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The Terminal Regions of Adenovirus and Minute Virus of Mice DNAs Are Preferentially Associated with the Nuclear Matrix in Infected Cells

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The interaction of viral genomes with the cellular nuclear matrix was studied by using adenovirus-infected HeLa cells and minute virus of mice (MVM)-infected A-9 cells. Adenovirus DNA was associated with the nuclear matrix both early and late in infection, the tightest interaction being with DNA fragments that contain the covalently bound 5′-terminal protein. Replicative forms of MVM DNA were also found to be exclusively matrix associated during the first 16 to 20 h of infection; at later times viral DNA species accumulated in the soluble nuclear fraction at different rates, suggesting a saturation of nuclear matrix-binding sites. MVM DNA fragments enriched in the matrix fraction were also derived from the terminal regions of the viral genome. However, only the subset of fragments which possess a covalently bound 5′-terminal protein (i.e., DNA fragments in which the 5′ palindromic DNA sequences are in the extended duplex rather than the hairpin conformation) were matrix associated. These observations suggest that the DNA-matrix interactions are, at least in part, mediated by the viral terminal proteins. Since these proteins have previously been shown to be intimately involved in viral DNA replication, our results further indicate that an association with the nuclear matrix may be important for viral genome replication and possibly also for efficient gene transcription.

Both the *Adenoviridae* and autonomous *Parvoviridae* contain DNA genomes with proteins covalently bound to the 5′ termini of both plus and minus DNA strands that play pivotal roles in the initiation of viral DNA replication (3, 52; for recent reviews, see references 27 and 65). Minute virus of mice (MVM), an autonomous parvovirus, encapsidates only the minus strand of DNA within the mature virion, in a form which is usually devoid of the terminal protein (TP) (3, 16). In addition, the MVM-TP is bound only to extended conformers of the palindromic sequences at the ends of the linear MVM replicative-form (RF) DNA duplexes and not to RF DNA molecules in which the terminal palindromic sequences are in the hairpin conformation (3, 16). In contrast, the adenovirus TP is present on virion DNA molecules and on all RF DNA species (52, 53).

The nuclear matrix is a proteinaceous nuclear substructure which can be isolated by sequential treatment of nuclei with nonionic detergents, nucleases, and high-salt concentrations (4, 19, 22, 33, 43) or by a low-salt (LIS) extraction procedure (17, 40). The nuclear matrix has been increasingly implicated in dynamic processes, such as chromatin organization, DNA replication, gene transcription, or RNA processing (for recent reviews, see references 4, 8, 18, 29, 30, 32, and 42). Specific DNA sequences within defined cellular genes have also been identified (19, 40, 46, 59) which exhibit highly specific interactions with protein components of the nuclear matrix, suggesting that such interactions may delineate both structural and functional DNA domains (8, 17, 29, 40, 59). The concept that the basic subunit for eucaryotic cell DNA replication is a DNA domain (or loop) with an average size of 50 kilobase pairs (kbp) has been widely accepted, and several models of DNA replication which invoke both stable and dynamic interactions with the nuclear matrix have been proposed elsewhere (8). Such models postulate that sequences constituting cellular DNA replication origins are preferentially associated with the nuclear matrix, where many of the cellular DNA replication enzymes appear to be sequestered (1, 22, 33, 38, 43). Although cell replication origins appear to be stably associated with the nuclear matrix (1, 13, 23, 50), the identification of functional cellular replication origins has proven extremely difficult, and this hypothesis has not been directly tested experimentally. In contrast, numerous viral DNA replication origins have been well characterized. If viral DNA replication mimics DNA replication of the host cell with regard to nuclear compartmentalization, one would predict that regions of the viral genome that function as replication origins would be selectively enriched in the nuclear matrix fraction. Although previous studies had demonstrated that adenovirus and herpes simplex virus DNAs are matrix associated throughout the cycle of lytic infection in HeLa cells (7, 60, 67), as are several important early viral polypeptides, such as the adenovirus E1A proteins (14, 26, 56) and the herpes simplex virus ICP4, ICP5, and ICP8 proteins (5, 7, 47, 49), no selective enrichment of origin DNA fragments was reported. Here we have studied the interaction between the nuclear matrix and adenovirus DNA or MVM DNA as a function of time postinfection (PI). Infected-cell nuclei were treated in situ with restriction enzymes, nucleoplasm and nuclear matrix fractions were prepared, and the viral DNA fragments in each fraction were analyzed. Our results, which show that terminal DNA fragments containing the viral replication origin sequences are highly enriched in the matrix fraction, suggest that such an association is important for viral DNA replication and that the interactions between matrix and viral DNA are mediated in part by the covalently bound TPs.

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MATERIALS AND METHODS

Cell culture and virus infection. MVM(p), the prototype strain of MVM, was grown on the A-9 variant of mouse L cells (61). Cell cultures were maintained and virus stocks were prepared and purified as described previously (61). Cells were parasynchronized by a single thymidine block by the method of Thilly (62), washed, infected with 20 to 40 PFU of virus per cell, and suspended in spinner medium.

HeLa S3 cells were grown and infected with adenovirus type 2 (Ad2), at a multiplicity of infection of 10 to 20 PFU per cell, as previously described (45).

Infected HeLa and A-9 cells were harvested at various times PI and analyzed as described below.

Cell fractionation and preparation of nuclear matrices. The preparation of nuclear matrix fractions from infected HeLa and A-9 cells was done as described by Razin et al. (51) and modified by Bodnar et al. (9). Briefly, cells were suspended at a concentration of 3×10^6 cells per ml in buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol [vol/vol]), and lysed by the addition of 0.5% Nonidet P-40 and 0.1% Triton X-100. After 15 to 30 min on ice, nuclei were collected by low-speed centrifugation and the cytoplasmic fractions were saved for analysis. The nuclei were then suspended in buffer A at 10^7 cells per ml and digested with endonucleases. For digestion with micrococcal nuclease (Sigma Chemical Co.), the nuclei were adjusted to 10 mM $CaCl_2$ and incubated with the enzyme (2 U/ml) for 15 min at 37°C. For restriction enzyme digestion, the nuclei were first diluted 1:2.5 with water to reduce the overall salt concentration, 1/10th volume of a 10× buffer for the appropriate restriction enzyme was added, and samples were incubated for 2 h at 37°C; restriction enzyme concentrations varied from 0.05 to 50 U/μg of DNA as described in Results. The nuclei were again pelleted, and the supernatants were saved. The nuclei were suspended at 3×10^6 cells per ml in buffer B (100 mM NaCl, 20 mM Tris hydrochloride [pH 9.0], 20 mM EDTA, 0.2% Nonidet P-40, 10% glycerol [vol/vol]) and adjusted to 2 M NaCl by the addition of 5 M NaCl. Alternatively, nuclei were adjusted to 2 M NaCl immediately after restriction enzyme digestion by the addition of 5 M NaCl. After incubation on ice for 1 h, nuclear matrix fractions were pelleted by low-speed centrifugation and both the supernatants and pellets were kept for further analysis.

