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Distorted DNA Structures Induced by HMGB2 Possess a High Affinity for HMGB2[1]

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HMGB2 (HMG2) protein binds with DNA duplex in a sequence-nonspecific manner, then bends and unwinds the DNA. In DNA cyclization analyses for the bending activity of HMGB2, two unidentified bands, denoted α and β, were observed in addition to monomer circular DNA (1C) on the gel. Re-electrophoresis and proteinase K digestion revealed that α and β are complexes of circularized probe DNA (seeming 1C) with HMGB2 ($K_d \simeq 10^{-10}$ M). The DNA components of α and β (α- and β-DNA) showed higher affinities to HMGB2 than did the linear probe DNA ($K_d \simeq 10^{-7}$ M). The DNAs have distorted structures containing partial single-stranded regions. Nicked circular molecules presumably due to severe DNA distortion by HMGB2 were observed in α- and β-DNA, in addition to closed circular double-stranded molecules. The α and β bands were not formed in the presence of sole DNA binding regions which are necessary for DNA bending, indicating that the acidic C-tail in the HMGB2 molecule is necessary for inducing the peculiar distorted structures of higher affinity to HMGB2. HMGB2 binds with linker DNA and/or the entry and exit of nucleosomes fixed at both ends likewise mini-circles similar to α-DNA and β-DNA. Thus, the distorted structures present in α-DNA and β-DNA should be important in considering the functional mechanisms in which HMGB2 participates.

Key words: DNA bending, DNA structural distortion, HMGB2, HMG box.

High mobility group box (HMGB) 1 and 2 proteins are members of a highly abundant class of nonhistone chromosomal proteins in nuclei of higher eukaryotic cells. These proteins have highly conserved primary sequences and tertiary structures (*1–4*). They each have two DNA-binding domains called HMG boxes A and B with non-identical amino acid sequences and a unique carboxyl terminal domain (C-tail) (*5, 6*). HMGB1 and 2 bind with duplex B-type DNA in a sequence-nonspecific manner (*7, 8*). These proteins show a preferential interaction with supercoiled plasmid DNA (*9–11*) and non–B-type DNA such as four-way junction (*12–15*), cruciform DNA (*9, 10, 16, 17*), cisplatin modified DNA (*18*), and B-Z junction (*19*). In addition, HMGB1 and 2 proteins bind with mini-circular DNA cooperatively (*20–22*). Other HMGB proteins such as HMG-D and NHP6A also show a preferential binding to mini-circular DNA (*23, 24*).

HMGB proteins have been reported to play various important vital roles in transcription, replication, recombination, and cellular differentiation (*1, 25–28*). For example, HMGB1 functions as a quasi-activator of transcription (*1, 3, 4, 25, 26, 29*). HMGB1 and HMGB2 can substitute for DNA-binding regulatory components of DNA-dependent protein kinase (DNA-PK) (*30*) and regulate transcription (*31–33*), replication (*34*), recombination (*35–37*), and cellular differentiation (*38, 39*) as coregulatory proteins. In these vital functions, DNA structural distortions such as DNA bending and unwinding in their target sites should be required. Thus, the participartion of HMGB1 and 2 in biological functions may be accompanied by DNA structural distortion caused by these proteins (*40, 41*). Other HMG box proteins, such as SRY and UBF, may also cause DNA structural distortions in expressing their functions (*42–45*). The structural distortion is caused by the intercalation of a "wedge" of amino acids of the HMG box into the main DNA chain (*11, 46, 47*). HMG box A lacks an intercalatable residue (such as 102F in HMG box B) to induce the DNA structural distortion. However, both HMG boxes are required for full activity of the DNA structural distortion. In the case of HMG2, HMG box A functions as a mediator of the DNA distortion together with box B (*48*).

In our previous studies, HMGB2 induced unidentified products (α and β) in DNA cyclization assay using T4 DNA ligase (*48*). The present study shows that α and β are stable DNA-HMGB2 complexes formed in the DNA ligation step. The DNA ligation products have unique structures showing high affinity to HMGB2. These structures were formed only in the presence of the full-length HMGB2 molecule containing the acidic C-tail, not in the presence of a single

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Abbreviations: HMGB, high mobility group box; SDS, sodium dodecylsulfate; bp, base pair(s); DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate.

HMG box or two boxes. Thus, the structure may be important in considering the functions of these proteins.

MATERIALS AND METHODS

HMGB2 Protein—HMGB2 protein was prepared from pig thymus as described previously (*49*).

DNA Topoisomerase I—DNA topoisomerase I was prepared from pig thymus nuclei as described for calf thymus (*50*). The partially purified fraction eluted from the second hydroxyapatite column was used.

