AN ABSTRACT OF THE THESIS OF

Chin-Hwa Hu for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on January 29, 1990.

Title: Structural Requirements for Adenovirus DNA Replication.

Abstract approved: Redacted for Privacy

Dr. George D. Pearson

The adenovirus replication origins reside in the inverted terminal repetition (ITR) sequences. Replication proceeds unidirectionally by a strand-displacement mechanism from either or both origins to produce duplex daughter DNA plus a displaced parental strand. The displaced strand can be duplicated by two different routes. The *cis* pathway involves intramolecular hybridization between the inverted terminal repeats to form a panhandle replicative intermediate, where synthesis of the complementary strand is initiated from the newly created duplex origin on the panhandle. Alternatively, complementary strands arising from replication on different molecules can directly hybridize intermolecularly to reform a duplex viral genome. This path is called *trans* replication.

Using plasmid mini-chromosomes as model systems, the structural requirements for adenovirus DNA replication and the relationship between the *cis* and *trans* pathways for complementary strand replication was investigated. Plasmids containing single or dual adenovirus origins, with or without inverted repeats, were specially constructed to mimic the structures of the adenovirus genome. Linear plasmids which contain exposed adenovirus origins can engage in adenovirus helper-dependent strand-displacement replication. A special class of panhandle intermediates (foldback structures), arising from the replication of symmetrical dimers and multimers generated by end-to-end ligation during transfection, was identified by two-dimensional gel electrophoresis and physically characterized. Foldback molecules provide the first evidence for the existence of the adenovirus *cis* replication pathway. Comparing the yields of *cis* replication products from different

plasmids, it is clear that the efficiency of *cis* replication increases with longer inverted repeats. In addition, work presented here demonstrates that the conversion of displaced strands into duplexes occurs simultaneously and independently by both the *cis* and the *trans* pathways.

The ability of embedded replication origins to direct adenovirus DNA replication was investigated in transfected cells using the plasmid mini-chromosome model system. Plasmids with origins embedded in circular or linear templates gave rise to replication-proficient linear molecules. Inverted repeats were necessary and sufficient in order to rescue displaced strands from circular or linear templates by the *cis* pathway. In general, the efficiency of replication on linear templates with embedded origins was higher than on circular templates, but was lower when compared to linear templates with exposed origins. This suggests that the creation of a 3'-end, essential for the *cis* replication pathway, on displaced strands arising from cicluar molecules may be rate limiting. Alternatively, this might imply that the efficiency of initiation on linear templates is higher than on circular templates.

A mutant adenovirus helper was used to investigate the role of homologous recombination in activating plasmids with wild-type adenovirus origin sequences. Recombination between the exposed origins on helper DNA and the embedded origins in linear plasmids might be one possible mechanism to activate embedded origins. However, recombination is clearly not necessary. Other mechanisms must be involved in the replication of templates which contain embedded origins. The production of tandemly repeated multimers from circular templates suggests that randomly initiated displacement reactions might proceeed continuously around the circles. Subsequently, the displaced strands could be converted into replication-proficient molecules by the *cis* replication pathway.

Structural Requirements for Adenovirus DNA Replication

by

Chin-Hwa Hu

A THESIS

submitted to

Oregon State University

in partial fullfillment of
the requirements for
the degree of

Doctor of Philosophy

Completed January 29, 1990

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Chairman of the Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of the Graduate School

Date thesis is presented January 29, 1990

ACKNOWLEDGEMENTS

I would like to express my great appreciation to my major professor, Dr. George Pearson, for his support, advice, and encouragement throughout the course of my research. Without his full support and general encouragement, it would have been impossible to finish the work presented here. Furthermore, I want to extend my thanks to my other committee members, Dr. Gary Merrill, Dr. Pui Shing Ho, Dr. Dennis Hruby, and Dr. Peter Bottomley, for their generous help. I also want to express my sincere respect and appreciation to Dr. Kensal van Holde for his great encouragement throughout the past years.

I would like to acknowledge and thank my colleagues, including Dr. Kai Wang, Dr. Kevin Ahern, Dr. Fu-Yun Xu, Kate Mathews, Margaret Nervik, Kelly Bennett, Kawai Lau, Jasmine Ng, and Andrew Pearson for their contributions and valuable discussions. Dr. Frank Graham generously provided the Mac-type adenovirus. I also would like to express my appreciation to other professors and friends in the Department and on this campus. They always gave their full support when I needed them. Sincere thanks to Hope Leman for her critical reading and patience while correcting this thesis.

Finally, I would like to acknowledge the support provided by all my family members, especially my wife Chi-Mei for her understanding, trust, and tolerance during the past years.

As I finish this research work, my lovely wife and I are also cheerfully awaiting a new life coming to our family. May she or he join with us to welcome another wonderful tomorrow.

TABLE OF CONTENTS

	Page
CHAPTER I	1
INTRODUCTION	
Adenovirus DNA structure and DNA replication	2
In vitro and *in vivo* studies of DNA replication	4
Initiation of adenovirus DNA replication	7
Complementary strand synthesis in adenovirus DNA replication	9
Transformation and DNA recombination	9
CHAPTER II	12
MATERIALS AND METHODS	
Enzymes and reagents	12
Plasmids and bacteria	12
Cell culture and adenovirus DNA purification	14
DNA transfection	17
DNA extraction	17
Replication assay by *Dpn*I and *Mbo*I restriction	18
Southern blot analysis	18
Two-dimensional agarose gel electrophoresis	18
Radioactive-labeling of plasmid DNA by random-priming	19
End-labeling of oligonucleotide probes	19
DNA-DNA hybridization	19
CHAPTER III	
CHARACTERIZATION OF A FOLDBACK MOLECULE DURING ADENOVIRUS DNA REPLICATION	21

INTRODUCTION 21

RESULTS AND DISCUSSION 23

Adenovirus mini-chromosomes participate in *cis* and *trans* replication 23

Foldbacks are intermediates in the replication of symmetric dimers 26

Identification of foldbacks by two-dimensional gel electrophoresis 26

Efficient *cis* replication depends on the length of inverted repeat sequences 29

Implications for the genomic organization of adenovirus 31

CHAPTER IV

ADENOVIRUS DNA REPLICATION DIRECTED BY EMBEDDED ORIGINS 34

INTRODUCTION 34

RESULTS AND DISCUSSION 35

The replicative fate of DNA containing an embedded adenovirus origin 35

Replication of circular DNA requires inverted repeats 38

Strand-displacement synthesis around circular templates 41

Replication of linear DNA with an embedded adenovirus origin 41

Possible replication mechanisms using embedded adenovirus origins 46

Possible mechanisms to displace strands from circular molecules 50

CHAPTER V

CONCLUSIONS 53

The relationship between *cis* and *trans* replication pathways 54

Identification of a special class of panhandle structures 53

Efficient *cis* replication depends on the length of inverted repeat sequences 55

Inverted repeats are required for the replication of circular DNA 56

Strand-displacement synthesis around circular DNA templates 56

Replication of linear DNA containing embedded origins 56

Activation of embedded adenovirus origins by homologous recombination 57

Possible mechanisms to replicate circular DNAs 57

BIBLIOGRAPHY 59

LIST OF FIGURES

Figure		Page
I.1	Overall scheme for adenovirus DNA replication	3
II.1	Structures of plasmids T4, pADIRK2, pIRLT, and pLIRLT	13
II.2	Construction of plasmids pIRST and pLIRST	15
II.3	Construction of plasmids pIRLT and pLIRLT	16
III.1	Transfections involving plasmids T4 and pLIRLT	24
III.2	Interactions between strands of plasmids T4 and pLIRLT	25
III.3	Transfection with *Eco*RI-linearized plasmid T4	27
III.4	Replication of symmetrical T4 dimers	28
III.5	Analysis of plasmid T4 replication by two-dimensional gel electrophoresis	30
III.6	Analysis of *cis* replication by two-dimensional gel electrophoresis	32
IV.1	Replication of linear T4	37
IV.2	Replication of circular DNA which contained adenovirus origin sequences	39
IV.3	Cotransfection of circular T4 and linear pLIRLT	42
IV.4A	Replication of linear pLIRLT	44
IV.4B	Replication of linear pIRLT	45
IV.5	Replication of linear T4 and pLIRLT	47
IV.6	Activation of plasmid mini-chromosome replication by homologous recombination with helper adenovirus DNA	48
IV.7	Terminal sequence conversion during the replication of *Bgl*II-cleaved pLIRLT cotransfected with helper Mac-adenovirus DNA	49
IV.8	Two-dimensional gel electrophoresis analysis of linear pLIRLT replication	51

STRUCTURAL REQUIREMENTS FOR ADENOVIRUS DNA REPLICATION

CHAPTER I

INTRODUCTION

Adenoviruses comprise a class of mammalian DNA viruses which infect human, cattle, sheep, pigs, monkeys, mice, horses, and several other animals (for review see Ishibashi and Yasue, 1984). Since the first adenovirus was described by Rowe *et al.* (1953) and later by Hilleman and Werner (1954), at least 42 distinct serotypes (species) have been isolated and characterized from the human population, and numerous different types have been isolated from other animal species (Ishibashi and Yasue, 1984). Human adenoviruses cause a variety of acute respiratory, ocular, gastrointestinal, and urinary diseases (for review see Straus, 1984). Based on various criteria, including hemagglutination properties (Rosen, 1960), GC content (Pina and Green, 1965), DNA homology (Green *et al.*, 1979), restriction maps (Wadell *et al.*, 1980), and oncogenicity (Green 1970), the serotypes of human adenovirus have been divided to six subgroups. The genomic sequence of human type 2 adenovirus (Ad2) has been completely determined and contains 35,937 base pairs (bp) (Gingeras *et al.*, 1982). Adenoviruses have been used as model systems to study DNA replication, gene expression, and oncogenic transformation.

Adenovirus type 12 (Ad12) was the first adenovirus reported to induce tumors when inoculated into newborn hamsters (Huebner *et al.*, 1962; Trentin *et al.*, 1962). Similar studies showed that adenovirus serotypes differed in tumorigenicity (for review see Green, 1970). Ad12, Ad18 and Ad31 are highly oncogenic and produce tumors in most animals within two months (Huebner *et al.*, 1962; Trentin *et al.*, 1962; Pereira *et al.*, 1965). Oncogenic adenoviruses also transform cells, and adenovirus DNA is integrated into cellular chromosomes (Lichtenberg *et al.*, 1987; Vogel *et al.*, 1986; Rosahl and Doerfler, 1988). The integrated adenovirus DNA is amplified several-fold to hundred-fold in transformed cells (Doerfler and Eick, 1982). The mechanism of amplifying integrated virus DNA is still unclear.

Adenovirus DNA structure and DNA replication

The adenovirus genome is a linear, double-stranded DNA. The sequence of the Ad2 genome has been completely determined (Gingeras *et al.*, 1982). There are two important structural features of the adenovirus genome which are essential for DNA replication. First, the sequence at both ends of the molecule are identical inverted repeats. The length of the inverted terminal repeat (ITR) sequence ranges from 63 to 166 bp in different adenovirus serotypes (Stillman *et al.*, 1982; Steeberg *et al.*, 1977; Shinagawa *et al.*, 1983; for review see van Ormondt and Galibert, 1984). There are conserved or highly homologous sequences in all ITRs (Steeberg *et al.*, 1977; Shinagawa *et al.*, 1983; Arrand and Roberts, 1979; for review see van Ormondt and Galibert, 1984). Second, the virus-encoded preterminal protein (pTP) is covalently linked to the first nucleotide, usually a C residue, at each 5'-end through a phosphodiester bond (Robinson *et al.*, 1973; Robinson and Bellett, 1974; Rekosh *et al.*, 1977; Carusi, 1977; Stillman *et al.*, 1982). During virus assembly, the 80-kd pTP is processed by a viral-encoded protease to a 55-kd terminal protein (TP) (Challberg *et al.*, 1980; Stillman *et al.*, 1981; Challberg and Kelly, 1981; Smart and Stillman *et al.*, 1982).

Adenovirus DNA replication initiates at the 5'-end of either or both strands. The origins of replication are located within the terminal 55 to 60 bp of the ITRs (Wang and Pearson, 1985; Hay, 1985a, 1985b; Bernstein *et al.*, 1986; Challberg and Rawlins, 1984; Tamanoi and Stillman, 1983a, 1983b; Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984b; Pearson *et al.*, 1983; Hay *et al.*, 1984; Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983). The pathways of adenovirus DNA replication are diagrammed in Fig. I.1 (Lechner and Kelly, 1977; Kelly and Lechner, 1978). Several virus-encoded as well as cellular proteins are involved in the replication of adenovirus DNA (described in detail below).

Adenovirus DNA is replicated in two stages. In the first stage, pTP serves as primer to initiate replication which then proceeds unidirectionally to 3' end by a strand-displacement mechanism (Lechner and Kelly, 1977; Kelly and Lechner, 1978). The initiation process involves the creation of a protein-nucleotide complex by forming a phosphodiester linkage between the β-OH of a serine residue in pTP and the α-phosphoryl group of the first dCMP residue in the new DNA strand (Challberg *et al.*, 1980). Elongation

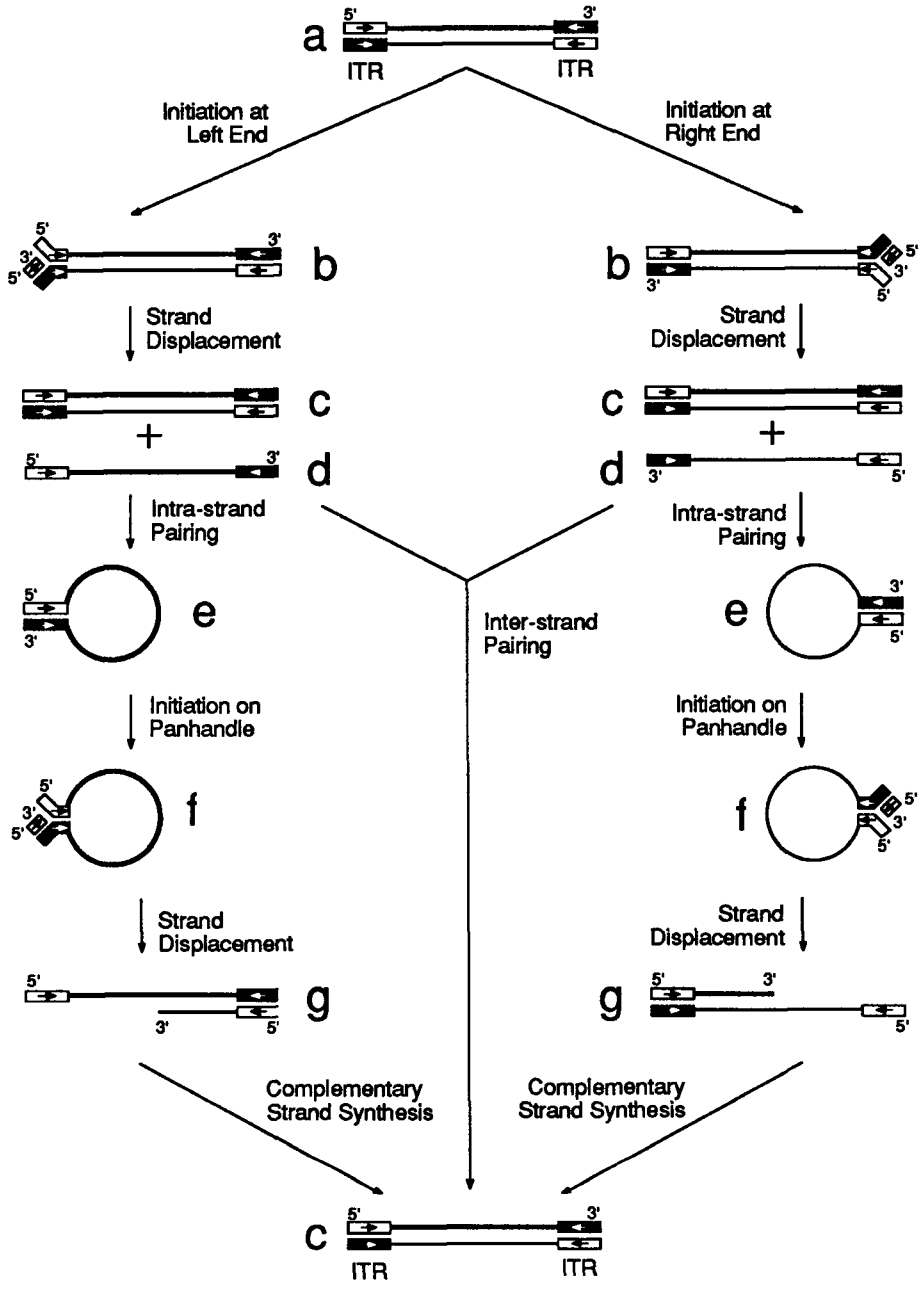

Figure I.1. Overall scheme for adenovirus DNA replication. Inverted terminal repetitions (ITRs) are shown as open and closed boxes with black and white arrows to indicate orientation. Complementary DNA strands are depicted as thick and thin lines. The polarity of DNA strands are designated by 3' and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

of the pTP-dCMP complex proceeds uninterruptedly to the end of the template strand with concomitant displacement of the nontemplate strand (Rekosh *et al.*, 1977). In the second stage, there are two alternate pathways to convert the displaced parental strand into a double-stranded daughter molecule (Ahern *et al.*, 1990). The *cis* pathway involves intramolecular base-pairing between complementary ITR sequences to regenerate a double-stranded replication origin on a "panhandle" replication intermediate (Lechner and Kelly, 1977; Kelly and Lechner, 1978). The *trans* pathway allows intermolecular hybridization between complementary displaced strands to reform double-stranded molecules directly (Ahern *et al.*, 1990).

In vitro and *in vivo* studies of DNA replication

Adenovirus DNA replication has been one of the most completely studied systems as a model for eukaryotic DNA replication. Many features of adenovirus DNA replication were initially established by *in vivo* work using infected cells (for review see Winnacker, 1978). However, profound insights into the biochemistry and enzymology of adenovirus DNA replication have come from the development of a cell-free system supporting *in vitro* DNA replication (Challberg and Kelly, 1982). For recent reviews of adenovirus DNA replication see Challberg and Kelly (1989) and Stillman (1989).

Early studies of the adenovirus replication cycle focused mostly on the events that occur in Ad2- or Ad5-infected cultured human (HeLa or KB) cells. Adenovirus infection is initiated by viral fibers attaching to specific cellular receptors (Philipson *et al.*, 1968). The virions enter cell by endocytosis or direct penetration of cytoplasmic membrane (Chardonnet and Dales, 1970a, 1970b; Lonberg-Holm and Philipson, 1969; Mogan *et al.*, 1969). The viral capsid proteins are mostly removed in the cytoplasm of infected cells (Philipson *et al.*, 1968; Lonberg-Holm and Philipson, 1969; Mogan *et al.*, 1969), and the rest of the coat proteins are removed in the nucleus, yielding "almost" protein-free viral genomes (Mogan *et al.*, 1969; Chardonnet and Dales, 1970a, 1970b).

Replication of viral DNA is first detected about 6 to 8 hr after infection. Early in infection, viral DNA synthesis proceeds by a semiconservative mechanism (Pearson and Hanawalt, 1971; Bellett and

Younghusband, 1972; van der Vliet and Sussenbach, 1972; van der Eb, 1973) and reaches a maximal rate about 19 hr after infection (Green *et al.*, 1970). Approximately 10^5 to 10^6 progeny viral genomes are produced per cell by 24 hr, but only 20% are ultimately packaged to form progeny viral particles (Green *et al.*, 1970). Cellular DNA replication is inhibited at 6 to 8 hr after infection and is virtually absent by 12 hr after infection (Pina and Green, 1965; Mantyjarvi and Russell, 1969).

Viral replicative intermediates, first identified by pulse-labeling methods (Pearson and Hanawalt, 1971; van der Vliet and Sussenbach, 1972; Burlinghan and Doerfler 1971; Pearson 1975), contain extensive regions of single-stranded DNA (ss DNA). The basic structural features of adenovirus replicative intermediates have been defined by several different methods. Electron microscope analysis showed two basics types of replicative intermediates, a type I intermediate and a type II intermediate. Accordingly, a model for adenovirus DNA replication was advanced (Ellens *et al.*, 1974; Lechner and Kelly, 1977). Type I molecules, composed of a unit-length, double-stranded segment with one or more single-stranded branches, are replicative intermediates engaging in strand-displacement synthesis (Lechner and Kelly, 1977; Kelly and Lechner, 1978). Type II molecules, unbranched, unit-length, linear molecules containing both double- and single-stranded segments, are replicative intermediates in the process of synthesizing the complementary strand (Lechner and Kelly, 1977; Kelly and Lechner, 1978). Pulse-labeling and electron microscopic techniques mapped the adenovirus origins as well as the termini of DNA replication at or near the ends of the genome.

The biochemistry of adenovirus DNA replication has also been studied *in vivo* by using transfection assays. The Ad2 terminal repeat sequences were cloned into plasmid vectors (Stow, 1981; Hay *et al.*, 1984). After sequence-specific endonuclease cleavage at or near the adenovirus origin sequences, the linearized plasmids were cotransfected into cells with purified Ad5 DNA as helper. It was found that the plasmids replicated as adenovirus mini-chromosomes in the transfected cells (Hay *et al.*, 1984). This provided a new strategy to study the mechanisms of adenovirus DNA replication. This approach will be discussed in detail below.

Recently, profound insights into the biochemistry and enzymology of adenovirus DNA replication have come from the development of a cell-free

DNA synthesis system (Challberg and Kelly, 1979). Various studies demonstrated that the overall mechanism of DNA replication *in vitro* reflects the mechanism deduced from studies *in vivo*. The crude cellular extract from infected cells has been fractionated to identify viral and cellular factors required for adenovirus DNA replication.

