AN ABSTRACT OF THE THESIS OF

Kuan-Chih W. Chow for the degree of Doctor of Philosophy in
Biochemistry and Biophysics presented on June 8, 1984.
Title: In Vitro Adenovirus DNA Replication.

Redacted for Privacy

Abstract approved:_____
George D. Pearson

XD-7, a 5.7-kb recombinant plasmid, contains the left terminal
XbaI-E fragment from adenovirus type 2 (Ad2) DNA inserted into the
EcoRI site of pBR322. As measured by electron microscopy, an average
of 9% of input XD-7 DNA replicated as rolling circles with displaced
single-stranded tails in the cell-free adenovirus replication system.
The replication origin was mapped on XD-7 DNA to the left boundary of
the cloned adenovirus DNA segment. An internally standardized assay
was also developed to study the function of the cloned adenovirus
origin. The first 20 nucleotides of the Ad2 inverted terminal
repetition (ITR), a region containing a sequence conserved among
human, murine, simian and avian adenoviruses, were found to be
essential for the initiation of adenovirus DNA replication. Deletions
removing or penetrating from either direction into the conserved
sequence inactivated replication. A point mutation within the
conserved sequence diminished the replication frequency, but point
mutations outside the conserved sequence had no effect. The conserved

sequence thus functions as an effective origin for the initiation of adenovirus DNA replication.

High resolution electrophoresis mapping located three sets of site-specific nicking which might in turn be related to the in vitro replication of adenovirus DNA. The first set occurs on the l-strand of the adenovirus left inverted terminal repetition (ITR). The nick site has the concensus G-rich sequence:

GGRGYG↓GGNRNGTG

The second set occurs at the center of the palindromic BamHI linker on the r-strand within the Ad2 insert in XD-7 deletion mutants. The size of the palindrome and the neighboring sequences affect recognition of the nick site. A cellular endonuclease presumably introduces both classes of nicks in an ATP-independent reaction. The third set is apparently adenovirus-specific. Nicking occurs at the junction between the Ad2 insert and vector sequences. However, the nicking signal is weak and an aberrant nick appears between the third T and the fourth C of Ad2 ITR. These could be due to an imperfect palindrome in the substrate DNA.

A ^{32}P-transfer assay was developed to identify the enzyme which is involved in the nicking reaction. The assay was essentially based on the covalent bonding between the DNA backbone and the enzyme. After removing unreacted DNA by DNase I digestion, ^{32}P-radioactivity which had been previously incorporated into the DNA backbone could then be transferred to the enzyme. Type I DNA topoisomerase (topo I) was identified by this simple, specific, and quantitative assay. The level of topoisomerase I in adenovirus-infected and adenovirus-

transformed 293 cells is at least ten-fold higher than in uninfected HeLa cells. Adenovirus early 1A gene products might be involved in stimulating the activity of cellular topoisomerase I or the expression of the topoisomerase I gene. Cellular topoisomerase I may be required for adenovirus DNA replication.

In <u>Vitro</u> Adenovirus DNA Replication

by

Kuan-Chih W. Chow

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for
the degree of

Doctor of Philosophy

Completed June 8, 1984

Commencement June 1985

APPROVED:

Redacted for Privacy
_____
Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Chairman of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

Date thesis is presented _____ June 8, 1984 _____

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

In Vitro Adenovirus DNA Replication

CHAPTER I

AN OVERVIEW

STRUCTURAL FEATURES OF THE ADENOVIRAL GENOME

The adenovirus (Ad) genome is a nonpermuted, linear, double-stranded DNA molecule containing 35,000 to 45,000 base pairs (bp) (1) in different serotypes. Adenovirus DNA has two specialized terminal features that appear to play a role in DNA replication: (a) Inverted terminal repetitions (ITRs), ranging from 102 bp to 162 bp (2-4), are precisely identical at both ends. The sequences of the ITRs of different adenovirus show a high degree of homology, especially nucleotides 9 through 17, which are conserved in all adenoviruses examined to date. (b) A terminal protein (TP) is covalently linked to the 5' terminus of each adenovirus DNA strand, and, in the case of adenovirus type 5 (Ad5), the molecular weight of terminal protein is 55,000-dalton (5-7). The protein-DNA linkage has been shown to be a phosphodiester bond between the β-OH of a serine residue in the terminal protein and the 5'-phosphate of the terminal deoxycytidine residue in the DNA (8).

REPLICATION IN VIVO

Replicating adenovirus DNA molecules have been characterized by electron microscopy (9,10), by analysis of the nature of the single-stranded DNA in replicating forms of the viral DNA, and by

elucidation of the temporal order of synthesis of specific regions of the viral genome.

Two basic replicative intermediates have been identified: first, linear duplexes with one or two single-stranded branches (type I molecules; Figure I.1), and second, unbranched full length molecules with a single- and a double-stranded region (type II molecules; Figure I.1). The double-stranded portions of both type I and type II molecules were shown by partial denaturation mapping to arise with equal frequency from both ends of the viral genome (10). A small fraction of molecules consisted of structures containing features of both type I and II molecules. Together, these three forms account for 85-90% of the total pool of replicative intermediates.

Based on these observations, the process of adenoviral DNA replication can be summarized in the following: The adenovirus DNA-protein complex (Ad DNA-pro) replicates by a strand-displacement mechanism. Replication of the double-stranded templates may initiate at either end of the DNA molecule. Elongation of the nascent strand proceeds continuously in a 5' to 3' direction along the template parental strand. Replication of the displaced strand then initiates at its 3' terminus. It has been proposed (Figure I.1) (11) that the complementary sequences of the ITRs at the opposite ends of the displaced single strands may form a circular "panhandle" structure, and that the generation of this short duplex region, indistinguishable from the termini of the double-stranded genome, may enable DNA replication to be initiated by the same mechanism. Circular, single-stranded replicative intermediates ("panhandle" stuctures) have not been detected, and there is no direct evidence

Figure I.1. The "protein-priming" mechanism for adenovirus DNA replication (11). Double thin lines represent the parental Ad2 DNA template. Replication is initiated at or near either end of the template. Following initiation, daughter strand synthesis (as shown by the heavy line with arrowhead) proceeds in the 5' to 3' direction with concomitant displacement of the parental strand with the same polarity. This produces a type I replicative intermediate. Displacement synthesis proceeds to the end of the Ad2 duplex and results in the formation of a daughter duplex and a parental single strand. The Type II replicative intermediate is produced by "fork annihilation" or by initiation from the self-complementary duplex region in the "panhandle intermediate".
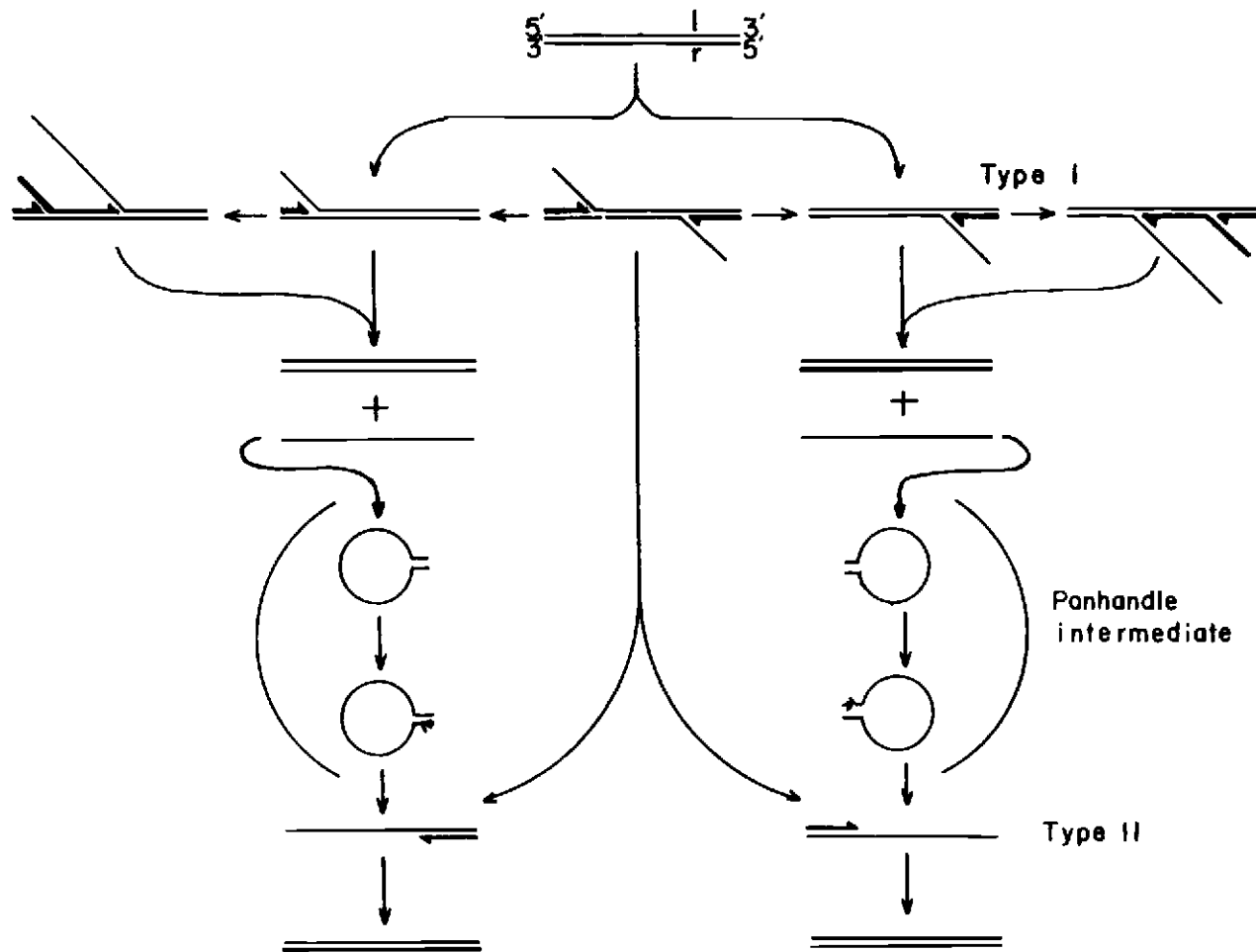
Figure I.1. The "protein-priming" mechanism for adenovirus DNA replication (11).

for such a structure occurring _in_ _vivo_. However, in a study of the infectivity of adenovirus deletion mutants by transfection of 293 cell lines, Stow (12) has demonstrated that genomes lacking 11, 40 or 51 nucleotides from their left end, or containing an additional 18 deoxyguanosine residues linked to this position were infectious, and analysis of the progeny viral genomes showed that the structure of the modified termini had been restored to normal. Deletion mutants in which the entire ITR region had been removed were noninfectious. These results suggest that the ITRs present at opposite ends of transfecting DNA molecules are important for the repair of the nonlethal deleted sequences and enable DNA synthesis to initiate on the repaired duplexes. The nascent strands which were initiated at the end of the "panhandle" elongate until a full-length, progeny, double-stranded adenovirus DNA-protein complex molecule has been formed (9,12,13). It is clear from the mechanism described above that a discontinuous mode of DNA synthesis is not required during any phase of adenovirus replication. All nascent strands initiate from the 5' end toward the 3' end, and can therefore be elongated without the synthesis or subsequent joining of Okazaki fragments (13-16).

REPLICATION IN VITRO

_In_ _vitro_ studies of adenovirus DNA replication first used isolated nuclei or subnuclear replication complexes (17,18). In these systems, replication appears to be a continuation of synthesis on replicative intermediates formed _in_ _vivo_. Nascent strands could be completed _in_ _vitro_, but _de_ _novo_ initiation of new DNA strands was not observed. The availability of a soluble enzyme system that replicates

exogenously added adenovirus DNA-protein complex (Ad DNA-pro) has made it possible to characterize the factors involved in replication. Furthermore, the DNA sequences that are required for the initiation of adenovirus DNA replication and the interaction of these sequences and the (protein) factors can be studied in detail. The original in vitro system (19) was a nuclear extract prepared from adenovirus-infected HeLa cells to which 10 mM hydroxyurea had been added 2 hours after infection. The addition of hydroxyurea blocks DNA synthesis, but permits the accumulation of viral proteins required for replication. DNA synthesis in this system required Ad DNA-pro complex, $MgCl_2$, ATP and the infected nuclear extract. The conditions are listed in Table I.1. No synthesis occurred with protease- or alkali-treated Ad DNA-protein complex or with an extract prepared from uninfected cells. Replication in vitro resembles the in vivo mechanism in that initiation occurred at either terminus of the template, the nascent chain was elongated continuously until a full-length progeny genome was formed, and the reaction required protein-linked DNA templates (20,21).

PROTEINS INVOLVED IN THE ADENOVIRUS DNA REPLICATION

In order to elucidate the mechanism of replication and the factors involved in adenoviral DNA synthesis, the initial fractionation work of the in vitro system was done by Hurwitz's laboratory (21). The first step divided the cell-free system into two components: nuclear and cytoplasmic extracts.

Two virus-encoded proteins required for replication were purified from the cytoplasmic extract. One was the adenovirus single-stranded

TABLE I.1   Comparative study of _in vitro_ replication conditions

Laboratory	Van Bergen(30)	Tamanoi(59)	Challberg(19)	Lichy(50)		
Total volume	30 μl	20 μl	100 μl	50 μl		
Hepes/KOH	40 mM	25 mM	50 mM	25 mM		
$MgCl_2$	4 mM	5 mM	5 mM	5 mM		
DTT	0.4 mM	2 mM	0.5 mM	2 mM		
ATP	1.7 mM	3 mM	2 mM	3 mM		
$[\alpha-^{32}P]dCTP$	8 μM	0.85 μM	NR	0.5 μM		
Creatine-PO_4	5 mM	----	----	----		
Creatine Phosphokinase	5 μg/ml	----	----	----		
Nuclear Extract	7-11 μl	6-10 μl	25 μl	70 μg prot		
Cytosol	----	----	----	15 μg prot		

Template

	Van Bergen(30)	Tamanoi(59)	Challberg(19)	Lichy(50)		
Ad DNA-prot	3.3 μg/ml (0.14 pmol/ml)	80 ng (3.5 fmol)	150 ng (6.5 fmol)	150 ng (6.5 fmol)		
Plasmid	16.5 μg/ml (4.4 pmol/ml)	80 ng ----	----	----		
$[\alpha-^{32}P]$ dCMP incorporation	3.2 pmol/ml	0.5 fmol	20-25 pmol	$\frac{0.67^a}{0.5}$	$\frac{0.93}{1}$	$\frac{1.4}{4}$
% Initiation	7 0.23(plasmid)	7	6	5	7	10

[a] fmol/hr.   NR   no record

DNA binding protein (Ad DBP), a 72,000-dalton (72K) phosphoprotein which is encoded by early region E2a on the viral genome (1). The Ad DBP is a multifunctional protein, having a role in DNA replication (22,23), as well as regulation of early mRNA levels in infected cells and late gene expression (24). The other virus-encoded protein purified from the cytoplasmic extract is the terminal protein precursor (pTP) (25-27), an 80,000-dalton protein which is also synthesized from early region E2b on the viral genome. This protein is covalently bound to all intracellular viral DNA, and is cleaved to TP during virion morphogenesis by a virus-encoded protease (26). The pTP becomes covalently attached to the 5' dCMP residue of nascent DNA strands replicated in vitro. In the standard in vitro reaction, dCTP can be covalently coupled to pTP to form a pTP-dCMP complex if the specific DNA sequences are at the replication origin (28-31). The purified pTP is tightly bound to a 140,000-dalton (140K) protein which contains a DNA polymerase activity (32); pTP has not been demonstrated to contain any known enzymatic activity. The 140K Ad DNA polymerase has also been identified as a product of early region E2b on the adenovirus genome (33). This DNA polymerase (Ad pol) differs from host DNA polymerase α, β and γ in its template preference and sensitivity to inhibitors. The purified pTP preparation contained both pTP and Ad pol. The two proteins were present in about equimolar amounts. The purified pTP/Ad pol complex supported the replication of Ad DNA-pro complex when added to reaction mixtures containing the Ad DBP and uninfected nuclear extract. The Ad pol retains enzymatic activity in the absence of pTP and is absolutely required for the formation of the pTP-dCMP complex and the elongation of nascent DNA

chains. In addition, the purified pTP/Ad pol fraction had the ability, not possessed by the crude extract, to synthesize pTP-dCMP complex and short chains linked to the pTP using a variety of single-stranded templates (34).

Both E2a (encoding the Ad DBP) and E2b (encoding the pTP and Ad pol) gene regions have the same promotor, and the mRNA produced from these regions by different splicing of precursor RNA might be coordinately regulated (26,33,35). Transcription of the E2a gene is regulated by an early region Ela gene products (1,24,36), and the relative levels of E2a and E2b mRNA may also be regulated at the level of transcription termination (37). Thus, it is interesting to find all three virus encoded-proteins that are required for DNA replication to be coordinately synthesized and regulated throughout lytic infection at the level of transcription.

Two factors, purified from the uninfected nuclear extract, are also important for adenovirus DNA replication. Nuclear factor I, a 47,000-dalton protein, is absolutely required for the formation of the pTP-dCMP complex in vitro in the presence of Ad DBP, although Ad DBP is not required for the initiation reaction (21). Factor I does not display any known enzymatic activity, but it does bind to a specific DNA sequence within the Ad ITR (nucleotides 17 through 48 from the termini) (38). In the presence of pTP, Ad pol, Ad DBP and factor I elongation of DNA replication terminates randomly, approximately one third the way along the genome. Nuclear factor II, also purified from uninfected nuclear extract, facilitates the completion of DNA replication on type I replication intermediates in vitro (39). This protein contains a DNA topoisomerase I-like

activity. Furthermore, type I DNA topoisomerase purified from HeLa cells or calf thymus tissue will completely substitute for nuclear factor II complementing activity in the in vitro adenovirus DNA replication system, but bacterial topoisomerase I will not. The native molecular weight of the active protein, however, is between 25,000 and 45,000 daltons, considerably smaller than the 100,000-dalton topoisomerase I isolated from either HeLa cells (40) or calf thymus. This size discrepancy may reflect proteolysis or the presence of a different topoisomerase. It is interesting to find a requirement for a topoisomerase activity for the replication of a linear adenovirus DNA molecule.

INITIATION MECHANISM

It is well established that none of the known eukaryotic DNA polymerases (41) can initiate de novo DNA synthesis without a pre-existing primer. Several mechanisms for the priming of DNA synthesis at replication origins have been proposed to circumvent such paradoxes: for example, the priming by an RNA synthesized either by primase (42,43) or by RNA polymerase (44) and the self-priming of replication by the template DNA (45).

In the case of the linear adenovirus genome, the classic DNA priming hypotheses are not applicable, because, after removal of the putative RNA primer, the original problem of requiring a primer at the extreme 5' end still exists. In terminally redundant linear molecules, like λ and T4 phages, these difficulties can be solved by the formation of circular or concatemeric intermediates through the redundant 3' single-stranded tails. The 3'-OH ends of the growing

daughter strands provide the primers for DNA polymerase to fill in the gaps left after removal of RNA primers. However, this solution cannot be adopted by adenovirus DNA which is not terminally redundant or permuted.

A hairpin self-priming mechanism, originally suggested by Cavalier-Smith (46), hypothesizes that the presence of foldback or palindromic sequences at the respective 3' termini of the viral DNA permits the transient formation of a self-priming hairpin loop (47) to provide the 3'-OH for the elongation reaction. In fact, the sequence of the inverted terminal repetition (2-4) excludes such a priming mechanism (48), since no hairpin structure can be formed in the regions of ITRs.

Rekosh et al. (5) postulated that the terminal protein may serve as a primer for DNA replication. The idea is that after the free protein molecule binds to the molecular end of the viral DNA at the first step of initiation, it then becomes covalently bound to a deoxycytidine or dCTP residue which in turn provides the 3'-OH terminus required for the subsequent chain elongation (49).

It has been demonstrated that the 5' end of nascent adenovirus DNA strands synthesized in the in vitro system are linked to the 80K terminal protein precursor (pTP) by a phosphodiester bond between the β-OH of a serine residue in the protein and the 5'-phosphate of the terminal deoxycytidine residue in the DNA (25). Moreover, by incubating the infected nuclear extracts with $[\alpha-^{32}P]$ dCTP as the only deoxynucleoside triphosphate, the transfer of ^{32}P-dCMP to the 80K pTP can be detected (50-52). However, the transfer of ^{32}P-dCMP to pTP has at least one alternative explanation (see Figure I.2).

Figure I.2. Mechanism for ^{32}P-transfer from [α-^{32}P]dCTP to terminal protein. A. The sequence through the covalently joined Ad2 ITRs. B. Terminal protein binds at the recognition site (conserved sequence, nucleotides 9 to 17 from replication origin), and nicks between G and C on the top strand. The β-OH of a serine residue then forms a phosphodiester bond with C, and the 3'-OH of the G at the nick site serves as the primer for DNA synthesis. C. The first phosphodiester bond containing ^{32}P comes from [α-^{32}P]dCTP (heavy letter P represents α-^{32}P label). D. Radioactivity is transferred to terminal protein during subsequent cleavage. E. After DNase I digestion, ^{32}P-transfer from the DNA to the protein can be detected by gel electrophoresis. Bold face p's represent radioactive ^{32}P.
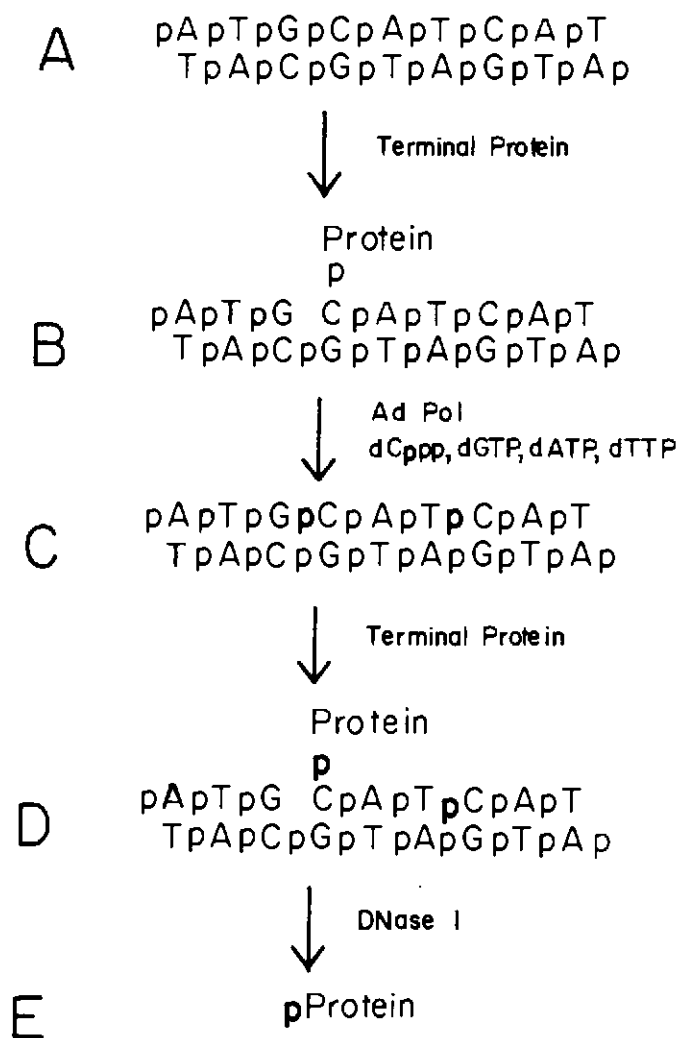
Figure I.2. Mechanism for ^{32}P-transfer from [α-^{32}P]dCTP to terminal protein.

An attractive priming mechanism is possible if adenovirus DNA is a circular, double-stranded molecule (the sequence through the replication origin is shown in Figure I.2A). Circular replicative complexes have been found in ΦX174 (53), Col El (54), SV40 (55), poliovirus RNA (56) and hepatitis-B virus DNA (57). The palindromic replication origin might facilitate the recognition of the conserved sequence (nucleotides 9 to 17) by the terminal protein as an initiation signal for the replication reaction (as shown in Figure I.2B). Terminal protein nicks between G and C at the replication origin and forms a phosphodiester bond with C through the β-OH of a serine residue. The 3'-OH of G at the nick site then serves as primer for subsequent DNA synthesis (as shown in Figure I.2C). In contrast to the examples of ΦX174, Col El and SV40 DNA, replicating adenovirus DNA, even in pulse as short as 60 seconds, has never been found as a covalently joint structure (58). The failure of finding the circular molecule could be due to the possibility that conventional methods used to isolate replicative intermediates would activate the enzyme to break such structures (7,59).

If adenovirus DNA replicates by a covalently closed circular intermediate, the essential features of such a mechanism might be: (a) The replication precursor is a covalently closed circle which contains a giant palindrome signaling for the initiation of the DNA synthesis. (b) There is a protein (perhaps terminal protein precursor) which can bind at a specific recognition site (conserved sequences within ITR). (c) Terminal protein precursor catalyzes the breakage on one strand of the DNA at a fixed distance from the recognition site (as shown in Figure I.2B). The active site on this

protein for cleavage could be different from that for recognition, or there could be two different proteins, one for recognition and one for cleavage. (d) Single strand breakage results in the covalent attachment of the protein to the 5'-phosphate, and the 3'-OH end now serves as the primer for chain elongation by a strand displacement mechanism (as shown in Figure I.2C).

The in vitro replication system (19) and plasmids with a cloned adenovirus origin were used to test these hypotheses. In the next chapter, the replication pattern and the replication origin of the XD-7 molecule which were observed by electron microscopy are discussed. XD-7 replicated as rolling circles with displaced single-stranded tails, and the origins of the tails were mapped to the boundary between the adenovirus ITR and the vector sequences. The significance of the conserved sequences (nucleotides 9 to 17) in the initiation reaction is also discussed in this chapter. Site-specific nicking within the adenovirus inverted terminal repetition, at the center of palindromic sequences and at the replication origin of adenovirus DNA are demonstrated in Chapters III and IV. In Chapter V, the effort to find the protein which is responsible for the nicking, and the ^{32}P-transfer assay for the activity of type I DNA topoisomerases in adenovirus-infected, adenovirus-transformed and uninfected cells are discussed. Chapter VI will show the attempt to clone a plasmid with palindromic sequences which contains ITRs from both termini of adenovirus DNA.

REFERENCES

1. Flint, S.J.,and Broker,T.R. (1980) Lytic infection by adenovirus. in Tooze, J. (ed.) Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratory, New York, pp.443-546.

2. Van Ormondt, H., Maat, J., and Dijkema, R. (1980) Comparison of nucleotide sequences of the early Ela regions for subgroups A, B, and C of human adenovirus. Gene 12, 63-76.

3. 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.

4. Alestrom, P., Stenlund, A., Li, P., and Pettersson, U. (1982) A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18, 193-197.

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

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

7. Robinson, A.J., Younghusband, H.B., and Bellett, A.J.D. (1973) A circular DNA-protein complex from adenoviruses. Virology 56, 54-69.

8. Desiderio, S. V., and Kelly, T. J., Jr. (1981) Structure of the linkage between adenovirus DNA and the 55,000-dalton molecular weight terminal protein. J. Mol. Biol. 145, 319-337.

9. 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.

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

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

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

13. Horwitz, M. S. (1971) Intermediates in the replication of type 2 adenovirus DNA. Virology 8, 675-683.

14. Horwitz,M.S. (1976) Bidirectional replication of adenovirus type 2 DNA. J. Virol. 18: 307-315.

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

16. Ariga, H.,and Shimojo, H. (1979) Incorporation of uracil into the growing strand of adenovirus 12 DNA. Biochem. Biophys. Commun. 87: 588-597.

17. Brison, O., Kedinger, C., and Wilhelm, J. (1977) Enzymatic properties of viral DNA replication complexes isolated from adenovirus type 2 infected HeLa cell nuclei. J. Virol. 24, 423-443.

18. Kaplan, L.M., Kleinman, R.E.,and Horwitz, M.S. (1977) Replication of adenovirus type 2 in vitro. Proc. Natl. Acad. Sci. USA 74, 4425-4429.

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

20. Challberg, M.D.,and Kelly, T.J.,Jr. (1982) Eukaryotic DNA replication: viral and plasmid model systems. Ann. Rev. Biochem. 51, 901-934.

21. Lichy, J. H., Nagata, K., Friefeld, B. R., Enomoto,T., Field, J., Guggenheimer, R.A., Ikeda, J.-E., Horwitz, M.S., and Hurwitz, J. (1983) Isolation of proteins involved in the replication of adenoviral DNA in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 731-740.

22. Kaplan, L. M., Ariga, H., Hurwitz, J., and Horwitz , M. S. (1979) Complementation of the temperature-sensitive defect in H5ts125 adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci. USA 76, 5534-5538.

23. Van Bergen, B.G.M., and van der Vliet, P.C. (1983) Temperature-sensative initiation and elongation of adenovirus DNA replication with nuclear extracts from H5ts36-, H5ts149-, and H5ts125-infected HeLa cells. J. Virol. 46: 642-648.

24. Babich, A., and Nevins, J.R. (1981) The Stability of early adenovirus mRNA is controlled by the viral 72 kd DNA-binding protein. Cell 26, 371-379.

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

26. 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.

27. Enomoto, T., Lichy, J. H., Ikeda, J.-E., and Hurtwitz, J. (1981) Adenovirus DNA replication in vitro: Purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. USA 78, 6779-6783.

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

29. 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.

30. 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.

31. Stillman, B.W.,and Tamanoi, F. (1983) Adenoviral DNA replication: DNA sequences and enzymes required for initiation in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 741-750.

32. Lichy, J. H., Field, J., Horwitz, M. S., and Hurwitz, J. (1982) Seperation of the adenovirus terminal protein precursor from its associated DNA polymerase; role of both protein in the initiation of adenovirus DNA replication. Proc. Natl. Acad. Sci. USA 79, 5225-5229.

33. Stillman, B.W., Tamanoi, F.,and Mathews, M.B. (1982) Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. Cell 31, 613-623.

34. Ikeda, J. -E., Enomoto, T., and Hurwitz, J. (1982) Adenoviral protein-primed initiation of DNA chains in vitro. Proc. Natl. Acad. Sci. USA 79, 2442-2446.

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

36. Berk, A.J., Lee, F., Harrison, T., Williams, J., and Sharp, P.A. (1979) Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNA. Cell 17, 935-944.

37. Stillman, B. W. (1983) The replication of adenovirus DNA with purified protein. Cell 35, 7-9.

38. Nagata, K., Guggenheimer, R.A., and Hurwitz, J. (1983) Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80, 6117-6181.

39. 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.

40. Liu, L.F., and Miller, K.G. (1981) Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa nuclei. Proc. Natl. Acad. Sci. USA 78, 3487-3491.

41. Weissbach,A. (1975) Vertebrate DNA polymerases. Cell 5, 101-108.

42. Stayton, M. M., Bertsch, L., Biswas, S., Dixon, N., Flynn, J. E., Fuller, R., Kaguni, J., Kobori, J., Kodaira, M., Low, R., and Kornberg, A. (1983) Enzymatic recognition of DNA replication origins. Cold Spring Harbor Symp. Quant. Biol. 47, 693-700.

43. Brown, D. R., Reinberg, D., Schmidt-Glenewinkel, T., Roth, M., Zipursky, S.L., and Hurwitz, J. (1983) DNA structures required for ϕX 174 protein-directed initiation and termination of DNA replication. Cold Spring Harbor Symp. Quant. Biol. 47, 701-715.

44. Fuller,C.W., Beauchamp, B.B., Engler,M.J., Lechner, R.L., Matson, S.W., Tabor, S., White, J.H., and Richardson, C.C. (1983) Mechanisms for the initiation of bacteriophage T7 DNA replication. Cold Spring Harbor Symp. Quant. Biol. 47, 669-679.

