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Linker histones affect patterns of digestion of supercoiled plasmids by single-strand-specific nucleases

(histone H1/DNA topology/superhelicity)

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ABSTRACT **The effect of histone H1 binding on the cleavage of superhelical plasmids by single-strand-specific nucleases was investigated. Mapping of P1 cleavage sites in pBR322, achieved by *Eco*RI digestion after the original P1 attack, showed an intriguing phenomenon: preexisting susceptible sites became "protected," whereas some new sites appeared at high levels of H1. Similar results were obtained with another single-strand-specific nuclease, S1. Disappearance of cutting at preexisting sites and appearance of new sites was also observed in a derivative plasmid that contains a 36-bp stretch of alternating d(AT) sequence that is known to adopt an altered P1-sensitive conformation. On the other hand, H1 titration of a dimerized version of the $d(AT)_{18}$-containing plasmid led to protection of all preexisting sites except the $d(AT)_{18}$ inserts, which were still cut even at high H1 levels; in this plasmid no new sites appeared. The protection of preexisting sites is best explained by long-range effects of histone H1 binding on the superhelical torsion of the plasmid. The appearance of new sites, on the other hand, probably also involves a local effect of stabilization of specific sequences in P1-sensitive conformation, due to direct H1 binding to such sequences. That such binding involves linker histone N- and/or C-terminal tails is indicated by the fact that titration with the globular domain of H5, while causing disappearance of preexisting sites, does not lead to the appearance of any new sites.**

It has been known for years that the lysine-rich or "linker" histones bind preferentially to supercoiled DNA, compared with linear or relaxed circular DNA (1, 2). Recent experiments have suggested that this preference might be due to the propensity of the protein to bind to the DNA crossovers that exist in superhelical plasmids (3). A similar preference is shown for DNA four-way junctions (4, 5), which structurally resemble DNA crossovers (6). Alternatively, superhelical DNA could be preferred in view of the intrinsic differences in twist between these molecules and the relaxed ones. In any event, the preference for superhelical DNA carries the implication that H1 binding might influence the tension existing in superhelical molecules. Effects on superhelicity have been observed with some other chromatin proteins, such as HMG1 (7–9) and HMGI/Y (10).

We have approached this question by examining the effect of histone H1 binding on the cleavage of specific sites in supercoiled plasmids by single-strand-specific nucleases, such as S1 and P1. These nucleases cleave at a variety of non-B DNA structures in double-stranded DNA, such as cruciforms and B-to-Z junctions, by recognizing conformations of the phosphodiester backbone that differ from the standard double-helical B structure (11). The existence and stability of such cleavage sites is a function of the energy stored in DNA supercoiling. Thus, changes in the pattern of these sites can be used to monitor changes in DNA supercoiling that may accompany ligand binding to topologically constrained DNA.

Depending on the ionic conditions, temperature, and superhelical density, a hierarchy of sensitive sites may be detected in plasmid pBR322. Some of these have been identified as stably unwound A+T-rich regions (12); others are extruded into cruciforms (13, 14). Z-DNA stretches have also been recognized in negatively supercoiled pBR322 (15–17); these are expected to be sensitive to single-strand-specific nucleases at the regions of B- to Z-DNA transitions (11). Other sensitive sites have also been observed but remain uncharacterized (18).

The positions of such nuclease-sensitive sites can be easily mapped by the procedure shown in Fig. 1. We have used his technique to demonstrate that titration with histone H1 can progressively change the pattern of P1- and S1-sensitive sites found in supercoiled plasmids, by first apparently protecting some sites and then inducing the appearance of new sites.

