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Replication of plasmid molecules containing a single adenovirus terminal sequence and specially arranged inverted repeat sequences were studied. When the plasmids were linearized to expose the adenovirus terminal sequence and transfected into 293 cells with helper adenovirus DNA, a larger molecule was detected. The new molecule contained adenovirus terminal sequences at both ends of the molecule. This approach provides a sensitive assay to determine which adenovirus terminal sequences are required in cis for replication

Plasmids carrying deletion mutations within cloned adenovirus terminal sequences have been studied to map the replication origin. The replication origin is contained entirely within the first 67 bp of the adenovirus inverted terminal repeat (ITR). This region could be further subdivided into two functional domains: a minimal replication origin and an adjacent auxilliary region which

boosted the efficiency of replication by more than 100-fold. The minimal origin occupies the first 18 to 21 bp and includes sequences conserved between all adenovirus serotypes. The adjacent auxilliary region extends past nucleotide 36 but not past nucleotide 67, and contains the binding sites for nuclear factor I and nuclear factor III.

Complementary strand replication has been studied in vivo by using plasmid molecules containing a single adenovirus replication origin and different sizes of inverted repeat sequences. Inverted repetitious sequences 33 bp or longer are required for complementary strand replication. Complementary strand synthesis cannot be detected when plasmids contain inverted repeat sequences 28 bp or smaller.

Adenovirus Complementary Strand Synthesis *In Vivo*

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CHAPTER 1

INTRODUCTION

Since the first adenovirus was described in 1953 by Rowe *et al.*. (1), 42 distinct serotypes (species) of adenoviruses have been isolated from the human population, and numerous different types have been isolated from other animal species (2). Adenoviruses have been widely studied as model systems for virus assembly (see 3 for review), gene transcription (see 4 for review), mRNA splicing (see 4 for review, 5), oncogenic transformation (see 6 for review) and DNA replication (see 7-11 for recent reviews).

GENOME STRUCTURE AND DNA REPLICATION

Human adenoviruses have a linear double-stranded DNA molecule of approximatly 36,000 base pairs (12). All adenovirus genomes examined to date have two distinctive features: an inverted terminal repetition (ITR) (13,14), and a virus-coded protein, called "terminal protein", covalently attached to each 5' end (15-17). The size of the ITR ranges from 63 bases for the avian CELO virus (18) to 166 bases for the tree shrew adenovirus (19). Although ITRs differ in size, all the ITRs share conserved or

highly homologous sequences (see 20 for review). These unusual terminal structures are important for virus DNA replication (for recent reviews see 7-11).

Based on electron microscopic (EM) and biochemical studies, the current model for adenovirus DNA replication can be summarized in Figure 1-1 (21). There are two distinct features in this replication model. One is a "protein-priming" mechanism which is proposed as the initiation step, and the other is the "panhandle structure" which is used for displaced single-stranded DNA to generate functional replication molecules. Adenovirus DNA replication origins lie at both ends of the linear genome. Replication initiates from either (or both) end(s) of the molecule (22, 23). The initiation process involves the formation of a phosphodiester linkage between the serine residue in the pre-terminal protein and the terminal nucleotide dCMP (24-26). The 3' end of dCMP in this protein-nucleotide complex can be used as a primer for viral DNA polymerase (27-29). The formation of protein-nucleotide primer is a sequence-dependent reaction (30-41). It occurs only in the presence of the adenovirus DNA replication origin. After initiation, viral DNA is elongated by a strand displacement mechanism (23, 42). Displacement synthesis proceeds to the end of the duplex molecule and generates a daughter duplex and a parental single strand. Molecules engaged in displacement

Figure 1-1. Proposed model for adenovirus DNA replication. Adapted from lechner and Kelly (21).

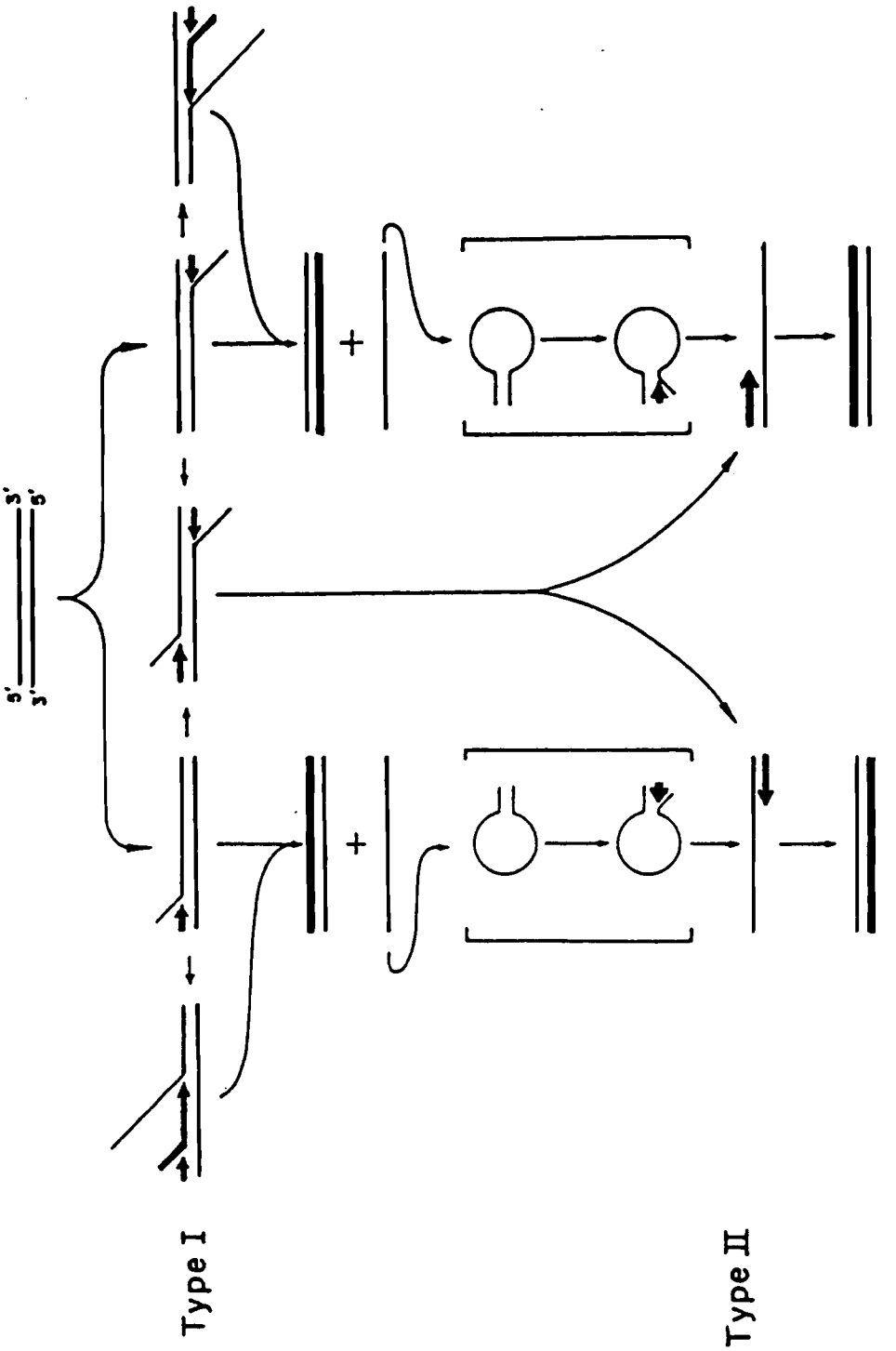

Figure 1-1

synthesis are called type I molecules (21). The step to convert displaced single strands to double strands is called complementray strand synthesis. Although no evidence shows that complementary strand synthesis is used by the virus, EM studies and some in vivo experiments strongly suggest that ITRs of displaced single strands can form a "panhandle structure". The initiation of complementary strand replication within the double-stranded ITR region of the panhandle would be identical or very similar to the first initiation reaction, but subsequent elongation may be different from strand displacement synthesis. Presumably, elongation of the initiated panhandle intermediate results in the formation of type II molecules, which appear by electron microscopy as partially duplex and partially single-stranded molecules of unit length. A composite replicating molecule, called a type I/ II molecule, can also be generated if initiation occurs on a type II molecule before completing complementary synthesis.

PROTEINS IN ADENOVIRUS DNA REPLICATION

Several experimental approaches have been developed in the past few years to study adenovirus DNA replication both in vitro and in vivo. Detailed information about the biochemistry and enzymology of adenovirus DNA replication

have come from the development of cell-free DNA replication systems (43, 44). The system mixes either adenovirus DNA-terminal protein complex or cloned adenovirus terminal fragments with crude extracts from adenovirus-infected cells. Several methods have been used to measure the replication of adenovirus in cell-free replication systems. The most popular method is to measure initiation by the formation of pre-terminal protein-nucleotide complex (dCMP) (25-28). Electron microscopy can also be used to measure both initiation and elongation (41, 45). Crude cell extracts have been fractionated to identify viral and cellular factors that are required for adenovirus DNA synthesis. At least three viral coded proteins (viral DNA polymerase, pre-terminal protein and DNA-binding protein) have been isolated and identified as being required for adenovirus DNA replication.

VIRAL-CODED PROTEINS

Adenovirus terminal protein is a 55 Kd protein which is covalently linked to the 5' ends of adenovirus DNA. This unique property of terminal protein has been widely used in *in vitro* replication systems to measure the level of initiation of DNA replication (25-28). Using *in vitro* replication systems, it was found that the 5' end of nascent viral DNA is linked to a protein with a molecular

weight of 80 Kd instead of the 55 Kd terminal protein (24, 27). Later it was shown that the 80 Kd protein is the precursor of the 55 Kd terminal protein (46). The 80 Kd pre-terminal protein is processed to the 55 Kd terminal protein by a virus-coded protease during the assembly of the viron (46-48). Pre-terminal protein is coded in the viral early 2b (E2b) region (49, 50).

Viral DNA polymerase is a 140 Kd protein that is also coded in E2b region (50, 51). Like other viral and prokaryotic polymerases, it has 3' to 5' exonuclease activity for proofreading capability (52). But unlike other DNA polymerases, it elongates RNA-primed DNA poorly, and it is the only polymerase tested capable of labeling pre-terminal protein with radioactively labeled dCTP in vitro (53). Although viral polymerase uses double-stranded DNA templates, evidence shows that it can also use any single-stranded DNA as a template. However, the polymerization reaction is extremely inefficient when single-stranded DNA templates are used (36). Viral DNA polymerase is sensitive to N-ethylmaleimide and cytosine arabinosyl CTP, but insensitive to aphidicolin (31). Both in vivo and in vitro evidence shows that the viral DNA polymerase forms a tight complex with pre-terminal protein (36, 37, 51, 54, 55).

Adenovirus DNA-binding protein has a molecular weight of 59 Kd (this protein was referred to as 72 Kd due to its

unusual electrophoretic mobility in gels) and is coded in the early 2a region (E2a) of the genome (56). By using temperature-sensitive mutants, it has been shown that viral DNA-binding protein is required for virus replication (57, 58). EM studies indicate that the single-stranded DNA produced during viral DNA replication is completely coated by the DNA-binding protein (42). Besides the DNA binding property, evidence suggests that it also plays some role in the regulation of viral early and late gene expression (59). From the in vitro replication system, it has been shown that DNA-binding protein is not necessary for the initiation of DNA replication, but is required for subsequent chain elongation (58).

CELLULAR PROTEINS

So far at least three host proteins are involved in adenovirus DNA replication. They are called nuclear factor I, nuclear factor II and nuclear factor III. Nuclear factor I(NF I) binds the sequence $TGG(N)_{6-7}GCCAA$ found in the ITRs of most adenovirus serotypes (34, 35). Nuclear factor I can stimulate the initiation of replication both in vivo (60, 61) and in vitro (35). The stimulation from NF I probably arises from either providing the binding site for other adenovirus

replication proteins by unwinding the DNA (28, 35, 62) or stablizing the viral replication initiation complex by protein-protein interactions (35). NF I or NF I-like proteins have been found in organisms ranging from yeast to humans (63). NF I binding sites occur in cloned HeLa cell DNA at a frequency of approximately 1 per 100,000 bp (64). Based on the requirement of NF I for adenovirus DNA replication and the apparent frequency of the NF I binding site in the HeLa cell genome, a role for NF I in cellular DNA replication is suggested (64). It has also been shown that the NF I binding site exists in the middle of some of the adenovirus genomes (position 10,767 in type 2; position 10,611 in type 7 (K. G. Ahern, personal communication)), in the regulatory region of the human c-myc oncogene (65), in the chicken lysozyme gene (66, 67), within the immunoglobulin gene (68), in the BK viral enhancer (69), in the MMTV LTR (69), and the major early gene of human cytomegalovirus (70). These findings suggest that NF I may also be involved in the control of gene expression.

Nuclear factor II (NF II) has topoisomerase I activity, and can be replaced by eukaryotic topoisomerase I (71). In the absence of this factor only 25% of the length of the genome can be replicated <u>in vitro</u> by using purified components (32, 71). Its primary function in adenovirus replication is probably not in the initiation

step, but at the level of elongation.

Recently, Pruijn et al. (72) reported that in addition to NF I there is another host protein, designated nuclear factor III (NF III), which can stimulate the initiation of adenovirus replication in vitro by 4- to 6-fold. DNase I footprinting shows that this factor binds to nucleotides 36 through 54. This region includes the partially conserved nucleotides TATGATAATGAG. Several possible binding sites have been found by using sequence searching computer programs. These possibilities include promotor elements of the histone H2B gene, the human interferon gene, the immunoglobulin gene, and the mammalian U1 and U2 small nuclear RNA genes.

There are other cellular factors involved in the adenovirus DNA replication. A heat-stable, ribonuclease-sensitive fraction from the cytosol of uninfected HeLa cells can stimulate the initiation of adenovirus DNA replication in vitro by 3-fold (73). The role of this RNA in adenovirus DNA replication is not clear at this time. A protein factor, called factor pL, was isolated from uninfected cell extracts (32, 38, 74). Factor pL stimulates adenovirus replication in vitro when protein-free templates (without terminal protein linked at 5'end) are used (32, 38, 74). Evidence shows that this factor is a 5' to 3' exonuclease, and it activates the template by degrading the 5'-end of the displaced strand. This action

exposes the 3'-end of the template strand. The partially duplex template still requires NF I for efficient initiation (74). This suggest that NF I may stimulate the initiation reaction both by unwinding the strands and by protein-protein interactions. From the results of studying factor pL, NF I, and adenovirus DNA polymerase, there are indications that a short single-stranded terminal region may be needed for the initiation of adenovirus DNA replication.

SEQUENCE DEPENDENCE OF ADENOVIRUS REPLICATION.

Analysis of the sequence requirements for adenovirus replication have been done both in in vitro (35, 38-41, 45, 75)and in vivo (60, 61, 76-78). The results from these experiments all agree that the first 67 nucleotides contain all the sequences required for the initiation of DNA replication. Origin sequences required for replication can be mapped by mutations. It has been found that for efficient initiation of adenovirus DNA replication, the template molecule must be linearized to expose the adenovirus terminus. Neither circular plasmids containing the same adenovirus origin nor plasmids linearized with internal adenovirus terminal sequences will function in the pre-terminal protein labeling assay (35, 38-41, 75). But embedded origins do give some level of initiation and elongation in vitro when assayed by

electron microscopy (41, 45). In vivo experiments also indicate the initiation of embedded viral origins (76, Ahern, K. G., Wang, K. and Pearson, G. D., manuscript in preparation). Experiments show that viral DNA replication requires a conserved sequence between nucleotides 9 to 18. Deletions or mutations which affect any of the sequences between nucleotides 9 and 18 greatly inhibit viral DNA replication both in vitro and in vivo. The sequence from nucleotides 25 to 45 contains the nuclear factor I binding site (32, 35). In the presence of this region the initiation of adenovirus DNA replication can be stimulated up to 30-fold (35). The nuclear factor III binding site is from nucleotide 36 to 54. This region can only stimulate in vitro replication from 4- to 6-fold (72). No in vivo experiments on the role of the NF III binding site have yet been done. It has been proposed that the sequences from 9-18 and 19-67 constitute two functional domains for adenovirus replication (61). The first domain is necessary for binding the pTP-Adpol complex and is absolutely required for initiation of replication. The second domain contains the NF I and NF III binding sites and is not necessary for initiation, but serves to stimulate the level of initiation. The sequences beyond the NF III binding site (beyond nucleotide 54) are GC-rich and contain short regions of sequences conserved between serotypes. Some of these

sequences have homology to sequences in polyoma virus and BK virus. The significance of these homologies is still unknown. This region also contains binding sites (GGGCGG) for transcription factor SP 1 which has been identified and isolated by Tjian's laboratory (79). The function for SP 1 binding sites in adenovirus DNA replication, if any, is still unknown.

To explain the utilization of an embedded origin *in vivo*, Hay *et al.* (76) suggested that sequences within the adenovirus ITR have the function to position the initiation event at a defined distance from the recognition site at the terminal C nucleotide of the genome. Thus, the use of an embedded origin does not require a specific cleavage by a host- or viral-coded factor(s) to expose the origin sequence.

All the factor and sequence requirements studied to date involve the initiation of strand-displacement synthesis, but the requirements for complementary strand synthesis may differ considerably.

COMPLEMENTARY STRAND SYNTHESIS

Although it has been widely accepted that displaced single strands in adenovirus DNA replication will be converted to double strands by a process referred to as complementary strand replication, there has been no direct

evidence for a mechanism. The development of *in vitro* adenovirus DNA replication systems has provided much valuable information for understanding adenovirus replication. Complementary strand synthesis, however, has not been demonstrated *in vitro* yet, and the mechanism of this process is virtually unknown. Several mechanisms for complementary strand replication have been proposed.

CIS-REPLICATION

Some indirect evidence suggests that displaced strands replicate via a panhandle-like structure. The replication process involving a panhandle structure as an intermediate is referred as *cis*-replication (see Fig. 1-1).

Electron microscopy has revealed three types of replicating adenovirus molecules: Y-shaped molecules (type I molecules), partialy single-stranded/partialy double-stranded molecules (type II molecules) and composite molecules with both characteristics (type I/II molecule). A replication model, based on the above observations, was proposed by Lechner and Kelly (see Fig. 1-1). They proposed that initiation with elongation via a strand-displacement mechanism at the end of viral genome will generate type I molecules. The ITRs in the displaced single strand will pair to form a panhandle structure. This will generate a double-stranded ITR region identical

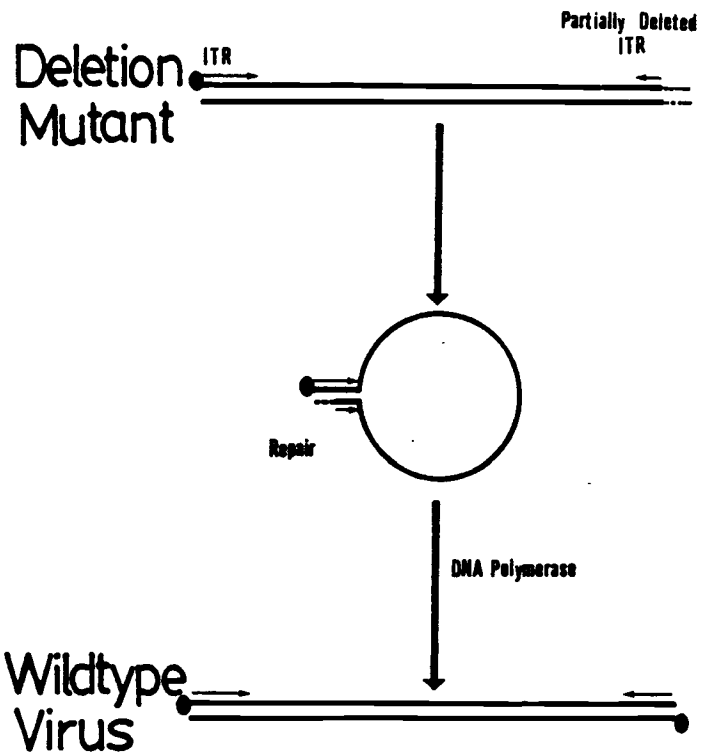

Figure 1-2. Proposed mechanism for repairing the ITR (81). Black-circles represent terminal proteins, arrows represent inverted terminal repeats (ITR).

or very similar to the double-stranded viral DNA. Initiation will arise from this double-stranded region and generate a type II molecule. However, electron microscopic analysis of replicating molecules that were photocrosslinked and then denatured revealed no panhandle-like structures (80).

The first indirect evidence for a panhandle structure was obtained by Nigel Stow (81). He made deletions to remove part or all of one molecular end of intact adenovirus DNA. He found that deletions removing as many as 51 base pairs at an end, when transfected into cells, resulted in the production of infectious virus with completely intact ends. Deletion mutants where one of the entire ITRs had been removed produced no infectious virus when transfected into cells. A likely explanation for this repair process is that during viral DNA replication the two ITRs at the end of the molecule have to be paired to form a panhandle structure. The deleted part of the ITR is repaired by using the wild-type copy of the ITR as a template. The paired, double-stranded region can then be initiated, and will generate complete ITRs at both ends of the molecule (Fig. 1-2). Deletions that remove one of the ITRs will not be able to form a panhandle structure. Thus, the repair and the second initiation can never happen.

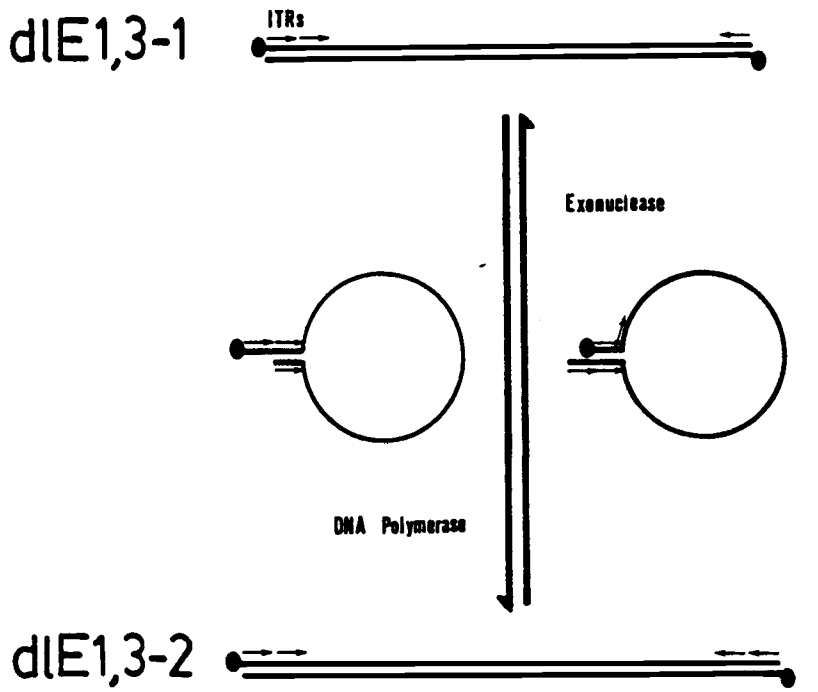

Figure 1-3. Mechanism proposed by Haj-Ahmad and Graham(85) on the interconversion of multiple ITR mutants. Black-circles represent terminal proteins and arrows represent ITRs.

Recent in vivo replication experiments (61, 76) also suggest that a panhandle structure is involved as an intermediate in adenovirus replication. Both Hay (76) and Wang and Pearson (61) used specially constructed plasmids to study adenovirus DNA replication in vivo. These plasmids contain a single adenovirus terminal sequence followed by a sequence repeated in an inverted orientation at the end of the molecule. When these molecules were transfected into cells with helper DNA, the plasmid generated a larger product which contained another adenovirus terminal sequence on the other end of the molecule (see Chapter 2, Fig. 2-4). A likely way to generate this larger molecule is by having the displaced single strand form a panhandle structure using the artifical inverted repetition. Subsequent repair of the panhandle generates an adenovirus origin by using the single-stranded adenovirus sequence as a template. Reinitiation on this terminus generates a linear molecule with adenovirus termini at each end. Whether panhandle structures are obligatory intermediates in adenovirus DNA complementary strand synthesis is still unknown.

Adenovirus mutants with duplicated ends have been isolated or constructed by various groups (82-84). Recently, Haj-Ahmad and Graham (85) isolated an adenovirus type 5 mutant which carried embedded inverted terminal repeats when they were constructing an adenovirus cloning

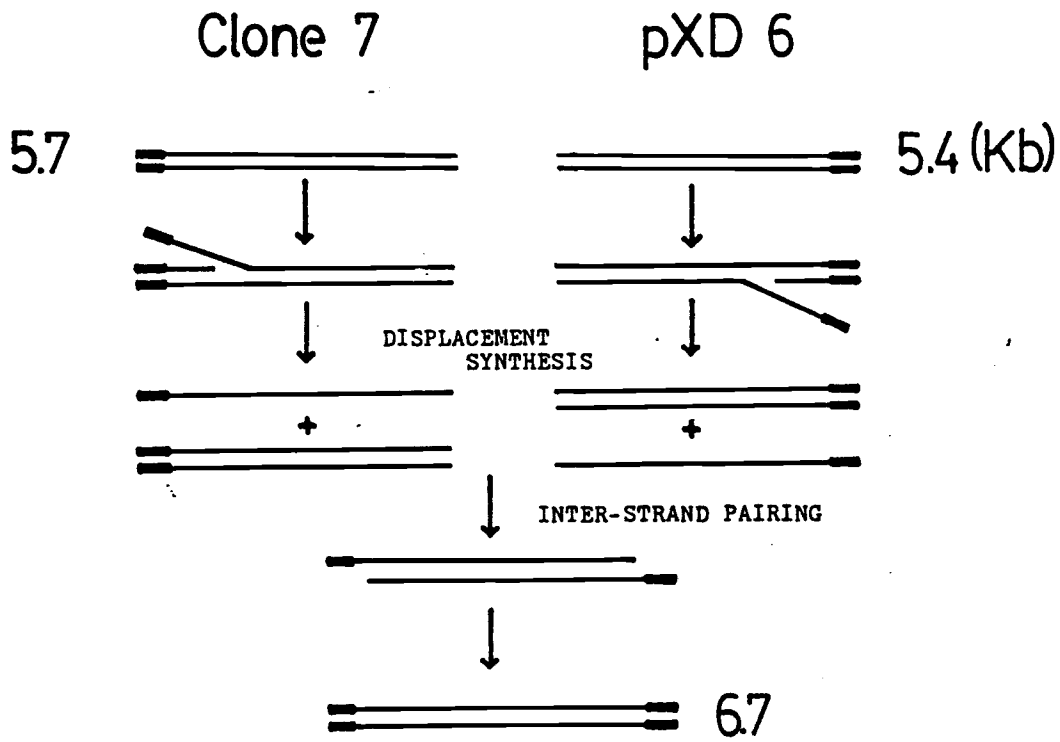

Figure 1-4. Scheme for Trans-replication. Clone 7 and pXD6 contain left and right adenovirus terminal sequences respectively. The thick line represents the adenovirus sequences and the thin line corresponds to the pBR322 backbone.

vector. dlE1,3-1 had a direct repeat of viral DNA terminal sequences attached to the left end of the genome, and dlE1,3-2 had duplicated terminal sequences at both ends of the viral genome. dlE1,3-1 can be converted to dl1,3-2 at high frequency and vice versa. Fig. 1-3 diagrams the interconversion using panhandle intermediates.