45. Astell, C. R., Thomson, M., Chow, M. B., and Ward, D. C. (1983) Structure and replication of minute DNA of mice DNA. Cold Spring Harbor Symp. Quant. Biol. 47, 751-762.

46. Cavalier-Smith, T. (1974) Palindromic base sequences and replication of eukaryotic chromosome ends. Nature 250, 467-470.

47. Wu, M., Roberts, R.J., and Davidson, N. (1977) Structure of the inverted terminal repetition of adenovirus type 2 DNA. J. Virol. 21, 766-777.

48. Sussenbach, J.S., and Kuijk, M.G. (1978) Initiation of adenovirus DNA replication does not occur via a hairpin mechanism. Nucl. Acids Res. 5, 1289-1295.

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

50. Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981) Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP in vitro. Proc. Natl. Acad. Sci. USA 78, 2678-2682.

51. Challberg, M. D., Ostrove, J. M., and Kelly, T. J., Jr. (1982) Initiation of adenovirus DNA replication: detection of covalent complexes between nucleotide and the 80 kd terminal protein. J.

Virol. 41, 265-270.

52. Pincus, S., Robertson, W., and Rekosh, D. (1981) Characterization of the effect of aphidicolin on adenovirus DNA replication: evidance in support of a protein primer model of initiation. Nucl. Acids Res. 9, 4919-4937.

53. Langeveld, S. A., van Mansfeld, A.D.M., Baas, P. D., Jansz,H. S., van Arkel, G.A., and Weisbeek, P.J. (1978) Nucleotide sequence of the origin of replication in bacteriophage ΦX174 RF DNA. Nature 271,417-420.

54. Tomizawa, J., Ohmori, H., and Bird, R.E. (1977) Origin of replication of colicin E1 plasmid DNA. Proc. Natl. Acad. Sci. USA 74, 1865-1869.

55. Kasamatsu, H.,and Wu, M. (1976) Structure of a nicked DNA-protein complex isolated from simian virus 40: covalent attachment of the protein to DNA and nick specificity. Proc. Natl. Acad. Sci. USA 73, 1945-1949.

56. Wu, M., Davidson, N.,and Wimmer,E. (1978) An electron micrposcope study of the proteins attached to polio virus RNA and its replicative form. Nucl. Acids Res. 5, 4711-4723.

57. Summers, J., and Mason, W.S. (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29, 403-415.

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

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

CHAPTER II

IN VITRO REPLICATION OF
A CLONED ADENOVIRUS ORIGIN
STUDIED BY ELECTRON MICROSCOPY

ABSTRACT

XD-7, a 5.7-kb recombinant plasmid, contains the left terminal XbaI-E fragment from adenovirus type 2 (Ad2) DNA inserted into the EcoRI site of pBR322. An average of 9% of input XD-7 DNA replicated as rolling circles with displaced single-stranded tails in the in vitro replication system. The replication origin was mapped on XD-7 DNA to the left boundary of the cloned adenovirus DNA segment. An internally standardized assay was also developed to study the function of the cloned adenovirus origin. The first 20 nucleotides of the Ad2 inverted terminal repetition (ITR), a region containing a sequence conserved among human, murine, simian and avian adenoviruses, were found to be essential for the initiation of adenovirus DNA replication. Deletions removing or penetrating from either direction into the conserved sequence inactivated replication. A point mutation within the conserved sequence diminished the replication frequency, but point mutations outside the conserved sequence had no effect. The conserved sequence within the first 20 nucleotides thus constitutes the required signal for the initiation of adenovirus DNA replication.

INTRODUCTION

In vitro systems are necessary to dissect mechanisms of DNA replication (1,2,3). Several aspects of DNA synthesis can be studied in detail using these system: (a) the biochemical processes involved in the initiation, elongation and termination of DNA chains; (b) the protein(s) required for these processes; and (c) the DNA sequence(s) or DNA structure(s) essential for these processes.

In this chapter, the template requirements for the in vitro adenovirus replication system (4,5) have been investigated by using cloned terminal sequences of adenovirus genome (6). The cloned Ad2 origin, inserted into pBR322 or pUC9 vectors, was efficiently recognized in reaction mixtures containing extracts from adenovirus-infected, but not uninfected, HeLa cells. Input supercoiled, protein-free molecules replicated as rolling circles with displaced single-stranded tails. The origin of the displaced tails was mapped on XD-7 DNA by electron microscopy to the boundary of the left terminus of the Ad2 insert. Nucleotide sequence analysis has shown that within the ITR there is a sequence, nucleotides 9 to 17 from both ends, conserved among human (7), simian (8), murine (9) and avian (10) adenoviruses. It is tempting to speculate that this sequence plays a role in the initiation of adenovirus DNA replication. Deletions and point mutations within or adjacent to this conserved block were thus constructed, and an internally standardized, quantitative assay was developed to study the effect of these mutations on the function of the putative adenovirus replication origin.

MATERIALS AND METHODS

Equipment and Reagent Treatment.

All glassware, Eppendorf tubes and pipette tips employed in the following procedures were autoclaved at 121°C for 30 minutes. Phenol was distilled and equilibrated with TE buffer (10 mM Tris-base, pH 7.9, 1 mM Na$_2$EDTA). Where appropriate, reagents and buffers were filtered through either 0.45 μm Acrodisc (Gelman Science, Inc.) or Nalgene filter units (Sybron/Nagel) or autoclaved for 30 minutes at 121°C.

Preparation of substrate DNAs.

A. Plasmid: E. coli C600 or JM83 was grown, transformed and selected by standard techniques (11). Where appropriate, selection was in the presence of ampicillin (50 μg/ml) or tetracycline (15 μg/ml) or both. Prior to plasmid DNA isolation, plasmid DNA was amplified by growing cells in 150 μg/ml chloramphenicol. Plasmid DNA was purified by either ethidium bromide-CsCl density gradient centrifugation or ethidium bromide-agarose electrophoresis. XD-7, a pBR322 derivative which has the 1342 bp adenovirus XbaI-E fragment inserted into EcoRI site of pBR322 (as shown in Figure II.1.), was provided by Dr. J. L. Corden. All the deletion and point mutants were derived from this parental molecule (see below).

B. M13 phage: JM101 or JM103 was grown, infected (or transfected) and selected by standard procedures (BRL user manual). The 1.35-kb EcoRI fragment from XD-7 was cloned into the EcoRI site of M13mp9 in both orientations. The M13 "+" strand which has the Ad2 l-strand insert is named mKC96. The M13 "+" strand which contains the r-strand

insert is named mKC93. Likewise, the HindIII-SmaI fragment from XD-7,
and HindIII-PstI and HindIII-BamHI fragments from deletion mutants
were subcloned into M13mp8.

Deletion and point mutations

Deletion and point mutants were provided by Dr. M.D. Challberg.
The procedure to construct these mutants will be described briefly
below:

A. Deletions were generated by BAL31 digestion of linearized DNA.
To remove approximately 5 base pairs per minute, linearized DNA
(5 μg) was incubated at 30°C with 0.05 unit of BAL31 nuclease in
150 μl of buffer containing 20 mM Tris-HCl (pH 8.1), 600 mM NaCl,
12 mM $CaCl_2$, 12 mM $MgCl_2$, and 1 mM Na_2EDTA (12). The reaction was
stopped by phenol extraction. BAL31-digested DNA was recircularized
by incubating at 20°C for one hour with 0.1 unit of T4 DNA ligase in
20 μl of buffer containing 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$,
10 mM DTT, and 0.5 mM ATP. XD-7 was digested with HincII and SmaI and
recircularized by blunt-end ligation to remove the EcoRI site at the
right end of the Ad2 insert. A 5.1-kilobase (kb) plasmid, named
pMDC5, was isolated by tetracycline selection. pMDC5 was modified by
inserting a 10-basepair (bp) BamHI linker at the SstII site to form
pMDC7. Deletion mutants were constructed by trimming sequences
unidirectionally from the SstII site within the cloned adenovirus DNA
segment of pMDC7 (Figure II.3) to penetrate variable distances toward
the inverted terminal repetition (ITR). The BAL31-trimmed molecules
were ligated to BamHI linkers, cleaved with BamHI, and fractionated
by gel electrophoresis. Fragments ranging in size from 375 bp to

450 bp were recovered from the gel and cloned into BamHI-cut pMDC7. Unidirectional deletion mutants, identified by DNA sequence analysis, were named according to the number of Ad2 terminal sequences that remained; that is, dl30 retains nucleotides 1 through 30 of Ad2 terminal sequences linked by a BamHI linker to Ad2 nucleotide 358. Each of the deletion mutants was roughly 4.7 kb, resistant to tetracycline only, and has a single EcoRI and a single HindIII site.

B. C to T transitions within adenovirus terminal sequences were constructed by directed mutagenesis with sodium bisulfite (see below). Plasmid pMDC7 was linearized at the HindIII site, 31 base pairs away from the EcoRI site at the junction between Ad2 and pBR322 sequences. After exonucleaseIII (exoIII) treatment (13), the resulting DNA was exposed to sodium bisufite under conditions to deaminate 10% of the deoxycytidine residues in single-stranded DNA: 3 M sodium bisulfite for 1 hour at 37°C. The DNA was then concentrated by ethanol precipitation and repaired with DNA polymerase I from Micrococcus luteus in a reaction mixture (100 μl) consisting 1 μg DNA, 70 mM Tris-HCl (pH8.0), 7 mM $MgCl_2$, 100 μM each of the four dNTPs, and 10 units of M. luteus DNA polymerase. The small EcoRI-BamHI fragment containing viral terminal sequences was then cloned into pUC9 (14). Plasmid DNA from individual colony isolates was screened for point mutations near the viral terminus by DNA sequencing. The wild type EcoRI-BamHI fragment from pMDC7 was also inserted into pUC9, creating plasmid pMDC10, which is ampicillin resistant but defective for β-galactosidase complementation. The designation pm n refers to a point mutant with a C to T transition at the n-th nucleotide of the adenovirus terminal sequence.

Sodium bisulfite reactions (15). A solution of 4 M bisulfite (pH 6.0) was prepared immediately prior to use by dissolving 156 mg of $NaHSO_3$ and 64 mg of Na_2SO_3 in 0.43 ml of deionized water. The final incubation mixture contained 3 volumes of this solution, 1 volume of DNA solution (approximately 50 µg/ml in 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), and 0.04 volume of 50 mM hydroquinone, with all components mixed at 0°C. The reaction mixture was sealed and incubated in the dark at 37°C. The reaction was terminated by dialyzing the reaction mixture against the following buffers: (a) 1000 volumes of 5 mM potassium phosphate (pH 6.8), 0.5 mM hydroquinone at 0°C for two hours; (b) repeat of (a); (c) 1000 volumes of 5 mM potassium phosphate (pH 6.8) at 0°C for 4 hours; (d) 1000 volumes of 0.2 M Tris-HCl (pH 9.2), 50 mM NaCl, 2 mM EDTA at 37°C for 16 to 24 hours; (e) 1000 volumes of 2 mM Tris-HCl (pH 8.0), 2 mM NaCl, 0.2 mM EDTA at 4°C for 6 to 12 hours. The water used to prepare the first four dialysis buffers was degassed by vigorous boiling prior to use.

DNA sequencing

Three methods were used for DNA sequence determinations.

A. Dideoxynucleotide chain-terminator method (16). Primers were 24-bases (BRL) or 15-bases (P.L. Biochemicals) long. Primer was hybridized to the M13 single-stranded template. $[\alpha-^{32}P]dATP$ (800 Ci/mmol, New England Nuclear), Klenow fragment of DNA polymerase I, and dideoxy-terminators were reacted to determine the sequence of the DNA insert.

B. ExoIII-dideoxy, second enzyme method (13). This method was

used for deletion mutants only. Plasmid DNA was linearized with PvuII and digested with exonuclease III (exoIII) from E. coli in the presence of 90 mM NaCl for 20 min. The digestion with exoIII gave a family of DNA molecules with 3'-hydroxyl ends shortened to variable distances. These 3'-hydroxyl ends serve as primers in the subsequent reaction. The exoIII treated DNA was subjected to dideoxy-terminator sequencing reactions for 30 min, and then digested completely with HindIII restriction enzyme. After fractionation on a denaturing polyacrylamide gel, the DNA sequences of the shorter fragments were well resolved, and the longer fragments stay at the top of the gel. The short PvuII fragment within Ad2 insert was completely digested by exoIII. It did not interfere with the reading of sequencing results.

C. ExoIII-labeled primer-dideoxy method. This method was used for most point mutants. The synthetic 15-base primer was 5'-end labeled by incubating with polynucleotide kinase in the buffer containing 100 μM [α-^{32}P]ATP (2,500-6000 Ci/mmol, ICN), 70 mM Tris-HCl (pH 7.6), 10 mM MgCl$_2$, and 5 mM DTT at 37°C for one hour, and hybridized to the plasmid DNA which had been linearized with HindIII and digested with exoIII to expose the area to be sequenced. The DNA sequence was determined with dideoxy-terminators and unlabeled dNTPs.

Gel electrophoresis was at 1000 to 1400 volts on 8% (weight/volume) polyacrylamide gels (19:1, acrylamide:bisacrylamide) containing 8 M urea, 100 mM Tris-borate (pH 8.3) and 2 mM Na$_2$EDTA. Gel dimensions were 25 cm wide X 35 cm long X 0.3 mm thick. The running buffer contained 50 mM Tris-borate (pH 8.3), 1 mM EDTA and 8 M urea. After electrophoresis, the gel was dried and autoradiographed.

Cells and Virus

HeLa S3 cells were grown at 37°C in suspension culture in Eagle's minimal essential medium supplemented with 7% fetal bovine serum (volume:volume) (Sterile System ur Grand Island Biological Co.). Adenovirus type 5 (Ad 5) was obtained from the American Type Culture Collection.

Preparation of Nuclear and Cytoplasmic Extract

One liter of cells at density of 4-6 X 10^5 cells/ml was infected with adenovirus type 5 at a multiplicity of 10 to 50 plaque forming units per cell. At two hours after infection, hydroxyurea was added to the suspension culture at a final concentration of 10 mM. At 18 hours after infection, the cells were collected by centrifugation at 3,000 X g for 5 min and washed once with 20 ml of cold hypotonic buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol) containing 0.2 M sucrose. The cells were resuspended in 5 ml of cold hypotonic buffer without sucrose, allowed to swell for 10 min on ice, and then lysed by 10 strokes of a type A Dounce homogenizer (Glenco). The resulting lysate was centrifuged at 2,000 X g for 5 min. The nuclear pellet was resuspended in 2.5 ml of 50 mM Hepes, pH 7.5, 10% sucrose and frozen in 200 µl aliquots in liquid nitrogen. The supernatant was clarified by centrifugation at 25,000 X g for 20 min at 4°C and the resulting cytoplasmic extract (cytosol) was frozen in liquid nitrogen. The protein concentration of the cytoplasmic extract from both Ad5-infected and uninfected HeLa cells was about 15 mg/ml as determined by the method of Lowry (17).

Uninfected HeLa S3 cells were treated with 10 mM hydroxyurea and processed in an identical fashion. All fractions were stored at -63°C.

Preparation of Nuclear Extract

An aliquot (200 μl) of frozen nuclei was allowed to thaw on ice. After addition of 6 μl of 5 M NaCl, the suspension was incubated on ice for one hour. During the procedure, the nuclei maintained typical morphology (as determined by light microscopy) and the suspension was nonviscous. The suspension was then centrifuged at 15,000 x g for 20 min at 4°C. The clear supernatant was removed with a micro-pipette and stored on ice. The protein concentration of the nuclear extract from both Ad5-infected and uninfected HeLa cells was 4-6 mg/ml.

Conditions for Cell-free DNA Synthesis

The standard reaction mixture for cell-free DNA synthesis contained 50 mM Hepes-NaOH (pH 7.5); 5 mM $MgCl_2$; 0.5 mM dithiothreitol; 50 μM each dATP, dCTP, dGTP and dTTP; 4 mM ATP (Sigma Chemical Co.); 150 ng DNA; 10 μl of nuclear extract and 10 μl of cytosol in a total volume of 50 μl. Incubations were carried out at 37°C for 30 min. To isolate the product of the reaction following incubation, Na_2EDTA was added to a concentration of 10 mM, the reaction mixture was incubated with 0.1 mg/ml pancreatic ribonuclease XA (Sigma Chemical Co.) at 37°C for 10 min, and then 1 mg/ml Pronase (Grade B, Calbiochem) was added in the presence of 0.5% sodium dodecyl sulfate (SDS) for further digestion at 37°C for 30 min. The resulting solution was extracted twice with one half volume of phenol

equilibrated with TE buffer. The DNA was precipitated with two volumes of 95% ethanol at -20°C for 30 min, and resuspended in 10 µl TE buffer.

Electron Microscopy

Formamide Technique

In order to distinguish the displaced single-stranded DNA from the parental double-stranded DNA, the formamide monolayer technique (18,19) was used to mount DNA samples. The hyperphase consisted of 5 µl of the DNA sample (concentration 5-15 µg/ml), 5 µl of cytochrome $\underline{C}$ solution (1 mg/ml in 1 M Tris, pH 8.5, 0.1 M Na_3EDTA), 20 µl of double-distilled, deionized water and 20 µl of formamide (Bethesda Research Lab. or MCB). Half of the 50 µl hyperphase was spread onto a 27 ml hypophase (double-distilled water) in a 6 cm Petri dish (Falcon). One to two minutes after spreading, the DNA-protein film was adsorbed to a parlodion-coated 200-mesh copper grid. The samples were stained for 30 seconds with uranyl acetate (50 mM in 90% ethanol, 50 mM HCl), washed twice by immersion in 90% ethanol for 5 seconds, air dried and then rotary-shadowed with platinum-pallad-ium. In general, with the formamide technique, staining alone does not give adequate contrast except for dark field electron microscopy. However, the contrast for light field electron microscopy is adequate if the DNA is just shadowed. Specimens were examined with a Zeiss 10A electron microscope operating at accelerating voltage of 60,000. Most of the grids were scored using a double-blind protocol. Micrographs were taken at an instrumental magnification of 6,000 times on 35 mm film (Eastman 5302). Molecular lengths were measured by a

calculator-driven digitizer on photographic prints enlarged to a final magnification of 60,000 times. Single-stranded lengths were converted to double-stranded lengths using the correction factor of 1.20. This correction factor is obtained from measurement of heteroduplexes (see below).

Reagents and Grids

Cytochrome C (Calbiochem, equine heart, salt free, Grade A) was dissolved into double-distilled water at a concentration of 20 mg/ml. The solution was allowed to stand at 4°C for five days to one week and then filtered through an Acrodisc (0.45 μm) that was washed extensively with distilled water. The concentration of the stock solution was measured spectrophotometrically where the absorbance at 410 nm of a 1 mg/ml solution is about 10. This solution was then diluted with 2 M Tris-HCl (pH 8.5) and 0 2 M Na_3EDTA to make a solution containing 1 mg/ml cytochrome C, 1 M Tris-HCl (pH 8.5) and 0.1 M Na_3EDTA.

Drying of solid parlodion strips (Pellco) was accomplished in a vacuum desiccator at 25°C for several days. The dried parlodion was then dissolved into amyl acetate (J. T. Baker Chemical Co. or Pellco) to make a 3.5% (w/v) parlodion solution which was stored over molecular sieve. It usually took several days to dissolve completely. Parlodion-coated grids were prepared as follows: A wire screen was placed in a shallow container (e.g. Petri dish). The container was filled with distilled water above the level of the screen. Copper grids (200 mesh, Pellco) were cleaned free of dirt and grease with acetone, air dried and placed below the surface of water onto the

wire screen. One drop of the parlodion solution was used to clean the surface of the water; after the amyl acetate had evaporated, the parlodion film was stripped off to remove dirt. Another drop of parlodion solution was applied to the surface, and after one minute or after the surface becomes a shining silver color, the wire screen was lifted, allowed to dry in vacuum or in air 2 hours before use. Support films prepared in this way are fairly strong and generally withstand the electron beam at crossover without a carbon coating.

Preparation of heteroduplex

A heteroduplex molecule is constructed by annealing together two related yet different single strands of DNA to form a duplex structure. Complementary single strands form a double-stranded region and unpaired single strands remain single-stranded. By visualizing the molecule in the electron microscope under conditions that allow discrimination between double- and single-stranded DNA, the region(s) of base complementarity and noncomplementarity can be identified, measured, and mapped. Experimentally, heteroduplex molecules are constructed by mixing together the two DNA molecules, denaturing them, and then allowing them to renature. The reaction mixture will consist not only of heteroduplex molecules, but also of renatured parent homoduplex molecules and unrenatured single-stranded molecules. All of these molecules are easily distinguishable. In the formamide monolayer technique, renaturation of single-stranded DNAs is usually done in 50% formamide at room temperature (20). The working procedure is as follows: Restriction endonuclease-linearized DNA molecules (0.25 μg of each DNA) in 40 μl of 25 mM Na_4EDTA (made

by adding 2 moles of NaOH to one mole Na_2EDTA) are denatured by the addition of 5 µl of 1 N NaOH. After 10 min at room temperature, the solution is neutralized by the addition of 5 µl of 1.8 M Tris-HCl, 0.2 M Tris-base (pH 8.5). Renaturation is accomplished by adding 50 µl formamide at room temperature for one hour. The DNA/formamide solution is cooled on ice and immediately mounted for electron microscopy as described above.

RESULTS

Construction of plasmids.

XD-7 DNA was provided by Dr. J. L. Corden. XD-7 was constructed as follows. The XbaI-E fragment (1342 bp; map position 0-3.85) from the left terminus of proteinase K-digested Ad2 DNA was repaired with DNA polymerase I and cloned into the DNA polymerase I-repaired EcoRI site of pBR322 by blunt end ligation with T4 DNA ligase. XD-7 is a 5.7-kb plasmid, carries markers for resistance to ampicillin and tetracycline, and releases a 1.35 kb fragment when cleaved with EcoRI. Figure II.1 gives the structure, restriction endonuclease maps, and DNA sequences at the junctions between pBR322 and the Ad2 insert. The sequences at both junctions were further verified by cloning the Ad2 insert into the EcoRI site of M13mp9 in both orientations, and sequencing by dideoxynucleotide chain termination method. The results are presented in Figure II.2. A 1-kb HindIII-SmaI fragment of XD-7 was also subcloned into the HindIII/SmaI site of M13mp8 to determine the orientation of the Ad2 fragment with respect to pBR322 sequences in XD-7 (shown in Figure II.4I).
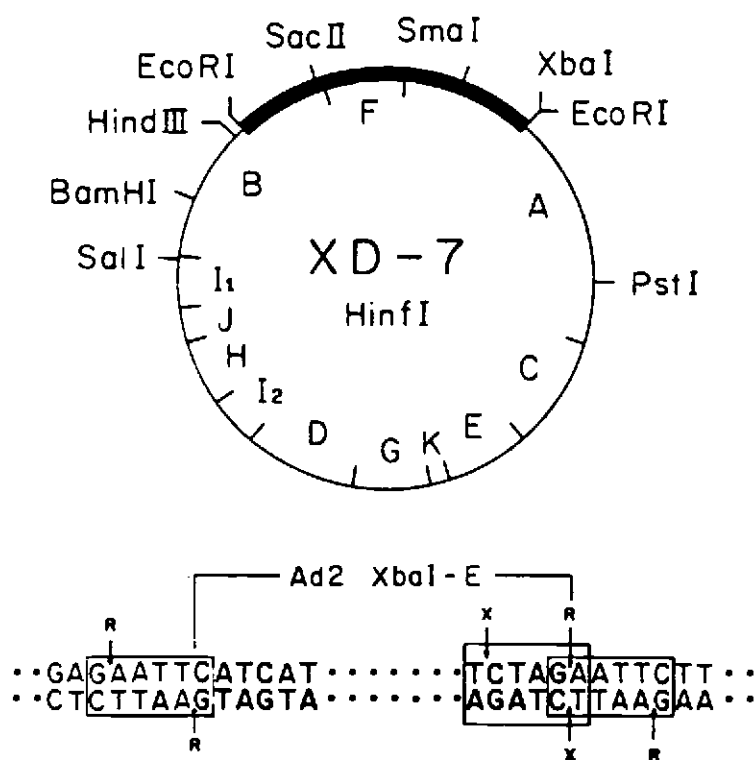
Figure II.1. Restriction endonuclease maps of XD-7 DNA. The thick segment represents the entire XbaI-E fragment from the left terminus of Ad2 DNA inserted into the EcoRI site of pBR322. Some single-cut endonuclease sites are located around the outside of the circular map. HinfI cleavage sites are indicated around the inside of the map, marking fragments A through K. DNA sequences at the junctions between pBR322 DNA and the inserted XbaI-E fragment of Ad2 DNA in XD-7 are presented below the map. Ad2 DNA sequences are indicated by boldface letters and pBR322 DNA sequences by regular letters. The boxes enclose the restriction edonuclease recognition sequences for EcoRI and XbaI. R = cleavage sites for EcoRI. X = cleavage sites for XbaI. The top strand is the l-strand of adenovirus DNA and the bottom strand is the r-strand.

Figure II.2. Sequence analysis of both ends of the Ad2 XbaI-E fragment contained in XD-7. The 1.35-kb EcoRI fragment from XD-7 was subcloned in both orientations into the EcoRI site of M13mp9. Orientation A gives the sequence of the Ad2 l-strand at the left terminus of the Ad2 genome (mKC93). The dot indicates the 5'-C residue to which terminal protein is covalently attached in the mature adenovirus chromosome. Orientation B gives the sequence of the Ad2 r-strand starting at the Ad2 XbaI site (mKC96).
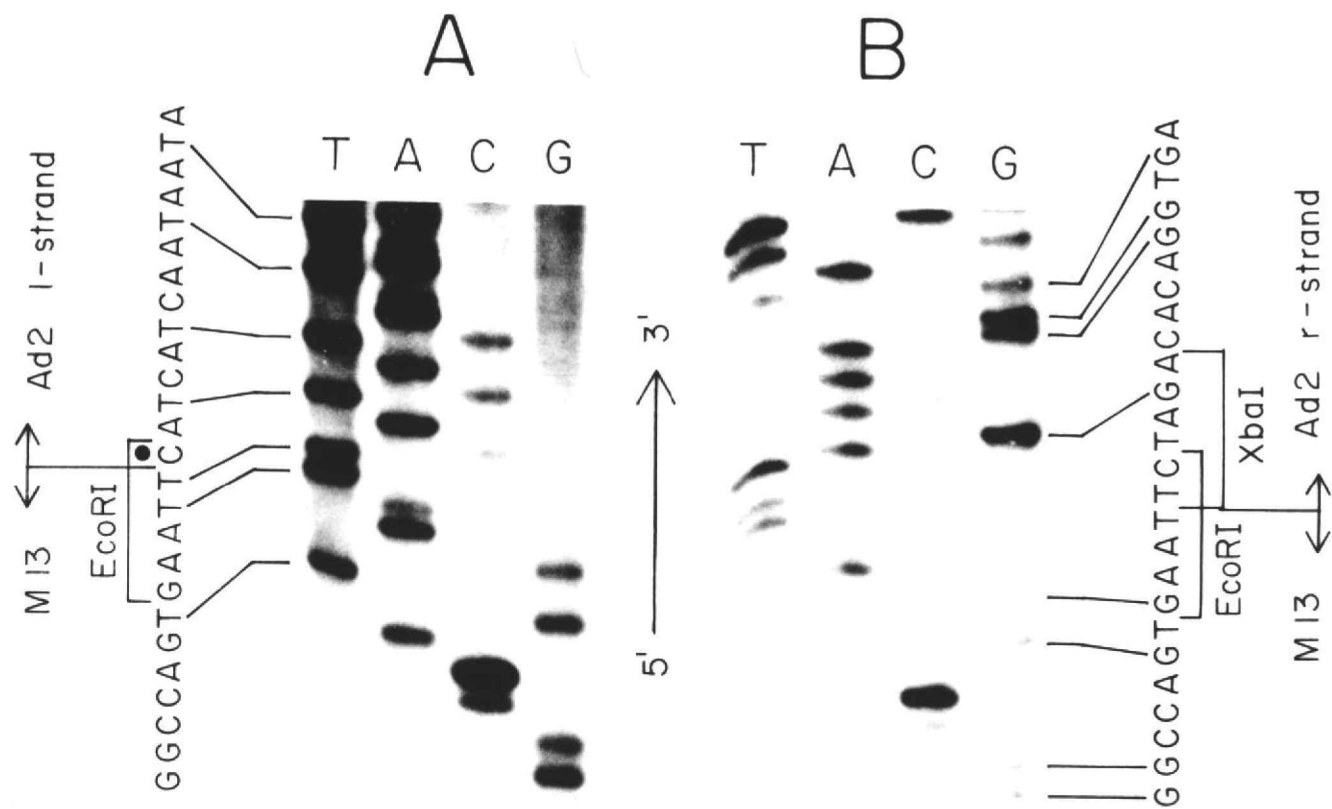
Figure II.2. Sequence analysis of both ends of the Ad2 XbaI-E fragment contained in XD-7.
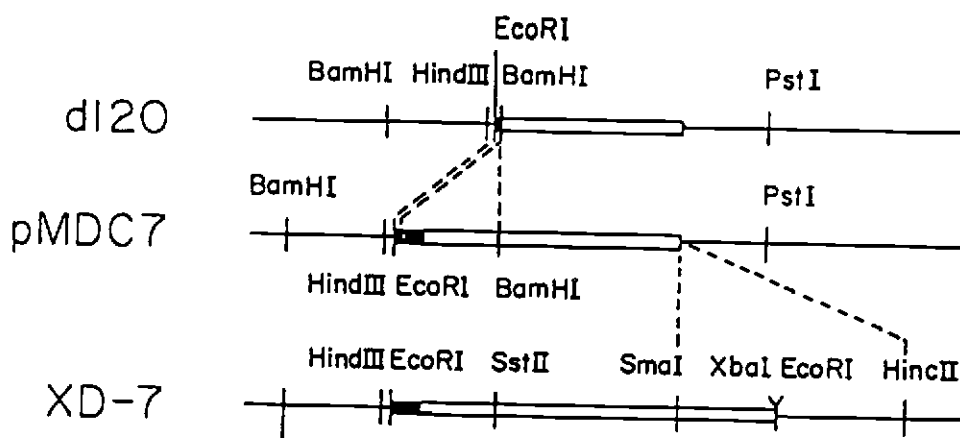
Figure II.3. Exonucleolytically constructed deletion mutants of XD-7. Open bar, Ad2 sequences; solid bar, Ad2 inverted terminal repetition; thin line, pBR322 sequences; dashed lines, extent of deletions. Only the region between BamHI and PstI is shown. pMDC7 has a BamHI linker at the SstII site. dl20 has only the first 20 bp of the adenovirus inverted terminal repetition.

Figure II.4. Autoradiogram of sequencing gels of mKM4 and deletion mutants. I. Sequencing of mKM4 by dideoxynucleotide chain-termination method. The 1-kb HindIII-SmaI fragment from XD-7 which contains the Ad2 ITR sequence was cloned into M13mp8 cut with HindIII and SmaI (mKM4). The restriction site is labelled by an asterisk in the middle of the sequence. mKM4 was used as internal marker for deletion mutants. II. Sequence of deletion mutant dl30 DNA. III. Sequence of deletion mutant dl18. The number represents the number of nucleotides remaining from the Ad2 ITR. The thin letters represent the adenovirus DNA sequence. The BamHI linker regions are bracketed.

