Development And Characterization Of A Human Adenovirus Type 5 Cloning Vector

By

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ABSTRACT

This thesis describes studies designed to develop and utilize human adenovirus type 5 (Ad5) as a cloning and expression vector in mammalian cells. The experimental approach taken in this work was first to determine the limits on the amount of DNA which could be packaged into virion capsid. To this end I have used mutants of Ad5 derived from insertion of a bacterial transposable element (Tn5) into the left end (the transforming region) of the viral genome. Attempts to rescue various sized derivatives of these Tn5 insertion mutants into full length infectious Ad5 DNA indicated that not more than about 2 kb of insert DNA could be rescued in this way. To extend this limit, a helper-independent Ad5 cloning vector has been constructed in which most of early region 3 (E3), from map co-ordinates 78.5 to 84.7, and essentially all of early region 1 (E1), from 1.0 - 10.6, have been deleted. E3 is non-essential for adenovirus replication in cultured cells and E1 is non-essential when the virus is propagated in 293 cells which constitutively express the E1 gene products. The resulting new virus, dlE1,3 was about 5.5 kb shorter than wt Ad5 and therefore should be able to accept up to 7.5 kb in foreign DNA. To test the usefulness of this vector, the Herpes Simplex Virus type 1 (HSV.1) thymidine kinase gene (tk) along with its regulatory sequences was inserted into the unique XbaI site of dlE1,3 (at map position 78.5/84.7). The resulting recombinant virus, Adtk, expressed the HSV tk at a low level (as compared to levels induced by HSV.1) in infected

cells; however, tk expression was markedly enhanced when Adtk infected cells were super-infected with a tk^- mutant of HSV. Furthermore, the Adtk virus efficiently transformed tk^- mouse cells (line K4 and its progenitor the LTA) to the tk^+ phenotype. At a low efficiency, it was also possible to transform tk^- human cells (line 143), and tk^+ transformants of both mouse and human origin have been established as permanent lines.

Lastly, during construction of dlE1,3, we isolated a variant, dlE1,3-1, which had a direct repeat of viral DNA terminal sequences attached to the left end of the genome. Analysis of this variant with restriction enzymes and by hybridization of Southern blots with specific probes indicated that the extra terminal segment contained the left 2.6% (920 bp) of Ad5 joined to 352 bp of pBR322 which in turn was linked to the left end (minus 21 bp) of dlE1,3. During replication of dlE1,3-1 the extra terminal segment was found to transfer to the right end of the genome resulting in a second variant, dlE1,3-2, having duplicated terminal sequences at both ends of the viral genome. DlE1,3-2 was shown to revert back to dlE1,3-1 at high frequency. Although evidence was obtained indicating that the extra segment could be lost from the left end at low frequency, spontaneous mutants which had lost direct repeats from both ends were never isolated. It was, however, possible to remove the extra terminal repeat of dlE1,3-1 by cleavage with a restriction enzyme and to isolate dlE1,3 containing wt termini. The rearrangements occurring during replication of dlE1,3-1 and dlE1,3-2 may be the consequences of the mode of replication of Ad5 DNA.

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TABLE OF CONTENTS

FIGURES

TABLES

LIST OF ABBREVIATIONS

Ad 2, 3, 4, 5, etc.	adenovirus type 2, 3, 4, 5, etc.
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
Ci	curie
cm	centimeter
cpe	cytopathic effect
cpm	counts per minute
DBP	DNA binding protein
dCMP	deoxycytosine monophosphate
dATP, dCTP, dGTP, dTTP	deoxynucleoside triphosphate
DHFR	dihydrofolate reductase
dl	deletion mutant
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E1 - E4	early region 1 - 4 of adenovirus genome
EDTA	ethylenediamine tetracetic acid
et al	and co-workers
EtBr	ethidium bromide
Fig.	figure
g	gram
HAT	hypoxanthine, aminopterin, thymidine
HBV	hepatitis B virus
HPV	human papilloma virus
hr(s)	hour(s)
HS	horse serum
HSV	herpes simplex virus
In	insertion mutant
ITR	inverted terminal repeat
Kb	kilobase pairs
Kd	kilodaltons
L1 - 5	late region 1 - 5 of adenovirus genome
l	liter
M	molar
min	minute
$MgCl_2$	magnesium chloride
MEM	minimal essential medium
ml	milliliter
MLP	the adenovirus major late promoter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
MoMLV	Maloney murine leukemia virus
mRNA	messenger ribonucleic acid
MSV	murine sarcoma virus
mu	map units
MW	molecular weight
NaCl	sodium chloride
Neo^r	neomycin resistance

NP-40	nonidet P-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFU (or pfu)	plaque forming unit
PI (or pi)	post infection
pTP	precursor of the terminal protein
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rotation per minute
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SV40	simian virus 40
Sub	substitution mutant
TCA	trichloroacetic acid
TE	tris-EDTA buffer
tk	thymidine kinase
^{3}H TdR	tritiated thymidine
TP	terminal protein
Tris	tris (hydroxymethyl) aminomethane
ts	temperature sensitive mutant
UV	ultraviolet radiation
VA-RNAI, VA-RNAII	virus associated RNAI, RNAII
wt	wild type virus
ZnCl	zinc chloride
^{32}P	phosphorous-32

CHAPTER I: INTRODUCTION

1.1 Discovery and Epidemiology of Adenoviruses

Adenoviruses were discovered over three decades ago by Rowe et al. (1953), and the first prototypes were isolated shortly afterwards from human adenoid tissues (Enders et al., 1956), from which their name was derived. They are ubiquitous agents, and one or more serotypes will have infected most individuals by the age of 15. Clinically, they appear to cause mild respiratory and gastrointestinal infections, occasional epidemics of acute respiratory disease in military recruits, and outbreaks of bronchiolitis among children; certain serotypes have also been shown to cause keratoconjunctivitis (Table 1.1; reviewed by Straus, 1984). The adenoviruses gained much attention when Trentin et al. (1962) reported tumor formation after injection of a newborn hamster with one member of these agents. Work on adenoviruses in subsequent years led to major advances in the field of virology, molecular biology and mammalian cell transformation; recently, they are being used as gene transfer vectors in mammalian cells. This chapter will summarize our current knowledge of the molecular biology of adenoviruses and viral vectors, and their possible use in mammalian cells as a prelude to a description of my studies aimed at developing human adenovirus type 5 into a cloning and

Table 1 -]

HUMAN ADENOVIRUS HOMOLOGY CLASSES

Class	Representative Serotypes	Hemag-glutination Group *	% DNA Homology +	% G + C	Oncogenicity in Rodents	Target Tissue	Epidemiology
A	12, 18, 31	IV	a 48-69% b 8-20% c 50-80%	48%	high	gastrointestinal tract	cryptic gastrointestinal infection
B	3, 7, 11, 21	I	a 89-94% b 9-20% c 50-80%	51%	weak	pharynx lungs (upper & lower respiratory tract) hemorrhagic cystitis (lower urinary tract) conjunctivits (eye)	acute epidemic infection
C	1, 2, 5, 6	III	a 99-100% b 10-16% c 50-80%	58%	nil	pharynx (upper respiratory tract)	latent throat infection; cryptic gastrointestinal infection
D	8, 9, 19	II	a 94-99% b 4 -23%	58%	nil	keratoconjunctivitis (eye)	acute epidemic infection
E	4	III		58%	nil	upper respiratory tract	
F	EA				nil	gastrointestinal tract	enteritis-associated enteric infection

* I. Complete agglutination of monkey erythrocytes; II. Complete agglutination of rat ethryocytes; III, Partial agglutination of rat erthrocytes; IV. Minimal agglutination response.

+ a. Homology of members of same group; b. Homology of members of different groups; c. DNA sequence homology of members of different groups (0-4.5 map units and 15-17 map units).

Reprinted from Sambrook et al. (1981)

expression vector in mammalian cells, and at developing efficient methods for insertion of genes into adenovirus DNA.

1.2 Adenoviruses

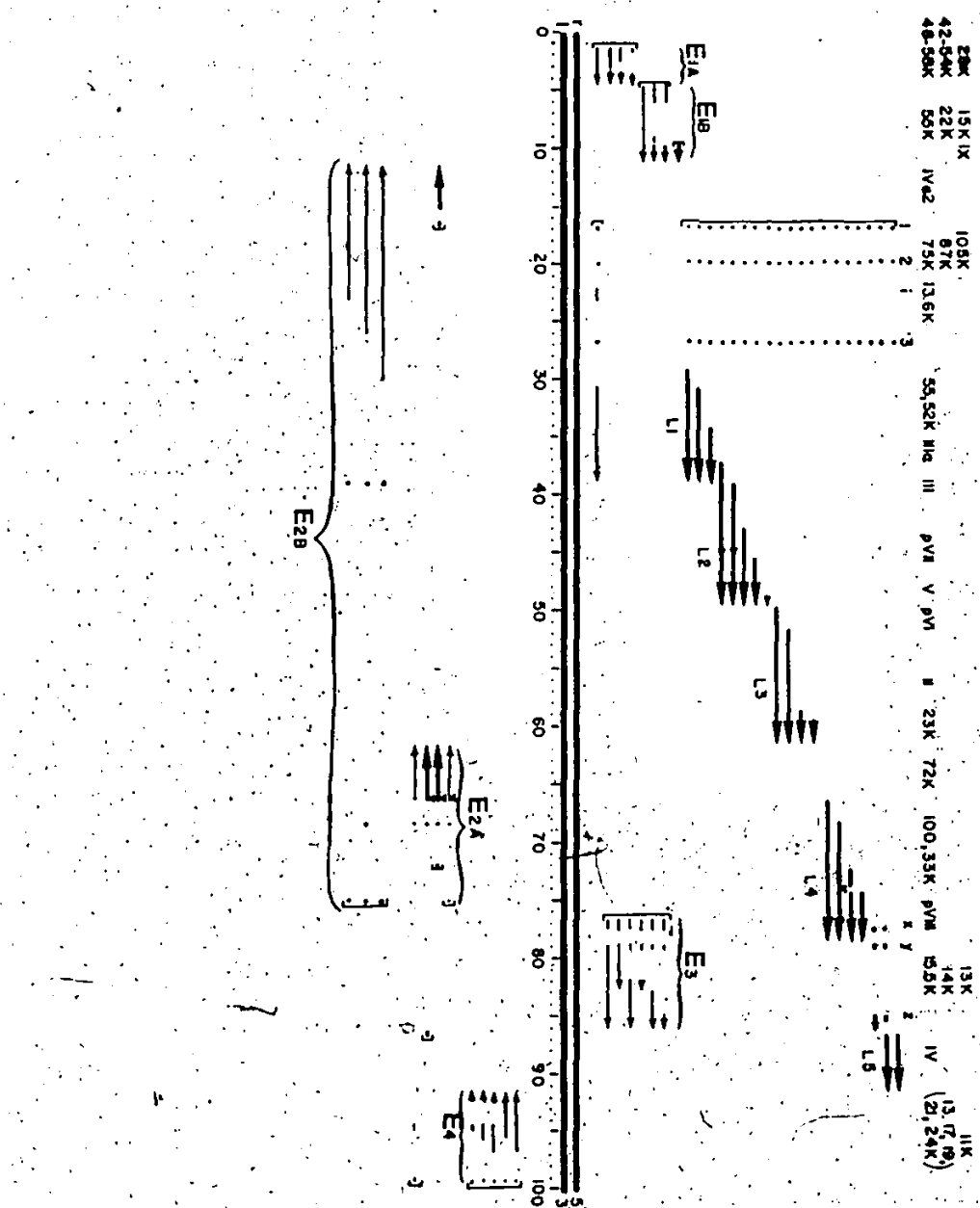
1.2.1 Classification

The adenovirus family comprises over 80 serotypes, of which 39 are human isolates and the rest are of animal origin. Based on at least four different criteria, the human serotypes have been classified into six different groups (Table 1:1). The most widely used subgrouping scheme is based on the ability of specific serotypes to partially or completely hemagglutinate rhesus or rat erythrocytes (Rosen, 1960). Other classification schemes have been based on oncogenicity (Green, 1970), percentage of G and C content (Pina and Green, 1965), genome homology (Green et al., 1979), morphology (Norrby, 1969) and on restriction endonuclease cleavage patterns (Wadell et al., 1980). Of the 39 human serotypes, adenovirus type 2 and type 5 (Ad2 and Ad5) are the most intensively studied, and therefore the remainder of this section will focus on them.

1.2.2 Genome Structure and Organization

The genome of adenoviruses is a linear double-stranded DNA molecule ranging in size from 30 to 36 kb. Figure 1.1 shows the principal transcriptional organization of the Ad2 genome, which is generally very similar for different adeno serotypes. It consists of several non-contiguous blocks of early genes (transcribed early in infection) and one major late transcription unit (expressed late in

Figure 1-1 A schematic drawing, showing the principal transcriptional organization of the Ad2 genome. The genome is subdivided into 100 map units (mu). The r-strand and l-strand are transcribed into RNA right- and left-ward, respectively. Arrow heads show the 3' polyadenylation sites while the promoters are indicated by brackets. Gaps in arrows represent intervening sequences in the RNA transcripts; the early RNA's are indicated as E1a, E1b, E2a, E2b, E3 and E4, whereas the late RNA's are designated by L1, L2, L3, L4, and L5. Proteins are designated by their molecular weights in Kilodaltons (Kd) or by Roman numerals. Also shown are the three segments which are spliced to form the tripartite leader (1,2,3) and the VA-RNA I and II. modified from Tooze (1981).


E1A
E1B
E2B
E2A
E3
E4
L1
L2
L3
L4
L5
0
10
20
30
40
50
60
70
80
90
100

infection). All of the adenovirus serotypes studied so far contain a short (102 to 164 bp) inverted terminal repetition (ITR) (Garon et al., 1972; Wolfson and Dressler, 1972; Van Ormondt et al., 1978; Steenbergh and Sussenbach, 1979, Shinagawa and Padmanabham, 1980). In addition, a 55 Kd protein is covalently attached to each 5' end of the adenovirus genome (Robinson et al., 1973; Rekosh et al., 1977; Desiderio and Kelly, 1981). Lastly, by convention, the adenovirus genome is divided into left and right halves based on its GC-content, and the strand transcribed to the right is called the r-strand whereas the leftward-transcribed strand is designated as the l-strand; the genome has been further divided into 100 map units (mu) from left to right as illustrated in Fig 1.1.

1.2.3 The Lytic Cycle

The vast majority of studies on the lytic cycle of adenoviruses (reviewed by Tooze, 1981; and Sharp, 1984) have been carried out in Ad2 or Ad5 infected human cell lines (KB or HeLa) in culture. The productive cycle (Fig. 1.2) is initiated when the viral DNA reaches the infected cell nucleus, approximately half an hour post adsorption. Transcription from early regions E1a, E1b, E3 (located on the r strand), and E2a, E2b and E4 (on the l-strand) commences in a defined order prior to the onset of viral DNA replication (Fig. 1.2). Transcripts from E1a (pre-early), and the promoter proximal segment (L1) of the major late promoter have been detected as early as 45 minutes postinfection (pi), followed by those of early regions E1b, E2 (from a promoter located at map position 75) E3 and E4 at about 1.5 to 2 hr pi (Jones and Shenk, 1979b; Berk et al., 1979; Nevins, 1981).

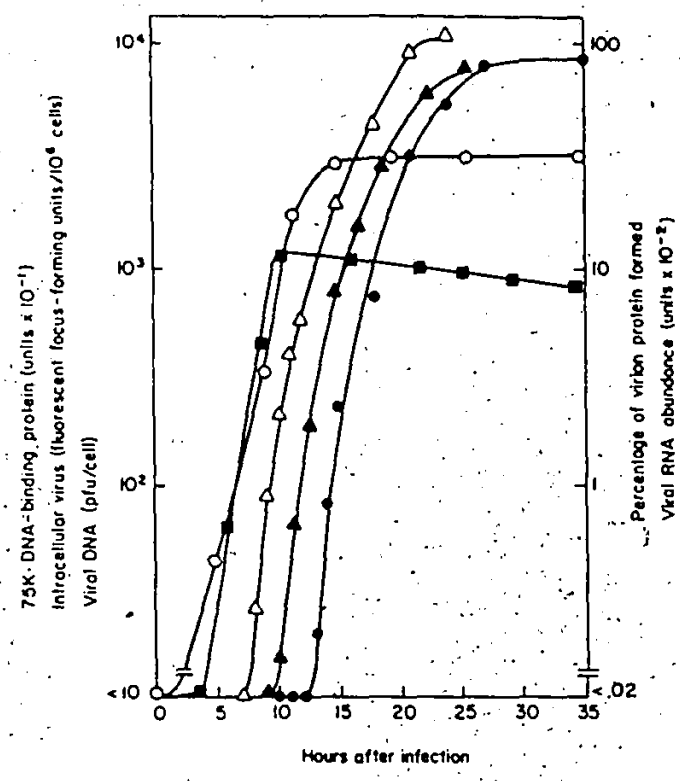


Figure 1-2 Time course of adenovirus-2 productive infection in suspension culture of KB cells.(●) intracellular virus measured as fluorescent focus-forming units/10^6 cells; (O) total virus-specific RNA measured by hybridization of labelled RNA to adenovirus-2 DNA; (Δ) synthesis of viral DNA (data from Green et. al., 1971); (▲) virion protein (hexon antigen measured by complement fixation; (■) 75K DNA binding protein. (Reprinted from Tooze, 1981).

All the viral genes are transcribed by the cellular RNA polymerase II, except for the virus-associated (VA) RNAI and VA-RNAII, which are transcribed by RNA polymerase III. Each of these primary transcripts gives rise to a family of mRNA species upon RNA splicing (Fig. 1.1), a mechanism found in all higher eukaryotes (Breathnack and Chambon, 1981) and first detected in the adenovirus system (Chow et al., 1977; Kitchingman et al., 1977; Berget et al., 1977). All of these early mRNAs are translated to give rise to early proteins (the function of the early products will be discussed subsequently), with the sole exception of the VA-RNA and the L1 transcript. Approximately six to eight hours post infection, with the onset of viral DNA replication, transcription from all the early regions increases three to tenfold. This increase is thought to be due to the augmentation in the number of templates (Shaw and Ziff, 1982).

Viral DNA replication (reviewed by Challberg and Kelly, 1982, Kelly, 1984, and described in more detail in chapter 6) proceeds by a semi-conservative, strand-displacement mechanism, initiating at either end of the genome, and reaches its maximum rate at about 19 hr pi (Fig. 1.2, and 1.3). By 24 hr, approximately 10^5-10^6 progeny viral genomes are made, of which only about 20% are ultimately packaged into viral particles (Green et al., 1970). Viral DNA replication (Fig. 1.3) requires three 1-strand encoded early proteins. These are the E2a encoded 72 Kilodalton (kd) DNA binding protein (DBP) (Chow et al., 1979a; Lewis et al., 1976), the E2b encoded 87 Kd pre-terminal proteins (pTP) (Stillman et al., 1981; Alestrom et al., 1982; Gingeras et al., 1982; Smart and Stillman, 1982) and the E2b encoded viral DNA polymerase (140 Kd) (Stillman et al., 1982a; Lichy

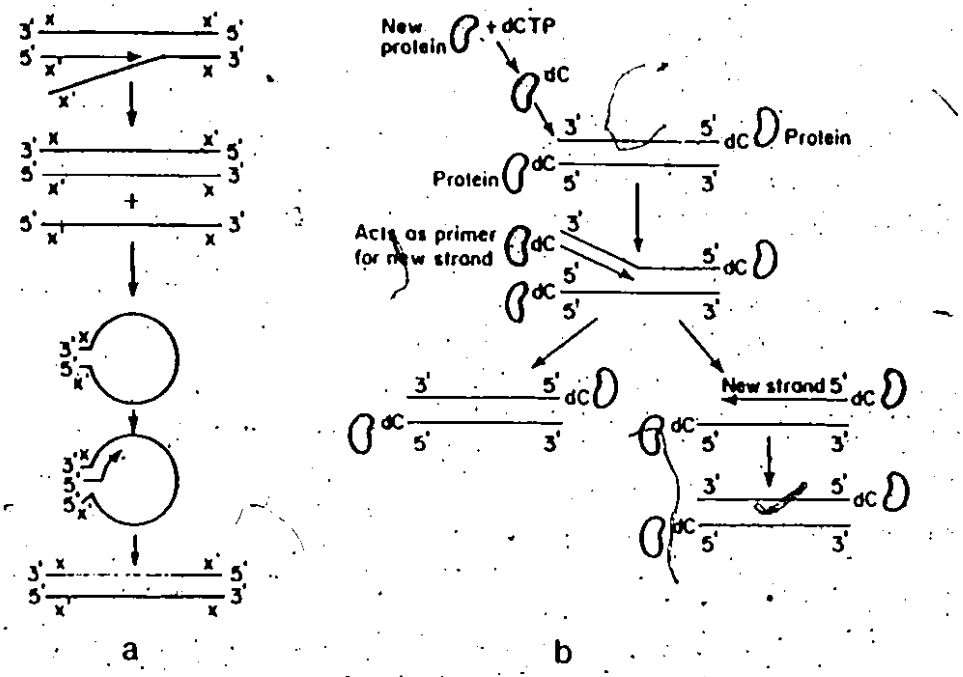


Figure 1-3 (a) A model of synthesis of adenoviral DNA (Daniell, 1976) in which synthesis of a new strand displaces one parental strand. If the sequences of the inverted terminal repetition base-pair to form the panhandle intermediate shown, then the double-stranded panhandle has the same terminal structure as parental viral DNA and will presumably be recognized by the enzyme complex responsible for initiation of viral DNA synthesis. Although not shown here, r-strands displaced during the first step in synthesis could obviously form an analogous panhandle intermediate, with the same terminal double-stranded sequence.(b) A similar displacement mechanism in which the possible role of the 5'-terminal protein in initiation of adenoviral DNA synthesis is illustrated, (redrawn from Rekosh et. al., 1977). (Reprinted from Tooze, 1980).

et al., 1982). Daughter strands are initiated at or near the termini and are presumably primed by a dCTP residue, covalently linked to the pTP (Rekosh et al., 1977; Pincus et al., 1981). The DBP appears to be multi-functional. Firstly, it is involved in initiation and elongation (Van der Vliet and Sussenbach, 1975; Van der Vliet et al., 1977; Horwitz, 1978), and secondly, in the down-regulation of its own as well as other early gene expression, presumably by decreasing mRNA stability (Babich and Nevins, 1981; Nevins and Winkler, 1980; Carter and Blanton, 1978a, 1978b).

With the onset of viral DNA replication, the transcription pattern from E2 and the major late promoter (MLP) changes. Transcription of the E2 region switches to a second promoter located at map position 72. On the other hand, transcripts from the MLP, which normally terminate at map position 40 early in infection (Lewis and Mathews, 1980; Shaw and Ziff, 1980; Akusjarvi and Persson, 1981) extend to include the entire r-strand following the onset of DNA replication (Fraser et al., 1979). Upon processing, these long transcripts are joined to a common tripartite leader sequence, with each primary transcript giving rise to one of five families of late mRNA each with a common 3' terminus generated by endonucleolytic cleavage and polyadenylation within the primary transcript (Fraser et al., 1979; Shaw and Ziff, 1980). The tripartite leader encompasses 203 nucleotides of 5' untranslated messages, including a sequence analogous to the Shine-Dalgarno (1974) sequence in prokaryotes (Tooze, 1981). A fourth leader segment known as the "i" leader is incorporated into some mRNAs (Chow et al., 1979b; Akusjarvi and Persson, 1981). Maximal rates of late protein synthesis occur at

approximately 15 hours pi (Russell and Skehel, 1972; Walter and Maizel, 1974). During the late phase, messages with the tripartite leader are preferentially translated, and the relative rate of their translation is directly proportional to the abundance of the mRNA in the infected cells' cytoplasm (Lawrence and Jackson, 1982).

The low molecular weight non-translated RNAs, VA-RNAI and VA-RNAII accumulate to very high levels during the late phase of infection. It has been suggested (Mathews, 1980) that the VA-RNAs play a role in the RNA splicing reaction by acting as an adaptor, by binding to the 5' and 3' sequences of exons leading to the formation of a loop, thus facilitating subsequent cleavage and ligation reactions. Recently, however, Thimmappaya et al.(1982) have shown that a mutant virus lacking VA-RNAI was defective for efficient translation of late viral mRNA, and only 1/20th of wt levels of late proteins were synthesized. On the other hand, a mutant virus defective in VA-RNAII grew as well as the wt virus. This led to the proposition that the VA-RNAI is essential for efficient translation of late mRNA. How the VA-RNAI exerts such an effect is not clear as yet.

1.2.3.1 Virus Assembly and Packaging Constraints

The assembly of virions (reviewed by Philipson, 1984) commences at about 12 to 15 hours post infection in the nuclei of infected cells. The process by which the structural polypeptides are put together has not yet been well characterized. Analysis of viral DNA sequences associated with particles of densities intermediate between empty and complete virions suggested that packaging of the viral

genome proceeds with left end polarity (Tibbetts, 1977; Hammarskjold and Weinberg, 1980; Hearing and Shenk, 1983) into a preformed, immature, isometric capsid (Sundquist et al., 1973; Edvardsson et al., 1976). Maturation of virions requires the proteolytic cleavage of several virion components; these are pVI, pVII, pVIII and the preterminal protein (Weber, 1976).

One of the fundamental structures of all virions is their capsid which encloses the nucleic acid. Capsids are made up of identical protein subunits. There are two ways in which identical asymmetrical subunits can be assembled to build stable, regular capsids: helical or isometric assemblies. In the case of adenoviruses, the capsid is isometric (an icosahedron). The stability of the final assembly depends largely on the number and strength of the bonds between the subunits in the capsid, and the internal volume of the capsids is rarely much greater than the volume of the genome it must hold. Viewed in these terms, the tolerance for packaging greater amounts of nucleic acid than the viral genome size is rather limited when the isometric capsids are considered. Analysis of the genomic size of various adenovirus mutants has suggested that, with respect to the lower limit, there seems to be no extreme constraint since a number of deletions have been isolated or constructed that range from as little as a few base pairs (reviewed by Young et al., 1984) to as much as 5.5 Kb or about 15% of the viral genome (this thesis). Furthermore, a defective adeno-SV40 hybrid has been isolated with a net deletion of 12 Kb or 33% of the viral genome (Tooze, 1981).

In contrast, there seem to be severe constraints on the maximum size of a DNA molecule which can be accommodated within the

virion. This conclusion is supported by the following observations: first, insertions of SV40 DNA sequences in SV40-Adeno hybrids have resulted in concomitant deletion of adeno sequences (usually in early region 3) such that the net change in viral DNA size is relatively small (reviewed by Tooze, 1981; Klessig, 1984). Jones and Shenk (1978) have isolated several substitutions and insertion mutants that resulted in a net insertion of about 0.4 to 1.7 Kb or 1.2 to 4.7%. On the other hand, Graham (1984b) has succeeded in inserting up to 2.2 Kb or 6% into an Ad5 mutant (dl309); this currently represents the maximum amount of DNA that has been successfully packaged into infectious Ad5 virions. Generally it has been observed that isometric capsids can package only up to about 5% over the normal genomic size, a finding which is well documented for the lambdoid bacteriophages (Hendrix et al., 1982). In contrast to viruses with icosahedral capsids, viruses lacking icosahedral symmetry such as poxviruses (Dales and Pogo, 1981) can package more than 5% over and above the normal genomic size. For instance Smith and Moss (1983) have succeeded in inserting 24.7 Kb into the vaccinia virus genome (187 Kb) or 13.2%.

1.2.3.2 Host-Cell Metabolism During Infection

Infection with an adenovirus causes a severe inhibition of host cell protein and DNA synthesis (Ginsberg et al., 1967; Pina and Green, 1969). This selective inhibition is coincident with the onset of viral DNA replication, starting 6 to 8 hr pi and is virtually complete by 12 hr after infection. On the other hand, the host cell transcription machinery is not inhibited and cellular RNA accumulates

in the nucleus during infection suggesting a preferential transport of viral mRNA to the cytoplasm (Price and Penman, 1972; McGuire et al., 1972). Thus, adenoviruses seem to rely on an efficient mechanism to redirect the host cell machinery to the synthesis of their macromolecules.

1.2.4 Early Region Gene Product Functions

1.2.4.1 Early Region 1

Figure 1-4 summarizes the genetic and physical map of early region 1 (E1); its structure and transcription map have been reviewed by Tooze (1981), Graham et al.(1983), and Sharp (1984). E1 which maps to the left-most end of the genome (1.3 to 11.1 map units) on the r-strand contains two independently promoted early transcription units: E1a and E1b. As is the case with adenovirus transcripts in general, the use of the coding capacity of this region is maximized by the process of RNA splicing to generate multiple mRNA species.

E1a encodes 3 mRNAs, 9s, 12s and 13s; all three mRNAs share a common 5' cap site (located at position 499 from the left end) and a 3' poly (A) addition site (positioned at 1609 bp from the left end) and differ only in the size of the intervening sequences removed by splicing. Based on the DNA sequence, the 13s and 12s messages should theoretically generate two proteins, 289 and 243 amino acids long (MW 32 Kd and 26 Kd respectively), differing only in the presence of a 46 amino acid segment unique to the larger protein. Viral proteins in general, and those encoded by E1 in particular, have been identified by using a variety of techniques (reviewed by Graham et al., 1983,

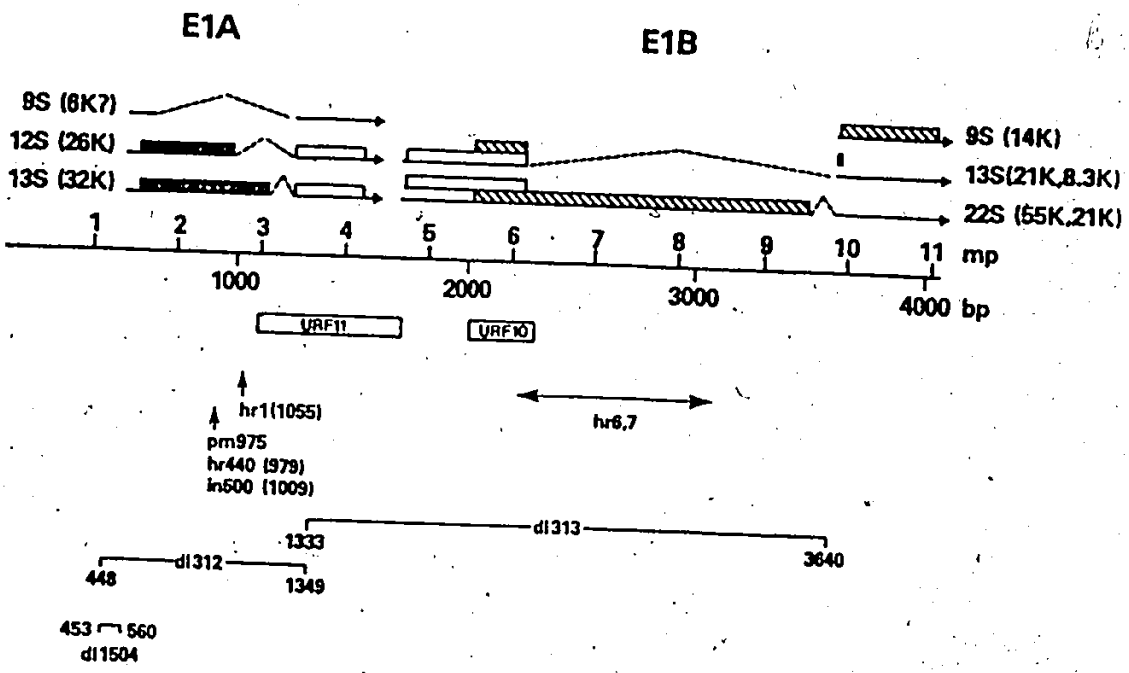


Figure 1-4 Organization of early region E1. Genetic and physical map of the left end 11.5% of E1 from group C adenoviruses. Top: r-strand transcripts; open translation reading frames 1, 2 and 3, represented by open, closed, and hatched bars respectively. Bottom: Unassigned reading frames URF10 and URF11 represent two large open reading frames on the l-strand. Theoretical molecular weights for E1 proteins are indicated in brackets, also indicated below are several E1 mutants of Ad5 (reprinted from Graham, 1984a).

1984a), including immunoprecipitation of viral antigens from transformed or infected cells using antisera from tumor bearing animals or sera raised against synthetic polypeptides. These studies have indicated that the 12s and 13s messages give rise to families of acidic proteins (Smart et al., 1981; Rowe et al., 1983): the 12s generates at least 3 proteins, one major (MW = 50 Kd) and two minor species (MW = 45 Kd and 35 Kd), and similarly, the 13s generates one major (52 Kd) and two minor species (48.5 Kd and 37.5 Kd). At present, it is not clear what is the biological significance of these multiple species, nor which post translational modification is responsible for this heterogeneity. In contrast to the very early appearance of the 12s and 13s, the Ela 9s mRNA is detected late in infection and its translation generates a 6.1 Kd protein (Virtanen and Petterson, 1983).

Transcription of Elb gives rise to a single early transcript, the processing of which generates two messages with sedimentation coefficients of 22s and 13s, again with common 5' and 3' ends. Both of these messages encode a theoretical 21 Kd protein (often referred to as 19 K) initiating from the first AUG in reading frame 1. The 22s message also encodes a 55 Kd protein (known as 58 K) in reading frame 3, starting from an internal AUG (position 2017) (Lassam et al., 1979; Green et al., 1979; Yee et al., 1983; Rowe et al., 1984). The relative amounts of these two messages change during the lytic cycle, with very little of the 13s species present early, and abundant amounts present late in infection (Spector et al., 1978; Wilson et al., 1979; Wilson and Darnell, 1981). More recently, however, Anderson et al. (1984) have identified two additional proteins (a 16

Kd, and a 18 Kd), which appear to have the same amino-end as the 58 K protein (Anderson et al., 1984). While the role of the 19 K and the 58 K proteins in mammalian cell transformation is well established (reviewed recently by Branton et al., 1985), their function as well as the function of the two newly identified polypeptides (the 16 and 18 Kd) during the lytic cycle remain unclear.

However, the E1 proteins are known to be involved in the oncogenic transformation of mammalian cells by adenoviruses (reviewed by Graham, 1984a; Branton et al., 1985). In addition, the E1 and more precisely the E1a gene product(s) are required for the activation of most, if not all, of the remaining viral genes (reviewed by Logan and Shenk, 1982; Sharp, 1984). Regulation by the E1a proteins was first detected by Berk et al. (1979) and Jones and Shenk (1979a,b) during the characterization of Ad5 host range mutants. These and other E1 mutants (reviewed by Young et al., 1984) grow as well as the wild type virus in cells constitutively expressing E1 gene products (293 cells, Graham et al., 1977; Aiello et al., 1979; Spector et al., 1980), but grow very poorly in other cultured human cell lines such as HeLa.

One or more of the E1a gene products facilitate expression of E1b, E2, E3, E4 and L1 mRNAs. This was indicated by the analysis of steady state levels of mRNA in virus infected HeLa cells (Berk et al., 1979; Jones and Shenk, 1979b). Viruses with mutations within E1a produced dramatically reduced levels of all other early mRNAs and, as expected, the proteins encoded by these early messages were also reduced (Ross et al., 1980). Nonetheless, the E1a products are not absolutely required for the expression of the other viral transcription units. Normal virus yields are produced when HeLa cells

are infected at high multiplicities (over 500 plaque forming units per cell) with E1a mutants (Shenk et al., 1980), and even at low multiplicities. These mutants are capable of growth after long periods of time as compared to the wild type (Nevins, 1981). It seems clear that control by E1a occurs at the level of transcription initiation but beyond this, the mechanism remains obscure.

A third independently promoted region in E1 is located on the r-strand at the 3' end of E1b (9.7 to 11.2 mu). A 9s unspliced mRNA is transcribed from a promoter (positioned at 3546) lying within the intron common to both the 13s and 22s E1b messages. This message shares the 3' poly (A) site with the E1b transcripts (position 4061) and encodes polypeptide IX (Alestrom et al., 1980). Although the entire coding sequence for protein IX is present in both the 13s and 22s messages, protein IX is not made from either of these messages. Protein IX is a minor constituent of the adenovirus capsid (Anderson et al., 1973); it is expressed at an intermediate time after infection and is found to be associated with the hexons that constitute the adenovirus icosahedron capsid (Maizel et al., 1968b; Everitt et al., 1973). Boulanger et al. (1979), and Oostrum and Burnett (1985) have estimated that there are about 280 protein IX molecules per virion, and that they are located within the large cavities created by hexon molecules symmetrically arranged on each facet (Fig. 1.5). Therefore, it has been suggested that protein IX plays a cementing (or stabilizing) role in the capsid structure (Everitt et al., 1975). Nonetheless, deletion mutants of Ad5 have been described that are capable of assembling virus particles despite the absence of protein IX (Colby and Shenk, 1981). Mutant virions are, however, heat labile,

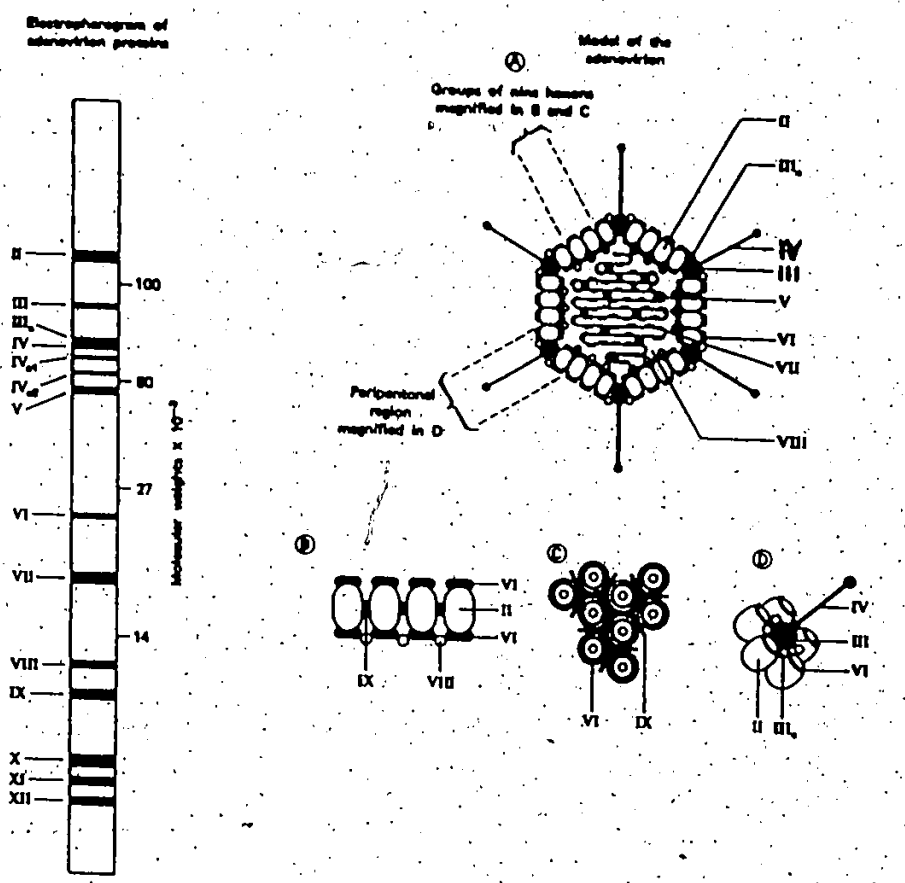


Figure 1-5 Adenovirus proteins. On the left is an idealized electrophoretic separation of the virion proteins. On the right is a hypothetical model of the virion, and the location of its proteins in the particle. Proteins V and VII (present in a molar ratio of 1 to 5) are internal (core) basic polypeptides.

(A) Schematic view of a vertical section of the virion, with the various proteins indicated by Roman numerals.

(B) A vertical section through a group of nine hexons showing the tentative location of proteins VI, VIII, and IX.

(C) A horizontal view from the outside of the group of nine hexons showing the tentative location of proteins VI and IX.

(D) A magnification of the peripentonal region showing the proteins II (hexon), III (penton base), IIIa, IV (fiber), and VI. Reprinted from Luria et al. (1978).

thus reinforcing the idea that protein IX is involved in the maintenance of the adenovirus capsid structure possibly by strengthening the bonds between the hexon capsomers.

1.2.4.2 Early Region 2

Early region 2 (E2) is located between map coordinates 75 and 11 on the l-strand (Fig. 1.2). Transcription from this region initiates from two alternative promoters, at 75 map units early in the lytic cycle, and at map coordinates 72 late in infection (Chow et al., 1979b). It is not clear why different promoters are used before and after the onset of viral DNA replication. E2 has been subdivided into two regions, E2a and E2b. E2a encodes a 72 Kd DNA-binding protein whereas E2b encodes two polypeptides, the 87 Kd precursor for the terminal protein, and the viral DNA polymerase (Stillman et al., 1981). E2, therefore, appears to resemble an operon for proteins involved in viral DNA replication. The function of the E2 products has been discussed in detail along with viral DNA replication (earlier in this section and in chapter 6).

1.2.4.3 Early Region 3

Early region 3 (E3) is located between map coordinates 76.6 and 86.0 on the r-strand, and encodes at least eight mRNA species (Chow et al., 1979b; Kitchingman and Westphal, 1980; Persson et al., 1980). All of the E3 messages share a common 5' leader (from map coordinates 76.6 to 77.6) joined to an acceptor site at map position 78.6 whereas the 3' ends of these transcripts vary (see Fig. 1-6; reviewed by Sussenbach, 1984). Furthermore, nucleotide sequence analysis of this

region (Herisse et al., 1980) revealed that a TATA box is located at map position 76.5, and that two poly (A) addition sites are located at map coordinates 82.6 and at 85.6 (Cladaras and Wold, 1985; Cladaras et al., 1985).

In light of the large numbers of messages assigned to E3, it is surprising that thus far only two proteins have been identified, namely a 14 Kd protein and a 19 Kd glycoprotein (reviewed by Pettersson, 1984). An additional 14.5 Kd polypeptide has also been assigned to E3, but it is unclear whether the 14 and 14.5 Kd proteins are structurally related or not (Persson et al., 1980). Furthermore, DNA sequence analysis has revealed the existence of several open translational reading frames which could theoretically encode polypeptides greater than 10 Kd (Herisse and Galibert, 1981; Herisse et al., 1980; Cladaras and Wold, 1985; Cladaras et al., 1985).

Immunoprecipitation with a monospecific serum against the 19 Kd glycoprotein and the 14 Kd polypeptide revealed that the synthesis of these products begins 2 hr after infection, and achieves a maximal rate at 4-6 hrs post infection, and later declines. It has been proposed that the 19 Kd glycoprotein is associated with the class I major histocompatibility antigens in transformed cells and in productive infection (Kvist et al., 1978; Signas et al., 1982). This suggestion is based on the fact that antisera against either the 19 Kd or the Heavy Chain of clase I histocompatibility antigens, precipitate a complex between the two components. Furthermore, it has been proposed that this form of interaction forms the basis for the recognition of adenovirus infected cells by cytotoxic T cells (reviewed by Pettersson, 1984). The 14 Kd protein is located in the

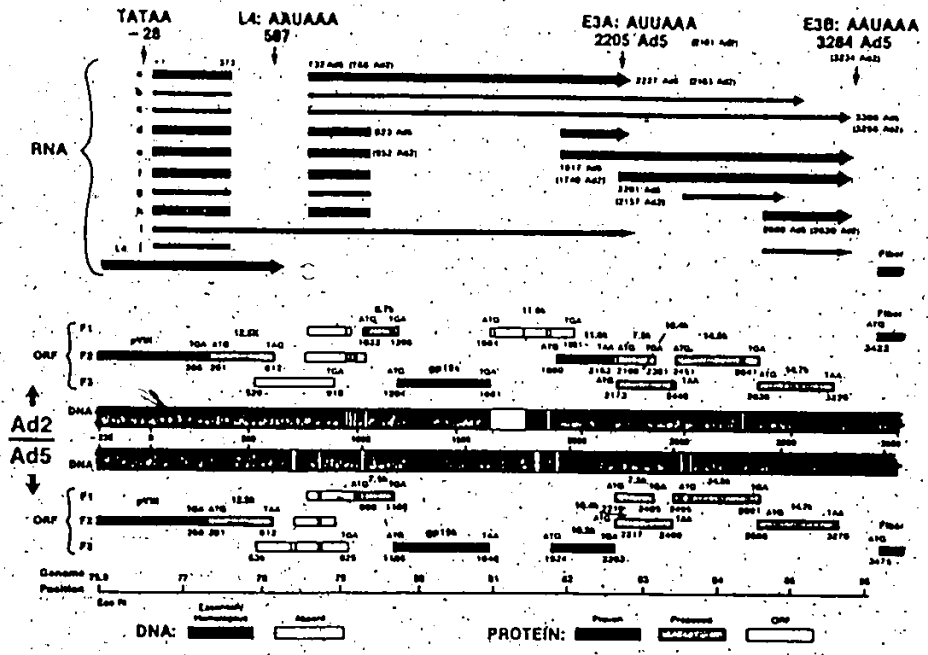


Figure 1-6 Schematic representation of the E3 transcription unit of Ad5 and Ad2. The DNA sequences of Ad5 and Ad2 are represented by heavy black bars. The gaps indicate regions where differences exist between Ad2 and Ad5. The bars above and below the DNA sequence represent open reading frames (ORFs) for proteins that are conserved in both viruses. Black bars are detected proteins whereas stippled bars indicate theoretical proteins. The location of the 3' ends, 5' ends, and spliced sites are also indicated. This information is from the l-strand (the sense strand), from Cladaras et. al., (1985).

cytoplasm and is completely unrelated to the 19 Kd glycoprotein; the two products are encoded by non-overlapping regions of E3.

