Article published online: 2021-05-10

AIMS Genetics, 3(4): 252-279.
DOI: 10.3934/genet.2016.4.252
Received: 08 October 2016
Accepted: 23 November 2016
Published: 02 December 2016

http://www.aimspress.com/journal/Genetics

Review

Nucleosome dynamics: HMGB1 facilitates nucleosome restructuring and collaborates in estrogen-responsive gene expression

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Abstract: The genome in the human cell is extraordinarily compacted in the nucleus. As a result, much of the DNA is inaccessible and functionally inert. Notwithstanding the highly efficient packaging, mechanisms have evolved to render DNA sites accessible that then enable a multitude of factors to carry out ongoing and vital functions. The compaction is derived from DNA complexation within nucleosomes, which can further consolidate into a higher-order chromatin structure. The nucleosome and nucleosomal DNA are not static in nature, but are dynamic, undergoing structural and functional changes as the cell responds to stresses and/or metabolic or environmental cues. We are only beginning to understand the forces and the complexes that engage the nucleosome to unearth the tightly bound and inaccessible DNA sequences and provide an opening to more accessible target sites. In many cases, current findings support a major role for the action of ATP-dependent chromatin remodeling complexes (CRCs) in providing an avenue to factor accessibility that leads to the activation of transcription. The estrogen receptor α (ERα) does not bind to the estrogen response element (ERE) in the canonical nucleosome. However, evidence will be presented that HMGB1 restructures the nucleosome in an ATP-independent manner and also facilitates access and strong binding of ERα to ERE. The features that appear important in the mechanism of action for HMGB1 will be highlighted, in addition to the characteristic features of the restructured nucleosome. These findings, together with previous evidence, suggest a collaborative role for HMGB1 in the step-wise transcription of estrogen-responsive genes. In addition, alternate mechanistic pathways will be discussed, with consideration that “HMGB1 restructuring” of the nucleosome may generally be viewed as a perturbation of the equilibrium of an ensemble of nearly isoenergetic nucleosome states in an energy landscape that is driven by conformational selection by HMGB1.

Keywords: nucleosome dynamics; HMGB1; estrogen receptor α; conformational selection; energy landscape

Abbreviations

ER: estrogen receptor; ERE: estrogen response element;
cERE: consensus estrogen response element; RE: response element;
CRC: chromatin remodeling complex; HMGB1: high mobility group protein 1;
HAT: histone acetyltransferase; HDAC: histone deacetylase;
H3K9: histone H3, with modification at lysine 9; FoxA1: Forkhead-box protein A1;
BRG-1 & hBrm: ATPase subunits in human CRCs; BAF: BRG1-asscoiated factor;
PIC: preinitiation complex; TF: transcription factor;
HSF: heat shock factor; NF1: nuclear factor1;
TBP: TATA-binding protein; Max: myc-associated factor X;
MMTV: mouse mammary tumor virus; TRF1: TTAGGG repeat binding factor-1;
LEF-1: lymphoid enhancer binding factor-1; PR: progesterone receptor;
GR: glucocorticoid receptor; TSS: transcription start site;
MCF-7: Michigan Cancer Foundation cell line 7; TNF-α: tumor necrosis factor alpha;
ISWI: imitation SWI; Kd: dissociation constant; KD: knock down.
USF: up-stream stimulating factor; Sp1: specificity protein1;
NF-κB1: nuclear factor kappa-light chain enhancer of activated B cells;
Transcriptional coactivators: CBP, p300, pCAF, PBP, p160;
ACF/CHRAC: ATP-utilizing chromatin remodeling factor/chromatin remodeling and assembly complex

1. Nucleosome structure and variability

The genetic material in a human cell—three billion base pairs (bps) of DNA—which when extended is 2 meters in length, is enormously compacted in the relatively microscopic cellular nucleus (10 microns (10×10^{-6} m) in diameter). The genome is overwhelmingly sheltered and protected from effective interaction with a vast array of factors by complexation within millions of nucleosomes that make up chromatin. Although this strategy permits the efficient packaging of the DNA, essential functions carried out on DNA—such as replication, transcription, DNA repair and genetic recombination—all require a multitude of complexes to gain access to specific sites on the functional template—nucleosomal DNA. In many of these transactions, remodeling, restructuring, eviction and/or translocation of nucleosomes is necessary to gain access and facilitate these functional programs. Chromatin remodeling complexes (CRCs) that contain an ATPase activity appear to be major functional units that can make DNA targets more accessible in transcriptional activation [1-3]. However, alternate or cooperative factors that are not catalytic and do not contain ATPase activities can restructure nucleosomes and collaborate in "opening-up" target sites [4]. This minireview will summarize evidence that high mobility group protein 1 (HMGB1) restructures the nucleosome and can play a collaborative role in the transcription of estrogen-responsive genes that are activated by the action of ERα.

The substrate for processing functional activities on the genetic material is not naked DNA, but DNA that is complexed within the repeating unit in the nucleus called the nucleosome. The nucleosome contains ~200 bp of DNA of which 147 bps of the DNA are bound within the nucleosome core particle,

wrapping 1¾ times on the exterior of a disk-shaped core of 8 highly basic histone proteins, two of each of the core histones—H2A, H2B, H3 and H4. The additional 20–80 bps is linker DNA which is more loosely bound and can associate with a single molecule of histone H1 that is important for condensation of nucleosomes into a less accessible and higher-order chromatin structure [5,6]

The structure of the core nucleosome establishes a helical ramp for DNA, which makes an irregular left-handed supercoil as the DNA backbone interacts with the histone core. Each of the core histones has a conserved structural motif called the histone-fold domain that resides within the nucleosome. These proteins assemble into head-to-tail histone-fold heterodimers—(H3-H4) and (H2A-H2B)—that interact to form the fundamental structural units that are responsible for protein-protein and the protein-DNA interactions and much of the stability of the nucleosome. The histone-fold domains organize about 130 bps of the central DNA, with the two heterodimers of (H3-H4) forming a tetramer that binds the central ~70–80 bps of DNA, while the two (H2A-H2B) heterodimers interact with the 40–50 bps nearer the ends of the DNA [7-9].

The most prominent interactions, 14 of them, are non-specific in nature and found between the histone main-chain amides and the DNA phosphate groups at about 10 bp intervals in the minor groove as it faces the histone octamer [7]. This is in line with the findings that proteins that bind nonspecifically with DNA often interact in the minor groove [10,11]. The histone-fold DNA binding sites are of two types. The α1α1 type formed from α–helical regions and L1L2 types formed from β-loops. There is a 2–3 fold difference in the crystallographic B-factors for phosphates that are bound to the histones and those facing the solvent, reflecting their enormous difference in mobility. In addition, the bending exhibited in the minor groove is facilitated by the insertion of an arginine residue into the minor groove at all 14 sites in contact with the histone octamer [7,9,12].

There is a delicate balance in the DNA-histone interactions, in which changes of just a few residues can alter the stability of the nucleosome significantly [13]. It is estimated that the energetics at the 14 DNA-histone interactions are in the range of about 12–14 kcal/mol, which is the energetic debt that must be expended to break the major contacts (~1 kcal/contact) [14]. The detailed structure also reveals an enormous number of waters and ions (>3000 waters & 18 ions) that help to stabilize the structure, including a "spine of hydration" in the minor groove that may be important when considering HMGB1 interactions [15].

