Plant Molecular Biology **53:** 281–295, 2003.

Mini-review

Chromatin-associated HMGA and HMGB proteins: versatile co-regulators of DNA-dependent processes

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Received 25 September 2003; accepted 9 October 2003

Key words: architectural protein, chromatin, gene regulation, HMGA, HMGB

Abstract

High-mobility-group (HMG) proteins are small and relatively abundant chromatin-associated proteins, which act as architectural factors. In plants, two groups of chromosomal HMG proteins have been identified, namely the HMGA family, typically containing four A/T-hook DNA-binding motifs, and the HMGB family, containing a single HMG-box DNA-binding domain. The HMGA proteins are structurally flexible and bind A/T-rich DNA stretches. By orchestrating multiple protein-protein and protein-DNA interactions, they assist the formation of higher-order transcription factor complexes, regulating gene expression. The HMGB proteins bind DNA non-sequence-specifically, but specifically recognise DNA structures. Due to their remarkable DNA bending activity, they can enhance the structural flexibility of DNA, facilitating the assembly of nucleoprotein structures that control various DNA-dependent processes such as transcription and recombination.

Abbreviations: HMG, high-mobility group

Introduction

The large genomic DNA of eukaryotic organisms is packaged in the nucleus in a highly complex and dynamic nucleoprotein structure known as chromatin. This compaction of the DNA into chromatin is brought about primarily by the histones. The condensation of the genomic DNA serves, besides structural functions, as a means to keep gene activity in a generally repressed state. Consequently, activation of gene expression requires remodelling of chromatin, which obviously constitutes an important control mechanism for gene activity. Numerous non-histone proteins are involved in these transient changes in chromatin structure priming the DNA for gene expression (Narlikar *et al*., 2002; Reyes *et al*., 2002). The high-mobility-group (HMG) proteins are a family of relatively abundant and ubiquitous non-histone proteins associated with eukaryotic chromatin. Their presence in all tissues of eukaryotes favours the possibility that the HMG proteins are required for proper cellular function. They act as architectural factors in the nucleus, facilitating various DNA-dependent processes such as transcription and recombination. Recent advances have improved our understanding of how HMG proteins contribute to the regulation of these essential nuclear events, which has renewed the interest in this versatile class of chromosomal proteins. About 30 years ago, HMG proteins were originally defined based on their extractability from calf thymus chromatin by 0.35 M NaCl, their solubility in 2% trichloroacetic acid or 5% perchloric acid, and their high content of charged amino acid residues (Goodwin *et al*., 1973). Because of their characteristic primary structures, mammalian HMG proteins have been subdivided into three distinct families (Bustin and Reeves, 1996). Recently, the nomenclature of the three HMG families has been revised (Bustin, 2001; and http://www.informatics.jax.org/mgihome/nomen/genefamilies/hmgfamily.shtml) to (1) facilitate interactions

among laboratories, (2) expedite literature searches, and (3) avoid confusion owing to similarity in the names of unrelated proteins. The three HMG protein families comprise the

- HMGA proteins (formerly HMGI/Y) containing A/T-hook DNA-binding motifs
- HMGB proteins (formerly HMG1/2) containing HMG-box domain(s)
- HMGN proteins (formerly HMG14/17) containing a nucleosome-binding domain

In plants, proteins belonging to the HMGA and HMGB families have been identified and characterized over the past few years (reviewed by Grasser, 1995; Spiker, 1988), but there is no evidence for the existence of HMGN proteins, which so far have been found exclusively in vertebrates. The pioneering work on plant HMG proteins was done by S. Spiker and co-workers, who have identified and characterized the HMG proteins from wheat germ, revealing structural differences between the plant and animal HMG proteins (Spiker, 1984, 1988; Spiker and Everett, 1987). Isolation and characterization of HMG proteins (and of the cDNAs encoding these proteins) from various mono- and dicotyledonous species largely confirmed the initial findings obtained for the wheat germ proteins (Grasser, 1995). This review summarizes the characteristic features of the HMGA and HMGB proteins, focusing in particular on the plant proteins and recent advances.

HMGA proteins

Structure, expression and post-synthetic modifications

Plant HMGA proteins (ca. 20 kDa) have a domain organization that differs from that of HMGA proteins of other sources. They typically contain four copies of the A/T-hook DNA-binding motif, while the mammalian counterparts have three A/T-hooks (Grasser, 1995). The A/T-hook motif is a short positively charged sequence containing the invariant GRP core, which is usually flanked by arginine and proline residues (Bustin and Reeves, 1996). In addition, the plant HMGA proteins have an N-terminal domain of ca. 65 amino acid residues, which shares sequence similarity to the globular domain of linker histones (Grasser, 1995). The evolutionary relation of these two plant gene families is supported by the presence of a single intron in the plant *hmga* genes, which is localised within the region encoding the domain similar to the linker histones. The position of this intron is conserved between *hmga* genes and the genes coding for linker histones (Krech *et al*., 1999). While the N-terminal linker histone-like domain contains a few aromatic residues, the A/T-hook domain is essentially free of aromatic amino acid residues. In general, the plant HMGA proteins contain a high proportion of proline and alanine residues. The amino acid sequences of HMGA proteins from various species are relatively conserved (Figure 1), sharing 40–80% amino acid sequence identity (Gupta *et al*., 1997b; Yamamoto and Minamikawa, 1997b). In the absence of DNA, the nuclear magnetic resonance spectrum of the A/T-hook region of human HMGA indicates random coil, but upon DNA binding, the part of the protein that contacts the DNA becomes ordered and adopts a well defined conformation. Thereby, the RGR motif of the A/T-hooks presents a narrow concave surface that perfectly fits into the minor groove of A/T-rich tracts, without causing severe perturbation of the B-DNA conformation. The minor groove bound by the A/T-hooks is only slightly wider than in classical B-DNA (Huth *et al*., 1997).

Depending on the species, plant genomes contain one (e.g. *Arabidopsis*, pea (Gupta *et al*., 1997a, b)) or two (e.g. *Canavalia gladiata*, soybean (Laux *et al*., 1991; Yamamoto and Minamikawa, 1997b)) genes coding for HMGA proteins. As examined by immunoblot and northern analyses, as well as by analysis of *hmga* gene promoter-reporter gene fusions in transgenic plants, the *hmga* genes are expressed ubiquitously (Gupta *et al*., 1997a, b, 1998; Yamamoto and Minamikawa, 1997a, b; Zhang *et al*., 2003b). These studies revealed, however, that the *hmga* expression levels vary significantly between different tissues. It is likely that the expression of the plant *hmga* genes correlates with the proliferative state of the cells (Gupta *et al*., 1997b; Zhang *et al*., 2003b). For mammalian *hmga* genes, it is well established that their expression is up-regulated in undifferentiated, rapidly proliferating cells or during embryonic development, whereas they are expressed only at low levels in fully differentiated or non-dividing cells (Reeves and Beckerbauer, 2001). In maize, there are increased levels of HMGA during endoreduplication in developing endosperm tissue, suggesting that HMGA is involved in the formation of a more open chromatin configuration facilitating transcription and/or replication (Zhao and Grafi, 2000). It should be stated that plant genomes

