Human Fragile Site FRA16B DNA Excludes Nucleosomes in the Presence of Distamycin*

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Human fragile sites are weak staining gaps in chromosomes generated by specific culture conditions. The short CCG repeating DNA derived from folate-sensitive fragile sites has been shown to exclude single nucleosomes. To test whether this nucleosome exclusion model provides a general molecular mechanism for the formation of fragile sites, a different class of fragile site, the 33-base pair AT-rich repeating DNAs derived from the rare distamycin-inducible site, FRA16B, was examined for its ability to assemble single nucleosomes and nucleosome arrays using in vitro nucleosome reconstitution methods. The FRA16B DNA fragments strongly exclude nucleosome assembly only in the presence of distamycin, and increasing the number of 33-bp repeats increases the effect of distamycin in the destabilization of the nucleosome formation, suggesting a common mechanism for the formation of fragile sites.

Fragile sites are chromosomal abnormalities in humans and are linked to the incidence of certain cancers and other severe disorders. Cytologically they are defined as sites of poor staining, gaps, or DNA strand breakage in metaphase chromosomes when cells are treated under specific culture conditions. To date, more than 100 different types of fragile sites have been identified in the human genome, classified as common or rare and further divided according to the agents used to identify them in cultured cells (1). The rare fragile sites are classified into the folate-sensitive, distamycin A-inducible and bromodeoxyuridine-inducible groups, whereas the common fragile sites include the aphidicolin-, 5-azacytidine-, and bromodeoxyuridine-inducible groups (1). Although the first fragile site was discovered in 1965 (2), the molecular nature of fragile sites is not clear. It has been shown that the locations of many fragile sites are cytogenetically correlated to those of deletion and translocation breakpoints in cancer cells (3). Also, fragile sites have been mapped at the molecular level to the integration sites of oncogenic viruses (4). Therefore, it is critical to investigate the nature of the DNA sequence and structure where fragile sites are located in order to understand how viruses find the fragile site and why fragile sites are preferential loci for deletion and translocation.

FRAXA, one of the folate-sensitive fragile sites, has been studied extensively because of its direct association with fragile X syndrome, one of the most common forms of inherited mental retardation (5–7). In FRAXA, as with four other folate-sensitive sites: FRA11B (8), FRA16A (9), FRAXE (10), and FRAXF (11), the DNA sequences reveal expanded CCG repeats in individuals expressing those fragile sites. To investigate the molecular basis of these fragile sites, we (12, 13) and others (14) have examined the ability of DNA containing long blocks of CCG triplet repeats to assemble into nucleosomes. Using a combination of electron microscopy (EM)† and competitive nucleosome reconstitution assays, we showed that expanded CCG repeats strongly exclude nucleosomes in vitro (12) and that methylation of the CCG repeats further enhances this exclusion (13), thus providing a possible molecular explanation for the basis of fragile sites in chromosomes. Further, we showed that a DNA motif(CCGNN)n based on the CCG repeat excludes nucleosomes in vitro (15) and is a DNase I hypersensitive site in yeast (16). Therefore, we suggest that the formation of the folate-sensitive fragile site results from the inability of DNA at this site to fold properly or compactly into chromosomes during metaphase.

To examine whether this model of nucleosome exclusion is specific for the folate-sensitive sites or a common mechanism for other classes of fragile sites, DNAs from a distamycin A-inducible fragile site, FRA16B, were tested for their ability to assemble into nucleosomes. FRA16B is a rare distamycin A-inducible fragile site located on chromosome 16 and found in about 1 in 40 chromosomes in the European population (17). Recently, DNA sequencing of FRA16B has identified a 33-bp AT-rich repeat (ATATATTATATATATATCTAATAATATATCTAATAATATTATATCTAATAATATATAAT3') and 5'-(ATATATTATATATATATATCTAATAATATATCTAATAATATATAAT-3') were synthesized, annealed to generate a duplex with 4 nucleotide cohesive ends, and ligated head-to-tail. Monomers, dimers, and trimers of the

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‡ The abbreviations used are: EM, electron microscopy; nt, nucleotide(s); PCR, polymerase chain reaction; RSF, remodeling and spacing factor.
accomplished by slowly lowering the salt in increments of 0.1 M to a was carried out as described (20). Briefly, nucleosome assembly was labeled calf thymus DNA and 2.5/ H9262 Procedures.