In addition, about 25% of the mass of the histone proteins is made up of evolutionarily conserved and highly charged tails (N-terminal tails of all histones and the C-terminal tail for H2A) that extend outside the nucleosome and are unresolved in the X-ray structure [7]. Being much more exposed to the solvent, specific tail residues are the target of a variety of enzymatic posttranslational modifications, which includes adding or removing groups (writing or erasing) by acetylation, methylation, sumoylation, ubiquitination and phosphorylation. The histone code hypothesis suggests that a collection of these post-translational modifications are "sign-post" to be "read" by coregulators to negotiate their interactions and thereby function to direct specific transcriptional programs. These modifications can change specific residues in the histone tail domains to become more hydrophobic or alter their electrostatic potential. These temporally regulated changes can exert a major impact on the histone interactions with DNA that may alter nucleosome structure and contribute to changing the nucleosome energy landscape, including perhaps the compaction of nucleosomes into higher-order chromatin structure [16-21]. Of particular note is the role of histone acetyltransferase (HATs) and histone deacetylases (HDACs) that act on lysine residues in these tail domains. Unacetylated lysine residues can interact strongly with the DNA, while acetylation frees up these electrostatic constraints,

acts to recruit coactivators and facilitates transcription factors (TFs) a greater access to nucleosomal DNA. For example, acetylation of H3K9, in addition to other modifications, has been generally correlated with transcriptionally active chromatin regions [22-24]. It was shown that different CRCs recruited to these sites can act on the different substrates and produce different nucleosome outcomes [25]. Moreover, variant histones, such as H3.3, H2A.Z and H2ABbd can also substitute in the nucleosome and mediate nucleosome stability and fine-tune its functional state [26-28]. Together, these core histone modifications and/or substitutions yield a collection of similar nucleosomes with different compositions and intramolecular forces and support the notion of an enormously diverse population of nucleosome states, which will influence, both qualitatively and quantitatively, the character of their interactions and serve as recruitment platforms for additional coregulators.

Crystallographic studies have added enormously to our picture of the intramolecular interactions within the nucleosome and its overall structure. However, as previously pointed out, the structure of the isolated (canonical) nucleosome must be viewed as just one particular conformer—the one that preferentially crystallizes out of solution under the experimental conditions [29]. Certainly additional forms of nucleosomes with alternate structural features that do not permit them to crystalize may be more prevalent in solution and more closely resemble the biologically relevant conformation *in vivo*.

2. Activation of transcription: gaining access to and binding to nucleosomal DNA target sites

The conventional model for transcriptional activation proposes that a regulatory or activator protein initiates the process by binding to its DNA response element embedded in the nucleosome or in a “nucleosome-free” region. This activator/DNA complex then provides the initial “target” to recruit a variety of factors that, as noted above, enzymatically modify the core histones by posttranslational modification which then provide the specific platforms to further recruit ATP-dependent CRCs and coregulators. In all cases, one subunit in the heterogeneous multisubunit CRCs exhibits an essential ATPase functionality, with the ATP hydrolysis providing the energy for a variety of remodeling activities that can include changing the structure or composition of the nucleosome or moving the histone octamer to a different DNA sequence. In the human CRCs, the catalytic ATPase resides in either the BRG-1 or hBrm protein, which is in association with about 10–12 BRG1/Brm-associated factors (BAFs) [30]. Although the timing of the recruitment steps will most likely vary depending on the context of the cell, this activator/DNA complex is then in a position to further recruit coactivator complexes, including the essential Mediator complex, which provides a physical linkage to RNA polymerase II and the preinitiation complex (PIC) at the proximal promoter to initiate transcription by RNA pol II. Mediator interacts with a multitude of diverse gene-specific activators and as a result, exhibits an intrinsic heterogeneity in its composition and conformational flexibility to efficiently accommodate a particular communication network [31-33].

A major challenge in understanding the nature of transcriptional activation and initiation is to clarify how the structural architectures of the TF, nucleosomal DNA and the nucleosome, and the variety of functionally different coregulators interact with, adjust to, or restructure in large nucleoprotein assemblies during the early steps in transcription. Notwithstanding the conventional model for transcriptional activation outlined above, one of the major questions that remains unresolved concerns the mechanism(s) by which an activator or regulatory protein gains access to and binds to its response element (RE) to initiate the subsequent events in the transcriptional

program. The importance of a mechanism to gain access is highlighted by genome-wide location analysis that showed that, for all transcription factors examined, a very small percentage of consensus sites is occupied, supporting the notion that the majority are inaccessible [34]. One also finds that the binding of transcription factors to their cognate sites is markedly reduced in nucleosomal DNA compared to binding in DNA (Table 1 below). Consequently, there is currently no general consensus as to how the activator can gain access to the obstructed or inaccessible RE. Although pioneer proteins can decondense or "open up" chromatin to make specific regions more accessible, it still remains that many activators cannot bind to their RE within the nucleosome.

A critical and underlying aspect to gaining access to the RE, by any and all of a variety of routes, is a mechanism to loosen or release the DNA from the grip of its interactions within the nucleosome. In the case of an isolated mononucleosome, the binding affinity will depend primarily on two factors. First, the sequences in the helical nucleosomal DNA exhibit different levels of accessibility, primarily dependent on the translational position and the (helical) rotational phasing of the DNA sequence within the nucleosome. The nucleosome is defined to be translationally positioned if the nucleosome occupies a fixed position relative to the underlying DNA sequence. The DNA is rotationally phased when an orientation of the DNA sequence within the helix is fixed relative to the histone octamer surface. The rotational orientation of the sequences in the RE, in terms of facing inward or outward from the core histones, strongly influences TF accessibility. When the major groove of the response element is oriented toward the core histones, it is effectively inaccessible. On the other hand, one would imagine that if the major groove faces outward, it would be much more accessible, if not in the optimum orientation for strong binding. Secondly, the activator proteins have different binding requirements. The extent to which the TF binds its RE within nucleosomal DNA, as opposed to naked DNA, will also be strongly influenced by whether the protein-DNA interactions require the TF to interact solely with the exposed surface within the RE or involve more extensive interactions with the DNA, such as wrapping around the DNA, a requirement for additional nucleotides outside the RE or the help of additional factors. In addition, TF binding bends the DNA sequence in a specific direction and so the ability to form a strong interaction within the nucleosome may also be tempered by the level of bending constraints on the local DNA by the core histone octamer [35,36].

The binding of a number of TFs to nucleosomal DNA, as compared to naked DNA, shows enormous variability in their ability to gain access and bind to their cognate sites in nucleosomal DNA. Table 1 shows this differential binding affinity, in which HSF, NF1, TBP and Max dimers effectively do not bind their RE in nucleosomal DNA, while virtually all other TFs exhibit a range of reduced affinities when their RE is incorporated within a nucleosome. These findings underscore the extent to which TFs experience the different levels of energetic and bending constraints on DNA within the grasp of the nucleosome.

As will be discussed below, HMGB1 binds in the minor groove of DNA. As a result, it is of interest that pyrrole-imidazole (py-im) polyamides were shown to be fully accessible to and bind with high affinity (nM range) and in a sequence-specific manner to sites in the minor groove of DNA that are facing away or partially facing away from the histone octamer. The crystal structure showed that although the stable binding interaction introduced modest distortion in the nucleosomal DNA at the interaction sites, there was no evidence for significant alteration in the DNA-protein interactions. The stretching and widening of the minor groove by poly (py-im), as observed in the crystal structure was, however, not detected in solution by DNase I digestion. As with the canonical nucleosome

structure, the crystallographic B-values were low for phosphates in direct contact with the main chain of the histone proteins and higher for those exposed to the solvent [50,51].

Table 1. Preferential binding affinity of transcription factors to DNA compared to nucleosomal DNA.