Figure II.4. Autoradiogram of sequencing gels of mKW4 and deletion mutants.
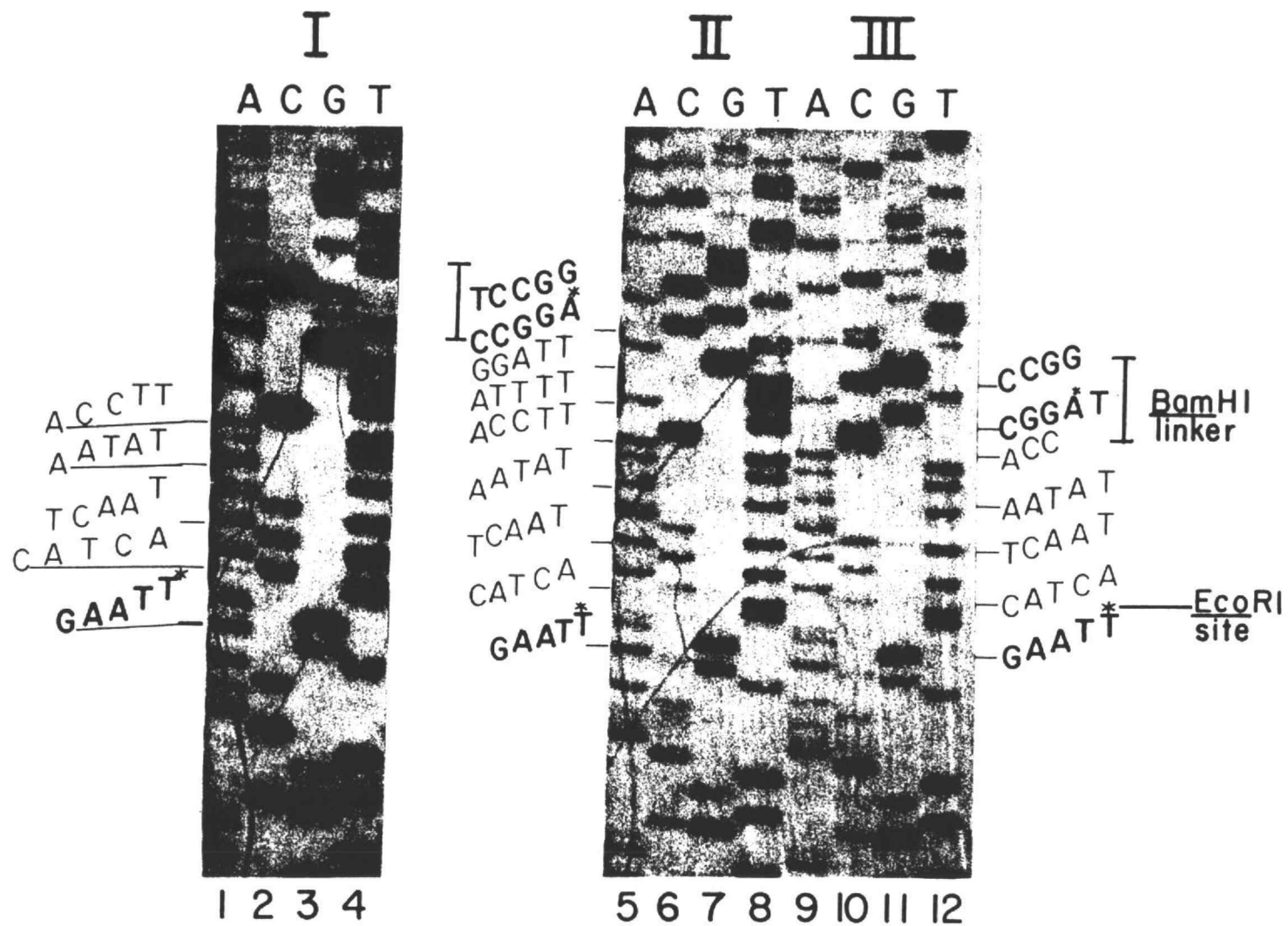
A collection of deletion and point mutants within the adenovirus inverted terminal repetition were constructed starting within XD-7 (see MATERIALS AND METHODS). Each mutant was characterized by DNA sequence analysis. HindIII-PstI fragments from some deletion mutants were inserted into M13mp8. If the HindIII-PstI fragment was from dl30 plasmid, and the phage vector was M13mp8, then the recombinant is named mKC8dl30HP. The sequences were determined by the dideoxynucleotide chain termination method. The results are presented in Figure II.4II and III. The sequences of point mutants were determined by the exoIII-labeled primer-dideoxy method (as shown in Figure II.5). The sequences of deletion and point mutations within or adjacent to the conserved sequences are listed in Figure II.6. The mutated sequences are indicated by regular letters, and the Ad2 sequences 1 to 22 on XD-7 are indicated by boldface letters. Clones 16Z, ZRI and 28 were provided by Dr. R.E. Enns of this laboratory. Clone 16Z has a 26-bp deletion removing the first 14-bp of the adenovirus ITR with tandem EcoRI linkers at the site of the deletion. Clone ZRI, like clone 16Z, has a 14-bp deletion from the left terminus of Ad2 DNA, but with only one EcoRI linker and restored pBR322 sequences. Clone 28 has a 38-bp deletion removing nucleotides 1 through 15 from the Ad2 ITR.

Rolling circles as replicative intermediates.

Reaction mixtures containing XD-7 DNA and nuclear and cytoplasmic extracts from adenovirus-infected HeLa cells were examined by electron microscopy after incubation under standard conditions. In 598 molecules scored, 91% were supercoiled monomers and 9% were rolling circles with displaced single-stranded tails (as shown in
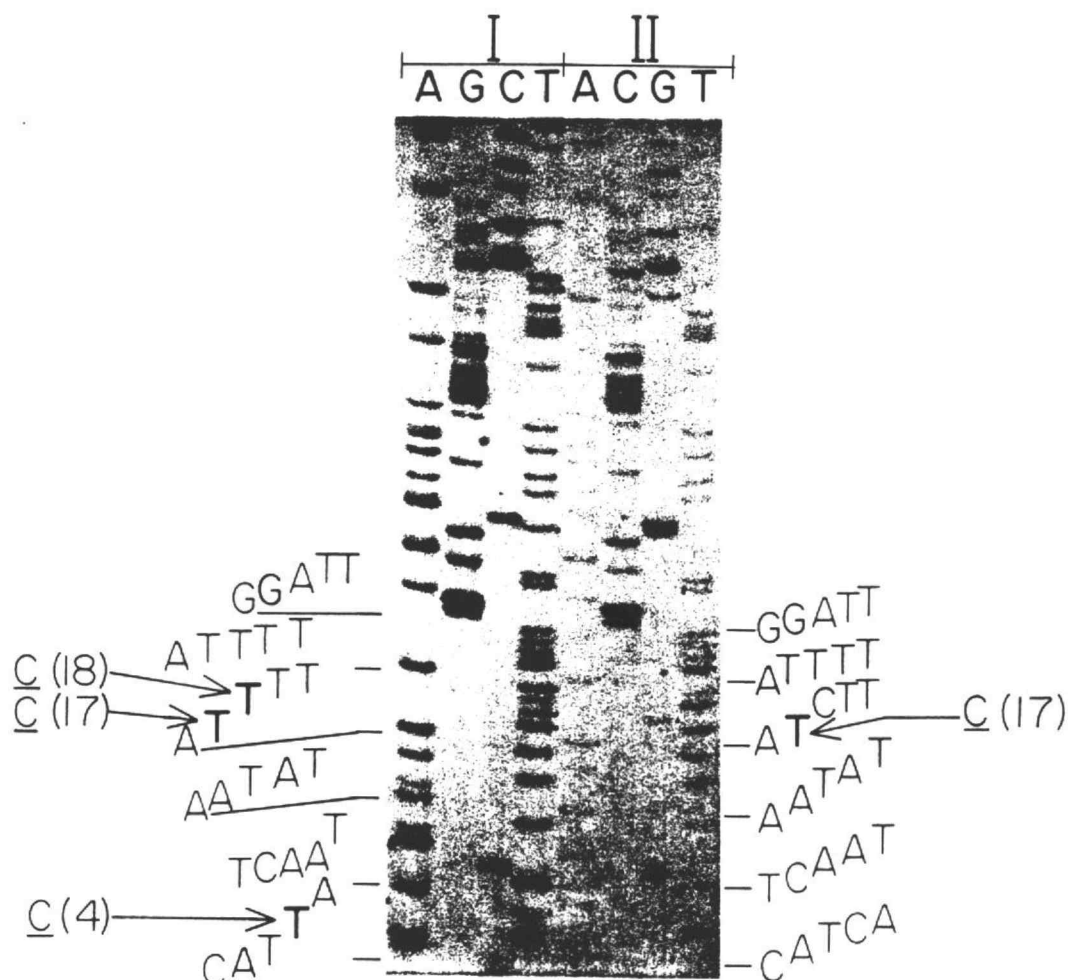
Figure II.5. Sequencing of point mutants by the ^{32}P-labeled primer-dideoxy-terminator method. I. Sequence of point mutant pm4-17-18. II. Sequence of point mutant pm17. The heavy letters represent the point substitution from C to T. The letters underlined represent the original nucleotides. The numbers bracketed represent the nucleotide numbers from the left end of the Ad2 sequence.

CONSERVED

	1	10	20
XD-7		CATCATCAATAATATACCTTAT	
dl 7		CATCATCCCGGATCCGGGGGGA	
dl 12		CATCATCAATAACCGGATCCGG	
dl 18		CATCATCAATAATATACCCCGG	
dl 20		CATCATCAATAATATACCTTCC	
Clone 16Z		AATTCCGGAATTCCTACCTTAT	
Clone ZRI		AACATGAGAATTCCTACCTTAT	
Clone 28		TAAAGCTTATCGATGACCTTAT	
pm 4		CATTATCAATAATATACCTTAT	
pm 18		CATCATCAATAATATACTTTAT	
pm 4-18		CATTATCAATAATATACTTTAT	
pm 7-18		CATCATTAATAATATACTTTAT	
pm 17		CATCATCAATAATATATCTTAT	
pm 17-18		CATCATCAATAATATATTTTAT	
pm 4-17-18		CATTATCAATAATATATTTTAT	

Figure II.6. Nucleotide sequences of deletion and point mutants defining the adenovirus origin. Adenovirus sequences are indicated by boldface letters and mutated sequences by regular letters. The horizontal bracket identifies nucleotides conserved between human, simian, murine, and avian adenovirus.

Figure II.7, A-D). No single-stranded circles were detected. Length measurements on XD-7 rolling circles showed that single-stranded tails ranged from 0.02 unit length to 1.10 unit length. No rolling circles or single-stranded DNA were observed after XD-7 was incubated with nuclear and cytoplasmic extracts from uninfected HeLa cells under otherwise identical conditions. Table II.1 shows that the percentage of XD-7 molecules found as rolling circles was reproducible over several experiments using independently prepared extracts. The percentage of molecules scored as rolling circles was 9% ± 1% of input XD-7 DNA. Reaction mixtures containing pBR322 DNA and nuclear and cytoplasmic extracts from Ad2-infected cells were also analyzed after incubation under the standard conditions. Roughly 1% of the input pBR322 molecules appeared as rolling circles (as shown in Table II.1).

Mapping of the replication origin on XD-7 DNA.

Four origins for the single-stranded tails were located on XD-7 molecules by length measurements with respect to the SalI cleavage site. After incubation in the standard reaction mixture, XD-7 DNA was cleaved with SalI and examined by electron microscopy. Inspection of between 500 to 1000 molecules revealed 44 branched molecules (roughly the frequency of rolling circles summarized in Table II.1), each containing two double-stranded arms and a single-stranded arm. The total length of the double-stranded arms in each branched molecule equaled the length of XD-7 DNA. Single-stranded arms ranged from 0.02 unit length to 1.30 unit length. Figures II.7F through II.7H present examples of branched molecules. All except four

Figure II.7. Study of replicative DNA intermediates by electron microscopy. The bar represents 1 kb. Single-stranded DNA lengths were corrected to double-stranded DNA lengths by a factor of 1.2 as described in MATERIALS AND METHODS. Panels A through D, XD-7 molecules replicating as rolling circles with displaced single-stranded tails. Tails were 0.83 unit length (A), 1.10 unit length (B), 0.43 unit length (C), and 0.84 unit length (D). Panel E, heteroduplex formed between SalI-cut XD-7 and SalI-cut pBR322 DNAs. Panels F through H, replicating XD-7 molecules after digestion with SalI endonuclease. Displaced, single-stranded tails were 0.07 unit length (F), 0.10 unit length (G), and 1.03 unit length (H).
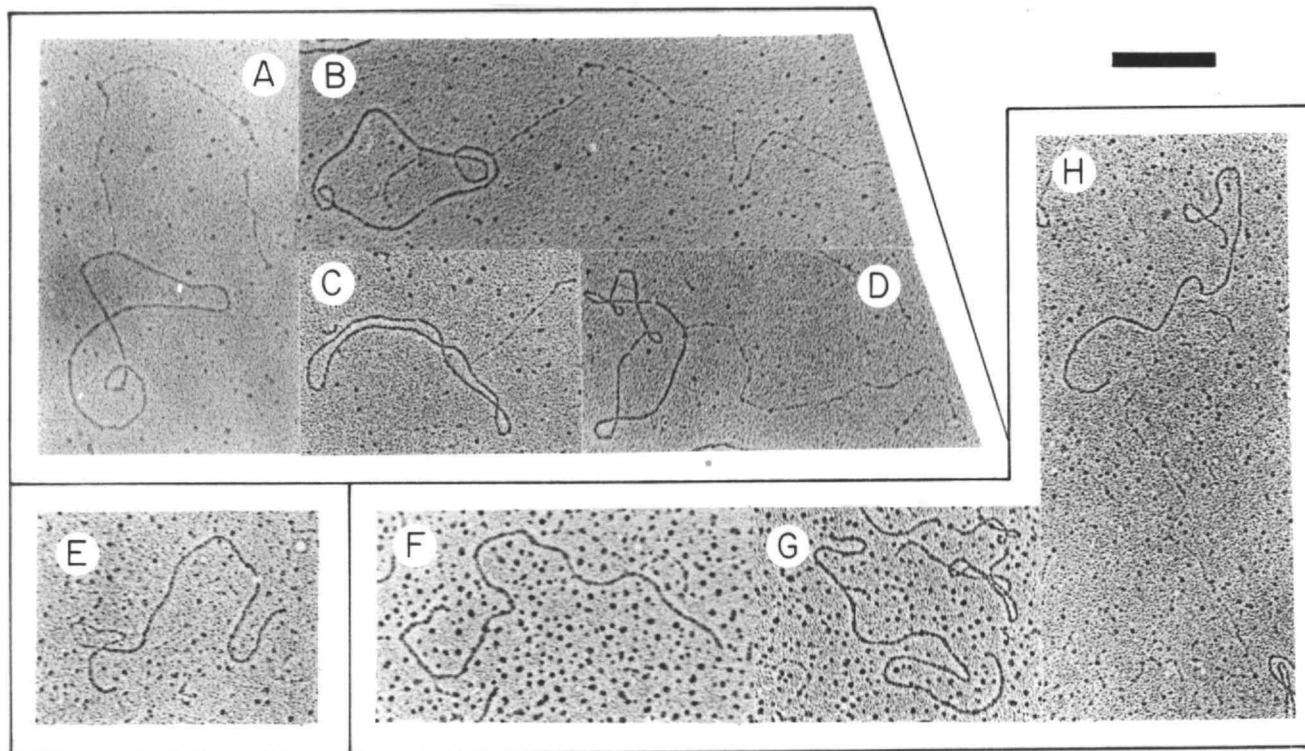
Figure II.7. Study of replicative DNA intermediates by electron microscopy.

TABLE II.1

Frequency of initiation during in vitro replication

Reaction	Template [a]	Number of molecules (rolling circles/total)	% Rolling circles [b]
1	XD-7	52/598	9
2	XD-7	29/314	9
3	pBR322	2/167	1
4	XD-7 pBR322	12/167 1/113	7 1

[a] Reaction mixtures with two templates contained 150 ng of each DNA. The two types of molecules were scored by electron microscopy on the basis of length differences.

[b] Expressed to the nearest percentage.

branched molecules could be sorted into at least four groups (Figure II.8). As shown in Figure II.8A, the major group (n=26) had single-stranded tails mapping at 651 ± 114 bp from the SalI site (coordinate 11.4 ± 2.0 on the SalI-linearized XD-7 map) corresponding to the junction between pBR322 sequences and left end of adenovirus DNA in XD-7. This was verified by direct measurements on heteroduplexes between XD-7 and pBR322 (Figure II.7E) where the shorter distance from the SalI site to the adenovirus insertion loop was 648 ± 40 bp (n=14). The sequenced distance is 656 bp (21). The alternative location of the major origin at coordinate 88.6, entirely within pBR322 sequences, could be excluded since no origin could be detected 650 bp fron the SalI site on pBR322 (see Figure II.9). Figure II.8A also shows that displacement replication from the major origin proceeded unidirectionally to the right, an indication that the adenovirus l-strand was displaced during replication.

The remaining minor origins were mapped on XD-7 at 23 ± 120 bp (n=7, Figure II.8B), 1976 ± 97 bp (n=4, Figure II.8C), and 1519 ± 23 bp (n=3, Figure II.8D) from the SalI cleavage site. Since the minor origins were common to both XD-7 and pBR322 (Figure II.9), they could be assigned to coordinates 0.4 ± 2.1 (Figure II.8B), 65.4 ± 1.7 (Figure II.8C), and 73.4 ± 0.4 (Figure II.8D) on the SalI-linearized XD-7 map. Replication originating at coordinates 65.4 and 73.4 (Figures II.8C and D) clearly proceeded to the left. Figure II.8F summarizes the distribution of the major, adenovirus-specific origin (solid histogram) and the minor, pBR322-specific origins (cross-hatched histograms) on XD-7 DNA.

After incubation in the standard reaction mixture, pBR322 DNA

Figure II.8. Gallery of replicating XD-7 molecules. Molecules were isolated after incubation in the cell-free replication system, cleaved with SalI endonuclease, and analyzed by electron microscopy (see Figure II.7, F through H). Thick lines represent the double-stranded, linearized, unit-length XD-7 DNA. Thin lines represent displaced, single-stranded tails. Coordinates are in percent XD-7 length. Panel A, tails mapping at coordinate 11.4 ± 2.0. Panel B, tails mapping at coordinate 0.4 ± 2.1. Panel C, tails mapping at coordinate 65.4 ± 1.7. Panel D, tails mapping at coordinate 73.4 ± 0.4. Panel E, map of XD-7 DNA linearized at the SalI cleavage site. Ad2 sequences (open box) extend from coordinate 11.4 to coordinate 34.9. The solid portion of the box represents the Ad2 inverted terminal repetition. pBR322 sequences are shown as a line. Panel F, distribution of the ends of displaced, single-stranded tails on replicating XD-7 molecules. In the histogram, bars labeled a through d correspond to molecules in panels A through D, respectively. The solid bars represent the adenovirus origin. The hatched bars indicate the minor pBR322 origins.
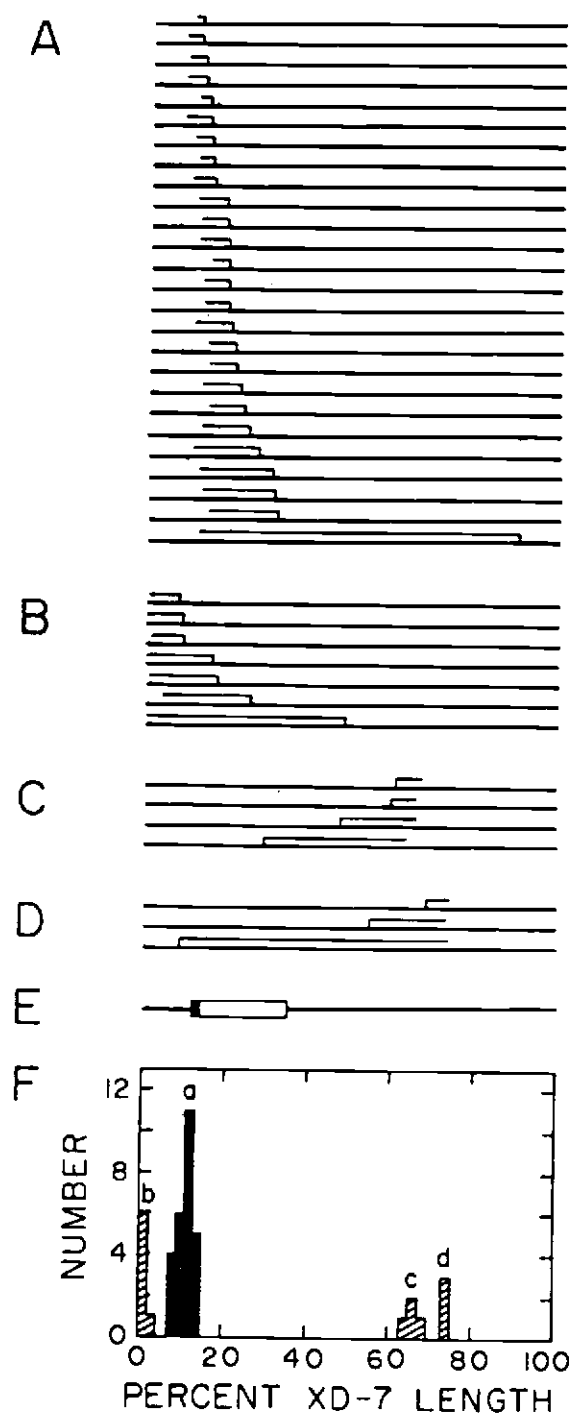
Figure II.8. Gallery of replicating XD-7 molecules.

was cleaved with SalI and examined by electron microscopy. A survey
of between 500 to 1000 molecules identified 14 branched molecules.
The double-stranded arms totaled to the length of pBR322 DNA. All but
two branched molecules could be assigned to at least three groups
(Figure II.9). The origins were mapped on pBR322 at 34 ± 74 bp (n=6,
Figure II.9A), 2181 ± 114 bp (n=4, Figure II.9B), and 1576 ± 23 bp
(n=2, Figure II.9C) from the SalI cleavage site. After adjusting for
the difference in length between pBR322 DNA and XD-7 DNA and
expressing coordinates in XD-7 length units, the origins for
strand-displacement replication were located on pBR322 at coordinate
0.6 ± 1.3 (Figure II.9A), coordinate 61.8 ± 2.0 (Figure II.9B), and
coordinate 72.4 ± 0.4 (Figure II.9C). Figure II.9E summarizes the
distribution of pBR322-specific origins. Thus, the pBR322 origins
coincided with the minor origins identified on XD-7, and they
functioned with the same efficiency on both DNAs. Clearly, factor(s)
in the extracts from adenovirus-infected, but not uninfected, cells
initiated displacement replication at low frequency at specific
pBR322 sequences. Sequences centered around the SalI cleavage site
can be arranged into extensive secondary structure, perhaps the
signal for replication at or near the SalI site on pBR322 and XD-7.
Two blocks of pBR322 sequences show limited homology to the sequences
conserved at the ends of the adenovirus DNA. Using the numbering
system of Sutcliffe, the blocks are located at nucleotide 2606 and
nucleotide 2324 (coordinate 65.8 and coordinate 70.7, respectively,
on the SalI-linearized XD-7 map). Since both pBR322 blocks are
oriented oppositely with respect to the adenovirus sequence at
coordinate 11.4, an orientation consistent with leftward replication,
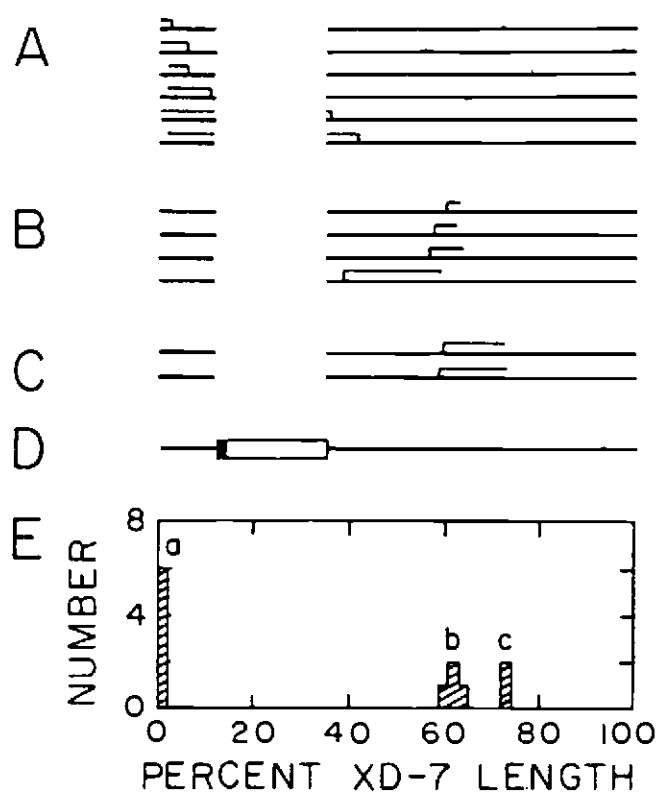
Figure II.9. Gallery of replicating pBR322 molecules. Molecules were prepared as described in Figure II.8. Thick lines represent double-stranded DNA and thin branches represent single-stranded DNA. pBR322 molecules have been adjusted to XD-7 length by leaving a gap corresponding to the length and position of the adenovirus insert. Coordinates are in percent XD-7 length. Panel A, tails mapping at coordinate 0.6 ± 1.3. Panel B, tails mapping at coordinate 61.8 ± 2.0. Panel C, tails mapping at coordinate 72.4 ± 0.4. Panel D, map of XD-7 DNA linearized at the SalI cleavage site (compare with Figure II.8E). Panel E, distribution of the ends of displaced, single-stranded tails on replicating pBR322 molecules. In the histogram, bars labeled a through c correspond to molecules in panels A through C, respectively.

they probably constitute the remaining minor origins on pBR322 and XD-7. The normal pBR322 origin did not function since rightward replication starting at coordinate 67.0 was never detected.

Figure II.10 summarizes the origins, directions of the displaced single-stranded tails and relative frequencies of finding tails on XD-7 and pBR322 in the in vitro adenovirus replication system.

DISCUSSION

XD-7 DNA replicated as rolling circles with single-stranded tails ranging up to unit length and longer, an indication of extensive strand-displacement synthesis, in a reaction mixture containing nuclear and cytoplasmic extracts from adenovirus-infected, but not uninfected, cells. Origins for displacement replication were mapped on XD-7 molecules by electron microscopy. The major origin on XD-7 was located at the left boundary of the cloned adenovirus segment, sequences corresponding to the left molecular end of adenovirus DNA. Since replication proceeded unidirectionally to the right, it can be concluded that the adenovirus l-strand was displaced during replication. Replication at the major origin was clearly due to adenovirus sequences in XD-7 since pBR322 showed no replication originating from positions at or near the EcoRI site. Displacement replication at the major XD-7 origin mimicked authentic adenovirus replication with respect to location, direction, and mode of replication. Three minor origins for strand-displacement replication were common to both XD-7 and pBR322. In all cases, these origins
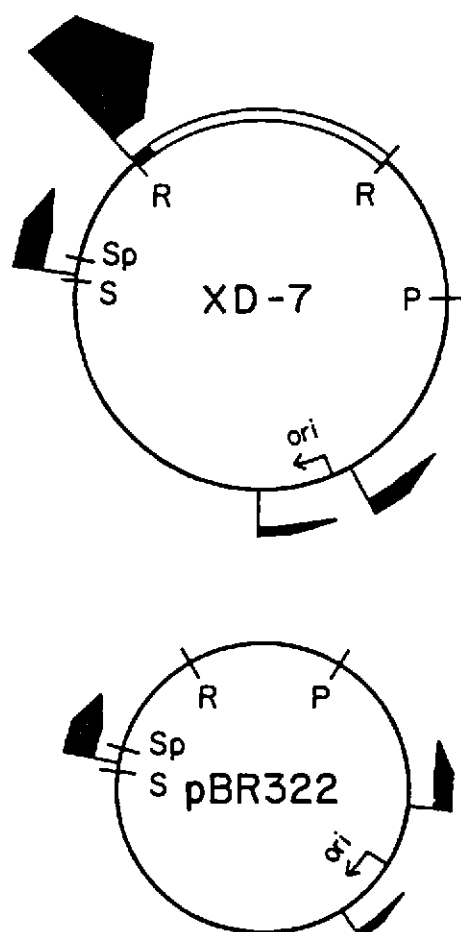
Figure II.10. Summary diagram of adenovirus-specific origins of replication mapped on XD-7 and pBR322. S, SalI; Sp, SphI; R, EcoRI; P, PstI; ori, pBR322 origin; open double-line segments, adenovirus sequences; filled-in segments, adenovirus inverted terminal repetition; single-line segments, pBR322 sequences. The black arrows indicate the location of adenovirus-specific origins and the direction of replication. The width of the arrows is proportional to the frequency of initiation at each origin.

functioned 5-fold to 10-fold less efficiently than the major origin on XD-7. At least two of these minor origins may share limited homology with conserved adenovirus sequences at the major origin.

The frequency of initiation (9% ± 1%) of input XD-7 DNA compares favorably with the frequency observed for initiation on adenovirus DNA-terminal protein complex (4,5). A standardized, quantitative assay has been developed for adenovirus replication by using XD-7 molecules as internal standards in the cell-free system to control for day-to-day and extract-to-extract variations. This assay has been used to study the in vitro replication properties of mutants with alterations within and around a cloned adenovirus replication origin. Deletion mapping located the adenovirus origin within the first 20 bp of the ITR, a region containing the longest conserved sequence shared between the ITRs of human, simian, murine, and avian adenovirus. Deletions removing or penetrating from either direction into the conserved sequence (nucleotides 9 through 17) inactivated the cloned adenovirus origin. A point mutation at position 17 within the conserved sequence markedly impaired the function of the adenovirus origin, but point mutations at position 4, 7, or 18 outside the conserved sequence had little or no effect. These results strongly suggest that the conserved sequence alone constitutes the signal for the adenovirus origin.

Tamanoi and Stillman (22) have developed an assay which detects the transfer of ^{32}P radioactivity from $[\alpha-^{32}P]dCTP$ to the 80,000-dalton precursor of terminal protein in the presence of a cloned adenovirus origin provided that the plasmid is linearized such that the adenovirus origin in at the end of the molecule. This

reaction has been taken as a measure of the initiation of adenovirus replication as first suggested by Rekosh et al (23). After linearization with EcoIRI, most of the deletion and point mutants described here were assayed by the terminal protein-labeling method. Results from the two assays agreed in general. For example, deletions removing the conserved sequence, such as dl7, neither replicated at high frequency nor allowed ^{32}P transfer to terminal protein precursor. In addition, pm4 behaved as wild-type, while pm17 was markedly inhibited in both assays. The major differences between the two assays involved the role of nucleotides 18 through 20. First, pm18 was as depressed as pm17 in the terminal protein-labeling assay. Second, dl18 behaved as wild-type in electron microscopic assay. These differences may simply reflect the structural requirements for initiation alone as measured by the terminal protein-labelling assay andfor both initiation as well as chain elongation as measured by the replication assay. Nevertheless, only the first 20 nucleotides of Ad2 ITR are essential for the initiation of replication in both assays.

The Ad2 origin compares in size with other well-characterized prokaryotic and eukaryotic viral origins. The ϕX174 origin for viral strand synthesis, a site-specific nick created by the action of gene A protein (24), is located in a 30-bp region conserved between phages ϕX174, G4, St-1, U3, α3, and G14 (25). Although a synthetic decadeoxyribonucleotide containing the nick site can be efficiently cleaved by gene A protein (26), supercoiled recombinant plasmids containing up to 20 bp spanning the nick site are not cleaved (27). Thus, more than 20 bp of the conserved ϕX174 origin region may be required for initiation of replication on supercoiled molecules.

