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Mononucleosomes Assembled on a DNA Fragment Containing $(GGA/TCC)_n$ Repeats Can Form a DNA–DNA Complex

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A DNA fragment of 163 bp containing 11 GGA repeats formed two-end positioned mononucleosomes as efficiently as that of CTG repeats. However, the rotational positioning of the GGA fragment was weak because clear DNase I cleavage patterns with 10-base periodicity were not seen near the center of the GGA fragment but were detected in the entire region of the CTG fragment. Incubation of the GGA mononucleosomes with the same fragment provided the DNA–DNA complex, which had been shown by using naked DNA fragments. DNase I digestion of the complex exhibited protection in the GGA repeats and in flanking sequences of about 30 bp at both sides, suggesting that both the repeat and flanking regions were involved in the association. Interestingly, histone H1, which enhanced DNA–DNA association on naked DNA, did not affect the complex formation on mononucleosomes. These results imply that GGA microsatellites in genomes could associate with one another at multiple sites and that the association may play a role in functional organization of higher order chromatin architecture.

Key Words: **chromatin structure; DNA–DNA complex formation; GGA repeats; histone H1; reconstituted mononucleosome.**

Minisatellites and microsatellites are a type of tandemly repeated DNA and most of them are not clear of the biological significance. Telomeric DNA is an exceptional example of a minisatellite which comprises hundreds of copies of the TTGGGG motif in yeast and of the TTAGGG motif in humans and mice (1). The former telomeric repeats can form a four-stranded structure, called a G-quartet, *in vitro* (2). Telomeres mark the ends of chromosomes, function in DNA replication and repress transcription of nearby genes. Other tandem repeats may also influence DNA transaction through constituting unique chromatin structures due to repetition of the same or a similar sequence unit. Recent studies of transgenes with tandem repetition in plants and mammals revealed that packaging of the transgenes into similar nucleoprotein complexes induces transcriptional silencing (3–5). This gene silencing can be ascribed to interaction between homologous structures in a stretch of repeated DNA which may be facilitated through DNA–protein–DNA or DNA–protein–protein–DNA interactions (5, 6). Homologous interaction has been also reported between alleles on separate chromosomes in Drosophila (7, 8). FISH analysis visualized association of $Brown^{dominant}$ (Bw^D) locus and Bw^w and centromeric heterochromatin of chromosome 2 in $Bw^D/+$ flies where the Bw^D locus has an insertion of about 2 Mb of AAGAG satellite DNA existing near the centromeric end of chromosome 2 (7, 9). This association is due to the repeat-repeat communication which leads to silencing of the wild-type *Bw* allele (5, 7, 9).

Interaction between homologous sequences could be mediated directly by DNA–DNA interaction. We previously reported DNA–DNA complex formation of a microsatellite consisting of (GGA/TCC)n repeats *in vitro* (10). The repeats consisting of polypurine/polypyrimidine sequence like the AAGAG satellite described above show three characteristics. First, oligonucleotide GGA repeats formed parallel-orientated homoduplex (11). Second, triplexes formed between double-stranded (ds) DNA fragments containing (GGA/TCC)n repeats and single-stranded oligonucleotide GGA or GGT repeats (12). The GGT-(GGA/TCC)n triplex was formed by typical Hoogsteen-type hydrogen-bonding, while the GGA-(GGA/TCC)n triplex showing a D-loop structure was due to Watson–Crick type in-

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teraction (12). Third, DNA fragments containing (GGA/TCC)n repeats formed homodimers of two paired double-stranded DNA (10). Interestingly, the formation of triplexes and DNA–DNA complexes was promoted by HMG-box and histone H1 (10, 13).

We have now extended our previous observations of DNA–DNA complex formation through (GGA/TCC)n repeats by using reconstituted mononucleosomes. In this paper, we describe the ability of the mononucleosomes to form DNA–DNA complexes with naked DNA carrying (GGA/TCC)n repeats. This suggests an implication for the DNA–DNA association through microsatellite DNA in functional organization of higher order chromatin architecture which may affect gene expression.

