Rearrangements of Adenovirus Genome.

REARRANGEMENTS OF THE ADENOVIRUS GENOME

INDUCED BY

EMBEDDED INVERTED TERMINAL REPEAT SEQUENCES

By

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TITLE: Rearrangements of the Adenovirus Genome Induced by Embedded Inverted Terminal Repeat Sequences

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ABSTRACT

The adenovirus genome is a linear double stranded DNA molecule and the current widely accepted model of viral DNA replication proposes linear viral DNA intermediates at all stages of replication. Although the experimental evidence for this mechanism of replication is very strong, circular forms of adenovirus serotype 5 (Ad5) DNA molecules have also been detected in both permissive and non-permissive cell lines. In some experiments the circular structures were detected before the onset of viral DNA replication and thus suggested a possible role for circular forms of Ad5 DNA in the life cycle of the virus after infection.

This study was undertaken to better understand the role of circular forms of the adenovirus genome in virus replication. The approach was to subclone the viral junction internally into the linear genome thus creating mutant viruses with embedded terminal sequences and to study the effect of such inserts on DNA structure. A total of five mutant viruses were constructed containing a variety of inserts and viral DNA from infected cells and banded viruses was analyzed by Southern Blot hybridization. The data clearly showed that embedded viral junctions have biological activity in that they generated novel, rearranged viral DNA molecules, and that

embedded single ITR sequences were also biologically active, but to a lesser extent. It was also observed that the copy numbers of the rearranged molecules were variable. It appeared that the embedded viral junction was active in recombination and replication of the viral genome, creating the rearrangements through these two processes. However, the results suggested that circular forms are not obligatory intermediates in DNA replication.

The analysis of banded viral DNA in this study suggested that the encapsidation signal from the left end of the linear genome was required in cis for packaging of the viral genome, confirming previous results which identified an encapsidation signal for viral DNA packaging. The banding experiments also showed that truncated viral DNA molecules containing 75% of the viral genome were packaged.

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In memory of my grandparents.

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LIST OF ABBREVIATIONS

Ad2, 5, 12, etc.	adenovirus serotypes 2, 5, 12 etc.
ATP	adenosine triphosphate
Ap^R	ampicillin resistant
ARS	autonomously replicating sequences
bp	base pairs
BSA	bovine serum albumin
°C	degrees centigrade
cDNA	complementary DNA
Ci	curie
cpe	cytopathic effect
cpm	counts per minute
DBP	DNA binding protein
dATP, dTTP, dGTP, dCTP	deoxynucleoside triphosphates
dl	deletion mutant
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreotol
E1, E2, E3, E4	early regions 1 to 4 of adenovirus
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis tetra-acetic acid
EtBr	ethidium bromide
µg, mg, g	microgram, milligram, gram
gp	glycoprotein
hr(s)	hour(s)
HS	horse serum

HSV	herpes simplex virus
IR	inverted repeat
ITR	inverted terminal repeat
kb	kilobase pairs
kd	kilodaltons
µl, ml, l	microlitre, millilitre, litre
l-strand	leftward transcribed strand of adenovirus genome
min(s)	minute(s)
MLP	major late promoter of adenovirus
moi	multiplicity of infection (pfu/cell)
mRNA	messenger RNA
µM, mM, M	micromolar, millimolar, molar
mu	map units
neo^R	neomycin resistant
NF	nuclear factor
nm	nanometre
OD	optical density
ORP	origin recognition protein
pfu	plaque forming units
pi	post infection
pTP	pre-terminal protein
R	amino acid residues
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
r-strand	rightward transcribed strand of adenovirus genome
S	Svedberg units

sec(s)	second(s)
SDS	sodium dodecyl sulfate
ss	single strand
SV40	simian virus 40
TP	terminal protein
tRNA	transfer RNA
ts	temperature sensitive
UV	ultra violet
V	volts
VA-RNAI, VA-RNAII	virus associated RNA I and II
wt	wild type

1. INTRODUCTION.

1.1 THE ADENOVIRIDAE.

1.1.1 General History.

The adenoviridae family is composed of viruses with a linear, double-stranded DNA genome enclosed in an icosahedral protein shell and is divided into two genera; Mast- and Avi-adenoviruses (reviewed in Straus, 1984). The mastadenoviruses infect a wide range of mammalian hosts including higher primates (eg. humans and chimpanzees), but the aviadenoviruses have only been isolated from a limited number of avian species, mainly fowls. Classification into the two genera is based on the presence (Mast-) or the absence (Avi-) of common antigenic determinants which are detected by either complement fixation assays or agar-gel precipitin tests (reviewed in Ishibashi and Yasue, 1984). The human adenoviruses belong to the Mastadenovirus group.

The first member of the adenovirus family was isolated from a Wisconsin fox farm population in 1930 (Green *et al*, 1930) and was subsequently shown to be the etiological agent responsible for encephalitis in foxes and was thus named fox encephalitis virus.

The first human adenoviruses were isolated approximately 35 years ago from tonsils of infected individuals (Hilleman and Werner, 1954; Rowe et al, 1953) and are now associated with a number of respiratory illnesses and inflammations of various tissues and organs of the body (Table 1) (reviewed in Straus, 1984). They are classified into subgroups (or categories) based on a number of physical properties as shown in Table 1 (Sambrook et al, 1980): hemaglutination (four subgroups), % DNA homology, % G+C (three subgroups), oncogenicity in rodents (three subgroups), target tissue and epidemiology. The majority of the research on adenoviruses has centred on human adenoviruses, particularly serotypes 2 and 5 (Ad2 and Ad5) from group C and serotype 12 (Ad12) from group A, and therefore, the information presented in this chapter will focus on these viruses.

1.1.2 Structure of the Virus.

The adenovirus genome is enclosed in an icosahedral protein shell made up of 240 hexon molecules and 12 pentons (Fig. 1). The hexons with their six-fold symmetry make up the triangular faces of the icosahedron, and the pentons with their five-fold symmetry and attached fibre protein make up the vertices (van Oostrum and Burnett, 1985). A hexon is composed of three identical polypeptides each with molecular weight of about 100 kilodaltons (kd) and are held together in groups of nine (GONs) by polypeptide IX which is positioned

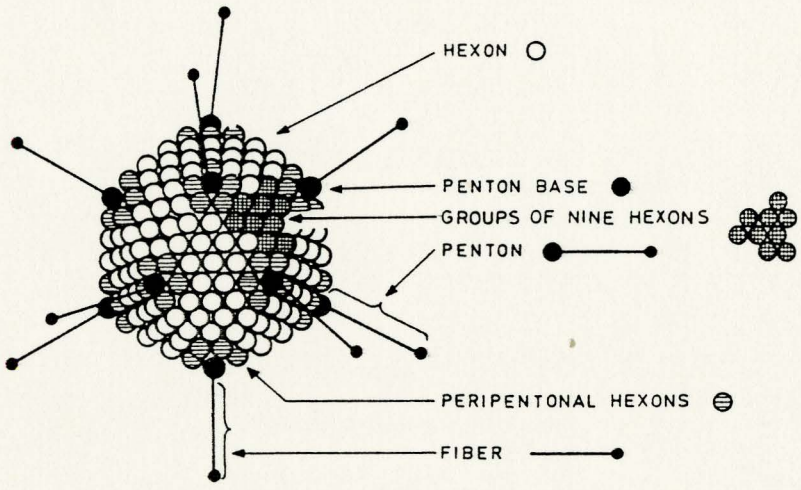
TABLE 1

HUMAN ADENOVIRUS HOMOLOGY CLASSES

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

FIGURE 1. Adenovirus serotype 5 capsid.

Faces of the icosahedron are made up of hexons held together in groups of nine by protein IX which is positioned between the hexon molecules. Vertices of the icosahedron are made up of pentons and the fibre protein. See text for details. Philipson, 1983.

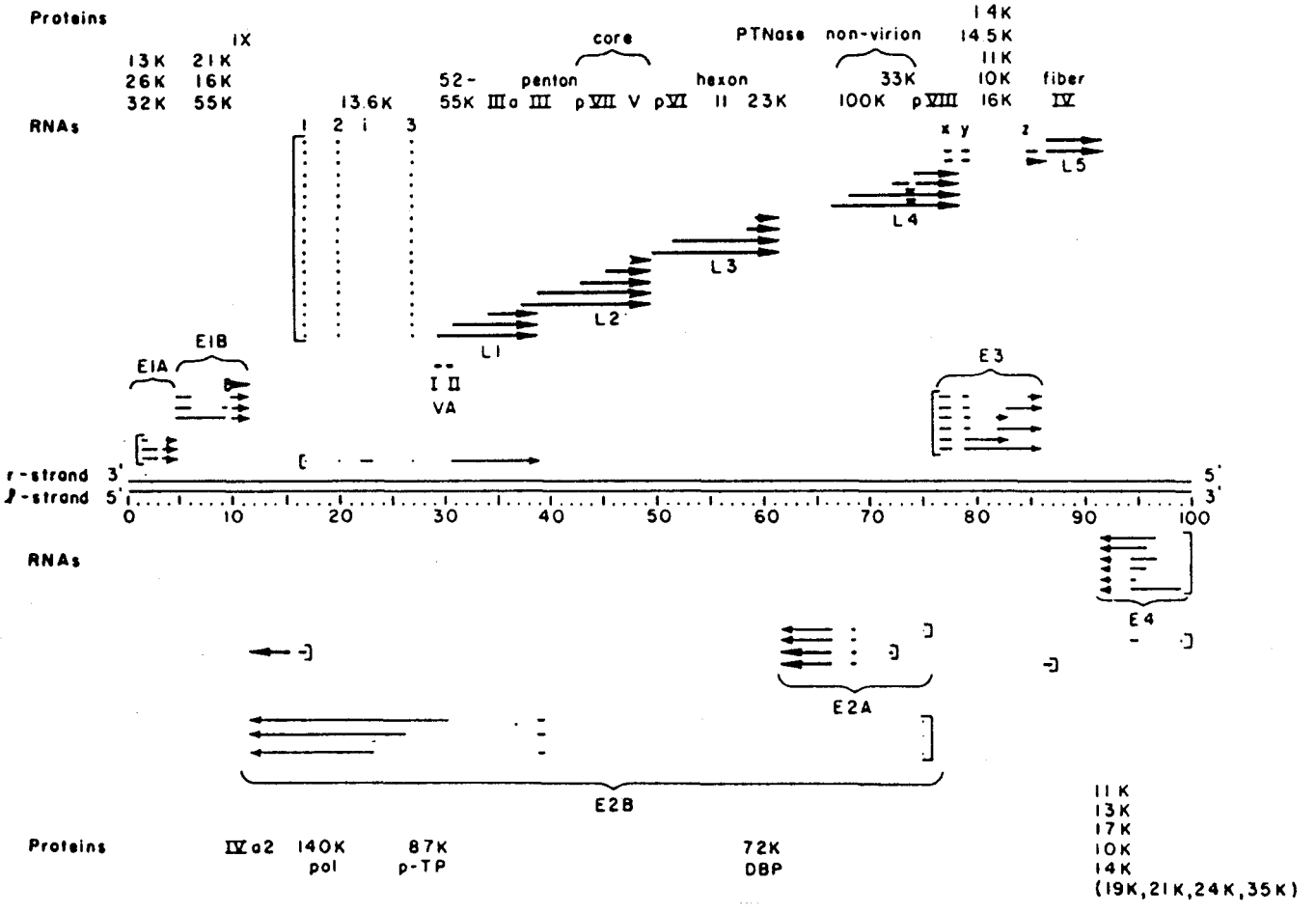

HEXON
PENTON BASE
GROUPS OF NINE HEXONS
PENTON
PERIPENTONAL HEXONS
FIBER

between the hexons (van Oostrum and Burnett, 1985). A penton is composed of multimers of penton base polypeptide, fibre polypeptide, and five hexon molecules (van Oostrum and Burnett, 1985).

The viral genome is a linear double-stranded DNA molecule of approximately 36 kb (Fig. 2) which exists in a nucleosome-like structure inside the viral capsid (Sussenbach, 1984). The ends of the DNA molecule are characterized by short inverted terminal repeat sequences ranging in length from 103 bp (Ad2, Ad5) to 163 bp (Ad12) (Sussenbach, 1984) and a 55 kd protein which is attached to the 5' dCMP molecule at the very end of each ITR sequence through a covalent bond (Desiderio and Kelly Jr., 1981). The genome is divided into left and right halves based on high GC content and low GC content respectively, and is designated to be 100 map units (mu) long (Sussenbach, 1984). The two strands of the double helix are separated into l- and r-strands based on leftward and rightward transcription, respectively, and serve as the template for nine RNA polymerase II transcription units, five expressed early in infection and four expressed either during or after viral DNA replication (Fig. 2). There are also two RNA polymerase III transcription units, virus-associated RNA-I and II (Sharp, 1984).

FIGURE 2. Transcription map of adenovirus serotype 5.

The linear genome is divided into 100 map units, and separated into r- and l-strands for rightward or leftward transcription respectively. There are 10 transcription units distributed through out the genome, serving as templates in viral mRNA transcription. The viral transcripts are classified into early and late mRNAs based on their time of appearance during infection. The early mRNAs are transcribed before viral DNA replication from five genes E1a, E1b, E2, E3, and E4, and the late mRNAs are transcribed after viral DNA replication from five transcription units, IX, IVa2,E2(late), virus-associated RNA I and II, and major late promoter. Promoter elements are represented by brackets and 3' polyadenylation sites are represented by arrowheads. Intervening sequences removed during processing of the primary transcripts are indicated by gaps in the arrows representing the mRNAs. Molecular weights and Roman numerals represent viral proteins. From Pettersson and Roberts (1986).


Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
52-55K IIIa
penton III
core pVII V pVI
hexon II 23K
PTNase
non-virion 33K 100K pVIII
14K 14.5K 11K 10K 16K
fiber IV
RNAs
1 2 i 3
x y z
L1 L2 L3 L4 L5
E1A E1B
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)

1.1.3 Lytic Infection.

A productive infection by adenoviruses occurs in cells permissive for viral DNA replication and late gene expression, and in non-permissive cells a block in either of these two steps leads to non-productive infection characterized by negligible viral yields (reviewed in Graham, 1984a; Johnston et al, 1985). In a semi-permissive infection, the virus yield is significantly below the output from a permissive infection.

Lytic infection begins with the virus adsorbing to the cell membrane, a function that likely involves the fibre, hexon and penton proteins of the icosahedral capsid (Falgout and Ketner, 1987; Wohlfart et al, 1985). After entering the cell, the viral capsid is removed and the viral core with its DNA enters the nucleus where the viral genes are transcribed temporally by DNA-dependent RNA polymerase II and DNA-dependent RNA polymerase III and where the DNA is replicated. These transcripts are grouped into four classes based on their time of appearance after infection; immediate-early, early, intermediate and late (detailed below). The immediate-early transcripts appear first after the viral genome reaches the cell nucleus, early transcripts generally appear before the viral DNA replication cycle and late transcripts appear after the viral DNA replication cycle. The intermediate transcripts appear during viral DNA replication. The synthesis of late gene products is followed by encapsidation of the viral DNA synthesized de novo.

1.1.3.1 Early Gene Expression.

There are five transcription units transcribed by RNA polymerase II before the onset of viral DNA replication. These five early genes can be divided into two classes, the immediate-early gene Ela and the early genes Elb, E2 early, E3 and E4 (Fig. 2) (Berk, 1986a).

1.1.3.1.1 Region El.

The Ela gene (1.3 - 4.2 mu), regulated by enhancers and a promoter positioned between nucleotides 200 and 480, is transcribed from the r-strand beginning approximately a half-hour to an hour after infection. To date, five mRNAs (13S, 12S, 11S, 10S, and 9S) with a common 5' start (position 499) and 3' termination (position 1632) have been mapped to the Ela gene, all produced from a single primary transcript through differential splicing (Berk and Sharp, 1978; Perricaudet et al, 1979; Stephens and Harlow, 1987; Ulfendahl et al, 1987) (Fig. 3). All five mRNAs have a common 3' splice acceptor at nucleotide 1229 but one of two 5' splice donors and in some messages (11S and 10S), a second intron is removed (Stephens and Harlow, 1987; Ulfendahl et al, 1987; Perricaudet et al, 1979). Each of the five mRNAs produce one protein (289R, 243R, 217R, 171R, and 55R respectively) and it appears that at least the two larger Ela proteins and probably all are post-translationally modified, possibly by phosphorylation of serine residues (Tremblay, Ph.D. Thesis, McMaster University, 1988). The Ela proteins are required in adenovirus transformation (reviewed in Berk, 1986b; Graham, 1984a;

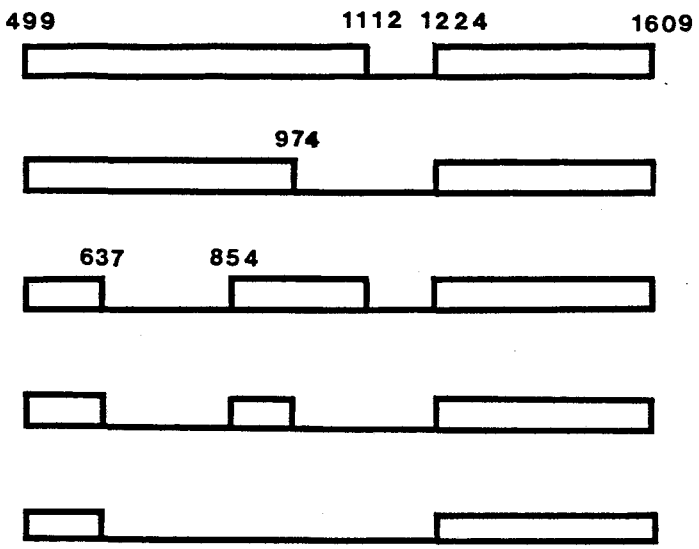
Branton et al, 1985), and are involved in transactivation of other adenovirus genes and some cellular genes such as the 70 kd heat-shock protein and beta-tubulin (reviewed in Berk, 1986b). As well as regulating the expression of other genes, the 13S product from E1a is believed to be involved in autoregulation (Hearing and Shenk, 1985).

The E1b transcription unit is located between 4.5 and 11.2 mu on the r-strand with upstream regulatory sequences, located in the coding sequences of the E1a gene (nucleotide 1350 - 1650), directing initiation of transcription at nucleotide 1702 (Parks et al, 1988). Five mRNA species are produced from a single primary transcript, two major (22S and 13S) and three minor, using an identical 3' splice acceptor, different 5' splice donors and removal of second introns (Fig. 3). Each of the four largest mRNAs produce one common (175R) and one unique polypeptide by initiating translation at different initiation codons and using different reading frames (Lewis and Anderson, 1987; Bos et al, 1981). Post-translational modification of E1b proteins has been observed for the 175R protein (amide linked acylation with palmitate and myristate - McGlade et al, 1987), and the 495R protein (phosphorylation - Malette et al, 1983). Mutation studies show E1b product(s) are required for both virus- and DNA-mediated oncogenic transformation of rodent cells and productive infection by adenoviruses (White and Stillman, 1987; Graham, 1984a). Also shown is that cells transformed with only the E1a region do not have the properties of fully transformed cells. Infection of cells by viruses with

FIGURE 3. Early region 1 mRNAs of human adenovirus group C serotypes.

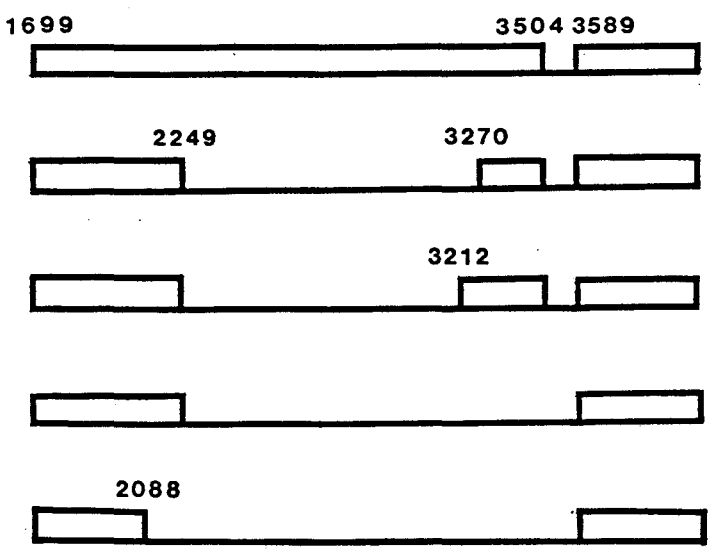
Early region 1 is composed of 2 transcription units E1a and E1b, both coding for five mRNAs. The E1a messages (13S, 12S, 11S, 10S, and 9S) are generated from a single primary transcript through differential splicing, and similarly the five E1b messages are also generated from a single primary transcript. Open blocks represent the sequences retained in the transcripts and the single lines represent sequences removed as introns. The numbers above each transcript indicate the genomic positions of the splice sites. The molecular weights of the polypeptide(s) made from each transcript are shown to the right of the mRNA sizes. See text for details.

E1A



13S	289R
12S	243R
11S	217R
10S	171R
9S	55R

E1B



22 S	175R
	495R
14 S	175R
	155R
14 S	175R
	92R
1.02 kb	175R
	82R
0.86 kb	168R

mutations in the E1b region results in degradation of total DNA with enhanced cytocidal effect (White and Stillman, 1987 and references therein; Graham, 1984a). Recent studies have shown that one of the products of E1b may be a regulatory protein, perhaps involved in the regulation of E1a expression. In particular, it has been suggested that the 19K product may have transactivating properties (Yoshida et al, 1987).

1.1.3.1.2 Region E2.

The E2 (early) gene extends from 75 mu to 11 mu on the 1-strand with a regulatory region that contains two TATA-box like elements at -30 and -50 and an enhancer-like element at -262. It produces three mRNAs early in infection from a single primary transcript through different splicing patterns (Berk, 1986a). The transcription is enhanced by the E1a 13S product (Nevins et al, 1979) through an E1a-activated cellular transcription factor E2F which has been identified in HeLa cells (Kovesdi et al, 1986). The three mRNAs produce one protein each that are essential to viral DNA replication: the 72 kd single strand DNA binding protein (DBP), the 80 kd pre-terminal protein, and the 140 kd viral DNA polymerase.

1.1.3.1.3 Region E3.

The E3 gene of adenovirus extends from 76 mu to 86 mu on the r-strand with regulatory sequences composed of a TATA box and a putative Sp1 binding site (Berk, 1986a and references within), and produces from a single primary transcript at least 8 mRNAs with identical 5' starts and one

of two 3' termini (Cladaras et al, 1985). Three proteins have been mapped to the E3 gene and they are the 19 kd glycoprotein (Ross and Levine, 1979), the 11.6 kd protein (Wold et al, 1984) and the 14.7 kd protein (Tollefson and Wold, 1988). Functions of the two latter proteins are unknown, but the former protein is found in association with the major histocompatibility complex class I antigens of mouse and man (Kvist et al, 1978). Although the function of this glycoprotein in viral replication is uncertain since adenovirus mutants with deletions in the E3 region grow as well as the wild type in tissue cultured cells, it may be required for persistent infection by adenoviruses (Burgert and Kvist, 1987).

1.1.3.1.4 Region E4.

The E4 gene is located between 91 mu and 100 mu on the 1-strand with a regulatory region composed of an Ela-like enhancer, and two putative and one identified Spl binding site (Berk, 1986a). Two proteins from the E4 transcription unit have been identified by immunoprecipitation and they are the 14 kd (or 11 kd) protein (Downey et al, 1983; Sarnow et al, 1982) and the 25 kd protein which is found in a complex with the Elb 58 kd protein (Sarnow et al, 1984). The E4 products are believed to be required for shut-off of host protein synthesis, transition of viral gene expression from early to late, and indirectly, viral DNA encapsidation (Weinberg and Ketner, 1986).

1.1.3.2 Late Gene Expression.

The late messenger RNAs of adenovirus are transcribed during or after viral DNA replication by RNA polymerase II or III (VA - RNA I and II only) and originate from six transcription units: protein IX from the 3' end of E1b, E2 (late), IVa2, VA - RNA I and II, and major late promoter (MLP) (Fig. 2). The major late promoter is located at 16.5 mu on the r-strand, directing very high levels of transcription, in part due to increased copy number of template from viral DNA replication (Thomas and Matthews, 1980), starting at 6039 in Ad2 and 6049 in Ad5 (Fig. 2). All the mRNAs from the MLP have the tripartite leader starting at 6039/6049 (Ad2/Ad5) spliced onto sequences near the translation initiation codon of each mRNA with one of five different polyadenylation signals. Based on the position of the polyadenylation signals the late mRNAs are grouped into five classes, L1 through to L5. The viral genes transcribed late in infection code for all of the proteins associated with the viral capsid as well as some non-structural polypeptides such as the viral protease, 100 kd polypeptide and the 33 kd phosphoprotein (Pettersson, 1984). Causes for non-permissive infections include the block of transcription from the MLP (Johnston et al, 1985), and the inability to transport mRNA to the cytoplasm.

1.2 DNA REPLICATION.

DNA replication is one the most important processes in molecular biology, required for faithful transmission of genetic information to daughter cells during mitosis and to

gametes during meiosis. Because DNA replication occurs in all dividing cells and its control may be altered in oncogenically transformed cells, the understanding of eukaryotic DNA replication is important not only for advances in studying cell proliferation and cell division, but also for understanding the mechanisms of oncogenic transformation.

While the study of DNA replication in higher eukaryotic cells has been hampered by the complexity of the system and the difficulty in isolating suitable mutants in DNA replication, the study of replication in lower eukaryotes has been more rewarding due to their relatively simple molecular biology. For analysis of DNA replication mechanisms in mammalian cells the replication of genomes of mammalian DNA viruses in cell culture has been used extensively as a model system for cellular DNA replication.

1.2.1 Eukaryotic DNA Replication.

Replication of a DNA molecule in prokaryotes is a complex enzymatic process requiring precise co-ordination between numerous proteins (reviewed by Nossal, 1983). In eukaryotes, this is further complicated by the chromatin structure and the size of the genome, requiring a very long S-phase to replicate the entire genome. To get around the first problem, the chromatin may undergo localized decondensation around the region of replication closely followed by condensation after replication. The second problem may be solved by dividing the eukaryotic genome into

many replication units or replicons, each with its own origin of replication (Darnell *et al*, 1986).

Eukaryotic DNA replication, like prokaryotic DNA replication, can be divided into three events; initiation, elongation, and termination. The majority of the current information about the molecular biology of eukaryotic DNA replication has come from studies of *in vitro* reconstituted systems and cell-free extract systems often utilizing viral chromosomes as templates.

The reconstitution studies using purified replication proteins suggest that initiation of DNA replication in eukaryotic cells is carried out by a primase that is a part of the replication complex called 'replicase', a multiprotein complex capable of supporting initiation and elongation of DNA templates. Primase in association with the replicase complex appears capable of producing the oligo-ribonucleotide primers required in both continuous and discontinuous replication (reviewed in Huberman, 1987).

The *cis* requirements for initiation of replication appear to vary depending on the source of the replication system used in the experiments. The *Xenopus* oocyte replication system used by Blow and Laskey (1986) shows no requirement for a specific *cis* acting sequence or DNA structure (Blow and Watson, 1987; Mechali and Kearsey, 1984) but the mouse cell system (Ariga *et al*, 1987) and other rodent cell systems (Carroll *et al*, 1987) do show such a requirement. This relaxed stringency for a specific origin of replication observed in the *Xenopus* oocyte replication system may reflect

the different level of developmental commitment between the oocytes and terminally differentiated transformed cells. On the other hand, the absence of requirement for autonomous replicating sequences (ARSs) in amphibian oocytes may be due to a higher level of plasticity exhibited by the average amphibian cell compared to the average mammalian cell or it may also be that the in vitro system does not faithfully mimic the in vivo situation.

The elongation step appears to be bidirectional and semi-conservative, and is probably carried out by DNA polymerase alpha and the auxiliary proteins of the replicase complex which provide topoisomerase and DNA ligase activities among others (Huberman, 1987).

The ends of linear cellular DNA present a problem in DNA replication because no polymerase enzyme has a 3' - 5' polymerase activity. Therefore without a primer at the very end of the DNA molecule, information at the ends would be lost after each round of DNA replication. A number of linear DNA viruses have overcome this problem by providing primers at the end(s) of their genome in different ways: parvoviruses use a 3' hairpin structure, and adenoviruses use a terminal protein linked to the terminal nucleotide. Although these two mechanisms have served as models for replication of cellular DNA telomeres, due to the unique structures of their respective viral DNA termini and of cellular telomeres, the models did not truely mimic the events in eukaryotic cells. Therefore, telomere replication in the ciliated protozoan Tetrahymena has been studied as a model for replication of

telomeres in higher eukaryotes because telomere structures are believed to be very similar.

A model for telomere replication has been proposed based on data gathered from replication of Tetrahymena telomeres in yeasts (Shampay et al, 1984). Based on this model (Fig. 4), telomere replication requires a terminal transferase-like activity (Greider and Blackburn, 1985) which adds specific sequences to the 3' ends of duplexes creating a 3' overhang. This 3' overhang can then act as a template for Okazaki replication of telomere sequences faithfully replicating the telomeres and accounting for tandem repeats of telomeric sequences that have been reported.

1.2.2 Replication of Eukaryotic DNA Viruses.

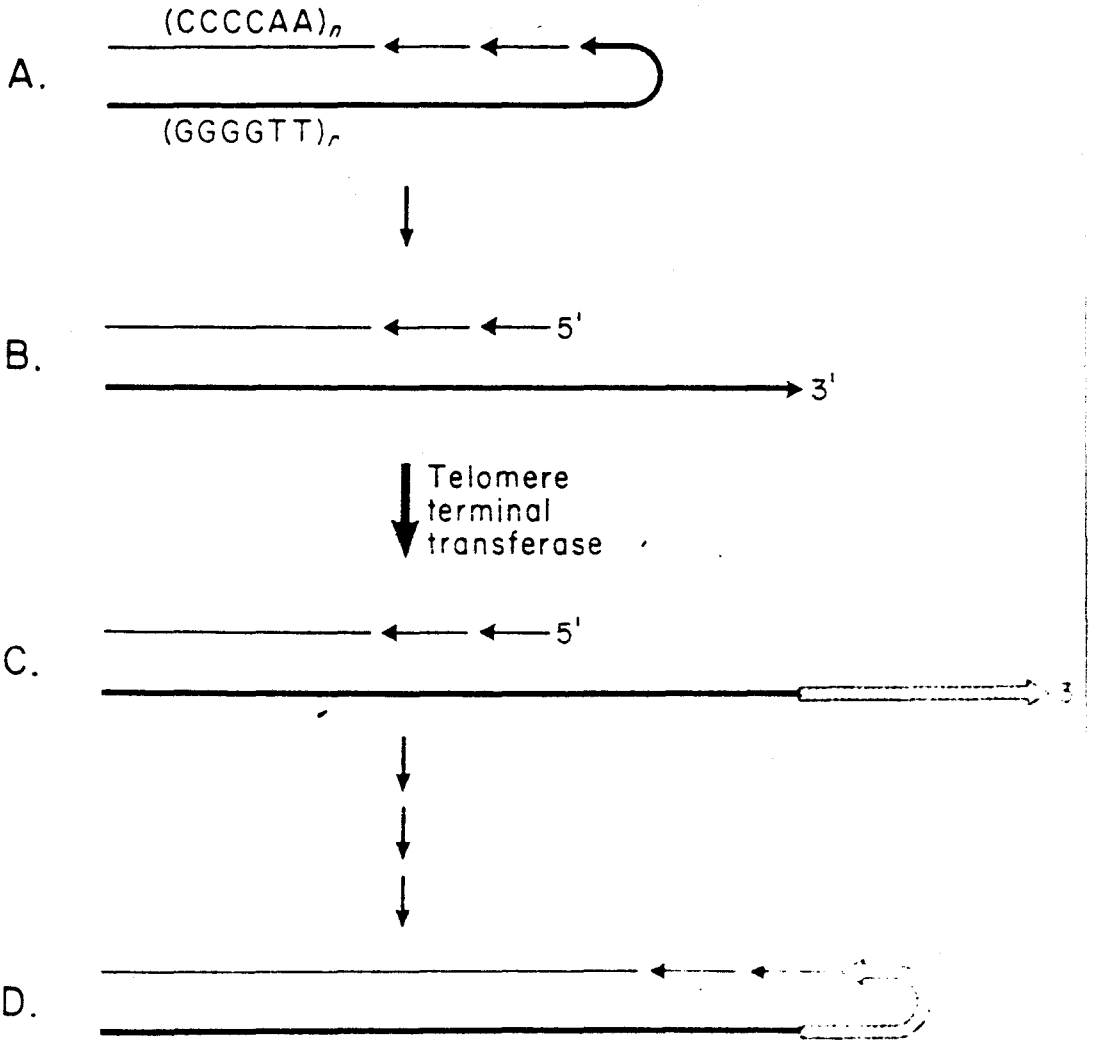
1.2.2.1 Parvovirus DNA Replication.

Parvovirus DNA replication has been used as a model in studying the replication of ends of linear, duplex DNA molecules, and therefore is reviewed in this section.

The autonomous parvovirus genome is a minus-sense, single-stranded DNA molecule approximately 5100 bp long with palindromic sequences at the two ends with no terminal protein. In adeno-associated viruses, a defective parvovirus, the genome is also single-stranded and linear, but both plus- and minus-sense strands are encapsidated into viral particles. The palindromes found at the ends of autonomous parvoviral DNA have been sequenced and analyzed by restriction digestion, and from the data two characteristics are evident.

FIGURE 4. Telomere replication in Tetrahymena.