Likewise, the SV40 origin of replication has been mapped by deletion and point mutations within a 60 bp region spanning binding sites for T antigen (28-30).

The mechanism for initiating rolling circle replication in the cell-free adenovirus DNA replication system is not yet known. Since the replication intermediates of XD-7 DNA observed by electron microscopy resemble that of ϕX174 DNA (31-36), the replication mechanism of adenovirus might be similar to that of ϕX174 phage. Two essential features are: (a) adenovirus DNA replicates in a covalently closed circular form and, (b) terminal protein (or its precursor) acts as an origin-specific topoisomerase. Ruben et al. (37) have demonstrated that covalently closed circular Ad5 DNA could be detected intracellularly as early as 3 to 5 hours post-infection. Circularization of incoming viral DNA could occur before the onset of viral replication, and that the circularization might be a process required for transcription and replication. Template topology has been shown to be essential for both trancription (38) and replication (39,40). The linear adenovirus genome cannot produce the topological torsion which is required for these processes. Circularization of DNA at an early stage of infection is an attractive explanation.

Regardless of the mechanism to initiate adenovirus replication, it is clear that the cell-free replication system directed by a cloned adenovirus origin has yielded valuable information about sequences controlling the initiation signal of adenovirus-specific replication.

REFERENCES

1. Kornberg, A. (1980) DNA replication (Freeman, San Francisco)

2. Wickner, S.H.(1978) DNA replication proteins of Escherichia coli. Ann. Rev. Biochem. 47, 1163-1191.

3. Stillman, B. W. (1983) The replication of adenovirus DNA with purified proteins. Cell 35, 7-9.

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

5. Challberg, M. D., and Kelly, T. J., Jr. (1979) Adenovirus DNA replication in vitro: origin and direction of daughter strand synthesis. J. Mol. Biol. 135, 999-1012.

6. Wasylyk, B., Kedinger, C., Corden, J., Brison, O., and Chambon, P. (1980) Specific in vitro initiation of transcription on conalbumin and ovalbumine genes and comparison with adenovirus-2 early and late genes. Nature 285, 367-373.

7. 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.

8. Tolun, A., Alestrom, P., and Pettersson, U. (1979) Sequence of inverted repetitions from different adenoviruses. Demonstration of conserved sequences and homology between SA7 termini and SV40 DNA. Cell 17, 705-713.

9. Temple, M., Antoinie, G., Delius, H., Stahl, S., and Winnacker, E.-L. (1981) Replication of mouse adenovirus strain FL DNA. Virology 109, 1-12.

10. Alestrom, P., Stenlund, A., Li, P., and Pettersson, U. (1982) A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18, 193-197.

11. Panayotatos, N., and Truong, K. (1981) Specific deletion of DNA sequences between preselected bases. Nucl. Acids Res. 9, 5679-5688.

12. Schleif, R.F., and Wensink, P.C. (1981) Practical methods in molecular biology. (Springer-Verlog, New York).

13. Guo, L.-H.,and Wu, R. (1982) New rapid methods for DNA sequencing based on exonucleaseIII digestion followed by repair synthsis. Nucl. Acids Res. 10, 2065-2084.

14. Vieira, J., and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19,259-268.

15. Shortle, D., and Nathans, D. (1978) Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. Proc. Natl. Acad. Sci. USA 75, 2170-2174.

16. Sanger, F., Nicklen, S., and Coulsen, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

17. Lowry, O.H., Rosebrough, N.J. Farr, A.L.,and Randall, R.J. (1951) Protein measurement with the Folin phosphate reagent. J. Biol. Chem. 193, 265-275.

18. Kleinschmidt , A. K. (1968) Monolayer technique in electron microscopy of nucleic acid molecules. in: Methods in Enzymology Vol.XIIB, (Academic Press). pp361-377.

19. Ferguson, J.,and Davis, R.W. (1978) Quantitative electron microscopy of nucleic acids. in: Adevanced technique in biological electron microscopy Vol.II, pp. 123-171.

20. Westmoreland, B.C., Szybalski, W., and Ris, H. (1969) Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163, 1343-1348.

21. Sutcliffe, J. G. (1979) Complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.

22. 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.

23. Rekosh, D.M.K., Russell, W. C., Bellett, A.J.D., and Robinson, A.J. (1977) Identification of a protein linked to the ends of adnovirus DNA. Cell 11,282-295.

24. Langevelt, S.A., van Mansfeld, A.D.M., Baas, P.D., Jansz, H.S., van Arkel, G.A., and Weisbeek, P.J. (1978) Nucleotide sequence of the origin of replication in bacteriophage ϕX174 RF DNA. Nature 271, 417-420.

25. Heidekamp, F., Langevelt, S.A., Baas, P.D.,and Jansz, H.S. (1980) Comparison of ϕX174, G4 and St-1 phage for site of action of A protein. Nucl. Acids Res. 8, 2009-2021.

26. Van Mansfeld, A.D.M., Langevelt, S. A., Baas, P.D., Jansz, H.S., van der Marel, G.A., Veeneman,G.H., and van Boom, J.H. (1980) Recognition sequence of bacteriophage ϕX174 gene A protein - an initiator of DNA replication. Nature 288, 561-566.

27. Heidekamp , F., Baas, P. D., van Boom, J. H., Veeneman, G. H. , Zipursky, S.L., and Jansz, H.S. (1981) Construction and characterization of recombinant plasmid DNAs containing sequences of the origin of bacteriophage ΦX174 DNA replication. Nucl. Acids Res. 9, 3335-3354.

28. Shortle, D., and Nathans, D. (1979) Regulatory mutants of simian virus 40: constructed mutants with base substitutions at the origin of DNA repication. J. Mol.Biol. 131, 801-817.

29. Myers, R. M., and Tjian, R. (1980) Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. USA 77, 6491-6495.

30. DiMaio, D., and Nathans, D. (1982) Regulatory mutants of simian virus 40. Effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. J. Mol. Biol. 156, 531-548.

31. Eisenberg, S., Griffith, J.,and Kornberg, A. (1977) ΦX174 cistron A protein is a multifunctional enzyme in DNA replication. Proc. Natl. Acad. Sci. USA 74,3198-3202.

32. Koths, K.,and Dressler, D. (1978) Analysis of the ΦX DNA replication cycle by electron microscopy. Proc. Natl. Acad. Sci. USA 75, 605-609.

33. Van der Ende, Langevelt, S.A., Teertstra, R.,van Arkel, G.A., and Weisbeek, P.J. (1981) Enzymatic properties of the bacteriophage ΦX174 A protein on superhelical ΦX174 DNA : a model for thetermination of rolling circle DNA replication. Nucl. Acids Res. 9, 2037-2053.

34. Aoyama, A., Hamatake, R,K., and Hayashi, M. (1981) Morphogenesis of ΦX174: in vitro synthesis of infectious phage from purified viral components. Proc. Natl. Acad. Sci. USA 78, 7285-7289.

35. Shlomai, J., Polder, L., Arai, K.,and Kornberg, A. (1981) Replication of ΦX174 DNA with purified enzymes. J. Biol. Chem. 256, 5233-5238.

36. Arai, N., Polder, L., Arai, K., and Kornberg, A. (1981) Replication of ΦX174 DNA with purified enzymes. J. Biol. Chem. 256, 5239-5246.

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

38. Harland, R.M., Weintraub, H.,and McKnight, S.L. (1983) Transcription of DNA injected into Xenopus oocytes is influenced by template topology. Nature 302, 38-43.

39. Gellert, M. (1981) DNA topoisomerase. Ann. Rev. Biochem. 50, 879-910.

40. Hagen, C.E. and Warren, G.J. (1983) Viability of palindromic DNA is restored by deletion occuring at low but variable frequency in plasmids of <u>Escherichia</u> <u>coli</u> Gene 24, 317-326.

CHAPTER III

SITE-SPECIFIC NICKING WITHIN
THE ADENOVIRUS
INVERTED TERMINAL REPETITION

ABSTRACT

Site-specific nicking occurs on the l-strand, but not on the r-strand, of the adenovirus left inverted terminal repeat. Nicks are presumably introduced into double- or single-stranded DNA by a cellular endonuclease in an ATP- independent reaction. The consensus nick site has the sequence:

$$GGRGYG \overset{\downarrow}{} GGRNRNGTG$$
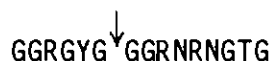
INTRODUCTION

DNA molecules from all adenovirus serotypes have an inverted terminal repetition (ITR) (1,2). Many adenovirus ITRs have recently been sequenced: human adenovirus serotypes 2, 3, 4, 5, 7, 9, 10, 12, and 31 (3-10); simian adenovirus SA7 (4); mouse adenovirus FL (11); avian adenovirus CELO (12,13); equine adenovirus (13); and infectious canine hepatitis virus ICHV (13). ITRs differ between serotypes in both sequence and length, but all ITRs share conserved or highly homologous sequences. The longest conserved sequence, ATAATATAC (nucleotides 9 through 17 from the ends), has been shown to control adenovirus replication (14) in a cell-free replication assay (15). Another conserved sequence, TGACGT, is located at or near the boundaries of the ITRs, but the function, if any, remains as yet undefined. In this chapter we show that highly homologous G-rich sequences within the ITR are nicked at specific sites. The results suggest that a cellular endonuclease nicks double- or single-stranded DNA in an ATP-independent reaction. The consensus nick site has the sequence GGRGYG$\overset{\downarrow}{}$GGRNRNGTG. Although the functional significance of

site-specific nicking within the ITR is not yet known, G-rich sequences similar to the adenovirus nick sites are also found at or near papovavirus replication origins (16).

MATERIALS AND METHODS

Materials.

Restriction endonucleases, E. coli DNA polymerase I (Klenow fragment), E. coli exonuclease III, dNTPs, and ddNTPs were from Bethesda Research Laboratories. The enzymes were used as recomended by the supplier. The synthetic 15-base primer was from P-L Biochemicals. $[\alpha-^{32}P]$dATP (800 Ci/mmol) was from New England Nuclear. Purified calf thymus topoisomerase I was generously provided by Dr. Leroy F. Liu.

Construction of mKM4.

XD-7 (15) and M13mp8 (17) have already been decribed. The 1-kb HindIII-SmaI fragment of XD-7 (Figure III.1) was cloned into M13mp8 cut with HindIII and SmaI. The 8.3-kb recombinant phage, called mKM4 (Figure III.1), was grown in E. coli JM103. Single-stranded, circular mKM4 DNA was purified by electrophoresis on a horizontal 0.8% agarose gel.

Labeling the adenovirus l-strand.

The annealing reaction (2.4 μl) contained 300 ng mKM4 DNA, 0.5 ng primer, 100 mM NaCl, 17 mM Tris-HCl (pH 7.5), 8 mM MgCl$_2$, and 0.8 mM EDTA. The solution was heated to 100°C for 3 min and then cooled to
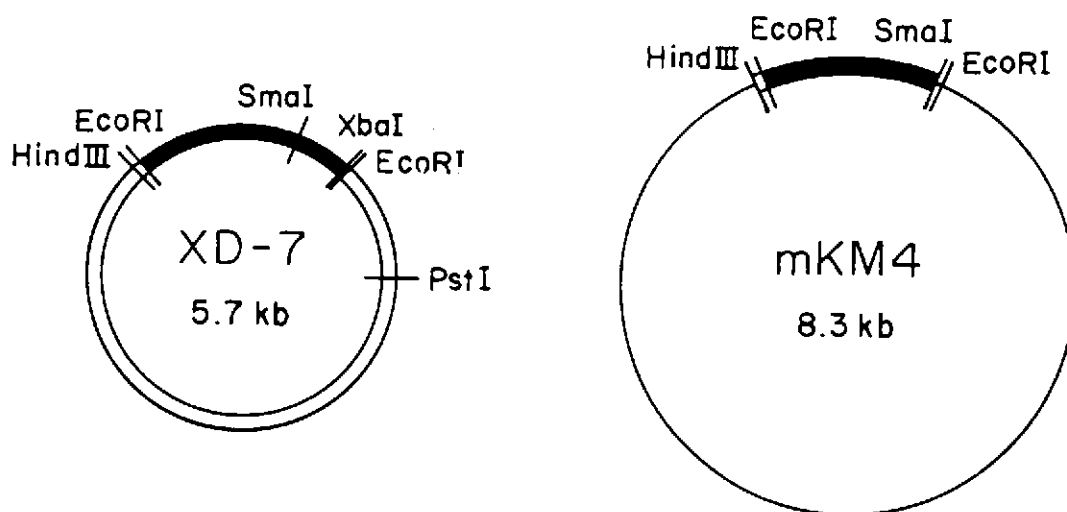
Figure III.1. Restriction endonuclease maps of XD-7 (left) and mKM4 (right). Double lines indicate pBR322 sequences, the thick line represents adenovirus sequences, and the thin line shows M13mp8 sequences. XD-7 contains the entire 1.35-kb XbaI-E fragment from the left end of type 2 adenovirus DNA inserted into the EcoRI site of pBR322. mKM4 contains the 1-kb HindIII-SmaI fragment of XD-7 inserted between the HindIII and SmaI sites of M13mp8. Single-stranded mKM4 has the adenovirus r-strand. The primer site lies just to the left of the HindIII site on mKM4.

22°C over a period of 45 min. Primer extension was carried out in a reaction mixture (5 µl) containing 300 ng mKM4 DNA annealed to 0.5 ng primer; 120 mM NaCl; 18 mM Tris-HCl (pH 7.5); 14 mM $MgCl_2$; 4 mM dithiothreitol; 0.4 mM EDTA; 25 µM each of dCTP, dGTP, and dTTP; 0.5 µM $[\alpha-^{32}P]dATP$ (2 µCi); and 0.2 unit E. coli DNA polymerase I (Klenow fragment). After incubating the solution for 10 min at 37°C, the dATP concentration was adjusted to 80 µM and incubation continued for 10 min. Approximately 600 bases were polymerized under these conditions. Dideoxy sequence ladders were synthesized on mKM4 as described by Sanger et al. (18) and used as length standards.

Labeling the adenovirus r-strand.

HindIII-cut XD-7 or pBR322 DNA (1 µg) was digested for 30 min at 22°C with 5 units of E. coli exonuclease III in a 15 µl reaction mixture containing 90 mM NaCl, 13 mM Tris-HCl (pH 7.4), and 5 mM $MgCl_2$. The reaction was stopped by phenol extraction. Approximately 400 bases were removed from each end of the linear DNA under these conditions (19). Exonuclease III-digested DNA was repaired in a 5 µl reaction mixture containing 1 µg DNA; 25 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 2 mM dithiothreitol; 50 µM each dGTP, dCTP, and dTTP; 1 µM $[\alpha-^{32}P]dATP$ (4 µCi); and 0.15 unit E. coli DNA polymerase I (Klenow fragment). After incubating the solution for 10 min at 37°C, the dATP concentration was adjusted to 80 µM and incubation continued for 10 min.

Nicking reaction.

Nuclear and cytoplasmic extracts were prepared from uninfected

and adenovirus-infected HeLa cells as previously described (15). The protein concentration in the nuclear extracts ranged from 4 to 6 mg/ml, and the concentation in the cytoplasmic extract was 15 mg/ml. The standard reaction mixture contained 90 mM NaCl; 60 mM Hepes (pH 7.5); 9 mM Tris-HCl (pH 7.5); 12 mM $MgCl_2$; 6 mM dithiothreitol; 100 µM each ddCTP, ddGTP, ddATP, and ddTTP; 2 µl nuclear extract (8 to 12 µg protein) from adenovirus-infected or uninfected HeLa cells; and 300 ng mKM4 DNA (or 1 µg XD-7 or pBR322 DNA) in total volume of 10 µl. Some reactions also contained 1 µl cytosol (15 µg protein). After incubation for 30 min at 37°C, the reaction was stopped by phenol extraction.

Topoisomerase I reaction.

The reaction mixture contained 40 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 ng mKM4 DNA, and 150 ng calf thymus topoisomerase I in a total volume of 10 µl. Incubation was for 30 min at 37°C.

Gel electrophoresis.

Gel electrophoresis was carried out at 1000 V on 8% polyacrylamide gels containing 8 M urea (25 cm wide x 35 cm long x 0.3 mm thick). The running buffer contained 8 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA. The gel was dried and autoradiographed after electrophoresis.

RESULTS

Site-specfic nicking on the l-strand.

The strategy to map site-specific nicking on the l-strand of the cloned adenovirus left inverted terminal repetition is detailed in Figure III.2. ^{32}P-labeled l-strand has the sequence shown below where the synthetic primer occupies the first fifteen nucleotides, the EcoRI site is underlined, and the arrow indicates cleavage by EcoRI:

$$\begin{array}{ccc} & \downarrow & 1 \\ \text{CCCAGTCACGACGTT...G} & \text{AATTC...} \\ 1 \qquad\qquad 15 & 67 & 72 \end{array}$$
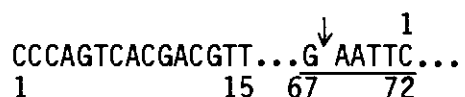
Numbers below the sequence indicate nucleotides from the 5' end of the primer whereas numbers above the sequence indicate nucleotides from the 5' end of adenovirus DNA (i.e., nucleotide 72 corresponds to adenovirus nucleotide 1). To test the strategy, mKM4 substrate containing ^{32}P-labeled adenovirus l-strand was cut with EcoRI. As expected, a 67-base fragment, designated r, was produced (Figure III.3, lane 2) which migrated with the ddG-terminated fragment within the EcoRI sequence (Figure III.3, lane G). When EcoRI-cut mKM4 substrate was subsequently incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells, the EcoRI cohesive site was partially repaired as shown in Figure III.3 (lane 1) by fragments ranging in size from 67 bases (fragment r) to 71 bases (fragment r'). A faint ladder extending down at nucleotide intervals from fragment r to the size of the synthetic primer indicated limited exonucleolytic damage to the 3' end of fragment r, presumably due to exonuclease III-like activity. Addition of ddNTPs to the reaction mixture prevented repair of the EcoRI cohesive site, but did not eliminate
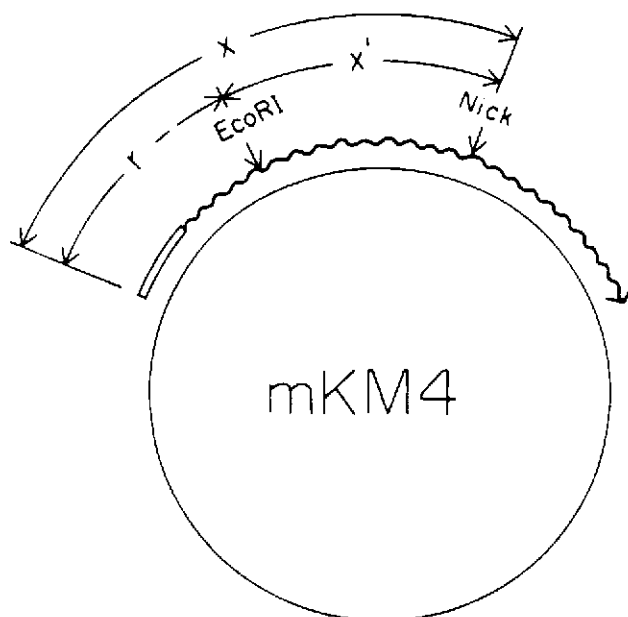
Figure III.2. Strategy to map site-specific nicking on the adenovirus l-strand. A 15-base primer was annealed to single-stranded mKM4 and elongated with the Klenow fragment of DNA polymerase I using [α-^{32}P]dATP and unlabeled dCTP, dGTP, and dTTP. ^{32}P-Labeled DNA corresponds to the adenovirus l-strand. After incubation in a reaction mixture containing nuclear extract, nicks were located by the size of specific fragments on sequencing gels. The locations of nicks were further verified by subsequent cleavage wth EcoRI as diagrammed: x = x' + r where r = 67 bases. The thin line represents single-stranded, circular mKM4 DNA; double lines indicate the 15-base primer; and the thick, wavy line shows the ^{32}P-labeled adenovirus l-strand.

Figure III.3. Analysis of site-specific nicking on the adenovirus
l-strand. Gel electrophoresis was for 3 hr. Lanes labeled G, C, A,
and T contain dideoxy sequence ladders respectively terminating in
ddG, ddC, ddA, and ddT. Lane 1: mKM4 cut with EcoRI and incubated
with uninfected nuclear extract. Lane 2: mKM4 cut with EcoRI only.
Lane 3: mKM4 incubated with uninfected nuclear extract containing
ddNTPs and 4 mM ATP. Lane 4: mKM4 incubated with uninfected nuclear
extract containing ddNTPs but no added ATP. Lane 5: Heat-denatured
mKM4 incubated as described in Lane 3. Lane 6: Heat-denatured mKM4
incubated as described in Lane 4. $r = 67$ bases, $r' = 71$ bases,
$x = 123$ bases, $y = 127$ bases, $z = 146$ bases, $u = 153$ bases, $v = 159$
bases, and $w = 164$ bases.

71
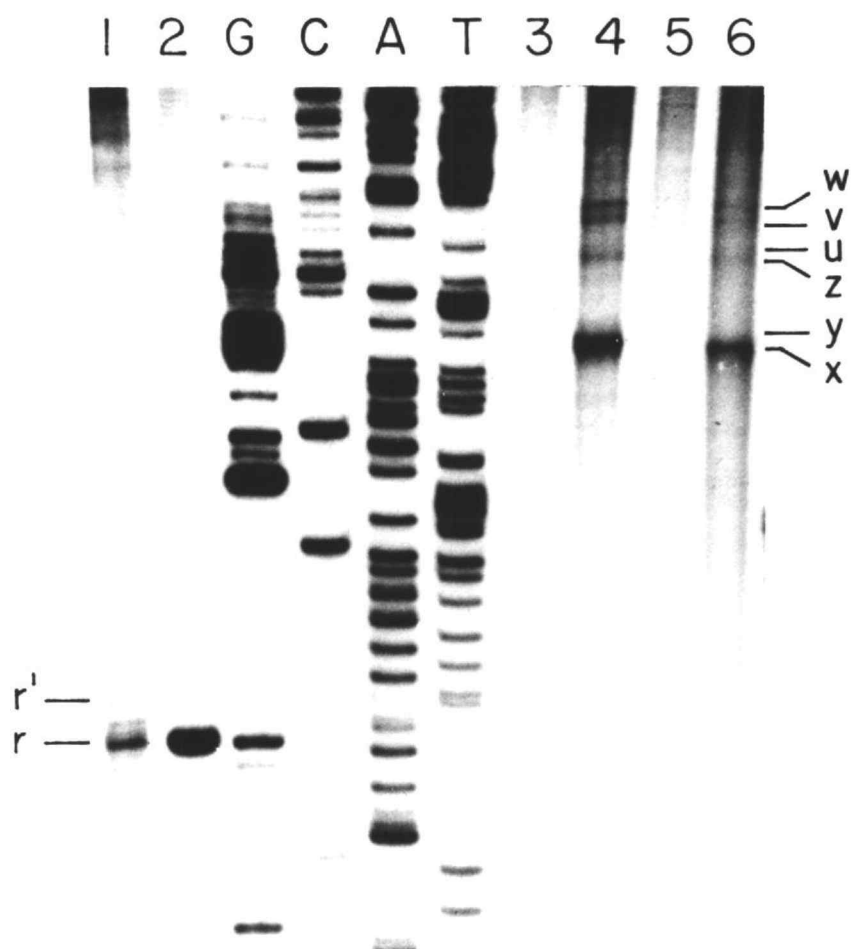
Figure III.3. Analysis of site-specific nicking on the adenovirus 1-strand.

3'-exonucleolytic activity (not shown).

Figure III.3 also shows that a set of specific fragments was generated when ^{32}P-labeled mKM4 substrate was incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells and ddNTPs. Six of the specific fragments, designated x, y, z, u, v, and w, arise from cleavages within the adenovirus ITR. Identical fragments were produced regardless of whether the mKM4 substrate was native (Figure III.3, lane 4) or denatured (Figure III.3, lane 6). Curiously, adding 4 mM ATP to the reaction mixture eliminated evidence of site-specific nicking (Figure III.3, lanes 3 and 5). Based on the concentration of ATP in nuclei isolated from uninfected or adenovirus-infected HeLa cells (20), we estimate that the endogenous ATP concentration in the nicking reaction is less than 0.2 µM. Even the addition of 0.5 mM ATP to the reaction completely abolished evidence of nicking (not shown). In contrast, the optimal ATP concentration for in vitro adenovirus DNA replication is 2 mM (20). It is not clear whether ATP directly inhibits the nicking enzyme or an ATP-dependent process repairs the nicks or degrades the fragments.

High-resolution mapping (Figure III.4A) located the 3' ends of fragments x, y, z, u, v, and w at nucleotides 52, 56, 75, 82, 88, and 93 respectively within the ITR. Moreover, Figure III.4A shows that identical fragments were produced regardless of whether the nuclear extract was made from uninfected HeLa cells (lane 3) or adenovirus-infected HeLa cells (lane 1) or the nuclear extract was supplemented with a cytoplasmic extract (lane 2). Neither the concentration of protein in the reaction (range: 0.8 to 2.7 mg/ml)

Figure III.4. High-resolution mapping of adenovirus l-strand nicking. (A) Gel electrophoresis was for 8 hr. Lane G: Dideoxy sequence ladder terminating in ddG. Lane 1: mKM4 incubated with nuclear extract from adenovirus-infected cells. Lane 2: mKM4 incubated with nuclear and cytoplasmic extracts from adenovirus-infected cells. Lane 3: mKM4 incubated with nuclear extract from uninfected cells. (B) Gel electrophoresis was for 3 hr. Lane 1: mKM4 incubated with uninfected nuclear extract. Lane 2: mKM4 incubated with uninfected nuclear extract and then cut with EcoRI. $r = 67$ bases, $x' = 56$ bases, $y' = 60$ bases, $z' = 79$ bases, $u' = 86$ bases, $v' = 92$ bases, and $w' = 97$ bases.
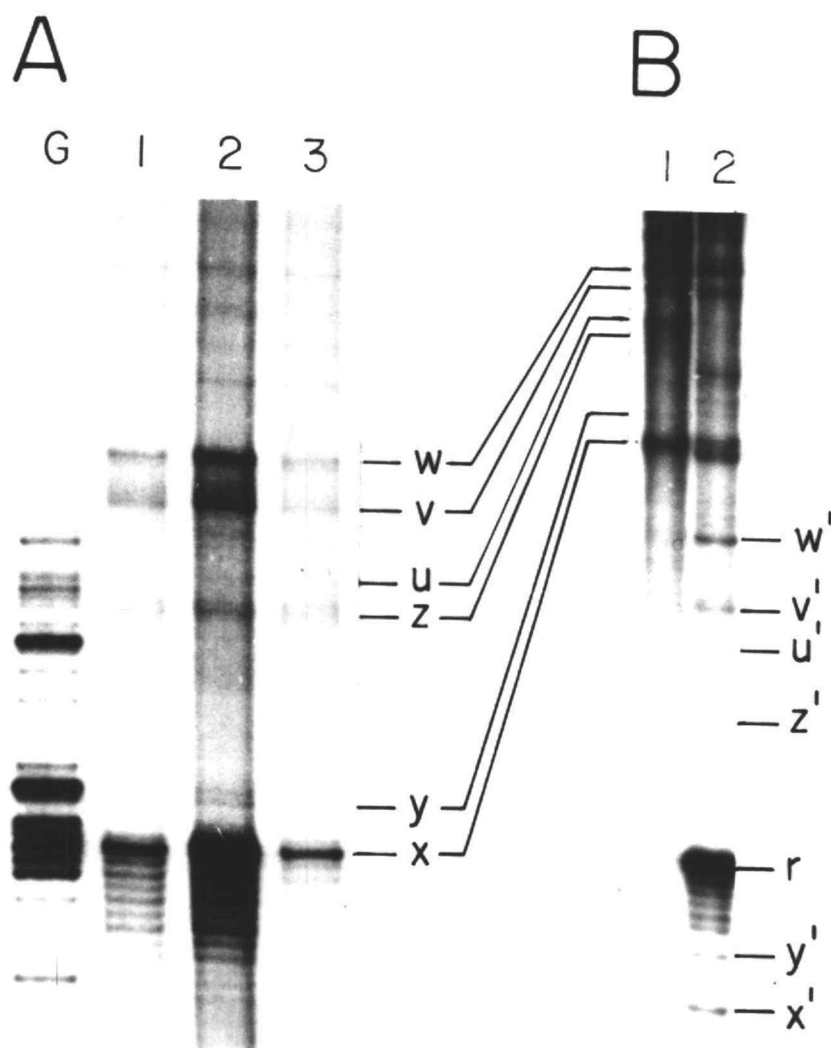
Figure III.4. High-resolution mapping of adenovirus l-strand nicking.

nor the length of incubation (up to 60 min) affected the production of specific fragments. However, reactions with higher protein concentrations (compare lanes 1 and 2 in figure III.4A with lane 3) or longer incubation times (not shown) showed increased exonucleolytic damage. We therefore conclude that specific fragments are created by endonucleolytic cleavages rather than by exonucleolytic damage. The locations of the nicks could be further verified by subsequent cleavage with EcoRI as shown in Figure III.4B (lane 2). A new set of fragments, identified as x', y', z', u', v', and w', was produced where each new fragment was exactly 67 bases shorter than the corresponding parental fragment. Only fragments cleaved by EcoRI could be located unambiguously. Whether nicking in the nuclear extract or cleavage by EcoRI occurred first is not important since a longer exposure of Figure III.3 (lane 1), where the order was reversed, also revealed fragments x', y', z', u', v', and w' (not shown). The sequences surrounding the nick sites are listed below where the arrows indicate the nicks and the numbers in parentheses identify the adenovirus nucleotides on either side of the nick:

$$
\begin{array}{lll}
x: & TGAGGG{\downarrow}GGTGGAGTT & (52\text{-}53) \\
y: & GGGGTG{\downarrow}GAGTTTGTG & (56\text{-}57) \\
z: & GGCGCG{\downarrow}GGGCGTGGG & (75\text{-}76) \\
u: & GGCGTG{\downarrow}GGAACGGGG & (82\text{-}83) \\
v: & GGAACG{\downarrow}GGGCGGGTG & (88\text{-}89) \\
w: & GGGGCG{\downarrow}GGTGACGTA & (93\text{-}94) \\
 & GGRGYG{\downarrow}GGRNRNGTG & consensus \\
\end{array}
$$

All cleavages occur between adjacent G residues. In each case at

least nine of the surrounding fifteen nucleotides are G residues, and these neighboring G-rich sequences appear to be related as indicated by the derived consensus sequence. This is all the more surprising since these sequences overlap extensively as shown in Figure III.7 below. The role, if any, that neighboring sequences play in determining the specificity or frequency of cleavage is not yet known. If the intensity of fragment r, produced by EcoRI, is taken as a measure of complete cleavage, it is clear that the yield of fragment x is much lower. In fact, fragments y through w, which span the x cleavage site, could not be detected at all if x were completely cleaved. Consequently, cleavages at sites distal to fragment x from the primer are underestimated using the strategy outlined in Figure III.2.

The r-strand is not nicked.