MATERIALS AND METHODS

Oligonucleotides and DNA fragments. Oligonucleotides were synthesized on a Beckmann Oligo 1000 DNA synthesizer. DNA fragments were prepared by PCR amplification of plasmids pUC118 containing 11 (GGA/TCC) repeats (named pUC-GGA11) and 11 (CTG/CAG) repeats (named pUC-CTG11) (schematically shown in Fig. 1).

The plasmid DNAs were used as templates in PCR to produce unlabeled, uniformly labeled or 5′-end labeled DNA fragments using a set of primers as follows: 230 bp and 127 bp (named M1) fragments of pUC-GGA11, F118A (5′-ATGTGCTGCAAGGCGATTA-3′) and R118A (5′-GTATGTTGTGTGGAATTGTGAGCGG-3′) and F118B (5′-GTTTTCCCAGTCACGAC-3′) and R118B (5′-ATTCGAGCTCGGTACCCGG-3′), respectively; 230- and 163-bp fragments of pUC-CTG11, F118A (see above) and R118A (see above) and F118B (see above) and R118B (see above), respectively.

5′-end labeled DNA fragments were prepared as follows. PCR products were purified on 5% polyacrylamide gel electrophoresis (PAGE), labeled at the 5′-ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, and again purified on 5% PAGE after cutting the DNA fragments with *Eco*RI or *Xba*I. 163 bp uniformly-labeled ^{32}P-DNA fragments were synthesized by PCR amplification of pUC-GGA11 and pUC-CTG11 with primers F118A (see above) and R118B (see above) in the presence of [α-^{32}P]CTP, and were purified on 5% PAGE.

Reconstitution of mononucleosome. Mononucleosome reconstitution was carried out essentially as described previously (14). Briefly, core histones prepared from HeLa cell nuclei (1.25 μg) were mixed with ^{32}P-labeled DNA fragments and 1.5 μg of carrier DNA (λ/*Hin*dIII DNA) in buffer A [TE (10 mM Tris (pH 7.4)–1 mM EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM β-mercaptoethanol (β-ME)] containing 2 M NaCl. Samples were sequentially dialyzed at 4°C against 2 M NaCl buffer A overnight, 1.5 M NaCl buffer A for 1 h, 1.0 M NaCl buffer A for 4 h, 0.75 M NaCl buffer A for 4 h and finally buffer A overnight.

DNA–DNA complex formation. Association reaction of DNA–DNA complex formation between 1 nM ^{32}P-labeled DNA (naked or mononucleosome) and 100 nM unlabeled dsDNA in the presence and absence of 50 nM histone H1 were carried out essentially as described previously (10), except for 20 mM Hepes (pH 7.9) and 50 mM NaCl used as the reaction mixture.

Micrococcal nuclease treatment. Mononucleosomes reconstituted on the uniformly labeled 163-bp fragments of pUC-GGA11 and pUC-CTG11 were digested with 0.15 or 0.6 units of MNase (Pharmacia) for 3 min at 22°C in the presence of 1 mM $CaCl_2$. Reactions were terminated with the addition of a buffer containing 5 mM EGTA, 0.3 M NaCl and 10 μg/ml yeast RNA. After 30 min treatment with proteinase K at room temperature, the reaction mixtures were extracted with phenol/chloroform and chloroform, followed by ethanol precipitation. Recovered DNA fragments were separated on 6% PAGE and autoradiographed. ^{32}P-labeled *Hpa*II-digested fragments of pBR322 were used as size markers.

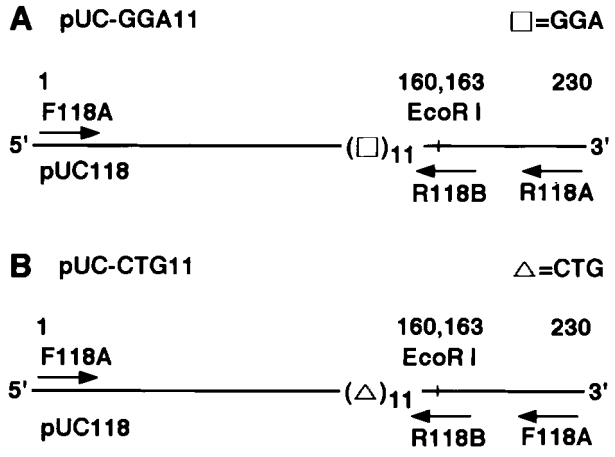

FIG. 1. Schematic representation of plasmid DNAs used as the templates in PCR to produce DNA fragments: (A) pUC-GGA11 and (B) pUC-CTG11. Numbers indicate the nucleotide positions and restriction sites relative to the ^{32}P-labeled 5′-end. Sites of the primer sets used are shown by arrows.

