Nucleosome Assembly on Methylated CGG Triplet Repeats in the Fragile X Mental Retardation Gene 1 Promoter*

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Expansion and methylation of CGG repeat sequences is associated with Fragile X syndrome in humans. We have examined the consequences of CGG repeat expansion and methylation for nucleosome assembly and positioning on the Fragile X Mental Retardation gene 1 (FMR1) gene. Short unmethylated CGG repeats are not particularly favored in terms of affinity for the histone octamer or for positioning of the reconstituted nucleosome. However, upon methylation their affinity for the histone octamer increases and a highly positioned nucleosome assembly with the repeat sequences found adjacent to the nucleosomal dyad. Expansion of these CGG repeats abolishes the preferential nucleosome assembly due to methylation. Thus, the expansion and methylation of these triplet repeats can alter the functional organization of chromatin, which may contribute to alterations in the expression of the FMR1 gene and the disease phenotype.

The most common form of inherited mental retardation is associated with a fragile site on the long arm of the X chromosome at Xq27.3 (1–3). This site contains an unstable CGG repeat at the 5’ end of Fragile X Mental Retardation gene 1 (FMR1) (4). Normal individuals have less than 60 CGG repeats, whereas affected individuals can have an amplification of repeat number to more than 200 copies (4–10). In all affected individuals, the CGG repeats are methylated (11, 12), and the FMR1 gene is transcriptionally inactive (13–15). The CGG repeat is also the site of preferential chromosome breakage (16). The molecular mechanisms responsible for expansion of CGG repeats, their selective methylation, transcriptional repression dependent on expansion, and chromosomal fragility have not been determined.

It is possible that alterations to chromatin structure associated with the CGG repeats might contribute to these various phenomena (17, 18). Expansions of the CTG repeat sequences associated with a range of other inherited human diseases (19) lead to alterations in chromatin structure in vivo (20) and the assembly of nucleosomes of unusually high stability (21–23). Evidence exists for the assembly of specialized chromatin structures on methylated DNA (24). H1 is enriched in nucleosomes that contain 5-methylcytosine in vivo (25). However, no differences have been detected for H1 binding to methylated DNA versus unmethylated DNA in reconstituted chromatin (26, 27) and core histone octamers do not in general discriminate between methylated or unmethylated DNA (28, 29). Nevertheless, sequence-specific methylation-dependent binding of linker histones (30, 31) and core histones (32) to particular DNA sequences can occur.

In this work we demonstrate that methylation of 13 CGG repeats associated with the FMR1 promoter in a normal individual increases the affinity of the histone octamer for a DNA sequence containing these repeats. Our results suggest that short segments of methylated CGG triplet repeats have similar properties to CTG sequences in that they are highly favored binding sites for the histone octamer (23), but that expansion of these triplet repeats leads to marked differences in their association with the core histones.

MATERIALS AND METHODS

DNA Constructs—Plasmids containing a portion of the human FMR1 gene were obtained by polymerase chain reaction amplification of patient DNA and contained the 252-bp fragment of the gene including 13 repeats of CGG (i.e. normal range) (Fig. 1). This region was cloned into the Smal site of pBluescript (Stratagene); the plasmid incorporating this fragment is referred to as pCGG13. Another fragment, which was amplified, using the same primers, from a Fragile X patient, contained 74 CGG repeats; this was cloned into the EcoRI site of vector pSV2neo (Clontech) and is referred to as pCGG74. Plasmid pCGG10 was as described previously (23). The Xenopus borealis somatic 5 S RNA gene was contained in plasmid XP-10 (33).

DNA Methylation—Ten μg of plasmid DNA was incubated with 10 units of SssI methylase (New England Biolabs) in the supplied buffer for 16 h at 37 °C in a volume of 50 μl. Methyl group donor S-adenosyl methionine was used at 160 μM. SDS and EDTA were added to final concentrations of 0.5% and 5 mM, respectively, to stop the reaction.

