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Transient HMGB protein interactions with B-DNA duplexes and complexes

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ABSTRACT

HMGB proteins are abundant, non-histone proteins in eukaryotic chromatin. HMGB proteins contain one or two conserved "HMG boxes" and can be sequence-specific or nonspecific in their DNA binding. HMGB proteins cause strong DNA bending and bind preferentially to deformed DNAs. We wish to understand how HMGB proteins increase the apparent flexibility of non-distorted B-form DNA. We test the hypothesis that HMGB proteins bind transiently, creating an ensemble of distorted DNAs with rapidly interconverting conformations. We show that binding of B-form DNA by HMGB proteins is both weak and transient under conditions where DNA cyclization is strongly enhanced. We also detect novel complexes in which HMGB proteins simultaneously bind more than one DNA duplex.

Double-stranded DNA is among the least flexible biopolymers. The local stiffness of DNA is reflected in its persistence length, *P*, (~150 bp) [1]. The global flexibility of naked DNA in solution is insufficient to allow the degree of compaction required for packaging within cells, or for deformed structures involved in DNA replication, recombination, and transcriptional control. While some controversy exists concerning the accuracy of predictive models for DNA flexibility over short lengths [2–4], it is clear that short lengths of DNA require proteins to induce strongly bent and looped conformations.

The apparent physical properties of DNA may differ in vitro and in vivo [5,6]. Activation over short distances in assays of eukaryotic transcription activation in vitro raised the possibility of apparent DNA flexibility enhancement by nuclear factors [7]. Indeed, heat-resistant HMGB proteins in nuclear extracts were found to dramatically enhance the cyclization of short DNA restriction fragments by DNA ligase [8].

Eukaryotic HMGB proteins are abundant small non-histone chromosomal proteins [9–12] that share one or two copies of an ~80-amino acid HMG box domain. Fig. 1A shows the amino acid alignment of HMG box domains, illustrating the partial conservation of intercalating residues [13]. As shown in Fig. 1B, this domain specifies three α-helices (red) whose L-shaped fold engages one face of DNA while delivering one or two intercalating residues into the minor groove [14–16]. Biophysical studies have provided some insights into the thermodynamic basis of DNA binding [17–20]. HMGB proteins to induce DNA kinking or bending of 60–90° per HMG domain [21–25], and bind preferentially to distorted DNA structures [16,21,26]. HMGB proteins may stabilize DNA bending either by sequence-specific DNA binding or through interaction with sequence-specific proteins [10,13,27,28].

Studies by Johnson and co-workers [29] have shown that eukaryotic HMGB proteins can functionally replace bacterial architectural proteins in reconstituted in vitro recombination systems that require enhanced DNA bending. This and subsequent work [30–36], suggests a model to explain how HMGB proteins enhance the rate of ligase-catalyzed DNA cyclization (Fig. 1C). A short DNA fragment (Fig. 1C, left) will have a large average end-to-end distance and slow cyclization rate. HMGB proteins (Fig. 1C, triangles) create an ensemble of more compact DNA conformations, each with a reduced end-to-end distance and higher probability of cyclization.

This model raises two important questions. First, how flexible is the DNA kink caused by HMGB? Are its properties more like those of a static kink or a flexible hinge? Second, what are the lifetimes of the HMGB/DNA complexes involving non-distorted B-form DNA?

Experimental procedures

Recombinant HMGB proteins

Rat HMGB1 (HMG boxes A and B) and human HMGB2 (HMG box A) proteins were cloned with amino-terminal hexahistidine tags and expressed in derivatives of *Escherichia coli* strain BL21(DE3). Proteins were purified to homogeneity by Nickel-chelate chromatography followed by C_{18} HPLC.

