.3 Harvest of Plaque Isolates

Once plaques were visible and had grown to approx. 3mm or more in size well isolated plaques were picked using a sterile pasteur pipet and transferred to 0.5ml of PBS++ with 10% glycerol. The plaque isolates were then frozen at -70°C until required. To analyze the viral DNA of a plaque isolate 250ul of the viral solution was used to infect a 293 dish prepared for viral infection at about 85-90% confluency (as described in Section 2.11.1). The virus was allowed to adsorb for 30-60min and then the dish was refed with F11 supplemented with P-S, L-glutamine, and 5% HS.

2.10.4 Harvest of Liquid Infections

Once the liquid infections had reached complete cytopathic effect (CPE) the viral DNA was harvested as follows. The dish was placed in the flow hood and any floating cells were allowed to settle for 15min. After this time the medium was carefully removed, leaving as many cells as possible on the dish, using a pasteur pipet and transferred to a 4 dram glass vial containing 0.5ml of 100% glycerol and stored at -70°C . To the remaining cell monolayer and cell debris was added 0.5ml of

pronase-SDS and the dish returned to the incubator for overnight digestion.

2.10.5 Analysis of Viral DNA

To isolate and analyze the viral DNA, the now viscous cell lysate was transferred to a 1.5ml eppendorf tube and extracted once with phenol. The aqueous phase was transferred to a fresh tube and the viral DNA was precipitated twice with 2vol of ice cold 95% ethanol. The DNA pellet was then dried at 37°C and resuspended in 100ul of 0.1x SSC. A 20 ul aliquot was used for restriction enzyme digests with HindIII or HindIII and BamHI prior to electrophoresis on agarose gels.

2.10.6 Plaque Purification of Viral Recombinants

After identification of a correct viral recombinant the virus-containing medium from the original liquid infection was used as a stock from which to plaque purify the virus. The stock was serially diluted in PBS++ and appropriate dilutions used to infect 60mm dishes of 293 cells. After 7-8 days a well isolated plaque was picked and used for a liquid infection of 293 cells. From this infection the viral DNA was reanalyzed, and if correct, the process was repeated a second time. After the second round the virus medium harvested was transferred to a 4 dram vial containing 0.5ml of glycerol, freeze/thawed three times to release all viral particles from cells, and then stored at -70°C to

serve as a stock for assay of plaque forming units per millilitre (pfu/ml).

2.11 Determination of Virus Titres

2.11.1 Preparation of Cells for Virus Infection

293 or HeLa cells to be used for virus infection were split as described in such a way to give 60mm dishes that were approximately 85-90% confluent the next day. To this end a near confluent 150mm dish of 293 cells was generally split into 6x60mm dishes and a near confluent dish of HeLa cells was split into 15-20x60mm dishes.

2.11.2 Viral Infection of 293 and HeLa Cells

To determine the plaque forming units of a viral stock serial dilutions of the stock were made in PBS++ ranging from 10^{-1} to 10^{-9} . 293 and HeLa cells prepared as described above for viral infection had their media removed and duplicate plates were infected with 250ul for each dilution. After addition of virus the plates were incubated at 37°C for 30-60min to allow virus adsorption. The plates were then overlaid with F11 supplemented with 2%HS, P-S, fungizone, L-glutamine, yeast extract and 0.5% agarose. Plaques were counted starting at 7 days post-infection and counting continued until no more plaques appeared, usually about 9 days post-infection. Results were tabulated as pfu/ml

for both 293 and HeLa cells and expressed as the ratio between 293 and HeLa cells relative to the ratio between 293 and HeLa cells for the wild type virus d1309.

Results

D.S. Bautista has constructed Ela insertion mutants and assayed them for the ability to transcriptionally activate other early viral gene promoters and the ability to repress the expression of enhancer-linked genes. As described in Section 1.6 the insertion mutants produce in-frame insertions of 13aa or 2aa or out of frame insertions resulting in the premature termination of translation (Figure 5). The purpose of this project was to analyze the insertion mutants for transformation of rodent cells and for viral replication on HeLa cells and to correlate these results with those on transactivation and repression.

3.1 Rescue of Mutated Ela Plasmid Into Elb Plasmid

In order to assay the Ela mutants for the DNA-mediated transformation of primary baby rat kidney cells (BRK cells) it was necessary to clone the mutants contained in the Ela plasmid pKH101 (Figure 4) into a plasmid containing all of the El region. The El plasmid used was pXC38, a derivative of pXC1 (McKinnon et al, 1982) which has had the BamHI site removed (D.S. Bautista, personal communication) and contains the left end of the Ad5 genome up to 16mu and therefore provides an intact and wild type Elb.

Figure 5. Oligonucleotides used by D.S. Bautista for the insertion mutagenesis and amino acids coded by the insertions. Two types of oligonucleotide linkers were used, one with blunt ends and one with HpaII ends, depending on the type of enzyme used to generate the insertion. Each linker is flanked by BamHI sites (G-G-A-T-C-C). Below the linker sequences is shown the amino acids coded by the inserts for each of the possible three reading frames in either the open reading frame or closed reading frame.

1) BamHI Linkers with HpaII Ends

Orientation A: Closed Reading Frame

5'-CGGATCCTGACCCAATTGTGAGCGGATAACAATTGGATC-3'
3'-CTAGGACTGGGTTAACAACCTCGCCTATTGTTAACCTAGGC-5'

I: C-GLY-SER-Stop
II: C-G-ASP-PRO-ASP-PRO-ILE-VAL-SER-GLY-Stop
III: ARG-ILE-LEU-THR-GLN-LEU-Stop

Orientation B: Open Reading Frame

5'-CGGATCCAATTGTTATCCGCTCACAATTGGGTCAGGATC-3'
3'-CTAGGTTAACAATAGCGGAGTGTTAACCCAGTCCTAGGC-5'

I: C-GLY-SER-ASN-CYS-TYR-PRO-LEU-THR-ILE-GLY-SER-GLY-SER
II: C-G-ASP-PRO-ILE-VAL-ILE-ARG-SER-GLN-LEU-GLY-GLN-ASP-PRO
III: ARG-ILE-GLN-LEU-LEU-SER-ALA-HIS-ASN-TRP-VAL-ARG-ILE-C

2) BamHI Linkers with Blunt Ends

Orientation A: Closed Reading Frame

5'-GGATCCTGACCCAATTGTGAGCGGATAACAATTGGATCC-3'
3'-CCTAGGACTGGGTTAACAACCTCGCCTATTGTTAACCTAGG-5'

I': GLY-SER-Stop
II': G-ASP-PRO-ASP-PRO-ILE-VAL-SER-GLY-Stop
III': G-G-ILE-LEU-THR-GLN-LEU-Stop

Orientation B: Open Reading Frame

5'-GGATCCAATTGTTATCCGCTCACAATTGGGTCAGGATCC-3'
3'-CCTAGGTTAACAATAGCGGAGTGTTAACCCAGTCCTAGG-5'

I': GLY-SER-ASN-CYS-TYR-PRO-LEU-THR-ILE-GLY-SER-GLY-SER
II': G-ASP-PRO-ILE-VAL-ILE-ARG-SER-GLN-LEU-GLY-GLN-ASP-PRO
III': G-G-ILE-GLN-LEU-LEU-SER-ALA-HIS-ASN-TRP-VAL-ARG-ILE-C

To accomplish this pXC38 was digested with the restriction endonucleases KpnI and EcoRI for a minimum of 4 hours at 37°C then run on a 1% agarose gel overnight (Figure 6). The large fragment containing the Ad5 left end from 2050nt to 16mu and pBR sequences was purified from the agarose gel as described in Section 2.7.1 and resuspended in 0.1x SSC at a final concentration of approximately one microgram per microlitre.

The pKH101 plasmid containing the linker insertion was digested with EcoRI and KpnI for 4 hours at 37°C and the enzymes were then inactivated by heating at 65°C for 30 minutes. An aliquot of the digest was then added to a ligation mixture containing one microgram of the purified large fragment of pXC38 and one unit T4 DNA ligase and then incubated for 16 to 20 hours at 15°C (Figure 6). The ligated DNA was then used to transform competent E. coli HMS 174 cells. The resulting colonies were screened by preparing mini-preps of plasmid DNA according to the method of Birnboim and Doly (1979) followed by digestion with EcoRI and KpnI and then by EcoRI and BamHI and electrophoresis on agarose gels. Recombinant clones were identified by the increased size of the plasmid evident from the EcoRI/KpnI digest (Figure 7). Final confirmation of the isolates was provided by the EcoRI/BamHI digest since the introduction of a novel BamHI site due to the linker insertion results in a band equal in size to the site of the insertion (Figure 7). For example the plasmid containing the insertion at nt1056 of the Ad5 genome when restricted with EcoRI/BamHI would have two bands on an

Figure 6. Cloning protocol used to rescue insertion mutants contained in the Ela plasmid into a plasmid containing the remainder of the El coding sequences. The plasmid pXC38, containing the sequences of the Ad5 genome from 0mu to 16mu (Ad sequences are indicated by solid bars), was digested to completion with EcoRI and KpnI and the large fragment containing the Ad5 sequences from 5.7mu to 16mu plus the plasmid sequences was purified from an agarose gel. The plasmid containing the insertion mutation, the example here being pEla-884c, was digested with EcoRI and KpnI and then ligated with the large fragment from pXC38 to form the plasmid pX884c.