DNase I footprinting. DNase I footprinting was performed for naked DNAs, mononucleosomes, DNA–DNA complexes and DNA–DNA complexes formed on mononucleosomes. ^{32}P-labeled DNA or complexes were partially digested with various units (0.0125 to 2) of DNase I (Takara, Kyoto) in the presence of 5 mM $MgCl_2$ for 1 min at 22°C. After incubation, the reactions in most experiments were stopped with the addition of a buffer containing 10 mM Tris (pH 7.4), 0.3 M NaCl 0.3% SDS and 10 μg/ml yeast RNA, followed by phenol/chloroform extraction and ethanol precipitation. In some experiments of ^{32}P-mononucleosomes used, the digested nucleosomes were purified from polyacrylamide gel after 5% PAGE and then subjected to phenol/chloroform extraction. The ^{32}P-DNA recovered was separated by 6% polyacrylamide/8 M urea gel electrophoresis. Size markers were made by the extension from the 5′-end labeled primers, which was labeled at the same site as the 5′-end ^{32}P-labeled DNA fragment used for DNase I footprinting, with a *Taq* thermal cycle dideoxy DNA sequencing system (Takara, Kyoto).

Gel mobility shift assay. Samples were separated by electrophoresis on a 5% native polyacrylamide gel in a buffer of 1× TBE containing 10 mM $MgCl_2$ and 50 mM NaCl at 100 V for 4 h at 4°C or on a 0.7% agarose gel in a buffer of 0.5× TBE at 100 V for 2 h and 10 min at room temperature. After electrophoresis, gels were dried and autoradiographed.

RESULTS

Mononucleosome Assembly on DNA Fragment Containing GGA Repeats

Figure 1 schematically shows two different plasmid DNAs containing an array of GGA or CTG repeats. They were used as templates in PCR to produce DNA fragments containing the repeats. Mononucleosomes were reconstituted on the DNA fragments by the method of salt gradient dialysis (14). CTG-repeats are known to be efficiently wrapped around the histone octamers (15, 16) and hence used as a control in this study.