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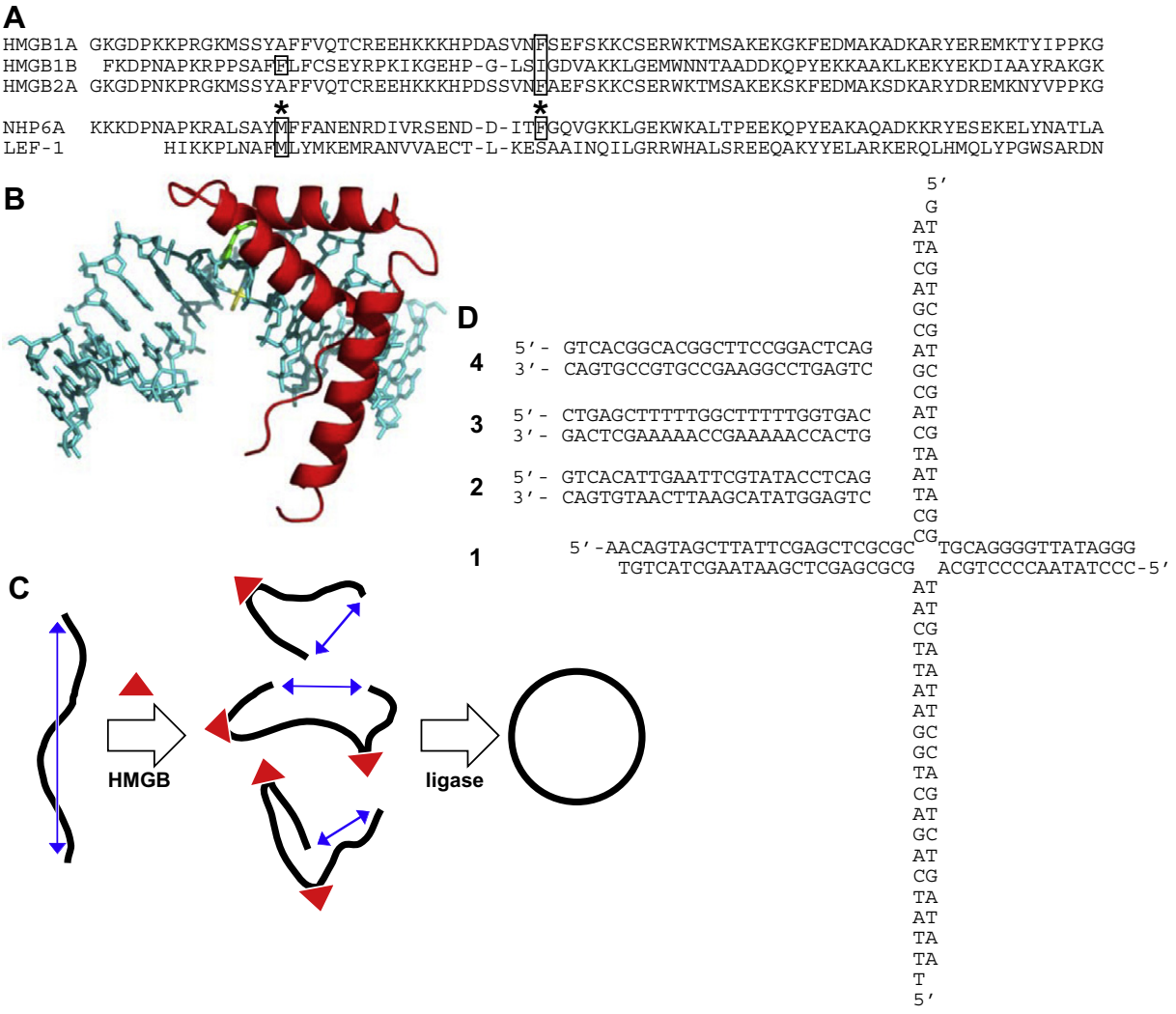

Fig. 1. HMGB protein overview and materials. (A) Conserved domains HMGB proteins. Intercalating residues shown by (∗). (B) Crystal structure [16] of HMGB1 (box A; red) bound to DNA (cyan) kinked by a chemical cross-link (yellow) with intercalation of F37 (green). (C) Model for random and transient binding of HMGB proteins (red) to form an ensemble of DNA conformations with reduced end-to-end distance (blue arrows) enhancing of the rate of ligase-catalyzed DNA cyclization. D. DNA molecules tested in this study. DNA **1** is a 4-arm junction [45]. DNA duplexes **2–3** contained 75% A/T bp, either dispersed or as two unphased A_5 tracts, respectively. Duplex **4** contained 25% A/T bp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Preparation of DNA targets

DNA duplexes are shown in Fig. 1D. Some oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). Cruciform DNA was prepared as described [37]. Biotinylated oligonucleotides were prepared using reagents from Glen Research.