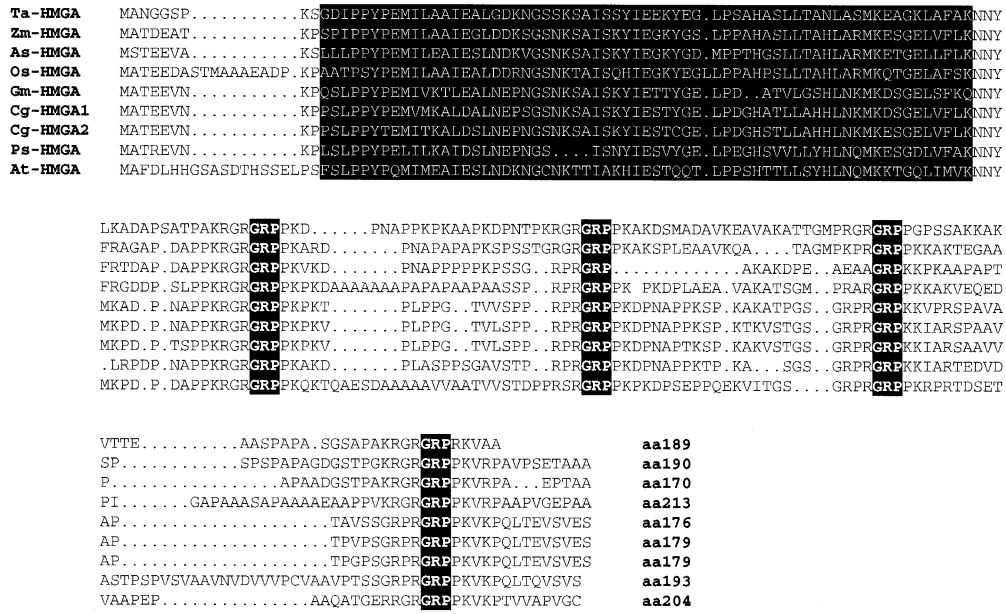

Figure 1. Alignment of amino acid sequences of plant HMGA proteins. The alignment contains the sequences of the HMGA proteins deduced from cDNAs of *Triticum aestivum* (AF502250), *Zea mays* (sequence has been modified, AJ131371), *Avena sativa* (L24391), *Oryza sativa* (L24390), *Glycine max* (X58246), *Canavalia gladiata* (D83070, D83071), *Pisum sativum* (X89568) and *Arabidopsis thaliana* (X99116). The region sharing sequence similarity with the globular domain of linker histone H1 and the GRP centre of the A/T-hook motifs are highlighted in black, and the total length of the polypeptides is indicated as well. The invariant GRP is mainly flanked by arginine and proline residues.

encode also proteins of higher molecular mass, which are structurally related to HMGA proteins containing a larger number of A/T-hook motifs (Meijer *et al*., 1996; Reisdorf-Cren *et al*., 2002; Tjaden and Coruzzi, 1994). Moreover, A/T-hook motifs have been found in subunits of the yeast RSC chromatin remodeling complexes (Cairns *et al*., 1999).

Mammalian and insect HMGA proteins occur as highly modified proteins *in vivo*. They are phosphorylated by several protein kinases, and they are acetylated and methylated (Reeves and Beckerbauer, 2001). Thus, the cell cycle-dependent CDC2 kinase, the mitogen-activated protein (MAP) kinase, as well as the CK2 and PKC kinases contribute to the phosphorylation of HMGA proteins (Banks *et al*., 2000; Schwanbeck *et al*., 2001). The phosphorylation events differentially affect the DNA and nucleosome interactions of the HMGA proteins, and may therefore play crucial roles in the regulation of functional properties of HMGA proteins (Banks *et al*., 2000; Schwanbeck *et al*., 2001). In the case of the human interferone-β (*IFN-*β) gene, it was found that acetylation of a specific lysine residue of HMGA by the CBP acetyltransferase results in destabilization of a protein complex formed at the enhancer region, leading to shut-down of transcription, whereas acetylation of another lysine residue by the PCAF/GCN5 acetyltransferase potentiates transcription by stabilizing the enhanceosome (Munshi *et al*., 2001). Currently, little is known about post-translational modifications of plant HMGA proteins. Analysis of HMGA isolated from pea shoots by mass spectrometry suggested that the protein might be modified post-translationally (Webster *et al*., 1997). Analysis of native HMGA proteins is complicated, however, by the fact that plant HMGA is susceptible to proteolytic degradation. Based on *in vitro* phosphorylation experiments, maize endosperm HMGA is phosphorylated (depending on the developmental state of endosperm) to different extents by the CDC2 kinase, implying that differential phosphorylation may contribute also to the regulation of plant HMGA proteins (Zhao and Grafi, 2000). Considering the importance of the complex post-synthetic control mechanisms of mammalian HMGA (Reeves and Beckerbauer, 2001), it is important to learn more about the regulation of the plant counterparts.

DNA and chromatin interactions

In several cases, HMGA proteins have been first recognised in electrophoretic mobility shift assays and footprinting studies by specifically interacting with

A/T-rich stretches frequently occurring in plant promoter regions (Pedersen *et al*., 1991; Nieto-Sotelo *et al*., 1994; Gupta *et al*., 1997b; Webster *et al*., 1997; Yamamoto and Minamikawa, 1997a; Martinez-Garcia and Quail, 1999; Zhao and Grafi, 2000; Zhang *et al*., 2003a). The HMGA proteins can interact with various affinities with different A/T motifs in double-stranded DNA. A random oligonucleotide selection experiment with pea HMGA revealed that HMGA binds to stretches of A/T-rich DNA of five or more bases (Webster *et al*., 1997). As measured by surface plasmon resonance, HMGA has a ca. 7-fold higher affinity for an immobilized A/T-rich DNA fragment than the structurally unrelated HMGB proteins (Webster *et al*., 2000). Mediated by the A/T-hook motifs, HMGA interacts with the DNA predominantly contacting the minor groove, and at least two A/T-hook motifs are required for efficient DNA binding (Nieto-Sotelo *et al*., 1994; Webster *et al*., 1997; Martinez-Garcia and Quail, 1999; Zhang *et al*., 2003a). A detailed study of the wheat HMGA protein has demonstrated recently that the four A/T-hooks do not contribute equally to the DNA binding of HMGA, since the N-terminal A/T-hooks play a major role in binding to a DNA fragment of the *PetE* promoter. Regions flanking the A/T-hook motifs can modulate the DNA binding properties, and therefore may account for the somewhat different DNA binding of the individual A/T-hook regions (Zhang *et al*., 2003a). Structural studies of various A/T-hook regions have also demonstrated that the regions flanking the core of the A/T-hooks contact the DNA to different extents (Huth *et al*., 1997), explaining the observed differences seen in DNA-binding experiments. Since plant HMGA proteins have four A/T-hooks, which are separated by unstructured linker regions, the individual A/T-hooks may contact several potential binding sites of a DNA substrate. Hence, through multiple A/T-hook-DNA contacts HMGA proteins are able to 'read the bar code' of DNA targets that are composed of several binding sites separated by varying numbers of base pairs (Reeves and Beckerbauer, 2001). Although the individual H1-like N-terminal domain specific for plant HMGA proteins does not bind DNA, it still enhances DNA binding of the full-length protein (Zhang *et al*., 2003a). By means of circular permutation analysis, rice HMGA (termed PF1) was shown to severely bend a fragment of the *phyA* gene promoter by ca. 80° (Martinez-Garcia and Quail, 1999). In contrast to this finding, no evidence for DNA bending was found in circularization studies with a DNA fragment of the plastocyanin gene enhancer and pea HMGA (Webster *et al*., 2001). Depending on the DNA substrate, binding of mammalian HMGA proteins can bend, straighten, unwind and loop the DNA molecules *in vitro* (Reeves and Beckerbauer, 2001).

