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Title: Intramolecular and Intermolecular Strand Hybridization during Adenovirus DNA Replication

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Adenovirus initiates strand-displacement DNA replication from origins located in identical inverted terminal repetitions (ITRs). Panhandle structures, formed by base-pairing between ITRs on the displaced strands, have been proposed as replication intermediates for complementary strand synthesis. Plasmid molecules containing a single adenovirus terminal sequence and specially arranged inverted repeat sequences were used as a model system to study the length and sequence integrity of panhandles. By making a series of unidirectional deletions in the panhandle sequence, it is possible to show that 31 bp or longer are sufficient for panhandle formation, but 28 bp or smaller are not. Removal of long stretches of unpaired 3' nucleotides distal to the panhandle is extremely efficient and rectification of certain kinds of mismatched sequences in the panhandle region occurs readily. These results argue for the formation of panhandles during adenovirus DNA replication and provide a mechanism for maintaining sequence identity between distantly located inverted repetitions. These size constraints may also explain why the adenovirus ITRs are larger than viral DNA replication origins. Moreover, when multiple copies of the inverted repeat sequences are introduced into the plasmid molecules, it appears that there is no preferential selection of specific pairs of inverted repeats for panhandle formation.

Two different pathways have been found to be involved in adenovirus complementary strand replication. *Cis* replication occurs by intramolecular base-pairing of complementary sequences within a displaced single strand to form a panhandle structure, and *trans*

replication involves intermolecular hybridization of complementary sequences from different displaced single strands to form a heteroduplex to complete the replication cycle. In order to study the relative participation of the *cis* and *trans* mechanisms in the adenovirus replication cycle, plasmid molecules were constructed with adenovirus origins and inverted repeat sequences that can pair distinguishably both within and between displaced strands. The results to date demonstrate that both *cis* and *trans* pathways function simultaneously during adenovirus DNA replication. Moreover, it appears as little as two copies of a 89-bp complementary sequence support *trans* replication although a single copy of 89 bp does not. Both *cis* and *trans* pathways show efficient sequence rectification of terminal non-homology at the 3' ends of strands, a phenomenon not detected in eukaryotic cells before.

Intramolecular and Intermolecular Strand Hybridization during Adenovirus DNA Replication

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CHAPTER I
INTRODUCTION

Adenovirus was first discovered and described in 1953 (Rowe *et al.*, 1953). Since then, many different types of adenovirus have been isolated from several animal species, and at least 42 distinct serotypes have been identified from the human population (Ishibashi and Yasue, 1984). Adenovirus quickly became one of the most extensively studied eukaryotic model systems for probing the mechanisms that operate in virus assembly (Nermut, 1984), oncogenic transformation (Graham, 1984b), gene transcription (Sharp, 1984), mRNA splicing (Montell *et al.*, 1984), RNA polyadenylation (Fraser *et al.*, 1982), DNA recombination (Munz and Young, 1984; Volkert and Young, 1983), and DNA replication (Challberg and Kelly, 1989; Field *et al.*, 1984a; Fütterer and Winnacker, 1984; Kelly, 1984).

The Adenovirus Genome

The human adenovirus genomes are linear, double-stranded DNA molecules with a size of approximately 36,000 bp; the exact size depends on the specific serotype (Sussenbach, 1984; Winnacker, 1978). All adenovirus DNAs examined to date have two distinctive features: (1) a terminal protein is covalently attached to the 5' end of each DNA strand (Robinson and Bellett, 1975; Robinson *et al.*, 1973), and (2) inverted terminal repetitions (ITRs), with identical DNA sequences spanning 102 to 166 bp, are located at the ends of the DNA molecule (Garon *et al.*, 1972; Wolfson and Dressler, 1972). Although ITRs differ between serotypes both in sequence and length, all ITRs share conserved or highly homologous sequences, especially nucleotides 9 through 18 (Shinagawa and Padmanabhan, 1980; Stillman *et al.*, 1982b). These two unusual terminal structures play important roles during virus DNA replication (Challberg and Kelly, 1989; Field *et al.*, 1984a; Fütterer and Winnacker, 1984; Kelly, 1984).

General Scheme of Adenovirus DNA Replication

The process of adenovirus DNA replication is outlined in Figure I.1 (Lechner and Kelly, 1977). Adenovirus DNA replication origins lie within

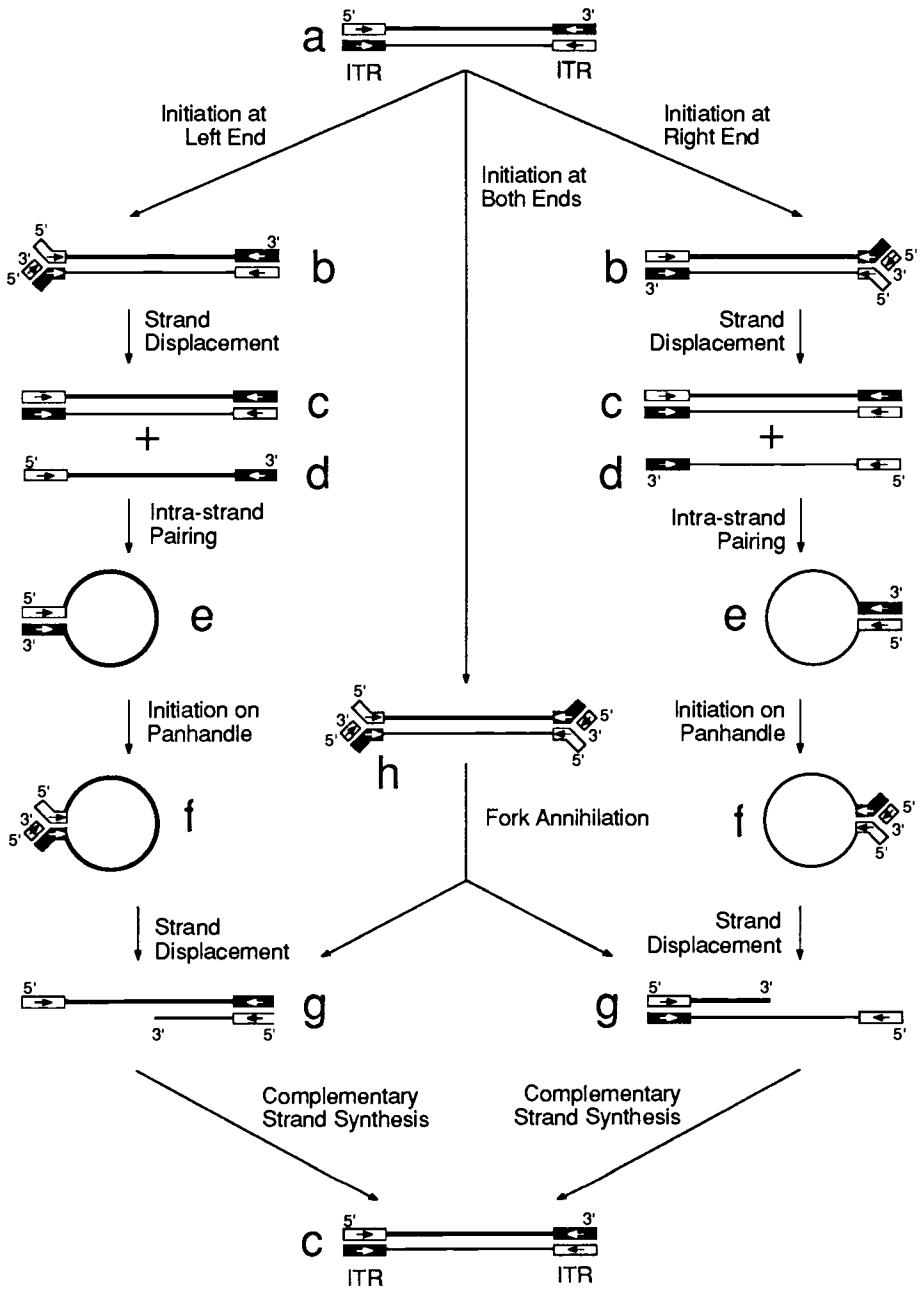

Figure I.1. Overall scheme for adenovirus DNA replication. Inverted terminal repetitions (ITRs) are shown as open and closed boxes with black and white arrows to indicate orientation. Complementary DNA strands are depicted as thick and thin lines. The polarity of DNA strands are designated by 3' and 5'. (a) Parental double-stranded molecule. (b) Type I replication intermediate. (c) Daughter double-stranded molecule. (d) Displaced parental strand. (e) Panhandle intermediate. (f) Initiation on a panhandle intermediate. (g) Type II replication intermediate. (h) Doubly initiated type I intermediate.

the ITRs at the extreme ends of the linear DNA molecule (Ellens *et al.*, 1974; Lechner and Kelly, 1977). Initiation of DNA replication takes place by using either one of the two replication origins at the ends of DNA molecule with about equal frequency (Lechner and Kelly, 1977). Replication can be divided into two phases: strand-displacement replication and complementary strand synthesis (Lechner and Kelly, 1977).

Two distinctive features characterize these two replication phases: (1) initiation of DNA replication is primed by terminal protein (Challberg *et al.*, 1980; Enomoto *et al.*, 1981; Tamanoi and Stillman, 1982), and (2) ITRs form panhandle structures as replicative intermediates for complementary DNA strand synthesis (Lechner and Kelly, 1977). Adenovirus employs a virus-encoded terminal protein as a primer for the initiation of DNA replication (Challberg *et al.*, 1980; Enomoto *et al.*, 1981; Tamanoi and Stillman, 1982). The critical step in the initiation process involves the formation of a phosphodiester bond between a serine residue in the pre-terminal protein (pTP) and the α-phosphate of dCTP to yield a covalent pTP-dCMP complex (Challberg *et al.*, 1982; Lichy *et al.*, 1981; Stillman and Bellett, 1979). The 3' hydroxyl group of dCMP in this protein-nucleotide complex serves as a primer for subsequent chain elongation by viral DNA polymerase (Challberg *et al.*, 1982; Guggenheimer *et al.*, 1984a; Lichy *et al.*, 1981; Stillman and Bellett, 1979). It has been shown that the initiation process depends on the presence of specific nucleotide sequences within the replication origin and requires the participation of several viral proteins and host nuclear factors (NFs) to reach maximum efficiency. These proteins include the adenovirus-encoded DNA polymerase and the pre-terminal protein (Challberg *et al.*, 1980; Enomoto *et al.*, 1981), and two cellular proteins called nuclear factor I (NFI) (Jones *et al.*, 1987; Nagata *et al.*, 1983b) and nuclear factor III (NFIII) (Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987).

After initiation, DNA elongation proceeds in a 5' to 3' direction by a strand displacement mechanism (Kedinger *et al.*, 1978; Lechner and Kelly, 1977). Two additional proteins, adenovirus DNA binding protein and host nuclear factor II (NFII), are required in order to complete the synthesis of full-length DNA strands (Nagata *et al.*, 1983a). Strand displacement replication generates a daughter duplex DNA molecule (Figure I.1c) and a displaced parental single-stranded DNA molecule (Figure I.1d). Molecules

engaging in displacement synthesis can be identified by electron microscopy as type I molecules (Figures I.1b and I.1h), branched structures with two double-stranded arms and one or more single-stranded arms (Lechner and Kelly, 1977).

The process converting displaced single strands into double-stranded daughter DNA molecules is called complementary strand synthesis (Lechner and Kelly, 1977). To date, the mechanism for complementary strand synthesis remains a mystery, but an attractive proposal is that displaced single strands form panhandle structures (Figure I.1e) by base pairing between complementary sequences within the ITRs (Lechner and Kelly, 1977). The duplex region of the panhandle regenerates a replication origin which is identical to the origins on parental double-stranded molecules. Thus, all initiation events involved in both strand displacement replication and complementary synthesis may be the same (compare Figures I.1b and I.1f). Furthermore, elongation on displaced single DNA strands results in the formation of type II molecules (Figure I.1g) which appear by electron microscopy as non-branched unit length structures containing both double- and single-stranded regions (Lechner and Kelly, 1977). Composite replicating molecules, called type I/II molecules (not shown in Figure I.1), can also be created if initiation occurs on a type II molecule prior to termination of complementary synthesis (Lechner and Kelly, 1977).

Adenovirus DNA Replication Proteins

Detailed insights about the biochemistry and enzymology of adenovirus DNA replication have come from the landmark development of a cell-free DNA replication system (Challberg and Kelly, 1979a; Challberg and Kelly, 1979b). Either adenovirus DNA-terminal protein complex or proteinase-treated adenovirus DNA lacking terminal protein can direct adenovirus-specific DNA replication in crude extracts from adenovirus-infected cells. Subsequent refinement of the *in vitro* system also allowed the initiation of replication on cloned adenovirus terminal fragments or terminal protein-free templates (Tamanoi and Stillman, 1982), but the efficiency of initiation is much lower on those molecules than on adenovirus DNA-terminal protein complex. The initiation reaction is detected by the formation of pre-terminal protein-dCMP complex (Challberg

et al., 1982; Lichy *et al.*, 1981; Stillman and Bellett, 1979). Crude cell-free extracts have been fractionated to identify and to purify both viral and cellular factors required for adenovirus DNA replication. As a result, three adenovirus-encoded proteins and three cellular factors have been isolated and identified to be important for adenovirus DNA replication (Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987; Stillman, 1983).

Virus Encoded Proteins

Three virus-encoded proteins play central roles in adenovirus DNA replication: adenovirus pre-terminal protein (pTP), adenovirus DNA polymerase (Ad pol), and adenovirus DNA-binding protein (Ad DBP). The mRNAs for all three proteins are produced from the same viral transcription unit, but arise by differential splicing of a common precursor transcript (Stillman *et al.*, 1981).

Adenovirus pre-terminal protein. The presence of a protein on the termini of viral DNA was first detected by electron microscopy. Adenovirus DNA molecules isolated by a process that did not involve protease treatment or phenol extraction appeared circular in form, but could be linearized by treatment with protease (Robinson and Bellett, 1975; Robinson *et al.*, 1973). The size of the terminal protein linked to DNA in mature adenovirus particles is about 55 kd (Stillman and Bellett, 1979). From *in vitro* studies, it was found that the 5' termini of nascent replicating adenovirus DNA strands are linked to a protein with a molecular weight of 80 kd instead of the 55-kd terminal protein (Challberg *et al.*, 1980; Enomoto *et al.*, 1981; Tamanoi and Stillman, 1982). The 80-kd protein is the precursor of the 55-kd terminal protein (Desiderio and Kelly, 1981), and is called precursor terminal protein or pre-terminal protein (pTP). The 80-kd pTP is processed to the 55-kd terminal protein by proteolytic cleavage mediated by a virus-encoded protease just prior to the packaging of viral DNA into virions (Challberg and Kelly, 1981).

Adenovirus terminal protein is absolutely required for the initiation of virus DNA replication (Challberg *et al.*, 1980; Enomoto *et al.*, 1981; Tamanoi and Stillman, 1982). It has been demonstrated *in vitro* that pre-terminal protein serves as the primer during initiation by forming a pTP-dCMP complex through a phophodiester bond between a serine residue in the pTP and the α-phosphate of the terminal nucleotide dCTP (Challberg

et al., 1982; Lichy *et al.*, 1981; Stillman and Bellett, 1979). The reaction is sequence-dependent and only occurs in the presence of the adenovirus DNA replication origin (Ikeda *et al.*, 1982; Lichy *et al.*, 1981; Stillman and Tamanoi, 1982; Tamanoi and Stillman, 1982). Other experiments have shown that pTP forms a tight, but not covalent, complex with Ad pol (Enomoto *et al.*, 1981; Ikeda *et al.*, 1982; Lichy *et al.*, 1982; Stillman *et al.*, 1982a).

Pre-terminal protein is encoded in the viral early 2b (E2b) region (Stillman *et al.*, 1981). Recently, the genomic sequence containing the large open reading frame from the adenovirus pre-terminal protein gene has been successfully cloned and the encoded protein expressed in an enzymatically-active form (Pettit *et al.*, 1988; Stunnenberg *et al.*, 1988). More detailed information about the adenovirus pre-terminal protein is therefore expected to be obtained soon.

Adenovirus DNA polymerase. That adenovirus encodes its own DNA polymerase (Ad pol) became clear only after it was shown that a 140-kd protein extensively co-purifies with the adenovirus pre-terminal protein (Enomoto *et al.*, 1981; Lichy *et al.*, 1982; Stillman *et al.*, 1982a). Ad pol is required for both initiation as well as chain elongation of viral DNA synthesis. Ad pol has an associated 3'-to-5' exonuclease activity for editing or proofreading of newly incorporated nucleotides just like prokaryotic DNA polymerases (Field *et al.*, 1984b). Polymerase activity can be inhibited by dideoxyribonucleotides (Field *et al.*, 1984b). Unlike eukaryotic DNA polymerase α, Ad pol elongates RNA-primed DNA poorly (Dekker and van Ormondt, 1984) and is insensitive to aphidicolin (Lichy *et al.*, 1983). Ad pol is the only polymerase known to catalyze the formation of pTP-dCMP complex, a reaction diagnostic for the initiation of viral DNA replication (Dekker and van Ormondt, 1984; Enomoto *et al.*, 1981). Ad pol can also use single stranded DNA as a template (Ikeda *et al.*, 1982). Single-stranded DNA molecules, even those lacking specific adenovirus origin sequences, direct the formation of pTP-dCMP complex with varying degrees of efficiency (Challberg and Rawlins, 1984; Guggenheimer *et al.*, 1984b; Tamanoi and Stillman, 1983). However, subsequent polymerization proceeds with extremely low efficiency (Challberg and Rawlins, 1984; Ikeda *et al.*, 1982).

Adenovirus DNA polymerase is also coded in the E2b region of virus

genome (Stillman *et al.*, 1981). The open reading frame of the Ad pol gene has also been cloned into a eukaryotic expression vector and the protein product expressed from this vector is functional (Hassin *et al.*, 1986; Shu *et al.*, 1987; Stunnenberg *et al.*, 1988). Mutational analysis of this cloned gene has just started in an effort to elucidate the functional domains of Ad pol (Chen and Horwitz, 1989). Although both pTP and Ad pol can be transported from the cytosol to the nucleus independently, pTP and Ad pol also interact to form a complex, and the complex is subsequently translocated to the nucleus with higher efficiency (Zhao *et al.*, 1988).

Adenovirus DNA-binding protein. The adenovirus DNA-binding protein (Ad DBP) gene was the first adenovirus replication gene to be identified (van der Vliet *et al.*, 1975; van der Vliet and Sussenbach, 1975). It was initially characterized in studies of a temperature-sensitive mutant defective for viral DNA replication (van der Vliet *et al.*, 1975; van der Vliet and Sussenbach, 1975). The availability of a wide range of mutants facilitated the dissection of the functions of Ad DBP both *in vivo* and *in vitro* (Friefeld *et al.*, 1983; Levine *et al.*, 1975; Nagata *et al.*, 1983a; van Bergen and van der Vliet, 1983). It has been shown that viral DNA-binding protein is required for viral DNA replication (Friefeld *et al.*, 1983; Levine *et al.*, 1975; Nagata *et al.*, 1983a; van Bergen and van der Vliet, 1983) and plays one or more roles in regulating the expression of other viral genes (Handa *et al.*, 1983). The Ad DBP binds tightly and cooperatively to single-stranded DNA generated by adenovirus replication (Kedinger *et al.*, 1978; Levine *et al.*, 1975). Ad DBP can not be functionally replaced by the *E. coli* single-stranded DNA-binding protein (Field *et al.*, 1984b), and it does not stimulate the activity of other DNA polymerases such as the HeLa DNA polymerase α (Field *et al.*, 1984b). It appears that Ad DBP may stimulate initiation several-fold, but the protein is clearly not essential for the initiation of replication; instead, it is required, together with the adenovirus DNA polymerase, for subsequent chain elongation (Friefeld *et al.*, 1983; Levine *et al.*, 1975; van Bergen and van der Vliet, 1983). With poly (dT) as template and oligo (dA) as primer, DNA synthesis by the Ad pol-pTP complex can be stimulated up to 100-fold in the presence of Ad DBP (Field *et al.*, 1984b). Ad DBP is encoded in the early 2a (E2a) region of the genome (Kruijer *et al.*, 1981). Although the actual molecular weight of the protein is 59 kd, its apparent size is 72 kd. Its abnormal mobility during

polyacrylamide SDS gel electrophoresis is due to the presence of a large number of proline residues (Levine *et al.*, 1975; Linne *et al.*, 1977; Sugawara *et al.*, 1977). Even though a 34-kd fragment from the carboxyl end of the Ad DBP is able to substitute for the intact protein during viral DNA replication, the amino-terminus of Ad DBP is thought to stabilize the binding of the carboxyl-end to DNA (Krevolin and Horwitz, 1987).

Cellular Factors

At least three host proteins are involved in adenovirus DNA replication: nuclear factor I (NFI), nuclear factor II (NFII), and nuclear factor III (NFIII).

Nuclear factor I. Nuclear factor I, a 47-kd protein, is a site-specific DNA binding protein required for efficient initiation of virus DNA replication both *in vivo* and *in vitro*. It was purified from uninfected host cells based on the observation that nuclear extracts from uninfected cells appeared to stimulate the formation of pTP-dCMP complex (Ostrove *et al.*, 1983; van Bergen *et al.*, 1983). Purified NFI can stimulate the initiation of adenovirus DNA replication *in vitro* at least 30-fold (Wides *et al.*, 1987). NFI binds to the sequence $TGG(N)_{6-7}GCCAA$ which is composed of TGG (motif 1) and GCCAA (motif 2) separated by a 6 or 7 bp spacer region. This binding site is found in the ITRs of virtually all adenovirus serotypes (de Vries *et al.*, 1985; Gronostajski, 1987; Jones *et al.*, 1987; Nagata *et al.*, 1983b). DNase I footprint analysis has shown that NF1 binds DNA sequences from nucleotides 24 to 41 (Wides *et al.*, 1987). Inversion of the NFI binding site does not change the NFI-dependent stimulation of DNA replication. NFI binds as a dimer on one side of the DNA helix, and the principal contact points are in the major groove of the DNA (de Vries *et al.*, 1987). How NFI stimulates the initiation of replication, apart from binding at the origin, is still unclear. The stimulation may arise by facilitating the binding of other adenovirus replication proteins, by unwinding the DNA strands at the viral origin, or by stablizing the viral replication initiation complex by protein-protein interactions (Nagata *et al.*, 1983b; Wides *et al.*, 1987). NFI and NFI-like proteins have been found in organisms ranging from yeast to humans (Schneider *et al.*, 1986). NFI binding sites exist in the regulatory regions of several eukaryotic transcriptional units, and even in the middle of some of the adenovirus genomes. Thus, NFI may also be

involved in the regulation of some cellular and viral genes.

Nuclear factor II. Nuclear factor II exhibits topoisomerase I-like activity although it is actually much smaller than eukaryotic topoisomerase I (Nagata *et al.*, 1983a). In the presence of NFII, pTP-dCMP complex can be elongated into full length adenovirus strands, but in the absence of this factor, only 25% of the length of the genome can be synthesized (Nagata *et al.*, 1983a). NFII presumably functions exclusively during chain elongation, but is not required for initiation of replication. Purified eukaryotic, but not prokaryotic, topoisomerase I can substitute for NFII (Nagata *et al.*, 1983a).

Nuclear factor III. In addition to NFI, a novel factor designated nuclear factor III (NFIII) was identified in uninfected extracts which stimulates adenovirus DNA replication 4- to 6-fold by increasing the initiation efficiency *in vitro* (Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987). NFIII binds immediately adjacent to the NFI site (Rosenfeld *et al.*, 1987; Wides *et al.*, 1987). DNase I footprinting shows that NFIII protects nucleotides from 36 to 54 in the adenovirus origin (Pruijn *et al.*, 1986). The core sequences for NFIII binding include the partially conserved nucleotides TATGATAAT in the ITR (Pruijn *et al.*, 1986). NFIII binding involves all base pairs of the core sequence in both major and minor grooves and, in contrast to NFI, the contact points are not confined to one side of the DNA helix (Pruijn *et al.*, 1988). The NFIII binding site overlaps the NFI binding site for at least one nucleotide (Pruijn *et al.*, 1988). Both NFI and NFIII contact the same AT base pair at position 39 in the adenovirus origin. NFIII has been highly purified as a 92-kd protein(O'Neill and Kelly, 1988). The mechanism operating to stimulate initiation by NFIII is not yet known, and no requirement for NFIII *in vivo* has been demonstrated. Potential cellular binding sites for NFIII, containing the octamer sequence ATGCAAAT, have been found in the promoter elements of several eukaryotic genes. Thus, NFIII may function as an activator of viral DNA replication as well as a regulator of cellular transcription.

Other cellular factors. A heat-stable, ribonuclease-sensitive fraction from the cytosol of uninfected HeLa cells stimulates the initiation of adenovirus DNA replication by 3-fold (van der Vliet *et al.*, 1984). This fraction appears to contain several RNA components which complement each other. The role of RNA molecules in adenovirus DNA replication is not clear at this time.

An additional host protein, called factor pL, has been identified and isolated from uninfected cell extracts. It enhances adenovirus DNA replication *in vitro* when terminal protein-free templates are used (Guggenheimer *et al.*, 1984a). pL factor is a 44-kd polypeptide with an associated 5'-to-3' exonuclease activity. It facilitates replication by degrading the 5' end of the DNA strand to expose the 3' end of the template as a single-stranded origin region (Guggenheimer *et al.*, 1984a). Other partially single-stranded templates, constructed using synthetic oligonucleotides, have been found to support the initiation reaction in the absence of the pL factor. This opens the possibility that a short single-stranded terminal region might be exposed during the initiation of adenovirus DNA replication (Challberg and Kelly, 1989; Guggenheimer *et al.*, 1984a).

Recently a protein, designated ORP-A, has been reported to bind at the end of the adenovirus DNA replication origin (Rosenfeld *et al.*, 1987), but its function is unclear.

Sequence Dependence of Adenovirus DNA Replication

Adenovirus replication origins lie at the termini of the linear viral genome (Lechner and Kelly, 1977). Further analysis of the sequence requirements for adenovirus replication have been done both *in vitro* (Challberg and Rawlins, 1984; Enns *et al.*, 1983; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983; Wides *et al.*, 1987) and *in vivo* (Bernstein *et al.*, 1986; Hay, 1985a; Hay and McDougall, 1986; Wang and Pearson, 1985). The results from these studies all agree that the first 51 nucleotides of the adenovirus genome contain all the sequences required for optimal initiation of DNA replication (Hay, 1985a; Rawlins *et al.*, 1984; Wang and Pearson, 1985).

Origin sequences required for replication can be mapped by deletion and base substitution mutations. The most common approach is to study the affects of mutations on the replication of plasmid templates containing cloned DNA segments derived from the termini of the viral genome. These experiments show that a conserved sequence between nucleotides 9 and 18 of the ITR is essential for viral DNA replication. Deletions or substitutions which affect any of the sequences within this region greatly inhibit viral DNA replication both *in vitro* (Challberg and Rawlins, 1984; Tamanoi and

Stillman, 1983; Wides *et al.*, 1987) and *in vivo* (Hay, 1985a; Wang and Pearson, 1985). The first 18 base pairs of the viral genome is sufficient to support a limited degree of initiation of DNA replication, and thus constitutes the minimal replication origin. Sequences beyond the conserved region seem to be required for maximal levels of DNA replication. Sequences spanning nucleotides 25 to 41 contain the NFI binding site (Wides *et al.*, 1987) and sequences from nucleotides 36 to 54 comprise the binding site for NFIII (Wides *et al.*, 1987). Coupling these binding sites to the minimal origin stimulates the initiation of DNA replication more than 30-fold (Rawlins *et al.*, 1984; Wides *et al.*, 1987). Recent *in vitro* studies have shown that the entire replication origin can be wholly defined within the first 51 bp of the viral genome (Wides *et al.*, 1987). It has been proposed that the adenovirus origin can be divided into at least three functionally distinct domains: A, B, and C. Domain A is the minimal origin consisting of the first 18 nucleotides. A conserved sequence, ATAATATACC, located between nucleotides 9 to 18 within this region, has been suggested to be important for the binding of the Ad pTP-pol complex to the end of the adenovirus DNA, and therefore is absolutely required for the initiation of DNA replication (Wides *et al.*, 1987). Domain B contains the NFI binding site from nucleotides 19 to 40. Domain C includes the recognition site for NFIII between nucleotides 41 to 51. Together domains B and C are not absolutely required for initiation, but serve to enhance the efficiency of initiation strongly (Rosenfeld *et al.*, 1987; Wides *et al.*, 1987). The distance between the minimal origin and the two adjacent accessory domains is critically important for efficient initiation. Mutations that alter the spacing between domain A and the auxiliary domains by even one or two bp dramatically decrease the initiation efficiency (Rosenfeld *et al.*, 1987; Wides *et al.*, 1987). Thus, precise spatial interactions between viral replication proteins and host nuclear factors may be required during the initiation reaction. In addition to the three nuclear factors described above, another cellular factor, called ORP-A, binds site-specifically to the first 12 bp within the minimal viral origin (Wides *et al.*, 1987). The function of ORP-A still remains unknown. These experiments provide evidence that the initiation of adenovirus DNA replication involves multiple protein-DNA, and perhaps protein-protein, interactions at the origin. Although *in vivo* studies have established that molecules containing just the terminal 45 bp of the ITR can

replicate as efficiently as molecules with the entire ITR (Bernstein *et al.*, 1986; Hay, 1985a; Hay and McDougall, 1986; Wang and Pearson, 1985) and the stimulatory effect of domain C has only been observed *in vitro*, the general picture of the organization and extent of the adenovirus replication origin is remarkably consistent between *in vitro* and *in vivo* experiments.

Based on the properties of pL factor and the observation that single-stranded or partially single-stranded templates can support the formation of the pTP-dCMP complex, a two-step process for initiation of DNA replication has been suggested (Challberg and Kelly, 1989). The first step involves the unwinding of the terminal region of the viral genome to expose a short single-stranded region. The second step is the Ad pol catalyzed reaction of pTP with dCTP to form the pTP-dCMP complex. The available data suggest that terminal protein (or pre-terminal protein) attached at the ends of the DNA may play a central role during strand unwinding. However, it is also possible that NFI, NFIII, pTP, and Ad pol may themselves accelerate opening.

The first half of the ITR, including the replication origin, is AT-rich whereas the remainder of the ITR is GC-rich (Stillman *et al.*, 1982b). What role, if any, this GC-rich region plays during viral DNA replication is unclear. The GC-rich region contains short sequences conserved between several human serotypes and some nucleotides in this region are found to be related to sequences in polyoma virus and BK virus (Dynan and Tjian, 1985). The significance of these homologies is not yet apparent. At least one GC-rich sequence, GGGCGG, has been identified as the consensus binding site for transcription factor SP1 (Dynan and Tjian, 1985). SP1 is not known to affect adenovirus DNA replication. It therefore becomes much more interesting to understand why the adenovirus ITRs are always larger than the replication origins.

