CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT HUMAN ADENOVIRUS TYPE 5 VECTORS

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ABSTRACT

In recent years adenovirus (Ad) vectors have been used for the expression of foreign genes in mammalian cells, have been studied as vaccine vectors and more recently as gene transfer vectors for gene therapy. A number of properties make the Ad system a good candidate for each of these applications not the least of which is the extensive understanding of their structure and biology that has been gained through their use as a model system for studying all aspects of gene expression and DNA replication. The construction of recombinant Ad vectors involves the insertion of foreign DNA sequences into the Ad genome usually in place of compensating deletions made in early region 1 (E1) or E3. Deletions of up to 2.9 kb have been made in E1, which is not required for viral replication in complementing 293 cells, allowing the construction of conditional helper independent vectors with a capacity of 4.7-4.9 kb. A variety of deletions have been made in E3, a region which is nonessential for viral replication in any normally permissive cells. The most commonly used deletion of 1.9 kb allows the construction of nonconditional helper independent vectors with a capacity of 3.7-3.9 kb.

Even with our extensive knowledge of Ads a more detailed understanding of vectorology is required before recombinants can be confidently used in humans for vaccine and gene therapy purposes. For example it was generally assumed that adenovirus vectors are relatively stable and can package DNA up to 105% of the wt genome length. To more thoroughly address this issue the genetic stability of Ad5 vectors with E3 substitutions representing net insertions of up to 8.3% of the Ad5 genome was analyzed after serial passages in 293 cells. This investigation revealed

that vector stability correlated with net genome size and vectors with the largest genomes rearrange extremely rapidly. This observation prompted the development of a new vector system (the BHG system) with larger deletions in E1 (3.2 kb) and E3 (2.7 or 3.2 kb) that can accommodate larger inserts. This system is the most versatile yet developed and allows the insertion of genes into E1 or E3 or both and mutations or deletions can be readily introduced elsewhere in the viral genome. The system provides a capacity of 8.3 kb.

Using the information gained in vector construction, and the new vectors systems that were developed, vectors were constructed that expressed various simian immunodeficiency virus (SIV) genes. There should be little doubt that the development of a safe, effective vaccine for human immunodeficiency virus (HIV) will provide the most secure and cost effective means of controlling HIV and AIDS. The infection of macaque monkeys with SIV provides one of the most relevant animal models for studying AIDS and for developing vaccines (Desrosiers and Ringler, 1989; Fultz, 1993). Information gained through the development and characterization of Ad/SIV vectors will have important implications in terms of developing a potential Ad/HIV vaccine.

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ABBREVIATIONS

aa	amino acid
Ab	antibody
Ad	adenovirus
ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
Ap^r	ampicillin resistance
araC	cytosine β-D-arabinofuranoside
bp	base pair
cpe	cytopathic effect
CR	conserved region
CRS	cis-acting repressor sequence
CTL	cytotoxic T lymphocytes
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
dNTP	dATP + dCTP + dGTP + dTTP
dTTP	deoxythymine triphospate
E1	early region 1
E2	early region 2
E3	early region 3
E4	early region 4
EGF	epidermal growth factor
g	grams
gp	glycoprotein
HBSAg	hepatitis B surface antigen
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
In	intranasal
Ip	intraperitoneal
IP	immunoprecipitation
ITR	inverted terminal repeat
K	kilodaltons
kb	kilobases
Kn^r	kanamycin resistance
LacZ	β-galactosidase
LTR	long terminal repeat
MHC	major histocompatibility complex

min	minutes
ml	millilitres
MLP	major late promoter
moi	multiplicity of infection
mu	map units
MW	molecular weight
nm	nanometres
Ori	origin of replication
pA	polyadenylation signal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	placque forming units
pro	promoter
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	seconds
SIV	simian immunodeficiency virus
TNF	tumour necrosis factor
TP	terminal protein
U	units
VSV	vesicular stomatitis virus
v/v	volume per volume
wt	wild type
w/v	weight per volume

INTRODUCTION

A. ADENOVIRUSES

1. General Background

Human adenoviruses (Ads) were first isolated over four decades ago by Rowe *et al.* (1953) as a transmissible agent responsible for the degeneration of primary cultures of tonsil and adenoidal tissues. Since that time they have proven to be very useful as a model system for studying such cellular processes as DNA replication, transcription, RNA processing and protein synthesis in mammalian cells (reviewed in Ginsberg, 1984). This work has led to an extensive understanding of the genomic organization and molecular biology of adenovirus and the realization that they have great potential for use as vectors to express foreign proteins in mammalian cells (Berkner, 1992; Graham and Prevec, 1991), as recombinant vaccines (Graham and Prevec, 1992), and more recently as gene transfer vectors for gene therapy (Siegfried, 1993; Trapnell, 1993).

The adenoviruses belong to the family Adenoviridae, which contains two genera, *Mastadenoviruses* which infect mammalian species and *Aviadenoviruses* which infect birds, as well as a minor group which infects poikilothermic animals. Over 100 distinct serotypes have been isolated, 47 of which are of human origin (Straus, 1984; and Hierholzer *et al.*, 1988). The human serotypes have been categorized into six subgenera (A-F) based on a number of biological, chemical, immunological and structural criteria which include hemaglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity (Table 1) (Straus, 1984; Horwitz, 1990a). The best characterized serotypes to date have been Ad5 and Ad2 (subgroup C), Ad7 (subgroup B) and Ad12 (subgroup

Table 1: Classification of Human Adenoviruses

Subgroup	Serotypes	Tumours in Animals	G+C	Hemagglutination
A	12, 18, 31	High	48-49%	IV (little agglutination)
B	3,7,1114, 16,21,34,35	Moderate	50-52%	I (complete)
C	1, 2, 5, 6	Low/none	57-59%	III (partial)
D	8-10,13,15,17, 19,20,22-30,32, 33,36-39,42-47	Low/none	57-61%	II (complete)
E	4	Low/none	57-59%	III (partial)
F	40, 41	Unknown	57-59%	III (partial)

(modified from Horwitz, 1990a)

A). Ad12 was the first serotype reported to have the potential to induce tumours in new born rodents (Trentin *et al.*, 1962; Huebner *et al.*, 1962) but Ads have never been linked to naturally occurring malignancies in any animal and surveys of human tumours have failed to find any virus specific sequences (Gilden *et al.*, 1970; McAllister *et al.*, 1972; Mackey *et al.*, 1976; Graham, 1984a).

The various Ad serotypes can infect and replicate at a number of locations in the body including the upper respirator tract, gastrointestinal tract, the eye and the urinary bladder (Straus, 1984; Horwitz, 1990b). Only about one third of the serotypes are associated with disease in humans while most infections are subclinical. Infection with adenovirus usually results in mild illness with symptoms which can include coryzal illness (rhinorrhea, nasal congestion, and sneezing) or pharyngoconjunctival fever (fever, sore throat, conjunctivitis) (Horwitz, 1990b). The pathogenesis and pathology of the various human Ad serotypes are reviewed in Horwitz 1990b.

2. Adenovirus Structure

Adenoviruses are nonenveloped, icosahedral viruses with diameters ranging from 65-80 nm depending on the serotype (Horne *et al.*, 1959; Horwitz, 1990a). The capsid is composed of at least 7 viral proteins which surround the linear double stranded DNA genome and at least two other viral proteins (Fig. 1) (Nermut, 1984; Horwitz, 1990a). The length of the genome (30-40 kb) and of the inverted terminal repeats (ITR's) found at its ends (100-200 bp) also depends on the serotype (For reviews see Ginsberg, 1984 and Horwitz, 1990a). The Ad5 genome, which was used for the construction of all vectors in this thesis, is 35935 bp in length with inverted terminal repeats of 103 bp (Chroboczek *et al.*, 1992). The viral genome also has a virus-encoded 55K terminal protein covalently linked to dCMP at each 5' end. The genome (Fig. 2) is divided into

Figure 1. Adenovirus virion.

A schematic illustration of the adenovirus is shown with each of the proteins in the virion designated. The configuration of the viral DNA is not representative of the actual structure in the virion. Reproduced from Horwitz, 1990a.

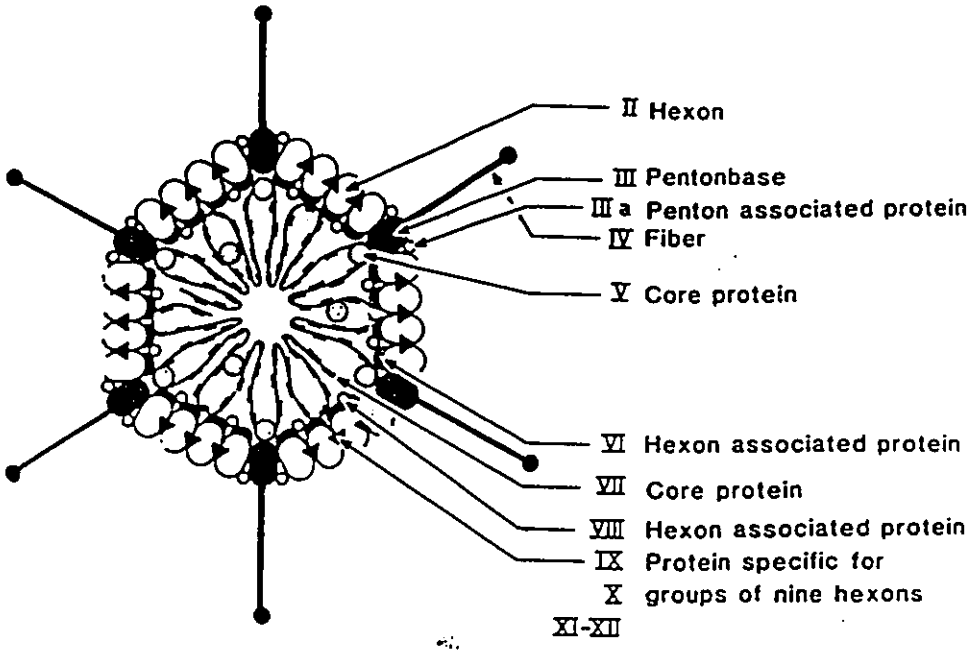
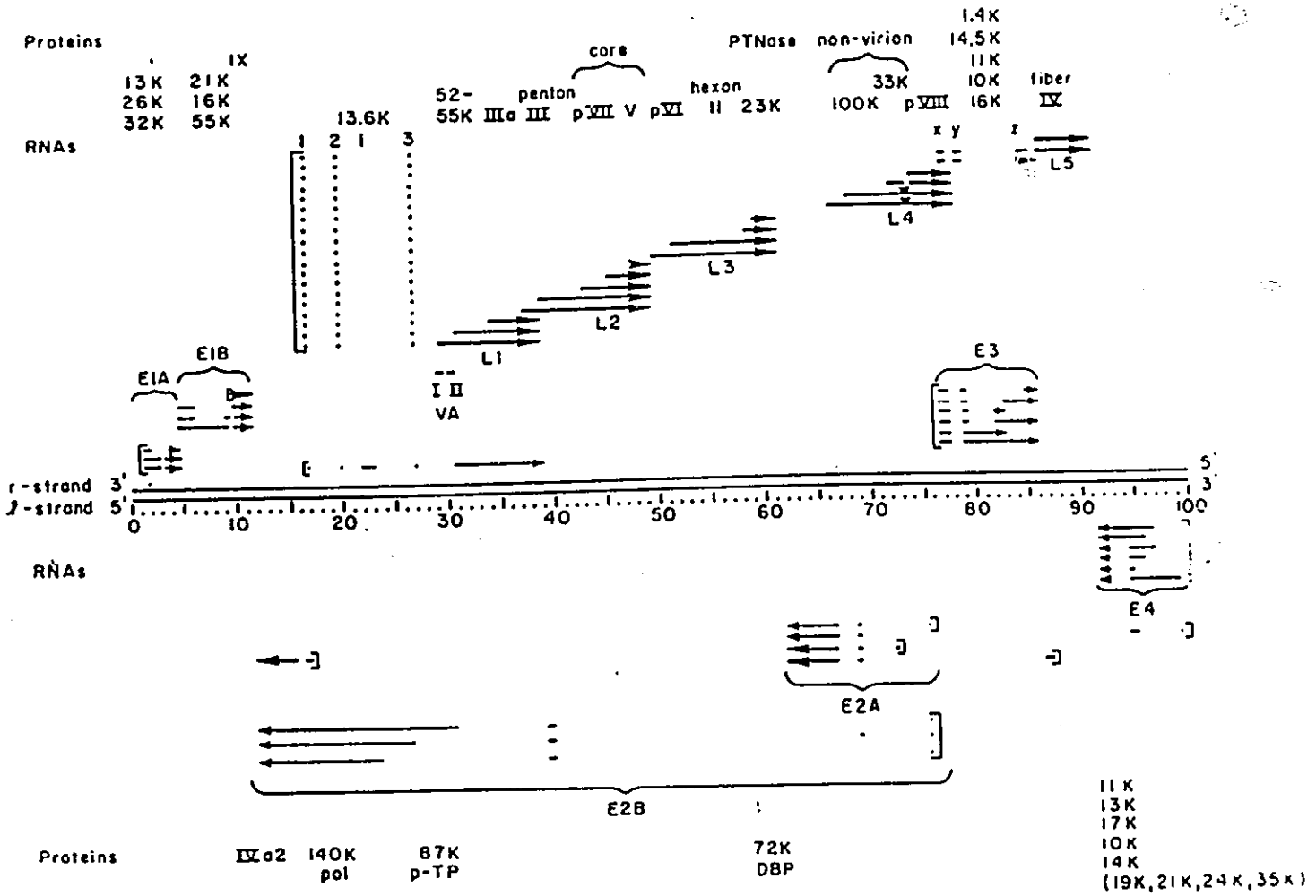

II Hexon
III Pentonbase
IIIa Penton associated protein
IV Fiber
V Core protein
VI Hexon associated protein
VII Core protein
VIII Hexon associated protein
IX Protein specific for
X groups of nine hexons
XI-XII

Figure 2. Adenovirus transcription map.

The linear genome is divided into 100 map units as well as into r- and l- strands which designate the direction of transcription. Early transcription units are designated with an E and are active prior to viral DNA replication. Late transcription units are designated with and L and are active primarily after DNA replication. Promoters are represented as brackets and polyadenylation sites as arrowheads. The tripartite leader is designated 1, 2, and 3. Virion structural proteins are represented as roman numerals and nonstructural viral proteins in kilodaltons (K). Reproduced from Horwitz, 1990a.


Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
RNAs
1 2 1 3
52- penton core hexon
55K IIIa III pVII V pVI II 23K
PTNase non-virion
33K
100K pVIII
1.4K
14.5K
11K
10K
16K
fiber
IX
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3' 5'
l-strand 5' 3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K pol
87K p-TP
72K DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

100 map units (mu) with the two strands designated l- or r- for left or rightward transcription in reference to the genes it encodes. The early region 1 (E1) A, E1B, pIX, the major late and the E3 transcription units are transcribed from the right strand of the genome while E2, IVa_2, and E4 are transcribed from the left strand (Ginsberg, 1984; Nevins, 1987).

3. Adenovirus Lytic Infection

Adenovirus infects cells through the attachment of fiber (Fig. 1) to a specific receptor on the cell surface (there are approximately 10^4 virus receptors per Hela cell) (Londberg-Holm and Philipson, 1969) and the virus/receptor complex is internalized through clathrin coated pits (Horwitz, 1990a). The acidic pH of the endosome (pH 5.5) is thought to alter the surface structure of the virion resulting in the rupture of the endosome and release of the virion into the cytoplasm. The virion is then transported by microtubules to the nucleus where transcription and replication of the viral genome occurs (Philipson *et al.*, 1968; Dales and Chardonnet, 1973; Sharp, 1984). After polyribosome translation in the cytoplasm, polypeptides are transported into the nucleus for virion assembly (Velicer and Ginsberg, 1970). In a productive lytic infection 1000 to 10000 virus particles can be produced per cell.

The life cycle of the virus is divided into two phases, early and late, corresponding to events before or after the initiation of DNA replication (reviewed in Horwitz, 1990a). In the early phase, six separate promoters are active, producing transcripts from early region 1 (E1), using promoters in subregions E1a and E1b, E2 (early), E3, E4 and the major late transcription unit (early), (Fig. 2). After the beginning of viral DNA replication, approximately 6 to 9 h post infection, the major late promoter (MLP) is responsible for the majority of viral transcription but transcripts are also produced from transcription units IX, IVa2, E2 (late), and virus-associated

(VA) RNA's I and II (Sharp, 1984). All viral messages are transcribed by RNA polymerase II except for VA RNA's I and II which are transcribed by RNA polymerase III (Sharp, 1984). In general multiple transcripts are produced from the various transcription units due to alternate splicing and polyadenylation site usage (Horwitz, 1990a; Sharp, 1984).

Replication of the adenovirus genome can initiate at either end of the genome and is primed by a protein complex composed of the precursor terminal protein (pTP) which becomes covalently linked to a dCMP molecule in a reaction catalyzed by the viral DNA polymerase and cellular DNA binding proteins (Lichy *et al.*, 1982). The daughter strand is then generated by strand displacement, involving the viral polymerase and DNA binding proteins, resulting in a duplex (daughter/parent) and single (parental) stranded DNA product. The single stranded parental molecule can then act as a substrate for replication, possibly through the annealing of its ITRs to form a panhandle structure (Daniell, 1976; Lippe and Graham, 1989). The pTP remains attached to the 5' ends of the newly replicated DNA and is cleaved to its mature form (TP) by the viral encoded protease during encapsidation. For a more complete description of adenovirus DNA replication see Tamanoi and Stillman (1983), Futterer and Winnacker (1984), and Kelly (1984).

Adenoviruses have a restricted host range and do not replicate to the same extent in all cells. Human adenoviruses grow well in most human epithelial cells and some human fibroblast lines which are said to be permissive for replication, but can exhibit poor (semipermissive) or no (nonpermissive) replication in other cell types such as human peripheral blood lymphocytes (Horvath and Weber, 1988), African green monkey (Klessig, 1984), rhesus macaque, mouse and canine cells (Graham and Prevec, 1992). The block to productive infection differs for the different cell types. In human peripheral lymphocytes it seems to be an inability to adhere to the cells (Horvath and Weber, 1988; Silver and Anderson, 1988) while in African green monkey

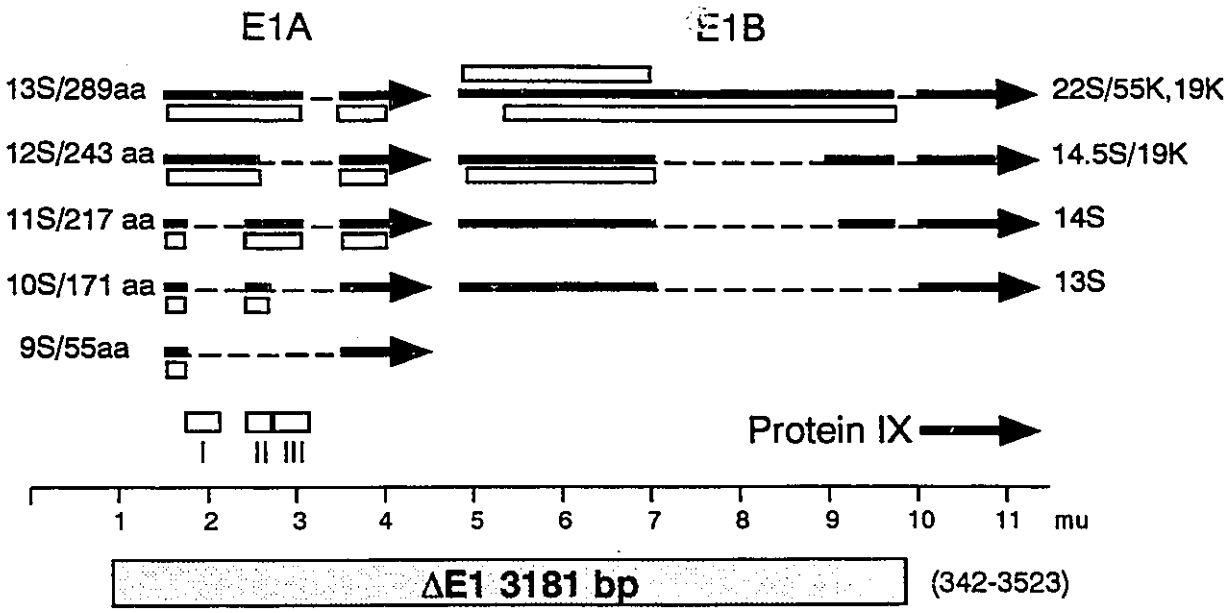
(Klessing, 1984; Horvath and Weber, 1988; Silverman and Klessig, 1989) and canine cells (Prevec *et al.*, 1989; Martins, 1993) it appears to be an inability to produce late proteins. In mouse cells the entire growth cycle is affected resulting in reduced early protein expression, DNA replication and late protein synthesis (Blair *et al.*, 1989; Silverstein and Strohl, 1986). Although adenovirus cannot undergo fully permissive replication in all cell types it is still able to infect a wide variety of cells both dividing and quiescent (Graham and Prevec, 1992).

4. Adenovirus Transcription Units

Early region 1 (1.3-11.2 mu) contains three transcription units: E1A (1.3-4.5 mu), E1B (4.6 to 11.2 mu) and the protein IX transcription unit (9.8-11.2 mu) (Fig.2 and 3). The E1a gene products are the first to be synthesized after infection and quickly target to the nucleus. Five proteins are produced in this region designated according to the mRNA's encoding them: 13S (289 aa), 12S (243 aa), 11S (217 aa), 10S (171 aa) and 9S (55 aa). Three regions of the E1A proteins are well conserved between different Ad serotypes and are designated conserved region 1 (CR1), CR2 and CR3 (Fig. 3) (van Ormondt *et al.*, 1980). These regions have been associated with specific cellular functions (for a review see Branton *et al.*, 1985; Berk, 1986). Of the five E1A proteins the 289 aa and 243 aa proteins are the best characterized and differ only in the presence of CR3 which is unique in the 289 aa protein. The 289 aa E1a protein is responsible for the transactivation of the other early transcription units (Jones and Shenk, 1979; Ricciardi *et al.*, 1981; Berk *et al.*, 1979) but both the 289 and 243 aa proteins can transactivate cellular promoters and transrepress both viral and cellular promoters (reviewed in Nevins 1987; Grand, 1987; Berk, 1986). E1A from all serotypes can immortalize primary cells in culture (Houweling *et al.*, 1980) and in cooperation with E1B proteins or with activated oncogenes (such as *ras*) can fully

Figure 3. Early region 1 of Ad5.

The structure of the E1 region (1.3-11.2 mu) in wt Ad5 is shown. E1A is located from 1.3-4.5 mu, E1B from 4.6-11.2 mu and protein IX from 9.8-11.2 mu. Horizontal arrows indicate the structures of the mRNA's made in this region, with the solid lines representing exons, dashed lines representing introns and arrowheads representing polyadenylation sites. Shaded bars above the arrows represent protein coding sequences. Conserved region 1 (CR1), CR2 and CR3 are indicated for E1A. The sequences removed by the 3.2 kb E1 deletion (Bett *et al.*, 1994) are indicated. This deletion dose not interfere with the ITR (1-103 bp), the packaging signal (194-358 bp) or the coding sequences for protein IX.


E1A
E1B
13S/289aa
12S/243 aa
11S/217 aa
10S/171 aa
9S/55aa
22S/55K,19K
14.5S/19K
14S
13S
Protein IX
I
II III
1
2
3
4
5
6
7
8
9
10
11
mu
ΔE1 3181 bp
(342-3523)

transform cells (Ruley, 1983; Graham, 1984a; Zerler *et al.*, 1986). For an excellent review of cellular transformation see Bayley and Mymryk (1995).

The E1B region produces two proteins designated 19K and 55K that have a number of other functions in addition to effecting full transformation (Jones and Shenk, 1979; Graham 1984a; Barker and Berk, 1987) such as the cytoplasmic accumulation of late viral mRNA (Babiss *et al.*, 1985), the protection of viral and cellular DNA sequences from nucleolytic degradation (Pilder *et al.*, 1984; Lai Fatt and Mak, 1982; Subramanian *et al.*, 1984; White *et al.*, 1984) and the shutoff of host message transport and translation (Babiss *et al.*, 1985; Babiss and Ginsberg, 1984). The E1B-19K protein may also protect human cells from lysis by tumour necrosis factor (see E3 transcription unit described below) (Gooding *et al.*, 1991a)

The protein IX transcription unit produces a single unspliced transcript encoding a minor capsid protein. Protein IX is not essential for virion particle assembly (Colby and Shenk, 1981, Ghosh-Choudhury *et al.*, 1987), but viruses lacking this protein are thermolabile and are deficient for packaging full length viral genomes (Ghosh-Choudhury *et al.*, 1987).

The E2 region (E2A from 67.0-61.5 mu and E2B from 29-14.2 mu on the l-strand) contains two promoters, one at 76 mu which functions early and one at 72 mu which functions after the initiation of DNA replication (Chow *et al.*, 1979; Horwitz, 1990a). E2A encodes a 72K DNA binding protein, and E2B encodes the 140K viral DNA polymerase and a 80K preterminal protein (pTP) all of which are required for viral DNA replication (Kelly, 1984). As well as being involved in viral DNA replication the 72K protein has been found to repress E4 transcription (Nevins and Winkler, 1980; Klessig and Quinlan, 1982). The 80K preterminal protein is the precursor to the 55K protein covalently associated with the ends of the Ad DNA molecule.

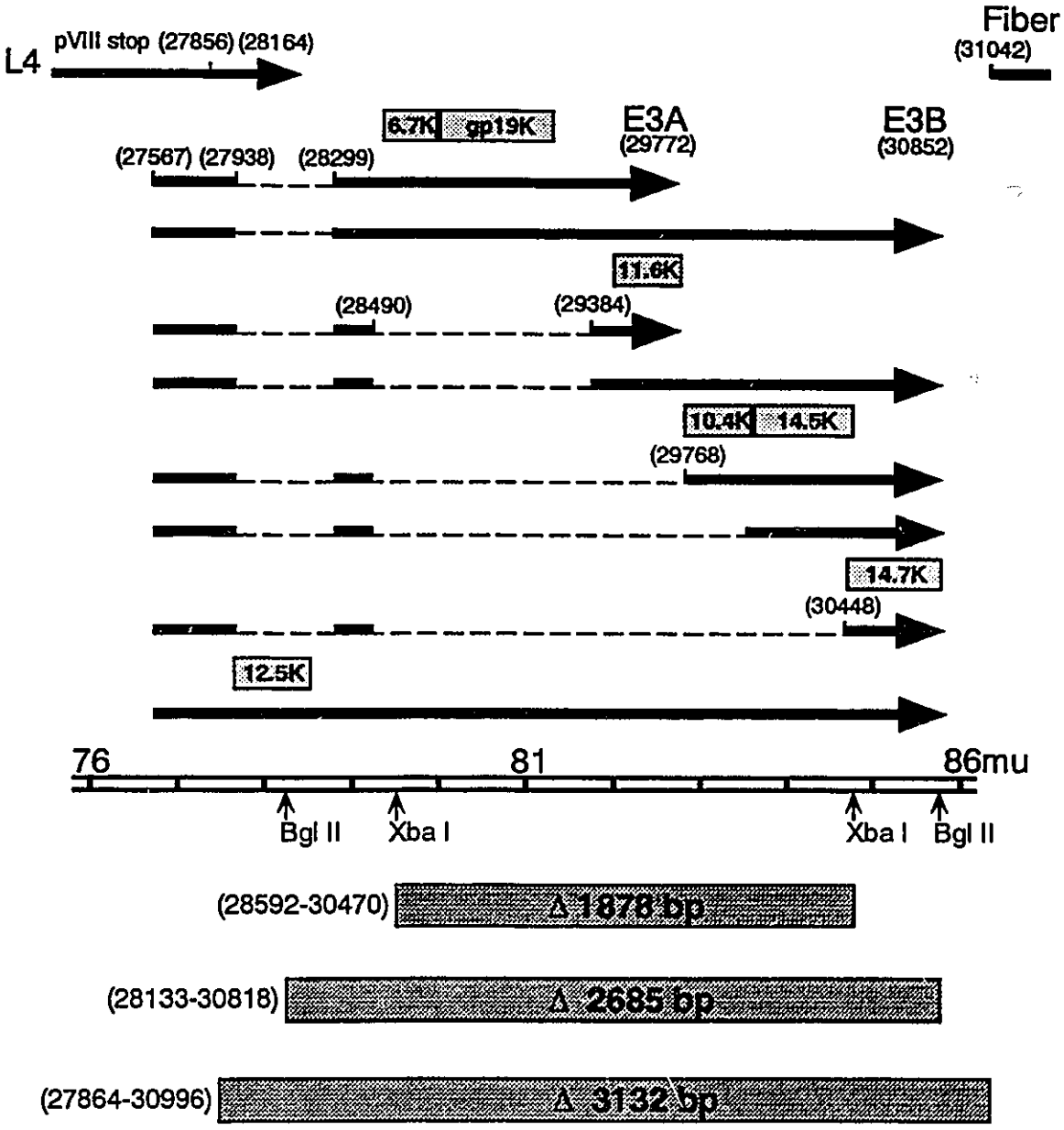
The E3 region (76.0 to 86.0 mu on the r-strand) is also divided into two subregions, E3a

and E3b, based on polyadenylation site usage (Fig. 4). The E3 promoter is active early in infection but at late times the E3 region can be transcribed using the MLP (Chow *et al.*, 1977, 1979; Chow and Broker, 1978). Sequencing data suggest that 9 proteins may be encoded in this region (Chow *et al.*, 1979; Cladaras and Wold, 1985). To date seven proteins have been identified: gp19K (Persson *et al.*, 1980a, 1980b), 14.7K (Tollefson and Wold, 1988; Wang *et al.*, 1988), 14.5K (Tollefson *et al.*, 1990a), 12.5K (Hawkins and Wold, 1992), 11.6K (Wold *et al.*, 1984), 10.4K (Tollefson *et al.*, 1990b), and 6.7K (Wilson-Rawls *et al.*, 1990) (Fig. 4). The proteins of the E3 region appear to be involved in the evasion of the host immune system (reviewed in Wold and Gooding, 1991; Mullbacher, 1992). Although the E3 region is not required for viral replication in tissue culture (Anderson *et al.*, 1976; Berkner and Sharp, 1983) its maintenance in natural isolates indicates its importance in natural infections. gp19K is a transmembrane glycoprotein found in the endoplasmic reticulum in infected cells which appears to protect infected cell from lysis by class I-restricted cytotoxic T cells. gp19K noncovalently associates with HLA/H-2 class I heavy chain molecules in cells transformed or infected with subgenera B, C, D, and E adenoviruses (Kvist *et al.*, 1978; Signas *et al.*, 1982; Paabo *et al.*, 1986a, 1986b). This association blocks the transport of class I antigens to the cell surface (Andersson *et al.*, 1985; Burgert and Kvist, 1985; Cox *et al.*, 1991; Lippe *et al.*, 1991) decreasing the efficiency of viral antigen presentation and recognition by cytotoxic T cells. Gp19K has been shown to have different affinities for different class I antigens (Burgert and Kvist, 1987; Tanaka and Tevethia, 1988; Rawle *et al.*, 1989; Cox *et al.*, 1990) suggesting that susceptibility to Ad infection in humans may depend on MHC haplotype. Adenoviruses of the A subgenus prevent MHC class I expression by reducing class I mRNA levels (Paabo *et al.*, 1986a, 1986b, 1989).

Another method by which the host may destroy virus infected cells involves the cytokine

Figure 4. Early region 3 of Ad5.

The structure of the E3 region (76.6-86.0 mu) in wt Ad5 is shown. The horizontal arrows indicate the structures of the E3 mRNA's with solid lines representing exons, dashed lines representing introns and arrowheads representing polyadenylation sites. Shaded bars above the arrows represent protein coding sequences. The locations of transcription initiation sites, splice donor and acceptor sites and polyadenylation sites are given in bp. Ad5 sequence positions refer to the sequence of wt Ad5 (Chroboczek *et al.*, 1992). To designate Ad5 E3 proteins Ad2 molecular weights were used. The sequences removed by the 1.88 kb (Berkner and Sharp, 1983; Haj-Ahmad and Graham, 1986), 2.7 kb (Bett *et al.*, 1993) and the 3.1 kb (Bett *et al.*, 1994) E3 deletions are indicated.


L4
pVIII stop (27856) (28164)
Fiber
(31042)
6.7K
gp19K
E3A
(29772)
E3B
(30852)
(27567) (27938)
(28299)
11.6K
(28490)
(29384)
10.4K
14.5K
(29768)
14.7K
(30448)
12.5K
76
81
86mu
Bgl II
Xba I
Xba I
Bgl II
(28592-30470)
Δ 1878 bp
(28133-30818)
Δ 2685 bp
(27864-30996)
Δ 3132 bp

tumour necrosis factor (TNF). TNF is produced by activated macrophages and lymphocytes and modulates many aspects of the immune response (Beutler and Cerami, 1989). TNF has been shown to be cytotoxic to certain tumour cells and inhibit the replication of a number of DNA and RNA viruses (Gooding *et al.*, 1991; Schutze *et al.*, 1992). How TNF mediates cell lysis is not well understood but the process involves binding of TNF to its cellular receptor, internalization of the receptor/ligand complex, intracellular signalling and finally lysis of the cell. Mouse cells infected with an Ad E3- mutant are lysed by TNF while uninfected cells and wt Ad5 infected cells were not (Gooding *et al.*, 1988). Using double deletion mutants (an E3 deletion plus other regions) it was shown that the E1A-289 aa or E1A-243 aa proteins induced the TNF sensitivity (Duerksen-Hughes *et al.*, 1989) and that the E3 14.7K protein protects most mouse cell lines against TNF cytolysis (Gooding *et al.*, 1988, 1990, 1991b; Horton *et al.*, 1990). 14.7K is the only adenovirus protein that can protect mouse C3HA cells from cytolysis by TNF but in many other mouse cell lines a complex of 10.4K/14.5K can also protect against cytolysis by TNF (Gooding *et al.*, 1991b). In human cells it appears that E1B-19K as well as 14.7 and 10.4K/14.5K prevents lysis of infected cells by TNF (Gooding *et al.*, 1991a). At this time the mechanism of action of these TNF-protecting proteins in unknown.

Epidermal growth factor receptor (EGF-R) is a plasma membrane receptor with tyrosine kinase activity. Binding of EGF to its receptor results in autophosphorylation of the receptor as well as phosphorylation of a number of cellular proteins (Ullrich and Schlessinger, 1990) resulting in cellular DNA synthesis and mitosis. Binding of EGF to its receptor also results in the internalization and degradation of the receptor/ligand complex. Ad infection can mimic the response to EGF by inducing the internalization and degradation of the EGF receptor (Carlin *et al.*, 1989; Tollefson *et al.*, 1991; Hoffman *et al.*, 1992) and it has been shown that the 10.4K and

14.5K proteins are responsible (Carlin *et al.*, 1989; Tollefson *et al.*, 1991). The internalization of the EGF-R is dose-dependent indicative of a fairly direct interaction (Hoffman *et al.*, 1992) but the exact mechanism is not yet understood. The E3 10.4K and or 14.5K proteins may also be responsible for the downregulation of E1a and late protein expression seen in various infected cell lines of mouse and human origin (Zhang *et al.*, 1991; 1992; 1994). Mouse MHC-restricted CTL response are primarily directed against E1A proteins with a minor role by E2A and late virion proteins (Mullbacher *et al.*, 1989). The down regulation of E1A expression, which appears to occur at the translational level (Zhang *et al.*, 1994), has been shown to result in reduced lysis of infected cells by Ad specific CTL's (Mullbacher *et al.*, 1989; Zhang *et al.*, 1991, 1994). Functions have not yet been found for the E3-12.5, 11.6 and 6.7 K proteins.

The E4 region (96.8 and 91.3 mu on the l-strand) contains seven open reading frames (Vitanen *et al.*, 1984; Sharp *et al.*, 1984) but only three E4 proteins have been identified: 14K (Sarnow *et al.*, 1982; Downey *et al.*, 1983), 34K (Sarnow *et al.*, 1984), and 19.4 K (Cutt *et al.*, 1987). The 34K product associates with E1B 55K and appears to play a role in the early to late switch in gene expression and the down regulation of host gene expression (Sarnow *et al.*., 1984; Hemstrom *et al.*, 1988; Yoder and Berget, 1986; Bridge and Ketner, 1990). 19.4K along with E1A proteins transactivate E2 transcription by binding and activating the E2 promoter-specific cellular transcription factor E2F (Hemstrom *et al.*, 1991). Deletions in E4 have complex phenotypes showing effects on viral DNA replication, the shutoff of host cell protein synthesis (Halbert *et al.*, 1985), the transition of viral gene expression from the early to the late phase (Yoder and Berget, 1986) and virion assembly (Falgout and Ketner, 1987).

The MLP, located at 16.5 mu on the r-strand, initiates transcripts both early and late (Sharp, 1984; Horwitz, 1990a). During early times the transcription levels are low and transcripts

terminate near mu 39 (L1 transcripts), while at late times transcription increases 1000X , likely due to higher genome copy number (Shaw and Ziff, 1980) and transcripts run the length of the genome, terminating near mu 99 (Sharp, 1984). These primary transcripts are then spliced to form five different families of messages, L1 to L5. Each family has its own polyadenylation site and individual mRNA's are spliced at their 5' ends to the tripartite leader which is positioned upstream from the translation initiation site for each protein (Berget *et al.*, 1977; Chow *et al.*, 1977). The tripartite leader, named for the three leader elements at 17, 20 and 27 mu which are spliced together to construct it, is thought to be responsible for efficient translation of Ad messages at late times (Logan and Shenk, 1984; Berkner and Sharp, 1985). The protein products produced from these late messages comprise most of the proteins associated with the viral capsid and some nonstructural proteins such as the viral protease, 100K protein and a 33K phosphoprotein (Pettersson, 1984).

Protein IVa2 (16.1 to 11 mu on the l-strand) is produced late in infection and is a scaffold protein involved in the morphogenesis of virions (Sussenbach, 1984). Two small RNA's (VA RNAs I and II) are synthesized from the r-strand at 29 mu by RNA pol III (Celma *et al.*, 1977a,b). The VA RNAs I and II have been shown to be required for efficient translation of viral messages (Thimmappaya *et al.*, 1982).

5. Regions For Insertion Of Foreign DNA Sequences

The construction of adenovirus recombinants involves the introduction of foreign DNA sequences into the adenovirus genome. The adenovirus virion can package up to 105% of the wild type genome length (Bett *et al.*, 1993) which allows for the insertion of approximately 1.8 to 2.0 kb of excess DNA. Three regions of the adenovirus genome have been used to accept

insertions of foreign genes, the E1 region, the E3 region and a region between E4 and the right ITR (reviewed in Graham and Prevec, 1991, 1992). In order to insert larger fragments compensating deletions must be made in the adenovirus genome. The two regions most commonly deleted to accommodate larger inserts are E1 and E3.

Deletion of the E1 region produces conditional helper independent viruses that must be grown in complementing 293 cells which contain and express the left end of the adenovirus genome (Graham *et al.*, 1977). For proper viral viability and maximum packaging capacity deletions in this region must not affect the ITR (1-103 bp), packaging signals (194-300 bp) (Hearing *et al.*, 1987; Grable and Hearing, 1990; 1992) or protein IX (Ghosh-Choudhury *et al.*, 1987). Deletions of up to 3.2 kb can be made in this region without affecting viral growth on 293 cells (this thesis) allowing for insertion of from 5.0 to 5.2 kb, (Fig. 3). Foreign genes inserted in the E1 deletion must be driven by a promoter introduced as part of the insert and typically the Ad2 MLP (Berkner and Sharp, 1984; Davis *et al.*, 1985; Massie *et al.*, 1986; Alkhatib and Briedis, 1988; Alkhatib *et al.*, 1988; Eloit *et al.*, 1990; Lamarche *et al.*, 1990; Levrero *et al.*, 1991) or the human cytomegalovirus immediate early promoter have been used (Jacobs *et al.*, 1992; Wilkinson and Akrigg, 1992; Fooks *et al.* 1995) although other promoters such as the E1 promoter (Ballay *et al.*, 1985; Karlsson, *et al.*, 1986), the herpes simplex virus ICP4 promoter (Spessot *et al.*, 1989) and the RSV LTR have been utilized. The level of expression obtained from inserts in the E1 region can be dependent on the orientation of the insert. Generally speaking, higher levels of expression (5 to 7X) are obtained when inserts are oriented so that transcription is E1 parallel (ie. directed rightward) (Hitt *et al.*, 1995). However some inserts in the rightward orientation have been found to produce aberrantly spliced messages likely involving down stream Ad sequences (Berkner and Sharp, 1984; Davidson and Hassel, 1987). The level of expression is also found to

vary with the inserted gene.

The E3 region is not required for viral replication in cultured cells (Klessig, 1984; Anderson *et al.*, 1976; Berkner and Sharp, 1983) and can be deleted to generate non-conditional helper independent viruses that can replicate in any normally permissive cells (Berkner and Sharp, 1983). The size of deletions in this region is limited by the late gene families (L4 and L5) flanking E3. The most frequently used E3 deletion for vector construction is one of 1.88 kb between the *Xba*I sites at 79.6 and 84.8 mu in Ad5 (Fig. 4) (Berkner and Sharp, 1983; Haj-Ahmad and Graham, 1986) although other E3 deletions have also been utilized including a 1.65 kb deletion from 79.4 to 84.0 mu (Dewar *et al.*, 1989), a 2.29 kb deletion from 78.5-84.3 mu (Shenk and Williams, 1984; Thimmappaya *et al.*, 1982), a 2.48 kb deletion from 78.8 to 85.7 mu (Cheng *et al.*, 1992), and a 3.0 kb deletion from 77.7 to 86.1 mu (Ranheim *et al.*, 1993). Two E3 deletions were created in the course of this thesis work: one of 2.7 kb from 78.3 to 85.8 mu, and one of 3.1 kb from 77.5 to 86.2 mu. In many vectors the SV40 promoter has been introduced as part of the expression cassette in this region but the thymidine kinase promoter has also been used (Haj-Ahmad and Graham, 1986; Schneider *et al.*, 1989). Heterologous promoters are not required in replication competent vectors with inserts in the E3 parallel orientation since in many cases transcripts appear to originate from the E3 promoter or the MLP. In at least one case the SV40 promoter has been shown to provide a cryptic 3' splice acceptor site for transcripts initiating from either the E3 or MLP (Johnson *et al.*, 1988). An analysis of regulatory sequences required to obtain high level expression from inserts in replication competent vectors was performed as part of this thesis work using β-galactosidase and firefly luciferase as reporter genes (Mittal *et al.*, 1994). The results of this study are presented in results section C.

6. Construction Of Recombinant Ad5 Vectors

A number of strategies have been developed to construct Ad vectors which all involve the manipulation of subgenomic fragments of the Ad genome (reviewed in Berkner, 1988, 1992; Graham and Prevec, 1991, 1995). The first Ad vectors were constructed by ligation of viral DNA or by recombination between viral DNA and subgenomic viral sequences contained in bacterial plasmids (Berkner and Sharp, 1983; Haj-Ahmad and Graham, 1986; Stow, 1981). Although these strategies are often satisfactory they have several drawbacks which include the time and technical difficultly involved in production of viral DNA, the background of infectious parental virus which makes screening more labour intensive and, in the case of direct ligation the limited availability of useful restriction sites due to the relatively large size of the adenovirus genome. A more useful and versatile strategy has proven to be recombination between two plasmids which together contain sequences comprising the entire Ad genome but which are noninfectious separately. A number of such plasmid systems have been developed for rescuing inserts into E1 (McGrory *et al.*, 1988) or E3 (Ghosh-Choudhury *et al.*, 1986; Mittal *et al.*, 1993) making the construction of vectors simpler and reducing the number of subsequent analyses required to identify recombinant viruses. The steps involved in rescuing foreign inserts into Ad are basically the same for all of the above mentioned systems. Briefly, the gene of interest plus appropriate regulatory sequences is first introduced into a shuttle plasmid containing a subsegment of the viral genome. This shuttle plasmid will contain either right or left end viral sequences with appropriate deletions and cloning sites into which the foreign gene can be inserted. The next step involves the cotransfection of the recombinant plasmid into mammalian cells, usually 293 cells, with overlapping viral DNA sequences that can reconstitute an infectious viral genome. The viral DNA can be either restricted viral DNA as mentioned previously or viral DNA contained in a second

plasmid (Graham and Prevec, 1990, 1995). The recombinant virus is then generated through *in vivo* recombination between the cotransfected plasmids or plasmid and viral DNA in the recipient cells (Fig. 5). As mentioned above direct ligation prior to cotransfection can also be used. The various systems available for rescuing inserts into virus have recently been reviewed by Graham and Prevec (1995) and Hitt *et al.* (1995).

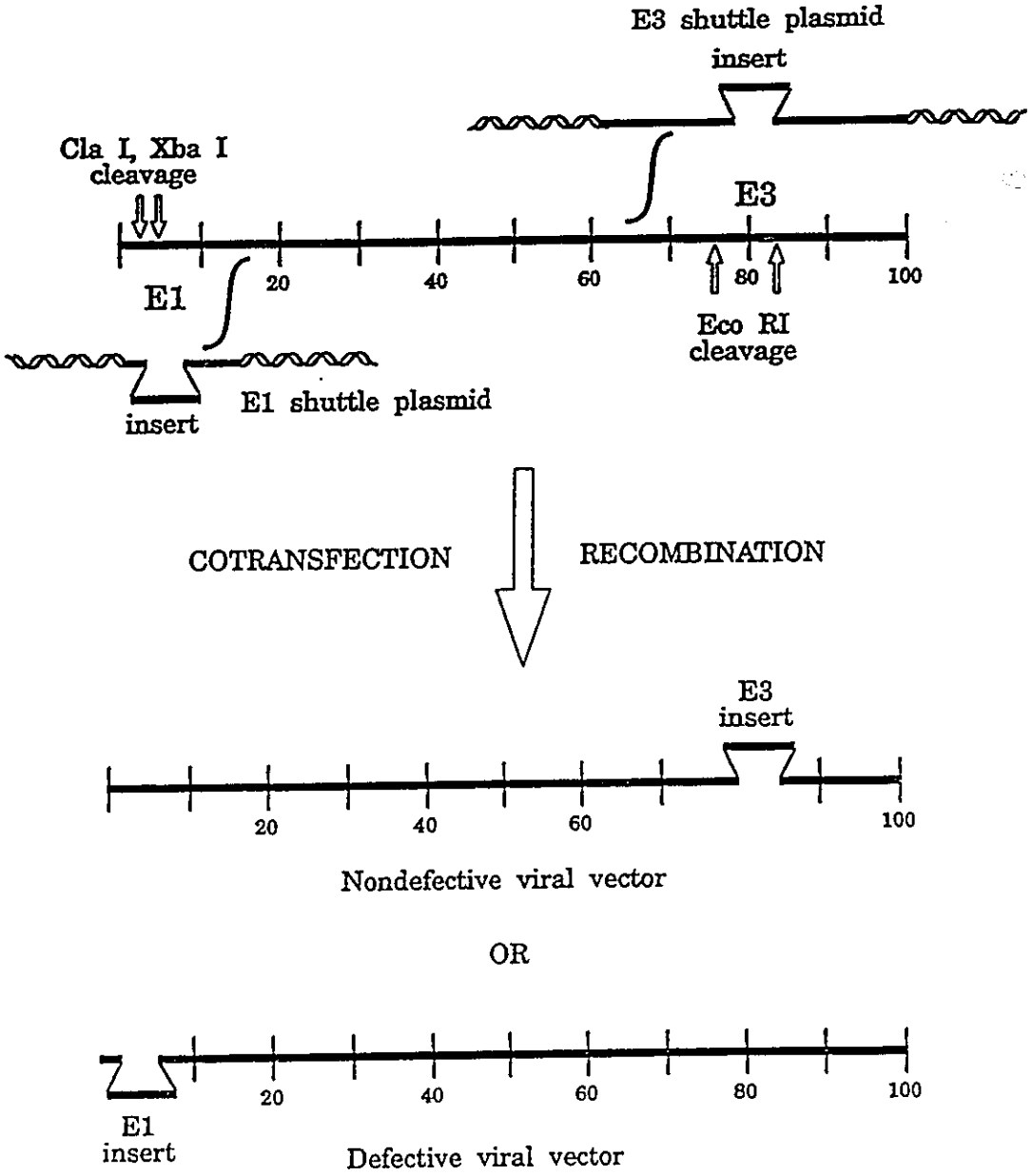
Among these methods for rescuing inserts into virus there was no simple procedure for generating vectors that utilize both E1 and E3 deletions. As part of this thesis work a new vector system was designed and developed (the pBHG system) which allows the construction of Ad5 vectors that combine E1 and E3 deletions or substitutions. This system is described in results section B.

7. Adenovirus As A Vaccine Vector

In addition to the extensive understanding of the structure and biology of adenoviruses there are other features of the system that make Ad vectors an excellent system for expression of foreign genes and especially for use as recombinant viral vaccines. As described above the rescue of inserts into recombinant adenovirus requires only a few relatively simple recombinant manipulations, and inserts of up to 8.0 kb can now be rescued, a size which should accommodate most genes along with regulatory sequences. Also high level expression can be obtained from inserts in both proliferating and quiescent cells and the virus infects cells from a variety of animals including human and other primates, canine, bovine and rodent. During permissive infection from 1000 to 10000 plaque forming units are produced per cell and the virus remains cell associated allowing easy harvest and concentration so that high titered stocks are readily obtained. Ad recombinants also appear to be stable, with no sequence loss or rearrangement

Figure 5. Strategy for rescue of foreign DNA into the Ad5 genome.

Rescue by in vivo recombination is shown schematically for inserts in E1 (below genome map) or E3 (above map). A recombinant E1 or E3 shuttle plasmid (a plasmid containing either left end or right end Ad 5 sequence respectively with the desired deletion and cloning sites into which the foreign gene has been inserted) is cotransfected into 293 cells with appropriate viral DNA (viral DNA can be either restricted viral DNA or viral DNA contained in a second plasmid). The recombinant virus is generated by in vivo recombination between the cotransfected plasmids or plasmid and viral DNA in the recipient cells. Ad5 sequences are indicated by hatched lines, inserts by solid lines, and plasmid sequences linked to viral DNA in E1 and E3 shuttle plasmids, by jagged lines.


E3 shuttle plasmid
insert
Cla I, Xba I
cleavage
E3
E1
20
40
60
80
100
Eco RI
cleavage
E1 shuttle plasmid
insert
COTRANSFECTION
RECOMBINATION
E3
insert
20
40
60
100
Nondefective viral vector
OR
20
40
60
80
100
E1
insert
Defective viral vector

during successive rounds of replication, as long as insert size does not exceed the capacity of the system (Bett *et al.*, 1993) and the vectors are properly propagated. It should be pointed out however that recombination of replication defective vectors, with deletions or insertions in E1, with the E1 sequences in 293 cells can result in the generation of replication competant virus. This problem can be minimized by proper propagation of viral stocks. Many foreign genes have already been successfully expressed in helper dependent and independent vectors (reviewed in Berkner, 1988 and Graham and Prevec 1991 and 1992) and shown to have the correct post-translational processing and to be biologically active (Ballay *et al.*, 1985; Davis *et al.*, 1985; Schaffhausen *et al.*, 1987; Hutchinson *et al.*, 1992, 1993; Hanke *et al.*, 1990). Most importantly, information on the use of adenoviruses as vaccines has already been obtained from the use of unattenuated types 4 and 7 to prevent epidemics of Acute Respiratory Disease in military recruits (Top *et al.*, 1971a, 1971b; Top, 1975; Chaloner-Larsson *et al.*, 1986). When Ad4 and Ad7 were administered in enterically-coated capsules 70-82% of the vaccinees given Ad4 capsules developed neutralizing antibodies Chanock *et al.*, 1966) while individuals given a placebo were susceptible to infection (Edmondson *et al.*, 1966).

Adenovirus vectors have also proven effective in inducing both humoral and cell mediated immune responses to their expressed antigens in vaccinated animals and in many cases to protect the animals from lethal challenge. Recombinants expressing inserts of herpes simplex virus type 1 (HSV-1) glycoprotein B (gB), vesicular stomatitis virus (VSV) or rabies glycoprotein in the E3 region have been shown to raise neutralizing antibodies in rhesus monkeys, cows, pigs, dogs, foxes, striped skunks, raccoons and mice (Graham and Prevec, 1992). Intranasal instillation of hamsters with an Ad5 vector containing hepatitis B virus surface antigen (HBSAg) in E3 was shown to induce a good antibody response against HBSAg (Morin *et al.*, 1987). Vectors

expressing VSV or rabies glycoprotein raised high levels of virus-neutralizing antibody in intraperitoneally immunized mice and induced protection from challenge with VSV given intravenously (Prevec *et al.*, 1989) or rabies virus intracerebrally (Prevec *et al.*, 1990). Two of four rhesus macaque monkeys given two subcutaneous injections of a vector containing HIV-1 p24 in the E3 region were shown to develop measurable levels of serum anti-p24 antibodies (Prevec *et al.*, 1991). Ad5 recombinants expressing HSV-1 gB as an E3 insert, administered to mice intraperitoneally (Ip) were able to protect the animals from a lethal challenge with HSV-2 via foot pad (McDermott *et al.*, 1989). Ad vectors expressing truncated forms of gB were also used to show that gB contains a major epitope recognize by H-2^b-restricted anti-HSV cytotoxic T lymphocytes (CTL) (Witmer *et al.*, 1990; Hanke *et al.*, 1991). A similar vector expressing HSV-1 gB was used to show that intranasal (IN) immunization of mice elicited good levels of serum anti-HSV gB IgG antibodies, secretory IgA antibodies as well as cell mediated immunity which protected the mice from challenge with HSV-2 given intranasally (Gallichan *et al.*, 1993). Recombinant Ad4, 5 and 7 vectors expressing either HIV envelope glycoprotein (gp120) or gag genes in E3 were found to be immunogenic in chimpanzees immunized by the oral and intranasal routes, inducing low titer neutralizing antibodies, secretory IgA antibodies and cell-mediated immune responses (Lubeck *et al.*, 1994; Natuk *et al.*, 1993). An Ad5 recombinant containing the gene for Tick-borne encephalitis virus (TBEV) nonstructural protein (NS1) in E1 under the control of the HCMV promoter was able to induce a good antibody response to the protein in mice and protect mice from challenge with TBEV (Jacobs *et al.*, 1992). A similar E1 replacement vector containing measles virus nucleocapsid protein driven by the HCMV promoter was shown to induce a good humeral, MHC class I-restricted antigen-specific cytotoxic T cell response and protection against challenge with measles virus in Ip immunized mice (Fooks *et al.*, 1995).

The success obtained in expressing foreign genes in recombinant adenoviruses, the extensive use and documented safety of Ad4 and Ad7 as vaccines, the numerous examples of efficient elicitation of both humoral and cell mediated immune responses and particularly the demonstration of the development of mucosal immunity (Gallichan et al., 1993) by adenovirus vectors indicates that this system may have potential application in the production of a safe, effective, recombinant adenovirus vaccine for protection against HIV infection. Due to the lack of suitable animal models for studying and testing potential HIV vaccines we have chosen to generate vectors expressing potentially immunogenic segments of SIV gene products. These vectors can then be assessed for their ability to elicit immune responses in macaque monkeys and mice. The infection of macaques with SIV provides an excellent model for studying potential vaccines (Desrosiers and Ringler, 1989; Fultz, 1993).

B. HUMAN AND SIMIAN IMMUNODEFICIENCY VIRUSES

1. General Background

Since the initial isolation of human immunodeficiency virus type 1 (HIV-1) in 1983 (Montagnier *et al.*, 1984; Gallo *et al.*, 1984; Levy *et al.*, 1984) much progress has been made towards understanding the nature of the virus and its pathogenic process (For an excellent general review of the pathogenesis of AIDS see Levy, 1994). It is now generally accepted that acquired immunodeficiency syndrome (AIDS) is the end-stage clinical result of immune system abnormalities resulting from infection with HIV. Two related but distinct viruses have been shown to cause AIDS in humans, HIV-1 and HIV-2 (Clavel *et al.*, 1986) which are retroviruses of the genus lentivirus. HIV-1 is the prototype AIDS virus prevalent in North America, Central

Africa, Europe and throughout most of the world whereas HIV-2 is largely confined to regions of West Africa (Wong-Staal, 1990). In order to study infection by HIV-1 and HIV-2 and the progression to AIDS, and to develop potential vaccines, nonhuman primates have been used extensively (Desrosiers and Ringler, 1989; Gardner and Luciw, 1988; Flutz, 1993). Infection of chimpanzees and gibbons with HIV-1 is one model being utilized but this model has a number of drawbacks including the lack of an AIDS like disease in these animals and the expense and restrictions on the use of these endangered species. Although the basic characteristics of HIV-1 infection in chimpanzees are similar to those of humans, because chimpanzees do not develop symptoms of AIDS or AIDS related conditions after infection disease prevention can not be assessed (Flutz, 1993). This is a major problem since the prevention of disease rather than infection may be a potential goal and acceptable endpoint for an effective vaccine (Shafferman *et al.*, 1992; Hirsch *et al.*, 1994). The infection of macaques with simian immunodeficiency virus (SIV) resembles the infection of humans with HIV-1 in many respects including loss of $CD4^+$ cells, development of lymphomas (Feichtinger, *et al.*, 1990), central nervous system involvement (Lackner *et al.*, 1991; Chakrabarti *et al.*, 1991) and the development of similar types of opportunistic infections (Letvin and King, 1990; Baskin *et al.*, 1988). The simian immunodeficiency viruses (SIV's) are the closest known relatives of the HIV's and are similar in both genomic organization (Fig. 6) and biological properties (Table 2) (reviewed in Desrosiers, 1988; Desrosiers *et al.*, 1989; Gardner and Leciw, 1988). So far nonhuman primate lentiviruses have been isolated from nine different species: chimpanzees (SIV_{cpz}), African green monkeys (SIV_{agm}), sooty mangabey monkeys (SIV_{smm}), mandrill baboons (SIV_{mnd}), Sykes monkeys (SIV_{sky}) and captive rhesus monkeys (SIV_{mac}) (Desrosiers, 1992). Based on sequence analysis the various primate lentiviruses have been placed into five groups all of which are approximately equidistant

Figure 6. Genomic organizations in the various primate lentiviruses.

The genomic organization of the various human and nonhuman primate lentiviruses are shown. Vertical lines represent termination codons. Reproduced from Desrosiers, 1992.

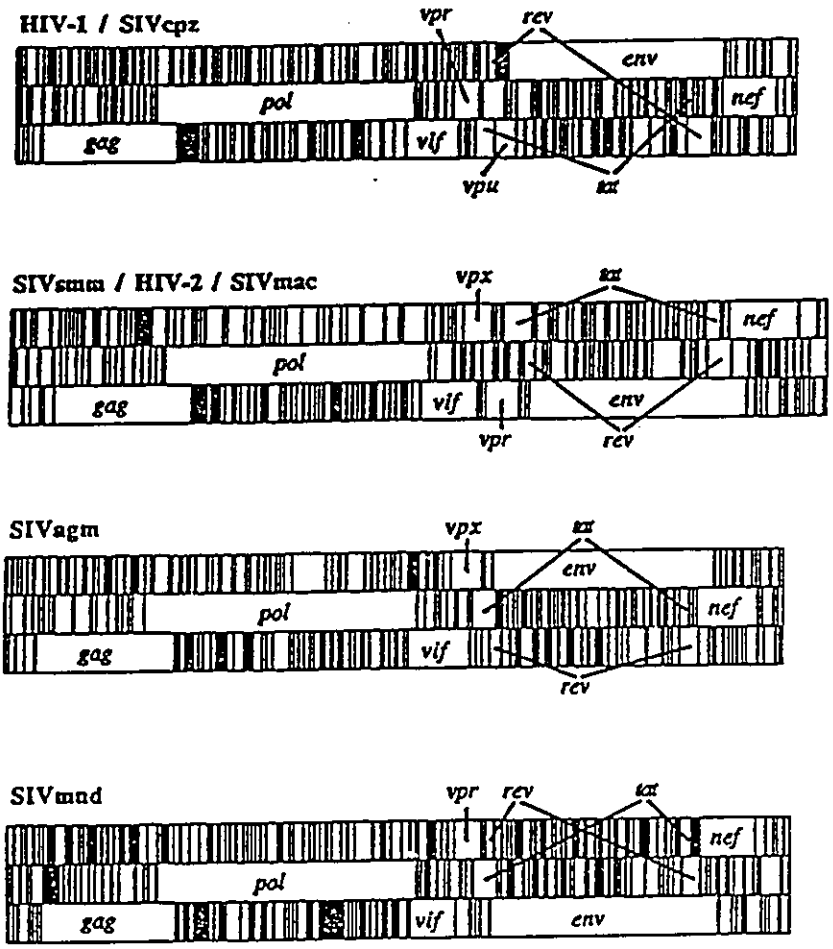

HIV-1 / SIVcpz
vpr
rev
env
pol
nef
gag
vif
vpu
tat
SIVsmm / HIV-2 / SIVmac
vpx
tat
nef
pol
gag
vif
env
vpr
rev
SIVagm
vpx
tat
env
pol
nef
gag
vif
rev
SIVmnd
vpr
rev
tat
nef
pol
gag
vif
env

Table 2. Properties of lentiviruses

Property	HIV	SIV
Morphogenesis/morphology	Lenti	Lenti
Macrophage tropism	Yes	Yes
T4 lymphocyte tropism	Yes	Yes
Receptor	CD4	CD4
Cytopathic	Yes	Yes
Natural mode of transmission	Sex, blood	?
Additional genes	Yes (at least 6)	Yes (at least 6)
Persistent infection	Yes	Yes
Chronic, debilitating disease	Yes	Yes
Immunodeficiency (AIDS)	Yes	Yes

from one another with 40-50% sequence homology. These groups include 1: (SIV_{smm}, SIV_{mac}, and HIV-2); 2: (SIV_{agm}); 3: (SIV_{mnd}); 4: (SIV_{sky}), and 5: (HIV-1 and SIV_{cpz}) (Flutz, 1993). Whereas African green monkeys, sooty mangabey monkeys, mandrill baboons and Sykes monkeys appear to be infected in nature and do not get an AIDS like disease, macaque monkeys are highly susceptible to disease induction by SIV and develop AIDS within a time frame suitable for study (Desrosiers, 1988, 1990). Thus, as mentioned previously, the macaque/SIV model provides an excellent system for vaccine development.

2. The Molecular Biology Of Primate Lentiviruses

Numerous reviews have been written on the molecular biology of both HIV (Hammarskjoid and Rekosh, 1989, Wong-Staal, 1990; Vaishnav and Wong-Staal, 1991; Levy, 1994) and SIV (Desrosiers, 1988; Desrosiers *et al.*, 1889). What follows is a brief description of the molecular biology of these viruses in order to discuss viral proteins which may be important in viral immunity. Any differences between the HIV's and SIV's have been noted. For a more detailed description of their molecular biology the reader is directed to the references cited above.

The HIV's and SIV's contain two copies of the positive-strand RNA genome encased by a cone shaped core which is surrounded by a membranous lipid envelope (Fig. 7) (Wong-Staal, 1990). Their proviruses, like all retroviruses, are flanked at each end by long terminal repeats (LTR's) and contain the structural genes *gag*, *pol* and *env* which are essential for viral replication (Fig. 6). The *gag* gene, translated from full-length viral mRNA, codes for a polyprotein precursor ($p55^{gag}$) consisting of components of the virion (p17: the myristylated matrix protein (MA); p24: capsid protein (CA); and p9 and p6: the nucleocapsid proteins (NC)) (Fig. 8). The *pol* gene is also translated from genome length transcripts into a polyprotein precursor designated $p160^{gag\text{-}pol}$.

Figure 7. HIV virion.

A schematic illustration of an HIV virion is shown with the structural and other virion proteins indicated. MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface glycoprotein; TM, transmembrane glycoprotein. Reproduced from Levy, 1994.

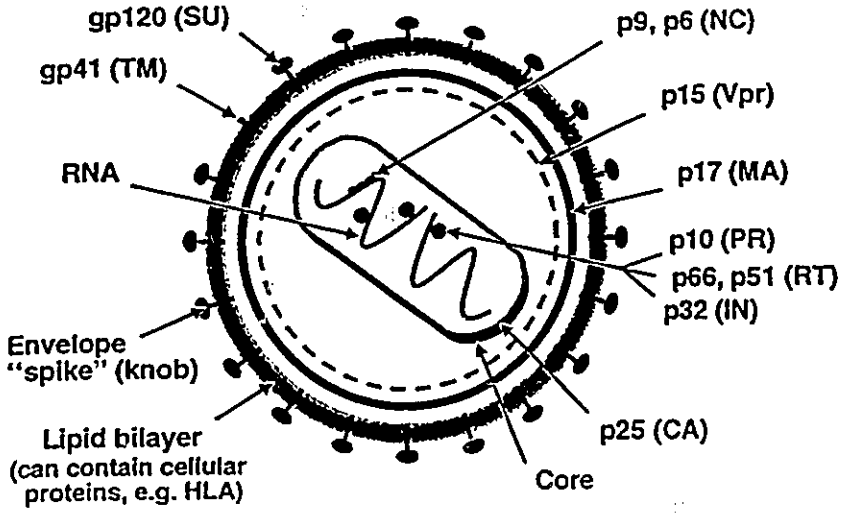
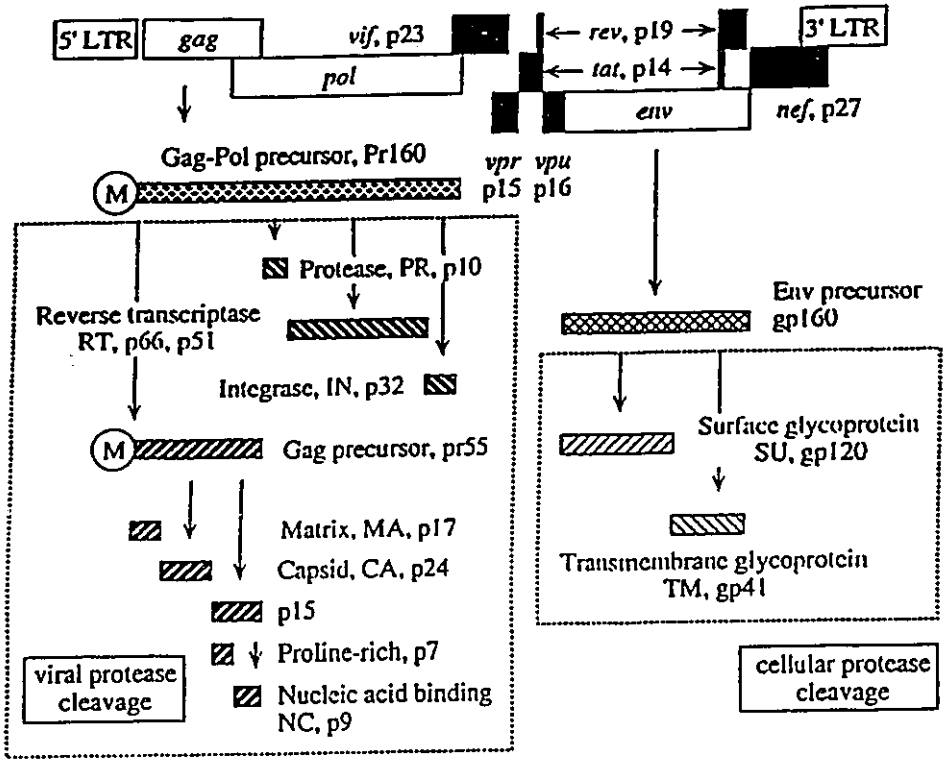

gp120 (SU)
gp41 (TM)
RNA
Envelope
"spike" (knob)
Lipid bilayer
(can contain cellular
proteins, e.g. HLA)
p9, p6 (NC)
p15 (Vpr)
p17 (MA)
p10 (PR)
p66, p51 (RT)
p32 (IN)
p25 (CA)
Core

Figure 8. Processing of HIV and SIV viral proteins.

The processing of HIV and SIV viral proteins is illustrated. Some viral proteins are further processed by viral and cellular protease following translation. The 55K gag precursor and 160K gag-pol precursor are processed by the viral protease into gag proteins (MA, CA, NC) protease (PR), reverse transcriptase (RT) and integrase (IN). The env precursor (gp160) is processed by a cellular protease into the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41). The viral regulatory and accessory proteins (Tat, Rev, Nef, Vif, Vpr, Vpu, Vpx) are not processed. The circled M at the amino terminus of the gag and gag-pol precursor represents a myristylation. Reproduced from Levy, 1994.


5' LTR
gag
vif, p23
pol
rev, p19
tat, p14
env
3' LTR
nef, p27
Gag-Pol precursor, Pr160
vpr vpu
p15 p16
M
Protease, PR, p10
Reverse transcriptase
RT, p66, p51
Integrase, IN, p32
M
Gag precursor, pr55
Matrix, MA, p17
Capsid, CA, p24
p15
Proline-rich, p7
Nucleic acid binding
NC, p9
viral protease
cleavage
Env precursor
gp160
Surface glycoprotein
SU, gp120
Transmembrane glycoprotein
TM, gp41
cellular protease
cleavage

The *gag* and *pol* genes overlap by approximately 200 bp and a ribosomal frame shift (frequency of 5-10%) which occurs in this region, results in the production of the *gag-pol* precursor (Wilson *et al.*, 1988). The *pol* gene encodes the viral protease (PR), reverse transcriptase (RT), and integrase (IN) enzymes (Fig. 8) (Muesing *et al.*, 1985; Ratner *et al.*, 1985; Wain-Hobson *et al.*, 1985). Both the *gag* and *gag-pol* precursors are myristylated at their amino terminus and associate with the cellular plasma membrane. The precursors are cleaved to the mature *gag* and *pol* products by the viral protease during assembly of virus particles.

The *env* gene is translated from singly spliced transcripts of full length viral mRNA into a glycosylated polypeptide precursor (gp160) that is processed by cellular enzymes into the external surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41) (McCune *et al.*, 1988). gp120 and gp41 remain noncovalently attached and are transported to the cell surface where they aggregate into trimers and tetramers (Earl *et al.*, 1990; Ozel *et al.*, 1988).

In addition to the *gag*, *pol* and *env* genetic elements the HIV's and SIV's also contain several accessory genes that play roles in viral replication in various cell types (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* and *vpx*). These proteins are translated from multiply spliced mRNA's. Four of the seven auxiliary genes are present in all primate lentiviruses, *tat*, *rev*, *nef*, and *vif*, but the presence or absence of *vpr* and *vpu* depends on the primate lentivirus group (Fig. 6) (Levy, 1994; Flutz, 1993).

The *tat* (transactivating protein) gene encodes a small nuclear protein absolutely required for viral replication (Dayton *et al.*, 1986). It is thought to act both transcriptionally and posttranscriptionally to up regulate HIV replication (Arya *et al.*, 1985; Sodroski *et al.*, 1985). Tat acts through a cis-acting sequence +1 to +44 (+1 marks the beginning of transcription and CAP

site) in the LTR called TAR (Tat responsive element) (Hauber and Cullen, 1988). *Rev* (regulator of viral protein expression) is another nuclear protein required for replication (Feinberg *et al.*, 1986) that interacts through a cis-acting RNA loop structure called the Rev responsive element (RRE) located in the viral envelope mRNA (Rosen *et al.*, 1988). Rev$^-$ mutants do not efficiently transport unspliced genome length mRNA encoding *gag-pol* or singly spliced mRNA for *env* to the cytoplasm for translation but rather only multiply spliced mRNA's encoding the auxiliary proteins in which the RRE is removed (Feinberg *et al.*, 1986). Evidence suggests that the mRNA encoding the structural proteins may be retained in the nucleus due to the presence of cis-acting repressor sequences (CRS) in the transcripts that contribute to retention (Rosen *et al.*, 1988) and also possibly because of inefficient splicing signals contained in the mRNA (Chang and Sharp, 1989). *rev* is thought to promote the transport of RRE-containing unspliced mRNA from the nucleus to the cytoplasm. The *nef* (negative factor) gene encodes a myristylated protein that is associated with the inner plasma membrane (Kan *et al.*, 1986). The exact function of *nef* has not yet been determined and its role is somewhat controversial. *Nef* has been reported by some investigators to down regulate virus expression (Terwilliger *et al.*, 1986; Ahmad and Venkatesan 1988) while others have reported no effect or up-regulation (Kim *et al.*, 1989; Kestler, 1991). The effect of *nef* may not be clear *in vitro* but *in vivo nef* appears to be essential for vigourous viral replication and full pathogenic potential (Kestler, 1991; Desrosiers, 1992).

Functions for the other viral proteins, *vif*, *vpr*, *vpu* and *vpx* have not been well established but they may play a role in infectivity, replication in certain cell types, and virion assembly and release, (Vaishnav and Wong-Staal, 1991; Desrosiers, 1992). Virus replication and expression is thought to involve an interplay between these various regulatory proteins (for reviews see Greene, 1991; Vaishnav and Wong-Staal, 1991).

The HIV's and SIV's primarily infect cells expressing CD4 on their surface such as helper T-lymphocytes, monocytes and macrophages which are essential to appropriate functioning of the immune system. Infection occurs following contact of virion envelope glycoprotein gp120 with the cellular CD4 receptor (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984) which results in fusion of the viral envelope with the cell membrane. Infection of a host cell can also occur by fusion with an infected cell expressing gp120 on its surface leading to syncytia formation. Fusion is thought to be mediated by the hydrophobic amino terminus of gp41 (Kowalski *et al.*, 1987). Once the virus core enters the cell, the viral RNA is reverse transcribed and the provirus DNA integrates randomly into the genome of the infected cell. Virus expression leads to the production of progeny virus by budding from the plasma membrane.

3. Protective Mechanisms Against Infection.

Transmission of HIV/SIV can be accomplished by free virus or virus infected macrophages or lymphocytes either through a break in the skin or by contact with mucosal membranes. One of the highest priorities in vaccine research for AIDS has been to identify the correlates of immunity and the immune mechanisms that will protect the host from disease but to date there is no consensus on what these correlates are. To find which regions of HIV and SIV stimulate immune responses many researchers have mapped antigenic epitopes in the viruses. It is hoped that mapping these epitopes will contribute to the development of an effective vaccine as well as to the understanding of the immune response to the virus. To date numerous antigenic epitopes recognized by antibodies, cytotoxic T cells, helper T cells and in antibody-dependent cellular cytotoxicity (ADCC) have been mapped (Nixon *et al.*, 1992; Norley *et al.*, 1993). While it is traditionally thought that neutralizing antibodies are important or essential for preventing viral

infection they might not be effective in clearing cell associated virus. For this reason it is presumed that an effective vaccine against the AIDS viruses will induce neutralizing antibodies and also must stimulate effector cells (Cytotoxic T cells, T-helper cells and antibody dependent cellular cytotoxicity (ADCC)) which will eliminate virus-infected cells.

The envelope glycoprotein (gp120/gp41) has been a major target in vaccine development due to its primary role in infection through interaction with the cellular CD4 receptor (Warren and Dolatshahi, 1993). In HIV-1 the third variable region (V3) of gp120 is thought to contain the principal neutralizing determinant (PND) of HIV-1 (Goudsmit *et al.*, 1988; Palker *et al.*, 1988; Matsushita *et al.*, 1988). The V3 loop, so named because it is located within a disulphide cross-bridged loop in the third variable domain of the envelope glycoprotein, is thought to be involved in the process of virus fusion to the host cell and the binding site for the cellular CD4 molecule (Putney *et al.*, 1986; Robey *et al.*, 1986; Freed *et al.*, 1991; Hwang *et al.*, 1991). The PND in HIV-1 appears to be a linear epitope since peptides to this region can induce neutralizing antibodies (Palker *et al.*, 1988; Robey *et al.*, 1986) comparable in titer to those raised in chimpanzees experimentally infected with HIV. V3 region antibodies have been found to block syncytium formation induced by both cell-free virus and cell-associated virus without blocking the primary gp120-CD4 binding event. Neutralizing antibodies are also detected to other regions of the envelope glycoprotein including the region involved in binding to CD4 (Sun et al., 1989) and to a portion of gp41 (Chanh *et al.*, 1986; Dalgleish *et al.*, 1988).

In SIV a number of neutralizing domains have also been mapped and although some appear analogous to those found in HIV-1 (Kodama *et al.*, 1991; Kent *et al.*, 1991) others are not (Kent *et al.*, 1991). For example although the PND in SIV has been localized to the same region (a region encompassing the analogous V3 loop) as in HIV-1 the epitope is not linear and requires

the tertiary structure of the protein (Javaherian *et al.*, 1992).

Although a neutralizing antibody response may be important to combat cell free virus it is likely that a cell mediated response will be important to combat cell associated virus. One of these defence mechanisms is ADCC. In the ADCC reaction antigen-specific antibodies bind to their epitope on the infected cell and induce killing by major histocompatibility complex (MHC)-unrestricted $CD16^+$ cells (Fc receptor bearing effector cells). HIV-specific ADCC activity has been found in the majority of sera from HIV-1-infected individuals (Ljunggren *et al.*, 1987; Lyerly *et al.*, 1887). A number of ADCC epitopes have been mapped to the envelope glycoprotein in HIV-1 (Nixon *et al.*, 1992; Norley *et al.*, 1993) but as of yet none have been defined for SIV.

A second cell mediated response important in controlling cell associated virus is the CTL response. CTL's recognize processed viral fragments (peptides) presented on the surface of an infected cell in association with an MHC molecule. Most CTL are $CD8^+$ T lymphocytes and recognize viral peptide fragments bound to class I MHC molecules. CTL are known to be crucial in the recovery from viral infections and appear early in the immune response (Yap et al., 1978). During HIV infection CTL's are detected early and it is thought that they are responsible for the drop in viral burden following initial infection (Walker et al., 1989; Walker, 1990). CTL epitopes are located in most of the viral components, both structural and regulatory (Clerici *et al.*, 1991; Culmann *et al.*, 1991; Walker *et al.*, 1988). Many CTL epitopes have now been mapped for both HIV and SIV (Norley *et al.*, 1993).