Adenovirus encodes three proteins that are directly required for the replication of its DNA: the 80-kd precursor of terminal protein (pTP) (Stillman *et al.*, 1981; Alestrom *et al.*, 1982), the 140-kd DNA polymerase (Ad pol) (Alestrom *et al.*, 1982), and the 59-kd single-strand DNA binding protein (Ad DBP) (Kruijer *et al.*, 1981). The biochemistry of the adenovirus replication proteins has been studied in detail. Both pTP and Ad pol have been expressed functionally after transfection with plasmid expression vectors (Shu *et al.*, 1987; Pettit *et al.*, 1988) or after infection with recombinant vaccinia virus (Stunnenberg *et al.*, 1988). A recent study indicates that the nuclear localization of the adenovirus DNA polymerase is facilitated by its interaction with pTP (Zhao and Padmanabhan, 1988). Thus, pTP and Ad pol may enter the nucleus as a complex initially formed in the cytoplasm. Recently, nuclear factor I (NFI) and Ad DBP were shown to interact cooperatively at the viral replication origin (Cleat and Hay, 1989).

By using reconstitution of replication as an assay, three cellular proteins, called nuclear factors (NF), required for efficient adenovirus DNA replication have been identified and characterized: NFI (Hay, 1985b; Wang and Pearson, 1985; Rawlins *et al.*, 1984), NFIII (Pruijn *et al.*, 1986), and NFII (Nagata *et al.*, 1983). Interestingly, NFI and NFIII also have transcriptional activities. NFI is identical to mammalian transcription factor CTF (Jones *et al.*, 1987) and NFIII is itentical to the cellular enhancer and promoter binding factor OTF-1 (Octamer transcription factor; also called Oct-1 or OBP-100; O'Neill *et al.*, 1988). OTF-1 is a member of the POU-protein family (Herr *et al.*, 1988). The apparent involvement of transcription factors in eukaryotic DNA replication is not understood.

Plasmids containing cloned adenovirus terminal sequences support viral-specific DNA replication *in vitro* provided that the plasmids are linearized to place the origin sequences at the ends of the DNA molecules (Guggenheimer *et al.*, 1984a, 1984c; Tamanoi and Stillman, 1983a, 1983b; Challberg and Rawlins, 1984). Initiation of replication on linearized plasmid

DNAs *in vitro* requires a cellular protein, factor pL, in addition to the previously defined viral and cellular replication proteins (Guggenheimer *et al.*, 1984b; Kenny *et al.*, 1988). pL has 5'-to-3' exonuclease activity required to remove sequences from the 5' end of the non-template strand to expose the 3'-dG residue in the template strand. This facilitates efficient formation of the pTP-dCMP complex (Guggenheimer *et al.*, 1984b). Linear plasmids containing the adenovirus origin have been used to define the sequences required for initiation of replication *in vitro*.

Initiation of adenovirus DNA replication

Adenovirus DNA replication origins have been mapped at or near the ends of the viral genome using *in vivo* and *in vitro* assays (Wang and Pearson, 1985; Hay, 1985a; Bernstein *et al.*, 1986; Challberg and Rawlins, 1984; Tamanoi and Stillman, 1983a; Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984b; Pearson *et al.*, 1983; Hay *et al.*, 1984; Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983). The precise extent of the viral origin was elucidated by analyzing the replication properties of plasmids that contained deletion or substitution mutations within the cloned viral terminal sequences (Tamanoi and Stillman, 1983a; van Bergen *et al.*, 1983; Challberg and Rawlins, 1984; Wides *et al.*, 1987).

The sequence organization of the viral origin of replication is complex (Tamanoi and Stillman, 1983a; Challberg and Rawlins, 1984; Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984a; Pruijn *et al.*, 1986; Wides *et al.*, 1987). There are at least three functionally distinct sequence domains that contribute to the overall efficiency of the initiation reaction. Domain A consists of the first 18 bp of the viral genome, constitutes the minimal origin of replication, and supports the basal level of the replication reaction (Wides *et al.*, 1987). A 10-bp sequence, ATAATATACC, is absolutely conserved within this domain in all adenovirus serotypes (Stillman *et al.*, 1982; van Bergen *et al.*, 1983; Shinagawa *et al.*, 1983). The conserved sequences are recognized by the pTP-Ad pol complex (Rijinders *et al.*, 1983). Mutating or deleting nucleotides between nucleotides 9 and 18 prevent initiation (Challberg and Rawlins, 1984; Guggenheimer *et al.*, 1984b; Rawlins *et al.*, 1984). Conversely, substitutions or deletions within the terminal 8 bp did not have any apparent effect on DNA replication (Challberg and Rawlins, 1984; Guggenheimer *et al.*, 1984b, Hu and Pearson, unpublished data).

Domain B consists of nucleotides 19 through 39, including the NFI binding site (nucleotides 25 to 39) (Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984c; de Vries *et al.*, 1985; Wides *et al.*, 1987). Domain C encompasses nucleotides 40 through 51, the binding site for factor NFIII (Pruijn *et al.*, 1986, Wides *et al.*, 1987). The presence of domains B and C enhances the efficiency of initiation of Ad2 or Ad5 DNA replication about 30-fold.

Circular or linear DNA containing embedded viral origins do not support initiation of adenovirus DNA replication *in vitro* (Guggenheimer *et al.*, 1984b, Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983). However, circular plasmid DNA containing two viral origins in inverted orientation give rise in transfected cells to a replicating linear molecule where the nucleotides flanking the two origins were lost in the product (Hay *et al.*, 1984). Circular adenovirus DNAs linked head-to-tail have been found in infected cells (Ruben *et al.*, 1983; Graham, 1984). The circular forms of the viral DNA are as infectious as linear forms. Circular plasmids containing the whole viral genome also replicate in transfected cells and produce infectious virus particles, even when several nucleotides have been deleted at both viral termini (Graham, *et al.*, 1989). Earlier studies produced no evidence for infectious virus arising from circular plasmid DNA (Hanahan and Gluzman, 1984). The reason for this discrepancy is still unknown.

The replication of linear molecules containing embedded adenovirus origins has also been studied. Plasmid pXAd, which contains the entire adenovirus genome, was restricted in such a way that both viral origins were embedded. Infectious virus particles were recovered after transfection into cells. Sequences flanking the embedded origins were lost in the progeny viral genomes (Hanahan and Gluzman, 1984). Plasmids containing two viral origins were linearized to leave both origins embedded. Linear replicating molecules lacking flanking sequences were detected in cells cotransfected with helper adenovirus DNA (Hay *et al.*, 1984; Vasudevachari *et al.*, 1987). Adenovirus mutants containing various sequences inserted in front of the left terminus were constructed by Stow (1981; 1982). These mutants yielded wild type progeny virus lacking the inserted sequences (Stow, 1982). Several adenovirus mutants have been isolated or constructed which contain an extra copy of the ITR sequences duplicated either at the left or right end of the genome (Brusca and Chinnadurai, 1983; Hanahan and Gluzman, 1984; Haj-Ahmad and Graham, 1986). The extra copy of the

ITR was either lost to create a wild type progeny adenovirus or, less frequently, duplicated at the other end to produce a variant containing four copies of the ITR. These events can be understood in terms of intramolecular interactions between the multiple ITR copies. Although embedded ITR sequences were considered unlikely to direct adenovirus DNA replication, the possibility of internal initiation from the embedded origins was not ruled out (Brusca and Chinnadurai, 1983; Hanahan and Gluzman, 1984).

Complementary strand synthesis in adenovirus DNA replication

Adenovirus DNA replication initiates from either or both origins within the ITR sequences (Lechner and Kelly, 1977). Once initiated, replication proceeds to the end of template using a strand-displacement mechanism to produce a daughter duplex and a displaced non-template (or parental) strand. Although the mechanism of the sequence-dependent initiation of replication has been intensively studied *in vitro*, the mechanism of the synthesis of the complementary strand is still not understood very well. It has been proposed that intramolecular base-pairing between the complementary sequences of the ITRs could regenerate a double-stranded origin on a "panhandle" replication intermediate (Lechner and Kelly, 1977). Initiation of replication on the regenerated origin of the "panhandle" intermediate is mechanistically identical to initiation on linear, double-stranded molecules. This pathway has been termed *cis* replication to emphasize the involvement of intramolecular base-pairing. In addition to *cis* replication, Ahern *et al.* (1990) discovered an alternative pathway to convert displaced complementary strands into progeny double-stranded molecules. In this pathway, called *trans* replication, complementary strands simply hybridize intermolecularly to reform linear, double-stranded DNA.

Transformation and DNA recombination

Ad12 was the first adenovirus serotype discovered to induce tumors when inoculated into newborn hamsters (Trentin *et al.*, 1962). Similar inoculation studies soon showed that adenovirus serotypes differed in tumorigenicity (Green, 1970): highly oncogenic group A viruses (Ad12, Ad18, Ad31), weakly oncogenic group B viruses (Ad3, Ad7, Ad11, Ad14, Ad16, Ad21), and nononcogenic viruses of group C (Ad1, Ad2, Ad5, Ad6), group D

(Ad8-Ad10, Ad13, Ad15, Ad17, Ad19, Ad20, Ad22-Ad30), and group E (Ad4). Both oncogenic and nononcogenic groups are able to induce morphological transformation of cells in culture (McBride and Weiner, 1964; Casto, 1968; McAllister *et al.*, 1969a, 1969b). The transformed cells can usually be expanded into lines of cells capable of dividing indefinitely. However, the process of transformation of cells in culture is not identical to tumor induction in animals. Many cell lines transformed by tumor viruses cannot induce tumors following inoculation into immunologically appropriate animals. In particular, rat cells transformed by group C adenoviruses do not induce tumors in rats, unlike their Ad12-transformed counterparts.

Although adenovirus-induced tumors were free of virus, virus-specific antigens and mRNA were frequently found in the tumors or transformed cells (Girardi *et al.*, 1964; Fujinaga and Green, 1966, 1967). The continued presence and expression of viral information may therefore be necessary for the maintenance of the transformed phenotype. Several studies showed that all Ad2-transformed rat cell lines always contain the left end of the genome regardless of the presence of other viral sequences (Sharp *et al.*, 1974; Gallimore *et al.*, 1974; Flint *et al.*, 1976; Johansson *et al.*, 1977, 1978), and viral DNA is always integrated into host chromosomal DNA (Green *et al.*, 1970). Most rodent cells transformed by Ad2 and Ad5 contain only limited portions of the viral genome. In certain cell lines transformed by host-range mutants or temperature-sensitive mutants of Ad5, the entire viral genome is integrated into cellular chromosome (Ruben *et al.*, 1982). In contrast, the entire Ad12 genome is usually integrated into host DNA in tumors and transformed cells (Green *et al.*, 1976; Ibelgaufts *et al.*, 1980; Kuhlmann *et al.*, 1982).

In situ hybridization has been used to localize integrated viral sequences on specific chromosomes in Ad12-transformed hamster cells as well as in human cells productively infected with Ad12 (Vogel *et al.*, 1986). About 20 copies of Ad12 DNA were found in an integrated form, predominantly located on different chromosomes, in various hamster cell lines (Vogel *et al.*, 1986). Few of these copies remained intact and colinear with viral DNA; most integrated viral genomes possessed deletions, inversions, or both in the right molecular end (Vogel *et al.*, 1986). Spontaneously arising morphological revertants of Ad12-transformed cells frequently have lost all or part of the viral DNA. The mechanism of

integration is unclear. In transformed cells, left and right molecular ends of viral DNA are frequently observed to be joined together in a head-to-tail joint (Sambrook *et al.*, 1979; Stabel *et al.*, 1980; Visser *et al.*, 1981, 1982). Such a structural arrangement suggests that a circular form of viral DNA could be an intermediate in the process of integration. DNA amplification and DNA rearrangement may also occur after integration (Doerfler and Eick, 1982).

CHAPTER II

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases, *Escherichia coli* (*E. coli*) DNA polymerase I (Klenow fragment), T4 DNA ligase, and S1 endonuclease were purchased from Bethesda Research Laboratories (BRL), Boehringer Mannheim Biochemicals, or International Biochemicals, Inc. (IBI). Pronase was purchased from Calbiochem. Lysozyme was purchased from Sigma. All the enzymes were used as recommended by the manufacturers. All chemicals and reagents were molecular biology grade (nuclease- and protease-free) and were purchased from Sigma, Merck, or Boehringer Mannheim Biochemicals. [α-^{32}P]deoxyribonucleotide triphosphates (specific activity 3,000 Ci/mmol) and [γ-^{32}P]ATP (specific activity > 6,000 Ci/mmol) were purchased from New England Nuclear or ICN.

Plasmids and bacteria

E. coli RR1 and *E. coli* DH5α, supplied as competent cells, were purchased from BRL and treated according to the supplier's specifications for high efficiency transformation. Transformed cells were grown in modified YT medium (containing per liter: 5 g yeast extract, 10 g Casein enzyme hydrolysate, and 5 g NaCl). Selection of cells transformed with recombinant plasmids was carried out in medium containing one or more of the following antibiotics: ampicillin (50 μg/ml), tetracycline (15 μg/ml), or kanamycin (50 μg/ml). Isolation of plasmid DNA was by the alkaline extraction procedure (Birnboim and Doly, 1979) followed by ethidium bromide-CsCl density gradient centrifugation.

Plasmids T4 and pADIRK2 were constructed by Kai Wang and Kevin G. Ahern, respectively. The structures are illustrated in Fig. II.1. In T4, the left terminal 338 bp of type 2 adenovirus DNA, excised from pMDC10 (Enns *et al.*, 1983) by *Eco*RI-*Ssp*I cleavage, were cloned into pUC19 (Messing *et al.*, 1979) to substitute for the vector *Eco*RI-*Ssp*I segment. Digesting with *Eco*RI positions viral terminal sequences at the end of the linear T4 molecule and digesting with *Pvu*II leaves the origin sequences embedded 230 bp within the linearized T4 molecule (Fig. II.1). In

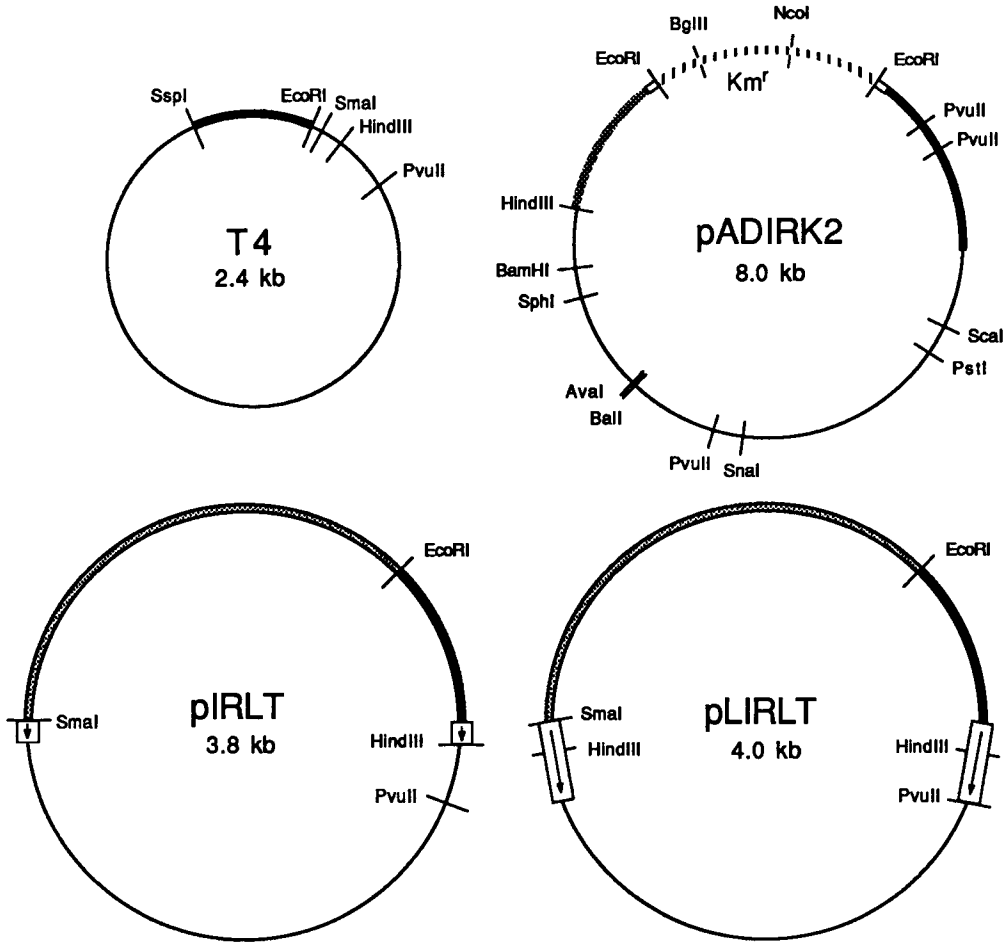

Figure II.1. Structures of plasmids T4, pADIRK2, pIRLT and pLIRLT. Plasmids T4, pIRLT, and pLIRLT are drawn to scale. The thin lines represent plasmid vector sequences. The adenovirus replication origins cloned into each plasmid are immediately adjacent to *Eco*RI sites. The thick line in plasmid T4 represents the left adenovirus terminus. Left and right adenovirus termini in pADIRK2 are indicated respectively by thick black and stippled lines where viral origins are shown as small open boxes (in inverted orientation with respect to each other). The hatched line in pADIRK2 indicates the kanamycin-resistance gene. The symbols for pIRLT and pLITLT are the same: thick lines are the same left adenovirus terminus found in plasmid T4; the thick stippled lines represent the kanamycin-resistance gene; and open boxes indicate inverted repeat sequences where the arrows show the orientation of the repeats. The repeats are 37 bp in pIRLT and 230 bp in pLIRLT.

pADIRK2, 1,423 bp of the left terminus and 1,000 bp of the right terminus of Ad2 DNA were cloned into pBR322 in opposite orientations. The kanamycin gene (1.4 kb) separates the two viral terminal sequences (Fig. II.1). Digesting with *Eco*RI excises the kanamycin gene from pADIRK2 and leaves exposed viral termini at both ends of the large remaining fragment (Fig. II.1). Digesting with *Bgl*II linearizes pADIRK2 leaving both viral origins embedded 420 bp and 1000 bp from the molecular ends (Fig. II.1).

Plasmid pLIRST (Fig. II.2) was constructed by Andrew Pearson. T4 was digested with a combination of *Ssp*I-*Pvu*II or *Ssp*I-*Sma*I endonucleases. The smaller and larger fragments, respectively, were purified from each digestion, ligated by T4 DNA ligase, and transformed into *E. coli* DH5α cells. Plasmid pIRST was constructed in a manner similar to the construction of pLIRST (Fig. II.2). T4 was cleaved with a combination of *Ssp*I-*Hind*III or *Ssp*I-*Sma*I endonucleases. The smaller and larger fragments, respectively, from each digestion were ligated together. Plasmids pIRLT and pLIRLT (Fig. II.3) were derived from pIRST and pLIRST, respectively. The 1.3-kb *Eco*RI-*Sma*I kanamycin fragment from pKAT21 was inserted into the large fragments of *Eco*RI-*Sma*I digestions of pIRST and pLIRST, respectively. The size of pIRLT is 3.8 kb and the size of pLIRLT is 4.0 kb. The newly constructed plasmids were isolated under kanamycin selection. *Eco*RI-cleavage positions the viral DNA replication origin at the molecular end of either pLIRLT or pIRLT. Cleavage by *Bgl*II embeds the replication origin by 420 bp on both plasmids (Fig. II.3).

Cell culture and adenovirus DNA purification

HeLa S3 cells were maintained in suspension culture in Joklik's minimal essential medium (GIBCO) containing 5% fetal calf serum (Hyclone). Type 5 and Mac-type adenovirus were grown in HeLa S3 cells. Mac-type adenovirus has been described previously (Lippe and Graham, 1989). The sequence differences between Mac-type and type 2 adenoviruses occur within the first seven nucleotides of the respective termini. The Mac-type termini start with the sequence 5'-CTATTCTAATAATATACC...3' which differs from the type 2 sequence 5'-CATCATCAATAATATACC...3'. Virus and viral DNA were isolated and purified as previously described (Pettersson and Sambrook, 1973). The viral DNA was digested with pronase and extracted with phenol/chloroform to remove the associated

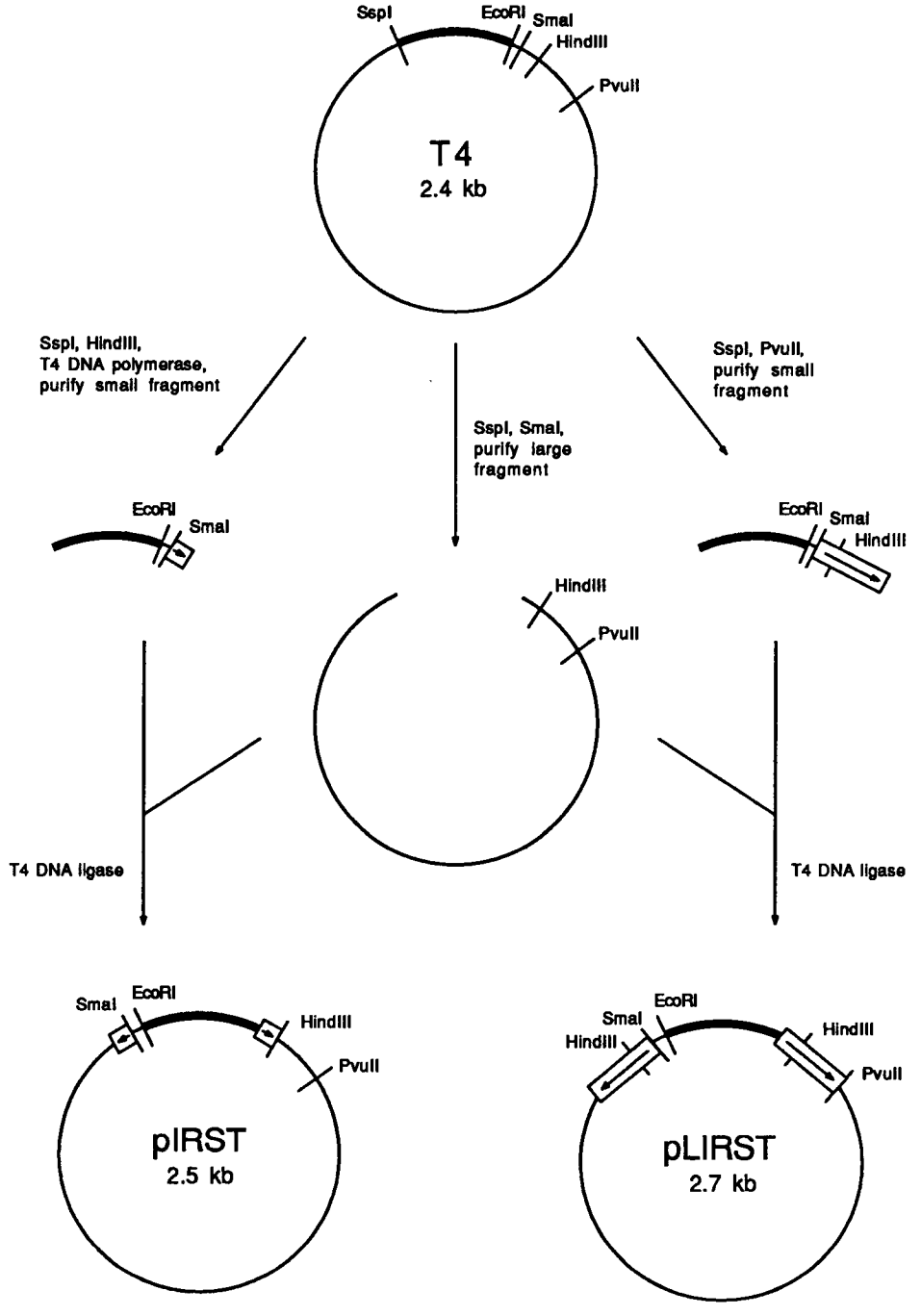

Figure II.2. Construction of plasmids pIRST and pLIRST. Symbols are as shown in Fig. II.1. pIRST has 37-bp inverted repeats derived from the polylinker region of pUC19. pLIRST has 230-bp inverted repeats that include the same 37-bp repeat sequences in pIRST. Both plasmids have 14 bp between the *Sma*I and *Eco*RI sites, and both plasmids carry 338 bp of the left adenovirus terminus (from plasmid T4) containing the viral replication origin. See text for experimental details.