Unexplained Aspects of Adenovirus DNA Replication

Early *in vitro* studies on the sequence requirements for replication showed that the template molecule must be linearized to expose the adenovirus terminus for efficient initiation of adenovirus DNA replication (Challberg and Rawlins, 1984; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983). Circular plasmids containing an adenovirus origin or plasmids linearized with an adenovirus origin located internally fail to direct

pTP-dCMP complex formation (Challberg and Rawlins, 1984; Enns *et al.*, 1983; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983). Nevertheless, electron microscopy has shown that strand-displacement replication can occur *in vitro* even when the adenovirus origin is embedded in circular molecules (Enns *et al.*, 1983; Pearson *et al.*, 1983). Several *in vivo* experiments also suggest that embedded origins can be used to direct viral DNA replication. Circular adenovirus molecules have been isolated from infected cells (Ruben *et al.*, 1983). Transfected circular adenovirus molecules are just as infectious as transfected linear adenovirus DNA (Graham, 1984a; Graham *et al.*, 1989). Adenovirus ITRs, including bracketed sequences, can be excised from flanking DNA in circular plasmids and amplified by replication as a linear molecule (Hay *et al.*, 1984). These experiments point out that a mechanism to liberate linear molecules from circles must exist. This process is likely the result of repeated cycles of precisely initiated adenovirus DNA replication. Thus, the utilization of an embedded origin does not require a specific cleavage to expose the replication origin sequence (Hay *et al.*, 1984).

Initiation and chain elongation during adenovirus strand-displacement replication has been described above. The basic features of strand-displacement replication are now reasonably clear. However, the process for converting displaced single strands to double-stranded DNA molecules, referred to as complementary strand synthesis, has not yet been described and will be discussed below.

Complementary Strand Synthesis

Although the development of *in vitro* systems has provided much valuable information for understanding adenovirus strand-displacement replication, complementary strand synthesis has not been demonstrated *in vitro*. Thus, there is no detailed knowledge about this process.

Cis replication pathway. Panhandle structures (Figure I.1e) have been proposed as replicative intermediates for adenovirus complementary strand replication (Lechner and Kelly, 1977). Displaced strands can form panhandle structures by base pairing between complementary sequences within the ITRs. The process is therefore called *cis* replication because it involves intramolecular hybridization on the same DNA strand. The creation of type II molecules (Figure I.1g) can be envisioned as the result of

initiation on panhandle intermediates (Figure I.1f) coupled with subsequent chain elongation through the panhandle. Although it has been widely accepted that adenovirus replication utilizes a panhandle intermediate in its replication cycle, there is no direct evidence for it (Revet and Benichou, 1981). However, indirect evidence has been found to support the existence of the *cis* replication pathway (Haj-Ahmad and Graham, 1986; Stow, 1982). Molecules with deletions removing part of one ITR, even as many as 51 bp, on one end of the adenovirus genome, can still yield infectious virus with completely intact ITRs at both ends when transfected into cells (Stow, 1982). The total deletion of one entire ITR is lethal for the production of infectious virus. A likely explanation for this repair process is that a panhandle structure is formed during complementary strand synthesis and the intact copy of the ITR serves as a template to repair the partially deleted ITR. Loss of an entire ITR prevents panhandle formation and interrupts the replication cycle.

Other studies also suggest that a panhandle structure is involved as an intermediate in adenovirus DNA replication (Hay *et al.*, 1984; Wang and Pearson, 1985). Specially constructed plasmids with a single adenovirus terminal sequence followed by a sequence repeated in an inverted orientation at the end of the molecule can give rise to replication-proficient molecules when transfected into cells by regenerating adenovirus origins at both ends. The mechanism to generate this larger replication-proficient molecule is due to the formation and repair of a panhandle intermediate specified by the synthetic inverted repetitions. Thus, the one copy of the adenovirus origin serves as the template for the synthesis the second adenovirus origin at the other end of the plasmid DNA molecule.

Adenovirus mutants with duplicated ends have been isolated or constructed (Haj-Ahmad and Graham, 1986). Mutant *dl*E1,3-1, with a direct repeat of viral DNA terminal sequences attached to the left end of the genome, and mutant *dl*E1,3-2, with duplicated terminal sequences at both ends of the viral genome, can interconvert to each other with high frequency (Haj-Ahmad and Graham, 1986). This interconversion is consistent with the involvement of panhandle replicative intermediates.

Recently, a special class of panhandles (hairpin structures) have been characterized (C.-H. Hu *et al.*, unpublished results). Hairpin structures apparently arise as intermediates in the replication of symmetrical dimers

and multimers, generated by end-to-end ligation of input plasmids during transfection. The detection of hairpins *in vivo* is strong evidence for the idea that panhandle structures may really be involved in the adenovirus DNA replication cycle.

Trans replication pathway. An alternative pathway for complementary strand replication, called *trans* replication, has also been proposed (Ahern *et al.*, 1990). Two linearized plasmids sharing the same DNA backbone, but carrying different adenovirus terminal sequences (either right or left) in opposite orientations, were studied. The two displaced single strands, arising separately from the input plasmids, can actually hybridize to each other through complementary DNA sequences. After a necessary repair at the ends of these hybrid DNA molecules to generate functional adenovirus replication origins, subsequent replication will lead to the generation of new replication-proficient double-stranded DNA molecules. This study was the first observation to imply the existence of a novel mechanism for complementary strand synthesis where intermolecular hybridization of complementary sequences from different displaced single strands forms a heteroduplex intermediate to complete the replication cycle.

Other replication pathways. At least other two pathways for complementary strand replication to copy the displaced strands can be imagined. Since displacement synthesis can be initiated at either end of the genome, one possibility is that before completion of one round of displacement replication initiated at one end of the genome, a second initiation will occur at the opposite end. Thus, both parental strands will serve as templates for daughter strand synthesis and two type II molecules can be formed as replication intermediates (Figure I-1 h and I-1g).

Another possibility is that complementary strand replication may just initiate at the 3' end of completely displaced single adenovirus strands without recourse to panhandle structures. Adenovirus DNA polymerase can use single stranded DNA as a template for DNA synthesis even though the efficiency is much lower and specific initiation is rare.

Present Work

The aim of the research described here is focused on studies of the replication mechanism(s) used in adenovirus complementary strand

replication *in vivo*. Two important questions have been asked: (1) what roles do ITRs play during viral DNA replication, and (2) what happens to displaced strands during adenovirus DNA replication? A model system has been developed to study the requirements for the formation of stable panhandle structures during adenovirus replication *in vivo*; this will be described in Chapter III. The relative participation between the *cis* and the *trans* replication pathways during the adenovirus DNA replication cycle will be discussed in Chapter IV.

CHAPTER II
MATERIALS AND METHODS

Reagents and enzymes

Restriction endonucleases, the Klenow fragment of *Eschericia coli* (*E. coli*) DNA polymerase I, and bacteriophage T4 DNA ligase were purchased from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, Pharmacia, Promega or IBI. Pronase was from Calbiochem-Boehringer Corp. Ribonuclease T1 and lysozyme were from Sigma. All the enzymes were used as recommended by the manufacturers. ^{32}P-labeled deoxyribonucleotides (3,000 Ci/mmol) were from New England Nuclear.

Plasmids and bacteria

Plasmids pBR322 (Bolivar *et al.*, 1977), pUC19 (Messing, 1979), pMDC10 (Enns *et al.*, 1983), pIR10 (Wang and Pearson, 1985), and 58Δ (Hay, 1985b) have been described previously. Plasmid DNA was isolated by an alkaline extraction procedure (Birnboim and Doly, 1979) and purified by ethidium bromide-CsCl density gradient centrifugation. *E. coli* RR1 and *E. coli* DH5α, supplied as competent cells, were from Bethesda Research Laboratories and were treated according to the supplier's specifications for high efficiency transformation. *E. coli* JC9387 *rec*BC*sbc*B (Leach and Stahl, 1983) was grown, transformed, and selected by standard procedures (Maniatis *et al.*, 1982). Transformed cells were grown in modified YT medium (containing per liter: 5 g yeast extract, 10 g Casein Enzyme Hydrolysate, and 5 g NaCl). Selection for cells transformed with recombinant plasmids was carried out in medium containing one or more of the following antibiotics: ampicillin (50 μg/ml), tetracyline (15 μg/ml), or kanamycin (50 μg/ml). If necessary, plasmid DNA was amplified by adding chloramphenicol (100 μg/ml) to the medium 12-16 hr prior to harvest of the cells (Maniatis *et al.*, 1982).

Preparation of DNA fragments

DNA was first digested with appropriate restriction enzymes. The fragments were then separated by gel electrophoresis on 1% agarose gels. The DNA band of interest was cut from the gel and the gel slice was minced into tiny pieces. The sliced gel was mixed with an equal volume of phenol

(pH 7.5) and frozen at -80°C for at least 15 min. The frozen mixture was then spun in a microfuge for 10 min. The top layer was collected and DNA was purified by multiple phenol extractions followed by ethanol precipitation.

Preparation of blunt-ended DNA

Reactions to blunt cohesive restriction enzyme sites on DNA fragments or plasmid backbones were carried out by treating 1 μg of DNA with 2 units of the Klenow fragment of *E. coli* DNA polymerase I in 50 μl of a solution containing 20 μM dATP, 20 μM dCTP, 20 μM dGTP, 20 μM dTTP, 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM dithiothreitol, and 50 μg/ml bovine serum albumin (BSA) at 37°C for 30 min. Prior to ligation, blunt-ended DNA was purified by phenol extraction and ethanol precipitation. If necessary, the DNA was eluted through a spun-column of Sephadex G-50 to remove the unincorporated dNTPs.

DNA ligation

Ligation reactions in 20 μl contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5% polyethylene glycol, 1 mM ATP, 1 mM dithiothreitol, 1 unit of bacteriophage T4 DNA ligase, and 1 μg DNA where the molar ratio of vector-to-insert was 1:3. Reactions were incubated for 4 hr to overnight at room temperature (23-26°C). Ligated DNA was diluted 5-fold before addition to competent cells for transformation.

Transformation of bacterial cells

A small scale reaction was used for transforming ligated DNA into competent *E. coli* RR1 cells or DH5α cells. Only 1 μl of diluted DNA (about 10 ng) was added to 20 μl of competent cells. The cells were shaken for approximately 5 sec immediately after the addition of DNA, incubated on ice for 30 min, and then shocked by heating for 45 sec at 42°C. The heat-shocked cells were immediately placed on ice for 2 min. Cells were diluted with 80 μl of SOC medium (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose), agitated at 225 rpm for 1 hr at 37°C, and spread on a plate containing YT medium with selective antibiotics. Plates were incubated at 37°C for 16 hr.

DNA sequencing

A rapid method for dideoxyribonucleotide chain termination

sequencing, applied directly to double-stranded supercoiled plasmid DNA, was used (Zagursky *et al.*, 1985). The technique employs avian myeloblastosis virus (AMV) reverse transcriptase instead of the Klenow fragment of *E. coli* DNA polymerase I for the labeling reaction. The sequencing kit was purchased from New England Biolabs. Double-stranded plasmid DNA (about 1 μg) was first denatured in 0.2 N NaOH and then renatured in 0.45 M potassium acetate buffer (pH 4.5) together with 100 ng of the reverse sequencing primer. The primer-DNA mixture was further precipitated and resuspended in 20 μl of sequencing buffer containing 10 mM Tris-HCl (pH 8.3), 40 mM NaCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol. At the same time, 20 units of AMV reverse transcriptase and 20 mCi of either [α-^{32}P]dATP or [α-^{32}P]dCTP (specific activity 600 Ci/mmol) were also added to the mixture. The mixture was divided into four parts (4 μl) to combine with each of the four appropriate dideoxyribonucleotide-deoxyribonucleotide solutions (1 μl). After incubation at 42°C, the sequencing reaction was stopped by adding 5 μl of stop solution (90% formamide). Boiled samples were loaded onto an 0.4-mm thick 8% polyacrylamide sequencing gel containing 8M urea. Electrophoresis was carried out at 1200 V for 2-3 hr in TBE buffer containing 89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA (pH 8.0).

Cells and virus

HeLa S3 cells were maintained in suspension culture in Joklik's minimal essential medium (GIBCO) containing 5% fetal calf serum (HyClone). Adenovirus type 5 was grown in HeLa S3 cells. Virus and viral DNA were isolated and purified as previously described (Pettersson and Sambrook, 1973). 293 cells (Graham *et al.*, 1977) were propagated in monolayer culture in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal calf serum, 1% penicillin/streptomycin and glucose at a concentration of 1 mg/ml. The same medium, but with glucose at 4 mg/ml, was used to grow 293 cells in preparation for transfection because the higher level of glucose greatly enhanced attachment of 293 cells to tissue culture plates during transfection.

Transfection

293 cells were transfected by the standard DNA-calcium phosphate coprecipitation technique as previously described with minor modifications

(Graham and van der Eb, 1973). Each 60 mm tissue culture plate was inoculated with 7×10^5 cells 48 hr prior to transfection. Transfection utilized 5 μg of each plasmid DNA and 5 μg of helper adenovirus type 5 DNA for each plate. The transfection was performed by gently mixing 125 μl of 0.5 M $CaCl_2$ solution containing the 10 μg of DNA (plasmid and helper) with an equal volume of 2 × HBS buffer (pH 7.1) containing per milliliter 16.36 μg NaCl, 0.4 μg Na_2HPO_4, and 11.9 μg Hepes. After no more than 20 min, the mixture was then added to 293 cells. After incubation at 37°C in a CO_2 incubator for another 20 min, 5 ml of growth medium was added to each plate. Cells were shocked for 1 min with 15% glycerol in 1 × HBS buffer 4-6 hr after transfection. Total DNA was extracted around 60 hr post transfection.

Isolation of DNA

DNA was isolated from transfected 293 cells by a modified rapid isolation method (Hirt, 1967; Wilkie, 1973). Transfected cells were scraped from plates with a rubber spatula and centrifuged for several min at 3,500 rpm. Cell pellets were first rinsed with 1 ml of PBSd buffer (pH 7.5; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4) and then resuspended in 400 μl of 0.6% sodium dodecyl sulfate (SDS) and 10 mM EDTA (pH 8.0). The mixture was digested with Pronase (1 mg/ml) and ribonuclease (1 mg/ml) at 37°C overnight. Total DNA was purified by phenol extraction and ethanol precipitation. Purified DNA was dissolved in 300 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Approximately 1/4 to 1/8 of the total DNA from each plate was used for subsequent Southern blot analysis. Sometimes DNA was digested with various restriction enzymes, such as *Dpn*I or *Mbo*I, prior to electrophoresis and blotting.

Southern blot analysis

Southern blotting with a quick alkaline transfer step was used (Chomczyhski and Qasba, 1984; Reed and Mann, 1985). DNA molecules isolated from transfected cells were first separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate and 2 mM EDTA). Following electrophoresis, DNA was transferred from the gel onto a Zeta-probe membrane for 16 hr using 0.4 N NaOH as the transfer solution. After transfer, the membrane was neutralized by washing three times for 10

min with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.1% SDS. The neutralized membrane was dried at room temperature using filter paper sheets. Prehybridization was performed for 2 to 16 hr at 37°C by placing the membrane in a plastic sealing bag together with 5 × SSC buffer (10 ml per 100 cm^2) containing denatured salmon sperm DNA (250 μg/ml), 50 mM sodium phosphate (pH 6.7), 5% dextran sulfate, 0.5% non-fat dry milk, 1% SDS and 50% formamide. Hybridization was carried out by incubating the membrane in a plastic sealing bag with radioactively labelled DNA probes (30 ng, specific activity > 1×10^8 cpm/μg) overnight in 5 × SSC containing denatured salmon sperm DNA (100 μg/ml), 20 mM sodium phosphate (pH 6.7), 5% dextran sulfate, 1% SDS and 50% formamide. After hybridization, the membrane was washed twice for 15 min at 37°C in 2 × SSC (approximately 50 ml per 100 cm^2) with 0.1% SDS, then 2-3 times in the same solution at 65°C for a total of 1-2 hr, and finally in 0.1 × SSC with 0.1% SDS for 30 min at 65°C. After washing, the membrane was autoradiographed using Kodak X-ray film.

Preparation of probe

Radioactively labeled probes were synthesized with an oligonucleotide labeling kit (Pharmacia) using [α–^{32}P]dCTP (New England Nuclear). pUC19 plasmid was first digested with *Pvu*I. DNA fragments (30 ng) were denatured by boiling in water for 10 min and immediately cooling on ice for 5 min. DNA was radiolabeled in a reaction (50 μl) containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 200 mM Hepes (pH 6.6), hexadeoxyribonucleotides (6 A_{260} units/ml), bovine serum albumin (0.2 mg/ml), and 5 μl [α–^{32}P]dCTP (3,000 Ci/mmol). After the reaction, unincorporated nucleotides were removed by chromatography on Sephadex G-50 (Pharmacia) or Nu-Clean D50 (IBI). Radiolabeled probe was denatured by boiling just before using.

CHAPTER III
CIS REPLICATION PATHWAY

Overview

The genomes of the human adenoviruses are linear, double-stranded DNA molecules having a terminal protein covalently attached to the 5' end of each strand (Robinson and Bellett, 1975; Robinson *et al.*, 1973), and containing an inverted terminal repetition (ITR) of about 100 bp (Garon *et al.*, 1972; Wolfson and Dressler, 1972). The replication origins are located at the extreme ends of the adenovirus genome within the ITR region (Ellens *et al.*, 1974; Lechner and Kelly, 1977). Extensive analysis of both *in vitro* (Challberg and Rawlins, 1984; Enns *et al.*, 1983; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983; Wides *et al.*, 1987) and *in vivo* (Bernstein *et al.*, 1986; Hay, 1985a; Hay and McDougall, 1986; Wang and Pearson, 1985) adenovirus DNA replication has established that only the terminal 51 bp of the ITRs are need for optimal initiation of DNA replication and the initiation of viral DNA replication is primed by the virus-encoded terminal protein (Challberg and Kelly, 1989; Field *et al.*, 1984a; Fütterer and Winnacker, 1984; Kelly, 1984). Adenovirus replicates by a strand-displacement mechanism from the origins. Subsequently, complementary strand synthesis converts the displaced single strand into double-stranded DNA to finish one viral replication cycle (Lechner and Kelly, 1977). Although studies using the *in vitro* replication system have provided considerable information about the adenovirus origin and strand-displacement replication (Challberg and Kelly, 1989), knowledge about the precise mechanism(s) involved in complementary strand synthesis has not yet been established. One attractive hypothesis is that panhandle structures, created by base-pairing between sequences of ITRs in the displaced strands, are formed as replicative intermediates for complementary strand synthesis (Lechner and Kelly, 1977). Deletion mutants which carry different sizes of ITRs have been used to test the proposed role of the panhandle intermediate. Stow (Stow, 1982) has demonstrated that viral terminal deletion mutants, lacking as many as first 51 bp of one of the ITRs but not the entire ITR, are still viable and able to generate wildtype virus with two complete ITRs at both ends. However, removal of one entire copy of the ITR is lethal. This result strongly suggests

that the deleted origin is restored by DNA repair synthesis on a panhandle structure, where the remaining wildtype copy of the ITR serves as a template to restore the deleted one. Deletion mutants lacking an entire copy of the ITR cannot form a panhandle to regenerate the missing origin, and therefore are not viable. A model system has been developed to define sequences required for the adenovirus replication origin *in vivo*. This approach separates origin sequences from those involved in panhandle formation (Hay *et al.*, 1984; Wang and Pearson, 1985). Linearized plasmids with a single adenovirus terminus but containing inverted repeats, when cotransfected with helper adenovirus DNA into cells, generate new, larger, replication-proficient DNA molecules with adenovirus termini at both ends (Hay *et al.*, 1984; Wang and Pearson, 1985). In order to study the size required for stable panhandles, plasmids with different sizes of inverted repetitious sequences derived from all or part of the polylinker sequences of plasmid pUC19 were been constructed and tested. Experiments showed that inverted repeats as small as 33 bp were sufficient to form panhandles, but repeats 28 bp or smaller were not (Wang *et al.*, 1990). In an effort to narrow the size requirement more precisely, a new plasmid carrying 31-bp inverted repeats has been made. It shows that 31 bp is also sufficient to support the formation of panhandles. Furthermore, the inverted repeats can be mutated by deleting or inserting sequences at specific restriction enzyme recognition sites within the polylinkers. Mismatched sequences can therefore be introduced into the panhandle region during intramolecular hybridization. Experiments demonstrate that as many as 4 bp of mismatched sequences have no effect on the formation of panhandles when the sizes of the inverted repeats are either 51 bp or 230 bp. This is true even when the 4 bp mismatch divides the 51-bp inverted repeats into two regions with each part less than 28 bp. It appears that the mismatched sequences can also be repaired in certain cases to restore perfectly double-stranded panhandles. These studies not only provide us useful information about sequence rectification inside living cells, but also help us to understand in more detail the base-pairing rules operating on adenovirus ITRs for maintaining identical inverted terminal repetitions. Plasmids containing multiple copies of inverted repeats in different orientations spaced at various distances from each other have also been constructed for testing the constraints placed on the selection of inverted repetitious

sequences for panhandle formation during complementary strand synthesis. The removal of 3' non-homologous sequences, a phenomenon never before observed in eukaryotic cells, has also been detected. The results indicate that there is no special preference on the selection of inverted repeats. The data here may explain why the adenovirus ITRs are larger than the viral DNA replication origins.

Construction of Plasmids

Plasmid with 31-bp inverted repeats. Figure III.1 shows the procedure for constructing a plasmid carrying 31-bp inverted repeats derived from the polylinker sequence of pUC19. The left adenovirus DNA replication origin was removed from pMDC10 as a 338-bp *Eco*RI-*Ssp*I fragment and cloned into the *Eco*RI and *Ssp*I sites of pUC19 to form plasmid T4. T4 was then cut with restriction enzymes *Ssp*I and *Hin*cII, and the small fragment was isolated and blunted with the Klenow fragment of *E. coli* DNA polymerase I. The purified fragment, which contains adenovirus origin sequences adjacent to part of the pUC19 polylinker region, was ligated with the blunted *Eco*RI-*Ssp*I pUC19 backbone. Plasmids in ampicillin-resistant *E. coli* RR1 colonies were screened by digestion with *Bam*HI or *Eco*RI-*Hin*dIII. A 2.5-kb plasmid, carrying the expected 390-bp *Eco*RI-*Hin*dIII fragment, was selected and named pIRHnc. The structure of pIRHnc is shown in Figure III.2 together with other plasmids which carry polylinker inverted repeats ranging from 14 bp to 51 bp. The 31-bp inverted repeats in pIRHnc were verified by DNA sequencing (one polylinker is 31 bp and the other is 51 bp).

Plasmids with mutated inverted repeats. The same strategy to construct plasmids with varying sizes of inverted repeats was applied to the assembly of plasmids with mutated inverted repeats (Figure III.1). Plasmid T4 was first linearized at *Kpn*I, *Xba*I or *Sph*I sites within the polylinker region followed by treatment with the Klenow fragment of *E. coli* pol I to blunt each cut site. Blunted plasmids were then recircularized to create intermediate plasmids called T"X" ("X" here represents mutations at either *Kpn*I, *Xba*I, or *Sph*I sites; 4 bp were deleted or inserted according to the nature of the altered cohesive site). The small, blunted *Ssp*I-*Hin*dIII fragment containing 338 bp of the adenovirus origin and 51 bp of pUC19 polylinker sequences from plasmid T4 was ligated with the large, blunted

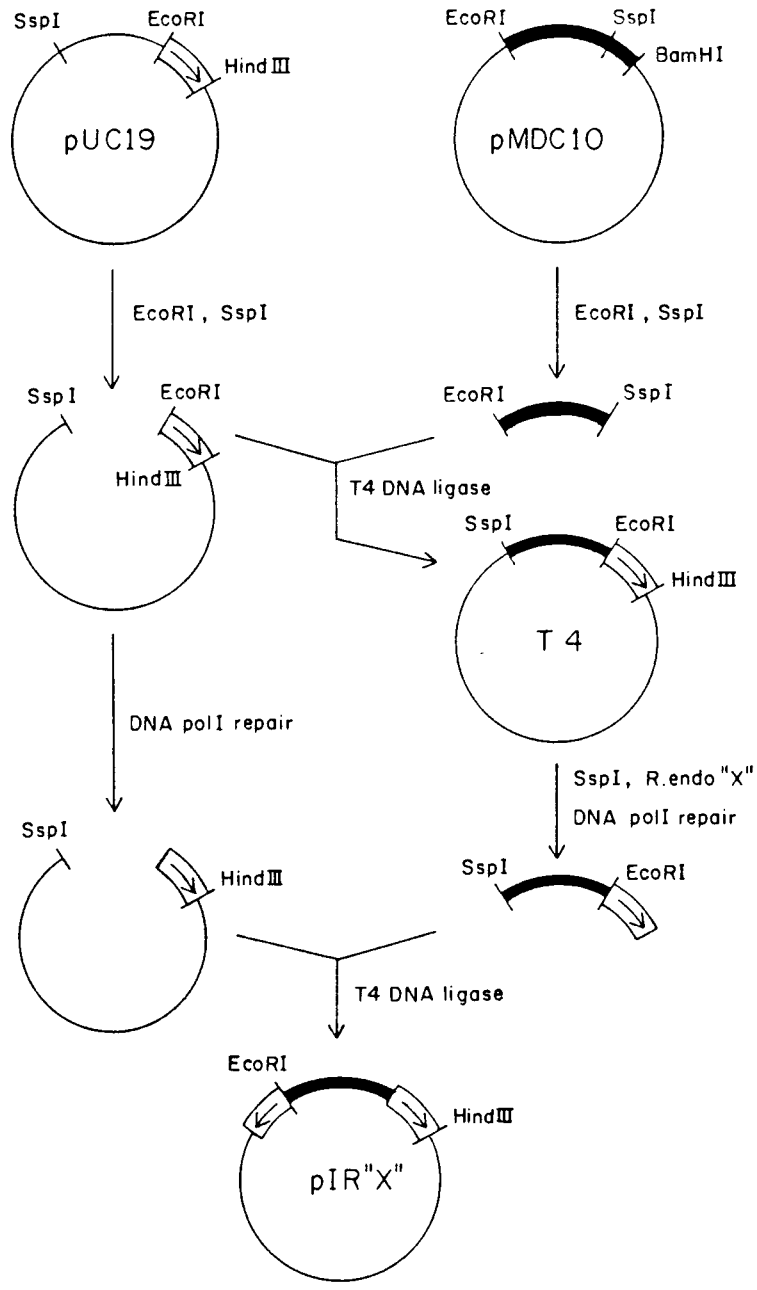

Figure III.1. Construction of plasmids containing inverted repeats derived from the polylinker region of pUC19. See text for details. R.endo "X" is any of the restriction endonucleases that cuts within the polylinker region.

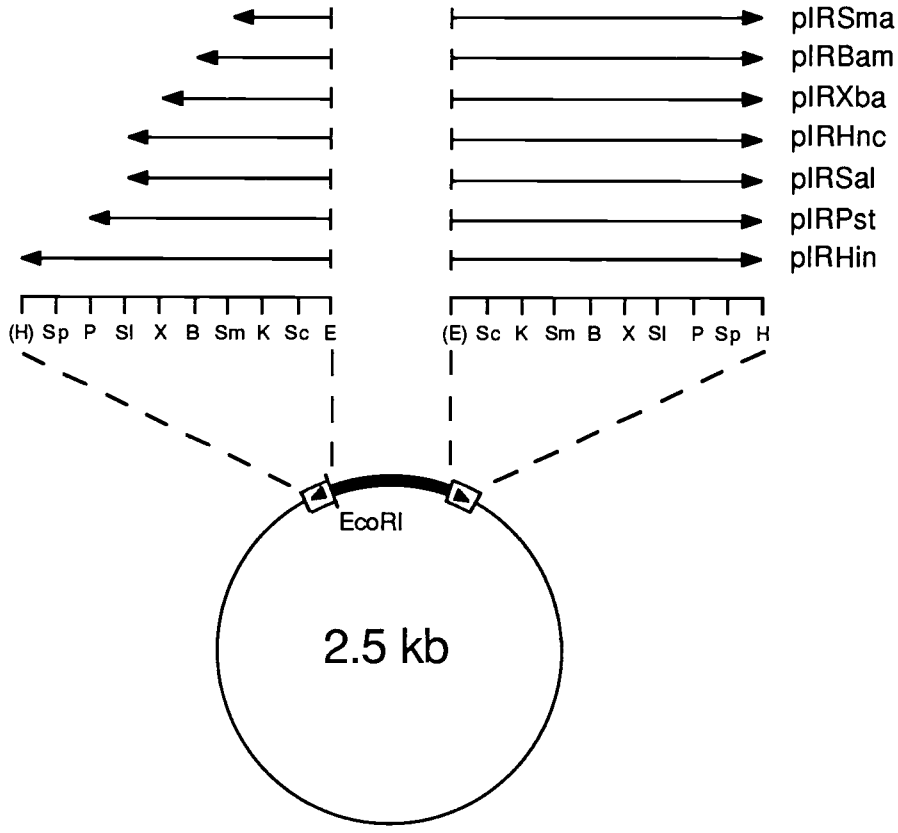

Figure III.2. Structures of the plasmids containing inverted repeats derived from the polylinker region of pUC19. Arrows show the orientation and extent of repeated sequences in each plasmid. E = *Eco*RI, Sc = *Sac*I, K = *Kpn*I, Sm = *Sma*I, B = *Bam*I, X = *Xba*I, Sl = *Sal*I, P = *Pst*I, Sp = *Sph*I, and H = *Hin*dIII. Parentheses enclose cleavage sites destroyed during construction of the plasmids. The adenovirus origin lies between the inverted repeats immediately next to the *Eco*RI site.

*Eco*RI-*Ssp*I backbones from different T"X" plasmids. Plasmids were screened by *Eco*RI-*Hin*dIII digestion, and those which gave a 390-bp fragment were selected. The structures of these 2.5-kb plasmids, designated pIR5'"X" (also referred to as 5' mutants) are shown in Figure III.3). The small, blunted *Ssp*I-*Hin*dIII fragments from T"X" plasmids could also be ligated with the large, blunted *Eco*RI-*Ssp*I T4 backbone to generate the 2.5-kb plasmids called pIR3'"X" (also known as 3' mutants; Figure III.3). Finally, the small, blunted *Ssp*I-*Hin*dIII fragment from each T"X" plasmid could be ligated back into the large, blunted *Eco*RI-*Ssp*I backbone of the same T"X" plasmid to make double mutants (pIR"X"D; Figure III.3). To introduce the *Kpn*I mutation on bigger inverted repeats (230 bp), each small *Bam*HI fragment from pIR5'K, pIR3'K and pIRKD was cloned into the corresponding *Bam*HI sites in plasmid pLIRST (described in Chapter IV) to generate pLIR5'K, pLIR3'K, and pLIRKD, respectively.

Plasmids with multiple inverted repeats. Figure III.4 shows the scheme for construction of plasmids carrying multiple copies of inverted repeat sequences. A blunted 2.1-kb *Eco*RI-*Pvu*II fragment from pBR322 containing the tetracycline gene was cloned into the blunted *Eco*RI site of pUC19. The blunt-end reaction was carried out by using the Klenow fragment of *E. coli* DNA polymerase I. A 4.8-kb intermediate clone, called pRT19, was isolated under tetracycline selection. The insertion joined the *Pvu*II site on the tetracycline fragment to the blunted *Eco*RI site of pUC19 to regenerate a new *Eco*RI site. The regenerated *Eco*RI site on pRT19 was removed to yield plasmid pT3. A 2.2-kb *Sma*I-*Pvu*II fragment from pT3 was then cloned into the *Xmn*I site of pIR10 under tetracycline selection. Two 5.4-kb plasmids, called pIRT6 and pIRT7, differing only in the orientation of inserted tetracyline fragment, were recovered and characterized. Both plasmids, shown in Figure III.5, contain three copies of the 89-bp repeats derived from part of the *lacZ* gene in pUC19. The existence of the third copy of the inverted repeat was verified by DNA sequencing.