Analysis of Ad2 and MVM DNA. DNA was isolated by sodium dodecyl sulfate-pronase treatment, followed by phenol extraction and ethanol precipitation as previously described (45). DNA samples were separated on agarose gels, transferred to nitrocellulose, and probed with cloned MVM(p) RF DNA or Ad2 DNA as described by Merchlinsky et al. (2, 39). Adenovirus DNA was also labeled in vivo with [^{3}H]thymidine under conditions that preferentially label viral DNA, as previously described by Bodnar and Pearson (10). Fluorography of ^{3}H-labeled DNA was accomplished by soaking gels for 1 h in 1 M sodium salicylate before drying the gels for autoradiography.

RESULTS

Digestion of viral DNAs in isolated nuclei with restriction endonucleases. Our general approach for analyzing viral DNA interactions with the nuclear matrix was to digest adenovirus-infected HeLa cell nuclei or MVM-infected A-9 cell nuclei with restriction enzymes in situ, extract the nuclei with 2 M NaCl, and then determine the distribution of viral DNA fragments in the solubilized and nuclear matrix fractions. This experimental approach requires that the viral DNA be digested to completion by the enzyme. We, therefore, first titrated the amount of restriction enzyme necessary to obtain complete digestion. HeLa cells were labeled overnight with [^{3}H]thymidine, spun out of [^{3}H]thymidine-containing medium, and infected with Ad2. The cells were harvested at 18 h PI, and nuclei were prepared by lysis in buffer A containing 0.5% Nonidet P-40 and 0.1% Triton X-100 (see Materials and Methods). Various concentrations of restriction enzyme were then added to aliquots of the nuclei and incubated at 37°C for 2 h. Total DNA was prepared by sodium dodecyl sulfate-pronase treatment, followed by phenol extraction and ethanol precipitation. The DNAs were then separated on 1% agarose gels; the gels were treated with 1 M sodium salicylate, dried, and developed by autoradiography.

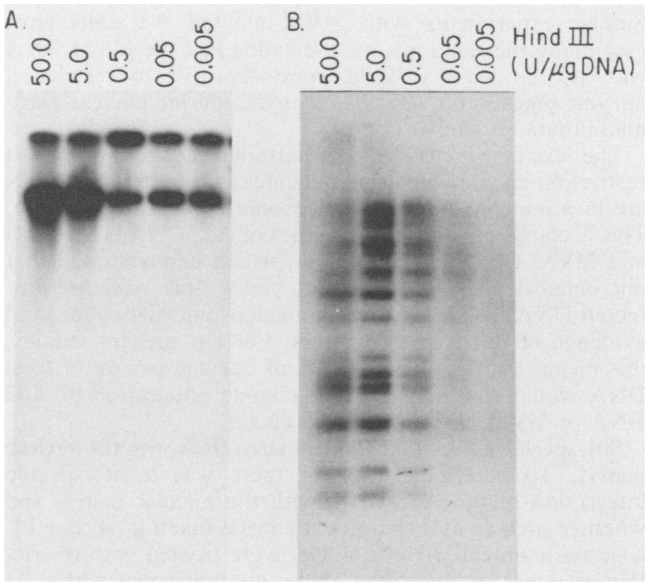

FIG. 1. In situ restriction enzyme digestion of cellular and Ad2 DNA in infected HeLa nuclei. Cells, prelabeled with [^{3}H]thymidine, were harvested 18 h PI, and nuclei were isolated as described in Materials and Methods. The nuclei were incubated with the indicated concentrations of the restriction enzyme *Hin*dIII for 2 h at 37°C. Whole-cell DNA was prepared by sodium dodecyl sulfate-pronase treatment, followed by phenol extraction and ethanol precipitation. The DNA was then fractionated by electrophoresis with a 1% agarose gel. (A) Size distribution of the HeLa cell DNA, detected by sodium salicylate-enhanced fluorography; (B) adenovirus DNA *Hin*dIII fragments, detected by Southern blotting using ^{32}P-labeled Ad2 virion DNA as the probe.

Figure 1 shows an experiment in which *Hin*dIII was used to digest the adenovirus-infected nuclei in situ. Even the sample with the highest concentration of *Hin*dIII showed very little cleavage of the cellular DNA, with the average fragment size remaining at least 20 kbp (Fig. 1A). When an identical gel was transferred to nitrocellulose and probed with nick-translated Ad2 virion DNA, it can be seen that in sharp contrast to the cellular DNA, the intranuclear Ad2 DNA was completely digested by *Hin*dIII at much lower enzyme concentrations (Fig. 1B). Indeed, the Ad2 DNA was over a 1,000 times more sensitive to digestion in situ than bulk chromatin. This hypersensitivity of intracellular Ad2 DNA to restriction enzymes was evident at 14, 18, or 22 h PI and with all restriction enzymes tested (data not shown).

Similar experiments with MVM-infected A-9 cells gave essentially the same results, indicating that the MVM DNA was also at least 100-fold more sensitive to restriction enzyme digestion in situ than was the murine nuclear chromatin (data not shown).

The extreme sensitivity of intranuclear viral DNA to restriction enzyme cleavage indicates that both viral DNAs are in a nonconventional nucleosome chromatin structure. This is consistent with prior studies of Ad2 DNA (12, 20, 21) and MVM DNA (24) chromatin, which demonstrated that micrococcal nuclease treatment yields only nuclease-protected DNA fragments of mononucleosome size, with little evidence of nucleosome ladders. For our present studies, this meant that as little as 10 U of enzyme per μg of total DNA would routinely achieve complete digestion of Ad2 DNA or MVM DNA in isolated nuclei.