$CD4^+$ T cells are responsible for helping many immunological responses following the recognition of viral peptides presented by MHC class II infected cells. Since HIV infects $CD4^+$ T cells the virus has the ability to alter the response of these cells and in fact the natural $CD4^+$ T-cell response against HIV is found to be weak (Nixon *et al.*, 1992). The inclusion of $CD4^+$

helper epitopes in an HIV vaccine may generate maximal help to CTL and antibody responses. T-helper epitopes have been located in many of the HIV structural and nonstructural proteins (Cease *et al.*, 1987; Clerici *et al.*, 1989; Wahren *et al.*, 1989; Hass *et al.*, 1991; Nixon *et al.*, 1992).

In the absence of certainty as to which immune mechanisms, cellular or humoral, might provide superior protection, we have attempted to construct adenovirus vectors that include both important humoral and cellular epitopes of SIV.

MATERIALS AND METHODS

A. BACTERIAL CULTURES

Escherichia coli strains DH5α (Hanahan, 1983), LE392 (Murray *et al.*, 1977) and HMS174 (Campbell, *et al.*, 1978) were grown in Lennox L (LB) broth (Becton Dickinson BBL LB broth base mix; 20 g LB broth base mix and 1 g glucose per liter H_2O, sterilized by autoclaving) or in Super-Broth (SB) (20 g LB broth base mix, 22 g tryptone, 15 g yeast extract, 1 g glucose and 5 ml 1N NaOH per liter H_2O, sterilized by autoclaving) or on solid LB agar (20 g bacto-agar per liter of LB) at 37°C. Bacteria containing plasmids with antibiotic resistance markers were grown in media supplemented with the appropriate antibiotic: ampicillin (60 μg/ml), kanamycin sulfate (50 μg/ml), tetracyclin (50 μg/ml). For long term storage 2ml of fresh overnight culture was diluted with an equal volume of 40% sterile glycerol and frozen at -80°C.

B. MAMMALIAN CELLS

The mammalian cell lines used in the course of these studies include: 293 cells (human embryonic kidney cells that constitutively express the left 12% of the Ad5 genome; Graham *et al.*, 1977), 293N3S cells (293 cells adapted to grow in spinner culture; Graham, 1987), KB cells (human oral epidermod carcinoma), Hela (human cervix epitheloid carcinoma), MRC-5 (normal human diploid lung fibroblasts), A549 (human lung carcinoma), LLCMK2 (Rhesus monkey kidney), BSC-1 (african green monkey kidney), Vero MT-SIV (african green monkey kidney cells

transfected with a plasmid containing a neomycin resistance gene and the $SIV_{mac}239$ genome under the control of the human metallothionein promoter), VSMFC1 (a clone similar to MT-SIV). Cell lines were passaged as monolayers in plastic 150 mm dishes (Falcon®, Nunclon®), unless otherwise stated.

293 cells were maintained in minimal essential medium (MEM) F11 containing 10% volume/volume (v/v) new born calf serum (Gibco BRL Life Technologies, Inc.), L-glutamine (2 mM final concentration), penicillin/streptomycin (100 Units (U)/ml and 0.1 mg/ml, final concentration respectively, Gibco BRL Life Technologies, Inc.), and fungizone (2.5 μg/ml final concentration; Bristol Myers Squibb Canada Inc.). 293 cells were dispersed for passaging by incubating with 1X citric saline (10 g KCl and 4.4 g sodium citrate/L H_20, sterilize by autoclaving) at 37°C.

Hela, MRC5, A549 and BSC-1 cells were maintained in α-MEM containing 10% v/v fetal bovine serum (Gibco BRL Life Technologies, Inc.), L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively), and fungizone (2.5 μg/ml final concentration). LLCMK2 cells were maintained in media as described above but were supplemented with 5% (v/v) new born calf serum in place of fetal bovine serum. Vero MT-SIV and VSMFC1 cells were maintained in dulbecco's modified eagles medium containing 10% (v/v) fetal bovine serum, L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively), fungizone (2.5 μg/ml final concentration) and Geneticin (600 μg/ml final concentration; Gibco BRL Life Technologies, Inc.). Vero MT-SIV and VSMFC1 cells were induced to express SIV genome products by incubating them for 24 h in the above medium containing 300 μM $ZnCl_2$ and 2 μM $CdCl_2$. The cell lines described above were dispersed for passaging by incubating with 0.5% trypsin (Gibco BRL Life Technologies,

Inc.) at 37°C.

293N3S and KB cells used in spinner culture were maintained in Joklik's modified MEM containing 10% (v/v) horse serum (Gibco BRL Life Technologies, Inc.), L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively) and fungizone (2.5 μg/ml final concentration).

For *in vitro* labelling of proteins 199-met (or -met-cys) media containing 5% (v/v) dialized fetal bovine serum (Gibco BRL Life Technologies, Inc.), L-glutamine (2 mM final concentration) and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively) was used. For in vitro labelling of viral DNA phosphate free MEM containing 5% (v/v) horse serum, L-glutamine (2 mM final concentration) and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively) was used. All serum was heat-inactivated at 56°C for 30 min prior to use in cell culture.

C. VIRUSES

Viruses used in the course of these studies include wild type human adenovirus 5 (wt Ad5), *dl*309 (containing small deletions which remove the Xba I sites at 29.5, and 79.5 map units (mu), and a deletion from approximately 83.5 to 85.6 mu that is substituted with a fragment of foreign DNA; Jones and Shenk, 1979, Bett *et al.* 1995), *dl*313 (containing a 2.3 kB E1 deletion extending into the protein IX gene; Jones and Shenk, 1979), Ad *dl*E3 (containing a 1.88 kb deletion in E3; Haj-Amad and Graham, 1986), Ad5 *dl*55 (containing a deletion from 5.5 to 9.2 mu; F. L. Graham unpublished), AdlacZ (containing a 3.82 kb insert consisting of the SV40 early promoter, β-galactosidase, and SV40 polyadenylation sequence inserted into a 1.88 kb E3 deletion; Prevec *et al.*, 1990), AdgB8 (containing a 4.1 kb insert consisting of the SV40 promoter and herpes simplex

virus type 1 (HSV-1) glycoprotein B in a 1.88 kb E3 deletion; D. C. Johnson and F. L. Graham, unpublished), AdgB10 (containing a 4.88 kb insert of HSV-1 glycoprotein B in a 1.88 kb E3 deletion; D. C. Johnson and F. L. Graham, unpublished), AdHTLV1gag2L (containing a 4.0 kb insert consisting of the human T-cell leukaemia virus type I gag region and an ampicillin resistance (Ap^r) gene, in the E3 antiparallel orientation, in a 2.69 kb E3 deletion; G. Dekaban and F. L. Graham, unpublished). Virus vectors constructed as part of this project are described in the subsequent chapters.

D. PLASMIDS

The following plasmids were used in the course of these studies for generating adenovirus vectors and for the development of new vector systems for generating adenovirus vectors. pBR322 (Bolivar *et al.*, 1977) is a *E. coli* cloning vector. pBRX (Haj-Ahmad, 1986) is a pBR322 derivative with an *Xba*I site at nucleotide 2066. pKN30 (Lee, 1989) is a small kanamycin resistant (Kn^r) plasmid. pUC19 (Yanisch-Perron *et al.*, 1985) is a small, high copy number cloning vector. pUC4K (Pharmacia Canada Inc.) is a pUC19 derivative containing the Kn^r gene flanked by various restriction sites. pSV2X3 (Prevec *et al.*, 1990) contains the complete SV40 early promoter and polyadenylation sequences separated by a multicloning site. pFG140 (Graham, 1984) is an infectious plasmid in single transfections of 293 cells which was derived from the *dl*309 viral genome (Jones and Shenk, 1979). It contains essentially the entire *dl*309 genome with an Ap^r and bacterial origin of replication (Ori) inserted at bp 1339 (3.7 mu) in Ad5 sequences. pJM17 is a (McGrory *et al.*, 1988) derivative of pFG140 which is noninfectious in single transfections of 293 cells since it contains a 4.4 kb insert (a derivative of pBR322) at bp 1339 (3.7 mu) in Ad5 sequences which makes the resulting viral genome too large to package. pFG173

(Hanke *et al.*, 1990; Mittal *et al.*, 1993) is a derivative of pFG140 which is noninfectious in single transfections of 293 cells since it contains a deletion of essential Ad5 sequences between bp 27334 (76.3 mu) and bp 30464 (85.0 mu) (bp and mu refer to the *dl*309 genome; Bett *et al.*, 1995) into which a Kn^r gene and bacterial Ori are substituted. pFG23 (McKinnon, 1982) is a pBR322 derivative containing the *Bam*HI B fragment (59.5 to 100 mu, minus 20 bp from the right end). pFGdX1 (Haj-Hmad and Graham, 1986) is a derivative of pFG23 from which the Ad5 sequences between the *Xba*I sites at 78.5 and 84.7 mu have been deleted. pFG144K3 (Bett *et al.* 1993) contains Ad5 left end sequences from 0 to 16.1 mu with a 3.47 kb deletion in E1 (1.0 to 10.6 mu) and right end sequences from 69.0 to 100.0 mu with a 1.88 kb deletion in E3 (79.6 to 84.8 mu) which contains a unique *Xba*I cloning site. pXC1 (McKinnon *et al.*, 1982) is a pBR322 derivative containing Ad5 sequences from bp 22 (0 mu) to bp 5790 (16.1 mu). pXC38 (Bautista *et al.*, 1991) was derived from pXC1 by deletion of a *Hind*III-*Bam*HI fragment from pBR322 sequences. pβ548-1 (Hitt and Graham, 1990) is a pXC1 derivative with a *Bam*HI site inserted at bp 548 in adenovirus sequences into which the β-Actin promoter was introduced. pΔ548IEP-1 is identicale to pβ548-1 except that it contains the HCMV promoter inserted at bp 548 and has the first 548 bp of Ad5 deleted (M. Hitt personal comunication). pKH188 (Bautista *et al.*, 1991; Bautista *et al.*, 1989) contains Ad5 sequences from bp 22 (0 mu) to 2048 (5.7 mu) with a *Bam*HI site inserted at 188 bp in the Ad5 genome. pNSS (donated by Connaught laboratories) is a pUC19 derivative containing the $SIV_{mac}239$ genome minus the long terminal repeats (from 1079 to 9482 bp) (numbering refers to the sequence puplished for $SIV_{mac}239$ by Kestler et al, 1990). pMT-SIV (donated by Connaught laboratories) is a pUC19 derivative containing the $SIV_{mac}239$ genome minus the long terminal repeats (from 1079 to 9482 bp) flanked at the 5' end by the human metallothionein promoter and at the 3' end by the SV40 poly A. In pMT-SIV a stop codon is

provided for the truncated env gene (it loses 4 aa) in downstream plasmid sequences. Plasmids constructed as part of this project are described in subsequent chapters.

E. ANTIBODIES

Antibodies used in the course of these studies include antisera to Ad2 protein IX (donated by W. Russell), used to immunoprecipitate Ad5 protein IX, anti-β-galactosidase monoclonal antibody (Promega corporation) used to detect *E. coli* β-galactosidase and rabbit anti-luciferase serum (donated by S. K. Mittal). Herpes simplex virus type 1 glycoprotein B was detected with 15βB2 monoclonal antibody (donated by D. C. Johnson). Ad5 E3 proteins were detected using appropriate antisera (donated by A. E. Tollefson and W. S. M. Wold): rabbit anti-6.7K raised against amino acids (aa) 47-61 of Ad2 6.7K (Wilson-Rawls *et al.*, 1990), rabbit anti-10.4K raised against aa 68-80 of Ad2 10.4K (Tollefson *et al.*, 1990), rabbit anti-14.5K raised against aa 118-132 of Ad5 14.5K (Tollefson *et al.*, 1991), rabbit anti-14.7K raised against a TrpE-fusion to Ad5 14.7K containing essentially the entire protein (Tollefson and Wold, 1988), rabbit anti-gp19K raised against the C-terminal 15 aa of Ad5 gp19K and rabbit anti-11.6K raised to the last 16 aa of Ad5 11.6K. Simian immunodeficiency virus (SIV) proteins were detected with SIV reference serum obtained from an SIV infected macaque (donated by Connaught laboratories), goat anti-SIV_{mac}239 gp130 (donated by N. L. Haigwood), rabbit anti-HIV-2 gag (donated by Connaught laboratories), anti-SIV_{agm}155 raised in pigtailed monkeys (Gravell *et al.*, 1989; Johnson *et al.* 1989), anti-SIV_{ssm}236 raised in rhesus monkeys and SIV_{mac} p27 monoclonal antibody (2F12) (Higgins *et al.*, 1993). Anti-SIV_{agm}155, anti-SIV_{ssm}236 and the SIV_{mac} p27 monoclonal antibody (2F12) were obtained through the AIDS research and reference reagent program. For Western blots hors radish peroxidase conjugated goat anti-human IgG, rabbit anti-goat IgG, goat anti-mouse

IgG and goat anti-rabbit IgG were obtained from Pierce.

F. ENZYMES

Modifying enzymes were purchased from either Boehringer Mannheim Canada, Ltd., New England Biolabs (NEB) Ltd., Gibco BRL Life Technologies, Inc., or Pharmacia Canada Inc.. Enzymes were used according to the manufactures recommendations with the reaction buffers provided.

G. RECOMBINANT DNA TECHNIQUES

1. DNA Restriction Digests

Restriction digests were performed for both cloning and diagnostic purposes. Typical reactions were carried out for 2-4 hours at the appropriate temperature for the enzyme (usually 37°C) in the buffer supplied by the manufacturer. 3-5 U of enzyme per μg of DNA was used. Enzymes were inactivated by incubating at 65°C for 15 min or by adding (10% v/v) loading buffer (20% glycerol, 2% SDS and 0.5% bromophenol blue) in preparation for gel electrophoresis.

2. Blunting 5' And 3' Overhangs

When it was necessary to obtain blunt ended DNA fragments after digestion with a restriction enzyme that produced 5' or 3' overhangs either the large fragment of DNA polymerase I (Klenow fragment) or nuclease S1 was used. The klenow fragment has a 5'-3' polymerase activity and a 3'-5' exonuclease activity but lacks the 5'-3' exonuclease activity possessed by the holo enzyme. Reactions typically contained 5 μg of DNA, 0.25mM of each dNTPs, nick translation buffer (50 mM Tris, pH 7.2, 10 mM $MgSO_4$, 0.1 mM dithiothreitol (DTT), 50 μg/mL

BSA) and 5 U of klenow, were carried out for 15 min at room temperature (RT) and inactivated for 15 min at 65°C. Nuclease S1 is a single strand specific nuclease used to remove 5' overhangs. Reactions typically contained 5 μg of DNA, S1 buffer (33mM NaAc, pH 4.6, 10 mM NaCl, 0.03 mM $ZnSO_4$, 0.1% SDS) and 8 U of nuclease S1, were carried out for 0.5 h at 37°C and inactivated by phenol extraction.

3. DNA Phosphorylation

Linker oligonucleotides were phosphorylated using T4 polynucleotide kinase which transfers the γ-phosphate group of ATP to the 5' hydroxyl termini of DNA. Typical reactions contained 1-5 μg of linker DNA, 1 mM ATP, 1X polynucleotide kinase buffer (70 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol) and 10 U T4 polynucleotide kinase. Reactions were carried out for 30 min at 37°C and inactivated for 15 min at 65°C.

4. Polymerase Chain Reaction

The polymerase chain reaction (PCR) amplification of DNA sequences was carried out in a Perkin Elmer Cetus DNA Thermal Cycler using the primers described in the results section. PCR reactions were carried out in a total volume of 100 μl and contained 1 μg linearized plasmid DNA, 100 pmols of each primer, PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) , 10 mM dNTPs and Tac DNA polymerase. Plasmid DNA was linearized by restriction enzyme digestion, and denatured by heating to 95°C for 10 min in the thermal cycler prior to the addition of Tac DNA polymerase. Thirty cycles were carried out using a denaturation temperature of 94°C for 1 min, an annealing temperature of between 37 and 56°C (depending on the primers used) for 1 min and an elongation temperature of 75°C for 2 min plus 3 s for every

cycle (Innis *et al.*, 1990). PCR products were analyzed by gel electrophoresis and purified for cloning using the Wizard PCR Preps Purification system® (Promega, Corporation).

5. Agarose Gel Electrophoresis

For the analysis of restriction digests and DNA fragments, horizontal 1% weight/volume (w/v) agarose (Gibco BRL Life Technologies, Inc.) gels were run submerged in TAE buffer (40 mM Tris, pH 7.9, 5 mM Na Acetate, 1 mM EDTA) in the presence of ethidium bromide. When labelled viral DNA was analyzed, vertical 1% agarose gels were run using TBE (45 mM Tris, 45 mM boric acid, 2 mM EDTA, pH 8.0). To determine the sizes of DNA fragments marker DNA samples were run on the gels consisting of *Hind* III digested wt Ad5 DNA or 1 kb DNA ladder (Gibco BRL Life Technologies, Inc.).

6. Isolation Of DNA Fragments From Agarose Gels

When isolating DNA fragments from agarose gels two methods were used. Approximately 20 μg of DNA was cut with the appropriate enzyme and electophoresed in the dark in TBE buffer in the presence of ethidium bromide. The fragment of interest was visualized under UV light and when using the first method a slit was made in the gel both in front and behind the band. Boiled dialysis tubing was inserted into the slits and high voltage applied to transfer the DNA from the gel to the dialysis membrane in the path of the DNA. When transfer was complete the dialysis membrane was removed (still under high voltage) and the DNA eluted from the membrane by washing repeatedly with 200 μl of "lysing buffer" (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.5% SDS). One tenth volume of 30% NaAc was added to the eluted DNA and it was then precipitated with 96% ethanol. After washing the pellet twice in ethanol, the pellet was dried and resuspended

in a small volume of 0.1X SSC. Using the second method the fragment of interest was electrophoresed through a 1% (w/v) low melting point agarose (Gibco BRL Life Technologies, Inc.) gels in the dark in TAE buffer in the presence of ethidium bromide. The band of interest was then excised from the gel and purified using the Wizard PCR Preps Purification system® (Promega, Corporation). Briefly the isolated band in approximately 300 µl (300µg) of agarose was transfered to a microfuge tube and incubated at 70°C until the agarose was completely melted. 1 ml of PCR Preps DNA Purification Resin was then added and the sample mixed gently. The DNA/Resin mixture was then pipetted into the barrel of a 3 ml disposable syringe attached to a Wizard minicolumn and the slurry pushed through the column with the syringe plunger. The column was then washed with 2 ml of 80% isopropanol using the same syringe. To dry the resin the minicolumn was then transfered to a microfuge tube and centrifuged for 20 seconds at 12000 xg. The column was then transfered to a new microfuge tube and the DNA eluted by adding 50 µl of ddH_2O, incubating 1 min at RT and then centrifuging at 12000 xg for 20 seconds.

7. DNA Ligation

T4 DNA ligase was used to catalyze the formation of phosphodiester bonds between the 5' phosphate and 3' hydroxyl termini of abutting duplex DNA. Ligation reactions contained 1X T4 DNA ligation buffer (10 mM $MgCl_2$, 10 mM DTT, 50 mM Tris, pH 7.6, 1 mM ATP) and 1 U T4 DNA ligase per µg of DNA and were carried out at RT for 0.5-3.0 h or at 14°C overnight. Reactions were inactivated at 65°C for 10 min.

8. Bacterial Transformation

Bacterial strains were transformed using $CaCl_2$ or by electroporation. For transformation using $CaCl_2$ DH5α, LE392 and HMS174 cells were made competent as follows: 0.5 ml of LB was inoculated with 5 ml of overnight culture and grown at 37°C with shaking to an OD_{600} of 0.5 to 1.0. Bacteria were then harvested by chilling on ice for 10 min and centrifuging at 4000 revolutions per minute (rpm) for 10 min at 4°C. The pellet was then washed twice by resuspending in 100 ml of $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM pipes, pH 7.0) and centrifuging at 2500 rpm for 5 min at 4°C. Before centrifuging after the second wash the cells were incubated on ice for 30 min. Following the second centrifugation the cells were resuspended in 2 ml of $CaCl_2$ solution. Cells were either transformed immediately or left on ice overnight to increase their competency. 250 µl aliquotes were made of the unused suspension in 0.5 ml microfuge tubes, frozen in a isopropanol-dry ice bath and stored at -80°C for future use.

For transformation 100-200 µl of newly competent or frozen competent bacteria were mixed with 0.1-1 µg of DNA in a round bottom test tube and incubated on ice for 10 min. The cells were then heat shocked at 42°C for 2 min, resuspended in 1 ml of LB and allowed to recover at 37°C with shaking for 1 hr. Transformations were then plated on LB agar using appropriate dilutions and antibiotic selection.

DH5α cells were made competent for electroporation as follows: 1 L of LB was inoculated with 1/100 volume of overnight culture and grown at 37°C with shaking to an OD_{600} of 0.5 to 1. Bacteria were then harvested by chilling the flask on ice for 10 min and centrifuging at 4000 rpm for 15 min. The pellet was then washed and recentrifuged twice in 0.5 L of 10% glycerol in cold water. The pellet was then resuspended in 20 ml of cold 10% glycerol, recentrifuged and resuspended in a final volume of 2-3 ml of 10% glycerol. 100 µl aliquots of this suspension were

then frozen in 0.5 ml microfuge in a isopropanol-dry ice bath and stored at -80°C for future use.

For electroporation 50 µl of cell suspension and 1-2 µl of plasmid DNA (20-100 ng) were mixed and added to an electroporation cuvette. The cuvette was then placed in a Bio Rad gene pulser® unit and pulsed at settings 25 µF, 2.25 kV and 200 ohms. Cells were resuspended in 1 ml of LB, allowed to recover at 37°C with shaking for 1 hr and then plated on LB agar using appropriate dilutions and antibiotic selection.

9. Small-Scale Plasmid Preparation

In order to screen for recombinant plasmids an adaptation of the rapid alkaline-lysis procedure of Birnboim and Doly (1979) was used. Briefly, colonies obtained after transformation were inoculated into 3 ml of LB plus appropriate antibiotic and grown overnight. 1.0 ml of the overnight cultures was then transferred into microfuge tubes, centrifuged for 1-2 min, and the media aspirated. Pellets were resuspended in 100 µl of resuspension buffer (100 µg/ml RNaseA, 50 mM Tris, 10 mM EDTA pH8.0), 200 µl of alkaline SDS (0.2N NaOH, 1% SDS) was added, mixed and samples incubated on ice for 5 min. 150 µl of 3M NaAc pH 4.8 was then added, mixed by shaking and incubated on ice for 15 min. Samples were then centrifuged for 5 minutes, and the supernatants transferred into new microfuge tubes containing 1 ml of isopropyl alcohol to precipitate the plasmid DNA, mixed and centrifuged for 10 min. Pellets were then washed in 0.5 ml of 70% ethanol, dried and plasmid DNA resuspended in 100 µl of ddH_20. Plasmid samples (5-10 µl) were analyzed by restriction digest and agarose gel electrophoresis.

10. Large Scale Plasmid Preparation

To obtain large quantities of plasmid DNA a scaled up version of the Birnboim and Doly procedure was used. Plasmid bearing bacteria were inoculated into 5 ml LB plus antibiotics, and incubated at 37°C for 6-8 hours with shaking. The 5 ml cultures were then added to 500 ml of LB or SB plus antibiotics and grown overnight. The next morning cultures were transfered to 500 ml centrifuge bottles, centrifuged at 6000 xg for 10 min and the pellets resuspended in 40 ml resuspension buffer. 80 ml of alkaline SDS was then added, mixed by swirling and samples incubated on ice for 5-10 min. Cellular debris were precipitated by mixing in 40 ml 5 M KAc, (made up according to Sambrook *et al.*, 1989: to 60 ml 5M KAc add 11.5 ml glacial acetic acid and 28.5 ml H_2O.), and incubating on ice for 15-30 min. 10 ml of H_2O was then added and samples centrifuged for 10 min at 6000 xg. Supernatants were collected by filtering through several layers of cheese cloth into a new 500 ml centrifuge bottle and the DNA precipitated by adding 100 ml of isopropanol. After sitting at RT for 30 min samples were centrifuged for 10 min at 6000 xg. The supernatant was then poured off and the centrifuge bottles inverted to allow the pellets to dry at RT for 5-10 min. Pellets were redissolve in 7.0 ml 0.1X SSC and transferred to 50 ml Corning tubes containing 8.6 g CsCl (ICN Biomedicals, Canada Ltd.). The CsCl was dissolved completely and samples incubated on ice for 30 min. Precipitated RNA and proteins were removed by centrifugation at 3200 xg for 15 min and the supernatants transferred to 13 ml Quick-Seal® centrifuge tubes (Beckman Instruments, Inc.). Ethidium Bromide (200 µl of a 10 mg/ml stock) was then added and the tubes were topped up with light paraffin oil and sealed. Plasmids were banded by equilibrium centrifugation for 16-18 h at 55000 rpm in a Beckman VTi 65.1 rotor. Plasmid bands were visualized under long wave length UV (lower band should be closed circular plasmid, upper band should be *E.coli* DNA, or nicked plasmid) and collected with

an 18 gauge needle and syringe through side of tube (in a volume of 1-2 ml). The ethidium bromide was removed from the DNA solution by extracting four times with CsCl saturated isopropanol in 25 mM Tris, 10 mM EDTA. The plasmid DNA was then precipitated by first mixing with 3 volumes of 0.1X SSC followed by 8 volumes of ethanol, (eg 1 ml DNA, 3 ml 0.1X SSC, 8 ml cold ethanol) and centrifugation at 4000 rpm for 10-15 min. DNA pellets were washed twice in 2 ml of 96% ethanol, dried at 37°C and redissolved in 0.5-1.0 ml of 0.1X SSC. Plasmid DNA concentration was estimated by absorbance readings at 260 nm or using a fluorometer.

11. DNA Sequencing

DNA sequencing reactions were based on the chain-termination method (Sanger *et al.*, 1977) and manual sequencing was performed following the DNA sequencing protocol described in the sequenase™ kit produced by US Biochemical. All oligo nucleotide primers were synthesized by the central facility of the Molecular Biology and Biotechnology Institute (MOBIX) at McMaster University, Hamilton, Ontario, Canada. Plasmid DNA was prepared for sequencing by alkaline denaturation: 2 µg of plasmid DNA in a volume of 20 µl ddH20 was added to an equal volume of 0.4 M NaOH and incubated at RT for 5 min. This reaction was neutralized with 4 µl of 5 M ammonium acetate, immediately precipitated with 100 µl of 96% ethanol and then incubated on ice for 10 min. The denatured DNA was pelleted by centrifugation at 15000 rpm for 10 min, the pellet washed in 1 ml 96% ethanol and dried at 37°C.

Template DNA was annealed with primer, by resuspending the denatured plasmid DNA in 6 µl of ddH20, 2 µl of 5X sequencing buffer (200 mM Tris, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 2 µl of primer (2 ng/µl). The tubes were then heated to 65°C for 2 min and cooled slowly to RT.

For the labelling reaction 1 µl of 0.1 M DTT, 2 µl of labelling mix (7.5 µM dGTP, 7.5 µM dTTP and 7.5 µM dCTP diluted 1:5 in ddH20) and 1 µl of [α-^{32}P]-dATP (Amersham Canada Ltd.) were added to the annealed template and primer. Polymerization was initiated by adding 2 µl of Sequenase® (diluted 1:8 in enzyme dilution buffer, 10 mM Tris, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) and allowed to continue for 5 min at RT. To terminate the labelling reaction 3.5 µl was transferred to each of 4 termination tubes containing 2.5 µl of one of the termination mixes (8 µM of either ddGTP, ddTTP, ddCTP or ddATP and 80 µM of each dNTP, 50 mM NaCl) and incubated for a further 5 min at 37°C. The termination reactions allow polymerization to continue until the addition of a dideoxynucleotide. 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) was then added and the tubes heated to 80°C for 2 min prior to loading on a sequencing gel.

Samples were run on 7% acrylamide/urea gels between glass plates 38 cm wide and 45 cm long using 0.4 mm spacers. Gels were pre run at 1800 volts for 45 min, 2 µl of each termination reaction loaded (in the order GATC) and the gels run at 2000 volts for approximately 2 h or until the bromphenol blue reached the bottom of the gel. When sequencing further then approximately 200 bp successive loadings were made. At the completion of electrophoresis gels were transferred to Whatman® chromatography paper and dried using a gel drier. Gels were then exposed to Kodak X-omat® AR film.

H. GENERATION, PURIFICATION and PROPAGATION OF ADENOVIRUS VECTORS

1. DNA Transfection For Rescue Of Recombinant Adenovirus Vectors: Calcium Phosphate Coprecipitation

293 cells were transfected using the $CaPO_4$ technique of Graham and van der Eb (1973). 293 cells in 60 mm dishes were 80-90% confluent at the time of transfection after being split one or two days previously. For each virus to be rescued twenty 60 mm dishes were set up. For each 60 mm dish to be transfected 0.5 ml 1x HEPES-buffered saline (HEBS) (21 mM HEPES, 0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose, pH 7.1) was prepared with 10 µg/ml of salmon sperm DNA (1 mg/ml stock solution) as a carrier and vortexed to shear the DNA. Experimental DNA was added to the HEBS and carrier DNA (5 or 10 µg of each plasmid used to generate the viral vector). To the Hepes, carrier DNA, and experimental DNA, 50 µl/ml of 2.5 M $CaCl_2$ was added for a final concentration of 125 mM, gently mixed and incubated at RT for 15 to 30 minutes. The mixture was then added to the cells without removing the growth medium and the cells were incubated at 37°C in a CO_2 incubator for 4 hours. After transfection cells were overlayed with MEM F-11 containing 0.5% agarose, 5% (v/v) horse serum, 0.1% yeast extract, L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively and fungizone (2.5 µg/ml final concentration) and then incubated at 37°C. Viral plaques usually appear with 5 to 14 days.

2. Screening Adenovirus Plaque Isolates

Viral plaques obtained from cotransfections were picked by punching out agar plugs with a sterile Pasteur pipet and stored in 1 ml of 1x PBS^{++} (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl supplemented with 0.01 vol 68 mM $CaCl_2$ and 0.01 vol of 50 mM

$MgCl_2$) 10% glycerol at -80°C for later analysis of viral DNA. For each plaque to be analyzed one 60 mm dish of 293 cells was set up to be 80-90% confluent at the time of infection. Medium was removed from the dish, 0.5 ml of virus added, allowed to adsorb at 37°C in a CO_2 incubator for 30 min and then the cells were refed with 5 ml of MEM F11 containing 5% (v/v) horse serum, L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively), and fungizone (2.5 µg/ml final concentration). Dishes were harvested when most of the cells were rounded up and floating, usually within 3 to 4 days. To harvest infected cells were alowed to settle for 15 min in the tissue culture hood, 4 ml of medium was removed and stored in a glass vial containing 0.5 ml of sterile glycerol at -80°C. The remaining medium was aspirated slowly to avoid removing infected cells and 0.5 ml of pronase-SDS (0.05% (w/v) pronase in 10 mM Tris, pH 7.5, 10 mM EDTA and 0.5% (w/v) SDS) was added to each dish and incubated at 37°C for 4-18 h. The viscous lysate generated was transferred to a microfuge tube, extracted once with phenol and precipitated with 1 ml of 96% ethanol. The pellet was then washed twice in 96% ethanol, dried and dissolved in 100 µl of 0.1X SSC. 5 to 10 µl of the viral DNA was then analyzed by digestion with Hind III to determine if the desired recombinant was obtained. Recombinants with the correct structure were plaque purified once and reanalyzed.

3. Plaque Assays: For Purification And Titration

For plaque purification and titration of virus stocks 293 cells were set up in 60 mm dishes to be 90-95% confluent at the time of infection. For plaque purifications dilutions were set up in PBS^{++} ranging from 10^{-4} to 10^{-8} using the medium saved from the initial viral DNA analysis, and for titrations dilutions ranging from 10^{-6} to 10^{-10} were used. Titrations were performed in

duplicate. 0.5 ml of each dilution was added to a 60 mm dish and allowed to adsorb for 30 min at 37°C in a CO_2 incubator and then the cells were overlayed. For plaque purifications well isolated plaques were picked and reanalysed. For titrations plaques were counted 7 and 10 days post infection.

4. Preparation Of High Titer Viral Stocks (Crude Lysates) From Cells In Monolayer

To prepare high titer viral stocks from crude lysates 8 or more 150 mm dishes of 293 cells were set up to be 90-95% confluent at the time of infection. Medium was removed and the desired amount of virus (usually between 1-10 plaque forming units (pfu)/cell) was added to the cells in 1.0 ml of 1x PBS^{++}. When preparing a new stock the 4 ml of medium saved after DNA analysis following plaque purification was used (1 ml of a 1:8 dilution in PBS^{++}). Virus was allowed to absorb for 30 min and the cells refed with 25 ml of MEM F11 containing 5% (v/v) horse serum, L-glutamine (2 mM final concentration), penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively), and fungizone (2.5 µg/ml final concentration). When most of the cells were rounded up and floating the virus was harvested by scraping the cells from the dishes, centrifuging the cells and medium at 800 x g for 15 min, and then resuspending the pellet in 1 ml PBS^{++}-10% glycerol per 150 mm dish. These crude stocks were frozen and thawed once before titration. Stocks were stored at -80°C.

5. Preparation Of High Titer Viral Stocks (Purified) From Cells In Suspension

To prepare stocks of replication-defective vectors 293N3S cells were grown to a density of 2-4 x 10^5 cells/ml and for replication competent vectors KB cells were grown to a density of 4-5 x 10^5 cells/ml. 2-4 L of cells were pelleted at 750 xg for 30 min, resuspended in 1/10th the

volume of original medium and virus added (usually 5-10 pfu/cell) and allowed to absorb for 1 hr at 37°C with gentle stirring. The cells were then returned to the original volume using 50% fresh and 50% original medium. The infection was monitored by inclusion body staining once a day starting 48 h post infection. Briefly, 5 ml of culture was removed and spun down at 750 xg for 10 min. The pellet was resuspended in 0.5 ml of 1% sodium citrate, incubated at RT for 10 min, 0.5 ml of Carnoy's fixative (3:1 methanol:glacial acetic acid) added and incubated for a further 10 min at RT. Next 1 ml of 1% sodium citrate was added, the sample centrifuged at 750 xg for 10 min and the pellet resuspended in a few drops of 1% sodium citrate. One drop of fixed cells was added to a slide, allowed to air dry for 1 h and one drop of Orcein (2% (w/v) Orcein dye in 50% (v/v) acetic acid) and a coverslip were added. Under the microscope inclusion bodies appear as densely staining nuclear structures resulting from the accumulation of virus and viral products late in infection. Virus was harvested when inclusion bodies were present in 80-90% of the cells (usually 72 h post infection) by centrifuging at 750 xg for 30 min in 1 L bottles. Pellets were resuspended and combine in 8-9 ml of 0.1 M Tris, pH 8.0 per L of infected cells and stored at -80°C.

To band virus, crude stocks were thawed and 1/10 vol of 5% sodium deoxycholate added and incubated at RT for 30 min. To the viscous lysate 1/100 vol of 2 M $MgCl_2$ and 1/20 vol of DNAase (1 mg/ml) was added and incubated at 37°C for 30-60 min, with mixing every 10 min. This treatment should reduce the viscosity of the solution to approximately that of water. Next 1.8 ml of saturated CsCl solution (sufficient CsCl is added at RT to 10 mM Tris, pH 8.0, 1 mM EDTA to saturate the buffer) was added for every 3.1 ml of virus suspension to give a final density of 1.35 g/ml. The sample was then transferred to Beckman 50Ti ultraclear quickseal tubes and spun in a beckman 50Ti rotor for 16-20 h at 4°C and 35000 rpm. Viral bands were collected

in a small volume, pooled and centrifuged in a Beckman SW50.1 rotor at 35000 rpm at 4°C for 16-40 h. Viral bands were collected in the smallest volume possible and dialysed against 2 changes of 500 volumes PBS^{++}-10% glycerol at 4°C over 8 h. Stocks were stored at -80°C.

I. ANIMAL IMMUNIZATIONS

The ability of recombinant Ad/SIV vectors to raise an immune respond was tested by immunizing 5 week old female BALB/c mice both intraperitonally (Ip) and intranasally (In). Mice were housed in a Level III facility and immunizations carried out in a laminar biological safety cabinet. For Ip immunizations mice were given either 1 or 5 x 10^8 pfu of recombinant virus in 100 µl of PBS^{++} using a 26G needle. For In immunization mice were anaesthetized, inverted and 1 or 5 x 10^8 pfu of recombinant virus in 30 µl PBS^{++} was placed in their nares for inhalation. Five mice were used at each dose for each recombinant tested. To obtain preimmune sera ten mice were eye bled prior to the first immunizations. Mice were then bleed two weeks post primary immunization, boosted and then bled one week post boost. Blood samples was allowed to clot at 4°C overnight, centrifuged and serum collected into new microfuge tubes.

J. PROTEIN EXPRESSION AND DETECTION

1. β-galactosidase Assays

To perform β-galactosidase assays cells in 60 mm dishes were counted and infected with a β-galactosidase reporter vectors at the desired multiplicity of infection (moi) and then cells were harvested at various times post infection. To harvest, cells were scraped from the dishes and transferred with the media to 15 ml corning tubes, centrifuged at 3200 rpm for 10 min, the media aspirated and the pellets frozen at -20°C. Cell pellets were then thawed and lysed in 200 µl of

PMSF/NP40 solution (250 mM Tris, pH7.8, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.5% NP40), centrifuged at 3200 rpm for 15 min at 4°C and the clarified extracts transferred to new microfuge tubes. Serial dilutions of the cell extracts were made in PMSF/NP40 solution (1/10, 1/100 and 1/1000 dilutions were typically made). 40 µl of each sample in the dilution series was then added to 350 µl of mercaptoethanol solution (10 mM KCl, 1 mM $MgSO_4$, 100 mM sodium phosphate, pH 7.5, 50 mM 2-mercaptoethanol) and incubated at 37°C for 5 min. 132 µl of ONGP solution (13.3 mM o-nitrophenol β-D-galactopyranoside (ONGP) (Sigma) in 100 mM sodium phosphate, pH 7.5) was then added and the samples incubated at 37°C for 1 h. β-galactosidase hydrolyzes colourless ONGP to o-nitrophenol which is yellow. Reactions were then terminated by adding 172 µl of 1M Na_2CO_3 and the absorbance measured at 420 nm. To quantitate expression β-galactosidase (Boehringer Mannheim Canada, Ltd.) was diluted in PMSF/NP40 solution and assayed as above. Activity is expressed as µg of β-galactosidase produced per 10^6 cells.

2. Cell Extracts

Both labelled and unlabelled cell extracts were prepared. Labelled cell extracts were generated using Trans[^{35}S]-methionine ,L-[^{35}S]-cysteine (ICN Biomedicals, Canada Ltd.) or [^{3}H]-myristic acid (Amersham Canada Ltd.). 293, MRC5 or A549 cells in 60 mm dishes were infected with virus at the desired moi. For labelled extract the medium was removed at the appropriate time post infection (ie. 7-11 h for early proteins or 22-28 h for late proteins) and cells incubated at 37°C in 3 ml of 199-met medium (or -met-cys medium; no preincubation or special medium was used when labelling with [^{3}H]-myristic acid) supplemented with 2% (v/v) dialysed fetal bovine serum (Gibco BRL Life Technologies, Inc.), L-glutamine (2 mM final concentration) and

penicillin/streptomycin (100 U/ml and 0.1 mg/ml, final concentration respectively) for 45 min. The 199-met medium was then replaced with 1 ml of 199-met medium containing 50 µCi of Trans[^{35}S]-methionine and cells were labelled at 37°C for the desired time. When labelling was complete or for harvesting unlabelled extracts, the medium was removed, the monolayers washed with 2.5 ml of PBS^{++} and 0.5 ml of radioimmunoprecipitation assay buffer (RIPA) plus inhibitors (50 mM Tris, pH 7.5, 150 mM NaCl, 1% (w/v) SDS, 1% (v/v) Triton X-100, 10 µg aprotinin/ml (Sigma), and 0.2 mM PMSF) was added to the dishes. Cell extracts were collected by scraping, transfered to microfuge tubes, vortexed for 5 seconds then incubated on ice for 30 min. Samples were then centrifuged for 30 min at 4°C at 30,000 x g, the clarified extracts transfered to new microfuge tubes and either stored at -80°C, run on SDS-PAGE gels or first immunoprecipitated.

3. Immunoprecipitations

For immunoprecipitations 2 to 5 µl of antibody was added to 0.25 to 1.0 ml of clarified labelled cell extract, with 100 µl of protein A sepharose suspension (0.5 g in 5 ml of RIPA buffer) and gently rotated at 4°C for 2-3 h. Immunoprecipitates were then recovered by centrifugation and washed 4 times with 0.5 ml of RIPA buffer. The final pellets were resuspended in 45 µl of 2x SDS-PAGE loading buffer (1 mM Urea, 83 mM Tris, pH 6.8, 2.5% SDS, 12.5% β-mercaptoethanol, 27% glycerol, bromphenol blue) and boiled for 3 min prior to loading on SDS-PAGE gels.

4. Protein Electrophoresis

Cell extracts and immunoprecipitates were separated by discontinuous SDS-Polyacrylamide gel electrophoresis using a ratio of acrylamide:*N,N'*-methylenebisacrylamide of 40:1. The

concentration of acrylamide in the gels ranged from 6 to 18 % depending on the size of the proteins being analyzed. The separating gels contained the acrylamide solution in 0.375 M Tris, pH 8.8, 0.1% SDS and the stacking gels contained 5% acrylamide in 0.12 M Tris, pH 6.8, 0.1% SDS. Gels were polymerized by adding ammonium persulfate to 0.05% and 0.5 µl/ml of TEMED (*N,N,N',N'*-tetramethylethylenediamine). The electrophoresis buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS. Marker proteins used were [^{14}C]-methylated protein molecular weight markers (Amersham Canada Ltd.) ranging from MW 2,350-46,000 or MW 14,300-200,000 or prestained protein markers (Amersham Canada Ltd.) from MW 14,300-200,000 for Western blots. Gels were run in a Bio Rad® Protean® II Slab cell gel apparatus at 5 mAmps overnight or at 18 mAmps during the day.

To enhance the detection of labelled proteins fluorography was used. Following electrophoresis gels were soaked in two changes of 300 ml dimethyl sulfoxide (DMSO) for 30 min, 400 ml of DMSO containing 22% 2,5-diphenyloxazole (PPO) for 1 h and then in ddH_2O for 15 min. Gels were then transferred to Whatman® chromatography paper, dried using a gel drier and exposed to Kodak X-omat® AR film at -80°C (Bonner and Laskey, 1974).

5. Western Blots

Proteins were transferred from acrylamide gels to nylon membranes using a BioRad® transfer tank. Following electrophoresis gels were soaked in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.0, 20% methanol) for 15 min. Simultaneously a piece of Immobilon P nylon membrane (Millipore) was cut to the size of the gel, wet with methanol, soaked in ddH_2O for 2 min and then in transfer buffer for 10 min. A gel/membrane "sandwich" was then assembled containing two pieces of Whatman® chromatography paper (soaked in transfer buffer), the gel,

the membrane and finally two more pieces of Whatman. Air bubbles were removed using a 10 ml pipet as a rolling pin and the sandwich placed in the plastic support and then into the transfer tank with the membrane facing the positive electrode. Proteins were transferred at 25 V overnight or at 90 V for 3 h at 4°C.

Following transfer membranes were blocked by incubating in PBS containing 5% skim milk powder with shaking for 2 h at RT or overnight at 4°C. Membranes were then incubated for 1 h at RT in PBS-5% milk containing primary antibody at an appropriate dilution. Membranes were then washed three times 5 min in PBS-0.1% Tween 20 (BioRad) and incubated for 1 h at RT in PBS-5% skim milk containing the appropriate secondary antibody (secondary antibody is directed against the primary antibody used) conjugated to horse radish peroxidase. Membranes were then washed as described above and the proteins detected by enhanced chemiluminescence (ECL). Briefly, membranes were placed on Saran Wrap, protein side up, and covered with 0.125 ml/cm^2 of an equal volume mixture of detection reagent one and detection reagent (Amersham Canada Ltd.) two for 1 min. The detection reagents are a mixture of hydrogen peroxide and luminol, which is oxidized by the horseradish peroxidase conjugated to the secondary antibody. Following oxidation luminol is in an excited state which decays to the ground state resulting in the emission of light. The detection reagents were when drained from the blot, the blot placed between two overhead transparencies and exposed to Kodak X-omat® AR film at RT.

6. ELISA

Enzyme linked immunosorbant assays (ELISA's) were performed to determine the titer of specific anti-SIV and anti-adenovirus IgG antibodies generated in immunized BALB-c mice. To determine the titer of anti-SIV antibodies generated in mice immunized with AdCAgR, AdCAV3R

and AdHCMVsp1LacZ 96-well polystyrene microtiter plates were coated with 20 μg of VSMFC1 cell extract (one half of each plate was coated with induced cell extract and the other half with uninduced cell extract) in 100 μl of PBS for 16 h at 4°C. Wells were then washed three times with Tris-T (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.05% Tween 20) and blocked with 200 μl of reagent diluent (R.D.) (10 mg/ml BSA in Tris-T buffer) for 30 min at 37°C and then washed three times with Tris-T. 100 μl of mouse serum serially diluted in R.D. (starting dilution was 1:20) was then added to each well and the plates were incubated at 4°C for 16 h and then washed five times with Tris buffered saline (10 mM Tris, pH7.4, 150 mM NaCl). 100 μl of biotin-labelled goat anti-mouse IgG (Southern Biotechnology Associates) diluted 1:500 in R.D. was then added to each well for 2 h at RT, the plates were washed five times with TBS, 100 μl of Streptavidin conjugated alkaline phoshatase (Sigma) was added for 1 h at RT, the plates were washed as above and 100 μl of diethanolamine buffer (10 mM diethanolamine, 1 mM $MgCl_2$/ ml, pH 9.8, containing 4 mM *p*-Nitrophenyl phosphate (Sigma)) was added to each well and incubated at RT for 1 h. The optical density of each well was measured with an ELISA reader (Titertek® Multiskan Plus MKII, ICN Biomedicals Canada Ltd.) Anti SIV p27 antibody was used as a positive control in these ELISA's. After subtracting the O.D. obtained using uninduced cell extract from that obtained using induced cell extract for each serum sample the serum dilution at which there was at least 0.1 O.D. reading above control sera (serum from mice immunized with AdHCMVsp1LacZ) was taken as the anti-SIV antibody ELISA titer. To determine the titer of anti-adenovirus antibodies 96-well polystyrene microtiter plates were coated with 5 μg of wt Ad5 infected A549 cell extract or uninfected A549 cell extract and processed as described above. Pre-immune mouse sera was used as a negative control in these ELISA's.

RESULTS

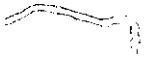
Much of the work described in this thesis has been published in four primary research articles. These publications are presented here, preceded in each case by a brief description of the experimental approach and followed by a list of my specific contributions and a synopsis of the main findings. Prior to and concomitant with the development of Ad vectors expressing various SIV genes a number of parameters important in vector construction and propagation were studied and improved systems for vector construction were developed. Studies investigating vector stability, expression of reporter genes in E1 and E3 and the development of the BHG vector system (an improved system for vector construction), all of which provided important insight on the development of the Ad/SIV vectors, are presented first followed by the description of the Ad/SIV vector work.

A. Packaging Capacity And Stability Of Human Adenovirus Type 5 Vectors (Bett *et al.*, 1993, J. Virol., 67:5911-5921).

1. Background

In this report adenovirus vectors with inserts in E3 were used to study the genetic stability of vectors in relation to net genome size. It had been generally accepted that adenovirus vectors are stable and that vectors with a net genome size of 105% of wt could be easily generated and propagated, although at the time this research project was initiated no careful or thorough

investigation of this question had been made. Ad vectors were constructed with inserts of 4.88 (HSV-1 gB), 4.10 (HSV-1 gB) or 3.82 kb (LacZ) combined with a 1.88 or 2.69 kb (the 2.69 kb deletion was newly generated and characterized in this paper) E3 deletion. The net excess of DNA over the wild type (wt) genome size ranged from 1.13 kb to 3.00 kb or 3.1% to 8.3%. The stability of these vectors was analyzed during serial passage in tissue culture.



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Packaging Capacity and Stability of Human Adenovirus Type 5 Vectors

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Adenovirus vectors are extensively used for high-level expression of proteins in mammalian cells and are receiving increasing attention for their potential use as live recombinant vaccines and as transducing viruses for use in gene therapy. Although it is commonly argued that one of the chief advantages of adenovirus vectors is their relative stability, this has not been thoroughly investigated. To examine the genetic stability of adenovirus type 5 vectors and in particular to examine the relationship between genetic stability and genome size, adenovirus vectors were constructed with inserts of 4.88 (herpes simplex virus type 1 gB), 4.10 (herpes simplex virus type 1 gB), or 3.82 (LacZ) kb combined with a 1.88-kb E3 deletion or with a newly generated 2.69-kb E3 deletion. The net excess of DNA over the wild-type (wt) genome size ranged from 1.13 to 3.00 kb or 3.1 to 8.3%. Analysis of these vectors during serial passage in tissue culture revealed that when the size exceeded 105% of the wt genome length by approximately 1.2 kb (4.88-kb insert combined with a 1.88-kb deletion), the resulting vector grew very poorly and underwent rapid rearrangement, resulting in loss of the insert after only a few passages. In contrast, vectors with inserts resulting in viral DNA close to or less than a net genome size of 105% of that of the wt grew well and were relatively stable. In general, viruses with genomes only slightly above 105% of that of the wt were unstable and the rapidity with which rearrangement occurred correlated with the size of the insert. These findings suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions and that exceeding the limit results in a sharply decreased rate of virus growth. The resultant strong selection for variants which have undergone rearrangement, generating smaller genomes, is manifested as genetic instability of the virus population.

For several years, human adenoviruses (Ads) have been used as vectors for expression of foreign genes in mammalian cells (1, 2, 5, 8, 11, 16, 19, 20, 25, 29, 32, 48, 50, 51, 57, 58) and studied for potential use in live viral vaccines (7, 9, 13, 20, 26, 28, 35, 37, 39–41, 57) and, more recently, for gene therapy (27, 34, 36, 42, 43, 45, 46, 52, 53, 56). A number of properties of the Ad system make it a good candidate for each of these applications, not the least of which is the extensive understanding of the structure and biology of Ads that has been gained through their use as a model system to study DNA replication, transcription, RNA processing, and protein synthesis (17). Other advantages include the ease with which the genome can be manipulated by simple recombinant DNA techniques, the high yields of virus that can be obtained and easily collected and concentrated, and the possibility of high-level expression of foreign DNA inserts (1, 19).

Construction of recombinant Ad vectors involves insertion of foreign DNA into the Ad genome, usually with compensating deletions of viral DNA. Previous work has shown that the Ad virion has the ability to package up to 105% of the wild-type (wt) genome length (15, 18), which allows for insertion of approximately 1.8 to 2.0 kb of excess DNA. By introduction of deletions in early region 1 (E1) or E3, vectors with correspondingly larger inserts can be constructed. E1 is not required for viral replication in complementing 293 cells which contain and express the left 11% of the Ad type 5 (Ad5) genome (22), and deletions of up to 2.9 kb have been made in this region (1, 18, 21, 51) to generate conditional helper-independent vectors with a capacity expected to be up to approximately 4.7 to 4.9 kb. The E3 region has been shown to be nonessential for viral replication in any normally permissive cells and can be deleted to produce nonconditional helper-independent vectors (3, 24). The maximum possible size of nonlethal deletions in the E3 region has not been determined, but they presumably cannot extend into the essential virion structural genes, those for pVIII and fiber, that flank this region. Many nonconditional helper-independent vectors have been developed by utilizing a 1.88-kb deletion in this region. Vectors of this type should have a capacity for inserts of approximately 3.7 to 3.9 kb (3).

Application of Ad vectors to gene therapy requires a more thorough examination of vector stability and packaging capacity than has yet been done. It is generally assumed that Ad vectors are relatively stable and can package DNA up to 105% of the size of the wt genome. To examine the genetic stability of Ad5 vectors and in particular to correlate genetic stability and genome size, a series of Ad vectors were constructed with E3 substitutions representing net insertions of up to 8.3% of the Ad5 genome. When the structures of these recombinant viruses were analyzed after serial passages in 293 cells, it was found that stability correlated with net insert size and vectors with the largest inserts rearranged extremely rapidly.

MATERIALS AND METHODS

Construction of recombinant plasmids. Enzymes used for recombinant DNA manipulations were purchased from Boehringer-Mannheim, Inc. (Laval, Quebec, Canada); New England BioLabs (Beverly, Mass.); or Bethesda Research Laboratories (Burlington, Ontario, Canada) and used in accordance with the supplier's recommendations. Plasmids

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were constructed by using standard protocols (47). Electroporation (12) was used to transform *Escherichia coli* DH5 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) with newly constructed plasmids. Plasmid DNA was prepared by the alkaline lysis method (4) and purified by CsCl-ethidium bromide density gradient centrifugation.

Cells and viruses. Cell culture media and reagents were obtained from GIBCO Laboratories (Grand Island, N.Y.). Ad vectors were titered and passaged on 293 cells, which constitutively express the left 11% of the Ad5 genome (22). The 293 cells were grown in monolayer in F-11 minimum essential medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2.5 μg of amphotericin per ml, along with 10% newborn calf serum for cell maintenance or 5% horse serum for virus infection. KB cells grown in spinner culture were maintained in Joklik's modified medium supplemented with antibiotics as described above and with 10% horse serum.

For one-step growth curves, KB cells were grown to a density of 2×10^5/ml, centrifuged, and resuspended in 1/10 of the volume of the original medium. Virus was then added at a multiplicity of infection (MOI) of 20 and allowed to adsorb for 1 h at 37°C with shaking. The cells were then returned to the original volume with 50% fresh medium and 50% original medium. At various times postinfection, 4-ml aliquots were taken, 0.5 ml of glycerol was added, and samples were stored at −70°C for assays of infectious virus by plaque titration.

Construction and growth of recombinant viruses. Recombinant viruses were isolated by cotransfection of 293 cells with appropriate plasmids (23). After 8 to 10 days, plaques were isolated and expanded and viral DNA was analyzed by restriction enzyme digestion as described previously (19, 24). Candidate viruses were then plaque purified once, and for stability studies, vectors were passaged by starting with medium from cells infected for viral DNA analysis after the first plaque purification. Semiconfluent monolayers of 293 cells in 60-mm-diameter dishes were infected with 0.5 ml of medium from each previous passage (an MOI of approximately 40), virus was allowed to adsorb for 0.5 h, and then the medium was replaced. Cells and medium were harvested when the cytopathic effect was complete, usually within 2 to 3 days postinfection.

^{32}P labelling and extraction of viral DNA. Semiconfluent monolayers of 293 cells in 60-mm dishes were infected with virus from passages to be analyzed, and at 24 h postinfection medium was removed and replaced with 1 ml of phosphate-free 199 medium containing 5% horse serum and 25 μCi of $^{32}P_i$ (Du Pont de Nemours & Co. Inc., Wilmington, Del.) per ml. After incubation of the infected cells for a further 6 h, they were harvested and DNA was extracted (19). Viral DNA was then digested with appropriate restriction enzymes and electrophoresed through 1% agarose gels, the gels were dried, and DNA bands were visualized by autoradiography.

^{35}S labelling of proteins. Semiconfluent monolayers of 293 cells in 60-mm dishes were infected at an MOI of 20. At 22 h postinfection, the medium was removed and replaced with 3 ml of methionine-free 199 medium supplemented with minimal essential medium vitamin solution (GIBCO), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2% dialyzed fetal calf serum and incubated at 37°C for 45 min. This medium was then removed and replaced with 1 ml of methionine-free medium containing 50 μCi of [^{35}S]methionine (Trans ^{35}S-label [1,000 Ci/mmol]; ICN Biomedicals Inc., Irvine, Calif.). The cells were labelled for 1 h at 37°C, washed with ice-cold phosphate-buffered saline; and harvested by scraping in 0.5 ml of ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 10 μg of aprotinin [Sigma, St. Louis, Mo.] per ml, 0.2 mM phenylmethylsulfonyl fluoride). The cell extracts were then vortexed and centrifuged for 30 min at 4°C and 30,000 × *g* and the supernatant was collected for analysis. Samples of cell extract were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8.0% gels, the gels were dried, and protein bands were visualized by autoradiography. Densitometry was performed with an LKB Ultroscan XL Enhanced Laser Densitometer.

RESULTS

A vector with a 39-kb genome is genetically unstable. As one of a series of Ad vectors expressing herpes simplex virus type 1 (HSV-1) glycoproteins, an Ad5 vector (AdgB10) with a 4.88-kb insert containing the HSV-1 gB gene in combination with an E3 deletion of 1.88 kb was constructed. The structure of this vector is illustrated in Fig. 1a. AdgB10 has a net insertion of 3.0 kb, representing 8.3% of the wt Ad5 genome, or approximately 1.2 kb more excess DNA than has been cloned in an Ad5 vector previously (1, 15). Initial findings indicated that AdgB10 replicated very inefficiently in early passages on 293 cells, requiring two to three times longer to induce a cytopathic effect following a typical infection of 293 cells compared with the wt virus or other vectors. After a few passages, a virus population which was able to replicate more rapidly than the original clone was obtained from the original AdgB10 plaque isolate. However, analysis of viral DNA from consecutive passages (Fig. 1b) indicated that the virus stock had undergone rapid rearrangement as determined by loss of the diagnostic 8.66-kb *Hin*dIII B′ fragment containing the HSV-1 gB insert (solid arrowhead in Fig. 1b). By passage 7, the viral DNA preparations no longer contained any detectable 8.66-kb *Hin*dIII fragment, which was replaced by a smaller fragment (open arrowhead in Fig. 1b) of about 3.2 kb that was first detected at passage 5. Thus, the increased growth rate seen as AdgB10 was serially passaged through 293 cells appeared to result from generation and outgrowth of a deletion variant with a net genome size approximately 95% of that of the wt. The faint bands seen between *Hin*dIII fragments A and C in the various passages shown in Fig. 1b are likely due to the presence of other deletion variants in the viral population. In two additional independent serial passages with AdgB10, two different rearrangements of the diagnostic 8.66-kb *Hin*dIII fragment were obtained, one in which a new fragment of 3.0 kb appeared at passage 2 and another in which a fragment of 3.4 kb was apparent by passage 4 (Fig. 1c). Since the *Hin*dIII B fragment containing just the 1.88-kb deletion results in a B′ fragment of 3.78 kb, it is apparent that in all three of these serial passages, AdgB10 had undergone a rearrangement which resulted in loss of all or part of the gB insert, as well as some of the surrounding Ad DNA. Because vectors made previously with smaller net insertions (less than 5.0% of the genome) had not demonstrated such severe instability (unpublished data), it seemed likely that the rapid appearance of rearrangements was due to the size of the AdgB10 genome. Since the packaging capacity of Ad virions is likely to be a limiting factor in the cloning and expression of some genes, we decided to develop vectors with an

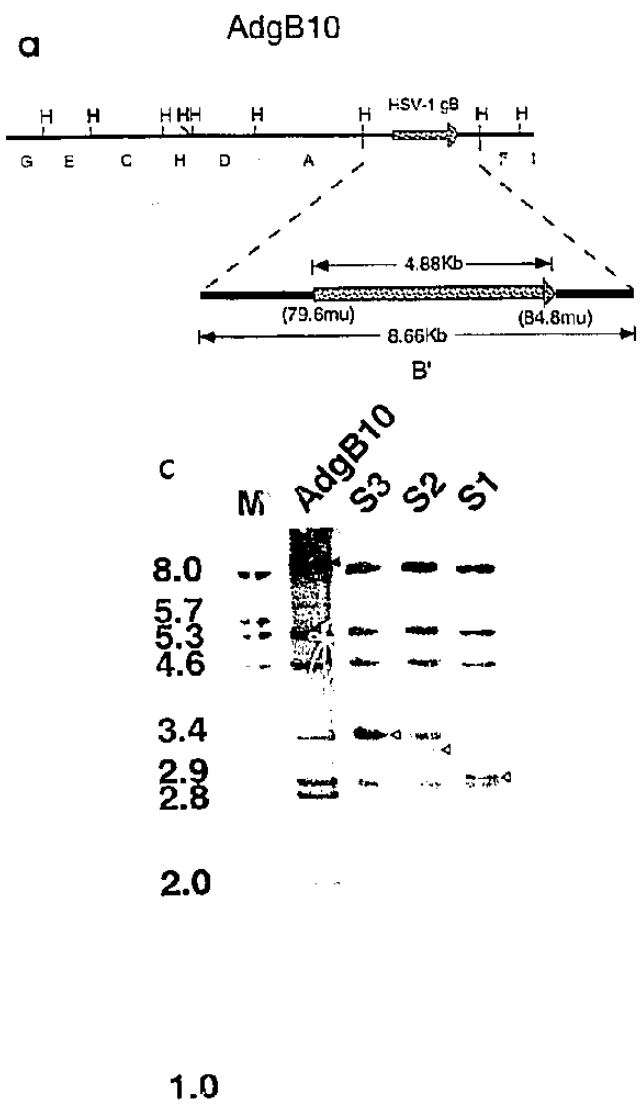
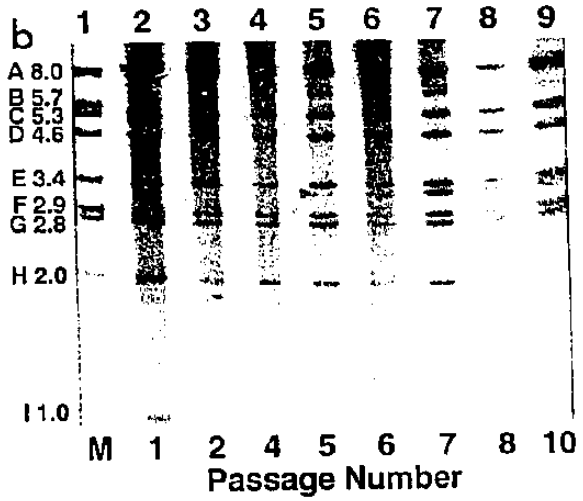


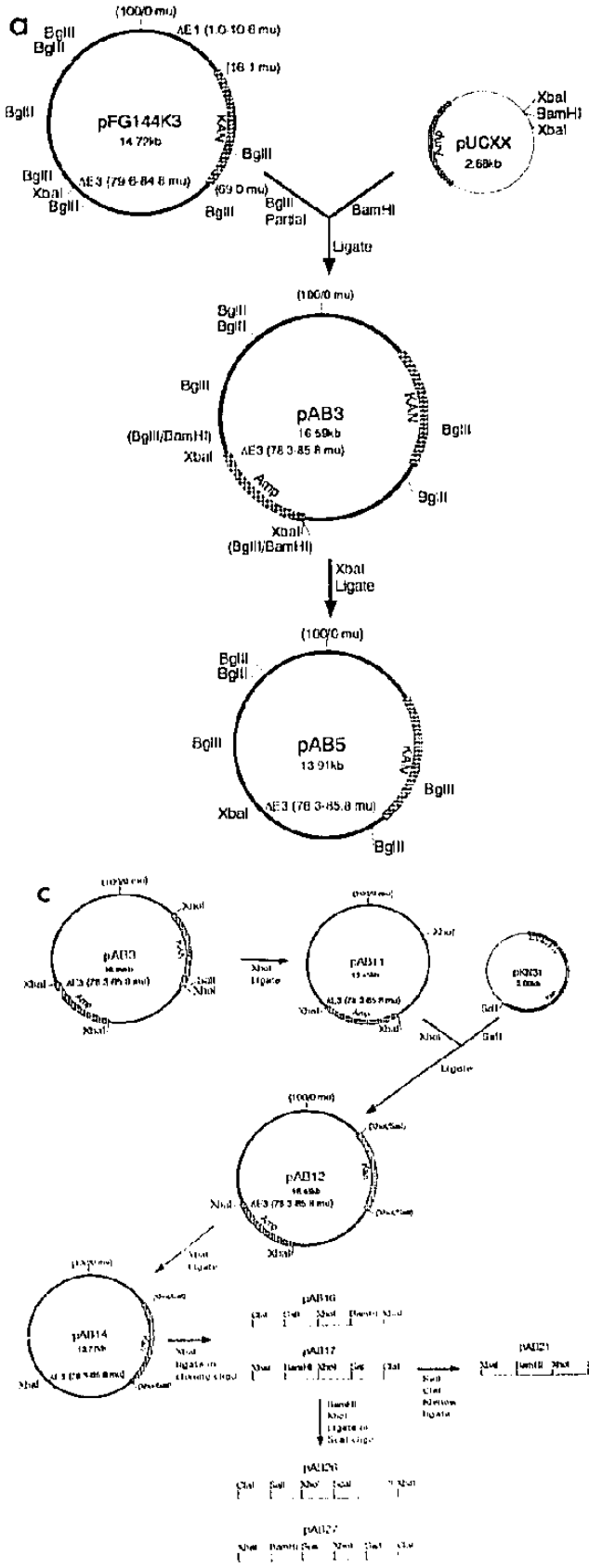
FIG. 1. Structure and genetic stability of AdgB10. (a) AdgB10 contains a 4.88-kb insert spanning the HSV-1 gB gene combined with an E3 deletion of 1.88 kb. *Hin*dIII restriction sites (H) are indicated above the line, and *Hin*dIII restriction fragments are labelled by letter below the line. Fragments A, C, D, E, F, G, and I are identical to wt Ad5 *Hin*dIII fragments, and B' is a diagnostic 8.66-kb fragment containing the 4.88-kb HSV-1 gB insert. Map units (mu) refer to positions on the Ad5 genome. The solid bars represent Ad5 sequences, and the stippled bars represent HSV-1 gB sequences. (b) *Hin*dIII digestion patterns for viral DNA from various passages of AdgB10. 293 cells were infected with virus from passages 1, 2, 4, 5, 6, 7, 8, and 10 for 24 h and then labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII, and separated by electrophoresis on a 1% agarose gel. The gel was then dried, and bands were visualized by autoradiography. Lane 1 contained wt Ad5 DNA digested with *Hin*dIII. Fragment sizes are indicated to the left in kilobases. Lanes 2 to 9 contained the passages of AdgB10 indicated below the lanes. In lane 2, the 8.66-kb fragment containing gB is indicated by a solid arrowhead. Over passages 2 to 5, the intensity of this fragment was reduced and replaced by a rearranged fragment of 3.2 kb, indicated by an open arrowhead in passage 5, lane 5. (c) *Hin*dIII digestion patterns for viral DNA representing the three rearrangements detected in three independent serial passages carried out with AdgB10. 293 cells were infected with virus from the initial plaque purification of AdgB10, passage 10 series 1, passage 10 series 2, and passage 8 series 3, for 24 h and then labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII, and separated by electrophoresis on a 1% agarose gel. The gel was then dried, and bands were visualized by autoradiography. Lane M contained wt Ad5 DNA digested with *Hin*dIII. Fragment sizes are indicated to the left in kilobases. The remaining lanes contained the series of AdgB10 indicated above. The 8.66-kb fragment containing gB is indicated by a solid arrowhead, and the rearranged fragments are indicated with open arrowheads. In series 3, a rearranged fragment of approximately 3.4 kb was present which ran as a doublet with *Hin*dIII fragment E, in series 2, a rearranged fragment of approximately 3.2 kb was seen; and in series 1, a rearranged fragment of approximately 3.0 kb was observed.



expanded E3 deletion, resulting in greater cloning capacity, and to study further the relationship between viral genome size and genetic stability.

Expansion of the E3 deletion and development of novel cloning vectors. To generate an expanded E3 deletion for use in vector construction, we started with plasmid pFG144K3 (Fig. 2a and reference 18a), which had been derived from pFG144 (14). Plasmid pFG144K3 contains Ad5 left-end sequences from 0 to 16.1 map units (m.u.) with a 3.47-kb deletion in E1 (1.0 to 10.6 m.u.) and right-end sequences from 69.0 to 100.0 m.u. with a 1.88-kb deletion in E3 (79.6 to 84.8 m.u.) which contains a unique *Xba*I cloning site. It is noninfectious but generates infectious virus following cotransfection with a second plasmid, pFG173 (25, 38) (see Fig. 3), resulting in rescue of genes cloned in the E3 region into viral vectors. pFG144K3 was chosen for further modifications designed to expand the E3 deletion and to introduce a variety of restriction enzyme sites to facilitate insertion of foreign DNA.

Figure 2a illustrates the strategy used to expand the E3 deletion in pFG144K3 from 1.88 to 2.69 kb. Examination of available restriction enzyme sites in the E3 region revealed that if the *Bgl*II sites at 78.3 and 85.8 m.u. were used, an E3 deletion of 2.69 kb could be generated that would not delete the essential virion structural genes, i.e., those for pVIII and fiber (Fig. 2b). Since pFG144K3 contains a total of seven *Bgl*II sites, partial digestion with *Bgl*II was performed and fragments of the appropriate size were gel purified and ligated with Amp^r plasmid pUCXX linearized with *Bam*HI. Restriction enzyme analysis was used to screen for the desired plasmid, pAB3, which contains pUCXX inserted, by fusion of *Bgl*II-*Bam*HI complementary ends, in place of the



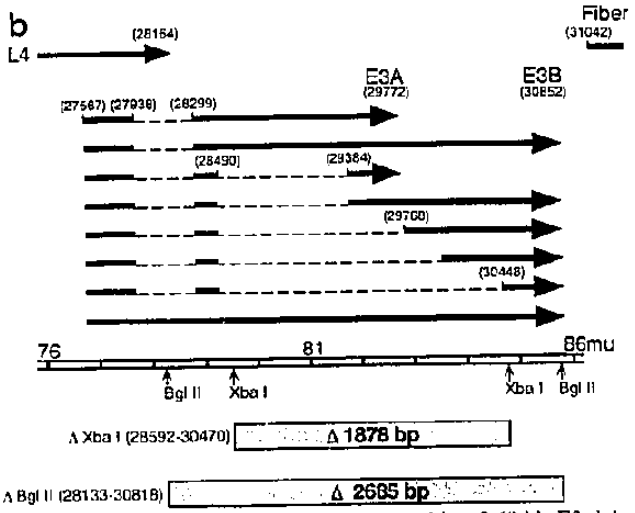


FIG. 2. Construction of shuttle vectors with a 2.69-kb E3 deletion. (a) To generate an E3 deletion of 2.69 kb between the *Bgl*II sites at 78.3 and 85.8 m.u., pFG144K3 was partially digested with *Bgl*II and fragments appropriate in size were gel purified and ligated with pUCXX linearized with *Bam*HI, generating pAB3 with pUCXX inserted in place of the deleted 800-bp *Bgl*II fragment in the E3 region. pAB3 was then digested with *Xba*I to remove pUCXX and recreate a single *Xba*I cloning site in the expanded E3 deletion in pAB5. (pUCXX was made from pUC19 by digestion with *Eco*RI and *Kpn*I and insertion of a synthetic oligonucleotide which recreated the *Eco*RI site, destroyed the *Kpn*I site, and introduced an *Xba*I site so that the *Bam*HI site was flanked by two *Xba*I sites.) (b) The structure of the E3 region for wt Ad5 is shown with the segments removed by the 1.88- and 2.69-kb deletions indicated below. The arrows indicate the structures of the E3 mRNAs, with solid lines representing exons, dashed lines represent introns, and arrowheads representing polyadenylation sites. The locations of transcription initiation sites, splice donor and acceptor sites, and polyadenylation sites are given in base pairs (10). (c) To facilitate insertion of foreign DNA segments into the expanded E3 deletion, additional cloning sites were introduced. pAB3 was digested with *Xho*I and religated to remove the Kan^r segment, generating pAB11, which was then digested with *Xho*I, and a new Kan^r plasmid, pKN31 (33), was inserted, generating pAB12. Digestion of pAB12 with *Xba*I and religation resulted in removal of the Amp^r segment to create pAB14. Finally, the polycloning sites indicated were introduced into the *Xba*I site of pAB14, generating pAB16, pAB17, pAB21, pAB26, and pAB27. Map units (mu) refer to Ad5 sequences: solid bars represent Ad5 sequences, and hatched bars represent Amp^r and Kan^r segments.

deleted 800-bp *Bgl*II fragment in the E3 region. pAB3 was then digested with *Xba*I and ligated to remove pUCXX sequences, generating pAB5. This step recreated a single *Xba*I cloning site in the expanded E3 deletion while leaving 20 bp of pUCXX DNA. The structure of the region spanning E3 for wt Ad5 and the deletions present in pFG144K3 and pAB5 are shown in Fig. 2b.

To facilitate insertion of foreign DNA segments into shuttle plasmids containing the expanded E3 deletion, additional cloning sites were introduced by the procedures outlined in Fig. 2c. So that *Sal*I and *Xho*I sites could be used as cloning sites, the Kan^r segment of pAB3 was removed by *Xho*I digestion and religation, generating pAB11, and a new Kan^r plasmid, pKN31, was inserted into the *Xho*I site of pAB11 by fusion of *Sal*I-*Xho*I complementary ends, generating pAB12. The Amp^r segment was then removed from the E3 region of pAB12 to generate pAB14. Various cloning

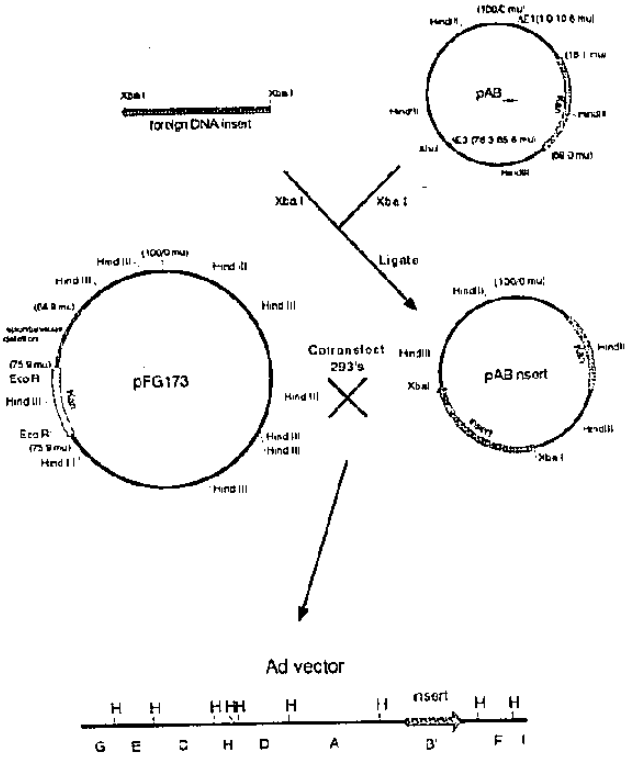


FIG. 3. Rescue of inserts into the E3 region with E3 shuttle vectors. The general strategy used to introduce foreign DNA inserts into either the 1.88- or 2.69-kb E3 deletions for rescue into virus is illustrated. The foreign DNA fragment insert was isolated with appropriate flanking restriction enzyme sites (*Xba*I in the example shown) for insertion into the E3 deletion of one of the pAB series of plasmids, generating a recombinant plasmid with the insert in the E3 parallel orientation. The recombinant plasmid, pABinsert, was then cotransfected with pFG173 (25, 38) into 293 cells, and infectious virus was generated by in vivo recombination.

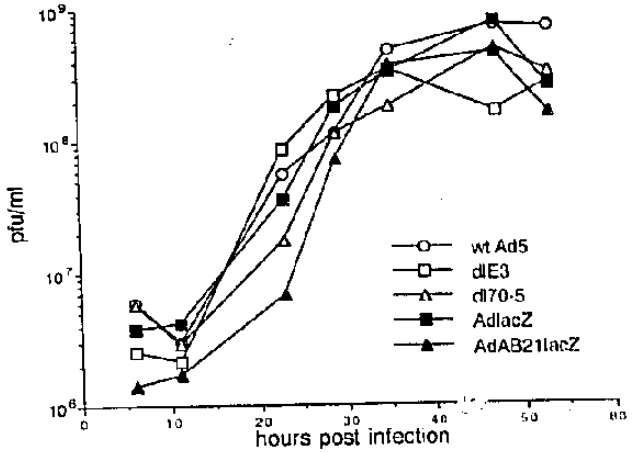


FIG. 4. Growth kinetics of viruses with the expanded E3 deletion. The growth properties of viruses with the 2.69-kb E3 deletion were studied and compared with those of wt Ad5 and viruses containing the 1.88-kb deletion by generating one-step growth curves. Spinner cultures of KB cells were infected at an MOI of 20 with wt Ad5, *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ, samples were taken at various times postinfection, and their titers were determined on 293 cells. *dl*70-5 contains the 2.66-kb E3 deletion, *dl*E3 contains the 1.88-kb E3 deletion, AdlacZ contains the *lacZ* gene flanked by the SV40 promoter and poly(A) sequences inserted in the 1.88-kb E3 deletion, and AdAB21lacZ contains the *lacZ* gene with SV40 poly(A) sequences inserted in the 2.66-kb E3 deletion. The *lacZ* inserts were introduced in the E3 parallel orientation.

sites were then introduced into the *Xba*I site of pAB14, generating shuttle vectors pAB16, pAB17, pAB21, pAB26, and pAB27.

All of the E3 deletion plasmids were sequenced in the region of the expanded deletion to confirm that they had the correct structures. The 2.69-kb E3 deletion present in the plasmids described above removed two additional elements that were not removed by the original 1.88-kb deletion. The 1.88-kb deletion removed the major parts of all E3 messages while leaving intact the E3 promoter and 5′ initiation site, the first set of E3 5′ and 3′ splice sites, and the E3 and L4 polyadenylation sites (3). As illustrated in Fig. 2b, the 2.69-kb E3 deletion, in addition to removing the major parts of all E3 messages, removed the first E3 3′ splice acceptor site and the L4 polyadenylation site (10).

Four of the plasmids described, pAB5, pAB14, pAB16, and pAB17, were rescued into virus by cotransfection with pFG173 (25, 38) into 293 cells, as illustrated in Fig. 3, generating viruses *dl*70-2, *dl*70-4, *dl*70-5, and *dl*70-6, respectively. These viruses were shown to have the expected structures by restriction analysis (data not shown).