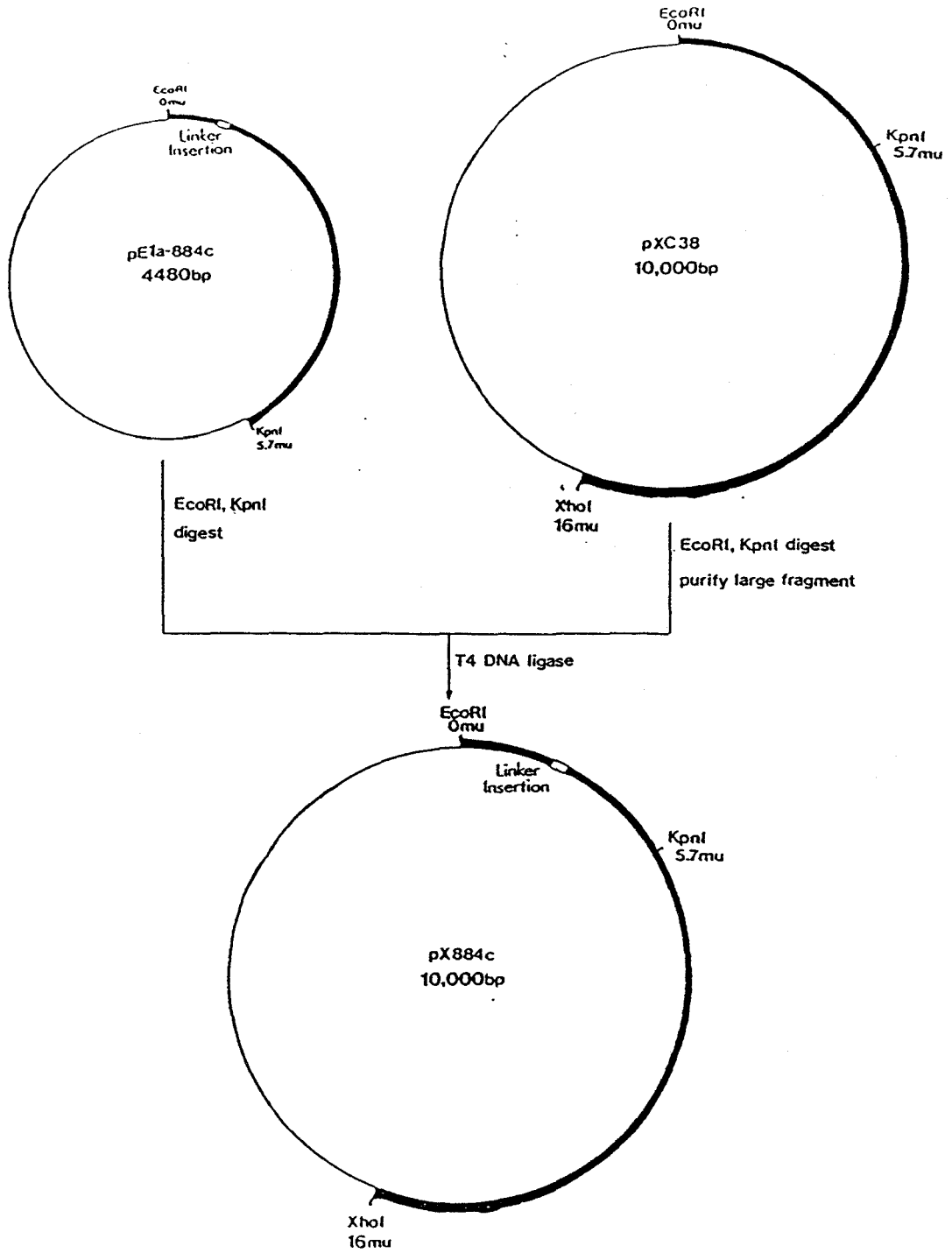
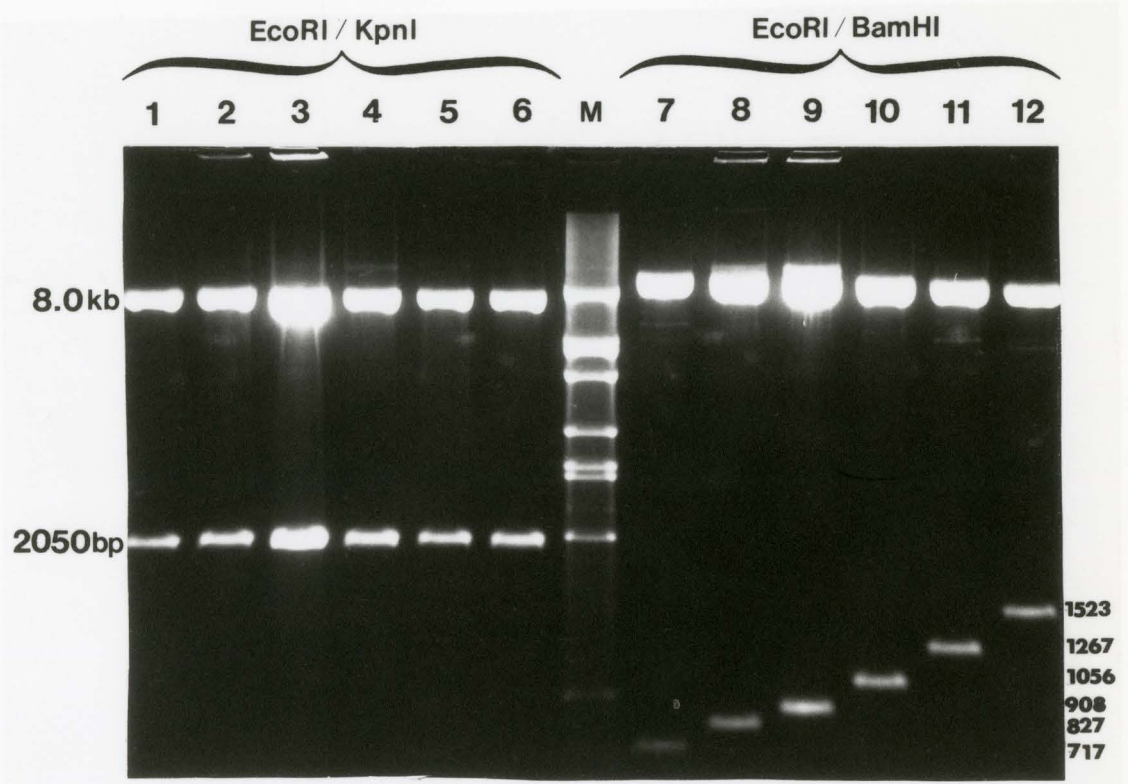


Figure 7. Restriction enzyme profile of selected E1 plasmids containing the insertion mutations. Lanes 1 through 6 indicate the plasmids pX717c, pX827c, pX908c, pX1056c, pX1267c and pX1523c, respectively, digested with EcoRI and KpnI. The sizes of the bands are indicated to the left of the gel picture in base pairs. The marker lane, designated M, is Ad5 DNA digested with HindIII. Lanes 7 through 12 correspond to the same plasmids as lanes 1 through 6 except the digestion was with EcoRI and BamHI. The introduction of the novel BamHI site due to the linker insertion results in the lower bands corresponding in size to the nucleotide site of the insertion as indicated by the numbers to the right of the gel picture.



agarose gel, the smaller of which would be equal to 1056bp (Figure 7). The efficiency of this cloning protocol was approximately 50%. Correct recombinants were streaked out on LB agar-ampicillin plates to obtain a well isolated colony and then reanalyzed and stored at -70°C . Each recombinant plasmid was designated as pX followed by the nucleotide site at which the insertion was made. The type of insertion was designated by either a (+) sign indicating a 39bp (or 13aa) insert in an open reading frame, a (-) sign indicating a 39bp insert resulting in a closed reading frame, or a small letter 'c' to indicate the 'collapsed' or 6bp (2aa) insert (Figure 8). For example, a 39bp ORF insert at nt 884 was designated pX884(+).

Because the insertion sites were generated randomly, each site had an insertion in one of three possible reading frames (Figure 5). In addition D.S. Bautista utilized two types of linker, one with blunt ends and one with HpaI ends resulting in the different possible reading frames (Figure 5). As shown in Figure 8 all the insertion mutants save two did not interrupt the codons for the flanking amino acids of the Ad5 genome. The exceptions are: the 1056 site where the aspartic acid residue at position 166 of the 289R protein was changed to glutamic acid followed by the amino acids of the insertion and then returning to wild-type coding sequences at residue 167; and the 1523 site where the serine residue at position 283 was changed to arginine.

Figure 8. List of insertion mutants and the predicted amino acid sequences resulting from the insertions. Insertion sites are given as the nucleotide site preceding the first nucleotide of the oligonucleotide linker. Thirteen amino acid in frame insertions are denoted by (+) sign, two amino acid in frame insertions are denoted by 'c' and closed reading frame insertions are denoted by (-). The amino acid insertions for each site are shown by the number of the amino acid position from the wild-type protein followed by the Roman numeral indicating the reading frame of the insertion (see Figure 5). The Roman numerals followed by the 'prime' sign e.g. II' indicate insertions made by the linker with blunt ends while all others were made with the linker with HpaII ends.

<u>Insertion Sites</u>	<u>Amino Acid Insertion</u>	<u>Insertion Sites</u>	<u>Amino Acid Insertion</u>
717(+)	53-II'-54	1008(+)	150-I-151
717(-)	53-II'	1008(-)	150-I
717c	53-Asp-Pro-54	1008c	150-Gly-Ser-151
812(+)	85-II-86	1039(+)	160-III-161
812(-)	85-II	1039(-)	160-III
812c	85-Asp-Pro-86	1039c	160-Arg-Ile-161
819(+)	87-I-88	1056(+)	165-Glu-II-167
819(-)	87-I	1056(-)	165-Glu-II
819c	87-Gly-Ser-88	1056c	165-Glu-Asp-Pro-167
827(+)	90-II-91	1267(+)	198-II-199
827(-)	90-II	1267(-)	198-II
827c	90-Asp-Pro-91	1267c	198-Asp-Pro-199
863(+)	102-II-103	1304(+)	210-II'-211
863(-)	102-II	1304(-)	210-II'
863c	102-Asp-Pro-103	1304c	210-Asp-Pro-211
882(+)	108-II'-109	1376(+)	234-I-235
882(-)	108-II'	1376(-)	234-I
882c	108-Asp-Pro-109	1376c	234-Gly-Ser-235
884(+)	109-II-110	1408(+)	245-II-246
884(-)	109-II	1408(-)	245-II
884c	109-Asp-Pro-110	1408c	245-Asp-Pro-246
906(+)	116-II'-117	1415(+)	247-II'-248
906(-)	116-II'	1415(-)	247-II'
906c	116-Asp-Pro-117	1415c	247-Asp-Pro-248
908(+)	117-II-118	1523(+)	282-Arg-II'-284
908(-)	117-II	1523(-)	282-Arg-II'
908c	117-Asp-Pro-118	1523c	282-Arg-Asp-Pro-284

3.2 Transformation of Primary BRK Cells

3.2.1 Variability of Transformation Assay

Preliminary studies were undertaken to optimize the reproducibility of the transformation assay. These studies revealed that the same DNA preparation employed in the same experiment varied by only 3.5% in transforming activity (Table 1). However the same DNA preps used in separate experiments varied in transformation rate by 22-122% (Table 1). Therefore the condition of the BRK cells and their ability to take up and express the transfected DNA introduces a source of uncontrollable variation. Despite efforts to standardize the preparation of the cells and their confluence upon transfection inherent variability in the number of cell types present eg. fibroblastic or epithelial, and the stage in each cell's growth cycle were beyond control. To overcome this variation each mutant was assayed at least three times (except for those which were completely defective which were assayed only twice) using 4x60mm dishes in each experiment and all results are expressed as a percentage of the wild type transformation rate for that experiment.

3.2.2 Linear Response of Colony Formation to DNA Concentration

To determine the linear response range of the transformation assay it was necessary to measure the colony forming capacity of several different concentrations of DNA. This was done to ensure that the

Table 1. Variability of DNA-mediated transformation assays. Comparisons were made between the average number of foci formed on 4 60mm dishes of BRK cells under different experimental conditions. The percent difference in colony numbers was calculated between the following: i) The same DNA preparation used in the same BRK cell preparation. ii) Different preparations of the same DNA plasmid used in the same BRK cell preparation. iii) The same DNA preparation used in different BRK cell preparations. iv) Different DNA preparations of the same plasmid used in different preparations of BRK cells.

PERCENT DIFFERENCE

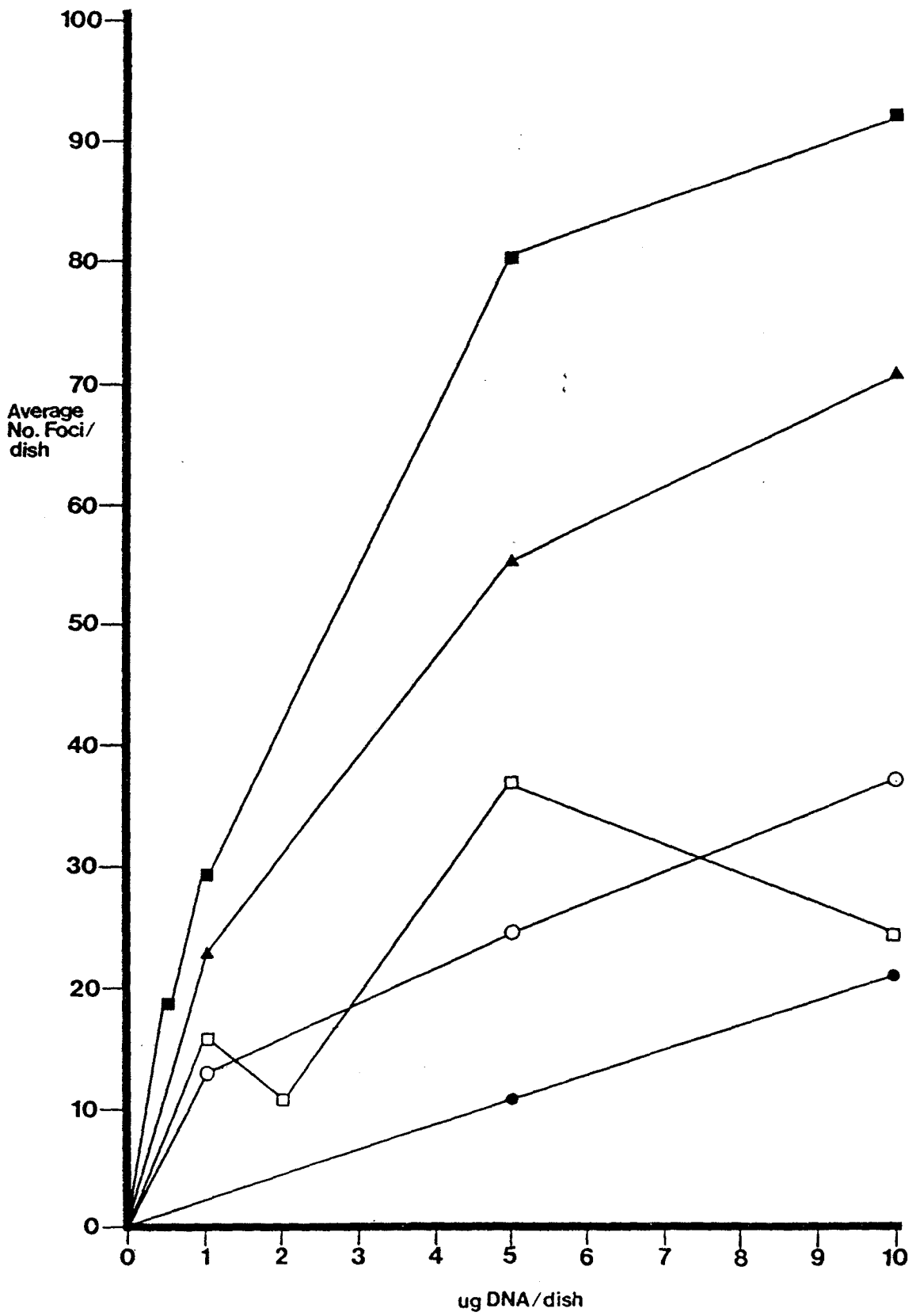
	Same DNA Preparation	Different DNA Preparation
Same BRK Preparation	3.5	12.1, 24.1
Different BRK Preparation	99.0, 122.6 45.6, 22.1	294.1