Site-specific association of adenovirus DNA with the nuclear matrix. To determine whether there was a site-specific interaction of the Ad2 DNA with the nuclear matrix and whether such an association varied as a function of time PI, adenovirus-infected HeLa nuclei were treated with restriction enzymes as described above and extracted with 2 M NaCl, and the solubilized and nuclear matrix-associated DNAs were analyzed. If the Ad2 DNA were associated with the nuclear matrix at specific sites, only those restriction fragments which were nuclear matrix bound would be expected to be enriched in the nuclear matrix fraction. If viral DNA-matrix interactions were random or nonexistent, one would expect to see no preferential enrichment of any DNA fragment. The 14, 18, and 22 h time points PI were chosen for the following reasons. (i) Fourteen hours is still early in the infection cycle but is several hours after viral DNA replication has begun. (ii) At 18 h the Ad2 DNA saturates the cellular replication machinery and DNA replication changes from a logarithmic to a linear phase (10). (iii) Twenty-two hours is late in infection but is before significant viral packaging. It should be noted, however, that packaged Ad2 DNA would not interfere with our analysis, since it is not susceptible to restriction enzyme cleavage in situ (data not shown).

Experimental conditions whereby intranuclear Ad2 DNA could be radiolabeled without significant labeling of cellular DNA have been established previously (10). It was therefore possible to analyze Ad2 DNA forms by fluorography without interference from cellular DNA and without needing to resort to Southern blot analysis. Cells were infected with Ad2 virions and [^{3}H]thymidine (0.5 μCi/ml) was added at 11 h PI, by which time cellular DNA synthesis is shut off. The cells were harvested at 14, 18, or 22 h PI, and nuclei were isolated as described above. The nuclei were digested with *Hin*dIII, with *Sma*I, or with *Eco*RI and *Bam*HI, and then extracted with 2 M NaCl on ice for 45 to 60 min. The nuclear matrix fractions were collected by low-speed centrifugation, extracted a second time with 2 M NaCl, repelleted by low-speed centrifugation, extracted a second time with 2 M NaCl, and collected again by low-speed centrifugation. Total cellular DNA was purified as described above and separated on agarose gels. Figures 2 and 3 show experiments with *Hin*dIII, *Sma*I, and *Eco*RI-plus-*Bam*HI digestions in situ after the gels were developed by fluorography; under the labeling conditions used, over 90% of all radioactivity exposed on these gels should represent Ad2 DNA (10). The nuclear matrix-associated and soluble DNAs isolated from nuclei digested in situ with *Hin*dIII and *Eco*RI-*Bam*HI at 14 h PI and with *Sma*I at 22 h PI are compared in Fig. 2. In all three cases, the internal Ad2 DNA fragments were solubilized according to their size, with the largest fragments associating more with the nuclear matrix. However, the terminal fragments were mainly matrix associated regardless of their size. In the *Hin*dIII digest (Fig. 2A), the termini are the G and K fragments, and these were both enriched in the matrix fraction in relation to internal fragments (e.g., H, I, and J) of about the same size. Although the left-end *Hin*dIII G fragment could not be resolved from the internal F fragment, the intensity of the bands was consistent with the G band being matrix associated and the F band being solubilized. This interpretation was confirmed in the *Eco*RI-*Bam*HI double digestion (Fig. 2A, lanes 3 and 4) in which the left-end E fragment was clearly matrix associated, as was the larger right-end A fragment. Again, note that the small internal fragments F, G, and H are in the supernatant fraction. In the *Sma*I digestion (Fig. 2B), the termini are the J and K fragments, which are the two smallest fragments, both being less than 1 kb long; the enrichment of these bands in the nuclear matrix fraction indicated that, unlike the internal fragments, the matrix association of the termini was independent of fragment size.

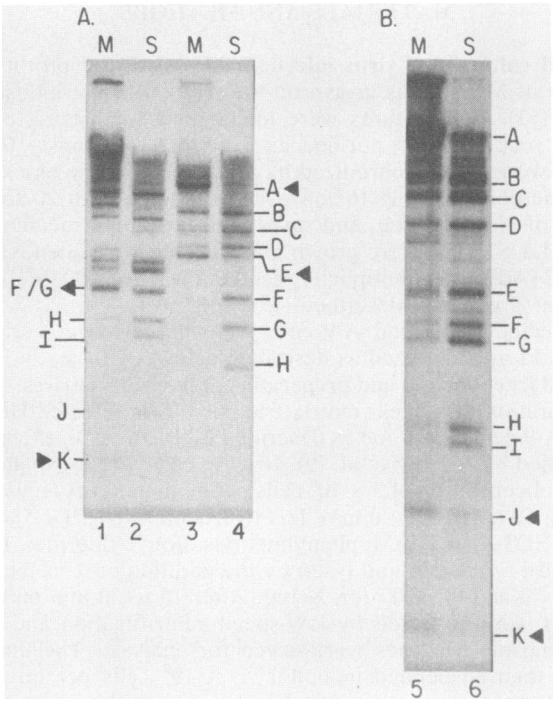

FIG. 2. Association of Ad2 DNA fragments with the nuclear matrix. HeLa cells were infected with Ad2, and [^{3}H]thymidine was added at 11 h PI to label the Ad2 DNA specifically. The cells were harvested at 14 (A) or 22 h PI (B), and the nuclei were isolated and digested in situ with the restriction enzyme(s) *Hin*dIII (lanes 1 and 2), *Eco*RI plus *Bam*HI (lanes 3 and 4), or *Sma*I (lanes 5 and 6) (10 U/μg of total DNA for 2 h). The nuclei were then extracted twice for 1 h on ice with 2 M NaCl. DNA remaining bound to the nuclear matrix fraction (M) and DNA solubilized in the salt extraction (S) were purified and separated on 1% agarose gels, and the Ad2 DNA fragments were detected by fluorography. The Ad2 DNA terminal fragments are indicated (▶).