As mentioned above, the newly created E3 deletion in the pAB series of shuttle plasmids lacked transcription signals that were present in earlier vectors with the 1.88-kb deletion. Therefore, it was of interest to analyze the growth properties of viruses containing this larger deletion. To do so, one-step growth curves were determined for several viruses containing either no deletion of E3 sequences (wt Ad5), the 1.88-kb deletion (*dl*E3), the 2.69-kb deletion (*dl*70-5), or a β-galactosidase gene inserted in the 1.88-kb deletion (AdlacZ) or in the 2.69-kb deletion (AdAB21lacZ) (Fig. 4). Spinner cultures of KB cells were infected at an MOI of 20, samples were taken at various times postinfection, and their titers were determined on 293 cells. The results shown in Fig. 4 indicated a somewhat lengthened eclipse phase for viruses with the 2.69-kb E3 deletion, but in other experiments this lag in virus replication was less pronounced (data not shown). Final yields of viruses with the 2.69-kb E3 deletion, with or without inserts, were always in the range of those obtained for wt Ad5 (data not shown).

To assess the effect of removal of the L4 polyadenylation site on expression of the L4 products, we compared the levels of 100K synthesized in 293 cells infected with wt Ad5, *dl*309 (31), *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ (Fig. 5). 293 cells were infected at an MOI of 20 and labelled with [^{35}S]methionine from 23 to 24 h postinfection, cell extracts were harvested, and samples were run on SDS–8.0% PAGE gels. The results shown in Fig. 5 indicated that there was, at most, a one-third reduction in the rate of synthesis of 100K for the viruses analyzed, suggesting that removal of the L4 polyadenylation signal by the 2.69-kb E3 deletion had not greatly affected the expression of L4 products. It is of interest that the level of fiber was reduced relative to those of other late proteins in vectors containing inserts in either the 1.88- or 2.69-kb E3 deletion. This reduction, which has been noted previously (38), seems to correlate inversely with the level of expression of the insert but does not appear to affect viral replication. Densitometry was used to more accurately determine the levels of 100K and fiber expressed by the various vectors. In Table 1, the results of two such analyses

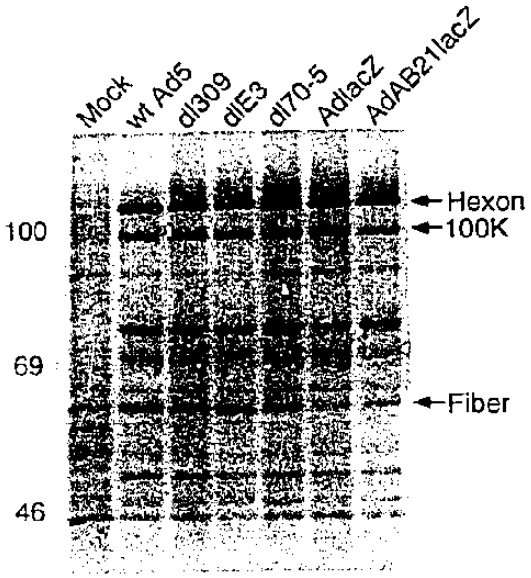


FIG. 5. 100K expression in viruses with the expanded E3 deletion. The rates of synthesis of 100K, an L4 product, for viruses with the 2.69-kb E3 deletion were determined and compared with those of wt Ad5 and viruses with the 1.88-kb deletion. 293 cells were either mock infected or infected at an MOI of 20 with wt Ad5, *dl*309, *dl*E3, *dl*70-5, AdlacZ, or AdAB21lacZ for 23 h and then labelled with [^{35}S]methionine from 23 to 24 h postinfection. Cell extracts were then prepared, and samples were separated by SDS-PAGE on an 8.0% gel. The gel was then dried, and bands were visualized by autoradiography. The relative levels of 100K present in the samples run on this gel were determined by densitometric analysis (Table 1). The lanes contained the samples indicated, and molecular weight markers are indicated on the left in thousands. *dl*70-5 carries the 2.66-kb E3 deletion, *dl*E3 has the 1.88-kb E3 deletion, AdlacZ contains the *lacZ* gene flanked by the SV40 promoter and poly(A) sequences inserted in the 1.88-kb E3 deletion, and AdAB21lacZ contains the *lacZ* gene with SV40 poly(A) sequences inserted in the 2.66-kb E3 deletion. The *lacZ* inserts were introduced in the E3 parallel orientation.

are shown; the first is from the autoradiogram presented in Fig. 5, and the second is from a gel on which recombinants with different inserts in the 2.69-kb E3 deletion were similarly analyzed (data not shown). In experiment 2 in Table 1, AdHTLV1gag2L is an Ad vector containing a 4.0-kb insert consisting of the human T-cell leukemia virus type 1 *gag* region and an Amp^r gene in the E3 antiparallel orientation (10a), and Ad5-luc11 and Ad5-luc11L are vectors containing a 1.7-kb insert of the luciferase gene in the E3 parallel and antiparallel orientations, respectively (37a). Densitometry revealed that viruses with the 1.88- or 2.69-kb E3 deletion or an insert in the 1.88-kb deletion expressed wt levels of 100K. In vectors that contain an insert in the 2.69-kb E3 deletion, the level of 100K varied from 92 to 67% relative to that in the wt, depending on the insert and its orientation. In viruses with the 2.69-kb deletion and no insert, it is possible that the E3b polyadenylation site can functionally substitute for the L4 polyadenylation signal (Fig. 2b). Differences in 100K levels seen with vectors carrying different E3 insertions may be due to the presence or absence of sequences in inserted genes that can act as fortuitous polyadenylation signals for L4 transcripts. The reduced levels of 100K and fiber synthesis seen with some vectors appear not to have significant effects on infectious virus yields, probably because viral late proteins are normally made in excess during viral replication. In a recent report by Ranheim et al. (44), a 3.0-kb E3 deletion was generated that also removed the L4 polyadenylation signal; it was also found that viruses with this deletion replicate to the same final titers as wt Ad5.

TABLE 1. Synthesis of 100K and fiber in cells infected with E3 deletion viruses[a]

Expt and virus	Size of E3 deletion (kb)	Expression level (%)[b]	
		100K	Fiber
1			
wt Ad5	0	100	100
*dl*309	Del-sub[c]	113	100
*dl*E3	1.88	102	135
*dl*70-5	2.69	98	87
AdlacZ	1.88	95	41
AdAB21lacZ	2.69	67	39
2			
*dl*309	Del-sub[c]	100	100
*dl*70-4	2.69	113	112
*dl*70-5	2.69	105	118
AdHTLV1gag2L	2.69	92	30
Ad5-luc11	2.69	68	38
Ad5-luc11L	2.69	86	92

[a] Cells infected with the indicated viruses were labelled with [^{35}S]methionine and cell extracts were prepared and run on polyacrylamide gels. The levels of 100K and fiber were determined by densitometric analysis.

[b] For each virus, the levels of 100K and fiber were determined relative to that of hexon and then expressed as a percentage of the wt Ad5 level (experiment 1) or the *dl*309 level (experiment 2).

[c] The deletion and substitution present in *dl*309 (31).

Construction and characterization of additional vectors. With the verification that the growth kinetics and expression of L4 products in vectors containing the expanded 2.69-kb E3 deletion were not significantly altered, it was decided to utilize the pAB shuttle vectors in further analyses of vector stability. By substituting inserts of various sizes for the 1.88- and 2.69-kb E3 deletions, vectors with a range of net genome sizes were easily generated. The DNA segments introduced into the two E3 deletions were a 4.88-kb fragment containing the transcriptional and translational information for HSV-1 gB, a 4.10-kb fragment containing the coding sequences for HSV-1 gB fused to the simian virus 40 (SV40) promoter, and a 3.82-kb fragment containing the coding sequences for LacZ flanked by the SV40 promoter and poly(A) signal. The inserts were introduced into shuttle plasmids containing the 1.88- or 2.69-kb E3 deletion and rescued into virus by cotransfection with pFG173 into 293 cells as outlined in Fig. 3.

Viral plaques obtained from cotransfections were isolated and plaque purified once, and viral DNA was analyzed by restriction enzyme digestion to ensure that the desired recombinant was obtained. The vectors constructed (Fig. 6) represent a series of viruses with net genome sizes ranging from 103.1 to 108.3% of the wt Ad5 genome length (Table 2).

As discussed previously, AdgB10, with a genome size of 108.3% relative to the wt, grew very inefficiently and underwent rapid rearrangement, resulting in the outgrowth of deletion variants able to replicate more rapidly. Detection of three different rearranged forms after three independent serial passages of the AdgB10 isolate (Fig. 1c) indicated that the appearance of rearrangements was not due to selection for a single variant preexisting in the virus population. Rather, AdgB10 was able to generate a number of different variants, each of which replicated sufficiently well to predominate after a few passages.

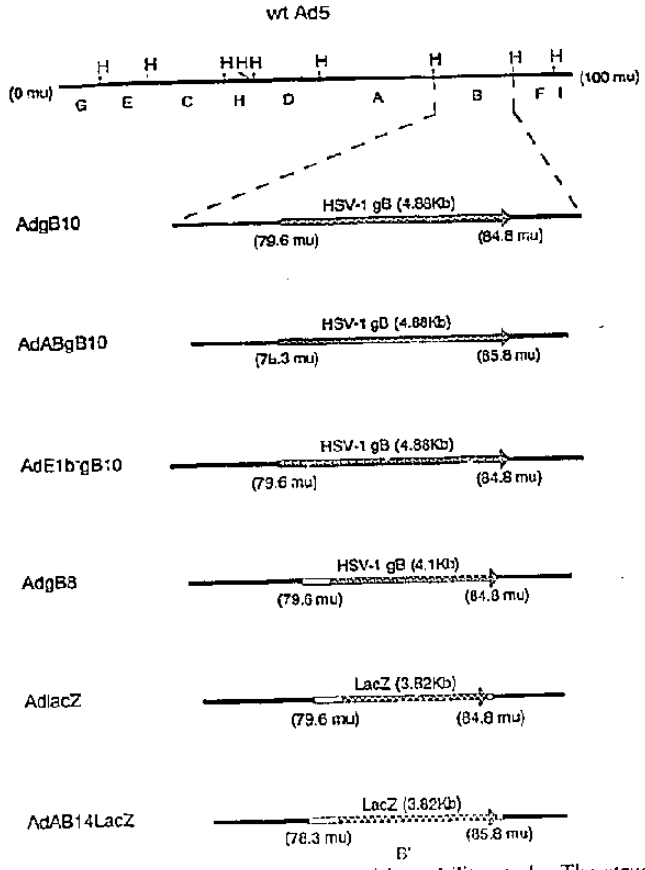


FIG. 6. Structures of viruses used in stability study. The structures of the viruses used in the analysis of vector stability are shown. *Hin*dIII restriction fragments are indicated by letter, and the *Hin*dIII B' fragment of each virus is expanded, showing the DNA segment inserted and the E3 deletion used. AdABgB10, AdgB8, AdLacZ, and AdAB14LacZ were all constructed by using basically the strategy outlined in Fig. 3. AdE1b⁻gB10 was created by using a different strategy and also contains a deletion of E1b sequences from 5.5 to 9.3 m.u. AdE1b⁻gB10 was created by digesting pFGdx1gB with *Spe*I and ligating it with Ad5*dl*55 viral DNA (18a) digested with *Spe*I and *Eco*RI (the latter enzyme was used to reduce the infectivity of parental *dl*55 DNA). The ligated DNA was then used to transfect 293 cells, and viral plaques were picked, expanded, and analyzed by restriction digestion to identify the desired E1b⁻ recombinants. Map units (mu) refer to Ad5 sequences. The solid bars represent Ad5 sequences, hatched bars represent inserts of HSV-1 gB or LacZ, as indicated, and open bars represent SV40 sequences.

To obtain a vector similar to AdgB10 but with a smaller net genome size, AdABgB10 was constructed by combining the 4.88-kb HSV-1 gB insert with the 2.69-kb E3 deletion (Fig. 7a). AdABgB10 has a net genome size of 106.1% of that of wt Ad5 and was found to replicate normally and induce a cytopathic effect following an incubation time similar to that required for wt Ad5 and other vectors during a typical infection of 293 cells. When AdABgB10 was serially passaged through 293 cells, it was found to be significantly more stable than AdgB10, taking severalfold more passages for rearrangements to appear. Figure 7b shows one of three independent stability assays performed with AdABgB10, in which the various passages shown were analyzed by restriction digestion with *Hin*dIII and *Xba*I. *Hin*dIII-*Xba*I double digestion was used in this case, since digestion with *Hin*dIII alone resulted in a B' fragment of 7.86 kb which ran as a doublet with *Hin*dIII fragment A, making analysis difficult. The 4.88-kb gB insert which was excised by *Xba*I is indicated by a solid arrow in Fig. 7b (lane 2). In this series, two deletion variants were detected, one at passage 14, when a fragment of 2.7 kb was first observed, and the second at passage 22, when a fragment of 1.9 kb appeared. These new fragments (indicated by open arrows in Fig. 7b) became more intense during successive passages as the 4.88-kb gB fragment became less intense. The first variant detected at passage 14 appeared to have deleted all or part of the gB insert and possibly some essential Ad sequences, thus generating a defective virus unable to completely overtake the viral population. Following low-MOI infections with virus from passage 14, only the parental AdABgB10 digest pattern was detected, consistent with elimination of a defective helper-dependent variant from the population (data not shown). The second variant detected in passage 22, which also appeared to have deleted gB insert sequences, was not defective, since in low-MOI infections with virus from passage 22 the variant pattern was detected, with complete loss of the 4.88-kb gB fragment (data not shown). In the other two stability assays done with AdABgB10, two different rearrangements were observed, which were first detected at passages 9 and 10 (Table 2). These rearrangements did not involve the gB insert in E3 but appeared to result in loss of essential sequences in *Hin*dIII fragment C (m.u. 17.4 to 32.2). That these variants represented defective, helper-dependent viruses was confirmed by further analysis, since they were eliminated following low-MOI infections and only the parental AdABgB10 digest pattern was detected (data not shown).

The detection of rearrangements involving *Hin*dIII fragment C in two of the stability assays performed with AdABgB10 seemed to suggest that the important factor causing the rearrangement of the vectors was the net genome size and not the insert or the flanking Ad sequences. To confirm that the difference in stability between AdgB10 and AdABgB10 was not due to the difference in the context of the

TABLE 2. Stability of Ad5 vectors having genomes of various sizes[a]

Virus	Deletion in E3 (kb)	Insert size (kb)	Net genome size (%)[b]	Rearrangement(s) detected; passage no(s).
Ad5			100	
AdgB10	1.88	4.88	108.3	S1, p2[c]; S2, p5; S3, p4
AdgB8	1.88	4.10	106.2	S1, p8; S2, p7; S3, p9
AdABgB10	2.69	4.88	106.1	S1, p14; S2, p10[c]; S3, p9[c]
AdlacZ	1.88	3.82	105.4	S1, p7; S2, p6
AdE1b⁻gB10[d]	1.88	4.88	104.6	No change to passage 20
AdAB14lacZ	2.69	3.82	103.1	S1, p19; S2, p20

[a] DNAs from Ad5 vectors serially passaged in 293 cells were analyzed by restriction enzyme digestion and gel electrophoresis.

[b] Net genome size represents the insert size minus the deletion of E3 sequences divided by the wt genome length (35,935 bp) and is expressed as a percentage.

[c] Novel bands were detected at the indicated passages (p#) indicating the appearance of viruses with rearranged genomes. S1 and S2, etc., represent independent serial passages starting from the same plaque isolate.

[d] Rearrangements involved loss of sequences in *Hin*dIII fragment C.

[e] Vector with deletions in both E1 and E3.

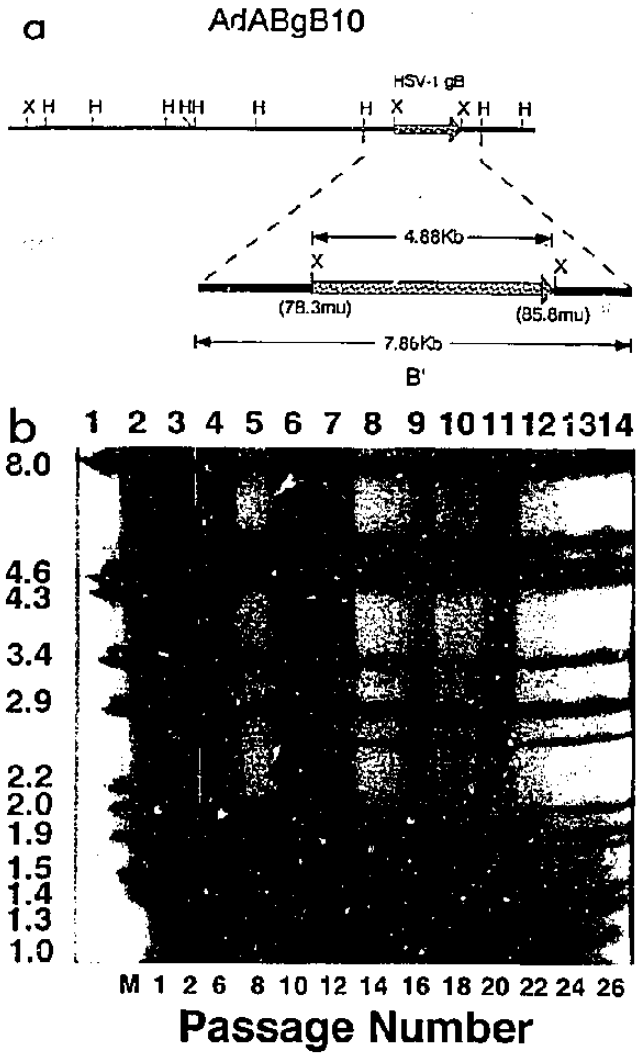


FIG. 7. Structure and genetic stability of AdABgB10. (a) AdABgB10 contains a 4.88-kb insert combined with a 2.69-kb E3 deletion. The 4.88-kb insert contains the transcriptional and translational information for HSV-1 gB. The *Hin*dIII and *Xba*I restriction sites are indicated. Map units (mu) refer to Ad5 sequences. The solid bars represent Ad5 sequences, and the stippled bars represent HSV-1 gB sequences. (b) *Hin*dIII-*Xba*I restriction analysis of passages of AdABgB10 are shown. 293 cells were infected with virus from various passages, as indicated at the bottom, and labelled with $^{32}P_i$ from 24 to 30 h postinfection. Infected cell DNA was extracted, digested with *Hin*dIII and *Xba*I, and separated by electrophoresis on a 1% agarose gel, the gel was dried, and bands were visualized by autoradiography. Lane 1 contained wt Ad5 DNA digested with *Hin*dIII and *Xba*I. Fragment sizes are indicated to the left in kilobases. Lanes 2 to 14 contained the passages of AdABgB10 indicated below the lanes. In lane 2, the 4.8-kb gB fragment is indicated with a solid arrowhead. Rearrangement was first detected at passage 14, when a fragment of 2.8 kb (open arrow, lane 8) began to appear, and a second rearrangement was detected at passage 22 (open arrow, lane 12), when a 1.9-kb fragment became apparent.

E3 region containing the gB insert in these vectors and to generate a vector with a smaller net genome size, AdE1b⁻gB10 was created. AdE1b⁻gB10 contained the same structure flanking the E3 region (4.88-kb HSV-1 gB insert in the 1.88-kb E3 deletion) as AdgB10 but also had a deletion of E1b sequences from 5.5 to 9.3 m.u., resulting in a net genome size of 104.6% of the wt Ad5 genome (Fig. 6 and Table 2). When viral DNA from serial passages of AdE1b⁻gB10 was analyzed, no rearrangements were detected to passage 20, indicating that the net genome size of AdgB10, rather than the sequences in the vicinity of the insert, was the cause of its genetic instability.

The results discussed above suggested that as the net genome sizes of the vectors decreased, the viruses became increasingly genetically stable. To analyze the stability of viruses with different inserts in the E3 deletions, the structures of three additional vectors, AdgB8, AdlacZ, and AdAB14lacZ, were analyzed following serial passage. AdgB8, which had a net genome size of 106.2% of the wt Ad5 genome (Fig. 6) was found to undergo rearrangements which appeared to involve the gB insert and surrounding Ad sequences, first detected at passages 8, 7, and 9 in the three independent stability assays performed with this vector (Table 2). AdgB8, whose genome size is very close to that of AdABgB10, was found to have essentially the same degree of stability. AdlacZ and AdAB14lacZ (Fig. 6) had net genome sizes of 105.4 and 103.1% of the wt Ad5 genome, respectively. AdlacZ was found to undergo rearrangements first detected at passages 7 and 6 in the two stability assays carried out with this vector (Table 2). The stability of AdAB14lacZ was found to be dramatically increased, with rearrangements not being detected until passages 19 and 20 (Table 2). The deletion variants detected for these two vectors appeared to have lost insert sequences and surrounding Ad DNA, were apparently nondefective, and overtook the viral population a few passages after they were first detected. The results obtained from the analysis of these additional vectors seemed to confirm that as the net genome size decreased, the stability of the virus increased.

To rule out the possibility that differences in the level of expression of gB protein obtained with the gB expression vectors affected stability, the amounts of gB protein produced by AdABgB10, AdE1b⁻gB10, and AdgB8 were compared by immunoprecipitation (data not shown). The levels of expression were similar for all three viruses and therefore did not appear to contribute significantly to the stability of the vectors. Because of the poor growth and rapid rearrangement of AdgB10, expression from this vector could not be analyzed.

DISCUSSION

In this study, the genetic stability of Ad5 vectors with net genome sizes ranging from 103.1 to 108.3% of the wt genome length was analyzed after serial passage in 293 cells. This range of genome sizes was obtained by constructing vectors with inserts of 4.88 (HSV-1 gB), 4.10 (HSV-1 gB), or 3.8 (LacZ) kb combined with a deletion in E3 of 1.88 or 2.69 kb. The results presented here show that the genetic stability of Ad5 vectors depends on net genome size. We have found that the larger the genome size over that of wt Ad5, the more genetically unstable the vector and the more rapidly it undergoes rearrangement.

Previous work has suggested that the Ad5 virion has the ability to package approximately 105% of the wt genome length, and this value is generally considered to be the maximum working capacity of the system (1, 15). Three of the largest genomes reported to date are found in Ad5in307 (30), Ad5in52 (15), and Ad5(pymT) (2), which have net genome sizes of 105.0, 105.7, and 106.0% of the wt Ad5 genome, respectively. AdgB10, the largest vector analyzed

in this study, exceeds 105% of the wt genome length by about 1.2 kb and was found to replicate much less efficiently than wt Ad5, requiring two to three times longer to induce a cytopathic effect following a typical infection of 293 cells. Analysis of viral DNA from serial passages of AdgB10 revealed that it was extremely unstable and underwent rearrangement within three to four passages (Fig. 1b and c). The other vectors analyzed in this study could be propagated with an efficiency indistinguishable from that of wt Ad5, even though they exhibited very different stabilities.

Vectors AdABgB10 (106.1%), AdgB8 (106.2%), and AdlacZ (105.4%), with net genome sizes much closer to 105% of the wt Ad5 genome, while more genetically stable than AdgB10, were still found to undergo rearrangement relatively quickly during serial passage. On average, rearrangements were first detected for these vectors by passage 9. Vectors AdE1b$^-$gB10 (104.6%) and AdAB14lacZ (103.1%), with net genome sizes below 105% of the wt genome length were significantly more stable. Rearrangement of AdAB14 lacZ was not detected until approximately passage 20 in the two series done with this vector, and rearrangement of AdE1b$^-$gB10 has not been detected to passage 20. These findings suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions and that exceeding this limit results in sharply decreased yields of infectious virus. The packaging limit of SV40, a virus that, like Ad, has an icosahedral capsid, has also been reported to be approximately 105% (54). In a study similar to the one presented here, in which the packaging capacities of SV40 capsids were analyzed, similar results were observed in that the stability of the SV40 genomes correlated with net genome size (6). The tight packaging constraints seen for Ads and SV40 are likely due to the icosahedral structure of the capsid. In a report by Chang and Wilson (6), it is suggested that the packaging limit be thought of as a steep gradient, with the probability of being packaged becoming reduced with increased genome size. It is unclear whether the poor growth properties of viruses with oversized genomes are strictly due to the inability of larger genomes to be packaged or whether packaging of oversized viral genomes also results in physical instability of the virion, which would contribute to the poor growth properties of such viruses. Whatever the cause, the decreased growth rate, which was very obvious for AdgB10 but less apparent for the other vectors analyzed, presumably results in strong selection for variants which have undergone rearrangement, generating smaller genomes.

The rearrangements detected for the vectors analyzed in this study appeared to result in viable deletion variants that lost insert sequences and some surrounding Ad DNA in all cases, except for the three serial passages of AdABgB10 in which defective variants that could not replicate in the absence of helper functions provided by the parental virus were generated. In two of the serial passages of AdABgB10, variants that had lost essential viral sequences in *Hin*dIII fragment C (m.u. 17.4 to 32.2) arose, and in the third series (Fig. 7b), one variant lost insert sequences and presumably essential viral sequences surrounding the insert, while a second variant appeared to be viable and would likely have gone on to overtake the viral population in subsequent passages. The detection of rearrangements occurring in *Hin*dIII fragment C for AdABgB10, rather than in the insert, supports the idea that it is neither the specific insert sequences nor the context of the E3 substitution that causes genetic instability. AdgB10 and AdE1b$^-$gB10, which contain the same gB insert and flanking E3 sequences, exhibited very different stabilities, which could only be attributed to net genome size. It seems likely that in the viral population, variants with deletions in all areas of the genome arise but the selection for nondefective variants which can replicate more efficiently is very strong and therefore most of the variants we detected have lost nonessential insert sequences. In view of this, it is surprising that we did not detect variants with deletions in the E1 region, which is nonessential in these vectors because they were passaged in 293 cells which complement E1 functions.

The differences in stability observed for the vectors that express gB (AdABgB10, AdE1b gB10, and AdgB8) could not be attributed to differences in expression levels, since it was found that all three vectors expressed comparable amounts of gB. It does seem likely, however, that other vectors with sequences whose presence or expression is deleterious to viral replication could result in rapid selection for variants in which the insert has been deleted.

The most frequently used E3 deletion for vector construction is a 1.88-kb E3 deletion which, on the basis of the data presented here, should allow construction of stable vectors with inserts of 3.7 to 3.9 kb. Other E3 deletions which have also been utilized include a 1.65-kb deletion from 79.4 to 84.0 m.u. (11), a 2.29-kb deletion from 78.5 to 84.3 m.u. (49, 55), a 2.48-kb deletion from 78.8 to 85.7 m.u. (8), and a 3.0-kb deletion from 77.7 to 86.1 m.u. (44). In this report, we have described the construction of a number of plasmids with a 2.69-kb deletion in E3 that contain various restriction enzyme sites that facilitate the introduction of DNA inserts for rescue into infectious virus. This deletion, in addition to removing the major portion of all of the E3 messages, removed the first E3 splice acceptor site and the L4 polyadenylation site. The removal of these transcription signals did not appear to affect the growth kinetics (Fig. 4), progeny virus yield, or 100K expression levels (Fig. 5 and Table 1) significantly which suggests that removal of the L4 polyadenylation site did not seriously affect expression of the L4 products. This expanded E3 deletion should increase the working capacity of nonconditional helper-independent vectors to 4.5 to 4.7 kb.

The findings described in this report have important implications for the construction and use of vectors for expression, in live viral vaccines studies, and as gene transfer vectors for gene therapy. We found that although vectors with net genome sizes of approximately 105 to 106% of the wt Ad5 genome length can be easily generated, they can be genetically unstable and rearrange to generate variants with smaller genomes during propagation. Therefore, it may be prudent to limit the size of inserts as much as possible and to monitor carefully the structure of viral DNA for vectors whose genomes approach or exceed the size limits defined by these analyses.

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2. Contributions To Bett *et al.* (1993)

(A) Construction of the E3 shuttle plasmids pAB5, pAB14, pAB16, pAB17, pAB21, pAB26 and pAB27 containing an expanded E3 deletion of 2.69 kb. These plasmids contain various cloning sites at the position of the deletion to facilitate the insertion of foreign DNA. All plasmids were sequenced in the region of the deletion to confirm that they had the correct structure.

(B) Generation of Ad vectors dl70-2, dl70-4, dl70-5, dl70-6, AdABgB10, AdE1b⁻gB10 and AdAB14LacZ. These vectors were used in this study to characterize the growth properties of viruses with the 2.69 kb E3 deletion, to analyze the expression of late products flanking the 2.69 kb deletion as well as to study the genetic stability of vectors in relation to net genome size.

(C) Serial passage in tissue culture and viral DNA analysis of all vectors used in this study.

3. Summary

In Bett *et al.* (1993) it was found that viruses with genomes only slightly above 105% of wt were unstable, and the rapidity with which rearrangement occurred correlated with the size of the insert. These observations suggest that there is a relatively tight constraint on the amount of DNA which can be packaged into virions, and exceeding the limit results in a sharply decreased rate of virus growth. The resultant strong selection for variants which have undergone rearrangement generating smaller genomes is manifested as genetic instability of the virus population.

These observations have important implications for the construction and use of vectors for expression purposes, live viral vaccines studies, and as gene transfer vectors for gene therapy. Although vectors can be easily generated with net genome sizes of approximately 105-106% of the wt Ad5 genome length they can be unstable and rearrange to generate variants with smaller

genomes during propagation. It therefore may be prudent to limit the size of inserts as much as possible and to monitor carefully the structure of viral DNA for vectors whose genomes approach or exceed 105%

In this paper we also described the construction of a number of E3 shuttle plasmids that can be used to construct Ad5 vectors with inserts in early region 3 (E3) by cotransfection of 293 cells with pFG173. These plasmids have an increased E3 deletion of 2.69 kb and contain various restriction enzyme sites to facilitate the introduction of foreign DNA inserts for rescue into infectious virus. This expanded E3 deletion increases the capacity of nonconditional helper independent vectors to 4.5-4.7 kb which was essential for the construction of the replication competent Ad/SIV vectors to be described in results section E.1.

B. An Efficient And Flexible System For Construction Of Adenovirus Vectors With Insertions Or Deletions In Early Regions 1 and 3 (Bett *et al.*, 1994, PNAS 91:8802-8806).

1. Background

As described in the introduction (section A.6) a number of strategies have been developed in the past to construct Ad vectors, the most useful being those based on recombination between two plasmids which together contain sequences comprising the entire Ad genome. The plasmid systems developed in the past allowed the rescue of inserts into E1 (McGrory *et al.*, 1988) or E3 (Ghosh-Choudhury *et al.*, 1986; Mittal *et al.*, 1993) but no simple procedure had been developed for combining both E1 and E3 deletions or substitutions in the same vector. To develop a system for construction of Ad5 vectors that combine E1 and E3 deletions or substitutions, and to simplify Ad vector production, we developed a new methodology based on a series of bacterial plasmids (pBHG) containing most of the viral genome in circular form, but lacking the DNA packaging signal. In this paper we describe the design and development of this new system and its use in generating a vector with an insert of 7.8 kb in the E1 region.

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An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3

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ABSTRACT **Human adenoviruses (Ads) are attracting considerable attention because of their potential utility for gene transfer and gene therapy, for development of live viral vectored vaccines, and for protein expression in mammalian cells. Engineering Ad vectors for these applications requires a variety of reagents in the form of Ads and bacterial plasmids containing viral DNA sequences and requires different strategies for construction of vectors for different purposes. To simplify Ad vector construction and develop a procedure with maximum flexibility, efficiency, and cloning capacity, we have developed a vector system based on use of Ad5 DNA sequences cloned in bacterial plasmids. Expanded deletions in early region 1 (3180 bp) and early region 3 (2690 or 3132 bp) can be combined in a single vector that should have a capacity for inserts of up to 8.3 kb, enough to accommodate the majority of cDNAs encoding proteins with regulatory elements. Genes can be inserted into either early region 1 or 3 or both and mutations or deletions can be readily introduced elsewhere in the viral genome. To illustrate the flexibility of the system, we have introduced a wild-type early region 3 into the vectors, and to illustrate the high capacity for inserts, we have isolated a vector with two genes totaling 7.8 kb.**

Construction of adenovirus (Ad) vectors involves insertion of foreign DNA into the Ad genome, usually with compensating deletions in early region 1 (E1) or early region 3 (E3). E1 is not required for viral replication in 293 cells (1), which express the left 11% of the Ad5 genome. For viral viability, deletions in this region must not affect the inverted terminal repeat (ITR; 1–103 bp) or packaging signals (194–358 bp) (2–5). In addition, deletions should not extend into the coding sequences for protein IX, which is essential for packaging of full-length genomes into functional virions (6). Since Ad virions can package ≈105% of the wild-type (wt) genome length (7), deletions of up to 2.9 kb in E1 (8–10) permit construction of defective vectors with inserts up to 4.7–4.9 kb.

E3 is nonessential for viral replication in any normally permissive human cell and can be deleted to produce nondefective vectors (11, 12). Deletions in E3 presumably cannot extend into essential virion structural genes, pVIII and fiber, flanking this region. Several E3 deletions have been used for vector construction, the most common resulting from removal of 1.88 kb of E3 sequences between *Xba* I sites at 79.6 and 84.8 map units (mu) in the Ad5 genome (11, 12), providing a capacity for inserts of 3.7–3.9 kb.

Among current methods for generating Ad vectors (8), there is no simple procedure for generating vectors with both E1 and E3 deletions. To construct Ad5 vectors that combine E1 and E3 deletions or substitutions and to simplify Ad vector production, we have developed a methodology based on a series of bacterial plasmids (pBHG) containing most of the viral genome in circular form but lacking the DNA packaging signals. In this paper, we describe this system and its use to generate vectors with a wt E3 region or with inserts of up to 7.8 kb of foreign DNA in the E1 region.

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MATERIALS AND METHODS

Construction of Recombinant Plasmids. Plasmids constructed by standard protocols (13) were used to transform *Escherichia coli* DH5 (13) by electroporation (14) or *E. coli* HMS174 by using $CaCl_2$. Plasmid DNA was prepared by the alkaline lysis method (15) and purified by CsCl–ethidium bromide density gradient centrifugation.

Cells and Viruses. Cell culture media were obtained from GIBCO. Ad vectors were propagated and titered on 293 cells as described (16). Recombinant viruses were isolated by cotransfection of 293 cells with appropriate plasmids or viral DNA (17). After 8–10 days, plaques were isolated and expanded, and viral DNA was analyzed by restriction enzyme digestion as described (12, 16). [^{35}S]Methionine labeling, immunoprecipitation, and SDS/PAGE were carried out as described (16, 18). Densitometry was performed using the LKB Ultroscan XL enhanced laser densitometer.

RESULTS

Generation of the Plasmid pBHG10. In developing the strategy to be described, advantage was taken of previous observations made by ourselves (10, 12, 19–21) and other investigators (2, 11, 22–24). One key finding is the fact that Ad DNA can circularize in infected cells (19) and that this phenomenon can be exploited to generate infectious circular Ad genomes that can be propagated as bacterial plasmids (10, 20, 21). Secondly, it has been shown that the cotransfection into mammalian cells of two plasmids with overlapping sequences can generate infectious virus with good efficiency (11, 12, 21). The third finding important to this strategy is that Ads carry a cis-acting sequence in the left end of the genome that is essential for encapsidation of viral DNA (2, 22–24). When this cis-acting signal, located from bp 194 to 358 in Ad5, is deleted, viral genomes cannot be packaged but are expected to replicate their DNA in transfected cells (2–5).

These findings led us to design and execute the strategy outlined in Fig. 1. The first step involved the construction of AdBHG, a virus that contains the Ad5 genome with the deletion of E3 sequences from bp 28,133 to 30,818 and the insertion of modified pBR322 at bp 1339. AdBHG was made by cotransfection of 293 cells with purified viral DNA from Ad5PacI, digested with *Cla* I and *Xba* I, and pWH3.

The next step involved the generation of a bacterial plasmid containing the entire AdBHG genome and subsequent identification of infectious clones. Baby rat kidney (BRK) cells were infected with AdBHG under conditions that result in the generation of circular Ad5 genomes (10, 19). At 48 h after infection DNA was extracted from the infected BRK

Abbreviations: Ad, adenovirus; E1 and E3, early regions 1 and 3, respectively; wt, wild type; ITR, inverted terminal repeat; mu, map unit(s); Ap^r, ampicillin resistance; Tet^r, tetracycline resistance; Km^r, kanamycin resistance; HCMV, human cytomegalovirus; HSV 1, herpes simplex virus type 1.

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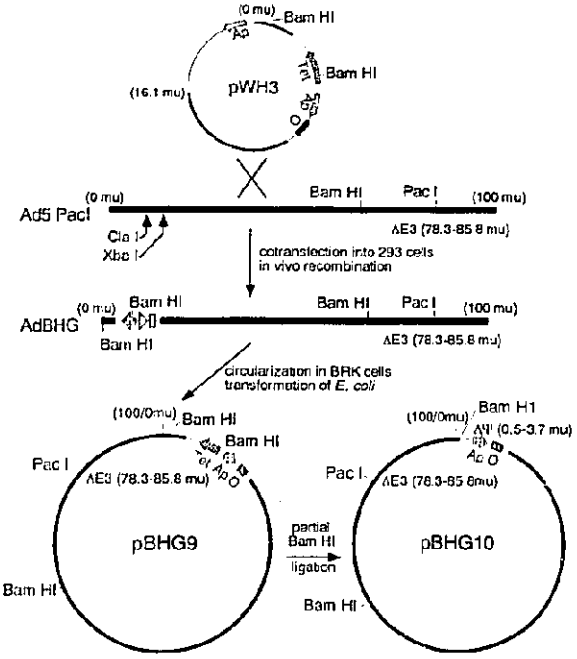

FIG. 1. Construction of pBHG10. pWH1 (not shown) was constructed from the plasmid pKH188 (2[*], 26) (derived by insertional mutagenesis of the E1A region in pKH101 resulting in the introduction of a *Bam*HI site at bp 188 in the Ad5 genome) by inserting pBRX (27) (a pBR322 derivative with an *Xba* I site at nt 2066) into the *Xba* I site at bp 1339 in Ad5 sequences. pWH1 was then combined with pXC38 (25) to include Ad5 sequences from 5.7 to 16.1 mu, generating pWH3. Ad5PacI [derived by cotransfection of 293 cells with pFG173 (18) and pAB14PacI, a modification of pAB14 (7) that substitutes a *Pac* I cloning site for 2.69 kb of E3] was digested with *Cla* I and *Xba* I and cotransfected into 293 cells with pWH3 to generate AdBHG. In the next step, the AdBHG genome was circularized by infecting BRK cells at multiplicity of infection of ≈20 under conditions that result in the generation of circular Ad5 genomes (10, 19). At 48 h after infection, DNA was extracted from the infected BRK cells and used to transform *E. coli* HMS174 to Ap[r] and Tet[r]. Small-scale plasmid preparations were made from the colonies obtained and screened by *Hin*dIII and *Bam*HI/*Sma* I digestion followed by gel electrophoresis (data not shown). Four candidates that appeared to possess a full AdBHG genome with intact junction regions were tested for infectivity and sequenced in the region of the junction. A single infectious clone was chosen, pBHG9. In the final step, the packaging signals were deleted from pBHG9 by partial *Bam*HI digestion and religation generating pBHG10. mu refers to Ad5 sequences, solid bars represent Ad5 sequences, and hatched bars represent Ap[r], Kn[r], and Tet[r] segments.

cells and used to transform *E. coli* HMS174 to ampicillin and tetracycline resistance (Ap[r] and Tet[r], respectively). From two experiments, plasmid DNA from a total of 104 colonies was screened by *Hin*dIII and *Bam*HI/*Sma* I digestion and gel electrophoresis (data not shown). Four candidate plasmids that appeared to posses a complete AdBHG genome were selected and all four were found to be infectious when transfected into 293 cells (data not shown). Since large palindromes, like that created by head to tail joining of the ITRs in these clones, are not compatible with plasmid replication in most strains of *E. coli* and result in rearrangements or deletions that disrupt the palindrome structure (10, 28, 29), the ITR junctions in each of the infectious clones were sequenced and analyzed. The number of nucleotides missing from the midpoint of the palindrome in each clone varied from as few as 4 bp (1 bp from the right ITR and 3 bp from the left) to as many as 19 bp (1 bp from the right ITR and 18 bp from the left). Because plasmids containing long palindromes tend to be unstable, we chose the clone missing 19 bp from the junction for further work. This plasmid was called pBHG9.

The final step involved generation of pBHG10 by deleting the packaging signals in pBHG9 by partial *Bam*HI digestion and religation (Fig. 1). Screening for pBHG10 was facilitated by the fact that removing the packaging signals also deleted the Tet[r] gene. pBHG10 contains Ad5 DNA sequences from bp 19 (left genomic end) to bp 188; bp 1339–28,133 and bp 30,818–35,934 (right genomic end). The left and right termini of the Ad5 genomes are covalently joined and a segment of plasmid pBR322 is present between Ad5 bp 188 and 1339 to allow propagation of pBHG10 in *E. coli*. A *Pac* I restriction enzyme site, unique in this plasmid, is present between Ad5 bp 28,133 and bp 30,818 to permit insertion of foreign genes. Because the packaging signal is deleted, pBHG10 is noninfectious but cotransfections with plasmids that contain the left-end Ad5 sequences including the packaging signal produce infectious viral vectors with an efficiency comparable to that obtained with pJM17 (20) (unpublished data and see below).

Additional Alterations to pBHG10: Vectors with wt E3 Sequences or with an Expanded Deletion in E3. The use of plasmids such as pBHG10 allows for rapid and relatively simple manipulation of the Ad genome. Two modifications of pBHG10 are described in this section. Since for some applications it may be desirable to generate Ad vectors with intact wt Ad5 E3 sequences, we reintroduced wt E3 sequences into pBHG10 (Fig. 2). First, pBHG10 was digested with *Spe* I, which cuts only at 75.4 mu in Ad5 sequences, and ligated with pFG23K also linearized with *Spe* I, generating pBHG10A that now contains the desired wt E3 sequences in tandem with the previous E3 region containing the 2.69-kb deletion. To remove repeated sequences, pBHG10A was partially digested with *Nde* I and religated, generating pBHG10B. The kanamycin-resistance (Kn[r]) segment was removed from pBHG10B by partial *Xba* I digestion and religation, generating pBHGE3. Except for the presence of a wt E3 region, pBHGE3 is identical to pBHG10 and is equally efficient for generation of Ad vectors with E1 substitution by cotransfection (unpublished).

For some applications, it may be desirable to have as large a deletion as possible within the E3 region. By utilizing a PCR and following a strategy similar to that described above for the construction of pBHGE3 (Fig. 2), we created a 3.13-kb E3 deletion and introduced it into pBHG10. The resulting plasmid pBHG11 is identical to pBHG10 except for an expanded E3 deletion that removes sequences from bp 27,865 to 30,995. Like pBHG10, pBHG11 contains a unique *Pac* I restriction enzyme site in place of the deleted E3 sequences to permit insertion of foreign genes. A detailed description of the construction of pBHG11 is available on request from the authors.

Construction of E1 Deletion Plasmids for Use in Cotransfections with pBHG Vectors. Plasmids pBHG10, pBHG11, and pBHGE3 were designed to contain all the essential Ad5 sequences required to produce infectious virus upon transfection of 293 cells except for the packaging signal (194–358 bp) needed to encapsidate viral DNA into viral particles. To generate infectious viral vectors, these plasmids or derivatives with an insert in E3 must be cotransfected into 293 cells with a second plasmid containing left end viral sequences including the packaging signal as illustrated in Fig. 3. To maximize the capacity of the BHG vector system, we required a plasmid with the largest possible E1 deletion for cotransfections with the BHG plasmids. Analysis of E1 sequences revealed that 3.2 kb could be deleted between an *Ssp* I site at 339 bp and an *Afl* II site at 3533 bp (Fig. 4). This

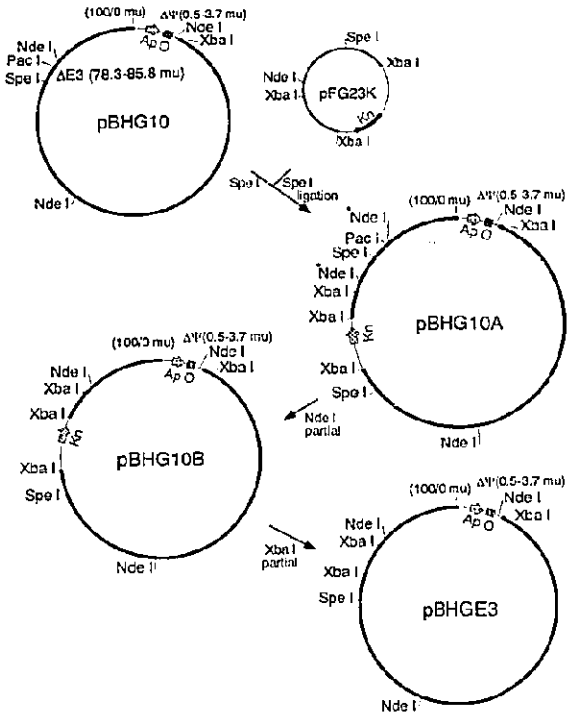

FIG. 2. Construction of pBHGE3. For insertion of wt E3 sequences, pBHG10 was digested with *Spe* I, which cuts at 75.4 mu in Ad5 sequences, and ligated with *Spe* I-digested pFG23K, generating pBHG10A. pFG23K was derived from the Ap^r plasmid pFG23 (30) (not shown), which contains Ad5 sequences from 60 to 100 mu. pFG23 was digested with *Xba* I, which cuts at bp 28,592 in Ad5 sequences (there is no cleavage at bp 30,470 due to Dam methylation in the *E. coli* strain used), and ligated with *Xba* I-digested pKN30 (31), a small Kn^r plasmid, generating pFG23AK (not shown). To remove Ad5 sequences that were not required and the Ap^r gene, pFG23AK was digested with *Afl* II and ligated, generating pFG23K. To remove the repeated sequences (shown between the asterisks), pBHG10A was partially digested with *Nde* I and religated, generating pBHG10B. In the final step, the Kn^r segment was removed from pBHG10B by partial *Xba* I digestion and religation, generating pBHGE3. mu refers to Ad5 sequences, solid bars represent Ad5 sequences, and hatched bars represent Ap^r and Kn^r segments.

deletion does not interfere with the ITR (1–103 bp), the essential core packaging signal (194–358 bp) (3–5), or coding sequences for protein IX, but does remove the Sp1 binding site (3525–3530 bp) from the protein IX promoter. Since the Sp1 binding site is thought to be essential for protein IX expression (32), it was reintroduced as a synthetic oligonucleotide that positioned the Sp1 site 1 bp closer to the protein IX TATA box (Fig. 4). To assess the effect of the 3.2-kb E1 deletion and the reintroduction of the Sp1 binding site on protein IX expression, 293 cells were infected at 10 plaque-forming units per cell with a number of different viruses. These include viruses with no deletion in E1 (wt Ad5), one with a 2.3-kb deletion extending into the protein IX gene (dl313) (33), one with the 3.2-kb deletion described above (dl70-3), and viruses with the 3.2-kb deletion containing the human cytomagalovirus (HCMV) immediate early promoter (AdHCMV) or the human β-actin promoter (AdβAct) in the E1-antiparallel orientation with or without the reintroduced Sp1 binding site. After labeling with [^{35}S]methionine, cell extracts were harvested and samples were immunoprecipitated with anti-Ad2 protein IX antibodies and analyzed by SDS/PAGE. The results (Fig. 5) indicate that variable levels

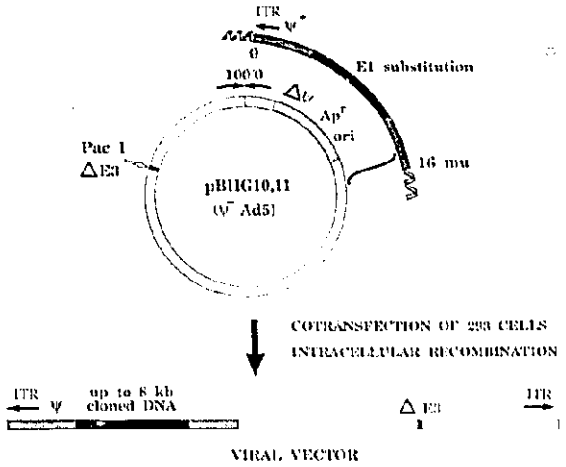

FIG. 3. Rescue using pBHG vectors. The general strategy used for generating infectious viral vectors using the BHG system is illustrated. Cotransfection of 293 cells with pBHG10, pBHG11, pBHGE3, or a pBHG derivative containing a foreign gene inserted in E3 plus a plasmid containing left-end viral sequences including the packaging signals results in the generation of infectious viral vectors by *in vivo* recombination.

of protein IX were expressed depending on the sequences upstream from the protein IX gene. With the reintroduced Sp1 site present, there was at most a 25% reduction compared to wt Ad5. The near-wt levels of protein IX expression obtained with mutant dl70-3 may be explained by sequences from left of the deletion (nt 333–338: GCGCGT, in Ad5 sequences) that fortuitously resemble and may act as an Sp1 site. The detection of reduced but significant levels of protein IX in cells infected by vectors containing only the HCMV or β-actin promoters, which have no potential Sp1 binding sites, suggests that the Sp1 binding site may not be absolutely essential for protein IX expression, in contrast to the findings of Babiss and Vales (32). Because protein IX is known to affect the heat stability of virions, we measured the infectious titers of wt Ad5 compared to dl313, dl70-3, AdHCMV2, AdβAct2, AdHCMVsp1, and AdβActsp1 after incubation at 45°C for 1 and 2 h. Of the six viral mutants tested, only dl313 differed significantly in heat lability from wt (Fig. 6). Even AdβAct2, which produces only 16% of wt levels of protein IX (Fig. 5), was as resistant to heat inactivation as was wt virus, suggesting that protein IX is likely made in excess during wt viral infection. We have also found that viruses containing the 3.2-kb E1 deletion replicate in 293 cells to the same final titers as wt Ad5 (data not shown).

Since the growth characteristics and stability of viruses with the 3.2-kb E1 deletion were not affected, this deletion was incorporated into pΔE1sp1A and pΔE1sp1B for use in cotransfections with the BHG plasmids (Fig. 4).

Testing the Efficiency and Capacity of the pBHG Vectors. To assess the ability of the BHG plasmids to generate infectious viral vectors, cotransfections with various left end plasmids were performed, and the efficiency of rescue was usually comparable to that obtained with pJM17 (20) (data not shown). Although pJM17 has been useful for rescue of E1 mutations or substitutions into infectious virus, because it is derived from dl309 (33), it has neither a wt E3 region nor a useful E3 deletion. Thus pJM17 will be superseded by the pBHG series of plasmids for most Ad5 vector constructions.

Use of pBHGE3, pBHG10, or pBHG11 combined with the 3.2-kb deletion in E1 should permit rescue of inserts of ≈5.2, ≈7.9, and ≈8.3 kb, respectively, into viral vectors. To test the capacity of the BHG system, we constructed an insert of

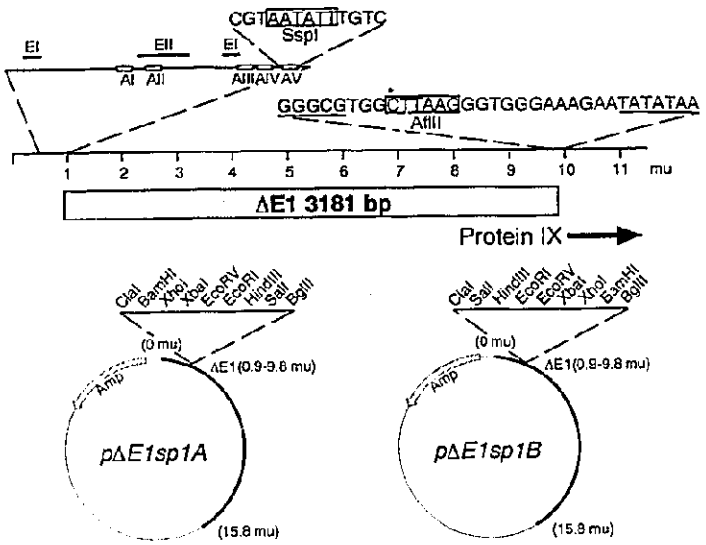

FIG. 4. Left-end shuttle plasmids with a 3.2-kb E1 deletion. The sequences removed by the 3.2-kb deletion are indicated. At the 5' end of the deletion the region from bp 190 to bp 348 has been expanded to show the position of repeated elements (AI–AV) involved in packaging and the enhancer elements (EI and EII). At the 3' end of the deletion, the region from bp 3525 to bp 3557 contains the protein IX promoter with the Sp1 binding site and TATA box (underlined). To create the E1 deletion, sequences were removed between the *Ssp* I (bp 339) and *Afl* II (bp 3533) restriction sites (boxed). This deletion does not interfere with the ITR (bp 1–103), the essential core packaging signal (bp 194–358), or coding sequences for protein IX, but does remove the Sp1 binding site (bp 3525–3530), which was subsequently reintroduced 1 bp closer to the protein IX TATA box. Thus the cytidine residue, indicated with the asterisk, is missing in plasmids pΔE1sp1A and pΔE1sp1B. In addition to the modified Sp1 site, plasmid pΔE1sp1A contains Ad5 DNA sequences from bp 1 to bp 341 and bp 3524 to bp 5790 with a polycloning oligonucleotide inserted between Ad5 bp 341 and bp 3524. Plasmid pΔE1sp1B is identical to pΔE1sp1A except that the restriction sites between the *Cla* I and *Bgl* II sites in the polycloning region are reversed.

7.8 kb consisting of the *lacZ* gene driven by the HCMV promoter (E1-antiparallel orientation) and the herpes simplex virus type 1 (HSV-1) *gB* gene driven by the simian virus 40 promoter (E1-parallel orientation) in the 3.2-kb E1 deletion. The 7.8-kb insert was constructed by inserting the 4.1-kb *Xba* I fragment from pgBdX17 (34) containing HSV-1 *gB* gene driven by the simian virus 40 promoter into the *Xba* I site in pHCMVsp1LacZ (35), generating pHlacZgBR (data not shown). After cotransfection of 20 60-mm dishes of 293 cells, 10 with 5 μg of pBHG10 and 5 μg of pHlacZgBR and the other half with 10 μg of each, one plaque was obtained. This plaque was isolated, expanded, analyzed by restriction digest with *Hin*dIII, and found to have the expected restriction pattern. The isolate, AdHlacZgBR, expressed both *lacZ* and HSV-1 *gB* at levels comparable to those obtained with vectors containing single inserts of these genes (data not shown).

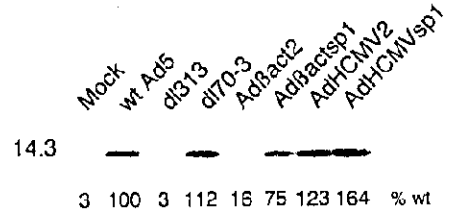

FIG. 5. Immunoprecipitation of protein IX from cells infected with viruses having a 3.2-kb E1 deletion. The levels of protein IX, a minor capsid protein required for the packaging of full-length viral genomes, were compared for wt Ad5 and viruses with a 3.2-kb E1 deletion with and without the reintroduced Sp1 binding site. The 293 cells were mock infected or infected at a multiplicity of infection of 10 with wt Ad5, dl313, dl70-3, AdβAct2, AdβActsp1, AdHCMV2, or AdHCMVsp1 and labeled with [^{35}S]methionine from 22 to 24 h after infection. Cell extracts were prepared and immunoprecipitated with anti-Ad2 protein IX antibodies, and samples were separated by SDS/PAGE. The gel was dried and bands were visualized by autoradiography. The lanes contain the samples indicated above and the molecular mass at 14.3 kDa is indicated on the left. Levels of protein IX were determined by densitometric analysis and are indicated below the samples relative to wt Ad5. dl313 contains a 2.3-kb deletion extending into the protein IX gene and, therefore, makes no protein IX; dl70-3 carries the 3.2-kb E1 deletion; AdβAct2 and AdHCMV2 contain the β-actin and HCMV promoters in the 3.2-kb E1 deletion, respectively; AdβActsp1 and AdHCMVsp1 contain the β-actin and HCMV promoters in the 3.2-kb E1 deletion with the Sp1 binding site reintroduced into the protein IX promoter. The β-actin and HCMV promoters were inserted in the E1-antiparallel orientation.

DISCUSSION

We have constructed and tested a vector system based on a series of bacterial plasmids (pBHG) that contain all the essential Ad5 sequences required to produce infectious virus upon transfection of 293 cells except for the packaging signal (bp 194–358) needed to encapsidate viral DNA. These plasmids are noninfectious in single transfections and must be cotransfected with a second plasmid containing left-end sequences including a packaging signal to generate infectious viral vectors (Fig. 3). This vector system is the most versatile system yet developed for generating Ad5 helper-independent vectors and will allow the construction of vectors with any combination of inserts, mutations, or wt sequences in both E1 and E3. Because with the pBHG system the entire viral genome is propagated as bacterial plasmids, manipulation of

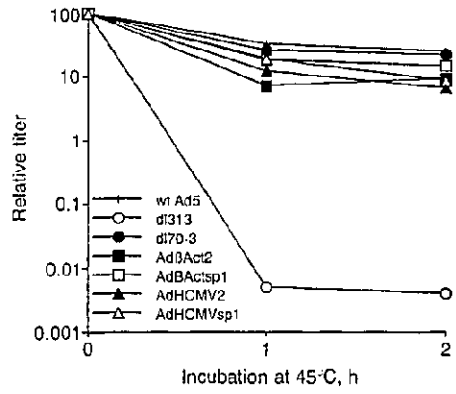

FIG. 6. Heat stability of viruses with the 3.2-kb E1 deletion. The heat stability of viruses with the 3.2-kb E1 deletion, with and without the reintroduced Sp1 binding site in the protein IX promoter, was compared to wt Ad5 and dl313. Viral stocks of wt Ad5, dl313, dl70-3, AdβAct2, AdHCMV2, AdβActsp1, and AdHCMVsp1 were titered on 293 cells prior to and after incubation for 1 and 2 h at 45°C. The structure of these viruses is explained in Fig. 5. The data presented for wt Ad5 and dl70-3 represent the average of four experiments and the data for dl313, AdβAct2, AdHCMV2, AdβActsp1, and AdHCMVsp1 represent the average of three experiments.

viral DNA sequences can be done rapidly and efficiently. To illustrate this and to generate two useful variants, we constructed pBHGE3 and pBHG11 from the original plasmid pBHG10. pBHGE3 permits construction of vectors with wt E3 sequences (Fig. 2), and pBHG11 increased the cloning capacity of resulting viral vectors. The 2.69-kb E3 deletion in pBHG10 removes the major portions of all E3 mRNAs, the first E3 3′ splice acceptor site, and the L4 polyadenylylation site but leaves the E3 promoter, the 5′ initiation site, the first E3 5′ splice donor site, and the E3b polyadenylylation site intact (36). Viruses with the 2.69-kb E3 deletion have the same growth kinetics and progeny virus yields as wt virus (7). The 3.1-kb E3 deletion in pBHG11 removes two additional elements not removed by the 2.69-kb E3 deletion: the first E3 5′ splice donor site and the E3b polyadenylylation site (36). This deletion does not interfere with the open reading frame for pVIII or any of the L5 family of mRNAs. Viruses containing the 3.1-kb deletion were found to give wt progeny yields in infected 293 cells (data not shown).

To maximize the capacity of the BHG system and to facilitate the introduction of inserts into the E1 region, we have constructed plasmids containing a 3.2-kb deletion of E1 sequences and multiple restriction sites for the insertion of foreign genes (Fig. 4*B*). This deletion leaves intact the left iTR and packaging signals and extends just past the Sp1 binding site of the protein IX promoter. The promoter for transcription of the protein IX gene is relatively simple, consisting of this Sp1 binding site and a TATA box. It has been reported that the Sp1 binding site is essential for expression of protein IX (32) and it was, therefore, reintroduced at a position 1 bp closer to the TATA box than in the wt promoter. However, neither the original 3.2-kb E1 deletion nor the deletion mutants containing the synthetic Sp1 site appeared to be significantly altered in protein IX expression (Fig. 5), heat stability (Fig. 6), or final progeny yields of viruses with this deletion. Although we did see a reduction in protein IX expression to ≈16% of wt levels for the virus containing the β-actin promoter combined with the 3.2-kb E1 deletion, even this level of expression appeared to be adequate for the formation of stable virions.

The Ad5 packaging signal, which overlaps the E1A enhancer region, has been found to consist of at least five AT-rich elements, which, by extensive mutational analysis, have been found to be functionally redundant (Fig. 4) (3–5). The E1A enhancer is composed of two functionally distinct enhancer elements, I and II (2, 37). Two repeats of enhancer element I flank element II and are responsible for regulating expression from the E1A gene (2). Enhancer element II regulates the transcription of all the early regions in the genome (37). The 3.2-kb E1 deletion does not interfere with the enhancer region but does remove the 3′ most packaging element. The removal of this element has been shown to have little or no effect on packaging (3–5) and should not, therefore, affect the packaging of recombinants that utilize the 3.2-kb E1 deletion.

One observation made when testing the BHG system was that the larger the insert being rescued in the E1 region the lower the efficiency of rescue. Although we have not systematically investigated the relationship between insert size and efficiency of recombination between cotransfected plasmids, we have observed that longer segments of foreign DNA seem to be more difficult to rescue into infectious virus than small inserts. This could be due to the inhibitory effect of heterologous sequences on recombination in agreement with the observations of Munz and Young (38).

When using pBHGE3, pBHG10, or pBHG11 in combination with the 3.2-kb deletion in E1, it should be possible to rescue inserts of up to 5.2, 7.9, and 8.3 kb, respectively, in conditional helper-independent vectors. To test the capacity of the system, we used pBHG10 to rescue a 7.8-kb insert consisting of the HSV-1 *gB* gene and *lacZ* gene in tandem, each with its own promoter. The vector obtained, AdHlacZgBR, was found to replicate efficiently and to express both *lacZ* and HSV-1 *gB* at levels comparable to that obtained with Ad vectors containing single inserts of these genes. The pBHG system has now been in use in our laboratory for ≈1 year and has facilitated the rescue of a variety of genes into E1 and E3. This vector system should have wide applications for the construction of Ad vectors for use as recombinant viral vaccines and for gene therapy transfer vectors.

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2. Contributions To Bett *et al.* (1994)

(A) Construction of all plasmids and viral vectors used to generate pBHG10 except for pWH1 and pWH3 which were constructed by W. Haddara.

(B) Construction of pBHG11 and pBHGE3.

(C) Construction of the E1 shuttle plasmids used in cotransfections with the pBHG plasmids.

(D) Generation of Ad vectors dl70-3, AdβAct2, AdβActsp1, AdHCMV2 and AdHCMVsp1.

(E) Characterization of vectors with the expanded 3.2 kb E1 deletion (with and without the Sp1 binding site reinserted in the protein IX promoter) by studying protein IX expression and heat stability.

3. Summary

In Bett *et al.*, (1994) the construction and testing of the pBHG vector system is described. The pBHG plasmids (pBHG10, pBHG11 and pBHGE3) contain all the essential Ad5 sequences required to produce infectious virus upon transfection of 293 cells except for the packaging signal (194-358 bp) needed to encapsidate viral DNA. These plasmids are therefore noninfectious in single transfections and must be cotransfected with a second plasmid containing left end sequences including a packaging signal to generate infectious viral vectors. This vector system is the most versatile system yet developed for generating Ad5 helper independent vectors and has several advantages over previous systems including: the possibility of combining relatively large E3 and E1 deletions to permit the cloning of genes up to 8.3 kb; the cloning of inserts into the E3 region in combination with E1 inserts of foreign DNA or with E1 deletions or mutations; and finally the possibility of constructing vectors with a wt E3 region and foreign gene inserts in E1. When using pBHGE3, pBHG10 or pBHG11 in combination with the 3.2 kb deletion in E1 it should be

possible to rescue inserts of up to 5.2, 7.9 and 8.3 kb respectively into conditional helper independent vectors. This new vector system should have wide applications for the construction of Ad vectors for use as recombinant viral vaccines and for gene therapy transfer vectors.

4. Construction Of pBHG11

A detailed description of the construction of pBHG11 could not be included in Bett *et al.*, 1994 due to constraints on the length of articles published in The Proceedings of the National Academy of Sciences. Therefore a complete description is provided here.

An analysis of the sequences in the E3 region of Ad5 (Cladaras and Wold, 1985; Cladaras *et al.*, 1985) revealed that it may be possible to expand the 2.69 kb E3 deletion present in pBHG10 (Bett *et al.*, 1994) to 3.13 kb by utilizing the technique of polymerase chain reaction (PCR). The procedure followed to create this deletion and introduce it into pBHG10 is outlined below and illustrated in Figure 9. The first step involved the selection of two sets of primers used in PCR to generate two fragments of the Ad5 genome that defined the left and right ends of the deletion (Fig. 9A). The first set of primers (AB3153 and AB3154) were used to generate a fragment of 842 bp corresponding to Ad5 sequences from 27043 to 27885 bp. The second set of primers (AB3155 and AB3156) were used to generate a fragment of 631 bp corresponding to Ad5 sequences from 30980 to 31611 bp. The template DNA for the PCR reactions was plasmid pFG23 (McKinnon *et al.*, 1982) which contains Ad5 sequences from 60 to 100 mu.

In the next step the PCR fragments were cloned on either side of the kanamycin resistance (Kn^r) gene in pABS.8 (A.B. and F.L.G. unpublished; see the appendix on the pBG vector system for details of its construction) (Fig. 9B). First pABS.8 was digested with *Eco*RI and *Pst*I and ligated with the 631 bp PCR fragment, also digested with *Eco*RI and *Pst*I, generating pABSPCR1.

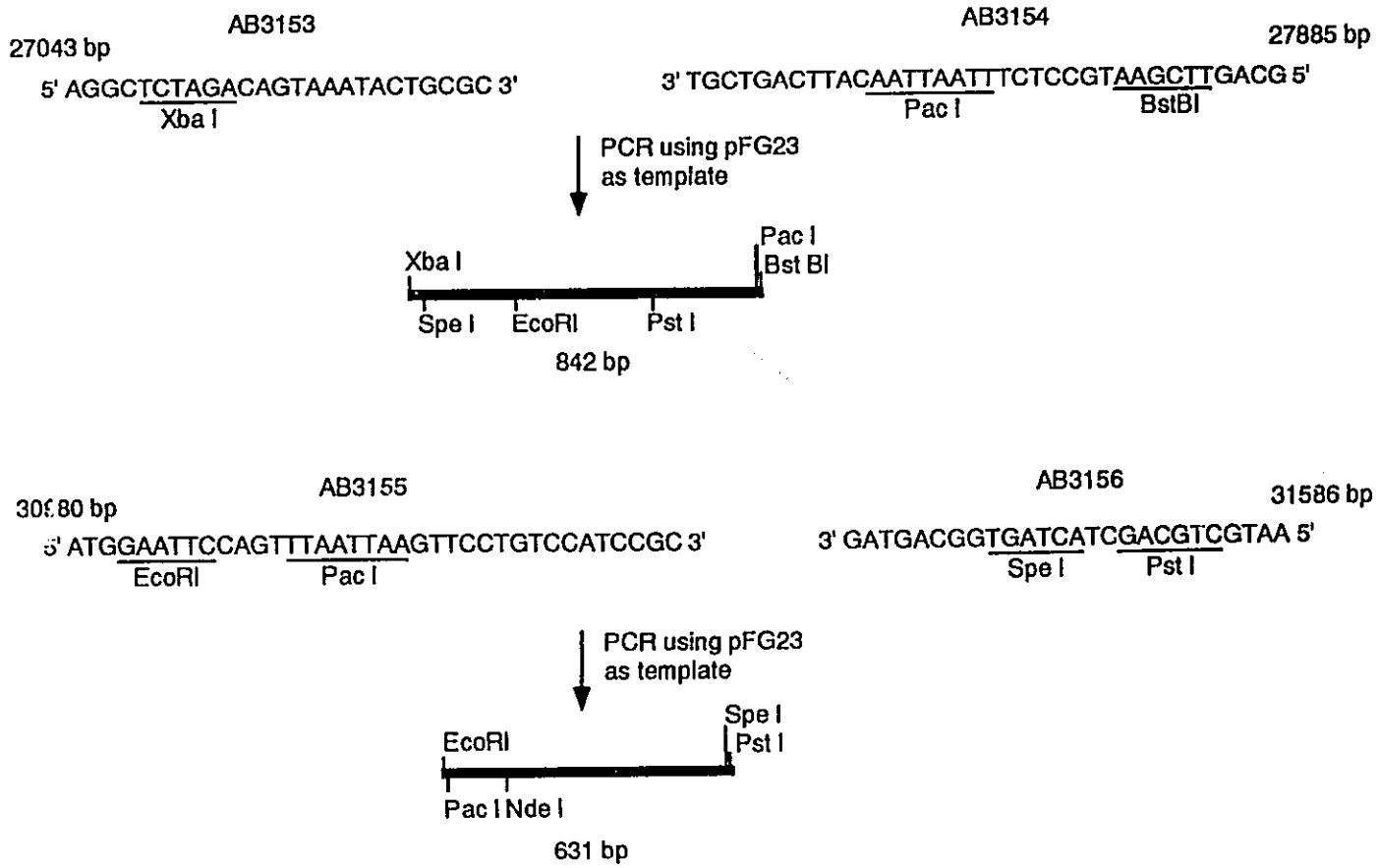
Figure 9. Construction of pBHG11

(A) In order to create the 3.13 kb E3 deletion PCR was used to generate two fragments of the Ad5 genome that would define the deletion. Two sets of primers were selected to generate these fragments. The first set of primers (AB3153 and AB3154) generated a fragment of 842 bp corresponding to Ad5 sequences from 27043 to 27885 bp. Primer AB3153 is 25 bp long and was designed to contain an *Xba*I restriction site for cloning purposes. Primer AB3154 is 36 bp long and was designed to contain both a *Pac*I and *Bst*BI site. The second set of primers (AB3155 and AB3156) generated a fragment of 631 bp corresponding to Ad5 sequences from 30980 to 31586 bp. Primer AB3155 is 37 bp long and was designed to contain an *Eco*RI and *Pac*I site. Primer AB3156 is 26 bp long and was designed to contain a *Spe*I and *Pst*I site.

(B) To facilitate the cloning of the 3.13 kb E3 deletion into pBHG10 the PCR products generated above were cloned of either side of the Kn^r gene in pABS.8. pABS.8 was digested with *Eco*RI and *Pst*I and ligated with the 631 bp PCR fragment which had also been digested with *Eco*RI and *Pst*I, generating pABSPCR1. In the next step pABSPCR1 was digested with *Xba*I and *Bst*BI and ligated with the 842 bp PCR fragment also digested with *Xba*I and *Bst*BI, generating pABSPCR2.

(C) In order to introduce the 3.13 kb E3 deletion into pBHG10 in the correct orientation pBHG10 was digested with *Spe*I, which cuts at 75.4 mu in Ad5 sequences, and ligated with pABSPCR2 also digested with *Spe*I, generating pBHGPCR2 which now contains the 3.13 kb E3 deletion in tandem with the previous E3 region containing the 2.69 kb deletion. In order to remove the repeated sequences containing the 2.69 kb deletion pBHGPCR2 was partially digested

A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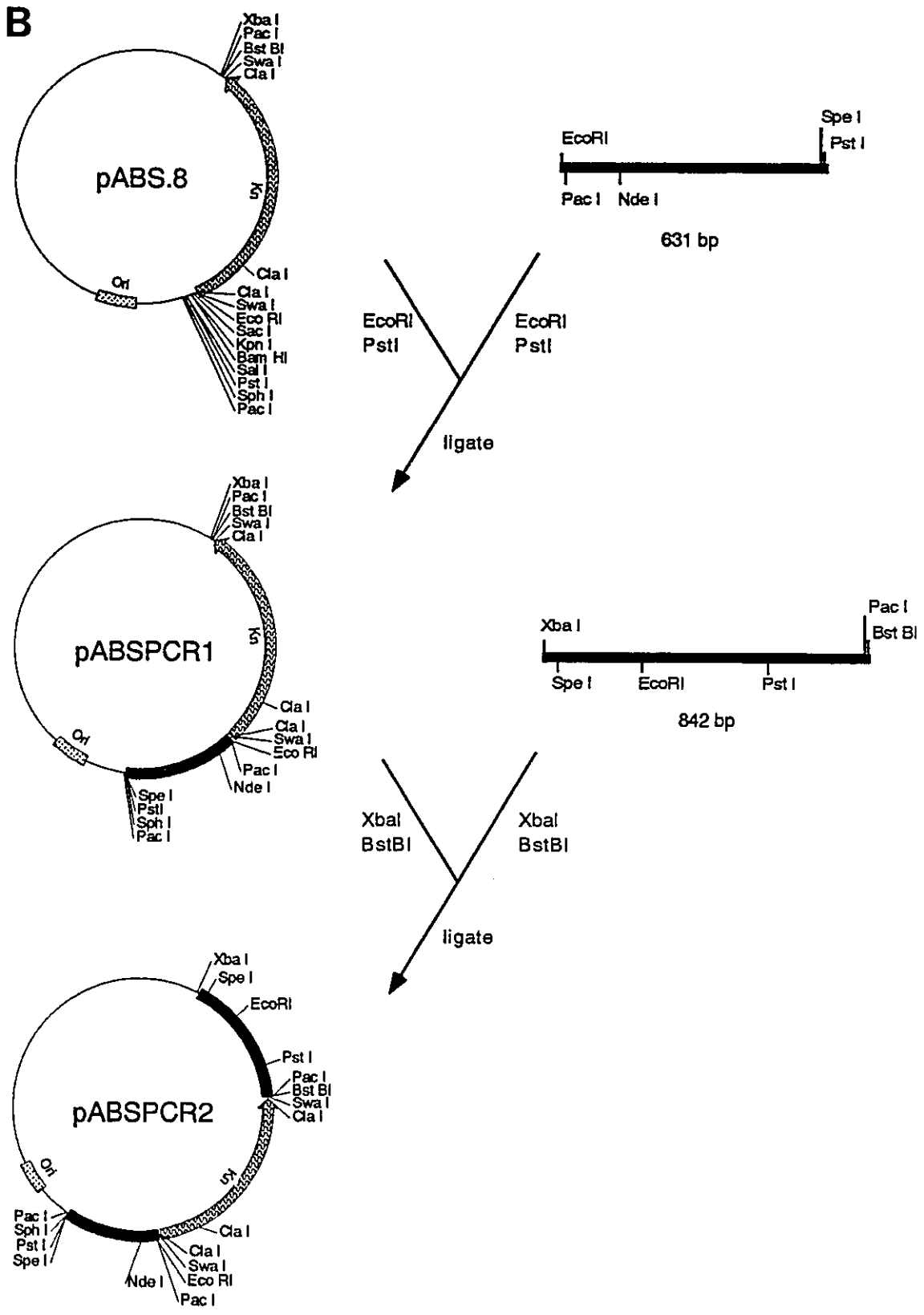
Xba I
AB3154
27885 bp
3' TGCTGACTTACAATTAATTTCTCCGTAAGCTTGACG 5'
Pac I
BstBI
PCR using pFG23
as template
Xba I
Pac I
Bst BI
Spe I
EcoRI
Pst I
842 bp
AB3155
30[illegible]80 bp
5' ATGGAATTCCAGTTTAATTAAGTTCCTGTCCATCCGC 3'
EcoRI
Pac I
AB3156
31586 bp
3' GATGACGGTGATCATCGACGTCGTAA 5'
Spe I
Pst I
PCR using pFG23
as template
EcoRI
Spe I
Pst I
Pac I Nde I
631 bp


B
pABS.8
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Pac I
Bst BI
Swa I
Cla I
KS
Ori
Cla I
Cla I
Swa I
Eco RI
Sac I
Kpn I
Bam HI
Sal I
Pst I
Sph I
Pac I
Spe I
EcoRI
Pst I
Pac I
Nde I
631 bp
EcoRI
PstI
EcoRI
PstI
ligate
pABSPCR1
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Pac I
Bst BI
Swa I
Cla I
KS
Ori
Cla I
Cla I
Swa I
Eco RI
Pac I
Nde I
Spe I
PstI
Sph I
Pac I
Pac I
Bst BI
Xba I
Spe I
EcoRI
Pst I
842 bp
XbaI
BstBI
XbaI
BstBI
ligate
pABSPCR2
Xba I
Spe I
EcoRI
Pst I
Pac I
Bst BI
Swa I
Cla I
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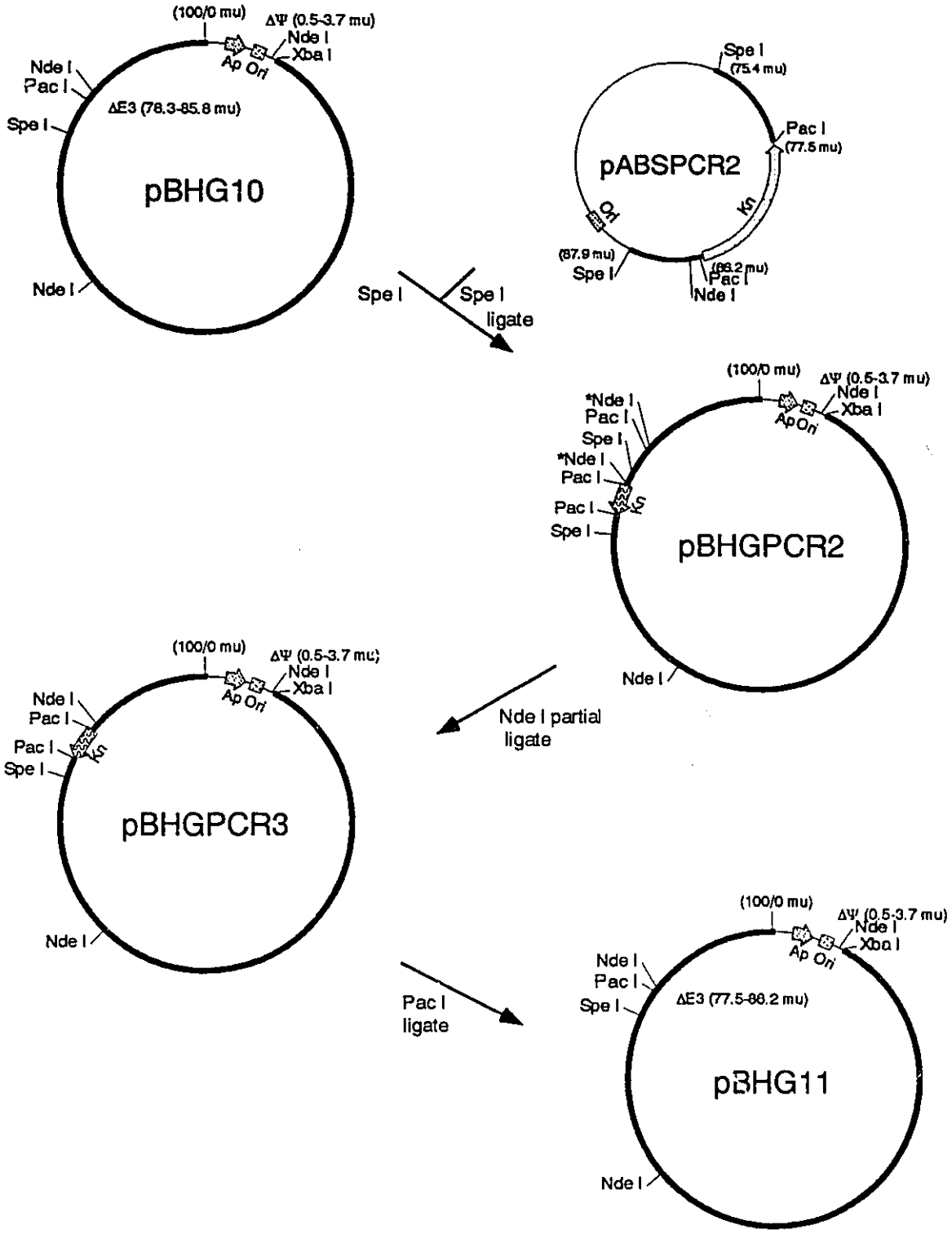
Cla I
Cla I
Swa I
Eco RI
Nde I
Pac I

C


(100/0 mu)
ΔΨ (0.5-3.7 mu)
Nde I
Xba I
Ap
Ori
Nde I
Pac I
Spe I
ΔE3 (78.3-85.8 mu)
pBHG10
Nde I
Spe I
Spe I
ligate
Spe I
(75.4 mu)
Pac I
(77.5 mu)
pABSPCR2
Ori
ks
(87.9 mu)
Spe I
(86.2 mu)
Pac I
Nde I
(100/0 mu)
ΔΨ (0.5-3.7 mu)
*Nde I
Pac I
Spe I
*Nde I
Pac I
Pac I
Spe I
ks
pBHGPCR2
Nde I
Nde I partial
ligate
(100/0 mu)
ΔΨ (0.5-3.7 mu)
Nde I
Xba I
Ap
Ori
Nde I
Pac I
Pac I
Spe I
ks
pBHGPCR3
Nde I
Pac I
ligate
(100/0 mu)
ΔΨ (0.5-3.7 mu)
Nde I
Xba I
Ap
Ori
Nde I
Pac I
Spe I
ΔE3 (77.5-86.2 mu)
pBHG11
Nde I

with *Nde*I (to remove the sequences between the asterisks) and religated, generating pBHGPCR3. In the final step the Kn^r segment was removed from pBHGPCR3 by *Pac*I digestion and religation, generating pBHG11. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences and hatched bars represent Ap^r, Kn^r and origin segments.

pABSPCR1 was then digested with *Xba*I and *Bst*BI and ligated with the 842 bp PCR fragment, also digested with *Xba*I and *Bst*BI, generating pABSPCR2. pABSPCR2 thus contains Ad5 sequences from 75.4 to 87.9 mu with a 3.13 kb deletion of E3 sequences from 77.5 to 86.2 containing a Kn^r gene.

