Identification of Specific Functional Subdomains within the Linker Histone H10 C-terminal Domain

Xu Lu‡ and Jeffrey C. Hansen‡§¶

Received for publication, October 15, 2003, and in revised form, December 5, 2003
Published, JBC Papers in Press, December 10, 2003, DOI 10.1074/jbc.M311348200

Linker histone binding to nucleosomal arrays in vitro causes linker DNA to form an apposed stem motif, stabilizes extensively folded secondary chromatin structures, and promotes self-association of individual nucleosomal arrays into oligomeric tertiary chromatin structures. To determine the involvement of the linker histone C-terminal domain (CTD) in each of these functions, and to test the hypothesis that the functions of this highly basic domain are mediated by neutralization of linker DNA negative charge, four truncation mutants were created that incrementally removed stretches of 24 amino acids beginning at the extreme C terminus of the mouse H10 linker histone. Native and truncated H10 proteins were assembled onto biochemically defined nucleosomal arrays and characterized in the absence and presence of salts to probe primary, secondary, and tertiary chromatin structure. Results indicate that the ability of H10 to alter linker DNA conformation and stabilize condensed chromatin structures is localized to specific C-terminal subdomains, rather than being equally distributed throughout the entire CTD. We propose that the functions of the linker histone CTD in chromatin are linked to the characteristic intrinsic disorder of this domain.

Linker histones comprise a family of small nucleosome-binding proteins that have a short N terminus, a central winged helix-like globular domain, and a long, highly basic C-terminal domain (CTD)1 (1–3). Binding of linker histones to nucleosomal arrays in vitro influences chromatin fiber structure at multiple levels. It has been widely observed that linker histones protect an additional 20 bp of DNA from micrococcal nuclease digestion (2–4). Upon direct examination, nucleosome protection appears to result from linker histone-linker DNA interactions involved in the formation of an apposed linker DNA stem motif (5), rather than from wrapping additional DNA around the nucleosome as was initially believed (2, 3). Linker histones also have important functions in chromatin condensation. They stabilize locally folded secondary chromatin structures, e.g., “30-nm diameter fiber” (2, 3, 6, 7), and facilitate the self-association of fibers into oligomeric tertiary chromatin structures thought to be relevant to global chromosomal fiber organization (6–8). The linker histone globular domain plays a major role in protecting additional DNA from nuclease digestion (2, 3). In contrast, the ability to stabilize extensively folded secondary chromatin structures lies exclusively in the linker histone CTD (9–11). The linker histone domains that facilitate fiber self-association have yet to be defined.

The biochemical properties of the linker histone CTD are enigmatic. In most isoforms, this domain consists of ~100 amino acid residues. There is no CTD sequence conservation among the linker histone isoforms (2, 3). However, ~40% of each somatic linker histone CTD consists of lysine residues that are very evenly distributed throughout the domain (12). The fact that peptides derived from the linker histone CTD have no detectable secondary structure in solution (3), together with the extensively basic character of this domain, has led to the proposal that the CTD functions in chromatin as an unstructured cationic stretch of amino acids that binds to linker DNA and neutralizes negative charges (11, 12).

There are, however, several hints that the structural features of the linker histone CTD may be more complex. In addition to the large percentage of basic amino acid residues, alanine, serine, threonine, and proline residues also are frequently found in the CTD of mouse H10, chicken H5, and all human somatic linker histones. By contrast, the CTDs of all linker histone isoforms are almost completely deficient in the acidic, aromatic, and highly hydrophobic amino acids. Protein domains having this distinctive amino acid composition are thought to possess “intrinsic disorder” (13–16), which is characterized by molten globule-like structure in the native state. Intrinsically disordered regions frequently assume classical secondary structure when interacting with other macromolecules (13–16). Consistent with this notion, the CTD peptides adopt a detectable α-helical structure when interacting with DNA (17, 18), and also in high salt solutions and organic solvents (17, 19–21). The CTD of all somatic linker histones also contain one or more S/TPKK DNA binding sequences (17, 19, 22, 23), which form β-turn motifs that bind to the DNA minor groove (22, 24) and mediate condensation of naked DNA in vitro (25–27).