TRANS-REPLICATION

An alternative pathway for complementary strand replication is trans-replication where intermolecular pairing events produce daughter duplex molecules from complementary displaced parental strands. Trans-replication was proposed by Kevin G. Ahern (Ahern, K. G., Wang, K. and Pearson, G. D., manuscript in preparation). Two linearized plasmids which have the same backbone but carry different adenovirus replication origins (either left or right) in opposite orientations were used in trans-replication experiments (Fig. 1-4). When these two plasmids were mixed and cotransfected with adenovirus DNA as a helper, a new molecule was generated corresponding to a recombinant between the input molecules. Further analysis has shown that this recombination event is not classical homologous recombination. Rather, the recombinant is formed by the intermolecular pairing of displaced strands from the input plasmids. This is the first evidence in vivo that the displaced strands can pair together to form a replication-proficient molecule.

Previous kinetic studies on adenovirus DNA replication also support this finding (86, 87). Whether this is a minor, a major, or the only pathway for adenovirus replication still needs to be investigated.

OTHER PATHWAYS

Other pathways for the complementary strand to replicate can be imagined. One of them is a consequence of the fact that displacement synthesis can be initiated at either end of the genome. Thus, there is a possibility that before completion of a round of displacement synthesis initiated at one end of the genome, a second initiation will occur at the opposite end. In such doubly initiated molecules, both parental strands serve as templates for daughter-strand synthesis and yield two type II molecules.

In vitro studies indicate that adenovirus DNA polymerase can use single stranded DNA as a template, though the efficiency is much lower and there is a loss of specificity in the initiation event. However, this suggests that complementary strand replication might just initiate from the displaced, single-stranded end without the formation of a panhandle structure.

The aim of the work described here is to study the replication mechanism(s) which are used in adenovirus complementary strand replication in vivo. An in vivo

adenovirus replication system and its use to determine the sequence requirement for *in vivo* replication are described in the next chapter. The inverted repetition sequence length requirement for efficient *cis*-replication will be discussed in Chapter 3. Other possibilities for complementary strand replication and the competition between *cis*- and *trans*-replication along with some other aspects of this study will be discussed in the final chapter.

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CHAPTER 2

ADENOVIRUS SEQUENCES REQUIRED FOR REPLICATION *IN VIVO*

INTRODUCTION

The type 2 adenovirus genome is a linear, double-stranded DNA molecule containing 35,937 base pairs (1). DNA molecules from all adenovirus serotypes have a termial protein (2) and an inverted terminal repetition (ITR) (3, 4). ITRs differ between serotypes both in sequence and length, but all ITRs share conserved or highly homologous sequences (5). These unusual terminal structures are important for adenovirus DNA replication (for recent reviews see refs. 6-8). Stow (9) has shown that the complete removal of one ITR renders adenovirus non-viable. However, it is not obvious which, if any, sequences within the ITR are required for viability since terminal deletions penetrating, but not removing, one of the ITRs can be repaired efficiently during replication to yield wild-type progeny. Recently, Hay *et al.* (10) demonstrated that linear plasmid DNA molecules containing adenovirus terminal sequences at each end replicate autonomously when introduced into cells together with adenovirus DNA as a helper. Linear molecules with a single adenovirus

terminus do not replicate under these conditions, but they can give rise to replication-proficient molecules by regenerating adenovirus termini at both ends. The regeneration of adenovirus ends requires specially constructed plasmids containing a specific geometrical arrangement of inverted repetitious sequences (these repeats need not even be adenovirus sequences). The production of replication-proficient plasmid molecules provides a sensitive assay to determine which adenovirus terminal sequences are required in cis for replication. A clear advantage of this assay is that the replication products differ in size from the input molecules. With this assay we locate the adenovirus replication origin entirely within the first 67 nucleotides of the ITR using unidirectional deletion mutations penetrating from within adenovirus sequences toward the terminus. Moreover, this region can be divided into at least two functional domains. The first domain, termed the minimal replication origin, spans the first 18 to 21 nucleotides and includes sequences highly conserved between all adenovirus serotypes (5). The second domain increases the efficiency of the minimal origin by more than 100-fold. This auxilliary region lies immediately adjacent to the minimal origin and encompasses sequences identified as the binding site for nuclear factor I (11, 12), a cellular protein required for efficient initiation of adenovirus

replication *in vitro* (12-15). We discuss the requirement for these adenovirus sequences for DNA replication *in vivo* and *in vitro* (12, 15-21).

MATERIALS AND METHODS

ENZYMES

Restriction endonucleases, *E. coli* DNA polymerase I (and the Klenow fragment), T4 DNA ligase, and S1 endonuclease were from Bethesda Research Laboratories. Pronase was from Calbiochem-Boehringer Corp. The enzymes were used as recommended by the suppliers.

CELLS AND VIRUS

The HeLa cell line was maintained in suspension culture in Joklik's minimal essential medium (GIBCO) containing 5% fetal calf serum. Adenovirus type 5 was grown in HeLa cells, and virus and viral DNA were purified as previously described (22). The 293 cell line (23) was maintained in monolayer culture in Dubecco's minimal essen tial medium (GIBCO) containing 10% fetal calf serum.

BACTERIAL AND PLASMIDS

E. coli JM83 (24) and *E. coli* JC9387 *recBC sbcB* (25) were grown, transformed, and selected by standard techniques (26). *E. coli* RRI was from Bethesda Research Laboratories and cultured as recommended. Selection was in the presence of ampicillin (50 µg/ml). Plasmids pUC8 (27), pMDC10 (12, 19, 21), and deletion mutants *dl*7 through *dl*67 (12, 19, 21) have been described. Prior to

plasmid DNA isolation (28), plasmid DNA was amplified by growth in chloramphenicol (100 µg/ml). Plasmid DNA was purified by ethidium bromide-CsCl density gradient centrifugation.

CONSTRUCTION OF PLASMIDS CONTAINING INVERTED REPEAT SEQUENCES

Figure 2-1 outlines the method to construct a plasmid containing cloned adenovirus terminal sequences flanked by inverted repeats. The scheme exploits the fact that re-cloning adenovirus terminal sequences and adjacent pUC9 sequences from pMDC10 into pUC8 will generate inverted repeats surrounding the adenovirus sequences. In order to prepare the appropriate vector backbone, pUC8 was cleaved with EcoRI, the cohesive sites were removed by S1 endonuclease treatment, and the blunt ends were polished with the Klenow fragment of DNA polymerase I. After cleavage with BamHI, the linear vector backbone terminating with a BamHI cohesive site and a blunt end was isolated. Cleavage of pMDC10 by the combined action of BamHI and PvuII yielded many fragments due to the presence of several PvuII sites. A 450 bp BamHI-PvuII fragment carrying adenovirus sequences together with adjacent pUC9 sequences was purified from the total digest and ligated to the specially prepared vector backbone. A 3.2-Kb recombinant plasmid, called pIR10 (Fig. 2-4), was identified in an ampicillin-resistant colony after

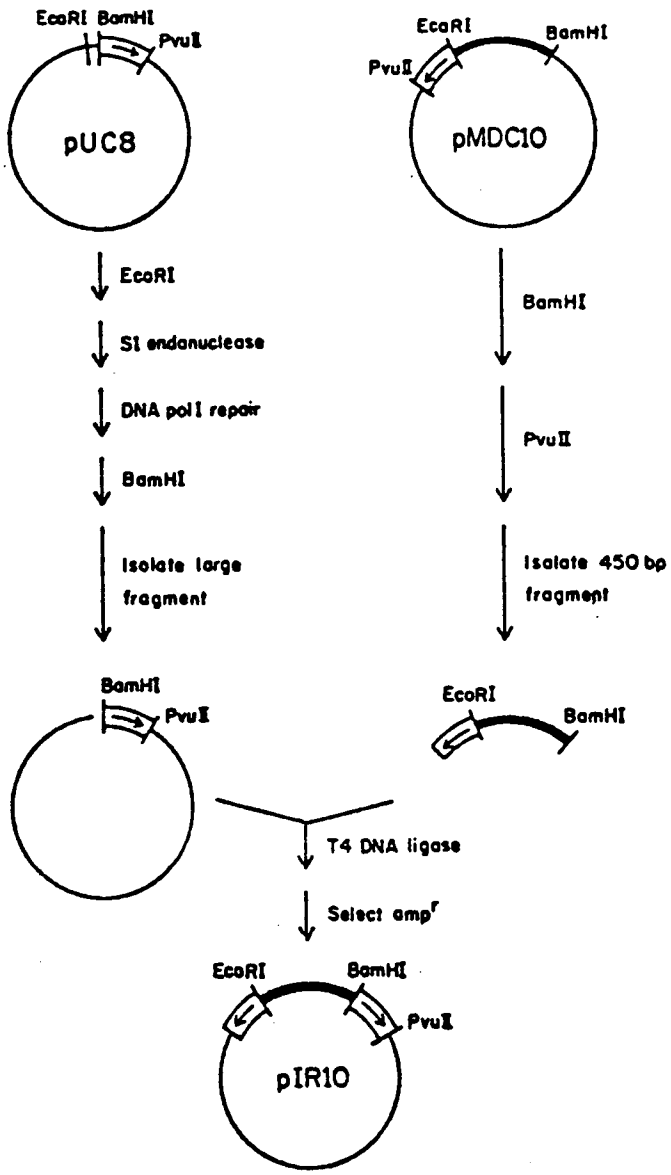

Figure 2-1. Construction of plasmid pIR10. pUC8 was cleaved with EcoRI, and the EcoRI site was blunted by treatment with S1 endonuclease followed by repair with the Klenow fragment of DNA polymerase. After cleavage with BamHI, the vector backbone was isolated and ligated to a 450 bp BamHI-PvuII fragment purified from pMDC10. The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA. pUC8 DNA is shown as the thin line.

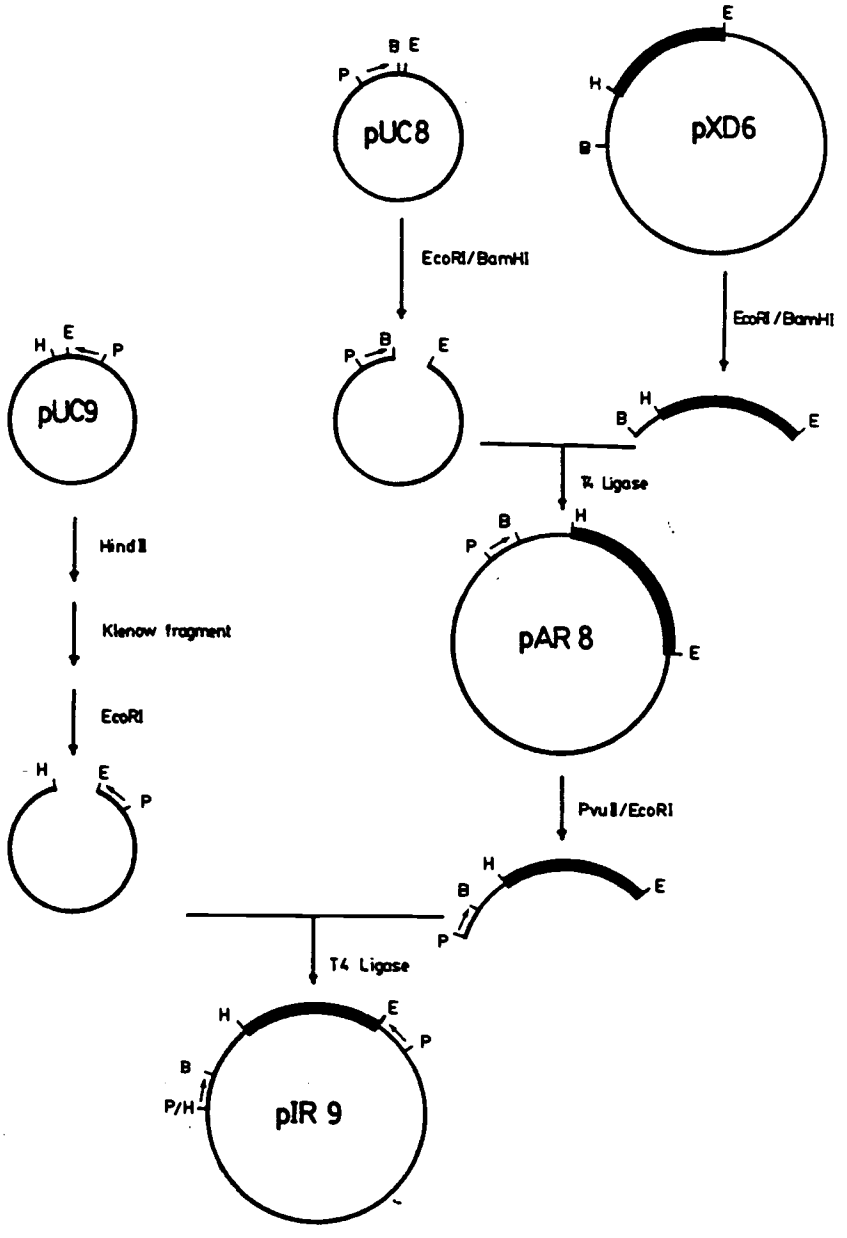

Figure 2-2. Construction of pIR9. The thick line reresents adenovirus sequences and the arrows represent the inverted repeat sequence. The plasmid backbone is shown as the thin line.

transformation of E. coli JM 83 with ligated DNA. Growth of pIR10 in E. coli JM83 generated at high frequency deleted plasmids lacking inverted repeats and adenovirus sequences. Re-cloning of pIR10 in E. coli JC9387 recBC sbcB prevented this problem (see Appendix A1).

Figure 2-2 illustrates the construction of pIR9 which has the same geometry as pIR10. However, pIR9 has 1.0-Kb of the right end of adenovirus and 346 bp of pBR322 sequences inserted between the 89 bp invert repeat sequence. The adenovirus DNA in pIR9 and pIR10 is in inverted orientation with respect to the vector backbone.

pXD6 was constructed by treating pIBIV (provided by Dr. Kathy Berkner) with HindIII and religating. pXD6 contains 1,000 bp from the right end of adenovirus type 2 DNA inserted between the HindIII to EcoRI sites of pBR322 in such a way that the ITR sequences are adjacent to the EcoRI site. The 1.3-Kb pXD6 EcoRI-BamHI fragment, which carries adenovirus sequences together with 346 bp of pBR322 sequences, was isolated and ligated with EcoRI- and BamHI-treated pUC8 backbone. A 4.0-Kb plasmid, called pAR8, was identified from an ampicillin-resistant E. coli RR1 colony. pUC9 was cleaved with HindIII, and the HindIII site was blunted by treatment with the Klenow fragment. After digestion with EcoRI, the pUC9 plasmid vector backbone was ligated with the 1.4-Kb EcoRI-PvuII fragment from pAR8. The 4.1-Kb pIR9 was identified and recloned

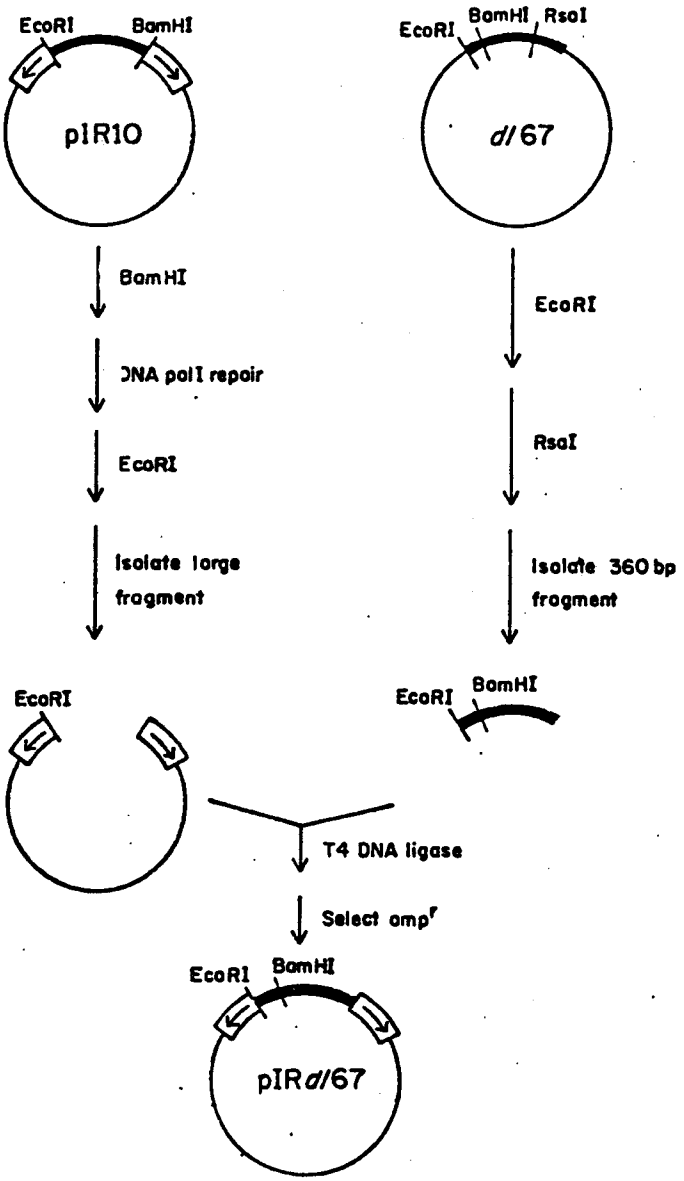

Figure 2-3. Construction of plasmid pIRdl67. The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA. Plasmid vector DNA is shown as the thin line.

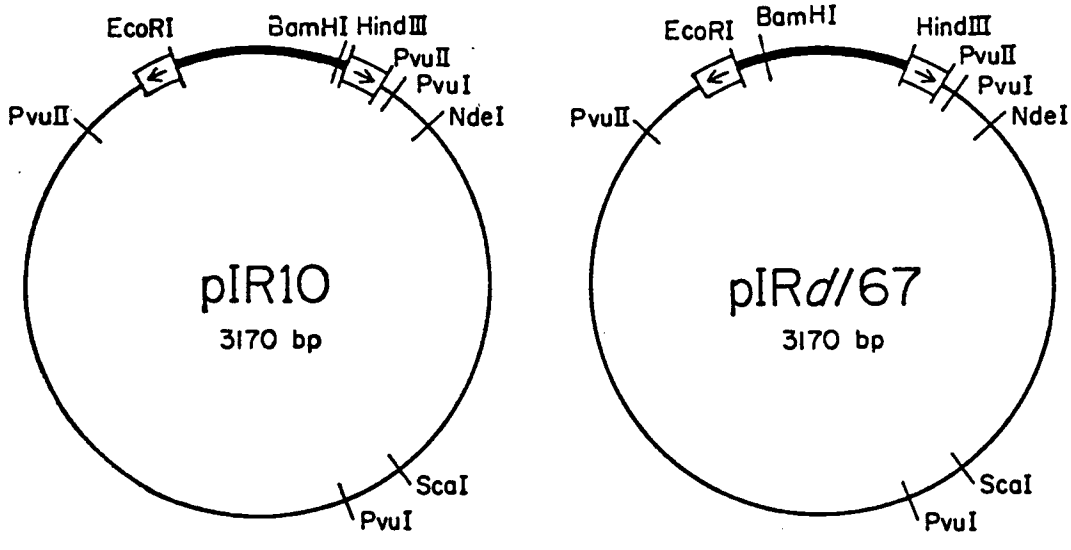

Figure 2-4. Restriction endonuclease maps of pIR10 and pIRdl67.

into E. coli JC9387.

Figure 2-3 illustrates the replacement of wild-type adenovirus terminal sequences in pIR10 with unidirectional deletion mutations penetrating from within adenovirus sequences towards the adenovirus terminus. pIR10 was cleaved with BamHI, the cohesive sites were repaired with the Klenow fragment of DNA polymerase I, and the adenovirus sequences were removed by further cleavage with EcoRI. Mutated adenovirus terminal sequences were excised from plasmids dl7, dl12, dl18, dl21, dl30, dl36, and dl67 by the combined action of EcoRI and RsaI (there are several RsaI sites in these plasmids). The appropriate EcoRI-RsaI fragment from each digest was isolated (the fragments ranged from 300 to 360 bp depending on the particular deletion mutant) and ligated to the specially prepared pIR10 backbone. As with pIR10, the initial cloning was done in E. coli JM83, but each plasmid was subsequently moved into E. coli JC9387. One of the clones, pIRdl67, is shown in Fig. 2-4. pIR10 and pIRdl67 have identical genome sizes.

TRANSFECTION

293 cells were transfected using the DNA-calcium phosphate coprecipitation method of Graham and van der Eb (29). Each 60mm plate was seeded with 4 x 10^5 cells 24 hr prior to transfection. Each plate received 3 µg of type 5 adenovirus DNA and 3 µg of plasmid DNA. Cells were

shocked for 1 min with 25% glycerol 4 hr after transfection.

EXTRACTION AND ANALYSIS OF DNA

Nuclei were prepared as described (30) from cells lysed with 1% NP-40 detergent in buffer containing 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4 Total DNA was extracted by incubating isolated nuclei for 6 to 8 hr at 37°C in 10 mM EDTA containing 0.6% sodium dodecyl sulfate (SDS) and Pronase at a concentration of 1 mg/ml. After phenol extraction and ethanol precipitation, purified DNA was suspended in buffer containing 10 mM Tris-HCl (pH7.5) and 1 mM EDTA. Approximately 1/5 of the DNA isolated from each plate was applied to each lane of a 1% agarose slab gel (12 x 20 cm). After gel electrophoresis, the DNA was transferred to a Gene Screen Plus membrane (New England Nuclear) by the method of Southern (31). Hybridization was for 18 hr at 68°C in 5 x SSC, 0.5% SDS and 1x Denhardt's solution (26). The probe was ^{32}P-labeled pUC8 DNA (specific activity > 1x 10^8 cpm per µg) prepared as previously described (32). After washing, the membrane was autoradiographed using Kodak SR-5 X-ray film. Autoradiograms were scanned with a Zeineh SL-5040XL soft-laser densitometer and band intensities were integrated by using an electrophoresis reporting integrator program (Biomed).

Figure 2-5. Generation of a replication-proficient plasmid with two adenovirus termini from a plasmid bearing a single viral terminus. (a) EcoRI-linearized 3.2-Kb pIR10 (see Fig. 2-4). The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA and the thin line shows pUC8 DNA. (b) Initiation of strand displacement replication at the single adenovirus terminus. (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 molecule using adenovirus sequences as a template. (f) Initiation of complementary replication at the adenovirus terminus of the panhandle molecule. (g) Complementary replication. (h) Production of a replication-proficient 3.6-Kb daughter molecule with two adenovirus termini at the conclusion of complementary replication.