The strategy to map site-specfic nicking on the r-strand of the cloned adenovirus left inverted terminal repetition is detailed in Figure III.5. When XD-7 substrate with ^{32}P-labeled adenovirus r-strand was incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells and ddNTPs, a set of specific fragments was generated (Figure III.6A, lane 2). However, none of the specific fragments was altered by subsequent cleavage with EcoRI although the expected 35-base fragment r" appeared (Figure III.6A, lane 1). We interpret this to mean that the fragments in figure III.6A arose from cleavages on the ^{32}P-labeled strand at the other end of the linearized XD-7 molecule and that the r-strand of the adenovirus ITR was not nicked. Lack of cutting of the r-strand

Figure III.5. Strategy to map site-specfic nicking on the adenovirus

r-strand and on pBR322. (A) XD-7 or pBR322 was linearized with

HindIII, digested with exonuclease III, and repaired with the Klenow

fragment of DNA polymerase I using $[\alpha-^{32}P]$dATP and unlabeled dCTP,

dGTP, and dTTP. ^{32}P-Labeled DNA (thick, wavy line) corresponds to

the adenovirus r-strand in XD-7. (B) After incubation in a reaction

mixture containing nuclear extract, nicks were located by the size of

specific fragments on sequencing gels. The locations of nicks were

further verified by subsequent cleavage with EcoRI as diagrammed:

$p = p' + r''$ where $r'' = 35$ bases.
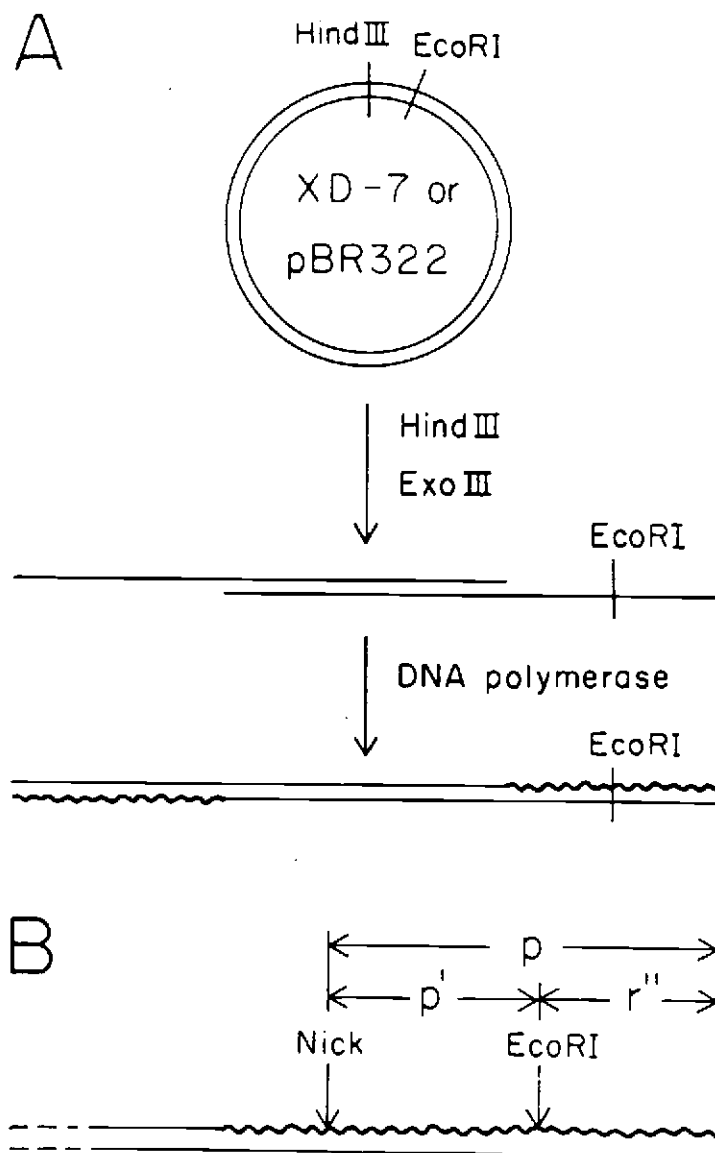
Figure III.5. Strategy to map site-specfic nicking on the adenovirus r-strand and on pBR322.
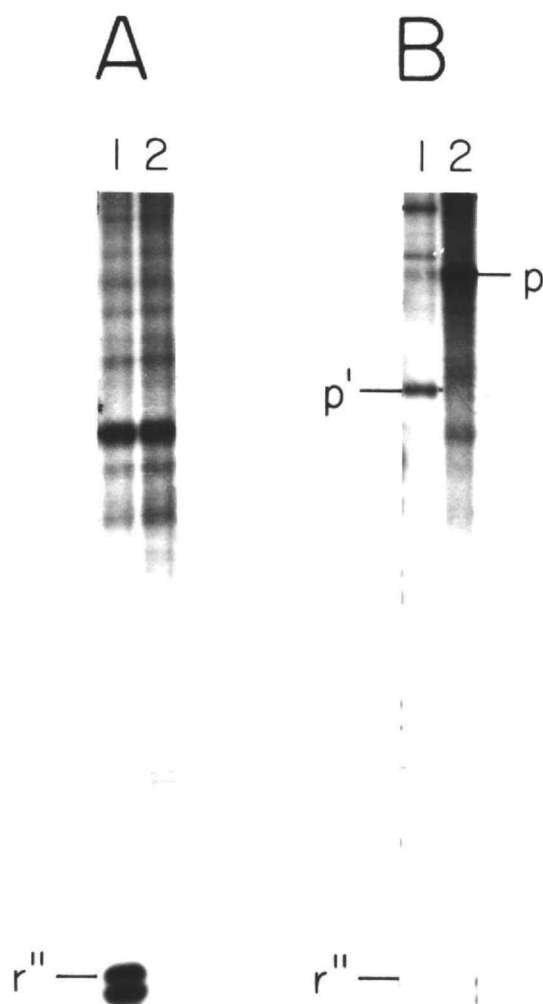
Figure III.6. Analysis of site-specific nicking on the adenovirus r-strand and on pBR322. Gel electrophoresis was for 3 hr. (A) Lane 1: XD-7 incubated with uninfected nuclear extract and then cut with EcoRI. Lane 2: XD-7 incubated with uninfected nuclear extract. (B) Lane 1: pBR322 incubated with uninfected nuclear extract and then cut with EcoRI. Lane 2: pBR322 incubated with uninfected nuclear extract. r" = 35 bases, p = 144 bases, and p' = 109 bases.

establishes that the l-strand cleavages are indeed nicks.

Failure to detect nicking on the r-strand of the ITR was not due to faulty experimental design. To test the strategy, ^{32}P-labeled pBR322 substrate was incubated in the nicking reaction. A set of specific fragments was produced (Figure III.6B, lane 2) which included all of the fragments displayed in Figure III.6A (lane 2) as well as at least one additional fragment designated p. Figure III.6B (lane 1) shows that a new fragment, called p', which was 35 bases shorter than p, appeared along with fragment r" after subsequent cleavage with EcoRI. Fragments p and p' locate a site-specific nick on pBR322 within the sequence AGGG GTT where the cleavage is between adjacent G residues at nucleotides 4251 and 4252 (21). Six of the seven nucleotides surrounding the pBR322 nick are identical to nucleotides around the nick defining fragment x.

Site-specific nicking is not due to topoisomerase I or topoisomerase II.

Site-specific nicking clearly cannot be caused by HeLa topoisomerase II since it requires ATP and makes double-stranded cleavages (22). To test for the involvement of topoisomerase I, purified calf thymus topoisomerase I was incubated with ^{32}P-labeled mKM4 substrate. Although a set of specific fragments was generated by topoisomerase I, none of the fragments coincided with fragments produced in the nicking reaction (not shown). Since calf thymus topoisomerase I and HeLa topoisomerase I cleave SV40 DNA at identical and specific sites (23), we conclude that site-specific nicking of the l-strand of the ITR is also not caused by HeLa topoisomerase I.

DISCUSSION

Site-specific nicking occurred on the l-strand, but not the r-strand, of the adenovirus left inverted terminal repetition in reaction mixtures containing extracts from uninfected as well as adenovirus-infected HeLa cells. The substrate for nicking could be either double-stranded or single-stranded DNA. The reaction did not require added ATP, and no nicks were observed in the presence of 4 mM ATP. Nicking clearly cannot be caused by HeLa topoisomerase II which requires ATP and makes double-stranded cleavages (22). Nicking is also probably not caused by HeLa topoisomerase I since a direct test eliminated calf thymus topoisomerase I, which cleaves SV40 at the same sites as HeLa topoisomerase I (23), as the nicking enzyme. The nature of the termini created at the nick site is not yet known. However, faint bands one nucleotide larger than the main cleavage fragments could often be detected. For example, a faint band is visible at position x+1 in Figure III.4A (lanes 1 and 3) and fragment y appears as a doublet differing by a single nucleotide in Figure III.4A (lanes 1 and 2). We interpret this to mean that one dideoxynucleotide residue can be added at the cleavage site, an indication perhaps that nicking yields a 3'-hydroxyl end. Nevertheless, other alternatives have not yet been excluded. For example, a phosphomonoesterase could convert 3'-phosphates into 3'-hydroxyls. We have detected a phosphomonoesterase activity which removes 5'-phosphates in the nicking reaction (not shown).

High-resolution mapping located the cleavage sites within the sequence of the adenovirus ITR. Figure III.7 shows that all cleavages occurred between adjacent G residues in highly homologous,
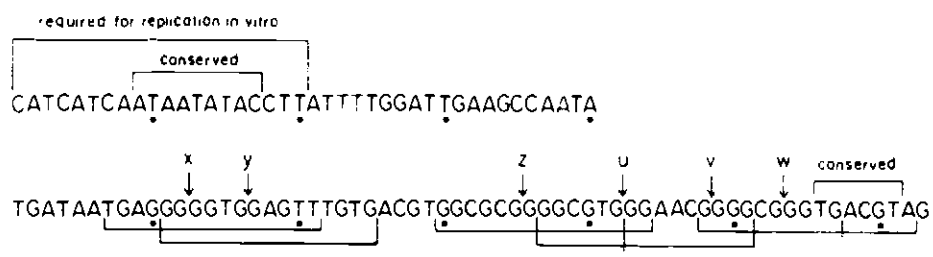
Figure III.7. Sequence of the l-strand of the type 2 adenovirus left inverted terminal repetition. Dots indicate every tenth nucleotide. Site-specific nicks are shown by the arrows. Brackets below the line enclose homologous G-rich sequences.

G-rich sequences which, surprisingly, overlap extensively. The consensus nick site has the sequence GGRGYG↓GGRNRNGTG. All adenovirus serotypes, except the avian adenovirus CELO, have related G-rich sequences in the ITR (3-13). Moreover, G-rich sequences similar to the adenovirus nick sites are also found at or near papovavirus replication origins (16). The functional significance, if any, of site-specific nicking within the ITR is not yet known. Deletions removing all of the nick sites in the ITR do not prevent adenovirus replication in a cell-free replication assay (14). However, all of the deletions are connected through a BamHI linker to a G-rich adenovirus sequence at the SstII site. Deletion mutant dl30 was subcloned into M13mp8 and tested for l-strand nicking (not shown). A site-specfic nick was mapped on dl30 within the sequence CCGGATCCGG↓GGGGA where the cleavage is between adjacent G residues, one G within the BamHI linker (underlined sequence) and the other G at adenovirus nucleotide 358. This G-rich sequence, present in all deletion mutants, may therefore be able to compensate for the loss of nick sites in the ITR.

REFERENCES

1. 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.

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

3. Steenbergh, 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.

4. Tolun, A., Alestrom, P., and Pettersson, U. (1979) Sequence of inverted repetition from different adenoviruses. Demonstration of conserved sequences and homology between SA7 termini and SV40 DNA. Cell 17, 705-713.

5. Dijkema, R., and Dekker, B.M.M. (1979) The inverted terminal repetition of the DNA of weakly oncogenic adenovirus type 7. Gene 8, 7-15.

6. Arrand, J.R., and Roberts, R.J. (1979) The nucleotide sequences at the termini of Ad2 DNA. J. Mol. Biol. 128, 577-594.

7. Shinagawa, M., and Padmanabhan, R. (1979) Nucleotide sequences at the inverted terminal repetition of Ad2 DNA. Biochem. Biophys. Res. Commun. 87, 671-678.

8. Shinagawa, M., and Padmanabhan, R. (1980) Comparative sequence analysis of the inverted terminal repetitions from different adenoviruses. Proc. Natl. Acad. Sci. USA 77, 3831-3835.

9. Sugisaki, H., Sugimoto, K., Takanami, M., Shiroli, K., Saito, I., Shimojo, Y., Sawada, Y., Uemizu, Y., Uesugi, S.-I., and Fujinaga, K. (1980) Structural and gene organization in the transforming Hind III-G fragment of Ad12. Cell 20, 777-786.

10. 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.

11. Temple, S., Antoine, G., Delius, H., Stahl, S., and Winnacker, E.-L. (1981) Replication of mouse adenovirus strain FL DNA. Virology 109, 1-12.

12. Alestrom, P., Stenlund, A., Li, P., and Pettersson, U. (1982) A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18, 193-197.

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

14. 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.

15. Pearson, G. D., Chow, K. -C., Enns, R. 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.

16. Seif, I., Khoury, G., and Dhar,R. (1979) The genome of human papovavirus BKV. Cell 18, 963-977.

17. Messing,J. (1981) M13mp2 and derivatives: A molecular cloning system for DNA sequencing, strand specific hybridization, and in vitro mutagenesis. in Walton, A.G. (Ed.) Proceedings of the Third Cleveland Symposium on Marcomolecules: Recombinant DNA. Elsevier, Amsterdam, pp. 143-153.

18. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

19. Guo, L.-H.,and Wu, R. (1982) Nem rapid methods for DNA sequencing based on exonucleaseIII digestion followed by rapair synthesis. Nucleic Acids Res. 10, 2065-2084.

20. De Jong, P.J., Kwant, M.M., van Driel, W., Jansz,H.S.,and van der Vliet, P.C. (1983) The ATP requirements of adenovirus type 5 DNA replication and cellular DNA replication. Virology 124, 45-58.

21. Sutcliffe, J. G. (1979) Complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.

22. Miller, K.G., Liu, L. F., and Englund, P. T. (1981) A homogenous type II DNA topoisomerase from HeLa cell nuclei. J. Biol. Chem. 256, 9334-9339.

23. Edwards, K.A., Halligan, B.D., Davis, J.L., Nivera, N.L.,and Liu, L.F. (1982) Recognition sites of eukaryotic DNA topoisomerase I: DNA nucleotide sequencing analysis of topo I cleavage sites on SV40 DNA. Nucleic Acids Res. 10, 2565-2576.

CHAPTER IV

SITE-SPECIFIC NICKING
AT THE CENTER OF
PALINDROMIC SEQUENCES

ABSTRACT

Site-specific nicking occurs at the center of palindromic sequences on the r-strand of XD-7 deletion mutant DNAs which have a 10-basepair BamHI linker. Nicks are not adenovirus-specific. Nicks also occur at the junction between pBR322 sequences and the adenovirus DNA ITR insert. These nicks are adenovirus specific.

INTRODUCTION

Adenovirus DNA molecules from all serotypes examined to date have two unusual structural features: An inverted terminal repetition about 100 to 160 base pairs long (1,2,3), and a 55,000-dalton protein, called terminal protein, covalently linked to the 5'-end of each strand (4,5,6). Recently, Ruben et al (7) identified covalently closed circles of adenovirus type 5 DNA in both infected BRK (Baby Rat Kidney) and HeLa cells. The joint structures were detected in small amounts as early as 3 hours after infection and were present in constant amounts (8-15%) from 5 to 120 hours after infection. A new model for the structure and replication of adenovirus DNA was proposed in Chapter I. This model postulates that adenovirus molecules are covalently closed circles, and that the linear DNA-protein complex may result from cleavage of the circles by terminal protein.

XD-7 DNA, a pBR322 clone which contains the XbaI-E fragment from the left end of Ad2 DNA at the EcoRI site, has been shown to replicate as rolling circles with displaced single-stranded tails (8) in an in vitro replication system. The frequency of initiation of replication was 9% of input XD-7 DNA molecules. When the initiation percentage was compared with that of adenovirus DNA in the in vitro replication system (9) or the percentage of circular DNA formed during infection (7), the percentages are all around 5-15%. It is not clear whether these three events are relevant to each other, nor is it clear whether circularization is necessary for the initiation of adenovirus DNA replication or whether circular structures are transposon-like intermediates formed during the process of

transformation (10). If circularization is an essential step of DNA replication or transformation, then in order to restore the organization of the viral genome, a novel enzyme is required to open the circle at the joint where two ends of DNA were linked together. Here I present evidence that there is indeed an enzyme in cells which is responsible for the nicking at the center of small palindromic sequences in several plasmids containing the adenovirus left inverted terminal repetition (ITR) insert. Since the nicking activity was found in both uninfected and Ad2-infected HeLa cells, it is not adenovirus-specific.

MATERIALS AND METHODS

General Procedures

The enzymes, the growth and purification of plasmid and M13 single-stranded DNAs, the preparation of nuclear extract, the labeling of DNA, the standard conditions of nicking reaction and the conditions of gel electrophresis have been described in Chapter III. Construction of deletion mutants has been described in Chapter II (as shown in Figure II.3). The designation dl n refers to a deletion mutant that has n nucleotides of the adenovirus terminal sequences left. For example, dl12 has only the first 12 base pairs of the Ad2 ITR.

RESULTS

Nicking at the center of palindromic sequences.

The strategy to map site-specific nicking on the adenovirus r-strand has been described in Chapter III. Deletion mutant d112, d121, d136, and d167, described in Chapter II, were tested for site-specific nicking. When the deletion mutant DNAs, which had been treated with exonuclease III and repaired with the Klenow fragment and $[\alpha^{32}P]dATP$, were incubated in the reaction mixture containing nuclear extract and dideoxynucleotides, a set of specific fragments was generated (Figure IV.1, lane 4, 6, 7, 9, 11,and 13). Several new fragments were generated by subsequent cleavage with EcoRI (Figure IV.1, lane 3, 5, 8, 10, 12, and 14). These fragments are all shortened by 35 bases (as shown in Figure IV.1), the size of the expected fragment r" which spanned sequences between HindIII and EcoRI sites of pBR322. The doublet r" fragments or the faint ladder extending down at nucleotide intervals indicated limited exonucleolytic damage to the 3' end of fragment r", presumably due to exonuclease III-like activity. Addition of dideoxynucleotides to the reaction mixture not only prevented the non-specific repair at nick site, but also partially inhibited 3'-exonucleolytic activity (data not shown).

Two specific bands, about 5 nucleotides apart, appear in each lane. Some appear as doublet bands. Because each of these bands shifted position on the gel by 35-bases after EcoRI cleavage, the nick sites could be accurately located at the molecular end which contains EcoRI site. The sequences surrounding the nick sites are listed below where the vertical arrows indicate the nicks, the BamHI

Figure IV.1. Analysis of nicking at the junction of palindromic sequences. Gel electrophoresis was for 3.5 hours. DNA circles were linearized with HindIII, digested with exonuclease III, and repaired with the Klenow fragment of DNA polymerase I using $[\alpha-^{32}P]$dATP and unlabeled dCTP, dGTP and dTTP. After incubation with nuclear extract, nicks were located by the size of the specific fragments on sequencing gels. The location of nicks were verified by subsequent cleavage with EcoRI. The HindIII-EcoRI fragment (r") is 35 bases long. The lane labeled T is a sequencing ladder used as a size standard. Substrate DNAs are named at the top of the Figure. Lanes labeled with N are reaction mixtures incubated with nuclear extract only. Lanes labeled with E are reaction mixtures cut with EcoRI after incubating with nuclear extract. Bands labeled with dots are sequences at the nick sites. Lane 1 and 2 are internal controls using primer-extended mKM4 as a substrate. r is 67 bases long.

Figure IV.1. Analysis of nicking at the junction of palindromic sequences.

linkers are overlined, the upper sequence is adenovirus l-strand, and the lower sequence is the r-strand:

```
                                    BamHI linker
      dl12: AGGAATTCATCATCAATAA CCGGA TCCGGGGGTACTTTGACC
            TCCTTAAGTAGTAGTTATT GGCCT AGGCCCCCATGAAACTGG
                               ↑     ↑

      dl21: TCATCAATAATATACCTTA CCGGA TCCGGGGGTACTTTGACC
            AGTAGTTATTATATGGAAT GGCCT AGGCCCCCATGAAACTGG
                               ↑     ↑

      dl36: CTTATTTTGGATTGAAGCC CCGGA TCCGGGGGTACTTTGACC
            GAATAAAACCTAACTTCGG GGCCT AGGCCCCCATGAAACTGG
                               ↑     ↑

      dl67: AGGGGGTGGAGTTTGTGAC CCGGA TCCGGGGGTACTTTGACC
            TCCCCCACCTCAAACACTG GGCCT AGGCCCCCATGAAACTGG
                               ↑     ↑
```

All cleavages on the r-strand occur right at the center of the BamHI linker and at the junction between Ad2 sequences and the BamHI linker. The BamHI linker itself is a 10-nucleotide palindrome. It is unlikely that this small palindrome adopts a transient cruciform conformation during the reaction, since the substrate is a linear molecule, but the nicking might recognize the nucleotide sequence of the palindrome. The surrounding sequences seems to be important in the nick site selection, since one side of the sequences is identical in all plasmids. The same reaction has been carried out on the l-strand of the deletion mutants by subcloning the HindIII-BamHI fragment, which contains part of the adenovirus ITR, into M13mp8. In this way, the sequences of the non-identical side were restored, and those of the identical side were eliminated. Also two nucleotides were inserted into the BamHI linker sequence to interrupt the perfect palindromic sequences. There was only a weak signal on the l-strand of dl30-subclone, mKC8dl30HB:

```
                                           ↓
      mKC8dl30HB: ACCTTATTTTGGATTCCGGATCCCC GGGAATTCGTA
                  TGGAATAAAACCTAAGGCCTAGGGG CCCTTAAGCAT
```

No nick was detected at the center of the BamHI site, that is, between nucleotide A and T. This may due to the decrease in the size of the palindrome, or the removal of the right hand sequence which is required for nick site recognition, or both. However, the nicking was found in the reaction with nuclear extracts from both uninfected and adenovirus-infected HeLa cells, the nicking is not adenovirus-specific.

Adenovirus specific nicking.

The experimental design to detect nicking on the adenovirus l-strand has been described in Chapter III. Two specific fragments, one 71 bases and the other 74 bases, were generated when ^{32}P-labeled mKM4 substrate was incubated in a reaction mixture containing nuclear extract from adenovirus-infected HeLa cells, ddNTPs, and 0.5 mM ATP (shown in Figure IV.2, lane 2). The sequences at the nick sites are shown below where the synthetic primer occupies the first 15 nucleotides, the EcoRI site is underlined, the dashes show the adenovirus sequences, and the vertical arrows indicate nicks:

```
                                 ↓   ↓
CCCAGTCACGACGTT......GGCCAGTGAATT CAT CATCAATAATATACC...
1             15                  71 --- adeno-sequence ---
```

These nicks are usually not detected or only weakly observed in the absence of added ATP (Figure IV.2, lane 1), but concentrations of ATP higher than 0.5 mM also appear to be inhibitory (Figure IV.2, lanes 3-5). Since these nicks have never been detected using extracts from uninfected HeLa cells, they appear to be adenovirus-specific.
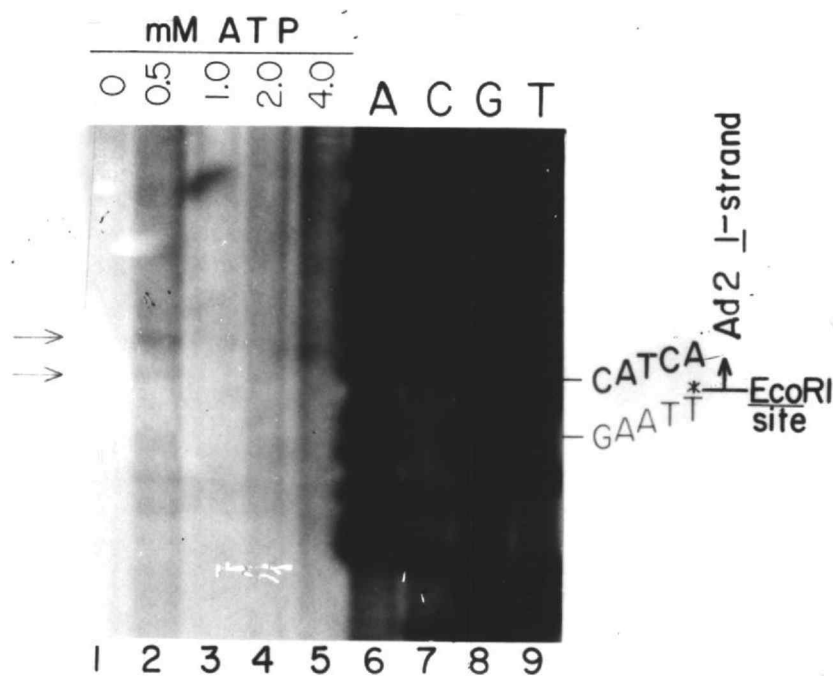
Figure IV.2. Adenovirus-specific nicking on mKM4. Gel electrophoresis was for 3 hours and the film was exposed for 80 hours. The reaction was run under standard conditions with the ATP concentrations in each reaction indicated at the top of each lane. Primer-extended mKM4 was used as the substrate DNA. Lanes labeled A, C, G, and T are dideoxy sequencing ladders which were used as size markers. Arrows show the adenovirus-specific nick sites.

DISCUSSION

Three types of site-specific nicking have been detected within the adenovirus inverted terminal repetition: (a) nicking at G-rich sequences (Chapter III), (b) nicking at the center of palindromic sequences, and (c) nicking at the junction between the adenovirus ITR and flanking vector sequences. Only the last class of site-specific nicks appears to be adenovirus-specific. Interestingly, this class of nicks shows a narrow dependence on ATP concentration which resembles the ATP-dependence of in vitro adenovirus DNA replication. As proposed in Chapter I, terminal protein may act as an origin-specific topoisomerase to catalyze a nick to prime DNA synthesis. In Chapter V, experiments to verify this hypothesis have instead identified HeLa type I topoisomerase. Type I topoisomerases do not require ATP for activity, so it is difficult to attribute nicking at the adenovirus origin to topoisomerase I.

Tamanoi and Stillman (11) have shown that some partially elongated products might have an alternative initiation signal in vitro in the presence of ddGTP. Because the first guanosine residue appears at the 26th nucleotide from the left end of Ad5 sequence, the elongation reaction can be terminated with the addition of ddGTP. The expected elongated product should be 26 nucleotides long. However, several minor fragments in addition to two major fragments appeared, and the two major fragments were 23 and 26 nucleotides long. These products correspond to the adenovirus-specific nicks discussed in this chapter. This could be due to the fact that ddGTP is an effective inhibitor of adenovirus-encoded DNA polymerase (12). ddNTPs

were in the reaction mixture in order to stop the elongation reaction after DNA was nicked.

REFERENCES

1. 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.

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

3. Stillman, B. W., Topp, W. C., and Engler, J. A. (1982) Conserved sequences at the origin of adenovirus DNA replication. J. Virol. 44, 530-557.

4. Robinson, A.J., Younghusband, H.B.,and Bellett, A.J.D. (1973) A circular DNA-protein complexes from adenoviruses. Virology 56, 54-69.

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

6. Carusi, E. A. (1977) Evidence for blocked 5'-termini in human adenovirus DNA. Virology 105, 357-370.

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

8. Pearson, G. D., Chow, K. -C., Enns, R. 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.

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

10. Chow, K.-C.,and Pearson, G.D. (1984) Site-specific nicking within adenovirus inverted terminal repetition. Nucl. Acids Res. 12, 1489-1500.

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

12. Lichy, J. H., Field, J., Horwitz, M. S., and Hurwitz, J. (1982) Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. Proc Natl. Acad. Sci. USA 79, 5225-5229.

CHAPTER V

^{32}P-TRANSFER FROM DNA TO
TOPOISOMERASE I

ABSTRACT

A simple and specific assay was developed to study the transfer of ^{32}P radioactivity from DNA to type I DNA topoisomerase. The assay was based on the formation of a covalent intermediate between DNA and the enzyme. Topoisomerase I binds preferentially to DNAs with the adenovirus type 2 inverted terminal repetition. The concentration of topoisomerase I is at least ten-fold higher in adenovirus-infected HeLa cells and in adenovirus-transformed cells (293 cells) than in uninfected HeLa cells.

INTRODUCTION

The adenovirus origin cloned into circular pBR322 plasmid DNA is efficiently recognized in reaction mixtures containing extracts from adenovirus-infected, but not uninfected, cells (1,2). Initiation at the cloned adenovirus origin resembles adenovirus replication with respect to location, direction, and mode of replication (see Chapter II). By high resolution gel electrophoresis, a site-specific nick was also located at the junction between Ad2 ITR and pBR322 sequences (see Chapter IV).

In order to identify what protein is responsible for the nicking at the replication origin, a simple assay was developed to study the ^{32}P-transfer from DNA to terminal protein (as described in detail in Figure I.2). The assay was essentially based on the covalent bonding between the DNA backbone and the protein. After subsequent removal of unreacted DNA, the ^{32}P radioactivity would be transferred to the protein. A 100K protein was identified with such characteristics.

The protein attaches more frequently to DNAs which contain sequences of the adenovirus type 2 inverted terminal repetition. The concentration of this 100K protein is at least ten-fold higher in adenovirus-infected and adenovirus transformed (293 cells) cells than in uninfected HeLa cells. Further characterization of this protein by partial proteolytic digestion and Western blotting using purified (calf thymus and HeLa) DNA topoisomerase I as a control, the protein was identified as HeLa DNA topoisomerase I.

DNA topoisomerases are enzymes which change the linking number of covalently closed circular DNA (3). Two types of topoisomerases have been identified. Type I DNA topoisomerases characteristically

introduce transient single-stranded DNA breaks and link covalently to the 3'-end of DNA at the nick site (4,5). The reaction is not energy dependent. Type II DNA topoisomerases catalyze DNA topoisomerization reactions by passing DNA double strands through enzyme-bridged double-stranded breaks with consumption of two ATP molecules per passage (6). The conventional assay for topoisomerase I involves measurement of the decrease in the linking number of supercoiled DNA on ethidium bromide-agarose gels (7). It requires purified enzyme, and can only detect the total activities of different topoisomerases in the reaction.

I have developed a simple, specific, quantitative assay for topoisomerase I which can be carried out without interference by contaminating nucleases in crude nuclear extracts. This assay not only can quantitatively estimate the endogenous concentration of topoisomerase I, but also can detect the activities of two topoisomerases in the same reaction if the enzymes have different molecular weights (or different mobilities on SDS-polyacrylamide gels).

MATERIALS AND METHODS

Materials.

Single-stranded M13mp8 and mKM4 DNAs were purified as described (8, also see Chapter III). E. coli DNA polymerase I (Klenow fragment) was from New England Biolabs, Inc.. Deoxyribonuclease I was from Worthington Biochemical Corporation. The synthetic 15-base primer, dNTPs and ddNTPs were from P-L Biochemicals. $[\alpha-^{32}P]dCTP$ (5000 Ci/mmol) was from New England Nuclear. Purified calf thymus and

HeLa topoisomerase I were generously provided by Dr. Leroy F. Liu.

Labeling of the substrate DNA.

The annealing reaction contained 120 ng/µl mKM4 DNA, 0.5 ng/µl primer, 10 mM NaCl, 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, and 0.8 mM Na_2EDTA. The solution was boiled for 3 minutes and then equilibrated to the ambient temperature for 45 minutes to hybridize the 15-base primer to the template DNA. Primer extension was carried out in a reaction mixture containing the primer-hybridized DNA, 120 mM NaCl, 20 mM Tris-HCl (pH 7.5), 14 mM $MgCl_2$, 4 mM dithiothreiotol, 0.4 mM EDTA, 25 µM each of dATP, dGTP, and dTTP, 0.5 µM $[\alpha\text{-}^{32}P]dCTP$ (125 µCi), and 1 unit of E. coli DNA polymerase I (Klenow Fragment). After incubating the solution at 37°C for 15 minutes, the dCTP concentration was adjusted to 80 µM and incubation was continued for another 15 minutes. About 600 to 2000 nucleotides were polymerized under these conditions. The reaction was then inactivated at 65°C for 10 minutes and the volume of the solution was adjusted to 100 µl with STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA). The unincorporated, free nucleotides were separated from the extended products by the spun-column method (9) using G-75 Sephadex as a gel filtration medium. The eluate was precipitated by adding 250 µl ethanol. The precipitate was resuspended in 40 µl TE, and 0.5 µl of this solution was analyzed by PEI cellulose thin layer chromatography to assure that there was no contamination by unincorporated $[\alpha\text{-}^{32}P]$ nucleotides.

