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The dynamics of HMG protein–chromatin interactions in living cells¹

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Abstract

The dynamic interaction between nuclear proteins and chromatin leads to the functional plasticity necessary to mount adequate responses to regulatory signals. Here, we review the factors regulating the chromatin interactions of the high mobility group proteins (HMGs), an abundant and ubiquitous superfamily of chromatin-binding proteins in living cells. HMGs are highly mobile and interact with the chromatin fiber in a highly dynamic fashion, as part of a protein network. The major factors that affect the binding of HMGs to chromatin are operative at the level of the single nucleosome. These factors include structural features of the HMGs, competition with other chromatin-binding proteins for nucleosome binding sites, complex formation with protein partners, and post-translational modifications in the protein or in the chromatin-binding sites. The versatile modulation of the interaction between HMG proteins and chromatin plays a role in processes that establish the cellular phenotype.

Keywords

chromatin; HMG proteins; protein–DNA interactions

Introduction

The dynamic and continuous interaction of nuclear regulatory factors and structural proteins with the chromatin fiber plays a key role in regulating the fidelity of gene expression and in establishing the cellular phenotype. Therefore, studies of the factors that regulate the interaction of chromatin-binding proteins with their targets provide insights into the molecular mechanisms that regulate chromatin function. One of the most perplexing aspects of chromatin biology is the mechanism of action and the cellular function of a group of

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nuclear proteins, known as architectural nucleosome-binding proteins. This class of proteins, which includes variants of the highly abundant linker histone H1 family (Kasinsky et al. 2001) and members of the high mobility group (HMG) protein superfamily (Bustin 1999; Bianchi and Agresti 2005), are structural proteins that bind to nucleosomes without any known specificity for the underlying DNA sequence. The interactions of architectural proteins with nucleosomes induce local and global changes in chromatin structure that affect various DNA-dependent activities, including transcription, recombination, and repair. These proteins may also play a role in epigenetic regulation, since their interaction with chromatin affects the levels of histone modifications (Lim et al. 2004, 2005; Fan et al. 2005).

Most of the information on the binding of the architectural proteins to chromatin was obtained from *in vitro* experiments with purified components or with reconstituted chromatin. However, *in vitro* studies cannot fully reflect the complex binding modes and cross interactions occurring in living cells. Fluorescence microscopy techniques that enable investigations on protein mobility and binding in living cells, in real time, provide new important insights into the interaction of nuclear proteins with chromatin. The results obtained with these approaches indicate that nuclear proteins in living cells are highly mobile, that their interaction with any specific site in chromatin is temporary, and that they move continuously among chromatin-binding sites in a stop-and-go fashion (Lever et al. 2000; Phair et al. 2004). Nevertheless, most of the time, most of the proteins are bound to chromatin because their dwell time on nucleosomes is longer than their transit time among nucleosome. Thus, at any particular binding site, there is a continuous turnover of the proteins, some of which compete with each other for binding to nucleosomes.

In vivo experiments have revealed that H1 and HMGs, and perhaps other nuclear components, form a dynamic network of proteins that continuously move among nucleosomes, constantly molding and reconfiguring their chromatin-binding site (Catez et al. 2004; Bustin et al. 2005). The chromatin interactions of members of this network are interdependent and are affected by structural and biochemical changes in the binding sites, as well as by changes in the levels and properties of the various members of the network. This network of continuous and dynamic interactions between architectural proteins and their nucleosomal binding sites is part of the mechanism that provides functional plasticity to the chromatin fiber.

Linker histone H1 is the major architectural protein family present in metazoan cells, most of which contain sufficient H1 to bind most of the nucleosomes. The major factors affecting the binding of H1 variants to chromatin have been previously summarized (Catez et al. 2006). The HMG protein superfamily, which consists of 3 families, named HMGA, HMGB, and HMGN, is also abundantly and ubiquitously expressed in most eukaryotic cells. Although each HMG family is clearly distinct and contains unique chromatin-binding motifs (Bustin 1999), they all have been shown to affect the properties of the chromatin fiber. The biochemical properties and the biological function of HMG proteins, and their involvement in the etiology of certain diseases, have been described in several reviews (Bustin 1999; Reeves and Beckerbauer 2001; Sgarra et al. 2004; Bianchi and Agresti 2005; Hock et al. 2007). Here, we review the information on the major factors that regulate the interaction of the 3 canonical HMG protein families (for definitions of canonical HMGs, see http://home.ccr.cancer.gov/metabolism/bustin/hmg_proteins.htm) with chromatin, and focus the attention on the more recent results obtained in studies with living cells. The functional motifs characteristic of the 3 HMG families are found in numerous nuclear proteins; therefore, elucidation of the factors that regulate the interaction of HMGs with chromatin may be of general relevance to understanding the molecular mechanisms by which architectural proteins affect chromatin function.

HMGA proteins

AT hooks

HMGA proteins are ubiquitously present in the nuclei of both plants and animals (Bustin and Reeves 1996; Reeves and Beckerbauer 2001). Their expression is related to cellular differentiation and proliferation, and they are involved in the regulation of gene expression, in differentiation processes, and in carcinogenesis (Reeves and Beckerbauer 2001; Sgarra et al. 2004; Fusco and Fedele 2007; Hock et al. 2007). The hallmark of the HMGA protein family is the AT hook, which serves as their major chromatin-binding motif. The canonical HMGA family is encoded by 2 distinct genes: HMGA1 and HMGA2. Alternative splicing of the HMGA1 transcript gives rise to 3 variants: HMGA1a, 1b, and 1c (Reeves and Beckerbauer 2001). The metazoan HMGAs have a molecular mass of ~11 kDa, contain 3 conserved AT hook DNA-binding domains, and have an acidic C-terminal tail (Fig. 1).