In spite of all the above, E3 appears to be dispensable for virus growth *in vitro*, since viruses lacking all or most of E3 are still viable and able to replicate in cultured cells (Tooze, 1981; Berkner and Sharp, 1983; Klessig, 1984; Saito et al., 1985; and in this work). However, the fact that this region remains undeleted in natural adenovirus populations, suggests that it may confer a selective advantage for growth or persistence *in vivo*.

1.2.4.4 Early Region 4

Early region 4 (E4) is located on the l-strand at the extreme right end of the viral genome between map positions 91.3 and 99.0. A large number of mRNAs have been identified by EM and nuclease-S1 analyses; all mRNAs appear to have common 5' and 3' sequences, but differ in the location of splice sites (Berk and Sharp, 1978). Translation of these mRNAs gives rise to 8 polypeptides ranging in size from 11 to 35 Kd. Mutant viruses with lesions in E4 appear to be defective in their expression of certain late proteins, suggesting that E4 might be involved in the regulation of some late genes (reviewed by Sussenbach, 1984; Pettersson, 1984). In addition, mutant viruses defective in one of the E4 gene products (a 24 Kd polypeptide) appear to be deficient in shutting off the host cells' protein synthesis (Challberg and Ketner, 1981); the phenotype of this mutant resembles the E1b mutant described by Babiss and Ginsberg (1984). Sarnow et al. (1982) have shown that the E1b 58 Kd protein forms a physical complex with the 24 Kd E4 polypeptide, during productive

infection of HeLa cells with Ad5; apparently, this physical complex between the two proteins may mediate the efficient shutoff of the host's protein synthesis (Babiss and Ginsberg, 1984).

1.3 Gene Transfer Into Mammalian Cells

Much of our current knowledge on eukaryotic gene expression can be attributed directly to our ability to isolate and manipulate in vitro the genetic information, and subsequently introduce altered DNA back into the cellular genome. At present there are several techniques available for transferring genes into mammalian cells (for review see Gluzman, 1982; Watson et al., 1983; Anderson, 1984; Old and Primrose, 1985). These are: 1) fusion of DNA-loaded membranous vesicles such as liposomes; this technique is the least well developed for DNA transfer and will not be discussed further; 2) electroporation; 3) microinjection; 4) the calcium phosphate technique; and recently 5) viral based vectors. Each of these techniques is valuable for certain types of experiments but none can yet be employed to efficiently deliver a gene into a specific chromosomal site in a target mammalian cell. Electroporation (Neuman et al., 1982) which is a relatively new procedure, involves the transport of DNA directly across a cell membrane made permeable by an electric current. It has been used to transfer a variety of genes into a number of different cells in vitro including the immunoglobulin K gene into B cells (Neuman et al., 1982; Brinster et al., 1983). Microinjection, on the other hand, has been most often used, for a number of years, for the transfer of genes into fertilized eggs (Gordon and Ruddle, 1981; Wagner et al., 1981a; Palmiter et al., 1982,

1983). However, in spite of the success of microinjection, this technique has its limitations. Firstly, only one cell at a time can be injected, thus treatment of very large numbers of cells (eg for biochemical studies) becomes an impossible task. Secondly, treatment of fertilized eggs by microinjection can produce deleterious effects because, as stated previously, there is no control over where the injected DNA will integrate in the genome (Lacy et al., 1983). Thirdly, such a procedure requires some fairly sophisticated equipment, and extensive practice in order to master this tedious technique.

The fourth mode of gene transfer, is via the calcium phosphate procedure of Graham and Van der Eb (1973). This technique is well established (Graham et al, 1980; Graham and Bacchetti, 1983; McKinnon and Graham, 1986), and is by far the most widely used procedure; it continues to be the method of choice for most gene transfers into mammalian cells in vitro. A large number of genes have been transferred using this technique, including the herpes simplex TK gene (Bacchetti and Graham, 1977; Wigler et al., 1977; Graham et al., 1980), the DHFR gene (Cline et al., 1980; Kaufman and Sharp, 1982; Carr et al., 1983) and the neo^r gene (Colbere-Garapin et al., 1981; Southern and Berg, 1982), to name a few. The efficiency of the process varies considerably with the type of recipient cells (Graham et al., 1980), and attempts to obtain transfected cells without selective pressure have generally failed. The major disadvantage of this procedure, however, is its inefficiency. Under optimal conditions, one cell in 10^3 to 10^4 can be transformed in monolayer, and an even lower efficiency is obtained with cells in

suspension (only about one cell in 10^6 or 10^7 becomes transformed) (Carr et al., 1983). Nonetheless, the calcium phosphate technique continues to be the most frequently used method for all gene transfers into mammalian cells in vitro, and it has been an important tool in much of the development and construction of eukaryotic viral vectors; its practical use in gene therapy, however, still remains to be demonstrated. Therefore, in order to develop a new approach for delivering genes efficiently both in vitro and in vivo , and into cells which are refractory to other methods, viral based vectors are receiving considerable attention. Recombinant viral vectors may offer several advantages over the physical and biochemical procedures described above. Among these advantages are a high efficiency of gene transfer in vitro , and possibly in vivo ; secondly, the ability to introduce one to many copies of a gene per cell. Thus, viral based vectors not only would facilitate studies on gene expression but also might improve the prospects for gene therapy, and bring the engineering of recombinant viral vaccines within reach.

1.3.1 Studies on Gene Expression

Some viruses lack an in vitro propagation system; examples are the human papilloma viruses (HPV) and the hepatitis B viruses (HBV). Therefore, most of the knowledge of their genomic structure and functions comes from the analysis of viral DNA cloned in bacteria (Tiollais et al., 1981; Kleid et al., 1981; Will et al., 1982). To study gene expression on episomally and actively replicating molecules that might mimic more closely the in vivo situations, virus based vectors offer distinct advantages over the previously mentioned

procedures for the functional analysis of eukaryotic genes (Will et al., 1984; Shih et al., 1984; Cochran et al., 1985; Davis et al., 1985).

In addition, the encapsidated pseudovirus will infect rapidly and reproducibly the great majority of the infected cell populations, and with viral infection it is possible to ensure that each host cell has a few to many copies of the foreign gene (Frankel et al., 1985; Asano et al., 1985). Moreover, since the molecular biology of DNA and RNA tumor viruses is extensively studied, their life cycle is well known and, depending on the infected host cells, they could either replicate or integrate into the host genome (reviewed by Tooze, 1981; Weiss et al., 1982). Therefore, a viral vector carrying a given gene can be used to either establish transformed cell lines that express the desired protein, or be employed to overexpress that protein upon lytic infection of permissive cells (Van Doren and Gluzman, 1984; Van Doren et al., 1984; Emerman and Temin, 1984; Berkner and Sharp, 1984, 1985; Yamada et al., 1985)

1.3.2 Gene Therapy

Gene therapy is the correction of a genetic defect by introducing a normal gene into an organism; such a procedure may be within reach, thanks to viral based vectors (reviewed by Anderson, 1984; Kolata, 1984). In recent years gene therapy has been carried out with some success in Drosophila (Spradling and Ruben, 1982), and in mice (Hammer et al., 1984). There are a number of advantages of vectors derived from viruses. Firstly, up to 100% of cells can be infected whether in suspension or in a monolayer. Secondly, under appropriate

conditions the DNA of such viral vectors can integrate as a single copy at a single albeit random site (reviewed by Panaganiban, 1985; Van Der Putten, 1985), whereas the previously described techniques often result in the insertion of multiple copies (Abraham et al., 1982; Miller and Temin, 1983). Thirdly, it is often possible to engineer the vector so that infection and long term harboring of the vector can be accomplished without harming the host cells, Using retrovirus based vectors, several groups have successfully transferred functional genes (neomycin, neomycin and the dihydrofolate reductase) into mouse hematopoietic progenitor cells (Joyner et al., 1983; Williams et al., 1984). However, as to how soon reliable gene therapy might be available for the treatment of some human genetic diseases depends to a large extent on the development of suitable delivery systems. Ultimately, an ideal vector not only would have to be stable but also tissue specific, and site specific. In other words, it should deliver the DNA to a predetermined chromosomal site. Thus, much work must be done before these goals can be met. While prospects for human gene therapy might be premature, the possible application of viral based vectors in the development of recombinant vaccines is not.

1.3.3 Vaccines

One exciting possibility within reach of clinical application is the use of virus-based vectors to generate specific immunity (Brown et al., 1986). For instance, a gene encoding a major surface antigen for the hepatitis B virus can be introduced into a viral based vector. Animals inoculated with such hybrid viruses should be able to produce

antibodies against the hepatitis B virus surface antigen, consequently, conferring immunity against the hepatitis B virus. Genetically engineered vaccines are especially attractive for the control of diseases caused by viruses or other pathogens which do not grow or grow poorly in tissue culture, and for viruses with oncogenic potential. Perhaps the most important aspect of this technology lies in the ability to produce antigens without the requirements to culture infectious pathogenic agents or to have the complete viral genetic information present.

Problems encountered with conventionally prepared viral vaccines are: firstly, ensuring attenuation of the vaccine virus strain, or ensuring a complete killing of the virus in the case of killed virus vaccines and secondly, ensuring that all potentially oncogenic genetic materials are removed. Thirdly, some viruses either do not grow in tissue culture such as the hepatitis B virus, or cannot be grown to titres high enough to provide a sufficient antigenic mass for effective vaccination, such as for the foot-and-mouth disease virus. All or at least most of the above problems can be obviated when genetically engineered vaccines are used (Old and Primrose, 1985). The main problem that might be associated with recombinant vaccines (Bittle et al., 1982), is the cloning of a suitable immunogenic polypeptide into a viral vector and expression in such a way that a maximal and long lasting immunity is achieved.

Vaccinia and related pox viruses have been used to immunize against smallpox for almost two centuries, and have been enormously successful in controlling smallpox. Recently, a number of laboratories have begun to use vaccinia to develop vaccines against diseases caused

by viruses such as hepatitis B (Smith et al., 1983), influenza (Panicali et al., 1983) and HSV (Paoletti et al., 1984). The basic strategy is as follows: firstly, the gene to which immunity is sought (e.g. the glycoprotein D gene of HSV-1) is inserted into a plasmid under control of a vaccinia virus promoter, then is rescued into the vaccinia virus genome by *in vivo* homologous recombination (as described subsequently, in section 1.4.2). The efficacy of the recombinant vaccinia as an HSV immunogen was tested by inoculating mice intraperitoneally, and subsequently, challenging by the same route with HSV-1. It was found that 100% of vaccinated mice survived as compared to 45% of the unvaccinated controls (Paoletti et al., 1984). While recombinant vaccinia has efficacy in protecting mice against HSV-1 challenge, the question is whether this type of vaccine would be effective in humans, since many people have already been exposed to this virus vector by vaccination against smallpox.

There may in any case be advantages in the use of a variety of different vaccination strategies for different agents since it is unlikely that a single vector would suffice to immunize against many agents. Therefore, other viral based vectors are currently under active consideration, such as the adenoviruses. Since the early 1970's, a bivalent (Ad4 and 7), live, enteric vaccine has been regularly used by the military around the world, with demonstrated safety and efficacy; on an average the live enteric vaccines have reduced adenovirus infection in recruits by over 80% (Edmondson et al., 1966; Smith et al., 1970; Top et al., 1971).

1.4 Current State of Development of Viral Based Vectors

As outlined above, the development of mammalian virus vectors has been motivated by a variety of interests. Most of these interests relate to the prospect of reintroducing cloned genes into particular cell types in order to either study gene expression, or to correct a genetic disease or to use these vectors to genetically engineer vaccines. In addition, with the recognition that a number of biologically and commercially important proteins require postranslational modification unique to animal cells, there also has been considerable interest in the use of mammalian vectors to produce high levels of gene products in animal cells. Viral based vectors can be classified under two groups, the retrovirus based vectors and the DNA virus based vectors.

1.4.1 Retrovirus Vectors

Retroviruses are single-stranded RNA viruses. The details of the retrovirus life cycle have been reviewed recently (Varmus and Swanstrom, 1982; Risser et al., 1983). Briefly, during infection, the viral RNA acts as a template for the reverse transcription of the genetic information into double-stranded DNA. This DNA with its long terminal repeats (LTR's) can integrate as a single copy (called a provirus) at a random location in the host genome. The host cell RNA polymerase transcribes the proviral DNA; a portion of the transcript is translated to give the viral proteins required for the generation of viral progeny, and the other portion is packaged as genomic RNA. Viral particles bud off from the host cell and can then reinfect other cells.

Based on the observation of a number of naturally occurring defective viruses as well as from experimental studies, it is known that almost all of the regions encoding viral proteins (gag.,pol., and env.) can be substituted by foreign DNA sequences. Secondly, the infection and long term harbouring of the virus usually does not harm the host cells. Thirdly, retroviruses have a broad host range in vitro and in vivo . These aspects make retroviruses attractive vectors for the delivery of exogenous DNA into vertebrate cells. Various laboratories have used retroviruses for the delivery of genes into mammalian cells in vitro (Perkins et al., 1983; Shimotohno and Temin, 1981; Tabin et al., 1982; Wei et al., 1981; Joyner et al., 1983; Bandyopadhyay and Temin, 1984a, b; Berger and Bernstein, 1985, to name a few).

The general approach is as follows: initially, the proviral DNA for the desired retrovirus (commonly either Maloney murine leukemia virus (MoMLV) or murine sarcoma virus (MSV)) is isolated and cloned into a suitable plasmid; subsequently, the viral genes (gag., pol., and env.) are replaced with a foreign DNA of choice. This chimeric construct is used to transfect cells (usually NIH 3T3) by the calcium phosphate technique (Graham and Van der Eb, 1973). Following infection with a wt helper virus, infectious viral particles possessing both the retroviral vector and the helper virus will bud off into the media.

An improvement on this procedure was introduced by Mann et al. (1983). They constructed a helper virus lacking the packaging sequence (ψ^-), which was thus capable of producing all of the viral proteins but not packaging its own genome. In order to use the

$\bar{\psi}$ helper virus most efficiently, a line of NIH 3T3 cells was established with the helper proviral DNA permanently integrated (Mann et al., 1983). Transfection of this cell line (called ψ-2) with the retroviral vector DNA results 48 hr later in the release of the retroviral vector with the packaging sequence in the supernatant (Williams et al., 1984).

Experimental evidence indicates that retroviruses can be used as a reasonably efficient delivery system. However; a major drawback of this system is that the retroviruses tend to totally or partially delete or rearrange inserted sequences during virus replication (Bandyopadhyay and Temin, 1984a,b; Emerman and Temin, 1984). In addition, with regard to potential application in gene therapy and recombinant vaccines, retroviruses carry strong outward directed promoters in their long terminal repeats (LTRs) which can cause neoplastic transformation by a promoter insertion mechanism (Fung et al., 1981; Hayward et al., 1981; Neel et al., 1981; Payne et al., 1981). These drawbacks might limit the usefulness of the retrovirus based vectors, therefore, other viruses are presently under active consideration.

1.4.2 DNA Virus Vectors

Viruses such as SV40 have been employed as gene transfer vectors for several years (Mulligan et al., 1979; Hamer and Leder, 1979) but in recent years other viruses, such as the bovine papilloma virus (BPV), vaccinia, adeno-associated viruses and herpes simplex viruses have been similarly used (for review see Gluzman, 1982, Old and Primrose, 1985).

A number of laboratories have investigated the possibility of using SV40 as a cloning vector through construction procedures involving recombinant DNA approaches (review by Elder, 1981). In the first approach, a portion of the late region of SV40 was replaced by foreign DNA and the chimeric virus molecule was propagated in the presence of a helper virus with a ts mutation in the early genes (Goff and Berg, 1976; Mulligan et al., 1979). In a variation of the above procedure, foreign DNA was linked to a subset of viral DNA sequences specifying *cis* replication functions and the recombinant DNA was also propagated along with the wt SV40 helper virus (Ganem et al., 1976; Nusbaum et al., 1976; Muzyczka, 1980).

In contrast to SV40 transformed cells, BPV (reviewed in Tooze, 1981) transformed cells contain the viral genome extra-chromosomally so that BPV-based vectors may prove to be useful for maintaining genes in cells in a non-integrated manner. However, because many eukaryotic genes of potential interest often exceed the size limit of several Kb that could be accommodated within the SV40 or BPV virion, it seemed logical to develop virus vectors derived from larger DNA-containing animal viruses, such as HSV and vaccinia.

The relatively small sizes of SV40, BPV, Ad and retroviruses have facilitated the *in vitro* construction and manipulation of recombinant DNA molecules. Although genetic engineering of large viruses is more difficult, *in vitro* and *in vivo* procedures have been developed for the insertion of foreign DNA into the large 187 Kb vaccinia virus genome. Moss et al. (1983) constructed a plasmid that facilitates the routine insertion and general expression of foreign genes and selection for the recombinant virus. Expression is achieved

by fusing foreign protein-coding sequences to the vaccinia virus tk promoter. Subsequent to infection with wt vaccinia virus, the cells are transfected with the chimeric plasmid, and the tk^- recombinant viruses that are generated by homologous recombination with the endogenous vaccinia tk gene, are selected in the presence of BudR in 143 cells (tk^- cell line) (Mackett et al., 1982; Moss et al., 1983).

Vaccinia virus based vectors appear to have several advantages over other eukaryotic vectors. Most noteworthy is that recombinant virus infectivity is not impaired by insertion and expression of foreign genes in contrast to defective SV40, BPV, retrovirus vectors, and to some adeno-based vectors (Mulligan et al., 1979; Wei et al., 1981; Thummel et al., 1981; Solnick, 1981). However, since this virus replicates in the cytoplasm of infected cells (Moss, 1978; Dales and Pogo, 1981) it cannot be used for delivery of genes into the nucleus in vitro or in vivo.

Lastly, recent years have seen the use of HSV-derived vectors. The efficient introduction of foreign DNA sequences into eucaryotic cells was initially demonstrated with a recombinant plasmid (amplicon) of an overall size of 8 to 10 Kb containing two HSV-derived cis replication functions as well as bacterial plasmid DNA sequences (Frenkel et al., 1982; Spaete and Frenkel, 1982; Stow, 1982; Barnett et al., 1983; Stow and McMonagle, 1984; Kwang and Frenkel, 1984). Co-transfection of cells with this chimeric plasmid together with the helper virus DNA resulted in the generation of virus stocks containing defective virus genomes derived from the input chimeric plasmid in addition to the helper virus genome. The packaged chimeric defective

genomes were similar to the helper virus DNA in overall size (150 Kb) but consisted of multiple reiterations of the amplicon sequences. In a second approach, similar to that described above to rescue foreign DNA sequences into the vaccinia virus, and to Smiley's procedure (Smiley, 1980) the hepatitis B virus surface antigen has been rescued and expressed in HSV (Shih et al., 1984). The major drawback of such a vector system stems from the fact that in the first approach the vector is defective and therefore contamination with wt HSV is unavoidable. In any case herpes viruses are causes of a variety of serious diseases in humans and in some cases have been implicated as causes of malignancies (for recent review see Rawls, 1985). Consequently, the use of such a vector system will be considerably limited. Recently, however, a number of laboratories have investigated the possible use of adenoviruses as cloning vectors in mammalian cells.

1.4.3 Adenovirus Vector Systems

Adenoviruses are particularly well suited as gene transfer vectors in mammalian cells for several reasons. Firstly, they are widely studied and well characterized (c.f. Ginsberg, 1984); secondly, they are easy to grow and manipulate, and they exhibit a broad host range *in vivo* and *in vitro*; thirdly, copious amounts of virus and viral gene products can be produced in lytically infected cells. In addition, because only a small portion of the viral genome appears to be required *in cis* for replication and packaging (Tooze, 1981; Challberg and Kelly, 1982; Pearson et al., 1983; Hearing and Shenk,

1983), adenovirus derived vectors may ultimately offer excellent potential for the substitution of large fragments once cell lines have been developed which can provide most of the essential viral functions in trans. Moreover, most adenovirus infections are relatively mild (Straus, 1984) and no adenovirus has been implicated in the etiology of any cancer either in animals or in man. Many of these and other properties offer distinct advantages for adenoviruses over vectors derived from other DNA viruses or from retroviruses. In particular, with regard to potential application in gene therapy (review by Anderson, 1984), it is important that adenovirus-based vectors, unlike those derived from retroviruses, do not carry strong outward-directed promoters like the retrovirus's long terminal repeats.

Two general approaches have been tried in the development of these vectors. In the first approach taken by Solnick (1981), and Thummel et al. (1981, 1982, 1983) the general strategy was to replace in vitro a segment of the adenovirus genome with a DNA segment such as a promoter-less SV40 early region placed under the control of an adeno promoter. In order to select for the hybrid virus in a mixed population, advantage was taken of the ability of the SV40 T antigen to help adenovirus growth in monkey cells. Four hybrids have been isolated in this way and their genome structures determined. In some hybrids the SV40 early region and the Ad2 MLP were in the same orientation and, as expected, the large T and small t antigens were expressed from the MLP. In the remaining hybrids, the SV40 sequences were in the opposite orientation to the MLP, but still the SV40 antigens were expressed from the E2 and E4 promoters (Solnick, 1981; Thummel et al., 1981, 1982, 1983). Using this basic procedure

(outlined above), Solnick (1981), Thummel et al. (1981, 1982, 1983), and Mansaur et al. (1985) were able to rescue and overexpress the three polyoma virus tumor antigens, and recently, Yamada et al.(1985) were able to overexpress the HSV tk gene. However, this approach yields defective recombinant viruses which, therefore, have to be grown in the presence of a wt helper virus.

An alternative approach taken by several groups to develop adenovirus-based vector systems relies on deleting non-essential sequences from the adenovirus genome, namely E1 and E3. As mentioned previously, E3 is not essential for virus growth in cell culture conditions and E1 is dispensable when the virus is propagated in the human cell line 293. Thus, viruses lacking either E1 (Van Doren et al., 1984) or E3 (Berkner and Sharp, 1983; Saito et al., 1985; this thesis) or both (this thesis) grow very well in 293 cells. Lastly, some of the advantages of these vectors are that they are helper-independent, easy to manipulate, and at present the adenovector dlE1,3 (described in this thesis), which is 5.5 Kb shorter than the wt virus, should be able to accept up to 7.5 Kb in foreign DNA.

1.5 Purpose of Investigation

The aim of this work was to explore the use of human adenovirus type 5 as a cloning vector in mammalian cells. The approach taken has been firstly to determine the upper limit on the size of the DNA molecule that can be successfully packaged into infectious virions, secondly, to delete the non-essential regions (E1, E3) of the Ad5 genome, and thirdly, to test the usefulness of the newly constructed vector (dlE1,3), by inserting into it the herpes simplex virus

thymidine kinase gene, and examining: 1) tk expression and 2) the ability of the adeno vector to transform tk^- mammalian cells to the tk^+ phenotype. Lastly, an indirectly related project involved studies on the effect of additional embedded inverted terminal repeats on viral DNA replication and genome stability.

CHAPTER II: MATERIALS AND METHODS

2.1 Radiochemicals

The radiochemicals used in this work were α-^{32}P-dCTP and γ-^{32}P-ATP, ^{32}P-labelled orthophosphoric acid, ^{3}H-thymidine, and ^{14}C-thymidine; all were purchased from Amersham or NEN.

2.2 Mammalian Cell Culture

2.2.1 Cells and Viruses

Wild type (wt) Ad5 (Harrison et al., 1978) and mutant dl309 (Jones and Shenk, 1979a) were grown in HeLa or KB cells and titrated on 293 cells as described previously (Graham et al., 1977). Newly constructed Ad5 insertion, deletion, and substitution mutants were propagated and titrated on 293 cells. This cell line was maintained in Joklik's modified medium supplemented with 10% horse serum (HS). The tk^- human cell line 143 (obtained from K. Huebner and C. Croce and described in Bacchetti and Graham, 1977) was grown in alpha-minimum essential medium (MEM) (Stanners et al., 1971) supplemented with 10% fetal bovine serum. Herpes simplex virus type 1 (HSV-1) strain KOS and a tk^- mutant of HSV-1 lacking 875 bp of tk coding sequences (Smiley, 1980) were a gift from J. Smiley.

2.2.2 Viral Growth and Purification of Viral DNA.

To prepare purified virus, infected (as described in the following sections) cell monolayers were scraped at 48 hrs postinfection in phosphate-buffered saline lacking Ca^{++} and Mg^{++} (PBS^{--}) into 50 ml Corning tubes, pelleted at 1000 rpm for 20 min and resuspended in 1 ml (per 150 mm dish) of 15 mM Tris pH 8. The cells were sonicated for 60 sec (Biosonic III, setting 35, 10 sec bursts) then 1.8 ml of saturated CsCl was added to every 3.2 ml of sonicated cells. Virus particles were banded at 35,000 rpm for 20 hr at $4^{o}C$. Viral bands were collected by puncturing the side of the tube with a 21 gauge needle, pooled, and rebanded in CsCl as described above. The final banded virus was dialyzed extensively against 50 mM Tris HCl (pH 8.0). Viral DNA was then purified by pronase-sodium dodecyl sulfate (pronase stock 5mg/ml pretreated at $56^{o}C$ for 10 min and $37^{o}C$ for 60 min) treatment, followed by phenol and chloroform extraction and finally by ethanol precipitation. The DNA pellet was resuspended in a minimum volume of 0.3 M NaOAC, reprecipitated with 2 volumes of ethanol, then washed with 96% ethanol, dried, and finally resuspended in an appropriate volume of 10 mM Tris pH 7.5, 1mM EDTA (TE buffer).

2.2.3 <u>In vivo</u> Labelling and Extraction of Viral DNA

Semiconfluent monolayers of 293 cells in 60 mm dishes were infected with 5 PFU/cell of a given virus; after 1 hr adsorption, 4 ml of MEM supplemented with 5% HS was added to each dish. Twenty to twenty-four hr post-infection, the medium was removed and 2 to 4 ml of

phosphate free MEM supplemented with 5% HS and 25 uCi/ml of ^{32}P-labelled orthophosphoric acid was added to each dish. After a further incubation of 8 to 12 hours, infected cells were harvested and viral DNA was isolated by the Hirt extraction procedure (Hirt, 1967), digested with appropriate restriction endonucleases and electrophoresed on 1 to 1.2% agarose gels. The gels were dried on Whatman paper under vacuum and exposed on Kodak X-omat R films XR1 or XR5.

2.2.4 Mammalian Cell DNA Purification

Carrier DNA used for transfection and transformed cell DNA for Southern blot hybridization analyses were isolated from cells growing as a monolayer in 150 mm petri dishes. Cell monolayers were rinsed once with PBS^{--} and digested with 500 ug/ml pronase in 10 mM Tris (pH 7.4), 10 mM EDTA and 0.6% SDS. Cell lysates were scraped into 50 ml Corning tubes and incubated at 37^{o}C for 6 to 12 hrs, then gently extracted with buffer saturated phenol. After centrifugation at 3000 rpm for 10 min the aqueous phase was extracted once with chloroform:isoamyl alcohol (24:1), dialyzed extensively against 0.1 X SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate pH 7.0) and overnight against TE buffer for use as carrier DNA in DNA transfection. For Southern transfer, DNA was ethanol precipitated after chloroform:isoamyl alcohol extraction, dried, and redissolved in the appropriate volume and RNAse treated. RNAse digestion was carried out at 37^{o}C for 20 to 30 min using 1 ug/ml of enzyme. Pancreatic RNAse A (Sigma) was dissolved in 10 mM Tris (pH 7.5) and 15 mM NaCl, after heating it to 100^{o}C for 2 min in order to inactivate

contaminating DNAses; stock solutions were aliquoted and stored at $-20^{o}C$ in a final concentration of 2 mg/ml.

2.2.5 DNA Transfection

Transfection of 293 cells was carried out as described previously by Graham and Van der Eb (1973), except that four to five hours after transfection the cells were treated with glycerol as described by Frost and Williams (1978), and overlaid with F11-MEM supplemented with 5% HS and 0.5% agarose. Plaques were picked eight to ten days after transfection, and used to infect 293 cells in 60 mm petri dishes. When cytopathic effect was clearly visible, the cultures were labelled with ^{32}p-orthophosphate (25 uCi/ml), and infected cells' DNA was extracted one to two days later as described previously, and analyzed with appropriate restriction enzymes.

2.2.6 Viral Infection and TK Transformation Assay

LTA , K4 or 143 cells were plated at densities around 1.5×10^5 cells per 60 mm dish and were infected 24 hr later. Wt Ad5, dlE1,3 or Adtk were used to infect cells at multiplicities of infection ranging from 0.1 to 20 PFU/cell. Each 60 mm dish was infected with 0.2 ml of virus in phosphate buffered saline (PBS) at $37^{o}C$ and after 1 hr adsorption, 5 ml of MEM plus 10% fetal calf serum was added. Twenty four hours later, the medium was removed and replaced with fresh MEM supplemented with 10% FCS and HAT (15 ug/ml hypoxanthine, 1 ug/ml aminopterin and 5 ug/ml thymidine (Szybalski and Szybalska, 1962)) and thereafter the medium was changed every three days. At two to three weeks postinfection, the dishes were fixed and

stained for determination of transformation efficiencies, and in some experiments tk^+ colonies were isolated and cell lines established.

2.3 Measurement of TK Activity

2.3.1 ^{3}H-Thymidine Incorporation

In order to measure the incorporation of ^{3}H-thymidine into replicating viral or cellular DNA (an indirect assay for tk activity) cells were infected with the appropriate viruses, namely dlE1,3 or Adtk at 10 PFU/cell, or HSV at 1 PFU/cell. Superinfection with HSV tk^- was carried out 24 hrs post Ad infection. Briefly, the medium was removed, cells were superinfected with HSV tk^- virus at 1 PFU/cell in a total volume of 0.2 ml/60 mm dish and after 1 hr adsorption at 37°C, 5 ml MEM with 5% HS was added to each dish. Subsequently, cells were pulse labelled with ^{3}H-Thymidine (1uCi/ml) at various times for 2 hrs. Infected cell monolayers were rinsed with PBS^{--}, then lysed in buffer containing pronase and sodium dodecyl sulfate as described previously. The nucleic acids were then ethanol precipitated twice and dried at 37°C for 1 hr; scintillation fluid was added and the samples were counted in a scintillation counter (Beckman LS6900).

2.3.2 TK Enzymatic Assay

The enzymatic assay for tk activity in cell-free extracts was carried out as described by Summers et al. (1975). Briefly, cell monolayers in 60 mm dish were rinsed once with PBS^{--}, the cells were scraped and resuspended in 300 ul of TK extraction buffer (20 mM

Tris pH 8.1; 1 mM 2-mercaptoethanol; 0.05 mM thymidine), then sonicated for 90 sec (10 sec bursts) and centrifuged at 15K for 15 min at 4°C. The supernatant was collected, and 50 ul was mixed with 150 ul of TK assay buffer (150 mM Tris pH 7.8; 16 mM $MgCl_2$; 16 mM ATP; 25 mM NaF; 8 mM Creatine phosphate; 1 unit Creatine kinase/ml; 10 ul of ^{14}C thymidine/ml) and incubated at 37°C. Samples of 30 ul were taken at 0, 30 min, 1, 2 and 3 hr intervals, and were spotted on a DEAE-80 filter (previously saturated with 1.2 mg/ml thymidine and dried). Filters were dried at 37°C and washed 3 times in 1 mM ammonium formate pH 3.6, 2 times in water and once in ethanol, and dried. Scintillation fluid was added and samples were counted in a scintillation counter. Values obtained were normalized (to cpm/ug of total cellular protein) on the basis of quantitative measurements of the protein concentration (Lowry, 1951) in each sample.

2.4 DNA:DNA Hybridization

2.4.1 Southern Blot

DNA samples from 143 or Adtk transformed cell lines (T143, TK4 and TLTA) were digested with various restriction endonucleases. Ten to fifteen ug of each DNA digest was then electrophoresed on 0.7 to 0.8% agarose horizontal slab gels. One or five genome equivalents of Adtk DNA per cell were mixed with appropriate amounts of 143 DNA, digested in the same way as test DNA, and used as a marker. Gels were run at thirty to fifty volts overnight, then treated and the DNA was transferred from the gels to nitrocellulose filters (Schleicher and Schwell, BA 85 R597) according to the Southern transfer technique

(Southern, 1975). DNA-DNA hybridization was performed at 45°C in the presence of formamide and dextran sulfate according to Wahl et al. (1979); autoradiography was on Kodak X-Omat R films XR1 or XR5.

2.4.2 Hybridization to DNA in Agarose Gels Using Oligonucleotide as a Probe

Synthetic 15-mer oligonucleotides, complementary to the ends of Tn5, to the Ad5 ITR or to the left end of Ad5 were synthesized on a SAMI automated DNA synthesizer (Biosearch) and were used as probes. Oligomers were end-labelled using ^{32}P-ATP and T_4 polynucleotide kinase (Bethesda Research Laboratories) as described by Maxam and Gilbert (1980). Digested DNA's were run on 1% agarose gels, stained with ethidium bromide and photographed. The gels were dried onto a dialysis membrane and stored dry at 4°C. Prior to hybridization, gels were rehydrated in 2X SSC for 5 min at room temperature (RT), peeled away from the dialysis membrane, denatured for 20 min at RT with 0.15 M NaCl, 0.4 M NaOH, and neutralized in 0.15 M NaCl, 0.5 M Tris-HCl pH 7.4 for 20 min. Hybridization was carried out at RT overnight in a 100 ml of hybridization solution (6X SSC and 0.5% NP40). The gel membrane was then washed twice with 100 ml of 6X SSC for 10-20 min at RT, wrapped in Saran Wrap and exposed on Kodak X-Omat film XR1 or XR5.

2.5 Bacterial Cell Culture and Recombinant DNA Techniques

2.5.1 Bacterial Strains

Escherichia coli strain LE392 was used as a host for most of

the recombinant plasmids constructed. Its genotype is: F^-, hsd R514 (r_k^-,m_k^+); sup E44, sup F58, lac Y1 or (lac IZY) 6, gal K_2, gal T22, met B1, trp R55, λ^-. LE392 was prepared by L. Enquist and is a derivative of strain ED8654 (Brock et al., 1976). Also a methylation negative (dam^-) E. coli strain GM199 was used to construct pFGdX1 plasmid. Its genome is: F', dam^-, dam3 metB, gal K_2, gal^-122, lac Y1, tsx 18, SupE44.

2.5.2 Propagation and Maintenance of Bacterial Strain

All bacteria were grown in Luria-Bertani (LB) broth with continuous shaking in either a floor shaker incubator for large scale cultures, or in a shakerbath for cultures of 10 ml or less. For selection of antibiotic resistance, the media were supplemented with 40 ug/ml of Ampicillin (Amp, Ayerst Laboratories) and/or Kanamycin sulphate (Kan, Sigma) and/or tetracycline (Tet, Sigma). Stocks of antibiotic solutions (20 mg/ml of ddH_2O) were sterilized by filtration (Nalgene millipore filters) and stored at -20^oC in 2 ml aliquots.

In order to obtain a clonal isolate of a bacterial population, overnight liquid cultures were streaked on agar plates containing the appropriate antibiotics. For short term storage bacteria were stored at 4^oC on agar plates whereas for long term storage, fresh overnight cultures were diluted with an equal volume of sterile 40% glycerol and 2 ml aliquots were frozen at -70^oC. Viable bacteria were recovered by thawing these cultures and by transferring a loop of bacteria to a liquid medium.

2.5.3 Transformation

Transformation was carried out essentially as described by Goodman and MacDonald (1979). Fresh overnight cultures of LE392 were diluted 1:100 in a sterile medium without antibiotics and grown to log phase (usually 2.5 to 3 hrs), then centrifuged at 4000 rpm for 5 min at 4°C. Cell pellets were resuspended in 25 ml of 75 mM $CaCl_2$, 5 mM Tris-HCl (pH 7.6) and maintained in ice either for 20 min or overnight. Subsequently, the bacteria were centrifuged again as above and resuspended in 2 ml of Tris $CaCl_2$. Plasmid DNA was added and the bacteria were incubated on ice for 40 to 60 min with intermittent shaking, then heat shocked for 2 min at 42°C, diluted 1:10 in warm Luria broth without antibiotics, and incubated at 37°C for 30 min. Finally, serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were carried out, and bacteria were plated on Luria agar plates (.2 ml/plate) with appropriate antibiotics. Transformation efficiency was routinely 1-5 x 10^6 colonies per ug of plasmid DNA.

2.5.4 Analytical Plasmid DNA Preparations

Screening for recombinant clones was essentially carried out according to the method of Birnboim and Doly (1979). Briefly, colonies were picked from agar plates and used to inoculate 3 ml of LB plus the appropriate antibiotics, then incubated overnight in a shakerbath at 37°C. One and a half ml of the culture was transferred to a 1.5 ml Eppendorf centrifuge tube; the bacteria were centrifuged for 20 sec, resuspended in 100 ul of lysozyme solution (50 mM glucose, 10 mM EDTA, 25 mM of Tris.Cl pH8 plus 5 mg/ml lysozyme were prepared fresh) and incubated for 30 min in ice. Spheroplasts

were then lysed and cellular proteins were denatured by adding freshly prepared 0.2 N NaOH, 1% SDS (alkaline SDS); after 5 min 150 ul of 3M Na acetate (NaOAC, pH 4.8) was added, and after 60 min of incubation on ice, the tubes were centrifuged for 5 min. The clear supernatant fluid was then transferred to fresh tubes containing 1 ml of 96% ethanol. After incubation at -70°C for 10 min, plasmid DNA was recovered by centrifugation; the pellet was resuspended in 100 ul of 50 mM Tris pH 8, 100 mM NaOAC, and reprecipitated with 2 volumes of ethanol. The final pellet was washed once with 96% ethanol, dried, then resuspended in 50 ul TE. Usually a 5 ul aliquot of this plasmid DNA solution was sufficient for analysis by restriction enzyme digestion and gel electrophoresis.

2.5.5 Preparative Scale Plasmid Preparations

For large scale plasmid DNA purifications, cultures were grown to mid log phase at which time 170 ug/ml of Chloramphenicol was added to them. Extraction of plasmid DNA was once again performed according to Birnboim and Doly (1979), except that the bacteria were centrifuged at 5000 rpm for 10 min at 4°C and resuspended in 8 ml of lysozyme buffer, followed by the addition of 16 ml of alkaline-SDS then 12 ml of 3M NaOAC as described earlier. The final DNA pellet was dried and dissolved in 8 ml of 10 mM Tris (pH 8) 1 mM EDTA plus 1 g/ml of CsCl, transferred to a Beckman nitrocellulose tube and overlaid with .5 ml of 10 mg per ml ethidium bromide (EtBr); finally, the tubes were capped, mixed by inversion and centrifuged at 35,000 rpm for 48 hrs at 15°C in a fixed angle rotor (Ti 50, Beckman). Covalently closed circular DNA was collected from the gradient using a 21 gauge needle.

The EtBr was extracted using CsCl-saturated isoamyl alcohol (usually 3 changes), and the DNA was dialyzed extensively against .1X SSC, or recovered by adding 2-3 volumes of TE and then ethanol precipitated. The plasmid DNA yield varied from .5 to 1 mg per 750 ml culture, depending on the type of plasmid grown.

All recombinant DNA work was carried out under level B biological containment conditions as specified by the Medical Research Council of Canada Guidelines (1980).

2.5.6 Restriction Endonuclease Digestions

All restriction enzyme digestions were carried out at 37°C for several hrs or overnight. The reaction mixture contained 50 mM KCl, 10 mM NaCl, 1 ug of DNA (dissolved in TE) and .1 to 1 unit enzyme per ug of DNA. Enzyme inactivation after digestion was performed either by phenol extraction or by heating at 50°C for 20 min except when the reaction mixture was used for gel electrophoresis in which case the samples were mixed with (10% v/v) loading buffer (20% glycerol, 2% SDS, and 0.5% bromophenol blue) and immediately loaded onto the gel.

2.5.7 DNA Ligation

Ligations were carried out either at 14°C overnight or for 1.5 to 2 hrs at RT in 10 mM $MgCl_2$, 20 mM dithiothreitol, 50 M Tris (pH 7.5) and 1 mM ATP with .1 to 1 units of T_4 DNA ligase (Bethesda Research Laboratories) per ug of DNA.

2.6 Gel Electrophoresis

Electrophoresis of DNA fragments through 1 to 1.2% of agarose (Miles Laboratories) gels was at 1 to 2 volts per cm (horizontal gels) or at 5 volts per cm for vertical gels, in a buffer consisting of 40 mM Tris (pH 7.9), 5 mM NaAc and 1 mM EDTA. The samples to be electrophoresed were loaded into submerged wells after the addition of 10% v/v stopper. During or after electrophoresis, the gels were stained using 0.2 ug/ml of ethidium bromide (Sigma) and then photographed with a mounted Polaroid land camera with Polaroid type 59 film and a monochromatic red filter under UV.

2.6.1 Polyacrylamide Gel Electrophoresis

The electrophoresis buffer used was either Tris-acetate (40 mM Tris pH 7.9; 5 mM NaAc and 1 mM EDTA) or Tris-borate (100 mM Tris pH 7.9, 80 mM boric acid and 1 mM EDTA). Acrylamide stocks were filtered using Nalgene millipore filters and stored at 4°C. Ammonium persulfate stock solution (10% in ddH_2O) was prepared weekly and also stored at 4°C. Polymerization of the acrylamide solution catalysed by ammonium persulfate was initiated by the addition of N, N, N', N'-tetramethylethylene diamine (TEMED), and immediately after, the acrylamide solution was poured into the apparatus. Electrophoresis was at 3 to 5 volts/cm; bands were visualized for photography by UV light after EtBr staining as described earlier.

2.6.2 Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis

Immunoprecipitation was carried out as described by Rowe et al.(1983). Briefly, labelled cells were washed with PBS^{--} and lysed in 1 ml of precipitation buffer (20 mM Tris pH 7.5, 1.0% Triton X-100, 1.0% sodium deoxycholate, 150 mM NaCl, 0.2% SDS) per 150 mm plate (1×10^7 cells). After 20 min at 4°C, the cell lysates were sonicated (Biosonic III setting 30) and centrifuged at 5000 rpm for 10 min. Usually 5 to 20 ul of antiserum and 30 ul of equilibrated settled protein A-Shepharose beads were added per ml of supernatant. The mixtures were then placed on a rotating wheel at 4°C for 6 hrs and immunoprecipitates were recovered by low speed centrifugation. Subsequently, the precipitates were washed three times with equal volumes of washing buffer (50 mM Tris pH 7.5, 250 mM LiCl, and 0.1% 2-mercaptoethanol), and resuspended in 50 ul of loading buffer (625 mM Tris pH 6.8, 0.2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue). Finally, prior to loading on gels, samples were submerged in boiling water for 5 min. The labelled proteins were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis as previously described by Rowe et al.(1983).