Competitive Reconstitutions—Plasmids were cut with the indicated restriction enzymes, labeled using the Klenow fragment (New England Biolabs), and the appropriate fragments were isolated from gels. Reconstitution of nucleosome cores under competitive conditions were as described (34) with modifications (23). Hydroxyl radical reactions have been detailed previously (35) with modifications and data manipulation described elsewhere (23).

Translational Mapping—DNA (0.5 mg) from methylated pCGG13 was cut with BamHI and PstI to liberate a 252-bp fragment. This was resolved by electrophoresis in a 1.5% agarose gel and recovered by electroelution of the gel slice in dialysis tubing. This was used to reconstitute nucleosomes by dialysis (36). Histones were purified from HeLa cells (37) and were used at a ratio of 0.8 mg of histone/1 mg of DNA. Modifications to salt dialysis and subsequent micrococcal nuclease digestion were as described (23). The resulting fragments were end-labeled with 32P using polynucleotide kinase and resolved on a 6% polyacrylamide gel for analysis.

RESULTS AND DISCUSSION

Methylation of the FMR1 Gene Facilitates In Vitro Nucleosome Assembly on a Normal Range CGG Triplet Repeat—We have shown previously that the Xenopus Mental Retardation Gene 1 (FMR1) 1 promoter in a normal individual increases the affinity of the histone octamer for a DNA sequence containing these repeats. Our results suggest that short segments of methylated CGG triplet repeats have similar properties to CTG sequences in that they are highly favored binding sites for the histone octamer (23), but that expansion of these triplet repeats leads to marked differences in their association with the core histones.

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† The abbreviation used is: bp, base pair(s).
FIG. 1. Map of the FMR1 promoters used in this study (38). The start site of transcription (+1) is indicated by the hooked arrow (47). Distances are shown in base pairs. The CGG trinucleotide repeat sequences are shown as solid bars, both in the normal range and in the expanded form found in affected individuals.

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The strongest known naturally occurring nucleosome positioning sequence (23). This gene contains two closely spaced runs of triplet repeats, CGG, and CTG. We sought to investigate the positioning capabilities, if any, of the CGG repeats in a different system where they would not be influenced by adjacent CTG tracts. We chose the human FMR1 gene, which contains between 6 and 60 repeats of the CGG trinucleotide in normal individuals juxtaposed to the promoter elements of the gene (Fig. 1; see also Ref. 38). Expansion of these CGG repeats is known to correlate with the onset of Fragile X syndrome (5, 6, 16).

We obtained a FMR1 gene clone, which contained 13 repeats of CGG and used this in competitive nucleosome reconstitution assays to determine its propensity for nucleosome formation in vitro (34). This involves an exchange reaction from purified donor chromatin onto the labeled fragment of interest in the presence of cold competitor DNA. The X. borealis somatic 5 S rRNA gene has typically been used as a standard sequence favored for association with the histone octamer (39, 40). We and others have more recently described the very high affinity for histone octamers that DNA sequences containing CTG triplets possess (21–23). We therefore made use of both the 5 S RNA gene and the human DRPLA (dantatorubral-pallidolysian atrophy) gene, which contains 10 CTG repeats, as standards in the competitive nucleosome reconstitution assay.

It can be seen that the FMR1 gene containing 13 CGG repeats does not have a strong affinity for histone octamers relative to the human DRPLA gene and Xenopus 5 S rRNA gene (Fig. 2, Table I). We then methylated the FMR1 gene using a commercially available prokaryotic methylase (SsmI), which adds a methyl group to cytosine residues. This modification can be seen to have a pronounced effect on the overall affinity of the sequence containing the CGG repeats for the histone octamer (Fig. 2, compare lanes 2 and 3). This effect is at least 3-fold when comparing a ratio of nucleosomal DNA to free DNA and accounts for a change in relative free energy of formation ($\Delta \Delta G$) between 0.6 and 0.7 kcal/mol (Table I). Methylation of the CGG repeats causes the degree of affinity for the histone octamer to increase to a level nearly identical to a comparable sized run of CTG trinucleotides in the DRPLA gene (Fig. 2, compare lanes 2 and 4).