Limited digestion of chromatin in intact maize nuclei with micrococcal nuclease resulted in the release of HMGA from the highly nuclease-sensitive chromatin, indicating that HMGA is enriched in open chromatin configurations, for instance, associated with transcriptional activity. Moreover, HMGA could be released from chromatin with distamycin A, a reagent that intercalates into A/T-rich DNA (Lichota and Grasser, 2001). This finding is consistent with distamycin A competition experiments performed on the binding of pea HMGA to naked DNA (Webster *et al*., 1997), suggesting that HMGA binds A/T-rich DNA both in the absence of nucleosomes and in the chromatin context. Plant HMGA can specifically bind isolated mononucleosome particles, both core particles (containing 146 bp of DNA, lacking internucleosomal linker DNA) (Arwood and Spiker, 1990), as well as nucleosomes containing ca. 165 bp of DNA (including linker DNA) (Lichota and Grasser, 2001). Specific nucleosome interaction of HMGA was affected by the presence of H1 linker histones, but HMGA could still bind the H1 containing particles (Lichota and Grasser, 2003). Moreover, HMGA and H1 (and HMGB) display preferential structure-specific binding to four-way junction DNA, as evident from the competition of the proteins for the DNA substrate in electrophoretic mobility shift assays (Zhang *et al*., 2003a, b).

HMGB proteins

Structure, expression and post-synthetic modifications

HMGB proteins that have been identified from plants share a conserved overall structure, which is different from that of HMGB proteins of other organisms (Grasser, 1995). The common feature of all HMGB proteins is the presence of one or two copies of the non-sequence-specific HMG-box DNA-binding domain of ca. 75 amino acid residues. The HMG-box domain has a conserved L-shaped fold with an angle of ca. 80° between the arms, consisting mainly of three α-helices (Travers, 2000; Thomas and Travers, 2001). In mammals, a number of proteins other than HMGB proteins have been identified, which also contain HMG-box domain(s), and many of them proved to be

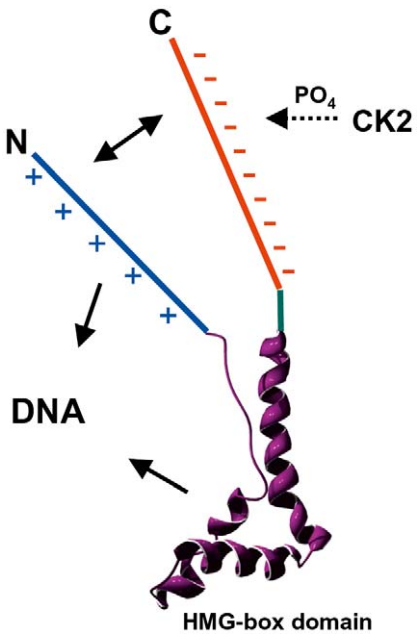

Figure 2. Schematic representation of plant HMGB proteins, and their interaction with DNA. Since to date no plant HMGB structure has been solved, the structure of the rat HMGB1 B-domain (amino acid residues P4-A72) (Weir *et al*., 1993) essentially consisting of three α-helices has been adapted here (in purple), as the global fold of the HMG-box domain is well conserved (Travers, 2000). In plant HMGB proteins, the basic N-terminal and the acidic C-terminal domain protrude from the same side of the central HMG-box domain, bringing them into proximity. The basic N-terminal domain (in blue) and the acidic tail (in red) are schematically indicated as straight lines (not drawn to scale). As demonstrated by intramolecular cross-linking, the basic N-terminal domain and the acidic C-terminal domain interact (indicated by a double-headed arrow), which is facilitated by protein kinase CK2-mediated phosphorylation of serine residues in the acidic tail (Thomsen and Grasser, unpublished). The enhanced intramolecular interaction can explain the observed reduced binding to linear DNA of phosphorylated HMGB1, as well as its increased thermal stability (Stemmer *et al*., 2002b). The DNA binding properties of the individual HMG-box domains of maize and rice HMGB1 resemble those of the full-length proteins, as the basic and acidic domains functionally neutralize each other. In the absence of the basic N-terminal region, the acidic tail most likely interacts with the HMG-box domain, presumably interfering with the DNA binding of the HMG-box domain, which can explain the severely reduced ability of N-terminally truncated HMGB1 to bind DNA (Ritt *et al*., 1998a; Wu *et al*., 2003b). When bound to DNA, both the concave face of the HMG-box domain and the basic N-terminal domain of (non-phosphorylated) full-length HMGB1 interact with the DNA (indicated by arrows). While the HMG-box domain predominantly contacts the minor groove, the basic N-terminal domain may bind the major groove of the DNA (Masse *et al*., 2002).

sequence-specific transcription factors such as LEF-1 and SRY (Bustin and Reeves, 1996). The global fold of the HMG-box domain is conserved between sequence-specific and non-sequence-specific HMG-box domains, but certain amino acid residues within the domain have been identified that differ between the two groups of proteins (Murphy *et al*., 1999; Masse *et al*., 2002; Klass *et al*., 2003). In the plant HMGB proteins, there is a single central HMG-box domain that is flanked by a basic N-terminal domain and a highly acidic C-terminal domain. While the HMG-box domain is relatively conserved between different plant HMGB proteins, the basic N-terminal and the acidic C-terminal domains are rather variable both in length and in amino acid sequence (Grasser, 1998). Therefore, the differences in the terminal domains mainly account for the variability in theoretical pI and in size of the plant HMGB proteins, ranging from ca. 13 kDa to 27 kDa (see below). The N- and C-terminal domains of plant HMGB proteins protrude from the same side of the central HMG-box domain bringing them into proximity (Figure 2). Recent studies using CD and fluorescence spectroscopy on full-length and truncated versions of the maize HMGB1 protein indicate that the terminal domains of the protein interact. In line with this finding, the basic N-terminal and the acidic C-terminal domains could be intramolecularly cross-linked using a zero-length cross-linker, demonstrating that terminal domains of maize HMGB1 (and other plant HMGB proteins) can interact (Thomsen and Grasser, unpublished).

