Published online 31 August 2012 *Nucleic Acids Research, 2012, Vol. 40, No. 20* ***10161–10171***
doi:10.1093/nar/gks815

Nucleosome dynamics: HMGB1 relaxes canonical nucleosome structure to facilitate estrogen receptor binding

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Received January 6, 2012; Revised July 25, 2012; Accepted August 2, 2012

ABSTRACT

High mobility group protein 1 (HMGB1) interacts with DNA and chromatin to influence the regulation of transcription, DNA repair and recombination. We show that HMGB1 alters the structure and stability of the canonical nucleosome (N) in a nonenzymatic, ATP-independent manner. Although estrogen receptor (ER) does not bind to its consensus estrogen response element within a nucleosome, HMGB1 restructures the nucleosome to facilitate strong ER binding. The isolated HMGB1-restructured nucleosomes (N′ and N″) remain stable and exhibit characteristics distinctly different from the canonical nucleosome. These findings complement previous studies that showed (i) HMGB1 stimulates *in vivo* transcriptional activation at estrogen response elements and (ii) knock down of HMGB1 expression by siRNA precipitously reduced transcriptional activation. The findings indicate that one aspect of the mechanism of HMGB1 action involves a restructuring of the nucleosome that appears to relax structural constraints within the nucleosome.

INTRODUCTION

The activity of all DNA transactions requires that sequence-specific factors—be they associated with replication, transcription, DNA repair or recombination—gain access to their target sites to perform critical functions (1). DNA within the nucleus of a eukaryotic cell is much more inaccessible than free DNA because it is wrapped on the surface of histone octamers to form nucleosomes within the context of a chromatin framework (2). To gain a deeper understanding of the regulation of gene expression, it is essential to discern the cellular strategies by which regulatory transcription factors can gain functional access and bind to their cognate response elements in the nucleosome (3,4). The combination of ATP-dependent chromatin remodeling complexes (CRCs) and histone modifying enzymes (5–8) is thought to play an important role in fine-tuning the dynamics of nucleosome/chromatin structure and factor accessibility. The CRCs may alter DNA-core histone interactions to loosen structural constraints and/or translocate nucleosomal DNA to provide more accessible target sequences. A variety of binding or enzymatic assays have been used to highlight and characterize this altered or remodeled state (9,10).

High mobility group protein 1 (HMGB1) is a ubiquitous, abundant and highly conserved nuclear, 'architectural' protein that exhibits a tripartite structure. It utilizes its highly basic A- and/or B-box to bind transiently and nonspecifically to the minor groove in DNA (11–13), whereas the negatively charged C-terminal domain interacts with TBP (14) and core histones *in vitro* (15). HMGB1 has been shown to generate and stabilize continuous distortions in DNA structure and increase its dynamic flexure (16,17) and to enhance the binding affinity of 'HMGB1-sensitive' transcription factors, including TBP, p53, Oct proteins, HOX D proteins, sterol regulatory element binding factor and steroid hormone receptors. In many cases, the level of transcriptional activity closely parallels the increased binding activity (14,16,18–25). It has also been shown that HMGB1 facilitates the assembly of large nucleoprotein complexes (26), increases the kinetics of CHRAC activity (27) and plays an essential role in enhancing RAG1/RAG2 activity in V(D)J recombination (28). Evidence suggests that HMGB1 may be directly involved in local changes in chromatin architecture in the transcriptional activation at the estrogen-responsive pS2 promoter (29), whereas the similar HMGB2, and presumably HMGB1, enhances transcriptional elongation on chromatin templates (30). Our lab has recently shown that HMGB1 enhances *in vitro* estrogen receptor (ER) binding to the canonical estrogen response element (cERE), in addition to ERE

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half-sites (hcERE) and a spectrum of nonconventional EREs (ncEREs). *In vivo* luciferase reporter assays demonstrate that these EREs drive transcriptional activation and the level of activation is enhanced by the presence of HMGB1. Knock down (KD) of HMGB1 gene expression using siRNA further showed that HMGB1 plays a powerful role in estrogen-responsive gene expression (21).

The *S. cerevisiae* HMG box protein, Nhp6, alters nucleosome structure, especially when in combination with Spt16-Pob3 to form yFACT (31). Interestingly, human FACT was shown to dissociate H2A/H2B from nucleosomes to facilitate enhanced transcriptional elongation by RNA pol II (32).

A primary aim of this study was to determine whether the mechanism of HMGB1 action might be associated with the restructuring of the nucleosome to enhance ER/cERE binding and therefore lead to an increase in transcriptional activation. We find that ER does not bind to the cERE in the canonical nucleosome. However, HMGB1 alters the nucleosome structure and facilitates strong sequence-specific ER binding to cERE in the HMGB1-restructured nucleosome. Initial characterization of two forms of HMGB1-restructured nucleosomes indicates that these nucleosome states can exist in a subset of stable structures that have distinctively different characteristics than the canonical nucleosome. The findings suggest that there is a family of stable nucleosomes that are in equilibrium, that these structures and their functional activities are influenced by HMGB1 and that the ensemble of nucleosome states is a sensitive function of the immediate micro-environment or context.

MATERIALS AND METHODS

Proteins

HMGB1 protein was isolated and purified from calf thymus as described (19). Bacculovirus-expressed ER was purchased from Panvera Corp (Madison, WI). Antibodies for the core histones (H2A, H2B and H3) and the polyclonal HMGB1 antibody were purchased from Millipore, and the antibody for core histone H4 was purchased from Active Motif.

Construction of 161 bp 2E2 DNA fragment and end-labeling of DNA

Plasmid pGEM-Q2 was reconstructed from pGEM3Zf (+) (33) by replacing the Ava I site with 5′-CTCGGG-3′, with subsequent sequential restriction cloning of nucleosome positioning sequences (NPS) fragment A (5′-TCG GTG TTA GAG CCT GTA AC-3′) and fragment B (5′-TCG GGG TTA GAG CCT GTA AG-3′) to generate a construct with an asymmetric Ava I site in the middle of four NPS (between two fragment A and two fragment B). A cERE insert (strand 1: 5′-TCG GT***A GGT CAC AGT GAC CT***A GCC TGT AAC-3′, strand 2: 5′-CCC GAG TTA CAG GCT ***AGG TCA CTG TGA CCT*** A-3′) with AvaI overhangs on either (5′- and 3′-end) was designed and purchased from Integrated DNA Technologies, hybridized to the cut pGEM-Q2 plasmid and ligated in the AvaI site to generate the pGEM-Q2-2E2 plasmid. This construct contains a 161 bp DNA within the EcoRI at 5′-end and HindIII at 3′-end. By this procedure, the 15 bp cERE (bold italicized above) was positioned between two NPS at both the 5′- and the 3′-ends. The DNA was labeled with γ-^{32}P-(ATP) at EcoRI or both EcoRI and HindIII ends as previously described (19).