A time course experiment of the Ad2 DNA-nuclear matrix association indicated that Ad2 terminal fragments were selectively enriched in the nuclear matrix fraction throughout infection. This was evident for both the *Hin*dIII (Fig. 3A) and *Sma*I (Fig. 3B) digestions in situ. Although the extent of

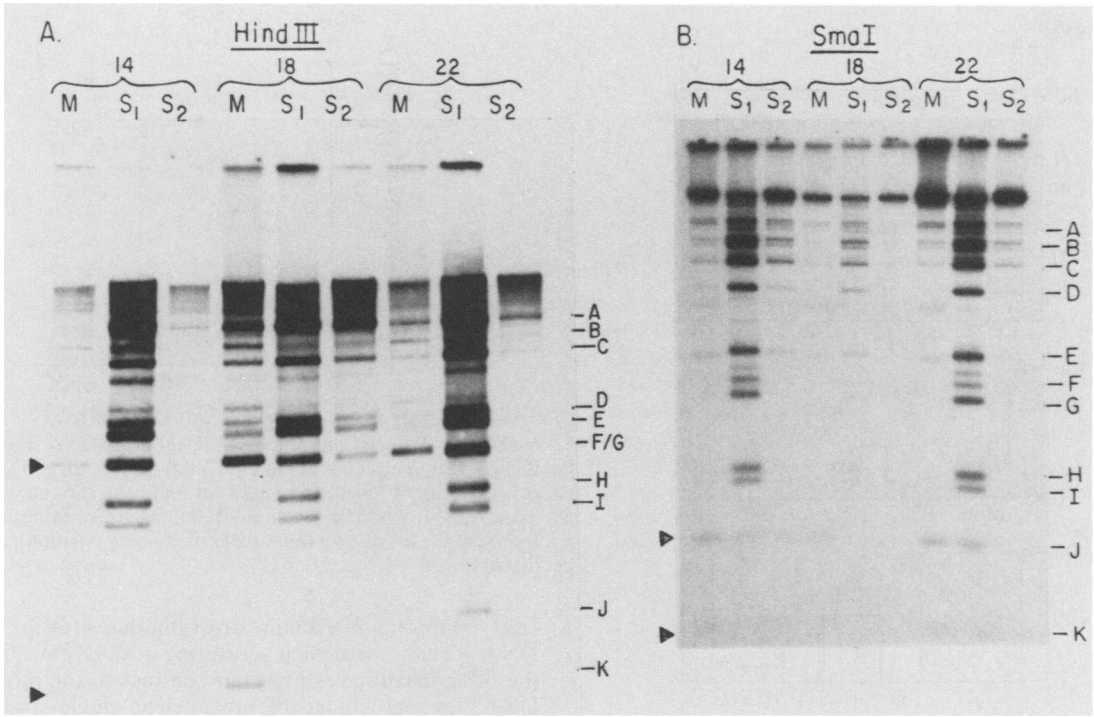

FIG. 3. Association of Ad2 DNA *Hin*dIII and *Sma*I fragments with the nuclear matrix as a function of time PI. Nuclei were prepared from Ad2-infected HeLa cells at 14, 18, and 22 h PI and digested in situ with *Hin*dIII (A) or *Sma*I (B) (10 U/μg of total DNA for 2 h). Nuclei were then extracted twice for 1 h on ice with 2 M NaCl, and the DNA remaining bound to the nuclear matrix fraction (M) and DNA solubilized in the first (S_1) and second (S_2) salt extractions were analyzed further, as described in the legend to Fig. 2. The Ad2 DNA terminal fragments are indicated (▶).

matrix enrichment varied within each time course experiment, the termini were clearly enriched compared with internal fragments of the same size. To better quantitate this enrichment, densitometry was performed on data obtained from several independent experiments to determine the relative distribution of each restriction fragment in the nucleoplasm and nuclear matrix fractions (Fig. 4). In all cases, the terminal fragments were preferentially enriched in the nuclear matrix, while the majority of the internal fragments were solubilized by the 2 M NaCl washes. By using several different enzymes, it was possible to map the specific interaction sites to the ends of the Ad2 DNA quite unambiguously. For example, when *Sma*I was used, the predominant fragments retained in the nuclear matrix were the *Sma*I J and K fragments (900 and 810 bp, respectively), which correspond to the right and left ends of the genome, respectively.

Mirkovitch et al. (40) and Small et al. (59) performed similar extractions with *Drosophila* nuclei after restriction enzyme treatment and reported that the retention of restriction fragments in the nuclear matrix seems to depend on the size of the fragment—the larger the fragment, the greater the percentage associated with the matrix fraction. These observations could be interpreted in several ways. For example, large DNA fragments could possess multiple matrix-binding sites, thus increasing the stability of the matrix-DNA interaction. Alternatively, large DNA fragments could exhibit a higher degree of nonspecific binding to matrix components and therefore reflect DNA contamination. The selective enrichment of Ad2 DNA terminal fragments of Ad2 DNA did not show this size dependency. For example, the right-end fragment was the largest fragment in the *Hin*dIII digests and the second to smallest in the *Sma*I digests (compare Fig. 2 and 3), yet both were highly enriched (>80%) in the nuclear matrix fraction (Fig. 4). The enrichment of terminal DNA fragments was consistently observed in multiple experiments; there was no apparent difference in the amount of enrichment at any time PI nor any apparent saturation of matrix attachment sites. In contrast, the matrix-associated percentage of internal Ad2 DNA fragments, although always lower than that of the terminal fragments, showed a nearly linear relationship between percent matrix bound and fragment size (Fig. 5). It is unclear, however, whether this observation reflects specific or nonspecific DNA-matrix interactions.

The data presented here indicate that Ad2 DNA is preferentially bound to the nuclear matrix at or near both of its molecular termini and that this interaction applies to all DNA species synthesized between 14 and 22 h PI.

Intranuclear distribution of MVM DNA species. The interaction of MVM DNA with the nuclear matrix had not been studied previously, in contrast to adenovirus DNA (60, 67) or herpes simplex virus DNA (5, 7); thus the intranuclear distribution of intact MVM RF DNA species was analyzed first. Mouse fibroblasts (A-9 cells) were grown in suspension and partially synchronized by blocking once with 0.2 mM thymidine (62). The cells were released from the block and infected with MVM at a multiplicity of infection of 20 to 50 PFU per cell. Cells were harvested at various times, ranging from 8 to 40 h PI, and the intranuclear distribution of the MVM DNA was analyzed after in situ restriction enzyme digestion. Since the MVM genome does not contain *Bam*HI restriction sites, infected-cell nuclei were incubated with this enzyme before fractionation to reduce the viscosity of nuclear matrix samples. The cytoplasmic, nuclear supernatant,