In contrast to other eukaryotes (which express only a smaller number of different HMGB proteins), higher plants express a variety of structurally different HMGB proteins. Thus, five HMGB proteins (HMGB1–HMGB5) have been characterized (Table 1), each from the monocot plant maize (Stemmer *et al*., 1999) and from the dicot plant *Arabidopsis* (Stemmer *et al*., 1997). Based on northern and western blot analyses, the *hmgb* genes are considered to be expressed ubiquitously in the plant (O'Neill and Zheng, 1998; Yamamoto and Minamikawa, 1998; Stemmer *et al*., 1999; Wu *et al*., 2003a), although there are no expression data at cellular resolution. The five HMGB proteins are present in markedly different amounts in maize (HMGB1 and HMGB2/3 are about 20 times more abundant than HMGB4 and HMGB5), and the relative amounts of the proteins differ between the analysed maize tissues (Stemmer *et al*., 1999). In the short-day plant *Pharbitis nil*, the expression of an *hmgb* gene is regulated by an endogenous circa-

Table 1. Variability of HMGB proteins in *Arabidopsis* and maize

Protein[a]	Length (amino acids)	Mass (Da)	Accession number	AGI locus	Expression[b]
At-HMGB1	178	20 265	Y14071	At3g51880	cDNA,EST
At-HMGB2	144	15 982	Y14072	At1g20693	cDNA,EST
At-HMGB3	141	15 681	Y14073	At1g20696	cDNA,EST
At-HMGB4	138	15 364	Y14074	At2g17560	cDNA,EST
At-HMGB5	125	14 203	Y14075	At4g35570	cDNA,EST
At-HMGB6	241	26 964	AY086023	At5g23420	cDNA,EST
	221	25 761	–	At5g05330	–
	151	17 481	AY084626	At2g34450	cDNA,EST
	149	16 997	AY052244	At5g23405	cDNA,EST
Zm-HMGB1	157	17 146	X58282	–	cDNA,protein
Zm-HMGB2	139	15 316	Y08297	–	cDNA,protein
Zm-HMGB3	138	15 007	Y08298	–	cDNA,protein
Zm-HMGB4	126	14 104	Y08807	–	cDNA,protein
Zm-HMGB5	123	13 637	AJ006708	–	cDNA,protein

[a]Name of protein, in cases where the (native and/or recombinant) protein has been characterized.
[b]Indicates the evidence proving that the gene is expressed.

dian rhythm, whereas another *hmgb* gene is not under control of photoperiod or an endogenous rhythm, suggesting that some HMGB proteins may be involved in the circadian-regulated gene expression (O'Neill and Zheng, 1998).

Searching the available DNA sequence of the *Arabidopsis* genome (http://www.arabidopsis.org/) for sequences displaying sequence similarity to HMGB proteins has revealed that *Arabidopsis* encodes (in addition to the five previously identified HMGB proteins) a number of further HMGB candidates (Grasser *et al*., unpublished). They contain regions with similarity to HMG-box domains, and may therefore belong to the HMGB proteins. In particular, there are four genes encoding proteins that share the typical overall structure of plant HMGB proteins by having a central HMG-box domain, which is flanked by a basic N-terminal and an acidic C-terminal region (included in Table 1). Since the amino acid sequences of these proteins display some differences relative to the previously characterised group of *Arabidopsis* HMGB proteins (Stemmer *et al*., 1997), a representative of this group of 'novel' HMGB-type proteins has been functionally characterized. This protein proved to be localized to the nucleus, it contains a highly α-helical HMG-box domain, and it recognises DNA structure (Grasser *et al*., unpublished). Accordingly, this protein (now termed HMGB6) and possibly its relatives can be regarded members of the HMGB protein family. The HMGB6 protein of 27 kDa is the largest plant HMGB protein identified to date, which is mainly due to its unusually extensive N-terminal region of 109 amino acid residues. Together with the five previously characterized HMGB proteins (Stemmer *et al*., 1997), the three 'novel' HMGB proteins (for one of the four above-mentioned genes identified in the database search, evidence for expression is lacking, Table 1) sum up to eight HMGB proteins, which are expressed in *Arabidopsis*, providing a wide repertoire of these chromatin-associated proteins. In addition to the HMGB proteins, there may be other plant proteins containing one or more HMG-box DNA-binding domains (as mentioned above), but currently experimental proof for their functionality is lacking.

Vertebrate HMGB proteins are subject to a variety of post-synthetic modifications such as acetylation, methylation, ADP-ribosylation, and glycosylation, but the functional significance of these modifications is largely unknown (van Holde, 1989). Acetylation of an N-terminal lysine residue of mammalian HMGB1 can enhance its binding to DNA structures (Ugrinova *et al*., 2001). Insect HMGB proteins are phosphorylated by protein kinases PKC and CK2, altering the DNA-binding properties of the proteins (Wisniewski *et al*., 1994, 1999). For HMGB proteins

purified from plant tissue it was initially found that the masses calculated from the amino acid sequences are significantly less than the masses determined experimentally by mass spectrometry, indicating that the proteins are post-synthetically modified *in vivo* (Webster *et al*., 1997; Stemmer *et al*., 1999). Treatment of the HMGB1 and HMGB2/3 proteins isolated from maize immature kernels and suspension cultured cells with alkaline phosphatase (which can dephosphorylate phosphoproteins) resulted in a decrease of the masses of the HMGB proteins essentially to the values predicted from the amino acid sequences (Stemmer *et al*., 2002b, 2003). Therefore, native maize HMGB proteins are phosphoproteins, which occur in different phosphorylation states. The HMGB1 protein, for instance, exists in the mono-, double- and tetra-phosphorylated form, and to a minor extent in the double-phosphorylated form, while the non-phosphorylated protein is not detectable in the mass spectra (Stemmer *et al*., 2003). Serine residues within the acidic C-terminal domain have been mapped as the phosphoacceptor sites, and the same amino acid residues are phosphorylated by recombinant protein kinase CK2α *in vitro* (Stemmer *et al*., 2002b). Hence, most likely protein kinase CK2 is the enzyme catalysing the phosphorylation of the HMGB proteins in plant tissue. Protein kinase CK2 is a conserved eukaryotic Ser/Thr-kinase that occurs in various isoforms in the plant nucleus and cytosol (Riera *et al*., 2001). CK2 is involved in cell growth and proliferation, but also in stress response and cell survival (Litchfield, 2003; Meggio and Pinna, 2003). Currently, the involvement of the HMGB proteins in these important biological events is still elusive, but as architectural factors they may contribute to proper control of gene expression in these processes (see below). CK2-mediated phosphorylation of the maize HMGB proteins increases protein stability and reduces the affinity of the proteins for linear DNA, whereas the structure-specific recognition of DNA minicircles is not affected (Stemmer *et al*., 2002b). Mass spectrometric analyses also indicated that the HMGB proteins are modified by enzymes other than CK2 (Stemmer *et al*., 2002b, 2003). Therefore, the post-synthetic modifications further increase the number of plant HMGB protein variants, which have distinct properties and may be adapted to act in different DNA-dependent nuclear processes.