DNA Probe—The 75-bp DNA probe [GATCCCCGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGG] has unique *Kpn*I, *Xho*I, *Hinc*II, *Hin*dIII, *Eco*RI, and *Pst*I restriction sites, and sticky *Bam*HI ends (Fig. 3). The probe was labeled at the 5′ end with [γ-^{32}P]ATP (111 Bq/mmol) with T4 polynucleotide kinase.

DNA Cyclization Assay—An aliquot of HMGB2 solution was mixed with 2.5 ng of DNA in a reaction buffer containing 10 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM dithiothreitol, 66 μM ATP, 10% glycerol, and 0.1 μg/μl bovine serum albumin. The solution in a total volume of 10 μl was kept at 25°C for 30 min. Then the reaction mixture was incubated with 20 units of T4 DNA ligase (Takara) at 16°C for 16 h. Resultant DNA was extracted with phenol and chloroform and precipitated with ethanol. The samples were applied to 5% polyacrylamide gel electrophoresis with 0.5 × TBE buffer (1 × TBE buffer contained 89 mM Tris-borate and 2 mM EDTA, pH 8.0). After electrophoresis, the gel was dried and processed for autoradiography.

Preparation of DNA Ligation Products from Polyacrylamide Gel—The bands separated by electrophoresis were cut from the gel by referring to its autoradiograph. The gel slices were diced and soaked in crush-and-soak solution (0.1% SDS, 1 mM EDTA, 10 mM $MgCl_2$, and 0.5 M ammonium acetate) for 3 h at 37°C. The DNA ligation products were precipitated with ethanol, treated with proteinase K at 37°C for 60 min, and again precipitated with ethanol.

Gel Retardation Assay for DNA Ligation Products—An aliquot of HMGB2 solution was mixed with 0.1 ng of DNA ligation products, kept at 25°C for 30 min, and applied to a 5% polyacrylamide gel electrophoresis with 0.5 × TBE buffer. After electrophoresis, the gel was dried and processed for autoradiography.

Nuclease Digestion and Topoisomerase I Treatment of DNA Ligation Products—The DNA ligation products of 1C, α-DNA, and β-DNA were digested with restriction enzymes (*Bam*HI, *Kpn*I, *Xho*I, *Hinc*II, *Hin*dIII, and *Eco*RI), nuclease S1 and exonuclease III at 37°C for 16 h for full digestion, or treated with topoisomerase I at 37°C for 16 h. The DNA samples were separated by 5 or 12% polyacrylamide gel electrophoresis with 0.5 × TBE buffer, and processed for autoradiography.

Analyses with Chemical Probes—The modifications of DNA ligation products by osmium tetraoxide (OsO_4; Sigma), diethyl pyrocarbonate (DEPC; Sigma), and dimethyl sulfate (DMS; Aldrich) were performed as described previously (*51*). The modified products were digested with *Bam*HI and *Pst*I for the analyses of the top strand or with *Bam*HI and *Kpn*I for the analyses of the bottom strand. The top and bottom strands were separated from undigested products by electrophoresis and isolated by the crush-and-soak method. They were digested with piperidine at 90°C for 30 min, separated by urea-denatured polyacrylamide gel electrophoresis with 0.5 × TBE buffer as described below, and processed for autoradiography.

Urea-Denatured Gel Electrophoresis—DNA samples of 0.5 ng were incubated in 10 μl of denaturation buffer containing 40% formamide at 95°C for 5 min and rapidly chilled on ice. The samples were separated by urea-denatured polyacrylamide gel (6% polyacrylamide gel containing 0.3% bisacrylamide and 42% urea) electrophoresis with 0.5 × TBE buffer at 70 V/cm and processed for autoradiography.

RESULTS

Analyses of DNA Ligation Products in DNA Cyclization Reaction—In the previous analyses of the DNA bending activity of HMGB2 (*48*), two unidentified bands, denoted α and β, were observed in addition to a series of linear and closed circular molecules as shown in Fig. 1. The α and β