2.7 Purification of DNA Fragments

DNA restriction endonuclease fragments were isolated after electrophoresis on agarose or polyacrylamide gels essentially as described by Maxam and Gilbert (1980). Briefly, bands were visualized by UV light, and were excised with a scalpel. In the case of agarose

gels, the excised DNA was electroeluted into 0.5 ml of electrophoresis buffer inside a small dialysis membrane bag, at about 150 volts for 2-4 hrs, then recovered by ethanol precipitation. Small DNA fragments were isolated from polyacrylamide gels. The gel slice containing the desired DNA fragment was placed in a 1.5 ml Eppendorf tube and minced in the presence of 0.5 ml elution buffer (.5 M ammonium acetate, 0.1 M magnesium acetate, 1 mM EDTA, .1% SDS), then incubated at 37°C for 12-18 hrs. Subsequently, the acrylamide was sedimented by centrifugation in an Eppendorf centrifuge for 10 min. The aqueous phase was collected and the acrylamide pellet washed with 300 ul of elution buffer and centrifuged again. The supernatants were pooled, filtered through glass wool, ethanol precipitated twice and, finally, the DNA pellet was dried and dissolved in an appropriate volume of TE buffer.

CHAPTER III: PACKAGING CONSTRAINTS IN AD5

3.1 Introduction

As an approach to determining the upper limit on the size of a DNA molecule which can be successfully packaged into an adeno virion, we chose to make use of a series of plasmids containing the left end of Ad5 into which a bacterial transposable element (Tn5) had been randomly inserted (McKinnon et al., 1982, McKinnon, 1984). Tn5 is a composite element made up of two inverted repeats (1534 bp each) (Berg et al., 1982) surrounding a central unique region (2400 bp) encoding the Kanamycin (or neomycin) resistance determinant (Jorgensen et al., 1979; Beck et al., 1982). The Kan^r determinant is also a dominant selectable marker in eukaryotic cells ($G418^r$) (Colbere-Garapin et al., 1981; Southern and Berg, 1982). The unique region of Tn5 also encodes streptomycin resistance expressed in Rhizobium meliloti (Putnoky et al., 1983). The right repeat, IS50R, encodes both the transposase protein (Rothstein et al., 1980), and the repressor protein (Johnson et al., 1982; Lowe and Berg, 1983). The left repeat, IS50L, is identical to the IS50R except for a single base pair difference which causes both premature termination of transcription of the repressor and transposase proteins, and creates a

promoter for the kahamycin gene (Rothstein and Reznikoff, 1981).

Tn5 provides a convenient approach to study packaging constraints in Ad5. Firstly, Tn5 is 5.7 Kb in size (about 16% of Ad5) and all of its sequences are known (Beck et al. 1982; Mazodier et al. 1985). Secondly, it contains several convenient restriction enzyme sites, allowing one to engineer various size inserts, and moreover, as mentioned earlier it encodes resistance to a dominant selectable marker in prokaryotes (Kan^r) and in eukaryotes ($G418^r$; when placed under an appropriate eukaryotic promoter). Thus, a mutant virus with an insert carrying the Kan^r gene can be used to establish various cell lines (analogous to the 293; Graham et al., 1977), which might prove useful in complementing various regions of the viral genome.

As mentioned previously (chapter 1), there seems to be no extreme constraint with respect to the lower limit whereas severe limitation seems to exist on the upper limit. These conclusions were based on data obtained from studies carried out on the Ad-SV40 hybrid families (reviewed in Tooze, 1981; and by Kiessig, 1984). It should be borne in mind, however, that all of the Ad-SV40 non-defectives were isolated from virus stock generated from a single plaque formed on monkey cells, and consequently may not be a representative sample of all the types of insertions that can occur (Tooze, 1981). Secondly, these non-defective hybrids were selected, primarily, on the basis of their ability to grow in monkey cells, and not on the basis of their genomic size. In addition, the various substitution and insertion mutants that have been isolated by Jones and Shenk (1978), all appear to have net insertions ranging from about 0.43 to 1.73 Kb (or 1.2 to

4.8% of the adeno genome). Again, these mutants were isolated using a biochemical procedure designed primarily to select for variants with fewer restriction endonuclease cleavage sites than the wt populations. Therefore, the general consensus that 5% above the genome size might be the maximum packageable size was not based on experiments specifically designed to examine this parameter and might be incorrect. For instance, in the Vaccinia virus, the largest fragment rescued was less than 4 Kb (or 2.1% of the viral genome), until Smith and Moss (1983) succeeded in inserting about a 25 Kb fragment (or 13.4% of the viral genome). A precise determination of the maximum genomic size which could be packaged was a prerequisite for the development of adeno into a cloning vector in mammalian cells (discussed in chapters 1 and 4).

This chapter describes the systematic and deliberate attempt to rescue various size inserts derived from Tn5 insertion plasmids using the method of Stow (1981). This section also contains restriction enzyme analyses of the resulting mutants, which constitute a series of insertion and substitution mutants in the E1a of Ad5.

3.2 Construction of recombinant plasmids with variable size inserts

The plasmid pHE16::Tn5 (Fig. 3.1 top) contains the Ad5 HpaI E-fragment (from -21 to 1575 bp) cloned into the BamHI to PvuII region of pBR322; Tn5 has been transposed into position 1272 as determined by DNA sequencing (McKinnon, 1984). This plasmid contains 2 XmaI sites:

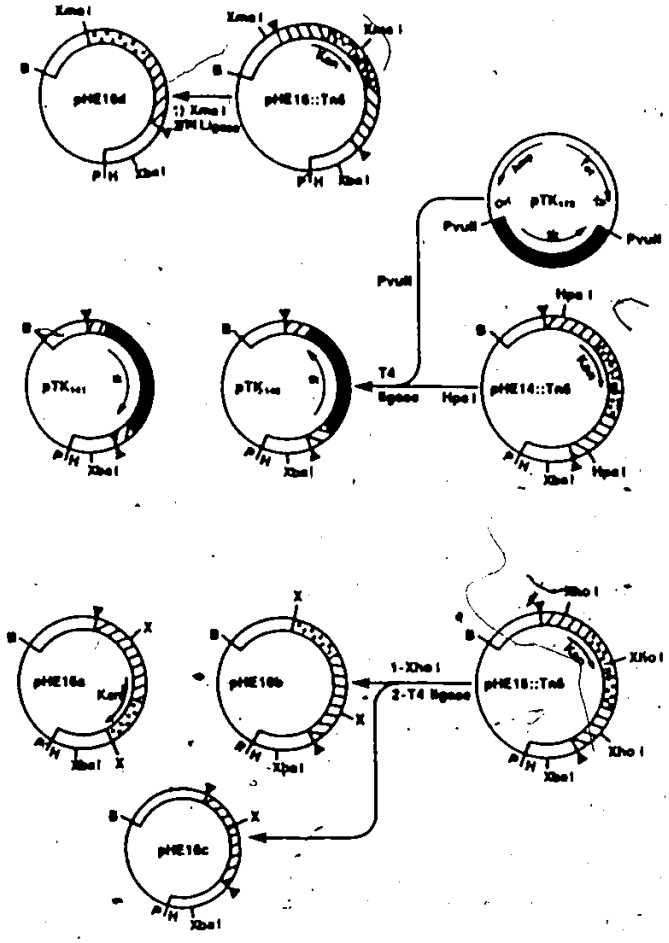


Figure 3.1 Construction of derivative Tn5 insertion plasmids.

Top: The plasmid pHE16::Tn5 (described in the text) was digested with XmaI then religated to generate the plasmid pHE16d.

Middle: The PvuII fragment carrying the HSV tk gene was purified by gel electrophoresis and ligated to HpaI digested pHE14::Tn5 DNA; after transformation of E.coli LE392 with the ligated DNA and screening of colonies the two indicated plasmids were obtained (McKinnon, 1984).

Bottom: The plasmid pHE16::Tn5 was digested with XhoI and religated, and was used to transform E.coli LE392. Colonies were replicated on Amp or on Amp + Kan plates, to distinguish between those which are Amp and/or Kan resistant. Subsequent analysis of the plasmid DNA yielded the three indicated plasmids.

one located in the adeno sequences at position 1009 and the other in the unique region of Tn5. Thus, by simply cleaving with XmaI and religating, the plasmid pHE16d was obtained. The total Tn5 DNA sequence in this plasmid (pHE16d) was about 3.3 Kb, but since there was a deletion of 263 bp of the adeno sequence extending from the XmaI site (at position 1009) to the Tn5 transposition site (at position 1272), the net insert size in pHE16d therefore, was about 3.1 Kb.

Plasmids pTK141 and pTK142 were constructed by McKinnon (1984) as outlined in Fig. 3.1 (middle). These plasmids contain a net insert of 2.4 Kb, made up of the HSV1 tk gene (PvuII fragment = 2043 bp) and 370 bp of Tn5 sequences (185 bp at either end of the tk gene).

The plasmid pHE16::Tn5 described above contains three XhoI sites (Fig. 3.1, bottom), two located in the arms, and the third in the middle of the unique region of Tn5. By digesting pHE16::Tn5 with XhoI and religating, plasmids pHE16a, pHE16b and pHE16c were obtained (Fig. 3.1, bottom). Plasmid pHE16a contained the leftmost arm and about half of the unique region of Tn5 carrying the Kan^r gene. The net insert size in pHE16a was about 3.3 Kb. Plasmid pHE16b contained the rightmost arm of Tn5 and again half of the unique region of Tn5, with a net insert of about 3.5 Kb. On the other hand, the third plasmid pHE16c, was presumably the by-product of an *in vivo* rearrangement caused by the instability of large palindromes in prokaryotes (Lilley, 1981; Collins et al., 1982). A palindrome of 970 bp would be generated upon *in vitro* ligation of the two arms of Tn5 at the XhoI site. Restriction enzyme analysis and oligonucleotide hybridizations carried out on pHE16c, revealed that it retained the XhoI restriction enzyme site, and contained two inverted repeats of

about 250 bp each (the right inverted repeat was missing the end of Tn5), separated by 150 bp of unique sequences; thus, pHE16c had a net insert of only about 650 bp, instead of 970bp.

3.3 Rescue of Various E1a Mutants Into Infectious Virus

Fig. 3.2 illustrates the strategy employed for rescuing various inserts into infectious viral DNA. The virus d1309 is an Ad5 mutant with a single XbaI site at map position 3.8 (or 1339), but wt for viral replication on HeLa cells (Jones and Shenk, 1979a). The viral (d1309) DNA was digested with XbaI, and ClaI (which cuts once at map coordinate 2.6, and was used to reduce the background). Similarly, plasmid DNA (with the insert to be rescued) was linearized with XbaI. Following ligation of viral and plasmid DNA, 293 cells were transfected as outlined in Materials and Methods, and 7 to 10 days later plaques were picked and progeny viral DNA was analyzed using diagnostic restriction enzymes and agarose gel electrophoresis. The extra DNA to the left of map position 0 is presumably removed via a correction mechanism using the right hand inverted repeat (Stow, 1982, and discussed in chapter 6). Using this approach the following plasmids were rescued: pHE16d, pTK141, pTK142, pHE16a, pHE16b, pHE16c and pHE144d.

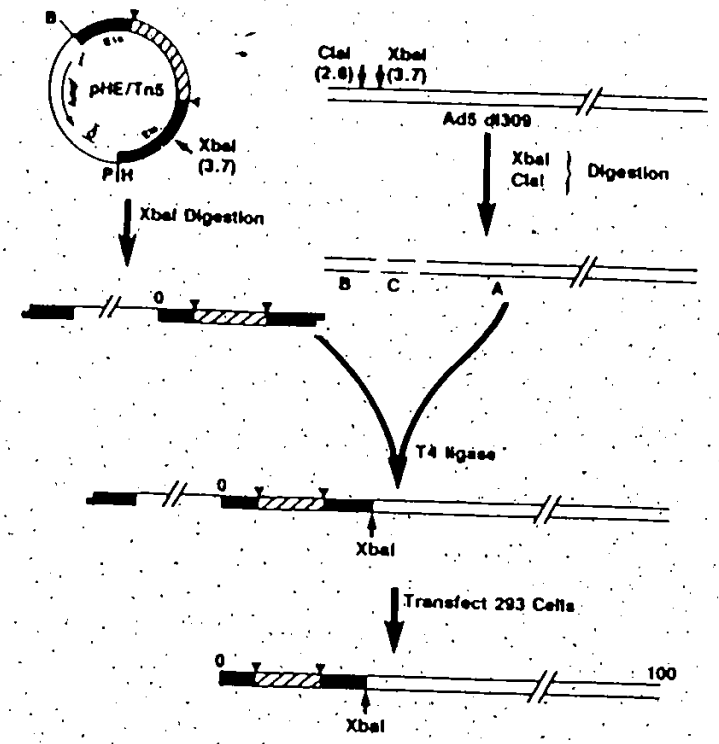


Figure 3.2 Procedure for rescuing portion of Tn5 insertions into Ad5. The plasmid pHE/Tn5 (a prototype) was digested with XbaI then ligated to XbaI and ClaI digested dl309 DNA (an Ad5 variant having a single XbaI site at map position 3.8 which is otherwise wt for viral replication on HeLa cells, Jones and Shenk, 1979a). ClaI cuts once at map position 2.6, and was used in order to reduce background. The ligation mixture was used to transfect 293 cells' monolayers as described in Materials and Methods. A week later plaques were picked and analyzed using diagnostic restriction enzymes and agarose gel electrophoresis. The excess amount of DNA to the left of map position 0 is normally removed by a correction mechanism using the right hand inverted terminal repeat (see Chapter 6 for details).

3.4 Restriction Enzyme Analysis of Isolated Mutants

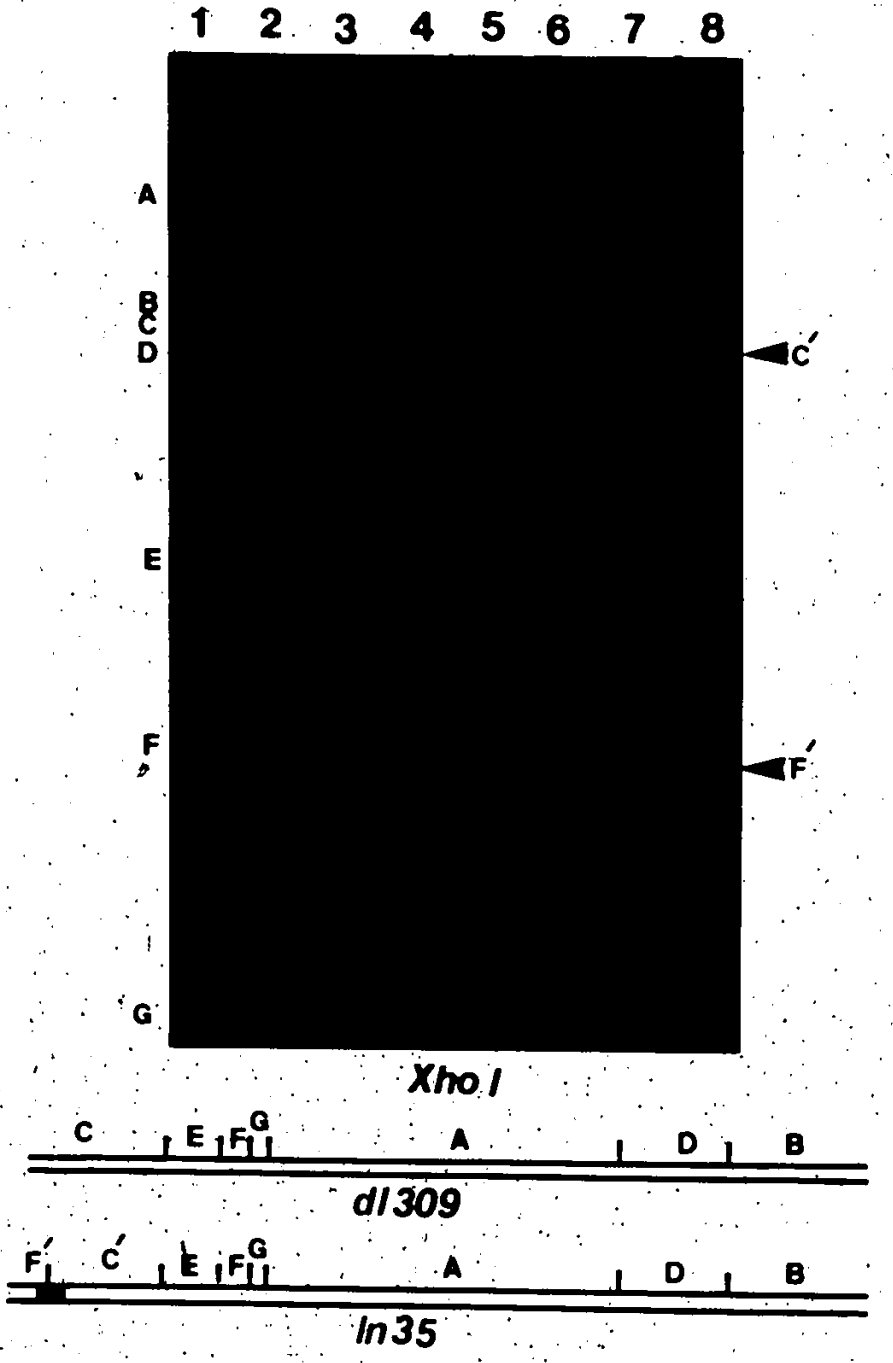
Viral DNA was ^{32}P-labelled and extracted as outlined in Materials and Methods. The viral DNA was digested with an appropriate restriction enzymes and electrophoresed on 1% agarose. The choice of a restriction enzyme was based on whether the enzyme cut in the insert thus generating a new fragment, or did not cut the insert, thus causing a particular fragment to become larger in size.

A) Rescue of Small Inserts into Virus

Fig. 3.3 shows the XhoI restriction enzyme pattern of 7 insertion mutants rescued using plasmid pHE16c (shown in Fig. 3.1 bottom). As mentioned previously, this plasmid contained an insert of about 650 bp and possessed an XhoI enzyme site. Therefore, mutant viruses rescued using this plasmid were expected to contain an extra XhoI site, and consequently, a new fragment should have been generated upon XhoI digestion of these mutants. Indeed, such an extra fragment did appear (F') just below the XhoI F-fragment. The XhoI C-fragment also was expected to be smaller in size, and to comigrate with the XhoI D-fragment but it was difficult to distinguish the new fragment (C') from the XhoI D-fragment on this gel. However, since the C'-fragment should contain a portion of the right arm of the Tn5 insert, hybridization to a 15-mer oligonucleotide probe (specific for the ends of Tn5) was expected to light up this band. In order to test this prediction, DNAs from these mutants were cleaved with XhoI and electrophoresed on 1% agarose gel, stained with EtBr, and photographed

FIGURE 3.3 XhoI restriction enzyme analysis of 7 insertion mutants rescued using the plasmid pHE16c with a small insert (Fig. 3.1)

Labelled DNA was digested with XhoI and eletrophoresed on 1% agarose gel. The 650 bp insert contains an XhoI restriction enzyme site; consequently, a new fragment appears (arrow head F') and the XhoI C-fragment becomes smaller (arrow head C'). In lane 1, d1309 DNA was digested with XhoI and run as a marker. In lanes 2-8 are the XhoI restriction enzyme pattern of 7 insertion mutants. XhoI restriction enzyme sites are illustrated below for both d1309 and In35 (which is a prototype of the remaining 6 viruses).

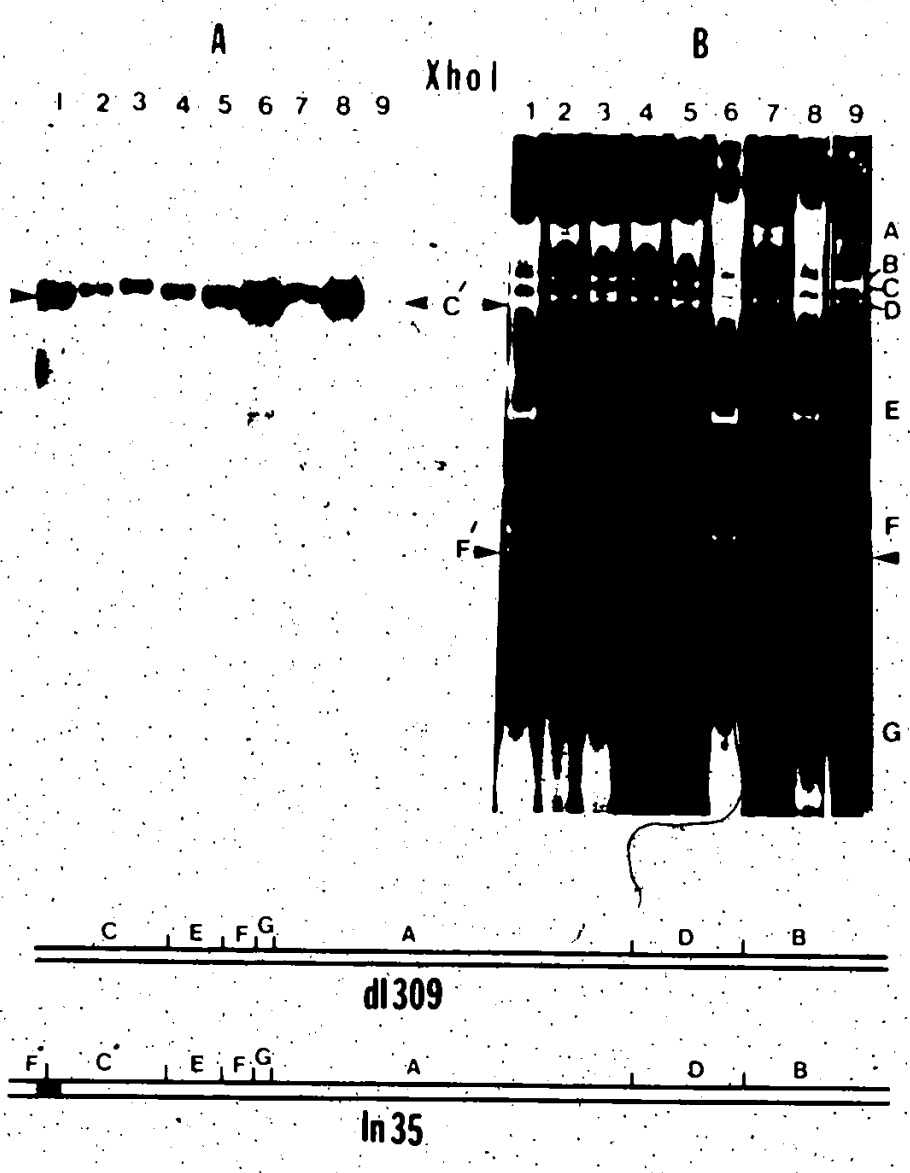

1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
C′
F′
Xho I
C E F G A D B
dl309
F′ C′ E F G A D B
In35

(Fig. 3.4, panel B) as described in Materials and Methods. Subsequently, the gel was treated (as described in Materials and Methods) and hybridized to the Tn5 probe. The results presented in Fig. 3.4 (panel A) were in agreement with the prediction. The hybridization was very specific, in that only the bands corresponding to the XhoI C'-fragment were lit up in all of the mutants, and no hybridization was detected in the d1309 control (lane 9). Although the XhoI C'-fragments (and to some extent the other XhoI fragments) did not appear to comigrate as expected in this particular gel, they did so on other gels (Fig. 3.5 and Fig. 3.6, lanes 2, 3, 4, and 5), when approximately equal amounts of DNA were applied in each lane. The mutant in lane 3 (Fig. 3.4), however, was an exception and seemed to have a larger insert than expected (by about 100 bp).

B) Rescue of Inserts Larger than 2 Kb

When plasmids containing inserts ranging in size from 2.4 Kb (as in pTK141 and pTK142) to about 3.5 Kb (as in pHE16a, pHE16b, and pHE16d) were rescued into infectious virions, all of the mutants isolated were found to have rearrangements in and around the inserts (Table 3.1). This conclusion was based on the results of analysis with several restriction enzymes (HinIII, KpnI, XbaI, ClaI, and SmaI) and of oligonucleotide hybridizations using the Tn5 probe. The HindIII restriction enzyme pattern of some of these mutants is shown in Fig. 3.5. In all of these mutants the HindIII G-fragment should be altered in size due to inserts. Indeed this has happened (arrow heads Fig. 3.5), but in no case was the expected size observed. Instead of modified HindIII G-fragments of about 6 Kb (G'= G (2.8 kb) + the

FIGURE 3.4 XhoI restriction enzyme analysis and oligonucleotide hybridization of insertion mutants rescued using the plasmid pHE16c (Fig. 3.1). Viral DNA was digested with XhoI and electrophoresed on a 1% agarose gel. The gel was stained using 0.2 ug/ml ethidium bromide and then photographed under UV light (panel B). Subsequently, the gel was dried, treated as described in Materials and Methods and hybridized to a 15-mer oligonucleotide specific for the first 15 bp of the Tn5. As expected, the oligonucleotide hybridized specifically to the XhoI C'-fragment. In lane 9 (panels A and B) d1309 was run as a marker. Arrow heads indicate the location of the modified C-fragment (C') and the new fragment F'. XhoI restriction enzyme maps for d1309 and In35 are shown below.


A
B
XhoI
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
C'
F'
A
B
C
D
E
F
G
C E F G A D B
dl309
F C E F G A D B
In35

insert (3.0 kb) = 5.8 kb), the latter fragments appeared to have increased in size only by from several hundred bp (Fig. 3.5, lanes 10, and 11) up to about 1.7 Kb (Fig. 3.5 lanes 9, 13, 18, and 19). In addition, the mutants shown in lanes 6 to 9 , and 14 to 19 should all have an extra fragment, because the original inserts possessed a HindIII site (located in the arm of Tn5 at position 1195). Instead, only one of them appeared to have an extra fragment (lane 16).

The KpnI restriction enzyme patterns (Fig. 3.6) basically confirmed the results obtained with HindIII digestion. Since KpnI does not cut in any of the original inserts, the KpnI H-fragment was expected to be larger and migrate slightly below the KpnI D-fragment in lanes 10-13, representing rescue from pTK141 and pTK142 with a net insert of 2.4 Kb, or just above KpnI H-fragment for lanes 6-9 and 14-19 containing inserts rescued from pHE16a, pHE16b, and pHE16d with a net insert of about 3 Kb. However, in no case did the modified KpnI H-fragments (H', arrow heads, Fig. 3.6), migrate as predicted, had no rearrangements taken place during rescue. Thus, in contrast to the results obtained from the rescue of smaller inserts (0.7 Kb) Fig. 3.4, an attempted rescue of larger inserts (2.4 to 3.5 Kb) resulted in a high frequency rearrangement of the rescued DNA segment. The results from these analyses were summarized in Table 3.1. The data from these experiments suggest that in no case did the observed insert size correspond to the expected insert size except when a 0.7 Kb was rescued.

Figure 3.5 HindIII restriction enzyme pattern of various insertion mutants. In lanes 1 and 20 are the d1309 HindIII restriction enzyme pattern run as a marker; the d1309 HindIII restriction enzyme map is illustrated below. Insertion mutants were rescued using the plasmid pHE16c (lanes 2-5), pHE16d (lanes 6-9), pTK141 and pTK142 (lanes 10-13), pHE16a and pHE16b (lanes 14-19). Arrow heads point to the location of the modified HindIII G-fragment which contains the insert. In lane 16, the insert appears to have a HindIII site.

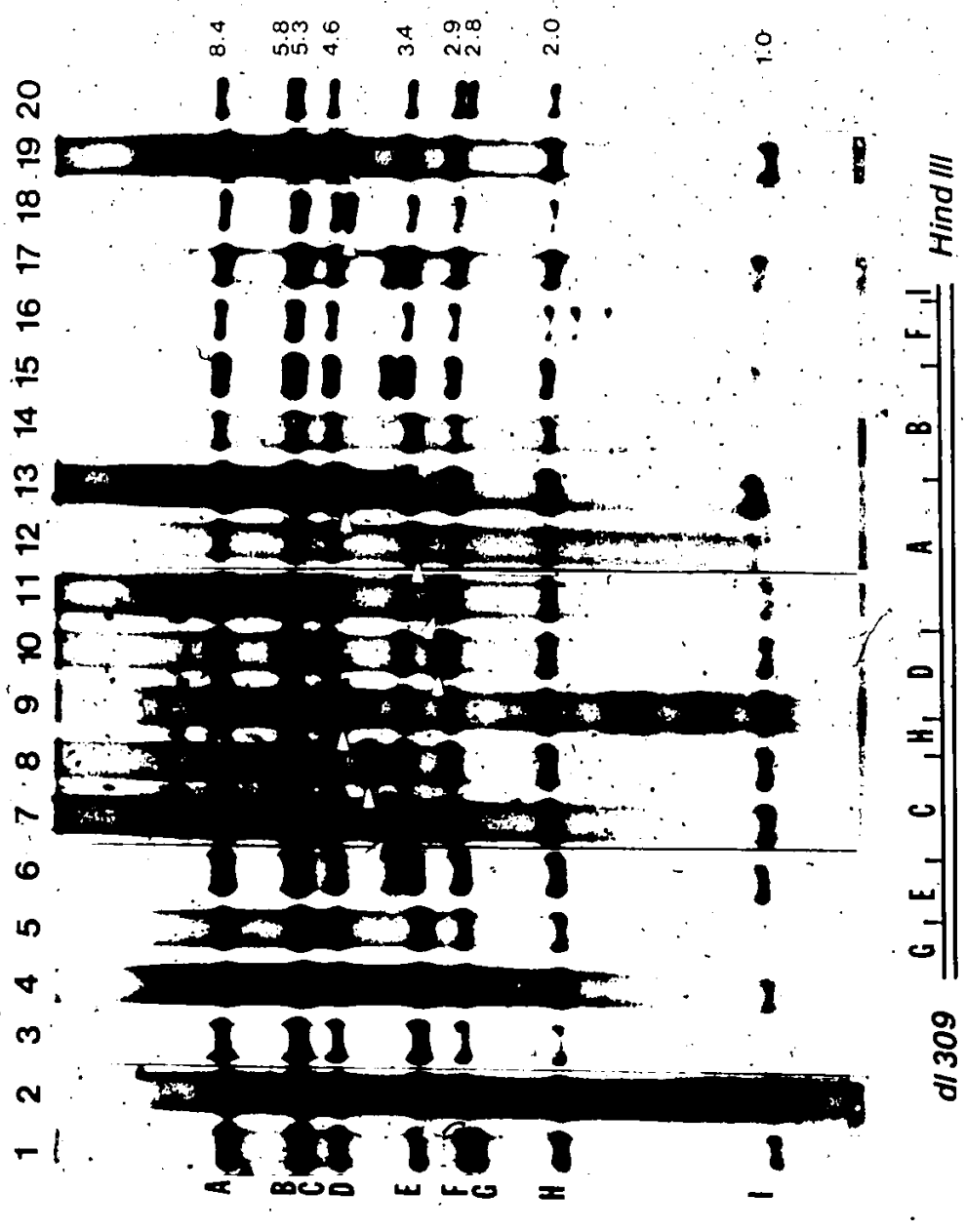

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
8.4
5.8
5.3
4.6
3.4
2.9
2.8
2.0
1.0
A B C D E F G H I
dl 309
G E C H D A B F
Hind III

Figure 3.6 KpnI restriction enzyme pattern of various insertion mutants. The order of these mutants is identical to that in Fig. 3.5. Arrow heads point to the modified KpnI H-fragments.

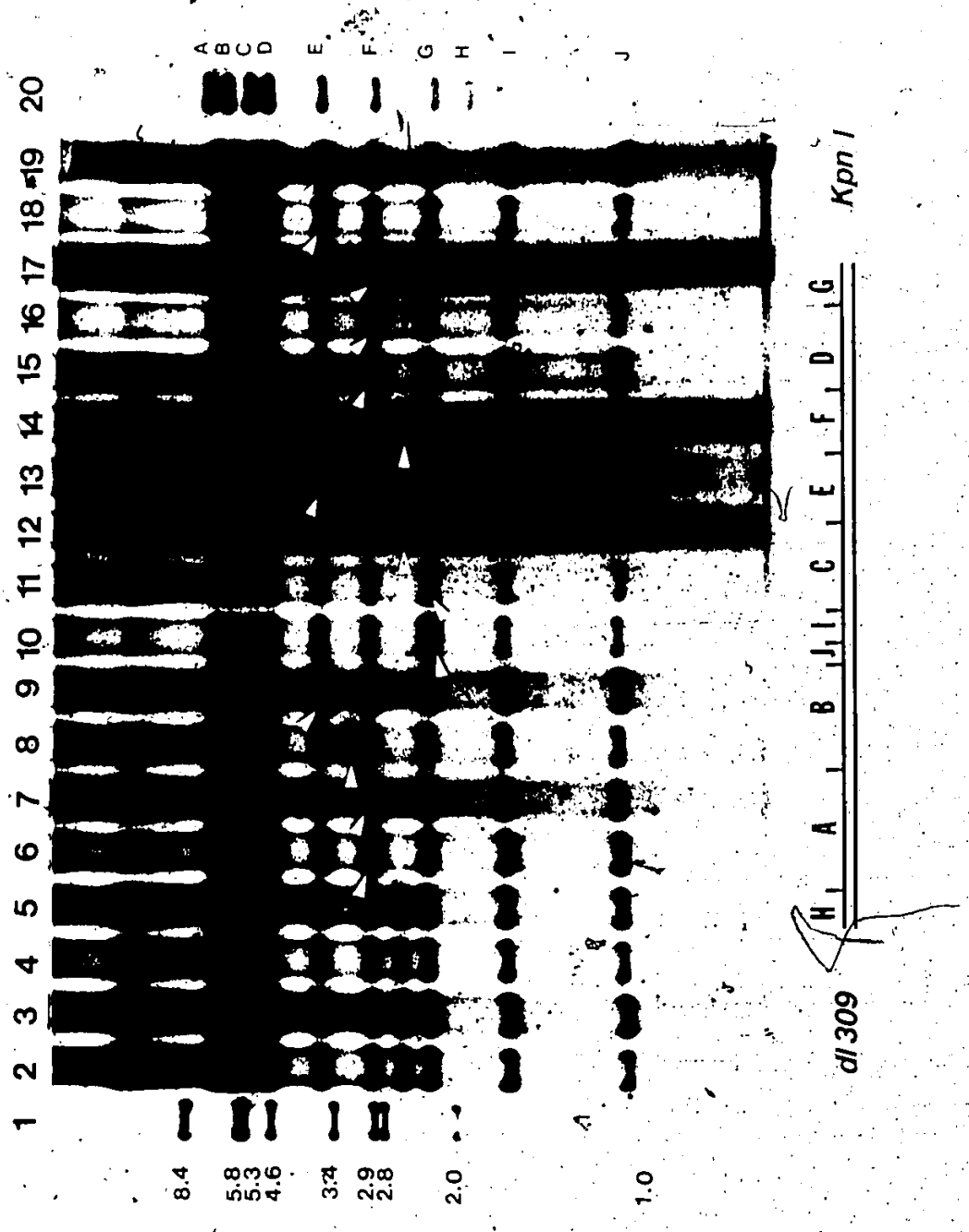

A
B
C
D
E
F
G
H
I
J
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
8.4
5.8
5.3
4.6
3.4
2.9
2.8
2.0
1.0
H
A
B
J
I
C
E
F
D
G
Kpn I
dl 309

Table 3.1 Summary of Rescued Mutants.

Plasmid	Expected insert size (in Kb)	No. of plaque isolates analyzed	Observed insert size (in Kb)	No. of mutants in each size range[a]
pHE16c	0.7	12	0.7	9
			0.8	1
pHE141 and pHE142	2.4	14	- 0.3	1
			0.4	1
			0.6	1
			0.7	1
			1.2	3
			1.3	1
			1.6	1
pHE16d	3.1	11	- 0.2	1
			0.3	1
			0.7	1
			1.2	2
			1.6	1
			1.7	2
pHE16a	3.3	12	- 0.2	1
			0.6	2
			1.0	1
			1.3	2
			1.4	1
			1.5	1
pHE16b	3.5	9	0.3	2
			0.6	1
			1.4	2
			1.7	1

The indicated plasmids were rescued as described in Fig. 3.2. Plaques were picked and analyzed on agarose gels for the presence of inserts as described earlier. The restriction enzyme patterns of some mutants are shown in Figs. 3.3, 3.4, 3.5, 3.6 and 3.7. A negative sign indicates a net deletion.

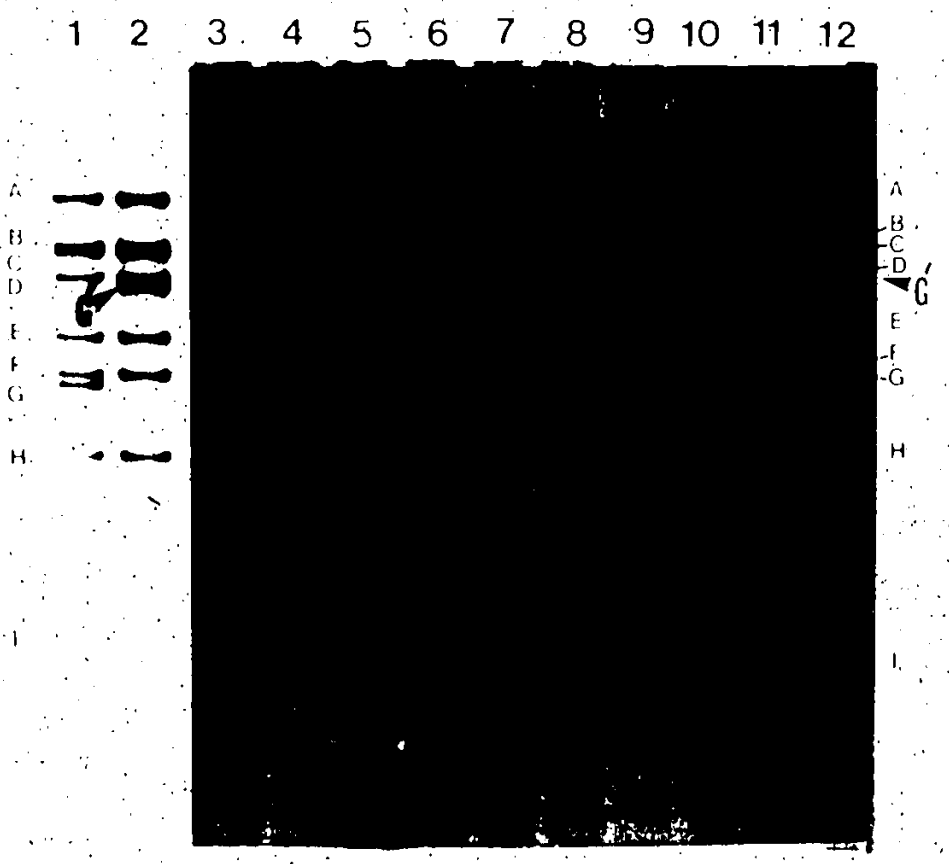
[a] The difference between the sum of numbers in the last column and in the 3rd column correspond to wt isolates.

3.5 Stability of Larger Inserts

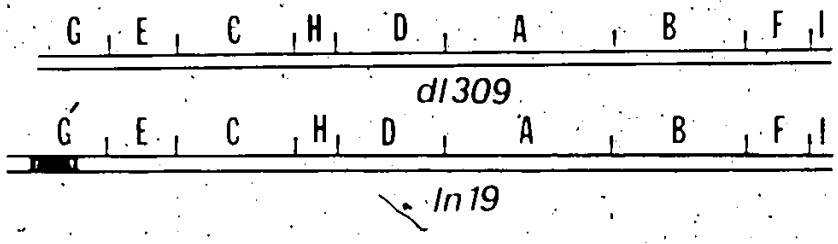
The data described in the previous sections indicated that gross rearrangements had occurred in and around the inserts. Such rearrangements could have occurred in the very first step during the rescuing of an insert, or, subsequently during replication of the recombinant virus. In the latter case, a virus with a large insert should be unstable and upon passaging should give rise to new variants with smaller inserts. In order to test this prediction, a mutant virus with an insert of about 1.7 Kb was passaged 5 times in 293 cells. Subsequently, the virus stock was plaqued, and 24 plaque isolates were examined. The HindIII restriction enzyme patterns of 9 of these isolates are shown in Fig. 3.7. It is clear that no detectable alteration occurred during the successive passages of the mutant virus in 293 cells (the remaining 15 plaque isolates were identical to those shown in Fig. 3.7). Therefore, it appears that deletions and rearrangements take place early during mutant rescue, and that once rescued the inserts and resulting viral mutants were relatively stable.

Figure 3.7 Stability of large inserts.

HindIII restriction enzyme pattern of 10 plaque isolates (lanes 3-12) of an Ad5 mutant with an insert of 1.8 Kb in E1a. The new HindIII G-fragment is indicated by arrow head as G'. The HindIII restriction enzyme map of d1309 and In19 are illustrated below. Lanes 1 and 2 are short exposure time, thus showing the doublet made up of the HindIII D and G'-fragments.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
I
G'

Hind III


G E C H D A B F I
dl309
G' E C H D A B F I
In 19

3.6 Phenotype of Mutant Virus

One mutant from each group (grouping is based on the parental plasmid used for rescue) was titrated on HeLa and 293 cells. The results in Table 3.2 suggest that these mutants, whether substitution or insertion mutants, exhibited a host range phenotype. In other words, their titre on 293 cells was comparable to that of a wt virus, whereas titres on HeLa cells were several logs lower than that of wt. This was in agreement with other workers' findings (Young et al., 1984). As expected for mutants defective in the Ela region, none of the viruses produced any detectable Ela gene products (Fig. 3.8). This was determined by immunoprecipitations of KB infected (with some of the rescued mutants) cell lysates, using sera raised against synthetic oligopeptides specific for the amino and carboxy terminal ends of the Ela gene products (Fig. 3.8), (these sera were kindly provided by S.P. Yee).