Preparation of canonical and tailless oligonucleosomes

Erythrocyte nuclei isolated from chicken blood (Pel-Freez) were limit digested with micrococcal nuclease to produce oligonucleosomes. H1-free oligonucleosomes were obtained by fractionated on a Sepharose CL-4B column (34). Tailless oligonucleosomes were similarly prepared after limited digestion of H1-free oligonucleosomes with trypsin (T) at 0.5 ug/mL, followed by addition of trypsin inhibitor (TI) at 10 ug/ml (35). The oligonucleosomes were then separated from T and TI on a Sepharose CL-4B column. The nature of the core histones in the canonical and tailless oligonucleosomes was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of canonical and tailless nucleosomes

Canonical and tailless nucleosomes were prepared with ^{32}P-labeled 161 bp 2E2 DNA by reconstituting canonical and tailless oligonucleosomes by the salt dilution, histone exchange method (36). Reconstituted nucleosomes were then isolated by centrifugation in a 5–30% sucrose gradient containing 10 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 mM phenylmethylsulfonyl fluoride with a Beckman SW55Ti rotor at 36 000 rpm for 16 hr at 4°C. After centrifugation, 200 µl fractions were collected from the top using a 50% sucrose 'push' solution applied to the bottom of the tube. Samples were run on a nondenaturing 4% polyacrylamide gel to monitor the distribution of free DNA and nucleosomal DNA. Nucleosome or tailless nucleosome fractions contained no free-labeled DNA. Purified canonical nucleosomes (~10 nM) were incubated with 1600 nM HMGB1 on ice for an hour and loaded onto a 5–30% sucrose gradient and centrifuged, samples collected and monitored as described earlier.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed as previously described (19). Briefly, ~5–10 nM nucleosomes, in the absence or presence of HMGB1, were incubated with ER in ER binding buffer (80 mM KCl, 10% glycerol, 14 mM Tris–HCl (pH 8.0), 0.2 mM EDTA and 0.4 mM dithiothreitol) in the presence of 2 ng/µl poly (dI-dC) and 0.1 µg/µl bovine serum albumin. The samples were incubated for 30 min at 4°C before electrophoresis. The gels were dried and exposed to phosphoimager screen and scanned with Molecular Dynamics or photographic film for autoradiography. The Image Quant Software was used to measure the band intensities and the Kd was determined (19). The percentage of complex was plotted against the concentration of ER to generate the binding curves. The best fit of

the data was derived using SigmaPlot from three independent determinations.

DNase I and exonuclease III digestions

Nuclease digestions were carried out as previously described (19). Canonical nucleosomes (N), HMGB1-restructured nucleosomes (N′/N″) and free DNA were incubated with or without HMGB1 on ice for 1 hr. The samples were brought to room temperature for digestions. DNase I, Mg^{+2} and Ca^{+2} were added to final concentrations of 0.2 U/mL, 2 mM and 0.5 mM, respectively. Exonuclease III and Mg^{+2} were added to final concentrations of 0.4 U/mL and 10 mM, respectively. Digestions were carried out for the times indicated in the captions. Samples were analysed on 6 or 8% denaturing polyacrylamide gels.

HMGB1-restructured nucleosomes challenged by heat, NaCl and DNA

Canonical and HMGB1-restructured nucleosomes were incubated (i) *for heat challenge:* at different temperature for increasing time; (ii) *for NaCl challenge:* with increasing concentrations of NaCl at 4°C for 30 min; immediately prior to loading, the NaCl concentration in the reaction mixture was diluted to 50 mM; and (iii) *for DNA challenge:* with increasing concentrations of cold 161 bp 2E2 DNA on ice for 30 min. Immediately after incubation, the samples were analysed on a non-denaturing 4% polyacrylamide gel.

Comparative cosedimentation of N′/N″ with mononucleosomes (M) and dinucleosomes (D)

Nuclei were digested with micrococcal nuclease and fractionated in a 5–30% sucrose gradient. The fractions that contained primarily M and D were collected and mixed with radiolabeled N′/N″, dialyzed into reaction buffer and resedimented in a 5–30% sucrose gradient. The position of the N′/N″ fractions was identified by cpms. The DNA from each fraction was purified and the peak fractions that contained the DNA from the unlabeled mono- and di-nucleosomes were identified by nondenaturing gel electrophoresis.

Atomic force microscopy

Canonical nucleosomes (N) and HMGB1-restructured nucleosomes (N′/N″) from a 5–30% sucrose gradient fraction were fixed with 0.5% glutaradehyde for 6 hr at 4°C and dialyzed into TE buffer at 4°C overnight with one change of buffer. The slide was prepared in a freshly cleaved mica surface treated with 1 mM spermidine as described earlier (37) and atomic force microscopy was conducted accordingly.

Determination of HMGB1 levels in sucrose gradient

HMGB1 levels in the nucleosome fractions of the sucrose gradient were determined by fractionating the gradient and carrying out a quantitative Western blot on the fractions using rabbit anti-HMGB1 (1 μg/ml) and the anti-rabbit secondary antibody tagged with IR-800CW. The intensities for the standard curve and the fraction concentrations were scanned at intensity 5 in 800 channels with the Odyssey Licor scanner.

RESULTS

HMGB1 restructures the nucleosome to facilitate ER binding

To extend our previous studies with ER binding to ERE (16,19,21), a 161 bp DNA containing the consensus estrogen response element (cERE), was constructed from the pGEM-Q2 plasmid. Li and Wrange used this DNA construct to show that the glucocorticoid receptor (GR) binds strongly to its cGRE at the dyad axis in nucleosomal DNA (33). Within the nucleosome, the cERE was rotationally phased and translationally positioned at the dyad axis, having two nucleosome-positioning sequences (NPSs) on each side (Figure 1A). Nucleosomes were assembled by the salt dilution procedure and purified by sedimentation in a 5–30% sucrose gradient. In marked contrast to the GR/cGRE binding results (33), Figure 1B and C show there is virtually no ER/cERE binding to nucleosomal DNA (N) up to about 200 nM ER (estimate Kd ~300 nM), even though the major groove of cERE was rotationally phased outward from the histone octamer for optimum ER accessibility. On the other hand, in the presence of 400 nM HMGB1, ER binds strongly to nucleosomal DNA, with a Kd value of 52 nM, exhibiting ca. a 6-fold enhancement in binding affinity. The DNase I footprint (Figure 1D) verifies ER protection at the cERE, indicative of sequence-specific binding. The effect of HMGB1 is to greatly facilitate ER binding to the cERE, with a binding affinity that is only ~10-fold weaker than ER binding directly to free DNA (Kd ~5 nM) (14). This suggested that the HMGB1 effect on ER binding affinity is primarily directed to the nucleosome structure because HMGB1 facilitates less than a 2-fold increase on ER binding to its cERE in free DNA (19).

In support of this hypothesis, the EMSA in Figure 1E shows that in the presence of increasing levels of HMGB1, the mobility of the EMSA band is increasingly reduced, reflecting a changed structure and/or a mixed population of nucleosomes. The band mobility for the canonical nucleosome is unaffected if equal levels of BSA are used in lieu of HMGB1, and importantly, the addition of 3 mM ATP likewise had no effect. Therefore, the altered mobility for the canonical nucleosome is a direct result of HMGB1 interaction and does not require energy derived from the hydrolysis of ATP.

