Methylation of Expanded CCG Triplet Repeat DNA from Fragile X Syndrome Patients Enhances Nucleosome Exclusion*  

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Long tracts of CCG trinucleotide or CCGNN pentanucleotide repeats in DNA have previously been shown to resist assembly into nucleosomes. This may provide a molecular explanation for the nature of certain rare, folate-sensitive fragile sites in human chromosomes that contain expanded CCG triplet tracts. Further, it is known that methylation of CpG dinucleotides at or near these fragile sites enhances the fragile phenotype. Here DNAs containing 76 tandem CCG triplets or 48 CCGNN pentanucleotide repeats were methylated with SssI methylase at three different levels of methylation. Using competitive nucleosome reconstitution/gel shift assays, the ability of these DNAs and a mixed sequence DNA from the pUC19 plasmid were compared in their ability to assemble into nucleosomes. DNA methylation had no significant effect on nucleosome formation over the pUC 19 fragment. However, the highly methylated DNAs containing 76 CCG triplets or 48 CCGNN pentanucleotide repeats were 2.0 ± 0.2-fold and 2.1 ± 0.3-fold less efficient in nucleosome assembly than the respective unmethylated forms, and 4.4 ± 0.4-fold and 12.6 ± 1.6-fold less efficient than a pUC19 fragment of similar length.

In eukaryotic cells methylation of CpG dinucleotides by DNA methyltransferase can directly inhibit gene expression (reviewed in Ref. 1). This enzyme, which places a methyl group on the cytosine residues of 5′-CpG-3′ dinucleotides, has been isolated from several species and shown to play an important role in embryonic development (2). The inhibition of transcription by methylation has been shown to be mediated by the binding of a methyl-CpG-binding protein (MeCP-1)† to DNA sequences containing methylated CpG dinucleotides, with the degree of inhibition depending on the degree of methylation (3, 4). DNA methylation also plays a direct role in certain pathological processes, for example in the fragile X syndrome (FRAX). This disease results from the inactivation of the FMR-1 gene, which encodes an RNA-binding protein (5, 6). A block of repeating CCG triplets, which varies from 5 to 50 repeats in normal individuals, was mapped to the 5′-untranslated region of this gene (7, 8). In carriers of FRAX, the triplet block frequently contains 60–190 triplet repeats and in individuals with the frank disease, expansions to 200 or more repeats are common. Here there is a strong correlation between the severity of the disease and the degree of methylation of CpG dinucleotides in the region of the expanded CCG repeats, with higher levels of methylation being more deleterious (9, 10).

The fragile X syndrome was named for the presence of a rare, folate-sensitive fragile site on the X chromosome (FRAXA) whose appearance correlates with the disease. Fragile sites are defined cytologically as loci that exhibit properties of unstable chromatin, and they stain poorly and exhibit uncondensed gaps or enhanced DNA fragmentation. At FRAXA, the CCG repeat block itself is the site of preferential chromosomal breakage (7, 8). Recently, expanded CCG triplet blocks have been correlated with four other rare, folate-sensitive fragile sites in the human genome, FRAXE, FRAXF, FRA16A, and FRA11B (11–15). In all five cases, the expression of the fragile site depends directly on the size of the triplet repeat block and the degree of methylation of the CCG repeats and adjacent CpG islands.

To investigate the molecular basis of these fragile sites, we recently examined the ability of DNA containing long blocks of CCG triplet repeats to assemble into nucleosomes. Using a combination of electron microscopy and competitive nucleosome reconstitution/gel retardation assays, we showed (16) that blocks of >50 contiguous CCG repeats strongly exclude nucleosomes, providing a possible explanation for the unstable chromatin phenotype. To test a specific model of nucleosome exclusion, we synthesized a model DNA containing repeating CCGNN pentanucleotides and demonstrated that a tract of 48 such repeats also strongly excludes nucleosomes (17).

Because the expression of the five fragile sites described above depends on the degree of methylation, it seemed important to examine the effects of DNA methylation on the ability of DNAs containing CCG or CCGNN repeats to assemble into chromatin. In this study, we have used competitive nucleosome reconstitution/gel retardation assays to investigate this issue and show that methylation inhibits nucleosome formation 2-fold beyond the inherent exclusion due to the sequences themselves. This observation provides further support for a model in which nucleosome exclusion creates unstable chromatin at these fragile sites, which may further facilitate the binding of the MeCP-1 protein to inhibit transcription from this region.

