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DNase I cleavage of adenoviral nucleoprotein

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ABSTRACT

Cleavage products resulting from DNase I treatment of adenoviral nucleoprotein were examined by gel electrophoresis, Southern blotting and hybridization to cloned restriction fragments derived from various regions of the viral genome. DNase I produced specific double-stranded cleavages in DNA of purified adenoviral cores and in DNA of intranuclear viral chromatin at early and late times of infection. At least some of these sites were also cleaved by DNase I in purified viral DNA, showing that sequence specificity of DNase I cleavage may contribute to the observation of specific double-stranded DNase I cleavage sites in adenoviral nucleoprotein. In addition, sites were observed which were specific either for cores or for intranuclear chromatin. In contrast to many cellular genes which have been characterized, there was no obvious relationship between DNase I cleavage sites and other features of the viral genome such as promoters or polyadenylation sites.

INTRODUCTION

The use of nucleases to probe accessiblity of DNA in nucleoprotein has yielded information about levels of structure in chromatin ranging from specific protein-DNA interactions within and between nucleosomes (1-3) to the accessibility to nucleases characteristic of actively expressed genes (4-7). Preferential cleavage by DNase I of genes undergoing active transcription relative to non-transcribed sequences is characteristic of all genes so far examined [reviewed by Mathis et al. (8)]. In addition to the general nuclease sensitivity typical of DNA in active regions of chromatin, cleavage at specific sites adjacent to structural genes has been observed following very mild digestion by DNase I [reviewed by Elgin (9)].

In studies reported here, we use DNase I cleavage to probe accessibility of adenoviral nucleoprotein during various phases of viral chromatin activity. In contrast to SV40 and polyoma, which package viral DNA as a histone-DNA complex, adenovirus DNA is associated with virally encoded histone-like proteins. Within the virus particle, adenoviral DNA, 35 kb in length, occurs in a complex with virally coded histone-like proteins. The major core protein, VII

($M_r \cong 18,000$ daltons) is present in about 1070 copies per virion. The minor core protein, V ($M_r \cong 43,000$ daltons) is present in about 180 copies per virion. The mass of these proteins together in the virus core is about equal to the mass of the DNA (10). This complex of viral DNA and core proteins is thought to penetrate the nucleus upon infection to generate the substrate for early viral transcription (11-13).

The major core protein is synthesized in the form of a precursor, pVII, which contains 20 additional hydrophobic and possibly acidic residues at its amino terminus (14-15). pVII is synthesized during the late phase of infection and is found in association with newly replicated DNA in immature virions. The processing of pVII to VII accompanies virion maturation (16). These viral proteins are arginine-rich (10, 17) and do not undergo reversible internal acetylation although we have shown that the amino-termini of pVII and V are acetylated (18). Adenoviral nucleoprotein differs from the nucleosomal structure characteristic of cellular and some viral chromatin (19) and yet functions in a mammalian cell nucleus.

Although adenoviral chromatin differs from eukaryotic chromatin in protein content, we found that DNase I yielded specific double-stranded fragments upon digestion of purified viral nucleoprotein. We undertook exploration of this phenomenon in order to determine the structural basis of this cleavage specificity and its relationship to chromatin activity. DNase I cleavage patterns found in purified viral cores were compared to those obtained with parental viral chromatin early in infection, with newly replicated viral chromatin late in infection and with purified viral DNA.

MATERIALS AND METHODS

Cells, virus, infections, virus and core purification and nuclei isolation

Virus infections of HeLa monolayers, virus particle purification and isolation of nuclei were all carried out as has been described (18, 20). Analysis of parental viral chromatin was performed using nuclei prepared at 3 hr post-infection. Analysis of late viral chromatin was carried out at 24 hr post-infection. Viral cores were prepared after the method of Prage et al. (21) as described (22).

Nuclease digestions and DNA purification

DNase I (E.C.3.1.4.5, Worthington Biochemicals, 2,812 units/mg) was prepared as a stock of 20 U/µl in 0.15 M NaCl. Aliquots were frozen at -20°C and diluted into cold 0.15 M NaCl just before use. Aliquots were thawed only once and were discarded after one use. Digestions of nuclei were carried out at a

concentration of 1.5 x 10^7 to 2 x 10^7 nuclei per ml of Buffer A (15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol) with 0.34 M sucrose, 0.2 mM PMSF and 5 mM $MgCl_2$ at 37°C. Purified viral cores and DNA were digested at a DNA concentration of 20 μg/ml, unless otherwise indicated, in the same buffer that was used for nuclei. Viral cores were obtained in the appropriate buffer by passage of cores, in pooled sucrose gradient fractions, over a Sephadex G-25 column (Pharmacia), equilibrated in Buffer A with 0.34 M sucrose and 0.2 mM PMSF. 5 mM $MgCl_2$ was added immediately before digestion. Reactions were terminated by the addition of EDTA to 10 mM and SDS to 0.25% (wt/vol).

Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. The conditions specified by the supplier were used for restriction enzyme digestions. Reactions were stopped by the addition of EDTA to 10 mM and incubation at 68°C for 5 min.

S_1 nuclease from Aspergillus oryzae (105,300 units/mg, Sigma) was used at a concentration of 10 units per 10 μg of DNA in 200 μl of 0.25 M NaCl, 30 mM $NaCH_3CO_2$, 1 mM $ZnSO_4$. Digestions were carried out at 42°C for 45 min. An equivalent amount of heat denatured DNA was virtually completely digested under these conditions.