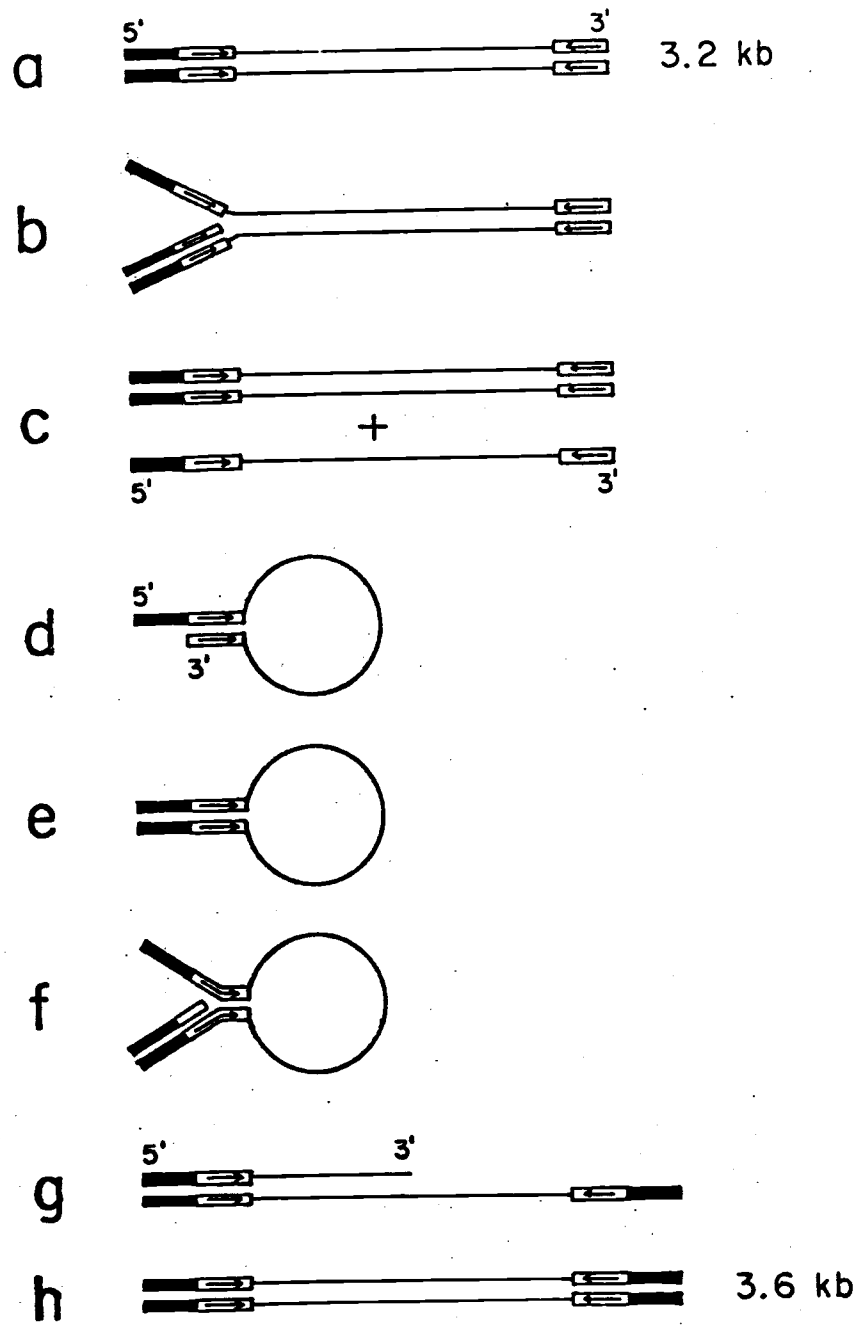

Figure 2-5

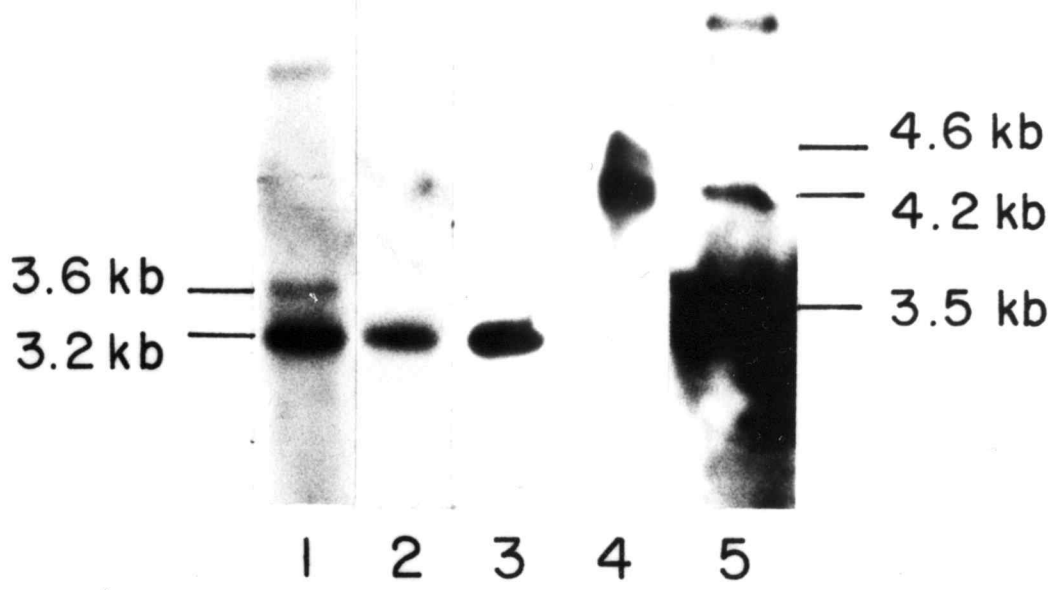

Figure 2-6. Replication of plasmids containing a single copy of adenovirus terminal sequences. DNAs were isolated 48 hours after transfection, and the total DNA analyzed by gel electrophoresis and Southern blotting. Lane 1: EcoRI-cut pIR10. Lane 2: BamHI-cut pIR10. Lane 3: EcoRI-cut pMDC10. Lane 4: EcoRI-cut pIRM. Lane 5: EcoRI-cut pIRBH.

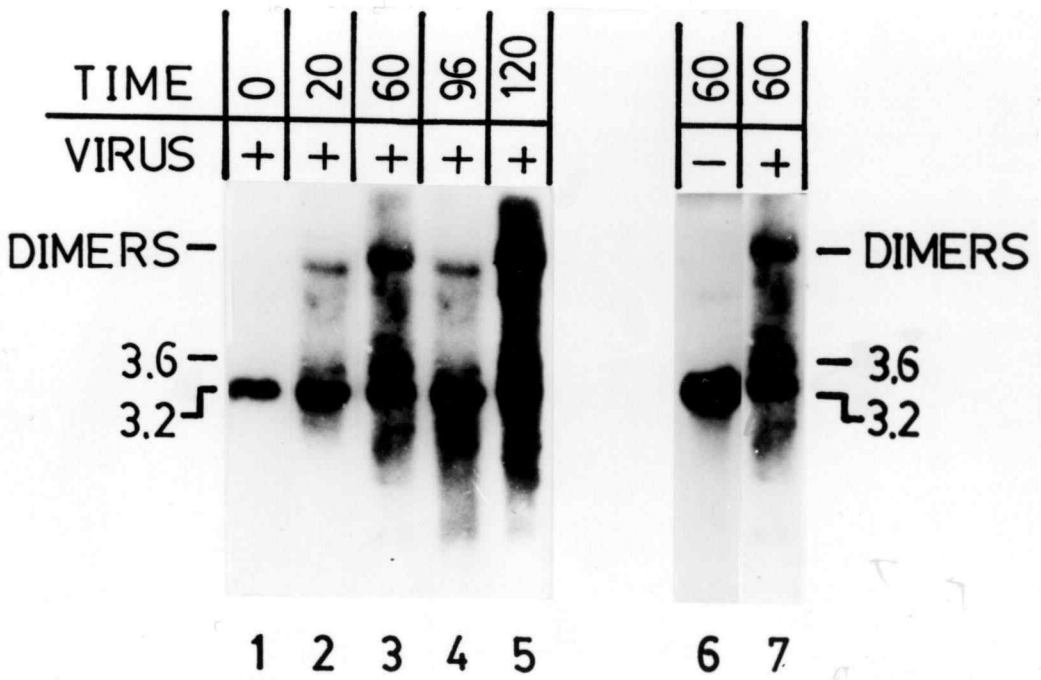

Figure 2-7. Replication assay from 0 to 120 hr for EcoRI-linearized pIR10. Except for lane 6, all the DNAs were cotransfected with adenovirus DNA as a helper. Sizes are given in kilobase pairs.

RESULTS AND DISCUSSION

Hay et al. (10) have shown that linear plasmid DNA containing adenovirus terminal sequences at each end replicates autonomously when introduced into cells together with adenovirus DNA as a helper. Linear molecules with a single adenovirus terminus do not replicate under these conditions, but they can give rise to replication-proficient molecules with adenovirus termini at each end if they additionally contain a specific geometrical arrangement of inverted repeat DNA. Fig. 2-5 diagrams the postulated sequence of events leading to the production of replication-proficient molecules. To test this scheme, 293 cells were transfected with EcoRI-linearized pIR10 DNA (Fig. 2-4) and helper adenovirus DNA. Fig. 2-6 shows the Southern blot analysis of total DNA isolated 48 hr after transfection. Two prominent bands were visualized with the nick-translated pUC8 probe (lane 1). The 3.2-Kb band, identical to the input pIR10 DNA, could be detected throughout infection without significant change in intensity (Fig. 2-7). The 3.6-Kb band was the exact size expected for the replication-proficient product arising from pIR10. It was not visible early after transfection, but its intensity increased with time and by 60 hr equaled or exceeded the intensity of the 3.2-Kb band (compare Fig. 2-6, lane 1 with Fig. 2-9, lane 1; also see Fig. 2-7). As

expected, the appearance of the 3.6-Kb band depended on the presence of helper adenovirus DNA (Fig. 2-7, lane 6) to supply in *trans* viral encoded replication proteins (DNA polymerase, DNA binding protein, and pre-terminal protein). pIR10 must be linearized at the adenovirus origin since *Bam*HI cut pIR10 (Fig. 2-6, lane 2) and circular pIR10 (not shown) did not give rise to the 3.6-Kb band. Plasmids lacking inverted repeat DNA (pMDC10; Fig. 2-6, lane 3) or adenovirus terminal sequences (pIRBR; Fig. 2-10) also failed to generate replication-proficient molecules. These experiments clearly establish a *cis*-requirement both for adenovirus terminal sequences as well as inverted repeat sequences in the same molecule, and suggest that adenovirus replication is necessary both to produce and to amplify replication-proficient plasmid molecules. Fig. 2-6 also shows the results of experiments exploring changes in the architecture of plasmid molecules. pIRM contains a 1.1-Kb insertion at the *Nde*I site which increases the distance between the inverted repeat sequences. This 4.2-Kb molecule efficiently generated a new, 4.6-Kb molecule with two adenovirus termini (Fig. 2-6, lane 4). pIRBH contains a 346-bp insertion between the *Bam*HI and *Hind*III sites which increases the length between the adenovirus terminus and one copy of the inverted repeat sequences. This 3.5-Kb molecule also efficiently produced a new, 4.2-Kb molecule

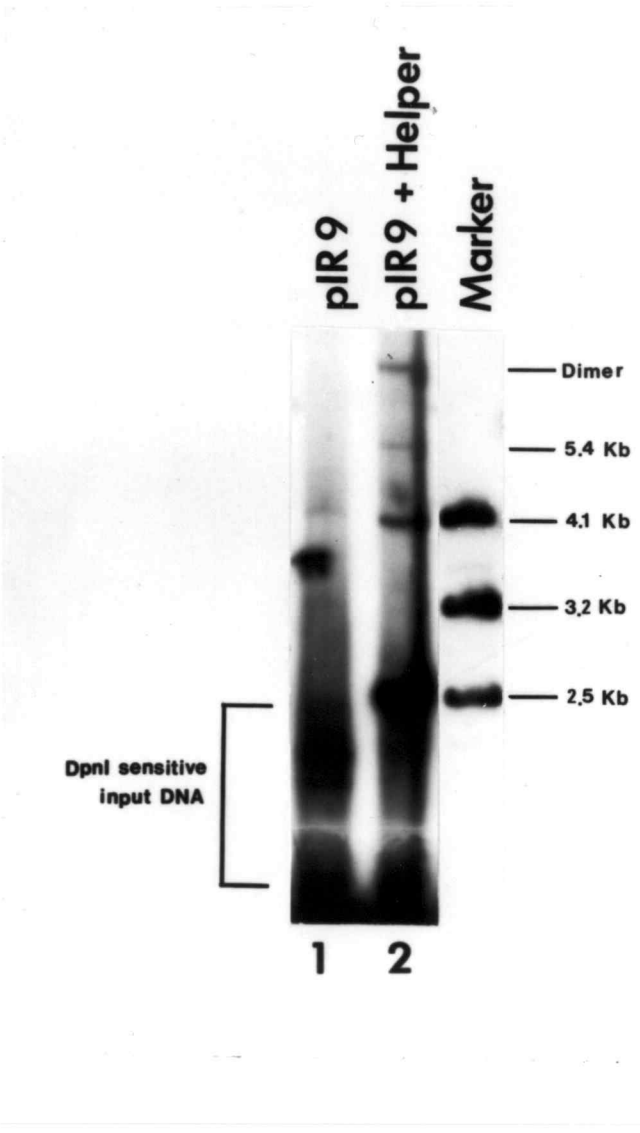

Figure 2-8. The replication of pIR9.

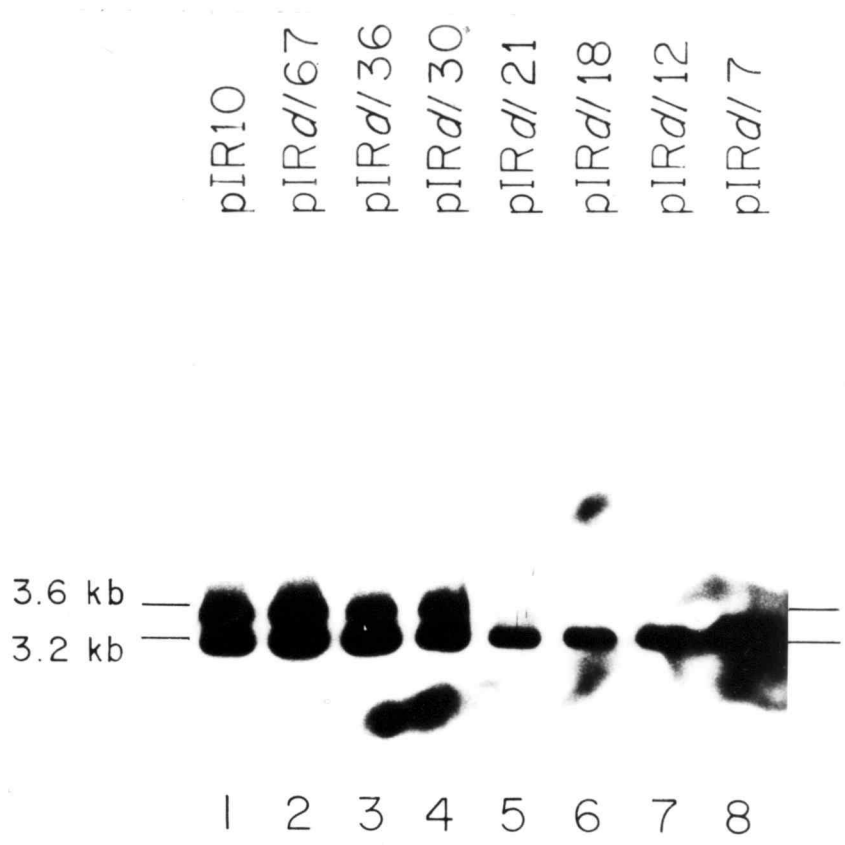

Figure 2-9. Replication of plasmids containing deletion mutations within the single copy of adenovirus terminal sequences.

Replication efficiencies of deletion mutants

Plasmid	Intensity of 3.6-Kb band[a] Experiment 1	Experiment 2
pIR10	(100)	(100)
pIRdl67	83	102
pIRdl36	23	20
pIIdl30	42	40

[a]. The data are expressed as precent of the intensity of the 3.6-Kb pIR10 band in each experiment. The intensity of each 3.6-Kb band (in arbitrary scanning units) was normalized to the intensity of the 3.2-Kb band in the same lane to correct for variable efficiencies of transfection, extraction, and blotting of plasmid molecules. The data in Experiment 1 came from a normal exposure of Figure 2-9 where the normalized intensity of the 3.6-Kb pIR10 band was 11,500 units. The normalized intensity of the 3.6-Kb pIR10 and in Experiment 2 was 27,900 units. Other deletion mutants could not be quantitated, but we estimated that the 3.6-Kb band of pIRdl21 could be no more than 1% of the wild-type level.

Table 2-1. Replication efficiencies of deletion mutants.

with two adenovirus termini (Fig. 2-6, lane 5). The newly generated adenovirus terminus in this case was 700 bp long compared to 360 bp for pIR10 or pIRM.

To test whether right adenovirus terminal sequences would also generate the replication-proficient molecules. EcoRI-linearized pIR9 (containing the right adenovirus ITR) was cotransfected into 293 cells with helper adenovirus DNA. Total DNA was isolated 60 hours after transfection. Prior to the loading, DNA was cut with DpnI which removes the unreplicated input DNA. Figure 2-8 shows that two bands were visualized with the pUC8 probe: a 4.1-Kb band corresponding to the input DNA and a 5.4-Kb band corresponding to the replication-proficient molecule (lane 2). Thus, the right adenovirus ITR can supply cis-required origin sequences.

The production of replication-proficient plasmid molecules provides a sensitive assay to determine which adenovirus terminal sequences are required in cis for replication. A clear advantage of this assay is that the replication products differ in size from the input molecules. Unidirectional deletion mutations penetrating from within adenovirus sequences toward the terminus were moved into plasmid molecules containing inverted repeat sequences as detailed in Fig. 2-3. Each of the EcoRI-linearized deletion mutants (Fig. 2-4) was tested for replication by transfecting 293 cells together with helper

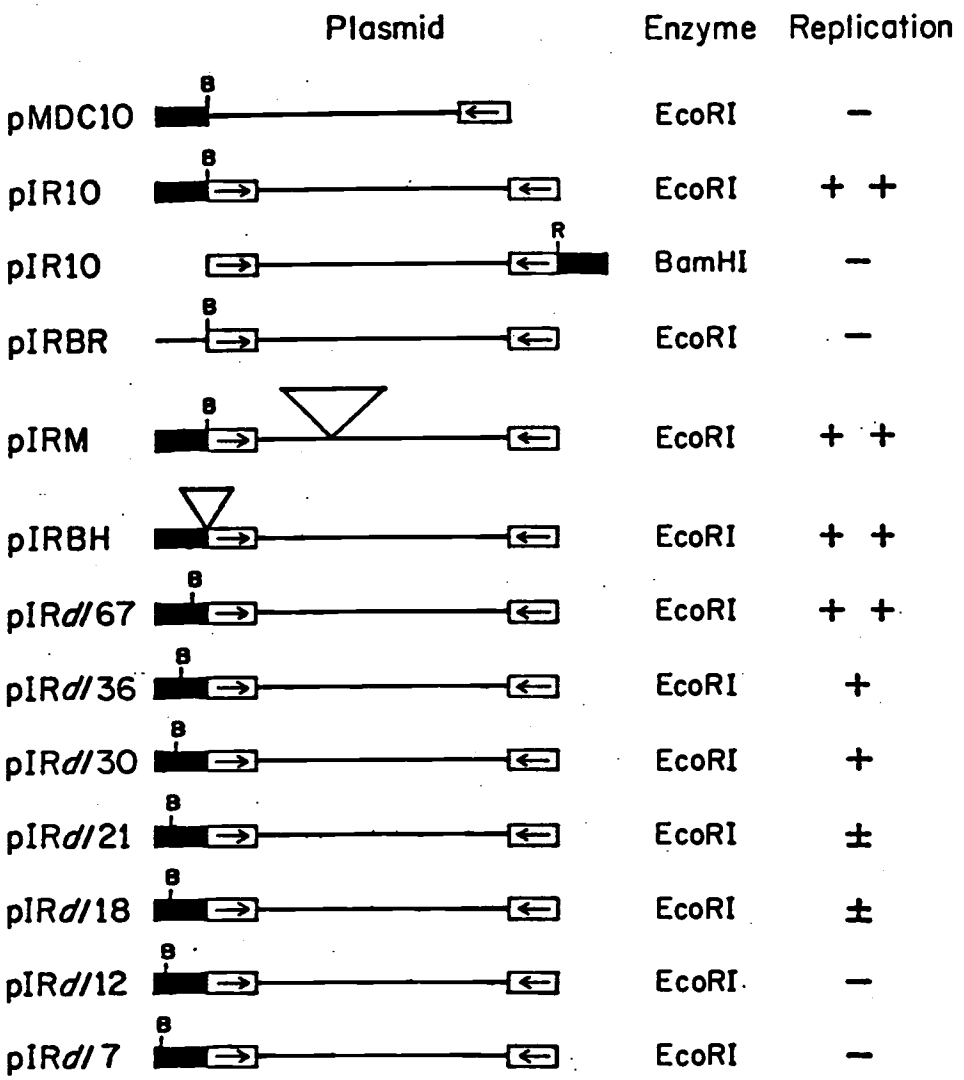

Figure 2-10. Summary of sequences required for adenovirus DNA replication in vivo.

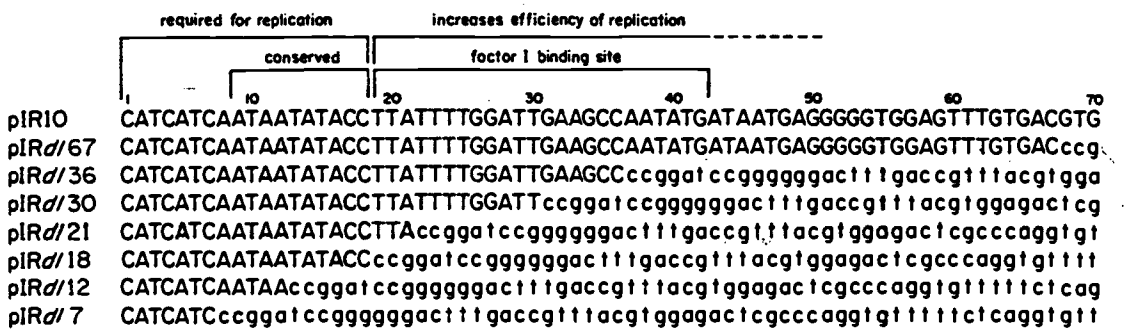

Figure 2-11 Nucleotide sequences of deletion mutations defining the adenovirus origin.

adenovirus DNA. Fig. 2-9 shows the Southern blot analysis of total DNA isolated 60 hr after transfection. Each input molecule was detected as a 3.2-Kb band after visualization with the pUC8 probe. Moreover, each of the 3.2-Kb bands, including the band for input pIR10, exhibited roughly equal intensities as expected. However, the mutants produced markedly different yields of replication-proficient molecules as measured by the intensity of the 3.6-Kb band in each lane of Fig. 2-9. The mutants could be sorted into at least four groups on this basis. pIRdl67 (lane 2), which retains the terminal 67 nucleotides of adenovirus, replicated fully as well as wild-type (pIR10, lane 1; see table 2-1). pIRdl36 (lane 3) and pIRdl30 (lane 4) both clearly gave rise to 3.6-Kb molecules, but the yield in each case was less than 50% of the wild-type level (Table 2-1). pIRdl21 (lane 5) and pIRdl18 (lane 6) were severely limited in the production of 3.6-Kb molecules. pIRdl21 generated no more than 1% of the wild-type level, and pIRdl18 produced an even lower level (visible on the original autoradiograph, but difficult to detect on the photographic reproduction). No 3.6-Kb molecules could be detected after transfection with pIRdl12 (lane 7) or pIRdl7 (lane 8). These results, summarized in Fig. 2-10, locate the adenovirus origin within the first 67 nucleotides of the adenovirus inverted terminal repetition. Futhermore, this region can be

divided into at least two functional domains as shown in Fig. 2-11. The first domain, termed the minimal replication origin, spans the first 18 to 21 adenovirus nucleotides and includes sequences highly conserved between all adenovirus serotypes (5). The minimal origin is absolutely required in *cis* for adenovirus replication. Deletion mutations penetrating (pIRdl12) or removing (pIRdl7) the conserved sequence in the minimal origin totally prevent adenovirus replication. The second domain increases the efficiency of the minimal origin by more than 100-fold. This auxilliary region lies immediately adjacent to the minimal origin and extends past nucleotide 36 but not past nucleotide 67. It encompasses sequences identified as the binding site for nuclear factor I (11, 12), a cellular protein required for efficient initiation of adenovirus replication *in vitro* (12-15). Mutants with altered binding sites for nuclear factor I do not initiate replication *in vitro* in factor I-dependent assays (12, 15), but do replicate in crude extracts (16-21). The entire binding site is apparently not required for efficient use of the minimal origin *in vivo* since removal of as much as the distal half of the site (pIRdl30) only reduced replication 2- to 3-fold (Table 2-1). Alternatively, sequences flanking deletions in pIRdl30 and pIRdl36 may partially restore the site for binding nuclear factor I *in vivo* (12). This may explain

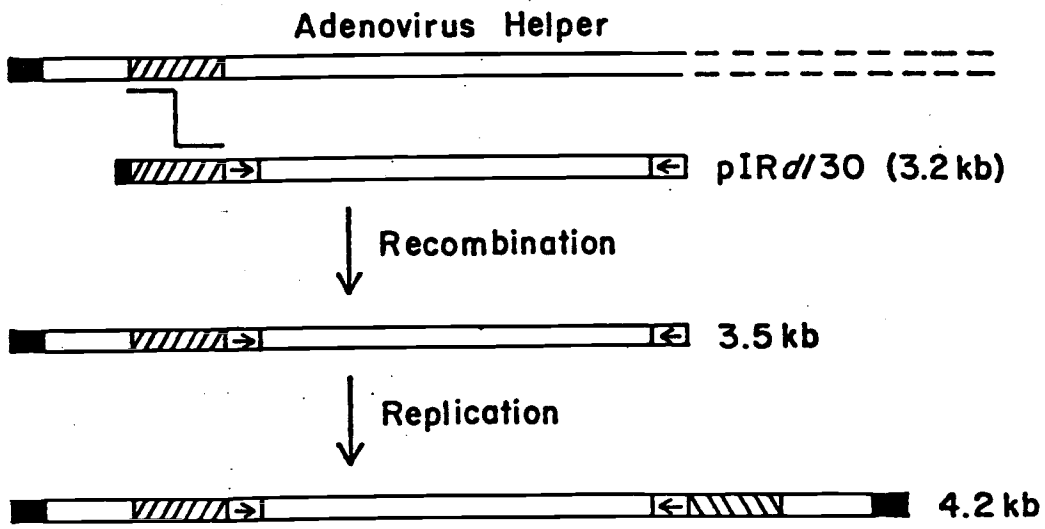

Figure 2-12. Hypothetical structure and replication properties of a recombinant plasmid produced by homologous recombination between pIRdl30 and helper adenovirus.

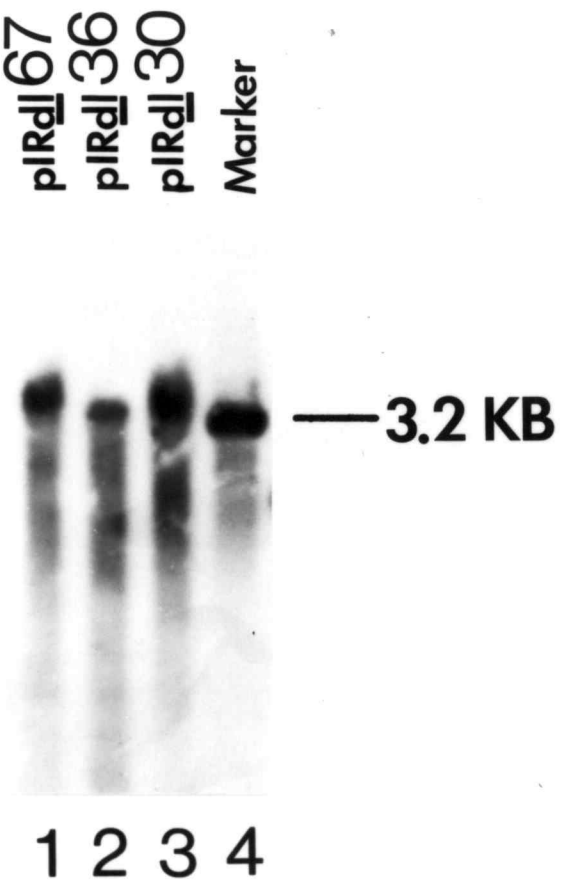

Figure 2-13 The transfection result with "ITR-less" plasmids.

why pIRdl30, with less of the binding site, replicated twice as well as pIRdl36 (Table 2-1). Nevertheless, the binding site for nuclear factor I clearly constitutes an important element of the adenovirus origin. In fact, serotypic (5) and host-specific (20) differences in the efficiency of adenovirus DNA replication may be due to variable binding of nuclear factor I to the adenovirus origin.

