
Linear adenovirus DNA is organized into supercoiled domains in virus particles

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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.

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 (1). 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

(2,3). 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 (2). 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 (3,4,5), in contrast to the result obtained using pentonless particles (2). These results together with the observations of a 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 (5,6).

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 (7) or by the ion-etching technique (8). These studies suggest that adenovirus DNA is organized in 8-12 DNA loops inside the virus particles. These findings are interesting because loop-domain organization has been found in the genomes of both prokaryotic and eukaryotic cells (see reviews in ref. 9,10). 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.

In the present report 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 (11). 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.

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 (12). The virus particles were purified by buoyant density centrifugation twice in a CsCl density gradient (density=1.34). Pentonless virus particles were prepared essentially as described (2) by dialyzing adenoviruses against 5 mM Tris-acetate, pH 6.4 at 40C overnight. The dialyzed virus particles were heated at 560C 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 300C 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 370C 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 370C 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 nitrocellulose paper 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 Ad5 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 (5mg/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 onto a hypophase of 10% formamide, 10 mM Tris-HCl, 1 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.

RESULTS:

Adenovirus DNA in the pentonless particles 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 (11). We used this property of Bal31 to probe the topological state of adenovirus DNA in the virus particles. To make virion DNA accessible to Bal31 nuclease, we removed the penton bases from the virus particles by the procedure of Corden et al. (2). 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 Ad5 DNA derived from either the right- or left-hand end, discrete bands were observed even at the earliest time point of digestion (Fig.1). In contrast, only a smear of DNA characteristic of exonuclease activity of Bal31 was observed when purified Ad5 DNA was digested with Bal31 under various conditions and probed with either right- or left-handed probes (Fig.2). These results

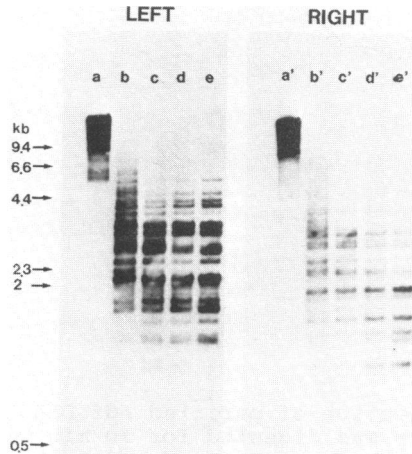


FIG. 1. Bal31 digestion pattern of Ad5 virion DNA in the pentonless virus particles. The pentonless particles were digested with Bal31 for 0.5 hr (lane b, b'), 1 hr (c, c'), 2.5 hr (d, d'), and 3.5 hr (e, e'), or self-incubated without Bal31 for 3.5 hr (a, a'). The digested DNA was purified, electrophoresed 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').

indicate that Ad5 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 adenovirus virion DNA only if the cleavages are due to a supercoiled conformation. As shown in Fig.3, endonuclease cleavages of Ad5 virion DNA by Bal31 was indeed inhibited by the pretreatment of the pentonless particles. 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

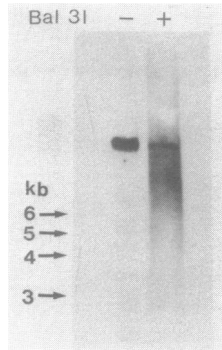


FIG. 2. Bal31 digestion of purified Ad5 DNA. Ad5 DNA purified from virus particles was digested for 30 min with Bal31 as in fig. 1 and probed with the left hand end probe.