Factor	Ref.	(DNA/N)
HSF	[37]	does not bind N
NF1	[38,39]	does not bind MMTV in N
TBP	[40]	~10^{+4}
Max homodimers	[41]	~10^{+4}
NF-kB-1	[42]	~50
USF	[42]	~30
Sp1	[43]	~10–20
LEF-1	[42]	~8–10
TRF1	[44]	~6
PR	[45]	~6
Fos/Jun (AP-1)	[46]	~4–5
NF-kB	[47]	~4 (bound at edge of nucleosome; not accessible at response element at dyad)
Amt1	[48]	~3
GR	[49]	~2–4

In the case of nucleosomes within chromatin, TF binding becomes even more problematical. A number of model strategies have been proposed to address this additional level of regulation. One model proposes that some TFs associate with and bring in a remodeling complex, in both yeast and mammalian cells, not only to activate transcription, but also to initially gain access to its target promoter [52]. In another model, the SWI/SNF complex is proposed to introduce transient changes randomly throughout chromatin, but only in the presence of a TF does a targeted remodeling occur. There is evidence that the BRG1 CRC (mammalian SWI/SNF) includes a BAF57 subunit that has an HMG box domain. Although it is not essential for nucleosome disruption *in vitro,* it was suggested that this HMG box may provide additional specificity in some targeting interactions [24,53,54]. A third model proposes that there are DNA sequences that promote inherently unstable nucleosomes (AT-rich stretches) or disfavor higher-order compaction (CpG islands). These sequences result in maintaining chromatin in a state in which TFs can at least gain access transiently [55]. Yet another model suggests that TFs such as Forkhead box protein A1 (FoxA1), AP1 and Sp1, which are found to have sites in the immediate vicinity of a subset of the EREs, can cooperatively bind with ER to facilitate its binding to an energetically favorable ERE that otherwise would be inaccessible. Recent studies indicate that in the case of FoxA1 participation, there is a dynamic interchange in chromatin in that while FoxA1 can reorganize chromatin to induce both ER and glucocorticoid receptor (GR) binding, the binding of ER or GR can likewise facilitate FoxA1 binding [56-62].

In light of these models, we shall summarize the evidence suggesting that the HMGB1 protein can restructure the nucleosome to facilitate ER binding, in addition to function in estrogen-responsive gene expression. Our data on nucleosome restructuring can then be generally considered in either of two models that describe the effect of a ligand interaction on the structure or

conformation of a protein or nucleoprotein assembly. The change in the nucleosome conformation by HMGB1 interaction can be conceptually viewed as a structural change due to (1) an "induced fit" of the nucleosome by the interaction with HMGB1 or (2) an alteration of the nucleosome population in the equilibrium of an ensemble of preexisting nucleosome states. The restructured nucleosome then exhibits an accessible cERE that now provides an effective pathway to ER binding. In addition, evidence indicates that ER interacts with HMGB1 in solution (see below) and can act as a "chaperone" for HMGB1 to help facilitate binding to the ERE site. In an independent study on estrogen-responsive gene expression, HMGB1 was also shown to be an active participant in one of the steps in transcriptional activation at the pS2 promoter [63].

3. Estrogen receptor α and transcriptional activation at the estrogen-responsive pS2 promoter: Capsule profile of the current paradigm

Estrogen receptor (ERα: NR3A1) is a ligand-activated transcription factor that has a modular structure, with six structural domains (A-F), each of which has specific functions (Figure 1).

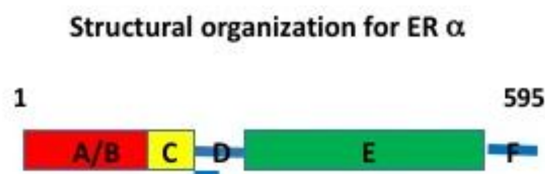

Figure 1. Domain structure for the 595 residue estrogen receptor α. The structural and functional domains include the N-terminal domain (A/B), the DNA binding domain (DBD) (C), the C-terminal extension (CTE) region of the DBD, that extends into the flexible hinge region (D), the ligand binding domain (LBD) (E), and the 42 residue C-terminal portion that makes up the F domain.

The central DNA binding domain (DBD) and the C-terminal extension (CTE) are the most relevant for our purposes, in which the essential DBD interacts directly with the cERE. ERα acts as a dimeric TF, with each subunit interacting directly in the major groove of each half-site of the 15 bp palindromic consensus ERE (cERE), AGGTCANNNTGACCT, in which N can be any nucleotide. In addition, the CTE resides immediately adjacent to the DBD and interacts with HMGB1. The CTE is followed by the flexible hinge region that extends to the E domain that contains the hydrophobic ligand binding domain (LBD), in which estrogen (E2) and a variety of agonists and antagonist can also bind. The binding of the dimeric (E2/ER) complex to the cERE within nucleosomal DNA then serves as a platform to recruit coactivators to remodel chromatin and provide the communication network with the basal transcriptional machinery at the promoter [64-67].

Although a vast collection of work has focused on the role of ERα in estrogen-responsive gene expression, detailed studies of the transcription process at the pS2 (trefoil factor-1; TFF1) promoter in MCF-7 cells (a cancerous cell line) now represent the most comprehensive model for elucidating the factors, coregulators and the multiple steps and cyclical nature associated with the activation of an estrogen-responsive gene. Only a very brief overview of specific aspects will be highlighted from a highly reviewed subject [68-70].

The anatomy of the pS2 promoter has been mapped in detail to reveal a single ERE positioned 400 bp upstream from the transcription start site (TSS). There are two adjacent rotationally phased and translationally positioned nucleosomes, one containing the ERE (NucE), while the TATAA box is within the downstream nucleosome, NucT. The ERE is precisely positioned near the 5'-edge of NucE (−405 to −392), while the TATAA box is on the 3'-edge of NucT (−30 to −24). The authors suggest that, similar to the glucocorticord receptor (GR) interaction, the rotational phasing of the ERE has the major groove (i.e., the binding site) positioned for optimum binding [69].

The transcriptional process was shown to be a cyclical progression of the assembly and disassembly of the transcriptional complex. After the addition of E2, ER binds to its ERE, followed by the recruitment of a family of p160 coactivators, CBP, p300, pCAF and PBP, with histone acetylation occurring to form a transcriptional complex. Importantly, this complex is involved in a dynamic process in which it cycles on and off the pS2 promoter to lead to the formation of a productive complex and initiation of transcription. It was also found that E2 stimulates the association of the BRG-1 containing CRC that targets the ERE and is involved in the transcriptional activation [71,72]. The collection of these studies suggests that two distinct chromatin modifying mechanisms – that involving histone acetylation/deacetylation and ATPase-dependent CRCs may be functionally linked and take part cooperatively in the control of ER-dependent transcriptional activation. Subsequently, it was reported that there are as many as 5000 genes that respond to induction by tumor necrosis factor alpha (TNF-α) that appear to exhibit these same cyclical changes in gene expression, suggesting that this cyclical nature may be more general and extend beyond estrogen-responsive gene expression [73].

In the most comprehensive works, the Gannon lab determined the sequence of events that occur on the pS2 promoter that lead to the induction of gene expression by ERα. The assays indicated that after E2 induction, there were as many as 30 different proteins associated with the pS2 promoter. The stepwise activation and deactivation showed an ordered recruitment and cyclical waves of assembly/disassembly of the many diverse multisubunit complexes. This cycling defines a "transcriptional clock" that ultimately drives the assembly of a transcriptionally productive complex, which is subsequently followed by the repressive phase. By the use of "kinetic" ChIP assays, they were able to identify the enzymes that modify the tails in the core histones, including (histone acetyltransferase (HAT), histone methyltransferase (HMT), histone-arginine methyltransferase (CARM1) or protein arginine methyltransferase (PRMT1), in addition to members of the p160 family of coactivators. These modifications then serve as targets for recruitment of human SWI/SNF that functions to remodel the target nucleosome. The stepwise process leads to a continuous change in the architecture of the nucleosome and the transcriptional complex. It was suggested that although the ERE in NucE is occluded, ER binds cERE transiently and this is sufficient to recruit SWI/SNF which then relocates the nucleosome so that the ERE is translocated outside the nucleosome proper [70,74-77]. Although these studies represent the most comprehensive work to date, the ChIP assays did not include a probe to determine if the HMGB1 protein was involved in any of the steps.