Following DNase I digestion, DNA from nuclei or viral cores was purified as described (19). DNA, pelleted by centrifugation, was dried and resuspended in 10 mM Tris-HCl (pH 7.9), 1 mM EDTA at a concentration of 0.2 μg/μl. Nuclear DNA samples were treated with RNase. Samples were brought to 0.1% SDS and 0.5 mg/ml pancreatic RNase (pre-incubated at 80°C for 10 min, Worthington). Samples were then incubated for 1 hr at 37°C and retreated with pronase, extracted and ethanol precipitated.

CsCl purified virus particles in 5 mM Tris-HCl,pH 7.5, prepared as has been described (20), were brought to 0.25% SDS and 2 mM EDTA and incubated with 1 mg/ml pronase for 1 hr at 37°C. Phenol extractions were performed as described (19). DNA was precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 7.9), 1 mM EDTA.

Cloned fragments of adenoviral DNA were the kind gifts of Arnold Berk and Phil Sharp. A fragment was subcloned from a pBR322 construct containing an insertion of the Ad2 HindIII C fragment. The Ad2 HindIII C fragment extends from 7.9 map units to 17.0 map units. Sequences between 7.9 and an XhoI site at 15.5 map units were deleted in the subclone leaving an insertion extending from 15.5 to 17.0 map units. Because of the extensive homology between adenovirus serotype 2 and serotype 5, cloned restriction fragments from both sero-

types were used interchangeably. Plasmids carrying insertions of adenovirus restriction fragments are described in detail in Fig. 2. Plasmid DNA was prepared after the method of Davis et al. (23).

Preparation of labeled DNA

[^{3}H]-thymidine labeled adenovirus type 5 for use as marker in preparative sucrose gradients and for quantification of nuclease digestions was prepared by adding 50 µCi of [^{3}H]thymidine (52 Ci/mmol, Schwarz/Mann) to 5 ml medium per 100-mm petri dish at 16 hr post-infection. An additional 5 ml of non-radioactive medium was added at 36 hr post-infection. Cells were harvested at 48 hr post-infection and virus particles were purified as has been described (20). This labeling regime resulted in a specific activity of approximately 3 x 10^5 cpm/µg of viral DNA.

Plasmid DNA was labeled by nick translation after the method of Rigby et al. (24). 1 µg of plasmid DNA was combined with 50 µCi or 72 µCi of each of thymidine, deoxyguanosine, deoxycytidine and deoxyadenosine 5'-[α-^{32}P]-triphosphates (410 Ci/mmol, Amersham) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 50 µg/ml BSA with 20 units of DNA polymerase (New England Biolabs) and 0.5 ng DNase I (2000 units/mg, Sigma) in a total volume of 100 µl. The reaction mixture was incubated for 1 to 1.5 hr at 15°C and was stopped by the addition of 20 µl of 0.5 M EDTA and labeled DNA was separated from unincorporated triphosphates. A specific activity of approximately 1 x 10^8 cpm/µg of DNA was usually obtained.

Agarose gel electrophoresis, blotting and hybridization

Vertical slab gels (10 by 13 by 0.3 cm) were prepared by dissolving agarose (Sigma Type II, electrophoresis grade) in E Buffer (25). Electrophoresis was carried out at 4°C at 60 V for 3 hr. Horizontal slab gels (23 by 16 by 0.5 cm) were prepared using E Buffer or TBE Buffer (26). Gels in E Buffer were electrophoresed at 50 V for approximately 16 hr at 4°C. Gels in TBE Buffer were electrophoresed at 70 V for 16 hr at 4°C. Five microliters of 50% (wt/vol) sucrose, 0.02% (wt/vol) bromophenol blue, 100 mM EDTA was added to samples in 10 mM Tris-HCl, pH 7.9, 1 mM EDTA or restriction enzyme buffer just before loading onto gels. Gels were stained with 5 µg/ml ethidium bromide for 20 to 45 min and photographed using long wave ultraviolet light and Polaroid type 55 film with Kodak No. 29 red and No. 15 yellow filters.

Blotting of agarose gels was carried out essentially according to the method of Southern (27). After being stained and photographed, gels were rocked in 2 volumes of 0.5 M NaOH, 1.5 M NaCl twice for 15 min to denature the DNA. They were then rinsed briefly with deionized water and neutralized

by incubation in 2 volumes of 0.5 M Tris-HCl pH 7.4, 3 M NaCl twice for 15 min. Depurination by incubation in 0.25 M HCl was omitted in the interest of optimizing retention and resolution of small fragments at the possible risk of lowering recovery of large fragments.

Transfer to nitrocellulose membrane filter was carried out in 20 x SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) using a reservoir of buffer. Blots were rinsed briefly in 20 X SSC and baked at 65°C for 12 to 16 hr. They were stored dessicated at 4°C until needed for hybridizations.

Alternatively, ammonium acetate buffer (1 M $NH_4C_2H_3O_2$, 0.2 M NaOH) was used in place of 0.5 M Tris-HCl pH 7.4, 3 M NaCl for gel neutralization and in place of 20 x SSC for transfer to nitrocellulose as described by Smith and Summers (28). Instead of using a reservoir, elution of fragments from gels onto nitrocellulose was carried out using 3 sheets of ammonium acetate buffer-saturated filter paper.