concentration of DNA used to measure transformation was one which fell within the linear response of the assay. As shown in Figure 9 the number of colonies formed per dish increased approximately in a linear fashion up to a point somewhere beyond 5ug after which it either started to plateau or, as in one case, actually decreased at 10ug per dish. From this data it was decided to use 5ug of DNA per dish for all subsequent assays for two reasons: 1) this concentration seemed to be within the linear response and 2) the number of colonies produced at this concentration was statistically significant and easily countable. The average number of colonies formed by 5ug of the wild type plasmid pXC38 was 37.1 per dish (from 7 experiments) with a standard deviation of 22.5 and standard error of 9.2. For transformation assays done with ras, 5ug of the Ela plasmid was cotransfected with 5ug of the pEJ-ras plasmid.

3.2.3 Transformed Colony Morphology

Colonies formed by the wild type Ad5 plasmid pXC38 were of two types, one type consisting of epithelial shaped cells in a flat, well adhered form while the second type consisted of somewhat smaller rounded cells growing to a high density in a piled-up manner. The frequency of appearance of the two colony types did not depend on the DNA sample but varied from experiment to experiment, an observation consistent with the findings of other investigators (Gallimore et al, 1974; van den Elsen et

Figure 9. Colony formation on BRK cells by pXC38 (wt E1) relative to DNA concentration. Units on the y-axis are the average number of foci formed per dish from four dishes in each experiment. The x-axis indicates the ug of pXC38 DNA per 60mm dish used in the transfection. Results from 5 separate experiments are shown.



al, 1983b). Colonies formed by the Ela mutant plasmids showed the same variation in colony types formed.

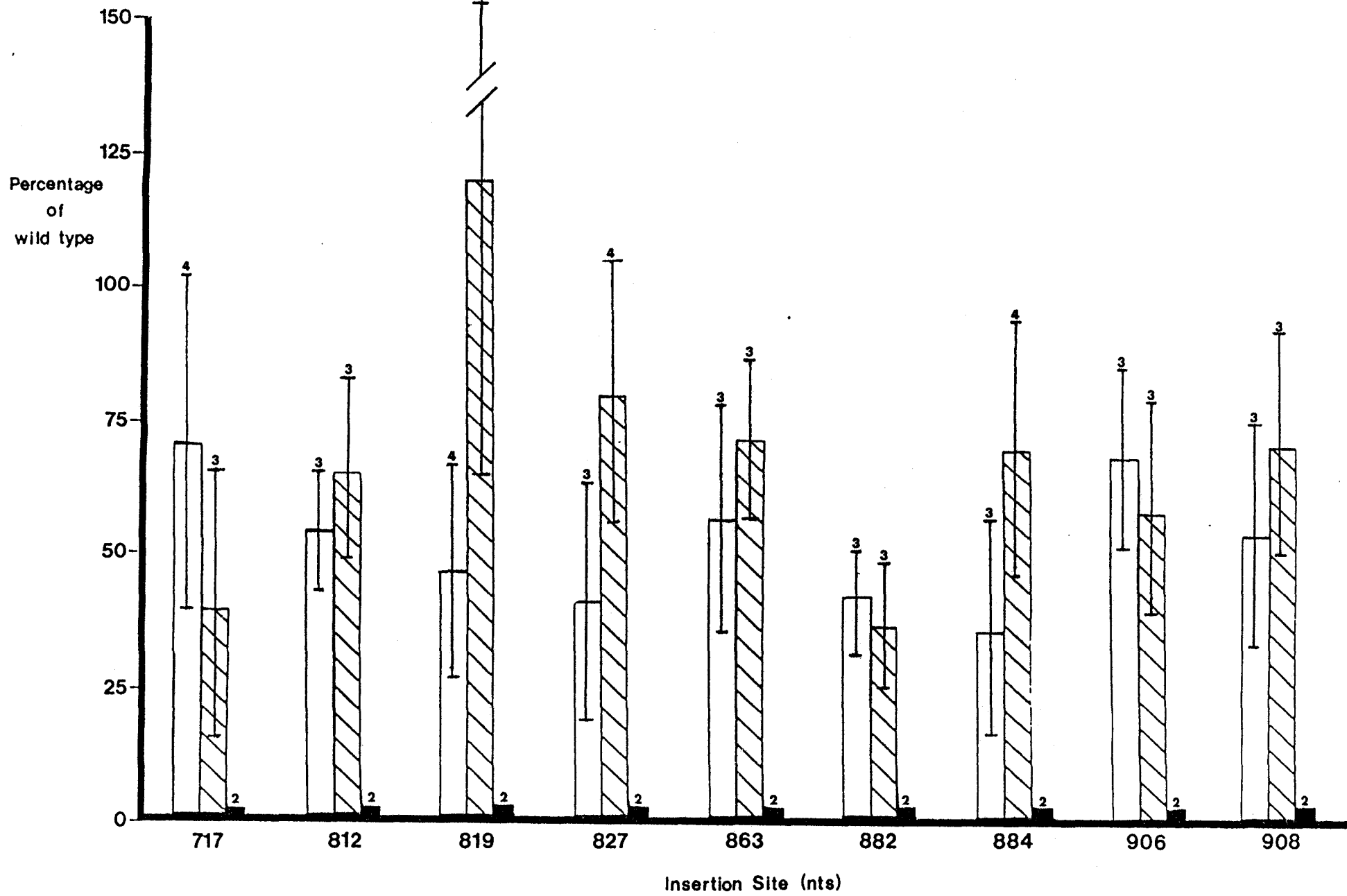
3.2.4 Transformation by Ela Insertion Mutants

In each transformation assay four dishes of BRK cells were transfected with 5ug each of the desired plasmid DNA. A complete table of the transformation results is presented in Appendix 1. Figures 10 and 11 illustrate the average values for transformation by each mutant from several experiments. The values for transformation of each mutant were obtained by taking the average number of colonies from 4 dishes in an experiment and expressing this average as a percentage of the wild type transformation rate from 4 dishes in the same experiment. A final average percentage of wild type transformation was computed for each mutant from the number of experiments indicated in Figures 10 and 11.

3.2.5 Transformation by Insertion Mutants Upstream of the Unique Region

The region of exon 1 common to both the 13S and 12S protein products is known to play an important role in transformation (Lillie et al, 1987; Whyte et al, 1988; Moran et al, 1986a; Schneider et al, 1987; Zerler et al, 1987; Lillie et al, 1986). This is supported by the results in Figure 10 which show that all insertion mutants in the closed reading frame in this region were completely defective for transformation. However, insertions in the open reading frame resulting

Figure 10. Transformation of BRK cells by insertion mutants located upstream of the unique region of Ela. The values for transformation were calculated by taking the average number of colonies from 4 60mm dishes and expressing that as a percentage of wild type for each experiment. Cumulative averages from each experiment were calculated to yield the final transformation value. Vertical lines extending from each bar represent the standard error and the numeral above each bar indicates the number of experiments (the asterisk above pX819c denotes that the error bar extends to 174%). Open bars represent 13aa in frame insertions, hatched bars represent 2aa in frame insertions and solid bars represent closed reading frame insertions.



in either a 13aa or 2aa insert in this region did not abolish the transforming ability of the proteins (Figure 10). Although transforming activity by these mutants, with the exception of pX819c, was on average less than wild-type it is difficult to determine the biological significance of these in-frame mutations. Statistical analyses of the results using Student's t distribution (Table 2) revealed that the mutants pX812(+), pX819(+), pX827(+), pX882(+), pX882, and pX884(+) were all significantly less than wild type in transformation activity. However, none of these mutants were consistently less than than 50% of wild type (Appendix 1) and in an assay such as this a difference of 2 fold or greater could often be observed within the 4 replicate plates of a single mutant in the same experiment. Therefore it is difficult to quantitatively determine the biological effect on transformation by these mutants, but it may be assumed that the insertion in the protein asserts a conformational change that acts to impair the transforming function. Results with pX819c indicate that a 2aa insert at this site in the protein has no effect on transformation since this mutant averaged greater than wt in 2 out of 4 experiments.

Table 2. Student's 't' test of the statistical significance of the transforming activity of the Ela insertion mutations. The Null hypothesis states that the average transformation value of a given mutant is equivalent to 100% of wild type. This hypothesis is either rejected or accepted depending on the value of 't' and the degrees of freedom. Rejection of the Null hypothesis indicates that the mutant in question is statistically less than wild type in transforming activity. Analyses were not performed on the CRF mutants upstream of the unique region since all of these were completely defective in transformation.

<u>Plasmid</u>	<u>Value of 't'</u>	<u>Degrees of Freedom</u>	<u>Null Hypothesis</u>
pX717(+)	-0.98	3	Accept
pX717c	-2.86	2	Accept
pX812(+)	-4.95	2	Reject
pX812c	-2.34	2	Accept
pX819(+)	-3.10	3	Reject
pX819c	0.37	3	Accept
pX827(+)	-3.09	2	Reject
pX827c	-1.06	3	Accept
pX863(+)	-2.40	2	Accept
pX863c	-2.07	2	Accept
pX882(+)	-7.73	2	Reject
pX882c	-6.56	2	Reject
pX884(+)	-3.77	2	Reject
pX884c	-1.50	3	Accept
pX906(+)	-2.26	2	Accept
pX906c	-2.71	2	Accept
pX908(+)	-2.62	2	Accept
pX908c	-1.65	2	Accept
pX1008(+)	-8.94	3	Reject
pX1008(-)	-68.5	3	Reject
pX1008c	-48.1	3	Reject
pX1039(+)	-34.9	2	Reject
pX1039(-)	-32.3	2	Reject
pX1039c	-1.07	2	Accept
pX1056(+)	-4.63	4	Reject
pX1056(-)	-15.8	3	Reject
pX1056c	-7.39	3	Reject