It is important to emphasize that we never observed homologous recombination between input plasmids and helper adenovirus molecules. Experiments with the deletion mutants provide a sensitive test for recombination. Fig. 2-12 also shows that recombination would alter the size of the input plasmids from 3.2-Kb to 3.5-Kb. In fact, such hypothetical recombinant plasmids would resemble pIRBH (Fig. 2-6, lane 5) both in size as well as structure and, like pIRBH, give rise to 4.2-Kb replication-proficient molecules. We did not detect 3.5-Kb bands after transfection with deletion mutants (Fig. 2-9). Moreover, we tested for recombination directly. ITR sequences were removed from all of the deletion mutants by cutting each plasmid with EcoRI and BamHI (see Fig. 2-4). Such "ITR-less" plasmids still contain adenovirus nucleotides 358 through 639. Replication-proficient molecules could not be rescued by recombination between helper adenovirus and any of the "ITR-less" deletion mutants (Fig. 2-13).

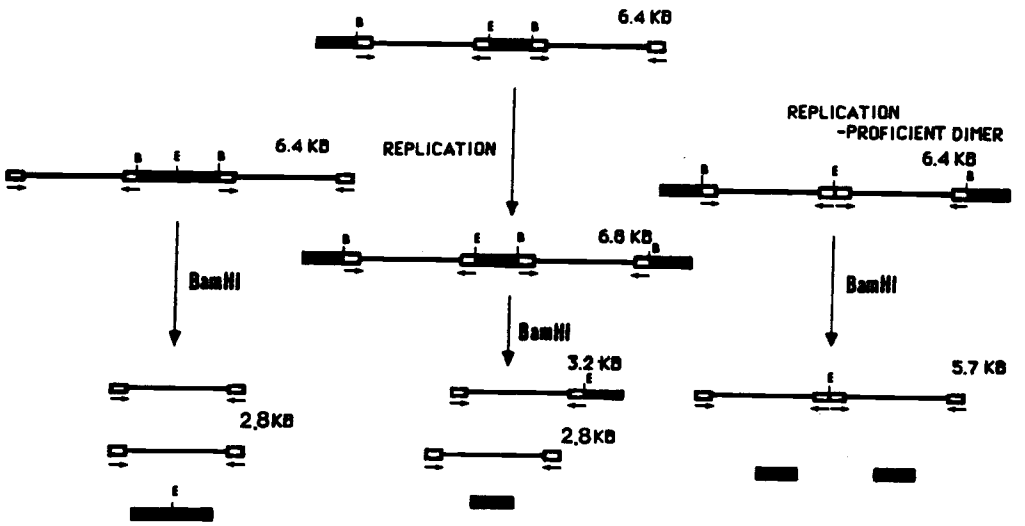

Figure 2-14. Diagram of possible dimer structures and BamHI digestion products.

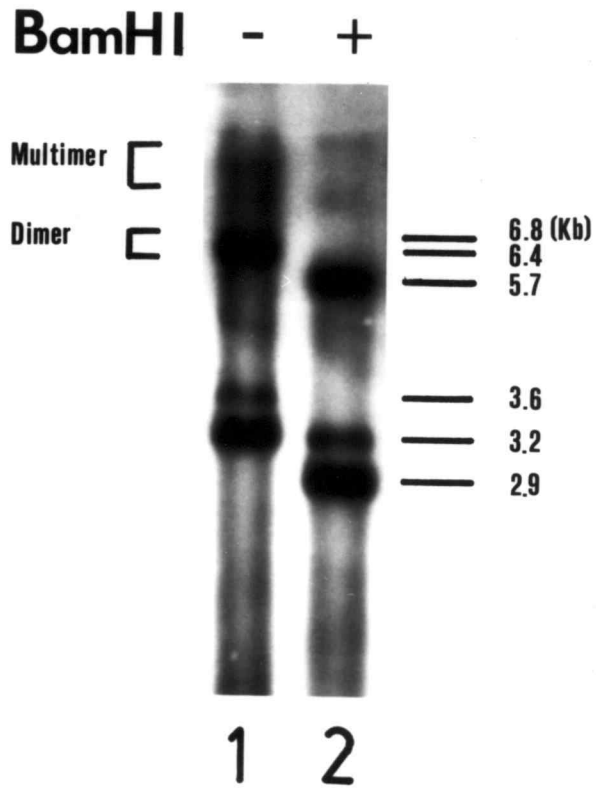

Figure 2-15. Analysis the dimer structure by cutting with BamHI

Fig. 2-9 also shows that the pUC8 probe visualized higher molecular weight products which correspond in size to oligomers of the input 3.2-Kb plasmids. The oligomers presumably arise from end-to-end ligation during transfection rather than by homologous recombination (see above). Only a small fraction of the input molecules apparently become joined together since oligomers were not detected after transfection with plasmids that replicate poorly (lanes 5 and 6) or not at all (lanes 7 and 8). In contrast, oligomers accumulated to observable levels after transfection with plasmids that replicate with wild-type (lanes 1 and 2) or near wild-type efficiencies (lanes 3 and 4). The polarity of joining may be restricted in order to form replication-proficient oligomers. Clues to the probable structure of oligomers come from the observation of 6.4-Kb as well 6.8-Kb dimer-sized molecules (6.4-Kb bands are visible in lanes 1 through 4, but the faint 6.8-Kb bands in the same lanes are difficult to detect on the photographic reproduction of the autoradiogram). A replication-proficient 6.4-Kb molecule could form by head-to-head joining (see Fig. 2-14) to give a simple dimer with adenovirus termini at each end. A simple, tail-to-tail dimer would not be expected to replicate since adenovirus termini would be fused together internally. On the other hand, a head-to-tail dimer could become replication-proficient only by generating a new

adenovirus terminus via the pathway outlined in Fig. 2-5. Such a molecule would be 6.8-Kb long. To test the hypothesis, DNA from cells which had been transfected with EcoRI-linearized pIR10 and adenovirus DNA were digested with BamHI. When hybridized with nick-translated pUC8 probe, both the 3.2-Kb input DNA and the 3.6-Kb replication-proficient molecules will generate the same 2.8-Kb band after cutting with BamHI. The head-to-head 6.4-Kb replication-proficient dimer will give a 5.7-Kb band. The tail-to-tail dimer will only give the 2.8-Kb band. But the 6.4-Kb head-to-tail dimer and the 6.8-Kb replication proficient dimer generated from head-to-tail joining give 3.2-Kb and 2.8-Kb fragments. Figure 2-15 shows the result of the Southern blot analysis. Lane 1, without BamHI digestion, shows the 3.2-Kb input, 3.6-Kb replication-proficient band, the 6.4-Kb dimer and a very faint 6.8-Kb replication proficient dimer. Lane 2 shows the effect of BamHI digestion. As predicted, only three bands, 2.8-Kb, 3.2-Kb and 5.7-Kb could be seen. Because several dimer molecules will generate the 2.8-Kb fragment, the intensity of the 2.8-Kb band is much stronger than other bands. Figure 2-15 also shows that some higher multimers are formed during the transfection process. Once cut with BamHI, all higher multimers give the same bands as the dimers.

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CHAPTER 3

ITR LENGTH REQUIREMENT FOR EFFICIENT CIS-REPLICATION

INTRODUCTION

The genome of human adenoviruses is a linear, double-stranded DNA molecule containing an inverted terminal repetition (ITR) of 63-166 bp (1, 2). The size depends on the serotype. A terminal protein is covalently attached to each 5' end (3). Adenovirus replicates by a strand displacement mechanism from origins of replication which are located within the first 67 nucleotides of the ITR region (4, 5 and see Chapter 2). Studies using the in vitro replication system have provided a considerable amount of information about the adenovirus origin and strand-displacement replication (see 4 for a recent review), but the system has not shed light on the mechanism for complementary strand synthesis. A panhandle structure, formed by pairing of the ITRs in the displaced single strand, was proposed by Lechner and Kelly (6) as an intermediate for complementary strand replication. Deletion mutants which carry different sizes of the ITR can be used to test the proposed role of the panhandle intermediate. Stow (7) has demonstrated that viral deletion mutants lacking the first 51 nucleotides of one

of the ITRs are still viable and generate virus with wild-type ITRs. Complete removal of one of the ITRs, however, gives no viable virus. This suggested that the ITR structure is required for viral replication, and the panhandle structure is an obligatory intermediate in DNA replication to allow the single wild-type copy of the ITR to serve as a template for the repair of the deleted ITR. Hay et al. (8) developed the first simple in vivo replication system to study adenovirus DNA replication. He showed that linear plasmid DNA containing adenovirus terminal sequences at both ends replicated autonomously when introduced into a cell with helper adenovirus DNA. They also reported that if a specially constructed plasmid containing a single adenovirus terminus, which was flanked by an inverted repeat sequence, was linearized to expose the adenovirus terminal sequence and cotransfected with helper virus DNA, the plasmid would then generate a larger product containing an additional adenovirus terminal sequence. The regeneration event signals the existence of a panhandle replication intermediate. Later, this system was adapted by Wang and Pearson (5, see Chapter 2) to determine the replication origin for adenovirus in vivo. A clear advantage of this system is that the requirement for a functional adenovirus origin is separated from size constraints on inverted sequences involved in panhandle formation. This method provides a

chance to study the length requirement of the ITR in adenovirus DNA replication without destroying the replication origin. Using plasmids carrying varying lengths of the adenovirus ITR, Hay showed that the replication origin was in the first 45 nucleotides of the ITR (9). Stow's and Hay's experiments indicated that ITRs as small as 45 bp would still replicate, but the minimum size was still unknown. Plasmids that carry the same terminal sequence but different sizes of inverted repetitous sequences were constructed. Linearized plasmids were cotransfected with adenovirus DNA into 293 cells. The generation of larger, repaired, replication-proficient bands can only be detected when a plasmid carrying an inverted repeat of 33 nucleotides or longer is used. This suggests that in order to form a stable panhandle structure, the inverted repeat sequence must not be less than 33 nucleotides. The state of replication for input DNA and the replication-proficient molelcules has also been monitored by sensitivity toward restriction endonucleases DpnI and MboI, a technique first described by Nathans et al. (10).

MATERIALS AND METHODS

REAGENTS AND ENZYMES

^{32}P-labeled deoxynucleotides (3,000 Curies/mmole) were from New England Nuclear. Restriction endonucleases,

E. coli DNA polymerase I, T4 DNA ligase, S1 endonuclease and exonuclease III were purchased from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim Biochemicals or Pharmacia. Pronase was from Calbiochem. Ribonuclease T1 and lysozyme were from Sigma. All of the enzymes were used according to the manufacturer's specifications.

CELLS AND VIRUS

The 293 cell line (11) was grown in Dulbecco's modified Eagle's medium with 5% (V/V) fetal calf serum. The Hela cell line was maintained in Joklik's minimal essential medium containing 5% (V/V) fetal calf serum. Adenovirus type 5 was grown in Hela cells; virus and viral DNA were isolated by the method of Pettersson and Sambrook (12).

PLASMIDS AND BACTERIA USED IN CONSTRUCTION OF CLONES

Plasmids pIR10 (5), pMDC10 (13), and pUC19 (14) have been described previously. Plasmid DNAs were isolated by the alkaline extraction method (15), and purified by ethidium bromide-CsCl density gradient centrifugation. E. coli RR1 was purchased from Bethesda Research Laboratories and used as recommended by the supplier. E. coli JC9387 recBC sbcB (16) were grown, transformed, and selected by standard procedures (17).

CONSTRUCTION OF DELETION MUTANTS

pIR10 molecules were linearized with EcoRI and

treated with exonuclease III at a concentration of 15-20 units/pmole of DNA. At this concentration, 10 to 15 nucleotides/min were removed (18). At various times, samples were removed and treated with S1 nuclease at a concentration of 40 units/pmole of DNA for 15 min at room temperature to remove single-stranded tails. The DNA was then treated with DNA polymerase I (Klenow fragment) to blunt the ends. DNA from different time points were ligated to an EcoRI linker and cloned in E. coli RR1 bacteria. Plasmid DNA from each ampicillin-resistant colony was digested with EcoRI and PvuII, and then loaded on a 4% polyacrylamide gel to screen for possible deletions. Since the deletion process extends bidirectionally from the original EcoRI site, part of the adenovirus terminal sequence in pIR10 was also deleted. To restore adenovirus sequences, the small, EcoRI-BamHI fragment from pIR10 was cloned back into the EcoRI-BamHI backbone of each deletion mutant.

CONSTRUCTION OF INVERTED REPETITIOUS POLYLINKER PLASMIDS

Figure 3-1 shows the procedure for constructing plasmids carrying inverted repeat sequences derived from the polylinker sequence of pUC19. The EcoRI-SspI fragment from pMDC10, containing adenovirus terminal sequences, was ligated to the pUC19 EcoRI-SspI backbone. An intermediate plasmid, called pT4, with 333 bp of the left end of adenovirus DNA and a pUC19 polylinker region (57 base

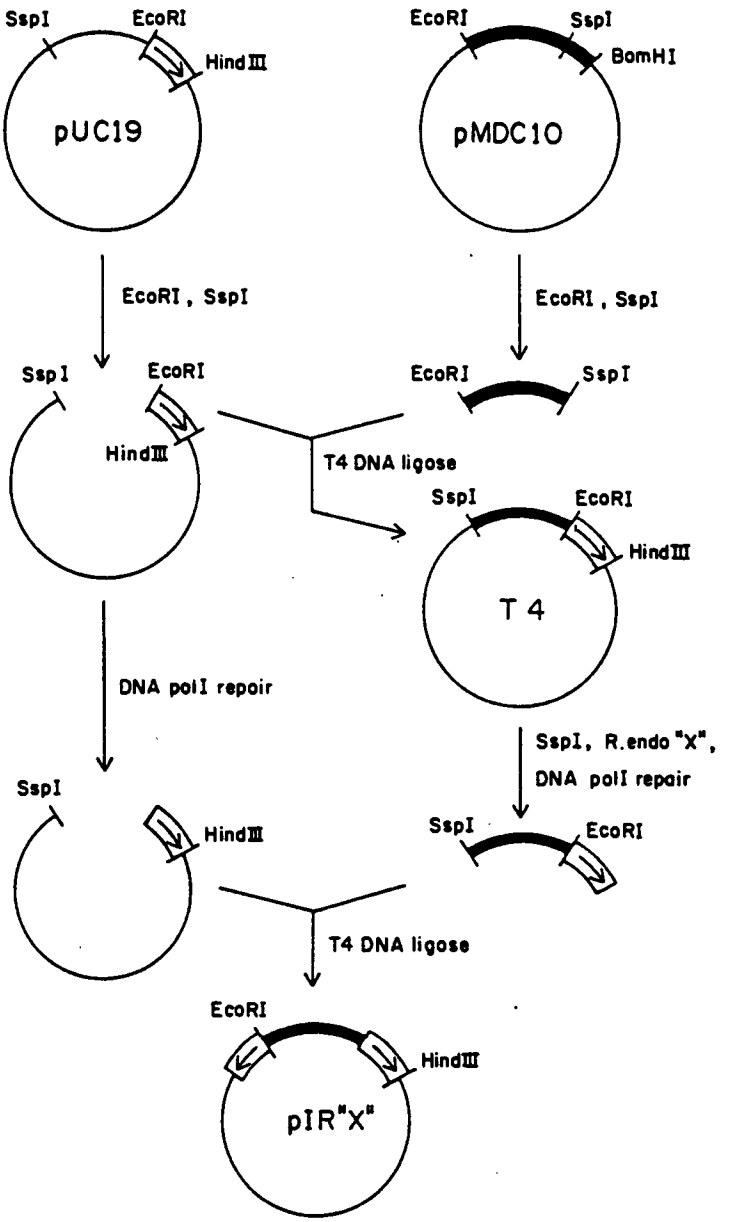

Figure 3-1. The construction of plasmids containing inverted repeats derived from the polylinker region of pUC19.

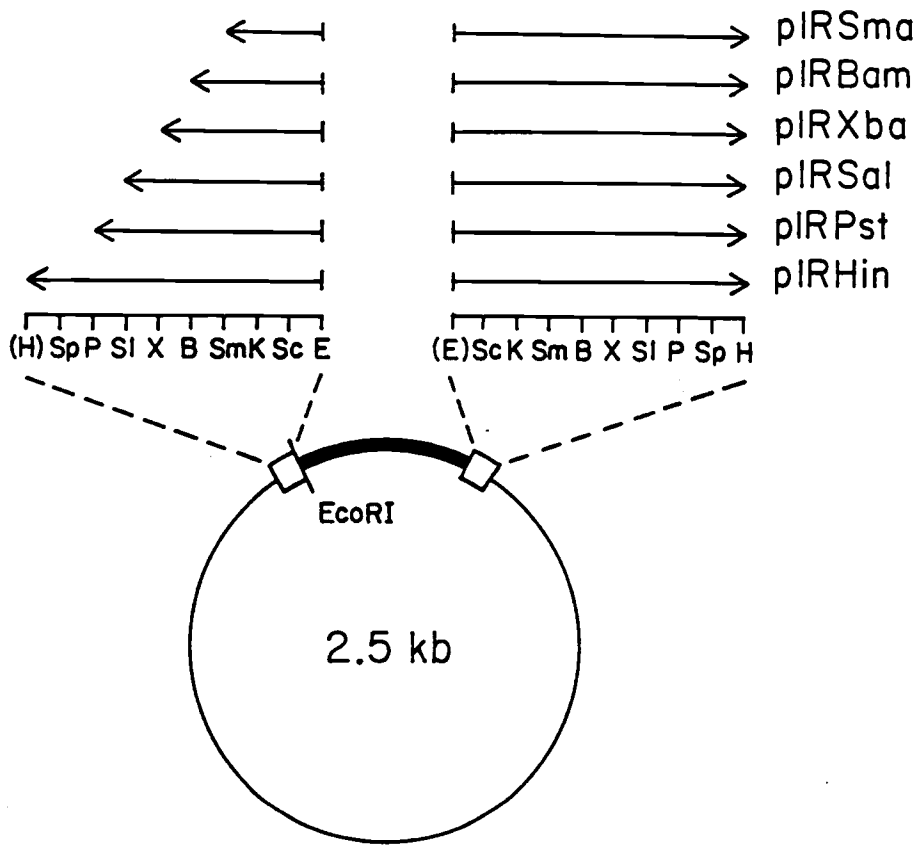

Figure 3-2. The structures of plasmids containing inverted repeats derived from the polylinker region of pUC19.

pairs) was selected from E. coli RR1 ampicillin-resistant clonies. pT4 was then cut with SspI and one of the restriction enzymes (restriction endonuclease "X") cutting within the polylinker region. The small fragment was isolated and blunted with DNA polymerase I. The purified fragment, which contains adenovirus terminal sequences and part of the pUC19 polylinker region, was ligated with the blunted pUC19 EcoRI-SspI backbone. E. coli RR1 was used for the transformation. Ampicillin-resistant clonies were screened with EcoRI and HindIII digestion. Clones that gave a 390-bp fragment were selected. These plasmids carried inverted repetitions ranging from 14 to 51 bp. In each case, inverted repetitions were separated by 336 bp of adenovirus left terminal sequences. All the plasmids were recloned into E. coli JC 9387 to stabilize the inverted repeat configuration. The structures of these plasmids are shown in Figure 3-2.

DNA SEQUENCING

Dideoxynucleotide chain termination sequencing was used to determine the sizes of inverted repetitious sequences in deletion clones. In brief, Avian Myeloblastosis Virus (AMV) reverse transcriptase was used in a dideoxy sequencing reaction to sequence double-stranded plasmid DNA (19). Double-stranded plasmid DNA (1μg) was denatured in 0.2 N NaOH, and renatured in 0.45 M potassium acetate (pH 4.5) in the presence of 100 ng of

the reverse sequencing primer (New England Biolab.). The DNA-primer mixture was precipitated with alcohol and resuspended in reverse transcriptase sequencing buffer which contained 10 mM Tris-HCl (pH 8.3 at 42°C), 40 mM NaCl, 5 mM DTT, and 10 mM $MgCl_2$. Reverse transcriptase (20 units) and 20 µCi of ^{32}P-labeled dATP (specific activity 600 Ci/mmol) were added to each sample. The mixtures were then divided into four tubes that already had the appropriate dideoxynucleotide-nucleotide concentrations. Reactions were carried out at 42°C. After incubation, 6 µl of stop solution containing 90% formamide were added to each reaction mixture, then samples were boiled for 3 min before being loaded onto an 8% polyacrylamide gel (25 x 35 x 0.03 cm) containing 8 M urea. Electrophoresis was carried out at 1200 V for 2 hr. The gel was then dried and autoradiographed.

TRANSFECTION

293 cells were transfected according to the standard DNA-calcium phosphate coprecipitation method (20). Transfections utilized 5 µg of the plasmid DNA and 5 µg helper adenovirus DNA. Each 60 mm plate was inoculated with 4 x 10 ^{5} cells 24 hours before transfection. Four hr after transfection, cells were shocked for 1 min with 25% glycerol.

EXTRACTION AND ANALYSIS OF DNA

Total DNA was isolated from transfected 293 cells by

a rapid nuclear isolation method (21), followed by a modified Hirt extraction (22). In brief, cells were harvested from the plate using a rubber spatula and spun-down on a desk top centrifuge for 10 minutes at 1000 rpm. After washing with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4), the cell pellet was resuspended in buffer containing 0.01 M NaCl, 0.01 M Tris-HCl (pH 7.4), 0.0015 M $MgCl_2$, and 0.5% NP-40, and placed on ice for 10 min. The cell suspension was centrifuged in a microfuge for 1 min in a cold room. Total DNA was extracted by resuspending the nuclear pellet in 500 µl of 0.6% Sodium dodecyl sulfate (SDS), 10 mM EDTA and Pronase at a final concentration of 1 mg/ml, and incubating at 37°C for 6 to 8 hr. After phenol extraction and ethanol precipitation, purified DNA was resuspended in 300 µl of TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Approximately 1/5 of the DNA isolated from each plate was applied to each lane of a 1% agarose gel. Before loading, some of the samples were cut with EcoRI to reduce viscosity, and some of the samples were cut with other restriction enzymes, or DpnI, or MboI. After gel electrophoresis, DNA was transferred to a Gene Screen Plus membrane (New England Nuclear) by the method of Southern (23). After prehybridization for 10 to 16 hr at 65°C in 5X SSC, 0.5% SDS and 5X Denhardt's solution, the membrane was hybidized with denatured, nick-translated pUC8 DNA

Figure 3-3. DpnI and MboI recognition sequences.

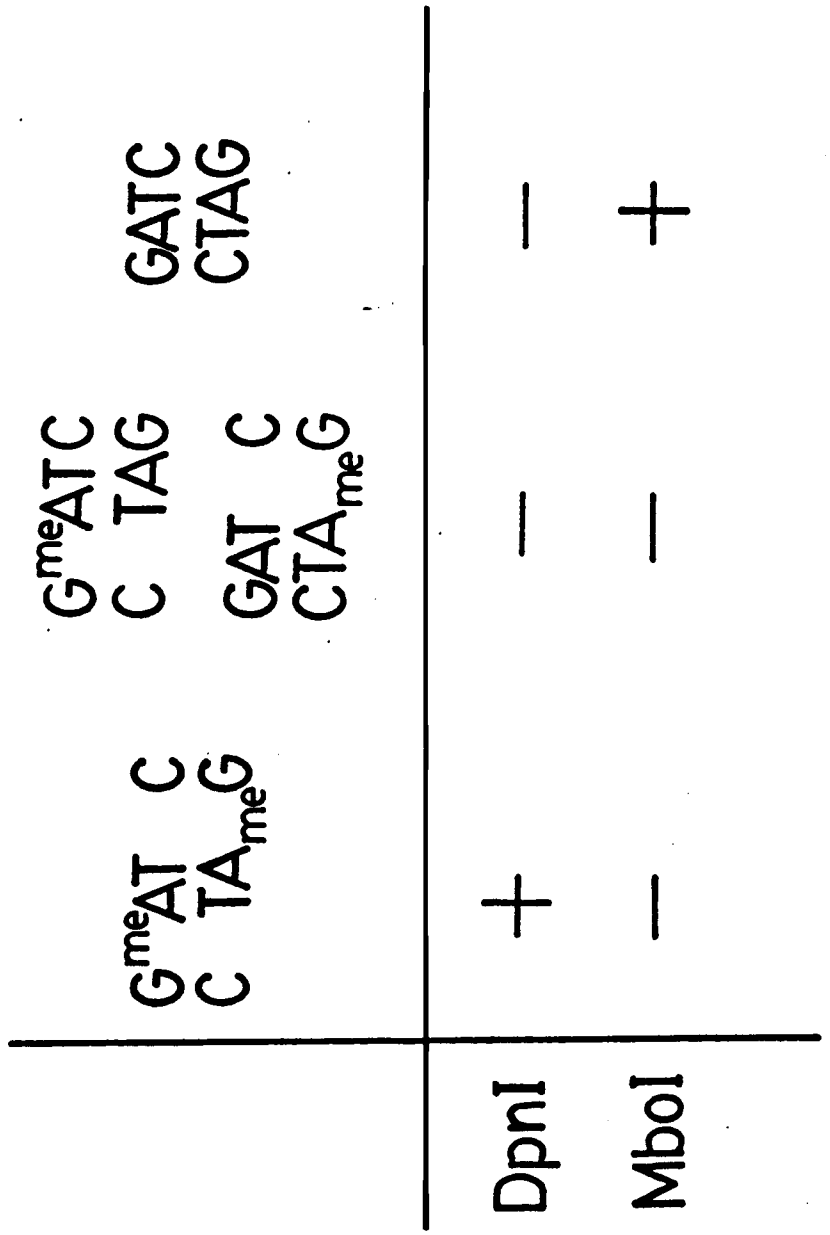

Figure 3-3

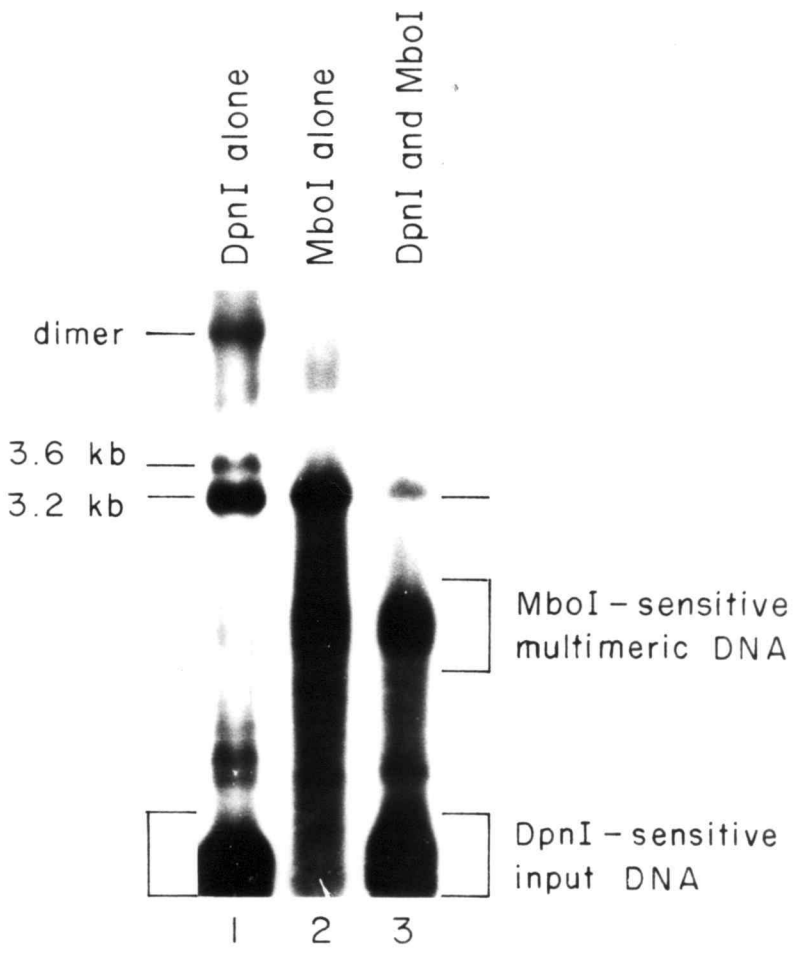

Figure 3-4. Southern blot analysis of pIR10 replication in vivo. DNA was isolated 60 hours after transfection with helper adenovirus DNA. Lane 1: DNA cut with DpnI alone. Lane 2: DNA cut with MboI alone. Lane 3: DNA cut with both DpnI and MboI.

