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Relationship between higher order structure of adenovirus genome and gene expression, and involvement of topoisomerases in the life cycle of adenovirus

Wong, Min-Liang, Ph.D.

City University of New York, 1990

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Relationship between Higher Order Structure of Adenovirus Genome and Gene Expression, and Involvement of Topoisomerases in the Life Cycle of Adenovirus

by

Min-Liang Wong

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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5/11/1990
Date

Ming-Ta Hsu, Ph.D.
Chair of Examining committee

5/11/1990
Date

Terry A. Krulwich, Ph.D.
Executive Officer

Selina Y. Chen-Kiang, Ph.D.

Edward M. Johnson, Ph.D.

Peter Palese, Ph.D.

Thomas Shenk, Ph.D.

Supervisory Committee

The City University of New York

Abstract

Relationship between Higher Order Structure of Adenovirus Genome and Gene Expression, and Involvement of Topoisomerases in the Life Cycle of Adenovirus

by

Min-Liang Wong

Advisor: Dr. Ming-Ta Hsu

The genome of human adenovirus is a linear, double-stranded DNA approximately 36 kilobase pairs long. DNA inside the virion particle is complexed with virus-encoded proteins (mostly VII and V). Using different approaches, the structure of viral nucleoprotein complexes has been investigated by many researchers, but a consensus has not been reached. We used two methods to study the structure of virion nucleoprotein complexes. First, electron microscopic analysis of bis-psoralen crosslinked adenovirus DNA shows that linear adenovirus DNA is organized into supercoiled domains. The supercoiled conformation of DNA inside the virion is also demonstrated by sensitivity of the adenovirus core to supercoiling-dependent endonucleolytic activity of Bal31 nuclease. Our data suggest that each supercoiled domain is equal to about 12% of adenovirus genome and that 8 loop-domains can be formed in the viral particles. However, regions at two ends (2%) of viral genome are not included in

supercoiled domains.

Based on the compact, superhelical arrangement of nucleoprotein complexes inside virus particles, The conformation change of viral nucleoprotein complexes following virus entry into the host was studied. Sucrose gradient sedimentation and electron microscopic analyses indicate that adenovirus genome gradually unfolds during the early phase of infection. Unfolding of viral templates of the E1a deletion mutants, dl312, is delayed in HeLa cells, while their unfolding proceeds normally in 293 cells, which expresses endogenous E1a gene products. The result indicate that E1a gene products are required for efficient unfolding of the highly condensed, supercoiled DNA. In addition, inhibition of transcription by alpha-amanitin also blocks unfolding of viral templates.

If a linear DNA is arranged in loops, each loop can be considered as a circular region containing topological constraints. Replication or transcription within a loop can produce topological tension. DNA topoisomerases are enzymes capable of solving the topological tension produced during DNA replication or transcription. Since linear adenovirus DNA is organized into supercoiled domains in the virion, we tested the possible roles of DNA topoisomerases in the life cycle of adenovirus in HeLa cells. Using different topoisomerases inhibitors, it was found that different topoisomerases activities are involved in replication, transcription, and packaging of the linear adenovirus genome. Topoisomerase I and II

inhibitors induce single- and double-stranded cleavages, respectively, at specific sites of adenovirus DNA in early times of infection. These results suggest that topoisomerases are associated with early viral templates at specific sites. Topoisomerase I, not II, is needed for adenovirus replication. Both topoisomerase I and II inhibitors block adenovirus transcription; topoisomerase I inhibitor causes premature termination of major late transcription. The packaging of adenovirus DNA into the virion is blocked by topoisomerase II inhibitor. This result suggests that folding of linear virus DNA into supercoiled conformation is mediated by topoisomerase II. Electron microscopic data provide direct visualization of topologically independent supercoiled domains of intracellular adenovirus DNA-protein complexes.

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Wong, M.-L., and M.-T. Hsu. 1988. Psoralen-cross-linking study of the organization of intracellular adenovirus nucleoprotein complexes. J. Virol. 62: 1227-1234.

Wong, M.-L., and M.-T. Hsu. 1989. Linear adenovirus DNA is organized into supercoiled domains in virus particles. Nucleic Acids Res. 17: 3535-3550.

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Wong, M.-L., and M.-T. Hsu. 1990. Involvement of topoisomerases in replication, transcription, and packaging of the linear adenovirus genome. J. Virol. 64: 691-699.

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Wong, M.-L., and M.-T. Hsu. 1990. Requirement of E1a and RNA synthesis for the unfolding of adenovirus templates following virus entry. submitted.

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Chapter 1: Introduction

I. Human adenoviruses.

A. Structure of the human adenovirus.

Human adenoviruses belong to the genus mastadenovirus of the family adenoviridae (Matthews,1982). The first human adenovirus was isolated independently by Rowe et al. and Hilleman and Werner from human adenoids (Hilleman and Werner,1954; Rowe et al.,1953). Human adenovirus type 2 and 5 are the best studied among 41 distinct serotypes. The virion is nonenveloped and icosahedral (65-80 nm) with "fibers" projecting from 12 vertices (Nermut,1984). It contains at least 9 different structural proteins, designated II to IX according to mobilities in SDS-polyacrylamide gel (Maizel et al.,1968a,1968b; van Oostrum and Burnett,1985). The major components of the protein coat (capsid) are polypeptides II (hexon), III (penton), and IV (fiber). The genome of adenovirus type 2 or 5 is double-stranded DNA of 36 kilobases (11-13 micrometer). The DNA inside the virion is covalently bound with virus-coded 55 K terminal protein (TP) at each 5' end of the linear DNA (Rekosh et al.,1977). Polypeptides V(180 copies) and VII (1070 copies) are

noncovalently associated with viral DNA. Protein VII contains about 22% arginine, which can neutralize the negative charge of DNA. Relative to VII, protein V is easily dissociated from the DNA-protein complex (Brown et al.,1975; Laver,1970; Sung et al.,1983; Vayda et al., 1983). Another low molecular weight nucleocapsid protein, μ, has been reported (Anderson et al.,1989; Hosokawa and Sung,1976), but whose function is poorly understood. Identities and functions of some small polypeptides (X, XI, and XII) revealed on SDS-polyacrylamide gel remain unclear (Weber and Anderson,1988).

B. Adsorption and penetration.

The attachment of adenovirus to human cells (HeLa or KB) has been studied. The fibers, which project from vertices of virion, attach to the putative receptor complex on the cell membrane (Svensson et al.,1981). In KB cells, this receptor complex contains three polypeptides of 78 K, 42 K, and 34 K (Hennache and Boulanger,1977). After the attachment, the mechanism of virus entry into the cell is not clear. Electron microscopic data suggest that virions enter the cell either by low pH dependent endocytosis (Chardonnet and Dales,1970; Pastan et al., 1986) or by direct penetration of the cell membrane (Brown and Burlingham, 1973). Uncoating of viral capsid proteins starts in the cytoplasm, and then the virus

is, probably facilitated by microtubule in the cytoplasm, transported into the nucleus (Dales,1973; Dales and Chardonnet,1973; Miles et al.,1980).

C. The organization of genome and functions of viral proteins.

By convention, the double-stranded 36 kbp DNA of adenovirus are divided into 100 map units from left (E1 region) to right (E4 region). Viral RNA can be transcribed from either strand of the double-stranded DNA. The rightward-transcribed strand is designated the r-strand and the leftward-transcribed strand is the l-strand. R-strand includes E1A, E1B, IX, VA RNA I&II, ML(L1-L5), and E3 regions. All others (E4, E2(A,B), and IVa2) are in the l-strand (Fig. 1). The genomic locations and functions of major viral proteins are briefly listed below.

Gene	Major products	Functions	Reference
E1A	289 R	Transcription activation	Lillie et al.,1987
	243 R	Transcription repression. Transformation.	Lillie et al.,1987
E1B	55K	Transformation. Facilitation of transport of viral	Barker and Berk,1987

		mRNA.	Leppard and Shenk,1989
		Formation of complex with 34 K of E4.	Sarnow et al.,1984
		Shut-off of host protein synthesis	Babiss and Ginsberg, 1984
	19 K	Protection of viral and cellular DNA.	Pilder et al.,1984 White et al.,1984
		Transformation.	Barker and Berk,1987
E2A	72 K	Viral DNA replication.	Friefeld et al.,1983
		Increasing turnover of early viral mRNA.	Babich and Nevins,1981
E2B	140 K	Virus DNA polymerase.	Stillman et al.,1982
	80 K	Precursor of terminal protein.	Challberg et al.,1980
E3	gp19 K	Blocking of surface expression of class I MHC. Protection of Ad-infected cell against cytotoxic T cell.	Burgert et al.,1987
	14.7 K	Countering antiviral effects of TNF.	Gooding et al.,1988
E4	34 K	Formation of complex with 55 K of E1B.	Sarnow et al.,1984
		Shut-off of host protein synthesis	Halbert et al.,1985
IX	IX	Virion protein.	Everitt et al.,1973
		Maintenance of capsid structure.	van Oostrum and Burnett,1985
IVa2	IVa2	Maturation protein in viral morphogenesis.	Persson et al.,1979
L1	52 K, 55 K	Assembly of virions.	Hasson et al.,1989

pIIIa		Vertex region of virion.	van Oostrum and Burnett,1985
L2	III	Penton base.	Pettersson and Hoglund,1969
	V	Core protein.	Alestrom et al.,1984
	pVII	Core protein.	Sung et al.,1983
	X	Core protein	Weber and Anderson,1988
L3	II	Hexon, major structural protein.	Akusjarvi et al.,1984
	pVI	Association with hexon (inside).	Akusjarvi and Persson, 1981
	23 K	Proteinase	Yeh-Kai et al.,1983
L4	100 K	Assembly of hexon trimers.	Cepko and Sharp,1982
	pVIII	Association with hexon.	Everitt and Philipson, 1974
L5	fiber (IV)	capsid vertices.	Dorsett and Ginsberg, 1975

D. Replication.

The genome of human adenovirus is a linear double-stranded DNA (36 kb) with inverted terminal repeats (103 bp for adenovirus type 2 and 5; Shinagawa and Padmanabhan,1980). The origins of DNA replication are located at two ends of the linear DNA. Initiation of viral DNA replication is a protein-priming reaction. The beta-OH of a serine residue in pTP forms

a ester bond with alpha-phosphoryl group of dCMP, the first nucleotide of nascent DNA (Challberg et al.,1980; Desiderio and Kelly,1981; Hay and Russell,1989; Lichy et al.,1981; Stillman,1989). The elongation of nascent DNA proceeds with the addition of nucleotides to 3'-OH of the pTP-dCMP complex. The inverted terminal repeats at two ends allow each single-stranded DNA to form a panhandle structure, which is exactly like the ends of double-stranded adenovirus DNA and is probably able to initiate DNA replication. Electron microscopic data suggest two types of replicating DNA. Type I replicating intermediate is a double-stranded template with the displacement of two parental single-stranded DNA by nascent DNA. Type II replicating intermediate is a panhandle single-stranded template with the elongation of nascent DNA along the single-stranded perental DNA (Kelly et al.,1988; Lechner and Kelly,1977; Leegwater et al.,1988).

Adenovirus DNA polymerase (Ad Pol) and single-stranded DNA binding protein (DBP) are two viral proteins involved in DNA replication (Friefeld et al.,1983; Kruijer et al.,1981; Stillman et al.,1982). Ad pol, which forms a heterodimer with pTP, is essential for initiation and elongation. DBP stimulates Ad pol elongation, but does not participate in the initiation reaction (Friefeld et al.,1983). Besides these virus coded proteins, at least three host nuclear proteins are required for optimal replication of adenovirus. Nuclear factor I and III (NF I and III), stimulating the initiation reaction, are sequence-specific DNA binding proteins (de Vries et al.,1985;

Pruijn et al.,1986; Pruijn et al.,1988). Nuclear factor II (NF II), containing topoisomerase I activity, is required for full length DNA synthesis (Nagata et al.,1983). The recognition sites of NF I and III are located in the origin of replication (nucleotides 19-39 and 39-50 respectively). Recently, the properties of NF I and III have been shown to be indistinguishable from transcriptional factor CTF (CCAAT) and OTF1 (ATGCAAT) respectively (Jones et al.,1987; O'Neill et al.,1988).

E. Transcription.

Adenovirus transcription is a series of complicated events during lytic infection and can be divided into four stages: pre-early, early, intermediate, and late stages. Transcription occurs from six regions (E1A, E1B, E2, E3, E4, and ML) of the genome before the onset of DNA replication. Pre-early and early genes, occurring at 0-2 and 2-6 hours postinfection respectively, include E1A, E1B, E2, E3, and E4 regions. In the intermediate stage, 6-12 hours postinfection, the activities of promoters of IVa2, IX, VAI, and VAII genes reach maxima. After DNA replication, the five late genes, L1-L5, accomplish full transcription (Nevins,1987; Glenn and Ricciardi,1988; Sharp,1984). Transcription of E1A and E1B continues to the late phase of infection, but transcription of E4 is rapidly repressed by 72K protein of E2A (Handa et al.,1983; Nevins and Winkler,1980).

Viral E1A gene products are the first proteins synthesized during lytic infection. All other viral genes, including the polymerase III-transcribed VAI and VAII genes, are under trans-activational control by E1A proteins in the presence of cellular factor(s). The mechanisms of E1A transactivation are not clear. It is likely that E1A activates the promoter by interacting with or by modifying cellular transcription factors (Berk,1986; Flint and Shenk,1989; Moran and Mathews,1987; Nevins,1987). The close association of E1A protein with the promoter, perhaps by interacting with a DNA-bound protein, has been reported (Lillie and Green,1989). Although the major late promoter is active in early infection, only part of the transcription units of late genes is transcribed(Nevins and Wilson,1981; Shaw and Ziff,1980). The onset of DNA replication marks the early to late transition. The expression of late genes is greatly amplified after DNA replication. Using a superinfection experiment of different serotypes adenoviruses, it has been demonstrated that expression of late genes depends on the replicated template, and the unreplicated template in a late infected cell does not express late gene products(Thomas and Mathews,1980). The molecular basis of early to late transition is not clear, but the cis-acting role of viral DNA is essential.

Synthesis of adenovirus mRNA from most transcription units is controlled by complex mechanisms that operate at different levels. All six major transcription units (E1A, E1B, E2, E3, E4, ML) are able to produce multiple mRNA by differential selections of donor/acceptor sites for splicing.

Three of them (E2, E3, ML) contain multiple poly(A) addition sites (Ziff,1985). Two alternative promoters are used in E2A transcription; promoter located at 75 map units is used in early phase of infection, whereas promoter at 72 map units is active after DNA replication (Baker and Ziff,1981; Chow et al.,1979). Relative to transcription units mentioned above, the production of mRNA of IVa2 and IX seems simpler; the former contains only a single intron, and the latter has no intron (Chow et al.,1979; Alestrom et al.,1980). One of the special features of E3 and IX genes is that they are entirely nested in ML(L4-L5) and E1B regions, respectively. Early in infection, E3 is actively transcribed, when primary transcription of ML is down to L3 region only (Shaw and Ziff,1980). Similarly, transcription of E1B is active at early times of infection, while IX gene is transcribed only after DNA replication (Vales and Darnell,1989).

Another important regulation of adenovirus gene expression is the preferential transport of viral mRNA from nucleus to cytoplasm (Beltz and Flint,1979; Flint,1986). Late in infection, transcription and processing of host pre-mRNA in the nucleus occur normally, but no newly synthesized host mRNA can be detected in the cytoplasm. Furthermore, newly synthesized 28S rRNA is also absent in the cytoplasm (Castiglia and Flint,1983). Because pre-mRNA and pre-rRNA are made by RNA polymerases II and I respectively in different nuclear locations and are processed differently, the only step shared by them in controlling their

maturation is the transport from nucleus into cytoplasm. Although the viral 55K protein of E1B has been involved in the control of viral mRNA transport through an intranuclear step in viral mRNA metabolism (Leppard and Shenk,1989), the molecular mechanism is poorly understood.

After transport, the stability of mRNA in the cytoplasm is important in determining the proper viral gene expression. The viral 72K DBP appears to mediate the decay of early mRNAs. There is a overproduction of early mRNAs in the absence of DBP (Babich and Nevins,1981).

F. Translation.

It was observed in early studies that lytic infection of HeLa or KB cells with human adenoviruses eventually leads to production of large amounts of viral capsid proteins and shut-off of the host protein synthesis (Bello and Ginsberg,1967; White et al.,1969). During late times of infection, the pre-existing host mRNA is not degraded, yet cannot be translated (Babich et al.,1983). One of the key components, governing efficient translation of the viral mRNA and shut-off of the host protein synthesis, has been shown to be virus-encoded small RNA, VAI (about 160 nucleotides), which is made in large amounts by RNA polymerase III at late times after infection (Schneider et al.,1984; Thimmappaya et al.,1982). Extensive studies on the relationship between VAI RNA and host translation machinery were

reported, and a tentative model was proposed; however, the interactions among virus and host components are very complicated, and an unambiguous mechanism needs to be established.

Protein synthesis is primarily regulated at the stage of initiation (Hershey et al.,1986). One of the important steps in initiation is the modification of eIF-2. Protein synthesis can be blocked by specific phosphorylation at serine(51) residue of α subunit of eIF-2 by protein kinase HRI (heme-regulated inhibitor) or DAI (double-stranded RNA-activated inhibitor). After completing a round of initiation, eIF-2 is released from the ribosome as eIF-2:GDP complex. In order to recycle eIF-2:GDP complex, the GDP moiety must be converted into GTP, a reaction catalyzed by eIF-2B. If the α subunit of eIF-2 is phosphorylated, it will trap the eIF-2B and eventually, itself is unable to enter another round of initiation (Hershey,1989).

It has been proposed that the primary function of VAI RNA is to prevent the phosphorylation of eIF-2 by DAI kinase and consequently facilitating the translation of viral mRNA (O'Malley et al.,1986; Reichel et al.,1985; Schneider and Shenk,1987). Recently, DAI kinase has been purified and can be bound and inhibited by VAI (Kostura and Mathews,1989). However, the mechanism of preferential translation of viral mRNA remains unknown. A model of two compartments of protein synthesis machinery within the infected cell has been proposed to explain this mystery (O'Malley et al.,1989). One compartment contains viral mRNA and VAI RNA; in the presence of VAI RNA, eIF-2 is not phosphorylated and viral protein

synthesis is active. The other compartment contains host mRNA, but no VAI RNA; DAI kinase can be activated to phosphorylate eIF-2 and protein synthesis is inactive. This two compartments model is speculative and needs to be verified.

Human adenovirus with deletion in VAI gene grows more poorly than wild type. Recently, growth complementation of adenovirus with VAI deletion in cells expressing a serine-to-alanine mutant eIF-2 was reported (Davies et al.,1989). The mutant eIF-2, containing serine-to-alanine mutation, is resistant to phosphorylation by DAI kinase. This result supports that the primary function of VAI RNA is the inhibition of phosphorylation of eIF-2 by DAI kinase.

However, VAI RNA is not the sole viral component involved in shut-off of host translation. It has been demonstrated that both 55K protein of E1B and 34K protein of E4 are required for shut-off of host protein synthesis (Babiss and Ginsberg,1984; Halbert et al.,1985). Since some mutants of E1B and E4 display similar phenotypes and 55K protein can form a complex with 34K protein, the shut-off of host protein synthesis may be a result of collaboration of these two proteins.

G. Assembly.

During the late times of adenovirus infection of HeLa or KB cells, the host protein synthesis is shut off and large amounts of viral proteins are synthesized and transported into nuclei (Bello and Ginsberg,1967; Beltz and Flint,1979). Hexon, penton base, and fiber are three main capsid proteins. The hexon capsomer is a trimer of identical polypeptide. Formation of the hexon trimer is mediated by a virus-coded 100 K protein, which is not present in the mature virion (Cepko and Sharp,1982). The penton capsomer is composed of trimers of the penton base and the fiber, respectively (Devaux et al.,1982;Sundquist et al.,1973). The empty capsid is formed prior to the entry of viral DNA. Only 20% of hexon trimers are assembled into virions and only 10% of viral DNA are inserted into virions (Philipson and Lindberg,1974).

The packaging of adenovirus DNA into virion is not a random process. A cis-acting packaging signal is located between 194 to 358 nucleotides from the left end of viral genome (Hammarskjold and Winberg,1980; Hearing et al.,1987; Tibbetts,1977). It is not solved completely whether naked DNA or core proteins associated DNA enters the virion (Edvardsson et al.,1976; Moncany et al.,1980;).

Proteolytic cleavages of some virion polypeptides are the last stage of maturation. At least five virion proteins are present as precursors (pVI, pVII, pVIII, pIIIa, and pTP), which are cleaved by an endopeptidase within

the virion (Bhatti and Weber,1979; Tremblay et al.,1983; Webster et al.,1989a, 1989b).

II. DNA topoisomerases.

A. Properties of the enzymes and inhibitors.

DNA topoisomerases are enzymes that control the topology and conformation of DNA. According to reaction mechanisms, DNA topoisomerases are classified into type I and II. Type I DNA topoisomerases transiently break one strand of DNA, pass another strand, and then rejoin the cleavage. Type II DNA topoisomerases transiently break both strands of DNA, pass another duplex DNA, and then rejoin the cleavages. Because these transient breakage and rejoining reactions can occur intra- or intermolecularly, a variety of interconversions between different topological forms of DNA can occur. Reactions of type I topoisomerases include relaxation of supercoiled DNA, knotting/unknotting of single-stranded circular DNA, duplex formation of two single-stranded circular DNA, and catenation/decatenation of two double-stranded circular DNA (with one of them containing a single-stranded break). Reactions of type II topoisomerases include relaxation/supercoiling of DNA, knotting/unknotting of double-stranded circular DNA, and

catenation/decatenation of two double-stranded circular DNA (Maxwell and Gellert,1986; Wang,1985). Both types of DNA topoisomerases have been isolated from prokaryotic and eukaryotic cells. Type I topoisomerases of prokaryotic and eukaryotic cells are different in reaction mechanisms and unrelated in their amino acid sequences (Wang,1987). Bacterial type I topoisomerases preferentially relax negatively supercoiled DNA, whereas eukaryotic type I topoisomerases are able to relax positively or negatively supercoiled DNA. The bacterial type I topoisomersase covalently links to the 5' phosphoryl group of DNA in the transient cleavage reaction (Depew et al., 1978). In contrast, the eukaryotic type I topoisomerase is linked to the 3' phosphoryl group of DNA (Champoux, 1977; Hsiang et al.,1985). Type II topoisomerases of prokaryotic and eukaryotic cells have similarities in amino acid sequences. ATP is required for reactions mediated by the prokaryotic or eukaryotic type II topoisomerase, but only the bacterial gyrase can use the ATP hydrolysis energy to introduce negative supercoiling into DNA (Gellert et al.,1976). An unusual type I topoisomerase, named reverse gyrase, has been isolated from a thermophilic bacterium Sulfolobus acidocaldarius. It is able to introduce positive supercoiling into DNA in an ATP-dependent process (Kikuchi and Asai,1984; Nakasu and Kikuchi,1985).

The transient cleavage intermediates of DNA by topoisomerase I or II can be trapped in the presence a strong protein denaturant, SDS or alkali,

and are greatly enhanced by inhibitors of topoisomerases (Drlica and Franco,1988; Liu,1989). Many inhibitors of topoisomerase I or II have been identified. They are very useful in studying cleavage sites of DNA by topoisomerases, molecular mechanisms of topoisomerases reactions, and roles of topoisomerases in DNA replication and transcription. Some of the inhibitors are listed below:

drug	inhibitor of topoisomerase I or II
novobiocin	bacterial gyrase B subunit
nalidixic acid	bacterial gyrase A subunit
oxolinic acid	T4 phage topoisomerase II
camptothecin	eukaryotic topoisomerase I
m-AMSA	eukaryotic topoisomerase II
ellipticine	eukaryotic topoisomerase II
VP-16	eukaryotic topoisomerase II
VM-26	eukaryotic topoisomerase II

In clinical applications, inhibitors of bacterial topoisomerases are antibiotics, and inhibitors of eukaryotic topoisomerases have been shown to be promising in cancer chemotherapy (Drlica and Franco,1988; Liu,1989).

B. Possible roles of topoisomerases in replication and transcription.

Since either prokaryotic or eukaryotic DNA inside the cell is conplexed with proteins and has higher order structure, it is very likely that the topology and conformation of DNA in the cell may influence replication and transcription processes. It has been reported that DNA gyrase is required for the initiation of E. coli DNA replication in vivo and in vitro (Baker et al.,1986; Filutowicz and Jonczyk 1983). From studies using top2 gene mutant in yeast, type II topoisomerase is indispensable for the resolution of intertwined progeny DNA (DiNardo et al.,1984). Topoisomerase I and II are involved in the elongation of SV40 DNA replication in vitro, but only topoisomerase II is able to segregate the multiple- interlocked molecules in the final stage of replication (Yang et al.,1987).

