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Histones H1 and H5 interact preferentially with crossovers of double-helical DNA

(pBR322/supercoiled DNA)

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ABSTRACT **The interaction of the linker histones H1 and H5 from chicken erythrocyte chromatin with pBR322 was studied as a function of the number of superhelical turns in circular plasmid molecules. Supercoiled plasmid DNA was relaxed with topoisomerase I so that a population with a narrow distribution of topoisomers, containing from zero to five superhelical turns, was obtained. None of the topoisomers contained alternative non-B-DNA structures. Histone–DNA complexes formed at either 25 or 100 mM NaCl final concentration and at histone–DNA molar ratios ranging from 10 to 150 were analyzed by agarose gel electrophoresis. The patterns of disappearance of individual topoisomer bands from the gel were interpreted as an indication of preference of the linker histones for crossovers of double-helical DNA. This preference was observed at both salt concentrations, being more pronounced under conditions of low ionic strength. Isolated H5 globular domain also caused selective disappearance of topoisomers from the gel, but it did so only at very high peptide–DNA molar ratios. The observed preference of the linker histones for crossovers of double-helical DNA is viewed as a part of the mechanism involved in the sealing of the two turns of DNA around the histone octamer.**

The lysine-rich histones (H1 and its variants) interact with the linker DNA between nucleosomes, sealing two turns of DNA around the nucleosome core (1). They are also involved in forming higher-order structures of the chromatin fiber (for reviews, see refs. 2 and 3). Recent evidence shows that the lysine-rich histones are involved in the regulation of gene transcription (reviewed in refs. 4–7).

One of the main features of the interaction of H1 and DNA is the preference of this histone for superhelical DNA. However, the original work from Singer's laboratory (8–10), which indicated that H1 had a higher affinity for superhelical than for relaxed circular or linear DNA, has been questioned on a number of occasions (see ref. 6). While direct competition experiments confirmed the claimed preference for superhelical DNA molecules in the case of H1 (11, 12) and $H1^0$ (12), the issue of whether histone H5 [the H1 variant specific to nucleated erythrocytes (13, 14)] possesses such a property is still a matter of controversy (11, 15).

Previous studies of this phenomenon are subject to a general criticism. In most cases plasmid or viral DNA preparations were used that were poorly characterized with respect to their topological state. Either total populations of closed circular DNA were used directly as isolated from bacterial or eukaryotic cells or high levels of supercoiling were induced by incubating nicked circular molecules with ethidium bromide and then ligating or treating with topoisomerase in the presence of the intercalator. In such preparations the superhelical density is not precisely known, and it is unclear how much torsional deformation of the DNA may accompany supercoiling. More importantly, in the highly supercoiled DNA populations used in previous studies alternative non-B-DNA conformations such as cruciforms or Z-DNA might be expected to form at specific nucleotide sequences. Histone H1 might preferentially bind to such non-B-DNA structures; thus, the apparent preference for supercoiling, *per se*, might be illusory.

The experiments described in this paper have been designed so as to avoid the above complications and to address specifically the effect of supercoiling on linker histone binding.

MATERIALS AND METHODS

Histones H1 and H5. The histones were prepared under nondenaturing conditions as described (16).

Preparation of H5 Globular Domain (GH5). The method for preparing GH5 was derived from the methods of Banchev *et al.* (17) and Thomas *et al.* (18). Ten milligrams of H5 (about 0.5 mg/ml) in 0.5 M NaCl/10 mM Tris·HCl, pH 7.5, was digested with trypsin (Sigma) at an enzyme/substrate wt/wt ratio of 1:250 for 20 min at 25°C. The mixture was diluted to 0.3 M NaCl, phenylmethylsulfonyl fluoride (PMSF) was added to 0.5 mM, and the solution was loaded onto a 10 × 0.7 cm CM-Sephadex C-25 column previously equilibrated with 0.3 M NaCl/10 mM Tris·HCl, pH 7.5/0.5 mM PMSF. The column was washed until the absorbance at 230 nm was below 0.04. GH5 was eluted with a 100-ml linear gradient of 0.3–1.0 M NaCl in 10 mM Tris·HCl, pH 7.5/0.5 mM PMSF in 50-drop fractions. Aliquots were checked on SDS/polyacrylamide gels (19) before pooling fractions that were extensively dialyzed versus 10 mM Tris·HCl, pH 7.5, at 4°C before use.

