ANALYSIS OF ADENOVIRUS TYPE 5 E1A INSERTION MUTANTS

To my wife Ma, Zhongyu and our son Bao, Dalin

To our friends : Susan Jolly

Lucy Aitchison

Lavinia Inbar

ANALYSIS OF ADENOVIRUS TYPE 5 EARLY REGION 1 A INSERTION MUTANTS

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ABSTRACT

Adenovirus Early region 1A(E1A) plays an important role in viral lytic infection and serves as a useful tool in the study of molecular mechanisms of gene regulation and gene expression. In addition to the ability of E1A functions to induce transactivation and transrepression of gene expression, E1A products play a critical role in transformation by adenovirus.

A technique employing linker insertion mutagenesis scanning most of the E1A major regions was developed in this laboratory to systematically study the effect of E1A mutations on transcriptional transactivation and trans-repression functions as well as transforming ability (Bautista 1989). Results obtained showed that the unique region was the region primarily for transactivation in agreement with a wealth of other data and that transrepression was sensitive to insertions in CRII (conserved region II) and a region immediately following the unique region at the beginning of Exon II. However, transformation was not affected by repression-sensitive mutants.

This thesis describes further studies carried out to create additional insertion mutants within conserved region of E1A to confirm and extend the results obtained by

Bautista. A synthetic oligonucleotide of 39 base pair (13 aa) was introduced into two additional restriction sites in this region. As in previous work, the oligonucleotide could be inserted in either of two possible orientations. In one orientation, all three reading frames were open whereas all three reading frames of the other orientation contained stop codons which resulted in truncation of the E1A protein at the site of insertion. In addition, the insertion oligomer was designed with flanking BamHI sites to provide the opportunity of collapsing the full length insertion to a 6 base pair (2 aa) insertion. This allowed comparison to be made between mutations containing 13 aa insertions and those with 2 aa inserts at the same site to see how different oligopeptide segments would influence different functions. Trans-repression assays utilized a novel β-galactosidase assay developed by Bautista for characterizing CRII mutants expressing only the 12S product. The results suggested that CRII was an important region in terms of ability of E1A to transrepress. This negative regulatory function was sensitive to full length 13 aa insertions but not 2 aa inserts for those mutants made to date. Transforming ability of all CRII mutants was more or less impaired. Transactivation activity was not reduced by insertion at CRII except for mutants which were in the reverse orientation and had stop codons in their reading frame thus terminating the translation upstream of the unique region.

In an effort to identify functional temperature sensitive mutants, DNA-mediated transformation assays of mutants within E1A functional regions were carried out at different temperatures. Two transformation temperature sensitive mutants were identified which failed to produce transformed colonies at 38.5°C but transformed efficiently at 32°C. No replication temperature sensitive mutants were identified among 61 mutant viruses.

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ABBREVIATIONS

A_{420}	absorbence at wavelength 420nm
Ad	adenovirus
Amp	ampicillin
ATP	adenosine triphosphate
BIS	N,N'-methylene-bis-acrylamide
bp	base pairs
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleotide triphosphate
ddTTP	dideoxythymidine triphosphate
Df	degree of freedom
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol

dTTP	deoxythymidine triphosphate
E1A	adenovirus early region 1A
E1B	adenovirus early region 1B
E2A	adenovirus early region 2A
E3	adenovirus early region 3
E4	adenovirus early region 4
EtBr	ethidum bromide
EtOH	ethanol
FBS	fetal bovine serum
HS	horse serum
mu	map unit
NCS	new born calf serum
NEB	New England Biolabs
NEN	New England Nuclear
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TAE	tris.acetate EDTA
TBE	tris.borate EDTA
TE	tris.EDTA
TEMED	N,N,N',N',-tetramethylethylenediamide
Xgal	5-bromo-4-chloro-3-indoy-βgalactopyranoside
Z buffer	Lac-Z buffer

INTRODUCTION

1.1 History of Adenovirus Research

During the past three decades, adenoviruses have attracted considerable attention in molecular virology research. The viruses have been employed not only as tools for the study of cellular transformation *in vitro* and tumorigenicity *in vivo* but also have served as an excellent model system for the study of regulation of gene expression. RNA splicing was first recognized as a result of studies with adenovirus and later it was confirmed that this discovery had universal biological significance (Berget et al., 1977; Green 1986). More recently, attention has focussed on adenoviruses as potential recombinant vaccines (Prevec et al.,1990).

Epidemiologically, although the recorded history of acute respiratory disease syndrome was first reported about 100 years ago (Dingle and Langmuir 1968), the close relationship between the syndrome and the adenoviruses was not recognized until the 1950s'. In 1953, adenovirus was first isolated as a transmissible cytopathic agent by Rowe and his coworkers (Rowe et al., 1953). This ubiquitous agent was then named ADENOVIRUS in 1956 (Enders et al., 1956). Of the

adenoviruses which have been identified to date, 41 different serotypes have humans as their natural host and a large number of serotypes have been isolated from other species.

Adenoviral infections arise worldwide in humans and are now known to cause a variety of different diseases. Adenovirus has been found associated with epidemic keratoconjunctivitis (EKC) (Kemp et al.1983), acute respiratory infection (Dingle et al., 1968), pneumonia (Simila et al., 1971), acute haemorrhagic cystitis (Harnett et al., 1982) and gastrointestinal disease (Wigand et al., 1983). Moreover, some species of adenoviruses have been isolated from AIDS patients in a few cases (Horwitz et al., 1990).

Researchers showed greater interest in this infectious agent when it was shown that some adenoviruses were oncogenic. Trentin and coworkers set a milestone in the study of adenovirus in 1962 by showing that serotype 12 (Ad12) adenovirus injected into rodents could cause tumours (Trentin et al., 1962). Later, Ad18 was proved to be a second member in this oncogenic adenovirus list (Huebner et al. 1962). Though not all adenoviruses could induce tumours in vivo, they did transform some cell lines *in vitro*. In 1964, Ad12 was shown to transform new born hamster cells (McBride and Wiener 1964), after which many serotypes of adenovirus were tested to see if they share the same property. The results showed that all tested adenovirus could transform cells including

those from the non-oncogenic group (McAllister et al., 1969; Casto 1968; Freeman et al., 1967). In many cases, cells transformed by adenoviruses could cause tumours if they were injected into rodents (Casto 1969; Kitamura et al., 1964) but the mechanism of oncogenicity of transformed cells was still unknown. There have also been some indications that tumorigenicity may be related to the immunogenicity of transformed cells in injected animals (Pina and Green 1965; Gallimore 1972; Gallimore et al., 1974) however, the importance of immunogenicity is still under investigation.

There has never been direct convincing evidence to show that adenovirus infection can induce any cancer in humans, though there was a report that an RNA sequence similar to that of adenovirus had been found in human neurogenic tumours (Ibelgaufts et al., 1982).

1.2 Classification of adenovirus

Adenoviruses share a common general structure and each serotype has its own specific properties as well (Pettersson 1984; Sussenbach 1984). Adenoviruses are classified based on their hemaglutination properties (Rosen et al.1962), DNA GC content (Pina and Green 1965), restriction enzyme profiles (Wadell et al., 1980), variation in molecular weights of virion proteins (Wadell et al., 1979) and oncogenicity (Huebner 1967). In addition to the above classifications,

adenovirus have been divided into group A through group E according to the DNA genome homologies among individual serotypes (Green et al., 1979). Homology is less than 20% between groups and usually more than 90% within groups (Green et al., 1979). Adenoviruses grouped according to DNA homology have similar degrees of oncogenicity in rodents. According to adenovirus DNA homology classification, group A containing Ad12, Ad18 and Ad31 is highly oncogenic. Group B viruses composed of Ad3,Ad7,Ad11,Ad14,Ad16 and Ad21 are intermediate in oncogenicity. Group C including well studied Ad2 and Ad5 (Green et al., 1979) and groups D through E are non-oncogenic.

There is relatively good correspondence between different classification methods. The grouping by DNA homology agrees well with the classification by electrophoretic mobility properties of virion proteins. Although initially, increasing oncogenicity appeared to correlate with decreasing GC content of the viral DNA (Pina and Green 1965), no convincing explanation for this phenomenon has been made, and further studies including nonhuman viruses studies now indicate that there is no relation between overall GC content and tumorigenicity. On the other hand, evidence indicates that a small GC rich fragment from a GC poor adenovirus may be responsible for the transformation by that serotype (Graham et al., 1974).

1.3 Structure, Replication and transcription of Human adenovirus

Human adenovirus is a member of mastodenovirus in the family of adenoviridae (Dimmock and Primrose 1988). The complete sequence of 35,937 nucleotide (nt) of Ad2 and sequences of many parts of other adenovirus genomes are available (Roberts et al., 1986).

The capsid of adenovirus with its 20 triangular facets and 12 vertices represents a form of regular icosahedron (Horne et al., 1959) and the diameter of a fully hydrated virus particle is about 88nM (Devaux et al., 1983). The viral particle shell is composed of 252 capsomers. Three kinds of soluble adenovirus proteins designated hexon, penton, and fiber are fractionated by DEAE chromotography (reviewed by Pettersson and Roberts 1986). The molecular weight of hexon, the first viral protein ever crystallized (Pereira et al., 1968), is 103K (von Bahr-Lindstrom et al., 1982). Each penton consists of a penton base and a fibre of length 10-37 nm (Valentine and Prreira 1965,) which was initially visualized by electron microscopy as an antenna-like projection. The molecular weight of penton is 365 Kilodalton (K) consisting of the penton base (245K) and fibre (120K) (Devaux et al., 1982).

Viral attachment begins when the fibre of the virus

fits to the proper receptor on the cell membrane. There are about 100,000 fibre receptors per cell. Successful attachment onto the cell surface results in penetration initiation. The peripentonal hexons are important in viral penetration, since this initiation of viral infection can be neutralized by antibodies either against the fibre or the hexon (Philipson et al,. 1968; Londberg-Holm and Philipson 1969). One possible mechanism for viral entry is that the viral DNA penetrates the cell leaving the penton capsomer at the surface. (Philipson et al., 1968). Alternatively. some electron micrographs also suggest that the virus enters the cell by pinocytosis (Chardonnet and Dales 1970).

About 13% of the adenovirus particle is DNA and viral proteins make up the rest of the virion (Green and Pina 1963). The double stranded DNA of Ad5 is 36Kb long with 103 base pair inverted terminal repeats (ITR) (Van der Eb and van Kesteren 1966; Garon et al., 1972). The size of the adenovirus ITR is variable depending on different serotypes. The viral DNA in viruses is a linear molecule and viral DNA replication in infected cells proceeds through linear intermediates. However, a small fraction of DNA also circulizes shortly after infection because of the joining of ITRs at their ends (Ruben et al., 1983) and these covalently closed circles are infectious (Graham 1984). The linear viral DNA in virions has TPs (terminal protein), attached at its 5 ' ends through a

phosphodiester linkage (Robinson et al.1973; Rekosh et al., 1977, Keegstra et al., 1977; Schaack and Shenk 1989), which play a role in replication of adenovirus DNA by functioning in protein primed initiation of DNA synthesis (Stillman 1983; Kelly 1984). Viral DNA replication also requires cellular factors such as NFI (nuclear factor I) and NFIII (van der Vliet et al., 1988). The precursors of TPs, the preterminal proteins (pTPs) are covalently attached to viral DNA as a result of the mechanism of DNA replication, and later are processed into TPs by proteolytic cleavage during virion maturation (Stillman et al., 1981). The infectivity of viral DNA-protein complexes is much higher than that of deproteinized viral DNA because of these terminal proteins (Sharp et al., 1976) though they are not absolutely necessary for viral DNA replication *in vitro* (Tamanoi and Stillman 1982) or for viral DNA infectivity. Replication of adenovirus in human cells is very efficient and as many as 10^6 progeny molecule could be produced 20 to 30 hours postinfection (van der Vliet et al., 1988).

As indicated by the transcription pattern of the Ad genome shown in Figure 1.1, during the lytic cycle of adenoviral infection, a group of viral genes encoded by the early regions of the virus genome are transcribed before DNA replication (Figure 1.1). These early genome products are critical in the initiation of viral infection. Genes expressed

after the onset of viral DNA replication are classified as late genes.

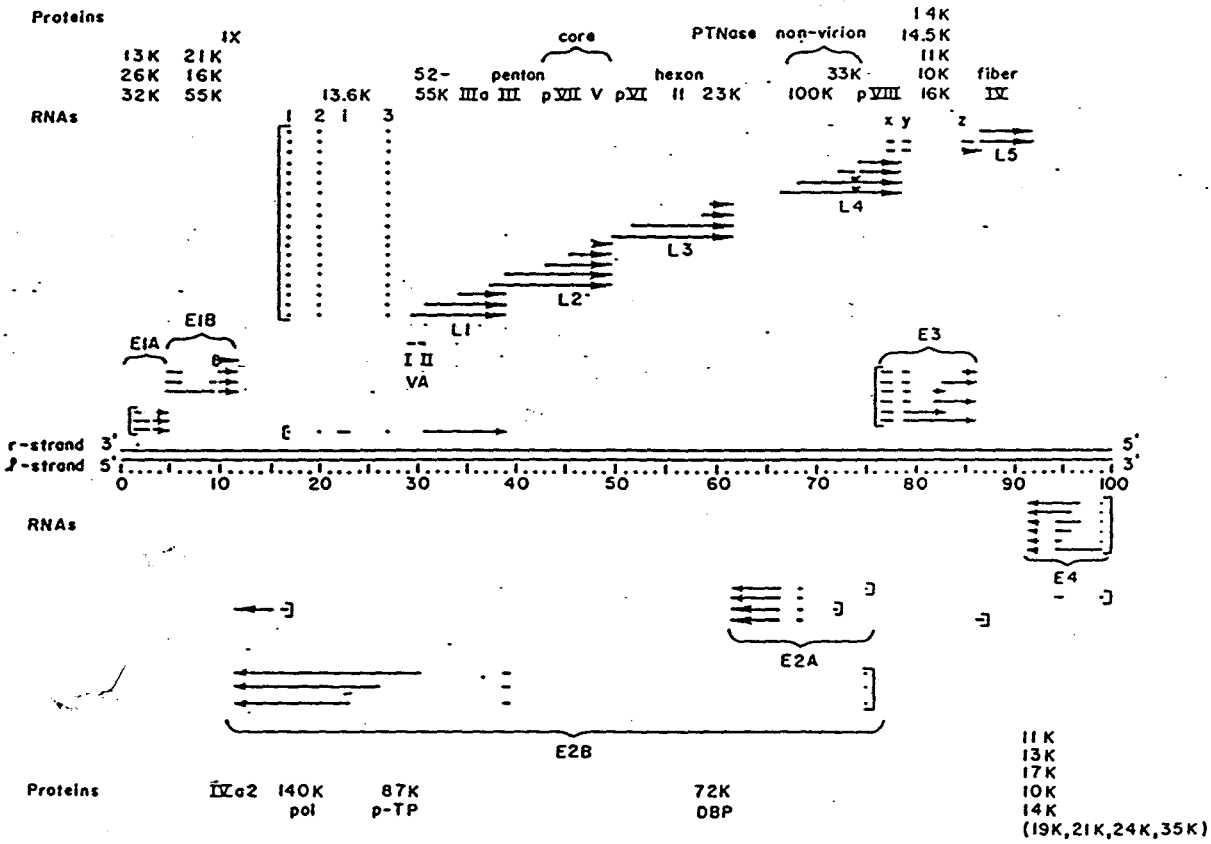
Early genes include L1, E1, E2, E3, E4, and intermediate genes-IVa2,IX (Persson and Philipson 1982).

L1 was originally designated a late gene because in early studies L1 transcripts were first detected late in infection. Later studies showed that L1 was also transcribed early, and in fact it was one of the first regions transcribed in the lytic cycle (Shaw and Ziff 1982). The L1 early region extends from map unit (mu) 29 to 34 on the r strand and its early mRNAs are initiated at the MLP start site (Nevins and Wilson 1981; Shaw and Ziff 1982). E1 and E3 regions are on the r strand transcribed rightward from mu 1.3 to 11.2 and 76.6 to 86.2 respectively. E4 and E2 are encoded on the leftward transcribed strand, the l strand, from mu 96.8 to 91.3 for E4, mu 67.9 to 61.5 for E2A and 29 to 14.2 for E2B.

Late phase gene expression begins after viral DNA synthesis (Figure 1.1), with a cis-acting mechanism being involved in the early to late switch (Thomas and Mathews 1980). In this stage viral mRNAs are about 10 fold higher than that in the early stage because of the general transactivation effects of early proteins (Horwitz 1990) and increased template copy numbers. Transcription from the r strand is four times more efficient than that from the l strand (Green et al., 1971). Late mRNAs mainly encode virion components. All

Figure 1.1 Transcription Pattern of the Ad genome

The Ad genome is presented by map units (mu.) 0-100. The linear genome is separated into r-strand and l-strand for rightward or leftward transcription, respectively. Early regions E1A, E1B, E2A,E2B, E3 and E4 transcribed before DNA replication are indicated by single lines and all 3' termini are indicated by arrowheads. Late gene families (L1-L5) originating at m.u. 17 are indicated by arrows and each late message is adjoined to the tripartite leader, indicated as 1, 2, 3, at mu.s 17, 20 and 27, respectively. L1 is also found to be one of the early genes (not shown in this figure). The gaps in the arrows represent sequences spliced out during mRNA formation (From Chow et al., 1979, modified by Pettersson and Roberts 1986).


Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
52-55K IIIa III
core
penton
pVII V pVI
hexon II 23K
PTNase
non-virion
33K
100K pVIII
14K
14.5K
11K
10K
16K
fiber IV
RNAs
1 2 i 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3'
l-strand 5'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K pol
87K p-TP
72K DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

late mRNAs have a common initiation site at mu 16.3 and the same standard tripartite leader. Each of five late gene families has its own common 3' terminus at mu 38[L1], 50[L2], 61.5[L3], 78[L4] and 94[L5], respectively (Berget et al., 1977; Fraser et al., 1979 and Chow et al., 1979). Viral RNAs are transported to the cytoplasm after processing (Nevins and Darnell 1978).

1.4 Early Region I

Early region 1 is located in the left 11% of the adenoviral genome. The E1 sequences of a number of human adenovirus serotypes are known (Dijkema et al., 1980b; 1981; Bos et al., 1981) and some cloned and sequenced cDNAs of E1 messages are available (Perricaudet et al., 1979; 1980). There are two distinct transcription units in the E1 region: E1A and E1B (Berk and Sharp 1978; Chow et al., 1979; Wilson et al., 1979; Perricaudet et al., 1979).

The E1 region is necessary and sufficient for cell transformation *in vitro* (Graham et al., 1974; Van der Eb et al., 1979; Graham 1984) and E1 products also play major roles in regulating viral gene expression. Both E1A and E1B major products are phosphorylated (Branton et al., 1985; McGlade et al., 1989). Serine is the only phosphorylated residue detected in Ad12 E1A polypeptides (Lucher et al., 1985). E1A products and the E1B 55K product are both found in the nucleus and

cytoskeleton. E1A appears in the nuclear matrix and nucleoplasm whereas E1B products are found in the nuclear envelope (Feldman and White et al., 1984).

1.4.1. The E1A Coding Region and its Products

The coding region of E1A extends from mu 1.3 to 4.5 on the r strand. A map of the Ad5 E1A region is shown (Figure 1.2). The E1A promoter region has a classical TATA box which determines the start site of transcription, and extensive deletional analysis of the promoter region shows that sequences far up stream are also necessary for the regular functioning of this promoter (Osborn et al., 1982; Osborn and Berk 1983; Hearing and Shenk 1983b; 1986). It is one of the earliest promoters expressed in viral lytic infection, driving transcription before any viral proteins are produced. Recent evidence indicates that E1A is autoregulated late in infection.

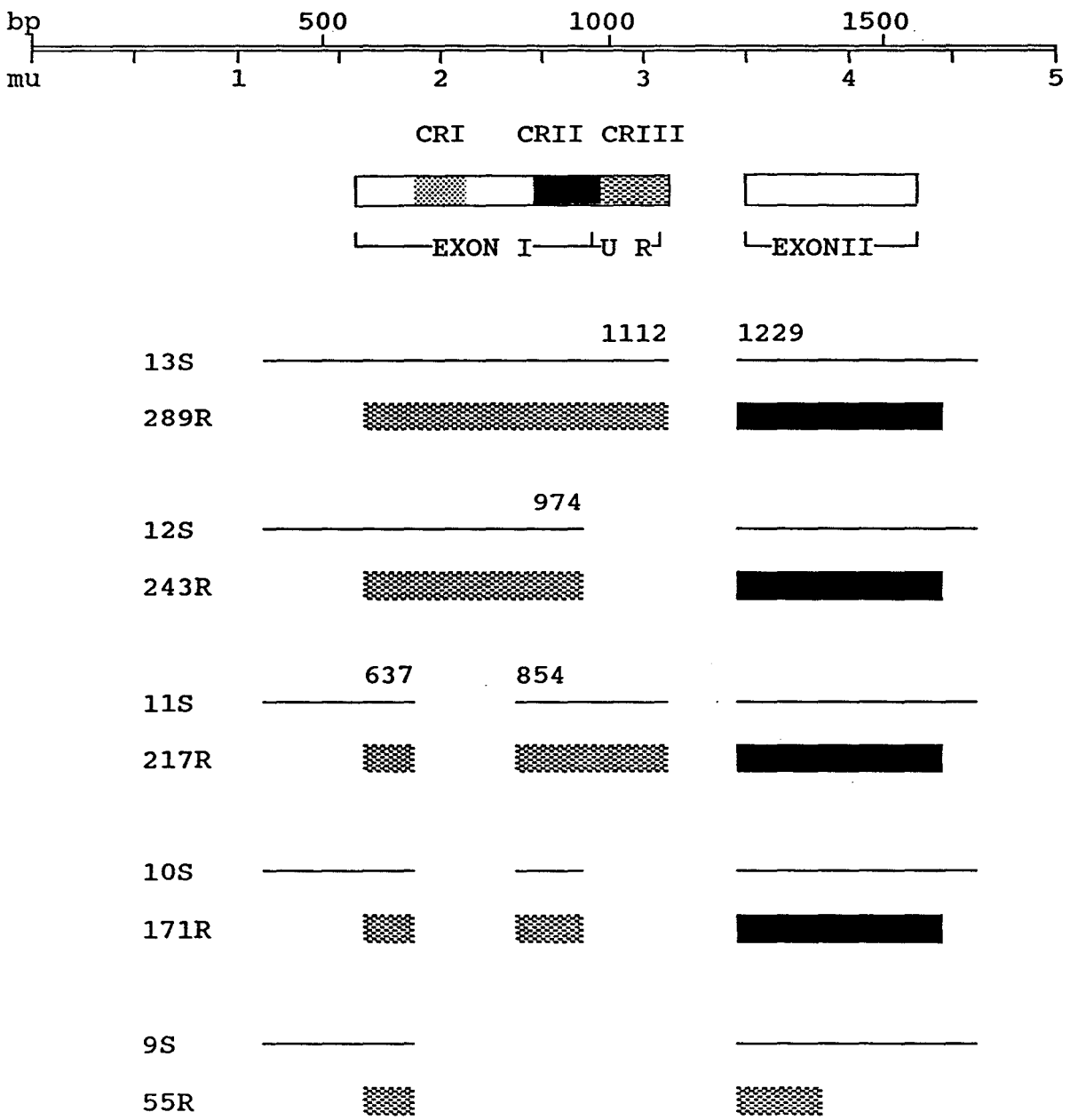
There are at least five E1A mRNAs formed by differential RNA splicing from a single RNA precursor. They are usually denoted 13S (1100 nt), 12S(960 nt),11S(880 nt), 10S(740) and 9S (620 nt) mRNAs according to their sedimentation coefficients. These mRNAs all share the same reading frames except for the 9S message which has a different reading frame in exon 2 resulting from differential splicing (Figure 1.2). The 13S and 11S mRNAs share the same splice

Figure 1.2 Map of Adenovirus Type 5 Early Region 1A (E1A)

E1A is at the left end of Ad5 genome up to mu 4.5. A scale of left end of Ad5 genome marking off from left to right in bp.'s and mu.'s is illustrated by a double line at the top of the diagram. Open boxes displayed under the genome represent E1A exon regions of I,II,III (light, dark and grey shading) indicating three highly conserved regions between adenoviruses of different serotypes, respectively. The range of each exon is shown under the blank box and unique region is labelled as UR. 5 E1A mRNAs are derived from a common precursor via differential splicing. All of the mRNAs contain a cap site from nt 499 and end at a polyadenylation site at nt 1632. The messenger RNAs are represented by single lines with their arrowheads pointing to 3' end. Numbers above lines are splice donor and acceptor sites, respectively. Translation of E1A products starts from nt 560 resulting in five E1A products with predicted sizes of 289R, 243R, 217R, 171R and 55R are shown. Translation products from these mRNAs are represented by stippled or filled bars referring to different reading frames caused by differential splicing of E1A mRNA.

Figure 1.2

MAP OF AD5 E1A REGION



donor site at nt 1112 and splice acceptor site at nt 1229, but the 11S species has an additional splice between nt 637 to 854 (Stephens and Harlow 1987). A similar structural relationship exists between the 12S and 10S mRNAs which share the same splice donor site at nt 974 and splice acceptor site at nt 1229, but with sequences between nt 637 to 854 deleted for the smaller mRNA (Stephens and Harlow 1987). The third minor mRNA, 9S mRNA, is potentially able to encode a 55 residue protein. This 9S transcript only appears late in infection and has never been found in transformed cells (Wilson et al., 1979; Spector et al., 1980).

The accumulation of E1A messages in the cytoplasm begins at 0.5 h postinfection. Major messages are 13S and 12S transcripts with only a small amount of the other species. The larger product of E1A 13S, has a half life of 35 to 55 minutes and the smaller 12S product 90 minutes (Spindler et al., 1984; Branton and Rowe 1985). Though the 9S message does not reach the level of two larger mRNAs until 8 to 12 h postinfection, it has a much longer half life than those of 13S and 12S (Spector et al., 1978; Wilson et al., 1979).

Because the products of E1A 13S, 12S, 11S and 10S transcripts share a common reading frame, they encode a set of highly related proteins which share a number of special characteristics, being rather acidic with pI 4-5 and rich in proline (16%) suggesting that E1A proteins may be rather rigid

molecules. As many as 40 to 60 E1A polypeptide species have been identified by 2D gel electrophoresis (Harlow et al., 1985). The diversity of E1A products may be caused in part by multiple phosphorylation sites and/or by some as yet uncharacterized modifications (Ferguson et al., 1985).

The two major E1A products are 289 residue (translated from the 13S mRNA) and 243 residue (translated from the 12S transcript) products also called 13S and 12S products, corresponding to their mRNA origins. These two proteins differ in that the smaller one lacks 46 amino acid residues which are unique to 13S product and are encoded in the region spliced out of the 12S mRNA (Perricaudet et al., 1979). The major 13S products migrate in SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) as polypeptides with molecular weights 53K and 41K, and the major 12S products as 47K and 35K, respectively. Products of 11S and 10S products are detectable in infected cells. The 11S product functions as a efficient transcriptional activator in transient expression assay (Ulfendahl et al., 1987), but the roles of minor species in viral replication are not clear since mutants which express neither 10S nor 11S products grow normally in Hela cells. E1A protein moves rapidly to the nucleus, requiring a short sequence at the carboxyl terminus of E1A for nuclear localization (Ferguson et al., 1985; Krippl et al., 1985; 1986).

Amino acid sequence comparisons have identified three regions which are highly conserved in E1A between different serotypes of adenoviruses (Moran and Mathews 1987). Conserved region I (CRI) from residue 46 to 77, has a structural similarity to a portion of large T antigen of SV40. CRII extends from residue 108 to 139 and this region is involved in binding of a cellular protein required for transformation (Egan et al.,1989; 1990). CRI and CRII appear in all known E1A products except the 9S product. CRIII from residue 142 through 188, the region unique to 13S and 11S products, contains a potential Zn finger structure which has several repeats of two cystine and two histine residues coordinating a zinc ion with two aromatic residues and one luecine residue in between (Berg 1986). Although the Zn finger of E1A does bind to Zn, it is not clear whether this binding is significant for E1A function such as transactivation, since genetic studies showed that any Cys of the E1A Zn finger could be substituted by another amino acid without affecting Zn binding though transactivation ability was reduced (Culp et al., 1988). The degree of conservation between different adenovirus serotypes is 50%, 50% and 46% for CRI, CRII and CRIII (Moran and Mathews 1987). These conserved regions seem closely related to various E1A activities such as transformation, transactivation, repression, induction of DNA synthesis, induction of mitosis and induction of PCNA expression (Velcich and Ziff 1985; Moran

et al., 1986; Lillie et al., 1986; 1987; Zerler et al., 1987; Smith and Ziff 1988; Whyte et al., 1988; Howe et al.,1990).

1.4.2. E1A and its Regulatory Functions

The best documented function of E1A is its transactivation ability though it is unclear how it transactivates. E1A can transactivate all other adenovirus early genes which share common promoter sequences, the activating transcription factor (ATF) binding site (Lee et al., 1987; Lillie et al., 1989). Possibly the E1A transcriptional transactivation function comes from its ability to enhance binding of a factor to this upstream promoter sequence (Kovesdi et al., 1987); however, many E1A inducible promoters do not have this binding site. Some data suggest that the TATA-box may be enough for E1A transactivation function (Wu et al., 1987; Simon et al., 1988). A construct fusing the E1A and sequence-specific DNA binding domain of GAL4 induces much higher transcription from promoters with GAL4 binding sequences than those lacking such sequences, suggesting that E1A may function directly at the promoter (Lillie et al., 1989).

E1A products activate transcription from various promoters, both *in vivo* and *in vitro*. These promoters are adenoviral promoters, including the E1A promoter itself, (Berk et al., 1979; Ferguson et al., 1984; Nevins and Wilson 1981;

Rossin 1983; Imperliale et al., 1983; Weeks and Jones, 1983; Spangler et al., 1987,), the c-myc and c-fos promoters (Sassone Corsi and Borrelli 1987), the HSV gD and tk promoters (Everett et al., 1984; Weeks and Jones 1985), the preproinsulin promoter (Gaynor et al., 1984) and the HTLV I and II promoters (Chen et al., 1985). They also transactivate the β-tubulin gene (Stein and Ziff 1984), the LTR of IAP (intracisternal A particle) (Luria and Horowitz 1986), the cellular human HSP70 gene (Kao and Nevins 1983), the MHC H-2K gene (Rothenthal 1985) as well as interferon-stimulated genes (Reich et al., 1988).

The product of the 13S message is the major, if not the only, E1A protein which plays a role in transactivation in infected cells (Carlock and Jones et al., 1981; Guilfoyle et al 1985; Glenn and Ricciardi 1985; 1987 and Green et al., 1988). The 13S product's transactivating function resides in 46 amino acid residues encoded by the unique region. Even a synthetic polypeptide mimicking this fragment of E1A could efficiently transactivate an E1A inducible promoter (Lillie et al., 1987). Transactivation may not be solely due to the 13S product, regions within CRII or upstream of CRII are also responsible for transactivation in a few cases (Osborn et al., 1984; Zerler et al., 1987). Transactivation performed by E1A regions other than the unique region may function via a different mechanism from that of the 13S product.