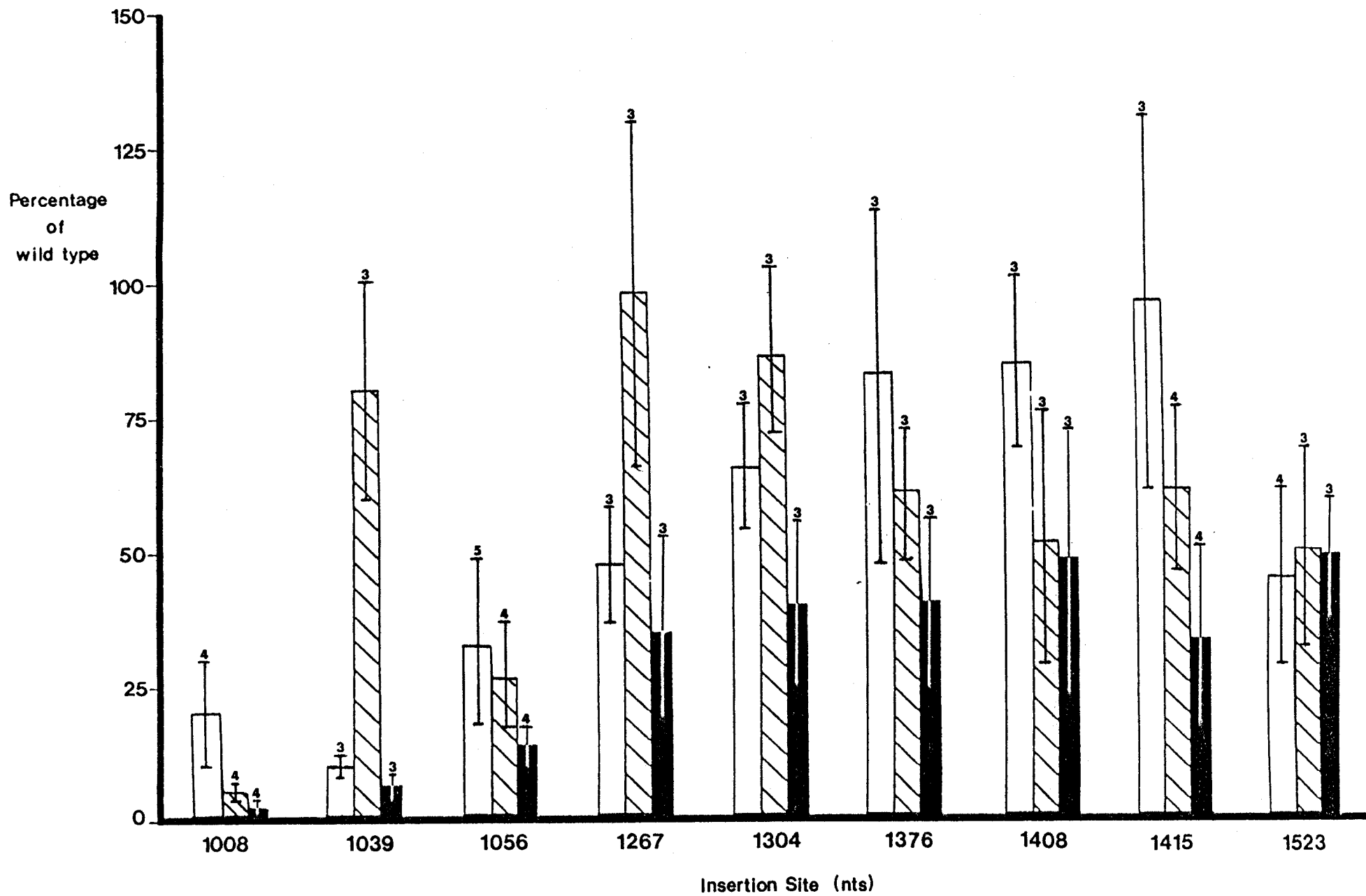
Table 2 (cont'd)

<u>Plasmid</u>	<u>Value of 't'</u>	<u>Degrees of Freedom</u>	<u>Null Hypothesis</u>
pX1267(+)	-5.29	2	Reject
pX1267(-)	-5.83	2	Reject
pX1267c	-0.07	2	Accept
pX1304(+)	-3.46	2	Reject
pX1304(-)	-4.52	2	Reject
pX1304c	-1.05	2	Accept
pX1376(+)	-0.56	2	Accept
pX1376(-)	-4.38	2	Reject
pX1376c	-3.85	2	Reject
pX1408(+)	-1.14	2	Accept
pX1408(-)	-2.60	2	Accept
pX1408c	-2.52	2	Accept
pX1415(+)	-0.12	2	Accept
pX1415(-)	-4.34	3	Reject
pX1415c	-2.81	3	Reject
pX1523(+)	-3.70	3	Reject
pX1523(-)	-5.18	2	Reject
pX1523c	-3.03	2	Reject

3.2.6 Transformation by Unique Region Insertion Mutants

Although the major activity of the unique region is the transactivation of other early viral genes it also plays a role in transformation (Babiss et al, 1986; Carlock and Jones, 1981). Several groups have found that mutants in the unique region are defective for both transactivation and transformation with Elb (Carlock and Jones, 1981; Graham et al, 1978; Bos et al, 1983a) while other unique region mutants transform at normal levels in conjunction with ras (Schneider et al, 1987). Still other mutants in the unique region are transactivation negative and transformation negative with ras (Schneider et al, 1987; Whyte et al, 1988). Figure 11 shows that insertion mutants in the unique region are also defective for transformation with Elb except for pX1039c which is near wild-type. pX1008(+), pX1056(+), pX1056(-) and pX1056c averaged between 15 and 35% of wild-type transformation while pX1008(-), pX1008c, pX1039(+) and pX1039(-) were consistently less than 15% of wild-type. Of interest is the fact that pX1039(+) which encodes the 13aa insert is defective in transformation while pX1039c which encodes the 2aa insert at the same site is unaffected for transformation. Also, the insertions at nt 1056 in the unique region appear to be slightly more capable of transformation relative to those at nt1008 and 1039, excepting 1039c. This may represent a functional border between sequences required for transformation with Elb.

Figure 11. Transformation of BRK cells by insertion mutants located in the unique region and exon 2 of Ela. The values for transformation were calculated by taking the average number of colonies from 4 60mm dishes and expressing that as a percentage of wild type for each experiment. Cumulative averages from each experiment were calculated to yield the final transformation value. Vertical lines extending from each bar represent the standard error and the numeral above each bar indicates the number of experiments. Open bars represent 13aa in frame insertions, hatched bars represent 2aa in frame insertions and solid bars represent closed reading frame insertions.



A possible explanation for the reduced transformation by UR mutants is that the transactivation of Elb by Ela is less efficient and therefore transformation is reduced. It has been shown that efficient expression of Elb is dependent on transactivation by Ela (Vaessen et al, 1987; Berk et al, 1979; Jones and Shenk, 1979; Nevins, 1981; Bos and ten Wolde-Kraamwinkel, 1983) and it is also known that Ela levels in transformed cells are higher when Elb is present (van den Elsen et al, 1983a). Therefore it may be possible that these mutants lack the ability to transactivate Elb which in turn results in lower levels of Ela and hence a lower rate of transformation. A possible mechanism by which Elb increases Ela expression may be the Elb 19K protein which can alleviate the enhancer repression function of the Ela proteins (Yoshida et al, 1987) which may be acting on the Ela promoter itself. In contrast some mutants in the unique region such as the group I mutant *hrl* (Graham et al, 1978) which is reduced in transforming activity, is still able to express wild-type levels of the Elb 58K and 19K proteins in transformed cells (Ruben et al, 1982) suggesting a direct role for the UR in transformation. An alternative explanation is that the insertion mutants in the unique region alter the ability of the 289R product to positively stimulate its own transcription, a role that this protein is known to play (Hearing and Shenk, 1985). In this case the reduced transformation would be a result of lower levels of Ela due to loss of a self-transactivating activity.

To help resolve this the transformation assay was performed using selection with MEM and 5% FCS instead of JOK and 5%HS. The former is a less stringent selection media which allows transformation by Ela alone while the latter requires expression of at least part of Elb (Graham et al, 1984). Results from this assay showed that the insertion mutants in the unique region, with the exception of pX1008(-), transform at about the same level as Ela alone when MEM plus 5%FCS is used for selection (Table 3). The mutants also transform at approximately the same frequency in either the MEM plus 5% FCS selection media or the Jok plus 5% HS media. This would suggest that it is not the autostimulation of Ela that is being disrupted but rather the lack of transactivation of Elb that is causing the reduced transformation efficiency. Because the UR mutants can transform, albeit at low levels, in the Jok plus 5% HS media while Ela alone can not, a low level of Elb is likely being expressed by the mutants. However this level of Elb expression is not sufficient to produce transformed foci at the same frequency as the wild type El. It is evident from Table 3 that the plasmid containing Ela alone transformed cells at about one-tenth of the wild type El suggesting again that the absence of Elb in transformed cells reduced the frequency of appearance of foci.

To further resolve this question the transformation assay was carried out using cotransfection of the UR mutant plasmids in an Ela only background with the EJ-ras oncogene. Several groups have found that transformation with unique region mutants is wild type with ras

<u>Plasmid</u>	<u>Colonies/dish</u>	
	<u>MEM plus 5%FCS</u>	<u>Jok plus 5%HS</u>
pXC38(wt E1)	40,59,57,49	58,55,59,50
pKH101(wt Ela)	9,4	0,0
pX1008(+)	5,3	4,4,4,5
pX1008(-)	0,1	0,1,1,1
pX1008c	3,7	1,0,3,2
pX1039(+)	5,10	ND
pX1039(-)	4,5	ND
pX1039c	10,18	ND
pX1056(+)	2,6	4,3,3,5
pX1056(-)	5,7	8,6,6,5
pX1056c	7,5	8,6,10,11
Neg. Con.	0,0	0,0

Table 3. Transformation of BRK cells by Unique Region Mutants Under Different Selective Media. The transformation assay was carried out as described using either selection with MEM plus 5%FCS or Jok plus 5%HS. Results are shown as the number of colonies formed on each 60mm dish.

(Schneider et al, 1987; Velcich and Ziff, 1988; Moran et al, 1986a) possibly since ras is not dependent on Ela for transactivation (Sassone-Corsi and Borrelli, 1987). The results showed that the unique region mutants in an Ela background were able to transform with ras at levels equal to or higher than wild type Ela with ras (Table 4). Therefore the integrity of the unique region does not seem to be important for Ela to transform rodent cells in conjunction with ras but is required for efficient transformation with Elb. This further suggests that the reduced transformation activity observed with most of the unique region mutants is not in fact directly due to Ela but a result of the reduced activity of Elb. The mutant pX1008(-) was severely reduced in all three types of transformation assays possibly because this is the earliest truncation in the UR and the sequences lost play a role in transformation or the protein product may be unstable.

3.2.7 Transformation by Exon 2 Insertion Mutants

Insertion mutants in exon 2, whether open reading frame or closed, retained the ability to transform (Figure 11). Analysis of the results by the Student's t test revealed that the mutants contained within pX1267(+), pX1267(-), pX1304(+), pX1304(-), pX1376(-), pX1376c, pX1415(-), pX1415c, pX1523(+), pX1523(-) and pX1523c were significantly less than wild type in transforming activity (Table 2). However, similar to the results discussed in Section 3.2.5 none of these mutants

Table 4. Transformation by Unique Region Mutants in an Ela only background with ras. 5ug of the indicated plasmids were cotransfected onto BRK cells as described. Shown are the number of colonies formed in each experiment and the transforming ability of each mutant expressed as the average percentage of wild-type (pKH101 + ras) from the two experiments.

<u>Plasmid</u>	<u>Expt .1</u>	<u>Expt .2</u>	<u>Average %wild-type</u>
pKH101(Ela only)	0,0,0,0	ND	0
pEJ-ras alone	0,0,0,0	ND	0
Neg. Control	0,0	0,0,0,0	0
pKH101+ras	5,6,4,6	3,2,4,4	100
pEla-717(+)+ras	1,6,4,5	3,2,0,2	65
pEla-884(-)+ras	0,0,0,0	0,0,0,0	0
pEla-1008(+)+ras	10,14,17,12	4,7,12,6	237
pEla-1008(-)+ras	3,4,4,2	2,3,2,5	77
pEla-1008c +ras	4,5,8,9	7,4,6,5	146
pEla-1039(+)+ras	12,12,13,11	10,11,10,6	256
pEla-1039(-)+ras	10,14,7,9	13,9,12,5	245
pEla-1039c +ras	12,9,10,8	4,9,8,9	208
pEla-1056(+)+ras	14,14,8,10	11,16,10,8	282
pEla-1056(-)+ras	8,5,4,3	8,7,8,6	159
pEla-1056c +ras	11,10,16,10	12,13,7,9	269

were consistently less than 50% of wild type in transformation (Appendix 1) thus it is difficult to draw definitive conclusions on the biological significance of these results. Evidence has been presented showing that exon 2 mutations have no effect on transformation (Velcich and Ziff, 1988; Schneider et al, 1987; Kuppaswamy and Chinnadurai, 1987). The insertion mutants that result in a truncated protein lose the carboxy terminus pentapeptide sequence that acts as a rapid nuclear localization sequence (Krippel et al, 1985; Lyons et al, 1987). The loss of this sequence appears not to drastically reduce transformation indicating the proteins have a sufficient half-life to allow their transport to the nucleus and exert their influence on the cell's growth cycle.

