MeCP2-Chromatin Interactions Include the Formation of Chromatosome-like Structures and Are Altered in Mutations Causing Rett Syndrome*

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hMeCP2 (human methylated DNA-binding protein 2), mutations of which cause most cases of Rett syndrome (RTT), is involved in the transmission of repressive epigenetic signals encoded by DNA methylation. The present work focuses on the modifications of chromatin architecture induced by MeCP2 and the effects of RTT-causing mutants. hMeCP2 binds to nucleosomes close to the linker DNA entry-exit site and protects ∼11 bp of linker DNA from micrococcal nuclease. MeCP2 mutants differ in this property; the R106W mutant gives very little extra protection beyond the ∼146-bp nucleosome core, whereas the large C-terminal truncation R294X reveals wild type behavior. Gel mobility assays show that linker DNA is essential for proper MeCP2 binding to nucleosomes, and electron microscopy visualization shows that the protein induces distinct conformational changes in the linker DNA. When bound to nucleosomes, MeCP2 is in close proximity to histone H3, which exits the nucleosome core close to the proposed MeCP2-binding site. These findings firmly establish nucleosomal linker DNA as a crucial binding partner of MeCP2 and show that different RTT-causing mutations of MeCP2 are correspondingly defective in different aspects of the interactions that alter chromatin architecture.

Sporadic mutations in hMeCP2 (human methylated DNA-binding protein 2) cause the majority of cases of the X-linked neurodevelopmental disease known as Rett syndrome (RTT), a severe autism spectrum disorder (reviewed in Refs. 1–3). The disease results in a diverse range of debilitating physical and neurological symptoms that typically make their initial appearance in the first 6–18 months of life in affected girls. Although most RTT cases result from a loss of function effect, mutations that increase the MeCP2 dose may also give rise to similar symptoms, a finding seen also in mice engineered to synthesize additional MeCP2 (4). Extensive research has suggested that MeCP2 acts as a transmitter of epigenetic information by binding to symmetrically methylated CpG dinucleotides, recruiting complexes that include histone deacetylase and methyltransferase, and leading to local transcriptional repression. However, there is also considerable evidence that MeCP2 governs additional processes such as chromatin condensation (5, 6) and that its function is not restricted to transcriptional repression (3).

To better understand MeCP2 function, we are studying the basic interactions between MeCP2 and its DNA and chromatin substrates. In addition to a methylation-dependent interaction with DNA that is mediated by the methylated DNA-binding domain (MBD), MeCP2 has a C-terminal chromatin-binding region as well as additional DNA-binding regions (5, 6). MeCP2 binding contributes to the formation in nucleosomal arrays of a distinctive structural motif in which the entering and exiting linker DNA segments are brought into close juxtaposition forming a “stem.” The stem motif appears very similar to structures induced by histone H1 on mono-nucleosomes and polynucleosomes (7, 8) and is thought to initiate the zigzag conformation and compaction of H1-containing chromatin. MeCP2 shares with H1 the ability to induce chromatin compaction, but the multiple chromatin-binding regions of MeCP2 lead to a higher level of condensation (9).

A distinctive property of H1-containing chromatin is the protection from micrococcal nuclease (Mnase) digestion of ∼20 bp of DNA beyond the ∼146 bp of the nucleosome core particle (NCP). There is considerable evidence that the globular domain of H1 binds near the linker entry-exit site of the nucleosome (reviewed in Ref. 10). However, the location of the ∼20 bp of protected DNA with respect to the parent nucleosome is influenced by the underlying DNA sequence (11), and the detailed molecular structure of the H1-containing unit, termed the chromatosome (12), remains controversial (10).

In the present study we show that hMeCP2, like H1, provides specific protection of linker DNA. However, the two proteins differ significantly in the length of protected DNA. With the
nucleosome-positioning sequence used here, MeCP2 preferentially protects one linker, whereas H1 protects the two linker DNA segments symmetrically. RTT-inducing mutations of MeCP2 differ in their ability to protect linker DNA from Mnase in a manner consistent with their altered chromatin binding properties. We also show that linker DNA is essential for MeCP2 binding and that histone H3 is a partner in MeCP2-nucleosome complexes. These results are discussed in terms of MeCP2-dependent changes in chromatin architecture and their relevance to MeCP2 dysfunction leading to RTT.