Hybridizations to DNA blotted onto nitrocellulose filters was performed using the procedure described by Wahl et al. (29), using an amount of labeled plasmid DNA corresponding to 0.5 to 1 x 10^7 cpm per 200 cm^2 of filter. Filters were incubated with probe for 6 to 16 hr at 42°C. They were then rinsed three times for 15 min at room temperature with 2 x SSC, 0.1% SDS and twice at 60°C with 0.1 x SSC, 0.1% SDS. Filters were dried at 65°C before autoradiography using Kodak XAR-5 film. When necessary to obtain sufficient exposure, film was pre-flashed with a Vivitar electronic flash with a Wratten No. 22 filter and a Dupont Cronex intensifying screen was used at -70°C.

RESULTS

DNase I cleavage of adenoviral cores and purified viral DNA yields specific double-stranded fragments

When viral cores (22) were digested with low levels of DNase I and the DNA products were electrophoresed into non-denaturing agarose gels, specific bands of variable size and intensity were seen (Fig. 1). With increasing times of digestion, the sizes of the high molecular weight bands remained the same while lower molecular weight bands appeared. At higher enzyme to substrate ratios (lane A, Fig. 1) the intensity of the bands relative to the background diminished. At least 15 prominent bands were distinguishable under these digestion conditions ranging from nearly genome sized (35 kilobase pairs) to less than 5 kilobase pairs.

A similar but less distinct pattern appeared following DNase I digestion of purified viral DNA. To achieve the same extent of digestion of DNA as of

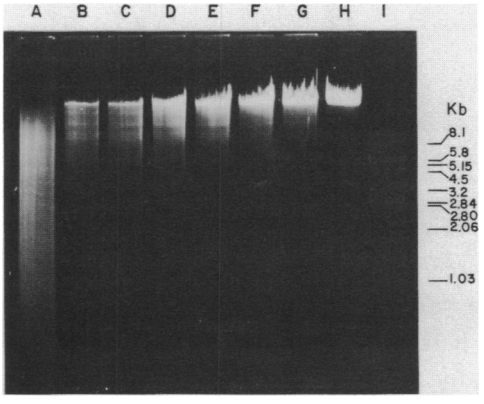

FIGURE 1. Ethidium bromide staining patterns of the products of DNase I digestion of adenoviral cores. Each lane contains 1 μg of DNA. The digestion conditions include 2U/ml of DNase I with 10 μg/ml DNA for 15 sec at 37°C (lane A), and 0.2U/ml of DNase I with 40 μg/ml of DNA at 37°C for 6 min (lane B), 4 min (lane C), 2 min (lane D), 1 min (lane E), 30 sec (lane F), 15 sec (lane G) and no digestion (lane H). Lane I contains HindIII digested Ad5 DNA co-electrophoresed as a size marker. Electrophoresis was carried out in 0.8% agarose vertical slab gels. Gels were stained for 30 min with 5 μg/ml of ethidium bromide.

viral cores it was necessary to use less nuclease (unpublished data).

In these experiments with adenoviral cores, digestion conditions were chosen such that most of the viral DNA migrated with the full length genome of 35 kilobases and very little migrated more rapidly than the Ad5 HindIII A fragment containing 8.3 kilobases. Investigations of hypersensitive sites in eukaryotic chromatin generally employ a level of DNase I cleavage that produces an average fragment size of 10 to 15 kilobase pairs as estimated by agarose gel mobility under non-denaturing conditions. Experiments with SV40 and polyoma commonly employ digestion conditions that produce an average of 1 cleavage event per 5 kilobase genome. With the extent of digestion displayed on these gels, no DNA has been rendered soluble in 10% TCA; over 30-fold more nuclease was required to achieve measurable TCA solubilization.

To compare cleavage patterns of cores and purified DNA, the DNA was blotted onto nitrocellulose following electrophoresis and hybridized with particular adenoviral restriction fragments. The results using the HpaI E fragment, which contained sequences at the leftmost end of the genome (see Fig. 2), are shown in Fig. 3A. Some prominent bands that appeared in the products of DNase I digestion of cores (C, D, H) were also found following DNase I digestion of DNA (H, I, J) although they were less pronounced. The

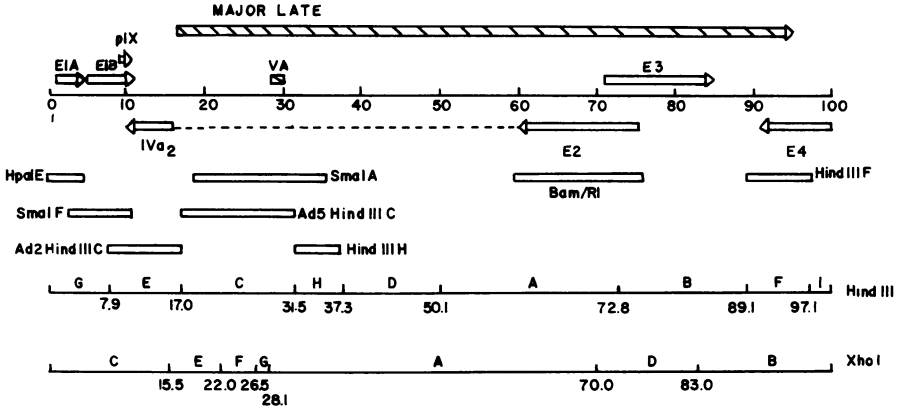

FIGURE 2. Transcription map of the adenovirus genome and locations of restriction fragment used in the analysis of DNase I cleavage sites. The map is divided into 100 units of about 355 base pairs each. Transcription proceeds in the direction of the arrows from promoters located at the arrow origins. Cross-hatched areas represent the major transcription units active late in infection. Open arrows represent the major transcription units active before the onset of DNA replication. The major late promoter is active at early times as well as late times. The dashed line represents a continuation of transcription from the E2 promoter that occurs at low frequency. The transcription data are derived from Ziff (30).