Isolation and characterization of HMGB1-restructured nucleosomes

In an attempt to isolate a distinct population of the restructured nucleosomes, canonical nucleosomes were incubated in the presence or absence of 1600 nM HMGB1 and then separately fractionated in 5–30% linear sucrose gradients. The fractionation profiles in Figure 2A show that the canonical and HMGB1-restructured nucleosomes exhibit virtually identical sedimentation characteristics. However, the presence of

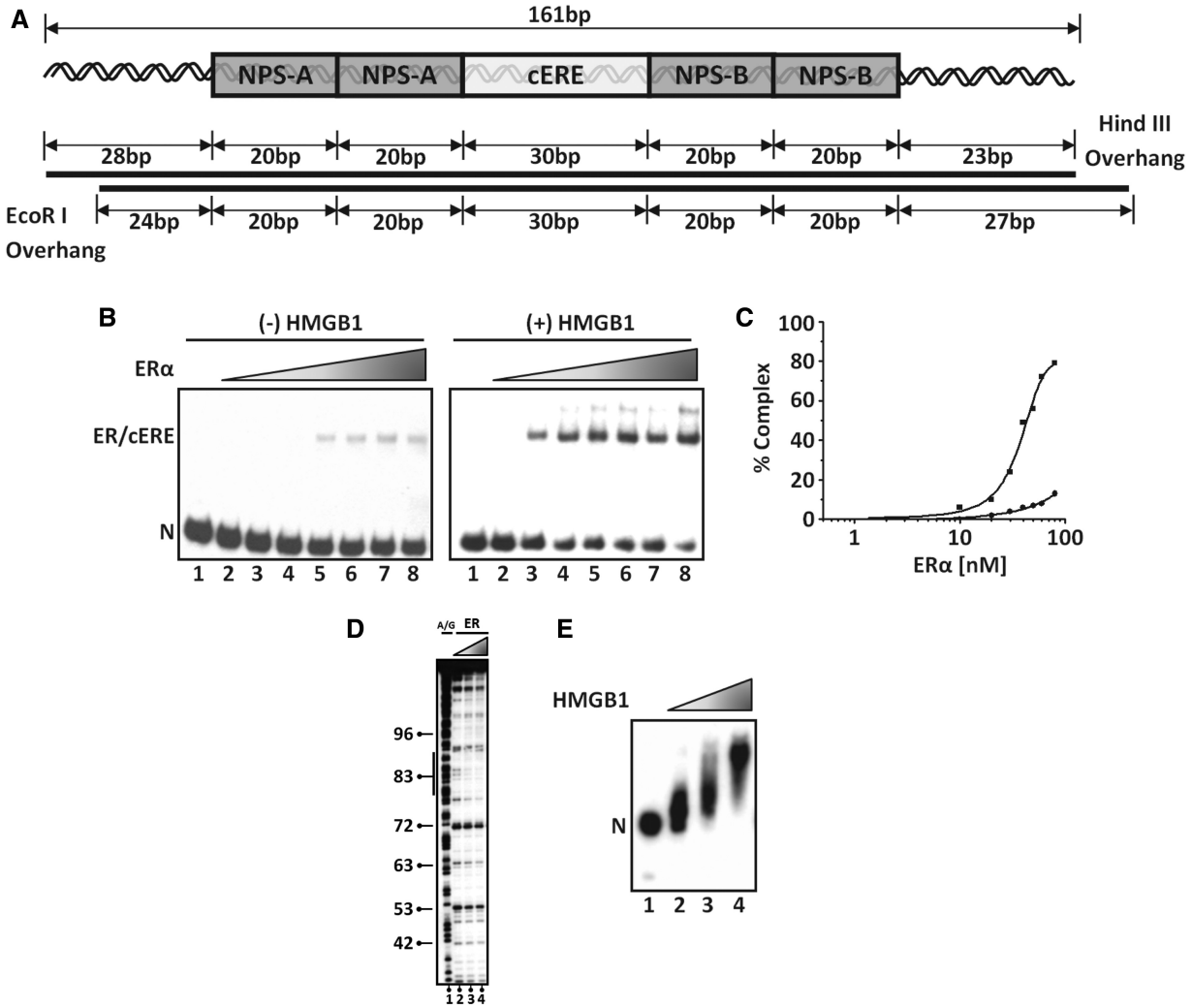

Figure 1. Influence of HMGB1 on the binding affinity of ER on cERE in nucleosomal DNA. (**A**) Schematic diagram of 161 bp DNA containing four nucleosome positioning sequences and the cERE at the dyad axis. The 161 bp DNA is excised by EcoRI and HindIII digestion of pGEM-Q2-2E2. The 30 bp ERE insert contains cERE, which is flanked by two nucleosome positioning sequences (NPS) on each side (**B**) EMSA of ER binding to cERE in nucleosomal DNA. Binding of ER to the cERE in the canonical nucleosomes (N) in the absence and presence of 400 nM HMGB1. ER concentrations are 0, 10, 20, 30, 40, 50, 60 and 80 nM, respectively. (**C**) The ER binding profile in the absence (closed circles) and presence (closed squares) of 400 nM HMGB1. (**D**) DNase I footprint on ER binding to cERE within the nucleosome. Lane 1, A/G ladder; lanes 2–4, nucleosomes were incubated on ice for 30 min with 400 nM HMGB1 and ER concentrations of 0, 50 and 100 nM, respectively. Reactions were set at room temperature for 5 min and digested with 0.04 U DNase I for 1 min. The bar on the left of the gel denotes the position of the cERE. (**E**) The effect of increasing levels of HMGB1 on electrophoretic mobility of nucleosomes. EMSA for canonical nucleosomes incubated with increasing concentrations of HMGB1. Lanes 1–4 canonical nucleosomes (N) in the presence of 0, 400, 800 and 1600 nM HMGB1, respectively. Larger sample amounts and longer exposure times were used compared to (b) to emphasize the differences resulting in the HMGB1 concentrations.

HMGB1 produced two EMSA bands of lower mobility than that of the canonical nucleosome (Figure 2B), indicating that HMGB1 treatment and subsequent sedimentation yielded two distinct nucleosome populations exhibiting the same sedimentation characteristic. We refer to these two HMGB1-restructured nucleosome forms as N′ and N″. To determine the composition of the restructured nucleosomes, the nucleosomes were reacted with antibodies for each core histone and HMGB1 (Figure 2C). Both the canonical nucleosomes (N) and the HMGB1-restructured nucleosomes (N′, N″) were supershifted by the antibodies for each of the core histones, indicating that the core histones are retained in the restructured nucleosomes. On the other hand, anti-HMGB1 did not produce a supershift for either nucleosome form, either prior to or after sedimentation, consistent with HMGB1 not being a stable component of the complex. This finding is similar to previous reports and suggests that the HMGB1 interaction with DNA is transient and most likely functions in a 'hit-and-run' mechanism (19).