In the present study we use biochemically defined model systems to probe the mechanistic basis of linker histone CTD function in chromatin. We initially compared the biochemical properties of native chicken erythrocyte H5 and recombinant mouse H10. Subsequently, the structural and functional effects of incremental deletions of the mouse H10 CTD were determined. Our results indicate that distinct subdomains within the CTD are responsible for mediating linker histone effects on linker DNA conformation, and stabilization of condensed chromatin fiber structures. A revised mechanism for CTD function in chromatin is proposed.
EXPERIMENTAL PROCEDURES

Construction of Mouse Histone H10 C-terminal Deletion Mutants—

The *Escherichia coli* strain XL-1 Blue and BL21(DE3) pLysS transformed with the plasmid pET-H10 were gifts from Dr. Susan Wellman. pET-H11-11d consists of the wild type mouse H10 cDNA cloned into expression vector pET-11d (28). DNA sequences encoding C-terminal deletion mutants H10CΔ24, H10CΔ48, H10CΔ72, and H10CΔ97 (see Fig. 2A) were synthesized by PCR using the following oligonucleotide primers: TACATTGGTACCGAGAACTCC and TTGGACCGAGGTGAGCTAGG for H10CΔ24; TACATTGGTACCGAGAACTCC and TTGGACCGAGGTGAGCTAGG for H10CΔ48; TACATTGGTACCGAGAACTCC and TTGGACCGAGGTGAGCTAGG for H10CΔ72; and TACATTGGTACCGAGAACTCC and TTGGACCGAGGTGAGCTAGG for H10CΔ97. PCR products were subcloned into pET-11d using standard procedures (29) to yield expression vectors pET-H10CΔ24-11d, pET-H10CΔ48-11d, pET-H10CΔ72-11d, and pET-H10CΔ97-11d, and propagated in *E. coli* strain XL-1 Blue. The identities of the constructs were confirmed by restriction mapping and sequencing of purified plasmid DNAs.

Histone Purification—Core histone octamers and native chicken erythrocyte linker histone H5 were purified as described previously (30, 31). To obtain full-length and mutant H10 proteins, pET-H11-11d, pET-H10CΔ24-11d, pET-H10CΔ48-11d, and pET-H10CΔ72-11d were transformed into *E. coli* BL21(DE3) pLysS-competent cells. The transformed cells were grown at 37 °C, harvested, and washed as described (28). The cells were sonicated, and the H10 proteins were purified as described (32, 33) with minor modifications. Cell pellets were sonicated in 3 volumes of lysis buffer (25 mM Tris-HCl, 2.5 mM sodium chloride, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA). The pH of the lysis buffers were 8.3, 8.3, 8.0, 8.0, and 7.8 for H10, H10CΔ24, H10CΔ48, H10CΔ72, and H10CΔ97, respectively. Sonicated cells were incubated on ice for 30 min, and then pelleted. The NaCl concentration of the supernatants was decreased to 0.3 M by dilution, the supernatants were mixed with pre-hydrated CM-Sephadex C-25 (Sigma), and the mixtures gently rocked for 3 h at 4 °C to allow the binding of the H10 proteins to the C-25. The mixtures were centrifuged to pellet the C-25, the resin was washed in 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 300 mM NaCl at the same pH as that of the lysis buffer, and the pelleted resin was loaded onto a C-25 column (2.5 × 25 cm) pre-equilibrated with the appropriate buffer. The proteins were eluted from the C-25 column with a 0.3–1.0 M NaCl gradient in 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride at the same pH as the lysis buffer. At the completion of these steps, H10, H10CΔ48, and H10CΔ97 required no further purification. However, we routinely observed that the fractions containing H10CΔ24 and H10CΔ72 were contaminated with degradation products. Consequently, C-25 fractions containing either H10CΔ24 or H10CΔ72 were combined, adjusted by dilution to 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, ~300 mM NaCl at the same pH as that of the lysis buffer, and loaded onto a 5-ml HiTrap SP HP column (Amersham Biosciences). The mutant H10 proteins were then eluted with the same gradients used for the C-25 column. The purity of all H10 proteins was determined by SDS-PAGE and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. The concentrations of the purified proteins were determined using a BCA protein assay kit (Pierce).

Assembly of Chromatin Model Systems—The 208-12 DNA template containing 12 tandem 208-bp repeats of *Lytechinus variegatus* 5 S rDNA (34, 35) was purified as described (6). Preparations of 208-12 nucleosomal arrays consisting of ~30% saturated arrays (12 nucleosomes/template) and ~50% unsaturated arrays (10–11 nucleosomes/template) were assembled by salt dialysis as described (6). Binding of H5 and H10 to 208-12 nucleosomal arrays was achieved by incubating linker histone with nucleosomal arrays (150 μg/ml) in 10 mM Tris, 0.25 mM EDTA, 50 mM NaCl, pH 7.8, for 3 h on ice at various molar ratios (r) of linker histone to 208-bp DNA repeat followed by dialysis of the samples against 10 mM Tris, 0.25 mM EDTA, 2.5 mM NaCl, pH 7.8 (TEN), overnight at 4 °C (6, 7).