Results and Discussion

Adenovirus-specific replication of linear plasmids *in vivo*. A model system for studying adenovirus complementary strand DNA synthesis (Hay *et al.*, 1984; Wang and Pearson, 1985) has been adapted in my research. In this system, plasmid DNA molecules containing cloned adenovirus origin

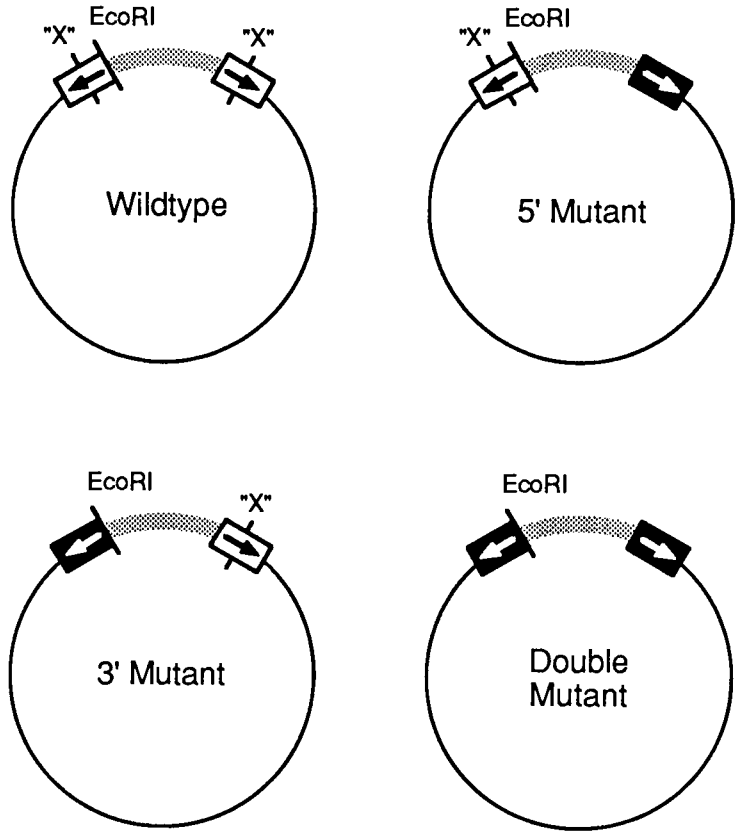

Figure III.3. Polylinker inverted repeat (IR) plasmids with mutations in the polylinker regions. The thin lines represent the plasmid vector sequences. The adenovirus sequences containing the viral DNA replication origin are shown as thick gray lines. The open boxes are unmutated inverted repeats. The black boxes are mutated inverted repeats. The arrows show the orientation of the inverted repeats. "X" indicates the unmutated restriction enzyme sites of either *Kpn*I, *Xba*I or *Sph*I.

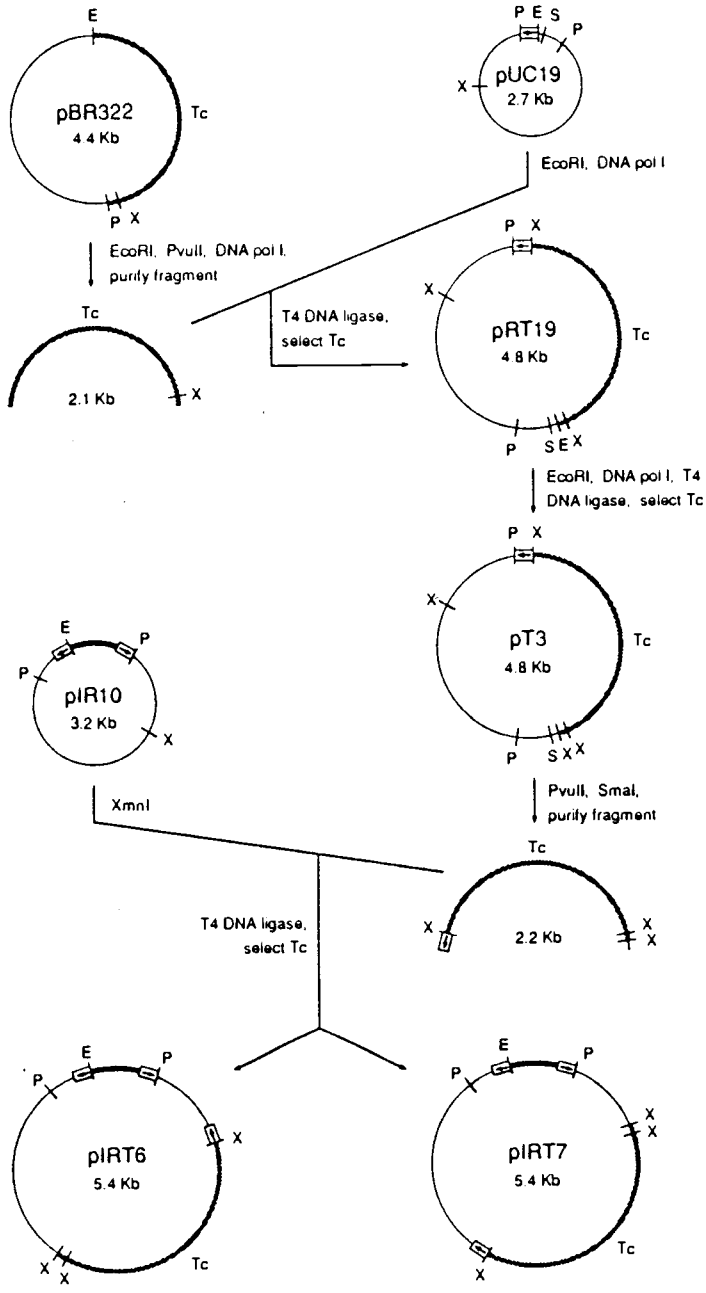

Figure III.4. Construction of plasmids containing multiple copies of inverted repeat sequences. Tc (cross-hatched line) indicates gene for tetracycline resistance. E = *Eco*RI, P = *Pvu*II, X = *Xmn*I, and S = *Sma*I. Not all restriction endonuclease sites are shown.

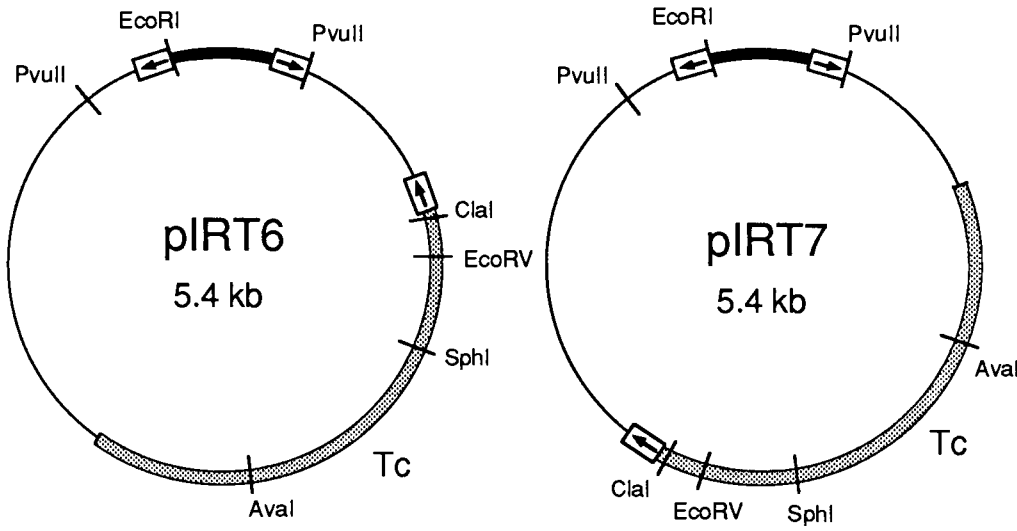

Figure III.5. The structures of pIRT6 and pIRT7. The thin lines represent the plasmid vector sequences. The thick black lines represent the adenovirus sequences containing the viral DNA replication origin. The open boxes are the 89-bp inverted repeats. The arrows indicate the orientation of inverted repeats. The thick stippled lines represent the gene conferring resistance of tetracycline. The plasmids can be truncated by cutting at *Cla*I, *Eco*RV, *Sph*I, or *Ava*I.

fragments, instead of the whole adenovirus genome, were used as the substrates to study viral DNA replication *in vivo*. Previous experiments demonstrated that linear plasmid DNA molecules carrying adenovirus origin sequences at both ends could replicate autonomously when introduced into cells together with adenovirus DNA as a helper (Hay *et al.*, 1984). Linear plasmids with a single adenovirus origin, when cotransfected with helper adenovirus DNA, do not amplify and only engage in strand-displacement replication (Hay, 1985a). However, if the specially constructed plasmids, besides bearing a single adenovirus terminus, also containing an inverted repetitious sequence (not necessarily an adenovirus sequence) with a specific geometrical arrangement, were linearized to expose the adenovirus origin, the plasmids could replicate to give rise to new, larger, replication-proficient molecules with adenovirus origins at both ends (Hay *et al.*, 1984; Wang and Pearson, 1985). The generation of an additional adenovirus origin at the other end of the DNA molecule is consistent with the participation of panhandle structure as a replicative intermediate for complementary strand replication (Lechner and Kelly, 1977). Two obvious advantages of this assay are: (1) the requirement for a functional adenovirus origin is separated from size constraints on inverted sequences involved in panhandle formation, thus this method provides an opportunity to study the length requirement of the ITR in adenovirus DNA replication without destroying the replication origin; (2) whether replication occurs or not can be simply monitored by the presence or absence of novel replication-proficient DNA molecules which differ in size from the input molecules. The 2.5-kb plasmids pIR"X," containing a solo adenovirus origin but with different sizes of non-viral inverted repeats, were constructed to test the size requirement for panhandles during complementary strand synthesis. The plasmids can be linearized by *Eco*RI to expose the adenovirus DNA replication origin. When plasmids pIR"X" were transfected into cells together with wildtype adenovirus DNA as a helper to provide all the virus-encoded proteins required for DNA replication, novel 2.8-kb replication-proficient molecules could be detected if the sizes of inverted repeats were large enough (Wang *et al.*, 1990). Figure III.6 diagrams the proposed events leading to the production of replication-proficient molecules. First, strand-displacement replication on a linearized pIR"X" DNA molecule will generate a double-stranded daughter DNA molecule and

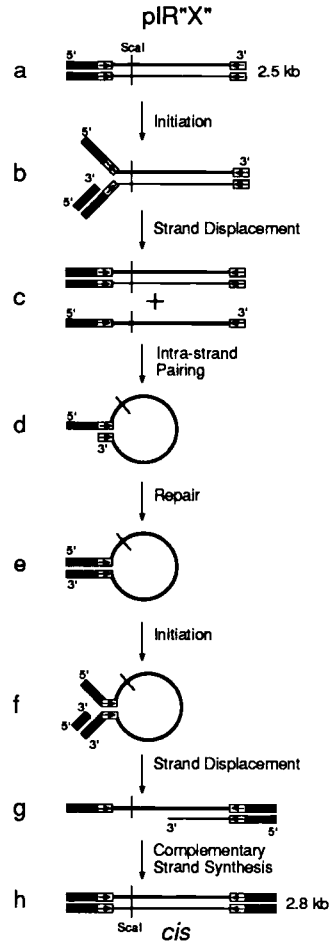

Figure III.6. Generation of a replication-proficient plasmid with two adenovirus origins from a plasmid bearing a single viral origin and inverted repeat sequences. (a) *Eco*RI-linearized 2.5-kb pIR"X". The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The closed boxes correspond to adenovirus DNA sequences containing the replication origin. The thin lines show plasmid DNA. (b) Initiation of strand displacement replication at the single adenovirus origin. (c) Production of a daughter double-stranded molecule and a displaced parental strand at the conclusion of displacement replication. (d) Formation of a panhandle structure by base pairing between inverted repeat sequences on the displaced strand. (e) Extension of the 3'-end of the panhandle molecules using adenovirus sequences as a template. (f) Initiation of complementary replication at the adenovirus origin of the panhandle molecule. (g) Complementary replication. (h) Production of a replication-proficient 2.8-kb daughter molecule with two adenovirus origins at the conclusion of complementary replication.

a displaced single strand. The displaced single strand can form a panhandle structure by base-pairing between the two artificial inverted repeats. DNA repair using one copy of the existing adenovirus terminal sequences as a template regenerates a double-stranded viral replication origin. Subsequent initiation and elongation on this repaired panhandle intermediate leads to the production of a replication-proficient DNA molecule with adenovirus termini at both ends.

A strategy (Peden *et al.*, 1980) has also been used to determine the state of replication for input DNA and replication-proficient molecules by measuring the sensitivity to cleavage with *Dpn*I and *Mbo*I. Plasmid DNA, propagated in Dam$^+$ *E. coli* hosts, will have the sequence GATC converted to GmeATC by site-specific methylation on adenine. GATC is the recognition site for both *Dpn*I and *Mbo*I; however, *Dpn*I cuts the site only when both strands are methylated, while *Mbo*I cuts the site only when both strands are totally unmethylated. Eukaryotic cells lack the specific methylase to methylate the sequence GATC. Input plasmid DNA, which does not replicate after transfection into mammalian cells, remains methylated on both strands and therefore sensitive to *Dpn*I but resistant to *Mbo*I. Plasmid DNA which undergoes only strand-displacement replication becomes both *Dpn*I- and *Mbo*I-resistant due to hemi-methylation. Only replication-proficient molecules with two newly synthesized strands are resistant to *Dpn*I but sensitive to *Mbo*I. It is clear that this assay can clearly distinguish the products designated a, c, and h in Figure III.6.

Determination of panhandle size. Figure III.7 shows the Southern blot analysis of the replication of plasmids containing different sizes of inverted repeat sequences. The plasmids have inverted repeats ranging from 51 bp (pIRHin) to 14 bp (pIRSma). Each of the *Eco*RI-linearized input inverted polylinker plasmids was detected as a 2.5-kb band. If the size of inverted repeat was big enough to support the formation of a panhandle structure, replication-proficient molecules generated from the displaced single strands could be detected as a 2.8-kb band. However, the generation of *Dpn*I-resistant input molecules as well as 2.8-kb replication-proficient molecules depended on the presence of helper adenovirus DNA. Without helper, *Dpn*I-resistant products were not detected (Figure III.7, lanes 1, 4, 7, and 10). Previous studies indicated that plasmids with inverted repeats of 33 bp or larger were able to form panhandle intermediates and give rise to

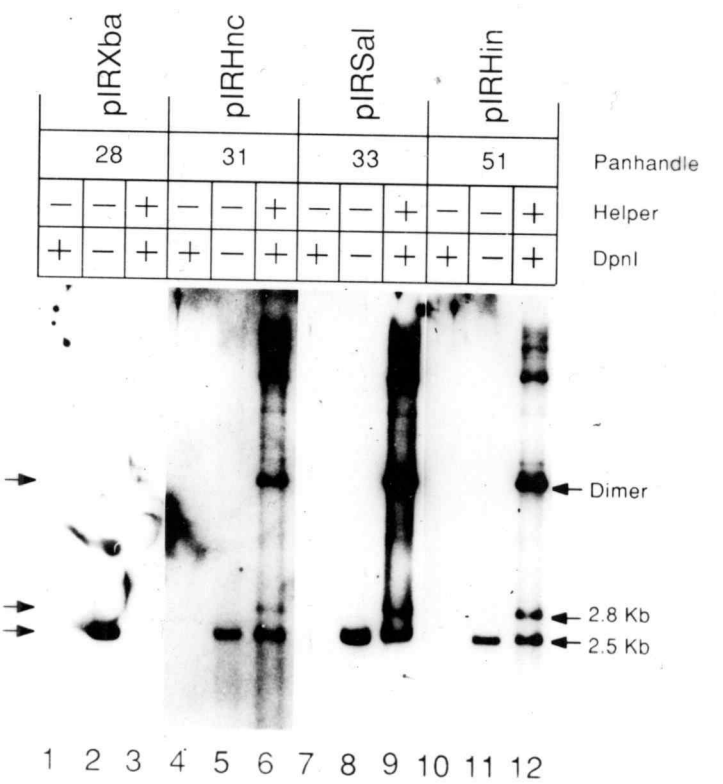

Figure III.7. Southern blot analysis of the replication of *Eco*RI-cut plasmids containing different sizes of inverted repeat sequences. See Figure III.2 for structures. DNA was isolated 60 hrs after transfection with (lanes 3, 6, 9, and 12) or without (all other lanes) helper adenovirus DNA. DNA was cut with *Dpn*I (except in lanes 2, 5, 8, and 11) before loading onto the gel. It should be noted that unreplicated input DNA is sensitive to *Dpn*I cleavage, but DNA that replicates in mammalian cells is not cleaved by *Dpn*I. Radioactive probe was plasmid vector DNA. Panhandle sizes are given in base pairs. See Figure III.6 for an explanation of the *cis* pathway leading to the creation of a 2.8-kb product from a 2.5-kb input molecule.

new 2.8-kb replication-proficient DNA molecules, but plasmids with inverted repeats of 28 bp or smaller failed (Wang *et al.*, 1990). As expected, plasmids pIRHin or pIRSal, with inverted repeats of 51 bp and 33 bp respectively, displayed 2.8-kb bands (Figure III.7, lanes 9 and 12), but pIRXba, with 28-bp repeats, did not (Figure III.7, lane 3). Using pIRHin, pIRSal, and pIRXba as the controls, it appears that plasmid pIRHnc, with 31-bp inverted repeats, also generated the 2.8-kb band. Thus, as little as 31 bp are sufficient for panhandle formation. Since pIRHnc contains the same adenovirus origin as all other polylinker plasmids, the differences in the ability to generate replication-proficient molecules are not due to different rates of producing displaced single strands. The 31-bp inverted polylinker sequences have a GC content of 64.5% which is within the range of 57.3% to 71.4% for the other polylinker plasmids. The base composition likely has no effect on panhandle stability. Based on the previous kinetic studies, it has been shown that the rate of replication is not a limiting factor for producing replication-proficient molecules. No 2.8-bp molecules were detected from pIRXba even when the molecules were assayed as late as 120 hr after transfection. Furthermore, the size of the panhandle loop also appears not to influence the yield of replication-proficient molecules since both 1.9-kb and 4.8-kb loops size are equally effective (see below). The evidence suggests that efficient panhandle formation depends on the size of the inverted repeats and that a minimum length of 31 bp is required. The minimum length is actually much shorter than the adenovirus replication origin (*i. e.*, the first 50 bp of the ITR). Understanding the function of the rest of the adenovirus ITR therefore becomes much more interesting.

Selection between multiple copies of inverted repeats. Plasmids carrying multiple copies of inverted repeat sequences have been constructed to study whether any special mechanism is involved in regulating the selection of inverted repeats to form panhandles during *cis* replication. Both pIRT6 and pIRT7 (Figure III.5) contain three copies of the 89-bp inverted repeat found in pIR10 (Wang and Pearson, 1985). When there are two possible choices for panhandle formation, it is interesting to know whether one copy of the inverted repeats on the displaced single DNA strand always selects another one which is farther away or nearer to form the panhandle, or whether both selections happen simultaneously. Figure III.8A diagrams two possible panhandle intermediates that could be formed in the displaced

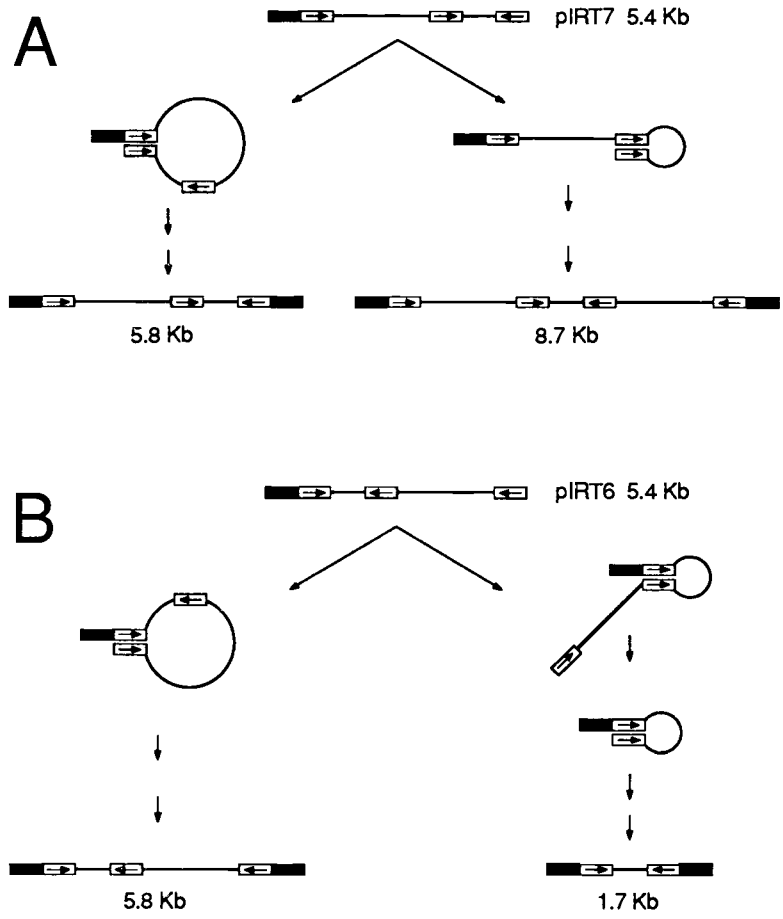

Figure III.8. Formation and fate of panhandle structures on displaced strands bearing multiple copies of inverted repeat sequences. (A) pIRT7. (B) pIRT6. The formation of a 1.7-kb molecule requires the loss of unpaired sequences at the 3' end of the displaced strand.

strand of pIRT7. Repair of the postulated panhandles would produce 5.8-kb and 8.7-kb replication-proficient molecules. Figure III.9 (lane 4) shows that both 5.8-kb and 8.7-kb bands expected for the two new replication-proficient molecules were detected in approximately equal intensities. This indicates that all available inverted repeats can be used for panhandle formation, and loops of 1.9 kb or 4.8 kb and the repair length of 400 bp or 3.3 kb in the panhandle structure do not appear to favor one structure over another. The middle copy of the inverted repeat on plasmid pIRT6 is in an opposite orientation to that on pIRT7. Two possible ways for forming panhandles on pIRT6 displaced single strands are diagramed in Figure III.8B. One of the structures would yield a 5.8-kb molecule, similar but not identical to the one produced by pIRT7. The other structure would generate a 1.7-kb replication-proficient molecule if 4000 unpaired bases at the 3' end of the displaced strand could be removed. Figure III.9 (lane 2) confirms the presence of the 5.8-kb product, and as expected, no 8.7-kb band was detected. The 1.7-kb replication-proficient molecule cannot be seen on this blot due to obscuring *Dpn*I digestion products, but the 1.7-kb band can be clearly seen in another blot (Figure III.10, lane 1). pIRT6, truncated with *Cla*I, *Eco*RV, *Sph*I, or *Ava*I, produced only 1.7-kb bands without regard to the size of the input molecules (Figure III.10, lanes 2 through 5). The displaced single strands from truncated pIRT6 form panhandles with unpaired 3' tails ranging from 23 bases to 1425 bases. These results also show that the 1.7-kb and 5.8-kb replication products arising from full-length pIRT6 had roughly equal intensities, indicating that both panhandles were efficiently used even though more than 4000 unpaired bases had to be removed from the 3' end of one panhandle structure. Neither loops as small as 700 bp nor 3' non-homologous tails as long as 4000 bases appeared to inhibit the yield. Panhandle structures have been proposed in the generation of sub-genomic defective molecules (Daniell, 1976). Displaced strands from plasmids like pIRT6 and pIRT7 resemble postulated intermediates in the formation of defective adenovirus genomes. Large inverted repeat sequences in the adenovirus genome (Munroe, 1983) may be targets for this process. The structure of pIRT6 is also equivalent to an adenovirus genome where new terminal sequences have been substituted for one of the adenovirus replication origins. Repair of these sequences to regenerate the origin could occur if the size of ITRs exceeded roughly 80 bp.

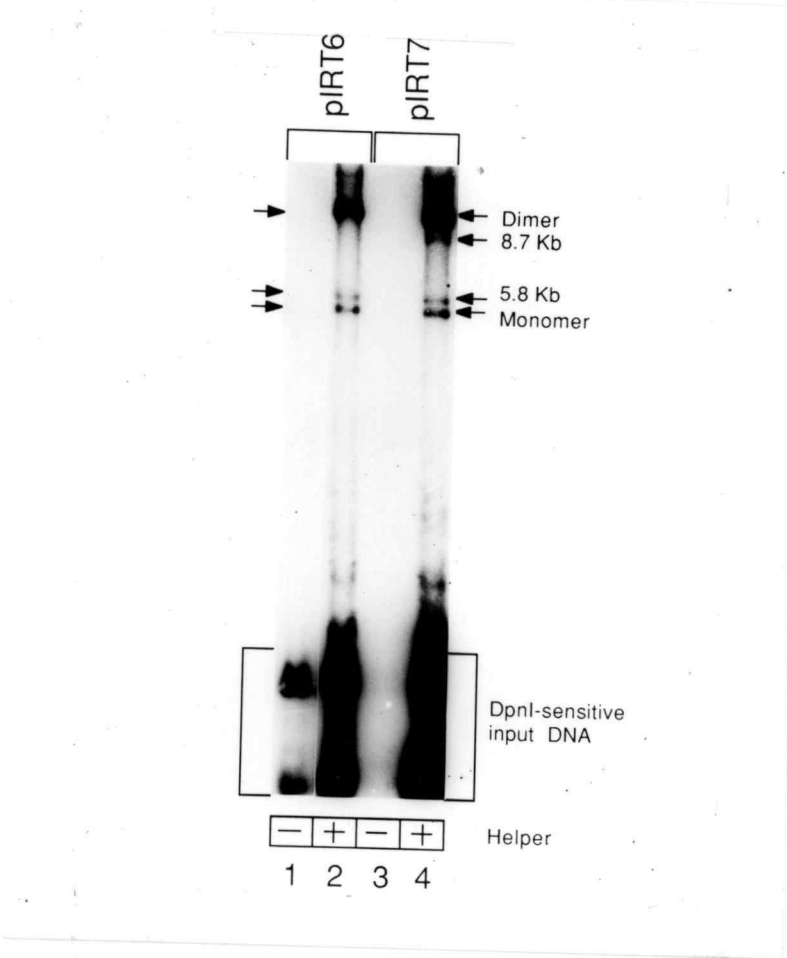

Figure III.9. Southern blot analysis of the replication of plasmids containing multiple copies of inverted repeat sequences. DNA was isolated 60 hrs after transfection with (lanes 2 and 4) or without (lanes 1 and 3) helper adenovirus DNA. DNA was cut with *Dpn*I before loading onto the gel. Lanes 1 and 2: pIRT6. Lanes 3 and 4: pIRT7.

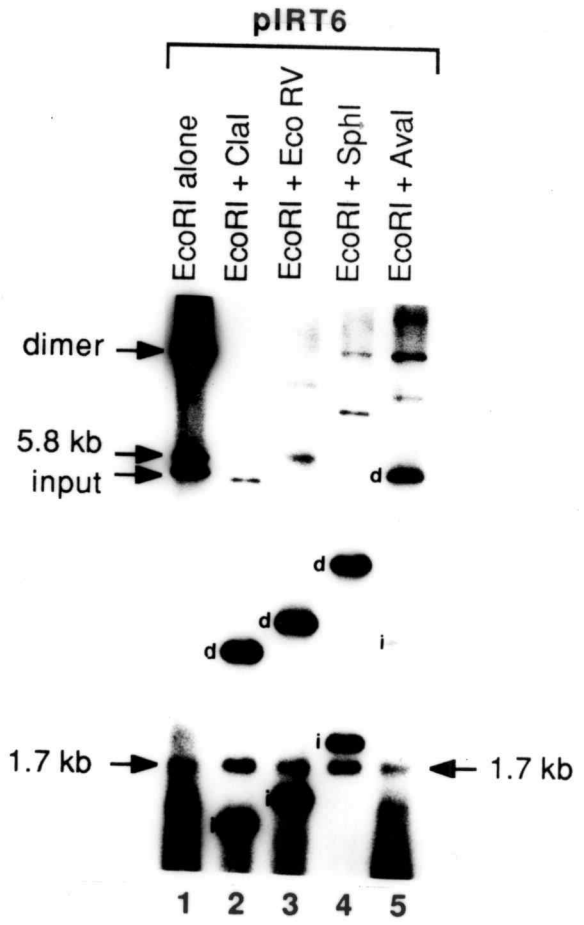

Figure III.10. Southern blot analysis of pIRT6 cut with *Eco*RI alone (lane 1) or *Eco*RI together with *Cla*I (lane 2), *Eco*RV (lane 3), *Sph*I (lane 4), or *Ava*I (lane 5). The 3' unpaired tails are over 4000, 23, 185, 562, and 1425 nucleotides, respectively. Samples were digested with *Dpn*I prior to gel electrophoresis. Bands corresponding to the truncated input plasmids are indicated by the letter "i" and the dimer bands are labelled with the letter "d" in lanes 2 through 5. The 1.7-kb cis product is seen in every lane, but the 5.8-kb cis product is only seen in lane 1.

Thus, these studies support the idea that evolutionary pressure may be exerted on the size of adenovirus ITRs in order to repair at high efficiency potentially lethal origin mutations.