Besides being able to transactivate, E1A products are capable of repressing different enhancers (Lillie et al., 1986; Borrelli et al., 1984). The 12S product is probably responsible for this negative regulatory function. E1A represses class I HLA expression in transformed primary human cells (Vasavada 1986), immunoglobulin heavy chain gene (Hen et al., 1985), myogenic differentiation (Webster et al., 1988),insulin gene expression (Stein and Ziff 1987), promoters of polyomavirus and SV40 (Velcich and Ziff 1985; Velcich et al., 1986) as well as the E1A promoter itself. Interestingly, E1A products are able to either transactivate or repress the same enhancer depending on assay conditions (Borrelli et al., 1986; Hen et al., 1985).

Both E1A products can induce cellular DNA synthesis (Ohshima and Shiroki 1986; Kaczmarek et al., 1986; Matsuzaki et al., 1987; Howe et al., 1990) and differentiation of F9 teratocarcinoma cells (Montano and Lane 1987). The E1A major products also have the ability to cause progression through the cell cycle at a similar rate (Nakajima et al., 1987).

1.4.3 E1A and Transformation

As mentioned previously, adenoviruses can induce tumours when injected into rodents, and can also morphologically transform cultured cells (Trentin et al., 1962; Huebner et al., 1962). This has led to studies on the

mechanism of oncogenic transformation employing the adenovirus family as a model system.

In 1974, the genes responsible for adenovirus-induced transformation were first mapped to the left end of the adenoviral genome (Graham et al., 1974; Gallimore et al., 1974). Further studies by Graham and his coworkers indicated that only the first 2800 base pairs (mu 7.8) near the left end within the E1 region (the HindIII-G fragment) were sufficient to transform primary rat embryo cells (Graham et al., 1975). These results were confirmed by many later reports (van der Eb et al., 1977;1979; Mak et al., 1979; Byrd et al., 1982; Rowe et al., 1984; Gallimore et al., 1985). E1A alone can immortalize primary cells, but E1B is also required for the fully transformed phenotype (Houweling et al., 1980; Ho et al., 1982; van den Elsen et al., 1982; van den Elsen et al., 1983; Montell et al., 1984; Shiroki et al,1986). Several oncogenes, such as Ha-ras and middle T antigen of polyomavirus, also have the ability to cooperate with E1A in transformation (Land et al., 1983b; Jochemsen et al., 1986; Zerler et al., 1986). E1A has many functional similarities with other tumour-associated proteins such as HPV16 E7 (Phelps et al., 1989), including sequence homology and ability to co-operate with ras. Great strides have been made following the pioneer works of using a variety of mutants to map the region within E1A critical for transformation. Results from several

laboratories indicate that exon I (containing CRI and CRII) is indispensable for transformation whereas exon II is not. The amino terminal 140 amino acids cooperate with ras to transform and to immortalize at detectable levels (Zerler et al., 1986), though there is no consensus as to whether the whole of exon I is needed for this function.

Studies have shown that deleting amino acid residues 86 to 120 has little effect on lytic infection or transformation whereas deleting residues 120 to 150, which includes all of CRII, significantly impairs both functions (Moran et al., 1986). In an attempt to narrow down the critical region for transformation Whyte et al have shown that deletion of residues 121 to 127 results in a total loss of transforming ability. In addition, amino acid residues 1 to 85 were shown to be essential for transformation (Whyte et al 1988). A further study demonstrated that even single amino acid substitutions at 124 or 135 impair the transformation function of E1A (Moran et al,1986).

In contrast, Jelsma et al have suggested that CRI and CRII are responsible for transformation (Jelsma et al., 1989) and other regions in exon 1 are dispensable. They have suggested that their mutants which are transformation defective are also defective for repression, implying that these two functions are related.

Many different, sometimes contradictory conclusions

have been drawn from results with various deletion, insertion and site specific mutations in various regions of E1A. Some suggest that transactivation is likely to contribute to the mechanism of transformation (reviewed by Berk 1986). Other investigators, on the other hand, have constructed mutants which has lost the ability to transactivate but can still transform, indicating that transactivation may not be required for transformation (Schneider et al., 1987). Also, Lillie and his coworkers reported a transformation-defective mutant which could transactivate but not repress transcription of target genes (Lillie et al., 1986), suggesting that transactivation and transformation are separate functions of E1A. E1A transactivation, however, can indirectly improve the efficiency of transformation by increasing the level of E1B transcription. E1A may also play a role in transformation by increasing the expression of cellular genes encoding growth factors during adenovirus infection of baby rat kidney cells. Growth factors are produced which induce DNA synthesis and cellular proliferation (Quinlan et al., 1987; 1988).

Other evidence indicates that the transrepression rather than the transactivation function of E1A may be involved in transformation. Suppression of cellular gene activities has been observed in adenovirus-transformed cells (van der Eb et al., 1989). Some evidence suggests that the 12S product of E1A may be more responsible for transformation than

the 13S product (Bellett et al; 1985, Ohshima and Shiroki 1986; Velcich and Ziff 1988). An independently isolated mutant, H5dl520, which only expresses 12S mRNA can transform both CREF and BRK cells 40 to 100 times more efficiently than can wt (Haley et al., 1984).

Other evidence suggests that both 12S and 13S expression are required for efficient and complete transformation (Winberg and Shenk 1984). One study has shown that the 289 residue protein can mediate immortalization of primary cells, while the 243 residue protein is capable of eliciting the transformation (Hurwitz and Chinnadurai 1985).

1.4.4. E1B Structure and Functions

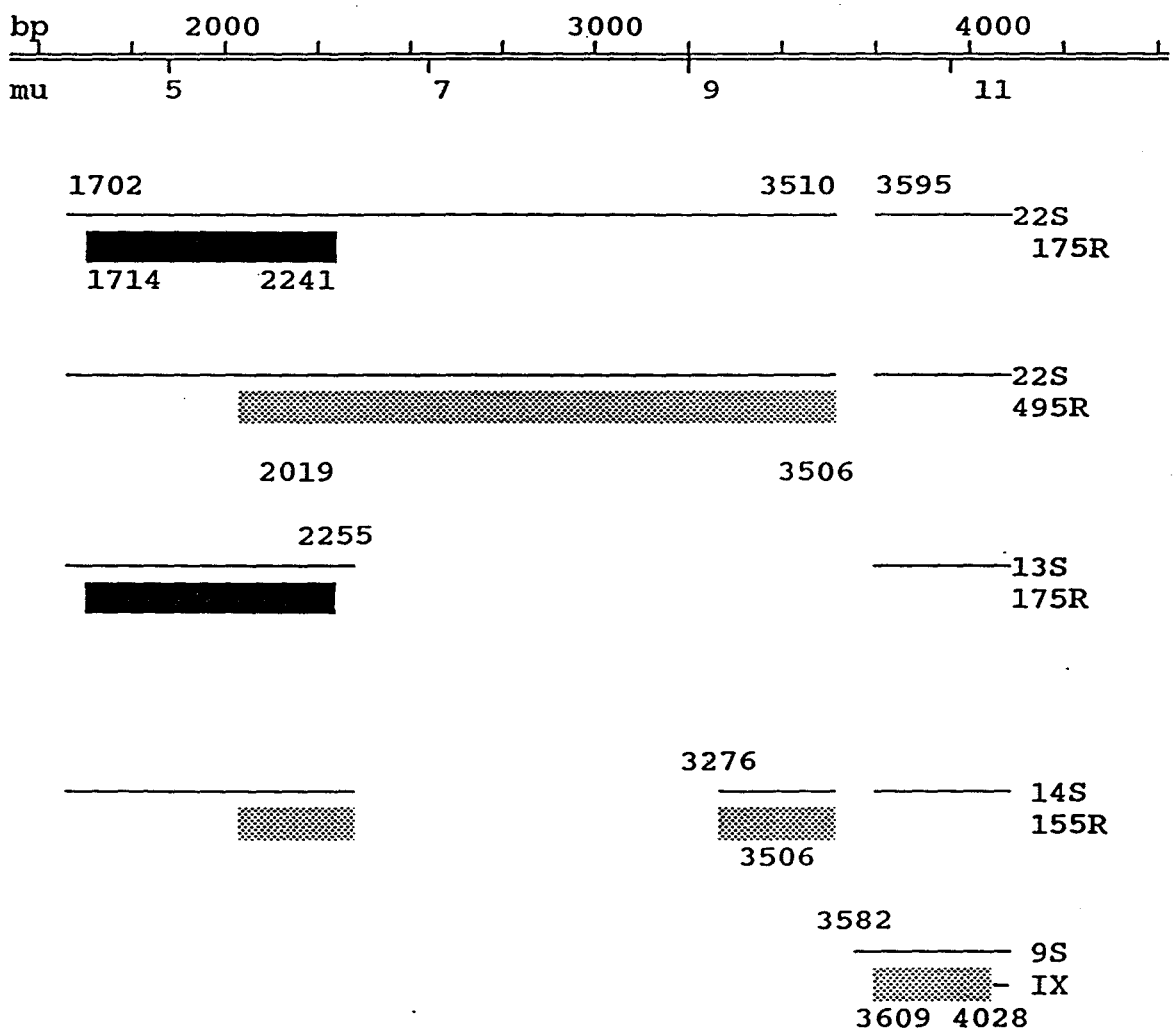
The E1B coding sequences are located downstream of the E1A region at mu 4.5 to 11.5 of Ad5 (Figure 1.3). The upstream regulatory sequence of E1B is located within E1A from nt 1350 to 1650 (Parks et al., 1988). The upstream promoter region contains Sp1 and CAAT-box transcription factor binding sites (Berk 1986). The distance between the E1B promoter and transcription initiation site varies depending on different species of adenoviruses (Berk 1986; Baker and Ziff 1981; Gingeras et al., 1982). In Ad5, the E1B transcription start site is at nt 1702 and the polyadenylation signal is at nt 4070. The half life of E1B mRNA is more than 4 hours in

Figure 1.3 Map of Adenovirus Type 5 Early Region 1B (E1B)

The E1B region is at the left end of Ad5 viral genome from mu 4.5 to 11.5 and extends from nt 1702 to nt 4070. The scale of the Ad5 genome is indicated at the top of the diagram by bp's and mu's. Messenger RNAs are shown by single lines with spliced parts gapped and arrowheads at their 3'ends. The numbers above the lines are splice donor or splice acceptor sites. The 22S mRNA encodes two products of 175R and 495R respectively translated from different ATGs. The 175R product can also be translated from another message: the 13S mRNA. A 155R protein is translated from one of two minor and intermediated sized mRNA species (only one is shown). The smallest 9S mRNA encoding a structural protein, protein IX, is also within E1B coding region. Sizes of different E1B products are indicated and predicted E1B amino acid coding sequences are shown by filled bars or stippled bars according to different reading frames.

Figure 1.3

MAP OF AD5 E1B REGION



infected cells which is much longer than E1A mRNA.

There are five mRNA species transcribed from the E1B coding region, four of which are coterminal at the 5' end. The overlapping 22S and 13S messages differ in that the 13S mRNA has a larger intron (from nt 2255 to nt 3590) than the 22S transcript (from nt 3510 to 3590)(Perricaudet et al 1980). Two intermediate-sized mRNA species have been identified, one of which encodes a 17K protein (Anderson et al., 1984) and is colinear with the 22S message with an intron extending from nt 2256 to 3275. The fifth mRNA, 9S mRNA, is the only unspliced E1B mRNA (Wilson 1979). E1B mRNAs are detectable in the cytoplasm at about 1.5 to 2 hours postinfection (Nevins et al., 1979). A large amount of the 22S mRNA is produced in the early stage of viral infection while the 13S mRNA peaks later.

Two polypeptides of 58K and 19K are derived from the larger message but share no common reading frame. The 58K protein coding sequence of the 22s message (from nt 2019 to nt 3506) starts at the second ATG downstream from the E1B promoter region, whereas the 19K ORF 22S mRNA extends from nt 1714 to 2241. The 19K product is also translated from the 13S mRNA (Bos et al., 1981). The 17K product coding region shares both N and C terminal sequence with 58K product (Anderson et al. 1984). The 9S transcript has a separate promoter and encodes for a 14K structural product protein IX (Wilson et

al., 1979; Willams et al. 1986).

E1B functions by enhancing E1A at initiation (Jochemsen et al., 1987) and can transactivate the expression of human ß interferon gene (Shiroki and Toth 1988).

Though the E1A gene is important in viral transformation, it is unable to produce completely transformed cells without the participation of E1B (van den Elsen et al., 1982; Byrd et al., 1988). The minimum requirement for complete transformation requires the cooperation of E1A protein(s) and those E1B products encoded by the first 1100 bp of the E1B gene, presumably the 19K product and possibly also a truncated form of the 58K product.

There may be different mechanisms involved in adenoviral or DNA-mediated transformation, since the E1B 58K protein is required for the former but not the latter (Graham et al., 1978; Jochemsen et al., 1982; McKinnon et al., 1982; Rowe and Graham 1983; Babiss and Ginsberg 1984).

The E1B 58K protein is phosphorylated (Branton et al 1984; Schughart et al., 1985) but the significance of this modification is unclear. Genetic studies showed that this product was the factor responsible for the normal viral mRNA accumulation, transport of viral RNA and turning off the transport of cellular mRNA to the cytoplasm (Babiss and Ginsberg 1984; Babiss et al., 1985; Williams et al., 1986; Pilder et al., 1986). It has been suggested that in adenoviral

lytic infection, the E1B 58K protein acts in mRNA transport as a complex with an E4 25K protein (Halbert et al., 1985).

A major proportion of the 19K product appears only after DNA replication. The intracellular location of 19K protein varies depending on different stages of viral infection. At the early stage of viral infection, the 19K protein localizes to the cell nuclear envelope, cytoplasmic membrane and the cell surface and disappears from the cytoplasmic membrane at the late stage (Grand and Gallimore 1984; White et al., 1984; Stillman 1986).

The 19K product no doubt plays an essential role in both virus-mediated or DNA-mediated transformation (Bernards et al., 1983; Chinnadurai 1983; Mak and Mak 1983; Babiss et al., 1984; Pilder et al., 1984). Insertion mutants with large inserts in the 19K coding region totally eliminate transforming ability (Mckinnon et al 1982) and the protein may be involved in the efficiency of transformation (Mak et al., 1984). Infection of primary human cells by mutants with a defect in 19K protein generates sharp-edged large plaques instead of small plaques with a hazy border (Takemori et al., 1968). This mutant produces an enhanced cytopathic effect as well (reviewed by Stillman 1986). The 19K product can activate all the adeno early promoters like E1A, E1B,E2, E3, E4 and a cellular HSP70 promoter, but none of the late promoters such as those of IX, 1Va2, MLP or E2L (Herrmann et al, 1987; White

et al., 1988). The enhancer activation activity of 19K protein can generally mask the E1A enhancer repression function.

1.5 Project Outline

1.5.1. A Series of E1A Insertion Mutants

A convenient way to study E1A functions is to make mutations at appropriate functional regions within E1A. A human embryonic kidney cell line, 293, which expresses the E1 region of Ad5, permits the replication of host range mutants, and has been invaluable for the isolation of E1 mutants by complementation (Graham et al., 1977). More recently, a simple recombination technique has been developed to rescue mutated E1 sequences back into adenovirus (McGrory et al., 1988).

To help elucidate the role of E1A in transformation, transactivation and transrepression, and to determine how these functions relate to each other, plasmids containing insertional mutations at multiple sites within the E1A gene were constructed, rescued into virus, and characterized by functional analysis (Bautista 1989; McGrory 1988).

The scheme for generating E1A mutants involved insertion of a cassette containing the lac operator sequence at various restriction sites in E1A (Figure 1.4). The lac operator sequence, if present at high copy number in the bacterial host, will bind all the available lac repressor molecules, resulting in induction of expression of the

bacterial β-glactosidase operon. This cassette design facilitated screening of recombinants, since clones carrying the insert produced blue colonies when plated in the presence of X-gal. In one orientation, this 39 bp lac operator insert introduced an additional 13 aa codons without altering the reading frames of E1A. In the opposite orientation, the insert carried stop codons in all three reading frames, thus truncating the rest of the E1A coding information. The insertion cassette, flanked by *Bam*HI restriction sites, could be collapsed to give a 2 codon insert. Thus, three different kinds of insertions were created at each restriction site: a 39 nt larger insertion (open), an insertion causing truncation (closed) and a 6 nt smaller insertion (collapsed). The ability of these mutant proteins to function as transactivators or transrepressors was tested by transient expression assays in Hela cells. The transient expression assays utilized a reporter plasmid containing either the E1A, E3, E2, or E4 promoter upstream of the β-galactosidase gene. The mutants were also tested for the ability to transform primary baby rat kidney cells.

Briefly, the results of the experiments were in agreement with many of those discussed earlier, in that, mutations affecting the unique region of E1A can drastically reduce transactivation and transformation (in E1 mediated transformation), whereas other mutations seem to have little

Figure 1.4 Lop-Mutator Insertion Cassette

LacZ operator (lop) sequence labelled insertion cassette with either blunt ends or 5' CG overhangs are shown. These cassettes are used to construct E1A insertion mutants. Besides lop core sequence (underlined), they all have *Bam* HI sites flanking the cassette. The predicted amino acid sequences of the cassettes in different reading frames in either orientation are shown.

Figure 1.4

1. Insertion cassette with blunt ends.

Orientation A: Open reading frame

5' GGATCCAATTGTTATCCGCTCACAATTGGGTCAGGATCC 3'
3' CCTAGGTTAACAATAGGCGAGTGTTAACCCAGTCCTAGG 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

III. GG-Ile--Gln--Leu--Leu--Ser--Ala--His--Asn--Trp--Val--Arg--Ile

Orientation B: Closed reading frame

5' GGATCCTGACCCAATTGTGAGCGGATAACAATTGGATCC 3'
3' CCTAGGACTGGGTTAACACTCGCCTATTGTTAACCTAGG 5'

I. Gly--Ser--End

II. G-Asp--Pro--Asp--Pro--Ile--Val--Ser--Gly--End

III. GG-Ile--Leu--Thr--Gln--Leu--End

2. Insertion cassette with sticky ends.

Orientation A: Open reading frame

5' CGGATCCAATTGTTATCCGCTCACAATTGGGTCAGGATC 3'
3' CTAGGTTAACAATAGGCCAGTGTTAACCCAGTCCTAGGC 5'

I. C-Gly--Ser--Asn--Cys--Tyr--Pro--Leu--Thr--Ile--Gly--Ser--Gly

II. CG-Asp--Pro--Ile--Val--Ile--Arg--Ser--Gln--Leu--Gly--Gln--Asp

III. Arg--Ile--Gln--Leu--Leu--Ser--Ala--His--Asn--Trp--Val--Arg--Ile

Orientation B: Closed reading frame

5' CGGATCCTGACCCAATTGTGAGCGGATAACAATTGGATC 3'
3' CTAGGACTGGGTTAACACTCGCCTATTGTTAACCTAGGC 5'

I. C-Gly--Ser--End

II. CG-Asp--Pro--Asp--Pro--Ile--Val--Ser--Gly--End

III. Arg--Ile--Leu--Thr--Gln--Leu--End

effect (McGrory 1988; Bautista 1989).

One mutant was found particularly interesting. This mutant contained a 39nt insertion upstream of CRII at nt908 (aa117) which reduced enhancer repression activity without affecting transformation ability. The smaller 6bp insertion at the same site did not significantly affect either repression or transformation. Two conclusions could be drawn from these facts: 1) the repression function could be separated from transformation, and 2) repression may be sensitive to local conformation changes to different degrees. Surprisingly, a mutant with the large insertion at nt 906 displayed wild type levels of repression, suggesting that the boundary for one repression region may be at 908 upstream of CRII. Bautista also found that any insert at nt 1267 reduced the repression function of the mutant, suggesting that this repressing region may be extended to the beginning of exon II.

1.5.2 Construction of CRII mutants and Screening of Temperature sensitive Mutants

I have constructed 9 new E1A insertion mutants at nt 918 (aa 120) and nt950 (aa131) within CRII using the same strategy employed by Bautista and McGrory. These mutants were analyzed to determine whether CRII (just down from nt 908) is required for repression and whether this region plays a role in transformation. In addition, we wished to determine if any

of the unique region insertion mutants were defective for replication or transformation, possibly induced by conformational changes caused by insertion. To accomplish the latter, I have screened by plaque assay, at three temperatures, all 60 insertion mutants scanning the whole E1A gene and 1 E1B insertion mutant previously obtained by Bautista et al. in an attempt to identify any temperature sensitive mutants. I have also screened 11 insertion mutants to identify any mutants temperature sensitive for transformation.

The results of my analyses of repression and transformation by CRII mutants, as well as the results of my screening for temperature sensitive mutants showed that insertion within CRII did affect repression and transformation, but not transactivation by E1A. The extent to which repression and transformation were affected depended largely on the size of insertion. Further results suggested that repression and transformation may be separate E1A functions. A 6 nt in frame insertion at nt 950 did not affect repression, but abolished the E1A transformation function.

Some mutants were characterized as host range mutants based on their plaque forming ability on 293 and Hela cells while others were not. However, none of the mutants were temperature sensitive for replication. Two mutants, pXC1008+ and pXC1039+, were temperature sensitive for transformation.

They both have a 39 bp open insertion in the unique region. pXC1008+ and 1039+ transformed BRK cells as efficiently as wild type did at 32°C but transformed very poorly at 38.5°C.

MATERIALS AND METHODS

2.1. Mammalian Cell Lines

Hela and 293 cells (Graham et al.,1977) were used for the propagation and titration of virus stocks. Hela cells were grown in α-MEM (α-minimal medium) with 10% newborn calf serum while 293 cells were grown in Joklik medium with 10% horse serum. All serum used were complement inactivated at 56°C for 30 min. Both cell lines were routinely grown in medium supplemented with 1% L-Glu (v/v), Penicillin-Streptomycin 100 μg/ml. (Gibco 100x solution contains 10000 μg/ ml each), Fungizone 2.5 μg/ ml (Squibb 100 X solution contains 0.25 mg/ ml made from 50mg/am package). Additionally, 0.2% yeast extract was added in medium for cell culture in plaque assays (5% solution as 25 X stock).

Both Hela and 293 cells were cultured in petri dishes and maintained in Forma Scientific Steri-Cult incubators with controlled conditions, namely 37°C (or 33°C and 39°C for temperature sensitive analysis), 95% humidity, and 5% Co_2. Petri dishes were 60 mm or 100 mm from Corning and 150 mm from Nunclon. Other reagents used in tissue culture were: PBS^{--} (1x, 80 g NaCl, 2 g KCl, 11.45 g Na_2HPO_4, 2g KH_2PO_4 in 1L sterile redistilled water served as 10 X phosphate buffered saline

stock) supplemented with routine penicillin and streptomycin; 1 X PBS^{++} (PBS supplemented with 10mg/ ml $CaCl_2$ and 10mg/ ml $MgCl^2$); pronase-SDS (0.5 mg/ ml pronase in lysing buffer); 2 X trypsin (2.0 g/100 ml EDTA in Sterile redistilled water) and 1 X Saline-EDTA (made from 10 X stock: 2 g glucose, 2 g di-Na-EDTA, 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 2 g KH_2PO_4 in 1 ml sterile redistilled water).

Cells about 70%-80% confluent were passaged. Cells to be passaged were washed with PBS^{--} after the medium had been removed by vacuum aspiration. 1 ml 1 X trypsin-EDTA was added to each dish of Hela cells and incubated at room temperature for about 4 min. 10 ml fresh medium was added after the cells had detached and the cell suspension then was diluted as desired and distributed onto new dishes. Passaging of 293 cells was followed the same method except 1 X saline EDTA was used instead of trypsin-EDTA to detach cells. This usually took 10 min at room temperature.

2.2. Bacteria

Bacterial strains of K12 *Escherichia Coli* included: LE392(F^-*hsd*R17($r_k^-m_k^-$)*sup*E44 *sup*F58 *lac*Y1 or (*lac*IZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55$^-$; Murray et al.,1977), E5014 ([*lac-pro*A]XIII, Spc^R, SuE[F,*lac*+ *pro*AB]; Reznicoff,pers.comm.) and GM48. The following were used for liquid cell culture: Luria Bertani (LB.10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl

and redistilled water to 1L, pH7.4, sterilized),B broth (10g bacto-tryptone, 8 g NaCl and redistilled water to 1 L. sterilized) and M9 (1 L contained 100 ml 10 X M9 salt (10 X M9 salt stock: 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl 10 g NH_4Cl and sterile redistilled water to 1 L, sterilized, stored at 4°C), 1 ml 1 M $MgSO_4.7H_2O$, 1 ml 1% Vitamin B1, 10 ml 10 mM $CaCl_2$, 10 ml 20% glucose and sterile redistilled water to 1 L. 40 μg/ ml X-gal was added if necessary). Ampicillin was used for bacterial antibiotic resistance selection at a final concentration of 40 μg/ml. For long term storage, bacterial cells were kept in 20% glycerol at -70° as stocks or at 4°C over night for short storage.

2.3. Plasmids

The plasmid pKH101 was used for the construction of E1A CRII insertion mutant plasmid. pE1A*Lac* and pE3*Lac* were used as reporter plasmids in β-galactosidase assay for E1A function studies. Plasmid pXC38 was used as a parent vector for the introduction of E1 background into E1A CRII insertion mutant for the purpose of transformation assay (Bautista 1989).

2.4. Enzyme

Restriction enzymes used in all experiments were supplied by Bethesda Research Laboratories (BRL),New England

Biolabs (NEB), Pharmacia and International Biotechnological Institutes (IBI). Restriction enzyme digestion was carried out primarily in a universal restriction buffer at the final concentration of 10 mM Tris.HCl (pH7.5), 50 mM KCl, 10 mM $MgCl_2$. The buffer was prepared as a 10 X stock stored at 4°C for normally no more than 4 months. In some cases, such as for DraIII or double restriction digestion, the restriction buffer suggested by the manufacturer was used to obtain optimal enzyme activity.

Restriction enzyme digestion was carried out at 37°C for 2 hr usually in a volume of 20 to 30 μl containing 1 to 5 μl of DNA sample, 2 to 3 μl of 10 X restriction buffer or manufacturer's suggested buffer, enzyme(s) at 2 units per μg DNA and sterile redistilled water brought to final volume. In the case of double restriction digestion, heat-inactivation was used to eliminate the enzyme activity used in previous step, usually at 70°C for 15 min to prevent the expression of activity of the first enzyme in the following digestion. Partial digestion to cut multicuttable plasmids only once was done by limiting the incubation time. The duration of the incubation of partial digestion varied depended on each enzyme and DNA sample used.

Klenow fragment, the large fragment of DNA polymerase I, supplied by BRL, was used to blunt the staggered ends of some linearized plasmids as a necessary step either to create

a restriction site or to eliminate an unnecessary restriction site of the parent plasmid. Klenow polymerase I blunted ends either by filling in the recessive 3'ends or by exonucleolytic digestion of protruding 3'termini. The reaction buffer for Klenow treatment was supplied by the manufacturer. This 5 X concentrated buffer was stored at -20°C until used. The following 20 ul Klenow reaction was incubated at room temperature for 15 to 20 min: 1 ug DNA sample, 1 unit of Klenow fragment and 0.5 mM of each dNTPs (dGTP, dATP, dTTP and dCTP each was prepared as a 10 mM solution in 10 mM Tris.HCl pH 8, stored at -20°C). Heat inactivation for 15 min at 70°C was used to stop the reaction . The solution was ethanol precipitated and dried prior to ligation.

DNA fragments were ligated using T4 DNA ligase (BRL) according to the required procedure for construction of new plasmids. A manufacturer suggested ligation buffer was used. The ligation buffer was stored in small aliquots at -20°C. DNA fragments obtained either from electroelution or Gene Clean were used for in vitro DNA fragments ligation. In a standard 10 ul ligation reaction, 200 ng DNA fragment(s), 1 unit T4 DNA ligase and 0.5 mM ATP(5.0mM as 10 X stock stored at -20°C) were incubated in an Eppendorf tube in ligation buffer 14 to 16 hr at 14°C. The molar ratio of DNA fragments to be ligated was kept at 1:1 if their sizes were close. In the case of linker insertion, the ratio of insertion cassettes

to parent vector was usually higher, up to 15 to 1.

The oligonucleotides for insertion were supplied by the Central Facility of the Institute for Molecular Biotechnology, McMaster University. They were dissolved in sterile redistilled water, usually at 2 O.D.,then stored at -20°C.

2.5. Bacterial Transformation

2.5.1. Calcium Technique

A modified E.Coli-transformation technique (Maniatis et al., 1982) was employed to transform bacteria with desired recombinant DNAs.

The bacterial cell line to be transformed was started with a loop of frozen cells in 2 ml of liquid media, either LB or M9, and grown at 37°C over night with vigorous shaking. A 1 ml over night culture was diluted in 100 ml of fresh LB broth and the growth was continued until turbidity reached O.D. 600 0.5. The cells were pelleted and resuspended in 40 ml of ice cold transformation buffer (75 mM $CaCl_2$ and 5 mM Tris. PH7.6) and incubated on ice for at least 1 hr. Finally the cells were centrifuged and resuspended in 2 ml of transformation buffer. The competent cells could be used immediately after 1 hr on ice incubation or could be kept on ice for up to 48 hr.

The recombinant DNA used for transformation was added

to 0.2 ml competent cells and incubated on ice for 40 min following by a 2 min heat-shock at 42°C. The cells were then diluted in 3 ml of B broth prewarmed at 37°C, after which the mixture was incubated at 37°C for 45 min with mild shaking every 15 min to allow the expression of antibiotic resistance. Cells were serially diluted then were plated onto agar dishes with proper antibiotics. The dishes were moved to 37°C and turned upside down after 20 min and incubation continued over night. Colonies usually appeared after 6 to 8 hr on LB plates and the next morning on M9 minimal agar plates.

2.5.2. Electroporation Technique

In some cases. electroporation was used for transformation. This high efficiency technique employed a physical method involving a high voltage to shock of pre-prepared *E.Coli* cells to transport DNA molecule into cells. This method generally gave 10^6 to 10^8 transformants per μg of religated plasmid DNA.

Bacteria strain E5014 was used for electroporation. A 10 ml over night culture was diluted in 1000 ml LB solution and grown with vigorous shaking at 37°C until the OD 600 reached 0.5. The cells were washed , pelleted twice with 1000 ml cold Sterile redistilled water and once with 20 ml 10% glycerol. Cells resuspended in 2 ml 10% glycerol were ready to use. Aliquots of 40 μl in Eppendorf tubes were stored at

-70°C until use.

Stored aliquots were moved to room temperature to thaw for electro-transformation. One μl to 5 μl of ligation reaction were added to the cells and allowed to sit on ice for 5 min. The mixture was transferred and shaken to the bottom of a cold electroporation cuvette and the cuvette was placed in a pre-chilled cuvette holder of gene pulser apparatus (Bio-Rad) between the two electrodes of the electroporation machine. The voltage was set at 2.5 KV, the controller at 200Ω and 25 μf. Immediately after the shock, the cells were suspended in 1 ml of LB and then incubated at 37°C for 1 hr for the expression of antibiotic resistance. The cells were plated on appropriate dishes as in calcium-mediated transformation.