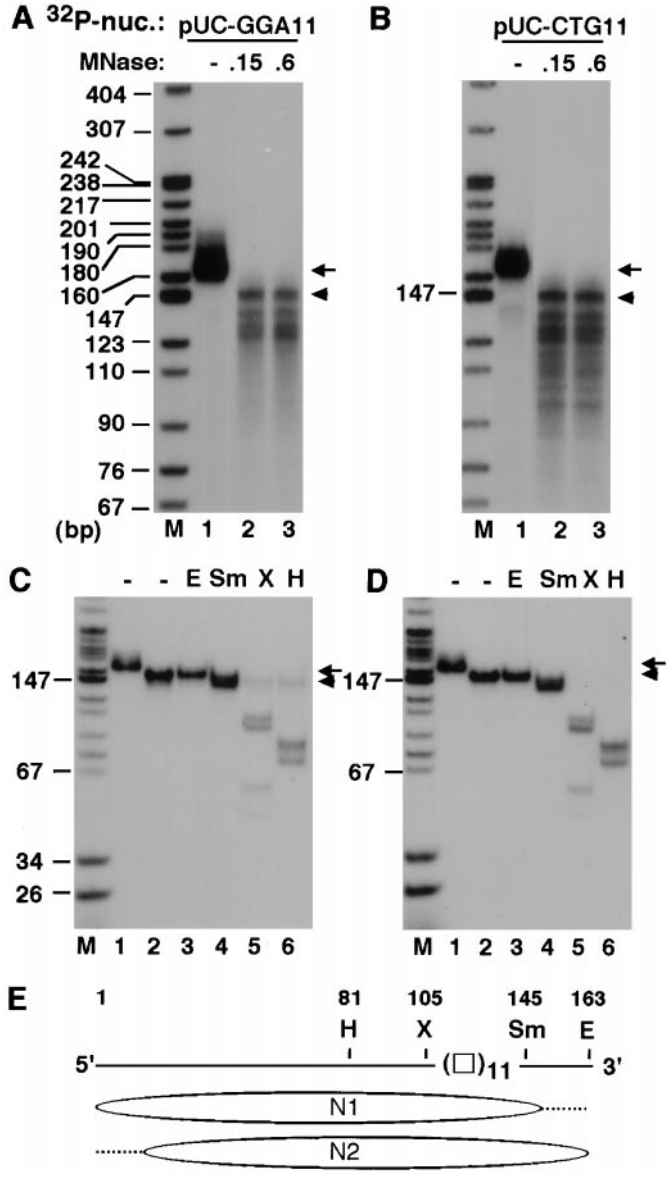

FIG. 2. MNase trimming and translational positioning of reconstituted mononucleosomes on 163-bp DNA fragments. Mononucleosomes reconstituted on uniformly labeled 163-bp fragments of pUC-GGA11 (A) and pUC-CTG11 (B) were treated with MNase as described under Materials and Methods. An arrow and an arrowhead indicate positions of the original DNA fragments and nucleosome core DNA fragments, respectively. The bands of MNase-resistant 146-bp fragments derived from mononucleosomes on pUC-GGA11 (C) and pUC-CTG11 (D) were extracted and digested with (lanes 3–6) and without (lane 2) restriction enzyme as indicated. The digests were electrophoresed on 6% native PAGE together with ^{32}P-labeled pBR322/*Hpa*II as size markers. H, X, Sm, E, and squire represent *Hin*dIII, *Xba*I *Sma*I, *Eco*RI, and GGA-repeat, respectively. (E) Results of translational positionings of mononucleosomes are schematically shown. Nucleosome positions and MNase-digested sequences are represented as ovals and broken lines, respectively.

Figures 2A and 2B show gel electrophoretic patterns of the micrococcal nuclease (MNase) digestion of mononucleosomes reconstituted on uniformly labeled 163-bp DNA fragments of pUC-GGA11 and pUC-CTG11, respectively. Both mononucleosomes yielded MNase-resistant DNA fragments at the position of the 147 bp marker (indicated by arrowheads), and smaller fragments between 140 and 100 bp. Such cutting pattern is similar to that of the nucleosome reconstituted on 5S rDNA fragment reported by An *et al.* (17). Naked DNA of the fragments was completely digested under the same conditions (data not shown). The results indicated that the DNA fragment containing GGA repeats was able to be wrapped around the histone octamers. Translational positioning was examined of mononucleosomes (18). MNase-resistant 146-bp core DNA fragments were recovered from the 147-bp band areas in polyacrylamide gel (arrowhead in Figs. 2A and 2B) and cleaved with *Eco*RI, *Sma*I, *Xba*I, or *Hin*dIII. Figures 2C and 2D show PAGE of the DNA digests of the GGA and CTG repeats, respectively. Cleavage patterns of both repeats were essentially the same. *Xba*I (lane 5) and *Hin*dIII (lane 6) enzymes cleaved the DNA into four (100, 95, 60, and 50 bp) and two (80 and 70 bp) fragments, respectively. A small fragment of 20 bp was yielded in the digest of *Sma*I (lane 4), but no cleavage was detected with *Eco*RI digestion (lane 3). These restriction cleavage patterns were the same to those that were supposed to arise from a mixture of two different types of mononucleosomes; one was located at the 5′ end of the fragment and the other at the 3′ end, which are schematically shown as N1 and N2 in Fig. 2E.

Figure 3 shows patterns of limited digestion of mononucleosomes with DNase I, representing the rotational positioning (18). The mononucleosome reconstituted on 5′-end-labeled DNA fragment of pUC-GGA11 provided a ladder of bands and the band pattern was distinct from that of naked DNA (compare Fig. 3A, lanes 6 and 7 with lanes 2 and 3). The band ladder showed alternating patterns of cleavage and protection with roughly 10 bp intervals (indicated by arrows in Fig. 3A) except for a repeat-flanking region (indicated by broken line). On the contrary, the mononucleosome reconstituted on pUC-CTG11 provided cleavage patterns with clear 10-base periodicity in the entire DNA region including the repeat (lanes 6–8 in Fig. 3B). This is compatible with the previous observation that CTG repeats are efficiently wrapped around the histone octamers (15, 16).