The terminal hairpin structure (A) is removed by single-strand specific nicking which generates a 3' overhang (B). A telomere specific terminal transferase enzyme then extends the 3' overhang by addition of palindromic sequences (C). This palindrome allows the 3' overhang to snap back, producing a primer for elongation and regeneration of the telomere sequences (D).


A.
(CCCCAA)n
(GGGGTT)n
B.
5'
3'
Telomere
terminal
transferase
C.
5'
D.

First, the terminal palindromes are not repetitive as in the ITRs of adenoviruses and while the 3'palindrome is unique and exists in only one orientation for every strain the 5'palindrome exists in two orientations. Secondly, the ends of the genome form stable hairpin structures, with the 3' end likely forming a "T" or a "Y" structure (reviewed in Hauswirth, 1984). In adeno-associated viruses, the terminal palindromes are inverted repeats, containing large and small palindromic sequences. The smaller palindrome exists within the larger palindrome (Berns and Hauswirth, 1984). The replication of viral DNA occurs in actively dividing cells, likely associated with cellular chromosomes and requires viral proteins as well as cellular replication proteins.

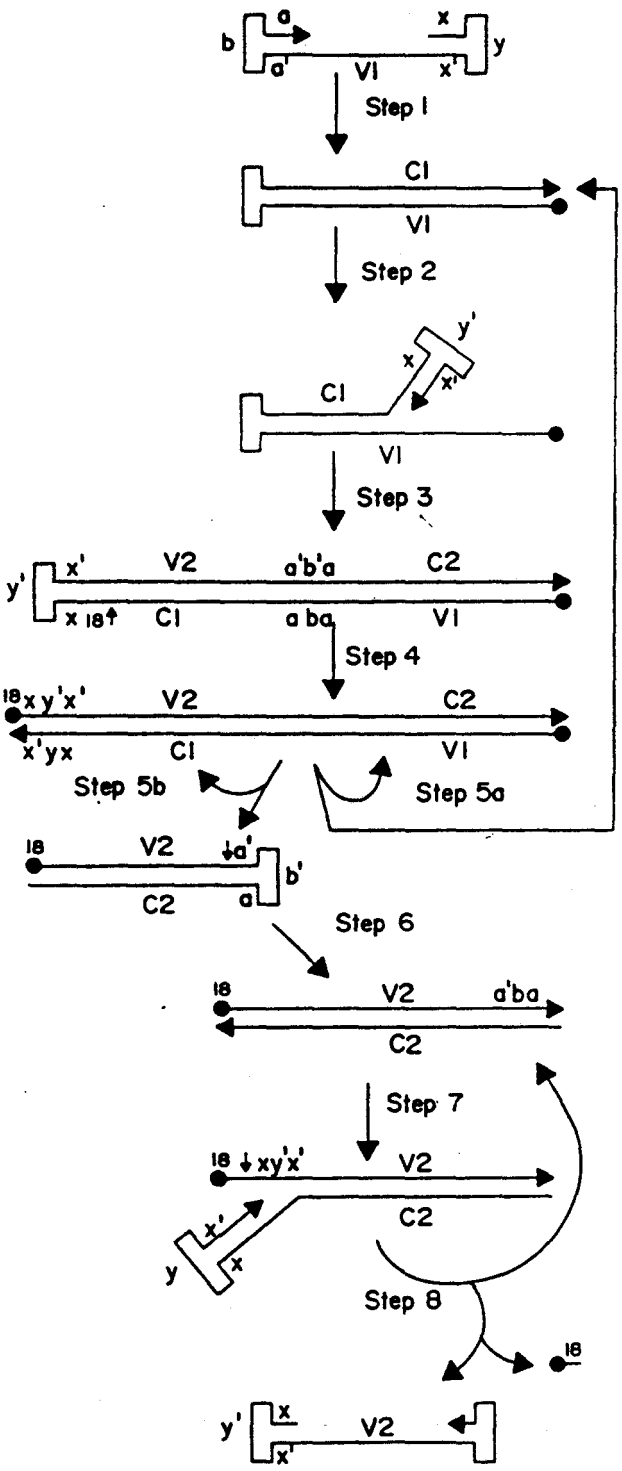
The initial step in replicating viral DNA is the formation of a double-stranded monomer by elongation from a primer created by the hairpin structure at the 3' terminus (Fig. 5). This duplex monomer RF is then converted to a dimer by strand displacement elongation from the hairpin structure at the 3'-OH of the monomer. The duplex dimer then goes on to regenerate itself and produce a monomer RF (Hauswirth, 1984). This monomer derived from the dimer is then used to generate viral DNA molecules with a unique 3' end sequence and 5' end sequences which can be in either of two possible orientations.

1.2.2.2 Adenovirus DNA Replication.

Viral DNA is replicated between early gene expression and late gene expression. The products of the E2-early gene

FIGURE 5. Model for Parvovirus replication.

The single-stranded parental genome (V1) is converted to a double-stranded dimer by 2 elongation events primed by the 3' terminal hairpin structure (Steps 1 to 3). The hairpin structure in the double-stranded dimer is removed by site-specific cleavage and repair (Step 4) and from this linear, double-stranded molecule two duplex monomers are generated (Steps 5a and 5b). The monomer from 5a is converted into the double-stranded dimer with the hairpin structure. The monomer generated through 5b serves as the template in the production of daughter viral strands. Solid triangles on DNA strands 'represent 3' ends, and solid circles represent covalently attached terminal protein molecules. The terminal repeat sequences are represented by xyx' and aba'. Figure taken from Hauswirth (1984).

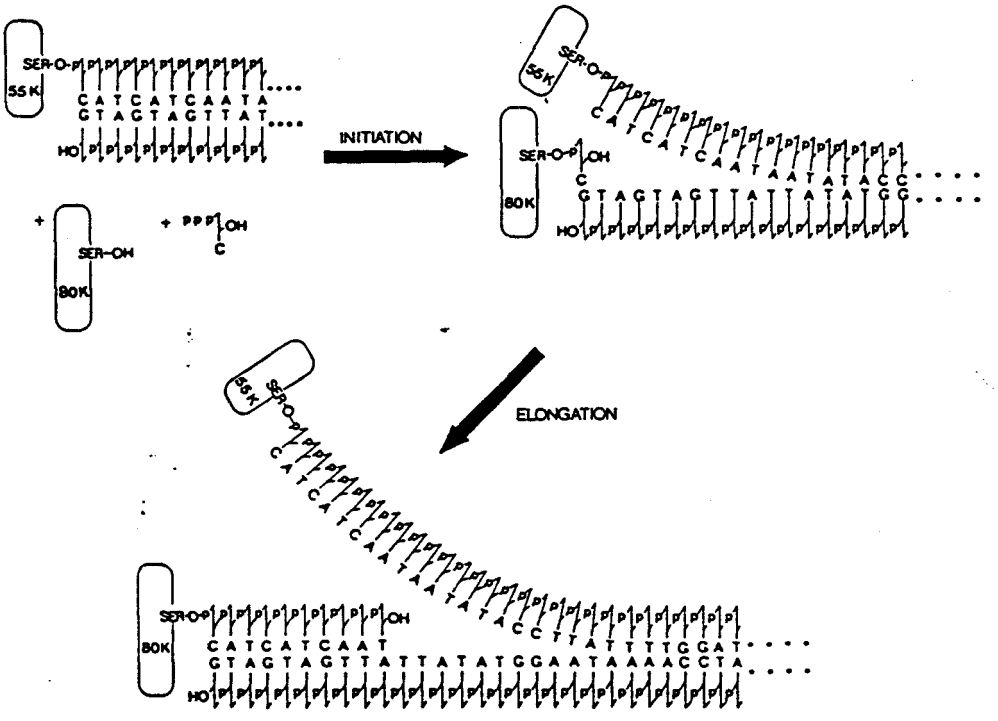

b
a
x
y
a'
V1
x'
Step 1
C1
V1
Step 2
y'
x
x'
C1
V1
Step 3
y'
x'
V2
a'b'a
C2
x 18
C1
a ba
V1
Step 4
18 x y'x'
V2
C2
x'yx
C1
V1
Step 5b
Step 5a
18
V2
a'
b'
C2
a
Step 6
18
V2
a'ba
C2
Step 7
18
xy'x'
V2
C2
x'
y
x
Step 8
18
y'
x
V2
x'

(see above) are necessary for DNA replication since mutations in this region abolish viral DNA replication, and temperature-sensitive mutants in the E2(early) gene produce significantly reduced amounts of viral DNA at the restrictive temperature, the extent of reduction being determined by the leakiness of the ts mutation (reviewed in Kelly Jr., 1984).

Replication of the viral DNA is initiated by formation of a protein primer composed of a virus-encoded 80 kd pre-terminal protein (pTP) and a dCMP molecule (Fig. 6), as demonstrated by Challberg and co-workers who showed that pTP is attached to 5' ends of newly synthesized viral DNA through a phosphodiester bond (Challberg et al, 1980), and the detection of pTP-dCMP complex synthesis in an in vitro replication system (Lichy et al, 1981). Formation of this primer complex requires the viral DNA polymerase to catalyze the transfer of dCMP to pTP as shown by the requirement for the 140 kd polymerase in complementation assays with partially purified pTP (Lichy et al, 1982). It also requires the presence of a single-stranded DNA template (Kelly Jr., 1984). Creation of a single-stranded region near the end of the ITR is likely carried out by nuclear factor I as demonstrated by its requirement in replication in the presence of adenovirus DBP (Nagata et al, 1982) and its ability to stimulate transcription (Jones et al, 1987). Priming of replication is then completed by the protein primer displacing a terminal protein-dCMP complex of the parental DNA under the guidance of viral DNA polymerase, viral DBP, and cellular nuclear factors. Replication then proceeds from the primer by strand

FIGURE 6. Initiation of adenovirus DNA replication.

The 80 kd preterminal protein (pTP) and a dCMP molecule are joined covalently de novo in the presence of viral DNA polymerase to form the pTP-dCMP complex. This complex then displaces the 5' strand of the infecting parental DNA by hybridizing to the terminal G in the 3' strand and initiates strand displacing elongation. Taken from Kelly Jr. (1984).


SER-O-P
55K
CATCATCAATA
GTAGTAGTTAT
HO
INITIATION
SER-OH
80K
+ PPP
OH
C
ELONGATION
CATCATCAATAATATACCTATTTTGGAT
GTAGTAGTTATTATATGGAATAAAACCTA

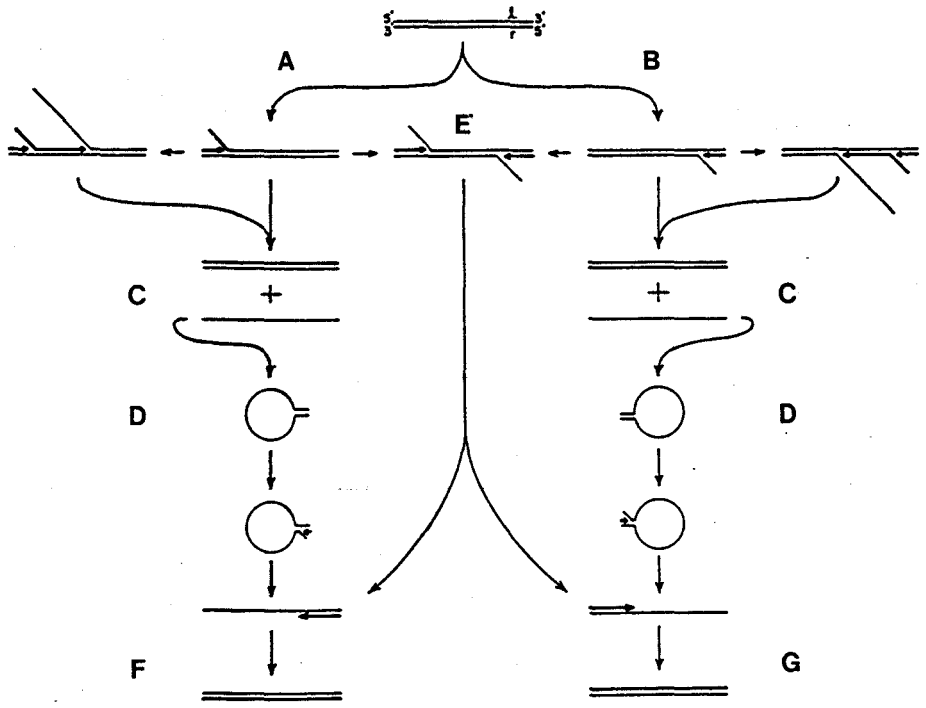
displacement elongation to produce the daughter molecule (Lechner and Kelly, 1977).

Viral DNA replication can be classified as Type I or Type II (Fig. 7). Type I replication (A and B) is characterized by initiation of viral DNA replication at either ITR followed by strand displacement elongation extending to the other ITR, thus generating a double-stranded daughter viral DNA molecule and a single-stranded parental molecule (C). The displaced ss-parental DNA is then replicated, perhaps by forming a panhandle structure with a double-stranded ITR region (D) to facilitate initiation of replication, followed by elongation to generate the second double-stranded daughter molecule (F and G). Type II replication is characterized by simultaneous initiation of replication at both ITRs (E), followed by strand displacement elongation giving rise to two linear replicative intermediates (F and G). Completion of the elongation process generates the two daughter viral DNA molecules.

Replication of all adenoviruses require the ITR sequences at the ends of the linear genome (Kelly Jr., 1984). Within the ITR there is a minimum sequence requirement for replication which varies between serotypes (Hay, 1985) and between *in vivo* and *in vitro* replication systems (Hay and McDougall, 1986; Wides *et al*, 1987). The results from *in vivo* studies with viruses that have deletions in the ITR suggest only the first 36 to 46 bp are required for efficient viral DNA replication (Hay and McDougall, 1986), whereas *in vitro* studies have shown that the 3' boundary for the origin of

FIGURE 7. The adenovirus replication pathway.

Type I replication is initiated at either the left (A) or the right (B) ITR, followed by strand displacing elongation which generates one double-stranded daughter molecule and one single-stranded parental molecule (C). The single-stranded parental DNA then forms a panhandle structure and serves as a template for type II replication (D). Type II replication is characterized by initiation at both ITRs (E) followed by strand displacing elongation to produce the two replicative intermediates (F and G) which become double-stranded daughter viral DNA molecules. Reprinted from Lechner and Kelly (1977).


A
B
E
C
+
+
C
D
D
F
G

replication lies between nucleotide 46 and 51 (Wides et al, 1987; Rosenfeld et al, 1987). The 51 bp in vitro origin of replication can be separated into 3 distinct blocks stretching from position 1 - 18 (A), 19 -40 (B), and 41 - 51 (C) (Fig. 8). Based on deletion studies, it appears that A is essential for replication and represents the minimal origin of replication, while B and C increase the efficiency of replication.

Both virus-encoded and host-encoded factors required for efficient initiation of viral DNA replication have been identified and some have been purified to homogeneity (Rosenfeld et al, 1987; Rosenfeld and Kelly Jr., 1986; Kelly Jr., 1984; Nagata et al, 1982). The viral proteins are the 140 kd viral DNA polymerase, 80 kd preterminal protein, and 72 kd DNA binding protein, all from the early region 2 transcription unit. The cellular proteins include topoisomerase I, nuclear factor I - a cellular nuclear protein from uninfected HeLa cells (Nagata et al, 1983; Wides et al, 1987), and origin recognition proteins-A (ORP-A) and -C (Rosenfeld et al, 1987) (ORP-C, also called nuclear factor III, Pruijn et al, 1986). NF-I binds to the B sequences (Wides et al, 1987) to participate in initiation of DNA replication in the presence of Ad-DBP (Nagata et al, 1982), and ORP-A and ORP-C bind to A and C sequences respectively, to enhance the efficiency of replication (Rosenfeld et al, 1987).

1.2.2.3 Papovavirus DNA Replication.

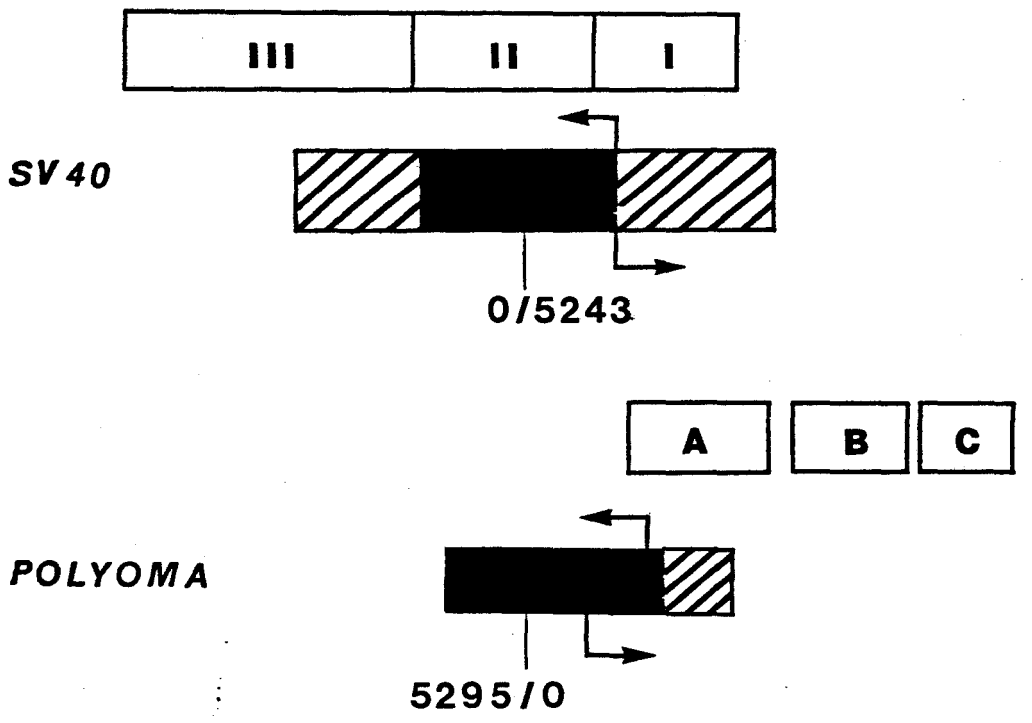
Papovavirus replication has served well as a model for replication of eukaryotic replicons because due to the limited coding capacity of their genome papovaviruses rely entirely on the cellular replication enzymes and auxiliary proteins with the exception of the large T-antigen (DePamphilis and Bradley, 1986).

SV40 and polyoma, the best characterized papovaviruses, are DNA tumor viruses with double-stranded, covalently closed circular genomes of about 5300 bp. Most of the information on papovavirus DNA replication has come from *in vivo* and *in vitro* studies on the replication of SV40 and polyoma viruses. By studying the replication of wild type and mutant viruses, both *trans* and *cis* factors necessary for viral DNA replication have been identified.

Initiation of replication occurs at a single origin of replication which is composed of two elements, the core sequences which are absolutely required for DNA replication and the auxiliary sequences which enhance the efficiency of replication (DePamphilis and Bradley, 1986). The core origin of SV40 is 64 bp long, extending from nucleotide 5209 through 5243/0 to 29 and is flanked by two blocks of auxiliary sequences at position 5164 to 5208 and 30 to 72. The polyomavirus core origin is 66 bp long, extending from position 5274 through 5295/0 to 44 with auxiliary sequences extending 20 bp toward the early gene side (Fig. 8) (DePamphilis and Bradley, 1986). These papovavirus replication origins share some similarities and have some

FIGURE 8. Origins of viral DNA replication.

The origins of viral DNA replication from three DNA tumor viruses are shown below. The papovavirus origin of replication contains a core sequence (solid box) which is necessary for DNA replication and an auxillary sequence(s) (hatched box) which enhances DNA replication. The positions of the core and the auxillary sequences are shown for SV40 and polyoma virus along with their T-antigen binding sites (open box) as reference. The adenovirus origin of replication has been localized to the first 51 nucleotides of the ITR and contains three distinct protein binding sequences A, B and C, of which A is necessary for replication and B and C enhance the efficiency of replication. See text for details.


III
II
I
SV40
0/5243
A
B
C
POLYOMA
5295/0
AD2/5
A
B
C
0
18
41
51

differences. The common structural features are a 14 bp inverted repeat, a 23 - 34 bp palindrome with high G-C content and a 15 - 20 bp A-T stretch, and the differences are the presence of a second palindrome in the SV40 core origin and a part of the enhancer sequence in the polyomavirus origin of replication (Hendrickson et al, 1987b; DePamphilis and Bradley, 1986). The mutants mapping to these regions are defective in viral DNA replication suggesting that these sequences are required for replication of SV40 and polyoma viral DNA (Tang et al, 1987; Prives et al, 1987; DePamphilis and Bradley, 1986; Luthman et al, 1984).

After initiation, parental DNA is replicated bidirectionally with continuous replication on the forward strand (3' to 5' on the template) and by discontinuous or Okazaki replication on the retrograde strand (5' to 3' on the template) (Hendrickson et al, 1987a). The site of transition from continuous replication to discontinuous replication has been mapped for the two viruses. In the SV40 virus, the transition points for the two strands are located directly opposite each other and are the junction between the core origin sequences and the auxiliary origin sequences (Hay and DePamphilis, 1982). In the polyoma virus, the transition points on the two strands are offset by 16 nucleotides with the transition point for the strand coding the early mRNAs downstream of the transition point for the late mRNA strand (Hendrickson et al, 1987b).

The elongation of the oligoribonucleotide primer requires DNA polymerase alpha, from mouse cells for polyoma

viruses and from HeLa cells for SV40 viruses suggesting that the host-range effect may be mediated through the DNA polymerase-primase complex (Murakami et al, 1986) and topoisomerase II, which appears to be unnecessary for chain elongation in SV40 DNA replication (Richter et al, 1987) but required in polyoma DNA replication (Sheinin et al, 1985). Termination of elongation can occur at several different specific sites in the viral DNA resulting in accumulation of the late replicative intermediates with gaps in the daughter DNA molecules which are filled either before or after sibling separation (decatenation) to generate either form I or form II daughter molecules. In both viruses, this separation requires the action of topoisomerase II (DePamphilis and Bradley, 1986).

1.4 RATIONALE FOR STUDY

Although it is widely accepted that the adenovirus genome replicates as a linear molecule in infected cells, there is evidence demonstrating that circular forms of the viral DNA also exist suggesting that these forms, along with the linear forms, may have some significance in the life cycle of the virus.

Fifteen years ago, non-covalently closed circular forms and linear concatamers of adenovirus DNA were detected in DNA samples extracted from infected cells, using procedures that left the terminal proteins attached to the ITRs of the linear viral DNA molecule. However, these novel forms were not detected in DNA samples extracted using a procedure that resulted in the removal of the terminal protein from the DNA

(Robinson and Bellett, 1974; Robinson et al, 1973). Based on these observations, Robinson and Bellett (1974) proposed that circular DNA-protein complexes had a role in viral DNA replication. This model has since been replaced by the linear replication model proposed by Lechner and Kelly Jr. (1977). Later, covalently closed circular forms of viral DNA were detected in DNA extracted from infected cells using a procedure that removed the terminal protein (Ruben et al, 1983). These circular forms were detected in infected cells as early as four hours pi in host range 1 infection of non-permissive primary baby rat kidney cells and 8 hours pi in wt infection of HeLa cells.' Utilizing its ability to circularize, the adenovirus genome was cloned into bacterial plasmids and subsequently these plasmids were shown to be infectious, that is, capable of generating infectious virus after transfection into 293 cells (Graham, 1984b). The specific infectivity of the plasmids was not significantly different from that of linear, deproteinized viral DNA (Graham, 1984b) suggesting that the ITR junction in these infectious plasmids was biologically active, perhaps serving as an origin of viral DNA replication.

The purpose of this study was to determine the biological activities of the viral junction in order to gain a better understanding for the role of circular forms of adenovirus DNA molecules in the molecular biology of adenovirus, specifically in viral DNA replication.

The approach we took to investigate the biological significance of the circular forms of adenovirus genome in

viral infection was to insert the viral junction from pFG140 (Graham, 1984b) internally into the linear genome. Although the efficient conversion of the infectious plasmids to infectious viruses suggested that at least in the former the viral junctions served efficiently as an origin of viral DNA replication, it did not provide any information about the relative importance of circular forms of adenovirus DNA in viral DNA replication during infection because only molecules which had successfully initiated DNA replication could go on to generate infectious virus. Due to this selection pressure, one could not determine whether during the course of a normal infection, circular forms are used efficiently during viral DNA replication. Therefore, it was reasoned that if viral junctions were inserted within the linear viral genome, we might obtain insights on biological function of circular forms by analyzing the effects of inserts in viral DNA structure during viral replication.

The results from this study suggested that embedded terminal sequences are biologically active in generating rearrangements of the viral genome mostly through replication through the panhandle formation. The results also suggested, however, that the circular forms of adenoviral DNA likely have an insignificant role in viral DNA replication.

2. METHODS AND MATERIALS

2.1 BACTERIAL CULTURE TECHNIQUES.

2.1.1 Bacterial Strains.

Bacterial strains Escherichia coli HMS174 and HB101 (Maniatis et al, 1982) were used as hosts for recombinant plasmid DNA molecules.

2.1.2 Propagation and Maintenance of Bacterial Strains.

Bacteria were propagated in both liquid and solid nutrient media. Bacteria harbouring plasmids with antibiotic resistance markers (beta-lactamase and/or neomycin phosphotransferase) were grown in Luria-Bertani (LB) broth (Gibco) supplemented with ampicillin (100 µg/ml, Ayerst Laboratories) and/or kanamycin sulfate (80 µg/ml, BMC) and bacteria used as recipients of recombinant plasmids were grown in LB broth containing no antibiotics. Bacterial stocks for long-term storage were prepared by mixing 2 mls of an overnight culture with 1 ml of 50% sterile glycerol and storing at -80°C.

2.1.3 Bacterial Transformation.

Transformation of bacteria (E. coli HMS174 or HB101) with DNA was carried out according to protocols described by either Hanahan (1983) or Mandel and Higa (1970) as indicated.

Using the Hanahan procedure, a 50 - 100 ml culture of SOB^{++} medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) was inoculated with 0.01 volume of an overnight culture in SOB^{++} and grown to log phase (OD_{550} = 0.45 - 0.55) at 37°C. The cells were cooled on ice for 15 min then centrifuged at 3000 rpm for 10 mins. The pellet was resuspended in 1/3 volume of ice cold Transformation Buffer (TB - 10 mM K-MES, pH 6.2; 100 mM RbCl; 45 mM $MnCl_2$; 10 mM $CaCl_2$; 3 mM Hexamine cobalt $IIICl_3$), incubated on ice for 20 mins, and repelleted by centrifugation under identical conditions. The pellet was resuspended in 1/12.5 original volume of TB and incubated on ice with 35 µl/ml of DMSO for 5 mins. To this mixture, 35 µl/ml of 2.25 M dithiothreotol (in 40 mM KOAc, pH 6.0) was added and incubated for 10 mins on ice, then incubated further for 5 mins on ice after adding 35 µl/ml DMSO. After DNA was added to the cells, the mixture was incubated on ice for 30 mins, then the cells were heat-shocked for 90 sec at 42°C and put on ice for 2 mins. The cells were diluted to 1/5 volume with SOB^{++}; 20 mM D-glucose and incubated at 37°C with agitation.

For transformation by the Mandel and Higa procedure (1970), bacteria were grown in LB broth inoculated with 0.01 volume of an overnight culture to log phase then pelleted by centrifuging for 10 min at 3000 rpm, 4°C. The pellet was then

resuspended to 1/2 the original culture volume in calcium chloride buffer (75 mM $CaCl_2$; 5 mM Tris-HCl, pH 7.6) and incubated on ice for 30 mins. Bacteria were again pelleted and resuspended in 1/25 volume of $CaCl_2$ buffer and incubated on ice overnight. The competent cells were incubated with the desired DNA at 0°C for 30 to 60 mins, then heat-shocked at 42°C for 2 mins. Heat-shocked cells were again incubated on ice for 30 mins, then diluted with LB Broth, and incubated at 37°C for 1 hr with shaking.

Transformed bacteria were plated onto LB agar plates with the appropriate antibiotic(s) and allowed to grow overnight at 37°C. The transformants were screened using the rapid alkaline-lysis procedure of Birnboim and Doly (1979) followed by restriction digestion with appropriate restriction endonuclease and gel electrophoresis.

2.1.4 Preparative Plasmid DNA Isolation.

Large quantities of plasmid DNA were prepared by a modified form of the Birnboim and Doly procedure followed by banding in a cesium chloride (Pharmacia Fine Chemicals) density gradient in the presence of ethidium bromide. Form I DNA was extracted from the gradient and the ethidium bromide was removed by repeated extractions with CsCl-saturated propanol. The plasmid DNA solution was mixed with 3 volumes of sterile 0.1X SSC (15 mM NaCl; 1.5 mM NaCitrate) and 8 volumes of cold 95% ethanol to precipitate the plasmid DNA. The DNA pellet was redissolved in 0.1X SSC and reprecipitated with 2 volumes of cold 95% ethanol to remove the remaining

CsCl prior to use. The pellet from the second ethanol precipitation was washed with 95% ethanol to remove remaining traces of salts and debris.

2.2 RECOMBINANT DNA TECHNIQUES.

2.2.1 Quantitation of DNA.

Plasmid DNA used in this study was quantified by either the modified diphenylamine assay (Giles and Myers, 1965) or by absorbance readings at 260 nm using the Beckman Du-7 Spectrophotometer.

2.2.2 DNA Fragment Isolation and Purification.

DNA molecules were extracted from agarose gels by a modification of the procedure of Girvitz et al (1980). Briefly, the procedure was as follows. A large amount (approx. 100 µg) of plasmid DNA was digested with the appropriate restriction endonuclease and electrophoresed in the dark in 1X Tris-Borate-EDTA buffer (45 mM Tris-base; 45 mM boric acid; 2 mM EDTA, pH 8.0) with 0.1 µg/ml EtBr. DNA bands were visualized under a long wavelength UV light and a cut was made just in front of the desired band into which a length of boiled dialysis tubing was inserted horizontally, eliminating air bubbles between the gel and the membrane. High voltage was applied to transfer the DNA from the gel matrix to the dialysis membrane and when the DNA had migrated from the gel to the membrane, the membrane was carefully lifted from the gel while the high voltage was maintained.

The DNA was eluted off the membrane by repeated washes with 200 µls of elution buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA) and the eluant was extracted once each with phenol and chloroform. The DNA was precipitated with 1/2 volume of 7.5 M NH_4Ac and 3 volumes of cold 95% ethanol. After centrifugation the pellet was washed twice with 95% ethanol, dried at 37°C and resuspended in 0.1X SSC.

2.2.3 DNA Modifications.

For the purpose of constructing recombinant plasmids used in this study, in some experiments the termini of DNA fragments were modified with DNA modifying enzymes.

2.2.3.1 Removal of 5' or 3' Overhangs.

5' overhangs left by some restriction endonucleases (eg. XbaI) were filled in using the Klenow fragment and dNTPs. The Klenow fragment is the large fragment from subtilisin digestion of E. coli DNA Polymerase I and contains 5' - 3' polymerase activity and 3' - 5' exonuclease activity (Maniatis et al, 1982). 3' overhangs were removed by the 3' - 5' exonuclease activity of T4 DNA Polymerase enzyme (Maniatis et al, 1982). The enzymes were inactivated by heating the reaction for 30 mins at 65°C and excess deoxynucleotides and salts were removed by microdialysis using a 0.05 micron Millipore filter (VM) against 10 mM Tris-HCl,1 mM EDTA (TE buffer) at room temperature for 30 - 60 mins or phenol extraction and ethanol precipitation if the quantity of DNA was sufficient.

2.2.3.2 Dephosphorylation.

Removal of 5'-phosphate groups from the vector DNA molecules used in construction of recombinant DNA molecules was carried out by incubating the vector DNA with Calf Intestinal Alkaline Phosphatase (CIAP, BMC) according to manufacturer's recommendations. The enzyme was inactivated by adding EGTA to a final concentration of 5 mM and heating for 30 mins at 65°C. The mixture was desalted by microdialysis or phenol extraction followed by ethanol precipitation.

2.2.3.3 Ligation.

Ligations of DNA fragments were performed overnight at 14°C using T4 DNA Ligase (BRL) in ligation buffer (37.5 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 10 mM Dithiothreotol; 1 mM Spermidine; 100 µg/ml BSA), with 1 mM ATP (added to the buffer at ligation). The concentrations of insert and vector in the ligation reactions were adjusted according to the recommendations of Legerski and Robberson (1985). With some reactions, an aliquot of the reaction was analyzed by agarose gel electrophoresis before use in a bacterial transformation.

2.3 MAMMALIAN CELL CULTURE TECHNIQUES.

2.3.1 Cell Lines.

293 cells were used in this study either as a monolayer culture or as a spinner culture. The 293 cells are Ad5 transformed human embryonic kidney cells containing the left 12% (E1 gene) of the Ad5 genome stably integrated in the cellular genome and expressing those E1 proteins required to maintain the fully transformed phenotype (Aiello et al, 1979; Graham et al, 1977). Due to the constitutive expression of the E1 genes and efficient uptake of calcium phosphate-DNA complexes, this cell line is ideal for rescuing viruses from transfected viral DNA and for propagation of viruses unable to express E1 proteins. 293 cells for the spinner cultures (293N3S) were derived from the 293 cells by three successive passages in nude mice (Graham, 1987). The two cell lines were maintained in Joklik's medium containing 10% heat-inactivated horse serum (Gibco), 1% L-glutamine, and 1% Penicillin-Streptomycin (Gibco) at 37°C in a carbon dioxide incubator (5%). The 293 cells were passaged at 70 - 90% confluency at either 1:2 or 1:3 ratio and the 293N3S cells were passaged in 1:10 ratio.