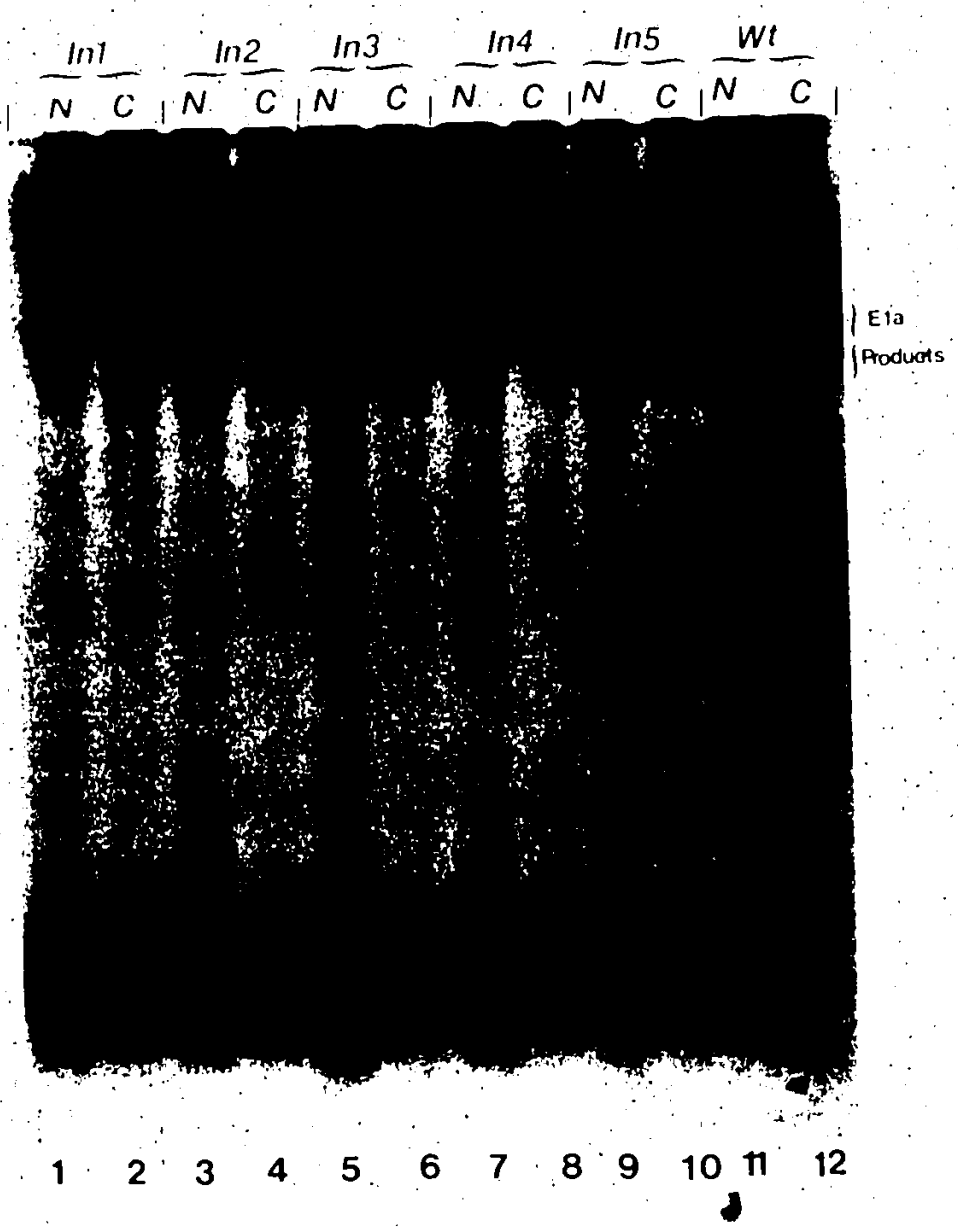
Table 3.2 Titration of Mutant Viruses on HeLa and 293 cells.

Virus	Pfu/ ml on 293	Pfu/ ml on HeLa
In30	7.5×10^9	1.5×10^5
In35	3.6×10^9	1.0×10^5
In58	3.5×10^9	1.8×10^6
In61	5.2×10^9	1.2×10^5
In86	6.1×10^9	3.5×10^5
In45	1.6×10^9	4.3×10^5
Sub143	1.3×10^9	2.5×10^6
d1309	3.5×10^9	4.3×10^9

Monolayers (in 60 mm dishes) of the indicated cells were infected with .2 ml of serially diluted virus stock in PBS^{++}, and after 60 min. of absorption at 37°C , F11 MEM supplemented with 5% HS and 0.5% agarose was overlaied.

Approximately 5 to 7 days later plaques were counted. To aid the visualizing of plaques on HeLa cells, an additional 5 ml of F11 MEM (+5% HS, and 5% agarose) supplemented with neutral red (1% final concentration) was added 4 to 5 days post infection.

Figure 3.8 Autoradiogram of a 12% polyacrylamide gel electrophoresis of immunoprecipitates, from wt, and In1 to In5 (Ad5 E1a insertion mutants) infected KB cells. Infected cells were pulse labelled with ^{32}P-orthophospate for 2 hrs from 7 to 9 hours p.i. Labelled polypeptides were immunoprecipitated with sera against the E1a carboxy (C) and the E1a amino (N) ends.


In1
In2
In3
In4
In5
Wt
N C N C N C N C N C N C
E1a
Products
1 2 3 4 5 6 7 8 9 10 11 12

3.7 Conclusions

A number of observations have been described in this chapter, and they can be summarized as follows:

1. First, it is clear from the above that not more than 2 Kb of extra DNA can be packaged in an adeno capsid.

2. Second, deletions and rearrangements occurred when attempts were made to rescue large inserts (over 2 Kb), and that such rearrangements were rare when a small (less than 1 Kb) insert was rescued.

3. Third, inserts once rescued into viable virions remain stable and unmodified upon successive passagings of the virus in 293 cells.

4. Lastly, and as expected, all of these mutants exhibit a host range phenotype with regard to lytic growth in HeLa and 293 cells. The studies described in this chapter provided the basis for development and use of an Ad5 vector as described in the next chapters.

CHAPTER IV: CONSTRUCTION OF THE AD5 dlE1,3 VECTOR

4.1 Introduction

Adenoviruses are particularly suitable as gene transfer vectors for various reasons which have been discussed previously (Chapter 1). The approach taken in our laboratory in the development of an adenovirus vector was initially to determine the packaging constraints of Ad5 (Chapter 3). Data was obtained suggesting that a maximum of about 1.7 Kb of extra DNA could be inserted into the wild type viral genome. In order to increase this limit and thus enhance the usefulness of adeno as a vector in mammalian cells, I have undertaken the development of deletion mutants lacking most of E3 (dlE3) or lacking both E1 and E3 (dlE1,3). As discussed in Chapter 1, early region 3 appears to be non-essential for virus growth in all types of cultured cells since viruses lacking or defective in E3 are still viable (Kelly and Lewis, 1973; Anderson et al., 1976; Berkner and Sharp, 1983; Saito et al., 1985 ; and work to be presented in this chapter) and E1 is not required when the virus is grown in 293 cells (Graham et al., 1977). Consequently, the virus vector dlE1,3 is about 5.5 Kb shorter than wt and yet is helper independent when propagated in 293 cells. The vector dlE1,3 is about 30.5 Kb in size and

therefore, theoretically should be able to accept inserts of up to 7.5 Kb in size. This chapter describes the construction and characterization of dlE3 and dlE1,3. The data presented in this chapter as well as in the next chapter have been published recently (Haj-Ahmad and Graham, 1986).

4.2. Construction of dlE3

Fig. 4.1 illustrates the strategy employed for the deletion of region E3 of Ad5. The plasmid pFG23 contains the BamHI B-fragment [(59.5-100 mu, minus 25 bp from the extreme right end) (D. Bautista, personal communication)] of wt Ad5 cloned into the BamHI site of pBR322 DNA. This plasmid contains two XbaI sites in the Ad5 sequences in early region E3 at map positions 78.5 and 84.7 mu. Since the site at map position 84.7 mu is sensitive to methylation (Berkner and Sharp, 1983), the plasmid pFG23 was grown in dam$^-$ *E. coli*. Deletion of early region 3 was then achieved by cleaving pFG23 with XbaI followed by ligation, thus eliminating the sequences from 78.5 to 84.7 mu. The resulting new plasmid, pFGdX1, contains a unique XbaI site into which additional foreign DNA can be inserted and subsequently rescued into virus. The plasmid pFGdX1 DNA was digested with BamHI, and dl309 virion DNA was digested with both BamHI and EcoRI (the latter digestion was carried out in order to decrease the likelihood of reconstitution of dl309); after inactivation of the restriction enzymes by heating, the digests were mixed and ligated, and the ligation reaction mixture was used to transfect 293 cells as

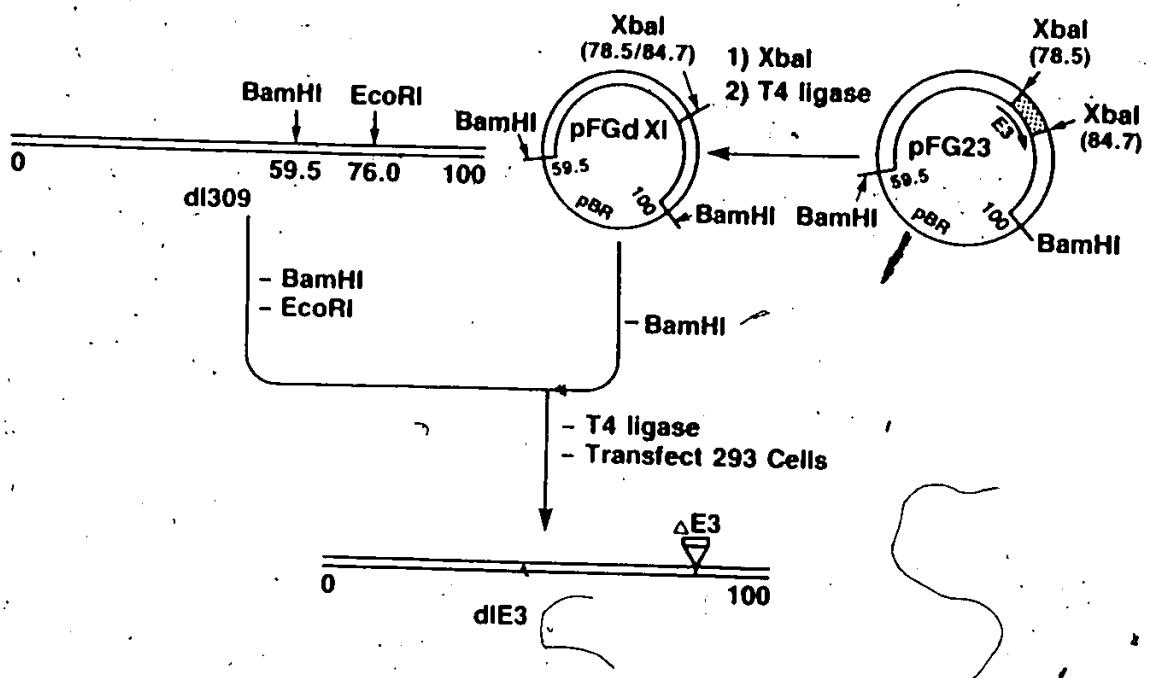


Figure 4.1 Strategy for construction of dlE3.

The plasmid pFG23 was digested with XbaI then religated to generate the plasmid pFGdxl with deletion of most of E3 (dotted box; from map positions 78.5 to 84.7). Subsequently, pFGdxl was digested with BamHI and ligated to BamHI and EcoRI digested viral DNA. The ligation mixture was used to transfect 293 cells' monolayers as described in Materials and Methods.

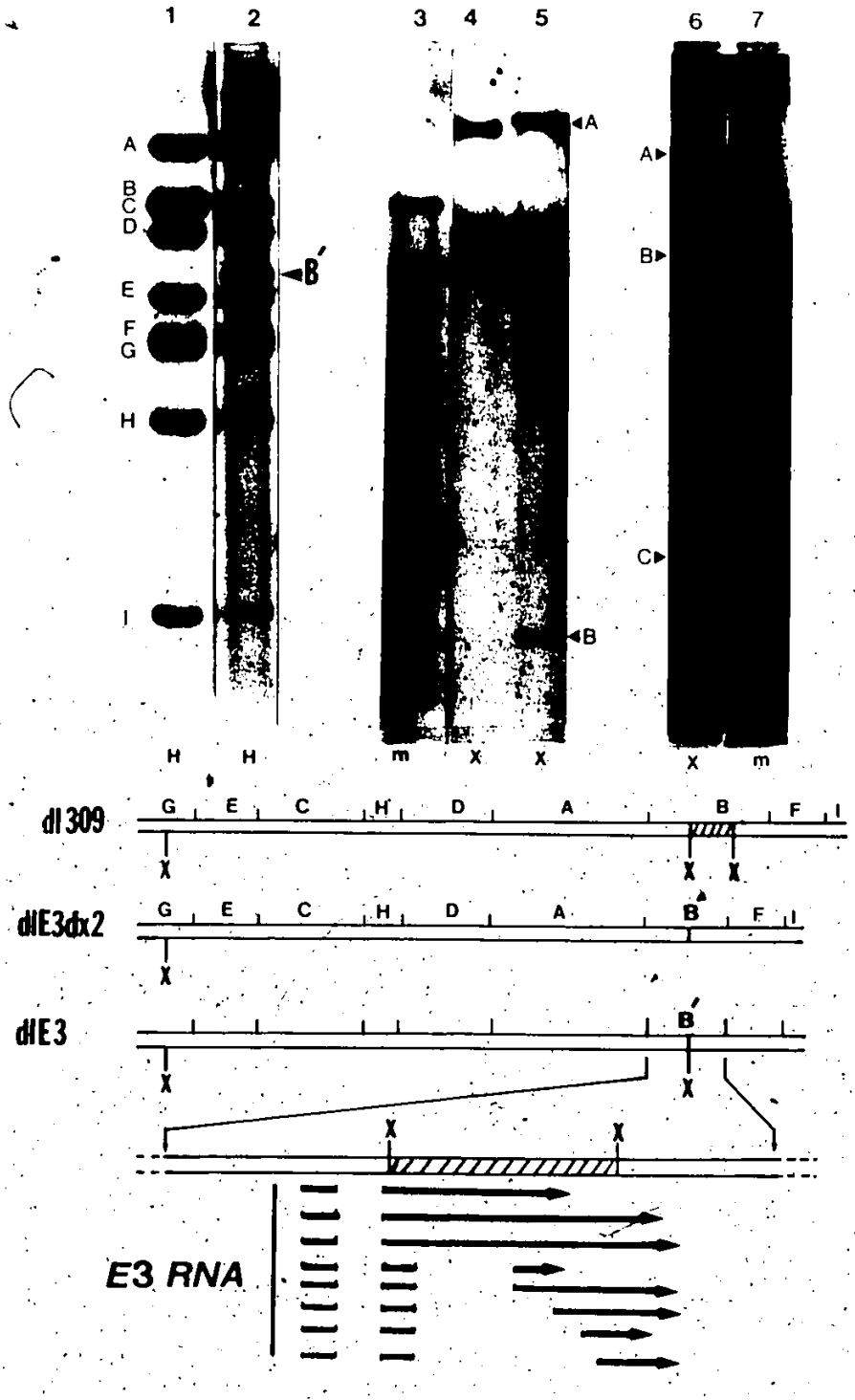
described in Materials and Methods. A total of 40-50 plaques were obtained and 3 of these were picked and their structure analyzed by restriction enzyme digestion and gel electrophoresis. All three isolates were found to have the desired structure (Fig. 4.2 , Channels 2 and 6).

The E3 deletion which extends from map positions 78.5 to 84.7 removes the major parts of all 9 E3 messages leaving the promoter and 5' initiation site, the polyadenylation sites and one set of 5' and 3' splice sites (Fig. 4.2, bottom). These signals have been shown to be utilized in vivo as determined by S1 mapping (Berkner and Sharp, 1983).

Figure 4.2 HindIII and XbaI restriction enzyme pattern of d1309, dlE3 and dlE3dx2.

The E3 deletion reduced the HindIII B-fragment size resulting in a new fragment (B') migrating slightly above the HindIII E-fragment as indicated by the arrow in lane 2. In lanes 1, 3, and 7 are the HindIII digests of d1309 DNA (run for comparison, and as a molecular weight marker). In lanes 4, 5, and 6 are the XbaI digests of dlE1,3, dlE3dx2 (for details of this mutant see appendix) and dlE3 respectively. The XbaI digests of dlE3 gave the expected 3 fragments A, B, and C (lane 6), whereas dlE3dX2 gave the 2 expected fragments A and B (lane 5).

HindIII cleavage sites are indicated above the maps, and the XbaI (X) sites are located below the maps, for the indicated viruses (illustrated below). Also a schematic representation of the E3 transcription units (from Cladaras and Wold, 1985) is shown; the shaded regions between the 2 XbaI sites represent the deleted portion from dlE3 and dlE3dx2. Introns in the E3 transcripts are represented by gaps.


1
2
3
4
5
6
7
A
B
C
D
E
F
G
H
I
B'
A
B
A
B
C
H
H
m
X
X
X
m
dl309
dlE3dx2
dlE3
G
E
C
H
D
A
B
F
I
X
B'
E3 RNA

4.3 Construction of dlE1,3

As mentioned previously, early region 1 (E1) is non-essential when a virus lacking this region is grown in the 293 cell line, which constitutively expresses the E1 gene products (Graham et al., 1977; Aiello et al., 1979; Berk et al., 1979). To delete E1, I followed the strategy illustrated in Fig. 4.3. The plasmid pXC1 which contains the Ad5 XhoI C-fragment (0 to 15.8 mu) minus 21 bp from the extreme left end (Mckinnon, 1984) was partially digested with SacII which cuts at 1.0, 10.6 and 15.2 mu and religated to generate pXCd11 lacking sequences between map positions 1.0 and 10.6. Infectious dlE1,3 was generated by co-transfecting 293 cells with pXCd11 DNA and dlE3 DNA which had been digested with ClaI (single cut at 2.6 mu) and treated briefly with S1. The S1 nuclease treatment was carried out in order to eliminate or reduce the likelihood of reconstitution of dlE3 by in vivo ligation. Approximately eight days after transfection 6 plaques were isolated and used to infect 293 cells. Following the development of CPE, infected cell DNA was extracted and viral DNA analyzed using restriction endonucleases as described in Materials and Methods. One out of 6 plaque isolates was found to have the expected deletion (Fig. 4.4, Channels 2, 3 and 4). The deletion between map positions 1.0 and 10.6 removed all of E1a and E1b, and about half of the coding sequences for protein IX (see Fig. 4.4, bottom). As expected, dlE1,3 was a host range for lytic growth in that it grew well in 293 cells but failed to replicate in HeLa cells.

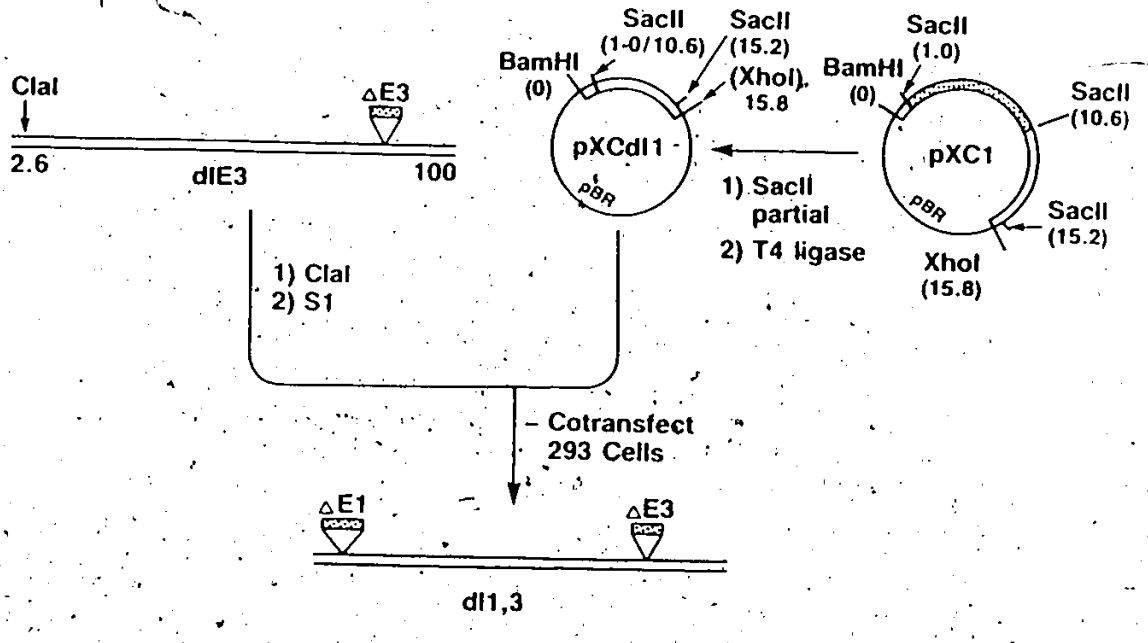
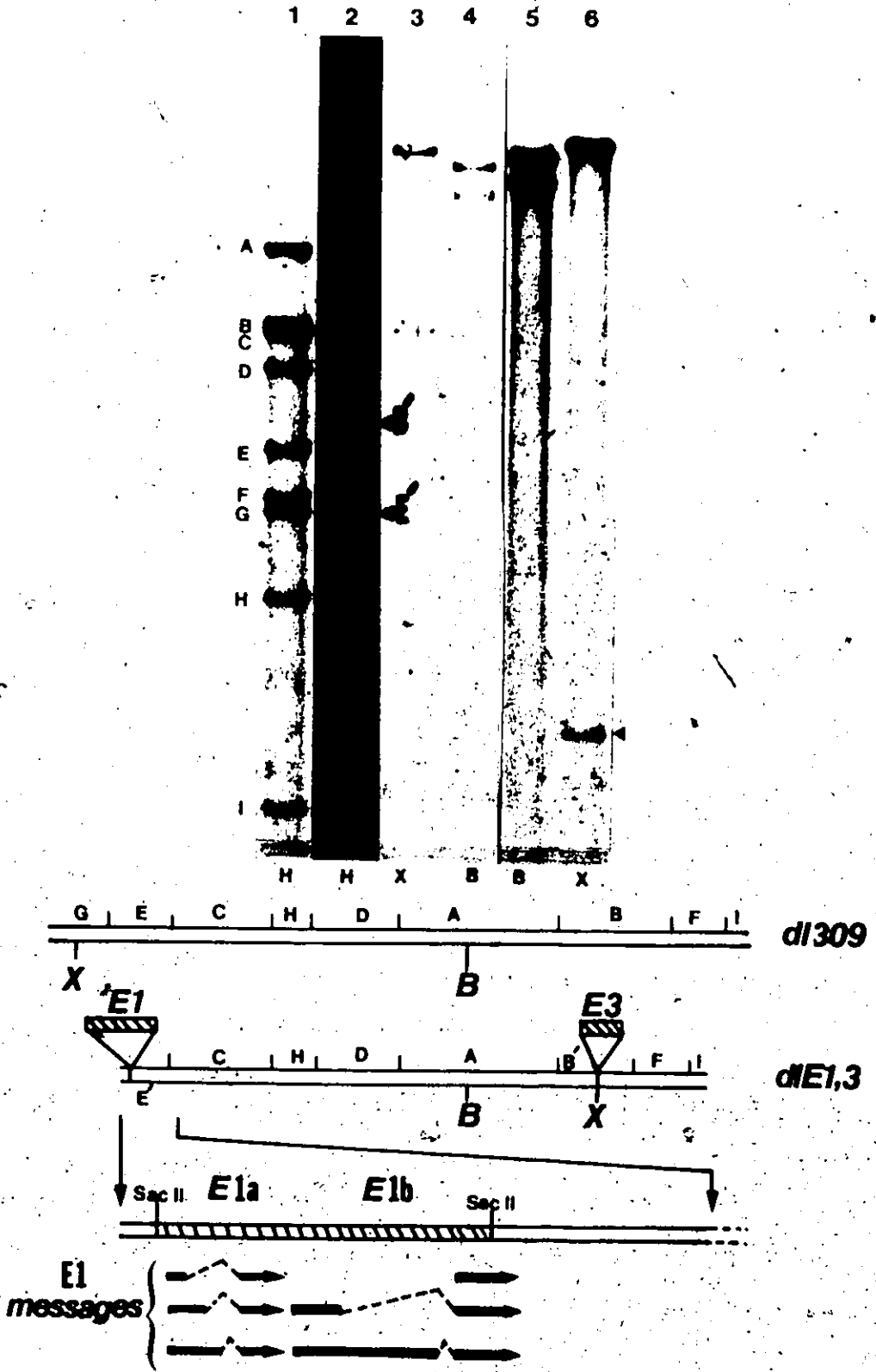


Figure 4.3 Strategy for construction of dlE1,3.

The plasmid pXC1 containing the left 15.8% of the Ad5 genome was cut by SacII (partial digestion) followed by religation to generate pXCdl1 lacking essentially all of E1 from map positions 1.0 to 10.6. Co-transfection of 293 cells with a mixture of ClaI digested, and S1 treated dlE3 DNA, and undigested pXCdl1 DNA yielded dlE1,3, presumably by in vivo recombination.

Figure 4.4 Restriction enzyme analysis of dlE1,3.

In vivo ^{32}P-labelled DNA was digested and electrophoresed on 1% agarose gel as described in Materials and Methods. Deletion of E1 removed sequences from the HindIII fragments G and E resulting in a fusion fragment migrating slightly ahead of the wt HindIII G-fragment (lane 2 arrow E'). Lanes 3 and 6 are the XbaI digests of dlE1,3 and dl309 respectively. The E1 deletion makes the BamHI fragment A smaller, whereas the E3 deletion makes the BamHI fragment B smaller; consequently, both the BamHI fragments A and B of dlE1,3 migrated faster than their counterparts in dl309. The XbaI (X), HindIII (H) and BamHI (B) restriction enzyme maps for dl309 and dlE1,3 are illustrated below, as well as a schematic representation of E1 transcription units. The shaded region between the two SacII sites represents the segment deleted from E1 in dlE1,3. Introns in E1 transcripts are represented by dashed lines.


1 2 3 4 5 6
A
B
C
D
E
F
G
H
I
H H X B B X
G E C H D A B F I
dl309
X
B
E1
E3
C H D A B' F I
dlE1,3
E
B
X
Sac II
E1a
E1b
Sac II
E1
messages

4.4 Growth of Deletion Mutants in HeLa and 293 Cells

The deletion mutants described in this chapter were all constructed and propagated in 293 cells (Ad5-transformed human embryonic kidney cells). The growth of these deletion mutants in 293 and in HeLa cells was compared to assess their host range phenotypes. Briefly, stocks of the indicated viruses were titrated by plaque assay on both cell types (Table 4.1). The wt; and dlE3 mutants did not exhibit host range phenotypes; they produced about equivalent numbers of plaques on both cell types. This was in agreement with what has been reported previously, that early region 3 appears to be non-essential for the adenovirus life cycle at least in cultured cells (Jones and Shenk, 1978, 1979a,b; Berkner and Sharp, 1983; Saito et al., 1985). Indeed, deletion of this region not only fails to block viral growth in HeLa or in 293 cells, but on the contrary it has been reported that a mutant lacking E3 has a growth advantage relative to the wt virus (Berkner and Sharp, 1983).

The virus dlE1,3 produced wild type levels of plaques on 293 cells but was markedly defective for growth in HeLa cells; where no detectable plaques were observed. However, a multiplicity of 1 and 10 PFU per cells, were cytotoxic for HeLa cell monolayers but no individual plaques were observed; this was in agreement with a previous report (Jones and Shenk, 1979a) for a deletion mutant (dl313) lacking all of E1b and the carboxy-terminus of E1a.

Table 4.1 Titration of deletion mutants on HeLa and 293 cells.

Virus[a]	Pfu/ ml on 293	Pfu/ ml on HeLa
dlE1,3	2.1×10^9	N.D.[b]
dlE1b,3	1.3×10^9	N.D.
dlE3dx2	2.4×10^9	1.7×10^9
dlE3	3.1×10^9	3.4×10^9
dl309	1.5×10^9	1.2×10^9

[a] Titration was carried out as outlined in Materials and Methods and in Table 3.2.

[b] Plaques were not detectable at any of the dilutions used ($1x10^{-2}$ to $1x10^{-7}$), this might be due to cell death at the lower dilutions.

4.5 Growth Characteristics and thermolability of dlE1,3

It has been reported previously that deletion of the coding sequences for protein IX does not prevent the replication of adenovirus but capsids lacking protein IX are less stable than wt virions (Colby and Shenk, 1981). Since dlE1,3 lacked more than half of protein IX's coding sequences, its growth rate as well as its thermolability were examined and compared to wt virus. Fig. 4.6 shows the growth kinetics and the yield of dlE1,3 in 293 cells as compared to d1309 (wt). No significant differences were observed between the two viruses for either parameters. Essentially, dlE1,3 grew as well as wt in 293 cells.

Moreover, in order to assess the degree of thermolability viral stocks of dlE1,3 and d1309 (wt) were diluted (10^{-1} to 10^{-9}) in PBS, and incubated at 45^{0}C. Samples were removed at 0 hr (just before submerging samples in a 45^{0}C water bath), and at 1 hr and 3 hr. Infectivity was then measured by plaque assay on 293 cells. In agreement with Colby and Shenk' (1981) findings, dlE1,3 virions are more heat labile than wt virions (Fig. 4.7).

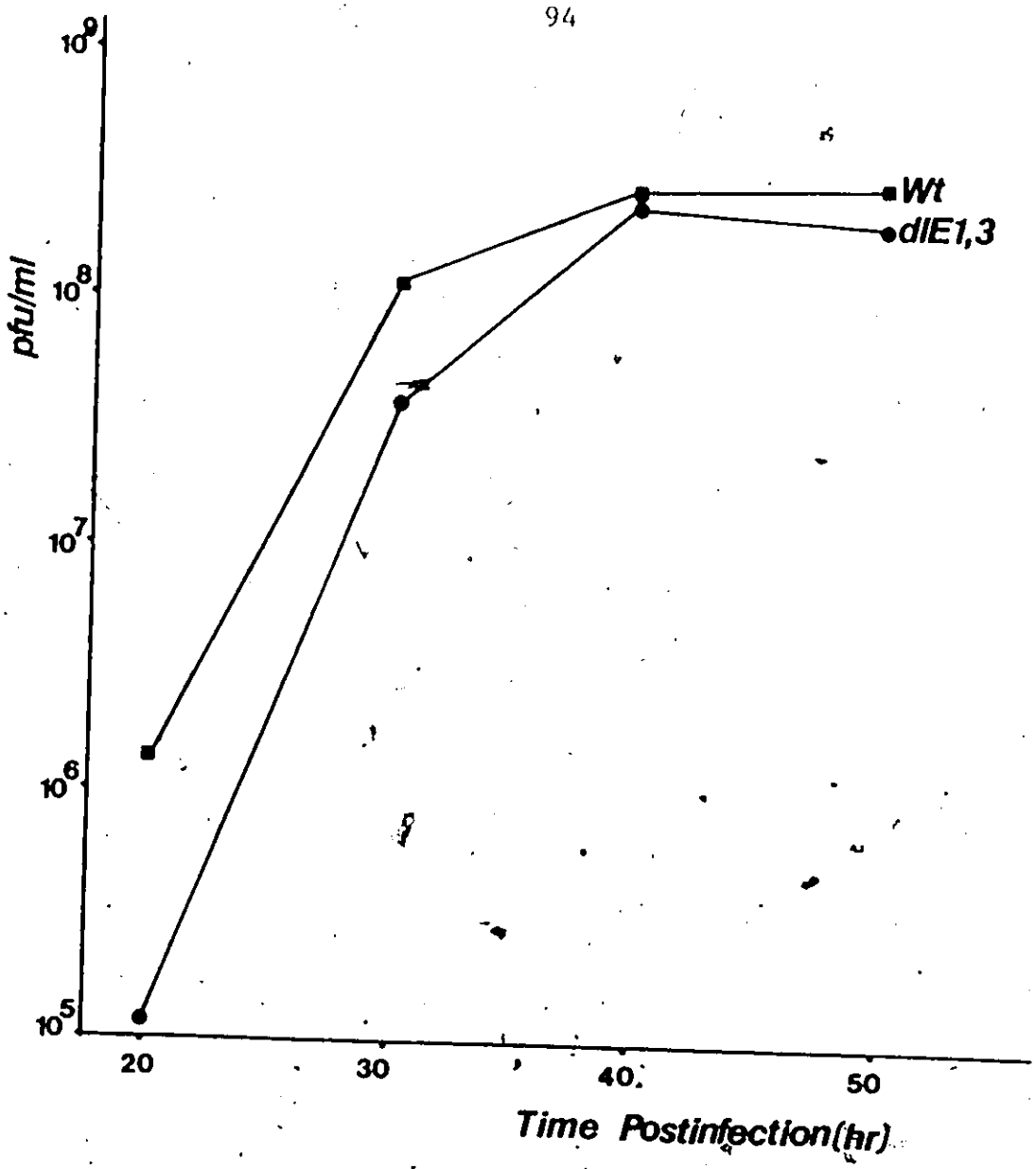


Figure 4.6 Growth curves of dl309 (wt) and dlE1,3, in 293 cells. Cells were infected at a multiplicity of 5 pfu per cell. After absorption for 60 min. at 37°C, cell monolayers were washed once with PBS^{--}, and medium containing 5% HS was added. Cultures were harvested at the indicated times, and the virus titre was measured by plaque assay on 293 cells.

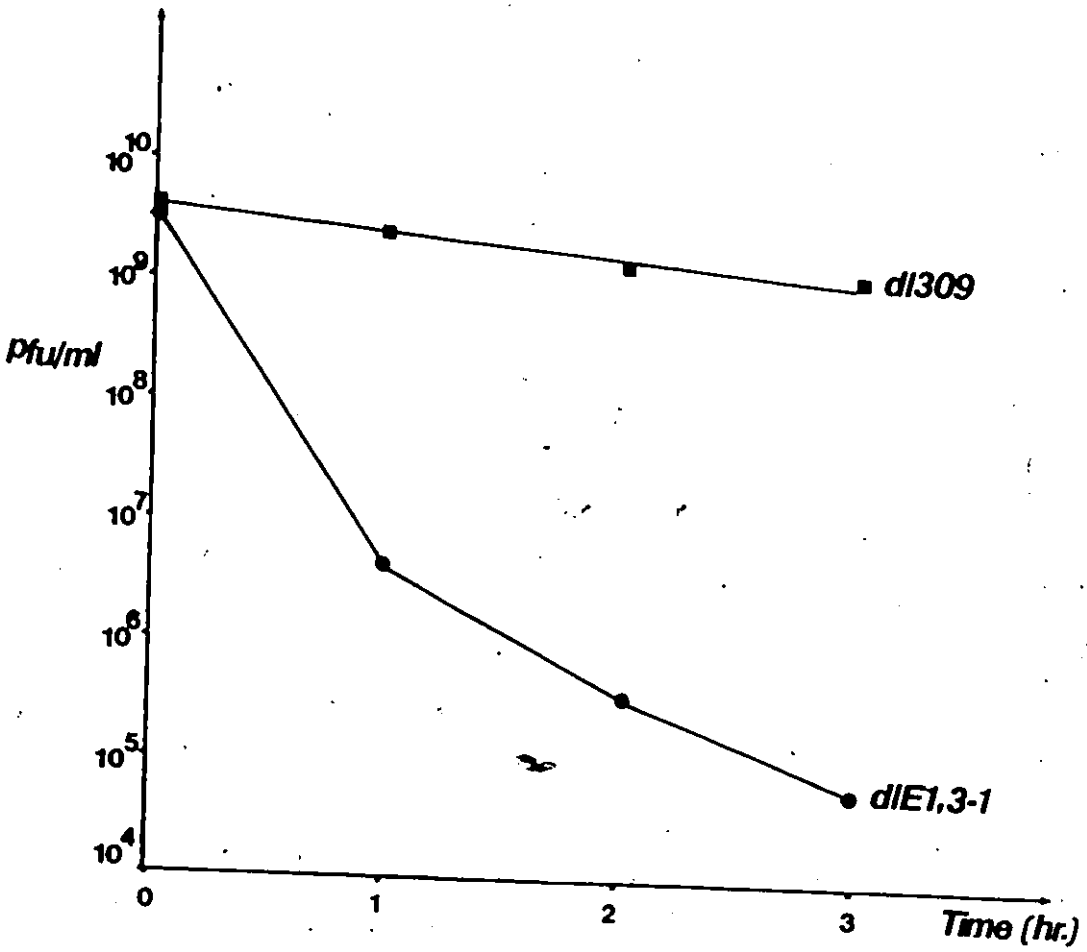


Figure 4.7 Heat inactivation of dl309 (wt) and mutant dlE1,3.

Virus stocks were diluted in PBS^{++}, and submerged in a water bath at 45°C. Samples were removed at the indicated times and used to infect 293 cells monolayers to assess infectivity by plaque assay.

4.6 Conclusions

The data presented and discussed in this chapter can be summarized as follows:

1. I have constructed a virus lacking most of E3 (dlE3). This virus has a wt phenotype, and is about 2 kb shorter than wt virion and therefore should be able to accept inserts of up to 4 kb in size.

2. A helper-independent vector (dlE1,3) which lacks all of E1 and most of E3, was also constructed. The vector dlE1,3 exhibits a host range phenotype with regard to growth on HeLa and on 293 cells. Although this virus is thermolabile, this property is not likely to hamper its usefulness as a vector since most biological experiments are performed at 37°C, at which temperature the virus appears to be stable.

3. In addition to its helper-independence, the genome of dlE1,3 is about 5.5 kb shorter than wt and should be able to theoretically accept inserts of up to 7.5 kb. At present dlE1,3 is the only adenovector with such a capacity. Moreover, the flexibility in cloning foreign DNA into dlE1,3 makes it an attractive cloning vector in mammalian cells. In the next chapter data are presented in which dlE1,3 is used as a vector to clone and express the HSV TK gene in mammalian cells.

CHAPTER V: INSERTION OF THE HSV1 TK GENE INTO DLE1,3 VECTOR

5.1 Introduction

The deletion mutant dlE1,3 (described in Chapter 4) was constructed to be used as a helper-independent cloning vector in mammalian cells. This vector contains a unique XbaI site located at the junction of map position 78.5/84.7. Thus, foreign DNA can be inserted at this site by following one of three different strategies. Briefly, a given gene can be easily inserted at the XbaI site of pFGdX1, then rescued into virion DNA by following a strategy similar to that shown in Fig. 4.1. Alternatively, after inserting a given gene into pFGdX1, the newly constructed plasmid can be co-transfected along with dlE[redacted] into 293 cells thus relying on in vivo recombination, similar to the approach used to construct dlE1,3 from dlE1,3 and plasmid pXCd11. A third possible strategy for insertion of DNA into dlE3 involves cleavage with XbaI and trimolecular ligation (c.f. Fig. 5.1).

In order to demonstrate the usefulness of dlE1,3 as a vector, I have inserted into its unique XbaI site the HSV tk gene in both orientations. The HSV tk gene was chosen for the following reasons: it is very well characterized, and its expression is easily detected;

in addition, it was available as a recombinant clone.

This chapter describes the construction and characterization of Adtk2 (with the HSV tk gene in the same orientation relative to the deleted E3 transcription), and Adtk4 (with the HSV tk gene in the opposite orientation relative to the deleted E3) using two different approaches, and it includes data on the ability of Adtk to transform tk^- cells to the tk^+ phenotype. Furthermore, this chapter includes results on the measurement of tk enzyme activity in Adtk transformed and infected cell lines.

5.2. Construction of Adtk

Fig. 5.1 illustrates the first strategy employed for the insertion of the HSV tk gene into the unique XbaI site of dlE1,3. In order to generate Adtk4, pTK123 (in which the two PvuII sites flanking the HSV tk gene in pTK173 (McKinnon, 1984) had been replaced by XbaI sites) was cut with XbaI and ligated to XbaI digested dlE1,3; the ligation mixture was then transfected onto 293 cells. Approximately eight days later, plaques were picked and progeny viral DNA was analyzed by restriction enzyme digestion. Three out of 39 plaques isolated and analyzed were found to have the tk gene inserted in the XbaI site (78.5/84.7); in all three cases the tk gene was inverted with respect to the direction of transcription of E3 (Fig. 5.2 lanes 2, 5, and 7, and Fig. 5.3 Channels 1, 2 and 3).

The construction of Adtk2 designed to carry the tk gene in the second orientation was carried out as follows: initially, the HSV tk

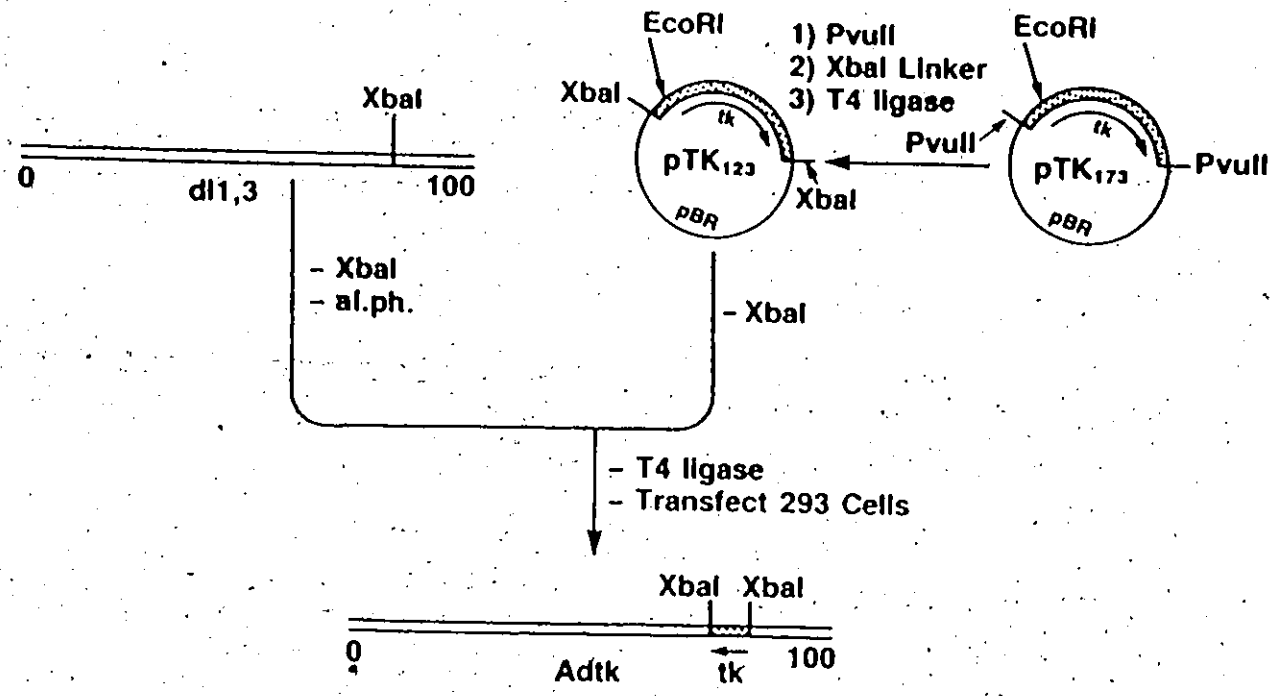


Figure 5.1 Construction of Adtk.

The plasmid pTK173 contains the entire HSV-1 tk gene (PvuII fragment) cloned into the pBR322 at the PvuII site. The two PvuII sites flanking the tk gene were converted to XbaI sites by ligating PvuII digested pTK173 in the presence of synthetic XbaI linker to generate pTK123. The tk gene was then inserted into the unique XbaI site of dlE1,3 via a trimolecular reaction in which pTK123 was digested with XbaI and ligated to XbaI digested dlE1,3. The resulting new virus had the tk inserted in the orientation indicated by the arrow. In addition, the HSV-1 tk gene was also inserted in the opposite orientation via a strategy similar to that outlined in Fig. 4.1, where the tk was inserted first into pFGdxl and subsequently rescued into dlE1,3.

gene was inserted into the XbaI site of pFGdX1, and plasmids were screened for one having the tk gene inserted in the direction of E3 transcription. The resulting new plasmid (pYHtk4) was digested with BamHI, and the dlE1,3 virion DNA was digested with both BamHI and EcoRI (the latter digestion was carried out in order to decrease the likelihood of reconstituting dlE1,3); after inactivation of the restriction enzymes, the digests were mixed and ligated, and the ligation reaction mixture was used to transfect 293 cells as described in Materials and Methods. A total of 6 plaques were obtained and their structure analyzed by restriction enzyme digestion and gel electrophoresis. Five out of the six isolates were found to have the desired structure (one is shown in Fig. 5.3 lanes 4, 5 and 6), and one was found to be dlE1,3; this might have been due to incomplete digestion with BamHI, EcoRI or both. Most of the characterization of tk containing adenovirus mutant was done on Adtk4, the first such isolate obtained. In the following sections, this mutant will be referred to simply as Adtk.