The sedimentation behavior for N′/N″ is distinctly different than the remodeled nucleosomes produced by the chromatin-remodeling complex, RSC, and those characterized as putative dinucleosomes (37). To address the question of whether (N′/N″) were restructured mononucleosomes, the sedimentation behavior of (N′/N″) was compared with that of mononucleosome (M)

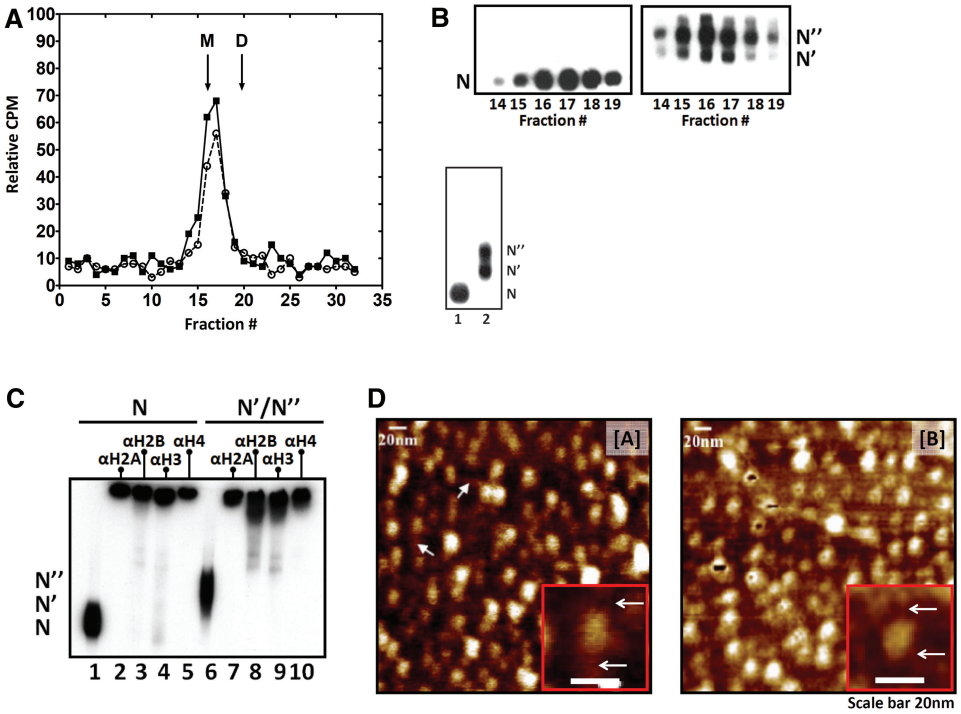

Figure 2. Fractionation and EMSA characterization of canonical (N) and HMGB1-restructured nucleosomes (N'/N″). (**A**) Sedimentation profiles. Fractions from a sucrose gradient and their relative counts per minute (CPM) for the canonical nucleosome (closed square) and HMGB1-restructured nucleosome (open circle). The arrow marks with M and D represent the position of mono- and dinucleosome in a sucrose gradient when co-sedimented with canonical nucleosomes and HMGB1-restructured nucleosomes. (**B**) EMSA profiles. EMSA bands for the canonical nucleosome (N), fractions 14–19 and HMGB1-restructured nucleosomes (N′/N″), fractions 14–19. EMSA of canonical nucleosome (N) (Lane 1, fraction 17) and HMGB1-restructured nucleosomes (N′/N″), (Lane 2, fraction 17) run on the same gel. (**C**) The effect of anti-histone antibodies (αH2A, αH2B, αH3, αH4) on canonical (N) and HMGB1-restructured nucleosomes (N′/N″). Lane 1: N; Lanes 2–5: N treated with αH2A, αH2B, αH3 and αH4, respectively. Lane 6: N′/N′; Lanes 7–10: N′/N″ treated with αH2A, αH2B, αH3 and αH4, respectively. (**D**) Atomic force microscopy of N and N′/N″. (A) Canonical mononucleosomes (N) on spermidine-treated mica. (B) HMGB1-restructured nucleosomes (N′/N″) seen as mononucleosomes on spermidine-treated mica. DNA tails, where visible, are indicated by arrows. Insets showing enlarged view of distinct mononucleosomes in both (A) and (B). Scale bar = 20 nm.

and dinucleosome (D) fractions derived from a limited micrococcal nuclease digestion of nuclei. The N′/N″ fraction was found to cosediment with the mononucleosome fraction and distinctly different than that for the dinucleosome fraction. The positions for M and D are indicated in Figure 2A. Furthermore, atomic force microscopy of the canonical and HMGB1-restructured nucleosomes (Figure 2D, A and B, respectively) shows only the presence of mononucleosomes, with no evidence for a dinucleosome.

We next wanted to determine whether there was HMGB1 protein in the N′/N″ fraction after the sedimentation and whether the presence of HMGB1 was essential for the stability of the N′/N″ nucleosome states. Using the same conditions as earlier, we determined the levels of HMGB1 in individual sucrose gradient fractions by western blot analysis, comparing band intensities with HMGB1 standard concentrations (Figure 3A and B). We find that HMGB1 levels in the two collected fractions containing N′/N″ (Figure 3C, lanes 7 and 8) were ~50 nM and undetectable, respectively, yielding an HMGB1 level of ~25 nM. This is about two times the level of nucleosomes in the preparations. However, because reactions of ER with the nucleosomes were prepared with equal volumes of ER and nucleosomes, the HMGB1 concentration in all ER reactions was ~10 nM HMGB1. To further characterize the effect of HMGB1 on the N′/N″ population, the nucleosomes were dialyzed (using 100 kDa cutoff membrane) against reaction buffer to eliminate or further reduce the presence of HMGB1. The presence of the two EMSA bands for N′/N″ and their positions remained unchanged. We also determined that incubation of canonical nucleosomes with 10 nM HMGB1 for extended times did not restructure the nucleosome (did not alter the EMSA mobility) and did not facilitate ER binding (data not shown). Furthermore, because the binding affinity of HMGB1 for DNA is in the micromolar range and the nucleosome concentration is also very low (nM), this low level of HMGB1 is both unable to restructure the nucleosome and does not appear essential to maintain the restructured nucleosomes. Importantly, the HMGB1-restructured nucleosomes can be kept and maintained for months at −20°C in TE/sucrose (gradient) buffer.

An inherent characteristic of canonical nucleosomes is the DNase I cutting pattern, with DNA cuts in the exposed minor groove spaced approximately every 10 bp. ATP-dependent CRCs disrupt the phased periodicity of the DNA backbone and/or translocates the DNA. This leads to a more heterogeneous cutting pattern that tends

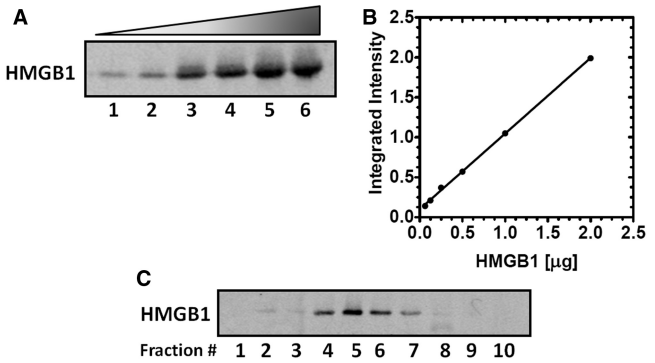

Figure 3. Determination of HMGB1 concentration in nucleosome population. (**A**) Western blot for HMGB1. Lanes 1–6; HMGB1 concentrations are 62, 125, 250, 500, 1000 and 2000 ng HMGB1, respectively. (**B**) Standard curve for HMGB1 concentrations. (**C**) HMGB1 levels in fractions from the sucrose gradient. Fractions 7 and 8 represent the HMGB1 levels present in the nucleosomal fractions 16 and 17 (Figure 2A) after sucrose gradient fractionation.