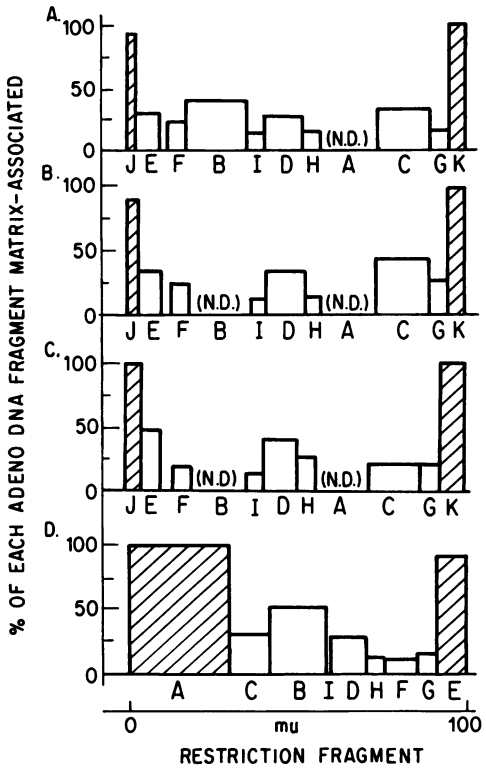

FIG. 4. Association of Ad2 (adeno) DNA fragments with the nuclear matrix. Densitometric analysis was performed on the fluorograms of Ad2 DNA fragments separated by gel electrophoresis after fractionation into matrix and supernatant subsets. Panels A, B, and C show the fraction of each *Sma*I restriction fragment that was matrix associated at 14, 18, and 22 h PI, respectively. In each case, the data represent the average of four independent experiments. Panel D shows the relative fraction of each *Bam*HI-*Eco*RI double-digest DNA fragment that is matrix associated at 22 h PI. Data represent average of two independent experiments. N.D., Not determined.

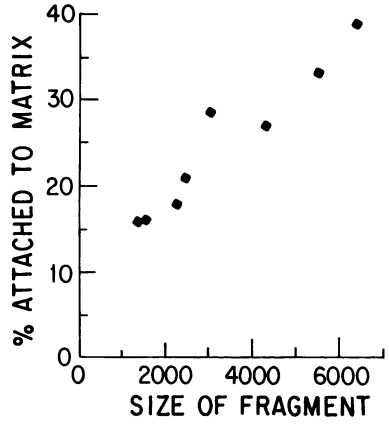

FIG. 5. Association of internal adenovirus DNA fragments with the nuclear matrix as a function of fragment size. Densitometry results (Fig. 4) were replotted to indicate the nuclear matrix-associated fraction of each nonterminal Ad2 restriction enzyme fragment versus its size (regardless of genome position). See text for discussion.

high-salt washes, and nuclear matrix pellets were then analyzed for the presence of viral DNA by Southern blotting. Although some of the single-stranded viral DNA was found in the cytoplasmic fraction, particularly at later times in the infection (data not shown), all of the other RF DNA species were predominantly localized within the nucleus. The distribution of MVM DNA in the nuclear matrix and nucleoplasmic fractions as a function of time PI is shown in Fig. 6 and 7.

Several general conclusions can be made from this experiment. First, essentially 100% of the monomer RF DNA is matrix associated during the first 18 to 20 h PI. Although the total pool of this DNA increases at later times in the infection, the amount of monomer RF DNA matrix bound remains relatively constant, indicating that the DNA-binding sites on the matrix have become saturated. By analyzing the intensity of the experimental hybridization signals relative to those observed with known viral DNA concentration standards, we estimate that there are ~5,000 monomer RF DNA-binding sites per infected nucleus. Second, both single-stranded viral DNA and dimer RF DNA are also exclusively matrix associated during the early stages of infection (~8 to 12 h PI). Again, both DNA species give matrix binding profiles which reach saturation points between 12 to 16 and 32 to 36 h PI, respectively. Thus, MVM-infected A-9 cells exhibit a limited number of binding sites for each viral DNA species, with each saturating at different times during the infection. Interestingly, in contrast to the monomer RF DNA, the percentage of the nuclear single-stranded viral DNA that is matrix bound remains fairly constant (at ~20%) after the saturation point is reached; the rest of this DNA species accumulates in the cytoplasm. Finally, a minor viral DNA component, termed the 8-kb DNA (25), which occurs here late in infection (36 to 40 h PI) shows very little, if any, association with the nuclear matrix fraction.

Collectively, the observations described above provide strong evidence that the MVM DNA-nuclear matrix interactions do not result from nonspecific binding, DNA aggregation, or coprecipitation during the high-salt extraction step used to prepare the nuclear matrix fraction. To further characterize the nature of these matrix-DNA interactions, infected A-9 cell nuclei were treated in situ with *Hae*III, *Eco*RI, or *Pst*I, enzymes which cut duplex MVM DNA at two or more sites. Nuclear matrix and nucleoplasmic fractions were prepared as described above, and the relative distribution of viral DNA fragments in each fraction was assessed by Southern blotting. The results of such an analysis after *Eco*RI digestion are shown in Fig. 8. MVM RF DNA is cut by this enzyme at map coordinates 20.9 and 69.9, generating an internal fragment (fragment A) of 2.5 kb and two end fragments B (1.5 kb) and C (1.1. kb), each of which exhibit two conformations termed t (turnaround) and e (extended); only the e conformer contains the covalently bound TP (3, 15). At all time points examined, ranging from 12 to 40 h PI, the vast majority, if not all, of the internal A fragment was in the nucleoplasmic fraction. In contrast, at 12 and 16 h PI, essentially all of the B and C fragments were found in the nuclear matrix fraction. By 20 h PI, both of these terminal fragments, predominantly in the e conformation, began to partition into the nucleoplasm in a fashion similar to that seen during the analysis of intact RF DNA species. The analysis of MVM DNA fragments generated by *Hae*III or *Pst*I digestion of infected-cell nuclei leads to the same conclusion, i.e., only terminal DNA fragments in the extended conformation are matrix associated (data not shown).