EXPERIMENTAL PROCEDURES

601 DNA and NAs—Substrates (termed 208–12 DNA) were based on the “601” nucleosome-positioning sequence and contained 12 repeats of the following 207-bp sequence (lowercase letters denote linker DNA added to the core) inserted into pUC19: agatcgcgacctatacgcgGCGCCCTGGAGAATCCCCGGTGCG- AGGCCCGCTCAATTGGTGTAGCAAGCTCTAGCACCC- GCTTAAAACGCACGTACGCCTGTCGCCCGCCGGTTTT- AACCGCGAAGGGGATTACTCCCTAGTCAGGGCA- CGTGTCAAGATATATACatacctgtgcatgtggatccgaattcatattaatataact.

This sequence was derived from the previously published 213-bp-long nucleosome-positioning template (29) and contained a centered nucleosome-positioning region from the 601 DNA sequence (13), flanked by EcoRV sites (see Fig. 1). Also included were NotI and BtsCI sites close to the NCP in each linker segment. 12-Nucleosome arrays were constructed, propagated, purified, and methylated, and reconstituted with histone octamers obtained from chicken erythrocyte chromatin as described (5). All of the reconstitutions were initially made with a range of histone:DNA ratios, and the products were routinely analyzed for nucleosome count and spacing using EM imaging. At the optimal input ratios of histone and DNA, the majority of the arrays contained 12 regularly spaced nucleosomes and migrated as a single band in agarose gels.

MeCP2 and H1—Wild-type human MeCP2 and selected RTT-causing mutations, and human H18 were expressed and purified as described (5, 19).

Mnase Digestion—Nucleosomal arrays (NAs) were incubated with the MeCP2 or H18 in 50 mM NaCl, 10 mM HEPES, pH 8.0, 0.25 mM EDTA, 0.025% Nonidet P-40 for 30 min at 23C, followed by Mnase digestion with 0.2–0.4 units Mnase (nuclease S7; Boehringer) per μg of DNA in the presence of 0.7 mM CaCl2. After the digestion times indicated, EDTA was added to 3 mM, and the reactions were placed on ice. DNA was extracted by adding SDS to 1% and proteinase K to 0.2 mg/ml and incubating for 1 h at 55C. Loading buffer (30 mM Tris–HCl, pH 7.0, 1% SDS, 5% sucrose, 2.5% β-mercaptoethanol, 0.005% bromphenol blue) was added, and the samples were applied to SDS discontinuous polyacrylamide gels (6% stacking gel, 13% resolving gel, 35:1 acrylamide:bis) and run for 3 h 50 min at a constant current of 24 mA. The gels were stained with gelRed (Biotium Inc).

Mapping Gels—DNA was purified from Mnase-digested chromatin fragments and run on 2% agarose gels, material in the 120–250 bp size range was cut out, and DNA was extracted and purified (Wizard SV gel and PCR Clean-Up kit; Promega). DNA fragments were 5′ end-labeled with 32P using T4 Kinase (Invitrogen), and the 3′-recessed ends were filled in with Klenow DNA polymerase (Roche Applied Science) according to manufacturer’s instructions and purified by phenol–chloroform extraction and ethanol precipitation. The labeled DNA was separated into three parts. One part was left intact, and the remainder two parts were cut with either StyI or AluI (New England Biolabs). The radioactive samples were mixed (3:2 w/w) with sequencing stop solution (U.S. Biochemicals, Cleveland, OH) and separated on 40-cm-long 6% polyacrylamide/urea “sequencing” gels. The gels were dried and exposed to a PhosphorImager (Amersham Biosciences) for autoradiography. Where noted, the gel lanes were straightened by selecting points along the center of the lane, fitting a cubic spline to them, as described (41) implemented in ImageJ (rsb.info.nih.gov/ij/).

Mononucleosome Preparation and EMSA Analyses—Methylated or unmethylated 601-12 DNA was cut to completion either with EcoRV, NotI, or BtsCI (New England Biolabs) according to the supplier’s instructions. The resulting DNA fragments were purified, reconstituted with core histones, and incubated with MeCP2, and gel shifts were carried out as described (5).