DNA cyclization assay

A radiolabeled 195-bp *Acc*65I restriction fragment from plasmid pJ673 (G $_2$0A27T in Ref. [8]) was studied. Reactions were prepared with 0.1 nM DNA, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA, and 0.08 U of T4 DNA ligase. Where indicated, 1 μL of 0.1 mg/mL heat-treated HeLa nuclear extract or recombinant HMGB protein was added. Reactions were incubated 45 min at room temperature. 10 μL aliquots were removed and analyzed by electrophoresis. Preliminary experiments with Bal31 exonuclease confirmed the identities of circular products. Gels were dried and imaged by storage phosphor technology (Molecular Dynamics).

Electrophoretic assays of protein/DNA interactions

Binding reactions (10 μL) contained 20 mM Hepes, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 200 μg/mL BSA, and 0.5 nM of the radiolabeled duplex DNA target. HMGB proteins were added after dilution in 20 mM Hepes, pH 7.5, 100 mM KCl, 1 mM EDTA, 100 μg/mL BSA containing 1 mM THP. In some cases, unlabeled competitor DNAs were added during or after the 45-min incubation at 25 °C. Electrophoresis time was short (typically 1 h) to minimize decomposition of complexes in the gel [21]. In kinetic experiments, samples were withdrawn from a master binding reaction at various times after addition of an excess of competitor DNA and were loaded onto a continuously running gel.

Results and discussion

Enhancement of DNA flexibility by HMGB proteins

HMGB proteins in heat-treated HeLa cell nuclear extract dramatically enhance the apparent flexibility of DNA restriction

fragments that are otherwise too short to cyclize in the presence of DNA ligase [8]. Fig. 2A compares cyclization enhancement by heat-treated HeLa nuclear extract (lanes 2–3), recombinant rat HMGB1(A+B) (lanes 5–9) and recombinant human HMGB2(A) (lanes 11–15). As previously observed, HeLa extract enhances ligase-mediated cyclization (Fig. 3, lane 3). In agreement with previous reports [24,31], HMGB2(A+B) enhances DNA cyclization at moderate concentrations (Fig. 3, lane 7). Interestingly, increasing HMGB1(A+B) concentrations inhibits DNA cyclization (Fig. 3, lanes 8–9). In contrast to HMGB1(A+B), the single HMG box domain of HMGB2(A) shows little activity in cyclization enhancement (Fig. 3, lanes 10–15). These data are consistent with previous reports that isolated box A deforms DNA relatively weakly [24,31].

Specific HMGB binding to cruciform DNA

HMGB proteins preferentially bind distorted DNA molecules (e.g. cruciforms, strained minicircles [16,21,26,38]) where complexes can be stable enough to be resolved by native polyacrylamide gel electrophoresis. A cruciform DNA substrate (Fig. 1D, DNA 1; [37]) was incubated with HMGB1(A+B) and complexes were examined by gel electrophoresis (Fig. 2B). Low nanomolar concentrations of HMGB1(A+B) bind tightly to this distorted DNA and yield a single discrete complex (*). Increasing HMGB1 concentration above 50 nM gives nonspecific binding seen as a smear of higher molecular weight complexes in the gel (Fig. 2B, lanes 9–10). Binding data from is fit to the Hill equation in Fig. 2C, providings an equilibrium dissociation constant of ~10 nM.

