Selectivity in the Interaction of Various DNA Sequences with H1 Histone

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ABSTRACT: An assay for the binding of H1 histone by DNA was developed based on extraction with phenol, which partitions free DNA into the aqueous layer and aggregates of H1 histone–DNA complexes into the phenol layer and interface. When this assay was performed on fragments of simian virus 40 (SV40) DNA, fragments containing the 21-bp repetitive element and a portion of the origin of replication were resistant to H1 binding. This result was corroborated when an endonuclease protection assay showed that the origin was poorly protected by H1 compared to other sites. DNase I protection mapping demonstrated that H1 "underprotected" sites immediately to either side of the AT element, which lies in the origin of replication. These sites were also hypersensitive to attack by hydroxyl radical in the absence of histone, probably indicative of some conformation aberration such as minor-groove distension. The same DNA sequences resistant to binding H1 histone resisted binding to H4 histone but showed much less selectivity, if any, in binding polylysine. These results clearly demonstrate that the interaction of DNA and H1 (and H4 histone) is more complicated than just charge neutralization and probably involves the conformation of the DNA.

Evidence is mounting that H1 histone may serve as a nonspecific yet carefully regulated transcriptional repressor [Croston et al., 1991; Wolfe, 1989; reviewed in Zlatanova (1990)]. The mechanism by which the deposition of H1 is regulated is not entirely understood, but the affinity of different DNA sequences for H1 may play a role. Although it seems certain that all kinds of DNA will bind H1 histone, there have been reports that some sequences bind H1 better than others. For this selectivity to have physiological relevance, one necessary condition is that the DNA within the cell competes for limiting amounts of H1. Since the H1/nucleosome ratio does in fact seem to be significantly less than unity (Bates & Thomas, 1981), this condition is indeed met. Moreover, there is evidence that H1 histones are nonuniformly distributed in chromatin (Huang & Cole, 1984; Mohr et al., 1989) and that their exchange within chromatin is restricted (Jin & Cole, 1986). Therefore, it is possible that, for example, the superiority of supercoiled DNA over relaxed DNA in binding H1 histone (Singer & Singer, 1978) could be meaningful. Similarly, the general preference of H1 for binding AT-rich over GC-rich sequences (Sponar & Sromova, 1972; Renz & Day, 1976) may have been put to use in blocking transcription from the 5S RNA oocyte gene in *Xenopus laevis* without substantially blocking the 5S RNA somatic gene (Jerzmanowski & Cole, 1990). Other bases for selectivity may also be at play. For example, Sevall (1988) reported that the selectivity of H1 for a particular sequence was strong enough to override the general preference of H1 for AT-rich sequences, and we found that H1–DNA interactions could be modulated by methylation of the DNA (Higurashi & Cole, 1991).

In order to further explore the range of selectivity in DNA–H1 histone interactions, we undertook a study of the interaction between H1 and SV40[1] DNA. The SV40 genome was chosen because it met several criteria: (1) it is a small (5 kb) eukaryotic genome; (2) it is a natural target for H1; and (3) the life cycle of SV40 and the organization of its genome are understood in molecular detail. In particular, the expulsion of H1 from intracellular virions in the final stage of maturation that precedes cell lysis (Coca-Prados et al., 1980; LaBella & Vesco, 1980) and the depletion of nucleosomes from the regulatory region of the genome [see Ambrose et al. (1986) and references therein] suggest that the placement of H1 along the SV40 genome, both temporally and spatially, is subject to regulation.

The first approach we used was to compare protection by H1 at several restriction sites in SV40 DNA complexed to H1. The second approach was to generate several sets of SV40 DNA fragments and measure the ability of each fragment in a mixed population to compete for limiting amounts of H1 histone. In each case we found evidence that a portion of the regulatory region near the origin and early transcriptional start sites has less affinity for H1 histone than do other parts of the genome. DNase I protection assays identified at least two sites in the early portion of the regulatory region which had a lower affinity for H1 than surrounding DNA. These sites were also found to be hypersensitive to scission by hydroxyl radical.

MATERIALS AND METHODS

Materials. Histone H1 was prepared from calf thymus by a modification of the method of Johns (1964). In brief, H1, 90–95% pure, was isolated from trimmed steer thymus by two extractions in 5% perchloric acid, acidification of the resulting supernatant to 0.3 N HCl, and finally acetone precipitation of the H1 overnight; the entire procedure was performed at 4 °C. Poly(L-lysine), 15–30 kDa, was purchased from Sigma. For the endonuclease protection assays, Form I SV40 DNA was purchased from Bethesda Research Labs, and the restriction enzymes were from New England Biolabs or Boehringer Mannheim. Restriction fragments of SV40 DNA labeled *in vivo* with [^{3}H]dTTP (Linn & Snapka, 1981) or 3′-end-labeled with [^{32}P]dideoxyATP (Amersham; 3000 Ci/mmol) by terminal transferase (Boehringer Mannheim) were used in the partitioning assay. We obtained Sea Plaque low

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[1] Abbreviations: SV40, simian virus 40; bp, base pairs; kb, kilobase pairs; kDa, kilodaltons; dTTP, thymidine triphosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; np, nucleotide position; SDS, sodium dodecyl sulfate.

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melting temperature agarose from FMC Bioproducts (Rockland, ME).

Two recombinant plasmids bearing the regulatory region of SV40 were used in this work. We constructed pFH411B by inserting the 366-bp *Hin*dIII/*Asp*718 fragment of SV40 into the polylinker of pUC119 (a plasmid derived from pUC19). pL16n (Allwine & Kelly, 1985) contains the regulatory region with a 6-bp deletion in the origin of replication and was a gift from Professor M. Botchan (University of California, Berkeley).

Partitioning Assay. Binding reactions were initiated by vortexing 1.5 μg of ^{3}H- or ^{32}P-labeled DNA fragments in 3.0 μL of TE buffer (10 mM Tris, pH 7.5, plus 1 mM EDTA) into 7.0 μL of buffer A (final concentration 10 mM Tris, pH 7.5, 1 mM EDTA, 0.2 M NaCl, and 5% glycerol) containing varying amounts of H1 or polylysine. After 30 min at room temperature, each reaction was brought to 40 μL with buffer A and immediately extracted with an equal volume of phenol buffered with 10 mM Tris, pH 7.5, and 1 mM EDTA. The phenol layer and the interface were discarded. The aqueous layers, containing unaggregated DNAs, were extracted with ether to remove residual phenol. Large (>300 bp) fragments, which had been generated from tritium-labeled DNA, were run on 1.2% low-melt agarose slab gels. DNA bands were visualized with ethidium bromide, photographed, and then carefully excised, melted and mixed with scintillation fluid. Radioactivity was counted in duplicate samples. The data points shown in Figure 4A,D are each the average values from two gels. Small (<300 bp) fragments, which had been ^{32}P-end-labeled, were resolved by electrophoresis through nondenaturing polyacrylamide gels (40 cm × 8 cm × 0.3 mm) at 700 V for 10–15 h. Gels were then transferred to 3 MM paper (Whatman), dried under vacuum at 80 °C, and exposed to Kodak X-omat RP film.

Restriction Endonuclease Protection Assay. In each reaction, 0.5 μL of intact or linearized SV40 DNA, as indicated, was preincubated in 10 μL of restriction buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 100 mg/mL BSA) containing varying amounts of H1 or polylysine. After 30 min at room temperature, 0.5 μL (2.5–6 units) of the appropriate restriction enzyme was added and the reaction mixture was incubated at 37 °C for 1 h. H1 or polylysine was removed from the DNA and the restriction enzyme was inactivated by the addition of 180 μL of 0.07% SDS/1.4 M ammonium acetate followed by extraction with an equal volume of phenol buffered with 10 mM Tris, pH 7.5, and 1 mM EDTA. Following ether extraction, 1.0 μL of 20 mg/mL glycogen and 400 μL of ethanol (–20 °C) were added to the aqueous layer. The DNA recovered by precipitation at 4 °C for 5 min was run on 1.0% agarose slab gels and stained with ethidium bromide.