MeCP2-associated Proteins—MeCP2 was conjugated with the sulfo-SBED reagent (Pierce) at a 1:1 molar ratio according to the manufacturer’s directions, dialyzed extensively, and then allowed to interact with NAs under standard conditions before initiating protein–protein cross-linking with UV light (Spectrashine lamp XX-15B 325 ± 5 nm) for 10 min at a distance of ~5 cm. After treatment with dithiothreitol to release MeCP2, the NAs were separated using 16% SDS-PAGE, and the proteins were stained with Coomassie Blue. Biotinylated proteins were detected using the ECL blotting system (Amersham Biosciences) with PVD membranes (Immobilon IPVH20200) and streptavidin-horseradish peroxidase.

Electron Microscopy of Mononucleosomes—Mononucleosomes were incubated with MeCP2 as described above, fixed by the addition of an equal volume of 0.2% glutaraldehyde for 4 h on ice, and then dialyzed against buffer only at 4°C overnight. Fixed samples were applied to glow-discharged carbon films and stained as described (5, 43). Dark field images were digitally recorded at 100 kV using a Tecnai 12 electron microscope (FEI Corp., Hillsboro, OR) with an F224 2K slow scan CCD camera (Tietz Video and Image Processing Systems, Gauting Germany). Digitized images of mononucleosomes were sorted and measured using software tools from EMAN (44) and SUPRIM (45).

RESULTS

To provide a fully defined substrate for studying MeCP2-chromatin interactions, we constructed 207 bp of DNA containing the 601 sequence, which accurately and uniquely positions histone cores with high affinity (13). The sequence (Fig. 1A) contains 18 CpG units distributed over the linker and NCP regions and has unique restriction endonuclease (RE) sites in both the NCP and in the linker DNA segments. Methylated and unmethylated NAs containing 12 tandemly arranged 601 sequences were created by reconstituting with core histones, and EMSAs were used to monitor interactions with recombi-
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Linker DNA Protection by RTT-causing Mutants of MeCP2—All RTT-inducing MeCP2 mutants examined bind very effectively to NAs but nevertheless differ markedly in their ability to induce chromatin compaction and generate specific structural motifs (5). The R106W mutant, which is defective in both chromatin compaction and stem formation, gave a Mnase protection pattern very much like NAs alone (Fig. 2B, lanes 1–6). In contrast, the R294X mutation, which lacks a large C-terminal portion of the 486-amino acid protein, produced a digestion pattern very similar to wild type (Fig. 2B, lanes 7 and 8), showing that the C-terminal region is not required for this aspect of MeCP2-chromatin interaction. The other MBD mutants tested alone was digested to much smaller fragments (Fig. 2A, lanes 1 and 2), indicating that the results seen with NAs were not due to the sequence preference of the enzyme (14–16). For NAs and NA-protein complexes, the base pair resolution of the acrylamide gel system reveals that a series of products is formed during the course of Mnase digestion. With NAs alone (lanes 3–6), four principal bands are seen at the 3-min time point, but by 10 and 15 min, the major persisting band is ~146 bp, corresponding to the protected NCP (white arrow). The larger fragments are short-lived intermediates, the largest corresponding to ~207 bp (1 cut/207-bp unit). By 15 min, some degradation of the NCP DNA is evident from the appearance of a ~132-bp fragment, which corresponds to the strong protection afforded by the H3/H4 tetramer.

When NAs are complexed with wild type MeCP2, four major bands are seen (Fig. 2A, lanes 7–10), the largest again resulting from a single cut in each 207-bp unit of the array, and rapidly degraded. Now, the most persistent band is ~158 bp (double asterisks). This is slowly further digested to the ~146-bp NCP, consistent with an MeCP2-dependent “pause” in digestion. In contrast, histone H10 produces an even more robust pause, corresponding to the ~168-bp chromatosome (lane 11, shaded arrow).