The relationship between DNA topology and transcription has been investigated in the prokaryotic system as well as in the eukaryotic system (Pruss and Drlica,1989; Wang,1985). The role of supercoiling in leucine operon transcription of Salmonella typhimurium was demonstrated by genetic study. Mutations in topA gene (encoding topoisomerase I) increased negative supercoiling and activated the transcription of leucine operon (Margolin et al.,1985). Bacterial gyrase can convert relaxed circular DNA to a negatively supercoiled form. Synthesis of gyrase itself was controlled by DNA supercoiling. The synthesis of gyrase in vivo was

increased when gyrase was blocked by novobiocin or coumermycin A1 (Menzel and Gellert,1983). This homeostatic regulation occurred at the transcriptional level. Several studies indicated that negative supercoiling of DNA template is important for efficient transcription of some of the eukaryotic genes. It was shown that some fraction of SV40 minichromosomes, which are transcriptionally active, can be relaxed by topoisomerase I, implying that active chromosomes are under supercoiled tension (Luchnik et al.,1982). For the 5S gene of Xenopus, the supercoiled DNA was found to be a better template than the relaxed one, and the formation of superhelical "dynamic" chromatin was mediated by topoisomerase II (Glikin et al.,1984; Ryoji and Worcel,1984). Using transfection, it was also reported that supercoiled DNA containing enhancers produced higher level of transcription than linear DNA (Weintraub et al.,1986). Moreover, The DNase I sensitivity of active beta-globin genes is promoted by superhelical tension of DNA, and novobiocin, a topoisomerase II inhibitor, can reverse the DNase I sensitivity of beta-globin genes (Villeponteau et al.,1984). However, not all studies support the template supercoiling dependence of transcription. It was found that relaxation of supercoiled rDNA by topoisomersase I is essential for its transcription in vitro, and that camptothecin, a topoisomerase I inhibitor, diminishes the transcription of supercoiled rDNA but has no significant effect on the transcription of linearized rDNA (Garg et al.,1987). Indeed

topoisomerase I was found to be associated with transcribed regions of Drosophila cells by in vivo protein-DNA photocrosslinking technique. This finding may reflect a biological role of topoisomerase I in the regulation of transcription (Gilmour et al.,1986). In contrast to the association of topoisomerase I with sequences internal to a gene, topoisomerase II cleavage sites are located in nontranscribed spacer regions and close to the 5' and 3' boundaries of gene (Udvardy et al.,1985). In yeast, it was suggested that rRNA synthesis requires an active topoisomerase to relieve tension; interestingly, topoisomerase I and II can substitute for each other in the reaction (Brill et al.,1987). In summary, the role of template supercoiling in transcription of eukaryotic genes is still controversial, but the accumulating data do support that topoisomerase I and/or II participate in the regulation of transcription.

The influence of DNA topology on transcription has been studied by many researchers. But how transcription affects DNA topology was less studied. Recently Liu and Wang proposed a model that local and temporal waves of supercoiling can be produced during transcription (Liu and Wang,1987). In this model, the relative rotation of RNA polymerase and its nascent RNA around the DNA in transcription will produce a positively supercoiled domain in front of RNA polymerase and a negatively supercoiled domain behind it. This hypothesis has been verified both in prokaryotic and eukaryotic cells (Figueroa and Bossi,1988; Giaever and

Wang,1988; Wu et al.,1988). These data emphasized the importance of the interplay between DNA topology and transcription.

III. Chromatin structure and its relationship with gene expression.

Chromatin structure is one of the factors which influence eukaryotic gene expression. At least two structural features of chromatin can influence the regulation of gene expression. First, at the nucleosome level, the 5' and 3' ends of active genes have been shown to be hypersensitive to DNase I or micrococcal nuclease (Elgin,1988; Gross and Garrard,1988; Samal et al.,1981; Wu,1980). These nuclease sensitivity of 5' end sequences indicates that these regions are more accessible to RNA polymerase and other transcription factors. Second, the conformation of chromatin is correlated with the control of gene expression. In the classic example of lampbrush chromosomes, the transcriptionally active regions of chromosomes stretch out and form loops to facilitate RNA synthesis while other dormant regions are maintained in the condensed state (Callan,1986). Another well-known example of chromosome loops is the fruitfly. The polytene chromosome structure in salivary gland cells of the fruitfly contains expanded domains called puffs, which are actively engaged in transcription (Bonner and Pardue,1977). Furthermore, DNA loop structures have been well-studied in several systems. Both electron microscopic visualization and

change of sedimentation rate after exposure to ethidium bromide suggest that the chromosomes of E. coli or HeLa cells contain supercoiled loop domains (Gasser and Laemmli,1987; Paulson and Laemmli,1977; Worcel and Burgi,1972). Loop sizes are in the range of 30 to 100 kbp of DNA, depending on different systems. The fact that DNA loops are preserved even in chromosomes of metaphase stage, which lacks a nuclear membrane, supports the idea that DNA loops are anchored to some scaffold components. The components that fasten bases of the loops are non-histone proteins, and topoisomerase II turns out to be the major component of scaffold proteins. Roughly three copies of topoisomerase II are associated with one DNA loop in human metaphase chromosome (Gasser et al.,1986). The anchorage sequences of DNA loops have been identified and named scaffold-associated regions (SARs) or matrix association regions (MARs). They are AT-rich sequences of several hundred base pairs long and contain topoisomerase II cleavage consensus sequences (Adachi et al.,1989; Cockerill and Garrard,1986; Gasser et al.,1989; Mirkovitch et al.,1984; Sperry et al.,1989).

IV. Specific aims.

Relative to whole cell chromosomes, animal viruses provide simple genomes whose structure and regulation can be easily studied. The

genome of eukaryotic cell is highly condensed in the nucleus. This raises questions of whether there is a specific arrangement of DNA in the nucleus and what is the effect of the organization of DNA on gene expression. Using human adenovirus as a model system, we would like to study the relationship between the higher order structure of DNA and gene expression.

Like cell DNA, the linear adenovirus DNA is highly condensed inside the virion, but contrasting with cell DNA, which is associated with histones, virion DNA is complexed with virus-coded basic proteins (mostly VII and V). Despite the amount of studies done by many researchers, the higher order structure of nucleoprotein complex inside the virus particle has not been unequivocally solved. The higher order organization of nucleoprotein complex inside the virion was the first problem that we had studied. There are few studies on the role of unpackaging of the highly condensed animal virus genome in the control of viral gene expression. The next goal was to examine the conformation change of the viral nucleoprotein copmplexes from early to late stages of infection and its relationship with gene expression. Finally, based on our supercoiled loop domains model of linear adenovirus DNA, the roles of topoisomerases in replication, transcription, and packaging of adenovirus during lytic infection were investigated.

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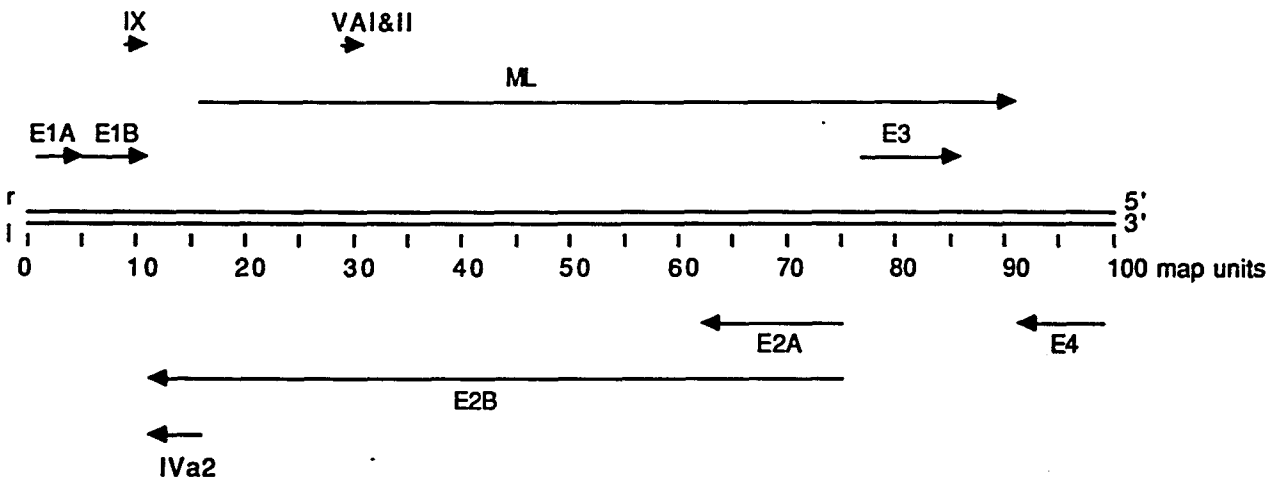
H. Figure legend.

Fig. 1. Schematic representation of transcription map of human adenovirus. The genome is divided into 100 map units from left to right. Viral RNA can be transcribed from either one strand of the double-stranded DNA (r: rightward transcription; l: leftward transcription). The functions of viral genes are described in the text.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Chapter 2

Linear adenovirus DNA is organized into supercoiled domains in virus particles

Min-Liang Wong and Ming-Ta Hsu

Department of Microbiology
Mount Sinai School of Medicine of City University of New York
New York, New York 10029

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A. Abstract.

Electron microscopic analysis of bis-psoralen crosslinked adenovirus type 5 virion DNA revealed supercoiled domains in an otherwise linear DNA. The existence of supercoiled arrangement in all the virion DNA was demonstrated by the sensitivity of Ad5 DNA in pentonless virus particles to the supercoiling-dependent endonucleolytic activity of Bal31 and S1 nucleases. These nucleases were found to cleave Ad5 virion DNA at specific sites. The observation of stable cleavage sites in the limit digestion of virion DNA by Bal31 suggests that cleavage sites represent boundaries of core proteins which impede the exonuclease activity of Bal31. These data suggest that specific arrangement of core proteins on Ad5 virion DNA. Based on this analysis we determined positions of core proteins in viral genome using indirect end labeling technique. The size of supercoiled domains of virion DNA was estimated by electron microscopy and also by boundaries of mutually exclusive Bal31 cleavage sites at limit digestion condition. Our data suggest each supercoiled domain is equal to about 12% of Ad5 genome length and about 8 loops can be accommodated in Ad5 virion. However sequences at two extreme ends of the viral genome were found to be outside of supercoiled domains. An interesting correlation

between supercoiled domains and gene domains of Ad5 genome was noticed.

B. Introduction.

The genome of human adenovirus is a linear double-stranded DNA about 36 kilobase pairs in length. In contrast to that of polyoma virus and papillomaviruses, DNA in the adenovirus particles is not associated with cellular histones. Instead, it is complexed with the virus-encoded core proteins, V, VII, and Mu (Nermut,1984). The association of adenovirus DNA with viral core proteins provides an interesting model for studying DNA packaging and organization other than the nucleosome found in all eukaryotic cell genomes.

The organization of adenovirus nucleoprotein complex in the virus particles has been investigated using micrococcal nuclease (Corden et al.,1976; Mirza and Weber,1982). Based on the pattern of micrococcal nuclease digestion of adenovirus DNA in pentonless particles, Corden et al. suggested that the adenovirus DNA is organized into a nucleosome-like structure with the subunit particle containing about 200 bp of viral DNA complexed with six copies of protein VII and one copy of protein V (Corden et al.,1976). While adenovirus cores prepared by the deoxycholate and the pyridine methods have been observed to contain the "bead-on-string" structure, supporting this nucleosome model of organization, digestion of the cores by micrococcal nuclease revealed no distinct nucleosome-like

DNA ladder (Mirza and Weber,1982; Vayda et al.,1983; Nermut, 1979), in contrast to the result obtained using pentonless particle (Corden et al.,1976). These results, together with the observations of rod-like structure in adenovirus cores under electron microscope, have prompted Nermut to propose a continuous model in which the adenovirus DNA is wound around a continuous superhelical filament of core proteins (Nermut,1979; Nermut,1980).

The higher order organization of adenovirus DNA-protein complex in the virus particles has been analyzed by electron microscopic techniques using virus cores prepared either by detergent lysis of virus particles (Brown et al.,1975) or by the ion-etching technique (Newcomb et al.,1984). These studies suggest that adenovirus DNA is organized in 8-12 DNA loops inside the virus particle. These findings are interesting because loop-domain organization has been found in the genomes of both prokaryotic and eukaryotic cells (Pettijohn and Sinden,1985; Gasser and Laemmli,1987). The simple structure of adenovirus makes it attractive as a model system for studying the mechanisms of loop-domain organization and the relationship between genome higher order structure and gene expression.

We probed the higher order structure of adenovirus DNA-protein complex in the virus particles using Bal31 nuclease and psoralen derivatives. Bal31 nuclease has been shown to exhibit two types of nuclease activity: a double-stranded DNA exonuclease activity and a

supercoiling-dependent endonuclease activity (Lau and Gray,1979). We took advantage of the latter property of Bal31 to show that adenovirus virion DNA is organized in torsionally stressed, supercoiled loops inside virions. The supercoiling conformation of intravirion adenovirus DNA is supported by the inhibition of Bal31 cleavage by calf thymus topoisomerase I and by the observation of highly supercoiled loops in adenovirus DNA extracted from virus particles crosslinked with psoralen. Our data are consistent with the loop-domain organization of adenovirus DNA. Bal31 digestion experiments further showed that the core proteins are positioned at defined locations on virion DNA.

C. Materials and methods.

Viruses and the preparation of pentonless virus particles: Human adenovirus type 5 was extracted from infected HeLa cell nuclei with ammonium sulfate (Wilhelm et al.,1976). The virus particles were purified by buoyant density centrifugation twice in a CsCl density gradient (density=1.34). The preparation of dl312 mutant was essentially same as that of wild type adenovirus type 5 except 293 cells were used for infection. Pentonless virus particles were prepared essentially as described (Corden et al.,1976) by dialyzing adenoviruses against 5 mM Tris-acetate, pH 6.4 at

4oC overnight. The dialyzed virus particles were heated at 56oC for 3 minutes to facilitate the removal of penton bases.

Digestion of pentonless adenovirus particles with nucleases: For digestion with Bal31 nuclease the reaction was performed in 12.5 mM CaCl2, 12.5 mM MgCl2, 600 mM NaCl, 50 mM Tris, pH 7.4 at 30oC for the periods indicated in the legends to the figures. The ratio of Bal31 to DNA is about 1 unit of enzyme per ug of adenovirus DNA. S1 and Mung Bean nuclease digestions were carried out at 37oC in 50 mM sodium acetate, 2 mM ZnSO4, pH 4.5, and in 10 mM Tris-HCl, pH 7.4, respectively. Digestion of pentonless particles with restriction endonucleases was carried out using the buffer supplied by the manufacturer. All the nucleases except calf thymus topoisomerase I (BRL) were purchased from Boehringer-Mannheim. After digestion the reactions were stopped by adding sufficient EDTA and/or EGTA to chelate the divalent ions, and the solutions were brought to 1% SDS. After digesting the proteins with 100 ug/ml proteinase K at 37oC for 2 hours viral DNA was purified by extraction with phenol and chloroform.

Gel electrophoresis and Southern blotting analysis: Adenovirus DNA samples were analyzed by horizontal gel electrophoresis in 1.2% agarose gels immersed in TEA buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.4). The DNA was blotted onto Nytran paper (Schleicher &

Schuell) and hybridized with nick-translated 32P labeled probes as indicated in the legends to the figures. The plasmid probe, pE1a, containing adenovirus sequences from 311 to 1764, was a gift from I. Kovesdi and J. Nevins.

Crosslinking of adenovirus virion DNA with bis-AMT: Bis-AMT was synthesized by the reaction between the free amino group of AMT (aminomethyltrioxsalen, Calbiochem) and the N-succinamide groups in Dithiobis(succinimidylpropionate) (Pierce). AMT (10 mg/ml) was incubated with Dithiobis (5 mg/ml) in N, N- dimethylformamide at room temperature overnight in the dark. The formation of bis-AMT was characterized by thin layer chromatography (solvent: 90% toluene and 10% acetone). Crosslinking of the adenovirus virion DNA was performed by irradiating the mixture of adenoviruses and bis-AMT (6 ug/ml) with 365 nm ultraviolet light (Spectroline) at a rate of 70 J/M2 per second for one hour. The drug was replenished at 20-minute intervals during irradiation. After crosslinking, adenovirus DNA was extracted as described above. The structure of the crosslinked DNA was examined in the Phillips EM 201 electron microscope. Crosslinked DNA was spread in 50% formamide, 100 mM Tris-HCl, 10 mM EDTA, pH 8.5. The grids were stained with 5 micromolar uranyl acetate and rotarily shadowed with platinum-palladium (80:20). In the experiments

for determining the handedness of DNA supercoiling, the grids were first shadowed from one direction and then rotarily shadowed.

D. Results.

Adenovirus DNA in the pentonless particle is highly sensitive to the endonuclease activity of Bal31 nuclease in a supercoiling-dependent manner. Bal31 nuclease has been shown to exhibit endonuclease activity towards supercoiled DNA with torsional stress (Lau and Gray,1979). We used this property of Bal31 to probe the topological state of adenovirus DNA in virus particles. To make virion DNA accessible to Bal31 nuclease, we remove penton bases from the virus particles by the procedure of Corden et al. (Corden et al.,1976). Digestion of pentonless particles with Bal31 rapidly produced small DNA fragments ranging from 1-8 kb in length. When the digested products were probed with adenovirus DNA derived from either the right- or left-hand end, discrete bands were observed even at the earlist time point of digestion (Fig. 1). In contrast, only a smear of DNA characteristic of exonuclease activity of Bal31 was observed when purified adenovirus DNA was digested with Bal31 and probed with left-hand probe (Fig. 2). These results indicate that adenovirus DNA in the pentonless particles was cleaved by the endonuclease activity of Bal31 and that cleavages occurred at specific sites. The endonuclease cleavage of

adenovirus virion DNA in the pentonless particles could reflect that the presence of DNA in a supercoiled conformation or alternatively, the presence of single-stranded regions located at specific sites. To distinguish between these two possibilities, we pretreated the pentonless particles with calf thymus topoisomerase I before Bal31 digestion. Since topological tension of supercoiled DNA is removed by topoisomerase I, this treatment should inhibit the endonuclease cleavages of virion DNA only if the cleavages are due to a supercoiled conformation. As shown in Fig. 3, endonuclease cleavages of adenovirus virion DNA by Bal31 was indeed inhibited by the pretreatment of pentonless particles with topoisomerase I. This result convincingly demonstrates the supercoiled conformation of adenovirus virion DNA.

Observation of supercoiled DNA loops in linear adenovirus virion DNA after psoralen crosslinking. The presence of supercoiled conformation in adenovirus virion DNA was also confirmed using psoralen crosslinking technique. A bi-functional psoralen reagent, bis-aminomethyltrioxalen (bis-AMT, see Materials and methods) was synthesized and used to fix the conformation of DNA in virus particles. When adenovirus DNA extracted from bis-AMT crosslinked virions was examined in the electron microscope, a novel structure with highly supercoiled loops in a linear DNA molecule was observed in about 6-10% of the crosslinked DNA molecules (Fig. 4, a

and b, arrows). No supercoiled conformation was ever found when purified adenovirus DNA was crosslinked in the same manner. The supercoiled portions of the crosslinked molecules were found to resist denaturation by glyoxal, in contrast to the linear parts of the molecules (Fig. 4, c and d). Furthermore, the supercoiled loops could be partially relaxed under partially denaturing conditions. These properties are reminiscent of those of covalently closed, supercoiled plasmid or viral DNA molecules (Vinograd et al.,1965). Despite our ignorance of the nature of chemical crosslinks that prevent the topological equilibrium between the linear and the supercoiled portions of the crosslinked molecules, the unusual coexistence of supercoiled and linear conformations in a single DNA molecule can only be due to the presence of supercoiled DNA loops inside adenovirus particles. This result, therefore, supports those obtained using Bal31.

The size distribution of the supercoiled loops in the crosslinked virion DNA is shown in Fig. 5. Peaks of distribution were found at about 12%, 24%, and 36% of genome length. Because of substantial tangling of large supercoiled loops, the size of loops larger than 36% could not be measured accurately. The distribution shown in Fig. 5 suggests a fundamental unit of supercoiled loop equal to about 12% of adenovirus genome length. The handedness of supercoiling was determined by shadowing the molecules unidirectionally (Iwamoto and Hsu,1983) and examined the direction of crossover of supertwisted DNA in comparison with form I SV40 DNA

included in the crosslinked adenovirus DNA as an internal standard. The direction of supercoiling was found to be the same as that of SV40 DNA, from which we conclude that adenovirus is supertwisted in a left-handed fashion inside the virus particles (Fig. 6).

Determination of core protein positions on adenovirus DNA. As described above, Bal31 nuclease cleaves adenovirus DNA in the pentonless virus at discrete sites (Fig. 1). Since Bal31 is also an exonuclease one would expect the free ends generated by the endonuclease activity of Bal31 to become progressively shortened as digestion is prolonged. This is not found in the kinetic analysis of Bal31 digestion of pentonless particles (Fig. 1, compare lanes d and e). Whereas purified adenovirus DNA became progressively shortened by the treatment with Bal31. This result suggests that the stable Bal31 cleavage sites in virion DNA may be generated by the inhibition of Bal31 exonuclease activity by proteins bound to adenovirus DNA. This is reminiscent of the Bal31 strong stops at nucleosomes (Scott et al.,1984) and of exonuclease III strong stops in mapping protein positions bound to DNA (Wu,1984). This interpretation is further supported by the finding that S1 nuclease cleaved adenovirus DNA in the pentonless particles at sites about 50-100 bp away from the Bal31 cleavage sites (data not shown). Since there is no exonuclease activity associated with S1 nuclease, the 50-100 bp extra sequences seen in S1 digestion presumably

correspond to the DNA removed by the exonuclease activity of Bal31. Based on this argument, we mapped the positions of core proteins in different regions of adenovirus DNA using the indirect labeling technique (Wu,1980). The result, summarized in Fig. 7, suggests that core proteins are located at defined positions on adenovirus DNA in virus particles.

To exclude the possibility that the specific cleavage is due sequence-specific cleavages by Bal31, we analyzed the Bal31 cleavage sites in supercoiled plasmid pE1a containing the E1a gene. Although specific cleavage sites could be discerned at early times of digestion, these sites were found to be different from those mapped in the virion DNA. Furthermore, the specific cleavage sites in the plasmid DNA appeared only transiently as DNA is eventually degraded by the exonuclease activity of the enzyme.

Core protein positions are not changed in dl312 deletion mutant. The results shown above indicate a specific arrangement of core proteins on adenovirus DNA inside the virion. To examine whether deletion in viral DNA may perturb the organization of virion nucleoprotein complex, we analyzed the Bal31 digestion pattern of the core of dl312 mutant, deleted in the E1a gene from nucleotides 448 to 1349 (Jones and Shenk,1979). When the left end probe was used to map the Bal31 digestion pattern of dl312 core DNA, the cleavage sites were found to shift about 0.9 kbp

toward the left end of adenovirus DNA as compared to the wild type cleavage sites (Fig. 8). Since the 0.9 kbp shift observed in the mutant corresponds to the deletion in dl312, the result suggests that core protein positions relative to viral DNA are not changed in dl312. Consistent with this interpretation, we found that cleavage sites in the right end of dl312 genome are indistinguishable from that of wild type (Fig. 8).

E. Discussion.

Supercoiled conformation of adenovirus DNA in virus particles. We have presented several types of evidence that adenovirus DNA in the virus particles is supercoiled. First, supercoiled loops were directly visualized after fixing the DNA conformation inside the virus particles by crosslinking. Second, DNA of virus cores was found to be sensitive to the endonuclease activity of Bal31 nuclease. The endonuclease activity of Bal31 towards double-stranded DNA has been shown to depend on the supercoiled conformation of DNA (Lau and Gray,1979). Furthermore, relaxation of supercoiling by topoisomerase I inhibited the digestion of adenovirus virion DNA by Bal31. The supercoiled conformation of intravirion adenovirus DNA has also been suggested by the transient appearance of supercoiled loops during lysis of adenovirus by sarkosyl (Brown et al.,1975).

Supercoiling of a linear genome was also reported for bacterial T4 and lambda phages (Sinden and Pettijohn,1982; Virrankoski-Castrodeza and Parish,1980). These observations, together with our present evidence, indicate that supercoiled conformation is not restricted to covalently closed circular DNA molecules. However, the supercoiling of a linear DNA must be maintained by restricting the rotation of the double helix in such a way that the topological tension of supercoiling is not released through rotation of the free ends. This could conceivably be achieved by one of the following ways: 1) tight-binding proteins at boundaries of supercoiled loops in the linear molecule; or 2) a cross-hybridized form of an inverted repeat sequence at the boundaries of supercoiled loops, as has been observed in plasmid DNA after denaturation and renaturation (Broker et al.,1977). Because only the linear adenovirus DNA is extracted from virions after proteinase digestion, the supercoiled conformation described in this report is most likely due to the restriction of DNA double helix rotation by tight-binding proteins.

The DNA in the nucleosomes is supercoiled but does not have topological tension (Sinden et al.,1980). Since our data indicate that the supercoiling of adenovirus DNA in virus particles is not restrained, the virion DNA-protein complex is most likely not organized in a nucleosome-like structure. The supercoiling of adenovirus virion DNA, therefore, is probably the result of gyrase-like activity such as that found in the organization of

bacterial chromosomes (Drlica,1984). Since unpackaged adenovirus DNA in infected cells at late times after infection is present in linear conformation (Wolgemuth and Hsu,1981), supercoiling of viral DNA must occur during the packaging process. This consideration suggests that gyrase-like activity is accociated with the virion packaging machinery, a hypothesis that can be tested by studying the effect of topoisomerase II inhibitors on adenovirus virion assembly.

The fixation of supercoiled conformation of adenovirus virion DNA by bis-AMT is presumably due to the crosslinking of two adjacent double helices at the base of the supercoiled loops. However, we also found that the supercoiled loops could be fixed with AMT alone albeit at lower efficiency than that of bis-AMT. AMT was also found to be able to transform linear lambda phage DNA, condensed in 50% ethanol solution, into supercoiled molecules (unpublished observation). These results suggest that the bis-AMT or AMT crosslinked sequences at bases of supercoiled loops must lie very close to each other, perhaps forming a tatrahelix. It is also possible that the fixation of supercoiled loops by bis-AMT is not due DNA-DNA crosslinks but to the crosslinking of two adjacent double helices mediated by proteins that are bound to these sequences. Although we could not exclude the protein-mediated crosslinking reaction at the present time we consider it rather unlikely for the following reasons. First, adenovirus virion DNA was extensively treated

with proteinase K and extracted with phenol and chloroform before examination in the electron microscope. Second, the quantum yield of photoreaction between psoralen and protein molecules is generally much lower that that of reaction between psoralen and nucleic acids (Cimino et al.,1985). Despite our ignorance of the nature of the chemical bonds involved in the fixation of supercoiled loops in virion DNA the method nevertheless has proved very useful in studying the topological arrangement of DNA.