2.3.2 Tranfection of Cells in Monolayer.

Transfection of DNA into 293 cells was carried out according to the $CaPO_4$ precipitate technique of Graham and van der Eb (1973). Briefly, sample DNA and carrier DNA (sheared hamster genomic DNA) in 1X HEPES buffer (pH 7.4) were mixed

with 0.125 M $CaCl_2$ and incubated for 20 mins at room temperature to form a precipitate of DNA and $CaPO_4$. A 0.5 ml aliquot of this mixture was overlayed on to a monolayer of cells at 60 - 80% confluency in 60 mm tissue culture dish containing 5 mls medium and incubated for 4 - 5 hrs at 37°C. The medium was removed by aspiration and the cells were overlayed with 10 mls F-11 containing 0.5% agarose and 5% horse serum, then incubated at 37°C and observed for plaques. Viruses isolated from plaques were stored in 1X PBS^{++} - 10% glycerol at -70°C.

2.3.3 Infection of Cells in Monolayer.

Viral infections were carried out in 60 mm dishes containing a monolayer of cells at 70 - 90% confluency. Medium from the dish was aspirated and the desired moi of the virus in 1X PBS^{++} was put on the cells and allowed to adsorb at 37°C for 30 - 45 mins. Monolayers were refed with fresh medium and incubated until the desired cpe was reached at which time cells were harvested for either virus or DNA.

2.3.4 Infection of Spinner Cells.

At a cell density of 2 - 3 $X10^5$ cells/ml, 293N3S spinner cells were pelleted by centrifugation, then resuspended in 0.1X original volume of fresh medium. Viruses were added to the cells at an moi of 10 pfu/cell and allowed to adsorb for one hour at 37°C while spinning. The volume was then expanded to the original volume using 50% fresh medium and 50% medium from the original culture and the infection was

monitored by staining for inclusion bodies. When 90 - 100% of the cells contained inclusion bodies, the infected cells were pelleted by centrifugation, resuspended in 0.1 M Tris-HCl (pH 7.5) (10 ml/1L culture) and stored at -70°C.

2.3.5 Inclusion Body Staining.

Inclusion body staining was carried out to determine the progress of the infection in 293N3S suspension cultures. A 5 ml sample of the infected cells was pelleted by low speed centrifugation (10 mins at 1000 rpm, 4°C) and the medium was removed by aspiration. The pellet was resuspended in 0.5 ml of 1% sodium citrate and incubated for 10 mins at room temperature. The cells were then fixed by adding 0.5 ml of Carnoy fixative (3:1, methanol:glacial acetic acid) and incubating for 10 mins at room temperature. The final volume was brought up to 3 mls by adding 2 mls of Carnoy fixative and the cells were again pelleted by low speed centrifugation. The supernatant was removed by aspiration and the pellet was resuspended in 3 drops of Carnoy fixative. A drop of the suspension was put on a glass slide and dried for at least one hour at room temperature. The cells were stained with a few drops of 2% orcein in glacial acetic acid, covered with a glass cover slip and examined under phase-contrast microscope. The percentage of cells infected as reflected by the number of cells with stained inclusion bodies was noted and if the percent inclusion body was greater than about 90% the cells were harvested.

2.3.6 Virus Titration.

Virus stocks were titrated on monolayer cultures of 293 cells in 60 mm dishes after they were freeze-thawed three times. Serial dilutions were prepared in 1X PBS^{++}. Each 60 mm dish was infected with either 0.20 or 0.25 ml of virus (four dishes/ dilution), adsorbed for 30 to 40 min at 37°C, then overlayed with 10 mls F-11 containing 0.5% agarose-5% horse serum. The dishes were incubated at 37°C, 5% CO_2 for seven days, then scored for plaques by peeling off the agarose overlay and staining and fixing the remaining monolayer cells with crystal violet in formaldehyde and methanol. Excess stain was removed by washing in water, and the dishes were dried at 37°C overnight.

2.3.7 CsCl Density Gradient Banding of Virus.

Suspension culture 293N3S cells were grown to a concentration of 2 - $3X10^5$ cells/ml in spinner flasks and infected at an moi of 10 pfu/cell. Progress of infection was monitored by inclusion body staining, and the cells were harvested by centrifugation (10 mins at 5000 rpm, 4°C) in Cryofuge-8000 (Heraeus-Christ, CanLab Inc.) with swinging bucket rotor. The pellet was resuspended in 10 mls of 0.1 M Tris-HCl (pH 7.5) per 1 L of cells and stored at -70°C until banding.

Frozen infected cells were thawed on ice, ruptured by sonication (5 X 30 sec pulses) on ice, after which divalent cations were added (5 mM $MgCl_2$; 5 mM $CaCl_2$). To this mixture, DNase and RNase (Sigma Chemicals) were added to a final

concentration of 50 µg/ml each and incubated for 90 mins at 37°C. To this cocktail, 1 ml of 5% sodium deoxycholate was added and incubated on ice for 20 mins then put through an Ultra-Turrax tissuemizer (Janke-Kunkel Ika-Werk, Telmak Co.) until the viscosity of the mixture was reduced to slightly higher than the viscosity of water. CsCl-saturated 0.1 M Tris-HCl (pH 7.5) was added to the mixture at a ratio of 1.8 mls CsCl to 3.2 mls virus then put into a 12.5 mls Beckman Polyallomer Ultracentifuge tube and centrifuged for 20 hrs at 35,000 rpm (4°C) in a Beckman 50Ti rotor. Tubes were removed and visually checked for an opaque band made up of virions containing full-size genome.

2.3.8 Fractionation of CsCl Gradients.

500 µl fractions from the gradient were collected dropwise from the bottom, and the fractions containing the viral band were noted. Fractions were then dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA on ice overnight and collected in 1.5 ml eppendorf tubes. DNA from each aliquot was extracted according to the protocol below.

2.3.9 Viral DNA Extractions.

Viral DNA was extracted either from infected cells in monolayer, or from banded viral particles. Viral DNA samples from banded virions were prepared by treating an aliquot of the dialyzed samples with pronase-SDS (1 mg/ml pronase; 0.4% SDS) in lysis buffer (10 mM Tris-HCl, pH 8.0; 10 mM Na_2EDTA; 0.4% SDS) for 3 - 4 hrs at 37°C. Pronase-SDS treated samples were phenol extracted once, and the supernatant was mixed with 3 volumes of cold 95% ethanol and 1/2 volume of 7.5 M ammonium acetate to precipitate the DNA. The DNA precipitates were pelleted by centrifugation in an Eppendorf centrifuge, washed twice with 95% ethanol, dried at 37°C, then resuspended in 50 - 100 µls of 0.1X SSC and stored at 4°C.

The protocol for preparation of viral DNA from infected cells (total intracellular DNA) was identical to that for banded viruses except for the following modifications. The lysis buffer was not supplemented with SDS, and 7.5 M ammonium acetate was not used during the initial precipitation of DNA.

2.4 ANALYSIS OF DNA.

2.4.1 Restriction Endonuclease Digestion.

DNA samples were digested with restriction endonucleases as indicated in the text following the manufacturers' recommendations. The endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim Canada, New England Biolabs, or Pharmacia Chemicals.

2.4.2 Agarose Gel Electrophoresis.

DNA samples were analyzed after digestion by restriction endonuclease(s) by agarose gel electrophoresis in 1X Tris-Acetate-EDTA (40 mM Tris-acetate; 2 mM EDTA) buffer containing 0.1 µg/ml of ethidium bromide as a DNA stain. Gels were electrophoresed under a current of 1.5 V/cm with wtAd5 DNA digested with HindIII as a molecular weight marker. DNA bands in the gel were visualized by exposure to short wavelength UV radiation (Fotodyne UV Transilluminator Model 3-3000) and permanent records of the gels were made by photographing the gels with Polaroid MP4 Land Camera/545 Land Film Holder/Toshiba R60 filter assembly on to Polaroid 57 Land Film.

2.4.3 Southern Blot Hybridization.

2.4.3.1 Transfer of DNA.

DNA was tranferred from the agarose horizontal slab gels to nitrocellulose membranes by the method of Southern (1975). Gels containing DNA fragments over 15 kb were treated with 0.25 M HCl for 5 - 10 mins to allow depurination of the DNA. The denatured DNA molecules were transferred by capillary action overnight at room temperature, then fixed onto the membrane by heating at 80°C for two hrs.

2.4.3.2 Hybridization and Autoradiography.

Hybridizations of Southern blotted membranes with nick-translated probes were carried out according to the

procedure of Southern (1975) with modifications as suggested by the membrane manufacturer (Transfer and Immobilization of Nucleic Acids to S&S Solid Supports, Schleicher and Schuell Inc., 1987). Briefly, membranes pre-soaked in 1X SSPE (0.18 M NaCl; 10 mM $NaPO_4$, pH 7.7; 1 mM EDTA); 0.1% SDS were incubated in pre-hybridization solution [5X SSPE; 5X Denhardt's solution (0.1% Ficoll, polyvinylpyrrolidone, BSA); 50% deionized formamide, (EM Science); 0.5% SDS; 200 µg/ml sheared, denatured salmon sperm DNA] overnight at 42°C with shaking, then hybridization was carried out in the same buffer by adding alkali denatured probe to a final concentration of 10^6 cpm/ml and incubating overnight at 42°C.

Hybridizations of membranes with end-labelled oligonucleotides were performed as recommended by the manufacturer (Schleicher and Schuell). Membranes were pre-hybridized for a minimum of 2 hrs, and hybridizations were carried out overnight using 200 µg/ml tRNA and 5X Denhardt's solution in the hybridization solution. Membranes were washed in 0.1X SSPE; 0.1% SDS at the appropriate temperature and dried at room temperature. Autoradiographs of the membranes were produced using Kodak X-Omat RP-1 or AR-5 with an intensifying screen at -80°C.

2.4.4 Oligonucleotides.

Oligonucleotides used in this study were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada.

2.4.5 Radio-isotope Labelling of DNA.

All probe DNAs were radioactively labelled with ^{32}-P, purchased from New England Nuclear or ICN Biochemicals. The probe DNAs were nick-translated according to Rigby et al (1977) using α-^{32}P-dCTP and unincorporated isotopes were separated from labelled DNA using Sephadex G-50 (Pharmacia Fine Chemicals) spin-columns (Maniatis et al, 1982). Oligonucleotides were end-labelled (Maniatis et al, 1982) using γ-^{32}P-ATP and unincorporated isotopes were separated from the oligonucleotides by chromatography through a Sephadex G-50 column.

2.4.6 Quantitation of Autoradiographs.

2.4.6.1 Measurement of Optical Density.

Quantitative analyses of autoradiographs were performed by measuring the optical densities of the DNA bands with a Hoefer Scanning Densitometer, Model GS-300 (courtesy of Dr. R. Rachubinski, Dept. of Biochemistry, McMaster University). The areas under the peaks were calculated by the accompanying software GS-350 with a Zenith Data Systems PC micro-computer.

2.4.6.2 Pre-flashing of X-ray Films.

Autoradiographs to be scanned by the densitometer were produced on fresh X-Omat AR-5 X-ray films (Kodak) pre-flashed to raise the background optical density at 550 nm to 0.2 OD units as recommended by Laskey and Mills (1977). X-ray films were pre-flashed using a Simmon Omega D2 photography enlarger with the diaphragm of the condenser placed 89 cm above the X-ray film. An X-ray film was placed on top of the yellow paper sleeve from film containers, and exposed to filtered white light for 0.2 sec at aperture setting of f/32. The pre-flashed films were immediately exposed to hybridized membranes at -80°C.

2.4.6.3 Liquid Scintillation Counting.

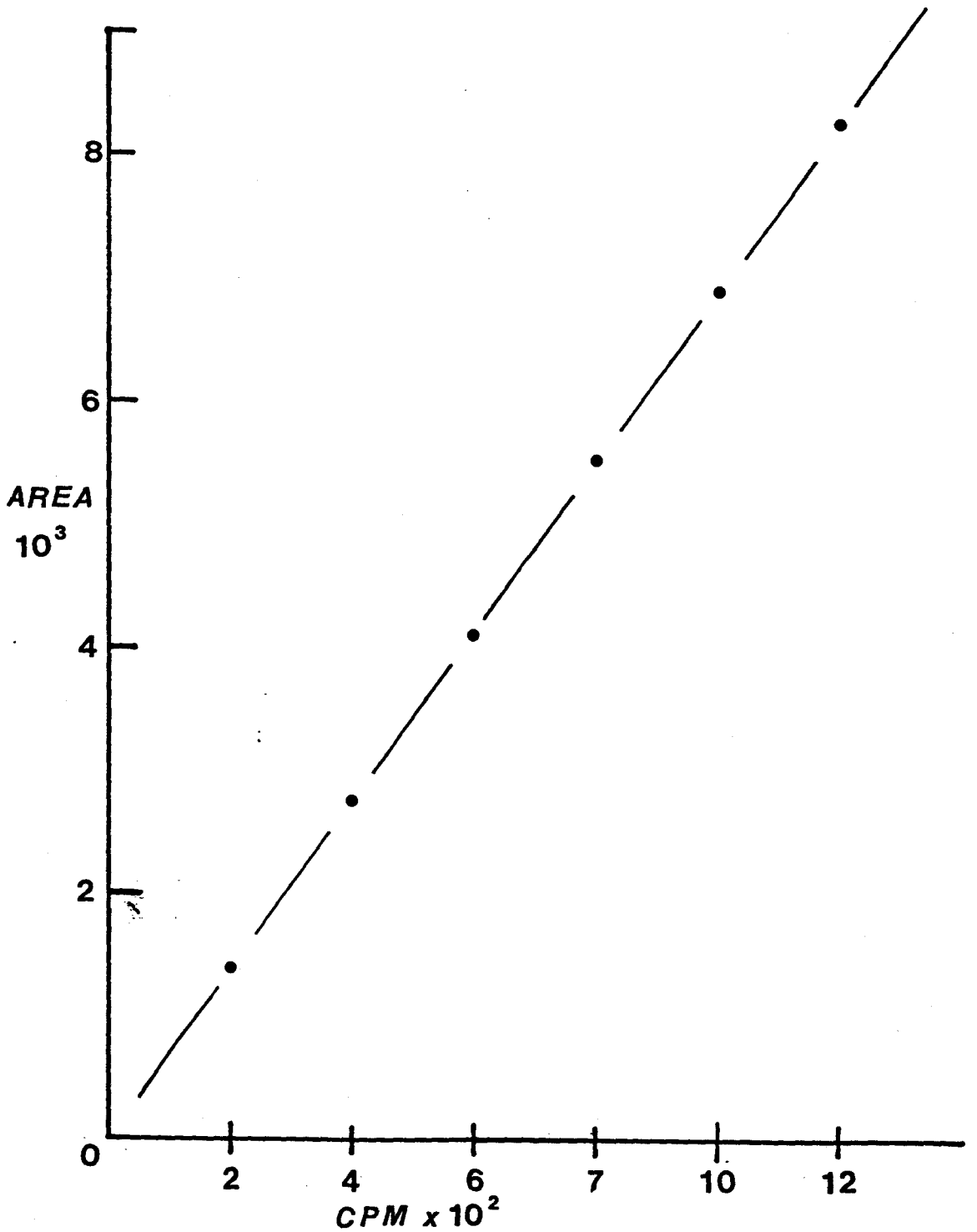
Sections of the nitrocellulose membrane with the marker DNA were excised and placed in 4 mls of toluene based scintillation fluid (0.4% Omnifluor, w/v). Each sample was counted ten times in a Beckman Liquid Scintillation Counter, Model LS-1801 and the arithmetic mean value was used in the establishment of a standard curve.

2.4.6.4 Standard Curve Plot.

A graph of cpm versus optical density (represented as area under a peak in a scan) was plotted to establish a standard curve required for determining cpm values from a given area (optical density) (Fig. 9). The best fitting line was determined using the least-squares approximation method (Steele and Torrie, 1980).

FIGURE 9. A standard curve plot of Area vs Cpm.

An autoradiograph of marker DNA bands was scanned using a Hoefer Scanning Densitometer (Model GS-300) and the area under each peak was calculated by the supporting software GS-350. The base line of the densitometer was set at 5% absorbance and the maximum absorbance was set at 95%. The Kodak X-Omat AR-5 X-ray film was pre-flashed as described in Chapter 2.


8
6
AREA
10³
4
2
0
2
4
6
7
10
12
CPM x 10²

3. RESULTS.

3.1 CONSTRUCTION OF MUTANT VIRUSES.

As mentioned in Chapter 1, we wished to insert various viral DNA segments containing ITR sequences (the viral junction and left end) into the E3 region, then analyze the structure of progeny viruses for effects of these embedded ITR sequences on viral DNA structure and replication. The viral junction from pFG140 was chosen from the two available (pFG140 and pFG141) because, although the pFG140 viral junction contained a larger deletion of sequences from ITR (Fig. 10), pFG140 was found to have a specific infectivity comparable to that of viral DNA when transfected into human cells, whereas pFG141 had a lower specific infectivity than that of viral DNA (Graham, 1984b). pUCIG contains this viral junction subcloned from pFG140 into the unique XbaI site in the multiple cloning region of pUC19 (Fig. 11). In addition to insertion of the viral junction, a virus vector was constructed with the left 1.25% of Ad2 DNA (from pTR00X, Fig. 11) to serve as a control for activity(ies) associated with a single terminus. The Ad2 left end was used because it had already been cloned as a segment flanked by convenient restriction endonuclease sites and the terminal 450 nucleotides at the left end of Ad2 and Ad5 are identical except for 7 mismatches scattered throughout the 347 bp region outside the 103 bp ITR. Because of this

FIGURE 10. Nucleotide sequence of wild type ITRs and viral junctions found in infectious plasmids pFG140 and pFG141.

Nucleotide sequences of the left and right ITRs of wtAd5 joined to form a junction are shown along with the nucleotide sequences of the viral junction isolated from infectious plasmids pFG140 and pFG141. The viral junction from pFG140 contains a deletion of 13 bp (10 bp from right ITR and 3 from left ITR) and the viral junction from pFG141 contains an insertion of 3 bp at the right ITR and a deletion of 5 bp at the left ITR. Modified from Graham (1984b).

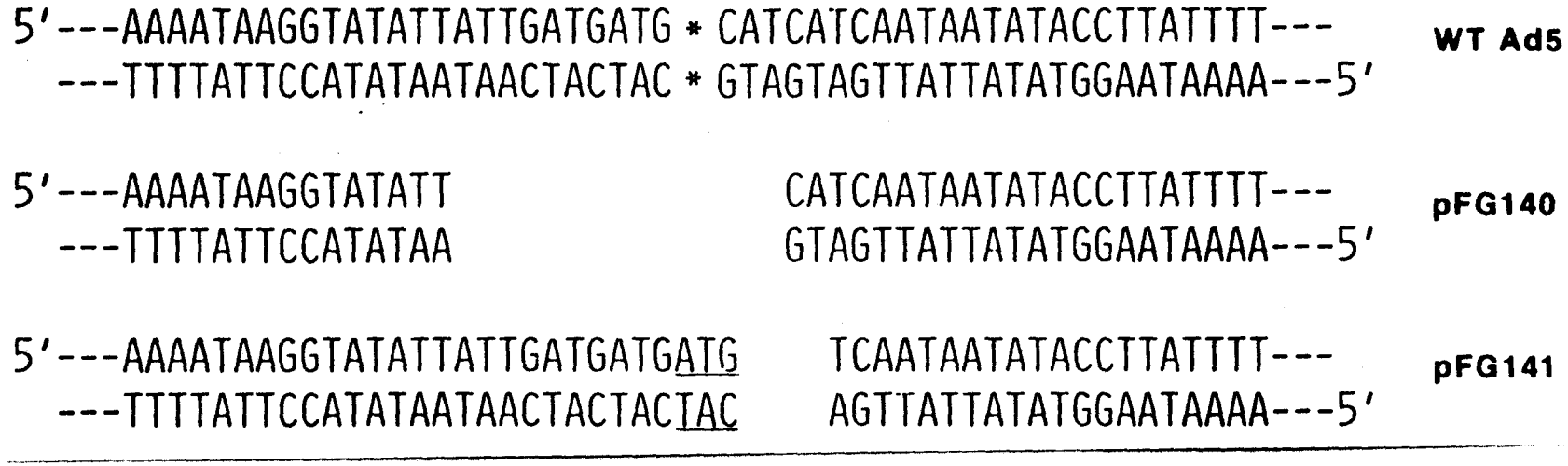
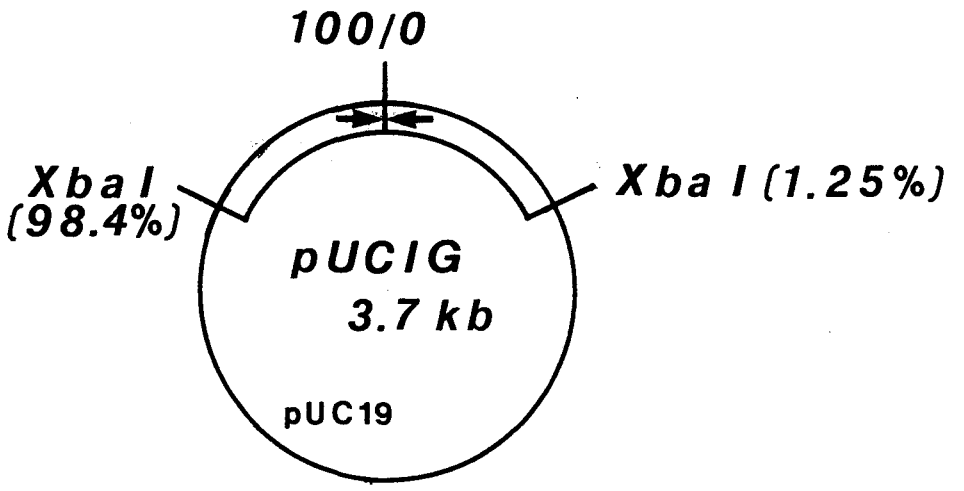
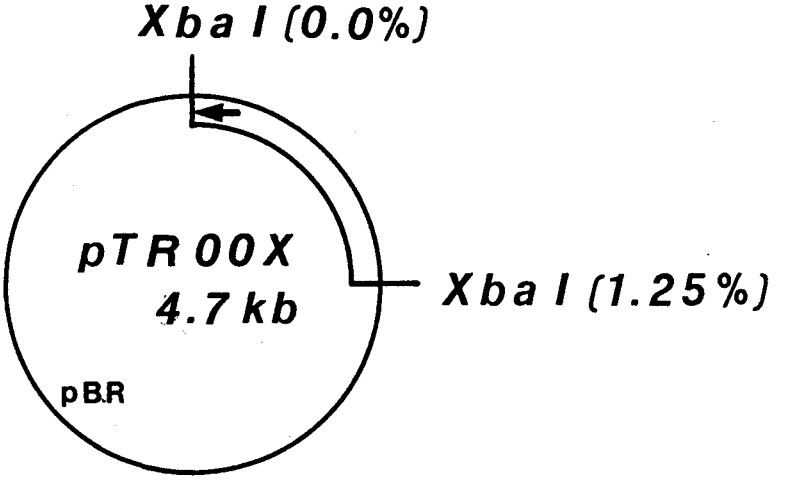

5′---AAAATAAGGTATATTATTGATGATG * CATCATCAATAATATACCTTATTTT---
---TTTTATTCCATATAATAACTACTAC * GTAGTAGTTATTATATGGAATAAAA---5′
WT Ad5
5′---AAAATAAGGTATATT CATCAATAATATACCTTATTTT---
---TTTTATTCCATATAA GTAGTTATTATATGGAATAAAA---5′
pFG140
5′---AAAATAAGGTATATTATTGATGATGATG TCAATAATATACCTTATTTT---
---TTTTATTCCATATAATAACTACTACTAC AGTTATTATATGGAATAAAA---5′
pFG141

FIGURE 11. Plasmids carrying the viral junction from pFG140 and left end of Ad2 DNA.

pUCIG has a 1 kb Ad5 DNA fragment containing the viral junction subcloned from infectious plasmid pFG140, inserted at the XbaI site in the multiple cloning region of pUC19. pTR00X has a 450 bp Ad2 DNA fragment from the left end of the linear viral genome inserted at the XbaI site in pBR322X, an XbaI site containing derivative of pBR322. Arrows indicate the orientation of the ITR sequences.


100/0
Xba I
(98.4%)
Xba I (1.25%)
pUCIG
3.7 kb
pUC19


Xba I (0.0%)
pTR00X
4.7 kb
Xba I (1.25%)
pBR

high homology, it was assumed that the biological activity of the Ad2 left end (0.0 - 1.25%) would be very similar, if not identical to the left end of Ad5.

Two different strategies were attempted during the construction of the mutant viruses. The first strategy involved manipulations of viral DNA from dl309 (Jones and Shenk, 1979) in a manner similar to that used by Stow (1981) and the second strategy involved the use of adenovirus-based shuttle vectors based on the dlE1,3 genome cloned in the form of an infectious bacterial plasmid (Ghosh-Choudhury et al, 1986). A total of five mutant viruses were constructed for this study, all having the genetic background of Ad5dlE1,3 (Haj-Ahmad and Graham, 1986b; Haj-Ahmad, Ph.D. Thesis, McMaster University, 1986a).

3.1.1 Attempts to Rescue Mutant Viruses Using dl309 DNA.

The conventional strategies for rescuing insert DNA into the viral genome relied on either in vivo recombination in co-transfected cells or ligation of viral DNA fragments followed by transfection (Haj-Ahmad and Graham, 1986b; Thummel et al, 1982). The approach that was first used in this study in an attempt to construct the mutant viruses with embedded terminal sequences was the latter strategy, ligation of viral DNA followed by transfection. This approach was used in this study in the following manner.

The 1.0 kb fragment of viral DNA containing the viral junction (98.4% - 100.0/0.0% - 1.25%) subcloned from the infectious plasmid pFG140 (Graham, 1984b) was isolated from

pUCIG (Haj-Ahmad, Ph.D. Thesis, McMaster University, 1986a) and inserted into a plasmid containing the right 40.5% of Ad5 genome, pFGdX1 (Haj-Ahmad and Graham, 1986b), at the unique XbaI site in the E3 region (pFGdX1 contains a deletion of 1.8 kb within E3 resulting from ligation of the XbaI sites at 78.5 mu and 84.7 mu, and deletion of the XbaI D fragment). Similarly, the left 1.25% of Ad2 DNA from pTR00X was inserted into the XbaI site of pFGdX1 (Fig. 12). The two plasmids, pFT and pFJ, were digested with BamHI, mixed with purified dl309 DNA which had been digested with BamHI and EcoRI to completion, and incubated with T4 DNA Ligase. The ligation products were then transfected into 293 cells and the dishes were observed for the presence of plaques for up to two weeks. The general scheme is outlined in Fig. 13. The plaques were isolated, expanded in 293 cells and viral DNA from each plaque isolate was extracted and analyzed on 1% agarose gels after complete digestion with diagnostic restriction endonuclease HindIII. A total of five plaques were isolated from the transfections and based on the restriction digest patterns (data not shown), all were parental dl309 genomes. The lack of success did not appear to be due to low ligase activity or poor transfection efficiency because the ligation products gave a ladder pattern after gel electrophoresis and positive control transfections with deproteinized, intact dl309 DNA gave numerous plaques. The insert size should not have been a problem in the rescue of these recombinant viruses since they were 1.0 kb (viral junction) and 0.45 kb (left end), well within the packaging limits of dlE1,3 (Haj-Ahmad and Graham,

FIGURE 12. Construction of pFT and pFJ.

pFT was constructed by inserting a 450 bp Ad2-left end fragment (0.0 - 1.25 mu) from pTR00X into the unique XbaI site of pFGdX1 which contains the right end of Ad5 genome (59.5 - 100 mu) with a 2.3 kb deletion (78.5 - 84.9 mu) in the E3 gene. pFJ was constructed by inserting a 1 kb Ad5 viral junction from pUCIG into the unique XbaI site in the E3 deletion of the Ad5 sequences in pFGdX1. The single lines represent bacterial plasmid sequences, the open boxes viral sequences, and solid boxes the insert DNA containing the ITR sequences. The arrows indicate the orientation of the ITR.

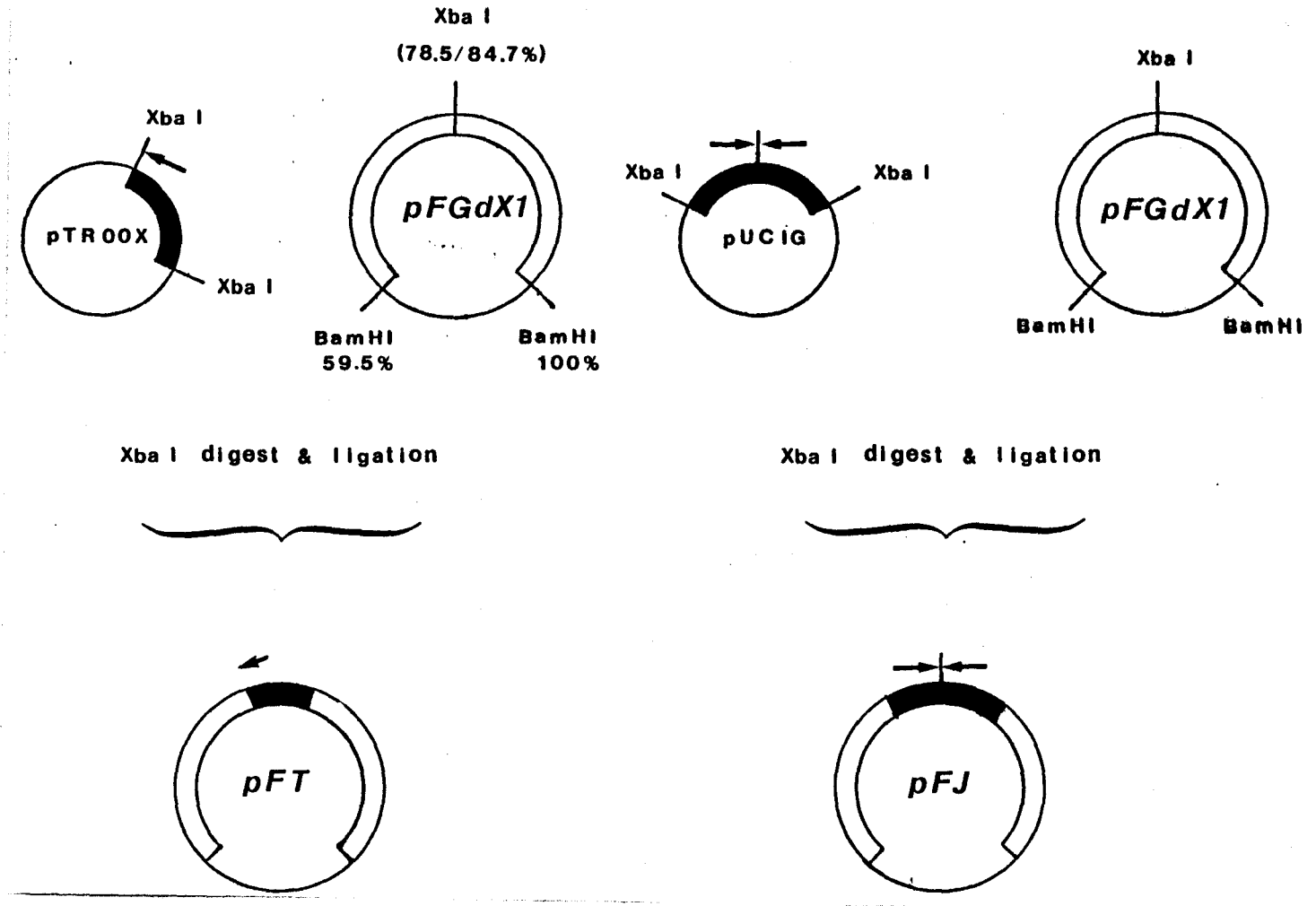
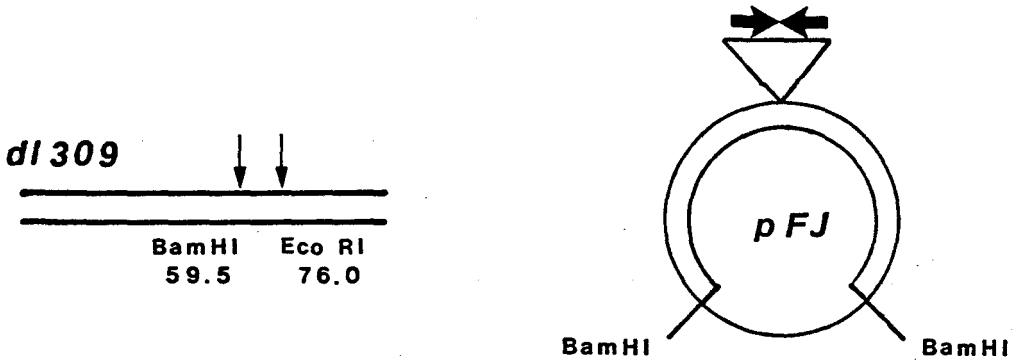

Xba I
(78.5/84.7%)
Xba I
pTR00X
Xba I
pFGdX1
BamHI
59.5%
BamHI
100%
Xba I
Xba I
pUC iG
Xba I
pFGdX1
BamHI
BamHI
Xba I digest & ligation
Xba I digest & ligation
pFT
pFJ

FIGURE 13. Conventional stategy for rescuing DNA fragments into the E3 deletion of linear viral genomes.