MATERIALS AND METHODS

Preparation of Plasmid DNA and Histone H1. Two types of plasmids were used: pBR322 and pGCP-36AT, in which a synthetic stretch of $(AT)_{18}$ was inserted at the *Hin*dIII site of pBR322 (provided by G. P. Schroth, Oregon State University). A spontaneously dimerized form of pGCP-36AT was also used. DNA was prepared by CsCl purification, followed by phenol extraction and ethanol precipitation (19). DNA was preincubated overnight in the appropriate incubation buffer (see below) at 16°C to allow the extrusion of cruciforms (13). Histones H1 and H5 from chicken erythrocyte nuclei were purified under nondenaturing conditions (20) and checked for purity by SDS-containing polyacrylamide gel electrophoresis (21). The globular domain of histone H5 (GH5) was prepared as outlined (3). The concentration of H1 was determined spectrophotometrically by using an extinction coefficient of 1.85 $ml \cdot cm^{-1} \cdot mg^{-1}$ at 230 nm (22). The concentrations of the stock solutions of H5 and GH5 were determined by scanning of Coomassie-stained polyacrylamide gels, using histone H1 of known concentration as a standard.

Single-Strand-Specific Nuclease Cutting: Mapping the Cutting Sites. Increasing amounts of histone H1 (0–0.8 μg) were added to 0.4 μg of DNA in 60 μl of 20 mM Mes·NaOH, pH 6.6/0.1 mM EDTA/50 mM NaCl. The ratio of the number of molecules of histone added to the number of base pairs defines the input ratio. P1 (GIBCO/BRL) digestion was carried out with about 2 units of the enzyme for 1 h at 16°C. DNA was phenol/chloroform-purified, ethanol-precipitated, and subjected to restriction nuclease digestion with either *Eco*RI or

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Abbreviation: GH5, globular domain of histone H5.

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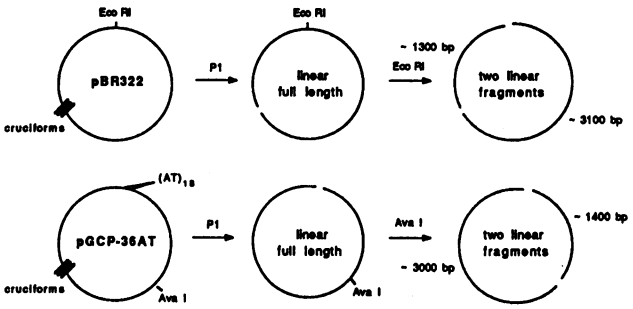

FIG. 1. Scheme of the procedure used in this work to map the position of initial P1 (or S1) cutting in pBR322 or its derivative plasmid pGCP-36AT. The single-strand-specific nuclease sensitive sites were mapped by using *Eco*RI for pBR322 or *Ava*I for pGCP-36AT.

*Ava*I (19). For digestion with S1 (GIBCO/BRL), 2 μg of DNA was incubated with increasing amounts of histone H1 in 10 μl of 10 mM Tris·HCl, pH 7.45/25 mM NaCl/1 mM $ZnCl_2$ for 15 min at room temperature. Ninety microliters of 33 mM sodium acetate, pH 4.6/55 mM NaCl was added and incubation was continued to 30 min at 37°C. Finally, 8 units of S1 was added and digestion was allowed to proceed for 10 min at 37°C. The reaction was stopped by addition of 100 mM Tris·HCl, pH 8.0/20 mM EDTA, and the DNA was purified and treated with restriction nucleases as above.

Preparation of Topoisomers of Different Average Linking Number. To prepare partially relaxed topoisomers, plasmids were incubated for 4 h at 37°C with wheat germ topoisomerase I (GIBCO/BRL) in the presence of different concentrations of EtdBr (23). The reaction conditions were as recommended by the manufacturer. After incubation, EtdBr was extracted with butanol, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Gel Electrophoresis. The products of digestion were analyzed by electrophoresis on 1% agarose gels in TBE buffer (19) at 3 V/cm. Two-dimensional electrophoresis was performed in Tris-acetate/EDTA (19) or in the same buffer containing 20 mM sodium acetate (10); the concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature.