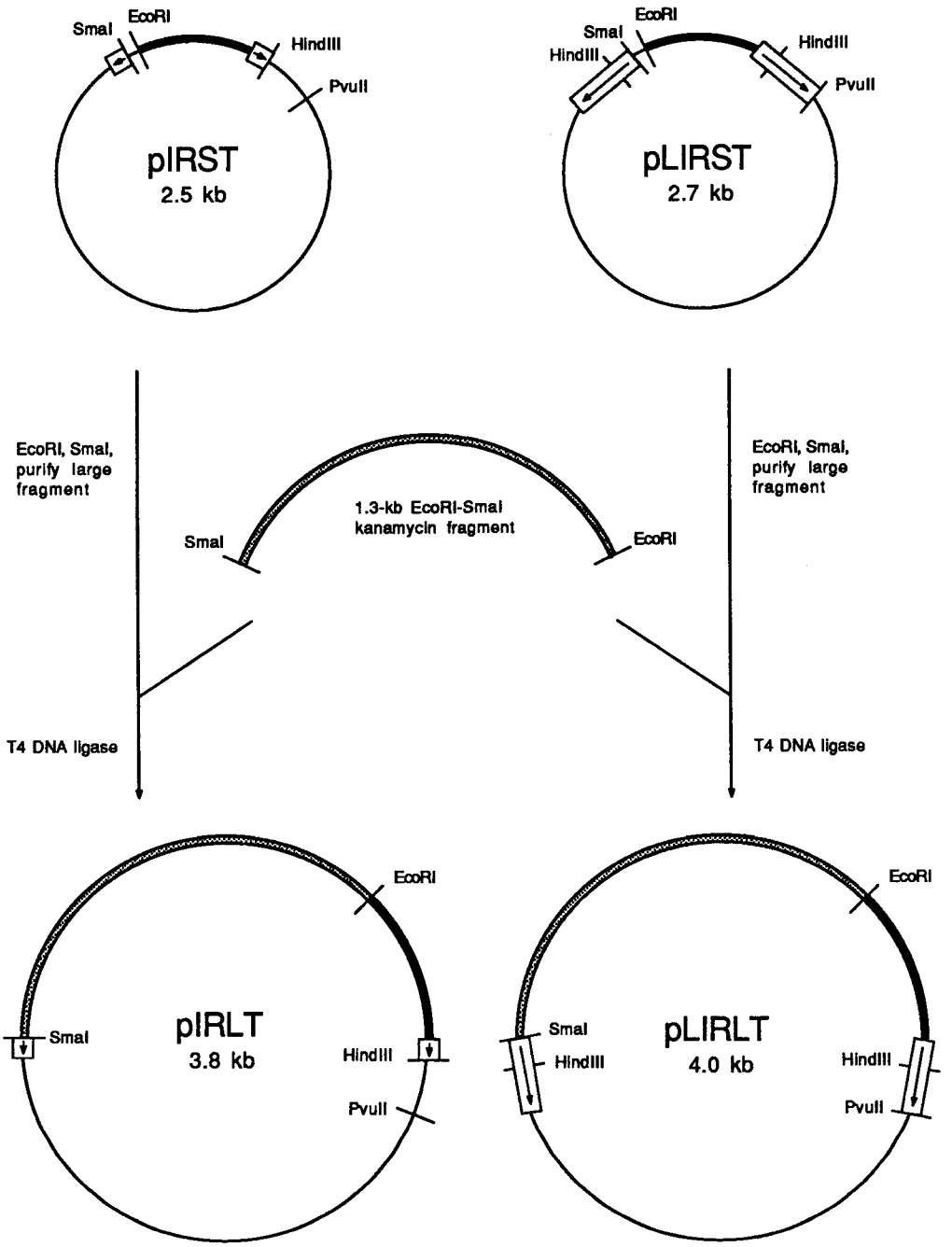

Figure II.3. Construction of plasmids pIRLT and pLIRLT. Symbols are as shown in Fig. II.1. pIRLT has 37-bp inverted repeats derived from the polylinker region of pUC19. pLIRLT has 230-bp inverted repeats that include the same 37-bp repeat sequences in pIRLT. Both plasmids have a 1.3-kb kanamycin-resistance gene inserted between the *Sma*I and *Eco*RI sites, and both plasmids carry 338 bp of the left adenovirus terminus (from plasmid T4) containing the viral replication origin. See text for experimental details.

proteins. The purity of viral DNA was examined by 1% agarose electrophoresis. The 293 cell line (Graham *et al.*, 1977) was grown in monolayer culture in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal calf serum, 1% penicillin/streptomycin, and glucose at 1 mg/ml. Forty-eight hrs before transfection, 10^6 293 cells were seeded into 60 mm tissue culture plates using the same medium supplemented with a higher concentration of glucose (4 mg/ml) to enhance cell attachment to the tissue culture plates.

DNA transfection

Transfection was performed with a modified procedure of the DNA-calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). Each plate received linearized plasmid DNA (4 μg) transfected together with helper adenovirus DNA (4 μg). The transfection was performed by gently mixing 125 μl of 0.5 M $CaCl_2$ solution containing the plasmid and helper DNA with 125 μl of 2X HBS buffer (pH 7.05). 1X HBS contains 280 mM NaCl, 50 mM HEPES, and 1.5 mM Na_2HPO_4. After 20 minutes, the mixture was then added to cells. After incubating at 37°C in the CO_2 incubator for another 20 minutes, 5 ml of medium, with glucose at 4mg/ml, was added to each plate. To enhance the efficiency of transfection, cells were shocked six hours after tranfection with 15% glycerol in 1X HBS buffer for 2 min (Frost and Williams, 1978). Cells were harvested after another 66 hr at 37°C in CO_2 incubator in order to isolate the total DNA.

DNA extraction

Total DNA was extracted from transfected cells after three days incubation (Hirt, 1967; Wilkie, 1973). The cells were concentrated by centrifugation for 5 min at 3,500 rpm. Cell pellets were first rinsed with 1 ml of PBSd buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH7.5) and then resuspended in 0.4 ml lysis solution (0.6% SDS, 10 mM EDTA, pH 8.0). The lysate was digested with pronase (1 mg/ml) at 37°C overnight. The sample was then mixed with 50 μl 5 M NaCl and the total DNA was then precipitated with 1 ml of 95% ethanol. The DNA sample was extracted with an equal volume of phenol three times, followed by extraction twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). The purified DNA was reprecipitated

with ethanol, and resuspended in 200 µl water. The concentration of nucleic acid was calculated using the A_{260} value (Maniatis *et al.*, 1982).

Replication assay by *Dpn*I and *Mbo*I digestion

Total DNA (40 µg) extracted from transfected cells was digested with *Dpn*I, *Mbo*I, or both. *Dpn*I-*Mbo*I digestion was used to assay eukaryotic DNA replication (Peden *et al.*, 1980). *Dpn*I and *Mbo*I recognize the same GATC sequence. However, *Dpn*I cuts the site only when both strands are methylated, while *Mbo*I cuts the site only when both strands are unmethylated. Site-specific methylation of plasmid DNA propagated in Dam^+ *E. coli* host cells converts the sequence GATC to GmeATC, a *Dpn*I-sensitive site. Mammalian cells lack the methylase and cannot methylate newly replicated DNA at GATC sites. Consequently, DNA replicated in mammalian cells is sensitive only to *Mbo*I. Hemimethylated DNA cannot be cut by either *Dpn*I or *Mbo*I.

Southern blot analysis

Southern blotting using alkaline transfer has been described (Chomczyhski and Qasba, 1984; Reed and Mann, 1985). Total DNA (40 µg) isolated from transfected cells was first separated by 1% agarose gel electrophoresis in TAE buffer (40mM Tris-acetate, 2mM EDTA, pH 8.5) at 50 mA for 11 hr, then transferred from the gel onto a "Zeta-Probe" nylon membrane (Bio Rad) in 0.4N NaOH overnight.

Two-dimensional agarose gel electrophoresis

Total DNA (80 µg) extracted from transfected cells was treated with various enzymes, such as *Dpn*I, *Mbo*I, or S1 nuclease. Two-dimensional agarose gel electrophoresis was performed as modified from the method of Modak and Beard (1980). The digested DNA was divided into two equal amounts, loaded onto 1% agarose gels, and electrophoresed in TAE buffer for 10 hr at 50mA. One of the duplicate samples and linearized plasmid (1-5 ng) loaded in the adjacent lane were used as a markers. After electrophoresis, the gel was soaked in alkaline denaturing solution (30mM NaOH, 2mM EDTA, pH > 12) for 45 min, and then marker lanes were cut off. The rest of the gel, which contained one of the duplicate loadings of total DNA, was turned 90° to leave the previously resolved DNA at the top

of the gel. Alkaline electrophoresis in the second dimension was for 15 hr at 35 mA. The DNA in the gel was then transferred to a "Zeta-Probe" nylon membrane as described above.

Radioactive-labeling of plasmid DNA by random-priming

CsCl-banded pUC19 DNA was labeled with [α-^{32}P]dGTP at room temperature overnight using the random-priming method (Feinberg and Vogelstein, 1983; 1984). The reaction solution (50 µl) contained 31 µl DNA (about 100 ng, heat-denatured), 10 µl reagent mix (250 mM Tris-HCl, pH 8.0; 250 mM $MgCl_2$; 0.1 µM each of dTTP, dATP, and dCTP; 1 M Hepes, pH 6.6; and hexadeoxyribonucleotides at 27 A_{260} units/ml); 2 µl bovine serum albumin (5 mg/ml); 5 µl [α-^{32}P]dGTP (50 µCi; 3,000 Ci/mmol), and 2 µl Klenow fragment of DNA polymerase I (2 to 5 units). After synthesis, unincorporated nucleotides were removed by Nuclean-50 spin column chromatography (IBI). Probe DNA was denatured in boiling water for 5 min just before using.

End-labeling of oligonucleotide probes

The termini of Mac-type and type 2 adenoviruses were differentially detected by hybridization with labeled oligonucleotides specific for either of the 5'-terminal sequences. The 15-mer oligonucleotides were synthesized by Dr. R. H. McParland in the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University using an Applied Biosystems automated DNA synthesizer. The sequence of Mac-oligo was 3'-GATAAGATTATTATA-5' and the sequence of wildtype-oligo (WT-oligo) was 3'-GTAGTAGTTATTATA-5'. The oligonucleotides were end-labeled with [γ-^{32}P]ATP (specific activity 6,000 Ci/mmol) using T4 polynucleotide kinase as described by Maxam and Gilbert (1980). The oligomer molecules were then purified by chromatography through Nuclean-25 spin-columns (IBI) and stored at -15°C.

DNA-DNA hybridization

Radiolabeled plasmid probe hybridization was performed as follows. The blotted filters were rinsed with wash solution (2X SSC, 0.1 % SDS, pH7.0) until the pH was below 8.0. 1X SSC contains 150 mM NaCl and 15

mM sodium citrate, pH 7.0. After neutralization, the filter was incubated in 50 ml of prehybridization solution (5X SSC; heat-denatured salmon sperm DNA at 0.25 mg/ml; 50 mM sodium phosphate, pH 6.7; 50% formamide; 0.5% non-fat dry milk; and 1% SDS) for 4 hr at 37°C. Hybridization was carried out by incubating the prehybridized membrane with radioactively labelled DNA probes (specific activity > 10^8 cpm/μg) overnight at 37°C in 12.5 ml of hybridization solution (5X SSC; heat-denatured salmon sperm DNA at 0.1 mg/ml; 20 mM sodium phosphate, pH 6.7; 10% dextran sulfate; 50% formamide; and 1% SDS). After hybridization, the membrane was first rinsed twice for 15 min at room temperature with wash solution, followed with a stringent rinse using the same wash solution at 65°C for 4 hrs. Autoradiography was performed with X-ray film (Kodak X-OMAT XAR-5) for 24 to 120 hours utilizing an intensifying screen at -20°C.

Hybridization with end-labeled oligonucleotide probes was performed as modified from the method of Zeff and Geliebter (1987). The filter was neutralized as above described. Then it was incubated in 50 ml of prehybridization solution (5X SSC; 20 mM sodium phosphate, pH 7.0; 10X Denhardt's solution; 7% SDS; and heat-denatured salmon sperm DNA at 0.1 mg/ml) for 4 hr at 29°C (5 to 7°C below the dissociation temperature). 1X Denhardt's solution contained Ficoll at 0.2 mg/ml, polyvinylpyrrolidone at 0.2 mg/ml, and BSA (Pentax Fraction V) at 0.2 mg/ml. The dissociation temperature (T_d) of the oligonucleotide probes was determined using the formula: T_d (°C) = 2(A + T) + 4(G + C) where A, T, G, and C are the number of residues in the oligonucteotide (Suggs *et al.*, 1981). T_d for the WT-oligo was 36°C and 34°C for the Mac-oligo. The filter was then placed in 12.5 ml of the hybridization solution (5X SSC; 20 mM sodium phosphate, pH 7.0; 10X Denhardt's solution; 7% SDS; 10% dextran sulfate; and heat-denatured salmon sperm DNA at 0.1 mg/ml), and incubated at same temperature overnight. After hybridization, the filter was rinsed twice with wash solution (2X SSC, 0.1% SDS, pH 7.0) for 15 min at room temperature and once for 30 min at 29°C. If necessary, the membrane was rewashed at a higher temperature for more stringent probe-specificity.

CHAPTER III

CHARACTERIZATION OF A FOLDBACK MOLECULE DURING ADENOVIRUS DNA REPLICATION

INTRODUCTION

The adenovirus genome is a linear, double-stranded DNA which contains about 36,000 bp (Doerfler and Kleinschmidt, 1970; Green et al., 1967; van Ormondt and Galibert, 1984). The adenovirus-encoded terminal protein (TP) is covalently bound to the first nucleotide at both 5'-ends by a phosphodiester bond (Robinson et al., 1973; Robinson et al., 1974; Rekosh et al., 1977; Carusi, 1977; Stillman et al., 1982). The viral genome contains identical inverted terminal repetitions (ITRs) ranging from 100 to 150 bp (Stillman et al., 1982; Garon et al., 1972; Wolfson and Dressler, 1972; Steenberg et al., 1977). There are several conserved or highly homologous sequences, including the replication origin and the recognition sequences of several DNA-binding proteins, within the ITR (Steenberg et al., 1977; Arrand and Roberts, 1979; Shinagawa et al., 1983; Stillman et al., 1982; Hay, 1985a, 1985b; Bernstein et al., 1986; Rawlins et al., 1984; for review see Challberg and Kelly, 1989). Initiation of replication occurs at either or both molecular ends. Viral DNA replication takes place in two stages: replication proceeds in the first phase by a strand displacement mechanism to produce a daughter duplex and a displaced parental strand, and the displaced strand is converted into the other daughter duplex in the second round of DNA synthesis (Lechner and Kelly, 1977; Kelly and Lechner, 1978; Ellens et al., 1974).

Although the initiation of adenovirus DNA replication has been studied intensively using *in vitro* replication systems, the study of the synthesis of the complementary strand has been hampered by the lack of sensitive assays. The structures of adenovirus replication intermediates have been determined by electron microscopy (Lechner and Kelly, 1977; Kelly and Lechner, 1978; Ellens *et al.*, 1974; Daniell, 1976; Revet and Benichou, 1981). A "panhandle" structure, formed by intramolecular hybridization between complementary inverted terminal sequences on the displaced parental strand to regenerate a duplex replication origin, has been

proposed as an intermediate in the conversion of the displaced strand to a duplex (Lechner and Kelly, 1977; Kelly and Lechner, 1978; Daniell, 1976). An artificially constructed panhandle molecule has been shown to support the initiation of strand-displacement replication as efficiently as double-stranded adenovirus DNA (Leegwater *et al.*, 1988). However, no direct evidence exists for the involvement of panhandle intermediates in the process of adenovirus DNA replication *in vivo* (Revet and Benichou, 1981). Recently an alternative mechanism for the duplication of the displaced parental single strand has been discovered: complementary displaced strands generated from different replicating molecules can simply hybridize intermolecularly to reform double-stranded heteroduplexes (Ahern *et al.*, 1990). The two pathways have been termed *cis* and *trans* replication to emphasize intra- and inter-molecular hybridization, respectively (Ahern *et al.*, 1990). Early in adenovirus replication, the *cis* pathway is thought to be the major mechanism to synthesize the complementary strand. Later, when viral genomes have been amplified to high concentrations, the *trans* pathway predominates. However, the relationship between the *cis* and *trans* replication pathways, if any, is still unclear.

Plasmid-based adenovirus mini-chromosomes have been used as models to study viral DNA replication *in vivo* (Hay *et al.*, 1984; Wang and Pearson, 1985; Hay, 1985; Bernstein *et al.*, 1986). In the presence of helper adenovirus DNA, linear plasmid molecules containing adenovirus replication origins can replicate in a viral-specific manner in transfected cells. A special class of panhandles (fold-back structures) arising from the replication of symmetrical dimers and multimers generated during the process of plasmid transfection has been successfully identified and characterized using two-dimensional gel electrophoresis. These fold-back structures have all the properties predicted for replicative intermediates and therefore provide physical evidence for the *cis* replication pathway. Experiments also show that the *cis*- and *trans*-replication pathways can operate simultaneously to ensure the synthesis of complementary strands. Moreover, sequence conversion can be demonstrated during both *cis* and *trans* replication.

RESULTS AND DISCUSSION

Adenovirus mini-chromosomes participate in *cis* and *trans* replication

Fig. II.1 displays the structures of plasmids T4 (2.4 kb) and pLIRLT (4.0 kb). A schematic representation of the linearized plasmids is shown at the top of Fig. III.2. Both plasmids have identical vector and adenovirus sequences, but the viral sequences are oriented in opposite directions relative to vector DNA in each plasmid. pLIRLT additionally contains 230-bp inverted repeats and a 1.4-kb insert carrying the gene for resistance to kanamycin. *Eco*RI cleavage linearizes both plasmids at the adenovirus replication origin. To study the relationship between the *cis* and *trans* replication pathways, T4 and pLIRLT, each alone and both together, were cotransfected with helper DNA and assayed by Southern blot hybridization using *Dpn*I-resistance as a measure of adenovirus-specific replication. Fig. III.1 (lane 1) shows that *Dpn*I-resistant monomeric, dimeric, and higher oligomeric bands were detected when T4 was transfected alone. The origin of these *Dpn*I-resistant bands will be descibed below. pLIRLT alone gave rise not only to *Dpn*I-resistant monomeric, dimeric, and higher oligomeric bands, but to a molecule with a size (3.0 kb) expected for the *cis* product (Fig. III.1, lane 2). When T4 and pLIRLT were cotransfected together, in addition to the collection of all *Dpn*I-resistant bands previously found in each single transfection, a novel 2.8-kb *trans* molecule was also detected (Fig. III.1, lane 3). Fig. III.2 diagrams the molecular associations occurring within and between displaced strands after cotransfection with T4 and pLIRLT. The formation of the smaller *cis* product involves the removal of 1.4-kb unpaired sequences at the 3' end of the displaced pLIRLT strand coupled with the repair of 400 bp of origin sequences on the panhandle intermediate. This process is identical to sequence conversion (Wang *et al.*, 1990; Bennett *et al.*, in preparation). The displaced strand of T4 lacks inverted repeats and therefore cannot participate in *cis* replication, but it shares 2.0 kb of complementary sequences with the displaced strand of pLIRLT. Inter-strand hybridization creates a heteroduplex intermediate which still has 1.6 kb of unpaired sequences at the 3' end of the pLIRLT strand. The size of the observed *trans* molecule suggests that these unpaired sequences, which are longer than the unpaired sequences removed in the formation of the *cis* product, are also readily lost. Thus, both *cis* and

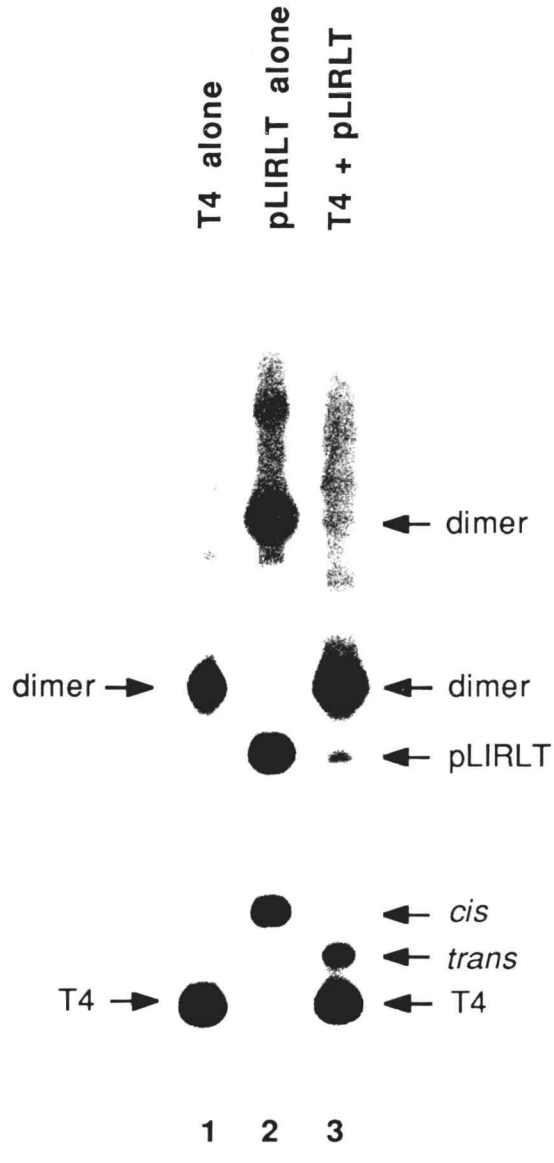

Figure III.1. Transfections involving plasmids T4 and pLIRLT. Plasmids were linearized by *Eco*RI prior to transfection. Total DNA was isolated 72 hrs after cotransfection with plasmids and helper adenovirus DNA, digested with *Dpn*I, and analyzed by Southern blot hybridization using a vector probe. Arrows designate the positions of input plasmids, dimers of input plasmids, and the products of the *cis* and *trans* pathways. Lane 1: Plasmid T4 alone. Lane 2: pLIRLT alone. Lane 3: pLIRLT and plasmid T4 together.