(specific activity > 1 x 10^8 cpm/μg) in 5X SSC, 0.5% SDS and 1X Denhardt's solution. After washing, the membrane was autoradiographed using Kodak XAR-5 X-ray film.

RESULTS AND DISCUSSION

Hay et al. (8, 9) and Wang and Pearson (5, see Chapter 2 for details) have shown that linear plasmid DNA containing a single adenovirus terminal sequence and a specific geometrical arrangement of inverted repeat sequences will generate replication-proficient molecules in the presence of helper adenovirus. To investigate this process further, a strategy adapted from Nathans et al. (10) has been used. Plasmid DNA propagated in dam$^+$ E. coli hosts will have the sequence GATC converted to GmeATC by site-specific methylation. GATC is the recognition site for the restriction endonucleases DpnI and MboI. However, DpnI cuts the site only when both strands are methylated, while MboI cuts the site only when both strands are unmethylated (Fig. 3-3). After transfection into mammalian cells, which do not methylate the sequence GATC, input plasmid DNA which has not or can not replicate will be digested with DpnI but not MboI. On the other hand, input DNA undergoing strand-displacement replication becomes DpnI-resistant, but also remains MboI-resistant. Replication-proficient molecules, are sensitive to MboI but not DpnI. To test this scheme, EcoRI-linearized pIR10

DNA was transfected into 293 cells with helper adenovirus DNA. Total DNA was isolated 60 hours after transfection. The DNA was cut with DpnI alone, MboI alone, or both enzymes together before being loaded onto a gel. Figure 3-4 shows the result of Southern blot analysis. Several bands were visualized with the nick-translated pUC8 probe when the DNA was cut with DpnI alone (lane 1). The 3.2-Kb band corresponds to the input pIR10 DNA which has gone through at least one round of strand displacement replication. The 3.6-Kb replication-proficient molecules are also resistant to DpnI digestion as predicated. Bands in the lower part of the gel arise from non-replicated input DNA still sensitive to DpnI cleavage. Lane 2 shows the result of MboI digestion. Since the 3.2-Kb input pIR10 DNA, whether it has been replicated or not, has at least one strand still carrying methylated GATC sequences, it is always resistant to the restriction endonuclease MboI. The 3.6-Kb replication-proficient molecules can be either hemi-methylated (resistant to MboI), or totally unmethylated (sensitive to MboI). Only a very strong 3.2-kb input DNA could be seen when the DNA was cut with MboI alone (lane 2). There was no detectable 3.6-kb MboI-resistant band. This indicates that the process to form the panhandle structure and to generate the replication-proficient molecules is a rate limiting step. Once molecules carrying adenovirus terminal sequences at both

ends have been generated, multiple rounds of replication occur on these molecules, and the contribution of the original displaced parental strand is diluted out. This observation supports the assumption that the 3.6-Kb replication-proficient band is detectable due to amplification by adenovirus-specific replication. Lane 3 indicates the result of combining DpnI and MboI digestion. Only molecules carrying hemi-methylated GATC sites can be seen. As expected, only the 3.2-Kb input DNA that has gone through at least one round of displacement replication can be seen. This experiment also shows that dimers of input pIR10 DNA can also go through the replication cycle to generate DpnI-resistant molecules as shown in lane 1. The DpnI-resistant dimer molecules are sensitive to MboI (lanes 2 and 3). This indicates that the dimer band in lane 1 is the product of a head-to-head joint which generates a 6.4-Kb replication-proficient molecule. This experiment also suggests that the dimerization event during transfection is not an efficient process since no MboI-resistant dimer bands can be detected. Thus, DpnI-resistant dimers seen in lane 1 arise as the result of replication to amplify the head-to-head ligation products.

The production of replication-proficient molecules needs not only a replication origin on the plasmid but also a suitable length of inverted repetitious sequence. This system provides a sensitive way to determine

Figure 3-5. DNA sequences of pIRD1 and pIR10.

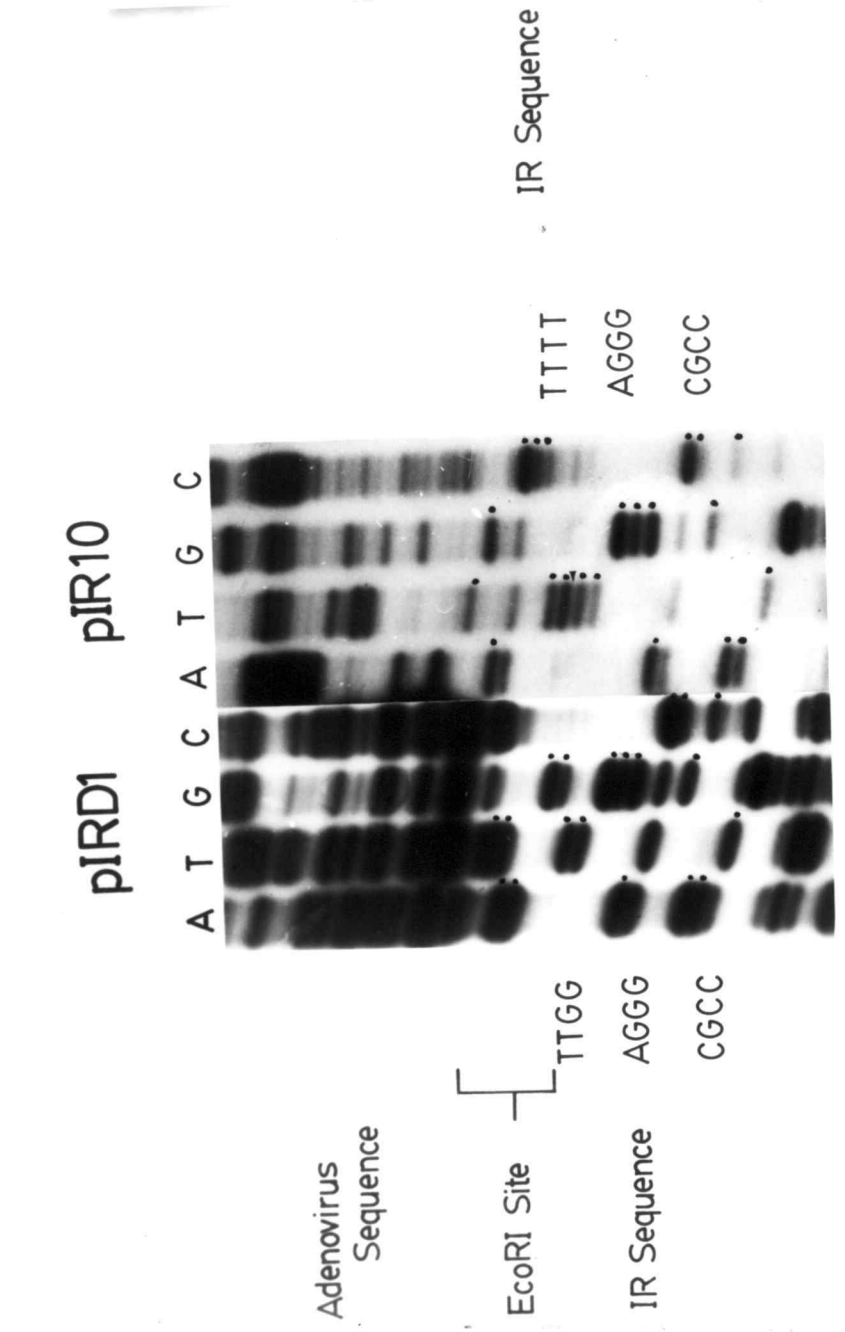
pIRD1
pIR10
A T G C A T G C
IR Sequence
TTTT
AGGG
CGCC
Adenovirus Sequence
EcoRI Site
TTGG
IR Sequence
AGGG
CGCC

Figure 3-5

Figure 3-6. The sequences of pIRSal and pIRXba. The arrows indicate the end of polylinker sequence.

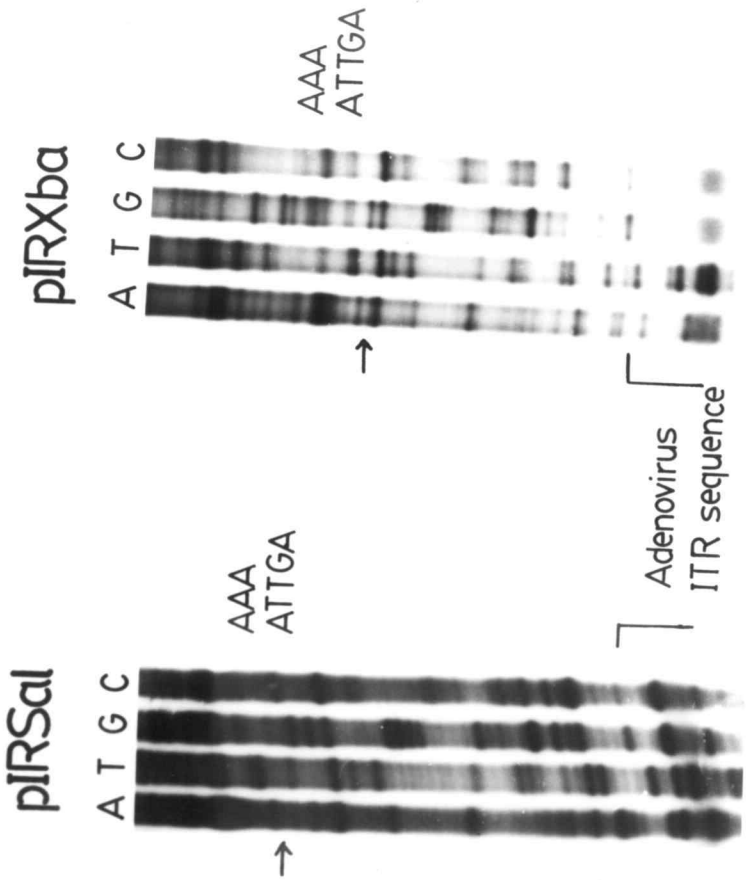
pIRXba
A T G C
AAA
ATTGA
pIRSal
A T G C
AAA
ATTGA
Adenovirus
ITR sequence

Figure 3-6

Figure 3-7. Nucleotide sequences of inverted repetitious sequences.

PLASMIDS	IR SEQUENCES
pIR10	GCTGGCGAAGGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGAGGGCCAGTG
pIRD1	GCTGGCGAAGGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTT
pIRHin	CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCT
pIRPst	CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACC
pIRSal	CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA
pIRXba	CGAGCTCGGTACCCGGGGATCCTCTAGA
pIRBam	CGAGCTCGGTACCCGGGGATC
pIRSma	CGAGCTCGGTACCC

Figure 3-7

adenovirus sequences required for the initiation of replication (see Chapter 2). It also provides a sensitive method to investigate the length of the inverted repetitious sequence believed to be required for complementary strand synthesis. Evidence to date shows that a plasmid carrying 45 bp of the terminal inverted repetitious sequence can replicate (9). Two attempts have been made to determine the minimum size required for panhandle formation. One approach is to use exonuclease III and S1 endonuclease to make deletions in one of the inverted repetitious sequences in pIR10. A deletion mutant, called pIRD1, was constructed and contains 54 bp of inverted repeat sequences in contrast to 89 bp in pIR10. The lengths of the inverted repeat sequences have been determined by DNA sequencing (Fig. 3-5). Since the procedure to construct deletions in pIR10 is complicated and the sizes of deletions are difficult to verify except by DNA sequencing, a new approach was made by using the polylinker region from pUC19 as the inverted repetitious sequences. Two obvious advantages for this procedure are that it is easy to verify the size of the inverted repeat and easy to construct deletion or insertion mutants within the inverted repeat region to study repair mechanisms for maintaining the size of the inverted repetitious sequence in adenovirus. Restriction endonuclease sites in the polylinker region which have been selected for the

Figure 3-8. Southern blot analysis of the replication of plasmids containing different sizes of inverted repeat sequences. DNA was isolated 60 hours after transfection. Samples were cut with DpnI before loading onto the gel.

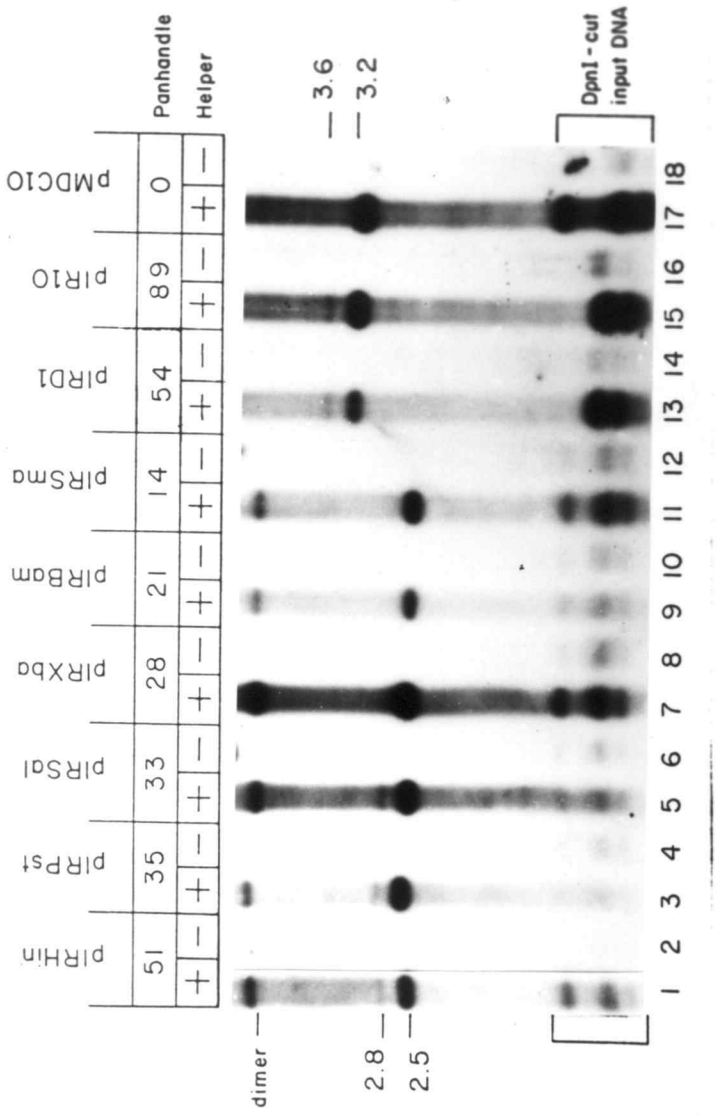
Panhandle
Helper
pMDC10
pIR10
pIRD1
pIRSma
pIRBam
pIRXba
pIRSal
pIRPst
pIRHin
0
89
54
14
21
28
33
35
51
3.6
3.2
DpnI-cut input DNA
dimer
2.8
2.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3-8

construction of plasmids are HindIII, PstI, SalI, XbaI, BamHI and SmaI. These sites will give inverted repetitious sequences ranging from 51 bp with HindIII to 14 bp with SmaI (see Figs. 3-2 and 3-7). All the plasmids carry the same adenovirus terminal sequences, but have different lengths of inverted repeat sequence. The structures of these plasmids are shown in Figure 3-2. The adenovirus origin can be exposed by cutting at the single EcoRI site in each plasmid. The size of the inverted repeat has been determined by restriction endonuclease digestion and confirmed by DNA sequencing. Figure 3-6 shows the inverted repeat sequences of pIRSal and pIRXba. Base composition should not affect the stability of the short duplex regions in the panhandle structure because these plasmids have inverted repeats with roughly the same GC content (see Table 3-1). The distal half of the adenovirus inverted terminal repeat also has a similar GC content.

Each of the EcoRI-linearized plasmids (Fig. 3-2) was tested for its ability to generate replication-proficient molecules by transfecting 293 cells with and without adenovirus DNA. Figure 3-8 shows the Southern blot analysis of total DNA isolated 60 hr after transfection. Samples were treated with the restriction enzyme DpnI before loading. Each of the input polylinker plasmids was detected as a 2.5-Kb band in the presence of helper

Figure 3-9. Kinetics of generating replication-proficient molecules from plasmids with different sized panhandles.

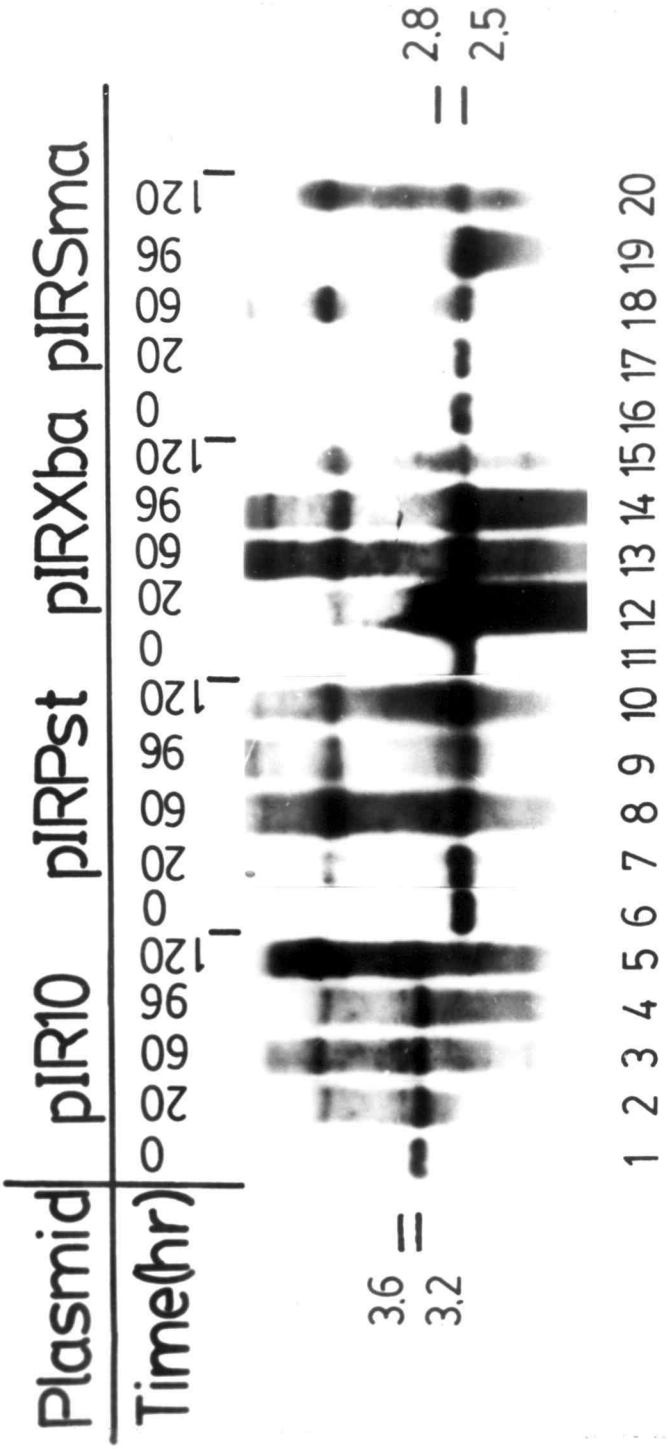
Plasmid
Time(hr)
pIR10
pIRPst
pIRXba
pIRSma
0
20
60
96
120
3.6
3.2
2.8
2.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 3-9

adenovirus. Displaced single strands will generate replication-proficient 2.8-Kb molecules if inverted repeat sequences are long enough to form stable panhandle intermediates. pMDC10, which has the same geometry and sequence as pIR10 (except for the inverted repeat configuration), was used as a negative control (lanes 17 and 18). pIRD1 (lanes 13 and 14) has 54 bp of inverted repeat sequence and replicated just like pIR10 (lanes 15, 16). It also generated 3.6-Kb replication-proficient molecules despite the removal of 35 bp from the inverted repetitious sequence. Polylinker plasmid pIRHin, which has 51 bp of inverted repeat sequence, replicated as well as pIRD1 and pIR10 did (lanes 1 and 2), even though the inverted repeat sequences are different in these clones. pIRPst and pIRSal, which contain 35 bp and 33 bp of inverted repeat sequence respectively, also replicated as well as pIRHind (lanes 3 through 6). No 2.8-Kb band could be detected after transfection with pIRXba, pIRBam and pIRSma (lanes 7 through 12). The generation of DpnI-resistant input molecules as well as replication-proficient molecules depends on the presence of adenovirus helper (Fig. 3-8; compare even and odd lanes). Differences in the ability to generate replication-proficient molecules are not due to different rates of producing displaced, single strands since all the polylinker plasmids contained the same adenovirus terminal

Table 3-1 Size and GC-content of inverted repetitious sequences in various plasmids.

PLASMID	SIZE	GC-CONTENT (%)	REPLICATION
pIRHin	51	62.7	+
pIRPst	35	65.7	+
pIRSal	33	63.6	+
pIRXba	28	66.6	−
pIRBam	21	71.4	−
pIRSma	14	71.4	−
pIR10	89	57.3	+
pIRD1	54	59.2	+

Table 3-1

sequences. This is supported by fact that the DpnI-resistant input 2.5-Kb bands were roughly equal in intensity despite the different capabilities to generate the 2.8-Kb replication-proficient bands. The difference in panhandle sizes between pIRSal, which yields 2.8-Kb molecules, and pIRXba, which does not, is only 5 bp. Kinetic studies show that rate of producing replication-proficient molecules is independent of the sequence or the length of the inverted repeat so long as the length is equal or longer than 33 bp (Fig. 3-9). This result indicates that there is a minimum length requirement for the double-stranded panhandle region. It is interesting to notice that the length requirement to form a stable panhandle structure is only 33 bp and this is much shorter than the inverted repeat sequence found in adenovirus. The function for the rest of the inverted repeat region now becomes more interesting. Although the process to form replication-proficient molecules is not efficient, since most the replication-proficient molecules are MboI-sensitive, this may not be the case for adenovirus itself. The ITR regions in displaced strands are associated with viral DNA polymerase (probably by protein-protein interactions between terminal protein and adenovirus DNA polymerase) during displacement synthesis (24). Thus, the local concentration of ITR sequences may be much higher than in ordinary hybridization. This may increase the

efficiency of forming panhandle structures. Several proteins have been found to bind to the inverted repeat region. These proteins may not only regulate the efficiency of replication (such as NF-I and NF-III) but also facilitate the formation and stabilization of the panhandle structure.

Bacteriophage ø 29, like adenovirus, has a linear DNA molecule with a terminal protein covalently attached to the 5' end of each strand. It also uses a strand displacement mechanism to replicate (see 25 for a recent review). However, it has only 6 bp of inverted terminal repeat sequence, which is much smaller than the minimum number of 33 bp determined here. This suggests that other replication mechanisms, instead of panhandle formation, may be involved in the replication of the displaced strand of ø 29. A replication mechanism called trans-replication, which involves an interstrand pairing event between two displaced, single-stranded, complementary strands, has been proposed recently for adenovirus complementary strand replication (K. G. Ahern, et al. manuscript in preparation). This trans-replication mechanism does not require a panhandle intermediate and might be used by bacteriophage ø 29.

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CHAPTER 4

CONCLUSIONS

Most studies on adenovirus DNA replication have been done *in vitro* using plasmid DNA containing a single adenovirus terminal sequence. These experiments provide information largely about displacement synthesis, 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 separating displacement synthesis from complementary strand synthesis *in vivo*.

The work presented here uses an approach developed by Hay *et al.* (1) to study the replication of displaced single strands (complementary strand replication) *in vivo*. This replication system not only provides a sensitive method to detect the complementary strand replication but also reveals a possible mechanism for the replication of displaced, single-stranded DNA.