DNA sequence rectification. Since adenovirus complementary strand synthesis proceeds through a postulated panhandle intermediate, the repair of mismatched sequences within inverted repeats can be readily studied. Such adenovirus-specific sequence rectification is formally equivalent to gene conversion (which has been defined as "... any process in which gene B acts as a sequence donor and remains unaffected, while gene A, which shares extensive homology with B, receives a block of B sequence and undergoes variation" (Kourilsky, 1986). When one copy of the inverted repeats is mutated, mismatched nucleotides are automatically introduced into the panhandle structure. Do mismatched sequences affect complementary strand synthesis? Because pUC19 polylinker sequences are used as inverted repeats, mutations can be made easily within one or the other or both of the inverted repeats by modifying any restriction enzyme site. Furthermore, the fate of mismatched sequences can be determined simply by monitoring the appropriate restriction enzyme cleavage pattern. As shown in Figure III.3, plasmids with mutated *Kpn*I, *Xba*I, or *Sph*I restriction sites in either copy or both copies of the inverted repeats have been constructed. Both deletions (4 bp) and insertions (4 bp) have been created. If any of these mutations inhibits panhandle formation, replication-proficient molecules will not be produced. If mutations have no effect on panhandle formation and the mismatched sequences on panhandle structures can be repaired or tolerated, replication-proficient molecules will be generated. Mutated restriction sites in the inverted repeat just next to the adenovirus origin are called 5' mutants. Mutated restriction sites in the inverted repeat at the other end of the plasmid molecule are called 3' mutants. Figure III.11 outlines the *cis* replication of a 5' mutant. Three possible *cis* replication molecules may be created. Molecules marked "a" (totally resistant to enzyme X) can be made by a 5'-IR to 3'-IR conversion process. Molecules designated "b" (cut once by enzyme X) arise if no conversion occurs during *cis* replication. Molecules labeled "c" (cut twice with enzyme X) originate by 3'-IR to 5'-IR sequence conversion. Figure III.12 diagrams three possible *cis* replication molecules produced by 3' mutants. In contrast to 5' mutants, replication-proficient molecules named

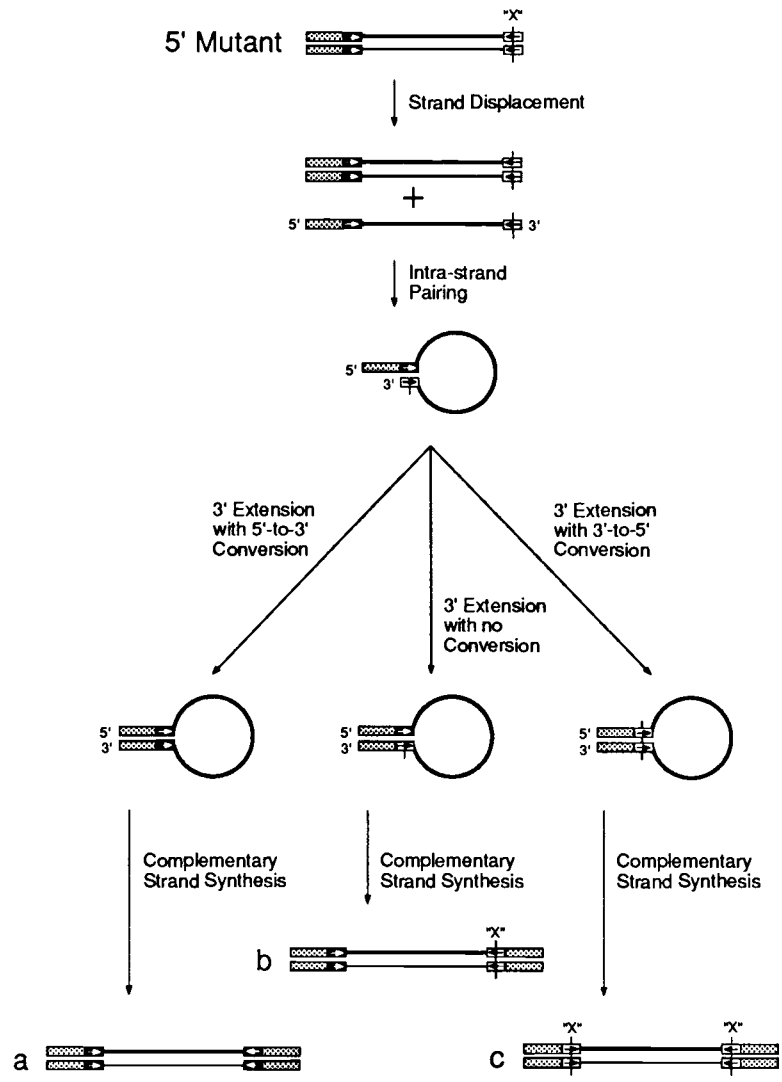

Figure III.11. Formation and fate of panhandle structures on displaced strands bearing mutations in the 5' inverted repeat sequence. The plasmids can be linearized by *Eco*RI. The thick stippled lines represent the adenovirus sequences containing the viral DNA replication origin. The open boxes are the unmutated inverted repeats. The black boxes are mutated inverted repeats. "X" indicates the cleavable restriction sites of *Kpn*I, *Xba*I or *Sph*I in unmutated inverted repeats. 3'-to-5' conversion means that sequences in the 3' inverted repeat are donated to the 5' inverted repeat. 5'-to-3' conversion means that sequences in the 5' inverted repeat are donate to the 3' inverted repeat. "a" identifies the replication-proficient molecules arising from the *cis* replication pathway totally resistant to restriction enzyme "X". "b" are the *cis* replication products cut once with "X". "c" are the *cis* replication products cut twice with "X".

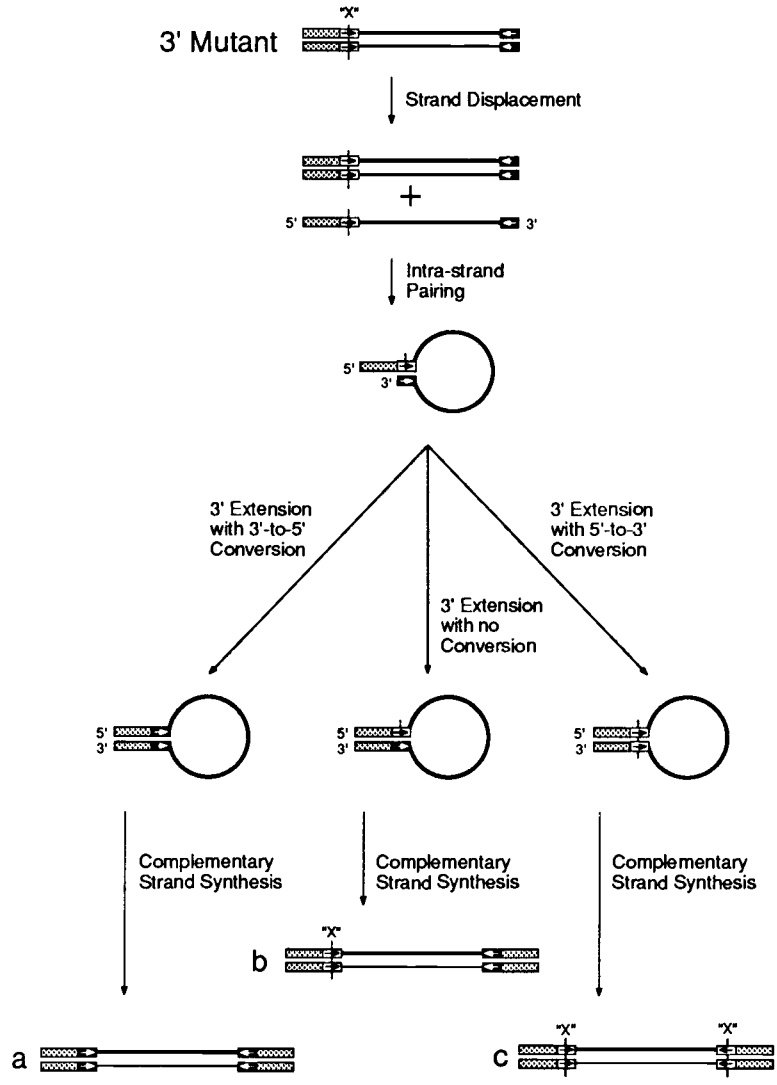

Figure III.12. Formation and fate of panhandle structures on displaced strands bearing mutations in the 3' inverted repeat sequence. See Figure III.11 for an explanation of the symbols.

"a" (uncut by enzyme X) are created as a consequence of 3'-IR to 5'-IR conversion. Molecules termed "b" (cut once by enzyme X) exhibit no conversion during *cis* replication. Molecules called "c" (cut twice by enzyme X) are produced by 5'-IR to 3'-IR sequence conversion. Figure III.13 shows the Southern blot results from transfection of pIRHin. pIRHin contains two unmutated 51-bp inverted repeats and therefore forms perfectly paired panhandles. All pIRHin *cis* molecules should be cut twice by any restriction enzyme having a site within each inverted repeat. Figure III.13 (lane 6) shows the 2.5-kb input pIRHin band, the 2.8-kb *cis* molecule, the dimer and higher oligomer bands. Lanes 1 through 5 show transfected pIRHin cut with various restriction enzymes. After digestion by *Sac*I, *Kpn*I, *Sma*I, *Xba*I, or *Bam*HI, DNA molecules from the pIRHin transfection dropped to the 2.2-kb size expected for doubly cut molecules. However, some of the molecules behaved as dimers where only terminal restriction sites were cleaved (especially noticeable with *Sac*I). One possibility is that dimers are symmetrical after end-to-end ligation of the input DNA (Perucho *et al.*, 1980), and subsequent replication of the dimer proceeds through a special case of the panhandle replicative intermediate, a fold-back structure (Hu *et al.*, 1990). A small single-stranded loop may exist at the turning point of the fold-back structure. Replication errors in this region of the fold-back might alter or eliminate some of the restriction enzyme sites in the center of the dimers. Therefore, the double-cut dimer from the 5' mutant dimer can be predicted. *Sac*I sites, closest to the center of the dimers, likely have a greater probability to be lost compared to other sites such as *Bam*HI, the furthest site from the center.

Figure III.14B shows the results from pIR5'K transfection. The displaced single strands from pIR5'K will lead to the formation of panhandle intermediate with a 4-bp deletion at 3' end of the panhandle region. This creates a 4-bp loop which divides the 51-bp polylinker panhandle into two parts of 39 bp and 8 bp (see Figure III.18). Besides the 2.5-kb input DNA molecule, dimer, and higher oligomers, the 2.8-kb *cis* replication product was detected (Figure III.14B, lane 1; marked "a"). It is clear that the 5' *Kpn*I deletion does not prevent *cis* replication. As a control, *Sac*I was used to show the double digestion of the non-mutated restriction sites within the inverted repeats (Figure III.14B, lane 2). Since the *Sac*I site is beyond the *Kpn*I site, the *Kpn*I mutation should have no effect. A faint band at position

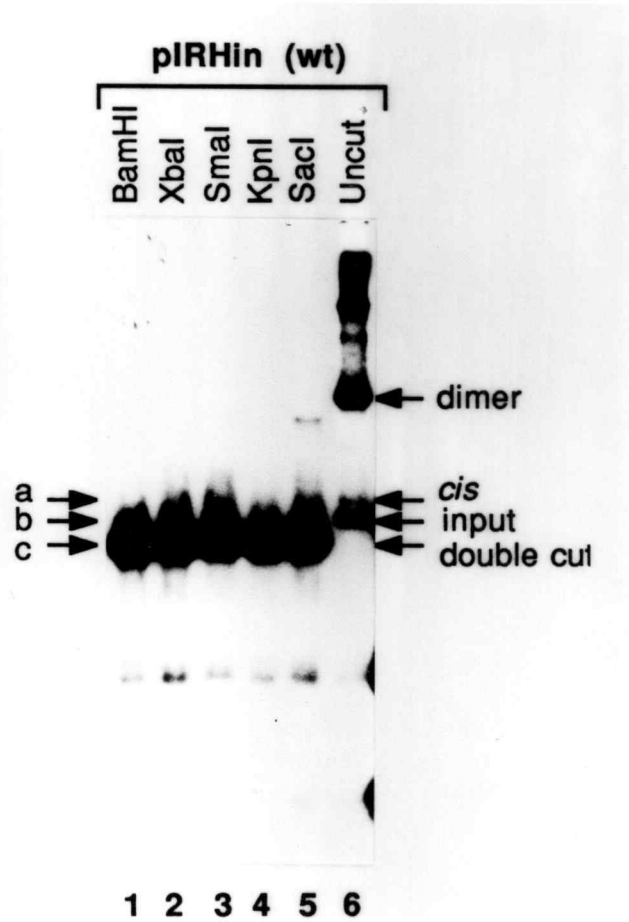

Figure III.13. Southern blot analysis of pIRHin. All of the DNA samples were cut with *Dpn*I before being loaded onto the gel. The samples were further digested with additional restriction endonucleases as indicated in each lane. "a" indicates the position of totally resistant *cis* molecules. "b" represents the position of input molecules or *cis* molecules cut once. "c" identifies the position of molecules cut twice (See also Figures III.11 and III.12).

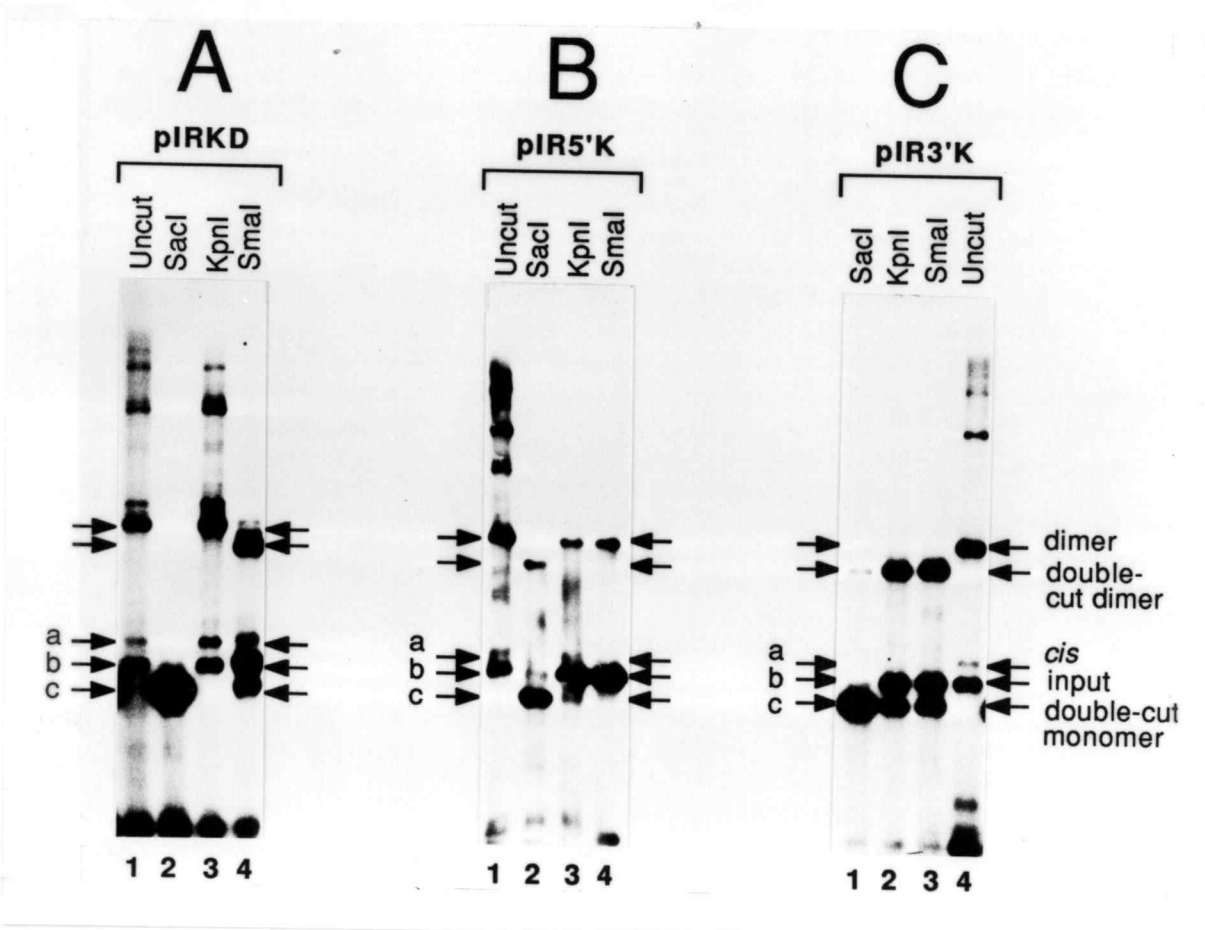

Figure III.14. Southern blot analysis of pIRKD, pIR5'K, and pIR3'K, shown respectively in A, B, and C. DNA samples were digested with *Dpn*I prior to loading onto the gel. Transfected DNA molecules were cut separately with *Sac*I, *Kpn*I, or *Sma*I as indicated in each lane. Bands labeled "a", "b", and "c" have been defined in Figure III.13.

"b" in lane 2 may represent the incomplete *Sac*I digestion. When the samples were cut by *Kpn*I (Figure III.14B, lane 3), the input monomer did not change size as expected, but oligomers dropped to the input size. However, a doubly *Kpn*I-resistant band "a" and a new doubly *Kpn*I-sensitive band "c" were also detected (Figure III.14B, lane 3). Since *Kpn*I and *Sma*I sites overlap by 2 bp, altering *Kpn*I site also mutates the *Sma*I site, thus the restriction digestion pattern after *Sma*I cleavage should mimic *Kpn*I cutting as shown in Figure III.14B, lane 4. These results strongly indicate that both 5'-IR to 3'-IR conversion and 3'-IR to 5'-IR conversion occur on pIR5'K. Figure III.14C shows the results after transfection with pIR3'K. The displaced single strands could form a panhandle with the 4-bp deletion at the 5' end of the neck region (see Figure III.19). The 3' *Kpn*I mutation also did not inhibit *cis* replication. In addition to the 2.5-kb input molecule, the dimer, and higher oligomers, the 2.8-kb *cis* molecule was also produced (Figure III.14C, lane 4). Lane 1 shows that *Sac*I dropped DNA molecules to the double-cut monomer size except for some molecules left at the double-cut dimer position. When samples were treated with *Kpn*I or *Sma*I (Figure III.14C, lanes 2 and 3), totally resistant *cis* molecules were not detected, but bands at the position of input DNA (presumably singly cut *cis* molecules) and smaller (cleaved input DNA) were seen. Although it is technically difficult to detect 5'-IR to 3'-IR conversion with the 3' mutant unambiguously, it clear that 3'-IR to 5'-IR conversion does not happen. Figure III.14A shows the results from the pIRKD transfection. Both *Kpn*I sites are deleted in pIRKD, but only the 3', not the 5', *Sma*I site is altered as the result of a cloning error. Thus, displaced strands from pIRKD could form a panhandle with a deletion loop smaller than 4 bp at 5' end of the neck region. As shown in lane 1, this deletion did not prevent the *cis* replication. Lane 2 shows that *Sac*I digested all the DNA to the position of double-cut molecules. Land 3 verifies the complete resistance to *Kpn*I cutting as expected. However, when the sample was cut by *Sma*I (Figure III.14A, lane 4, the pattern differed significantly from the expectation for a 3' *Sma*I mutant. Unlike the 3' *Kpn*I mutant (Figure III.14C), the 3' *Sma*I mutant produced totally resistant *cis* molecules. This indicates that 3'-IR to 5'-IR conversion occurs when the 3' deletion is smaller than 4 bp. A possible mechanism will be further discussed later. When the 5' *Kpn*I and 3' *Kpn*I deletions were introduced into longer inverted repeats (230 bp including the

51 bp polylinker sequences), the cleavage patterns were identical to those seen with shorter inverted repeats. Both pLIR5'K and pLIR3'K function in complementary strand synthesis and can give rise to *cis* replication products (Figures III.15A and III.15B). pIR5'S contains a deleted *Sph*I site at 5' end of the DNA molecule. The resulting 4-bp deletion loop divides the panhandle region into two parts with only 6 bp of the panhandle remaining toward to the single strand loop side and 41 bp on the other side (see Figure III.18). pIR3'S differs from the pIR5'S by having the deleted *Sph*I site at the 3' end of the DNA molecule (see Figure III.19). pIRSD has both *Sph*I sites deleted. As shown in Figure III.16, none of these mutations prevented *cis* replication. Although the *Sph*I cleavage patterns of pIRSD (Figure III.16A) and pIR3'S (Figure III.16C) resembled the *Kpn*I digests of pIRKD and pIR3'K respectively, the results with pIR5'S (Figure III.16B) differed from those with pIR5'K. In particular, no totally *Sph*I-resistant *cis* molecules were generated from pIR5'S transfections, an indication that only 3'-IR to 5'-IR conversion repairs the mismatched region on pIR5'S panhandles (see Figure III.18). Finally, another set of mutant plasmids with alterations at the *Xba*I site were constructed and tested. The *Xba*I mutations are 4-bp insertions (actually 4-bp tandem duplications) instead of deletions. pIRXD has both *Xba*I sites altered, and the *cis* product was totally resistant to cleavage by *Xba*I as expected (Figure III.17A, lane 2). The panhandle structure of pIR5'X actually resembles the panhandles of pIR3'K and pIR3'X (see Figure III.19) due to the nature of the mutation (*i. e.*, tandem duplication instead of deletion). The mutation divides the panhandle into either 27 bp and 24 bp regions or 23 bp and 28 bp regions since the 4-bp non-homology loop can move by branch migration over the tandem duplication in the panhandle. Interestingly, this mutation did not inhibit *cis* replication (Figure III.17B, lane 3). When transfected pIR5'X was analyzed by *Xba*I cleavage, doubly *Xba*I-sensitive, but no doubly *Xba*I-resistant, molecules were see (Figure III.17B, lane 2). This result suggests that only 3'-IR to 5'-IR conversion is taking place (see Figure III.19), precisely the event never seen with pIR3'K, pLIR3'K, or pIR3'S. This difference will be discussed further below.

In conclusion, the results can be summarized as follows. The displaced single strands from pIR5'K, pLIR5'K, and pIR5'S form panhandle structures depicted as "a" in Figure III.18. The displaced single strands

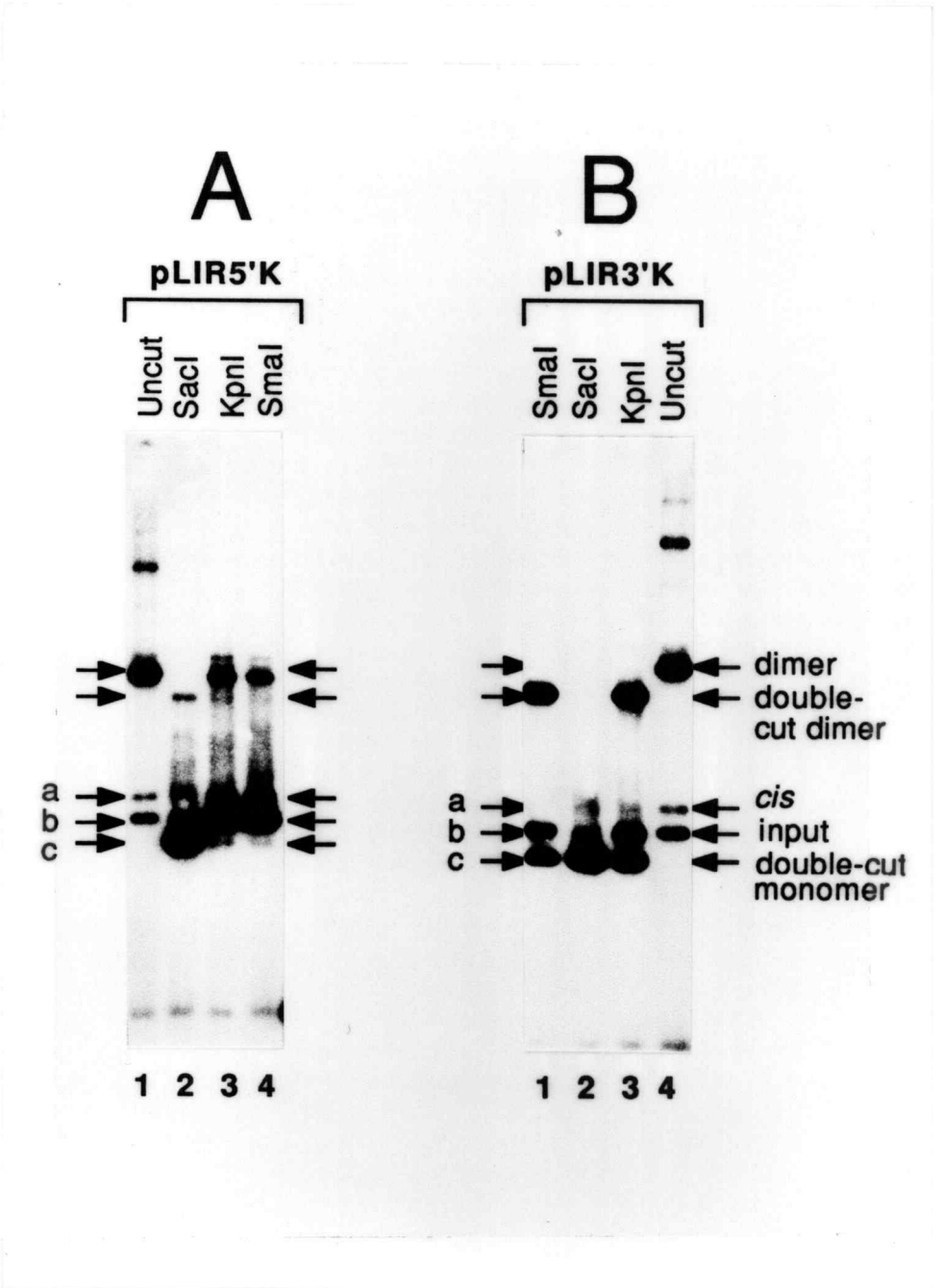

Figure III.15. Southern blot analysis of pLIR5'K and pLIR3'K, shown respectively in A and B. See Figures III.13 and III.14 for details.

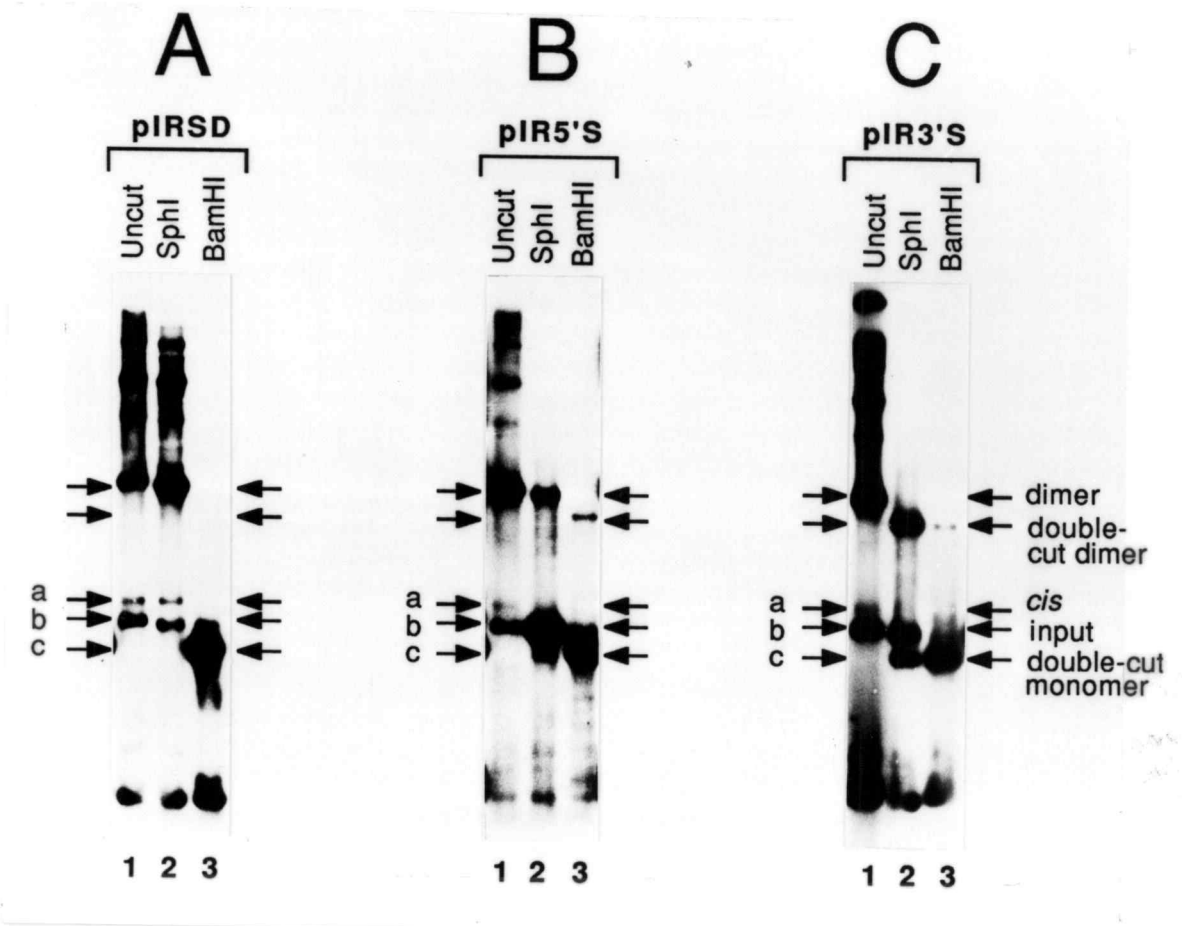

Figure III.16. Southern blot analysis of pIRSD, pIR5'S and pIR3'S, shown respectively in A, B, and C. See Figures III.13 and III.14 for details.

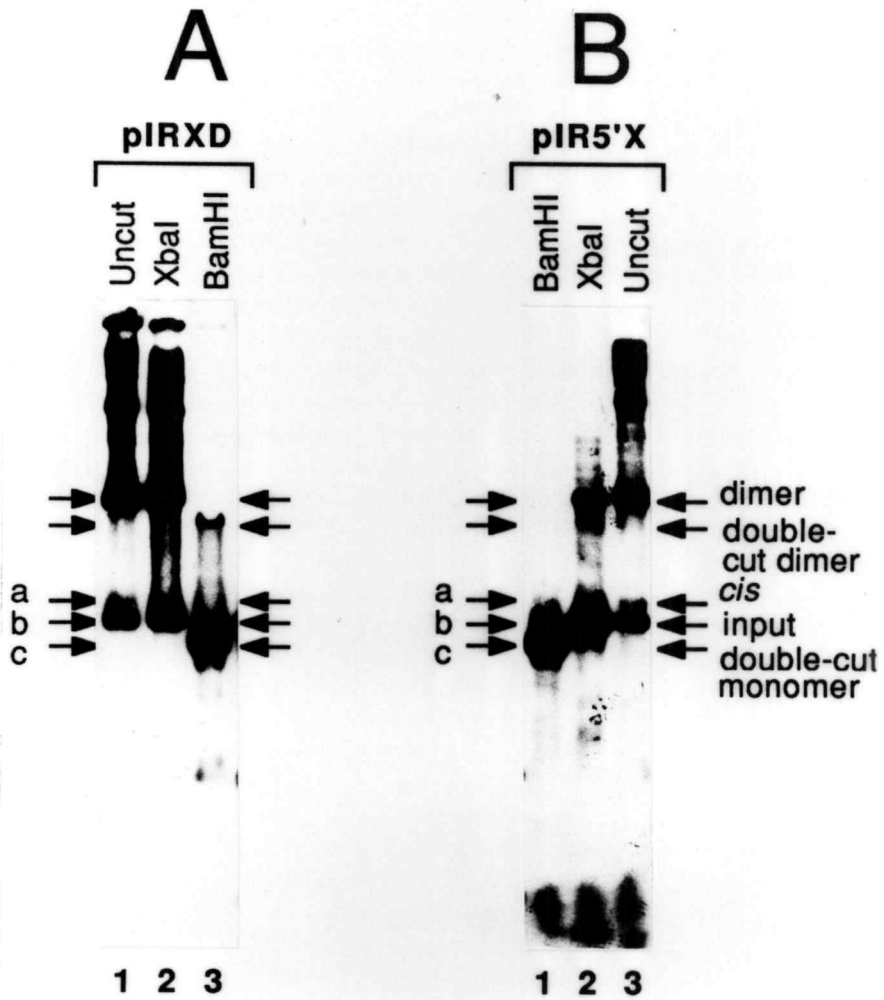

Figure III.17. Southern blot analysis of pIRXD, and pIR5'X, shown respectively in A and B. See Figures III.13 and III.14 for details.