The AT-hook domain is a small motif containing the consensus PRGRP sequence flanked by additional positively charged residues (Reeves and Nissen 1990; Huth et al. 1997). This consensus sequence is necessary and sufficient for the specific binding of the proteins to DNA (Reeves and Nissen 1990). The central RGR tripeptide penetrates into the minor groove of AT base pairs, where the Arg residues form electrostatic and hydrophobic contacts with the floor of the minor groove DNA (Huth et al. 1997). Early footprinting studies suggested that HMGA proteins bind to any short run of AT-rich DNA, and that the AT hook motifs bind preferentially to AT-rich B-form DNA (Solomon et al. 1986; Reeves and Nissen 1990); however, more recent studies identified 2 consensus sequences for HMGA2 binding, suggesting some degree of sequence specificity for their binding (Cui and Leng 2007). Indeed, in living cells, the proteins display specific effects on the cellular transcription profile and affect distinct cellular processes (Merika and Thanos 2001; Reeves and Beckerbauer 2001; Sgarra et al. 2004; Hock et al. 2007). In addition, the AT-hook motif facilitates the binding of HMGA proteins to DNA substrates with altered structures, including synthetic 4-way junctions (Claus et al. 1994; Hill and Reeves 1997; Hill et al. 1999; Banks et al. 2000), supercoiled plasmids (Nissen and Reeves 1995), and isolated nucleosomes (Reeves and Nissen 1993; Reeves and Wolffe 1996; Li et al. 2006).

Upon binding to their targets, the HMGA proteins undergo conformational changes (Huth et al. 1997), which facilitate their ability to bend DNA (Reeves and Beckerbauer 2001). The enthalpy-entropy compensation associated with the binding of HMGA to DNA is similar to that of minor groove-binding drugs, such as netropesin, distamycin A, and Hoechst 33258 (Cui et al. 2005), which compete with HMGA proteins for chromatin-binding sites both in vitro and in vivo (Reeves and Nissen 1990; Radic et al. 1992). Although a single AT hook is sufficient for the binding of the protein to DNA, the simultaneous binding of 2 or more hooks increases the strength of this interaction (Claus et al. 1994; Maher and Nathans 1996; Yie et al. 1997; Frank et al. 1998). Efficient binding of HMGA proteins to DNA is dependent on the number, the arrangement, and the proper phasing of their AT hooks (Thanos and Maniatis 1995; Kim and Maniatis 1997; Reeves and Beckerbauer 2001).

Photobleaching analyses of living cells revealed that HMGA is included in the pool of chromatin-binding proteins that interact with their targets transiently and constantly move through the cell nucleus (Catez et al. 2004; Harrer et al. 2004). Although the binding of a particular HMGA molecule to any particular site is temporary, most of the HMGA proteins are bound to heterochromatin; in mitotic cells, the protein remains associated with the metaphase chromatin. Detailed analyses of HMGA point mutants revealed that, in living cells, the Arg residues in the AT hook play a key role in their chromatin interaction. Mutations of a single Arg residue weakened, but did not abolish, the preferential interaction of the protein with heterochromatin. Any double point mutant bearing Arg mutations in 2 different AT

hooks did not localize to heterochromatin, and was diffusely distributed throughout the entire nucleus (Harrer et al. 2004). Thus, the AT hooks are the major determinants affecting the interaction of HMGA with chromatin both in vivo and in vitro.

Multiple post-translational modifications modulate the binding of HMGA to chromatin

Beyond the AT hooks, the binding of HMGA proteins to chromatin is modulated by a large number of post-translational modifications. HMGA proteins are among the most heavily and extensively modified proteins in the cell nucleus; they are known to be modified by phosphorylation, acetylation, methylation, ribosylation, and sumoylation (Zhang and Wang 2008). The modification patterns change during the cell cycle in response to changes in the physiological state of a cell, and are also cell-type specific (Banks et al. 2000; Diana et al. 2001; Reeves and Beckerbauer 2001; Edberg et al. 2004, 2005; Jiang and Wang 2006; Zou et al. 2007; Cao et al. 2008).

In vitro studies demonstrated that several kinases, including CDC2, PKC, casein kinase 2, and HIPK2, modify distinct residues in HMGA proteins, and that these modifications alter the binding of the proteins to their targets (Schwanbeck et al. 2000; Reeves and Beckerbauer 2001; Zhang and Wang 2007). During mitosis, PKC and CDC2 hyperphosphorylate Ser and Thr residues next to each AT hook, suggesting that some of the modifications are cell-cycle related (Nissen et al. 1991). Photobleaching experiments demonstrated that, in living cells, the Ser and Thr residues that are modified by these kinases do indeed play a role in regulating the interaction of HMGA1 with chromatin. Inhibition of PKC or CDC2 kinase activities increased the HMGA1a mobility preferentially in areas containing compacted chromatin, indicating that suppression of HMGA1 phosphorylation reduces its binding to heterochromatin. Fluorescence recovery after photobleaching (FRAP) studies using point-mutated proteins of mitosis-specific phosphorylation sites support the conclusion that phosphorylation promotes the binding of HMGA1 proteins to mitotic chromosomes and to interphase heterochromatin. Furthermore, the phosphorylation patterns seem to fine tune HMGA1 dynamics and regulate their binding specificity to either DNA or nucleosomes (Harrer et al. 2004).