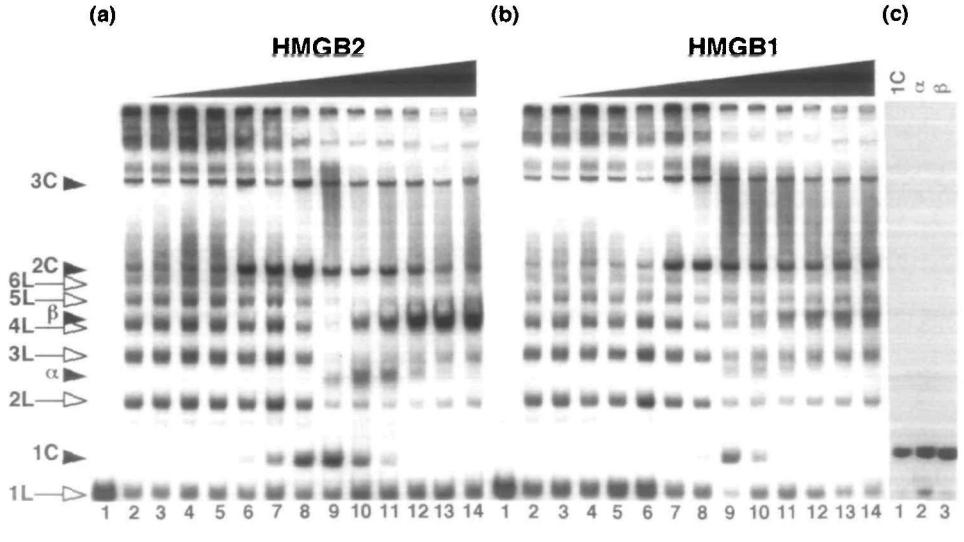

Fig. 1. **DNA cyclization assay of the 75-bp DNA probe.** Molar ratios of HMGB2 (a) and HMGB1 (b) proteins to DNA were: 0, 0.66, 1.32, 2.00, 6.60, 13.2, 20.0, 66.0, 132, 200, 660, 1,320, and 2,000 (lanes 2–14). The 75-bp DNA probe was run as control (lane 1). Two unidentified bands, α and β, were observed in the panel (a) for HMGB2. The α band lies between the dimer linear DNA (2L) and the trimer linear DNA (3L) in lanes 9–12 of panel (a). The β band is proximal to the tetramer linear DNA (4L) in lanes 10–14 of panel (a). (c) The DNAs isolated from α and β bands were re-electrophoresed together with isolated 1C.

bands were produced clearly in ligation reactions in the presence of HMGB2 (Fig. 1a), but faintly in the presence of HMGB1 (Fig. 1b). To analyze the nature of α and β, these products were isolated from the electrophoretic gel by a crush-and-soak method. Re-electrophoresis of the α and β showed similar migrations to that of monomer circular (1C) (Fig. 1c).

Component Analyses of α and β—The above results suggested that HMGB2 may have remained in α and β due to insufficient deproteinization with phenol and chloroform and been removed in a step of extraction from the gel. To examine this possibility, gel retardation assay of 1C with HMGB2 was performed. The 1C-HMGB2 complexes (cpl. 1 and cpl. 2) migrated similarly to α and β, respectively (Fig. 2a, lanes 11 and 12). Proteinase K digestion of ligation products was then performed after phenol and chloroform extraction. The bands of α and β (Fig. 2a, lanes 7–9) disappeared after the digestion (lanes 19–21), showing that α and β were 1C-HMGB2 complexes.

Binding of HMGB2 with 1C, α-DNA, and β-DNA—Our previous studies showed that the dissociation constant (K_d) of 75-bp linear DNA (1L) with HMGB2 is approximately 5.0×10^{-7} M (*48*). SPR measurements showed that the K_d value using 30-bp linear DNA is approximately 6.0×10^{-7} M (*11*). On the other hand, the K_d value of 1C with HMGB2 was appropriately 4.9×10^{-10} M (Fig. 2b). Also, the K_d values of the DNA components of α and β, named α-DNA and β-DNA, with HMGB2 were 2.4×10^{-10} M and 5.3×10^{-11} M, respectively. These results showed that HMGB2 binds with 1C, α-DNA, and β-DNA with K_d values in the order of 10^{-10} M, which are lower by a factor of approximately 10^{-3} compared with that of 1L.