3.3 Rescue of Mutated El Plasmid into Infectious Virus

3.3.1 Construction of pJM17

As well as studying the effect of the Ela insertion mutants on the transformation of BRK cells it was also of interest to determine what effect they would have on the ability of the virus to replicate in a human cell line, such as HeLa cells. Because of the number of mutants involved it was deemed necessary to develop a method of constructing recombinant viruses that was both simple and efficient. A plausible solution was based on observations that two non-infectious plasmids could recombine efficiently in 293 cells to produce infectious virus (Ghosh-Choudhury et al, 1987) and that the adenovirus capsid seemed to

be capable of packaging only about 2kb in excess of its genome (Ghosh-Choudhury et al, 1987). It was reasoned that an insert of greater than 4kb in the Ela region of the infectious plasmid pFG140 (Graham, 1984) would result in a non-infectious plasmid that could recombine with a second plasmid in vivo to produce infectious virus.

To this end the protocol shown in Figure 12(A) was employed (McGrory et al, 1988). The infectious plasmid pFG140 (Graham, 1984) which is a derivative of the phenotypically wild type virus dl309 (Jones and Shenk, 1979) was digested with BamHI and a kanamycin resistant plasmid pPBdxI (Ghosh-Choudhury et al, 1986) was inserted at the BamHI site at 59.5mu. Recombinant plasmids were identified by their resistance to both ampicillin (due to the pMX2 insert) and kanamycin. Of the 36 colonies screened, 32 that showed double resistance were not of the predicted conformation, presumably due to rearrangements in vivo caused by homologous recombination between sequences within the pMX2 and pPBdxI inserts. The remaining 4 plasmid preps were of the predicted configuration and one, designated pJM15 (Figure 13), was chosen for further work. pJM15 was digested with XbaI to excise the pMX2 sequences and then religated with pBRX (Haj-Ahmad, 1986), a pBR322 derivative coding for ampicillin and tetracycline resistance. Only one recombinant, called pJM16, was obtained in this experiment. pJM16 showed resistance to ampicillin, tetracycline and kanamycin and had the predicted restriction endonuclease pattern (Figure 13). Finally pJM16 was

Figure 12. The construction of pJM17 and its use in the rescue of Ela insertion mutants. A) The infectious Ad5 plasmid pFG140 (Graham, 1984), containing the 2.2kb ampicillin resistant plasmid pMX2 inserted at the unique XbaI site at 3.7mu of the Ad5 genome, was restricted with BamHI and ligated to the kanamycin resistant plasmid pPBdx1 (Ghosh-Choudury et al, 1986) to give pJM15. The pMX2 insert was excised by XbaI digestion followed by insertion of a 4.3kb plasmid, pBRX (Haj-Ahmad, 1986) encoding ampicillin and tetracycline resistance, at the unique Ad5 XbaI site. The resulting plasmid, pJM16, was then partially digested with BamHI to remove pPBdx1 yielding pJM17 of approximate size 40.3kb. J denotes the junction of the left and right ends of the viral genome, X and B denote XbaI and BamHI restriction sites. Relevant positions on the Ad5 genome are indicated in map units. B) A possible mechanism for the in vivo recombination event between pJM17 and a plasmid containing an insertion mutation in Ela. It should be noted that although both plasmids in this drawing are represented as linear they are in fact transfected as circular DNA. In pJM17 the ends of viral DNA are joined (indicated by arrows) whereas the El sequences in the pMutant DNA are joined by plasmid sequences. pJM17 DNA may become linearized in transfected 293 cells prior to recombination with overlapping sequences from the plasmid containing El. Taken from McGrory et al (1988).

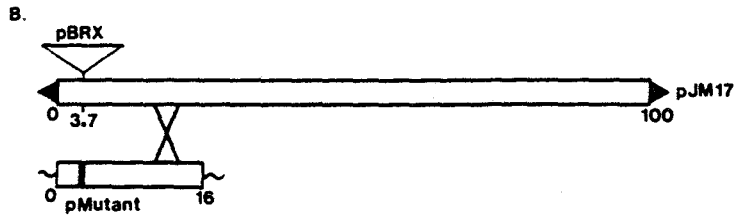
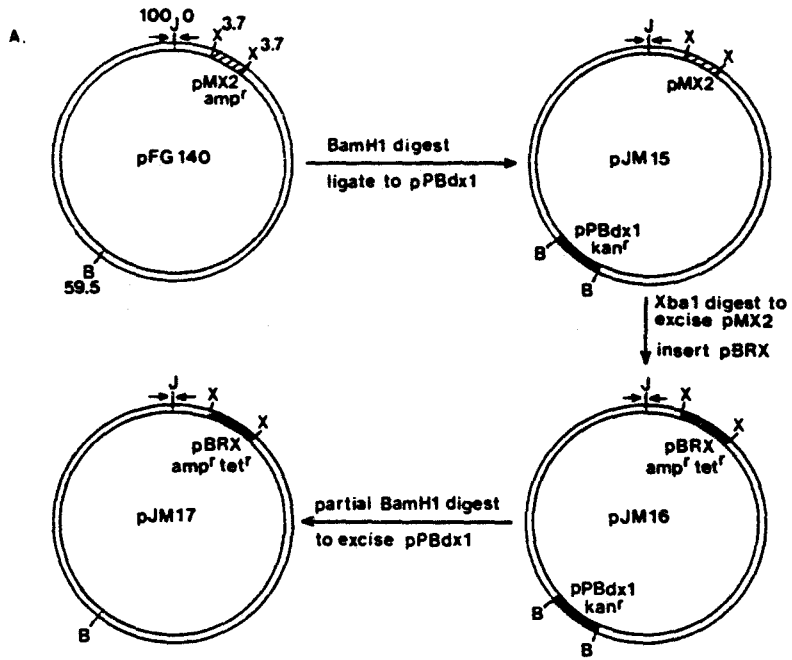
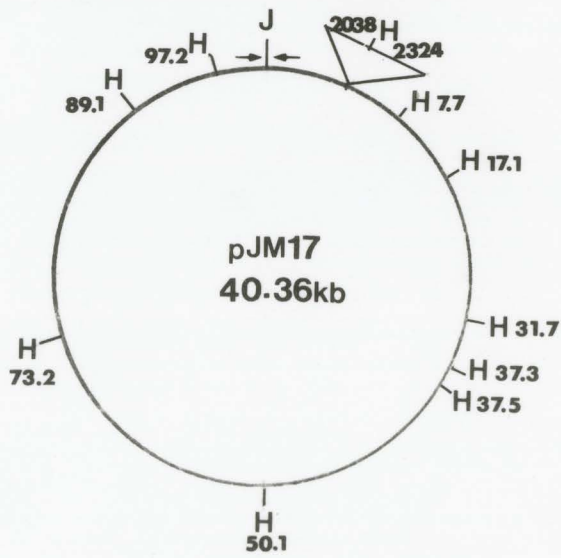


Figure 13. Restriction enzyme profile of pFG140, pJM15, pJM16 and pJM17. The lanes designated M show the HindIII profile of Ad5, the fragments are indicated to the left of the picture and the sizes in kb are indicated to the right. The plasmids pFG140, pJM15, pJM16 and pJM17 are all digested with HindIII. Shown below is a map of pJM17 indicating the HindIII sites and their location in map units of the Ad5 genome, J denotes the junction of the ends of the viral genome. The pBRX insert at the XbaI site at 3.7 mu is shown, the numbers above the insert indicate the length in base pairs from the ends of the insert to the HindIII site located within the pBRX sequences.



subjected to a partial digest with BamHI to remove the pPBdx1 sequences from the BamHI site at 59.5mu but retain the pBRX sequences inserted at nt 1338 in Ela. Of 150 colonies screened 6 were resistant to tetracycline and ampicillin and sensitive to kanamycin. Of these 6, 3 had the expected configuration and 3 had rearranged, again presumably due to homologous recombination between the two bacterial plasmid sequences. One of the correct recombinants was subcloned and the resulting plasmid, pJM17 (Figure 13), was expected to be noninfectious in mammalian cells because the size of the insert, 4.3kb, was beyond the predicted packaging capabilities of the virion capsid (Ghosh-Choudury et al, 1987). However, pJM17 was in fact slightly infectious (Table 5) but only at low frequencies due to the requirement of the resulting virus to rearrange in order to reduce its genome to a packageable size. In contrast cotransfection of pJM17 with a bacterial plasmid containing the left 16% of the Ad5 genome (Figure 12B) produced plaques at a much higher frequency (Table 5).

3.3.2 Transfection with pJM17

Because of the low infectivity of pJM17 and the high recombination frequency between plasmids in 293 cells it was possible to rescue the plasmids containing the insertion mutants into infectious virus simply by cotransfecting with pJM17 onto 293 cells as described in Materials and Methods. pJM17 provides wild type viral sequences rightward of the

Table 5. Plaquing Efficiency of pJM17 and E1 mutant plasmids cotransfected on 293 cells. pJM17 and the indicated mutant plasmid were cotransfected onto 293 cells at various plasmid DNA concentrations. Indicated are the total number of plaques from 2 60mm dishes scored at 11 days post-transfection. Taken from McGrory et al (1988).

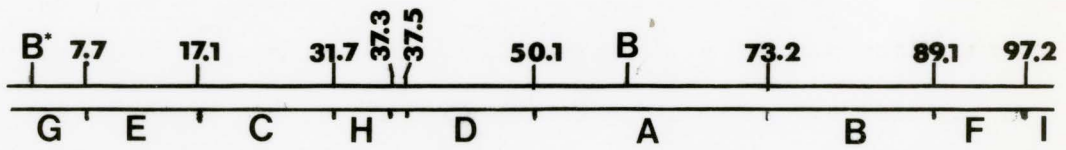
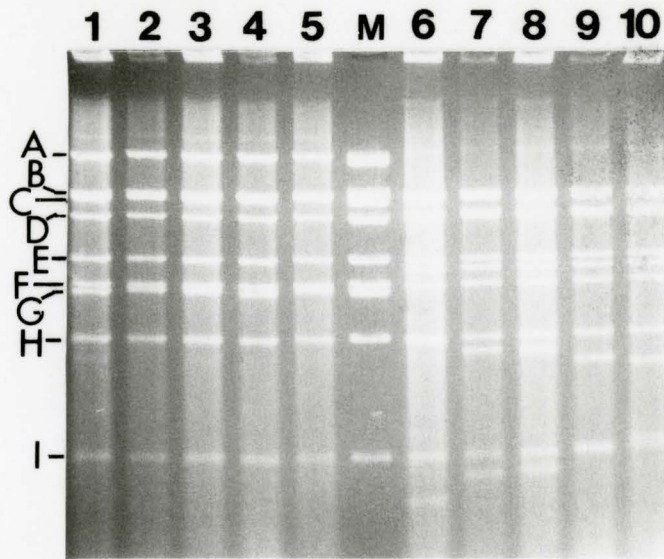
E1 Plasmid

	pX1056c (ug/dish)					pX1376c (ug/dish)				
	0	0.5	1.0	2.0	5.0	0	0.1	0.25	1.0	2.0
pJM17 (ug/dish)										
0.5	0	1	1	0	1	0	1	0	2	1
1.0	0	2	6	11	5	0	3	1	5	5
2.0	0	5	9	10	11	0	0	5	4	4
5.0	8	13	32	35	37	0	3	4	13	18

pBRX insert while the E1 plasmid containing the insertion mutation provides an Ela region only slightly larger (39bp or 6bp) than normal. Recombination between the two plasmids (Figure 12B) results in a viral recombinant that is well within the packaging capabilities of the virion capsid. The recombination process may occur according to the model of Holliday (1964) where a single double-stranded homologous exchange of sequences between the two plasmids followed by replication results in the recombinant virus or it may involve the strand invasion model postulated by Meselson and Radding (1975) whereby a single strand of DNA is generated by strand displacement during replication of one of the plasmids. Because 293 cells constitutively express early region 1 of Ad5 they are able to complement any host range mutations and allow the virus to replicate.

