Difference in HMG1-induced DNA Bending Among Microsatellites

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Abstract

Sequence-dependency of high-mobility group protein (HMG) 1-induced DNA bending is examined for microsatellites using a circularization assay which can measure the extent of bending. Fragments of 133 bp containing (GGA/TCC)u in the middle showed greater bending than those harboring (GAA/TTC)n and (GT/AC)i7 repeats, and fragments possessing (GA/TC)i7 exhibited only slight bending. Differences were not detected for fragments having the repeats near the end. Filter binding assays showed no difference in their binding affinity, suggesting that GGA/TCC repeats are more flexible than the other three repeats as concerns HMG1-induced bending. These results suggest that the mammalian genomes comprise flexible and inflexible regions of microsatellites which might play roles in chromatin architectures and in dynamic packaging of genomic DNA during the cell division cycle.

Key words: DNA bending; microsatellite DNA; HMG1; chromatin architecture; circularization assay

1. Introduction

Mammalian genomes contain varying amounts of tandemly repeated DNA sequences. Some of them are located in regions containing heterochromatin such as centromeres and telomeres, and other tandem repeats including microsatellites are dispersed throughout the genome. In the cell nucleus, genomic DNA of unique and repetitive sequences are tightly folded as nucleoprotein complexes that maintain the ordered packaging of DNA. There is a possibility, however, that repetitive sequences may not be packaged at the same order as unique sequences. It is known that the mouse satellite DNA has an inherent curvature that is important for packaging during centromeric heterochromation condensation. Since the mammalian genomes contain a wide variety of sequences contributing to sequence-directed DNA curvature, such curved DNA might confer different levels of DNA packaging in the cell nucleus.

On the other hand, protein-induced bending of DNA is also known. There are various proteins capable of bending DNA including high mobility group proteins, HMG1 and HMG2. HMG1/2 are abundant in the nucleus and are associated with chromatin. They bind to DNA in a sequence-independent manner, preferring DNA fragments with preexisting bends such as those induced by cisplatin lesions or synthetic Holliday recombination intermediates. HMG1/2-induced DNA bending can facilitate the formation of higher-order nucleoprotein complexes, and hence the proteins are regarded as an architectural element. The higher order structure is thought to involve a variety of DNA transactions such as transcriptional control by assisting interaction between protein-DNA complexes.

Binding preference and bendability of HMG1/2 have been investigated extensively but the effect of DNA sequence on HMG1/2-induced bending has received little attention. This study was conducted to address this issue. As modulators of bending, we chose four microsatellites of two different types: polypurine/polypyrimidine stretches of GGA/TCC, GAA/TTC and GA/TC repeats can form H-DNA in vitro, and GT/AC repeats take Z-DNA conformation. These two types were chosen because they contain tandem repeats that are abundant in the genome and because they possess such unique structural conformations. We examined the bending of DNA containing the repetitive sequences using GST-fusion protein of the box-B of HMG1. The extent of bending was determined by a circularization assay. The results show that HMG1-induced DNA bending depends on sequences in microsatellites and that GGA/TCC repeats are more flexible than GAA/TTC, GA/TC and GT/AC repeats. This suggests that GGA/TCC repeats undergo a high HMG1-induced bending, which might
Curvatures of DNA Induced by HMG1

2. Materials and Methods

2.1. Synthesis of GST-box B of HMG1

The plasmid capable of directing the synthesis in E. coli of box-B of HMG1 was constructed by cloning the relevant sequences of the mouse cDNA18 in pGEX2T.19 GST-fusion protein containing amino acids N92-K172 was prepared and used for the circularization assay.

2.2. Probe DNA

Double-stranded DNA of repeats, and A/T-rich and unique sequences were synthesized. The DNA had single-stranded DNA, GATC and CTAG, at the ends for cloning. Their sequences are (GGA/TCC)n, (GAA/TTC)n, (GA/TC)i7, (GT/AC)i7, (GGA/TCC)5, (GAA/TTC)5, 5'-GATCCGGGAGACTGAGAACAAAGCGCTCTCTAG-3' (A/T-1) and 5'-GATCCGGGAGACTGAGAACAAAGAACAAAGCGCTCTCTAG-3' (A/T-2). Two random unique sequences created by a computer program are: us-1, 5'-GCGAAAGGCCGAACGAGATAACTTCTCAGATG-3'; and us-2, 5'-TGCTTTTATCATGGAGATGCATGTACTGT-3'. The (GGA/TCC)5 and (GAA/TTC)5 DNA had GA(GGA)4 and AA(GAA)4 sequences in the place of the 14-bp A/T-rich sequence (AACAAAGAACAAAG) of A/T-2 DNA.