Reaction conditions.

Nuclear and cytoplasmic extracts were prepared from uninfected and adenovirus-infected HeLa cells and 293 cells as previously described (10). The protein concentrations in the nuclear extracts were measured at A_{595} using 2 mg/ml bovine serum albumin as a standard (Bio-Rad protein assay kit), and they ranged from 4 to 6 mg/ml. The concentrations in the cytoplamic extracts ranged from 13.5 to 17 mg/ml. The standard reaction mixture contained 30 mM NaCl, 60 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 µM ddCTP, 1 mM ATP, 2 µl nuclear extract (or cytoplamic extract or purified topoisomerase I), and 300 ng DNA substrate in a total volume of 10 µl. After incubation at 37°C for 30 minutes, 1 µl DNase I (1 mg/ml) was added and the incubation continued for 10 minutes. The reaction was stopped by adding 11 µl of double-strength Laemmli buffer (11). Samples were boiled for 3 minutes and resolved by electrophoresis on 10% polyacrylamide/$NaDodSO_4$ (SDS) gels with a 6% stacker (using 50 mM Tris-borate, pH 8.3, with 1 mM EDTA and 0.1% SDS as running buffer). The electrophoresis was carried out at 120 volts for one hour and 45 minutes. After fixation in 50% methanol, gels were stained with silver nitrate, dried and autoradiographed at -20°C overnight. The autoradiogram was scanned with a Zeineh soft laser densitometer (model SL-504-XL, Biomed Instrument Inc.) and analyzed with a electrophoresis reporting integrator program (ERIP-V3A, Biomed Instruments Inc.). The intensity of the radioactive band which was scanned and integrated was corrected to account for the protein concentration in each sample.

RESULTS

^{32}P-transfer from DNA backbone to topoisomerase I.

The strategy of ^{32}P-transfer from DNA to topoisomerase I is detailed in Figure V.1. As expected, a specific radioactive band appeared between 85,000-dalton (85K) and 120,000-dalton (120K) protein markers (Figure V.2). The molecular weight is approximately 100,000-dalton. The radioactive band shown on the autoradiogram of the SDS-polyacrylamide gel suggests that the radioactivity was obtained from the DNA directly. In order to verify this point, the purified, primer-extended DNA was analyzed by PEI cellulose thin layer chromatography, with 1 M LiCl (pH 7.5) and autoradiographed for the same length of time as used for the exposure of the gel after electrophoresis. No radioactive spot corresponding to the input nucleotide ([α-^{32}P]dCTP) was observed (data not shown). Thus, free [α-^{32}P]dCTP in the reaction mixture cannot account for the labeling of the 100K polypeptide. To determine whether the 100K band reflects a segment of DNA which is protected from DNase I digestion, some samples were treated with 1 mg/ml Pronase in the presence of 0.1% SDS at 37°C for 1 hour after DNase I digestion. Pronase (Figure V.3) or trypsin (Figure V.7) digestion eliminated the ^{32}P-labeled 100K polypeptide. Although the reaction does not require ATP (Figure V.4, lane 1), the ^{32}P-transfer reaction was marginally stimulated by the addition of ATP (as shown in Figure V.4, lanes 2 and 3).

Molecules containing the Ad2 ITR sequence (as shown in Figure V.5) appear to be more efficiently used as substrates when the 100K band in each lane was quantitated by laser scanner densitometry. However, no radioactive band was detected when the substrate DNA was

Figure V.1. Strategy to detect transfer of phosphate from DNA to topoisomerase I. A. Thin line represents template DNA, either M13mp8 or mKM4. The 15-base primer, designated by the open bar, was hybridized to the template DNA to synthesize the complementary strand by the primer extension method. Labeled DNA molecules were separated from free nucleotides by gel filtration (as described in detail in MATERIALS AND METHODS). The adenovirus l-strand is labeled if the template DNA is mKM4. B. ^{32}P-labeled DNA reacts with topoisomerase I to form a DNA-protein complex with the enzyme linked covalently to the 3'-end of the nicked strand (5). The reaction mixture was·then treated with DNase I to remove unreacted DNA, and the labelled phosphate group in the DNA backbone was thus transferred to topoisomerase I. C. The phosphate-protein complex was resolved on SDS-PAGE. If the enzyme is HeLa topoisomerase I, the radioactivity will be located at 100K region on the autoradiogram. If the input enzyme is calf thymus topoisomerase I, the 82K band will be labeled. If both enzyme are used, then both bands would be labeled.

A

Primer

M13mp8
or
mKM4

B

HO—(topo I)

(5')··pN^{32}pC···(3')
(3')···N p Gp··(5')

^{32}p–O–(topo I)

(5')··pN   C···(3')
(3')···N p Gp··(5')

DNase I

(pN)$_?^{32}$p–O–(topo I)

C

HeLa   Mixed   Calf

←— 100 kd
←— 82 kd
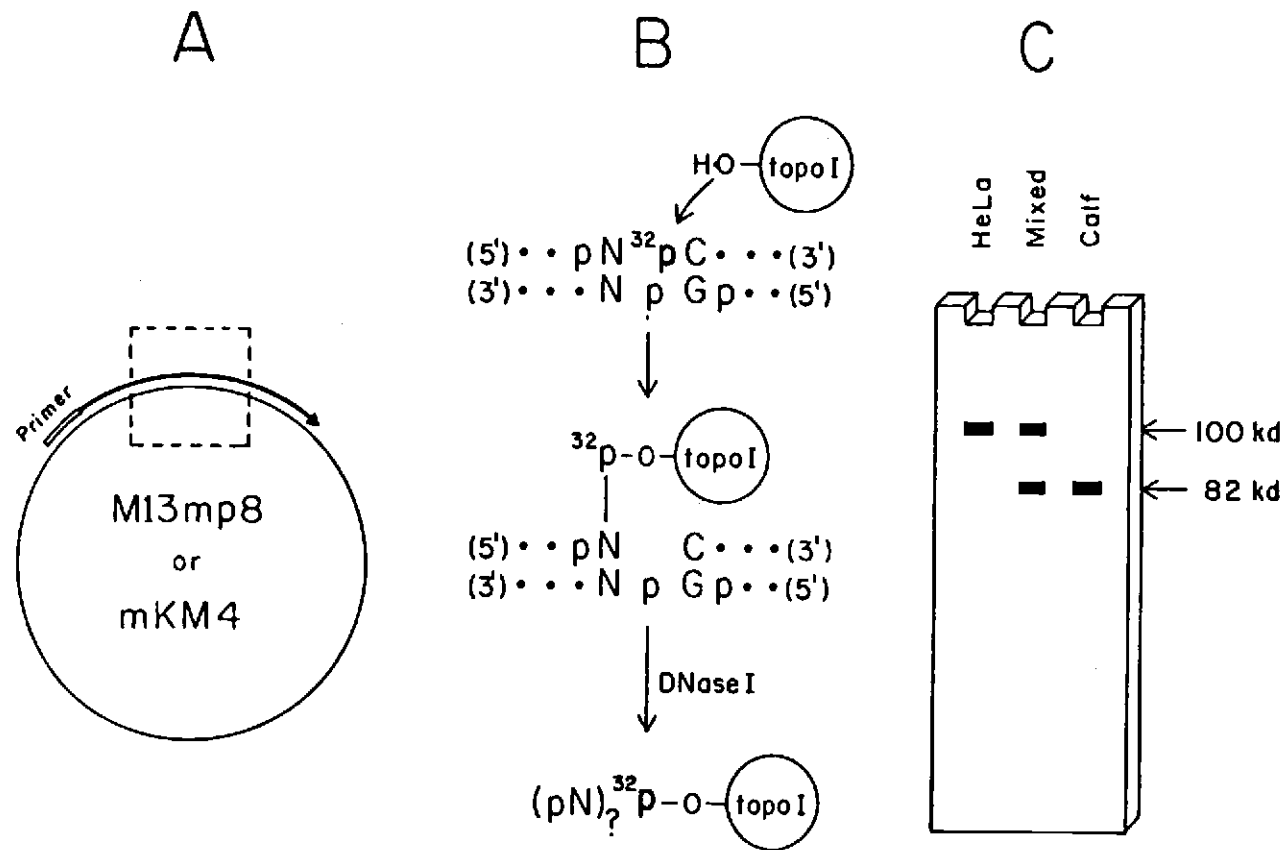
Figure V.1. Strategy to detect transfer of phosphate from DNA to topoisomerase I.

Figure V.2. Transfer of ^{32}P-radioactivity from DNA to a 100K protein analyzed by SDS-polyacrylamide gel electrophoresis. The l-strand of adenovirus DNA was synthesized on mKM4 using [α-^{32}P]dCTP and other unlabed dNTPs. The purified labeled-DNA was incubated in a standard in vitro reaction (2, 8). Samples were digested with 100 µg/ml pancreatic DNase I for 10 minutes at 37°C. The reaction was stopped by adding 2X Laemmli buffer, and the material was analyzed by SDS-polyacrylamide gel electrophoresis (11) (Ad2 capsid proteins were used as markers). The gel was stained with silver nitrate, dried and the radioactivity was localized by autoradiography. UNINF: the reaction mixture contained nuclear extract from uninfected HeLa cells. INF: the reaction mixture contained nuclear extract from Ad2-infected HeLa cells.
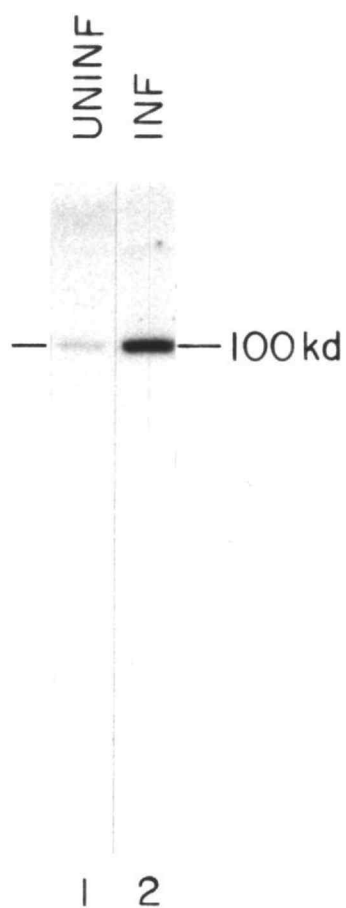
Figure V.2. Transfer of ^{32}P-radioactivity from
DNA to a 100K protein analyzed by
SDS-polyacrylamide gel electrophoresis.
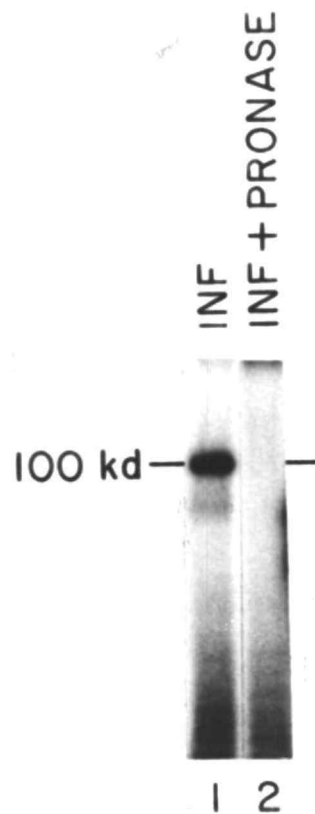
Figure V.3. Pronase treatment of the _in vitro_ reaction product. After samples were treated with DNase I (as described in Figure V.2.), one of the samples was further digested with 1 mg/ml Pronase in the presence of 0.1% SDS at 37°C for one hour (as shown in lane 2). The nuclear extract used in this experiment was from adenovirus-infected HeLa cells. Lane 1, control. Lane 2, Pronase digestion.

mM ATP

0 0.5 1 2 4

— 100 kd

1 2 3 4 5
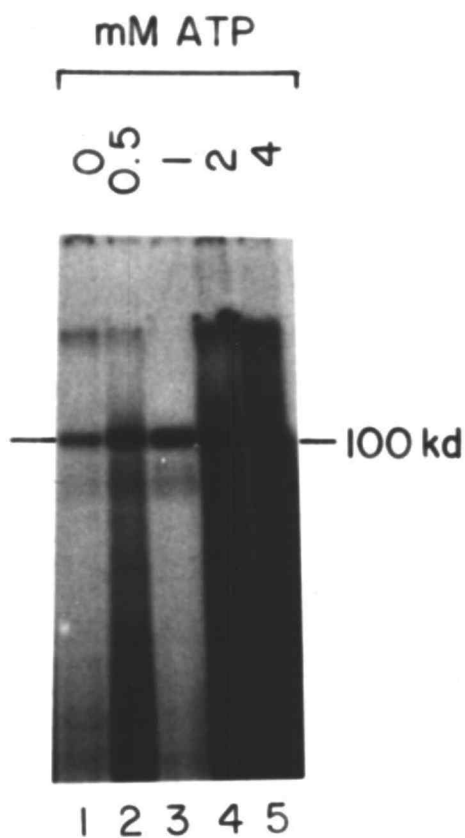
Figure V.4. Effect of ATP concentration on the phosphate transfer reaction. The assay was performed under standard conditions (as described in MATERIALS AND METHODS) except that the ATP concentration was varied from 0 to 4 mM (as indicated at the top of each lane). Nuclear extract used in this assay was from adenovirus-infected HeLa cells.

Figure V.5.   Sequence preference in the phosphate-transfer reaction.
A. The asterisk represents α-^{32}P-labeled nucleotide used in the
primer extension reaction. mKC96 is a single-stranded DNA template
which contains the XbaI-E fragment of adenovirus type 2 DNA cloned
into EcoRI site of M13mp9 in the orientation such that when the
complementary strand is synthesized, the r-strand of adenovirus DNA
is labeled. XD-7 and mKM4 have been described (8). After primer
extension and gel filtration, some of the substrate DNA was treated
with either EcoRI or HindIII restriction enzymes (as indicated at the
top of each lane) to linearize the partially double-stranded DNA
before adding it to the reaction mixture. XD 7-C* was nick-translated
relaxed circular DNA. In this experiment, nuclear extract was from
adenovirus-infected cells. B. [α-^{32}P]dCTP was used for primer
extension. INF: Nuclear extract from Ad2-infected HeLa cells. UNINF:
Nuclear extract from uninfected HeLa cells. CALF represents purified
calf thymus topoisomerase I used as an internal control.

Figure V.5. Sequence preference in the phosphate-transfer reaction.

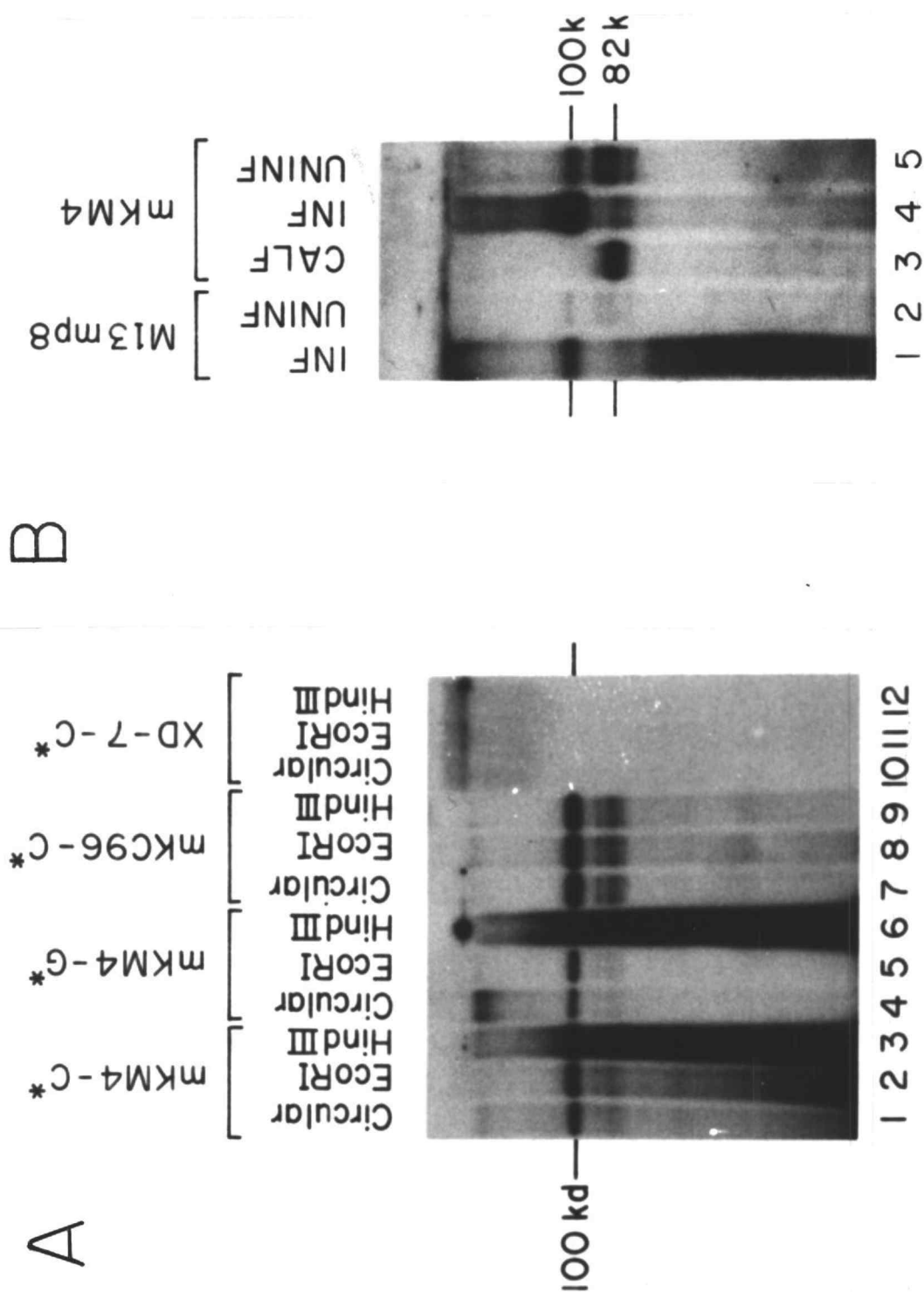a nick-translated XD-7 molecule (Figure V.5A, lanes 10-12). Also, the ^{32}P-transfer reaction is not [α-^{32}P] dCMP-specific (Figure V.5). When the base composition ratio of the first 200 nucleotides in the sequence of l-strand of the Ad2 DNA was analyzed, the percentage of A, C, G, and T is 22.5%, 13.5%, 37.5%, and 26.5%, respectively. If the protein attackes the DNA backbone randomly, the intensity of the 100K band in each lane should be in the ratio of 2:1:3:2. But this is not the case. There is no significant variation in the intensity of the 100K band in each lane (data not shown). As shown in Figure V.5B, the intensities of the 100K bands in lanes 4 and 2 are in the ratio of 4:1 and the composition ratio of dCMP in the DNA is 1:2. The binding of the 100K protein to the DNA is obviously nonrandom. The 100K protein was characterized further in order to differentiate between HeLa topoisomerase I (100K) and adenovirus pre-terminal protein (87K). The mechanism of the topoisomerase I reaction is similar to the mechanism which has been proposed for terminal protein (or its precursor). The different mobilities, 100K instead of 87K (10), or 80K (14,15), could be due to the SDS-polyacrylamide gel system and standard markers used for analysis. The partial trypsin digests (Figure V.7A) did not really establish whether the 100K protein was a DNA topoisomerase I or terminal protein precursor. The partial trypsin-digested fragments of DNA topoisomerase I are 82K, 67K, and 57K; those of terminal protein precursor are 80K, 62K, and 55K. The 100K protein was also analyzed by Western blotting (Figure V.7B). The nuclear extract (from Ad2-infected HeLa cells) was loaded onto 10% SDS-polyacrylamide gels directly without incubation with labelled DNA substrate. Nuclear extracts were also partially digested
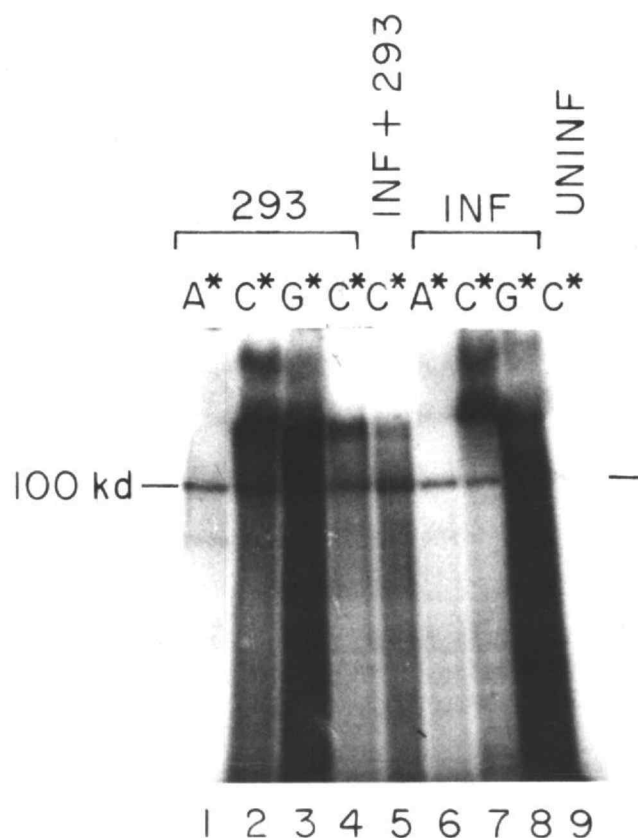
Figure V.6. Deoxynucleotide preference in the phosphate-transfer reaction. Capital letters with asterisks represent the labeled nucleotides; e.g., A^* is $[\alpha^{-32}P]$dATP which was used in the primer extension reaction. The labeled DNA was separated from unincorporated, free nucleotides by gel filtration and reacted with nuclear extract (described in MATERIALS AND METHODS). 293: Nuclear extract from 293 cells. INF: Nuclear extract from adenovirus-infected HeLa cells. UNINF: Nuclear extract from uninfected HeLa cells.
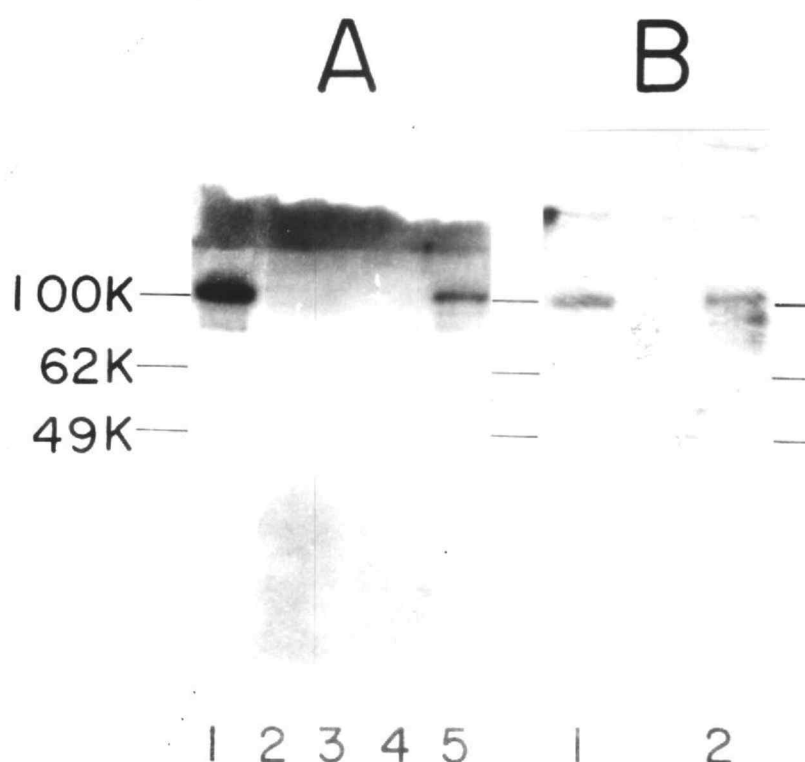
Figure V.7. Characterization of the 100K protein. A. Partial trypsin digestion of the 100K protein. Lane 1, 100K protein labeled in the standard reaction. After the reaction, the 100K protein was treated at room temperature with trypsin at concentration 100 μg (lane 2), 10 μg (lane 3), 1 μg (lane 4) or 100 ng (lane 5) per reaction. B. Western blotting of the 100K protein in the nuclear extract detected by antibody against HeLa topoisomerase I. Lane 1, crude nuclear extract only. Lane 2, nuclear extract was treated with 100 ng of trypsin before electrophoresis. 49K and 62K are positions of protein markers run on the parallel lanes.

with trypsin before loading onto SDS-gel. After transferring the proteins from SDS-gel to a nitrocellulose membrane, antibody which was raised against HeLa DNA topoisomerase I was applied, and the [^{125}I] A protein was used to detect antigen-antibody complexes. Antibody to HeLa topoisomerase I identified a 100K polypeptide (compare Figure V.7A, lane 1 with Figure V.7B, lane 1). Moreover, partial trypsin digestion of ^{32}P-labeled 100K protein generated polypeptide fragments which comigrated with partial trypsin fragments visualized with topoisomeras eantibody (compare Figure V,7A, lane 5 with Figure V.7B, lane 2). This identifies the 100K polypeptide as HeLa topoisomerase I.

Quantitation of topoisomerase I activity

Purified calf thymus topoisomerase I with molecular weight of 82,000 was reacted with ^{32}P-labeled substrate DNA. Figure V.8 shows that when calf thymus topoisomerase I was assayed alone, only the two highest concentrations of a serial dilution showed activity (lane 8 and 9). However, when the enzyme was diluted into nuclear extract before starting the reaction, the enzyme was active down to a concentration of 75 ng per reaction (lane 7). The intensities of the 82K bands were analyzed by soft laser scanning densitometry and plotted against the amount of the input enzyme. The intensity of the 82K band was linearly proportional to the amount of the input topoisomerase I (Figure V.8B.). The concentration of endogenous HeLa topoisomerase I (100K band in lanes 2 to 7) can be extrapolated from this curve. The intensity of the 100K band in lane 3 through 7 is identical to that in lane 2 which does not have exogenously added

116

Figure V.8. Stoichiometric estimation of topoisomerase I. Lanes 8-12
show the reactions of serially diluted calf thymus topoisomerase I.
Lanes 3-7 show the reactions of calf topoisomerase I diluted into
nuclear extract from adenovirus-infected HeLa cells. Lane 2 shows the
reaction with nuclear extract from adenovirus-infected HeLa cells.
Lane 1, nuclear extract was from 293 cells. The number at the top of
each lane is the amount of purified calf thymus topoisomerase I added
to each reaction. The intensities of 100K band in lanes 2-7 are
identical by soft laser scanner densitometry. The intensity of the
82K band is proportional to the amount of the input enzyme (as shown
in B). B. The band intensity of 82K topoisomerase I is plotted
against the amount of the input enzyme.
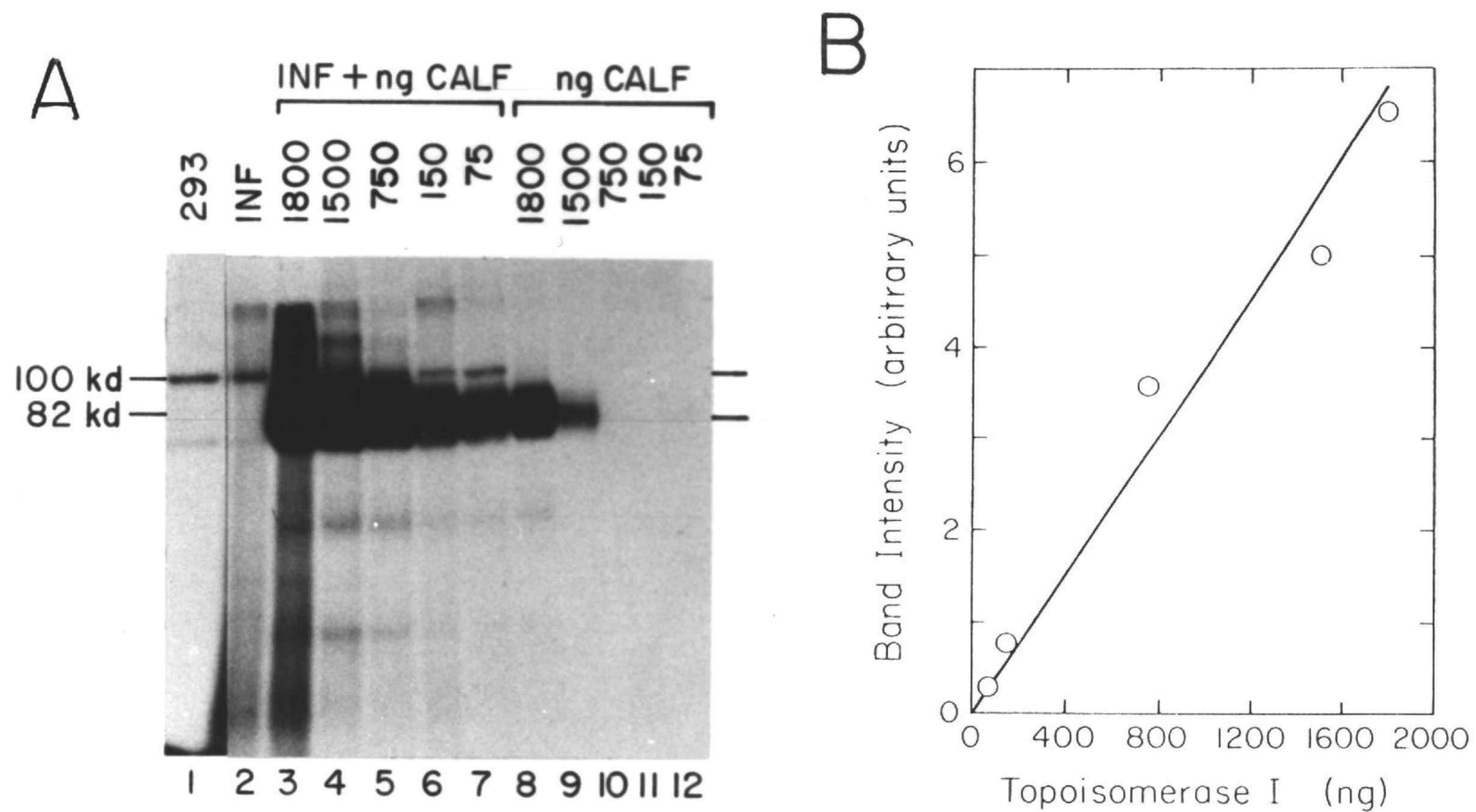
Figure V.8. Stoichiometric estimation of topoisomerase I.