The majority of the unrestricted single-stranded viral DNA is also in the soluble fraction as observed earlier in this

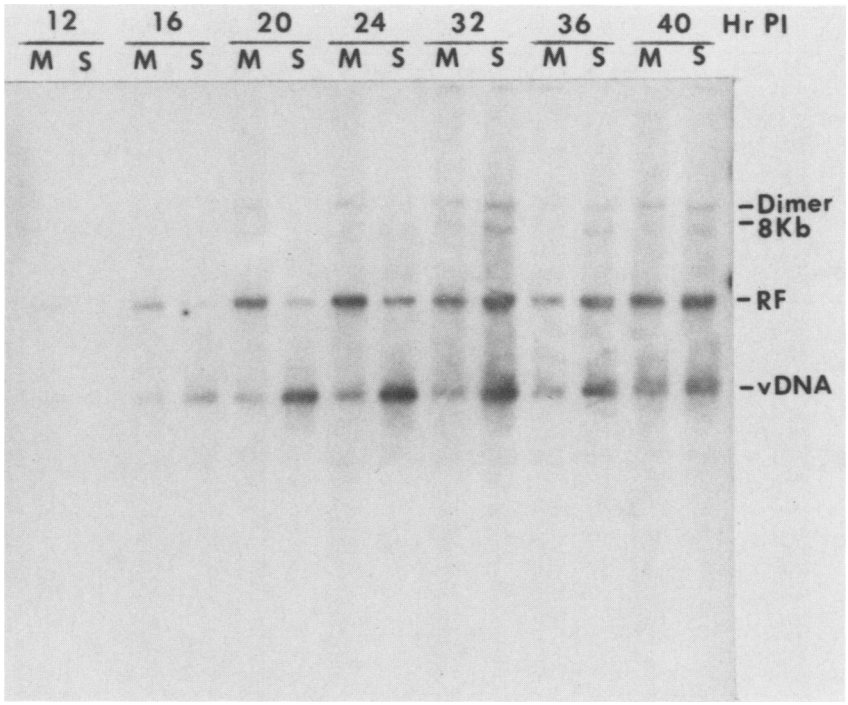

FIG. 6. Association of MVM DNA with the nuclear matrix. Nuclei from MVM-infected A9 cells were prepared at various times (in hours PI [Hr PI]) as described in Materials and Methods, and DNA in the nuclear matrix (M) and soluble (S) fractions were separated on agarose gels. The MVM DNA was detected on Southern blots, using cloned MVM RF DNA as the probe. MVM DNA forms are as follows: vDNA, single-stranded virion DNA; RF, monomer RF DNA; dimer, dimer RF DNA; and 8Kb, the 8-kbp partially replicated MVM DNA.

report. Interestingly, the B_t and C_t conformers, which begin to accumulate late in the infection (32 to 40 h PI), are not found in the matrix fraction. Since only the terminal DNA fragments in the extended conformation, here B_e and C_e, contain the covalently bound TP, these observations provide strong evidence that the matrix-MVM DNA interactions are largely mediated via the TP, rather than via a specific nucleotide sequence present in these fragments.

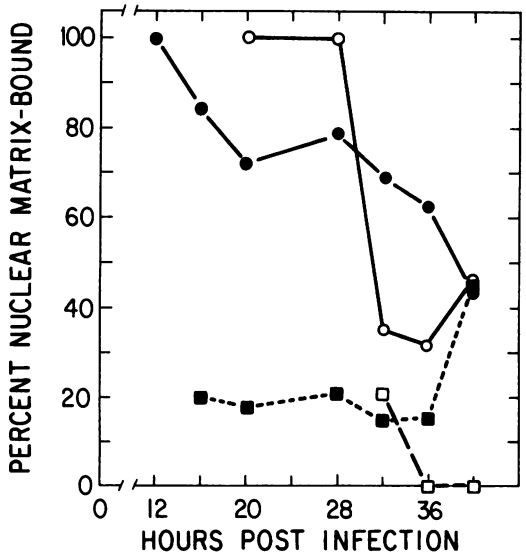

FIG. 7. Association of MVM DNA forms with the nuclear matrix. Densitometric analysis was performed on the autoradiogram shown in Fig. 6 to quantitate the nuclear matrix-associated fraction of each MVM DNA form. The DNA forms were single-stranded virion DNA (■), monomer RF DNA (●), dimer RF DNA (○), and the 8-kbp partially replicated MVM DNA (□).

DISCUSSION

Ad2 and the prototype strain of MVM (MVMp) have been used as model systems to study the specificity of DNA-nuclear matrix interactions. Although the genomes of these two viruses differ significantly in size, 35 versus 5 kb, both Ad2 DNA and MVM RF DNA are linear duplex molecules with proteins covalently bound to their 5′ termini. Previous studies had shown that adenovirus DNA is quantitatively bound to the nuclear matrix of HeLa cells between 4 and 24 h PI (60, 67). Adenovirus RNA transcription and pre-mRNA processing events are also quantitatively associated with the nuclear matrix in infected cells (36, 37) and several adenovirus early proteins, including the E1A transcriptional activators, become matrix bound (14, 26, 55, 56). Indeed, adenovirus infection causes major rearrangements in the structure of the nuclear matrix (58, 64, 68), suggesting that the altered matrix provides a structural framework upon which viral DNA replication, transcription, and viral assembly can occur. In contrast, before the studies reported here, little was known about MVM DNA-nuclear matrix interactions. It is interesting to note, however, that adenoviruses can serve as helper viruses for the DNA replication of both defective (6) and autonomous parvoviruses (35; E. Fox and J. W. Bodnar, unpublished results).

The data presented here demonstrate that both Ad2 DNA and MVM DNA are associated with the nuclear matrix during infection. Most of the Ad2 DNA is matrix bound throughout the full infectious cycle, in accord with previous reports (60, 67). In contrast, MVM DNA is predominantly