Linker DNA Protection by wild type MeCP2 and H10. Lanes 1 and 2, the 207-12 DNA substrate is rapidly digested by Mnase. Lanes 3–6, NAs digested with Mnase for 3–15 min show a persistent band at ~146 bp representing the NCP (open arrow). Lanes 7–10, in the presence of wild type MeCP2, a resistant ~158-bp fragment (**) is obtained in addition to the ~146 NCP band. Lane 11, NAs reconstituted with H10 yield a predominant ~168-bp DNA, representing the chromatosome (shaded arrow). Lane 12, the ~168-bp chromatosome band predominates in NAs reconstituted with both H10 and MeCP2, but additional shorter fragments are also seen. B, Mnase protection by RTT-causing mutations. Lanes 1–4, controls with NA alone and with wild type MeCP2. The open arrow indicates the ~146-bp NCP. Lanes 5 and 6, the R106W mutation shows almost the same digestion pattern as NAs alone. Lanes 7 and 8, the R294X truncation mutation shows a protection pattern very similar to the wild type. ** indicates the ~158-bp protection characteristic of MeCP2. Lanes 9–14, the mutations R133C and F155S give Mnase protections only slightly different from the wild type, with a persistent band at ~158 bp. Lanes M, size markers prepared from 207-bp DNA, and a pair of RE fragments (149 and 58 bp) derived from it. The figures are composites using standards to align groups of lanes. All of the gels included controls with NAs alone and/or with wild type MeCP2. Accurate bp values were determined using sequencing gels (Fig. 3).
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for Mnase protection were R133C, which interferes with binding to methylated DNA, and F155S, which is generally deficient in inducing compaction as measured by EMSA. Both of these mutants showed only subtle differences from wild type MeCP2 (Fig. 2, lanes 9–14). For mapping the positions of protected bands within the 207-bp unit, optimal digestion times were selected from Mnase series such as those shown in Fig. 2.

Mapping of Protection with RE Digestion and Sequencing Gels—Fig. 3 illustrates our strategy for mapping locations of Mnase-protected regions through the use of RE digestion (see “Experimental Procedures” for details). As expected, NAs alone show the strong protection of ~146 bp conferred by the NCP (top lane), and when reconstituted with human H1, the ~168-bp band characteristic of the chromatosome is seen (second lane). Subsequent digestion by StyI produces pairs of bands (Fig. 3, lower lanes) that can be used to locate the protected DNA within the 207-bp sequence. The application of this approach to NAs exposed to wild type and mutant MeCP2 is shown in Fig. 4A, and the results are summarized in Fig. 4B (note that StyI has a staggered cut, which has to be taken into account). The central location of the NCP, extending from bases ~28 to ~175 with the dyad at ~100, matches the expectation based on the properties of the 601 positioning sequences (17). The linker DNA protection afforded by wild type MeCP2 covers ~11 bp and occurs predominantly at the 5′ end of nucleosomal DNA, although there is an additional weaker protection of ~11 bp at the 3′ end, seen as a short-lived 168-bp intermediate in the Mnase digest pattern (Fig. 2A, lanes 7–10).

The R294X truncation mutant provides protection very much like the wild type, whereas the R106W mutant, which has lost its ability to bind DNA tightly and influence chromatin architecture, afforded very little protection beyond the NCP. Nucleosomal arrays reconstituted with one histone H1 per nucleosome show a different pattern of protection from Mnase. An ~11-bp linker protection is seen at the 5′ end of the NCP, similar to that observed with MeCP2. However, H1 also results in a symmetrical protection of ~11 bp at the 3′ end of the core particle DNA (Fig. 4B). As noted above, the total protection with H1 is ~168 bp, representing the canonical chromatosome.

In a study of the Mnase protection conferred by Xenopus laevis MeCP2 (65% identity with hMeCP2) on mononucleosomes assembled on a 55 rDNA sequence from Xenopus borealis, Chandler et al. (18) observed linker protection, but it was asymmetrical and identical to that of H1. These differences may be related to the source of MeCP2 as well as to the much higher protein:nucleosome ratios (typically 10:1) used in the earlier work. Even with 2 MeCP2/nucleosome, we observe only a minor protection of ~168 bp in addition to the major ~158 bp (Fig. 4A, No RE, WT MeCP2 lane). Further increases of the MeCP2 input ratio lead to increased protection of ~168 bp as well as protection of the complete 207-bp repeat and aggregation of NAs (not shown).