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed using either a Beckman XL-A or XL-I ultracentrifuge as described (6, 7, 36). Boundaries were analyzed by the method of van Holde and Weischat (37) using Ultrascan (version 5.0) software. Data were plotted as boundary fraction versus sedimentation coefficients, S(s). Average sedimentation coefficients were obtained at boundary fractions equal to 0.5 of the S(s) plot. For folding experiments, arrays were mixed with an equal volume of 2 × MgCl₂ stock solutions prior to sedimentation to obtain the desired final MgCl₂ concentration. The final absorbance at 280 nm of all samples was 0.6–0.8.

Agarose Multigel Electrophoresis—Electrophoretic mobilities, μ, were measured in a multigel composed of 9 individual agarose gels running in concentration from 0.2 to 1.0%. The casting and running buffer was 40 mM Tris acetate (pH 7.8), 0.25 mM Na₂EDTA. Samples were loaded and electrophoresed at 1.33 V/cm for 6 h. The temperature was 24 ± 3 °C. The gels were stained with ethidium bromide and the gel image was digitized. For each individual band, the migration was measured from the center of the well to the center of the band using NIH Image software and subsequently converted to μ. The effective macromolecular radius (Rₑ) and free-electrophoretic mobility (μₑ) were obtained from μ as described (6, 7).

Self-association Assay—The salt-dependent self-association of H5 and H10-bound nucleosomal arrays was determined using a differential centrifugation assay as described (6, 36). For each salt concentration assayed, data were plotted as the percentage of the initial sample absorbance that remained in the supernatant after centrifugation at 16,000 × g for 5 min in a microcentrifuge.

RESULTS

The Ability of Chicken Erythrocyte H5 and Recombinant Mouse H10 to Bind to Nucleosomal Arrays, Alter Linker DNA Conformation, and Stabilize Condensed Secondary and Tertiary Chromatin Structures, Is Indistinguishable—The experiments described below extend our previous studies of linker histone structure-function relationships (6, 7, 38) by investigating how specific deletion mutations in the linker histone CTD influence the primary, secondary, and tertiary structure of biochemically defined nucleosomal arrays. The linker histone used in our earlier experiments was H5 purified from chicken erythrocytes. However, given that recombinant chicken erythrocyte H5 is expressed very poorly in bacterial cells (39), this linker histone isoform is a poor candidate for mutagenesis studies. Because the H10 isoform is considered to be the mammalian functional homologue of chicken H5 (40, 41), and mouse H10 can be readily expressed and purified from *E. coli* (28), recombinant mouse H10 was chosen for all studies involving recombinant proteins.

To determine whether the in vitro properties of purified chicken H5 and recombinant mouse H10 were the same, we directly compared the binding of these linker histone isoforms to biochemically defined nucleosomal arrays, and characterized their respective abilities to stabilize condensed chromatin structures in salt (Fig. 1). Binding was assayed by determining the increase in average S(ro) of 208-12 nucleosomal arrays in low salt TEN buffer as a function of the molar linker histone input ratio (r[H]). Under these low salt conditions, nucleosomal arrays and chromatin fibers are unfolded, and there is no contribution to the S(ro) from higher order folding. Instead, the increase in S(ro) results from a combination of the mass of the bound linker histone, and a decreased array frictional coefficient (i.e. shortening of the overall length of the unfolded 12-mer array) because of linker histone-dependent formation of an apposed linker DNA stem motif (5). Our previous studies using native chicken erythrocyte H5 showed that binding of approximately one H5 per nucleosome was achieved at r[H] = 1.3, and led to an increase in average S(ro) in low salt from 29 to 36 S (Refs. 6 and 7; also see Table 1). Binding of native chicken erythrocyte H5 and recombinant mouse H10 to 208-12 nucleosomal arrays is shown in Fig. 1A. Consistent with our previous studies, both linker histone isoforms caused an increase in average S(ro) with increasing r[H], until a narrow plateau region was achieved at r[H] = 1.2–1.3. The average S(ro) in this plateau region was 36.8 ± 0.7 S for chicken erythrocyte H5 and 35.9 ± 0.3 S for recombinant H10. We previously have shown that the plateau is narrow because the average S(ro) increases rapidly at higher r[H] values because of nonspecific linker histone binding and aggregation (Fig. 1A) (6, 7). The data in Fig. 1A demonstrate that, within experimental error, stoichiometric
were mixed with equal volumes of 2× H10-bound 208-12 chromatin fibers in 0.25 and 0.5 mM MgCl2. H5- and H10-bound nucleosomal arrays were assembled with each linker histone at a ratio (r), and the average s20,w determined by analytical ultracentrifugation as described under "Experimental Procedures." Each data point represents the mean ± S.D. of two to three experiments. The G(s) plots of H5 and H10 arrays in TEN ± 0.25 or 0.5 mM MgCl2 are shown in Fig. 1B. In TEN, ~50% of both the H5- and H10-bound arrays sedimented at 36–37 S (boundary fraction ≥50%), indicating that this fraction consisted of 208-12 DNA templates stoichiometrically bound with 12 histone octamers/template and 1 linker histone per nucleosome (see Fig. 1A) (6, 7). The remainder of the templates (boundary fraction ≤50%) contained substoichiometric amounts of histone octamers and (or) linker histones, rendering this fraction of the samples uninformative for fiber stability studies (6, 7). The G(s) plots of the saturated H5- and H10-bound arrays in the presence of 0.25 and 0.5 mM MgCl2 were essentially identical (Fig. 1B). Notably, in 0.5 mM MgCl2, the saturated fraction of both the H5 and H10 arrays formed homogeneous populations of extensively condensed ~55 S particles, as indicated by the nearly vertical G(s) plots. These data indicate that native chicken erythrocyte H5 and recombinant mouse H10 were equally effective at stabilizing the highly folded 55 S secondary chromatin structures formed by 12-mer nucleosomal arrays in 0.5 mM MgCl2.