Individual plaques were picked and their DNA analyzed as described in Materials and Methods. Initial screening was done by digestion with the endonuclease HindIII which was used to eliminate those viruses that had undergone rearrangements during the recombination event or during subsequent replication. The HindIII pattern of recombinants on agarose gels was identical to that of wild type virus since the size of the insert would not significantly alter the size of the left most HindIII fragment (approximately 2.8kb) (Figure 14). To confirm that the recombinant virus contained the insert, digestion with both HindIII and BamHI was carried out. Identification of a recombinant was verified by the addition of a new BamHI site within the HindIII-G fragment creating

Figure 14. Example of restriction enzyme profile of viruses constructed by rescue with the pJM17 technique. A representative sample of viruses rescued using pJM17 shown digested with HindIII or HindIII and BamHI. Lanes 1 through 5 show HindIII digests of the viruses in717c, in863(-), in882(-), in1008c and in1056(+), respectively. The HindIII pattern of the recombinant viruses is identical to that of the wild type Ad5 pattern shown in the lane denoted M. Lanes 7 through 11 correspond to the same viruses as lanes 1 through 5, respectively, except digestion is with both HindIII and BamHI. The correct recombinants are identified by the addition of a BamHI site in the HindIII-G fragment resulting in the loss of the G fragment and the generation of two new bands, one proportional in size to the location of the insertion site and one equal to the size of the G fragment minus the sequences from Omu to the insertion site. Shown below is a composite HindIII restriction map of the recombinant viruses. HindIII sites are shown by their location in map units while BamHI sites are shown by the letter B. The asterisk indicates the BamHI site resulting from the linker insertion. Below the map the HindIII fragments of the virus are given.



two new bands, one corresponding to the length from the left viral end to the BamHI site and the other corresponding to the distance from the BamHI site to the HindIII site at nt 2798 (Figure 14). The presence of a BamHI site at 59.5mu in the pFG140 parental virus results in the loss of the large HindIII-A fragment and the addition of two new bands of sizes 5040bp and 3240bp.

Using this technique a rescue success of 70-80% was routine and often the success rate would approach 100% in some experiments. Attempts to rescue virus using this technique with plasmids containing only the Ela sequences up to 2050bp was less efficient, about 40-50%, presumably due to the decreased amount of homologous sequences. Once recombinant viruses were obtained they were plaque purified and expanded. Virus nomenclature was similar to that used for the plasmids except the prefix 'in' was used to denote insertion mutant, e.g. in717(+).

3.4 Plaquing Efficiency of Recombinant Viruses

3.4.1 Plaque Formation on 293 and HeLa Cells

Viral stocks prepared as described in Section 2.10.6 of Materials and Methods were assayed for their ability to form plaques on both 293 cells and HeLa cells. The 293 cells acted as a positive control because they contain and express the products of the El region (Graham et al, 1977). Conversely the HeLa cells provided a measure of the mutation's

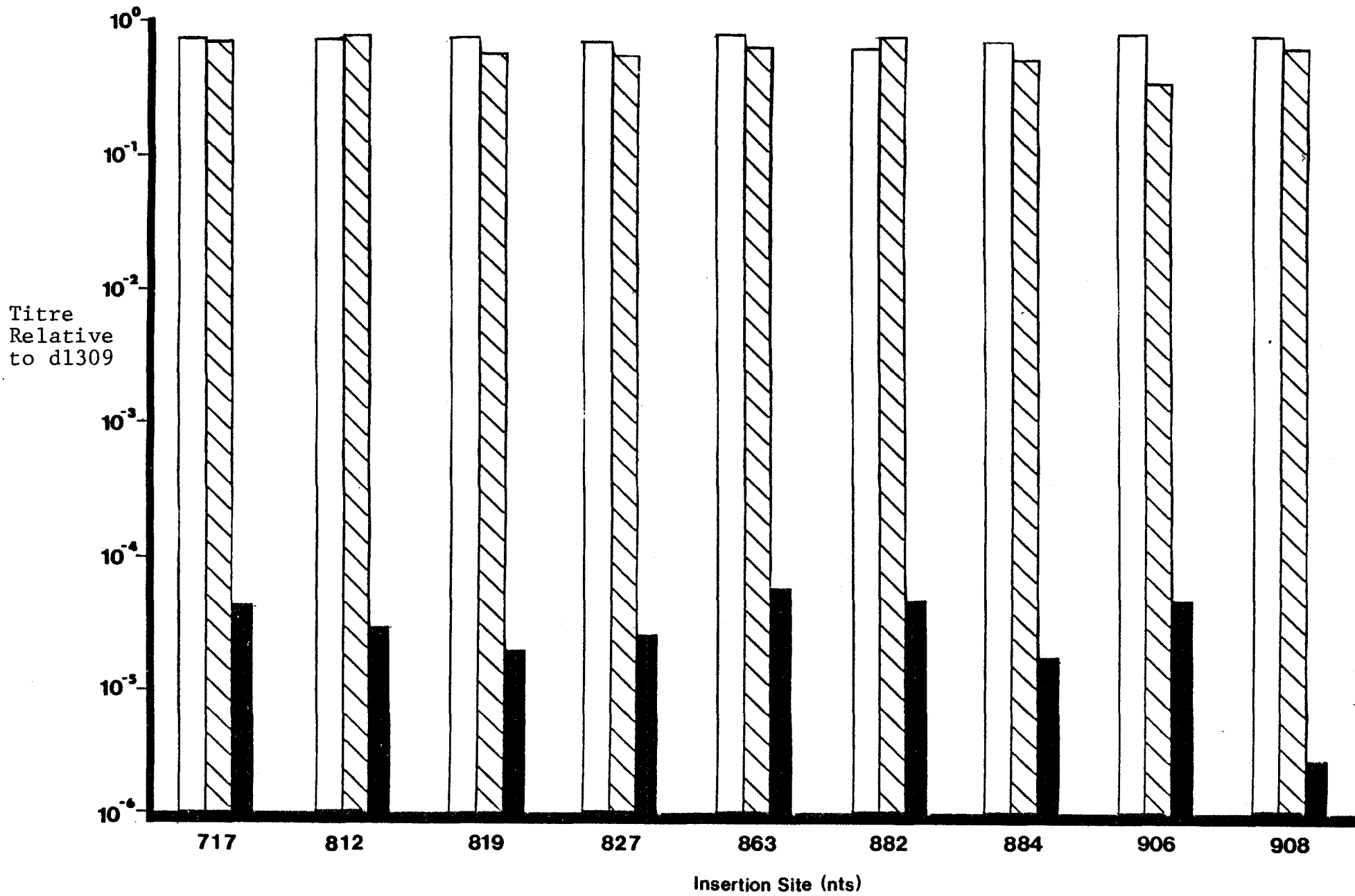
effect on the relative ability of the virus to lytically infect a human cell line. Titre results were determined by infecting two 60mm dishes of each cell type for each dilution of the viral stock. Plaques were counted from 7 days post-infection up to 11 or 13 days. In all cases results were tabulated at the point where no more plaques appeared and are expressed as the ratio of pfu/ml between 293 and HeLa cells for the mutant virus relative to the ratio of pfu/ml between 293 and HeLa cells for the wild type virus dl309 (see Appendix 2 for raw data). Viral stocks assayed for plaquing efficiency in separate experiments showed a variation of less than 2 fold in their relative ability to form plaques on 293 cells compared to HeLa cells.

3.4.2 Plaquing Efficiency of Insertion Mutants Upstream of the Unique Region

All closed reading frame insertion mutants in the region of exon 1 common to both the 12S and 13S messages were defective for growth on HeLa cells, generally producing 10^4 to 10^5 fewer plaques compared to 293 cells (Figure 15). This was expected since the truncated proteins would all lack the unique region of the 289R protein which has been shown to be necessary for efficient viral replication (Montell et al, 1982; Ricciardi et al, 1981).

In contrast open reading frame insertion mutants in either the 13aa or 2aa form were all effectively wild type in their ability to form plaques on HeLa cells (Figure 15).

Figure 15. Plaquing efficieny of viruses containing insertion mutations upstream of the unique region of Ela. Results are expressed as the ratio of plaque forming units per ml (pfu/ml) between 293 and HeLa cells for each mutant divided by the same ratio for the wild type virus dl309. Open bars represent 13aa in frame insertions, hatched bars represent 2aa in frame insertions and solid bars represent closed reading frame insertions.

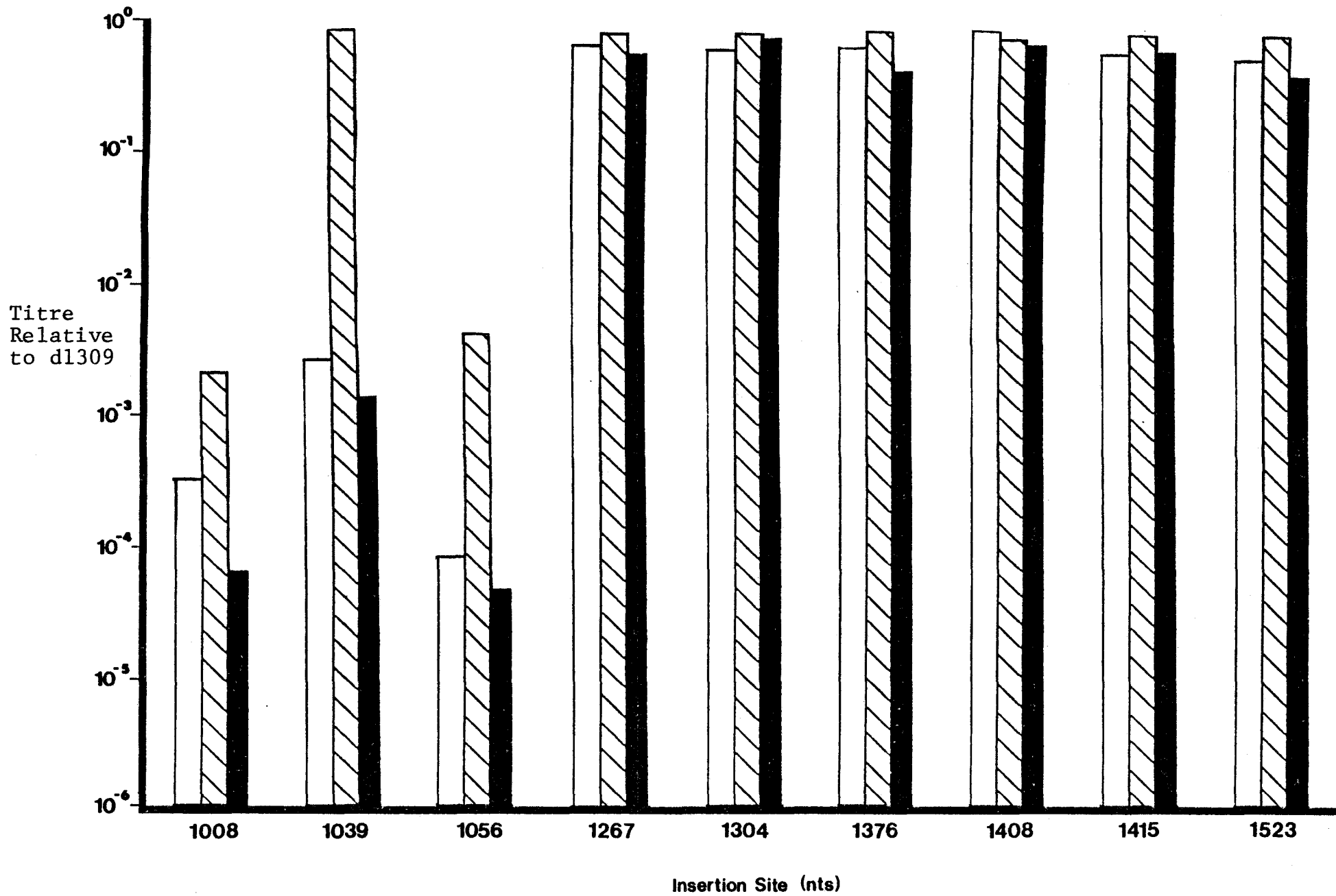


3.4.3 Plaquing Efficiency of Unique Region Insertion Mutants

As expected insertion mutants in the unique region severely reduced the virus' replication, between 10^3 and 10^7 , on HeLa cells (Figure 16). One notable exception was the in1039c virus which was wild type for growth on HeLa cells, an indication of its wild type transactivation ability. This same mutation in plasmid form was wild type for transformation, which was concluded to be the result of a normal level of E1b expression. These results taken together suggested that the 1039c insertion mutant did not impair transactivation and therefore could replicate in HeLa cells and transform rodent cells with E1b as well as wild type. All other mutations in the unique region were defective in both aspects. Comparing 13aa to 2aa inserts in the unique region the latter were about two times more efficient for viral growth than the former while 13aa inserts and closed reading frame insertions were about the same.