DNA and chromatin interactions

In general, HMGB proteins bind non-sequence-specifically to double-stranded DNA, but they can bind certain DNA structures (four-way junctions, DNA minicircles, *cis*-platinated DNA, etc.) with high affinity compared to linear DNA (Grasser, 1998; Travers, 2000; Thomas and Travers, 2001; Agresti and Bianchi, 2003). As studied by electrophoretic mobility shift assays, DNA footprinting and surface plasmon resonance, plant HMGB proteins bind to a variety of (A/T-rich) promoter regions (Pedersen *et al*., 1991; Grasser *et al*., 1994; Webster *et al*., 1997, 2000; Wu *et al*., 2003b). With a random oligonucleotide binding site selection assay, it was found that a pea HMGB protein binds preferentially to structurally flexible sites in linear DNA containing deformable dinucleotide steps (Webster *et al*., 1997). In line with that, upon DNA binding plant HMGB proteins can severely bend the DNA, as demonstrated by circularization experiments (Grasser *et al*., 1994; Ritt *et al*., 1998b; Webster *et al*., 2001; Wu *et al*., 2003b). In the circularization assays the HMGB proteins can mediate covalent intramolecular ring closure of short DNA fragments (<150 bp) catalysed by DNA ligase, whereas (due to the inflexibility of the DNA) formation of DNA minicircles does not occur in the absence of a DNA bending protein. Maize HMGB1 could facilitate the formation of DNA mini-circles as small as 70 bp, demonstrating its ability to bend the DNA remarkably (Grasser *et al*., 1994). HMGB proteins of various sources have been shown to bend DNA by over 90°, and the bending activity is an important feature of the HMGB proteins in their function as architectural factors (Travers, 2000; Thomas and Travers, 2001; Agresti and Bianchi, 2003). The concave face of the HMG-box domain primarily contacts the minor groove of the DNA, and a hydrophobic wedge usually consisting of four spatially close amino acid residues, is inserted deep into the minor groove. One residue partially intercalates between two base pairs introducing a kink, which contributes significantly to the overall bend (Travers, 2000; Thomas and Travers, 2001). The potential primary intercalating residue of the characterized plant HMGB proteins is a well-conserved phenylalanine residue (e.g. F49 in maize HMGB1), while some of the 'novel' *Arabidopsis* HMGB proteins (mentioned above) have other residues in the corresponding position (Figure 3). There are even greater differences in the potential secondary intercalation site (Travers, 2000; Thomas

and Travers, 2001), since all the previously characterized plant HMGB proteins have a valine residue at that position (e.g. V69 in maize HMGB1), while there is no amino acid residue conserved among the 'novel' HMGB proteins. The intercalating residues of HMG-box domains play a critical role in DNA binding and modulating DNA structure by bending and supercoiling (Stros and Muselíková, 2000; Klass *et al*., 2003). Therefore, the DNA-binding properties of the 'novel' *Arabidopsis* HMGB proteins (as well as related proteins of other species) may differ to some extent from the HMGB proteins characterized previously, and need to be further analysed.

It is well established that plant HMGB proteins preferentially bind certain DNA structures such as DNA mini-circles, four-way junctions, and supercoiled DNA, relative to linear DNA (Grasser *et al*., 1994; Stemmer *et al*., 1997; Ritt *et al*., 1998a; Webster *et al*., 2001; Wu *et al*., 2003b; Zhang *et al*., 2003b). Detailed studies of the maize and rice HMGB1 proteins revealed that the basic N-terminal domain significantly stimulates the binding to linear DNA, whereas the acidic C-terminal domain has the opposite effect. However, the domains flanking the central HMG-box DNA-binding domain have only little influence on the binding to DNA mini-circles. Full-length HMGB1 displays DNA-binding properties similar to those of the individual HMG-box domain, suggesting that the terminal domains functionally neutralize each other (Ritt *et al*., 1998a; Wu *et al*., 2003b). This finding is in line with the interaction of the basic N-terminal and the acidic C-terminal domains in maize HMGB1 (Figure 2). Moreover, this interaction can explain the negative effect of CK2-mediated phosphorylation of residues within the acidic tail of maize HMGB1 on binding to linear DNA, while the phosphorylation does not affect the affinity for DNA mini-circles (Stemmer *et al*., 2002b). Phosphorylation of the acidic tail of HMGB1 by protein kinase CK2 enhances the interaction with the basic N-terminal domain (Thomsen and Grasser, unpublished) and, therefore, limits the positive effect of the basic domain on interactions with linear DNA, while the basic region is dispensable for binding DNA mini-circles (Ritt *et al*., 1998a; Wu *et al*., 2003b). The basic N-terminal domain can support DNA binding of the plant HMGB proteins by directly interacting with the DNA (Ritt *et al*., 1998a). The yeast HMGB protein, NHP6A, consists of an HMG-box domain and a basic N-terminal region, which facilitates DNA binding (Yen *et al*., 1998). Solution of the structure of the NHP6A/DNA complex revealed that the HMG-box domain is primarily bound to the minor groove, whereas the basic N-terminal domain wraps over the DNA backbone from the minor groove into the major groove of the DNA (Masse *et al*., 2002). This situation is likely to be similar for many plant HMGB proteins, as they also contain the proline residue between the N-terminal basic region and the HMG-box domain (P^{38} in maize HMGB1), which seems to be critical for directing the N-terminal domain into the major groove (Yen *et al*., 1998; Masse *et al*., 2002).

HMGA and HMGB proteins from plants can bind to A/T-rich promoter regions, and in some cases they even bind overlapping sites within these promoter regions (Pedersen *et al*., 1991; Webster *et al*., 1997), which raised the question of a possible interference between the two structurally unrelated HMG protein families upon DNA binding. Moreover, both HMGA and HMGB proteins bind preferentially certain DNA structures such as four-way junction DNA (Zhang *et al*., 2003b), while only HMGB proteins bind DNA mini-circles with high affinity (Webster *et al*., 2001). In the case of pea HMGA, it was demonstrated that an HMGB protein could directly interact with HMGA and enhance the binding of HMGA to an A/T-rich enhancer element. Thereby, the N-terminal region of the HMGB protein containing the HMG-box domain interacted with the C-terminal A/T-hook region of the HMGA protein (Webster *et al*., 2001). The interaction of maize HMGA and HMGB1 is mediated by contacts between the acidic tail of HMGB1 and the A/T-hook region of HMGA (Grønlund and Grasser, unpublished). The significance of the interaction between members of the two HMG protein families requires further elaboration.