Loop-domain organization of adnovirus DNA in virus cores. In the limit digestion of adenovirus DNA with Bal31 in the pentonless particles, a stable pattern of digestion products was observed (see Fig. 1). Since the larger products contained the Bal31 cleavage sites that generated the smaller products, this result indicated that the cleavage sites in the limit digestion were mutually exclusive. A simple explanation of this result is that these mutually exclusive cleavage sites are located in the same supercoiled domain. Any cleavage would eliminate the supercoiled conformation and thereby prevent the other sites from being cleaved by Bal31. Alternatively, it is possible that mutually exclusive cleavages observed are due to the existence of different types of nucleoprotein organization in the virus population. Although we cannot exclude the latter possibility, we favor the

supercoiling model because of our finding of the supercoiled conformation of adenovirus DNA in the virus particles.

If the mutually exclusive Bal31 cleavages are indeed due to supercoiling conformation, then the cleavage sites must all be present in the same supercoiled domain. Therefore, the size and location of the supercoiled domain can be derived from the locations of the mutually exclusive cleavage sites. We estimate that the supercoiled domains at the two ends of adenovirus genome are about 12% of the viral genome. This size is similar to the repeat unit of supercoiled loops observed in the bis-psoralen crosslinked DNA. Assuming adenovirus DNA is organized in supercoiled loops of 12% genome length, then there would be eight supercoiled loops in the virion DNA. This type of arrangement of virion DNA is consistent with the 8-12 DNA loops observed previously in adenovirus disrupted with sarkosyl (Brown et al.,1975). We have also observed eight DNA loops in adenovirus cores prepared by the deoxycholate method (Fig. 9).

An interesting correlation between supercoiled loop-domains and gene arrangement of adenovirus DNA can be made based on the organization of adenovirus DNA into eight loops. This is shown in Fig. 10. For example, the first loop, from coordinate 2-14, accommodates the E1a and E1b transcription units. This correlation between genetic and physical organization of adenovirus genome suggests a topological mechanism for regulation of adenovirus gene expression. We propose that the viral

genome is present as a compact supercoiled structure after virus uncoating and that the expression of adenovirus genes at early times after infection is governed by the unfolding of viral templates. Recently, we have analyzed the structures of adenovirus templates at early times after virus infection by sedimentation and electron microscopic analysis. Indeed, we found that the viral templates at early times after infection underwent conformational unfolding from a compact supercoiled structure to an extended form. We are currently investigating the relationship bwtween the observed conformational changes and viral gene expression.

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G. Figure legends.

Fig. 1. Bal31 digestion pattern of Ad5 virion DNA in the pentonless virus particles. The pentonless particles were digested with Bal31 for 0.5 hr (lane b, b'), 1 hr (c, c'), 2.5 hr (d, d'), and 3.5 hr (e, e'), or self-incubated without Bal31 for 3.5 hr (a, a'). The digested DNA was purified, electrophoresed in a 1.2% agarose gel, and probed with a left hand end probe (nucleotide 311-1764, left panel, a-e) or with a right hand end probe (nucleotide 34933-35937, Hind III-I fragment, a'-e').

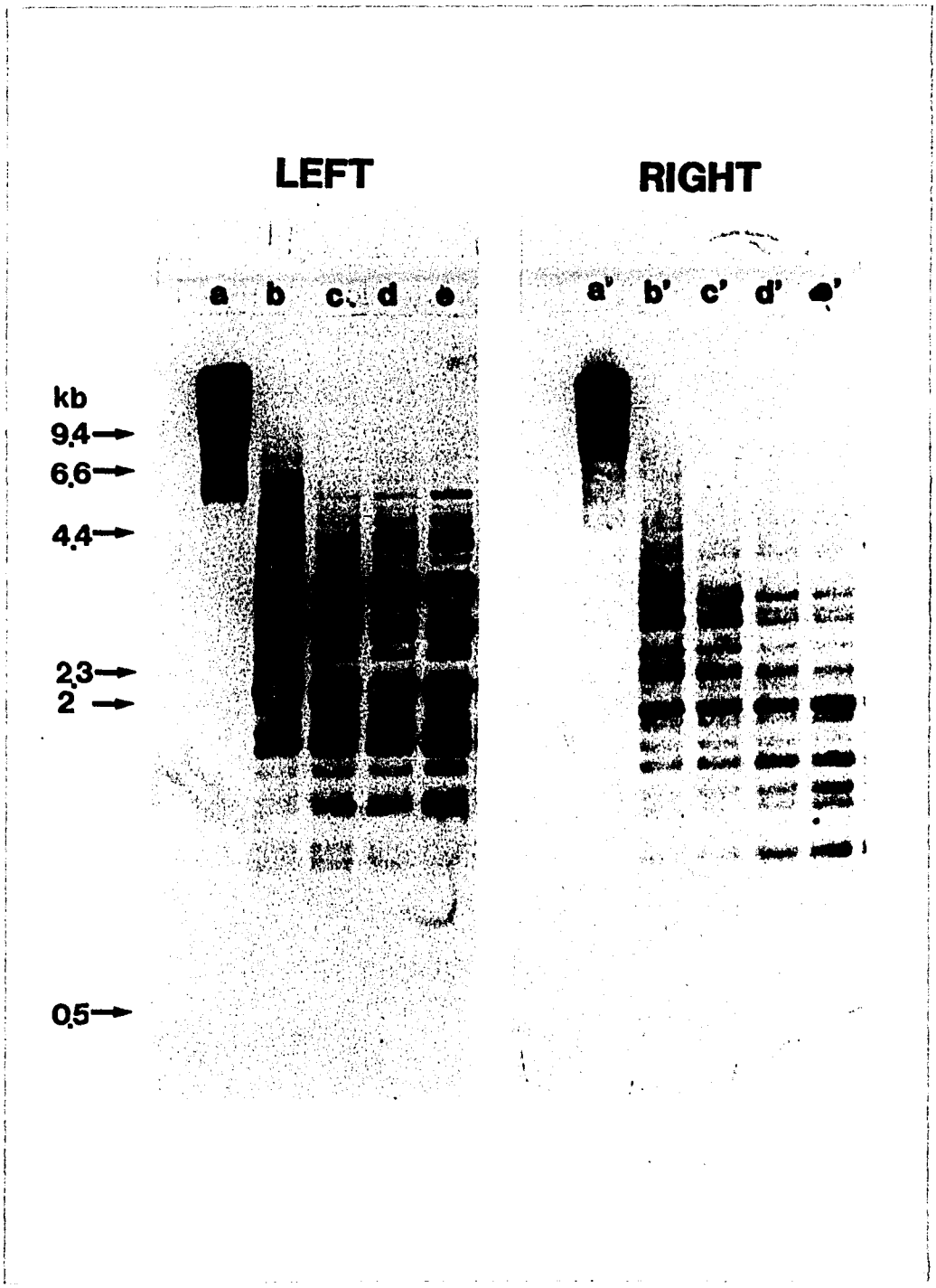

LEFT
RIGHT
a b c d e
a' b' c' d' e'
kb
9.4
6.6
4.4
2.3
2
0.5

Fig. 2. Bal31 digestion of purified Ad5 DNA. Ad5 DNA purified from virus particles was digested for 30 min with Bal31 as in Fig. 1 and probed with the left hand probe.

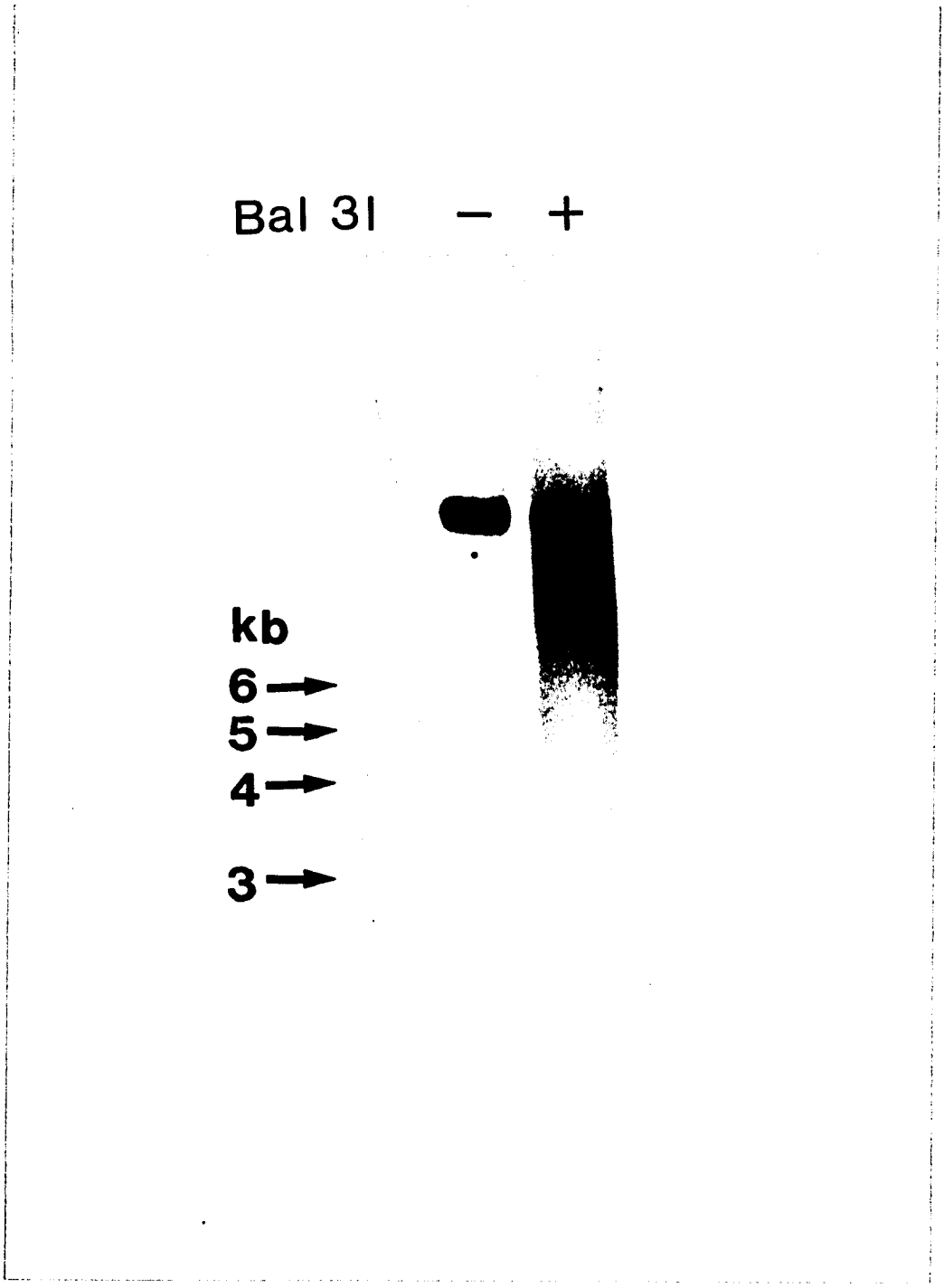

Bal 31 − +
kb
6
5
4
3

Fig. 3. Inhibition of Bal31 cleavages of Ad5 pentonless particles DNA by calf thymus topoisomerase I. Pentonless particles were digested with Bal31 (lane b) or without (lane a) pretreatment with 60 units of calf thymus topoisomerase I. The amount of undigested full length Ad5 DNA was increased in the topoisomerase I treated sample (lane b).

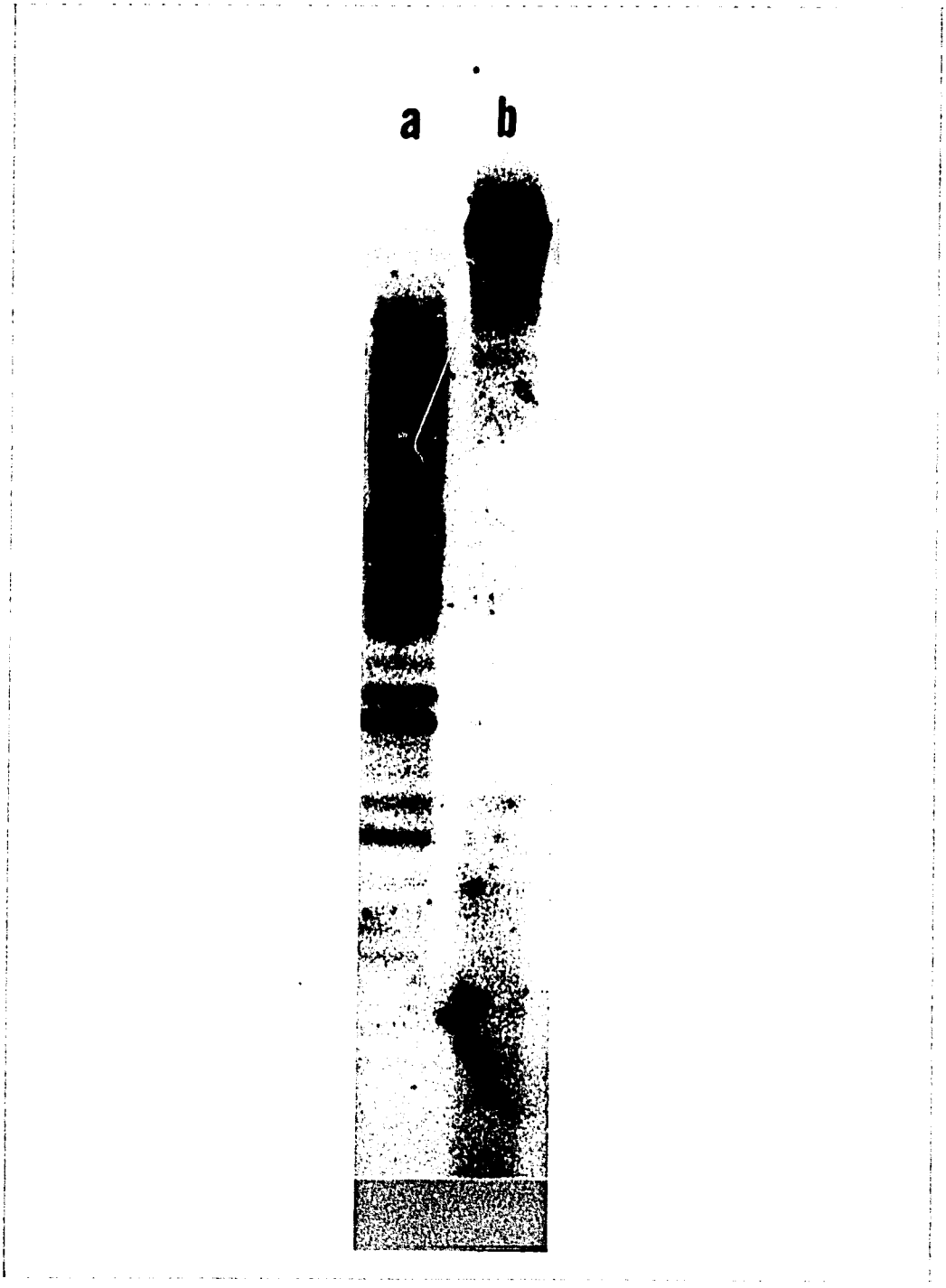

a
b

Fig. 4. Electron micrographs of Ad5 virion DNA crosslinked with bis-AMT showing supercoiled loops in the linear Ad5 DNA. Ad5 DNA in the intact virus particle was crosslinked with bis-AMT and the viral DNA was purified and spread in 60% formamide without further treatment (panels a and b) or after the DNA was denatured with glyoxal and formamide (panels c and d). The supercoiled loops are indicated by arrows. Note that in panels c and d the linear portion of the crosslinked DNA shows denaturation bubbles bounded by crosslinks whereas supercoiled loops remain double-stranded. The bars represent 1 micrometer.

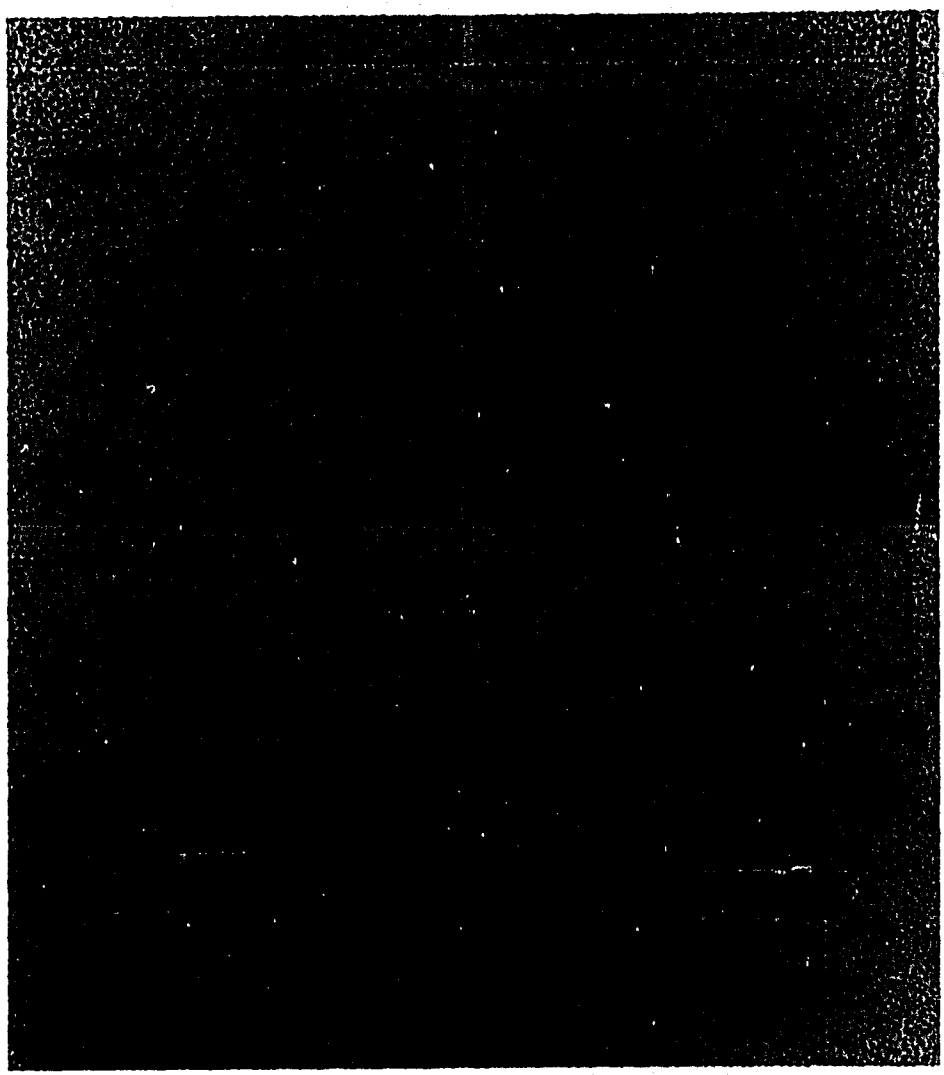


Fig. 5. Histogram of length distribution of supercoiled loops in crosslinked Ad5 virion DNA. Because of the difficulty of tracing highly supertwisted loops, the lengths of supercoiled loops were estimated by substracting the linear portions of the molecules from an intact Ad5 linear DNA nearby. A total of 65 molecules were measured.

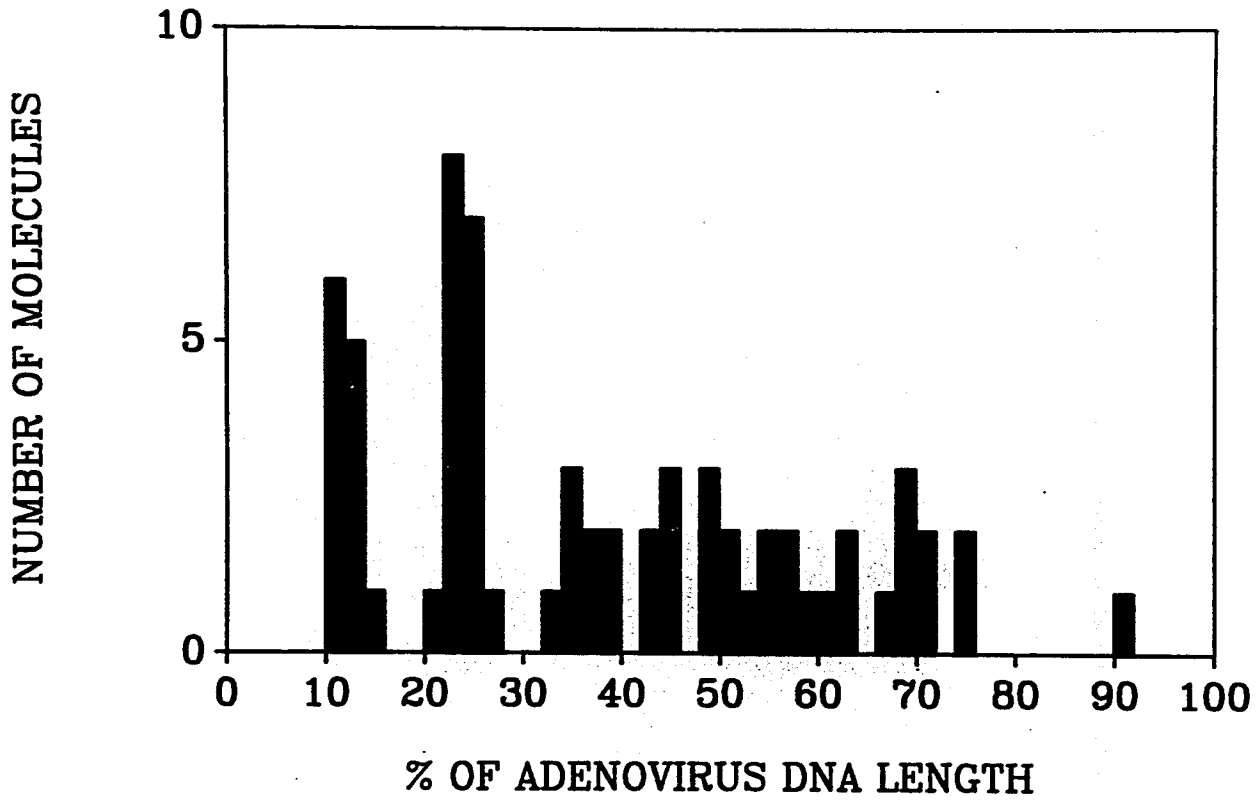

NUMBER OF MOLECULES
10
5
0
0
10
20
30
40
50
60
70
80
90
100
% OF ADENOVIRUS DNA LENGTH

Fig. 6. Determination of handedness of Ad5 DNA supercoiling with unidirectional shadowing. The handedness of crosslinked Ad5 virion DNA (panels A and B, bars = 1 micrometer), was compared with that of form I SV40 DNA (panel C, bar = 0.1 micrometer) and they showed the same direction of supercoiling (panel D).

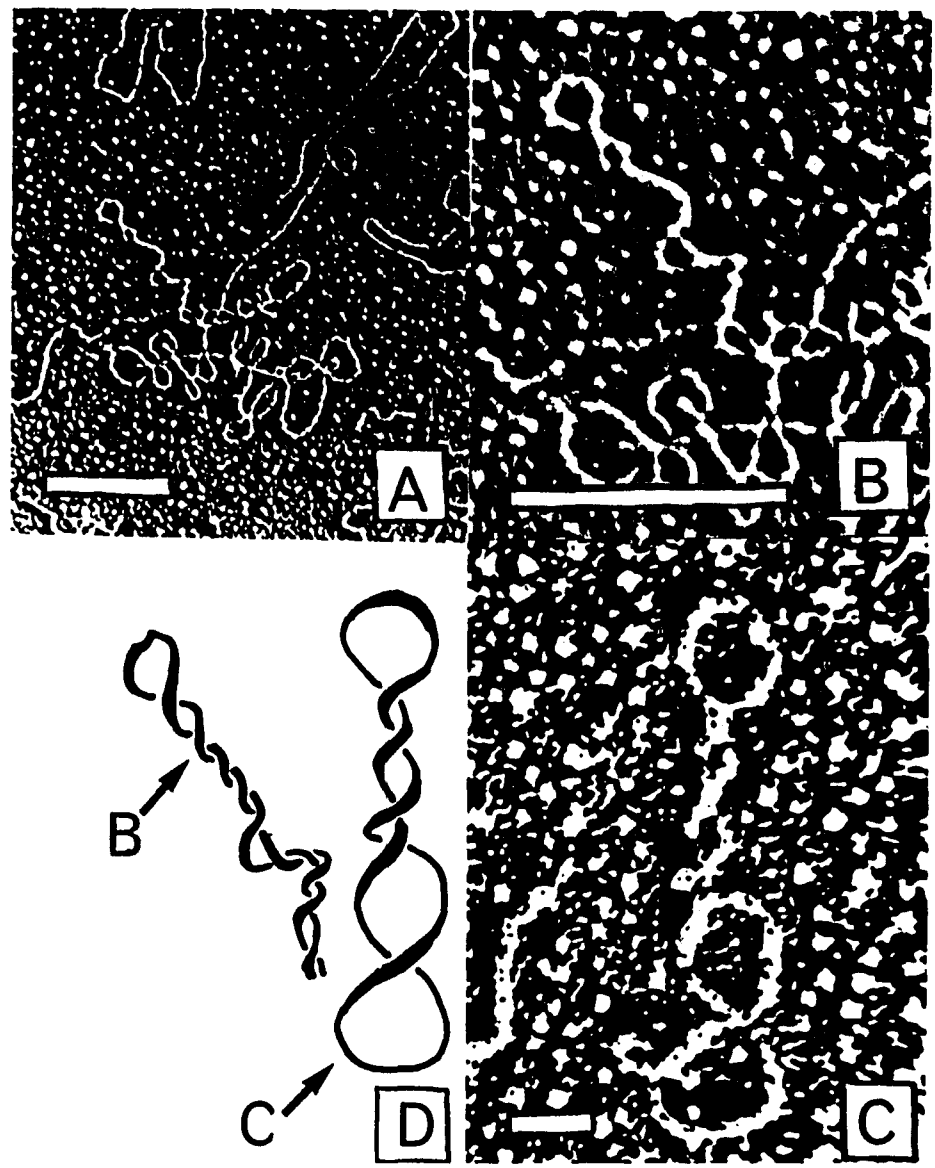

A
B
C
D
B
C

Fig. 7. Summary of the map position of Bal31 strong stops in the Ad5 virion DNA. The probes used (leftward and rightward directions) are indicated in the graph. Longer vertical lines above the map represent the major sites while shorter lines represent the minor ones. Map positions are expressed in kilobases starting from the left hand end of Ad5 genome. The internal probes are generated by combination of two different restriction enzymes digestion of Ad5 DNA (Sal I-Hind III, 9.68-11.34 kb. Sal I-Hind III, 16.52-18.04 kb. Bam HI-Kpn I, 21.42-22.07 kb. Xho I-Eco RI, 29.81-30.06 kb.).

