Basic and Acidic Regions Flanking the HMG-Box Domain of Maize HMGB1 and HMGB5 Modulate the Stimulatory Effect on the DNA Binding of Transcription Factor Dof2†

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ABSTRACT: The chromatin-associated high-mobility group (HMG) proteins of the plant HMGB family are characterized by a central HMG-box domain that is flanked by a basic N-terminal and an acidic C-terminal domain. By functional interaction with certain transcription factors, HMGB proteins contribute to transcriptional regulation. Previous work has shown that the maize HMGB5 protein is markedly more efficient than other HMGB proteins in stimulating the binding of transcription factor Dof2 to DNA target sites. Here we examine the structural requirements that determine the particular efficiency of HMGB5. The HMG-box domains of HMGB1 and HMGB5 (which mediate the interaction with Dof2) promoted Dof2−DNA binding to a similar extent, indicating that the terminal domains modulate the interaction with Dof2. Analysis of full-length, truncated, and chimeric HMGB1/5 proteins revealed that the acidic C-terminal domains positively influence the stimulation of Dof2−DNA binding, while the basic N-terminal domains have a rather negative effect. In particular, the C-terminal domain of HMGB5 has a striking positive effect and may account for the efficient stimulation mediated by full-length HMGB5. Interestingly, recombinant HMGB protein variants that have a relatively low affinity for linear DNA (such as proteins lacking the basic N-terminal domain) efficiently assist Dof2−DNA binding.

In eukaryotes, transcript initiation is controlled by the concerted action of sequence-specific transcription factors binding their DNA recognition sequences in promoter regions. The DNA-bound factors form higher-order nucleoprotein structures that regulate the expression of many genes in a tissue- and developmental stage-specific manner. In these complexes, multiple protein−DNA and protein−protein interactions provide the precision required for efficient and controlled gene transcription in higher eukaryote genomes (*1*). Because of the limited structural flexibility of the DNA double helix, the assembly of the regulatory complexes often is assisted by architectural DNA-bending proteins. Among these architectural proteins are the chromatin-associated high-mobility group (HMG)[1] proteins of the HMGB family (*2*−*4*). Typically, HMGB proteins contain one or two copies of the ~75-amino acid HMG-box DNA-binding domain. They bind DNA non-sequence-specifically, but they display a high affinity for various DNA structures (*2*−*4*). HMGB proteins do not act as classical transcriptional regulators, but they can assist transcription factor function. By direct interactions with sequence-specific transcriptional regulators, they can be recruited to target sites. Thus, HMGB proteins of various sources have been found to interact functionally with a variety of transcription factors, including members of the Rel, HOX, p53, octamer, and SREBP families as well as TFIID/TFIIA and steroid hormone receptors (*5*−*12*).

Higher plants express several chromosomal HMGB proteins, which contain a central HMG-box domain that is flanked by a basic N-terminal and an acidic C-terminal domain (*13*, *14*). The N- and C-terminal domains modulate the DNA interactions of the HMGB proteins (*15*, *16*). The plant HMGB proteins are structurally variable, in particular within their basic and acidic terminal domains. Accordingly, they differ from each other in their chromatin association, in their post-translational modifications, and in some of their DNA interactions (*17*−*20*). Plant HMGB proteins bind DNA non-sequence-specifically, but they recognize certain DNA structures, including four-way junctions and DNA minicircles (*16*, *19*, *21*, *22*). Moreover, upon DNA binding they severely bend the DNA, which is a typical property of the HMG-box DNA-binding domain (*15*, *16*).

To date, HMGB proteins have been shown to interact with plant transcription factors of the bZIP and Dof families. Thus, a wheat HMGB protein [termed HMGb, which is closely related to maize and rice HMGB1 (*23*)] can stimulate the binding of the bZIP transcription factor EmBP-1 to its DNA target site, whereas other tested HMG proteins are not effective (*24*). The plant-specific family of Dof transcription factors (DNA binding with one finger) contain a conserved Dof domain that may form a single C_2-C_2 zinc finger (*25*).

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[1] Abbreviations: HMG, high-mobility group; Dof, DNA binding with one finger; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; CD, circular dichroism.