DNase I Protection Assay. Three DNA fragments 5′-end-labeled on one strand only were used as targets: the inserts of pFH411B and pL16n 5′-end-labeled at the *Hin*dIII site and the insert of the former 5′-end-labeled at the *Asp*718 site. Each binding reaction was initiated by the addition of 1.0 μL of DNA (20 fmol, or about 4 ng) to a 49-μL solution of 16 ng of unlabeled calf thymus DNA and up to 120 ng of H1 in buffer A. After 1 h at room temperature, 50 μL of 10 mM $MgCl_2$ and 10 mM $CaCl_2$ was added, followed by 2.0 μL of 5.0 μg/mL DNase I (Worthington, DPFF grade) in water. After 60 ± 5 s, 1 volume of 2× stop solution was added, and the reaction was placed on ice; the 2× stop solution was 4 M ammonium acetate, 20 mM EDTA, and 2 mg/mL glycogen. To remove H1, 0.1% SDS was added prior to extraction with 1 volume of TE-saturated phenol. This was followed by ether extraction and three rounds of ethanol precipitation. The final pellet was resuspended in 8.0 μL of dye mixture (90% deionized formamide, 1× TBE, and 0.02% each xylene cyanol and bromophenol blue), plus 4.0 μL of 0.1 N NaOH to aid in the denaturation of the probe. TBE was 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. These DNA samples were twice heated at 100 °C for 5 min before being loaded on 6% polyacrylamide (38:2 acrylamide:bisacrylamide) and 8 M urea sequencing gels. Electrophoresis was carried out at 1200 V for 135 min; autoradiography was performed as described above.

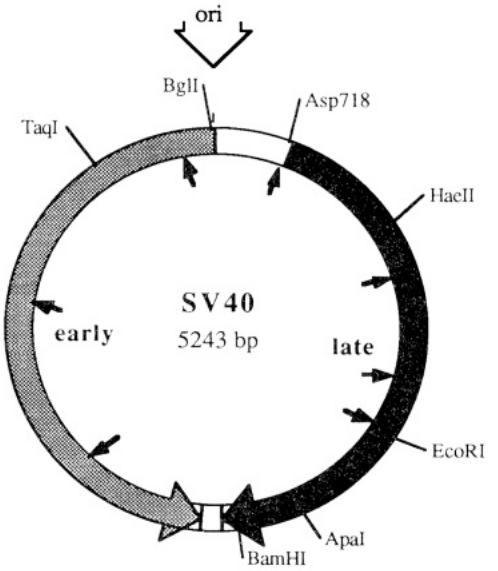


FIGURE 1: SV40 genome. The positions of the early and late transcribed regions and the directions of transcription are depicted by the two long, filled arrows. The open region at the top of the circle depicts the regulatory region, which contains the origin of replication (large arrowhead), both the early and late promoters, and *cis*-acting elements. The open region at the bottom represents the termination site for both replication and transcription. Some unique restriction sites are shown on the outside of the map, and the small arrows inside point to the six *Hin*dIII sites and single *Kpn*I/*Asp*718 site.

Interpretation of the DNase I digestion products was facilitated by running chemical sequencing reaction products alongside them. The reactions we used were the G and G + A reactions of Maxam and Gilbert (1980) and the A reaction described by Iverson and Dervan (1987).

Hydroxyl Radical Cleavage. The inserts of pFH411B and pL16n 5′-end-labeled at the *Hin*dIII site were also subjected to the hydroxyl radical cleavage reaction described by Tullius and Dombrosky (1986).

RESULTS

A Restriction Site near the SV40 Origin Was Not as Well Protected by H1 Histone as Other Restriction Sites. Using a simple restriction endonuclease protection assay, we compared protection by H1 at seven unique restriction sites scattered about the SV40 genome (Figure 1). Unique restriction sites were chosen because cleavage with each of the corresponding enzymes would yield the same product, i.e., a linearized genome. Interpretation of the data would thus be straightforward: the extent of protection by H1 would be correlated with the ratio of supercoiled to linear molecules. The data in Figure 2 strongly suggest that when the DNA was combined with 1.5 weight equiv (essentially the same as charge equivalents) of H1, the histone protected the *Taq*I, *Asp*718, *Hin*dIII, *Eco*RI, and *Apa*I sites on all but a small fraction of molecules. The *Bam*HI site, however, was protected to a lesser

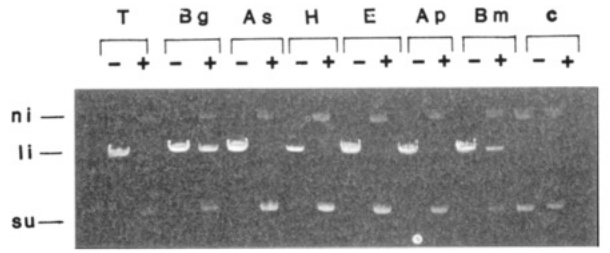
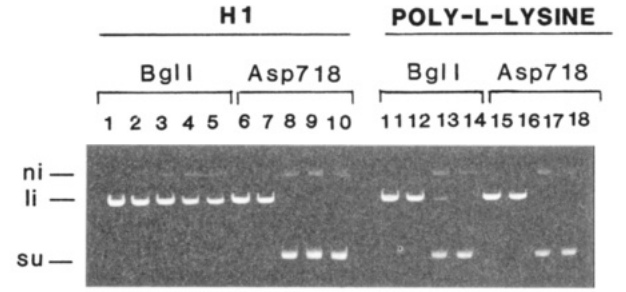


FIGURE 2: Protection of unique restriction sites in the SV40 genome by H1. Preincubation of 500 ng of intact SV40 DNA with (+) or without (–) 750 ng of H1 was followed by treatment with *Taq*I (T), *Bgl*I (Bg), *Asp*718 (As), *Hae*II (H), *Eco*RI (E), *Apa*I (Ap), or *Bam*HI (Bm) or mock digestion with 50% glycerol (c). H1 was stripped from the DNA as described in the text and the DNA was run on 1.0% agarose slab gels. The positions of nicked (ni), linear (li), and supercoiled (su) forms are given alongside the photograph.



PROTECTION OF THE ORIGIN AND LATE PROMOTER OF LINEARIZED SV40

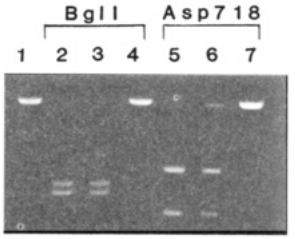


FIGURE 3: Protection of the *Bgl*I and *Asp*718 sites of SV40 DNA. (Top) Protection of intact SV40 DNA by H1 and polylysine. Parallel sets of assays were done in which 500-ng aliquots of intact SV40 DNA were preincubated with increasing amounts of H1 (lanes 2 and 7, 200 ng; lanes 3 and 8, 400 ng; lanes 4 and 9, 600 ng; lanes 5 and 10, 750 ng) or polylysine (lanes 12 and 16, 150 ng; lanes 13 and 17, 300 ng; lanes 14 and 18, 450 ng) or just buffer (lanes 1 and 11). This was followed by treatment with *Bgl*I or *Asp*718. The positions of the linear (li), nicked (ni), and supercoiled (su) forms are indicated to the left. (Bottom) Protection of linearized SV40 DNA by H1. Aliquots of 500 ng of SV40 DNA linearized at the *Bam*HI site were preincubated with buffer (lanes 1, 2, and 5) or with H1 (lanes 3 and 6, 200 ng; lanes 4 and 7, 500 ng) and then treated with *Bgl*I or *Asp*718 or mock-digested with 50% glycerol (lane 1).

degree by this amount of H1. Finally, protection was poorest at the *Bgl*I site. It should be noted that complete linearization of the DNA was achieved with all the nucleases in the absence of histone.