2.6. Small Scale Plasmid DNA Preparation

Small scale DNA preparation was used to screen recombinant DNA transformants (Birnboim and Doly 1979). Well isolated colonies were picked and transferred to 2 ml LB broth and grown with shaking over night at 37°C. Cells were transferred and collected at the bottom of 1.5 ml Eppendorf tubes and resuspended in ice cold lysozyme (Boeringer-Mannheim, 5 mg/ ml in 10 mM EDTA, 50 mM glucose and 25 mM Tris, pH 8.0). After 30 min incubation on ice, 200 μl of alkaline-SDS (0.2N NaOH, 1% SDS) was added and the lysate was

kept on ice for 5 min. 150 μl of 3M NaOAc (pH4.8) was then added and the incubation was continued on ice for 60 min. After 10 min spinning in a benchtop Eppendorf centrifuge, the supernatant was collected and precipitated with 2 volumes of ethanol and then washed 3 times with 96% ethanol. The final pellet was dried at 37°C for 15 min and then dissolved in 40 μl of 0.1 SSC. 5 μl of the final sample was digested with an appropriate restriction enzyme and run on an agrose gel for analysis by electrophoresis. Colonies with plasmids having expected fragments were streaked on plates with antibiotics to obtain well isolated colonies, then grown up and kept at -70°C as a stock.

2.7. GeL Electrophoresis

Boiled TAE buffer (0.4 M Tris-Acetate, 0.002 M EDTA) containing EtBr at 0.1 μg per ml of electrophoresis buffer and the required amount of agarose was cooled to 44°C before pouring the gel. The mixture was poured into a horizontal gel apparatus with removable comb and allowed to set about 30 min at room temperature to solidify. To run the gel, the loading mixture including DNA restriction reaction, 1/6 volume of loading buffer (20% glycerol, 2% SDS and 0.5% bromophenol blue) up to a final volume 20-30 ul, was loaded into each gel well. Gels were run 14 to 16 hr at 1V/cm and HindIII digested Ad5 DNA was employed as a standard marker. All gels were

photographed under UV light using a mounted Polaroid camera with a red filter. Auto developed high speed Polaroid 57 films were kept as records.

2.8. Large Scale Plasmid DNA Preparation

Bacteria containing plasmids were grown up overnight in 10 ml LB broth with appropriate antibiotic. The cells were then transferred to 700 ml LB in a 2000 ml flask and agitated until the O.D.600 reached around 0.4. Chloramphenicol was then added at a final concentration of 50 μg/ml. After incubation over-night centrifugation was carried out at 5000 rpm for 10 min in a Sorval RC-5B refrigerated centrifuge. The pellet was resuspended in 40 ml lysozyme (5 mg/ml). After 30 min incubation on ice, 80 ml Alkaline SDS was added to each pellet and set on ice for 10 min. 40 ml 5M KAc (pH4.8) was added and the preparation was allowed 15 min incubation at 0°C. 10 ml sterile redistilled water had been added before centrifugation for 10 min at 9K in a Sorval GS3 rotor. Precipitates were discarded and the supernatant was filtered through cheese cloth. Finally, 0.6 Vol(100ml) isopropanol was added, dissolved, and incubated at room temperature for 30 min and centrifuged 10 min at 10 K at room temperature. The pellet was drained for 15 min then was suspended in 7 ml 0.1 X SSC and transferred to a 50 ml tube. 8.5 g CsCl was added, dissolved, and incubated 30 min on ice, then centrifuged at 3.7 K for 10

min. The supernatant was carefully rescued into a Vti 65.1 13 ml quick seal tube (Beckman) and 400 µl 6mg/ ml EtBr was added. The tube was then topped with light paraffin oil and balanced. After heat-sealing, ultracentrifugation was performed at 55 K for 16 to 22 hr at 14°c. The band containing plasmid DNA, visualized with long wave UV, was collected with needle and syringe. EtBr was extracted off with isopropanol saturated CsCl (in 25 mM Tris, 10 mM EDTA) and DNA was diluted 3 X in TE then precipitated and washed with ethanol. Dried DNA was dissolved in about 1 ml of 0.1 X SSC, depending on the size of pellet. The DNA solution was kept either at -20°C or 4°C.

2.9. Determination Of DNA Concentration

The diphenylamine method was used to determine the concentration of DNA in plasmid preparations. DNA samples were diluted serially to a total volume of 300 µl. 1 ml of diphenylamine regent was added to each Eppendorf tube and incubated in boiling water for 10 min. Then these tubes were allowed to be slowly cooled down to room temperature. OD 595 was taken and concentration of DNA samples were derived according to a standard curve derived using DNA of known concentration.

2.10. Agarose Gel DNA fragment Purification

TBE buffer (12.1 Tris-HCl, 5.135g Boric Acid and 0.372g Na2-EDTA in 1 L Sterile redistilled water) was used to make agarose gels for isolation of desired DNA fragments. The gel was run in the dark. In order to get well isolated DNA fragments, usually it took a longer time than analytical gel electrophoresis depending on the size of the fragment one wanted. The gel was cut using a new scalpel blade immediately ahead of the desired band visible under UV. Agarose gel sections containing larger bands were removed and a dialysis membrane (pre-treated with 4 mM EDTA in boiling water for 10 min) was inserted into the cut and the gel was run at 150 V to 300 V to let the DNA to be captured by the membrane. The DNA was washed off the membrane onto a piece of parafilm with lysing buffer (10 mM Tris pH7.4, 10 mM EDTA and 0.4% SDS). It then was transferred to an Eppendorf tube. The solution was phenol and chloroform extracted, ethanol precipitated and washed. It was then resuspended in an appropriate volume of 0.1 SSC.

2.11. Dideoxy Sequencing Technique

The Sanger dideoxy termination sequencing technique was used for analysis of insertion mutants sequence (Sanger et al.,1977).

Oligonucleotides AB663, AB690 and AB691 (supplied by the Central Facility of the institute for Molecular Biology

and Biotechnology, McMaster University) were used as sequencing primers. The primer AB663 corresponded to the adenovirus 1 strand from nt 1000 to 984 (5'to 3'). AB690 and AB691 were the oligos used to form a full size lac-labelled insertion cassette. The sequencing gel was run on vertical IBI gel apparatus. The radio-labelled α-^{32}P-dATP used for sequencing was purchased from NEN (3000Ci/mmole, 50mCi/ml). The radioactive materials were stored in lead canisters at-20°c.

Sequencing gels were prepared on the same day of sequencing. Sequencing gel mixture (6.65 g acrylamide, 0.35 g bis and 42 g urea) was dissolving in 80 ml freshly made 1 X TBE at 65°C for about 5 min. The solution was filtered through a 0.45u Nalgene filter after cooling to room temperature, then the volume was brought to 100 ml using 1 X TBE and it was kept on ice until casting the gel.Generally, the cooler the gel mixture, the longer the time of polymerization.

Two glass plates were detergent washed and polished with ethanol. The inner side of the longer plate was coated using 5 ml 2% repel silane (in 1,1,1, trichloroethane) by polishing. The two plates were spaced 0.4 mm apart. 1 ml of fresh 10% ammonium persulfate and 40 μl TEMED were added to sequencing mixture, immediately before casting the gel. The gel was allowed to lay horizontally about 60 min for

polymerization and was then pre-run at 1800 V and 100 mA,60 min before loading to warm the sequencing apparatus plates.

A Sequenase kit (USB) was used for all sequencing. Two μg DNA were used for dideoxy sequencing. The DNA sample was dried in a vacuum centrifuge. The pellette was dissolved in 40 μl denaturation buffer (NaOH, 0.2 mol/L; EDTA,0.2 mmol/L; pH8.0) and set at room temperature for 5 min. Four μl of 2M ammonium acetate (pH4.5) were added to the reaction for neutralization before the DNA was precipitated with 100 μl 96% ethanol. After pelleting, the DNA was washed again with 70% ethanol. The vacuum dried DNA was dissolved in 7 μl of sterile redistilled water for immediate use, or kept at -20°C for a few days until needed. A single annealing reaction was carried out for each of four sequencing lanes. Seven μl of DNA solution preprepared for sequencing were mixed with 1 μl primer,2 μl sequencing reaction buffer and allowed to incubate at 65°C for 2 min. The capped Eppendorf tube was then slowly cooled to 30°C over a period of no less than 30 min. At this time, labelling reactions were carried out as follows: 1 μl 0.1 M DTT, 2 μl diluted labelling mix (1:15, labelling buffer: Sterile redistilled water) 0.5 μl α-^{32}P-dATP and 2 μl diluted sequenase (1:8 sequenase:enzyme diluting buffer) were added in the tube containing the template-primer reaction and incubated at room temperature for 5 to 10 min. 2.5 μl of each termination mixture containing ddGTP, ddATP, ddTTP and ddCTP

was added to tubes labelled G, A, T, C respectively. At the end of the labelling reaction, 3.5 μl of each reaction solution was transferred to the termination tubes G, A, T, C pre-warmed at 37°C accordingly. Incubation was continued for 5 to 10 min at 37°C after centrifuging to collect the solution at the bottom of the tube. At this point 4 μl stop solution was added to each tube and they were then heated for two min at 75°C to 80°C immediately before loading. The sequencing gel was usually run at 2200v and 100mA.

The time needed for the running of sequencing gel varied depending on the voltage, electric current, the acrylamide gel concentration and the distance from the 3' end of the sequencing primer to the desired sequencing region. When the sequencing dye front ran far enough, the gel chamber was removed from the IBI apparatus. The longer glass plate was separated off leaving the gel attached to the shorter plate. The shorter plate was removed after transferring the gel to a piece of filter paper. The gel was dried on a gel dryer (Bio-Rad) at 80°C for 120 min and was exposed to X-ARP 5 film in cassette for 3 to 18 hr, depending on the level of radioactivity. The film was developed in a Cachine auto-developing machine and usually as long as 130 base pairs were readable for a single run. The sequence ladder could be read down to about the 4th nt from the 3' end of the primer.

2.12. PAGE Gel Analysis Of Mutants by *Hpa*II Restriction

To establish that there were no undesired modifications made during E1A CRII insertion mutation construction, PAGE with acrylamide concentration 6% (w/v) was used for nucleic acid analysis of multiply cut mutant plasmids. An appropriate amount of acrylamide and BIS were dissolved in TBE buffer and filtered through a 0.45 μm Nalgene filter. After adding ammonium persulfate to a final concentration of 0.1% (w/v) and TEMED, the gel mixture was poured into a vertical gel apparatus to be polymerized. HpaII is a restriction enzyme whose recognition site C\CGG appears frequently in pKH101 especially in its GC rich E1A region. This enzyme was chosen to cut insertion mutants and wild type plasmid pKH101, pKH105 and run on PAGE gel. The gel was run at 250 V after 1 hr polymerization. After electrophoresis, the gel was EtBr stained and photographed under UV.

2.13. Transfection of Mammalian cells

Hela cells used in transfection assays were passaged twice within 4 days before carrying out the assay. Transfection was usually carried out on cells at 70 to 80% confluency in 100mm petri dishes.

Calcium phosphate transfection was employed to assay the trans-regulatory functions of E1A insertion mutants employing a colorimetrically measured enzymatic activity

system based on β-galactosidase activity.

The transfection cocktail contains carrier DNA, test plasmid reporter plasmid and TE buffer (pH 8.0) with the total volume of 1.8 ml. 500 μl of 1 M $CaCl^2$ was added to each 15 ml corning tube and the solution was collected from the tube bottom by centrifugation. This solution was added dropwise to another 15 ml Corning tube containing 2 ml of 2 X Hepes (8.0 g NaCl, 0.37 g KCl, 0.1 g NA_2HPO_4, 0.5 g Hepes and 1.0 g Glucose in 500 ml sterile redistilled water, pH7.1, autoclaved) as air bubbles were introduced (Wigler et al 1979). This 4 ml mixture was incubated at room temperature for 30 min then 1 ml of this transfection cocktail was added to each dish of actively growing cells at the desired confluency. These dishes then were returned to 37°C incubator. After 5 hr incubation, the medium was removed and 2 ml of complete medium with 10% glycerol was added to each dish. After 1 min, 8 ml fresh medium was added and all medium was removed after 40 sec. 10 ml of complete medium was added to each dish for a final 43-hr incubation.

After removing the medium from transfected cells, PBS^{--} was used to wash the cells and the cells were then collected with a rubber scraper in 1 ml PBS^{--} to a 1.5 ml Eppendorf tube. Cells were pelleted and resuspended in 200 μl of FT buffer (10 mM Tris pH 7.4, 10 mM Na_2EDTA, 25 mM sucrose) and submitted 4 circles of freezing and thawing (liquid

nitrogen (0.5 min) and 37°C water bath (5 min)) to burst the cells. Cellular debris were pelleted at 4°C and discarded, and the supernatant was transferred to a sterile tube. The solution was kept at -70°C until needed or was immediately tested for enzyme activity.

2.13.1. Determination Of Protein Concentration

Protein concentrations of transfected cell extracts were determined using a Bio-Rad protein assay. Well mixed 20 μl of cell lysate and 1 ml of BioRad solution were set at room temperature for 5 min, then OD was read at 595 nm on a Beckman DU-7 Spectrophotometer. The protein concentration of each tube was derived by comparing OD 595 of each reaction to the standard curve produced by bovine plasma gamma globulin of known concentration.

2.13.2. β-galactosidase Assay

The level of expression of β-galactosidase activity under the control of a transactivation sensitive promoter was determined based on the hydrolysis of colourless ONPG (o-nitrophenyl-β-D-galactopyranoside) to O-nitrophenolate with a yellow colour. 50 μl of the cell extract was added to a mixture of 150 μl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4,10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercapto-ETOH, pH7.0) and 40 μl of ONPG (Sigma, 4mg per ml of 60 mM Na_2HPO_4 and 40 mM NaH_2PO_4). The

reaction was stopped by adding 0.1 ml of 1 M Na_2CO_3 following 30 to 60 min incubation at 37°C. The absorbance of the reaction at 420 nm was determined on a Beckman DU-7 Spectrophotometer and the enzyme activity was calculated using the following formula:

$$\text{Sp.Activity (units/mg)} = \frac{\text{AB.420 / 0.0045}}{\text{incubation time(min) X mg of protein}}$$

2.14. Transformation Of BRK Cells (Primary Baby Rat Kidney Cells)

One week old Hooded-Lister baby rats (originally from the Chester Beattie Institute) were obtained from the Central Animal Facility, McMaster University. The rats were killed by cervical dislocation, the kidneys were carefully removed sterilely and placed in PBS^{--}. Excess membranes and blood vessels were removed and kidneys were washed with PBS^{--} twice.The kidneys in 0.5 ml PBS^{--} then were minced for approximately 10 min, 30 ml 2 X trypsin-EDTA was added, and the mixture was stirred at room temperature for 20 min. The supernatant was pipetted off into a bottle with 10 ml of FCS to stop the trypsin digestion at room temperature. Addition of 2 X trypsin-EDTA to the remaining kidney mixture continued the extraction, and this procedure was repeated twice. The kidney cells were then collected by centrifugation at 3000 rpm for 5 min in 50 ml Corning centrifuge tubes. Cells were

resuspended in 40 ml of α-MEM supplemented with 10% FCS and incubated at 37°C for 15 min after removing the supernatant. The cells were then filtered through two layers of sterilized cheese cloth and brought to the desired volume with α-MEM supplemented with 10% FCS. The cells were seeded into 60mm petri dishes and refed using fresh medium the following morning. Usually one could obtain 6 to 10 dishes of 70% confluent cells next morning from each pair of kidneys.

A modified calcium phosphate transfection technique (Graham and van der Eb 1973) was employed for transformation. 30 μg of Hela carrier DNA, which served as carrier for the transfection, was added to a 15 ml Corning polystyrene tube containing the desired amount of plasmid DNA to be transfected and the volume was brought to 0.9 ml using sterile redistilled water before adding 100 μl of 2.5 M $CaCl_2$ to a final volume of 1 ml. It then took about 40 sec to add this cocktail dropwise to another 15 ml Corning polystyrene tube containing 1 ml of 2xHepes with continuously air bubbling (Wigler et al., 1979). This mixture was kept at room temperature for 30 min to precipitate DNA. To each 60 mm dish was added 0.5 ml of this mixture and uptake of DNA was allowed overnight at 37°C. The medium was changed to fresh α-MEM supplemented with 10% of FCS next morning and refed with the same medium on the third day. Subsequently cultures were shifted to Joklik's modified MEM, supplemented with 10% horse serum and refed every 3 days until

14 days post-transfection for the selection of transformed cell colonies. On the 14th day transformed cell colonies were identified visually and confirmed microscopically after fixing with Methanol:Acetic acid (3:1) for 30 min and staining with Giemsa (Fischer Scientific, diluted 1:20 with 0.1 X PBS).

2.15. Rescue Of E1A Mutants Into Virus

A recombinant plasmid pJM17 was employed (McGrory et al.,1989) to rescue E1A insertion mutants into intact virus by calcium phosphate transfection. The transfection cocktail contained 0.5 ml 2 X Hepes, 10 μg pJM17, 5 μg to 10 μg of the pXC plasmid which had the left 16 mu of Ad5 with an insertion at E1A. The mixture was added to a 15 ml polystyrene tube and 100 μl of 2.5 M $CaCl_2$ was added after the volume was brought up to 0.9 ml using sterile redistilled water. Then 0.5 ml of the transfection cocktail was added to a 60mm dish of 293 cells at 70% to 80% confluency after the mixture was allowed to sit in room temperature for 30 min and the dishes were incubated at 37°C for 5 hr. Finally, the dishes were overlaid with 0.5% agarose in F11 supplemented with 5% horse serum, Penicillin-streptomycin, fungizone, L-glutamine and yeast extract. They were left at room temperature for about 25 to 30 min to allow solidification of agarose then moved back to the 37°C incubator. Plaques were normally visible within 7 days.

Well isolated plaques were picked using a sterile pipet and suspended in 0.5 ml of PBS^{++} containing 10% glycerol. This virus suspension was stored at -70°C until required. To screen for the desired viral DNA, 250 μl of the viral solution was used to infect 293 cells at about 80% confluency. The viral DNA was harvested when infected cells exhibited complete cytopathic effect (CPE), usually within 3-4 days. The dishes were placed undisturbed in the hood for 20 min and the medium was transferred to a 4 dram glass vial containing 0.5 ml of 10% glycerol and stored at -70°C. Pronase-SDS (0.5 mg/ ml pronase in lysing buffer) was added to the remaining cells (0.5ml/dish) and the dish was incubated at 37°C for 5 hrs. At this time, the lysate was collected by a rubber scraper and transfered to a 1.5 ml Eppendorf tube and extracted once with phenol. The aqueous phase was precipitated twice with ice cold 95% ethanol and dried at 37°C for 15 min. The viral DNA was resuspended in 100 μl of 0.1 X SSC. Screening of recombinants involved restrict digestion of 10 μl of the viral DNA with HindIII and BamHI.

The media from the original viral liquid infection were used for virus plaque purification. Serially diluted virus preparations were used to infect 293 cells, plaques were isolated and the viral DNA was reanalysed. Isolates with the correct restriction pattern were freeze/thawed between -70°C and room temperature three times and served as a stock for

further studies.

2.16. Screening Of Temperature Sensitive E1A Mutant Virus

Plaque assays at different temperatures were used to determine whether there were any temperature sensitive mutants created due to conformational changes caused by insertion of oligopeptides in E1A protein.

Hela and 293 cells were used for the determination of virus PFU. They were passaged from 150mm petri dishes into 60mm dishes and grown in fresh medium over night usually resulting in 70% to 80% confluency for assay next morning. A 90% confluent 150 mm dish of Hela cells generally could be used to prepare 20 X 60 mm dishes and one 150 mm dish of 293 cell, 6 X 60 mm dishes.

Virus dilutions were prepared in PBS^{++} from 10^{-1} to 10^{-9}. Each viral dilution was added on duplicate 70% to 80% confluent dishes of cells (250 μl/dish). After 45 min adsorption, the dishes were overlaid as in rescuing of mutant virus mentioned previously. Plaques usually appeared at the 6th day and new plaques forming after the 12th day were considered secondary plaques. Plaque forming ability was calculated as the number of plaques per ml virus used for infection. PFUs of mutant viruses on 293 cells and Hela cells at different temperature were compared statistically.

2.17. Data Analysis

Computation were performed on a IBM compatible personal computer-the Zenith Data System. The analyses of DNA and protein structure were carried out using MicroGenie (Beckman) software. Data derived from various experiments were statistically treated according to Pipkin (1984) (1985). Most calculations were performed using QUATTRO spreadsheet software, and a pocket Texas calculator.

RESULTS

3.1 Construction of E1A CRII Insertion Mutants

The objective of this study was to make insertion mutations in the E1A CRII region in order to examine the relationship between various E1A functions and the primary structure of CRII. There were two restriction sites that could be used for these insertions: a *ClaI* site at nt 918 and a *DraIII* site at nt 953. A DNA structure map and protein sequence of Ad5 CRII region is shown in Figure 3.1.

Construction of the mutants employed a simple and efficient strategy: cassette insertion of a synthetic sequence containing the *LacZo* sequence to facilitate screening of colonies carrying a mutagenized plasmid. As described in the INTRODUCTION, this 39 nt synthetic oligonucleotide insert (the "lop mutator") (Figure 1.4) contained a *Lac* repressor binding region the operator ($lacZ_o$) which when present at high copy number, bound to and titrated out the *Lac* repressor (lac i) resulting in constitutive expression of *the lac* operon containing the β-galactosidase gene in the host cell. β-glactosidase expression was monitored by growing cells on agar plates containing X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). Hydrolysis of this substrate by β-

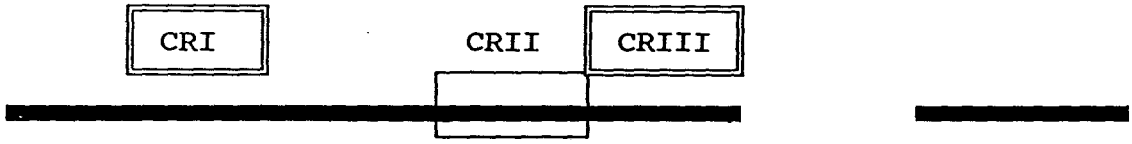
Figure 3.1 DNA Sequence Of Ad5 CRII Region

The local sequence of Ad5 E1A CRII region is shown in detail in this figure. At the top of the panel, genes of 13S and 12S products are shown by horizontal bold bars and the number of residues of each protein are shown in brackets. The spliced regions of different gene products are indicated by gaps between internal ends of each bar. The E1A CRII region from amino acid residue 108 to 140 is boxed on the 13S transcript and enlarged in the square below. The detailed sequence of the CRII region is given. The lower case letters starting from nt 975 are the sequences spliced from the 12S transcript. The two restriction sites, *Cla*I at nt 918 and *Dra*III site at nt 953 are underlined and the restriction point is shown by "\". Restriction recognition sites of endonuclease *Cla*I and *Dra*III are presented at the bottom of the figure.

Figure 3.1

DNA SEQUENCE OF AD5 CRII REGION

Gene of 13S product (289 amino acid residue)



Gene of 12S product (243 amino acid residue)

```
nt                                                        918
GGG CCG GTT TCT ATG CCA AAC CTT GTA CCG GAG GTG AT/C GAT
Gly Pro Val Ser Met Pro Asn Leu Val Pro Glu Val Ile Asp
aa                                                        120
                                              953
CTT ACC TGC CAC GAG GCT GGC TTT CCA CCC A\GT GAC GAC GAG
Leu Thr Cys His Glu Ala Gly Phe Pro Pro Ser  Asp Asp Glu
                                    130

          974
GAT GAA GAG Ggt  gaggagtttgtgttagattatgtggagcaccccgggca
Asp Glu Glu
```

ClaI site AT\CGAT

DraIII site CACNNN\GTG

galactosidase caused a change in colour from clear to blue. The colour indication provided a simple visual detection of successfully transformed colonies by E1A mutants bearing *Lac* operator sequence insertions.

As mentioned previously, this insertion cassette also had some other features designed for easy cloning. The cassette was constructed with either blunt ends or with 2 nt overhangs to fit whatever target DNA was selected. The fact that this oligonucleotide, whether it had blunt ends or sticky ends, was flanked by *Bam*HI restriction sites, offered the advantage of allowing formation of a smaller cassette insertion mutant at the same site by collapsing these sites via restrictive digestion with *Bam*HI and religation to form a 6 nt *Bam*HI linker insertion. Potentially, recutting the collapsed small insertion mutant also could create an opportunity to insert additional sequences having *Bam*HI restriction sites at their ends.

The oligonucleotides could be inserted in either of two orientations and the sequence was designed so that when read in one direction all reading frames were open, but, when read in the other orientation, all three closed reading frames were closed. Therefore, one could use this oligonucleotide to make three kinds of insertion at the same restriction site: two 39 nt insertions with ORF (open reading frame) or CRF (closed reading frame) and one small 6 nt insertion derived

from collapsing the *Bam*HI sites originally at the ends of the 39 nt cassette.

3.1.1 The Cassette for the Insertion Mutagenesis

Initially, mutagenesis was carried out using oligos obtained from D. Bautista(Bautista 1989). The insertion cassettes were assembled from five small oligomers two of which were lacZ core sequences, the others comprising flanking sequences (Figure 3.2). The formation of the insertion cassettes was performed by mixing and annealing different oligonucleotides in equimolar amounts. Generally, 39 nt insertion cassettes inserted during this strategy proved to have the expected structure (Bautista 1989). However, during the construction of one DraIII site insertion, a mutant, pKH950d, with a concatemeric lop mutator insertion at nt 950 was identified (see 3.1.4 for detailed strategy). The insert contained two almost complete lop sequences with inverted orientation connected by a *Bam*HI sequence (FIgure 3.3 and Figure 3.4). Two more *Bam*HI sites flanked the insert. There seemed to be one C missing on the r strand of the E1A sequence, 5' to the lop insertion cassette, according to the sequencing gel. pKH950d was the first nt 950 insertion mutant obtained and sequenced, after mutagenesis using a lop mutator composed of small oligos which annealed together to generate the full length 39 mer. Subsequently, we synthesized two

Figure 3.2 Cassette Oligonucleotides

The full length (39 bp) insertion mutator (1) was assembled from up to 6 oligonucleotides (Bautista 1989). Two of these (A and B) comprise the core that contains the *lacZo* sequence. The flanking sequences (NNNNNNN) are variable (C to J).

The cassette with blunt termini flanked by *Bam*HI sites was assembled using oligomers C, D, E and F and the cassette with 5' CG-overhangs flanked by *Bam*HI sites was derived from assembly of oligomers G, H, I and J.

Figure 3.2 Cassette Oligonucleotides

1: Full length lop mutator

5'-NNNNNNTGACCCAATTGTGAGCGGATAACAATTNNNNNN-3'
3'-NNNNNNACTGGGTTAACACTCGCCTATTGTTAANNNNNN-5'

2: Oligos to assemble core

(A) 5'-CCCAATTGTGAGCGGATA-3'
(B) 3'-ACACTCGCCTATTGTTAA-5'

3. Flanking oligos with *Bam*HI site and blunt ends

(C) 5'-GGATCCTGA-3' 5'-ACAATTGGATCC-3' (E)
(D) 3'-CCTAGGACTGGGTTA-5' 3'-CCTAGG-5' (F)

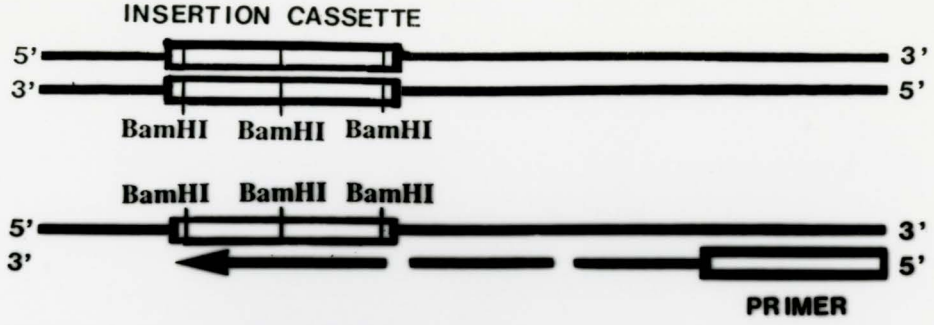
4. Flanking oligos with *Bam*HI site and 5'-CG overhang

(G) 5'-CGGATCCTGA-3' 5'-ACAATTGGATC-3' (I)
(H) 3'-CTAGGACTGGGTTA-5' 3'-CCTAGGC-5' (J)

modified from Bautista 1989

Figure 3.3 Sequencing Of A Double Cassette Concatemeric Insertion At Position Nt 950

A concatemer lop mutator insertion was identified by sequencing. This double cassette insertion has three *Bam*HI sites, two sites flanking the insert, and a third *Bam*HI site in the middle of the insert. The sequence contains two core sequences with one "-" at its 5' end followed by a "+" at its 3'end. The l strand sequence of the double cassette insertion and its flanking region are shown. The top lane of the figure is a cartoon showing a double cassette inserted and three *Bam*HI sites are indicated. The middle lane shows the direction of sequencing and the location of the primer. Note that there are three "?" at the top right of the sequence labelling. These are sites too close to be distinguished in this sequencing gel. Combining the data derived from other sequencing gels (data not shown), the sequence should be read from the first "?" as (5') GGTCAGG'ATCC'GTG. There appeared to be one G (in the l strand) missing from the insertion cassette and which would cause a frame shift.