Relaxed pBR322 DNA. Plasmid DNA was obtained by the alkaline lysis procedure (20) and further purified by either cesium chloride gradient (20) or according to a modification of the protocol as described (21). In the second procedure, the DNA was phenol extracted, precipitated with ethanol and treated with DNase-free RNase. The plasmid was then separated from the RNA degradation products on an A15 M (Bio-Rad) gel filtration column (3 × 45 cm) in 0.5 M NaCl/10 mM Tris·HCl, pH 7.5/0.25 mM EDTA. Fractions containing the plasmid were precipitated with ethanol, and the pelleted DNA was dissolved in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. The DNA was treated with calf thymus topoisomerase I (BRL/GIBCO) under the conditions recommended by the manufacturer, extracted with phenol–chloroform, and precipitated by ethanol.

Formation and Analysis of Histone–DNA Complexes. Relaxed DNA was dissolved in binding buffer [10 mM Tris·HCl,

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Abbreviation: GH5, globular domain of histone H5.

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pH 7.5/1 mM EDTA/1 mM dithiothreitol/5% (vol/vol) glycerol (22)], modified by addition of 0.5 mg of bovine serum albumin (Sigma) per ml, and 0.6 M NaCl. The appropriate amount of histone in the same binding buffer without NaCl was mixed slowly with the high-salt DNA solution. Mixing in a Vortex mixer assured that the reaction mixture was homogeneous with respect to NaCl concentration at all times and that the molarity of salt was lowered smoothly to a desired value (25–30 or 100–125 mM). The mixed samples were left at room temperature for 30 min and then loaded onto 1% agarose gels; electrophoresis was performed in 0.04 M Tris acetate, pH 7.7/1 mM EDTA for 1300 V·hr. The DNA in the gel was visualized by ethidium bromide; photographs were taken on Polaroid 55 Professional Instant Sheet Film, and the negatives were scanned on a Zeineh SL-504-XL densitometer. Densitograms were enlarged by 100% on a photocopier, and the peaks were cut out and weighed. The amount of DNA in a band was taken to be proportional to the weight of the corresponding peak.

The binding of the isolated GH5 to pBR322 was assayed by gel filtration of the mixture through Chroma Spin-100 columns (Clontech) that were preequilibrated with binding buffer containing 25 mM NaCl.

RESULTS AND DISCUSSION

Interaction of Histones H1 and H5 with Topoisomers of Low Superhelical Density. To study the interaction of the linker histones H1 and H5 with DNA molecules of different superhelical densities at the resolution of single topoisomers, the initial superhelical pBR322 population was relaxed with topoisomerase I at 37°C. DNA topoisomer populations were obtained that contained about 65% of completely relaxed (and also some nicked) DNA circles and DNA circles with one superhelical turn (these two entities were usually not resolved on the electrophoretic gel, but see lanes 5 and 6 in Fig. 2*B* for an exception) and decreasing amounts of circular molecules with two, three, four, and in some cases five superhelical turns (about 20%, 11%, 3–4% and less than 1% of the total DNA in the sample, respectively). These topoisomers could be well resolved on a 1% long agarose gel (Fig. 1, lane 0). The small amount of linear molecules comigrated with the topoisomer containing three superhelical turns.

None of these topoisomers were expected to contain non-B-DNA structures, as even in the most highly supercoiled topoisomers the superhelical density σ was less than -0.012. Vasmel (23) has shown that even at $\sigma = -0.069$, at least 98% of all bases in pBR322 are fully base-paired, and the conformation of the sugar-phosphate backbone is essentially identical to that of linear DNA. No stable unwinding of A·T-rich sequences was observed in naturally supercoiled pBR322 populations at 23°C (24); similarly, no S1 nuclease-sensitive structures were found in pBR322 at superhelical density ≤ 0.036 (25) (the endonucleolytic cleavage by single strand-specific S1 nuclease reveals cruciform structures, B-DNA/Z-DNA junctions, stably unwound regions, triplex sites, etc.; for a review, see ref. 26). Our own experiments with S1 nuclease digestion of the relaxed population showed that no S1-sensitive sites were present as the amounts of topoisomers with two, four, and five superhelical turns remained unchanged upon digestion; the small increase in the linear form, which comigrated with topoisomer 3, corresponded roughly to the amount of nicked circles present (data not shown).