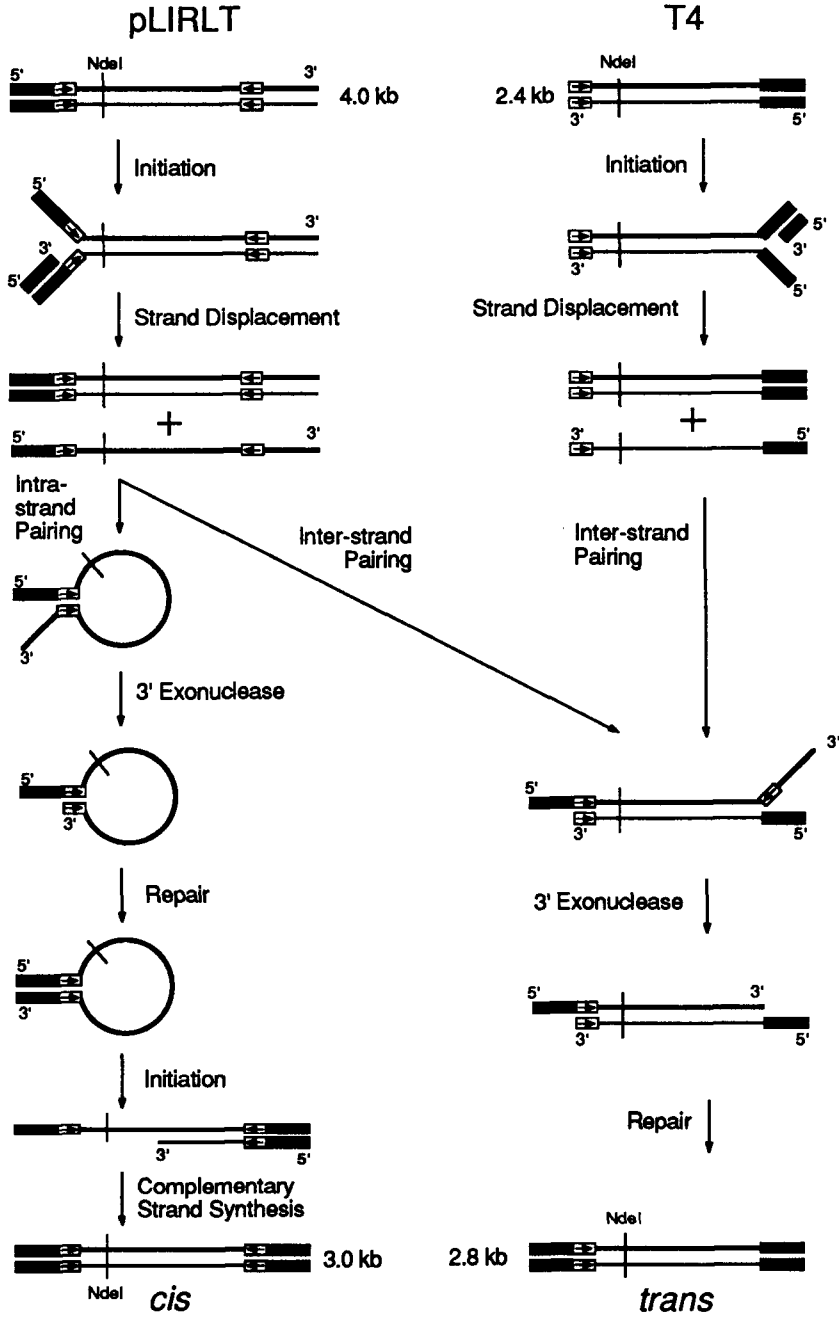

Figure III.2. Interactions between strands of plasmids T4 and pLIRLT. pLIRLT can participate in the *cis* pathway to form a 3.0-kb molecule. A 1.4-kb unpaired tail must be removed exonucleolytically from the panhandle intermediate. Plasmid T4 lacks inverted repeats and therefore is not able to form a panhandle intermediate in the *cis* pathway. Intermolecular hybridization between displaced strands of pLIRLT and plasmid T4 form a heteroduplex intermediate. Repair of the intermediate, including removal of 1.6-kb unpaired sequences at the 3' end of the pLIRLT strand, creates a 2.8-kb *trans* molecule.

trans replication, with concurrent sequence conversion, occur simultaneously in cotransfected cells. It is important to emphasize that the formation of the *trans* molecule is mechanistically quite different from homologous recombination (Ahern *et al.*, 1990).

Foldbacks are intermediates in the replication of symmetric dimers

As illustrated above in Fig. III.2, the helper-driven replication of plasmid T4 alone should be limited to the displacement of a single strand under the direction of the single adenovirus origin. Although some or all of the input T4 DNA can become *Dpn*I-resistant as a consequence, replicative amplification of monomeric T4 should not happen since the displaced strands lack inverted repeats necessary to form panhandle intermediates. However, monomeric T4 molecules appear to be amplified in the presence of helper DNA (Fig. III.3, compare lanes 1 and 2), and are substantially, but not completely, *Dpn*I-resistant (Fig. III.3, lane 3) and *Mbo*I-sensitive (Fig. III.3, lane 4). The residual *Dpn*I-sensitive, *Mbo*I-resistant monomers presumably constitute unreplicated input T4 DNA. These results suggest that both strands of most monomers have replicated. Fig. III.3 also shows that *Dpn*I-resistant, *Mbo*I-sensitive T4 dimers and multimers accumulated in the presence of helper DNA. Oligomerization of input DNA by end-to-end ligation commonly occurs during transfection (Perucho *et al.*, 1980; Hay *et al.*, 1984; Vasudevachari *et al.*, 1987). Fig. III.4 outlines a possible pathway for the replication of T4 dimers. Only dimers containing adenovirus origins at both molecular ends (symmetrical dimers) can amplify by adenovirus-specific DNA replication. Replication from either dimer origin generates identical, completely self-complementary displaced strands that anneal intramolecularly to form foldbacks, a special class of panhandle structures. Foldbacks, in turn, are intermediates in the synthesis of new symmetrical dimers.

Identification of foldbacks by two-dimensional gel electrophoresis

As intermediates in the replication of dimers, foldbacks should exhibit several characteristic properties: (a) the same mobility as duplex monomers during non-denaturing gel electrophoresis, (b) the mobility of dimer-length single strands during denaturing gel electrophoresis, (c) total resistance to *Dpn*I digestion, (d) complete sensitivity to *Mbo*I cleavage, and

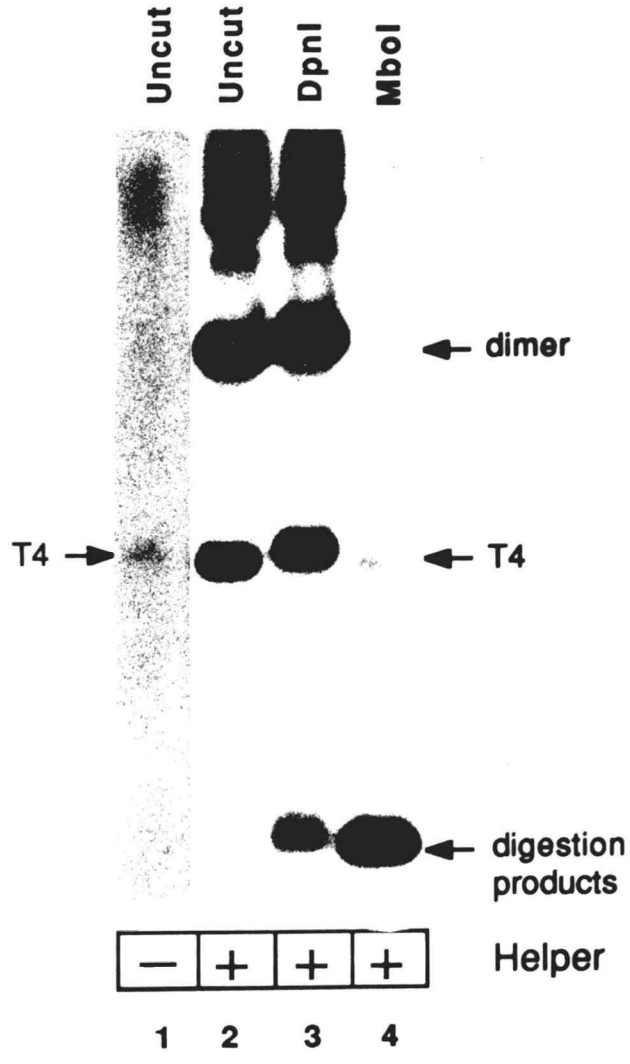

Figure III.3. Transfection with *Eco*RI-linearized plasmid T4. Plasmid T4 was transfected without (lane 1) or with (lanes 2 through 4) helper adenovirus DNA. Total DNA was extracted 72 hrs after transfection and analyzed by Southern blot hybridization using a vector probe. Arrows designate the positions of the input plasmid and dimers of the input plasmid. Lanes 1 and 2: No restriction endonuclease digestion of the extracted DNA. Lane 3: Digestion with *Dpn*I endonuclease. Lane 4: Digestion with *Mbo*I endonuclease.

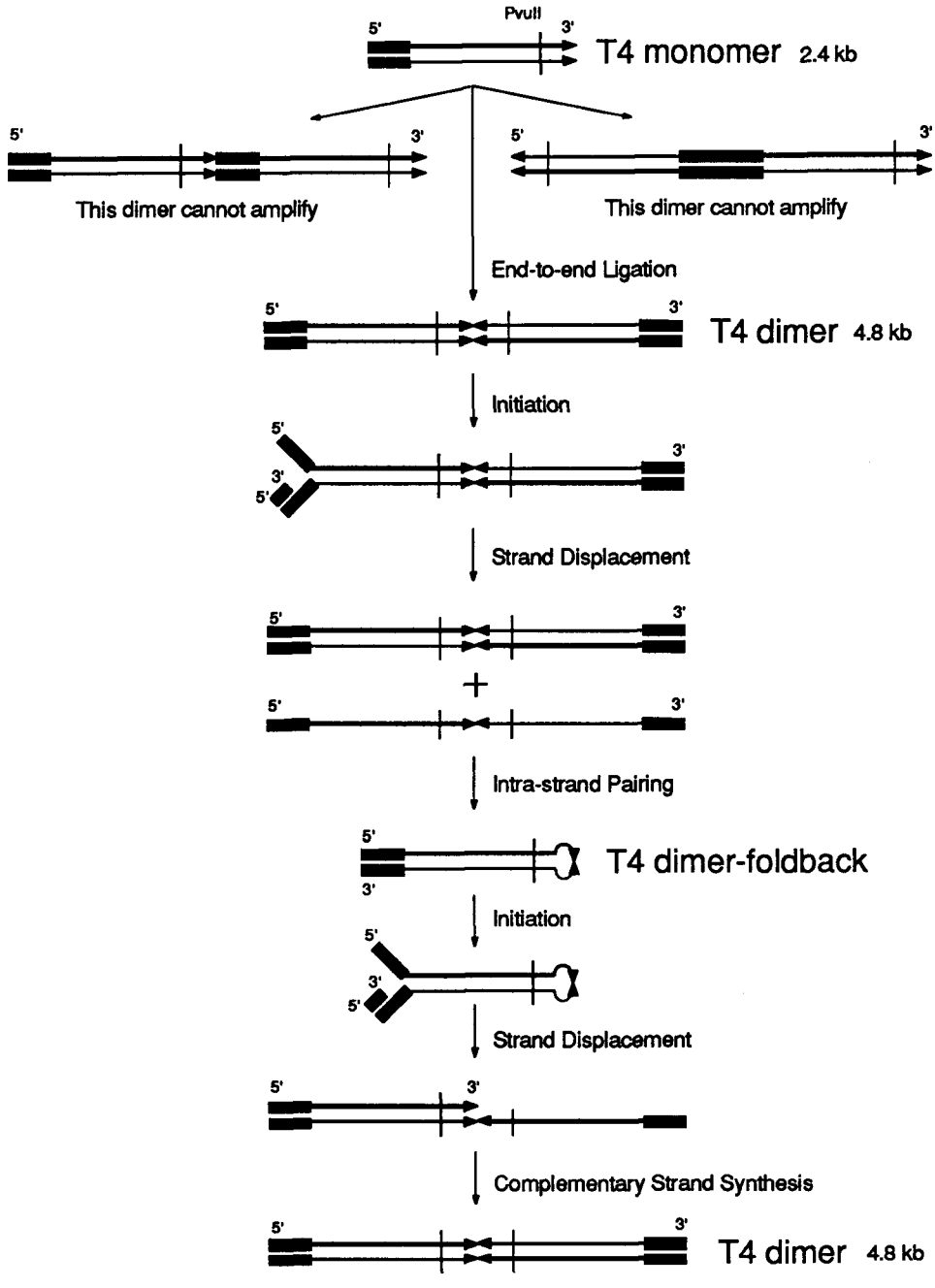

Figure III.4. Replication of symmetrical T4 dimers. Dimers are generated by end-to-end ligation during the process of transfection. Only symmetrical dimers with adenovirus origins at both ends can engage in adenovirus-specific DNA replication. Although initiation is shown occurring only at one end of the symmetrical dimer, initiation at the other end also leads to the formation of an identical T4 dimer foldback intermediate. The dimer foldback has the same mobility as input T4 DNA in a neutral gel, but behaves as a dimer-length single strand in an alkaline gel. The loop at one end of the dimer foldback is sensitive to the single-strand-specific S1 endonuclease.

(e) susceptibility to attack by the single-strand-specific S1 endonuclease within the small, single-stranded loop facilitating the foldback junction.

Dimer foldbacks were identified using two-dimensional gel electrophoresis (Fig. III.5). DNA was initially separated as double-stranded molecules in the first dimension by gel electrophoresis at neutral pH. Subsequent alkaline gel electrophoresis at right angles resolved DNA as single-stranded molecules in the second dimension. Total DNA extracted from cells cotransfected with plasmid T4 and adenovirus helper was fractionated by two-dimensional gel electrophoresis and visualized by Southern blot hybridization using a vector probe. As shown in Fig. III.5 (panel A), monomeric and dimeric plasmid molecules fell on a diagonal line as expected. In addition, a novel molecule was detected above the diagonal. This molecule moved with the same mobility as duplex monomeric plasmid DNA in the first dimension, but migrated as dimer-length single strands in the second dimension. Moreover, the new molecule was *Dpn*I-resistant (Fig. III.5, panel B), *Mbo*I-sensitive (Fig. III.5, panel C), and S1 endonuclease-sensitive (Fig. III.5, panel D). These properties clearly identify it as a T4 dimer foldback molecule. The existence of foldbacks provides the first physical evidence for the *cis* pathway in adenovirus DNA replication. Since foldbacks comigrate with input T4 DNA in one-dimensional gels, they also explain the apparent replicative accumulation of monomeric T4 DNA seen above in Fig. III.3. In fact, adenovirus-specific replication actually displaces only one strand of input T4 DNA, not both, as demonstrated by the *Dpn*I- and *Mbo*I-resistant monomers on the diagonal (Fig. III.5, compare panels B and C).

Efficient *cis* replication depends on the length of inverted repeat sequences

Symmetrical dimers are perfect inverted repeats with completely self-complementary foldback replicative intermediates. Consequently, T4 dimers accumulate very rapidly. Dimers could not be detected at all in cells transfected in the absence of helper (Fig. III.3, lane 1), but clearly emerged as a major constituent in cells cotransfected with helper (Fig. III.3, lane 2). The minimum inverted repeat must at least contain the adenovirus replication origin, roughly 50 bp long. Previous experiments found little or no replicative advantage when inverted repeats varied in length from 31 bp to 89 bp (Wang *et al.*, 1990). In order to address this question more

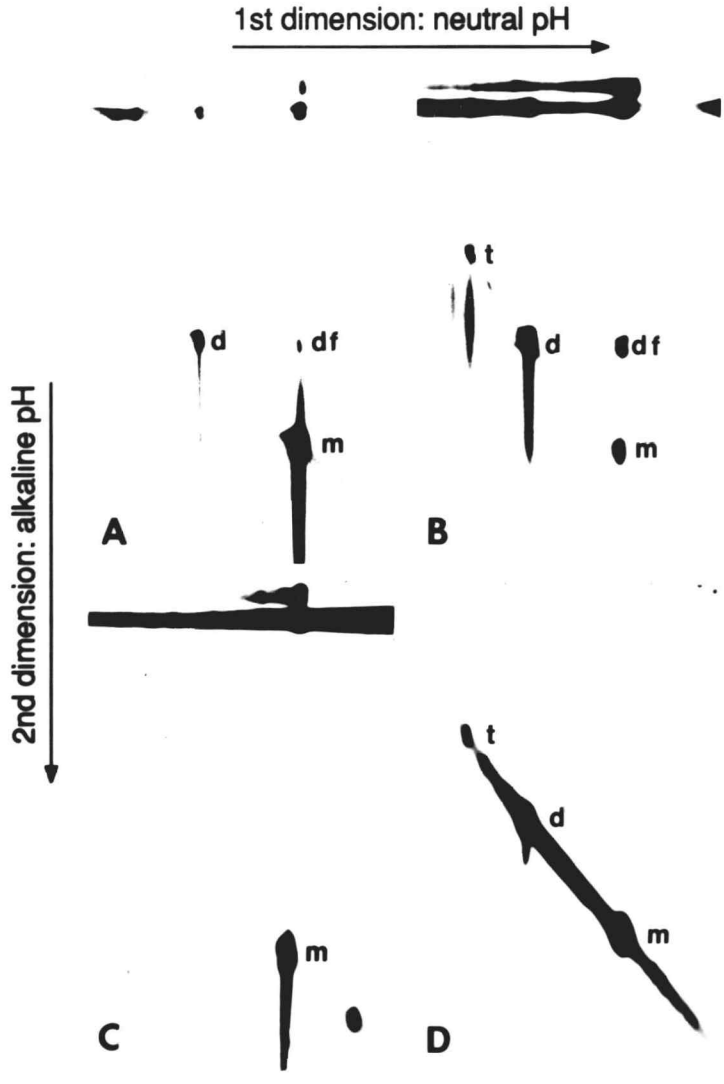

Figure III.5. Analysis of plasmid T4 replication by two-dimensional gel electrophoresis. Cells were cotransfected with *Eco*RI-cut T4 plasmid and adenovirus helper DNA. Total DNA was analyzed 72 hrs after transfection by two-dimensional gel electrophoresis without further treatment (panel A) or after digestion with *Dpn*I (panel B), *Mbo*I (panel C), or S1 endonuclease (panel D). Gels were loaded at the top left in each panel. Arrows show the direction of electrophoresis in both dimensions. The length of exposure during autoradiography varies in each panel. The top two lanes in panels A through C are one-dimensional marker gels containing, respectively, T4 DNA alone and the corresponding samples resolved in each two-dimensional gel. No marker lanes are shown in panel D. Symbols: t = symmetrical tetramers, d = symmetrical dimers, m = monomers, and df = dimer foldbacks.

thoroughly, the helper-driven replication of plasmids pIRST and pLIRST (Fig. II.2) and pIRLT and pLIRLT (Fig. II.3) was investigated. The plasmids differ in the length of inverted repeats (37 bp or 230 bp) and the length of unpaired sequences at the 3' ends of the displaced strands (14 bp or 1.4 kb). The *cis* replication pathway for pLIRLT (and similarly for the other plasmids) has already been diagrammed in Fig. III.2. Fig. III.6 shows the two-dimensional gel analysis of *Dpn*I-resistant DNA isolated from cells transfected with pLIRLT (panel A), pIRLT (panel B), pLIRST (panel C), and pIRST (panel D). The *cis* bands from pLIRLT and pLIRST (panels A and C) were nearly as intense as their respective dimers and decidedly more intense than the *cis* bands of pIRLT and pIRST (panels B and D), indicating that longer inverted repeats strongly increase the efficiency of adenovirus-specific replication. In fact, the *cis* bands of pIRLT and pIRST were markedly less intense than their respective dimers. The lengths of the unpaired sequences at the 3' ends of the displaced strands also appeared to influence the yield of *cis* products. This is most clearly seen by comparing the relative intensities of *cis* bands of pLIRLT (panel A) and pLIRST (panel C) with their corresponding monomer and foldback bands. The shorter unpaired 3' tail of pLIRST is apparently less a barrier to the formation of a replication-competent panhandle intermediate. The unpaired tail is presumably removed by the 3'-exonucleolytic activity of the adenovirus-encoded DNA polymerase (Field *et al.*, 1984; Bernad *et al.*, 1989).