INSERTION CASSETTE
5'
3'
3'
5'
BamHI BamHI BamHI
BamHI BamHI BamHI
5'
3'
3'
5'
PRIMER

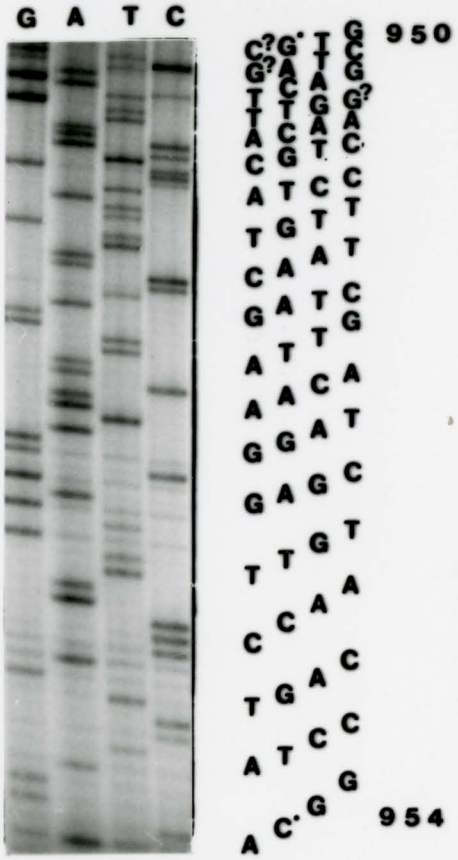
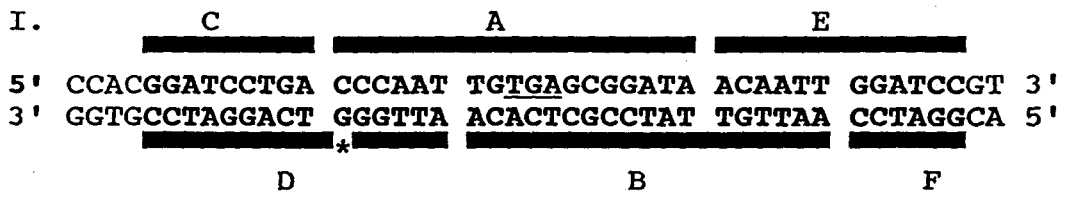

G A T C
950
954

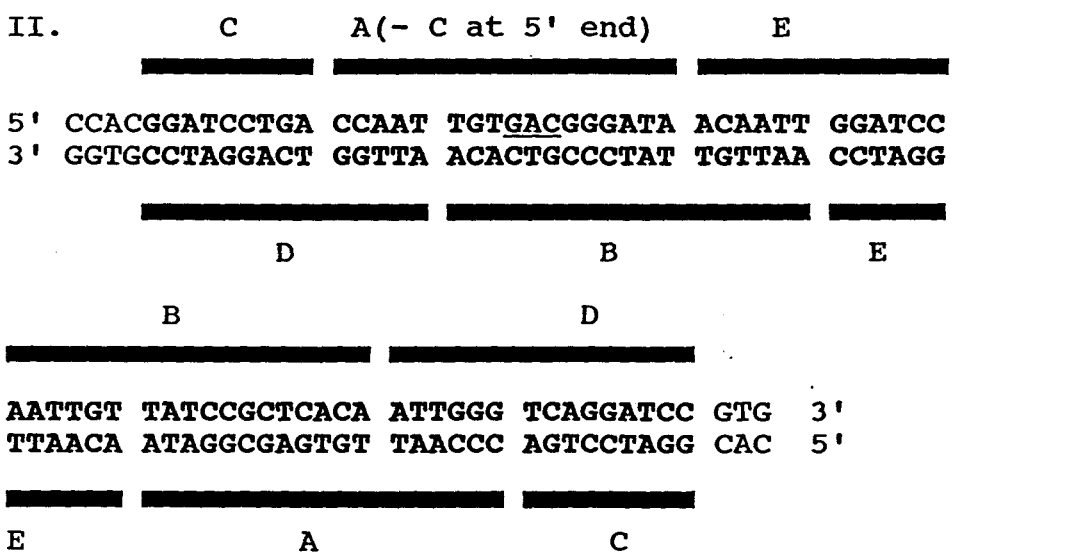
Figure 3.4 DNA structure of A Double Cassette Insertion At Nt 950

Sequence I is the structure of a normal "-" lop cassette insertion at nt 950. A stop codon TGA terminates the expression of the gene. The blunt end insertion cassette was assembled from several oligomers in the sequence of C A E. Sequence II is a double cassette
D B F.
insertion with two lop sequence, a "-" form followed by a "+" form connected by a common *Bam*HI site. There is no stop codon appeared as in sequence I resulted from a frame shift (see below). At the equivalent position of the stop codon in sequence I, a GAC codon is underlined in sequence II. The larger insertion cassette appears to have been formed by oligomers in the order of
C A E B D. The C/G bp labelled by * in sequence I
D B E A C.
is missing in the first lop sequence of sequence II. This results in a frame shift so that the first lop in sequence II is no longer a "-" form of the lop mutator. The 73 nt insertion results a frame shift in CRII after the insertion.

Figure 3.4 DNA Structure Of Double Cassette Insertion

(A) 5'-CCCAATTGTGAGCGGATA-3'
(B) 5'-AATTGTTATCCGCTCACA-3'
(C) 5'-GGATCCTGA-3'
(E) 5'-ACAATTGGATCC-3'
(D) 5'-ATTGGGTCAGGATCC-3'
(F) 5'-GGATCC-3'





complete, full length, oligonucleotides for insertional mutations at the *Dra*III site. However, the composite oligo designed for insertion into sites with 2 bp overhang was used for insertion mutagenesis at *the Cla*I site and gave satisfactory results (see below).

3.1.2. The Plasmid Used for the Construction of Insertion Mutants

Plasmid PKH101 (Bautista 1989, Figure 3.5) was employed to construct E1A insertion mutants at conserved region II. The parent vector of this plasmid was pUC which had a high copy number. The *Kpn*I H fragment of Ad5 from nt 22 to 2050 was introduced into pKH101 (pUC derived plasmid with the lac region including the Lac operator sequence deleted). This wt E1A plasmid when expressed in proper *LacZ+* host cells forms white colonies on X-gal agar plates which could be distinguished from those with plasmid containing a *lac-* labelled insert which could titrate out all lac repressor molecules in the *LacZ+* host.

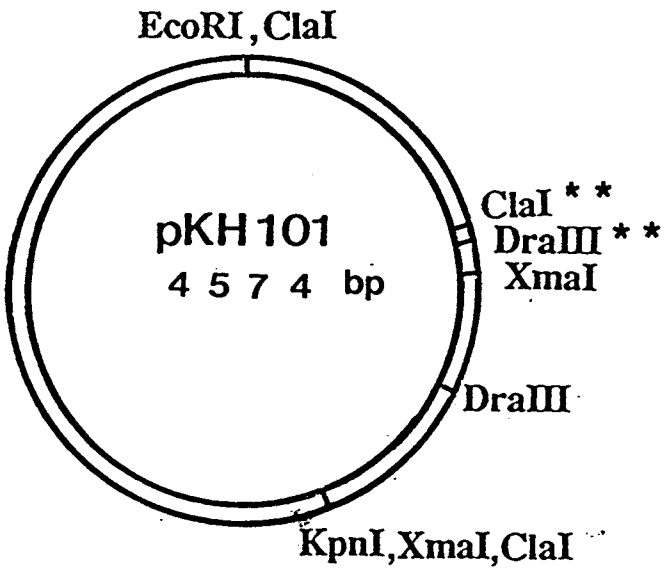
3.1.3. Construction of E1A Insertion Mutants at Nt 918

A *Cla*I restriction site at nt 918 in CRII of the Ad5 sequence in pKH101 was chosen for the construction of insertion mutants. Unfortunately, there were two additional *Cla*I sites in the plasmid pKH101 at nt -30 and nt 2060 just

Figure. 3.5 Map Of PKH101 And Its DNA Sequence

pKH101 (Bautista 1989) was used as the parental plasmid for the construction of E1A insertion mutants including the generation of CRII mutants in this study. The sequence of pKH101 in this figure is numbered starting from its *Eco*RI site according to its parental plasmid pBR322. The sequence from nt 1 to 41 is a pBR322 segment with *Hind*III and *Bam*HI sites deleted. The sequence following it from nt 42 to nt 78 inclusive is an unknown sequence introduced during molecular cloning of the left end of Ad5 (McKinnon PH.D. thesis 1982). The *Kpn*I H fragment of Ad5 sequence nt 22 to nt 2053 has been cloned in this plasmid (conversion of pKH101 nucleotide positions to Ad5 positions can be achieved by subtracting 57). The major restriction sites of pKH101 related to this study are labelled by "* *" in the graph. The complete sequence of the plasmid is given and the restriction sites mentioned in the drawing are underlined and labelled.

To simplify the discussion, the numbering of the insertion sites will use adenovirus sequence numbering as a standard everywhere else in this thesis other than the pKH101 numbering in this figure.


EcoRI, ClaI
pKH101
4 5 7 4 bp
ClaI * *
DraIII * *
XmaI
DraIII
KpnI, XmaI, ClaI

pKH101 4574

EcoRI ClaI

```
        10         20         30         40         50         60         70
GAATTCTCAT GTTTGACAGC TTATCATCGA TAAGCTGATC CGGGCCCCCA TTTCCCCTCC CTTCCAGCTC

        80         90        100        110        120        130        140
TCTGCCCCTT TTGGATTGAA GCCAATATGA TAATGAGGGG GTGGAGTTTG TGACGTGGCG CGGGGCGTGG

       150        160        170        180        190        200        210
GAACGGGGCG GGTGACGTAG TAGTGTGGCG GAAGTGTGAT GTTGCAAGTG TGGCGGAACA CATGTAAGCG

       220        230        240        250        260        270        280
ACGGATGTGG CAAAAGTGAC GTTTTTGGTG TGCGCCGGTG TACACAGGAA GTGACAATTT TCGCGCGGTT

       290        300        310        320        330        340        350
TTAGGCGGAT GTTGTAGTAA ATTTGGGCGT AACCGAGTAA GATTTGGCCA TTTTCGCGGG AAAACTGAAT

       360        370        380        390        400        410        420
AAGAGGAAGT GAAATCTGAA TAATTTTGTG TTACTCATAG CGCGTAATAT TTGTCTAGGG CCGCGGGGAC

       430        440        450        460        470        480        490
TTTGACCGTT TACGTGGAGA CTCGCCCAGG TGTTTTTCTC AGGTGTTTTC CGCGTTCCGG GTCAAAGTTG

       500        510        520        530        540        550        560
GCGTTTTATT ATTATAGTCA GCTGACGTGT AGTGTATTTA TACCCGGTGA GTTCCTCAAG AGGCCACTCT

       570        580        590        600        610        620        630
TGAGTGCCAG CGAGTAGAGT TTTCTCCTCC GAGCCGCTCC GACACCGGGA CTGAAAATGA GACATATTAT

       640        650        660        670        680        690        700
CTGCCACGGA GGTGTTATTA CCGAAGAAAT GGCCGCCAGT CTTTTGGACC AGCTGATCGA AGAGGTACTG

       710        720        730        740        750        760        770
GCTGATAATC TTCCACCTCC TAGCCATTTT GAACCACCTA CCCTTCACGA ACTGTATGAT TTAGACGTGA

       780        790        800        810        820        830        840
CGGCCCCCGA AGATCCCAAC GAGGAGGCGG TTTCGCAGAT TTTTCCCGAC TCTGTAATGT TGGCGGTGCA

       850        860        870        880        890        900        910
GGAAGGGATT GACTTACTCA CTTTTCCGCC GGCGCCCGGT TCTCCGGAGC CGCCTCACCT TTCCCGGCAG
                                                                    ClaI
       920        930        940        950        960        970        980
CCCGAGCAGC CGGAGCAGAG AGCCTTGGGT CCGGTTTCTA TGCCAAACCT TGTACCGGAG GTGATCGATC
                          DraIII
       990       1000       1010       1020       1030       1040       1050
TTACCTGCCA CGAGGCTGGC TTTCCACCCA GTGACGACGA GGATGAAGAG GGTGAGGAGT TTGTGTTAGA
              XmaI
      1060       1070       1080       1090       1100       1110       1120
TTATGTGGAG CACCCCGGGC ACGGTTGCAG GTCTTGTCAT TATCACCGGA GGAATACGGG GGACCCAGAT

      1130       1140       1150       1160       1170       1180       1190
ATTATGTGTT CGCTTTGCTA TATGAGGACC TGTGGCATGT TTGTCTACAG TAAGTGAAAA TTATGGGCAG

      1200       1210       1220       1230       1240       1250       1260
TGGGTGATAG AGTGGTGGGT TTGGTGTGGT AATTTTTTTT TTAATTTTTA CAGTTTTGTG GTTTAAAGAA

      1270       1280       1290       1300       1310       1320       1330
TTTTGTATTG TGATTTTTTT AAAAGGTCCT GTGTCTGAAC CTGAGCCTGA GCCCGAGCCA GAACCGGAGC

      1340       1350       1360       1370       1380       1390       1400
CTGCAAGACC TACCCGCCGT CCTAAAATGG CGCCTGCTAT CCTGAGACGC CCGACATCAC CTGTGTCTAG
                                                                    DraIII
      1410       1420       1430       1440       1450       1460       1470
AGAATGCAAT AGTAGTACGG ATAGCTGTGA CTCCGGTCCT TCTAACACAC CTCCTGAGAT ACACCCGGTG

      1480       1490       1500       1510       1520       1530       1540
GTCCCGCTGT GCCCCATTAA ACCAGTTGCC GTGAGAGTTG GTGGGCGTCG CCAGGCTGTG GAATGTATCG
```

RESULTS

```
      1550        1560        1570        1580        1590        1600        1610
AGGACTTGCT TAACGAGCCT GGGCAACCTT TGGACTTGAG CTGTAAACGC CCCAGGCCAT AAGGTGTAAA

      1620        1630        1640        1650        1660        1670        1680
CCTGTGATTG CGTGTGTGGT TAACGCCTTT GTTTGCTGAA TGAGTTGATG TAAGTTTAAT AAAGGGTGAG

      1690        1700        1710        1720        1730        1740        1750
ATAATGTTTA ACTTGCATGG CGTGTTAAAT GGGGCGGGGC TTAAAGGGTA TATAATGCGC CGTGGGCTAA

      1760        1770        1780        1790        1800        1810        1820
TCTTGGTTAC ATCTGACCTC ATGGAGGCTT GGGAGTGTTT GGAAGATTTT TCTGCTGTGC GTAACTTGCT

      1830        1840        1850        1860        1870        1880        1890
GGAACAGAGC TCTAACAGTA CCTCTTGGTT TTGGAGGTTT CTGTGGGGCT CATCCCAGGC AAAGTTAGTC

      1900        1910        1920        1930        1940        1950        1960
TGCAGAATTA AGGAGGATTA CAAGTGGGAA TTTGAAGAGC TTTTGAAATC CTGTGGTGAG CTGTTTGATT

      1970        1980        1990        2000        2010        2020        2030
CTTTGAATCT GGGTCACCAG GCGCTTTTCC AAGAGAAGGT CATCAAGACT TTGGATTTTT CCACACCGGG

      2040        2050        2060        2070        2080        2090        2100
GCGCGCTGCG GCTGCTGTTG CTTTTTTGAG TTTTATAAAG GATAAATGGA GCGAAGAAAC CCATCTGAGC
         XmaI     ClaI
      2110        2120        2130        2140        2150        2160        2170
GGGGGGTACC CGGGGATCGA TCCCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT
      KpnI

     2180        2190        2200        2210        2220        2230        2240
TGGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG AGCGGTATCA

      2250        2260        2270        2280        2290        2300        2310
GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA

      2320        2330        2340        2350        2360        2370        2380
AAGGCCAGCA AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC

      2390        2400        2410        2420        2430        2440        2450
TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA AAGATACCAG

      2460        2470        2480        2490        2500        2510        2520
GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC CGACCCTGCC GCTTACCGGA TACCTGTCCG

      2530        2540        2550        2560        2570        2580        2590
CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT

      2600        2610        2620        2630        2640        2650        2660
CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC


      2670        2680        2690        2700        2710        2720        2730
TATCGTCTTG AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA

      2740        2750        2760        2770        2780        2790        2800
GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT ACACTAGAAG

      2810        2820        2830        2840        2850        2860        2870
GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC

      2880        2890        2900        2910        2920        2930        2940
GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAAG

      2950        2960        2970        2980        2990        3000        3010
GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG

      3020        3030        3040        3050        3060        3070        3080
GATTTTGGTC ATGAGATTAT CAAAAAGGAT CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA
```

RESULTS

```
      3090        3100        3110        3120        3130        3140        3150
TCAATCTAAA GTATATATGA GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT

      3160        3170        3180        3190        3200        3210        3220
CAGCGATCTG TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA

      3230        3240        3250        3260        3270        3280        3290
GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA

      3300        3310        3320        3330        3340        3350        3360
GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT

      3370        3380        3390        3400        3410        3420        3430
CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT

      3440        3450        3460        3470        3480        3490        3500
TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA

      3510        3520        3530        3540        3550        3560        3570
AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA

      3580        3590        3600        3610        3620        3630        3640
GAAGTAAGTT GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC

      3650        3660        3670        3680        3690        3700        3710
ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA

      3720        3730        3740        3750        3760        3770        3780
CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA

      3790        3800        3810        3820        3830        3840        3850
TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA GTTCGATGTA

      3860        3870        3880        3890        3900        3910        3920
ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA

      3930        3940        3950        3960        3970        3980        3990
GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT

      4000        4010        4020        4030        4040        4050        4060
TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA

      4070        4080        4090        4100        4110        4120        4130
AAATAAACAA ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT

      4140        4150        4160        4170        4180        4190        4200
ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTCT CGCGCGTTTC GGTGATGACG

      4210        4220        4230        4240        4250        4260        4270
GTGAAAACCT CTGACACATG CAGCTCCCGG AGACGGTCAC AGCTTGTCTG TAAGCGGATG CCGGGAGCAG

      4280        4290        4300        4310        4320        4330        4340
ACAAGCCCGT CAGGGCGCGT CAGCGGGTGT TGGCGGGTGT CGGGGCTGGC TTAACTATGC GGCATCAGAG

      4350        4360        4370        4380        4390        4400        4410
CAGATTGTAC TGAGAGTGCA CCATATGCGG TGTGAAATAC CGCACAGATG CGTAAGGAGA AAATACCGCA

      4420        4430        4440        4450        4460        4470        4480
TCAGGCGCCA TTCGCCATTC AGGCTGCGCA ACTGTTGGGA AGGGCGATCG GTGCGGGCCT CTTCGCTATT

      4490        4500        4510        4520        4530        4540        4550
ACGCCAGCTG GCGAAAGGGG GATGTGCTGC AAGGCGATTA AGTTGGGTAA CGCCAGGGTT TTCCCAGTCA

      4560        4570
CGACGTTGTA AAACGACGGC CAGT
```

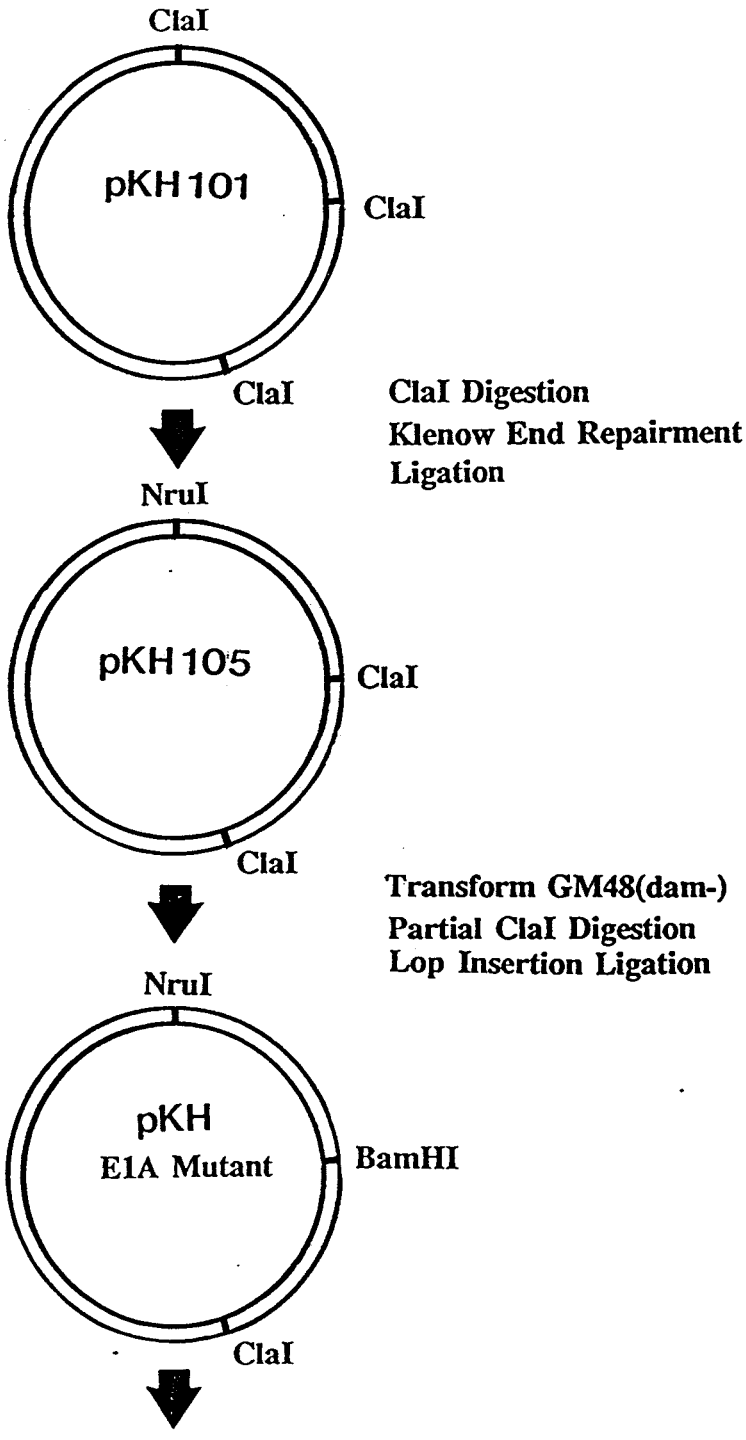
outside of the cloned adeno sequence. Thus the first step for the generation of nt 918 insertion mutants was to eliminate one of these two *Cla*I sites followed by partial *Cla*I digestion then lop-mutator insertion (Figure 3.6). The endonuclease *Cla*I recognizes the sequence AT/CGAT only when the adenosine residues are unmethylated. Dam+ host cell-derived pKH101 has two *Cla*I sites partly within the methylase recognition sequence GATC, one at nt 918 and the other at nt 2060, both of which are protected, by methylation, against *Cla*I endonuclease restriction. The third *Cla*I site (nt-30), which does not overlap with a dam methylase site, we wished to mutate such that it was no longer recognized by *Cla*I. Therefore to eliminate this *Cla*I site, *E.coli* bacterial strain LE392 (*dam+*) was transformed with pKH101. *Cla*I linearized DNA, with the sequence 5' AT CGAT 3' at the ends, was treated
TAGC TA
with the Klenow fragment of DNA polymerase to fill in the recessive 3' end and religated to generate a new sequence: 5' ATCGCGAT 3' (newly synthesized nts underlined) which contained a novel Nrul restriction site TCGCGA. This intermediate working plasmid was named pKH105. pKH105 was used for the construction of E1A CRII insertion mutants.

There were two possible ways to create insertion mutants at nt 918. The first possibility was to eliminate the *Cla*I site immediately following the E1A sequence using the

Figure 3.6 Construction of Insertion Mutants at Position nt 918

pKH101 was used for the construction of site nt 918 lop-mutator insertion. In the first step of mutant construction, a non-methylation protected *Cla*I site was eliminated by *Cla*I restriction (AT CGAT) and
TAGC TA
religation after Klenow end repairing. This results in the replacement of the *Cla*I site by a new NruI site ATCGCGAT (underlined). The resulting plasmid, pKH105, TAGCGCTA was used to transform *dam*⁻ *E.coli*. The plasmid was next partially digested with *Cla*I and mutated by lop-mutator insertion. The desired lop-mutator insertion was selected from blue clone candidates by verification of a novel *Bam*HI site generated after insertion of lop and generation of a single *Bam*HI band of expected size.

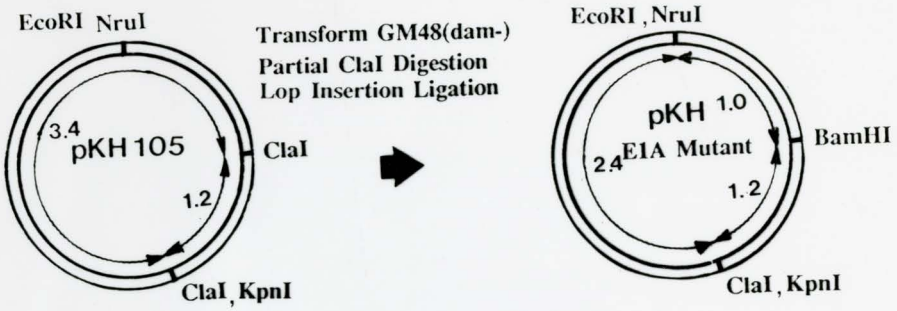
Construction of Full length Insertional Mutants at nt 918

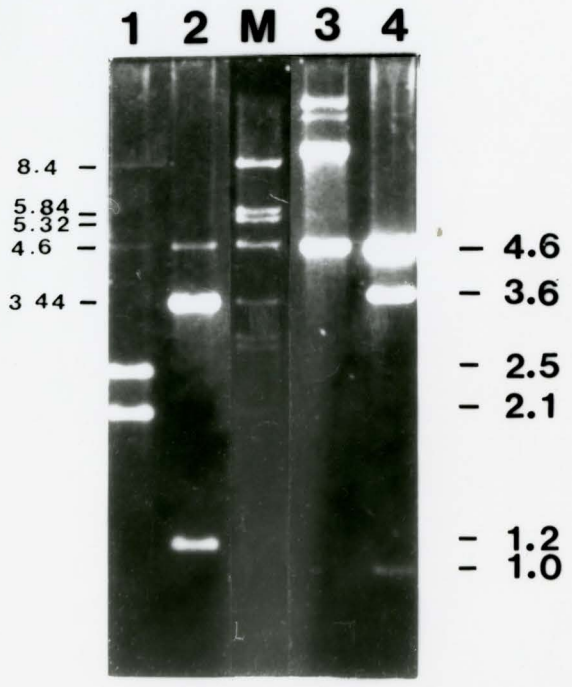


same strategy mentioned above. This would leave the *Cla*I site at 918 a unique *Cla*I site and readily available for lop mutator insertions. The second choice was to partially digest pKH105 cutting the plasmid only once at one or the other of the two remaining *Cla*I sites, then inserting the lop mutator. Both of these strategies required transforming the *dam-E.coli*.strain GM48 to generate methylation free *Cla*I sites. The latter method, partial digestion, was used to generate nt 918 insertion mutants since this approach would have been necessary in any case to eliminate the undesired *Cla*I site. A *Cla*I partial digestion of pKH105 prepared from dam- GM48 was carried out and the DNA was run on an agarose gel to purify full length DNA fragments. This linear DNA, containing a mixture of plasmid DNA cut at either 918 or 2060, was used as the target for the insertion by the *Lac*-labelled synthetic oligonucleotide. Screening of the recombinant transformants was accomplished by transforming *E.coli*. strain E5014 and visual screening of blue colonies as mentioned before. Restriction mapping was done to identify insertions at the desired site. The restriction patterns of *Bam*HI and *Eco*RI double digestion as well as *Kpn*I and *Eco*RI double digestion were diagnostic for the desired insertion mutants. Successful insertions were selected by confirmation of a newly created *Bam*HI site at the desired location by gel electrophoresis (Figure 3.7).

Figure 3.7 Screening Of E1A Mutant With Lop-mutator Inserted At Position Nt 918.

The screening of insertion mutants was based on visually identified blue colonies on X-gal gel (see text for details). Lane M is a marker lane using *Hind*III digested Ad5 DNA. The sizes of bands are shown at the left side of the gel. Lane 1 shows *Kpn*I and NruI double digested plasmid pKH105 DNA generating bands of expected sizes 2.1 plus 2.5 kbp. Lane 2 is *Cla*I digestion of dam- E.coli derived pKH105 showing the presence of two *Cla*I sites (with bands about 1.2 and 3.4 kbp). Lane 3 contains *Eco*RI plus *Bam*HI digested pKH105 DNA before lop insertion. The plasmid is only cut once showing a 4.6 kbp band since there was no *Bam*HI site in this plasmid. The upper bands in lane 3 are undigested excess DNA. Lane 4 is *Eco*RI and *Bam*HI digestion of pKH918- with two fragments of sizes about 1 kb and 3.6 kb showing expected *Bam*HI linker insertion. The extra band of 4.6 kbp is partially digested DNA resulting from excess DNA in digestion.


EcoRI NruI
3.4
pKH 105
1.2
ClaI
ClaI, KpnI
Transform GM48(dam-)
Partial ClaI Digestion
Lop Insertion Ligation
EcoRI, NruI
pKH 1.0
E1A Mutant
2.4
1.2
BamHI
ClaI, KpnI


1 2 M 3 4
8.4
5.84
5.32
4.6
3 44
4.6
3.6
2.5
2.1
1.2
1.0

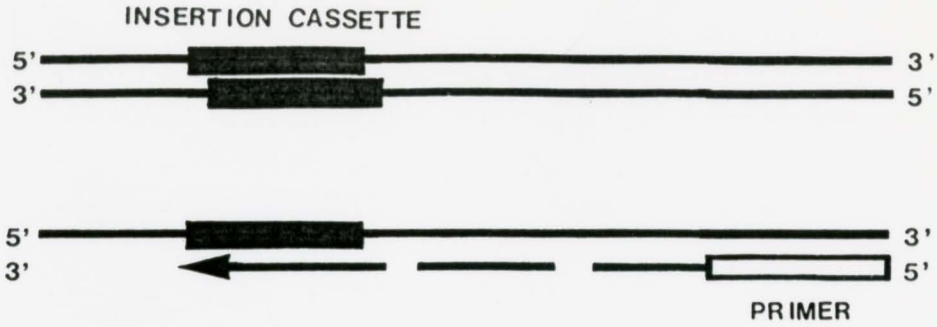
There was an equal probability of getting cassettes inserted in either orientation. According to the design of the inserted oligonucleotide, both open reading frame and closed reading frame insertion mutants would thereby be obtained. Upon confirmation of the orientation of the inserted oligonucleotide by sequencing (Figure 3.8 and Figure 3.9), the mutated plasmids were named pKH918+ (plus, with ORF) and pKH918- (minus, with CRF),respectively. Two smaller insertion mutants pKH918c and 918c' at this site were derived from collapsing each full size insertion with either "+" or "-" orientation (Figure 3.10). The collapsed mutants were sequenced and found to have the expected structure (Figure 3.11).

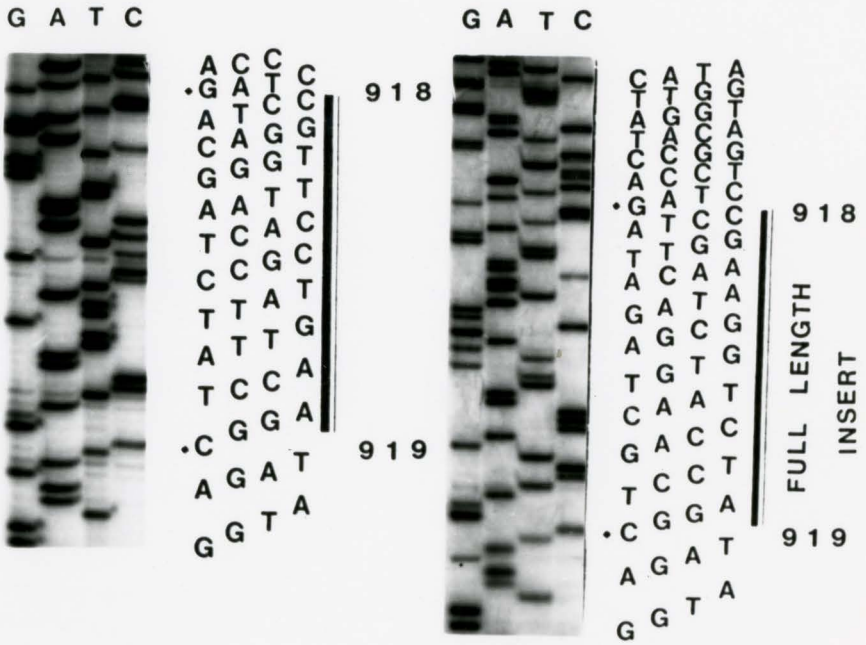
3.1.4. Construction of E1A Insertion Mutants at Nt 950

The second set of CRII mutants contained insertions at nt 950 (amino acid 130) of the E1A fragment which was introduced into pKH105. There were two *Dra*III sites in this plasmid, one cutting at nt 953 and the other at nt 1409. The strategy for construction of the insertion at site 950 was straightforward (Figure 3.12). A partial *Dra*III digestion was carried out to cut at a single *Dra*III site, then the linearized full length DNA fragment was blunt ended using Klenow fragment to eliminate nt 951 to 953 inclusive in the plasmid cut at nt 953. Blunt end full length insertion

Figure 3.8 Sequencing of a Lop Cassette Insertion at nt 918 to Determine the Insertion Orientation.