RESULTS

Plasmid pBR322 at Extractable-Negative Superhelical Density Contains a Multitude of P1-Sensitive Sites. Most pBR322 molecules extracted from *Escherichia coli* are highly supercoiled, with a small contamination by nicked molecules. Digestion of such DNA with the single-strand-specific endonuclease P1 converted all supercoiled molecules into nicked circles or linear duplexes (data not shown). That multiple P1-susceptible sites exist at the extractable superhelical density of pBR322 ($\sigma = -0.06$) is demonstrated by the mapping technique described above (Fig. 2*A*, lane 1, and *B*). The three cruciforms identified by Lilley (13) as major (position 3065 of the map), minor (position 3221), and subminor (position 3124), appear sensitive to P1 cleavage. Two additional fragments of 2.3 and 2.0 kbp probably result from cutting at position 2325, identified by Sheflin and Kowalski (18); the exact structure causing nuclease sensitivity at this location remains to be identified. In addition, several sites of minor frequency of cutting are indicated by fainter bands in the gel.

Histone H1 Protects Some Sites in Plasmid pBR322 from P1 Cutting and Causes a Gradual Shift to a New Pattern of P1-Sensitive Sites. Superhelical pBR322 was titrated with increasing amounts of histone H1 and then digested with P1 alone (data not shown) or P1 followed by *Eco*RI to map the P1 sites (Fig. 2*A*). It should be noted that the input ratios used to designate lanes in the figures do not represent true binding

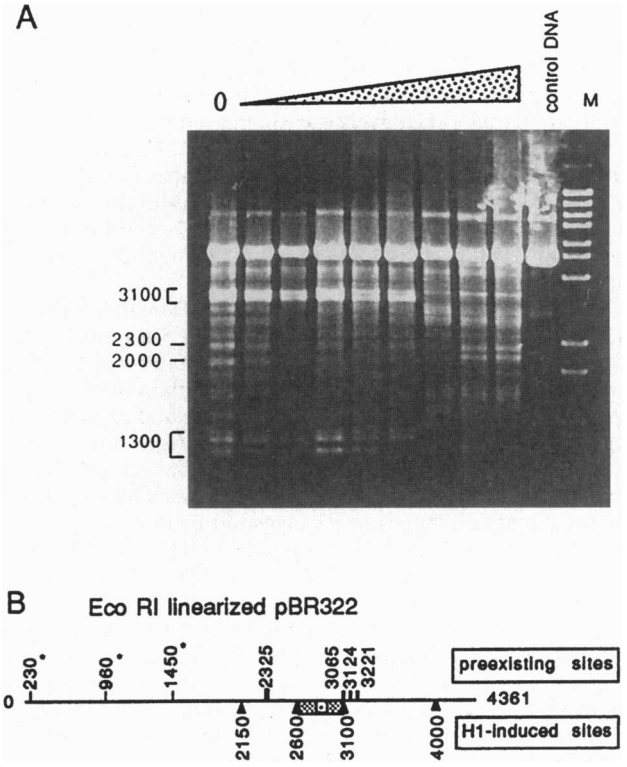

FIG. 2. (*A*) Mapping of the P1-sensitive sites in pBR322 by *Eco*RI digestion. pBR322 samples treated with P1 in the presence of increasing amounts of H1 were subjected to digestion with *Eco*RI and analyzed by 1% agarose gel electrophoresis. The amount of H1 was one molecule of H1 per 800 bp in lane 2 and was increased by a factor of 1.5 in each successive lane; the end point of titration had one molecule of H1 per 45 bp. Lane M contains *Bst*EII-digested λ DNA used as marker. Control DNA denotes *Eco*RI-digested pBR322 (no P1 digestion). The lengths of the major fragments are to the left in bp. (*B*) Scheme representing the major P1-sensitive sites existing in pBR322 at native superhelical density (designated above the *Eco*RI-linearized pBR322 map) and the major H1-induced sites (designated below the map). The group of sites at ≈3100 bp are the three cruciforms described by Lilley (13), site 2325 is the one identified by Sheflin and Kowalski (18); the sites marked by asterisks are the sites of potential Z-DNA formation; these are not P1-sensitive under the conditions of the present experiments. For simplicity, the H1-induced P1-sensitive sites are shown only in one of the two possible orientations (clockwise) with respect to the *Eco*RI site; pairs of fragments of the same length would be produced upon cutting at sites counterclockwise from the *Eco*RI site. However, comparison of the data in Figs. 2 and 4, obtained using two different restriction nucleases, strongly supports the clockwise positions given.