The transactivation results of D.S. Bautista were in complete agreement with the results obtained for virus replication. All mutant viruses that showed a decreased ability to replicate on HeLa cells were also defective in the transactivation of early viral promoters as measured in transient expression assays using plasmid DNA.

Figure 16. Plaquing efficiency of viruses containing insertion mutations in the unique region and exon 2 of E1a. Results are expressed as the ratio of plaque forming units per ml (pfu/ml) between 293 and HeLa cells for each mutant divided by the same ratio for the wild type virus d1309. Open bars represent 13aa in frame insertions, hatched bars represent 2aa in frame insertions and solid bars represent closed reading frame insertions.



3.4.4 Plaquing Efficiency of Exon 2 Insertion Mutants

None of the insertion mutants in exon 2 had any significant effect on the ability of the viruses to replicate on HeLa cells (Figure 16). The results of transient expression assays on these mutants by D.S. Bautista also showed effectively wild type levels of transactivation. Other mutations in exon 2 that affect the last amino acid of the unique region, position 185 (nt 1229 or 1230), severely reduce the ability of the 289R protein to transactivate other early genes during viral infection (Velcich and Ziff, 1988) or in transient expression assays (Jelsma et al, 1988; Schneider et al, 1987). However existing mutations in exon 2 that do not alter the UR have no effect on the ability of Ela to transactivate other early viral genes (Kuppuswamy and Chinnadurai, 1987; Velcich and Ziff, 1988; Jelsma et al, 1988; Schneider et al, 1987).

Discussion

4.1 Transformation of BRK Cells by Ela Insertion Mutants

Because of the variability of the transformation assay it was necessary to carry out a number of repetitions to determine the ability of each mutant to transform BRK cells. Expressing each mutant's transformation rate as a percentage of the wild-type transformation from these repetitions allowed conclusions to be made on the relative ability of each mutant to transform.

4.1.1 Transformation by Insertion Mutants Upstream of the Unique Region

It is clear that the region of exon 1 that is removed by the premature termination of translation in the closed reading frame mutants from nucleotide 717 to nt908 is essential for transformation (Figure 10). All of these mutants were completely defective for transformation. This agrees with past work where truncation of the Ela proteins upstream from CR2, located between nucleotides 920 and 967, results in a loss of transforming ability (Whyte et al, 1988). Point mutants, insertions or deletions in CR2 have been shown to be defective for transformation in conjunction with either Elb or ras (Kuppuswamy and Chinnadurai, 1987; Moran et al, 1986; Lillie et al, 1986; Schneider et al, 1987). It has

been postulated that CR1 located between nucleotides 695 and 790 is responsible for inducing DNA synthesis in cells and that CR2 then acts to allow the cells to continue on through mitosis and cell division (Zerler et al, 1987). Other investigators have concluded that CR2 is also needed for the efficient induction of DNA synthesis (Lillie et al, 1987). In either case it is obvious that the region of the Ela genome between nucleotide 717 and nt 1008 was absolutely necessary for transformation. The data obtained in this study indicates that only closed reading frame mutants at or downstream of nt1008 begin to show transforming activity.

The mutants which code for 13aa or 2aa insertions at the sites 717, 812, 819, 827, 863, 882, 884, 906, and 908 were all able to transform at significant levels. As discussed in Section 3.2.5 some of these mutants were statistically less than wild type yet none were completely defective (Figure 10). All of these sites except 717 are located between the conserved regions 1 and 2, a region which has been completely deleted with a similar effect on transformation (Zerler et al, 1987). Although the ORF insertions at nt 717 are within CR1 they were not significantly less than wild type in transforming ability. CR1 point mutants have been shown to be defective for transformation (Lillie et al, 1987) but these mutants were located downstream of the 717 site. Although it is difficult to compare the results between point mutants and insertion mutants, these results suggest that the actual sequences

necessary for transformation may not encompass the entire region conserved between adenovirus serotypes. Conversely it could be argued that the insertion mutations at nt 717 do not affect transformation simply because they have no effect on the secondary structure of the proteins in CR1.

4.1.2 Transformation by Unique Region Insertion Mutants

The role of the unique region in the transformation process is not well understood. It is clear that both the 289R and 243R proteins are required to induce the complete transformed phenotype: the ability to grow in low serum media, anchorage-independent growth, growth in soft agar, an epithelial morphology, and tumorigenicity (Ruben et al, 1982; Winberg and Shenk, 1984; Hurwitz and Chinnadurai, 1985; Babiss et al, 1986; Carlock and Jones, 1981; Haley et al, 1984; Moran et al, 1986b; Roberts et al, 1985). However various studies have yielded conflicting results on the actual role of the unique region in transformation. This is to some extent due to the different assays used to measure transforming ability. Early work was done mostly with intact virions or with purified viral DNA while later, with the advent of recombinant DNA techniques, it became convenient to work with plasmids containing the E1 region of the adenovirus in DNA-mediated transfections. This allowed the study of the transforming region in a more reproducible manner since variations in cytopathic effect by the virus were eliminated. The finding that E1a could co-operate with other oncogenes such as ras (Land

et al, 1983; Ruley, 1983) prompted the use of yet another assay using cotransfection of plasmids containing only Ela with the ras oncogene. Finally, a number of different cell types are also used some of which are primary such as BRK cells, while others are already immortalized such as cloned rat embryo fibroblast (CREF) cells. These multiple assays used by different researchers often makes comparisons of data complicated.

Work with group I host-range mutants of Ad5 located in the unique region showed this region is necessary for transactivation (Glenn and Ricciardi, 1985) and transformation of rodent cells by virus (Graham et al, 1978) despite that normal or near normal levels of Elb proteins are expressed in hr1 transformed cells (Ruben et al, 1982). This is at odds with the work of Montell et al (1984) who observed a high rate of transformation with hr1 virus but diminished levels of Elb messages.

Conversely the group I mutant hr3 can transform at wild-type levels in conjunction with ras (Zerler et al, 1987). Similar results were found with other unique region mutants which were unable to transactivate but could nonetheless transform with ras at high frequency (Velcich and Ziff, 1988; Moran et al, 1986a; Schneider et al, 1987). Unfortunately, these same transactivation negative mutants were not tested for their transforming ability with Elb.

Results with the mutants pX1008(+), pX1008(-), pX1008c, pX1039(+), pX1039(-), pX1056(+), pX1056(-) and pX1056c indicated they were reduced in transformation in assays with Elb (Figure 11). However these same

insertion mutants in an Ela only background were able to transform at levels equal to or greater than wild type Ela when co-transfected with ras (Table 4). Furthermore, when the transformation assay was conducted under selective media that allows transformation of BRK cells by Ela alone it was shown that the insertion mutants in an E1 background were able to transform cells at the same frequency as Ela alone (Table 3). This indicated that the mutants in the unique region were not expressing levels of Elb that would allow them to transform at the same level as wild type E1 (Table 3). However these mutants were not completely defective in Elb expression since they are able to transform, although at low levels, when selected in the Jok plus 5%HS media, conditions where Ela alone is unable to transform. Taken together these results suggest that the unique region had an indirect role in transformation, that is the transactivating activity of this region was required to express Elb and allow wild-type transformation. The insertion mutants in the UR are able to transform at wild-type levels with ras because the transactivation domain is not required for the expression of ras (Sassone-Corsi and Borreli, 1987). The 2aa insert in pX1039c was the only mutant in the unique region that was wild-type for transformation with Elb indicating this site is not affected by a 2aa insert but is defective when 13aa are inserted as in pX1039(+).

4.1.3 Transformation by Exon 2 Insertion Mutants

All mutants in exon 2, whether a 13aa insert, a 2aa insert or a closed reading frame insertion were able to transform to some degree (Figure 11). Again, some of these mutants were statistically less than wild type in transformation indicating the insertions are impairing the function of the proteins, perhaps by altering stability, conformation or both. Other investigators have shown that exon 2 mutations do not eliminate transformation but may reduce it somewhat (Velcich and Ziff, 1988; Schneider et al, 1987; Kuppuswamy and Chinnadurai, 1987; Jones and Shenk, 1979b).

4.2 Plaquing Efficiency of Ela Insertion Mutants

The ratio of plaques formed on HeLa cells to plaques formed on 293 cells is an indication of a mutation's effect on the ability of the virus to replicate. As mentioned 293 cells contain the Ad5 E1 region and constitutively express E1 products and can therefore efficiently complement any Ela mutations. The phenotypically wild-type virus dl309 acted as a positive control for the comparison of plaquing efficiency between 293 and HeLa cells.

4.2.1 Plaquing Efficiency of Insertion Mutants Upstream of the Unique Region

The closed reading frame insertion mutants in the region of exon 1 common to the 12S and 13S messages were all reduced for growth on HeLa cells by a factor of 10^4 to 10^5 (Figure 15). This reduction is most likely due to the loss of the unique region which is required for efficient early viral gene expression (Nevins, 1981; Ricciardi et al, 1981; Montell et al, 1982; Jones and Shenk, 1979). The 13aa or 2aa insertions located here had no significant effect on the ability of the virus to replicate since all of these viruses' titre in HeLa cells approached that of 293 cells (Figure 15). The two conserved regions in exon 1 have been shown to play a role in the induction of DNA synthesis (Lillie et al, 1987) which presumably makes for a hospitable environment for viral DNA replication. However since the plaquing assay was carried out on HeLa cells, a rapidly dividing cell line, this activity of exon 1 would not necessarily be required.

4.2.2 Plaquing Efficiency of Unique Region Insertion Mutants

Insertion mutants in the unique region of Ela were for the most part defective for replication on HeLa cells (Figure 16). The in1008(-), in1039(-) and in1056(-) viruses produced 10^4 , 10^2 and 10^4 fold fewer plaques, respectively, on HeLa cells than on 293 cells as

would be expected since they result in a premature truncation of the unique region. The 13aa inserts in the in1008(+), in1039(+) and in1056(+) were similarly defective for replication on HeLa cells indicating that the unique region domain is sensitive to insertion mutagenesis. Even the 2aa insert in the viruses in1008c and in1056c were sufficient to reduce plaquing efficiency by 10^2 fold which was, interestingly, almost exactly half of the host range effect exhibited by the 13aa inserts at the same sites. These results correlate well with both the transformation data and the transactivation data of D.S. Bautista suggesting that the loss of transactivating activity by these mutants lowers the ability of the 289R protein to activate Elb expression and therefore reduces the number of colonies formed on BRK cells. This same reduction in transactivation of other early viral genes in the virus result in the observed host range effect.

The 2aa insert in in1039c was the only unique region mutant wild type for replication on HeLa cells indicating that this particular mutation had no effect on the ability of the virus to grow. It should be noted that this same mutation in plasmid form, pX1039c, was the only unique region mutation that was also wild-type in transformation of BRK cells. This further supports the conclusion that the reduced transforming ability seen with the other unique region insertion mutants was due to their inability to transactivate Elb.

4.2.3 Plaquing Efficiency of Exon 2 Insertion Mutants

Insertion mutants of any type in exon 2 had no significant effect on the ability of the viruses to replicate (Figure 16). Similarly the results for these mutants compiled by D.S. Bautista using transient expression assays showed effectively wild type levels of transactivation. Existing mutations in exon 2 have no effect on the ability of Ela to transactivate other early viral genes (Kuppuswamy and Chinnadurai, 1987; Velcich and Ziff, 1988; Jelsma et al, 1988; Schneider et al, 1987).