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The Dof domain mediates the binding to the AAAG core motif found in the promoter regions of a variety of plant genes (*26*). Dof proteins as transcription factors play critical roles in various processes, including tissue-, hormone-, and light-regulated gene expression, as well as metabolic control (*27*−*34*). Maize HMGB1 has been shown to interact with transcription factors Dof1 and Dof2 (through their Dof DNA-binding domain) and promotes Dof−DNA binding (*35*). The HMG-box domain of HMGB1 mediates the interaction and the individual domain can stimulate Dof2−DNA binding. All maize HMGB proteins can cooperate with Dof2, but HMGB5 is clearly more effective than the other tested HMGB proteins (HMGB1−HMGB4) in promoting binding of Dof2 to its target DNA site (*36*). HMGB5 can also facilitate the binding of Dof2, when the binding sites were incorporated into reconstituted nucleosomes (*37*). The studies analyzing the functional interaction of maize HMGB proteins and Dof2 suggested that the transcription factor interacts preferentially with certain members of the HMGB protein family. Here, we have examined the specificity determinants of the HMGB−transcription factor interaction. The interaction of various recombinant versions of maize HMGB1 and HMGB5 with transcription factor Dof2 was studied biochemically, revealing that the basic and in particular the acidic domains flanking the HMG-box domain critically influence the HMGB-facilitated DNA binding of Dof2.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The region encoding the HMG-box domain of HMGB5 was amplified by PCR using DNA polymerase DeepVent (NEB), primers (1) 5′-AAGGATCCAAGAAGGTCGGCGGCGCCAA and (2) 5′-AATTAAGCTTAGGAGCTAGTGTTCTCCTTCTT, and plasmid pQE9cm-HMGe (containing the complete HMGB5 coding sequence) (*38*) as the template. The *Bam*HI−*Hin*dIII-digested DNA fragment was cloned into the *Bam*HI−*Hin*dIII-digested expression vector pQE9 (Qiagen), resulting in plasmid pQE9-HMGB5box. For the construction of the fusion of the HMGB5 N-terminal domain and the HMG-box domain of HMGB1, the sequence encoding the N-terminal region of HMGB5 was amplified by PCR using primers (3) 5′-CACAGAATTCATTAAAGAGGAGA and (4) 5′-AATTGGATCCGCGCTTGGCGCCGGTGGCCT and pQE9cm-HMGe as the template. The *Eco*RI−*Bam*HI-digested PCR fragment was cloned in frame with the sequence encoding HMGB1box of the *Eco*RI−*Bam*HI-digested plasmid pQE9-HMGa(G35-Y109) (containing the sequence encoding the HMG-box domain of maize HMGB1) (*15*), giving plasmid pQE9-B5N-HMGB1box. For the construction of the fusion of the C-terminal domain of HMGB5 with the HMG-box domain of HMGB1, the region encoding the C-terminal domain of HMGB5 was amplified by PCR using primers (*5*) 5′-AATTAAGCTTAAGAAGGCCAAAGCTGAT and (*6*) 5′-CAGATGGAGTTCTGAGGTCATT and pQE9cm-HMGe as the template. The *Hin*dIII-digested PCR fragment was cloned in frame with the sequence encoding HMGB1box of the *Hin*dIII-digested plasmid pQE9-HMGa(G35-Y109), giving pQE9-HMGB1box-B5C. To generate a construct, in which both the N- and C-terminal domains of HMGB5 are fused to the HMG-box domain of HMGB1, the plasmids pQE9-B5N-HMGB1box and pQE9-HMGB1box-B5C were digested with *Xho*I (which cleaves within the region encoding HMGB1box and in the plasmid backbone). The *Xho*I fragment containing the region encoding the N-terminal part of pQE9-B5N-HMGB1box was cloned in frame with the *Xho*I fragment encoding the C-terminal part of pQE9-HMGB1box-B5C, giving plasmid pQE9-B5N-HMGB1box-B5C. All plasmid constructs were confirmed by DNA sequencing.