The assembled sample was then mixed with an equal volume of buffer (A) or 100 μM distamycin (B) for 30 min at room temperature. These samples were further fixed with 0.6% glutaraldehyde and prepared for EM. Micrographs are shown in reverse contrast. A Cohu CCD camera attached to a Macintosh computer programmed with the NIH IMAGE software was used to form the images. The bar represents 160 nm. C, quantitative analysis. In fields of molecules, the number that represents protein-free DNA was scored against those bound by histone octamers.

66-bp AT-rich DNAs (2 copies of the 33-bp FRA16B DNA) were purified by gel electrophoresis and cloned into the pGEM3zf (+) vector (Promega). The sequences of the inserts in the recombinant plasmid pFRA16B1-3 were verified by direct DNA sequencing. HeLa cell histone octamers were isolated as previously described (19).

The plasmid containing a human FRA16B sequence was constructed by inserting a 921-bp fragment into the pGEM3zf (+) vector. The 921-bp fragment was generated by polymerase chain reaction (PCR) of human genomic DNA with primers 27 and 29 flanking the human FRA16B site (18) followed by AccI and BglII digestion. The 33-bp AT-rich FRA16B repeat was used in a nucleosome assembly reaction or prior to assembly (see text) for 30 min at room temperature. The free energy of nucleosome assembly was calculated according to the equation $E = RT\ln Q - RT\ln Q_{\text{free}}$ (12, 15) where $E$ represents the free energy of assembly of the 33-bp repeat-containing fragment, $K$ is the ratio of DNA in complex to free DNA for the pUC19 fragment, and $Q$ is the ratio of DNA in complex to free DNA for the 33-bp repeat-containing fragment. The free energy for the pUC19 DNA was defined as zero. The values are derived from three separate experiments.

**Distamycin Treatment**—Distamycin A (Sigma) at a concentration of 100 μM was added to an equal volume of DNA sample after nucleosome assembly or prior to assembly (see text) for 30 min at room temperature.

**Assembly and Analysis of Nucleosome Arrays**—Assembly reactions were performed as described in Loyola et al. (21) with 1 μg of plasmid DNA, 0.9 μg of histones, 0.15 μg of remodeling and spacing factor (RSF) (kindly provided by D. Reinberg, Robert Wood Johnson Medical School, Piscataway, NJ), 75 μg of bovine serum albumin, 3 mM ATP, 30 mM phosphocreatine, 0.2 μM of phosphocreatine kinase, 5 mM MgCl2, 60 μM KCl, 10 mM Hepes, pH 7.6, 0.2 mM EDTA, and 5% glycerol. Reactions were incubated at 30 °C for 16 h. The reaction mixtures were then partially digested with micrococcal nuclease (22), and the digestion patterns were analyzed by Southern blots, first probing with an oligonucleotide derived from nt 1572 to 1601 of pGEM3zf (+) (the vector probe). The reverse probing steps were also carried out, and the results showed no difference with the order of probing.

**RESULTS**

**Nucleosome-assembled FRA16B DNA Expels Nucleosomes upon Addition of Distamycin**—To examine the ability of the 33-bp AT-rich FRA16B DNA repeat to assemble into nucleosomes, a 250-bp DNA fragment containing 6 copies of the FRA16B repeat was used in a nucleosome assembly reaction and analyzed by electron microscopy. Fig. 1A shows a field with free DNA and DNAs bound by histone octamers with different translational positioning. The size of a nucleosome was determined as a histone octamer by comparing it to the size of nucleosomes in EM micrographs from a previous study (12) in which micrococcal nuclease was used to confirm the formation of legitimate nucleosomes. Results from scoring protein-free DNA versus histone octamer-bound DNA are shown in Fig. 1C.
FRA16B DNA Excludes Nucleosomes with Distamycin

Fig. 2. Competitive nucleosome reconstitution with DNAs containing various numbers of 33-bp AT-rich FRA16B repeats.