DNA–DNA Complex Formation on the Mononucleosome Containing GGA Repeats

The mononucleosome reconstituted on the fragment containing GGA repeats was used as a substrate for examining the ability to form DNA–DNA complex by DNase I footprinting and gel mobility shift assay. Figure 4A shows DNase I footprinting analyses of the DNA–DNA complexes that were formed on naked DNA fragment of pUC-GGA11 or on the mononucleosome

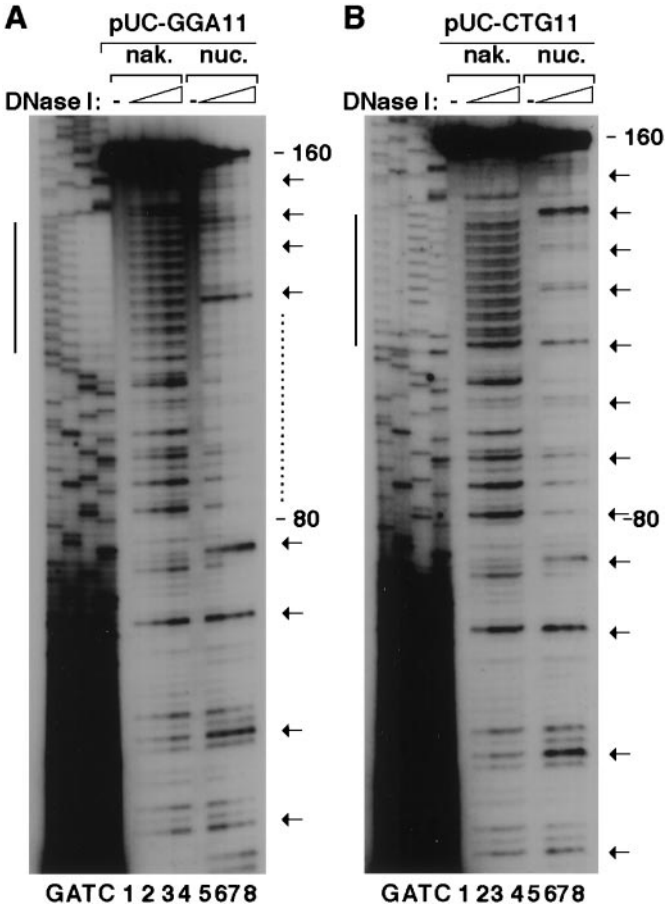

FIG. 3. DNase I digestion of mononucleosomes reconstituted on the fragments of pUC-GGA11 and pUC-CTG11. (A) 5′-end-labeled naked DNA of 160 bp were treated with 0.0125, 0.025, and 0.05 units of DNase I (lanes 2–4) and mononucleosome reconstituted on the same fragment were treated with 0.5, 1, and 2 units of DNase I (lanes 6–8), and separated on 6% polyacrylamide/8 M urea gels as described under Materials and Methods. Untreated samples are indicated as − (lanes 1 and 5). A vertical line indicates the region of GGA repeats. Arrows and a broken line indicate the clear and faint cleavage sites of DNase I digestion, respectively. (B) 5′-end labeled 160-bp fragment of pUC-CTG11 used. Experiments were performed as in A. A vertical line indicates the region of CTG repeats. Arrows indicate cleavage sites.

reconstituted with the same DNA fragment. The 5′-end-labeled 160-bp naked DNA provided a ladder of bands that were produced by limited DNase I digestion (lane 2), and the pattern did not change in the presence of dsDNA (lane 3) or histone H1 (lane 4) in the incubation mixture. However, when the naked ^{32}P-DNA was incubated in the presence of both dsDNA and histone H1 (lane 5), which is the condition giving DNA–DNA complex formation (see Fig. 4B), the GGA repeat region and flanking sequences of about 30 bp at both sides were protected from the digestion.