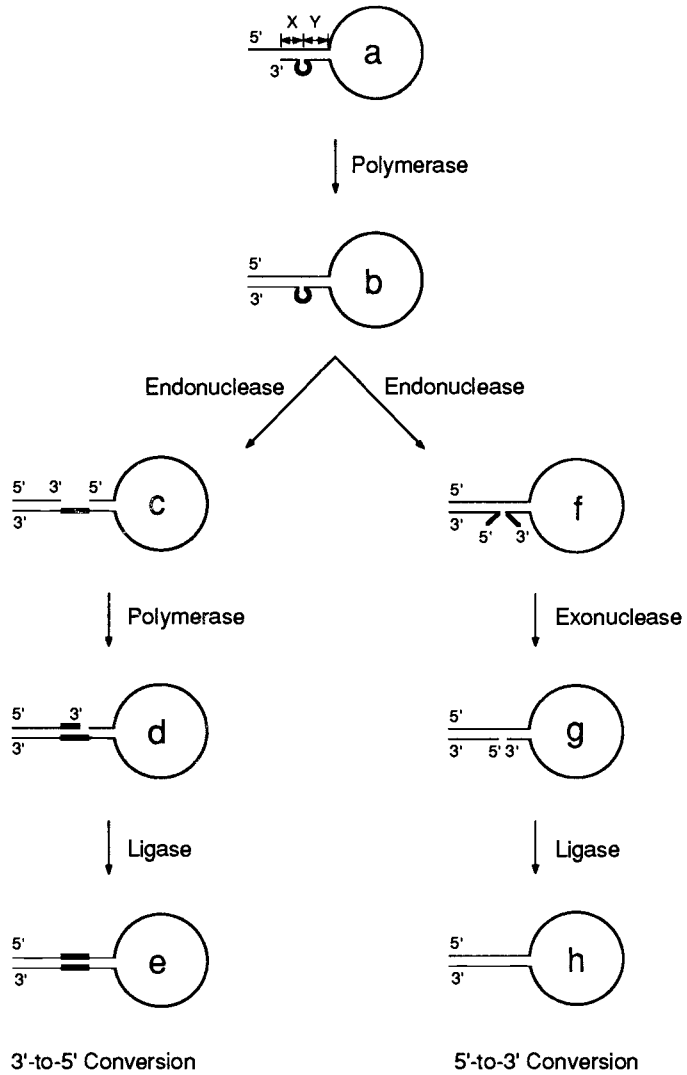

Figure III.18. Pathways to resolve a loop in the 3'-IR sequence. The pathway from a to e represents the repair of mismatched sequences by 3'-to-5' conversion which means that sequences in the 3' inverted repeat are donated to the 5' inverted repeat. The pathway from a to h represents the repair of mismatched sequences by 5'-to-3' conversion which means that sequences in the 5' inverted repeat are donated to the 3' inverted repeat.

from pIR3'K, pLIR3'K, pIR3'S, pIR5'X, and pIRKD (which is actually a 3' *Sma*I mutant) have panhandles diagramed as "a" in Figure III.19. None of these mutants inhibit *cis* DNA replication. It is clear that some, but not all, of the mismatched sequences on panhandles can be repaired. The generic pathways, called 3'-IR to 5'-IR conversion (which means that inverted repeat sequences at the 3' end of the displaced strand are used as a template to repair or correct sequences in the inverted repeat at the 5' end of the strand) and 5'-IR to 3'-IR conversion, are postulated for the repair of mismatched sequences on panhandles. Individual steps of the pathways most likely involve both viral enzymes (*e. g.*, adenovirus DNA polymerase and its associated 3'-exonuclease activity (Field *et al.*, 1984b) and cellular enzymes (*e. g.*, single-strand-specific endonuclease and DNA ligase). Both pIR5'K and pLIR5'K clearly participate in both conversion pathways shown in Figure III.18. 3'-IR to 5'-IR conversion (from a to e) requires a single-stranded cut in the strand immediately opposite the 4-base deletion loop. Subsequent repair by DNA polymerase and ligase restores *Kpn*I sites in both strands. Thus, the resulting *cis* molecule will have two *Kpn*I sites. The repair of deleted restriction endonuclease sites shows the accuracy of sequence rectification at the nucleotide level. 5'-IR to 3'-IR conversion (from a to h) can be initiated by endonucleolytic cleavage anywhere within the 4-base deletion loop. Ensuing exonucleolytic degradation of unpaired 3' and 5' tails leaves a nick which can be sealed by ligase. An alternate pathway for 5'-IR to 3'-IR conversion can also be imagined. Efficient excision of unpaired 3' tails on panhandles has already been demonstrated (see Figure III.10). Transit from f to h could therefore be accomplished by the combined action of the 3'-exonuclease activity of the adenovirus DNA polymerase to remove the unpaired 3' tail followed by strand-displacement synthesis from the now fully paired 3' primer to the end of the panhandle. The resulting *cis* product would loose both *Kpn*I sites. pIR5'S behaves differently compared to pIR5'K and pLIR5'K; no totally *Sph*I-resistant *cis* molecules are detected. This can be interpreted as a failure to undergo 5'-IR to 3'-IR conversion (from a to h). Structure g would have a panhandle only 6 bp long. Panhandle stability is not a likely explanation since pIR5'S does participate in 3'-IR to 5'-IR conversion and structure c also has a 6-bp panhandle. The short panhandle is presumably not long enough for DNA repair polymerization (panhandles must equal or exceed 31 bp for repair; see

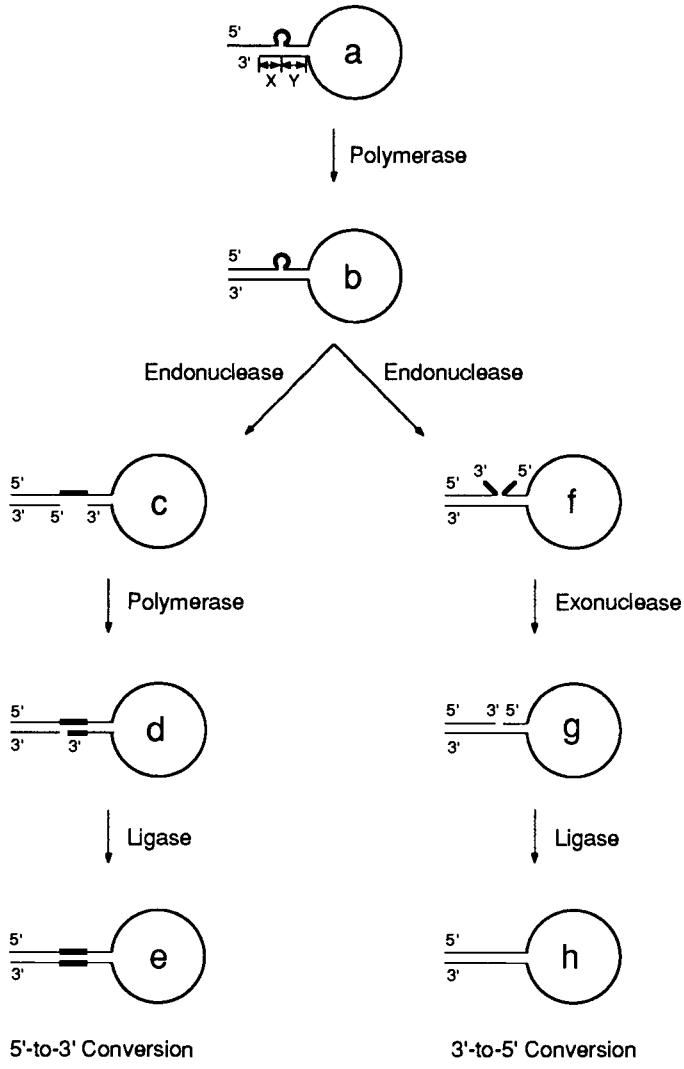

Figure III.19. Pathways to resolve a loop in the 5'-IR sequence. See Figure III.18 for details.

Figure III.7). Experiments with pIR3'K, pLIR3'K, and pIR3'S cannot be interpreted unambiguously because input molecules and *cis* molecules arising after 5'-IR to 3'IR conversion are cleaved to the same size. None of these mutants, however, appear to engage in 3'-IR to 5'-IR conversion (no totally resistant *cis* molecules) regardless of the size of the panhandle in structure f (Figure III.19, pathway a through h). One conjecture is that the 3' exonuclease of Ad pol first degrades the unpaired 3' tail on structure f, but subsequent strand-displacement synthesis simply disrupts the panhandle and linearizes the molecule without regenerating another adenovirus origin, a configuration incapable of further replication. Experiments with pIR5'X and pIRKD support this view. As discussed above, pIR5'X forms a panhandle intermediate similar to pIR3'K, pLIR3'K, and pIR3'S (Figure III.19), but unlike those plasmids it uses the 3'-IR to 5'-IR conversion pathway to generate *cis* molecules with two *Xba*I sites (Figure III.17B). Since the *Xba*I mutation is tandem duplication, some of the bases of the unpaired 5' tail in structure f can reanneal to the *Xba*I sequences on the other strand by branch migration. After excision of the unpaired 3' tail, the resulting nick on intermediate g can be sealed by ligase to yield structure h. However, pIR5'X cannot participate in 5'-IR to 3'-IR conversion since the size of the panhandle in structure c is at most only 28 bp, not long enough for DNA repair polymerization. Finally, pIRKD (actually a 3' *Sma*I mutant) proceeds through the 3'-IR to 5'-IR pathway to give totally *Sma*I-resistant *cis* progeny. The deletion loop in structure a (Figure III.19) is probably only a single nucleotide, but clearly not four bases, long. Endonucleolytic cleavage in this small unpaired region will increase the possibility that no 5' unpaired tail is produced. Thus, after excision of the unpaired 3' tail, subsequent ligation creates a perfectly double-stranded panhandle lacking *Sma*I sites.

Studies of the fate of mismatched sequences in panhandles open a window for investigating still unknown repair mechanisms operating in the eukaryotic cells. The repair of mismatched sequences on panhandles plays an important role during the adenovirus DNA replication cycle. Sequences within the adenovirus ITRs, especially the replication origins, must remain identical. From these studies, the size of the adenovirus ITRs is crucial in facilitating the repair of potentially lethal origin mutations.

CHAPTER IV
TRANS REPLICATION PATHWAY

Overview

Adenovirus complementary strand synthesis (*cis* replication), the conversion of displaced single-stranded DNA molecules into daughter duplexes, has been proposed to use panhandle replicative intermediates formed by hybridization between inverted terminal repetitions (ITRs) (Lechner and Kelly, 1977). The structure of the panhandle is identical to the ends of the duplex viral genome, and reconstitutes an origin of DNA replication. All viral initiation events, whether for strand-displacement replication or complementary strand synthesis, would therefore occur by the same mechanism. Although *cis* replication has been widely accepted as a mechanism for complementary synthesis, there are at least two alternatives to panhandle formation that could be considered in copying the displaced strand. One is that initiation may simply take place on completely single-stranded displaced adenovirus strands. There is tenuous evidence for this notion since non-adenovirus single-stranded DNA molecules, either with or without an adenovirus origin, can support the formation of pTP-dCMP complexes *in vitro* (Challberg and Rawlins, 1984; Guggenheimer *et al.*, 1984a; Tamanoi and Stillman, 1983). Another possibility is that replication initiates more or less simultaneously at both ends of the adenovirus DNA molecule. This pathway, termed fork annihilation, is diagramed in Figure I.1h. Examples of such intermediates with replication forks proceeding towards each other have been identified by electron microscopy (Lechner and Kelly, 1977). Recently, however, a novel mechanism, called *trans* replication, has been proposed (Ahern *et al.*, 1990). *Trans* replication resembles adenovirus overlap recombination (Chinnadurai *et al.*, 1979), but differs in several important ways: (a) it is driven by adenovirus-directed strand-displacement replication; (b) only one strand, the displaced strand, from each parental molecule participates in the formation of the heteroduplex intermediate; (c) the heteroduplex arises from direct intermolecular hybridization of displaced strands, so single strands need not sequentially invade a duplex to form a recombinational intermediate; (d) efficient excision of unpaired 3' ends, but not 5' ends, from the heteroduplex occurs pre-replicatively; and (e) internal non-homology

(*e.g.*, deletion or insertion loops) remains intact. Replicative intermediates formed by intermolecular hybridization of displaced strands can complete the adenovirus replication cycle without need for panhandles. The plasmids made to study *trans* replication did not contain inverted repeats and could not engage in *cis* replication (Ahern *et al.*, 1990). Just on kinetic principles alone, the unimolecular *cis* pathway might be expected to dominate the bimolecular *trans* pathway. It is interesting to determine if *cis* and *trans* replication can coexist and, if so, the relative contribution each process makes to the adenovirus DNA replication cycle. New molecules were designed to allow both *cis* and *trans* replication in the presence of appropriate partner plasmids. The replication products in each experiment could be distinguished from each other on the basis of size. As shown here, both *cis* and *trans* replication function simultaneously during adenovirus DNA replication. Furthermore, complementary strands must share a minimum length of sequence homology in order to participate in the *trans* pathway. Exactly how large a *single* sequence must be is not yet known, but it must exceed 89 bp. The *trans* pathway has never been described before, but its putative operation provides a fundamental explanation for several anomalies previously observed during adenovirus DNA replication (Bodnar and Pearson, 1980).

Construction of Plasmids

Plasmids capable of both *cis* and *trans* replication. Plasmids pBR322 (Bolivar *et al.*, 1977), 58Δ (Hay, 1985b), pMDC10 (Enns *et al.*, 1983), pIR10 (Wang and Pearson, 1985), pIR9 and KAT21 (Ahern *et al.*, 1990), and T4 and pIRHin (Chapter III) have been described previously. The procedure for constructing plasmid pIR9K is as follows. Plasmid 58Δ was cut first with *Hae*II, blunted by treatment with the Klenow fragment of *E. coli* DNA polymerase I, then cut with *Eco*RI. The small *Eco*RI-*Hae*II fragment containing the first 58 bp of the right end of adenovirus DNA was isolated and cloned into the large *Eco*RI-*Sma*I fragment of pIR9 to make plasmid pIR95. pIR95 was further digested with *Eco*RV and a blunted 1.5-kb *Eco*RI fragment from KAT21 containing the kanamycin gene was inserted to generate pIR9XS under kanamycin selection. The orientation of the insertion is such that the *Hin*dIII site of the kanamycin gene is closest to the adenovirus origin sequences. Finally, pIR9XS was cut partially with

*Hin*dIII and religated to remove the fragment containing non-origin adenovirus sequences. A new 4.7-kb construct, called pIR9K, with the orientation of the kanamycin fragment unaltered was selected. Plasmid pIR10K (4.5 kb) was made by inserting the 1.3-kb *Hin*dIII-*Sma*I kanamycin fragment from KAT21 into *Hin*dIII-*Hin*cII-digested pIRI0. pIRBH11 was built by cloning a 350-bp *Hin*dIII-*Bam*HI fragment from pBR322 into pIR10 cut with *Hin*dIII and *Bam*HI. pIRBH11 was characterized as a 3.9-kb molecule due to the insertion of two copies of the *Hin*dIII-*Bam*HI fragment in an adjacent inverted orientation. pMDC10, pIR10, pIR9K, pIR10K, and pIRBH11 are shown in Figure IV.3.

Pasmids with unpaired 3' tails during both *cis* and *trans* replication. Figure IV.1 shows the procedure for making plasmids pIRST and pLIRST. Plasmid T4 was cut separately with *Ssp*I and *Hin*dIII, *Ssp*I and *Sma*I, or *Ssp*I and *Pvu*II. The small, blunted *Ssp*I-*Hin*dIII fragment of T4 was ligated to the large *Ssp*I-*Sma*I fragment of T4. Plasmids in ampicillin-resistant colonies of *E. coli* RR1 were screened for the presence of a 390-bp *Eco*RI-*Hin*dIII fragment. The final 2.5-kb construct, named pIRST, contains 37-bp inverted repeats. Displaced single strands of pIRST would form an intermediate with a 37-bp panhandle and an unpaired 3' tail 14-bases long. A 2.8-kb plasmid, called pLIRST, was constructed by joining the small *Ssp*I-*Pvu*II fragment of T4 to the large *Ssp*I-*Sma*I fragment of T4. The correct plasmid was identified by *Hin*dIII digestion to give a 440-bp fragment. Displaced single strands of pLIRST would form an intermediate with a 230-bp panhandle and an unpaired 3' tail 14-bases long. Figure IV.2 shows the scheme for making plasmids pIRLT and pLIRLT. In brief, the 1.3-kb *Eco*RI-*Sma*I fragment bearing the kanamycin gene from KAT21 was cloned separately into *Eco*RI-*Sma*I-cleaved pIRST or pLIRST. Two plasmids, 3.8-kb pIRLT and 4.0-kb pLIRLT, were isolated under kanamycin selection and characterized. Displaced single strands of pIRLT and pLIRLT would form intermediates with a 37-bp or a 230-bp panhandle respectively, but both would have unpaired 3' tails 1300-bases long.

Results and Discussion

Intramolecular and intermolecular strand pairing operate together. In an effort to study the relative participation of *cis* and *trans* replication in the adenovirus DNA replication cycle, plasmid molecules were constructed

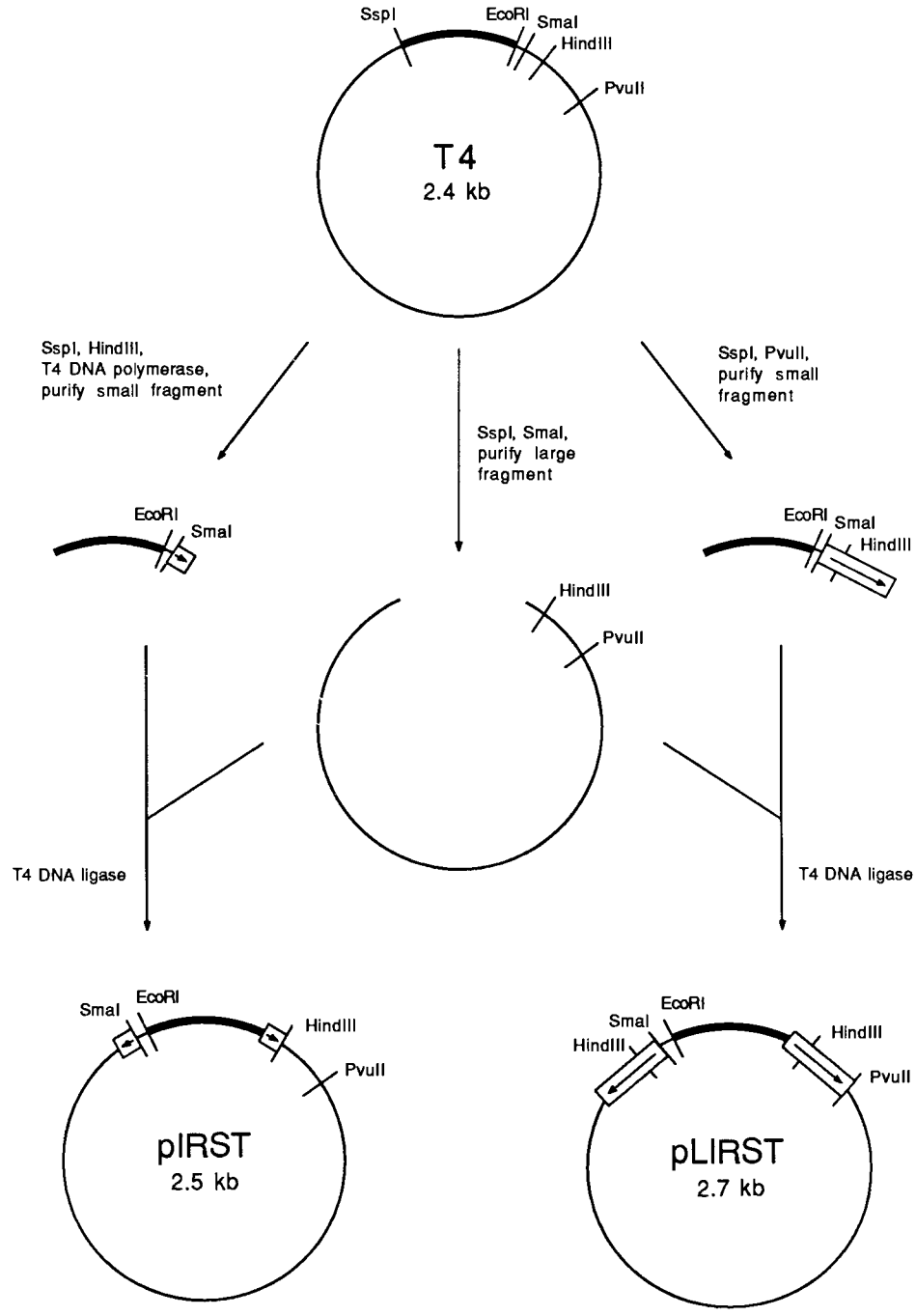

Figure IV.1. Construction of pIRST and pLIRST. The thin lines represent plasmid vector sequences. The thick black lines represent adenovirus sequences containing the viral DNA replication origin. The small open boxes are 89-bp inverted repeats, the larger open boxes are 230-bp inverted repeats. The arrows indicate the orientation of the inverted repeats.

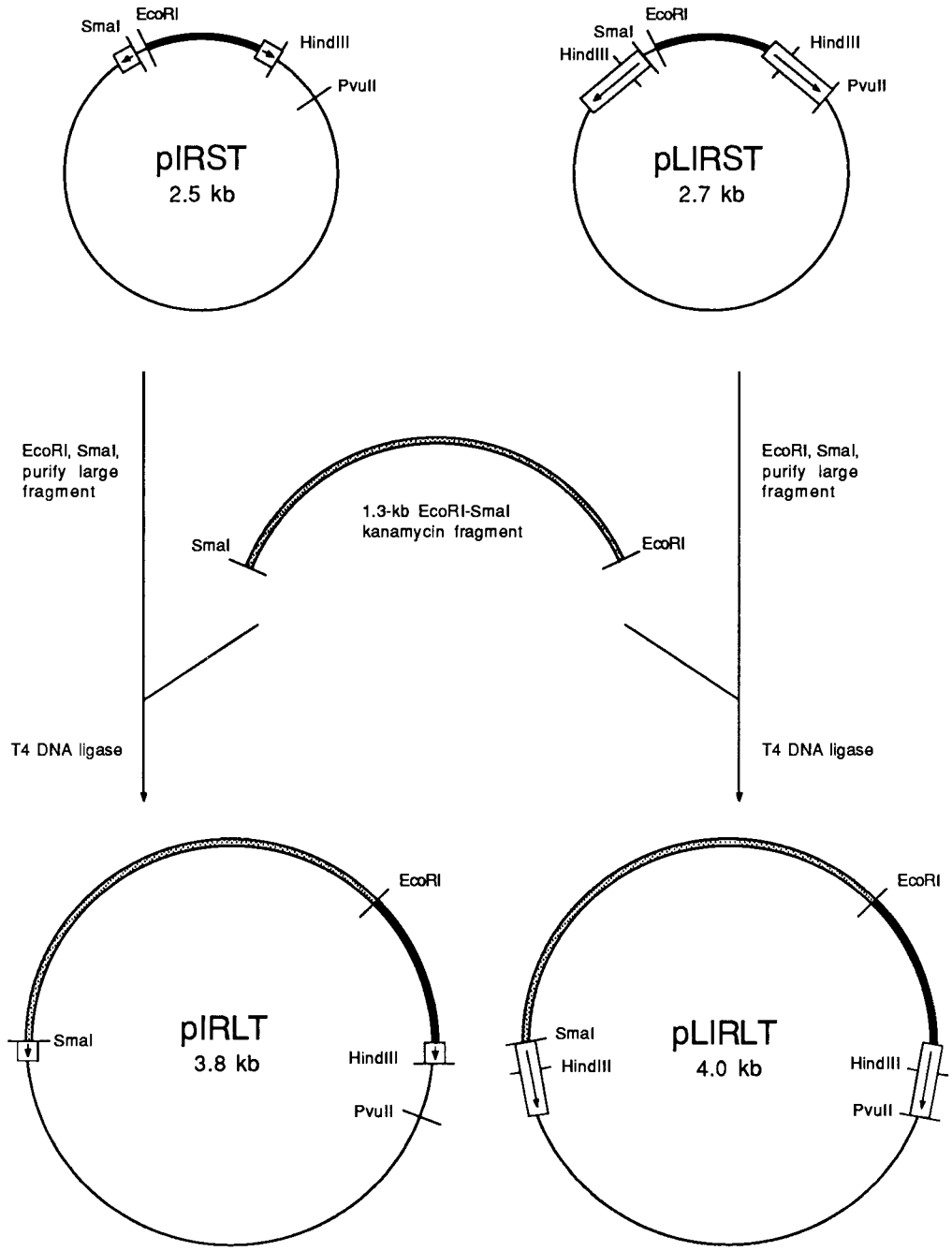

Figure IV.2. Construction of pIRLT and pLIRLT. Symbols are as described in Figure IV.1 except that the thick stippled line represents sequences containing the gene conferring resistance to kanamycin.

containing a single adenovirus origin and sequences capable of pairing both within and between displaced single strands to form either panhandle-like molecules or partial heteroduplexes. Figure IV.3 displays the structures of 3.2-kb pIR10 and 4.7-kb pIR9K. The two plasmids share identical vector sequences and two copies of 89-bp inverted repeats, but the sizes of DNA sequences inserted between the inverted repeats is different and the adenovirus replication origin is in an opposite direction relative to the vector sequences in each plasmid. When transfected individually, *Eco*RI-linearized pIR9K and pIR10 each gave rise to *Dpn*I-resistant DNA in the presence of helper adenovirus (Figure IV.4, lanes 2 and 5), but not in the absence of helper (Figure IV.4, lanes 1 and 4). Besides the input, dimer, and higher oligomer bands, *cis* replication products with sizes of 6.6-kb and 3.6-kb were detected (Figure IV.4, lanes 2 and 5). When pIR10 and pIR9K were transfected together with helper, in addition to the collection of all *Dpn*I-resistant bands previously found in the single transfections, a novel 5.1-kb DNA band, referred to as a *trans* molecule, was also detected (Figure IV.4, lane 3). The experimental results can be interpreted by the participation of replication pathways involving both intramolecular and intermolecular hybridization of displaced single strands after adenovirus-directed specific plasmid DNA replication (Figure IV.5). Since both pIR10 and pIR9K carry inverted repeat sequences themselves, each of them is capable of producing 3.6-kb or 6.6-kb *cis* molecules respectively by just pairing between the 89-bp inverted sequences within the displaced single strands. Because the replication of pIR10 and pIR9K also proceed in opposite directions, the displaced single strands from each are therefore able to undergo cross-hybridization between complementary sequences extending over 2.8-kb. After DNA repair to regenerate double-stranded replication origins at both ends of the heteroduplex intermediate, a 5.1-kb replication-proficient *trans* molecule is created. Figure IV.4, lane 3 also shows that the *trans* band and both of the *cis* bands from pIR10 and pIR9K have roughly equal intensities. This is the first direct evidence that both *cis* and *trans* replication pathways can operate simultaneously, and that *trans* replication may proceed as efficiently as *cis* replication.

Two copies of 89-bp are sufficient for intermolecular strand pairing. In order to determine if inverted repeats play a role during *trans* replication, experiments with pIR10K and pIR10 were carried out. The

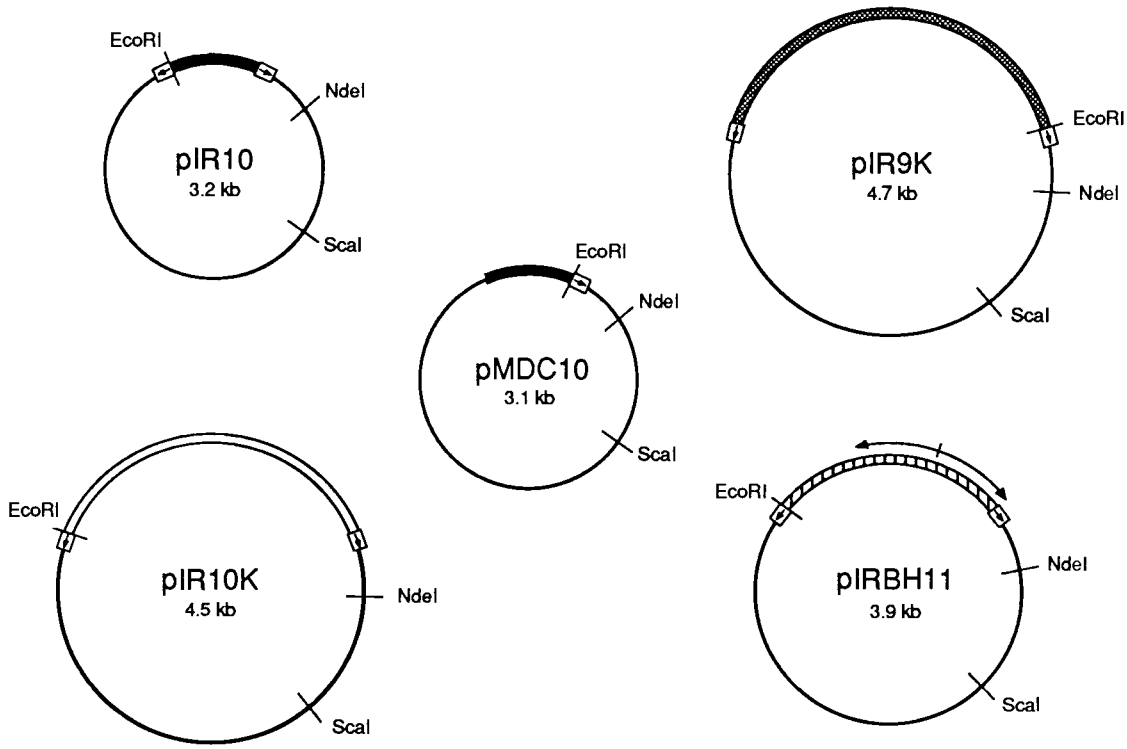

Figure IV.3. Structures of pIR10, pIR9K, pIR10K, pIRBH11, and pMDC10. The thin line represents plasmid vector sequences. The small open boxes are 89-bp inverted repeats where the arrows indicate the orientation. The thick lines (black, stippled, hatched, and open) specify regions of variable length and composition inserted between the inverted repeats. These regions also contain the adenovirus DNA replication origin located immediately adjacent to the *Eco*RI site on each plasmid. The origins are oriented to direct replication clockwise on pIR10, pIR10K, and pIRBH11, but counterclockwise on pIR9K and pMDC10. pIRBH11 has a large palindromic sequence shown by the double-headed arrow. Plasmids are drawn to scale.