Another prominent modification found in HMGA proteins is arginine methylation (Banks et al. 2000; Sgarra et al. 2003a, 2003b). Significantly, many of the methyltransferases target the Arg residues found in the AT-hook motifs. The type I protein arginine methyltransferases PMRT1, 3, and 6 have been shown to be involved in most of these methylations. PMRT1 and PMRT3 preferentially methylate Arg 23 and 25, located in the first AT hook of HMGA. PMRT6 was able to methylate all the Arg residues in all 3 AT hooks, but with some preference for the second AT hook (Miranda et al. 2005; Sgarra et al. 2006; Zou et al. 2007). Some of the methylations were linked to cellular changes, such as apoptosis (Sgarra et al. 2003a, 2003b). Arginine 25 methylation was described in HMGA1a, but not HMGA1b, suggesting functional specificity between these 2 splice variants (Zou and Wang 2007). Given that the Arg residues located in the AT hooks play an important role in stabilizing the interaction of HMGA with chromatin, it can be expected that methylation of these residues would affect the interaction of the proteins with their chromatin targets sites; however, the effect of this modification on chromatin binding has not been yet examined.

In addition, it is well documented that acetylation of lysine residues affects the binding of HMGA proteins to their target. Elegant studies on the activation of interferon- β revealed that the ordered acetylation of HMGA on specific lysine residues by PCAF/GCN5 and CBP plays an important role in the activation of endogenous promoters (Munshi et al. 1998, 2001). A correlation between the acetylation of Lys 64 and 70 and the metastasis state of breast cancer cells has also been reported (Edberg et al. 2004). Recent studies suggest that p300 and PCAF acetylate Lys residues at multiple positions with various efficiencies (Zhang

et al. 2007). FRAP analyses of cells treated with histone deacetylase inhibitors revealed that hyperacetylation increased significantly the dynamic properties of HMGA1 proteins in both heterochromatin and euchromatin (Harrer et al. 2004). Thus, acetylation of HMGAs and (or) their nucleosomal targets plays an important regulatory role in their chromatin interactions.

Interaction with regulatory proteins

The binding of HMGAs to chromatin is also regulated by their interactions with other chromatin components. One of the best examples is the role of HMGA1 in enhanceosome formation on the promoter of the interferon- β gene, where HMGA1 proteins form a multiprotein complex with specific transcription factors. Enhanceosome formation requires allosteric changes of the DNA, which are induced by HMGA1 binding and followed by protein-protein interactions between HMGA1 nuclear factor κ B and ATF2/c-Jun to stabilize the enhanceosome (Yie et al. 1997). Likewise, HMGA proteins target the formation of the origin recognition complex (ORC) via direct interaction with the components Orc1 and Orc6 of this protein complex (Thomae et al. 2008). Significantly, functional AT hooks are required for the targeting of ORC, an indication that the interaction of HMGAs with chromatin plays a major role in this targeting.

Competition with chromatin-binding proteins

In the living nucleus, HMGAs are highly mobile and continuously move among nucleosomes. Photobleaching experiments indicated that, in living cells, HMGA variants compete with histone H1 variants for chromatin-binding sites (Catez et al. 2004). Competition of HMGA proteins and histone H1 for binding sites could involve the conserved SPTXK motifs that play an essential role in the binding of H1 to the minor groove of DNA. This motif, which is repeated several times in the amino and carboxyl tails of histone H1, adopts a β -turn structure, similar to that in Hoechst 33258, and preferentially binds to AT-rich sequences (Churchill and Suzuki 1989), suggesting that it could compete with AT hooks for chromatin binding. Although not yet experimentally proven, it is likely that the HMGA variants compete among themselves, and perhaps also with other nuclear proteins, for chromatin-binding sites. Potentially, changes in these competitors could affect the interaction of the HMGAs with chromatin.

HMGB proteins

The HMG box

The high mobility group box (HMGB) protein family is the most abundant family of HMGs and a major nucleosome-binding constituent of the metazoan nucleus. This protein family has been shown to affect various chromatin-related processes, such as transcription and replication, and plays a role in determining the cellular phenotype (Bustin 1999; Thomas and Travers 2001; Agresti and Bianchi 2003; Bianchi and Agresti 2005; Hock et al. 2007). Interestingly, HMGB proteins also have important functions as extracellular signaling molecules (Lotze and Tracey 2005; Bianchi and Manfredi 2007). The canonical metazoan HMGB protein family consists of 3 major variants, HMGB1, HMGB2, and HMGB3, each encoded by a specific gene. The functional hallmark of this protein family is the HMG-Box, which serves as the major binding site of the proteins to DNA and chromatin. The canonical HMGB proteins have a molecular mass of ~25 kDa, contain 2 DNA-binding domains, called HMG box A and B, and a long acidic tail, which is a continuous array of ~30 acidic amino acids (Thomas and Travers 2001) (Fig. 2).

The HMGB box consists of 3 α -helices arranged in an L-like structure (Thomas and Travers 2001). In vitro studies using purified double-stranded DNA revealed that both box A and box B bind to and distort the minor groove of the DNA in a sequence-independent

manner. Although either HMG box A or B can bind to DNA by itself, the binding is enhanced by a tandem array of the 2 boxes (Yoshioka et al. 1999). The details of these interactions and the specific features of the various boxes are described elsewhere (Thomas and Travers 2001). The acidic tail of HMGB1 contacts the DNA-binding surfaces of both HMG boxes and reduces the affinity of HMGB1 to double-strand DNA, whereas it enhances the affinity of the protein to distorted DNA (Lee and Thomas 2000; Watson et al. 2007). It seems that the acidic tail modulates the biological functions of HMGBs (Aizawa et al. 1994; Shirakawa et al. 1997); however, its role in the chromatin binding of these proteins in living cells has not been fully clarified.