Implications for the genomic organization of adenovirus

Intact inverted terminal repeats (ITRs) are absolutely essential for the viability of adenoviruses (Stow, 1982). Evidence has been presented in this Chapter showing that adenovirus *cis* replication becomes more efficient as the size of inverted repeats increases. The maximum arrangement of inverted sequences is achieved in perfectly symmetrical molecules. Subgenomic defective adenovirus molecules with larger ITRs rapidly evolve during lytic infection (Daniell, 1976). Perfectly symmetrical defective adenovirus genomes have also been isolated and characterized (Deuring *et al.*, 1981; Gluzman and van Doren, 1983). It is not at all clear what factors, if any, maintain the normal or wildtype size of the adenovirus ITR (only 100

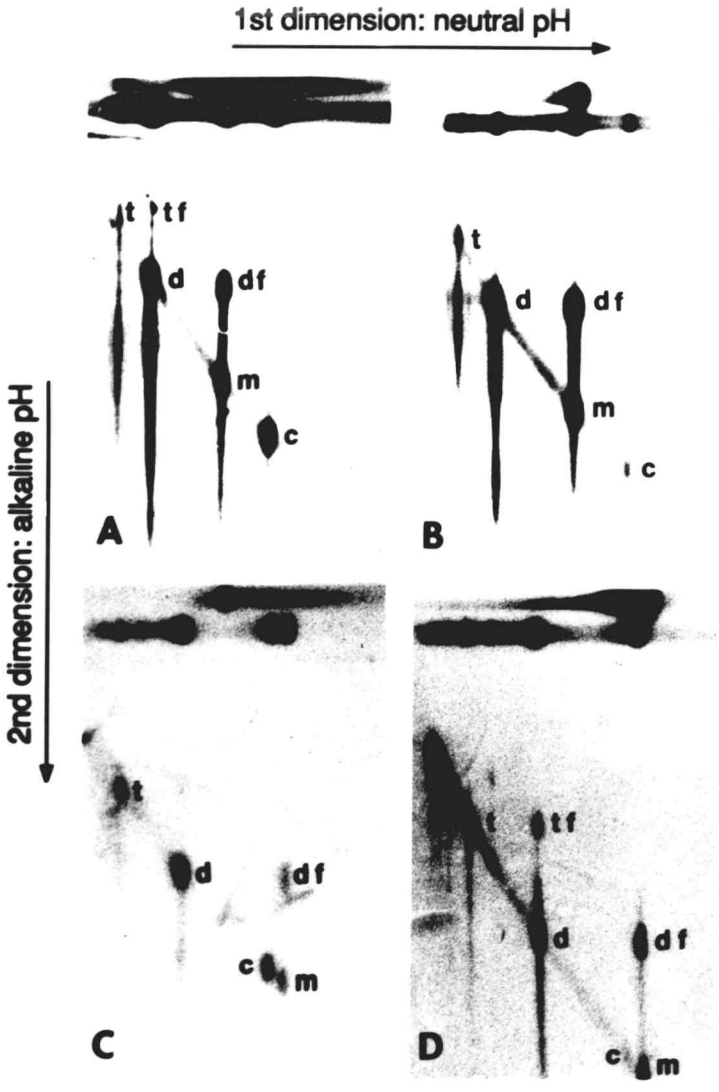

Figure III.6. Analysis of *cis* replication by two-dimensional gel electrophoresis. Cells were cotransfected with *Eco*RI-cut plasmids and adenovirus helper DNA. Total DNA was isolated from cells 72 hrs after transfection with pLIRLT (panel A), pIRLT (panel B), pLIRST (panel C), or pIRST (panel D) and analyzed by two-dimensional gel electrophoresis. Gels were loaded at the top left in each panel. Arrows show the direction of electrophoresis in both dimensions. The length of exposure during autoradiography varies in each panel. The top two lanes in panels A through D are one-dimensional marker gels containing, respectively, each plasmid alone and the corresponding samples resolved in each two-dimensional gel. Symbols: t = symmetrical tetramers, tf = tetramer foldbacks, d = symmetrical dimers, df = dimer foldbacks, m = monomers, and c = *cis* products.

bp out of a total genomic size of 36,000 bp).

Large extrachromosomal inverted duplications are involved in many specific eukaryotic gene amplification events (Ford and Fried, 1986; Yao *et al.*, 1987; Passananti *et al.*, 1987; Ruiz and Wahl, 1988). It would be interesting to know if the amplification of extrachromosomal DNA is mechanistically similar to adenovirus DNA replication.

CHAPTER IV

ADENOVIRUS DNA REPLICATION DIRECTED BY EMBEDDED ORIGINS

INTRODUCTION

The biochemistry and molecular enzymology of adenovirus DNA replication has been intensively studied *in vitro* using reconstituted cell-free systems. The involvement of the 80-kd precursor of the terminal protein (pTP) in the initiation of adenovirus DNA replication has been demonstrated *in vitro* by a [α-^{32}P]dCMP labeling assay (Challberg and Kelly, 1979; Stillman, 1981; for review see Stillman, 1989). Initiation of adenovirus DNA replication is template-dependent and requires recognition of origin sequences by pTP. The formation of a phosphodiester bond between pTP and the first nucleotide (dCMP) of the nascent DNA strand initiates viral DNA replication.

The origin sequences and structural requirements of the DNA template have been precisely defined for efficient adenovirus DNA replication in cell-free systems. These requirements have also been found to hold for *in vivo* systems. Mutations in the adenovirus inverted terminal repeats (ITRs) both within the viral genome and within plasmid mini-chromosmes that contain only the adenovirus ITR have been used in DNA transfection assays to study the specific sequence requirements for a functional replication origin (Graham and van der Eb, 1973; Wang and Pearson, 1985; Hay *et al.*, 1984; Hay, 1985a, 1985b; Bernstein *et al.*, 1986). The results of these studies show that the terminal 60 bp of adenovirus ITRs, including several highly conserved sequences and protein-binding sites, were necessary and sufficient to support DNA replication *in vitro* and *in vivo* (Tamanoi and Stillman, 1983; Challberg and Rawlins, 1984; Rawlins *et al.*, 1984; Wang and Pearson, 1985; Hay, 1985a, 1985b; Bernstein *et al.*, 1986). Experiments with plasmid mini-chromosomes require cotransfection with helper adenovirus DNA to supply virus-encoded replication proteins (Hay *et al.*, 1984; Wang and Pearson, 1985).

The position, as well as the sequence, of the replication origin are important for efficient initiation of adenovirus DNA replication. A replication origin embedded in the middle of linear DNA was observed to

inhibit initiation of replication *in vitro*, suggesting that the replication origin must be exposed at the end of linear DNA to be functional (Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983; Guggenheimer *et al.*, 1984a). However, other studies have shown that embedded adenovirus origins are apparently functional *in vivo*. Infectious circular forms of the adenovirus genome, in which the viral DNA termini are joined head-to-tail, have been found in virus-infected cells (Ruben *et al.*, 1983; Graham, 1984; Graham *et al.*, 1989). Although this head-to-tail arrangement effectively embeds the replication origin, linear progeny wildtype virus are recovered. In addition, transfected circular plasmids containing dual adenovirus origins oriented in opposite directions can give rise to linear replicating mini-chromosomes lacking sequences originally located between the two origins (Hay *et al.*, 1984).

The intact linear adenovirus genome in which the two origins are flanked by plasmid sequences produce infectious virions in transfected cells, but the flanking sequences in front of the embedded origins were not retained in the replicated virion genome (Hanahan and Gluzman, 1984). Similarly, linear plasmids that contain dual embedded adenovirus origins were found to replicate in host cells in the presence of helper adenovirus DNA (Hay *et al.*, 1984; Vasudevachari *et al.*, 1987). The mechanisms for replicating these DNAs containing embedded adenovirus origins are not yet clear.

The studies presented in this chapter provide evidence that embedded adenovirus origins do not inhibit strand displacement during replication by the virus-specific replication machinery. In the presence of inverted repeats, the displaced strand from the template containing an embedded adenovirus origin can be amplified by the *cis* replication pathway. Possible replication mechanisms are considered and discussed.

RESULTS AND DISCUSSION

The replicative fate of DNA containing an embedded adenovirus origin

In the presence of helper DNA, linear plasmids containing exposed adenovirus terminal sequences have been shown to engage in adenovirus-specific strand-displacement replication (Hay *et al.*, 1984; Wang and Pearson, 1985). The T4 plasmid (Fig. II.1) can be linearized by *Eco*RI to

expose its single adenovirus origin, or by *Pvu*II resulting in a linear plasmid with an embedded adenovirus origin. Cells were transfected by either of the linearized T4 plasmids along with helper adenovirus DNA. Cells were harvested after three days and the total DNA extracted. Plasmid replication was assayed by *Dpn*I-digestion as previously described (Peden *et al.*, 1980; see Chapter II). Three radiolabeled probes corresponding to three different regions of the plasmid sequence were used to indicate which regions of the plasmid have been properly replicated. Monomer-sized and dimer-sized *Dpn*I-resistant plasmid molecules were observed using all three probes after transfection with *Eco*RI-linearized T4 plasmid, in which the adenovirus origin was exposed. In Fig. IV.1, the monomer (labeled as input) and dimer bands are shown to hybridize to all three probes (a, b, and c) in the absence (lanes 1, 3, and 5) and in the presence (lanes 2, 4, and 6) of *Dpn*I digestion. This suggests that these *Dpn*I-resistant molecules resulting from adenovirus-specific replication contained the entire T4 plasmid sequence and had not been rearranged. However, in our previous studies (see Chapter III), we demonstrated that the amplified monomer-sized T4 molecules were replicative intermediates (dimer foldbacks) arising from replication of a dimer that resulted from end-to-end ligation during transfection. Dimer foldback molecules were previously identified by two-dimensional gel electrophoresis (Hu *et al.*, 1990; see Chapters II and III).

The *Pvu*II-cleaved T4 plasmid leaves the adenovirus origin embedded by 230 bp of flanking sequence (region c) at one end of the linear plasmid. Transfection of this plasmid resulted in two monomer-sized and one dimer-sized plasmid molecules that can be detected by probes a and b (Fig. IV.1, lanes 7-10). One of the monomer bands was the input plasmid, as shown by its sensitivity to *Dpn*I digestion (Fig. IV.1, compare lanes 7 and 8 and lanes 9 and 10). The second monomer band is smaller than the input plasmid and is *Dpn*I-resistant. The resulting dimer molecules are also *Dpn*I-resistant, and thus resulted from an end-to-end ligation of two T4 plasmids. The dimer, however, is smaller than twice the input plasmid. Probe c hybridizes with the input *Dpn*I-sensitive monomer band, but not to either of the *Dpn*I-resistant replication products (Fig. IV.1, compare lanes 11 and 12). These results suggest that the 230 bp sequence flanking the adenovirus replication origin is lost during replication. The structures of

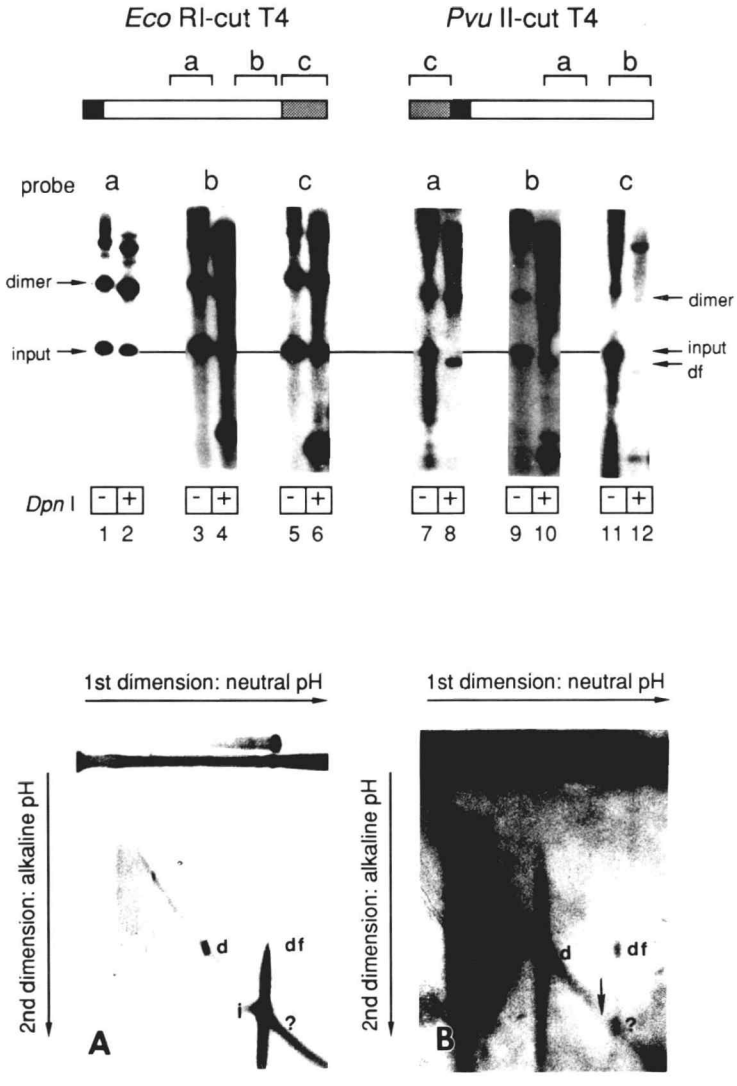

Figure IV.1. Replication of linear T4. Black box = adenovirus origin. Gray box = plasmid sequences between *Eco*RI and *Pvu*II. Plasmid T4 was cleaved by *Eco*RI or *Pvu*II endonucleases and transfected into cells with helper. Southern blot pUC19 probes: probe a = *Ava*II fragment (222 bp), probe b = *Pvu*II-*Taq*I fragment (278 bp), and probe c = *Eco*RI-*Pvu*II fragment (228 bp). Transfected DNA in lanes 7 to 12 is *Pvu*II-cleaved T4. Probe species: probe a = lanes 1, 2, 7, and 8; probe b = lanes 3, 4, 9, and 10, probe c = lanes 5, 6, 11, and 12. *Dpn*I digestion: lanes 2, 4, 6, 8, 10, and 12. Two-dimensional gel electrophoresis of *Pvu*II-cleaved T4 detected by intact pUC19 probe: no *Dpn*I digestion (panel A), *Dpn*I digestion (panel B). i = Input transfected molecules; d = symmetrical dimers; df = dimer foldbacks.

these monomer and dimer sized molecules were analyzed by two-dimensional gel electrophoresis and visualized by hybridizing with a probe corresponding to the entire plasmid sequence. Fig. IV.1 (panel A) shows the two-dimensional gel of the DNA, not digested with *Dpn*I, isolated after transfection with *Pvu*II-linearized T4. The input monomer molecule (i) and the symmetric dimer DNA (d) were separated along the diagonal. In addition, a faint band assigned to a dimer foldback (df) structure, corresponding to the smaller sized monomer DNA in the native gel (lanes 8 and 10), was observed above the diagonal. After *Dpn*I digestion (panel B), the input molecule was degraded, leaving the dimer and dimer foldback molecules clearly visible. This suggests that in the presence of helper DNA, the *Pvu*II-cleaved linear T4 can replicate in transfected cells using the adenovirus replication machinery. The symmetrical dimer structure arises from the end-to-end ligation during transfection. The presence of dimer foldbacks (a special class of panhandle replicative intermediates) suggests that the embedded origins do not prevent strand-displacement, and that the displaced single strand can be replicated by the *cis* replication pathway. We suspect that the additional band (labeled with a question mark in panels A and B) in the two-dimensional gel that migrates along the first dimension with the dimer foldback corresponds to a linear duplex resulting from nicking the dimer foldback.

Replication of circular DNA requires inverted repeats

In previous studies, circular plasmid pARKR containing dual adenovirus ITR sequences could be amplified *in vivo* in the presence of helper DNA (Hay *et al.*, 1984). It was proposed that the DNA is replicated out of the cirular plasmid by multiple rounds of precisely initiated strand-displacement synthesis directed by the internal origins. However, transfection of circular T4, which contains a single adenovirus origin, generated no *Dpn*I-resistant molecules and no oligomers since end-to-end ligation is not possible with circles (Fig. IV.2, lanes 2 and 3). This suggests that the circular T4 could not initiate replication in transfected cells. Transfection of the pARKR-like circular plasmid pADIRK2, with dual adenovirus origins orientated in opposite directions, resulted in smaller monomer- and multimer-sized *Dpn*I-resistant molecules (Fig. IV.2, lanes 8 and 9). The input DNA was totally sensitive to *Dpn*I digestion. The

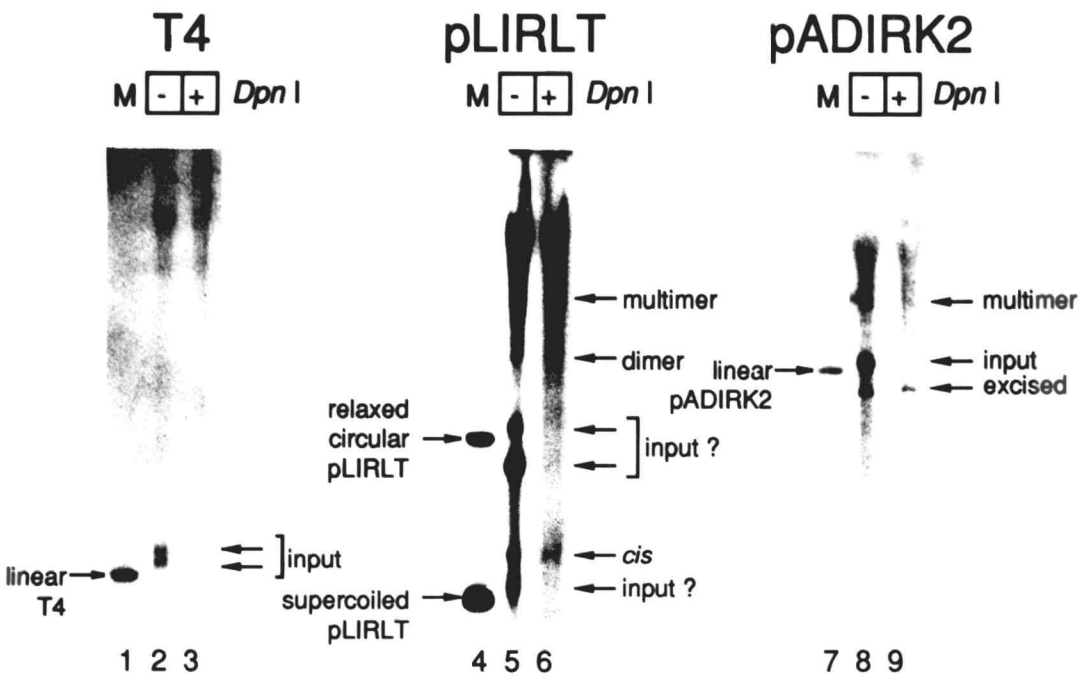

Figure IV.2. Replication of circular DNA which contained adenovirus origin sequences. Helper adenovirus DNA was cotransfected with circular T4 (lanes 2 and 3), circular pLIRLT (lanes 5 and 6), or circular pADIRK2 (lanes 8 and 9). DNA in lanes 3, 6, and 9 was treated with *Dpn*I prior to gel electrophoresis. Markers: 1 μg *Eco*RI-cleaved T4 (lane 1), 1 μg circular pLIRLT (lane 4), 2 μg *Bgl*II-cleaved pADIRK2 (lane 7).

*Dpn*I-resistant submonomer (labeled "excised") was about 1.4 kb smaller than linear pADIRK2 (Fig. IV.2, lane 7) and the subdimer (labeled multimer in lane 9) was smaller than twice the linear monomer duplex. This suggests that the kanamycin sequences between two adenovirus origins were removed in the smaller monomer-sized replication product. The size of the multimer corresponds to a tandem repeat structure, a nonsymmetrical dimer containing only one copy (not both) of the kanamycin sequences. The production of tandemly repeated multimers suggests that a long stretch of single-stranded DNA might have been displaced from the circular template. Such a rolling circle-like structure was observed by electron microscopy during *in vitro* replication of circular molecules containing an adenovirus origin (Pearson *et al.*, 1983). A pADIRK2-like circular plasmid, pARKR, containing two adenovirus origins in inverted orientation has also been shown to give rise to a linear replication-proficient mini-chromosome (Hay *et al.*, 1984). It was proposed that circular pARKR DNA replicated by multiple rounds of precisely initiated displacement synthesis directed by the embedded origins. In this model, the presence of two opposed replication origins in the circular DNA are critical for adenovirus-specific DNA replication.

Circular plasmid pLIRLT, with 230-bp inverted repeats and a single adenovirus origin, also produced a *cis* band and several multimer-sized *Dpn*I-resistant molecules (Fig. IV.2, lanes 5 and 6). Input DNA again was completely sensitive to *Dpn*I cleavage. Previous studies indicated that the *cis* band arises from complementary strand replication of the displaced monomer strand using the *cis* pathway (Fig. III.1). Dimers and multimers were smaller than symmetrical dimers and multimers arising from the transfection of linearized pLIRLT (see Fig. 4A below). This suggests that these dimers and multimers had tandemly repeated, nonsymmetrical structures. The presence of tandemly duplicated, *Dpn*I-resistant molecules is consistent with adenovirus-specific strand-displacement synthesis proceeding continuously around the circular pLIRLT template. The proposed mechanism of multiple precise internal initiations (Hay *et al.*, 1984) can not explain the replication of circular pLIRLT, which contains single adenovirus origin.