The remainder of the strategy involved the introduction of the 3.13 kb E3 deletion into pBHG10 in the correct orientation (Fig. 9C). In order to do this we followed essentially the same strategy used to create pBHGE3 (Bett *et al.*, 1994). Briefly pBHG10 was digested with *Spe*I, which cuts at 75.4 mu in Ad5 sequences, and ligated with pABSPCR2 also digested with *Spe*I, generating pBHGPCR2 which now contains the 3.13 kb E3 deletion in tandem with the previous 2.69 kb deletion. In order to remove the repeated sequences pBHGPCR2 was partially digested with *Nde*I and religated, generating pBHGPCR3. In the final step the Kn^r segment was removed from pBHGPCR3 by *Pac*I digestion and religation, generating pBHG11.

pBHG11 contains Ad5 DNA sequences from bp 19 (left genomic end) to bp 188; bp 1339 to 27864; bp 30996 to 35933 (right genomic end). As with pBHG10 the left and right termini of the Ad5 genomes are covalently joined and a segment of plasmid pBR322 is present between Ad5 bp 188 and 1339 to allow propagation of pBHG11 in *E. coli.* Also a *Pac*I restriction enzyme site, unique in this plasmid, is present between Ad5 bp 27864 and bp 30996 to permit insertion of foreign genes.

5. Construction Of E1 Shuttle Plasmids

In Bett *et al.* (1994) two left end shuttle plasmids were described, pΔE1sp1A and pΔE1sp1B, into which foreign genes can be cloned for rescue into virus by cotransfection with the pBHG plasmids (Bett *et al.*, 1994; Fig. 2 and 3) or pJM17 (McGrory *et al.*, 1988). The

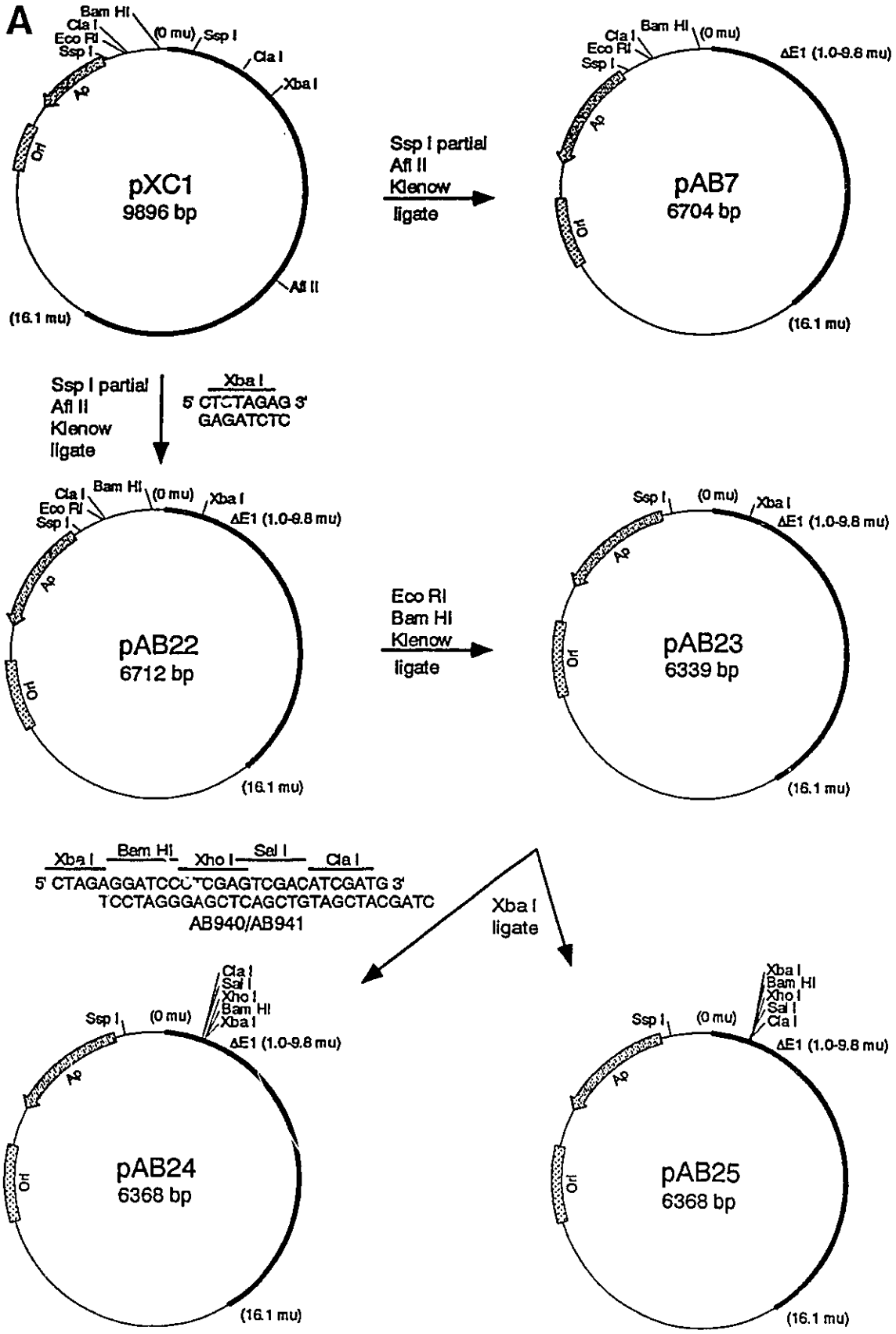
construction of pΔE1sp1A and pΔE1sp1B involved many manipulations that could not be reported due to constraints on the length of articles published in The Proceedings of the National Academy of Sciences. Also many other potentially useful E1 shuttle plasmids were generated during the construction of pΔE1sp1A and pΔE1sp1B which were not described in this paper. Therefore the complete strategy followed to construct pΔE1sp1A and pΔE1sp1B is described below and illustrated in Figures 10 and 11. To create the 3.2 kb E1 deletion pXC1 (McKinnon *et al.*, 1982) which contains the Ad5 *Xho*I-C fragment corresponding to the first 5788 bp (16.1 mu) of the genome was partially digested with *Ssp*I which cleaves at 339 bp and once in plasmid sequences and then digested completely with *Afl*II which cleaves at 3533 bp. Following digestion, the large fragment of *E.coli* DNA polymerase I (klenow) was use to obtain blunt ends and the DNA was either ligated generating pAB7 or ligated in the presence of an *Xba*I linker, generating pAB22 (Fig. 10A). To remove the *Bam*HI and *Cla*I sites in pAB22 plasmid sequences so that they could be reintroduced as cloning sites in the E1 deletion, pAB22 was digested with *Eco*RI and *Bam*HI, treated with klenow and ligated generating pAB23. Finally pAB23 was digested with *Xba*I and ligated with synthetic oligo AB940/AB941 which contains *Bam*HI, *Cla*I, *Sal*I and *Xho*I restriction sites, generating pAB24 and pAB25.

To generate E1 shuttle plasmids containing strong promoters that could be used to drive expression of foreign genes, the human cytomegalovirus (HCMV) immediate-early promoter (-299 to +76 bp) (Boshart *et al.*, 1985) (Fig. 10B) and the human β-actin (β-Act) promoter (-450 to +910 bp) (Gunning *et al.*, 1987) (Fig. 10C) were cloned into the 3.2 kb E1 deletion. Because some vectors constructed with inserts in the E1 parallel orientation have been found to produce aberrantly spliced messages involving down stream Ad sequences (Berkner and Sharp, 1984; Davidson and Hassel, 1987) it was decided to insert the promoters in the E1 antiparallel

Figure 10. Construction of E1 shuttle plasmids with a 3.2 kb deletion

(A) To create the 3.2 kb E1 deletion pXC1, an ampicillin resistant plasmid containing the Ad5 *Xho*I-C fragment representing the first 5788 bp of the genome, was partially digested with *Ssp*I to obtain cleavage at 339 bp and then digested completely with *Afl*II which cleaves at 3533 bp. Following digestion the DNA was treated with the large fragment of *E. coli* DNA polymerase I (klenow) to obtain blunt ends and then either ligated generating pAB7 or ligated in the presence of an *Xba*I linker generating pAB22. pAB22 was then digested with *Eco*RI and *Bam*HI, treated with klenow and ligated generating pAB23. The *Bam*HI and *Cla*I sites were then reintroduced into the *Xba*1 site of pAB23 as part of the synthetic cloning oligo (AB940/AB941) generating pAB24 and pAB25.

(B) To introduce the human cytomegalovirus (HCMV) immediate-early promoter (-299 to +76 bp) into the 3.2 kb E1 deletion so that it could be used to drive expression of foreign genes pAB24 was digested with *Xba*I, treated with klenow, digested with *Bam*HI and then ligated with pΔ548IEP1 which had been digested with *Eco*R1, treated with klenow and digested with *Bam*HI, generating pABE1HCMV. To remove the restriction sites upstream of the promoter pABE1HCMV was digested with *Xba*I and *Eco*R1, treated with S1 nuclease and ligated, generating pABE1HCMV2. Finally to introduce additional cloning sites downstream of the promoter, pABE1HCMV2 was digested with *Bam*HI and *Sal*I and ligated with synthetic oligo AB1531/AB1532 also digested with *Bam*HI, generating pABE1HCMV3.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Ssp I
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Xba I
Ap
Ori
pXC1
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Afl II
(16.1 mu)
Ssp I partial
Afl II
Klenow
ligate
Cla I
Bam HI
Eco RI
Ssp I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB7
6704 bp
(16.1 mu)
Ssp I partial
Afl II
Klenow
ligate
Xba I
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(0 mu)
Xba I
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB22
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(16.1 mu)
Eco RI
Bam HI
Klenow
ligate
Ssp I
(0 mu)
Xba I
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB23
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(16.1 mu)
Xba I
Bam HI
Xho I
Sal I
Cla I
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AB940/AB941
Xba I
ligate
Cla I
Sal I
Xho I
Bam HI
Xba I
Ssp I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB24
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(16.1 mu)
Xba I
Bam HI
Xho I
Sal I
Cla I
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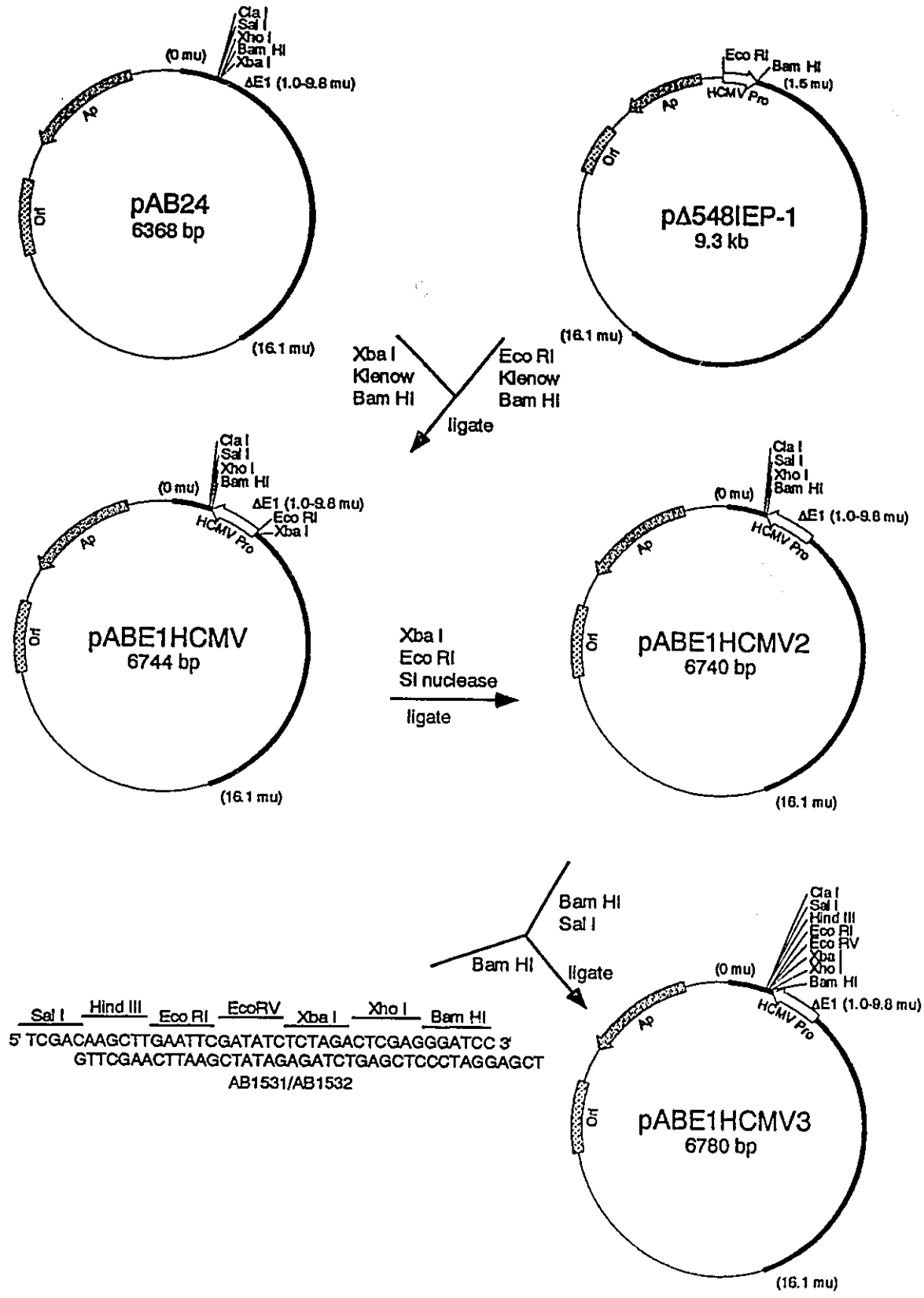
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB25
6368 bp
(16.1 mu)

B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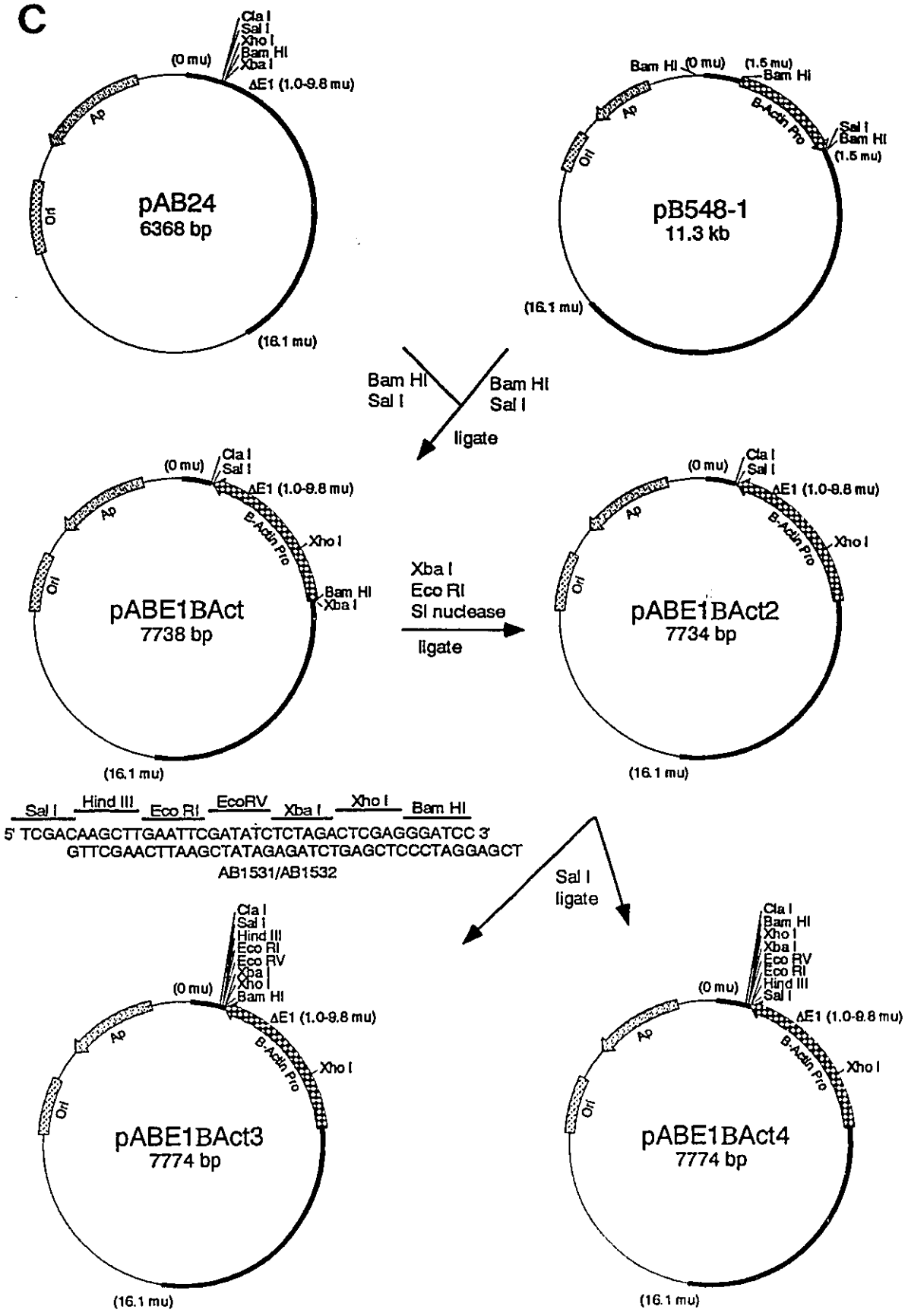
Xba I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
pAB24
6368 bp
(16.1 mu)
Eco RI
Bam HI
(1.5 mu)
HCMV Pro
pΔ548IEP-1
9.3 kb
Xba I
Klenow
Bam HI
Eco RI
Klenow
Bam HI
ligate
Eco RI
pABE1HCMV
6744 bp
Xba I
Eco RI
SI nuclease
ligate
pABE1HCMV2
6740 bp
Bam HI
Sal I
Bam HI
ligate
Hind III
Eco RV
Xba
pABE1HCMV3
6780 bp
Sal I
Hind III
Eco RI
EcoRV
Xba I
Xho I
Bam HI
5' TCGACAAGCTTGAATTCGATATCTCTAGACTCGAGGGATCC 3'
GTTCGAACTTAAGCTATAGAGATCTGAGCTCCCTAGGAGCT
AB1531/AB1532

(C) To introduce the β-Actin promoter into the 3.2 kb E1 deletion so that it could be used to drive expression of foreign genes, pAB24 was digested with *Bam*HI and *Sal*I and ligated with pβ548-1 also digested with *Bam*HI and *Sal*I, generating pABE1βAct. To remove the restriction sites upstream of the promoter pABE1βAct was digested with *Xba*I and *Eco*RI, treated with nuclease S1 and ligated, generating pABE1βAct2. Finally to introduce additional cloning sites downstream of the promoter, pABE1βAct2 was digested with *Sal*I and ligated with synthetic oligo AB1531/AB1532, generating pABE1βAct3 and pABE1βAct4. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r, origin and β-Actin promoter segments, and open bars represent HCMV promoter segments.


C
pAB24
6368 bp
(0 mu)
Cla I
Sal I
Xho I
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Ap
Ori
(16.1 mu)
pB548-1
11.3 kb
Bam HI (0 mu) (1.5 mu)
Bam HI
B-Actin Pro
Sal I
Bam HI
(1.5 mu)
Ap
Ori
(16.1 mu)
Bam HI
Sal I
Bam HI
Sal I
ligate
pABE1BAct
7738 bp
(0 mu)
Cla I
Sal I
ΔE1 (1.0-9.8 mu)
B-Actin Pro
Xho I
Bam HI
Xba I
Ap
Ori
(16.1 mu)
Xba I
Eco RI
SI nuclease
ligate
pABE1BAct2
7734 bp
(0 mu)
Cla I
Sal I
ΔE1 (1.0-9.8 mu)
B-Actin Pro
Xho I
Ap
Ori
(16.1 mu)
Sal I
Hind III
Eco RI
EcoRV
Xba I
Xho I
Bam HI
5' TCGACAAGCTTGAATTCGATATCTCTAGACTCGAGGGATCC 3'
GTTCGAACTTAAGCTATAGAGATCTGAGCTCCCTAGGAGCT
AB1531/AB1532
Sal I
ligate
pABE1BAct3
7774 bp
(0 mu)
Cla I
Sal I
Hind III
Eco RI
Eco RV
Xba I
Xho I
Bam HI
ΔE1 (1.0-9.8 mu)
B-Actin Pro
Xho I
Ap
Ori
(16.1 mu)
pABE1BAct4
7774 bp
(0 mu)
Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
ΔE1 (1.0-9.8 mu)
B-Actin Pro
Xho I
Ap
Ori
(16.1 mu)

orientation to prevent transcription through the foreign gene insert and into downstream Ad sequences. To insert the HCMV promoter, pAB24 was digested with *Xba*I, treated with klenow, digested with *Bam*HI and then ligated with pΔ548IEP-1 (M. Hitt personal communication) which had been digested with *Eco*R1, treated with klenow and digested with *Bam*HI, generating pABE1HCMV (Fig. 10B). pABE1HCMV was then digested with *Xba*I and *Eco*R1, treated with S1 nuclease and ligated, generating pABE1HCMV2. Finally to introduce additional cloning sites in front of the HCMV promoter, pABE1HCMV2 was digested with *Bam*HI and *Sal*I and ligated with the synthetic oligo AB1531/AB1532 also digested with *Bam*HI, generating pABE1HCMV3.

To insert the β-Actin promoter, pAB24 was digested with *Bam*HI and *Sal*I and ligated with pβ548-1 (Hitt and Graham, 1990) also digested with *Bam*HI and *Sal*I, generating pABE1βAct (Fig. 10C). pABE1βAct was then digested with *Xba*I and *Eco*RI, treated with nuclease S1 and ligated, generating pABE1βAct2. Finally to introduce additional cloning sites in front of the β-Actin promoter, pABE1βAct2 was digested with *Sal*I and ligated with synthetic oligo AB1531/AB1532, resulting in pABE1βAct3 and pABE1βAct4.

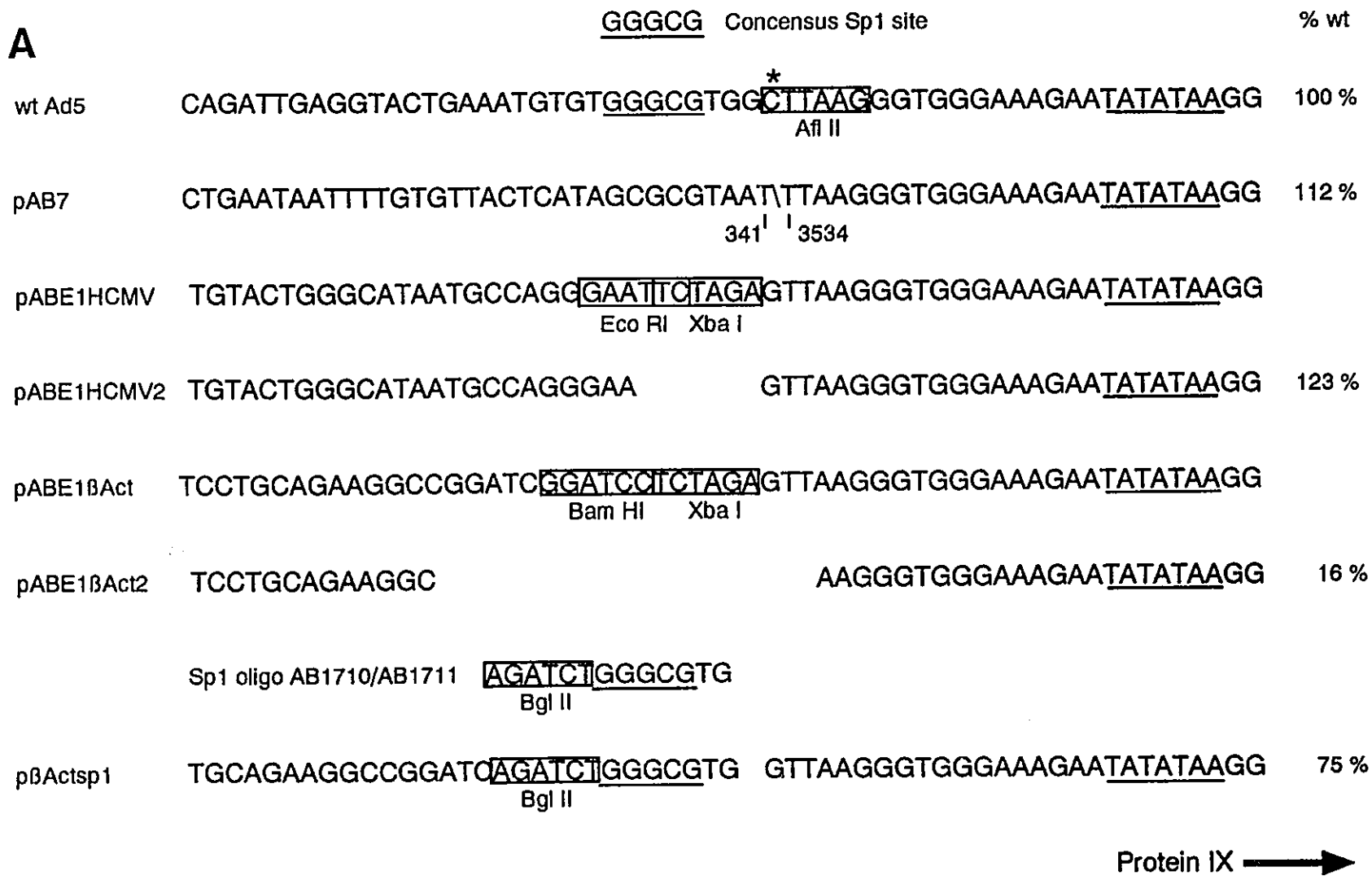
The E1 deletion contained in the plasmids described above does not interfere with the ITR (1-103 bp), the essential core packaging signal (194-358 bp) (Hearing *et al.*, 1987; Grable and Hearing, 1990; Grable and Hearing, 1992) or coding sequences for protein IX but it does remove the Sp1 binding site (3525-3530 bp) from the protein IX promoter (See Bett *et al.*, 1994 Fig. 4). Protein IX is required for packaging full-length genomes into functional virions (Ghosh-Choudhury *et al.*, 1987) and the Sp1 binding site is thought to be essential for protein IX expression (Babiss and Vales, 1991). Since initial attempts (described in results section F.2) to rescue inserts of SIV env, cloned into pABE1HCMV3 and pABE1βAct3 failed, it was suspected that protein IX expression might be low and the packaging of the recombinant genomes

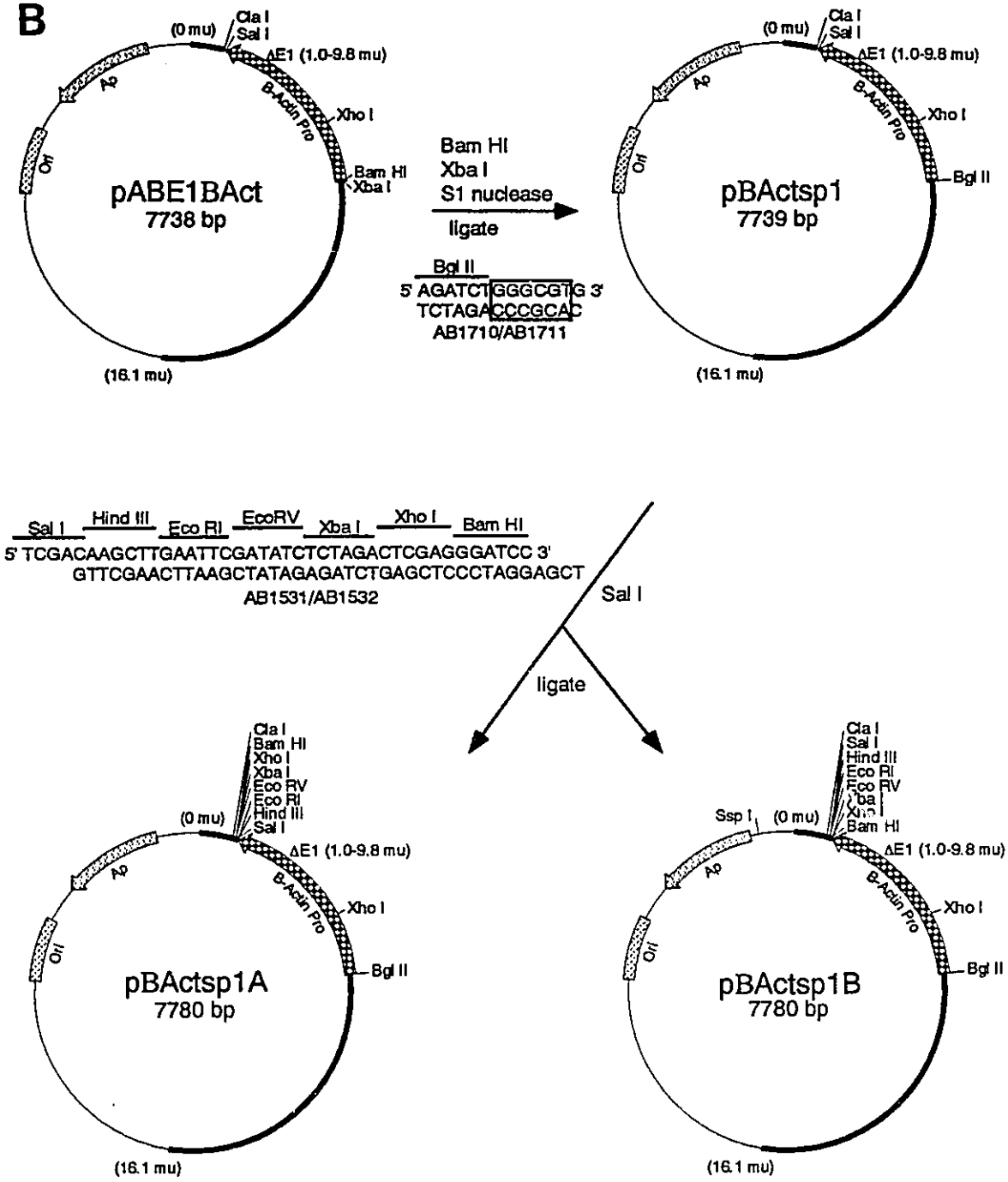
inefficient. To study the level of protein IX expression in vectors with the 3.2 kb deletion pAB7, pABE1HCMV2 and pABE1βAct2 were rescued into virus by cotransfection with pJM17 (McGrory *et al.*, 1988) generating Ad5 dl70-3, AdHCMV2 and AdβAct2 respectively. These vectors were found to have wt growth characteristic but expressed differing amounts of protein IX depending on the insert present (See Bett *et al.*, 1994; Fig. 5). The sequences in the region of the protein IX promoter for these vectors are shown in Figure 11A. dl70-3 which contains no insert expressed 112% of the wt level, AdHCMV2 expressed 123% while AdβAct2 expressed only 16% of the wt level (Fig. 11A). To ensure adequate protein IX expression in all vectors utilizing the 3.2 kb deletion, second generation E1 shuttle plasmids were constructed in which the Sp1 site was reintroduced into the protein IX promoter using a synthetic oligo. The strategy followed to create these plasmids is described below and illustrated in Fig. 11. Since the position of the Sp1 site relative to the TATA box can affect expression of protein IX (Babiss and Vales, 1991) we wanted to reintroduce the Sp1 site so that it was the same distance from the protein IX TATA box as in wt Ad5. A synthetic oligo (AB1710/AB1711) was designed so that when it was inserted into the *Eco*RI and *Xba*I sites in pABE1HCMV or the *Bam*HI and *Xba*I sites in pABE1βAct, made blunt by treatment with S1 nuclease, the Sp1 site would be in the correct position (Fig. 11A). A *Bgl*II site was also incorporated into the Sp1 oligo to facilitate the screening of clones. When we attempted to introduce the Sp1 oligo into pABE1HCMV only 3 out of 168 clones screened contained the insert. When the three clones were sequenced it was found that the Sp1 oligo was either in the wrong orientation or positioned too close to the protein IX TATA box due to over digestion with S1 nuclease. When we attempted to introduce the Sp1 oligo into pABE1βAct, 4 out of 48 clones screened contained the insert (Fig. 11B). Sequencing revealed that all four had the insert positioned 1 bp closer to the protein IX TATA box and only

Figure 11. Reinsertion of the Sp1 binding site into the protein IX promoter in the E1 shuttle plasmids

(A) The sequences in the region of the protein IX promoter are shown for wt Ad5, pAB7 (used to derive dl70-3), pABE1HCMV, pABE1HCMV2 (used to derive AdABE1HCMV2), pABE1βAct, pABE1βAct2 (used to derive AdABE1βAct2) and pβActsp1 (used to derive AdβActsp1). In wt Ad5 the Sp1 transcription factor binding site is located 20 bp from the protein IX TATA box. When the 3.2 kb E1 deletion contained in pAB7 and the pABE1 series of plasmids was created using the *Afl*II site at 3533 bp the Sp1 binding site was removed from the protein IX promoter. To reintroduce the Sp1 site back into the protein IX promoter in the correct position synthetic oligo AB1710/AB1711 was designed so that it could be inserted into the *Eco*RI and *Xba*I sites in pABE1HCMV or the *Bam*HI and *Xba*I sites in pABE1βAct made blunt by treatment with nuclease S1. After many attempts the best clone obtained, pβActsp1, contained the Sp1 site 1 bp closer to the protein IX TATA box then in wt Ad5 (the missing nucleotide is indicated with an asterisk). The protein IX TATA box and Sp1 sites are underlined. The nucleotides at the position of the deletion in pAB7 are indicated.

(B) To reintroduce the Sp1 site back into the protein IX promoter pABE1βAct was digested with *Bam*HI and *Xba*I, treated with nuclease S1 and then ligated with synthetic oligo AB1710/AB1711. Only 4 out of 48 clones screened from this ligation contained the Sp1 oligo and sequencing revealed that only one of these had the insert in the correct orientation. This clone was designated pβActsp1 and contains the Sp1 oligo positioned 1 bp closer to the protein IX TATA box then in wt Ad5 (Fig. 13A). To introduce additional cloning sites downstream of the β-Act promoter pBActsp1 was digested with *Sal*I and ligated with oligo AB1531/AB1532,


A
GGGCG Concensus Sp1 site
% wt
*
wt Ad5 CAGATTGAGGTACTGAAATGTGTGGGCGTGGCTTAAGGGTGGGAAAGAATATATAAGG 100 %
AfI II
pAB7 CTGAATAATTTTGTGTTACTCATAGCGCGTAATTTAAGGGTGGGAAAGAATATATAAGG 112 %
341 3534
pABE1HCMV TGTACTGGGCATAATGCCAGGGAATTCTAGAGTTAAGGGTGGGAAAGAATATATAAGG
Eco RI Xba I
pABE1HCMV2 TGTACTGGGCATAATGCCAGGGAA GTTAAGGGTGGGAAAGAATATATAAGG 123 %
pABE1ßAct TCCTGCAGAAGGCCGGATCGGATCCTCTAGAGTTAAGGGTGGGAAAGAATATATAAGG
Bam HI Xba I
pABE1ßAct2 TCCTGCAGAAGGC AAGGGTGGGAAAGAATATATAAGG 16 %
Sp1 oligo AB1710/AB1711 AGATCTGGGCGTG
Bgl II
pßActsp1 TGCAGAAGGCCGGATCAGATCTGGGCGTG GTTAAGGGTGGGAAAGAATATATAAGG 75 %
Bgl II
Protein IX


B
(0 mu)
Cla I
Sal I
ΔE1 (1.0-9.8 mu)
Ap
B-Actin Pro
Xho I
Ori
Bam HI
Xba I
pABE1BAct
7738 bp
(16.1 mu)
Bam HI
Xba I
S1 nuclease
ligate
Bgl II
5' AGATCTGGGCGTG 3'
TCTAGACCCGCAC
AB1710/AB1711
pBActsp1
7739 bp
Sal I
Hind III
Eco RI
EcoRV
Xba I
Xho I
Bam HI
5' TCGACAAGCTTGAATTCGATATCTCTAGACTCGAGGGATCC 3'
GTTCGAACTTAAGCTATAGAGATCTGAGCTCCCTAGGAGCT
AB1531/AB1532
Sal I
ligate
pBActsp1A
7780 bp
pBActsp1B
7780 bp
Ssp I

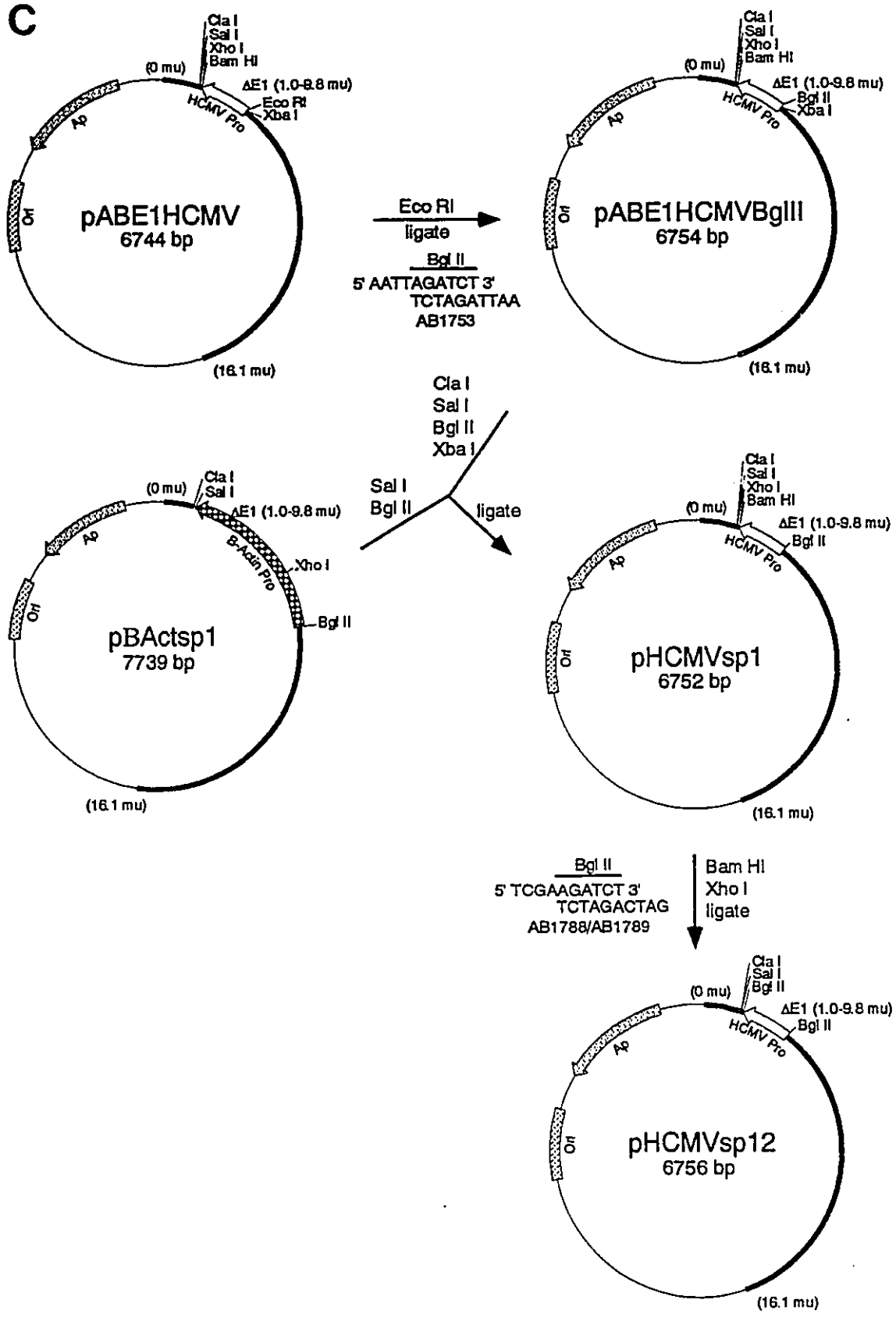
generating pβActsp1A and pβActsp1B.

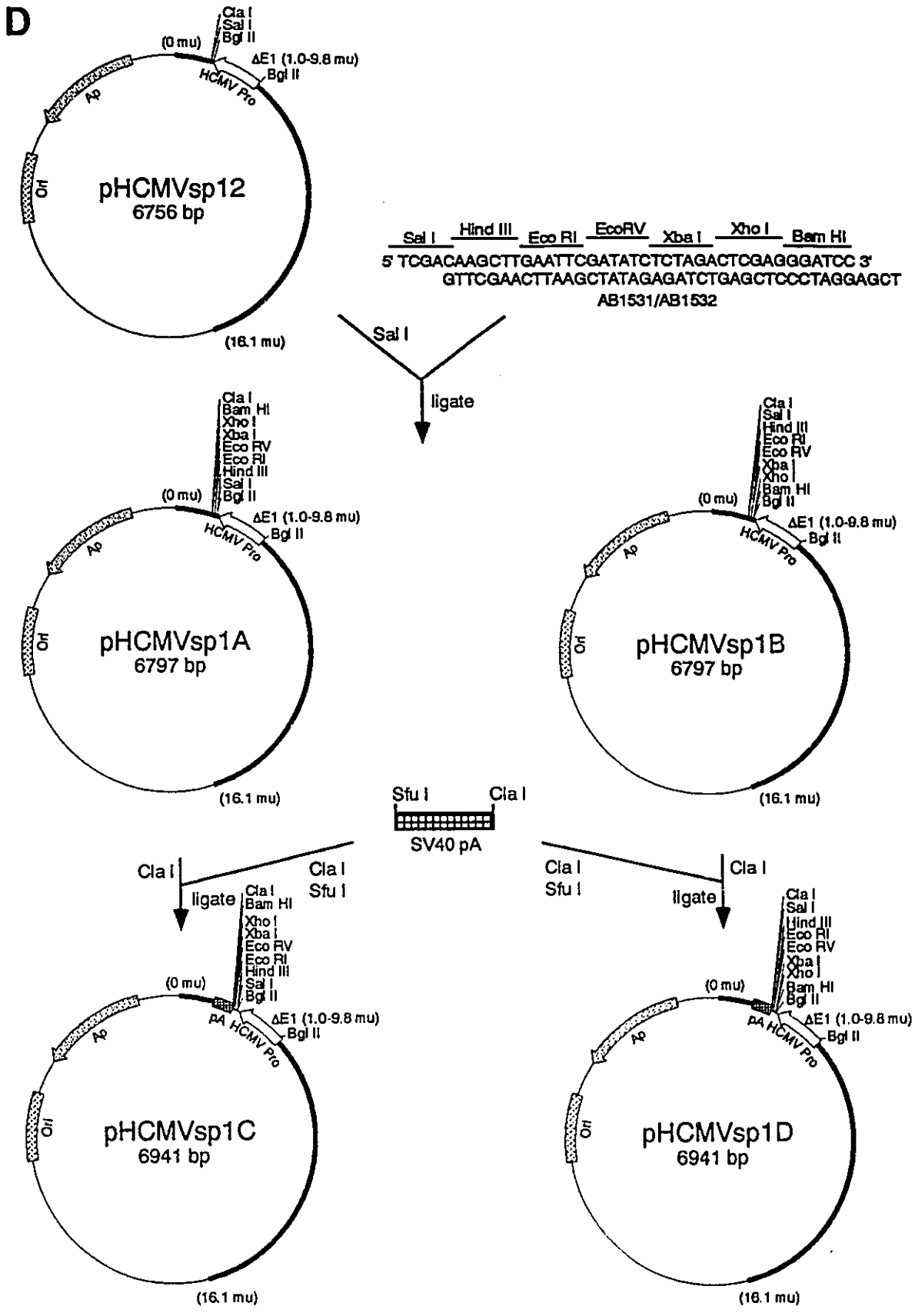
(C) Since reintroducing the Sp1 site into the protein IX promoter using the Sp1 oligo was proving difficult a new strategy was adopted to insert the Sp1 site into pABE1HCMV. pABE1HCMV was digested with *Eco*R1 and ligated with oligo AB1753, generating pABE1HCMVBglII. pABE1HCMVBglII was then digested with *Cla*I, *Sal*I, *Bgl*II and *Xba*I to obtain the *Sal*I/*Bgl*II fragment containing the HCMV promoter (*Cla*I and *Xba*I were used so the plasmid backbone could not recircularize or reincorporate the HCMV promoter) and ligated with pβActsp1 which had been digested with *Sal*I and *Bgl*II, generating pHCMVsp1. pHCMVsp1 was then digested with *Bam*HI and *Xho*I and ligated with the synthetic oligo AB1788/AB1789 containing a *Bgl*II site, generating pHCMVsp12.

(D) Two additional improvements were made to pHCMVsp12. First additional cloning sites were introduced downstream of the HCMV promoter by digesting pHCMVsp12 with *Sal*I and ligating with oligo AB1531/AB1532, generating pHCMVsp1A and pHCMVsp1B. Then PCR was used to generate a 150 bp fragment containing the SV40 poly A which was inserted downstream of the HCMV promoter and cloning sites. PCR primers, AB2633 (5' GGGATCGATCAACTTGTTTATTGC 3') and AB2634 (5'CTTTTCGAATTGCCTAGACGATCC 3') were designed to contain *Cla*I and *Sfu*I sites (underlined) respectively and pSV2X3 was used as the template DNA. pHCMVsp1A and pHCMVsp1B were digested with *Cla*I and ligated with the PCR fragment digested with *Cla*I and *Sfu*I generating pHCMVsp1C and pHCMVsp1D.

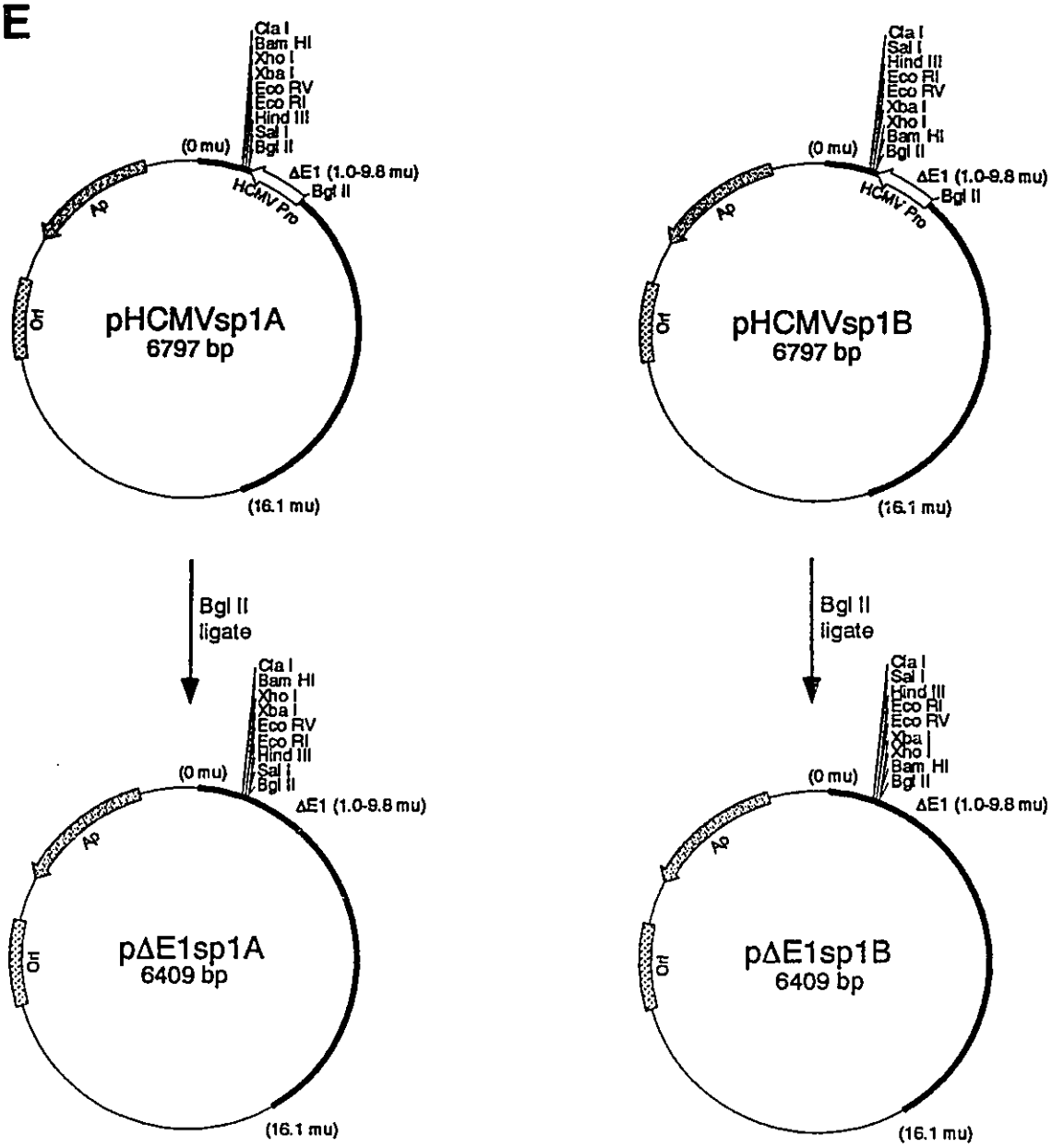
(E) To create E1 shuttle plasmids with the Sp1 correction and no heterologous promoter pHCMVsp1A and pHCMVsp1B were digested with *Bgl*II and religated, generating pΔE1sp1A and pΔE1sp1B respectively.

(F) To create an E1 shuttle plasmid containing the Ad2 major late promoter pΔE1sp1A was digested with *Cla*I and *Bam*HI and ligated with pPyMLPX-1 also digested with *Cla*I and


C
Cla I
Sal I
Xho I
Bam HI
(0 mu)
ΔE1 (1.0-9.8 mu)
Eco RI
Xba I
HCMV Pro
Ap
ori
pABE1HCMV
6744 bp
(16.1 mu)
Eco RI
ligate
Bgl II
5' AATTAGATCT 3'
TCTAGATTAA
AB1753
Cla I
Sal I
Xho I
Bam HI
(0 mu)
ΔE1 (1.0-9.8 mu)
Bgl II
Xba I
HCMV Pro
Ap
ori
pABE1HCMVBglII
6754 bp
(16.1 mu)
Cla I
Sal I
Bgl II
Xba I
Sal I
Bgl II
ligate
Cla I
Sal I
(0 mu)
ΔE1 (1.0-9.8 mu)
β-Actin Pro
Xho I
Bgl II
Ap
ori
pBActsp1
7739 bp
(16.1 mu)
Cla I
Sal I
Xho I
Bam HI
(0 mu)
ΔE1 (1.0-9.8 mu)
Bgl II
HCMV Pro
Ap
ori
pHCMVsp1
6752 bp
(16.1 mu)
Bgl II
5' TCGAAGATCT 3'
TCTAGACTAG
AB1788/AB1789
Bam HI
Xho I
ligate
Cla I
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
Bgl II
HCMV Pro
Ap
ori
pHCMVsp12
6756 bp
(16.1 mu)

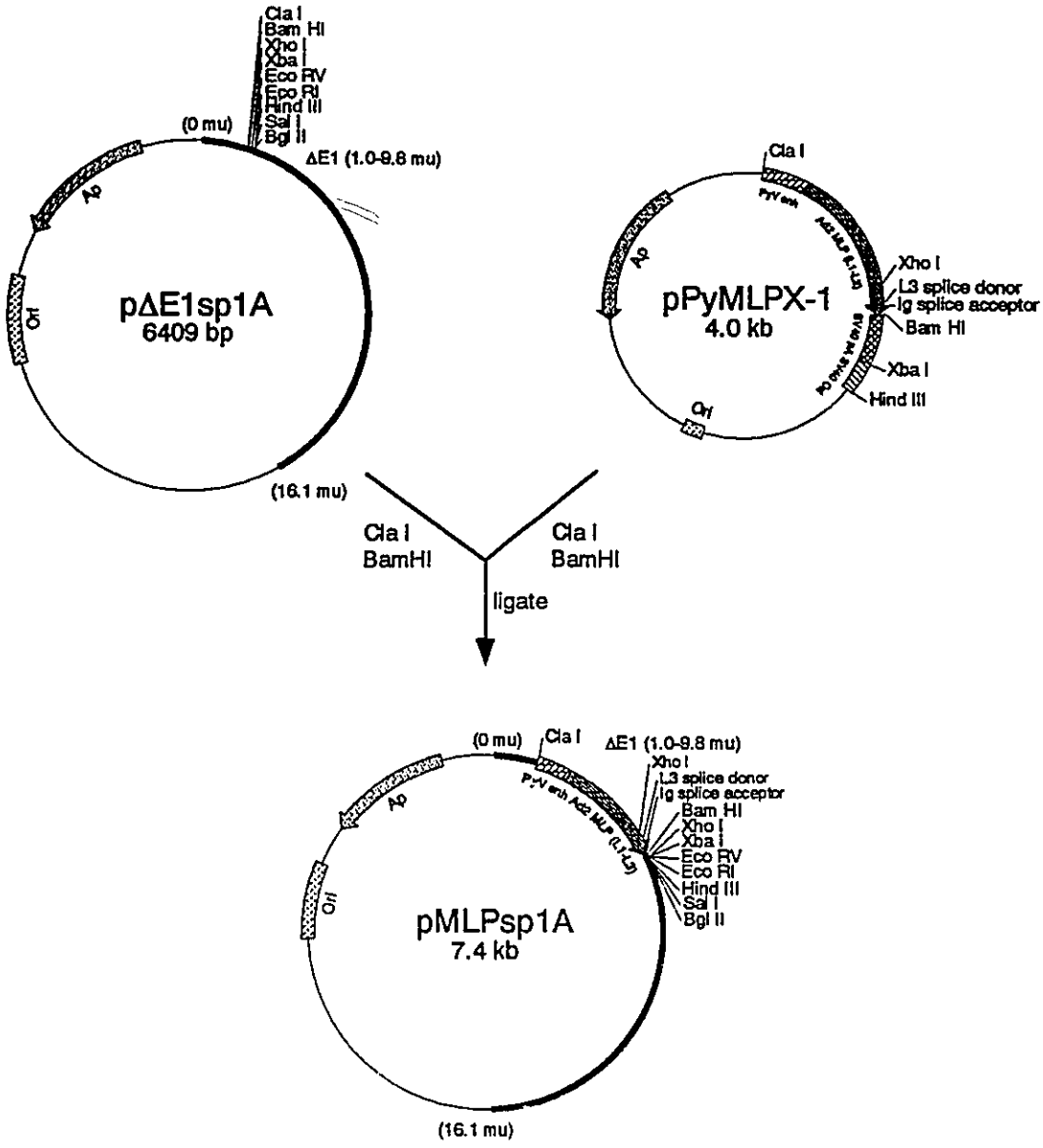

D
Cla I
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
Bgl II
HCMV Pro
Ap
ori
pHCMVsp12
6756 bp
(16.1 mu)
Sal I
Hind III
Eco RI
EcoRV
Xba I
Xho I
Bam HI
5' TCGACAAGCTTGAATTCGATATCTCTAGACTCGAGGGATCC 3'
GTTCGAACTTAAGCTATAGAGATCTGAGCTCCCTAGGAGCT
AB1531/AB1532
Sal I
ligate
Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
pHCMVsp1A
6797 bp
pHCMVsp1B
6797 bp
Sfu I
Cla I
SV40 pA
Cla I
Sfu I
ligate
pHCMVsp1C
6941 bp
pHCMVsp1D
6941 bp
PA

E


Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
HCMV Pro
Bgl II
Ap
Ori
pHCMVsp1A
6797 bp
(16.1 mu)
Cla I
Sal I
Hind III
Eco RI
Eco RV
Xba I
Xho I
Bam HI
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
HCMV Pro
Bgl II
Ap
Ori
pHCMVsp1B
6797 bp
(16.1 mu)
Bgl II
ligate
Bgl II
ligate
pΔE1sp1A
6409 bp
(16.1 mu)
pΔE1sp1B
6409 bp
(16.1 mu)

was digested with ClaI and BamHI and ligated with pPyMLPX-1 also digested with *Cla*I and *Bam*HI, generating pMLPsp1A. The promoter segment in pMLPsp1A contains the polyoma virus enhancer linked to the Ad2 major late promoter and tripartite leader. The third leader retains its splice donor which can splice to an Ig splice acceptor placed just upstream of the cloning sites. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r, origin, SV40 polyadenylation, β-Actin and Ad2 MLP promoter segments, and open bars represent HCMV promoter segments.

F


Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
pΔE1sp1A
6409 bp
(16.1 mu)
pPyMLPX-1
4.0 kb
Xho I
L3 splice donor
Ig splice acceptor
Bam HI
Xba I
Hind III
Cla I
BamHI
Cla I
BamHI
ligate
(0 mu)
Cla I
ΔE1 (1.0-9.8 mu)
Xho I
L3 splice donor
Ig splice acceptor
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
pMLPsp1A
7.4 kb
(16.1 mu)

one had the insert in the correct orientation. Because of the difficulty being encountered in inserting the Sp1 oligo into pABE1HCMV and pABE1βAct we decided to choose the βact clone containing the Sp1 oligo in the correct orientation but positioned one bp closer to the protein IX TATA box and designated this clone pβActsp1 (Fig. 11A and 11B). To introduce additional cloning sites downstream of the β-Actin promoter pβActsp1 was digested with *Sal*I and ligated with oligo AB1531/AB1532, generating pβActsp1A and pβActsp1B (Fig. 11B). pβActsp1A and pβActsp1B were constructed in collaboration with C. Addison.

Since introducing the Sp1 oligo was proving to be difficult a new strategy was designed to reintroduce the Sp1 site into pABE1HCMV (Fig. 11C). pABE1HCMV was digested with *Eco*R1 and ligated with oligo AB1753 which contains a *Bgl*II site, generating pABE1HCMVBglII. pABE1HCMVBglII was then digested with *Cla*I, *Sal*I, *Bgl*II and *Xba*I to obtain the *Sal*I/*Bgl*II fragment containing the HCMV promoter (*Cla*I and *Xba*I were used so the plasmid backbone could not recircularize or reincorporate the HCMV promoter) and ligated with pβActsp1 which had been digested with *Sal*I and *Bgl*II, generating pHCMVsp1. pHCMVsp1 was then digested with *Bam*HI and *Xho*I and ligated with the synthetic oligo AB1788/AB1789 containing a *Bgl*II site, generating pHCMVsp12. Additional cloning sites were incorporated by digesting pHCMVsp12 with *Sal*I and ligating with oligo AB1531/AB1532, generating pHCMVsp1A and pHCMVsp1B (Fig. 11D). In the final step the SV40 polyadenylation signal (poly A) was introduced downstream of the HCMV promoter and cloning sites. PCR was used to generate a 150 bp fragment containing the SV40 poly A. PCR primers, AB2633 and AB2634, were designed to contain *Cla*I and *Sfu*I sites respectively and pSV2X3 (Prevec *et al.*, 1990) was used as the template DNA. pHCMVsp1A and pHCMVsp1B were digested with *Cla*I and ligated with the PCR fragment digested with *Cla*I and *Sfu*I generating pHCMVsp1C and pHCMVsp1D (Fig. 11D).

In order to create E1 shuttle plasmids with the Sp1 correction and no heterologous promoter pHCMVsp1A and pHCMVsp1B were digested with *Bgl*II and religated, generating pΔE1sp1A and pΔE1sp1B respectively (Fig. 11E).

One final E1 shuttle plasmid was created containing the Ad2 major late promoter for driving expression of foreign DNA inserts (Fig. 11F). pΔE1sp1A was digested with *Cla*I and *Bam*HI and ligated with pPyMLPX-1 (Jelinek *et al.*, 1992) also digested with *Cla*I and *Bam*HI, generating pMLPsp1A. The promoter segment in this plasmid contains the polyoma virus enhancer linked to the Ad2 major late promoter and tripartite leader. The third leader retains its splice donor which can splice to an Ig splice acceptor placed just upstream of the cloning sites.

To determine if the reintroduction of the Sp1 site had positively affected the expression of protein IX, pβActsp1 and pHCMVsp1 were rescued into virus by cotransfection with pJM17, generating AdβActsp1 and AdHCMVsp1 respectively. When the level of protein IX expression obtained with AdβActsp1 and AdHCMVsp1 was analyzed and compared to AdβAct2 and AdHCMV2 it was found to have increased in both cases (See Bett *et al.*, 1994; Fig. 5). It should be noted that neither the original 3.2 kb E1 deletion nor the deletion mutants containing the synthetic Sp1 site appeared to be significantly altered in protein IX expression (Bett *et al.*, 1994; Fig. 5) heat stability (Bett *et al.*, 1994; Fig. 6) or final progeny yields. This suggests that the Sp1 site in the protein IX promoter is not absolutely required for adequate protein IX expression in contrast to the findings of Babiss and Vales (1991). The E1 shuttle vectors with the Sp1 site reintroduced or derivatives of these plasmids are now commonly used in cotransfections with pJM17 and the pBHG plasmids to ensure adequate levels of protein IX are produced.

6. Construction of E3 Shuttle Plasmids For Use With The pBHG Vector System

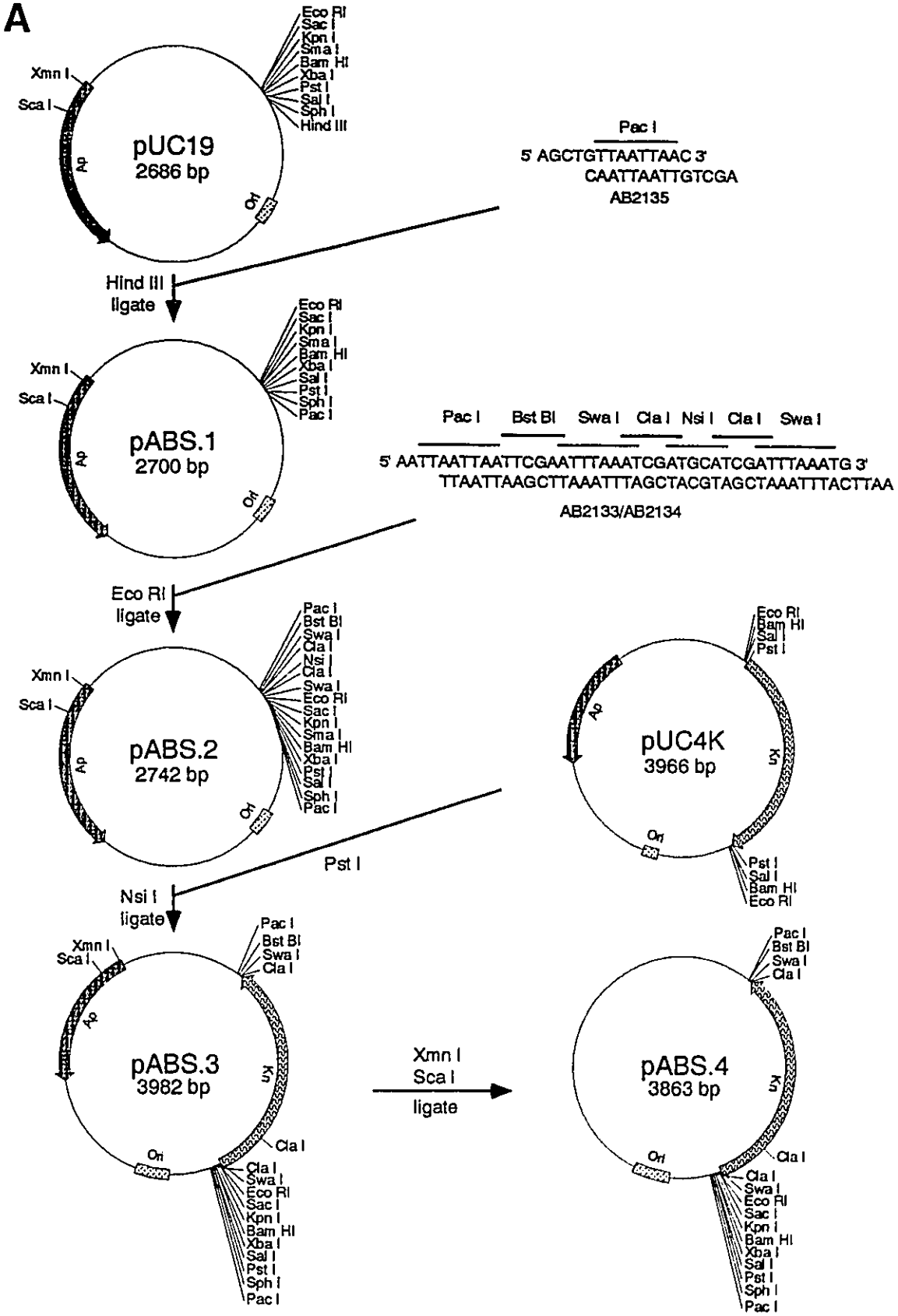
pBHG10 and pBHG11 were designed so that foreign DNA inserts could be cloned directly into the *Pac*I site at the position of their E3 deletions. In order to simplify the cloning procedure a Kn^r shuttle vector, pABS.4, was developed. The strategy followed to create pABS.4 and a description of its use are given below and illustrated in Figure 12. To create pABS.4, pUC19 was digested with *Hind*III and ligated with oligo AB2135, generating pABS.1 (Fig. 12A). Next pABS.1 was digested with *Eco*RI and ligated with oligo AB2133/AB2134 generating pABS.2. A Kn^r gene was then obtained from pUC4K (Pharmacia Canada Ltd.) by digesting with *Pst*I and ligated with *Nsi*I digested pABS.2, generating pABS.3. In the final step the Ap^r gene in pABS.3 was disrupted by digesting with *Xmn*I and *Sca*I and ligation, generating pABS.4.

The shuttle plasmid pABS.4 can be used to facilitate the transfer of foreign DNA inserts into the pBHG plasmids as follows: gene sequences are inserted into pABS.4 using the cloning sites *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Kpn*I, *Sac*I or *Eco*RI (Fig. 12B). The shuttle plasmid is then cut with *Pac*I and the insert/Kn^r containing fragment is inserted into the Ap^r pBHG plasmid making use of Ap+Kn double resistance to select for bacterial transformants carrying the desired plasmid. Subsequently the Kn^r gene can be removed by *Swa*I digestion and ligation resulting in a pBHG derivative ready for cotransfection with an appropriate E1 shuttle plasmid. The cloning sites present in pABS.4 and the use of Ap+Kn double selection greatly simplify the introduction of inserts into the E3 deletions in pBHG10 and pBHG11.

Figure 12. Construction and use of E3 shuttle plasmid pABS.4.

(A) To simplify the cloning of inserts into the *Pac*I sites at the position of the E3 deletions in pBHG10 and pBHG11, shuttle vector pABS.4 was developed. In the first step pUC19 was digested with *Hind*III and ligated with oligo AB2135, generating pABS.1. pABS.1 was then digested with *Eco*RI and ligated with oligo AB2133/AB2134 generating pABS.2. A Kn^r gene was then obtained from pUC4K by digesting with *Pst*I and ligated with pABS.2 digested with *Nsi*I, generating pABS.3. In the final step the Ap^r gene in pABS.3 was disrupted by digesting with *Xmn*I and *Sca*I and ligation, generating pABS.4.

(B) Shuttle plasmid pABS.4 can be used to facilitate the transfer of foreign DNA inserts into the E3 deletions in the pBHG plasmids as described below. In the first step the gene of interest is inserted into pABS.4 using the cloning sites *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Kpn*I, *Sac*I or *EcoR*I. In the example shown the foreign DNA insert is cloned into the *Eco*RI and *Sph*I sites generating pABSIN. Recombinant shuttle plasmid pABSIN is then cut with *Pac*I and the insert/Kn^r cassette is introduced into the Ap^r pBHG plasmid making use of Ap+Kn double resistance to select for bacterial transformants carrying the desired plasmid, pBHGINK. Subsequently the Kn^r gene can be removed by *Swa*I digestion and ligation generating pBHGIN. This pBHG derivative can now be used to generate infectious virus by cotransfection with an appropriate E1 shuttle plasmid. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r, Kn^r, origin, and insert segments.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Eco RI
Sac I
Kpn I
Sma I
Bam HI
Xba I
Pst I
Sal I
Sph I
Hind III
Xmn I
Sca I
Ap
Ori
Pac I
5' AGCTGTTAATTAAC 3'
CAATTAATTGTCGA
AB2135
Hind III
ligate
pABS.1
2700 bp
Pac I
Bst BI
Swa I
Cla I
Nsi I
Cla I
Swa I
5' AATTAATTAATTCGAATTTAAATCGATGCATCGATTTAAATG 3'
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AB2133/AB2134
Eco RI
ligate
pABS.2
2742 bp
pUC4K
3966 bp
Kn
Pst I
Sal I
Bam HI
Eco RI
Nsi I
ligate
pABS.3
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Xmn I
Sca I
Cla I
Swa I
Eco RI
Sac I
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Sal I
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ligate
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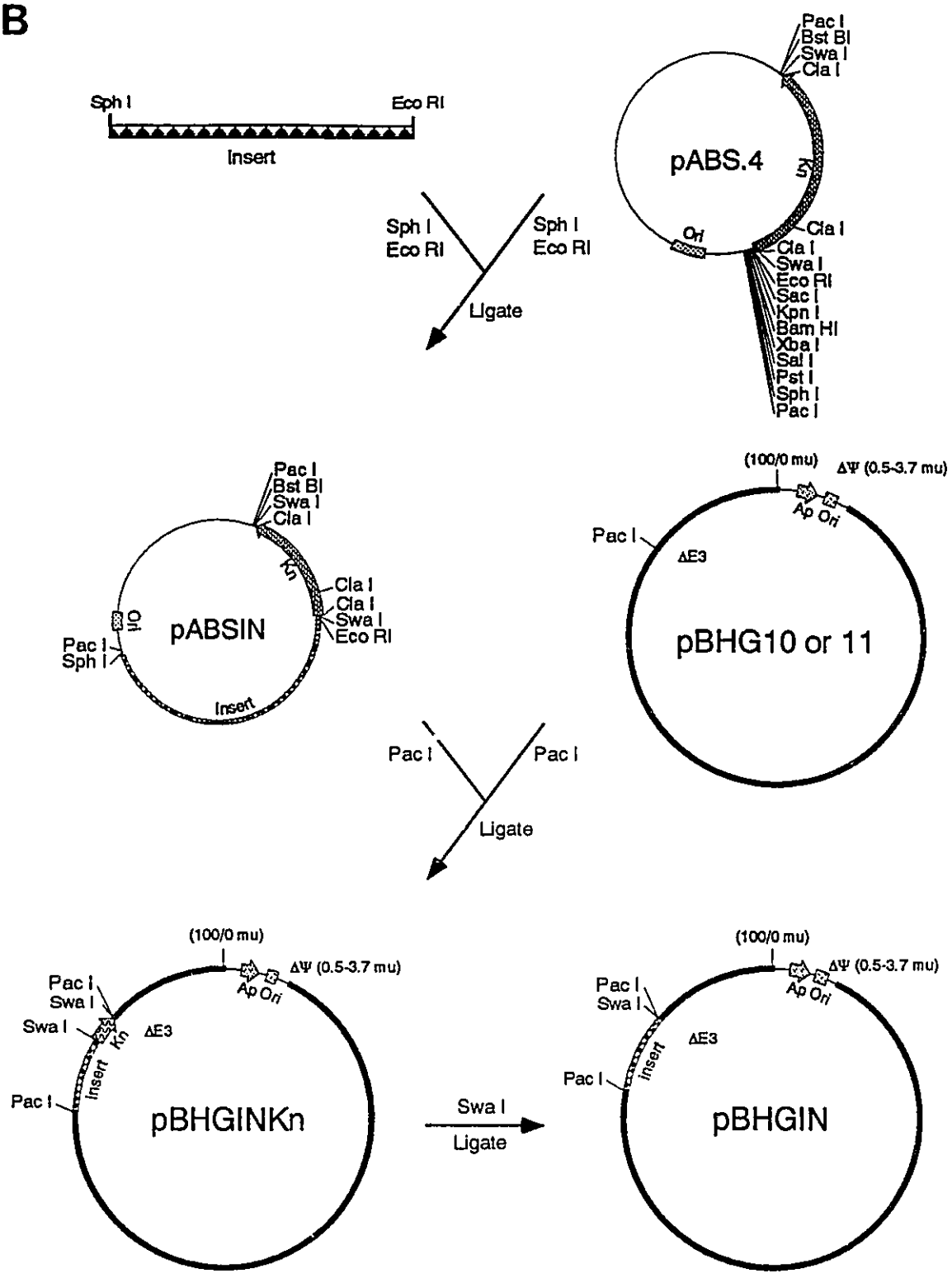
B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Eco RI
Insert
Sph I
Eco RI
Sph I
Eco RI
Ligate
pABS.4
Pac I
Bst BI
Swa I
Cla I
Ori
Cla I
Cla I
Swa I
Eco RI
Sac I
Kpn I
Bam HI
Xba I
Sal I
Pst I
Sph I
Pac I
pABSIN
Pac I
Bst BI
Swa I
Cla I
Kn
Cla I
Cla I
Swa I
Eco RI
Ori
Pac I
Sph I
Insert
(100/0 mu)
ΔΨ (0.5-3.7 mu)
Ap Ori
Pac I
ΔE3
pBHG10 or 11
Pac I
Pac I
Ligate
(100/0 mu)
ΔΨ (0.5-3.7 mu)
Pac I
Swa I
Swa I
Ap Ori
ΔE3
Kn
Insert
Pac I
pBHGINKn
Swa I
Ligate
(100/0 mu)
ΔΨ (0.5-3.7 mu)
Pac I
Swa I
Ap Ori
ΔE3
Insert
Pac I
pBHGIN

C. Foreign Gene Expression By Human Adenovirus Type 5 Vectors Studied Using Firefly Luciferase And Bacterial Beta-galactosidase Genes As Reporters. (Mittal, *et al.*, 1995, Virology, 210:226-230.)

1. Background

As described in the introduction many Ad vectors have been constructed containing foreign genes substituted for deletions in the E3 region generating helper independent vectors. However at the time that this research project was initiated little work had been done to determine the importance of regulatory sequences flanking the inserted gene, on gene expression. In Mittal *et al.* (1995) we set out to study the basic requirements needed to obtain optimum expression from foreign genes inserted into early region 3. To do this a series of Ad5 helper independent vectors were generated that contained the firefly luciferase gene or the bacterial β-galactosidase gene (LacZ) with or without simian virus 40 (SV40) regulatory sequences, in E3 deletions of 1.88 or 2.69 kb. Expression levels of luciferase and LacZ were determined and compared for the various vectors in Hela cells.