The chromatin-association of HMGB proteins was examined by extracting the proteins from isolated maize nuclei using various concentrations of NaCl, ethidium bromide and spermine. While the different HMGB proteins (detected by immunoblotting) were similarly released from chromatin by NaCl, there were clear differences in the extractability using ethidium bromide and spermine. Thus, ethidium bromide released preferentially HMGB2/3 and HMGB4, whereas spermine released exclusively HMGB1 (Lichota and Grasser, 2001). Limited digestion of the chromatin with micrococcal nuclease also resulted in a differential release of the HMGB proteins. HMGB2/3 appears to be associated primarily with the highly nuclease-sensitive chromatin, whereas HMGB1 and especially HMGB4 and HMGB5 were released only

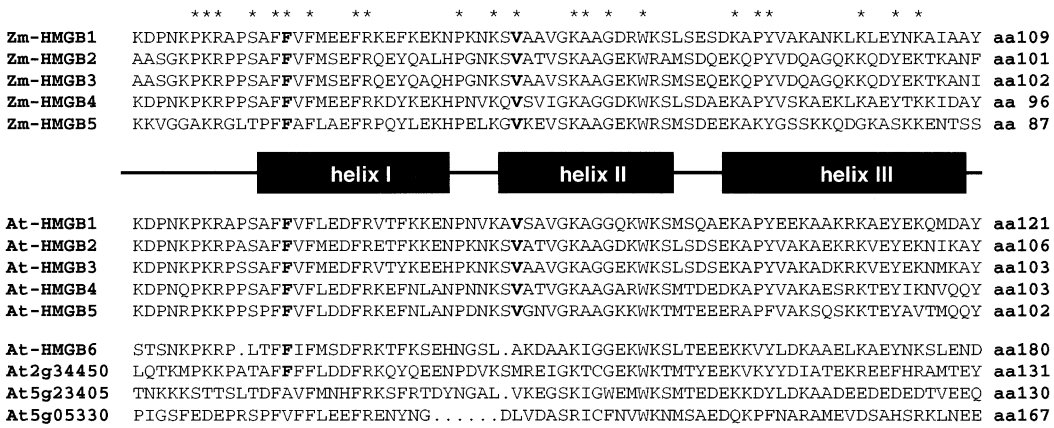

Figure 3. Alignment of the amino acid sequences of the HMG-box domains of the HMGB proteins from maize and *Arabidopsis*. The alignment contains the sequences of the five characterized HMGB proteins (HMGB1-HMGB5) from *Zea mays* (Ritt *et al*., 1998b) (top part), the five previously characterised HMGB proteins (HMGB1-HMGB5) from *Arabidopsis thaliana* (middle part), which display sequence and functional similarities to the maize proteins (Stemmer *et al*., 1997). The database accession numbers are included in Table 1. The positions of the three α-helices (cf. Figure 2) of the HMG-box domain (deduced by analogy from the solved structures of other HMGB proteins, (Thomas and Travers, 2001)) are indicated (helices I– III). In the bottom part, there are four sequences recently identified in the genome sequence of *Arabidopsis thaliana*, encoding potential 'novel' plant HMGB proteins. These sequences display several differences in conserved amino acid positions compared to the previously identified HMGB proteins (top and middle part of the alignment) both within and outside the HMG-box domain. Therefore, experimental evidence is needed to determine, whether these sequences actually encode typical HMGB proteins. The HMGB6 protein shares several key properties with the characterized plant HMGB proteins (Grasser *et al*., unpublished). For the sequence At5g05330, there is currently no evidence for expression (see Table 1). Highly conserved amino acid positions are indicated by asterisks above the sequences (conserved in at least nine of the ten maize and *Arabidopsis* HMGB1-HMGB5 sequences), while the conserved potential DNA-intercalating residues (phenylalanine, valine) are indicated (in bold). The amino acid positions of the C-terminal residue of the HMG-box domains within the full-length proteins are given next to the sequences.

after more extensive digestion of the chromatin (Lichota and Grasser, 2001). These results indicate that the various plant HMGB proteins are differentially associated with chromatin. Since the different HMGB proteins have similar DNA-binding properties (although there are subtle differences, which may prove to be functionally important) (Ritt *et al*., 1998b), in addition to DNA binding, interactions with histones and other chromosomal proteins are likely to be important determinants of the differential chromatin association of plant HMGB proteins. In electrophoretic mobility shift assays, maize HMGB proteins can bind isolated mononucleosomes containing DNA of ca. 165 bp. Removal of the core histone N-termini by limited tryptic digestion does not abolish the nucleosome binding of the HMGB proteins, but the specificity of the interaction appears to be affected. Analysis of the nucleosome interactions of full-length and truncated HMGB1 proteins demonstrated that only the full-length protein can bind nucleosomes specifically, as both deletion of the basic N-terminal domain and deletion of the acidic C-terminal domain abolish specific nucleosome interactions (Lichota and Grasser, 2001, 2003). Most likely, proper nucleosome interaction of the plant HMGB proteins requires simultaneous interactions of the HMG-box domain and the acidic tail with DNA and core histone(s), respectively. Recently, an intriguing novel function for HMGB proteins was proposed in the context of chromatin remodelling. Mammalian HMGB1 was found to facilitate the nucleosome sliding mediated by the ATP-dependent nucleosome remodelling complexes CHRAC and ACF. Presumably, HMGB1 can stimulate the nucleosome mobilization by assisting the initial rate-limiting distortion of the nucleosomal linker DNA (Bonaldi *et al*., 2002). Therefore, HMGB1 may act as DNA chaperone promoting chromatin dynamics, which is an important prerequisite for the efficient progress of various DNA-dependent processes (Bonaldi *et al*., 2002; Narlikar *et al*., 2002; Reyes *et al*., 2002; Agresti and Bianchi, 2003; Travers, 2003). Interestingly, several chromatin remodelling complexes contain subunits that also have an HMG-box DNA binding domain (Papoulas *et al*., 2001; Chi *et al*., 2002).