The right end of Ad5 genome (59.5 - 100 mu) with the DNA of interest inserted in the E3 deletion (78.5 - 84.7 mu) is excised from its plasmid vector by BamHI digestion and ligated to dl309 DNA digested with BamHI and EcoRI. The ligation products are then transfected into 293 cells and viral DNA samples, extracted from plaque isolates, are screened for the desired recombinant genome. Single line represents plasmid sequences and double line represents viral sequences.


dl309
BamHI
59.5
Eco RI
76.0
pFJ
BamHI
BamHI
BamHI Eco RI
digest
BamHI
digest
ligation
transfect 293 cells
screen plaque isolates

1986b). The five plaques may have originated from intact dl309 DNA that was not detected by ethidium bromide staining. Although Munz and Young (1987) reported that co-tranfected viral DNA fragments with compatible ends could be rescued into infectious viruses by *in vivo* ligation, it appeared that in the rescue of pFT and pFJ, such an *in vivo* ligation event did not occur. Because the preliminary attempts at rescuing E3 inserts were unsuccessful and at the time a novel adenovirus cloning vector system was developed in the laboratory, an alternative approach utilizing the new cloning vector system was adopted.

3.1.2 Adenovirus Cloning Vector System.

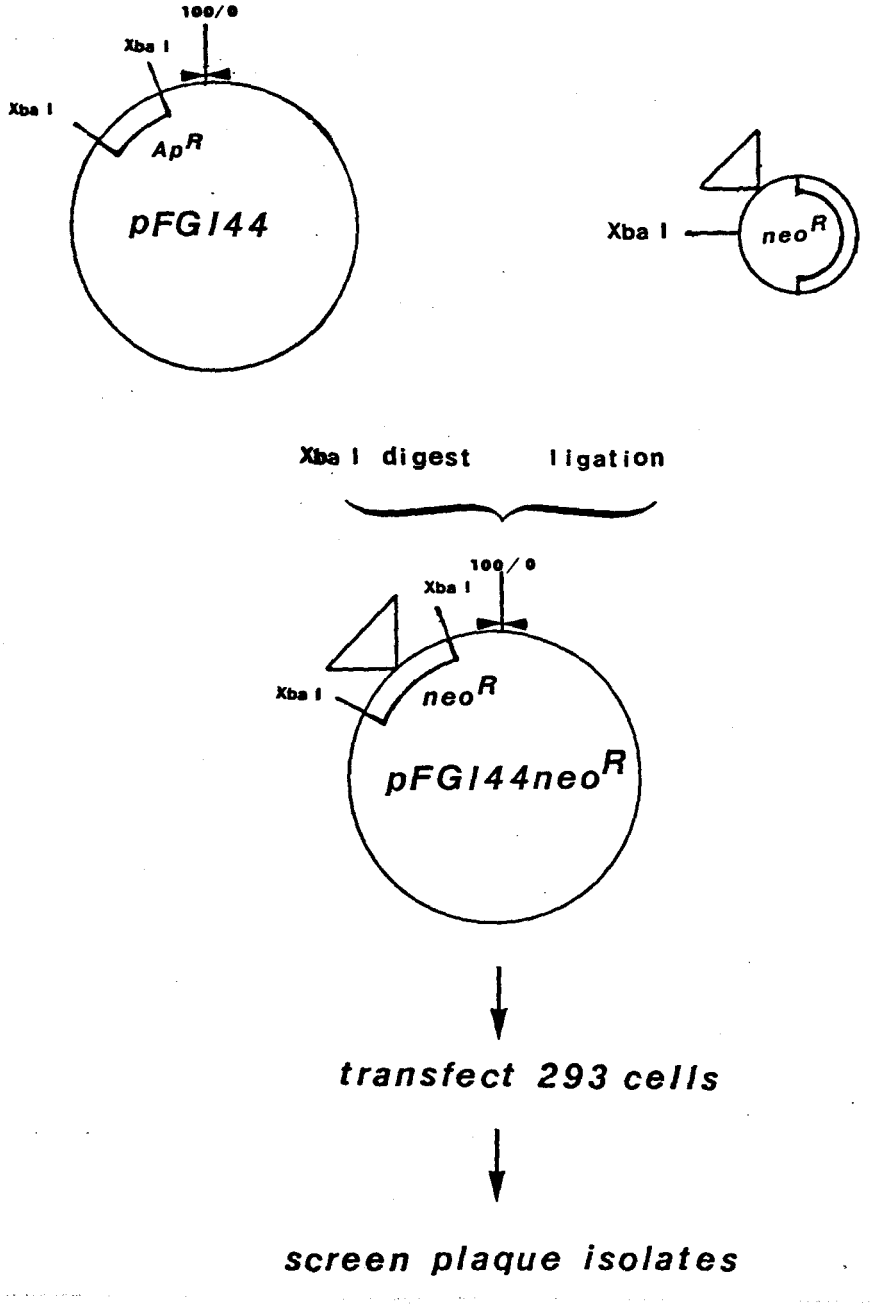
3.1.2.1 Construction of a Neo^R Plasmid - pKN30.

Recently an adenovirus based cloning vector system was developed (Ghosh-Choudhury *et al*, 1986). This system has a number of advantages over conventional systems, one of which is the ability to propagate recombinant DNA molecules in a bacterial host where they can be screened quickly and easily. The general strategy is shown in Figure 14. Plasmid sequences linked to an antibiotic resistance marker (Ap^R) in pFG144 are replaced by a second plasmid with the insert DNA linked to a different selectable marker (neo^R). Large quantities of this plasmid are purified, then transfected into 293 cells, and the plaque isolates are screened for the correct recombinants.

pKN30 was constructed to provide a small neo^R plasmid vector into which ITR sequences could be inserted for

FIGURE 14. Adenovirus cloning vector approach to rescuing DNA fragments into linear viral genomes. General Strategy.

Viral DNA from an infectious Ad5 plasmid with a selectable marker (eg. Ap^R) is digested with an appropriate restriction endonuclease (eg. XbaI) and ligated with a second plasmid vector with a different selectable marker (eg. neo^R) linked to the DNA fragment of interest. This Ad5 plasmid is then transfected into 293 cells and viral DNA samples from plaque isolates are screened for the desired recombinant viruses. ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome are represented by head-to-head arrows.


100/0
Xba I
Xba I
Ap^R
pFGI44
Xba I
neo^R
Xba I digest
ligation
100/0
Xba I
Xba I
neo^R
pFGI44neo^R
transfect 293 cells
screen plaque isolates

subsequent rescue into the viral genome using the approach described above. A small plasmid vector was required so that the final plasmid constructs with ITR sequence inserts would remain within the packaging limits of the adenovirus cloning vector system (Ghosh-Choudhury *et al*, 1986). Construction of pKN30 is shown in Figure 15. pAM112 was constructed by ligating a mixture of pML digested with *NruI* - *EcoRI* and pPB3 digested with *PvuII*(partial) - *EcoRI*(complete), then screening for Ap^R-neo^R colonies. pKN30 was constructed by digesting pAM112 with *DraI* and *AatII*, treating with Klenow fragment of *E. coli* DNA polymerase I and ligating prior to transforming and selecting for kan^R colonies. Screening of potential candidates by *PvuII* digestion is shown in Figure 16 and the correct recombinant was determined based on the increased mobility of the *PvuII*-A fragment. The upper band in lanes 4, 6, 10, and 12 represent the *PvuII*-A fragment from plasmids constructed by incomplete *DraI* digestion of the parent plasmid (pAM112). In lane 8, the upper bands are likely products of incomplete *PvuII* digestion. The first band below the upper band in all lanes is probably from over digestion of the plasmid by *PvuII* star activity.

FIGURE 15. Construction of a neo/G418^R plasmid - pKN30.

pPB3 is a kanamycin resistant plasmid with bacterial origin of replication and polycloning sites from pUC18. The polycloning sites are located just downstream from the kanamycin resistant marker. pML digested with EcoRI and NruI was ligated to pPB3 digested partially with PvuII and completely with EcoRI to create a neo^R-Ap^R plamid pAM112. pAM112 was then digested with DraI and AatII, blunt-ended, and ligated to delete the Ap^R marker and generate pKN30. The hatched box represents the SV40 early promoter and solid circle represents the bacteriophage lambda packaging signal cos. E - EcoRI, P - PvuII, S - SalI, X - XbaI.

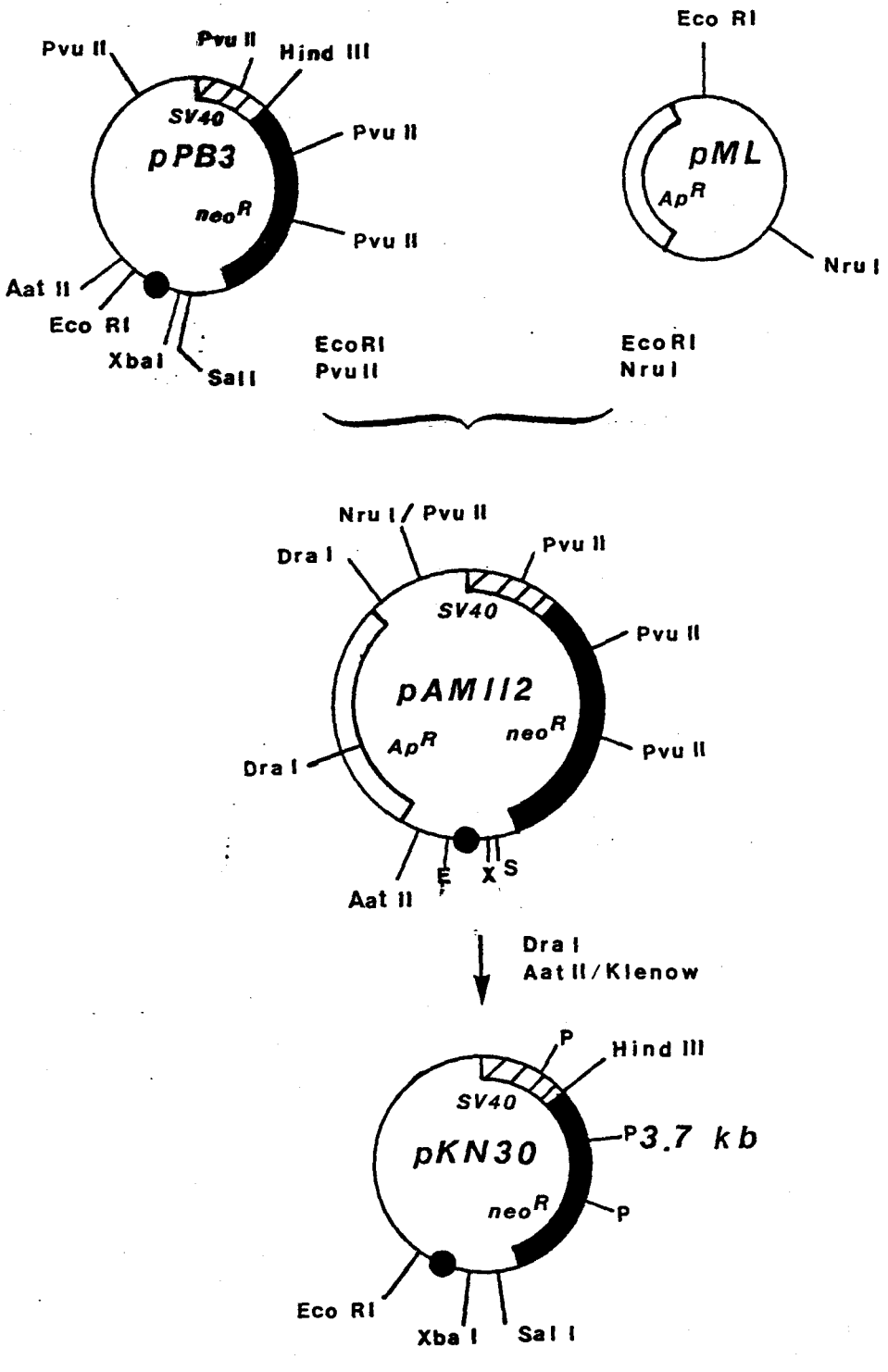
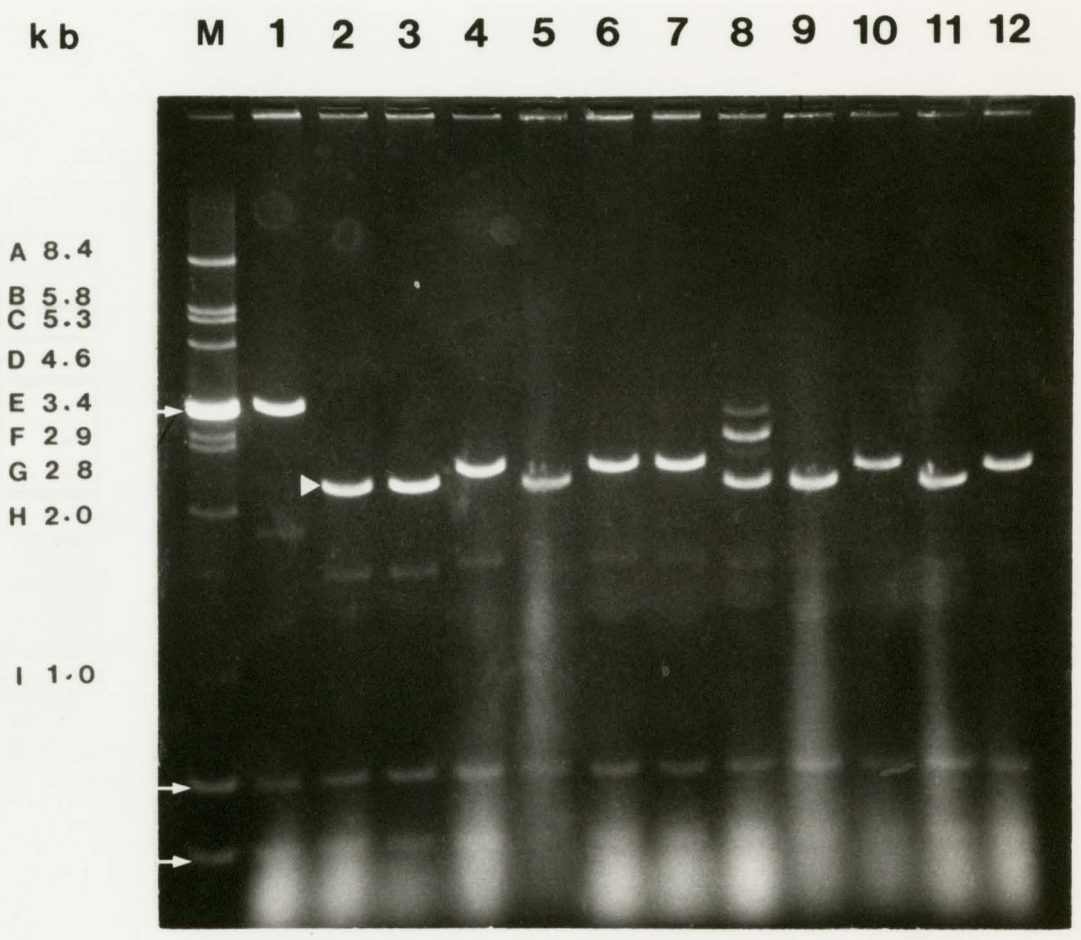

Pvu II
Pvu II
Hind III
SV40
pPB3
Pvu II
neo^R
Pvu II
Aat II
Eco RI
XbaI
SalI
Eco RI
pML
Ap^R
Nru I
EcoRI
PvuII
EcoRI
NruI
Nru I / Pvu II
Dra I
Pvu II
SV40
pAM112
Pvu II
Ap^R
neo^R
Dra I
Pvu II
Aat II
E
X
S
Dra I
Aat II/Klenow
P
Hind III
SV40
pKN30
P3.7 kb
neo^R
P
Eco RI
Xba I
Sal I

FIGURE 16. Screening of candidates for kan^R plasmid - pKN30.

pAM112 was restricted with restriction endonuclease DraI and AatII to delete sequences from Ap^R marker and the products of ligation were used to transform E.coli to kan^R. Analytical plasmid DNA samples were extracted from selected transformants and digested with restriction endonuclease PvuII before agarose gel electrophoresis. PvuII cleaves the parental plasmid (pAM112) into 3 fragments (A, B, and C) indicated by the white arrows in the marker lane. Deletion of sequences from the Ap^R marker generated a smaller, faster migrating A fragment (solid triangle, lane 2). Sizes of fragments B and C are not altered by the deletion of Ap^R marker. Lane M(arker) contains a mixture of HindIII digested wtAd5 DNA and PvuII digested pAM112 (white arrows). Lanes 1 to 12 inclusive are candidates for pKN30.


kb
M 1 2 3 4 5 6 7 8 9 10 11 12
A 8.4
B 5.8
C 5.3
D 4.6
E 3.4
F 2 9
G 2 8
H 2.0
I 1.0

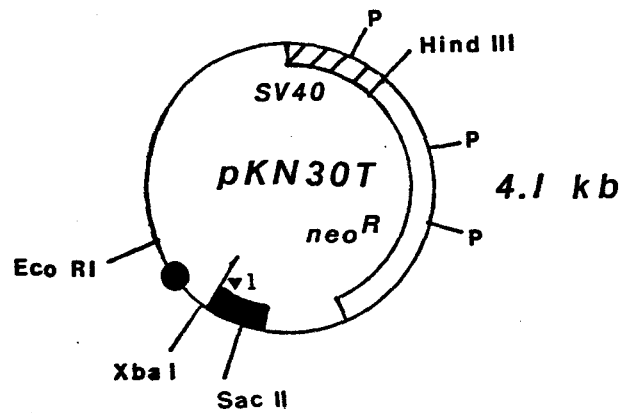
3.1.2.2 Construction of neo^R Plasmids Carrying Embedded Terminal Sequences.

As the first step in using the adenovirus cloning vector system to construct linear viruses with an embedded viral junction or an embedded single ITR, the two terminal sequences were inserted into pKN30. The viral junction and the left terminal sequences from pUCIG and pTR00X, respectively, were removed, blunt-ended with the Klenow fragment of E. coli DNA polymerase I and ligated to pKN30 linearized at the unique SalI site and blunt-ended (Fig. 17). Results from screening of Kan^R colonies for recombinant plasmids containing the terminal sequence inserts (single ITR or ITR junction) are shown in Fig. 18 (single ITR) and Fig. 19 (ITR junction). The desired recombinant plasmids were identified by a decrease in the mobility of PvuII-A fragment. The second band was likely the result of over digestion by PvuII star activity. Orientation of the terminal sequence inserts in pKN30T and pKN30J was determined by digesting the respective plasmids with SacII and HindIII as shown in the second panels of Fig. 18 and Fig. 19 respectively.

pKN30T contains the left 450 bp of Ad2 inserted in the orientation which positioned the ITR sequence distal to the 3' end of the neo/G418 resistance gene; pKN30J contains the viral junction with flanking viral sequences inserted in the orientation which positioned the left ITR proximal to the 3' end of the neo/G418 resistance gene.

FIGURE 17. Maps of pKN30T and pKN30J.

pKN30T was constructed by inserting the 450 bp Ad2-left end fragment from pTR00X into the unique SalI site of pKN30. pKN30J was constructed by inserting a 1050 bp Ad5 viral junction from pUCIG into the unique SalI of pKN30. The open box represents neomycin phosphotransferase gene, the hatched box represents the SV40 early promoter, the solid circle represents the bacteriophage lambda packaging sequence cos, the solid box represents adenoviral sequences, and the arrows indicate the orientation of ITR sequences. The 'l' and 'r' indicate the left and right ITR sequences respectively. P - PvuII.


P
Hind III
SV40
P
pKN30T
4.1 kb
neo^R
P
Eco RI
1
Xba I
Sac II

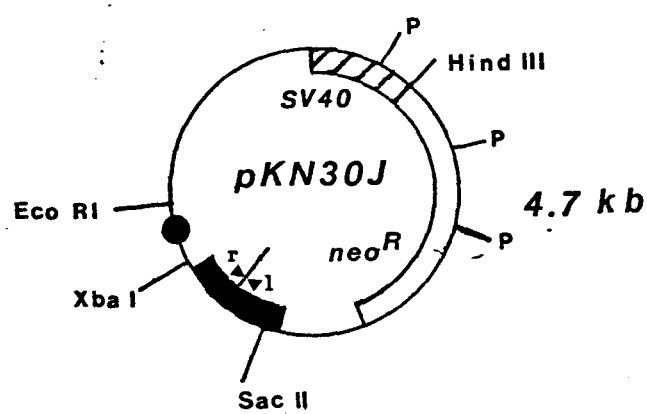
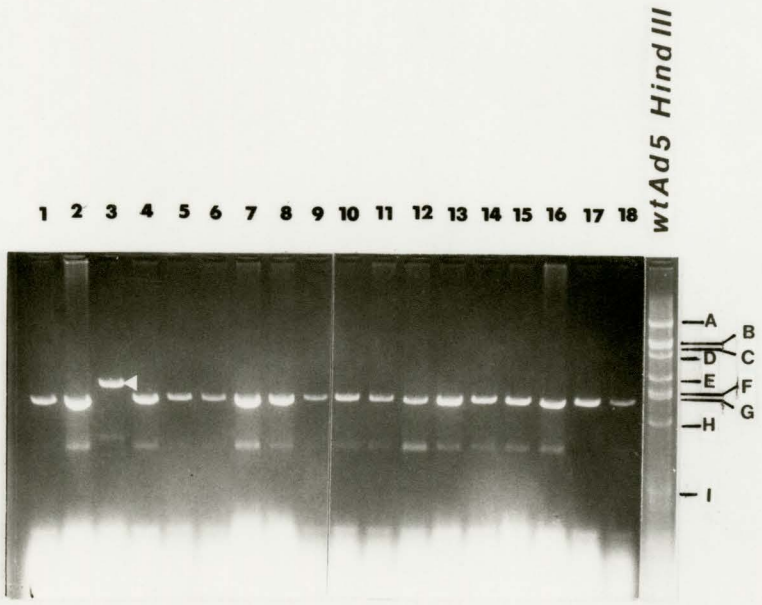

P
Hind III
SV40
P
pKN30J
4.7 kb
Eco RI
neo^R
P
r
1
Xba I
Sac II

FIGURE 18. Screening of candidates for pKN30T.

(Panel A) The 450 bp viral DNA fragment from the left end of Ad2 DNA purified from pTR00X was inserted into the SalI site of pKN30 and used in transformation of E.coli to kan^R. Plasmid DNA from selected transformants was prepared by modified Birnboim and Doly procedure and samples were digested with PvuII prior to agarose gel electrophoresis. Presence of the viral DNA insert at the SalI site is indicated by the shift-up in the mobility of PvuII-A fragment (lane 3). Lanes 1 through to 18 are candidates for pKN30T. (Panel B) The orientation of the viral DNA insert was determined by digesting pKN30T with SacII and HindIII. SacII cleaves in the viral DNA 350 bp from the left end. The restriction digest pattern shows that the left end insert is positioned such that the left ITR is in the same orientation as the neo/$G418^R$ gene.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
wtAd5 HindIII
A
B
C
D
E
F
G
H
I

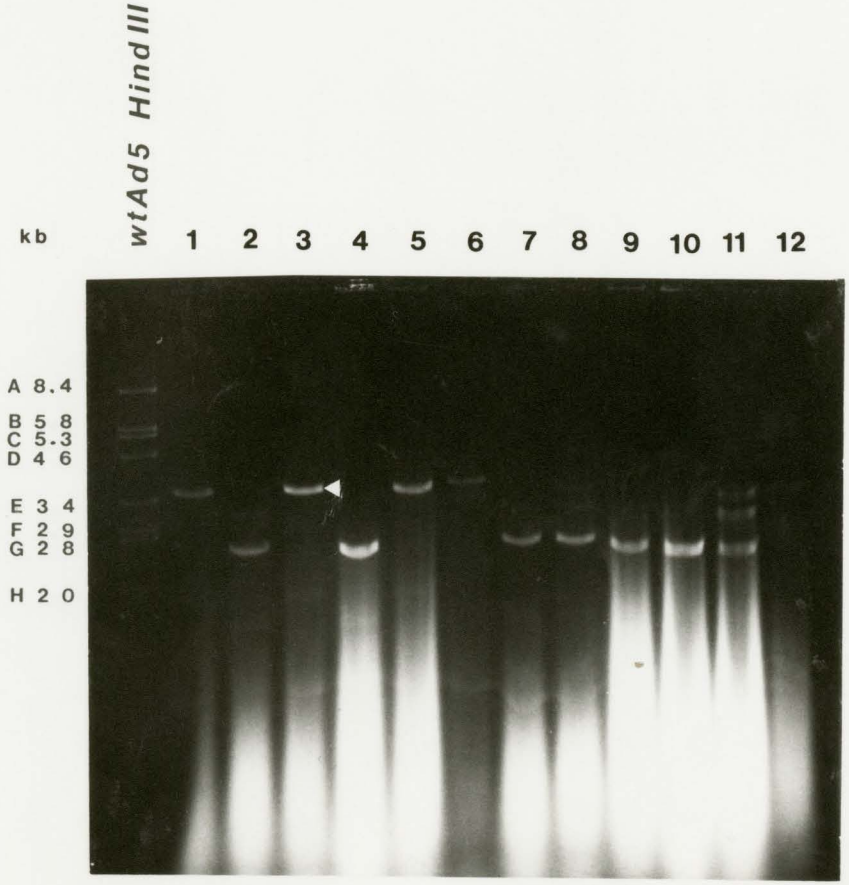
A


1
wtAd5 HindIII
A 8.4
B 5 8
C 5.3
D 4 6
E 3.4
F 2 9
G 2 8
H 2.0
I 1 0

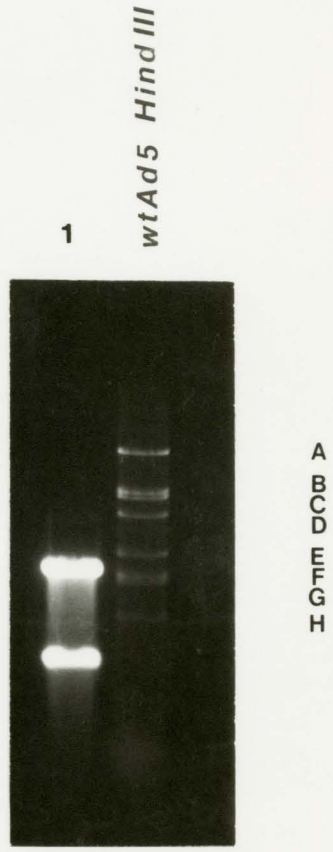
B

FIGURE 19. Screening of candidates for pKN30J.

The 1 kb viral DNA fragment containing the viral junction from pFG140 was purified from pUCIG and inserted into the unique SalI site in pKN30 as described in the text. The ligation product was used in transformation of E.coli to kan^R, and analytical plasmid DNA samples were prepared from selected transformants. Aliquots of plasmid DNA samples were digested with PvuII and separated by agarose gel electrophoresis. The desired recombinant plasmid DNA constructs were identified by the presence of a slower migrating PvuII-A fragment, its retardation in the gel due to an increase in the size of the A-fragment as a result of the presence of the 1 kb viral DNA fragment (lane 3). Lanes 1 through to 12 represent candidates from pKN30J. (Panel B) The orientation of the viral DNA insert in pKN30J was determined by digesting the plasmid with SacII and HindIII, similar to pKN30T. The restriction pattern indicates that the 1 kb viral DNA fragment containing the viral junction is positioned such that the left ITR is in the same orientation as the neo/$G418^R$ gene.


wtAd5 Hind III
kb
1 2 3 4 5 6 7 8 9 10 11 12
A 8.4
B 5 8
C 5.3
D 4 6
E 3 4
F 2 9
G 2 8
H 2 0

A


1
wtAd5 Hind III
A
B
C
D
E
F
G
H

B

3.1.2.3 Construction of an Ad5 Plasmid Carrying pKN30.

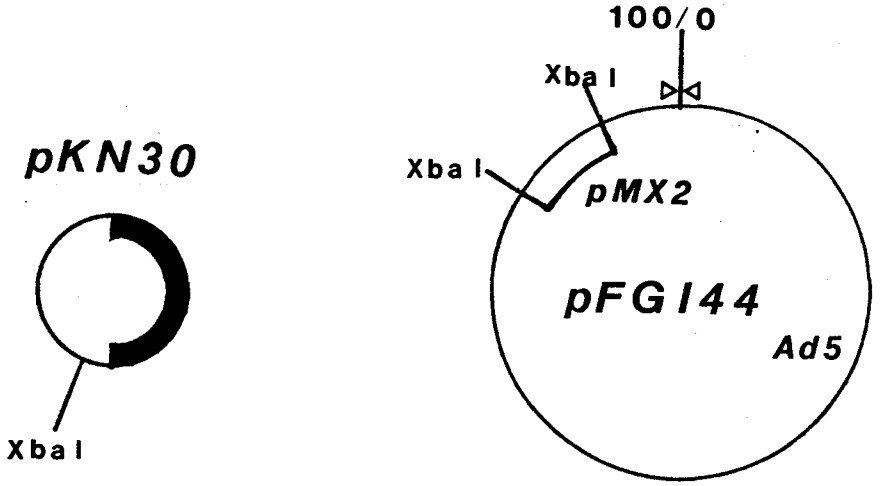
pKN30 was linearized at the XbaI site and an aliquot of the linearized plasmid was treated with Calf Intestinal Alkaline Phosphatase (CIAP) to prevent religation of the vector. An infectious plasmid pFG144 [dlE1,3 genome cloned in the unique XbaI site of pMX2 (Ghosh-Choudhury et al, 1986)] was digested with XbaI to remove pMX2 containing the Ap^R marker, then mixed with linearized pKN30 (CIAP treated and untreated) and ligated. The ligation products were then used to transform E. coli HMS174 to Kan^R by the Hanahan procedure (Chapter 2) (Fig. 20). Plasmid DNA from the transformants was extracted by the rapid alkaline-lysis method (Chapter 2) and HindIII digested DNA samples were analyzed by agarose gel electrophoresis (Fig. 21). Candidates with the correct structure, indicated by the presence of the HindIII-B' (4.2 kb) and B" (2.8 kb) fragments, were further analyzed by SmaI digestions to check for deletions in the viral junction of the Ad5 sequences (data not shown). The candidates with the correct HindIII and SmaI digestion patterns were streaked out on selective solid agar media to isolate single colonies.

3.1.2.4 Construction of Ad5 Plasmids Carrying Embedded Viral Junctions.

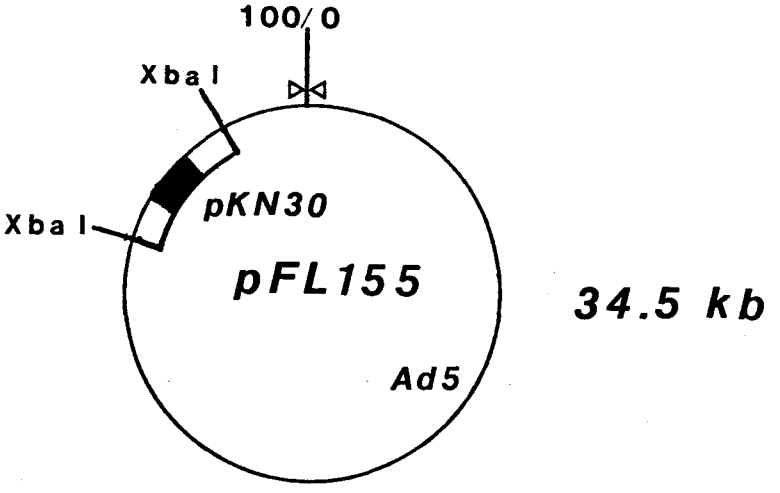
The Ad5 plasmids carrying pKN30J (neo^R plasmid with viral junction insert) were constructed using the same approach as that adopted for pFL155 as described above in detail (Fig. 20). Plasmid DNA from the transformants was digested with HindIII and the candidates containing the

FIGURE 20. Construction of pFL155.

The infectious Ad5 plasmid pFG144 contains the ampicillin resistant pMX2 inserted into the unique XbaI site at the deletion in the E3 gene (78.5 - 84.7 mu) of dlE1,3 genome (circularized by covalent joining of the ITRs). This plasmid was digested with XbaI to remove pMX2 and the viral sequences were ligated to XbaI digested pKN30, constructing pFL155. The open boxes represent plasmid DNA and solid boxes represent the SV40 early promoter - neo^R sequences. Ad5 plasmids containing embedded viral junction, pFL156 and pFL157, were constructed by the same strategy except pKN30J was inserted into E3-XbaI site in both orientations. ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome are represented by head-to-head arrows.