DNA was also confirmed using psoralen crosslinking technique. A bi-functional psoralen reagent, bis-aminomethyltrioxsalen (bis-AMT, see Materials and Methods) was synthesized and used to fix the conformation of the DNA in the virus particles. When adenovirus DNA extracted from bis-AMT crosslinked virions was examined in the

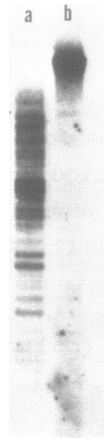


FIG. 3. Inhibition of Bal31 cleavage of Ad5 pentonless particles DNA by calf thymus topoisomerase I. pentonless particles were digested with Bal31 with (lane b) or without (lane a) pretreatment with 60 units of calf thymus topoisomerase I. The amount of undigested full length Ad5 DNA was increased in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

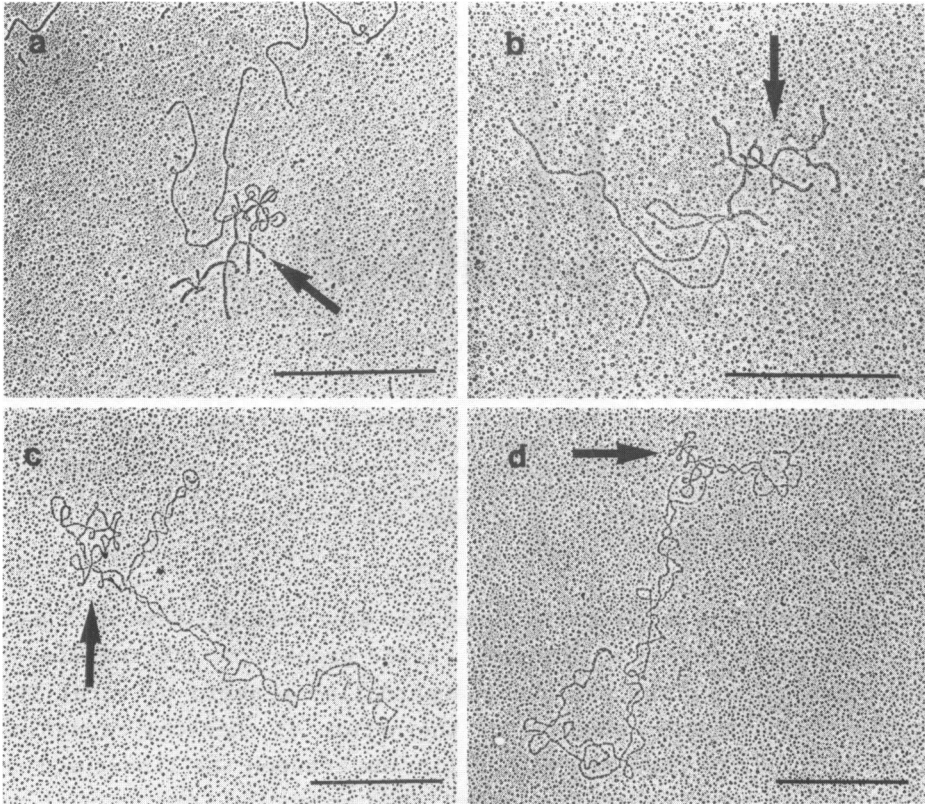


FIG. 4. Electron micrographs of Ad5 virion DNA crosslinked with bis-AMT showing supercoiled loops in the linear Ad5 DNA. Ad5 DNA in the intact virus particle was crosslinked with bis-AMT and the viral DNA was purified and spread in 60% formamide without further treatment (panels a and b) or after the 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.

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).

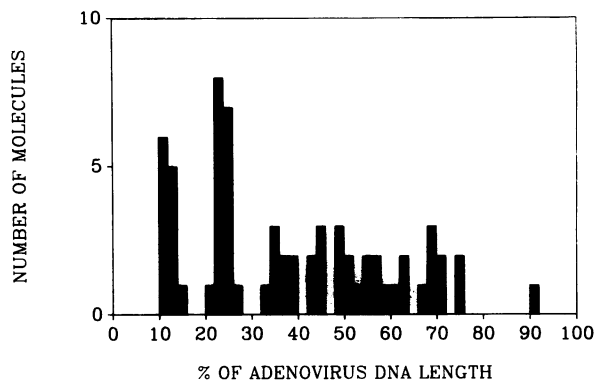


FIG. 5. Histogram of length distribution of supercoiled loops in crosslinked Ad5 virion DNA. Because of the difficulty of tracing highly supertwisted loops the lengths of supercoiled loops were estimated by subtracting the linear portions of the molecules from an intact Ad5 linear DNA nearby. A total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