AB663 which had the sequence corresponding to Ad5 sequence 1 strand nt 1000 to nt 984 was used as a primer for the sequencing of CRII insertion mutants. During the generation of 918 insertion mutants, DNA derived from blue colonies was sequenced to determine the insert orientation. All "blue clone" DNA sequenced contained full length cassette insertions, most of them with the open reading frame orientation. Figure 3.8 A is a cartoon for the model of cassette insertion and Figure 3.8 B shows the direction of sequencing. The sequences of two mutant candidates with different orientations at position 918 are shown at the bottom with "-" orientation at left (C) and "+" orientation at right (D) as labelled. All mutants had the expected structure with no modification of flanking sequences.


INSERTION CASSETTE
5'
3'
3'
5'
5'
3'
3'
5'
PRIMER


G A T C
AGACGATCTATCAG
CATAGACCTTCGGG
CTCGGTAGATCGAT
CCGTTCCTGAATA
918
919
G A T C
CTATCAGATAGATCGTCAG
ATGACCATTCAGGAACGGGT
TGGCGCTCGATCTACCGAT
AGTAGTCCGAAGGTCTATA
FULL LENGTH INSERT
918
919

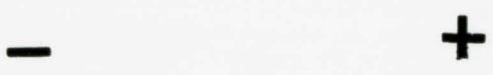

−
+

Figure 3.9 DNA Structure And Protein Amino Acid Sequence For Insertion Mutants At Position Nt 918.

1. Ad5 sequence from nt 914 to 961. The locations of *Cla*I and *Dra*III sites used for insertional mutation are shown.

2. Full size in frame Lop-mutator cassette insertion with ORF at nt 918. Note there are two *Bam*HI (GGATCC) sites flanking the insertion cassette.

3. Full size in frame Lop-mutator cassette insertion with CRF at nt 918. A stop codon is introduced into the coding sequence by the insertion cassette.

4.& 5. Small insertions derived by collapsing 918+ and 918-, respectively.

Figure 3.9

1. AD5 sequence from nt 914 to nt 964

```
     ClaI                                                  DraIII
   918|              930           940           950        |      960
GTG AT|CGAT CTT ACC TGC CAC GAG GCT GGC TTT CCA CCC A|GTGAC GAC
 Val Ile Asp Leu Thr Cys His Glu Ala Gly Phe Pro Pro Ser Asp Asp
```

2. 918+ (39 nt insertion at nt 918 with ORF)

```
   918                                                      919
GTG ATC GGA TCC AAT TGT TAT CCG CTC ACA ATT GGG TCA GGA TCC GAT
Val Ile Gly Ser Asn Cys Tyr Pro Leu Thr Ile Gly Ser Gly Ser Asp
```

3. 918- (39 nt insertion at nt 918 with CRF)

```
   918                                                      919
GTG ATC GGA TCC TGA CCC AAT TGT GAG CGG ATA ACA ATT GGA TCC GAT
Val Ile Gly Ser End
```

4. 918c (6 nt insertion derived from collapsing 918+)

Collapsing 918+

```
   918                                                      919
GTG ATC GG|A TCC AAT TGT TAT CCG CTC ACA ATT GGG TCA GG|A TCC GAT
Val Ile Gl|                                            y Ser Asp
Leu     BamHI                                       BamHI
```

```
   918      919
GTG ATC GGA TCC GAT CTT
Val Ile Gly Ser Asp Leu
```

5. 918c' (6 nt insertion derived from collapsing 918-)

Collapsing 918-

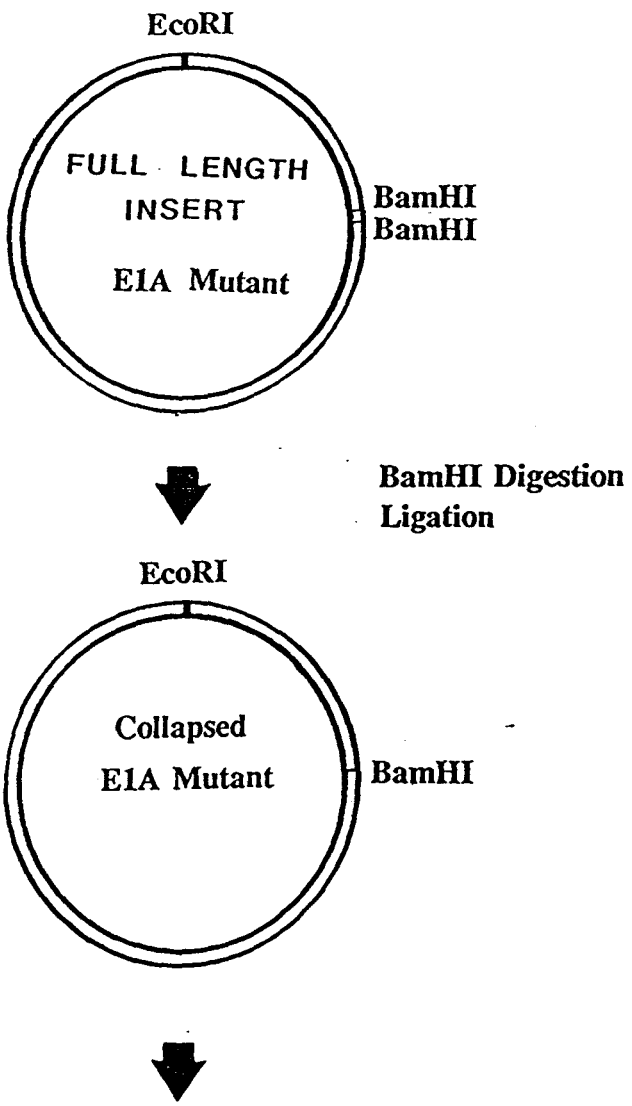
```
   918                                                      919
GTG ATC GG|A TCC TGA CCC AAT TGT GAG CGG ATA ACA ATT GG|A TCC GAT
Val Ile Gl|                                            y Ser Asp
Leu     BamHI                                       BamHI
```

```
   918      919
GTG ATC GGA TCC GAT CTT
Val Ile Gly Ser Asp Leu
```

Figure 3.10 Construction Of "Collapsed" Insertion Mutants

The lop mutator cassette used in the E1A insertional mutagenization has *Bam*HI sites flanking its ends. After getting full length insertions, one can easily "collapse" the 39 nt insertion by *Bam*HI restriction and religation, removing the core lacZ operator sequence. The resulting plasmid DNA produces white colonies on X-gal$^+$ plates.

Construction of E1A Mutants of Collapsed Form



Transform E5014
Visual Screening of White Colonies On X-gal^{+} Plate
Sequencing

Figure 3.11 Sequencing Analysis of "Collapsed" Insertion Mutant.

The sequence analysis at bottom left shows the result of "collapsing" a full length insert at 918/919, and at bottom right a collapsed insert at 950/954.

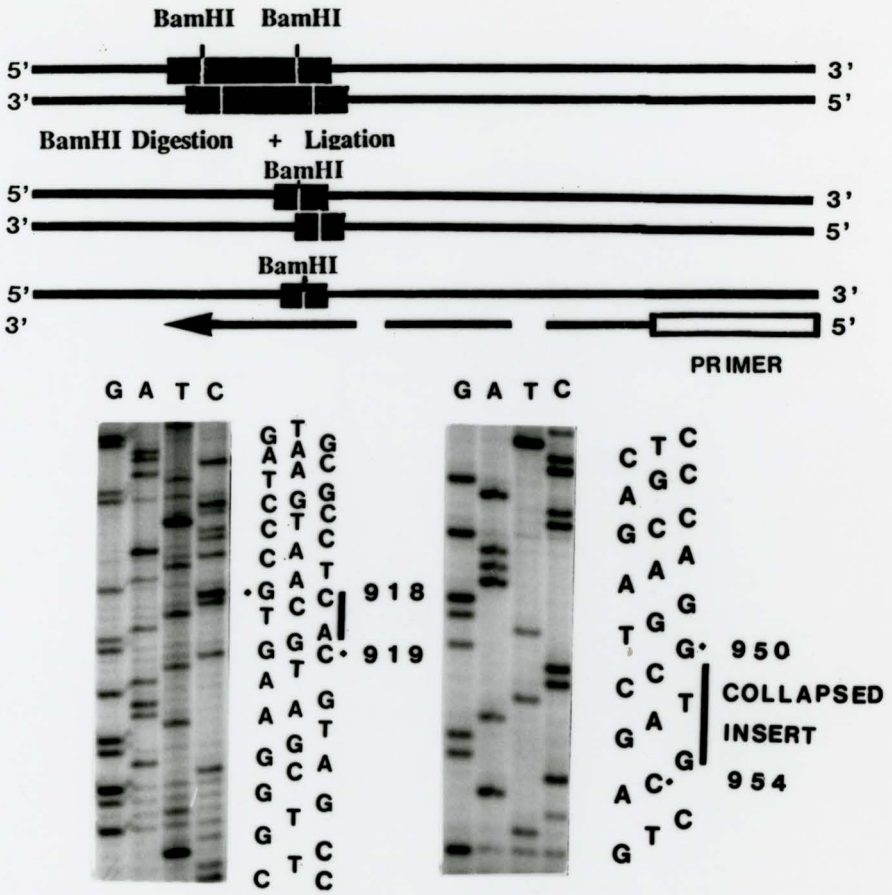

BamHI BamHI
5'
3'
3'
5'
BamHI Digestion + Ligation
BamHI
BamHI
PRIMER
G A T C
G A T C
918
919
950
COLLAPSED
INSERT
954

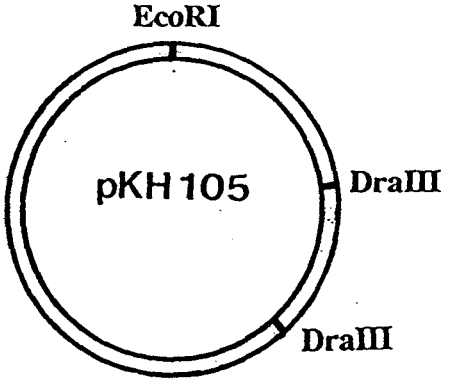
Figure 3.12 Construction of Insertion Mutants at Position nt 950

pKH105 was used for the construction of nt 950 lop-mutator insertion. Partial *Dra*III digestion of pKH105 was carried out to produce full sized linear plasmid DNA. The recognition site of *Dra*III is (5') CACNNN/GTG (3') in which N refers to any one of nucleotides G, A, T or C. There is a *Dra*III site CACCCAGTG in Ad5 sequence cut at nt953. When the plasmid was cut by *Dra*III, ends

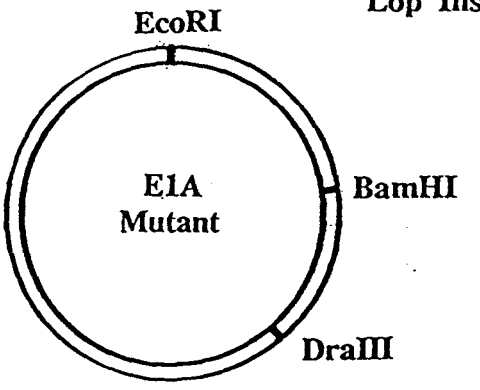
5' CACNNN GTG 3' were generated. Exonuclease
GTG NNNCAC

activity of Klenow fragment was used to "blunt" the ends. After the generation of the linker insertion by blunt end insertional ligation and transformation, blue colonies were isolated and screened for the new *Bam*HI site with right size *Bam*HI band and then sequenced to determine the insertion orientation.

Construction of Full length Insertional Mutants at nt 950



Partial DraIII Digestion
Klenow End Repairment
Lop Insertion Ligation



Transform E5014
Visual Screening of Blue Colonies On X-gal+ Plate
Sequencing

cassettes as described in Figure 1.4 were then ligated to this fragment. Insertion mutants were identified as before and the plasmid DNA was screened by double restriction digestion mapping to distinguish between insertions at nt 950 and at nt 1406. The restriction pattern of *Bam*HI plus *Eco*RI (Figure 3.13) indicated a successful insertion. Mutants with both orientations were identified by sequencing and named pKH950+ and pKH950- (Figure 3.14 and Figure 3.15). The collapsed forms were named pKH950c and pKH950c' according to the plasmid from which each was collapsed and both were sequenced and shown to have the expected structure (Figure 3.11).

3.1.5 PAGE Analysis of Mutant DNA Structure

To ensure that no unexpected rearrangements had occurred outside the insertion site, analysis of plasmid DNA structure was carried out. The restriction enzyme *Hpa*II was used for fine structure analysis of E1A mutant and wild type plasmids, since its short recognition sequence CCGG is present many times in pKH101. Digested DNA samples were run on a 6% polyacrylamide gel. From MicroGenie sequence analysis, all CRII mutant insertion sites should be located within a 100 bp fragment flanked by *Hpa*II sites 965 and 1065 of pKH101 (corresponding to adeno sequence 908 and 1008). Full length cassette insertions were expected to increase the size of this band to 139 bp. For example, in the PAGE analysis shown in

Figure 3.13 Screening of E1A Mutant Candidates With Lop-Mutator Inserted at Position nt 950

The screening of nt 950 insertions was performed by double digestion and restriction pattern comparison between mutant DNA and the DNA of pKH105. There are two *Dra*III sites in the plasmid pKH105. Lop-mutator insertion at position 950 replaces the *Dra*III site at 953 (951 to 953 inclusive was removed by Klenow treatment, see Figure 3.15) with a novel *Bam*HI site. Lane M is a marker lane using *Hind*III digested Ad5 DNA and sizes of bands are shown at the left of the gel. Lane 1 containing parental pKH105 plasmid DNA digested with *Eco*RI and *Dra*III before lop insertion shows bands of various sizes: 4.6 kbp, 3.6 kbp, 3.2 kbp, 1.4 kbp, 1 kbp and 0.4 kbp (not shown, this small fragment had run out of the gel). The 4.6 kbp band is linearized plasmid DNA. The 3.6 kbp band is the result of partial digestion by *Eco*RI and *Dra*III at site nt 953. DNA of lane 2 is a pKH105 plus a lop insertion at nt 950. The 3.6 kbp and 1 kbp band resulting from double digestion of EcoRI and BamHI show a expected *Bam*HI site insertion.

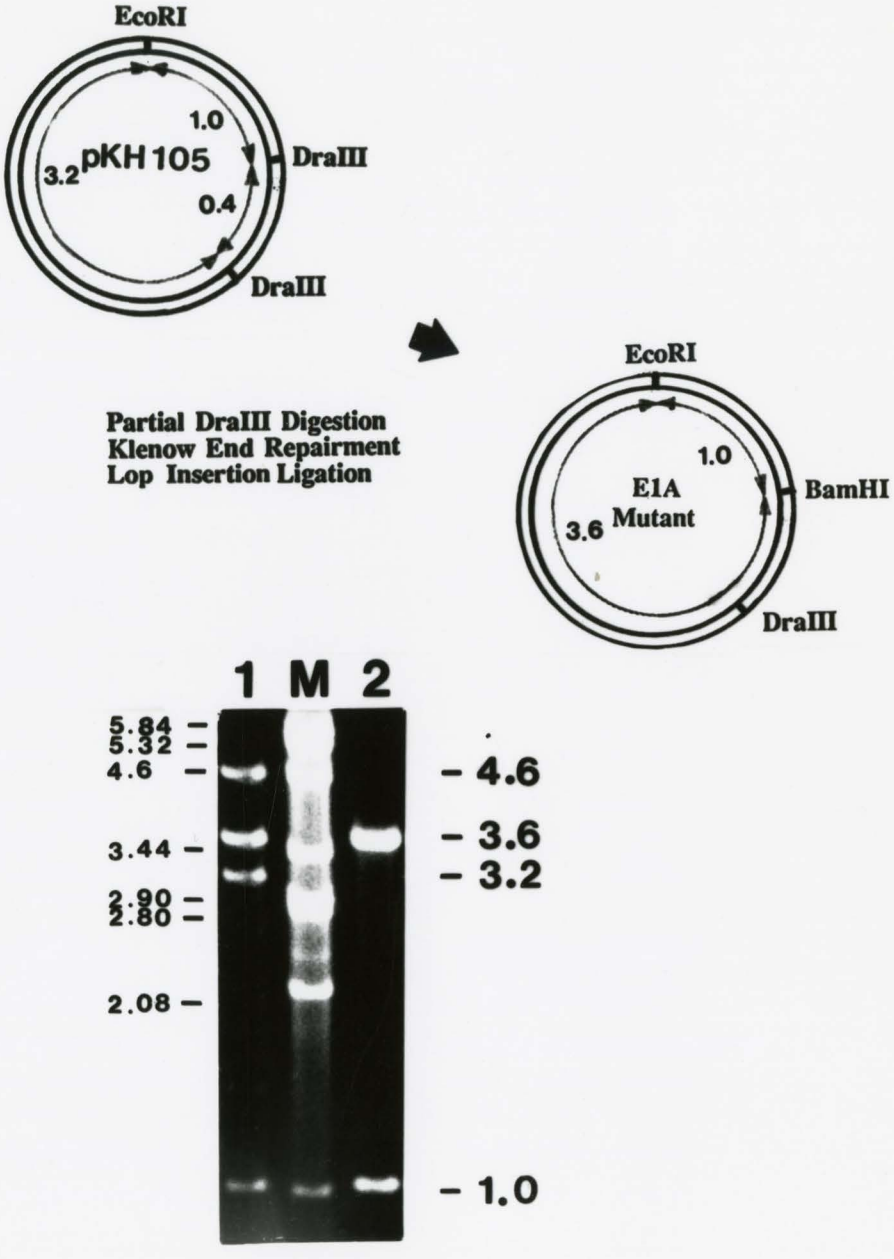

EcoRI
1.0
3.2
pKH 105
DraIII
0.4
DraIII
Partial DraIII Digestion
Klenow End Repairment
Lop Insertion Ligation
EcoRI
1.0
E1A
Mutant
3.6
BamHI
DraIII
1 M 2
5.84
5.32
4.6
3.44
2.90
2.80
2.08
- 4.6
- 3.6
- 3.2
- 1.0

Figure 3.14 Sequencing of Full Length Cassette Insertion at nt 950 to Determine the Insertion Orientation.

Insertion at site nt 950 was different from insertion at nt 918 in that the latter was a simple linker insertion whereas the former mutagenesis was a combination of two kinds of mutations: a 39 bp lop mutator insertion and a small deletion because of Klenow end repair after partial *Dra*III digestion. The first lane at the top of the figure is a cartoon for cassette insertion and the second lane shows the direction of the cassette sequencing. The figure shows the structure for a nt 950 insertion cassette in the "+" orientation (left panel) or "-" orientation (right panel).

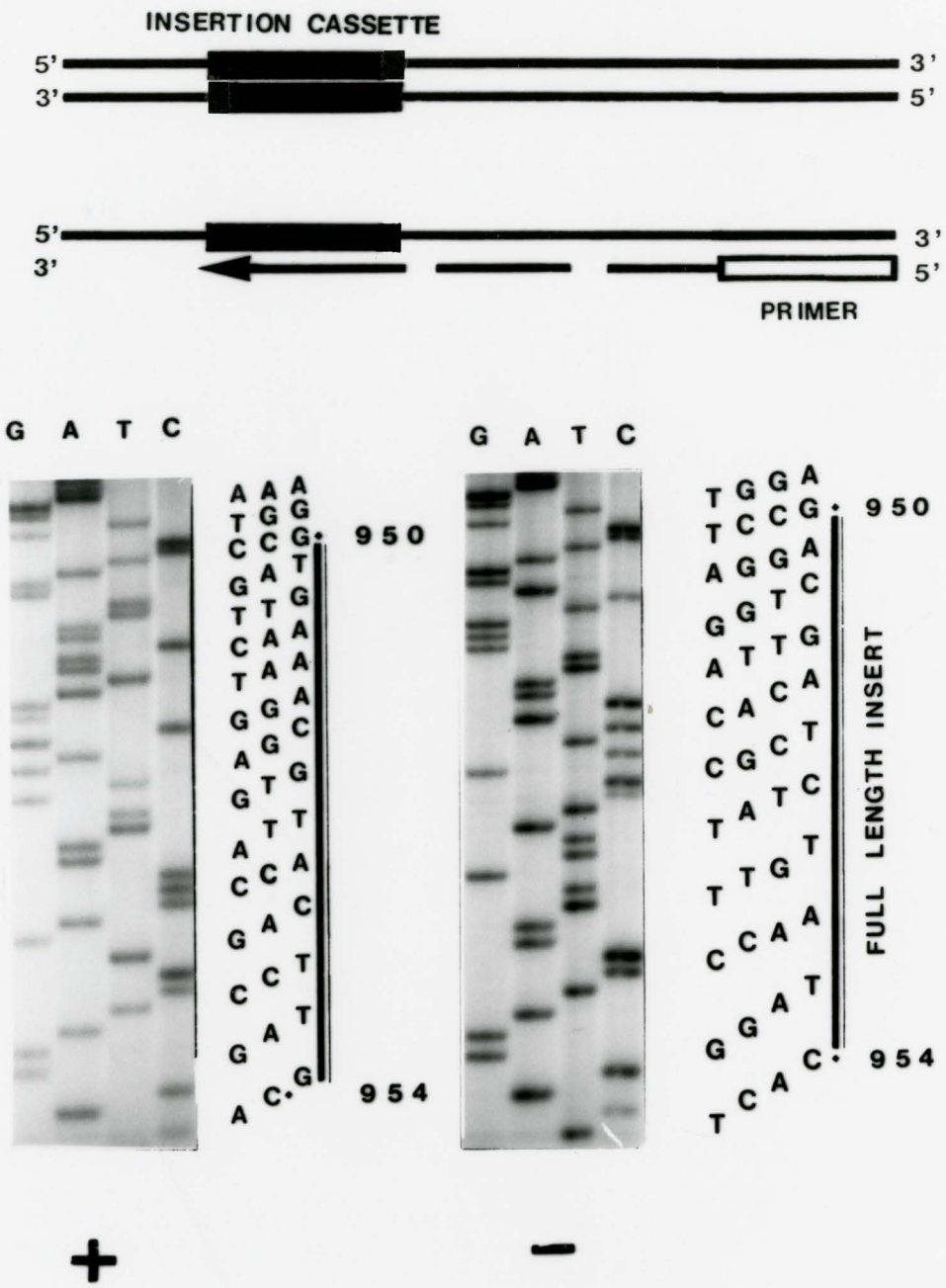

INSERTION CASSETTE
5'
3'
3'
5'
5'
3'
3'
5'
PRIMER
G A T C
950
954
G A T C
950
954
FULL LENGTH INSERT
+
-

Figure 3.15 DNA Structure And Protein Amino Acid Sequence For Insertion Mutants At Position Nt 950

1. Full size in frame lop-mutator cassette insertion with ORF at nt 950. Note nts 951 to 953 were removed by exonuclease activity of klenow fragment. The cassette insertion is immediately after nt 950.

2. Full size in frame Lop-mutator cassette insertion with CRF at nt 950. A stop codon was introduced into the coding sequence by the lop mutator.

3 & 4 Small insertions derived from collapsing 950+ and 950-, respectively.

5. Double cassette insertion with one "minus" orientation followed by a plus orientation (Discussed previously 3.1.1). Note in minus orientation one C is missing because of unknown reason, so in fact this mutant was a frame shift ORF insertion.

Figure 3.15

1. 950+ (39 nt insertion at nt 950 with ORF)

```
  950                                                          954
CCA CGG ATC CAA TTG TTA TCC GCT CAC AAT TGG GTC AGG ATC CGT GAC
Pro Arg Ile Gln Leu Leu Ser Ala His Asn Trp Val Arg Ile Arg Asp
```

2. 950- (39 nt insertion at nt 950 with CRF)

```
  950                                                          954
CCA CGG ATC CTG ACC CAA TTG TGA GCG GAT AAC AAT TGG ATC CGT GAC
Pro Arg Ile Leu Thr Gln Leu End
```

3. 950c (6 nt insertion collapsing from 950+)

Collapsing 950+

```
  950                                                          954
CCA CGG | ATC CAA TTG TTA TCC GCT CAC AAT TGG GTC AGG | ATC CGT GAC
Pro Arg |                                            | Ile Arg Asp
     BamHI                                          BamHI
```

```
  950      954
CCA CGG ATC CGT GAC
Pro Arg Ile Arg Asp
```

4. 950c' (6 nt insertion collapsing from 950-)

Collapsing 950-

```
  950                                                          954
CCA CGG | ATC CTG ACC CAA TTG TGA GCG GAT AAC AAT TGG | ATC CGT GAC
Pro Arg |                                            | Ile Arg Asp
     BamHI                                          BamHI
```

```
  950      954
CCA CGG ATC CGT GAC
Pro Arg Ile Arg Asp
```

5. 950d [71 nt (?) frame shift (?) insertion 950 with ORF]

```
   950
CCA CGG ATC CTG ACC AAT TGT GAC GGG ATA ACA ATT GGA TCC AAT
Pro Arg Ile Leu Thr Asn Cys Asp Gly Ile Thr Ile Gly Ser Asn

                                        954
TGT TAT CCG CTC ACA ATT GGG TCA GGA TCC GTG
Cys Tyr Pro Leu Thr Ile Gly Ser Gly Ser Val
```

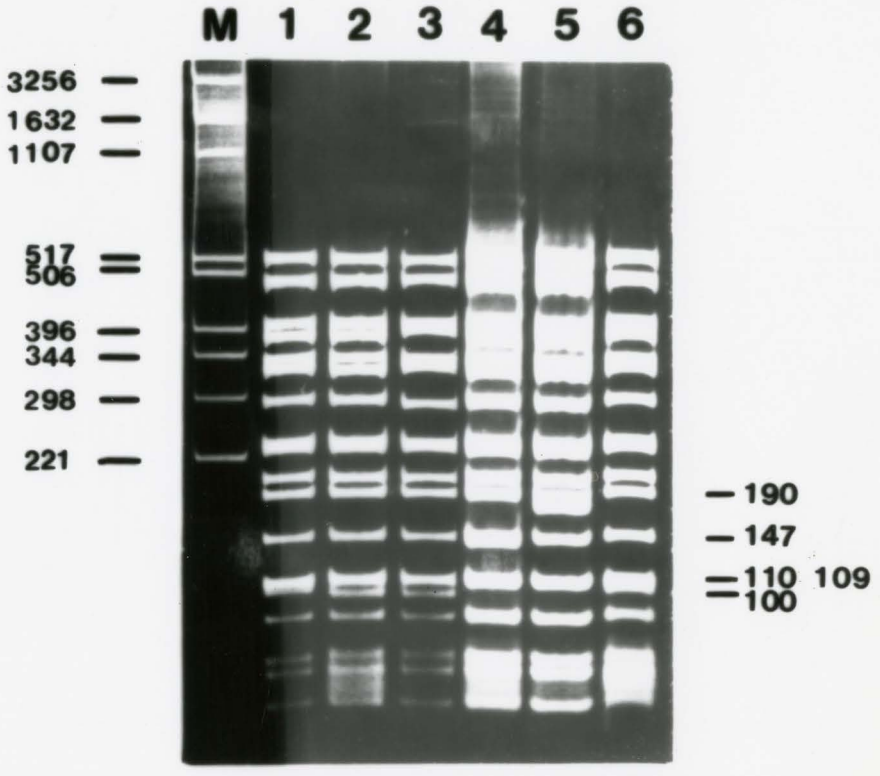
Figure 3.16, lane 4, a band of about 139 bp was present just below a band of 147 bp as predicted. However, a small 6 nt insertion only increased the band slightly to 106 bp (Figure 3.16 lane 1). In the case of the concatemer insertion at nt 950, the 100 bp band was shifted to about 171 bp (novel band just below 190 in lane 5). Except for pKH950d containing the concatemeric insertion, all mutants gave the expected PAGE pattern and no gross alterations had been made to the mutants.

3.2 The Study of Transactivation of the E3 Promoter by E1A Insertion Mutants

DNA transfection of Hela cells was used to assay transient expression of E1A transactivation function. CAT (chloramphenicol acetyltransferase) assays have been one of the most commonly used means to determine E1A trans-regulatory ability. In these assays, E1A regulatory ability was detected by its trans effects on an E1A responsive promoter of a co-transfected plasmid. The degree of CAT activity as a reporter signal for the trans regulatory ability of E1A (Weeks and Jones 1986) was dependent on the responsiveness of the promoter. Though modifications have been done to simplify and to improve the CAT assay, a more efficient and easier assay for the determination of transient trans-activation ability is still being sought. Recently, a colorimetric assay using β-galactosidase as a reporter gene

Figure 3.16 PAGE Gel Analysis Of Mutants Using Restriction Enzyme *Hpa*II

PAGE gel analysis was used to ensure that there were no unexpected modification has been made during the construction of insertion mutants. Lane M is a marker lane, a combination of digested fragments of HincII and HinfI restricted pBR322. Sizes of bands of the marker are shown at the left side of the gel. Lanes 1 to 6 are lanes pKH950c, pKH105, pKH101, pKH918+, pKH950d and pKH918c, respectively. All plasmids had predicted structures without unexpected modifications except the double cassette insertion mutant pKH950d discussed at the beginning of the results section. Both insertion sites nt918 and 950 are located within a 100 bp band. When a full length cassette was inserted as in the case of pKH918+ in lane 4, a novel 139 bp band appeared just below a band of 147 bp. A 6 nt insertion only shifted the 100 bp band slightly, bringing it some closer to the 109 and 110 bp band as shown in lane 1 and lane 6. The concatemeric insertion (lane 5, p950d) created a band of approximately 171 bp just below a band of 190 bp.