Competition between MeCP2 and H1—Because MeCP2 and H1 appear to occupy the same or overlapping sites on nucleosomes, we examined the effect of competition between the two proteins. Core histones were first reconstituted onto 601-12 DNA, and then H1 and MeCP2 were added simultaneously in 50 mM NaCl (19) for 30 min prior to Mnase digestion. Although both proteins are fully bound to DNA under these conditions (as demonstrated by the absence of either protein in the supernatants following centrifugation in the presence of 5 mM MgCl2 [not shown]), the H1 pattern of Mnase protection clearly dominated (Fig. 2A, lanes 11 and 12). Nevertheless, with H1 and MeCP2, protected fragments shorter than those obtained with H1 only were observed (Fig. 2A, lane 12), showing that under these in vitro conditions, MeCP2 does interfere somewhat with normal H1 binding and linker DNA protection.

Linker DNA Is Required for MeCP2 Interaction with Nucleosomes—The Mnase protection data showed that MeCP2 is able to protect a portion of linker DNA, presumably by binding strongly to it. It was therefore of interest to determine whether linker DNA interaction was required for chromatin–MeCP2 binding. We reconstituted mononucleosomes on the 601 sequence that contained either two linker segments, a single 5′ linker, a single 3′ linker, or no linker (Fig. 5A). The three linker-containing constructs had the same amount of DNA, but the linker segments differed in the number and locations of CpG units with respect to the NCP. The staggered NotI cutting site spans two CpGs (Fig. 1A), effectively removing both to create the single 3′ linker mononucleosome. These two sites occur close to the NCP in the single 5′ linker substrate (Fig. 5A). EMSA experiments showed that with two-linker and one-linker mononucleosomes, wild type MeCP2 induces a very strong interaction, with production of dramatic reductions in mobility featuring two prominent bands (asterisks), whereas linkerless core particles showed almost no interaction (Fig. 5B). Interestingly, mononucleosomes containing only one linker showed as robust an interaction as those with two linkers, and similar gel shift patterns occur both with the 5′ and the 3′ linker segments (Fig. 5E). We also examined the interactions between mononucleosomes and MeCP2 mutants. In contrast to wild type MeCP2, the shift induced by the R106W mutant was markedly reduced in magnitude (Fig. 5C). A similar effect is seen with 12-nucleosome arrays, where the R106W mutant induces weaker shifts than seen with wild type MeCP2 (5). The R294X mutant induced a different EMSA pattern. Because the truncated protein is 40% smaller than the wild type, the magnitude of the mobility shifts is correspondingly reduced. More importantly, the R294X mutation produces a single well
is involved in a variety of interactions with nucleosomes and that in its absence these do not occur.

**MeCP2 Induces Changes in Linker DNA Conformation**—Changes in electrophoretic mobility of DNA-protein complexes may be the result of differences in mass, conformation, charge, or a combination. To examine the interactions between mononucleosomes and MeCP2 in more detail, methylated and unmethylated mononucleosomes with 0, single 5', single 3', and two linker DNA arms (Fig. 5A) were prepared and allowed to interact with wild type MeCP2 under conditions that gave robust gel shifts (Fig. 5B) before fixation and preparation for EM examination. As expected, the predominant conformation of the two-linker mononucleosomes showed two DNA arms of approximately equal length (mean = 32.8 bp, S.E. = 0.8, S.D. = 5.6) extending from the NCP (not shown), and the one-linker mononucleosomes contained a single linker of the expected length (mean = 63.8 bp, S.E. = 1.7, S.D. = 7.5) (Fig. 6A, B, and C). Images of wild type MeCP2-nucleosome complexes revealed a variety of conformations, consistent with the broad range of mobility shifts (Fig. 5B). For example, although ~60% of one-linker mononucleosomes appeared as individual nucleosomes with a single linker arm (Fig. 6A and B), the remaining ~40% had linker DNA looped back to the NCP or existed as complexes containing two nucleosomes (Fig. 6D).