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 DNAs (13). 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 Ad5 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 Ad5 genome length. The handedness of supercoiling was determined by shadowing the molecules unidirectionally (14) and examined the direction of crossover of supertwisted DNA in comparison with Form I SV40 DNA included in the crosslinked Ad5 virion DNA as an internal standard.

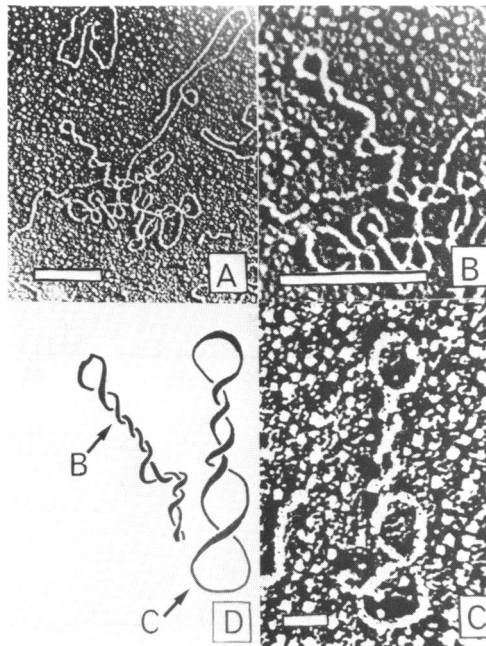


FIG. 6. Determination of handedness of Ad5 DNA supercoiling with unidirectional shadowing. The handedness of crosslinked Ad5 virion DNA (panels A and B, bars = 1 micrometer) was compared with that of Form I SV 40 DNA (panel C, bar = 0.1 micrometer), and they showed the same direction of supercoiling (panel D).

The direction of supercoiling was found to be the same as that of SV40 DNA, from which we conclude that Ad5 DNA is supertwisted in a left-handed fashion inside the virus particles (Fig.6).

Determination of core protein positions on adenovirion DNA.

As described above, Bal31 nuclease cleaves adenovirus DNA in the pentonless virus at discrete sites of Ad5 DNA (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 Ad5 DNA fragments became progressively shortened by the treatment with Bal31 (data not shown). This result suggests that the stable Bal31 cleavage sites in adenovirus virion DNA may be generated by the inhibition

