A Putative DNA Binding Surface in the Globular Domain of a Linker Histone Is Not Essential for Specific Binding to the Nucleosome*

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A fundamental step in the assembly of native chromatin is the specific recognition and binding of linker histones to the nucleoprotein subunit known as the nucleosome. A first step in defining this important interaction is the determination of residues within linker histones that are important for the structure-specific recognition of the nucleosome core. By combining in vitro assays for the native binding activity of linker histones and site-directed mutagenesis, we have examined a cluster of basic residues within the globular domain of H1(10), a somatic linker histone variant from Xenopus laevis. We show that these residues, which comprise a putative DNA binding surface within the globular domain, do not play an essential role in the structure-specific binding of a linker histone to the nucleosome.

Chromatin is the true substrate in all biological processes involving DNA within the eukaryotic cell nucleus. Recent work has clearly demonstrated that the organization of DNA within the chromatin complex can exert both positive and negative influences on these processes (1–3). In order to fully understand how chromatin complexes have been integrated into gene control mechanisms, it will be necessary first to fully characterize the protein and DNA structures within chromatin.

The primary unit of structure in chromatin, the nucleosome core, is comprised of two turns of DNA wound onto a spool of core histone proteins. The association of a single molecule of H1-type histone (i.e. linker histone) with the nucleosome core completes the formation of the nucleosome proper and stabilizes condensed forms of chromatin (4–7). Posttranslational modifications of linker histones have been proposed to have important roles in chromosome condensation, replication, and transcription (3, 8, 9). Biophysical studies have led to the proposal that linker histones bind somewhere on the exterior surface of the nucleosome core (6). Linker histones bind specifically and preferentially to nucleosomes over naked DNA (10).

We have developed a simple in vitro assay for detecting binding of linker histones to a model chromatin complex. This complex is based on a DNA fragment that contains a Xenopus borealis 5 S RNA gene. This fragment yields “positioned” nucleosomes upon reconstitution with purified core histone proteins in vitro (11, 12). Protein-DNA contacts and the details of the DNA structure have been precisely mapped within the 5 S nucleosome (11, 13, 14). Moreover, the binding of linker histones to reconstituted 5 S nucleosomes fulfills such classical criteria for the correct incorporation of these proteins into chromatin as the requirement for linker DNA flanking the nucleosome core and the generation of chromatosome-length DNA during micrococcal nuclease digestion of reconstituted complexes (10, 15). Thus the native interaction between linker histones and a nucleosome can be recapitulated in vitro (10, 15).

A protease-resistant ~80-amino acid residue globular domain within linker histones alone is responsible for specific recognition of the nucleosome (16). The three-dimensional structure of a globular domain has been solved by x-ray crystallography (17). Based on structural homology to several sequence-specific DNA-binding proteins, a region encompassing helix III in the globular domain has been proposed to bind in the major groove of DNA when linker histones are bound to nucleosomes. A second DNA binding surface, inferred from biophysical studies, has been predicted to be comprised of a cluster of basic residues on the side of the globular domain opposite helix III. However, the role of these basic residues in the structure-specific recognition of the nucleosome has not been determined. Here we have tested the relevance of these basic residues for the specific binding of a linker histone to a nucleosome.

EXPERIMENTAL PROCEDURES

DNA Fragments—A 238-bp HpaII-DdeI fragment derived from plasmid pXbs-1 containing the X. borealis somatic 5 S RNA gene was radiolabeled on the coding strand at the HpaII site 102 bp upstream from the initiation site for transcription of the 5 S gene (+1) as described (10). For micrococcal nuclease digestions, this fragment was internally labeled at the BboI cut site at position −23 as described previously (18).

Preparation of H1wt and Mutants—Xenopus laevis H1wta (hereafter referred to as H1(19, 20)) was cloned by polymerase chain reaction methods from erythrocyte mRNA into the pET3d vector (Novagen). Mutants were prepared by standard four-primer polymerase chain reaction techniques in the same vector. Proteins were expressed by treating BL21(DE3) cells, containing these vectors grown to early log phase, with 0.4 mM isopropyl-1-thio-D-galactopyranoside for 3 h at 37 °C. Cells were pelleted, treated with lysozyme, and sonicated, and insoluble components were separated by centrifugation at 5000 × g. Soluble proteins were fractionated by ion exchange chromatography (Bio-Rad Bio-Rex, 50–100 mesh), and H1(15) was eluted from the matrix with 20 mM Tris, pH 8.0, 2 mM EDTA, 1.0 mM NaCl. Proteins were refractometered over a second Bio-Rex column (100–200 mesh) in the same way and stored in the elution buffer at −80 °C. Correct folding of all bacterially expressed proteins was confirmed by comparing patterns of tryptophan sensitivity with native linker histones (results not shown). Preparations of mutant proteins were standardized with respect to the concentration of the wild-type preparation by densitometric analysis of the intensity of stained bands on protein gels (data not shown). The concentration of H1wt was determined assuming ε230 = 2.0 for 1 mg/ml of