Strand-displacement synthesis around circular templates

The presence of *Dpn*I-resistant tandemly arrayed multimers arising from transfected pADIRK2 and pLIRLT suggests that multimeric strands have been displaced from the circular templates. In the presence of inverted repeats, the long displaced strands can be converted into duplex form by the *cis* replication pathway including sequence conversion (see Fig. III.1). Accordingly, a displaced single-stranded DNA was also expected to be produced after transfection of circular T4. However, the displaced T4 strand cannot enter the *cis* pathway since it lacks inverted repeats. To identify the presence of the displaced T4 strand, circular T4 was cotranfected with linear pLIRLT. Previously in Chapter III, it was shown that the non-replicatable T4 strand could be rescued by hybridizing with the complementary sequences in the displaced pLIRLT strand to generate a replication-proficient *trans* molecule (Figs. III.1 and III.2). In the experiment described above, transfection of circular T4 alone did not produce any *Dpn*I-resistant molecules (Fig. IV.2, lanes 2 and 3). When circular T4 was cotransfected with *Eco*RI-cleaved pLIRLT, Southern blot analysis revealed, in addition to the pLIRLT replication products, a novel *Dpn*I-resistant *trans* band and several nonsymmetrical T4 multimers (Fig. IV.3). This suggests that strand-displacement replication proceeded continuously around the circular T4 at low frequency, and the resulting displaced strand could be rescued by hybridizing with the complementary strand arising from the replication of cotransfected linear pLIRLT. The sizes of the multimers suggest that they were also generated by *trans* hybridization between a displaced pLIRLT strand and a complementary, tandemly repeated T4 strand. Input circular T4 was degraded after *Dpn*I digestion (Fig. IV.3, lane 2). Thus, the production of a *trans* molecule and tandemly repeated multimers indicates the cryptic presence of a displaced T4 strand.

Replication of linear DNA with an embedded adenovirus origin

Cloned adenovirus genomes with embedded origins produce infectious virions with loss of sequences flanking the embedded origins after transfection (Hanahan and Gluzman, 1984). Similarly, a linearized plasmid (*Pst*I-cleaved p3.1), which contains dual embedded adenovirus origins, replicate in cells in the presence of helper adenovirus DNA (Hay *et al.*, 1984; Vasudevachari *et al.*, 1987). As mentioned above, Hay *et al.* (1984) proposed

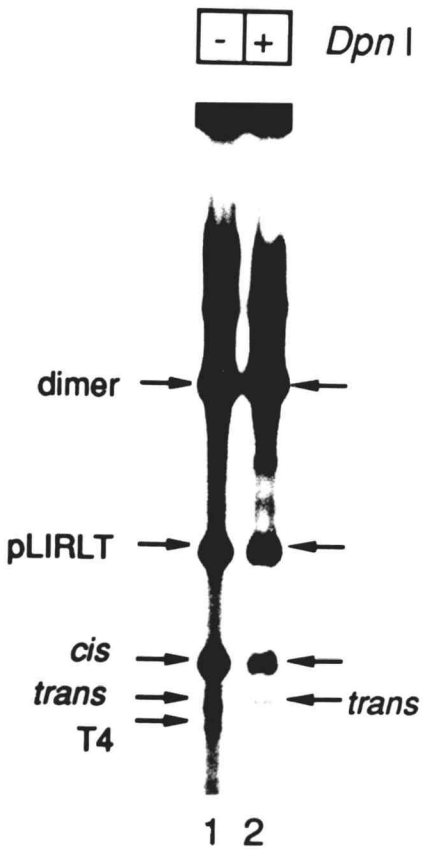

Figure IV.3. Cotransfection of circular T4 and linear pLIRLT. Total DNA extracted from transfected cells was analyzed by Southern blotting without (lane 1) and with (lane 2) prior *Dpn*I digestion. *cis* = *cis* replication product of *Eco*RI-cleaved pLIRLT; dimer = symmetrical dimer of *Eco*RI-restricted pLIRLT; *trans* = *trans* replication product of T4 and pLIRLT.

that the amplification of p3.1 occurs by precise internal initiation at either embedded origin followed by multiple rounds of replication. Here, several linear plasmids which contain a single embedded adenovirus origin were transfected into cells with helper DNA and assayed for adenovirus-specific replication. Plasmid pLIRLT can be linearized by *Eco*RI to expose the single adenovirus origin or by *Sma*I (or *Bgl*II) to embedded the adenovirus origin (Fig. II.1). *Eco*RI-cleaved pLIRLT can be amplified by two distinguishable *cis* replication pathways (see Fig. III.6): one path generates a novel *cis* molecule and the other a symmetrical dimer and dimer foldback molecule (Fig. IV.4A, lanes 1 and 2). Transfections of either *Sma*I- or *Bgl*II-cleaved pLIRLT produced several *Dpn*I-resistant molecules, including the *cis* band, smaller dimers, and corresponding dimer foldbacks (Fig. IV.4A, lanes 3-6). The presence of smaller dimers and corresponding dimer foldbacks suggests that they are amplified from symmetric dimers arising from the end-to-end ligation during transfection. However, the production of *cis* bands in transfections with *Sma*I- or *Bgl*II- cleaved pLIRLT suggests that displacement replication was not eliminated on these linear DNAs containing embedded origins. In the presence of inverted repeat sequences, the displaced monomer-sized strand could be duplicated by the *cis* replication pathway. Comparing the intensities of the *cis* bands arising from the replication of *Sma*I- or *Bgl*II-cleaved pLIRLT (Fig. IV.4A, lanes 4 and 6) and circular pLIRLT (Fig. IV.2, lane 6), it is clear that the replication efficiency on a linear template is higher than on a circular template. This suggests that the mechanisms of initiation on circular and linear templates might be different. Furthermore, it might also mean that releasing the displaced strand from the template might be critical for the synthesis of the complementary strand by the *cis* replication pathway.

The structure of pIRLT is nearly identical to pLIRLT, differing only in the length of the inverted repeats (37 bp compared to 230 bp; see Fig. II.1). pIRLT can be cleaved by *Eco*RI to expose or by *Sma*I or *Bgl*II to embed the single adenovirus origin (Fig. II.1). Like transfections with pLIRLT, these linearized plasmids produces *cis* bands, dimers, and corresponding dimer foldbacks (Fig. IV.4B). The intensities of the *cis* bands in the pIRLT transfections were less intense than the corresponding pLIRLT transfections (compare Figs. IV.4A and IV.4B), and less intense than the corresponding dimer foldbacks as well. This is a consequence of

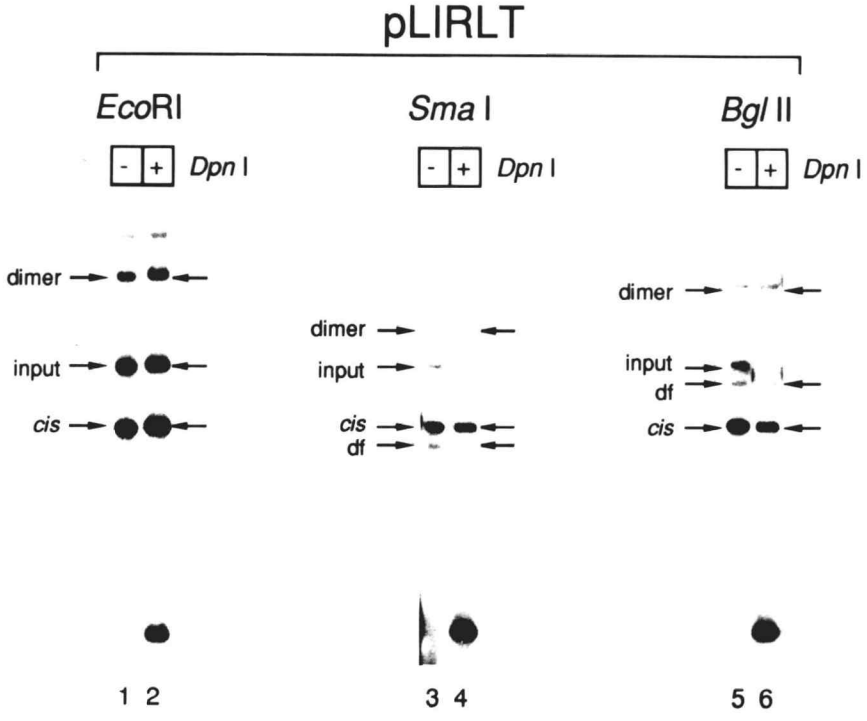

Figure IV.4A. Replication of linear pLIRLT. Helper adenovirus DNA was cotransfected with pLIRLT cut with *Eco*RI (lanes 1 and 2), *Sma*I (lanes 3 and 4), or *Bgl*II (lanes 5 and 6). Total DNA from the transfected cells was analyzed by Southern blot hybridization. Some samples were digested with *Dpn*I (lanes 2, 4, and 6) prior to gel electrophoresis. df = dimer foldback; *cis* = *cis* replication product.

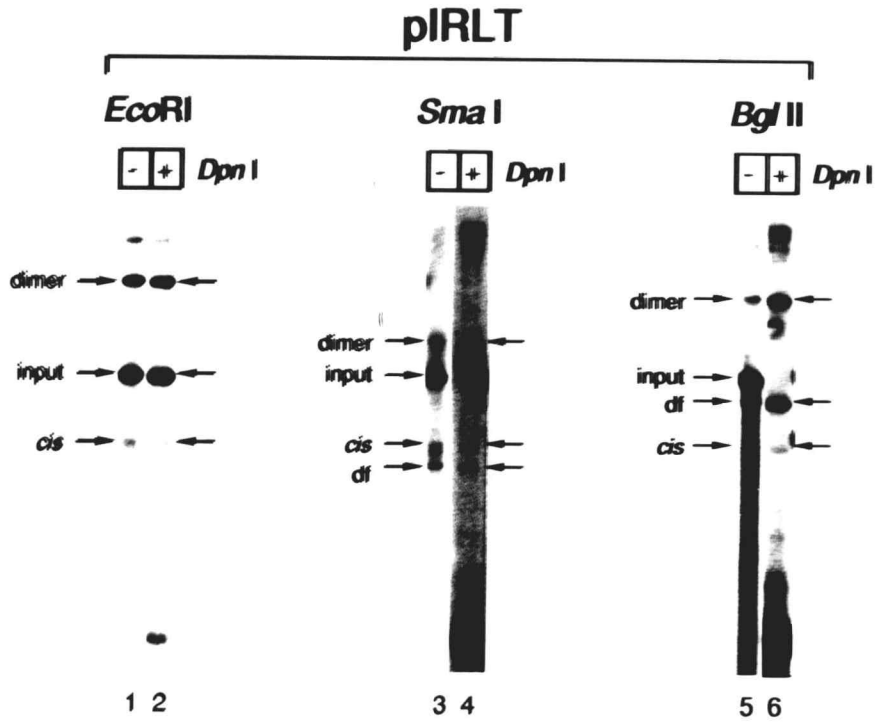

Figure IV.4B. Replication of linear pIRLT. Helper adenovirus DNA was cotransfected with pIRLT cut with *Eco*RI (lanes 1 and 2), *Sma*I (lanes 3 and 4), or *Bgl*II (lanes 5 and 6). Total DNA from the transfected cells was analyzed by Southern blot hybridization. Some samples were digested with *Dpn*I (lanes 2, 4, and 6) prior to gel electrophoresis. df = dimer foldback; *cis* = *cis* replication product.

the smaller inverted repeats (see Fig. III.6 also).

Cis and *trans* replication products can be abundantly detected when linearized palsmid T4 and pLIRLT (in all combinations of exposed and embedded origin configurations) were cotransfected with helper adenovirus DNA (Fig. IV.5).

Possible replication mechanisms using embedded adenovirus origins

Adenovirus recombination is polar with crossovers occurring more frequently at the molecular termini of the viral genome (Munz and Young, 1984). Homologous DNA recombination between the terminal sequences of the adenovirus helper and the embedded adenovirus origin on the plasmid could activate the replication of the plasmid mini-chromosome (Fig. IV.6). To study the contribution, if any, of recombination in the activation of plasmid replication, linear pLIRLT was transfected with a mutant adenovirus helper DNA. pLIRLT contains the left terminal 338 bp of type 2 adenovirus sequences. The mutant helper adenovirus (Mac-Ad) was isolated by Lippe and Graham (1989). The Mac-Ad and type 2 Ad genomes differ only in the terminal seven nucleotides. These terminal sequences could be distinguished with specific 15-mer oligonucleotide probes complementary to the 5' end of either adenovirus genome (see Chapter II). Mac-adenovirus replicates as well as type 2 adenovirus (Lippe and Graham, 1989).

In the presence of helper Mac-adenovirus DNA, *Bgl*II-cleaved pLIRLT produced several *Dpn*I-resistant molecules, including the *cis* band, dimers, and dimer foldback molecules (Fig. IV.7B). The terminal sequences of these replication products were analyzed by hybridization with a type 2-specific oligonucleotide probe (WT probe) and a Mac-specific oligonucleotide probe (Mac probe). The specificities of the probes were determined in Fig. IV.7A, lanes 2 and 4. Both lanes were loaded with linear pLIRLT (1 μg) and Mac-adenovirus DNA (2 μg). The type 2 terminal sequences in the plasmid could only be detected with the WT probe, but not with the Mac probe (lane 2). Similarly, the Mac-adenovirus terminal sequences in the helper DNA could only be detected with the Mac probe, but not with the WT probe (lane 4). There was no cross hybridization between these two sequence-specific probes. By analyzing with WT and Mac probes, both type 2 and Mac terminal sequences were found in all plasmid replication products, including

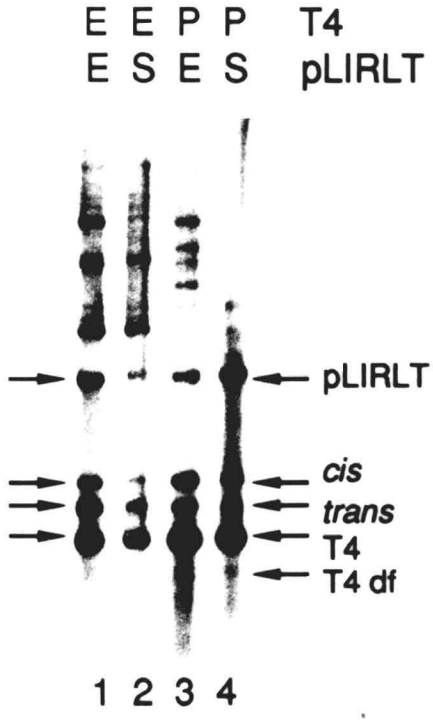

Figure IV.5. Replication of linear T4 and pLIRLT. T4 was linearized with *Eco*RI (E) or *Pvu*II (P) and cotransfected in the indicated combinations with pLIRLT cut with *Eco*RI (E) or *Sma*I (S). Total DNA from the transfected cells was analyzed by Southern blot hybridization. Lane 1: *Eco*RI-cut plasmid T4 and *Eco*RI-cut pLIRLT, lane 2: *Eco*RI-cut plasmid T4 and *Sma*I-cut pLIRLT, Lane 3: *Pvu*II-cut plasmid T4 and *Eco*RI-cut pLIRLT, lane 4: *Pvu*II-cut plasmid T4 and *Sma*I-cut pLIRLT. T4 df = T4 dimer foldback; *cis* = pLIRLT *cis* replication product; *trans* = *trans* replication product of T4 and pLIRLT.

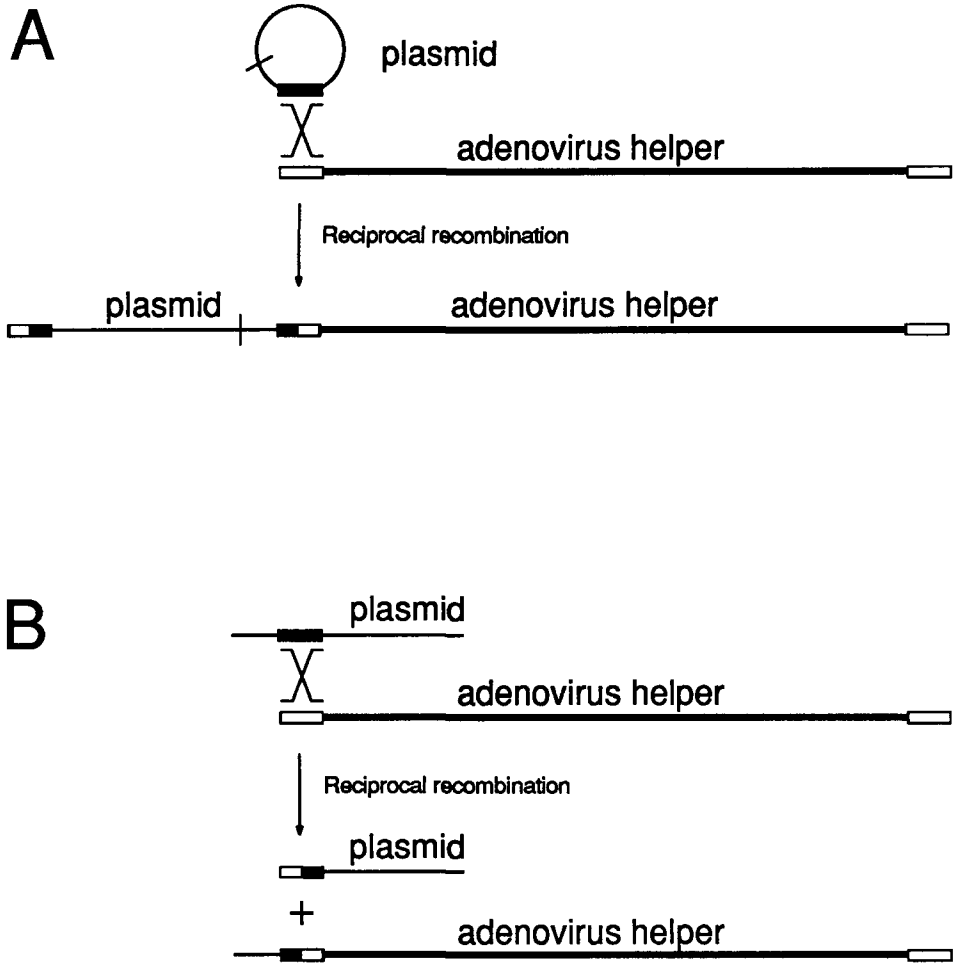

Figure IV.6. Activation of plasmid mini-chromosome replication by homologous recombination with helper adenovirus DNA. Panel A: Recombination between helper and ciecular plasmid molecules. Panel B: Recombination between helper and linear plasmid molecules. The open and closed boxes represent adenovirus origins in the helper and the plasmid, respectively.

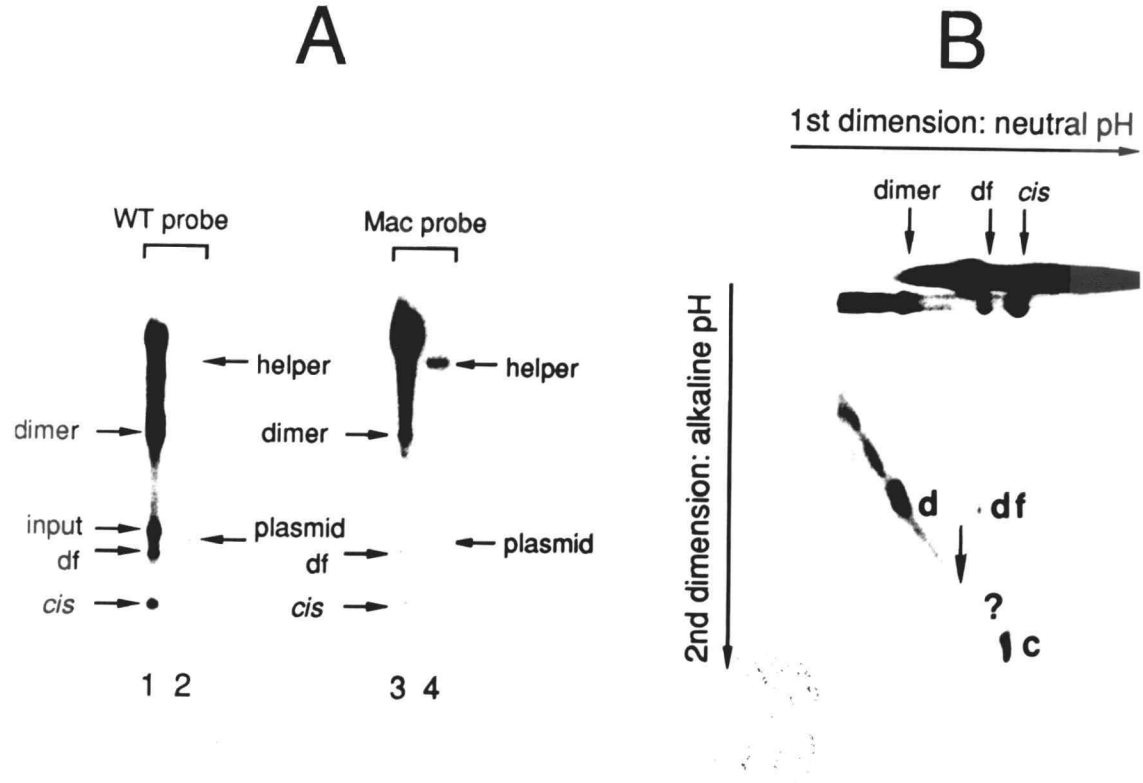

Figure IV.7. Terminal sequence conversion during the replication of *Bgl*II-cleaved pLIRLT cotransfected with helper Mac-adenovirus DNA. Panel A: one-dimensional gel electrophoresis analysis of linear pLIRLT replication *in vivo*. Southern blot analysis employed a WT oligonucleotide probe (lanes 1 and 2) or a Mac oligonucleotide probe (lanes 3 and 4). Panel B: Two-dimensional gel electrophoresis analysis of linear pLIRLT replication *in vivo*. Southern blot analysis employed a pUC19 plasmid probe. df = dimer foldback; c = *cis*-replication product; d = symmetrical dimer; ? = degradation of dimer foldback.

the *cis* band, dimers, and dimer foldback molecules (Fig. IV.7A, lanes 1 and 3). The presence of Mac terminal sequences in the replication products suggested that DNA recombination might have contributed to the activation of the embedded origin by recombination. However, we could not exclude the contribution of sequence conversion arising as a consequence of *trans* hybridization between displaced plasmid strands and helper strands to generate heteroduplex structures. Xu and Pearson (unpublished data) have preliminary evidence that the *trans* pathway requires more than 89 bp of complementary sequences (pLIRLT and helper adenovirus share 338 bp). Recently, *cis* sequence conversion of terminal sequences was demonstrated in the process of adenovirus DNA replication (Lippe and Graham, 1989). The replication products from transfections with helper Mac-adenovirus DNA together with *Eco*RI- or *Bgl*II-cleaved pLIRLT were also analyzed by two-dimensional gel electrophoresis (Fig. IV.8). After hybridization with WT and Mac probes, both types of terminal sequences were found in all replication products. However, the efficient retention of type 2 terminal sequences in the *cis* bands arising from both *Eco*RI- and *Bgl*II-cut pLIRLT (Fig. IV.7A, lane 1; Fig. IV.8, panels A and C) strongly implies that homologous recombination with the helper cannot be the sole mechanism for activating embedded origins.