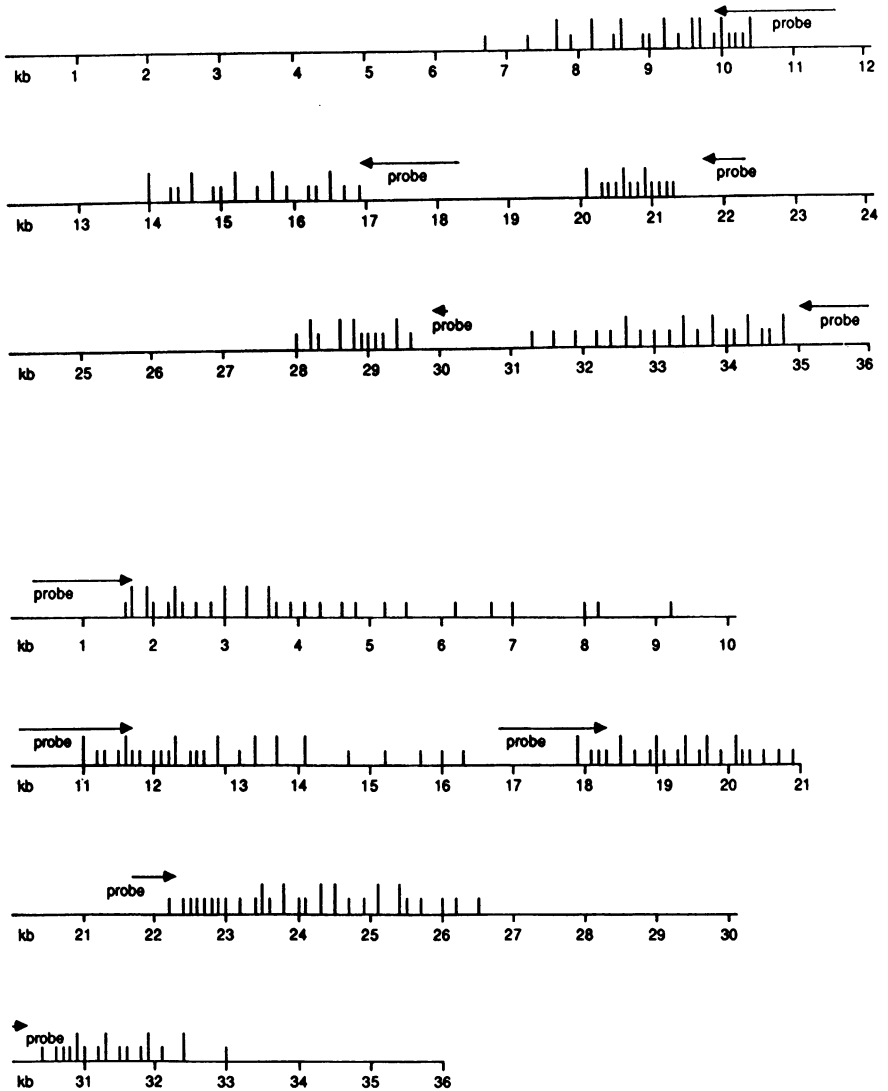


FIG. 7. Summary of the map positions of Bal31 strong stops in the Ad5 virion DNA. The probes used (leftward and rightward directions) are indicated in the graph. Longer vertical lines above the map represent the major sites while shorter lines represent the minor ones. Map positions are expressed in 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.).

of Bal31 exonuclease activity by proteins bound to Ad5 DNA. This is reminiscent of the Bal31 strong stops at nucleosomes (15) and of exonuclease III strong stops in mapping protein positions bound to DNA (16). This interpretation is further supported by the finding that S1 nuclease cleaved Ad5 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 (17). The result, summarized in Fig.7, suggests that core proteins are located at defined positions on adenovirus DNA in the virus particles.

To exclude the possibility that the specific cleavage is due to sequence-specific cleavages by Bal31, we analyzed the Bal31 cleavage sites in supercoiled plasmid pE1a DNA 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 Ad5 DNA (data not shown). Furthermore, the specific cleavage sites in the plasmid DNA appeared only transiently as the DNA is eventually degraded by the exonuclease activity of the enzyme.

DISCUSSION:

Supercoiled conformation of adenovirus DNA in the virus particles

We have presented several types of evidence that Ad5 DNA in the virus particles is supercoiled. First, supercoiled loops were directly visualized after fixing the DNA conformation inside the virus particles by crosslinking. Secondly, DNA in the Ad5 virus cores was found to be sensitive to the endonuclease activity of Bal31 nuclease. The endonuclease activity of Bal31 nuclease towards double stranded DNA has been shown to depend on the supercoiled conformation of DNA (11). Furthermore, relaxation of supercoiling by topoisomerase I inhibited the digestion of Ad5 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 (7).