Weak HMGB binding to non-distorted B-form DNA

We studied the affinity of HMGB proteins for non-distorted B-DNA duplexes of different sequence composition. Three 25-bp DNAs (**2**–**4**, Fig. 1D) were incubated with increasing concentrations of HMGB proteins. DNAs **2** and **3** contained 75% A-T bp, organized either randomly (DNA **2**) or in two unphased A_5 tracts (DNA **3**). DNA **4** contained 75% G/C base pairs. Results are shown in Fig. 3A. Binding of HMGB2(A) was similar for all DNAs tested (Fig. 3A, lanes 1–19). Protein/DNA complexes appeared as a diffuse smear. This pattern indicates weak, sequence nonspecific binding and dissociation in the gel even during the short electrophoresis experiment. Similar results were obtained for samples of HMGB1(A+B) protein (Fig. 3A, lanes 20–37). It is interesting to note that maximum cyclization enhancement in the ligation experiment (Fig. 2A) was seen for a concentration of HMGB1(A+B) below the lowest concentration tested in Fig. 3A.

Simultaneous HMGB binding to multiple DNA duplexes

Experiments involving the addition of unlabeled excess target DNAs to HMGB binding reactions that contain trace concentrations of labeled DNA yielded an unexpected observation: HMGB proteins

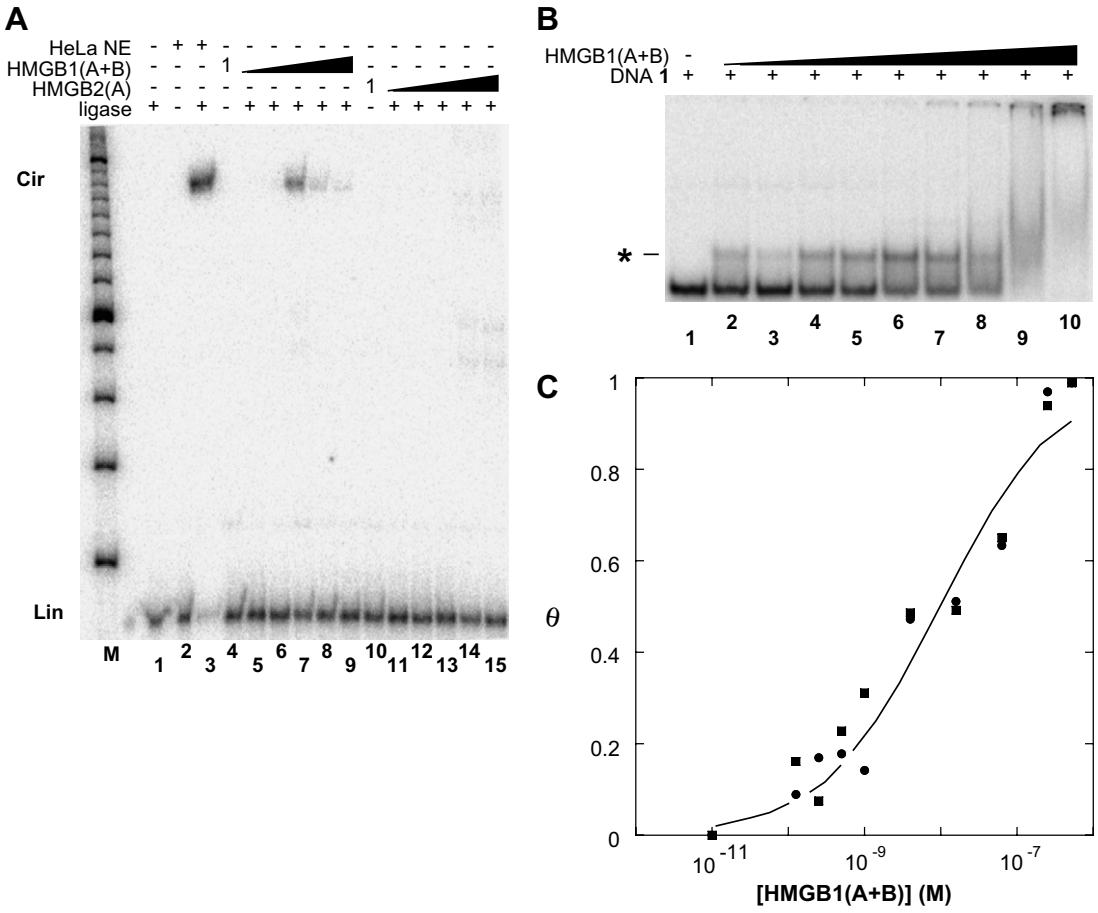

Fig. 2. HMGB cyclization enhancement and DNA binding. (A) Linearized, radiolabeled 195-bp DNA (L) was incubated with heat-treated HeLa nuclear extract (HeLa NE) or the indicated recombinant HMGB proteins in the absence or presence of ligase. Circular products are indicated (C). Gradient symbols reflect the following final concentrations of the indicated HMGB proteins: 1.6 nM (lanes 5 and 11), 8 nM (lanes 6 and 12), 40 nM (lanes 7 and 13), 200 nM (lanes 8 and 14), 1 μM (lanes 4, 9, 10, 15). (B) HMGB1(A+B) tight binding to four-way junction DNA. Gel shift analysis of radiolabeled 4-way junction DNA **1** forming a complex (*) upon incubation with increasing concentrations (0, 0.125, 0.25, 0.5, 1, 4, 16, 64, 256, and 512 nM) of HMGB1(A+B). (C) Binding isotherm for two repetitions (squares and circles) where θ indicates the fraction of radiolabeled DNA shifted fit to Hill binding equation.