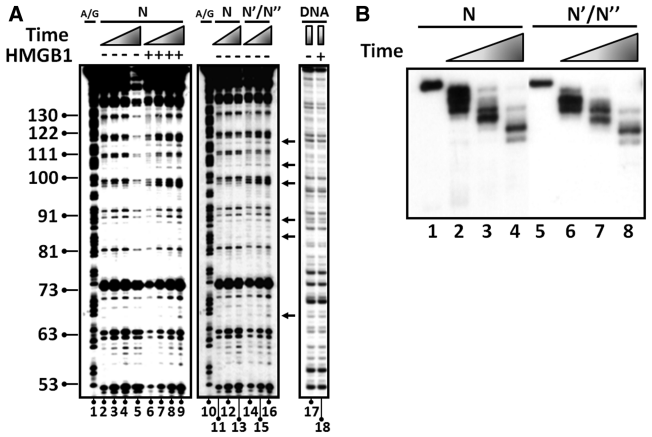

Figure 4. Nuclease digestion profile on canonical (N) and HMGB1-restructured nucleosomes (N′/N″). (**A**) DNase I digestion profile. Lanes 1 and 10: A/G ladder. Lanes 2–5: canonical nucleosomes without HMGB1. Lanes 6–9: HMGB1-restructured nucleosomes with 400 nM HMGB1. DNase I digestion was performed for increasing time, 30, 60, 90 and 120 s, respectively. Lanes 11–13: canonical nucleosomes. Lanes 6–9: HMGB1-restructured nucleosomes. DNase I digestion was performed for increasing time, 30, 60 and 120 s, respectively. Lanes 17 and 18: DNA, with and without 400 nM HMGB1. DNase I digestion was performed for 30 s. Arrows to the right indicate additional DNase I cuts as a result of HMGB1 interactions. (**B**) Exo III digestion profiles. Lane 1: canonical nucleosomes. Lanes 2–4: canonical nucleosomes digested with Exo III for 1, 2 and 4 min, respectively. Lane 5: Untreated HMGB1-restructered nucleosome. Lanes 6–8: HMGB1-restructured nucleosomes digested with Exo III for the same times.

to resemble more that observed on free DNA, consistent with a reduction of rotational constraints in nucleosomal DNA (9,38). Figure 4A shows the DNase I pattern on the canonical nucleosome (N), the nucleosome in the presence of 400 nM HMGB1, the sucrose gradient isolated forms (N′/N″) and free DNA. The DNase I 10 bp periodicity observed in the canonical nucleosome remains intact for nucleosomes in the presence of 400 nM HMGB1 (400 nM/N) and the N′/N″ forms. Although the cutting pattern for both 400 nM/N and N′/N″ restructured nucleosomes are similar to the canonical form, both these structures exhibit a modest increase in the number of bands for DNase I cutting, indicative of a modest disruption of internal histone–DNA interactions.

To determine whether there was any DNA translocation as a result of the structural change in N′/N″, the Exo III digestion patterns for the canonical and restructured nucleosomes were obtained. Figure 4B shows that the Exo III cutting patterns are essentially the same, indicating that the HMGB1 interactions do not lead to translocation of the DNA within the nucleosome and also that the action of HMGB1 has not altered the accessibility of the ends of the DNA fragment to Exo III.

Although the HMGB1-restructured nucleosomes (N′/N″) are stable for months at −20°C in TE/sucrose buffer, changes in solution conditions may provide a challenge to their integrity. To begin to characterize their intrinsic stability, N′/N″ were challenged by increasing heat and increased levels of NaCl or DNA. Figure 5A shows that incubation of the canonical nucleosome is unaffected by temperature change, whereas the N′/N″ fraction is unstable when incubated for increasing times at 37°C. N″ is destabilized within 60 min as it is converted to the more stable N′ state. However, the N′ state remains stable, even after overnight incubation. Therefore, the canonical nucleosome, N, and the restructured state, N′, have comparable thermal stability at 37°C, with the relative thermal stability of the three different nucleosome forms being N ~ N′ > N″. Figure 5B shows that incubation of N′/N″ with increasing concentrations of NaCl converts N″ into a mixture of N′ and N at ~25 mM NaCl, with N′ then progressively and completely converted to the canonical nucleosome at ~200 mM NaCl. This demonstrates again that N″ is the more unstable of the restructured nucleosomes and that the N′/N″ nucleosome states are differentially stable to increasing ionic strength, with presumably the electrostatic forces involved in stabilizing N′ and N″ being progressively disrupted. This also indicates that the electrostatic forces that are involved in the N′/N″ states are different than those in the canonical nucleosomes, with the latter forces being stable at high NaCl concentrations. Finally, Figure 5C shows that, in the presence of up to ~50 nM DNA, all three nucleosome states (N, N′ and N″) are present and stable. However, the addition of higher levels of unlabeled 161 bp DNA progressively destabilizes the N″, with N″ being undetectable at ~94 nM DNA. Up to ~94 nM DNA, the band intensities reveal that the population of N′ remains virtually unchanged, whereas the population of N″ decreases, with the concomitant increase in the canonical nucleosome. The N′ population is then completely converted to the canonical nucleosome at 940 nM DNA. Again, the stability of the canonical nucleosome and the N′ state appear comparable, until high levels of DNA challenge their stability. Overall, the order of stability is N″ < N′ < N. It should also be noted that since there is a low level of HMGB1 present in the solution, it can be expected that increasing NaCl levels will reduce the extent of any electrostatic interaction of HMGB1 with the nucleosomes, whereas increasing levels of DNA will