The locations of restriction fragments used to analyze DNase I cleavage sites are represented by rectangles. HindIII fragments were inserted into the HindIII site of pBR322. The fragment designated Bam/RI extends from a BamHI site at 59.5 map units to an EcoRI site at 75.6 map units and is inserted into the BamHI and EcoRI sites of pBR322. The HpaI E fragment is inserted into the PstI site of pBR322 by means of GC linkers. The SmaI fragments were inserted into the EcoRI site of pBR322 by means of EcoRI linkers. The entire plasmid DNA was nick-translated and used as hybridization probe. HindIII and XhoI restriction maps for the Ad5 genome are shown below.

complete pattern of fragments remained too complex for precise analysis. The relative intensity of the bands appeared similar throughout a range of digestion conditions.

This analysis was continued using restriction fragment probes (Fig. 2) selected to encompass the entire viral genome in order to estimate the density and number of DNase I cleavage sites (Table 1). The number of bands detected by hybridization to probes from different regions of the genome varied considerably. It seems clear, however, that more bands, covering a broader size range, were detected by the hybridization of probes near the ends of the genome. Probes from the middle of the genome revealed a smaller number of bands and the bands that were detected were of higher molecular weight. This observation is consistent with the conclusion that the fragments observed

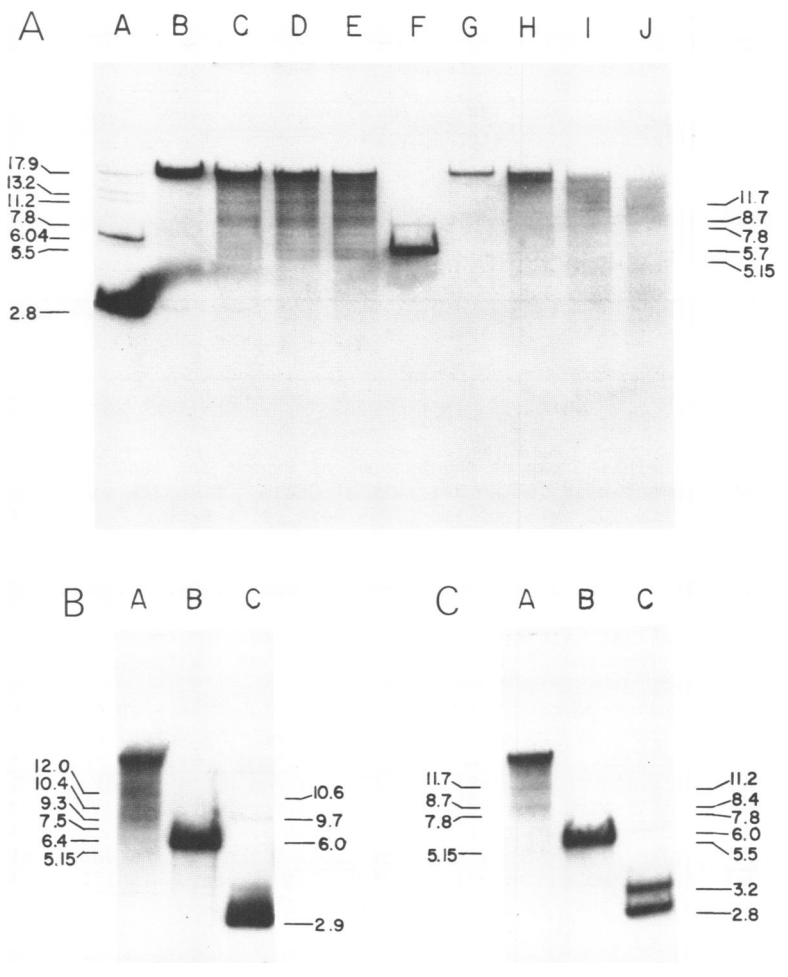

FIGURE 3. Products of DNase I digestion of viral cores and DNA hybridizing to cloned viral restriction fragments. Panel A displays the autoradiographic pattern obtained when the products of DNase I digestion are electrophoresed into an agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labeled plasmid DNA containing an insert of the Ad2 HpaI E fragment. Each lane contains 0.1 µg of DNA. Ad5 DNA digested with HindIII (lane A) or XhoI (lane F) was co-electrophoresed as marker. DNA from mock digested viral cores is shown in lane B. Cores were digested with 0.125 U/ml DNase I (lane C), 0.25 U/ml DNase I (lane D), or 0.5 U/ml DNase I (lane E) for 1 min at 37°C. DNA was digested with 0.025 U/ml DNase I (lane H), 0.05 U/ml DNase I (lane I), or 0.125 U DNase I (lane J). Mock digested DNA appears in lane G. Size markers are indicated on the left side of the panel and sizes of fragments produced from DNase I cleavage are indicated on the right.