Possible mechanisms to displace strands from circular molecules

In the presence of inverted repeats, circular pLIRLT and pADIRK2 both produced multimeric *Dpn*I-resistant molecules (Fig. IV.2, lanes 5, 6, 8, and 9), a result not consistent with precise internal initiation on embedded origins (Hay *et al.*, 1984). In the absence of inverted repeats, circular T4 did not produce *Dpn*I-resistant molecules (Fig. IV.2, lanes 2 and 3), an indication that the inverted repeats were critical to rescue replication-competent plasmid mini-chromosomes from circular input molecules. However, the presence of multimers in the replication of circular DNA hints that strand-displacement replication proceeded continuously around circular templates. Homologous recombination between circular plasmids and linear helper (Fig. IV.6) cannot explain the production of multimers. A plausible sequence of events is the use of 3'-hydroxyl groups at random nicks on the circular DNA as primers for helper-driven strand-displacement synthesis followed by release of the displaced strand at

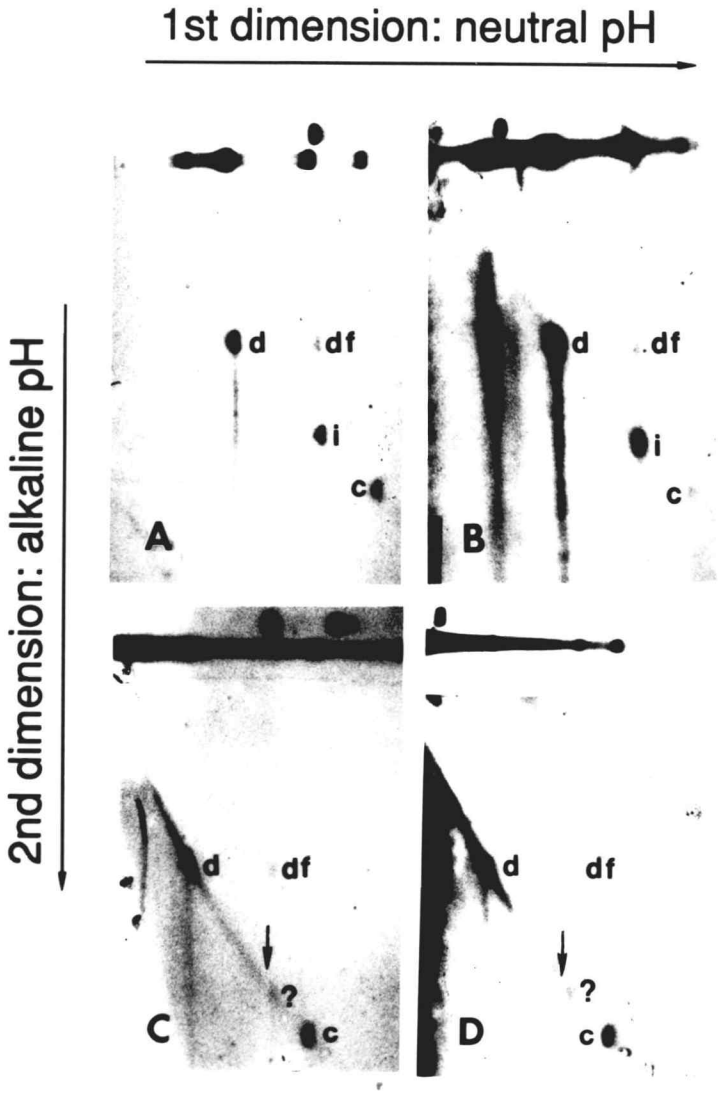

Figure IV.8. Two-dimensional gel electrophoresis analysis of linear pLIRLT replication. Mac-adenovirus helper DNA was cotransfected with pLIRLT cleaved with *Eco*RI (panels A and B) or *Bgl*II (panels C and D). Total DNA from transfected cells was analyzed by two-dimensional gel electrophoresis. Specific ITR sequences were identified by hybridization with a WT oligonucleotide probe (panels A and C) or a Mac oligonucleotide probe (panels B and D). Top lanes across each panel: probe-specific controls containing 1 μg of linear pLIRLT and 2 μg of Mac-adenovirus helper DNA (first lane) and one-dimension gels of each transfection (second lane). i = input monomer DNA; d = symmetrical dimer; df = dimer foldback structure, c = *cis*-replication product; ? = degradation of dimer foldback.

subsequent random nicks or by endonucleolytic cleavage of the single-stranded tail of the rolling circle. The released strand could be rescued and amplified by the *cis* replication pathway. Alternatively, complementary strands displaced individually from different circular templates could hybridize in *trans* to generate various partial duplex structures with potential for adenovirus-specific replication.

The yield of replication products was higher from linear DNA containing an embedded adenovirus origin than from circular DNA. The replication mechanisms on these two forms of template are likely different. A cellular protein, factor pL (a 5'-to-3' exonuclease), is involved in exposing embedded origins in linear templates to allow efficient initiation of adenovirus DNA replication *in vitro* (Guggenheimer *et al.*, 1984b; Kenny *et al.*, 1988; for review, see Stillman, 1989).

CHAPTER V

CONCLUSIONS

Adenovirus replication origins reside within the inverted terminal repeat sequences at both ends of the chromosome (Tamanoi and Stillman, 1982; Tamanoi and Stillman, 1983; Pearson *et al.*, 1983; van Bergen *et al.*, 1983; Hay *et al.*, 1984; Challberg and Rawlins, 1984; Rawlins *et al.*, 1984; Guggenheimer *et al.*, 1984a, 1984b; Wang and Pearson, 1985; Hay, 1985; Bernstein *et al.*, 1986). Replication proceeds by a strand displacement mechanism (Lechner and Kelly, 1977; Kelly and Lechner, 1978). Once replication is initiated, synthesis continues to the end and produces a duplex daughter plus a displaced parental strand. Subsequently, the displaced strand can be duplicated by two different pathways (Ahern *et al.*, 1990). In the *cis* pathway, a replication-proficient intermediate structure, which contains a duplex terminus and a single-stranded loop, is formed by the intramolecular hybridization between the inverted repeat sequences. The synthesis of the complementary strand is initiated from the regenerated duplex terminus by a new round of displacement synthesis to generate duplex DNA (Lechner and Kelly, 1977; Kelly and Lechner, 1978). Alternatively, in the *trans*-pathway, the complementary strands arising from replication on different molecules can hybridize directly to generate a linear duplex virus genome (Ahern *et al.*, 1990).

A plasmid model system, which was first developed by Hay *et al.* (1984), was used in this research to study the structural requirements of the template and the mechanism of complementary strand synthesis in adenovirus DNA replication. Special plasmids were constructed to mimic the structure of the adenovirus genome. Single or dual adenovirus replication origins were cloned into the plasmid vectors. Origin could then be exposed or left embedded by various endonuclease cleavages. In the presence of helper adenovirus DNA, a linear plasmid which contains an exposed adenovirus origin at both ends could be amplified by adenovirus-specific replication to produce *Dpn*I-resistant replication products in transfected cells. However, a linear plasmid containing a single exposed origin and sufficient long inverted repeats could be converted to a replication-proficient molecule by the *cis* replication pathway (Wang and

Pearson, 1985). This approach offers an excellent opportunity to study the mechanism(s) of complementary strand replication.

The relationship between *cis* and *trans* replication pathways

During adenovirus DNA replication, the displaced strands can be duplicated by two alternative pathways. In the *cis* pathway, the generation of a panhandle intermediate by intramolecular hybridization between the inverted repeat sequences is a concentration-independent reaction. However in the *trans* pathway, intermolecular hybridization between different complementary strands is dominated by the accumulation of the displaced strands from either templates. Accordingly, it has been proposed that in the early stage of adenovirus infections, virus genomes would be amplified by the *cis* pathway (Ahern *et al.*, 1990). Although the relationship between these alternative pathways in the late stage of virus infection is not yet clear, it is likelt that the *trans* pathway predominates.

Using plasmid mini-chromosomes as a model system, I have demonstrated that the duplication of the displaced single strand is processed by both the *cis* and *trans* pathways simultaneously. Linear pLIRLT, with inverted repeats, could be amplified in transfected cells by adenovirus-specific replication using the *cis* pathway. Lacking inverted repeats, the displaced strands from linear T4 could not be amplified via the *cis* pathway. However when the linear pLIRLT and T4 were cotransfected into cells simultaneously, a novel replication-proficient molecule was generated by hybridizing of the displaced T4 and pLIRLT strands. The coexistence of the *cis* replication products arising from the monomer pLIRLT template and the *trans*-replication products arising from the heteroduplex of pLIRLT and T4 strands suggested that the complementary strands were built up by the independent but additive *cis* and *trans* pathways.

Identification of a special class of panhandle structures

In the presence of helper DNA, linear plasmids containing a single adenovirus origin can displace only one strand during adenovirus-specific DNA replication. In the absence of inverted repeat sequences, the displaced strand DNA cannot be further amplified by the *cis* pathway. However, linear T4, which contains a single exposed adenovirus origin, was

apparently amplified in transfected cells and produced *Dpn*I-resistant and *Mbo*I-sensitive monomers. This suggests that both strands have been displaced during adenovirus-specific replication. Further analysis by two dimensional gel electrophoresis identified a dimer foldback structure arising from the replication of a symmetrical dimers that were generated by end-to-end ligation during transfection. The symmetrical dimers could be amplified by helper-driven replication. The completely self-complementary displaced parental strands from these dimers can efficiently anneal to form foldback molecules. Dimer foldbacks comigrate with monomers during non-denaturing gel electrophoresis but behave as dimer-sized single strands during denaturing gel electrophoresis. Further analysis found that the foldback structures were *Dpn*I-resistant and *Mbo*I-sensitive, as expected for a molecule containing newly synthesized strands. In addition, the presence of a small single-stranded loop at one end of the flodback was detected by S1 endonuclease digestion. The identification of a dimer foldback structure provides direct evidence for the *cis* pathway in adenovirus DNA replication.

Efficient *cis* replication depends on the length of inverted repeat sequences

Symmetrical dimers are perfect inverted repeats with completely self-complementary foldback replicative intermediates. The minimum inverted repeat must contain at least the adenovirus replication origin (roughly 50 bp long). Previous studies found little or no replicative advantage for inverted repeats that varied in length from 31 bp to 89 bp (Wang *et al.*, 1990). The structures of the replication products of other plasmids, including pIRST and pLIRST (Fig. II.2) and pIRLT and pLIRLT (Fig. II.3), have also been studied by two-dimensional gel electrophoresis. The plasmids differ in the length of their inverted repeats (34 bp or 230 bp) and the length of the unpaired sequences at the 3' ends of their displaced strands (14 bp or 1.4 kb). Comparing the intensities of the *cis* bands arising from these plasmids, it was clear that longer inverted repeats strongly increased the efficiency of adenovirus-specific *cis* replication. The lengths of the unpaired sequences at the 3' ends of the displaced strands also appeared to influence the yield of the *cis* products. In addition, symmetrical dimers, resulting from end-to-end ligation of input molecules, could be efficiently amplified by the adenovirus-specific replication by forming foldback intermediates, a special class of the panhandle intermediate structures.

Inverted repeats are required for the replication of circular DNA

Adenovirus origins are located exactly at both ends of the linear genome. Embedding the adenovirus origin prevents the initiation of adenovirus DNA replication *in vitro*. However, several circular forms of the adenovirus genome have been isolated from infected cells. The circular viral molecules could produce infectious virions containing linear chromosomes. This suggests that the embedded origins in these circular DNAs were not completely inert *in vivo*. Embedding the adenovirus replication origin in plasmids did not prevent the helper-dependent recovery of replicating mini-chromosomes with the size of *cis* molecules. This process, however, required the presence of inverted repeats in the plasmids as well as at least one viral origin. This suggests that the recovery of replicating mini-chromosomes occurs by the *cis* pathway.

Strand-displacement synthesis around circular DNA templates

Circular plasmids with inverted repeats also produced tandemly repeated *Dpn*I-resistant multimers. In the absence of inverted repeat sequences, no *Dpn*I-resistant molecules were produced from circular plasmids. The presence of tandemly repeated multimers suggests that a long stretch of single-stranded DNA has been displaced continuously around the circular templates. To identify strands displaced from circular templates, circular T4 and linear pLIRLT were cotranfected with helper into cells. In addition to the *cis* replication products from linear pLIRLT, a *trans* molecule was observed. A *Dpn*I-resistant multimer also had a size indicative of the *trans* rescue of a tandem T4-dimer strand by a complementary displaced strand from pLIRLT.

Replication of linear DNA containing embedded origins

Linear plasmids containing embedded adenovirus origins have also been assayed for DNA replication by the *in vivo* transfection technique. Linear plasmids with inverted repeats and an embedded origin could be amplified by two different *cis* pathways. The displaced strands from the monomers can be converted into duplex molecules by intramolecular hybridization between the inverted repeats. In addition, the displaced strands from symmetrical dimers generated during transfection were amplified as dimer foldbacks. However, the distance of the adenovirus

origin to a free linear end affects the efficiency of replication. The sequences flanking the embedded adenovirus origins are lost in the replication products. In general, the efficiency of replication on linear templates is higher than on circular templates. This suggests that the initiation mechanisms on circular and linear templates might be different. It might also imply that releasing the displaced strand from the template is critical for the synthesis of the complementary strand by the *cis* replication pathway.

Activation of embedded adenovirus origins by homologous recombination

DNA recombination between linear plasmids and helper DNA was studied by using a mutant adenovirus helper. Adenovirus origin sequences in the plasmids and the helper could be distinguished by sequence-specific oligonucleotide probes. Both wildtype (plasmid) and mutant (helper) terminal sequences coexisted in the plasmid replication products. The retention of the wildtype origin in min-chromosomes suggests that homologous recombination was not the exclusive mechanism used to activate embedded adenovirus origins. Other mechanism(s) must opperate to recover replication-proficient products from molecules with embedded adenovirus origins. The acquisition of mutant helper terminal sequences by plasmid mini-chromosomes indicates that either homologous recombination or sequence conversion has occurred. However, recombination between circular plasmids and helper cannot explain the production of tandemly arrayed multimers.

Possible mechanisms to replicate circular DNAs

Precise internal initiation followed by multiple rounds of replication have been proposed as a mechanism to liberate linear replication-proficient mini-chromosomes from circular molecules containing two adenovirus origins in inverted orientations (Hay *et al.*, 1984). However, this mechanism cannot explain the replication of circular pLIRLT which contains only a single adenovirus origin. The production of tandemly repeated oligomers suggests that continuous strand-displacement synthesis around the circular template might provide a supply of single strands able to enter the *cis* replication pathway. The displaced strand would be released as the replication fork on the rolling circle encountered a random nick. In the

presence of multiple copies of inverted repeat sequences, displaced strands longer than unit length could also be converted into replication-proficient multimers by the *cis* replication pathway. Alternatively, complementary strands arising from strand-displacement synthesis on different circular templates might directly hybridize to form partial duplex structures. Rescue of mini-chromosomes would then occur by the *trans* replication pathway.

BIBLIOGRAPHY

Ahern, K.G., Wang, K., Xu, F.-Y., Mathews, K.Z., and Pearson, G.D. (1990). Intermolecular hybridization of displaced strands during adenovirus DNA replication: a novel mechanism for overlap recombination. In preparation.

Alestrom, P., Akusjarvi, G., Pettersson, M., and Pettersson, U. (1982). DNA sequence analysis of the region encoding the terminal protein and the hypothetical N-gene product of adenovirus type 2. *J.Biol. Chem.* **257**, 13493-13498.

Arrand, J.R., and Roberts, R.J. (1979). The nucleotide sequences at the termini of Ad 2 DNA. *J. Mol.Biol.* **128**, 577-594.

Bellett, A.J.D., and Younghusband, H.B. (1972). Replication of the DNA of chick embryo lethal orphan virus. *J. Mol. Biol.* **72**, 691-709.

Bernad, A., Blanco, L., Lazaro, J.M., Martin, G., and Salas, M. (1989). A conserved 3'-5' exonuclease active site in prokaryotic and eukaryotic DNA polymerases. *Cell* **59**, 219-228.

Bernstein, J.A., Porter, J.M. and Challberg, M.D. (1986). Template requirements for *in vivo* replication of adenovirus DNA. *Mol. Cell. Biol.* **6**, 2115-2124.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7**, 1513-1523.

Brusca, J.S. and Chinnadurai, G. (1983). Structure and reversion of an adenovirus type 2 mutant containing a duplication of the left end of the genome at the right end. *Virology* **129**, 381-392.

Burlingham, B.T., and Doerfler, W. (1971). Three size classes of intracellular adenovirus DNA. *J. Virol.* **7**, 707.

Carusi, E.A. (1977). Evidence for blocked 5'-termini in human adenovirus DNA. *Virology* **76**, 380-394.

Casto, B. (1968). Adenovirus transformation of hamster embryo cells. *J. Virol.* **2**, 376-383.

Challberg, M.D., and Kelly Jr., T.J. (1979). Adenovirus DNA replication *in vitro*. *Proc. Natl. Acad. Sci. USA* **76**, 655-659.

Challberg, M.D., and Kelly Jr., T.J. (1981). Processing of the adenovirus terminal protein. *J. Virol.* **38**, 272-277.

Challberg, M.D., and Kelly Jr., T.J. (1982). Eukaryotic DNA replication:

viral and plasmid model systems. *Annu. Rev. Biochem.* **51**, 901-934.

Challberg, M.D., and Kelly, T.J. (1989). Animal virus DNA replication. *Annu. Rev. Biochem.* **58**, 671-717.

Challberg, M.D. and Rawlins, D.R. (1984). Template requirements for the initiation of adenovirus DNA replication. *Proc. Natl. Acad. Sci. USA* **81**, 100-104.

Challberg, M.D., Desiderio, S.V., and Kelly Jr., T.J. (1980). Adenovirus DNA replication *in vitro*: characterization of a protein covalently linked to nascent DNA strands. *Proc. Natl. Acad. Sci. USA* **77**, 5105-5109.

Chardonnet, Y., and Dales, S. (1970a). Early events in the interaction between adenoviruses and HeLa cells. I. Penetration of type-5 and intracellular release of the DNA genome, *Virology* **40**,462-477.

Chardonnet, Y., and Dales, S. (1970b). Early events in the interaction between adenoviruses and HeLa cells. II. Comparative observations on the penetration of types 1, 5, 7, and 12. *Virology*, **40**, 478-490.

Chomczyhski, P., and Qasba, P.K. (1984). Alkaline transfer of DNA to plastic membrane. *Biochem. Biophys. Res. Comm.* **122**, 340-344.

Cleat, P.H. and Hay, R.T. (1989). Co-operative interactions between NFI and the adenovirus DNA binding protein at the adenovirus origin of replication. *EMBO J.* **8**, 1841-1848.

Daniell, E. (1976). Genome structure of incomplete particles of adenovirus. *J. Virol.* **19**, 685-708.

De Vries, E., van Driel, W., Tromp, M., van Boom, J., and van der Vliet, P.C. (1985). Adenovirus DNA replication *in vitro*: site-directed mutagenesis of the nuclear factor I binding site of the Ad2 origin. *Nucl. Acids Res.* **13**, 4935-1952.

Deuring, R., Klotz, G., and Doerfler, W. (1981). An unusual symmetric recombinant between adenovirus type 12 DNA and human cell DNA. *Proc. Natl. Acad. Sci. USA* **78**, 3142-3146.

Doerfler, W., and Eick, D. (1982). Integrated adenovirus type 12 DNA in the transformed hamster cell line TG37: sequence arrangements at the termini of viral DNA and mode of amplification. *J. Virol.* **42**, 317-321.

Doerfler, W., and Kleinschmidt, A.D. (1970). Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. *J. Mol. Biol.* **50**, 579-593.

Ellens, D.J., Sussenbach, J.S., and Jansz, H.S. (1974). Studies on the

mechanism of replication of adenovirus DNA III: electron microscopy of replicating DNA. *Virology* **61**, 427-442.

Enns, R.E., Challberg, M.D., Ahern, K.G., Chow, K.-C., Mathews, C.Z., Astell, C.R., and Pearson, G.D. (1983). Mutational mapping of a cloned adenovirus origin. *Gene* **23**, 307-313.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochem.* **132**, 6-13.

Feinberg, A.P. and Vogelstein, B. (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. (Addendum to Feinberg and Vogelstein, 1983). *Analytical Biochem.* **137**, 266-267.

Field, J., Gronostajski, R.M., and Hurwitz, J. (1984). Properties of the adenovirus DNA polymerase. *J.Biol. Chem.* **259**, 9487-9495.

Flint, S. J., Sambrook, J., Williams, J., and Sharp, P.A. (1976). Viral nucleic acid sequences in transformed cells. IV. A study of the sequences of adenovirus 5 DNA and RNA in four lines of adenovirus 5 transformed rodent cells using specific fragments of the viral genome. *Virology* **72**, 456-470.

Ford, M., and Fried, M. (1986). Large inverted duplications are associated with gene amplification. *Cell* **45**, 425-430.

Frost, E., and Williams, J. (1978). Mapping temperature-sensitive and host range mutations of adenovirus type 5 by marker rescue. *Virology* **91**, 39-50.

Fujinaga, K., and Green, M. (1966). The mechanisms of viral carcinogenesis by DNA mammalian viruses. I. Viral specific RNA in polyribosomes of adenovirus tumor and transformed cells. *Proc. Natl. Acad. Sci. USA* **55**, 1567-1574.

Fujinaga, K., and Green, M. (1967). Mechanism of viral carcinogenes is by deoxyribonucleic acid mammalian viruses. IV. Related virus-specific ribonucleic acids in tumor cells induced by "highly" oncogenic adenovirus types 12, 18 and 31. *J. Virol.* **1**, 576-582.