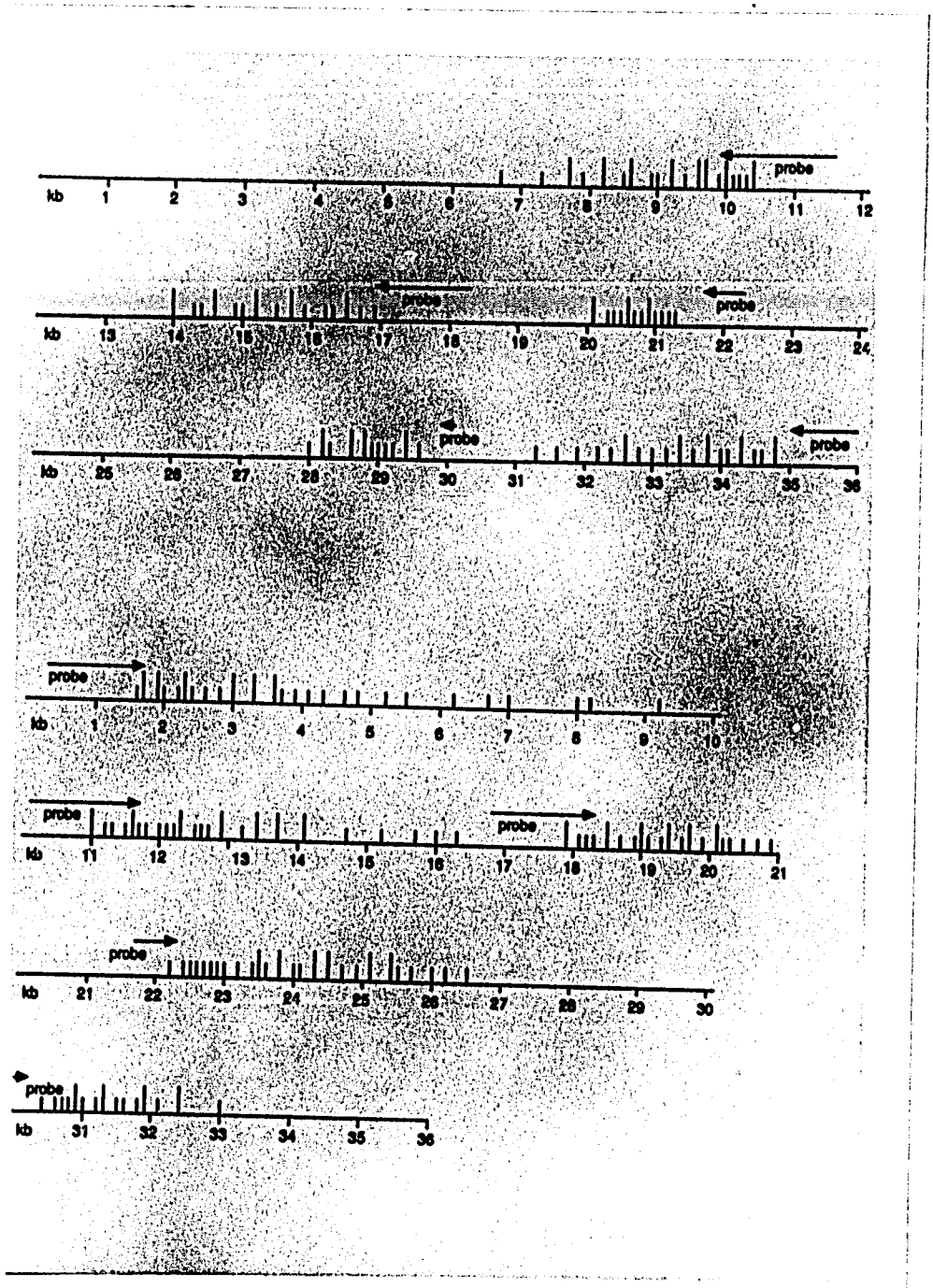
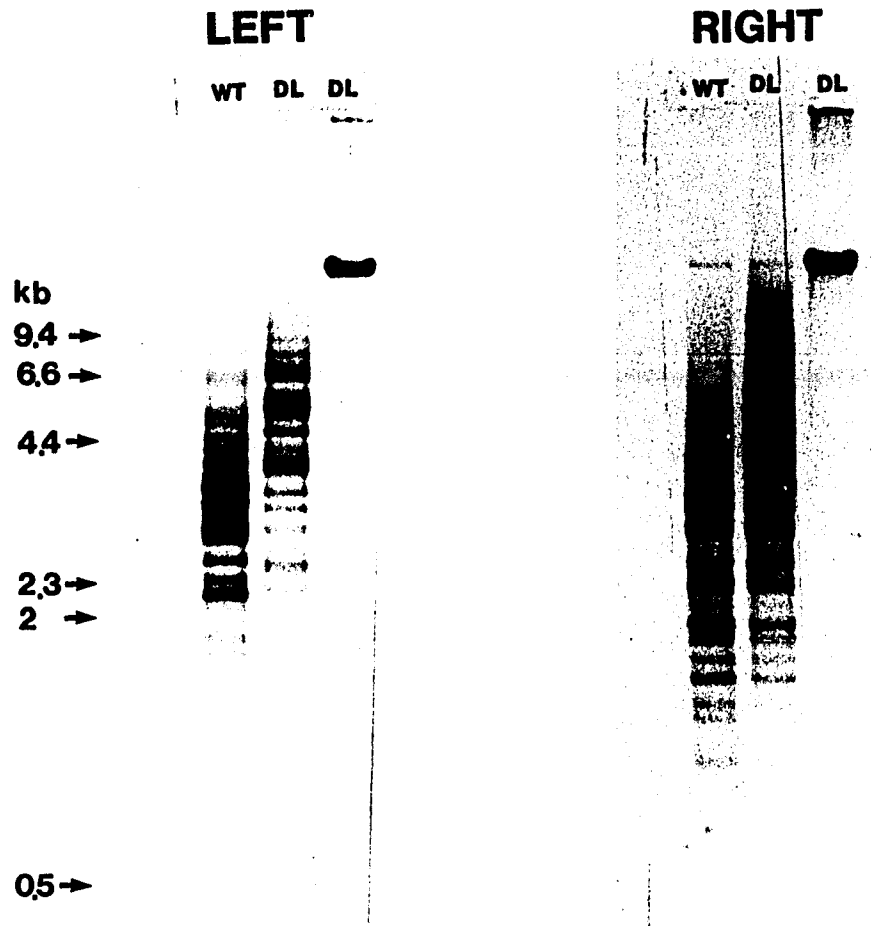

probe
kb 1 2 3 4 5 6 7 8 9 10 11 12
probe
probe
kb 13 14 15 16 17 18 19 20 21 22 23 24
probe
probe
kb 25 26 27 28 29 30 31 32 33 34 35 36
probe
kb 1 2 3 4 5 6 7 8 9 10
probe
probe
kb 11 12 13 14 15 16 17 18 19 20 21
probe
kb 21 22 23 24 25 26 27 28 29 30
probe
kb 31 32 33 34 35 36

Fig. 8. Comparison of Bal31 cleavage sites in wild type and dl312 virion DNA. The cleavage products were analyzed with the left end probe (left upper panel) or the right end probe (right upper panel). The cleavage sites at the right ends of the two viral genomes are identical. The relationship between the left ends cleavage sites of the two viruses is shown in the lower panel, which shows that cleavage sites in dl312 are shifted about 0.9 kb relative to that of wild type.


LEFT
RIGHT
WT
DL
DL
WT
DL
DL
kb
9.4
6.6
4.4
2.3
2
0.5

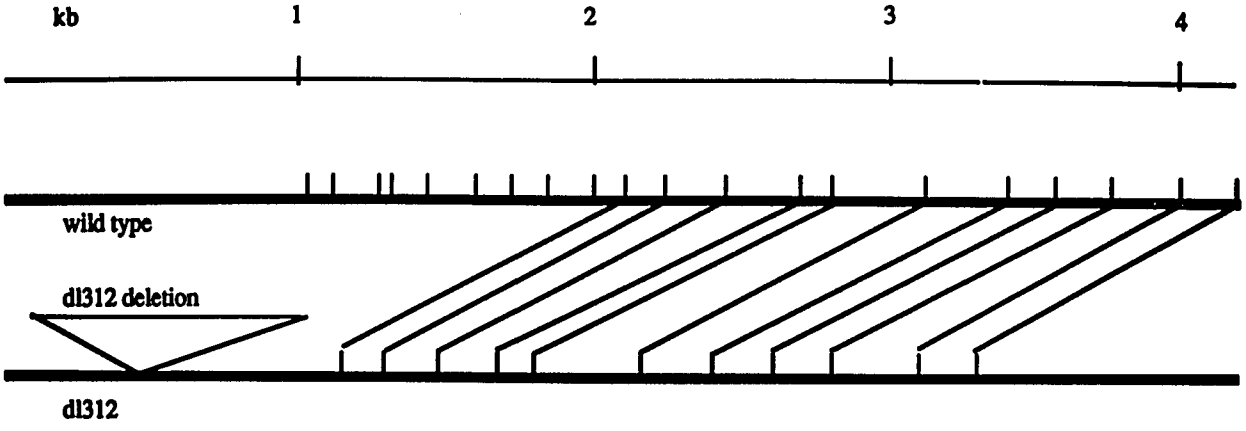

kb
1
2
3
4
wild type
dl312 deletion
dl312

Fig. 9. Electron micrographs of Ad5 virus core prepared by deoxycholate method. Highly condensed, supercoiled DNA loops (arrow heads) were seen and emerged from condensed centers of virus cores (panels A-D). A virus core with one end of the DNA unfolded is shown in panel A. The unfolded region is about 3 kb (8% genome length). The bar represents 0.5 micrometer.

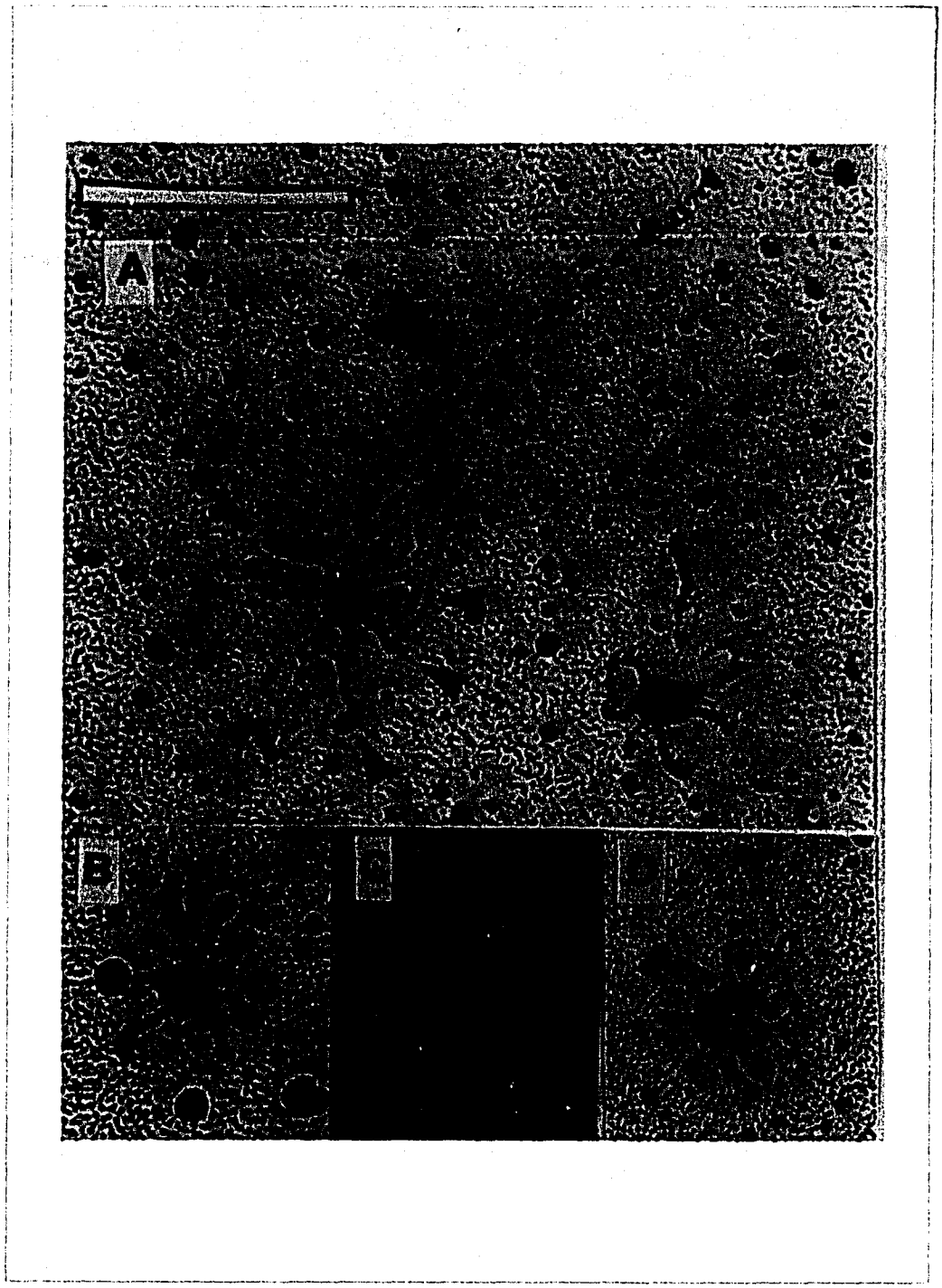
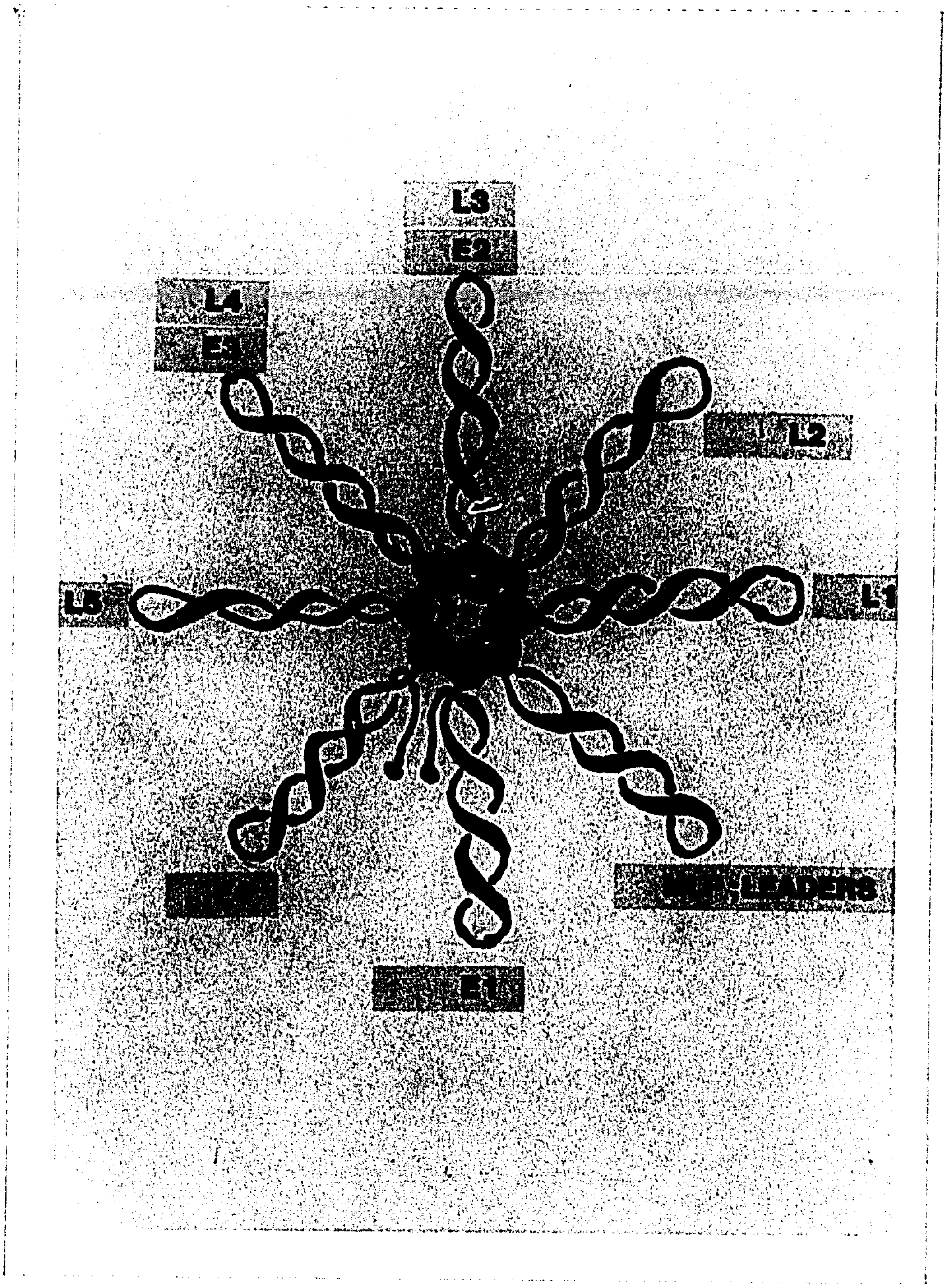

A
B

Fig. 10. A loop-domain model for the organization of Ad5 DNA in the virus particle based on the Bal31 nuclease digestion and electron microscopic data. Ad5 DNA is assumed to be organized into eight supercoiled loops and these loops are anchored to the center of the virus core by yet uncharacterized proteins. E1-E4 represent Ad5 early genes, L1-L5 represent late genes, and MLP is major late promoter. DNA at two ends of Ad5 genome containing promoters of E1a and E4 as well as replication origins are shown outside of supercoiled domains because of their insensitivity to Bal31 digestion. Assuming equal length for all supercoiled loops, the positions of loops can be estimated to be approximately from coordinates 2-14, 14-26, 26-38, 38-50, 50-62, 62-74, 74-86, and 86-98 respectively. Locations of Ad5 genes were found to have a strong correlation with the loop-domain in this model.


L3
E2
L4
E3
L2
L5
L1
LEADERS
E1

Chapter 3

Requirement of E1a and RNA Synthesis for the Unfolding of Adenovirus Templates Following Virus Entry

Min-Liang Wong and Ming-Ta Hsu

Department of Microbiology
Mount Sinai School of Medicine of City University of New York
New York, New York 10029

submitted

A. Abstract.

We have shown that DNA in human adenovirus type 5 virus particles is organized into supercoiled domains (M.-L. Wong and M.-T. Hsu, Nucleic Acids Res. 17:3535-3550, 1989). In the present study we investigated how the condensed, supercoiled adenovirus virion DNA is unfolded following entry into HeLa cells. Sedimentation and electron microscopic analyses showed that adenovirus DNA gradually unfolded during the early phase of infection. This unfolding of the viral templates was delayed in HeLa cells infected with an E1a deletion mutant, dl312. The delay of unfolding of dl312 templates coincided with the delayed expression of viral genes. The unfolding of dl312 templates proceeded normally in 293 cells which expressed endogenous E1a gene products. These results indicate that E1a gene products are required for the unfolding of condensed virion DNA following virus entry into the host. Inhibition of transcription by alpha-amanitin also prevented the unfolding of wild type adenovirus templates in HeLa cells and of dl312 templates in 293 cells. Since E1a gene products are required for the enhancement of adenovirus early gene transcription, these results suggest the transcription process is required for unfolding of the condensed virion templates after virus uncoating.

B. Introduction.

The genomes of viruses are highly condensed inside the virus particles. Following entry into the hosts, viral genomes must be unpacked to allow the expression of viral genes. Since viral genes are nonrandomly organized in viral genome, a specific, ordered unfolding of viral genome could potentially provide a mechanism for regulating viral gene expression during the early period of virus infection. Studies of DNA structure inside bacteriophage Mu particle suggested that phage DNA entered the cells in a polarized, ordered fashion (Breepoel et al., 1976; Inman et al.,1976). In T5 phage this ordered unpacking process has been shown to serve as a protection mechanism against the host restriction system (Brunel and Davison,1979; Lanni,1968; McCorquodale and Warner,1988). Only the left 7.9% T5 phage DNA, which is deficient of restriction sites, is injected into the host at the beginning of infection. The injected DNA encodes for factors which inactivate the host restriction system so that the subsequent transfer of the rest of the phage DNA is protected from nuclease attack.

In animal viruses, ordered and specific unpackaging of virion genome has not been demonstrated. There are also few studies on the role of unpackaging process in the regulation of viral gene expression during early infection of animal cells. In poxvirus infected cells, the immediate early viral

genes are transcribed from the virus cores after the outer envelope is removed and at least one of the immediate early gene products facilitates further unfolding of viral genome (Dales and Pogo,1981). This observation indicates that poxvirus gene expression at early times of infection could be regulated by the degree of uncoating and unfolding of viral genome. In order to gain further information of the role of viral templates unfolding in early gene expression of animal viruses we studied the unfolding of adenovirus genome during early phase of infection. Previous studies of early events in adenovirus infection indicate that uncoating of adenovirus starts in the cytoplasm and finishes at nuclear pores of the infected cell (Chardonnet and Dales,1970; Dales,1973; Dales and Chardonnet,1973; Mirza and Weber,1979).

In a previous report we have shown that the linear adenovirus DNA is packaged in supercoiled loop-domains inside the virus particle (Wong and Hsu,1989). The arrangement of viral genes in the loop-domains was found to correlate with the temporal order viral gene expression. This model suggests that unfolding of viral templates during early infection may play a role in the temporal regulation of adenovirus gene expression. To test this possibility, we investigated the unfolding of adenovirus DNA after the virus enters the cells. The present study showed that the condensed virion DNA was gradually unfolded during the early phase of virus infection. Furthermore, we showed that the unfolding of viral templates required the

pre-early E1a genes and transcription process. The implication of these observations with respect to the regulation of adenovirus gene expression during early infection will be discussed.

C. Materials and methods.

Cells and virus infection. Growth of HeLa and 293 cells and human adenovirus type 5 and dl312 mutant infection were as described previously (Graham et al.,1977; Jones and Shenk,1979; Wong and Hsu,1988). For inhibition of transcription, alpha-amanitin (Boehringer Mannheim) was added at 1 μg/ml. Protein synthesis was inhibited using 35 μg/ml of cycloheximide (Sigma).

Extraction of adenovirus nucleoprotein complexes and sucrose gradient analysis. Intracellular adenovirus nucleoprotein complexes were extracted from nuclei of adenovirus infected cells at the time indicated in the text using 300 mM ammonium sulfate, 0.2% Triton, 0.2 M NaCl, 40 mM Tris, 1 mM EDTA, pH 7.4. Viral nucleoprotein complexes were loaded on a 10-40% sucrose gradient in 50 mM Tris, pH 7.4 and centrifuged at 25,000 rpm for one hour at 18°C in a SW40 rotor. 400 μl fractions were collected and the viral DNA in each fraction extracted by phenol and chloroform extractions after digestion with 100 μg/ml proteinase K at 37°C for two hours. After precipitation in ethanol, DNA was loaded in a 1% agarose gel

in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.4) and electrophoresed at 100 volt for 1.5 hours. Then DNA was transferred to Nytran (Schleicher & Schuell) by the Southern blotting and hybridized with 32P-labeled adenovirus DNA. For the analysis of 3H-thymidine pulsed sample, the agarose gels were processed for fluorography as described previously (Wong and Hsu,1988). For the analysis of endogenous RNA polymerase activity, each gradient fraction was adjusted to 80 mM Tris (pH 7.9), 2.5 mM MnCl2, 2.5 mM MgCl2, 2 mM EDTA, 0.1 mM DTT, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and incubated at 30oC for 60 minutes in the presence of 30 μCi of 32P-UTP (New England Nuclear). The incorporation of 32P-UTP was determined by Cerenkov counting after precipitation with 10% trichloroacetic acid and the collection of precipitates on glass fiber filter (Millipore).

Electron microscopy. Adenovirus nucleoprotein complexes from different gradient fractions were examined in Zeiss 10CA electron microscope as described previously (Wolgemuth and Hsu,1981).

D. Results.

Unfolding of adenovirus DNA templates during infection. Adenovirus DNA exists in a highly condensed state inside the virus particle. Unfolding of

viral templates during infection should result in a decrease in the sedimentation rate of the viral nucleoprotein complexes (NPC) extracted from the infected cell nuclei due to an increase in the friction coefficient. Sedimentation analysis of adenovirus nucleoprotein complexes extracted from infected HeLa cells at 4, 8, and 16 hours after infection indeed showed a gradual decrease in the sedimentation rate of adenovirus templates (Fig. 1A, B, and C). The majority of adenovirus NPC extracted from cells at 4 hours after infection sedimented near the bottom of the gradient around 400 to 700S (Fig. 1A). As infection proceeded, the sedimentation rate of adenovirus NPC decreased to around 200S at 8 hours postinfection (Fig. 1B) and then to the top of the gradient around 40-70S at 16 hours postinfection (Fig. 1C). Since there was no significant increase in the number of viral DNA copies between 4 and 8 hours after infection (Fig. 2), the shift of sedimentation profile of viral nucleoprotein complexes at 8 hours postinfection can not be due to the synthesis of new viral templates by DNA replication. Thus the results indicate that the majority of the input viral templates underwent conformational change during early times after infection.