We next analyzed the cooperative self-association of nucleosomal arrays bound to chicken H5 and mouse H10. Self-association occurs independently of folding (43, 44) and has been proposed to be an in vitro manifestation of long-range fiber-fiber interactions that occur in intact chromosomes (8, 44). It has been shown previously that nucleosomal arrays bound to chicken H5 self-associate at a significantly lower MgCl2 concentration than nucleosomal arrays alone, i.e. 0.5 versus 2.0 mM, respectively (6). We found that the salt dependence of self-association of H5- and H10-bound arrays was identical within experimental error (Fig. 1C). Taken together, the data

binding of native chicken erythrocyte H5 and recombinant mouse H10 to 208-12 nucleosomal arrays was achieved at the same molar linker histone input ratio, and led to the same increase in sedimentation coefficient under low salt conditions.

We next examined the salt-dependent condensation of H5- and H10-bound nucleosomal arrays. In vitro condensation consists of intramolecular folding in the range of 0.1–0.5 mM MgCl2 followed by cooperative array self-association at higher MgCl2 concentrations (6, 7). Folding of the biochemically defined model systems was characterized by sedimentation velocity, and the data were analyzed by the method of van Holde and Weischedel (37) to yield the integral distribution of sedimentation coefficients, G(s), of the entire sample (6, 7, 42). The G(s) plots of H5 and H10 arrays in TEN ± 0.25 or 0.5 mM MgCl2 are shown in Fig. 1B. In TEN, ~50% of both the H5- and H10-bound arrays sedimented at 36–37 S (boundary fraction ≥50%), indicating that this fraction consisted of 208-12 DNA templates stoichiometrically bound with 12 histone octamers/template and 1 linker histone per nucleosome (see Fig. 1A) (6, 7). The remainder of the templates (boundary fraction ≤50%) contained substoichiometric amounts of histone octamers and (or) linker histones, rendering this fraction of the samples uninformative for fiber stability studies (6, 7). The G(s) plots of the saturated H5- and H10-bound arrays in the presence of 0.25 and 0.5 mM MgCl2 were essentially identical (Fig. 1B). Notably, in 0.5 mM MgCl2, the saturated fraction of both the H5 and H10 arrays formed homogeneous populations of extensively condensed ~55 S particles, as indicated by the nearly vertical G(s) plots. These data indicate that native chicken erythrocyte H5 and recombinant mouse H10 were equally effective at stabilizing the highly folded 55 S secondary chromatin structures formed by 12-mer nucleosomal arrays in 0.5 mM MgCl2.

We next analyzed the cooperative self-association of nucleosomal arrays bound to chicken H5 and mouse H10. Self-association occurs independently of folding (43, 44) and has been proposed to be an in vitro manifestation of long-range fiber-fiber interactions that occur in intact chromosomes (8, 44). It has been shown previously that nucleosomal arrays bound to chicken H5 self-associate at a significantly lower MgCl2 concentration than nucleosomal arrays alone, i.e. 0.5 versus 2.0 mM, respectively (6). We found that the salt dependence of self-association of H5- and H10-bound arrays was identical within experimental error (Fig. 1C). Taken together, the data

TABLE I

| Stoichiometrya | Stoichiometryb | Stoichiometryc | Stoichiometryd |
|----------------|----------------|----------------|----------------|
| NA              | 1.0            | 1.0            | 1.0            |
| H5              | 1.3            | 1.5            | 1.0            |
| H10             | 1.0            | 1.0            | 1.0            |
| H10:24          | 1.5            | 1.5            | 1.5            |
| H10:348         | 2.0            | 1.5            | 1.0            |
| H10:372         | 2.3            | 2.3            | 2.3            |
| H10:397         | 2.3            | 2.3            | 2.3            |

a Calculated total macromolecular charge.

b Stoichiometry = [m(octamer)]/[m(linker histone)]/[m(LH)]=1

c Parent 208-12 nucleosomal arrays.

d Values represent the mean ± S.D. of two to three determinations.