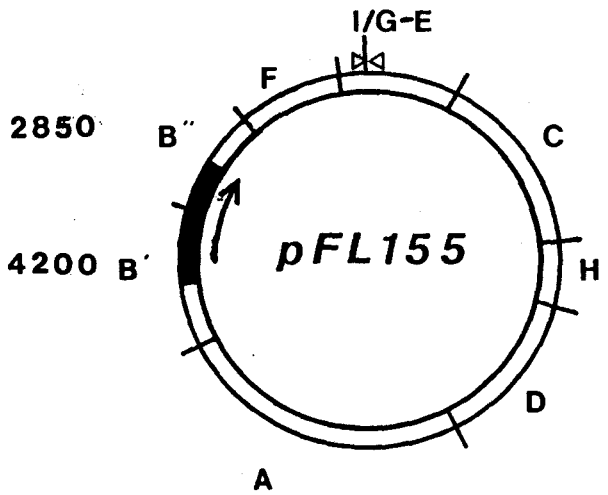
Xba I digest & ligation

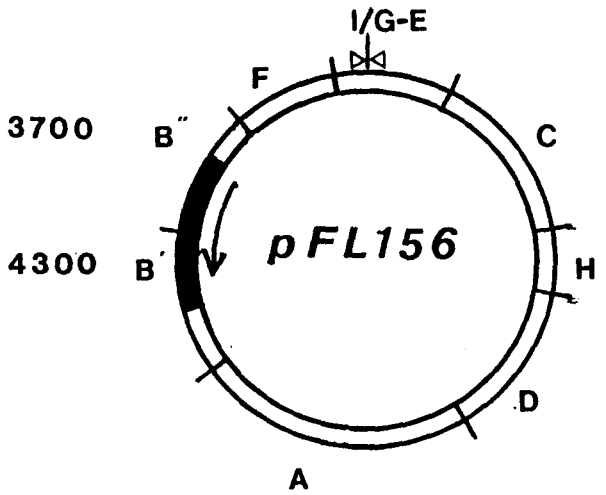


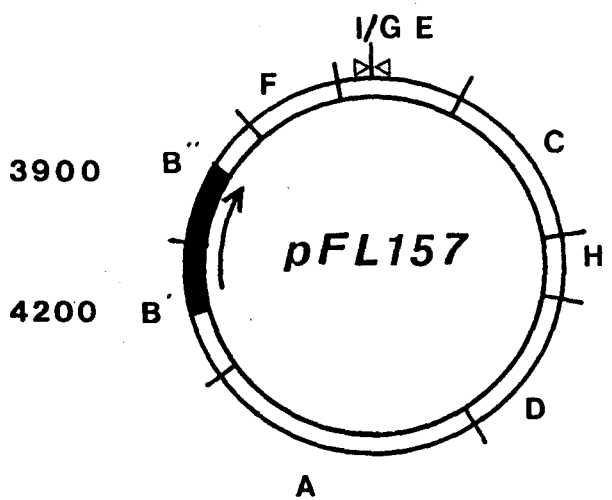
34.5 kb

FIGURE 21. Screening of candidates for pFL155, pFL156 and pFL157.

Analytical plasmid DNA samples were extracted as described in Chapter 2, digested with HindIII and electrophoresed. The correct recombinant clones were selected based on the appearance of two faster migrating B' (solid triangle) and B" (open triangle) fragments. HindIII restriction digest maps of pFL155, pFL156 and pFL157 are shown below. Solid blocks represent pKN30 (pFL155) and pKN30J (pFL156 and pFL157) sequences. ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome are represented by two open triangles at 0 and 100 mu. Lane 1 - pFL155 candidate; lanes 2 to 7 inclusive - pFL156 and pFL157 candidates; lane 8 - HindIII digested pFG144; lane 9 - HindIII digested wt Ad5 DNA.


I/G-E
F
2850
B''
C
4200
B'
pFL155
H
D
A


I/G-E
F
3700
B''
C
4300
B'
pFL156
H
D
A


I/G E
F
C
3900
B''
pFL157
H
4200
B'
D
A

pFL155
pFL156
pFL157
pFG144
wtAd5 HindIII
kb
A 8.4
B 5.8
C 5.3
D 4.6
E 3.4
F 2.9
G 2.8
H 2.0
1 2 3 4 5 6 7 8 9

desired recombinant plasmids were identified by the presence of B' and B" fragments in the restriction digest pattern (Fig. 21). pFL156 (lane 3) contains pKN30J inserted such that the G418 resistance gene is in opposite orientation to the E3 promoter and pFL157 (lane 4) has the plasmid in the opposite orientation.

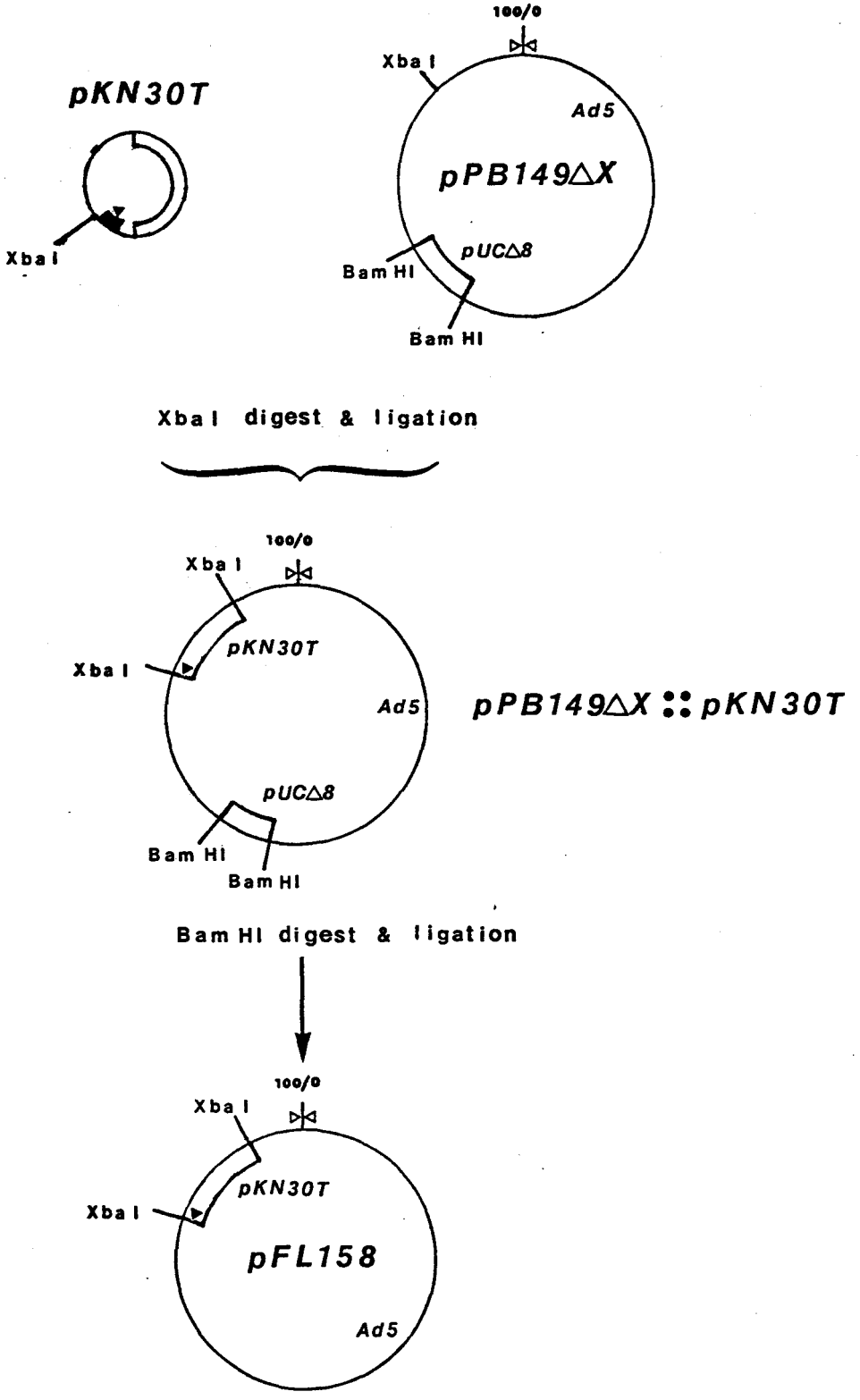
3.1.2.5 Construction of Ad5 Plasmids Carrying Embedded Single ITR Sequences.

During the construction of pFL155, pFL156, and pFL157, a large number of transformants were screened, of which 80% in pFL155 construction and 90% in pFL156 and pFL157 construction were reisolations of the neo^R vector, due to the absence of any selection against the parental vector. To decrease the number of transformants that needed to be screened an efficient selection mechanism was introduced by modifying the strategy used in construction of the previous three pFL plasmids, pFL155, pFL156 and pFL157.

This modified strategy used a plasmid pPB149ΔX (P. Brinkley, personal communication) (Fig. 22) which was derived from pGGC145 by insertion of a modified Ap^R-pUC8 (pUC8ΔEco) into the unique BamHI site of pGGC145. pKN30T was inserted into the unique XbaI site of pPB149ΔX and by selecting for Ap^R-Kan^R plasmids, pPB149ΔX::pKN30T-1 and -2 (both orientations of pKN30T) were created. Subsequently, the pUC8ΔEco sequences were deleted from pPB149ΔX::pKN30T-1 and -2 by digesting the plasmids with BamHI and religating (Fig. 22). Restriction endonuclease HindIII digest patterns of

FIGURE 22. Construction of pFL158.

A kanamycin-ampicillin double resistant recombinant plasmid was constructed by inserting pKN30T into the unique XbaI site of pPB149ΔX (which contains the dlE1,3 genome covalently closed at the ITRs cloned into unique BamHI site of modified pUC8, pUCΔ8), creating pPB149ΔX::pKN30T. This plasmid was then digested with BamHI and religated to remove ampicillin resistant pUC8ΔX, leaving pKN30T in the unique XbaI site at the E3 deletion. The hatched box in pKN30T represents the left end (0.0 - 1.25 mu) of Ad2 genome and head-to-head arrows represent the ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome.


pKN30T
Xba I
100/0
Xba I
Ad5
pPB149ΔX
pUCΔ8
Bam HI
Bam HI
Xba I digest & ligation
100/0
Xba I
pKN30T
Xba I
Ad5
pUCΔ8
Bam HI
Bam HI
pPB149ΔX :: pKN30T
Bam HI digest & ligation
100/0
Xba I
pKN30T
Xba I
pFL158
Ad5

potential candidates are shown in Fig. 23 and the presence of pKN30T in the E3 region is demonstrated by the presence of B' and B" fragments.

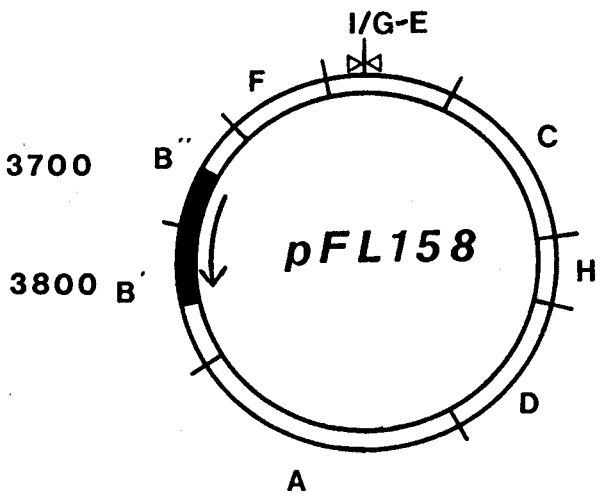
3.2 ISOLATION AND PRELIMINARY CHARACTERIZATION OF THE RECOMBINANT VIRUSES.

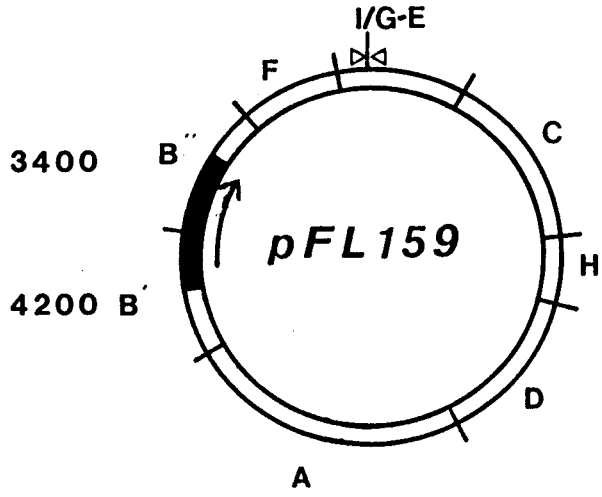
As described previously in this chapter, during the rescue of a DNA insert into the viral genome using the adenovirus cloning vector system, infectious viruses are generated from recombinant Ad5 plasmids by transfecting 293 cells. For Ad5 plasmids with inserts less than 4.3 kb in E3, this is a simple procedure (Ghosh-Choudhury et al, 1986), however, for Ad5 plasmids with inserts larger than 4.3 kb a second plasmid pXCΔ3 is required (Ghosh-Choudhury et al, 1987). pXCΔ3 contains the left 15% of Ad5 genome with an E1 deletion similar to that found in dlE1,3 except the deletion in the former does not extend into the coding sequences for protein IX, a protein required for packaging of full-length Ad5 genome. By co-transfecting with pXCΔ3, protein IX coding sequences can be incorporated into the viral genome by in vivo recombination and allow for the rescue of larger inserts (Ghosh-Choudhury et al, 1987).

The Ad5 plasmids containing embedded viral junction and left ITR (Fig. 24) were rescued into linear viral genomes by transfecting 293 cells with appropriate plasmids and screening the plaque isolates. Ad5neo3.7 was rescued from pFL155. Ad5TER-1, Ad5TER-2, Ad5J-1 and Ad5J-2 were rescued from pFL158, pFL159, pFL156 and pFL157 respectively by

FIGURE 23. Screening of candidates for pFL158 and pFL159.

Analytical plasmid DNA samples from potential clones were digested with HindIII and analyzed by gel electrophoresis. The replacement of the HindIII-B band with two faster migrating B' (solid triangle) and B" (open triangle) bands indicated the presence of pKN30T in the unique XbaI site at the E3 deletion. HindIII restriction maps for pFL158 and pFL159 are shown below. Solid blocks represent pKN30T sequences and head-to-head arrows represent the ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome. Lanes 1 to 4 inclusive - sample DNA; lane 5 - marker DNA. Note that in lane 4, fragment B" (solid triangle) comigrates with the G/E fusion fragment and in lane 5, B' (open triangle) comigrates with the HindIII D fragment.


I/G-E
F
C
3700
B''
H
3800
B'
pFL158
D
A


I/G-E
F
C
3400
B''
H
4200
B'
pFL159
D
A

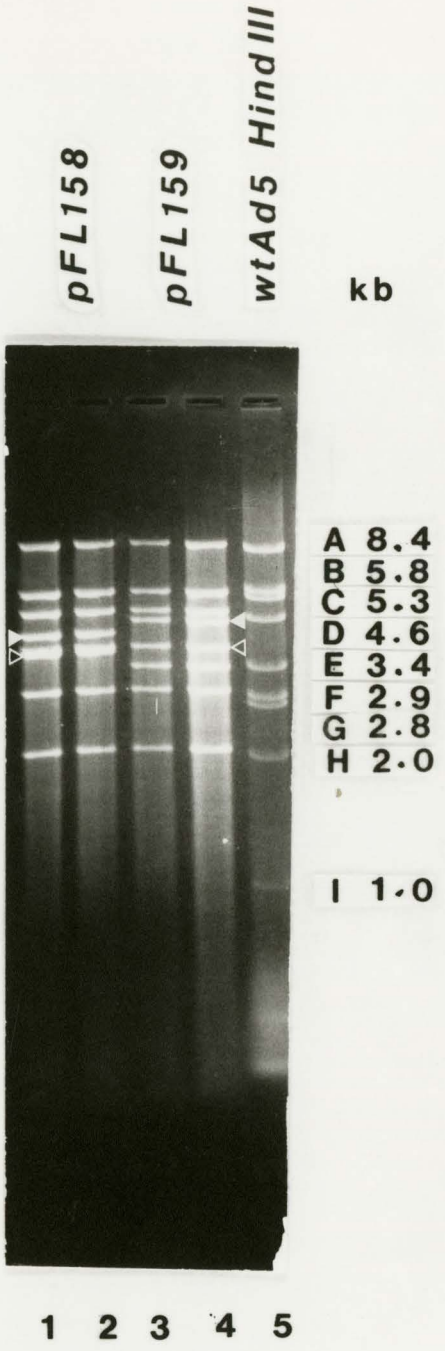
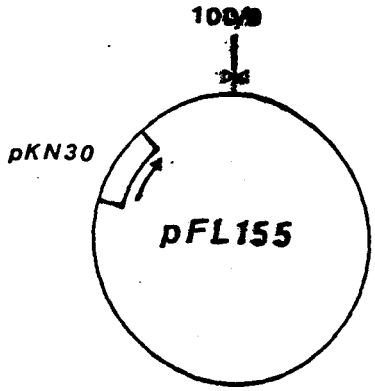
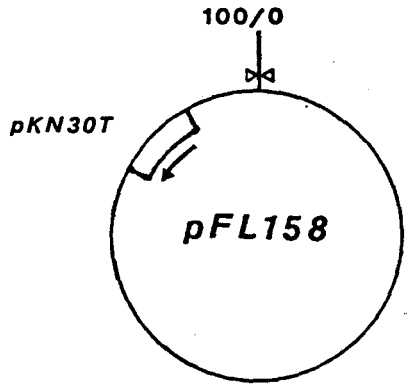
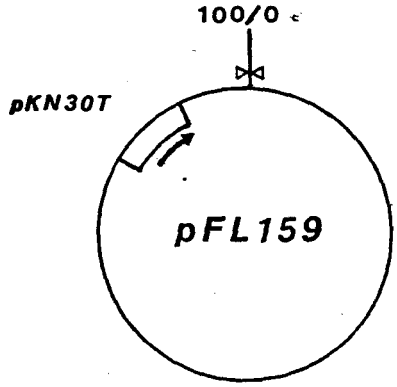

pFL158
pFL159
wtAd5 HindIII
kb
A 8.4
B 5.8
C 5.3
D 4.6
E 3.4
F 2.9
G 2.8
H 2.0
I 1.0
1 2 3 4 5

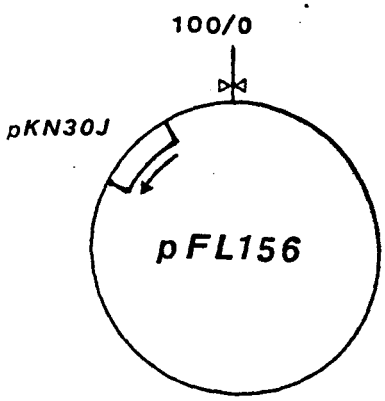
FIGURE 24. Maps of the five pFL plasmids used to generate viruses with various inserts.

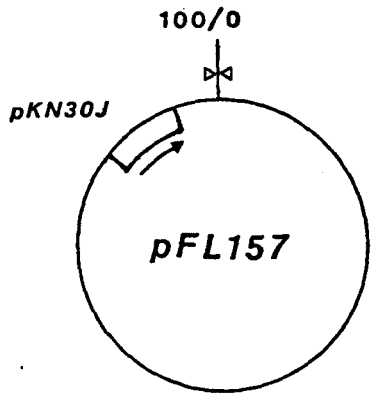
The single line represents viral DNA, open box represents plasmid sequences, and the arrows indicate the orientation of the neomycin/G418 resistance gene. ITRs covalently joined at 0.0 and 100 mu in the circularized viral genome are represented by head-to-head arrows.


100/0
pKN30
pFL155


100/0
pKN30T
pFL158


100/0
pKN30T
pFL159


100/0
pKN30J
pFL156


100/0
pKN30J
pFL157

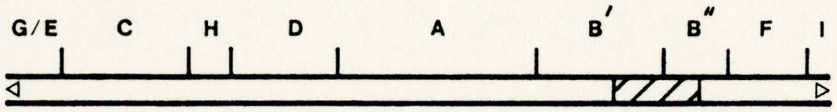
co-transfection with pXCΔ3. All the plaques from the co-transfection with pXCΔ3 had the correct recombinant viruses with the insert indicating that the efficiency of rescue of large inserts into recombinant viruses through co-transfection with pXCΔ3 was 100%. Figure 25 shows the HindIII digestion pattern of the five recombinant viruses. The presence of the protein IX gene at the left end of the latter four viruses (Ad5TER-1 and -2, and Ad5J-1 and -2) was verified by a decrease in the mobility of the left end fragment.

Figure 26 shows a schematic map of the five viruses indicating the orientation of the respective plasmid inserts in the E3 gene. In Ad5neo3.7, pKN30 is inserted such that the SV40 early promoter-neo/G418 construct is in the same orientation as the E3 promoter sequence. In Ad5TER-1, the SV40-neo/G418 construct is inserted in the opposite orientation to the E3 promoter which places the left ITR insert in pKN30T in the same orientation as the terminal left ITR. In Ad5TER-2 the orientation of pKN30T is reversed. Ad5J-1 contains the SV40-neo/G418 construct inserted in the opposite orientation to the E3 promoter, thereby placing the left end sequences (0.0 - 1.25%) of the viral junction in pKN30J distal to the E3 promoter element; Ad5J-2 has pKN30J inserted in the opposite orientation.

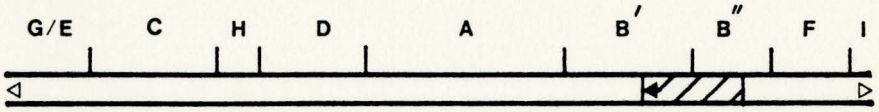
FIGURE 25. Restriction digest pattern of the five recombinant viruses.

Viral DNA samples from 293 cells infected with viruses rescued from the five Ad5 plasmids were digested with HindIII and separated by agarose gel electrophoresis. The presence of neo^R plasmid inserts in the E3-XbaI site is indicated by the replacement of the HindIII-B band present in the marker lane with a pair of faster migrating bands B' (solid triangle) and B" (open triangle). The presence of the protein IX gene in the genomes of Ad5TER-1, Ad5TER-2, Ad5J-1 and Ad5J-2 is indicated by the slower migrating G-E band (solid circle). HindIII restriction maps of the five recombinant viruses are shown below for reference. The hatched boxes represent the plasmid inserts, open triangles represent terminal ITRs and solid triangles represent embedded ITRs. Lane 1 - wtAd5 DNA; lane 2 - Ad5neo3.7; lane 3 - Ad5TER-1; lane 4 - Ad5TER-2; lane 5 - Ad5J-1; lane 6 - Ad5J-2.

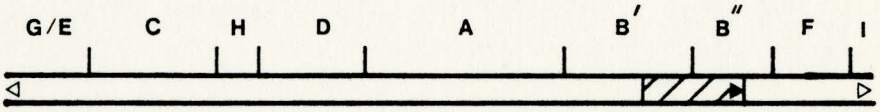
Ad5 neo3.7



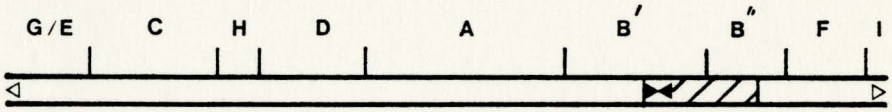
Ad5TER−1



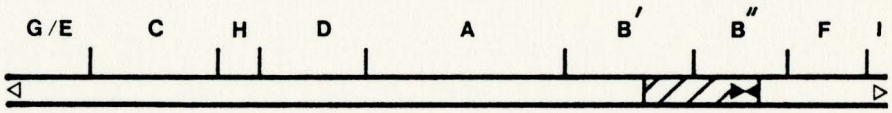
Ad5TER−2



Ad5J−1



Ad5J−2

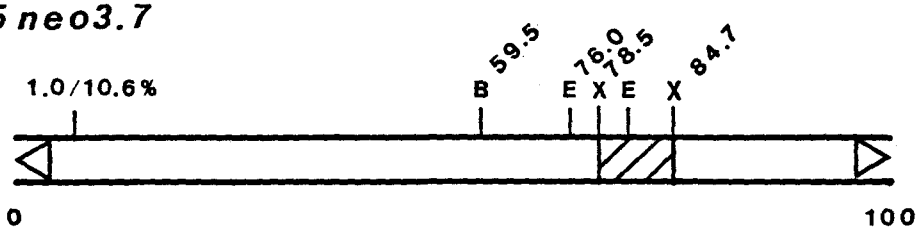


kb
wtAd5 HindIII
Ad5 neo3.7
Ad5TER—1
Ad5TER—2
Ad5J—1
Ad5J—2
A 8.4
B 5.8
C 5.3
D 4.6
E 3.4
F 2.9
G 2.8
H 2.0
1 2 3 4 5 6

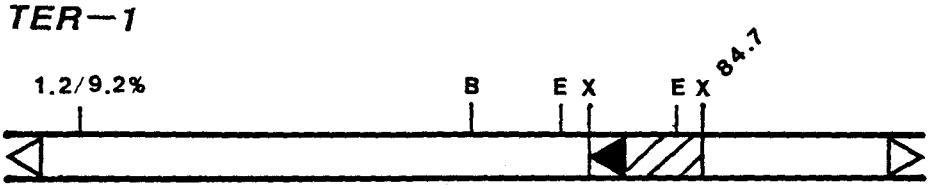
FIGURE 26. Restriction endonuclease cleavage maps of five recombinant viruses.

Restriction cleavage maps for BamHI (B), EcoRI (E), and XbaI (X) are shown for each recombinant virus. The E1 deletion in Ad5neo3.7 spans 1.0 mu to 10.6 mu, and the E1 deletion in the other four viruses spans 1.2 mu to 9.2 mu. Viral DNA is represented by a double line and the plasmid sequences are represented by hatched boxes. The open triangles represent the terminal ITR sequences, and the solid triangles represent ITR sequences internally embedded in the linear genome.

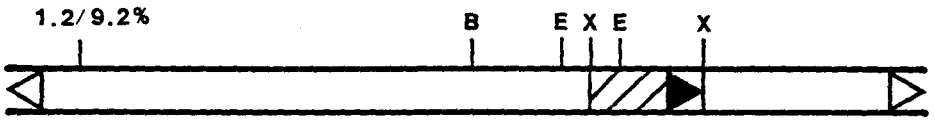
Ad5 neo3.7



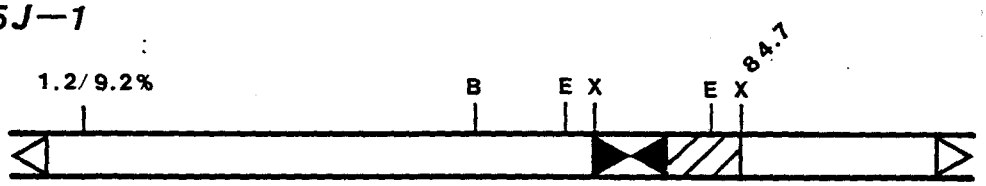
Ad5TER—1



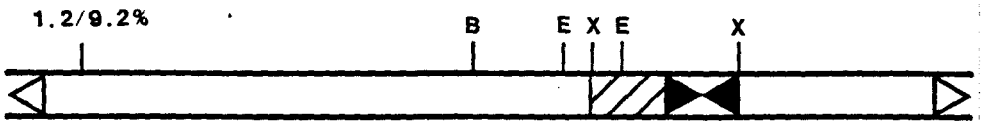
Ad5TER—2



Ad5J—1



Ad5J—2



3.3 ANALYSIS OF INTRACELLULAR VIRAL DNA.

3.3.1 Possible Mechanisms for the Generation of Genomic Rearrangements.

As mentioned previously (Chapter 1), there is evidence which suggests that the viral junction must have some biological activity. In particular, covalently closed circles of viral genome are infectious, suggesting a possible role in replication, or alternately, in recombination of the viral genome or as intermediates in integration during transformation. To study the biological activity of the viral junction from a circularized genome, the five mutant viruses were used to infect 293 cells and intracellular viral DNA was analyzed for rearrangements.

It may be useful to review at this time the genomic rearrangements generated by embedded repeat sequences in other systems since they could serve as models in predicting the rearrangements generated by the embedded terminal sequences. The systems I will briefly consider are isomerization of the HSV genome and the yeast 2 micron plasmid.

The genome of HSV is a linear double-stranded DNA molecule with no attached terminal proteins in contrast to the adenovirus genome, and is flanked at the ends by direct terminal repeat sequences called 'a' sequences which may be present in multiple copies at the left end and in only one copy at the right end of the linear molecule. The linear genome is divided into unique long (U_L) and unique short (U_S) segments with the above mentioned 'a' sequences at the

junction between the two unique segments (Roizman, 1979). As with the 'a' sequences at the left terminus, the copy number of the 'a' sequence at the junction may be greater than one (Fig. 27). The two unique segments of the viral genome are found in both possible orientations relative to each other in infected cells, thus giving rise to all four structural isomers of the viral genome in equal copy numbers (Fig. 27). It has been suggested that the isomerization of HSV genome occurs during viral DNA replication either through site-specific cleavage (Varmuza and Smiley, 1985) or through site-specific recombination (Chou and Roizman, 1985) at the 'a' sequences.

The yeast 2 micron plasmid is an episomal genetic element found in yeasts, and contains two embedded inverted repeat sequences (Fig. 27). Two structural isomers (A and B) of the plasmid, generated by site-specific intramolecular recombination at the inverted repeat sequences, exist in equal copy numbers (Broach, 1982). Along with the inverted repeat sequences which are required in *cis* for the isomerization of the plasmid, a protein product from a gene on the plasmid is required in *trans*.

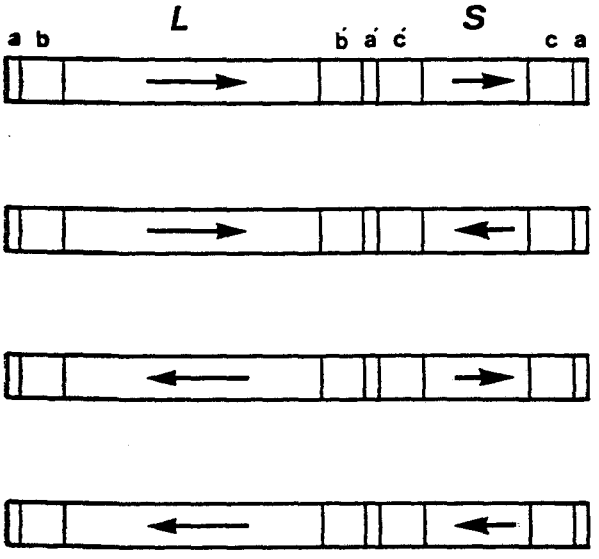
Based on these observations in other systems, it seemed possible that the recombinant adenoviruses constructed in this study could undergo rearrangements through homologous recombination. Along with rearrangements of parental genomes through homologous recombination, the viral DNA molecules may be rearranged during replication. In this case, the embedded ITR sequences could allow the formation of novel panhandle

FIGURE 27. A schematic diagram showing the structural isomers of HSV genome and yeast 2 micron plasmid.

The HSV genome is divided into 2 unique segments, long (L) and short (S). Each unique segment is flanked by inverted repeat elements (ab and b'a' for the L segment, and a'c' and ca for the S segment). The two segments are covalently joined to create b'a'c' junction. The copy number of the a sequence from the long segment and the a' sequence from the junction can vary from 1 to more than 10, however the copy number of the a sequence from the short segment is constant at 1. The four isomers of the genome are present in equal copy numbers in infected cells, and represent all combinations of orientations of the segments (indicated by the arrows).

The yeast 2 micron plasmid exists in two equimolar isomeric forms A and B, which differ by the orientation of the sequences between the inverted repeat (IR) sequences.

HSV



YEAST 2μ PLASMID

structures by the displaced parental strand during viral DNA replication. Such structures were postulated to explain the interconversion of Ad5dlE1,3-1 and Ad5dlE1,3-2 (Haj-Ahamd and Graham, 1986c). Ad5dlE1,3-1 and Ad5dlE1,3-2 are mutant viruses rescued during the construction of dlE1,3. dlE1,3-1 has an extra copy of the ITR at the left end and dlE1,3-2 has an extra ITR at both the left and right ends. The embedded viral junction in Ad5J-1 and Ad5J-2 could generate additional rearrangements of the parental genome by serving as an origin of viral DNA replication in both the linear or the circular form of parental DNA.

These possible mechanisms of rearrangement are discussed below with accompanying diagrams.

3.3.1.1 Homologous Recombination.

In Fig. 28 I have illustrated the possible rearrangements of parental DNA with embedded viral junction resulting from recombination using Ad5J-1 as an example. The recombination intermediates can be formed through homologous recombination (Holliday, 1964; Meselson and Radding, 1975) and resolution of this cross-over intermediate by nicking of the non-crossing strand opposite the site of cross-over, followed by subsequent ligation, would generate the two novel viral DNA molecules as shown in C. Other rearranged viral DNA molecules (as shown in Fig. 35) could be generated from Ad5J-1 through different alignments of the recombining parental genomes. Similarly, in Fig. 29 I have used Ad5TER-1 as an example to

FIGURE 28. Rearrangement of Ad5J-1 by homologous recombination.

Two recombining molecules are aligned by the homology between the embedded ITR sequences and the terminal ITR sequences (A). Strand exchange occurs (B), followed by nicking and exchange of the non-crossing strands. Subsequent ligation generates a truncated viral DNA molecule and a viral DNA molecule with a duplication of the genome. See text for details. The terminal ITRs of the parental virus are represented by open triangles and embedded ITRs of the parental virus are represented by solid triangles.