A similar experiment focused on protection at the *Bgl*I site and for comparison, at the nearby *Asp*718 site; the two lie ~400 bp apart at either end of the regulatory region (Figure 1). In this case the DNA was titrated with either H1 or polylysine prior to restriction. Figure 3 (top) shows that the *Bgl*I site was somewhat more poorly protected by polylysine than the *Asp*718 site: 0.6 weight equiv of polylysine completely protected the latter (lane 13) but left a significant fraction of SV40 DNA molecules accessible to cleavage at the *Bgl*I site

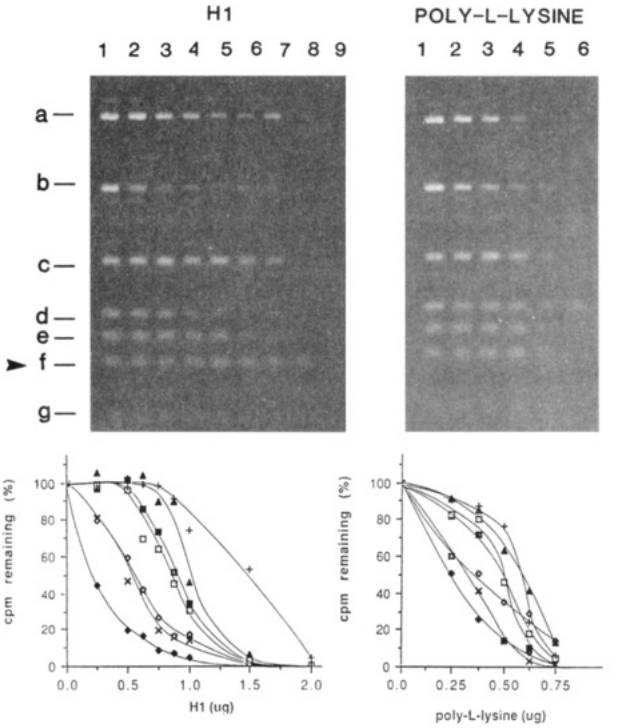


FIGURE 4: Titration of *Asp*718/*Hin*dIII-generated SV40 DNA fragments by H1 or polylysine. (Upper left) Unaggregated SV40 DNA fragments (*Asp*718/*Hin*dIII digest) were run on a 1.2% agarose slab gel after recovery from binding reactions in which [^{3}H]DNA fragments (1.5 μg) were incubated with increasing amounts of H1: lane 1, none; lane 2, 250 ng; lane 3, 500 ng; lane 4, 625 ng; lane 5, 750 ng; lane 6, 875 ng; lane 7, 1.0 μg; lane 8, 1.5 μg; lane 9, 2.0 μg. Fragment f (see arrowhead) contains the SV40 regulatory region. (Lower left) Radioactivity in each gel band was measured to determine how much of each DNA fragment (a, ×; b, ◆; c, ■; d, ◇; e, □; f, +; g, ▲) remained at each titration point. Upper right, lower right) In a control experiment, titrations were performed with increasing amounts of poly(L-lysine): lane 1, none; lane 2, 250 ng; lane 3, 375 ng; lane 4, 500 ng; lane 5, 625 ng; lane 6, 750 ng.

(lane 17). At 0.9 weight equiv, however, protection by polylysine at both sites was complete (lanes 14 and 18). For histone H1, the discrepancy between protection at these two sites was much greater than in the case of polylysine. Protection at the *Asp*718 site was essentially complete when 0.8 weight equiv of H1 were added (lane 8), but even at a ratio of 1.5 we estimate that somewhat less than half of the *Bgl*I sites were still accessible (lane 5). (These weight ratios were chosen so that a comparable range of protection at the *Asp*718 site was obtained for H1 and polylysine.)

When linearized SV40 DNA was assayed (Figure 3, bottom), the discrepancy between the levels of protection afforded by H1 at the *Bgl*I and *Asp*718 sites was much diminished compared to that which was observed for supercoiled molecules (see Figure 3, top). H1 was still less effective at protecting the *Bgl*I site as compared to the *Asp*718 site, since 0.4 weight equiv of H1 provided no detectable protection at the *Bgl*I site, whereas some degree of protection at the *Asp*718 site was seen (compare lanes 3 and 6). At an H1:DNA ratio of 1.0, though, both sites were completely protected (lanes 4 and 7). In contrast, on supercoiled molecules assayed under the same conditions [Figure 3 (top), lane 5] 1.5 weight equiv of H1 protected no more than about half of the *Bgl*I sites, whereas complete protection at the *Asp*718 site was attanied at an H1:DNA ratio of 0.8. The effect of topological relaxation on the relative affinities of the *Bgl*I and *Asp*718 sites for H1 suggests that some feature of the DNA promoted by supercoiling contributes to that discrepancy.