These fragments were cloned into BamHI and XbaI sites of pUC118 vector. Using these plasmid DNA as templates, 32P-labeled DNA fragments with ApaLI or EcoRI sticky ends were synthesized by polymerase chain reaction (PCR) in the presence of [α-32P]dCTP (Fig. 1). Sets of primers used were: F-ApaLI, 5'-AAGTGCACAGCGCCAGTGTCCAGGAC-3'; F-pUC 5'-AGTTCTGTTTTCCAGAGAGAGCTC-3' and R-EcoRI 5'-CACAGGAAACAGCAGCTGAC-3'.

Amplified DNA were cleaved with ApaLI or EcoRI, and fragments were purified from polyacrylamide gels after electrophoresis. The two probe fragments with ApaLI or EcoRI ends possess flanking sequences of 52 bp and 48 bp, or 25 bp and 74 bp, respectively. The Bgl II probe was also synthesized that had the same sequence as the probe with ApaLI except for the Bgl II site at the ends.

2.3. Circularization assay

The circularization assay was carried out essentially as described.20 The probe fragment (10 nM) was incubated without and with various amounts of GST-fusion proteins containing HMG1-box B in 20 μl of a buffer containing 50 mM Hepes-NaOH (pH 7.5), 50 mM potassium glutamate, 10 mM Mg-Acetate, and 1 mM ATP for 30 min at 30°C. T4 DNA ligase (0.6 Weiss units, Takara Shuzo, Japan) was then added and incubated for 30 min followed by inactivation of the ligase by shifting to 70°C for 10 min. Reactions were subsequently incubated in 50 μl of the buffer with 10 U of exonuclease III (Takara Shuzo) for 30 min at 37°C to remove linear ligation products.20 The reaction products were subjected to phenol extraction and precipitated with ethanol. An aliquot was electrophoresed in a 4.5% polyacrylamide gel containing a buffer of Tris-borate-EDTA (pH 8.3) unless specified otherwise. Gels were dried and autoradiographed.

2.4. Filter binding assay

The filter binding assay was basically performed as described.21,22 32P-labeled DNA was mixed with various amounts of the box-B of HMG1, GST-protein and bovine serum albumin (BSA) in a final volume of 0.1 ml.
in a buffer containing 20 mM Hepes-NaOH (pH 7.9), 50 mM MgCl₂, 0.2 mM dithiothreitol (DTT) and 6% glycerol. After incubation at 30°C for 30 min, the complexes were diluted twofold in the same buffer and filtered through prewashed, wet, nitrocellulose filter. The filters were washed three times with 1 ml buffer, air-dried, and the bound radioactivity was counted. The assays were carried out in triplicate.

3. Results and Discussion

3.1. HMG1-induced DNA bending of fragments having repeats at the middle

The T4 DNA ligase-catalyzed circularization assay allows the study of DNA bending in solution by covalent ring closure in the presence of T4 ligase. DNA molecules shorter than 150 bp are rather inflexible and cannot be circularized without the aid of additional factors or sequences that serve to increase flexibility. HMG1, one such accessory protein, has been demonstrated to fulfill this role by this method. We examined circularization of 133-bp fragments containing four kinds of repeats which had the repeat in the middle and the ApaLI cohesive site at both ends (Fig. 1). The results are shown in Fig. 2. Fragments containing the (GGA/TCC)n repeat produced ligation products of longer sizes than fragments carrying the GAA/TTC, GA/TC or GT/CA repeats; none of them showed a clear band at a concentration of 100 nM (lanes 11, 17 and 23, respectively). These results suggest that there is a difference in the level of HMG1-induced DNA bending among the repeats; fragments carrying the GGA/TCC repeat may be more flexible than fragments carrying the GAA/TTC and GT/CA repeats, and the GA/TC repeat is rather inflexible. Since some A/T-rich sequences contribute to a sequence-directed curvature, we synthesized and assayed the fragments containing one and two copies of an A/T-rich sequence, AACAAAG (see Materials and Methods). Fragments having one copy showed only a faint band whereas fragments carrying two copies exhibited a clear band (lanes 35 and 36), but both produced a smaller amount than fragments containing the GGA/TCC repeat. Two unique sequences, us-1 and us-2, which were randomly chosen, also showed different bendability; us-1 exhibited a bending similar to that of the GAA/TTC repeat (lanes 41 and 42) whereas us-2 displayed a resistance for bending (lane 48). Examination of fragments with the BglII cohesive site at both ends provided a similar difference between the GGA/TCC and
GAA/TTC repeats (data not shown), although bending required a large amount of HMG1 proteins, probably due to a difference in ligation efficiencies given by the ApoLI and Bgl II cohesive sites.