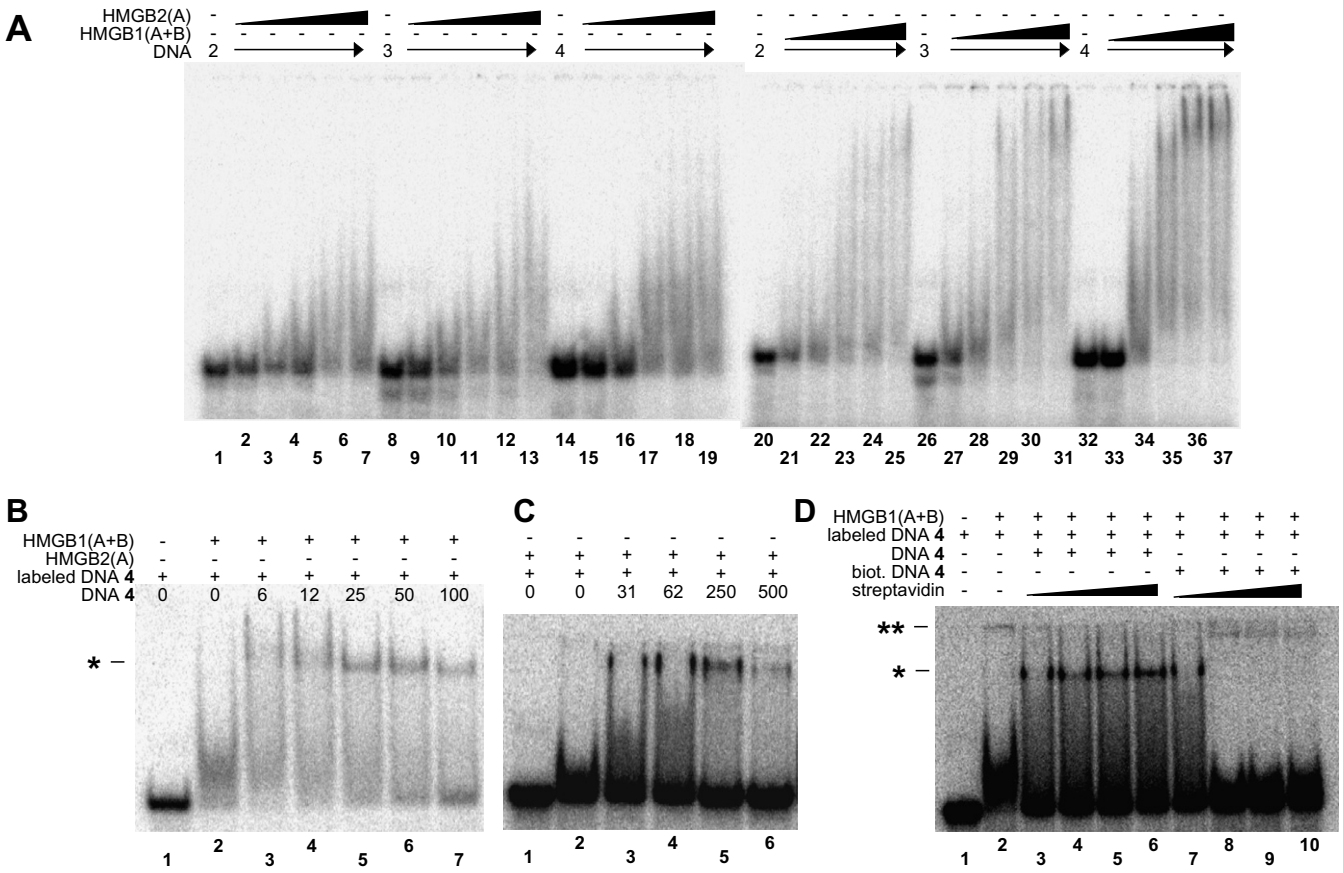

Fig. 3. HMGB weak nonspecific DNA binding and evidence for binding to DNA complexes. (A) Radiolabeled DNA duplexes (0.5 nM) were incubated with 0–800 nM of HMGB proteins and analyzed by electrophoretic gel mobility shift assay. Each set of six samples contained 0, 100, 200, 400, 600, and 800 nM HMGB protein, respectively. (B) Incubation of 400 nM HMGB1(A+B) with 0.5 nM labeled DNA **4** in the presence of increasing concentrations of unlabeled DNA **4** showing the formation of a higher molecular weight complex (∗). (C) Similar to panel (A) but for HMGB2(A). (D) As in A and B except biotinylated (biot.), unlabeled 100 nM DNA **4** supershifts the higher molecular weight complex (∗∗) of HMGB1(A+B) and 0.5 nM labeled DNA **4** in the presence of increasing concentrations (17, 34, 68 nM, respectively) of streptavidin.

form non-covalent complexes involving multiple DNA duplexes (Fig. 3B–D). Radiolabeled DNA **4** (Fig. 3B, lane 1) is shifted only into a diffuse smear when incubated with 400 nM HMGB1(A+B) (Fig. 3B, lane 2). However, upon addition of increasing amounts of unlabeled competitor DNA **4**, a new complex of higher molecular weight is observed (asterisk to the left of Fig. 3B). Only at the highest concentrations of unlabeled DNA **4** is radiolabeled DNA **4** displaced (Fig. 3B, lane 7). Intermediate concentrations of unlabeled DNA actually promote the new protein/DNA complex (e.g. Fig. 3B, lane 5). The novel species must represent HMGB1(A+B) participation in complexes containing more than one DNA **4**.

Fig. 3C shows similar results for HMGB2(A). Two HMG box domains are therefore not required for the formation of complexes involving multiple DNAs. We hypothesize that the complexes involve simultaneous HMGB binding to two DNA duplexes that have collided to form a transient "node". The DNA concentration-dependence of DNA/HMGB complexes (Fig. 3B–C) implies that the complexes contain both labeled and unlabeled DNA duplexes. To test this idea, the competing (unlabeled) form of DNA **4** was substituted with a biotinylated version of the same duplex. Electrophoresis was performed to show that complexes contained both labeled and biotinylated copies of DNA **4** (Fig. 3D). Streptavidin had no effect on the DNA-dependent complex in the absence of biotinylated DNA **4** (asterisk, Fig. 3D, lanes 3–6). Excess biotinylated DNA **4** also promoted complex formation (Fig. 3D, lane 7). Addition of streptavidin to binding reactions containing HMGB1(A+B), labeled DNA **4** and unlabeled biotinylated DNA **4** yielded detectable supershifted complexes (double asterisk, Fig. 3D, lanes 8–10).