The complexes of the "relaxed" plasmid populations with H1 or H5 were formed by gradual addition of the histone dissolved in low ionic strength binding buffer to the DNA solution in 0.6 M NaCl, so that the salt concentration gradually decreased from an ionic strength high enough to prevent interaction to the final desired one. This new procedure, which is alternative to the direct mixing or gradient dialysis used so far (6), was chosen to ensure conditions for the formation of more regular H1–DNA complexes than are formed by direct instant mixing at the final salt concentration (see, for instance, ref. 27).

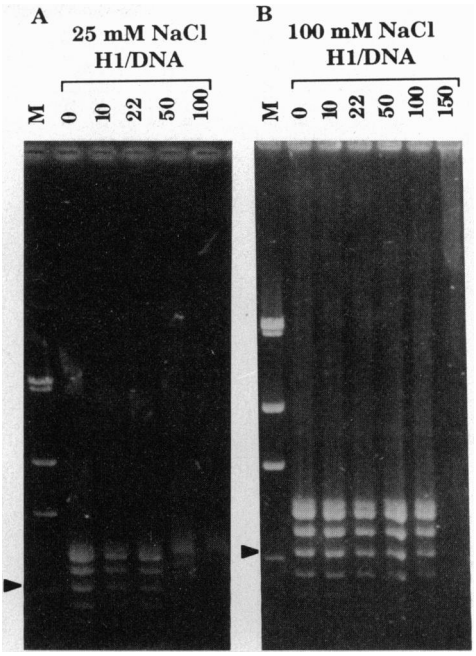

FIG. 1. Agarose gel electrophoresis of H1/DNA complexes formed at 25 mM (*A*) and 100 mM NaCl (*B*). The input histone/DNA molar ratio is denoted above the respective lane; lane M contains the molecular mass marker λ *Hin*dIII. The arrowheads indicate the location of the linearized plasmid molecules, which run directly above the 4.36-kilobase pair band of the marker.

Because the selectivity of H1 binding to DNA may depend on whether the binding is cooperative or not (6), the complexes were studied either at 25–30 or at 100–125 mM NaCl. As shown in our previous work (22), the interaction of H1 with DNA under the conditions used is still noncooperative even up to 50 mM NaCl and up to very high input H1/DNA ratios. The increase of NaCl concentrations to 100 mM or higher leads to cooperativity of binding. It should be noted that while H1 binding to DNA shows a transition from noncooperative to cooperative interaction upon increasing the ionic strength, the binding of H5 is cooperative even under low ionic strength conditions (28).

The histone–DNA complexes were analyzed by agarose gel electrophoresis. The results obtained with histone H1 for the two different salt concentrations are presented in Fig. 1; and those for H5, in Fig. 2. As can be clearly seen, gradual increase of the input histone/DNA ratio led to consecutive disappearance of DNA bands, starting from the most supercoiled, accompanied by the formation of aggregated material that did not enter the gel. A similar, but less pronounced gradual disappearance of consecutive bands was observed under the high-salt conditions (compare Figs. 1*B* and 2*B* to Figs. 1*A* and 2*A*). The gels were scanned (see *Materials and Methods*), and representative data are depicted in Fig. 3. The important point to note is that even at moderate H1- or H5-to-DNA ratios (i.e., 50), bands 4 and 5 can be completely depleted, while bands 0–1 are only partially reduced in intensity.