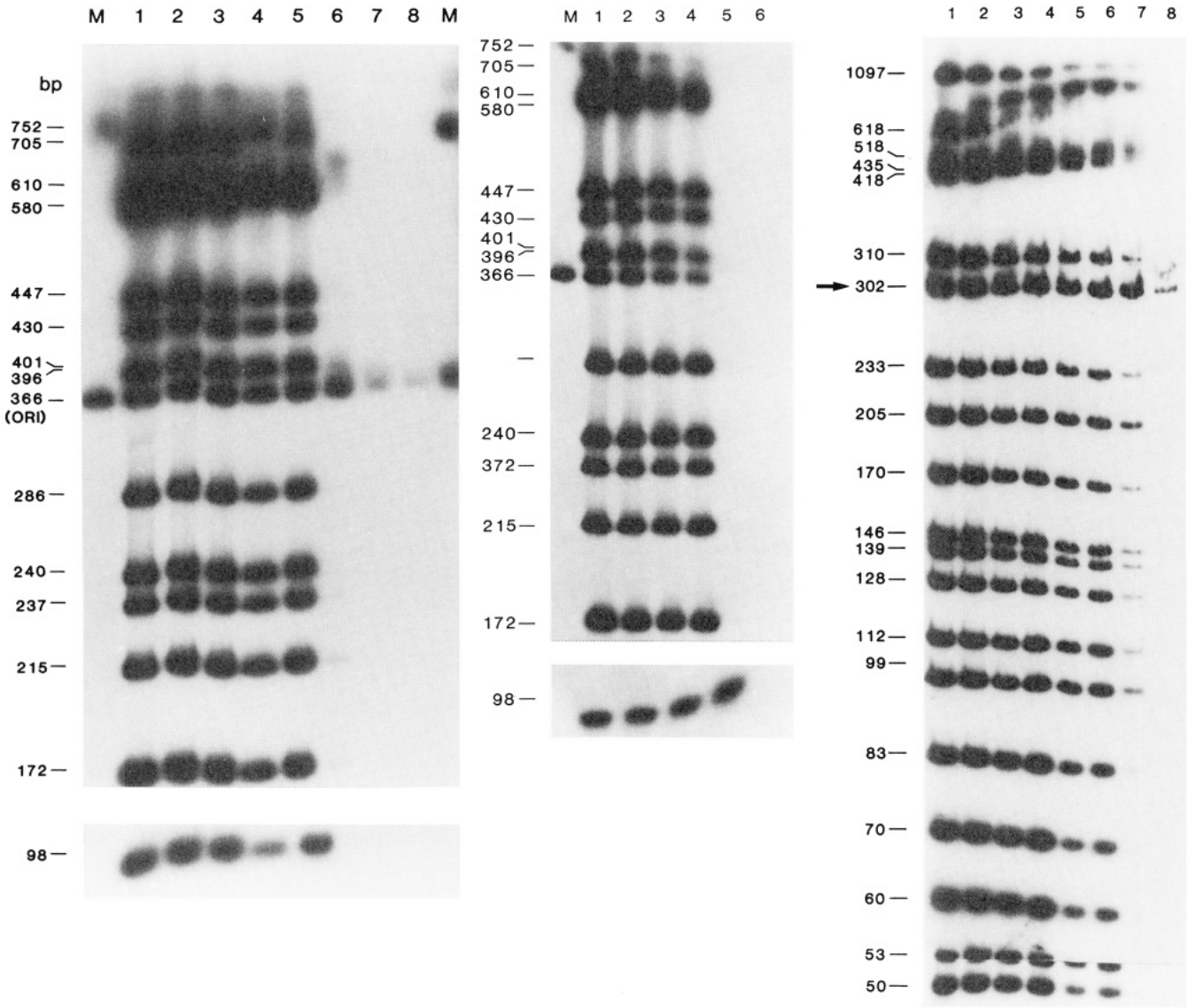


FIGURE 5: Titration of various restriction digests by H1 and polylysine. (Left) Titration of *Asp*718/*Hin*dIII/*Mbo*I-generated SV40 DNA fragments by H1. Treatment of SV40 DNA with *Asp*718, *Hin*dIII, and *Mbo*I yielded 15 fragments ranging in size from 705 to 60 bp. Unaggregated fragments were run on an 8.0% polyacrylamide nondenaturing gel after recovery from binding reactions in which 1.5 μg of calf thymus DNA plus 10 ng of ^{32}P-end-labeled fragments were incubated with increasing amounts of H1: lane 1, none; lane 2, 500 ng; lane 3, 750 ng; lane 4, 875 ng; lane 5, 1.0 μg; lane 6, 1.25 μg; lane 7, 1.35 μg; lane 8, 1.5 μg. The 60-bp fragment is not shown. In the lanes labeled M were two marker DNA fragments, 366 and 752 bp, prepared from SV40 DNA. (Center) Titration of *Asp*718/*Hin*dIII/*Mbo*I-generated SV40 DNA fragments by polylysine. Unaggregated fragments were run on an 8.0% polyacrylamide nondenaturing gel after recovery from binding reactions in which 1.5 μg of calf thymus DNA plus 10 ng of ^{32}P-end-labeled fragments were incubated with increasing amounts of polylysine: lane 1, none; lane 2, 250 ng; lane 3, 500 ng; lane 4, 625; lane 5, 700 ng; lane 6, 750 ng. Lane M contained two marker DNA fragments, 366 and 752 bp. (Right) Titration of *Dde*I/*Bgl*I-generated fragments of SV40 DNA by H1. Unaggregated *Dde*I/*Bgl*I-fragments were run on an 8.0% polyacrylamide nondenaturing gel after recovery from binding reactions in which 1.5 μg of calf thymus DNA plus 10 ng of ^{32}P-end-labeled fragments were incubated with increasing amounts of H1: lane 1, none; lane 2, 500 ng; lane 3, 750 ng; lane 4, 1.0 μg; lane 5, 1.1 μg; lane 6, 1.2 μg; lane 7, 1.3 μg; lane 8, 1.4 μg.

Most DNA Fragments Bind Histone and Aggregate Better Than Those from the Region near the SV40 Origin/Early Promoter. The interaction of H1 and the genome of SV40 was also studied by means of partitioning assays. This assay took advantage of our fortuitous observation that, in the presence of limiting amounts of H1, DNA is partitioned by phenol extraction between the organic phase and interface on the one hand and the aqueous phase on the other hand. This is apparently because H1–DNA complexes cannot be dissociated by simple phenol extraction if detergent or some other chaotropic agent is not added beforehand; some partially saturated DNAs were recovered in the aqueous layer along with free DNAs, but complexes at or near complete saturation with H1 remained in the phenol and interface layers. Since the fragments generated by a restriction digest are equimolar, the amount of any one restriction fragment recovered in the aqueous layer can be taken as a measure of the degree of H1 binding by that region of the SV40 genome.

When the interaction of H1 or polylysine with the SV40 genome was probed in a series of partitioning assays, a fragment bearing most of the regulatory region was resistant to aggregation by H1, compared to other fragments, but did not resist aggregation by polylysine. Cleavage of SV40 DNA at the six *Hin*dIII sites and single *Asp*718/*Kpn*I site (Figure 1; *Hin*dIII sites are denoted by the small arrows) generates seven fragments, a–g. Their order along the genome, going clockwise from the top of Figure 1, is f (a 366-bp fragment containing most of the regulatory region), c, e, g, a, d, and b. The left panels of Figure 4 show the result of an H1 titration of this set of fragments. It is immediately evident that these

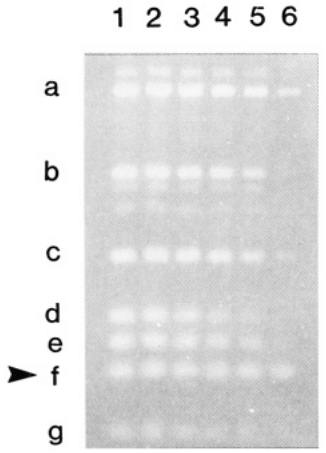


FIGURE 6: Titration of *Asp*718/*Hin*dIII-generated SV40 DNA fragments by histone H4. Unaggregated SV40 DNA fragments (identical to those described for Figure 4) were run on a 1.2% agarose slab gel after recovery from binding reactions in which DNA fragments (1.5 μg) were incubated with increasing amounts of H4: lane 1, none; lane 2, 750 ng; lane 3, 1.5 μg; lane 4, 3.0 μg; lane 5, 4.5 μg; lane 6, 6.0 μg.

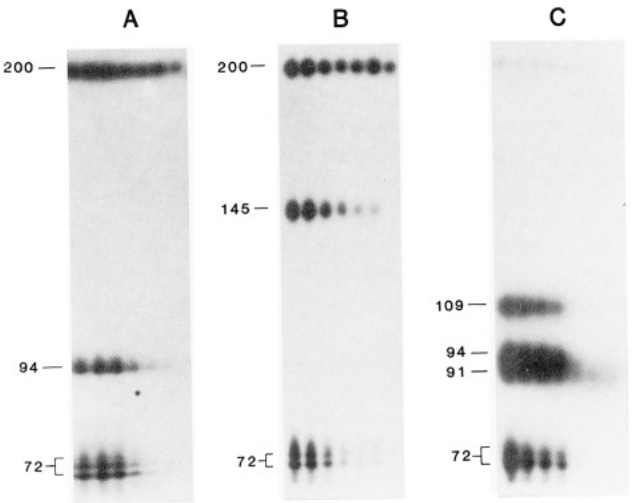


FIGURE 7: H1 titration of fragments of the pFH4111B and pL16n inserts. (A and C) Insert DNA excised from pFH4111B by *Hin*dIII and *Asp*718 was further treated with *Sph*I alone (panel A) or *Sph*I and *Nco*I (panel C). Unaggregated fragments were run on an 8.0% polyacrylamide nondenaturing gel after recovery from binding reactions in which 1.5 μg of calf thymus DNA plus 4 ng of ^{32}P-end-labeled fragments were incubated with increasing amounts of H1: lane 1, none; lane 2, 750 ng; lane 3, 1.0 μg; lane 4, 1.2 μg; lane 5, 1.4 μg; lane 6, 1.5 μg; lane 7, 1.6 μg. (B) *Sph*I-generated fragments of the pL16n insert, excised with *Hin*dIII and *Sal*I, were assayed as described above.