ratios, because some histone is lost to the walls of the containers (24), and a small fraction of that remaining may not be bound. The mapping gels show intriguing changes in the pattern of sites susceptible to the enzyme. The initial increase in the H1/DNA input ratio led to a gradual apparent protection of some sites, while the three cruciforms remained accessible. At higher H1 levels, cleavage at these cruciforms was lost as well. More remarkably, at still higher H1/DNA input ratios, new P1-hypersensitive sites started to appear (Fig. 2). A group of closely spaced bands in the region of 2600–3100 bp appeared first, followed at still higher H1/DNA ratios by a pair of distinct bands of ≈2150 and ≈2250 bp and a band of ≈4000 bp. These bands were never seen when plasmid with low (or zero) H1 bound was cut by P1. They do not map to any recognized sites of unusual DNA structure (but see *Discussion*).

A Similar Effect on the Pattern of Cutting of pBR322 Is Seen Using Another Single-Strand-Specific Endonuclease, S1. To determine whether the effects observed above reflect some

peculiarity of digestion with P1 or some intrinsic changes in the plasmid that occur upon histone H1 binding, experiments using S1 nuclease were also performed. As in the P1 studies, the bands present at zero H1 gradually disappeared, and new bands appeared as H1 levels were increased (data not shown).

In general, the changes observed with S1 were very similar to those with P1, with only minor differences in band intensities, most probably due to slight differences in the superhelical density of the plasmid preparations (25), to differences in incubation and digestion conditions, or to slight differences in enzyme specificity of cutting (11).

Titration of pBR322 with the Globular Domain of Histone H5 Leads to Disappearance of Preexisting Sites but No New Sites Appear. The linker histones are known to consist of three structural domains, a structured central domain flanked by N- and C-terminal portions that are very basic and probably lack regular structure. Despite considerable effort, the roles of these domains are still not clear (for a review, see ref. 26). To understand which portion of the molecule may be responsible for the peculiar changes in P1 digestion profile, we repeated the mapping experiments using purified GH5, with the intact protein as a control (Fig. 3). Histones H5 and H1 behaved similarly (compare Figs. 3*A* and 2*A*): titration with both proteins led to disappearance of preexisting P1-sensitive sites, followed by appearance of new sites. Moreover, the new sites mapped to exactly the same positions in the plasmid. In contrast, GH5 led to a gradual disappearance of the preexisting sites, but no new sites appeared. A possible interpretation of these results will be presented below.

Effect of Histone H1 on the P1 Digestion Patterns of Derivative Plasmids Containing Alternating $d(AT)_{18}$ Sequences. To further elucidate the differential effect of histone H1 binding on cutting of alternative P1-sensitive sites, the titration experiments were repeated with a derivative plasmid in which a stretch of $d(AT)_{18}$ had been inserted into pBR322 (pGCP-36AT). This sequence could potentially be extruded into a stable cruciform; alternatively, it has been reported to adopt an altered conformation that is different from A-, B-, or Z-DNA and is susceptible to a number of reagents and enzymes, including mung bean and S1 nucleases (27, 28).

The analysis was carried out by P1 cutting followed by digestion with *Ava*I instead of *Eco*RI, since the latter cleaves too close to the insert position to allow identification of fragments on electrophoretic gels (see Fig. 1). As shown in Fig. 4*A*, titration with histone H1 led to a gradual protection of the three cruciforms present in the native sequence of pBR322, as well as the $(AT)_{18}$ insert. Higher amounts of H1 again created new P1-sensitive sites; a well defined band at ≈3800 bp and a dense set of bands between ≈2900 and ≈3400 bp were observed. The former would correspond to the ≈2150 site seen in the *Eco*RI map of pBR322, whereas the latter could correspond to the region between ≈2600 and ≈3100 bp on the *Eco*RI map. Thus, the *Ava*I cleavage data are consistent with the *Eco*RI cleavage data.