Adenovirus NPC extracted at 16 hours postinfection have two major sedimentation forms. The fast sedimenting NPC of the 16 hour sample is due to the formation of virus particles as shown by their resistance to nuclease digestion. When adenovirus NPC, pulse- labeled with 3H-

thymidine from 13-16 hours postinfection, were analyzed, the labeled NPC were found to sediment near the top of sucrose gradient (40-70S). This result indicated that newly synthesized, unpackaged viral NPC have an unfolded conformation. Pulse-labeled adenovirus NPC could be completely digested by micrococcal nuclease indicating that they were not packaged into mature virus particles which are resistant to nuclease digestion (data not shown). In contrast to the total adenovirus NPC, pulse-labeled, nuclease-sensitive NPC were found mostly on the top of the sucrose gradient (Fig. 1F). Thus the data presented in Fig. 1 indicate that during adenovirus infection there is a gradual decrease of the sedimentation rates of viral NPC extracted from the infected HeLa cell nuclei and at late times after infection the newly synthesized viral templates are exclusively found at the top of the sucrose gradient around 40-70S.

The change in the sedimentation rate of adenovirus NPC during infection suggests that unfolding of viral templates during infection. Direct observation of the structure of adenovirus NPC in the electron microscope confirmed this interpretation. As shown in Fig. 3, the fast sedimenting NPC in the 4 hours postinfection sample were highly compact with occasional extension of one end of the viral DNA (Fig. 3A). The NPC with sedimentation rate of 200-400S were partially unfolded, whereas the NPC near the top of gradient were almost completely unfolded (Fig. 3B and 3C). The possibility that change in sedimentation rate of viral NPC is due to

alteration of the quantity of proteins bound to adenovirus DNA was excluded by the analysis of buoyant density of viral NPC in a metrizamide density gradient.

Conformation of adenovirus transcription complexes during viral infection. Results described above showed the sedimentation patterns of the bulk of intracellular viral templates during infection. To determine the sedimentation behavior of active adenovirus templates, we analyzed the sedimentation of adenovirus transcription complexes in the sucrose gradient. The positions of adenovirus transcription complexes in the sucrose gradients were determined by hybridization of 32P-UTP labeled RNA with adenovirus DNA. As shown in Fig. 4, adenovirus transcription complexes extracted at 4 hours postinfection had higher sedimentation rate than that of transcription complexes extracted at 16 hours postinfection. The positions of adenovirus transcription complexes at late times of infection coincided with the bulk of the viral templates, suggesting that the active viral templates also underwent gradual unfolding during infection.

Template unfolding of E1a deletion mutant dl312 was delayed in HeLa cells but proceeded normally in 293 cells. To examine the relationship of the unfolding of adenovirus templates during infection to viral gene expression we analyzed the unfolding of dl312 virus in HeLa cells. This mutant

contains a large deletion in adenovirus E1a gene which is required for the activation of other viral genes during adenovirus infection (Berk,1986). In the absence of E1a gene products, dl312 virus gene expression and replication are severely delayed in HeLa cells (Gaynor and Berk,1983; Nevins,1981). If unfolding of viral templates is dependent on viral gene expression or on E1a gene products then the unfolding of dl312 templates will be expected to be delayed in HeLa cells. As shown in Fig. 5A and 5B, dl312 NPC extracted from HeLa cells sedimented around 400-700S at 16 or 24 hours postinfection. At 48 hours postinfection slow sedimenting NPC around 40-70S began to be observable (Fig. 5C). In contrast, the kinetics of unfolding of dl312 in the permissive 293 cells is similar to that of the wild type virus in HeLa cells (compare Fig. 5D and Fig. 1C). These data indicate that the unfolding of dl312 templates was normal in 293 cells which provide complementing E1a gene products whereas unfolding was severely delayed in HeLa cells. The delay in the unfolding of dl312 templates in HeLa cells coincides with the delay in adenovirus gene expression (Gaynor and Berk,1983; Nevins,1981) and in adenovirus DNA replication as shown by the Southern blot analysis of the accumulation of dl312 viral DNA during infection.

Unfolding of adenovirus early templates requires transcription. The coincidence of delays in both the unfolding of dl312 templates and in viral

gene expression in HeLa cells suggests a close relationship between transcription of adenovirus templates and templates unfolding during early infection. To further examine the role of transcription in the unfolding of adenovirus templates, we studied the unfolding of wild type adenovirus templates in the presence of the transcription inhibitor, alpha-amanitin. Alpha-amanitin can bind to RNA polymerase II and inhibit mRNA synthesis (Faulstich,1980; Weinmann et al.,1974), and block adenovirus replication when added to intact cells(Chardonnet et al.,1972). As shown in Fig. 1D, unfolding of adenovirus templates was inhibited by 1 μg/ml of alpha-amanitin when it was added at the time of infection. At this concentration of the drug, adenovirus DNA replication after 24 hours of infection was reduced to 10% of the control (data not shown). Incorporation of 3H-uridine into adenovirus specific RNA in the presence of 1 μg/ml of alpha-amanitin was reduced to 30% of the control untreated cells during a three hours pulse labeling from 5 to 8 hours postinfection. These results indicate that transcription is necessary for the unfolding of adenovirus DNA at early time after infection. In addition, unfolding of adenovirus early templates could be inhibited partially by cycloheximide, a protein synthesis inhibitor (Fig. 1E)

To examine if E1a protein is involved directly in the unfolding of viral templates, we also analyzed the unfolding of dl312 templates in 293 cells in the presence of alpha-amanitin. As shown in Fig. 6B, treatment of 293 cells with alpha-amanitin at the beginning of infection with dl312 inhibited unfolding of the majority of dl312 templates assayed at 8 hours

postinfection. Since E1a gene products are present in 293 cells before virus infection, this suggests that E1a products per se are not sufficient for the unfolding of viral templates.

E. Discussion.

Using sedimentation velocity and electron microscopic techniques we have shown that the bulk of adenovirus templates undergo gradual unfolding during the early phase of infection. However, studies of early events of animal virus infection is complicated by the high particle to infectious unit ratio. Thus the observed biochemical events during early infection of animal viruses may not necessarily represent the processes leading to successful infection. In our analysis we showed that the majority of the input viral templates changed their sedimentation rate during infection. Furthermore, we showed that the adenovirus templates active in transcription of viral RNA also underwent similar unfolding as the bulk of the viral templates. The delay of the unfolding of E1a deletion mutant, dl312, in HeLa cells also showed that the unfolding of adenovirus templates is coupled to viral transcription. These observations indicate that the observed unfolding of adenovirus templates during early infection is relevant to the infection process of adenovirus.

The data presented here suggest that the unfolding of adenovirus templates during early phase of infection is coupled to the transcription of adenovirus genome. This is supported by the inhibition of unfolding of adenovirus templates by alpha-amanitin which blocks transcription by RNA polymerase II (Faulstich,1980; Weinmann et al.,1974). Since induction of transcription of other viral genes requires E1a gene products, the defective unfolding of E1a deletion mutant dl312 templates in HeLa cells, but not in 293 cells, further demonstrates the coupling of transcription and template unfolding.

Inhibition of transcription has been shown to cause condensation of chromatin in lampbrush chromosomes (Bucci et al.,1971; Izawa et al.,1963; Mancino et al.,1971), in polytene chromosomes (Beermann,1971) and in liver and kidney cells treated with alpha-amanitin (Fiume, et al.,1969; Marinozzi et al.,1971). In the latter example, cellular chromatin becomes highly condensed and nucleoli fragmented early after amanitin treatment. These observations together with the results described in this communication indicates a close relationship between transcription and higher order folding of chromatin. However, the mechanism by which inhibition of transcription induces alteration of chromatin or viral NPC folding is still not understood. One possible mechanism for transcription coupled unfolding of adenovirus templates would be that the relative movement of transcription machinery along adenovirus DNA physically unfolds the DNA.

However, since the majority of adenovirus DNA unfolds during infection, this hypothesis would have to assume that the bulk of input adenovirus DNA participates in transcription process at one time or another during early phase of infection. Although this is not impossible it is quite unlikely because only a very small percentage of adenovirus templates are engaged in transcription (Wolgemuth and Hsu,1981). An alternative explanation would be that transcription is needed to produce some viral or host gene products which are needed to unfold the templates. Further analysis is needed to identify factors required for unfolding of adenovirus early templates.

Acknowledgments.

This work was supported by an American Cancer Society grant to M.-T. H.

F. References.

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G. Figure Legends.

Fig. 1. Sucrose gradient sedimentation analysis of human adenovirus type 5 nucleoprotein complexes (NPC). The sedimentation rates of NPC decreased as infection proceeded from 4 to 16 hours after infection(A, B, and C). Inhibition of transcription by alpha-amanitin (ama) could block the unfolding of viral NPC, but inhibition of protein synthesis by cycloheximide (chx) caused a partial blocking of unfolding (D and E). The newly synthesized viral templates were found at top fractions of sucrose gradient (F). All samples were analyzed by Southern blot except F.

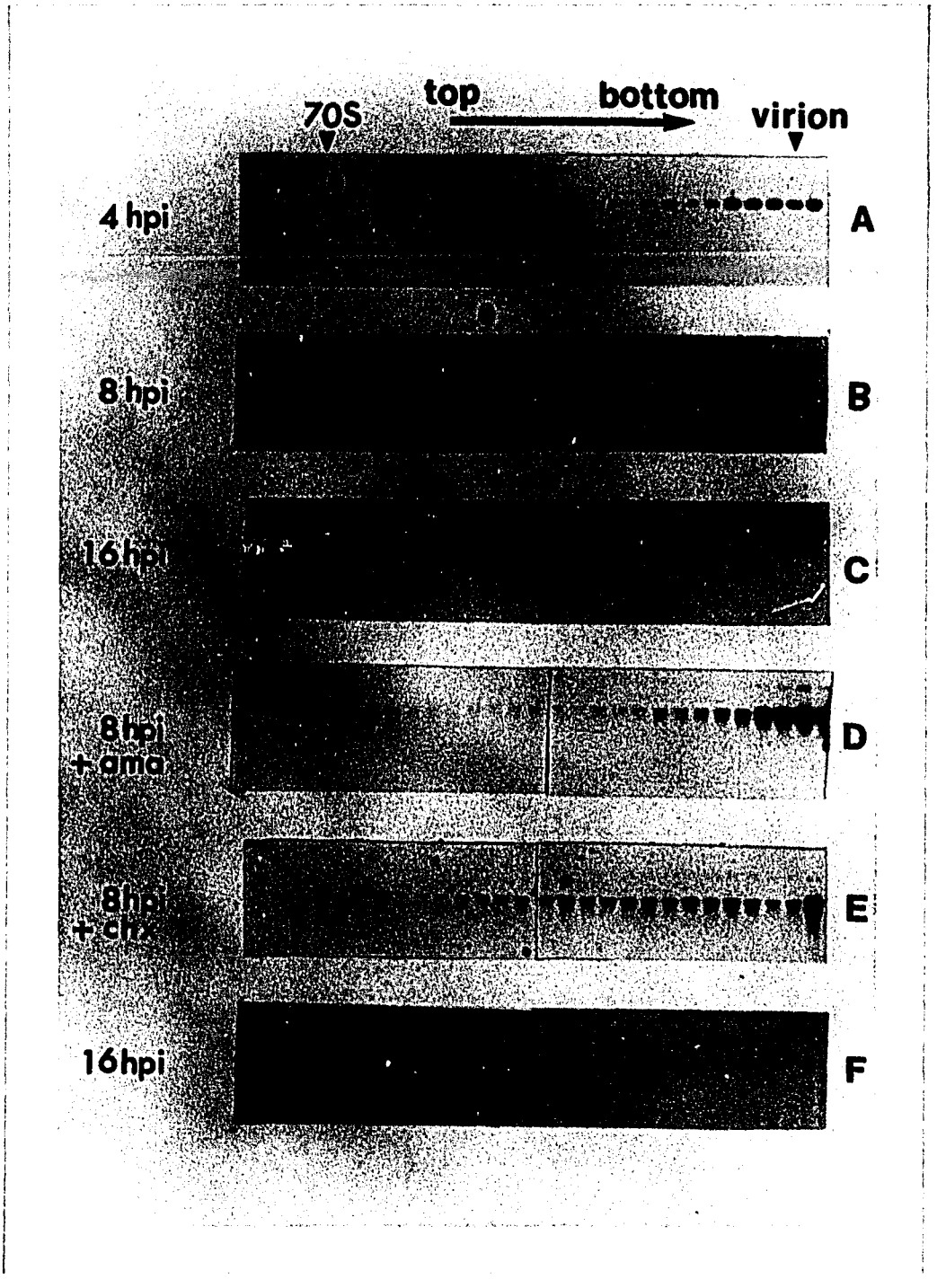

70S
top
bottom
virion
4 hpi
A
8 hpi
B
16 hpi
C
8 hpi
+ ama
D
8 hpi
+ chx
E
16 hpi
F

Fig. 2. Adenovirus DNA analyzed by Southern blot at 4, 8, and 16 hours postinfection. There was no significant increase in the number of DNA copies between 4 and 8 hours after infection.

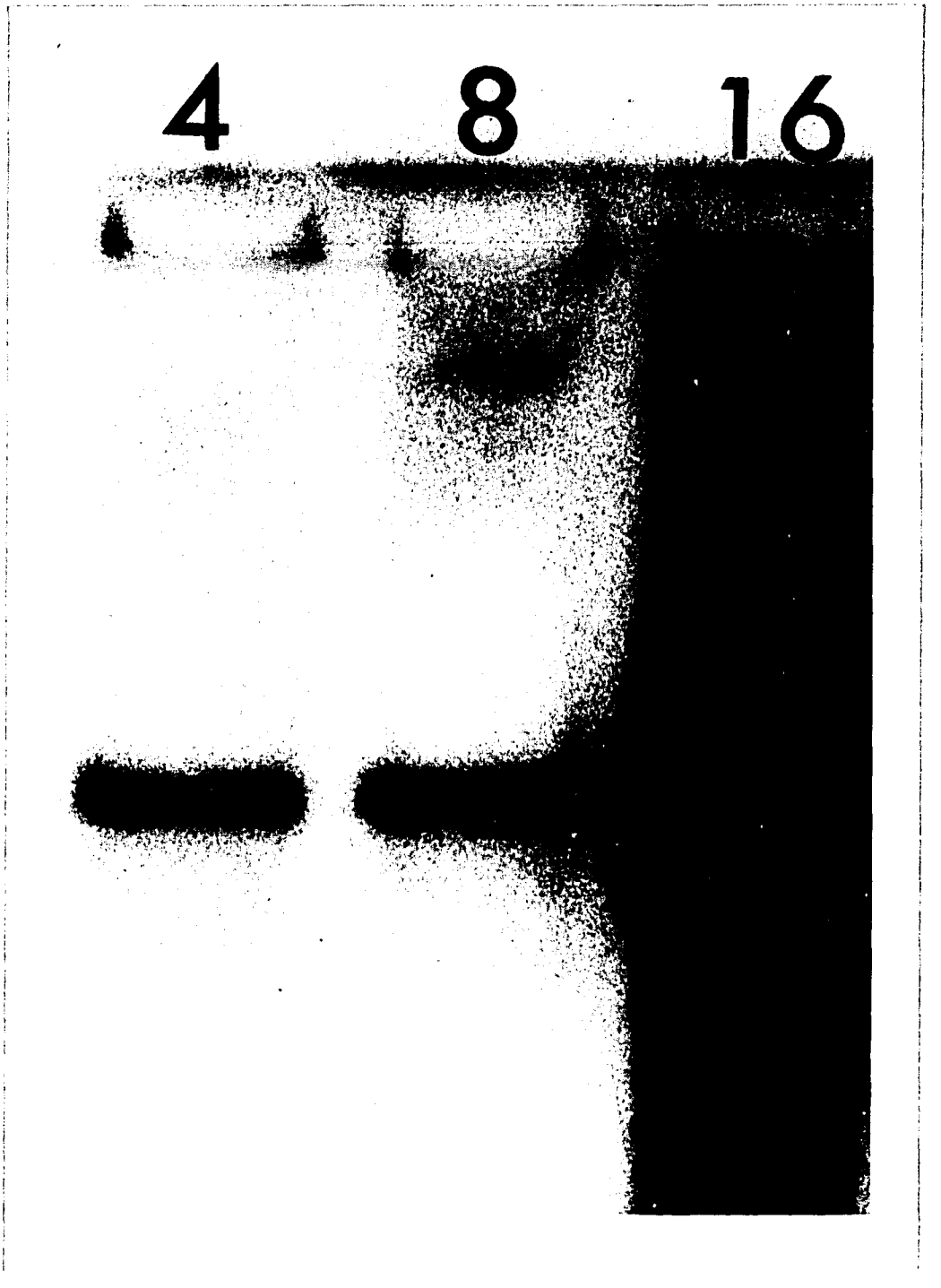

4
8
16

Fig. 3. Electron micrographs of adenovirus NPC showing different degree of unfolding. The fast sedimenting NPC was highly compact, occasionally with one end unfolded (A, arrow head). The NPC with sedimentation rate of 200-400S was partially unfolded (B), whereas the NPC near the top of gradient was almost completely unfolded (C).

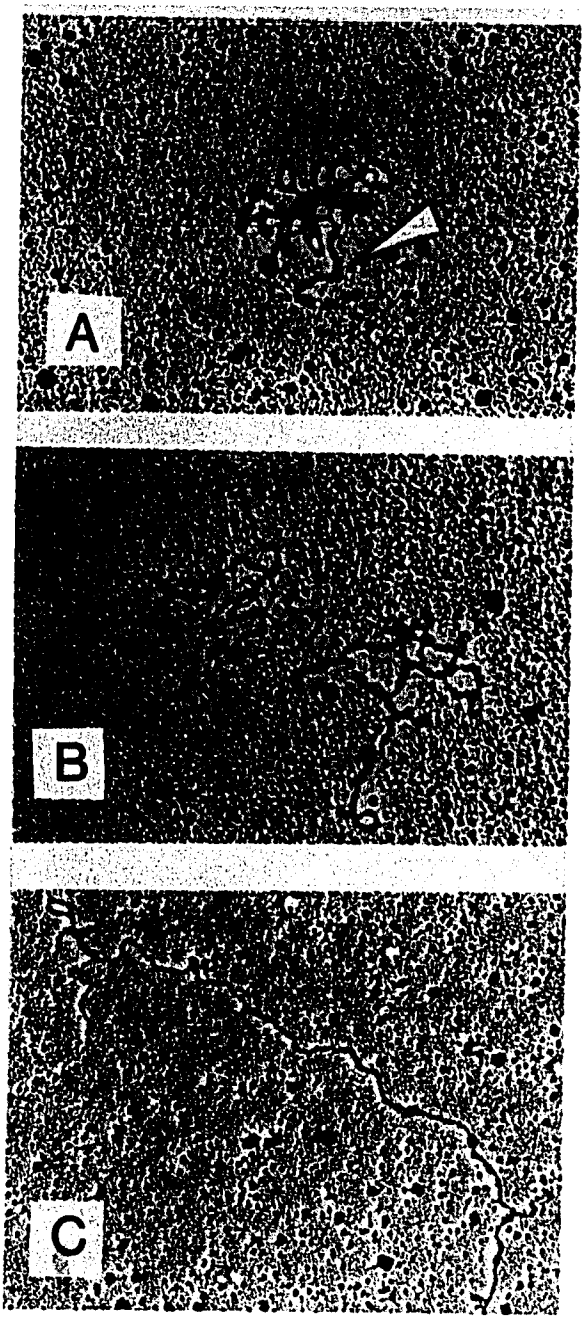

A
B
C

Fig. 4. Sucrose gradient sedimentation analysis of adenovirus transcriptional complexes at early (panel A) or late (panel B) times of infection. Adenovirus NPC were extracted and fractionated through sucrose gradient at early (4 h.p.i.) or late (16 h.p.i.). Each fraction was assayed for RNA polymerase activity, and the nascent RNA transcribed in vitro were hybridized with adenovirus DNA on the Nytran filter to ensure they were viral specific RNA.

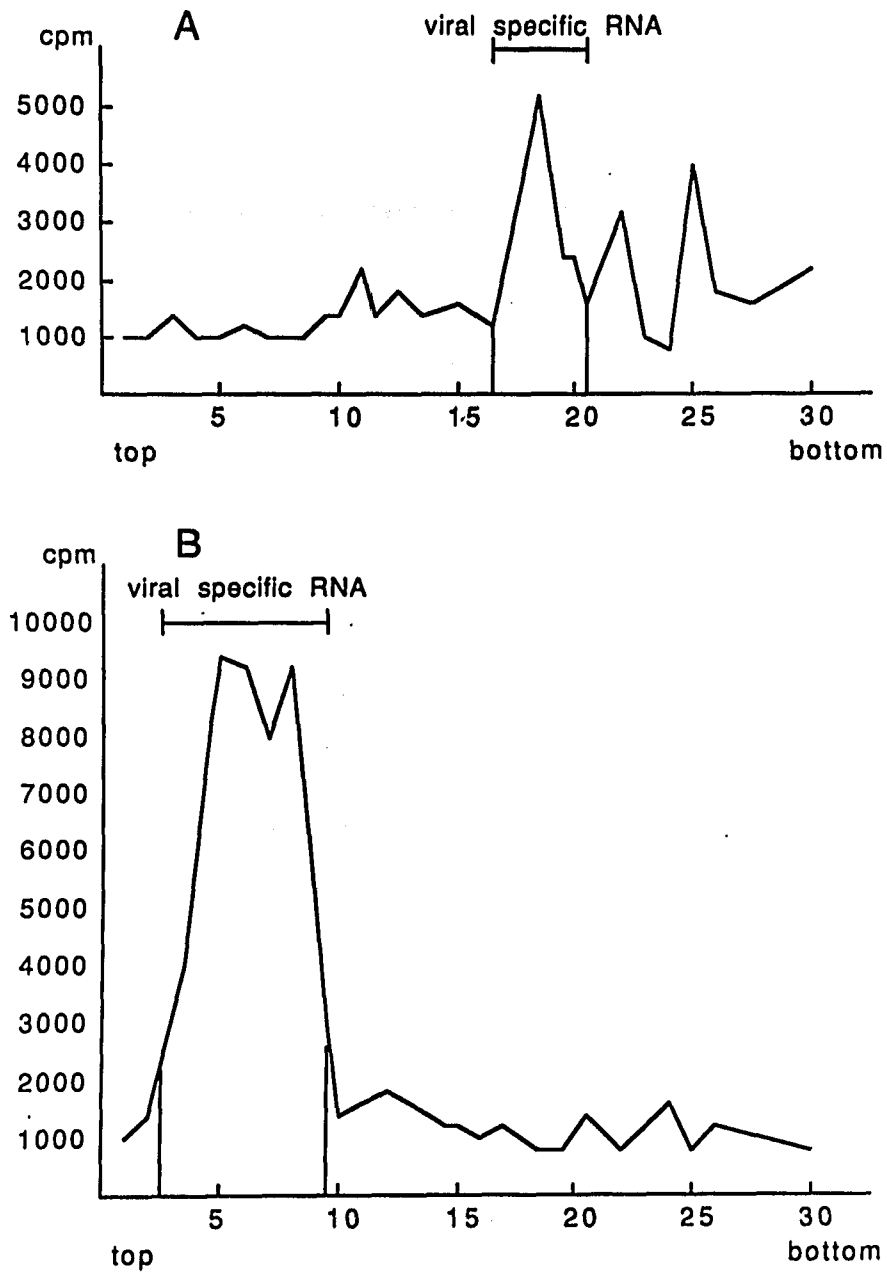

A
cpm
viral specific RNA
5000
4000
3000
2000
1000
5
10
15
20
25
30
top
bottom
B
cpm
viral specific RNA
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
5
10
15
20
25
30
top
bottom

Fig. 5. Sucrose gradient sedimentation analysis of NPC of dl312 mutant in HeLa or 293 cells. The dl312 mutant (a large deletion in E1a gene) failed to unfold in HeLa cells at 16 and 24 hours postinfection (A and B). The slow sedimenting NPC around 40-70S began to be observable at 48 hours postinfection in HeLa cells (C). On the other hand, the behavior of unfolding of dl312 in permissive 293 cells (D and E) is similar to that of wild type virus in HeLa cells (Fig. 1C).