With one-linker mononucleosomes in which bound MeCP2 was visible (Fig. 6A, panels 2 and 3, and B, panels 2 and 3), there was a significant difference (p < 0.001) between the 3' and the 5' constructs. With the 5' linker, which has a cluster of CpGs close to the NPC (Fig. 5A), 84% of bound MeCP2 molecules were proximal to the NCP, whereas with the 3' linker, the frequencies of distal and proximal MeCP2 were approximately equal (Fig. 6C). A noteworthy correlation was seen between the EM and the gel shift experiments, both of which revealed differences between the 3' and 5' linker constructs. With the 3' linker, two equally pronounced gel shift bands were seen at MeCP2' = 2.0, whereas with the 5' linker, the intensity of the lower band was much more pronounced (Fig. 5E), suggesting that the proximal location of MeCP2 resulted in the lower shifted band.

There were also significant (p < 0.0001) differences between complexes formed with wild type as compared with the R294X mutant. As noted, with wild type MeCP2, ~40% of nucleosome-MeCP2 structures were in the form of two-nucleosome complexes or one-nucleosome complexes with looped linker, whereas with R294X, only 5% of MeCP2-nucleosome interactions resulted in conformations of this type (Fig. 6F). The results with R294X are consistent with the EMSA data showing a single well defined mobility shift, in contrast to the broad range of shifts produced by the wild type (Fig. 5, B and D). Further, the dramatic reduction in the more complex interactions correlates well with the loss of the chromatin-binding C-terminal portion of MeCP2.

**Histone H3 Is a Primary Contact Partner in MeCP2-Mono- nucleosome Complexes**—To further examine the specificity of MeCP2-nucleosome binding, we employed a sulfo-SBED reagent that enables the transfer of biotin from one protein to a binding partner. MeCP2 was conjugated with the reagent and reacted with methylated two-linker mononucleosomes under...
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FIGURE 5. Linker DNA is required for MeCP2 binding to mononucleosomes. A, diagram of the four mononucleosome types prepared, with CpG units in the linker and close to the DNA dyad marked by arrowheads. B, mononucleosomes with two-, one- (3'), or zero-linker DNA arms were reacted with wild type MeCP2 at r = 2, and the products were separated on agarose gels. Mononucleosomes with one or two linker segments show dramatic shifts in mobility, with two prominent bands (asterisks), whereas most of the linkerless mononucleosomes remain unshifted. These mononucleosome preparations contained some free DNA migrating as minor species below the main band that bound MeCP2 as seen by their disappearance in the lanes with MeCP2. As seen from the zero-linker lanes (which have no nucleosome-MeCP2 complexes), these minor DNA contaminants contribute insignificantly to the chromatin gel shifts. C and D, EMSAs were performed as in B but with the R106W and R294X mutants, respectively. With two- and one-linker mononucleosomes, R106W induces a much weaker response than the wild type in terms of the magnitude of the gel shifts (squares). R294X induces a single prominent shifted band and lacks the broad range of interactions seen with the wild type. E, comparison of the interactions between wild type MeCP and nucleosomes with 3' or 5' linkers. The two linker types give very similar gel shifts over a range of input MeCP2 ratios. M indicates DNA marker lanes (1-kb ladder; Invitrogen).

standard conditions, and the complexes were exposed to a photo-cross-linking step in which any nucleosomal proteins in close proximity to MeCP2 become covalently bound. A final treatment to break S-S bonds removes the MeCP2, leaving a biotin marker on the protein partner that can be detected by blotting. As shown in Fig. 7, histone H3 gives a strong signal, suggesting that this is the nucleosome component that is closest in proximity to MeCP2 or actually bound to it. The N-terminal region of H3 exits the nucleosome close to the linker entry-exit site (22), a position that is close to the location of MeCP2 indicated by the Mnase protection data (Fig. 4B). In future, it will be important to determine the residue(s) in H3 that become biotinylated in MeCP2-nucleosome complexes.