Beyond the intrinsic structure of the proteins, the *in vitro* interaction of HMGBs with DNA and chromatin is affected by several factors, including post-translational modifications in the HMGs, interaction with regulatory factors, and DNA conformations (Bustin 1999; Thomas and Travers 2001; Bianchi and Agresti 2005; Assenberg et al. 2008). The organization of HMGB in the nucleus of live cells is highly dynamic and, like other nuclear proteins, HMGBs bind transiently to chromatin (Scaffidi et al. 2002; Phair et al. 2004). Factors known to affect the interaction of HMGBs with DNA *in vitro* also affected their chromatin binding in living cells. Thus, a mutant HMGB1, bearing mutations in conserved amino acid residues that are important for anchoring HMGB1 to the minor groove of DNA, does not bind to chromatin and mislocalizes to nucleoli (Agresti et al. 2005).

Competition experiments, in which HMGB1 was micro-injected into living cells expressing H1-GFP, indicated that HMGB1 competes with histone H1 for chromatin-binding sites. HMGB1 deletion mutants lacking the acidic C-terminal, which interacted strongly with DNA *in vitro*, competed efficiently with H1, while deletion mutants that did not interact with DNA *in vitro* did not compete (Catez et al. 2004). These results suggested that, *in vivo*, the binding sites for HMGB1 and histone H1 on the chromatin partially overlap, and that the HMG boxes are the main chromatin-binding domain of this protein family. These results are also in agreement with a previous *in vitro* experiment, which demonstrated that HMGB1 preferentially binds to the linker DNA rather than to nucleosome core particles (CPs) (Nightingale et al. 1996), and that an HMGB1 truncation mutant lacking the acidic tail can bind to nucleosome cores (Bonaldi et al. 2002). Nevertheless, it has been demonstrated that the acidic tail of HMGB1 interacts with histone H3 and facilitates the proper binding of HMGB1 to the linker DNA between adjacent nucleosome CPs (Ueda et al. 2004).

Post-translational modifications of HMGBs affect their chromatin interactions both *in vitro* and in living cells. Monomethylation at Lys42 located in HMG box A of HMGB1 weakens its DNA binding, and the protein relocalizes to the cytoplasm (Ito et al. 2007). In monocytes, acetylation of nuclear HMGB1 leads to relocation to the cytoplasm, indicating a weakening in chromatin binding (Bonaldi et al. 2003). Indeed, detailed *in vitro* analyses revealed that acetylation of specific Lys residues in HMGB1 plays a critical role in its binding to purified DNA (Assenberg et al. 2008). An interesting, albeit puzzling, finding was that HMGB1 binds strongly, almost irreversibly, to the chromatin in apoptotic cells, but not to the chromatin in necrotic cells (Scaffidi et al. 2002). The mechanism responsible for the strong binding of HMGB1 to apoptotic chromatin is not understood. In living cells, the binding of HMGB1 to mitotic chromatin is weaker than that to interphase chromatin (Pallier et al. 2003), a finding that is in full agreement with previous immunofluorescence analyses demonstrating significant depletion of HMGB1 from mitotic chromatin following fixation (Isackson et al. 1980). Thus, both post-translational modifications of HMGBs and the higher-order chromatin conformation affect the chromatin binding of HMGB in living cells.

Numerous studies, including reporter assays in tissue culture cells and *in vitro* binding assays, indicate that HMGBs interact with many proteins that are known to affect chromatin

function (Bustin and Reeves 1996; Bustin 1999; Agresti and Bianchi 2003). Obviously, these interactions may affect the binding of HMGB1 to chromatin *in vivo*. This possibility has been experimentally demonstrated by analyses of living cells expressing both fluorescently labeled HMGB1 and glucocorticoid receptors. Detailed FRAP and fluorescence resonance energy transfer studies revealed that, in living cells, these 2 proteins interact, but only when bound on chromatin. The chromatin interaction of this complex is more stable than could be predicted from the independent chromatin binding of the 2 proteins, and its disassembly requires energy (Agresti et al. 2005). This elegant study serves as a paradigm of the dynamic cooperative interactions between chromatin-binding proteins and their targets in living cells. However, given the large numbers of proteins that can potentially interact with HMGBs, it is still possible that some of these interactions occur prior to chromatin binding, and that, in some instances, HMGBs bind to chromatin as part of a protein complex.

HMGN proteins

The nucleosome-binding domain

HMGNs are the only nuclear proteins known to specifically bind to the 147 bp nucleosome CP, the building block of the chromatin fiber. This protein family consists of 5 major variants, named HMGN1, HMGN2, HMGN3a, HMGN3b, and HMGN4, all with a molecular mass of ~10 kDa (Bustin 2001; Bianchi and Agresti 2005). A much larger protein, named NSBP1 (or NBD-45), shares chromatin-binding characteristics with the HMGN family (Shirakawa et al. 2000; King and Francomano 2001). The hallmark of this family is the nucleosomal binding domain (NBD), which facilitates the specific binding of these proteins to the CP (Fig. 3). The NBD is a 30 amino acid domain that has been shown to be the minimum peptide able to bind specifically to nucleosomes, both *in vitro* and *in vivo* (Crippa et al. 1992; Ueda et al. 2008). Embedded in this domain is an 8 amino acid motif, **RRSARLSA**, which is encoded by a single exon and is absolutely conserved in all HMGNs. Within this sequence, each of the 4 amino acids in the R-S-RL sequence are absolutely necessary for the specific binding of the HMGN protein to the CP, both *in vivo* and *in vitro* (Ueda et al. 2008).