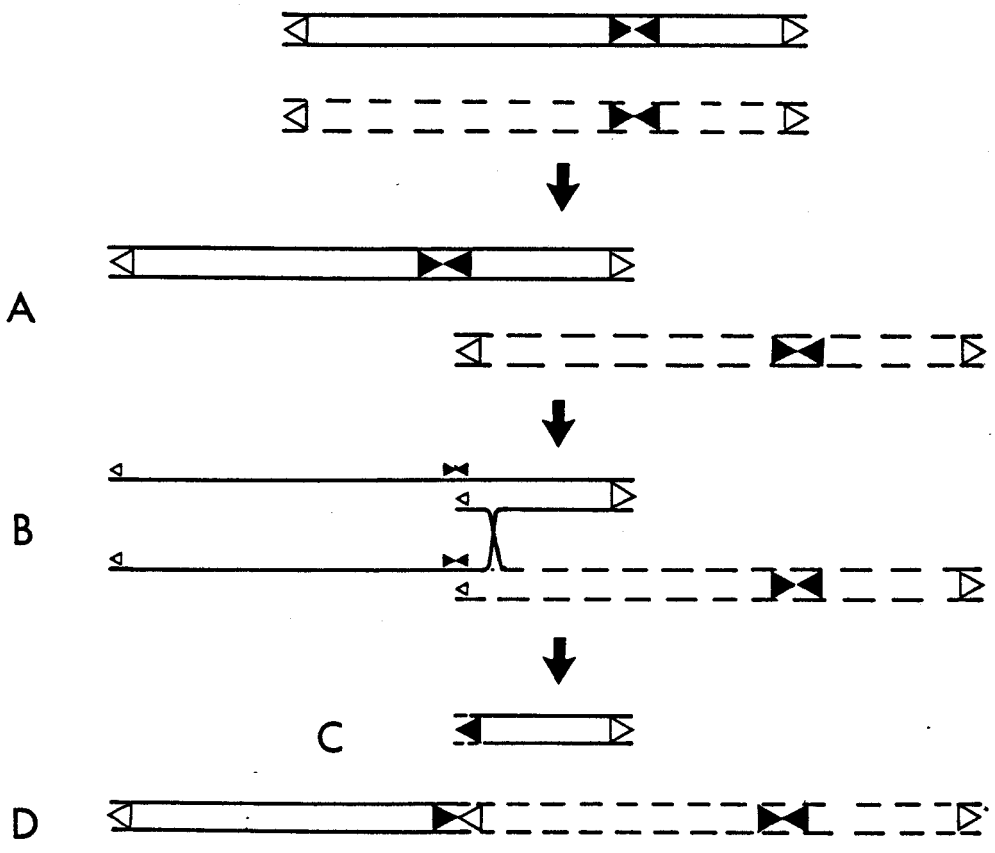
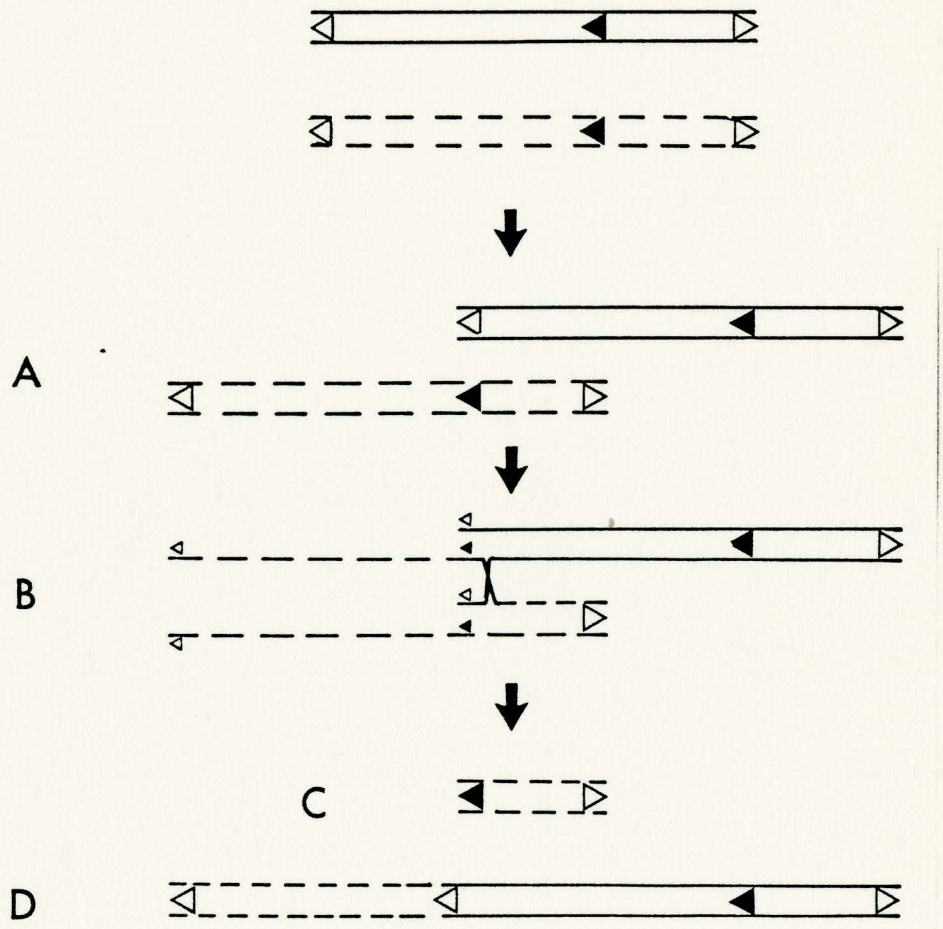

A
B
C
D

FIGURE 29. Rearrangement of Ad5TER-1 by homologous recombination.

Two recombining molecules are aligned by the homology between the embedded ITR sequences and the terminal ITR sequences (A). Strand exchange occurs (B), followed by nicking and exchange of the non-crossing strands. Subsequent ligation generates a truncated viral DNA molecule (C) and a viral DNA molecule with a duplication of the genome (D). The terminal ITRs of the parental virus are represented by open triangles and embedded ITRs of the parental virus are represented by solid triangles.


A
B
C
D

illustrate the possible rearrangements of parental DNA with an embedded single ITR.

3.3.1.2 Replication.

Using Ad5J-1, Figure 30 illustrates the possible rearrangements of parental DNA with embedded viral junctions resulting from viral DNA replication. Initiation of replication at the left end followed by strand-displacement synthesis of a double-stranded daughter molecule produces the single-stranded parental DNA. This ss-parental DNA can then form the three different possible panhandle structures and, depending on the panhandle structure formed by the second daughter molecule, can have any one of three different structures. Panhandle formation between the terminal ITRs would regenerate the parental form, whereas panhandle formation between a terminal ITR and an embedded ITR would generate either a deletion or a duplication of parts of the parental genome. By the formation of panhandle intermediates during replication, each mutant virus can generate novel viral DNA molecules identical to those generated by the recombination mechanism, except for molecules G and H as shown in Fig. 35. To illustrate the possible rearrangements of parental DNA with an embedded single ITR through viral DNA replication, Ad5TER-1 was used as an example in Fig. 31.

Besides the mechanism shown in Fig. 30, for Ad5J-1 and Ad5J-2, viral DNA could be rearranged as a result of initiation of replication at the embedded viral junction in the linear or circularized forms. These two alternative pathways are shown in Figs. 32 and 33 using Ad5J-1 as an

FIGURE 30. Rearrangement of Ad5J-1 by panhandle formation during replication.

Type I replication following initiation at the left end generates a double-stranded daughter molecule and a single-standed parental molecule. The single-stranded parental molecule then forms any one of three panhandle structures by hybridization between the terminal ITRs and the embedded ITRs (A and C) or between the terminal ITRs (B). Subsequent single-strand DNA specific nuclease activity (D) or DNA polymerase activity (E) removes the single strand overhangs. These panhandle structures then generate the rearranged viral DNA molecules F and H, and the parental molecule G. Rearranged viral DNA molecules that could be generated from the viruses with embedded terminal sequences through panhandle formation are shown in the next panel. Open triangles represent terminal ITRs and solid triangles represent embedded ITRs of the parental genome.

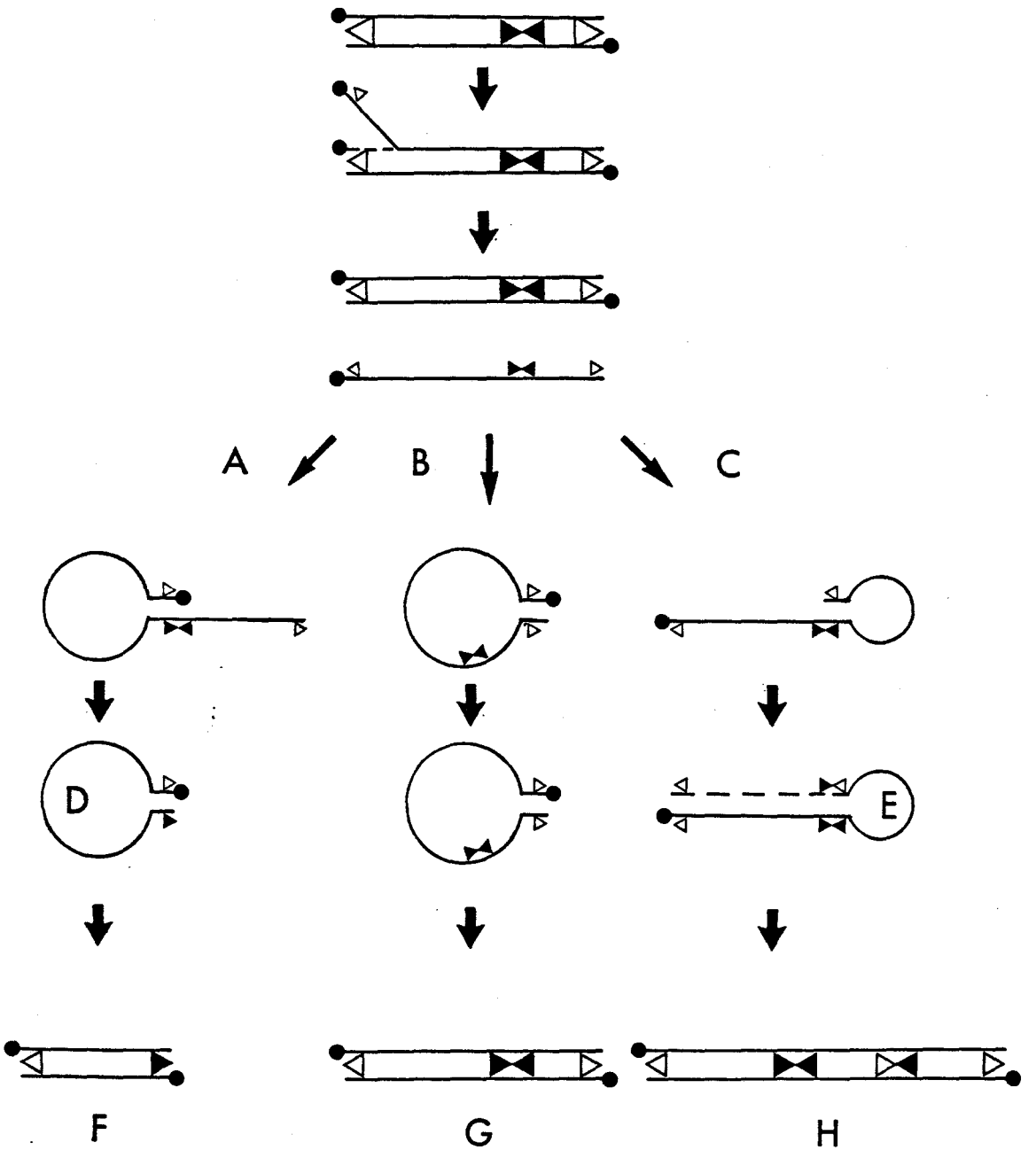

A
B
C
D
E
F
G
H

FIGURE 31. Rearrangement of Ad5TER-1 by panhandle formation during replication.

Type I replication following initiation at the left end generates a double-stranded daughter molecule and a single-standed parental molecule. The single-stranded parental molecule then forms one of two possible panhandle structures by hybridization between the right ITR and the embedded ITR (A) or between the terminal ITRs (B). DNA polymerase activity to remove the ss overhang followed by viral DNA replication (C) can generate a novel molecule (E). Parental DNA molecule (F) is regenerated through replication of the panhandle structure (D).

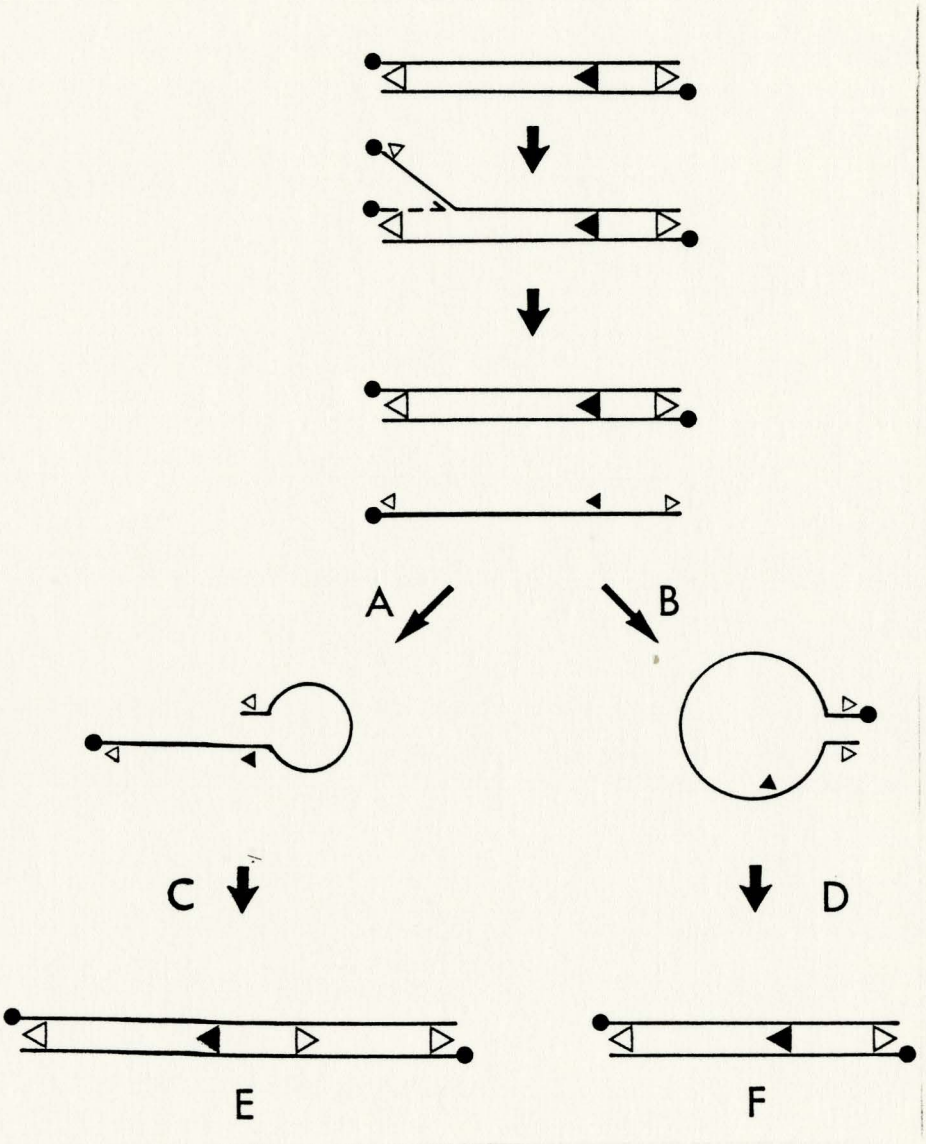

A
B
C
D
E
F

FIGURE 32. Generation of truncated viral DNA molecules through viral DNA replication at the embedded viral junction of Ad5J-1.

Initiation of viral DNA replication at the embedded viral junction in the linear form of parental genome followed by strand displacing elongation generates the truncated viral DNA molecules.

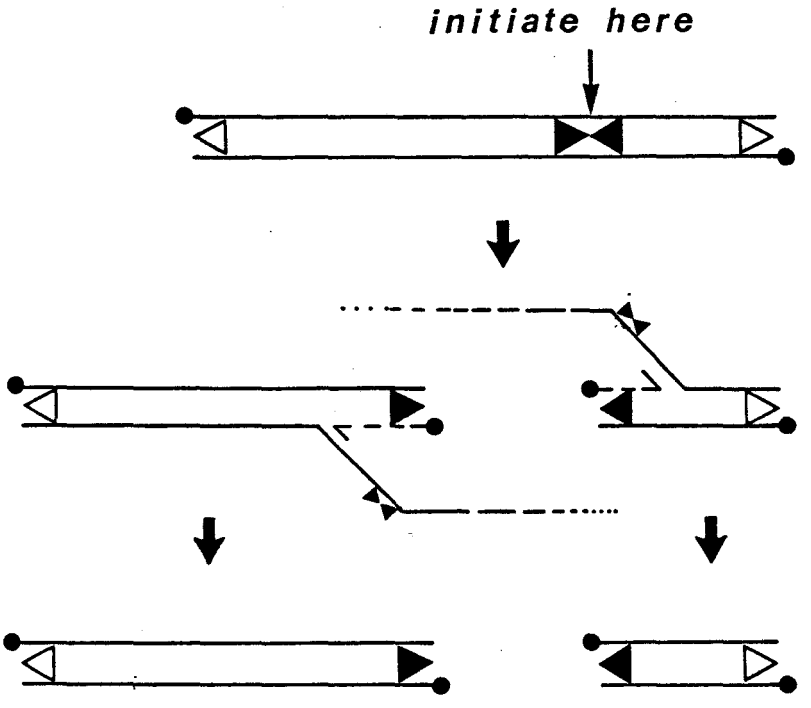
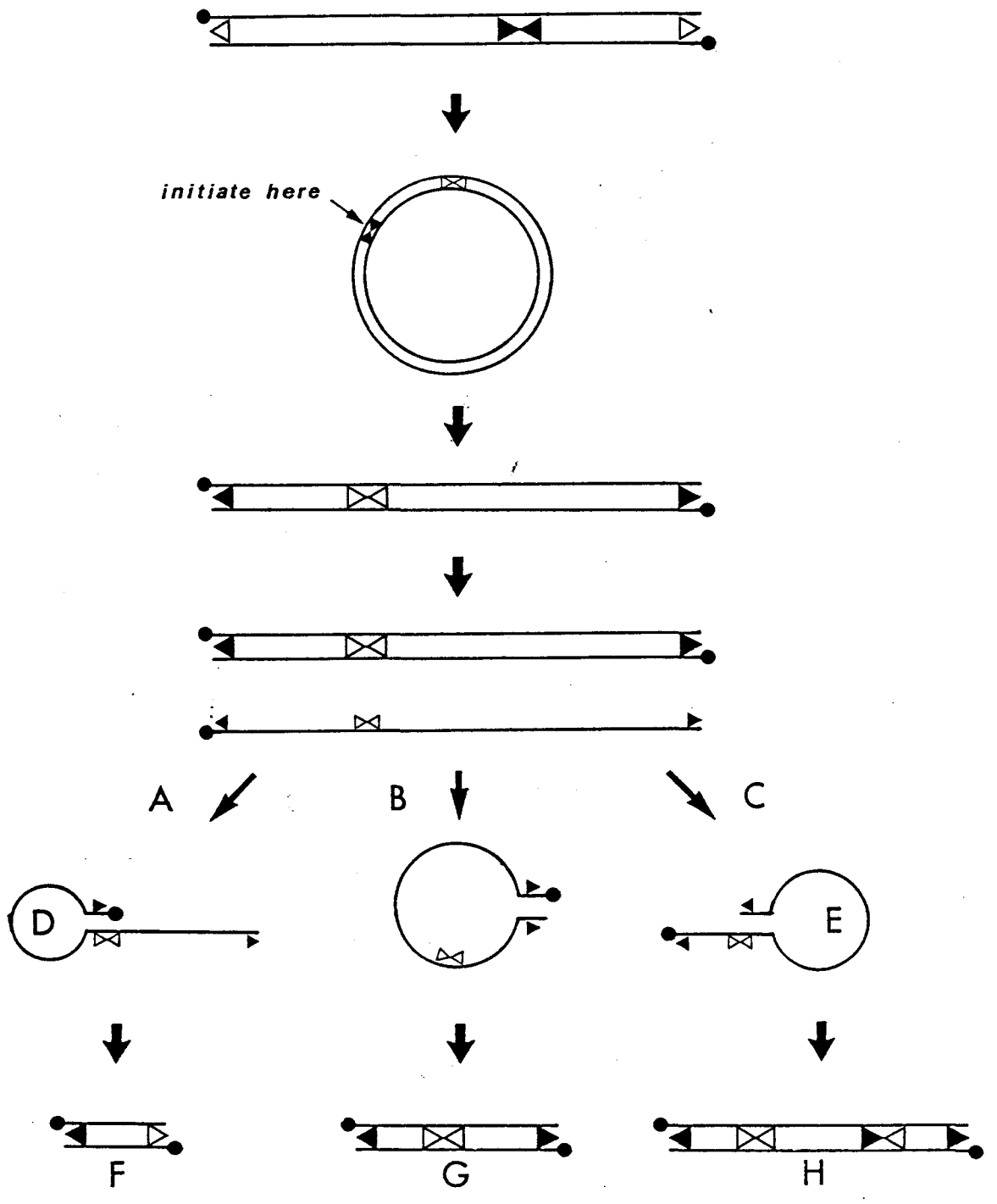

initiate here

FIGURE 33. Rearrangement of Ad5J-1 DNA through initiation of viral DNA replication at the embedded viral junction of the circular form.

Circularization of the linear form followed by initiation of viral DNA replication at the embedded viral junction and strand displacing elongation would generate a double-stranded daughter molecule that is a flip-flop isomer of the parental genome, and a single-stranded viral DNA molecule with the same flip-flopped orientation. The single strand form of the isomer can then form three different possible panhandle structures through hybridization of the ITR sequences (A, B, and C). Subsequent 3'-exonuclease activity (D) or 3'-extension activity (E) to remove the single-stranded region of the panhandle followed by initiation of replication at the panhandle and elongation, generates rearranged viral DNA molocules (F and H) as well as the flip-flop isomer parent molecule (G). Open triangles represent terminal ITRs and solid triangles represent embedded ITRs of the parental genome.


initiate here
A
B
C
D
E
F
G
H

example. In Fig. 32, initiation of replication at the embedded viral junction in a linear form would generate two truncated, defective viral DNA molecules (Fig. 32) capable of replication in the presence of replication proteins supplied in trans. In Fig. 33, initiation at the embedded viral junction of a DNA molecule in circular form would generate a unit-length molecule in which the parental DNA molecule has flipped about the vertical axis through the middle of the embedded viral junction. Replication of this molecule from the left end would generate a double-stranded daughter molecule and a single-stranded parental molecule which could then form three different possible panhandle structures. By the 3'-exonuclease or 3'-extension activities as described in the mechanism illustrated in Fig. 30, the three panhandle structures could generate the three novel viral DNA molecules G, H, and I. Other rearranged viral DNA molecules that can be generated from Ad5J-1 by this mechanism (Fig. 33) are shown in Fig. 35.

Rearrangements of parental viral DNA molecules that can be generated from Ad5J-1, Ad5J-2, and Ad5TER-1 and -2 through the four mechanisms discussed above are shown in Figures 34 (Ad5TER-1 and -2), 35 (Ad5J-1) and 36 (Ad5J-2). These figures show the novel viral DNA molecules produced from a single round of rearrangement through the four mechanisms and therefore, have the simplest structure possible. It should be noted that further rounds of rearrangement could generate novel viral DNA molecules larger than the 65.0 kb molecules and with yet more complex structures.

FIGURE 34. A schematic drawing of the rearranged viral DNA molecules which could be generated by Ad5TER-1 and Ad5TER-2.

The novel viral DNA molecules represented here were predicted through the rearrangement mechanisms discussed earlier (recombination - recomb, replication from the terminal ITRs - external replicat) after a single round of rearrangements (A and B - Ad5TER-1, C and D - Ad5TER-2). These molecules represent the hypothetical products from above mechanisms. The parental genomes are shown above the rearranged DNA molecules (Pa). The terminal and embedded ITRs of the parent genome are represented by open and closed triangles respectively. 'L,l' and 'R' indicate the left (L) and right (R) ITRs of the parental terminal and the embedded left ITR (l). Predicted sizes of the viral DNA molecules are given to the right of the molecules. The rearrangement mechanisms are listed to the right of the predicted sizes and the source of each viral DNA molecule is indicated by the plus sign (+).

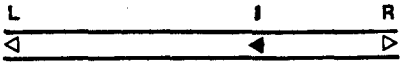
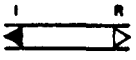
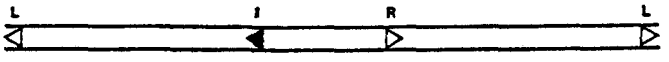
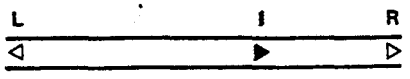
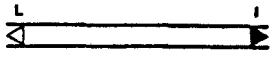
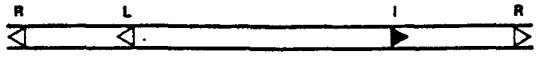
REARRANGEMENTS		kb	possible mechanisms: recomb	possible mechanisms: external replicat
Ad5TER 1				
Pa		34.9		
A		9.5	+	+
B		60.3	+	+
Ad5TER 2				
Pa		34.9		
C		29.5	+	+
D		40.3	+	+

FIGURE 35. A schematic drawing of the rearranged viral DNA molecules which could be generated by Ad5J-1.

The novel viral DNA molecules represented here were predicted through the rearrangement mechanisms discussed earlier (recombination - recomb, replication from the terminal ITRs - external replicat, and replication from the embedded ITRs) after a single round of rearrangements. These molecules represent the hypothetical products from above mechanisms. The parental genome is shown above the rearranged viral DNA molecules (Pa). The terminal and embedded ITRs of the parental genome are represented by open and closed triangles respectively. The upper and lower case 'L,l' and 'R,r' indicated the left and right ITRs of the parental terminal and embedded ITRs respectively. Predicted sizes of the viral DNA molecules are given to the right of the molecules. The rearrangement mechanisms are listed to the right of the predicted sizes and the source of each viral DNA molecule is indicated by the plus sign (+).

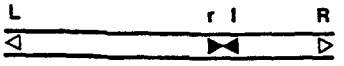
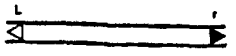
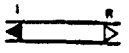
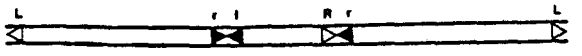
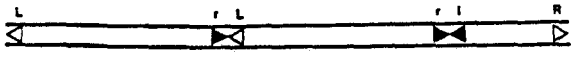
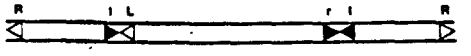
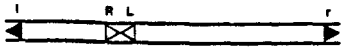
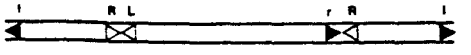
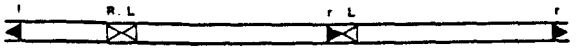
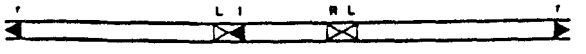
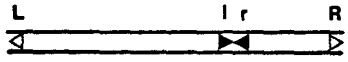
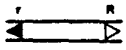
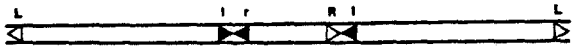
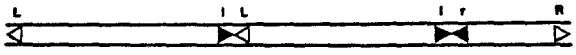
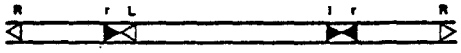
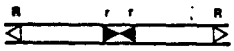
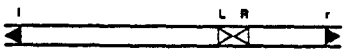
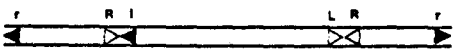
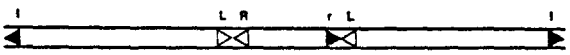
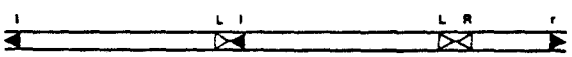
	REARRANGEMENTS	*kb*	possible mechanisms		
			recomb	external replicat	internal replicat
Pa		35.5			
A		26.0	+	+	+
B		9.5	+	+	+
C		61.5	+	+	-
D		61.5	+	+	-
E		44.0	+	+	-
F		44.0	+	+	-
G		52.0	+	-	-
H		19.0	+	-	-
I		35.5	-	-	+
J		44.0	-	-	+
K		44.0	-	-	+
L		61.5	-	-	+
M		61.5	-	-	+

FIGURE 36. A schematic drawing of the rearranged viral DNA molecules which could be generated by Ad5J-2.

The novel viral DNA molecules represented here were predicted through the rearrangement mechanisms discussed earlier (recombination - recomb, replication from the terminal ITRs - external replicat, and replication from the embedded ITRs) after a single round of rearrangements. These molecules represent the hypothetical products from above mechanisms. The parental genome is shown above the rearranged molecules (Pa). The terminal and embedded ITRs of the parental genome are represented by open and closed triangles respectively. The upper and lower case 'L,l' and 'R,ŕ' indicated the left and right ITRs of the parental terminal and embedded ITRs respectively. Predicted sizes of the viral DNA molecules are given to the right of the molecules. The rearrangement mechanisms are listed to the right of the predicted sizes and the source of each viral DNA molecule is indicated by the plus sign (+).

	REARRANGEMENTS	*kb*	possible mechanisms		
			recomb	external replicat	internal replicat
Pa		35.5			
A		29.5	+	+	+
B		6.0	+	+	+
C		65.0	+	+	-
D		65.0	+	+	-
E		41.5	+	+	-
F		41.5	+	+	-
G		59.0	+	-	-
H		12.0	+	-	-
I		35.5	-	-	+
J		41.5	-	-	+
K		41.5	-	-	+
L		65.0	-	-	+
M		65.0	-	-	+

3.3.2 Novel Viral DNA Molecules Are Detected in Infected Cells.

Predicted structure and sizes of the hypothetical rearranged viral DNA molecules generated by the embedded terminal sequences are shown in Figs. 34, 35, and 36. To detect novel viral DNA molecules in infected cells, 60 mm dishes of 293 cells were infected with the five mutant viruses at an moi of 10 and the total intracellular DNA was extracted as described in Chapter 2. The undigested intracellular viral DNA extracted from the five infected cell populations was analyzed by Southern blot hybridization using a nick-translated genomic probe, pFL157. Figure 37 shows the results from two separate hybridization experiments because due to the insufficient amount of total DNA loaded in certain lanes in one experiment (Panel A), some of the novel viral DNA molecules in infected cells were not detected, whereas in Panel B where more total DNA was used, these molecules were detected.

3.3.2.1 Ad5neo3.7.

In mock-infected cells (Fig. 37, Panel A - lane 3; Panel B - lane 2) no sequences were detected by the probe, indicating the absence of non-specific hybridization between pFL157 and cellular sequences. Although 293 cells contain the left 14% of the Ad5 genome (Aiello et al, 1979; Graham et al, 1977), the E1 deletion in the probe pFL157, along with the low copy number of the E1 gene in 293 cells probably prevented the detection of the Ad5 sequences in 293 cell DNA. In Ad5neo3.7

infected cells (Panel A - lane 4), a band co-migrating with wtAd5 DNA (lane 1) was detected as expected from the predicted size of Ad5neo3.7 DNA, suggesting that the band probably represents the parental viral DNA molecule. No other viral DNA sequences were detected in Ad5neo3.7 infected cells.

3.3.2.2 Ad5TER-1 and Ad5TER-2.

In Ad5TER-1 infected cells a total of three viral DNA bands were detected (Fig. 37, Panels A and B). Two are seen in lane 5 (Panel A) and a third very faint band migrating above the major band can be seen in Panel B (lane 4). Based on the migration rates of wtAd5 DNA (Panel A - lane 1) and Ad5neo3.7 DNA (lane 4 - Panel A), and the predicted size of the Ad5TER-1 parental DNA molecule, the major band in the infected cell DNA sample probably represented the parental unrearranged DNA molecule. Although there were no marker DNA molecules run as references in the blot shown in Panel B, based on migration rate relative to other viral bands the band migrating above the parental DNA in lane 4 (Panel B) was estimated to be about 60 kb, perhaps corresponding to molecule B predicted in Figure 34. For the band migrating below the parental DNA band, the size was estimated to be 9.5 kb based on the rate of migration of the marker DNA molecules, suggesting that the band could represent molecule A in Figure 34.

In Ad5TER-2 infected cells, three viral DNA bands were detected (lane 6, Panel A; lane 5, Panel B). The major band in the lane probably represented the parental genome based on

its migration rate relative to the marker DNA. Based on the migration rate of marker DNA, the size of the viral DNA band above the parental band was estimated to be 42 kb, corresponding to molecule D (Fig. 34). Similarly, the size of the band migrating below the parental band was estimated to be 30 kb, corresponding to molecule C (Fig. 34).

These results suggested that novel viral DNA molecules that were predicted by the recombination and terminal replication mechanisms were detected in Ad5TER-1 and Ad5TER-2 infected cells, and that these rearrangements could be correlated with the presence of an embedded ITR sequence in the linear genome.

3.3.2.3 Ad5J-1 and Ad5J-2.

A total of six viral DNA bands were detected by the genomic probe in Ad5J-1 infected cells, four visible in both Panels A (lane 7) and B (lane 6) of Fig. 37 and two bands migrating slower and faster than the major band detectable in the autoradiograph shown in Panel B. Based on the migration rate of other parental and marker DNA molecules, it appeared probable that the major band in the infected cell DNA sample represented the parental DNA molecule.

Two bands were detected above the parental DNA band. Although suitable marker DNA bands were absent from this blot, based on migration rate, the size of the viral DNA in the upper most band above the parental DNA (lane 6, Panel B, Fig. 37) was estimated to be 60 kb, which suggested that these molecules may correspond to molecules C, D, L and M in Fig.