Protein Production and Purification. Using the expression plasmids mentioned above and previously described expression plasmids (*15*, *38*), the six-His-tagged HMGB proteins were expressed in *Escherichia coli* and purified by three-step column chromatography (*15*, *39*). First, the proteins were purified by metal-chelate chromatography using Ni-NTA−agarose, followed by FPLC ion exchange chromatography using S Sepharose Fast Flow (Pharmacia). The final purification step was a FPLC ion exchange chromatography using a Resource Q column (for the proteins that contain an acidic C-terminal domain), while the proteins lacking an acidic tail were purified by FPLC hydrophobic interaction chromatography using phenyl Sepharose (Pharmacia) as previously described (*15*, *39*). The six-His-tagged and the GST-tagged versions of the Dof domain of Dof2 were expressed in *E. coli* and purified as previously described (*36*). Six-His-tagged full-length Dof2 was expressed in *E. coli* and purified by metal-chelate chromatography as previously described (*37*). Dof2 was further purified by cation exchange chromatography. The protein was loaded in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (1×TE) onto a CM Sepharose column (Pharmacia). Following a washing step with 1×TE containing 300 mM NaCl, the protein was eluted from the column with 1×TE containing 700 mM NaCl. The eluate was desalted by ultrafiltration, and after addition of 10% glycerol, the protein was stored at −80 °C.

Circular Dichroism. Proteins were diluted to a final concentration of 15 μM in buffer containing 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. Spectra were recorded essentially as previously described (*20*, *40*) at ∼20 °C over the wavelength range of 195−250 nm with measurements every 0.2 nm using a Jasco J-715 instrument and cuvettes with a path length of 1 mm. The spectra represent an average of 10 scans, which were subsequently processed for baseline subtraction.

Protein Cross-Linking. Protein interaction was examined by chemical cross-linking essentially as previously described (*36*). The six-His-tagged Dof domain of Dof2 (1 μg) was incubated for 5 min at 20 °C with an equimolar amount of the HMGB proteins in NaCl/P_i in a final volume of 200 μL. The cross-linking reaction was started by adding glutaraldehyde to a final concentration of 0.0125%. The proteins were precipitated with 25% trichloroacetic acid, washed twice with acetone, dried, and resuspended in SDS loading buffer. The proteins were separated by SDS−PAGE in 18% polyacrylamide gels and stained with Coomassie Blue.

GST Pulldown Assay. Protein interaction between the GST-tagged Dof domain of Dof2 and the HMG-box domains was analyzed by GST pulldown assays as previously described (*36*).

Electrophoretic Mobility Shift Assays (EMSAs). Protein binding to a double-stranded ^{32}P-labeled 21 bp Dof site oligonucleotide containing the Dof binding site (*35*) was examined using EMSAs. Binding reaction mixtures contained binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1