Panel B displays the bands produced by DNase I cleavage of cores hybri-

dizing to the cloned HindIII F fragment in lane A. Size markers are in lanes B and C, with sizes indicated on the right side of the panel.

Lane A of panel C displays the bands produced by DNase I cleavage of cores hybridizing to the cloned SmaI F fragment. Fragment sizes produced by DNase I cleavage are indicated on the left of the panel. Lanes B and C contain size markers of sizes indicated on the right side of the panel. In each case, cores were digested with 0.2 U/ml of DNase I at a concentration of 40 µg/ml of DNA for 15 sec at 37°C.

under these digestion conditions each result from a single DNase I cleavage and one end of each fragment is coincident with the end of the genome. If several cleavages took place on each DNA molecule, small fragments homologous to probes from the center of the genome would have been seen.

DNase I cleavage sites in adenovirus cores

Assuming that the length of a fragment reflects the distance of the cleavage site from one end of the genome, the most prominent bands hybridizing to the HpaI E probe (Figure 3A, 4), indicate cleavage sites located at approximately 33, 24.5, 22, 16 and 14.7 map units. When the Ad5 SmaI F fragment, is used as hybridization probe the resulting bands should overlap with those seen using the HpaI E fragment. As shown in Figs. 3C and 4, similar fragment sizes were observed.

The cloned Ad5 HindIII F fragment was used as hybridization probe to analyze another set of sites (Figs. 3B and 4). The size of a fragment

TABLE 1. Survey of DNase I Cleavage Sites in Adenovirus type 5 Cores

Hybridization Probe	Location (map units)	Number of Bands	Size of Smallest Fragment	
			Map Units	Base Pairs
Ad2 HpaI E	0-4.5	~14	<9	<3200
Ad5 SmaI F	2.85-10.7	~14	<12	<4200
Ad2 HindIII C	7.9-17.0	9	>9	>3200
Ad5 SmaI A	18.5-35.4	2	>20.3	>7200
Ad5 HindIII C	17-31.5	2	>43.5	>15,000
Ad5 HindIII H	31.5-37.3	0	--	--
Ad5 BamHI/EcoRI	59.5-75.9	4	>22.7	>8000
Ad5 HindIII F	89.1-97.3	~14	<8	<2850

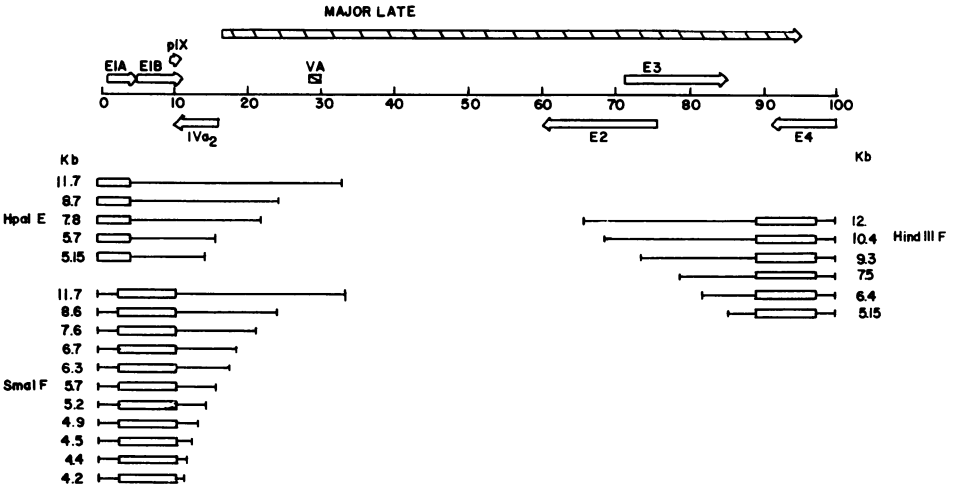

FIGURE 4. Summary of DNase cleavage sites in DNA of adenoviral cores. Cleavage sites were estimated by determining the fragment sizes of DNA in bands hybridizing to clone restriction fragment of the viral genome. Under the assumption that each fragment is the result of a single DNase I cleavage, one border of the fragment will coincide with with an end of the linear genome. The location of the hybridization probes, HpaI E, SmaI F, and HindIII F, are indicated by rectangles within the cleavage fragment representations. Some fragments are indicated in this figure that are not apparent in Fig. 3 because they were clearly observed in other experiments using modified electrophoresis conditions. By listing additional bands mapped using the Sma F probe, we do not mean to imply that these bands could not be detected using the Hpa I E probe (see Table 1). The resolution of some blots probed with Sma F simply allowed us to designate more cleavage sites with confidence.

hybridizing to this probe reflects the distance between the cleavage site and the righthand end of the genome. Fragment sizes of the most prominent bands of indicate cleavage sites near 66, 71, 74, 79, 82 and 85 map units.

Fragments that are not easily observed in the autoradiographs shown here were apparent in other experiments with different electrophoresis conditions and levels of exposure. The position of these additional fragments are indicated in Fig. 4, as well as those fragments apparent in the autoradiographs of Fig. 3. Many more bands were observed than the small number of prominent bands that we selected for cleavage site analysis. No precise conclusions can be drawn from analysis of these less prominent bands because of the low degree of resolution of such a large number of similarly prominent cleavage sites.