While the NBD determines the specificity of the interactions of HMGNs with nucleosomes, the C-terminal region contributes to the binding affinity. The binding of HMGN C-terminal truncation mutants to chromatin is significantly weaker than that of the intact protein, both *in vitro* and *in living cells* (Ueda et al. 2008).

Nucleosome interactions

All HMGNs bind to nucleosomes with very similar affinities (Ueda et al. 2008); however, their chromatin interactions are variant specific. Bimolecular fluorescence complementation assays in living cells (Cherukuri et al. 2008), chromatin immunoprecipitation analyses (Postnikov et al. 1997), and mobility shift assays of HMGN-CP complexes that have been supershifted with specific antibodies (Postnikov et al. 1997) reveal that, both *in vitro* and *in vivo*, 2 molecules of HMGN bind to CPs. These nucleosome-CP complexes contain either 2 molecules of HMGN1 or 2 molecules of HMGN2; mixed complexes containing 1 HMGN1 and 1 HMGN2 molecule on the same nucleosomes are not observed under physiological conditions (Postnikov et al. 1995; Cherukuri et al. 2008). The formation of homodimeric complexes raises the possibility that each HMGN variant produces unique structural modification in CPs. Site-directed cross-linking revealed that the proteins occupy distinct positions on the nucleosomes (Trieschmann et al. 1998; Ueda et al. 2008); however, the exact path of these proteins on the nucleosomal surface is not yet known.

Analyses of HMGN binding to chromatin in vivo by FRAP mobility and by visualization of their intranuclear organization, and in vitro by mobility shift assays suggest 2 different binding modes (Cherukuri et al. 2008; Ueda et al. 2008). The major mode is high-affinity binding to CPs, which involves specific interactions between the conserved NBD and the nucleosome CP, which leads to the formation of CP–HMGN homodimer complexes on chromatin. Mutation of any of the 4 amino acids in the R-S-RL motif described above abolishes this specific NBD-dependent nucleosome binding, resulting in FRAP recovery rates that are 10 times faster than those of the wild-type protein (Ueda et al. 2008). The minor mode is low-affinity binding to DNA, which probably does not involve homodimerization of the HMGN proteins. This type of binding is found in mitotic cells, where a high fraction of the HMGNs are phosphorylated (Cherukuri et al. 2008; Prymakowska-Bosak et al. 2001). Interestingly, when the specific binding of HMGN to nucleosomes is abolished, the protein mislocalizes to the nucleolus, an organelle from which the wild-type protein is excluded (Prymakowska-Bosak et al. 2001; Ueda et al. 2008). These findings suggest that the formation of homodimeric HMGN–CP complexes requires specific high-affinity interaction of these proteins with their nucleosomal targets.

Factors affecting the mobility and chromatin binding of HMGNs in live cells

Several factors were shown to affect the binding of HMGNs to nucleosomes in live cells: the primary structure of the HMGNs themselves, post-translational modifications, the structure of the chromatin fiber, and competition with other proteins for chromatin-binding sites.

Based on sequence similarity and in vitro chromatin-binding analyses, 3 distinct conserved elements were described in the primary structure of HMGNs: the NBD, the bipartite nuclear localization signals flanking the NBD, and a less well defined regulatory domain, which is located in the C-terminal region of the protein (Fig. 3). The regulatory domain was previously called the chromatin-unfolding domain (Bustin 2001); it was renamed because recent findings indicate that this region is involved in chromatin interactions and in modulating the HMGN-related effects on histone post-translational modifications in an HMGN-variant-specific manner (Ueda et al. 2006, 2008).

A comprehensive analysis of the kinetic and chromatin-binding properties of a battery of point and deletion mutants of HMGN2 revealed that several regions of the protein, most of which are located in the NBD and toward the C-terminal half, affect its chromatin affinity (Ueda et al. 2008). The C-terminal region affects the affinity of the protein for chromatin, while the specificity for nucleosome binding resides in the NBD. Detailed analyses of HMGN2 point mutants revealed that several residues in the NBD affect its binding to CPs; however, the specific interaction between HMGN2 and CP is dependent only on residues R₂₂S₂₄R₂₆L₂₇. Mutation in any of these 4 residues, which are absolutely conserved in all HMGN variants, will abolish the specific interaction of the HMGN proteins with CPs (Ueda et al. 2008). Previous biochemical studies (Crippa et al. 1992) and more recent FRAP analysis of HMGN swap mutants indicated that the NBD is an interchangeable chromatin-binding module that can act as an independent functional domain (Ueda et al. 2008). Thus, the NBD domain can be considered as a chromatin-binding module that specifically recognizes structural features in CPs, independent of DNA sequence or histone tail modifications.