calf topoisomerase I. Exogenously added topoisomerase I does not interfere the activity of the endogenous topoisomerase I. The extrapolation of endogenous concentration of topo I does not require any correction factor. About 6.2 ng topoisomerase I per reaction was quantitated in extract from adenovirus-infected HeLa cells corresponding to 4×10^5 molecules/infected cell. The sensitivity of this assay was 0.3 ng per band. Some minor bands appear below the 82K band. Their molecular weights are 62K and 30K, respectively. They are proteolytic fragments of the 82K protein (5). There is another band of which the molecular weight is equivalent to 170K. It might be DNA topoisomerase II. The concentration ratio of topoisomerase I to topoisomerase II in the purified calf thymus preparation is 50:1 (also see Figure V.5B, lane 4, the intensities of 100K and 170K proteins are in the ratio of 50:1). Figure V.9A shows the heat inactivation of HeLa topoisomerase I in nuclear extracts. The activity of HeLa topoisomerase I could be totally inactivated by incubating the nuclear extract at 60°C for 10 minutes or longer. Purified HeLa topoisomerase I was then diluted into this heat-inactivated nuclear extract before starting the reaction. The results are shown in Figure V.9B. The 100K band appeared as expected, and the intensity of the 100K band on the autoradiogram was proportional to the concentration of the input enzyme (compare lanes 3 and 4). Twice as much input enzyme gave twice the intensity of the 100K band. Some minor bands also appeared in Figure V.9B. Their molecular weights are 72K and 55K, respectively, and they are proteolytic products of HeLa type I DNA topoisomerase (5). The stabilizing effect of the nuclear extract on the purified enzyme was

Figure V.9. A. Inactivation of endogenous HeLa topoisomerase I activity. The nuclear extract was incubated at 60°C for a period of time, as indicated at the top of each lane (in minutes), before adding to the reaction mixture.   B. Stoichiometric estimation of exogenously added purified HeLa topoisomerase I. Lane 1 is from an overexposed film. UNINF: the nuclear extract was from uninfected HeLa cells. Lane 2, reaction with the purified enzyme only. Lane 3 and 4, the purified enzyme was diluted into the heat-inactivated nuclear extract before adding to the reaction mixture. The numbers at the top of lanes 2-4 give the amount in ng of purified enzyme used in each reaction.
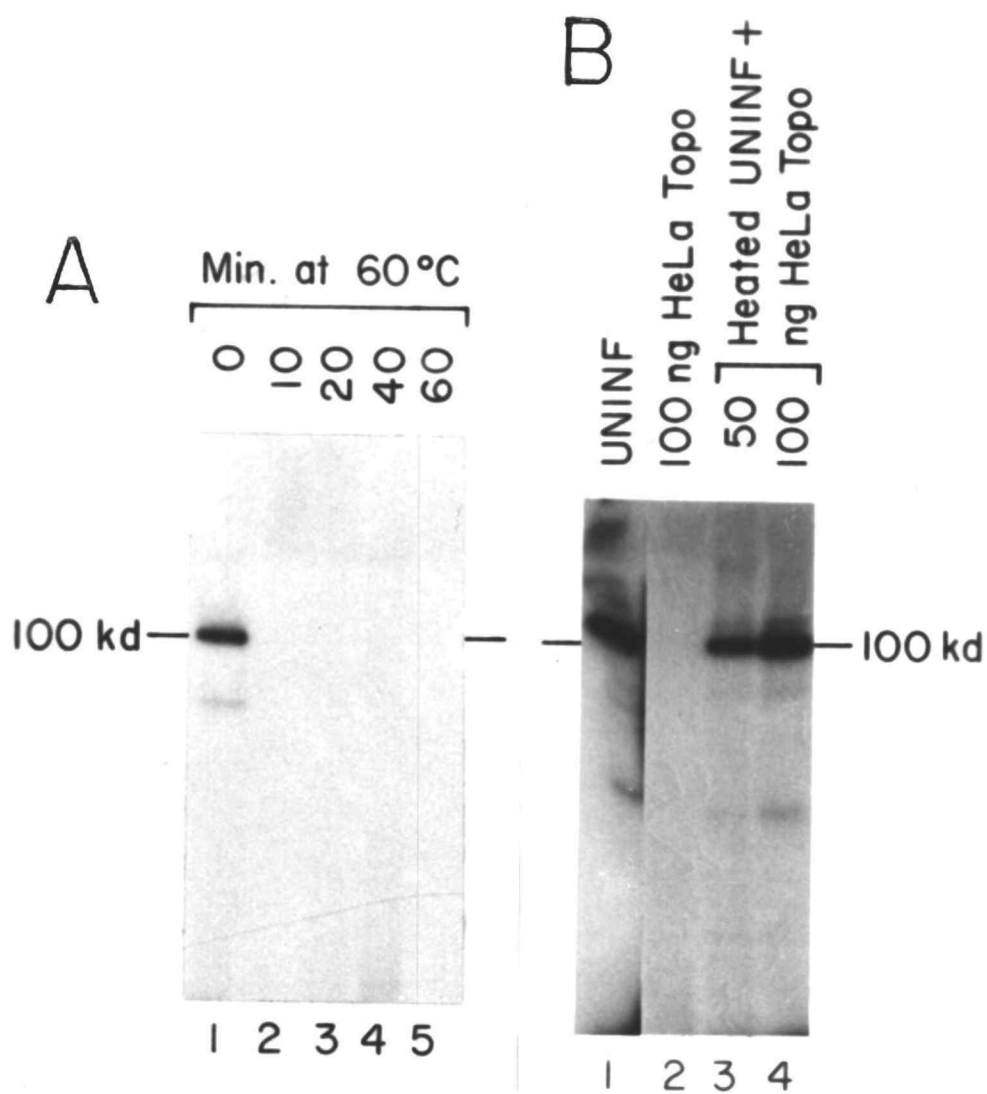
Figure V.9. Stoichiometric estimation of exogenously
added purified HeLa topoisomerase I.

still observed (compare lanes 2 and 3 in Figure V.9B). It is not yet known what the stabilizing factor is. Since the nuclear extract had been heat inactivated, the stabilizing factor is heat-stable and likely has no enzymatic activity. The endogenous concentration of HeLa topoisomerase I in uninfected HeLa cells is about 4×10^4 molecules/cell.

The level of HeLa topoisomerase I activity is at least 10-fold higher in adenovirus-infected and adenovirus-transformed cells than in uninfected HeLa cells. In order to determine whether this difference is caused by loss during the isolation of nuclei, activity was measured in both nuclear and cytoplasmic extracts. The results are present in Figure V.10. No topoisomerase I activity was observed in cytoplasmic extracts from either adenovirus-infected or uninfected HeLa cells (Figure V.10, lanes 1 and 3). On the other hand, the activity of topoisomerase I in the nuclear extract from adenovirus-infected cells was 40-times higher than in the nuclear extract from uninfected cells (Figure V.10, lanes 2 and 4).

DISCUSSION

The ^{32}P-transfer experiment was designed to demonstrate that terminal protein (or its precursor) is responsible for the initiation of in vitro replication on supercoiled DNA (1,2, and Chapter II) and the site-specific nicking at the junction between pBR322 and Ad2 ITR sequences (Chapter IV). Base on the model of initiation of replication proposed in Chapter I (see Figure I.1), it was expected that radioactivity would be transferred from ^{32}P-labeled DNA to terminal protein. However, instead of finding ^{32}P-terminal protein

Figure V.10. Comparison of topoisomerase I activity in cytoplasmic and nuclear extracts from uninfected or adenovirus-infected HeLa cells. INF: adenovirus-infected extracts. UNINF: uninfected extracts. CYTO: cytoplasmic extract. NUC: nuclear extract.
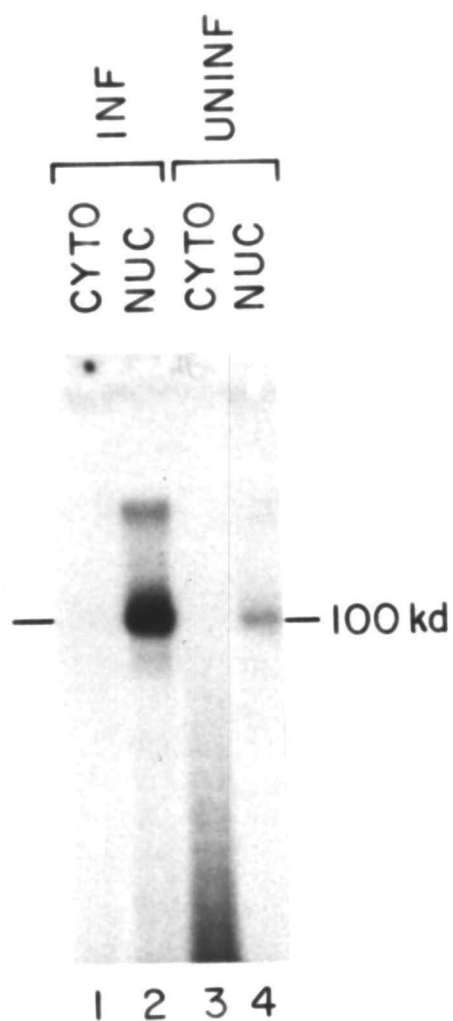
complex, ^{32}P-labeled topoisomerase I was identified in this experiment. Both topoisomerase I and terminal protein precursor give similar proteolytic fragments: 80-87K, 62-67K, and 55-57K (5,10,14,15,22). Because the protein which was labeled in the transfer reaction is ATP-independent (Figure V.4), comigrates with the purified topoisomerase I from HeLa cells (Figure V.9), has the identical reactivity of topoisomerase I (Figures V,8 and 9), and cross reacts with the antibody raised against HeLa topoisomerase I, I conclude that this protein is indeed topoisomerase I.

This assay for topoisomerase I is: (a) simple: activity can be assayed in a crude nuclear extract without any interference from contaminating nucleases; (b) specific: the assay is essentially based on the stoichiometric formation of a covalent intermediate between a tyrosine residue of the enzyme and the 3'-phosphate at the break in the DNA, only topoisomerase I and its functional proteolytic fragments will be detected; and (c) quantitative: by using an exogenous standard, the endogenous concentration of topoisomerase I in the reaction can easily be determined. The sensitivity of assay was 0.3 ng/band; that is, 3 fmol/band. The level of DNA topoisomerase I is at least ten-fold higher in adenovirus-transformed human cells (293 cells) and adenovirus-infected HeLa cells, than in uninfected HeLa cells when assayed by the ^{32}P-transfer method. In 293 cells and adenovirus-infected HeLa cells, the concentration of topoisomerase I is about 4 X 10^5 molecules/ cell, and in uninfected HeLa cells it is around 4 X 10^4 molecules/cell. Several possibilities which would cause this difference were ruled out: (a) It is not nonspecific damage to the nuclear membrane by the adenovirus infection. I have

demonstrated that no topoisomerase I activity was detected in cytoplasmic extracts from either adenovirus infected or uninfected HeLa cells (Figure V.10). (b) It is not due to the inhibition of cellular DNA synthesis after adenovirus infection. The level of topoisomerase I also increased in 293 cells which grow and synthesize their DNA normally. (c) It is not due to adenovirus DNA replication. When hydroxyurea, which inhibits viral DNA replication was added two hours postinfection, the level of topoisomerase I still increased. (d) In a time course study, the level of topoisomerase I increased six hours after viral infection (data not shown).

Only the E1A region of adenovirus genome is expressed in 293 cells.The adenovirus E1a gene is the first to be transcribed upon viral infection, and the transcription of the remaining viral genes requires one of the E1A gene products (13, 17). It also induces the synthesis of a 70K HeLa heat-shock protein (17), activates exogenous genes in trans and even overcome the cis-requirement for enhancer or activator sequences in transient expression assay (18-20). Thus it is tempting to speculate that E1A gene products induce the expression of the cellular topoisomerase I gene in this case.

During in vitro adenovirus DNA replication, the chain elongation terminates randomly approximately one third the way along the genome (21). Nuclear factor II, which contains a DNA topoisomerase I-like activity, facilitates the completion of full length DNA chains, and, in fact, nuclear factor II can be totally replaced by the purified topoisomerase I from either HeLa cells or calf thymus tissue (22). Therefore, the increase of topoisomerase I level in adenovirus-infected and adenovirus-transformed cells might be an

example of viral induction of a cellular factor which is required for viral transcription or replication.

REFERENCES

1. Pearson, G.D., Chow, K.-C., Corden, J.L., and Harpst, J.A. (1981) Replication directed by a cloned adenovirus origin, in: Ray, D.S. and Fox, C.F. (Eds.), ICN-UCLA symposium on molecular biology, Vol. 21, (Academic Press, New York), pp.581-595.

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

3. Gellert, M. (1981) DNA topoisomerase. Ann. Rev. Biochem. 50, 879-910.

4. Wang, J.C. (1971) Interaction between DNA and an Escherichia coli protein. J. Mol. Biol. 55, 523-533.

5. Halligan, B.D., Davis, J.L., Edwards, K. A., and Liu, L.F. (1982) Intra- and intermolecular strand by HeLa DNA topoisomerase I. J. Biol. Chem. 257, 3995-4000.

6. Cozzarelli, N.R. (1980) DNA gyrase and the supercoiling of DNA. Science 207, 953-960.

7. Keller, W. (1975) Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA 72, 4876-4880.

8. Chow, K.-C.,and Pearson, G.D. (1984) Site-specific nicking within adenovirus inverted terminal repetition. Nucleic Acids Research 12, 1489-1500.

9. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, (Cold Spring Harbor Lab., New York), pp. 464-467.

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

11. Laemmli, J. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-686.

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

13. Berk, A. J., Lee, F., Harrison, T., Williams, J., and Sharp, P.A. (1979) Pre-early adenovirus 5 gene product regulates synthesis of erly viral messenger RNAs. Cell 17, 935-944.

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

15. Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981) Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and the 5'-dCMP in vitro. Proc. Natl. Acad. Sci. USA 78, 2678-2682.

16. Carusi, E. A. (1977) Evidence for blocked 5'-termini in human adenovirus DNA. Virology 76, 380-396.

17. Nevins, J.R. (1982) Induction of the synthesis of a 70,000-dalton mammalian heat-shock protein by the adenovirus E1A gene product. Cell 29, 913-919.

18. Treisman, R., Green, M.R., and Maniatis, T. (1983) Cis and trans activation of globin gene transcription in transient assays. Proc. Natl. Acad. Sci. USA 80, 7428-7432.

19. Green, M.R., Treisman, R.,and Maniatis, T. (1983) Transcriptional activation of cloned human β-globin genes by viral immediate-early gene products. Cell 35, 137-148.

20. Imperiale, M.J., Feldman, L.T.,and Nevins, J.R. (1983) Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell 35, 127-136.

21. Lichy, J.H., Nagata, K., Friefeld, B. R., Enomoto, T., Field, J., Guggenheimmer, R.A., Ikeda, J.-E., Horwitz, M.S.,and Hurwitz, J. (1983) Isolation of proteins involved in the replication of adenoviral DNA in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 731-740.

22. Stillman, B. W. (1983) The replication of adenovirus DNA with purified proteins. Cell 35, 7-9.

CHAPTER VI

CLONING OF PALINDROMIC ITRs OF ADENOVIRUS 2 DNA
IN recBCsbcB STRAINS OF E. COLI

INTRODUCTION

Ruben et al. (1) have shown that 8-15% of intracellular Ad5 DNA exists as covalently closed circles, probably with palindromic ITRs. Although the precise nucleotide sequence through the joint (the covalent bridge between two ITRs) remains to be determined, joint structures were indeed detected as early as 3 to 5 hours post-infection. This suggests that the incoming molecules might be circularized before the onset of viral replication, and circularization could be a required process for viral DNA synthesis.

Supercoiled XD-7 DNA, which contains the Ad2 terminal XbaI-E fragment replicates in the in vitro system (2,3) as rolling circles with displaced single-stranded tails (strand-displacement mechanism). The origin of the displaced DNA tails was mapped by electron microscopy to the terminus of the Ad2 ITR insert (3). A specific nick was also located at the junction between vector (pBR322) and Ad2 ITR sequences (Chapter IV). However, the nicking signal was very weak and the predicted transfer of ^{32}P radioactivity from DNA to terminal protein (or its precursor) was not clearly observed (Chapter V).

The recBC gene product, exonuclease V, and the sbcB gene product, exonuclease I, have been shown to be responsible for genetic recombination and the excision of palindromic sequences in bacteria (4). For this reason, recBCsbcB strains of bacteria were used to attempt to clone palindromic ITRs of Ad2 DNA.

MATERIALS AND METHODS

Materials.

Restriction enzymes, T4 DNA ligase and alkaline phosphatase were from BRL. The enzymes were used as recommended by the supplier. Ampicillin and tetracyclin were from Sigma. pEcoRI B Ad5 (pIB5), which contains Ad5 EcoRI-B fragment cloned into EcoRI site of pBR322 (5), was provided by Dr. K. L. Berkner. JC9387 and JC11850, recBCsbCB strains of E. coli, were provided by Dr. F.W. Stahl. The recombination genotype of JC9387 is recB21recC22sbcB15, and that of JC11850 is recB21recC22sbcB15recF143 (6).

Methods.

The strategy of cloning is described in figure VI.1. CsCl purified, supercoiled DNA was cut· with HindIII and EcoRI. The completion of each restriction enzyme cleavage step was examined by 1% EtBr-agarose gel electrophoresis (VI.2A and B). The desired fragments were electroeluted and extracted with n-butanol equilibrated with TE buffer to remove EtBr. The vector fragment was further treated with alkaline phosphatase at 65°C for 4 to 12 hours. The purified fragment was then joined to the vector at a 3 to 1 ratio using T4 DNA ligase for 1 hour at 20°C. An aliquot of reaction mixture was examined by EtBr-agarose gel electrophoresis (figure VI.3B) to decide the amount of DNA to use for transformation. As shown in figure VI.3B, lane 1, the concentration of the palindrome-containing DNA was about 50 ng/10 μl. The concentration of DNA was adjusted to 1 ng, 5 ng and 10 ng per reaction before transforming

Figure VI.1. Strategy of cloning palindromic inverted terminal repetitions of Ad2 DNA into pBR322. (a) Clone 7 was linearized with both EcoRI and HindIII. (b) Linearized Clone 7 was treated with alkaline phosphatase at 65°C for 4 hours. (c) pEcoRI B Ad5 (pIB5) was restricted with HindIII and EcoRI. (d) A 0.9 kb fragment which contained the Ad5 ITR was selected by gel electrophoresis. (e) The selected fragment was ligated to the alkaline phosphatase-treated Clone 7 with T4 DNA ligase at 16°C for 4-12 hours. Clone 7 is a subclone of XD-7 (as shown in Figure II.1) with a deletion at the XbaI site of about 80 nucleotides. The thin line of the circle represents pBR322 vector. The thick line represents adenovirus DNA. The open box is the adenovirus ITR and arrowa point in the direction of ITR. The clone with the expected palindromic ITR sequence is displayed at the bottom of the Figure. Asterisks show the EcoRI sites. The EcoRI recognition sequence is underlined.
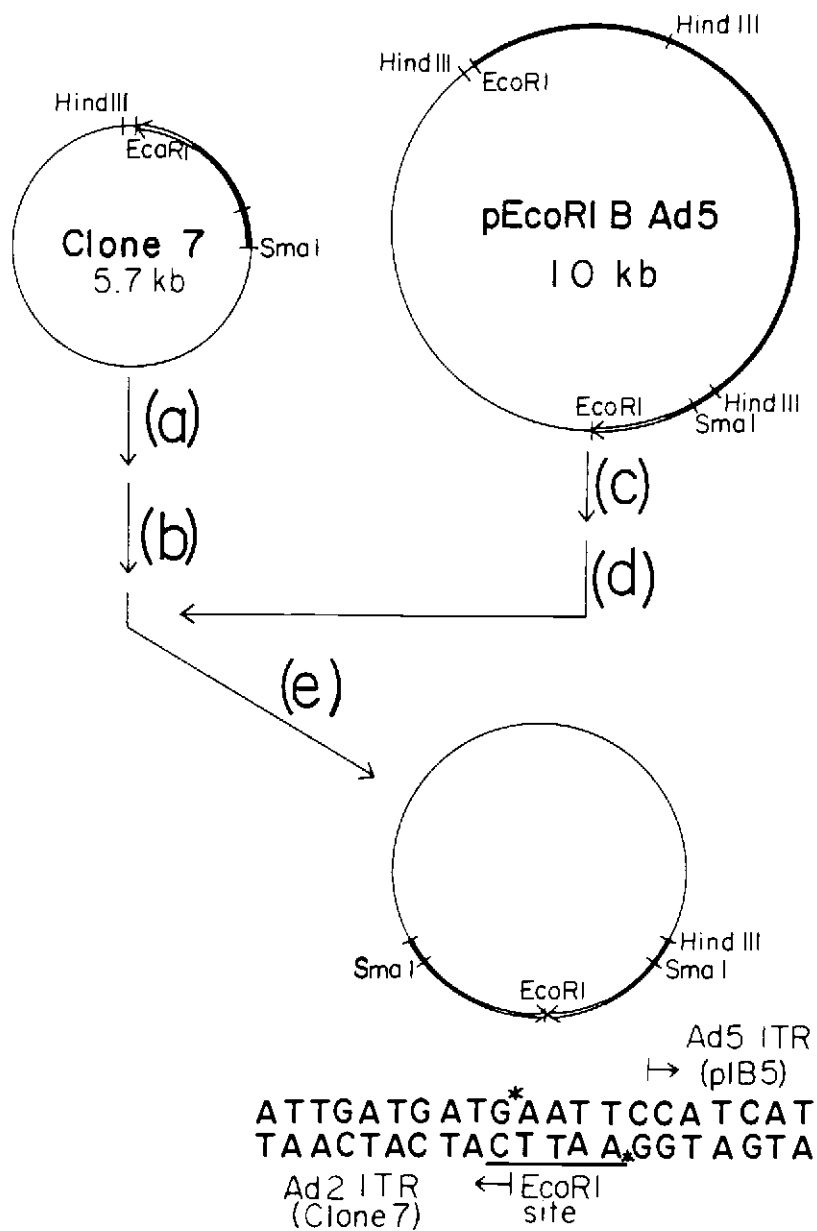
Figure VI.1. Strategy of cloning palindromic
inverted terminal repetitions of
Ad2 DNA into pBR322.
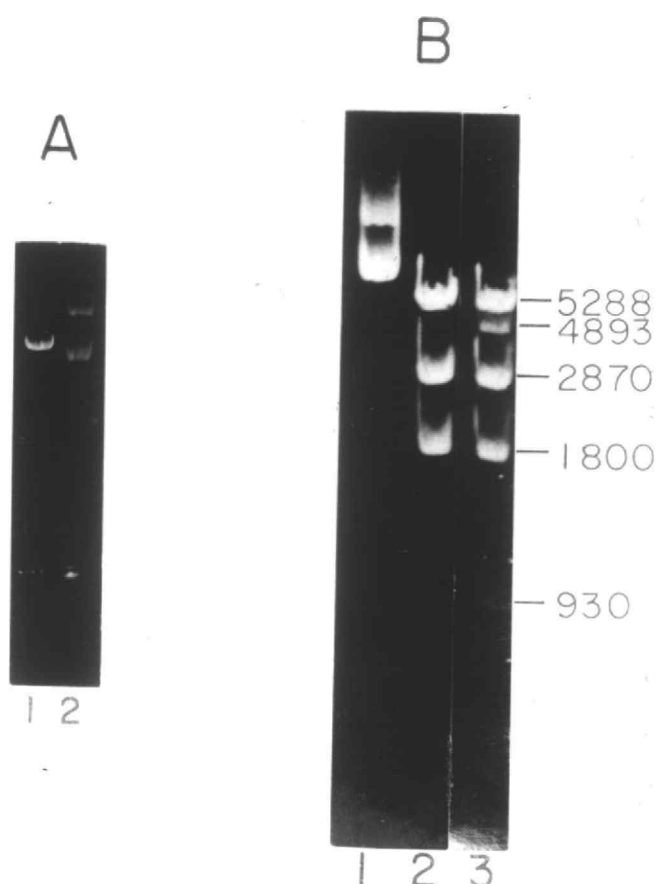
Figure VI.2. Electrophoretic analysis of restricted Clone 7 and pIB5. A. Lane 1, Clone 7 cut with EcoRI. Lane 2, uncut Clone 7. B. Lane 1, uncut pIB5. Lane 2, pIB5 cut with HindIII. Lnae 3, pIB5 cut with HindIII and EcoRI. Numbers indicate positions of DNA in base pairs.
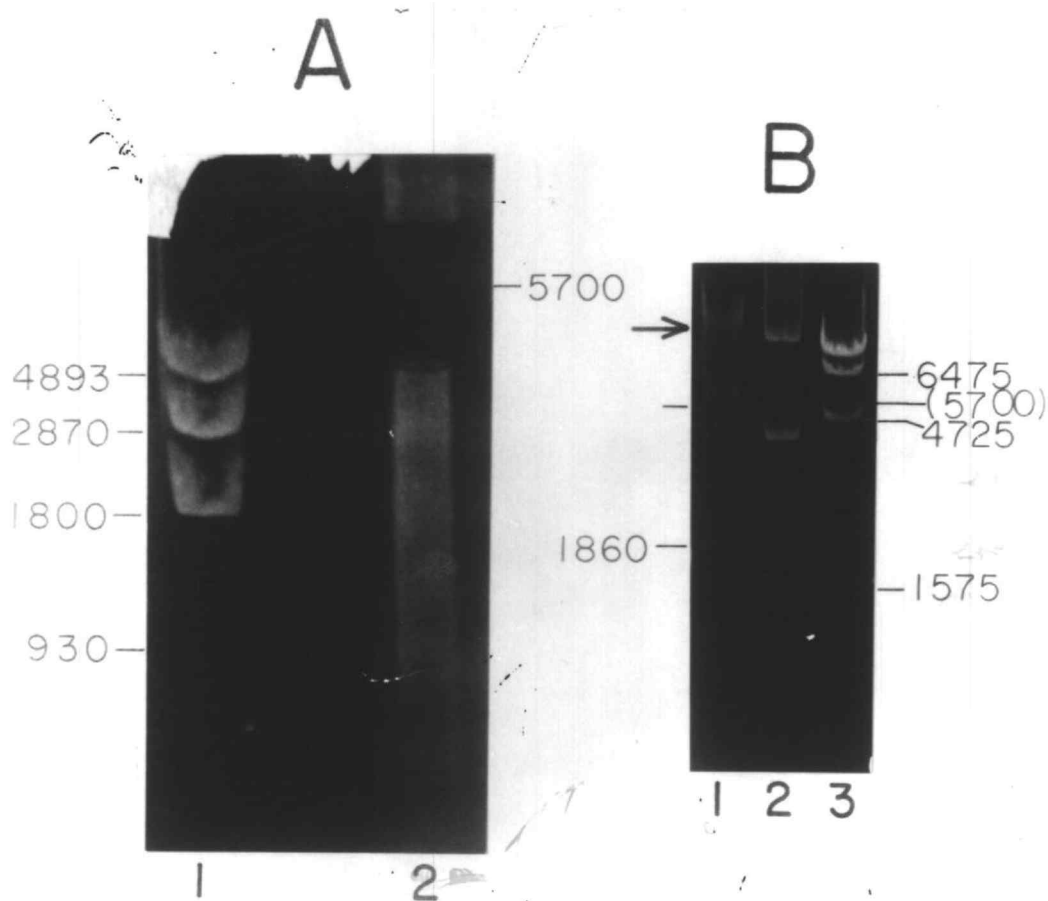
Figure VI.3. Identification of the intermediate fragments and the ligated products. A. Lane 1 shows the 930 bp HindIII-EcoRI fragment of pIB5. Lane 2 shows the position of linearized Clone 7. B. The arrow points to the expected ligation product should be. Lane 1, the ligated products. Lane 2, uncut Clone 7. Lane 3, HpaI cut Ad2 DNA used as a size standard. Numbers indicate positions of DNA in base pairs.

competent recBCsbcB bacteria. The transformants were selected on YT plates containing 50 μg/ml ampicillin, and counter selected on plates containing 15 μg/ml tetracycline. Ampicillin-resistant but tetracycline-sensitive colonies were grown up in 5 ml YT broth containing 50 μg/ml ampicillin.

RESULTS AND DISCUSSION

Amplicillin-resistant but tetracycline-sensitive colonies (92) were picked from three different recBCsbcB strains of bacterial hosts (the third recBCsbcB strain was from Dr. F. Graham, genotype not certain). Plasmid DNA was isolated by the alkali lysis method (7), and examined by agarose gel electrophoresis. Sixty of these DNA preparations did not contain any plasmid (data not shown). The other 32 DNA preparations showed only a trace of plamid DNA, but the bacterial DNA band, on the other hand, was very prominent. The sizes of the plasmids were also much smaller than markers, double-stranded M13mp8 RF (7.1 kb) or supercoiled vector, clone 7 (5.7 kb). The expected size of the plasmid with palindromic ITRs is 6.7 kb.

A perfect palindrome has the possibility of forming a cruciform structure. This conformation is favored by negative supercoiling, which in turn relaxes the DNA molecules (8). If circular DNA molecules were relaxed, the mobility of such DNA (in agarose gels) should be slower than that of supercoiled or linear DNA with the same molecular weight. However, this was not the case. The plasmid DNAs isolated in these experiments not only moved faster, but also were

resistant to EcoRI, HindIII, and SmaI cleavage. These observations suggest that although palindrome-containing plasmids could transform recBCsbcB bacteria, the palindrome might not be stable in the bacteria. Excisions of palindrome are frequently observed (5,6,9-12). The center of a cruciform structure is physically identical to a Holliday junction, a central intermediate in genetic recombination. Exonuclease V, the recBC gene product, might cut diagonally across a Holliday junction to generate recombinant DNAs (12). If such a cut were made across the base of a cruciform, the excision of the palindrome would thus occur.

It is still not clear why two-thirds of the colonies did not have plasmids, but exhibited drug resistance. One possibility might be that palindrome-containing plasmids were integrated into the bacterial genome. As a control, the host bacteria were streaked on ampicillin plates to examine whether any of these hosts carried drug resistance. None was found to be drug resistant. Competent bacteria were also transformed with alkaline phosphatase-treated linear vector or ligated vector, and only 2 to 3 colonies appeared on each plate (data not shown). The efficiency of transformation with 5 ng of recombinant DNA ranged between 250 to 300 colonies per plate. Under normal conditions, 1500 to 2000 colonies per plate could usually be isolated by using 1 ng DNA per transformation. There was a 40-fold decrease in the transformation efficiency.

No bacterial mutants so far have been found to accomodate plasmids with large palindromes. The cloning of a large palindrome remains a challenge.

REFERENCES

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

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

3. Pearson, G.D., Chow, K.-C., Enns, R.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.

4. Lungblad, V., Taylor, A. F., Smith, G.R., and Kleckner, N. (1984) Unusual alleles of recB and recC stimulate excision of inverted repeat transposons Tn 10 and Tn 5. Pro. Natl. Acad. Sci. USA 81, 824-828.

5. Berkner, K.l., and Sharp, P.A. (1983) Generation of adenovirus by transfection of plasmids. Nucl. Acids Res. 11, 6003-6020.

6. Leach, D.R.F.,and Stahl, F.W. (1983) Viability of phages carrying a perfect palindrome in the absence of recombination nucleases. Nature 305, 448-451.

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

8. Collins, J. (1981) Instability of palindromic DNA in Escherichia coli. Cold Spring Harbor Symp. Quant. Biol. 45: 409-416.

9. Collins, J., Volchaert, G., and Nevers, P. (1982) Precise and nearly precise exercision of the symmetrical inverted repeats of Tn 5; common features of rec A-independent deletion mutasnts in Escherichia coli. Gene 19, 139-146.

10. Mizuuchi, K., Mizuuchi, M., and Gellert, M. (1982) Cruciform structures in palindromic DNA are favored by DNA supercoiling. J. Mol. Biol. 156,229-244.

11. Hagen, C. E., and Warren, G. T. (1983) Viability of palidromic DNA is restored by deletions occuring at low but variable frequency in plasmids of Escherichia coli. Gene 24, 317-326.

12. Gilickman, B.W., and Ripley, L.S. (1984) Structural intermediate of deletion mutagenesis: a role for palindromic DNA. Proc. Natl. Acad. Sci. USA 81, 512-516.