M 1 2 3 4 5 6
3256
1632
1107
517
506
396
344
298
221
190
147
110 109
100

driven by various E1A responsible promoters has been employed for assaying the regulatory functions of E1A insertion mutants (Bautista 1989). This was a simpler, more accurate transient expression assay system than the CAT assay, and also did not require use of radioisotopes. In the β-galactosidase transient expression assay, the E1A carrying plasmid was co-transfected with another plasmid having the β-galactosidase reporter gene under the control of an E1A-responsible promoter. The β-galactosidase activity, which was assumed to directly reflect the trans-regulatory capability of the co-transfected E1A plasmid, was measured by its ability to hydrolyse the colourless chemical compound ONPG (o-nitrophenyl -β-D galactoside) into the yellow-coloured o-nitrophenol. The optical density of the reaction was determined by spectrophotometry at 420 nm. The higher the β-galactosidase activity, the yellower the colour, and the greater the transactivation function.

The reporter plasmid pTEQ4 used in β-galactosidase assays was constructed by Bautista. In this plasmid, a polycloning site was immediately upstream of the Promoterless β-galactosidase gene followed by the SV40 polyadenylation signal at its 3' end. Depending on the E1A function to be examined, various E1A responsive promoters were inserted in the polycloning cassette at the 5' end of the β-galactosidase gene.

The β-galactosidase assay was used to test the transactivation function of CRII insertion mutants. The reporter plasmid co-transfected with the mutated E1A plasmid in these assays was pE3*LacZ*, which had an E3 promoter introduced into pTEQ4 at the 5'side of the β-galactosidase gene as a target for E1A transactivation.

Each 150 mm dish of subconfluent Hela cells was cotransfected with 100 ng of pKH mutant plasmid and 10 μg of reporter plasmid pE3*LacZ*. PKH101 with the wt E1A sequence was included in all experiments as a positive control. Transactivation ability was calculated as a mutant's ability to transactivate the expression of β-galactosidase activity relative to the same activity induced by the wt E1A plasmid, and is given as a percentage (Table 3.1). Table 3.2 shows the transactivation activity data and Figure 3.17 presents a histogram of relative activities. The results suggest that with exception of the two truncation mutants pKH918- and pKH950-, all insertion mutants, whether the insertion was as large as 39 nt or as small as 6 nt, had no significant effect on the transactivation ability of E1A. The very low transactivation activity of the two minus mutants on the E3 promoter was not unexpected. These two mutants carried stop codons inserted in CRII, deleting the unique region which is thought to be responsible for the transactivation function of E1A. These results generally agreed with the study of other

Table 3.1

Example of Calculation of Transactivation Activity[a]

plasmid	AB420[b]	protein[c] (mg)	Sp.Ac[d]	Aver activated activity		% of wt
pKH101(wt)	0.21	0.21	7.4	8.2	6.9	100
	0.26	0.22	8.7			
	0.20	0.15	9.6			
	0.26	0.27	7.2			
pKH918+	0.27	0.27	7.2	6.7	5.4	78
	0.26	0.28	7.0			
	0.20	0.32	4.6			
	0.24	0.23	7.9			
pE3lacZ	0.038	0.26	1.1	1.3	0	0
(control)	0.045	0.22	1.5			
	0.047	0.35	1.0			
	0.037	0.17	1.6			

a. Transactivation activity of plasmids was examined by a β-galactosidase assay system.

b. AB420 refers to the OD read at 420 nm as mentioned in 2.13.2.

c. Amount of protein in mg in each reaction tube measured in a BioRad protein assay as in 2.13.1.. The incubation time in Bio-Rad assay was 30 minutes.

d. The specific activity (Sp.Ac) was the β-galactosidase activity expressed under different conditions (with or without co-transfecting transactivating plasmid). The specific activity of each plasmid was calculated according to the formula in 2.13.2 and was calculated as the mean of four

dishes.

$$\text{Transactivation Activity} = \frac{\text{Mutant Activity} - \text{Background Activity}}{\text{Wt Activity} - \text{Background Activity}}$$

Activity mentioned here was transactivation activity

Background Activity = pE3lacZ activity (when it was transfected alone).

Table 3.2

Transactivation Assay Data[a]

Exp	percent of wt 1	2	3	4	Avg
pKH884-		-1.1	1.3		1 ± 1.2
pKH908+		77.3	89.7	104	90 ±11
pKH918+	76.1	75.8	98.9	78.0	82.2 ± 9.7
pKH918-	9.1	-5.5	5.6	-4.0	1.3 ± 6.2
pKH918c	77.5	79.5	91.1	90.0	84.5 ± 6.1
pKH918c'		69.4	84.1	75.1	76.2 ± 6.1
pKH950+	84.9	86.2	94.6	71.7	84.3 ± 8.2
pKH950-	8.9	-6.4	2.7	-0.1	-1.5 ± 3.7
pKH950c	75.5	75.8	99.2	58	77.0 ±14.8
pKH950c'		72.6	103.9	66	80.9 ±16.5
pKH1008+				3.2	3.2

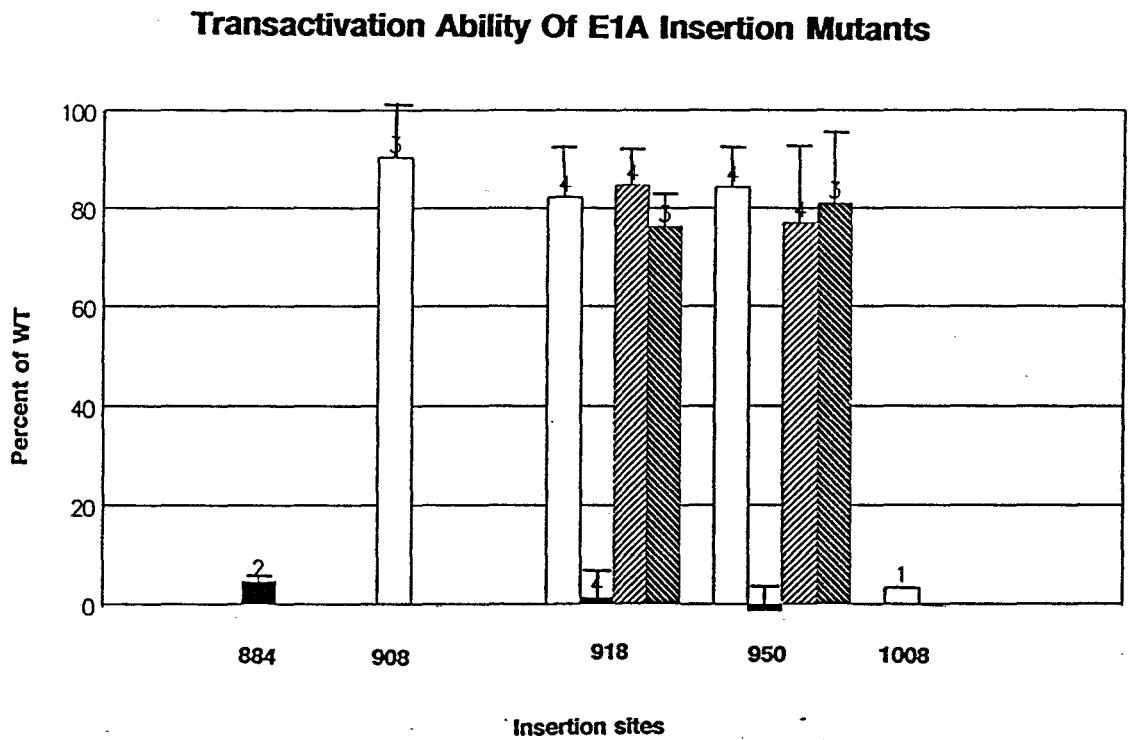
a. Transactivation activity of E1A CRII insertion mutants was tested using a β-galactosidase assay. Numbers given in the table are relative transactivation efficiencies of testing plasmid of Wild type plasmid pKH101. The standard deviations (±) are shown.

Figure 3.17 Trans-Activation Activity of E1A CRII Mutants

The histogram represents the transactivation activity of E1A CRII mutants on a co-transfected reporter plasmid pE3LacZ as percentage of wild type control pKH101 activity. Some E1A insertion mutants created earlier in this lab were included in different experiments for comparison.

Empty bar	39 nt lop-mutator insertion with ORF
Filled bar	39 nt lop-mutator insertion with CRF
// bar	6 nt insertion collapsed from 39 nt ORF
\\ bar	6 nt insertion collapsed from 39 nt CRF

Digits above CRII mutant bars are experiment numbers. Error bars indicate standard deviation from the mean.


Transactivation Ability Of E1A Insertion Mutants
Percent of WT
100
80
60
40
20
0
884
908
918
950
1008
Insertion sites

E1A insertion mutants done by Bautista in that all truncation insertion mutants upstream of the unique region significantly reduced the transactivation ability of E1A.

3.3 The Study of Repression of the E1A Enhancer by E1A Insertion Mutants

E1A not only functions as a universal activator but also as a repressor of enhancers of different viral and cellular genes. The 12S E1A product rather than 13S product is thought to be responsible for the E1A repression effect. Demonstration of E1A repression can only be obtained after eliminating the transactivation properties of the 13S product.

To eliminate activation by E1A, the CRII mutants used in repression assays were further mutated by inserting a terminator sequence in the unique region at nt 1007 to truncate the 13S protein (Figure 3.18 and Figure 3.19). Since this second insertion was in the 12S intron, presumably it would not impair the formation of 12S product. Most of these double insertion mutants would theoretically produce the 12S protein with a structural alteration at CRII and a nonfunctional truncated form of the normal 13S product. In the case of 12S CRF mutants there should be neither full-length 12S nor 13S E1A product produced.

This 13S truncating insertion cassette consisted of two single synthetic oligonucleotides, each of which was 15

Figure 3.18 A Terminator Insertion Cassette for the Construction of Mutants expressing only 12S products

A 15 nt termination insertion cassette was inserted at nt 1007 to create 12S mutants. This cassette introduces a stop codon and a novel *Hind*III site in the unique region when inserted in either orientation at nt 1007. The sequence of the terminator cassette with 5'CCGG overhangs is shown. The *Hind*III site is underlined (1). Wild type Ad5 local sequence with nt 1007 marked * and 1008, $, is shown (2). Terminator insertion at nt 1007, introduced a stop codon,in either orientation (3).

Figure 3.18

1. Annealed 13S terminator insertion cassette

HindIII

CCGGTTAAGCTTTAA
AATTCGAAATTGGCC

2. Ad5 DNA sequence from 998 to 1027 and its aa sequence

1007
*$
GTG GAG CAC CCC GGG CAC GGT TGC AGG TCT
Val Glu His Pro Gly His Gly Cys Arg Ser

3. 13S terminator generates stop codon in insertion of either orientation.

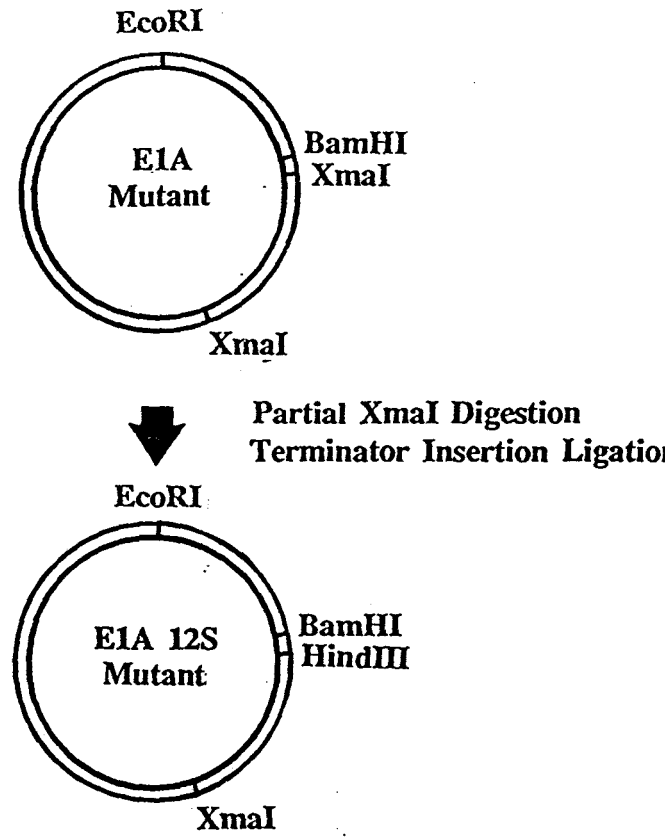
A.
```
                  *                        $
GTG GAG CAC CCC GGT TAA GCT TTA ACC GGG CAC
Val Glu His Pro Gly End
```

B.
```
                  *                        $
GTG GAG CAC CCC GGT TAA AGC TTA ACC GGG CAC
Val Glu His Pro Gly End
```

Figure 3.19 Construction of 12S Mutants.

All E1A mutants were converted to mutants producing only 12S product by inserting a terminator sequence at unique region to stop the expression of 13S transcript. Mutant plasmid, linearized by partial *Xma*I digestion was used as a target for the terminator insertion.

Construction of E1A 12S Mutant



nt long: (5') CCGGTTAAGCTTTAA (3') and CCGGTTAAAGCTTAA (Figure 3.18). This sequence also included a *Hind*III recognition sequence for rapid screening by *Hind*III restriction for the identification of recombinant plasmids. This terminator sequence carried the stop codon TAA when inserted in either orientation into the E1A sequence.

The terminator was introduced into the *Xma*I site at nt 1007 (Figure 3.19). The plasmid pKH105 contained two *Xma*I restriction sites, one at nt 1007 and another at 2111 just following the adeno E1A sequence in this plasmid. The desired terminator insertion eliminated the *Xma*I site at nt 1007 and left the site at 2111 intact. Recombinants were identified by digestion with *Hind*III, then confirmed by digestion with *Eco*RI plus *Xma*I (Figure 3.20). Several unique region terminator insertion mutants were sequenced to verify that they had the expected structure (Figure 3.21).

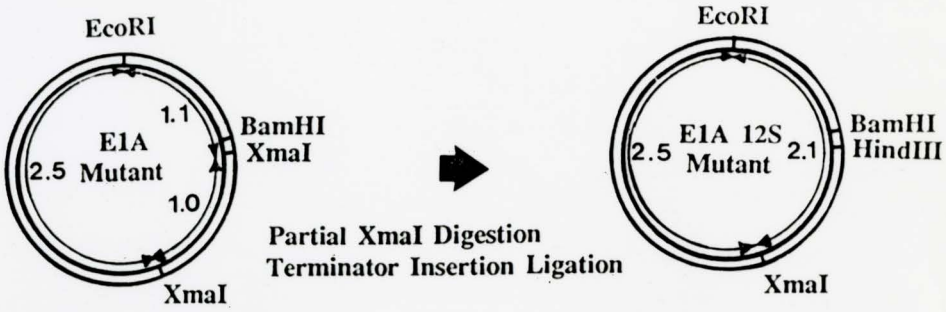
The reporter plasmid co-transfected with 12S E1A mutants in repression assays was pE1A*LacZ* whose enhancer was a repressible target of E1A product itself. To generate the reporter plasmid pE1A*LacZ*, the E1A enhancer region was introduced into the linker insertion cassette preceding the β-galactosidase gene of pTEQ4 (Bautista 1989).

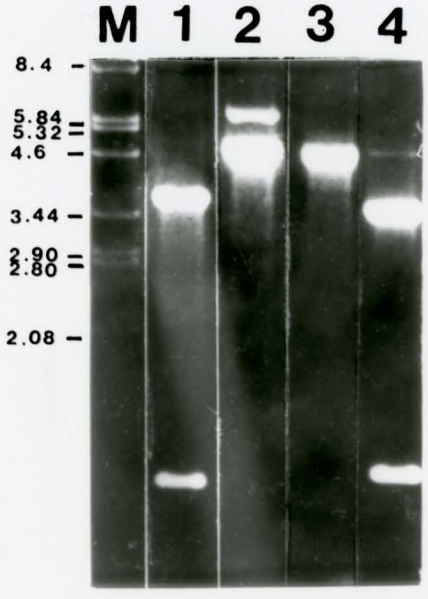
Each 150 mm dish of subconfluent Hela cells was cotransfected with 1 μg of 12S plasmid and 25 μg of reporter plasmid. The 12S only E1A plasmid pHP084, which contained a

Figure 3.20 Screening of 12S Mutants Candidates.

All 12S mutants were constructed by a terminator insertion within the unique region, at nt 1007 (see text for details). Successful terminator insertion was determined by detection of a newly introduced *Hind*III site at position nt 1007. Correct terminator insertion was verified by showing that there was only one *Xma*I site left and it was at nt 2111.

Lane M is *Hind*III digested Ad5 DNA as a marker. The sizes of the marker is shown at the left side of the gel. Lane 1 is pKH950 *Xma*I digestion showing two *Xma*I bands of about 1 kb and 3.6 kb. Lane 2 is p12S950+ digested by *Xma*I. The band of 4.6 kbp indicates the presence of a single *Xma*I site which was shown to be at 1008 by *Eco*RI and *Xma*I double digestion resulting in bands of 2.1 and 2.5 kbp (Lane 5) compared to 1.1 kb and 3.5 kbp bands generated by the same double digestion of undesired sited insertion at position nt 2117 (Lane 6). Lane 3 is *Hind*III digested 12S mutant DNA indicating a novel *Hind*III site. Lane 4 is double digestion of 12S918+ showing expected bands of 1.1 kb and 3.5 kb produced by *Eco*RI and the novel *Hind*III site at nt 1008.


EcoRI
1.1
BamHI
XmaI
E1A
2.5 Mutant
1.0
XmaI
Partial XmaI Digestion
Terminator Insertion Ligation
EcoRI
E1A 12S
2.5 Mutant
2.1
BamHI
HindIII
XmaI


M 1 2 3 4
8.4
5.84
5.32
4.6
3.44
2.90
2.80
2.08

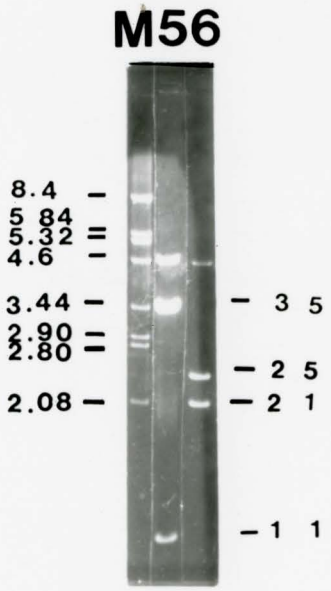
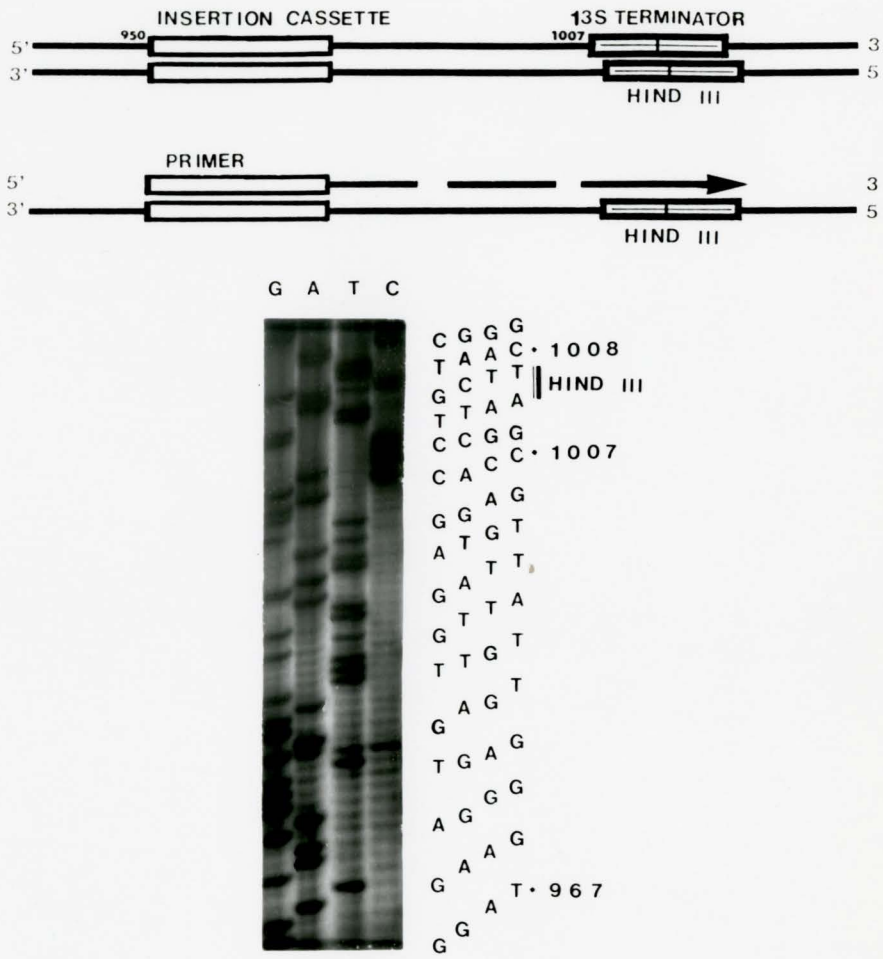

M56
8.4
5.84
5.32
4.6
3.44
2.90
2.80
2.08
3 5
2 5
2 1
1 1

Figure 3.21 Sequencing of a Terminator Insertion at Unique Region of 12S Mutant.

AB690, one of the two components used to build the full length lop mutator insertion (see Figure 1.4) was used as primer to sequence across the terminator insertion at nt 1007 of p12S950-. At the top of the figure is a cartoon showing relative location of 950 mutational insertion and 1007 terminator insertion. The second lane shows the terminator sequencing direction. The results showed that all the sequences were as predicted. The unique *Hind*III site designed within the terminator sequence is indicated.


INSERTION CASSETTE
13S TERMINATOR
950
1007
5'
3'
3
5
HIND III
PRIMER
5'
3'
3
5
HIND III
G A T C
C·1008
HIND III
C·1007
T·967

stop codon at nt 1008, was included in all repression assays as a wt control for maximum repression ability. The reporter plasmid pE1A*LacZ* alone was used as a control for repression-free β-galactosidase expression. 12S E1A insertion mutants which had insertion sites flanking CRII were included in each assay to control for any inhibition of repression which might have been introduced by cassette insertion irregardless of the site of insertion. The repression efficiency of the insertion mutants was determined by their ability to repress expression of the E1A promoter driven β-galactosidase gene relative to the amount of repression obtained in the presence of the wild type plasmid. An example of the calculation of repression activity is shown in Table 3.3.

The results of these repression assays are presented in Table 3.4 and in the bar diagram in Figure 3.22. These data showed that 12S CRII mutants with larger (39 nt) inserts, regardless of orientations, were all significantly reduced in the ability to repress E1A enhancers. The collapsed mutants with smaller insertions (6nt) all repressed β-galactosidase expression almost as efficiently as the wt plasmid did. The same results were obtained using mutants with insertions at nt 908 and nt 1267. Full size ORF insertions impaired repression, however, mutants with 2 aa insertions repressed efficiently.

Table 3.3
Example of Calculation of Repression Activity

Plasmid[a]	AB420[b]	protein[c] (mg)	Sp.Ac[d]	Aver	Repressed activity	% of wt
pHP084(wt)	0.03	0.24	0.46	0.5	2.2	100
	0.03	0.21	0.52			
	0.03	0.22	0.50			
	0.03	0.24	0.46			
pE1AlacZ	0.20	0.31	2.38	2.7	0	0
(control)	0.15	0.28	1.98			
	0.17	0.17	3.63			
	0.19	0.25	2.71			
p12S950+	0.13	0.21	2.22	2.2	0.5	22
	0.13	0.23	2.04			
	0.12	0.21	2.09			
	0.16	0.23	2.56			

a. Repression activity of plasmids was examined by a β-galactosidase assay system.

b. AB420 refered to the OD read at 420 nm as mentioned in 2.13.2.

c. The column of protein refered to the amount of protein in mg in each reaction tube derived in BioRad protein assay as in 2.13.1. The incubation time in Bio-Rad assay was 60 minutes.

d. The specific activity (Sp.Ac) was the β-galactosidase activity expressed under different conditions (with or without co-transfecting repressing plasmid). The specific activity of each plasmid was calculated according to the formula in 2.13.2

and took a mean of the value of four dishes.

$$\text{Repression Activity} = \frac{\text{Background Activity - Mutant Activity}}{\text{Background Activity - Wt Activity}}$$

Activity mentioned here was repression activity

Background Activity = Repression Activity of pE1AlacZ (when it was transfected alone)

Table 3.4

Trans-Repression Assay Data[a]

Exp	percent of wt					Avg
	1	2	3	4	5	
p12S908+	48.5	43.0		26.0		39.1 ± 9.6
p12S908-	32.5					32.5
p12S908c		68.3		69.3		68.8 ± 0.5
p12S918+	27.8	29.7	37.1	43.1	34.6	34.4 ± 5.4
p12S918-	6.4	21.4	24.3	46.4	21.3	24.0 ±12.8
p12S918c	77.9	83.2	88.7	95.4	85.7	86.2 ± 5.7
p12S918c'			99.0		87.9	93.4 ± 5.5
p12S950+	59.4	26.2	20.5	-1.5	13.9	12.0 ±10.9
p12S950-			28.3		22.0	25.1 ± 3.1
p12S950c	53.7	80.5	95.5	87.2	90.3	82.5 ±12.8
p12S950c'			89.9		72.6	81.2 ± 8.6
p12S1267+			33.2		39.7	36.5 ± 3.3
p12S1267c			72.5		72.8	72.6 ± 0.1

a. Repression activity of E1A CRII insertion mutants was tested using a β-galactosidase assay. Numbers given in the table are relative transrepression efficiencies of testing plasmids as a percentage of Wild type plasmid pKH084 activity. Standard deviations are shown after ± sign.

Figure 3.22 Trans-Repression Activity of E1A CRII Mutants

The histogram represents the repression activity of E1A CRII mutants on E1A enhancer of a co-transfected reporter plasmid pE1AlacZ as a percentage of wild type control (pHP084) activity. Some E1A insertion mutants created earlier in this lab sited flanking CRII region were included in different experiments for comparison (Bautista 1989).

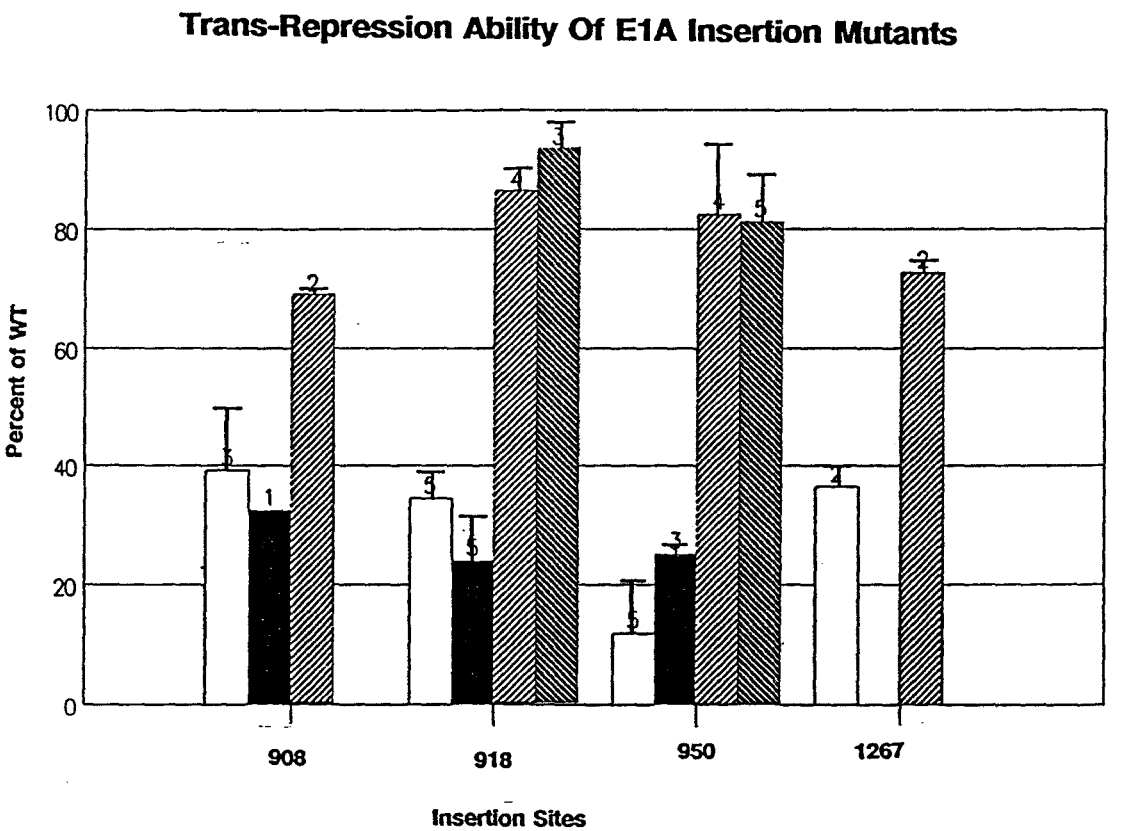
Open bar 39 nt lop-mutator insertion with ORF

Filled bar 39 nt lop-mutator insertion with CRF

// bar 6 nt insertion collapsed from 39 nt ORF

\\ bar 6 nt insertion collapsed from 39 CRF

Digits above CRII mutant bars are experiment numbers. Error bars indicate standard deviation from the mean.


Trans-Repression Ability Of E1A Insertion Mutants
Percent of WT
100
80
60
40
20
0
908
918
950
1267
Insertion Sites

3.4 Transformation of Baby Rat Kidney Cells by E1A Insertion Mutants with Wt E1B

In order to assay for complete transformation, E1A mutants were subcloned into a plasmid bearing the whole E1 region including both E1A and E1B. The plasmid pXC38, which contains the Ad5 XhoI-C fragment was chosen as a parent vector for the construction of new plasmids carrying a structural alteration in the E1A region as well as a normal E1B background (Figure 3.23). The subcloning strategy was straight forward: plasmid pXC 38 was digested with *Eco*RI and *Kpn*I and the 7.5 kb larger fragment of pXC38, with the E1A sequence deleted, was purified. Meanwhile the E1A mutant plasmid was cut using the same two enzymes and the 2.1 kb smaller DNA fragment containing mutated E1A region was also purified. These two fragments were then ligated. The sticky ends generated by *Eco*RI and *Kpn*I restriction share no common complementary sequence, so the pXC38 7.5kb and the mutagenized E1A 2.1kb fragment could religate with only one possible orientation. The successful insertion of the E1A mutation into pXC38 was indicated by the newly introduced unique *Bam*HI site, and was verified by *Eco*RI plus *Bam*HI double restriction (Figure 3.24). All CRII insertion mutants were successfully introduced into plasmids with a wt E1B background. They were named the pXC918 series and the pXC950 series, accordingly.