H5 stoichiometries calculated by the same method in previous studies were 1.3 ± 0.2 (6) and 1.4 ± 0.2 (7).

FIG. 1. Linker histones H5 and H10 function indistinguishably in chromatin in vitro. A, increases in the array average s20,w upon linker histone binding. Biochemically defined 208-12 nucleosomal arrays were incubated with increasing linker histone to nucleosome molar ratio (r), and the average s20,w determined by analytical ultracentrifugation as described under “Experimental Procedures.” Each data point represents the mean ± S.D. of two to three experiments. The inset shows the R2 of the same samples as a function of increasing r, as determined by agarose multigel electrophoresis. B, folding of H5- and H10-bound 208-12 chromatin fibers in 0.25 and 0.5 mM MgCl2. H5- and H10-bound chromatin fibers were assembled with each linker histone at r = 1.3 as described under “Experimental Procedures.” The complexes were mixed with equal volumes of 2× MgCl2 solution, and analyzed by sedimentation velocity. Shown are the integral distributions of sedimentation coefficients of the samples, G(s), plotted as boundary fraction versus s20,w. The data are representative of three independent experiments. Symbols: H5(TEN), □; H5 (0.25 mM MgCl2), ▪; H10 (0.25 mM MgCl2), ●; H10 (0.5 mM MgCl2), ▲; H10 (1.0 mM MgCl2), ○; and H10 (2.0 mM MgCl2), △. C, self-association of H5 (■) and H10 (▲) bound 208-12 nucleosomal arrays. Self-association was analyzed by differential centrifugation as described under “Experimental Procedures.” Each data point represents the mean ± S.D. of two to three experiments.
in Fig. 1, B and C, demonstrate that native chicken erythrocyte H5 and recombinant mouse H1° were equally effective at stabilizing salt-dependent secondary and tertiary chromatin structures in vitro.

Binding of H1° C-terminal Deletion Mutants to Nucleosomal Arrays—The mouse H1° C-terminal domain consists of 97 amino acid residues. To determine the extent to which the CTD functions through nonspecific DNA charge neutralization, four truncation mutants were created that incrementally deleted an approximately equal number of basic amino acid residues from the H1° CTD. Specifically, 24, 48, 72, or 97 amino acid residues were deleted beginning from the C-terminal end of H1°, yielding mutants termed H1°CΔ24, H1°CΔ48, H1°CΔ72, and H1°CΔ97 (Fig. 2A). After purification, the full-length H1° and H1° truncation mutants were judged to be homogeneous by SDS-PAGE (Fig. 2B).

Binding of the H1° truncation mutants to biochemically defined nucleosomal arrays was monitored by sedimentation velocity as in Fig. 1A. In marked contrast, the plateau s values increased as the H1° CTD was incrementally deleted; the middle of the plateau region for full-length H1°, H1°CΔ24, H1°CΔ48, H1°CΔ72, and H1°CΔ97 was 1.2, 1.3, 1.7, 2.2, and 2.2, respectively. These results suggest either that deletion of the CTD decreases the relative binding affinity of linker histones for nucleosomal arrays, or that there are substantive differences in linker histone stoichiometry at the respective plateau regions. Because the μs term obtained from agarose multigel electrophoresis is directly proportional to macromolecular surface charge density, linker histone stoichiometries can be estimated with reasonable accuracy using the measured μs values, and the total macromolecular charge of the respective linker histones and parent nucleosomal arrays (6, 7, 42). The stoichiometries calculated for the full-length and mutant arrays at their respective plateau regions are shown in Table 1. Whereas there is some scatter in the data, there is no evidence that full-length H1° or the H1° mutant CTD have a markedly greater stoichiometry at their plateau regions compared with native H5. The H1°CΔ97 mutant had the greatest stoichiometry (1.7 linker histones per nucleosome), but nevertheless, was unable to direct formation of the 36 S/22 nm conformation, removal of the C-terminal most 72 residues had a small effect at best, whereas removal of the entire 97-residue CTD completely abolished the ability of H1° to form the 36 S/22 nm conformation.
conformation. We therefore conclude that the affinity of linker histones for nucleosomal arrays is dictated in part by the CTD.