In another study, a similar "kinetic" ChIP approach was used and found that histone H1 bound to both nucleosomes (NucE & NucT) prior to and up until about 10 mins after E2 treatment. Then both type II topoisomerase β (topo IIβ) and poly(ADP-ribose) polymerase (PARP-1) were found complexed to NucE. This led to a transient DNA double-strand break which was required for E2-dependent activation. At this point, histone H1 is expelled from the complex at NucE and was

replaced by HMGB1 binding. Although previous studies have shown that HMGB1 binds to many TFs and activates transcription in many cases (see below), this is the first and clearest demonstration of an active role for HMGB1 at the ERE, in a specific step in estrogen-responsive gene expression [63].

4. HMGB1 (also referred to as amphoterin or melanoma-associated factor): its role in nucleosome dynamics and transcriptional activation of estrogen-responsive gene expression

HMGB1 appears to be a protein "for all seasons" or a "jack-of-all-trades" due to its dual "personality" as evidenced by its role in a vast number of both cellular and extracellular activities. Within the cell, it has been reported to have roles in DNA replication, DNA repair, transcription and V(D)J recombination. Unexpectedly, it was also found to be a (extracellular) cytokine that serves as a proinflammatory mediator. So collectively, it is not surprising that it has been associated with a multitude of normal health issues and disease states, such as cancer, sepsis and others [63,78-86].

HMGB1 is a small protein (ca 25 kDa) found primarily in the nucleus. It is highly conserved, ubiquitous and binds nonspecifically in the minor groove of DNA. It is a very abundant protein (ca. 1,000,000 copies/cell), with the estimation that there is about 1 HMGB1 for each 10 nucleosomes (1 HMGB1/10N). If one assumes that a significant population of nucleosomes is embedded or condensed in much higher-order structures, this ratio can be expected to be much higher. Since HMGB1 has been reported to be one of the most kinetically mobile proteins in the cell, it is not unexpected that it is found to bind transiently throughout the available chromatin [85,87].

From both structural and functional standpoints, HMGB1 can be considered a modular protein, with three domains (Figure 2). It contains two HMG boxes, the tandem N-terminal domain and the central domain (referred to as A- and B-boxes, respectively), which both contain about 80 residues that are highly basic in nature and bind to DNA. The boxes exhibit different binding preferences, but bind to distorted DNA (kinked, cruciform or underwound DNA) and have been shown to bend DNA as much as 90 degrees [88,89].

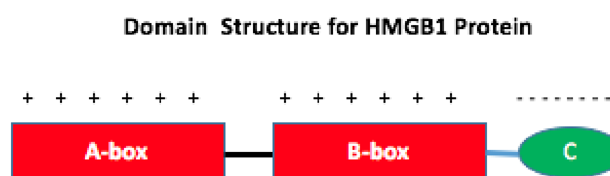

Figure 2. Structural and functional domains of the 215 residue HMGB1 protein. The tripartite structure includes the basic A- and B-boxes separated by a 10 residue spacer, with about a 20 residue spacer between the B-box and the acidic C-terminal domain, which is made up entirely of acidic (Asp/Glu) residues.

The transient nature of the HMGB1 interaction with DNA has been shown to produce a dynamic flexibility or flexure in the DNA which can reduce the binding energy for a TF by fashioning a spectrum of bends, some of which are more favorable to successful TF binding. This flexure permits the DNA to accommodate a broader and more diverse spectrum of nucleoprotein assemblies and is the reason HMGB1 is considered an "architectural" protein [88,90-92]. This has been considered a prominent mechanism by which HMGB1 facilitates or enhances the binding of

both (1) general or basal TFs bound to DNA in the PIC and (2) regulatory TFs to their cognate binding sites in DNA. The list of "HMGB1-sensitive TFs" includes the human TATA binding protein (TBP), p53, p73, HoxD9, Oct family of proteins, nuclear factor kappa beta (NF-κB), sterol regulatory element-binding protein, c-Rel proteins, hepatocyte nuclear factor-1α (HNF1α), ZEBRA and the steroid hormone receptors. The enhanced binding of some of these TFs has also been correlated with the activation of transcription [93-105].

In addition, the acidic C-terminal domain of HMGB1 contains a continuous stretch of 30 negatively charged residues that modulates its interaction with DNA by interacting with both the constituents proteins in the PIC and the core histones, which suggests that these interactions may play a role in transcription. While the C-terminal segment of human TBP binds directly to the TATA element in DNA, the acidic tail of HMGB1 interacts with the Q-tract in the nonconserved N-terminal part of TBP, which has no role in binding the TATA box. However, this interaction enhances TBP/TATA binding affinity 20-fold and influences the binding of TFIIB and TFIIA. This interaction could provide an additional avenue for an unusually high local concentration of HMGB1 at or near the pS2 promoter. *In vitro* and *in vivo* evidence also implicates the interaction of the C-terminal domain of HMGB1 with the N-terminal tail of histone H3. It was suggested that this may localize HMGB1 near the nucleosome dyad to interact and bend the DNA, which could destabilize the nucleosome and make it more accessible. Another study finds that the DDDDE sequence in the HMGB1 acidic tail also interacts with the N-terminal tail of histone H3 and this interaction is essential for transcriptional stimulation. These studies support the contention that deletion of the H3 tail or altering its character plays an important role in nucleosome structure, in that, it destabilizes the H2A/H2B dimer within the nucleosome and alters both histone-DNA contacts within the nucleosome core and nucleosome stability [93,106-110].

The *drosophila melanogaster* CRC, ACF/CHRAC (ATP-utilizing chromatin remodeling factor/chromatin remodeling and assembly complex) contains the ATPase ISWI (imitation SWI) that can catalytically slide the histone octamer on DNA. HMGB1 promotes ACF binding to the nucleosome and accelerates the mobility of the sliding process. Interestingly, the HMGB1 mutant lacking the C-terminal domain binds the nucleosome with such high affinity that it strongly inhibits nucleosome sliding [91].

Furthermore, HMGB1 was also found to bind to the CTE in ERα which provides a mechanism for ER to "chaperone" HMGB1 to the ERE. By this avenue, ERα could recruit an unusually high level of HMGB1 to the vicinity of any ERE [66] and this may be important for the influence of HMGB1 in aspects of transcription at the pS2 promoter. It was also shown that the mammalian SWI/SNF complex (BRG-1), that is required for activation by ER, contains a subunit, BAF57, which contains an HMG box and is recruited to the pS2 promoter in a ligand-dependent manner. It has been suggested that this may be a mechanism for the recruitment of the SWI/SNF complex to estrogen-responsive gene promoters [111].

5. Evidence for HMGB1 restructuring nucleosomes, facilitating ER binding in nucleosomal DNA and transcription

As noted above, HMGB1 has been shown to increase the binding affinity for a number of transcription factors to their cognate sites on DNA, including ER binding to its cERE. The binding affinity (Kd) of ER to cERE was 10 nM, while in the presence of 400 nM HMGB1, the affinity was

increased two-fold to 4 nM. The complete cERE was required for binding since ER did not bind to an ERE half-site (hcERE). In the absence of HMGB1, ER did not bind to an ERE half-site (hcERE). As a result, it was unanticipated when we found that in the presence of HMGB1, ER bound strongly to hcERE, with a binding affinity comparable to that for ER binding to cERE in the absence of HMGB1 [103-105].