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† The abbreviations used are: bp, base pair; wt, wild type.
solution.2

Reconstitution of Nucleosome Cores—Nucleosome core particles were prepared as described (21). Nucleosome cores were reconstituted onto radiolabeled 5 S DNA fragments by exchange with core particles (22) as described (18). The original 20-μl exchange reaction contained 1 mM NaCl, 1.5 μg of donor core particles, 0.3 μg of naked nonspecific DNA, and 50–100 ng of labeled 5 S fragment. After exchange and dilution, about 50–60% of the labeled 5 S fragments were assembled into mononucleosome cores without detectable dinucleosome complexes, as monitored by nucleoprotein electrophoresis (21).

Linker Histone Gel Shift Assay—Samples containing approximately 25 ng of nucleosome core particles and ≤1 ng reconstituted 5 S nucleosome cores were incubated with various amounts of linker histone (see Figs. 2, 4, and 5) in 10 μl of binding buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol). Samples were incubated at room temperature for 15–30 min and loaded directly onto running 0.7% agarose gels in 0.5 × TBE (1 × is 90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA). Control experiments in which linker histones were reconstituted onto 5 S nucleosome cores from chicken erythrocyte chromatin by free exchange in the same buffer showed identical shifted bands. After electrophoresis, the gels were dried and autoradiographed, bands were quantitated with a Molecular Dynamics, Inc. laser scanning densitometer, or the dried gels were directly quantitated by PhosphorImager analysis.

Micrococcal Nuclease Digestion—Samples reconstituted with internally labeled 5 S DNA containing a total of ~40 ng of nucleosome cores were incubated with linker histones as indicated in the legends to Figs. 2 and 6 for 15 min at 25 °C. Digestions were performed for 5 min with 0.15, 0.3, and 0.6 unit of enzyme per sample at 22 °C. CaCl2 was adjusted to 2.0 mM concomitantly with the addition of micrococcal nuclease. Digestions were terminated by the addition of EDTA (5 mM), SDS (0.25%, w/v), and proteinase K (1 μg/ml). The DNA was recovered, and fragments were separated by electrophoresis in nondenaturing 8% polyacrylamide gels (10).

Transcription Reactions—Dinucleosome complexes were reconstituted with a radiolabeled end-labeled DNA fragment derived from plasmid pX5319T–2 (23), which contains a tandem repeat of the 5 S DNA sequence, and were purified by sucrose gradient fractionation. 10 ng of purified 5 S dinucleosomes were incubated with various amounts of linker histones as indicated in the legend to Fig. 7 in 50 mM KCl at 22 °C for 20 min and then transcribed in an extract from Xenopus oocyte nuclei as described previously (23). Oocyte nuclear extract was prepared as described previously (24). Radiolabeled transcripts were extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis in 8% denaturing polyacrylamide gel. The radiolabeled 5 S DNA template served as internal control for recovery.

FIG. 1. Purification of bacterially expressed Xenopus H10. A, expression of H10 in bacterial cells. BL21(DE3) cells harboring a pET3d-H10 expression plasmid were induced with isopropyl-1-thio-galactopyranoside for 3 h. Whole bacterial cell lysates before and after induction were analyzed by protein gel electrophoresis. Lane 1, purified native calf thymus (Ct) H1s as a standard (arrow); lanes 2 and 3, before and after induction, respectively. B, purification of H10. Soluble extracts of induced cells were fractionated by Bio-Rex 70 ion exchange chromatography, and fractions were eluted with increasing NaCl concentrations, as indicated. H10 in the gel is indicated by the arrow (right).