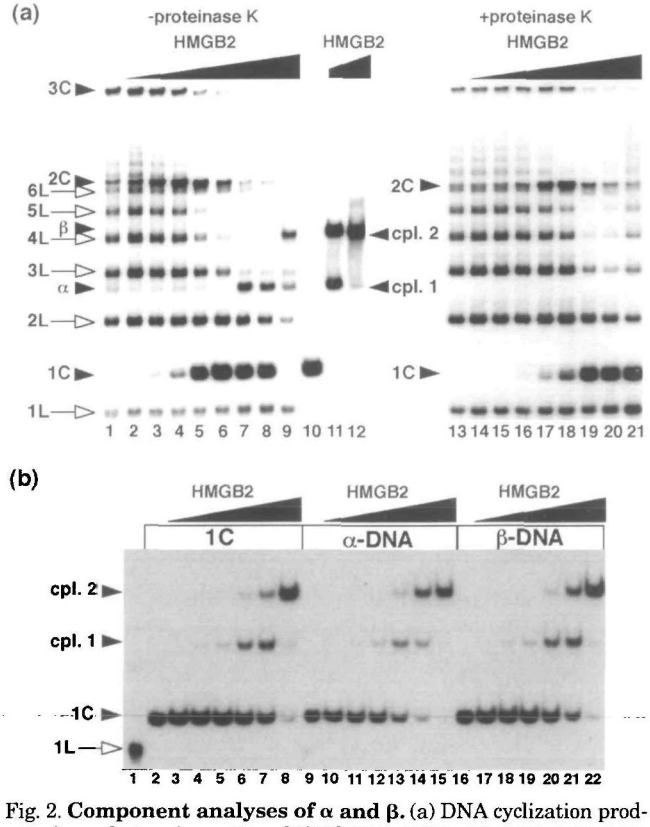

Fig. 2. **Component analyses of α and β.** (a) DNA cyclization products formed at molar ratios of HMGB2 to DNA of 0, 0.20, 1.00, 2.00, 10.0, 20.0, 100, 200, and 1,000 without (lanes 1–9) and with (lanes 13–21) proteinase K treatment were subjected to gel electrophoresis. For comparison, gel retardation of isolated 1C with HMGB2 at the concentrations of 5.0×10^{-9} M (lane 11) and 1.0×10^{-8} M (lane 12) was conducted on the same gel. Complexes cpl.1 and cpl.2 migrated similarly to α and β, respectively (lanes 11 and 12). (b) Gel retardation assays of isolated 1C (lanes 2–8), α-DNA (lanes 9–15), and β-DNA (lanes 16–22) with HMGB2 at different concentrations: 0, 2.5×10^{-11}, 5.0×10^{-11}, 1.0×10^{-10}, 2.5×10^{-10}, 5.0×10^{-10}, and 1.0×10^{-9} M. The 75-bp DNA probe was run as control (lane 1).

Analyses of 1C, α-DNA, and β-DNA with Nuclease and Topoisomerase I Treatments—The lower K_d values of interaction of 1C, α-DNA, and β-DNA with HMGB2 compared with that of 1L led us to expect that these DNAs have unique DNA structures out of simple closed circular DNA. To analyze the DNA structures, 1C, α-DNA, and β-DNA were digested with various restriction enzymes (*Bam*HI, *Kpn*I, *Xho*I, *Hin*cII, *Hin*dIII, and *Eco*RI) at 37°C for 16 h immediately after deproteinization by proteinase K digestion and phenol and chloroform extraction. These DNA components showed various degrees of resistance to the enzymes and were particularly resistant to *Hin*dIII and *Bam*HI (Fig. 3b, lanes 1–18), although the monomer linear DNA [equal to the 75-bp DNA probe (1L)] was completely digested by these enzymes (data not shown). The results suggested that parts of 1C, α-DNA, and β-DNA do not have a normal double-stranded structure. These products seemed to be resistant to exonuclease III digestion (Fig. 3c, lanes 2, 7, and 12). Nuclease S1 digested the DNAs partially (Fig. 3c, lanes 3, 8, and 13). Topoisomerase I treatment did not affect their electrophoretic mobilities (Fig. 3c, lanes 4, 9, and 14). When the α-DNA and β-DNA were applied to 12% polyacrylamde gel electrophoresis in the presence of chloroquine, they did not show any topoisomers (data not shown). These results suggested that the structures of 1C, α-DNA, and β-DNA are not normal closed circulars that are tightly base-paired, but similar to each other.

Analyses of 1C, α-DNA, and β-DNA with Chemical Probes—To analyze the fine structures of 1C, α-DNA, and β-DNA, analyses with chemical probes were performed to identify single- and triple-stranded DNA regions in the DNA structures. Experiments with OsO_4 and DEPC showed the existence of single-stranded regions at the periphery of the *Hin*dIII site, indicated by asterisks on the sequence of the 75-bp probe, in 1C, α-DNA, and β-DNA (Fig. 4a for top strand and Fig. 4b for bottom strand). The single-stranded regions common to both strands are summarized in Fig. 4c. The DMS probe showed no triple-stranded region in any of the DNAs (Fig. 4, a and b). These chemical analyses suggested that the fine structures of 1C, α-DNA, and β-DNA are very similar and have a single-stranded region at the periphery of the *Hin*dIII site.