On the other hand, the mononucleosome showed the pattern of DNase I sensitivity consisting of 10-bp intervals of cleavage and protection (lane 7), and the pattern did not change when incubated in the presence of histone H1 alone (lane 9). However, the incubation with either dsDNA (lane 8) or dsDNA plus histone H1 (lane 10) gave protection in the GGA-repeats and flanking regions (indicated by arrows). Lanes 11 and 12 show patterns of a similar experiment except that the purification of the DNase I-treated ^{32}P-mononucleosome–DNA complex by 5% PAGE prior to DNA extraction. Protection in the GGA-repeats and flanking regions was also seen. These results suggest that DNA–DNA complex formation occurs between the reconstituted mononucleosomes and naked DNA through the GGA repeats and probably nearby sequences.

Figure 4B shows a gel mobility shift assay of DNA–DNA complex formed on ^{32}P-labeled naked DNA or ^{32}P-labeled mononucleosome. ^{32}P-labeled naked DNA of 163-bp pUC-GGA11 fragment and the mononucleosome reconstituted on its fragment were each incubated with unlabeled fragment of the same DNA in a buffer containing histone H1 or without histone H1. After 1 h incubation at 37 or 4°C, proteinase K was added to the reaction mixtures to digest the proteins and then DNA–DNA associated products were subjected to 5% PAGE. As previously reported of the naked DNA (10), DNA–DNA complex was detected as a slowly migrating band only in the presence of both unlabeled DNA and histone H1 (lanes 1–5). The shifted band was proved to consist of DNA–DNA associated complex (10). Likewise, the mononucleosome provided the shifted band under the same incubation conditions (lanes 8 and 10). However, there was a difference between DNA and mononucleosomes; the addition of only cold dsDNA in the incubation at 37°C yielded the shifted band as to the nucleosome (lane 7). These results suggest that the mononucleosome containing GGA repeats are able to form DNA–DNA complexes and that the ability is stronger than that of naked DNA because the complex formation did not require histone H1.

The stability of mononucleosome during the incubation was examined. Incubation was carried out at 37°C for 1 h in a buffer with and without dsDNA and/or histone H1 (Fig. 4C). Only a trace amount of free DNA was released form ^{32}P-labeled reconstituted mononucleosome (marked as mononuc.). The observed stability of mononucleosomes excluded the possibility that DNA–DNA complex was formed from free DNA stripped from nucleosomes during the incubation.

DISCUSSION

DNA sequences can be separated into two groups as to the formation of nucleosome *in vitro* and *in vivo;* one dictates preferentially positioning of DNA around histone octamers and the other is not favored for the nucleosome formation. The former sequences include five (A/T)3NN(G/C)3NN repeats (19), repeated TATA-