FIG. 2. Nucleosome binding activity of recombinant H10. A, recombinant H10 binds preferentially to nucleosomes over naked DNA. Increasing quantities (0.3, 0.625, 1.25, 2.5, 5, and 10 ng) of native H1s purified from calf thymus (Ct) or recombinant (r) H10 were incubated with nucleosomes reconstituted with radioactively end-labeled 5 S DNA. Complexes were separated on agarose nucleoprotein gels, and the gels were autoradiographed. The positions of free DNA, nucleosomes (Nuc), and H1-nucleosomes (H1-Nuc) complexes are indicated. con, control. B, recombinant H10 efficiently promotes production of chromatosomes during a micrococcal nuclease digestion. Nucleosomes (40 ng) reconstituted with 5 S DNA internally labeled at position –23 within the core region (16) were incubated with 5 ng of the indicated linker histone protein, the complexes were digested with micrococcal nuclease, and the products of digestion were analyzed as described under “Experimental Procedures.” DNA fragments corresponding to nucleosome core (Core) and chromatosome (Ch) particles (146 and 168 bp, respectively) are indicated.

RESULTS

A recombinant linker histone, Xenopus H10, was expressed in bacterial cells and purified to homogeneity (Fig. 1, A and B). Previous studies have demonstrated that linker histones bind with higher affinity to DNA fragments reconstituted into nucleosomes than to the naked DNA (10). This preferential interaction is strong evidence for the recapitulation of the native structure-specific interaction between the linker histones and nucleosomes. Native linker histones isolated from calf thymus bind preferentially to reconstituted 5 S nucleosomes over naked 5 S DNA (Fig. 2A, lanes 1–6). We find bacterially expressed H10 binds reconstituted nucleosomes in an indistinguishable manner from the native calf thymus protein (Fig. 2A, lanes 8–13). Production of a stable intermediate during micrococcal nuclease digestion, which contains 168 bp of chromatosome-length DNA (4), has also been taken as diagnostic for the correct incorporation of H1 into chromatin complexes. We find that recombinant Xenopus H10 yields chromatosomes upon micrococcal nuclease digestion of reconstituted nucleosomes as efficiently as linker histones isolated from native chromatin (Fig. 2B).

The structure of the globular domain of the avian linker histone H5 has been solved by x-ray crystallography (17). Xenopus H10 (also referred to as Xenopus H5) shares significant

2 D. Clark, personal communication.
identity with avian H5 at both the nucleotide and amino acid levels (19, 25), especially within the globular domain region (~85% identity). Inspection of the structure of the globular domain of H5 reveals a cluster of relatively conserved amino acids on the side opposite of helix III (17) (Fig. 3). This cluster has been proposed to constitute a second DNA binding surface within the globular region, as inferred from biophysical studies of the globular domain binding to naked DNA (17, 26) (see “Discussion”). However, the physiologically relevant binding activity of linker histones involves the specific recognition and binding of a nucleoprotein substrate, not naked DNA.

To determine if this putative second DNA-binding domain is essential for specific binding of linker histone to the nucleosome core, we changed the basic amino acid residues at positions 40, 42, and 94 either singly or in combination to residues with uncharged side chains. We first tested the effects of the single substitutions on the preferential binding of mutated linker histones to nucleosomes by the gel shift assay (10). Three mutant proteins were prepared, K40M, R42V, and R94V, in the same way as the wild-type protein (see above). None of these single substitution mutations caused a loss of the preferential binding of H10 to nucleosomes over the naked DNA (Fig. 4A).

Interestingly, each of the three mutated proteins exhibits a detectable decrease in the well characterized propensity of linker histones to form aggregates with nucleic acids (25, 27). These aggregates are observed near the top of the nucleoprotein gel and ultimately do not enter the gel matrix (Fig. 4A). This is most apparent with the mutant K40M that does not form significant amounts of stable aggregates even at concentrations where H10wt completely aggregates all labeled material at the top of the gel (Fig. 4A, top left versus top right panel).

Nucleoprotein gels such as those presented in Fig. 4A were quantitatively analyzed to determine the relative nucleosome binding affinity of each of the mutant proteins. This analysis reveals that the affinity of these mutants for the 5 S nucleosome is not significantly different from that of wild-type H10. We find an apparent dissociation constant of 5 nM for H10wt, slightly higher than that previously reported for H1 isolated from calf thymus (Kd ~ 2 nM) (28). The apparent dissociation

Fig. 3. Model of H10 globular domain binding to DNA. An α-carbon backbone model of the globular domain of the avian H10 homologue H5, as determined by Ramakrishnan et al. (17), is shown. A, a potential DNA binding surface as predicted from homology of the GH5 structure to several sequence-specific DNA binding proteins for which the co-crystal structures have been solved (17). B, a cluster of several basic amino acid residue side chains that comprises a second putative DNA binding surface. This surface is located on the opposite side of the domain as that shown in A, and the critical residues are indicated by the numbered arrows and are pictured as space-filling models. This figure was generated with the program RasMol.