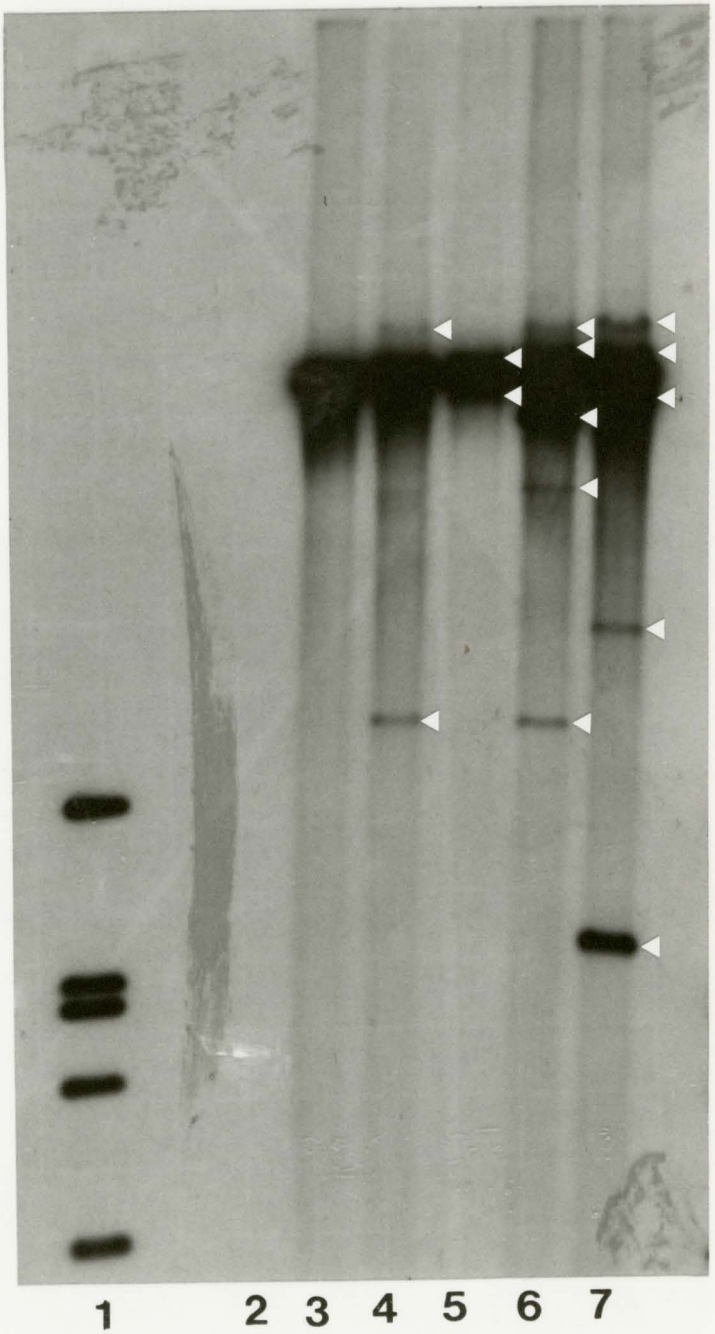
FIGURE 37. Autoradiograph of undigested intracellular viral DNA.

Panel A: 60 mm dishes of 293 cells were infected with each of the five viruses individually. The infections and the extraction of viral DNA were carried out as described in Chapter 2. The DNA samples were separated by 0.3% agarose gel electrophoresis and the Southern blot of this gel was probed with nick-translated pFL157, a genomic probe. Novel, rearranged viral DNA molecules are indicated by the solid triangles. The bands in lane 2 were generated by ligating the EcoRI fragments (A - 27 kb, B - 6 kb, C - 3 kb) from wtAd5 DNA. Lanes 1 - undigested, banded wtAd5 DNA; lane 2 - ligation of EcoRI digested wtAd5 DNA; lane 3 - mock-infected; lane 4 - Ad5neo3.7; lane 5 - Ad5TER-1; lane 6 - Ad5TER-2; lane 7 - Ad5J-1; lane 8 - Ad5J-2; lane 9 - HindIII digested wtAd5 DNA. Panel B: In a similar experiment, undigested total viral DNA from the infected cells was separated by 0.5% agarose gel electrophoresis and Southern blotted to a nitrocellulose filter membrane. The membrane was then probed with nick-translated pFL157, and the autoradiograph from the experiment is shown. The novel, rearranged viral DNA molecules are indicated by the solid triangles. Lane 1 - wtAd5 HindIII digest; lane 2 - mock-infected cell DNA; lane 3 - Ad5neo3.7; lane 4 - Ad5TER-1; lane 5 - Ad5TER-2; lane 6 - Ad5J-1; lane 7 - Ad5J-2.

A

kb
54
36
33
30
27
6
1
2
3
4
5
6
7
8
9
wtAd5
WtAd5 EcoRI ligation
mock—infected
Ad5 neo3.7
Ad5TER—1
Ad5TER—2
Ad5J—1
Ad5J—2
wtAd5 HindIII
8.4
kb

B


wtAd5 HindIII
mock–infected
Ad5neo37
Ad5TER–1
Ad5TER–2
Ad5J–1
Ad5J–2
1
2
3
4
5
6
7

35. The size of the viral DNA in the second band above the parental DNA was estimated to be 45 kb based on rate of migration, which is in close agreement with the predicted sizes of molecules E, F, J and K (45 kb) in Fig. 35. Similarly, the three viral DNA bands migrating faster than the parental DNA probably represented viral DNA molecules with lengths of 27 kb, 19 kb, and 9 kb respectively, close to the sizes of the predicted truncated molecules A, H and B in Fig. 35. The 9 kb fragment co-migrated with the smallest fragment of Ad5TER-1 as predicted from Figures 34 and 35, suggesting that the same mechanism(s) could have generated these molecules. Among the three bands representing truncated viral DNA molecules, the second band below the parental band was interesting because the estimated size of the band suggested that it may represent a covalently joined dimer of the 9.5 kb molecule (B, Fig. 35). From comparison of the lengths of the rearranged viral DNA molecules, it appeared that these novel molecules were produced by mechanism(s) that were capable of generating both deletions and duplications of the viral genome. Furthermore, based on the different intensities of the viral DNA bands it appeared that the rearranged molecules were not present in the same copy number.

Similar to the viral DNA from Ad5J-1 infected cells, a total of six viral DNA bands were detected in Ad5J-2 infected cells. Four bands were visible in both Panels A (lane 8) and B (lane 7) and two bands migrating above and below the major band in lane 7 (Panel B). Based on comparison of rates of migration of the two bands above the parental

band, the marker DNA molecules and viral DNA molecules from other infected cells, the upper band in the pair of bands migrating above the parental DNA (lane 7, Panel B; Fig. 37) was estimated to be 65 kb and the lower band in the pair (lane 8, Panel A; Fig. 37) was estimated to about 43 kb in length. These estimated lengths corresponded to those of the hypothetical rearranged molecules C, D, L and M (upper band) and E, F, J and K (lower band), and therefore, suggested as with the two bands above the parental band in Ad5J-1 infected cells, that these two bands likely contained rearranged viral DNA molecules with duplications of the viral genome. The lengths of viral DNA in the three bands migrating faster than the parental DNA were estimated to be about 30 kb, 12 kb and 6 kb respectively, and the bands probably represented the truncated viral DNA molecules A, H and B in Fig. 36 respectively. Co-migration of the 6 kb band with the smallest fragment from Ad5TER-2 as predicted from tables in Figures 34 and 36 supports the suggestion that the mechanism(s) discussed previously (Figs. 29 to 33 inclusive) probably generated these rearrangements. Similar to the second band below the parental band in Ad5J-1 infected cells, the estimated size of the corresponding band from Ad5J-2 infected cells suggested that it may represent dimers of the 6.0 kb truncated viral DNA molecule B (Fig. 36). As observed for the viral genome rearrangements in Ad5J-1 infected cells, comparison of lengths of rearranged molecules in Ad5J-2 infected cells further supported the speculation that these rearrangements were created by mechanism(s) capable of generating deletions and

duplications of parental genomes. Based on the intensities of novel viral DNA bands, it appeared that rearranged molecules, as observed previously, were not present in equal copy numbers.

Results presented in this section, together with the results from analysis of viral DNA from cells infected with Ad5neo3.7, Ad5TER-1, and Ad5TER-2, suggested that likely the efficiency of rearrangement of viral genome was higher with the embedded junction than with the embedded single ITR.

3.3.3 Quantitation of Rearranged Viral DNA Molecules.

As noted above, from visual examination of autoradiograms rearranged viral DNA molecules did not appear to be present in equimolar concentrations in infected cells. To quantitate the amount of rearranged viral DNA in infected cells, the concentrations of the rearranged molecules relative to the total intracellular viral DNA were determined by densitometer scans of an autoradiogram similar to those shown in Fig. 37.

Based on the results shown in Table 2, it appeared that in Ad5J-1 infected cells the concentration of the 27 kb molecule was similar to the concentration of unrearranged DNA suggesting that this truncated molecule was generated and propagated very efficiently. The high copy number of the 27 kb molecule is likely due to the presence of the adenoviral encapsidation signal near the left terminus (Hearing et al, 1987). This same signal is present near the left terminus of the 9.5 kb molecule (molecule B, Fig. 35), but this molecule

TABLE 2. Results from densitometric scanning of an autoradiograph of undigested viral DNA from Ad5J-1 and Ad5J-2 infected cells[a].

Lane	Band[b]	Area(O.D)	Cpm	% Total[d]
Ad5J-1	1	ND[c]	ND	ND
	2	ND	ND	ND
	Parental	18851	3055	53
	3	12135	2685	47
	4	ND	ND	ND
	5	ND	ND	ND
AD5J-2	1	ND	ND	ND
	2	ND	ND	ND
	Parental	21359	3462	34
	3	14356	2800	29
	4	ND	ND	ND
	5	3676	3411	35

[a] To determine the ratio of the viral band intensities in infected cell DNA samples, an autoradiograph similar to that shown in Fig. 37, Panel B was scanned using the Hoefer densitometer as described in Chapter 2. Where possible, the cpm value for each band was determined from its optical density value through interpolation on the standard graph shown in Chapter 2 (Fig. 9).

[b] The numbers refer to the band's position relative to the parental band (above - 1 and 2; below - 3, 4, and 5).

[c] Due to the insignificant size of the peaks, the area was not determined.

[d] Total = Σ calculated cpm values.

is not present in high copy number in infected cells. This suggested that perhaps there was a selection pressure against this molecule during viral DNA rearrangement or viral DNA replication.

In Ad5J-2 infected cells, Table 2 shows that the concentrations of the 30 kb and 6 kb molecules were 29% and 35% of the total DNA. This suggested that the 30 kb molecule was generated as efficiently as the 27 kb molecule from Ad5J-1. This molecule (30 kb) also contained the adenoviral encapsidation signal (Hearing et al, 1987), suggesting a correlation between the presence of encapsidation signal and amplification of the truncated molecule. It should be noted, however, that the molecule present in the highest copy number (band 5, Table 2 and molecule B, Fig. 36) does not contain the said signal.

3.3.4 Identification of Novel Viral Termini in Infected Cells.

Results from hybridizations of undigested viral DNA from infected cells showed the presence of novel, rearranged viral DNA molecules. From the estimated sizes of these novel viral bands, it appeared they may correspond to the hypothetical rearranged viral genomes predicted by the various mechanisms discussed previously (Figs. 28 to 33 inclusive). A sub-population of these hypothetical rearranged viral DNA molecules possessed novel termini (eg. molecules A, B, I to M - Fig. 35).

I wanted to determine whether the pool of novel viral DNA molecules generated by each mutant virus contained any

viral DNA molecules with novel termini as would occur if embedded ITR sequences of the parental genome were converted to terminal ITR sequences of the rearranged molecules. To do this, DNA from cells infected with each of the five viruses individually was digested with a number of restriction endonucleases and Southern blots were prepared. The filters were hybridized with different DNA probes to detect the restriction digest fragments diagnostic for the novel viral termini. The data from the hybridization experiments with these three DNA probes are presented below. The three probes used were a 1 kb viral DNA insert from pUCIG that contains the ITR junction (ITR probe), the neo/G418^R plasmid pKN30 (neo probe), and a 20 base oligonucleotide complementary to E3 promoter sequences (E3 probe) (Figs. 38, 39, and 40).

3.3.4.1 Ad5neo3.7.

The autoradiographs from hybridizations with the three DNA probes (Figs. 38, 39, and 40) showed that no restriction digest fragment diagnostic of the novel termini were detected, suggesting that Ad5neo3.7 did not generate any viral DNA molecules with novel termini. This observation was consistent with results obtained by probing blots of undigested viral DNA (Fig. 37). The band indicated by the solid circle (lanes 1 to 5, Fig. 38) was probably due to over digestion of parental genomes by EcoRI and does not represent a restriction digest product from a novel viral termini.

3.3.4.2 Ad5TER-1 and Ad5TER-2.

According to the possible structures predicted for rearranged viral DNA molecules generated from Ad5TER-1 and Ad5TER-2, only one novel terminal structure was expected (A and C, Fig. 34). This novel end would contain the left ITR sequence which was embedded internally in the parental genome.

A Southern blot of DNA samples from Ad5TER-1 and AD5TER-2 infected cells digested with EcoRI was probed with the ITR probe (Fig. 38, lanes 2 and 3). As well as detecting restriction fragments from the parental genomes (open triangles and solid circles), the probe detected a viral DNA fragment migrating at 3.6 kb in both samples (solid triangle) corresponding to a restriction digest fragment diagnostic of a novel viral terminus in the predicted rearranged viral DNA molecules. This suggested that viral DNA molecules with novel end(s) were indeed present in the infected cells.

To corroborate the results from the ITR probe hybridization experiment, a similar blot was probed with the neo probe. The autoradiogram (Fig. 39) shows that a viral DNA band migrating at 3.6 kb was again detected in DNA samples from Ad5TER-1 and Ad5TER-2 infected cells (lanes 7 and 8), suggesting that the 3.6 kb fragment detected by the ITR and the neo probes probably represented the same novel terminal fragment. These results were further substantiated by hybridization of a Southern blot of digested viral DNA from infected cells with the E3 probe (Fig. 40, lanes 2 and 3), which showed no novel viral DNA bands as expected. The data obtained from digestions with other restriction endonucleases

(BamHI and XbaI) confirmed the results from EcoRI digested DNA samples.

Detection of the restriction digest fragment diagnostic for the unique terminus in Ad5TER-1 and Ad5TER-2 infected cells suggested that the parental genome had been rearranged by a mechanism(s) that converted the embedded ITR into a terminal ITR of rearranged viral DNA molecules.

3.3.4.3 Ad5J-1 and Ad5J-2.

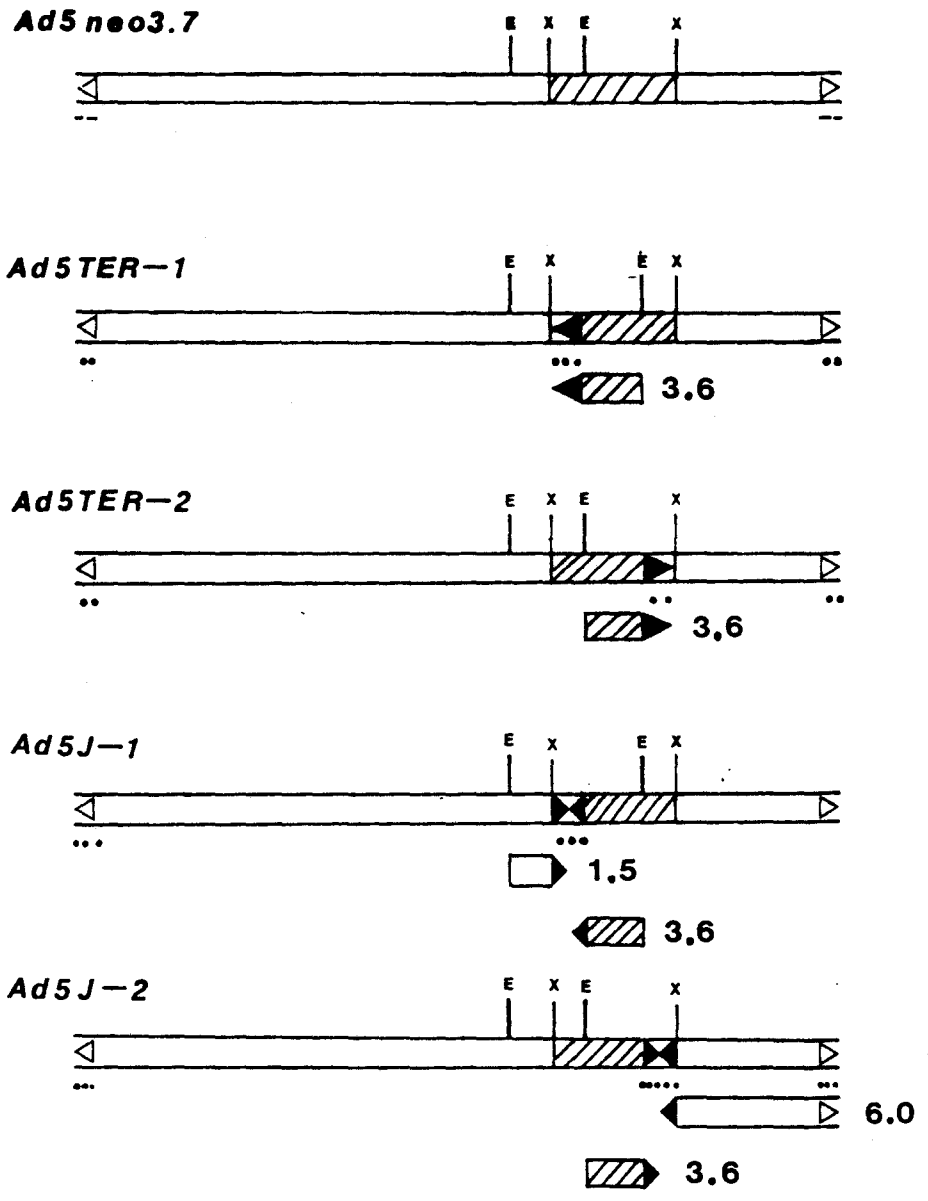
According to the predicted structures of the novel rearranged viral DNA molecules generated from Ad5J-1 and Ad5J-2, two novel terminal structures (A, B, and I to M inclusive; Figs. 34 and 35) might be expected. The predicted two novel termini would be present either on different novel viral DNA molecules (A and B, Figs. 34 and 35) or on the same viral DNA molecule (I, Figs. 34 and 35). To detect restriction fragments diagnostic for unique viral termini that were predicted from the structures of novel viral DNA molecules described previously in Section 3.3.1, the ITR probe was hybridized to a Southern blot of EcoRI digested DNA from Ad5J-1 and Ad5J-2 infected cells (Fig. 38, lanes 4 and 5).

In Ad5J-1 infected cells, the autoradiograph shows that the ITR probe detected restriction fragments from the parental genome (open triangles and solid circle) and two novel viral DNA bands migrating at 3.6 kb and 1.5 kb (solid triangles, lane 4, Fig. 38) as predicted. This suggested that they may represent the restriction fragments diagnostic for the predicted novel viral termini. To confirm this

FIGURE 38. Autoradiograph of digested intracellular viral DNA probed for ITR sequences.

Viral DNA from 293 cells infected with each of the five recombinant viruses individually was extracted and digested with EcoRI (lanes 1 - 5) and XbaI (lanes 6 - 10). A Southern blot after electrophoresis in a 0.8% agarose gel was probed for restriction digest fragments diagnostic for novel viral termini, with nick-translated 1 kb viral DNA fragment containing the junction of ITR sequences (ITR probe). The diagnostic restriction fragments predicted from an EcoRI digest are shown along with their lengths and the structures of the parental viruses as reference. The sequences which hybridize to the ITR probes are indicated by hatched boxes. The embedded ITR(s) and terminal ITRs of the parental genomes are represented by solid and open black triangles respectively. Sizes of the restriction fragments from EcoRI digest are from left to right: Ad5neo3.7 - 23.9, 1.4, 8.6 kb; Ad5TER-1 - 24.5, 4.5, 5.9 kb; Ad5TER-2 - 24.5, 1.4, 9.0 kb; Ad5J-1 - 24.5, 5.1, 5.9 kb; Ad5J-2 - 24.5, 1.4, 9.6 kb. E - EcoRI, X - XbaI.

The autoradiograph shows the presence of viral DNA band(s) representing the restriction digest fragment(s) (solid white triangles) from novel viral ends in viral DNA samples extracted from cells infected with viruses containing embedded ITR(s) and the absence of such fragment(s) in cells infected with Ad5neo3.7, a virus with no embedded ITR sequences. The open white triangles indicate the restriction fragments from the parental genomes, and the solid circles indicate the products from EcoRI over digestion of parental genomes. Lanes 1 and 6 - Ad5neo3.7; lanes 2 and 7 - Ad5TER-1; lanes 3 and 8 - Ad5TER-2; lanes 4 and 9 - Ad5J-1; lanes 5 and 10 - Ad5J-2; lane 11 - HindIII digested wtAd5 DNA.


Ad5 neo3.7
E X E X
Ad5TER−1
E X E X
3.6
Ad5TER−2
E X E X
3.6
Ad5J−1
E X E X
1.5
3.6
Ad5J−2
E X E X
6.0
3.6

1 2 3 4 5 6 7 8 9 10 11
Ad5 neo3.7
Ad5TER−1
Ad5TER−2
Ad5J−1
Ad5J−2
Eco RI
Ad5 neo3.7
Ad5TER−1
Ad5TER−2
Ad5J−1
Ad5J−2
Xba I
wtAd5 Hind III
kb
10
2.8

FIGURE 39. Autoradiograph of digested intracellular viral DNA probed for sequences from the kanamycin resistant plasmid pKN30.

A Southern blot of intracellular viral DNA digested with BamHI (lanes 1 - 5), EcoRI (lanes 6 - 10) and XbaI (lanes 11 - 15) individually and separated by 1.0% agarose gel electrophoresis was probed for the predicted restriction digest fragments diagnostic for novel viral termini with nick-translated neo/G418^R plasmid, pKN30. The diagnostic restriction fragments predicted from an EcoRI digest are shown along with their lengths and the structures of the parental viruses as reference. The sequences hybridizing to the probe are indicated by dashed lines. The embedded ITR(s) and terminal ITRs of the parental genomes are represented by solid and open black triangles respectively. The kb values down the righthand side refer to the sizes of various restriction fragments from parental genomes. Sizes of the restriction fragments from EcoRI digest are from left to right: Ad5neo3.7 - 23.9, 1.4, 8.6 kb; Ad5TER-1 - 24.5, 4.5, 5.9 kb; Ad5TER-2 - 24.5, 1.4, 9.0 kb; Ad5J-1 - 24.5, 5.1, 5.9 kb; Ad5J-2 - 24.5, 1.4, 9.6 kb. B - BamHI, E - EcoRI, X - XbaI.

The autoradiograph shows the presence of viral DNA band(s) representing the restriction fragment(s) (solid white triangles) from novel viral termini in viral DNA samples from cells infected with viruses containing embedded ITR(s) and the absence of such fragment(s) in cells infected with virus containing no embedded ITR sequences. The open white triangles indicate the restriction fragments from the parental genomes. Lanes 1, 6, 11 - Ad5neo3.7; lanes 2, 7, 12 - Ad5TER-1; lanes 3, 8, 13 - Ad5TER-2; lanes 4, 9, 14 - Ad5J-1; lanes 5, 10, 15 - Ad5J-2.

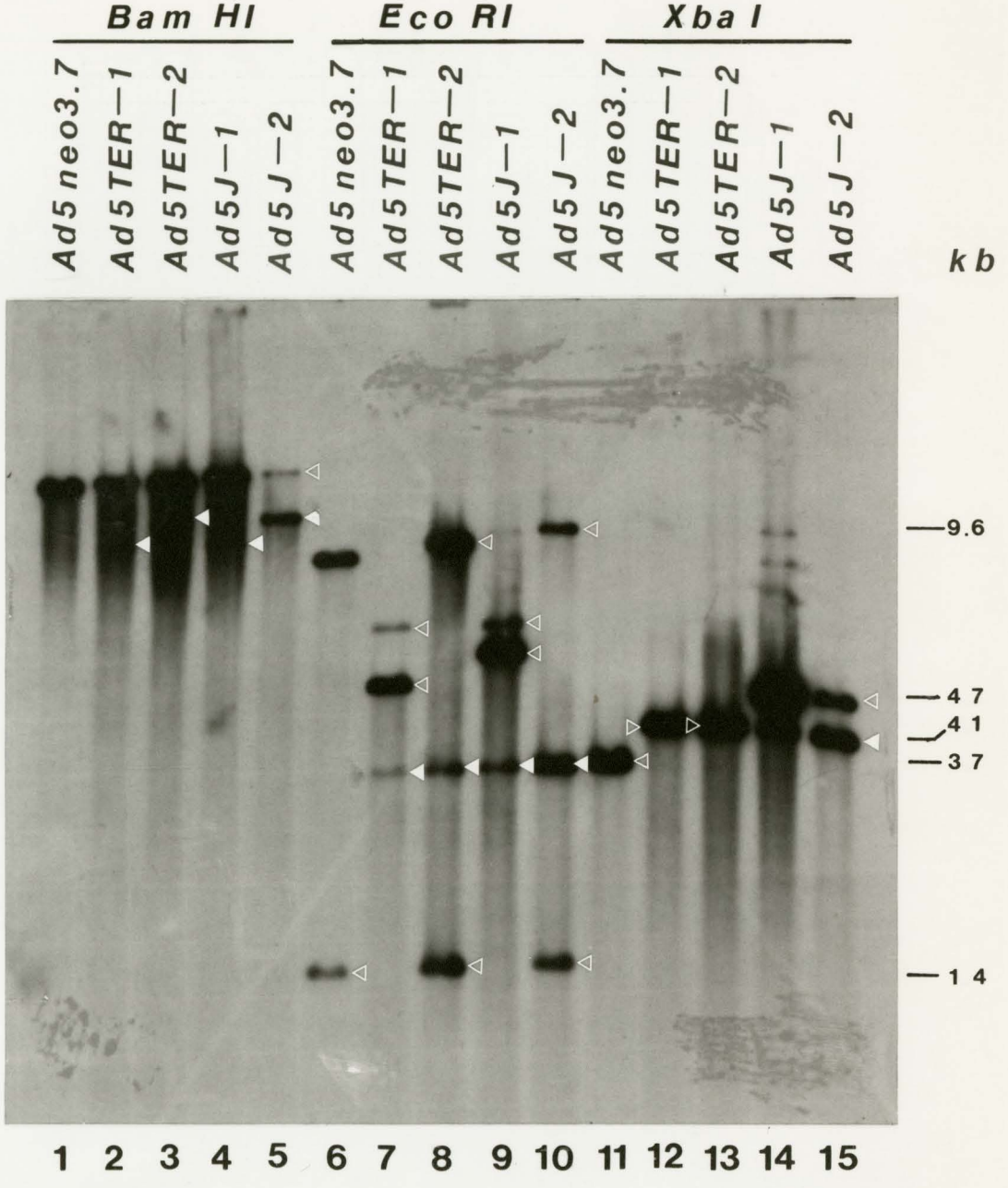
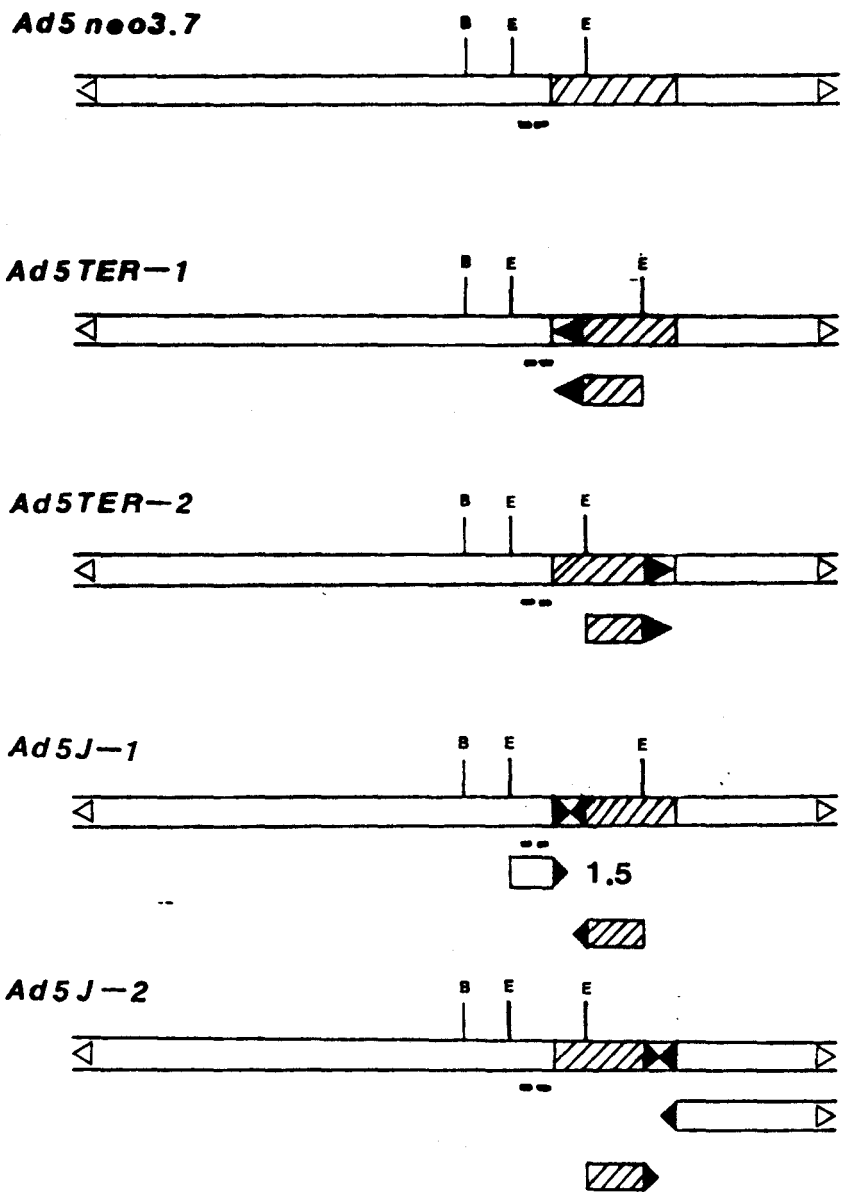

Bam HI
Eco RI
Xba I
Ad5 neo3.7
Ad5 TER—1
Ad5 TER—2
Ad5 J—1
Ad5 J—2
Ad5 neo3.7
Ad5 TER—1
Ad5 TER—2
Ad5 J—1
Ad5 J—2
Ad5 neo3.7
Ad5 TER—1
Ad5 TER—2
Ad5 J—1
Ad5 J—2
kb
9.6
4.7
4.1
3.7
1.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 40. Autoradiograph of digested intracellular viral DNA probed with an oligonucleotide hybridizing within the E3 promoter.

A Southern blot similar to Fig. 27, but containing BamHI digests (lanes 1 - 5) and EcoRI digests (lanes 6 - 10) was probed with a 20-mer oligonucleotide hybridizing to the E3 promoter sequences, to detect the predicted restriction digest fragments diagnostic for novel viral termini. The diagnostic restriction fragments predicted from an EcoRI digest are shown along with their lengths and the structures of the parental viruses as reference. The sequences hybridizing to the probe are indicated by hatched boxes. The embedded ITR(s) and terminal ITRs of parental genomes are represented by solid and open black triangles respectively. Sizes of the restriction fragments from EcoRI digest are from left to right: Ad5neo3.7 - 23.9, 1.4, 8.6 kb; Ad5TER-1 - 24.5, 4.5, 5.9 kb; Ad5TER-2 - 24.5, 1.4, 9.0 kb; Ad5J-1 - 24.5, 5.1, 5.9 kb; Ad5J-2 - 24.5, 1.4, 9.6 kb. B - BamHI, E - EcoRI.

The autoradiograph shows the presence of viral DNA band(s) (solid white triangles) representing the restriction fragment(s) from novel termini in cells infected with viruses carrying embedded ITR(s) and the absence of any novel viral band(s) in cells infected with virus carrying no embedded ITR(s). The open white triangles indicate the restriction fragments from the parental genomes, and the solid circles indicate the products from EcoRI over digestion of parental genomes. Lanes 1 and 6 - Ad5neo3.7; lanes 2 and 7 - Ad5TER-1; lanes 3 and 8 - Ad5TER-2; lanes 4 and 9 - Ad5J-1; lanes 5 and 10 - Ad5J-2; lane 11 - mock-infected cell DNA; lane 12 - HindIII digested wtAd5 DNA. E3 Probe - 5'-CGATCTGAAATGTCCCGTCC-3'


Ad5 neo3.7
B
E
E
Ad5TER—1
B
E
E
Ad5TER—2
B
E
E
Ad5J—1
B
E
E
1.5
Ad5J—2
B
E
E

1 2 3 4 5 6 7 8 9 10 11 12
Ad5 neo3.7
Ad5TER−1
Ad5TER−2
Ad5J−1
Ad5J−2
BamHI
Ad5 neo3.7
Ad5TER−1
Ad5TER−2
Ad5J−1
Ad5J−2
EcoRI
mock−infected
wtAd5 HindIII
kb
5.8
1.4

observation for the 3.6 kb fragment, a similar blot was hybridized with the neo probe which should detect only the diagnostic fragment containing the neo/G148 gene construct. Lane 9 of the autoradiograph in Fig. 39 shows that the neo probe hybridized only to the diagnostic fragment migrating at 3.6 kb, suggesting this fragment and the 3.6 kb fragment detected by the ITR probe (Fig. 38, lane 4) represented the same diagnostic fragment that was predicted by the structures of novel viral DNA molecules generated by different possible rearrangement mechanisms discussed earlier (Figs. 30 to 33 inclusive). To confirm that the second novel restriction fragment detected by the ITR probe represented the second novel viral terminus, a Southern blot similar to that used above was probed with the E3 probe. Fig. 40 (lane 9) shows that the probe detected a novel viral DNA band estimated to be about 1.5 kb, suggesting that this band represented the second predicted diagnostic fragment.