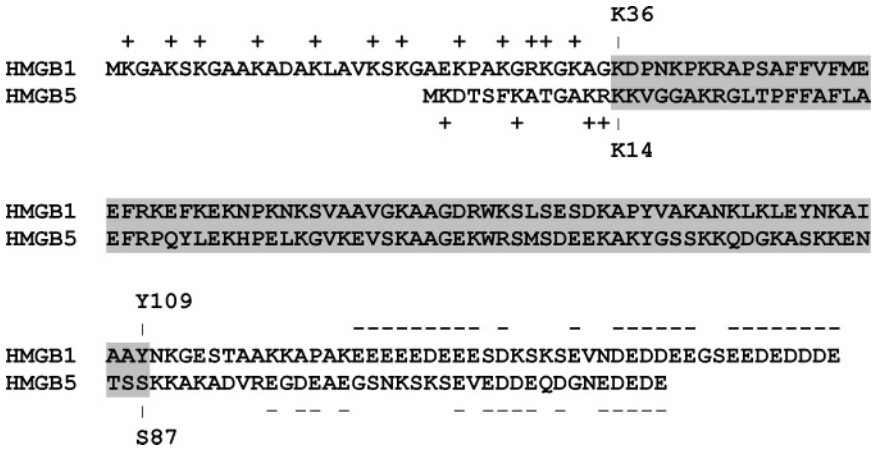

FIGURE 1: Alignment of the amino acid sequences of maize HMGB1 and HMGB5. The central HMG-box DNA-binding domains are highlighted, and the amino acid positions that delineate the HMG-box domain (and the flanking N-terminal and C-terminal domains) of the two proteins are indicated. The amino acid sequences of the HMG-box domains of HMGB1 and HMGB5 are only 35% identical, while the HMG-box domains of the HMGB1−HMGB4 proteins are more similar to each other, being 55−93% identical in terms of amino acid sequence. HMGB1 (157 amino acids, 17.1 kDa) and HMGB5 (123 amino acids, 13.6 kDa) are different in size, which essentially is due to differences in the basic N-terminal and acidic C-terminal domains (in which positively and negatively charged amino acid residues are indicated, respectively).

mM EDTA, 1 mM DTT, 5% glycerol, 0.05% bromophenol blue, and 0.05% xylene cyanol]. In the standard binding reactions, six-His-tagged Dof2 (50 nM) and the respective HMGB protein (50−400 nM) were incubated for 10 min before the oligonucleotide (3.5 nM) was added. After a final incubation for 10 min, the samples were loaded onto 7% polyacrylamide gels in 1×TBE. When electrophoresis was completed, the gels were dried under vacuum, scanned using a Typhoon 8600 phosphorimager (Molecular Dynamics), and analyzed quantitatively using Image Quant.

RESULTS

The Individual HMG-Box Domains of HMGB1 and HMGB5 Similarly Interact with Dof2. Previous work has shown that transcription factor Dof2 functionally interacts with maize HMGB proteins, and in case of the HMGB1 protein, the interaction (based on GST pulldown and protein cross-linking experiments) is mediated by the Dof domain of Dof2 and the HMG-box domain of HMGB1 (*35*, *36*). These experiments also revealed that HMGB5 is approximately 4−5-fold more efficient than the other tested HMGB proteins in promoting binding of Dof2 to its target DNA site. Here we have examined the structural features that determine the efficient interaction between Dof2 and HMGB5 relative to HMGB1. The two HMGB proteins are not closely related, sharing ∼36% overall amino acid sequence identity. While HMGB1 is the largest (157 amino acid residues) of the characterized maize HMGB proteins, HMGB5 (123 amino acid residues) is the smallest HMGB protein, which is due to the shorter N- and C-terminal domains (Figure 1). Moreover, the amino acid sequence of the HMG-box domain of HMGB5 displays only a moderate similarity to those of the HMG-box domains of the other maize HMGB proteins (e.g., ∼35% amino acid sequence identity between the HMG-box domains of HMGB1 and HMGB5), whereas the HMG-box domains of the HMGB1−HMGB4 proteins share more than 50% amino acid sequence identity (*38*). To examine the contribution of different domains of the HMGB1 and HMGB5 proteins to the functional interaction with Dof2, we have generated a number of expression vectors suitable for the production of full-length, truncated, and chimeric HMGB1/5 proteins (Figure 2A). The identity and purity of the recombinant proteins that were expressed in *E. coli* and purified by three-step column chromatography were analyzed by SDS−PAGE (Figure 2B) and MALDI-TOF mass spectrometry (data not shown).

The individual HMG-box domains of HMGB1 and HMGB5 were compared by CD spectroscopy. CD spectra were recorded in the wavelength range of 195−250 nm. The spectra display a shape typical of α-helical proteins with the characteristic negative peaks around 208 and 222 nm (Figure 3). Despite the differences in amino acid sequence, the spectra of the HMG-box domains of HMGB1 and HMGB5 are similar, indicating that both domains have a comparable α-helical content. Structural studies of HMG-box domains from various sources revealed that the overall fold of the domain is well-conserved (*4*).