Fig. 4. Effect of single substitutions within a putative DNA-binding domain of H10 on binding to nucleosomes. A, nucleoprotein gel shift assay of the nucleosome binding behavior of H10wt and the single substitution mutants. Linker histone binding was analyzed as in

Fig. 2A. The positions of free DNA, nucleosome (Nuc), and H10-nucleosome complexes (H10-Nuc) are indicated. Nucleosomes were incubated as indicated above the gels with 0, 0.3, 0.9, 2.5, 5, 10, and 20 ng of H10wt, R42V, or R94V or 0, 0.15, 0.3, 0.9, 2.5, 5, and 10 μg of K40M (lanes 1–7, respectively). B, binding titration of H10wt and single substitution mutants. The fraction of linker histone-bound nucleosomes observed on autoradiographs of several gel mobility shift experiments was determined as described and plotted against concentration (28). Protein concentrations were determined as described under “Experimental Procedures.” Boxed numbers represent the Kd from the estimated concentration at 50% saturation. Estimated random error is ±2 nM.
constant for the mutant R42V (8 nM) is only marginally greater (1.5-fold) than that measured for H10wt. Interestingly, the substitution K40M actually appears to slightly increase the stability of binding ($K_d = 2$ nM), whereas changing arginine at position 94 to an uncharged residue (R94V) has little effect on the apparent dissociation constant ($K_d = 5$ nM).

To increase the probability of observing changes in the nucleosome binding behavior of H10 as a result of these mutations, a double mutant (K40M/R42V) and a triple mutant (K40M/R42V/R94V) were prepared and tested in an extended binding titration with reconstituted 5S nucleosomes. The double and triple mutants were observed to bind preferentially and to reconstituted 5S nucleosomes with affinities nearly identical to that of the wild-type protein (Fig. 5, A and B).

The production of chromatosomes during micrococcal nuclease digestion has been taken as strong evidence for the correct incorporation of linker histones into chromatin (4). We first tested the effect of the single substitutions on the ability of H10 to bind 5S nucleosomes and generate chromatosomes upon digestion with micrococcal nuclease. In every case, chromatosome-length DNAs were clearly observed when the products of digestion were analyzed by native gel electrophoresis (Fig. 6A). Moreover, chromatosome-length DNA was also generated when nucleosomes were bound by either the doubly (K40M/R42V) or triply (K40M/R42V/R94V) mutated H10 (Fig. 6B).

We next tested the effect of the above mutations on the ability of H10 to repress transcription from a chromatin template. A short linear DNA construct that contains two tandemly repeated units of the Xenopus 5S RNA gene fragment has been shown to be efficiently transcribed by RNA polymerase III and associated class III factors in nuclear extracts prepared from Xenopus oocytes (23, 24). Likewise, the 5S genes within dinucleosome complexes reconstituted with this construct can be transcribed in these extracts. Previous work has shown that linker histones bind to dinucleosome complexes and significantly repress transcription. Furthermore, the degree of transcriptional repression observed closely parallels the affinity with which a particular linker histone variant binds to the dinucleosomal substrate (29). Therefore, we compared the ability of H10wt with the triple mutant K40M/R42V/R94V to repress transcription from the 5S dinucleosome template. When added at a ratio of about 1 molecule/nucleosome, both proteins efficiently repress transcription to approximately 10% of that observed in the absence of added linker histones (Fig. 7). At a ratio of 2 molecules/nucleosome, 5S RNA products are no longer detectable on the gel, indicating that transcription is...
FIG. 6. Substitution mutants of H1\(^{wt}\) still generate chromatosomes during micrococcal nuclease digestion. Wild type and mutant H1\(^{wt}\) with single (A) or double substitutions (B) as indicated above the lanes were incubated with nucleosome cores reconstituted with 5 S DNA containing an internal radioactive label. The resulting complexes were digested with micrococcal nuclease, and the products of digestion were analyzed on nondenaturing acrylamide gels. Concentrations are as in Fig. 2B. A, lanes 1, 4, 7, 10, and 13 were digested for 10 min; lanes 2, 5, 8, 11, and 14 were digested for 5 min; and lanes 3, 6, 9, 12, and 15 were digested for 2 min as described under “Experimental Procedures.” B lanes 1, 3, 6, and 9 were digested for 10 min; lanes 2, 4, 7, and 10 were digested for 5 min; and lanes 5, 8, and 11 were digested for 2 min. Lanes marked as Control were digested in the absence of linker histones. The positions of the 146-base pair core particle DNA (Core) and the 168-base pair chromatosome DNA (Ch) are indicated. 40/42, K40M/R42V; 40/42/94, K40M/R42V/R94V.