Post-translational modifications in either HMGNs or in chromatin were also shown to affect their interaction. HMGNs are phosphorylated (Thomson et al. 1999; Prymakowska-Bosak et al. 2001; Soloaga et al. 2003) and acetylated (Herrera et al. 1999; Bergel et al. 2000; Luhrs et al. 2002) at multiple sites (Zhang and Wang 2008). The levels of phosphorylation are low in interphase nuclei, but elevated during mitosis or during response to cellular stress. Although in vitro analyses suggest that the phosphorylation of several sites in HMGN inhibit

its CP binding (Prymakowska-Bosak et al. 2001), in vivo FRAP and fluorescence loss in photobleaching experiments suggest that only phosphorylation inside the NBD has significant effects on this binding (Prymakowska-Bosak et al. 2001; Catez et al. 2004; Lim et al. 2004). The most significant effect on chromatin interactions occurs upon phosphorylation of the conserved Ser residue, which is 1 of the 4 amino acids that determine the nucleosome-binding specificity of the protein (Prymakowska-Bosak et al. 2001; Catez et al. 2004; Lim et al. 2004; Ueda et al. 2008). Both HMGN1 and 2 are also acetylated on multiple residues (Bergel et al. 2000), a modification that has been shown to affect the interaction with CPs in vitro (Herrera et al. 1999). Interestingly, treatment of cells with histone deacetylase inhibitors reduces the binding of HMGN to chromatin in vivo. This treatment is known to elevate the acetylation of the nucleosomal histones; however, it is also possible that it leads to modifications in HMGNs, which in turn may affect their binding to chromatin (Luhrs et al. 2002; Ramirez et al. 2008).

Detailed comparison of in vivo and in vitro interaction between HMGNs and chromatin suggests that the major factors that affect the binding of HMGN to chromatin are operative at the level of the single nucleosome (Ueda et al. 2008). Nevertheless, the structure of the chromatin fiber and the degree of nucleosome compaction in chromatin seems to play a significant role in the intranuclear organization and their chromatin-binding affinity. In heterochromatin, where the CPs are relatively crowded, the mobility of HMGNs is at least 2 times slower and the FRAP recovery is less complete than in euchromatin, where the chromatin fiber is less compacted (Catez et al. 2002; Phair et al. 2004). Heterochromatin and euchromatin are also distinguished by their pattern of histone post-translational modifications (Ruthenburg et al. 2007; Trojer and Reinberg 2007), which might also affect the mobility of HMGN. Since intranuclear mobility is related to chromatin-binding affinity, it may seem that binding of HMGNs to the CPs in heterochromatin is stronger than to the CPs in euchromatin. However, it is also possible that the tight packing of the CPs in heterochromatin minimizes the relative time necessary to find a binding site, i.e., the “go” stage in HMGN mobility. Thus, at the level of the single CP, the interaction of HMGN with euchromatin may be similar to its binding to heterochromatin. In contrast, the interaction of the HMGNs with the chromatin fiber in the condensed mitotic chromosome seem to be fundamentally different than their binding to euchromatin (Cherukuri et al. 2008), perhaps because, in mitotic cells, a significant fraction of the HMGNs is phosphorylated (Prymakowska-Bosak et al. 2001).

FRAP analyses of living cells microinjected with various proteins revealed that HMGN variants compete among themselves and with variants of the linker histone H1 for chromatin-binding sites (Catez et al. 2002, 2004). These experiments suggest that HMGNs interact with chromatin as part of a network of chromatin-binding proteins, and that their binding to CPs may be affected by changes occurring in any member of the network. Thus, changes in the absolute levels, or even in the chemical properties of a competing component, will affect the binding of HMGN to chromatin. As elaborated elsewhere (Bustin et al. 2005), the competitive interaction among chromatin-binding proteins may lead to functional redundancy among these proteins, and may have significant effects on the cellular phenotype.

Perspective

Studies on the intranuclear organization of HMG proteins in living cells provided a new view on their intranuclear organization and into the mode by which these nuclear proteins interact with chromatin. Traditionally, these interactions were viewed as static, in which a specific molecule would continuously associate with a specific binding site until a specific stimulus actively caused its dissociation. In the new view, these interactions are highly

dynamic and stochastic. Similar to other nuclear proteins, HMG proteins constantly move throughout the nucleus, scanning the chromatin landscape for binding sites (Phair et al. 2004). Their interactions with chromatin are regulated by multiple factors, including chemical changes in the protein or in their chromatin targets, and by competition with other nuclear proteins for chromatin-binding sites.

HMG proteins bind to chromatin as part of a protein network in which they compete with histone H1 variants, and with variants of their own HMG family for nucleosome-binding sites (Hill and Reeves 1997; Zlatanova and van Holde 1998; Catez et al. 2004). Competition of HMGs with other nuclear proteins can also be envisioned; however, this has not yet been experimentally demonstrated. Conceivably, a change in the expression level or in the binding affinity of a competitor could affect the binding of H1 or other HMG variants and trigger compensatory adjustments that would establish a new steady state of chromatin interactions. For example, induction of cell migration alters H1 histone post-translational modifications and increases its binding to chromatin (Gerlitz et al. 2007), while the binding of HMGN2 to chromatin seems to weaken (G. Gerlitz and M. Bustin, unpublished data). Similar situations could occur during the cell cycle or differentiation. Indeed, the mobility of H1 in embryonic stem cells differs from that in fully differentiated cells (Meshorer et al. 2006).