A. Diagram showing the location of the 33-bp AT-rich FRA16B repeating sequences within the DNA fragments and the nucleotide sequence of the 33-bp repeat. Fragment 1 is the 262-bp pUC19 fragment, fragments 2–4 are DNA fragments containing 2, 4, and 6 copies of the 33-bp repeat, respectively, and fragment 5 is the 250-bp DNA containing 48 copies of the CCGNN motif (15). B. Autoradiogram of a competitive nucleosome reconstitution experiment. Lanes 1–5, DNA fragments 1–5 assembled with histones; lanes 6–10, DNA fragments processed as for samples in lanes 1–5 but serving as controls without histone octamers. DNA preparation, reconstitution of the fragments with histone octamers, and gel electrophoresis were as described under “Experimental Procedures.”

A pUC19 DNA fragment of 262 bp, representing a random DNA sequence, was also included as a control for nucleosome assembly. The (33bpFRA16B)$_4$ DNA, treated under the conditions described under “Experimental Procedures,” has 65% of the DNAs bound by histone octamer and a slightly better assembly ability compared with the pUC19 DNA, which shows 45% of the molecules associated with histone octamers.

FRA16B is one of the distamycin-inducible fragile sites, and to observe this site FRA16B-expressing cultured cells usually have to be treated with 100 μM distamycin prior to the preparation and examination of the metaphase chromosomes. Therefore, the above nucleosome-assembled DNA samples were mixed with an equal volume of 100 μM distamycin at room temperature for 30 min and scored by EM visualization (Fig. 1B). Interestingly, for the (33bpFRA16B)$_4$ DNA, only 21% of the total molecules are assembled into nucleosomes, which is about 3-fold lower compared with the same DNA without the distamycin treatment. In contrast, pUC19 DNA shows no significant differences upon the addition of distamycin.

To quantitate the strength of a DNA for nucleosome assembly, a competitive nucleosome reconstitution assay was used (Refs. 12–15, see “Experimental Procedures”). DNA fragments containing 2, 4, or 6 copies of the FRA16B DNA repeat (Fig. 2A) ranging from 250 to 267 bp were constructed. The same pUC19 DNA fragment of 262 bp described above was also included as the baseline for nucleosome assembly. In addition, a DNA fragment containing 48 copies of a CCGNN repeat, which was previously shown to exclude nucleosomes, was compared (15).

The nucleosome-assembled DNAs appear as retarded bands when the DNA samples assembled with histone octamers (Fig. 2B, lanes 1–5) are compared with the same set of DNAs assembled in the absence of histone octamers (Fig. 2B, lanes 6–10). Because the aforementioned EM analysis shows that only histone octamers (not dimers or tetramers) are observed in nucleosome-assembled DNAs, the multiple retarded bands seen in the FRA16B DNA samples are probably caused by DNAs bound to histone octamers with different translational positionings. The results summarized in Table I (two left columns) present the ratio of the nucleosome-assembled DNA to free DNA for the pUC19, FRA16B, and (CCGNN)$_{48}$ DNA fragments. The FRA16B DNA fragments were about 2–2.8-fold more effective in nucleosome assembly compared with the pUC19 fragment, regardless of the number of 33-bp repeats. The (CCGNN)$_{48}$ fragment was 3-fold less effective relative to the pUC19 fragment, agreeing with the previous observations (15).

Subsequently, the aforementioned nucleosome-assembled DNA samples were treated with an equal volume of 100 μM distamycin at room temperature for 30 min and analyzed on gels (Fig. 3A). The results are shown in Table I (two middle columns). This analysis showed that the addition of distamycin had only a minor measurable influence (~30% decrease) on nucleosome formation for the pUC19 fragment. In contrast, the addition of distamycin to the nucleosome-assembled FRA16B DNA had a dramatic effect on the energetics of nucleosome formation on these fragments, agreeing with the EM analysis. The 250-bp DNA containing 6 copies of the 33-bp FRA16B repeat is 46-fold less efficient in nucleosome assembly upon distamycin treatment, 14-fold less efficient than the pUC19 DNA with the same distamycin treatment, and 20-fold less than the pUC19 DNA without distamycin treatment. All three FRA16B DNA fragments show nucleosome exclusion in the presence of distamycin with an increasing ability to exclude nucleosomes in proportion to the number of 33-bp repeats (Fig. 3B). Indeed, DNAs containing 2 and 4 copies of the 33-bp repeat show about 4- and 9-fold less efficient nucleosome formation, respectively, upon the addition of distamycin. These results suggest that the 33-bp repeat sequence is responsible for the nucleosome exclusion.