Comparison of DNase I cleavage patterns among viral cores, viral DNA, and intranuclear viral chromatin early and late after infection

It was of interest to compare the nuclease cleavage pattern of DNA in virion cores with that of DNA in nuclei at different stages in infection, because viral chromatin structure and transcriptional activity change throughout the course of infection (19, 30). Nuclei prepared from cells late after infection were treated with DNase I. At this stage in infection, viral DNA replication and late gene expression are occurring, and newly synthesized intranuclear viral DNA is associated with the core protein precursor, pVII (13). The resulting DNA fragments were analyzed as described above by hybridization to the HpaI E probe from the leftmost end of the genome. DNA purified from nuclei prepared late in infection was treated with S_1 nuclease to remove single-stranded replication intermediates that might have obscured DNase I cleavage patterns. Core and deproteinized DNA were similarly treated. No significant changes in DNase I cleavage patterns resulted from S_1 nuclease treatment. A slight apparent increase in electrophoretic mobility of S_1 nuclease treated samples from nuclei, compared to untreated samples, probably reflects lower viscosity and more rapid entry into the gel due to the removal of the single-stranded DNA component. Because of the very different accessibilities of DNA to DNase I in these different structures, the amount of DNase I used in the digestion of each type of preparation varied by more than an order of magnitude and the equivalence of extent of digestion can only be approximate.

Comparison of the electrophoretic patterns from experiments with cores, nuclei and DNA (Fig. 5) revealed complex patterns of fragment sizes and intensities unique to each preparation. Some bands were common to cores and DNA only, to cores and late viral chromatin only, to late viral chromatin and DNA only or common to all three. These results are schematically represented in Fig. 6. Over the range of digestion conditions used, very few bands appeared to be unique to just one of the cleavage substrates. In general, bands generated from cores were much stronger than the analogous bands from DNA. It is clear that specific cleavage was enhanced in viral cores when compared to deproteinized DNA.

In addition to differences between the substrates in sizes of cleavage products, they varied in the relative abundance of fragments resulting from cleavage at a particular site or within a particular region. For example, in the region of the genome containing the major late promoter, more cleavage seemed to occur in DNA of nuclei late in infection than in cores or DNA. In

FIGURE 5. Comparison of DNase I cleavage of adenoviral cores, DNA and intranuclear chromatin at early and late times after infection. DNA was electrophoresed into a 0.8% agarose gel, blotted and hybridized to the cloned HpaI E fragment. Cores were digested with 0.125 U/ml DNase I and electrophoresed before (lane B) or after (lane C) treatment with S_1 nuclease. Lane D contains DNA purified from mock digested nuclei. DNA from nuclei digested with 10 U/ml DNase I was electrophoresed before (lane F) or after S_1 nuclease treatment (lane E). DNA from mock-digested nuclei was treated with S_1 nuclease (lane G). Purified viral DNA was treated with 0.025 U/ml DNase I and S_1 nuclease (lane H), or 0.025 U/ml DNase I only (lane I). HindIII digested Ad5 DNA was electrophoresed in lane A and XhoI digested Ad5 DNA was electrophoresed in lane J as markers. All DNase I digestion were carried out at 37°C for 1 min. S_1 nuclease treatment was described under Materials and Methods.

late viral DNA (lanes E and F of Fig. 5), intense hybridization was observed to fragments ranging in size from 7.8 to 5.7 kilobases which represent cleavage between 22 and 16 map units. Although the bands were more prominent in late viral chromatin, less intense analogs to these bands were observed in digestion products of viral cores and deproteinized DNA as well. Thus, no particular cleavage sites appeared to be unique to this region of late chromatin.

DNase I digestions were also performed on nuclei prepared from infected cells at 3 hr post-infection. Since no viral DNA replication would have occurred this early after infection, the DNA detected in this experiment was derived from the parental innoculum. The viral nucleoprotein in nuclei early

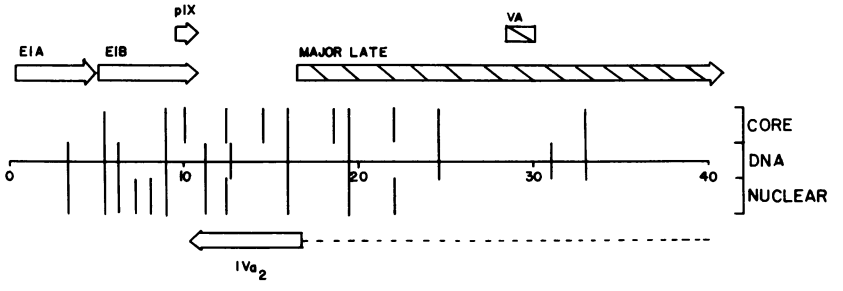

FIGURE 6. Schematic representation of DNase I cleavage sites of viral cores, DNA and intranuclear viral chromatin. Vertical lines represent DNAse I cleavage sites estimated from the sizes of fragments hybridized to ^{32}P-labeled HpaI E DNA. The data were derived from the Southern blot shown in Fig. 5. A cleavage site is indicated at 22 map units in late viral chromatin because it appeared prominently in DNA from nuclei treated with 2 U/ml of DNase I (data not shown) although it was not apparent in the sample treated with 10 U/ml DNase I that is shown in Fig. 5.

after infection differs from purified viral cores in that the genome has become transcriptionally active (30) and changes in sensitivity to micrococcal nuclease have occurred (19, 31). The DNA from this preparation was compared to the products of viral core digestion by hybridization to the Sma F fragment. Similar fragment sizes were observed although the intensities varied. The intensities of bands in intranuclear chromatin early in infection appeared more homogeneous than bands from digested viral cores, and individual bands are thus less prominent (data not shown).