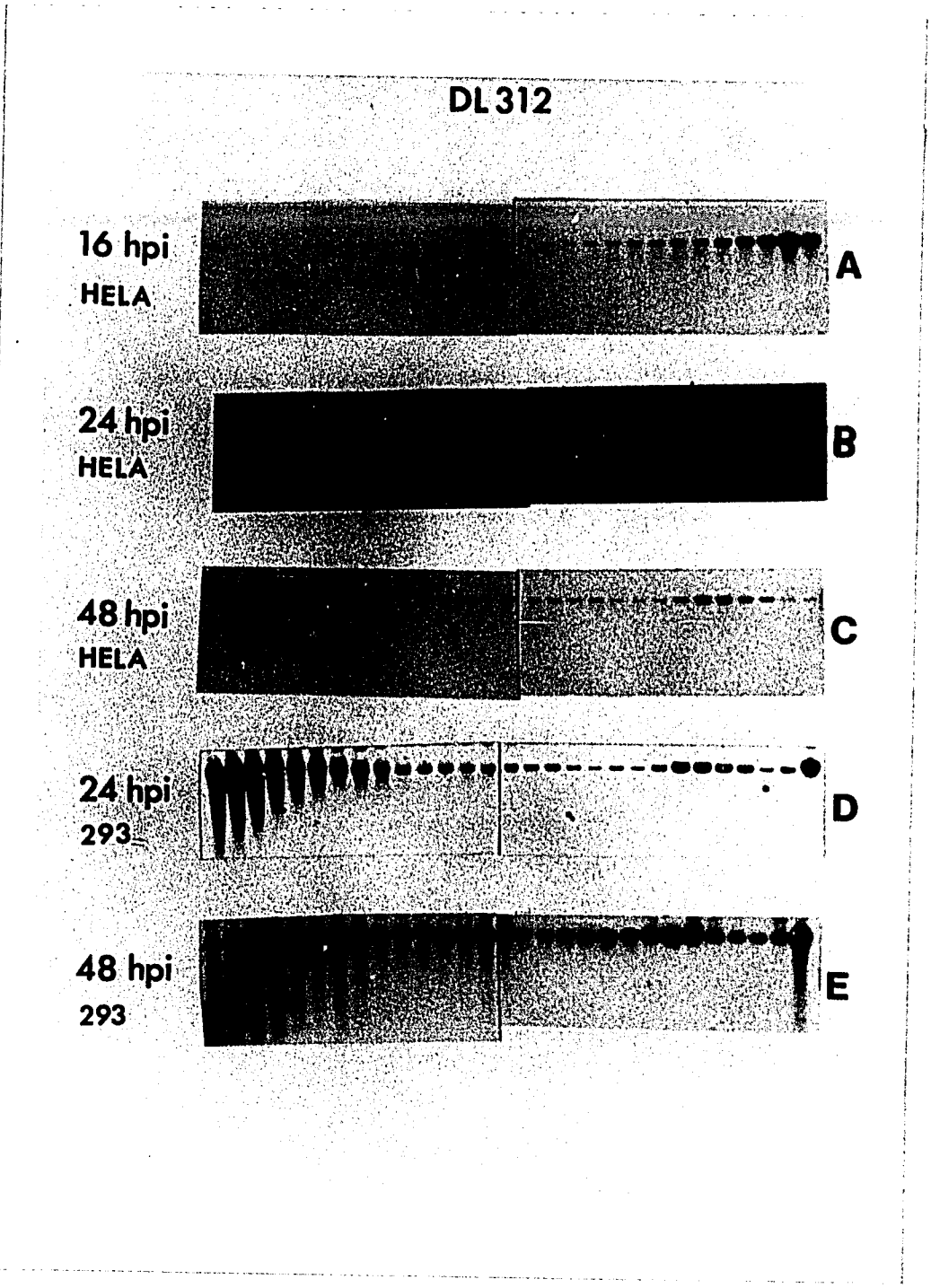
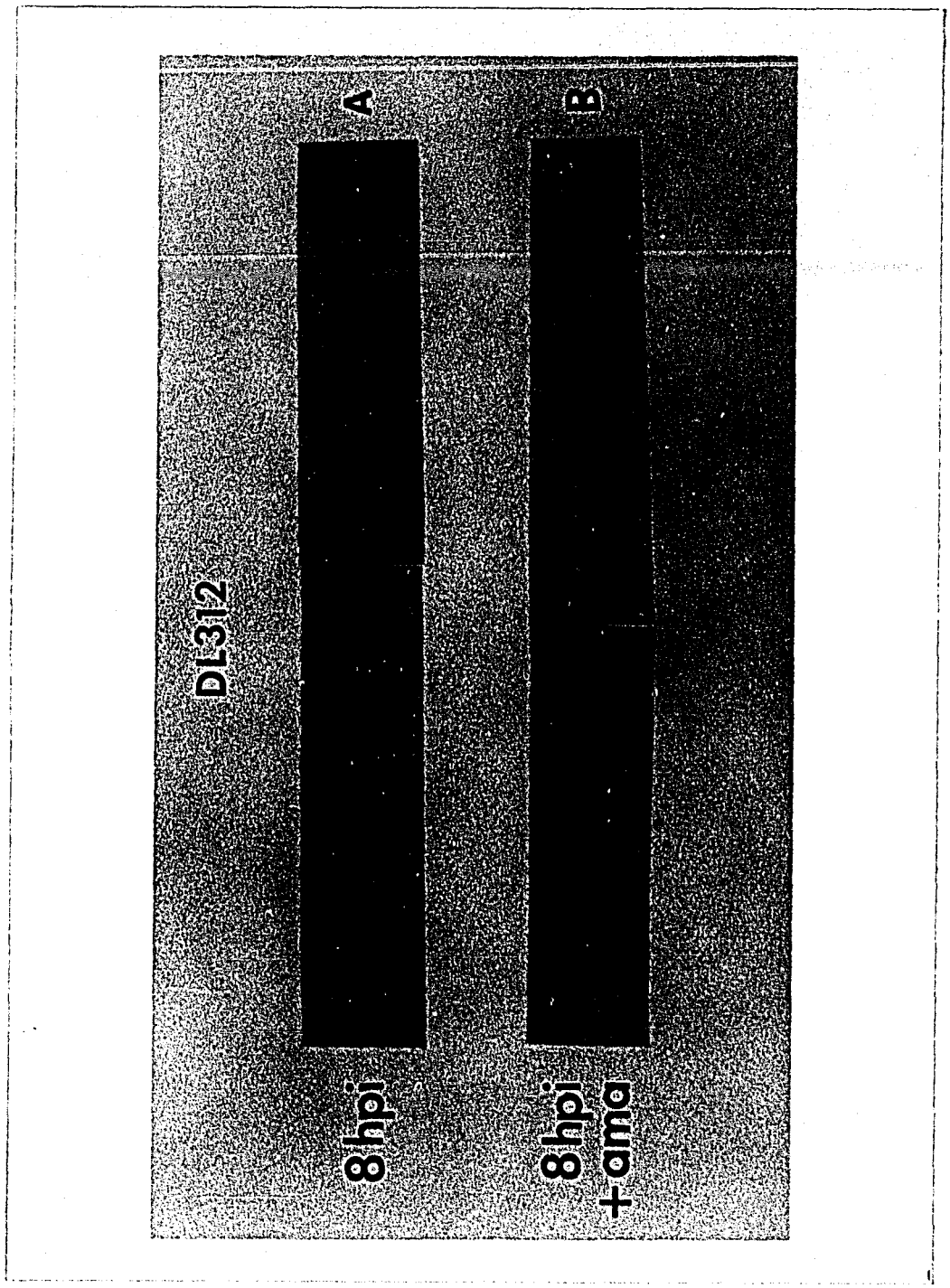

DL 312
16 hpi
HELA
A
24 hpi
HELA
B
48 hpi
HELA
C
24 hpi
293
D
48 hpi
293
E

Fig. 6. Sucrose gradient sedimentation analysis of NPC of dl312 mutant in 293 cells in the absence or presence alpha-amanitin. The unfolding of dl312 in 293 cells was inhibited with alpha-amanitin treatment (A, control; B, alpha-amanitin).


A
B
DL312
8hpi
8hpi
+ama

Chapter 4

Involvement of Topoisomerases in the Replication, Transcription, and Packaging of Linear Adenovirus Genome

Min-Liang Wong and Ming-Ta Hsu

Department of Microbiology
Mount Sinai School of Medicine of City University of New York
New York, New York 10029

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A. Abstract.

The role of topoisomerases (topo) in the replication of human adenovirus type 5 (Ad5) has been investigated using topoisomerase inhibitors. Both topo I and topo II inhibitors blocked adenovirus replication when added at the time of infection. Both types of inhibitors induced strand cleavages at specific sites in the adenovirus early templates. The cleavage sites were mapped near the 5' and 3' ends of the genes transcribed early during infection. At late times after infection, camptothecin, a topo I inhibitor, inhibited adenovirus DNA replication and induced the formation of single and double-stranded fragments with break points located at defined regions of the viral genome. The topo II inhibitors, VP-16 (etoposide) and ellipticine, did not block adenovirus DNA replication and did not induce an appreciable amount of double-stranded cleavages in the newly synthesized DNA. On the other hand, VP-16 promoted double-stranded cleavage at specific sites of the nonreplicating adenovirus DNA. The packaging of adenovirus DNA into virus particles, which contain supercoiled adenovirus DNA (M.-L. Wong and M.-T. Hsu. Nucleic Acids Res, 17: 3535-3550, 1989), was inhibited by topo II inhibitors. Transcription of adenovirus major late genes was inhibited with both topo I or topo II inhibitors. In addition, camptothecin caused premature termination of the major late transcription unit. Electron

microscopic analysis showed that adenovirus templates late after infection were arranged in topologically constrained loop-domains. Together, these data provide evidence for the requirement of topoisomerase activities in the replication, transcription, and packaging of the linear adenovirus genome.

B. Introduction.

Gene expression and replication of eukaryotic and prokaryotic DNA is influenced by the topology of DNA (see reviews in Drlica,1984; Pruss and Drlica,1989; Wang,1985). Topological problems of DNA are usually associated with covalently closed circular DNA, where the two strands of the double helix are topologically linked. However, a number of bacteriophages and animal viruses with linear double-stranded DNA genomes have been shown to require either the host or viral coded topoisomerases in their life cycles (Alonso et al.,1981; Benson and Huang,1988; Constantinou et al.,1986; De Wyngaert and Hinkle,1979; Itoh and tomizawa,1977; Liu et al.,1979; Poddar and Bauer,1986). These observations indicate that DNA topology also plays an important role in linear genomes which may be arranged in different topological forms through DNA-protein interactions inside the cells. The organization of linear DNA inside the cell and the effects of different topological arrangements on the replication and gene expression of such genome are poorly understood at the present time.

We recently showed that the linear genome of human adenovirus type 5 is organized in a supercoiled conformation inside the virus particles (Wong and Hsu,1989). Biochemical analysis of adenovirus DNA replication

in the cell-free system revealed that a host topoisomerase I activity is required for the elongation of adenovirus nascent DNA (Nagata et al.,1983). These observations suggest that DNA topology plays an important role in the life cycle of adenovirus. In the present communication, we investigate the role of topoisomerases in the replication of adenovirus type 5 in HeLa cells using topoisomerase inhibitors. Our results indicate that topoisomerase inhibitors block lytic infection of adenovirus in HeLa cells by interfering with viral DNA replication, transcription, and virus assembly.

C. Materials and methods.

Virus infection and treatment with inhibitors. Monolayer cultures of HeLa cells were infected with Ad5 virus as described previously (Wong and Hsu,1988). Camptothecin and ellipticine (Sigma) were dissolved in dimethyl sulfoxide and 10 mM HCl to a final concentration of 7.2 mM and 2.5 mM respectively. VP-16 (Bristol Lab) was obtained as 34 mM solution. The inhibitors were added to the infected cultures at 30 micromolar except when indicated in the text. In the control cultures equivalent amounts of dimethyl sulfoxide were added to the culture medium. Adenovirus DNA was extracted from infected cells by the filtration method (manuscript in preparation). Briefly, infected cells were lysed with lysis buffer containing 1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 7.4

followed by digestion with 100 ug/ml proteinase K (Boehringer-Mannheim) at 37 oC for 3 hours or longer. The viscous lysates were then filtered through glass wool plugged in a disposable syringe. High molecular weight cell DNA was trapped in the glass wool and adenovirus DNA was quantitatively recovered in the filtrate. The DNA in the filtrate was further purified by phenol and chloroform extraction.

Two-dimensional gel electrophoresis and fluorography. The methods for neutral-alkaline two dimensional agarose gel electrophoresis and for fluorography have been described (Wong and Hsu,1988). Briefly, DNA samples were run in the first dimension in a 1.2% agarose gel in 40 mM Tris-HCl (pH 7.4), 5 mM sodium acetate, 1 mM EDTA. The second dimension was run in a 1.4% agarose gel in 30 mM NaOH, 1 mM EDTA at 200 mA in a cold room. Alkaline buffer was changed every 8 to 12 hours during electrophoresis. For fluorography, gels were first dried and then incubated with 0.7 M sodium salicylate for 1 hour at room temperature.

Electron microscopy. Adenovirus DNA-protein complexes were spread from 50% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5 and 100 ug/ml ethidium bromide onto a hypophase with 10% formamide, 0.01M Tris, 0.001M EDTA

and 100 ug/ml ethidium bromide. After rotary shadowing the samples were examined in a Zeiss 10CR electron microscope.

D. Results.

Inhibition of adenovirus infection of HeLa cells by topoisomerase I or topoisomerase II inhibitors. Addition of topoisomerase I inhibitor (camptothecin) to HeLa cells at the time of infection blocked the replication of human adenovirus type 5 (Fig.1). Similarly, topoisomerase II inhibitors (ellipticine or VP-16) also blocked adenovirus replication. Addition of the drugs at later times after infection (2 and 8 hrs. p.i.) delayed the onset of viral DNA replication. Inhibition of adenovirus infection was initiated at concentrations of 0.5 and 2 micromolar of camptothecin and ellipticine, respectively. No appreciable cytotoxicity or change in cell morphology was observed at these concentrations of the inhibitors. Therefore, the effect of the drugs on the replication of adenovirus is probably not due to general nonspecific cytotoxicity of the drugs. To analyze the mechanisms of inhibition of adenovirus replication by the topoisomerase inhibitors, we studied their effects on viral DNA replication, transcription, and virion packaging as described below.

Induction of cleavage at specific sites in the early transcription units of adenovirus early templates by camptothecin or VP-16. The results described above indicate that topoisomerase activities are essential for adenovirus replication in HeLa cells. To determine if topoisomerase activities are directly involved in the macromolecular synthesis of adenovirus, we analyzed the effect of topoisomerase inhibitors on adenovirus DNA templates during viral infection. Topoisomerase inhibitors cause DNA strand breaks when the DNA-topoisomerase complex is trapped by treatment with SDS or alkali (Liu et al.,1983;Ross et al.,1979). Thus, strand breaks in adenovirus DNA would be observed if topoisomerases are associated with adenovirus templates during viral infection.

Treatment of adenovirus infected HeLa cells from 0 to 4 hours postinfection (p.i.) with camptothecin induced nicking of adenovirus DNA as shown by neutral-alkaline two-dimensional gel electrophoresis (Fig.2 panel A). From the distribution of intact and subgenomic DNA fragments one can calculate that on average about 4 nicks were found per adenovirus genome in the presence of camptothecin as compared to about 1 nick per genome in the untreated control. The nicks induced by camptothecin were found at specific locations in the viral genome. Using a probe derived from the left end of the viral genome, the major nicks were mapped to coordinates 6.4, 8.0, 10.3, and 15.0 (+ 0.6). Similarly, nicks at the right end of the viral genome were located at 85.6, 88.9, 90.6, 94.4, and 95.6. These nicks were

located within the E1 and E4 transcription units and at the 5' end of the major late transcription unit, which is active at early times after infection.

Treatment of adenovirus infected cells with a topoisomerase II inhibitor, VP-16, produced both double-stranded and single- stranded breaks in the viral DNA, as shown by neutral-alkaline two-dimensional gel electrophoresis (Fig.2 panel A). In this gel system, the double-stranded DNA fragments are displayed in the diagonal line whereas single-stranded breaks are manifested as vertical spots or line under the DNA fragment containing the nicks. The double-stranded cleavage sites were mapped to coordinates 4.4, 8.3, 12.2 , 17.2 and 22.8 (+ 0.6) using the left hand end probe and coordinates 75.0 and 85.0 using the right hand end probe. The former set of cleavage sites correspond to the 3' end of E1a and E1b and the 5' end of the major late transcription unit, respectively. The right end 85.0 cleavage site corresponds to the 3' end of the E3 and E4 units and the 75.0 site corresponds to the 5' end of E2 and E3. The major single strand cleavage sites mapped to the same sites as the double strand cleavage sites. The cleavage pattern of adenovirus DNA induced by VP-16 did not change between 0 and 6 hours postinfection. However, the frequency of cleavages increased as infection progressed (Fig.2 panels B and C).

In contrast to VP-16, ellipticine produced very few double strand breaks in Ad5 DNA at the same concentrations (data not shown). This was consistent with the finding that ellipticine or its derivative is a weaker

inducer of double strand cleavages of eukaryotic topoisomerase II in vitro (Pommier et al.,1985; Riou et al.,1986).

Inhibition of adenovirus DNA replication and induction of specific double and single-stranded fragments in adenovirus DNA with camptothecin. To determine whether topoisomerases are involved in the replication of linear adenovirus genome, we analyzed the effect of topoisomerase inhibitors on the incorporation of 3H-thymidine into newly synthesized Ad5 DNA at 16 hours postinfection. At a concentration of 30 micromolar of camptothecin the uptake of 3H-thymidine was not affected whereas 3H-thymidine incorporation into Ad5 DNA during a two-hour pulse was inhibited 80% .

To further examine the mechanism of inhibition of Ad5 DNA replication by camptothecin, we analyzed the structure of viral DNA synthesized during the drug treatment using two-dimensional neutral-alkaline gel electrophoresis. In this gel system double-stranded DNA species are displayed in a diagonal line whereas single-stranded species form a separate curve with faster gel mobility in the first (neutral) dimension. As shown in Fig.3A the control Ad5 DNA forms a single spot at the position of 36 kb. Ad5 DNA labeled during camptothecin treatment, however, was found to contain subgenomic double-stranded fragments, as indicated by the presence of subgenomic size spots in the diagonal line (Fig.3B). In addition, a DNA curve migrating faster than the double-stranded DNA in the

diagonal line was observed (Fig.3B, triangle). The single-stranded character of this DNA species was demonstrated by the its sensitivity to S1 nuclease digestion (data not shown). Furthermore, after HindIII digestion of Ad5 DNA labeled in the presence of camptothecin, the labeling of terminal fragments (HindIII G and I) was enriched (Fig.3 compare panels C and D). Since the origins of replication of adenovirus DNA are located at two termini, this result indicates that premature termination of Ad5 DNA replication occurs in the presence of camptothecin. Our results indicate that camptothecin induces double-stranded breaks at specific sites in newly synthesized adenovirus DNA and it also induces the formation of single-stranded DNA.

The positions of the double-stranded cleavages of adenovirus DNA induced with camptothecin was determined by Southern blotting using a DNA probe located at the left hand end of the viral genome (nucleotide 311 to 1764). As shown in Fig.4 panel B camptothecin induced specific cleavages at coordinates 4.2, 5.6, 6.4, 8.0, 9.4, and 11.9 (+ 0.6) from the left hand end. Hybridization with a right hand end probe (nucleotide 34933 to 35937) mapped the double-stranded cleavage sites on the right hand part of the genome at coordinates 87.8, 89.2, 90.6, 91.1, 94.7, and 95.6 respectively (Fig.4 panel B'). In addition, hybridization with the two probes revealed the presence of prominent single-stranded DNA fragments with similar lengths as the double-stranded DNA fragments.

Topoisomerase II inhibitor, VP-16, causes site-specific cleavages of nonreplicating adenovirus DNA at late times after infection. Treatment of Ad5 infected HeLa cells at late times after infection (24-26 hours p.i.) with 30 micromolar of ellipticine did not affect the incorporation of 3H-thymidine into newly synthesized Ad5 DNA (see Fig.8 lanes a and b, below). On the other hand, VP-16 reduced the incorporation of 3H-thymidine into Ad5 DNA by about 80% in a two-hour pulse (Fig.5, compare panels a and b). However, the reduced incorporation was found to be due to the inhibition of 3H-thymidine uptake by VP-16. Furthermore, no cleavage of newly labeled Ad5 DNA was found in the presence of VP-16 (Fig.5 panel b). In contrast, VP-16 produced double strand breaks in the bulk of Ad5 DNA during the same treatment period (Fig.5 panels c and d). Since VP-16 induces double strand cleavage by topoisomerase II, the absence of cleavages in newly labeled Ad5 DNA in the presence of VP-16 indicates that topoisomerase II activity is not associated with adenovirus DNA replication.

Inhibition of adenovirus transcription by camptothecin or ellipticine. Because ellipticine does not affect adenovirus DNA replication, and yet it inhibits viral replication when added at the time of infection, it is likely that ellipticine affects the expression of the adenovirus genes. Total incorporation of 3H-uridine into TCA precipitable RNA was reduced by

about 75% in the presence of ellipticine or camptothecin from 20 to 22 hrs. p.i. whereas the uptake of uridine was unaffected (Fig. 6). Adenovirus-specific RNA synthesis was also reduced by 75% as shown by the label hybridizable to adenovirus DNA (data not shown).

To ensure that the inhibition of 3H-uridine incorporation was not due to an effect of the drugs on the ribonucleotide triphosphate pool, we performed nuclear run-on assays of cells treated with the two drugs. As shown in Fig.7, overall adenovirus RNA transcription was greatly reduced in the camptothecin treated cells. The RNA transcribed from the nuclei of cells treated with camptothecin hybridized predominantly to HindIII-C fragment. This restriction fragment contains the adenovirus major late promoter. Hybridization to the promoter distal fragments such as HindIII A, B, H, or F fragments was greatly reduced relative to the control. Hybridization of 32P-UTP labeled RNA synthesized in the camptothecin treated cells to specific sense strands of XhoI-E fragment confirmed that the RNA transcribed in vitro and hybridized to XhoI-E has the same polarity as the major late RNA (data not shown). These results indicated that camptothecin caused premature termination of adenovirus major late transcription.

Treatment of adenovirus infected cells with ellipticine resulted in an overall reduction of viral RNA transcription in the isolated nuclei (Fig.7). Thus, unlike camptothecin which appeared to affect transcription elongation, the effect of ellipticine appeared to suppress viral transcription initiation.

Inhibition of adenovirus type 5 DNA packaging by ellipticine. We have shown that linear adenovirus DNA is present in supercoiled conformation inside virus particles (Wong and Hsu,1989). This result suggests that topoisomerase activities are required to package adenovirus DNA into the virus particles. To further examine this possibility, we determined the effect of ellipticine on the incorporation of newly synthesized Ad5 DNA into virus particles. Adenovirus DNA incorporated into the virus particles was assayed by the nuclease-resistant Ad5 DNA extracted from infected cell nuclei which were exhaustively digested with micrococcal nuclease. DNA in the mature virus particles is resistant to nuclease digestion whereas unpackaged or partially packaged viral DNA is degraded by extensive nuclease digestion. Electron microscopic and buoyant density analysis of the viral nucleoprotein present in the extensively digested nuclei confirmed that the nuclease-resistant DNA indeed represents DNA packaged in the mature virus particles (data not shown).

Treatment of cells from 24 to 26 hours postinfection with 30 micromolar of ellipticine has no effect on the total incorporation of 3H-thymidine into Ad5 DNA as described above (Fig.8 lanes a and b). However, the conversion of 3H-thymidine labeled viral DNA into nuclease-resistant virion DNA was inhibited by ellipticine (Fig.8 lanes c and d). To exclude the possibility that inhibition of packaging of newly synthesized viral DNA is due to the inhibition of synthesis of virion proteins, we analyzed the effect of

ellipticine on the synthesis of virion proteins in infected cells. No effect of ellipticine treatment on the quantity and the quality of the labeling of major adenovirus capsid proteins was observed during the two hours period of ellipticine treatment (data not shown). Thus the effect of ellipticine is probably due to the direct inhibition of topoisomerase II activity required for the packaging of adenovirus DNA into the virus particles.

Electron microscopic evidence for the presence of topologically constrained DNA loops in the intracellular adenovirus DNA. The results obtained using topoisomerase inhibitors suggest that intracellular adenovirus DNA templates are topologically constrained. If the viral templates are indeed topologically constrained, then the addition of agents that unwind double helix should result in the compensatory positive supercoiling of adenovirus DNA. To examine this possibility we treated adenovirus nucleoprotein complexes extracted from infected cells at 20 hours p.i. with 100 ug/ml of ethidium bromide and examined the DNA conformation with the electron microscope. About half of the molecules examined was found to contain supercoiled domains (Fig.9 panels A and B). Furthermore, both adenovirus type I and type II replication intermediates exhibited supercoiled domains (Fig.9 panels C and D). Treatment of the nucleoprotein complexes with proteinase K prior to the addition of ethidium bromide abolished the induction of supercoiling by ethidium bromide. These observations provide

direct evidence that intracellular adenovirus DNA is organized into topologically independent loop-domains.

E. Discussion.

The data described here indicate that replication, transcription, and packaging of linear adenovirus DNA are affected by drugs that have been shown to inhibit mammalian topoisomerases (reviewed in Drlica and Franco,1988). These results indicate that the linear adenovirus genome is topologically constrained in vivo, and unwinding of the DNA during replication and transcription generates topological tension. Consequently topoisomerase activities are required for resolution of this topological tension. This conclusion is supported by direct electron microscopic observation of topological constraints in intracellular adenovirus nucleoprotein complexes. Furthermore, topological constraints in adenovirus DNA replication has been demonstrated by the requirement of topoisomerase I activity for the replication of adenovirus DNA in a cell-free system (Nagata et al.,1983).

DNA topology also has been shown to play an important role in the life cycles of several phages or viruses with linear genomes. In T4 phage, the phage coded topoisomerase II (products of genes 39, 52, and 60) is involved in the initiation of phage DNA replication (Huang 1986;Liu et

al.,1979), in the expression of late genes (Mosig et al.,1983), and in the packaging of phage DNA (Zachary and Black,1986). In T5 and T7 phages the replication of phage DNA and the expression of certain phage genes require the host gyrase activity (Constantinou et al.,1986; De Wyngaert and Hinkle,1979; Itoh and Tomizawa,1977; Steck and Drlica,1985). B. subtilis bacteriophage SP01, which contains a linear double-stranded genome, also requires the host gyrase activity for replication (Alonso et al.,1981). In animal viruses, the association of type I topoisomerase with herpes simplex virus and the presence of topoisomerase gene in vaccinia virus imply a role for DNA topology in the replication of their linear genomes (Muller et al.,1985; Schuman and Moss,1987).

How do topological constraints arise in linear DNA? Topological tension in linear DNA generally can be dissipated through the free rotation of DNA termini. However, if the linear DNA is organized into loops with proteins tightly bound to the base of the loops, the loops will form pseudo-circles. The degree of topological constraints in these loops will depend on how tightly the rotation of the double helix is restricted by the DNA-protein interactions at the base of the loop. The existence of such topologically constrained loops in adenovirus particles has been demonstrated by psoralen crosslinking and Bal31 nuclease digestion (Wong and Hsu,1989). The presence of such an arrangement in intracellular adenovirus DNA was demonstrated by the observation of compensatory supercoiling of portions

of adenovirus DNA when the viral DNA double helix is unwound through the intercalation of ethidium bromide. Using similar strategy we also showed that mammalian cell DNA is arranged in topological independent loops.