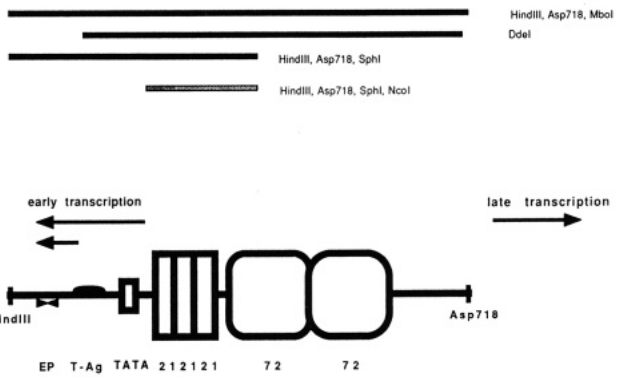


FIGURE 8: Restriction fragments of SV40 DNA resistant to H1-induced aggregation. At the top of the figure are lines representing restriction fragments of SV40 DNA that were strongly resistant (black) or weakly resistant (gray) resistant to aggregation by H1. They are aligned above a schematic depiction of the major functional elements of the regulatory region. Each fragment was generated by digestion of intact SV40 DNA (upper two lines) or pFH4111B (lower two lines) by the restriction enzymes listed to the right. The regulatory elements include the early promotor (EP), the T-antigen binding sight (T-Ag), the TATA box; three 21-bp repeats; and two 72-bp enhancers.

fragments differed in their ability to compete for H1 over a fairly broad range. What is especially striking, however, is that at an H1:DNA weight ratio of 1 (upper left panel, lane 8; lower left panel), near charge equity, H1 removed only half of the fragment f population from solution, though all but a few percent of the other fragments were aggregated at that ratio (lower left panel). When H1 was replaced by polylysine, fragment f was not especially resistant to binding and aggregation (Figure 4, right panels). Several fragments were similar to fragment f in resistance to binding (e.g., upper right panel, lane b).

The same selectivity was observed with different restriction digests. When SV40 DNA fragments generated by digestion with a combination of *Hin*dIII, *Asp*718, and *Mbo*I were titrated with H1, the fragment (366 bp) bearing most of the regulatory region was particularly resistant to aggregation (Figure 5, left). When these fragments were titrated with polylysine, however, the 366-bp fragment behaved normally (Figure 5, center). The 396-bp fragment, which showed slight resistance to aggregation by H1, terminates near the *Bam*HI site, which was shown earlier to be somewhat underprotected by H1 in the protection assay. A small degree of resistance was also shown by fragments of 215 and 610 bp for unknown reasons. The H1 titration of yet another restriction digest, by *Dde*I and *Bgl*I, again demonstrated (Figure 5, right) quite clearly the resistance of a 302-bp fragment to H1-induced aggregation. In this case, the fragment bearing the *Bam*HI site (418 bp) did not show unusual resistance. This titration revealed that an intact *Bgl*I site is not required for resistance to H1 binding and aggregation since the 289-bp fragment begins in the *Bgl*I site.

The selectivity in this DNA–histone interaction must lie to a great extent in the structure of the DNA rather than in that of the polycation: When a titration of the *Hin*dIII–*Asp*718 digest described above was done with histone H4 in place of H1, a qualitatively similar pattern of selectivity was observed (Figure 6). Again the outstanding feature was the resistance of fragment f to binding the histone. On the other hand, some constraint concerning protein structure must be responsible for the low level of selectivity observed when the same fragments were titrated with polylysine (Figure 4, right panels; Figure 5, center). It is well established (Liao & Cole, 1981 a–c) that H1 variants differ among themselves in their ability to condense DNA and it is conceivable that they would differ in their preferences for the DNA fragments in the present studies. This does not seem a very promising notion, however, since H1 and H4 behaved similarly in the current work. The dominant factor is more likely to lie in the conformations of the various DNA fragments.

Further Definition of the DNA Region Histone Binding and Aggregation: Partitioning Assays. The foregoing results indicate that the different fragments of DNA vary substantially in their binding of H1 histone but that fragments containing the SV40 origin stood well out from all the others in its resistance to H1 binding, and therefore we decided to focus on this region of SV40 DNA. A series of partitioning assays was performed on the inserts of pFH4111B and pL16n to further dissect the elements of the SV40 regulatory region with regard to their effect on its aversion toward H1 histone.