3.2. HMG1-induced DNA bending of fragments having repeats near the end

The method used here relies on the assumption that ligation efficiency reflects the position of an HMG1-mediated bend in the fragment; the bend located near the middle probably gives a strong effect, whereas the same bend will affect the efficiency only slightly if it occurs close to one of the ends. This assumption is supported by previous experiments.22,25 Probe fragments having repeats near the end were synthesized using another set of primers and hence these fragments possessed the same pUC sequence in the middle (Fig. 1). Figure 3 shows results of the circularization assay. Fragments having the GGA/TCC repeat exhibited a prominent band of circular product only at a box-B concentration of 200 nM (lane 6). The other seven probe fragments also showed similar patterns. Note that the fragments containing the GA/GC repeat, one copy of A/T-rich sequence and us-2 near the end gave circular products (lanes 18, 30 and 48, respectively), which contrasts with the results obtained by using fragments containing inserts at the middle (Fig. 2). This suggested that the ligation or bending efficiency was not much affected by the different inserts if the inserts were located at the end of fragments. This supports the implication given by the result in Fig. 2 that strong HMG1-induced DNA bending of fragments having the GGA/TCC repeat is due to the repeat integrated.

3.3. Dependence of bending on the number of repeat units

Repeat-number dependency of the bending was next examined. Two variant plasmid clones were constructed from the A/T-2 clone which had the GA(GGA)4 and AA(GAA)4 fragments in the place of the 14-bp A/T-rich sequence. Probe fragments with ApoLI sites at the ends were synthesized from the three plasmids and subjected to the ligation assay (Fig. 4). Fragments containing five copies of GGA/TCC and GAA/TTC gave products of a similar amount in 100 nM and 200 nM of HMG1 (lanes 5, 6, 11, 12). This efficiency was comparable to that of the A/T-2 probe (lane 18). These results suggest that the extent of HMG1-induced DNA bending is dependent on the copy number of repeat units.

3.4. Binding affinity of HMG1 to DNA containing repeats

Binding affinity of the box-B protein to DNA containing the different repeats was measured in a nitrocellulose filter binding assay.21,22 Fragments having GGA/TCC, GAA/TTC and GT/CA repeats and fragments harboring one and two copies of the A/T-rich sequence showed the same binding profile (Fig. 5). The profile was also the same as that for fragments harboring the us-1 and us-2 unique sequences (data not shown). GST-protein and BSA used as controls failed to bind to those DNA. These results suggest that the binding affinity was almost the same among the DNA probes used. This is consistent with the idea that HMG1 has no sequence specificity in binding.7-9
3.5. Difference of microsatellites in HMG1-induced DNA bending

In this study, the effect of microsatellite sequences on HMG1-induced DNA bending was examined. The extent of bending can be measured by the circularization of short DNA fragments in the presence of T4 DNA ligase. This assay is quantitative, since the circularization efficiency directly correlates with the degree of bending. The results obtained with such an assay show that the HMG1-induced DNA bending is influenced by DNA sequence and by the repeat number of microsatellites (Figs. 2 and 4). Fragments containing the GGA/TCC repeat in the middle showed a higher extent of bending than those harboring the GAA/TTC and GT/AC repeats, and fragments containing the GA/TC repeat were rather inflexible. The difference in bendability among the four repeats decreased if the repeat was located near one end of the fragments (Fig. 3), suggesting that the repeats are responsible for the differences of bending.