SUMMARY OF RESULTS

Specially constructed plasmids which carry a single adenovirus terminal sequence and inverted repeat sequences were linearized to expose the adenovirus terminal

sequence. The linear plasmid DNA was transfected into 293 cells with helper adenovirus DNA. Sixty hours after transfection, total DNA was isolated, electrophoresed, blotted, and hybridized with vector probe. In addition to the input DNA, a higher molecular weight molecule was detected. This newly generated molecule has adenovirus terminal sequences at both ends. It can replicate autonomously with the help of adenovirus. Generation of this replication-proficient molecule depends on the presence of helper adenovirus, a viral replication origin on the plasmid, and artificial inverted repeat sequences. The origin sequence needs to be exposed at the end of plasmid molecule. The artificial inverted repeat sequences need not be adenovirus sequences; part of the lac gene from pUC8 plasmid (such as pIR10, pIRD1, and pIR9) or polylinker region from pUC19 (such as pIRHin, pIRPst and pIRSal) have been used. The generation of replication-proficient molecules presumable occurs via a "panhandle" intermediate which forms by intrastrand pairing of the inverted repetitious sequences on the displaced single strand. This replication system separates the sequences controlling the initiation of replication from inverted repetitious sequences which are required to form "panhandles". It provides the opportunity to study these two requirements independently.

Using this approach, the replication origin for

adenovirus and the minimum length of the inverted repetitious sequences needed to generate replication-proficient molecules have been determined *in vivo*. The adenovirus replication origin was completely contained in the first 67 nucleotides of the adenovirus inverted terminal repeat (ITR) region. This region contains binding sites for nuclear factor I (NF-I) and nuclear factor III (NF-III), and has a highly conserved sequence found among different adenovirus serotypes. The origin can be divided into at least two parts, a minimal replication origin and a stimulatory region. The minimal origin is absolutely required for replication. It contains the conserved sequence encompassing nucleotides 9 to 18 of the ITR. Mutations or deletions which alter sequences in this region drastically inhibit replication. The stimulatory region contains NF-I and NF-III binding sites. In the presence of these stimulatory sequences, the efficiency of replication markedly increases both *in vitro* and *in vivo*.

The minimum length of the inverted repetitious sequences required for the generation of replication-proficient molecules has also been determined. Inverted repeats must be at least 33 nucleotides long. Inverted repeats shorter than 33 nucleotides will not generate the replication-proficient molecules. Kinetic studies indicate the replication-proficient molecules can be

detected as early as 24 hours after transfection. Plasmids carrying inverted repeats of 28 nucleotides or less will not generate the replication-proficient molecules, even 144 hours after transfection.

IMPLICATIONS OF THIS STUDY

The results of this study not only define the adenovirus replication origin and establish the ITR length requirement *in vivo*, but also reveal possible mechanisms of adenovirus complementary strand synthesis and the function(s) of adenovirus ITR. The presence of ITRs and terminal protein has been demonstrated in linear mitochondrial DNAs S1 and S2 from cytoplasmic male-sterile maize (2), linear plasmids from *Streptomyces rochei* (3), yeast (4) and bacteriphages (5). Most of the mechanisms of replication and initiation for these systems are unknown. Methods used in the studies here may also help to establish the modes of replication for these systems.

THE SIZE OF ADENOVIRUS ITR: OTHER FUNCTIONS IN THIS REGION

Most of the adenoviruses have ITR sequences longer than 67 bp (6). The results here show that first 67 bp of the ITR not only contains the entire replication origin but also is large enough to support complementary strand

replication. The sizes and sequences of ITRs in each adenovirus serotype have been highly conserved. Mutations in this region must be repaired. One might imagine that longer ITRs might be needed to form stable panhandle intermediates more efficiently. In this view, any mutation which increases the size of ITR might be preserved since the mutant panhandle would be more stable than that of the wild type. However, no trend toward increasing ITR length has yet been found for any adenovirus serotype. This suggests that the ITR, especially the second half of the ITR, might 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 which might be needed during virus infection or replication.

Graham et al. (7) reported that up to 15% of the adenovirus DNA isolated from adenovirus type 5 infected BRK or HeLa cells were covalently linked into head-to-tail circular DNA molecules. This circular form can be detected as early as 3 hr after infection and remains present as late as 120 hr post-infection. The circular adenovirus DNA can not be assembled into virions by the viral packaging system. Transfection of synthetic circular adenovirus DNA into HeLa or BRK cells generates wild-type linear virus (8). The replication of circular virus DNA may occur by a mechanism proposed by Hay et al.

(1). The formation of covalently linked circles may, therefore, be a part of the "normal" adenovirus replication cycle. Covalent circles may be one of the intermediate(s) of adenovirus DNA replication or important for gene transcription. The ITR sequences beyond the first 67 bp may have a special role involving the circular form of viral DNA or the formation of circular DNA. Mechanisms to form circular viral DNA and the function of this circular DNA still remain to be studied.

Recently, a very sensitive gel retention assay for the detection of DNA-binding proteins in crude cell extracts has been developed by Schneider et al. (9). They detected protein(s) which bind the ITR sequence between nucleotides 60 to 94. Sequences in this region are highly G-C rich (76%) and include transcription factor Sp1 binding sites (GGGCGG) (10). Whether Sp1 actually binds in the second half of ITR during the adenovirus lytic cycle is still unknown. Rijnders et al. (11) showed that the 5'-end of the displaced, single strand might be carried along by the adenovirus DNA polymerase during displacement synthesis. The efficiency of the formation of panhandle intermediates would be increased by this mechanism.

DNA HYBRIDIZATION IN VIVO

Extensive studies have been done on DNA hybridization

in vitro. However, litle is yet known about DNA hybridization *in vivo*. Single-stranded DNA hybridization is needed in the *in vivo* replication system discussed here. Using different synthetic oligonucleotides as inverted repeat sequences in the plasmids used in this replication system will provide a sensitive approach to study nucleic acid hybridization *in vivo*.

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. Studies have investigated the requirements for homologous sequences during recombination. By processively decreasing the length of homologous sequences, Rubnitz and Subramani (12) reported that 14 bp of homologous sequences were sufficient for recombination in CV1 cells. Ayares *et al.* (13) reported that 25 bp of homologous sequences were required for recombination in EJ human bladder carcinoma cells and COS-1 monkey kidney cells. In the *cis*-replication system at least 33 bp of inverted repeat sequences are required to form a stable or usable panhandle structure, an example of intrastrand hybridization. The homologous sequences needed in the replication system are longer than the requirement for *in vivo* interstrand hybridization which are detected by homologous recombination. Possible reasons for these

differences can be imagined: a) different lengths may be required for inter- and intra-strand hybridization; b) adenovirus DNA binding protein (see Chapter 1) may stablize single-stranded DNA and destablize the panhandle intermediate (a longer sequence may be required to overcome this effect so that a stable panhandle structure forms); c) 33 bp may not be the minimum length required to form a stable panhandle structure, rather a longer double-stranded region may be required for DNA polymerase (or other enzymes) to bind; or d) 33 bp may required to overcome cellular exonucleases which decrease the inverted repeat size after transfection. Specifically designed experiments to solve this puzzle are underway.

COMPETITION BETWEEN CIS- AND TRANS-REPLICATION

In contrast to the intrastrand pairing occurring during *cis*-replication, an interstrand pairing, called *trans*-replication, has also been demonstrated as a pathway used by adenovirus (see Chapter 1). It would be interesting to know whether *cis*-replication will be the sole pathway for replicating the displaced, single-stranded DNA when there is a choice between *cis*- and *trans*-replication. Preliminary experiments looking for competition have been done, using pIR10, which carries the left end of adenovirus, and pIR9, which carries the right end in the opposite orientation (see Chapter 2).

Unfortunately, the results are not clear. The molecules may need to be redesigned in order for the experiment to work. If the experiment shows that only one of the pathways is dominant in complementary strand replication, it will be interesting to see whether changes in the length, the sequence, or the geometry of the inverted repeats will effect replication.

SUMMARY

The work here clearly demonstrates that panhandle intermediates can be used to generate replication-proficient molecules during adenovirus replication. The replication origin of adenovirus and the minimum ITR length required for *cis*-replication have been determined *in vivo*. Though this provides a great deal information about complementary strand replication, it is still not clear whether adenovirus DNA replication actually utilizes a panhandle intermediate during the viral replication cycle and what factors are required for this process. Development of an *in vitro* complementary strand replication system may be required for further understanding of the biochemical and enzymological mechanisms involved in adenovirus complementary strand replication.

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APPENDICES

APPENDIX A1

ESCHERICHIA COLI recBC sbcB HOST PREVENTS THE EXCISION OF STEM-LOOP FORMING SEQUENCES

INTRODUCTION

It has long been known that large palindromic or inverted repetitious sequences are unstable in *Escherichia coli* (1-9), and the *E. coli* genome contains no naturally occurring palindromic sequences longer than 8 bp (10). It has been reported that short direct and inverted repeat sequences capable of forming secondary structure may mediate important biological functions (2, 11, 12). These findings suggest that mechanism(s) must exist in *E. coli* to remove longer palindromic regions in order to maintain proper sizes of secondary structures. Extensive studies have been conducted on the stability of inverted repeat sequences in *E. coli*. Excision of inverted repeats is the most common event to remove long palindromic sequences. Several deletion patterns have been reported: removal of the entire inverted repeat region, called precise or near precise excision (1-7); deletions which simply decrease the size of the inverted repeats (1-3, 6-8); or excision of one copy of the inverted repeat sequences (1-3). The efficiency of removing palindromic sequences depends on the size and sequence of the inverted

repeat (2), the sequences neighboring the inverted repeat region (1-8), and the temperature used to grow the bacteria (2). The recombination genotypes of bacteria have been shown to have different abilities to stabilize inverted repeat sequences (2, 3, 6, 8). Small direct repeats in or near the palindromic sequences have also been found to be targets for the deletion process (1-3, 8).

Plasmid pIR10 and other plasmids carrying stem-loop forming sequences were constructed to study adenovirus DNA replication in vivo (See Chapters 2 and 3). These plasmids carry inverted repeat sequences ranging from 14 to 89 bp. Some of the the inverted repetitions are separated by 330 to 1350 bp of adenovirus DNA. pIR10 was not stable in E. coli JM83, and generated deleted plasmids at high frequency. The deletion event removed both copies of the inverted repeats and all sequences between them. Plasmids with stem-loop forming sequences can be stabilized in E. coli strains which carry recBC and sbcB mutations. Introducing a recF mutation into this background decreases the stability of stem-loop forming sequences. A study of the stability of other plasmids also reveals that the deletion process(es) depends on the size of the inverted repeats and the distance between them. DNA sequence analysis and other experimental evidence also show that small direct repeats (7 bp)

flanking the inverted repeat region may also be involved in the process of removing the stem-loop configuration in E. coli.

MATERIALS AND METHODS

REAGENTS AND ENZYMES

Restriction endonucleases and enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, or Pharmacia and used according to the manufacturer's specifications. ^{32}P-labeled deoxynucleotides (3,000 Ci/mmole) were from New England Nuclear.

BACTERIA STRAINS AND GROWTH CONDITIONS

Escherichia coli strains and their recombination genotypes are listed in Table A1-1. E. coli JC9387, JC9937 and JC11850 were obtained from F. W. Stahl (6). E. coli RR1 (13) and HB101 (13) were purchased from Bethesda Research Laboratories. E. coli JM83 and JM103 have been described (14). YT-broth (0.8% tryptone, 0.5% yeast extract and 0.5% NaCl) with and without 1.5% agar were used as growing medium and agar plates respectively (13). Selective media contained either 50 µg/ml of ampicillin or 50 µg/ml of kanamycin. Bacteria were grown at 37^{o}C. All liquid cultures were incubated with shaking. Plasmid DNAs were isolated by the alkaline extraction method (15) and purified by ethidium bromide-CsCl density gradient centrifugation.

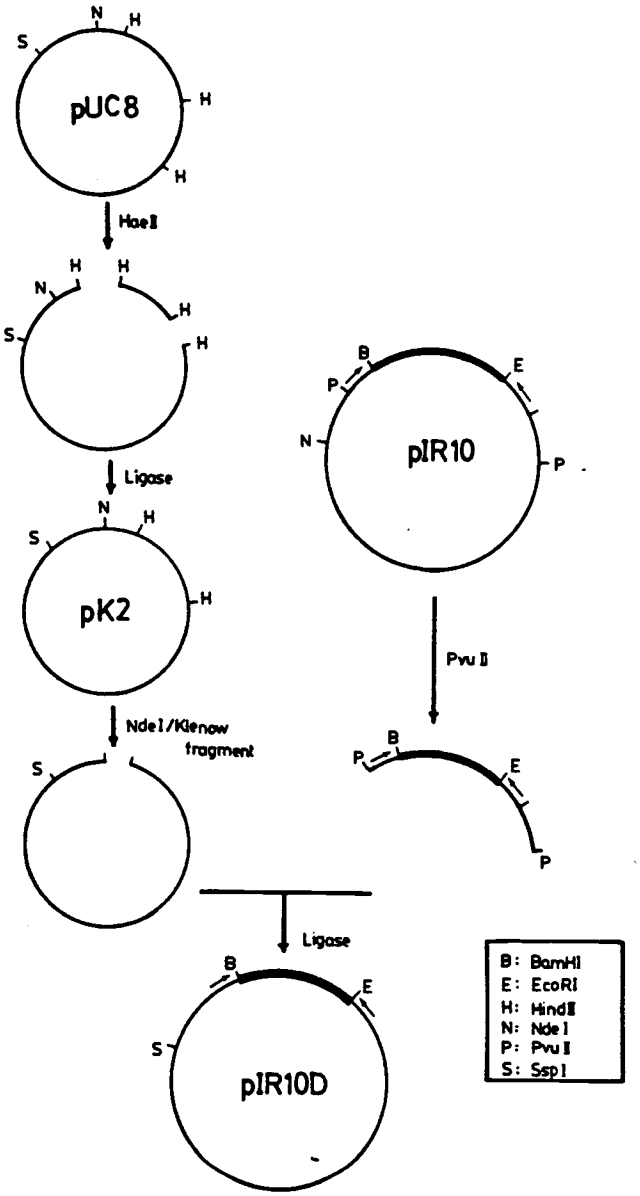

Figure A1-1. Construction of plasmid pIR10D. pUC8 was cleaved with HaeII. The largest and the smallest HaeII fragements were isolated and ligated. pK2 was isolated from ampicillin-resistant clones. pK2 was then cleaved with NdeI and the NdeI site was blunted by treatment with the Klenow fragment of DNA polymerase I. The vector backbone was ligated with small PvuII fragment of pIR10. The thick line represents adenovirus sequences, and the thin line corresponds to the plasmid backbone. The arrows indicate the orientations of the inverted repeat sequences.

PLASMID CONSTRUCTION

Plasmids pIR10, pIRBR, pIRBH and pIR9 have been described in Chapter 2, and plasmids pIRHin, pIRPst, pIRSal, pIRXba, pIRBam, pIRSma and pIRD1 have been described in Chapter 3. All of these plasmids were initially constructed in either *E. coli* JM83 or RR1.

pΔ1, one of the deletion products from pIR10, has been cloned in *E. coli* JM83. DNA sequence analysis shows that pIR10 not only has 89 bp of inverted repititious sequences, but also has small (7 bp) directly repeated sequences at or near the junctions of the inverted repeat sequences (Fig. A1-2). Plasmid pIR10D was constructed to eliminate the direct repeats. Figure A1-1 shows the method used to construct pIR10D. pUC9 was cut with *Hae*II, then the largest (1871 bp) and the smallest (370 bp) fragments were isolated and ligated together. Ampicillin-resistant *E. coli* RR1 colonies were screened for the 2.1-Kb plasmid pK2. Plasmid pK2 has the same geometry as pUC plasmids except that pK2 has no *lac* gene *Hae*II fragment insertion. The *Pvu*II fragment from pIR10, which carries the inverted repeat sequences, was inserted into the blunted *Nde*I site in pK2. pIR10D (2.8-Kb) was identified in ampicilln-resistant colonies. pIR10D has the same stem-loop forming sequences as pIR10 but lacks one of the small direct repeats at one of the inverted repeat junctions.

TRANSFORMATION

E. coli RR1 and HB101 were transformed following the protocol which was provided by supplier. E. coli JC9387, JC9937, JC11850, JM83 and JM103 were transformed by standard procedures (13). In brief, 0.5 ml of an overnight culture were inoculated into 50 ml of YT-broth and incubated at 37°C with vigorous shaking until the optical density at 550 nm reached 0.3. Cultures were chilled, centrifuged (600 x g, 10 min), and suspended in 25 ml of cold 50 mM $CaCl_2$ (pH 8.0). The cell suspension was placed on ice for 20 min, then centrifuged, and resuspended in 2 ml of 50 mM $CaCl_2$ solution. The cell solutions were kept at 4°C for 24 hr before use. $CaCl_2$-treated cells (0.2 ml) were added into the DNA solution (less than 10 µl), gently mixed, and kept on ice for 40 min. After incubation, cell-DNA mixtures were heated at 42°C for 2 min. 3X YT-broth (100 µl) was added to each tube after heating, and the tubes were further incubated at 37°C for 1 hr before the contents were spread onto YT-plates under appropriate selection. To determine the transformation efficiency, colonies were scored after 24 hr of incubation at 37°C, otherwise colonies were re-streaked on another plate for plasmid mini-screening. TE buffer (pH 8) alone, instead of DNA, was used as a control in the transformation assay. Total viable cells were counted on YT-plates without selection. Table 1

shows the results of two different experiments to measure transformation efficiencies of the bacteria. No matter what DNA was used, the transformation efficiency was the same for E. coli JM83 and JC9937, and slightly lower for JC9387. E. coli JC11850 has an even lower transformation efficiency, especially when DNA from JM83 was used in the transformation process. This may be due to the minor change(s) in DNA modification between JM83 and JC-serial bacterial strains.

DNA SEQUENCING

Two different DNA sequencing methods were used. Sequencing of pIRD1 has been described in Chapter 3. The sequence of p 1 was determined by the exonuclease III sequencing method (16). In brief, NdeI-cut p 1 DNA was treated with exonuclease III for 30 min at room temperature. The total volume of the reaction mixture was 40 µl and contained 75 mM Tris-HCl (pH 8.0), 90 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT and exonuclease III at a concentration of 30 units/pmole of DNA. To stop exonuclease III activity, 10 µl of 0.1 M EDTA-1.5 M NaOAc was added. Exonuclease III-treated DNA was purified and resuspended in water. The DNA was then divided into four tubes which contained 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 13 mM DTT, 4 µCi ^{35}S-labeled dATP, 0.15 units of the Klenow fragment of DNA polymerase I and appropriate deoxy-dideoxynucleotide mixtures. After 20 min incubation at

Figure A1-2. Sequences of the inverted repeat region in pIR10. The thick line corresponds adenovirus sequences and the thin line represents the plasmid backbone. The plasmid replication origin is marked as "ori" and the thick arrow indicates its orientation. The small arrows represent the 7-bp direct repeats and the large arrows indicate the orientation of the inverted repeat sequences.

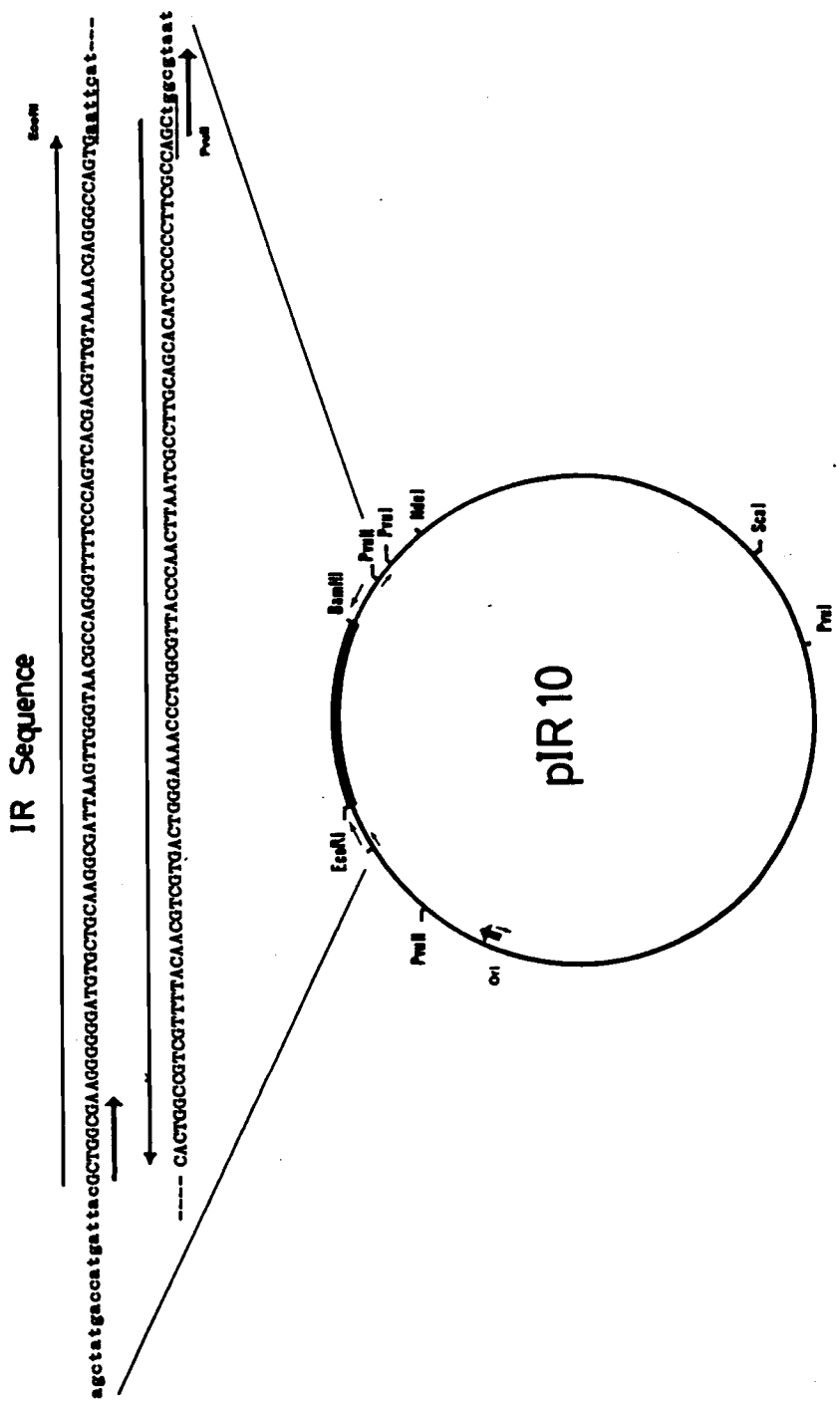

Figure A1-2

room temperature, 1 µl of chase solution containing 1 mM of each dNTP was added to each tube and incubated for another 5 min. Tubes were then heated at 65°C for 10 min to stop the sequencing reaction, and 1 unit of restriction endonuclease Pvu II was added and incubated at 37°C for 15 min. After enzyme digestion, 7 µl of 90% formamide with bromophenol blue (0.1%) was added, and heated at 90°C for 3 min just before being loaded onto an 8% polyacrylamide gel (25 x 35 x 0.03 cm) containing 8 M urea. Electrophoresis was carried out at 1200 V for 2 hr. The gel was then fixed in 5% methanol-15% acetic acid for 15 min and dried before autoradiography.

HETERODUPLEX MAPPING

ScaI-linearized pΔ 1 and pIR10 (100 ng of each) were mixed in TE buffer (pH 8) with 50% (V/V) of formamide. The solution was incubated at 70°C for 10 min to denature the DNAs, then NaCl was added to a final concentration of 0.14 M. For renaturation, the solution was left at 37°C for 40 min. After incubation, DNA was then diluted 3 times into 50% formamide (V/V) containing cytochrome C (100 µg/ml), and spread onto water. The DNA-protein film on the surface of the water was adsorbed onto parlodion-coated grids. Grids were stained with uranyl acetate, and rotary-shadowed with platinum-palladium. Samples were examined with a Zeiss EM-10A electron microscope operating at 40KV. Micrographs were taken at an instrumental

Bacterial strain	Recombination genotype[a]	Transformation frequency (colonies/μg DNA) x 10^{-5}			
		pIR10 (JC9387)		pMDC10 (JM83)	
		Exp 1	Exp 2	Exp 1	Exp 2
JM83	*rec*$^+$	2.2	0.36	2.6	1.3
JC9937	*rec*$^+$	1.3	0.33	-	0.98
JC9387	*recB21 recC22 sbcB15*	1.5	0.11	0.39	0.36
JC11850	*recB21 recC22 sbcB15 recF143*	0.6	-	-	0.02

Table A1-1. Transformation efficiency of various bacterial strains.

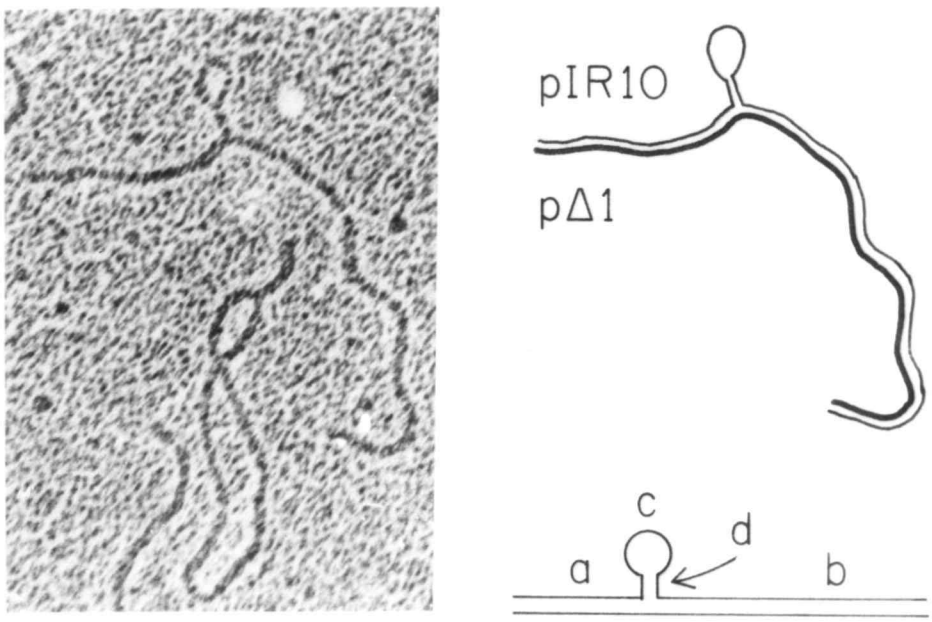

Figure A1-3. Analysis by electron microscopy of heteroduplexes formed between cloned pΔ1 and pIR10. Interpretive drawing of the heteroduplex is shown at right in which the thin line is pIR10 DNA and the thick line is pΔ1 DNA. Sections are lettered from a to d. The length of each section is given in Table A1-2.