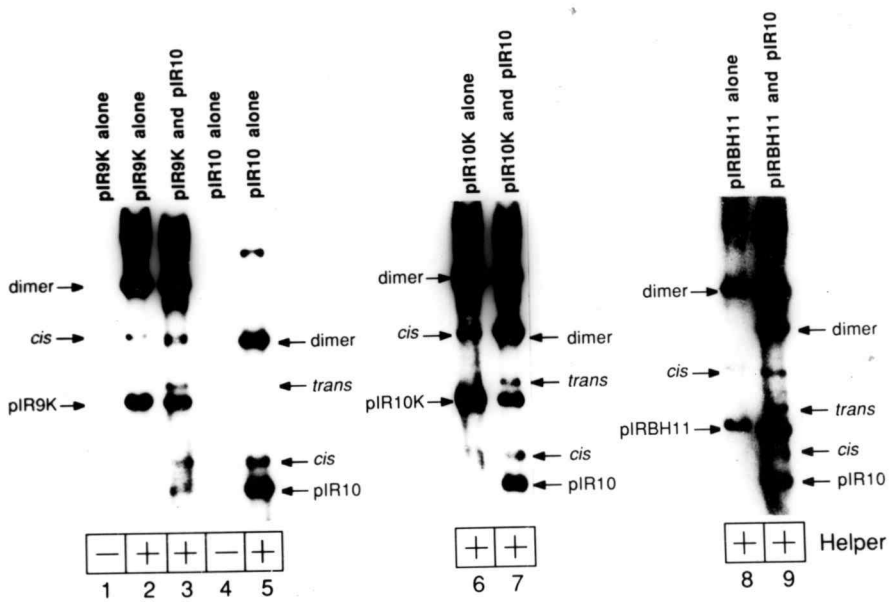

Figure IV.4. Detection of molecules generated by *cis* and *trans* pathways. All input plasmids were linearized by *Eco*RI cleavage. DNA was isolated 60 hours after transfection with (lanes 2, 3, 5-9) or without (lanes 1 and 4) helper adenovirus DNA, digested with *Dpn*I, and analyzed by Southern blot hybridization. Arrows designate the positions of input plasmids, dimers of input plasmids, and the products of the *cis* and *trans* pathways. Lanes 1 and 2: pIR9K alone. Lane 3: pIR9K and pIR10. Lanes 4 and 5: pIR10 alone. Lane 6: pIR10K alone. Lane 7: pIR10K and pIR10. Lane 8: pIRBH11 alone. Lane 9: pIRBH11 and pIR10.

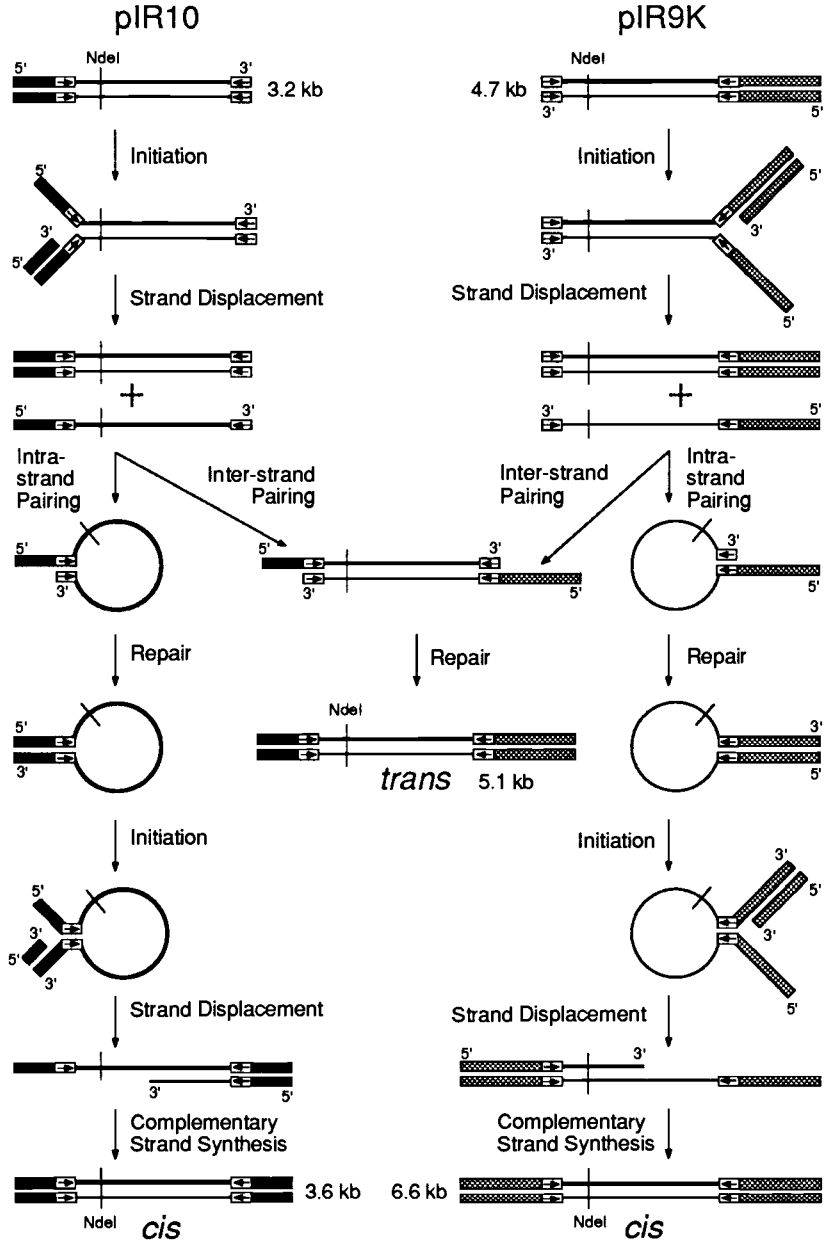

Figure IV.5. Diagram of the *cis* and *trans* pathways for pIR10 and pIR9K. Symbols are as described in Figure IV.1, except that thick and thin lines represent complementary strands. Each plasmid alone can participate in the *cis* pathway. Inverted repetitious sequences on displaced strands anneal intramolecularly to form a panhandle structure. Extension of the paired 3' end by DNA synthesis repairs the adenovirus origin. Subsequent adenovirus-specific replication of the repaired panhandle intermediate produces a *cis* molecule. The sizes of the *cis* molecules expected from pIR10 and pIR9K are 3.6 kb and 6.6 kb, respectively. Displaced strands can simply hybridize to form a heteroduplex intermediate. Extension of the paired 3' ends by DNA polymerization creates a "recombinant" molecule, called a *trans* molecule, 5.1-kb in size.

4.5-kb pIR10K (Figure IV.3) contains the same vector backbone, inverted repeats, and orientation of the adenovirus replication origin as pIR10 does. pIR10K differs from pIR10 only by having a larger size of DNA inserted between the inverted repeats. When *Eco*RI-linearized pIR10K was transfected into cells alone with helper, *Dpn*I-resistant input, dimer, higher oligomers, and a 6.2-kb *cis* product were detected (Figure IV.4, lane 6). However, cotransfection of pIR10K with pIR10 produced not only all of the *Dpn*I-resistant products expected from a single-plasmid transfection, but also generated a new 4.9-kb *trans* band (Figure IV.4, lane 7). This result can be understood as the consequences of intramolecular and intermolecular hybridization events exclusively involving the two copies of 89-bp inverted repeat (Figure IV.6). Although pIR10 and pIR10K are both capable of pairing between inverted repeats within their own displaced single strands to give *cis* products, only the route leading to the formation of the *trans* molecule is diagramed. As indicated in Figure IV.6, the only way for *trans* replication to happen is by intermolecular hybridization between the two copies of the 89-bp inverted repeat sequences in the displaced single strands from pIR10 and pIR10K. Since both pIR10 and pIR10K replicate in the same direction, all other vector sequences in the displaced strands are identical, not complementary. The resulting *trans* product probably consists of two different, but identically sized, molecules. It is worth noting that the intensities of the *trans* bands generated from pIR10 and pIR9K (Figure IV.4, lane 3) and pIR10 and pIR10K (Figure IV.4, lane 7) are approximately equal despite the large differences in the length of complementary sequences shared between partner plasmids; *i. e.*, 2.8 kb versus two copies of 89 bp. This supports the conclusion that homologous recombination, which depends greatly on the length of homology, does not play a major role in *trans* replication (Ahern *et al.*, 1990). It is surprising that as little as two copies of 89 bp support *trans* replication. This may indicate that not only entire complementary adenovirus displaced single strands, but adenovirus ITRs as well, may be involved in *trans* replication, and this makes it hard to distinguish *cis* and *trans* adenovirus DNA replication experimentally.

Large palindromic sequences are stably replicated. *Trans* molecules can be formed by intermolecular hybridization between two copies of 89 bp. To document further the role of inverted repeats in *cis* and *trans* replication, a new plasmid with two extra copies of inverted DNA sequences has been

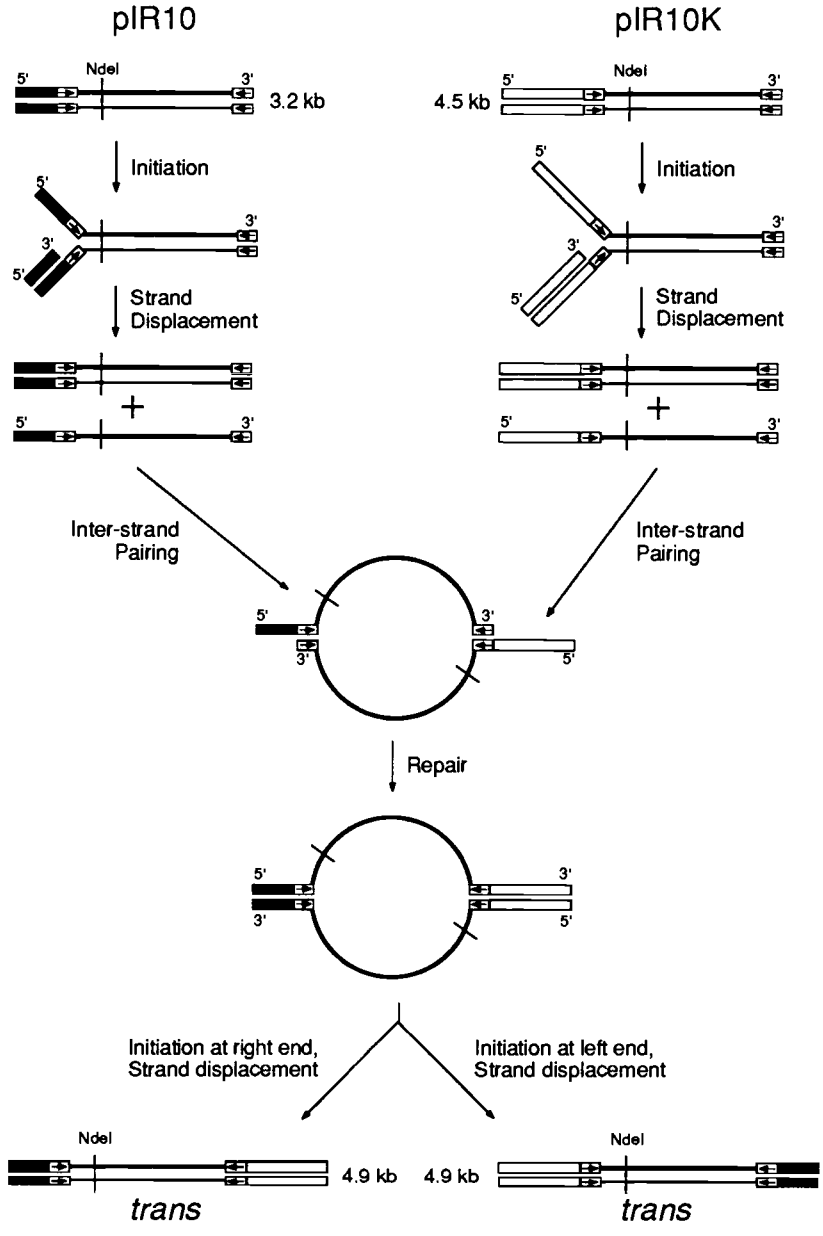

Figure IV.6. Diagram of the *trans* pathway for pIR10 and pIR10K. Although not shown in this diagram, each plasmid alone can participate in the *cis* pathway. The sizes of the *cis* molecules expected from pIR10 and pIR10K are 3.6 kb and 6.2 kb, respectively. A heteroduplex intermediate with displaced strands from the two plasmids can only form between the 89-bp inverted repeats. Extension of the paired 3' ends by DNA polymerization regenerates adenovirus origins at both ends of the heteroduplex. Subsequent rounds of viral-specific replication segregates two *trans* molecules of identical size (4.9 kb) but different sequence arrangement.

tested. pIRBH11 (Figure IV.3) differs from pIR10 only by containing two adjacent, but inverted, copies of a 350-bp sequence to create a 700-bp palindrome. When pIRBH11 was transfected alone with helper, a 5.0-kb *cis* molecule was readily formed (Figure IV.4, lane 8). The predicted intermediate used in *cis* replication is described in Figure IV.7A. When pIRBH11 and pIR10 were cotransfected, both 5.0-kb and 3.6-kb *cis* products from the two respective inputs as well as a 4.3-kb *trans* molecule were detected (Figure IV.4, lane 9). The putative *trans* intermediate involves intermolecular hybidization between only the 89-bp inverted repeats of pIRBH11 and pIR10 (Figure IV.7B). Thus, the *trans* molecule is most likely two 4.3-kb molecules, each having distinct structures (see Figure IV.6). The results clearly indicate that the additional secondary structure from the large palindrome adjacent to the panhandle stem does not prevent the formation or repair of pIRBH11 *cis* and *trans* intermediates, and large palindromes are surprisingly stable during subsequent rounds of DNA replication.

One copy of 89 bp is not sufficient for intermolecular strand pairing. pMDC10 (Figure IV.3) lacks inverted repeats, and therefore cannot generate a *cis* molecule (Figure IV.8, lane 1). When pMDC10 and pIR9K were cotransfected into cells, the 6.6-kb *cis* product expected from pIR9K was readily detected, but no new band corresponding to a *trans* molecule was found (Figure IV.8, lane 3). This rules out intermolecular hybridization between the displaced pMDC10 strand and either the displaced pIR9K strand or the displaced strand of the pIR9K *cis* product (Figure IV.9). It also eliminates homologous recombination between input pMDC10 and the pIR9K *cis* product as a mechanism to form the *trans* molecule. As a positive control, pIR10K transfected together with pMDC10, where the two displaced strands again share more than 2,000 bp of homology, yielded the expected 4.8-kb *trans* molecule and the 6.2-kb pIR10K *cis* product (Figure IV.8, lane 4). The *trans* pathway between pMDC10 and pIR10K, but not the pIR10K *cis* pathway, is diagramed in Figure IV.10. We do not yet know how large a *single* sequence must be in order to trigger the *trans* pathway, but it must exceed 89 bp. These experiments provide the first hint that intermolecular hybridization of displaced strands is at least sequential (or concentration-dependent) as predicted for a bimolecular reaction. The two heteroduplex intermediates (one with strands from pMDC10 and pIR10K,

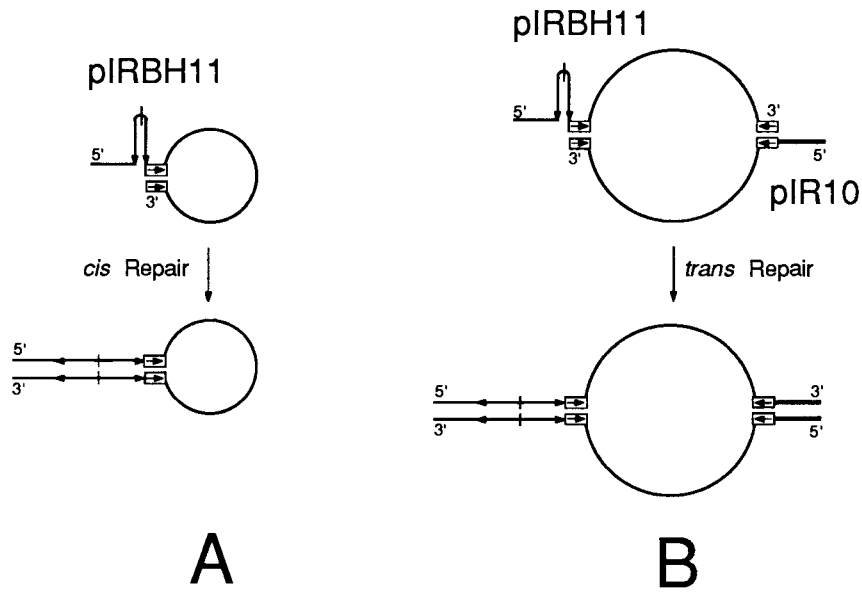

Figure IV.7. Intermediate structures in the *cis* and *trans* pathways involving pIRBH11. (A) Panhandle intermediate in the *cis* pathway showing the secondary structure from the large palindromic sequence in pIRBH11. (B) Heteroduplex intermediate between pIR10 and pIRBH11 displaced strands. The heteroduplex is held together by base-pairing between the 89-bp inverted repeats. The secondary structure from the large palindromic sequence in pIRBH11 is also shown. Subsequent rounds of viral-specific replication segregates two *trans* molecules with identical size (5.0 kb), but different sequence arrangement.

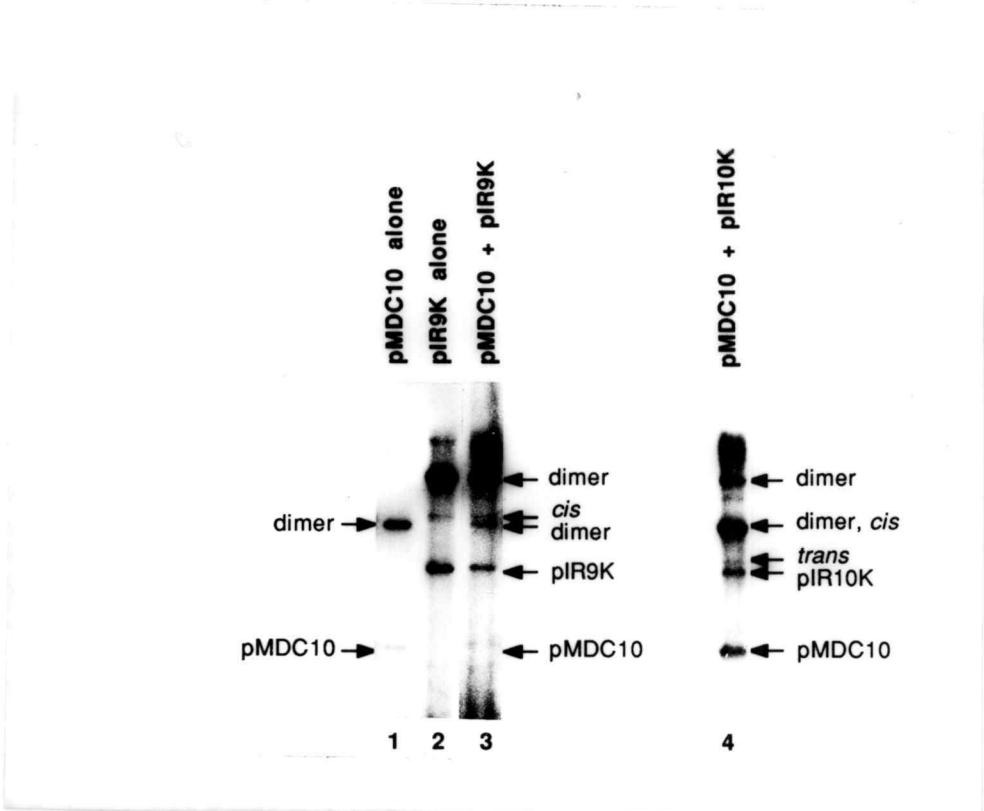

Figure IV.8. Transfections involving pMDC10, pIR9K, and pIR10K. DNA was isolated 60 hours after transfection with helper adenovirus DNA, digested with *Dpn*I, and analyzed by Southern blot hybridization. Arrows designate the positions of input plasmids, dimers of input plasmids, and the products of the *cis* and *trans* pathways. Lane 1: pMDC10 alone. Lane 2: pIR9K alone. Lane 3: pIR9K and pMDC10. Lane 4 : pIR10K and pMDC10.

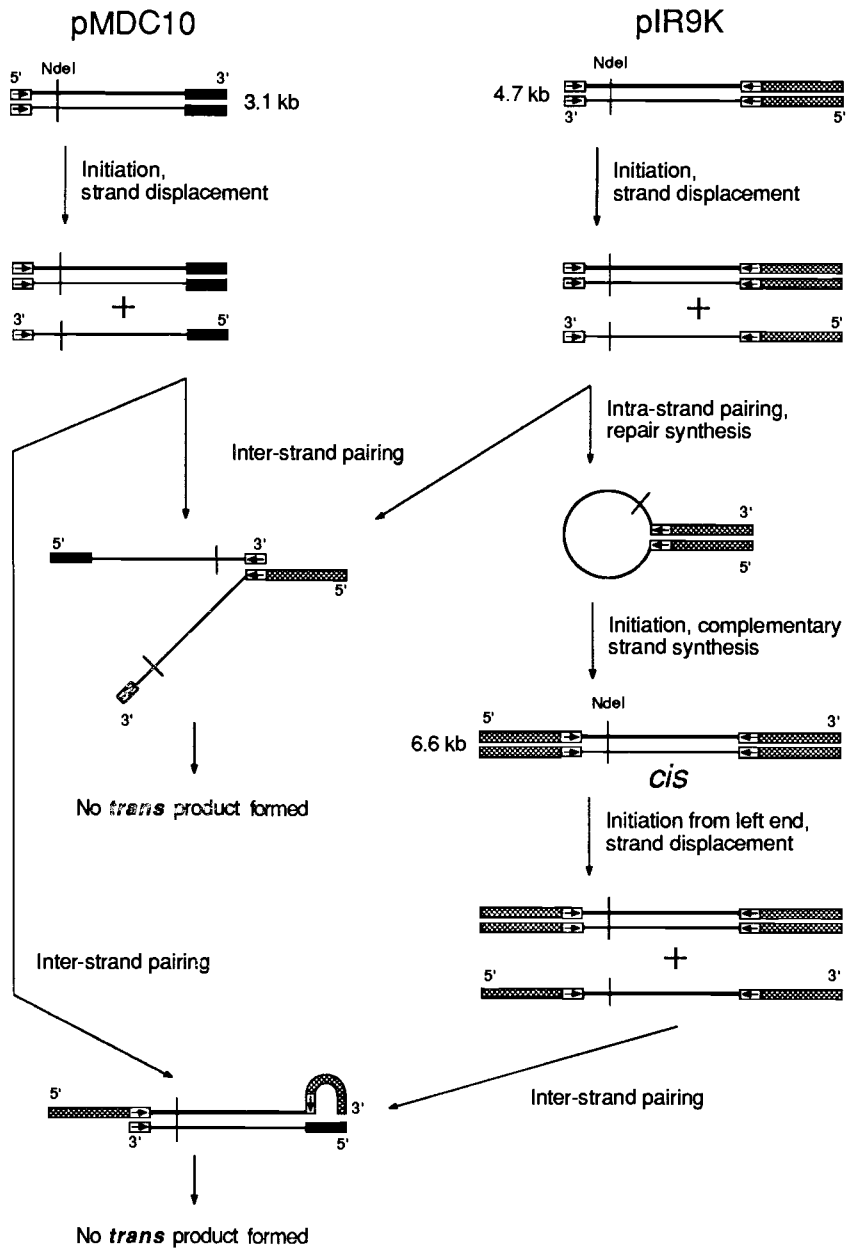

Figure IV.9. Interactions between strands of pMDC10 and pIR9K. pIR9K can participate in the *cis* pathway to form a 6.6-kb molecule. pMDC10 lacks inverted repeats and therefore is not able to form a panhandle intermediate in the *cis* pathway. Since no *trans* molecules were detected (see Figure IV.8), 89 bp apparently cannot specify intermolecular hybridization between displaced strands of pMDC10 and pIR9K. Base-pairing between the displaced strand of pMDC10 and strands from the pIR9K *cis* molecule also did not appear to occur.

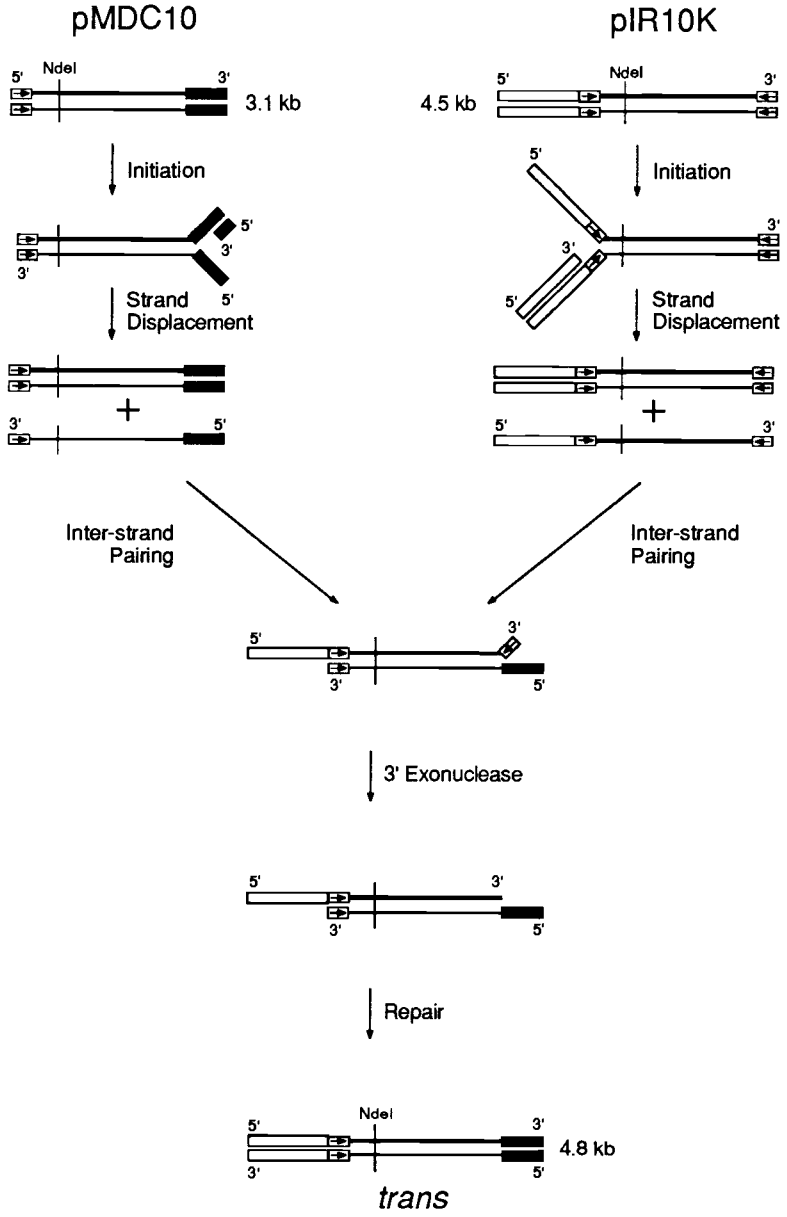

Figure IV.10. Interactions between strands of pMDC10 and pIR10K. Although not shown, pIR10K can participate in the *cis* pathway to form a 6.2-kb molecule. pMDC10 lacks inverted repeats and therefore is not able to form a panhandle intermediate in the *cis* pathway. Intermolecular hybridization between displaced strands of pMDC10 and pIR10K form a heteroduplex intermediate. Repair of the intermediate creates a 4.8-kb *trans* molecule.

the other with strands from pMDC10 and the *cis* product of pIR9K) are nearly identical in structure. The first yielded a *trans* molecule; the second did not, presumably because the pIR9K *cis* molecule did not accumulate sufficiently during the experiment.

Sequence rectification occurs in intramolecular and intermolecular pathways. We could show directly that the removal of unpaired 3' sequences in the heteroduplex intermediate is not a limiting factor in the *trans* pathway. Figure IV.11 displays the structures of plasmids T4 (2.4 kb) and pLIRLT (4.0 kb). Like pMDC10, plasmid T4 lacks inverted repeats and cannot participate in the *cis* pathway (Figure IV.12, lane 1). pLIRLT has inverted repeats of 220 bp and gives rise to a *cis* product 1.0 kb smaller than the input molecule (Figure IV.12, lane 2). When both T4 and pLIRLT are transfected together, all of the *Dpn*I-resistant bands in the individual experiments were found in addition to a 2.8-kb *trans* band (Figure IV.12, lane 3). Figure IV.13 summarizes the molecular transactions occurring after cotransfection with T4 and pLIRLT. The formation of the smaller *cis* product involves the removal of 1.4-kb unpaired sequences at the 3' end of the displaced strand of pLIRLT coupled with the repair of 400 bp of origin sequences on the panhandle intermediate. The displaced strand of T4 is complementary with 2.0 kb of the displaced strand of pLIRLT. The heteroduplex intermediate still has 1.6 kb of unpaired sequences at the 3' end of the pLIRLT strand. The size of the observed *trans* molecule suggests that these unpaired sequences are readily lost. Sequence rectification during *cis* and *trans* replication thus appear to be generally similar.

The *trans* pathway has never been described before, but its putative operation provides a fundamental explanation for several anomalies previously observed during adenovirus DNA replication (Bodnar and Pearson, 1980). Figure IV.14 diagrams the *cis* and *trans* pathways during adenovirus DNA replication. Early in infection, when there are few genomes per cell and the concentration of displaced single strands is low, *cis* replication prevails and molecules are synthesized semi-conservatively (Pearson and Hanawalt, 1971). Later during the cycle, as viral DNA accumulates significantly in the infected cells and the concentration of displaced single strands is high, *trans* replication begins to dominate. The *trans* pathway thus acts as a short-circuit in semi-conservative DNA replication (Bodnar and Pearson, 1980; D'Halluin and Milleville, 1984).

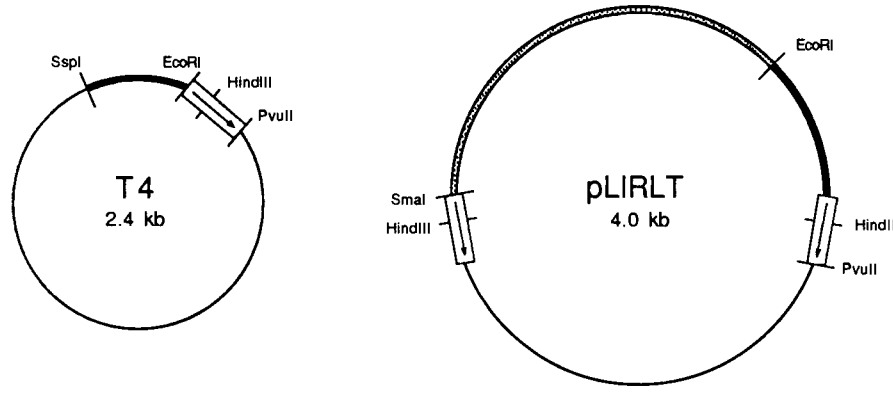

Figure IV.11. Structures of plasmids T4 and pLIRLT. Symbols are as described in Figure IV.1. Inverted repeats (230-bp) are open boxes where arrows indicate orientation. The thick stippled line represents sequences containing the gene conferring resistance to kanamycin. Plasmids are to scale.