Finally, a third plasmid, a dimer of the above plasmid, was subjected to the same type of analysis. Interestingly, although the dimer plasmid contains the same sequences as pGCP-36AT, *no* new P1-cleavable sites appeared upon H1 titration

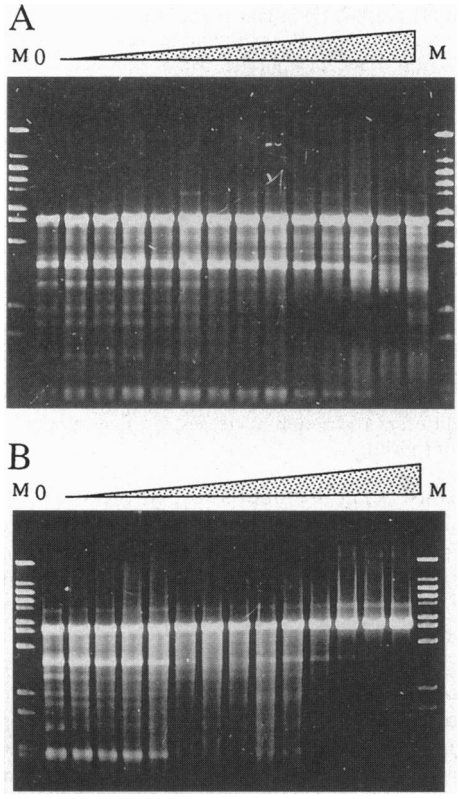

FIG. 3. Mapping of the P1-sensitive sites in pBR322 by *Eco*RI digestion. Titration was with the following: *A*, histone H5; *B*, GH5. The amount of H5 was increased 1.5-fold in each successive lane, starting at one molecule of H5 per 2250 bp and ending at one molecule per 15 bp. The amount of GH5 in each successive lane was also increased by the same factor, starting at one GH5 molecule per 950 bp and ending at one molecule per 7 bp. For further details, see Fig. 2.

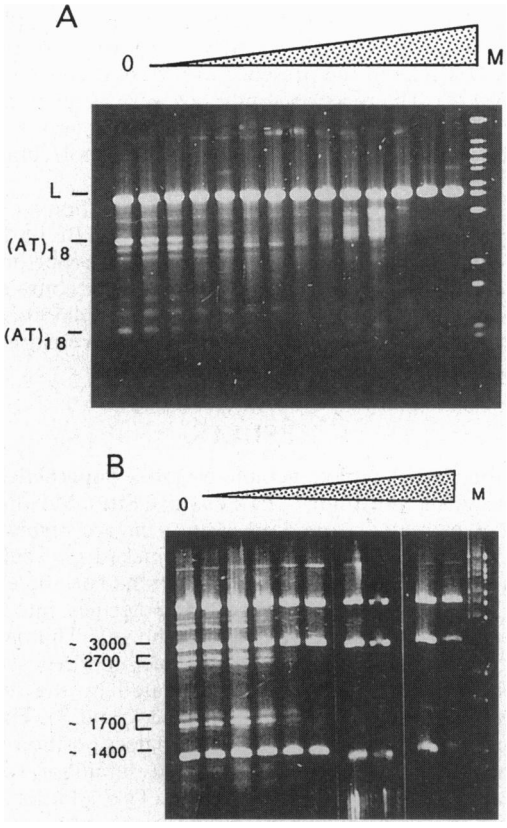

FIG. 4. Mapping of P1-sensitive sites in plasmid pGCP-36AT (*A*) or its dimerized version (*B*), in the absence and presence of histone H1. The plasmids were incubated with increasing amounts of histone H1 and cleaved with P1, followed by *Ava*I (see Fig. 1). H1 amount changed from one molecule per 2100 bp to one molecule per 17 bp, by increments of 1.5 in each successive lane in *A* and from one molecule per 1500 bp to one molecule per 60 bp in *B*. The products of digestion were analyzed by agarose gel electrophoresis. M is molecular mass marker (*Bst*EII-digested λ DNA). P1 cleavage at the $(AT)_{18}$ insert produced fragments of ≈3000 and ≈1400 bp, while cleavage at the cruciforms present in the native pBR322 sequence (13) gave fragments of ≈2700 and ≈1700 bp.