In Ad5J-2 infected cells the ITR probe detected restriction digest fragments from the parental DNA (open triangles and solid circle), and a 6.0 kb and 3.6 kb digest fragments (solid triangles, lane 5, Fig. 38). This suggested that the two bands may represent novel termini as predicted by structures of the hypothetical rearranged viral DNA molecules. The 3.6 kb band was confirmed as a novel end through the use of the neo probe (solid triangle, lane 10, Fig. 39). Similarly, it can be demonstrated that the 6.0 kb fragment detected by the ITR probe in Ad5J-2 infected cells represented the second diagnostic fragment for novel viral

termini.

Detection of both diagnostic restriction fragments for predicted unique viral termini suggested that, as in Ad5TER-1 and Ad5TER-2 infected cells, the parental genomes were rearranged by a mechanism(s) which converted the embedded ITRs to terminal ITRs of novel, rearranged viral DNA molecules.

3.3.5 Identification of Novel Viral Junctions in Infected Cells.

Structures of the hypothetical rearranged viral DNA molecules shown in Figs 35 and 36 predicted novel viral junctions composed of a terminal ITR and an embedded ITR (eg. r,L in D; Fig. 35), two identical embedded ITRs (eg. r,r in G; Fig. 35) or two terminal ITRs (eg. R,L in I; Fig. 35). Detection of these novel viral junctions would assist in identifying the novel viral DNA molecules that are present in infected cells, and therefore, suggest the possible mechanism(s) which generated the rearranged molecules.

In Ad5J-1 infected cells, novel viral junction from E (Fig. 35) appeared to be present (9.5 kb - lanes 9 and 14, Fig. 39), however, it should be mentioned that a co-migrating fragment can be generated from incomplete digestion of B with EcoRI or XbaI. Also, novel viral junction from J (R,l - Fig. 35; 9.5 kb - lanes 9 and 14, Fig. 38) and K (r,R - Fig. 35; 9.5 kb - lane 9, fig. 40) appeared to be present in infected cells, but again, it should be mentioned that a co-migrating fragment can be generated from incomplete restriction digests of B by EcoRI or XbaI. The inability to detect other novel

viral junctions suggested that rearranged viral DNA molecules harboring these novel junctions were not present in infected cells, although the possibility exists that these novel junctions were present in amounts below the sensitivity of the detection system.

In Ad5J-2 infected cells, no novel viral junctions were detected although the presence of novel viral junctions in amounts below the sensitivity of the detection system is clearly possible.

Absence of novel viral junctions from molecules G and H (Figs. 35 and 36) suggested that the second last novel band from the well (lanes 6 and 7, Fig. 37) previously suggested to be H (lane 6 - Fig. 35, lane 7 - Fig. 36) may be another novel viral DNA molecule perhaps generated during further rounds of rearrangements through replication and/or recombination. However, in consideration of low intensity of the second last band in each of the two lanes (6 and 7), the presence of novel junctions from G and H, and therefore, G and H is possible.

3.3.6 Kinetics of Rearrangement of Viral DNA.

Southern blots of undigested and digested viral DNA samples from cells infected with the four mutant viruses demonstrated the existence of rearranged viral DNA molecules. In cells infected with Ad5J-1 or Ad5J-2 it appeared that these novel molecules were not present in equimolar amounts. Therefore, to determine when after infection these molecules appeared and when their non-equimolar concentration could be

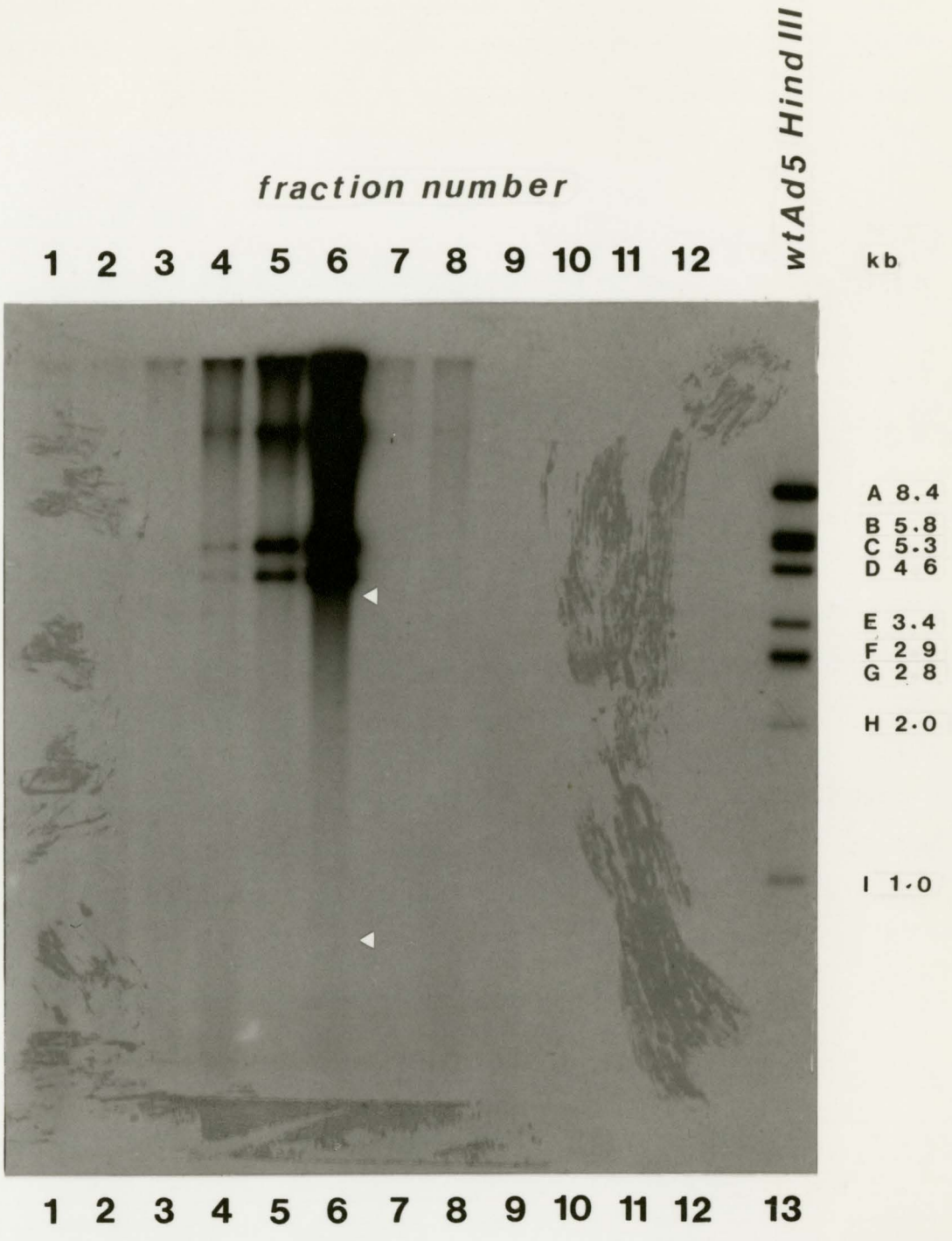
observed, viral DNA from cells infected with Ad5J-1 was extracted at various times after infection and the DNA samples were analyzed for rearranged viral DNA molecules by Southern blot hybridization.

To ensure that any rearranged molecules detected in the infected cells were being generated de novo, a homogeneous population of parental Ad5J-1 was needed. This homogeneous population was obtained by passaging Ad5J-1 twice through 293 cells at low moi (0.01) to select out any defective virions. Viruses from this stock were then used to infect 293N3S cells in suspension at an moi of 0.01. The viruses harvested from the infection were fractionated in a cesium chloride density gradient as described in Chapter 2 and DNA from the fractions digested with XbaI were screened for rearranged viral DNA molecules by Southern blot hybridization. Figure 41 shows that the fraction containing the majority of the parental viral DNA (fraction 6) was free of any contaminating defective genome. This is demonstrated by the absence of restriction fragments diagnostic for the truncated defective genomes in the location where it was expected to migrate (solid triangle). The autoradiograph suggested that fraction 6 contained a pure population of Ad5J-1 although the presence of rearranged molecules in amounts below the sensitivity of the detection system is clearly possible.

Viruses from the banded stock described above were used to infect 60 mm dishes of 293 cells at moi of 0.01 and 10, and at the indicated times post-infection, viral DNA was extracted. Viral DNA samples from both infections, digested

FIGURE 41. Cesium chloride density gradient banding of Ad5J-1.

In order to obtain the pure population of parental Ad5J-1 viruses required for studying the kinetics of rearrangement of the parental genome, a low moi passaged stock of Ad5J-1 was used to infect 293N3S cells in suspension at 0.01 pfu/cell. Viruses from the infection were fractionated and DNA from the fractions was extracted as described in Chapter 2. The viral DNA samples were digested with XbaI and the restriction digest fragments were separated by 1.0% agarose gel electrophoresis. A Southern blot of the gel was probed with nick-translated genomic probe (pFL157). The autoradiograph shows that restriction digest fragments from parental genome only were detected. Predicted location of restriction fragments from defective genomes are indicated by solid triangles. Lanes 1 through 12 represent fractions 1 (bottom of gradient) through to 12 (top), and lane 13 is the marker lane (HindIII digested wtAd5 DNA).


fraction number
1 2 3 4 5 6 7 8 9 10 11 12
wtAd5 HindIII
kb
A 8.4
B 5.8
C 5.3
D 4 6
E 3.4
F 2 9
G 2 8
H 2·0
I 1·0
1 2 3 4 5 6 7 8 9 10 11 12 13

with XbaI to produce restriction fragments diagnostic for rearrangements of parental genome, were separated by electrophoresis and a Southern blot of the gel was probed with a nick-translated genomic probe, pFL157.

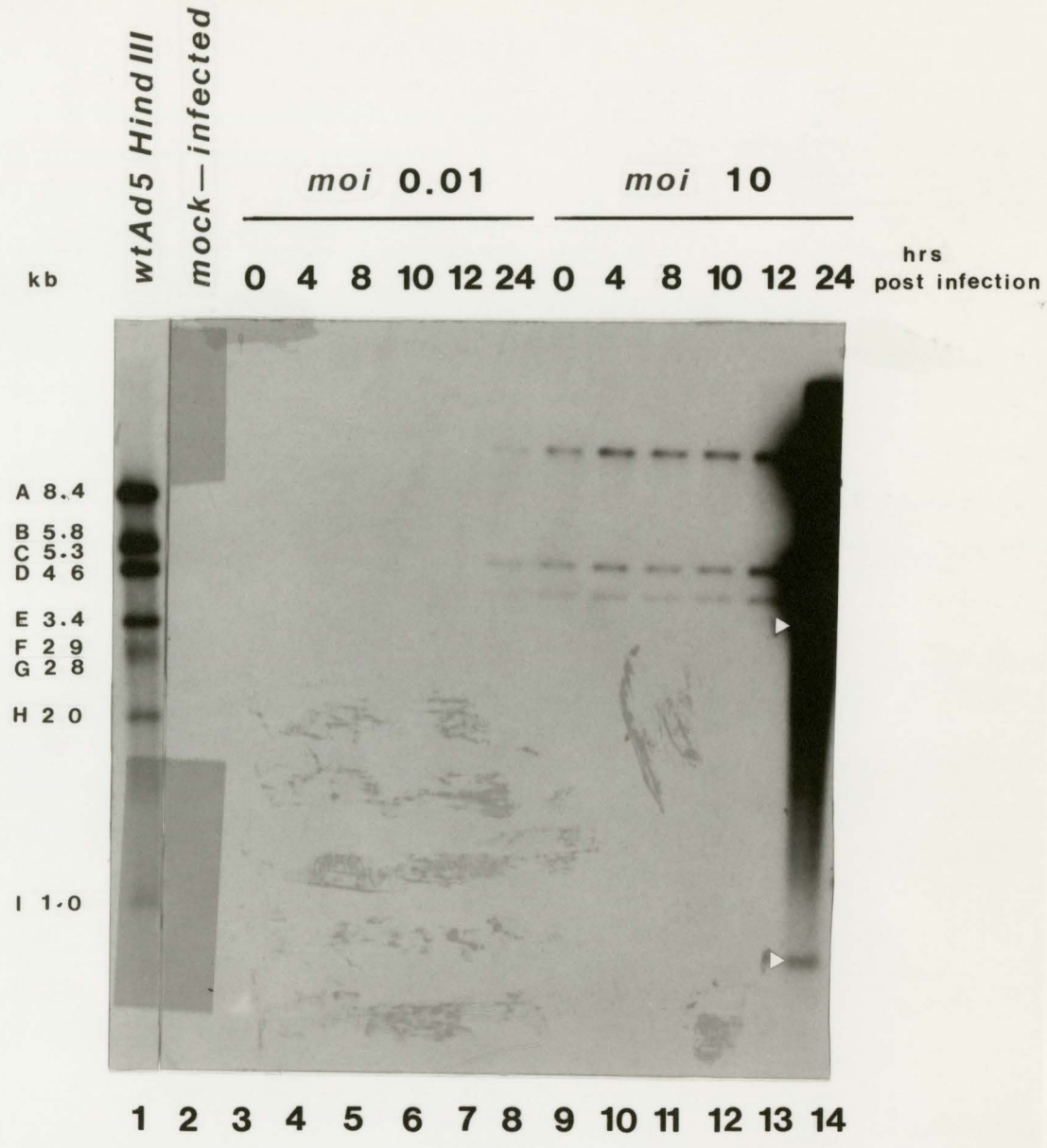
The autoradiograph (Fig. 42) shows the accumulation of viral DNA in the cells at 24 hrs pi in both high and low moi infections (lanes 8 and 14), and indicates that in cells infected at 10 pfu/cell the viral DNA had accumulated to much higher amounts relative to cells infected at 0.01 pfu/cell. Also, at 24 hrs pi in cells infected at 0.01 pfu/cell no rearranged viral DNA molecules were detected as demonstrated by the absence of diagnostic fragments, whereas in cells infected at 10 pfu/cell rearrangements of the parental viral DNA were detected, which suggested a correlation between the amount of input DNA and the amount of rearranged viral DNA molecules in infected cells. Inability to detect rearrangements of the viral DNA in low moi infected cells could be related to the low copy number of the parental viral DNA molecules, suggesting that perhaps the amounts of rearranged viral DNA molecules in these infected cells were below the sensitivity of the detection system.

3.4 CESIUM CHLORIDE DENSITY GRADIENT BANDING OF Ad5J-2.

Adenovirus DNA is packaged into viral capsids in the late stages of infection (Chapter 1), and it has been shown in many serotypes that the packaging of viral DNA requires the presence of DNA sequences from the left end of the linear genome (Hearing et al, 1987; Hammarskjold and Winberg, 1980;

FIGURE 42. A time course of replication and rearrangement of Ad5J-1 DNA.

The banded stock of parental Ad5J-1 was used to infect 293 cells at two different moi values and the viral DNA was harvested at the various time points. The viral DNA samples were digested with XbaI and separated by 1.0% agarose gel eletrophoresis. A Southern blot of the gel was probed with nick-translated pFL157 (genomic probe) for evidence of parental genome replication and rearrangements. The autoradiograph shows that viral DNA fragments indicating rearrangement of the parental genome (solid triangles) were detected at 24 hrs pi in cells infected at 10 pfu/cell. Lane 1 - HindIII digested wtAd5; lane 2 - mock-infected; lanes 3 to 8 - 0.01 moi infection; lanes 9 to 14 - 10.0 moi infection.


wtAd5 Hind III
mock—infected
moi 0.01
moi 10
kb
0 4 8 10 12 24 0 4 8 10 12 24
hrs post infection
A 8.4
B 5.8
C 5.3
D 4 6
E 3.4
F 2 9
G 2 8
H 2 0
I 1.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Tibbetts, 1977). In Ad5 DNA, this sequence has been localized to 32 bps, between nucleotide 238 and nucleotide 270 in the left end (Hearing et al, 1987) and it has been shown that this signal could be transferred to the right end and remain active as an encapsidation signal.

The embedded terminal sequences in all the mutant viruses contained the encapsidation signal described by Hearing et al (1987) and it was shown that these embedded ITR(s) can be converted to terminal ITR(s) of novel viral DNA molecules in infected cells. One predicted novel viral DNA molecule was the unit length viral DNA molecule which was generated by replication at the embedded viral junction of circularized Ad5J-2 genome (molecule I, Fig. 35). In this molecule the two viral ends would have novel structures. Theoretically this molecule would be capable of being packaged as efficiently as the parental genome. Had replication at the embedded viral junction of a circularized genome been efficient (that is, if the circular forms were obligatory intermediates in viral DNA replication), then this isomer of parental DNA would be present in the banded viral DNA. Finding evidence for the existence of such a molecule in banded viral DNA would have suggested that circular forms of viral DNA do have a significant role in viral DNA replication, perhaps as an obligatory replication intermediate.

During the analysis of intracellular viral DNA from cells infected with Ad5J-2 it was noticed that among the various rearranged viral DNA molecules, the 29.5 kb molecule (molecule A, Fig. 35) was present in highest concentration.

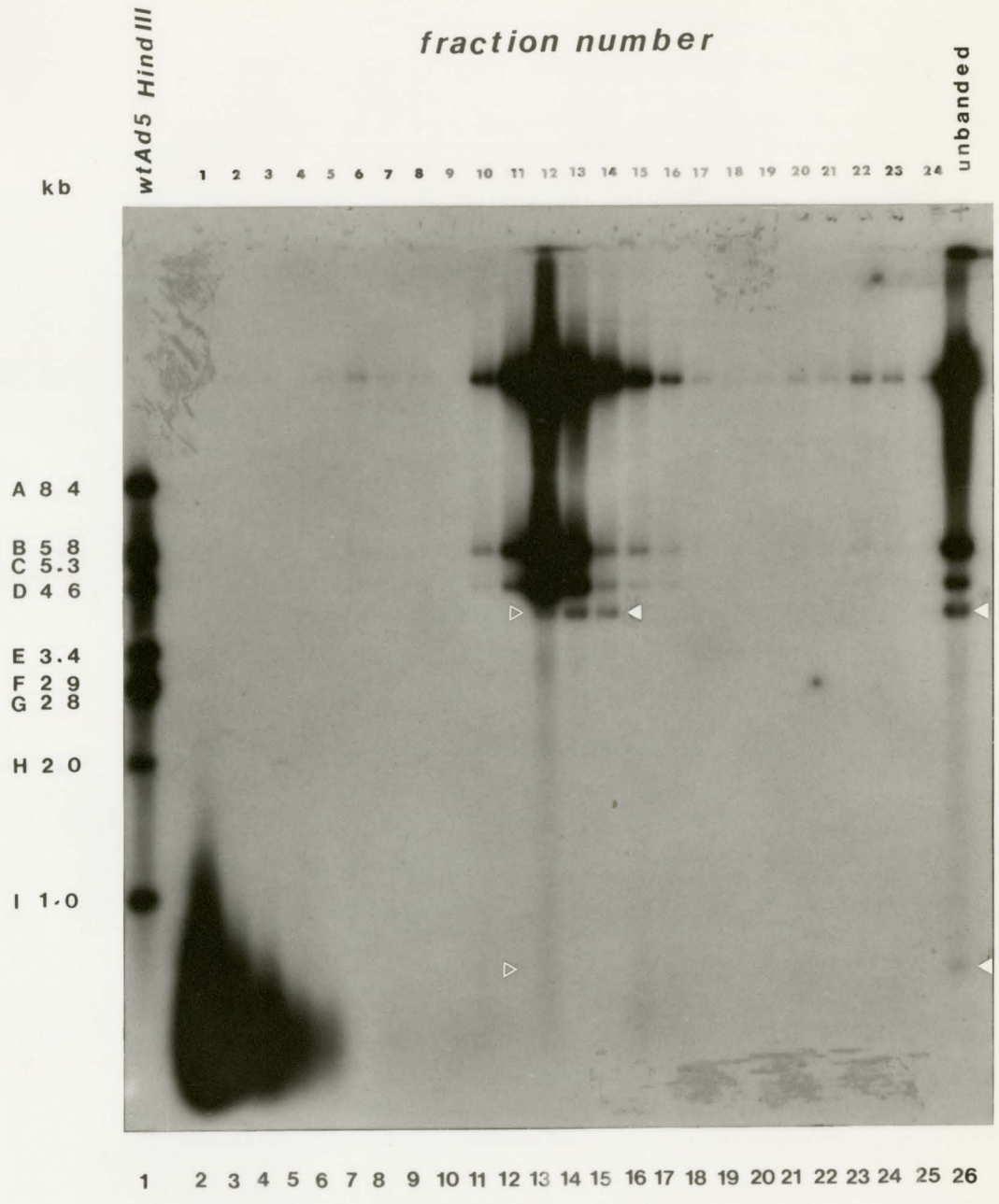
Based on the predicted structure of this molecule, it should have been packageable and thus capable of propagation as a defective virus.

In an attempt to detect the flip-flop isomer of the parental genome and to examine the mode of amplification of the 30 kb molecule, suspension culture of 293N3S cells were infected at 10 pfu/cell. Viruses from this infection were fractionated in a CsCl density gradient, and XbaI digested DNA from each fraction was electrophoresed. A Southern blot of the gel was then probed with a genomic probe and shown in Fig. 43.

The autoradiograph (Fig. 43) indicates that two species of viral DNA molecules were packaged into the viral capsid. Virions containing the parental genome was detected in fractions 10 to 15 inclusive, as indicated by the restriction digest fragments from the parental genome (top three bands, 25 kb, 5.4 kb, and 4.7 kb), with majority of the parental genome present in fraction 12. Along with restriction fragments from the parental genome, a restriction fragment diagnostic for a novel viral terminus was detected in fractions 12 to 14 inclusive (4.1 kb, solid triangle). Majority of this fragment was detected in fraction 13, above fraction 12 where majority of the parental genome was detected, indicating that density of the virion containing the source of the 4.1 kb band was lower than that of the virion with parental genome. This suggested that the 4.1 kb fragment was derived from novel viral DNA molecule(s) that was less than unit length. From the molecules shown in Figure 36 it

FIGURE 43. Determination of the packaging characteristics of the rearranged viral DNA molecules from Ad5J-2.

293 cells in suspension were infected with Ad5J-2 and harvested at full cpe as described in Chapter 2. The harvested viruses were fractionated in a cesium chloride density gradient and DNA from the fractions was digested with XbaI. After 1.0% agarose gel electrophoresis, the DNA was Southern transferred and probed with nick-translated pFL157 (genomic probe) to determine the packaging properties of the rearranged viral DNA molecules. A restriction digest fragment diagnostic for the 29.5 kb truncated viral DNA molecule was detected (solid triangles - lanes 13 to 16 inclusive) suggesting that this molecule is packaged into viral capsids. Predicted location of restriction digest fragments from flip-flop isomer of the parental genome are indicated by open white triangles. The 4.1 kb band in fractions 12 to 14 inclusive is probably from the 29.5 kb truncated viral genome. Lane 1 - HindIII digested wtAd5; lanes 2 through 24 - fractions 1 through to 23; lane 25 - XbaI digested unbanded intracellular viral DNA.


fraction number
wtAd5 HindIII
unbanded
kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A 8 4
B 5 8
C 5.3
D 4 6
E 3.4
F 2 9
G 2 8
H 2 0
I 1·0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

appeared that the novel end belonged to molecule A, a 29.5 kb truncated viral DNA molecule. This result also suggested that the high copy number of 29.5 kb molecule relative to other rearranged viral DNA molecules (except 6.0 kb molecule) in Ad5J-2 infected cells was most likely due to the packaging and propagation of the 29.5 kb molecule during the course of an infection.

Absent from the autoradiograph was evidence of the flip-flopped isomer of parental viral DNA in the fraction containing the majority of parental DNA. The existence of this isomer would have been indicated by the detection of two viral bands migrating at 4.1 kb and 0.6 kb (solid triangles) in the fraction containing the majority of parental genome (fraction 12). This showed that viral DNA replication initiating from the circular forms of viral genome was low, suggesting that circular forms of viral DNA do not have a significant role in this process. Also absent from the fractions were other rearranged molecules which had been previously identified in infected cell DNA preparations suggesting that these DNA forms were not packaged into virions either due to their larger than unit length genomes or to lack of the cis-acting encapsidation signal. The contamination of fractions containing the parental genome with the defective genome was probably due to the inability of the CsCl banding procedure to resolve the two particles.

4. DISCUSSION.

4.1 BIOLOGICAL ACTIVITY OF VIRAL JUNCTION.

Based on rearrangements of other viral and lower eucaryotic genomes with embedded repetitive sequences different possible mechanisms for rearrangement of adenoviral genomes with embedded ITR sequences were proposed and various novel viral DNA molecules were predicted from these mechanisms. One of the mechanisms was initiation of replication at the embedded viral junction of Ad5J-1 or Ad5J-2 covalently closed at the terminal ITRs which would have generated a novel viral DNA molecule that was a flip-flop isomer of the parental genome. In this model, the ITRs of the embedded viral junction in the parental genome would be terminal ITRs and the terminal ITRs of the parental genome would become embedded in the flip-flopped genome. This isomer of parental viral DNA was not detected in banded viral DNA population (Fig. 43) suggesting that circular forms of viral DNA are probably not obligatory intermediates in viral DNA replication. This conclusion is further supported by results from banding of Ad5J-1 (Fig. 41) which also showed that flip-flopped isomers of the parental genome were not present in the CsCl gradient fractions containing parental viral DNA. The absence of flip-flopped isomer could not be attributed to selection pressure against defective genomes because the

isomer should have been packageable. Also the flip-flop isomer cannot be considered as a defective genome since the viral DNA had linearized at the embedded ITR junction and thus is capable of regenerating the parental genome in infected cells through circularization followed by initiation of replication at the junction of parental ITRs. Therefore, circular forms of viral DNA probably do not play a significant role in viral DNA replication. It is possible, however, that circular forms may have other role(s) in viral infection or viral transformation. It has been suggested that circular forms of viral DNA may play a role in integration during viral transformation (Graham, 1984a) similar to the circular proviral DNA of retroviruses (reviewed in Panganiban, 1985). This question remains to be answered.

In Ad5TER-1 or Ad5TER-2 infected cells, both possible mechanisms (recombination and replication from ends) for generating rearrangements of parental genome predicted 2 novel viral DNA molecules from each virus, one larger and one smaller than unit length, the latter with a unique viral end. Analysis of undigested and digested DNA from infected cells showed that 2 novel viral DNA molecules were generated, and a subpopulation of novel viral DNA molecules had the unique viral terminus. From the results the source of these rearranged molecules could not be determined. The rearrangements could have been generated by either the recombination or the replication mechanism, or both.

In Ad5J-1 or Ad5J-2 infected cells, each possible mechanism for generating rearrangements of the parental

genomes predicted a variety of novel viral DNA molecules, of which some were larger or smaller than unit length and some had unique viral ends. The detection of putative novel junction from E, J, and K (Fig. 35) suggested the rearrangements were generated by recombination and/or replication. However, the absence of novel junctions from G and H (Figs. 35 and 36) generated only through intermolecular recombination suggested that recombination likely had a very minor role if any in rearrangement of parental genomes.

Besides recombination, other mechanisms could also be responsible for generating the novel viral DNA molecules detected. Among these, the panhandle mechanism was discussed earlier in this thesis (Figs. 32 and 33) and involves formation of different panhandle structures due to embedded ITR sequences followed by either extension or exonuclease acitvity at the 3' end to generate viral DNA molecules that were larger and smaller than parental genomes. This mechanism can be applied to all four viruses with embedded ITR(s), and theoretically, both the larger and the smaller novel viral DNA molecules would be generated at the same frequencies, although once they were created, the truncated molecules might be replicated faster than the larger molecules. The absence of novel viral junctions from larger than unit length viral DNA molecules may be related to its low copy number although it is possible that these molecules could have been generated from subsequent rounds of rearrangements.

The second mechanism, replication from the embedded viral junction in a linear parental virus was also discussed

earlier in this thesis (Fig. 34). This mechanism can generate only the two truncated viral DNA molecules and applies only to Ad5J-1 and Ad5J-2.

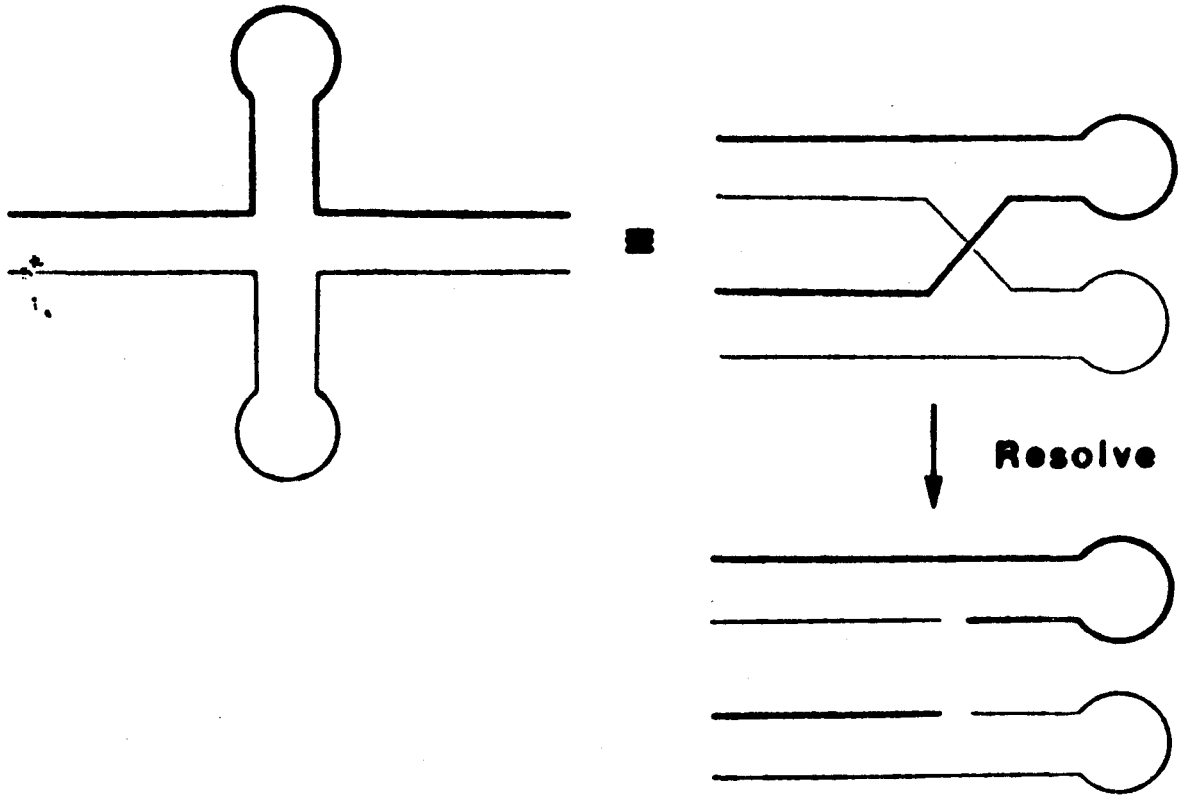
A third mechanism which could generate truncated viral genomes from Ad5J-1 and Ad5J-2 is a process similar to resolving of cruciform structures in prokaryotes reported by Lilley and Kamper (1984). This process resolves cruciform structure in a circular molecule to ends of the linear form of circular parent molecule (Fig. 44). A similar process has been proposed in eukaryotic cells with poxvirus telomeres (DeLange et al, 1986). This suggests that the embedded viral junction in Ad5J-1 or Ad5J-2 could form a cruciform structure which perhaps could be resolved to yield two truncated linear viral DNA molecules.

4.2 UNEQUAL COPY NUMBERS OF TRUNCATED MOLECULES IN Ad5J-1 AND Ad5J-2 INFECTED CELLS.

The autoradiograph (Fig. 37) clearly showed that in Ad5J-1 infected cells, the 26.0 kb molecules and the 9.5 kb molecules were not present in equal copy numbers. A similar observation was made between the 30 kb molecules and the 6.0 kb molecules in Ad5J-2 infected cells, although the difference in the copy numbers were not as dramatic when compared to the two truncated molecules in Ad5J-1 infected cells. The results from CsCl banding of viruses from Ad5J-2 infected cells suggested the high copy numbers of the 30 kb molecule in Ad5J-2 infected cells was due to amplification of the 30 kb molecule during successive rounds of replication as a result

FIGURE 44. Proposed mechanism for resolving of cruciform structures in prokaryotes.

The arms of the cruciform align with each other by their homology. One strand from each of the two hairpin duplex is nicked by T4 endonuclease. This is followed by strand exchange and resolution of this cross-over structure to yield a linear molecule. The ends of this linear molecule are made up of sequences from cruciform structure. Reprinted from Lilley and Kamper (1984).


Resolve

of packaging of the 30 kb molecule. The 30 kb molecule was expected to be packaged because it had the adenoviral DNA encapsidation signal required in cis (Hearing and Shenk, 1987; Hammarskjold and Winberg, 1980). This observation confirmed the reports that packaging of viral DNA molecules that are less than unit length occurs, regardless of their origin (Tibbetts, 1977).

4.3 CONCLUSIONS.

This study was carried out to better understand the biological activities of the viral junction isolated from covalently closed forms of adenovirus DNA by inserting the viral junction internally into a linear viral genome. Results from studying the rearrangements of viral DNA suggested that the viral junction was probably active in rearrangement of parental genomes through mostly panhandle formation during viral DNA replication although minor rearrangements through recombination probably occured. The results also suggested that circular forms of viral DNA probably play only a minor role,if any, in viral DNA replication. This study also showed that truncated viral DNA molecules containing 80% of the wild type genome could be efficiently packaged and propagated as a defective genome.

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