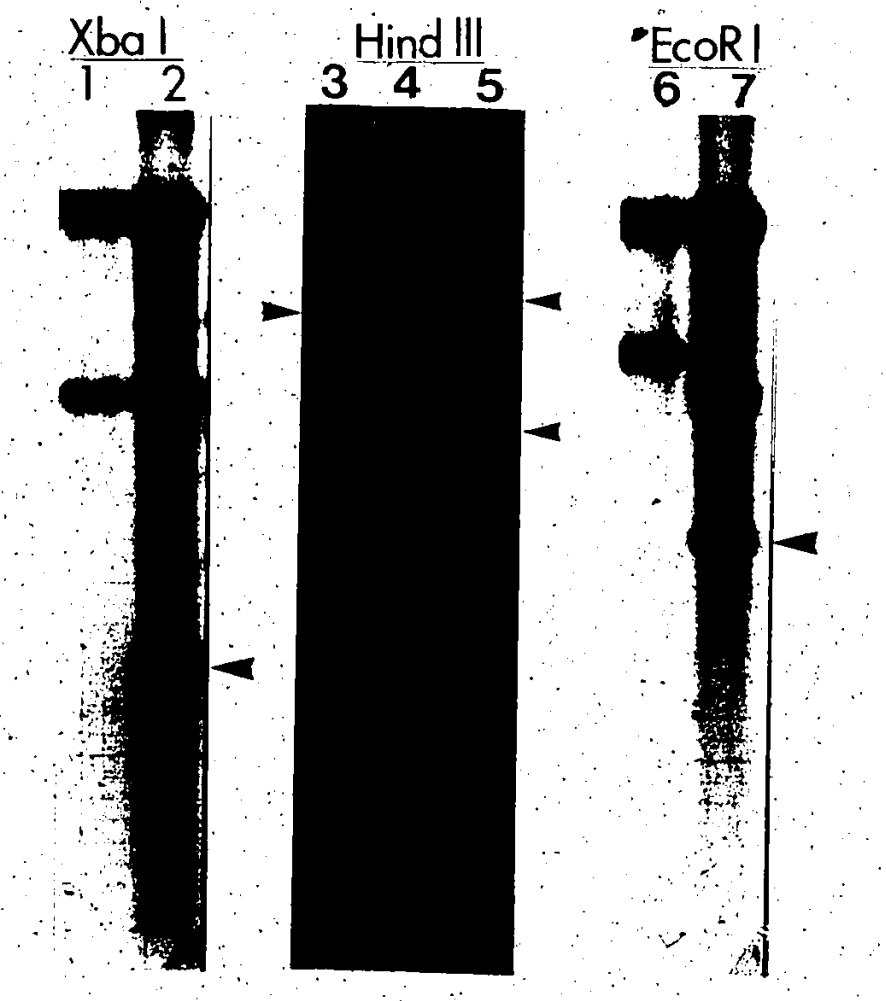
5.3 Phenotype of Adtk

As expected, Adtk has a host range phenotype identical to its progenitor dlE1,3, (i.e.) it grows as well as wt on 293 cells but does not grow in HeLa cells. It was observed that when Adtk and dlE1,3 were grown in 293 cells in a mixed infection, the ratio of Adtk to dlE1,3 seemed to decrease as a function of passage numbers. This might have been due to the difference in genomic size, (dlE1,3 being

Figure 5.2 Restriction enzyme analysis of Adtk4.

In vivo labelled viral DNA was digested with the indicated enzymes and electrophoresed on 1% agarose gel. Lanes 1 and 2 are XbaI digests of dlE1,3 and Adtk respectively; the tk containing band is indicated by arrow heads (lanes 2,5 and 7).

Lanes 3, 4 and 5 are HindIII digests of dl309, dlE1,3 and Adtk respectively; the top arrow indicates the location of the fragment carrying the tk gene (lane 5), while the lower arrow indicates the B'-fragment of dlE1,3 (lane 4). Lanes 6 and 7 are EcoRI digests of dlE1,3 and Adtk respectively. The arrow head (lane 7) points to the fragment containing the tk sequence. The EcoRI (E) and XbaI (X) restriction enzyme maps of dlE1,3 and Adtk are illustrated below.


Xba I
1 2
Hind III
3 4 5
EcoR I
6 7

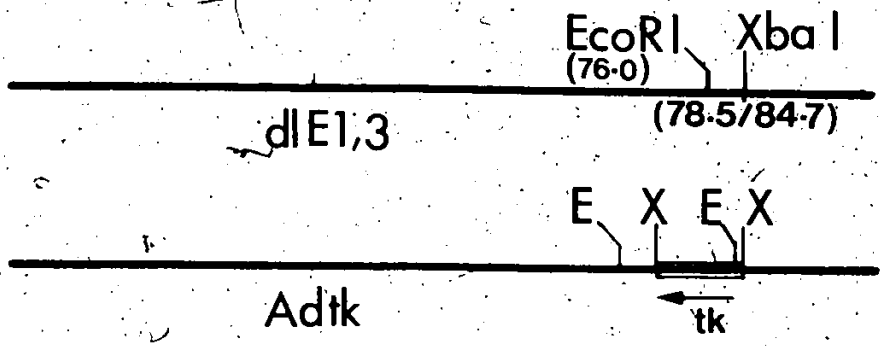
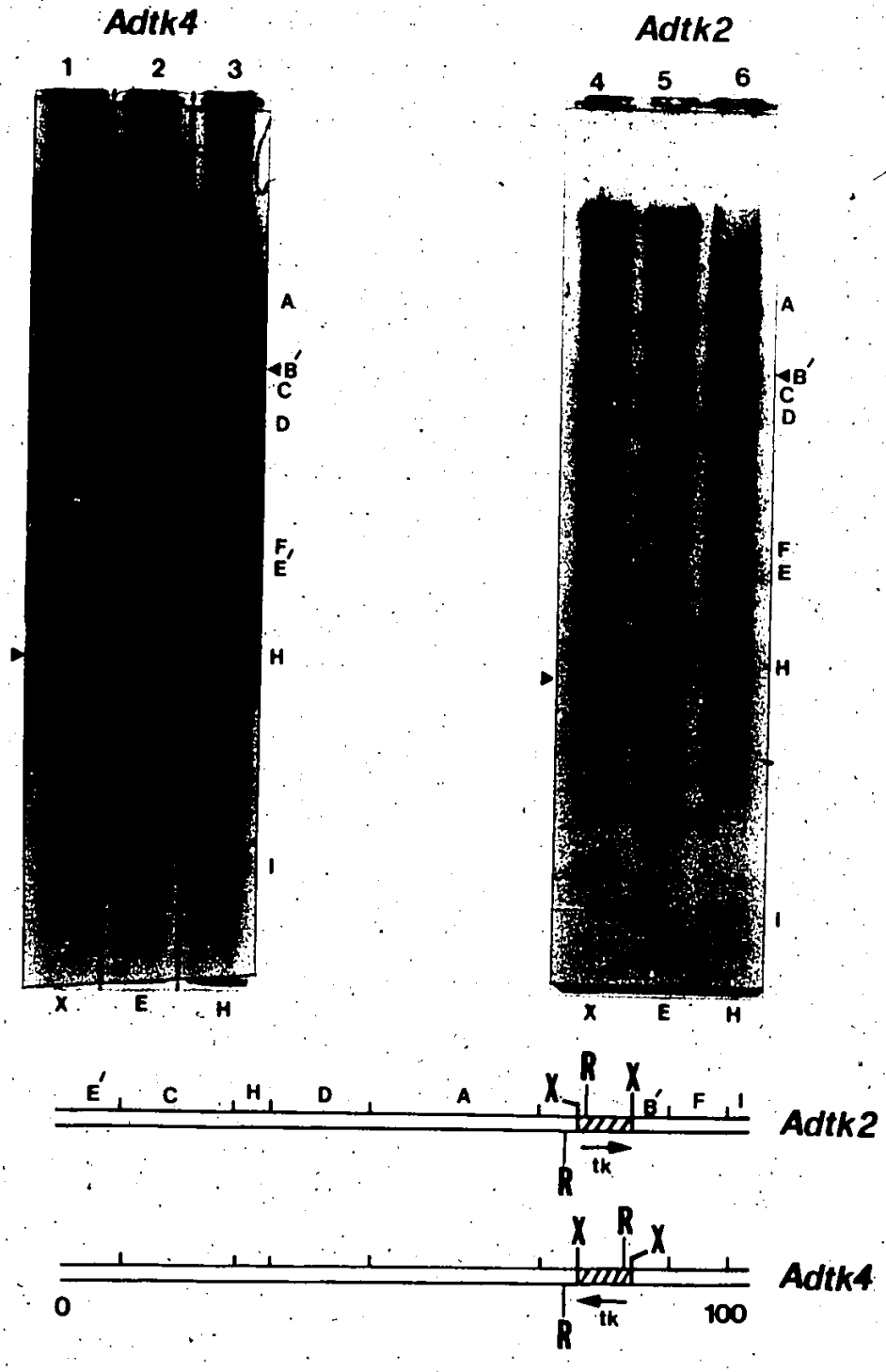

EcoR I
(76·0)
Xba I
(78·5/84·7)
dlE1,3
E X E X
Adtk
tk

Figure 5.3 Restriction enzyme analysis of Adtk2 and Adtk4.

In vivo labelled viral DNA was digested with the indicated restriction enzymes and electrophoresed on 1% agarose gel. Lanes 1, 2 and 3 are Adtk4 (Adtk) digested with XbaI (X), EcoRI (E) and HindIII (H). Lanes 4, 5 and 6 are Adtk2, digested with XbaI (X), EcoRI (E) and HindIII (H). The XbaI and HindIII patterns for both Adtk2 and Adtk4 (Adtk) were identical as expected whereas the EcoRI digest (lanes 2 and 5) discriminated between the 2 orientations of the insert. The HindIII (H), XbaI (X) and EcoRI (E) restriction enzyme maps for both Adtk4 (Adtk) and Adtk2 are shown below.


Adtk4
1
2
3
A
B′
C
D
F′
E
H
I
X
E
H
Adtk2
4
5
6
A
B′
C
D
F
E
H
I
X
E
H
E′
C
H
D
A
X
R
X
B′
F
I
tk
R
Adtk2
X
R
X
Adtk4
0
tk
100
R

about 2 kb shorter than Adtk) which might result in faster replication of dlE1,3.

5.4 Stability of the tk Insert in Adtk

To test the stability of the tk insert, Adtk4 was passaged five times in 293 cells, then plaqued on 293 cells. About 5 days later 12 plaques were picked and their DNA was analyzed on a 1% Agarose gel.

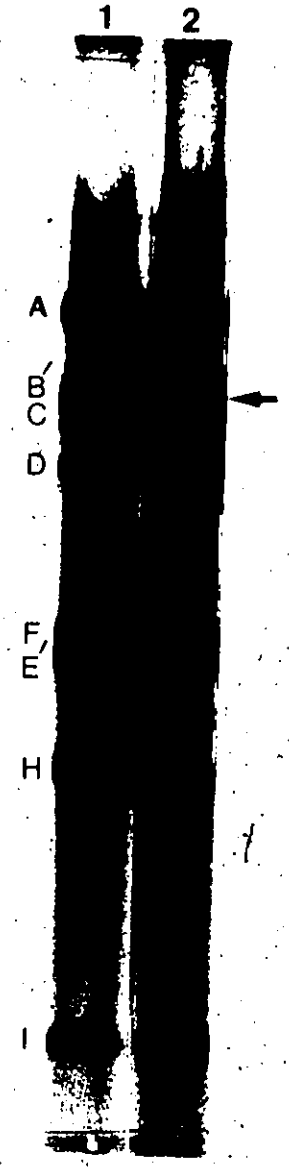
Figure 5.4 shows the HindIII restriction enzyme pattern of 2 such plaque isolates, however, identical patterns were obtained with all 12 plaques picked. Thus, the tk insert seemed to be very stable based on the limited number of plaques analyzed.

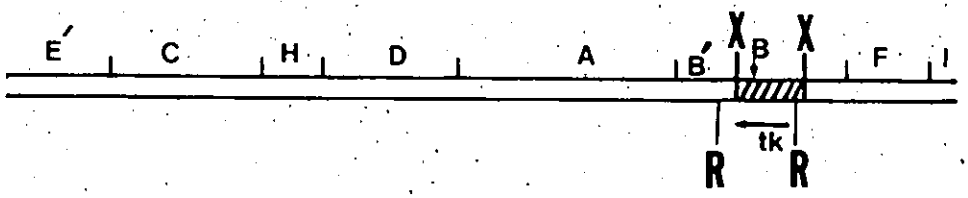
5.5 TK Expression in Adtk Infected Cells

In order for ^{3}H-thymidine to be incorporated into DNA the labelled substrate has to be phosphorylated by thymidine kinase. Thus, measurements of ^{3}H-thymidine incorporation into DNA can be used as an indirect measure of the level of tk activity. TK^- cells (143 or LTA) were infected with adenoviruses (dlE1,3 or Adtk) at 10 pfu/cell. Twenty-four hours later one half of the Adtk infected dishes were superinfected with HSV tk^- virus at 1 pfu/cell. Cells were then pulse-labelled with ^{3}H-thymidine (1 uCi/ml) for 2 hrs at 24, 27, 30, 36 and 48 hrs post adeno infection and the nucleic acids were extracted and radioactivity counted as described in Materials and

Figure 5.4 Stability of tk insert in Adtk4.

HindIII restriction enzyme pattern of 2 plaque isolates of Adtk4; all the remaining 10 plaque isolates are identical to these 2. The band containing the tk gene is indicated by an arrow. The HindIII restriction enzyme map of Adtk4 is illustrated below.





Adtk4

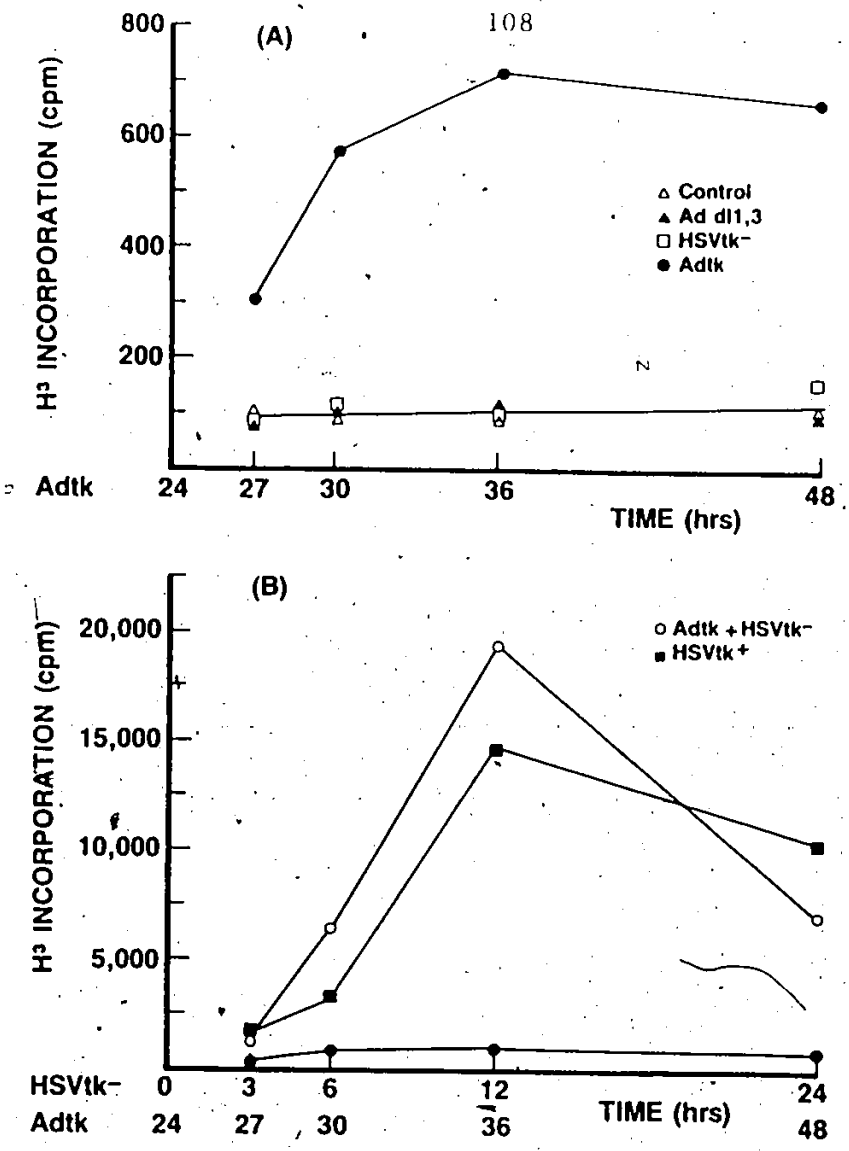


Figure 5.5 Kinetics of ^{3}H-thymidine incorporation (cpm) in LTA cells following viral infection. Cells were infected with 10 pfu/cell of Adtk or dlE1,3 or superinfected at 24 hours after Adtk infection with 1 pfu/cell of HSV (either tk^{+} or tk^{-}). At the indicated times post-infection, the cells were harvested and the levels of ^{3}H-incorporation were determined as described in Materials and Methods. The times indicated along the abscissa represent the time post adenovirus infection (all panels) and the time after infection or superinfection with HSV1 (panel B).

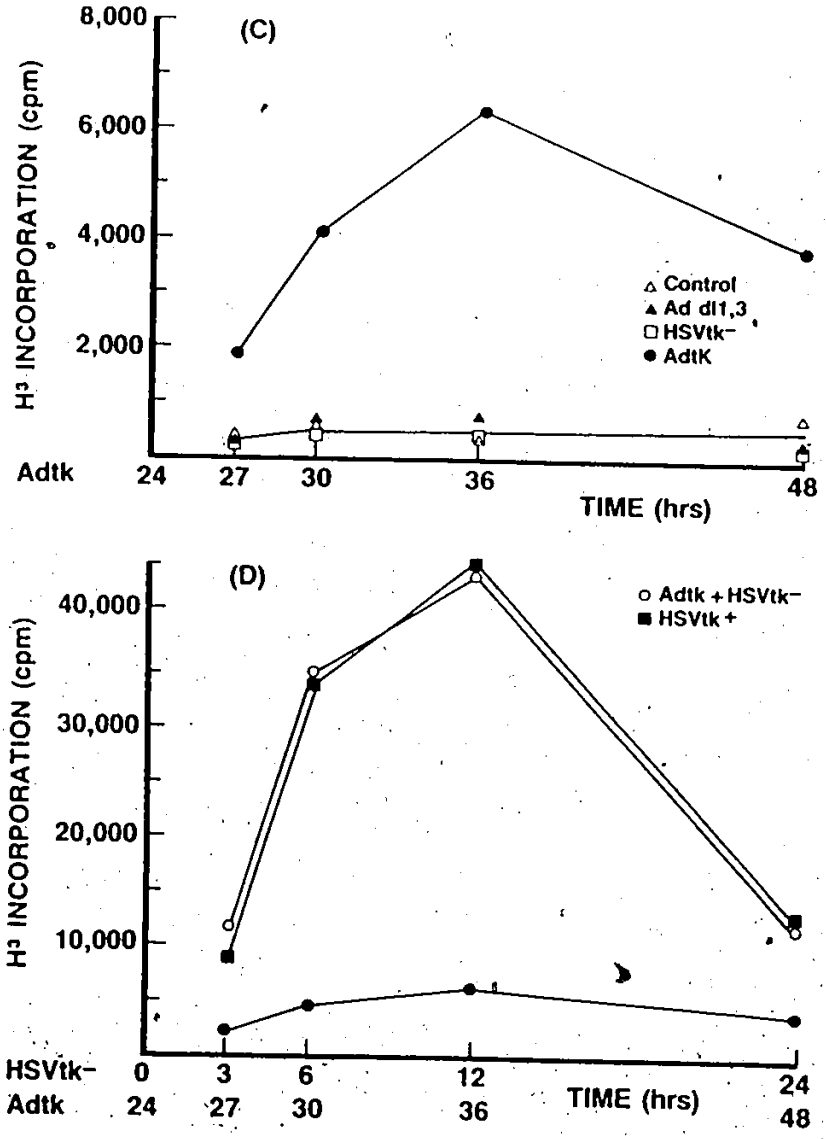


Figure 5.6 Kinetics of ^{3}H-Thymidine incorporation (cpm) in 143 cells following viral infection. The details are exactly as in Fig. 5.5.

Methods. The results obtained in LTA and 143 cells are shown in Figures 5.5 and 5.6 respectively. In both cell lines the rate of ^{3}H-thymidine incorporation reached a maximum at about 36 hrs after infection with Adtk virus. Although the absolute levels were approximately 10 fold higher in 143 cells (Fig. 5.6 panel C) than in LTA cells (Fig. 5.5 panel A), the ratio of tk activity in infected cells over tk activity in mock-infected cells was very similar (7 fold increase upon infection in LTA compared to 8 fold in 143 cells). The rate of incorporation presumably reflected both the levels of tk expression and the rate of DNA replication in infected cells. The relatively slow time course of ^{3}H thymidine incorporation was probably due to the fact that Adtk is defective for growth both in LTA and 143 cells as a result of the E1 deletion. This may also explain, in part, the apparent low levels of tk activity seen in Adtk infected cells. However, a more likely explanation for inefficient expression is that the HSV tk gene is under the control of its own regulatory sequences (which are early, or beta class), and under normal circumstances (i.e. during HSV infection), maximum tk gene expression requires one or more HSV immediate early or alpha functions (Tooze, 1981). That this may indeed be the case was indicated by the results shown in Fig. 5.5, panel B, and Fig. 5.6 panel D. In these experiments Adtk infected LTA (Fig. 5.5 panel A) or 143 (Fig. 5.6 panel B) cells were superinfected at 24 hrs with a tk^{-} mutant of HSV. This resulted in a large increase in tk expression (as measured by ^{3}H thymidine incorporation) raising it to levels comparable to those in cells singly infected with wt HSV. Furthermore, tk activity in Adtk2 was found to be similar to that of Adtk4 (Fig. 5.7),

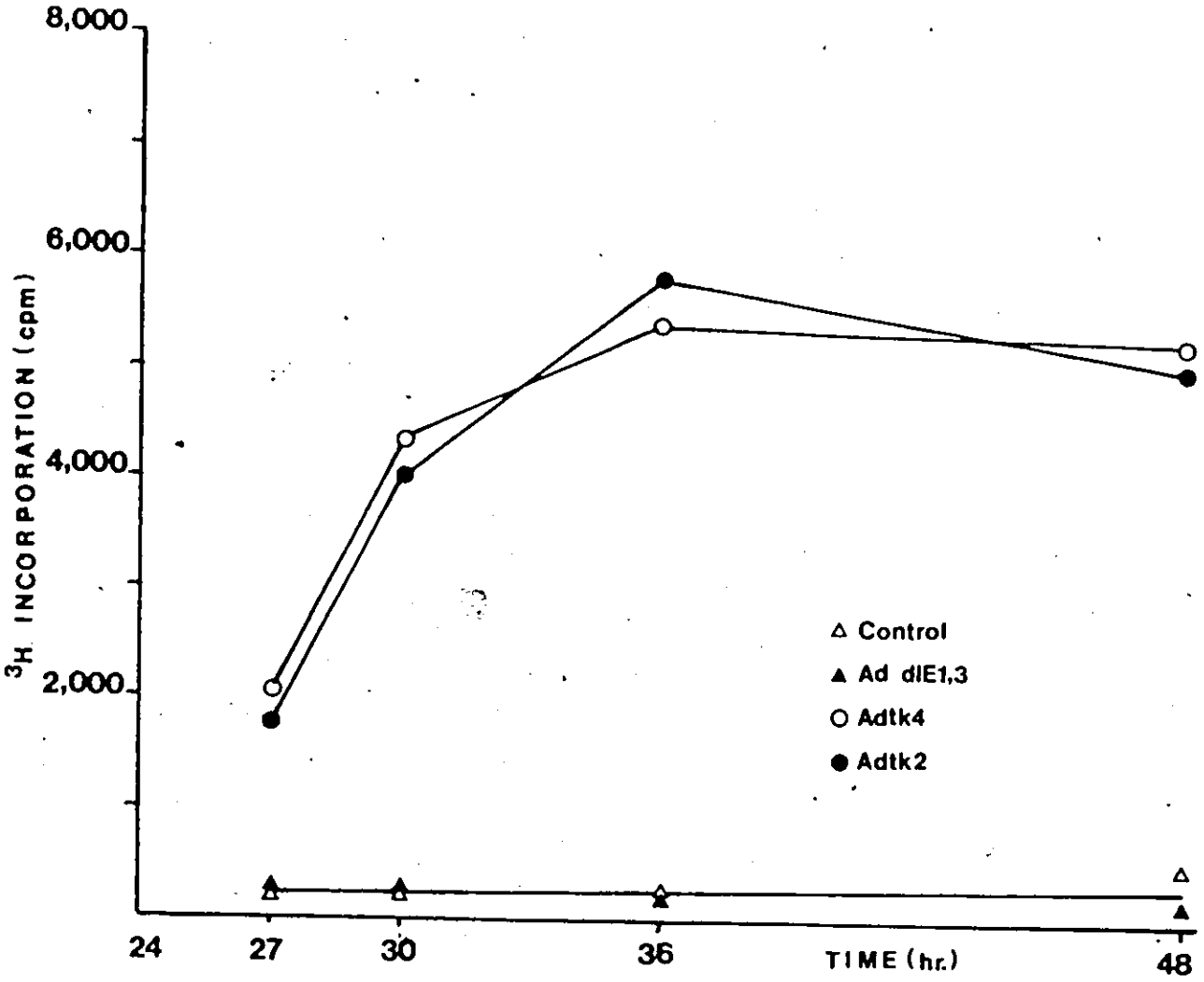


Figure 5.7 Kinetics of ^{3}H-thymidine incorporation (cpm) in 143 cells following viral infection. Cells were infected with 10 pfu/cell of dlE1,3 (as a control), and with either Adtk2 or Adtk4. At the indicated times post-infection, the cells were harvested and the levels of ^{3}H-incorporation were determined as described in Materials and Methods.

indicating that the orientation of the tk gene is irrelevant for tk expression.

Finally, the results obtained by direct measurement of tk activity in cell free extracts prepared from Adtk infected 143 cells (Table 5.1) were in qualitative agreement with the results obtained by assaying thymidine incorporation into DNA. In particular, the relative enhancement of tk activity in infected cell extracts relative to uninfected cell extracts was similar to that shown in Figures. 5.5 and 5.6 for the rates of incorporation. The residual levels of tk activity in mock infected cells might be due to mitochondrial tk activity.

5.6 Transformation of tk^- cells to tk^+ phenotype

Wt Ad5 will replicate in both mouse and human cells with the result that the lytic response typically prevents the detection and isolation of transformants. However, because the Adtk mutant lacked E1 it seemed likely that it could be used to transform tk^- cells to a tk^+ phenotype and to establish tk^+ cell lines.

Three tk^- cell lines, K4 (contains and constitutively expresses the HSV ICP4; Persson et al., 1985), LTA and 143 cells were used in transformation assays but the efficiency of transformation with 143 cells was very low and quantitative results were obtained only for both the K4 and the LTA cells. Colonies of K4 and LTA cells resistant to HAT were visible 1 to 2 weeks post-infection and were counted three weeks post-infection with Adtk. Table 5.2 shows the

Table 5.1 TK activity in infected 143 cells[a].

Viruses	Hours Post-Infection			
	6	12	24	36
Mock	ND	ND	90, 65	80, 90
Adtk	ND	ND	140, 120	300, 270
HSV	580, 450	950, 820	ND	ND

[a] Cells were infected with either 10 pfu/ cell of Adtk or 1 pfu/ cell of HSV1; at the times indicated post-infection, the cells were harvested and the specific, activity of tk in cell-free extracts was determined. Each value represents the average of two reactions calculated as cpm of ^{14}C-thymidine phosphorylated per ug of total cellular protein. The tk activity in mock infected cells might be due to mitochondrial tk.

transformation efficiency obtained in two independent experiments after infection of LTA cells with 0.1, 0.5, 10 and 20 pfu/cell. Table 5.3 shows the transformation efficiency obtained after infection of K4 cells. The results indicate that in both LTA and K4 cells, the transformation efficiency was approximately linear with respect to multiplicity of infection yielding 1-2 X 10^{-6} transformants per pfu in LTA, and 1-2 X 10^{-4} transformants per PFU in K4 cells; these values were similar to those reported by Van Doren et al. (1984) for an adenovirus mutant carrying the neomycin resistance gene (or the SV40 large T antigen, Van Doren and Gluzman, 1984). The transformation efficiency was also dependent upon the recipient cell used and, as mentioned above, the 143 cell line was transformed at a very low efficiency compared to LTA and K4 cells; in contrast to K4 and LTA cells for which up to 100 transformants/plate were readily obtained, only 1-2 transformants were detected in several dishes of 143 cells infected with Adtk. It seems likely that this difference resulted from differences in efficiency of integration or from some other factor rather than differences in the efficiency of delivery of the gene into the cells since levels of tk expression were higher in infected 143 cells than in the mouse lines.

Tk^+ cell lines of both mouse and human origin were established by picking single colonies and passaging them in a selective medium (HAT). Tk activity in the resulting transformed cells was measured by the enzymatic assay as described by Summers et al. (1975). The results shown in Table 5.4 indicated that the level of tk activity in Adtk transformed human (T143) and mouse cells (TLTA) was significantly higher than that of the respective tk^- parents

Table 5.2 Transformation of LTA Cells

Expt. No.	Virus[a]	moi	No. of foci[b]	Mean
1	Mock	-	0,0,0,0	0
	dlE1,3	0.5	0,0,0,0	0
		20	0,0,0,0	0
	Adtk	0.1	1,0,1,0	0.5
		10	48, 33, 63, 60	51
		20	80, 100, 93, 105	94
2	Mock	-	0,0,0	0
	dlE1,3	0.5	0,0,0	0
		20	0,0,0	0
	Adtk	0.1	1,0,0,0	0.3
		0.5	5,6,7,3	5
		10	23,19,26,16	21
		20	40,36,48,33	39

[a] Cells were infected with the virus and moi indicated, incubated in selective (HAT) medium, fixed and stained at two to three weeks post-infection.

[b] Colonies of transformed cells per 60 mm dish.

Table 5.3 Transformation of K4 cells.

Virus[a]	moi	No. of foci[b]	Mean
Mock	-	0,0,0,0	0
dlE1,3	1	0,0,0,0	0
	20	0,0,0,0	0
Adtk	0.1	26, 27, 22, 28	26
	1	400, 450, 455, 390	420
	10	TNTC	TNTC[c]
	20	TNTC	TNTC

[a] Cells were infected with the virus at moi's indicated, incubated in selective (HAT) medium, fixed and stained at two to three weeks post-infection.

[b] Colonies of transformed cells per 60 mm dish.

[c] TNTC= too numerous to count.

Table 5.4 Tk activity in Adtk-transformed or control cells

Cell Line	Specific Activity[a]
143	110, 90
T143[b]	970, 760
LTA	36, 40
TLTA[b]	780, 810
293	1100, 950

[a] Specific tk activity in cpm ^{14}C-thymidine phosphorylated per ug protein, average of two reactions.

[b] T143 and TLTA are tk^+ human and mouse cell lines established following transformations of tk^- 143 and LTA cells with Adtk.

(143 and LTA) and was comparable to levels expressed in a wt tk^+ human cell line (293).

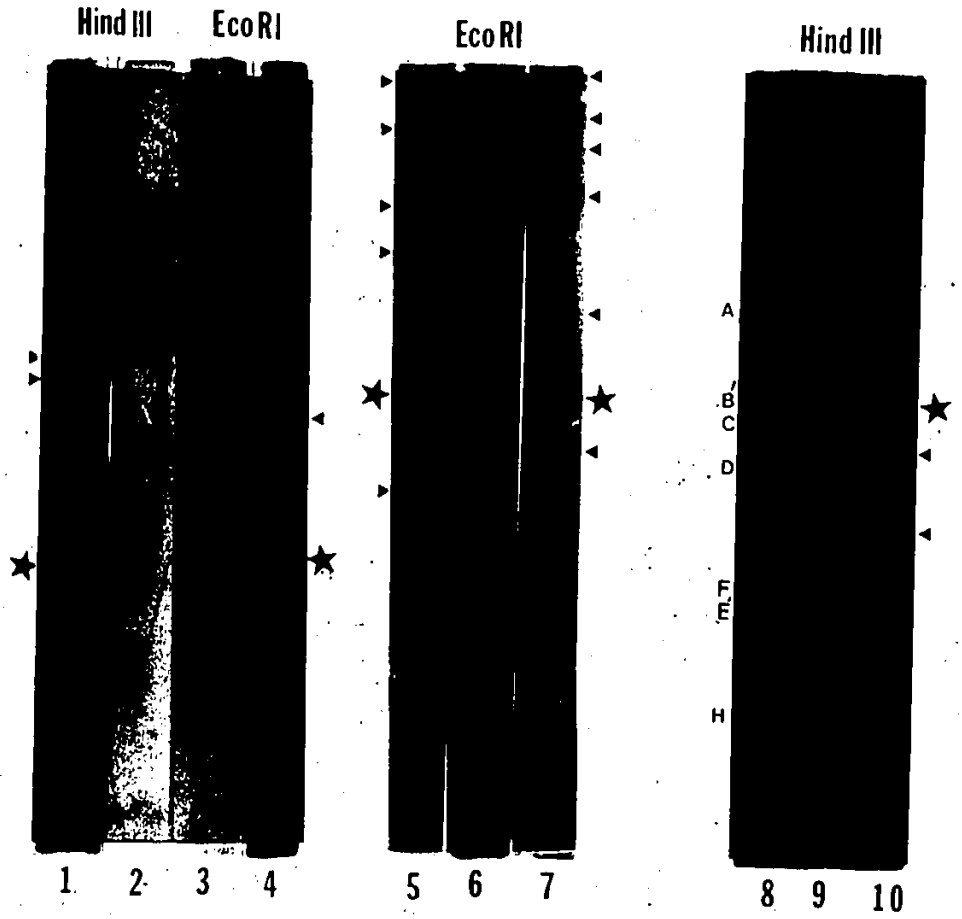
5.7 Structure of Adtk in Transformed Cell Line

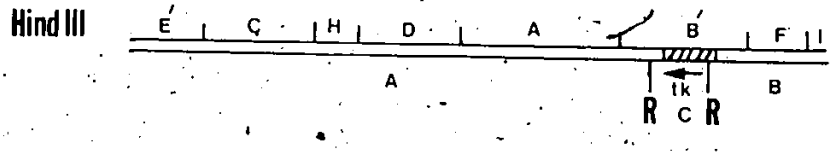
Cellular DNA was extracted from the TK4, TLTA and T143 transformed lines, digested with EcoRI or HindIII and electrophoresed on 0.8% agarose gels. The DNA was then denatured, transferred onto nitrocellulose filters and hybridized with nick-translated Adtk DNA as described in Materials and Methods. The results (Fig. 5.8) show that the DNA from all of the transformants contained most, if not all, of the viral DNA. Furthermore, from a reconstruction using 5 genome equivalents of virion DNA per genome equivalent of cell DNA (7 pg/mg) (lanes 2, 3, 6, and 8) it was concluded that about 2 to 5 copies of the viral genome were present per transformed cell. This conclusion was based on comparing the intensity of the restriction enzymes' bands in the control lanes (lanes 2, 3, 6, and 8) with the bands' intensity in the other lanes. Two additional points can be made: first, the viral DNA sequences appeared to be integrated at several sites in the host genome; this was clearly seen in lanes 5 and 7, where multiple junctions (between cellular and viral DNA) bands can be seen (arrow heads). Secondly, the tk containing bands (asterisks, lanes 1, 4, and 5) appeared to become more intense than the other viral bands within the same lanes, suggesting that these sequences had been amplified after successive passages of the transformed cells in selective medium (HAT-containing medium), whereas the intensity of the tk bands

(asterisks lanes 7 and 10) is about the same as the other viral bands in early passages of transformed cells (lanes, 7, 9, and 10). Therefore, it appears that in this type of transformation assay, integration may not require E1 gene products, an observation in agreement with the results reported by Van Doren et al. (1984).

Figure 5.8 Structure of Adtk sequences in Adtk transformed cell lines.

Southern gel analysis of Adtk transformed cells. DNAs were extracted as described in Materials and Methods, digested with the indicated restriction enzymes and electrophoresed on 0.8% agarose gel. Nick translated Adtk DNA was used as a probe. Lanes 1 and 2 are HindIII digested of TK4 (tk^- K4 cell line transformed to tk^+ by Adtk) and Adtk4 DNA respectively. Lanes 3 and 4 are EcoRI digests of Adtk4 and TK4 respectively. Lanes 5, 6, and 7 are EcoRI digests of TLTA (transformed LTA), Adtk4 and T143 DNA respectively. In lanes 1, 4, and 5, the DNA was isolated from the transformed cells (TK4, and TLTA) after 10 passages, whereas the DNA in lanes 7, 9, and 10 was isolated from the transformed cells (T143, and TLTA) after 4 passages. The HindIII (H) and EcoRI (E) map of Adtk4 are shown below. Arrow heads point to bands which do not appear in the control lanes (Adtk). Asterisks point to the tk containing bands.


Hind III
EcoRI
EcoRI
Hind III
A
B'
C
D
F'
E'
H
1 2 3 4
5 6 7
8 9 10


Hind III
E'
C
H
D
A
B'
F
I
A
1k
B
R
C
R

5.8 Conclusions

The data presented and discussed in this Chapter can be best summarized as follows:

1. Two different approaches were used to insert the HSV tk gene in both orientations in dlE1,3 to generate Adtk2 and Adtk4 (Adtk).

2. The phenotype of Adtk was found to be similar to that of the progenitor dlE1,3 with respect to growth in HeLa and 293 cells. More importantly, the tk insert appeared to be stable and unmodified following successive passagings of Adtk in 293 cells.

3. Although the tk gene was under its own regulatory sequences, and was not subjected to positive selection, tk activity in Adtk infected cells was expressed albeit at detectable low levels as compared to the HSV wt tk expression.

4. Adtk converted tk^- cells to tk^+ phenotype, with variable efficiencies depending on the cell line used.

5. Adtk transformed human and mouse cell lines appeared to contain the entire Adtk sequences, most likely integrated; these tk^+ transformed cell lines expressed tk as efficiently as a wt tk^+ human cell line.

CHAPTER VI: ISOLATION OF AN ADENOVIRUS TYPE 5 MUTANT WITH EMBEDDED INVERTED TERMINAL REPEATS

6.1 Introduction

Human adenoviruses have proven to be very useful as a model system not only for the study of oncogenic transformation (reviewed recently by Branton et al., 1985), and gene expression in mammalian cells (reviewed in chapter 1), but also for exploring the basic mechanisms of eukaryotic DNA replication. At the present time as a result of extensive studies carried out both *in vivo* and *in vitro*, the structure and replication of adenovirus DNA is probably better understood than for any other eukaryotic system (reviewed recently by Kelly, 1984). Each molecular end of the Ad5 genome possesses an identical inverted terminal repeat (ITR) of 103 bp (Tooze, 1981). The 5' end of each strand is covalently linked to a 55,000 (55kd) terminal protein (TP) via a deoxycytosine-to-serine phosphoryl bond (Robinson et al., 1973; Rekosh et al., 1977; Desiderio and Kelly, 1981). The 55kd TP is generated when an adeno-encoded protease specifically cleaves the early region E2B encoded 80kd preterminal protein (pTP) (Challberg and Kelly, 1981; Stillman et al., 1981). The pTP participates in the initiation of viral DNA replication (Challberg et

al., 1980, 1982; Lichy et al., 1981; Stillman et al., 1981) forming a complex with the adeno-encoded polymerase (Stillman et al., 1982a; Lichy et al., 1982), and becoming covalently linked to dCMP which then serves as a primer for chain elongation and becomes the first nucleotide of the daughter strand. After initiation, DNA replication proceeds in a continuous fashion along the template strand displacing the opposite strand of parental DNA. Finally, the displaced parental strand is thought to replicate by forming a panhandle structure by hybridization of the ITRs, thus generating a double-stranded origin of replication identical to that of a linear duplex (Daniell, 1976; Rekosh et al., 1977).

As mentioned above, each end of the viral chromosome possesses an identical ITR (Steenbergh and Sussenbach, 1979; Van Ormondt et al., 1978) where the origin of viral DNA replication has been localized (Challberg and Kelly, 1982; Wang and Pearson, 1985). One notable feature of the adenovirus' ITRs is the presence of a highly conserved 10 bp AT rich sequence (ATAATATACC) beginning 9 bp from the terminus and found in all human adenovirus serotypes examined (Tolun et al., 1979; Shinagawa and Padmanabham, 1980; Stillman et al., 1982b). This DNA sequence and the sequences from approximately 20 to 45 which contain the binding site for the cellular protein, nuclear factor I, constitute the origin of viral DNA replication (Leegwater et al.1985; de Vries et al., 1985; Wang and Pearson, 1985). For a viral DNA molecule to be infectious, it is not necessary that both ITRs be intact. Using various <u>in vitro</u> constructs of viral DNA, Stow (1981, 1982) has shown that deletions in the left ITR can be repaired to generate wild type (wt) progeny following transfection of permissive

cells provided the deletions do not remove the entire ITR. Deletions larger than the ITR, however, render the viral DNA non-infectious. These findings led Stow (1982) to propose that deletions in one ITR can be corrected in vivo from the homologous sequences in the other ITR via panhandle formation and repair synthesis.

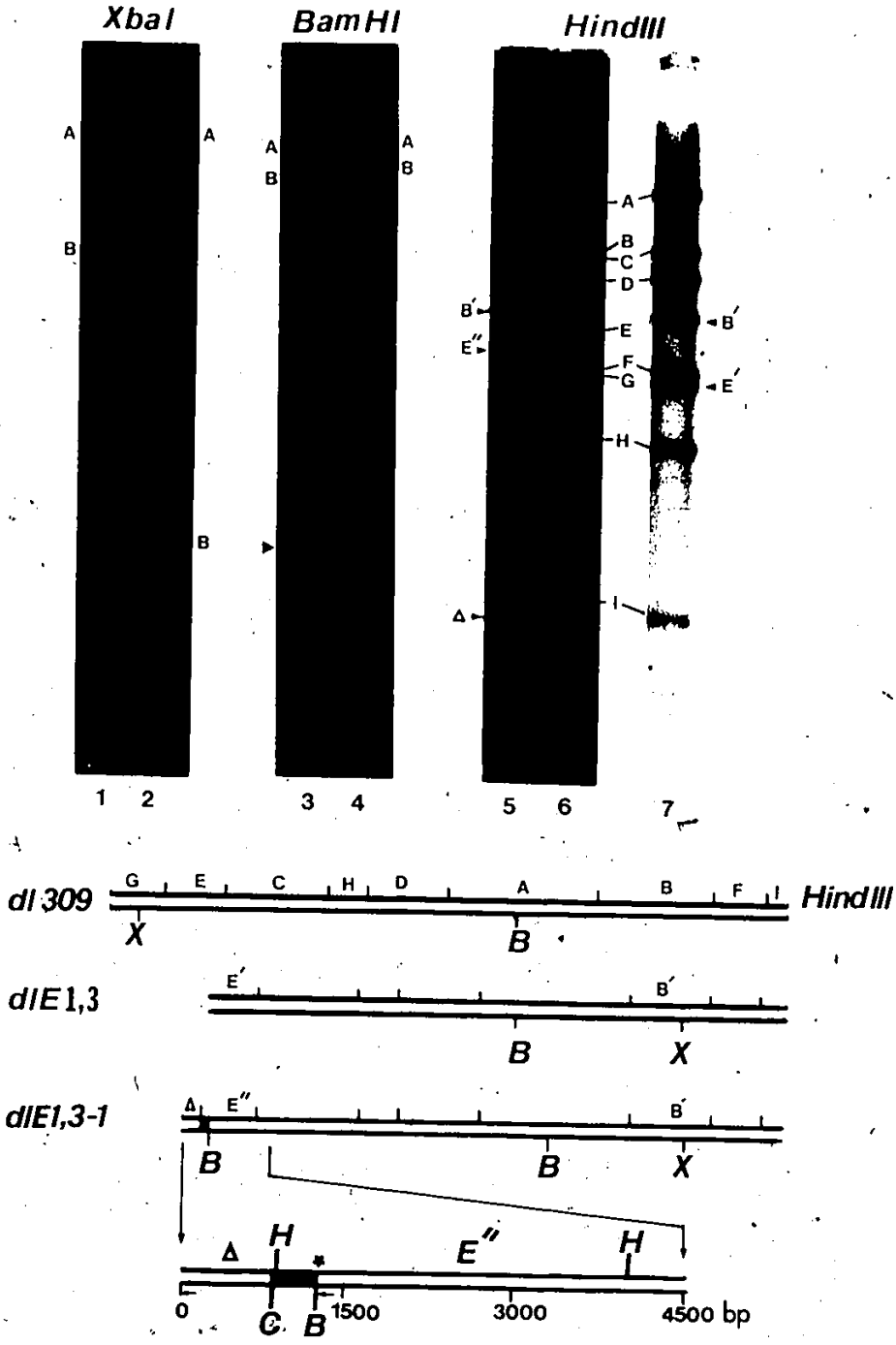
Adenovirus mutants with duplicated ends have been isolated by various groups (Hammarskjold and Winberg, 1980; Brusca and Chinnadurai, 1983; Hanahan and Gluzman, 1984). Generally these mutants have been found to be fairly stable, undergoing further rearrangements or reverting to wt either not at all or at very low rates. This chapter describes the isolation and characterization of a novel Ad5 mutant (dlE1,3-1) that had direct repeats of left end DNA sequences.