(Fig. 4*B*). However, it should be noted that in the dimer plasmid, the $(AT)_{18}$ inserts were not completely protected from cutting even at the highest H1/DNA input ratios tested. Apparently, the presence of two $(AT)_{18}$ sites allows opportunity for cleavage even at high H1 levels, and the persistence of these as readily cleavable sites prevents the observation of new sites (see *Discussion*).

Two-Dimensional Electrophoretic Analysis of the Topoisomers Susceptible to P1 Cleavage in the Absence and Presence of Histone H1. The presence of P1-sensitive sites in a supercoiled plasmid is dependent on the degree of superhelical stress, so that in any given distribution of topoisomers only molecules above a certain threshold of superhelical density will be expected to be cleaved by this enzyme. Indeed, when a uniform spread of negatively supercoiled pGCP-36AT topoisomers (Fig. 5*A*) were treated with P1 nuclease, only topoisomers with more than 15 or 16 negative superhelical turns disappeared from the electrophoretic gels, demonstrating their selective cleavage by the enzyme (Fig. 5*B*).

When the digestion was performed in the presence of increasing amounts of histone H1, the "tail" of highly negatively supercoiled topoisomers observed in the absence of P1 cleavage gradually reappeared (Fig. 5 *C–F*). This means that sites that were P1-sensitive in the absence of H1 became less and less accessible in its presence, exactly in accordance with the mapping experiments shown in Fig. 4. At still higher H1/DNA input ratios, the tail disappeared again, indicating that under these conditions the topoisomers in the tail again became susceptible to P1 cleavage (Fig. 5 *G–I*). This was again in accordance with the mapping data, which showed that new P1 sites appeared at these increased H1/DNA ratios.

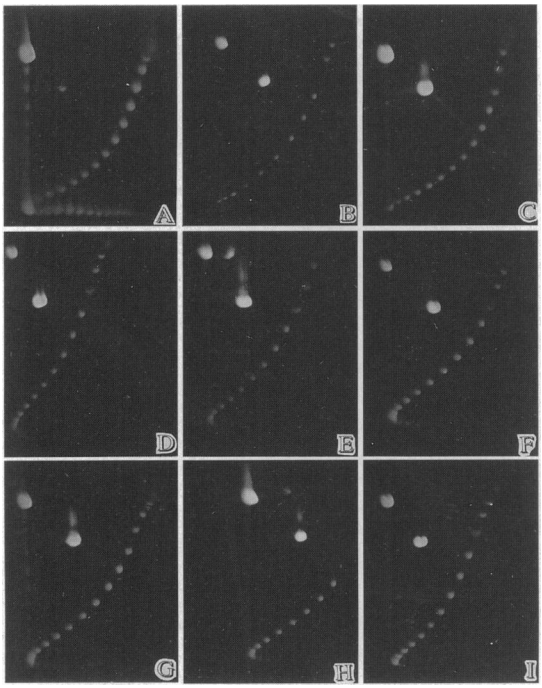

FIG. 5. Two-dimensional gel analysis of the changes in distribution of topoisomers in plasmid pGCP-36AT after P1 cleavage in the presence of increasing amounts of histone H1. A uniform spread of negatively supercoiled topoisomers over the range from 0 to ≈30 superhelical turns was prepared according to ref. 23. To that end, aliquots of pGCP-36AT were relaxed with topoisomerase I in the presence of various amounts of EtdBr, then the intercalated ethidium was extracted, and the individual samples were mixed to produce the control population shown in *A*. (*B*) Topoisomers resisting P1 digestion of the control topoisomer population, in the absence of added H1. (*C–I*) P1 digestion in the presence of increasing amounts of H1. The H1/DNA input ratios (one molecule of H1 per number of bp) were 1:1400, 1:700, 1:180, 1:105, 1:70, 1:45, and 1:33, respectively.

DISCUSSION

Two features of the effect of H1 binding on the digestion patterns require explanation: the disappearance of cleavage sites at moderate levels of H1 addition and the emergence of new sites at higher H1 levels. Disappearance of sites admits of at least two possible explanations. First, cleavage sites might simply be covered by bound histone and, thus, protected. However, this explanation is not consistent with Fig. 5, in which *A* and *B* show that only the higher topoisomers are P1-sensitive in the absence of histone H1. Note that as H1 is added, the first of these topoisomers to become resistant to cleavage are at the lower (less superhelical) end of the group that demonstrated P1 sensitivity in the absence of the protein. Since it has been shown that highly supercoiled plasmids exhibit the strongest H1 binding (29, 30), one would expect instead that the protection mechanism would be that the most supercoiled topoisomers would first be made P1-resistant upon H1 titration. We see exactly the opposite.