Salt-dependent Folding—Sedimentation velocity experiments in salt were used to assay the extent to which truncations in the CTD influenced the ability of H10 to stabilize folded secondary chromatin structures (6, 7). The G(s) plot in TEN of the samples used for these experiments is shown in Fig. 4A. Folding subsequently was determined in TEN containing 0.25 or 0.5 mM MgCl2 (Fig. 4, B and C). The lower MgCl2 concentration induces intermediate extents of folding, whereas the higher concentration stabilizes the saturated fraction of H10-bound 208-12 nucleosomal arrays in the maximally folded 55 S conformation (Fig. 1B) (6). Assuming a charge neutralization-based mechanism of CTD function (2, 3, 12), our expectation was that incremental deletions in the CTD would cause an incrementally reduced extent of folding at both MgCl2 concentrations. This would be indicated by successively left-shifted G(s) plots relative to those of H10-bound arrays. However, the G(s) plots of saturated full-length H10 and H10C/ H900424-bound arrays were identical in TEN buffer containing 0.25 (Fig. 4B) and 0.5 mM MgCl2 (Fig. 4C). Furthermore, whereas the extent of folding of H10C/ H900448-bound arrays was significantly reduced in both salt concentrations, the G(s) plots of the H10C/ H900472- and H10C/ H900497-bound arrays were superimposed. Finally, the G(s) plots of H10C/ H900497-bound arrays in 0.25 and 0.5 mM MgCl2 were essentially identical to those of the parent 208-12 nucleosomal arrays, indicating complete loss of linker histone-dependent stabilization of salt-dependent chromatin folding. The inability of the N terminus and globular domain alone to stabilize folded chromatin fibers has been observed previously (10, 11). The data in Fig. 4 demonstrate that the ability of the H10 CTD to stabilize folded secondary chromatin structures is not spread evenly throughout the entire domain. Instead, the sequences that mediate folding are localized to two discrete 24-amino acid stretches within the 97-amino acid domain (see Fig. 6).

Salt-dependent Self-association—The self-association of 208-12 nucleosomal arrays containing 208-12 nucleosomal arrays containing bound H10 proteins was analyzed by differential centrifugation as described under "Experimental Procedures." Each data point represents the mean ± S.D. of two to three experiments. Symbols: nucleosomal arrays (NA), ○; full-length H10, line; H10C/ H900424, ■; H10C/ H900448, ○; H10C/ H900472, ▲; H10C/ H900497, ▼.

FIG. 4. Salt-dependent formation of folded, secondary chromatin structure. 208-12 nucleosomal arrays containing bound H10 proteins were analyzed by sedimentation velocity in TEN alone (A) or TEN containing 0.25 (B) or 0.5 mM (C) MgCl2. Shown are the G(s) plots obtained from the sedimentation velocity data. Symbols in panels A–C: nucleosomal arrays, ○; full-length H10, dashed line; H10C/ H900424, ■; H10C/ H900448, ○; H10C/ H900472, ▲; H10C/ H900497, ▼. The r² of the samples used in these experiments was the same as indicated in Table I.

FIG. 5. Salt-dependent formation of oligomeric, tertiary chromatin structure. 208-12 nucleosomal arrays containing bound H10 proteins were analyzed by differential centrifugation as described under "Experimental Procedures." Each data point represents the mean ± S.D. of two to three experiments. Symbols: nucleosomal arrays (NA), ○; full-length H10, line; H10C/ H900424, ■; H10C/ H900448, ○; H10C/ H900472, ▲; H10C/ H900497, ▼.

FIG. 6. Different regions of linker histone H10 have different functions when interacting with DNA. The CTD regions identified in this study that are important for altering linker DNA conformation (L), stabilizing folding (F), and facilitating self-association (SA) are shown.
the ability of H10 to promote self-association resides in both the globular domain and the first 25 amino acids C-terminal to the globular domain. In contrast, the 72 most H10 C-terminal residues do not contribute to this process. MgCl$_2$, which is an intermediate concentration relative to that observed for arrays bound to full-length H10, H10C324, and H10C48. The H10C72-bound arrays did require a slightly greater amount of MgCl$_2$ to achieve 50% self-association, although whether this small difference is significant is difficult to discern because of the highly cooperative nature of the self-association transition (Fig. 5). In contrast, 50% self-association of H10C397-bound arrays occurred at 2.5 mM MgCl$_2$, which is an intermediate concentration relative to that observed for arrays bound to full-length H10 (1.5 mM MgCl$_2$), and nucleosomal arrays alone (3.3 mM MgCl$_2$) (Fig. 5). Thus, the ability of H10 to promote self-association resides in both the globular domain and the first 25 amino acids C-terminal to the globular domain. In contrast, the 72 most H10 C-terminal residues do not contribute to this process.