The nature of the proteins holding adenovirus DNA in the topologically independent loop-domains remains unknown. In mammalian cells topoisomerase II has been implicated in the formation of the loop-domains (Cockerill and Garrard,1986; Gasser and Laemmli,1986). Recently, nuclear scaffold proteins have been shown to organize yeast mating type genes into DNA loops (Hofmann et al.,1989). Some bacterial regulatory proteins have been shown to regulate genes from a distance by looping out the intervening DNA segment (see review in 24). The present observation of the induction of double strand cleavages in intracellular adenovirus DNA by VP-16 suggests that topoisomerase II may be used to organize the viral DNA in the nucleus, similarly to cellular DNA. We are currently testing this hypothesis using anti-topo II antibody to decorate intracellular adenovirus nucleoprotein complexes.

Topoisomerases have been shown to cleave DNA in the presence of their inhibitors (Horwitz and Brayton,1972; Liu et al.,1983; Pommier et al.,1985; Porter and Champoux,1989; Yang et al,.1985). Induction of degradation of adenovirus DNA during lytic infection by camptothecin has been observed by Horwitz and Brayton (Horwitz and Brayton,1972). The data presented here showed that the subgenomic viral DNA induced by

camptothecin was composed of both single and double-stranded DNA. Furthermore, we showed that the cleavage sites induced by camptothecin were located at specific sites of adenovirus genome. Since camptothecin has been shown to induce DNA breaks with topoisomerase I covalently linked to the 3' end of DNA (Hsiang et al.,1985), the specific breaks observed in adenovirus DNA suggests that topoisomerase I enzymes are located nonrandomly on adenovirus DNA. These locations may represent the swivel points for the replication or transcription of adenovirus DNA. Since topoisomerase I cleaves and rejoins only one of the two strands of DNA helix, the observation of double-stranded cleavages of adenovirus DNA in the presence of topoisomerase I inhibitor is somewhat unexpected. In order to generate double-stranded cleavages topoisomerase I has to be located opposite to a nick or a gap in the DNA. Such a structure can be found at the replication fork of the type I and type II adenovirus replication intermediates (Lechner and Kelly,1977). If topoisomerase I is located at the replication forks, then cleavage of type I and type II replication intermediates would generate both double-stranded and single-stranded DNA fragments, as observed in the present report. Cleavage of adenovirus DNA type I replication intermediates at the replication fork would generate both single and double-stranded DNA fragments of the same length. This provide an explanation of the observation that many of the subgenomic

double-stranded DNA fragments have the same length as the single-stranded DNA fragments (Fig.4).

The different responses of adenovirus DNA transcription to camptothecin and ellipticine are quite intriguing. Camptothecin causes premature termination of the major late transcription unit at the late times after infection. On the other hand, ellipticine causes a general reduction in adenovirus transcription. These results suggest that topo I is involved in the elongation step of transcription while topo II participates in the initiation step. Transcriptional process has been shown recently to generate positive supercoiling in the forward direction and negative supercoiling in the backward direction (Tsao et al.,1989; Wu et al.,1988). In linear DNA, these topological tensions should be dissipated by free rotations of the DNA termini. The inhibition of adenovirus transcription by topoisomerase inhibitors implies that the linear viral transcription template is folded into loop-domains with topological constraints. Further research is needed to delineate the relationship between the folding of adenovirus genome and the transcription of various adenovirus transcription units.

Studies in our laboratory have shown that the linear adenovirus DNA is organized into supercoiled domains inside the virus particles (Wong and Hsu,1989). This type of arrangement suggests that topoisomerase is involved in the packaging of adenovirus DNA into the virus particles. This prediction is supported by the present data. However, it is possible that

ellipticine but not camptothecin inhibits adenovirus assembly by blocking the synthesis of protein components necessary for virion assembly. Our data indicated that the major virion proteins were synthesized in normal amount in cells treated with ellipticine. However, we can not yet exclude the possibility that the synthesis or the modification of some minor but critical viral proteins is inhibited by ellipticine.

Note added in proof: Similar results have been obtained by J. Schaack, P. Schedl, and T. Shenk.

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G. Figure legends.

FIG. 1. Inhibition of adenovirus infection of HeLa cells by camptothecin or ellipticine added at the time of infection. Ad5 DNA was purified and digested with HindIII at 16 hours postinfection. lane a is control. lanes b-e are treated with 0.1, 0.5, 2, 5 μM of camptothecin, respectively. lanes f-i are treated with 0.1, 0.5, 2, 5 μM of ellipticine, respectively.

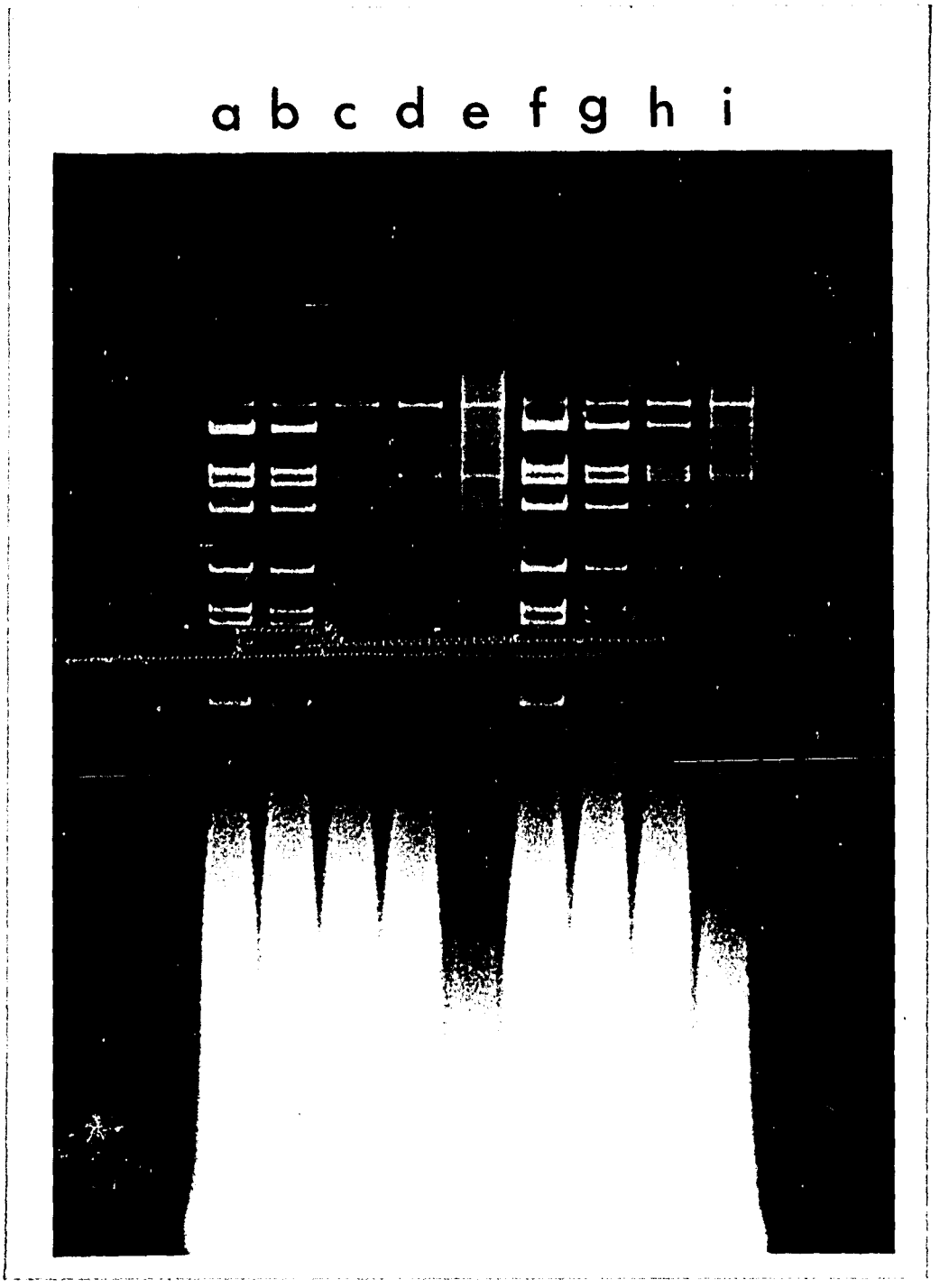

a b c d e f g h i

FIG. 2. Nicks and double-strand cleavages at specific sites of adenovirus early templates induced by camptothecin or VP-16. Treatment of Ad5 infected HeLa cells from 0 to 4 hours p.i. with camptothecin induced nicking of Ad5 DNA as shown by neutral(1-D)-alkaline(2-D) gel electrophoresis (panel A, arrows. CTR: control, CPT: camptothecin). the double-strand breaks of Ad5 DNA (arrows) induced with VP-16 is shown in panel B (mapped with left hand end probe, nucleotide 311-1746) and C (right hand end probe, nucleotide 34933-35937). Lanes a and a' are controls (6 hours p.i.). Lanes b, b', c, c', d, and d' are treated with VP-16 between 0-2 hours p.i., 2-4 hours p.i., and 4-6 hours p.i., respectively, and viral DNA is harvested at 2, 4, and 6 hours p.i., respectively. KB, Kilobases.

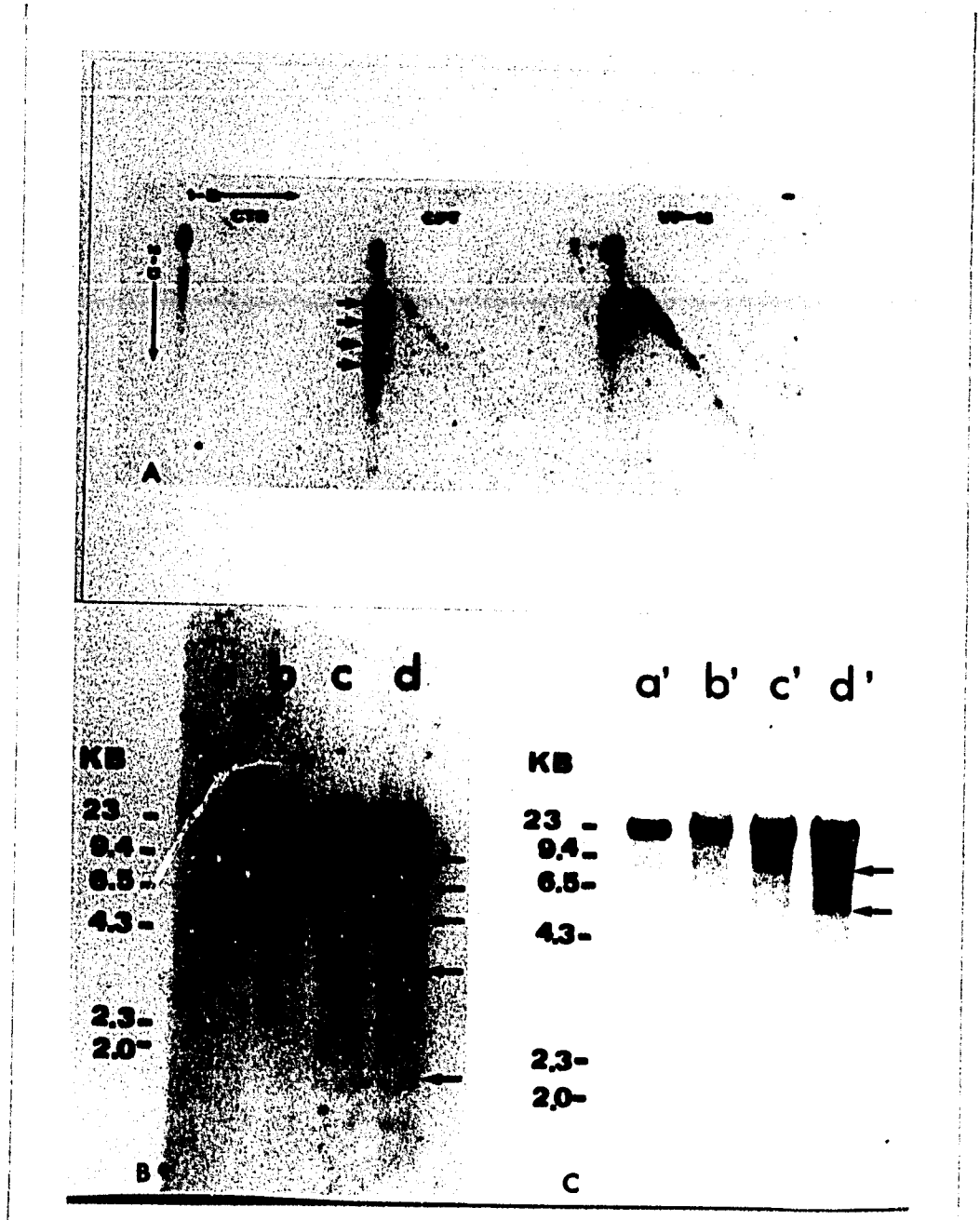

A
b c d
KB
23
9.4
6.5
4.3
2.3
2.0
B
a' b' c' d'
KB
23
9.4
6.5
4.3
2,3
2,0
C

FIG. 3. Inhibition of Ad5 DNA replication and induction of specific double- and single-stranded fragments in Ad5 DNA by camptothecin. The control Ad5 DNA forms a single spot (36 kb) after a two hours (16-18 hours p.i.) pulse with 3H-thymidine (panel A). However, subgenomic size spots are produced in the presence of camptothecin (panel B. single-stranded DNA curve is pointed with triangle). After HindIII digestion of Ad5 DNA, labeling of the camptothecin treated sample is enriched at terminal fragments G and I (compare panels C,control and D,camptothecin) indicated with arrows in panel D.

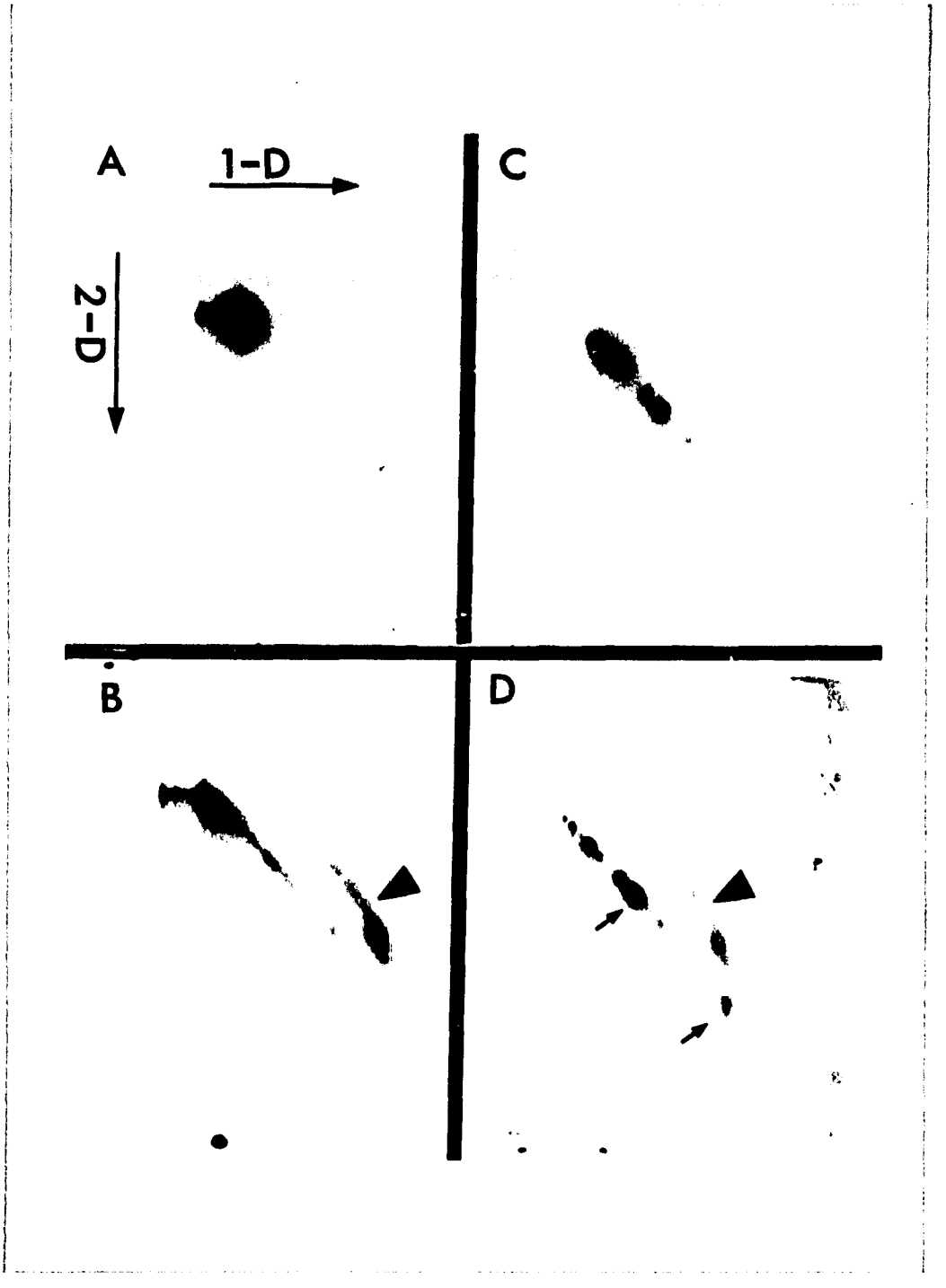

A
1-D
2-D
C
B
D

FIG. 4. Southern blot analysis of single- and double-strand cleavages of Ad5 DNA induced by camptothecin. Added at late times of infection (16 hours) camptothecin induced specific single and double-stranded cleavages of Ad5 DNA. Panels A and A' are controls. Panels B and B' are treated with camptothecin and hybridized with left hand end probe (nucleotide 311-1746) or right hand end probe (nucleotide 34933-35937), respectively. The positions of double-stranded cleavages are located at coordinates 4.2, 5.6, 6.4, 8.0, 9.4 and 11.9 (panel B, arrows) and 87.8, 89.2, 90.6, 91.1, 94.7, and 95.6 (panel B', arrows).

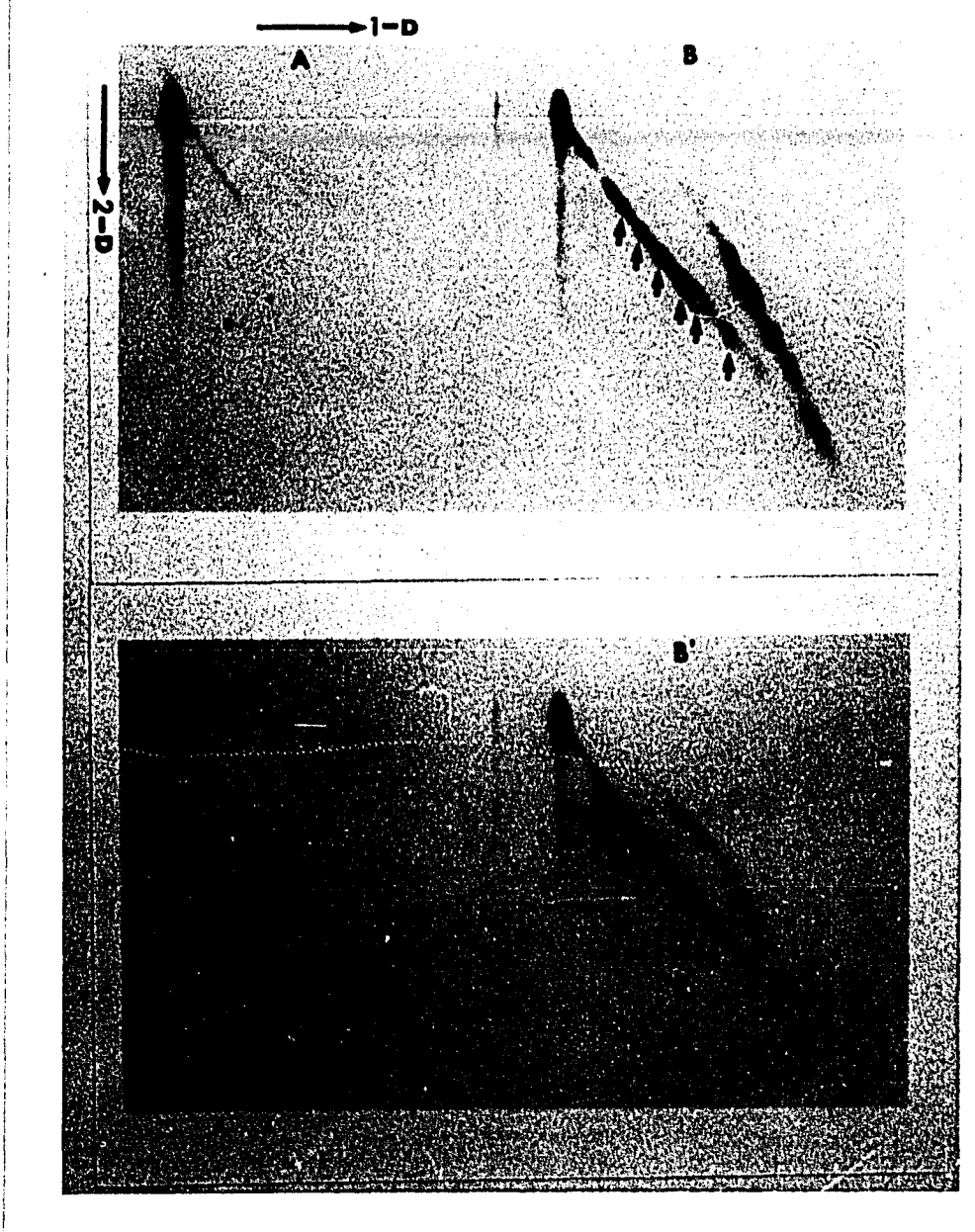

1-D
2-D
A
B
B'

FIG. 5. Induction of double-strand cleavages of nonreplicating Ad5 DNA at specific sites by VP-16 at late times of infection. During a two-hour pulse with 3H-thymidine, VP-16 did not induce cleavage of newly synthesized Ad5 DNA (compare panels a and b). The reduced incorporation in panel b was due to the inhibition of 3H-thymidine uptake with VP-16. In contrast, VP-16 produced double strand breaks in the bulk of Ad5 DNA (panel c, probed with left hand end probe; panel d, probed with right hand end probe).

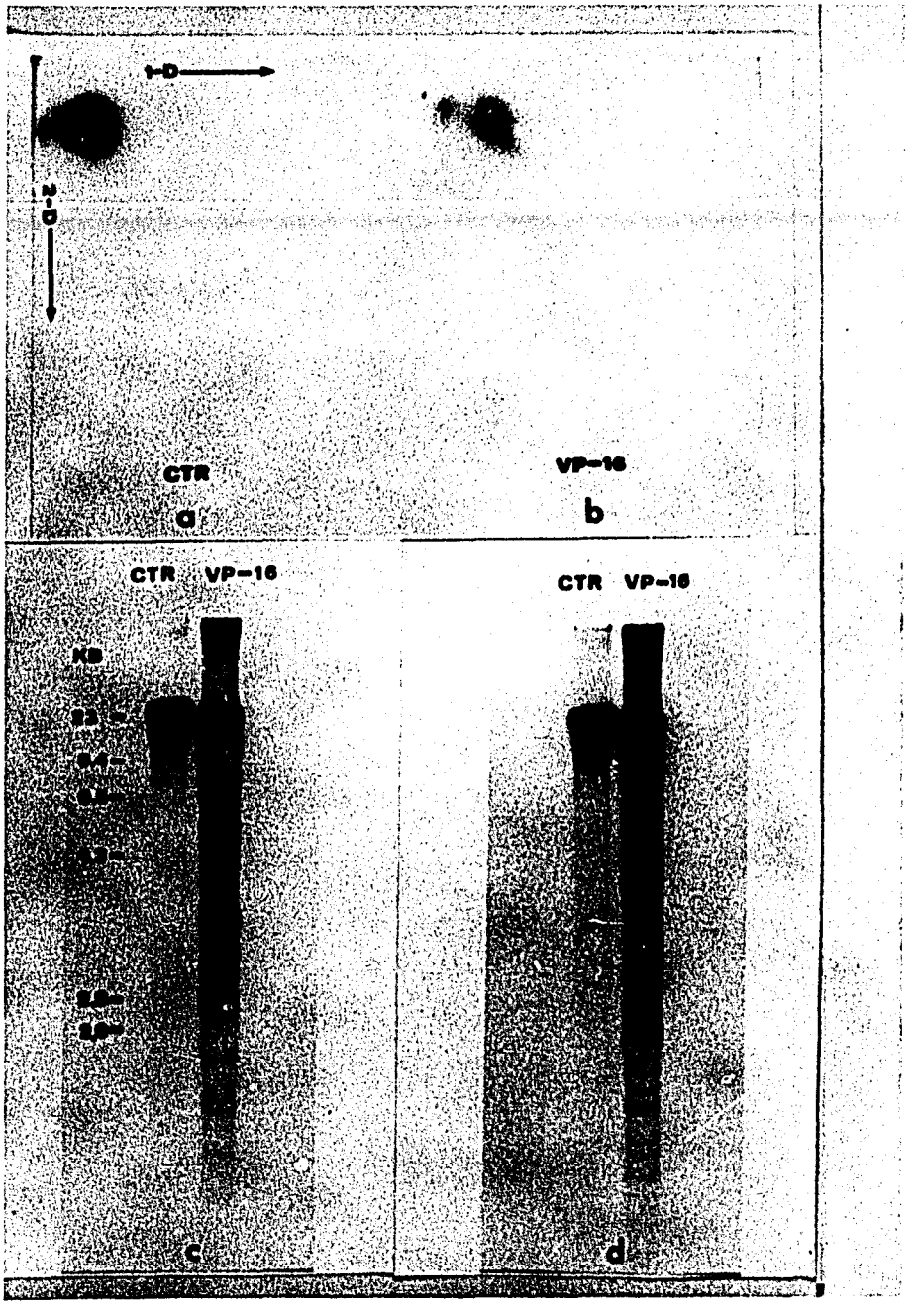

1-D
2-D
CTR
a
VP-16
b
CTR VP-16
CTR VP-16
c
d

FIG. 6. Reduction of incorporation of 3H-uridine into trichloroacetic acid-precipitable RNA by camptothecin or ellipticine. Treatment of Ad5 infected HeLa cells with either drug from 20 to 22 hours p.i. reduced the labeling of RNA by 75% (panel A. CTR: control, CPT: camptothecin, ELP: ellipticine) whereas uptake of 3H-uridine was essentially unaffected (panel B).