Furthermore, ER bound strongly to a spectrum of nonconcensus (ncEREs) sites (direct repeats, everted repeats and inverted repeats having different spacers). Following up on this, *in vivo* luciferase reporter assays in human osteosarcoma (U2OS) cells were used to show that ER interactions with these ncEREs drove transcription and the level of transcription was enhanced by the presence of HMGB1. Moreover, using siRNA technology, endogenous HMGB1 gene expression was knocked down (KD), sharply decreasing HMGB1 mRNA and decreasing HMGB1 proteins levels by 30%, which resulted in the luciferase activity dropping by over 80% [106]. Collectively, and in addition to the findings that HMGB1 binds in the pS2 promoter during the early stages of transcription [63], these collective findings provide evidence that HMGB1 plays a significant role in the regulation of this model estrogen-responsive system, in addition to estrogen-mediated gene expression at the pS2 promoter in MCF-7 cells.

These studies led directly into investigating whether the effect observed for HMGB1 on transcription could be directly linked to an alteration in the structure of the nucleosome and, if so, would this have an influence on ER/cERE binding. Nucleosomes were prepared with the 161 bp DNA designed with four nucleosome positioning sequences (Figure 3) so that the cERE was translationally positioned at the dyad axis and the DNA helix rotationally phased so that the major groove for the dimeric ER interaction was directed outward from the histone octamer for optimum accessibility. However, even under these conditions that should be optimal for binding, ER did not bind (Kd ~300 nM) the cERE in the canonical nucleosome [112]. On the other hand, the presence of 400 nM HMGB1 facilitated strong, sequence-specific ER binding to cERE, with a Kd value of 52 nM. This ER/cERE binding in nucleosomal DNA is ~6 times stronger than in the absence of HMGB1 and 5 times weaker than its binding affinity to free DNA (Kd = 10 nM). This also showed that the effect of HMGB1 on ER binding to nucleosomal DNA (6x) is much greater than its effect on binding to DNA (2x).

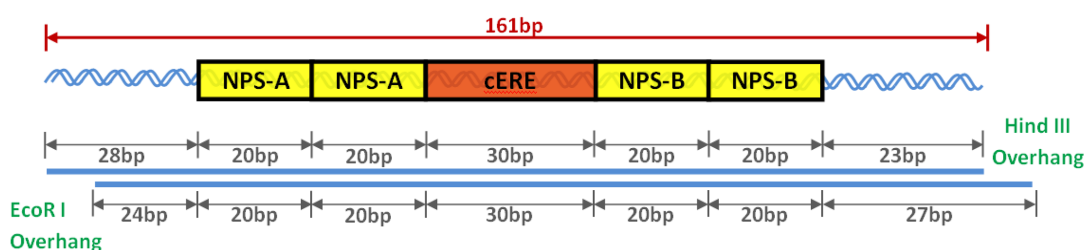

Figure 3. A schematic drawing of the 161 bp DNA containing four nucleosome positioning sequences (yellow boxes), that straddle the 30 bp consensus estrogen response element (cERE- red box) at the dyad axis. The 161 bp DNA was excised by EcoRI and HindIII digestion of the pGEM-Q2-2E2 plasmid [105].

Electrophoretic mobility shift analysis (EMSA) showed that as the HMGB1 level was increased from 400 to 800 to 1600 nM, the initial band for the canonical nucleosome, N, (with no HMGB1) progressively became an increasingly broader band, suggesting that additional nucleosome states may have developed as a result of the increased level of HMGB1. The reaction with 1600 nM

HMGB1 was then further investigated, with the products fractionated by sucrose gradient to determine the nature of the nucleosome population. This nucleosome fraction exhibited the identical sedimentation profile as the canonical nucleosome (N), but the EMSA revealed two distinctly different forms of the nucleosome (N’ and N”) with the mobilities reduced compared to that for N. Supershift experiments showed that these nucleosomes had the same histone composition as the canonical nucleosome, but the lack of a supershift for HMGB1 indicated that HMGB1 was not a stable component of the nucleosome. This “hit-and-run” mechanism for HMGB1 has been observed previously and is consistent with its transient interactions with DNA. Atomic force microscopy further showed that the restructured nucleosomes were mononucleosomes, with no evidence for dinucleosomes, which were produced by the remodeling action of SWI/SNF [113,114]. It is important to note that the HMGB1-restructured nucleosome population (N’ and N”) is stable for months at −20 °C, which readily permits more extensive characterization.

The concentration of HMGB1 in the nucleosome fraction was determined to be 25 nM, which was about twice the concentration of nucleosomes (10 nM). Using EMSA, the Kd for the binding affinity for ER with the N’/N” nucleosomes ([HMGB1] now at 12 nM) was ~30 nM, which was reduced from the 52 nM Kd value determined for the unfractionated reaction at 400 nM HMGB1 and only 3 times weaker than ER binding to cERE in DNA.

Canonical nucleosomes exhibit a characteristic 10 bp cutting pattern when nucleosomal DNA is digested with DNase I. The DNase I produces an endonucleolytic cleavage in the minor groove of the DNA that represents the repeating character of the DNA backbone exposed on the surface of the nucleosome. The digestion pattern observed for the N’/N” was very similar to that for the canonical nucleosome, with the addition of six modestly intense bands. This suggests that there were no major alterations of the internal DNA interactions with the histone octamer. Furthermore, digestion by Exo III provided a test to determine if the nucleosomal DNA had been translocated out from the histone octamer by the HMGB1 interaction. The digestion pattern was found to be identical to that for the canonical nucleosome and indicated that the HMGB1 interaction, which is ATP-independent, produced no translocation of the DNA and that the HMGB1/nucleosome interaction did not interfere with the cutting activity of Exo III on the DNA ends.

The stability of the N’/N” nucleosome population was examined after individual challenges by heat, increased NaCl and DNA levels. Although the canonical nucleosomes (N) were unchanged after hours, and even overnight incubation at 37 °C, the population of the N” state was destabilized after 60 min at 37 °C and converted into the N’ state (N” + N’ -- > N’), which remained stable after overnight incubation and did not revert to the N state. The relative thermal stability of the three nucleosome states is then N ~ N’ > N”, with the N’ restructured state having a comparable thermal stability to that for the canonical nucleosome. Increasing the NaCl concentration partially converted the N” state to N’/N states at about 25-50 mM NaCl (N” + N’ -- > N” + N’ + N) and then at ~200 nM NaCl, the remaining N”/N’ states were converted into the N state (N” + N’ + N -- > N). This supports the notion that there is a differential stability in the three nucleosome states, suggesting that electrostatic interactions in the restructured nucleosomes have been compromised by the increasing NaCl concentration, while the canonical nucleosome was unaffected. The addition of increasing levels of DNA similarly destabilized the N” state to N’ and N, which were equally stable until the highest DNA, at which point only the N state remained. The presence of DNA destabilizes the N” and N’ states by acting like an “HMGB1 sponge” as the DNA preferentially sequesters the HMGB1 and inhibits its interaction with the nucleosomes (N” + N’ -- > [N” + N” + N] -- > N),

similar to the effect of DNA competition studies reported previously [94]. It should be emphasized that at intermediate NaCl and DNA levels, the three states of the nucleosomes exist simultaneously in equilibrium. In addition, the effect of HMGB1 was unaffected by the presence of ATP, consistent with the lack of a recognized ATPase activity and a non-catalytic mode of action.

Since it was reported that the core histone tails influence TF binding to nucleosomal DNA, tailless nucleosomes (Ntl) were prepared in the absence of HMGB1 and their influence on ER binding was determined. Reaction of ER with the Ntl yielded a value for Kd ~45 nM, a binding affinity slightly stronger than that for ER binding in 400 nM HMGB1, but a weaker affinity than ER binding to the fractionated N'/N''. The collective data provide the initial evidence for a role of HMGB1 in 1) restructuring the nucleosome, 2) facilitating the binding of ER to cERE, in addition to showing the inhibitory role that the histone tails exert on the binding of ER to nucleosomal DNA.