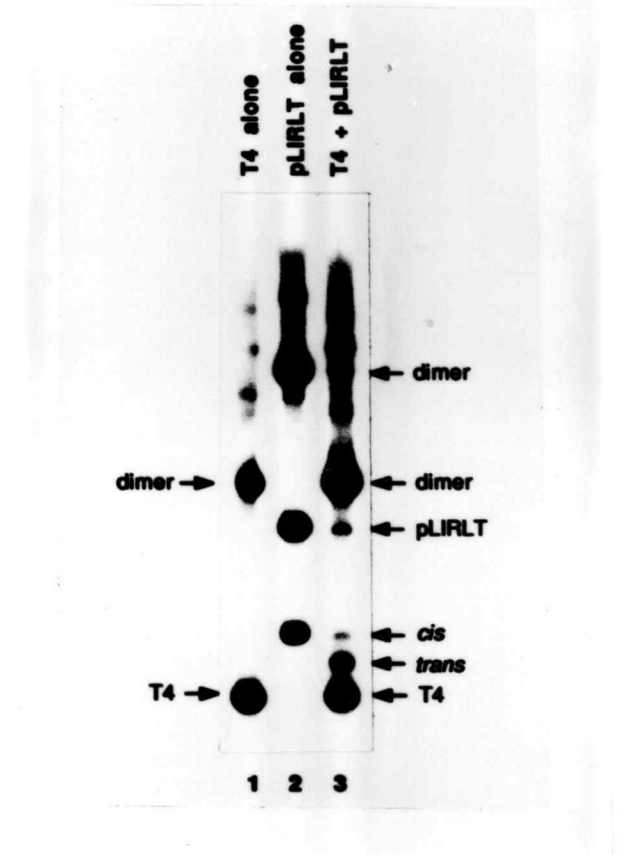

Figure IV.12. Transfections involving plasmids T4 and pLIRLT. DNA was isolated 60 hours after transfection with helper adenovirus DNA, digested with *Dpn*I, and analyzed by Southern blot hybridization. Arrows designate the positions of input plasmids, dimers of input plasmids, and the products of the *cis* and *trans* pathways. Lane 1: Plasmid T4 alone. Lane 2: pLIRLT alone. Lane 3: pLIRLT and plasmid T4.

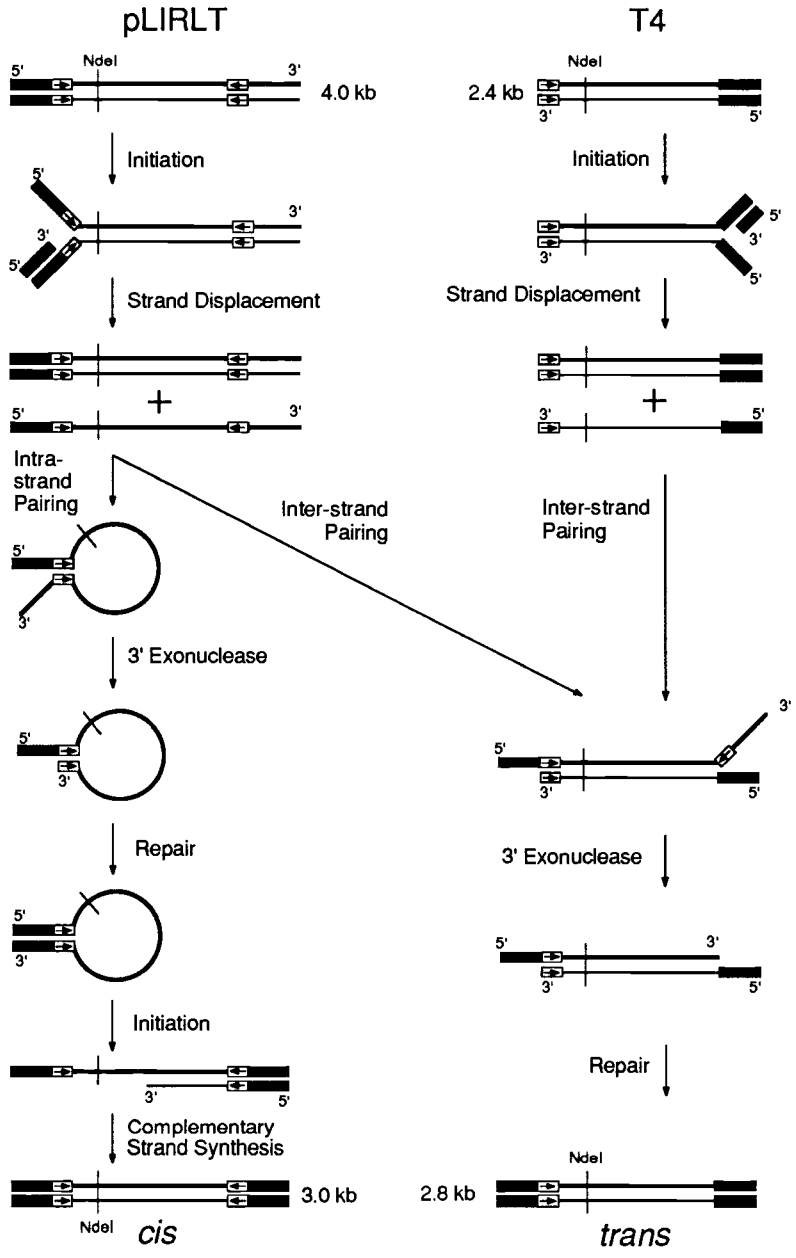

Figure IV.13. Interactions between strands of plasmids T4 and pLIRLT. pLIRLT can participate in the *cis* pathway to form a 3.0-kb molecule. A 1.4-kb unpaired tail must be removed exonucleolytically from the panhandle intermediate. Plasmid T4 lacks inverted repeats and therefore is not able to form a panhandle intermediate in the *cis* pathway. Intermolecular hybridization between displaced strands of pLIRLT and plasmid T4 form a heteroduplex intermediate. Repair of the intermediate, including removal of 1.6-kb unpaired sequences at the 3' end of the pLIRLT strand, creates a 2.8-kb *trans* molecule.

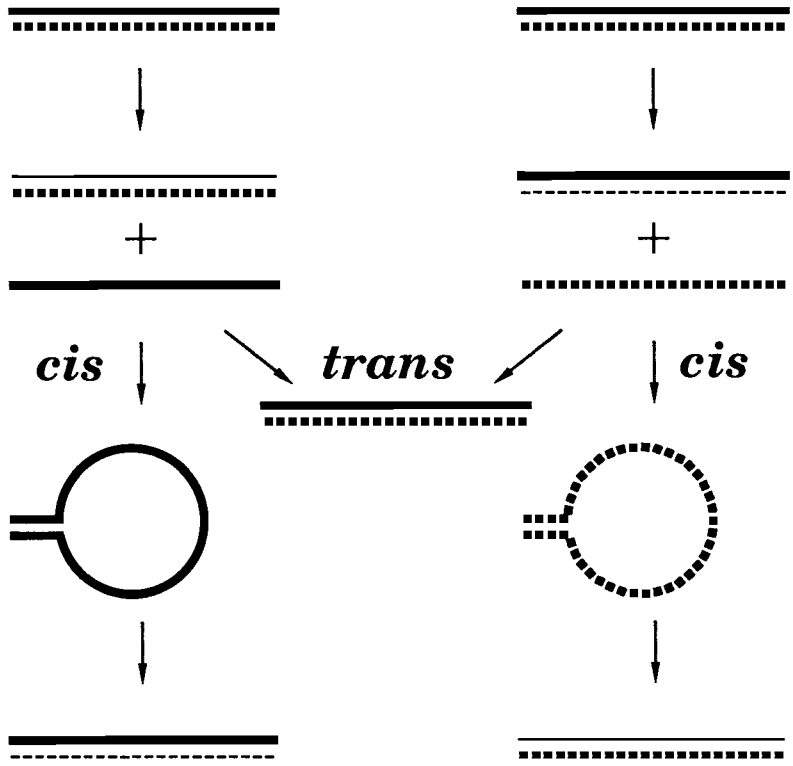

Figure IV.14. Schematic diagram of the *cis* and *trans* replication pathways of adenovirus. Complementary parental strands are shown as thick solid and thick dashed lines. Rightward displacement replication from the left origin displaces a thick, solid parental strand and yields a progeny molecule with a thin, solid daughter strand and a thick, dashed parental strand. Replication of the displaced strand by the *cis* pathway generates a progeny molecule with a thick, solid parental strand and a thin, dashed daughter strand. Leftward displacement replication from the right origin displaces a thick, dashed parental strand and yields a progeny molecule with a thin, dashed daughter strand and a thick, solid parental strand. Replication of the displaced strand by the *cis* pathway generates a progeny molecule with a thick, dashed parental strand and a thin, solid daughter strand. Semi-conservative DNA replication results in either case. If, however, displaced complementary strands can simply reanneal, the resulting duplex molecules will contain only parental strands. Although such reannealed molecules have actually replicated, they "look" like unreplicated parental molecules. *Trans* replication thus acts as a short-circuit in semi-conservative DNA replication. This short-circuit has been observed experimentally late in adenovirus infection.

Fully heavy molecules do not "appear" to be lost during density-shift experiments since heavy displaced strands simply reanneal to form fully heavy molecules again (Figure IV.14). Consequently, the CsCl gradient profiles exhibit abnormal density-shift patterns (Bodnar and Pearson, 1980). *Trans* replication also affects the accumulation of viral DNA in infected cells. During semi-conservative synthesis, one DNA molecule becomes two (or two DNA molecules become four), whereas in the *trans* pathway two DNA molecules yield three. In fact, the accumulation of viral DNA deviates from the initial logarithmic rate exactly at the time the *trans* pathway begins to predominate and the density-shift patterns become aberrant (Bodnar and Pearson, 1980).

Bacteriophage ϕ29, like adenovirus, has a linear DNA molecule with a terminal protein covalently attached to the 5' end of each strand, and it replicates with a strand displacement mechanism (Gutiérrez *et al.*, 1988; Serrano *et al.*, 1989). However, it has an ITR of only 6 bp, which is much shorter than the adenovirus ITR, and also smaller than the minimum number of 31 bp required for *cis* replication. Therefore, it seems that other replication mechanisms, instead of panhandle formation, may be used in ϕ29 replication. Obviously, *trans* replication, which involves interstrand hybridization between complementary strands and does not require a panhandle intermediate, might be used for bacteriophage ϕ29 DNA replication.

CHAPTER V
CONCLUSIONS

Adenovirus DNA replication has been studied intensively both *in vitro* and *in vivo*. Experiments using the *in vitro* replication system have provided information largely about sequences required for the adenovirus origin and initiation and elongation of strand-displacement replication, which is the first phase of adenovirus DNA replication. Knowledge of complementary strand synthesis has remained very limited due to the absence of suitable *in vitro* systems and the difficulty of distinguishing displacement synthesis from complementary strand synthesis *in vivo*. Displaced strands participate in two pathways that constitute adenovirus complementary strand synthesis: one involving intramolecular hybridization (*cis* replication) and the other using intermolecular hybridization (*trans* replication).

The work presented here uses an approach developed to study the fate of displaced single strands *in vivo* (Hay *et al.*, 1984; Wang and Pearson, 1985). Specially constructed plasmids which carry a single adenovirus origin and inverted repeat sequences were extensively studied in this replication system. The system separates the sequences controlling the initiation of DNA replication from the inverted repeats which are involved in forming "panhandles". Thus, the function of inverted repeats during complementary strand synthesis can be studied independently without destroying the adenovirus replication origin. Moreover, the products of complementary strand replication can be distinguished from molecules engaging in strand displacement replication by virtue of a different molecular size. This replication system not only provides a sensitive method to detect the complementary strand replication but also provides the opportunity to investigate possible mechanisms for the replication of displaced single-stranded DNA.

Plasmids carrying a single adenovirus terminal sequence with a series of unidirectional deletions in the polylinker inverted repeat sequences have been constructed to study the length and sequence integrity of panhandles. The minimum length of the inverted repetitious sequences required for the generation of stable panhandle structure was determined. Inverted repeats must be at least 31 bp long. Inverted repeats shorter than

31 bp do not support *cis* replication. It appears that when multiple copies of 89-bp inverted repeats are present, there is no preferential selection of particular copies of inverted repeats to form the panhandle intermediate, and removal of 3' non-homologous tails as long as 4000 bases distal to the panhandle is extremely efficient. Thus, neither the loop size of the panhandle nor a long, unpaired tail at the 3' end of the panhandle seems to be a limiting factor affecting the formation or fate of panhandle intermediates. Insertion and deletion mutations were also made within the polylinker inverted repeat sequences at different positions (*Kpn*I, *Xba*I, or *Sph*I sites). Mismatches can be consequently generated on the panhandles formed by displaced single strands. Certain kinds of mismatched sequences in the panhandle region were repaired. Several mechanisms for rectification of mismatched sequences on panhandle structures to regenerate perfectly double-stranded DNA were proposed. It is postulated that mismatched loops can be opened by as yet unknown endonuclease(s), and the repair of unpaired nucleotides presumably requires a 3'-exonuclease activity coupled with DNA polymerization. Double-strand DNA sequences remained on the panhandle region larger than 31 bp in order to let the 3' exonuclease activity function.

A novel mechanism for adenovirus overlap recombination, now termed *trans* replication, has been described previously (Ahern *et al.*, 1990). The *trans* replication pathway involves the intermolecular hybridization of displaced complementary single strands from different parental DNA molecules to generate a heteroduplex intermediate. In order to study the relative participation of the *cis* and *trans* mechanisms involved in the adenovirus replication cycle, plasmid molecules were constructed with adenovirus origins and inverted repeat sequences that can pair both within and, distinguishably, between displaced strands. The experiments demonstrated that both *cis* and *trans* pathways operate simultaneously during adenovirus replication. Furthermore, it appears as little as two copies of a 89-bp complementary sequence support *trans* replication, whereas a single copy of 89-bp does not. Sequence rectification of 3' unpaired nucleotides during *trans* replication has also been observed.

Homologous recombination in mammalian cells has been extensively studied. Although the mechanisms involved in recombination have not yet been fully elucidated, hybridization between homologous sequences is

believed to be required as an intermediate step. As little as 14 bp of homologous sequences are sufficient for recombination in CV1 cells (Rubnitz and Subramani, 1984), and 25 bp of homologous sequences were required for recombination in EJ human bladder carcinoma cells and COS-1 monkey kidney cells (Ayares *et al.*, 1986). From studies of sequence rectification in the 5' mutant of pIR5'S, 3'-IR to 5'-IR conversion could generate totally *Sph*I-sensitive, replication-proficient, *cis* molecules. However, this conversion would leave just 6 bp holding the displaced strand into a panhandle configuration during the repair process (the possibility that some protein factor may be involved in stablizing the panhandle structure cannot yet be ruled out). All the evidence suggests that the size for stable homologous DNA hybridization might be very small. Nevertheless, at least 31 bp of inverted repeat sequences are required to form a stable panhandle structure. Several possible reasons for this requirement can be imagined: (1) Adenovirus single-stranded DNA-binding proteins may impair panhandle formation, requiring a longer sequence to overcome this destablizing effect. (2) A longer double-stranded DNA region may be required for DNA polymerase (or other enzymes) to bind so that the DNA replication origin can be restored. (3) Cellular exonucleases may decrease the inverted repeat size after transfection. However, this is not very likely since the study of the 5'-*Kpn*I mutant has already eliminated the possibility of strong exonuclease damage to the end of the inverted repeat sequences. pIR5'K replication displayed both 3'-IR to 5'-IR and 5'-IR to 3'-IR conversions for repairing mismatched sequences in the panhandle. If exonuclease degraded as few as 8 bp at the end of the inverted repeat, only 5'-IR to 3'-IR conversion should be observed.

Adenovirus ITRs are believed to play an important role in panhandle formation during complementary strand synthesis. Studies have shown that inverted repeats as small as 31 bp are sufficient to support *cis* replication. Although 31 bp may not be the true minimum size required for panhandle formation, this size is much smaller than the adenovirus replication origin (roughly the terminal 50 bp of the adenovirus genome). These results indicate that the adenovirus terminal 50 bp not only contains the entire replication origin but also is long enough to support the formation of the panhandle replication intermediate. Most adenoviruses have 100-bp ITR sequences or longer, but never shorter than 67 bp (Sussenbach, 1984).

Therefore, it is very interesting to understand why adenoviruses always carry longer ITRs. The sizes and sequences of ITRs within each adenovirus serotype are identical. Any mutation or damage in this region must be repaired efficiently to maintain sequence identity. The extra 50 bp of ITR sequences may function to prevent lethal terminal deletions or origin substitutions. It is interesting to note that the avian CELO virus, the adenovirus with the shortest known ITR, is the only known adenovirus with a mismatch within the bounds of its ITR (Aleström *et al.*, 1982). The second half of the ITR might also serve other functions. Possible roles for this region might be to regulate gene expression, to regulate DNA replication, to assemble virions, or to form special structures needed for virus infection or replication. Some proteins bind ITR sequences between nucleotides 60 to 94 (Schneider *et al.*, 1986) and a transcription factor Sp1-binding site was found in the second half of the ITR (Dynan and Tjian, 1985). The actual role for these proteins is still unknown. ITR sequences beyond the first 50 bp may also have a role in the formation of circular DNA. Covalently linked adenovirus circles have been identified during adenovirus infection (Ruben *et al.*, 1983).

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APPENDIX

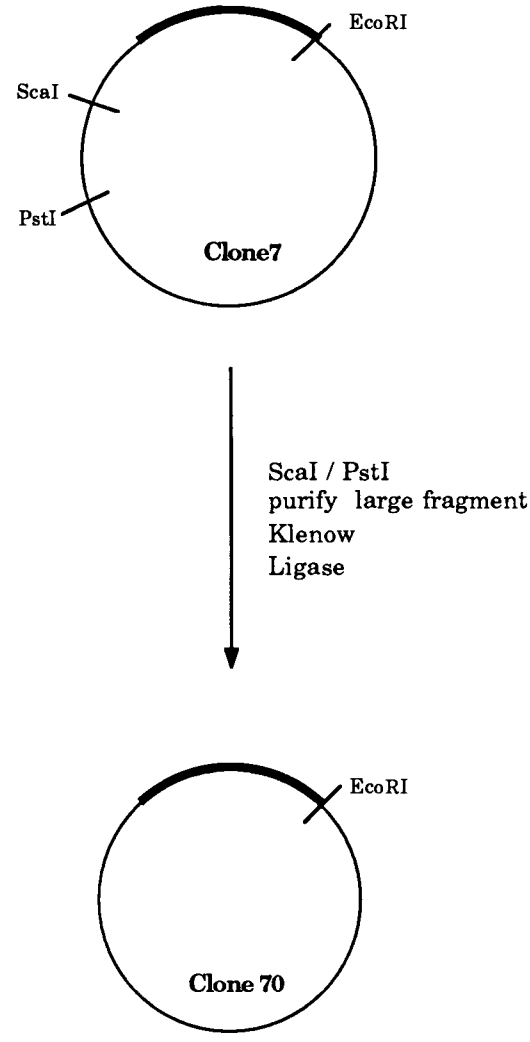

Figure A.1. Construction of clone 70. Clone 70 is a 5.5-kb tetracycline resistant clone which contains 1.3 kb of the left-end of adenovirus DNA. It was derived from clone 7 (Enns *et al.*, 1983) by deleting the small *Sca*I-*Pst*I fragment out of clone 7. This plasmid was made in an attempt to study DNA recombination during adenovirus replication *in vivo*. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences.

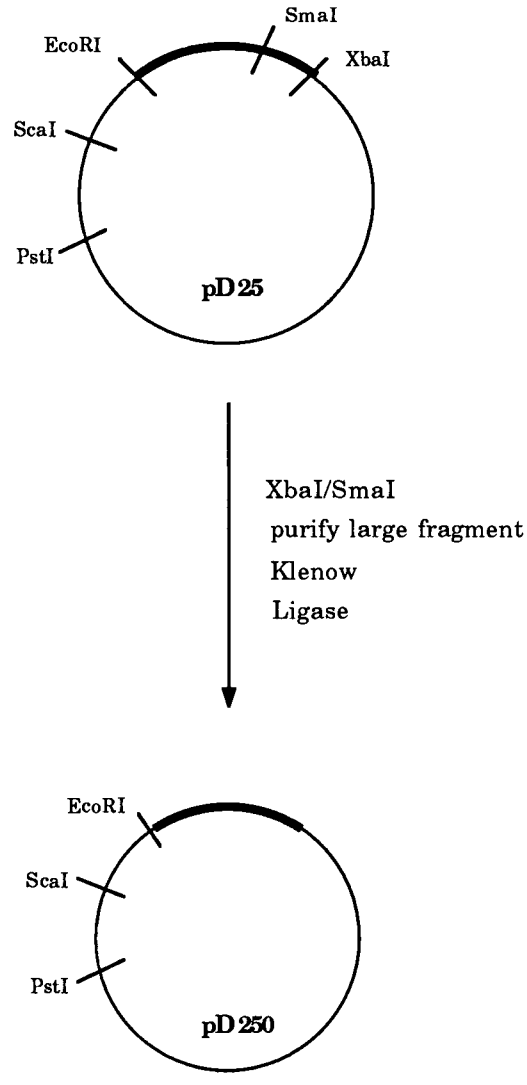

Figure A.2. Construction of pD250. pD250 is a 3.7-kb ampicillin-resistant clone which contains 1.0 kb of the left-end of adenovirus DNA. pD250 is a derivative of pD25 (Ahern, 1985) made by deleting the small *Sma*I-*Xba*I adenovirus sequences out of pD25. This plasmid was made in an attempt to study DNA recombination during adenovirus replication *in vivo*. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences.

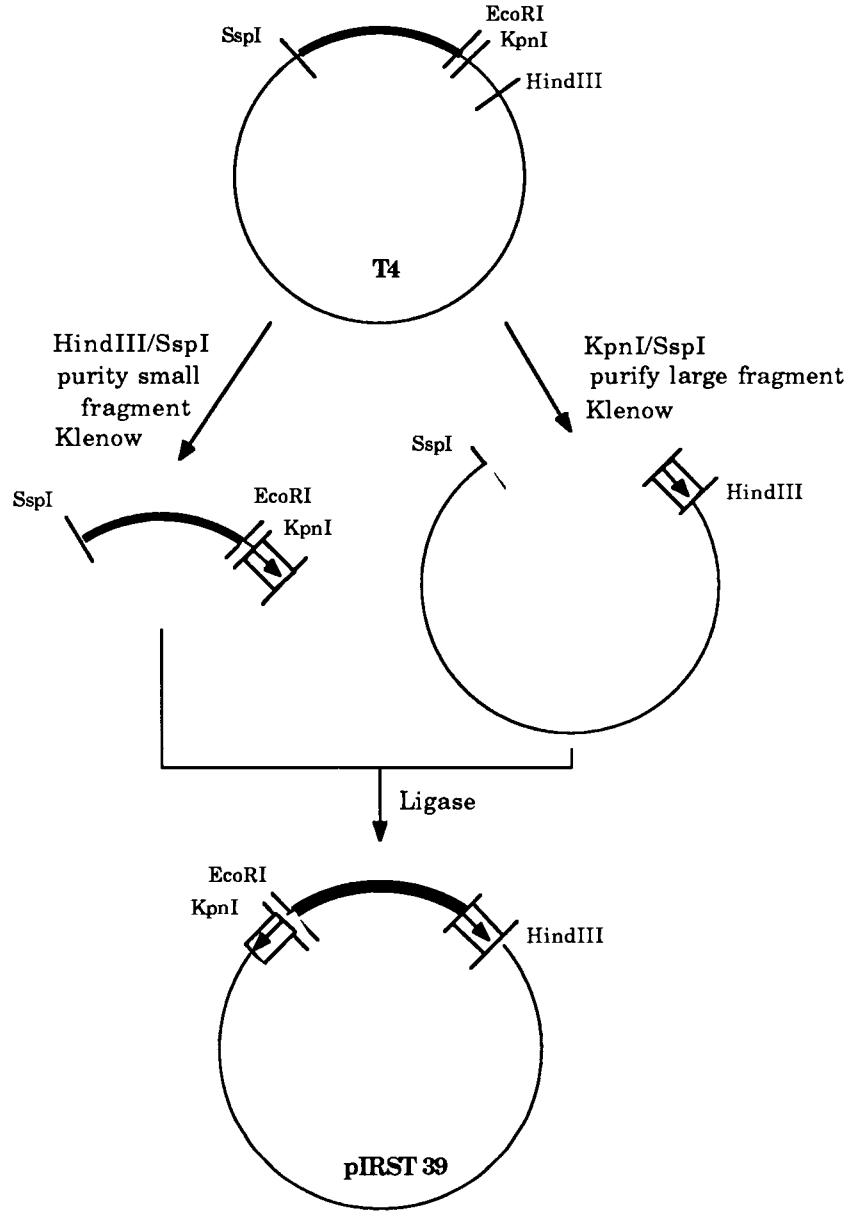

Figure A.3. Construction of pIRST39. pIRST39 is a 2.5-kb ampicillin-resistant clone which contains a single copy of 338 bp of the adenovirus left-terminal sequences and two 39-bp inverted repeats derived from the polylinker sequences of pUC19. The plasmid was made by ligating the small, blunted *Ssp*I-*Hin*dIII fragment from T4 (Wang, 1986) into the large, blunted *Ssp*I-*Kpn*I plasmid backbone of T4 with correct orientation. This plasmid was made in an attempt to study the size requirement for inverted repeats for repairing the 3' non-homologous nucleotides on shorter panhandles. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

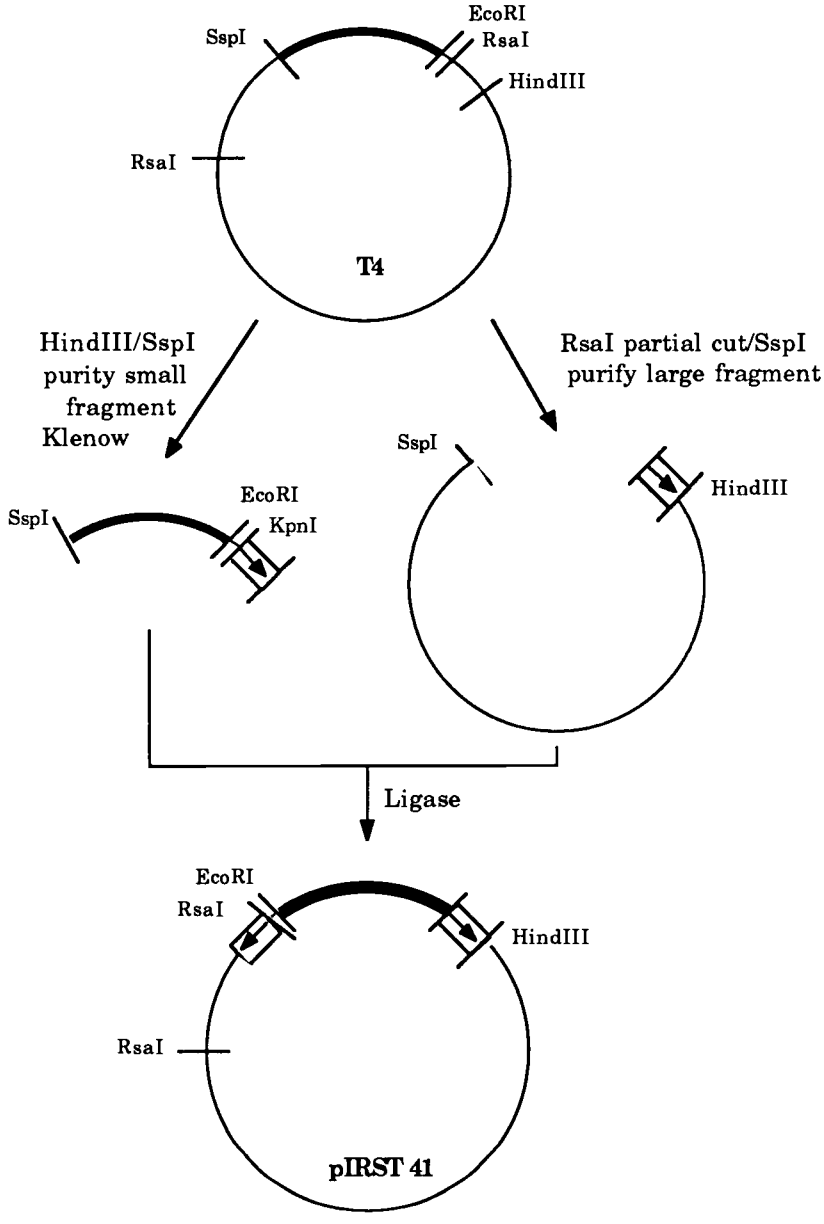

Figure A.4. Construction of pIRST41. pIRST41 is a 2.5-kb ampicillin-resistant clone which contains a single copy of 338 bp of the adenovirus left-terminal sequences and two 41-bp inverted repeats derived from the polylinker sequences of pUC19. The plasmid was made by ligating the small, blunted *Ssp*I-*Hin*dIII fragment from T4 into the large, blunted *Ssp*I-*Rsa*I plasmid backbone of T4 with correct orientation. This plasmid was made in an attempt to study the size requirement of inverted repeats for repairing the 3' non-homologous nucleotides on shorter panhandles. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

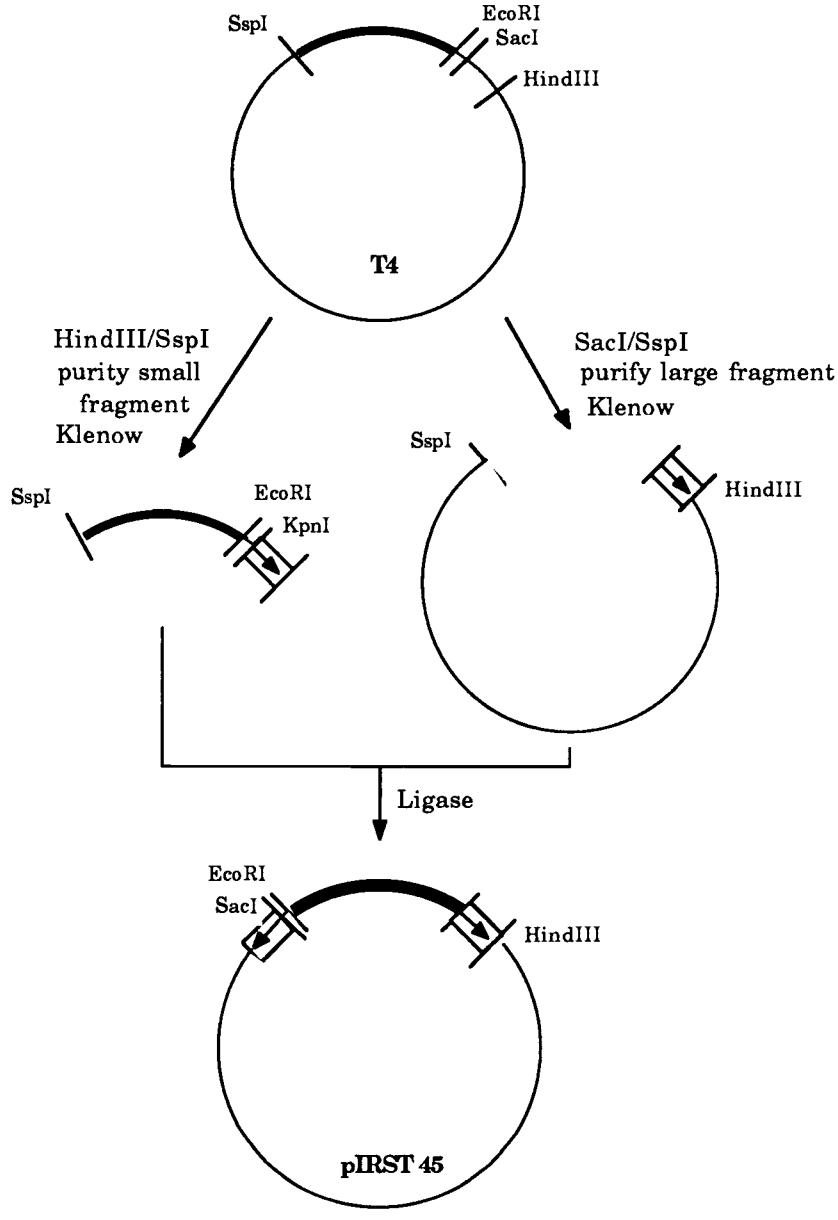

Figure A.5. Construction of pIRST45. pIRST45 is a 2.5-kb ampicillin-resistant clone which contains a single copy of 338 bp of the adenovirus left-terminal sequences and two 45-bp of inverted repeats derived from the polylinker sequences of pUC19. The plasmid was made by ligating the small, blunted *Ssp*I-*Hin*dIII fragment from T4 into the large, blunted *Ssp*I-*Sac*I plasmid backbone of T4 with correct orientation. This plasmid was made in an attempt to study the size requirement of inverted repeats for repairing the 3' non-homologous nucleotides on panhandles. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