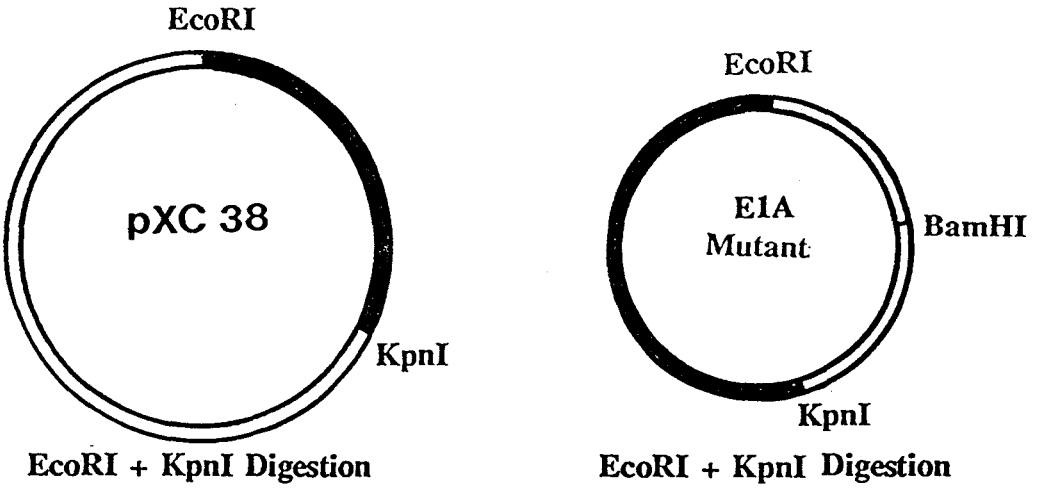
DNA mediated transformation of BRK (primary baby rat

Figure 3.23 Introduction Of E1A Mutants Into an E1 Background

E1A CRII mutants were subcloned into a plasmid pXC38 with E1 for BRK transformation assays.

E1A mutants and E1 plasmid pXC38 were digested individually with *Eco*RI and *Kpn*I and the smaller *Eco*RI-*Kpn*I fragment of the mutant (2.1 kb, open circle) containing mutagenized E1A and the larger *Eco*RI-*Kpn*I fragment of pXC38 (7.5 kb, open circle) having wild type E1A region deleted were fragment purified and ligated to replace the wt E1A region in pXC38 with a mutated E1A sequence.

Introduction of E1A Mutations into an E1 Background



Clone EcoRI-KpnI Fragment

Contains Mutated E1A region into pXC38

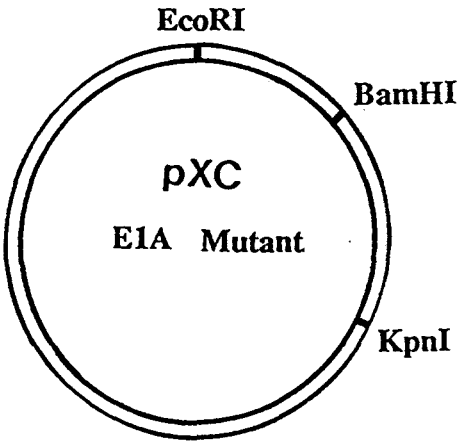
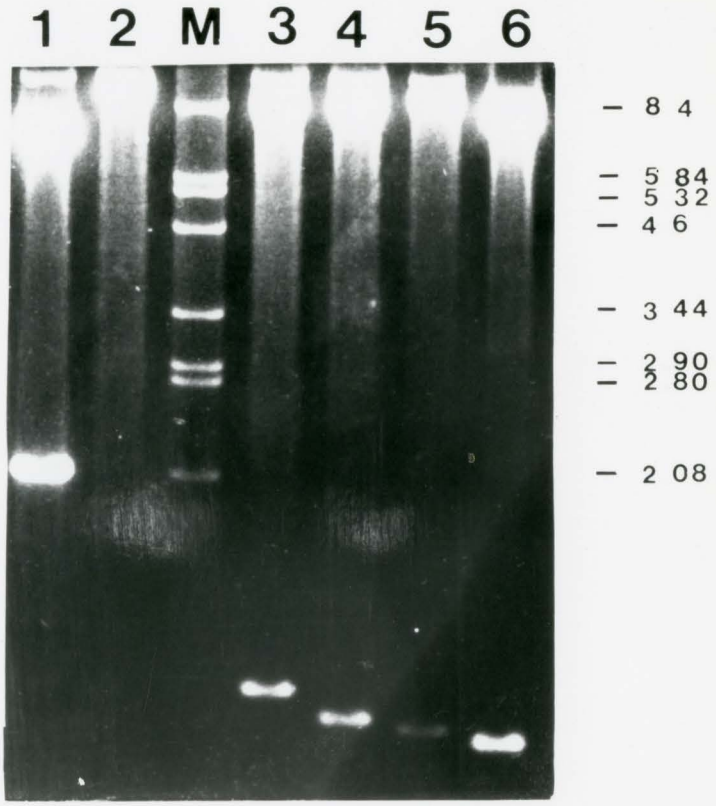


Figure 3.24 Examples of Restriction Profiles of E1A Mutants Placed In a pXC38 E1B Background.

Endonuclease double digestion was carried out to check the restriction pattern of E1 plasmids. Lane M is *Hind*III digested Ad5 DNA as a marker. Lane 1 contains wild type E1 plasmid pXC38 after *Eco*RI and *Kpn*I double digestion to generate bands of 2.1 kb and 7.6 kb. The top band in lane 1 is partially digested pXC38 DNA. Lane 2 contains *Bam*HI plus *Eco*RI digested DNA generating a full size fragment since there is no *Bam*HI site in pXC38. The *Eco*RI and *Bam*HI digestion of mutated E1 plasmids gives bands of different sizes according to the site of insertion. Lanes 3 to lane 6 contain E1 plasmid pXC38 with different mutagenized E1A regions. pXC1039c (Lane 3), pXC950c (lane 4), pXC918c (lane 5) and pXC884c (lane 6).


1
2
M
3
4
5
6
– 8 4
– 5 84
– 5 32
– 4 6
– 3 44
– 2 90
– 2 80
– 2 08

kidney) cells by E1A CRII insertion mutants was carried out to assay the transforming ability of these mutants. Primary cells were used because permanent cell lines (eg. Rat; CREF) are already immortalized and one of the functions of E1A in transformation is that of "immortalization". Each 60 mm dish of BRK cells was transfected with 5 μg of mutant plasmid utilizing the calcium phosphate technique.

Transformed BRK cells could be easily distinguished from primary BRK cells by their altered phenotypes and appearance after Giemsa staining, distinct colony shapes and different growth patterns. In a successful transformation, the first transformed colonies could be detected morphologically as early as 7 days post-transfection. Generally, wt transformants showed one of two different kinds of appearance. With one type, cells were spindle shaped, rather flat, and closely packed to form a circle with cells radiating outward, making an irregular edged island. The other type of colony formed a smooth edged island of transformed cells. No significant morphological differences were found between transformants of wt and mutant E1A plasmids.

Transformed colonies that survived in selection medium were counted after two weeks. The parent vector of new constructs, wt plasmid pXC38, and mutants with E1A insertions flanking CRII were included in all experiments as controls. The transformation efficiencies of various insertion mutants

were calculated as a percentage of their ability to transform BRK cells relative to that of wt E1A plasmid.

In these transformation assays, 5 μg of mutant DNA was used to transfect each dish of BRK cells. This concentration had been shown to be within the linear response of efficient transformation expression (McGrory 1988). The average number of foci formed per dish transfected with wild type E1A plasmid was 71.9 ± 13.5.

The results of the transformation assays are shown in Table 3.5 and Figure 3.25. The data indicated that all nine mutants, whether they had insertions at nt 918 or 950, reduced or totally abolished E1 transformation ability. Mutant plasmid pXC918+ severely reduced the E1 transformation ability. Although two collapsed mutants pXC918c and pXC918c' were impaired in their transforming ability, they still nevertheless transformed. The minus orientation insertion at nt 918, truncating E1A in the CRII region was unable to transform. The double cassette insertion mutant pXC950d transformed poorly whereas the other four pXC950 plasmids were totally transformation defective. Mutants with insertions at nt 908 and nt 1267 outside of CRII showed wt transformation ability. These results suggest that transformation was sensitive to insertion in CRII but not to inserts in its flanking regions.

Table 3.5

Transformation Assay Data[a]

	Foci per dish				Avg	% of wt
EXP No: 1						
pXC38	54	51	57	36	50	
pKH101	0	0	1	1	1	2
pXC918+	0	0	0	0	0	0
pXC918-	0	0	0	0	0	0
pXC918c	18	29	26	20	23	46
pXC950+	0	0	0	0	0	0
pXC950d	27	29	24	23	24	48
pXC950c	0	0	0	0	0	0
Control	0	0	0	0	0	0
EXP No: 2						
pXC38	180	186	172	184	181	
pKH101	0	0	1	1	1	1
pXC918+	0	0	1	1	1	1
pXC918-	0	0	0	0	0	0
pXC918c	121	131	114	115	121	67
pXC950+	0	0	0	0	0	0
pXC950d	15	22	32	11	20	11
pXC950c	0	0	0	0	0	0
Control	0	0	0	0	0	0
EXP No: 3						
pXC38	47	76	78	53	64	
pXC908+	56	49	58	62	56	88
pXC918+	4	2	0	0	2	3
pXC918-	0	0	0	0	0	0
pXC918c	12	22	51	18	26	41
pXC918c'	26	42	16	49	33	52
pXC950+	0	0	0	0	0	0
pXC950-	0	0	0	0	0	0
pXC950c	0	0	0	0	0	0
pXC950c'	0	0	0	0	0	0
pXC1267+	173	219	51	159	151	236
Control	0	0	0	0	0	0

Transformation Assay Data

		Foci per dish				Avg	% of wt
EXP No:	4						
	pXC38	30	60	58	43	48	
	pXC908c	24	26	11	14	19	40
	pXC918+	3	2	9	6	5	10
	pXC918-	0	0	0	0	0	0
	pXC918c	28	1	3	29	26	54
	pXC918c'	19	28	23	22	23	48
	pXC950+	0	0	0	0	0	0
	pXC950-	0	0	0	0	0	0
	pXC950c	0	0	0	0	0	0
	pXC1267-	19	10	24	11	16	33
	Control	0	0	0	0	0	0
EXP No:	5						
	pXC38	19	13			16	
	pXC918+	3	3			3	19
	pXC918-	0	0			0	0
	pXC918c	8	14			11	69
	pXC918c'	7	9			6	38
	pXC950+	0	0			0	0
	pXC950-	0	0			0	0
	pXC950c	0	0			0	0
	pXC1039+	4	7			6	38
	Control	0	0			0	0
EXP No:	6						
	pXC38	27	18			23	
	pXC918+	3	1			2	9
	pXC918-	0	0			0	0
	pXC918c	9	11			10	43
	pXC918c'	6	9			8	35
	pXC950+	0	0			0	0
	pXC950-	0	0			0	0
	pXC950c	0	0			0	0
	pXC1008+	4	1			3	13
	Control	0	0			0	0

*. DNA mediated transformation was employed to test the transformation ability of E1A CRII insertion mutants.

Figure 3.25 Transforming Ability of E1A CRII Mutants On BRK Cells

The histogram represents the transforming activity of E1A CRII insertion mutants as a percentage of the transforming activity of wild type plasmid pXC38. The E1A insertion mutants created earlier in this lab present in CRII flanking regions were included in each experiment for comparison (McGrory 1988).

Open bar 39 nt lop-mutator insertion with ORF

Filled bar 39 nt lop-mutator insertion with CRF

// bar 6 nt insertion collapsed from 39 nt ORF

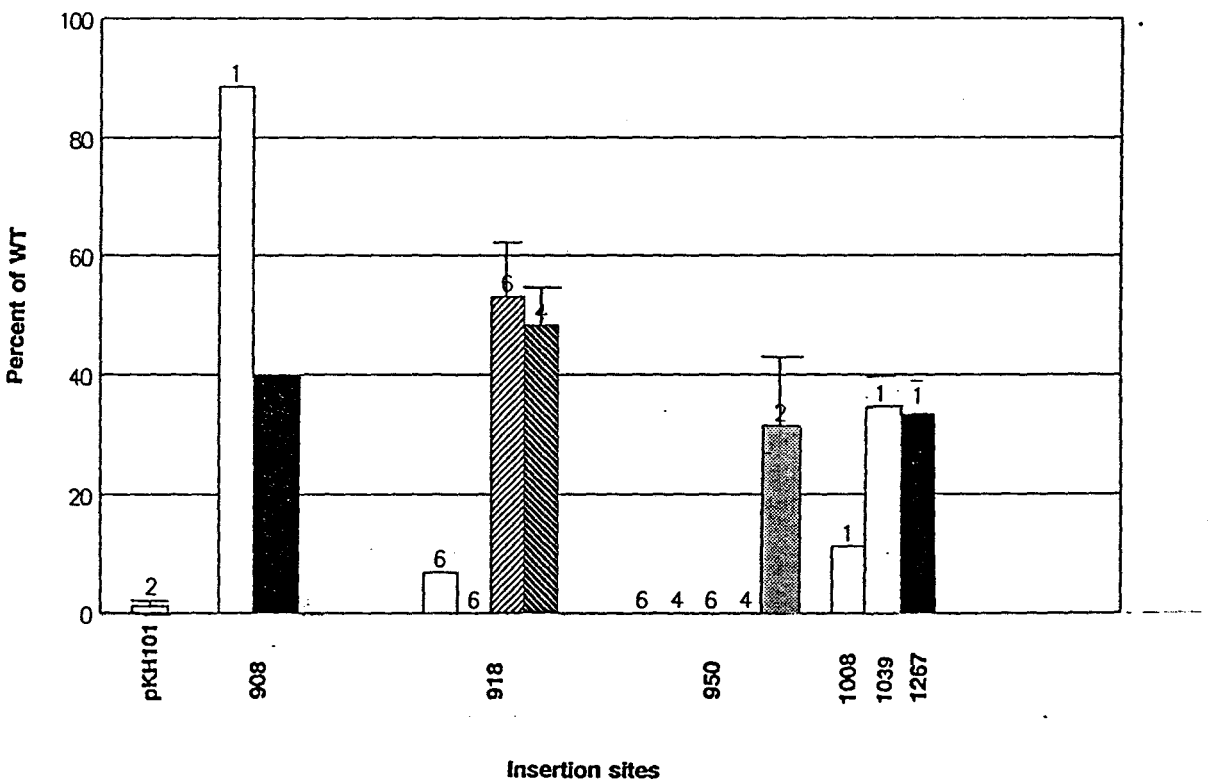
\\ bar 6 nt insertion collapsed from 39 CRF

Grey bar 71 (?) bp double cassette insertion

Digits above CRII mutant bars are experiment numbers.

Error bars indicate standard deviation from the mean.

Transformation By E1A Insertion Mutants On BRK Cells



3.5. Rescuing E1A Insertion Mutation into Viruses

A simple and efficient method employing the non-infectious plasmid pJM17 in cotransfection with mutant E1 plasmids to rescue E1A insertion mutants into infectious viruses has been developed (McGrory et al.,1989). All insertion mutants were rescued into viruses using homologous recombination in 293 cells between pJM17 and E1 plasmids with the E1A region mutated. Recombinant viruses were identified either by *Bam*HI restriction to map the the newly introduced *Bam*HI site or by *Hind*III plus *Bam*HI double digestion to generate diagnostic restriction patterns (Figure 3.26). Rescued viruses were plaque purified a minimum of two times on 293 cells before being used for further study.

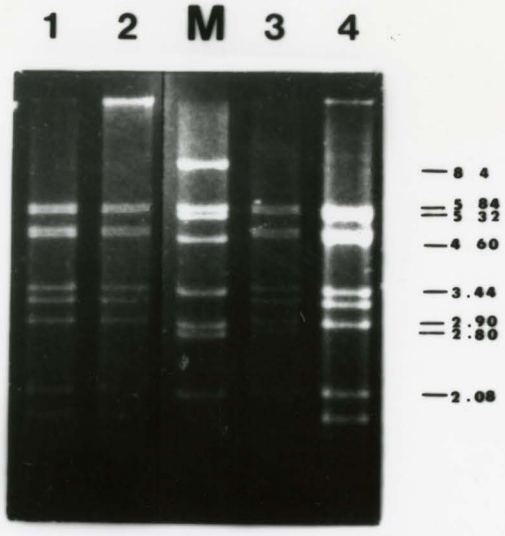
3.6. Screening of Temperature Sensitive Mutants from E1A Mutagenized Adenoviruses

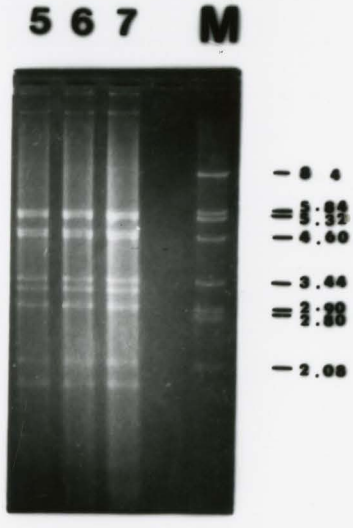
E1A mutants were generated in our laboratory with insertions at random sites which cover the whole E1A region. It was possible that the conformational changes caused by insertions of various sizes,39 nt (13aa) or 6 nt (2aa), may induce different structural alterations at different temperatures and thus affect certain functions in a temperature dependent manners. It would be very beneficial to have a temperature sensitive mutant in order to study E1A functions since by simply shifting temperature, some

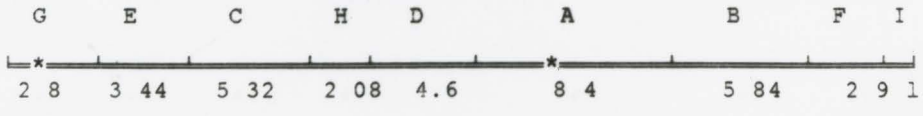
Figure 3.26 Restriction Profile Of E1A Insertion Mutants Rescued Back Into Viruses

The cartoon at the bottom shows the restriction map of a representative of mutant viruses. The horizontal double line represents the virus genome. *Hind*III sites are indicated by vertical lines on the genome and the size of each fragment is indicated and *Bam*HI sites are shown by asterisks. The *Bam*HI site in the *Hind*III G fragment is that produced by lop cassette insertion.

The DNA of rescued mutant viruses was digested using *Hind*III and *Bam*HI to screen rescued viral isolates. Lanes M are marker lanes using *Hind*III digested Ad5 DNA. Sizes of different bands of marker lanes are shown (I fragment not shown). Lanes 1 to 7 contain *Hind*III and *Bam*HI digested In918+, In918-, V38, In918c, In950c, In950- and In950+, respectively. V38 is a rescued wt virus. Note that in all mutant virus lanes, a fragment of 2.8 kb (G fragment) disappeared compared to lane 3, the wild type lane and a new smaller fragment is created resulting from *Bam*HI digestion because of the successful lop insertion in G fragment. The second novel small fragment was too faint to see in these photographs.


1 2 M 3 4
8 4
5 84
5 32
4 60
3.44
2.90
2.80
2.08


5 6 7 M
8 4
5.84
5.32
4.60
3.44
2.90
2.80
2.08


G E C H D A B F I
2 8 3 44 5 32 2 08 4.6 8 4 5 84 2 9 1

activities would be altered and the possible functional mechanisms could be studied.

Plaque assays were carried out to screen a total of 61 mutant viruses to see if any mutants would show significant differential reduction in replication at elevated temperatures. Plaque assays were performed at 33°C, 37°C and 39°C on Hela cells and 293 cells (Graham et al., 1977). Infections of 293 cells were used as positive controls, since 293 cells constitutively express E1A and thus complement viruses with mutated E1A regions. Plaque counting began on the 7th day to the 9th day and the plaque forming abilities of all E1A insertion mutants at the different temperatures were compared statistically. The Log of the ratios of plaque forming ability of mutants on Hela cells at each temperature relative to the plaque forming ability of the mutants grown on 293 cells at 37°C was chosen as a criteria to see whether any of these mutants was ts or replication deficient in the cells (if the log of the ratio was great than 2, the mutant was defective [host range]). The results showed that (Table.3.6, Figure 3.27, Figure 3.28 and Figure 3.29):

1. None of the mutants with any one of three types of insertions at any selected inserted site showed any significant temperature sensitive plaque forming ability (Table 3.6).

2. Mutants with insertions in minus orientation in exon I were

Table 3.6
Comparison Of E1A Insertion Mutants Infectivity

On Hela And 293 Cells At Different Temperatures (log)*

Mutant/ °C	Hela/Hela 33/39	Hela/293 33	37	39
Wt	0.02	-0.273	0.12	-0.30
717+	-1.26	-0.85	-0.59	0.41
717-	-0.86	**-6.25**	**-4.95**	**-5.39**
717c	-0.53	-0.60	0.07	0.07
812+	-0.62	-0.93	-0.46	-0.30
812-	0.09	**-4.70**	**-4.9**	**-4.8**
812c	-0.84	-0.59	-0.32	0.25
819+	-0.88	-0.40	-0.10	0.49
819-	-0.48	**-6.36**	**-5.76**	**-5.88**
819c	0.68	-0.88	-0.56	-1.56
827+	-0.33	-0.74	-0.29	-0.41
827-	ND	**-7.43**	**-6.13**	ND
827c	-0.67	-0.98	0.09	-0.30
863+	-1.18	-1.85	-0.45	-0.67
863-	0.06	**-5.65**	**-4.78**	**-5.71**
863c	0.88	-0.43	-0.43	-1.30
882+	0.32	-0.52	-0.58	-0.85
882-	0.28	**-4.89**	**-4.71**	**-5.17**
882c	-0.13	0.01	0.09	0.14

Comparison Of E1A Insertion Mutants Infectivity On Hela And 293 Cells At Different Temperatures (log)

Mutant/ °C	Hela/Hela 33/39	Hela/293 33	Hela/293 37	Hela/293 39
884+	0.05	-0.71	-0.51	-0.76
884-	0.12	**-5.10**	**-5.10**	**-5.22**
884c	0.03	-0.97	-0.55	-1
906+	-0.11	-0.68	-0.23	-0.58
906-	**-4.24**	**-6.68**	**-5.94**	**-5.67**
906c	0.56	-0.80	-0.73	-1.37
908+	-1.32	-1.56	-0.16	-0.24
908-	ND	ND	**-5.86**	ND
908c	-1.12	-1.47	-0.29	-0.34
918+	-1.49	-1.40	-1.07	0.09
918-	-1.40	**-7.19**	**-6.89**	**-5.79**
918c	-0.48	-2.14	-1.12	-1.66
950+	-0.69	-1.90	**-1.68**	**-1.21**
950-	**-5.09**	**-5.15**	-4.15	**4.55**
950c	-0.92	-1.61	-1.36	-0.69
1008+	0.78	**-3.06**	**-2.40**	**-3.84**
1008-	-0.52	**-4.82**	**-4**	**-4.30**
1008c	-1.16	**-3.96**	**-2.60**	**-2.79**

Comparison Of E1A Insertion Mutants Infectivity On Hela And 293 Cells At Different Temperatures (log)

Mutant/ °C	Hela/Hela 33/39	Hela/293 33	Hela/293 37	Hela/293 39
1039+	-1.38	**-4.54**	**-3.42**	**-3.15**
1039-	0.31	**-3.86**	**-3.44**	**-4.18**
1039c	-0.10	-0.56	-0.43	-0.46
1056+	-0.21	**-2.98**	**-2.06**	**-2.77**
1056-	-0.70	**-5.22**	**-4.11**	**-4.52**
1056c	-0.36	**-3.19**	-1.76	**-2.82**
1267+	-0.10	**-3.76**	**-3.34**	**-3.66**
1267-	0.38	-0.68	-0.51	-1.05
1267c	0.48	-0.73	-0.67	-1.21
1304+	-0.30	-0.73	-1.04	-0.43
1304-	0.41	-0.36	-0.19	-0.78
1304c	-0.45	-0.54	-0.15	-0.09
1376+	-1.08	**-2.22**	**-2.11**	-1.14
1376-	0.85	-0.51	-0.58	-1.36
1376c	0.11	-0.99	-0.85	-0.88
1408+	-0.08	-0.48	-0.27	-0.40
1408-	0.32	-0.92	-0.50	-1.24
1408c	0.02	-1.11	-0.62	-1.13

Comparison Of E1A Insertion Mutants Infectivity On Hela And 293 Cells At Different Temperatures (log)

Mutant/ °C	Hela/Hela 33/39	Hela/293 33	Hela/293 37	Hela/293 39
1415+	-0.72	-1.25	-0.25	-0.52
1415-	-0.11	-0.47	-0.07	-0.35
1415c	-1.56	-0.51	-0.11	1.06
1523+	0.32	-1.45	-1.15	-1.78
1523-	0.24	-1.22	-1.07	-1.46
1523c	0.71	-1.12	-1.19	-1.83
1969-	0.3	-0.44	-0.18	-0.74

a. The infectivity is the plaque forming ability per ml of each mutant virus at each different temperature. The infectivities of mutants on different cells at different temperatures are compared by log numbers. The darker values in the table are those log ratios greater than 2.

Figure.3.27 Plaquing Efficiency of Mutants of Plus orientation at Different Temperatures

Plaquing efficiency of E1A insertion mutants of plus orientation is presented as log number of their infectivities (Hela/293). The Infectivity here is the plaque forming ability per ml of rescued mutant viruses. Plaquing efficiencies of "+" mutants at different temperatures are shown as bars. Open bars are mutant virus plaquing efficiencies at 33°C, filled bars, 37°C and hatched bars, 39°C. Numbers below the graph are insertion sites of mutants.

The Plaquing Efficiency Of Plus Orientation

E1A Mutants at Different Temperatures

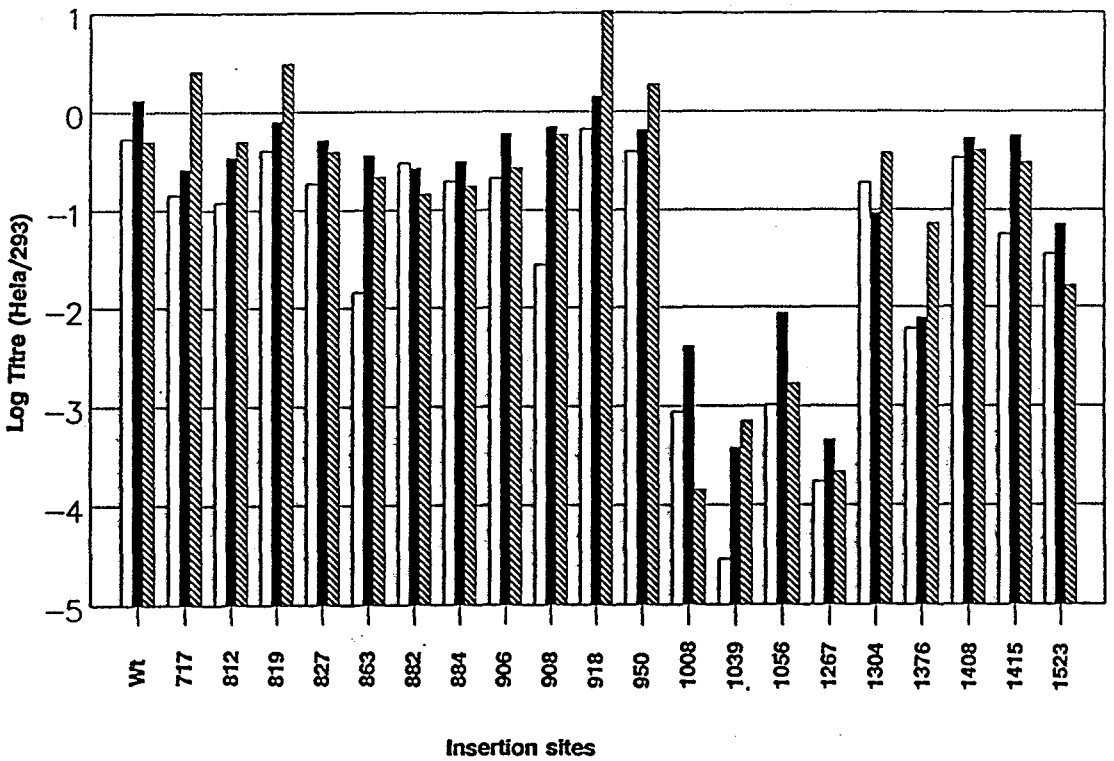


Figure.3.28 Plaquing Efficiency of Collapsed Mutants at different temperatures

Plaquing efficiency of collapsed E1A insertion mutants is presented as log number of their infectivities (Hela/293). The Infectivity here is the plaque forming ability per ml of rescued mutant viruses. Plaquing efficiencies of collapsed mutants at different temperatures are shown as bars. Open bars are mutant virus plaquing efficiencies at 33°C, filled bars, 37°C and hatched bars, 39°C. Numbers below the graph are insertion sites of mutants.