EXPERIMENTAL PROCEDURES  
DNAs—Plasmid p(CCGNN)76 contains four head-to-tail copies of 5′-CCGTTA CCGAT CCGAA CCGGA CCGCT CCGAG CCGTC CCGTG-3′. This 240-base pair (bp) repeat block is termed here (CCGNN)76. A 268-bp fragment containing this element and a few nucleotides from the vector was obtained by EcoRI and XbaI digestion of p(CCGNN)76 (17). A 282-bp fragment containing the (CCGNN)76 repeat was obtained by XmaI digestion of pRWR378 (18), and a 262-bp pUC 19 fragment was obtained by polymerase chain reaction amplification using primer pairs from nucleotide 239 to 263 and from nucleotide 477 to 500. Restriction endonuclease-treated fragments or polymerase chain reaction products were purified by electrophoresis on 5% polyacrylamide gels, eluted with a solution containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS, and the purified DNAs were dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs), then labeled with T4 DNA kinase (New England Biolabs) in the presence of [γ-32P]ATP (Amersham).  

Methylation of DNA—SssI methylase (New England Biolabs) was used

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† The abbreviations used are: MeCP-1, methyl-CpG-binding protein; FRAX, fragile X syndrome; bp, base pair(s); DHFR, dihydrofolate reductase.
to methylate cytosines in Cpg dinucleotides (19). Reaction buffer contains 160 μM S-adenosyl methionine, 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, and 10 mM EDTA and no MgCl2 was included in the reaction to avoid the topoisomerase activity in SsI methylase preparations (20). The reactions were carried out at 37 °C for 2 h using 0.25–6 units of SsI methylase, and DNAs were then extracted with phenol and chloroform and precipitated with ethanol. The degree of methylation was determined by resistance to cleavage with a methylation-sensitive restriction endonuclease HhaI (see “Experimental Procedures”). HhaI cleaves the unmethylated pUC19 fragment into 242- and 20-bp pieces and the unmethylated (CCG)76 fragment into 234- and 48-bp pieces. The small fragments are not shown. The amount of DNA in each band was determined by PhosphorImager scanning. The percentage of uncleaved DNA in each band of the HhaI-cleaved sample is designated as the degree of methylation.

RESULTS

Methylation of DNAs Containing CCG and CCGNN Repeats—To examine the influence of DNA methylation on nucleosome formation over DNAs containing long tracts of CCG, or CCGNN repeats, SsI methylase was used to place a methyl group on the C5 position of cytosines within the 5'-CpG-3' dinucleotides (19). Two parallel sets of experiments were carried out. In one, a 262-bp pUC19 fragment (lanes 1–6) and a 282-bp fragment containing the (CCG)48 repeat element (lanes 7–12) were treated with 0, 1, or 6 units of SsI methylase to methylate cytosines at Cpg dinucleotides and the degree of methylation determined by resistance to cleavage by the methylation-sensitive restriction endonuclease HhaI (see “Experimental Procedures”). HhaI cleaves the unmethylated pUC19 fragment into 242- and 20-bp pieces and the unmethylated (CCG)48 fragment into 234- and 48-bp pieces. The small fragments are not shown. The amount of DNA in each band was determined by PhosphorImager scanning. The percentage of uncleaved DNA in each band of the HhaI-cleaved sample is designated as the degree of methylation.

For the second set of DNAs, the pUC19 fragment was treated with 0, 0.25, or 1 units of methylase to achieve degrees of methylation of 0, 36%, and 43% and the (CCGNN)48 containing DNA was treated with 0, 0.5, and 4 units of methylase to methylate at 0, 12%, and 62%, respectively (data not shown).

Competitive Nucleosome Assembly—Competitive nucleosome reconstitution was used to measure the energetics of nucleosome formation over the CCG and CCGNN repeats as compared to a pUC19 DNA of the same size. For these assays, 50 ng of each DNA fragment (labeled with 32P) methylated at three different levels was mixed with a 200-fold excess of unlabeled calf thymus DNA (10 μg) and 2.5 μg of histone octamers in a solution containing 2 mM NaCl. The salt was slowly lowered in increments of 0.1 mM to a final concentration of 0.1 mM by adding a solution of 20 mM HEPES, 1 mM EDTA, pH 7.5 (5 min for each step at room temperature). The assembly mixtures were then directly electrophoresed on 5% polyacrylamide gels at 150 V for 4 h at room temperature to separate free DNA from the nucleosome-assembled DNA. Samples were visualized by autoradiography. The amounts of DNA in each band were determined by PhosphorImager scanning (Molecular Dynamics). Each DNA was reconstituted in three separate but identical experiments.