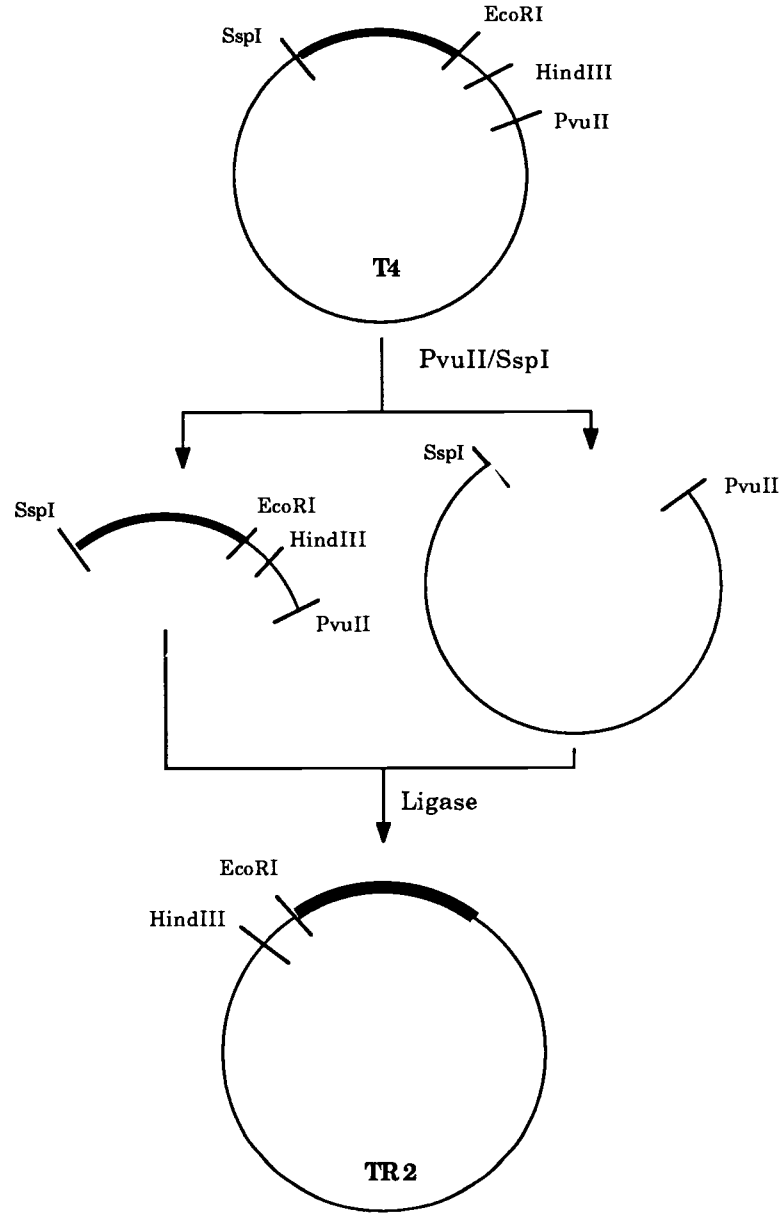

Figure A.6. Construction of TR2. TR2 is a 2.4-kb ampicillin-resistant clone. It was made by cutting T4 with *Ssp*I and *Pvu*II, then religating. TR2 as a result contains an adenovirus left-end fragment of 338 bp and part of the plasmid sequences in an inverted orientation relative to T4. This plasmid was made in an attempt to study whether a single copy of 230 bp can support *trans* replication or not. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences.

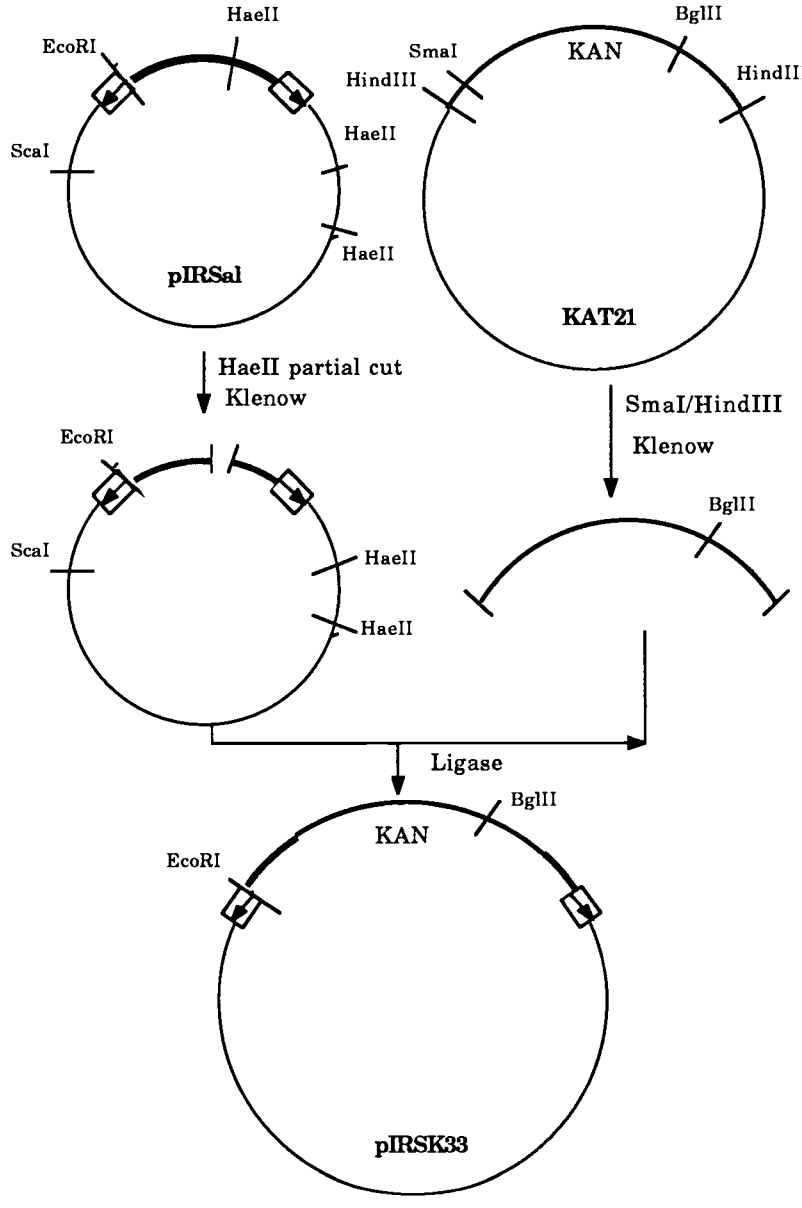

Figure A.7. Construction of pIRSK33. pIRSK33 is a 3.8-kb ampicillin- and kanamycin-resistant clone which contains a single copy of adenovirus left-terminal sequences and two 33-bp polylinker inverted repeats. However, a 1.3-kb blunted *Sma*I-*Hin*dIII kanamycin gene fragment from KAT21 (Ahern, 1985) was inserted into the blunted *Hae*II site located within the 338 bp of adenovirus terminal sequences on pIRSal. The single *Hae*II cut within the adenovirus sequences was made possible by partial digestion of pIRSal with *Hae*II. The orientation of kanamycin gene was verified as shown in the map. This plasmid was made in an attempt to study the roles played by two small inverted repeats during adenovirus *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus and kanamycin sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

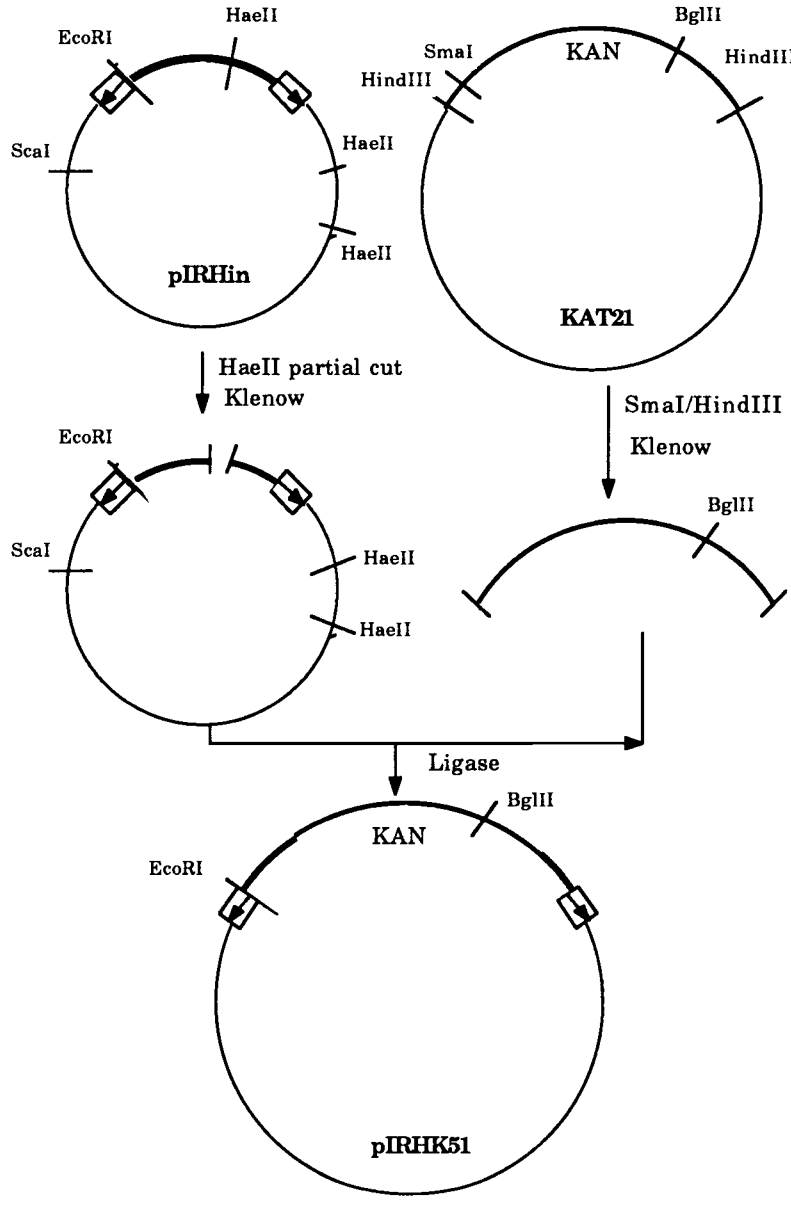

Figure A.8. Construction of pIRHK51. pIRHK51 is a 3.8-kb ampicillin- and kanamycin-resistant clone which contains a single copy of adenovirus left-terminal sequences and two 51-bp polylinker inverted repeats. However, a 1.3-kb blunted *Sma*I-*Hin*dIII kanamycin gene fragment from KAT21 was inserted into the blunted *Hae*II site located within the 338 bp of adenovirus terminal sequences on pIRHin. The single *Hae*II cut within the adenovirus sequences was made possible by partial digestion of pIRHin with *Hae*II. The orientation of the kanamycin gene was identified as shown in the map. This plasmid was made in an attempt to study the roles played by two small inverted repeats during adenovirus *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus and kanamycin sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

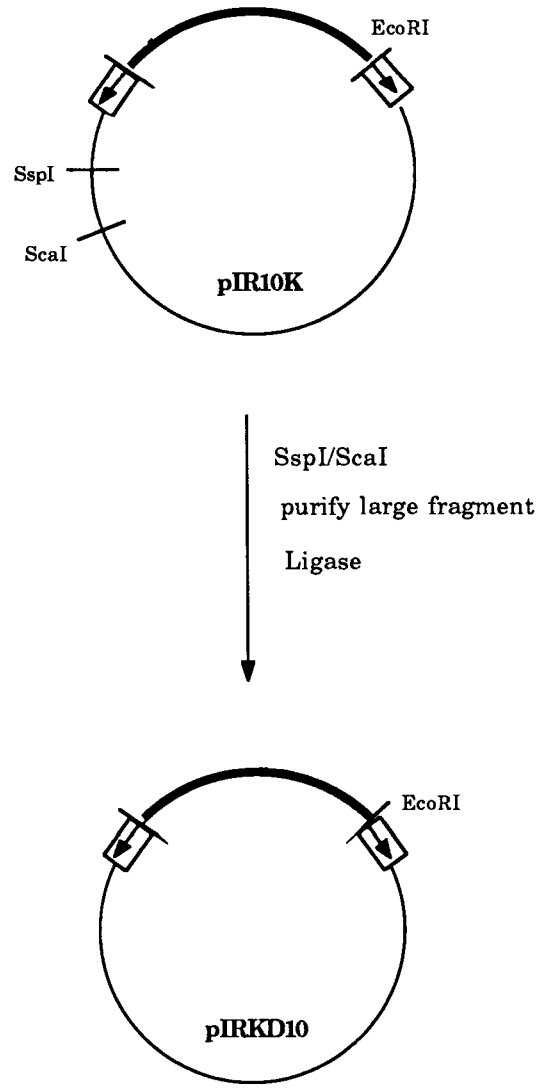

Figure A.9. Construction of pIRKD10. pIRKD10 is a 4.1-kb kanamycin-resistant clone which contains a single copy of 350 bp of adenovirus left-end sequences and a kanamycin gene fragment between the two copies of the 89-bp inverted repeats. The plasmid was made by deleting the small *Ssp*I-*Sca*I fragment out of the pIRK10. This plasmid could be used in distinguishing the two *trans* replication products formed by base-pairing between two copies of 89-bp inverted repeats. The thin line represents the plasmid vector sequences and the thick line corresponds to adenovirus and kanamycin sequences. The small open boxes show the inverted repeats where the arrows indicate the orientation.

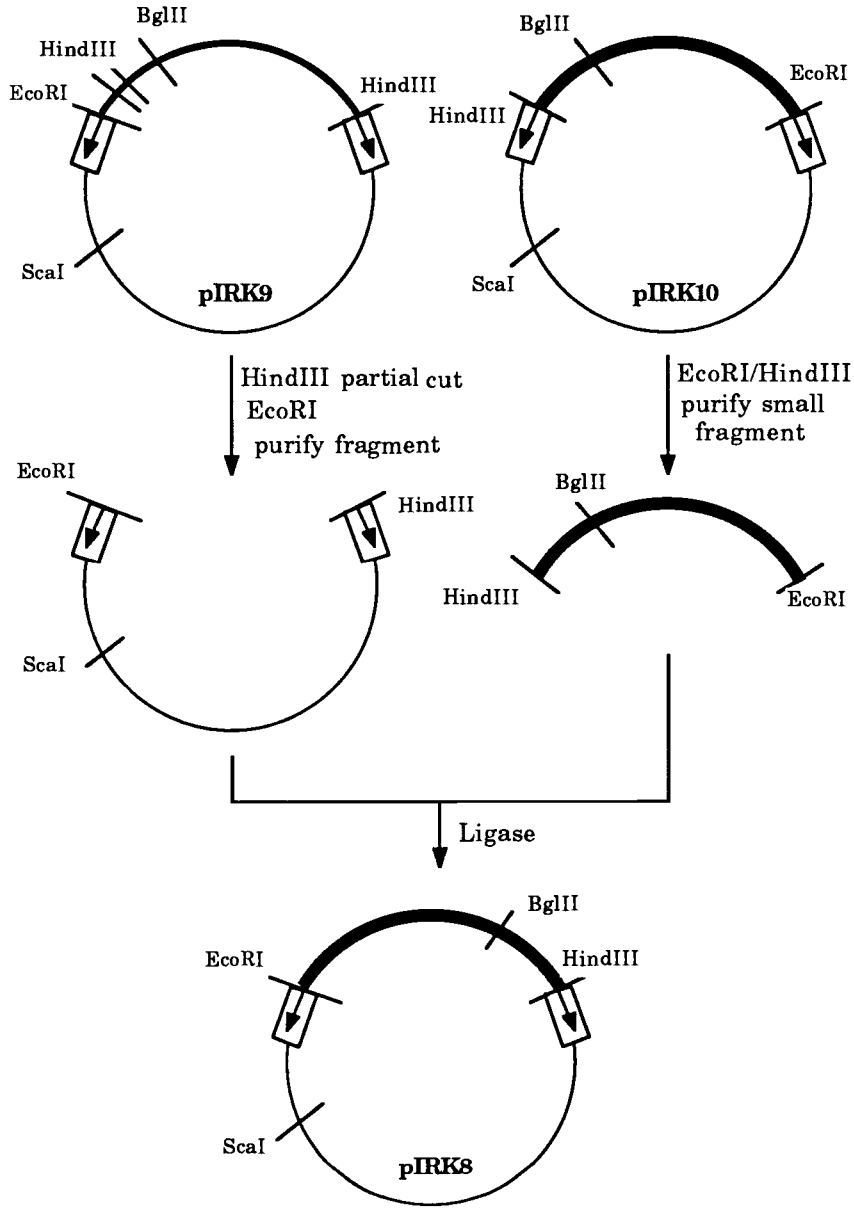

Figure A.10. Construction of pIRK8. pIRK8 is a 4.5-kb ampicillin- and kanamycin-resistant clone. It was made by moving the *Eco*RI-*Hin*dIII fragment carrying the 350 bp of left-end adenovirus sequences and a kanamycin-resistant gene from pIRK10 into the corresponding *Eco*RI- and *Hin*dIII-treated pIRK9 backbone. pIRK8 contains the exact same DNA sequences as pIRK10; however, the adenovirus replication origin on pIRK8 is in an opposite orientation relative to pIRK10. This plasmid was originally constructed in an attempt to study the roles of 350 bp adenovirus sequences in adenovirus *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus and kanamycin sequences. The small open boxes show the 89-bp inverted repeats where the arrows indicate the orientation.

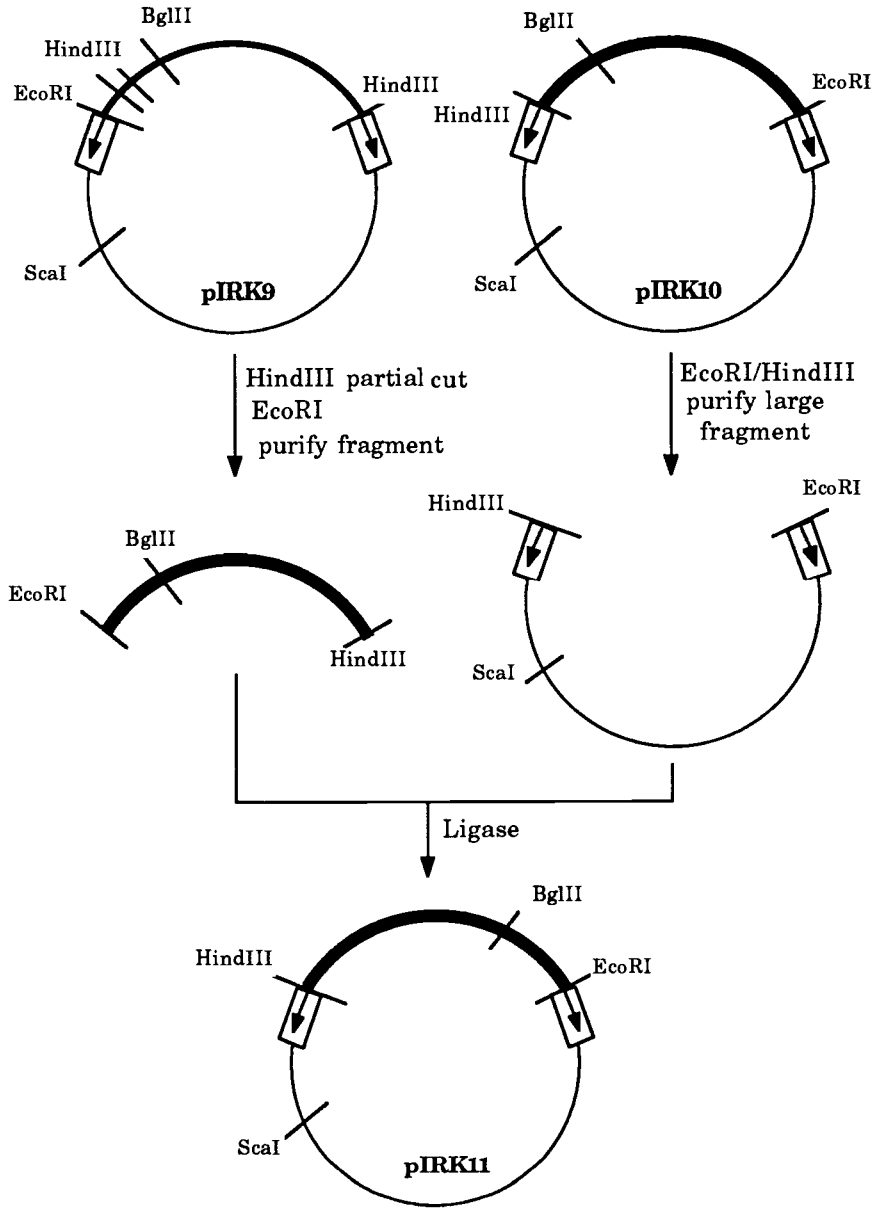

Figure A.11. Construction of pIRK11. pIRK11 is a 4.7-kb ampicillin- and kanamycin-resistant clone. pIRK9 was cut by *Eco*RI and then partially digested with *Hin*dIII. The *Eco*RI-*Hin*dIII fragment carrying 58 bp of the right-end adenovirus sequences and a kanamycin-resistant gene from pIRK9 was cloned into the corresponding *Eco*RI- and *Hin*dIII-treated pIRK10 backbone. pIRK11 contains the same DNA sequences as pIRK9; however, the adenovirus replication origin on pIRK11 is in an opposite orientation relative to pIRK9. This plasmid was constructed in an attempt to study the effects of 58 bp adenovirus sequences in adenovirus *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus and kanamycin sequences. The small open boxes show the 89-bp inverted repeats where the arrows indicate the orientation.

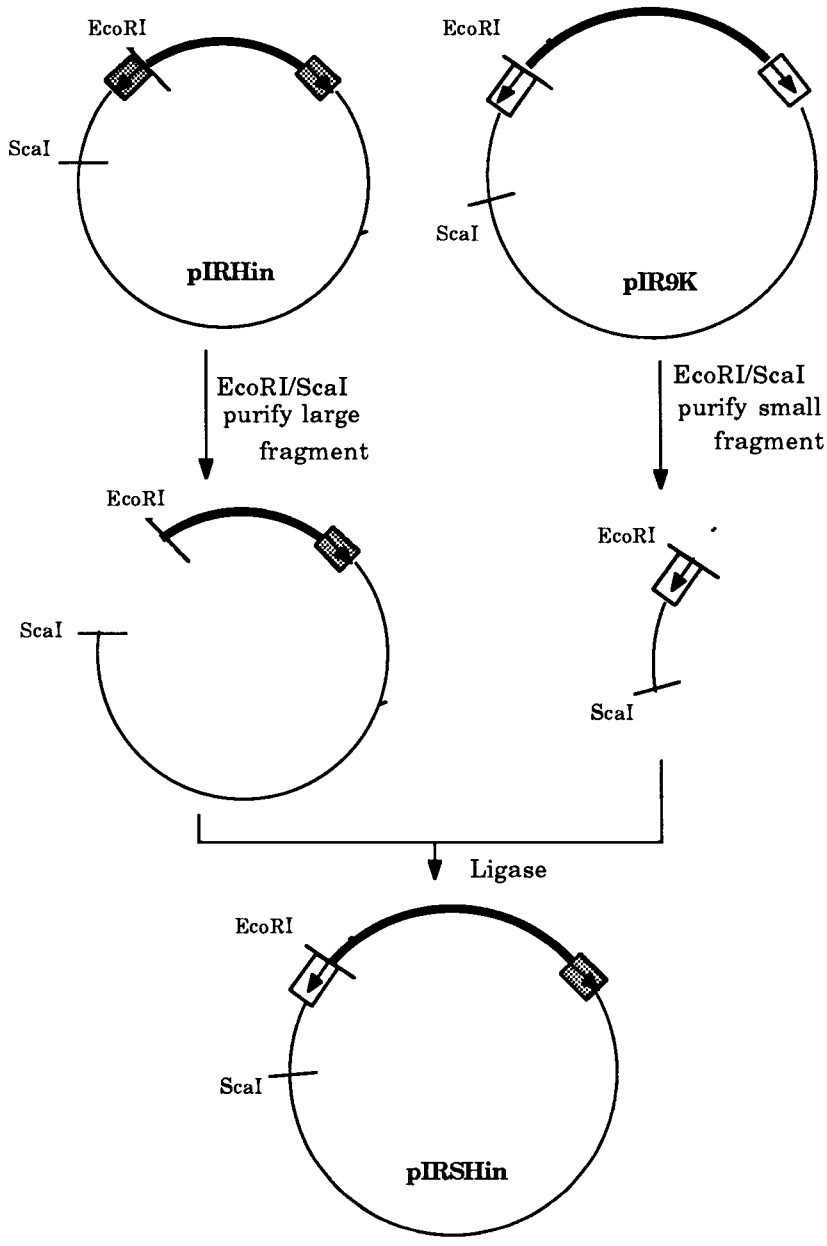

Figure A.12. Construction of pIRSHin. pIRSHin is a 3.1-kb ampicillin-resistant clone containing a single copy of 338 bp of the left-end adenovirus sequences and two distinctive DNA sequences. One is 51 bp of polylinker sequences derived from pIRHin and the other is 89 bp derived from pIRK9. The plasmid was made by replacing the small *Eco*RI-*Sca*I fragment of pIRHin with the corresponding *Eco*RI-*Sca*I fragment from pIRK9. This plasmid was made in an attempt to study the possibility of recombination during adenovirus replication and the size requirement for *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences. The filled boxes show the 51-bp inverted repeat sequences and the open boxes show the 89-bp inverted repeat sequences where the arrows indicate the orientation.

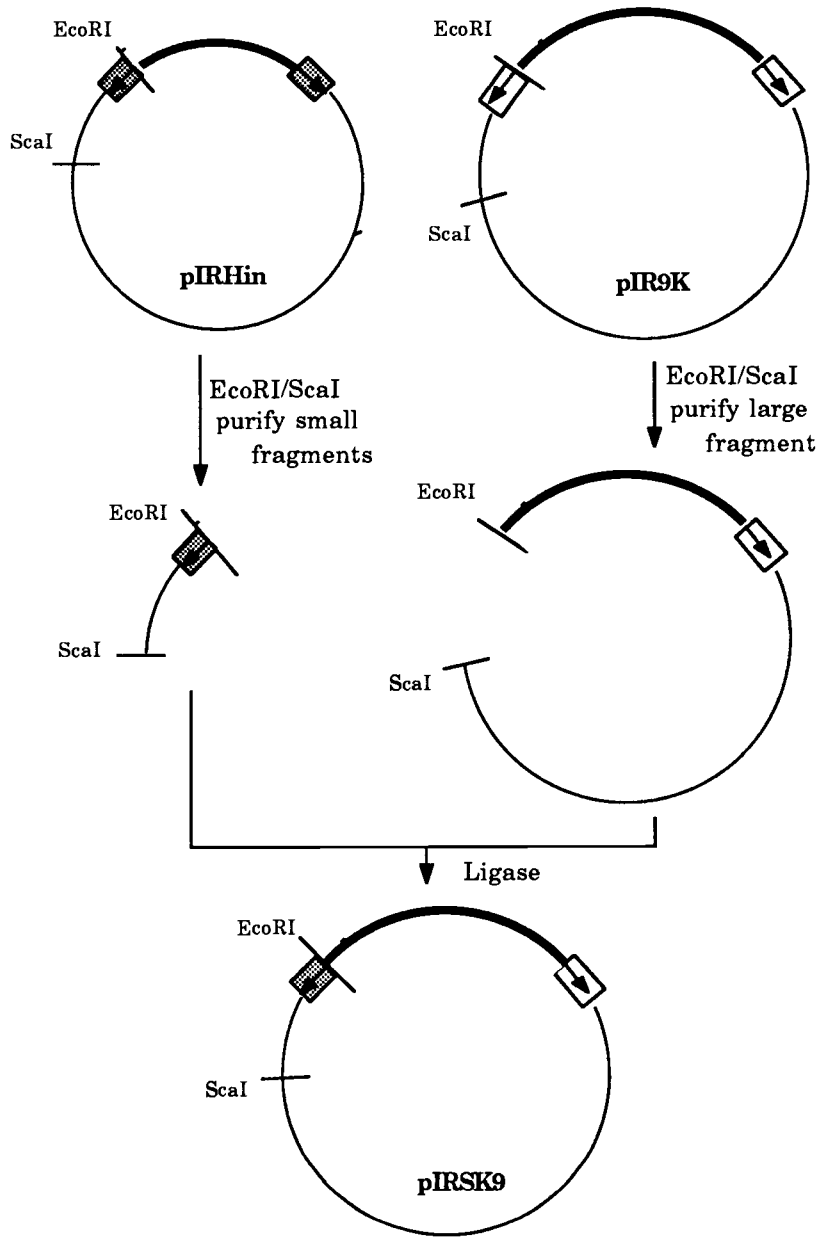

Figure A.13. Construction of pIRSK9. pIRSK9 is a 4.1-kb ampicillin-resistant clone containing a single copy of 58 bp of the right-end adenovirus sequences and two distinctive DNA sequences. One is 51 bp of polylinker sequences derived from pIRHin and the other is 89 bp derived from pIRK9. The plasmid was made by replacing the small *Eco*RI-*Sca*I fragment of pIRK9 with the corresponding *Eco*RI-*Sca*I fragment from pIRHin. This plasmid was made in an attempt to study the possibility of recombination during adenovirus replication and the size requirement for *trans* replication. The thin line represents the plasmid backbone and the thick line corresponds to adenovirus sequences. The filled boxes show the 51-bp inverted repeat sequences and the open boxes show the 89-bp inverted repeat sequences where the arrows indicate the orientation.

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