6.2 Isolation of dlE1,3-1 and Determination of its Genomic Structure

During the construction of an Ad5 cloning vector dlE1,3 (presented and discussed in chapter 4), which lacked most of E1 and E3, one of the first plaques isolated was found to contain 1270 bp of extra DNA attached to the left end of dlE1,3 genome. Fig. 6.1 shows the results of restriction enzyme analyses for this mutant (dlE1,3-1) compared to wt Ad5 (d1309) (odd and even numbered channels respectively). XbaI digestion of dlE1,3-1 DNA gave two bands (as would have been obtained with dlE1,3) whereas BamHI digestion gave two high molecular weight bands as expected plus a novel band of 1270 bp which migrated slightly faster than the XbaI B-fragment (1339 bp) of

Figure 6.1: Restriction endonuclease analysis of dlE1,3-1 and d1309 viruses.

^{32}P-labelled virion DNA was isolated as described in Materials and Methods, digested with restriction endonucleases and electrophoresed on 1% agarose gels. Odd numbered lanes contain digests of dlE1,3-1 DNA (except lane 7 which contains dlE1,3) and even numbered lanes contain d1309 DNA. Restriction enzyme maps of d1309, dlE1,3 and dlE1,3-1 are illustrated below (map units are from Sussenbach, 1984).. DlE1,3 (chapter 4) differs from d1309 in having a 3.5 Kb deletion of E1 sequences from map coordinates 1.0 to 10.6 which fuses HindIII G and E to generate E', and a 2.0 Kb deletion in E3 from map positions 78.5 to 84.7 which converts HindIII B of d1309 to HindIII B'. The map of the left end 4.5 Kb of dlE1,3-1 at the bottom was derived from analyses with XbaI, BamHI and HindIII as shown above as well as from several additional enzymes. HindIII cleavage sites are indicated above the maps, and, the sites for other enzymes are located below (B=Bam HI; C=ClaI; H=HindIII; X=Xba I). The solid bar in the dlE1,3-1 map represents pBR322 sequences.


XbaI
BamHI
HindIII
A
B
A
B
A
B
A
B
A
B
B′
E″
Δ
B′
E′
I
1 2
3 4
5 6
7
dl309
G E C H D A B F I
HindIII
X
B
dlE1,3
E′
B′
B
X
dlE1,3-1
Δ
E″
B′
B
B
X
Δ
H
E″
H
0
C
B
1500
3000
4500 bp

dl309. HindIII digestion generated two unexpected bands (E'' and Δ). E'' was approximately 350bp larger than, and appeared in place of, E' of dlE1,3 whereas Δ was an entirely novel band of 920 bp migrating just below the HindIII I-band of dl309 (1003 bp). Analysis of dlE1,3-1 DNA with additional enzymes (KpnI, SmaI, ClaI, XhoI, XhoI+BamHI, EcoRI) suggested that dlE1,3-1 had the structure shown at the bottom of Fig. 6.1. It appeared that the 920 bp ClaI B-fragment of Ad5 was linked to the ClaI-BamHI fragment (352 bp) of pBR322, followed by the left ITR of Ad5 dlE1,3. This structure was confirmed by experiments described below.

Fig. 6.2, which describes the strategy employed for the construction of dlE1,3 (chapter 4) helps to illustrate how the dlE1,3-1 variant may have been generated. Briefly, after digesting DNA of dlE3 (a deletion mutant lacking E3 sequences) with ClaI (single cut at 2.6 mu or 920bp) the DNA was treated with S1 nuclease to eliminate or reduce the likelihood of reconstitution of dlE3 by <u>in vivo</u> ligation. Plasmid pXCdl1, which contains the Ad5 XhoI C-fragment (0 to 15.8 mu) minus 21 bp from the extreme left end (McKinnon, 1984) and lacks sequences between map positions 1.0 and 10.6 (chapter 4) was then added and the DNA mixture was used to co-transfect 293 cells. Apparently, the ClaI endonuclease retained activity in the S1 buffer resulting in cleavage of a fraction of pXCdl1 molecules at the ClaI site of pBR322. In addition, a fraction of the ClaI sticky ends in both dlE3 and pXCdl1 DNA evidently escaped the S1 digestion. Finally, <u>in vivo</u> ligation of the ClaI B fragment of dlE3 to the ClaI site of pBR322, either prior to or after homologous crossover as illustrated in Fig. 6.2 would have generated dlE1,3-1. That such a sequence of

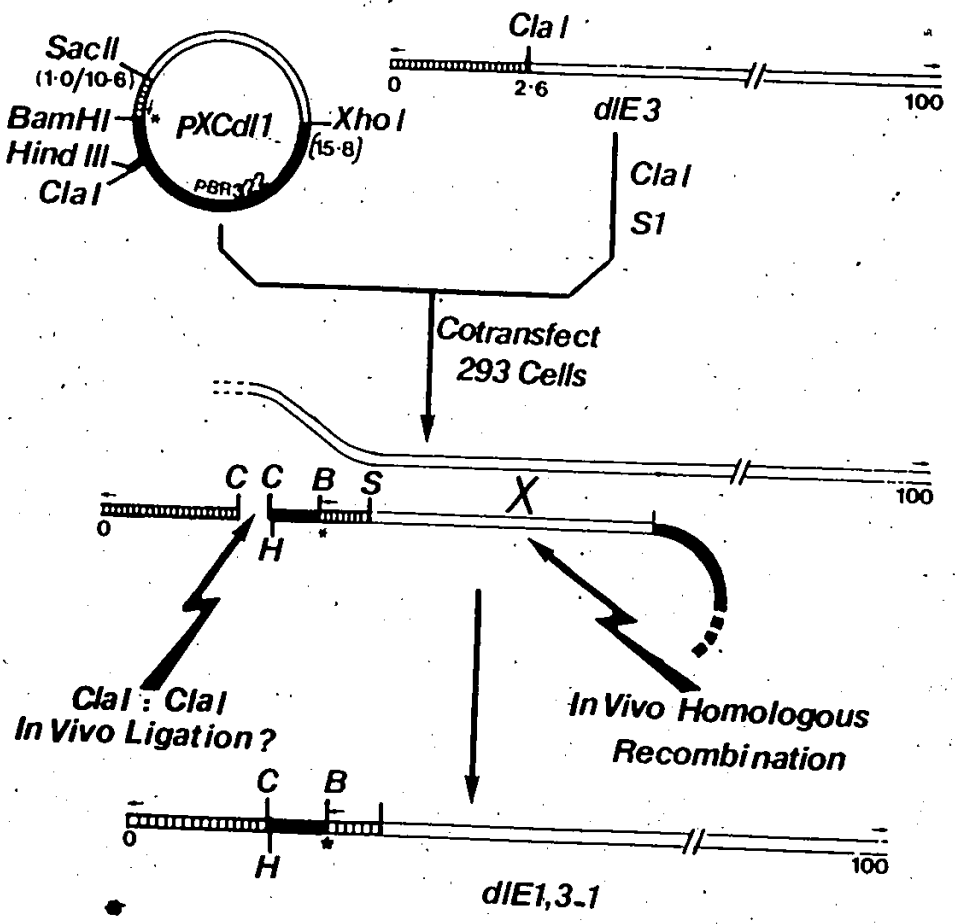


Figure 6.2: Outline of the construction of dlE1,3-1.

The plasmid pXCd11 contains the left 15.8% of the Ad5 genome, minus the first 21 bp of the ITR (McKinnon, 1984) and E1 sequences between map coordinates 1.0 and 10.5 (chapter 4). Cotransfection of 293 cells with a mixture of ClaI digested and S1 treated dlE3 DNA, and pXCd11 DNA yielded dlE1,3-1 by <u>in vivo</u> ligation and recombination.

events could have occurred may seem surprising; however, there seems to be no more reasonable explanation for the isolation of dlE1,3-1.

6.3 Verification of The Proposed Structure

In addition to restriction enzyme digestion, the proposed structure of dlE1,3-1 was tested in two different ways. First, to verify the presence of a 352 bp fragment of pBR322 (ClaI to BamHI) Southern blot hybridization analyses using pBR322 as probe was carried out. Fig. 6.3.A contains dlE1,3-1 cut with the indicated restriction enzymes; the odd numbered lanes were hybridized to ^{32}P-labelled Ad5 DNA. Subsequent to exposure the filter was washed to remove most of the viral probe and rehybridized to labelled pBR322 DNA (Fig. 6.3A, lanes 2, 4, and 6). The bands hybridizing with pBR322 were those predicted by the structure shown in Fig. 6.2 and at the bottom of Fig. 6.3: a 3150 bp fragment (E") in lane 2, a 352 bp fragment (pBR322 sequences) in lane 4, and a 1270 bp fragment (Δ + pBR322 sequences) in lane 6. Thus, the results fully agreed with the model shown in Fig. 6.2 and confirmed the presence of pBR322 sequences at the left end of dlE1,3-1. One additional feature of the analyses shown in Fig. 6.3.A was the presence of a faint band of around 1350 bp in lane 1 which also appeared to hybridize to pBR322 (lane 2, *). As will be described in more detail below, this fragment resulted from the transfer of the extra terminal sequences to the right end of the viral genome.

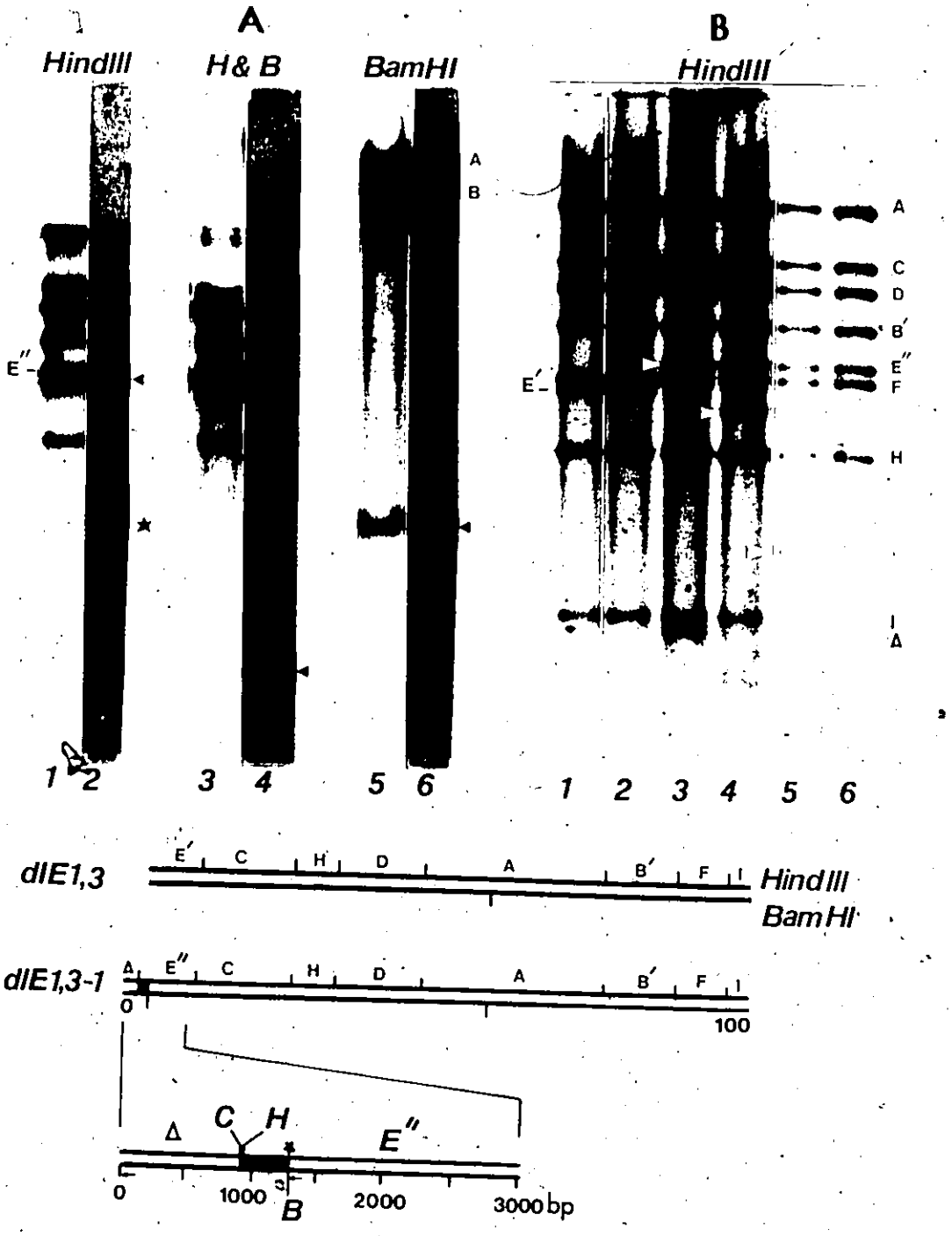
Another test of the proposed structure for dlE1,3-1 was based on

Figure 6.3: A) Southern blot analysis of dlE1,3-1, using Ad5 (lanes 1, 3, 5) or pBR322 DNA (lanes 2, 4 and 6) as probes.

Viral DNA was digested with either HindIII (lanes 1 and 2), BamHI plus HindIII (lanes 3 and 4), or BamHI (lanes 5 and 6), electrophoresed on 0.8% agarose gels, and blotted onto nitrocellulose filters. Initially the nitrocellulose sheet was hybridized with ^{32}P-labelled Ad5 DNA (lanes 1, 3 and 5); after autoradiography the filter was washed (label was partially retained thus leaving an internal marker) and rehybridized with ^{32}P-labelled pBR322 DNA (lanes 2, 4 and 6). The positions of the relevant fragments are indicated by arrows or asterisks. The HindIII and BamHI restriction maps of dlE1,3 and dlE1,3-1 are shown at the bottom of the Fig.; pBR322 sequences (352 bp ClaI to BamHI fragment) are indicated by the solid bars.

B) Generation of dlE1,3 and other variants from dlE1,3-1.

DlE1,3-1 DNA was digested with ClaI (which cuts once adjacent to Δ) and used to transfect 293 cells; a week later plaques were picked, viral DNA was extracted and labelled as outlined in Materials and Methods, then digested with HindIII and electrophoresed on a 1% agarose gel (lanes 2 to 6). Lane 1 contains dlE1,3 DNA, lane 6 contains parental dlE1,3-1 DNA, and arrows indicate the locations of novel bands among which are E'' (top arrow), and Δ (lowest arrow).


A
B
HindIII
H& B
BamHI
HindIII
E″
E′
A
B
C
D
B′
E″
F
H
I
Δ
1 2 3 4 5 6
1 2 3 4 5 6
dlE1,3
E′ C H D A B′ F I
HindIII
BamHI
dlE1,3-1
Δ E″ C H D A B′ F I
0
100
Δ C H
E″
0 1000 B 2000 3000 bp

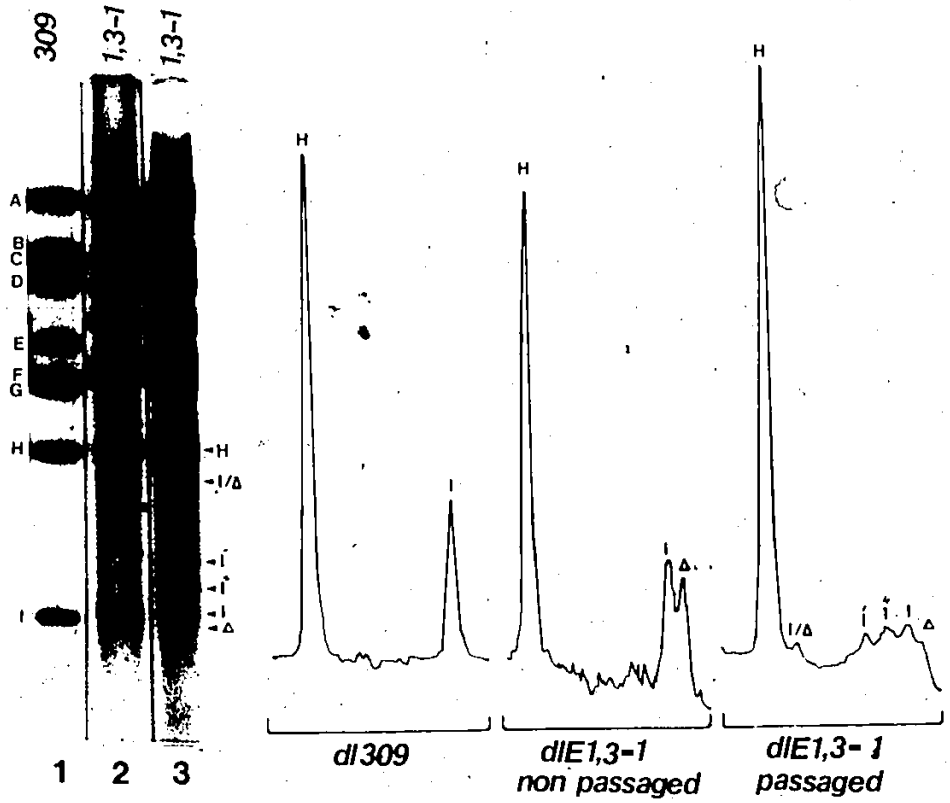
the observation of Stow (1982) that viral genomes with one terminus linked to plasmid DNA can give rise to virus with normal ends, presumably as a result of repair using the unaltered terminal sequences as a template. ClaI digestion of dlE1,3-1 DNA and transfection of 293 cells was therefore expected to give rise to virus which had lost the extra terminal fragment attached to the left end. Indeed, of 7 plaques analyzed, two had restriction cleavage patterns identical to that of dlE1,3. One of these is shown in lane 2 of Fig. 6.3B compared to dlE1,3 in lane 1. Two plaque isolates, one of which is shown in lane 3, were identical in structure to dlE1,3-1 (lane 6) and may have arisen by in vivo religation or from DNA which had not been cleaved by ClaI. Finally, two new and unexpected variants were isolated from this experiment (lanes 4 and 5). One variant (lane 5) had lost the HindIII I-fragment from the right end while retaining the Δ fragment and contained a new fragment of approximately 1350 bp. This virus (dlE1,3-2) will be discussed in more detail below. The virus shown in lane 4 had a structure similar to dlE1,3-1 but had lost the Δ fragment and exhibited a new fragment of approximately 2500 bp. The precise structure of this isolate is unknown and was not analyzed further, but the isolation of two novel viral mutants from this experiment indicated that the ends of dlE1,3-1 might be highly unstable. Later analyses described below proved this to be the case, and suggested that the isolation of dlE1,3-2 was probably unrelated to the ClaI digestion but instead was the result of pre-existing dlE1,3-2 molecules in the population.

6.4 Stability of dlE1,3-1 and dlE1,3-2

The unexpected structure of some of the isolates presented in Fig. 6.3B prompted me to examine the genomic stability of dlE1,3-1. Fig. 6.4 shows the HindIII restriction enzyme pattern of unpassaged dlE1,3-1 (lane 2), and of dlE1,3-1 virus after 5 successive passages in 293 cells (lane 3). From the autoradiograms and adjacent scans, it is clear that the intensities of the HindIII I-fragment and of the extra band (Δ) relative to the HindIII H-band in the passaged virus (lane 3) were quite different from those of the unpassaged virus in two respects. Firstly, the I and Δ bands of passaged virus DNA were reduced in intensity and secondly, three additional light bands were present in patterns of passaged viral DNA (arrows, lane 3). One was just below the H band and its size (approximately 1900 bp) was consistent with head to tail joining of viral DNA ends (i.e. Δ = 920 bp plus the I-fragment = 1000 bp). Evidence for such head to tail joining in adenovirus infected cells has been presented and discussed previously (Ruben et al., 1983; and Graham, 1984b). The second band, designated I', was 1350 bp in size, which corresponds to the HindIII I-fragment (1000 bp) plus the pBR322 sequences (352 bp). This would be generated if the left end extra fragment was duplicated at the right end as illustrated in Fig. 6.5 and corresponds to the type of isolate (dlE1,3-2) seen in channel 5 of Fig. 6.3B. Finally, a band (I") migrating between I' and I was also visible in lane 3. The size of this fragment was approximately 1200 bp, very close to the predicted molecular weight (1270 bp) of the fragment which would be

Figure 6.4: Stability of dlE1,3-1.

DlE1,3-1 DNA was labelled *in vivo*, digested with HindIII and electrophoresed on a 1% agarose gel followed by autoradiography. Lane 1 contains a HindIII digest of d1309 (as a marker), and lanes 2 and 3 contain unpassaged and passaged dlE1,3-1, respectively. New submolar bands are indicated by the arrows adjacent to lane 3. Densitometer scans are shown for regions of the gels from just above the HindIII H to slightly below the HindIII I fragments.


309
1,3-1
1,3-1
A
B
C
D
E
F
G
H
I
H
I/Δ
I
I
I
Δ
1
2
3
H
H
H
I
I
Δ
I/Δ
I
I
I
Δ
dl309
dlE1,3-1
non passaged
dlE1,3-1
passaged

generated by HindIII cleavage of DNA molecules with 2 tandem copies of the extra fragment at one end. The existence of viral DNA species with 2 tandem iterations of the 1270 bp segment linked to the right end was also suggested by analyses to be presented later. Thus starting with one extra terminal segment at the left end, as many as two additional copies could be generated at the right end.

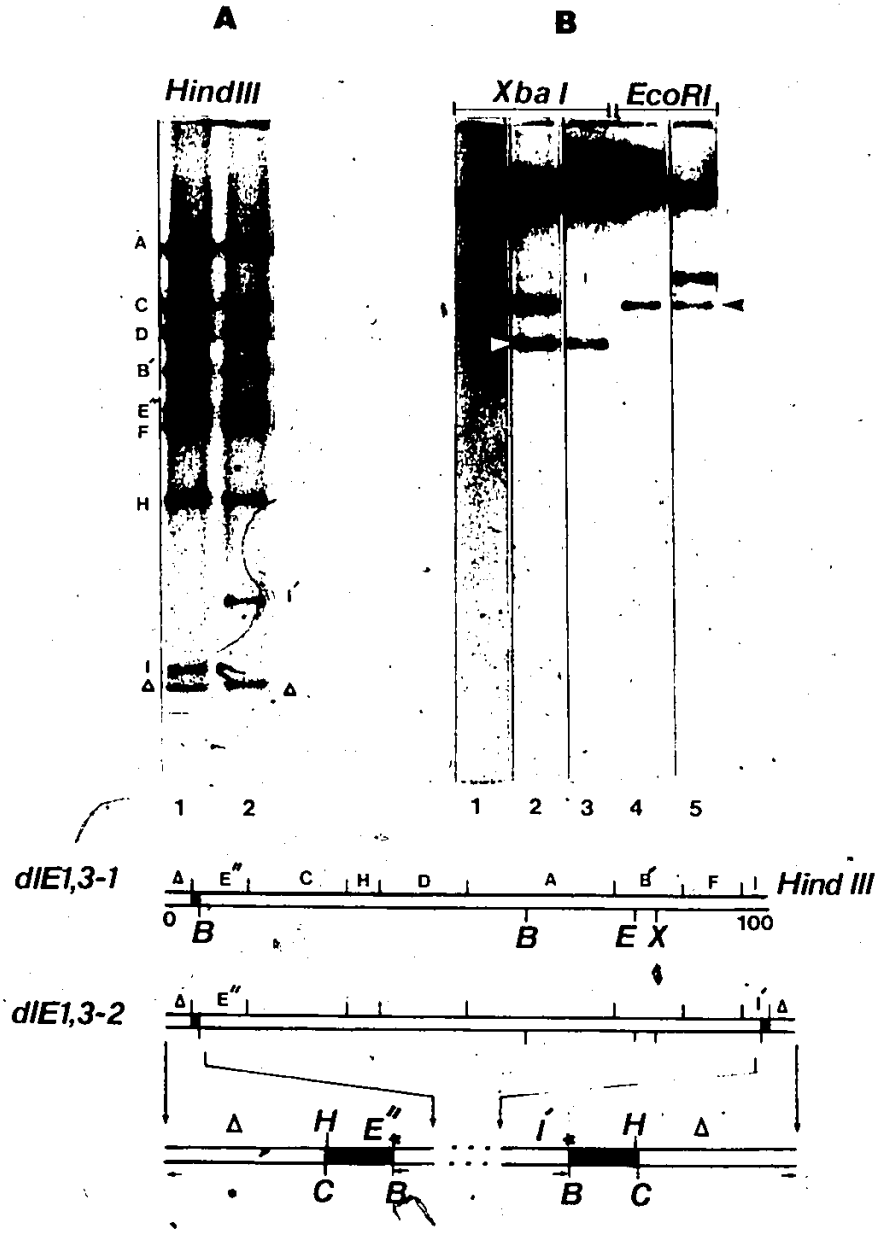
When passaged dlE1,3-1 virus was plaque purified and DNA of newly cloned virus stocks analyzed, 3 plaque isolates (eg. Fig. 6.5A, lane 2) out of 36 were found to have duplicated the fragment onto the right end (losing the Hind III I fragment and generating I') while retaining it at the left end (as indicated by the presence of fragment E''). The remaining 33 isolates had the structure of dlE1,3-1 (lane 1). Thus, about 8% of the progeny in passaged dlE1,3-1 stock seemed to have four ITRs; two at each end and two internally embedded within 1270 bp from each end (dlE1,3-2). However, when a dlE1,3-2 isolate was passaged and subsequently plaque purified, 13 plaque isolates out of 30, or 43% were found to have the structure shown in Fig. 6.5A lane 1 and thus represented revertants to dlE1,3-1. The remaining isolates (17) were dlE1,3-2.

Direct examination of the structure of viral DNA in passaged dlE1,3-2 virus preparations (Fig. 6.5B) revealed that the right end Δ fragment had been lost from about 1/3 of the viral genomes. This was indicated by restriction cleavage patterns obtained with XbaI and EcoRI digestion (Fig. 5B) as well as with HindIII digestion (not shown). Lane 1 (Fig. 6.5B) shows the XbaI pattern of dlE1,3-2 before passaging, and lane 2 the pattern after passaging. The fastest migrating band in lane 2 comigrates with the XbaI B-fragment of

Figure 6.5: Interconversion of dlE1,3-1 and dlE1,3-2.

A) Passaged dlE1,3-1 was used to infect 293 cells; 5 days later 36 plaques were picked, expanded on 293 cells, labelled *in vivo*, their DNA extracted, digested with HindIII and electrophoresed on a 1% agarose gel. Thirty-three out of the 36 plaques screened were found to be identical to the parental virus dlE1,3-1 (lane 1), and 3 out of 36 were found to have a larger HindIII I-fragment (I' lane 2).

B) *In vivo* labelled DNA of unpassaged (lane 1) and passaged (lane 2) XbaI digested dlE1,3-2 DNA; lanes 3 and 4 contain XbaI and EcoRI digested dlE1,3-1 DNA, respectively. Lane 5 contains an EcoRI digest of passaged dlE1,3-2 DNA, and novel bands generated by interconversion or reversion are indicated by arrows (lanes 2 and 5).


A
B
HindIII
Xba I
EcoRI
A
C
D
B'
E''
F
H
I'
I
Δ
Δ
1 2
1 2 3 4 5
dlE1,3-1
Δ E'' C H D A B' F I
Hind III
0
B
B
E X
100
dlE1,3-2
Δ E''
I' Δ
Δ H E''
I' H Δ
C B
B C

dlE1;3-1 (lane 3) and corresponds to a subpopulation of virus in which the extra fragment on the right end has been lost. The EcoRI digestions for passaged dlE1,3-2 (lane 5) compared to dlE1,3-1 (lane 4), supported the conclusions drawn from XbaI digestion. Passaged dlE1,3-2 contained two major species of viruses, those with a right terminus identical to that of dlE1,3-1 (about 30-40%), and those with the right terminus lengthened by the extra terminal segment copied from the left end. Therefore, based on analysis of mixed populations of viruses from passaged dlE1,3-1 and dlE1,3-2 viruses and from analysis of plaque isolates, there appeared to be a rapid interconversion between the two variants. The extra segment could be readily lost from or added to the right end of the genome but seemed to be stably maintained at the left end. Finally, a faint band was visible in lane 2 of Fig. 6.5B (top arrow), just above the XbaI B-fragment. The size of this minor species was exactly that predicted if a small fraction of dlE1,3-2 molecules contained 2 copies of the extra terminal fragment attached to the right end, in agreement with the appearance of the I" fragment seen in lane 3 of Fig. 6.4. Tandem repeats at the left end may also have occurred but would have gone undetected due to a limited resolution near the top of the gel.

6.5 Mode of Duplication, Interconversion and Reversion

Fig. 6.6 outlines the adenovirus DNA replication pathways, and presents a possible model to account for the rearrangements which were observed. Briefly, DNA replication could start at either the left

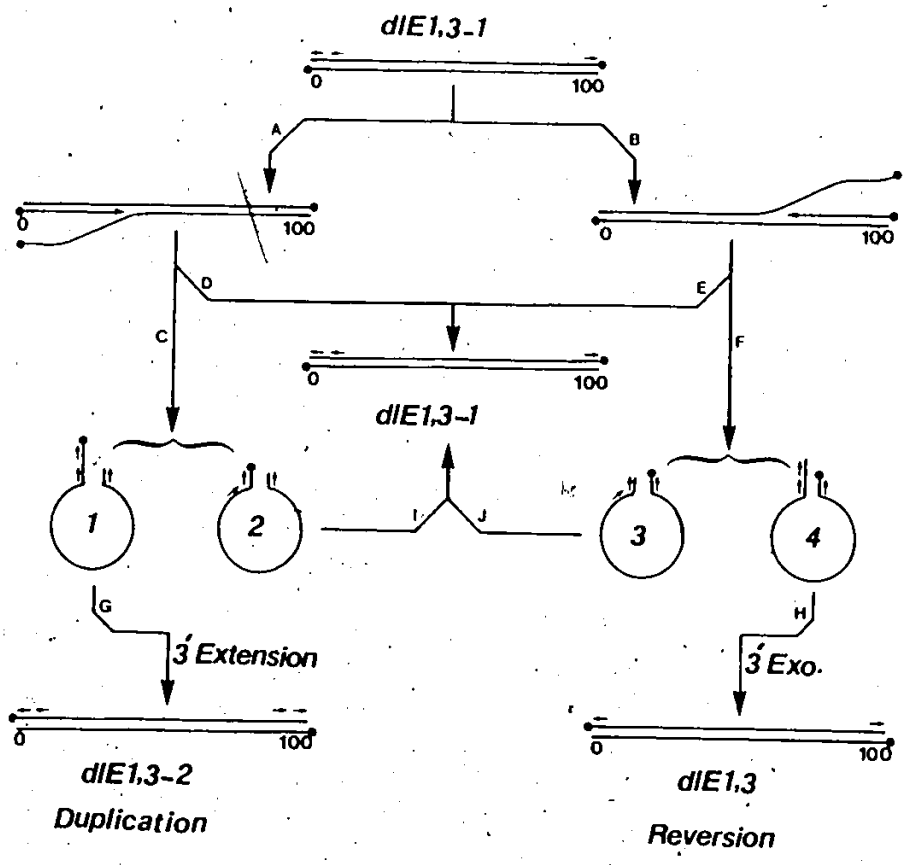


Figure 6-6: Schematic representation of Ad5 replication, and the mode by which transposition of the left end extra fragment to the right end occurs. DNA replication could start at either the left end (A) or at the right end (B); the replicated strand will regenerate dlE1,3-1 (D and E) whereas the displaced strand following hybridization of the ITRs will generate four alternate panhandle structures 1, 2, 3 and 4. 3'-extension in form 1 will generate dlE1,2-2 (duplication) whereas 3'-exo in form 4 will generate dlE1,3. (reversion), and replication of form 2 and 3 will regenerate dlE1,3-1.

(pathway A) or at the right end (pathway B) of the molecule. Replication of template strands along either pathway A-D or B-E would regenerate the parental genome (dlE1,3-1). However, the displaced strand in pathway A-C could form two alternative panhandle structures: 1 or 2. In form 1 the right ITR would hybridize to 82 bp of homologous DNA from the embedded ITR at the left end. The unhybridized 21 bp of the right ITR would be removed via 3'-exonuclease digestion followed by 3'-extension and subsequent replication (pathway G) to give rise to dlE1,3-2 with 4 ITRs: two complete ITRs, one at each molecular end, and two incomplete ITRs embedded internally. Alternatively, replication of form 2, in which the two external ITRs have hybridized would regenerate dlE1,3-1 (pathway I). Successive rounds of replication through the A-C-G pathway could result in multiple additions of the extra terminal sequence to the ends of viral DNA progeny in agreement with our detection of tandem repetition.

The displaced strand in pathway B-F could also form two alternative panhandle structures (forms 3 and 4). Form 3, like form 2, upon replication would regenerate the parental virus (pathway J). Replication of form 4 would require 3'-exonuclease activity to remove the unhybridized sequences followed by 3'-extension to replace the 21 bp missing from the embedded ITR. Replication along this pathway would generate dlE1,3.

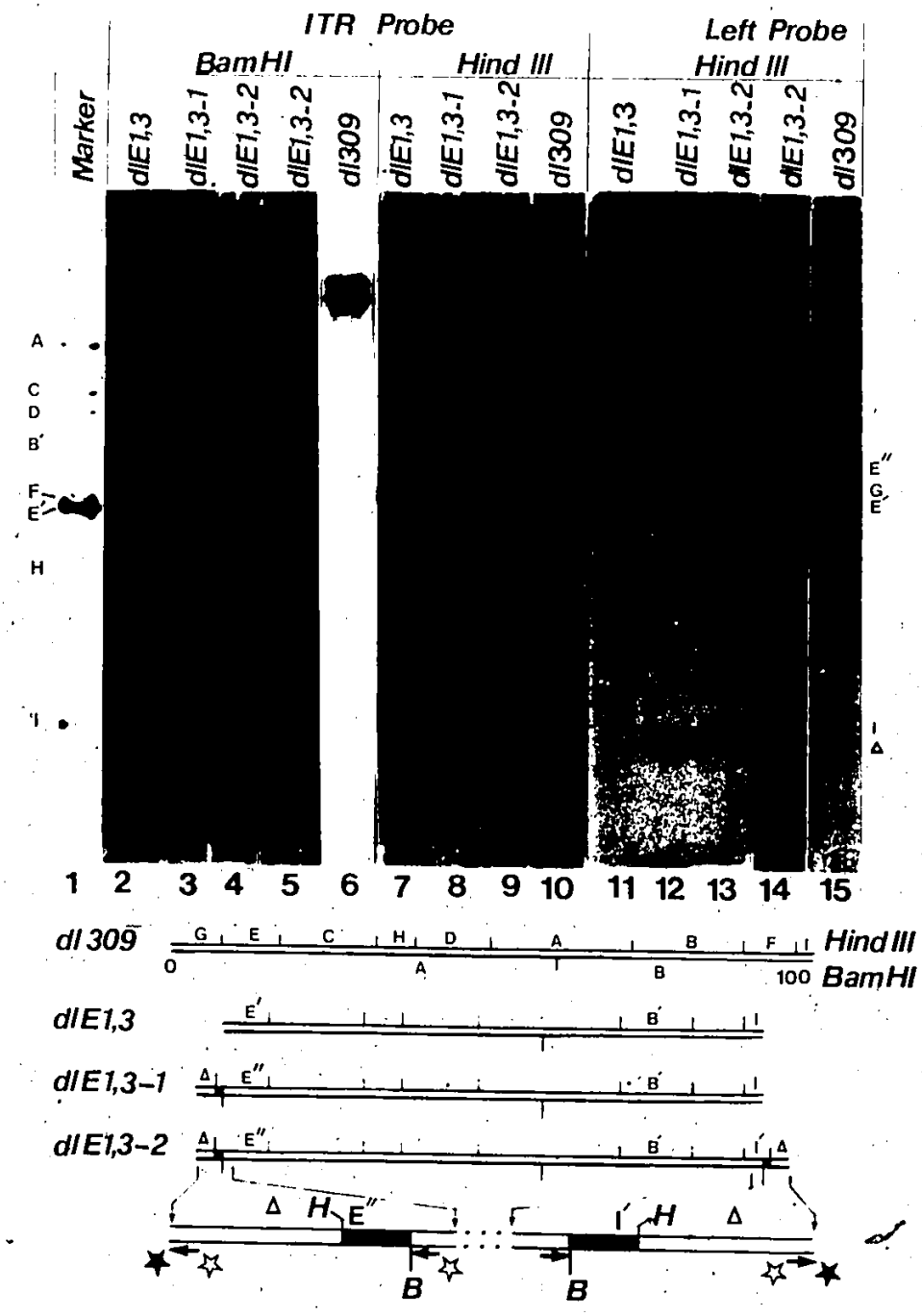
This model can account for all of the variants detected in the experiments described previously. However, it should be noted that the generation of dlE1,3-2 from dlE1,3-1 could also occur as a result of intermolecular (but not intramolecular) recombination.

6.6 Fine structure analysis of the segment transfer process

The postulated mechanism for generation of dlE1,3-1 shown in Fig. 6.2, and the model for dlE1,3-2 formation illustrated in Fig. 6.6 make very strong predictions about the fine structure of the embedded ITRs. Firstly, because the terminal 21 bp are deleted from the extreme end of Ad5 DNA in plasmid pXCd11, the viral sequences of the embedded ITR in dlE1,3-1 should start at nucleotide 22. Secondly, models based on recombination, and the model shown in Fig. 6.6 both predict that the 21 bp deletion in the embedded ITR should be transferred to the right end during duplication of the extra terminal fragment in dlE1,3-1 to generate dlE1,3-2. In particular, examination of pathway G in Fig. 6.6 suggests that if 21 bp of ITR information are missing from the embedded terminal sequences then 3'-extension requires prior 3'-exonucleolytic action to provide a base paired 3'-primer for chain elongation. To confirm these predictions hybridization analyses were carried out with probes consisting of oligonucleotides corresponding to the first 15 nucleotides of the viral termini (ITR probe) or 15 nucleotides just to the right of the left ITR of Ad5 (left probe). The results of these analyses are shown in Fig. 6.7. The ITR probe hybridized to both BamHI A (left end) and B (right end) fragments of dlE1,3 (lane 2) and wt viral DNA dl309 (lane 6). It hybridized only to the BamHI B-fragment of dlE1,3-1 (lane 3) and to the extra terminal fragment of 1270 bp attached to the left end of dlE1,3-1. This is in agreement with the structure predicted for dlE1,3-1 by the model shown in Fig. 6.2. Neither BamHI A nor B of dlE1,3-2 hybridized to the ITR probe when early passage viral DNA was analyzed (lane 4) in agreement

Figure 6.7: Oligonucleotide hybridization.

Viral DNAs were digested with the indicated enzymes, and electrophoresed on a 1% agarose gel. The gel was processed as described in Materials and Methods and hybridized against two different probes: an oligonucleotide (ITR probe, solid star) which hybridizes specifically to the first 15 bp of the ITR and an oligonucleotide (left probe, open star) hybridizing to sequences just to the right of the left ITR. Restriction maps of various forms of Ad5 DNA are illustrated below in the format used for Fig.6.1.


ITR Probe
Left Probe
BamHI
Hind III
Hind III
Marker
dlE1,3
dlE1,3-1
dlE1,3-2
dlE1,3-2
dl309
dlE1,3
dlE1,3-1
dlE1,3-2
dl309
dlE1,3
dlE1,3-1
dlE1,3-2
dlE1,3-2
dl309
A
C
D
B'
F
E'
H
I
E''
G
E'
I
Δ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
dl 309
G E C H D A B F I
Hind III
0 A B 100
BamHI
dlE1,3
E' B' I
dlE1,3-1
Δ E'' B' I
dlE1,3-2
Δ E'' B' I Δ
Δ H E''
H Δ
B
B

with predictions based on the model in Fig. 6.6, or on models involving intermolecular recombination. Instead the ITR probe hybridized exclusively to the 1270 bp fragment attached to each end of dlE1,3-2 as depicted at the bottom of Fig. 6.7. After several passages, dlE1,3-2 underwent further alterations resulting in reversion of part of the molecules back to dlE1,3-1 as indicated by hybridization of the ITR probe to BamHI fragment B (lane 5). Also apparent in lane 5 is a low level of hybridization to the left BamHI A-fragment of passaged dlE1,3-2 virus suggesting loss of the terminal duplication from the left end of a small fraction of molecules in this virus preparation.

When HindIII digests were analyzed using the ITR probe, it was found that the oligonucleotide hybridized to the HindIII I-fragment and to the 920 bp Δ fragment of dlE1,3-1 DNA (lane 8) and primarily to the Δ fragment of early passage dlE1,3-2 DNA (lane 9). A low level of hybridization to E' was also seen in lane 9. Since no comparable hybridization to the I-fragment was observed in this lane this result again suggests the loss of the terminal segment from the left end of molecules which retained the segment at the right end.

Hybridization analysis with the left probe specific for sequences just inside the left ITR essentially confirmed conclusions drawn from analysis using the ITR probe. The left probe hybridized to the HindIII E' of dlE1,3 (lane 11), to E" and Δ of dlE1,3-1 (lane 12) and to E", Δ , and E' (faintly) of preparations of dlE1,3-2 DNA (lanes 13 and 14). This is entirely consistent with previous results and with predictions based on the models in Figs. 6.2 and 6.6.

6.7 Conclusions

The results presented in this chapter describe the isolation and characterization of a viable Ad5 mutant (dlE1,3-1) with 1250 bp of extra DNA linked to its left end. A number of observations were made during the study of this mutant and can be summarized as follows:

1. Analysis of this variant with restriction enzymes and by hybridization of Southern blots with specific probes indicated that the ClaI C-fragment (the left 2.6%, or 920bp) of Ad5 was joined to 352 bp of pBR322 which in turn was linked to the left end (minus 21 bp) of dlE1,3.
2. During passaging of dlE1,3-1 the extra terminal segment was found to transfer to the right end of the genome, thus generating a second variant, dlE1,3-2. The latter mutant had 4 ITRs: one normal ITR at each molecular end, and two incomplete ITRs (each missing 21 bp) embedded internally.
3. Analysis of dlE1,3-2 revealed that it back reverted to dlE1,3-1 at a high rate. Although evidence was obtained indicating that the extra segment could be lost from the left end, spontaneous mutants which had lost direct repeats from both ends were never isolated.
4. It was possible to remove the extra terminal repeat of dlE1,3-1 by cleavage with ClaI, and to isolate dlE1,3 containing wt termini.

Chapter VII: Discussion

Recent years have seen the development of a variety of virus based vectors designed to facilitate the introduction of foreign genetic information into mammalian cells (reviewed by Gluzman, 1982; Watson et la., 1983; Old and Primrose, 1985). The impetus in this field has been motivated primarily by the notion that recombinant viral vectors might offer distinct advantages for the transfer and expression of genes as compared to DNA-mediated transfection. Firstly, infection of cells with recombinant viruses should, and does, provide a higher efficiency of gene transfer both in vitro and in vivo than the calcium technique and its various modifications (reviewed by Graham et al., 1980; Graham and Bacchetti, 1983; McKinnon and Graham, 1986). Secondly, viral vectors afford the possibility of controlling more accurately the copy number of genes introduced into recipient cells by varying the multiplicity of infection. Thirdly, expression of mammalian genes cloned in these vectors can be achieved in a mammalian host. Thus, both the factors controlling gene expression as well as the nature of post-translational modifications (which do not take place in bacteria) can be analyzed. In addition, virus vectors can be useful or even essential in the characterization of gene products from agents for which a culture system is not

available, such as the human hepatitis B virus and papilloma viruses. Lastly, viral vectors may be helpful in the development of recombinant vaccines and perhaps ultimately for gene therapy (Panicali and Paoletti, 1982; Panicali et al., 1983; Watson and Enquest, 1985; Anderson, 1984).