One further conclusion can be drawn from the fact that some bands disappear completely. This shows that the histones do not discriminate (at least in an all-or-none sense) between positive and negative superhelical turns, for it has been demonstrated that each electrophoretic band contains

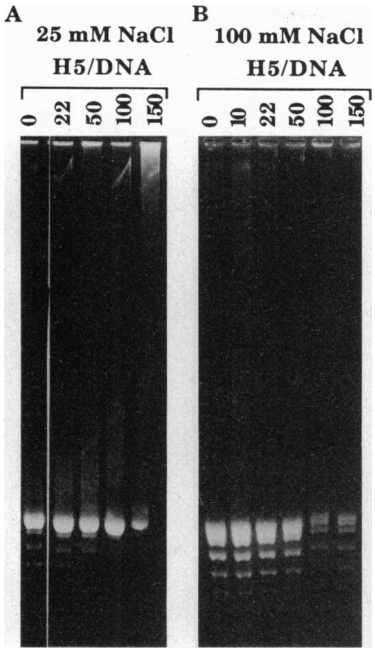

FIG. 2. Agarose gel electrophoresis of H5–DNA complexes formed at 25 mM (*A*) and 100 mM (*B*) NaCl. For other details see the legend to Fig. 1.

approximately equal amounts of both kinds of topoisomers; this observation is in accordance with previous reports (8, 9).

The sole difference in behavior between histones H1 and H5 lay in the fact that higher H5/DNA ratios were required to produce similar patterns of disappearance of consecutive topoisomers. This was rather unexpected, since in view of its higher net positive charge, H5 is known to bind to DNA more strongly than H1 (e.g., see ref. 28). However, the result can be interpreted as indicating that the observed preferential binding to topoisomers with more superhelical turns is determined by more specific structural features of the histone molecules (see below).

An intriguing observation concerns the critical H1/DNA ratios at which topoisomers with increasing numbers of superhelical turns begin to disappear from the electrophoretic gels. Under the low ionic strength conditions, when the preference of the histone to the more highly supercoiled topoisomers is more pronounced, molar ratios of H1 to DNA of only 10–20 were enough to cause almost complete disappearance of the DNA band with five superhelical turns. This is far below the molar ratio required for complete saturation of pBR322 by histone H1, which is about 140 as calculated on the basis of the size of the DNA binding site (27, 29). How the available histone molecules were distributed among the different topoisomers cannot be decided from our data. It is possible that the majority of histones were bound to the DNA in aggregates, as no retardation of the topoisomers that entered the gel was apparent. However, binding of a few molecules of H1 to so large a DNA (4362 base pairs) would