VIROLOGY 210, 226–230 (1995)

SHORT COMMUNICATION

Foreign Gene Expression by Human Adenovirus Type 5-Based Vectors Studied Using Firefly Luciferase and Bacterial β-Galactosidase Genes as Reporters

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Adenovirus (Ad) vectors have been used extensively to obtain high-level expression of foreign genes in mammalian cells and are currently being studied for use as live viral-vectored vaccines and as gene transfer vectors for gene therapy. Many Ad recombinants have been generated that express foreign genes inserted in early region 3 (E3); however, little has been done to study the importance for gene expression of regulatory sequences flanking the gene. We have generated a series of Ad5 helper-independent vectors that contain the firefly luciferase gene or the bacterial β-galactosidase gene (LacZ) with or without simian virus 40 (SV40) regulatory sequences, combined with E3 deletions of 1.88 or 2.69 kb. The greatest levels of luciferase expression were obtained with a vector containing the luciferase gene under the control of the SV40 promoter and polyadenylation signal inserted in a 1.88-kb E3 deletion. In contrast, LacZ expression was highest with a vector containing the LacZ gene with just the SV40 polyadenylation sequence combined with a 1.88-kb E3 deletion. It was also observed that regardless of the SV40 sequences flanking the reporter gene or the E3 deletion used, expression from the luciferase recombinants was dependent on viral DNA replication, whereas expression from the LacZ recombinants was only partially reduced when DNA replication was blocked. Analyses of RNA by dot blot hybridizations revealed that the levels of reporter gene-specific mRNA for various vectors in each series did not vary significantly. These results indicate that the kinetics and efficiency of expression of genes inserted into the E3 region, in nonconditional helper-independent vectors, may be more strongly dependent on the sequences in the foreign gene insert itself than on flanking regulatory sequences such as those used here, derived from SV40.

Adenoviruses (Ads) can be used as mammalian cell expression vectors (*1–3*) that have excellent potential as live recombinant vaccines (*1–3*) and as transducing vectors for gene therapy (*4–7*). In the human Ad genome, early region 1 (E1), E3, and a site upstream of E4 have been utilized as sites for introducing foreign DNA sequences to generate adenovirus recombinants (*8–10*). In the absence of compensating deletions in E1 or E3 a maximum of about 2 kb can be inserted into the Ad genome to generate viable virus progeny (*11, 12*). The E1 region is not required for viral replication in complementing 293 cells (*13*) and up to 3.2 kb can be deleted in this region to generate conditional helper-independent vectors with a capacity of 5.0–5.2 kb. In the E3 region, which is not required for viral replication in cultured cells (*9, 14*), deletions of various sizes have been utilized to generate nonconditional helper-independent vectors with a capacity of up to 4.5–4.7 kb (*12*).

Many foreign proteins have been successfully expressed when inserted into the E3 region including ones encoded by genes from herpes simplex virus (*15*), hepatitis B virus (*16*), vesicular stomatitis virus (*17*), respiratory syncytial virus (*18*), rabies virus (*19*), human immunodeficiency virus (*20, 21*), and simian immunodeficiency virus (*22*). Inserts in this region have been shown to be expressed in permissive as well as nonpermissive and semipermissive cell lines (*17*) and have also been found to elicit humoral, mucosal, and cellular immune responses to antigens encoded by the inserted foreign gene in a variety of different animals (*16, 17, 20, 23–25*). This suggests that Ad recombinants will be effective for vaccine use even in species in which the virus does not replicate well. Although much work has been done to develop and characterize recombinant Ad vectors with inserts in E3, little has been done to study the importance of upstream and downstream flanking sequences on gene expression. To begin to explore this we have compared levels of expression from two reporter genes, firefly luciferase and bacterial β-galactosidase, in a series of vectors with and without SV40 regulatory sequences and in the context of two E3 deletions, one of 1.88 kb and one of 2.69 kb.

To construct the recombinants used in this study the firefly luciferase cDNA (*26, 27*) or the LacZ gene coding

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0042-6822/95 $6.00

TABLE 1

Ad5-Recombinants

Virus	Reporter gene	E3 deletion[a]	SV40 reg. seq.		Maximal expression (μg/10⁶ cells)[b]
			Promoter	Poly(A)	
Ad5-Luc 3	Luciferase	1.88 kb	+	+	23.1
Ad5-Luc 13	Luciferase	2.69 kb	+	+	8.2
Ad5-Luc 1	Luciferase	1.88 kb	−	−	2.8
Ad5-Luc 11	Luciferase	2.69 kb	−	−	1.9
Ad5-Luc 5	Luciferase	1.88 kb	−	+	1.3
Ad5-LacZ 5	LacZ	1.88 kb	−	+	17.6
Ad5-LacZ 3	LacZ	1.88 kb	+	+	4.3
Ad5-LacZ 11	LacZ	2.69 kb	−	−	3.1
Ad5-LacZ 15	LacZ	2.69 kb	−	+	2.7
Ad5-LacZ 13	LacZ	2.69 kb	+	+	2.1

[a] E3 deletion into which reporter construct was cloned. The 1.88-kb deletion removes Ad sequences from 28592 to 30470 bp and the 2.69 kb deletion removes sequences from 28133 to 30818 bp (*12*).

[b] Maximal expression obtained with each virus expressed as micrograms of protein per million cells.

sequences, alone or flanked by the SV40 early promoter and/or the SV40 polyadenylation signal [poly(A)] were inserted into E3 transfer plasmids containing either a 1.88-kb (*12, 26*) or 2.69-kb (*12*) E3 deletion. The recombinant plasmids were then cotransfected with pFG173 (*26, 28*) into 293 cells and viruses generated by *in vivo* recombination (*2*). Recombinant virus were isolated and grown in 293 cells and the viral DNA was extracted, digested with *Hin*dIII, and analyzed on 1% agarose gels (*2*) (results not shown). The presence of the reporter gene inserts in the recombinant virus DNA was confirmed by the presence of diagnostic *Hin*dIII fragments and by enzyme assays. In all a total of 10 helper-independent Ad5-recombinants (5 Ad5-luciferase recombinants and 5 Ad5-LacZ recombinants) were generated (Table 1).

Ad5-Luc 1 (*26*) contains the luciferase gene combined with a 1.88-kb E3 deletion. Ad5-Luc 3 contains the luciferase gene linked to the SV40 promoter and poly(A) combined with a 1.88-kb E3 deletion. Ad5-Luc 5 contains the luciferase gene linked to the poly(A) combined with a 1.88-kb E3 deletion. Ad5-Luc 11 and Ad5-Luc 13 are identical to Ad5-Luc 1 and Ad5-Luc 3, respectively, except that in the former the inserts are combined with a 2.69-kb E3 deletion. The Ad5-LacZ recombinants Ad5-LacZ 3 (*12*), Ad5-LacZ 5, Ad5-LacZ 11, and Ad5-LacZ 13 (*12*) are similar to the Ad5-luciferase recombinants Ad5-Luc 3, Ad5-Luc 5, Ad5-Luc 11, and Ad5-Luc 13, respectively, except for the reporter gene used. Finally, Ad5-LacZ 15 contains the LacZ gene linked to the poly(A) rescued into a 2.69-kb E3 deletion (*12*). All inserts were introduced in the E3 parallel orientation.

The levels of luciferase expression in Ad5-luciferase recombinant-infected HeLa cells were measured at various times postinfection by standard enzymatic assays for luciferase activity (*26*) (Fig. 1A). For all vectors expressing luciferase, activity was first detected at 6 hr postinfection, increased gradually to a maximum between 30 and 36 hr, and levelled out or dropped thereafter. In infections with Ad5-Luc 1 and Ad5-Luc 11, which contain the luciferase gene without any flanking SV40 sequences, or Ad5-Luc 5, which contains just the SV40 poly(A) sequence, the levels of enzyme expression were similar and reached a maximum between 30 and 36 hr postinfection. The presence of both the SV40 promoter and poly(A) sequences increased the level of expression by fourfold (Ad5-Luc 13 vs Ad5-Luc 11) or eightfold (Ad5-Luc 3 vs Ad5-Luc 1). The levels of LacZ expression in Ad5-LacZ recombinant-infected HeLa cells were also measured at various times postinfection by LacZ assays (*29*) (Fig. 1B). In contrast to the luciferase expression data, the highest LacZ levels were obtained not from the recombinant containing the LacZ gene flanked by the SV40 promoter and poly(A), but rather with Ad5-LacZ 5 which contains the LacZ gene with just the SV40 poly(A) in a 1.88-kb E3 deletion. Expression levels using Ad5-LacZ 5 were approximately sevenfold higher than those obtained with Ad5-LacZ 11, Ad5-LacZ 13, and Ad5-LacZ 15 and fourfold greater than that obtained with Ad5-LacZ 3. Maximal expression levels attained in cells infected with these various vectors are summarized in Table 1.

To examine the dependence of reporter gene expression on viral DNA replication, infected cells were incubated in the presence or absence of 1-β-D-arabinofuranosyl cytosine (AraC), which blocks DNA replication (Table 2). In the presence of AraC, luciferase expression by the Ad5-luciferase recombinants (Ad5-Luc 1, Ad5-Luc 3, Ad5-Luc 11, and Ad5-Luc 13) at 24 hr postinfection was inhibited by an average of 97.6%. This suggests that the majority of luciferase expression was dependent on viral DNA replication. In contrast, LacZ expression from the Ad5-LacZ recombinants (Ad5-LacZ 3, Ad5-LacZ 5, Ad5-LacZ 11, Ad5-LacZ 13, and Ad5-LacZ 15) was only inhib

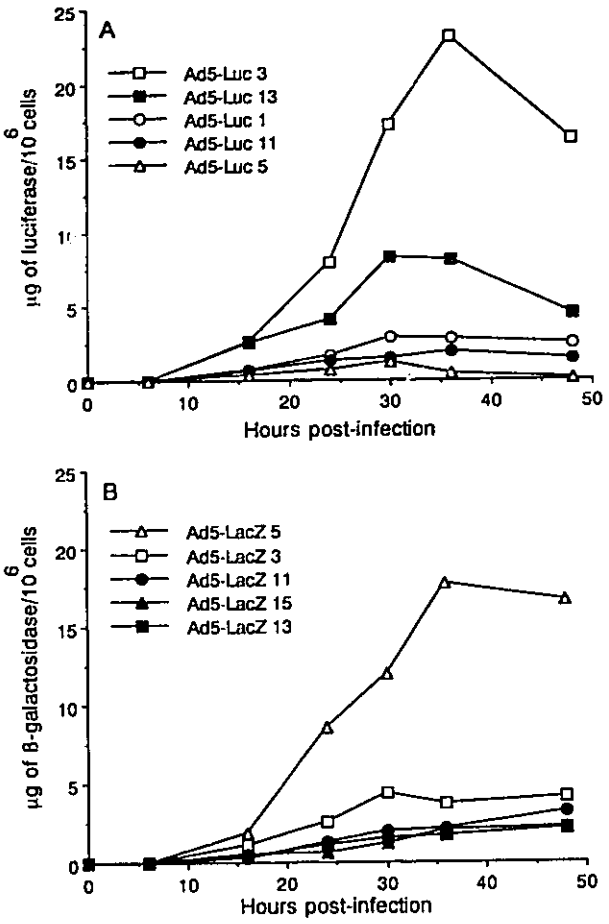

FIG. 1. Kinetics of luciferase and LacZ expression in HeLa cells infected with different Ad5-recombinants. (A) To assay luciferase expression HeLa cell monolayers in 60-mm dishes were infected with an m.o.i. of 50 PFU/cell of an Ad5 luciferase recombinant. Virus-infected cell pellets were collected at various times postinfection, homogenized in 100 m*M* potassium phosphate, pH 7.8, containing 1 m*M* dithiothreitol, and lysed by three cycles of freezing and thawing. The supernatants were saved and assayed in duplicate for luciferase activity as described in detail elsewhere (*26*). The luciferase expressed in HeLa cells infected with Ad5-Luc 1 (*26*), Ad5-Luc 3, Ad5-Luc 5, Ad5-Luc 11, or Ad5-Luc 13 is shown as μg luciferase/10^6 cells. The vectors have been listed in the key according to decreasing expression levels. (B) To assay LacZ activity HeLa cell monolayers in 60-mm dishes were infected with an m.o.i. of 50 PFU/cell of the various Ad5-LacZ recombinants. At various times postinfection virus-infected cell pellets were collected and lysed in 250 m*M* Tris · HCl, pH 7.8, containing 1 m*M* phenylmethylsulfonyl fluoride and 0.5% NP-40. The supernatants were saved and assayed in duplicate for LacZ activity (*29*). The β-galactosidase expressed in HeLa cells infected with Ad5-LacZ 3 (*12*), Ad5-LacZ 5, Ad5-LacZ 11, Ad5-LacZ 13 (*12*), or Ad5-LacZ 15 (*12*) is shown as μg β-galactosidase/10^6 cells. Ad5-LacZ 3, Ad5-LacZ 13, and Ad5-LacZ 15 were previously referred to as AdlacZ, AdAB14lacZ, and AdAB21lacZ, respectively (*12*). The vectors have been listed in the key according to decreasing expression levels.

ited an average of 34.2% by AraC (Table 2). This suggests that a large fraction of LacZ expression may be due to transcripts derived from the E3 promoter and that a less than twofold increase results after DNA replication to which the MLP may contribute. Alternatively, lower levels of LacZ production may simply be due to a reduced number of viral DNA molecules in infected cells treated with AraC. Differences in dependence of expression on viral DNA replication have also been reported for an insert containing the coding sequences for hepatitis B surface antigen (HBsAg) (*16*). When the coding sequences for HBsAg were inserted into the E3 region at 78.5 m.u. expression was unaffected by AraC but when the same insert was combined with an E3 deletion of 1.88-kb expression was greatly reduced in the presence of AraC.

To determine whether differences in protein synthesis as measured by enzymatic assays might be due to differences in levels of mRNAs containing LacZ or luciferase coding sequences, polyadenylated RNA from infected cells was used in dot blot hybridization assays with either LacZ or luciferase DNA as probes. There was at most a twofold difference in levels of reporter gene-specific mRNA as determined by dot blot hybridization for various Ad5-luciferase and Ad5-LacZ recombinants (Fig. 2), suggesting that the differences observed in the levels of luciferase or LacZ protein expression by both series of recombinant viruses were not due to gross differences at the level of reporter gene-specific mRNA production.

In an attempt to map the 5′ termini of reporter gene-specific RNA, RNA isolated from HeLa cells infected with Ad5-luciferase or Ad5-LacZ recombinants was analyzed by primer extension using 5′ ^{32}P-labeled oligonucleotides designed to bind specifically near the 5′ termini of the luciferase or LacZ coding sequences. A large number of primer extension products ranging in size from approximately 70 to >600 nt were produced with each RNA sample isolated from recombinant virus-infected cells (data not shown). Thus the results of primer extension

TABLE 2

Reporter Gene Expression in the Presence of AraC[a]

Virus	Expression 24 hr postinfection[b]		% Inhibition by AraC
	− AraC	+ AraC	
Ad5-Luc 1	1655.5	39.2	97.6
Ad5-Luc 3	7895.8	30.3	99.6
Ad5-Luc 11	1264.7	43.6	96.6
Ad5-Luc 13	4103.1	143.0	96.5
Ad5-LacZ 3	1281.0	977.8	23.7
Ad5-LacZ 5	9925.5	7019.6	29.3
Ad5-LacZ 11	747.5	420.0	43.8
Ad5-LacZ 13	485.2	293.1	39.6
Ad5-LacZ 15	544.3	354.7	34.8

[a] Hela cells in 60-mm dishes were infected at an m.o.i. of 50 with the various vectors and then incubated in the presence or absence of 50 μg of AraC/ml of medium for 24 hr. Luciferase or LacZ assays were then performed on cell extracts.

[b] Enzyme expression obtained with each virus expressed as nanograms of protein per million cells.

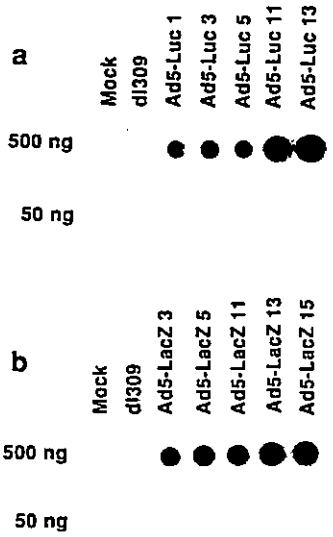

FIG. 2. Dot blot hybridization of mRNA isolated either from Ad5-luciferase or from Ad5-LacZ recombinant-infected cells, using a DNA fragment containing the luciferase or the LacZ gene, respectively, as a probe. HeLa cells were mock-infected or infected with Ad5dl309 (*32*), Ad5-luciferase, or Ad5-LacZ recombinants and harvested at 24 hr postinfection, and the poly(A)-selected RNAs were purified using a Pharmacia Quickprep Micro mRNA purification kit. mRNA (500 and 50 ng) from each sample was transferred onto a GeneScreen Plus membrane using the GIBCO BRL dot blot apparatus and hybridized with a fragment containing only (a) the luciferase gene or (b) the LacZ gene which was labeled with ^{32}P using a Pharmacia Oligolabeling Kit. Quantitative analysis of results were done using Image I software (Universal Imaging Corp.).

were uninformative, a not unexpected outcome, given the extensive transcription that occurs across this region during virus replication and the large number of splicing events that are possible.

Transcription of inserts in E3 is probably complex, with transcripts likely originating from the MLP or E3 promoter. Although in the past our laboratory has routinely included the SV40 promoter and poly(A) with the foreign genes inserted into E3 (*15, 19, 26*), we have obtained evidence suggesting that the SV40 promoter does not function significantly to drive transcription (*15, 21*). For the luciferase insert the presence of the SV40 promoter was found to enhance expression but the dependence on Ad5 DNA replication for expression suggests it was not functioning directly as a promoter. In the case of the LacZ insert the SV40 promoter did not enhance expression significantly. In some vectors the SV40 promoter has been found to provide a cryptic splice acceptor for transcripts arising upstream (*15*) but in others this is not the case (*21*). The luciferase and LacZ gene constructs have flanking sequences both 5′ and 3′ of the coding regions which differ for the two genes. We have not been able to identify any probable splice donor or acceptor sites in the upstream luciferase or LacZ sequences. Furthermore, even when a strong splice acceptor such as the Ad2 hexon splice acceptor was deliberately inserted 5′ to the reporter gene there was only a slight increase in enzyme expression (unpublished observations). A detailed analysis of transcription in the E3 region has shown that the E3 transcription unit is differentially expressed at early and late times during infection and that mutations or deletions in this region can alter splice site usage (*30*). It has also been shown that changes in exon sequences can alter the splicing pattern of a gene (*31*). These observations together probably account for the differences in expression observed for the various constructs we have analyzed and their response to AraC. An analysis of expression in the presence of AraC revealed that the two reporters were expressed differently in relation to viral DNA replication. Luciferase expression, regardless of the SV40 sequences flanking the reporter gene or the E3 deletion used, was strongly dependent on viral DNA replication, whereas LacZ expression was largely independent of DNA replication. The observation that expression of some inserts in E3 can be strongly dependent on DNA replication contraindicates the use of such vectors in species in which the virus does not replicate well.

One general observation that was made was that expression from the reporter constructs inserted in the 1.88-kb E3 deletion was as good as or better than expression obtained from similar constructs inserted in the 2.69-kb deletion. The higher levels of expression obtained when the 1.88-kb deletion was utilized could not be attributed to differences in growth characteristics between viruses with the 1.88- and 2.69-kb E3 deletions since we found that the replication of the various recombinants in HeLa cells was similar (data not shown). Also viruses with the 2.69-kb E3 deletion have been found to replicate with wt kinetics (*12*). One possible explanation for the reduced expression obtained when the 2.69-kb deletion was used is that this deletion has removed sequences which increase the rate of transcription or the stability of nascent RNA or possibly sequences that act as fortuitous splice acceptors. One potentially important difference between the 1.88- and 2.69-kb deletions is that the first E3 3′ splice acceptor site is removed by the 2.69-kb deletion (*12*).

Based on our results with two reporter genes, luciferase and β-galactosidase, design of vectors expressing maximal levels of protein from a gene introduced in the E3 region is likely to be an empirical process. We have generally obtained relatively efficient expression by including a cassette consisting of the SV40 large T antigen promoter, then the insert followed by the SV40 poly(A) signal, but for simplicity in cloning and to obtain a relatively high level of expression it appears that it may often be sufficient to insert just the coding sequences for the gene of interest. Expression of foreign genes without the control of any exogenous regulatory sequences is likely sufficient for live recombinant vaccines and for a number

of other purposes where exceptionally high levels of expression are not an absolute requirement. However, to optimize expression it may be useful to modify the sequences flanking the insert. In particular, it may be useful to explore the use of other strong promoters such as the human cytomegalovirus immediate early promoter or the human β-actin promoter to permit expression independent of Ad viral promoters.

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2. Contributions to Mittal *et al.* (1995)

(A) Generation of Ad vectors Ad5-LacZ 11, Ad5-LacZ 13 and Ad5-LacZ 15.

(B) LacZ and Luciferase assays performed with the various vectors.

3.Summary

In Mittal *et al.* (1995) we compared the levels of expression of firefly luciferase and bacterial β-galactosidase in a series of vectors with and without SV40 regulatory sequences and in the context of two E3 deletions, one of 1.88 kb, and one of 2.69 kb. It was found that the vector that gave the highest level of expression of luciferase (Ad5-Luc 3; luciferase gene flanked by the SV40 promoter and polyadenylation signal inserted in the 1.88 kb E3 deletion) differed from the vector that gave the highest expression of LacZ (Ad5-LacZ 5: LacZ gene with just the SV40 polyadenylation signal inserted in the 1.88 kb E3 deletion) (Mittal *et al.*, 1995, Fig 1). An analysis of expression in the presence of AraC revealed that the two reporters were expressed differently in relation to viral DNA replication. Luciferase expression, regardless of the SV40 sequences flanking the reporter gene or the E3 deletion used, was dependent on viral DNA replication whereas a significant portion of LacZ expression occurred in the absence of DNA replication (Mittal *et al.*, 1995, Table 2). This suggests that the kinetics and efficiency of expression of genes inserted in the E3 region in nonconditional helper independent vectors may be more strongly dependent on the insert sequences of the foreign gene than on the flanking regulatory sequences used. Based on the results presented in the paper, it appears that the design of vectors expressing maximal levels of protein from a gene introduced into the E3 region may be an empirical process. In general relatively good levels of expression were obtained for all the vectors tested and to simplify cloning it is usually sufficient to insert just the coding sequences

for the gene of interest. The SIV genes inserted into the 2.69 kb E3 deletion described in results section E.1 were introduced without flanking regulatory sequences and were found to be expressed to significant levels. The lack of a requirement for regulatory sequences allowed the maximum SIV gene sequences to be introduced.

4. Construction Of E3 Shuttle Vectors With A Splice Acceptor Upstream Of The Polycloning Region.

As discussed in Mittal *et al.*, 1995 our lab typically constructs vectors with inserts in E3 flanked by the SV40 promoter and poly A sequences (Johnson *et al.*, 1988; Prevec *et al.*, 1990; Mittal *et al.*, 1993). Evidence suggests that generally the SV40 promoter does not function significantly to drive expression (Johnson *et al.*, 1988; Prevec, *et al.*, 1991) but instead, in at least some vectors, acts as a cryptic splice acceptor for transcripts initiating upstream (Johnson *et al.*, 1988). In Mittal *et al.* (1995) we found that the SV40 promoter sequences enhanced the expression of luciferase but the dependence of expression on DNA replication suggests that these sequences do not function as a promoter directly. The presence of the SV40 promoter sequences did not have such a significant effect on LacZ expression. It was also noted in Mittal *et al.*, 1995 that overall, expression was better from inserts in the 1.88 kb E3 deletion compared to the 2.69 kb E3 deletion. One difference between these deletions is that the 2.69 kb deletion removes the first E3 3' splice acceptor site (Bett *et al.*, 1993). It therefore seemed possible that this splice acceptor plays a role in the production of mature transcripts for E3 inserts and that its removal results in reduced expression. In an attempt to improve expression from inserts in the 2.69 kb deletion a strategy was developed to provide an authentic 3' splice acceptor and branch site upstream of the cloning region in pAB16 and pAB26 (Bett *et al.*, 1993) (Fig. 13). A 60 bp

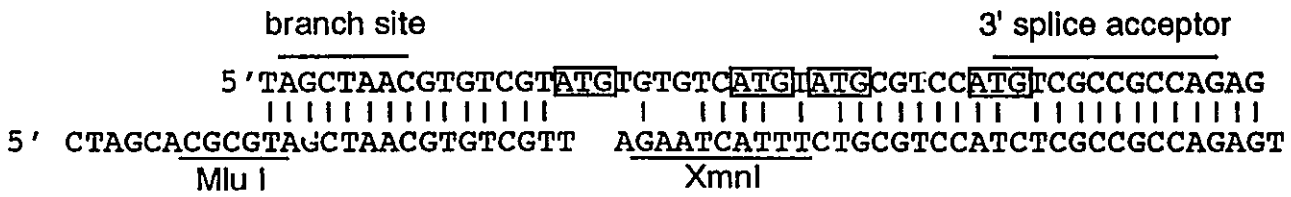
Figure 13. Insertion of the Ad5 hexon splice acceptor and branch site 5' of the E3 deletion in pAB16 and pAB26.

(A) The sequence for the Hexon splice acceptor and branch site oligo (AB1288/AB1289) inserted into pAB16 and pAB26 is shown in comparison to the actual Ad5 hexon splice acceptor sequences. Two unique restriction sites were incorporated into the oligo, *Mlu*I and *Xmn*I, so that the branch site or 3' splice acceptor site could be individually changed if desired. Changes to wt Ad5 sequences in the oligo were made to remove two ATG triplets that may have effected expression of inserts in the E3 deletion, and to incorporate the XmnI site.

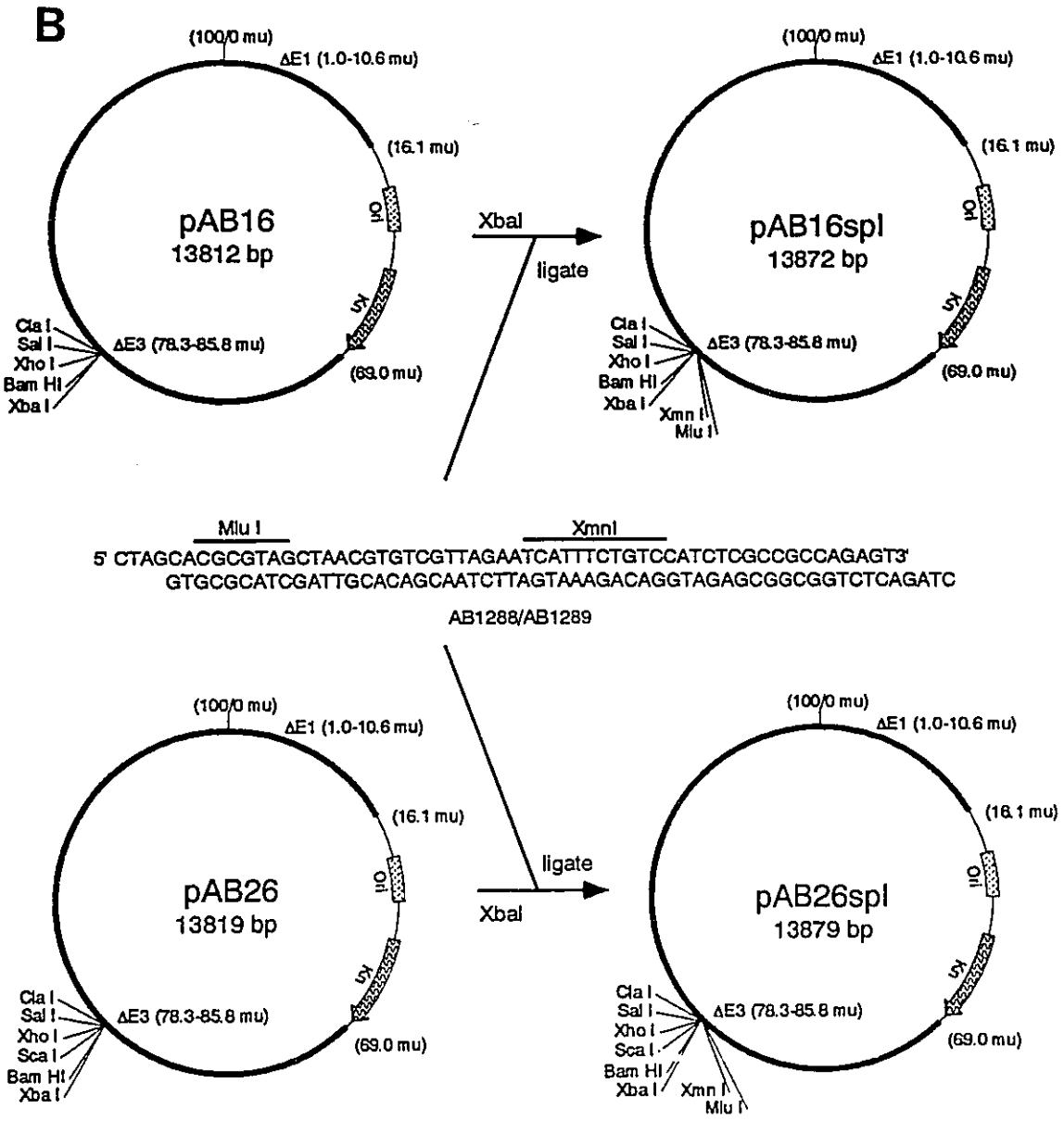
(B) The hexon splice acceptor and branch site oligo (AB1288/AB1289) was inserted into the *Xba*I sites in pAB16 and pAB26, generating pAB16spl and pAB26spl respectively. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences and hatched bars represent Kn^r and origin segments.

A

Ad5 Hexon 3' splice acceptor and branch site



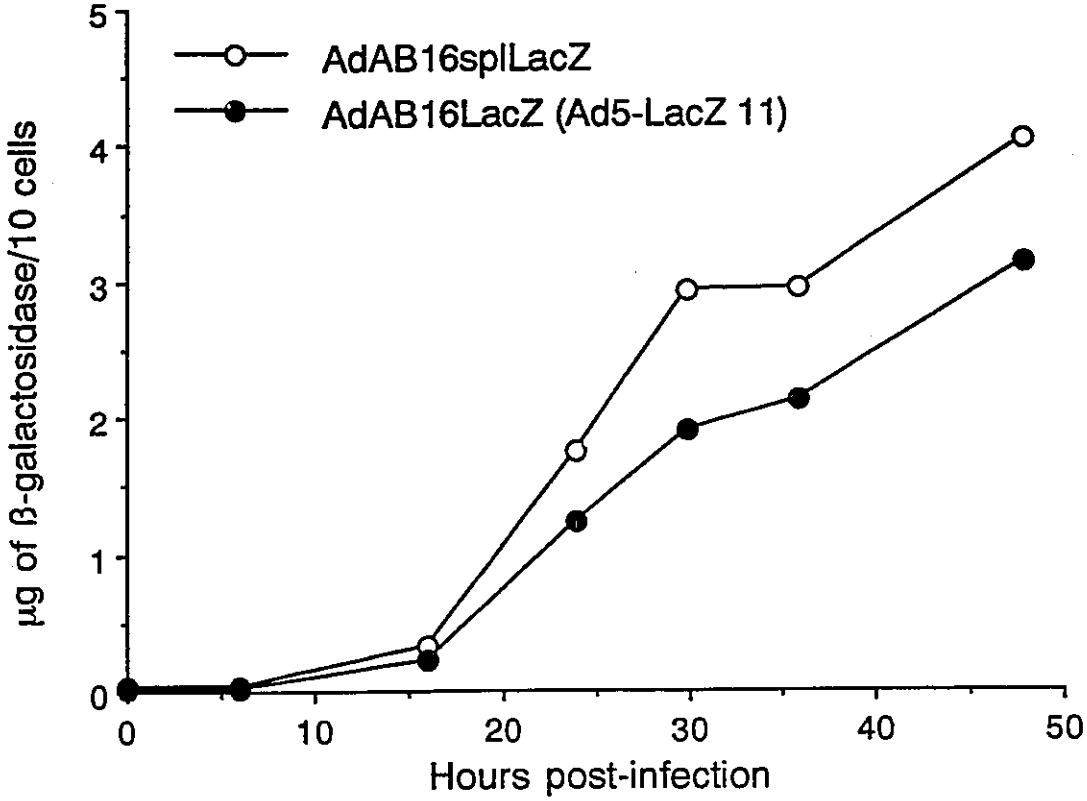
Hexon 3' splice acceptor and branch site oligo


B
(100/0 mu)
ΔE1 (1.0-10.6 mu)
(16.1 mu)
pAB16
13812 bp
Ori
K5
ΔE3 (78.3-85.8 mu)
(69.0 mu)
Cla I
Sal I
Xho I
Bam HI
Xba I
XbaI
ligate
pAB16spI
13872 bp
Xmn I
Mlu I
MluI
XmnI
5' CTAGCACGCGTAGCTAACGTGTCGTTAGAATCATTTCTGTCCATCTCGCCGCCAGAGT3'
GTGCGCATCGATTGCACAGCAATCTTAGTAAAGACAGGTAGAGCGGCGGTCTCAGATC
AB1288/AB1289
pAB26
13819 bp
Sca I
pAB26spI
13879 bp

synthetic oligo (AB1288/AB1289) was generated that represents the splice acceptor and branch sites for the main body of Ad5 hexon (Fig. 13A). Hexon transcripts are among the most abundant messages produced late in infection, making its 3' splice acceptor a good candidate to trap splicing from the MLP or E3 promoters to the insert in E3 (Chow *et al.*, 1977). Oligo AB1288/AB1289 was designed to contain two unique restriction sites, *Mlu*I and *Xmn*I, so that either the 3' splice acceptor or branch site could be individually changed at a later time. pAB16 and pAB26 were digested with *Xba*I and ligated with oligo AB1288/AB1289 generating pAB16spl and pAB26spl (Fig. 13B). To examine the effect of the synthetic splice acceptor on expression the coding sequences for lacZ were inserted into pAB16spl and rescued into virus by cotransfection with pFG173 generating AdAB16splLacZ (construction not shown). The level of LacZ expression obtained with this vector was compared to AdAB16LacZ (designated Ad5-LacZ 11 in Mittal *et al.*, 1995), the identical vector lacking the synthetic splice acceptor site (Fig. 14). The level of lacZ expression was not significantly increased by the presence of the splice acceptor site in AdAB16splLacZ. Less then a two fold increase was observed. Although the splice acceptor did not significantly increase the expression of LacZ it is possible that it may positively affect the expression of other inserts made in the 2.69 kb E3 deletion. However, no further studies were done to explore this approach.

Figure 14. Kinetics of LacZ expression in Hela cells infected with AdAB16LacZ and AdAB16splLacZ

To assay LacZ activity HeLa cell monolayers in 60 mm dishes were infected at an m.o.i. of 50 pfu/cell with AdAB16LacZ (Ad5LacZ 11) or AdAB16splLacZ. At various times post-infection virus-infected cell pellets were harvested and assayed in duplicate for LacZ activity (Sambrook *et al.*, 1989). β-galactosidase expression is shown as μg β-galactosidase per 10^6 cells.


AdAB16splLacZ
AdAB16LacZ (Ad5-LacZ 11)
µg of β-galactosidase/10^6 cells
0
1
2
3
4
5
0
10
20
30
40
50
Hours post-infection

D. β-galactosidase Expression Using The HCMV and β-Actin Promoters In Replication Defective Vectors.

As mentioned in the introduction inserts cloned into the E1 region require a strong promoter to drive expression. Two of the most commonly used promoters are the Ad2 MLP and the HCMV promoter. Previous studies had shown that up to 27 μg of β-galactosidase/60 mm diameter dish of MRC5 cells could be produced using the HCMV promoter (Wilkinson and Akrigg, 1992). As described in results section B.5, E1 shuttle plasmids were constructed containing either the human cytomegalovirus (HCMV) immediate-early promoter (-299 to +76 bp) (Boshart *et al.*, 1985) or the human β-actin (β-Act) promoter (-450 to +910 bp) (Gunning *et al.*, 1987) in the E1 antiparallel orientation followed by a number of cloning sites into which foreign genes can be introduced. As mentioned previously it was decided to insert the promoters in the E1 antiparallel orientation to prevent the production of aberrantly spliced messages sometimes seen when inserts are in the E1 parallel orientation (Berkner and Sharp, 1984; Davidson and Hassel, 1987). To more easily characterize the expression obtained with these promoters in various cell lines and to help determine which promoter to use in the construction of replication defective Ad/SIV recombinants it was decided to construct β-galactosidase reporter vectors. pHCMVsp1A and pβActsp1A were digested with *Sal*I and *Xba*I and ligated with pAB21LacZ (Bett *et al.*, 1993) also digested with *Sal*I and *Xba*I, generating pHCMVsp1LacZ (Morsey *et al.*, 1993) and pβActsp1LacZ respectively (Fig. 15). pHCMVsp1LacZ and pβActsp1LacZ were rescued into virus by cotransfection with pJM17 to obtain AdHCMVsp1LacZ (Morsey *et al.*, 1993) and AdβActsp1LacZ. To compare expression levels obtained with the reporter vectors in cells noncomplementing for replication, MRC5 and A549 cells were infected at an moi of 50 and cell extracts assayed for LacZ activity at various times post-infection (Fig. 16). The highest levels

Figure 15. Construction of reporter vectors AdHCMVsp1LacZ and AdβActsp1LacZ.

To construct reporter vectors containing LacZ driven by the HCMV or β-Actin promoters pHCMVsp1A and pβActsp1A were digested with *Sal*I and *Xba*I and ligated with pAB21LacZ (Bett *et al.*, 1993) also digested with *Sal*I and *Xba*I, generating pHCMVsp1LacZ and pβActsp1LacZ respectively. pHCMVsp1LacZ and pβActsp1LacZ were rescued into virus by cotransfection with pJM17 to obtain AdHCMVsp1LacZ and AdβActsp1LacZ.

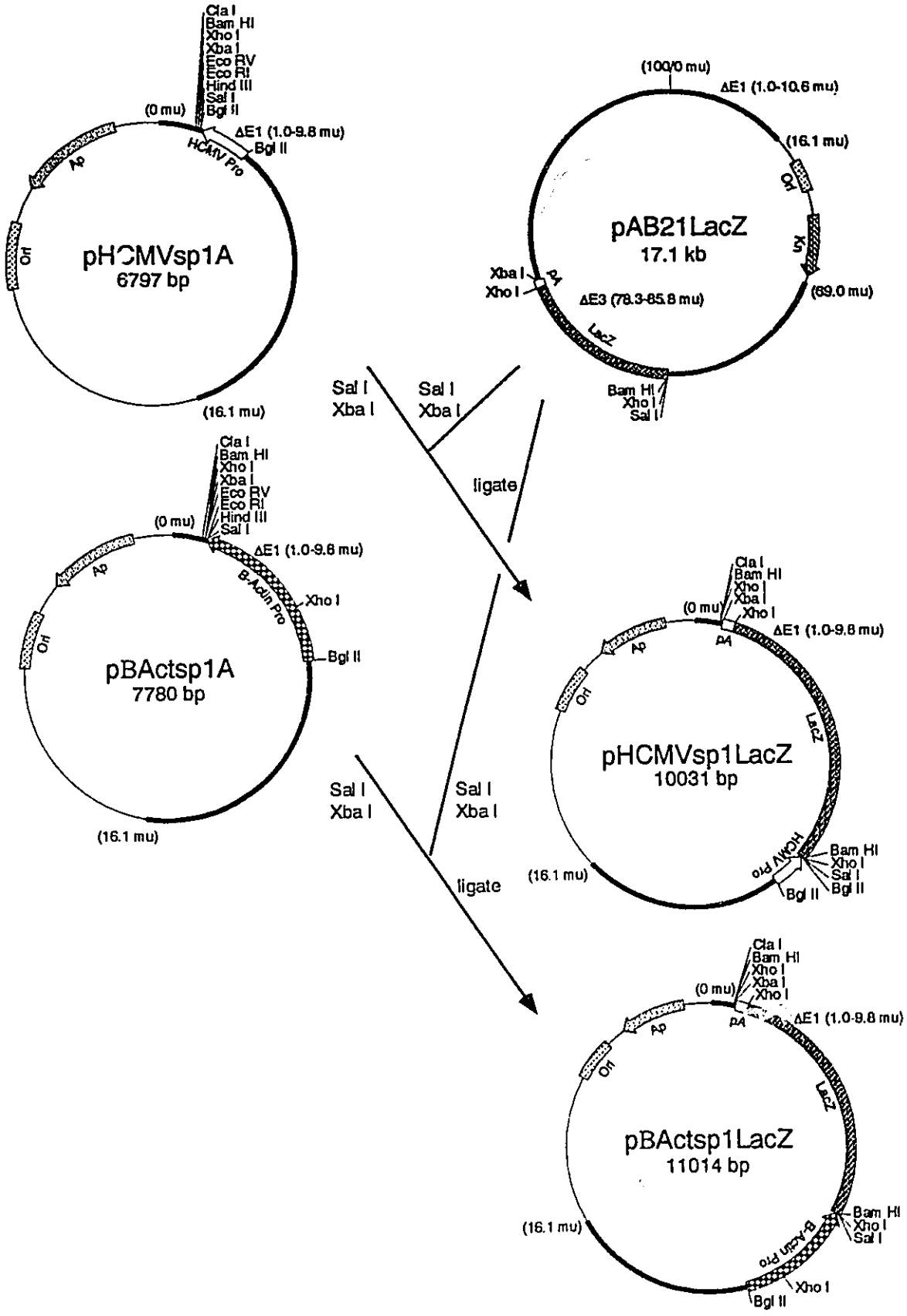
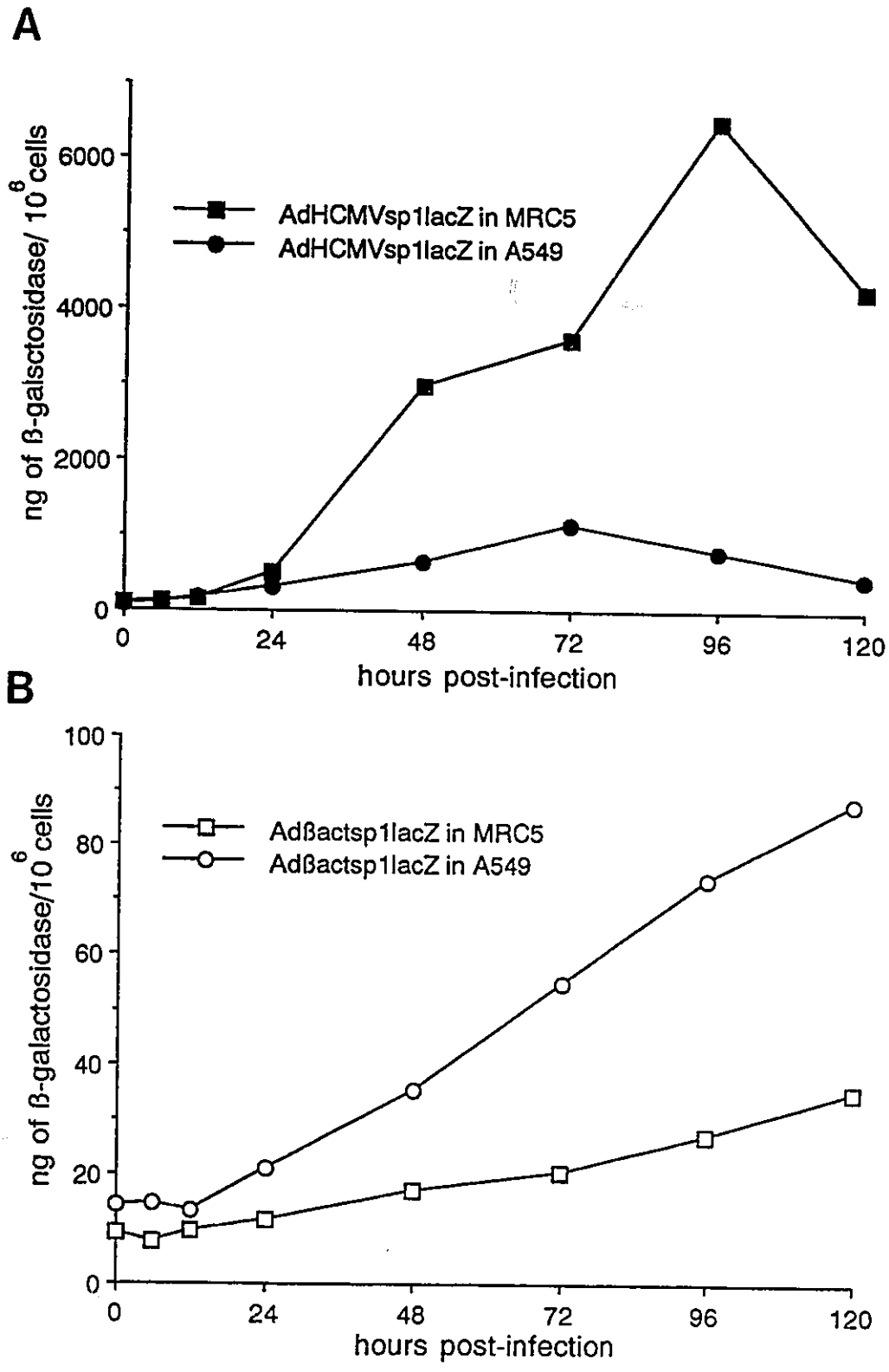

Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
HCMV Pro
Ap
Ori
pHCMVsp1A
6797 bp
(16.1 mu)
(100/0 mu)
ΔE1 (1.0-10.6 mu)
(16.1 mu)
Ori
pAB21LacZ
17.1 kb
Xba I
Xho I
pA
ΔE3 (78.3-85.8 mu)
LacZ
(69.0 mu)
Bam HI
Xho I
Sal I
Sal I
Xba I
Sal I
Xba I
ligate
Cla I
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
β-Actin Pro
Xho I
Ori
pBActsp1A
7780 bp
Bgl II
(16.1 mu)
Sal I
Xba I
Sal I
Xba I
ligate
Cla I
Bam HI
Xho I
Xba I
Xho I
(0 mu)
ΔE1 (1.0-9.8 mu)
pA
Ap
Ori
LacZ
pHCMVsp1LacZ
10031 bp
(16.1 mu)
HCMV Pro
Bam HI
Xho I
Sal I
Bgl II
Bgl II
Cla I
Bam HI
Xho I
Xba I
Xho I
(0 mu)
ΔE1 (1.0-9.8 mu)
pA
Ap
Ori
LacZ
pBActsp1LacZ
11014 bp
(16.1 mu)
β-Actin Pro
Bam HI
Xho I
Sal I
Xho I
Bgl II

Figure 16. Kinetics of LacZ expression in A549 and MRC5 cells infected with AdHCMVsp1LacZ or AdβActsp1LacZ.

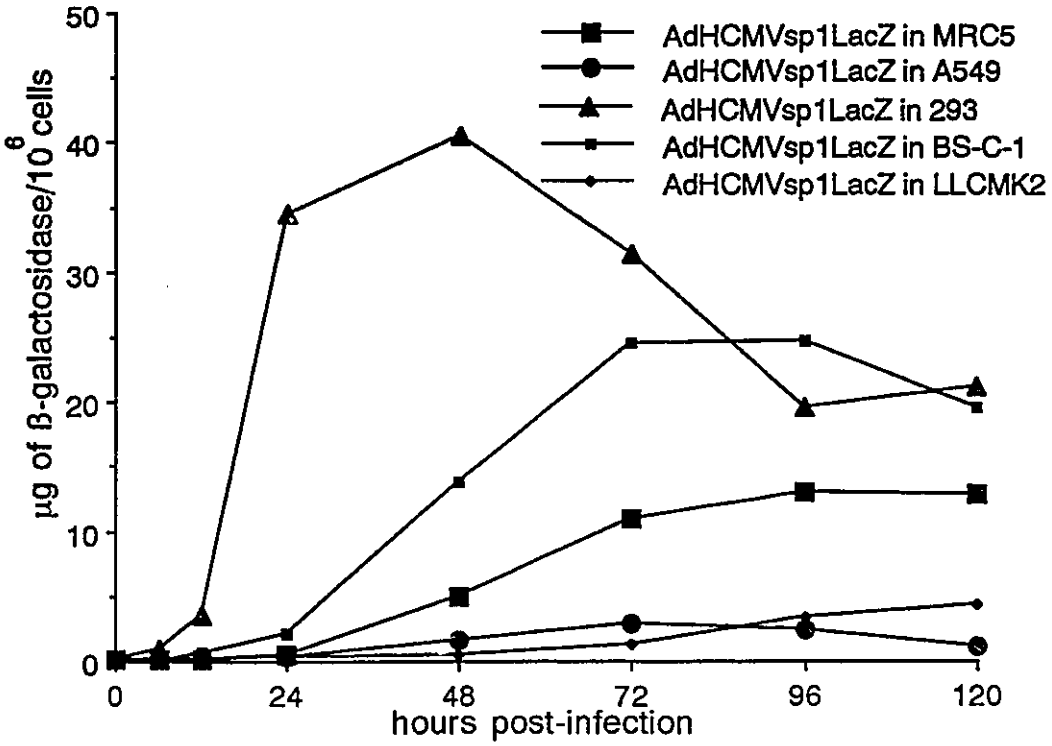
To assay LacZ activity MRC5 and A549 cells in 60 mm dishes were infected at an moi of 50 with either (A) AdHCMVsp1LacZ or (B) AdβActsp1LacZ. At various times post-infection virus-infected cell pellets were collected and assayed in duplicate for LacZ activity (Sambrook *et al.*, 1989). The β-galactosidase expressed in MRC5 and A549 cells infected with AdHCMVsp1LacZ and AdβActsp1LacZ is shown as ng β-galactosidase per 10^6 cells.


A
ng of β-galsctosidase/ 10^6 cells
6000
4000
2000
0
AdHCMVsp1lacZ in MRC5
AdHCMVsp1lacZ in A549
0
24
48
72
96
120
hours post-infection
B
ng of β-galactosidase/10^6 cells
100
80
60
40
20
0
AdßactsplacZ in MRC5
AdßactsplacZ in A549
0
24
48
72
96
120
hours post-infection

of lacZ expression were obtained with AdHCMVsp1LacZ in both cell lines: 7 $\mu g/10^6$ and 1 $\mu g/10^6$ cells of β-galactosidase in MRC5 and A549 cells respectively. 200 fold and 10 fold lower levels were obtained in MRC5 and A549 cells infected with AdβActsp1LacZ. In subsequent expression studies only AdHCMVsp1LacZ was used due to the relatively low level of expression obtained with the β-Actin promoter in the experiment described above and in studies carried out by Christina Addison in a variety of other cell lines. Because ultimately we wished to study the immunogenicity of the replication defective Ad/SIV vectors in mice and macaque monkeys the expression obtained with AdHCMVsp1LacZ was assayed in cell lines derived from these species. Expression was assayed in two monkey lines, LLCMK2 (a macaque kidney line) and BSC-1 (an African green monkey kidney line) and compared to that obtained in A549, MRC5 and 293 cells (Fig. 17). Various levels of expression were obtained depending on the cell line assayed, the highest levels being produced in 293 cells as expected since in these cells the vector can replicate. In 293 cells maximum expression (40 $\mu g/10^6$ cells) was obtained 48 h post-infection after which time β-galactosidase activity decreased steadily. This is due to the cytopathic effect of the virus on the cells most of which were lysed by 48 h post infection. In the non complementing cell lines expression generally increased throughout the length of the experiment but the level of expression was dependent on the cell type. Of the noncomplementing lines the BSC-1 cells gave the highest expression producing a maximum of 25 $\mu g/10^6$ cells by 72 h postinfection. Expression in the LLCMK2 cells was about 6 fold lower reaching a maximum of approximately 4 $\mu g/10^6$ cells. In this experiment approximately 13 $\mu g/10^6$ and 3 $\mu g/10^6$ cells of β-galactosidase was obtained in the infected MRC5 and A549 cells respectively. Expression studies performed by Christina Addison in a variety of mouse cell lines revealed that expression was approximately 50 to 100 fold lower than that seen in 293 cells. Presumably the differences in expression seen for the

Figure 17. Kinetics of LacZ expression in various cell lines infected with AdHCMVsp1LacZ.

To assay LacZ activity 293, MRC5, A549, LLCMK2 and BS-C-1 cells in 60 mm dishes were infected at an moi of 50 with AdHCMVsp1LacZ. At various times post-infection virus-infected cell pellets were collected and assayed in duplicate for LacZ activity (Sambrook *et al.*, 1989). The β-galactosidase expressed in the various cell lines tested is shown as μg β-galactosidase per 10^6 cells.


AdHCMVsp1LacZ in MRC5
AdHCMVsp1LacZ in A549
AdHCMVsp1LacZ in 293
AdHCMVsp1LacZ in BS-C-1
AdHCMVsp1LacZ in LLCMK2
µg of β-galactosidase/10^6 cells
50
40
30
20
10
0
0
24
48
72
96
120
hours post-infection

various cell lines reflects the presence or absence of transcription factors utilized by the HCMV promoter or possibly differences in the efficiency of infection. Since the HCMV promoter provided high level expression in the majority of cell lines tested it was used in the construction of the replication defective Ad/SIV vectors to be described below.

E. Adenovirus Simian Immunodeficiency Virus Recombinants

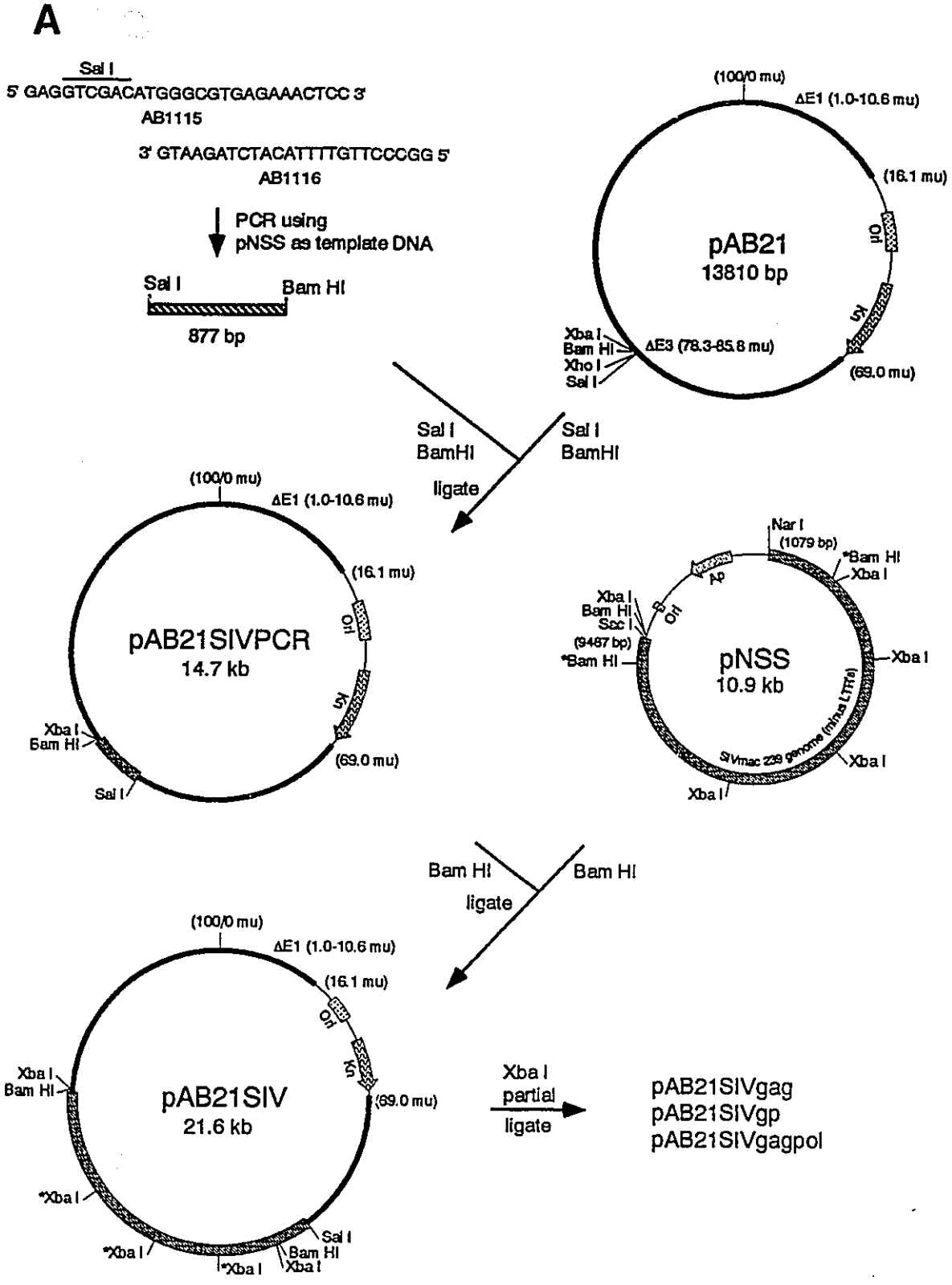
1. Construction And Characterization Of Replication Competent Vectors Containing SIV *gag-pol* Inserts In The E3 Region.

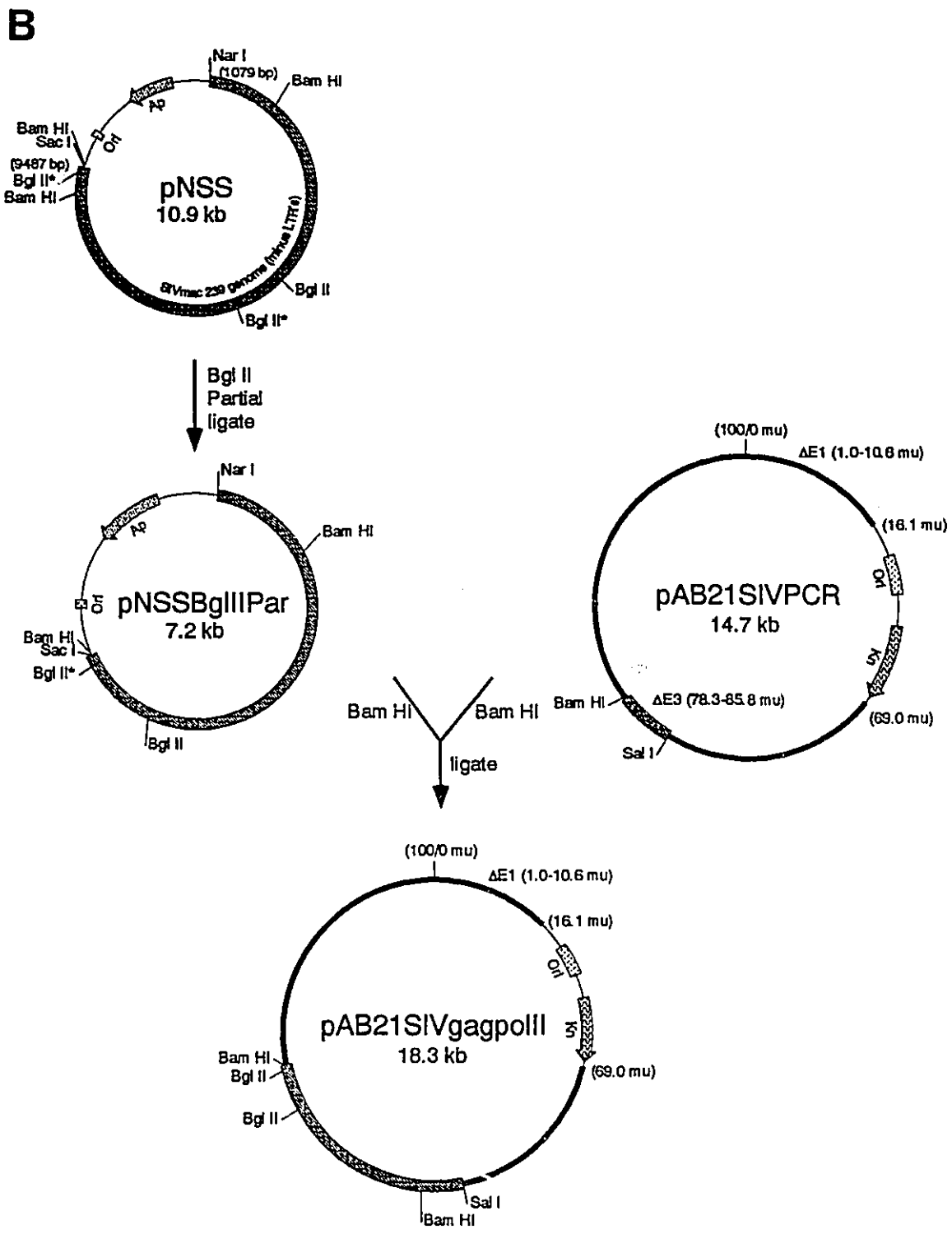
The development of a safe effective vaccine against HIV is a major goal in the fight against AIDS. In order to gain information on the development of an Ad based vaccine against HIV we decided to utilize the SIV/macaque model. Macaque monkeys are highly susceptible to disease induction by SIV, develop AIDS within a time frame suitable for study and have been shown to be an extremely important model system for studying AIDS and for vaccine development (Desrosiers, 1988, 1990). As discussed in the introduction the immune mechanisms that will protect against HIV infection and/or disease have not been well established and for this reason a number of Ad/SIV recombinants containing various SIV genes were constructed. The first series of Ad/SIV recombinants generated were similar to vectors previously constructed in our lab which express HIV-1 *gag* (p55) and the major capsid protein (CA or p24) (Prevec *et al.*, 1991). These replication competent vectors were shown to induce an immune response in inoculated mice and rhesus macaque monkeys. The first vectors expressing SIV proteins contained various segments of the *gag-pol* region of SIV inserted in the E3 region. The *gag* and *pol* genes contain potentially important CTL, ADCC and T helper epitopes (Nixon *et al.*, 1992; Norley *et al.*, 1993). Four inserts of various sizes were introduced into the 2.69 kb E3 deletion in the pAB series of E3 shuttle plasmids (Bett *et al.*, 1993). These inserts were introduced without flanking regulatory sequences so that expression would be dependent on the E3 and/or MLP. As shown in Mittal et al. (1995) relatively good levels of expression can usually be obtained without flanking regulatory sequences and the addition of heterologous promoters in replication competent vectors does not ensure improved expression. The construction of these vectors is outlined in Figure 18. The

Figure 18. Construction of Ad/SIV *gag-pol* recombinants.

(A) SIV sequences for $SIV_{mac}239$ representing the entire genome minus the LTR's were obtained from plasmid pNSS. PCR was used to generate an 877 bp fragment representing the first portion of the SIV *gag* region due to the lack of convenient restriction sites. The left primer (AB1115) was designed to contain a *Sal*I site just upstream of the gag ATG. The PCR product was gel purified, cut with *Bam*HI and *Sal*I and ligated with pAB21 also cut with *Bam*HI and *Sal*I, generating pAB21SIVPCR. Additional SIV sequences were cloned into pAB21SIVPCR by inserting the 6971 bp *Bam*HI fragment from pNSS (indicated with asterisks), generating pAB21SIV. pAB21SIV was then partially digested with *Xba*I to remove sequences between each of the three *Xba*I sites within the insert (indicated with asterisks) and the XbaI site at the 3' end of the insert, generating pAB21SIVgag, pAB21SIVgp, and pAB21SIVgagpol.

(B) To create a smaller insert containing the entire *gag-pol* region pNSS was partially digested with *Bgl*II to remove the *Bgl*II fragment between 5697 and 9374 bp (indicated with asterisks), generating pNSSBglIIPar. Next the *Bam*HI fragment of pNSSBglIIPar was inserted into the *Bam*HI site in pAB21SIVPCR, generating pAB21SIVgagpolII. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Kn', origin, and SIV segments.

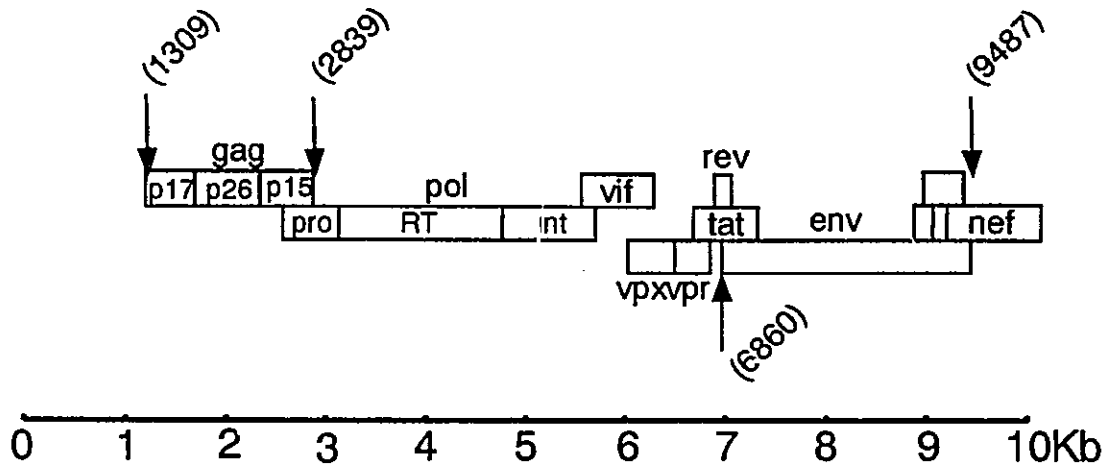

A
Sal I
5' GAGGTCGACATGGGCGTGAGAAACTCC 3'
AB1115
3' GTAAGATCTACATTTTGTTCCCGG 5'
AB1116
PCR using
pNSS as template DNA
Sal I
Bam HI
877 bp
(100/0 mu)
ΔE1 (1.0-10.6 mu)
(16.1 mu)
pAB21
13810 bp
Ori
K5
Xba I
Bam HI
Xho I
Sal I
ΔE3 (78.3-85.8 mu)
(69.0 mu)
Sal I
BamHI
ligate
Sal I
BamHI
pAB21SIVPCR
14.7 kb
Xba I
Bam HI
Sal I
Nar I
(1079 bp)
*Bam HI
Xba I
Ap
Ori
Xba I
Bam HI
Sac I
(9487 bp)
*Bam HI
pNSS
10.9 kb
Xba I
SIVmac 239 genome (minus LTRs)
Xba I
Xba I
Bam HI
ligate
Bam HI
pAB21SIV
21.6 kb
*Xba I
*Xba I
*Xba I
Sal I
Bam HI
Xba I
Xba I
partial
ligate
pAB21SIVgag
pAB21SIVgp
pAB21SIVgagpol


B
Nar I
(1079 bp)
Bam HI
Ap
Bam HI
Sac I
Ori
(9487 bp)
Bgl II*
Bam HI
pNSS
10.9 kb
SIVmac 239 genome (minus LTRs)
Bgl II
Bgl II*
Bgl II
Partial
ligate
Nar I
Bam HI
Ap
Ori
pNSSBglIIPar
7.2 kb
Bam HI
Sac I
Bgl II*
Bgl II
(100/0 mu)
ΔE1 (1.0-10.6 mu)
(16.1 mu)
Ori
pAB21SIVPCR
14.7 kb
Ap
Bam HI
ΔE3 (78.3-85.8 mu)
(69.0 mu)
Sal I
Bam HI
Bam HI
ligate
(100/0 mu)
ΔE1 (1.0-10.6 mu)
(16.1 mu)
Ori
pAB21SIVgagpolII
18.3 kb
Ap
(69.0 mu)
Bam HI
Bgl II
Bgl II
Sal I
Bam HI

plasmid pNSS containing coding sequences for $SIV_{mac}239$ representing the entire genome minus the LTR's (from 1079 to 9487 bp) was obtained from Joel Haynes, Connaught Laboratories. Nucleotide numbers refer to the sequence reported for $SIV_{mac}239$ (Kestler *et al.*, 1990). Because convenient restriction sites were lacking, in order to clone SIV sequences starting just upstream of the gag ATG an 877 bp fragment representing the first segment of *gag* was generated by PCR. This also ensured that the major 5' splice donor located upstream of the gag initiation site was not included since it might be expected to affect the expression of the inserts. For cloning purposes the left PCR primer (AB1115) was designed to contain a *Sal*I site just upstream of the *gag* ATG (Fig. 18A). After digestion of the PCR fragment with *Sal*I and *Bam*HI, it was cloned into the *Sal*I and *Bam*HI sites of pAB21 generating pAB21SIVPCR. Next, the remainder of the SIV genome was cloned into pAB21SIVPCR, by inserting the 6971 bp *Bam*HI fragment of pNSS, generating pAB21SIV. To generate the first three SIV inserts a partial *Xba*I digest was carried out on pAB21SIV generating pAB21SIVgag (a 2163 bp insert representing the entire SIV *gag* region and one third of the *pol* region), pAB21SIVgp (a 3685 bp insert representing the entire *gag* region and three quarters of the *pol* region) and pAB21SIVgagpol (a 5394 bp insert representing the entire *gag-pol* region and containing coding sequences for *vif* and *vpx* (Fig. 18A, 19). Since the 5394 bp *gag-pol* insert was slightly above the expected working capacity (4.5-4.7 kb) when using the 2.69 kb E3 deletion (Bett *et al.*, 1993), a smaller insert containing the entire *gag-pol* region was made (Fig. 18B). The first step involved a partial *Bgl*II digest of pNSS in order to remove the *Bgl*II fragment between 5697 and 9374 bp in the SIV sequences, generating pNSSBglIIPar. Next, the *Bam*HI fragment of pNSSBglIIPar was cloned into pAB21SIVPCR generating pAB21SIVgagpolII (a 4500 bp insert representing the entire *gag-pol* region). Figure

Figure 19. Segments of the SIV genome present in the AdSIV *gag-pol* recombinants.

The segments of the $SIV_{mac}239$ genome inserted into the 2.69 kb E3 deletion are indicated. pAB21SIVgag contains a 2163 bp insert representing the entire SIV *gag* region and one third of the *pol* region, pAB21SIVgp contains a 3685 bp insert representing the entire *gag* region and three quarters of the *pol* region, pAB21SIVgagpol contains 5394 bp insert representing the entire *gag-pol* region and containing coding sequences for *vif* and *vpx* and pAB21SIVgagpolII contains a 4500 bp insert representing the entire *gag-pol* region. The inserts rescued into virus by cotransfection with pFG173 are indicated with an asterisk.



*pAB21SIVgag (bp 1309 to bp3469)

*pAB21SIVgp (bp 1309 to bp 4991)

pAB21SIVgagpol (bp 1309 to bp 6700)

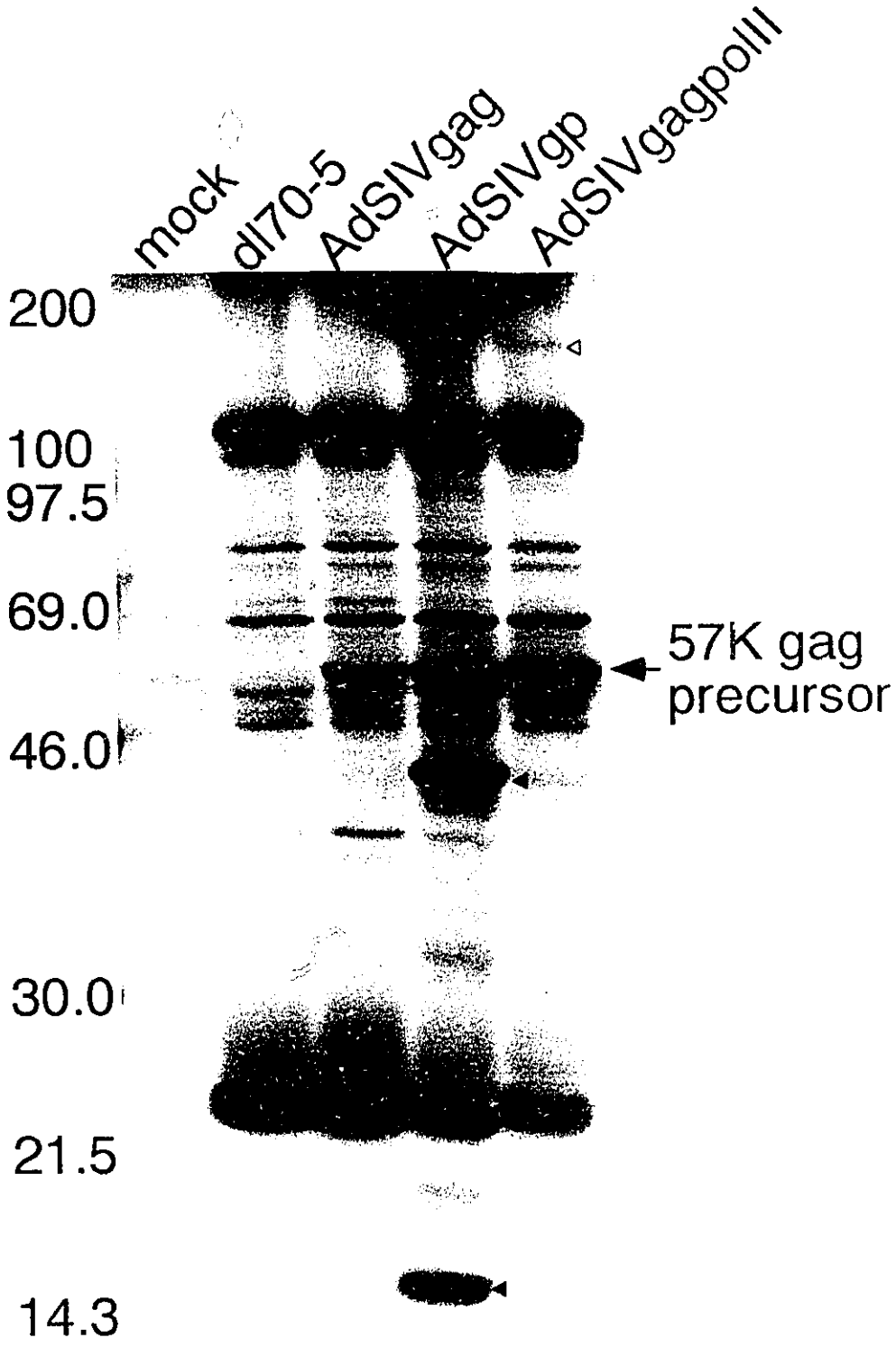
*pAB21SIVgagpolII (bp 1309 to bp 5702/ bp 9380 to bp 9487)

19 illustrates the segments of the SIV genome inserted in the various constructs. All inserts were sequenced at their 5' and 3' ends to ensure they possessed the correct structure.

The three smallest SIV inserts, (2163 bp, 3685 bp and 4500 bp) were rescued into virus by cotransfection with pFG173 generating AdSIVgag, AdSIVgp and AdSIVgagpolII respectively. The 5394 bp insert could not be rescued, presumably because of its size and if rescued would likely have generated an unstable vector (Bett *et al.*, 1993). The three vectors that were obtained were expected to express the 57K *gag* precursor and portions of the 160K *gag-pol* precursor. In AdSIVgag and AdSIVgp a stop codon is supplied for the truncated *gag-pol* protein in down stream Ad5 sequences resulting in a fusion protein (13 aa are picked up from the flanking Ad sequences) and it is likely that the E3b polyadenylation signal is utilized. The predicted molecular weights of the truncated *gag-pol* proteins are 81K for AdSIVgag and 140K for AdSIVgp and the entire 160K *gag-pol* precursor for AdSIVgagpolII. To detect expression of these SIV products, immunoprecipitations (IP's) were carried out using various radio labelled substrates and antisera. In the first IP (Fig. 20) 293 cells were either mock infected or infected at an moi of 20 with dl70-5 (Bett *et al.*, 1993), AdSIVgag, AdSIVgp or AdSIVgagpolII. Cells were then labelled from 22-24 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using serum from an SIV infected macaque (SIV reference sera) and analyzed by SDS-PAGE. A protein was detected for all three Ad/SIV recombinants that appeared to correspond to the *gag* precursor protein of 57K (Fig. 20). In AdSIVgp and AdSIVgagpolII infected cells high molecular weight species were also detected that may correspond to their respective *gag-pol* precursors. Surprisingly the species detected for AdSIVgp was larger and more abundant than the species seen for AdSIVgagpolII and it was also more abundant then the putative gag precursor. As explained in the introduction the *gag-pol* precursor is expected to be less abundant than the *gag* precursor

Figure 20. Immunoprecipitation of SIV proteins expressed by the Ad/SIV *gag-pol* vectors using SIV reference sera.

293 cells in 60 mm dishes were either mock infected or infected at an moi of 20 with dl70-5, AdSIVgag, AdSIVgp or AdSIVgagpolII for 22 h and then labelled with Trans[^{35}S]-methionine, from 22-24 h. Cell extracts were then prepared, immunoprecipitated with serum from an SIV infected macaque (SIV reference sera) and samples separated by SDS-PAGE on a 10.0% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. dl70-5 carries the 2.69 kb E3 deletion. The putative 57K *gag* precursor is indicated with an arrow. Additional proteins detected for AdSIVgp are indicated with solid arrow heads and the putative *gag-pol* precursor detected for AdSIVgagpolII is indicated with an open arrow head.