Progress in this field has been rapid especially with regard to the development of retrovirus based vectors which have been used to transfer a variety of different genes into cultured cells in vitro (Wei et al., 1981; Shimotohno and Temin, 1981, 1982; Miller et al., 1983; Joyner et al., 1983; to name a few). Among the positive characteristics of these vectors are the fact that they do not transform cells in vitro, and they grow to high titres without a cytopathic effect. In addition, they can accept up to 7.5 Kb of foreign DNA, an amount sufficient to accomodate most average genes. A disadvantage, on the other hand, is that DNA sequences cloned into these vectors frequently undergo rearrangements and deletions, a phenomenon which may limit the usefulness of the system (Shimotohno and Temin, 1982; Joyner and Bernstein, 1983; Bandyopadhyay and Temin, 1984a and b).

Several DNA viruses have also been used to construct eukaryotic vectors. Among these, the small papova viruses (such as simian virus 40 and bovine papilloma) have become very popular in the study of gene expression; their widespread application, however, is limited due to the small amount of foreign DNA (2 to 5 Kb) that can be rescued into them (Mulligan et al., 1979; Hamer and Leder, 1979; Gething and Sambrook, 1981; Asano et al., 1985). Large DNA viruses such as poxviruses and herpes viruses do not suffer from this drawback,

however, their large genomes make them difficult to manipulate *in vitro* (Panicali and Paoletti, 1982; Mackett et al., 1982, 1984; Cochran et al., 1985). In addition, poxviruses replicate in the cytoplasm of infected cells and thus are not suitable for the delivery of genes into the cell nucleus (Moss, 1978). Herpesviruses, on the other hand, are very cytopathic and can cause disease in animals (reviewed by Rawls, 1985)

Recently, a number of laboratories have undertaken the development of adenovirus vectors for the delivery and expression of genes into mammalian cells. The advantage of these viruses over those described above rests on several characteristics. As stated previously (chapter 1), adenoviruses are not only well characterized, easy to grow and manipulate, and exhibit a broad host range *in vitro* and *in vivo*, but also produce copious amounts of virus and viral gene products in lytically infected cells. In addition, because only a small portion of the viral genome appears to be required *in cis* for replication and packaging (Tooze, 1981; Hearing and Shenk, 1983), this group of viruses may ultimately offer excellent potential for the substitution of large DNA fragments of up to 30 Kb, once cell lines have been developed which can provide most of the essential viral functions *in trans*. Moreover, adenoviruses do not carry strong outward directed promoters like the retrovirus' long terminal repeats which might cause neoplastic transformation in humans, of considerable concern if retroviruses were to be used for gene therapy or in the engineering of recombinant vaccines. The various adenovirus vectors that have been developed in different laboratories over the last several years can be classified into two categories, helper-dependent

(Thummel et al., 1981, 1982, 1983; Yamada et al., 1985; Mansour et al., 1985), and helper-independent (Berkner and Sharp, 1983; Van Doren and Gluzman, 1984; Saito et al., 1985). Although, the amount of DNA that can be rescued into helper-dependent vectors is relatively large (over 10 Kb), the need for a helper virus is a clear disadvantage as compared to helper-independent vectors. Among the disadvantages are, first, determining the percentage of the defective virus every time the virus is grown, and secondly, the presence of the wt virus often complicates the interpretation of the results. Helper-independent vectors, however, cannot accept more than 4-5 Kb of foreign DNA, and often lack alternative cloning strategies, thus making their use somewhat limited.

The main aim of the work reported in this thesis was directed towards the development and use of the human adenovirus type 5 into a helper-independent vector for the transfer of genes into mammalian cells. As this work was initiated prior to the use of the adenovirus as a vector in other laboratories, the intial studies were aimed at determining the maximum amount of DNA which could be packaged into virion capsids. As described in the results section, several insertion mutants rescued within the E1a region using the method of Stow (1981) were analyzed with restriction endonucleases. These experiments indicated that no more than 1.7 Kb (or 5% of the wt Ad5 genome) of extra DNA could be rescued in this way, suggesting severe constraints on the size of packageable DNA molecules. These findings are in agreement with the results obtained with Ad-SV40 hybrids in which the net change in viral DNA size is relatively small (reviewed in Tooze, 1981; Klessig, 1984). They also conform to the results of Jones and

Shenk (1978) who have isolated substitution and insertion mutants with net increases in genomic size of about 0.43 to 1.73 Kb (or 1.2 to 4.8%). In one instance, however, Graham (1984b) was able to rescue a 2.2 kb (or 6%) plasmid DNA fragment into an adenovirus mutant (d1309); to date this represents the largest insert stably carried by an adenovirus. The inability to rescue large inserts into adenovirus is most likely related to the icosahedrical structure of the virion capsid. Polyhedron capsids rarely have an internal volume much larger than the genomic DNA (Lewin, 1985), and the viability of lambdoid bacteriophages, for example, decreases dramatically when DNA molecules larger than 105% of the wt genome are packaged (reviewed by Hendrix et al., 1982).

A second observation derived from the results presented in this work is that when attempts were made to rescue inserts of 2.4 Kb or greater, deletions and rearrangements occurred at a high frequency within or around the insert, in such a way that the resulting net insert size was always 1.7 Kb or less. It seems unlikely that this phenomenon is related to the DNA used (Tn5 in origin) and/or to the procedure, since small inserts of Tn5 DNA could be easily rescued without rearrangements. Rather such rearrangements might be the consequence of recombinational events which are known to occur soon after DNA mediated transfection, as well as between infecting adenovirus genomes (Wilson et al., 1982; Miller and Temin, 1983). In any case, once an insert was rescued it remained stable and unmodified upon successive passagings of the virus, indicating that once mutants were established as packageable viral genomes they could be propagated as stable viruses.

In order to increase the size of inserts and thus enhance the usefulness of the vectors, a mutant adenovirus lacking both early regions E1 and E3 (dlE1,3) was constructed. As mentioned previously, E3 is non-essential for virus growth in cultured mammalian cells (Berkner and Sharp, 1983; Saito et al., 1985); the deletion of E1 on the other hand renders the virus a host range mutant in that it grows normally in 293 cells but does not produce detectable cpe in other human cell lines unless high moi's are used. In dlE1,3 the deletion in E1 extends from nucleotides 354 to 3824; the sequences needed in cis for packaging are retained (Hearing and Shenk, 1983), but 243 base pairs from the region encoding the 5' end of the polypeptide IX mRNA are removed. Therefore, dlE1,3 (like dl313) does not synthesize polypeptide IX, a 14.3 Kd structural component of the adenovirus particle (Philipson, 1984; Oostrum and Burnett, 1985) which is responsible for stabilizing hexon-hexon interactions. Colby and Shenk (1981) have reported that an adenovirus mutant (dl313) lacking protein IX, is significantly more thermolabile than the wt virions and similar observations were made for dlE1,3. Heat sensitivity is not a drawback for the dlE1,3 vector, since almost all biological experiments are performed at a temperature at which the virus appears to be stable. One possibility which has not been examined, however, is whether the lack of protein IX might alter the packaging limits of the dlE1,3 vector in view of the fact that capsids lacking this component are less stable than wt virions.

The most important feature of dlE1,3 for our purposes is that its genome is physically smaller (85%) than the wt genome by approximately 5.5 kb (or 15 %). This reduction in size is unlikely in itself to

destabilize the virion since in the case of bacteriophage lambda, deletion mutants (lacking up to 20% of the genome) are more heat stable than wt bacteriophage (Parkinson and Huskey, 1971). Theoretically the dlE1,3 vector should be capable of accepting an insert as large as 7.5 kb, comparable to the insert size which can be rescued into retrovirus vectors; however, this upper limit has not yet been tested directly. Although a 7.5 Kb insert is large enough to encode most average proteins, its size could be further increased by deleting additional DNA sequences from the E3 region. In principle it should even be possible to delete up to 30 Kb of the wt genome, although this would involve several steps, of which the first and most difficult would be the development of cells capable of expressing almost all essential viral functions.

At present, dlE1,3 should have the largest capacity for foreign DNA of any conditionally helper-independent adenovectors. Several adeno-expression vectors engineered by Solnik (1981), Thummel et al. (1981, 1982, 1983) and Mansour et al. (1985) are missing essential information and therefore must be grown in the presence of a helper. Recently, a conditionally helper-independent adenovector was developed in which 2.9 kb had been deleted from the E1 region of d1309 (Van Doren and Gluzman, 1984). Inserts of foreign DNA of up to about 5 kb in size can theoretically be cloned in this vector. Other workers have deleted approximately 2 kb of DNA from the E3 region of Ad5 (Berkner and Sharp, 1983; Saito et al., 1985) to create vectors analogous to dlE3 which are non-conditionally helper-independent and should accept inserts of up to 4 kb.

In addition to a capacity for DNA fra[illegible] useful size, an

optimized gene transfer vector for mammalian cells must fulfill a number of requirements. Ideally, cloning of foreign DNA into such a vector should be simple, and should be possible through alternative cloning strategies. Second, the viral vector should permit the introduction of the cloned genes into a wide range of recipient cells at a high efficiency. Third, inserted genes should remain stable and unmodified upon successive passagings of the vector or of the cells transformed by it. This aspect is particularly important for potential applications in gene therapy (Anderson, 1984) and genetically engineered vaccines. To a large extent the adenovirus vector system described in this thesis fulfills these requirements.

For the cloning and expression of foreign genes, the mutants and procedures described in this work offer considerable flexibility. Three different possible strategies for the insertion of a DNA fragment into the unique XbaI site of dlE1,3 have been described, and each of these methods was used at different stages in this work. Firstly, genes can be inserted into the XbaI site of pFGdX1 and the resulting plasmid can be spliced to the BamHI A-fragment of Ad5 DNA (either wt or dlE1,3, or any of a variety of E1 mutants) to generate infectious virus, as was done for the construction of dlE3 and for the construction of Adtk2. It should be noted that splicing to the EcoRI A-fragment (left 76%) of Ad5 DNA is also an option if the inserted DNA has one or more BamHI sites. Secondly, a DNA fragment inserted into pFGdX1 can be rescued into infectious virus by co-infecting cells with overlapping viral DNA fragments and relying on in vivo recombination or marker rescue, as in the method used to obtain dlE1,3. Finally, genes flanked by XbaI sites can be inserted directly into dlE1,3 by

trimolecular ligation, the approach used to construct Adtk4.

In order to demonstrate the usefulness of dlE1,3 as a vector, the HSV tk gene was inserted into its unique XbaI site. This gene was chosen mainly because one could select for its expression in HAT-containing medium (Szybalska and Szybalski, 1962) or against its expression in BrdU-containing medium (Davidson et al., 1973). In addition, the level of tk expression can be measured quantitatively by biochemical assays (Summers et al., 1975). Two alternative strategies were used to insert the HSV tk gene in both orientations in dlE1,3; both strategies are relatively simple, and viruses containing the tk gene were readily obtained. Characterization of the new virus, Adtk, revealed that it behaved like its progenitor dlE1,3 with respect to its growth in various cell lines tested. Furthermore, limited analysis suggested that the tk insert remained stable and unmodified during successive passages of the recombinant Adtk.

The HSV tk gene is a delayed-early gene, and under normal circumstances (i.e. during HSV infection), it requires the prior synthesis and continued function of at least two HSV immediate early (IE) proteins, infected cell protein 4 (ICP4) and ICP0 (Preston, 1979; Leung et al., 1980; Watson and Clements, 1980; Gelman and Silverstein, 1985). This requirement, however, is not absolute since the isolated tk gene can transform tk^- cells to the tk^+ phenotype (Bacchetti and Graham, 1977; Wigler et al., 1977; Minson et al., 1978), and is expressed upon microinjection into Xenopus oocytes (McKnight et al., 1981). In agreement with these findings, determination of tk expression in Adtk infected tk^- cells revealed that the HSV tk gene, presumably still under control of its own

regulatory sequences, was expressed at low but detectable levels in the absence of any positive selection and irrespective of its orientation within the Ad genome. In transformed cells and in oocytes tk transcription appears to initiate at the authentic tk start site (McKnight et al., 1981; Wagner et al., 1981b), however, the level of expression of both tk mRNA transcripts (Smiley, personal communication) and proteins substantially increases upon superinfection of tk transformed cells with tk^- HSV (Lin and Munyon, 1974; Leiden et al., 1976). These findings indicate that the tk gene must still retain 5' target sequences which allow it to respond to the IE functions supplied by the incoming virus (Smiley et al., 1983). Transactivation of the tk gene is also seen in transient transcription assays in HeLa cells after superinfection with tk^- virus (ElKareh, et al., 1985), or in co-transfection with plasmids bearing either the ICP0 or the ICP4 genes (Gelman and Silverstein, 1985). Again in agreement with these findings, when Adtk infected cells were superinfected with tk^- HSV the level of tk activity was increased dramatically, indicating a response to <u>trans</u> acting signals (presumably ICP4 and/or ICP0) from the superinfecting virus.

However, besides the absence of HSV regulatory functions, other factors might also be responsible for the constitutive low level of tk expression in Adtk infected cells. Thus, in the case of the tk gene inserted in the opposite orientation to the deleted E3 promoter, it is possible that transcripts from the latter promoter might generate anti-sense tk messages which could interfere with tk mRNA translation. Recently, Kim and Wold (1985) have expressed anti-sense tk RNA as part of a chimeric dihydrofolate reductase transcript. In such a

construct, progressively higher levels of tk anti-sense RNA were obtained by selecting with increasing concentrations of methotrexate. As the level of anti-sense tk mRNA increased, the level of tk enzyme activity decreased by 80% to 90%. In addition, Izant and Weintraub (1984) using a transient expression assay, have obtained a significant reduction of transient tk expression in tk^- mouse L cells in the presence of a plasmid which allowed expression of the anti-sense tk DNA strand. In the case of the genes inserted in the same orientation as the E3 promoter, the principle of promoter exclusion could be in effect and transcription initiating from the E3 promoter could interfere with transcription initiating from the tk promoter. Bandyopadhyay and Temin (1984a,b) have in fact reported that when the chicken tk gene was placed in the same orientation as that of the LTR promoter of the spleen necrosis virus, tk activity was low. Nonetheless, the fact that the HSV tk gene in Adtk infected cells was expressed at all regardless of the absence of positive selection, transactivating functions and of its orientation in the viral genome, was a surprising and an encouraging finding. In the case of the Hepatitis B virus surface antigen which was cloned at the extreme right end of an adenovirus vector, a low level of transcription but no translation could be detected in infected cells (Saito et al. 1985).

However, if dlE1,3 is to be used as an expression vector to overproduce a given protein, further modifications would be necessary. For example, the E3 promoter could be deleted and a given gene could be inserted with its own regulatory sequences, or under the control of the adeno-major late promoter (MLP). During the late phase of the lytic cycle, transcription from the adeno MLP is greatly enhanced and

most of the cellular protein synthesis is shut off (Flint, 1982); both of these factors account for the fact that over 90% of newly synthesized proteins are viral in origin. Thus, placing a given gene under the control of the adeno MLP should result in very high rates of expression, especially if the complete promoter plus its tripartite leader is used in the construct. Using this strategy several groups have recently obtained high levels of expression of the simian virus 40 tumor antigen (Thummel, et al., 1981; 1982; 1983), the HSV tk gene, (Yamada, et al., 1985), the dihydrofolate reductase gene (Berkner and Sharp, 1984, 1985), and the hepatitis B surface antigen (Davis, et al., 1985). In a second approach, additional deletions in the E3 region could be engineered in such a way that an inserted promoter-less gene would be placed directly under the control of the E3 promoter. Both of the above mentioned possibilities are relatively easy to construct, however, it seems reasonable to assume that the first approach would lead to a greater expression of a given gene.

In its present structure dlE1,3 is, however, an efficient delivery vehicle (chapter 5), in that it can readily convert tk^- cells to the tk^+ phenotype. Transformation efficiency was found to be dependent upon the cell line used, with the human cell line 143 being transformed very inefficiently as compared with the mouse cell line LTA. It is interesting to note that 143 cells were also very inefficiently transformed to tk^+ in DNA-mediated transfection assays (Graham et al., 1980) and that similar differences between human and rodent cells have been observed for oncogenic transformation (Graham, 1977). The reasons for this difference between human and mouse cells are not known, but at least in the experiments reported

here, they are unlikely to be related to differences in the efficiency of delivery or expression of the tk gene. First, adenoviruses are known to infect human cells more efficiently than mouse cells, and second, the levels of tk activity in Adtk infected 143 cells were at least as high or even higher than in LTA cells.

In the present study we also observed that the mouse K4 cell line, an LTA derivative which constitutively expresses the HSV regulatory protein, ICP4 (Persson et al., 1985), could be transformed at higher efficiencies than its progenitor LTA. Although the observed difference could be merely due to clonal variation, an alternative hypothesis is that enhancement of tk expression in response to ICP4 might result in the increased survival of the transfected cells. In this context it is of interest that the human 293 cells, which contain and constitutively express the Ad5 E1 regulatory function (Graham et al., 1977), can also be transformed more efficiently than other human cell lines (Weeks and Jones, 1983). In addition, in transient expression assays transfected genes appear to express more efficiently in 293 cells than in any other cell line tested (Green et al., 1983; Treisman et al., 1983; Alwine, 1985). Since both ICP4 and E1a proteins are known to be capable of activating homologous and heterologous genes in trans, termed transactivators (Kingston et al., 1985), these observations support the hypothesis that enhanced expression of transfected genes in the early stages of transformation may directly affect transformation efficiency.

Alternatively, transformation efficiency might be related to the efficiency of integration of transfected genes into the host chromosome, a process which might be enhanced by functions such as E1

and ICP4. Support for this hypothesis comes from several lines of evidence. In the adenovirus system it has been known for some time that early in the lytic cycle, a minor but significant portion of the viral DNA integrates into the host genome (Schick et al., 1976). It has been reported that significantly lower levels of integration are detected when cells are infected with cytocidal (cyt) mutants, such as cyt 141, which map in the Elb region (D'Halluin et al., 1985). Moreover, host range II (hrII) viral mutants also defective in the Elb, are unable to transform primary cells (Graham et al., 1978), but transformation can be obtained with hrII mutant DNA (Rowe and Graham, 1983). All of the above observations suggest that the Elb gene products might be involved in integration or recombination events occurring between viral and host DNA upon viral infection of the cells. Such functions, however, are apparently dispensable for the integration of transfected DNA, a process which might be mechanistically different. Similar conclusions were drawn from studies with polyoma virus. Although virus tsA mutants (which affect the large T antigen) are greatly impaired in their ability to transform primary cells at the non-permissive temperature (Fried, 1965; DiMayorca et al., 1969; Eckhart, 1975), DNA fragments encoding only the small and middle T antigens are sufficient for DNA-mediated transformations (Hassell et al., 1980; Novak et al., 1980); this again has suggested that the large T antigen might be involved in promoting integration during viral infection and might thus increase the efficiency of transformation (Della Valle et al., 1981). Perhaps the strongest evidence supporting a direct correlation between integration and transformation efficiency comes from two recent studies. In the

first report, Ihara and Ben-Porat (1985) have shown that expression of G1, an immediate-early protein (analogous to ICP4) in pseudorabies virus was necessary for high frequency recombination. In the second study, Laanen et al. (1985) have shown that co-transfection with either the adenovirus E1 or the retrovirus long terminal repeat (LTR) was necessary for stable integration to occur in the tk^- 143 cells.

The mechanism(s) by which functions such as E1b, T antigen or the herpes virus IE proteins affect integration or recombination is at present unknown. In the case of retroviruses it has been demonstrated that the LTR enhance stable integration by affecting the level of expression of transfected genes (Kriegler and Botchan, 1983; Luciw et al., 1983). The retrovirus' LTRs contain strong enhancer elements (Blair et al., 1980; Chang et al., 1980), but they do not possess the sequences encoding the integrase protein, which are located at the 3' end of the polymerase gene (Donehower and Varmus, 1984; Panganiban and Temin, 1984). However, this model (stable integration based on stimulation of transcription of the transfected gene) does not explain the transformation defect of adenovirus E1b mutants which still express the E1a transactivating functions and have functional enhancers. Therefore, further studies are required to provide an explanation for the increased transformation efficiency of cells such as 293 and K4.

During the construction of dlE1,3, (chapter 4) a variant (dlE1,3-1) with a duplication of the left 920 bp of Ad5, linked by pBR322 sequences to the left end of the dlE1,3 genome, was isolated. Several lines of evidence have been presented suggesting that the left

end duplication had been generated by in vivo ligation of the Ad5 ClaI B-fragment to the ClaI site of pBR322 either before or after homologous recombination between the adeno sequences located between map coordinates 10.6 and 15.8. Although such an event might seem unlikely, studies carried out on DNA soon after transfection have indicated that the transfected cells mediate recombination and ligation of the transfected DNA very efficiently (Wilson et al., 1982; Miller and Temin, 1983).

Adenovirus mutants with embedded right ITRs have been previously isolated (Brusca and Chinnadurai, 1983; Hanahan and Gluzman, 1984). Analyses of these mutants gave differing results. Brusca and Chinnadurai (1983) reported that an Ad2 duplication mutant (dp 201), in which the left 1110 bp of the genome was duplicated at the right end reverts to wild type Ad2 at a low rate, whereas Hanahan and Gluzman (1984) reported that an Ad5 duplication mutant in which the left end 920 bp (ClaI B-fragment) was duplicated at the right end does not revert to wt. These latter workers concluded that the embedded ITR at the right end is entirely inactive, and that a virus with such a configuration is very stable.

In contrast to the above, the extra fragment in passaged dlE1,3-1 viral populations seemed to readily transfer to the right end of the genome leaving the original copy at the left end, thus generating dlE1,3-2. Moreover, mutants with an extra terminal segment at both ends (dlE1,3-2) readily back reverted to dlE1,3-1. Variants which had lost the extra segment at the left end were also detectable but seemed to be very rare, and revertants which had lost the extra fragment from both ends, though they may exist, were never detected as

plaque isolates. Likewise, variants containing two tandem repeats of the extra terminal segment were detectable, but were never isolated as pure plaque isolates. This might have been due to their presence at a low frequency; only a total of 60 plaques were examined and the probability of isolating such variants may have been small.

The interconversion of dlE1,3-1 and dlE1,3-2 can be explained on the basis of intermolecular recombination events, as a consequence of the mechanism of adenovirus DNA replication as outlined in Fig. 6.6, or as a combination of these processes. It is interesting, however, that complete loss of the extra terminal segment from the virus characterized in this study (as would have resulted from pathway B-F-H of Fig. 6.6) seems to be a rare event as compared to the frequency of interconversion between dlE1,3-1 and dlE1,3-2. The fact that of the 60 plaque isolates analyzed, none had lost the terminal duplication, suggests that this pathway is rarely utilized. This could suggest that viral DNA replication might initiate infrequently at the right molecular end, but there seems to be very clear evidence that initiation events occur at about the same frequency at both ends. Evidence for this came mainly from the following study: the Ad5 genome contains three clusters of AT-rich regions at map coordinates 0-15, 50-60 and 80-100 (Doerfler and Kleinschmidt, 1970), under suitable partial denaturation conditions, the left end can be distinguished from the right end. Analysis of partially denatured replicative intermediates (under an electron microscope) showed conclusively that initiations occur at about the same frequency at either end, and that a significant fraction of the molecules contained two replication forks moving in opposite directions (Lechner and Kelly, 1977).

Alternatively, it is possible that viral DNA molecules which have one or more repeats of the terminal segment have a selective advantage over molecules with no repeats. Viral molecules with extra repeats might be packaged more efficiently since the extra fragment contains the packaging sequence, thus, these genomes can be packaged either from left (like the wt) or from the right. However, this is unlikely, because those molecules with packaging sequences at either end (dlE1,3-2), back reverted to molecules with packaging sequences at the left end only (dlE1,3-1) at a high rate. Moreover, the chromosomes of variants with one or more extra repeats are physically larger than those with no extra repeats, suggesting that they should be at a slight disadvantage in terms of replication rate. Therefore, one is faced with the dilemma of accounting for the high rate of loss of the extra fragment from the right but not from the left end. The structure of the left end differs from the right end in two respects: first, the sizes of the direct repeats at the left end are larger (350 bp each) than those at the right end (80 bp each); second, the length of the segment separating the repeats at the left end is smaller (570 bp) than the one at the right end (1190 bp). It is possible that this difference in structure between the left and right ends might play a role in causing the loss of the extra fragment at the right but not at the left end, via an unknown mechanism.

In summary, as has been noted in chapter I, over the last two decades adenoviruses have and continue to serve in the field of molecular biology as an excellent model system for studies on oncogenesis, gene expression, and DNA replication and recombination;

furthermore, the principle aim of the studies presented in this thesis was to determine whether this group of viruses can also be used as gene transfer vectors in mammalian cells. Since the work reported in this thesis is among the earliest in this field, little was known about the packaging limits of adenoviruses. Therefore, the approach taken was to initially determine the upper limits on the size of a DNA molecule which can be packaged in an adenovirus capsid. The data presented in chapter III have demonstrated that not more than about 1.7 Kb (or about 5%) of extra DNA can be packaged in an Ad5 virion. Moreover, deletions and rearrangements occurred when large inserts were rescued whereas no such rearrangement was observed when a small insert was rescued.

Subsequently, this upper limit was increased by constructing a double deletion mutant, lacking early regions 1 and 3 (dlE1,3; chapter IV). The virus dlE1,3 is helper-independent when propagated in 293 cells, and its genome is 5.5 Kb shorter than wt and therefore should be able to theoretically accept inserts of up to 7.5 Kb. Like other early region 1 defective mutants, the dlE1,3 virus has a host range phenotype, and is thermolabile, due to its lack of protein IX.

The results presented in chapter V have demonstrated the usefulness of dlE1,3 as a vector in mammalian cells. Firstly, the HSV tk gene, along with its own regulatory sequences, was readily inserted in both orientations into the unique XbaI site of dlE1,3. Secondly, the HSV -tk gene was stably carried, and efficiently delivered into tk^- cell lines. However, transformation efficiency appeared to depend on the cell line used. It appeared that cells which are constitutively expressing a transacting function (such as the Ad5 E1

and the HSV IE) transformed more efficiently than the other cell lines tested. Thus, taken together, the present capacity of potentially accepting up to 7.5 Kb in foreign DNA, the helper-independence, the flexibility of cloning, the stability of inserts, and the high efficiency of gene delivery make dlE1,3 virus an attractive vector for use in mammalian cells *in vitro*. Of course, further work in this area is needed in order to exploit the full potential of adenoviruses not only as gene transfer and expression vectors in mammalian cells, *in vitro* and *in vivo*, but also for the engineering of recombinant vaccines.

Lastly, characterization of an Ad5 mutant with embedded inverted terminal repeats (ITRs) (chapter VI) indicated that the left end extra fragment in dlE1,3-1 transfers to the opposite end, thus generating a second variant (dlE1,3-2) with 4 ITRs. Analysis of both mutants indicated that reversion to wt (dlE1,3) might occur but at a very low rate whereas the interconversion between dlE1,3-1 and dlE1,3-2 occurs at a high rate. The rearrangements of adenovirus DNA seen in this study (chapter VI) are of interest not only for the information they may afford in relation to adenovirus DNA replication and recombination, but also for the insights these phenomena may provide into the general mechanism of DNA rearrangements in eukaryotic cells.

APPENDIX: CONSTRUCTION OF VARIOUS PLASMIDS AND VIRUSES

The appendix contains the details for the construction of various plasmids and viruses that were constructed at various periods of time during my tenure as a graduate student. Most if not all of these constructs did not appear in the studies described in this thesis. Since some of these plasmids and viruses might be useful to my colleagues, a comprehensive schematic drawing of all of these constructs was presented in this section. Details of the procedures and reaction conditions were not included for the sake of brevity, but they may be found in the Materials and Methods. However, the purpose of each construct was briefly stated in each figure's legend.

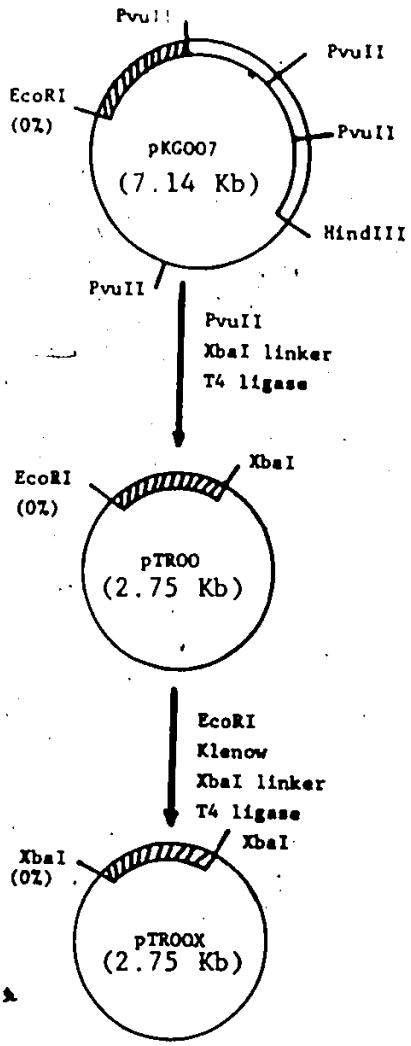


Figure A.1 Construction of pTR00X (Amp^+, Tet^-).

The objective of this construct was to introduce an XbaI restriction enzyme site in place of the EcoRI (at position 0), and the PvuII site (at position 454), in order to rescue the left end into the XbaI site of dl309, or dlE3. Thus, one could examine the effect of an internal ITR on viral DNA replication and rearrangement, as compared to the effect of an internal head to tail joint (described in the next Figure). The plasmid pKG007 contains the adeno HindIII G-fragment cloned into pBR322 at the EcoRI and HindIII sites(obtained from F.L. Graham). The hatchet areas represent adeno sequences from 0% to 1.3% (PvuII site).

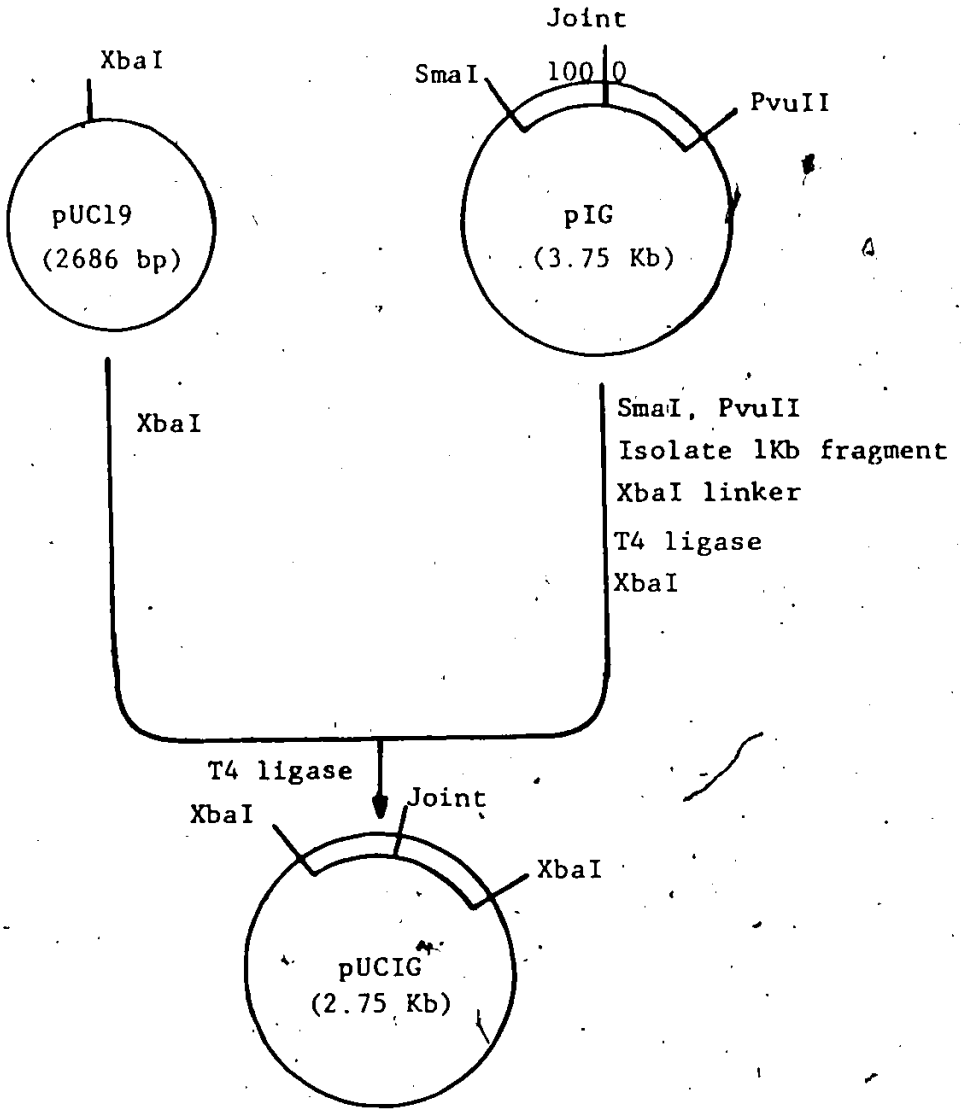


Figure A.2 Procedure for constructing pUCIG (Amp^+, Tet^-).

The purpose of this construct is similar to that described in Fig. A.1. The plasmid pIG (for details see Fig. A.4) is a derivative of pFG140 (Fig. A.3, Graham, 1984); it was constructed by digesting pFG140 with HindIII and ligating. An XbaI linker was ligated to the SmaI to PvuII fragment (the joint fragment) and subsequently subcloned into pUC19 to generate pUCIG. Thus, the joint flanked by two XbaI sites can be rescued into dlE3 or dl309 in order to assess its effect on viral DNA replication and rearrangement, as well as its effect on viral viability.

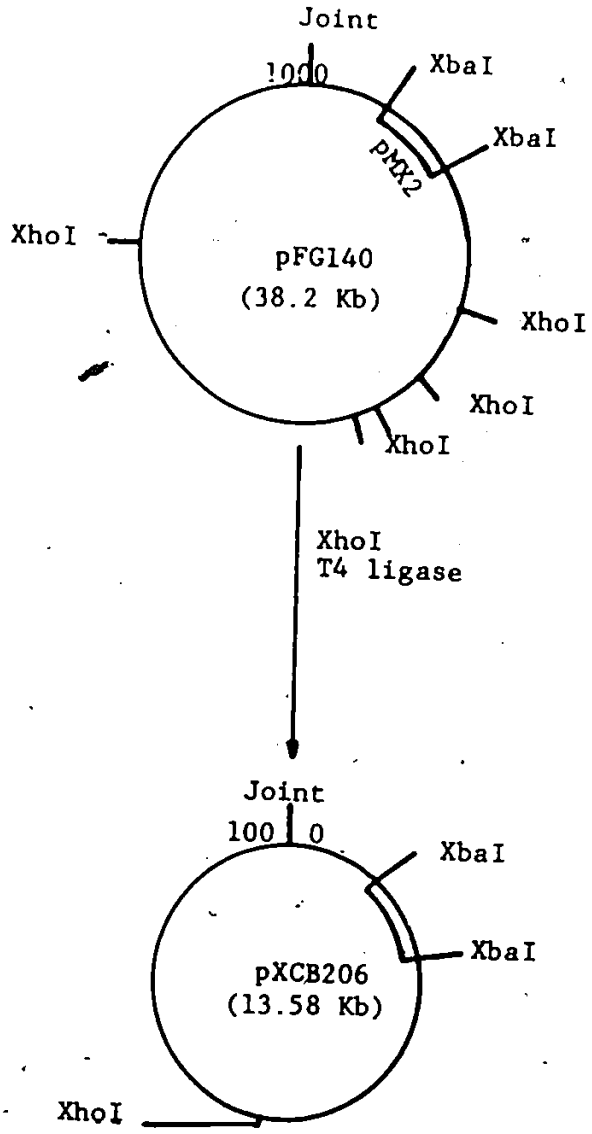


FIGURE A.3 - The plasmid pXCB206 (Amp^+), was constructed as outlined above. The rational for this construction was as follows: first, to insert foreign DNA containing a selectable marker and plasmid origin at the unique XhoI site, and subsequently, to delete the pMX2 from E1 inserted at the XbaI site of d1309; lastly to transfect T143, TLTA, and TK4 cell lines (these cell lines appear to contain most if not all of the Adtk sequences), and to determine whether the presence of the intact E1 region in this plasmid would induce the expression of essential viral products needed for its replication and packaging as a virion.

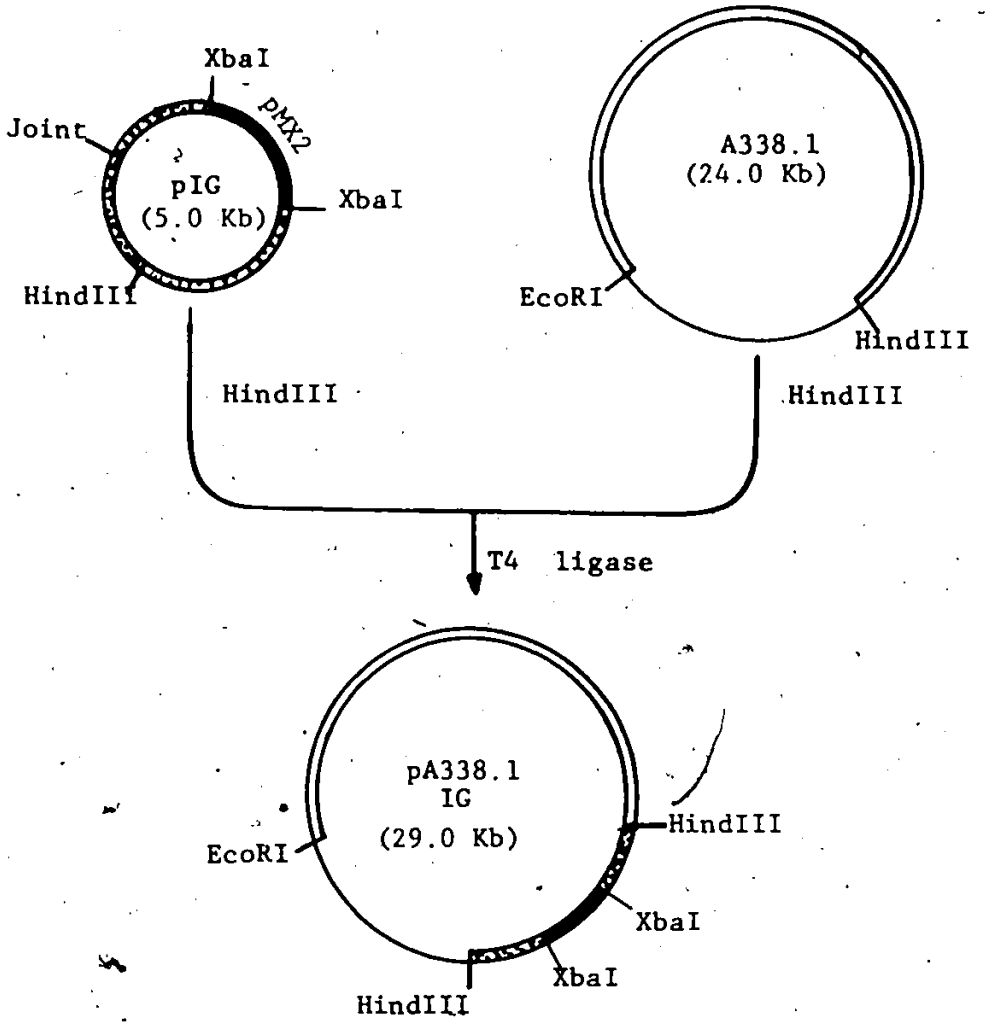


Figure A.4 The plasmid pA338.1-IG (Amp^+, Kan^+, Tet^-) was constructed as outlined in Figure above, for the same reasons as indicated in Fig. A.3. However, an additional step has to be carried out before using it, which is the deletion of pMX2 from the XbaI site of E1. The plasmid A338.1 (obtained from S. Bacchetti) contains the HSV reductase coding sequences, cloned into the EcoRI and HindIII sites of pKC7 (Maniatis et al., 1982).

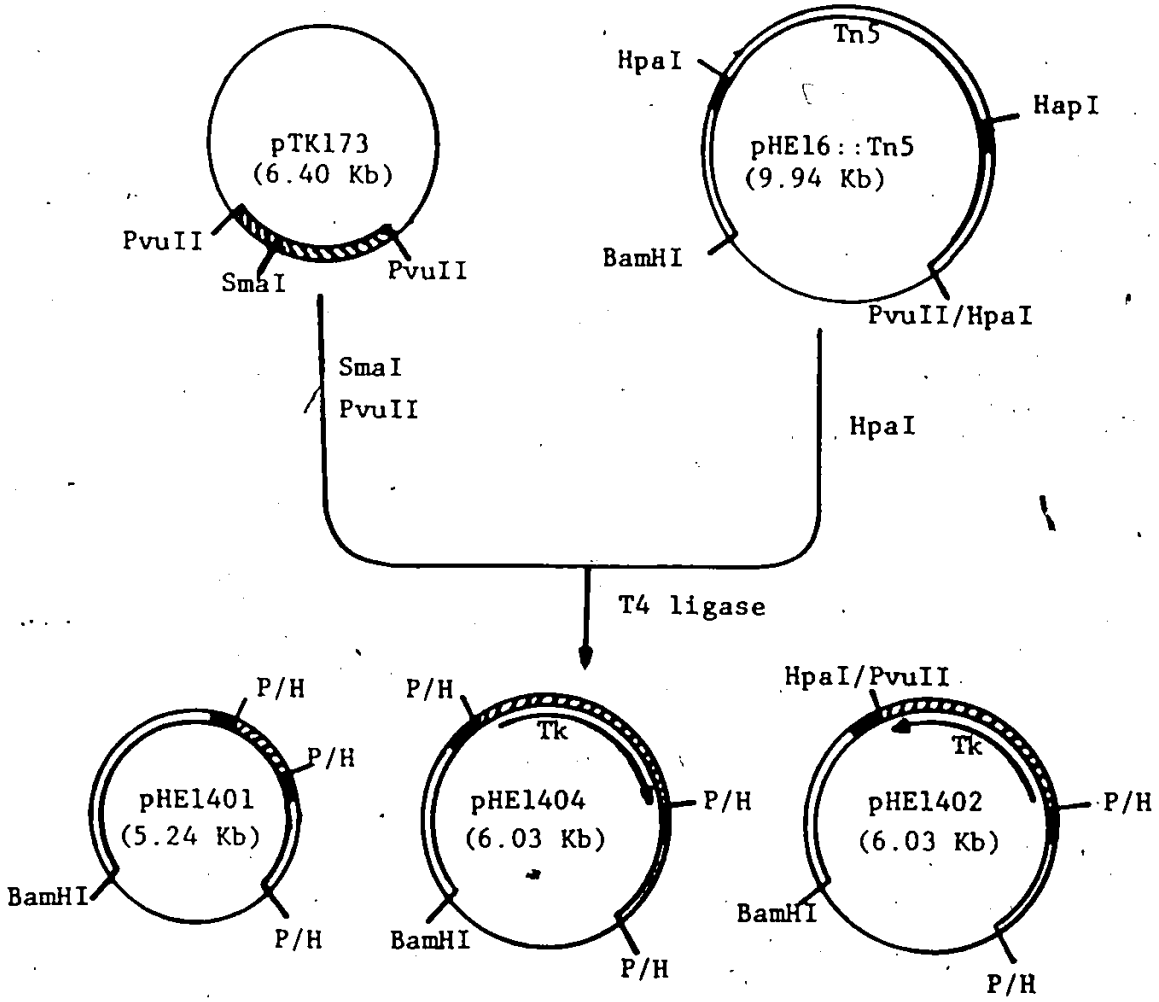


Figure A.5 Construction of plasmids pHE1401, pHE1404, pHE1402 (all are Amp^+). These plasmids were constructed for two reasons: first, to make the total insert small in size so that it can be rescued into dl309, without rearrangements; secondly, to assess the effect of the palandromes (black regions, each 185 bp in size) on viral DNA replications and rearrangements. The plasmid pHE16::Tn5 (McKinnon, 1984) contains the left 4.3% of the Ad5 genome (HepaI E-fragment) cloned into the pBR322 BamHI and PvuII sites, with Tn5 being transposed into the adeno sequences. The plasmid pTK173 (McKinnon, 1984) contains the HSV tk gene (PvuII fragment) cloned into the pBR322 PvuII site.