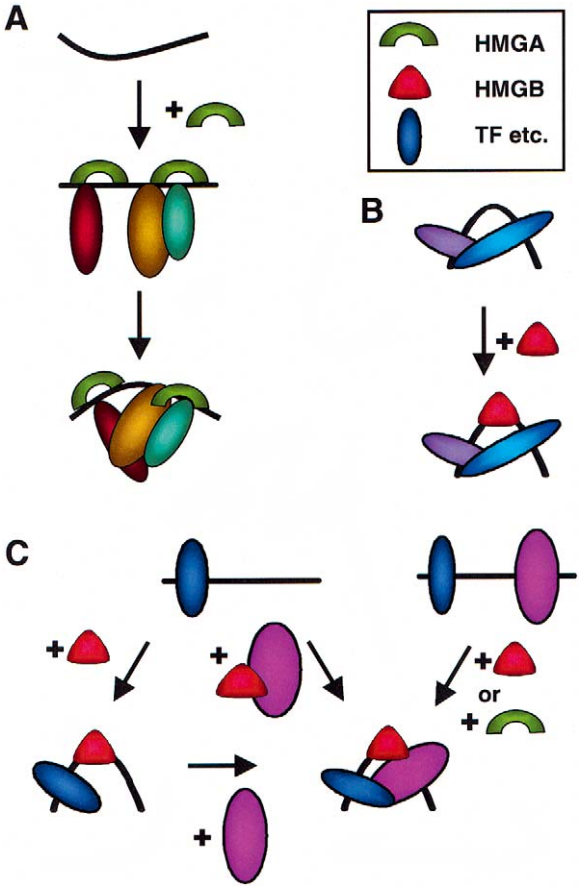

Figure 4. Model for the architectural function of HMGA and HMGB proteins in the formation of nucleoprotein structures. Both HMGA and HMGB proved to be versatile and functionally flexible proteins presumably involved in a variety of DNA-dependent processes, although most evidence has accumulated in the context of transcriptional regulation, but (as mentioned in the text) it is very likely that these proteins (especially HMGB) act also in other processes (recombination, DNA repair, etc.). A. Simplified view of the role of HMGA in the formation of enhanceosomes as typified by the human *IFN-β* enhanceosome. The target sites for several transcription factors NFκB(p50+p65), IRF-1, ATF-2/c-Jun (TF, indicated by ovals) in the DNA (indicated by a black line) are bound only with low affinity, due to an unfavourable intrinsic bend of the DNA. Initially, binding of HMGA to this region unbends the DNA and allows high-affinity interaction with the transcription factors (only a subset is shown). In a second step, multiple protein-protein interactions between the components of the complex (including HMGA) result in the formation of the remarkable stable enhanceosome (Yie *et al*., 1999). The stability of the enhanceosome is regulated by acetylation of specific lysine residues of HMGA (Munshi *et al*., 2001). B. Sequence-specific factors (site-specific recombinases, transcription factors, etc.) may bind their target sites creating a transient DNA bend, which represents a high-affinity binding site for HMGB proteins. Due to its DNA bending activity, the recruited HMGB protein can stabilise the final complex, and no protein-protein interactions between the HMGB proteins and the specific factors are required. The HMGB proteins may be part of the final assembly, like in the enhanceosome formed at the *BHLF-1* promoter (Ellwood *et al*., 2000). C. In other situations, the HMGB-induced DNA bending is essential for initiating complex formation (McKinney and Prives, 2002; Mitsouras *et al*., 2002). There may be different orders and mechanisms for HMGB recruitment, and HMGB can interact only transiently to establish the complex, or it can be a stable component of the assembly. Due to its abundance, HMGB binds the DNA randomly, eventually resulting in the formation of productive complexes (Grosschedl, 1995). Alternatively, HMGB proteins (in other biological contexts HMGA proteins may act in a similar way) are specifically recruited by protein-protein interactions with sequence-specific regulators. Free or DNA-bound regulators can recruit the architectural HMGA or HMGB proteins. As demonstrated for the maize HMGB proteins, various members of the HMGB family have different efficiencies to stimulate the formation of specific complexes (Stemmer *et al*., 2002a), and they interact differently with sequence-specific factors, which is in addition regulated by post-translational modifications of the HMGB proteins (Krohn *et al*., 2002). Therefore, it is likely that depending on the geometry of the complex and the factors involved, particular HMGB variants are recruited as architectural assistant factors.

Chromosomal HMGA and HMGB proteins as architectural cofactors of DNA-dependent processes

DNA-dependent processes such as transcription are under control of DNA-binding factors, which recognize specifically *cis*-acting DNA target sequences. In eukaryotes, usually various regulators bound to different DNA sequence elements interact to form higher-order nucleoprotein structures in which multiple protein-protein and protein-DNA contacts enhance the specificity and stability of the regulatory complexes (combinatorial control). During differentiation or in response to environmental signals, specific sets of genes within the large eukaryotic genomes have to be activated or repressed. This is achieved by assembling complex three-dimensional nucleoprotein structures containing various transcription factors bound to their cognate promoter/enhancer elements (also termed enhanceosomes) (Tjian and Maniatis, 1994; Merika and Thanos, 2001). Therefore, a gene can be activated by an enhancesosome only if all components of the final complex are simultaneously available. The correct assembly of these DNA-bound multiprotein complexes often requires the assistance of architectural factors such as HMGA and HMGB proteins that can modulate DNA structure and/or contribute to the protein interactions within the complex.

The best studied enhanceosome is that formed at the enhancer of the human virus-inducible *IFN-β* gene. The assembly of the *IFN-β* enhanceosome is inhibited by an unfavourable intrinsic DNA curvature,

which is responsible for the low affinity of the activators for their binding sites. Binding of HMGA to the promoter unbends the DNA and enhances the binding of several transcription factors to the promoter (Yie *et al*., 1999). This allosteric effect on the DNA results in a significantly increased affinity of the activators for their binding sites in the absence of protein-protein interactions (Figure 4A). Finally, the enhanceosome is stabilized by a complex network of mutual protein-protein interactions between activators and HMGA, leading to a remarkable stable nucleoprotein structure (Yie *et al*., 1999). Dynamic control of *IFN-β* gene expression requires the regulated assembly and disassembly of the enhanceosome. This is accomplished (as mentioned above) by differential acetylation of HMGA. Acetylation of HMGA by the acetyltransferase CBP destabilizes the complex, whereas acetylation by PCAF/GCN5 potentiates transcription by stabilizing the enhanceosome (Munshi *et al*., 2001). Therefore, the ordered and highly regulated acetylation of HMGA coordinates the transcriptional switch by causing either enhanceosome stabilization or destabilization. The enhanceosome instructs the recruitment of chromatin remodelling factors and general transcription factors. In the course of the promoter rearrangements, a nucleosome blocking the core promoter slides to a downstream position allowing transcriptional activation of the gene (Lomvardas and Thanos, 2002). There are numerous other examples for an involvement of HMGA in the formation of productive complexes contributing to transcriptional activation (Reeves and Beckerbauer, 2001). The role of HMGA in the assembly of complexes regulating gene transcription is to facilitate the DNA binding of transcription factors by inducing allosteric effects on DNA and/or by direct protein-protein interactions with the sequence-specific factors. In electrophoretic mobility shift assays, the rice HMGA protein (termed PF1) could enhance (ca. 10-fold) the binding of the transcriptional activator GT-2 to the *phyA* gene promoter. Since no direct physical contacts could be detected between HMGA and GT-2, the stimulation of GT-2 DNA binding was attributed to a pre-conditioning of the DNA target site by HMGA (Martinez-Garcia and Quail, 1999).

Another mode of action that has been proposed for HMGA concerns its involvement in modifying chromatin structure (Reeves and Beckerbauer, 2001). It was found that HMGA could cause transcriptional activation by displacing inhibitory proteins (such as linker histone H1) from A/T-rich scaffold attachment regions (SARs, also termed matrix attachment regions, MARs) (Zhao *et al*., 1993). Maize HMGA could also relieve *in vitro* the inhibitory effect exerted by H1 on transcription driven by an A/T-rich zein gene promoter, suggesting that transcription may be controlled to some extent by the interplay of HMGA and H1 (Zhao and Grafi, 2000). A/T-rich sequences can act as quantitative, non-tissue-specific enhancers of plant gene expression, and binding of HMGA to these sequences may play a critical role (Sandhu *et al*., 1998). More recently, it has been suggested that HMGA, in addition to assisting the DNA binding of transcription factors, may contribute to the stimulation of gene expression at the pea *PetE* enhancer by facilitating the interaction with the nuclear matrix and recruiting histone acetyltransferase activity, resulting in an altered chromatin structure favourable for transcription (Chua *et al*., 2003). In a genetic screen in yeast for proteins conferring resistance against nickel toxicity, maize HMGA was identified, since expression of HMGA enabled growth of the yeast cells in the presence of toxic nickel concentrations. HMGA appears to prevent nickel toxicity by modifying chromatin structure (Forzani *et al*., 2001).