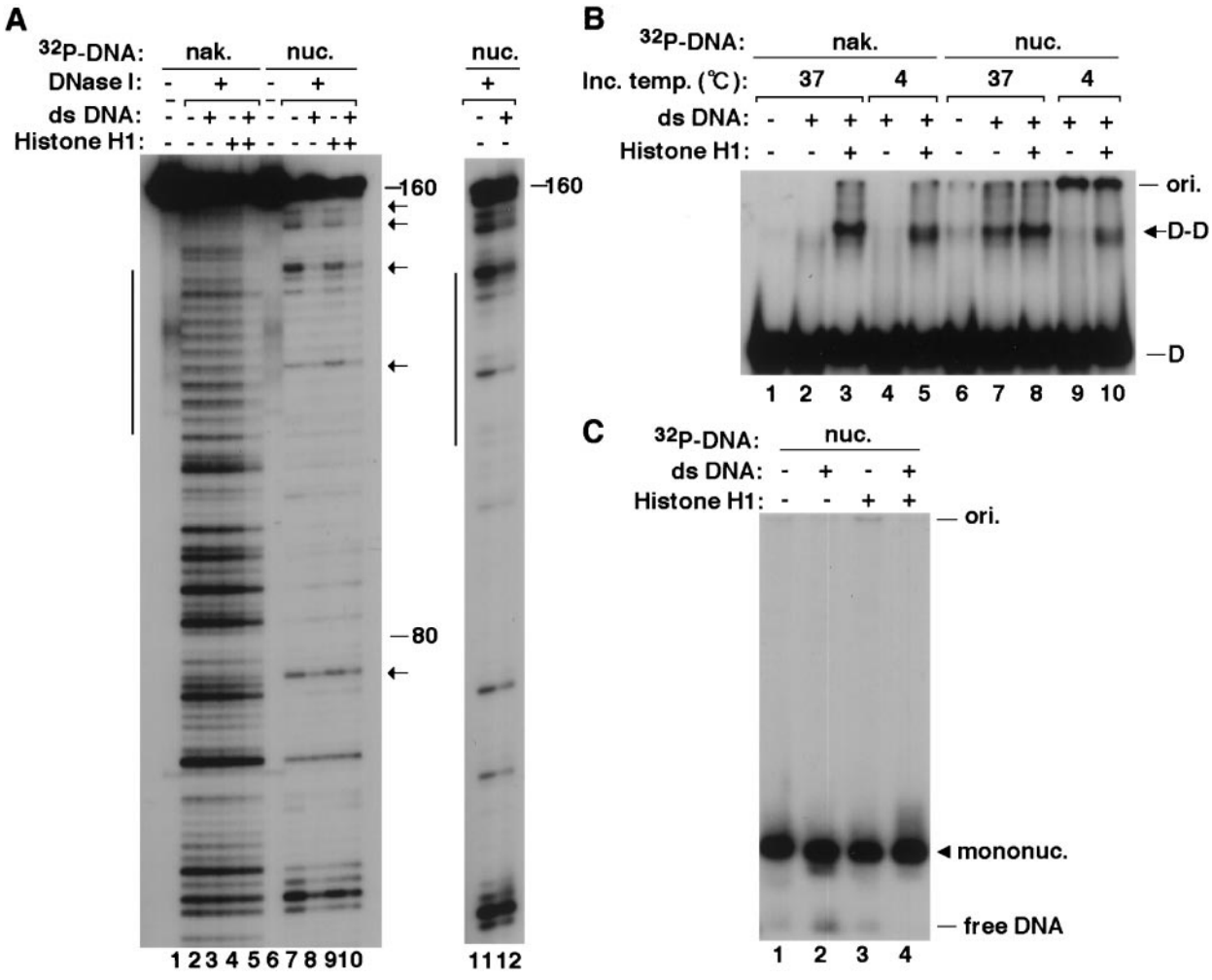

FIG. 4. DNA–DNA complex formation on the mononucleosome reconstituted with DNA fragment containing GGA repeats. (A) DNase I footprinting of DNA–DNA complexes. Mononucleosome reconstituted on 5′-end labeled 160 bp of pUC-GGA11 (lanes 6–12) as well as naked DNA labeled at the same 5′-end (lanes 1–5) were incubated with and without dsDNA in the presence or absence of histone H1 as indicated above each lane. After 1 h incubation at 37°C, 1 mM $MgCl_2$ and 0.1 (for naked DNA) or 1 (for nucleosome) unit of DNase I was added. The reaction mixtures were extracted with phenol/chloroform and ethanol precipitated except for lanes 11 and 12. Complexes in DNase I-treated reaction mixtures were isolated after 5% PAGE and then DNA was extracted (lanes 11 and 12). DNase I-sensitive sites were visualized by autoradiography after electrophoresis on 6% polyacrylamide/8 M urea gels together with sequence ladders. A vertical line indicates the region of GGA repeats. Arrows indicate the protected bands on the nucleosomes. (B) Formation of DNA–DNA complex (indicated as D–D) was analyzed with 5% PAGE. Uniformly labeled ^{32}P-mononucleosomes (lanes 6–10) as well as naked ^{32}P-DNA (lanes 1–5) were incubated with 100 nM nonlabeled cold dsDNA (lanes 2–5 and 7–10) in the presence (lanes 3, 5, 8, and 10) or absence of 50 nM histone H1 (lanes 1, 2, 4, 6, 7, and 9). Products were separated in 5% PAGE after incubation at 4 or 37°C for 1 h and additional incubation with proteinase K at 4°C for 2 h. (C) Gel electrophoresis of ^{32}P-labeled reconstituted mononucleosome after incubation at 37°C for 1 h. Incubates under the association conditions as indicated above the each lane were electrophoresed on an 0.7% agarose gel.