**Fig. 1.** Determination of the level of methylation by HhaI cleavage. A 262-bp pUC19 fragment (lanes 1–6) and a 282-bp fragment containing the (CCG)48 repeat element (lanes 7–12) were treated with 0, 1, or 6 units of SsI methylase to methylate cytosines at Cpg dinucleotides and the degree of methylation determined by resistance to cleavage by the methylation-sensitive restriction endonuclease HhaI (see “Experimental Procedures”). HhaI cleaves the unmethylated pUC19 fragment into 242- and 20-bp pieces and the unmethylated (CCG)48 fragment into 234- and 48-bp pieces. The small fragments are not shown. The amount of DNA in each band was determined by PhosphorImager scanning. The percentage of uncleaved DNA in each HhaI-cleaved sample is designated as the degree of methylation.

**Fig. 2.** Competitive nucleosome reconstitution with methylated DNAs. A, competitive nucleosome reconstitution (see “Experimental Procedures”) was carried out comparing a 262-bp pUC19 fragment to a 282-bp DNA containing 76 tandem CCG repeats at three different levels of methylation. Lanes 1–3, the pUC19 fragments methylated at 0, 95, and ~100% of the available sites, respectively; lanes 4–6, the (CCG)48 DNA fragment methylated at 0, 45%, and 89% of the available sites, respectively. B, comparison of the 262-bp pUC19 fragment to a 268-bp DNA containing 48 tandem CCGNN repeats at three different levels of methylation. Lanes 1–3, the pUC19 fragments methylated at 0, 36, and 43% of the available sites, respectively; lanes 4–6, the (CCGNN)48 DNA methylated at 0, 12, and 62% of the available sites, respectively.
Methylation of CCG Triplet DNA

The ratio of DNA bound by histones to free DNA for each sample was determined by measuring the amount of DNA in each radioactive band using PhosphorImager scanning. The ratios for all samples were normalized against that of the pUC19 DNA fragment with no methylation. The ratio for the pUC19 DNA fragment with no methylation was assigned a value of 100. The values are derived from three separate experiments. The free energy was calculated according to the equation:

\[ E = RT \ln(\text{ratio of DNA in complex to free DNA for pUC19 fragment}) - RT \ln(\text{ratio of DNA in complex to free DNA for the CCG (or CCGNN)-containing fragment}) \]

The free energy for the unmethylated pUC19 DNA was defined as zero. These efficiencies correspond to 425 ± 93 and 1498 ± 75 cal/mol differences in free energy, respectively.

**DISCUSSION**

In this study, the effect of methylation of CpG dinucleotides on the ability of DNAs containing CCG or CCGNN repeats to assemble into nucleosomes was investigated. This analysis showed that DNAs containing 228–240 bp of continuous repeats, which are already severalfold less efficient in nucleosome formation compared to the unmethylated (CCGNN)48 DNA and the pUC19 fragment, respectively. These efficiencies correspond to 425 ± 93 and 1498 ± 75 cal/mol differences in free energy, respectively.

Similar results were obtained when the pUC19 fragment was compared to the 268-bp fragment containing 48 tandem CCGNN repeats (Table I, panel B; Fig. 3). The (CCGNN)48 DNA fragment methylated at 62% of the available sites was 2.1 ± 0.3-fold and 12.6 ± 1.6-fold less effective in nucleosome formation compared to the unmethylated (CCGNN)48 DNA and the pUC19 fragment, respectively. These efficiencies correspond to 425 ± 93 and 1498 ± 75 cal/mol differences in free energy, respectively.