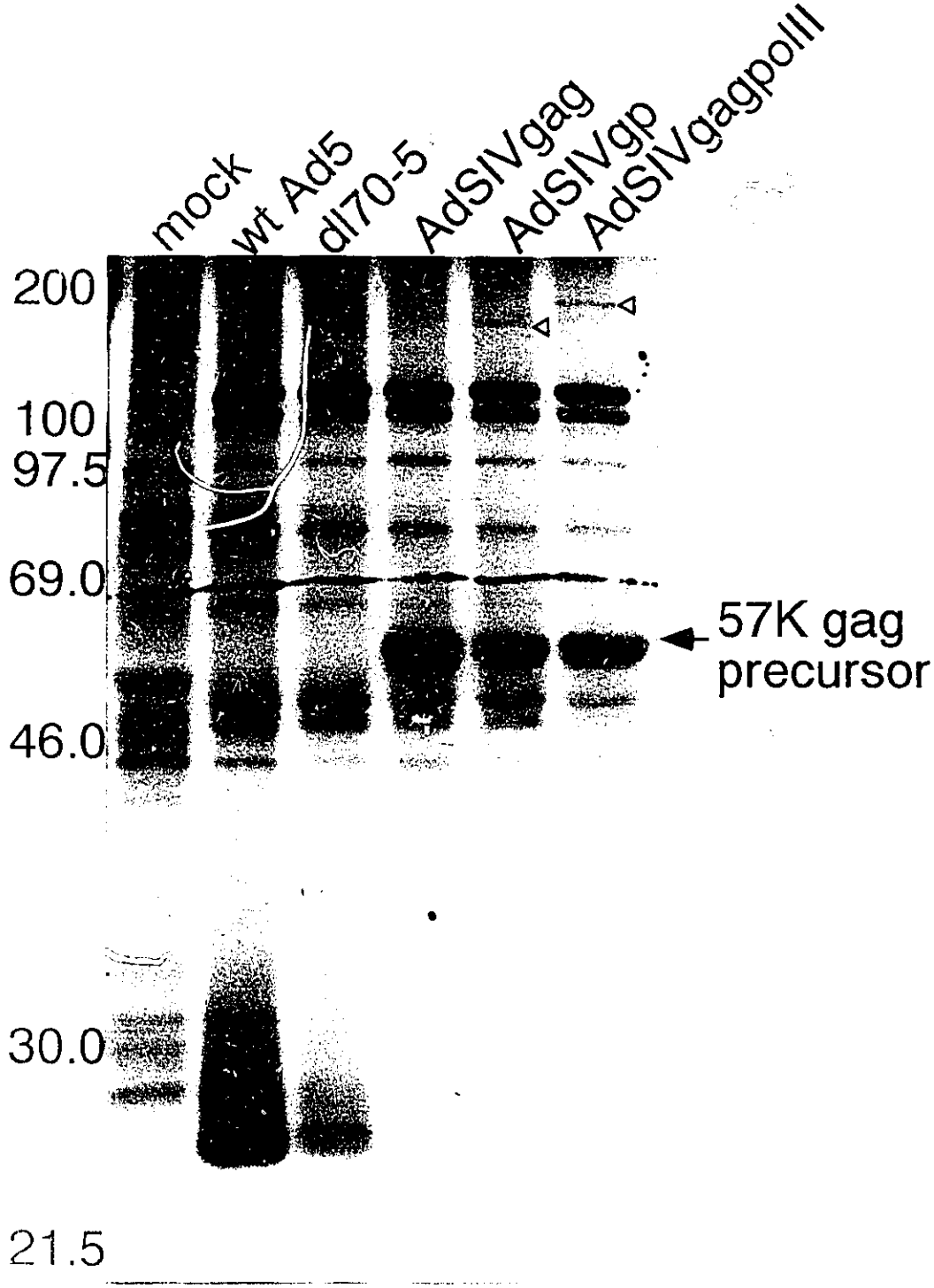

mock
dl70-5
AdSIVgag
AdSIVgp
AdSIVgagpolII
200
100
97.5
69.0
46.0
30.0
21.5
14.3
57K gag
precursor

since the ribosomal frame shift that results in the production of the *gag-pol* precursor occurs at a frequency of 5-10%. In AdSIVgp infected cells a major species of approximately 41K was also detected which may correspond to a second *gag* precursor. A *gag* precursor of this size is expressed by a recombinant vaccinia virus containing the *gag-pol* region of HIV (Flexner *et al.* 1988) as well as in HIV infected cells (Chassagne *et al.*, 1986). There did not appear to be any cleavage of the putative *gag* and *gag-pol* precursors to mature *gag* or *pol* products in either AdSIVgag or AdSIVgagpolII infected cells but a number of proteins from 15-17k were detected in AdSIVgp infected cells that may correspond to mature *gag* products. Absence of cleavage, or little cleavage to mature products has been reported for recombinant vaccinia viruses expressing the *gag-pol* region of HIV (Flexner *et al.*, 1988; Shioda and Shibuta, 1990) and this may be due to the fact that cleavage usually occurs during the maturation of SIV virus particles.

In an attempt to more clearly define the identity of the products being expressed by the Ad/SIV recombinants, a second IP was performed using anti-HIV-2 *gag* serum, which should cross react with our SIV *gag* and *gag-pol* products (Fig. 21). The 57K protein was again detected for all three recombinants suggesting that it was indeed the *gag* precursor. The results also suggested that the additional proteins detected in AdSIVgp infected cells were neither the *gag-pol* precursor (abundant high molecular weight species), a second *gag* precursor (the 41K band) nor processed *gag* products (the 15-17K bands) since they were not precipitated by the anti HIV-2 *gag* serum. Interestingly a new high molecular weight species was detected in AdSIVgp infected cells which migrated faster than the putative *gag-pol* precursor seen for AdSIVgagpolII. This product which is approximately as abundant as the AdSIVgagpolII species may not have been detected in the first IP (Fig. 20) due to the intensity of the band migrating just above it. Because of the size and relative mobilities of these high molecular weight species it is believed that they represent

Figure 21. Immunoprecipitation of SIV protiens produced by the Ad/SIV *gag-pol* vectors using anti HIV-2 *gag* antiserum.

To more clearly define the identities of the SIV proteins being produced by the Ad/SIV gag-pol recombinants an immunoprecipitation was performed as described in Fig. 20 except using anti-HIV-2 *gag* sera. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. The 57K *gag* precursor is indicated with an arrow. The *gag-pol* precursors for both AdSIVgp and AdSIVgagpolII are indicated with open arrow heads.


mock
wt Ad5
dl70-5
AdSIVgag
AdSIVgp
AdSIVgagpolIII
200
100
97.5
69.0
46.0
30.0
21.5
57K gag
precursor

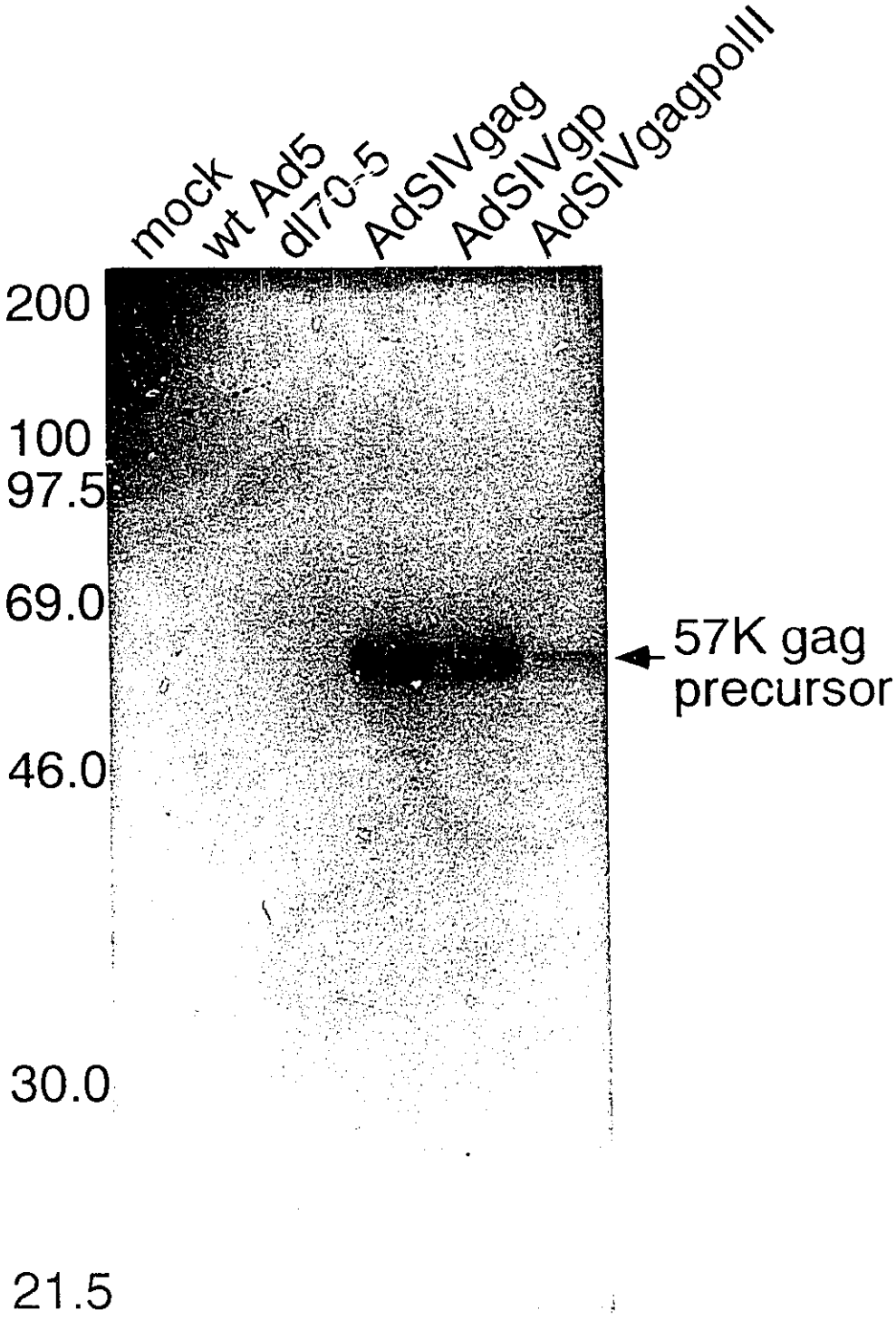
the 140K and 160K *gag-pol* precursors predicted to be produced by AdSIVgp and AdSIVgagpolII respectively.

To further characterize the products being produced a third IP was executed in which the Ad/SIV infected cells were labelled with [^{3}H]-myristic acid and immunoprecipitated using the SIV reference serum (Fig. 22). The *gag* and *gag-pol* precursors are myristylated at their amino terminus following translation. The results of this IP clearly demonstrate that the 57K protein is the *gag* precursor. In cells infected with AdSIVgp, the abundant high molecular weight protein, 41K species and 15-17K products detected with SIV reference sera in the first IP (Fig. 20) were not detected indicating that they do not contain the amino terminus of the *gag* precursor. The identities of these proteins have not been determined but they may represent translation products from aberrantly spliced messages. The *gag-pol* precursors seen in Figure 21 for AdSIVgp and AdSIVgagpolII were not detected in this IP likely because of less intense labelling.

Due to concerns about possible sero conversion to the proteins expressed by the replication competent Ad/SIV vectors and the problems that could be associated with this, work was discontinued with these vectors. It was decided that replication defective vectors might be more suitable as vaccine vectors for safety reasons and to ensure adequate expression of the SIV inserts. As reported in Mittal *et al.* (1994) the expression of some E3 inserts in replication competent vectors can be dependent on DNA replication which contraindicates their use in species in which the virus does not replicate well. Since we had planned to study the immunogenicity of our Ad/SIV vectors in mice and macaque monkeys, species whose cells are semipermissive and nonpermissive for virus replication respectively *in vitro*, we could not be sure that the replication competent vectors generated would provide adequate expression. For these reasons I began the construction and characterization of replication defective Ad vectors containing the SIV *env* gene

Figure 22. Immunoprecipitation of SIV proteins produced by the Ad/SIV *gag-pol* vectors after labelling with [^{3}H]-myristic acid.

293 cells were either mock infected or infected at an moi of 10 with wt Ad5, dl70-5, AdSIVgag, AdSIVgp or AdSIVgagpolII for 7 h and then labelled with [^{3}H]-myristic acid from 7-19 h. Cell extracts were then prepared, immunoprecipitated with SIV reference sera and samples separated by SDS-PAGE on an 10.0% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. The 57K *gag* precursor is indicated with an arrow.


mock
wt Ad5
dl70-5
AdSIVgag
AdSIVgp
AdSIVgagpolII
200
100
97.5
69.0
46.0
30.0
21.5
57K gag
precursor

or chimeric *gag-env* inserts driven by either the HCMV or β-Actin promoters. These studies are described in the subsequent sections.

2. Construction And Characterization Of Replication Defective Vectors Containing SIV *env* Inserts In The E1 Region.

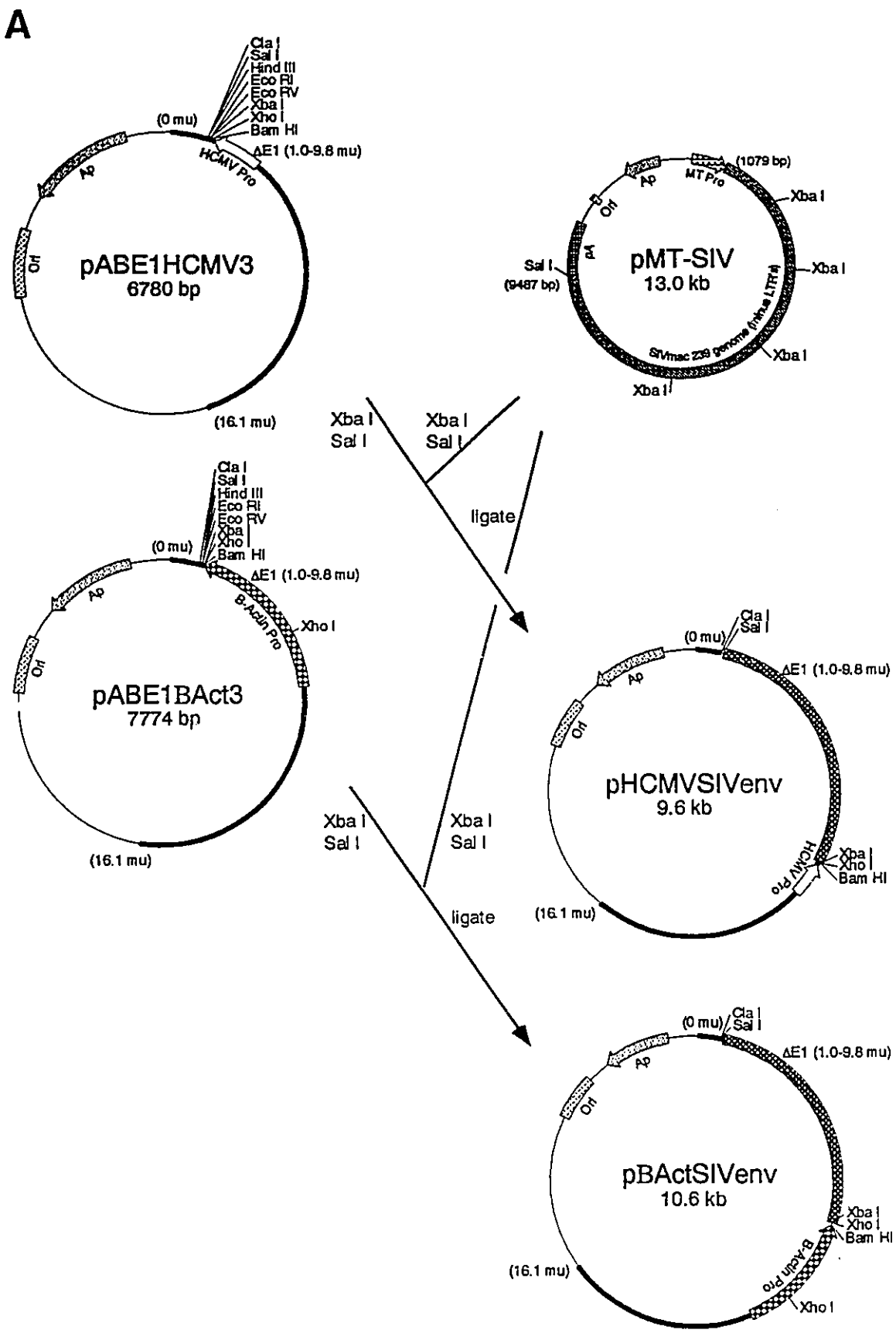
The first series of replication defective vectors constructed contained the coding sequences for the SIV envelope glycoprotein. As mentioned in the introduction the envelope glycoprotein has been the target of much research and has been included in many vaccine approaches due to its potential to raise neutralizing Ab but this protein also contains potential epitopes important for an efficient cellular immune response (Nixon *et al.*, 1992; Norley *et al.*, 1993). Replication defective Ad/SIV vectors were constructed containing the SIV *env* region driven by the HCMV and β-Actin promoters inserted in place of the 3.2 kb E1 deletion. The insert consisted of a 2805 bp fragment representing the coding sequences for SIV *env* and *rev* (Fig. 23). This fragment was obtained from pMT-SIV which contains the coding sequences for $SIV_{mac}239$ (entire genome minus the LTR's; 1079 to 9487 bp) with a stop codon provided for the truncated *env* gene in the flanking vector sequences (the last three aa of *env* are removed and replaced by three aa encoded in vector sequences). Sequence numbers refer to those reported for $SIV_{mac}239$ in Kestler *et al.*, 1990. pMT-SIV was digested with *Xba*I and *Sal*I and ligated with either pABE1HCMV3 or pABE1βAct3 DNA that had also been digested with *Xba*I and *Sal*I, generating pHCMVSIVenv and pβActSIVenv respectively (Fig. 23A). Initial attempts to rescue these inserts into virus by cotransfection with pJM17 failed, and this failure lead to the analysis of protein IX expression in vectors with the 3.2 kb E1 deletion and subsequent reinsertion of the Sp1 site into the protein IX promoter in the E1 shuttle plasmids (described in results section B.5). To ensure adequate protein

Figure 23. Construction of Ad/SIV *env* recombinants.

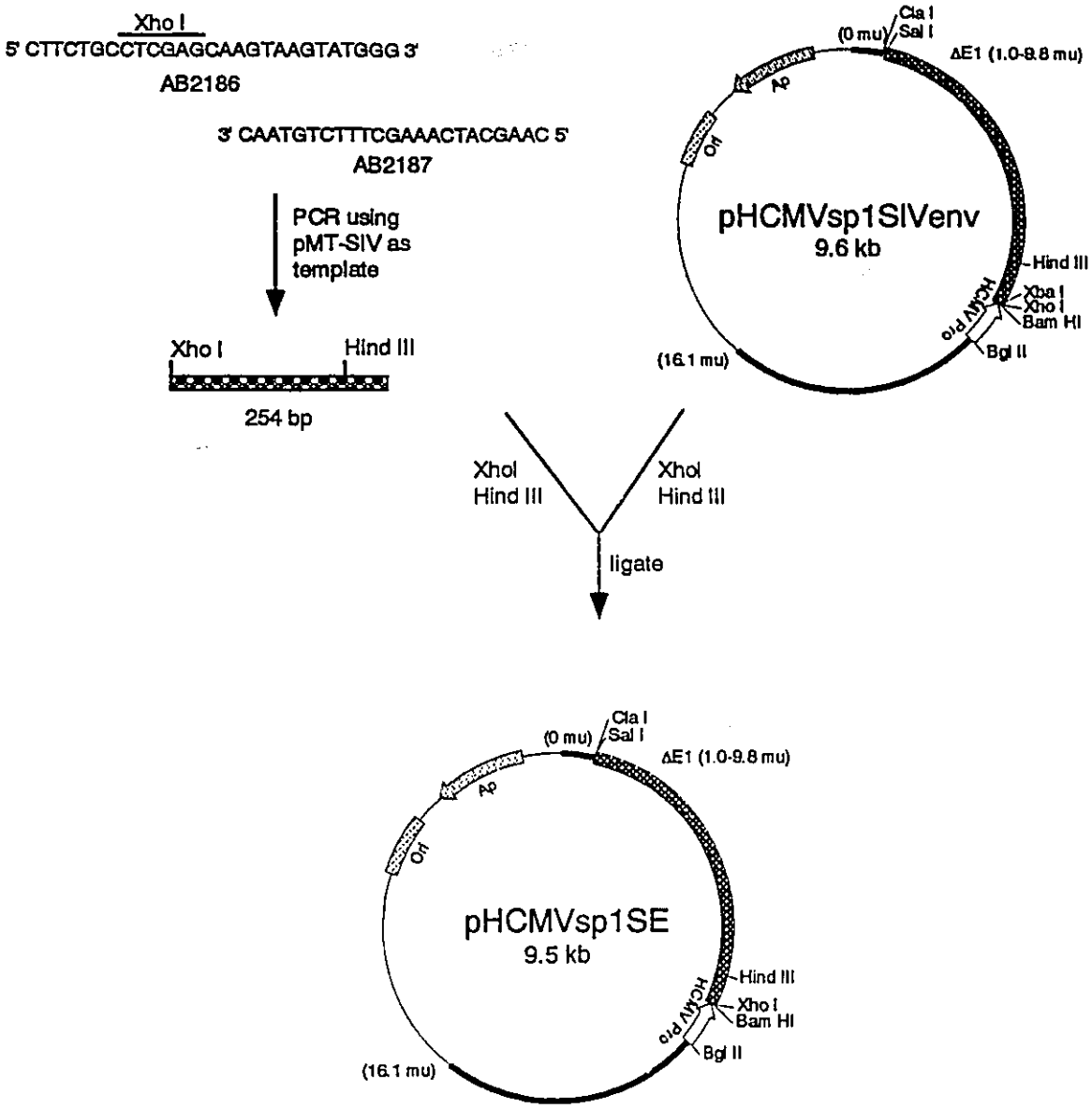
(A) A 2805 bp fragment representing the coding sequences for SIV *env* and *rev* was obtained from pMT-SIV (contains the entire $SIV_{mac}239$ genome minus the LTR's; 1079 to 9487 bp with a recovered stop codon for *env* in the flanking vector sequences) by digesting with *Xba*I and *Sal*I. This fragment was inserted into the *Xba*I and *Sal*I sites in pABE1HCMV3 and pABE1βAct3 generating pHCMVSIVenv and pβActSIVenv.

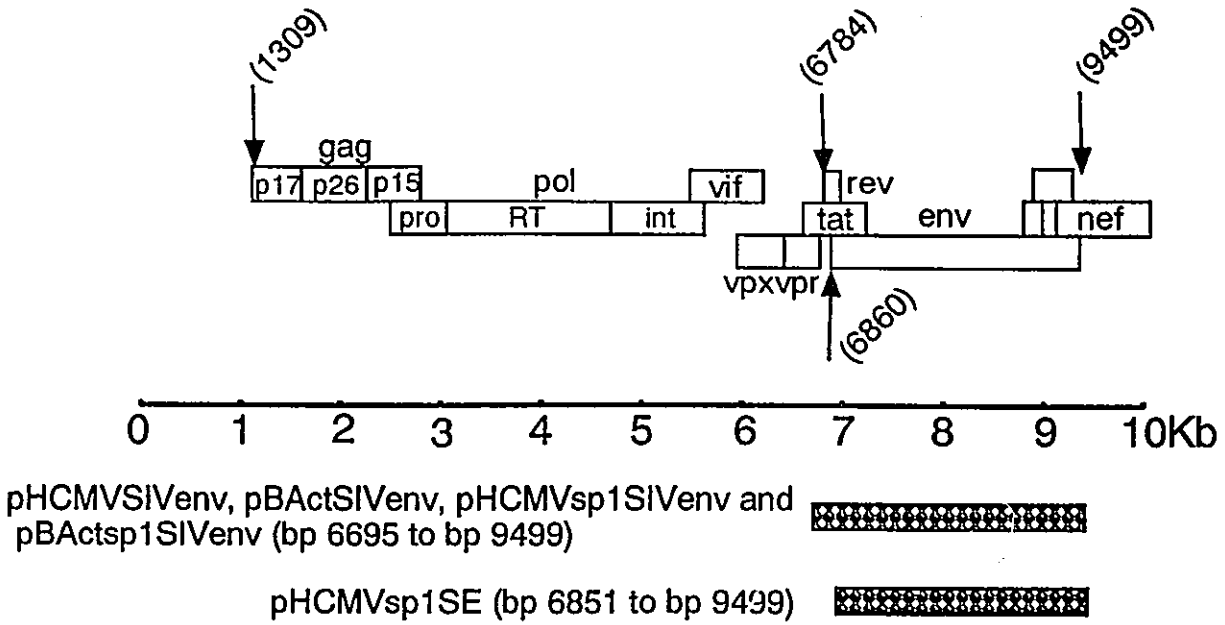
(B) To generate a construct containing just the coding sequences for SIV *env* the insert in pHCMVsp1SIVenv was shortened at the 5' end to remove *rev* coding sequences. Due to the lack of convenient restriction sites PCR was used to generate a 254 bp fragment corresponding to the first segment of *env* (bp 6838 to bp 7092). The 5' PCR primer (AB2186) was designed to contain an *Xho*I site and to disrupt the 5' splice donor found just upstream of the *env* ATG. After digestion of the PCR product with *Xho*I and *Hind*III it was ligated with pHCMVsp1SIVenv, also digested with *Xho*I and *Hind*III generating pHCMVsp1SE.

(C) The segments of the $SIV_{mac}239$ genome used in the construction of the Ad/SIVenv vectors are indicated. pβActSIVenv, pHCMVSIVenv, pβActsp1SIVenv and pHCMVsp1SIVenv all contain a 2805 bp fragment representing the coding sequences for *env* and *rev*. pHCMVsp1SE contains a 2648 bp fragment representing the coding sequences for *env*. pβActSIVenv, pHCMVSIVenv, pβActsp1SIVenv and pHCMVsp1SIVenv were rescued into virus by cotransfection with pJM17 while pHCMVsp1SE was rescued into virus by cotransfection with pBHG10.


A
pABE1HCMV3
6780 bp
pMT-SIV
13.0 kb
pABE1BAct3
7774 bp
pHCMVSIVenv
9.6 kb
pBActSIVenv
10.6 kb
ligate
ligate

B

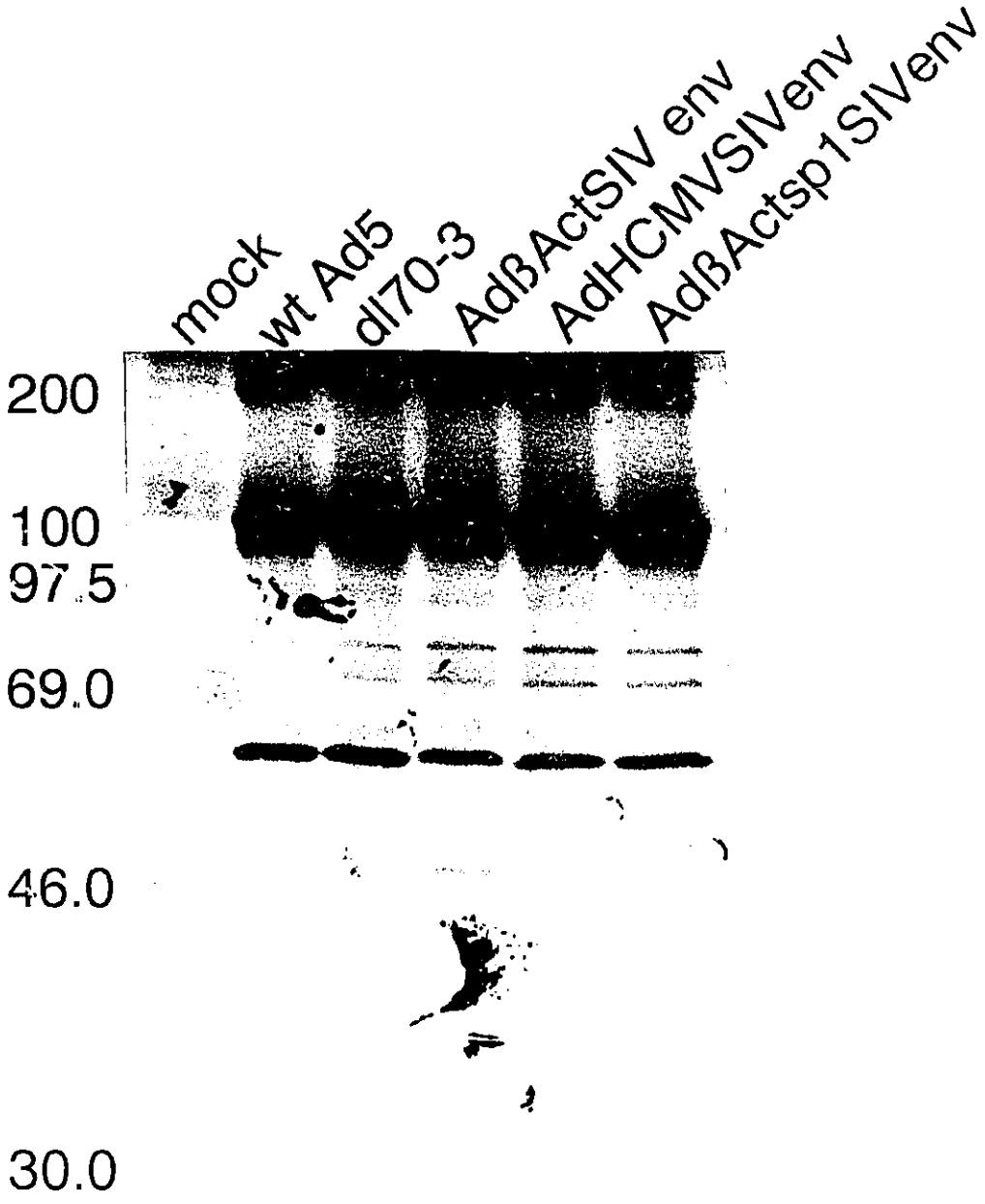

Xho I
5' CTTCTGCCTCGAGCAAGTAAGTATGGG 3'
AB2186
3' CAATGTCTTTCGAAACTACGAAC 5'
AB2187
PCR using
pMT-SIV as
template
Xho I
Hind III
254 bp
pHCMVsp1SIVenv
9.6 kb
Cla I
Sal I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
Hind III
Xba I
Xho I
Bam HI
Bgl II
HCMV P.o
(16.1 mu)
XhoI
Hind III
XhoI
Hind III
ligate
pHCMVsp1SE
9.5 kb
Cla I
Sal I
(0 mu)
ΔE1 (1.0-9.8 mu)
Ap
Ori
Hind III
Xho I
Bam HI
Bgl II
HCMV P.o
(16.1 mu)


(1309)
(6784)
(9499)
gag
p17
p26
p15
pol
vif
rev
pro
RT
int
tat
env
nef
vpxvpr
(6860)
0
1
2
3
4
5
6
7
8
9
10Kb
pHCMVSIVenv, pBActSIVenv, pHCMVsp1SIVenv and pBActsp1SIVenv (bp 6695 to bp 9499)
pHCMVsp1SE (bp 6851 to bp 9499)

IX expression the SIV *env* fragment was also cloned into pβActsp1 and pHCMVsp1 (E1 shuttle plasmids with the Sp1 site reintroduced). The *XhoI/SalI* fragment from pβActSIVenv (Fig. 23A) containing the *env* segment was cloned into the *SalI* site in both pβActsp1 and pHCMVsp1, generating pβActsp1SIVenv and pHCMVsp1SIVenv respectively (construction not shown). All inserts were sequenced at their 5' and 3' ends to ensure they had the proper structure. Ultimately all four constructs were rescued into virus through cotransfections with pJM17, generating AdHCMVSIVenv, AdβActSIVenv, AdHCMVsp1SIVenv and AdβActsp1SIV. As described above and illustrated in Figure 23C all four vectors contain the coding sequences for both *env* and *rev*. The product of the *rev* gene is a trans acting nuclear regulatory protein responsible for transporting unspliced messages encoding viral structural proteins from the nucleus to the cytoplasm through its interaction with the rev responsive element (RRE) (Hadzopoulou-Cladaras *et al.*, 1989; Hammarskjold *et al.*, 1989; Malim *et al.*, 1989). The coding sequences for rev were included in this construct since previous work had shown that coexpression of *rev* with *env* improves the expression of *env* (Cheng *et al.*, 1990). To detect expression of *env* products from the vectors immunoprecipitations were performed using SIV reference sera. 293 cells were either mock infected or infected at an moi of 10 with wt Ad5, dl70-3 (Bett *et al.*, 1994), AdβActSIVenv, AdHCMVSIVenv or AdβActsp1SIVenv (AdHCMVsp1SIVenv had not yet been titered when this IP was carried out). Cells were then labelled from 22-24 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated and separated by SDS-PAGE (Fig. 24). The results of this IP suggested that expression from the *env* insert was not occuring since neither the *env* precursor (gp160) nor processed *env* proteins (gp130 and gp41) were detected. In a further attempt to detect expression Western blots were performed using goat anti-SIV$_{mac}$239 gp130 (donated by N. L. Haigwood, Chiron). 293 cells were either mock infected or infected at an moi

Figure 24. Immunoprecipitation to detect expression from Ad/SIV *env* vectors.

293 cells in 60 mm dishes were either mock infected or infected at an moi of 10 with wt Ad5, dl70-3 (Bett *et al.*, 1994), AdβActSIVenv, AdHCMVSIVenv or AdβActsp1SIVenv (AdHCMVsp1SIVenv had not yet been titered when this IP was performed) for 22 h and then labelled with Trans[^{35}S]-methionine, from 22-24 h. Cell extracts were then prepared, immunoprecipitated with SIV reference sera and samples separated by SDS-PAGE on an 10.0% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. dl70-3 carries the 3.2 kb E1 deletion. Expression of *env* was not detected.

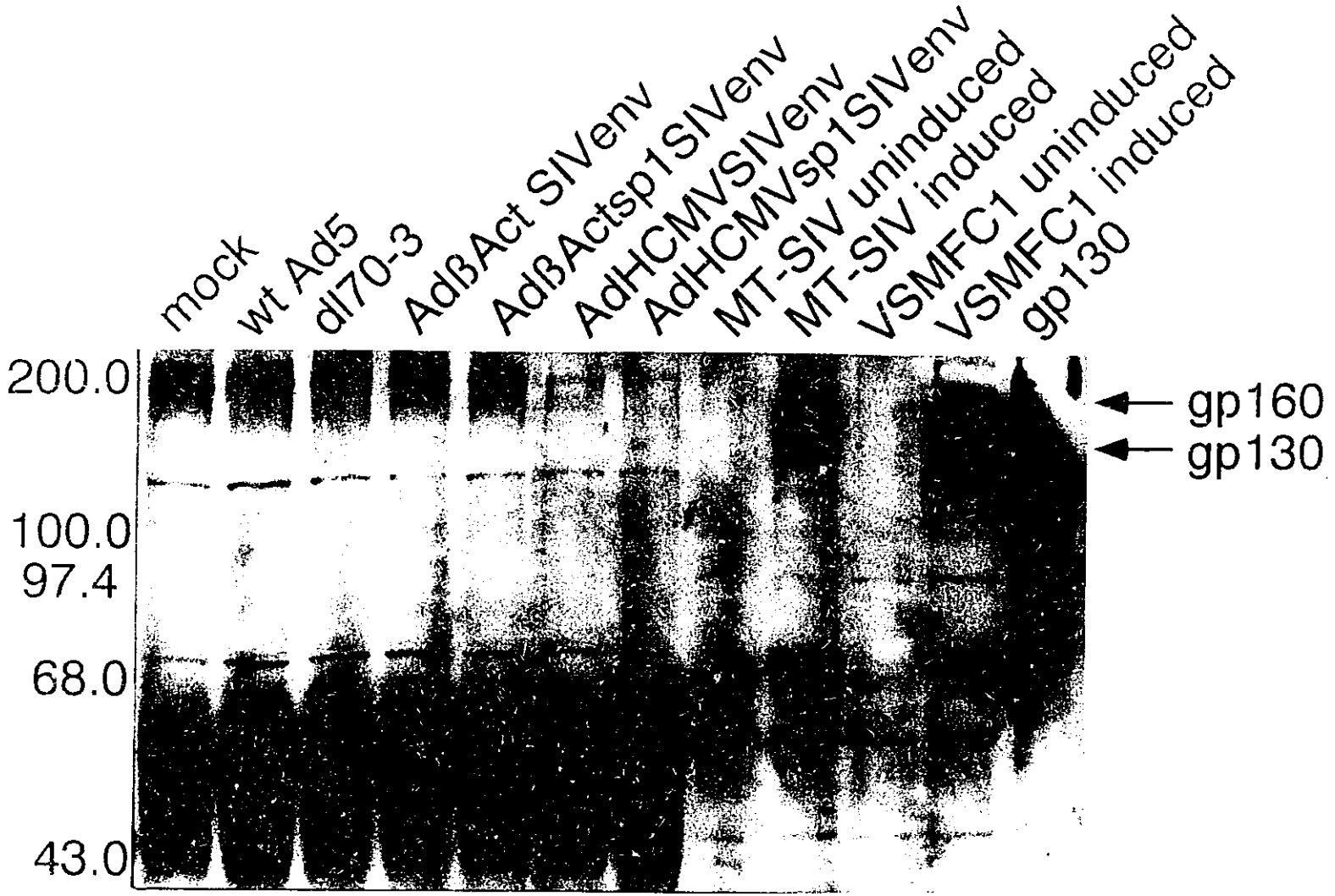

mock
wt Ad5
dl70-3
AdßActSIV env
AdHCMVSIVenv
AdßActsp1SIVenv
200
100
97.5
69.0
46.0
30.0

of 10 with wt Ad5, dl70-3, AdβActSIVenv, AdHCMVSIVenv, AdβActsp1SIVenv or AdHCMVsp1SIVenv and cell extracts were prepared 24 h post infection. As a positive control, cell extracts were also prepared for Vero MT-SIV and VMSFCI cells. Vero MT-SIV and VSMCF1 cells contain the $SIV_{mac}239$ genome under the control of the human metallothionein promoter and are induced to express all SIV proteins by incubating the cells in 300 μM $ZnCl_2$ and 2 μM $CdCl_2$ for 24 hours. Both uninduced and induced extracts were prepared for the two cell lines. As an additional positive control recombinant $SIV_{mac}239$ gp130 (donated by N. L. Haigwood) was included. Samples were separated by SDS-PAGE, transferred to a nylon membrane and the proteins detected using enhanced chemiluminescence (Fig. 25). Env proteins were not detected in cells infected with AdβActSIVenv, AdHCMVSIVenv, AdβActsp1SIVenv or AdHCMVsp1SIVenv but were detected for both positive controls. One possible explanation for the lack of expression from the Ad/SIV env vectors was that the *rev* gene in our inserts was not expressed or expressed at low levels. For this reason the Western blots were repeated using extracts from 293 cells infected at an moi of 100 with AdβActSIVenv, AdHCMVSIVenv, AdβActsp1SIVenv or AdHCMVsp1SIVenv or coinfected with the above vectors (moi 100) and AdRev2 (moi 50). AdRev2 is a replication competent vector containing HIV-1 *rev* in the 2.69 kb E3 deletion flanked by the SV40 promoter and poly A. HIV-1 *rev* has been shown to functionally substitute for the SIV *rev* protein (Lewis *et al.*, 1990). Again, no expression of SIV specific proteins was detectable from any of the infections (data not shown).

Another possible explanation for the lack of expression observed with the Ad/SIVenv vectors was interference from the sequences encoding *rev* up stream of the *env* ATG. For this reason a new vector was generated with the sequences upstream of the *env* initiation site removed (Fig. 23B, C). Due to the lack of convenient restriction sites PCR was needed to generate a 254

Figure 25. Western blot to detect expression from Ad/SIV *env* vectors.

293 cells in 60 mm dishes were either mock infected or infected at an moi of 10 with wt Ad5, dl70-3, AdβActSIVenv, AdHCMVSIVenv, AdβActsp1SIVenv or AdHCMVsp1SIVenv and cell extracts prepared 24 h post infection. Cell extracts were also prepared for Vero MT-SIV and VMSFCI cells (induced and uninduced) as a positive control. Recombinant $SIV_{mac}239$ gp130 was included as an additional positive control. Samples were separated by SDS-PAGE and transferred to a nylon membrane. The blot was incubated first in goat anti-$SIV_{mac}239$ gp130 sera (1:400 dilution), then in HRP-conjugated rabbit anti-goat IgG (1:2000 dilution) and proteins detected by enhanced chemiluminescence. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. Arrows indicate the position of gp160 and gp130 detected in the positive controls only.


mock
wt Ad5
dl70-3
AdßAct SIVenv
AdßActsp1SIVenv
AdHCMVSIVenv
AdHCMVsp1SIVenv
MT-SIV uninduced
MT-SIV induced
VSMFC1 uninduced
VSMFC1 induced
gp130
200.0
100.0
97.4
68.0
43.0
gp160
gp130

bp fragment corresponding to the first segment of *env* (bp 6838 to bp 7092). The 5' PCR primer (AB2186) was designed to include an *Xho*I site and to disrupt the 5' splice donor found just upstream of the *env* ATG. After digestion of the PCR product with *Xho*I and *Hind*III it was ligated with pHCMVsp1SIVenv, also digested with *Xho*I and *Hind*III generating pHCMVsp1SE. pHCMVsp1SE was then rescued into virus by cotransfection with pBHG10 (Bett *et al.*, 1994), generating AdBHGSE. Immunoprecipitations and Western blots similar to those described above were carried out to detect expression from AdBHGSE but again, no expression of SIV *env* specific protein was detected. Because these vectors did not contain a good polyadenylation signal I attempted to introduce the SV40 pA generated by PCR (described in results section B.5) without success. The cloning strategy involved the partial digestion of pHCMVsp1SIVenv and pHCMVsp1SE with *Cla*I which cut at the 3' end of the *env* insert and within the *env* gene. This cloning step could not be completed since it appeared that *Cla*I cleaved at the *Cla*I site within the *env* insert in preference to cleavage at the 3' end. The development of vectors expressing the SIV env gene was abandoned for a new strategy described below.

3. Construction And Characterization Of Replication Defective Vectors Containing Chimeric SIV gag/V3 Inserts In The E1 Region.

The new strategy that was adopted involved the construction of replication defective vectors that would express chimeric SIV proteins in order to combine epitopes potentially important for cellular immunity and the primary neutralization domain of the envelope glycoprotein. The chimeric inserts were constructed so that they contained the first portion of *gag* fused to one two or three tandem repeats of the third variable domain of *env* (V3 loop). Work that was proceeding in the lab at the time indicated that Ad vectors expressing from one to four tandem repeats of a

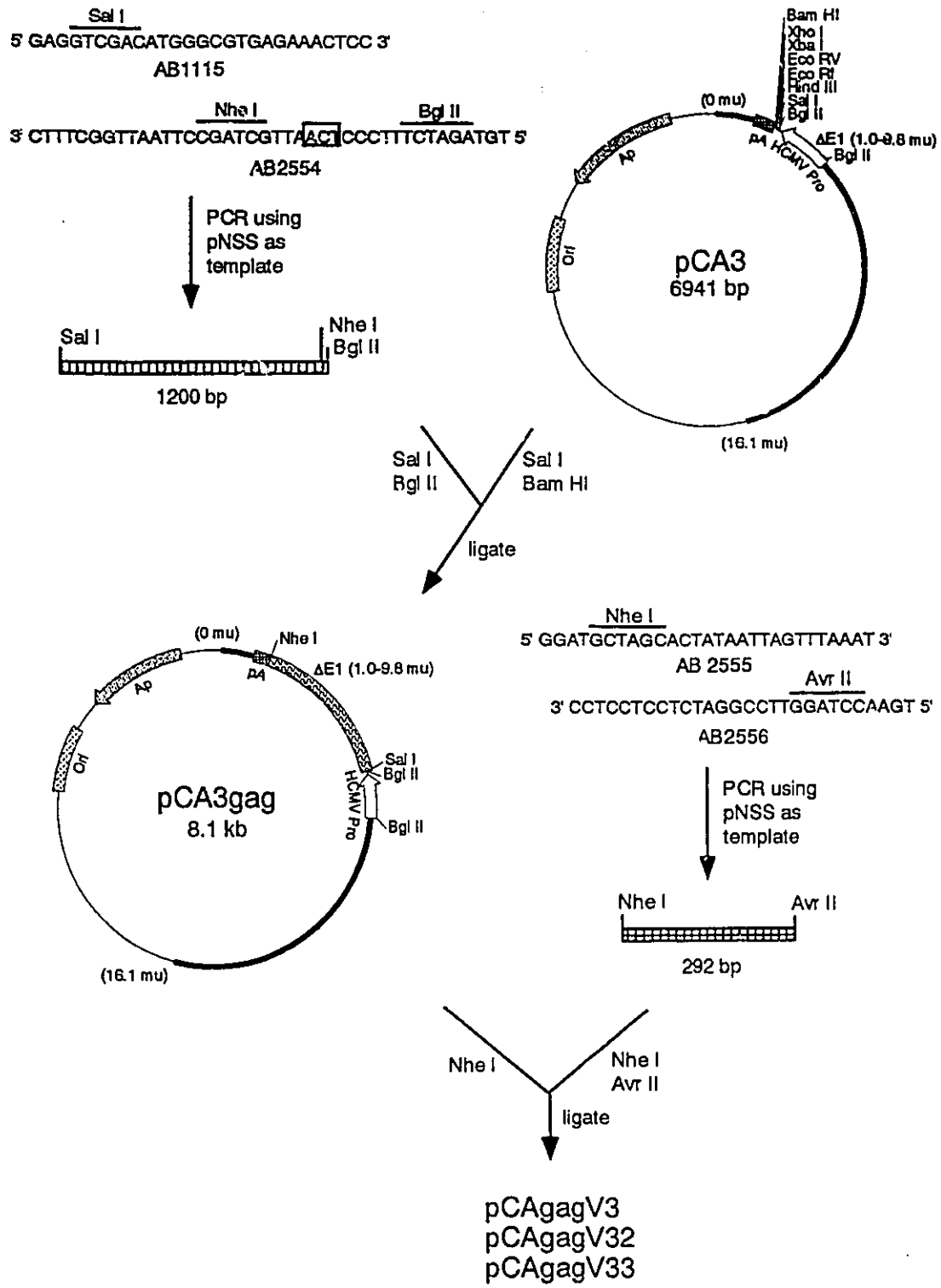
linear neutralizing epitope from HSV-1 gD fused to β-galactosidase generated neutralizing Ab in immunized mice and that the mean Ab titer increased with the number of epitope repeats expressed (Zheng *et al.*, 1993). Also vectors expressing four repeats of the epitope were as effective in protecting mice from a lethal dose of HSV-2 as vectors expressing the entire gD protein (Zheng *et al.*, 1993). Also HIV-1 and HIV-2 chimeric *gag-env* proteins were being constructed and expressed in the baculovirus system (Luo *et al.*, 1992). For our constructs we again chose the HCMV promoter to drive expression since it was shown to have the potential for high level expression of β-galactosidase (results section D). A description of the construction of the chimeric constructs is given below and in Figure 26. PCR was used to generate the *gag* and *env* fragments using pNSS as the template DNA (Fig. 26A). Primers AB1115 and AB2554 were designed to generate a 1200 bp fragment of *gag* encoding the matrix and capsid proteins but having a truncated nucleocapsid protein to eliminate the possibility that the chimeric proteins would retain RNA binding activity. For cloning purposes the 5' primer (AB1115) was designed to contain a *Sal*I site while the 3' primer (AB2554) was designed to contain *Nhe*I and *Bgl*II sites. The 3' primer also contained a stop codon for the chimeric proteins. The *gag* fragment was digested with *Sal*I and *Bgl*II and ligated with pCA3 that had also been digested with *Sal*I and *Bam*HI, generating pCAgag. pCA3 was constructed from pHCMVsp1A by Christina Addison and contains the SV40 pA down stream of the HCMV promoter and cloning region. Next a 292 bp fragment corresponding to the V3 loop region of *env* was generated using primers AB2555 and AB2556 (Fig. 26A). This fragment encompasses the two sets of cystine residues that join in disulphide bonds to produce the characteristic double loop structure of the V3 loop (Hoxie, 1991). This segment was expected to preserve the tertiary structure required to generate SIV neutralizing antibodies since in SIV the epitope is thought to be nonlinear (Javaherian, 1992). The 5' primer

Figure 26. Construction of chimeric *gag/V3* inserts.

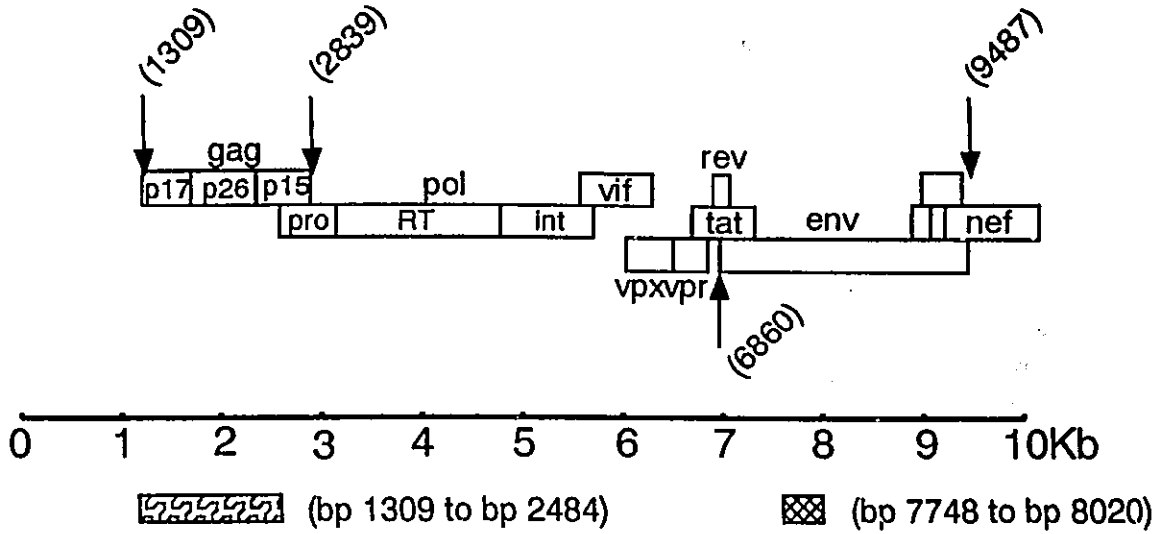
(A) PCR using pNSS as the template DNA was performed to generate both the *gag* and *env* segments used in the chimeric constructs. Primers AB1115 and AB2554 were designed to generate a 1200 bp fragment of *gag* representing the first three quarters of the protein. For cloning purposes the 5' primer (AB1115) was designed to contain a *Sal*I site while the 3' primer (AB2554) was designed to contain *Nhe*I and *Bgl*II sites and the stop codon for the chimeric proteins. The *gag* fragment was digested with *Sal*I and *Bgl*II and ligated with pC3 digested with *Sal*I and *Bam*HI, generating pCAgag. Next a 292 bp fragment corresponding to the V3 loop region of *env* was generated using primers AB2555 and AB2556. The 5' primer was designed to contain a *Nhe*I site while the 3' primer contains an *Avr*II site. The V3 loop fragment was digested with *Nhe*I and *Avr*II and ligated with *Nhe*I digested pCA3gag, generating pCAgagV3, pCAgagV32 and pCAgagV33 which contain one, two, or three repeats of the V3 loop fragment respectively.

(B) The segments of the $SIV_{mac}239$ genome used to generate the chimeric vectors is illustrated. All chimeric constructs contain a 1200 bp fragment of *gag* encoding the matrix and capsid proteins but having a truncated nucleocapsid protein to eliminate the zinc finger domain responsible for RNA binding activity. Constructs also contain either 0, 1, 2 or 3 repeats of a 292 bp fragment corresponding to the V3 loop region of *env*.

A


Sal I
5' GAGGTCGACATGGGCGTGAGAAACTCC 3'
AB1115
Nhe I
Bgl II
3' CTTTCGGTTAATTCCGATCGTTACGTCCCTTTCTAGATGT 5'
AB2554
PCR using pNSS as template
Sal I
Nhe I
Bgl II
1200 bp
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
(0 mu)
ΔE1 (1.0-9.8 mu)
Bgl II
HCMV Pro
Ap
Ori
pCA3
6941 bp
(16.1 mu)
Sal I
Bgl II
Sal I
Bam HI
ligate
(0 mu)
Nhe I
ΔE1 (1.0-9.8 mu)
Ap
Ori
Sal I
Bgl II
HCMV Pro
Bgl II
pCA3gag
8.1 kb
(16.1 mu)
Nhe I
5' GGATGCTAGCACTATAATTAGTTTAAAT 3'
AB 2555
Avr II
3' CCTCCTCCTCTAGGCCTTGGATCCAAGT 5'
AB2556
PCR using pNSS as template
Nhe I
Avr II
292 bp
Nhe I
Nhe I
Avr II
ligate
pCAgagV3
pCAgagV32
pCAgagV33

B


(1309)
(2839)
(9487)
gag
p17
p26
p15
pol
pro
RT
int
vif
rev
tat
env
nef
vpxvpr
(6860)
0
1
2
3
4
5
6
7
8
9
10Kb
(bp 1309 to bp 2484)
(bp 7748 to bp 8020)

was designed to contain a *Nhe*I site while the 3' primer contained an *Avr*II site. The V3 loop fragment was digested with *Nhe*I and *Avr*II and ligated with *Nhe*I digested pCAgag generating pCAgagV3, pCAgagV32 and pCAgagV33 containing one, two, or three tandem repeats of the V3 loop fragment respectively (Fig 26A). Shortly after the construction of these recombinant shuttle plasmids the poly A sequence in pCA3 was suspected to be incorrect since constructs generated by others in the lab failed to express significant levels of protein. For this reason the *gag* and *env* fragments were also cloned into pHCMVsp1A following the same strategy described above, generating pHCMVsp1gag, pHCMVsp1gagV3, pHCMVsp1gagV32 and pHCMVsp1gagV33 containing the gag insert and none, one, two or three tandem repeats of the V3 segment respectively (construction not shown). In the final step a fragment representing the SV40 poly A was generated by PCR and cloned into the *Cla*I at the 3' end of the inserts in pHCMVsp1gag, pHCMVsp1gagV3, pHCMVsp1gagV32 and pHCMVsp1gagV33 generating pHCMVsp1gagpA, pHCMVsp1gagV3pA, pHCMVsp1gagV32pA and pHCMVsp1gagV33pA. The primers and template DNA used to generate the poly A fragment are described in results section B.5. It should be noted that the SV40 sequence in pCA3 was subsequently shown to be correct and functional (See below; C. Addison personal communication). A summary of the chimeric constructs described above, which constructs were rescued into virus, and the expected molecular weights of the chimeric proteins is given in Table 3.

To study the expression of the Ad/SIV gag-env chimeric constructs immunoprecipitations were performed using SIV reference sera. 293 cells were either mock infected or infected at an moi of 50 with dl70-3, or the various chimeric vectors rescued. Cells were then labelled from 24-26 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated and separated by SDS-PAGE (Fig. 27). Proteins of the predicted molecular weights were seen for each of the

Table 3. Shuttle plasmids constructed containing chimeric SIV gag/V3 inserts in E1.

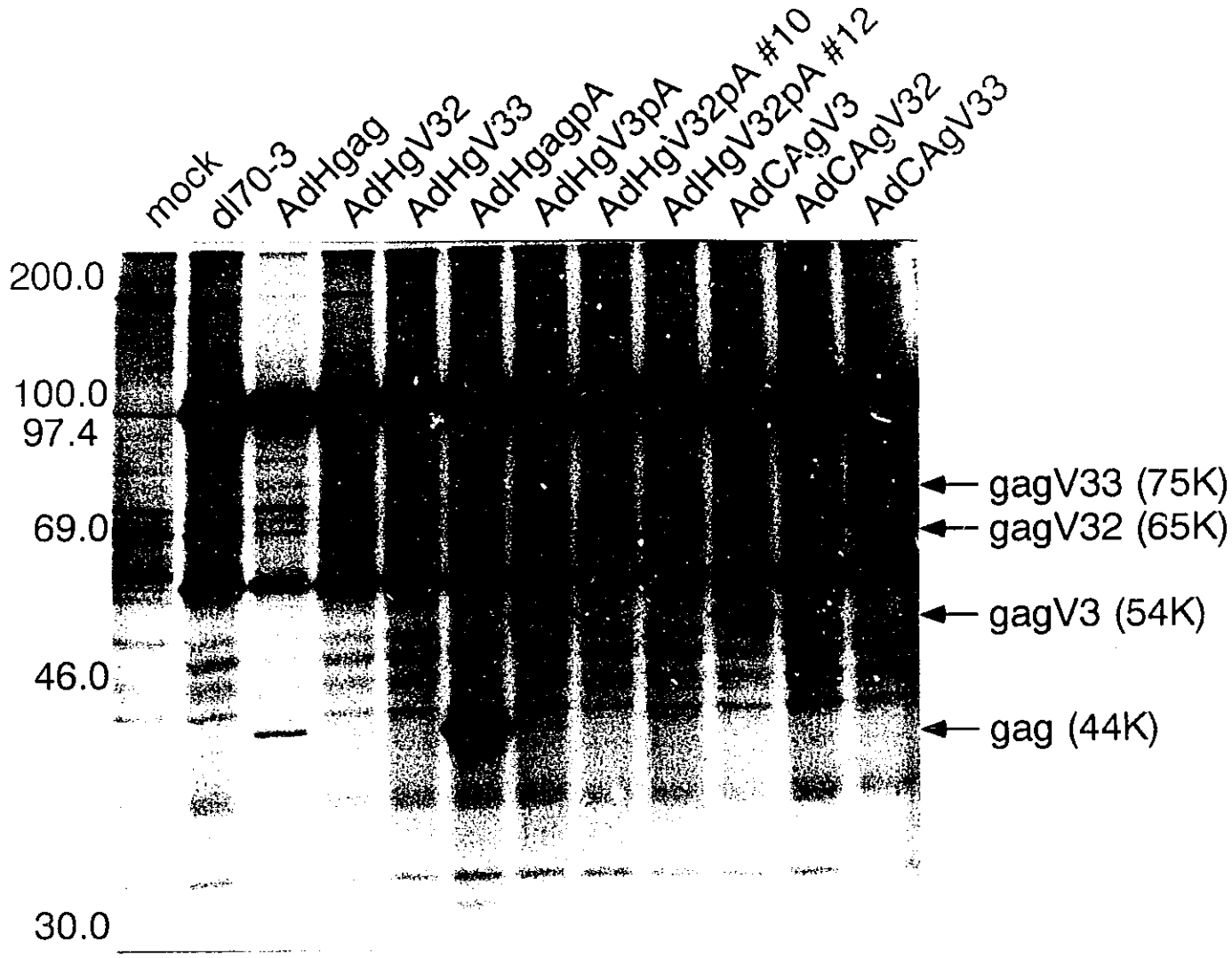
shuttle plasmid	insert size (bp)	rescued into virus[a]	mol. weight[b] (kd)
pCAgag	1730	no	44
pCAgagV3	2022	yes	54
pCAgagV32	2314	yes	65
pCAgagV33	2602	yes	75
pHCMVsp1gag	1580	yes	44
pHCMVsp1gagV3	1872	yes	54
pHCMVsp1gagV32	2164	yes	65
pHCMVsp1gagV33	2456	yes	75
pHCMVsp1gagpA	1730	yes	44
pHCMVsp1gagV3pA	2022	yes	54
pHCMVsp1gagV32pA	2314	yes	65
pHCMVsp1gagV33pA	2606	no	75

a Indicates inserts in shuttle plasmids rescued into virus by cotransfection with pJM17.

b The expected molecular weight in kilo daltons of the chimeric protein encoded by the vector.

Figure 27. Immunoprecipitation to detect expression of the chimeric Ad/SIV *gag/V3* proteins.

293 cells were either mock infected or infected at an moi of 50 with dl70-3, AdHCMVsp1gag, AdHCMVsp1gagV32, AdHCMVsp1gagV33, AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA (two different isolets were used), AdCAgagV3, AdCAgagV32 and AdCAgagV33. Cells were then labelled from 24-26 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using SIV reference sera and analyzed by SDS-PAGE on a 10% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. Proteins of the predicted molecular weights were seen for each of the vectors tested and are indicated with arrows to the right.

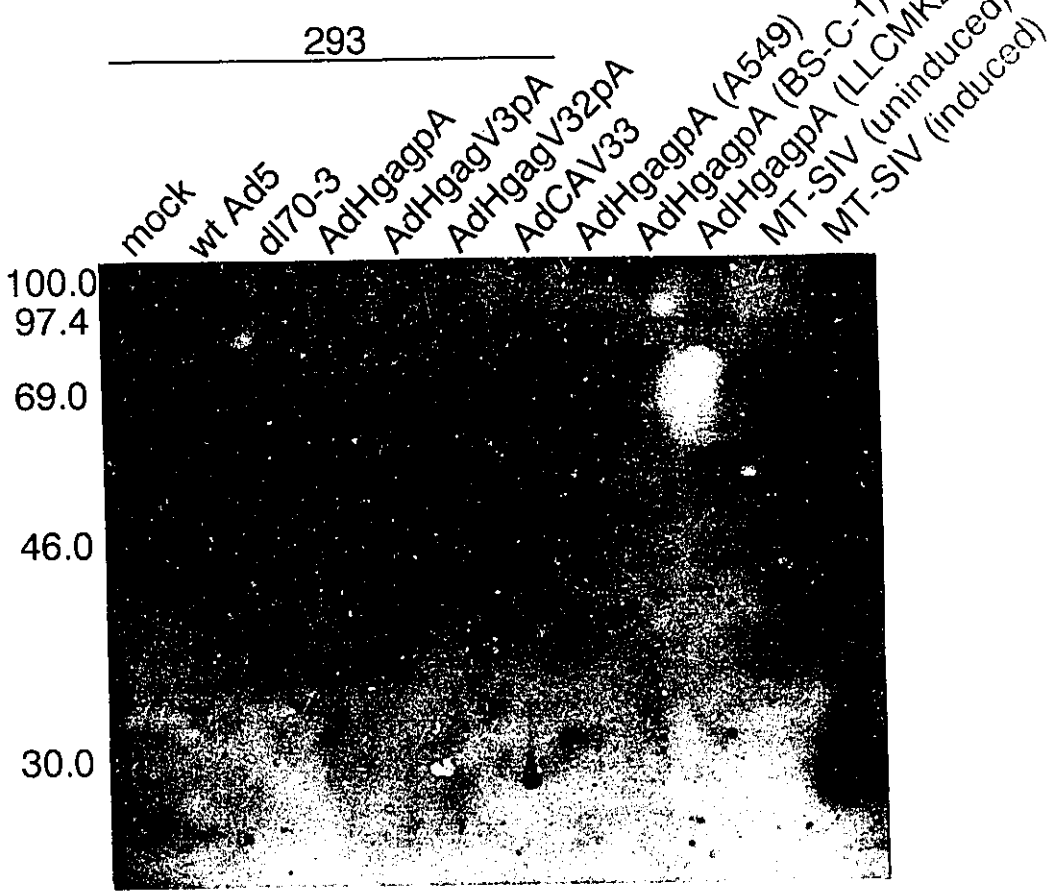

mock
dl70-3
AdHgag
AdHgV32
AdHgV33
AdHgagpA
AdHgV3pA
AdHgV32pA #10
AdHgV32pA #12
AdCAgV3
AdCAgV32
AdCAgV33
200.0
100.0
97.4
69.0
46.0
30.0
gagV33 (75K)
gagV32 (65K)
gagV3 (54K)
gag (44K)

vectors tested. As expected, constructs containing the SV40 pA expressed higher levels of chimeric proteins than similar vectors lacking the poly A sequence. Constructs generated using pCA3 appeared to express amounts of protein comparable to those expressed by the AdHCMV series of vectors containing the SV40 pA, indicating the pA sequence in pCA3 was functional. Both sets of vectors were used in subsequent studies. One other observation made in this experiment was that as the number of V3 repeats increased the level of expression of the chimeric protein decreased.

To characterize the expression of the chimeric proteins in non complementing cell lines Western blots were executed. 293, A549 (human lung carcinoma), LLCMK2 (Rhesus monkey kidney) and BSC-1 (african green monkey kidney) cells were either mock infected or infected at an moi of 50 with wt Ad5, dl70-3, AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA and AdCAgagV33 and cell extracts prepared 24, 48 or 96 h post infection. As a positive control both induced and uninduced cell extracts were prepared for the Vero MT-SIV cell line. Samples were separated by SDS-PAGE, transferred to nylon membranes and proteins detected using SIV reference sera. In contrast to the IP described above (Fig. 27) in which expression was detected for each of the chimeric vectors in 293 cells, expression could only be detected by Western blot in 293 cells infected with AdHCMVsp1gagpA. Expression could not be detected in A549, LLCMK2 or BSC-1 cells for any of the vectors tested. Figure 28 shows the results obtained in one of the blots in which several of the extracts were tested. The Western blots were repeated using the HIV-2 *gag* antisera but the same result was obtained, expression was only detected in 293 cells infected with AdHCMVsp1gagpA. It appeared that chimeric proteins were not being efficiently expressed in non complementing cell lines.

Figure 28. Western blots to detect expression of chimeric Ad/SIV *gag/V3* proteins.

293, A549, LLCMK2 and BS-C-1 cells were either mock infected or infected at an moi of 50 with wt Ad5, dl70-3, AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA and AdCAgagV33 and cell extracts prepared 48 post infection. As a positive control both induced and uninduced cell extracts were prepared for the Vero MT-SIV cell line. Samples were separated by SDS-PAGE on a 10% gel, transferred to nylon membranes, incubated in SIV reference sera (1:500 dilution) then in HRP-conjugated goat anti-human IgG (1:1000 dilution) and proteins detected using enhanced chemiluminescence. An arrow indicates the 44K *gag* protein detected in 293 cells infected with AdHCMVsp1gagpA.

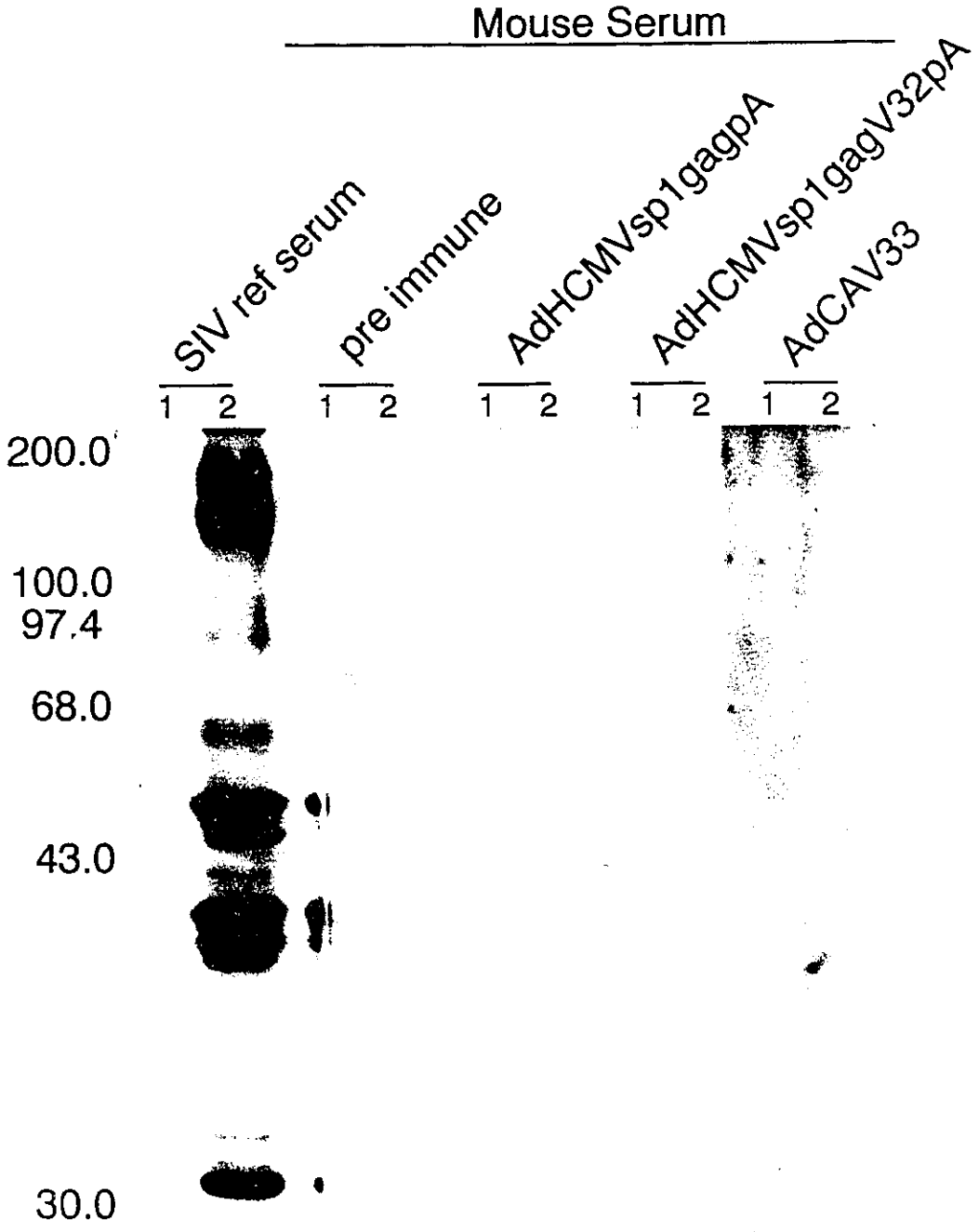

293
mock
wt Ad5
dl70-3
AdHgagpA
AdHgagV3pA
AdHgagV32pA
AdCAV33
AdHgagpA (A549)
AdHgagpA (BS-C-1)
AdHgagpA (LLCMK2)
MT-SIV (uninduced)
MT-SIV (induced)
100.0
97.4
69.0
46.0
30.0

4. Immunization Of Mice With Chimeric SIV gag/V3 Vectors

Because high levels of expression are not necessarily required to generate an immune response it was decided to test the immunogenicity of the chimeric proteins by administering the recombinant vectors to mice. Cesium chloride gradient purified stocks of AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA, AdCAV33 and AdHCMVsp1LacZ (Morsey *et al.*, 1993) (used as a negative control) were injected intraperitoneally or intranasally into female BALB/c mice at $1X10^8$ pfu per animal. Five animals were immunized for each vector. Blood samples were taken two weeks later, animals were reimmunized (boosted) and blood samples taken one week post boost. The sera from the boosted animals were pooled and tested for the presence of anti-SIV antibodies by Western blot. Both induced and uninduced MT-SIV cell extracts were prepared, separated by SDS-PAGE on a 10% gel and transferred to nylon membranes. The membrane was then cut into strips (each strip having both a lane containing uninduced and induced cell extract) and individual strips incubated with mouse sera from each immunization group (diluted 1:800). Figure 29 shows the results obtained using the sera collected from mice immunized intraperitonally. None of the mouse sera were positive for antibodies by Western blot using sera diluted 1:800. The Western blots were repeated using less dilute mouse sera (1:20) but again all groups were negative (data not shown). Since the vectors failed to induce an immune response in immunized mice and appeared to be expressing low levels of protein *in vitro* we decided to construct a new series of chimeric constructs in an attempt to improve expression.

Figure 29. Western blot to detect anti-SIV antibodies in mice immunized with chimeric Ad/SIV *gag/V3* vectors.

Female BALB/c mice (five mice in each group) were immunized either intraperitoneally or intranasally with $1X10^8$ pfu of cesium chloride purified AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA, AdCAgagV33 or AdHCMVsp1LacZ (used as a negative control). Blood samples were taken two weeks later mice were boosted with an additional $1X10^8$ pfu of virus and blood samples taken one week post boost (week 3). Serum samples taken at week 3 were pooled and tested for the presence of anti-SIV antibodies by western blot. Induced and uninduced MT-SIV cell extracts were prepared, separated by SDS-PAGE on a 10% gel and transferred to nylon membranes. The membrane was then cut into strips (each strip having both a lane containing uninduced and induced cell extract) and individual strips incubated with mouse sera from each immunization group (diluted 1:800). Blots were then washed and incubated with HRP-conjugated goat anti-mouse IgG (1:1000 dilution) and proteins detected by enhanced chemiluminescence. As a positive control one strip was incubated with SIV reference sera (1:1000 dilution) and then with HRP-conjugated goat anti human IgG (1:1500). In the blot shown serum was tested from mice immunized intraperitonally with the vectors indicated. Molecular weight markers are indicated on the left.


Mouse Serum
SIV ref serum
pre immune
AdHCMVsp1gagpA
AdHCMVsp1gagV32pA
AdCAV33
1 2
1 2
1 2
1 2
1 2
200.0
100.0
97.4
68.0
43.0
30.0

5. Construction Of Second Generation Chimeric SIV gag/V3 Vectors.

Two strategies were adopted in an attempt to improve the expression of the chimeric *gag/V3* inserts. The first strategy involved reversing the orientation of chimeric inserts in the E1 deletion. In all the SIV expression vectors described in the previous section the inserts are in the E1 antiparallel orientation. Data obtained by C. Addison using HCMV/β-galactosidase and HCMV/luciferase vectors indicated that 5-7 fold higher expression levels can be obtained from the same insert in the E1 parallel orientation as opposed to antiparallel (Hitt *et al.*, 1995). This is likely due to the presence of the E1 enhancer positioned upstream of the HCMV promoter when the cassette is in the E1 parallel orientation. For this reason the orientation of the chimeric *gag/V3* inserts was changed to E1 parallel. The second attempt at improving expression involved generating vectors with the Ad2 MLP driving expression of the chimeric inserts instead of the HCMV promoter.

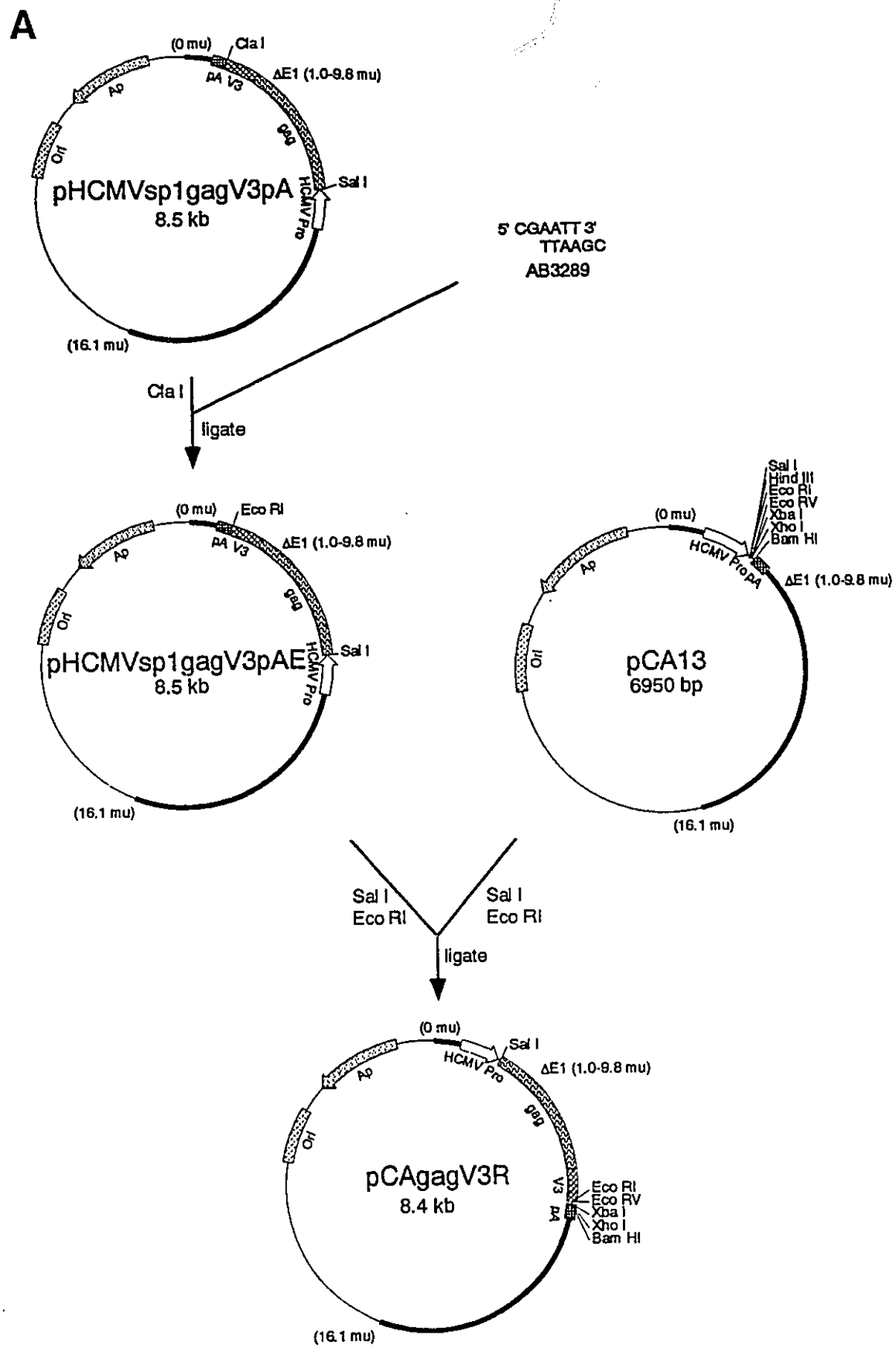
The strategy followed to reverse the orientation of the chimeric *gag/V3* inserts is described below and illustrated in Figure 30. First an oligo containing an *Eco*RI site (AB3289) was introduced into the *Cla*I site in pHCMVsp1gagpA, pHCMVsp1gagV3pA, pHCMVsp1gagV32 and pHCMVsp1gagV33, generating pHCMVsp1gagpAE, pHCMVsp1gagV3pAE, pHCMVsp1gagV32E and pHCMVsp1gagV33E. In the next step the *Sal*I/*Eco*RI fragments from pHCMVsp1gagpAE, pHCMVsp1gagV3pAE, pHCMVsp1gagV32E and pHCMVsp1gagV33E (containing the chimeric inserts) were introduced into the *Sal*I and *Eco*RI sites in pCA13, generating pCAgagR, pCAgagV3R, pCAgagV32R and pCAgagV33R respectively (Fig. 30A).