DISCUSSION

The work presented here demonstrates that a primary interaction between MeCP2 and chromatin results in the protection from Mnase of a specific ∼11-bp segment of linker DNA in defined nucleosomal arrays (Figs. 2–4). This, together with the requirement for a nucleosome with at least one linker arm, indicates that the linker entry-exit region of the nucleosome is a primary site of MeCP2-DNA contacts. The 601 sequence used in these studies was chosen for its strong rotational and translational nucleosome positioning properties, and, like most of the high affinity SELEX-derived positioning sequences (23), it has CpG dinucleotides close to the dyad axis (Fig. 1B). Interestingly, this also occurs in consensus sequences of naturally occurring nucleosomes (24), suggesting that CpGs near the dyad axis, accessible for both methylation and MeCP2 binding, are common in vivo. In the present study, clusters of methylated CpG dinucleotides, brought into proximity by the relationship between the NCP and the linker arms, enhance MeCP2 binding, as seen by the preference for proximal binding to constructs with the 5' versus the 3' linker (Fig. 6C). However, the strong binding of MeCP2 to unmethylated four-way junction DNA (20) supports the concept that DNA geometry at the nucleosome dyad favors MeCP2 binding regardless of the methylation state.

Mutations in MeCP2 that cause RTT differ in their linker protection capacities. Although the loss of 193 amino acids from the C-terminal region (R294X) has no appreciable effect on linker protection (Fig. 4), it is nevertheless defective in promoting nucleosome-nucleosome bridging (Fig. 6E) and is also required for full chromatin compaction (5). Thus, binding close to the nucleosome dyad and the concomitant protection of linker DNA is seen as necessary but not sufficient for normal MeCP2 function. Patients with the R294X mutation may exhibit the classical symptoms of RTT, and a mouse mutant engineered to express MeCP2 truncated at residue 306 is similarly afflicted (25). In contrast, linker DNA protection is abolished in the R106W mutation (Figs. 2B and 4A). The mutant protein does bind to nucleosomal arrays, but the mode of binding is evidently inappropriate for inducing the linker DNA protection and chromatin compaction observed with the wild type (5).

MeCP2 and Histone Modification—Of the core histones, only H3 is clearly identified in the biotin transfer experiments (Fig. 7). Although the region of H3 involved in this interaction has not yet been determined, it may involve the long N-terminal domain that exits the NCP in the linker DNA entry-exit region (22). Significantly, the H3 N terminus appears to be the primary target for MeCP2-mediated modification by histone deacetylase and histone methyltransferase (26). Moreover, modulation of H3K9 acetylation during neuronal maturation is correlated with MeCP2 levels in both RTT and autism brains (27).

Histone H1 and MeCP2 Share Common Features—Linker histones have long been recognized as contributing to chromatin higher order structure (10), and their abundance in chromatin is correlated with nucleosome repeat length (28). Although the precise locations of the domains of H1 in chromatin remain controversial, it is clear that the globular domain binds in the vicinity of the linker DNA entry-exit region of the nucleosome and, when bound, protects an additional ∼20 bp of nucleosomal DNA, forming the chromatosome. The ability to protect linker DNA at the nucleosome entry-exit site is shared by MeCP2 but not seen with other chromatin binding proteins such as the HMG family, HP1, or MENT (29). Based on the
work reported here, it appears that this property resides in the N-terminal 294 residues of MeCP2. Interestingly, with both H1 (30) and MeCP2, linker protection is retained after removal of a large C-terminal region.

With H1, the location(s) of linker protection appear to depend on DNA sequence. Hayes and Wolffe (31) reported that for the *Xenopus* 5 S rDNA, 15 bp was protected on one linker arm, and 5 bp was protected on the other. In contrast, An et al. (11) found asymmetrical protection with two nucleosome-positioning sequences: one based on the *Lytechinus variegatus* 5S rDNA, and the other containing Gal4 and USF sites. However, these two sequences resulted in H1 protection of different linker arms in each case. With the 601 positioning sequence used here, H1-dependent chromatosome protection appears to be almost completely symmetrical, extending to ~11 bp on each linker arm (Fig. 4). Thus, with H1, the amount of DNA protected in the chromatosome is constant, but the specific region(s) involved are sequence-dependent. With MeCP2, there is preferred protection of ~11 bp of the 5' linker, involving the same DNA sequence. Whether the location of linker DNA protection by MeCP2 is sequence-dependent will await exploration of NAs containing other DNA-positioning sequences.