Lastly, to determine whether the HMGB1 effect on ER binding was influenced by its position in the nucleosome, nucleosomes were prepared as described above, but with the cERE translationally positioned at either −20 or −40 bps from the nucleosome dyad. The ER binding affinity was the same at all sites, showing that ER binding affinity was independent of the cERE location in the nucleosome [115].

A summary of the Kd values for the different nucleosomes under the various conditions is shown in Table 2.

Table 2. Binding affinity of ER to DNA and nucleosomal DNA states.

ER Substrate	Kd (nM)
Canonical N	~300
N (w 400 nM HMGB1)	52
Ntl	45
Ntl (w 400 nM HMGB1)	30
N'/N'' (1600 nM HMGB1 followed by sed. to 12 nM)	30
DNA	10

6. Discussion

6.1. HMGB1 is an important factor in transcriptional activation for ERa at the pS2 promoter in the MCF-7 breast cancer cell line

Increasing evidence shows multiple roles for HMGB1 in transcription and especially transcription by ERα on estrogen-responsive genes [63,94,95,97-101,105,106,109]. It has been shown that at one point in the transcriptional activation at the pS2 promoter, HMGB1 competes successfully with prebound histone H1 and binds to NucE, in concert with the dissociation of histone H1. One of the clear differences between histone H1 and HMGB1 is that H1 promotes and stabilizes higher-order chromatin or compaction, which limits access and inhibits transcription, while HMGB1 is found in more open chromatin structures, can increase accessibility and facilitates transcriptional initiation [116]. So this finding is in line with the notion that the highly mobile histone H1 binding on the NucE contributes to transcriptional inhibition and that HMGB1 binding appeared to temporally stabilize a more active nucleosome state to facilitate subsequent steps in transcriptional activation. Recent

evidence shows that H1 binding on nucleosomes inhibits transcriptional activity and specifically in the case for ERα–mediated transcription initiation [117]. Although HMGB1 is one of the most mobile proteins in the cell, the findings at the pS2 promoter [63] differ from previous findings in which HMGB1 acts by a transient “hit-and-run” mechanism when examined in its interactions with only single factors—DNA or transcription factors. Within the cell, where HMGB1 may make multiple interactions within multi-protein complexes, and uses two or more of its three domains (A, B and/or C), the multiple constraints with other factors presumably increases its residence time on the nucleosome and permits detection by ChIP assays. As pointed out above, the A- and/or B-boxes are expected to interact with nucleosomal DNA, while the C-terminal domain has multiple targets that it may bind to. Within the nucleosome, it may bind to the histone tail domain to undo some of the electrostatic constraints between DNA and the histone tails, especially the tail of histone H3. In addition, the C-terminal domain may bind to the N-terminal segment of TBP [93] since it is positioned on NucT in the vicinity [63]. The detailed studies by the Gannon lab revealed many factors in the cyclical process during transcription at the pS2 promoter [70-75], but did not probe for the presence of HMGB1. As noted above, it would be of interest to revisit this stepwise, cyclical process, define the temporal presence of HMGB1 and integrate the results with those previously reported that identified HMGB1 binding at the pS2 promoter [63].

6.2. HMGB1 action restructures the nucleosomes (N’, N”) and facilitates strong ER binding: Characteristics of the novel nucleosome states

In an ATP-independent manner, HMGB1 alters the structure of the nucleosome from what can be considered the canonical state (N) to two alternate states of the nucleosome (N’/N”) which have the same sedimentation characteristics as N, but clearly have different electrophoretic mobilities and a few, but distinct additional bands observed in the DNase I cutting profile. This restructuring leads to a change in the character of the nucleosomal DNA, and in particular, changing an inaccessible cERE to one in which ER binds strongly. This appears to be an HMGB1 concentration-dependent perturbation. Although the presence of 1600 nM HMGB1 produces a distribution of new states, with the predominant formation of N’ and N”, sedimentation through a sucrose gradient indicates that after formation of the N’ and N” states, they remain stable and can be sustained in HMGB1 levels as low as 12 nM HMGB1 for extended periods of time (months at −20 °C in TE/sucrose gradient buffer).

6.2.1. The core histone tail domains

The influence of the histone tails on ER binding was enormous. Without the presence of HMGB1, the Kd values dropped from the inaccessible and effectively nonbinding Kd ~300 nM in the canonical (N) nucleosome to 45 nM without the histone tail domains (Ntl). While this binding affinity is in the same range as that found from the action of 400 nM HMGB1 on the canonical nucleosome, it cannot account completely for the stronger ER/cERE binding in the isolated N’/N” (Kd = 30 nM). This, and the moderate change in the DNase I profile of the N’/N”, suggests that although the interaction of the histone tails with the DNA strongly impedes ER binding, the interaction between the DNA and histones within the nucleosome core must also be weakened by HMGB1 interaction.

6.2.2. Location of the cERE in nucleosomal DNA

We have also established that the position of cERE within the nucleosome did not influence the effect of HMGB1 on the binding affinity of ER. With the cERE in each case rotationally phased outward from the octamer and positioned at the dyad axis and at −20 and −40 bps from the dyad axis, the ER binding affinity was the same at the three locations. This shows that the HMGB1 effect is independent of the cERE position in the nucleosome and suggests that the HMGB1 effect is not a localized restructuring, but a change in the global nature of the nucleosome.

6.2.3. ER/cERE binding: The HMGB1 effect differs from the "site exposure" model

The outcome of HMGB1 interaction with nucleosomes and the comparable binding affinity of ER at three different cERE positions on nucleosomal DNA is inconsistent with that expected for the uncatalyzed "site exposure" model for factor binding on nucleosomal DNA [129,130]. This model postulates that the ends of the DNA are in a rapid dynamic equilibrium between being bound to the histone octamer (fully wrapped) or partially unwrapped and the extent to which a site is exposed depends on its distance from the DNA end. In this model, sites that are closer to the ends would be less costly to dissociate from the octamer and therefore have a greater opportunity to become unwrapped than those at the dyad and as a result, would exhibit a greater binding affinity. In contrast to this model, our findings suggest that the HMGB1 interactions that produce the restructured nucleosomes do not produce a weakening of just localized DNA-histone constraints that are more pronounced near the ends of the nucleosomal DNA. We find that HMGB1 facilitates a global instability by disrupting, in an uncatalyzed manner, the constraining forces in and about the nucleosome in general.

6.3. The ABCs of HMGB1 interactions on nucleosomes

6.3.1. The basic A- and B-boxes of HMGB1

We propose that HMGB1 interacts transiently and continually with the nucleosome. It binds globally to restructure the nucleosome by altering both the nucleosomal DNA and the core histones and involves two, if not all, of its structural and functional domains. Specifically, the positively charged A- and/or B-box of HMGB1 will interact transiently in the minor groove of the DNA to reduce the rigidity of the DNA by creating an extraordinary bending activity that increases the flexure of the DNA and reduces or weakens the grip of the DNA-histone protein contacts [118]. The principle binding constraints released on nucleosomal DNA are at the positions in the minor groove where the L1L2 loops and the α1α1 helical structures contact the phosphate backbone of the DNA. In addition, the transient HMGB1 interactions will disrupt indirect DNA-protein interactions that occur through the mediation of bound waters and ions that help to accommodate DNA conformational variation, resulting in further destabilization of the canonical nucleosome structure [15]. These HMGB1 interactions will have similar effects to that proposed for the ATP-independent FACT complex on nucleosomes [4].