Methylation of an Expanded CGG Repeat in the FMR1 Gene Reduces the Efficiency of Nucleosome Assembly—We next examined the binding of a histone octamer to a FMR1 gene fragment containing expanded methylated CGG triplets (C$_m$GG). We used a clone that contained 74 repeats, isolated from a carrier of the Fragile X syndrome. The unmethylated DNA was used in nucleosome reconstitution experiments and was found to form nucleosomes with an efficiency nearly identical to the unmethylated, unexpanded gene (Fig. 2, lane 6; Table I). The multiple species resolved on the polyacrylamide gel suggest the existence of distinct translational positions of the histone octamer with respect to the DNA sequence containing the 74 CGG triplets (Refs. 41 and 42; see below). Upon methylation, however, the affinity of the expanded fragment for the histone octamer drops off sharply in contrast to the behavior exhibited by the normal-sized C$_m$GG repeat (Fig. 2, lane 5). This decrease is on the scale of an entire kilocalorie of free energy (Table I). Note that in Fig. 2 (lanes 5 and 6), both experiments with the expanded fragment were performed in the absence of competitor DNA in order to highlight the difference between the methylated and unmethylated C$_m$GG. The free energies of nucleosome formation for this sequence in methylated or unmethylated form were calculated from experiments done in parallel with the expanded fragments in the presence of competitor (data not shown). Thus methylation of the CGG repeats can have differing effects on nucleosome formation, dependent on the repeat number.

Reconstituted Nucleosomes Are More Effectively Positioned in a Single Location Using a DNA Fragment Containing the Methylated, Unexpanded CGG Repeat Compared to the Unmethylated Gene—We studied the precise nucleosome positioning capabilities of the normal range C$_m$GG repeat in addition to the overall affinity of the sequence for histone octamers. The rota-
Hydroxyl radical footprinting of reconstituted nucleosomes on the normal and expanded FMR1 gene. Constructs as described in Fig. 2 were labeled on the bottom strand using Klenow fragment either at the BamHI cleavage site (CGG13, lanes 1–6) or at the EcoRI site (CGG13, lanes 7–12). The latter was subsequently cleaved with DdeI to remove 51 bp from one end to ensure labeling of one strand only. (In all cases, G is the Maxam-Gilbert reaction cleaving DNA at G-residues, Oct is hydroxyl radical cleavage of nucleosomal DNA, and DNA is hydroxyl radical cleavage of naked DNA. The filled vertical bar shows area of CGG repeats. The dashed vertical bar between lanes 4 and 5 indicates sites of preferential cleavage by hydroxyl radical in the methylated CGG13 nucleosome.

Densitometric scanning (Fig. 4) shows line graphs of the data contained in Fig. 3. An open box representing the area of CTG repeats is marked. Marker denotes the G-reaction.

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CONCLUSIONS

We conclude that methylation of unexpanded CGG repeat sequences has a significant influence on the association of core histones with DNA. This result suggests that short methylated
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CGG repeats and CTG repeats might possess structural features in chromatin that contribute to a tendency toward expansion. A reduction in the processivity of DNA polymerase as a consequence of stable histone-DNA interactions might facilitate the expansion of triplet repeats (48). The presence of a stable nucleosome immediately downstream of the start site of transcription of the FMR1 gene might also contribute to the regulation of gene expression at this promoter. Nucleosomes are known to impede the processivity of RNA polymerase II and to potentially contribute to the regulation of transcription downstream from the start site of transcription (49, 50).

Expanded CTG and CGG triplet repeats as found in affected individuals differ markedly in their properties. While expanded CTG repeats continue to favor nucleosome assembly (21–23), expanded methylated CGG repeats do not continue to significantly favor nucleosome assembly. The methylated expanded CGG repeats assemble nucleosomes that have a stability comparable to nucleosomes including mixed sequence DNA (Table I). Thus nucleosomal instability per se is unlikely to contribute to chromosomal fragility.

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