4.3 Conclusions

It was evident from the results with the closed reading frame insertion mutants that the coding sequences of exon 1 between nt717 and 1008 of Ad5 Ela was necessary for the transformation of BRK cells. This is in agreement with other results that have shown that exon 1 and in particular the two conserved regions contained therein play an important role in the transformation process (Whyte et al, 1988; Kuppuswamy and Chinnadurai, 1987; Zerler et al, 1987; Lillie et al, 1986 and 1987; Velcich and Ziff, 1988; Moran et al, 1986a; Schneider et al, 1987). It is also evident from the 13aa and 2aa insertions upstream of the unique region that the Ela protein products are remarkably resistant to insertion mutagenesis since all of these mutants retained the ability to transform BRK cells and were effectively wild type for virus growth on

HeLa cells. Either the mutations, generated at random, happened to fall at sites in the proteins which are not important for the formation of a stable secondary structure or the 243R and 289R proteins simply do not have any well defined secondary structure requiring precise folding of exon 1. If the latter is true one could imagine that the active domains, such as CR1 and CR2, are resistant to mutations which do not delete or alter CR1 or CR2 sequences themselves.

Not surprisingly the mutations in the unique region of the 13S product showed greater phenotypic effects than anywhere else in Ela. All mutants here, except 1039c, were defective in both transformation and viral growth. It was shown that the unique region itself has no direct role in the transformation process, since mutants there can transform at wild-type levels in conjunction with ras, but appeared to be required only for the efficient activation of Elb. This confirms that Elb is necessary for the efficient transformation of BRK cells but the actual role of the Elb proteins is unknown. The presence of Elb in transformed cells results in a higher level of Ela (van den Elsen et al, 1983a; Vaessen et al, 1984). This activity may be the result of the Elb-19K protein which is known to relieve the repression activity of Ela proteins on their own promoter (Yoshida et al, 1987). It can be envisaged that Ela begins to repress its own transcription at some point and that Elb alleviates this block allowing higher expression of Ela and therefore higher transformation frequencies. Evidence for this is provided by the fact that the overall transforming ability of pKH101

(Ela) plus ras is about one third of the wild type pXC38's transformation frequency (Table 4). This is consistent with the observation of lower levels of Ela in cells transformed in co-operation with ras relative to Ela levels in cells transformed in conjunction with Elb (Vaessen et al, 1984). Conversely, the Elb proteins may play a direct role in the transformation process and require efficient activation by Ela to assume this role while ras, needing no Ela-mediated transactivation, can co-operate with unique region mutants as well as wild-type Ela to transform BRK cells.

Exon 2 of Ela was seemingly dispensable for the transformation of BRK cells and for efficient replication of virus in HeLa cells. This fact has been confirmed by others (Velcich and Ziff, 1988; Schneider et al, 1987; Kuppuswamy and Chinnadurai, 1987) and may be expected in light of exon 2 not being highly conserved between different adenovirus serotypes (Kimelman et al, 1985). The loss of the rapid nuclear localization signal located at the C-terminus (Krippel et al, 1985) of exon 2 does not appear to eliminate the ability of Ela products to induce transformation nor productively infect HeLa cells.

APPENDIX 1

Number of Foci Formed on 60mm dishes of BRK cells by Ela Insertion Mutants with Elb

Plasmid	Experiment							
	1	2	3	4	5	6	7	8
pXC38(wt)	10,9,13,12	23,25,51,58	82,84,70,89	37,35,43,31	58,55,59,50	36,31,38,31	33,44,50,39	16,22,17,17
pX717(+)	---	27,31,45,35	10,4,15,13	21,14,13,14	---	---	50,70,58,--	---
pX717(-)	---	---	0,0,0,0	---	---	0,0,0,0	---	---
pX717c	---	---	17,14,17,11	5,6,7,6	---	26,21,43,21	---	---
pX812(+)	---	24,21,27,29	50,57,49,45	17,13,11,10	---	---	---	---
pX812(-)	---	---	---	---	---	---	0,0,0,0	0,0,0,0
pX812c	---	---	---	14,11,11,13	---	30,20,29,24	33,39,49,20	---
pX819(+)	6,12,10,5	---	---	8,5,0,5	---	6,15,9,9	25,31,15,20	---
pX819(-)	0,0,0,0	---	---	---	---	0,0,0,0	---	---
pX819c	36,6,25,26	---	---	20,20,12,15	---	39,30,28,39	30,34,23,24	---
pX827(+)	---	21,21,38,36	---	11,14,19,4	---	1,5,2,2	---	---
pX827(-)	---	---	---	---	---	0,0,0,0	0,0,0,0	---
pX827c	---	41,34,60,55	---	11,6,14,8	---	34,32,31,40	27,31,22,25	---
pX863(+)	---	---	---	---	---	22,20,24,15	40,30,41,33	7,2,5,4
pX863(-)	---	0,0,0,0	0,0,0,0	---	---	---	---	---
pX863c	---	36,36,38,43	47,64,43,69	---	---	16,15,20,19	---	---
pX882(+)	---	---	24,36,33,20	15,9,10,14	---	21,18,21,17	---	---
pX882(-)	---	---	0,0,0,0	0,0,0,0	---	---	---	---
pX882c	---	---	29,29,22,27	10,9,7,3	---	27,11,18,17	---	---
pX884(+)	---	---	11,17,18,11	7,3,8,6	---	25,20,22,27	---	---
pX884(-)	---	---	0,0,0,0	---	---	0,0,0,0	---	---
pX884c	---	---	16,20,14,6	31,23,17,27	---	46,28,53,37	39,18,28,28	---

APPENDIX 1 (cont'd)

Plasmid	Experiment							
	1	2	3	4	5	6	7	8
pXC38(wt)	10,9,13,12	23,25,51,58	82,84,70,89	37,35,43,31	58,55,59,50	36,31,38,31	33,44,50,39	16,22,17,17
pX906(+)	---	37,34,35,33	28,37,36,29	---	---	28,19,27,25	---	---
pX906(-)	---	---	---	---	---	0,0,0,0	0,0,0,0	---
pX906c	---	---	---	---	---	10,19,12,12	37,43,27,40	10,4,6,10
pX908(+)	10,18,9,9	10,7,18,20	---	14,14,14,12	---	---	---	---
pX908(-)	0,0,0,0	0,0,0,0	---	---	---	---	---	---
pX908c	---	10,13,29,24	---	13,17,21,31	---	42,37,37,28	---	---
pX1008(+)	---	---	---	---	4,4,4,5	2,8,8,7	29,12,20,15	1,0,1,4
pX1008(-)	---	0,1,0,0	1,2,1,1	4,2,2,2	0,1,1,1	---	---	---
pX1008c	---	4,4,5,3	4,1,4,1	2,5,1,5	1,0,3,2	---	---	---
pX1039(+)	---	6,6,7,3	---	2,5,4,3	---	2,1,4,0	---	---
pX1039(-)	---	0,0,4,1	---	1,2,2,2	---	6,3,4,4	---	---
pX1039c	---	11,6,20,30	---	26,49,28,41	---	37,26,43,28	---	---
pX1056(+)	---	---	5,2,12,2	16,30,39,37	4,3,5,5	---	22,15,20,15	7,5,2,6
pX1056(-)	---	1,0,3,4	17,6,5,10	11,8,4,10	8,6,6,5	---	---	---
pX1056c	---	4,3,11,14	19,10,8,8	31,32,13,6	8,6,10,11	---	---	---
pX1267(+)	---	---	---	---	21,15,16,15	22,26,17,--	28,17,20,19	---
pX1267(-)	---	---	---	---	10,15,4,11	9,7,15,10	18,21,30,24	---
pX1267c	---	---	---	---	26,25,32,30	53,43,45,55	58,35,40,31	---
pX1304(+)	---	23,12,21,25	---	---	37,21,41,40	---	44,31,27,39	---
pX1304(-)	---	---	---	---	22,17,18,26	---	21,25,30,29	1,4,4,3
pX1304c	---	35,16,36,37	---	---	32,34,42,46	---	49,45,48,43	---
pX1376(+)	---	---	---	---	101,98,58,29	---	50,39,42,21	6,4,7,5
pX1376(-)	---	---	---	---	28,39,27,35	---	23,26,17,17	1,2,4,3
pX1376c	---	---	---	---	---	16,14,10,29	37,40,32,26	12,10,10,5

APPENDIX 1 (cont'd)

<u>Plasmid</u>	<u>Experiment</u>							
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>
pXC38(wt)	10,9,13,12	23,25,51,58	82,84,70,89	37,35,43,31	58,55,59,50	36,31,38,31	33,44,50,39	16,22,17,17
pX1408(+)	12,12,16,10	---	---	---	---	18,25,34,40	50,38,51,39	10,11,14,8
pX1408(-)	3,0,1,1	---	6,11,9,4	---	---	25,19,23,30	35,28,20,25	---
pX1408c	---	---	22,15,34,15	---	---	---	45,31,34,38	9,6,10,4
pX1415(+)	---	---	---	---	80,85,81,71	---	49,52,38,32	5,13,6,7
pX1415(-)	---	10,10,21,10	8,17,9,9	3,2,2,8	48,43,31,48	---	---	---
pX1415c	---	14,19,24,25	23,28,30,28	26,23,23,21	57,52,47,61	---	---	---
pX1523(+)	---	---	9,10,7,6	---	35,37,34,43	---	23,30,34,33	3,7,8,5
pX1523(-)	---	---	---	---	27,48,27,39	---	20,27,19,26	5,4,7,7
pX1523c	---	---	---	---	29,52,42,40	---	18,32,27,--	4,3,6,2

APPENDIX 2

Titre (pfu/ml) of viruses containing Ela Insertion Mutations.

<u>Virus</u>	<u>293/HeLa</u>	<u>293/HeLa dl309</u>	<u>Mutant Virus/dl309 ratio</u>
in717(+)	1.72	1.0	1.72
in717(-)	3.9×10^4	1.0	3.9×10^4
in717c	2.70	1.5	1.80
in812(+)	1.80	1.2	1.50
in812(-)	5.4×10^4	1.0	5.4×10^4
in812c	3.40	2.5	1.36
in819(+)	2.10	1.1	1.91
in819(-)	8.6×10^4	1.2	7.2×10^4
in819c	2.70	1.1	2.50
in827(+)	1.98	1.1	1.80
in827(-)	7.7×10^4	1.1	7.0×10^4
in827c	3.20	1.1	2.90
in863(+)	2.00	1.6	1.25
in863(-)	3.9×10^4	1.5	2.6×10^4
in863c	3.40	1.6	2.10
in882(+)	2.20	1.0	2.20
in882(-)	4.1×10^4	1.2	2.10
in882c	2.10	2.0	1.05
in884(+)	3.10	1.8	1.75
in884(-)	8.9×10^4	1.1	8.1×10^4
in884c	5.40	1.8	3.10
in906(+)	1.56	1.2	1.30
in906(-)	4.6×10^4	1.4	3.30
in906c	5.50	1.2	4.80
in908(+)	1.20	1.0	1.20
in908(-)	8.4×10^5	1.4	6.0×10^5
in908c	2.90	1.4	2.10
in1008(+)	9.5×10^3	1.9	5.1×10^3
in1008(-)	2.1×10^4	1.2	1.7×10^4
in1008c	8.7×10^2	1.2	7.3×10^2

APPENDIX 2 (cont'd)

<u>Virus</u>	<u>293/HeLa</u>	<u>293/HeLa dl309</u>	<u>Mutant Virus/dl309 ratio</u>
in1039(+)	6.3×10^2	1.1	5.7×10^2
in1039(-)	9.4×10^2	1.1	8.5×10^2
in1039c	1.50	1.1	1.36
in1056(+)	1.1×10^4	0.9	1.2×10^4
in1056(-)	3.1×10^4	0.9	3.4×10^4
in1056c	3.3×10^4	0.9	3.7×10^4
in1267(+)	3.20	1.4	2.29
in1267(-)	7.20	2.5	2.88
in1267c	1.60	1.0	1.52
in1304(+)	4.90	2.0	2.45
in1304(-)	4.50	2.8	1.61
in1304c	2.30	2.0	1.15
in1376(+)	3.70	1.4	2.74
in1376(-)	8.00	1.8	4.32
in1376c	1.20	1.0	1.20
in1408(+)	1.45	0.9	1.53
in1408(-)	2.60	1.1	2.36
in1408c	4.20	2.0	2.10
in1415(+)	3.30	1.1	2.87
in1415(-)	4.90	2.0	2.45
in1415c	1.50	1.0	1.50
in1523(+)	8.80	2.8	3.14
in1523(-)	13.7	3.1	4.42
in1523c	4.30	2.8	1.54

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