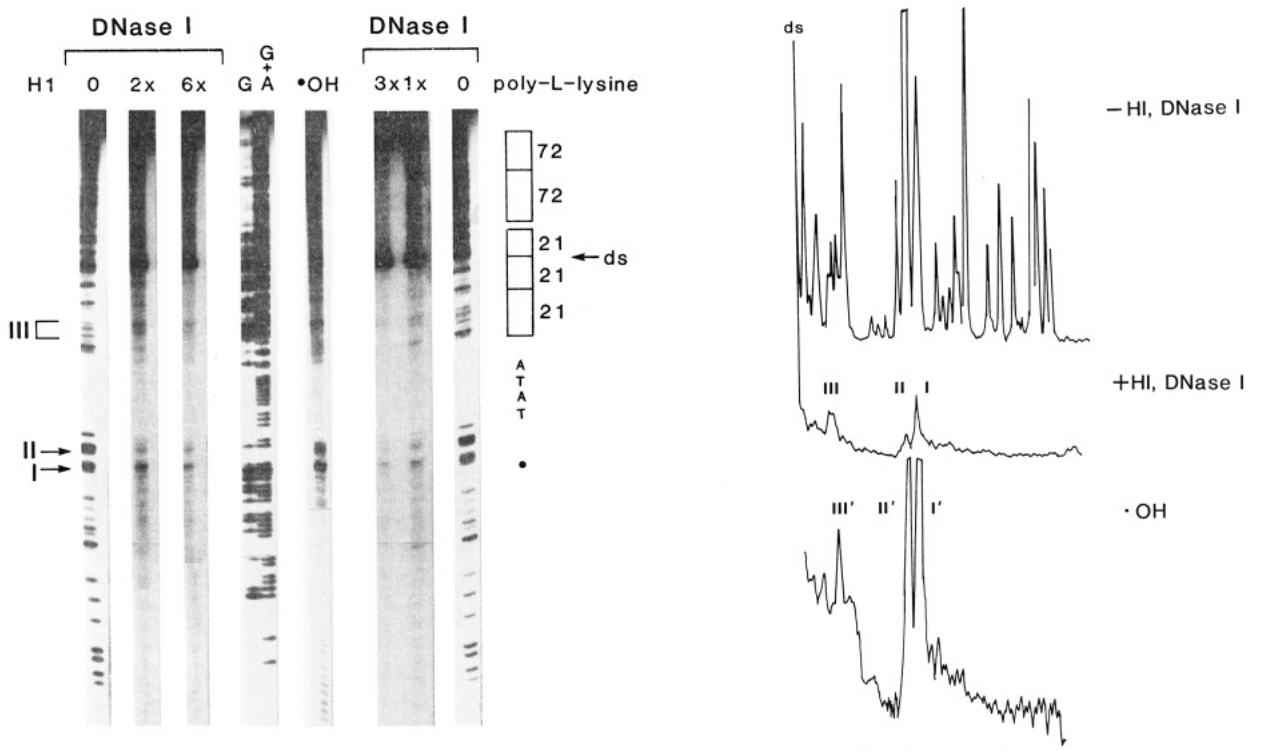


FIGURE 9: Enhanced hydroxyl radical cleavage sites and DNase I protection mapping of sites selectively underprotected by H1 along the top strand of the regulatory region of SV40. (Left) The insert of pFH4111B was 5′-end-labeled at the *Hin*dIII site; the lanes marked 0 show DNase I digestion of this probe in the absence of any protein. The second and third lanes in from the left show DNase I digestion in the presence of, respectively, a 2- or 6-fold excess (w/w) of H1 over total DNA, i.e., 40 or 120 ng of H1, respectively. Similarly, DNase I digestion in the presence of a 1- and 3-fold excess of polylysine is shown in the second and third lanes in from the right. The products of chemical sequencing reactions are shown in the lanes marked G and G + A, and the products of a reaction with hydroxyl radical are shown in the lane marked •OH. Along the left side of the figure are arrows and a bracket indicating the position of sites underprotected by H1 (I, II, and III). The arrow to the right points to the position of the double-stranded form of the probe, which was not always completely denatured. The positions of G1 (•), the AT element (TATA), the 21-bp repeats, and the 72-bp enhancers are also shown on the right. (Right) the 0× H1, 6× H1, and •OH lanes shown in the left panel were scanned at 600 nm on a Kratos SD3000 scanning densitometer from the ds signal downward to give the top (–H1, DNase I), middle (+H1, DNase I), and bottom (•OH) scans, respectively. The signal corresponding to the sites underprotected by H1 (I, II, and III) and enhanced hydroxyl radical cleavage sites (I′, II′, and III′) identified in the left panel (see text) are labeled accordingly.

The inserts were excised by *Hin*dIII and either *Asp*718, for pFkH4111B, or *Sal*I, for pL16n; and treatment with *Sph*I yielded three small (≤200 bp) fragments from the regulatory region. The order of these fragments along the genome, from left to right (i.e., early to late), is 200, 72, and either 94 or 145 bp. As panels A and B of Figure 7 show, levels of H1 that aggregated virtually all of the 72- and 94-bp (pFH4111B) or 72- and 145-bp (pL16n) fragments only partially aggregated the population of 200-bp fragments. The "200-bp" fragment from pL16n bore a 6-bp deletion within the *Bgl*I site and thus was actually 194 bp. Its resistance to aggregation by H1 implies, together with the results obtained from the *Dde*I/*Bgl*I-generated fragments, that the *Bgl*I site is not required for resistance to H1-induced aggregation, even though the endonuclease protection assays showed that it is contained within the region which is averse to binding H1.

The 200-bp *Sph*I fragment from the pFH4111B insert can be cleaved between the AT element and the 21-bp repeats by *Nco*I, yielding a 91- and a 109-bp fragment. When the pFH4111B insert was digested with *Sph*I plus *Nco*I and subjected to the same assay procedure as above, the 91-bp fragment containing the 21-bp repeats resisted H1-induced aggregation more than did the 109-bp fragment, although the difference between the two fragments was modest (compare the relative intensities of the 91-bp fragment bands in lanes 1 and 7 of Figure 7C with the relative intensities of the 200-bp fragment bands in lanes 1 and 7 of panel A).

Figure 8 schematically represents the results of the partitioning assays performed on H1-titrated SV40 DNA fragments of pFH4111B. The data are consistent with the view that the 21-bp repeats (although not necessarily the entire element) plus some sequence near the other end of the AT element are required for a high level of resistance to aggregation by H1. A contribution by the 72-bp enhancer element at the late end of the 200-bp *Sph*I fragment cannot be ruled out.

Further Definition of the DNA Region Resisting Histone Binding and Aggregation: DNase I Protection Assays. A series of DNase I protection assays revealed that sites to either side of the AT element are selectively underprotected by H1. First the 366-bp insert of pFH4111B was isolated and 5′-end-labeled with ^{32}P only at the *Hin*dIII site so that differential binding along the top strand (the strand that reads 5′-to-3′ going from the early to the late ends) of the regulatory region could be detected. After titration with H1, followed by limited DNase I digestion, electrophoresis and autoradiography revealed three potential underprotected sites, referred to as sites I, II, and III [Figure 9 (left), first three lanes]; in the 6× H1 lane they stand out clearly from surrounding cleavage sites. When the single-end-labeled insert was reacted with hydroxyl radical in the absence of any protein, the frequency of cleavage was enhanced at three sites that coincided with or abutted the three underprotected sites produced by H1. These hydroxyl radical enhanced cleavage sites will be referred to by primedroman numerals (I′, II′, and III′) to distinguish them from the sites underprotected by histone.

Comparison of the results of the H1 titration in the first three lanes of Figure 9 (left) with those of the polylysine

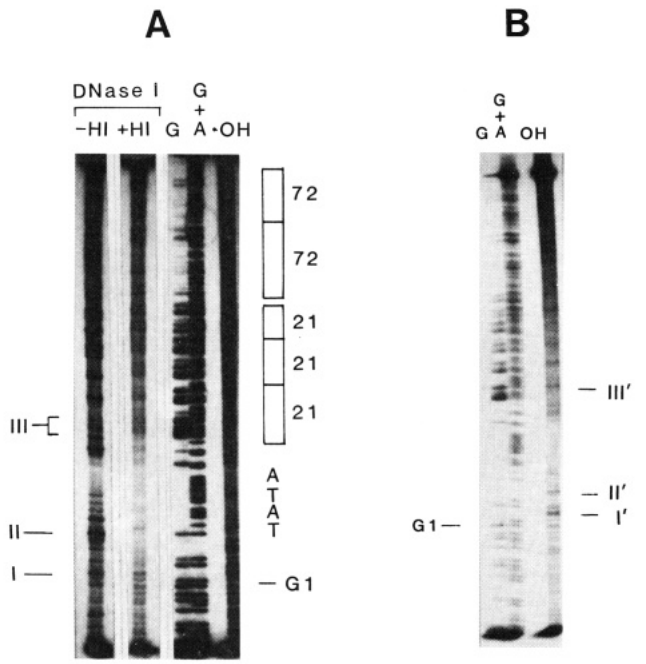
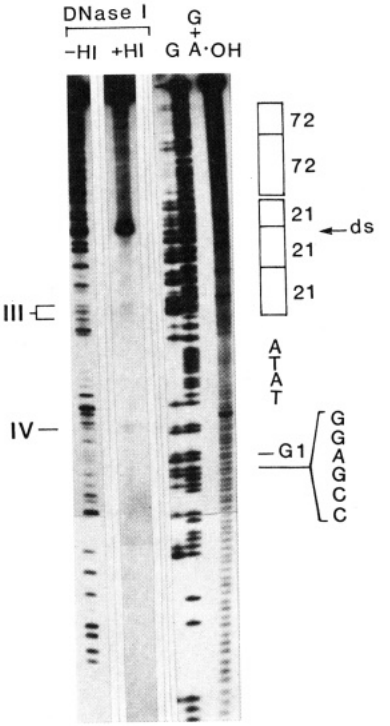


FIGURE 10: Formamide gel analysis of enhanced hydroxyl radical cleavage sites and DNase I protection mapping of sites selectively underprotected by H1 along the top strand of the regulatory region of SV40. (A) Some samples run on the gel shown in Figure 9 were selected to be run on a 40% formamide gel: 0× H1 and 6× H1 DNase I digestion products (now –H1 and +H1), the G and G + A sequencing reactions, and the •OH cleavage products. The positions of G1, the AT element, the 21-bp repeats, and the 72-bp enhancers are indicated on the right. (B) The last three lanes of the gel are shown on an autoradiogram produced by a briefer exposure than was used for that shown in panel A.

titration in the last three lanes again shows that protection by polylysine was less selective. The pattern of bands in the 1× polylysine lane is more of a reflection of the DNase I cleavage sites in the absence of polycations than are the patterns in the 2× or 6× lanes.