Detailed analysis of DNase I cleavage in the vicinity of the major late promoter

To obtain better resolution of cleavage sites, the region of the genome encompassing the adenoviral late promoter was selected for more detailed analysis. Following DNase I digestion of DNA or nucleoprotein, the DNA was purified and digested to completion with either HindIII or XhoI. Blots were hybridized with a cloned fragment extending from the XhoI site at 15.5 map units to the HindIII site at 17.0 map units (Fig. 2). Sites located between the HindIII sites at 7.9 and 17.0 map units could be detected by hybridization to the HindIII digested DNA. Similarly, hybridization to the XhoI digested DNA allowed the identification of sites within the XhoI fragment spanning 15.5 and 22 map units.

The use of this fragment to probe HindIII digested DNA from DNase I treated cores generated the hybridization patterns shown in Fig. 7. Clusters of cleavage sites might have contributed to the observation of a single site

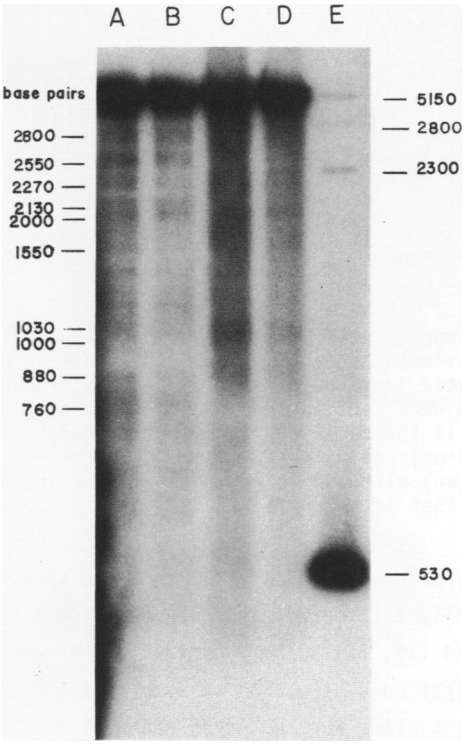

FIGURE 7. Fine mapping of DNase I cleavage near the adenoviral late promoter. This blot displays the hybridization pattern of a probe spanning 5.5 to 17 map units to HindIII digested DNAs from DNase I digested viral cores and DNA. Lanes A and B contain 0.2 μg each of DNA from cores digested at a DNase I concentration of 0.25 U/ml (lane A) or 0.125 U/ml (lane B). Lanes C and D contain 0.2 μg each of deproteinized DNA digested at a DNase I concentration of 0.025 U/ml (lane C) or 0.0125 U/ml (lane D). Lane E contains 0.04 μg of Ad5 DNA digested with both HindIII and XhoI. All DNase I digestions were carried out at a DNA concentration of 20 μg/ml for 1 min at 37°C. DNA was electrophoresed for 14 hr into a 1.2% agarose horizontal gel (23 x 16 cm) in TBE buffer at 4°C.

during gross mapping. If this was the case, the cleavage sites inferred rom large and small scale mapping were consistent with an accuracy of approximately 0.5 map units. The data from the higher resolution and low resolution mapping are represented schematically in Fig. 8. The pattern of fragments obtained from nuclear viral chromatin examined late in infection were not significantly different (data not shown). This is consistent with our failure to observe cleavage sites unique to any of the three substrates during gross mapping experiments. Regional differences in cleavage frequency would not be

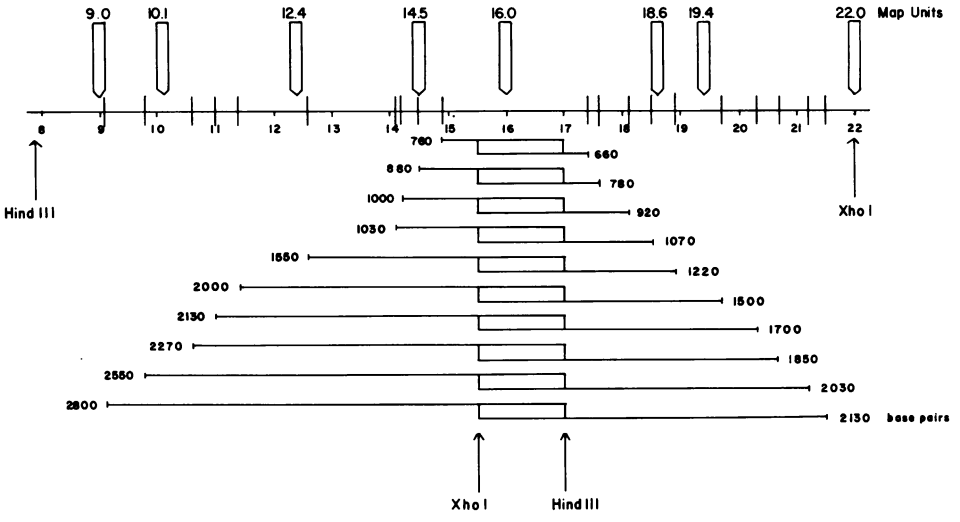

FIGURE 8. Schematic representation of DNase I cleavage sites near the adenoviral late promoter. Broad arrows represent cleavage sites inferred from results of low resolution mapping with HpaI E and SmaI F fragments. Vertical lines indicate cleavage sites inferred from sizes of fragments hybridizing to cloned sequences extending from 15.5 to 17.0 map units. Fragment sizes were determined from the Southern blots shown in Fig. 7. The rectangles reflect the sequences homologous to the hybridization probe.

apparent when analysis is confined to a small region.