HMGB/B-DNA complexes are transient

We monitored HMGB/B-DNA binding kinetics as has been done for HMGB complexes with deformed DNA [21]. Pre-formed HMGB/DNA complexes with labeled DNA **4** were challenged by excess unlabeled DNA **4** and the residual fraction of original HMGB/DNA complex was monitored by gel electrophoresis after various times. Samples loaded onto a running gel so that complexes from later time points migrate shorter distances (Fig. 4). Panels A and B of Fig. 4 compare the kinetics of DNA complexes with HMGB1(A+B) while panels C and D show DNA complexes with HMGB2(A). Panels A and C show conditions where only low molecular weight complexes form because the DNA concentration was low. Panels B and D show conditions that support the formation of the discrete complexes of higher molecular weight. The results show that HMGB/DNA complexes completely dissociate during <1 min of equilibration. We conclude that HMGB/DNA complexes are transient under conditions where apparent DNA flexibility is being enhanced.

Relevance of HMGB interactions with non-distorted DNA

HMGB proteins cause sequence non-specific DNA kinking, and consequently bind strongly and preferentially to distorted DNA structures resembling such kinked sites [16,21,23,26]. HMGB proteins can also increase the rate of ligase-mediated cyclization of B-form DNA fragments that lack any distortion [8,24,29,31]. We hypothesized that HMGB proteins interact transiently with