Using chemical protein cross-linking, the physical interaction between the Dof domain of Dof2 and full-length HMGB1 and HMGB5 and the individual HMG-box domains was analyzed. Equimolar mixtures of the Dof domain and the HMGB proteins were treated with glutaraldehyde for various times before the proteins were analyzed by SDS−PAGE (Figure 4A). Both HMGB1 and HMGB5 formed a distinct, cross-linked complex with the Dof domain, whereas no or only faint bands of cross-linked products were detected, when the proteins were individually treated with glutaraldehyde. Similarly, both individual HMG-box domains were cross-linked with the Dof domain. The binding of the Dof domain to the HMG-box domains was also examined using GST pulldown assays. In these experiments, both HMG-box domains bound to the Dof domain fused to GST, whereas no interaction was detected with GST alone (Figure 4B). Therefore, the protein cross-linking experiment and the GST pulldown assay demonstrate that both the HMG-box domains of HMGB1 and HMGB5 interact with the Dof domain of Dof2.

We tested the possibility that the sequence differences within the HMG-box domains of HMGB1 and HMGB5 cause the differential stimulation of Dof2−DNA binding.

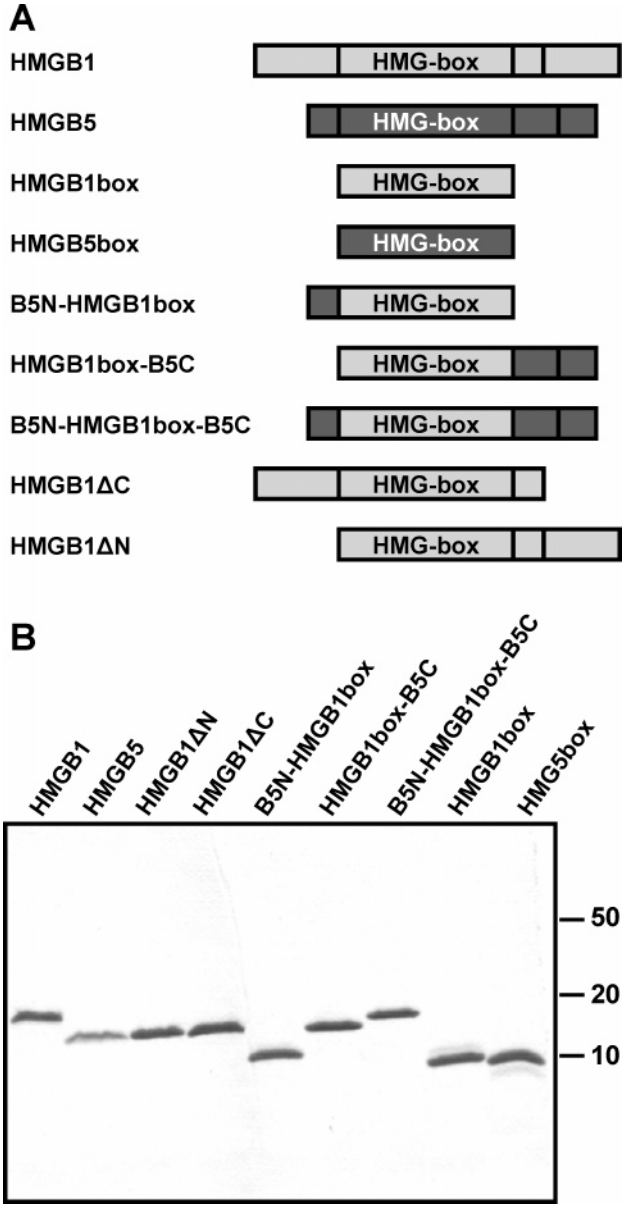

FIGURE 2: Schematic representation and SDS−PAGE analysis of the recombinant HMGB proteins used in this study. (A) Schematic representation of the full-length, truncated, and chimeric HMGB proteins, in which protein domains of the maize HMGB1 protein are indicated in light gray and domains of the HMGB5 protein are indicated in dark gray. The proteins comprise the full-length proteins (HMGB1 and HMGB5), the individual HMG-box domains (HMGB1box and HMGB5box), chimeric proteins consisting of HMGB1box and terminal domains derived from HMGB5 (B5N-HMGB1box, HMGB1box-B5C, and B5N-HMGB1box-B5C), and truncated HMGB1 proteins (HMGB1ΔC and HMGB1ΔN). The N- and C-terminal domains of the HMGB proteins that have been deleted or fused to HMGB1box are as specified in Figure 1. (B) The HMGB proteins listed in panel A were expressed in *E. coli*, purified by three-step chromatography, and analyzed by SDS−PAGE in a 18% polyacrylamide gel with Coomassie staining. The electrophoretic migration positions of marker proteins are indicated in kDa.