Electron microscopic length measurements

DNA segment[a]	Contour length[b]
a	840 ± 60 (45)
b	1790 ± 90 (45)
c	410 ± 40 (17)
d	130 ± 20 (17)
pΔ1	2470 ± 120 (27)

[a]See Figure A1-3.

[b]Length is expressed in bp relative to the length of pIR10 (3170 bp). Error limits are expressed as the standard error of the mean. The number of measurements are shown in parentheses.

Table A1-2. Electron microscopic length measurements.

Figure A1-4. Analysis of the pΔ1 deletion junction by the exonuclease III DNA sequencing method. Panel A shows the sequence of pΔ1. Boldface letters GC correspond to the deletion junction. Panel B shows the sequence of pIR10 and the deletion event.

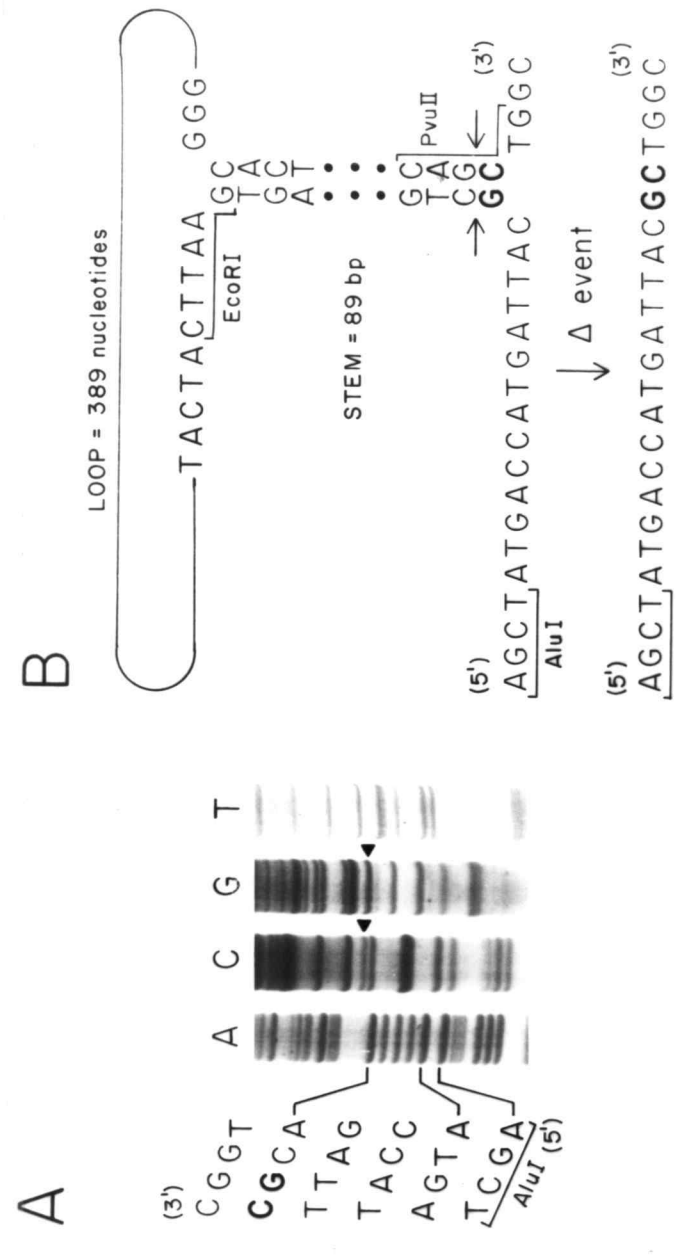
A
B
LOOP = 389 nucleotides
TACTTAA
EcoRI
GGG
STEM = 89 bp
PvuII
(5')
AGCTATGACCATGATTAC
AluI
(3')
TGGC
Δ event
(5')
AGCTATGACCATGATTACGCTGGC
(3')
AluI
(3')
CGGT
CGCA
TTAG
TACC
AGTA
TCGA
AluI (5')
A
C
G
T

Figure A1-4

magnification of 6,000-times on 35 mm film. DNA lengths were determined by a calculator-driven digitizer on photographic prints.

RESULTS AND DISCUSSIONS

Most of the plasmids carrying stem-loop forming sequences have been described either in Chapter 2 or Chapter 3. The structure and sequence of the inverted repeat region in pIR10 is shown in Figure A1-2. pIR10 has 89 bp of inverted repetitious sequences from the lac Z gene in pUC plasmids. The inverted repeat sequences are separated by 358 bp of left-end adenovirus sequences and 28 bp of pUC8 polylinker sequences. Distinct deletion bands can be seen when pIR10 is grown in E. coli JM83, a recombinational wild-type bacterial strain (Fig. A1-5A, lane 5, lane 9; and Fig. A1-5B, lane 11) . One of the deletion products is called pΔ1 and the other is called pΔ2. pΔ1 has been cloned in JM83 and propagated stably (Fig. A1-5A, lane 1). Several unsuccessful attempts have been made to clone pΔ2 under ampicillin selection. The cloned pΔ1 has been studied further. Restriction endonuclease analyses show that pΔ1, like pIR10, contains one ScaI, one NdeI, and two PvuI sites, but only one PvuII site. Neither EcoRI nor BamHI sites have been detected. The deletion in pΔ1 was mapped by heteroduplex formation between pΔ1 and pIR10. A stem-loop structure clearly can

be seen in the heteroduplex between pΔ1 and pIR10 (Fig. A1-3). Length measurements suggest that the stem-loop forming region has been deleted in pΔ1. The structure of pΔ1 determined by electron microscopy is in excellent agreement with the restriction endonuclease mapping data. The DNA sequence of pΔ1 is shown in Figure A1-4. It can be seen that the deletion process removes the inverted repeat sequences precisely except the last G/C nucleotides. The size pΔ2 is about 1Kb smaller than pIR10. Restriction endonuclease analyses show that pΔ2 does not contain EcoRI, BamHI and NdeI sites. The difficulty of cloning pΔ2 may be due to the deletion of part of the ampicillin resistant gene.

It has been reported that inverted repeat sequences are stable in recombination-deficient bacteria. Collins (2) showed that the removal of inverted repeats does not dependent on the host recA system, but can be prevented by introducing recB sbcB mutations. Leach and Stahl (6) also found that a recBC sbcB mutant could support the growth of lambda phage containing a 300-bp palindromic sequence. Introducing the recF mutation confired no additional stability compared to the recBC sbcB mutant. Astell and Boissy (8) demonstrated that an E. coli recBC sbcB recF mutant could stabilize palindromic sequences in a plasmid containing the genome of the minute virus of mice (MVM). These results indicate that gene products of recBC, sbcB,

Figure A1-5. Electrophoretic analysis of plasmid pIR10 in different bacterial strains. Lane 1 shows the cloned pΔ1 DNA from E. coli JM83. Lanes 2 through 9 show the stability of pIR10 in different strains. The transforming DNA was either from JC9387 (lanes 2 through 5) or JM83 (lanes 6 through 9). Lanes 10 and 11 show pIR10 in JM83 and JC9387 on an overloaded gel. Lanes 12 and 13 show pMDC10, which does not contain inverted repeats, in JM83 and JC9387.

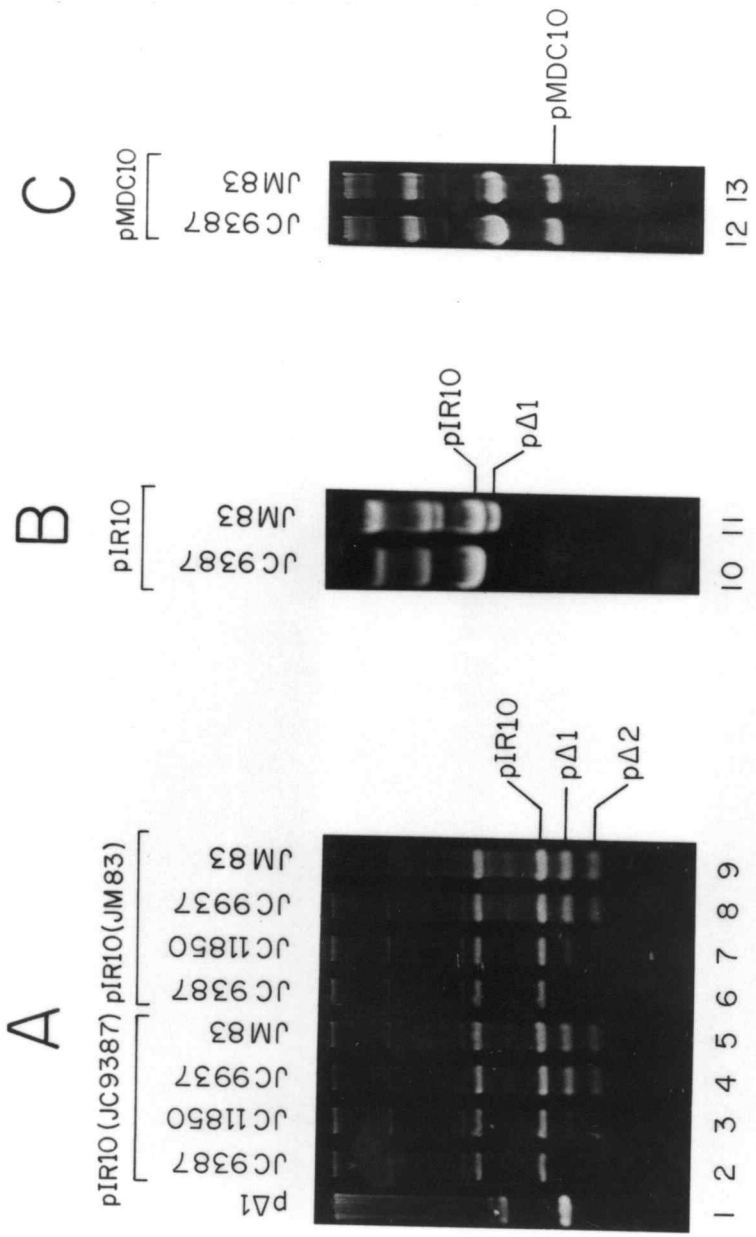
A
pIR10 (JC9387) pIR10(JM83)
pΔ1
JC9387
JC11850
JC9937
JM83
JC9387
JC11850
JC9937
JM83
pΔ2
pΔ1
pIR10
1 2 3 4 5 6 7 8 9
B
pIR10
JC9387
JM83
pΔ1
pIR10
10 11
C
pMDC10
JC9387
JM83
pMDC10
12 13

Figure A1-5

and recF may be involved in the process for deleting the inverted repetitious sequences.

In order to stabilize the stem-loop forming sequence in pIR10, the 3.2-Kb plasmid was purified by preparative agarose gel electrophoresis. The gel-purified pIR10 DNA was transformed into bacterial stains with different recombinational genotypes. Colonies that contained pIR10 were then grown up, and DNA was isolated and analyzed. The stability of pIR10 in these different bacteria is shown in Figure A1-5. About 50% of the total plasmid DNA isolated from E. coli JM83 and JC9937, which are both wild type for recombination, are the deleted plasmids (Fig. A1-5A, lane 8 and lane 9). E. coli JC11850 is a recB21 recC22 sbcB15 recF143 mutant. The stability of pIR10 in this strain is about 5-times greater than in wild-type bacteria (compare Fig. A1-5A, lane 7 to lanes 8 and 9). A recB21 recC22 sbcB15 E. coli mutant, JC9387, prevents any detectable amount of deletion products generated from pIR10 (Fig. A1-5A, lane 6). pIR0 from E. coli JC9387 was purified by gel electrophoresis and retransformed into different bacterial strains. This ensures that the deletion process is reproducible and that the deletion products are not generated by trace amounts of deletion plasmids cotransformed with pIR10. As expected, the retransformation results are the same pIR10 is not stable in E. coli JM83 and JC9937 (Fig. A1-5A, lanes 4 and 5).

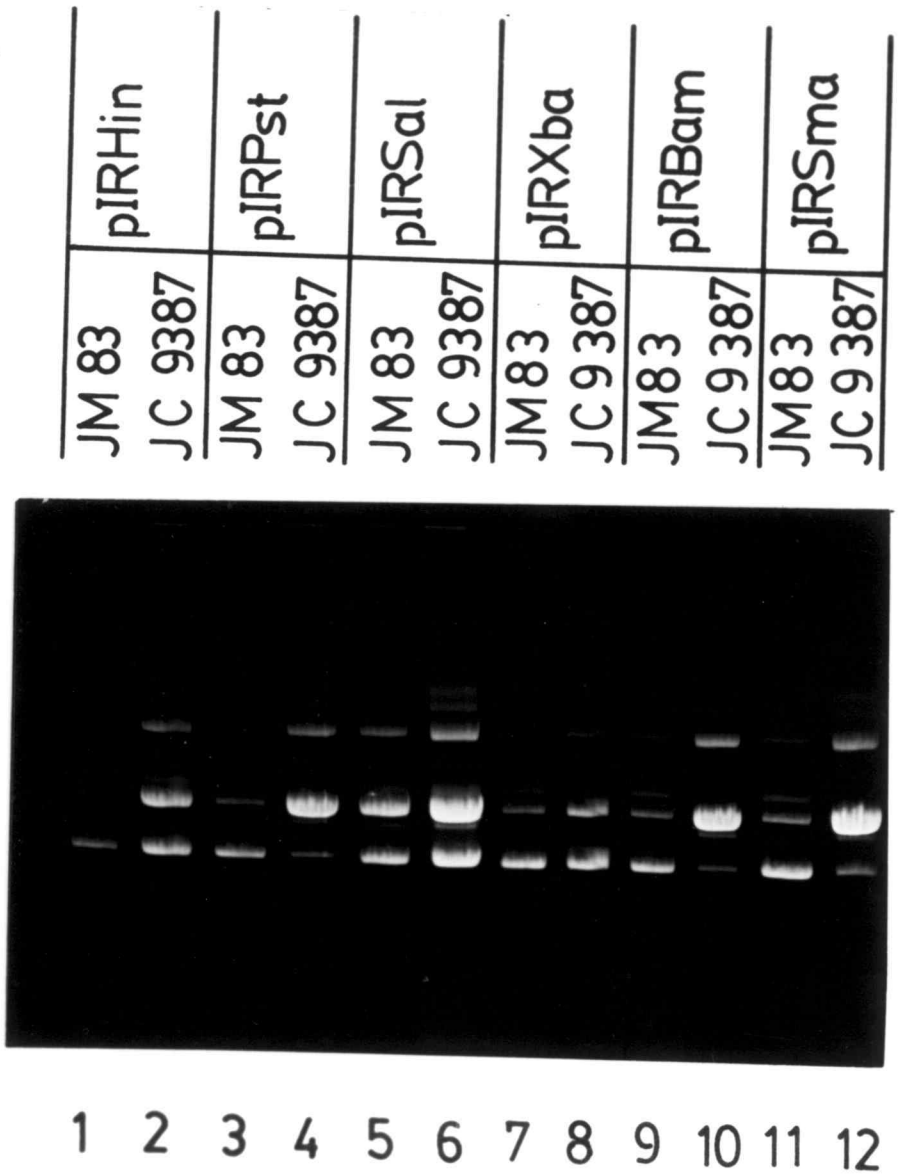

Figure A1-6. Electrophoretic analysis of clones with polylinker inverted repeats. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12 show plasmid DNA pIRHin, pIRPst, pIRSal, pIRXba, pIRBam, and pIRSma isolated from E. coli JM83 and JC9387 respectively.

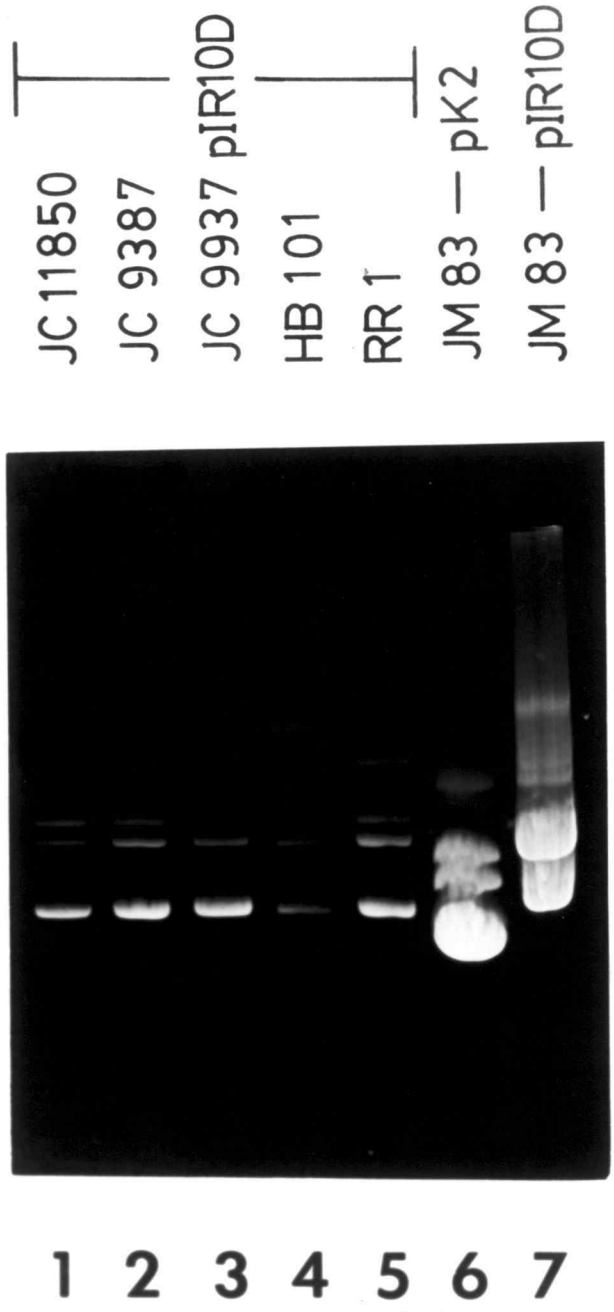

Figure A1-7. Electrophoretic analysis of pIR10D. Lanes 1 to 5 are pIR10D plasmid DNA isolated from different bacterial strains. Lane 6 shows pK2, which is the backbone of pIR10D, isolated from E. coli JM83. Lane 7 shows overloaded pIR10D DNA from JM83.

JC11850 increases the stability of pIR10, but some deleted plasmids can still be seen in the gel (Fig. A1-5A, lane 3). No deletion products can be detected in DNA isolated from E. coli JC9387 (Fig. A1-5A, lane 2). The deletion process depend on the inverted repeat configuration since no deletion products have been found in both E coli JM83 and JC9387 for pMDC10 which has the same sequence as pIR10 except for the inverted repeat configuration (Fig. A1-5C). Transforming pIR10 DNA from E. coli JC9387 into HB101, a recA mutant, does not stabilize the stem-loop configuration. This suggests that the deletion in pIR10 does not depend on the recA gene product, but that recBC and sbcB gene products are involved. However, introduction of a recF mutation increases the frequency of deletion, in contrast to other reports (6, 8).

Different plasmids carrying stem-loop forming sequences have been constructed to study adenovirus replication in vivo (See Chapters 2 and 3). The stabilities of these plasmids in different recombination environments have also been tested. Plasmids pIRHin, pIRPst, pIRSal, pIRXba, pIRBam, and pIRSma carry from 51 to 14 bp of inverted repetitious sequences derived from the polylinker region in pUC19. The polylinker inverted repeat sequences are separated by 342 bp of left-end adenovirus sequences. All of these plasmids have been shown to be stable in both E. coli JM83 and JC9387 (Fig.

A1-6). pIR9, which has the same inverted repetitious sequences as pIR10 but separated by 1.3-Kb right-end adenovirus sequences, yielded detectable deleted products in both JM83 and JC9387 (not shown). pIRD1 has the same geometry as pIR10, but only carries 54 bp of inverted repeat sequences. It generated no detectable deletion products on both JM83, and JC9387 (not shown). These results indicate that the deletion process depends on the length of the inverted repeat and the distance between the inverted repetitious sequences (compare pIR10, pIRD1 and pIR9).

It is interesting to notice that the deletion process did not remove all of the invert repeat sequences, but left the last G/C bp. Careful analysis the sequence at the deletion junction in pΔ1 revealed a single copy of a 7-bp direct repeat found in pIR10. Their relative positions have been marked on Figure A1-2. It is possible that the small direct repeats are the target for the deletion process. pΔ1 could be formed by the interaction of the two direct repeats at the junction of inverted repeat sequences. To determine the role of the small direct repeats, a pUC-like plasmid, pK2, was constructed, and the PvuII fragment from pIR10 was inserted into the NdeI site. pIR10D has almost the same structure as pIR10 except for one of the direct repeat sequences. The stability of this plasmid is shown in Figure A1-7. No detectable deletion

products were observed in any of the bacterial strains tested. This indicates the small direct repeats or the sequences around the small direct repeats which have been removed in pIR10D may be the actual target for the deletion event. These results suggest that the deletion of stem-loop forming sequences in the plasmids depends on the size of the inverted repeats (compare pIR10 and pIRD1), the distance between the invert repeats (compare pIR10 and pIR9), and the small direct repeats (compare pIR10 and pIR10D).

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APPENDIX A2

CONSTRUCTION OF CLONES

INTRODUCTION

The purpose of this section is to provide a comprehensive description of clones constructed during the course of this work.

Figure A2-1. Construction of pUCdl20. pUCdl20 is a 2.7-Kb ampillicin-resistant clone which contains the first 20 nucleotides of the left-end adenovirus. It was made by moving the EcoRI-BamHI fragment of dl20 (1) containing adenovirus terminal sequences into the EcoRI- and BamHI-cut pUC9 backbone. This plasmid was made in an attempt to study the sequence requirements for adenovirus DNA replication in vitro. The thin line represents the plasmid backbone and the thick line corresponds to the adenovirus sequence.

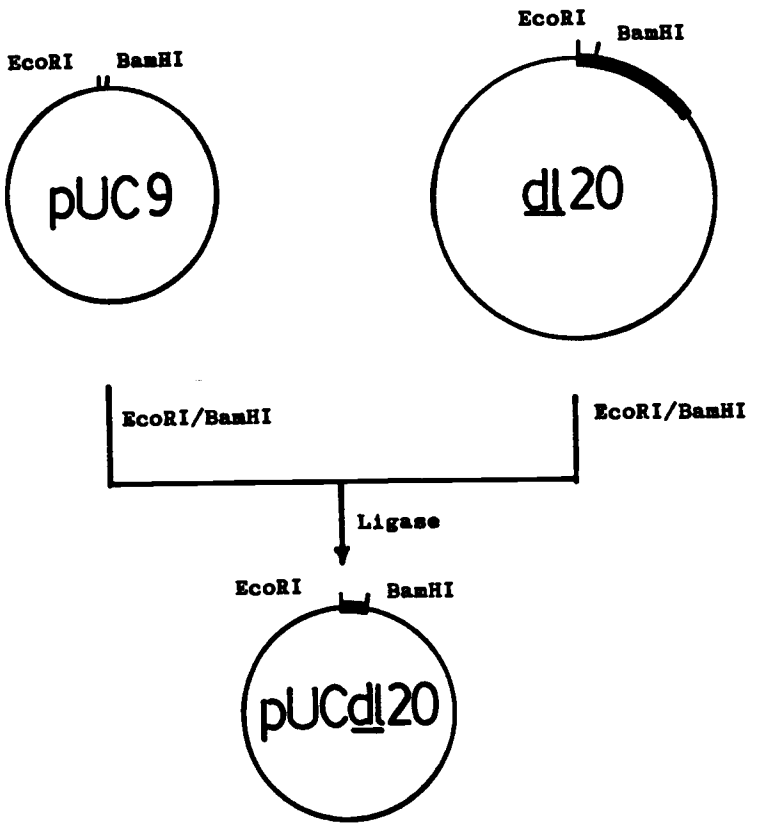
EcoRI
BamHI
pUC9
EcoRI
BamHI
dl20
EcoRI/BamHI
EcoRI/BamHI
Ligase
EcoRI
BamHI
pUCdl20

Figure A2-1

Figure A2-2. Construction of pIRpmX. These clones were made in an attempt to study the sequence requirement for adenovirus DNA replication in vivo. pIRpmX are 3.2-Kb ampicillin-resistant clones which contain 89 bp inverted repeat sequences. They also contain point mutations in the adenovirus ITR region. Clones were made by removing the left-end adenovirus sequences as EcoRI-BamHI fragments from pm4, pm17, pm18, pm4-18, pm17-18, and pm4-17-18 (1) into EcoRI- and BamHI-cut pIR10. The thin line represents the plasmid backbone. The thick line corresponds to adenovirus sequences and the arrows represent the inverted repeat sequences and their orientation.