6.3.2. The acidic C-terminal domain in HMGB1

The C-terminal domain is a stretch of 30 negatively charged aspartic or glutamic acid residues that will interact strongly with positively charged residues in the tails of the core histones. Since these residues are believed to interact with the negatively charged backbone of DNA, the HMGB1 interaction will reduce their electrostatic potential and diminish their residence times in contact with the DNA and widen the window of opportunity for ER interaction at cERE. This is consistent with the finding that the removal of the histone tails dramatically increases the binding affinity of ER to cERE. In addition, acetylation of histone residues eliminates their positive charge and has been shown to increase the accessibility of a wide variety of TFs to their REs [119-123]. This is exclusively a “histone tail-effect” since acetylation does not change the characteristic DNase I 10 bp pattern for the nucleosome. There is also accumulating evidence for an interaction between the C-terminal domain of HMGB1 and the H3 tails that may disrupt H3 tail-DNA contacts and there are proposals on how this may impact their role in aspects of transcription [107-109,124,125].

6.4. Additional factors influencing ER binding to nucleosomal DNA

6.4.1. The constrained bending orientation of the cERE in nucleosomal DNA is reduced by HMGB1

The binding of the ER DBD to ERE was determined to produce a bend of 34° [126]. Furthermore, it was shown that, in MCF-7 human breast cancer cell extract, full-length ER binding to the ERE induces a bend of 54°, with the ER interaction bending the ERE in DNA toward the major groove, which is toward ER [127]. Within the nucleosome, although our studies have the cERE facing the solvent and in the optimum orientation for maximum binding affinity, this bending orientation, constrained by interactions with the histone octamer, is just the extreme opposite to the natural bending direction for ER-ERE found in DNA. This constraint on the bend can provide a significant increase in the binding energy and impede successful ER binding, which can help to explain why ER cannot bind the canonical nucleosome. The transient interactions of HMGB1 within the minor groove of nucleosomal DNA induces a global flexure in the DNA, which conceivably releases a level of the DNA binding constraints. This would provide a greater “residence time” for a more favorable, and therefore more accessible DNA bending alignment. As a result, this provides ER a greater opportunity for its residues in the ER DBD to gain a far better alignment with the base pairs in the major groove of the ERE, reducing the binding energy, which rewards ER with a stronger binding interaction.

In support of this proposal, the binding affinity of p53, like many TFs, can be modulated by its orientation within the nucleosome. Interestingly, p53 is found to bind with a greater affinity in nucleosomal DNA than in free DNA. In the nucleosome, the p53 response element is facing toward the solvent, in addition to being “prebent” by interaction with the histone octamer in the same bending direction as found in the X-ray structure in the p53-DNA co-crystals. If this bend is in the opposite direction, the p53 binding affinity is strongly reduced and the p53-RE is effectively inaccessible [128].

6.4.2. HMGB1 binding to the C-terminal extension (CTE) of ERα

Notwithstanding the role of HMGB1 in restructuring the nucleosome to facilitate ER binding, the question remains as to how HMGB1 can facilitate this local event at the cERE to improve the targeting of ER to the cERE. HMGB1 is one of the most abundant proteins and can be regarded as continually sampling nucleosomes through out the nucleus. In this regard, the situation may be similar to the proposal that suggests that CRCs are continuously sampling nucleosomes to introduce transient changes randomly throughout chromatin, but only in the presence of a TF does the targeted remodeling occur [24,52-54]. However, in addition to a random sampling by HMGB1, HMGB1 binds to ERα through the CTE which is immediately adjacent to the ER DBD [102]. In this way, the local HMGB1 concentration can be effectively increased and the interaction of both ERα and HMGB1 at the cERE may be effectively a concerted manner of targeting to the cERE.

6.5. Alternate models for HMGB1 interaction with and "restructuring" of the nucleosome

6.5.1. Induced fit (IF) model: HMGB1 binding induces a change in the conformation of the nucleosome

This long-held model, which was directed initially at enzyme specificity, maybe perhaps the most obvious theoretic model to explain the effect of the HMGB1 interaction in converting the canonical nucleosome population to a new and different population of nucleosomes. The IF model proposes that the protein, enzyme, or nucleosome in this case, exists in a single, stable conformational state on the energy landscape and its plasticity to change to one or more alternate states is effectively energetically restricted. A new conformational state, however, can be induced or driven by the selective binding of a specific ligand at the target site [131]. Certainly, most of the data appear consistent with this model. However, points of concern that are assumed in this model include that (1) there is a singular, stable conformational state for the unbound (constrained) nucleosome; (2) the ligand (HMGB1) targets a specific site; (3) the change is local at the point of ligand binding and this change leads to a single different conformation. In contrast, our data suggest that (1) there is a global change in the conformation and not just one highly localized change; (2) HMGB1 interacts nonspecifically with both the minor groove of the DNA and the N-terminal ends of the core histones; and (3) HMGB1 binding leads to a new population of (at least two) conformers, not one. Since our findings are incompatible with the premise of the IF model, it cannot represent the primary framework to explain the HMGB1-nucleosme interaction.

Furthermore, increasing data suggest that large biomolecules or assemblies often do not exist in a single conformation, but preexist in an equilibrium of multiple states. With this premise in mind, we consider an alternate model as the primary model that appears more in line with our findings [132,133].

6.5.2. Conformational selection (CS) or population shift (PS) model: HMGB1 interaction resets the equilibrium in the ensemble of nucleosome states that alters the population of states on the free energy landscape

In this model, the nucleosome does not present as a singular energetic conformational state. The premise is that the nucleosome preexists as a collection of nearly isoenergetic conformational

isomers or states on the energy landscape. This ensemble of states is in an equilibrium that can be continuously sampled in conformational space by a ligand. The ligand binds preferentially to one or more favorable conformations (conformational selection), stabilizes it (them) and drives the selection of this conformation or subset of conformations. In this ligand-driven CS or PS model, the interaction of the ligand (HMGB1) resets a new population of conformations in the ensemble, which shifts the equilibrium of the ensemble of states so that a different nucleosome state now occupies the new free-energy minimum on the energy landscape. The ligand interaction may lead to a favorable (active) or unfavorable (inactive or inhibitory) conformation for subsequent reaction, depending on the character of the ligand. The data show that interaction of HMGB1 with the nucleosome population leads to the population shift to the newly "restructured" nucleosomes referred to as N' and N". Challenges or stress on these nucleosome states can occur by increasing levels of heat, NaCl or DNA. As a result of the latter two challenges, all three conformational states are detectable by EMSA and exist simultaneously in a delicate dynamic equilibrium. The original application of this model was directed to small ligands binding to proteins. However, it appears valid to extend this model to the effect of HMGB1 on the (detectable) ensemble of at least three different nucleosome conformers.

Figure 4A is a limited representation of two hypothetical, three-dimensional energy landscapes for the equilibrium that is envisioned for the three nucleosome conformational states—the canonical state, N, and the two HMGB1 "restructured" states, N' and N". Landscape I shows that the canonical nucleosome is the most stable and the dominant conformer under the standard conditions in which the nucleosome are isolated. The two other forms are kinetically trapped at higher energy states and are at concentrations that were not detectable by EMSA. The interaction of HMGB1 preferentially targets (conformational selection) and stabilizes the N' and N" states and perturbs or drives a population shift of the three conformers in the ensemble. As a result, the energy landscape is shifted from landscape I to II, in which there is a significant increase in the population of conformers N' and N", with the concomitant decrease in conformer N. All three nucleosome conformers have been isolated and are stable under the conditions outlined earlier. The stability of the nucleosome states was challenged by increasing heat, NaCl or DNA. The latter two challenges both presented conditions in which all three conformers were present and simultaneously in equilibrium with each other. This represents a simple example of the manner in which the immediate environment can change the population of states in an ensemble. These findings foretell that in the cell, an even more complex and dynamic population of states can be anticipated and envisioned, as conditions may abruptly change as environmental and metabolic cues fluctuate in an ongoing fashion.