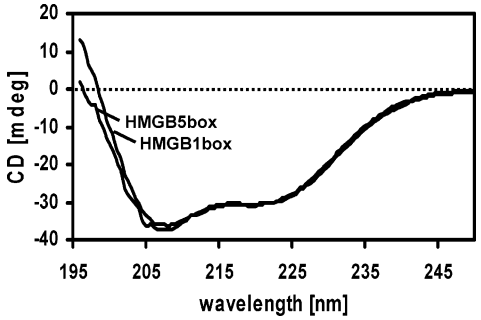

FIGURE 3: CD wavelength spectra of the HMG-box DNA-binding domains of HMGB1 and HMGB5. CD spectra were recorded for the HMG-box domains of HMGB1 and HMGB5 in the wavelength range of 195−250 nm, resulting in very similar spectra that indicate α-helical structure.

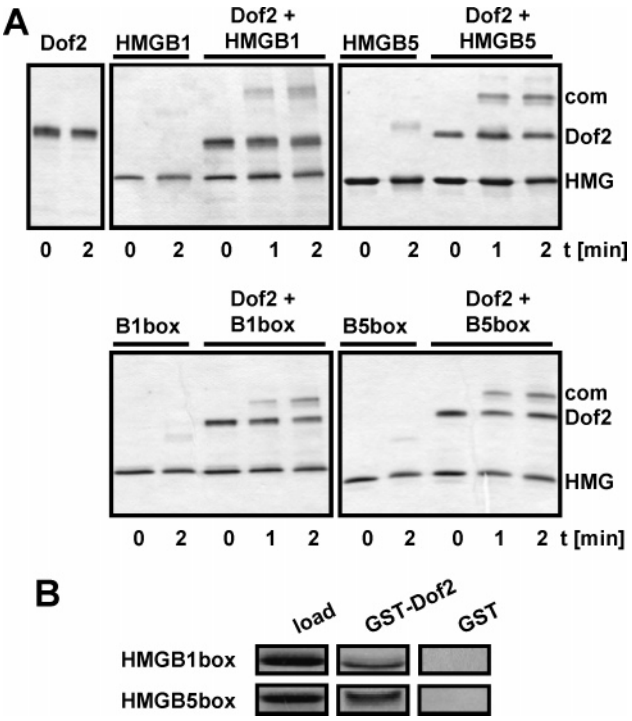

FIGURE 4: Full-length HMGB1 and HMGB5 as well as the individual HMG-box domains interact with Dof2. (A) The association of HMGB1/5 and the HMG-box domains with the Dof domain of Dof2 in solution was assessed by protein cross-linking. Dof2 and the HMGB proteins were either individually or as equimolar mixtures reacted with glutaraldehyde. Aliquots of the reaction mixtures were taken at the indicated times and analyzed by SDS−PAGE and Coomassie staining. The electrophoretic migration positions of the individual HMGB proteins (HMG), Dof2, and the HMGB−Dof complex (com) are indicated. (B) HMGB1box and HMGB5box interact with the Dof domain of Dof2 in GST pulldown assays. The GST−Dof2 fusion and as control GST alone were immobilized on glutathione-Sepharose beads and incubated with HMGB1box and HMGB5box. After the beads had been extensively washed, proteins were eluted with glutathione and analyzed by SDS−PAGE and Coomassie staining. One-tenth of the input protein (load) and the HMG proteins eluted from the GST−Dof beads are shown, while no binding was observed with GST alone.