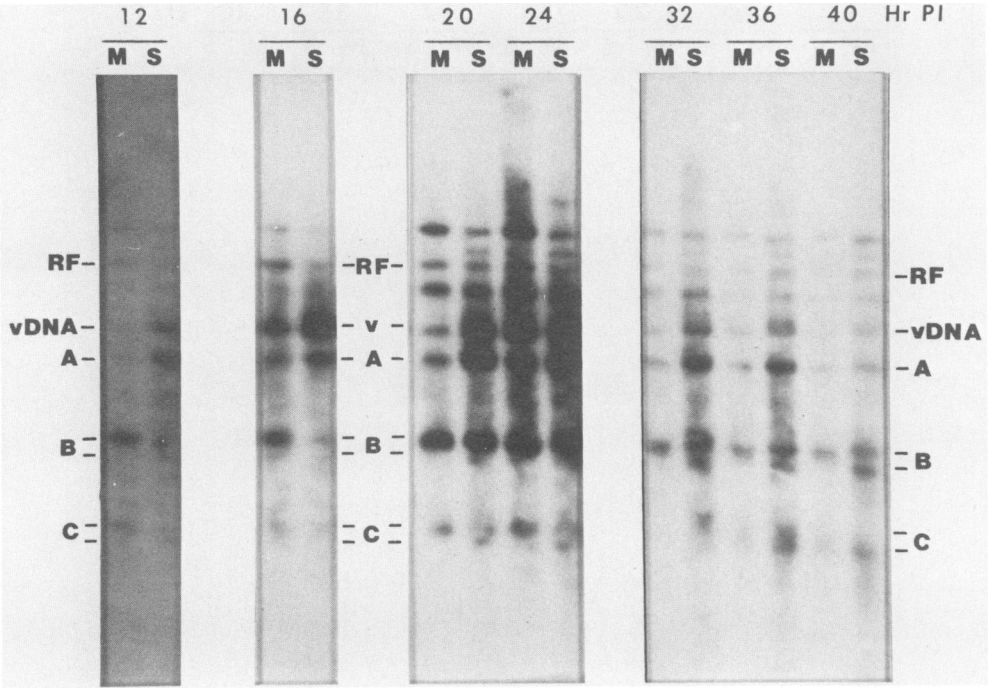

FIG. 8. Association of MVM DNA *Eco*RI restriction fragments with the nuclear matrix. Nuclei were isolated from MVM-infected mouse A9 cells, digested in situ with *Eco*RI, and extracted with 2 M NaCl and the nuclear matrix (M) and soluble (S) fractions were then separated as described in Materials and Methods. Total-cell DNA was purified and separated on agarose gels, and the MVM DNA was detected on Southern blots, using cloned MVM RF DNA as the probe (36). MVM DNA fragments are as follows: vDNA, single-stranded virion DNA (uncut with *Eco*RI); A, the *Eco*RI A fragment (20.9 to 69.9 map units); B, the *Eco*RI B fragment (69.9 to 100 map units); and C, the *Eco*RI C fragment (0 to 20.9 map units). For the B and C fragments, the upper line is the extended (TP-bound) conformer and the lower line is the turnaround (TP-free) conformer. Hr PI, Hours PI.

matrix bound early in infection (up to ~20 h); during the later stages of infection, a limited number of matrix attachment sites appear to become saturated such that the various molecular species of viral DNA accumulate in the nucleoplasm at different rates. However, both viral DNAs are preferentially associated with the nuclear matrix at or near their termini, and the restriction fragments which are most strongly matrix bound are those which contain the covalently bound TPs.

Since only those DNA fragments that were associated with the viral TPs were tightly bound to the nuclear matrix, we suggest that these high-avidity interactions are mediated via the TPs. This was especially evident for the MVM DNA for which the extended RF DNA terminal conformers (which are TP bound) were stably associated with the matrix while the turnaround conformers of the same fragments (which are TP-free) were not. Both Ad2 and MVM TPs are hydrophobic and relatively insoluble, which are characteristics one would expect for nuclear matrix-associated proteins. However, this raises the question whether the TP association is real or artifactual as a result of their stickiness. Since the methods to isolate both Ad2 and MVM DNA-TP complexes use detergents and denaturing conditions (16, 52, 53), reconstitution experiments would be hard to perform and very difficult to interpret. Nevertheless, several lines of evidence indicate that these observations are meaningful. First, similar results are obtained by using either high- or low-salt nuclear extraction methods. Additionally, adenovirus DNA-nuclear matrix interactions have been studied genetically (using temperature-sensitive adenovirus TP mutants) in combination with another mild extraction procedure, the lithium diiodosalicylate (LIS) method. In these studies, adenovirus DNA-nuclear matrix interactions were observed at permissive temperature but not when shifted to the nonpermissive temperature at which TP structure is perturbed (J. Schaack and T. Shenk [Princeton University, Princeton, N.J.], personal communication). Finally, the differential partitioning, both numerically and temporally, of different MVM RF species with bound TP between nuclear matrix and nucleoplasm cannot be rationalized on the basis of nonspecific interactions.

The characteristics of the viral DNA-nuclear matrix interactions are consistent with each genome being in a single DNA domain, with the most stable interactions occurring at or near the DNA termini (Fig. 9). The concept that the organizational and functional unit of eucaryotic DNA are DNA domains, with an average size of ~50 kbp, has been proposed by several researchers (for a recent review, see reference 8) and developed into a consolidated model of nuclear structure and function (8). In this model both chromosome replication and cell type-specific gene expression depend upon a combination of stable and dynamic DNA-nuclear matrix interactions, with the more stable of these defining the boundaries of the domain. The data presented here are consistent with the hypothesis that the viral DNA genomes of Ad2 and MVM are arranged within the nuclei of infected cells as miniature DNA domains, 35 and 5 kb, respectively, thus facilitating a coordinate regulation of both replication and transcription.

Study of the replication kinetics of both Ad2 and MVM have shown biochemical compartmentalization of DNA, which is consistent with solid-state chemistry on a fixed substrate, such as the nuclear matrix. Late in Ad2 infection, a DNA molecule which has just completed a round of DNA