Analysis of 1C, α-DNA, and β-DNA with Urea-Denatured Gel Electrophoresis—The DNA structures of 1C, α-DNA, and β-DNA were analyzed by urea-denatured gel electrophoresis. Each DNA band isolated from the urea-denatured gel was treated with exonuclease III and applied to native 12% gel electrophoresis in order to identify linear molecules (data not shown). The results showed that 1C consists of 75-base linear DNA (ss1l), 75-base circular DNA (ss1c), 75-bp circular DNA (ds1c), and an unidentified DNA with molecular mass of 90 bases (Fig. 5, lane 4). The α-DNA and β-DNA consist of ss1l, ss1c, ds1c, unidentified DNA with

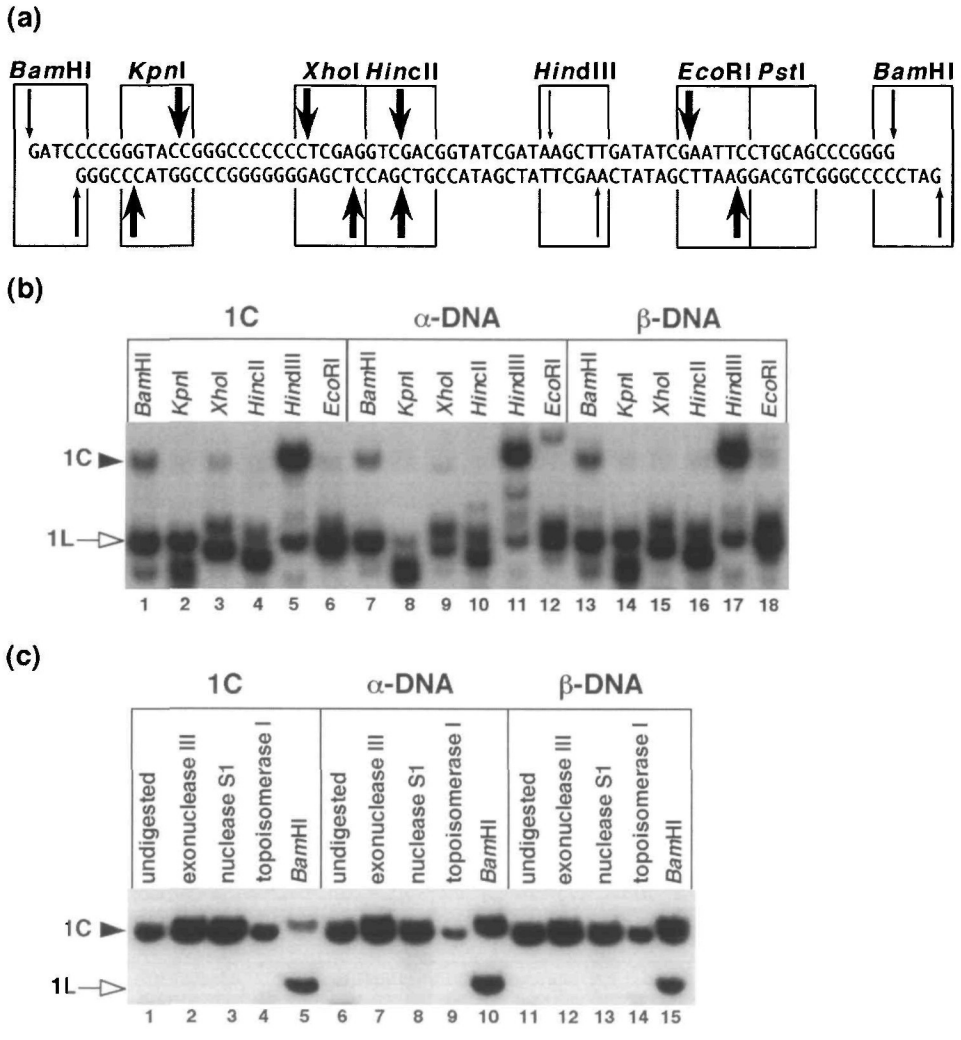

Fig. 3. **Structural analyses of 1C, α-DNA, and β-DNA.** (a) The DNA probe has unique restriction sites (*Kpn*I, *Xho*I, *Hinc*II, *Hin*dIII, *Eco*RI, and *Pst*I). (b) The isolated 1C (lanes 1–6), α-DNA (lanes 7–12), and β-DNA (lanes 13–18) were digested with the six restriction enzymes (*Bam*HI, *Kpn*I, *Xho*I, *Hinc*II, *Hin*dIII, and *Eco*RI). The degree of digestion by each enzyme is represented by line width of the arrow in panel (a). (c) The 1C (lanes 2–5), α-DNA (lanes 7–10), and β-DNA (lanes 12–15) were treated with exonuclease III, nuclease S1, topoisomerase I, and *Bam*HI. Undigested DNAs were run as control (lanes 1, 6, and 11).

molecular mass of 125 bases, and 150-base linear DNA (ss2l) (Fig. 5, lanes 5 and 6). The major components of α-DNA are ss1c and ss1l, and the major component of β-DNA is closed circular. The detection of double-stranded molecules on the denatured gel suggested the existence of katenated single molecules in α-DNA and β-DNA.