The difference in free energy of assembly between the (33-bp)$_n$-containing FRA16B DNA and the same fragment treated with distamycin is 2260 cal/mol. In the presence of distamycin, compared with the pUC19 DNA, the distamycin-treated (33bpFRA16B)$_n$ DNA is 1553 cal/mol less favorable in assembling nucleosomes. These results demonstrate that the 33-bp FRA16B repeating sequence expels nucleosomes efficiently when distamycin is added to the assembled samples.

Incubation of Distamycin with the FRA16B DNAs Prior to Nucleosome Assembly Excludes Nucleosomes—We have shown that distamycin disrupts nucleosomes assembled with FRA16B DNAs. Next we examined whether binding of distamycin to FRA16B DNAs affects their ability to assemble nucleosomes. The FRA16B DNA fragments were first mixed with distamycin at the same concentration as above, at room temperature for 30 min, then assembled with nucleosomes by the stepwise salt dilution method (same as above), and then analyzed for assembly on gels. Again, the (33bpFRA16B)$_n$ DNA showed strong nucleosome exclusion as efficient as for the addition of distamycin after assembly of nucleosomes and was about 46-fold less favorable in nucleosome formation than the same DNA in the absence of distamycin (Table I, two right columns). Therefore, we conclude that the 33-bp FRA16B repeat generates a strong nucleosome exclusion element in the presence of distamycin.

Interestingly, for the pUC19 DNA, the (33bpFRA16B)$_n$ DNA, and the (33bpFRA16B)$_n$ DNA, the preincubation with distamycin gives these DNAs 2.3–3.4-fold lower efficiency in nucleosome assembly relative to the addition of distamycin after assembly.
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 by a phosphorimager. The ratios for FRA16B DNA were normalized against that of the pUC19 DNA fragment. The ratio for the pUC19 DNA fragment in the absence of distamycin was assigned a value of 1. The free energy of nucleosome assembly was calculated according to the equation $E = RT\ln K - RT\ln Q$ (12, 13, 15) where $E$ represents the free energy of assembly of the 33-bp repeat-containing fragment, $K$ is the ratio of DNA in complex to free DNA for pUC19 fragment, and $Q$ is the ratio of DNA in complex to free DNA for the 33-bp repeat-containing fragment. The free energy for the pUC19 DNA was defined as zero. The values are derived from three separate experiments.

**Table I**

| DNA        | None | After assembly | Preincubated |
|------------|------|----------------|--------------|
|            | Complex DNA/ free DNA | Free energy | Complex DNA/ free DNA | Free energy | Complex DNA/ free DNA | Free energy |
| pUC19      | 0    | 0.68 ± 0.05    | 0.26 ± 0.01 | 798 ± 23 |
| (33bpFRA16B)2 | 1.97 ± 0.15 | 0.51 ± 0.09 | 402 ± 115 | 1111 ± 46 |
| (33bpFRA16B)4 | 2.79 ± 0.51 | 0.32 ± 0.12 | 702 ± 251 | 1181 ± 68 |
| (33bpFRA16B)6 | 3.23 ± 0.74 | 0.05 ± 0.02 | 1782 ± 291 | 1782 ± 120 |
| (CCGN148)  | 0.31 ± 0.04 | 673 ± 67    | NA           | NA         | NA           | NA         |

* NA: not applicable.

**FIG. 3.** Addition of distamycin to nucleosome-assembled FRA16B samples. A, the nucleosome assembly mixture from Fig. 2 for each DNA fragment was mixed with an equal volume of 100 $\mu$M distamycin at room temperature for 30 min and then analyzed on a 5% polyacrylamide gel. Lanes 1–4 and 5–8 are the DNA samples similar to lanes 1–4 and 6–9, respectively, shown in Fig. 2. B, the dependence of nucleosome assembly on the number of 33-bp repeats is plotted. 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 a phosphorimager.