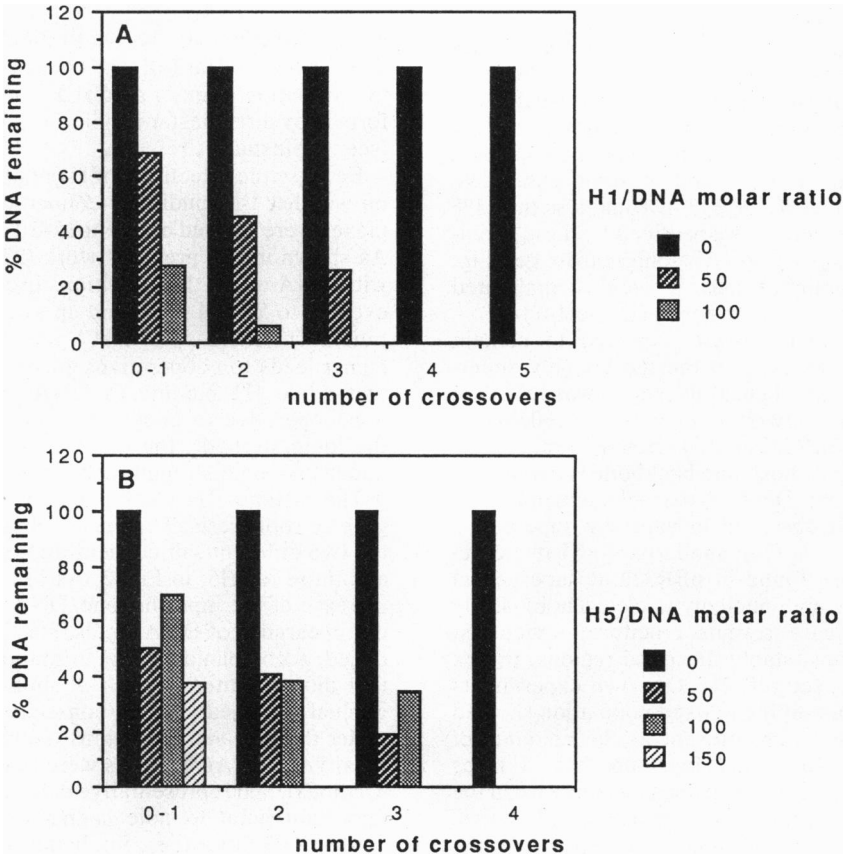

FIG. 3. Histograms quantifying the electrophoretic patterns of the linker histone–DNA complexes presented in Figs. 1*A* and 2*A* (25 mM NaCl). (*A*) Histone H1; (*B*) Histone H5. The negatives of ethidium bromide-stained gels were scanned, and the amount of DNA present in each band was determined from the area of the respective peak (see *Materials and Methods*). The absolute amount of each topoisomer in each successive lane was related to the amount of the same topoisomer in the lane where no H1 had been added (100%). The uppermost band contained both the relaxed DNA circles and those with one superhelical turn and was resolved into two closely situated bands only at loadings below the usually applied ones; higher loadings were necessary to attain reasonable amounts of DNA in the higher topoisomer bands. Qualitatively similar results were obtained in at least three independent experiments.

not be expected to produce a detectable mobility shift; thus, it is quite possible that molecules remaining on the gel have some histone bound. Furthermore, it is clear that while the higher topoisomers were the first to disappear, some binding occurred even on the fully relaxed circles, as between 30% and 50% of these molecules also disappeared from the gel upon histone addition to the levels used here.

An interpretation of these results has to take into account two separate phenomena: an initial preference of the histone for some structural features that distinguish the topoisomers of different electrophoretic mobilities, and subsequent aggregation that leads to exclusion of the complexes from the gel. The major structural distinction among the topoisomers of different electrophoretic mobility is the number of superhelical turns, for differences in twist could not be resolved by electrophoresis. Moreover, in none of the topoisomers are any non-B-DNA structures expected or observed because of their low superhelical density. Thus, it seems that the linker histones H1 and H5 possess selectivity for the crossovers of double-stranded DNA formed by the superhelical turns. Such an interpretation is not surprising when one bears in mind that both H1 and H5 are viewed as molecules sealing off the two turns of DNA around the histone octamer, creating the characteristic "zig-zag" appearance of chromatin fibers at low ionic strength. The preferential binding to crossovers is also indirectly supported by published experiments with highly supercoiled DNA molecules (30), which indicate that H1 protects supercoiled DNA from relaxation with a DNA-relaxing enzyme.

Interaction of GH5 with pBR322. The interpretation that the linker histones recognize and bind preferentially to DNA crossovers is in accordance with recent x-ray diffraction studies suggesting two specific DNA-binding sites in the globular regions of H5 (31). As the globular domains of H5 and H1 are evolutionarily conserved, it is highly probable that similar DNA binding sites exist in H1.

These observations raised the question of whether the globular part of a linker histone by itself could show the preference to crossovers observed with the intact protein molecules. Control experiments proved that under our conditions the globular domain by itself bound to DNA. A mixture of purified GH5 and pBR322 (molar ratio of 200) was spun through Chroma Spin-100 gel filtration columns that had been preequilibrated with the low-salt binding buffer. Any free GH5 would be retained in the column, while only bound GH5 would appear in the flow-through fraction. Indeed, as shown in Fig. 4*A*, the flow-through contained both DNA and GH5 (about 40% of the DNA and 60% of the protein from the initial input were not recovered because of losses to the walls of the vessels and to some retention by the resin).

An experiment comparable to that in Figs. 1 and 2 but using GH5 instead of the intact molecule is shown in Fig. 4*B*. This experiment demonstrates that the globular domain by itself can bring about preferential loss of the higher superhelical topoisomers but that this occurs only at very high molar ratios of the protein fragment to DNA (compare lanes 7 and 8 in Fig. 4*B*). In the case of H5, a molar ratio of 150 caused aggregation of about 70% of the total DNA in the gel; similar levels of aggregation were achieved at a molar ratio of 400–500 in the case of GH5.