DISCUSSION

HMGB2 binds with duplex DNA in a sequence-nonspecific manner by two HMG boxes, then bends and unwinds the DNA. We have analyzed the structural alteration of DNA caused by HMGB2, especially bending and unwinding (*48*). The analyses revealed that a tandem array of boxes A and B through linker (l) region is important for such structural distortion of DNA as bending and unwinding.

In the course of analyses, two unidentified bands, denoted α and β in the present paper, were observed (Fig. 1). Although ligation product 1C was formed in the presence of peptides 2AlB and 2AlBj containing the boxes A and B (*48*), α and β were not formed in the presence of these peptides (data not shown). This suggested the acidic C-tail is necessary for the formation of α and β. The acidic C-tail weakens the DNA binding ability of peptides 2AlB and 2AlBj (*11, 48*). The acidic C-tail in chicken HMGB1, HMGB2a, and HMGB2b weakens their DNA binding abilities to linear DNA and four-way DNA junction. The preferential binding of chicken HMGBs to 88-bp circular DNA relative to the linear molecule of the same length was also abolished by removal of the tail (*21*). These findings suggest that the acidic C-tail of HMGB2 is important for the formation of circular DNA and its binding with the DNA. Thus, it was of interest to analyze the participation of the acidic C-tail of HMGB2 in the formation of α and β.

The migrations on re-electrophoretic gel of α and β prepared from the first gel (Fig. 1a) were similar to that of 1C (Fig. 1c). The gel retardation assay of 1C with HMGB2 showed similar migrations to those of α and β (Fig. 2a). The α and β bands disappeared following proteinase K treatment of the reaction products and 1C appeared (Fig. 2a). In addition, the gel shift assay of α-DNA and β-DNA with HMGB2 gave bands similar to α and β (Fig. 2b). These results indicated that α and β are complexes of 1C (or seeming 1C) with HMGB2. The dissociation constant K_d of the complex of 1C and HMGB2 was 4.9×10^{-10} M as calculated from the gel retardation assay in Fig. 2b. The K_d values of α-DNA and β-DNA with HMGB2 were 2.4×10^{-10} M and 5.3×10^{-11} M, respectively (Fig. 2b). The K_d value for the interaction of 75-bp DNA probe with HMGB2 was approximately 6.0×10^{-6} M (*48*). These higher affinities of α-