Figure 4B is an overview of the reactions that occurs as ER reacts with the nucleosome under different conditions. Pathway 1 indicates that ER is inhibited from binding to the canonical nucleosome. Pathway 2 shows that the presence of 400 nM HMGB1 produces a population shift so that ER binds to the "restructured" nucleosomes, which are the N'/N" states. Pathway 3 shows the route to isolating N'/N". After treatment with 1600 nM HMGB1, the nucleosomes were sedimented through a sucrose gradient. This nucleosome fraction contained only N'/N" with the level of HMGB1 at 25 nM, which corresponded to about 2 HMGB1/nucleosome. ER binds strongly to these well-defined nucleosome states.

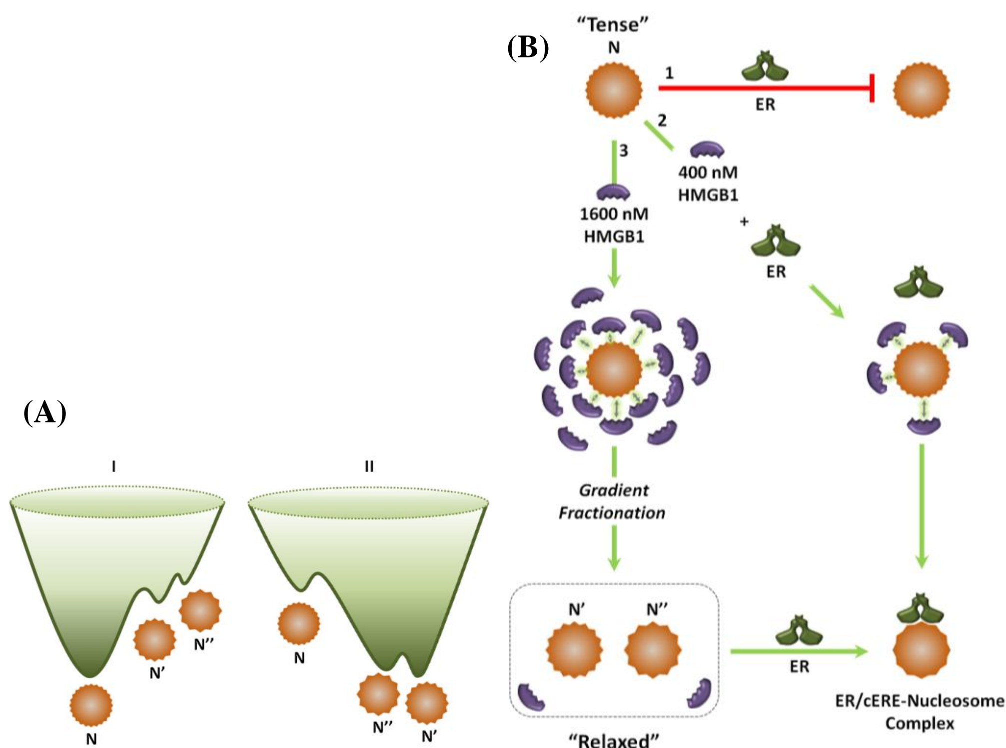

Figure 4. HMGB1 relaxes the canonical nucleosome structure and facilitates ER binding [112]. (A) Energy landscapes favoring canonical (I) or HMGB1-restructured nucleosomes (II). A hypothetical representation for the energy landscape of the canonical nucleosome, N, and the HMGB1-restructured nucleosomes, 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 preferentially interacts with a subset of the nucleosome population, 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 reset the population of 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 heat, NaCl or DNA. **(B)** Model for the interaction of the nucleosome with HMGB1 and ER. The canonical nucleosome (N) represents a 'tense' and relatively inaccessible conformational isomer (pathway 1) and so ER binding is inhibited from binding 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 double-headed arrows (< -- >), the intranucleosomal constraints are relaxed, which facilitates ER binding. ER binds to the nucleosome to form the ER/cERE nucleosome complex. In the presence of 1600 nM HMGB1 (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, but nevertheless maintain the more accessible and "relaxed' conformational isomers (N' and N") that permit ER binding.

So the data indicate that the nucleosome can occur in three different states in the absence of ER. ER cannot bind to the canonical nucleosome (N) because the cERE is inaccessible, while ER binds strongly to the cERE in the N' and N" states. It would seem especially applicable to use the terms "tense" and "relaxed" for the different conformational states of the nucleosome, N and N'/N", respectively. This is reminiscent of the narrative espoused by Perutz, almost 40 years ago, in the simplest description of the two conformations of deoxyhemoglobin and oxyhemoglobin, respectively [134]. Within the ensemble of nucleosomes, the canonical nucleosome can be viewed as a highly constrained, rigid, or "tense" state of the nucleosome. Core histone-DNA and histone tail-DNA interactions inhibit flexibility in the DNA and makes the cERE inaccessible to ER. HMGB1 binds preferentially to the nucleosome states (N' and N") in which the forces are different than those in the canonical nucleosome and more readily facilitate ER binding. These nucleosome states can be viewed as much less constrained, with enhanced flexibility and in a more "relaxed" state that facilitates less constraints globally on the nucleosomal DNA and locally on the cERE.

It is clear that the IF and CS models are not mutually exclusive and, in may cases, can work together to play a role in conformational change. For example, one can imagine that after the HMGB1 interaction leads primarily to a population shift, IF can play a secondary role by further optimizing one or more local interactions. Although the nature of the nucleosome conformation after ER binding has not been explored, subsequent sequence-specific interaction of ER binding to cERE may additionally involve IF leading to the final optimization of the local nucleosome conformation [132].

This simple picture of the energy landscape for nucleosomes can be regarded as only the first level of complexity in the ensemble of conformational states. If one could scan the nucleosome population within a cell, the impact of HMGB1 would be only one of a multitude of factors that could affect and alter the energetic state of the nucleosome. Environmental cues will drive changes in the nature of the local nucleosomes and extend into influencing the level of the higher-order chromatin structure. These factors include DNA methylation and the current and growing list of posttranslational modifications (epigenetic factors) on the core histones. Of course, if one considers the multiple combinations of modifications that can take place at any one time, the energies and the energy differential between these states on an energy landscape, together with the ongoing transient and temporal nature of the modifications, it can only be forecast to be enormously complex, with a very large and enormously heterogeneous population of nucleosome conformations. These factors will serve to fine-tune local and higher-order structure and function.

7. Conclusions

ER is incapable of binding to the cERE in the canonical nucleosome. HMGB1 interactions restructure the nucleosome to provide a more accessible cERE and facilitate strong ER/cERE binding. The binding affinity increased 10-fold, from a Kd ~300 nM in the canonical nucleosome to 30 nM in the restructured nucleosomes, which is only three times weaker than ER binding to naked DNA.

The structure of the "HMGB1 restructured" nucleosomes (N' and N") differs very little from the canonical nucleosome. The HMGB1 restructuring effectively readjusts the interactions of nucleosomal DNA with the proteins in the histone octamer. The main differences are in the reduced level of interaction between the minor groove of DNA and the core histones, together with the disruption of the positively charged residues in the N-terminal tails of the histones with the DNA. All conformers are nearly isoenergetic.

The ER/cERE binding affinities are essentially the same when the cERE is positioned at three locations within the nucleosome—the dyad and −20 and −40 bps from the dyad. This suggests that the effect of non-specific HMGB1 interactions with the nucleosome is global and not localized.

The stability and population of the nucleosome states in the ensemble are a delicate function of solution conditions.

The collective data from our studies on the effect of HMGB1 on nucleosomes and subsequent binding of ER to cERE in these nucleosomes, together with the detailed transcriptional studies focused on the pS2 promoter, provide evidence that HMGB1 plays a collaborative role in transcription of estrogen-responsive genes. While progress has been impressive, we are only starting to develop an understanding of the dynamic aspects of nucleosome and higher-order chromatin structure and their role in transcription. Future studies will continue to unveil many of the questions that will be interesting and important to address.

Conflict of interest

The authors declare there is no conflict of interest.

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