HMG1 and HMG2 are small acidic proteins extractable from chromatin with 0.35 M NaCl. They have two homologous segments of an 80-amino acid sequence, called HMG-box A and box-B. The HMG-box is a protein domain consisting of an L-shaped arrangement of three α-helices containing two independent DNA-binding surfaces. The DNA-binding domain is responsible for bending, and the bending angle can be as much as 130°. Our experiments do not address the molecular mechanism by which this domain gives different bendings to the microsatellites. However, there are three possible explanations. Firstly, it may be ascribed to differences in the amount of HMG1 protein binding to DNA. Filter binding assays, however, showed no difference in HMG1 binding among five different kinds of fragments harboring the GGA/TCC, GAA/TTC and GT/CA repeats, and the two A/T-rich sequences (Fig. 5). This is consistent with no or low binding specificity of HMG1. The result suggests that the high extent of bending of the GGA/TCC repeat is not caused by a difference in binding affinity. Secondly, the difference may be due to the curvature of the DNA sequences themselves. Fragments containing the GGA/TCC repeat showed more bending than that observed for the A/T-rich sequences, which are known to have a curvature. Thus, this possibility seems unlikely. Lastly, HMG1-induced bending may depend on the sequence of the repeats. Structural analysis suggests that a single HMG domain may cover 20 bp and potentially bend the DNA molecule through as much as 130°. It is possible that this curvature is affected by sequence. We think this possibility more likely.

The GGA/TCC, GAA/TTC and GA/TC repeats all consist of polypurine/polypyrimidine strands but only the GGA/TCC repeat shows unique properties. The re-
pept duplex is sensitive to digestion with S1-nuclease, indicating that two strands are locally melted.\textsuperscript{31,32} GGA-repeat oligonucleotides form homoduplexes, probably through guanine : guanine base pairing.\textsuperscript{33} Consistently, a triple-stranded complex is also detected by incubating fragments containing the GGA/TCC repeat with GGA-repeat oligonucleotides.\textsuperscript{34} In contrast, those properties are not found in fragments containing the GAA/TTC repeat. DNase I protection analysis revealed that binding of HMG1 to fragments containing the GGA/TCC repeat does not confer clear footprinting except for inducing slight hypersensitivity against DNase I in the GGA strand.\textsuperscript{35} Interestingly, this HMG1 binding enhances the triple-stranded complex formation possibly by exposing single-stranded regions.\textsuperscript{35} Such a melting property of the GGA/TCC repeat might be related to the high extent of HMG1-induced bending.

On the other hand, the sequence of non-repetitive sequences also influenced the ligation efficiency. The us-1 unique sequence showed greater HMG1-induced DNA bending than did another unique sequence, us-2. Since the bending between the GGA/TCC repeat and many other unique sequences was not investigated, it is not possible to give an absolute assessment of the flexibility of GGA/TCC-repeats. Therefore, the possibility still remains that the GA/TC repeats may be rather inflexible relative to a variety of unique sequences.

### 3.6. Biological relevance

HMG1 and HMG2 are abundant in the nucleus and are associated with approximately 5% of chromatin.\textsuperscript{36} Their half-life is about 10 min for binding.\textsuperscript{37} Accordingly, HMG1/2 are implicated in the dynamic packaging of DNA or nucleosomes.\textsuperscript{7,38} This packaging directly or indirectly may assist various proteins to bind to DNA, loop DNA to allow protein-protein interactions between proteins bound to distant DNA-binding sites, or mediate the positioning of nucleosomes by varying DNA bendability.\textsuperscript{7,11,12,20,24,39} On the other hand, the microsatellites examined here are also abundant and dispersed throughout the mammalian genomes.\textsuperscript{40,41} The copy number of GT/AC repeats is calculated to be 10\textsuperscript{5} in the human genome and those of GGA/TCC, GAA/TTC and GA/TC repeats are each 10.\textsuperscript{4} In vitro assay of HMG1-induced DNA bending demonstrated that there are differences in DNA bendability among fragments containing various microsatellites when HMG1 is bound. The results suggest that the genomes of mammals are composed of flexible and inflexible regions, as concerns HMG1/2-mediated DNA packaging. Such regions might play a role in the construction of chromatin architectures in interphase nuclei, which may assist replication, recombination and transcription, and in dynamic packaging of genomic DNA during the cell division cycle.

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