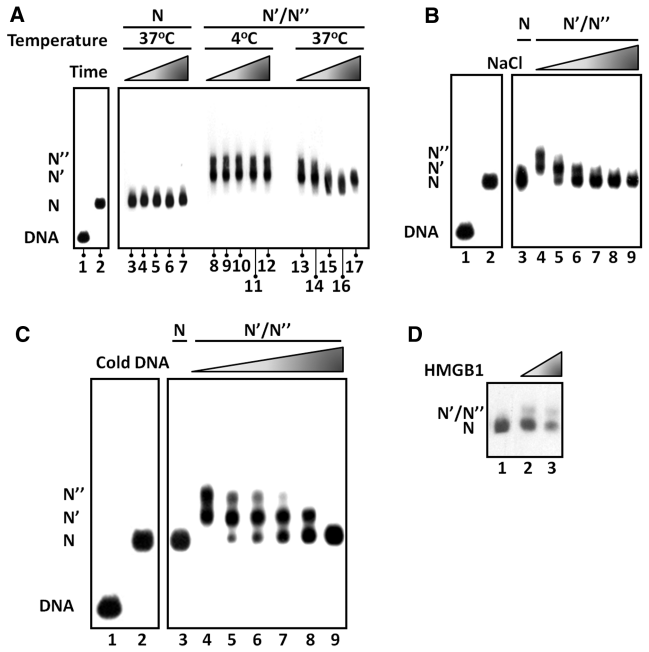

Figure 5. EMSA profiles showing the effect of heat, NaCl or DNA on the integrity and stability of HMGB1-restructured nucleosomes. (**A**) Effect of heat. Lane 1, DNA, Lane 2, canonical nucleosome, Lanes 3–7: canonical nucleosomes (N) incubated at 37°C for 0, 0.5, 1, 2 hr and overnight, respectively. Lanes 8–12: HMGB1-restructured nucleosomes (N′/N″) incubated at 4°C and lanes 13–17: HMGB1-restructured nucleosomes (N′/N″) incubated at 37°C for the same times as the canonical nucleosomes. (**B**) Effect of NaCl. Lane 1, DNA, Lane 2, canonical nucleosome, Lane 3: canonical nucleosome (N). Lane 4: Untreated HMGB1-restructured nucleosomes (N′/N″). Lanes 5–9: HMGB1-restructured nucleosomes treated with increasing concentrations of NaCl (25, 50, 100, 200 and 300 mM NaCl, respectively), with NaCl concentrations leveled to 50 mM immediately prior to electrophoresis in lanes 6–9. (**C**) Effect of DNA. Lane 1, DNA, Lane 2, canonical nucleosome, Lane 3: canonical nucleosome (N). Lane 4: HMGB1-restructured nucleosomes (N′/N″). Lanes 5–9: HMGB1-restructured nucleosomes treated with increasing concentrations of cold DNA (12, 24, 47, 94 and 940 nM, respectively). (**D**). Effect of HMGB1 concentration on the level of canonical and restructured nucleosome populations. Canonical nucleosomes were incubated with increasing concentrations of HMGB1 on ice for 1 hr and fractionated by sucrose gradient. Lane 1: canonical nucleosomes. Lane 2: Fraction of 400 nM HMGB1-treated canonical nucleosomes. Lane 3: Fraction of 800 nM HMGB1-treated canonical nucleosomes. The band for the restructured population of nucleosomes is indicated as N′/N″.

act as an 'HMGB1 sink' and likewise strongly inhibit its transient binding. The finding that the electrophoretic mobility of both forms of the restructured nucleosomes readily revert to that of the canonical nucleosome also supports the contention that the N′/N″ forms contain the same stoichiometry or the entire complement of core histones as the canonical nucleosome. In addition, there was no evidence for any dissociation of radiolabeled DNA from the restructured nucleosomes in the three challenge experiments. Collectively, these results indicate that the N′/N′ nucleosomes can be readily converted to the more stable canonical N state by altering solution conditions. In further support of this, Figure 5D also shows that the relative population of the canonical nucleosome and restructured nucleosomes is a function of the level of HMGB1. Nucleosomes were reacted with 0, 400 or

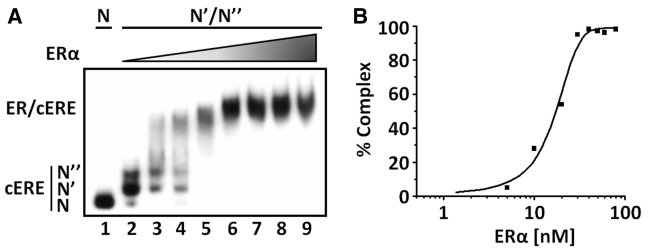

Figure 6. EMSA of ER binding to cERE in HMGB1-restructured nucleosomes. (**A**) *Binding of ER to the cERE in the HMGB1-restructured nucleosomes* (*N′/N″*). Lane 1: Canonical nucleosome (N). Lanes 2–9: HMGB1-restructured nucleosomes treated with increasing concentrations of ER (0, 10, 20, 30, 40, 50, 60 and 80 nM, respectively). (**B**) The binding profile of ER to HMGB1-restructured nucleosomes.

800 nM HMGB1 and then fractionated on sucrose gradients. The EMSA profile shows that the population of the restructured nucleosome states increases with increasing levels of HMGB1. It is important to emphasize that under the different solution conditions shown in Figure 5B (~25–50 mM NaCl), 5C (between 12 and 47 nM DNA) and in 5d (at 400 and 800 nM HMGB1), there is clearly an equilibrium distribution of canonical and restructured nucleosome states.

ER binds strongly to HMGB1-restructured nucleosomes

The binding affinity of ER to the isolated HMGB1-restructured nucleosome states (N′/N″) was examined and Figure 6A and B show that ER binds strongly, with a Kd of ~30 nM. This is comparable with what was observed in the presence of 400 nM HMGB1 and suggests that after isolation of these restructured states, they contain a genuinely accessible cERE that facilitates strong ER binding.

ER binds to tailless nucleosomes in the absence of HMGB1

Previous studies showed that removal of the core histone-tail domains from the nucleosome does not disrupt its structure as reflected by the DNase I 10 bp pattern (39). Because the presence of HMGB1 produced only a modest disruption of the DNase I pattern, we investigated the possibility that the HMGB1 effect, with its positive effect on ER binding, may, in part, be associated with disruption of the interaction of the positively charged histone tail domains with the DNA to facilitate greater accessibility and stronger ER binding. A limited trypsin digestion was used to cleave off the histone tail domains of oligonucleosomes, from which tailless nucleosomes were prepared and then gradient fractionated. Figure 7A compares the SDS-PAGE of the core histones and those in which the core histone tail domains have been removed by digestion with trypsin. Figure 7B and C show that ER binds strongly to the tailless nucleosomes, with a Kd ~45 nM, a comparable binding affinity to that determined for both (i) the canonical (tailed) nucleosomes in the presence of 400 nM HMGB1 and with (ii) N′/N″, and which markedly contrasts the lack of ER binding to the canonical nucleosomes.

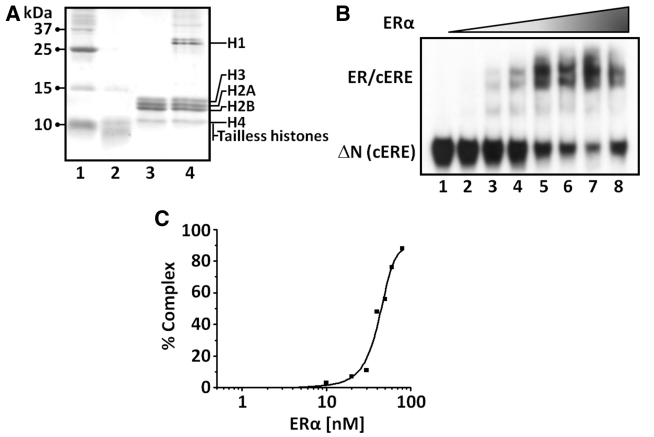

Figure 7. Characterization of tailless nucleosomes. (**A**) SDS-PAGE of histone proteins. Lane 1: molecular weight markers. Lane 2: tailless core histones from oligonucleosomes that were treated with trypsin. Lane 3: core histones. Lane 4: histone proteins from crude micrococcal nuclease digestion of nuclei. (**B**) EMSA profile for ER binding to the cERE in the tailless nucleosomes (ΔN). Lanes 1–8: Tailless nucleosomes treated with increasing concentrations of ER (0, 10, 20, 30, 40, 50, 60 and 80 nM, respectively). (**C**) The binding profile of ER to tailless nucleosomes.