The key determinants shown to affect the binding of HMGs to chromatin in living cells are essentially the same as those affecting the *in vitro* binding of purified HMGs to isolated chromatin or nucleosomes. These interactions are operative at the level of the single nucleosome; the higher-order chromatin structure does not seem to have major effects on the binding of HMG protein to chromatin. The functional motifs characteristic of the 3 HMGs families are crucial for chromatin binding of the protein both *in vivo* and *in vitro*. Thus, in living cells, disruption of the NBD in HMGNs (Ueda et al. 2008), of the AT hook in HMGAs (Harrer et al. 2004), and of the HMG-Box in HMGBs (Agresti et al. 2005) abolish the interaction of the proteins with their chromatin targets. Likewise, studies in both living cells and with purified components have demonstrated that post-translational modifications in HMG proteins or in their binding sites affect chromatin interactions.

A major remaining unanswered question is whether the binding of these proteins to chromatin is specific. So far, there is no evidence that the proteins bind to chromatin with any sequence specificity; in fact, most *in vitro* studies failed to detect any preferential binding of the protein to chromatin containing unique sequences. Yet, emerging evidence suggests that HMG variants may affect the expression of specific genes or specific cellular processes. Three possible mechanisms for the preferential interaction of the proteins with specific sites in chromatin can be envisioned. First, and most likely, HMG proteins may be targeted to specific regions as part of multiprotein complexes. Indeed, HMG proteins are known to form such complexes and can interact with specific regulatory factors (Reeves and Beckerbauer 2001; Lim et al. 2002; Bianchi and Agresti 2005). Second, an HMG and a regulatory factor interact only on the surface of chromatin, as was shown for HMGB1 and glucocorticoid receptors (Agresti et al. 2005). In this scenario, stochastic assembly of HMG and a regulatory factor results in mutual strengthening of the chromatin interaction of each individual component. Such an interaction may nucleate the assembly of additional regulatory factors in a fashion similar to the stepwise, dynamic, assembly of polymerase I transcription complexes on ribosomal genes (Gorski et al. 2008). The third possibility, which has not yet been experimentally demonstrated, is that the various HMG variants preferentially bind to nucleosomes bearing specific histone post-translational modifications.

Studies with genetically altered mice suggest that HMG proteins affect developmental processes and play a role in the etiology of certain diseases (Hock et al. 2007). Elucidation

of the factors that regulate the binding of the HMGs to the chromatin could provide insights into the molecular mechanisms by which these ubiquitous and abundant nuclear proteins affect the cellular phenotype.

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AT hooks bind to minor groove in AT rich DNA

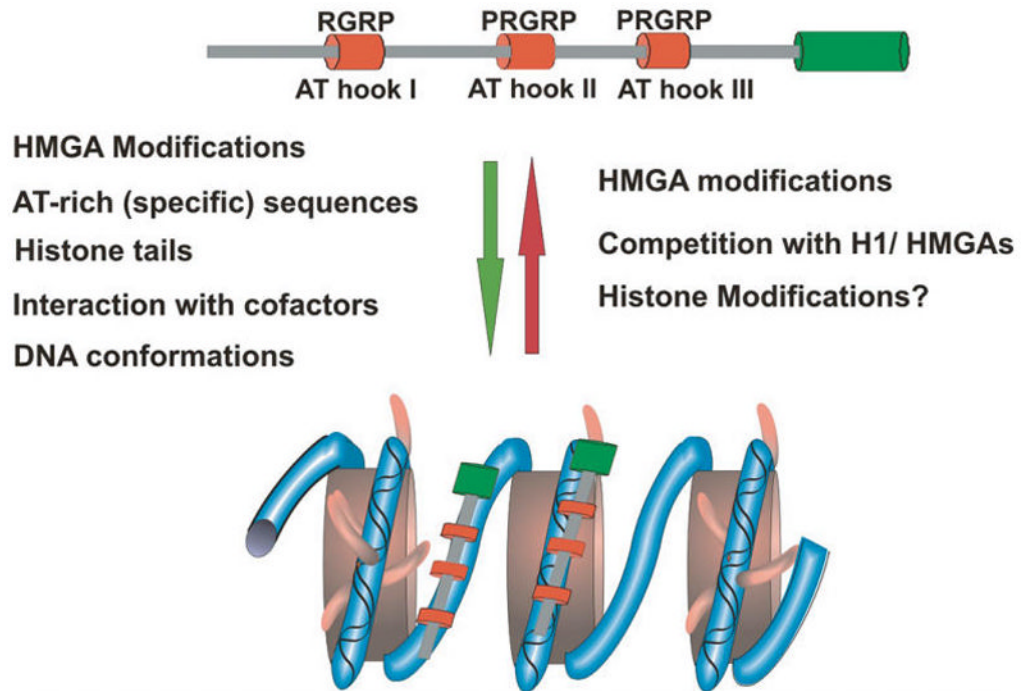


Fig. 1. Determinants of HMGA–chromatin interactions. Shown is a schematic diagram of HMGA proteins. The AT hooks appear as red cylinders. The factors listed to the left of the green arrow promote the interaction with HMGA with chromatin, while those listed to the right of the red arrow promote dissociation of HMGA from chromatin.