The Plaquing Efficiency Of Collapsed

E1A Mutants at Different Temperatures

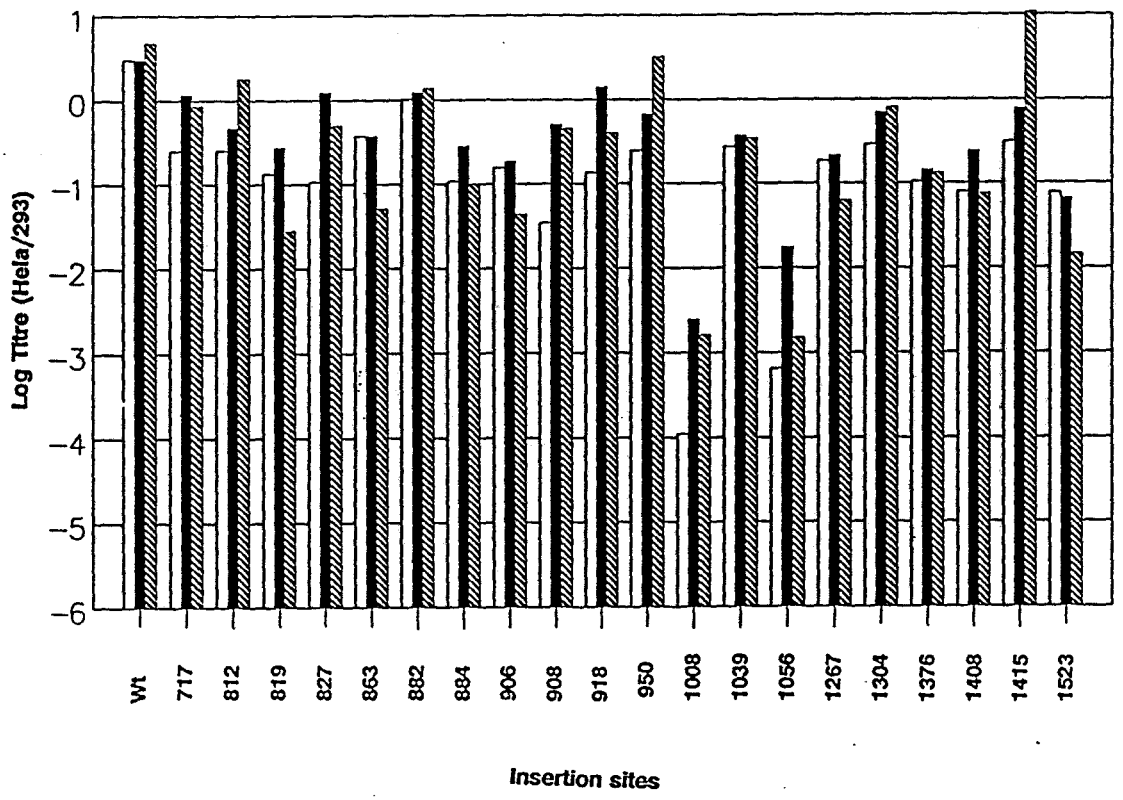
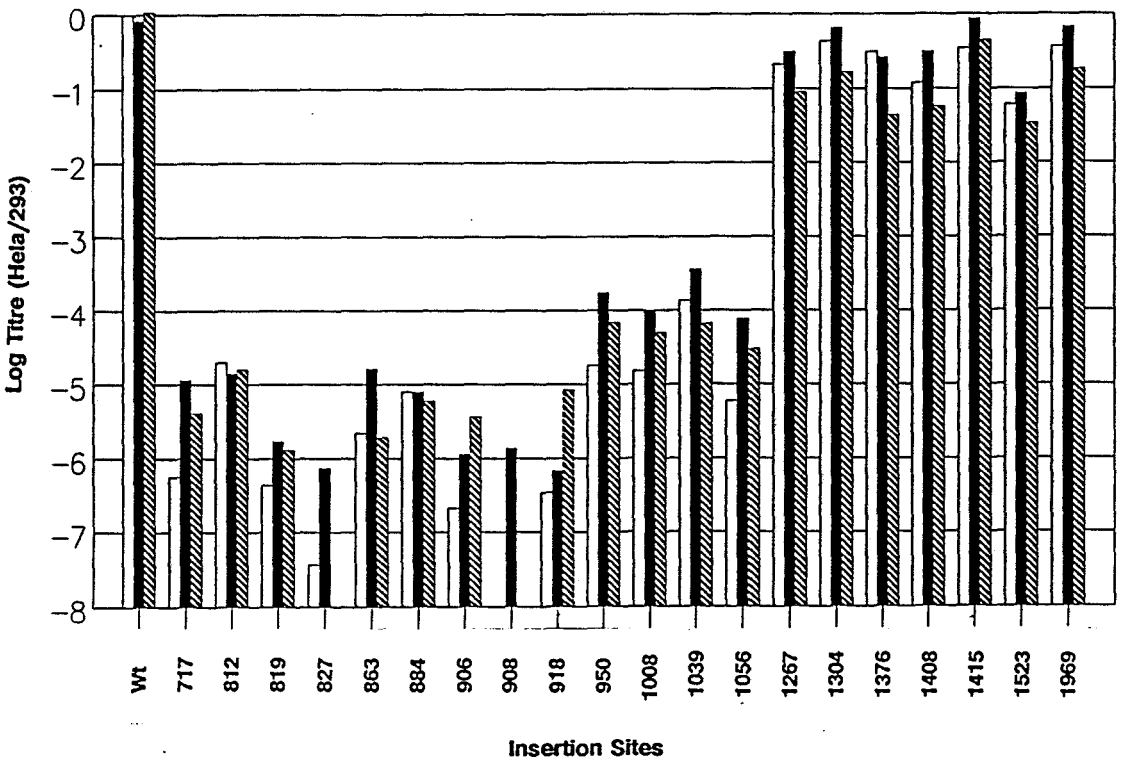


Fig.3.29 Plaquing Efficiency Of Mutants Of Minus Orientation At Different Temperatures

Plaquing efficiency of E1A insertion mutants of minus orientation is presented as log numbers of their infectivities (Hela/293). The Infectivity here is the plaque forming ability per ml of rescued mutant viruses. Plaquing efficiencies of "-" mutants at different temperatures are shown as bars. Empty bars are mutant virus plaquing efficiencies at 33°C, filled bars, 37°C and hatched bars, 39°C. Numbers below the graph are insertion sites of mutants.

The Plaquing Efficiency Of Minus Orientation

E1A Mutants at Different Temperatures



replication deficient in Hela cells relative to 293 cells at all three temperatures, 33°C, 37°C and 39°C.

3. All unique region mutants, whether they had the full size cassette insertion or the collapsed 6 nt insertions in either orientation, at all three temperatures showed the host range phenotype except the mutant with a 6 nt insertion at nt 1039.

4. At all three different temperatures, mutants with inserts at nt 1267 and nt 1376 in exon II showed the host range phenotype Except for 1376+ at 39°C.

5. E1B truncation mutant 1969- showed wild type infectivity.

The inability to obtain temperature sensitive mutants in replication indicated that this function may be insensitive to differences in temperature. However, it may still be possible to obtain temperature sensitive E1A insertion mutants via testing other E1A functions such as repression and transformation.

3.7. Transformation by E1A Insertion Mutant pXC1008+ and pXC1039+ Sensitive at 38.5°C And 32°C.

Though some evidence suggests that mutants which fail to transactivate do not transform, the results of studies with E1A insertion mutants (McGrory 1988, Bautista 1989 and this study) indicated that transactivation and transformation were not necessarily linked functions nor was repression and transformation. It was of some interest to see whether

transactivation or repression defective mutants with reduced transformation ability at 37°C, could transform at a higher (38.5°C) or lower (32°C) temperature. Therefore, experiments were carried out to screen for temperature sensitive transformation mutants. Selected mutants with inserts in CRI, CRII, the unique region or the beginning of exon II were chosen for analysis of possible temperature effects. Five μg of mutant plasmid DNA was used to transfect BRK cells and the transfected cells were maintained at 38.5°C or 32°C in α-MEM medium plus 10 % FBS, then shifted to selection medium, Joklik MEM plus 10% HS, on the fourth day post-transfection and treated as desired for the DNA-mediated transformation procedure mentioned previously. Colonies produced at 32°C were stained and counted on the 19th day rather than the 14th day post-transfections, since the cellular metabolic rate was much slower at the lower temperature. The results are shown in Table 3.7 and Figure 3.30. The transformation ability of some insertion mutants varied depending on the temperature. Two mutants with 39 bp ORF insertion in the unique region were sensitive for transformation at the high temperature, 38.5°C for transformation. These mutants which had an insert in the region encoding the potential Zn finger, produced no transformed colonies at 38.5°C, whereas they functioned as efficiently as wt at 32°C.

Table 3.7

Transformation Temperature Sensitive Assay Data[a]

	32°C				% of	38.5 °C				% of
	Foci per dish			Avg	wt	Foci per dish			Avg	wt
EXP No:	1									
pXC38	202	194	190	195		97	96	79	84	
pXC717c	134	143	144	140	72	76	97	66	80	95
pXC918+	86	87	98	90	46	0	1	0	0	0
pXC918-	0	0	0	0	0	0	0	0	0	0
pXC918c	53	57	57	56	29	3	4	4	4	5
pXC950+	0	0	0	0	0	0	0	0	0	0
pXC950-	3	1	2	2	1	0	0	0	0	0
pXC950c	0	0	1	0	0	0	0	0	0	0
pXC1008+	173	170	164	169	87	2	0	1	1	1
pXC1039+	161	162	164	162	83	5	6	8	6	7
pXC1267c	79	80	78	79	41	32	37	42	37	44
Control	0	0	0	0	0	0	0	0	0	0
EXP No:	2									
pXC38	56	45	50	50		55	50	51	52	
pXC717c	37	31	47	38	76	53	39	45	46	89
pXC918+	13	12	13	13	26	3	5	5	4	8
pXC1008+	44	39	59	47	94	0	1	0	0	0
pXC1039+	27	17	21	22	44	3	1	0	1	2
pXC1376+	49	54	43	49	78	54	41	47	47	90
Control	0	0	0	0	0	0	0	0	0	0

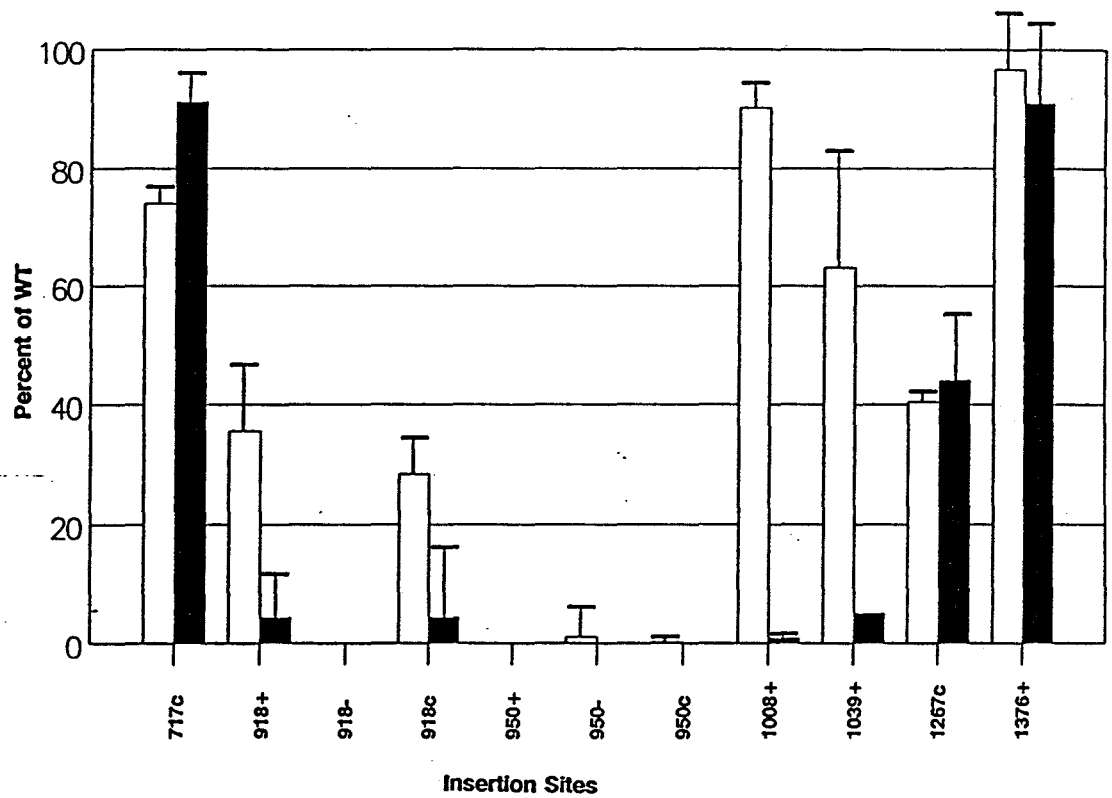
a. DNA mediated transformation technique was used to screen transformation temperature sensitive mutants.

Figure 3.30 Transforming Ability of E1A Mutants at Different Temperatures

Transformation ability of E1A insertion mutants of CRII (717c), CRII (918+, - and 950+, - and c), unique region (1008+ and 1039+) and exon II (1267c and 1376+) were tested at different temperatures using DNA mediated transformation. Transformation efficiencies are shown in bars for mutants relative to the activity of wt plasmid DNA. The Open bars represent transforming activity at 32°C and filled bars, 38.5°C. Error bars give the standard deviation from the mean.

Transformation Ability Of E1A

Mutants At Different Temperatures



DISCUSSION

In this study, I report the construction of 9 insertion mutants in CRII of the adenovirus E1A gene and the characterization of these mutants together with a sampling of other insertion mutants, previously generated in Dr. Graham's laboratory, which span the whole E1A region. Presumably, conformational alteration caused by insertion at a region crucial for the expression of a certain function would affect or even eliminate the function. The results of my thesis research reveal that CRII is a sensitive region as far as repression and transformation are concerned.

The insert employed in this study can induce different structural alterations at the same insertion site, depending on the orientation of the insert. In one orientation, the insert contains a 13 codon open reading frame (ORF). In the opposite orientation, the insert encodes a translation termination signal, forming a closed reading frame (CRF). When the 2 *Bam*HI sites of the insert are collapsed, a 2 codon insert is obtained. These different types of inserts were used to examine how local minor or major structural changes in E1A affected the optimal expression of various functions.

4.1 Structural Alterations Outside of the Unique Region Do Not Significantly Affect Transactivation Function

The E1A insertion mutant studies strongly support a model in which the unique region comprises the major domain responsible for the transactivation function. Two subdomains, one zinc finger from aa 140 to 178 and one transactivation region around aa 178 to 186 are considered responsible for the E1A transactivation function. The mechanism of E1A transactivation has not been elucidated. However, accumulation or activation of a cellular factor responsible for transactivation of the E2A promoter has been shown to depend on the presence of the E1A 13S product (Reichel et al., 1988). Transactivation of another Ad promoter, the E4 promoter, requires increasing expression of a cellular factor the level of which has been shown to increase in the presence of E1 (Gilardi and Perricaudet 1986).

The transactivation activities of the CRII insertion mutants showed no statistical difference relative to wild type with the exception of two mutants with termination codons upstream of the unique region (Appendix A1 and A2). All 6 CRII ORF insertion mutants, whether they had 13 aa or 2 aa nt insertions, were transactivation positive (Table 3.2; Figure 3.17). These results are consistent with previous studies on transactivation. Functional assays of insertion mutants

generated by Bautista using the same insertion strategy, indicate that the transactivation function is insensitive to ORF insertion outside unique region, including insertions at nt 950 and nt 1267 which immediately flank the unique region.

Results of assays in transactivation done in this study were in agreement with results obtained previously by Bautista.

4.2 Effect of Conformational Change In CRII On E1A Enhancer Repression Function

In order to study transrepression activity of the CRII mutants, it was first necessary to introduce a terminator sequence in the unique region of E1A at nt 1007 to truncate and thus inactivate the 13S encoded protein. This mutation disrupted the putative Zn finger (nt 980 to 1090), which may be important in interacting with some cellular factors to turn on transcription, and truncated the 13S product at this point. Eliminating the 13S protein's positive regulatory effect allowed detection of repression in the transient expression assay system employed in this study. The site nt 1007 was also chosen because it is not too close to the 12S messenger splice donor site at nt 974, thus avoiding any possibility of disabling the normal 12S splice required to produce a functional 12S product. In the transrepression assays reported here, the E1A gene with a terminator at nt 1007 does, in fact,

function to repress E1A promoter-driven β-galactosidase activity, indicating that our predictions were correct, regarding function of the termination mutant.

Results of transrepression assays using the CRII mutants carrying terminator sequences in the unique region indicated that the ability to repress was influenced by the size of the insert in CRII (Table 3.4 and Figure 3.22). Inserts of 39 nt in either orientation impaired repression by about 70% or more compared to wt whereas 6 nt insertions decreased repression less than 40%. The differences among the 3 mutants at the same site and between each of the mutants and wild type were significantly different from the wt activity (Appendix B1 and B2). One possible explanation for why all of these 39 nt insertions in CRII impaired repression, independent of orientation, might be that these large insertions were too close to the 12S product splice donor site at nt 974 to allow proper splicing. The smaller inserts, on the other hand, may not have inhibited splicing. However, it seems more likely that the defect is due to an alteration in 12S protein structure.

The region around the end of exon I of 12S and the beginning of exon II appears to be involved in repression. Insertions of 13 codons at nt 908(aa116), 918(aa120), 950(aa131) and nt 1267(aa197) of 12S product in CRII or the beginning of exon II show some impairment of repression

activity. Interestingly, the 13 codon insertion at nt 906 does not affect function at all (Bautista 1989). A specific conformation of the protein from aa 116 to aa 197 may be important for repression function. Mutations generated by inserting termination codons both at nt 918 and 950 eradicated repression implying that the polypeptide encoded by the deleted sequence may be important for maintaining the protein structure necessary for E1A repression function. The repression function was only sensitive to certain major conformational changes or deletions in CRII. Some minor conformational changes in the same area seemed not to impair repression at all, since all 2 aa insertion mutants repressed more efficiently than large insertion mutants. It is interesting that two kinds of insertions at the same site which differ by only 11 aa can give very different effects with respect to repression. The peptides encoded by the 39 nt insert are predicted to be neither predominantly hydrophobic nor predominantly hydrophilic, thus hydropathy seems to play no major role in this case. The mechanism of how E1A represses is still unclear. It seems that a local general structure contributed by a region of the E1A protein rather than a few amino acids may be important in the maintenance of the configuration necessary for repression. This configuration may tolerate small inserts such as 2 aa insertions, whereas larger insertions may alter conformation sufficiently to distort the

3 dimensional structure of the protein eliminating repression. If the ability of E1A to repress certain enhancers does map to the region from nt 908 to 1267, as appears from results with 39 nt insertion mutants, then one must assume that the protein structure required for repression ability is not absolutely rigid, since 2 aa insertions in this region of the protein seem to have little effect.

In each repression assay, mutants generated by insertions at sites flanking CRII (Bautista, 1989) were included for comparison. Most of our results generally agree with each other. The only major difference was that the repression activity of mutant 1267c was reduced in transrepression assay of this study. The reason for the discrepancy is not known, although the result, in the present assay, was reproducible.

4.3. Transformation Impaired by Insertion at CRII of E1A

The regulation of transformation ability differs from that of transactivation in that transactivation is due mainly to the region unique to the 13S protein. In contrast, transformation has been found to be sensitive to structural alterations involving several regions of E1A (Lillie et al., 1986; Velcich and Ziff 1988, Howe et al.,1989; Jelsma et al.,1989). CRII is one of these key transformation regions. This CRII insertion mutant study showed that all CRII

insertion mutants reduced E1A transformation ability, except the 2 aa insertion at nt 918 which partially retained this ability (Table 3.5; Figure 3.25; Appendix C1 and C2). The fact that the CRII truncation mutants were unable to transform implies that at least some portion of the truncated sequences from CRII to the C terminus, are needed for transformation. There might be a function-related reason why the sequence of CRII is highly conserved among different adeno serotypes, considering that an insertion 10 nt upstream of CRII (nt 908) does not affect the transformation capability of E1A at all (Bautista 1989). That four out of five mutants which had insertions at nt 950 were completely defective for transformation suggests that this particular site may be more sensitive to structural alterations than nt 918 as far as transformation is concerned. Even the 6 bp insertions at 950 resulted in the failure to morphologically transform BRK cells.

Mutational analyses of CRII carried out by other laboratories also suggest that CRII is an important region for transformation (Kuppuswamy et al., 1987; Moran et al., 1986; Lillie et al., 1986; Schneider et al., 1987). Whyte and his colleagues found that deletion of the region between nt 920 and 967 reduced transformation (Whyte et al., 1989). Amino acids 121 to 127 of CRII comprise one of the regions responsible for binding of the Rb-105 kDa cellular protein

and this binding may be important for oncogenic transformation (Egan et al., 1988; 1989; Whyte et al., 1988). The conclusion derived from my CRII insertion mutant study are consistent with these results. It is possible that the insertion at CRII affects transformation because the insertion interrupts the local conformation necessary for binding a protein needed for transformation.

Transformation is not only sensitive to local structural alterations in CRII caused by linker insertion, but the extent of sensitivity also varied with different sizes of insertions. For example, a 39 bp insertion in either orientation at nt 918 totally abolished transformation, whereas the corresponding collapsed forms of the inserts at the same site were partially active. This result is interesting since it suggests that E1A transformation ability may be more sensitive to large than to small changes in conformation. The transformation results of all control plasmids agree with McGrory's results using the same plasmid (McGrory 1988).

4.4 Replication Ability of E1A Insertion Mutants is Not Temperature Sensitive

As described previously in the RESULTS, the E1A insertion mutants generated in this laboratory contained cassette inserts of different sizes at different sites and

orientations covering the whole E1A region. It seemed very likely that the structural alteration caused by some of these insertions may have occurred in a region necessary for stability or activity of the protein. An insertion in such a region could alter the properties of the encoded protein such that the protein might be more sensitive to environmental conditions such as a temperature. Evidence has shown that even a single amino acid substitution can transform a wild type virus into a temperature-sensitive mutant virus (Kruijer et al., 1983, Nakajima et al.,1986). Unfortunately, it is usually impossible to predict what mutation will induce a temperature-sensitive phenotype. Though temperature sensitive (ts) mutants would be very useful to study E1A function, no ts E1A mutants are currently available.

The principle behind the screening for ts mutants was to find a mutant that fails to function at elevated temperature. A ts replication mutant should be defective for plaque formation at the non-permissive temperature but replicate efficiently at the permissive temperature. Because wt E1A is not ts, this phenotype would have to be generated by mutating E1A, as for example, by introducing insertions as described in this thesis. If a ts E1A mutant were detected by screening, it could be used to further explore the mechanism of replication. However, no significant difference between plaquing efficiencies of E1A insertion mutants at different

temperatures was seen in this study. Failure to identify any temperature sensitive mutants by plaque assay may be due to several reasons. It could simply mean that the replication function is not sensitive to any conformational change in E1A induced by the insertions except those in the unique region. Insertion in the unique region damages the transactivation function and reduces the replication rate. This damage seems to be unaffected by differences in temperature. Alternatively it may be that E1A proteins are so rigid in their conformation that insertions of 13 aa or 2 aa do not sufficiently alter the shape of the proteins to affect transactivation in a temperature dependent fashion.

The results of my plaque assays generally agreed with J.McGrory's data (McGrory 1988). One major difference was that the plaque forming ability of mutant 1267+ was very poor at all three temperatures in this study; however, its efficiency was as high as that of wild type in J.McGrory's study. The reasons for this discrepancy are not known and require further study.

4.5 pXC1008+ and pXC1039+ Transformation Temperature Sensitive.

I have screened out two Ad5 E1A mutants which were temperature-sensitive for DNA-mediated transformation (Table 3.7; Figure 3.30; Appendix D1). These two plasmids, pXC1008+

and pXC1039+, were generated by Bautista (Bautista 1989), and each contains a 39 nt lacZ-labelled cassette ORF insertion in the unique region. When performing transformations at 38.5°C, only a few transformed colonies were found, whereas at 32°C more than 100 transformed colonies were visible per 60 mm dish. Functionally, the unique region might be subdivided into one region encoding a zinc finger (nt 980 to nt 1090) and another encoding a potential transcription regulator region (nt 1090 to nt 1240). pXC1008+ and pXC1039+ all have ORF in frame inserts located just within the sequence encoding the zinc finger. This structure alteration seems to completely inhibit DNA-mediated transformation at 38.5°C. Transforming ability was almost destroyed at 37°C, exhibiting only 20% of wt's activity (McGrory 1989). The interesting point is that at 32°C, they transformed BRK cells as well as wt. The fact that only these mutants were found to be ts for transformation suggested that this phenotype may be related to structural change induced in the Zn finger by the 13 aa insertion. The consensus aa sequence for Zn fingers is Cys-X_{2-5}-Cys-X_{12}-His-X_{2-3}-His. (X stands for any possible amino acid). The sequence of nt 1008 full cassette insertion encoded one Cys residue. At higher temperature, this Cys as part of the insertion cassette, might interrupt the normal arrangement of metal binding amino acids thus preventing a wild type level of transformation function expression. A conformational change

at the lower temperature might shift this Cys to a position just sufficient to compensate for the structural alteration caused by lop insertion, thus rescuing Zn binding. If the Zn finger in the unique region does contribute to transformation in wt E1A transfection, it is understandable that foreign sequences inserted in this region could interrupt local structure thus reducing transformation ability. However, this disturbance seemed not to interfere with the Zn finger when at lower temperature as far as transformation was concerned.

It was surprising to see that these two transformation temperature sensitive unique region insertion mutants did not show any ts phenotype in relation to replication (Table 3.6 and Figure 3.27). This event was very hard to explain. There could be some different mechanisms involved in the DNA-mediated transformation and replication.

4.6 Transformation Ability Relatively Independent of Either Transactivation or Transrepression

From the results of the E1A CRII insertion mutant studies employing various functional assays, it is clear that none of these E1A functions are highly correlated. Transactivation is to some extent important, at least indirectly, for transformation, possibly by transactivating E1B to levels required for transformation. However, transformation is not exactly parallel to transactivation

ability. In this study all non-truncation CRII mutants transactivated more or less as well as wt but all had their transformation proficiency diminished, or even totally abolished. It seems likely that transactivation is not directly required for transformation but acts indirectly by regulating E1B function.

Trans-repression could contribute to transformation via repression of cellular genes resulting in cell phenotype changes. In this insertion mutant study, however, results show that although insertion in the CRII region more or less affects both repression and transformation abilities, both functions are not always affected to the same extent. Collapsed insertions at either site in this conserved region did not significantly reduce the repression ability of E1A proteins, but the transformation ability was severely impaired. This result agrees with the results of other E1A insertion mutant studies in this laboratory (Bautista 1989), which have shown that insertion at nt 908 did not affect the transformation ability of E1A proteins whereas the 39 nt aa cassette insertion at the same site did reduce E1A repression ability. All this evidence suggests that transformation and repression are separate E1A functions (Velcich and Ziff 1988)

4.7 Conclusions

An insertional mutation study has been carried out to

study E1A functions involved in transactivation, repression and transformation. Evidence presented here suggests that CRII is an important region for transformation, consistent with other studies, and that some major conformational changes in the CRII region can affect the repression function. In agreement with most other reports, this region is not a critical region for transactivation. This study also shows that transformation is a separate E1A function from both transactivation and repression. Two transformation temperature sensitive mutants with 39 bp inserts in the unique region have been identified. No mutants were detected which were temperature sensitive for replication.

APPENDIX

Student's T test was employed to examine the statistical significance of differences between E1A mutants generated at the same site and between E1A mutants and corresponding E1A wt plasmid in E1A functions of transactivation, trans-repression and transformation. The Null hypothesis suggested that there was no significant difference between them. The hypothesis would be rejected if the P value was less than 0.05. DF: degrees of freedom, derived by the number of experiments - 1. SD: standard deviation. R: Null hypothesis rejected; N: Null hypothesis not rejected.

Appendix A1. Evaluation of Null Hypothesis of Transactivation Differences between E1A CRII Mutants Generated at the Same Site

A.pKH 918+							
B.pKH 918-	R						
C.pKH 918c	N	R					
D.pKH 918c'	N	R	N				
E.pKH 950+							
F.pKH 950-					R		
G.pKH 950c					N	R	
H.pKH 950c'					N	R	N
Mutant	A	B	C	D	E	F	G

* The results showed that transactivation ability of "-" mutant was significantly different from other forms of mutants generated at the same site

Appendix A2. Student's T Test for the Transactivation Differences between E1A Mutants and Wt Plasmid

MUTANT DNA	Df	SD	P value	Null hypothesis
pKH 884-	1	1.1	<0.01	Rejected
pKH 908+	2	11	>0.05	Not Rejected
pKH 918+	3	9.7	>0.05	Not Rejected
pKH 918-	3	6.2	<0.01	Rejected
pKH 918c	3	6.0	>0.05	Not Rejected
pKH 918c'	2	6.1	>0.05	Not Rejected
pKH 950+	3	8.2	>0.05	Not Rejected
pKH 950-	2	3.7	<0.01	Rejected
pKH 950c	3	14.8	>0.05	Not Rejected
pKH 950c'	2	16.5	>0.05	Not Rejected

* The results showed that P value of all "-" mutants were less than 0.01. The Null hypothesis was rejected and their transactivation ability was significantly different from that of wt.

Appendix B1. Evaluation of Null Hypothesis of Trans-repression Differences between E1A CRII Mutants Generated at the Same Site

A.12S 918+							
B.12S 918-	R						
C.12S 918c	R	R					
D.12S 918c'	R	R	N				
E.12S 950+							
F.12S 950-					R		
G.12S 950c					R	R	
H.12S 950c'					R	R	N
Mutant	A	B	C	D	E	F	G

* The results showed that all mutants generated at the same site differed significantly from each other in transrepression with the exception that "collapsed" mutants of different sources (whether they were derived from "+" or "-" mutants) showed no significant difference each other.

Appendix B2. Student's T Test for the Transrepression Differences between E1A Mutants and Wt Plasmid

MUTANT DNA	Df	SD	P value	Null hypothesis
12S 908+	2	9.6	<0.01	Rejected
12S 908c	1	0.5	<0.05	Rejected
12S 918+	4	5.4	<0.01	Rejected
12S 918-	4	12.8	<0.01	Rejected
12S 918c	4	5.8	>0.05	Not Rejected
12S 918c'	1	5.5	>0.05	Not Rejected
12S 950+	4	10.9	<0.01	Rejected
12S 950-	1	3.1	<0.05	Rejected
12S 950c	4	12.8	>0.05	Not Rejected
12S 950c'	1	8.6	>0.05	Not Rejected
12S 1267+	1	3.3	<0.05	Rejected
12S 1267c	1	0.1	<0.05	Rejected

* The results showed that all but 918, 950 collapsed mutants had Null hypothesis rejected. Their transrepression ability differed significantly compared to wt plasmid.

Appendix C.1 Evaluation Of Null Hypothesis of Transformation Differences between E1A CRII Mutants Generated at the Same Site

A.pXC 918+							
B.pXC 918-	N						
C.pXC 918c	R	R					
D.pXC 918c'	R	R	N				
E.pXC 950+							
F.pXC 950-					N		
G.pXC 950c					N	N	
H.pXC 950c'					N	N	N
Mutant	A	B	C	D	E	F	G

* The results showed that all mutants containing inserts at nt 950 had no difference among them in their transformation. However, transforming ablities of "collapsed" mutants inserted at nt 918 had significant differences when compared to mutants of other forms at this site ("+ or ").

Appendix C2. Student's T test for the Transformation Differences between E1A Mutants and Wt Plasmid

MUTANT DNA	Df	SD	P value	Null hypothesis
pKH 101	1	0.4	<0.01	Rejected
pXC 918+	5	6.6	<0.01	Rejected
pXC 918-	5	0.0	<0.01	Rejected
pXC 918c	3	10.1	<0.01	Rejected
pXC 918c'	5	6.8	<0.01	Rejected
pXC 950+	5	0	<0.01	Rejected
pXC 950-	3	0	<0.01	Rejected
pXC 950c	5	0	<0.01	Rejected

* The results showed that all experimental groups had Null hypothesis rejected. Their transforming ability differed significantly from wt plasmid.

Appendix D. Comparison of Variable Transformability of E1A Mutants at Different Temperatures

	32°C	38.5°C	P Value	Null Hypothesis
* pXC38	100	100		
* pXC717c	108	91	>0.05	not rejected
* pXC918+	45	4	>0.05	not rejected
pXC918-	1	0	>0.05	not rejected
pXC918c	39	4	>0.05	not rejected
pXC950+	0	0	>0.05	not rejected
pXC950-	1	0	>0.05	not rejected
pXC950c	0	0	>0.05	not rejected
* pXC1008+	107	1	<0.05	rejected
* pXC1039+	72	5	<0.05	rejected
pXC1267c	56	44	>0.05	not rejected
pXC1376+	79	91	>0.05	not rejected

* The transforming ability of plasmid pXC1008+ and pXC1039+ (which had full length cassettes inserted in the unique region of Ad5 E1A), showed a significant difference between the two temperatures tested, 32°C and 38.5°C, $P<0.05$. The " *" labelled

experiments were repeated. Values given in the table were normalized using pXC38 as a standard for easy comparison.

Equivalency of E1 plasmids used for temperature sensitive transformation

This study	J.McGrory
pXC717c	pX717
pXC1008+	pX1008+
pXC1039+	pX1039+
pXC1267+	pX1267+
pXC1376c	pX1376

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