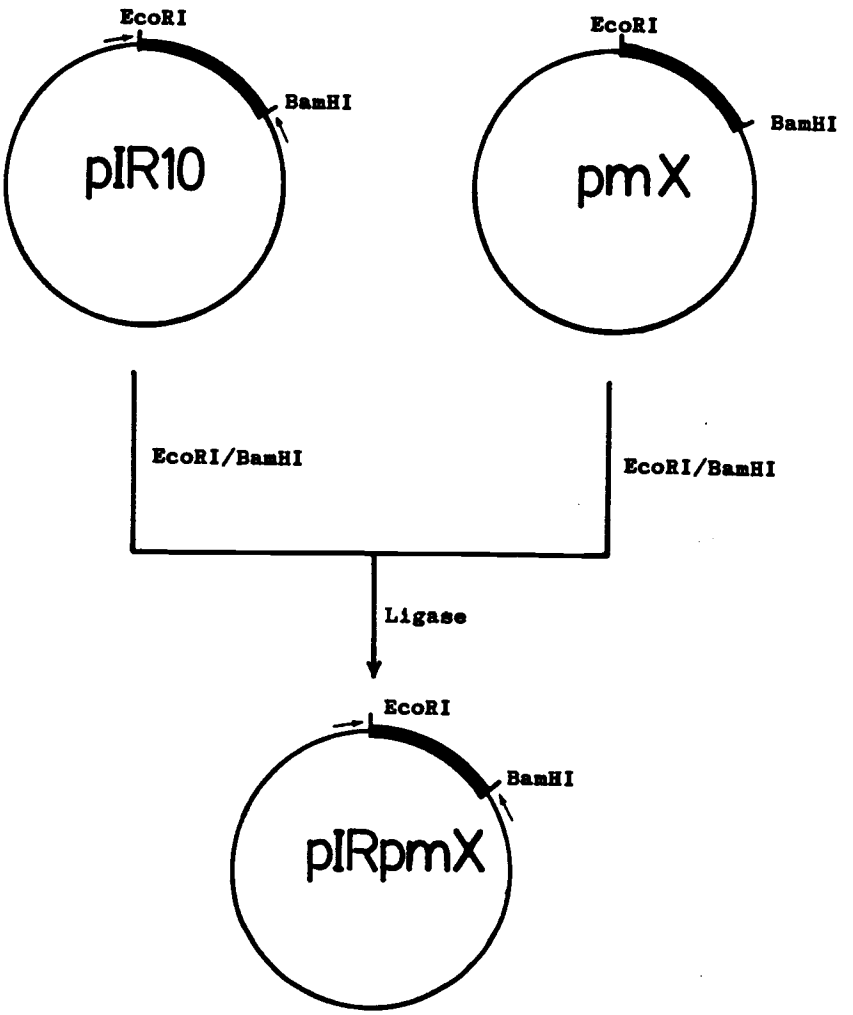
EcoRI
BamHI
pIR10
EcoRI
BamHI
pmX
EcoRI/BamHI
EcoRI/BamHI
Ligase
EcoRI
BamHI
pIRpmX

Figure A2-2

Figure A2-3. Construction of pUCT18. pUCT18 is a 4.1-Kb ampicillin- and tetracycline-resistant clone. It was made by removing the EcoRI-BalI fragment carrying the tetracycline resistant gene into the EcoRI- and SmaI-treated pUC18 backbone. This clone is one of the intermediates to construct a pIR10-like molecule with multiple inverted repeat sequences.

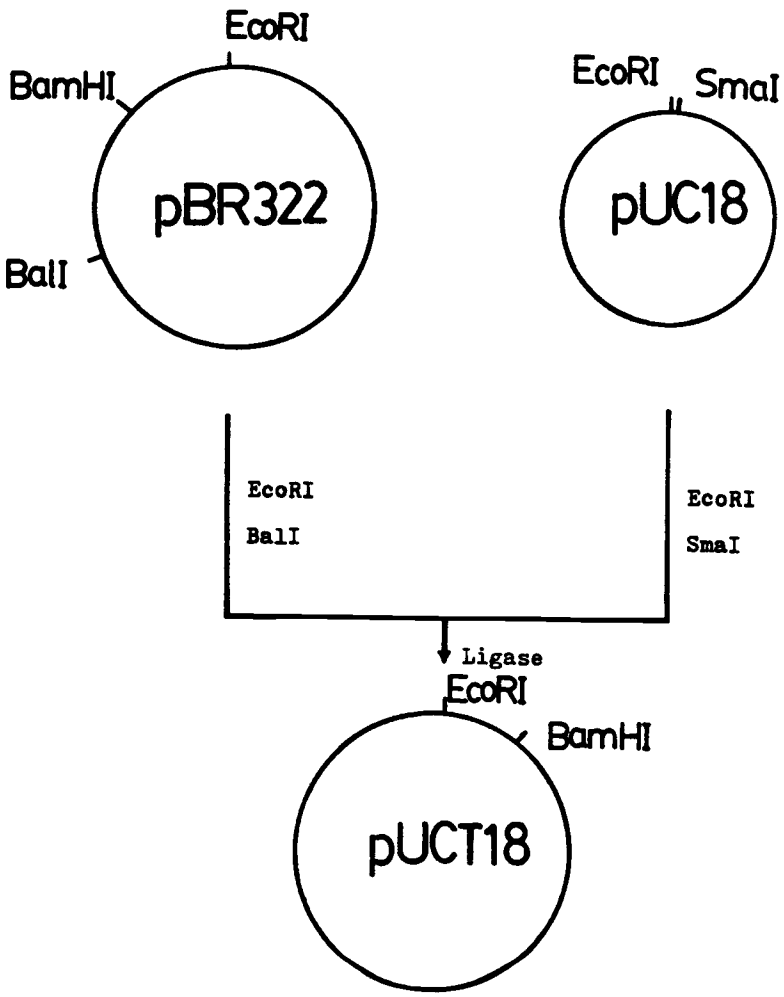
EcoRI
BamHI
pBR322
BalI
EcoRI
SmaI
pUC18
EcoRI
BalI
EcoRI
SmaI
Ligase
EcoRI
BamHI
pUCT18

Figure A2-3

Figure A2-4. Construction of MPRL102. The thin line corresponds to the plasmid backbone and the thick line represents adenovirus sequences. The arrows represent the adenovirus ITRs and their orientation. This plasmid contains both the left- and right-end of adenovirus. The ITRs are separated by 500 bp of right-end and 900 bp of left-end adenovirus DNA. The ITRs can be exposed by EcoRI. MPRL102 was made by cutting pRL102 (2) with EcoRI and the EcoRI fragment was inserted into the M13-mp18 EcoRI site. It was made in an attempt to study adenovirus DNA replication *in vivo* by generating a large amount of single-stranded adenovirus DNA *in vitro*.

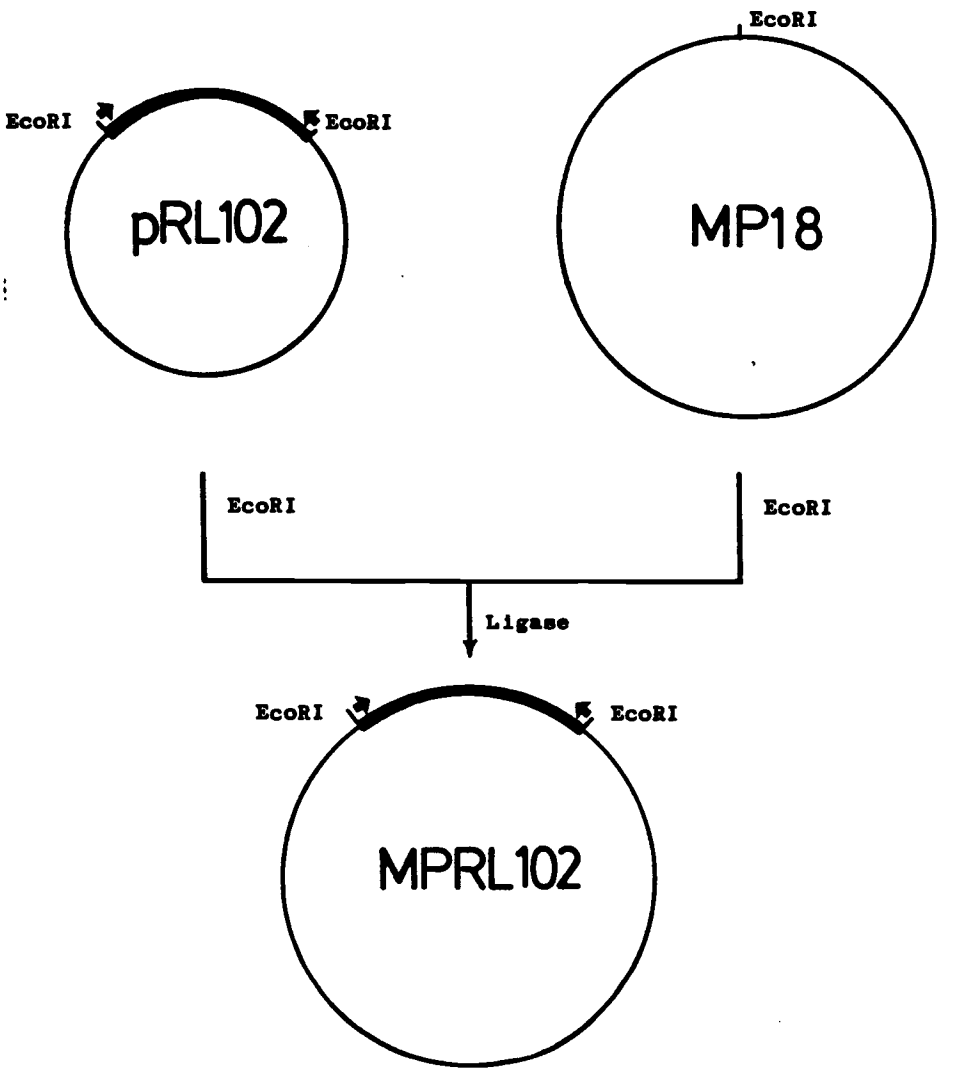
EcoRI
EcoRI
pRL102
EcoRI
MP18
EcoRI
EcoRI
Ligase
EcoRI
EcoRI
MPRL102

Figure A2-4

Figure A2-5. Construction of pXD6. pXD6 was made by cutting pIBIV (provided by Dr. Kathy Berkner) with HindIII and religating. pXD6 contains 1000 bp of the right-end of adenovirus. It is one of the parent clones of pRL102 and pADIRK.

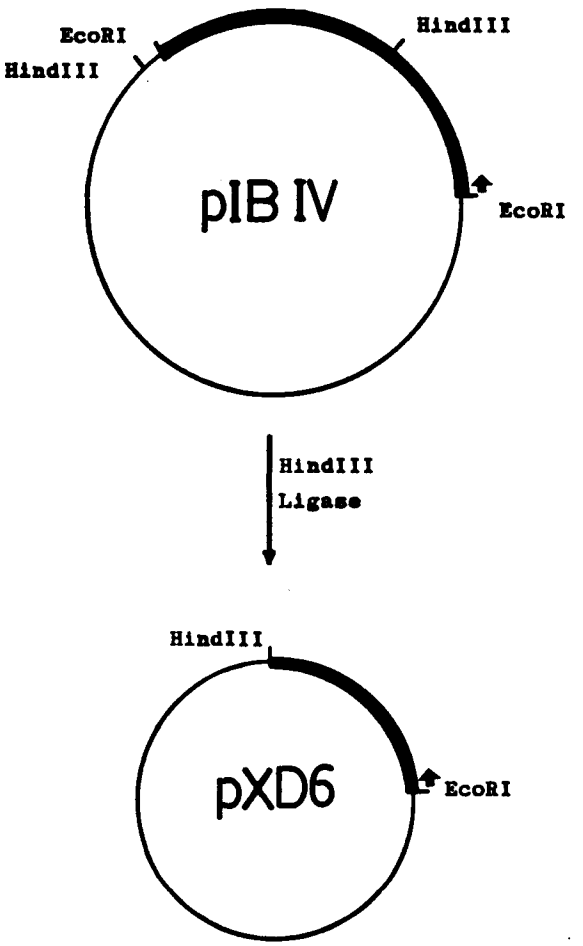
EcoRI
HindIII
HindIII
pIB IV
EcoRI
HindIII
Ligase
HindIII
pXD6
EcoRI

Figure A2-5

Figure A2-6 Construction of pADIR. pADIR was made by ligating the small EcoRI-PstI fragment from Clone 7 (containing the left-end of adenovirus) (2) to EcoRI- and PstI-cut pXD6 and selecting for resistance to ampicillin. pADIR was made to study adenovirus DNA replication *in vitro* and *in vivo*. The ITRs are separated by an EcoRI linker. The thick line corresponds to the adenovirus sequences and the arrows represent the ITRs and their orientation. The thin line represents the plasmid backbone.

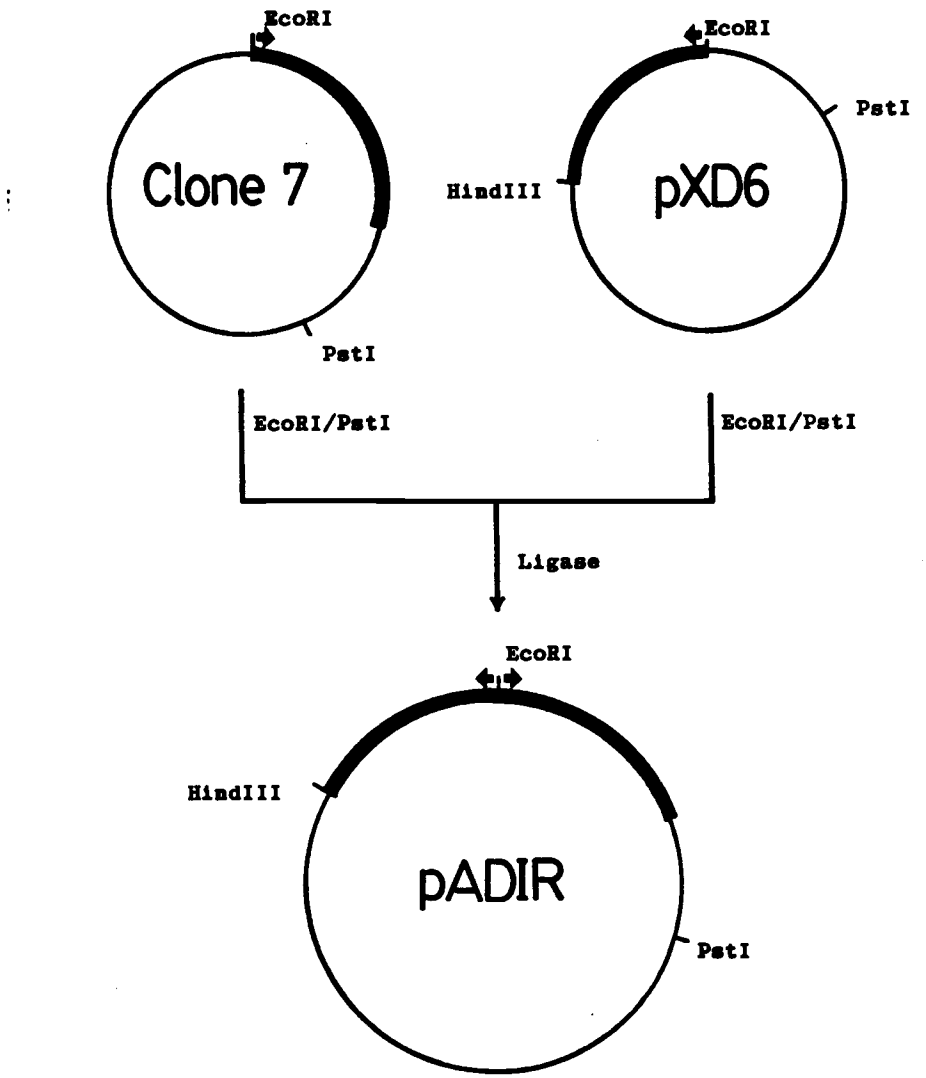
EcoRI
Clone 7
PstI
EcoRI
pXD6
PstI
HindIII
EcoRI/PstI
EcoRI/PstI
Ligase
EcoRI
HindIII
pADIR
PstI

Figure A2-6

Figure A2-7. Construction of pUCK19. pUCK19 is one of the intermediates to construct a series of clones to study the sequence homology requirement in trans-replication. It was made by ligating the EcoRI fragment containing the kanamycin gene from pKT14 (2) to the EcoRI-cut pUC19 backbone and selecting for ampicillin- and kanamycin-resistant clones.

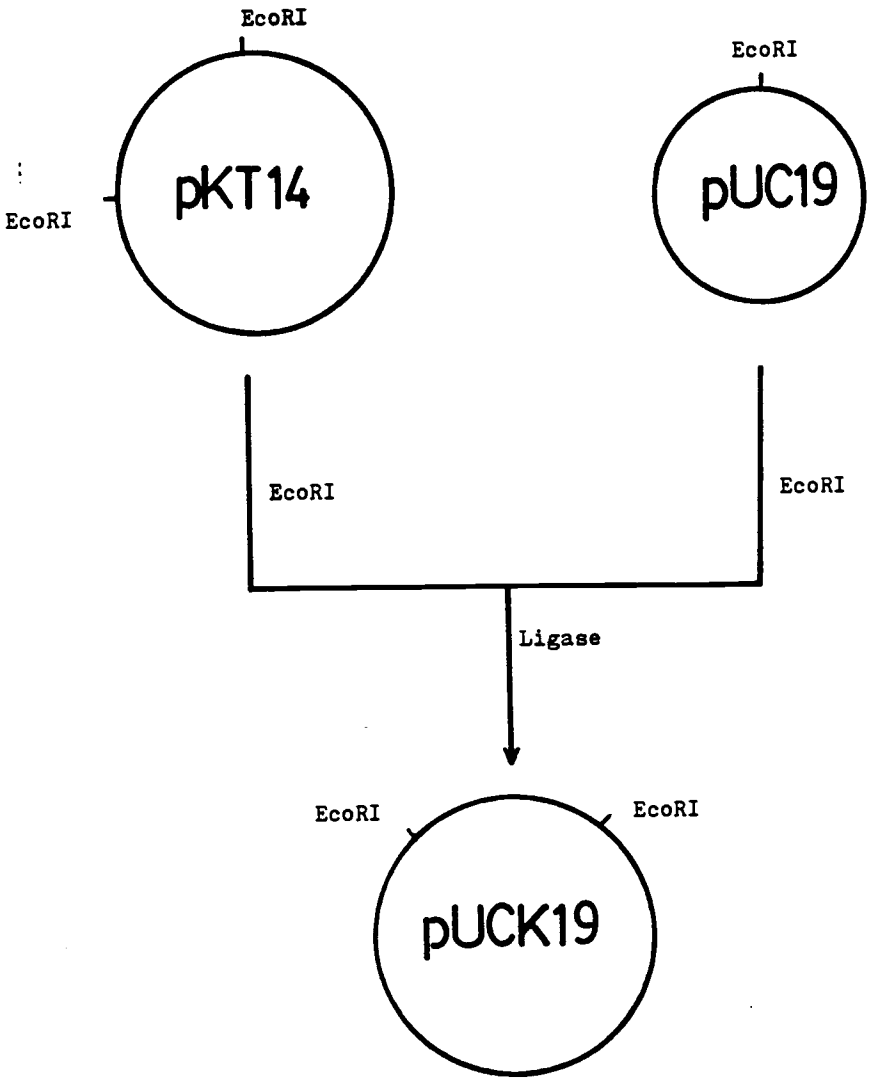
EcoRI
EcoRI
pKT14
EcoRI
pUC19
EcoRI
EcoRI
Ligase
EcoRI
EcoRI
pUCK19

Figure A2-7

Figure A2-8. Construction of pXD64. pXD64 was constructed to study the possibility of using adenovirus type 4 as a helper in trans-replication. pXD64 contains the adenovirus type 4 ITR. It was made by ligating the EcoRI-HaeII fragment of p4A2 (containing the adenovirus type 4 ITR; provided by Dr. R. Hay) into the EcoRI- and SmaI-cut pXD6 backbone. The ITR sequence is exposed by cleavage with EcoRI.

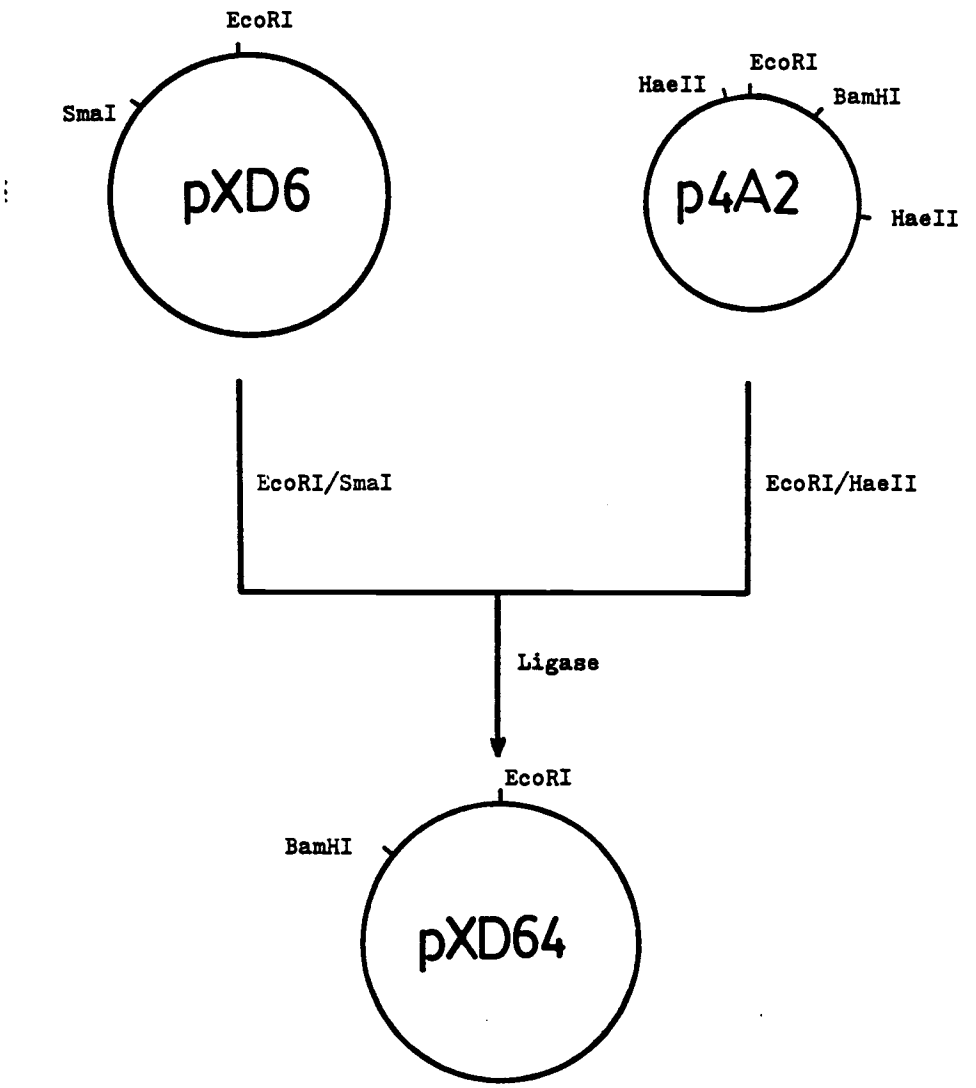
EcoRI
SmaI
pXD6
HaeII
EcoRI
BamHI
p4A2
HaeII
EcoRI/SmaI
EcoRI/HaeII
Ligase
EcoRI
BamHI
pXD64

Figure A2-8

Figure A2-9. Construction of pXD658. pXD658 is a derivative of pXD6 made by ligating the EcoRI-HaeII fragment of 58A (containing 58 nucleotides of the right-end of the adenovirus type 2 ITR; provided by Dr. R. Hay, 3) into the large EcoRI-SmaI fragment of pXD6. It contains the first 58 nucleotides of the right-end of the adenovirus type 2 ITR. It was designed to study the sequence requirement for trans-replication.

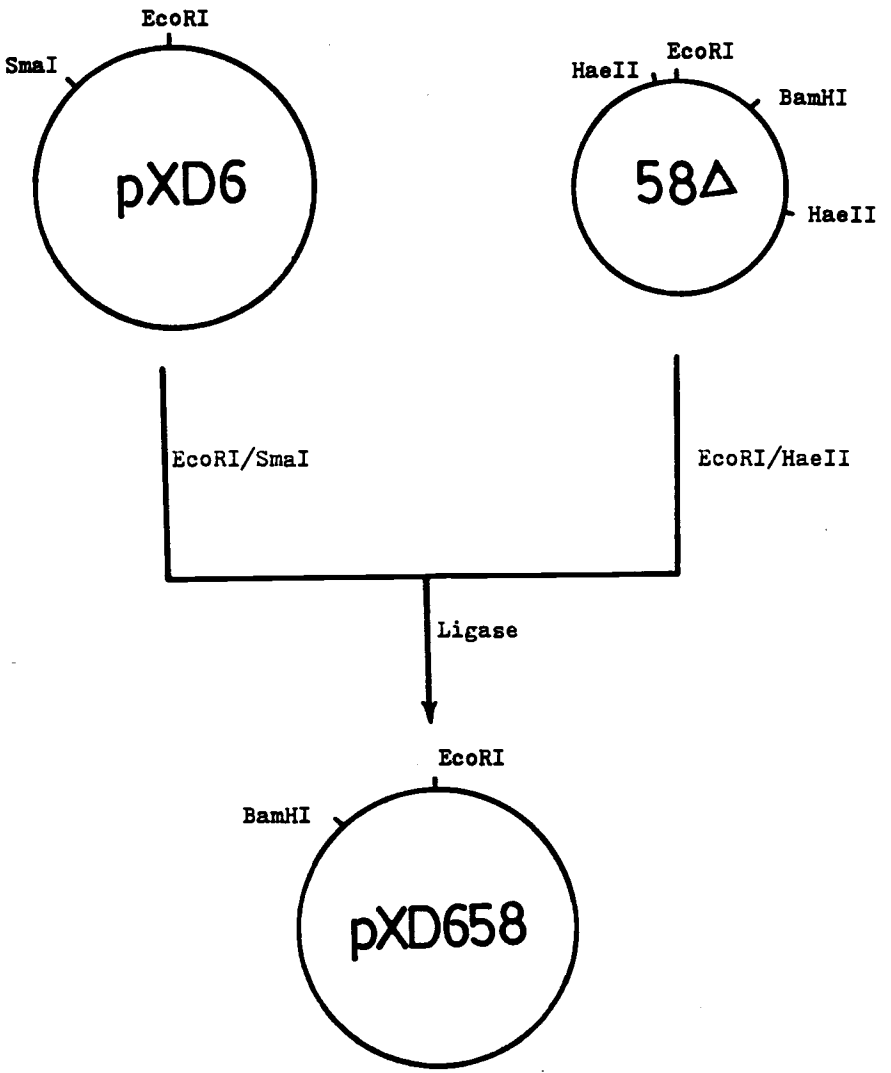
EcoRI
SmaI
pXD6
HaeII
EcoRI
BamHI
58Δ
HaeII
EcoRI/SmaI
EcoRI/HaeII
Ligase
EcoRI
BamHI
pXD658

Figure A2-9

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2. Ahern, K. G. (1985). Adenovirus replication in Trans: A new replication pathway. Ph.D. Thesis, Oregon State University.

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