Supercoiling of a linear genome has also been reported for bacterial T4 and lambda phages (18,19). 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 the supercoiling is not released through the rotation of the free ends. This could conceivably be achieved by one of following ways: 1) tight-binding proteins at the boundaries of the supercoiled loops in the linear molecule; or 2) a cross-hybridized form of an inverted repeat sequence at the boundaries of the supercoiled loop, as has been observed in plasmid DNA after denaturation and renaturation (20). Because only 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 (21). Since our data indicate that the supercoiling of adenovirus DNA in the 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 (22). Since unpackaged adenovirus DNA in infected cells at late times after infection is present in the linear conformation (23), supercoiling of viral DNA must occur during the packaging process. This consideration suggests that gyrase-like activity is associated 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 Ad5 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 the base of the supercoiled loops must lie very close to each other, perhaps forming a tetrahelix. It is also possible that the fixation of the supercoiled loops by bis-AMT is not due to 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, Ad5 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 than that of the reaction between psoralen and nucleic acids (24). Despite our ignorance of the nature of the chemical bonds involved in the fixation of supercoiled loops in Ad5 virion DNA the method nevertheless has proved very useful in studying the topological arrangement of DNA.

Loop-domain organization of Ad5 DNA in the virus cores.

In the limit digestion of Ad5 DNA with Bal31 in the pentonless particles, a stable pattern of digestion products was observed (see Fig.1). Since the larger products contain 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

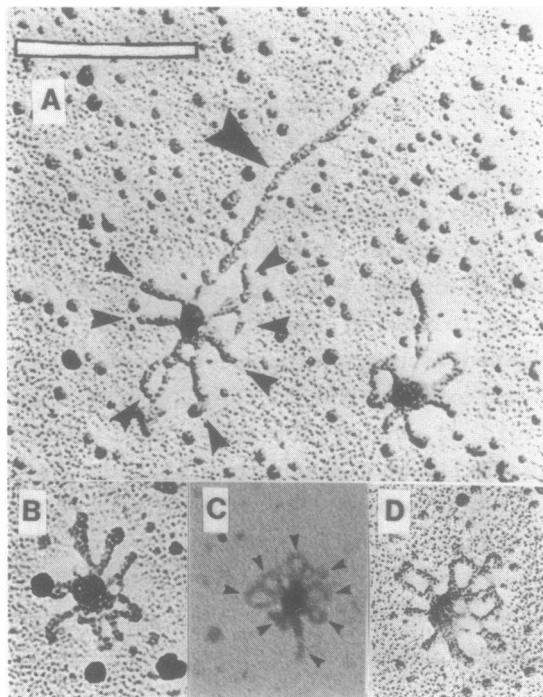


FIG. 8. Electron micrographs of Ad5 virus cores prepared by the deoxycholate method. Highly condensed supercoiled DNA loops (arrow heads) were seen and emerged from a condensed center of the virus cores (panels A-D). A virus core with one end of the viral DNA uncoiled is shown in panel A. The uncoiled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

that the supercoiled domains at the two ends of adenovirus genome to be 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(7). We have also observed eight DNA loops in adenovirus cores prepared by the deoxycholate method (Fig.8).

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

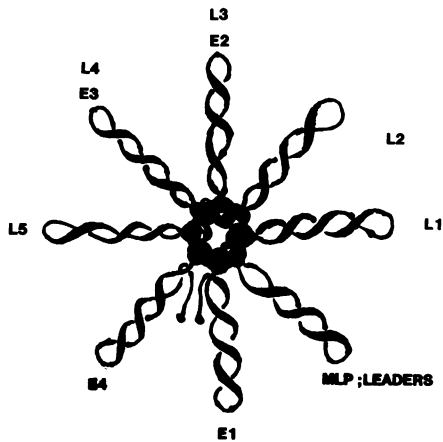


FIG. 9. A loop-domain model for the organization of Ad5 DNA in the virus particles based on the Bal31 nuclease digestion and electron microscopic data. Ad5 DNA is assumed to be organized into eight supercoiled loops and these loops are anchored to the center of the virus core by yet uncharacterized proteins. 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 coordinate 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.

Fig.9. For example, the first loop, from coordinate 2-14, accommodates the E1a and E1b transcription units. This correlation between the 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 between the observed conformational changes and viral gene expression.

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