**DISCUSSION**

Our results have provided new insight into the molecular mechanism of action of the linker histone CTD in chromatin. Key findings are summarized in Fig. 6. Historically, there is a deeply ingrained perception that the CTD behaves as an unstructured polycationic domain that binds linker DNA and neutralizes negative charge, thereby permitting close approach of neighboring nucleosomes and stabilizing condensed chromatin structures (2, 11, 12, 45). However, evidence in favor of this model is largely theoretical (12, 45). By constructing specific deletion mutants of the H10 CTD and assembling the mutants into biochemically defined model systems, we have performed the first direct experimental test of the charge neutralization hypothesis in a bona fide chromatin environment. A charge neutralization-based mechanism predicts that the functions mediated by the CTD, i.e., the intrinsic disorder does not automatically equate to unstructured polypeptide coils, but is thought to often reflect stretches of polypeptides that have molten globule-like properties in their native state (13–16). One of the most common characteristics of intrinsically disordered regions is that they become

**Table II**

| Isoform | Lys | Ala | Pro | Ser | Thr | Val | Gly | Arg | Glu | Asp | Phe | Ile | Leu | Asn | Gln | Met | His | Cys | Trp | Tyr | O | O | O |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| cH5     | 32  | 16  | 9   | 11  | 3   | 3   | 2   | 13  | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| mH10    | 40  | 17  | 12  | 7   | 5   | 9   | 1   | 2   | 2   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| hH10-1  | 41  | 17  | 12  | 7   | 6   | 6   | 1   | 1   | 2   | 1   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| hH10-2  | 41  | 25  | 15  | 4   | 6   | 5   | 6   | 1   | 0   | 0   | 0   | 0   | 0   | 1   | 0   | 1   | 0   | 0   | 0   | 0   | 0   | 0   |
| hH10-3  | 45  | 37  | 13  | 2   | 3   | 4   | 4   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| hH10-4  | 43  | 35  | 14  | 3   | 4   | 1   | 4   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| hH10-a  | 37  | 19  | 11  | 7   | 10  | 6   | 3   | 2   | 0   | 0   | 0   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |

The total number of each amino acid residue in the CTDs of chicken linker histone H5 (cH5), mouse H10 (mH10), human H10 (hH10), and somatic human linker histones (46) is shown. The amino acids that promote intrinsic disorder, promote order, and are neutral (13–16) are indicated as D, O, and N, respectively.
structured upon binding to their macromolecular partners (13–16). Thus, the properties associated with intrinsic disorder are consistent with our empirical evidence suggesting that regions of the CTD become structured upon binding to linker DNA, and that these subdomains subsequently mediate alteration of linker DNA structure and chromatin condensation. A primary functional advantage of intrinsic disorder stems from its conformational malleability (13–16), which in principle allows a single protein domain to mediate several different functions by participating in different specific macromolecular interactions. Ultimately, our results suggest that a closer examination of the role of intrinsic disorder and interaction-induced secondary structure is warranted, and is likely to reveal important relationships between the structure and function of the linker histone CTD.

From previous results, one possible explanation for the clustering of function (Fig. 6) is that the subdomains identified in our studies contain S/T/PPKK motifs. These sequences form β-turns that bind the minor groove of DNA (22, 24), and are known to mediate CTD-dependent condensation of naked DNA (25–27). The CTD of both chicken H5 and mouse H10 contains canonical S/T/PPKK motifs. Interestingly, the two H10 S/T/PPKK sequences are found in each of the C-terminal subdomains (residues 97–121 and 146–169, respectively) needed to stabilize extensively folded secondary chromatin structures (Fig. 6). Whereas it is attractive to propose a functional role for the S/T/PPKK motifs, we note that chicken H5 has three separate S/T/PPKK sequences, and yet the H5 CTD performs identical functions as the H10 CTD in vitro. Thus, the involvement of S/T/PPKK sequences in CTD function in intact chromatin remains to be determined.

In summary, the studies reported here have led to new insights into linker histone CTD function in chromatin, and have focused attention on the intrinsic disorder of this domain. They have also laid the foundation for future studies of biochemically defined chromatin model systems assembled from recombinant core and linker histone mutants and isoforms. Through such experiments, it will be possible to dissect the complex macromolecular events involved in chromatin condensation.

Acknowledgements—We are grateful to Dr. Susan Wellman for providing the H11 expression vector. We thank Karolin Luger and Steve McBryant for critical reading of the manuscript, and Bob Woody for helpful discussions about intrinsic disorder in proteins.