In electrophoretic mobility shift assays (EMSAs), a concentration of Dof2 was used that resulted in a relatively weak retarded band corresponding to the Dof2−DNA complex (Figure 5A). To examine the stimulatory effect of the HMGB proteins, increasing amounts of HMGB1 and HMGB5 were added to a fixed concentration of Dof2 and of the oligonucleotide containing the Dof2 site. As previously observed (*36*), HMGB5 is significantly more efficient than HMGB1 in promoting the binding of Dof2 to the oligonucleotide containing the Dof2 recognition site (Figure 5A, top panels, compare lanes 3 and 7). When the individual HMG-box domains of HMGB1 and HMGB5 were added to the binding reaction mixtures, they promoted the formation of the Dof2−DNA complex to a similar extent (Figure 5A, bottom panels),

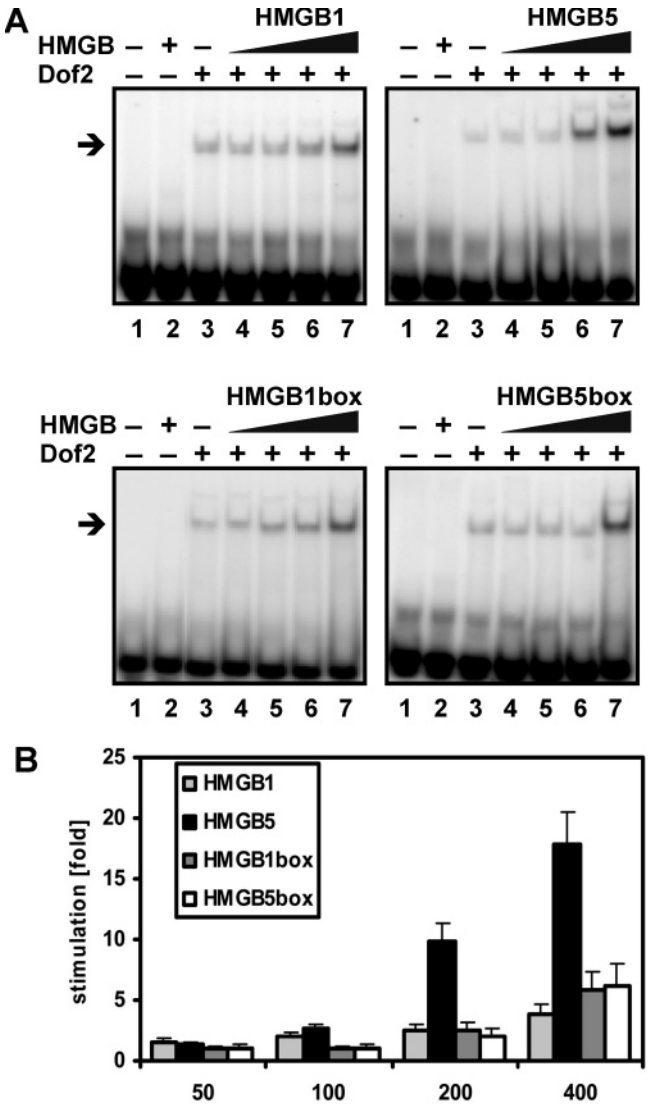

FIGURE 5: Full-length HMGB1 and HMGB5 as well as the individual HMG-box domains can promote Dof2−DNA binding. (A) In electrophoretic mobility shift assays (EMSAs), a fixed concentration of Dof2 (50 nM) was preincubated either alone (lanes 3) or in the presence of increasing concentrations of the indicated HMGB protein (50, 100, 200, and 400 nM in lanes 4−7, respectively), before the ^{32}P-labeled Dof site oligonucleotide was added. The electrophoretic migration of the Dof site oligonucleotide in the absence of protein is shown in lanes 1. The migration position of the Dof2−DNA complex is denoted with an arrow. The migration of the protein−DNA complex formed by Dof2 alone (lane 3) is indistinguishable from that of the HMGB-stimulated Dof2−DNA complex (lanes 4−7) (*36*). (B) HMGB1 and the individual HMG-box domains of HMGB1/5 similarly stimulate Dof2−DNA binding, but HMGB5 is clearly more effective. Protein−DNA complexes were quantified from polyacrylamide gels of EMSA experiments (as shown in panel A) using a phosphorimager. The amount of protein−DNA complex formed with 50 nM Dof2 alone was defined arbitrarily as 1, and the fold stimulation by different concentrations of HMGB proteins that were added to the binding reaction mixture was calculated relative to this value. The data displayed in the histogram represent means of three independent experiments, and the error bars indicate the standard deviation of the mean.