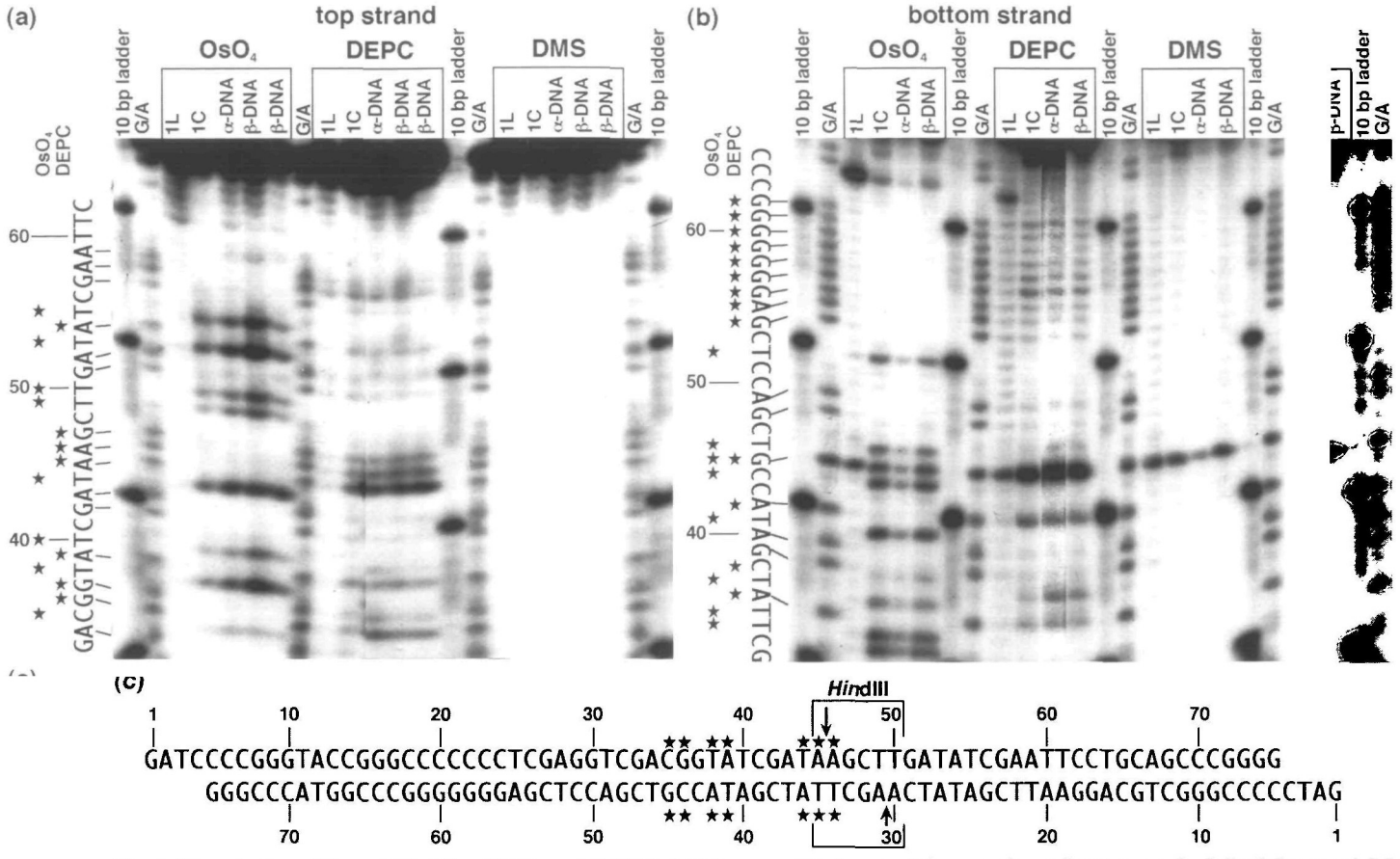

Fig. 4. **Chemical probing of 1L, 1C, α-DNA, and β-DNA.** (a) Fine mapping of OsO_4, DEPC, and DMS sites on the top strand of 1L (equal to 75-bp DNA probe) as control, 1C, α-DNA, and β-DNA. The second β-DNA lane was isolated from the gel at the higher HMGB2 concentration to avoid contamination by linear DNA (4L, Fig. 2). The modified nucleotides are shown by stars at the left of the panel. (b) Mapping of OsO_4, DEPC, and DMS sites on the bottom strand. (c) Modified nucleotides common to the top and bottom strands with OsO_4 and DEPC are shown by stars. The arrows on the top and bottom strands indicate *Hin*dIII sites.

DNA and β-DNA with HMGB2 might cause strong binding that does not readily dissociate upon phenol and chloroform extraction. These results showed that the ligation reaction of 1L DNA in the presence of HMGB2 induced some peculiar structures which were maintained in the ligated molecule(s).

The deproteinization of α and β gave bands similar to 1C. The simple explanation for this fact is that 1C is a primary DNA product formed by the ligation, and that α and β are stable complexes formed in the presence of a large amount of HMGB2. The exonuclease III digestion and topoisomerase I treatment of 1C, α-DNA, and β-DNA did not show any detectable difference between them (Fig. 3c). Electrophoresis on 12% polyacrylamide gel in the presence of chloroquine did not derive any change in linking numbers of these structures. Restriction enzyme digestion showed that 1C, α-DNA, and β-DNA are resistant to cleavage at the *Hin*dIII site (Fig. 3b). The presence of a single strand in the DNAs was observed at the periphery of *Hin*dIII site (Fig. 4c). This bubble-shaped DNA region may be an AT-rich sequence at the periphery of the *Hin*dIII site on the 75-bp DNA probe due to the structural alteration by HMGB2.