Gallimore, P.H. (1974). Interactions of adenovirus type 2 with rat embryo cells: permissiveness, transformation and *in vitro* characteristics of adenovirus transformed rat embryo cells. *J. Gen. Virol.* 25, 263-273.

Garon, C.F., Berry, K.W. and Rose, J.A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. *Proc. Natl. Acad. Sci. USA* **69**, 2391-2395.

Gingeras, T.R., Sciaky, D., Gelinas, R.E., Jiang, B.-D., Yen, C.E., Kelly, M.M., Bullock, P.A., Parsons, B.L., O'Neill, K.E., and Roberts, R.J. (1982). Nucleotide sequences from the adenovirus-2 genome. *J. Biol. Chem.* **257**, 13475-13491.

Girardi, A.J., Hilleman, M.R., and Zwickey, R.E. (1964). Tests in hamsters for oncogenic quality of ordinary viruses including adenovirus type 7. *Proc. Soc. Exp. Biol. Med.* **115**, 1141-1155.

Gluzman, Y., and van Doren, K. (1983). Palindromic adenovirus type 5-simian virus 40 hybrid. *J. Virol.* **45**, 91-103.

Graham, F.L. (1984). Covalently closed circles of human adenovirus DNA are infectious. *EMBO J.* **3**, 2917-2922.

Graham, F.L., and van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456-467.

Graham, F.L., Rudy, J. and Brinkley, P. (1989). Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. *EMBO J.* **8**, 2077-2085.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus 5. *J. Gen. Virol.* **36**, 59-72.

Green, M. (1970). Oncogenic viruses. *Annu. Rev. Biochem.* **39**, 701-756.

Green, M., Chinnadurai, G., Mackey, J., and Green, M. (1976). A unique pattern of integrated viral genes in hamster cells transformed by highly oncogenic human adenovirus 12. *Cell* **7**, 419-428.

Green, M., Mackey, J.K., Wold, W.S. M., and Rigden, P. (1979). Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based upon DNA genome homologies. *Virology* **93**, 481-492.

Green, M., Parsons, J.T., Pina, M., Fujinaga, K., Caffier, H., and Landgraf-Leurs, I. (1970). Transcription of adenovirus genes in productively infected and in transformed cells. *Cold Spring Harbor Symp. Quant. Biol.* **35**, 803-818.

Green, M., Pina, M., Kimes, R., Wensink, P.C., MacHattie, L.A. and Thomas, C.A. (1967). Adenovirus DNA I: Molecular weight and conformation. *Proc. Natl. Acad. Sci. USA* **57**, 1302-1309.

Guggenheimer, R.A., Nagata, K., Lindenbaum, J., and Hurwitz, J. (1984a). Protein-primed replication of plasmids containing the terminus of the adenovirus genome. I: characterization of an *in vitro* DNA replication system dependent on adenoviral DNA sequences. *J. Biol. Chem.* **259**,

7807-7814.

Guggenheimer, R.A., Nagata, K., Kenny, M., and Hurwitz, J. (1984b). Protein-primed replication of plasmids containing the terminus of the adenovirus genome. II. Purification and characterization of a host protein required for the replication of DNA templates devoid of the terminal protein. *J. Biol. Chem.* **259**, 7815-7825.

Guggenheimer, R.A., Stillman, B.W., Nagata, K., Tamanoi, F. and Hurwitz, J. (1984c). DNA sequences required for the *in vitro* replication of adenovirus DNA. *Proc. Natl. Acad. Sci. USA* **81**, 3069-3073.

Haj-Ahmad, Y. and Graham, F.L. (1986). Characterization of adenovirus type 5 mutant carrying embedded inverted terminal repeats. *Virology* **153**, 22-34.

Hanahan, D. and Gluzman, Y. (1984). Rescue of functional replication origins from embedded configurations in a plasmid carrying the adenovirus genome. *Mol. Cell. Biol.* **4**, 302-309.

Hay, R.T. (1985a). The origin of adenovirus DNA replication: minimal DNA sequence requirement *in vivo*. *EMBO J.* **4**, 421-426.

Hay, R.T. (1985b). Origin of adenovirus DNA replication: role of the nuclear factor I binding site *in vivo*. *J. Mol. Biol.* **186**, 129-136.

Hay, R.T., Stow, N.D., and McDougall, I.M. (1984). Replication of adenovirus mini-chromosomes. *J. Mol. Biol.* **175**, 493-510.

Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingragam, P.A., Rosenfeld, M.G., Finney, M., Ruvkun, G., and Horvitz, H.R. (1988) The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and Caenorhabditis elegans unc-86 gene products. *Genes and Develop.* **2**, 1513-1516.

Hilleman, M.R., and Werner, J.R. (1954). Recovery of a new agent from patients with acute respiratory illness. *Proc. Soc. Exp. Biol. Med.* **85**, 183-188.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**, 365-395.

Hu, C.-H., Xu, F.-Y., Pearson, A.N., and Pearson, G.D. (1990). Characterization of a foldback molecule during adenovirus DNA replication. In preparation.

Huebner, R.J., Rowe, W.P., and Lane, W.T. (1962). Oncogenic effects in hamsters of human adenovirus types 12 and 18. *Proc. Natl. Acad. Sci. USA* **48**, 2051-2058.

Ibelgaufts, H., Doerfler, W., Scheidtmann, K.H., and Wechsler, W. (1980). Adenovirus type 12-induced rat tumor cells of neuroepithelial origin: persistence and expression of the viral genome. *J. Virol.* **33**, 423-437.

Ishibashi, M., and Yasue, H. (1984). Adenoviruses of animals. *In* "The Adenoviruses", (H.S. Ginsberg, ed.), Plenum Press, New York and London, pp. 497-562.

Johannsson, K., Persson, H., Lewis, A.M., Pettersson, U., Tibbetts, C., and Philipson, L. (1978). Viral DNA sequences and gene products in hamster cells transformed by adenovirus type 2. *J. Virol.* **27**, 628-639.

Johansson, K., Pettersson, U., Philipson, L., and Tibbetts, C. (1977). Reassociation of complementary strand-specific adenovirus type 2 DNA with viral DNA sequences of transformed cells. *J. Virol.* **23**, 29-35.

Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J., Tjian, R. (1987). A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. *Cell* **48**, 79-89.

Kelly Jr., T.J. and Lechner, R.L. (1978). The structure of replicating adenovirus DNA molecules: characterization of DNA-protein complexes from infected cells. *Cold Spring Harbor Symp. Quant. Biol.* **43**, 721-728.

Kelly, T.J., Wold, M.S., and Li, J. (1988). Initiation of viral DNA replication. *Adv. Virus Res.* **34**, 1-42.

Kenny, M.K., Balogh, L.A., and Hurwitz, J. (1988). Initiation of adenovirus DNA replication. I. Mechanism of action of a host protein required for replication of adenovirus DNA templates devoid of the terminal protein. *J. Biol. Chem.* **263**, 9801-9808.

Kruijer, W., van Schaik, F.M.A., and Sussenbach, J.S. (1981). Structure and organization of the gene coding for the DNA binding protein of adenovirus type 5. *Nucl. Acids Res.* **9**, 4439-4458.

Kuhlmann, I., Achten, S., Rudolph, R., and Doerfler, W. (1982). Tumor induction by human adenovirus type 12 in hamsters: loss of the viral genome from adenovirus type 12-induced tumor cells is compatible with tumor formation. *EMBO J.* **1**, 79-86.

Lechner, R.L., and Kelly Jr., T.J. (1977). The structure of replicating adenovirus 2 DNA molecules. *Cell* **12**, 1007-1020.

Leegwater, P.A.J., Rombouts, R.F.A. and van der Vliet, P.C. (1988). Adenovirus DNA replication *in vitro*: duplication of single-stranded DNA containing a panhandle structure. *Biochim. Biophys. Acta* **951**, 403-410.

Lichtenberg, U., Zock, C., and Doerfler, W. (1987). Insertion of adenovirus type 12 DNA in the vicinity of an intracisternal A particle genome in Syrian hamster tumor cells. *J. Virol.* **61**, 2719-2726.

Lippe, R., and Graham, F.L. (1989) Adenoviruses with nonidentical terminal sequences are viable. *J. Virol.* **63**, 5133-5141.

Lonberg-Holm, K., and Philipson, L. (1969). Early events of virus-cell interactions in an adenovirus system. J. Virol. 4, 323-335.

Maniatis, T., Fritsch, E., and Sambrook, J. (1982). "Molecular cloning. A laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mantyjarvi, R., and Russell, W.C. (1969). Studies on DNA synthesis in cells infected with adenovirus. *J. Gen. Virol.* **1**, 1010-1017.

Maxam, A.M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**, 499-560.

McAllister, R.M., Nicolson, M.O., Lewis Jr., A.M., Macpherson, I., and Huebner, R.J. (1969a). Transformation of rat embryo cells by adenovirus type 1. *J. Gen. Virol.* **4**, 29-36.

McAllister, R.M., Nicolson, M.O., Lewis Jr., A.M., Macpherson, I., and Huebner, R.J. (1969b). Transformation of rodent cells by adenovirus 19 and other group D adenoviruses. *J. Natl. Cancer Inst.* **43**, 917-923.

McBride, W.D., and Weiner, A. (1964). *In vitro* transformation of hamster kidney cells by human adenovirus type 12. *Proc. Soc. Exp. Biol.* **115**, 870-874.

Modak, S.P., and Beard, P. (1980). Analysis of DNA double- and single-stranded breaks by two-dimensional electrophoresis: action of micrococcal nuclease on chromatin and DNA, and degradation *in vivo* of lens fiber chromatin. *Nucl. Acids Res.* **8**, 2665-2678.

Messing, J. (1979). "Recombinant DNA Technical Bulletin, National Institutes of Health Publ. No. 79-99." National Institutes of Health, Bethesda, MD.

Mogan, G., Rosenkranz, H.S., and Medris, B. (1969). Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. *J. Virol.* **4**, 777-788.

Munz, P.L., and Young, C.S.H. (1984). Polarity in adenovirus recombination. *Virology* **135**, 503-514.

Nagata, K., Guggenheimer, R.A., and Hurwitz, J. (1983). Adenovirus DNA

replication *in vitro*: synthesis of full-length DNA with purified proteins. *Proc. Natl. Acad. Sci. USA* **80**, 4266-4270.

O'Neill, E.A., Fletcher, C., Burrow, C.R., Heintz, N., Roeder, R.G., Kelly, T.J. (1988). Transcriptional factor OTF-1 is functionally identical to the DNA replication factor NFIII. *Science* **241**, 1210-1213.

Passananti, C., Davies, B., Ford, M., and Fried, M. (1987). Structure of an inverted duplication formed as a first step in a gene amplification event: implications for a model of gene amplification. *EMBO J.* **6**, 1697-1703.

Pearson, G.D. (1975). Intermediates in adenovirus type 2 replication. *J. Virol.* **16**, 17-26.

Pearson, G.D., Chow, K.-C., Robert. E.E., Ahern, K.G., Corden, J.L. and Harpst, J.A. (1983). *In vitro* replication directed by a cloned adenovirus origin. *Gene* **23**, 293-305.

Pearson,G.D., and Hanawalt, P.C. (1971). Isolation of DNA replication complexes from uninfected and adenovirus-infected HeLa cells. *J. Mol. Biol.* **62**, 65-80.

Peden, K.W.C., Pipas, J.M., Pearson-White, S., and Nathans, D. (1980). Isolation of mutants of an animal virus in bacteria. *Science* **209**, 1392-1396.

Pereira, M.S., Pereira, H.G., and Clarke, S.K. (1965). Human adenovirus type 31: a new serotype with oncogenic properties. *Lancet* **1**, 21-23.

Perucho, M., Hanahan, D., and Wigler, M. (1980). Genetic and physical linkage of exogenous sequences in transformed cells. *Cell* **22**, 309-317.

Pettersson, U., and Sambrook, J. (1973). Amount of viral DNA in the genome of cells transformed by adenovirus type 2. *J. Mol. Biol.* **73**, 125-130.

Pettit, S.C., Horwitz, M.S., and Engler, J.A. (1988). Adenovirus preterminal protein synthesized in COS cells from cloned DNA is active in DNA replication *in vitro*. *J. Virol.* **62**, 496-500.

Philipson, L., Lonberg-Holm, K., and Pettersson, U. (1968). Virus-receptor interactions in an adenovirus system. *J. Virol.* **2**, 1064-1072.

Pina, M., and Green, M. (1965). Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analyses of 28 human adenoviruses. *Proc. Natl. Acad. Sci. USA* **54**, 547-551.

Pruijn, G.J.M., van Driel, W., and van der Vliet, P.C. (1986). Nuclear factor III, a novel sequence-specific DNA binding protein form HeLa cells stimulating adenovirus DNA replication. *Nature* **322**, 455-459.

Rawlins, D.R., Rosenfeld, P.J., Wides, R.J., Challberg, M.D., and Kelly Jr.,

T.J. (1984). Structure and function of the adenovirus origin of replication. *Cell* **37**, 309-319.

Reed, K.G., and Mann, D.A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acids Res.* **13**, 7207-7221.

Rekosh, D.M.K., Russell, W.C., and Bellett, A.J.D. (1977). Identification of a protein linked to the ends of Ad DNA. *Cell* **11**, 283-295.

Revet, B., and Benichou, D. (1981). Electron microscopy of AD4 replicating molecules after *in vivo* photocrosslinking with trioxsalen. *Virology* **114**, 60-70.

Rijinders, A.W.M., van Bergen, B.G.M., van der Vliet, P.C., and Sussenbach, J.S. (1983). Specific binding of the adenovirus terminal protein precursor-DNA polymerase complex to the origin of DNA replication. *Nucl. Acids Res.* **11**, 8777-8789.

Robinson, A.J. and Bellett, A.J.D. (1974). A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. *Cold Spring Harbor Symp. Quant.Biol.* **39**, 523-531.
Robinson, A.J., Younghusband, H.B. and Bellett, A.J.D. (1973). A circular DNA-protein complex from adenoviruses. *Virology* **56**, 54-69.

Rosahl, T., and Doerfler, W. (1988). Predominant association of adenovirus type 12 DNA with human chromosome 1 early in productive infection. *Virology* **162**, 494-497.

Rosen, L. (1960). A hemagglutination-inhibition technique for typing adenoviruses. *Am. J. Hyg.* **71**, 121-128.

Rowe, W.P., Huebner, A.J., Gilmore, L.K., Parrott, R.N., and Ward, T.G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.* **84**, 570-573.

Ruben, M., Bacchetti, S., and Graham, F.L. (1982). Integration and expression of viral DNA in cells transformed by host range mutants of adenovirus type 5. *J. Virol.* **41**, 674-685.

Ruben, M., Bacchetti, S. and Graham, F. (1983). Covalently closed circles of adenovirus 5 DNA. *Nature* **301**, 172-174.

Ruiz, J.C., and Wahl, G.M. (1988). Formation of an inverted duplication can be an initial step in gene amplification. *Mol. Cell. Biol.* **8**, 4302-4313.

Sambrook, J., Botchan., M.B., Gallimore, P.H., Ozanne, B., Pettersson, U., Williams, J.F., and Sharp, P.A. (1979). Viral DNA sequences in cells transformed by simian virus 40, adenovirus type 2 and adenovirus type 5.

Cold Spring Harbor Symp. Quant. Biol. **39**, 615-632.

Sharp, P.A., Pettersson, U., and Sambrook, J. (1974). Viral DNA in transformed rat cells using specific fragments of the viral genome. *J. Mol. Biol.* **86**, 709-726.

Shinagawa, M., Ishiyama, T., Padmanabhan, R., Fujinaga, K., Kamada, M., and Sato, G. (1983). Comparative sequence analysis of the inverted terminal repetition in the genomes of animal and avian adenoviruses. *Virology* **125**, 491-495.

Shu, L., Horwitz, M.S., and Engler, J.A. (1987). Expression of enzymatically active adenovirus DNA polymerase from cloned DNA requires sequences upstream of the main open reading frame. *Virology* **161**, 520-526.

Smart, J.E., and Stillman, B.W. (1982) Adenovirus terminal protein precursor. *J. Biol. Chem.* **257**, 13499-13506.

Stabel, S. Doerfler, W., and Friis, R.R. (1980). Integration sites of adenovirus type 12 DNA in transformed hamster cells and hamster tumor cells. *J. Virol.* **36**, 22-40.
Steeenbergh, P.H., Maat, J., van Ormondt, H., and Sussenbach, J.S. (1977). The nucleotide sequence at the termini of adenovirus type 5 DNA. *Nucl. Acids Res.* **4**, 4371-4389.

Stillman, B.W. (1981). Adenovirus DNA replication *in vitro*: a protein linked to the 5' end of nascent DNA strands. *J. Virol.* **37**, 139-147.

Stillman, B.W. (1989). Initiation of eukaryotic DNA replication *in vitro*. *Annu. Rev. Cell Biol.* **5**, 197-245.

Stillman, B.W., Lewis, J.B., Chow, L.T., Mathews, M.B., and Smart, J.E. (1981). Identification of the gene and mRNA for the adenovirus terminal protein precursor. *Cell* **23**, 497-508.

Stillman, B.W., Topp, W.C., and Engler, J.A. (1982). Conserved sequences at the origin of adenovirus DNA replication. *J.Virol.* **44**, 530-537.

Stow, N.D. (1981). Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. *J. Virol.* **37**, 171-180.

Stow, N.D. (1982). The infectivity of adenovirus genomes lacking DNA sequences from their left-hand termini. *Nucl. Acids Res.* **10**, 5105-5119.

Straus, S.E., (1984). Adenovirus infections in humans. *In* "The adenoviruses", (H.S. Ginsberg, ed.), Plenum Press, New York and London, pp. 497-562.

Stunnenberg, H.G., Lange, H., Philipson, L., van Miltenburg, R.T., and van der Vliet, P.C. (1988). High expression of functional adenovirus DNA polymerase and precursor terminal protein using recombinant vaccinia virus. *Nucl. Acids Res.* **16**, 2431-2444.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B. (1981). ICN-UCLA Symp. Mol. Cell. Biol. 231, 683-691.

Tamanoi, F. and Stillman, B.W. (1982). Function of adenovirus terminal protein in the initiation of DNA replication. *Proc. Natl. Acad. Sci. USA* **79**, 2221-2225.

Tamanoi, F. and Stillman, B.W. (1983a). Initiation of adenovirus DNA replication *in vitro* requires a specific DNA sequence. *Proc. Natl. Acad. Sci. USA* **80**, 6446-6450.

Tamanoi, F., and Stillman, B.W. (1983b). The origin of adenovirus DNA replication. *Curr. Topics Microb. Immunology* **109**, 75-87.

Trentin, J.J., Yabe, Y., and Taylor, G. (1962). The quest for human cancer viruses. *Science* **137**, 835-841.

Van Bergen, B.G.M., van der Ley, P.A., van Driel, W., van Mansfeld, A.D. M., and van der Vliet, P.C. (1983). Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. *Nucl. Acids Res.* **11**, 1975-1989.

Van der Eb, A.J. (1973). Intermediates in type 5 adenovirus DNA replication. *Virology* **51**, 11-23.

Van der Vliet, P.C., and Sussenbach, J.S. (1972). The mechanism of adenovirus DNA synthesis in isolated nuclei. *Eur. J. Biochem.* **30**, 584-592.

Van Ormondt, H., and Galibert, F. (1984). Nucleotide sequences of adenovirus DNAs. *Curr. Topics Microb. Immunology* **110**, 73-142.

Vasudevachari, M.B., Natarajan, V., and Salzman, N.P. (1987). Cotransfection with adenovirus DNA enhances transcription from linear DNA containing eukaryotic promoters. *Mol. Cell. Biol.* **7**, 1063-1069.

Visser, L., Reemst, A.C.M.B., van Mansfeld, A.D.M., and Rozijn, T.H. (1982). Nucleotide sequence analysis of the linked left and right hand l regions of adenovirus type 5 DNA present in the transformed rat cell line 5RK20. *Nucl. Acids Res.* **10**, 2189-2198.

Visser, L., Wassennaar, A.T.D.C., van Maarschalkerweerd, M.W., and Rozijn, T.H. (1981). Arrangement of integrated viral DNA sequences in cells transformed by adenovirus type 2 and 5. *J.Virol.* **39**, 684-693.

Vogel, S., Rosahl, T., and Doerfler, W. (1986). Chromosomal localization of integrated adenovirus DNA in productively infected and in transformed mammalian cells. *Virology* **152**, 159-170.

Wadell, G., Hammarskjold, M.-L., Winberg, G., Varsanyi, T.M., and Sundell, G. (1980). Genetic variability of adenoviruses. *Annu. N. Y. Acad. Sci.* **354**, 16-31.

Wang, K., and Pearson, G.D. (1985). Adenovirus sequences required for replication *in vivo*. *Nucl. Acids Res.* **13**, 5173-5187.

Wang., K., Xu, F.-Y., Ahern, K.G., and Pearson, G.D. (1990). Base-pairing requirements for functional panhandles in adenovirus complementary strand replication. In preparation.

Wides, R.J., Challberg, M.D., Rawlins, D.R., and Kelly, T.J. (1987). Adenovirus origin of DNA replication: sequence requirements for replication *in vitro*. *Mol. Cell. Biol.* **7**, 864-874.

Wilkie, N.-M. (1973). The synthesis and substructure of Herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. *J. Gen. Virol.* **21**, 453-467.

Winnacker, E.-L. (1978). Adenovirus DNA: structure and function of a novel replicon. *Cell* **14**, 761-773.

Wolfson, J., and Dressler, D. (1972). Adenovirus-2 DNA contains an inverted terminal repetition. *Proc. Natl. Acad. Sci. USA* **69**, 3054-3057.

Yao, M.-C., Zhu, S.-G., and Yao, C.-H. (1987). Gene amplification in *Tetrahymena thermophila*: formation of extrachromosomal palindromic genes coding for rRNA. *Mol. Cell. Biol.* **5**, 1260-1267.

Zeff, R.A., and Geliebter, J. (1987). Oligonucleotide probes for genomic DNA blots. *Focus* **9**, 1-2.

Zhao, L., Irie, K., and Padmanabhan, R. (1988). Nuclear transport of adenovirus DNA polymerase is facilitated by interaction with preterminal protein. *Cell* **55**, 1005-1015.