The effect seen in Fig. 5 suggests that the loss of sensitivity to single-strand nucleases upon H1 binding is a consequence of a change in superhelical torsion. If histone H1 binding absorbs some of the negative superhelical stress in the molecule, less stress will be available to create single-strand-specific sites. One would then expect that those topoisomers at the lower limit of superhelicity consistent with P1 sensitivity would be the first to lose that sensitivity, precisely as observed. Thus, we conclude that the apparent "protection" of sites seems more likely to be due to their disappearance, as a consequence of stress relaxation. Indeed, independent results from our research indicate that H1 can nominally be defined an "unwinding" ligand (30), in a manner similar to HMG1 (7–9).

To understand the creation of new sites, we must consider their peculiar properties. (*i*) The new sites never appear until most of the original sites have been lost. (*ii*) Some level of superhelical torsion is apparently required, for they do not appear on relaxed plasmids, no matter now much H1 is bound (Fig. 5 and data not shown). (*iii*) The appearance of new sites seems to require the binding of intact linker histone. Note that no new sites appear when the globular domain alone is used. (*iv*) We have found that when the unwinding agent EtdBr is used in the absence of H1, no new sites are generated even at very high ethidium levels (30). Thus, these are sites that require both some level of superhelical torsion and the presence of bound linker histones to be susceptible to P1 digestion.

A reasonable hypothesis concerning the nature of such sites is that they represent regions rich in A (or T) tracts. The B-DNA structure in poly(dA-dT)·poly(dA-dT) or poly(dA)·poly(dT) has been shown to be destabilized by interaction with the DNA-binding motif SPKK, which is present in the C-terminal tail of the linker histones (31). [The SPKK motif is present twice in the chicken histone H1 family members, with a third degenerate copy also present; chicken histone H5 contains three SPKK motifs and two degenerate copies (26).] Inspection of the sequences containing the new P1-sensitive sites in the broadly defined region between 2600 bp and 3100 bp clockwise from the *Eco*RI site shows an abundance of long A tracts, consisting of four, five, or seven consecutive A (or T) residues. The regions around bp 2150 and 4000 also contain concentrations of A tracts. That the C-terminal tail is involved in the creation of new sites is supported by the observation that the globular domain by itself is not capable of creating such sites (Fig. 3*B*).

If linker histone binding to such sites can induce P1 sensitivity, such sensitivity should be present at relatively low H1 levels, since A+T-rich regions are known be preferred sites for H1 binding (32). Why, then, are they not seen early in the H1 titration? One possibility is that cleavage at the "classical" sites, such as cruciforms, is more likely to cause two single-strand cuts (which are what we measure) because these

structures do not locally relax immediately after one strand is cut. It is important to realize that under our conditions only one stress-related site may be seen in a given molecule, since after the initial cutting the stress is relaxed. Thus, the complex pattern of P1 cutting sites seen in the mapping gels is the result of different single sites cut in different molecules of the DNA population. Only after the most stable preexisting structures have been relaxed by extensive H1 binding will cleavage at the A tracts be observable.

That superhelical stress is required for new site cleavage may indicate that the DNA double helix in these structures has to be somewhat modified to allow P1 cleavage. H1 binding may stabilize the P1-sensitive conformation enough to make simultaneous double cuts in the opposite strands possible in the absence of the relatively more stable cruciforms.

In conclusion, binding of linker histone to superhelical plasmids causes a complex alteration in the patterns of sites susceptible to single-strand-specific nucleases. This alteration may reflect both changes in superhelicity and stabilization of some P1-sensitive sites by direct H1 binding. Production of changes in DNA superhelicity by H1 binding must be taken into account in considering the conformational changes in chromatin fibers resulting from H1 binding or release.

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