**Distamycin Affects Nucleosome Arrays Assembled on Long FRA16B DNA**—We have demonstrated that distamycin abolishes the ability of FRA16B DNA to assemble a single nucleosome. Next we investigated the effect of distamycin on the assembly of multiple nucleosomes with a cloned human FRA16B sequence under physiological conditions. A 921-bp human FRA16B DNA was cloned into the pGEM3zf(+) vector, and it contains 14 copies of the 33-bp repeat and some imperfect repeats with AT-rich flanking sequences. This plasmid was used to assemble chromatin by RSF, which has been shown to assemble and space nucleosomes (21, 23). The assembled plasmid was treated with distamycin or buffer, digested with micrococcal nuclease, and analyzed by Southern blots. Without distamycin, the FRA16B sequence (Fig. 4, lane 2) and the upstream vector sequence (Fig. 4, lane 5) can form regularly spaced nucleosome arrays as demonstrated by micrococcal nuclease digestion. However, with addition of distamycin the nucleosome array was lost from the FRA16B sequence (Fig. 4, lane 3), but spaced nucleosomes are still present in the vector region of the same plasmid (Fig. 4, lane 6). These results agree with what we observed in the assembly of single nucleosomes and argue that increasing the number of 33-bp repeats will increase the effect of distamycin in the destabilization of the nucleosome array.

**FIG. 4.** The effect of distamycin on the formation of nucleosome arrays on a plasmid containing a 920-bp FRA16B sequence. RSF-assembled plasmids (lanes 1 and 4, the vector only; lanes 2 and 3 and 5 and 6, the plasmid containing the FRA16B sequence) were mixed with distamycin (lanes 3 and 6) or buffer (lanes 1 and 2 and 4 and 5), partially digested by micrococcal nuclease, and analyzed by Southern blotting first with the FRA16B probe (lanes 1–3), then re-probing with the vector probe (lanes 4–6). The vector probe is 1535 and 1625 bp from the AccI and BglII sites of the FRA16B sequence, respectively.

In this study we demonstrated that with addition of distamycin to the nucleosome-assembled FRA16B DNAs, or to these DNAs prior to nucleosome assembly, the FRA16B fragments strongly exclude nucleosomes compared with pUC19 DNA with the same treatment and expel nucleosomes up to 46-fold compared with the same fragment without distamycin. As the number of 33-bp repeats increases the ability to exclude nucleosomes also increases. These results suggest that both the 33-bp AT-rich repeating sequence and the presence of distamycin contribute to nucleosome exclusion by the FRA16B fragments, which would explain the basis for the generation of distamycin-inducible fragile sites.

Although low copy numbers of 33-bp repeats, which are not within the range of repeats expressing the fragile site, were
examined in this study, the phenomenon of nucleosome destabilization in the presence of distamycin could be amplified in FRA16B-expressing cells with the 33-hp repeat for as many as 2000 copies for the following reasons. First, the tendency to exclude nucleosomes is likely to increase in strength as the size of the repeat tract lengthens (Fig. 3). Second, the distamycin-dependent destabilization is not limited to single nucleosomes but also extends to nucleosome arrays (Fig. 4). Moreover, the lack of the formation of nucleosome arrays could affect the higher order organization of chromosome regions. In addition, distamycin has been shown to inhibit the binding of AT-rich DNA sequences to histone H1 and nuclear scaffolds (24), which would result in further improper packaging or premature un-wrapping of chromatin.

We also realize that the free energy differences in nucleosome assembly were measured in vitro and are relatively low (Table I). However, in the cell and in the presence of histone H1 (see aforementioned reasons), these differences may be large enough to have strong biological effects. Indeed, the strong explanation for nucleosome exclusion by the AT-rich FRA16B DNA sequences to histone H1 and nuclear scaffolds (24), which lack of the formation of nucleosome arrays could affect the higher order organization of chromosome regions. In addition, distamycin has been shown to inhibit the binding of AT-rich DNA sequence in yeast (16) and the unfavorable ener- eny and unwrapping of chromatin.

de not be able to adopt a rotational positioning favorable for nucleosome assembly. This interpretation would also account for the increasing nucleosome exclusion ability as the number of FRA16B AT-rich repeats increases (Fig. 3).

In summary, the results from this study provide strong support for a general model in which nucleosome exclusion creates unstable chromatin at these fragile sites and leads to the formation of gaps, DNA strand breakage, or poor staining in metaphase chromosomes.

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