References

1. Hartman, P. G., Chapman, G. E., Moss, T., and Bradbury, E. M. (1977) Eur. J. Biochem. 77, 45–53
2. Wolfe, A. (1998) Chromatin: Structure and Function, 3rd Ed., Academic Press, San Diego, CA
3. Van Holde, K. E. (1988) Chromatin, Springer-Verlag, New York
4. Simpson, R. T. (1978) Biochemistry 17, 5524–5531
5. Bednar, J., Horowitz, R. A., Grigoryev, S. A., Carruthers, L. M., Hansen, J. C., Koster, A. J., and Woodcock, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14173–14178
6. Carruthers, L. M., Bednar, J., Woodcock, C. L., and Hansen, J. C. (1998) Biochemistry 37, 14776–14787
7. Carruthers, L. M., and Hansen, J. C. (2000) J. Biol. Chem. 275, 37285–37290
8. Fletcher, T. M., and Hansen, J. C. (1996) Crit. Rev. Eukaryotic Gene Exp. 6, 149–188
9. Thomas, J. O. (1999) Curr. Opin. Cell Biol. 11, 312–317
10. Allan, J., Hartman, P. G., Crane-Robinson, C., and Aviles, F. X. (1986) Nature 288, 675–679
11. Allan, J., Mitchell, T., Harborne, N., Bohm, L., and Crane-Robinson, C. (1986) J. Mol. Biol. 187, 591–601
12. Subirana, J. A. (1990) Biopolymers 29, 1351–1357
13. Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Rutliff, C. M., Hippe, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chu, W., Garner, E. C., and Obradovic, Z. (2001) J. Mol. Graph. Model. 19, 26–59
14. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Biochemistry 41, 6573–6582
15. Dunker, A. K., Brown, C. J., and Obradovic, Z. (2002) Adv. Protein Chem. 62, 25–49
16. Wright, P. E., and Dyson, H. J. (1999) J. Mol. Biol. 293, 321–331
17. Vila, R., Ponte, I., Collado, M., Arrendado, J., Jimenez, M. A., Rico, M., and Suaup, P. (2001) J. Biol. Chem. 276, 30898–30903
18. Vila, R., Ponte, I., Collado, M., Arrendado, J. L., and Suaup, P. (2000) Protein Sci. 9, 627–636
19. Hill, C. S., Martin, S. R., and Thomas, J. O. (1989) EMBO J. 8, 2591–2599
20. Clark, D. J., Hill, C. S., Martin, S. R., and Thomas, J. O. (1988) EMBO J. 7, 69–75
21. Suzuki, M. (1989) EMBO J. 8, 797–804
22. Suzuki, M., Gerstein, M., and Johnson, T. (1993) Protein Eng. 6, 565–574
23. Churchill, M. E., and Suzuki, M. (1989) EMBO J. 8, 4189–4195
24. Bharath, M. M., Ramesh, S., Chandra, N. R., and Rao, M. R. (2002) Biochemistry 41, 7617–7627
25. Khadake, J. R., and Rao, M. R. (1997) Biochemistry 36, 1041–1051
26. Khadake, J. R., and Rao, M. R. (1997) FEBS Lett. 400, 193–196
27. Wellman, S. E., Song, Y., Su, D., and Mannon, N. M. (1997) Biotechnol. Appl. Biochem. 26, 117–123
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Garcia-Ramirez, M., Leuba, S. H., and Ausio, J. (1990) Protein Expression Purif. 1, 40–44
30. Hansen, J. C., Ausio, J., Stanik, V. H., and van Holde, K. E. (1989) Biochem. 28, 9129–9136
31. Carter, G. J., and van Holde, K. (1998) Anal. Biochem. 263, 79–84
32. Cerf, C., Lippens, G., Muyldermans, S., Segers, A., Ramakrishnan, V., Wodak, S. J., Hallenga, K., and Wyns, L. (1993) Biochemistry 32, 11345–11351
33. Guelz, P., Demeler, B., Gorter, E. J., Paule, M. R., and van Holde, K. E. (1995) J. Biol. Chem. 268, 1947–1954
34. Simpson, R. T., Thoma, F., and Brubaker, J. M. (1985) Cell 42, 799–808
35. Schwarz, P. M., and Hansen, J. C. (1994) J. Biol. Chem. 269, 16284–16289
36. Van Holde, K. E., and Weissch, W. O. (1978) Biopolymers 17, 1387–1403
37. Horn, P. J., Carruthers, L. M., Logie, C., Hill, D. A., Solomon, M. J., Wade, P. A., Imbalzano, A. N., Hansen, J. C., and Peterson, C. L. (2002) Nat. Struct. Biol. 9, 263–267
38. Gerchman, S. E., Graziano, V., and Ramakrishnan, V. (1994) Protein Expression Purif. 3, 242–251
39. Pepehrson, J. R., and Cole, R. D. (1980) Nature 285, 43–44
40. Pepehrson, J. R., and Cole, R. D. (1981) Biochemistry 20, 2298–2301
41. Carruthers, L. M., Schiir, V. R., Demeler, B., and Hansen, J. C. (2000) Methods Enzymol. 321, 66–80
42. Tse, C., and Hansen, J. C. (1997) Biochemistry 36, 11381–11388
43. Hansen, J. C. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 361–392
44. Clark, D. J., and Kimura, T. (1996) J. Mol. Biol. 211, 883–896
45. Parsegian, M. H., Henschel, A. H., Krieglstein, K. G., and Hamkalo, B. A. (1994) Protein Sci. 3, 575–587