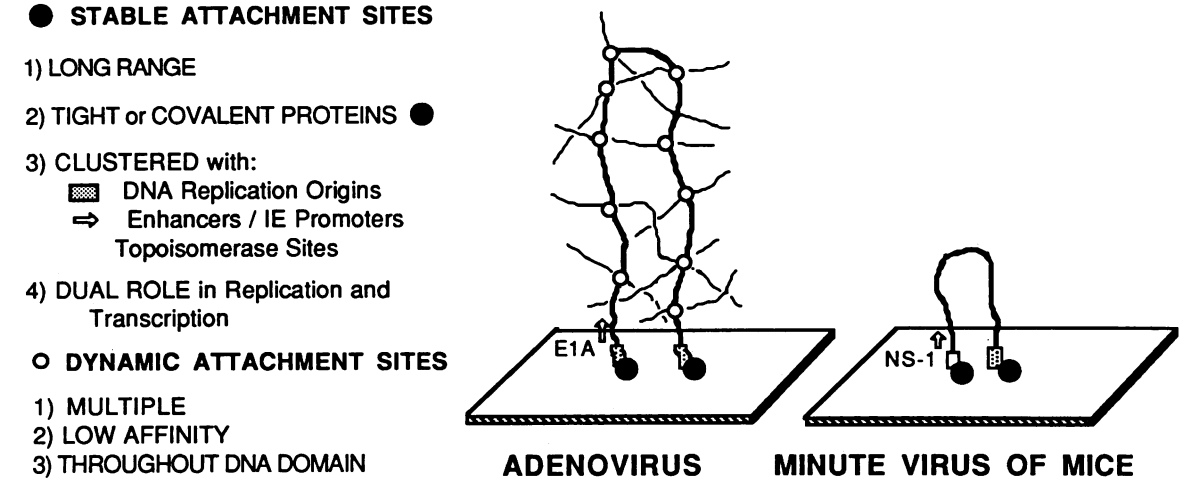

FIG. 9. Proposed domain organization of adenovirus and MVM DNA. As hypothesized in the domain model for eucaryotic DNA (8), both Ad2 and MVM DNAs are single DNA domains stably associated with the nuclear matrix (represented schematically as the box) via the interactions with proteins covalently bound at the termini of their genomes. The model predicts that these stable attachment sites are clustered with functional DNA sequences, including DNA replication origins, enhancers or immediate-early (IE) promoters, and topoisomerase sites (see text). Additionally, Ad2 DNA because of its larger size, has further association with the nuclear matrix at multiple low-affinity (but cooperative) sites (O) scattered throughout the rest of the DNA domain.

replication has 7 times the probability of replicating again as an average Ad2 DNA, and reinitiation of a displaced single-stranded Ad2 DNA occurs at least 50 times as fast as the original initiation event on the double-stranded DNA (10); both these results are consistent with the domain model in Fig. 9, in that both Ad2 DNA ends remain associated with replication complexes throughout DNA replication by association with the nuclear matrix. For MVM, DNA replication can be modeled by assuming equal access of viral DNAs to the host replication machinery early in infection. However, beginning between 20 and 24 h PI (when the nuclear matrix attachment sites are saturated), the extended conformers of MVM RF DNAs reach a maximum concentration and further buildup of the RF DNAs is mainly in the form of non-matrix-associated turnaround conformers (Fig. 7; K. McHenry and J. W. Bodnar, manuscript in preparation). Additionally, before 20 h PI, MVM DNA is localized primarily in the host nucleoli, but after that time it begins to accumulate throughout the entire nucleus (64a).

In the domain model we expect that important DNA regulatory sequences required for transcription will also be found in a cluster near the stable attachment sites; this is evident in the DNA organization of both Ad2 and MVM (Fig. 9). In both cases the promoters and enhancers for the immediate-early genes, as well as topoisomerase nick sites, are found close to the DNA termini. For adenovirus, the E1A and E1B promoters and enhancers are found within 1 kb on the viral DNA left end (for recent reviews, see references 27 and 57), while specific topoisomerase 1 nicking sites have been mapped to within the viral inverted terminal repetitions (15). The NS-1 promoter of MVM DNA is within 200 bp of the DNA left end, while specific DNA sites for nicking-closing events in replication are found at each terminal palindrome (3). We suggest that early transcriptional events for both Ad2 and MVM depend on localization of the immediate-early promoters and enhancers on the nuclear matrix by their clustering near the stable attachment sites. The recent genetic experiments of Schaack and Shenk (personal communication) support this concept as well. Additionally, supercoiling appears to be important in activation of DNA for transcription (54, 63); we suggest that the stable attachments of Ad2 and MVM DNAs at their termini may serve as anchorage points, which can allow supercoiling of the DNA throughout the DNA domains of these viral genomes.

The clustering of DNA regulatory sequences near stable attachment sites may represent a common mechanism for activation of a DNA for both replication and transcription, and therefore one might expect that some of the DNA sequences within that cluster serve a dual role in replication and transcription (8). For adenovirus, this has been shown in that nuclear factor I (NF-I) which regulates adenovirus replication is identical to a protein which binds the CAAT transcriptional signal (34). Also, an octanucleotide which is functional in immunoglobin enhancers (41, 44) is the same DNA sequence recognized by NF-III, an adenovirus replication regulator (48).

Another aspect of the domain model relates to the extension of the domain at multiple dynamic (low-affinity) nuclear matrix attachment sites distributed throughout the domain to facilitate high-efficiency transcription. Our data with Ad2 are also consistent with this aspect of the domain model. Whereas Ad2 terminal fragments were predominantly matrix bound, the interior fragments were matrix associated to a much lower degree. The fraction of the interior fragments associated with the nuclear matrix was proportional to their size and did not select for any particular region of the genome (Fig. 5). It is unclear whether the size-dependent association of Ad2 internal fragments is specific or nonspecific. However, there is evidence to suggest that this property may be the result of attachment at multiple, specific, low-affinity DNA-binding sites. Small and co-workers (59) have found a 3.5-kb *Eco*RI fragment at the 5′ end of the *Drosophila* actin gene which is preferentially associated with the nuclear matrix, yet no smaller subfragment in that region is matrix associated. They have suggested that there are multiple specific sequences in that region which cooperatively bind DNA to the nuclear matrix. To account for the observed pattern of matrix association of the Ad2 internal fragments, this hypothesis would require the presence of

specific DNA-binding sequences every few hundred base pairs. Computer analysis of the Ad2 genome has indicated that such highly recurring sequence elements are found within adenovirus DNA and the DNAs of MVM, several other viruses, and cellular genes (11).

Proteins covalently or tightly bound to viral or cellular genomes have been identified on mammalian cell DNA, herpesvirus DNA, hepatitis B virus DNA, poliovirus RNA, ϕ29 DNA, ϕX174 DNA, as well as several more animal, plant, or bacterial viral DNAs or RNAs (9, 28, 31, 66; for a recent review, see reference 65). These proteins as a class are hydrophobic and insoluble; we suggest that they all serve a similar function in anchoring the DNA or RNA genomes to the nuclear matrix (in eucaryotic cells) or bacterial membrane (in procaryotic cells). Since study of cellular DNA domains is hampered by their large size—up to 2×10^6 base pairs (for a recent review, see reference 8)—the continued study of model viral DNA domains, which are found in high copy number and are relatively short, should provide insight into the mechanisms of higher-order DNA organization and nuclear matrix binding in eucaryotic DNA regulatory events.

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