The fact that preferential aggregation of higher topoisomers can be observed even with the globular portion of H5 must mean that the ability to bind to two DNA duplexes is retained even in this truncated protein. However, the fact that much higher levels of GH5 are required to produce comparable effects suggests that the C-terminal and/or N-terminal tails of linker histones strongly reinforce such interactions.

The model that emerges from these studies can be summarized as follows. As salt concentration is decreased from

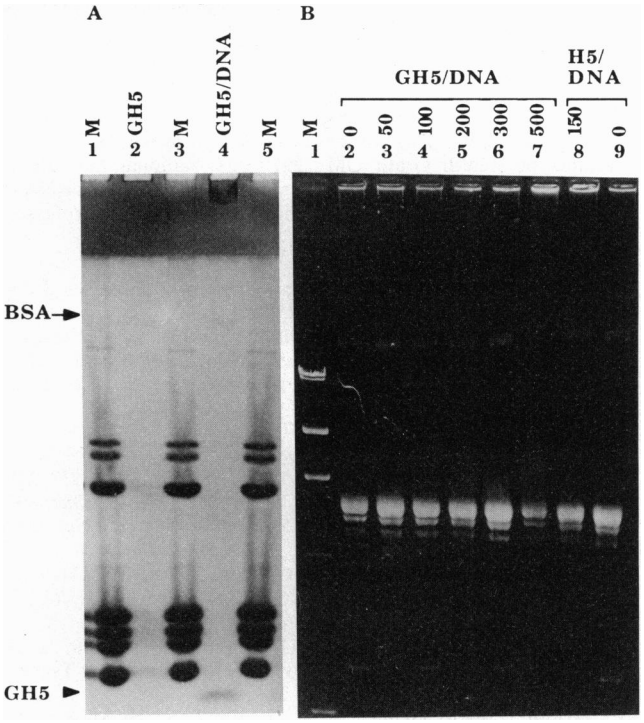

FIG. 4. Interaction of GH5 with relaxed pBR322. (*A*) SDS/polyacrylamide gel electrophoretic analysis of the flow-through fractions of Chroma Spin-100 columns. GH5 was allowed to interact with pBR322 in binding buffer containing 25 mM NaCl at a molar ratio (GH5/DNA) of 200, and the complex was spun through the column and equilibrated with the same buffer. Lanes: 1, 3, and 5, total chicken erythrocyte histones as markers; 2, flow-through fraction of a control sample containing GH5 and no DNA; 4, flow-through fraction of a sample containing both GH5 and DNA. The arrowheads point to the band of bovine serum albumin (BSA) present in the binding buffer and to the GH5 as marked. (*B*) Agarose gel electrophoresis of GH5–DNA complexes formed at 25 mM. Lanes: 1, molecular mass marker; 2 and 9, free DNA; 3–7, GH5–DNA complexes at the molar ratios indicated above the lanes; 8, intact H5–DNA complex in a molar ratio of 150.

0.6 M toward the final value, linker histones begin to associate with the DNA, binding first to the most favorable sites—namely, the crossovers. Further histone binding is cooperative. The linker histone binding, nucleated at crossover sites, proceeds via cooperative addition of more linker histones, which will have only one DNA-binding site occupied. The additional histone molecules can provide crosslinks to adjacent DNA molecules, causing the observed aggregation. Protein–protein interactions among bound histone molecules can also contribute to the aggregation process. The higher topoisomers are preferentially lost to aggregation because they nucleate cooperative binding preferentially. Dilution to 25 mM salt "locks in" this distribution. When the salt-dilution is stopped at about 100 mM and the samples are incubated in this medium, redistribution of linker histones occurs (32). This will have the effect of blurring the original distribution of the histone between topoisomers.

Our view of how the molecules of the linker histones interact with superhelical DNA is reminiscent of that of Singer and Singer (10). Their experiments with isolated fragments 72–217 and 106–212, respectively composed of most of the globular domain and the whole C terminus and just the C terminus, indicated that the globular domain was involved in the recognition of superhelicity. However, these experiments did not address the issue of which feature(s) of superhelical DNA was actually recognized. Singer and Singer (10) postulated two components in the interaction: initial

recognition of the superhelicity by the globular domain and subsequent stabilization of the interaction in consequence of the binding of the strongly charged C-terminus.

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