To generate constructs with the Ad2 MLP driving expression of the chimeric *gag/V3* inserts pCAgagR, pCAgagV3R, pCAgagV32R and pCAgagV33R were digested with *Sal*I and *Bgl*II (to obtain the insert flanked at the 3' end by the SV40 poly A) the insert was purified and ligated

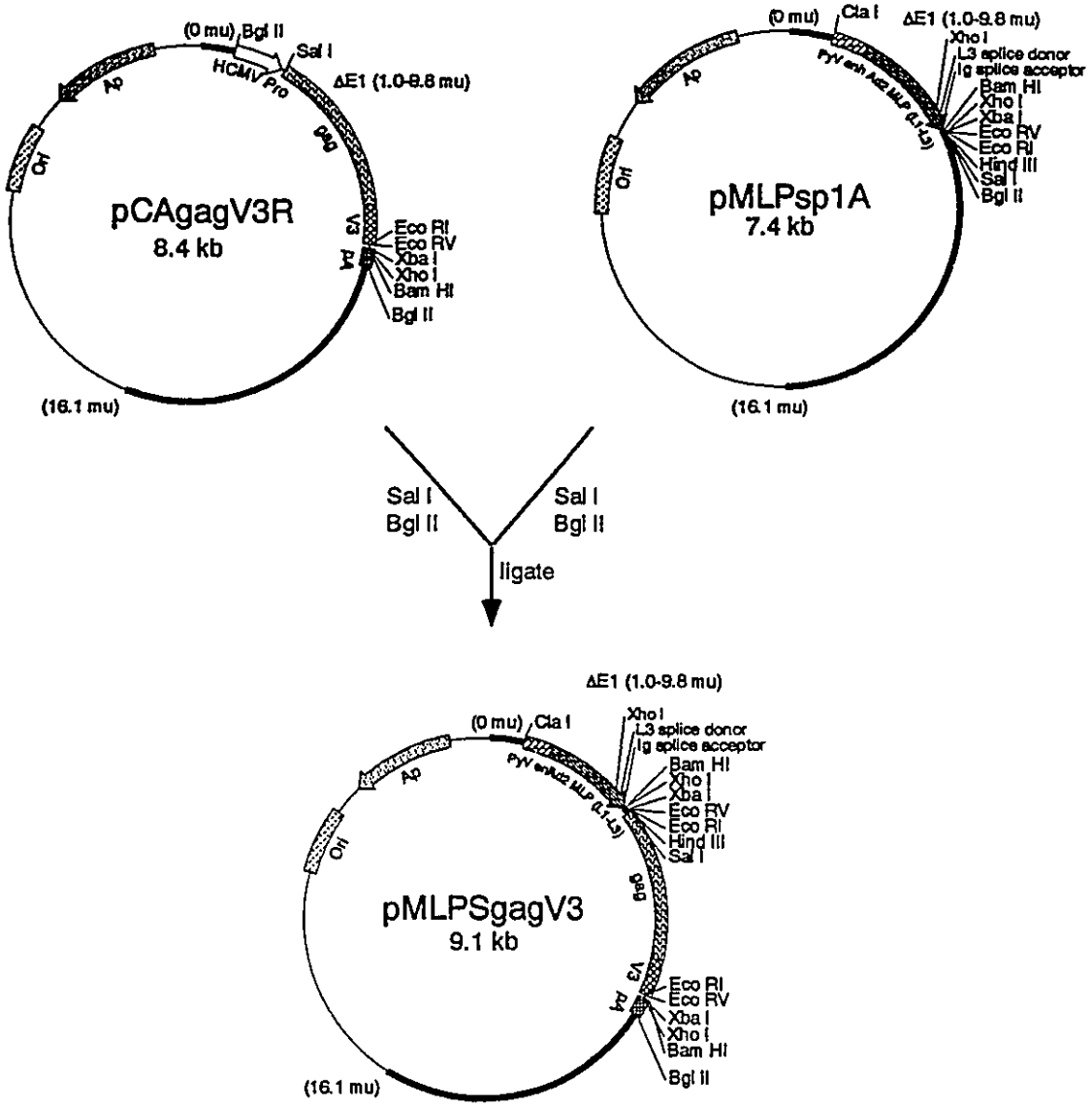
Figure 30. Construction of chimeric Ad/SIV *gag/V3* inserts in the E1 parallel orientation.

(A) In an attempt to improve the expression of the chimeric *gag/V3* proteins the orientations of the inserts was reversed making them E1 parallel. The same strategy was followed to reverse the orientation of all the chimeric *gag/V3* inserts and is only described for the construct containing the gag fragment with one V3 repeat here. First oligo AB3289 was introduced into the *Cla*I in pHCMVsp1gagV3pA, generating pHCMVsp1gagV3pAE. Next the *Sal*I/*Eco*RI fragment from pHCMVsp1gagV3pAE (containing the insert) was purified and ligated with *Sal*I and *Eco*RI digested pCA13, generating pCAgagV3R.

(B) The strategy followed to generate constructs in which the Ad2 MLP was driving expression of the chimeric inserts is shown for the *gag/V3* insert only but was the same for all the chimeric genes. pCAgagV3R was digested with *Sal*I and *Bgl*II, the fragment containing the insert purified and then ligated with pMLPsp1A also digested with *Sal*I and *Bgl*II, generating pMLPSgagV3.


A
(0 mu)
Cla I
ΔE1 (1.0-9.8 mu)
pA
V3
gag
Ap
Ori
HCMV Pro
Sal I
pHCMVsp1gagV3pA
8.5 kb
(16.1 mu)
5' CGAATT 3'
TTAAGC
AB3289
Cla I
ligate
(0 mu)
Eco RI
ΔE1 (1.0-9.8 mu)
pA
V3
gag
Ap
Ori
HCMV Pro
Sal I
pHCMVsp1gagV3pAE
8.5 kb
(16.1 mu)
Sal I
Hind III
Eco RI
Eco RV
Xba I
Xho I
Bam HI
(0 mu)
HCMV Pro
pA
ΔE1 (1.0-9.8 mu)
Ap
Ori
pCA13
6950 bp
(16.1 mu)
Sal I
Eco RI
Sal I
Eco RI
ligate
(0 mu)
Sal I
HCMV Pro
ΔE1 (1.0-9.8 mu)
Ap
gag
Ori
pCAgagV3R
8.4 kb
V3
pA
Eco RI
Eco RV
Xba I
Xho I
Bam HI
(16.1 mu)

B


(0 mu) Bgl II
Sal I
HCMV Pro
ΔE1 (1.0-9.8 mu)
Ap
Ori
gag
V3
R
Eco RI
Eco RV
Xba I
Xho I
Bam HI
Bgl II
pCAgagV3R
8.4 kb
(16.1 mu)
(0 mu) Cla I
ΔE1 (1.0-9.8 mu)
Xho I
L3 splice donor
Ig splice acceptor
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Bgl II
pMLPsp1A
7.4 kb
(16.1 mu)
Sal I
Bgl II
Sal I
Bgl II
ligate
ΔE1 (1.0-9.8 mu)
(0 mu) Cla I
Xho I
L3 splice donor
Ig splice acceptor
Bam HI
Xho I
Xba I
Eco RV
Eco RI
Hind III
Sal I
Ap
Ori
gag
V3
R
Eco RI
Eco RV
Xba I
Xho I
Bam HI
Bgl II
pMLPSgagV3
9.1 kb
(16.1 mu)

with pMLPsp1A also digested with *Sal*I and *Bgl*II, generating pMLPSgag, pMLPSgagV3, pMLPSgagV32 and pMLPSgagV33 (Fig 30B). Table 4 gives a summary of the new chimeric constructs described above, indicating which constructs were rescued into virus, and listing the expected molecular weights of the chimeric proteins.

Immunoprecipitations were done to compare the level of expression obtained with the various parallel and antiparallel inserts in various cell lines. In the first IP AdHCMVsp1gagpA (insert antiparallel) was compared to AdCAgagR (insert parallel). AdMLPSgagV3 was also included in this experiment but could not be compared directly to the analogous HCMV driven construct since they had not yet been rescued into virus. MRC5, LLCMK2, BSC-1 and NIH 3T3 cells were either mock infected or infected at an moi of 500 with AdHCMVsp1gagpA, AdCAgagR, AdMLPSgagV3. Cells were then labelled from 48-50 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using SIV reference sera and separated by SDS-PAGE on a 10% gel (Fig. 31). The results indicated that better expression of the HCMV gag insert was obtained with the insert in the E1 parallel orientation as compared to antiparallel in LLCMK2 and BSC-1 cells in agreement with the data obtained from studies on β-galactosidase expression (Hitt *et al.*, 1995). In MRC5's the difference was not as apparent. Very little but detectable expression was observed in the mouse cell line NIH3T3 for both orientation. Expression was detected for the gag/V3 insert driven by the MLP in all cell lines but the level varied with the cell line. Additional IP's were performed to compare directly the expression levels obtained with the MLP and HCMV promoters driving the same insert. One of the original replication competent vectors, AdSIVgag (containing coding sequences for the entire p57 gag precursor in E3), was also included in this experiment to compare the expression levels obtained with replication competent and replication defective vectors in the various cell lines. 293, MRC5,

Table 4. Shuttle plasmids constructed containing chimeric SIV gag/V3 inserts in the E1 parallel orientation.

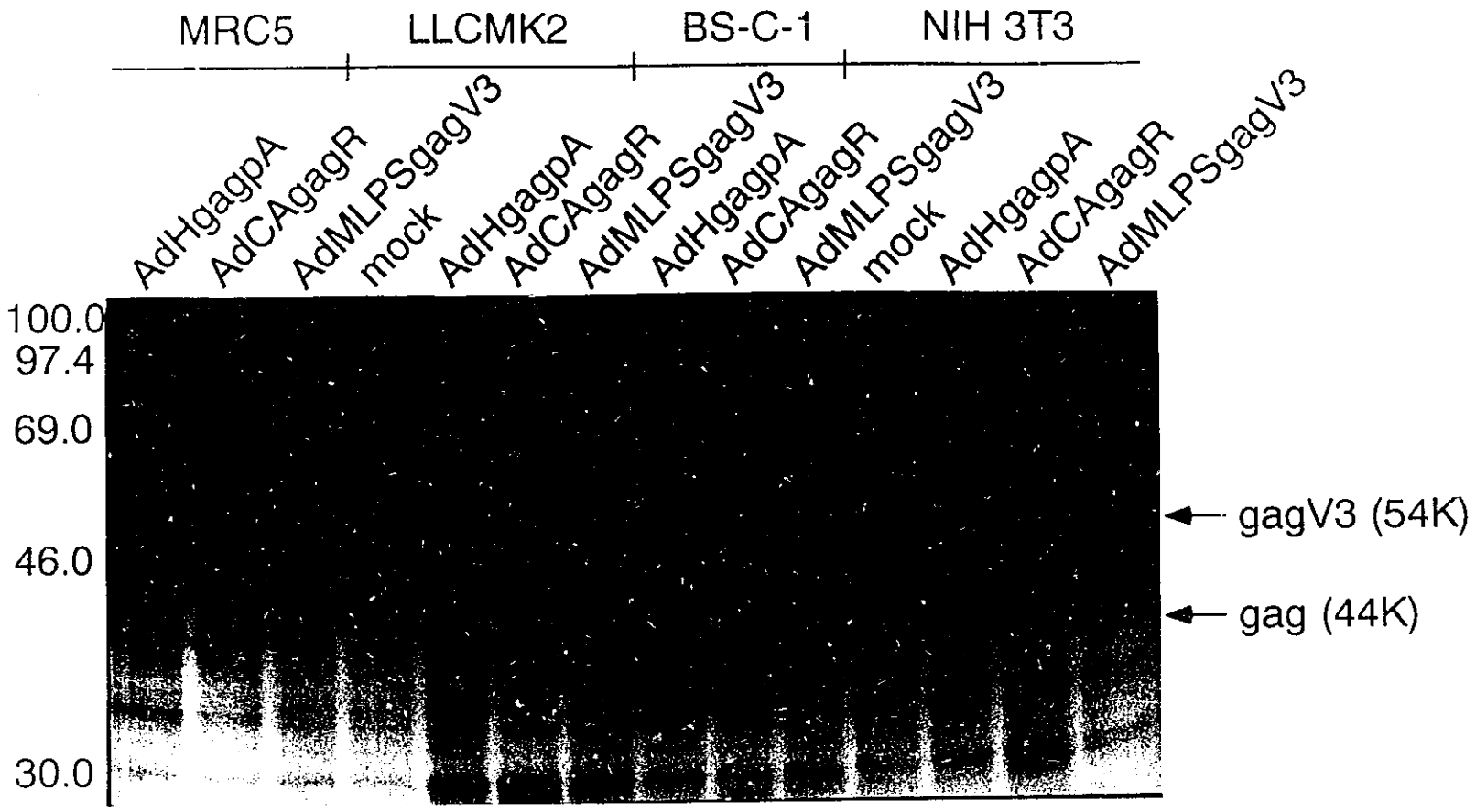
shuttle plasmid	insert size (bp)	rescued into virus[a]	mol. weight[b] (kd)
pCAgagR	1730	yes	44
pCAgagV3R	2022	yes	54
pCAgagV32R	2314	no	65
pCAgagV33R	2606	yes	75
pMLPSgag	2313	no	44
pMLPSgagV3	2605	yes	54
pMLPSgagV32	2897	no	65
pMLPSgagV33	3189	no	75

a Indicates inserts in shuttle plasmids rescued into virus by cotransfection with pBHG11.

b The expected molecular weight in kilo daltons of the chimeric protein encoded by the vector.

Figure 31. Comparison of expression levels obtained with AdHCMVsp1gagpA and AdCAgagR in various cell lines.

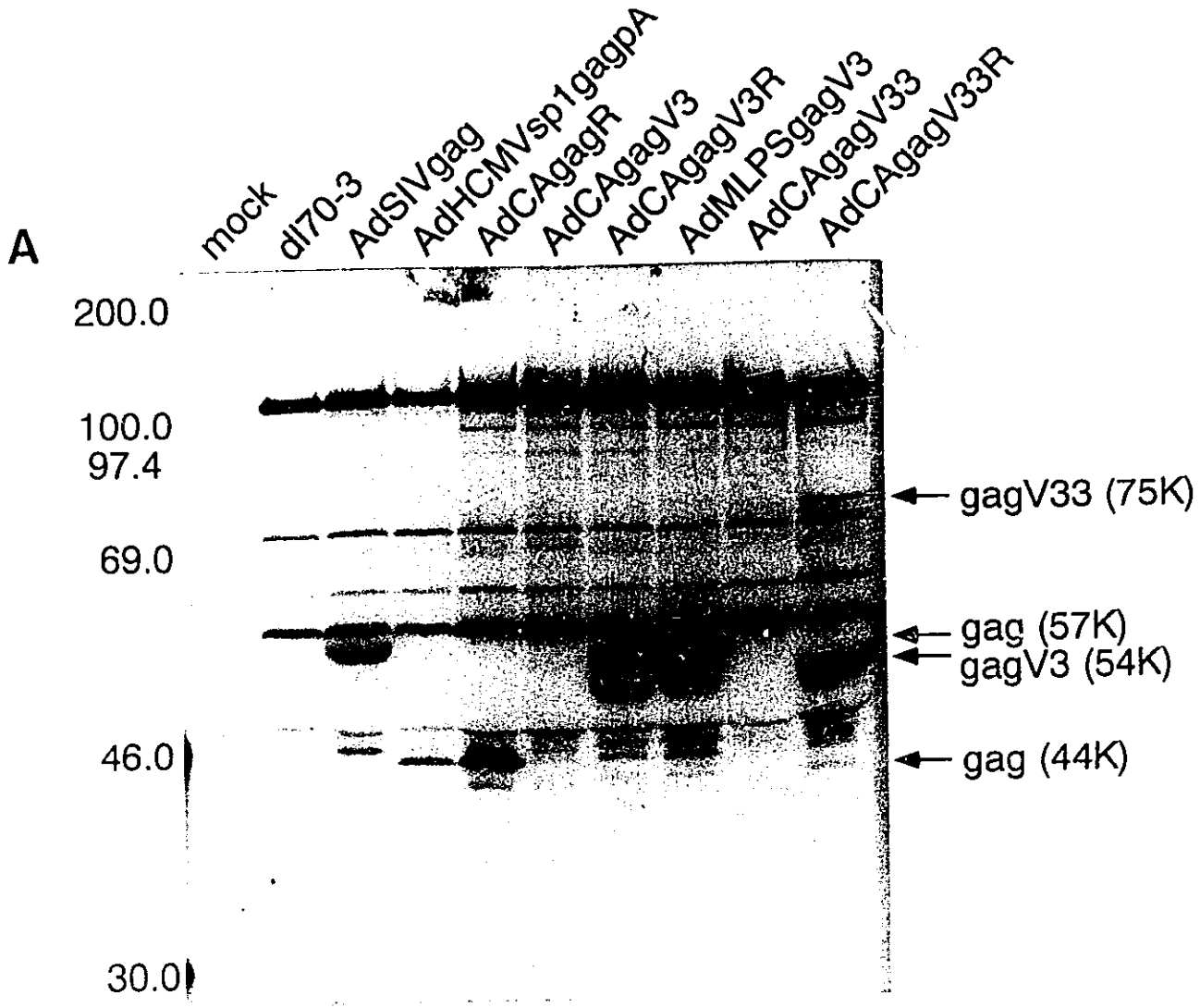
MRC5, LLCMK2, BSC-1 and NIH 3T3 cells were either mock infected or infected at an moi of 500 with AdHCMVsp1gagpA, AdCAgagR or AdMLPSV3. Cells were then labelled from 48-50 h post infection using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using SIV reference sera and separated by SDS-PAGE on a 10% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above (AdHgagPA represents AdHCMVsp1gagpA) and molecular weight markers are indicated on the left. Proteins of the predicted molecular weights were seen for each of the vectors tested and are indicated with arrows to the right.

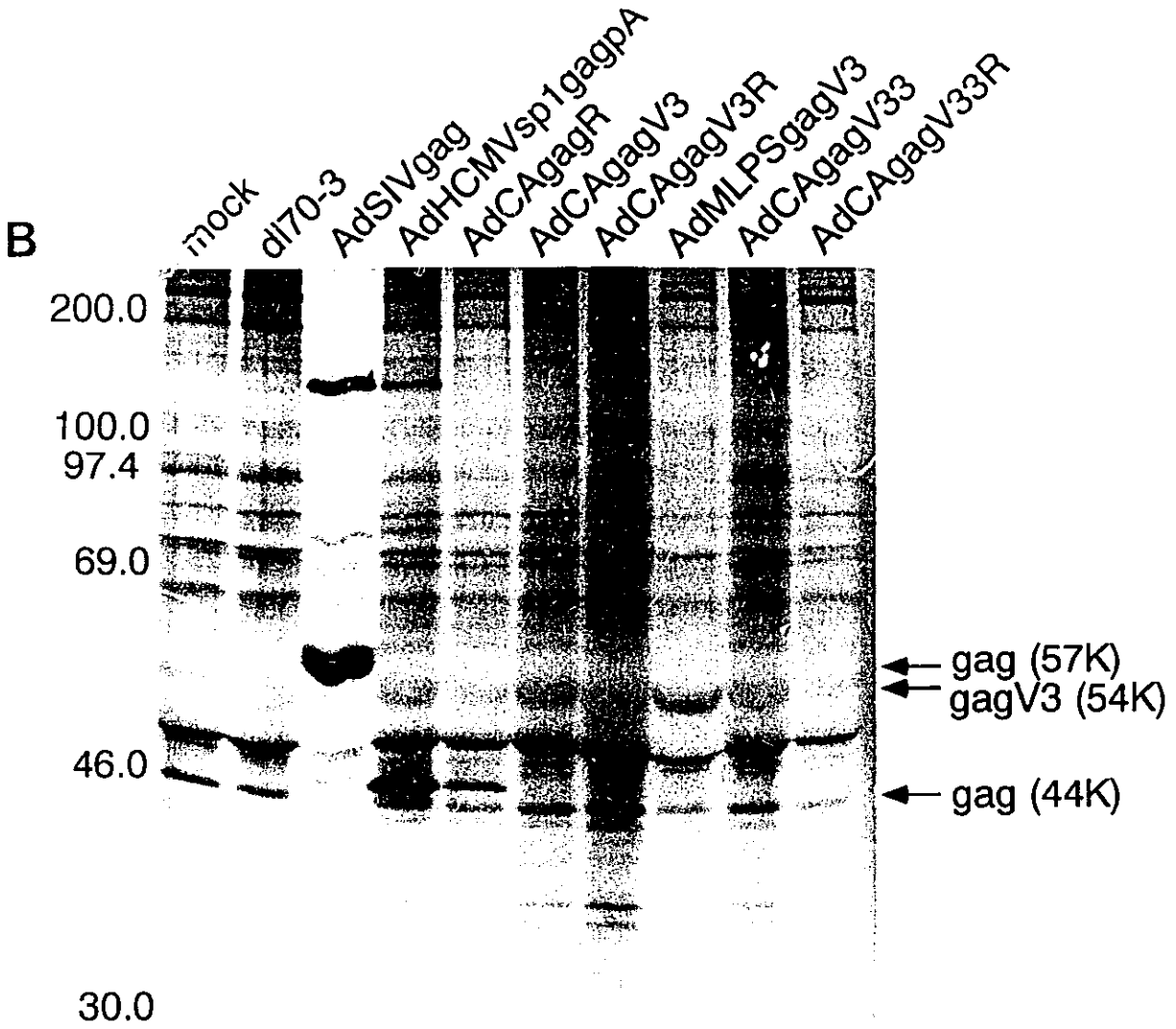

MRC5
LLCMK2
BS-C-1
NIH 3T3
AdHgagpA
AdCAgagR
AdMLPSgagV3
mock
AdHgagpA
AdCAgagR
AdMLPSgagV3
AdHgagpA
AdCAgagR
AdMLPSgagV3
mock
AdHgagpA
AdCAgagR
AdMLPSgagV3
100.0
97.4
69.0
46.0
30.0
gagV3 (54K)
gag (44K)

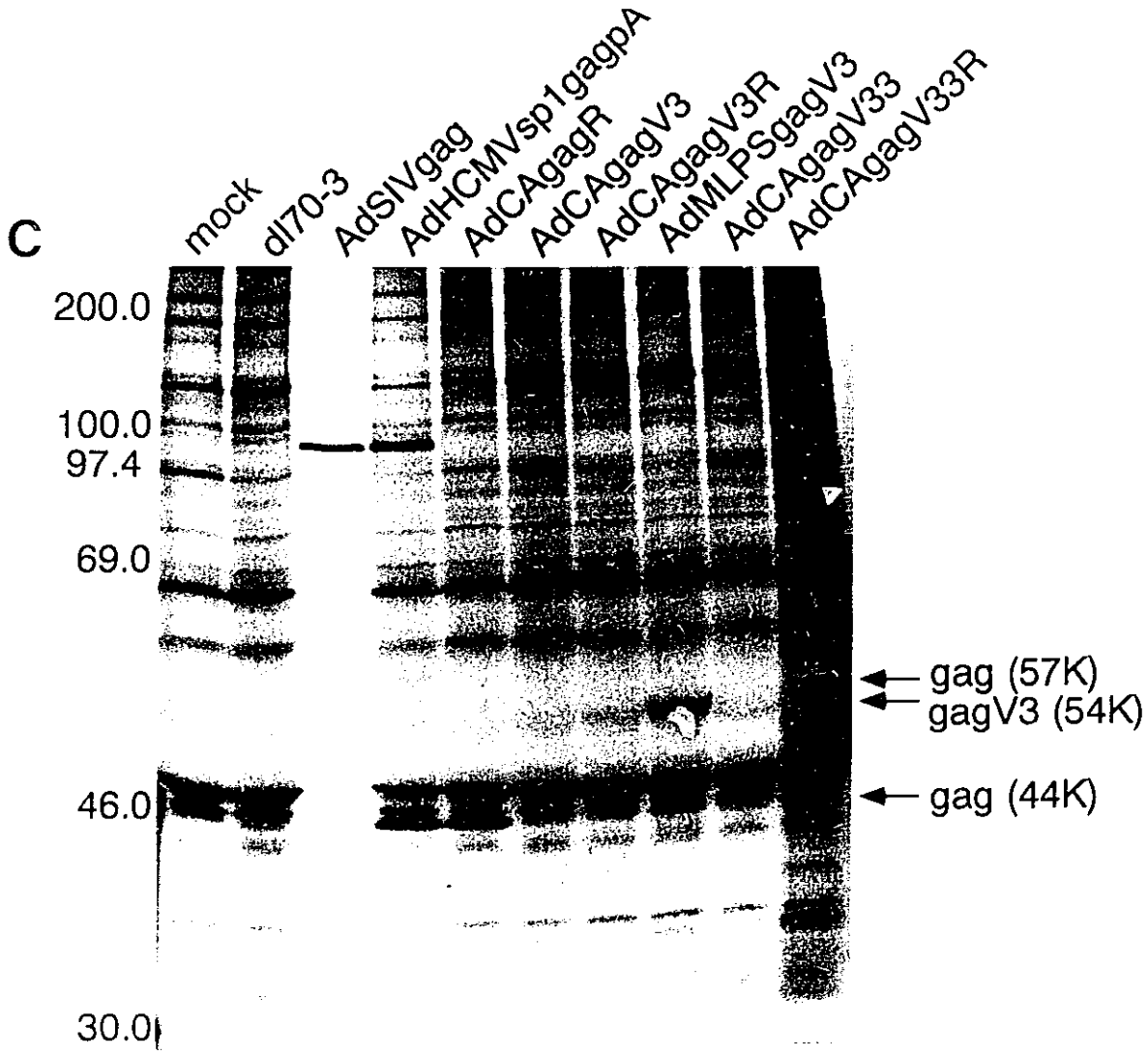
LLCMK2 and BSC-1 cells were either mock infected or infected at an moi of 100 with dl70-3, AdSIVgag, AdHCMVsp1gagpA, AdCAgagR, AdCAV3, AdCAV3R, AdMLPSgagV3, AdCAV33 and AdCAV33R. Cells were then labelled from 68-70 h post infection (293 cells were labelled 22-24 h post infection) using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using SIV reference sera and separated by SDS-PAGE on 10% gels (Fig. 32). In 293 cells expression of inserts driven by the HCMV promoter was higher in the E1 parallel orientation (Fig. 32A, compare lanes 4 and 5; 6 and 7; 9 and 10) but the highest level of expression of the *gag/V3* insert was obtained with the vector utilizing the MLP (Fig. 32A, compare lane 7 and 8). Expression from the replication defective vectors using the MLP or HCMV promoter (E1 parallel) was as good as or better than that obtained with the replication competent vector AdSIVgag if inserts of approximately the same size were compared (Fig. 32A, compare lane 3 with lanes 7 and 8). It should be pointed out that in 293 cells infected with AdCAgagV3R, AdMLPSgagV3 and AdCAgagV33R (inserts in the E1 parallel orientation) an additional product of approximately 52K was detected (indicated with an open arrow head in Fig. 32A) which was presumably produced due to aberrant processing of the insert mRNA and may involve splicing to down stream Ad sequences. This product was not seen for inserts in the E1 antiparallel orientation in longer exposures of the autoradiogram (not shown). As mentioned in the introduction inappropriate splicing of inserts in the E1 parallel orientation has been observed by others (Berkner and Sharp, 1984; Davidson and Hassel, 1987). In this experiment, as in the IP shown in figure 27, it was apparent that as the number of tandem repeats of the V3 segment increased the expression level of the corresponding chimeric protein decreased. In MRC5 (Fig. 32B), BSC-1 (Fig. 32C) and LLCMK2 (Fig. 32D) cells expression was only detected consistently following infection with AdHCMVsp1gagpA and AdCAgagR indicating that in these noncomplementing lines expression

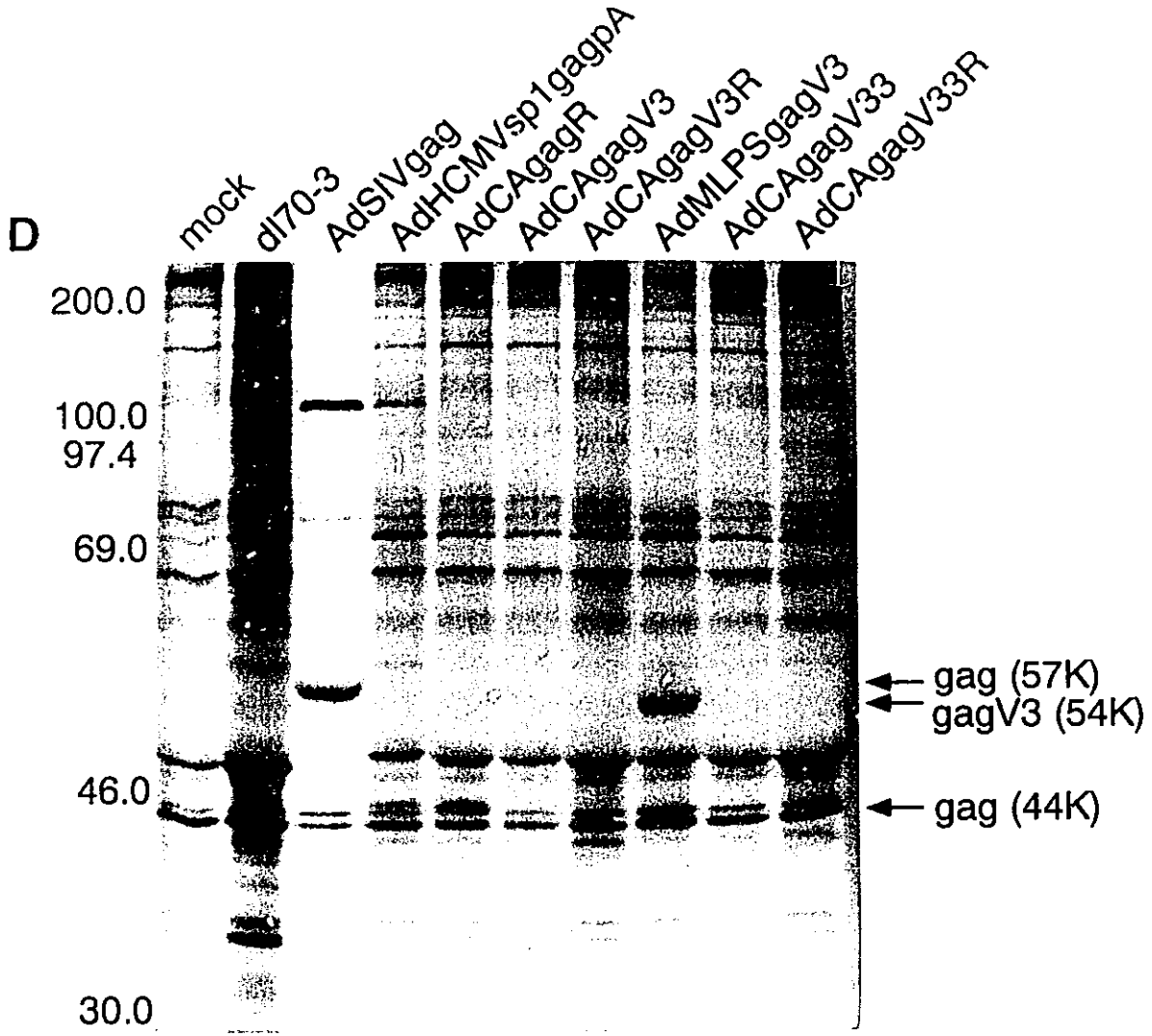
Figure 32. Comparison of expression levels obtained with the Ad/SIV chimeric vectors in various cell lines.

293 (A), MRC5 (B), LLCMK2 (C) and BSC-1 (D) cells were either mock infected or infected at an moi of 100 with dl70-3, AdSIVgag, AdHCMVsp1gagpA, AdCAgagR, AdCAV3, AdCAV3R, AdMLPSV3, AdCAV33 and AdCAV33R. Cells were then labelled from 68-70 h post infection (293 cells were labelled 22-24 h post infection) using Trans[^{35}S]-methionine, cell extracts prepared, precipitated using SIV reference sera and separated by SDS-PAGE on a 10% gel. Gels were then dried and bands visualized by autoradiography. The lanes contain the samples indicated above and molecular weight markers are indicated on the left. The chimeric proteins that were detected are indicated with arrows to the right. In (A) the additional product of approximatly 52K produced in cells infected with AdHCMVgagV3R, AdMLPSgagV3 and AdHCMVsp1gagV33R is indicated with an open arrowhead.


A
mock
dl70-3
AdSIVgag
AdHCMVsp1gagpA
AdCAgagR
AdCAgagV3
AdCAgagV3R
AdMLPSgagV3
AdCAgagV33
AdCAgagV33R
200.0
100.0
97.4
69.0
46.0
30.0
gagV33 (75K)
gag (57K)
gagV3 (54K)
gag (44K)


B
mock
dl70-3
AdSIVgag
AdHCMVsp1gagpA
AdCAgagR
AdCAgagV3
AdCAgagV3R
AdMLPSgagV3
AdCAgagV33
AdCAgagV33R
200.0
100.0
97.4
69.0
46.0
30.0
gag (57K)
gagV3 (54K)
gag (44K)


C
mock
dl70-3
AdSIVgag
AdHCMVsp1gagpA
AdCAgagR
AdCAgagV3
AdCAgagV3R
AdMLPSgagV3
AdCAgagV33
AdCAgagV33R
200.0
100.0
97.4
69.0
46.0
30.0
gag (57K)
gagV3 (54K)
gag (44K)


D
mock
dl70-3
AdSIVgag
AdHCMVsp1gagpA
AdCAgagR
AdCAgagV3
AdCAgagV3R
AdMLPSgagV3
AdCAgagV33
AdCAgagV33R
200.0
100.0
97.4
69.0
46.0
30.0
gag (57K)
gagV3 (54K)
gag (44K)

was still relatively low. The levels of expression observed for AdHCMVsp1gagpA and AdCAgagR in the three cell lines were approximately equal in contrast to the results shown in Figure 31 in which expression using AdCAgagR (insert in the E1 parallel orientation) was higher. This may be due to the fact that the stock of AdHCMVsp1gagpA used in this experiment contained replication competent virus as indicated by the presence of a band corresponding to hexon in cells infected with AdHCMVsp1gagpA (Fig. 32B, C, D lane 4). The hexon band was not detected for the other replication defective vectors analyzed but was seen for the replication competent vector AdSIVgag (Hexon is a late protein which should not be detected in cells that cannot support the replication of the defective vectors). In MRC5, LLCMK2 and BSC-1 cells expression seemed to be higher with the MLP (Fig. 32B, C, D, compare lanes 7 and 8) as compared to the HCMV promoter. The level of expression obtained using the MLP in the defective vector AdMLPSgagV3 appeared to be equal to or higher then that obtained with the replication competent vector AdSIVgag in the BSC-1 and LLCMK2 cells. Over all the results indicate that both strategies used to improve the expression of the chimeric *gag/V3* inserts were successful. In vectors utilizing the HCMV promoter the production of the chimeric proteins were improved by reversing the orientation of the expression cassette to E1 parallel. Also the MLP proved to be as good or better than the HCMV promoter in the E1 parallel orientation.

6. Immunization Of Mice With Second Generation Chimeric SIV gag/V3 Vectors

With the demonstration *in vitro* that the expression levels of the chimeric SIV *gag/V3* vectors had been improved it was decided to test the immunogenicity of two of the new vectors in mice. Female BALB/c mice were immunized either intraperitoneally or intranasally with $5X10^8$ pfu per animal of cesium chloride purified AdCAgagR, AdCAgagV3R or AdHCMVsp1LacZ

(Morsey *et al.*, 1993) (used as a negative control). The vector utilizing the MLP was not tested in this experiment since it had not been demonstrated to provide higher expression levels then the HCMV promoter at the time the immunizations were initiated. Five animals were immunized with each vector and two weeks later animals were bled, reimmunized (boosted) and bled again two weeks later. Serum samples were pooled within each immunization group and samples collected at week 2 and week 4 were tested for the presence of anti-SIV antibodies by ELISA and Western blot. With neither of these techniques could anti-SIV antibodies be detected (data not shown) whereas controls using SIV reference sera were positive. ELISA's performed to detect anti-adenovirus antibodies were positive using serum collected at week 2 and the antibody ELISA titer increased after the boost (week 4). The failure to detect an antibody responses in mice to the chimeric vectors is discussed in the subsequent section.

DISCUSSION

The aims of the studies carried out throughout this thesis work was to generate and characterize Ad recombinants expressing various segments of the SIV genome. Prior to and concomitant with the development of Ad vectors expressing various SIV genes a number of parameters important in vector construction and propagation were studied and improved systems for vector construction were developed. These studies not only provided important insights on, and aided the development of the Ad/SIV vectors generated but also provided important information for the development of Ad vectors for all purposes. A complete discussion of the studies on vector stability, the BHG vector system (an improved system for vector construction), and expression of reporter genes in replication competent vectors are provided as part of the published reprints and in the summaries following the reprints presented in the results section and will not be provided here. A discussion of the construction and characterization of the Ad/SIV vectors generated as part of this thesis work is provided below.

As mentioned in the introduction a clear understanding of the immune mechanisms that will provide protection against HIV infection and/or disease have not been determined. It is currently believed that an effective vaccine against the AIDS viruses will induce both a strong humoral and cellular immune response. The first series of Ad/SIV recombinants generated were replication competent vectors expressing various segment of the *gag-pol* region of SIV. The *gag* and *pol* genes contain potentially important CTL, ADCC and T helper epitopes (Nixon *et al.*, 1992; Norley

et al., 1993). The three vectors constructed, AdSIVgag, AdSIVgp and AdSIVgagpolII, contained increasingly larger segments of the *gag-pol* region combined with the newly generated 2.7 kb E3 deletion (Bett *et al.*, 1993) (Fig. 19). The utilization of the larger E3 deletion allowed the coding sequences for the entire *gag-pol* region to be introduced. The expression of the SIV proteins in these constructs relies on transcription from the E3 and/or MLP since a heterologous promoter was not included in these constructs. AdSIVgag, AdSIVgp and AdSIVgagpolII were each found to express the 57K *gag* precursor in infected 293 cells and AdSIVgp and AdSIVgagpolII, which contained larger *gag-pol* inserts, were found to express a *gag-pol* polyprotein (results section E.1 Figs 20-22). Also the *gag* polyprotein produced by these vectors was shown to be properly myristylated at its amino terminus (Fig. 22). Work was discontinued with the replication competent Ad/SIV vectors due to concerns about possible sero conversion to the proteins expressed by the vectors and since it was decided that replication defective vectors may be more suitable for vaccine purposes. In Mittal *et al.*, (1995) it was demonstrated that the expression of some inserts in the E3 region in replication competent vectors can be dependent on viral DNA replication. The use of a vector with an insert whose expression is dependent on DNA replication may be ill advised in species in which the virus does not replicate well. Since ultimately we planned to test the immunogenisity of the Ad/SIV vectors in mice and macaque monkeys, species whose cells are semipermissive and nonpermissive for viral replication respectively *in vitro*, we were concerned that adequate expression may not have been obtained. Additionally expression studies utilizing replication defective vectors containing HCMV/β-galactosidase reporter constructs had demonstrated the potential for high level expression in a number of cell lines (results section D). For these reasons it was decided that replication incompetent vectors should be generated in which the expression of the SIV inserts were driven by strong heterologous promoters (discussed

below). Replication competent Ad/SIV vectors similar to those described above have been reported in the literature which contain and express either the 57K gag precursor or the p27 core protein in a 1.88 kb E3 deletion (Caravokyri *et al.* 1993). To improve the replication of these vectors in simian cells the inserts were rescued into a virus background containing a point mutation in the E2A 72K DNA-binding protein which confers an increased growth potential in simian cells (Klessig and Grodzicker, 1979). Infection of Hela cells with the vector expressing the 57K precursor was shown to result in the generation of virus like particles in the culture supernatant (Caravokyri *et al.*, 1993). Previous studies had shown that the HIV-1 *gag* and *gag-pol* polyproteins can self-assemble and exit the cell in the absence of either retroviral genomic RNA or envelope proteins when expressed using the baculovirus system (Gheysen *et al.*, 1989), the vaccinia virus system (Karacostas *et al.*, 1989; Haffar *et al.*, 1990; Shioda and Shibuta, 1990); the SV40 system (Smith *et al.*, 1990) and the Ad vector system (Vernon *et al.*, 1991). Caravokyri *et al.*, (1993) reported that SIV 57K gag precursor was detected in the culture supernatant at approximately 50% of the level seen in the intracellular fraction from the same cultures. Due to the similarity between the replication competent Ad/SIV vectors constructed as part of this thesis work with those reported by Caravokyri *et al.* 1993, it is expected that infected cells would also produce SIV like particles and release them into the culture medium although this was never tested directly. Various groups are investigating the use of these virus like particles as potential vaccines.

Since it was decided that replication defective vectors might be more suitable in general for vaccine purposes I began the construction and characterization of replication defective Ad vectors expressing SIV *env* and chimeric *gag/V3* proteins. The first series of replication defective vectors constructed contained the coding sequences for the SIV *env* gene. As mentioned in the

introduction the envelope glycoprotein has been included in many vaccine approaches due to its potential to raise neutralizing Ab but this protein also contains potential epitopes important for an efficient cellular immune response (Nixon *et al.*, 1992; Norley *et al.*, 1993). Indeed it has been shown that immune responses to the surface glycoproteins of HIV and SIV can protect chimpanzees and macaques respectively against subsequent live virus challenge (Berman *et al.*, 1990; Hu *et al.*, 1992). The first replication defective Ad/SIV *env* vectors constructed (AdβActSIVenv, AdHCMVSIVenv, AdβActsp1SIVenv and AdHCMVsp1SIVenv) contained a 2805 bp fragment representing the coding sequences for SIV *env* and *rev* driven by the HCMV or β-Actin promoters inserted E1 antiparallel in place of a 3.2 kb E1 deletion (Fig. 23). As mentioned previously the coding sequences for *rev* were included in these constructs since Cheng *et al.* (1990) had shown that coexpression of *rev* with *env* improves the expression of *env* in the SV40 system. Attempts to detect expression from the Ad/SIV *env* vectors by immunoprecipitation and Western blot failed. To determine if the *rev* coding sequences located upstream of the *env* ATG could be interfering with *env* expression an additional Ad/SIV *env* vector was constructed (AdBHGSE) with the *rev* coding sequences 5' of the *env* initiation site removed. Attempts to detect expression of *env* with AdBHGSE also failed even when *rev* was supplied by coinfection with a second Ad vector expressing HIV-1 *rev*. The inability to detect expression of *env* products in these vectors may be attributed to the lack of a good polyadenylation signal following the *env* inserts. Attempts to introduce the SV40 pA at the 3' end of the *env* inserts were unsuccessful and the corresponding vectors were not generated. Replication competent Ad/SIV vectors have been reported in the literature that contain the coding sequences for either *env* or *env* and *rev* in the E3 region (Cheng *et al.*, 1992). The vector containing coding sequences for both *env* and *rev* showed

enhanced expression of the envelope glycoprotein (Cheng *et al.*, 1992). Replication competent vectors which express HIV-1 *env* have also been constructed (Dewar *et al.*, 1989; Chanda *et al.*, 1990) in which the level of expression of *env* is enhanced in the presence of the rev protein (Chanda *et al.*, 1990).

Due to the lack of expression detected with the SIV *env* vectors a new strategy was adopted which involved the construction of replication defective vectors expressing chimeric SIV proteins consisting of the first portion of the *gag* polyprotein fused to one two or three tandem repeats of the third variable domain of *env* (V3 loop). This approach was adopted in an attempt to combine potentially important epitopes for cellular immunity and virus neutralization. As mentioned earlier work that was proceeding in the lab at the time indicated that Ad vectors expressing from one to four tandem repeats of a linear neutralizing epitope from HSV-1 gD fused to β-galactosidase generated neutralizing Ab in immunized mice and that the mean Ab titer increased with the number of epitope repeats expressed (Zheng *et al.*, 1993). Vectors expressing four repeats of the epitope were as effective in protecting mice from a lethal dose of HSV-2 as vectors expressing the entire gD protein (Zheng *et al.*, 1993). Also HIV-1 and HIV-2 chimeric *gag-env* proteins were being constructed and expressed in the baculovirus system (Luo *et al.*, 1992). The first set of chimeric constructs generated (Table 3) contained HCMV promoter driven inserts in the E1 region in the E1 antiparallel orientation. These vectors were shown to produce chimeric proteins of the predicted molecular weight in infected 293 cells (Fig. 27) in which the vectors can replicate but very little expression was detected in non-complementing lines such as MRC5, BSC-1 and LLCMK2 by Western blot. These results paralleled those seen in the β-galactosidase expression studies (Fig. 16 and 17) in which expression of β-galactosidase was highest in 293 cells and lower in the noncomplementing cells. Although it appeared that we were getting much less expression

of the chimeric proteins than of β-galactosidease. Also it was observed that as the number of tandem repeats of the V3 segment increased the expression of the corresponding chimeric protein decreased. Reasons for this are discussed below.

Even though expression of the chimeric proteins appeared to be low it was decided to test the immunogenicity of several of the chimeric constructs in mice. Female Balb C mice were immunized either intraperitonally and intranasally with $1X10^8$ pfu of cesium chloride gradient purified virus (either AdHCMVsp1gagpA, AdHCMVsp1gagV3pA, AdHCMVsp1gagV32pA, AdCAV33) and then boosted two weeks later. Serum collected one week post boost was assayed for the presence of antibodies to the chimeric proteins by Western blot but no antibody response could not be detected (Fig. 29). The inability to detect an antibody response in the immunized mice was somewhat surprising since antibodies have been raised in mice immunized with both replication competent and replication defective Ad vectors expressing a variety of proteins including HIV-1 gag (Prevec *et al.*, 1991), HSV-1 gB (McDermott *et al.*, 1989), VSV glycoprotein (Prevec *et al.*, 1989), rabies glycoprotein (Prevec *et al.*, 1990; Yarosh, 1994), the tick-borne encephalitis virus nonstructural protein (Jacobs *et al.*, 1992) and the measles virus nucleocapsid protein (Fooks *et al.*, 1995). It was felt that the inability to detect an antibody response in mice immunized with the chimeric Ad/SIV constructs was likely due to the low level of expression obtained with these vectors. It should be pointed out that in the HCMV/β-galactosidase expression studies described in results section D it was found that HCMV driven expression of β-galactosidase was lowest in mouse cells.

In an attempt to improve the expression of the chimeric constructs two strategies were adopted. The first strategy involved reversing the orientation of the expression cassettes in the E1 deletion so that they were in the E1 parallel orientation. As mention earlier C. Addison had

shown that a 5-7 fold increase in expression could be obtained from HCMV/β-galactosidase and HCMV/luciferase expression cassettes when they are positioned in the E1 parallel orientation as opposed to antiparallel. The second strategy to improve expression involved generating vectors in which the MLP directed the expression of the chimeric proteins. As mentioned in the introduction the Ad2 MLP has been used to drive expression of a number of heterologous proteins in Ad vectors. Table 4 lists the shuttle vectors which were generated and which constructs were rescued into virus. Both strategies were found to improve the expression of the chimeric proteins *in vitro* (Fig. 31, 32). Reversing the orientation of the expression cassettes so that they were E1 parallel (AdHCMVsp1gagpA (E1 antiparallel) compared to AdCAgagR (E1 parallel) in Figure 31) improved the expression in MRC5, LLCMK2 and BSC-1 cells although the overall increase in expression depended on the cell line tested. In MRC5 cells there was not a significant increase in the level of expression, but in monkey cell lines (LLCMK2 and BSC-1) there appeared to be an approximately 5 to 10 fold increase in the level of expression when the insert was E1 parallel. Expression was just detectable in the mouse cell line, NIH3T3, for vectors containing inserts in both orientations with no apparent difference between the two (Fig. 31). Expression using the MLP was higher than that obtained with the HCMV promoter in all the cell lines tested when vectors containing the same insert are compared (Fig. 32). It should be noted that in 293 cells infected with AdCAgagV3R, AdMLPSgagV3 and AdCAgagV33R, which contain their inserts in the E1 parallel orientation, an additional product of approximately 52K was detected (Fig. 32A). This product was not detected from similar vectors containing inserts in the E1 antiparallel orientation and is presumably the result of aberrant processing of the primary transcript. Aberrant expression of inserts in the E1 parallel orientation has been noted by others (Berkner and Sharp, 1984; Davidson and Hassel, 1987).

It was observed for the vectors containing inserts in the E1 parallel orientation as with the original vectors with E1 antiparallel inserts that as the number of tandem repeats of the V3 segment increased the expression of the corresponding chimeric protein decreased. One possible explanation for this is that the V3 segment represents a strong cis-acting repressive sequence (CRS). As explained in the introduction in the absence of the rev protein the genomic and singly spliced forms of mRNA encoding the *gag*, *pol* and *env* proteins are retained in the nucleus while the multiply spliced forms encoding regulatory proteins are transported to the cytoplasm (Emerman *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Hammarskjold *et al.*, 1989). The nuclear retention of the unspliced mRNA's it thought to be due to the presence of cis-acting repressive sequences located throughout the coding sequences for the structural genes (Rosen *et al.*, 1988; Dayton *et al.*, 1988; Maldarelli *et al.*, 1991; Cochrane *et al.*, 1991; Schwartz *et al.*, 1992). One such CRS element was mapped to a 270-nt segment of *pol* which was shown to repress gene expression when present in the 3' untranslated portion of an mRNA transcript (Cochrane *et al.*, 1991). Rev relieves the block to expression by allowing the transport of the nuclear entrapped RNA to the cytoplasm through its interaction with the cis-acting RRE element located in the env coding sequences (Malim *et al.*, 1990; Olsen *et al.*, 1990). If the V3 segment used in our vectors represents a CRS, then introducing additional copies of this element may be expected to adversely effect the expression of the chimeric proteins. Due to the fact that our chimeric constructs do not contain the RRE we could not determine if the addition of the Rev would improve the expression of the chimeric proteins. The presence of CRS elements throughout the SIV sequences present in our chimeric vectors may help explain the relatively low levels of expression obtained with the vectors. An additional explanation for the decrease in expression with the increase in the number of tandem V3 repeats is that the V3 segment somehow causes

protein instability.

With the demonstration that the level of expression of the chimeric proteins had been improved, mice were immunized with two of the vectors, AdCAgagR and AdCAgagV3R. AdMLPSgagV3 was not used in this experiment even though the MLP was shown to provide higher levels of expression in a number of cell lines (Fig. 32) since at the time the immunizations were performed expression using the MLP had not yet been directly compared to that of the HCMV promoter using the same insert. For the second series of immunizations $5X10^8$ pfu per animal of cesium chloride gradient purified virus was administered either IP or IN and then boosted two week later. Serum collected two weeks post boost was assayed for the presence of antibodies to the chimeric proteins by Western blot and ELISA but again no antibody response could not be detected. We suspect that the inability to detect an antibody response to the chimeric proteins is due to the low level of expression of the chimeric proteins in mouse cells. Although the chimeric vectors failed to raise an immune response in mice this does not suggest that they will be inefficient when tested in the macaque model. Results obtained using the HCMV/β-galactosidase reporter vectors and studies on expression of the chimeric proteins revealed that expression levels are significantly higher in monkey cells (BSC-1 and LLCMK2) as compared to mouse cells. Plans are currently under way to test several of the chimeric vectors in macaque monkeys.

APPENDIXES

A. DNA Sequence Of The Deletion/Insertion In Early Region 3 Of Ad5 *dl*309 (Bett *et al.*, 1995, Virus Research, 39:75-82).

1. Background

*dl*309 is an Adenovirus type 5 (Ad5) mutant with an altered *Xba*I cleavage pattern (Jones and Shenk, 1978, 1979) that has been extensively utilized for construction of Ad5 mutants in early region 1 (E1), in developing vectors for use as viral vaccines, and in development of gene transfer vectors for gene therapy. It contains only one *Xba*I site at 3.7 mu as compared to wt Ad5 which contains four (3.7, 29.5, 79.5, and 84.8 mu), making it useful in a number of strategies for rescuing mutations and inserts in E1. The increased use of Ad vectors for vaccine and gene therapy purposes requires a complete understanding of the sequences contained in potential vectors. In this paper the sequence of the mutations in the E3 region which remove the *Xba*I sites at 79.5 and 84.8 mu are reported as well as an analysis to determine which E3 products are expressed by *dl*309.

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Virus Research

DNA sequence of the deletion/insertion in early region 3 of Ad5 *dl*309 *

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Abstract

*dl*309 is an adenovirus type 5 (Ad5) mutant that has been extensively utilized for construction of Ad5 mutants in early region 1 (E1), in developing vectors for use as viral vaccines, and in development of gene transfer vectors for gene therapy. Ad5 *dl*309 has been useful for vector construction because of its altered *Xba*I restriction pattern and lends itself to a variety of strategies for rescuing inserts or mutations into E1. It contains only one *Xba*I site at 3.7 map units (m.u.) as compared to wt Ad5 which contains 4 (3.7, 29.5, 79.5, and 84.8 m.u.). The loss of the sites at 29.5 and 79.5 m.u. is due to deletions of a few bp but the loss of the site at 84.8 m.u. was the result of a deletion from approximately 83 to 85 m.u. and substitution with a fragment of foreign DNA. Because of the widespread use of *dl*309 and derivatives of this mutant in the construction of Ad5-based vectors and the need to have precise genetic information on the sequences present in vectors to be used as vaccines and in gene therapy, we have sequenced the alterations in *dl*309 which affect the *Xba*I sites at 79.5 and 84.8 m.u. and have determined which E3 proteins are expressed by this virus. The deletion that removes the *Xba*I site at 84.8 m.u. extends from Ad5 bp 30005–30750 and is substituted with 642-bp of heterologous DNA that shows homology to salmon DNA. This alteration deletes all or part of the coding sequences for the E3 14.7K, 14.5K and 10.4K proteins and these proteins were not detected in *dl*309 infected cells. The loss of the *Xba*I site at 79.5 m.u. is the result of a 6-bp deletion which removes two internal amino acids (18 and 19) from the E3 6.7K protein. The E3 6.7K protein and other E3 proteins whose coding

* The accession number for the insertion/deletion in *dl*309 is U22898.

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sequences are unaffected by the alterations in *dl*309 (gp19K, 12.5K and 11.6K) were expressed in *dl*309 infected cells.

Keywords: Adenovirus; Viral vector; Early region 3; DNA sequence; Prolactin

Adenoviruses (Ads) have been used extensively to express heterologous proteins in mammalian cells (Graham and Prevec, 1991, 1992; Berkner, 1992) and are currently attracting considerable attention because of their potential use as live viral vectored vaccines (Berkner, 1992; Graham and Prevec, 1992) and as gene transfer vectors in gene therapy (Siegfreid, 1993; Trapnell, 1993). A number of strategies have been used for the construction of Ad vectors for these purposes, all of which involve the manipulation of subgenomic fragments of the Ad genome (Graham and Prevec, 1991; Berkner, 1992). Due to the relatively large size of the Ad5 genome and the lack of unique restriction sites that can be used in vector construction, *dl*309 (Jones and Shenk, 1979), an Ad5 mutant with an altered *Xba*I restriction pattern, has been extensively used in place of wt Ad5. This mutant contains only one (at 3.7 map units (m.u.)) of the 4 (3.7, 29.5, 79.5, and 84.8 m.u.) *Xba*I sites present in the wt genome.

*dl*309 was obtained by selection for variants in a wt Ad5 population that lacked restriction endonuclease sites (Jones and Shenk, 1978, 1979). Briefly Ad5 DNA was digested with the restriction enzyme of interest and the DNA was religated then transfected into 293 cells (Graham et al., 1977) to obtain viral plaques which were then screened for mutants lacking cleavage sites. This procedure enriched for variants in the viral population that had a reduced number of cleavage sites, since fewer ligation events were required to regenerate the genome (Jones and Shenk, 1978, 1979). Using this procedure *dl*309 was obtained in two steps. First variants were selected which lacked one of the *Eco*RI restriction sites, present at 76.1 and 83.6 m.u. in wt Ad5 DNA. This resulted in the isolation of *sub*304 (Jones and Shenk, 1978) which had lost the *Eco*RI site at 83.6 m.u. due to a deletion from approximately 83–85 m.u. and substitution with foreign DNA of unknown origin. Because the deletion in *sub*304 also removed the *Xba*I site at 84.8 m.u. this mutant was then used to select for viruses that lacked additional *Xba*I restriction sites (Jones and Shenk, 1979). Successive rounds of selection generated mutant 308 (missing the *Xba*I site at 79.6 m.u.) which was then used in a further round of selection to obtain mutant 309 which had lost the *Xba*I site at 29.5 m.u. Because of the position of the remaining *Xba*I site at 3.7 m.u., *dl*309 DNA has been used in direct ligation (Stow, 1981) and cotransfection strategies (Berkner and Sharp, 1983; Haj-Ahmad and Graham, 1986) for rescuing mutations or inserts into early region 1 and was the parent virus for the development of a number of systems for Ad5 vector construction based on bacterial plasmids (Graham, 1984; Ghosh-Choudhury et al., 1986; McGrory et al., 1988).

To sequence the alteration at 79.6 m.u. and the deletion/insertion between 83 and 85 m.u., pFG140 (Graham, 1984) (derived from *dl*309) was partially digested with *Xho*I and religated to generate pFGdX8. pFGdX8 contains *dl*309 sequences

from bp 4 (left genomic end) to bp 5788 (m.u. 16.1) and from bp 24796 (m.u. 69.0) to the right genomic end save for the last 10-bp. The left and right termini of the genome are covalently joined and an ampicillin resistant plasmid pMX2 inserted at the *Xba*I site at bp 1339 allows propagation of pFGdX8 in *Escherichia coli*. DNA sequencing reactions were based on the chain-termination method (Sanger et al., 1977) and manual sequencing following the DNA sequencing protocol described in the Sequenase™ kit produced by US Biochemical. [α-^{32}P]dATP was obtained from Amersham Canada Ltd. All oligonucleotide primers were synthesized by the central facility of the Molecular Biology and Biotechnology Institute (MOBIX) at McMaster University, Hamilton, Ontario, Canada.

The alteration affecting the *Xba*I site at 29.5 m.u. was previously determined to be the result of a 2-bp deletion (Ad5 bp 10594 and 10595) (Thimmappaya et al., 1979). This 2-bp deletion is located 22–23 bp before the initiation site for VAI(A) RNA and prevents its expression, but has no effect on the VAI(G) RNA which initiates 3-bp downstream of VAI(A) (Thimmappaya et al., 1979). Sequencing from the present study showed that the loss of the *Xba*I site at 79.6 m.u. was the result of a 6-bp deletion (Ad5 bp 28597–28602) which affects the coding sequences for

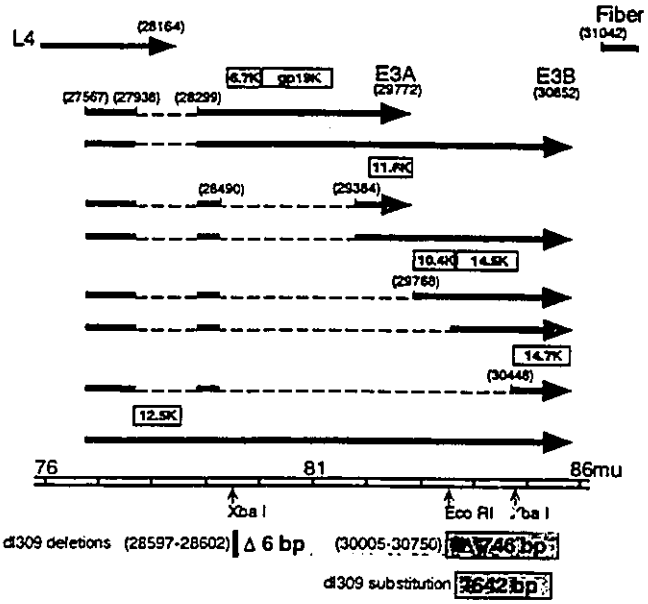

Fig. 1. The structure of the E3 region in wt Ad5 is shown with the locations of the alterations in *dl*309 indicated below. The horizontal arrows indicate the structures of the E3 mRNAs with solid lines representing exons, dashed lines representing introns and arrowheads representing polyadenylation sites. Shaded bars above the arrows represent protein coding sequences. The locations of transcription initiation sites, splice donor and acceptor sites and polyadenylation sites are given in bp. Ad5 sequence positions throughout this article refer to the sequence of wt Ad5 (Chroboczek et al., 1992). To designate Ad5 E3 proteins Ad2 molecular weights were used.

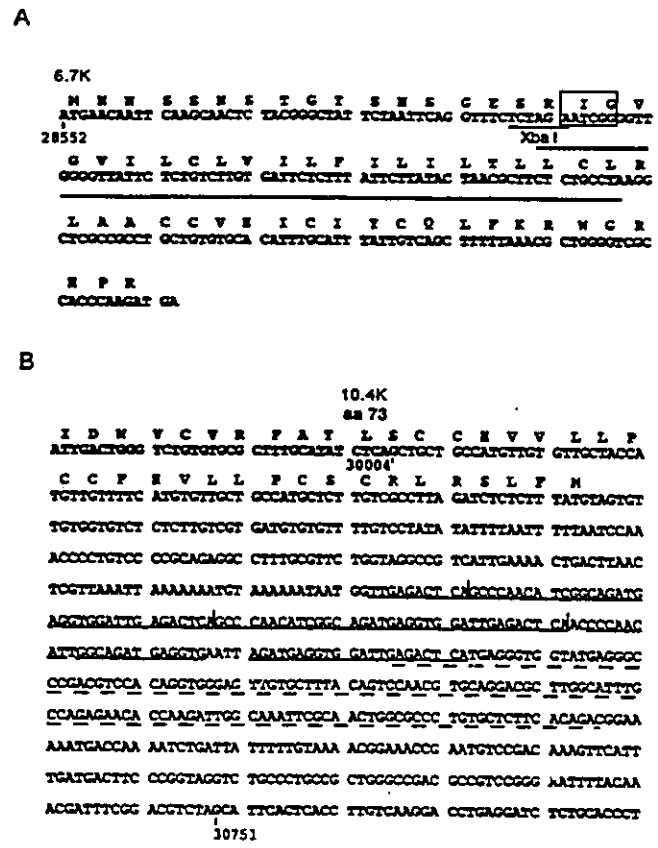

Fig. 2. DNA sequence alterations in the E3 region of *dl*309 are shown. A: the coding sequences for the E3 6.7K protein which span the *Xba*I site at 79.6 m.u. are shown. The loss of the *Xba*I site was due to a 6-bp deletion (Ad5 bp 28597–28602) which removes two amino acids (18 and 19) from the internal hydrophobic region (underlined in bold) which is thought to function in membrane insertion and retention (Wilson-Rawls and Wold, 1993). The 6 bp and two amino acids which are deleted in *dl*309 are boxed. B: the DNA sequence of the deletion/insertion in E3 of *dl*309 is shown. Ad5 sequences flanking the foreign DNA insert are indicated in bold with the last Ad5 nucleotide on each side of the deletion indicated below. The E3 10.4K protein (91 aa in length) is truncated at amino acid 73 but would be predicted to gain 27 amino acids from the insert sequences. Amino acids (single letter code) for the E3 10.4K protein 5' to the deletion are indicated in bold. The 140-bp segment homologous to the chinook salmon genomic clone of prolactin is indicated with a dashed line. Complete and partial repeat sequences of 35-bp are underlined, and vertical lines indicate the start and end of complete repeats.

the E3 6.7K protein (Fig. 1 and Fig. 2A). 6.7K is a type III integral membrane protein that is retained in the endoplasmic reticulum (Wilson-Rawls and Wold, 1993). The 6-bp deletion removes two amino acids (18 and 19) from the internal hydrophobic region of the protein which is thought to function in membrane insertion and retention (Wilson-Rawls and Wold, 1993). Although 6.7K was

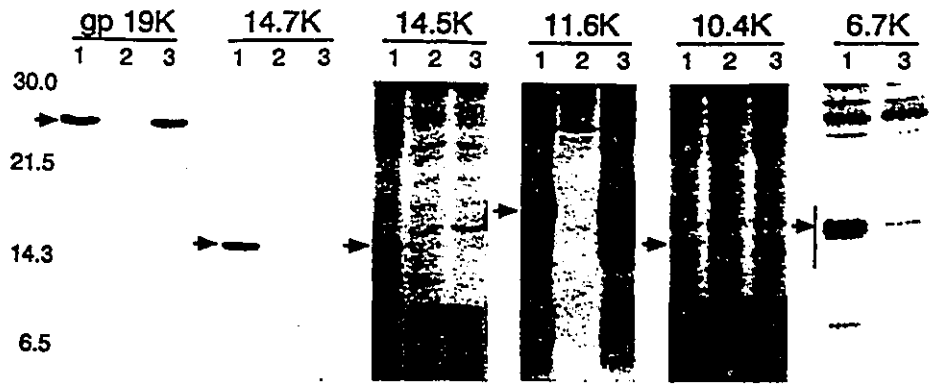

Fig. 3. Immunoprecipitation of E3 proteins from *dl*309-infected A549 cells. The presence of the E3 proteins gp19K, 14.7K, 14.5K, 11.6K, 10.4K and 6.7K was determined for *dl*309 by immunoprecipitation with appropriate antisera (gift of A. E. Tollefson and W. S. M. Wold). A549 cells were infected at an m.o.i. of 50 with wt Ad5, *dl*70-4 which contains a 2.7-kb E3 deletion which disrupts or deletes the coding sequences for all E3 proteins (Bett et al., 1993), or *dl*309 for 7 h and then labelled with 50 μCi each of [^{35}S]methionine and [^{35}S]cystine from 7 to 11 h or infected for 22 h and labelled from 22 to 26 h (to prepare cell extracts for immunoprecipitation of 11.6K only). Cell extracts were then prepared, immunoprecipitated with appropriate antiserum and samples separated by SDS–PAGE on an 18.0% gel after which the gel was dried and bands visualized by autoradiography. Immunoprecipitated extracts obtained from wt Ad5, *dl*70-4 and *dl*309-infected cells were run in lanes marked 1, 2, and 3, respectively, using antisera to the protein indicated above the lanes. Arrows indicate protein bands and molecular weight markers are shown on the left in thousands. The autoradiograms for gp19K and 14.7K represent 6-h exposures while those for 14.5K, 11.6K, 10.4K and 6.7K represent 120-h exposures.

expressed by *dl*309 (Fig. 3) only one of the three 15–16K glycosylated forms found in a wt infection (Wilson-Rawls and Wold, 1993) was detected in *dl*309 infected cell extracts.

The location and sequence of the deletion/insertion in *dl*309 are shown in Figs. 1 and 2B, respectively. The deletion was found to extend from Ad5 bp 30005 to 30750 inclusive, a deletion of 746-bp. Substituted for the deletion is an insertion of 642-bp of non-viral DNA. This alteration disrupts the coding sequences for the E3 10.4K, 14.5K and 14.7K proteins (Cladaras and Wold, 1985). The E3 14.7K protein (Tollefson and Wold, 1988) has been found to protect Ad infected cells from lysis by tumour necrosis factor (Gooding et al., 1988; Gooding et al., 1990; Horton et al., 1991). The 10.4K (Tollefson et al., 1990a) and 14.5K (Tollefson et al., 1990b) proteins form a complex (Tollefson et al., 1991) and have been found to protect cells from lysis by tumour necrosis factor (Gooding et al., 1991) as well as to down-regulate the expression of epidermal growth factor receptor (Carlin et al., 1989; Tollefson et al., 1991) in adenovirus infected cells. Previous studies have shown that the 14.7K protein is not detected in cells infected with *sub*304 (Tollefson and Wold, 1988), the mutant used to derive *dl*309 as described above, in agreement with our sequence (Fig. 1), and expression data (Fig. 3). The 14.5K protein is also not detected in *dl*309 infected cells (Fig. 3). From our sequence

data, the 10.4K protein, normally 91 amino acids long, is predicted to be a fusion protein with the last 18 amino acids removed and 27 amino acids added from the substituted sequences (Fig. 2B). 10.4K could not be detected in *dl*309 infected cells (Fig. 3) with the antisera used in this study. The serum used was raised against a peptide corresponding to aa 68–80 of Ad2 10.4K part of which is deleted in *dl*309. Other E3 proteins that are unaffected by the alterations in the *dl*309 genome, such as gp19K (Persson et al., 1980) and 11.6K (Wold et al., 1984), were detected in *dl*309 infected cells (Fig. 3). The 12.5K protein (Hawkins and Wold, 1992) is also expressed by *dl*309 (W.S.M. Wold, personal communication).

In the study in which *sub*304 was derived, 3 other mutants were obtained with non-viral DNA inserts. When insert sequences in two of these mutants, *sub*305 and *sub*307, were used in reassociation experiments with uninfected HeLa cell DNA, the results suggested that the heterologous DNA was of human origin (Jones and Shenk, 1978), although the percent reassociation was not as high as expected. When the 642-bp of foreign DNA substituted in the E3 region of *dl*309 were used in a homology search of gene bank sequences it was found that a 140-bp stretch was 90% homologous to sequences 3′ to the coding sequences for prolactin in a genomic clone of chinook salmon DNA (Xion et al., 1992) (Fig. 2). The sequence of the human prolactin cDNA (Cooke et al., 1981) is only 28.9% homologous to the sequence of the chinook prolactin clone and the sequences 3′ to the coding region would be expected to be even less well conserved. This suggests that the origin of the foreign DNA in *dl*309 may be that of salmon DNA. The heterologous sequences present in the various insertion mutants obtained in the original study may have originated from the salmon sperm DNA used as a carrier during the transfections performed to select for the mutants (Jones and Shenk, 1978). Previous studies that have analyzed transgenes in cells transformed with exogenous DNA have revealed that in many cases, the transgene DNA is found linked to the carrier DNA used in the transfection (Perucho et al., 1980; Weston et al., 1982). Presumably the exogenous DNA and carrier DNA introduced into the cell using the calcium phosphate technique (Graham and Van der Eb, 1973), are ligated within the cell. The non-viral DNA insert in *dl*309 also contained two direct repeats of 35-bp, flanked by partial repeats, that showed patchy homology (up to 75%) to immunoglobin genes from human and mouse. The presence of sequences in the insert that are partially homologous to human DNA could explain the low level of reassociation obtained using *sub*305 and *sub*307 with HeLa genomic DNA in the original study (Jones and Shenk, 1978), if the inserts in those mutants were also of salmon origin.

Acknowledgements

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Contributions To Bett *et al.* (1995)

(A) All manual sequencing of pFGdX8.

(B) Immunoprecipitations performed to determine which E3 proteins were expressed in *dl*309 infected A549 cells.

3. Summary

In Bett *et al.* (1995) the DNA sequence of the mutations in the E3 region of *dl*309 which remove the *Xba*I sites at 79.5 and 84.8 mu were reported along with data describing which E3 products are produced by the virus. The deletion that removes the *Xba*I site at 84.8 mu extends from Ad5 bp 30005 to 30750 and is substituted with 642-bp of heterologous DNA that shows homology to salmon DNA. This alteration deletes all or part of the coding sequences for the E3 14.7K, 14.5K and 10.4K proteins and these proteins were not detected in *dl*309 infected cells. The loss of the *Xba*I site at 79.5 mu is the result of a 6-bp deletion which removes two internal amino acids (18 and 19) from the E3 6.7K protein. The E3 6.7K protein and other E3 proteins whose coding sequences are unaffected by the alterations in *dl*309 (gp19K, 12.5K and 11.6K) were expressed in *dl*309 infected cells. Knowledge of the sequences present in *dl*309 and the E3 proteins expressed by this virus is important because of the large number of vectors made using *dl*309 or its derivative pJM17 (McGrory *et al.*, 1988). If vectors based on *dl*309 were to be used for vaccine and gene therapy purposes the sequences across E3 would be essential.

B. THE pBG VECTOR SYSTEM

As was mentioned earlier, most strategies for rescuing inserts into E1 and or E3 involve cotransfection of subgenomic fragments of the Ad genome into 293 cells and generation of infectious vectors relies on *in vivo* recombination between the fragments (Berkner, 1992; Graham and Prevec, 1992). The BHG vector system (Bett *et al.*, 1994) is one example of this approach using cotransfection of two plasmids which separately are noninfectious but together comprise the sequences necessary to generate an infectious vector. Although in vivo recombination between two plasmids has proven to be a very useful means of generating recombinants, the efficiency of this process can be low. In order to simplify and to improve the efficiency of vector construction we attempted to developed a new vector system (the pBG system) based on use of infectious adenovirus DNA sequences in the form of a single bacterial plasmid. The Ad5 sequences in the pBG plasmids contain deletions in both E1 (3180 bp) and E3 (3130 bp) with unique restriction sites in each of these regions into which foreign DNA inserts can be cloned. Theoretically the transfection of a pBG plasmid or a derivative containing an insert in E1, E3 or in both regions (total capacity of 6.2-6.4 kb) would result in the generation of an infectious recombinant virus. Although such plasmids were generated and shown to produce plaques in single transfections of 293 cells, the resulting viruses grew poorly and stocks could not be obtained with titers higher than $1x10^6$ pfu/ml. Subsequent analysis revealed that these vectors were producing an aberrant form of fiber which may explain their poor growth characteristics. The strategy followed to generate the pBG plasmids is described below in hopes that the problem may be corrected and that some of the plasmids generated may be useful for other purposes.

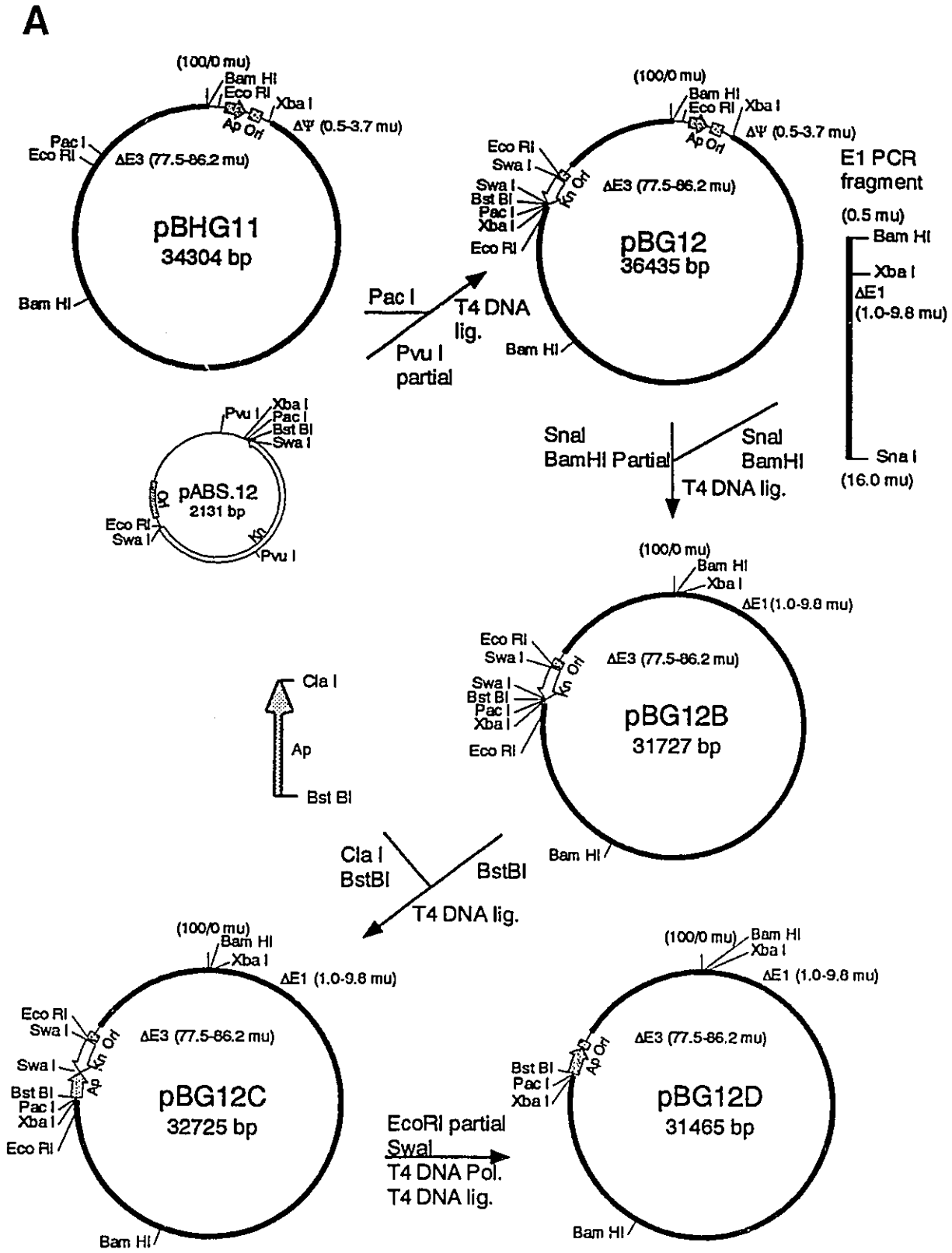
1. Construction Of pBG16, pBG17 And pBG18.