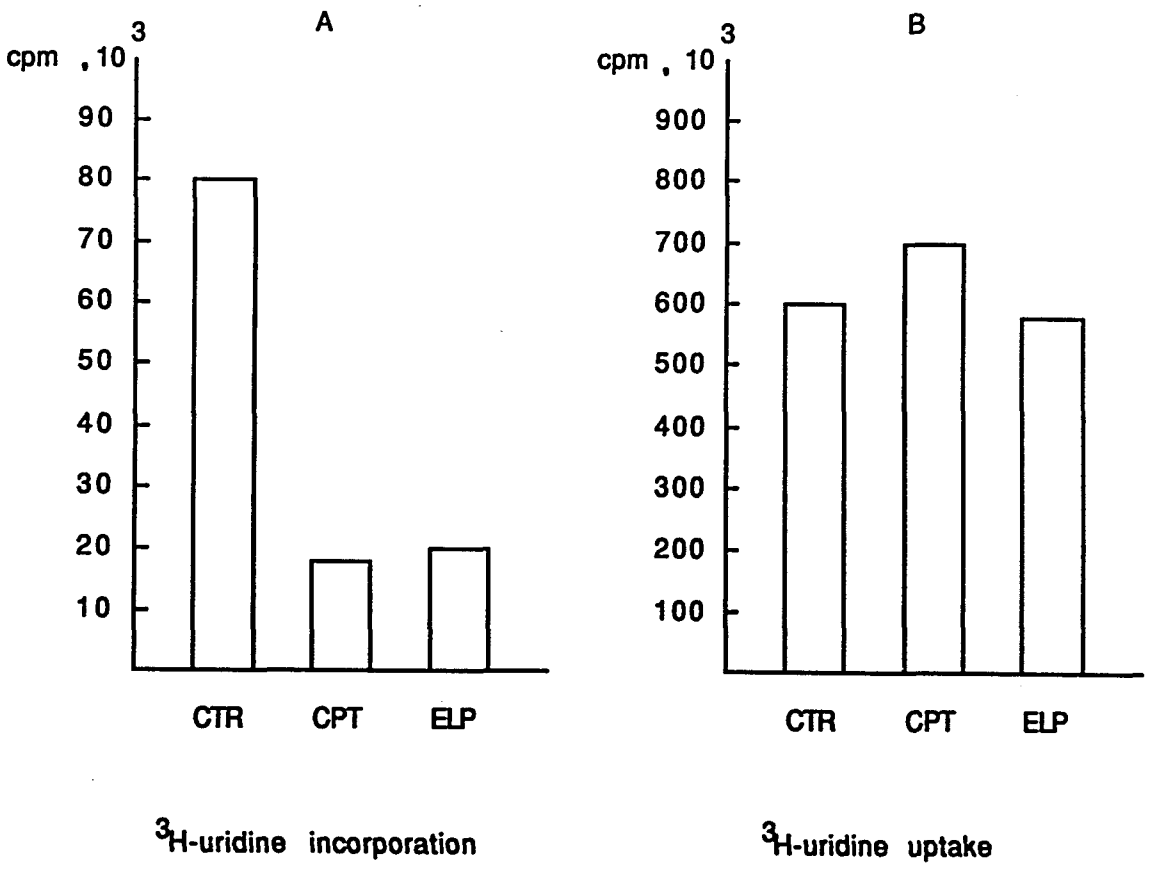

A
cpm , 10 3
90
80
70
60
50
40
30
20
10
CTR
CPT
ELP
3H-uridine incorporation
B
cpm , 10 3
900
800
700
600
500
400
300
200
100
CTR
CPT
ELP
3H-uridine uptake

FIG. 7. Effects of topoisomerase inhibitors on adenovirus transcription analyzed by in vitro nuclear run-on assays. In vitro 32P-UTP labeled Ad5 RNA from control, camptothecin, or ellipticine treated cells was hybridized to HindIII digested Ad5 DNA. Adenovirus RNA synthesized in the presence of camptothecin hybridized preferentially to HindIII-C fragment. Ellipticine produced an overall reduction in viral RNA synthesis.

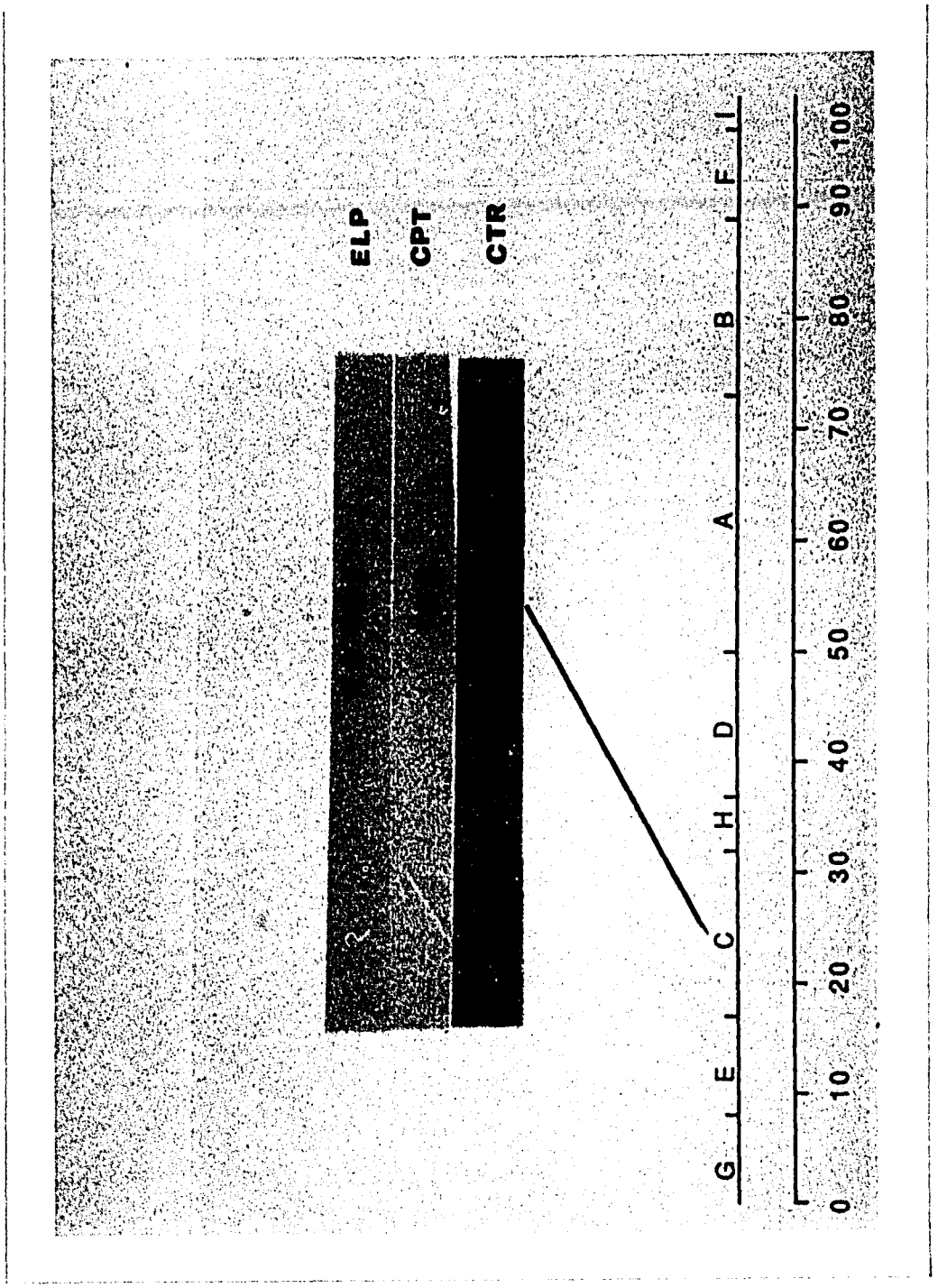

ELP
CPT
CTR
G
E
C
H
D
A
B
F
I
0
10
20
30
40
50
60
70
80
90
100

FIG. 8. Inhibition of Ad5 DNA packaging by ellipticine. Shown are fluorographs of HindIII digested total intracellular Ad5 DNA (lanes a and b) and Ad5 DNA packaged into mature virions (lane c and d). Ad5 DNA were labeled with 3H-thymidine for two hours (24-26 hours p.i.) in the presence (lanes b and d) or absence (lanes a and c) of 30 uM ellipticine. Ad5 DNA in mature virions were resistant to micrococcal nuclease digestion (lanes c and d). In the presence of ellipticine (lane d), formation of the mature virion was dramatically reduced.

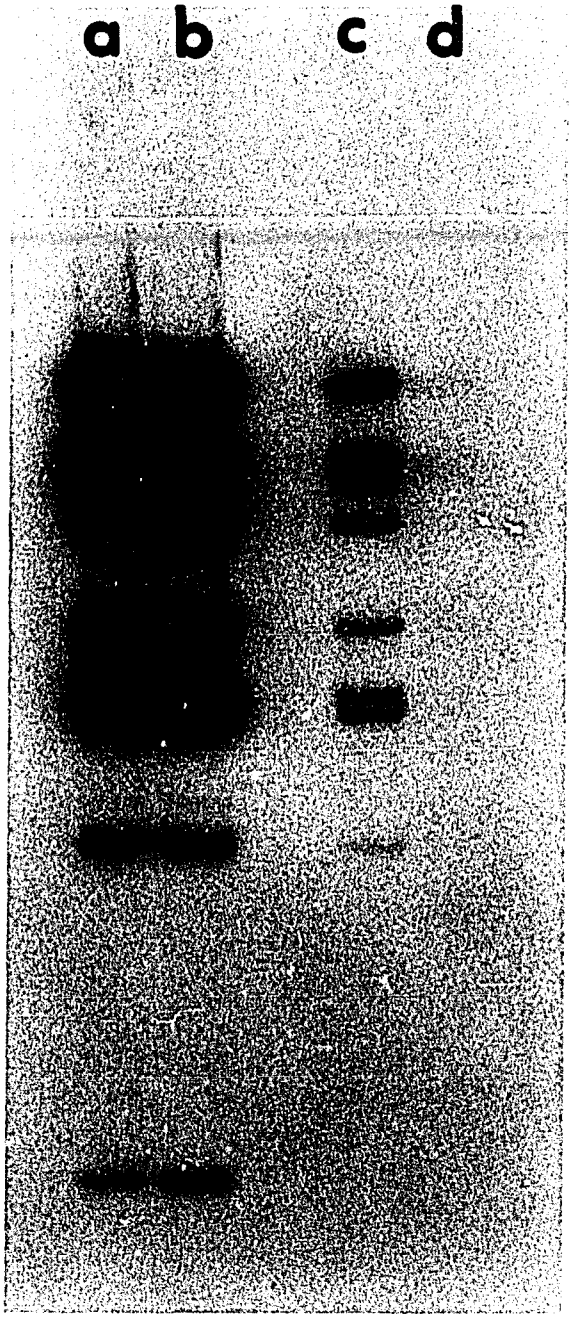
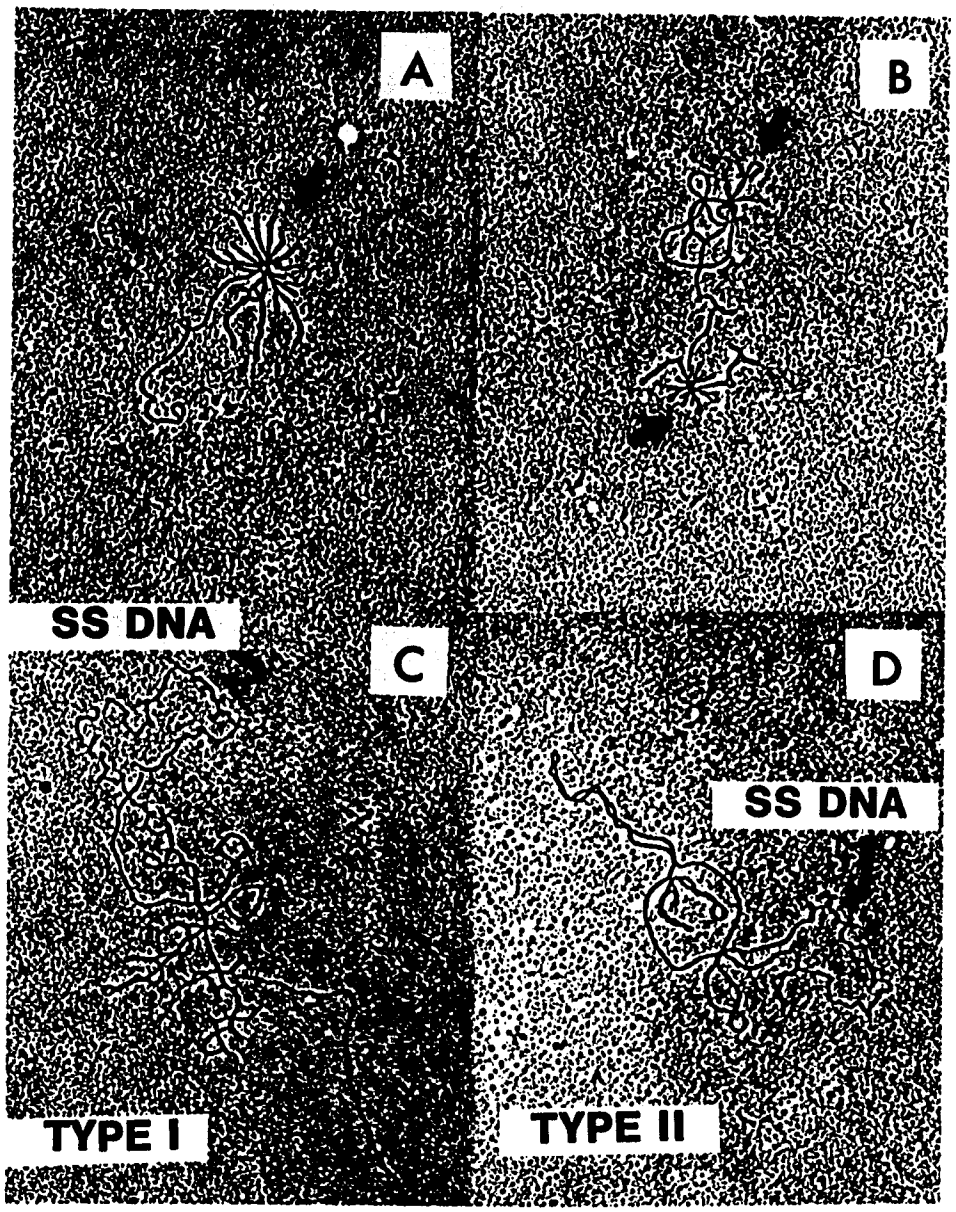

a b c d

FIG. 9. Electron micrographs of intracellular Ad5 DNA-protein complexes with supercoiled domains (A and B, arrows) induced by ethidium bromide. Both types I and II replication intermediates exhibited supercoiled domains (C and D). SS, single-stranded.


A
B
SS DNA
C
D
SS DNA
TYPE I
TYPE II

Chapter 5: Discussion

A: Supercoiled loop domains of DNA-protein complexes in adenovirus particles.

Several lines of evidence indicated that the higher order structure of DNA-protein complexes in adenovirus particles is arranged in supercoiled loop domains. First, supercoiled loops were directly visualized by electron microscopic analysis of bis-psoralen crosslinked adenovirus DNA. Second, viral DNA of adenovirus cores is sensitive to the supercoiling-dependent endonuclease activity of Bal31 nuclease. Third, relaxation of supercoiling of virion DNA by topoisomerase I inhibits the cleavages of viral DNA by Bal31. Fourth, eight DNA loops of the adenovirus core prepared by deoxycholate were directly observed using electron microscope. The supercoiled conformation of virion DNA is also demonstrated by transient appearance of supercoiled loops during treatment of adenovirus particles with sarkosyl (Brown et al.,1975).

From our data, we propose that linear adenovirus DNA is organized into 8 supercoiled loops, except regions at two extreme ends of viral genome, and that each loop is approximately equal to 12% of adenovirus genome.

This model suggests an interesting correlation between supercoiled loop-domains and the organization of viral genes (shown in Fig. 10 of chapter 2). This correlation between loop-domain structure and gene organization of adenovirus DNA also implies a mechanism of control of gene expression by conformation and topology of viral DNA. We proposed that the compact, supercoiled adenovirus DNA sequentially unfolds from two ends of the genome after uncoating, and that unfolding of the viral template plays a role in regulating viral gene expression at early phase of infection. The data, supporting this hypothesis, is presented in chapter 3.

Supercoiling of a linear genome has been reported for bacterial T4 in vivo and lambda phages in vitro (Sinden and Pettijohn,1982; Virrankoski-Castrodeza and Parish,1980). These reports, together with our data, indicate that supercoiled conformation is not restricted to covalently closed circular DNA molecules. The supercoiling conformation of a linear DNA can be maintained by proteins binding tightly to stems of supercoiled loops, or by cross-hybridization of inverted repeat sequences. Because only linear DNA is observed after proteinase digestion of adenovirus particles, the supercoiled structure of DNA inside the virion is likely maintained by tight-binding proteins. Conserved sequences has been found near the stems of loop-domains, and they are probably binding sites of the putative tight-binding proteins. The core feature of these conserved sequences is GGGCGT, which is almost same as the core motif (GGGCGG) of trans-

acting factor Sp1 (Kadonaga et al., 1986). This raises the possibilities that Sp1 or a Sp1-like factor is involved in the control of higher order structure of adenovirus nucleoprotein complexes in virion and in infected cells and that these conserved elements can serve as regulatory sites for virus gene expression. On the other hand, when covalently circular closed SV40 DNA was incubated with adenovirus core, it was observed that different topoisomers were generated (data not shown). This finding implies that host or virus-encoded topoisomerases are associated with adenovirus cores and may play a role in organizing the supercoiled structure of adenovirus genome. Although these two findings are either speculative or preliminary, the putative role of Sp1 or Sp1-like factor and the identity and properties of topoisomerases in virus particles are worthy of studying in the future.

Since unpackaged adenovirus DNA molecules in infected cells at late times of infection display unfolded, linear structure, the formation of highly condensed, supercoiled viral DNA must occur during the packaging stage, and may be mediated by topoisomerase II. In chapter 4, we demonstrated that a topoisomerase II activity was required for packaging of linear adenovirus DNA into the protein coat.

B. Roles of E1a and transcription for unfolding of adenovirus DNA after uncoating.

We showed that the bulk of adenovirus templates underwent gradual unfolding during early times of infection by sucrose gradient sedimentation and electron microscopic analyses. Unfolding of viral templates of the E1a deletion mutants, dl312, was delayed in HeLa cells, while their unfolding proceeded normally in 293 cells, which expressed endogenous E1a gene products. This finding indicates that E1a gene products are necessary for unfolding of the highly condensed, supercoiled DNA. Furthermore, our data suggest that unfolding of adenovirus templates during early times of infection is coupled to transcription of viral genome. This is supported by blocking of unfolding of adenovirus templates by alpha-amanitin, which inhibits transcription by RNA polymerase II (Faulstich,1980; Weinmann et al.,1974). Since of E1a gene products are required for proper transcription of other viral genes, the delayed unfolding of E1a deletion mutant, dl312, templates in HeLa cells, but not in 293 cells, also demonstrates the coupling of transcription and unfolding of templates.

The relationship between transcription and unfolding can be further investigated by using the hr1 mutant of E1a gene, which is unable to grow efficiently in the HeLa cell (Harrison et al.,1977). Unlike dl312, which contains severe deletion in E1a, hr1 has a single base pair deletion at position 1055. The physical length of E1a of hr1 mutant is basically intact, but the 51K trans-activating protein is truncated to a dysfunctional 28K protein due to a shift in reading frame (Ricciarddi et al., 1981). Therefore,

during the infection of HeLa cells with hr1, E1a can be transcribed, but due to the production of a truncated protein, the transcription of other viral genes is blocked. If the template unfolding is coupled to transcription, we will expect that the E1a region is preferentially unfolded, while the rest of viral genome is maintained in a compact structure. The preferential unfolding of E1a region will result in the fast sedimentation rate of hr1 nucleoprotein complexes in HeLa cells and preferential hybridization of RNA transcribed in vitro to E1a region.

Our data showed that E1a gene is required for the unfolding of adenovirus early templates, but we cannot exclude the participation of other viral early genes in template unfolding. For instance, the role of E4 gene, located at the right hand end of adenovirus genome, in unfolding can be tested by infecting HeLa or W162 cells with dl366, a deletion mutant of E4. The dl366 mutant, lacking the majority of E4 gene, can be grown only in the W162 cell, a monkey kidney (Vero) cell line which expresses E4 gene products (Weinberg and Ketner,1983; Halbert et al.,1985). It could be expected that the unfolding of dl366 templates in W162 cells is similar to that of wild type virus templates in HeLa cells. However, the conformation of dl366 in HeLa cells can be either unfolded or condensed. If it is unfolded, this is very likely a result of transactivation of other viral genes (E1b, E2, E3, ML) by E1a, and E4 gene is redundant in unfolding of virus templates. In contrast, if the conformation of dl366 is still highly condensed,

we can conclude that E4 gene also plays an important role in unfolding of virus templates.

Inhibition of transcription can cause condensation of chromatin in lampbrush chromosomes (Izawa et al.,1963; Mancino et al.,1971) and polytene chromosomes (Beermann,1971). These observations, together with our results, indicate a close relationship between transcription and higher order folding of chromatin or viral nucleoprotein complexes, although the mechanism is not known yet. One possible mechanism for coupling of transcription and unfolding of adenovirus templates could be that the relative movement of transcription machinery along DNA physically unfolds DNA. However, since the majority of adenovirus DNA unfolds during infection, this hypothesis would have to assume that the bulk of input adenovirus DNA engages in transcription during early phase of infection. This may be not impossible, but it is quite unlikely because only a very small percentage of adenovirus DNA are engaged in transcription (Wolgemuth and Hsu,1981). An alternative explanation could be that transcription is needed for producing some viral and/or host gene products, which are required for unfolding. Identification of factors required for unfolding of adenovirus templates needs further studies.

C. Involvement of topoisomerases in the life cycle of adenovirus.

Topological problems are usually pertinent to covalently closed circular DNA, and DNA topoisomerases are enzymes capable of resolving these topological problems. However, a number of bacteriophages and animal viruses with linear double-stranded DNA need either host- or virus-encoded topoisomerases in their life cycles (Alonso et al.,1981; Benson and Huang,1988; Constantinou et al.,1986; De Wyngaert and Hinkle,1979; Itoh and Tomizawa,1977; Liu et al.,1979; Poddar and Bauer,1986). These observations implicate that DNA topology also plays an important role in higher order organization, replication, and transcription of a linear genome, which can be organized into different conformations and topological structures through DNA-protein interactions inside the cell. Taking the above observations and the existence of supercoiled loop-domains of DNA in adenovirus particles (Wong and Hsu,1989) into consideration, we tested the roles of topoisomerases in the life cycle of adenovirus in HeLa cells by using different topoisomerases inhibitors. Shown in chapter 4, It was found that different topoisomerases activities are required for replication, transcription, and packaging of linear adenovirus DNA. We found 1) Topoisomerase I, not II, is needed for adenovirus replication; this finding is consistent with the requirement of topoisomerase I activity for adenovirus replication in vitro (Nagata et al.,1983). 2) Both topoisomerase I and II inhibitors block transcription of adenovirus, and topoisomerase I inhibitor causes premature termination of major late transcription. 3) The packaging

of adenovirus DNA into virion is blocked by topoisomerase II inhibitor. These findings suggest that adenovirus genome is topological constrained in infected cells and that topological tension can be produced by unwinding of DNA topological constraints during replication and transcription.

For a linear double-stranded DNA, topological constraints can be produced by loop formation of DNA through DNA-protein interactions at stems of loops. The nature of proteins maintaining adenovirus DNA in topological constrained loop-domains in infected cells is unknown. Topoisomerase II has been suggested in controlling formation of loop-domains in mammalian cells (Cockerill and Garrard,1986; Gasser and Laemmli,1986). A nuclear scaffolding protein has been shown to organize DNA of yeast mating type gene into loop in vitro (Hofmann et al.,1989). We observed that VP-16, a topoisomerase II inhibitor, can induce significant double-stranded cleavages of the bulk of adenovirus DNA in early and late times of infection. This indicates that topoisomerase II is associated with the viral DNA and may be involved in the organization of adenovirus DNA into topological constrained loops in the nucleus.

The existence of supercoiled loop-domains of DNA inside adenovirus virion implies that topoisomerases is probably essential for packaging of the linear viral genome into a highly condensed, supercoiled conformation in ther virion. Our data showed that ellipticine, a topoisomerase II inhibitor, can block the packaging of viral DNA into virion, while it does not affect the

synthesis of adenovirus DNA. This indicates that topoisomerase II activity is essential for packaging of viral DNA and formation of supercoiled conformation in virion. Although the amounts of synthesized major virion proteins are not changed by ellipticine, we can not exclude the possibility that ellipticine affects the synthesis or modification of some minor proteins important for viral DNA packaging.

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