DISCUSSION

The association of a single molecule of a linker histone with the core histone octamer-DNA complex completes the formation of the nucleosome proper. Studies of linker histones have shown that these proteins bind specifically to the exterior surface of the nucleosome, and the globular domain of H1 alone is responsible for this structure-specific recognition (6, 10, 14). Unfortunately, little is known about the details of this important interaction, and several models have been proposed (6, 15, 25).

Biophysical studies of linker histones demonstrate that these proteins bind strongly and cooperatively to naked DNA (30). The complexes formed appear to include several DNA strands linked together by a central core of linker histone proteins in a structure referred to as a tramtrack configuration. This cooperative binding behavior is evidence of the existence of at least two sites for DNA interaction within the linker histone protein (30). Clearly the relatively unstructured N- and C-terminal tail domains in linker histone, which contain the majority of the excess positive charges in these proteins, are likely candidates for some of the sites of interaction with DNA. Interestingly, cooperative aggregation of naked DNA to form tramtrack-like structures also has been demonstrated with the globular domain of linker histone alone (26, 31). Furthermore, the globular domain has been shown to bind with high affinity to immobile 4-way junctions (32). The simplest explanation for these observations is that at least two surfaces exist on this small structured domain that can interact with naked DNA. Inspection of the structure of the globular domain of the avian linker histone variant H5 has led to the identification of two potential DNA binding surfaces (17) (see Fig. 2). One surface encompasses helix III, and a second is comprised of a cluster of basic residues on the opposite face of this domain (see Fig. 2).

Linker histones specifically recognize and bind to a nucleoprotein substrate in vitro (6). Moreover, H1 does not bind cooperatively to chromatin under the same conditions that it binds cooperatively to DNA (33). The binding of H1 in chromatin may also involve specific protein-protein interactions (15, 27, 34). Furthermore, the binding of a single molecule of H1 to mono-nucleosomes occurs preferentially over the cooperative binding of multiple H1s to naked DNA (10). These observations suggest that the binding of linker histones in chromatin occurs by a drastically different mechanism than the binding to naked DNA.

Several different models exist for the specific interaction of linker histones on the surface of the nucleosome core. A crucial difference between these models is the predicted number of contacts to DNA by the globular domain of the linker histone. One model predicts that at least two (and perhaps three) separate surfaces of the globular domain interact with DNA, consistent with the mode of H1 binding to naked DNA (16). Alternative models based on hydroxyl radical footprinting, protein-DNA cross-linking, and neutron diffraction data suggest that only a single surface of the globular domain is involved in binding to DNA in chromatin (15, 27, 35). This model suggests that the globular domain makes a single contact with DNA and also contacts histone H2A within the nucleosome core (27).

Evidence suggests that the surface of the globular domain, which includes helix III, interacts with DNA when linker histones are bound in chromatin. Several conserved lysines within or in the vicinity of helix III are relatively protected from reductive methylation by association with the nucleosome core (36), and mutation of one of these residues (Lys-85) reduces the ability of the protein to generate a chromatosome stop (37). However, little is known about the relevance of basic residues at positions 40, 42, and 94, which comprise a second putative DNA binding surface for the specific binding of linker histones in chromatin. We have assessed the relevance of these residues for the physiological binding activity of a somatic linker histone variant from Xenopus, H1\(^{\circ}\). We find that these residues are neither individually nor in combination essential for the specific nucleosome binding activity. These results may imply that this putative DNA binding surface, although possibly important for cooperative binding and aggregation of DNA in vitro, does not play a role in the structure-specific recognition and binding of the nucleosome by linker histones in vitro. Indeed, mutation of lysine at position 40 to an uncharged amino acid residue results in a protein that exhibits a much reduced ability to aggregate nucleic acids but an increased affinity for binding to the nucleosome core. These results support a new model for
the specific interaction of linker histones with the nucleosome core that envisions only a single interaction between DNA and the globular domain of linker histones (27).

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Addendum—Goytisolo et al. (38) recently reported the effects of similar mutations in a polypeptide spanning the globular domain of histone H5. Contrary to our results with a full-length linker histone protein, these authors do not observe a chromatosome stop when this peptide is mixed with bulk oligonucleosomes containing mixed sequences. Future experiments will address the basis for this discrepancy.

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