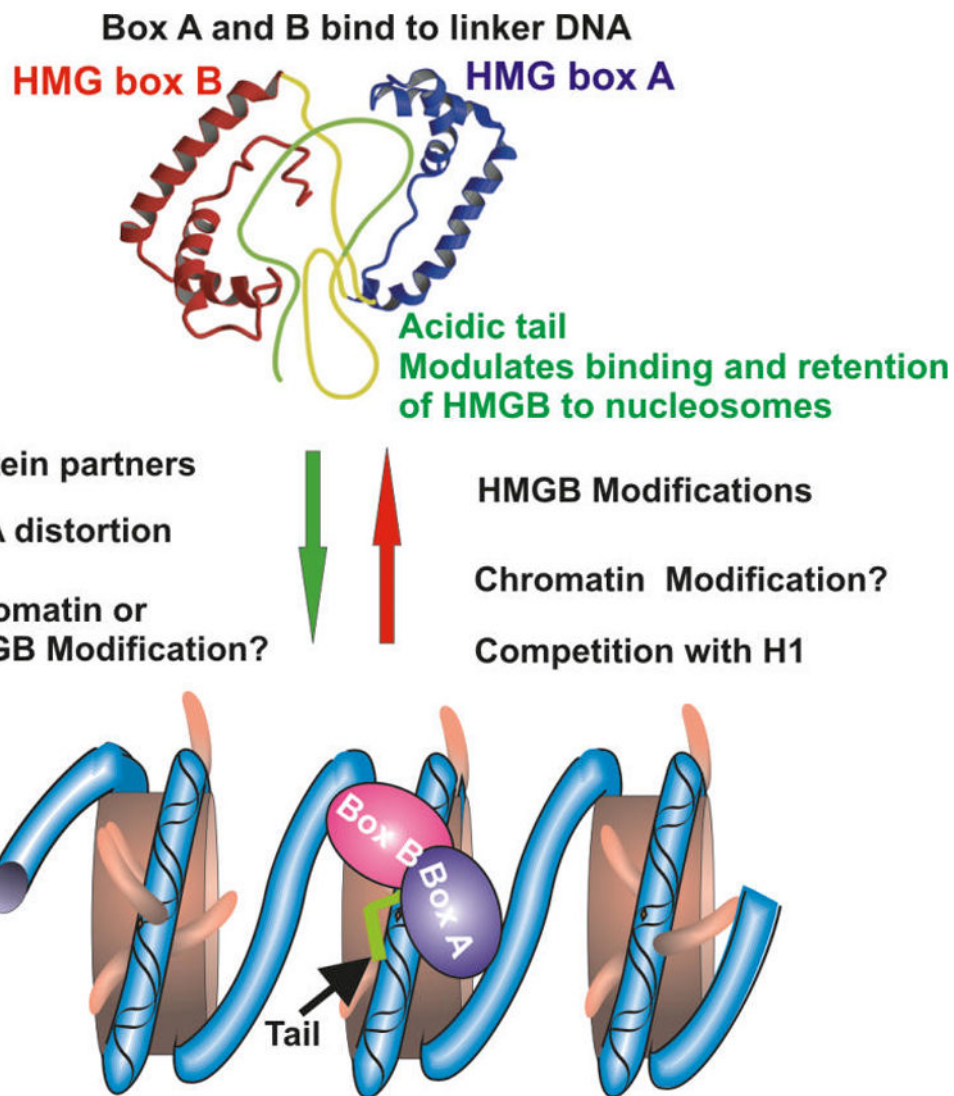


Fig. 2. Determinants of HMGB–chromatin interactions. Shown is a schematic diagram of HMGB proteins. The 3 structural domains of HMG box A and HMG box B are indicated. The factors listed to the left of the green arrow promote the interaction of HMGB with chromatin, while those listed to the right of the red arrow promote dissociation of HMGB from chromatin. HMGB structure was adapted from Lee and Thomas (2000).

The NBD binds specifically to nucleosome cores

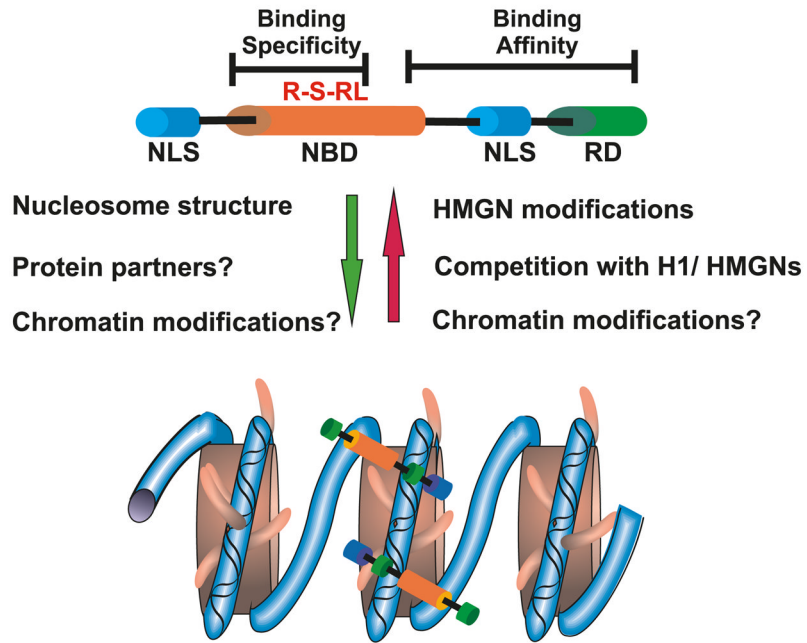


Fig. 3. Determinants of HMGN–chromatin interactions. Shown is a schematic diagram of HMGN proteins. The R-S-RL listed above the nucleosomal binding domain (NBD) denotes amino acid residues that specify the interaction of HMGN proteins with nucleosome core particles. The factors listed to the left of the green arrow promote the interaction of HMGN with chromatin, and those listed to the right of the red arrow promote dissociation of HMGN from chromatin. NLS, nuclear localization signal, RD, regulatory domain.