The stimulatory effect of HMGB proteins on the formation of complex nucleoprotein structures was initially characterized with bacterial site-specific recombination reactions as model systems. In these reactions, HMGB proteins could stimulate the assembly of productive synaptic complexes, which is an essential step in the reaction catalysed by the recombinases (Grosschedl, 1995). Maize HMGB proteins can facilitate the site-specific β-recombination reaction *in vitro* and *in vivo* without physically contacting the recombinase (Figure 4B). Most likely, the DNA bending activity of the HMGB proteins can assist the correct three-dimensional formation of the synaptic complex. Depending on the DNA substrate, the various maize HMGB proteins display different efficiency in stimulating β recombination, indicating that the members of the HMGB family have somewhat different abilities to support the assembly of nucleoprotein structures (Stemmer *et al*., 2002a). HMGB proteins interact with various transcription factors facilitating their sequence-specific DNA binding (Figure 4C). The protein-protein interactions with transcription factors explain how the non-sequence-specific HMGB proteins are recruited to their sites of action (Thomas and Travers, 2001; Agresti and Bianchi, 2003). In other cases such as the Epstein-Barr virus *BHLF-1* promoter, HMGB is recruited (analogous to the

site-specific recombination reactions) without physical interactions with the transcription factors. The transcription factors (two ZEBRA dimers, in the case of the *BHLF-1* promoter) bind the target DNA weakly, creating a transient bend, which represents a high-affinity binding site for HMGB (Figure 4B), resulting in HMGB recruitment (Ellwood *et al*., 2000). HMGB is part of the final assembly, stabilising the enhanceosome. At the *BHLF-1* enhancer, HMGB displays a different mode of action, binding the target DNA only transiently to establish binding of two molecules of the viral activator Rta by a charperone mechanism. Here, the DNA-bending activity of HMGB is the primary determinant of the stimulatory effect (Figure 4C), since other, even structurally unrelated bending proteins such as bacterial HU could substitute for HMGB in promoting enhanceosome assembly, whereas mutants affected in HMGB-mediated bending failed to stimulate complex formation (Mitsouras *et al*., 2002). The importance of HMGB-induced DNA bending has been also demonstrated in a study examining the functional interaction of HMGB1 and the tumor suppressor protein p53. HMGB1 stimulates the binding of p53 to linear DNA, but not when the target sequence occurs in pre-bent DNA (McKinney and Prives, 2002). HMGB proteins enhance the DNA binding of various transcription factors, but in many cases they are not detectable in the final complex with DNA. Therefore, depending on the promoter/enhancer geometry and on the transcription factors involved, HMGB proteins may leave the assembly after initiating formation of the transcription factor-DNA complex.

Maize HMGB1 can stimulate reporter gene expression in transient co-transformation experiments in protoplasts (Grasser *et al*., 1993), and there are also a few examples for functional plant HMGB-transcription factor interactions. Thus, a wheat HMGB protein can stimulate the binding of the bZIP transcription factor EmBP-1 to its DNA target site (Schultz *et al*., 1996). Maize HMGB1 was found to interact with the zinc-finger transcription factors Dof1 and Dof2 (through their Dof DNA binding domain), and to facilitate Dof DNA binding (Yanagisawa, 1997). The HMG-box domain of HMGB1 mediates the interaction and the individual domain is sufficient for stimulating Dof2 DNA binding. Although all maize HMGB proteins can cooperate with Dof2, they do so with different efficacy (Krohn *et al*., 2002). Thus, HMGB5 is clearly most effective, and can stimulate the binding of Dof2 to its target site >30-fold in naked DNA, and it can also facilitate Dof2 binding to the target site in the nucleosomal context (Cavalar *et al*., 2003). Moreover, phosphorylation of HMGB1 and HMGB2/3 by protein kinase CK2α (mentioned above) abolishes the functional interaction with Dof2 (Krohn *et al*., 2002). Since HMGB1 and HMGB2/3 occur as phosphoproteins in maize (Stemmer *et al*., 2002b, 2003), HMGB5 is presumably the primary assistant factor of Dof2.

Studies on the inactivation of *hmg* genes in yeast and mammals have confirmed that these proteins have important cellular roles. Knockout of the mouse *hmga1* gene revealed that HMGA1 is required for normal sperm development, while inactivation of the *hmga2* gene results in the mouse pygmy phenotype (Zhou *et al*., 1995; Liu *et al*., 2003). The lack of HMGB1 causes pleitropic defects in mice and they die soon after birth, but cell lines can grow normally without HMGB1 (Calogero *et al*., 1999). Mice lacking HMGB2 (which is ca. 80% identical to HMGB1) are viable, but male mice have reduced fertility, since HMGB2 seems to play a role in germ cell differentiation (Ronfani *et al*., 2001). In yeast, knockout of one of the two *nhp6a/b* genes (encoding HMGB proteins) did not result in a phenotype distinct from the wild type, but the inactivation of both genes lead to growth aberrations such as temperature-sensitive growth and various morphological defects (Costigan *et al*., 1994). Analyses of the gene expression in the strain lacking both HMGB proteins (NHP6A and NHP6B) revealed that the induction of transcription and the expression levels of a variety of genes is altered relative to the control strain (Paull *et al*., 1996; Moreira and Holmberg, 2000). The expression of the majority of genes (6144 genes tested) is not affected, but certain genes are up-regulated (1.9% of the genes tested), whereas other genes are down-regulated (1.4% of the genes tested) in the double-mutant strain (Moreira and Holmberg, 2000).

In summary, the analyses of the role of HMGA and HMGB proteins in the formation of higher-order nucleoprotein structures and the *hmg* gene knockout experiments indicate that the HMGA and HMGB proteins act as architectural factors that contribute to proper transcriptional control. They can be involved in both the up- and down-regulation of gene expression (Moreira and Holmberg, 2000; Reeves and Beckerbauer, 2001). In addition to their role in gene transcription, the HMGA proteins can facilitate the integration of viral DNA in host cell chromosomes, while the versatile HMGB proteins can also assist recombination reactions, DNA replication, transpos-

ition, and DNA repair (Bustin and Reeves, 1996; Bustin, 1999; Reeves and Beckerbauer, 2001; Thomas and Travers, 2001; Zayed *et al*., 2003). Although there are some parallels in the action of HMGA and HMGB proteins (indicated in Figure 4), the two protein families also have distinct features. Due to their unusual structural flexibility, the HMGA proteins can act as a sort of molecular 'glue', stimulating the cooperative assembly of nucleoprotein complexes by orchestrating multiple protein-DNA and protein-protein interactions (Merika and Thanos, 2001; Reeves and Beckerbauer, 2001). The HMGB proteins have a remarkable DNA-bending activity, which can facilitate DNA conformational changes providing the DNA flexibility that is required for folding the DNA into the appropriate three-dimensional nucleoprotein structures (Ross *et al*., 2001; Thomas and Travers, 2001; Agresti and Bianchi, 2003). In certain cases, the assembly of these complexes requires also protein-protein interactions between the HMGB proteins and specific regulators. Thus, the HMGA and HMGB proteins in cooperation with sequence-specific regulators form (in response to signal transduction pathways) complexes, in which the synergy of multiple protein-DNA and protein-protein interactions provides the necessary precision for the proper control of DNA-dependent processes in complex eukaryotic genomes.

Acknowledgements

Research in my laboratory is supported by grants from the Danish Research Council. I would like to thank Anne Lentz and Jesper Grønlund for comments on the manuscript.

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