To obtain a clearer picture of how the DNase I cleavage patterns compare in the absence and presence of H1 the, first (0× H1) and third (6× H1) lanes, along with the sixth (•OH) lane of the autoradiogram were scanned at 600 nm on a Kratos SD3000 scanning densitometer. All were scanned downward (3′ to 5′ on the labeled strand) from a fixed postion within the band corresponding to the double-stranded form of the probe (ds). To produce Figure 9 (right), the scans were aligned using the ds signal as a reference. Of the three signals above the baseline in the middle scan, only peak III was not a major DNase I cleavage site in the absence of H1. All three peaks align well with the three highest signals in the bottom (•OH) scan.

Examination of the sequencing reaction lanes (G and G + A) of Figure 9 (left) indicated that band compression had occurred at G1 (marked by a dot on the right side of the figure). Sites I and II and I′ and II′ were therefore suspected of being apocryphal, the results of band compression rather than an enhanced frequency of cleavage by DNase I in the presence of H1 and by hydroxyl radical in the absence of protein. To address this question we ran the same reactions shown in the 0× H1, 6× H1, G, G + A, and •OH lanes of Figure 9 (left) on a gel containing 40% (v/v) formamide. As seen in Figure 10, this successfully resolved the doublet of guanidinyl residues at np 5243 and 1 from that at np 6 and 7 in the sequencing reaction lanes. Also, residual double-stranded probe molecules that were seen in the previous autoradiogram were dissociated. Careful consideration of the



DELETION MUTANT, TOP STRAND

FIGURE 11: Enhanced hydroxyl radical cleavage sites and DNase I protection mapping of sites selectively underprotected by H1 along the top strand of the regulatory region bearing a 6-bp deletion at the *Bgl*I site. The *Hin*dIII/*Sal*I insert of pL16n was isolated from vector DNA and 5′-end-labeled at the *Hin*dIII site. The first two lanes show the products of DNase I digestion of this probe in the absence of any protein (–H1) and in the presence of a 4-fold excess of H1 by weight (+H1). Also shown are sequencing reaction products (G and G + A) and hydroxyl radical cleavage products (•OH). The positions of sites IV and III are indicated along the left side of the figure. The positions of the deleted sequence (CCGAGG), G1, the AT element, the 21-bp repeats, the double-stranded form of the probe, and the 72-bp enhancers are indicated on the right.

two DNase I lanes shown in panel A of Figure 10 led us to conclude that sites I and II do not result from band compression. Site I is apparent at np 3 and 4 and so is adjacent to the *Bgl*I site; site II is at np 14. It can now be seen that site II is not a preferred site for DNase I cleavage in the absence of H1, as Figure 9 implies; site I may merely be a trace of the cleavage site strongly preferred by DNase I in the absence of protein. Site III is at np 41–47, in the first GC cluster of the first 21-bp repeat.

Panel B of Figure 10 shows the autoradiogram of the •OH and sequencing reactions produced by a briefer exposure of the corresponding lanes of panel A; the briefer exposure reduced the background in the •OH lane. Site I′ lies between the G-doublets at np 5243 and 1 and np 6 and 7. Site II′ is at np 9 and 20. Site III′ is at np 46 and 47 within the first GC cluster of the first 21-bp repeat; the frequency of cleavage at analogous positions in the other GC clusters was also high.

A DNase I protection assay performed on the insert of pL16n (Figure 11) showed that the deletion of six base pairs at np 5236–5241 eliminated the nearby enhanced hydroxyl radical cleavage site (I′) and apocryphal H1 negative footprint (site I). The deletion also eliminated the secondary structure in the labeled strand that caused the band compression at G1 in its wild-type counterpart seen in Figure 9 (left). Clearly present was site II′, the enhanced hydroxyl radical cleavage site at np 9 and 10, but a site underprotected by H1 at np 14

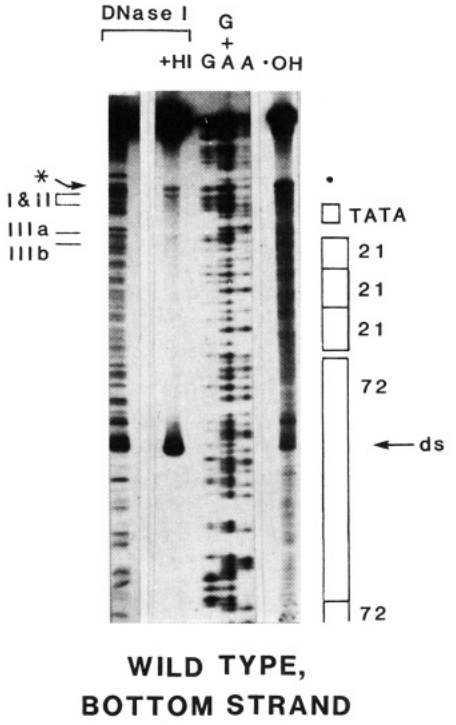


FIGURE 12: Enhanced hydroxyl radical cleavage sites and DNase I protection mapping of sites selectively underprotected by H1 along the bottom strand of the regulatory region of SV40. The *Hin*dIII/*Asp*718 insert of pFH4111B was isolated as before, but 5′-end-labeled at the *Asp*718 site rather than at the *HindIII* site. The first two lanes show the products of DNase I digestion of this probe in the absence of any protein (–H1) and in the presence of a 6-fold excess of H1 by weight over total DNA (+H1). Also shown are sequencing reaction products (G and G + A) and hydroxyl radical cleavage products (·OH). The positions of sites I, II, and III are indicated along the left side of the figure. The positions of G1 (•), the AT element, the 21-bp repeats, the double-stranded form of the probe, and the 72-bp enhancers are indicated on the right. Along the left side of the figure are arrows and a bracket indicating the position of sites underprotected by H1 (I, II, and III). The asterisk denotes an apparent underprotected site that is actually the result of band compression.

(site II) was not observed. Instead, there was a new site underprotected by the histone (IV) at np 8, midway between sites I and II on the wild-type DNA. The site underprotected by H1 and the enhanced hydroxyl radical cleavage site in the first GC cluster of the first 21-bp repeat (sites III and III′) were unaffected by the deletion.

The pFH4111B insert 5′-end-labeled at the *Asp*718 site was assayed to detect underprotected sites and enhanced hydroxyl radical cleavage sites on the other (bottom) strand. The result is shown in Figure 12. The band (marked by an asterisk) which is present in each lane is the result of band compression. Two H1-underprotected sites were observed. One (ii) was positioned opposite site II on the strand. The other (iiia,b) overlapped with site III on the top strand. At the position of iiia and throughout the 21-bp repeats, a somewhat elevated frequency of cleavage by hydroxyl radical was observed. Another site of enhanced reactivity is evident in the 72-bp enhancer just above the ds band.

The results of the DNase I protection mapping and hydroxyl radical reaction experiments are summarized in Figure 13. The GC-rich regions to either side of the AT element contained sites that were less likely to be protected from DNase I by H1 and were more likely to be cleaved by hydroxyl radical than others. The correlation between hydroxyl radical hypersensitivity and aversion to histone binding suggets an unusual DNA conformation as a common cause.

DISCUSSION

Both the endonuclease protection experiments and the partitioning assay for H1-induced aggregation reveal that the region of DNA near the SV40 origin and early promoter is peculiarly averse to binding H1. The affinity for H1 shown by DNA fragments containing this region stands out clearly from the spread of affinities shown by all the other DNA fragments studied here. Although there have been some reports which documented site-selective binding by H1 (Servall, 1988; Ristiniemi & Oikarinen, 1989; Izaurralde et al., 1989; Yaneva & Zlatanova, 1992), this is the first observation of its aversion to a site, to the best of our knowledge.