DISCUSSION

A combination of approaches shows that HMGB1 restructures the canonical nucleosome to facilitate strong ER binding. There are at least three distinct and stable states of the nucleosome (N, N′ and N″) that are in equilibrium, with the stability and population of the states sensitive to differential solution (microenvironment) conditions.

Although the cERE is centered at the dyad axis, with the major grooves of the cERE rotationally phased outward to provide ER optimum access to bind nucleosomal DNA, ER does not bind. This difficulty may be related, in part, to the finding that ER binding bends the cERE toward ER (40), which is opposite to the direction the DNA is constrained to by interactions with the core histones. The transient interaction of HMGB1 with the nucleosome, increases not only DNA flexure but also generates and/or stabilizes a nucleosome conformer that is effectively less constrained, more accessible and can be considered a more 'relaxed' form on the nucleosome energy landscape. ER alone is not capable of surmounting this energetically restricted barrier in the canonical, 'tense' form. In the presence of 400 nM HMGB1, ER binding affinity is increased about 6-fold (Kd = 50 nM), whereas the EMSA mobility and the DNase I 10 bp cutting pattern are both only marginally changed. The lack of major disruption of the DNase I 10 bp pattern suggests that the DNA interactions within the inner core of the histones are only marginally disrupted, with the rotational phasing of the DNA experiencing little or no change in the presence of 400 nM HMGB1. Because cleaving off the core histone tail domains also does not affect the DNase I 10 bp pattern, we hypothesized that disruption or alteration of their interactions with DNA on the nucleosome surface may contribute to the enhancement of ER binding affinity. The stability of the restructured nucleosomes, N′/N″, decreases with increases in NaCl levels, similar to that observed for the altered nucleosome produced by the CRC, RSC (41). NMR evidence further supports this in that the N-terminal domains of H3 and H4 remain tightly bound to the DNA in core particle up to ~0.3 M NaCl (42), which is the same level of NaCl that converts the N′/N″ states to the canonical nucleosome. Finally, the increased binding affinity of ER on the tailless nucleosomes ($K_d \sim 45$ nM) is consistent with the tails inhibiting ER binding and supports this hypothesis because this binding affinity is comparable with that for nucleosomes in the presence of 400 nM HMGB1.

The Exo III digestion pattern is not altered for nucleosomes in the presence of 400 nM HMGB1 indicating that (i) the HMGB1 interaction does not preferentially bind to the ends of the DNA and (ii) there is no significant movement of the DNA relative to the histone octamer. The lack of a translocation activity on nucleosomes is not unexpected because HMGB1 exhibits no known ATPase activity, in contrast to the ATP-dependent CRCs, which can affect DNA translocation (43).

Although additional experiments are essential to understand the action of HMGB1 and to gain a more complete understanding of this and other alternate nucleosome structures (10,37,38,41,44), we propose a model in which the highly charged HMGB1 interacts in the minor groove to enhance DNA flexure, disrupt electrostatic interactions and reduce core histone-DNA interactions. HMGB1 further reduces the residence times of the positively charged histone tails with DNA and thereby widens the window of opportunity for greater ER access to the cERE. This hypothesis is in line with other findings that showed the removal of, or alternatively, acetylation of the histone tail domains, did not alter the DNase I 10 bp pattern, but did increases transcription factor accessibility to their target sites (6,39,45,46).

The lack of ER binding to DNA in the canonical nucleosome also challenges the generally held view that members of the steroid receptor family have similar binding characteristics and bind strongly to their response elements within a nucleosome (33,47). Although the *in vitro* binding of GR or ER to their respective response elements within free DNA is comparable, their binding affinity to nucleosomal DNA is distinctly different. Although the GR/cGRE binding affinity to DNA and nucleosomal DNA remain comparable (33), the ER/cERE binding affinity in (canonical) nucleosomal DNA is markedly reduced by as much as 60-fold from that in DNA. This indicates that DNA within the nucleosome exerts a very different influence on the binding behavior of these two steroid hormone receptors. This is consistent with previous findings that showed that although HMGB1 enhanced GR binding to the GRE in MMTV DNA, the influence of HMGB1 on GR binding to MMTV chromatin was minimal (48).

From the EMSA mobility data as a function of HMGB1 levels and the effect of both NaCl and unlabeled DNA on the distribution of nucleosome conformers, it is clear that these states (N, N′ and N″) can simultaneous