Ten fragments were observed in the region corresponding to the XhoI E fragment spanning 15.5 to 22.0 map units. These fragments had molecular weights between 660 base pairs and 2130 base pairs (Fig. 8). A fragment produced by cleavage at 16 map units would extend from the XhoI site at 15.5 map units to 16 map units and would therefore be only 177 base pairs long and too small to detect. The fragment of 2130 base pairs indicates cleavage 6 map units to the right of 15.5, at 21.5 map units, which is consistent with the observation of a cleavage site at 22 map units in experiments using the HpaI E and SmaI F hybridization probes (Figs. 3 and 4). A fragment larger than 2130 base pairs would not have been resolved from the XhoI E fragment itself so it was not possible to determine whether the site inferred from the fine mapping experiment is the only site near 22 map units. Observation of five prominent but broad bands between 900 and 1700 base pairs was consistent with cleavage sites at 18.6, 18.9 and 19.4 map units.

DISCUSSION

These studies show that double-stranded cleavage of adenoviral core DNA

by DNase I occurs at many locations on the viral genome. Limited digestion of purified adenoviral cores produced multiple bands upon electrophoresis of digestion products into non-denaturing agarose gels, visualized by ethidium bromide fluorescence. At least 28 bands could be detected through the use of hybridization probes containing sequences derived from the ends of the genome. Over twice as many bands in the vicinity of the major later promoter were detectable through fine-scale mapping as were detected in this region during a low resolution survey of the whole genome. Furthermore, many bands could have escaped detection because they overlapped with the hybridization probe only partially or were too large to be resolved from genome-sized DNA. These observations suggest that double-stranded cleavage by DNase I may occur at frequencies of greater than one site per kilobase pair throughout the adenovirus genome.

Not all sites were conclusively localized in relation to more than one reference point. However, the following data support the notion that each band detected with hybridization probes resulted from a single DNase I cleavage event that defines one end of the fragment, the other end of the fragment being the end of the genome. Firstly, these experiments were carried out at very low levels of DNase I digestion. Secondly, hybridization to sequences derived from locations throughout the genome resulted in labeling of bands of the sizes expected if the fragments extended from the probe to one end of the genome. It seems certain that these fragments resulted from double-stranded DNase I cleavage because they were detected under non-denaturing conditions. Furthermore, no significant changes in fragment sizes followed S_1-nuclease treatment. Therefore, the termini probably do not consist of extensive single-stranded regions, although they may not be blunt-ended.

We found no striking correlations between DNase I cleavage sites and known regulatory loci in the viral genome. Cleavage sites were detected in introns and exons, near promoters and near the ends of transcription units. Spacing between sites appeared regular in some regions and random in others. Regions that appeared devoid of cleavage sites in these studies probably resulted from the resolution and size constraints inherent in the hybridization probes that were used rather than an absence of cleavage. It is therefore difficult to draw conclusions regarding the biological significance of sensitivity of these sites to double stranded cleavage by DNase I.

Variation in intensity of bands was observed, suggesting that sites differed in susceptibility to DNase I cleavage. DNase I also produced double-

stranded cleavages in purified DNA resulting in the visualization of multiple bands in non-denaturing gels through ethidium bromide fluorescence. The affinity of DNase I for particular sequences has not been well characterized at low levels of digestion, although some sequence specificity is apparent from analyses of cleavage products following exhaustive digestion of calf thymus or E. coli DNA (32-33).

Much less nuclease was required to achieve an equivalent extent of digestion when digestion of DNA was compared to digestion of viral cores. Bands produced from DNA generally appeared against a higher background and were less intense when compared to the same amount of DNA from digested cores. It therefore seems likely that the proteins associated with viral DNA reduced accessibility of DNA to nuclease and enhanced the specificity of cleavage.

A complex pattern of double-stranded cleavage occurred in viral DNA when nuclei prepared at early and late times of digestion were treated with DNase I. In this case, a concentration of DNase I more than 10-fold greater than that used in core digestions and more than 100-fold greater than that used in DNA digestions was required to achieve an equivalent extent of digestion. Each substrate produced a unique pattern of fragment sizes and fragment intensities, although few fragments appeared in just one of the substrates examined.

This is the first analysis reported of DNase I sensitive sites in chromatin which differ from cellular (nucleosomal) chromatin. Adenoviral cores contain no histones or high mobility group proteins. Instead, the viral DNA is associated with virally encoded proteins. It appears that the distribution of such sites in adenoviral DNA is different from those which have been characterized in cellular chromatin. Specific double-stranded cleavage seems to occur much more frequently in viral DNA and no obvious correlations can be made between locations of viral regulatory loci and DNase I cleavage sites.

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