**Table I**

| Sample       | Methylation % | Complex DNA/Free DNA | Free energy cal/mol |
|--------------|---------------|----------------------|---------------------|
| A. pUC19 (262 bp) |               |                      |                     |
| 0            | 100           | 55 ± 54              |                     |
| 95           | 90 ± 9        | 55 ± 54              |                     |
| 100          | 105 ± 7       | −21 ± 36             |                     |
| (CCG)76 (262 bp) |               |                      |                     |
| 0            | 45 ± 2        | 475 ± 15             |                     |
| 45           | 37 ± 2        | 595 ± 32             |                     |
| 89           | 23 ± 2        | 880 ± 54             |                     |
| B. pUC19 (262 bp) |               |                      |                     |
| 0            | 100           | 49 ± 46              |                     |
| 36           | 91 ± 9        | 49 ± 46              |                     |
| 43           | 98 ± 11       | 0 ± 59               |                     |
| (CCGNN)48 (268 bp) |               |                      |                     |
| 0            | 16 ± 1        | 1074 ± 20            |                     |
| 12           | 13 ± 0        | 1209 ± 0             |                     |
| 62           | 8 ± 1         | 1498 ± 75            |                     |

**Fig. 3.** Dependence of nucleosome assembly on methylation of CpG dinucleotides. Data from the competitive nucleosome reconstitution experiments (Fig. 2, Table I) are shown. Each DNA was reconstituted in three separate but identical experiments, and the fraction of DNA in the nucleosome-assembled and nucleosome-free DNA bands was measured by PhosphorImager scanning.

around the histone octamer to form nucleosomes. Sequence match analysis of the GenBank data base against 240 bp of a (G/G)3NN48 repeating sequence revealed many matches, and 75 examples showed ≥85% sequence match over 200 bp of this motif. Many of these were present in or near the control regions for eukaryotic genes (17). Among them, the 5’ control regions of the human dihydrofolate reductase (DHFR) and ETS-2 genes have been probed for nuclease-hypersensitive sites in vivo (often identified as nucleosome-free regions) and the nuclease-hypersensitive regions (27, 28) were found to overlap with the regions containing the (G/G)3NN48 motif. It remains unknown how heavily methylated these loci may be, however from the findings described here, methylation of these regions could influence gene expression by further promoting nucleosome exclusion.

The DHFR, ETS-2, and several other genes that show sequence matches with the (C/G)3NN48 motif are genes regulated by promoters lacking a TATA box. The “TATA-less” family includes many housekeeping genes, several oncogenes, and genes encoding growth factors and transcription factors (29). We suggested that the (C/G)3NN48-like sequences in these
genes may provide a mechanism that keeps the promoter regions accessible to transcription factors in part by excluding nucleosomes. Pugh and Tjian (30) have demonstrated that Sp1 is required in the initiation of transcription from TATA-less promoters by TFIIID. Sp1 is a general transcription factor that recognizes the consensus sequence: 5′-G/TGGCGCGG/G/A/G(T)-3′ (31). This sequence is very G/C-rich and thus resembles the ((G/C)\(n\))\(_{m}\) motif. Indeed a GenBank sequence analysis revealed many sites that had been shown to be Sp1 binding sites within the sequences identified as having high analysis revealed many sites that had been shown to be Sp1

genes(17) cited above argue that the free energy of nucleosome

ences may be large enough to have strong biological effects and are likely to increase in strength as the size of the repeat tract

in the presence of histone H1 and other factors, these differences

methylated and unmethylated DNA are relatively low (Table I). However, in the cell, at physiological ionic strength and in the presence of histone H1 and other factors, these differences may be large enough to have strong biological effects and are likely to increase in strength as the size of the repeat tract

lengthens. Furthermore, little has been known about how these values translate into nucleosome stability under physiological salt conditions. However, the examples of the DHFR and ETS-2 genes (17) cited above argue that the free energy of nucleosome formation measured in vitro by gel retardation methods are physiologically relevant. In addition, the strong correlation between the unstable chromatin phenotype of the rare folate-sensitive fragile sites and the unfavorable energies of nucleo-

some formation of repeating CCG DNA, in particular when methylated, further argues that the range of free energies measured in vitro provide insight into the range of chromatin stability in vivo.

Myotonic dystrophy is a human genetic disease also linked to expansions of repeating triplets, in particular a block of CTG expansions of repeating triplets, in particular a block of CTG

tracts of these two triplet repeats.

The data presented here support a general proposal for the role of CCG repeats and methylation in the generation and expression of fragile sites containing these triplet repeats (Fig. 4). Long repeating CCG triplet blocks inhibit chromatinization of DNA (16), which would make the accessibility of these regions to DNA methyltransferase. Methylation in turn would further repress chromatinization and make the DNA more available for binding by methyl-CpG-binding proteins, which have been shown to repress transcription (3, 4). Thus, these changes promoted by methylation would lead to the simultaneous generation of a fragile site and the repression of nearby genes such as the FMR-1 gene.

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