AACGCC motifs (20), triplet CTG repeats and methylated CGG repeats (15, 16, 21). The latter involves the sequences carrying telomeric repeats (1, 22), TGGA repeats (23) and poly(dA) or poly(dT) tracts (24, 25). GGA-repeat sequences are considered to belong to the latter group in the following reasons. First, DNA fragment of 163 bp containing the 11 GGA repeat near the end was able to form two-end-positioned mononucleosomes as examined by micrococcal nuclease treatment (Fig. 2). On the other hand, the 163-bp DNA fragment having the repeat in the middle could not form stable mononucleosomes but the fragment having 11 CTG repeat in the position showed stable mononucleosome formation (data not shown). Second, DNase I cleavages near the repeat region were weaker in mononucleosomes reconstituted on pUC-GGA11 than in those on pUC-CAG11 (compare Fig. 3A with 3B). This suggests that rotational setting of the region containing GGA repeats are rather random on the nucleosomes. These *in vitro* property of GGA repeats on nucleosomes suggests that GGA/TCC sequences in the genome tend to impair nucleosome formation *in vivo* and the impair-

ment may render the region more accessible to proteins and DNA molecules.

We previously reported that naked DNA fragments containing GGA repeats have an ability to form DNA–DNA complexes and that the formation is promoted by HMG proteins and histone H1 (10). Hydrogen bonding between guanine stretches is probably involved in the complex formation, since DNA fragments containing GAA repeats fail to form such DNA–DNA complex (our unpublished data). In this paper, we have demonstrated that the DNA–DNA complexes are formed even on the mononucleosomes reconstituted on both synthetic and genomic DNA fragments (Figs. 3 and 4). Two methods were used, one being DNase I footprinting and the other gel mobility shift assay. Both demonstrated the complex formation but there was a slight difference between their results in the relative levels of DNA–DNA complex formation (Figs. 4A and 4B). Limited DNase I digestion of the DNA–DNA complexes showed that the GGA-repeat region and flanking regions were specifically protected from the enzyme attack. The protection patterns of naked DNA and mononucleosome were almost complete (compare Fig. 4A, lane 2 with 5 and lane 7 with lane 8). On the other hand, about one fifth of labeled fragments gave DNA–DNA complexes in gel mobility shift assay (Fig. 4B, lanes 3 and 8). This may be due to different sensitivities in the detection of complex formation, the former being more sensitive than the latter. It is also possible that some complexes dissociated during deproteinization and subsequent electrophoresis.

It is interesting to note that GGA repeats may form the complex more efficiently in a chromatin context than in naked DNA. Gel mobility-shift assay showed that histone H1 enhanced DNA–DNA association between mononucleosome and naked DNA as well as that between naked DNA and naked DNA (Fig. 4B, lanes 3 and 8). One difference in the histone H1 involvement is that the former case did not require histone H1 for the DNA–DNA complex formation (compare Fig. 4B, lane 2 with lane 7). The complexes in the presence of histone H1, however, did not change the pattern of DNase I digestion (Fig. 4A, lanes 4 and 9). This suggests that the histone H1 itself is not directly involved in the binding but influences the association efficiency. The mechanism of enhancement is not clear but the enhancing activity is similar to the function of a protein chaperon which stabilizes a protein in a conformation for assembly (26).

The ability of GGA/TCC repeats to associate one another provides a possibility of the *in vivo* interaction between the homologous or similar sequences which could function as architectural elements in the nucleus. Our preliminary study of the chromosomal location of genomic GGA/TCC repeats has shown that such sequences are dispersed on chromosomes but not clustered in particular regions like telomeres or centromeres (unpublished data). Therefore, possible chromosomal interactions can occur in any parts of chromosomes and within the same chromosome or on separate chromosomes. In the interphase nucleus chromosomes occupy discrete patches referred to as chromosome territories and within individual territories the chromatin fiber is highly contorted, looped back and forth between the nuclear interior and the periphery (27). It is possible that when DNA–DNA association through GGA repeats occurs between neighboring two regions it may participate in the formation of chromosome territories through DNA loop formation.

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