These experiments also showed that the origin/early promoter region of SV40, though averse to binding H1, has an affinity for polylysine within the range exhibited by other parts of the genome. An analogous situation is revealed when one compares H1 and polylysine with respect to preferential binding by some AT-rich DNAs. Sponar and Sromova (1972) and Renz and Day (1976) demonstrated that, at 0.15 M NaCl,

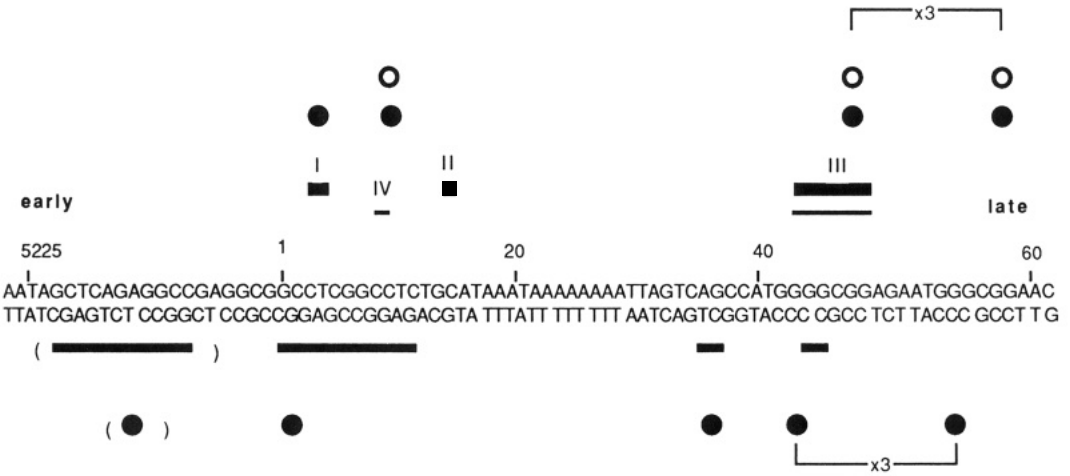


FIGURE 13: Representation of sites selectively underprotected by H1 and of sites of enhanced hydroxyl radical cleavage within the regulatory region of SV40. Underprotection was determined using DNase I (lines). Enhanced hydroxyl radical cleavage is indicated by circles. Symbols above the sequence denote sites on the top strand; those below denote sites on the bottom strand. Thick lines and closed circles depict sites found on the wild-type probe; thin lines and open circles represent sites found on the probe bearing a 6-bp deletion at the *Bgl*I site. The sequence of only one 21-bp repeat is shown; the presence of a pair of enhanced hydroxyl radical cleavage sites in each repeat is denoted by the ×3 in the bracket. The sites in parentheses are probably artifacts caused by band compression. The roman numerals refer to the sites underprotected by H1 on the top strands of both probes.

the polynucleotide aggregated by the least amount of H1 was the most AT-rich of the DNAs tested. Polylysine has been shown to have a preference for AT-rich DNA at 1 M NaCl, but none was observed at 0.1 M NaCl (Leng & Felsenfeld, 1966). Thus, at near-physiological ionic strength, although some DNA sequences may have enhanced or, as in our studies, diminished affinity for histone H1 relative to nucleotide sequences in general, this is not true for polylysine. It is clear, however, that A-T base pairs per se do not have a higher affinity for H1 than do G-C base pairs. Renz and Day found that, of eight synthetic or genomic DNAs tested, poly(dA)·poly(dT) needed the least H1 to be 90% aggregated, but poly(dG)·poly(dC) was the second most readily aggregated and required less H1 for 90% aggregation than poly[d(AT)]. Equally important is the work by Blumenfeld et al. (1978) which showed that *Drosophila virilis* histone H1 preferentially aggregated satellite III DNA over satellite II DNA even though these are composed of repeated AT-rich heptanucleotides that are anagrams of each other and are therefore of identical base composition.

The region of the SV40 genome that resists H1 is remarkable for its richness in G + C. Flanking a 17-bp sequence of all A + T are 31 bp to the 5′ side that is 77% G + C, and 71 bp to the 3′ side that is 70% G + C. It is tempting therefore to conclude that the selectivity demonstrated in the present work is simply another expression of the preference of H1 for binding and aggregating some AT-rich sequences over GC-rich sequences. Although this preference may be a factor, more is involved in the aversion of the SV40 origin toward H1 than its G + C content. For example, a sequence of 38 bp at the late end of the regulatory region (np 331–368) is 76% G + C and one of 23 bp (np 2701–2723) is 70% G + C, but neither detectably resisted H1. A few other regions of about 15 bp were about 70% G + C, but with the exception of the sequence np 2521–2534 near the *Bam*HI site, none tested as avoiding H1. Intriguingly, the *Bam*HI site is in the noncoding, termination region of the genome and might have a conformation distinct from coding sequences.

That the origin/early promoter region should respond similarly to lysine-rich H1 and arginine-rich H4 yet differently to polylysine than to H1 may seem surprising. A precedent may be found in the work of Ong et al. (1976). They found that copolymers of leucine and lysine induced changes in the circular dichroism of DNA when DNA–polymer complexes were formed and that a block polymer $(Lys_{85}Leu_{15})_n$ was much less effective than the random polymer $(84Lys,16Leu)_n$. The net charge cannot be the whole story, hydrophobic interactions, charge distribution, and steric hindrance may each play a role in interactions between DNA and polycations or histones.

We imagine then that aversion will occur in general wherever a DNA sequence presents the polypeptide in question with potential surface contacts that are less energetically favorable than the contacts offered by typical base sequences. The relevant surfaces might be in one of the grooves, where specific or nonspecific base contacts could be made, or along the sugar–phosphate backbone.

Naturally occurring DNA contains many local deviations from average B-DNA values of propeller twist, base tilt, minor and major groove width, and other parameters (Dickerson, 1983). Churchill and Travers (1991) discussed protein motifs, common to H1 histone and other DNA binders, that recognize particular DNA conformations preferentially. Two of our observations are consistent with the presence some local conformational aberration in the origin/early promoter region of SV40. First, to either side of the AT element there are sites with enhanced sensitivity to hydroxyl radical (Figure 8). Though only about the size of a water molecule, hydroxyl radical is sensitive to the width of the minor groove. In particular, the rate of cleavage is diminished to poly(dA)·poly(dT) tracts (Berkhoff & Tullius, 1987), and crystallographic studies indicate that the minor groove of this sequence is only 9 Å rather than 12 Å across (Nelson et al., 1987) as is typical of B-DNA. So it may be that the minor groove at either end of the AT element is, conversely, distended and highly accessible to hydroxyl radical. That an opening up of the minor groove should discourage binding by H1 is in fact consistent with evidence that structural motifs in H1 recognize a narrow minor groove (Turnell et al., 1988; Churchill & Suzuki, 1989). The second relevant observation is that the difference between the *Bgl*I and *Asp*718 sites in affinity for H1 was markedly greater on supercoiled SV40 DNA than on linearized molecules (Figure 3). Since negative supercoiling thermodynamically facilitates the formation of gross structural deformations such as left-handed Z-DNA (Singleton et al., 1982; Peck et al. 1982) and cruciform DNA (Geller et al., 1978), it is reasonable to suppose that it can also promote more subtle deviations.

Although these simple histone–DNA complexes are not much like chromatin, it would not be surprising to find that the physicochemical effects revealed in the present study have some manifestation *in vivo*. In this regard, we note that nucleosomes are depleted or absent from the regulatory region of SV40 minichromosomes [see Ambrose et al. (1986) and references therein]. It is conceivable that the ineffectiveness of the SV40 origin/early promoter region in competing for H1, H4, or other histones has been put to use by the virus to keep this region clear of nucleosomes.

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