The urea-denatured gel electrophoreses indicated that 1C consists of ss1l, ss1c, ds1c, and a small amount of unidentified DNA with a size of 90 bases (Fig. 5). The DNA length of 75-bp is below the persistence length to ligation in the absence of HMGB2. The detection of a ds1c molecule on the urea-denatured gel suggested the existence of katenated single molecules, showing that the 75-bp probe DNA was ligated end-to-end in the presence of HMGB2 (Fig. 5). The detection of similar amounts of ss1c and ss1l in 1C may indicate that one strand of the probe DNA was ligated but the other strand not. That is, 1C may consist of a mixture of closed circular double-stranded DNA (dsDNA) and nicked circular dsDNA at one of the strands. The band migrating at around 90 bases may be a katenated ss1c molecule. The α-DNA may be a mixtures of closed circular dsDNA, a large amount of nicked circular dsDNA, and unidentified DNA structures migrating at around 125 and 150 bases. The ss2l DNA of 150 bases might be the result of cross-ligation in one of the strands between two DNA probes. The amount of 125-base DNA was equal to that of ss2l, suggesting that the structure is nicked ds2c. The β-DNA may be a mixture of closed circular dsDNA, a small amount of cross-ligation products containing ss1c and ss2l DNA, and structures migrating at around 110–150 bases. Al-though the detailed structure of the smeared bands could not be solved, they may be variously katenated ss2c molecules of 125 bases. The incomplete ligation products in 1C, α-DNA, and β-DNA may be the result of partial unfit-

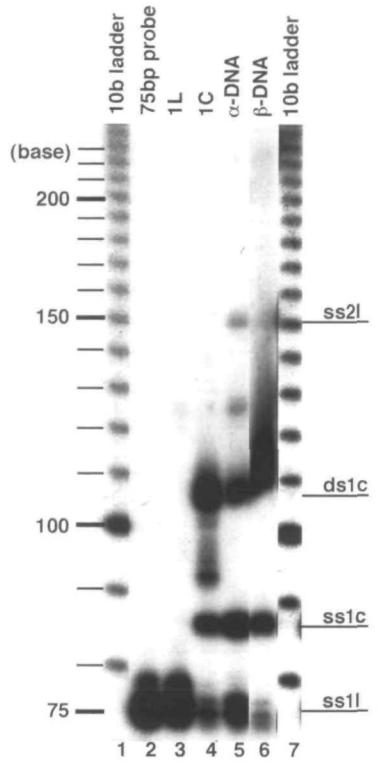

Fig. 5. **Urea-denatured gel analyses of components of 1C, α-DNA, and β-DNA.** The 75-bp DNA probe (lane 2), isolated 1L (unligated product, lane 3), 1C (lane 4), α-DNA (lane 5), and β-DNA (lane 6) were heat-denatured and subjected to urea-denatured gel electrophoresis. A 10-bp ladder marker was also denatured and applied to the gel (lanes 1 and 7). The ss1l (75-base band), ss2l (150-base band), ss1c (85-base band), and ds1c (110-base band) indicate single-stranded 1L, 2L, single-stranded 1C, and double-stranded 1C, respectively.

ting of sticky *Bam*HI ends due to severe bending and unwinding by HMGB2.

The HMG box binds with DNA from minor groove to contact at the concave surface (*47, 52, 53*). The binding of an HMG box with DNA induces a bend of ~80–100° (*54, 55*) and twists the DNA strand by 27° (*56*). HMGB2 as well as HMGB1 may induce the DNA bending by about 140° (*47*). As described above, 1C (seeming 1C) was formed even in the presence of peptides 2AlB and 2AlBj. The DNA structure in 1C (seeming 1C) might be bent and unwound before the ligation reaction. The torsional stress induced by the bending and unwinding should be maintained in the ligation product after the deproteinization. The DNA structure thus induced and maintained in 1C (seeming 1C) may be preferable to the binding of HMGB2 molecules, as shown in Fig. 2a. On the other hand, the ligation products α and β were observed only in the presence of full-length HMGB2. This fact suggests that the acidic C-tail is concerned in formation of the peculiar structures maintained in the ligation products. As far as we know, the DNA binding structure of full-length HMGB has not been clarified. However, one possible explanation is that the highly acidic C-tail interacts with bases in the DNA to change the bending and unwinding angles. If that is the case, the structures of α-DNA and β-DNA should be different from that of 1C. These peculiar structures in α-DNA and β-DNA might be maintained after deproteinization and possess higher affinity to HMGB2.

HMGB2 as well as HMGB1 binds with linker DNA (*57, 58*) and/or the entry and exit region of nucleosomes (*59*). Linker DNA and the entry and exit DNA region are fixed at both ends with nucleosomes likewise mini-circles ligated at both ends. When the nucleosome structures are formed after HMGB2 binding, the torsionally-stressed DNA structure will be maintained in the chromatin structure. When the HMGB2 binds to the linker DNA and/or entry and exit DNA region, the binding may induce the peculiar DNA structures which are bent and unwound. Therefore, the DNA structures found in this study to be induced and maintained by full-length HMGB2 should be important in considering the mechanisms of replication, recombination and repair involving HMGB2.

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