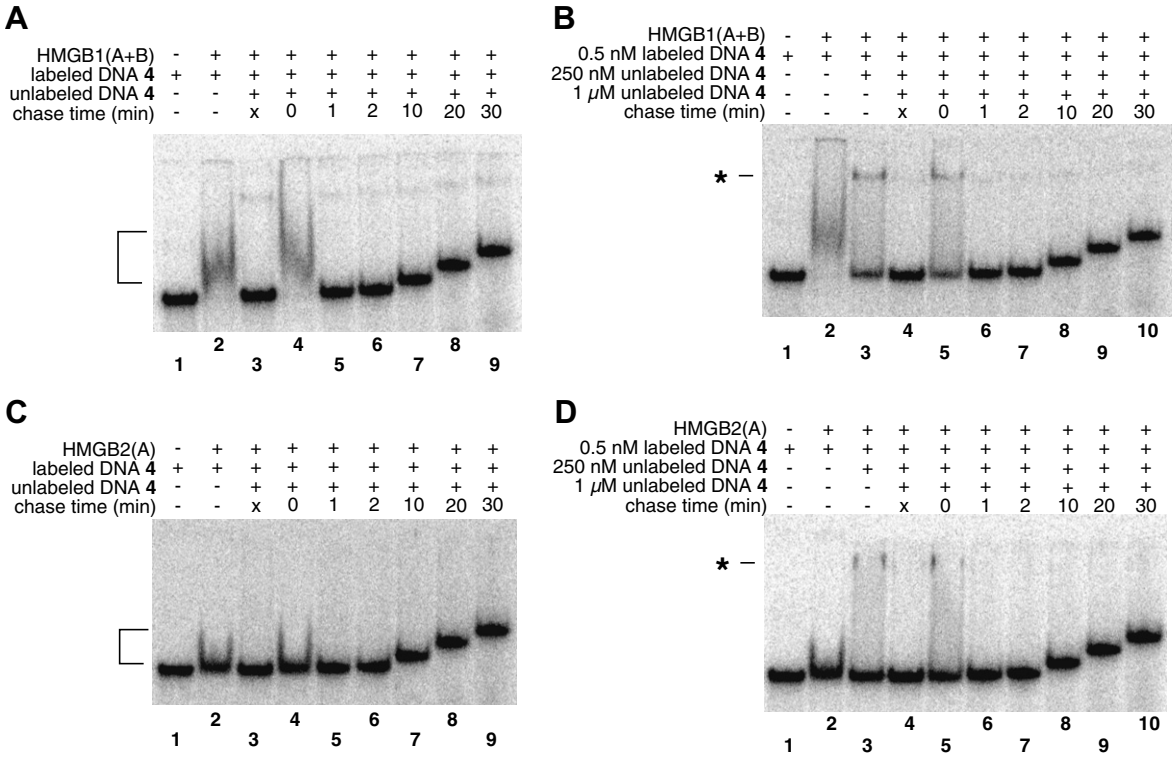

Fig. 4. Rapid dissociation of HMGB proteins from DNAs and DNA complexes. (A and B) HMGB1(A+B) kinetic assays. (C and D) HMGB2(A) kinetic assay. (A and C) 0.5 nM labeled DNA **4** incubated in the absence or presence of HMGB protein. Pre-formed complexes were challenged by the addition of 1 μM unlabeled DNA **4** and the survival of labeled complexes was monitored by loading samples onto the running gel after the indicated times. In panels B and D, 0.5 nM labeled DNA **4** was incubated in the absence or presence of HMGB protein with or without 250 nM unlabeled DNA **4** to promote a higher molecular weight complex before challenge by the addition of 1 μM unlabeled DNA **4**.

non-distorted DNA molecules, resulting in a DNA ensemble rapidly sampling distorted geometries that would be unavailable from thermal energy [8]. Our results confirm that DNA and HMGB proteins interact weakly and transiently under conditions that strongly enhance apparent DNA flexibility. Maximal enhancement of apparent DNA flexibility (Fig. 2A) is observed for an HMGB1(A+B) concentration that results in few complexes detectable by gel shift assay (Fig. 3A, lanes 21, 27, 33). DNA compaction by enhanced apparent flexibility suggests one general activity of HMGB proteins in vivo: reducing the effective persistence length of bulk DNA [39]. Such an effect would be important for stabilizing highly strained or distorted DNA conformations such as are encountered in recombination [29]. We have recently shown that bacterial cells lacking the architectural DNA binding protein HU are significantly disabled in *lac* operon repression looping [40], and expression of ectopic HMGB proteins can partially substitute for HU [40].

Comparison with results of single-molecule experiments

The observations in Fig. 3B–D point to novel complexes where HMGB proteins bridge between multiple DNA duplexes. This result was obtained when the total concentration of DNA duplexes was raised into the tens of nM, promoting intermolecular DNA collisions. Evidence for HMGB bridging of duplex DNA segments has been observed in single molecule experiments where force-induced dissociation of DNA loops was detected when phage λ DNA was stretched after incubation with HMGB2(A) [41]. Such HMGB-stabilized loop complexes might also reflect nodes where DNA duplexes cross, creating preferred HMGB binding sites. Such binding sites may be the physiologically relevant targets for HMGB function, and experimental recognition of distorted DNA structures could simply reflect their similarity to natural DNA node structures.

The results shown in Fig. 2 suggest that there is an optimal HMGB concentration for enhancement of apparent DNA flexibility. This result is consistent with a "filament" binding mode on DNA, as observed in single molecule experiments [41]. Filaments were stiff and likely to be poor substrates for DNA ligase. A similar binding mode has been observed for HU protein [42].

HMGB mechanisms

The present data support a model in which DNA compaction and enhancement of ligase-catalyzed cyclization are promoted by HMGB proteins through random brief static DNA kinks. The maximum lifetime of each kink event is less than one minute. A "flexible hinge" model is not excluded. Structures of HMGB/DNA complexes suggest that induced flexibility would likely be anisotropic [14,16]. HMGB proteins share some features with bacterial IHF [43] and X-ray structures of complexes of the similar HU protein with DNA suggest that a range of induced DNA bend angles are possible [44]. Results from atomic force microscopy for DNA/HU complexes also suggest a range of protein-induced bend angles rather than a fixed geometry [42].

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