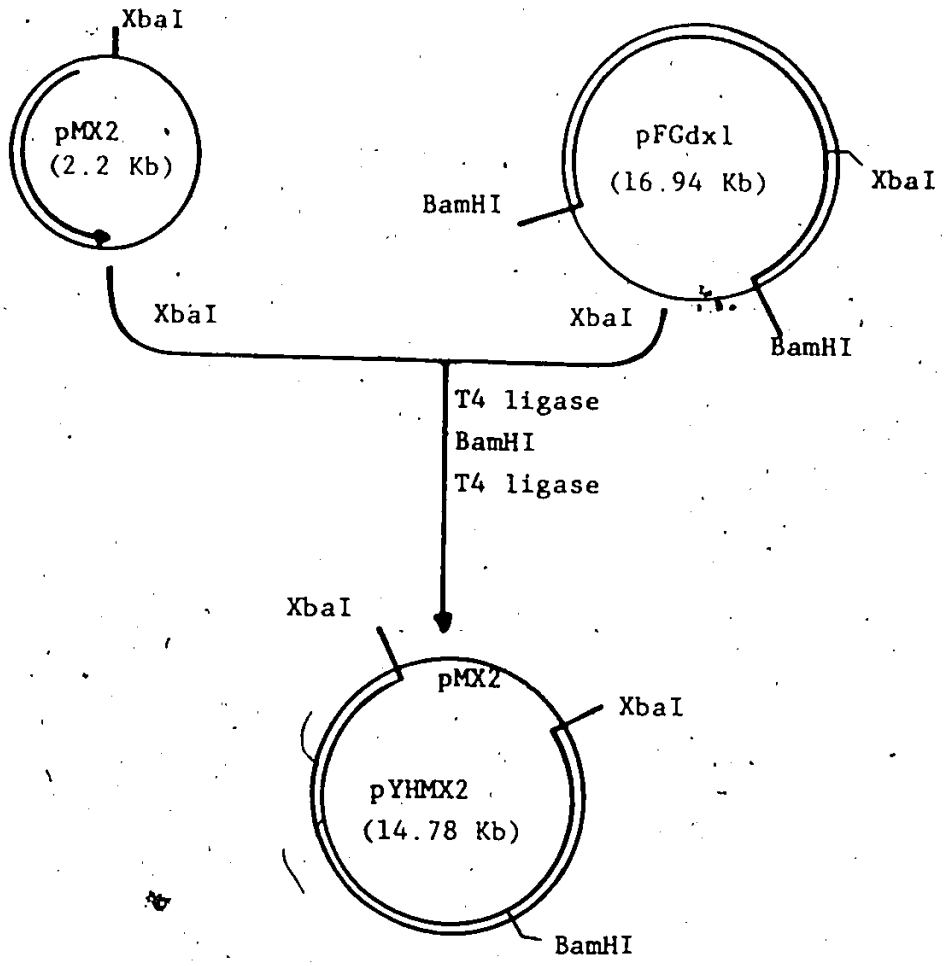


Figure A.6 Construction of pYHMX2 (Amp^+). This plasmid was initially constructed to be rescued into dlE1,3 virion, and subsequently, to use the newly constructed virus to generate an infectious dlE1,3 in a plasmid form analogous to pFG140 (Graham, 1984). The plasmid pFGdx1 was described in detail in Chapter 4.

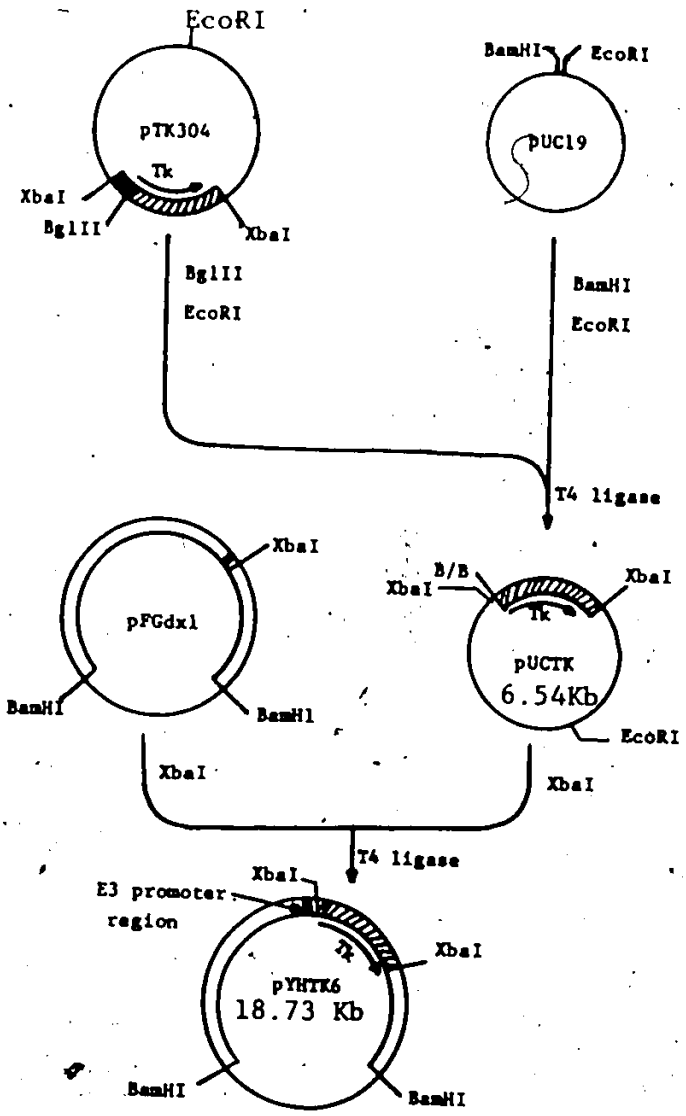


Figure A.7 Construction of pYHTK6 (Amp^+).

This plasmid was constructed (as outlined in Figure above) in order to test in a tk transfection assay, as to wehther the HSV tk gene can be expressed from the E3 promoter or not; if yes, then to rescue it into the dlE1,3 vector. The rational for the construction of such a virus is the following: if the tk gene expresses at a high rate from the E3 promoter, then it should be possible to rescue foreign genes into this virus, by co-transfecting viral DNA and pFGdx1 (with an insert in E3). DNA and selecting for recombinat in the presence of BudR as described by Smiley (1980), thus, making the rescue into dlE1,3 a simple procedure.

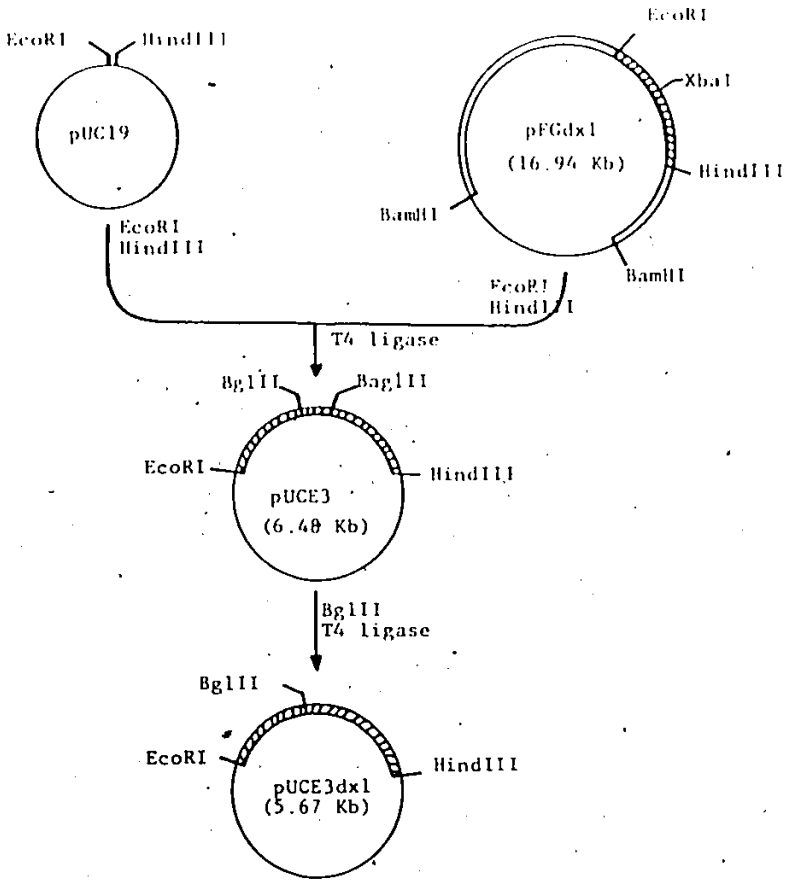


Figure A.8 Construction of pUCE3dxl (Amp^+).

The purpose of the construct outlined in this Figure was twofold: first to increase the deletion in E3 by about 1 Kb, from BglII to BglII. Secondly, in one case, to introduce at this unique BglII site XbaI and ClaI sites, and subsequently to reinsert this modified fragment (from EcoRI to HindIII) back into pFGdxl. In the second case, to insert at the BglII site the PvuII to EcoRI fragment (as outlined in Fig. A.9, containing the SV40 promoter-enhancer, and pUC19 multicloning sites), and again to reinsert this modified EcoRI to HindIII fragment back into pFGdxl. In both instances cloning into pFGdxl would be simpler, and in the second situation the SV40 promoter will be useful for the insertion of promoterless genes.

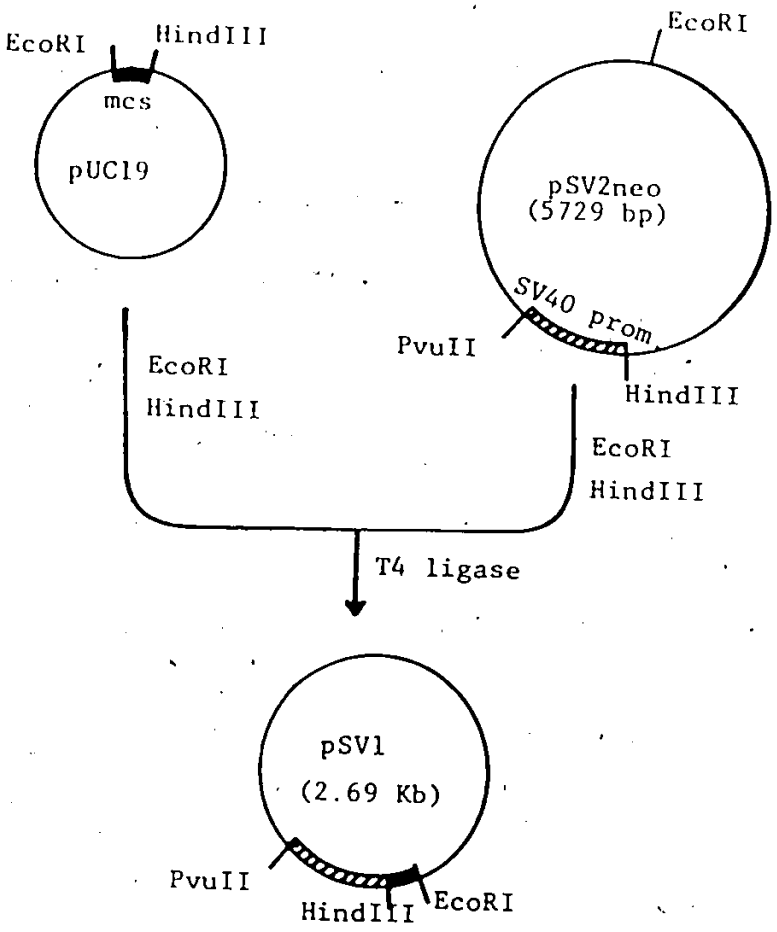


Figure A.9 Construction of pSV1 (Amp^+).

The objective of the construct outlined above was to insert a multicloning site (black area) next to the SV40 promoter-enhancer, and subsequently to insert the PvUII to the EcoRI fragment into pFGdx1, as outlined in Fig. A.8.

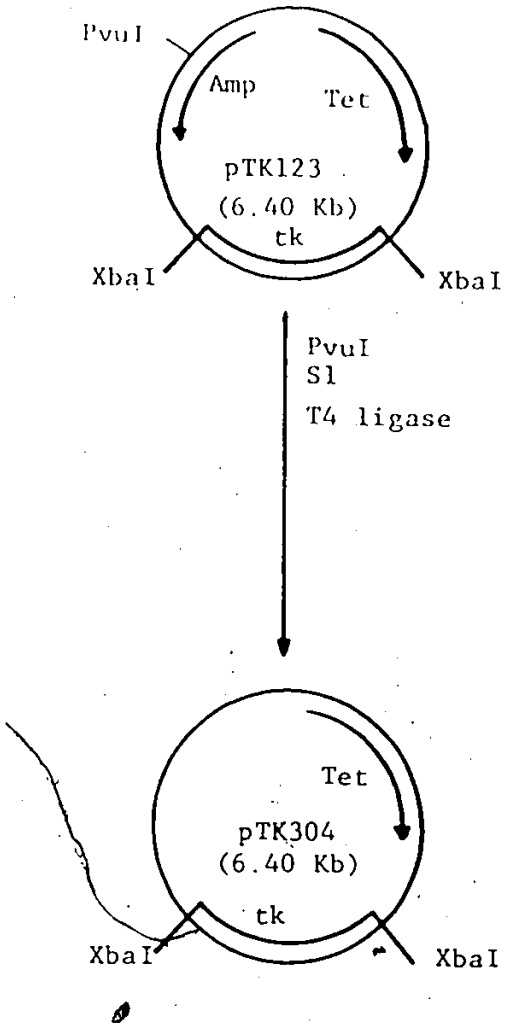


Figure A.10 Construction of pTK304 (Amp^-, Tet^+).

The objective of this construct was to facilitate the insertion of the tk gene into pFGdxl. Thus, by cutting pFGdxl (Amp^+, Tet^-), pTK304 with XbaI, and religating, followed by transformation and selection on Amp plates, only pFGdxl, with or without an insert, would give colonies.

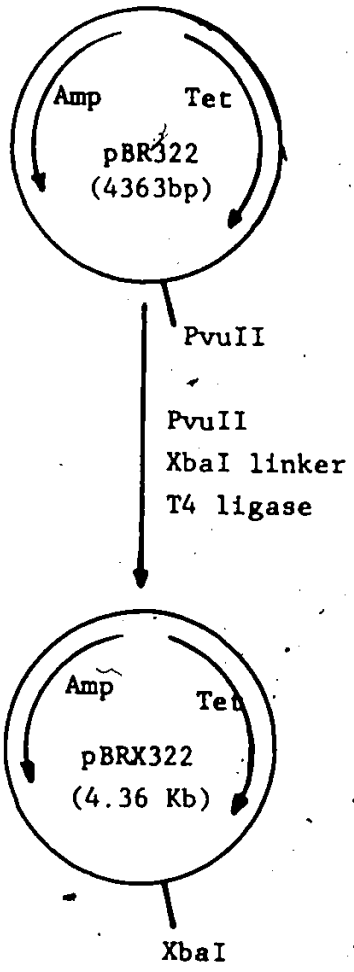
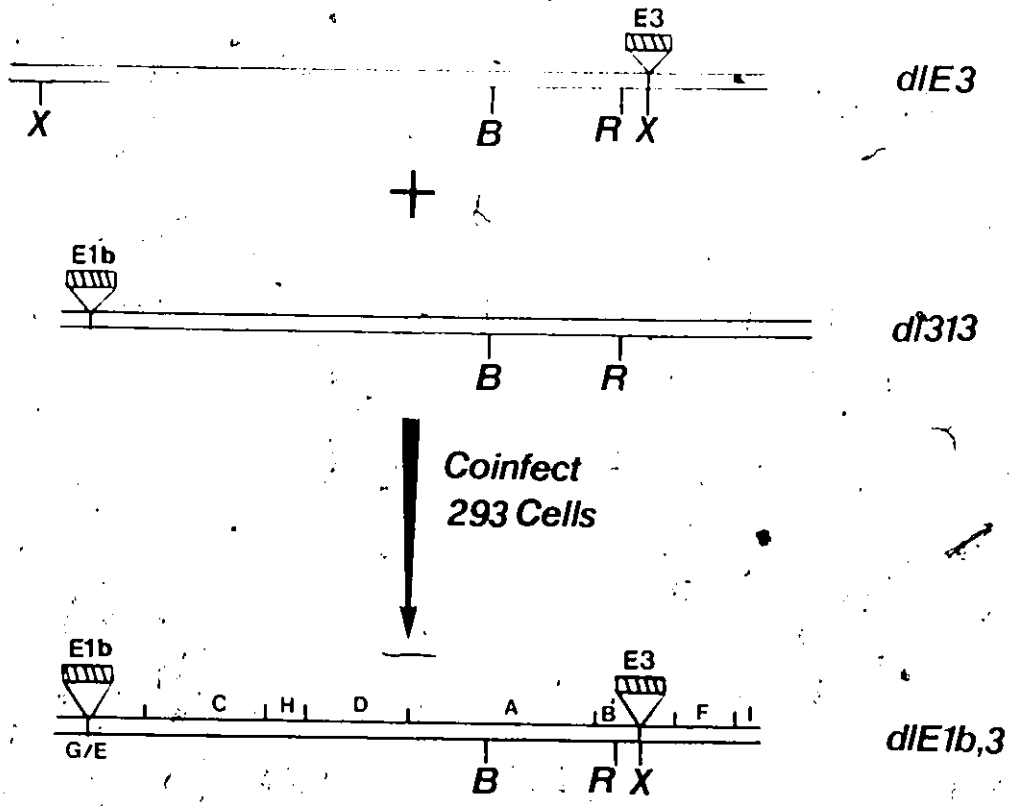


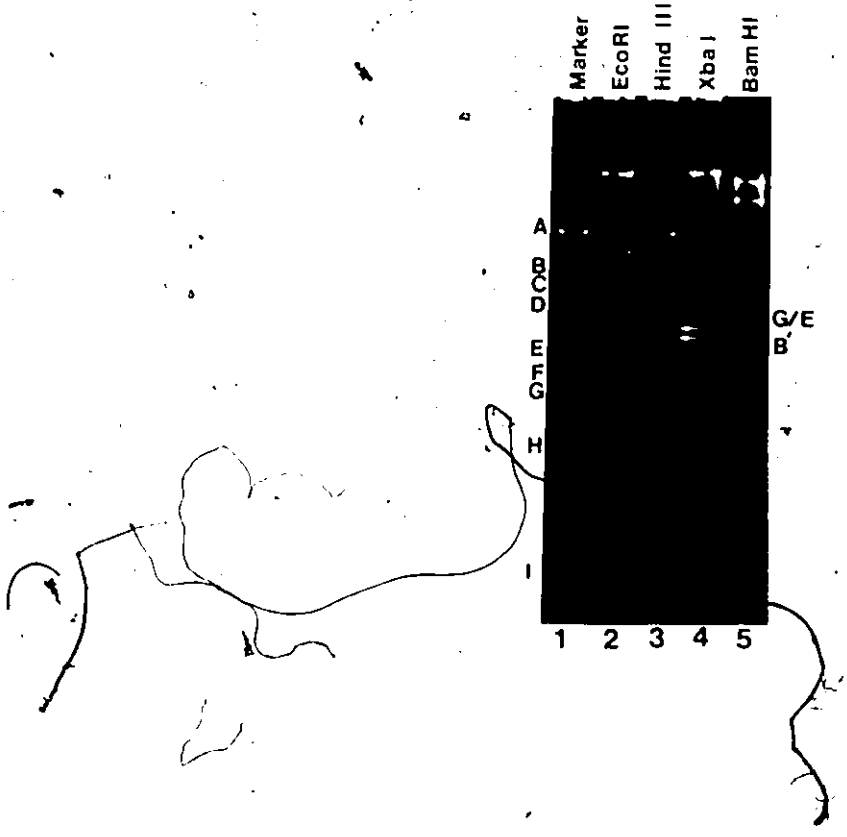
Figure A.11 Construction of pBRX322 (Amp^+, Tet^+).

This plasmid is identical to pBR322 except that it has an XbaI in place of a PvuII site. This plasmid was generated when the PvuII sites bracketing the tk gene were converted into XbaI sites (for details, see Chapter 5).

Figure A.12 Construction of dlE1b,3.

After co-infection of 293 cells with dlE3 and dl313, progeny viruses were screened (using restriction enzymes) for the presence of the double deletion of E1b and E3 (indicated by shaded triangles). The restriction enzyme pattern of such a double deletion mutant (dlE1b,3) is shown in the middle of the Figure. An ethidium bromide stained gel showing the restriction enzyme pattern of dlE1b,3 digested with the indicated enzymes is shown at the bottom of the Figure. In lane 1 is the HindIII digest of dl309, run as a marker.


E3
X
B
R X
dlE3
E1b
B
R
dl313
Coinfect
293 Cells
E1b
E3
C
H
D
A
B
F
G/E
B
R X
dlE1b,3


Marker
EcoRI
Hind III
Xba I
Bam HI
A
B
C
D
E
F
G
H
I
G/E
B'
1 2 3 4 5

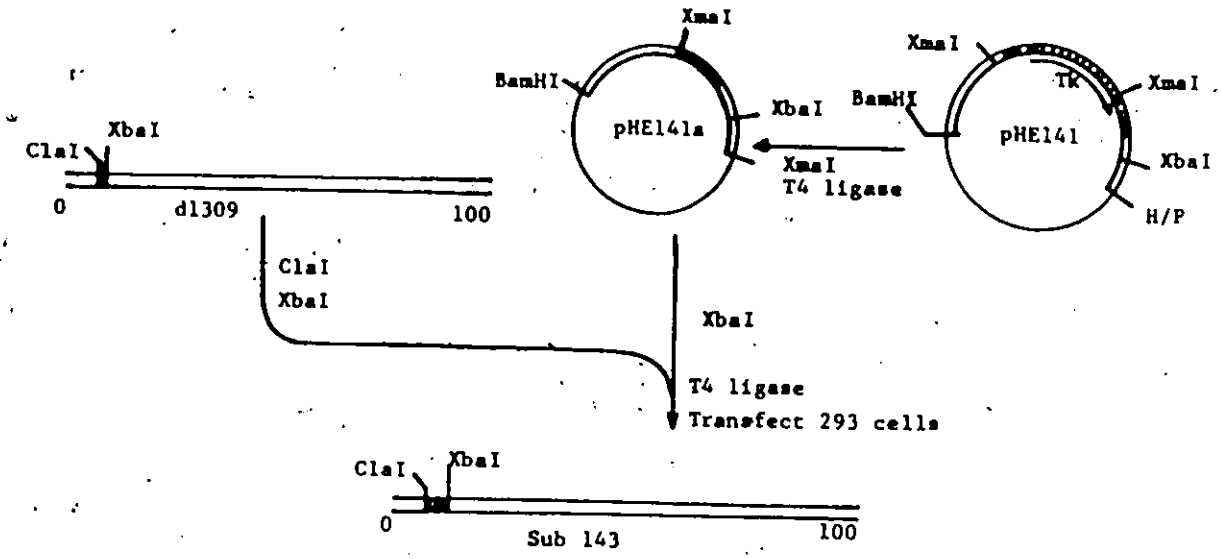


Figure A.13 Deletion of the E1a 13s message splice donor site, and the construction of Sub143.

The plasmid pHE141 was digested with XmaI then religated to generate the plasmid pHE141a. The dark box indicates the 185 bp Tn5 sequence whereas the region shaded with slashes indicates the HSV tk sequence (from SmaI to PvuII sites). The open box represents the adeno left end sequence from -21 bp (at the BamHI site) to the HpaI site at position 1575. Subsequently, the plasmid pHE141a was rescued into d1309 exactly as described in Chapter 3.

Figure A.14 Restriction enzyme analysis of d1309 and sub143.

293 cells were infected with either of the above viruses, in vivo labelled DNA was extracted as outlined in Materials and Methods, digested with restriction endonucleases and run on a 1% agarose gel. Lanes 1 to 4 are d1309 digested with HindIII (H), XbaI (X), XhoI (Xh) and ClaI (C). Similarly, in lanes 5 to 8 the sub143 was digested with the above enzymes. The substitution resulted in the deletion of 102 bp from the adeno sequence and a net insetion of 685 bp. Consequently, the new HindIII G-fragment (G' would be larger by 685 bp and should comigrates with the HindIII E-fragment, arrow G', line 5). Similarly, the XbaI B'-fragment would also be greater in size (arrow lane 6). The Cla B fragment is not expected to change in size due to ClaI cutting to the left of the insert. The restriction enzyme map of d1309 and sub143 are illustrated below. The black box is the 185 bp Tn5 sequence; the dotted box represents a portion of the HSV-1 tk sequence. Introns in the E1a 12s as well as the 13s transcript are represented by dashed lines.

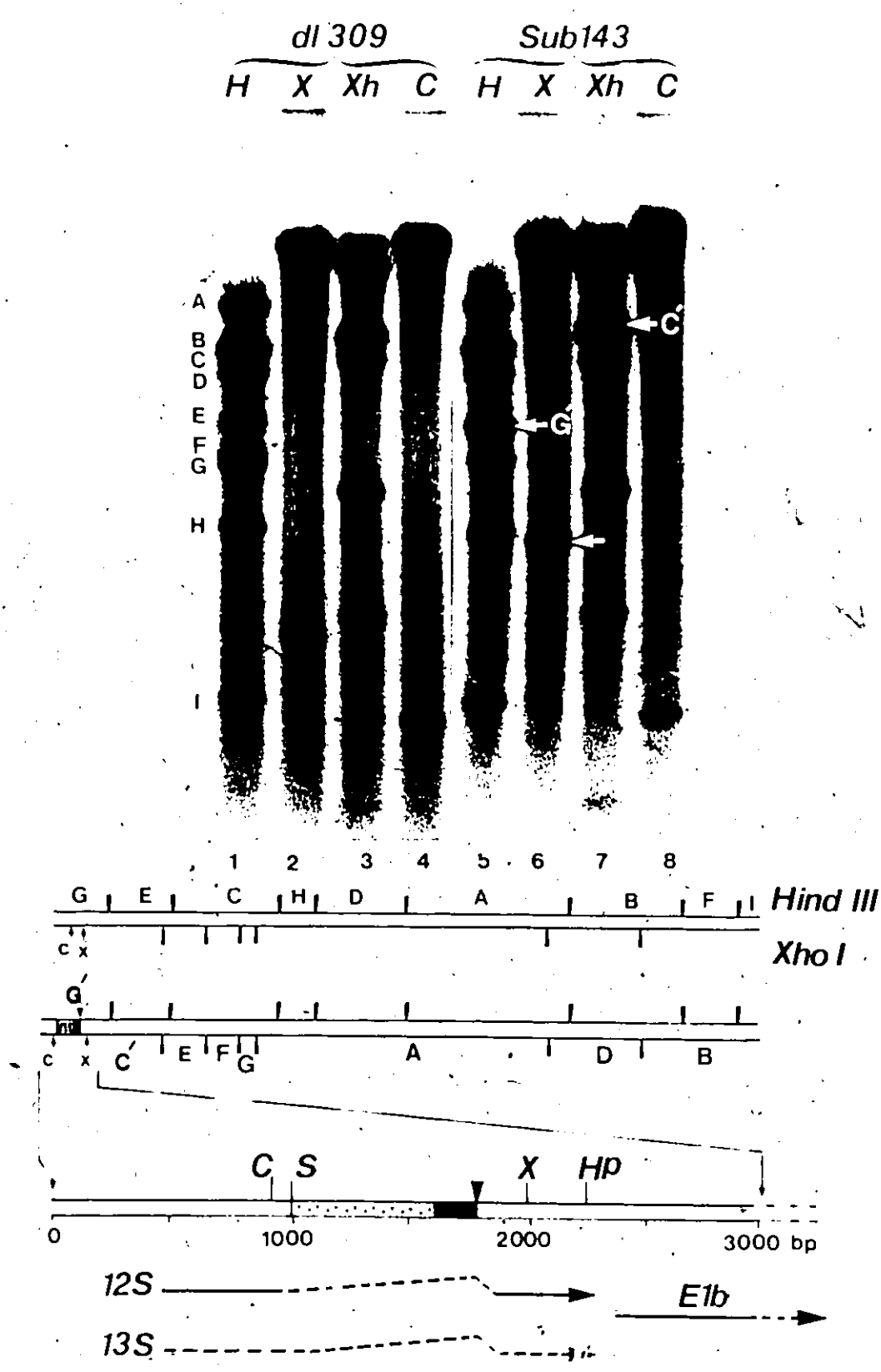
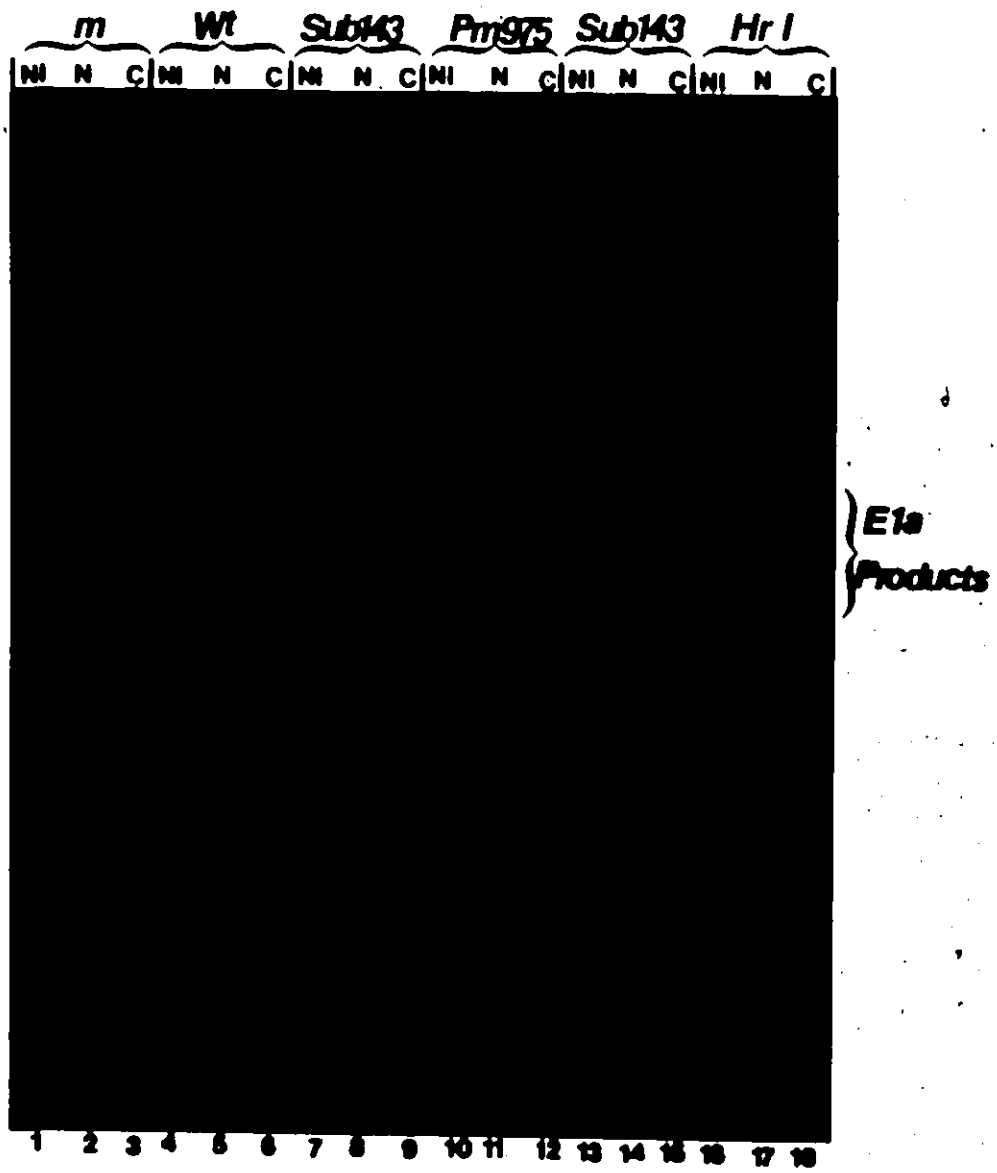

dl 309
Sub143
H X Xh C H X Xh C
A
B
C
D
E
F
G
H
I
C'
G'
1 2 3 4 5 6 7 8
G E C H D A B F I
Hind III
c x
Xho I
G'
c x C' E F G A D B
C S X Hp
0 1000 2000 3000 bp
12S
E1b
13S

Figure A.15 Autoradiogram of a 14% polyacrylamide gel analysis of immunoprecipites from mock, wt, sub143, pm975 and HrI infected KB cells. All cells were pulse labelled with ^{32}p-orthophospate for 2 hrs from 7 to 9 hrs pf. Labelled polypeptides were immunoprecipitated with serum against the carboxy (C) or the animo (N) ends; NI = non immune serum; m=mock. These sera were kindly provided by Yee, S.P.


m
Wt
Sub143
Pm975
Sub143
Hr I
NI N C NI N C NI N C NI N C NI N C NI N C
E1a
Products
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

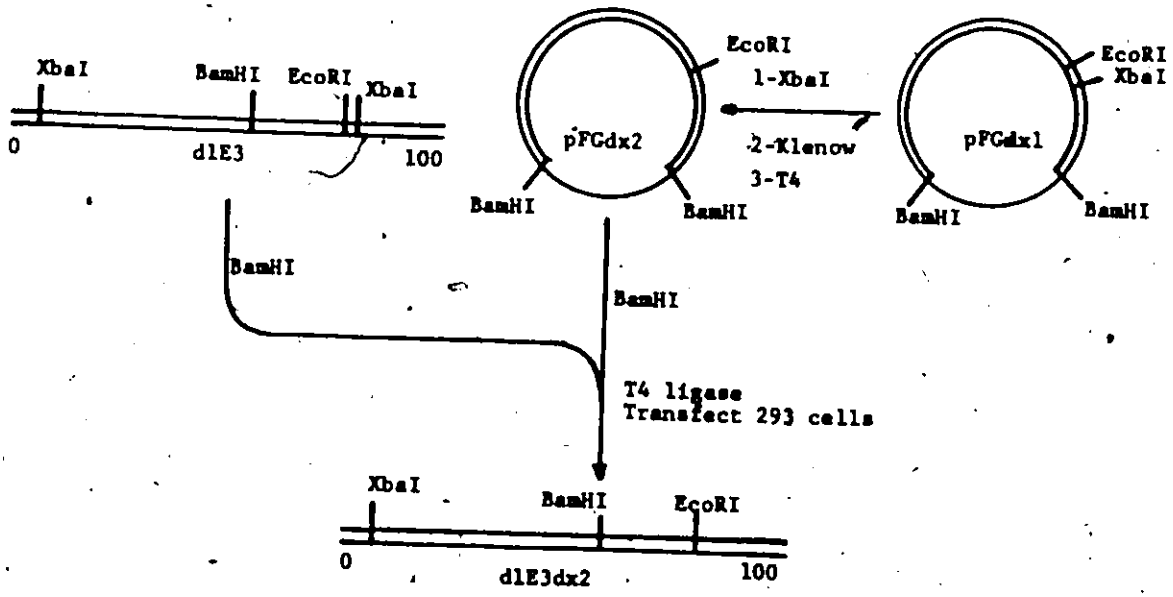


Figure A.16 Construction of dlE3dx2.

This virus is identical to dlE3, except that it has a single XbaI site at position 1339 whereas dlE3 has two XbaI restriction enzyme sites, one at position 1339 and the other in E3 (for details, see Chapter 4). This virus is useful for rescuing foreign genes at the left end by using a procedure similar to that described in Chapter 4 (for the construction of dlE1,3), or by using one identical to that described in Chapter 3.

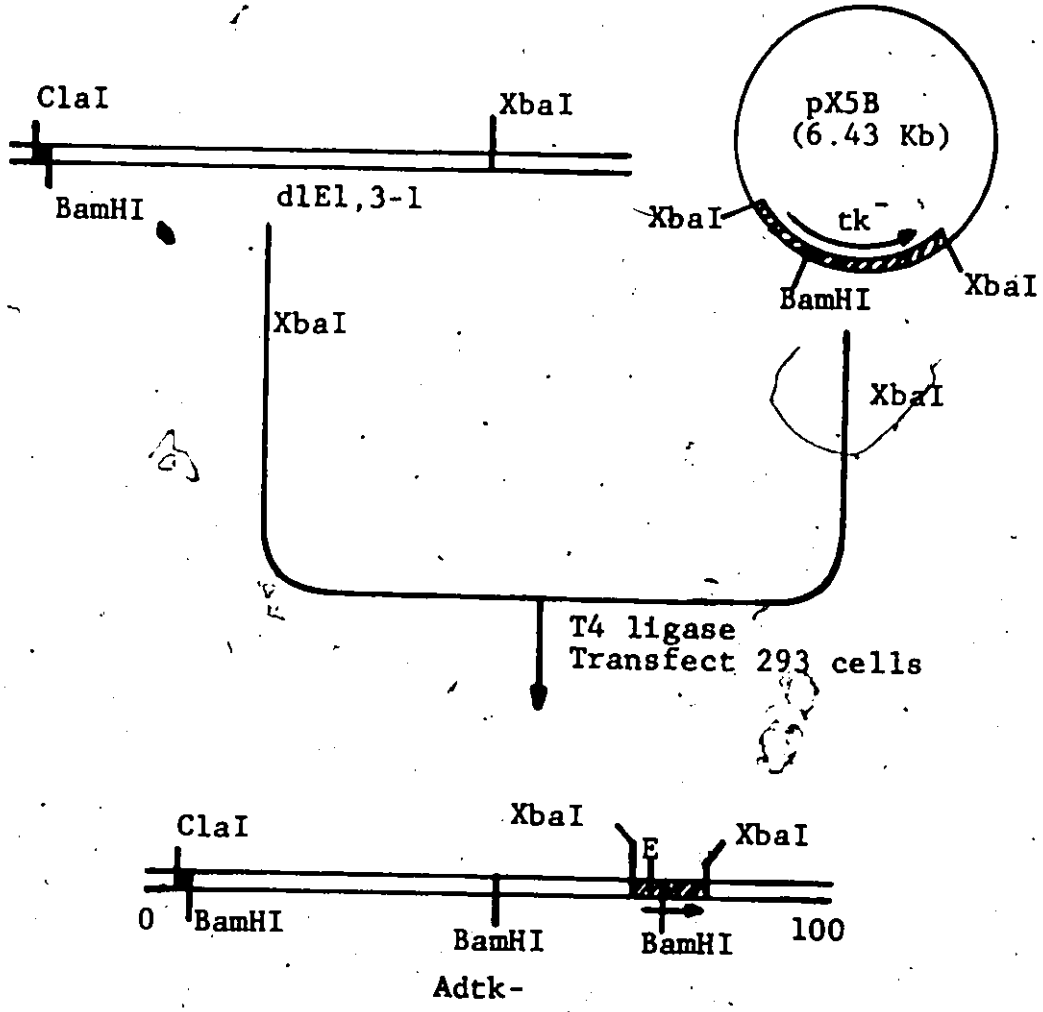
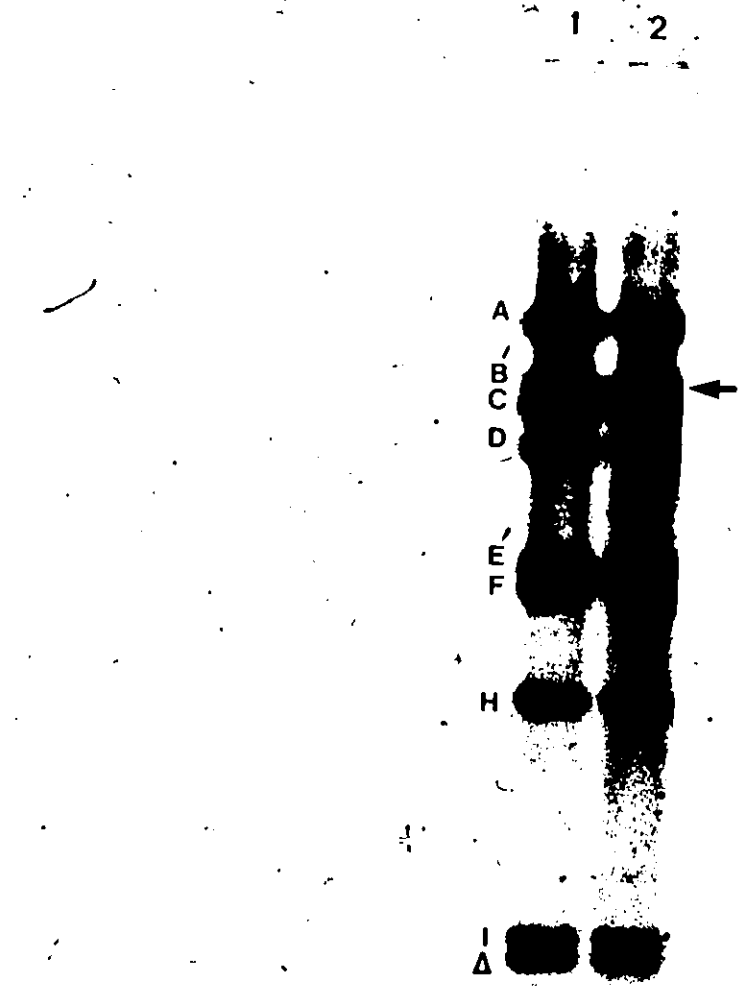
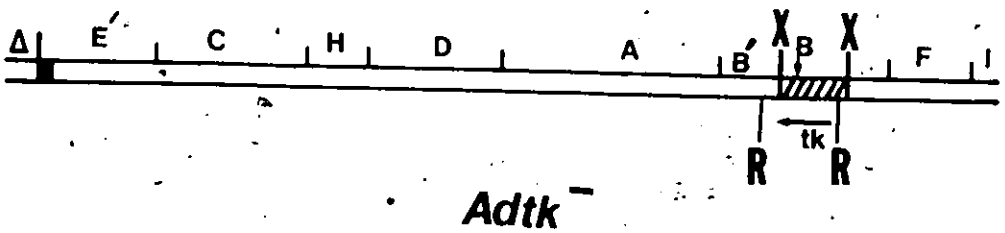


Figure A.17 Construction of Adtk⁻.

The plasmid pX5B (constructed by Susannah Varmuza, personal communication) contains a BamHI linker inserted at the AvaI site (in the 5' end) in the tk sequences; consequently, the tk gene is inactive (Susannah Varmuza, Ph.D. thesis 1985, McMaster University). The latter plasmid was rescued into dlE1,3-1 via a trimolecular ligation procedure as outlined in Chapter 5. This virus was constructed (along with Susannah) in order to carry out site specific recombination experiments in a cell line containing an HSV tk gene with a BamHI linker inserted in the 3' end of the tk gene.

Figure A.18 HindIII restriction enzyme pattern of 2 plaque isolates of Adtk$^-$. The tk gene-containing band is indicated by an arrow. The HindIII restriction enzyme map of Adtk$^-$ is illustrated below. The black box to the left of the genome represents pBR322 sequences. In other words, Adtk$^-$ has an extra end indicated by the delta which migrate just below the HindIII I-fragment, and an embedded ITR at the left end (for more information, see Chapter 6). Also an Adtk$^+$ was constructed which is identical in structure to Adtk$^-$, except that it has a functional tk gene.





Adtk^{-}

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