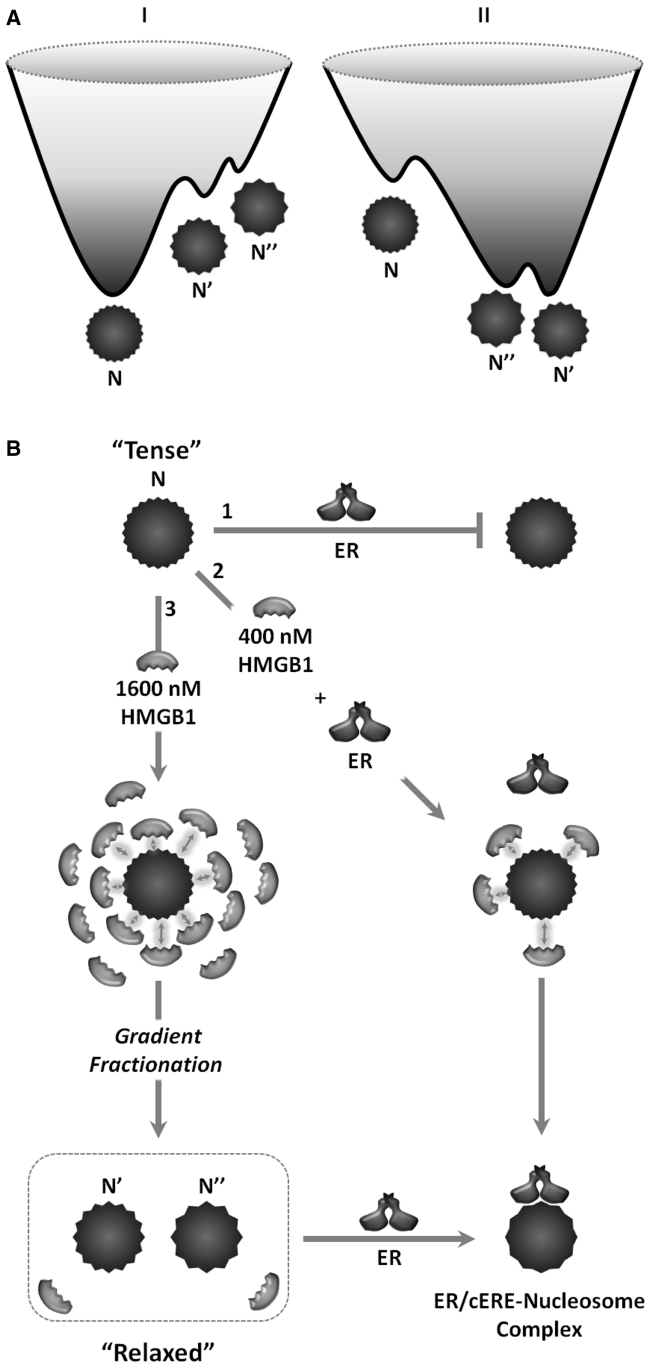

Figure 8. HMGB1 relaxes the canonical nucleosome structure and facilitates ER binding. (**A**) Energy landscapes for canonical (I) and HMGB1-restructured nucleosomes (II). A hypothetical representation for the energy landscape of the canonical nucleosome, N, and the HMGB1-restructured nucleosomes, N′ and N″. Using conventional isolation protocols, the canonical nucleosome, N, is the predominant and thermodynamically most stable conformation. N′ and N″ are in low abundance, higher energy conformational isomers that are kinetically trapped near the bottom of energy landscape I. HMGB1 interaction with N reduces intranucleosomal constraints, which resets the energy landscape (II), resulting in a population shift in which the N population significantly decreases and the population of the more 'relaxed' and accessible N′ and N″ states increases. The more unstable form, N″, sets in a shallower potential well than that for N′. Although interactions with HMGB1 provide the driving force to restructure N into these states, these forms remain stable and although in equilibrium with the canonical state under many solution conditions, can revert to the canonical nucleosome on challenge with increasing concentrations of NaCl and DNA. (**B**) Interaction of the nucleosome with HMGB1 and ER. The canonical nucleosome (N) represents a 'tense' and relatively inaccessible conformational isomer (pathway 1) and ER does not bind to the canonical nucleosome state. In the presence of 400 nM HMGB1 (pathway 2), due to the transient and dynamic 'hit and run' interaction of HMGB1 with the nucleosome, represented by arrows (↔), the intranucleosomal constraints are relaxed, which facilitates ER binding. ER binds to the nucleosome to form ER/cERE nucleosome complex. In the presence of 1600 nM (pathway 3), the intranucleosomal constraints are relaxed due to increased 'hit and run' interaction of HMGB1. After gradient fractionation, the restructured nucleosomes (N′ and N″) are isolated and contain only low nM levels of HMGB1, which maintain the more accessible and 'relaxed' conformational isomers (N′ and N″) that permit ER binding.

exist and may be considered in the context of a dynamic equilibrium of multiple populations of conformational isomers on a nucleosome energy landscape (49). This paradigm suggests that there is a statistical ensemble of nucleosome conformers in equilibrium, of which the population of states and the energy barriers between them is sensitive to the immediate microenvironment and to interactions from binding factors, such as HMGB1. The interaction of HMGB1 with the nucleosome can be regarded as a 'ligand-driven' conformational selection of states, or what is referred to as a population shift (49). This population shift may be further influenced by ER binding.

Figure 8A portrays a limited picture of two hypothetical energy landscapes for the three nucleosome conformational states—the canonical N and the HMGB1-remodeled N′ and N″ states. Using the conditions for the conventional preparation/isolation of nucleosomes (energy landscape I), the canonical nucleosome, N, is by far the predominant species, with N′ and N″ being kinetically trapped and at levels undetectable by EMSA. The HMGB1 interaction with the nucleosome transitions the energy landscape from I to II, as it perturbs this distribution of states and drives a population shift, in which the levels of N′ and N″ increase significantly, with a concomitant decrease in the population of canonical nucleosomes. Once isolated, all three states are stable at low temperatures, but N′, and especially N″, can be converted to N under a variety of 'stressful' conditions. Figure 8B provides a general picture for the absence of a significant reaction of ER with the canonical, 'tense' form of the nucleosome (pathway 1). Treatment of the canonical nucleosome with HMGB1 (pathways 2 and 3) reduces constraints to permit ER binding to the HMGB1-restructured nucleosome. Gradient fractionation eliminates the initial high (1600 nM) levels of HMGB1, whereas the two stable forms of the 'relaxed' nucleosome to which ER binds strongly, maintain their integrity in low levels of HMGB1.

This collection of nucleosome forms can be considered as only the first level in the ensemble of nucleosome states. In this paradigm, influences such as DNA methylation, posttranslational modifications of the core histone proteins, histone variants, SIN mutations and the level of chromatin compaction may each contribute to a

multitude of additional energy states within the chromatin network. All these factors can potentially alter intra- and internucleosomal forces and establish a different or more extended ensemble of nucleosome conformational states, and therefore further fine-tune the functional activities. This is consistent with the notion of a heterogeneous population of nucleosomes within chromatin, all in a dynamic state and able to respond to continuous changes from environmental ques. In light of the obvious comparison of dynamic nucleosome restructuring to the simple manipulation and controlled reshaping of a toy 'stress ball', (accomplished by the pressure from your hand), we refer to this dynamic nucleosome model, in which diverse forces and binding interactions affect the dynamics of nucleosome states, as the 'stress ball' model.

These findings show for the first time that the chromosomal protein, HMGB1, restructures nucleosomes, or resets the population distribution of states in an ATP-independent manner. This is evident from multiple assays that reveal (i) an altered EMSA mobility, (ii) a modestly disrupted DNase I 10 bp pattern, (iii) facilitated ER access and binding to cERE and (iv) a challenge by heat, increased NaCl and excess DNA. These altered characteristics clearly show that the HMGB1-restructured nucleosome can have a significant contribution to ER gaining access to its cognate site (21). Although it is well established that HMGB1 does not bind DNA in a sequence-specific manner, it was recently shown that HMGB1 could interact with steroid hormone receptors, PR and ER, prior to their binding to DNA (50). If a subpopulation of ER is 'chaperoned' to its ERE/DNA by HMGB1, this ER/HMGB1 association may provide a chaperone-assisted mechanism that would increase the local concentration of HMGB1 and increase the effectiveness of targeting access to estrogen response elements.

Some of the characteristics exhibited by the HMGB1-dependent restructured nucleosomes are reminiscent of those produced by the ATP-dependent CRCs. However, there is no evidence for an HMGB1-induced DNA translocation. In addition, the HMGB1-restructured states, unlike those produced by CRCs, are stable for extended periods of time – at least months. One may speculate that the nonenzymatic role of HMGB1 in remodeling nucleosomes and in facilitating ER binding to its ERE in nucleosomal DNA may, in some cases, be one of the steps in the transcription initiation or the elongation process (16,18–25,30,51,52). In this light, it is of interest to determine further how these seemingly disparate activities—HMGB1, ATP-dependent CRCs and post translational modifications—might complement each other and/or work together in a step-wise or concerted manner to modify chromatin structure for optimum transcriptional activity. Certainly, HMGB1-restructured nucleosomes can play an important role in gaining new insight and understanding into the diverse mechanistic avenues used to alter or remodel nucleosomes within a chromatin context that lead to a greater accessibility and functional activity.

ACKNOWLEDGEMENTS

The authors thank O. Wrange for the pGEM-Q2 plasmid and H. Peter Lu for assistance with the AFM experiments.

FUNDING

National Institutes of Health [GM054357-04 to W.M.S.]. Funding for open access charge: Waived by Oxford University Press.

Conflict of interest statement. None declared.

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