To construct plasmids which could be used for insertion of foreign DNA fragments and which could directly generate infectious viral vectors following single transfections of 293 cells, we took advantage of the previously described plasmid pBHG11 (Bett *et al.* 1994). pBHG11 contains Ad5 DNA sequences from bp 19 (left genomic end) to bp 188; bp 1339 to 27864 and 30996 to 35934 (right genomic end). The left and right termini of the Ad5 genome are covalently joined and a segment of plasmid pBR322 is present between Ad5 bp 188 and 1339 to allow propagation of pBHG11 in *E. coli.* Also a *Pac*I restriction enzyme site, unique in this plasmid, is present between Ad5 bp 27864 and bp 30996 to permit insertion of foreign genes into the E3 deletion. Because the packaging signals (Ad5 bp 194-358) are deleted, pBHG11 is noninfectious in single transfections and must be cotransfected with plasmids containing left end Ad5 sequences, including the packaging signal, in order to produce infectious viral vectors. Thus reconstitution of the packaging signal in pBHG11 DNA, and incorporation of appropriate restriction sites for cloning foreign DNA might result in a plasmid with increased utility for Ad vector isolation. The original strategy designed to create such plasmids is outlined in Figure 33. The strategy utilized alternating selection with ampicillin and kanamycin to facilitate each of the desired manipulations. In the first step the small Kn^r plasmid pABS.12 was partially digested with *Pvu*I and ligated with *Pac*I digested pBHG11, generating pBG12 (Fig. 33A). pABS.12, whose construction is illustrated in Figure 33B, was specially constructed so that it contained minimal sequences in order to maximize the capacity of the pBG plasmids. Thus only essential sequences such as an origin of replication, Kn^r gene and an *Xba*I, *Bst*BI and a new *Pac*I restriction site were retained in pABS.12. Next, the technique of polymerase chain reaction was used to generate a fragment of the Ad5 genome that contained the packaging signals and viral sequences to the right of the 3.2 kb E1

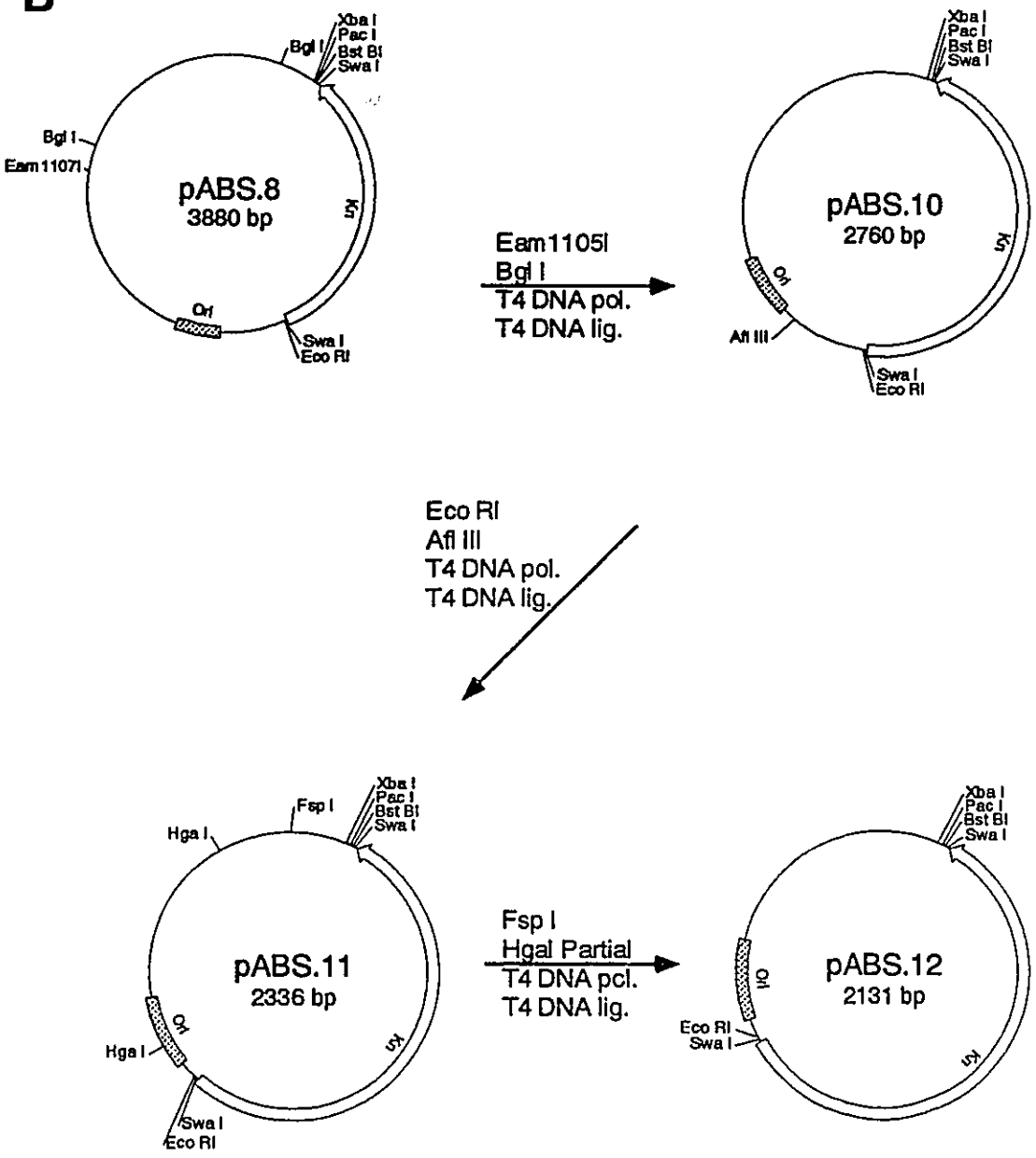
Figure 33. Construction of pBG plasmids (Strategy 1)

(A) To modify the E3 region in pBHG11 so that it would contain two additional restriction sites for cloning, (*Xba*I and *Bst*BI in addition to the *Pac*I site), and minimal plasmid sequences so the capacity of the pBG plasmids would be maximized, pBHG11 was digested with *Pac*I and combine with pABS.12 (Fig. 33B), partially digested with *Pvu*I, generating pBG12. Next PCR was used to generate a fragment of the Ad5 genome containing the packaging signals (194-358 bp) and a 3.2 Kb E1 deletion (Fig. 33C). The gel purified PCR fragment was digested with *Sna*I and *Bam*HI and ligated with a gel purified fragment of pBG12 generated by complete digestion with *Sna*I and partial digestion with *Bam*HI (to obtain cleavage at 0.5 mu) but the desired clone pBG12B could not be obtained. pBG12B was likely too large to replicate efficiently, without rearranging to reduce its size, when transformants were grown under Kn selection. The cloning strategy was modified as shown in Figure 34. Had pBG12A been obtained it would have been digested with *Bst*BI and ligated with a 1014 bp PCR generated fragment containing the coding sequences for the Ap^r gene (Fig. 34B) in order to generate pBG12C. The Kn^r segment would then have been removed from pBG12C by partial digestion with *Eco*RI, complete digestion with *Swa*I, treatment of the DNA with T4 DNA polymerase and religation, to obtain pBG12D. pBG12D would have contained all the Ad5 sequences required to generate infectious virus following transfection into 293 cells.

(B) To maximize the capacity of the pBG system we wanted only essential none Ad5 sequences (an origin of replication and resistance gene) in the pBG plasmids. For this reason a small Kn^r plasmid was constructed with all extraneous sequences removed, that would introduce an *Xba*I, *Bst*BI and *Pac*I site into the E3 deletion in pBHG11. We started with pABS.8 (A.B. and


A
(100/0 mu)
Bam HI
Eco RI
Xba I
Ap Ori
ΔΨ (0.5-3.7 mu)
Pac I
Eco RI
ΔE3 (77.5-86.2 mu)
pBHG11
34304 bp
Bam HI
Pac I
T4 DNA lig.
Pvu I partial
(100/0 mu)
Bam HI
Eco RI
Xba I
Ap Ori
ΔΨ (0.5-3.7 mu)
Eco RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Ks Ori
ΔE3 (77.5-86.2 mu)
pBG12
36435 bp
Bam HI
E1 PCR fragment
(0.5 mu)
Bam HI
Xba I
ΔE1
(1.0-9.8 mu)
Sna I
(16.0 mu)
Pvu I
Xba I
Pac I
Bst BI
Swa I
Ap
pABS.12
2131 bp
Eco RI
Swa I
Ks
Pvu I
SnaI
BamHI Partial
SnaI
BamHI
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1(1.0-9.8 mu)
Eco RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Ks Ori
ΔE3 (77.5-86.2 mu)
pBG12B
31727 bp
Bam HI
Cla I
Ap
Bst BI
Cla I
BstBI
BstBI
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Eco RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Ks Ori
Ap
ΔE3 (77.5-86.2 mu)
pBG12C
32725 bp
Bam HI
EcoRI partial
SwaI
T4 DNA Pol.
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Bst BI
Pac I
Xba I
Ap Ori
ΔE3 (77.5-86.2 mu)
pBG12D
31465 bp
Bam HI

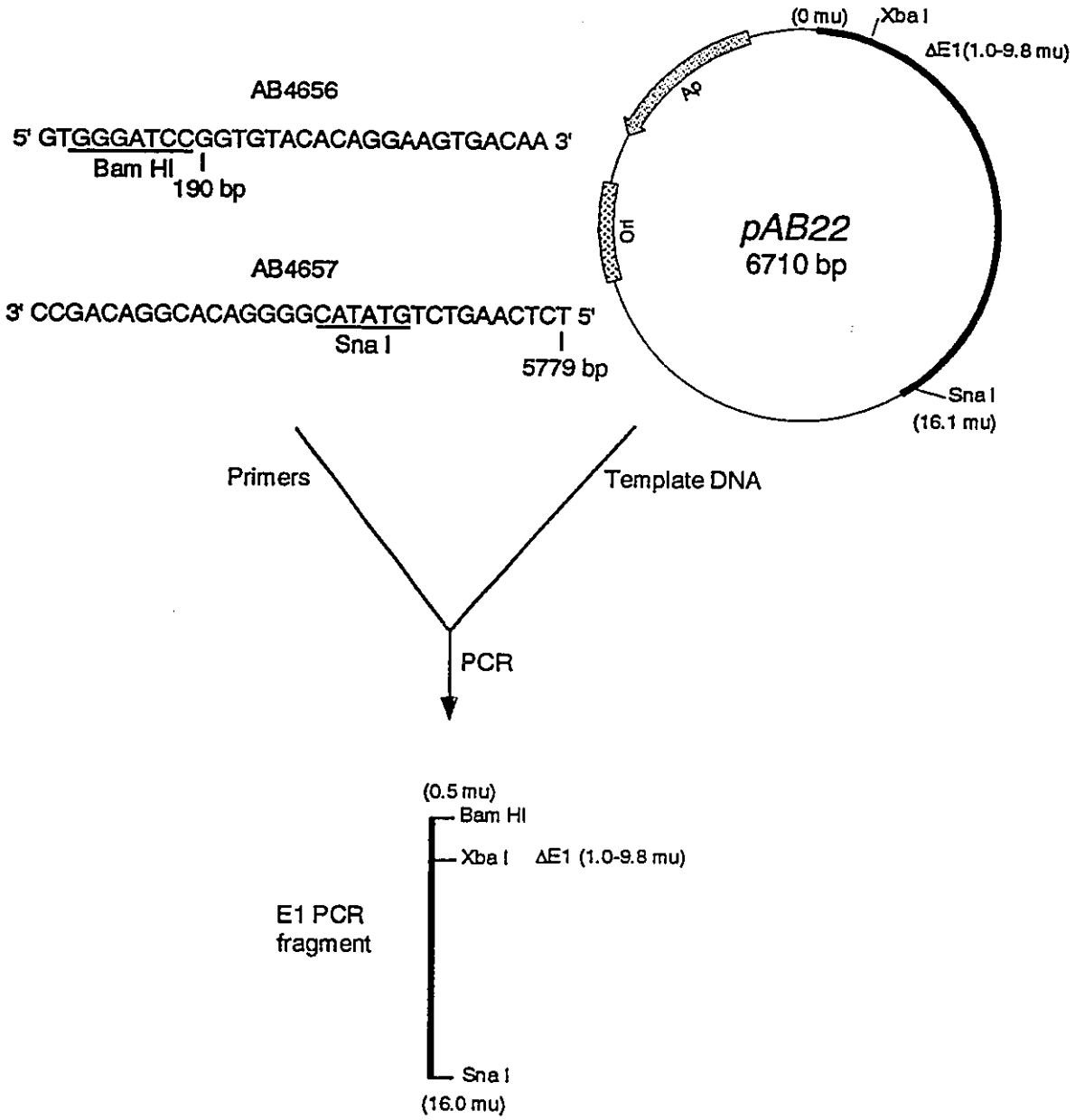
B


Xba I
Pac I
Bst BI
Swa I
Bgl I
Eam1107I
pABS.8
3880 bp
Ori
Swa I
Eco RI
Eam1105I
Bgl I
T4 DNA pol.
T4 DNA lig.
pABS.10
2760 bp
Ori
Afl III
Swa I
Eco RI
Eco RI
Afl III
T4 DNA pol.
T4 DNA lig.
Fsp I
Hga I
pABS.11
2336 bp
Ori
Hga I
Swa I
Eco RI
Fsp I
Hgal Partial
T4 DNA pcl.
T4 DNA lig.
pABS.12
2131 bp
Ori
Eco RI
Swa I

F.L.G. unpublished) which was digested with Eam1105I and *Bgl*I, the DNA treated with T4 DNA polymerase and ligated, generating pABS.10. pABS.10 was then digested with *Eco*RI and *Afl*II, the DNA treated with T4 DNA polymerase and ligated, generating pABS.11. Finally pABS.11 was digested with *Fsp*I, partially digested with *Hga*I, the DNA treated with T4 DNA polymerase and ligated, generating pABS.12.

(C) The technique of polymerase chain reaction was used to generate a fragment of the Ad5 genome that contained the packaging signals and the 3.2 Kb E1 deletion. The primers selected (AB4656 and AB4657) generated a fragment of 2415 bp corresponding to Ad5 sequences from 190 to 341 and from 3534 to 5779 bp, and an *Xba*I restriction site at the position of the deletion (ie. between nt 341 and 3534). The 5' PCR primer was designed to contain a *Bam*HI restriction site 5' to Ad5 bp 190. The template DNA for the PCR reaction was plasmid pAB22 (described in results section B.5) which contains Ad5 sequences from 0 to 16.0 mu and the E1 deletion described above. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r and origin segments, and clear bars represent Kn^r segments.

C

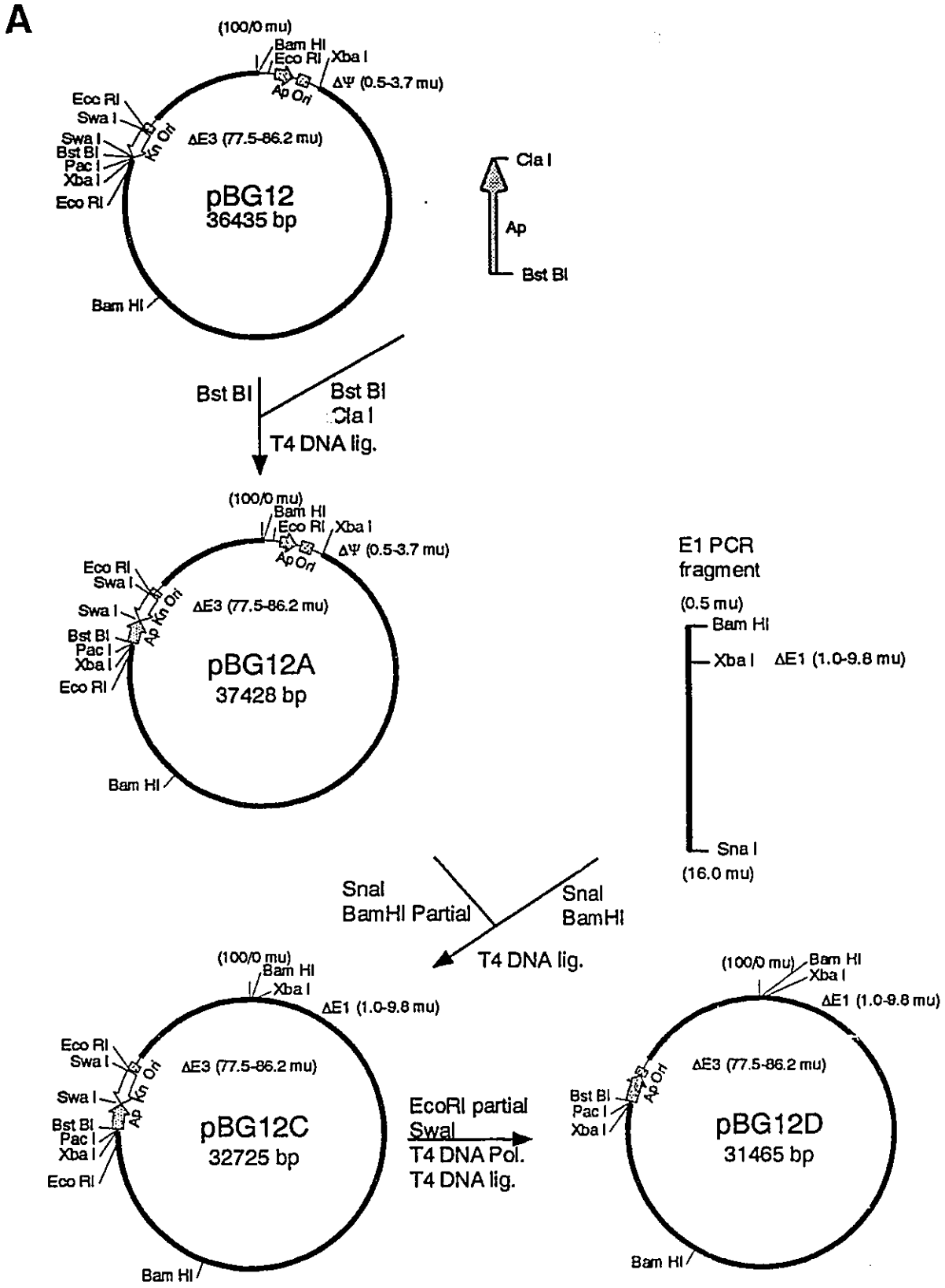

AB4656
5' GTGGGATCCGGTGTACACAGGAAGTGACAA 3'
Bam HI
190 bp
AB4657
3' CCGACAGGCACAGGGGCATATGTCTGAACTCT 5'
Sna I
5779 bp
(0 mu)
Xba I
ΔE1(1.0-9.8 mu)
Ap
ori
pAB22
6710 bp
Sna I
(16.1 mu)
Primers
Template DNA
PCR
(0.5 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
E1 PCR
fragment
Sna I
(16.0 mu)

deletion (Fig. 33C). The primers selected (AB4656 and AB4657) generated a fragment of 2415 bp corresponding to Ad5 sequences from 190 to 341 and from 3534 to 5779 bp, and an *Xba*I restriction site at the position of the deletion (ie. between nt 341 and 3534). The 5' PCR primer was designed to contain a *Bam*HI restriction site 5' to Ad5 bp 190. The template DNA for the PCR reaction was plasmid pAB22 which contains Ad5 sequences from 0 to 16.1 mu and the E1 deletion described above (A.B. and F.L.G. unpublished). The gel purified PCR fragment was digested with *Sna*I and *Bam*HI and ligated with a gel purified fragment of pBG12 generated by complete digestion with *Sna*I and partial digestion with *Bam*HI (to obtain cleavage at 0.5 mu) but the desired clone pBG12B could not be obtained. This cloning step was attempted in four separate experiments in which a total of 111 colonies were screened. Reasons why we could not obtain the plasmid pBG12B are unknown. Since pBG12B would have been 31727 bp in length, it may have been too large to replicate when the transformants were grown under Kn selection without rearranging to reduce the size of the plasmid. Because pBG12B could not be isolated the remainder of the strategy proposed in Figure 33A had to be abandoned. For this reason the original strategy was altered as shown in Figure 34. So that the Ap^r gene would not be eliminated from pBG12 when the E1 PCR fragment was introduced, PCR was used to generate a 1014 bp fragment encoding the Ap^r gene (Fig. 34B) which was introduced into the *Bst*BI site in pBG12 generating pBG12A (Fig. 34A). The 3' primer, AB4746, was designed to contain a *Bst*BI site while the 5' primer AB4747 was designed to contain a *Cla*I site which is compatible with *Bst*BI. pUC19 was used as template DNA for the PCR reactions (Fig. 34B). Next the gel purified E1 PCR fragment was digested with *Sna*I and *Bam*HI and ligated with a gel purified fragment of pBG12A generated by complete digestion with *Sna*I and partial digestion with *Bam*HI (to obtain cleavage at 0.5 mu) but again the desired clone pBG12C was not obtained following

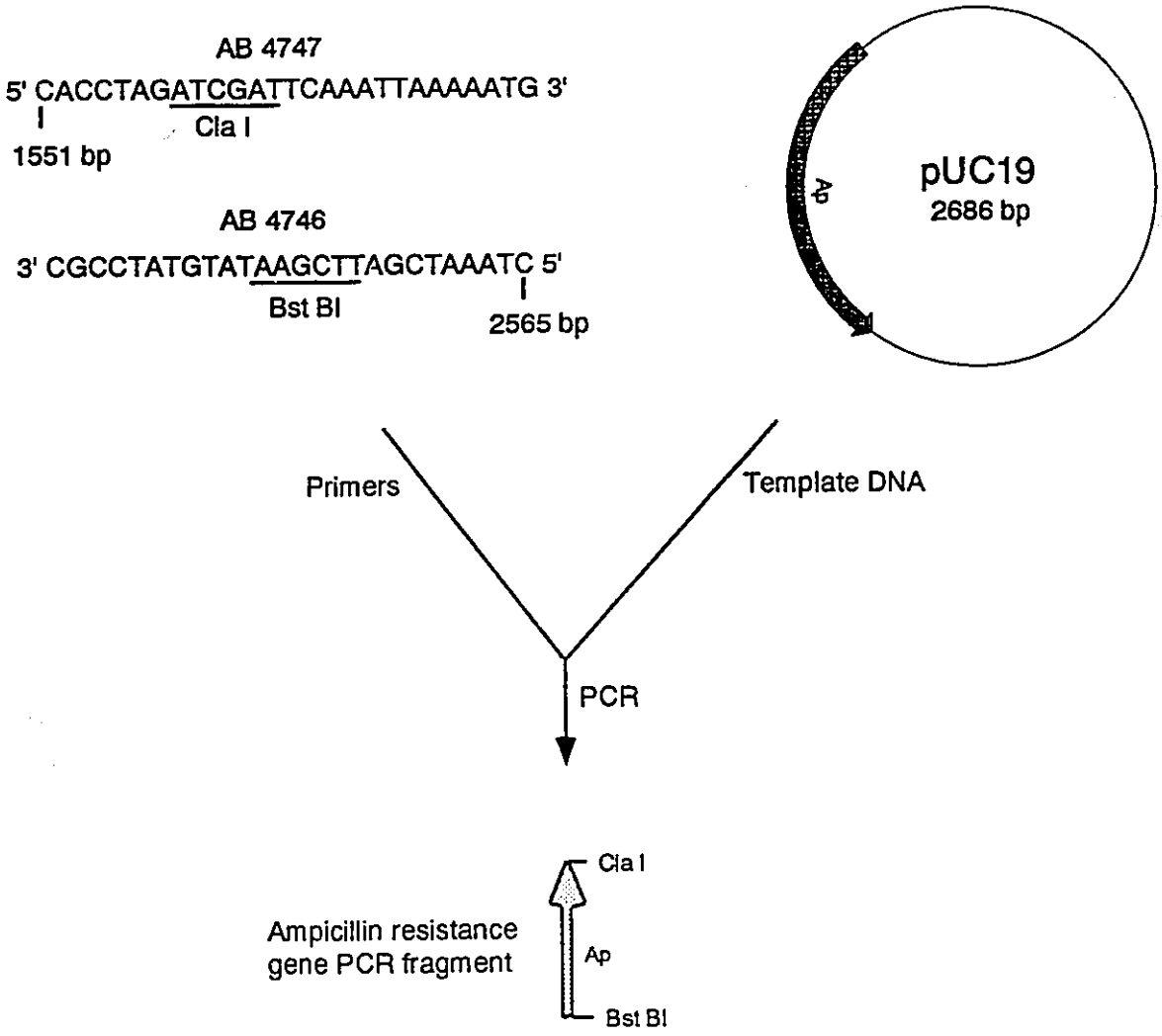
Figure 34. Construction of pBG plasmids (Strategy 2)

(A) Since we could not obtain pBG12B (Fig. 33A), the strategy was altered so that the Ap^r gene would not be eliminated from pBG12 when the E1 PCR fragment was introduced. pBG12 was digested with *Bst*BI and ligated with a PCR fragment of 1014 bp containing the Ap^r gene (Fig 34B), generating pBG12A. Next pBG12A was digested with *Sna*I and partially digested with *Bam*HI (to obtain cleavage at 0.5 mu) and fragments of appropriate size were purified from agarose. Ligations were set up with the purified *Bam*HI/*Sna*I pBG12A fragment and the *Bam*HI/*Sna*I digested E1 PCR fragment (Fig. 33C) but the desired clone pBG12C was not obtained. This cloning step was attempted several times with either the PCR generated E1 fragment or the E1 fragment contained in pBRE1PCR (Fig. 34C). Had pBG12C been obtained, the Kn^r segment would have been removed by partial digestion with *Eco*RI, complete digestion with *Swa*I, treatment of the DNA with T4 DNA polymerase and religation, generating pBG12D. pBG12D would have contained all the Ad5 sequences required to generate infectious virus following transfection into 293 cells.

(B) PCR was used to generate a 1014 bp fragment encoding the Ap^r gene which was introduced into the *Bst*BI site in pBG12 generating pBG12A (Fig. 34A). The 5' primer used for generating the Ap^r gene (AB4746) was designed to contain a *Bst*BI site and the 3' primer (AB4747) to contain a *Cla*I site which is compatible with *Bst*BI. Puc19 was used as the template DNA for the PCR reactions. bp refer to pUC19 sequences.

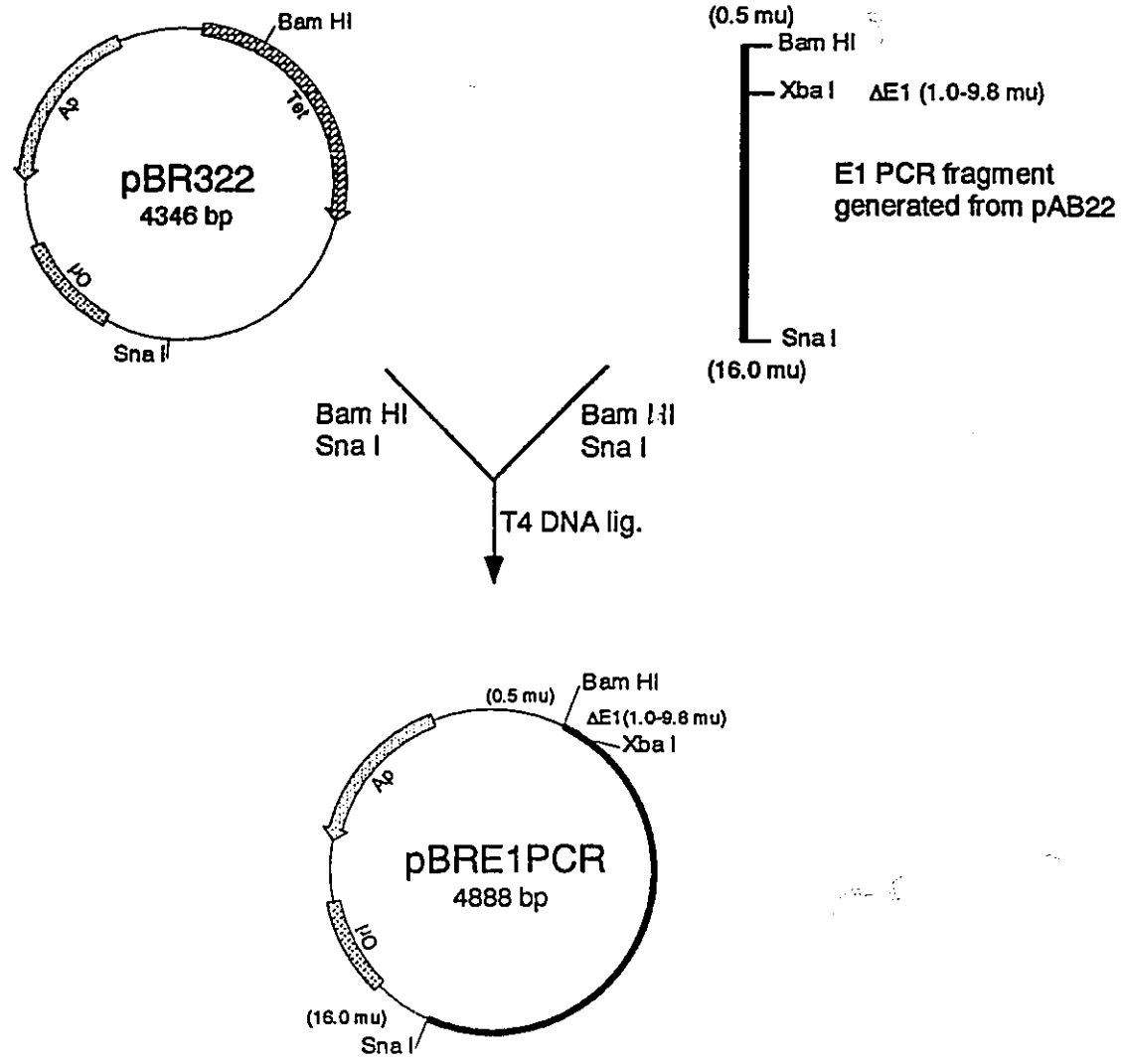

A
(100/0 mu)
Bam HI
Eco RI
Xba I
ΔΨ (0.5-3.7 mu)
Ap Ori
Ecc RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Kn Ori
ΔE3 (77.5-86.2 mu)
pBG12
36435 bp
Bam HI
Cla I
Ap
Bst BI
Bst BI
Bst BI
Cla I
T4 DNA lig.
(100/0 mu)
Bam HI
Eco RI
Xba I
ΔΨ (0.5-3.7 mu)
Ap Ori
Eco RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Kn Ori
ΔE3 (77.5-86.2 mu)
pBG12A
37428 bp
Bam HI
E1 PCR
fragment
(0.5 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Sna I
(16.0 mu)
SnaI
BamHI Partial
SnaI
BamHI
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Eco RI
Swa I
Swa I
Bst BI
Pac I
Xba I
Eco RI
Kn Ori
Ap
ΔE3 (77.5-86.2 mu)
pBG12C
32725 bp
Bam HI
EcoRI partial
SwaI
T4 DNA Pol.
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Bst BI
Pac I
Xba I
Ap Ori
ΔE3 (77.5-86.2 mu)
pBG12D
31465 bp
Bam HI

B


AB 4747
5' CACCTAGATCGATTCAAATTAAAAATG 3'
Cla I
1551 bp
AB 4746
3' CGCCTATGTATAAGCTTAGCTAAATC 5'
Bst BI
2565 bp
pUC19
2686 bp
Ap
Primers
Template DNA
PCR
Ampicillin resistance
gene PCR fragment
Cla I
Ap
Bst BI

(C) Since the PCR reactions performed to generate the E1 fragment containing the packaging signals and 3.2 Kb E1 deletion (Fig. 33C) were giving poor yields it was decided to subclone the PCR fragment into pBR322. The E1 PCR fragment was subcloned into pBR322 by digesting both with *Bam*HI and *Sna*I and ligating, generating pBRE1PCR. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r and origin segments, and clear bars represent Kn^r segments.

C


Bam HI
Ap
Tet
pBR322
4346 bp
ori
Sna I
(0.5 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
E1 PCR fragment
generated from pAB22
Sna I
(16.0 mu)
Bam HI
Sna I
Bam HI
Sna I
T4 DNA lig.
(0.5 mu)
Bam HI
ΔE1(1.0-9.8 mu)
Xba I
Ap
pBRE1PCR
4888 bp
ori
(16.0 mu)
Sna I

transformation of *E. coli*. In the first attempt 12 clones were screened but all had an incorrect structure. Because it was found that the PCR reactions used to generate the E1 fragment, described above, were providing low yields it was decided to subclone the E1 PCR fragment into pBR322 as shown in Figure 34C. Several attempts were made to ligate the E1 fragment generated by digestion of pBRE1PCR (Fig. 34C) with *Bam*HI and *Sna*I with the gel purified *Sna*I/*Bam*HI fragment of pBG12A described above without success. A total of 123 colonies were screened but again we failed to isolate a plasmid having the desired structure.

At this point it was decided that the two halves of the pBG genome would have to be manipulated separately and later rejoined to reconstitute the viral genome and a third strategy was developed as shown in Figure 35. To generate a plasmid containing the desired right half of the Ad5 genome pBG12A was digested with *Bam*HI and religated, generating pBHG12AR (Fig. 35A) The Kn^r gene was then removed from pBHG12AR by partial digestion with *Eco*RI and complete digestion with *Swa*I and religation, generating pBG13AR.

To generate a plasmid containing the desired left half Ad5 sequences pBHG11 was digested with *Bam*HI and religated, generating pBHG11L (Fig. 35B). Since the insertion of the E1 PCR fragment into pBHG11L would remove the Ap resistance gene and origin of replication the small kanamycin resistant plasmid pABS.12 was introduced into the *Xba*I site in pBRE1PCR, generating pBRE1PCRK (Fig. 35C). pBHG11L was then digested with *Bam*HI and *Sna*I and ligated with pBRE1PCRK that had also been digested with *Bam*HI and *Sna*I, generating pBG13BL (Fig. 35B).

To reconstitute the entire Ad5 genome (minus engineered deletions in E1 and E3), pBG13AR and pBG13BL were joined together by digesting each with *Bam*HI and religating, generating pBG14 (Fig 35D). To remove the Kn^r gene from the E1 deletion, pBG14 was partially digested with *Xba*I and religated, generating pBG15. In the final step, to create two derivatives

Figure 35. Construction of pBG plasmids (Strategy 3)

(A) To generate a plasmid containing the desired right half of the Ad5 genome pBG12A was digested with *Bam*HI and religated to obtain pBHG12AR. The Kn^r gene was then removed from pBHG12AR by partial digestion with *Eco*RI and complete digestion with *Swa*I and religation, generating pBG13AR.

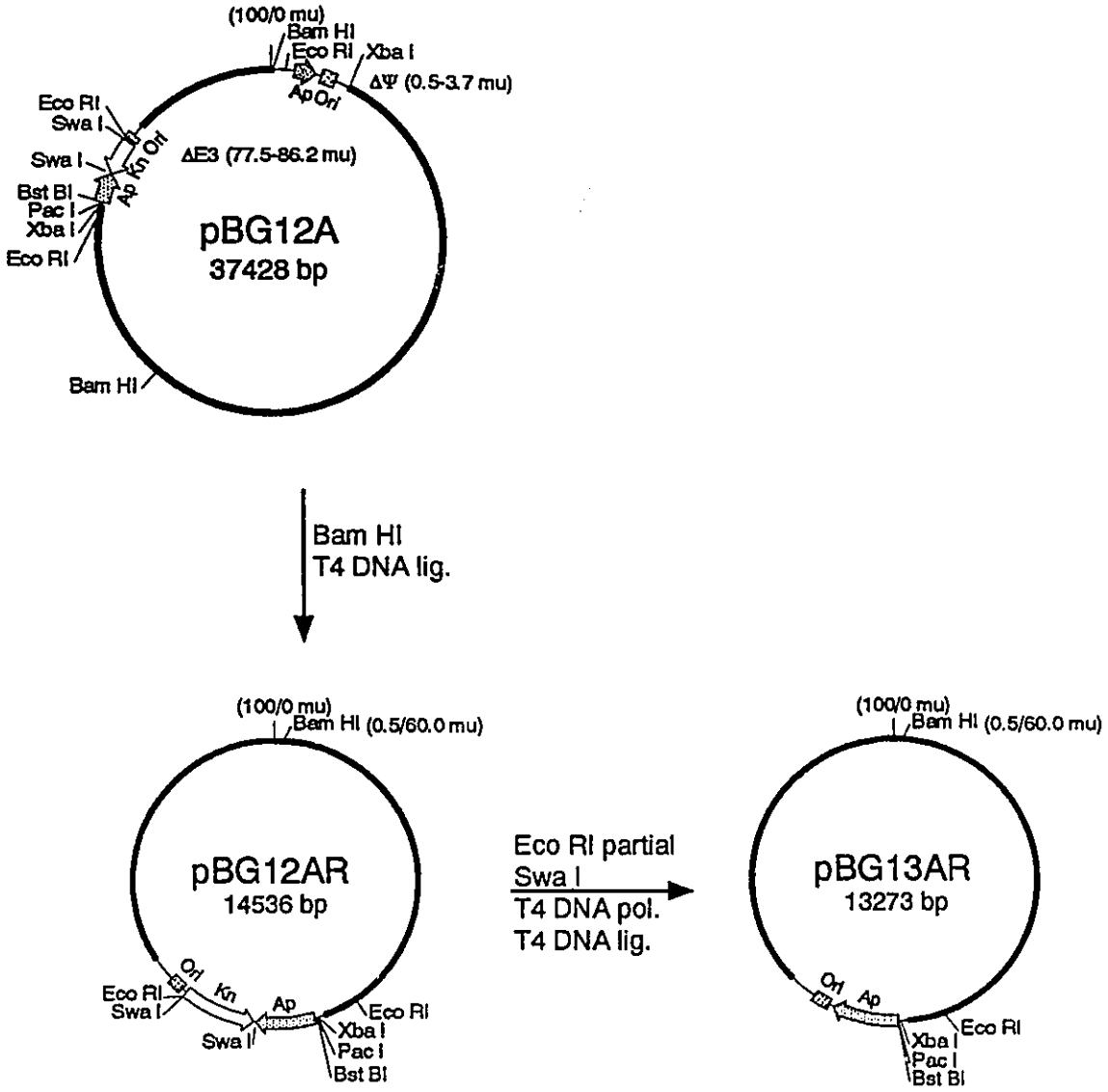
(B) To generate a plasmid containing the desired left half Ad5 sequences pBHG11 was digested with *Bam*HI and religated, generating pBHG11L. pBHG11L was then digested with *Bam*HI and *Sna*I and combine with pBRE1PCRK (Fig. 35C) also digested with *Bam*HI and *Sna*I, generating pBG13BL.

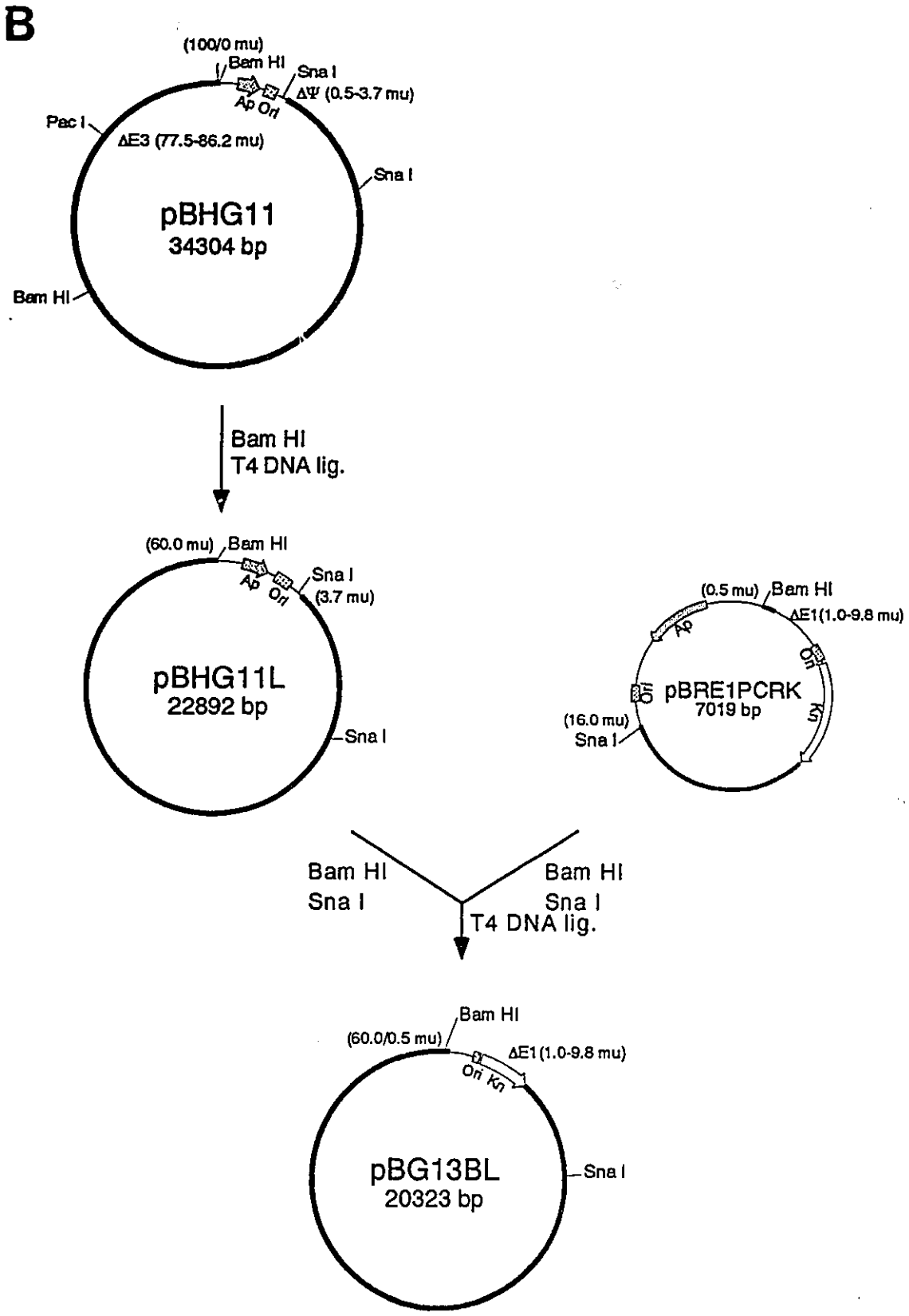
(C) Since the insertion of the E1 PCR fragment into pBHG11L would remove the Ap resistance gene and origin of replication the small kanamycin resistant plasmid pABS.12 was introduced into the *Xba*I site in pBRE1PCR, generating pBRE1PCRK.

(D) To create the infectious pBG genome pBG13AR and pBG13BL were each digested with *Bam*HI and ligated, generating pBG14. The Kn^r segment was then removed from the E1 deletion in pBG14 by partial *Xba*I digestion and religation, generating pBG15.

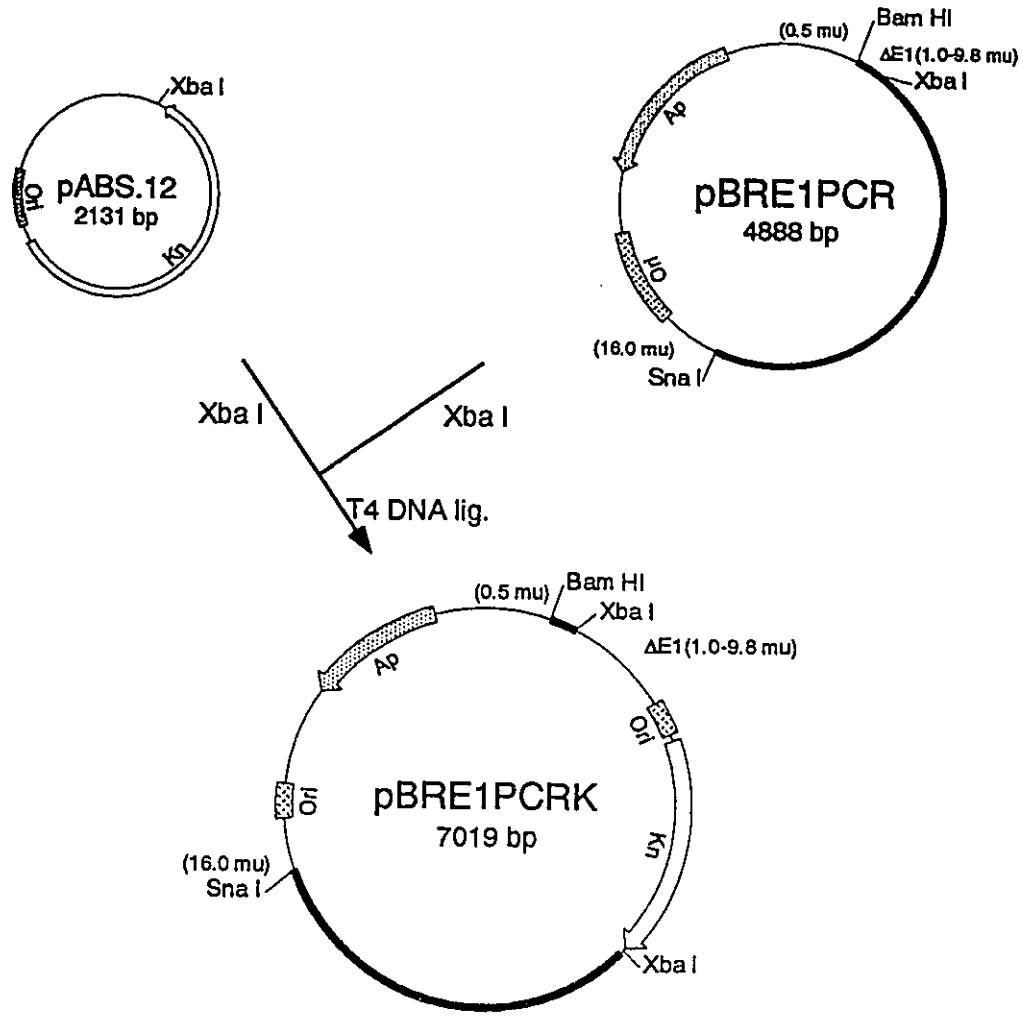
(E) To create two derivatives of pBG15, with a unique *Xba*I cloning site in either the E1 or E3 deletion, pBG15 was partially digested with *Xba*I, the DNA treated with T4 DNA polymerase and then religated, generating pBG16 and pBG17. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences, hatched bars represent Ap^r and origin segments, and clear bars represent Kn^r segments.

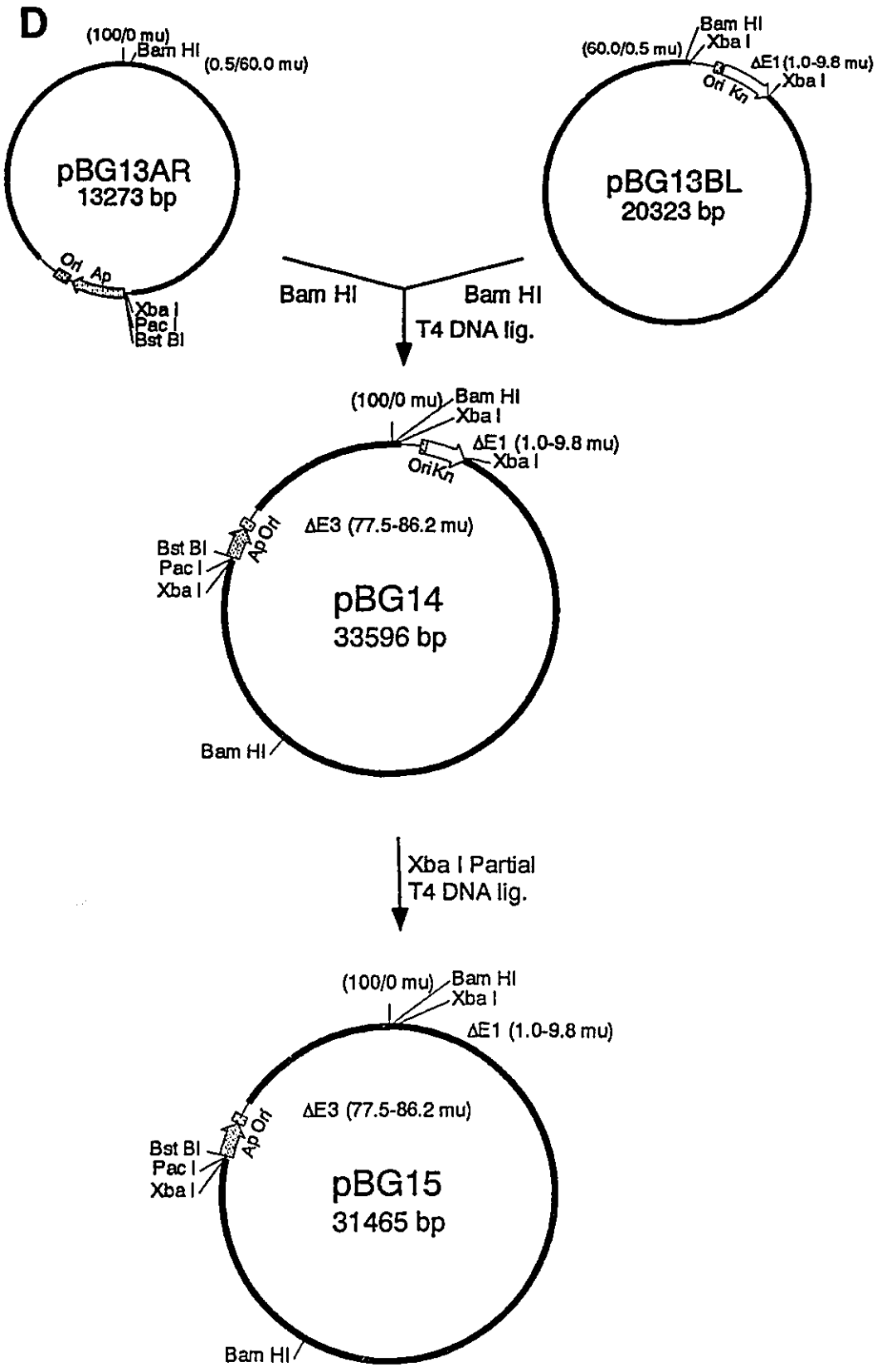
A


(100/0 mu)
Bam HI
Eco RI
Xba I
ΔΨ (0.5-3.7 mu)
Ap
Ori
Eco RI
Swa I
Swa I
Kn
Ori
ΔE3 (77.5-86.2 mu)
Bst BI
Pac I
Xba I
Ap
Eco RI
pBG12A
37428 bp
Bam HI
Bam HI
T4 DNA lig.
(100/0 mu)
Bam HI (0.5/60.0 mu)
pBG12AR
14536 bp
Ori
Kn
Eco RI
Swa I
Ap
Eco RI
Xba I
Swa I
Pac I
Bst BI
Eco RI partial
Swa I
T4 DNA pol.
T4 DNA lig.
(100/0 mu)
Bam HI (0.5/60.0 mu)
pBG13AR
13273 bp
Ori
Ap
Eco RI
Xba I
Pac I
Bst BI

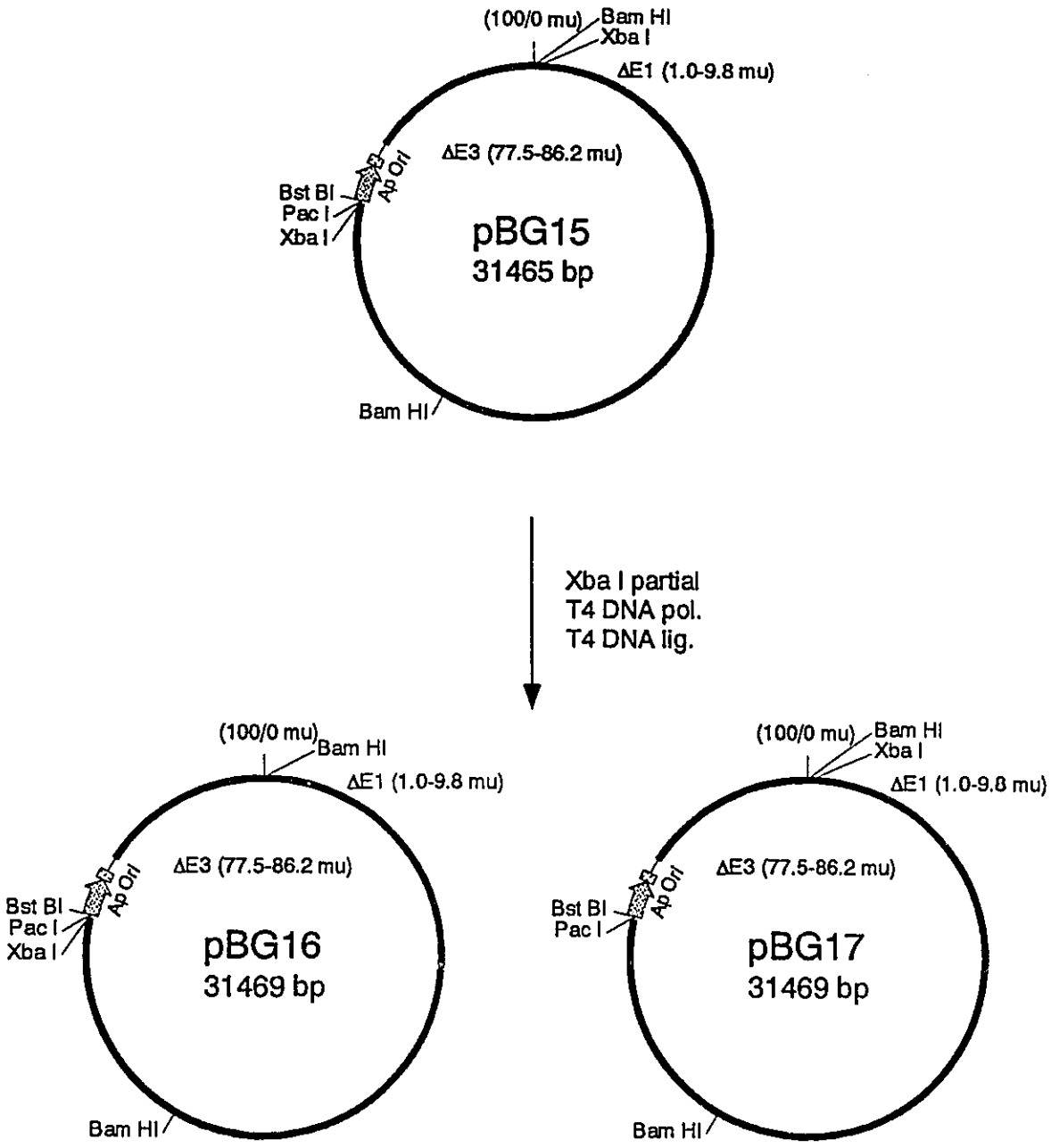

B
(100/0 mu)
Bam HI
Sna I
ΔΨ (0.5-3.7 mu)
Ap
Ori
Pac I
ΔE3 (77.5-86.2 mu)
Sna I
pBHG11
34304 bp
Bam HI
Bam HI
T4 DNA lig.
(60.0 mu)
Bam HI
Ap
Ori
Sna I
(3.7 mu)
pBHG11L
22892 bp
Sna I
(0.5 mu)
Bam HI
ΔE1(1.0-9.8 mu)
Ap
Ori
pBRE1PCRK
7019 bp
Kn
(16.0 mu)
Sna I
Bam HI
Sna I
Bam HI
Sna I
T4 DNA lig.
Bam HI
(60.0/0.5 mu)
ΔE1(1.0-9.8 mu)
Ori
Kn
pBG13BL
20323 bp
Sna I

C


Xba I
pABS.12
2131 bp
Ori
Kn
(0.5 mu)
Bam HI
ΔE1(1.0-9.8 mu)
Xba I
Ap
pBRE1PCR
4888 bp
Ori
(16.0 mu)
Sna I
Xba I
Xba I
T4 DNA lig.
(0.5 mu)
Bam HI
Xba I
ΔE1(1.0-9.8 mu)
Ap
Ori
Ori
pBRE1PCRK
7019 bp
Kn
(16.0 mu)
Sna I
Xba I


D
(100/0 mu)
Bam HI
(0.5/60.0 mu)
pBG13AR
13273 bp
Ori
Ap
Xba I
Pac I
Bst BI
Bam HI
Xba I
(60.0/0.5 mu)
ΔE1 (1.0-9.8 mu)
Ori Kn
Xba I
pBG13BL
20323 bp
Bam HI
Bam HI
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
Ori Kn
Xba I
ΔE3 (77.5-86.2 mu)
Bst BI
Pac I
Xba I
Ap Ori
pBG14
33596 bp
Bam HI
Xba I Partial
T4 DNA lig.
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
ΔE3 (77.5-86.2 mu)
Bst BI
Pac I
Xba I
Ap Ori
pBG15
31465 bp
Bam HI

E

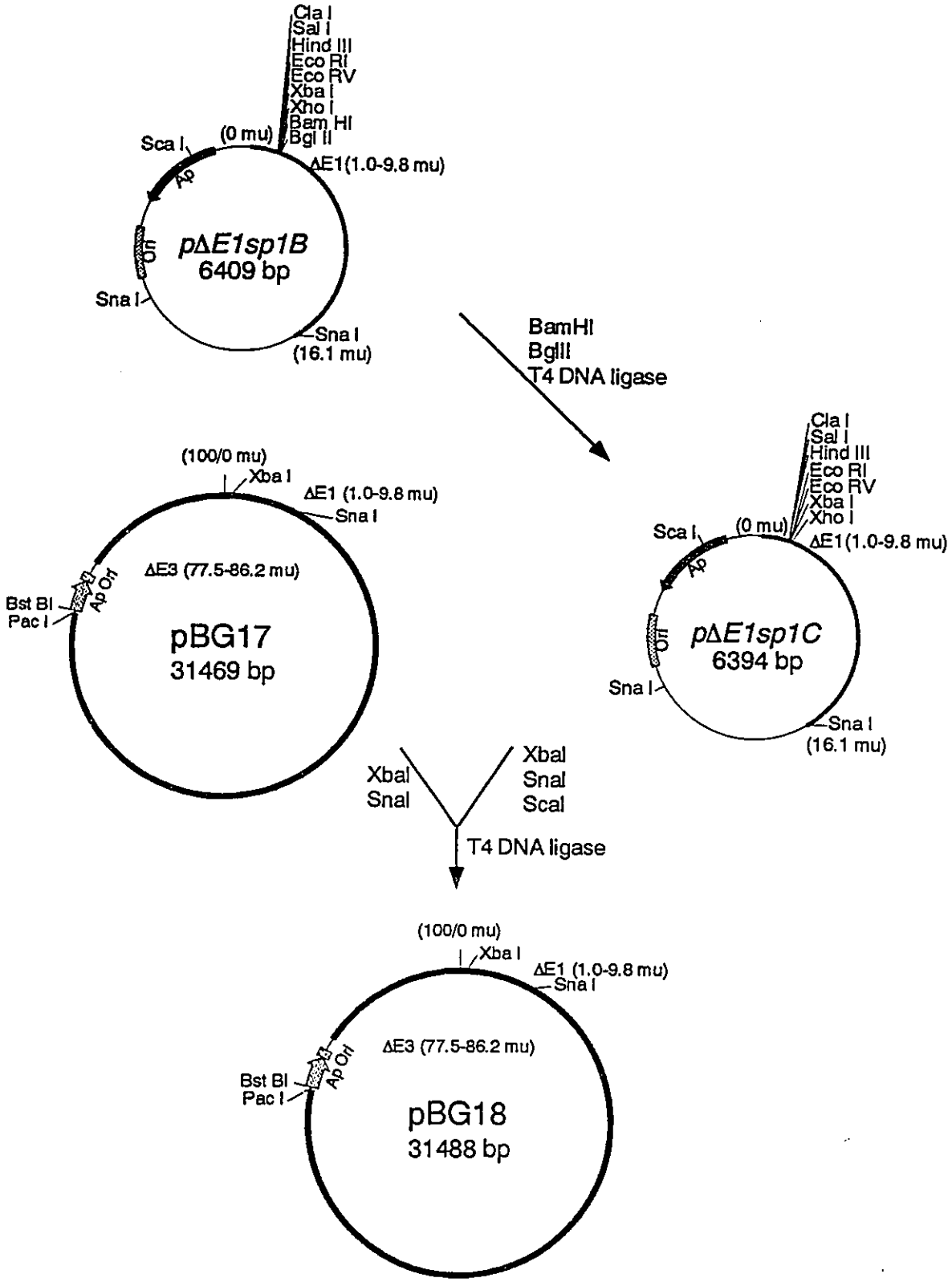

(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
ΔE3 (77.5-86.2 mu)
Ap
Ori
Bst BI
Pac I
Xba I
pBG15
31465 bp
Bam HI
Xba I partial
T4 DNA pol.
T4 DNA lig.
(100/0 mu)
Bam HI
ΔE1 (1.0-9.8 mu)
ΔE3 (77.5-86.2 mu)
Ap
Ori
Bst BI
Pac I
Xba I
pBG16
31469 bp
Bam HI
(100/0 mu)
Bam HI
Xba I
ΔE1 (1.0-9.8 mu)
ΔE3 (77.5-86.2 mu)
Ap
Ori
Bst BI
Pac I
pBG17
31469 bp
Bam HI

with a unique *Xba*I cloning site in either E1 or in E3, pBG15 was partially digested with *Xba*I, the DNA treated with T4 DNA polymerase, and then religated, generating pBG16 and pBG17 (Fig. 35E). pBG16 and pBG17 contain Ad5 sequences from bp 19 (left genomic end) to bp 341; bp 3534 to 27864 and 30996 to 35934 (right genomic end). A *Bam*HI site is inserted between Ad5 bp 189 and 190. The left and right termini of the Ad5 genome are covalently joined and 1874 bp of DNA containing the pUC19 origin of replication and ampicillin resistance gene are located between Ad5 bp 27864 and 30996 to allow propagation of these plasmids in *E. coli*. pBG16 contains unique *Xba*I, *Pac*I and *Bst*BI restriction enzyme sites in place of E3 sequences to permit insertion of foreign genes. pBG17 contains a unique *Xba*I site at the location of the E1 deletion and unique *Pac*I and *Bst*BI sites have been substituted for E3 to permit insertion of foreign genes.

The E1 deletion present in the pBG plasmids removes the Sp1 binding site from the protein IX promoter which is located at the 3' end of the E1 deletion. Protein IX is essential for packaging of full length genomes into functional virions (Ghosh-Choudhury *et al.*, 1987). Previous studies have shown that variable levels of protein IX are expressed depending on the sequences upstream from the protein IX gene when no Sp1 site is present (Bett *et al.*, 1994). It is likely that with pBG16, in which no insert can be introduced into E1, the level of protein IX will be close to wt as seen with previous vectors with this same deletion (Bett *et al.*, 1994). To ensure that adequate levels of protein IX are expressed when inserts are introduced into the E1 region in pBG17 the sp1 site was reintroduced into the protein IX promoter (Fig. 36). To reintroduce the sp1 site advantage was taken of the E1 shuttle plasmid pΔE1sp1B which contains the same E1 deletion found in the pBG plasmids but has previously had the sp1 site reintroduced (Bett *et al.*, 1994). To remove the *Bam*HI and *Bgl*II restriction sites from the polycloning region

Figure 36. Reinsertion of Sp1 site into the protein IX promoter in pBG17.

To ensure adequate protein IX expression is obtained from the protein IX promoter when inserts are cloned into the E1 deletion the Sp1 site was reintroduced into the protein IX promoter. To do this advantage was taken of the plasmid pΔE1sp1B (Bett *et al.*, 1994), which contains the same E1 deletion as the pBG plasmids but has already had the Sp1 site reintroduced. To remove unwanted restriction site so they would not be transferred to the pBG plasmid, pΔE1sp1B was digested with *Bgl*II and *Bam*HI and religated, generating pΔE1sp1C. pΔE1sp1C was then digested with *Xba*I, *Sna*I and *Sca*I and the *Xba*I/*Sna*I fragment containing the protein IX sequences was gel purified. This gel purified fragment was then ligated with the large gel purified *Xba*I/*Sna*I fragment from pBG17, generating pBG18. Map units (mu) refer to Ad5 sequences; solid bars represent Ad5 sequences and hatched bars represent Ap^r and origin segments.


Cla I
Sal I
Hind III
Eco RI
Eco RV
Xba I
Xho I
Bam HI
Bgl II
Sca I
(0 mu)
ΔE1(1.0-9.8 mu)
Ap
Ori
pΔE1sp1B
6409 bp
Sna I
Sna I
(16.1 mu)
BamHI
BglII
T4 DNA ligase
(100/0 mu)
Xba I
ΔE1 (1.0-9.8 mu)
Sna I
ΔE3 (77.5-86.2 mu)
Bst BI
Pac I
Ap Ori
pBG17
31469 bp
Cla I
Sal I
Hind III
Eco RI
Eco RV
Xba I
Xho I
Sca I
(0 mu)
ΔE1(1.0-9.8 mu)
Ap
Ori
pΔE1sp1C
6394 bp
Sna I
Sna I
(16.1 mu)
XbaI
SnaI
XbaI
SnaI
ScaI
T4 DNA ligase
(100/0 mu)
Xba I
ΔE1 (1.0-9.8 mu)
Sna I
ΔE3 (77.5-86.2 mu)
Bst BI
Pac I
Ap Ori
pBG18
31488 bp

in pΔE1sp1B, the plasmid was digested with *Bam*HI and *Bgl*II and religated, generating pΔE1sp1C. pΔE1sp1C was then digested with *Xba*I, *Sna*I and *Sca*I and the *Xba*I/*Sna*I fragment containing the protein IX sequences was gel purified. This fragment was then ligated with the large *Xba*I/*Sna*I fragment from pBG17, generating pBG18.

After these many manipulations of viral DNA grown in bacterial plasmids it was important to determine whether the final plasmids were able to generate infectious Ad virions following transfections of 293 cells. To assess the ability of the pBG plasmids to generate infectious viral vectors, the infectivity of pBG15 was compared to pFG140 (Graham, 1984). pFG140 is an infectious circular Ad 5 genome derived from *dl*309 (Jones and Shenk, 1979) and is used as a control for transfection efficiency. Unlike the pBG plasmids described above inserts cannot be cloned into pFG140 since it contains neither useful deletions in E1 or E3 nor suitable cloning sites in the form of unique restriction sites in regions that can accept inserts without interference with viral replication. When 60 mm dishes of 293 cells were transfected with either 5 or 10 μg of pBG15 or pFG140 the number of plaques obtained was 1.1 plaques/μg and 5.9 plaques/μg respectively. Although plaques were efficiently formed by pBG15 DNA the plaques grew very slowly. When the plaques were analyzed very little viral DNA was obtained but all plaques analyzed had the predicted *Hind*III restriction pattern (data not shown) and the virus generated was designated AdBG15. When 150 mm dishes of 293 cells were infected with AdBG15 in order to generate high titered stocks, cpe was very slow in forming and stocks could not be generated with titers higher then $1x10^7$ pfu/ml.

2. 100K And Fiber Expression In The pBG Vectors

Since it had been previously observed that inserts in E3 can cause a reduction in the level of expression of 100K and fiber (Mittal *et al.*, 1993; Bett *et al.*, 1993) we decided to compare the level of 100K and fiber synthesized in 293 cells infected with wt Ad5, AdBG15 and AdBHGΔE3.1 (a virus containing the same 3.1 kb E3 deletion present in AdBG15). 293 cells were infected at an moi of 1 and cells were labelled with [^{35}S]methionine from 46-48 h post infection, cell extracts were harvested and samples run on 8.0% SDS-PAGE gels. The results shown in Fig. 37A indicate that there was a reduction in the rate of expression of fiber for AdBG15 and AdBHGΔE3.1 as compared to wt Ad5. When densitometry was used to more accurately determine the levels of 100K and fiber expressed by these viruses (Table 5) it was found that 100K expression was not greatly affected (115% and 90% as compared to wt Ad5 for AdBHGΔE3.1 and AdBG15 respectively) but fiber expression was reduced 2-3 fold (40% and 27% as compared to wt Ad5 for AdBHGΔE3.1 and AdBG15 respectively). Similar reductions in fiber expression were noted previously (Bett *et al.*, 1994) for vectors containing a 2.69 kb E3 deletion substituted with various foreign DNA inserts but were not found to significantly effect the yield of infectious viral progeny. This is likely because viral late proteins are normally made in excess during viral replication. Therefore the reduced level of fiber produced by AdBG15 likely does not explain it poor growth. Interestingly an additional higher molecular weight product was present above fiber in the AdBG15 lane (Fig. 37A). In an attempt to determine if this additional band represented an aberrant form of fiber samples of the cell extracts generated above were immunoprecipitated using anti-fiber antisera (Ascites fluid 8C6-7-3, obtained from Dr. A Wandiler, Nepean, Ont. by Dr. Prevec). The results presented in Fig. 37B show that this higher molecular weight species was precipitated suggesting that it is indeed an aberrant form of fiber.

Figure 37. 100K and Fiber expression in AdBG15 infected cells.

(A) The level of 100K and fiber expression in AdBG15 infected cells was studied and compared to wt Ad5 and AdBHGΔE3.1 (generated by cotransfection of pBHG11 with pXC1). 293 cells were either mock infected or infected at an moi of 1 with wt Ad5, AdBHGΔ3.1, and AdBG15 for 46 h and then labelled with [^{35}S]methionine from 46-48 h. Cell extracts were then prepared and samples separated by SDS-PAGE on an 8.0% gel. The gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above. Molecular weight markers are indicated on the left. The relative levels of 100K and fiber present in the samples run on this gel were determined by densitometric analysis. The novel band seen in the AdBG15 lane is indicated with an solid arrow head.

(B) To determine if the novel band running above fiber in the lane containing the AdBG15 cell extracts (Fig. 37A) was an aberrant form of fiber immunoprecipitations were performed using fiber specific antisera (Ascites fluid 8C6-7-3, obtained from Dr. A. Wandiler, Nepean, Ont., by Dr. Prevec). Immunoprecipitates were separated on an 8% SDS-PAGE gel, the gel was then dried and bands visualized by autoradiography. The lanes contain the samples indicated above. Molecular weight markers are indicated on the left.

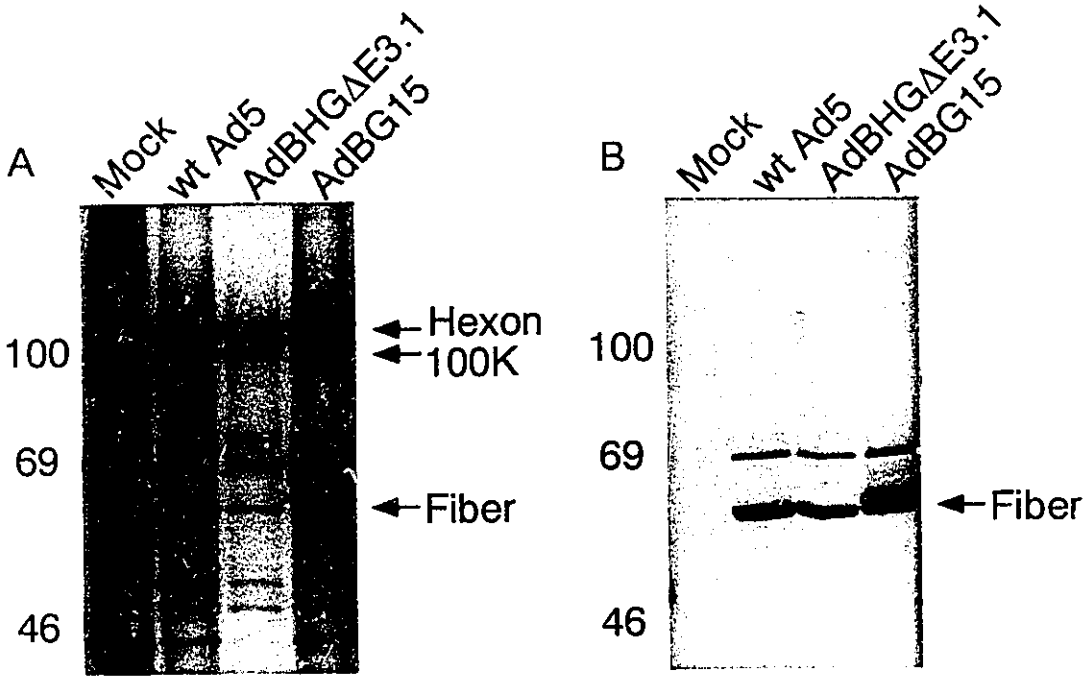

A
Mock
wt Ad5
AdBHGΔE3.1
AdBG15
100
69
46
Hexon
100K
Fiber
B
Mock
wt Ad5
AdBHGΔE3.1
AdBG15
100
69
46
Fiber

Table 5. Synthesis of 100K and fibre in cells infected with AdBG15[a]

virus	E3 deletion	expression level (%)[b]	
		100K	fiber
wt Ad5	none	100	100
AdBHGΔE3.1	3.1kb	90	40
AdBG15	3.1kb	115	27

a Cells infected with the indicated viruses were labelled with [S^{35}]methionine,cell extracts prepared and run on polyacrylamide gels. The levels of 100K and fibre were determined by densitometric analysis.
b For each virus the level of 100K and fibre was determined relative to hexon and then expressed as a percent of the wt Ad5 level.

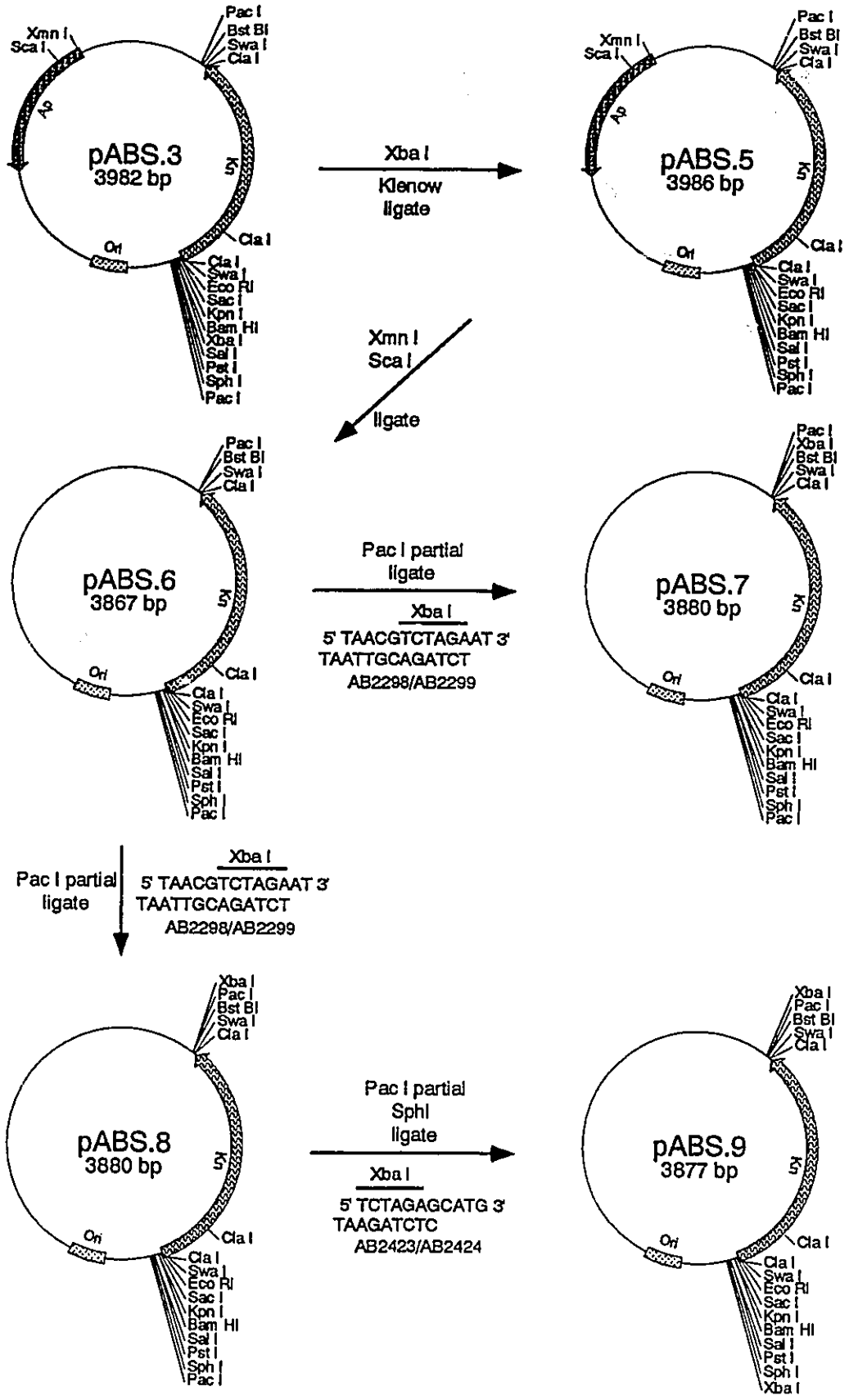
This product may be produced due to incorrect splicing involving the Ap insert in the E3 region in the pBG plasmids and explain the poor growth of vectors derived from the pBG plasmids. Since other vectors with inserts in the 3.1 kb E3 deletion grow with wt kinetics it is possible that if the Ap insert in the pBG plasmids is altered, for example by switching its orientation, splicing to fiber and therefore vector propagation would be normal.

3. Shuttle Plasmids For Use With The pBG Vectors.

In order to simplify the cloning of inserts into the E1 and/or E3 deletions present in the pBG plasmids three shuttle plasmids similar to pABS.4 (results section B.6) were generated. The construction of these plasmids is described below and illustrated in Figure 38. First pABS.3 was digested with *Xba*I treated with klenow and religated, generating pABS.5. The Ap^r gene in pABS.5 was then disrupted by digesting with *Xmn*I and *Sca*I followed by ligation, generating pABS.6. pABS.6 was then partially digested with *Pac*I and ligated with oligo AB2298/AB2299, generating pABS.7 and pABS.8. Finally PABS.8 was partially digested with *Pac*I, digested completely with *Sph*I and ligated with oligo AB2423/AB2424, generating pABS.9. pABS.6, pABS.7 and pABS.9 can be used to clone inserts into the *Pac*I, *Bst*BI and *Xba*I sites present at the positions of the E1 and/or E3 deletions in the pBG plasmids using the same strategy described for using pABS.4 to clone inserts in the E3 deletions in pBHG10 and pBHG11 (results section B.6). Briefly inserts can be cloned into the *Sph*I, *Pst*I, *Sal*I, *Bam*HI, *Kpn*I, *Sac*I or *Eco*RI restriction sites in the appropriate shuttle plasmid. The shuttle plasmid is then cut with one or two combinations of *Xba*I, *Pac*I or *Bst*BI and the fragment containing the insert/Kn^r cassette is inserted into the Ap^r pBH plasmid. This step is facilitated by using Ap+Kn double selection for bacterial transformants carrying the desired plasmid. Subsequently the Kn^r gene can be removed by

Figure 38. Shuttle plasmids used to facilitate the cloning of inserts into the pBG plasmids.

To simplify the cloning of inserts into the pBG plasmids three new shuttle plasmids similar to pABS.4 were constructed, designated pABS.6, pABS.7 and pABS.9. To construct these plasmids pABS.3 was digested with *Xba*I, treated with klenow and religated, generating pABS.5. The Ap^r gene in pABS.5 was disrupted by digesting with *Xmn*I and *Sca*I and ligating, generating pABS.6. pABS.6 was then partially digested with *Pac*I and ligated with oligo AB2298/2299, generating pABS.7 and pABS.8. pABS.8 was then partially digested with *Pac*I, digested completely with *Sph*I and ligated with oligo AB2423/AB2424, generating pABS.9. Hatched bars represent Ap^r, Kn^r and origin segments.


pABS.3
3982 bp
Xmn I
Sca I
Pac I
Bst BI
Swa I
Cla I
Ap
5'
Ori
Cla I
Cla I
Swa I
Eco RI
Sac I
Kpn I
Bam HI
Xba I
Sal I
Pst I
Sph I
Pac I
Xba I
Klenow
ligate
pABS.5
3986 bp
Xmn I
Sca I
ligate
pABS.6
3867 bp
pABS.7
3880 bp
Pac I partial
ligate
Xba I
5' TAACGTCTAGAAT 3'
TAATTGCAGATCT
AB2298/AB2299
pABS.8
3880 bp
pABS.9
3877 bp
Pac I partial
SphI
ligate
5' TCTAGAGCATG 3'
TAAGATCTC
AB2423/AB2424

digestion with *Cla*I or *Swa*I and ligation. The recombinant pBG plasmid can then be transfected into 293 cells in order to generate an infectious Ad vector.

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