CHAPTER VII

CONCLUSION

Considerable information is already known about the structure and replication of adenovirus. The genome of adenovirus is a double-stranded DNA molecule with inverted terminal repetitions (ITRs) about 100 bp to 162 bp in length (1,2). A terminal protein is covalently linked to the 5'-deoxycytidine of each strand (3,4). The replication of adenovirus DNA in vivo or in vitro (Figure I.1) starts at or near either end of the viral DNA to synthesize the daughter strand in the 5' to 3' direction with concomitant displacement of the parental strand of the same polarity (strand displacement mechanism) (5). The displaced strand is replicated by complementary synthesis which begins from the 3' end of the strand, presumably involving a proposed "panhandle" intermediate (6,7), and proceeds in the 5' to 3' direction. Only five proteins are involved in the replication of the adenoviral genome. Three of these are adenovirus-encoded: the 72K single-stranded DNA binding protein (Ad DBP), the 80K terminal protein precursor (pTP) and the 140K DNA polymerase (Ad pol). The other two are host factors: the 47K nuclear factor I, which binds to nucleotides 17 to 48 of the adenovirus ITR and is absolutely required for pTP-dCMP complex formation; and nuclear factor II (ranging from 15 to 30K), which prevents premature termination of DNA chain elongation. It contains topoisomerase I-like activity (8,9,10). Sequence analysis within the ITRs of adenoviruses has shown that a

specific region, from nucleotide 9 through 17, is conserved among several species (1,2,11-14). Because of the linearity of the viral genome, classic RNA priming schemes are not directly applicable for the initiation of adenovirus DNA replication (15,16). Nucleotide analysis of ITRs (1,2,11-14) has also shown that a hairpin structure does not exist (17) and that the hairpin self-priming hypothesis is unlikely.

In considering other mechanisms that might be involved in the initiation of adenovirus replication, covalently closed, circular template would be an attractive possibility. However, no circular replicative intermediates have ever been isolated (18). The failure of finding circular template DNA could be due to the extremely short life of these replicative intermediates or to the fact that conventional methods used to isolate DNA would activate the enzyme to break such structures. A special plasmid, XD-7, was thus constructed and used in the in vitro replication system (19) to study processes which might be involved in initiation on a circular molecules.

XD-7 molecules, which contain the XbaI-E fragment of Ad2 DNA, replicated as rolling circles with displaced single-stranded tails, an indication of extensive strand displacement synthesis, in a reaction mixture containing nuclear and cytoplasmic extracts from adenovirus infected HeLa cells. Origins of the displaced tails were mapped on XD-7 molecules by electron microscopy to the left boundary of the cloned adenovirus insert, sequences corresponding to the left terminus of the adenovirus genome. The conserved sequence, nucleotides 9 to 17, was also demonstrated essential for adenovirus-specific replication by an internally standardized, quantitative

assay of electron microscopy (TABLE II.1). From the results in Chapter II, the replication mechanism of XD-7 DNA resembles that of adenovirus DNA with respect to location, direction and mode of replication.

In an effort to locate the specific cleavage site which might in turn provide the 3'-OH for the initiation of replication, two groups of nicking sites were found to be adenovirus-specific. The first group, as shown in Figure III.7, was distributed from nucleotides 52 to 94 within the adenovirus ITR, very different from the replication origin, the conserved sequence (nucleotides 9-17) and the binding site of nuclear factor I (nucleotides 17-48). The functional significance of site-specific nicking within the ITR is not yet known. Deletions removing sequences which contain all the nick sites do not prevent adenovirus DNA replication in a cell-free system (20-24). However, the presence of the segment containing the nick sites greatly enhances the efficiency of the initiation reaction (21). The second group was located at the junction between the adenovirus insert and the vector (as shown in Figure IV.2). The nicking signal was weak when compared with the nicking signals within the ITR or at the center of palindromes.

The pTP-dCMP transfer assay was originally developed by Challberg et al. (25). The assay was based on the "protein-priming" hypothesis (3) that (a) the terminal protein precursor (pTP) is covalently coupled to dCTP in the absence of the other three deoxynucleoside triphosphates to form a pTP-dCMP complex; (b) the 3'-OH of the bound dCMP residue in this complex then acts as a primer for DNA chain elongation (4,26); and (c) the formation of pTP-dCMP complex requires

Ad DNA-protein complex, dCTP, terminal protein precursor, 140K adenovirus-encoded DNA polymerase and 47K nuclear factor I from host cells (8-10,15) (also see TABLE I.1).

Although most of the characterization of the purified enzymes as well as the identification of template sequences which are required for adenovirus replication have been determined by the in vitro replication assay, there are still several points to be clarified in order to determine the role the pTP-dCMP complex plays in the mechanism of initiating DNA synthesis: (a) The molecular weight of the terminal protein precursor (pTP) varied from 80K to 87K, depending on the gel system used (20,25,26); (b) When linearized plasmid DNA is used as template, an inhibitory factor present in the nuclear extract (26) apparently prevents initiation, but not always (25); (c) Nuclear factor I is required for pTP-dCMP complex formation when double-stranded adenovirus DNA is used as template, but not when single-stranded DNA is used (27); (d) Nuclear factor I is absolutely required for the formation of pTP-dCMP complex, but binds to a specific sequence within the ITR (nucleotides 17 to 48) which is dispensable for the initiation of adenovirus DNA synthesis (8,9,20-24,27); (e) Single-stranded DNA can support pTP-dCMP formation, and this reaction, like the reaction with duplex DNA as template, occurs in response to an adenovirus-specific nucleotide sequence (25,27); and (f) A topoisomerase activity is required for full-length elongation of the linear adenovirus DNA (28).

Experiments were designed to test the hypothesis that terminal protein nicked the adenovirus origin (Chapter V). However, the assay detected HeLa topoisomerase I instead. The assay is essentially based

on the stoichiometric formation of a covalent intermediate between the enzyme and the ^{32}P-labelled DNA substrate (Figure V.1). After removing the unreacted DNA by DNase I digestion and resolving the ^{32}P-labeled protein by gel electrophoresis, the level of type I DNA topoisomerase can be quantitatively determined in a crude nuclear extract without any interference from the extraneous nucleases. Using this assay, I have shown that the level of topoisomerase I activity in adenovirus-transformed and adenovirus-infected cells is at least ten-times higher than in uninfected cells (chapter V). Because only E1A gene expression is common to both adenovirus- transformed and adenovirus-infected cells, it is tempting to speculate that E1A gene products activate the expression of the host topoisomerase I gene.

The adenovirus E1A region is the first to be transcribed upon viral infection, and the transcription of the remaining viral genes requires one of the E1A gene products (29). E1A gene products also induce the synthesis of a 70K mammalian heat-shock protein (30), and activate exogenous genes in <u>trans</u> and even overcome the <u>cis</u>-reqirement for enhancer or activator sequences in transient expression assays (31-34). Furthermore, adenovirus E1A gene products provide functions required for the polyoma virus middle-T and the T24 Harvey ras-1 genes to transform primary cells following DNA mediated gene transfer (35). Transformation of primary cells requires at least two separate functions: an establishment function and a transformation function. An establishment function, which is concerned with immortalization of cells, can be provided by the expression of adenovirus E1A genes, polyoma large T antigen or viral or cellular <u>myc</u> genes (35,36). The proteins encoded by adenovirus E1A

and the oncogenes myc or myb are structurally related with 15-21% of identical amino acids in pairwise comparisons (37). Primary diploid cells usually have a limited lifespan and will reproducibly undergo approximately 20 population doublings in culture before losing their proliferative potential. The establishment functions enable primary cells to grow indefinitely in culture. The fundamental change from a limited lifespan to immortality is that primary cells escape the controls of DNA replication (38). Topoisomerase activity is required for DNA replication, transcription, recombination and DNA repair. Adenovirus DNA chain elongation terminates randomly, approximately one third the way along the genome, without nuclear factor II (28). Nuclear factor II contains a topoisomerase I-like activity, and facilitates the completion of DNA replication. The increase of topoisomerase I activity might be due to adenovirus regulation of the expression of the topoisomerase I gene.

REFERENCES

1. 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.

2. Van Ormondt, H., Maat, J., and Dijkema, R. (1980) Comparison of nucleotide sequences of the early Ela regions of subgroups A, B and C of human adenoviruses. Gene 12, 63-76.

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

4. Desiderio, S. V., and Kelly, T. J., Jr. (1981) Structure of the linkage between adenovirus DNA and the 55,000-dalton molecular weight terminal protein. J. Mol. Biol. 145, 319-337.

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

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

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

8. Stillman, B. W. (1983) The replication of adenovirus DNA with purified proteins. Cell 35, 7-9.

9. Lichy, J. H., Nagata, K., Friefeld, B.R., Enomoto, T., Field, J., Guggenheimer, R.A., Ikeda, J.-E. Horwitz, M.S., And Hurwitz, J. (1983) Isolation of proteins involved in the replication of adenoviral DNA in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 731-740.

10. Ostrove, J. M., Rosenfeld, P., Williams, J., and Kelly, T.J., Jr. (1983) In vitro complementation as an assay for purification of adenovirus DNA replication proteins. Proc. Natl. Acad. Sci. USA 80, 935-939.

11. Alestrom, P., Stenlund, A., Li, P., and Pettersson, U. (1982) A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18, 193-197.

12. Temple, M., Antoine, G., Delius, H., Stahl, S., and2 Winnacker, E.-L. (1981) Replication of mouse adenovirus strain FL DNA. Virology 109, 1-12.

13. Tolun, A., Alestrom, P., and Pettersson, U. (1979) Sequence of inverted terminal repetitions from different adenoviruses. Demonstration of conserved sequences and homology between SA 7 termini and SV40 DNA. Cell 17, 705-713.

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

15. Challberg, M. D., and Kelly, T. J., Jr. (1982) Eucaryotic DNA replication: viral and plasmid model systems. Ann. Rev. Biochem. 51, 901-934.

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

17. Sussenbach, J.S., and Kuijk, M.G. (1978) Initiation of adenovirus DNA replication does not occur via a hairpin mechanism. Nucl. Acids Res. 5, 1289-1295.

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

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

20. 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.

21. Rawlins, D.R., Rosenfeld, P.J., Wides, R.J., Challberg, M.D., and Kelly, T.J., Jr. (1984) Structure and function of the adenovirus origin of replication. Cell 37, 309-319.

22. 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.

23. Stillman, B.W., and Tamanoi, F.(1983) Adenoviral DNA replication: DNA sequences and enzymes required for initiation in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 741-750.

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

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

26. 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.

27. Nagata, K., Guggenheimer, R. A., Enomoto, T., Lichy, J. H., and Hurwitz, J. (1982) Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA 79, 6438-6442.

28. 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.

29. Berk, A.J., Lee, F., Harrison, T., Williams, J. and Sharp, P.A. (1979) Pre-early adenovirus 5 gene product regulates synthesis of erly viral messenger RNAs. Cell 17, 935-944.

30. Nevins, J.R. (1982) Induction of the synthesis of a 70,000-dalton mammalian heat-shock protein by the adenovirus E1A gene product. Cell 29, 913-919.

31. Treisman, R., Green, M.R. and Maniatis, T. (1983) Cis and trans activation of globin gene transcription in transient assays. Proc. Natl. Acad. Sci. USA 80, 7428-7432.

32. Green, M.R., Treisman, R.,and Maniatis, T. (1983) Transcriptional activation of cloned human β-globin genes by viral immediate-early gene products. Cell 35, 137-148.

33. Imperiale, M.J., Feldman, L.T.,and Nevins, J.R. (1983) Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell 35, 127-136.

34. Gaynor, R.B., Hillman, D., and Berk, A.J. (1984) Adenovirus early region 1 A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. Proc. Natl. Acad. Sci. USA 81, 1193-1197.

35. Ruley, H. E. (1983) Adenovirus early region 1 A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304, 602-606.

36. Land, H., Parada, L. F., and Weinberg, R. A. (1983) Tumorigenic conversin of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304, 596-602.

37. Ralston, R., and Bishop, J.M. (1983) The protein products of the myc and myb oncogenes and adenovirus Ela are structurally related. Nature 306, 803-809.

38. Newbold, R.F., and Overell, R.W. (1983) Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature 304, 648-651.

BIBLIOGRAPHY

Alestrom, P., Stenlund, A., Li, P., and Pettersson, U. (1982) A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18, 193-197.

Aoyama, A., Hamatake, R,K. and Hayashi, M. (1981) Morphogenesis of φX174: in vitro synthesis of infectious phage from purified viral components. Proc. Natl. Acad. Sci. USA 78, 7285-7289.

Arai, N., Polder, L., Arai, K. and Kornberg, A. (1981) Replication of φX174 DNA with purified enzymes. J. Biol. Chem. 256, 5239-5246.

Ariga, H., and Shimojo, H. (1979) Incorporation of uracil into the growing strand of adenovirus 12 DNA. Biochem. Biophys. Res. Commun. 87, 588-597.

Arrand, J.R., and Roberts, R.J. (1979) The nucleotide sequences at the termini of Ad2 DNA. J. Mol. Biol. 128, 577-594.

Astell, C.R., Thomson, M., Chow, M.B., and Ward, D.C. (1983) Structure and replication of minute virus of mice DNA. Cold Spring Harbor Symp. Quant. Biol. 47, 751-762.

Babich, A., and Nevins, J. R. (1981) The stability of early adenovirus mRNA is controlled by the viral 72 kd DNA-binding protein. Cell 26, 371-379.

Berk, A.J., Lee, F., Harrison, T., Williams, J., and Sharp, P.A. (1979) Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNA. Cell 17, 935-944.

Berkner, K.l., and Sharp, P.A. (1983) Generation of adenovirus by transfection of plasmids. Nucl. Acids Res. 11, 6003-6020.

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

Brison, O., Kedinger, C., and Wilhelm, J. (1977) Enzymatic properties of viral DNA replication complexes isolated from adenovirus type 2 infected HeLa cell nuclei. J. Virol. 24, 423-443.

Brown, D.R., Reinberg, D., Schmidt-Glenewinkel, T., Roth, M., Zipursky, S.L., and Hurwitz, J. (1983) DNA structures required for φX174 A protein-directed initiation and termination of DNA replication. Cold Spring Harbor Symp. Quant. Biol. 47, 701-715.

Caruci, E.A. (1977) Evidence for blocked 5'-termini in human adenovirus DNA. Virology 105, 357-370.

Cavalier-Smith, T. (1974) Palindromic base sequences and replication of eukaryotic chromosome ends. Nature 250, 467-470.

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

Challberg, M.D., and Kelly, T.J., Jr. (1979) Adenovirus DNA replication in vitro: origin an direction of daughter strand synthesis. J. Mol. Biol. 135, 999-1012.

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

Challberg, M.D., Ostrove, J.M., and Kelly, T.J., Jr. (1982) Initiation of adenovirus DNA replication: detection of covalent complexes between nucleotide and the 80 kd terminal protein. J. Virol. 41, 265-270.

Challberg, M.D., and Kelly, T.J., Jr. (1982) Eukaryotic DNA replication: viral and plasmid model systems. Ann. Rev. Biochem. 51, 901-934.

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.

Chow, K.-C., and Pearson, G.D. (1984) Site-specific nicking within adenovirus inverted terminal repetition. Nucl. Acids Res. 12, 1489-1500.

Collins, J. (1981) Instability of palindromic DNA in Escherichia coli. Cold Spring Harbor Symp. Quant. Biol. 45, 409-416.

Collins, J., Volchaert, G., and Nevers, P. (1982) Precise and nearly precise exercision of the symmetrical inverted repeats of Tn 5; common features of rec A-independent deletion mutants in Escherichia coli. Gene 19, 139-146.

Cozzarelli, N.R. (1980) DNA gyrase and the supercoiling of DNA. Science 207, 953-960.

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

De Jong, P.J., Kwant, M.M., van Driel, W., Jansz, H.S., and van der Vliet, P.C. (1983) The ATP requirements of adenovirus type 5 DNA replication and cellular DNA replication. Virology 124, 45-58.

Desiderio, S.V., and Kelly, T.J., Jr. (1981) Structure of the linkage between adenovirus DNA and the 55,000-dalton molecular weight terminal protein. J. Mol. Biol. 145, 319-337.

Dijkema, R., and Dekker, B.M.M. (1979) The inverted terminal repetition of the DNA of weakly oncogenic adenovirus type 7. Gene 8, 7-15.

DiMaio, D. and Nathans, D. (1982) Regulatory mutants of simian virus 40. Effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. J. Mol. Biol. 156, 531-548.

Edwards, K.A., Halligan, B.D., Davis, J.L., Nivera,N.L., and Liu,L.F. (1982) Recognition sites of eukaryotic DNA topoisomerase I: DNA nucleotide sequencing analysis of topo I cleavage sites on SV40 DNA. Nucl. Acids Res. 10, 2565-2576.

Eisenberg, S., Griffith, J. and Kornberg, A. (1977) φX174 cistron A protein is a multifunctional enzyme in DNA replication. Proc. Natl. Acad. Sci. USA 74,3198-3202.

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, K.Z., Astell, C.R., and Pearson, G.D. (1983) Mutational mapping of a cloned adenovirus origin. Gene 23, 307-313.

Enomoto, T., Lichy, J.H., Ikeda, J.-E., and Hurtwitz, J. (1981) Adenovirus DNA replication in vitro: Purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. USA 78, 6779-6783.

Ferguson, J. and Davis, R.W. (1978) Quantitative electron microscopy of nucleic acids. in: Adevanced technique in biological electron microscopy Vol.II, pp.123-171.

Flint, S.J., and Broker, T.R. (1980) Lytic infection by adenovirus. in Tooze, J. (ed.) Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratory, New York, pp.443-546.

Fuller, C.W., Beauchamp, B.B., Engler, M.J., Lechner, R.L., Matson, S.W., Tabor, S., White, J.H., and Richardson, C.C. (1983) Mechanisms for the initiation of bacteriophage T7 DNA replication. Cold Spring Harbor Symp. Quant. Biol. 47, 669-679.

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, 2381-2395.

Gaynor, R.B., Hillman, D., and Berk, A.J. (1984) Adenovirus early region 1A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. Proc. Natl. Acad. Sci. USA 81, 1193-1197.

Gellert, M. (1981) DNA topoisomerase. Ann. Rev. Biochem. 50, 879-910.

Gilickman, B.W., and Ripley, L.S. (1984) Structural intermediate of deletion mutagenesis: a role for palindromic DNA. Proc. Natl. Acad. Sci. USA 81, 512-516.

Green, M.R., Treisman, R., and Maniatis, T. (1983) Transcriptional activation of cloned human β-globin genes by viral immediate-early gene products. Cell 35, 137-148.

Guo, L.-H. and Wu, R. (1982) New rapid methods for DNA sequencing based on exonucleaseIII digestion followed by repair synthsis. Nucl. Acids Res. 10, 2065-2084.

Hagen, C.E., and Warren, G.T. (1983) Viability of palidromic DNA is restored by deletions occuring at low but variable frequency in plasmids of Escherichia coli. Gene 24, 317-326.

Halligan, B.D., Davis, J.L., Edwards, K.A., and Liu, L.F. (1982) Intra- and intermolecular strand by HeLa topoisomerase I. J. Biol. Chem. 257, 3995-4000.

Harland, R.M., Weintraub, H. and McKnight, S.L. (1983) Transcription of DNA injected into Xenopus oocytes is influenced by template topology. Nature 302, 38-43.

Heidekamp, F.,Langevelt, S.A., Baas, P.D. and Jansz, H.S. (1980) Comparison of φX174, G4 and St-1 phage for site of action of A protein. Nucl. Acids Res. 8, 2009-2021.

Heidekamp, F., Baas, P.D., van Boom, J.H., Veeneman, G.H., Zipursky, S.L. and Jansz, H.S. (1981) Construction and characterization of recombinant plasmid DNAs containing sequences of the origin of bacteriophage φX174 DNA replication. Nucl. Acids Res. 9, 3335-3354.

Horwitz, M.S. (1971) Intermediates in the replication of type 2 adenovirus DNA. Virology 8, 675-683.

Horwitz, M.S. (1976) Bidirectional replication of adenovirus type 2 DNA. J. Virol. 18: 307-315.

Ikeda, J.-E., Enomoto, T., and Hurwitz, J. (1981) Replication of the adenovirus DNA-protein complex with purified proteins. Proc. Natl. Acad. Sci. USA 78, 884-888.

Ikeda, J.-E., Enomoto, T., and Hurwitz, J. (1982) Adenoviral protein-primed initiation of DNA chains in vitro. Proc. Natl. Acad. Sci. USA 79, 2442-2446.

153

Imperiale, M.J., Feldman, L.T., and Nevins, J.R. (1983) Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell 35, 127-136.

Kaplan, L.M., Kleinman, R.E., and Horwitz, M.S. (1977) Replication of adenovirus type 2 DNA in vitro. Proc. Natl. Acad. Sci. USA 74, 4425-4429.

Kaplan, L.M., Ariga, H., Hurwitz, J., and Horwitz, M.S. (1979) Complementation of the temperature-sensitive defect in H5ts125 adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci. USA 76, 5534-5538.

Kasamatsu, H., and Wu,M. (1976) Structure of a nicked DNA-protein complex isolated from simian virus 40: covalent attachment of the protein to DNA and nick specificity. Proc. Natl. Acad. Sci. USA 73, 1945-1949.

Keller, W. (1975) Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA 72, 4876-4880.

Kleinschmidt, A.K. (1968) Monolayer technique in eletron microscopy of nucleic acid molecules. in: Methods in Enzymology Vol.XIIB, (Academic Press). pp.361-377.

Kornberg, A. (1980) DNA replication, (Freeman, San Francisco).

Koths, K., and Dressler, D. (1978) Analysis of the ϕX DNA replication cycle by electron microscopy. Proc. Natl. Acad. Sci. USA 75, 605-609.

Laemmli, J.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-686.

Land, H., Parada, L.F., and Weinberg, R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304, 596-602.

Langeveld, S.A., van Mansfeld, A.D.M., Baas, P.D., Jansz, H.S., van Arkel, G.A., and Weisbeek, P.J. (1978) Nucleotide sequence of the origin of replication in bacteriophage ϕX174 RF DNA. Nature 271,417-420.

Leach, D.R.F., and Stahl, F.W. (1983) Viability of λ phages carrying a perfect palindrome in the absence of recombination nucleases. Nature 305, 448-451.

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

Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981) Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and the 5'-dCMP in vitro. Proc. Natl. Acad. Sci. USA 78, 2678-2682.

Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981) Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP in vitro. Proc. Natl. Acad. Sci. USA 78, 2678-2682.

Lichy, J.H., Field, J., Horwitz, M.S., and Hurwitz, J. (1982) Seperation of the adenovirus terminal protein precursor from its associated DNA polymerase; role of both protein in the initiation of adenovirus DNA replication. Proc. Natl. Acad. Sci. USA 79, 5225-5229.

Lichy, J.H., Nagata, K., Friefeld, B.R., Enomoto, T., Field, J., Guggenheimer, R.A., Ikeda, J.-E., Horwitz, M.S., and Hurwitz, J. (1983) Isolation of proteins involved in the replication of adenoviral DNA in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 731-740.

Liu, L.F., and Miller, K.G. (1981) Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa nuclei. Proc. Natl. Acad. Sci. USA 78, 3487-3491.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, (Cold Spring Harbor Laboratory, New York), pp. 464-467.

Messing, J. (1981) M13mp2 and derivatives: A molecular cloning system for DNA sequencing , strand specific hybridization, and in vitro mutagenesis. in Walton, A.G. (Ed.), Proceedings of the Third Cleveland Symposium on Macromolecules: Recombinant DNA. Elsevier, Amsterdam, pp.143-153.

Miller, K.G., Liu, L.F., and Englund, P.T. (1981) A homogeneous type II DNA topoisomerase from HeLa cell nuclei. J. Biol. Chem. 256, 9334-9339.

Mizuuchi, K., Mizuuchi, M., and Gellert, M. (1982) Cruciform structures in palindromic DNA are favored by DNA supercoiling. J. Mol. Biol. 16,229-244.

Myers, R.M. and Tjian, R. (1980) Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. USA 77, 6491-6495.

Nagata, K., Guggenheimer, R.A., Enomoto, T., Lichy, J.H., and Hurwitz, J. (1982) Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA 79, 6438-6442.

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.

Nagata, K., Guggenheimer, R.A., and Hurwitz, J. (1983)) Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80, 6117-6181.

Nevins, J.R. (1982) Induction of the synthesis of a 70,000-dalton mammalian heat-shock protein by the adenovirus Ela gene product. Cell 29, 913-919.

Newbold, R.F., and Overell, R.W. (1983) Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature 304, 648-651.

Ostrove, J.M., Rosenfeld, P., Williams, J., and Kelly, T.J., Jr. (1983) In vitro complementation as an assay for purification of adenovirus DNA replication proteins. Proc. Natl. Acad. Sci. USA 80, 935-939.

Panayotatos, N. and Truong, K. (1981) Specific deletion of DNA sequences between preselected bases. Nucl. Acids Res. 9, 5679-5688.

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

Pearson, G.D., Chow, K.-C., Corden, J.L., and Harpst, J.A. (1981) Replication directed by a cloned adenovirus origin. in: Ray, D.S. and Fox, C.F. (Eds.), ICN-UCLA symposium on molecular and cellular biology, Vol. 21 (Academic Press, New York) pp.581-595.

Pearson, G.D., Chow, K.-C., Enns, R.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.

Pincus, S., Robertson, W., and Rekosh, D. (1981) Characterization of the effect of aphidicolin on adenovirus DNA replication: evidence in support of a protein primer model of initiation. Nucl. Acids Res. 9, 4919-4937.

Ralston, R., and Bishop, J.M. (1983) The portein products of the myc and myb oncogenes and adenovirus Ela are structurally related. Nature 306, 803-809.

Rawlins, D.R., Rosenfeld, P.J., Wides, R.J., Challberg, M.D., and Kelly, T.J., Jr. (1984) Structure and function of the adenovirus origin of replication. Cell 37, 309-319.

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

Robinson, A.J., Younghusband, H.B., and Bellet, A.J.D. (1973) A circular DNA-protein complexes from adenoviruses. Virology 56:54-69.

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

Ruley, H.E. (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304, 602-606.

Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Schleif, R.F. and Wensink, P.C. (1981) Practical methods in molecular biology. (Springer-Verlog, New York).

Seif, I., Khoury, G., and Dhar, R. (1979) The genome of human papovavirus BKV. Cell 18, 963-977.

Shinagawa,M., and Padmanabhan, R. (1979) Nucleotide sequences at the inverted terminal repetition of Ad2 DNA. Biochem. Biophys. Res. Commun. 87, 671-678.

Shinagawa, M., and Padmanabhan, R. (1980) Comparative sequence analysis of the inverted terminal repetitions from diferent adenoviruses. Proc. Natl. Acad. Sci. USA 77, 3831-3835.

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

Shlomai, J., Polder, L., Arai, K. and Kornberg, A. (1981) Replication of φX174 DNA with purified enzymes. J. Biol. Chem. 256, 5233-5238.

Shortle, D. and Nathans, D. (1978) Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. Proc. Natl. Acad. Sci. USA 75, 2170-2174.

Shortle, D. and Nathans, D. (1979) Regulatory mutants of simian virus 40: constructed mutants with base substitutions at the origin of DNA repication. J. Mol.Biol. 131, 801-817.

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

Stayton, M.M., Bertsch, L., Biswas, S., Burgers, P., Dixon, N., Flynn, J.E., Fuller, R., Kaguni, J., Kobori, J., Kodaira, M., Low, R., and Kornberg, A. (1983) Enzymatic recognition of DNA replication origins. Cold Spring Harbor Symp. Quant. Biol. 47, 693-700.

Steenberg, 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., 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., Tamanoi, F., and Mathews, M.B. (1982) Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. Cell 31, 613-623.

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.

Stillman, B.W., and Tamanoi, F. (1983) Adenoviral DNA replication: DNA sequences and enzymes required for initiation in vitro. Cold Spring Harbor Symp. Quant. Biol. 47, 741-750.

Stillman, B.W. (1983) The replicaton of adenovirus DNA with purified protein. Cell 35, 7-9.

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

Sugisaki, H., Sugimoto, K., Takanami, M., Shiroli, K., Saito, I., Shimojo, Y., Sawada, Y., Uemizu, Y., Uesugi, S.-I., and Fugonaga, K. (1980) Structural and gene organization in the transforming HindIII-G fragment of Ad12. Cell 20, 777-786.

Sutcliffe, J.G. (1979) Complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.

Summers, J., and Mason, W.S. (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29, 403-415.

Sussenbach, J.S., and Kuijk, M.G. (1978) Initiation of adenovirus DNA replication does not occur via a hairpin mechanism. Nucl. Acids Res. 5, 1289-1295.

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

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

Temple, M., Aotonie, G., Delius, H., Stahl, S. and Winnacker, E.-L. (1981) Replication of mouse adenovirus strain FL DNA. Virology 109, 1-12.

Tolun, A., Alestrom, P. and Pettersson, U. (1979) Sequence of inverted repetitions from different adenoviruses. Demonstration of conserved sequences and homology between SA7 termini and SV40 DNA. Cell 17, 705-713.

Tomizawa, J., Ohmori, H., and Bird, R.E. (1977) Origin of replication of colicin E1 plasmid DNA. Proc. Natl. Acad. Sci. USA 74, 1865-1869.

Treisman, R., Green, M.R., and Maniatis, T. (1983) Cis and trans activation of globin gene transcription in trasient assays . Proc. Natl. Acad. Sci. USA 80, 7428-7432.

Van Bergen, B.G.M., and van der Vliet, P.C. (1983) Temperature-sensative initiation and elongation of adenovirus DNA replication in vitro with nuclear extracts from H5ts36-, H5ts149-, and H5ts125-infected HeLa cells. J. Virol. 46, 642-648.

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 Ende, Langevelt, S.A., Teertstra, R., van Arkel, G.A. and Weisbeek, P.J. (1981) Enzymatic properties of the bacteriophage φX174 A protein on superhelical φX174 DNA : a model for the termination of rolling circle DNA replication. Nucl. Acids Res. 9, 2037-2053.

Van Mansfeld, A.D.M., Langevelt, S.A., Baas, P.D., Jansz, H.S., van der Marel, G.A., Veeneman,G.H. and van Boom, J.H. (1980) Recognition sequence of bacteriophage φX174 gene A protein - an initiator of DNA replication. Nature 288, 561-566.

Van Ormondt, H., Maat, J., and Dijkema, R. (1980) Comparison of nucleotide sequences of the early E1a regions for subgroups A, B, and C of human adenovirus. Gene 12, 63-76.

Vieira, J., and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Wang, J.C. (1971) Interaction between DNA and an Escherichia coli protein. J. Mol. Biol. 55, 523-533.

Wasylyk, B., Kedinger, C., Corden, J., Brison, O. and Chambon, P. (1980) Specific in vitro initiation of transcription on conalbumin and ovalbumine genes and comparison with adenovirus-2 early and late genes. Nature 285, 367-373.

Weissbach, A.(1975) Vertebrate DNA polymerases. Cell 5, 101-108.

Westmoreland, B.C., Szybalski, W. and Ris, H. (1969) Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163, 1343-1348.

Wickner, S.H. (1978) DNA replication proteins of Escherichia coli. Ann. Rev. Biochem. 47, 1163-1191.

Wimmer, E. (1982) Genome-linked proteins of viruses. Cell 28, 199-201.

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

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

Wu, M., Roberts, R.J., and Davidson, N. (1977) Structure of the inverted terminal repetition of adenovirus type 2 DNA. J. Virol. 21, 766-777.

Wu, M., Davidson, N., and Wimmer, E. (1978) An electron micrposcope study of the proteins attached to polio virus RNA and its replicative form. Nucl. Acids Res. 5, 4711-4723.