Both H1 and MeCP2 are considered to be chromatin architectural proteins based on the compaction they induce (reviewed in Refs. 32 and 33). Contributing to the compaction mechanism in both cases is the stem conformation of nucleosomal linker DNA arms (5, 34). However, the modes of chromatin compaction effected by H1 and MeCP2 differ significantly. H1 tends to result in a zigzag arrangement of nucleosomes and linker DNA, leading to compact chromatin fibers (32), whereas MeCP2 tends to produce globular clusters of nucleosomes and a higher overall compaction ratio (9). The high potency of MeCP2 is most likely related to its possessing at least two independent DNA-binding regions, including one in the transcriptional regulatory domain (6), and we have suggested that both are needed for forming stem structures in chromatin and side-by-side aggregation of naked DNA (5). Full compaction, however, requires the nucleosome-binding C-terminal region. The failure of MeCP2 to induce gel shifts with linkerless mononucleosomes (Fig. 5) suggests that MeCP2 requires protein-free DNA for productive binding. Importantly, the property of binding more strongly to cruciform
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(four-way junction) than linear DNA, shared by both H1 (21) and MeCP2 (20), indicates that binding is strongly modulated by DNA conformation. In some respects, cruciform DNA resembles the linker entry-exit site of nucleosomes (35), suggesting that this conformation is key to both H1 and MeCP2 binding.

Because H1 and MeCP2 induce similar Mnase protections when bound to chromatin, it is likely that they compete for binding sites. Based on their affinities for chromatin, H1 (which typically requires >500 mM monovalent ions for release) might be expected to be more tightly bound than MeCP2 (which is eluted from nuclei in the 300—400 mM range). Indeed, with chromatin containing both H1 and MeCP2, the Mnase protection pattern was similar to the H1 only situation (Fig. 2A). The addition of MeCP2 does, however, result in additional smaller DNA fragments, suggesting some degree of H1 displacement. Thus, in a situation where H1 and MeCP2 are in competition, it is likely that the stronger affinity of H1 for chromatin will result in a higher H1 occupancy of nucleosomal sites. In the nucleus, H1 and MeCP2 have similar mobilities (36, 37), and the number of nucleosomes exceeds the number of H1 molecules in most cells (28), suggesting that there will always be free binding sites for both proteins. However, the present work does suggest that MeCP2 may compete poorly for binding to H1-containing nucleosomes in vivo and require a less than full occupancy of H1 for productive binding to a specific region of chromatin.

Implications for Rett Syndrome—The work reported here, together with earlier studies, supports the concept that the interaction between MeCP2 and chromatin has multiple components. The linker DNA entry-exit region of the nucleosome is clearly a very potent binding site for MeCP2 and requires the presence of extranucleosomal DNA. Binding to this region via the MBD is probably an early event in MeCP2–chromatin interaction that proceeds in the absence of the C-terminal portion of the protein, as seen from the results with the R294X mutation. Failure of this interaction in RTT mutations such as R106W compromises subsequent events. Following the initial binding, MeCP2 brings the linker DNAs in nucleosomal arrays together in the stem conformation through a second DNA-protein interaction (5, 6). This important conformational change is not inhibited in the R294X mutation and thus does not require the C-terminal portion of MeCP2. The C-terminal region is, however, required for a third interaction that involves MeCP2 binding to nucleosomes and is essential for maximal compaction of chromatin (5).

Our finding that RTT-causing mutations of MeCP2 show fundamental differences in their interactions with DNA and chromatin suggests that RTT should be considered to be a family of diseases with shared symptoms but very different root causes. These include failure to bind to methylated DNA (R133C), failure to induce nucleosome-nucleosome interactions (R294X), and multiple failures (R106W). The latter mutation, which is ineffective both in protecting linker DNA from Mnase (Figs. 2 and 4) and in compacting chromatin (5), merits further detailed study. The location of the mutation within the MBD does not appear to directly affect the DNA-binding site (38), and there is no obvious a priori cause for its severe loss of function. A failure of R106W either to fold properly (39) or to undergo the normal conformational changes upon substrate binding is a possibility under investigation. Single amino acid substitutions tend to result in altered dynamics and energetics rather than an overall change in three-dimensional structure (40), and examining these properties is an important future goal. Understanding the different defects in MeCP2 mutations that lead to reduced interactions with DNA and chromatin as reported here will provide information critical to the identification of small molecules that may restore function and lead to effective mutation-targeted RTT therapies.

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