but it clearly was less efficient than full-length HMGB5 and slightly more efficient than full-length HMGB1 (Figure 5B). As seen previously for the HMGB−Dof2 interaction [and other HMGB−transcription factor complexes (*5*, *11*, *41*)], despite the formation of a ternary complex consisting of the

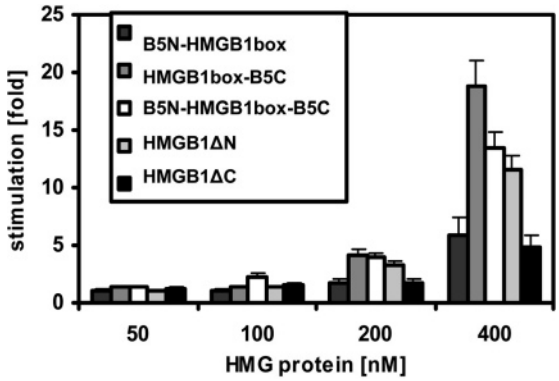

FIGURE 6: Presence of the acidic C-terminal tail of HMGB1 and in particular of HMGB5 positively influences the DNA binding of Dof2. The stimulation of Dof2−DNA binding was examined using various chimeric HMGB1/5 proteins (B5N-HMGB1box, HMGB1box-B5C, and B5N-HMGB1box-B5C) and truncated HMGB1 proteins (HMGB1ΔN and HMGB1ΔC). Protein−DNA complexes were quantified from polyacrylamide gels of EMSAs using a phosphorimager. The amount of protein−DNA complex formed with 50 nM Dof2 alone was defined arbitrarily as 1, and the fold stimulation by different concentrations of HMGB proteins that were added to the binding reaction mixture was calculated relative to this value. The data displayed in the histogram represent means of three independent experiments, and the error bars indicate the standard deviation of the mean.

oligonucleotide, HMGB, and Dof2, the complex displays the same electrophoretic mobility as the Dof2−DNA complex in the absence of HMGB (*36*). Our EMSAs indicate that the differential stimulation of Dof2−DNA binding seen with HMGB1 and HMGB5 is not due to sequence differences in their HMG-box DNA-binding domains.

The Basic and Acidic Regions Flanking the HMG-Box Domain Modulate the HMGB-Mediated Stimulation of Dof2−DNA Binding. To study the possible effect of the basic N-terminal and acidic C-terminal domains of the HMGB proteins on the functional interaction with Dof2, we examined chimeric HMGB proteins. The N-terminal and C-terminal domains of HMGB5 were individually or both in combination fused to the HMG-box domain of HMGB1 (replacing the corresponding HMGB1 domains), giving B5N-HMGB1box, HMGB1box-B5C, and B5N-HMGB1box-B5C, respectively (cf. Figure 2A). Moreover, truncated HMGB1 proteins lacking either the basic N-terminal domain (HMGB1ΔN) or the acidic C-terminal domain (HMGB1ΔC) were analyzed. In chemical protein cross-linking experiments, the five tested HMGB proteins are cross-linked to the Dof domain of Dof2 by glutaraldehyde (data not shown), indicating that all the tested chimeric and truncated HMGB proteins can interact with Dof2.

The effect of the recombinant HMGB proteins on binding of Dof2 to its DNA target site was examined using EMSAs. To test the stimulatory effect of the HMGB proteins on Dof2−DNA binding, increasing concentrations of the HMGB proteins were added to a fixed concentration of Dof2 and of the oligonucleotide containing the Dof2 site. The binding reaction mixtures were separated by polyacrylamide gel electrophoresis, and the amount of protein−DNA complex was quantified. Fusion of the basic N-terminal domain of HMGB5 to the HMG-box domain of HMGB1 in B5N-HMGB1box did not significantly change the stimulatory effect seen with HMGB1box (cf. Figures 5B and 6). By contrast, fusion of the acidic C-terminal domain of HMGB5

to the HMGB1 HMG-box domain markedly increased the stimulation of Dof2−DNA binding (Figure 6). HMGB1box-B5C is approximately as efficient as full-length HMGB5 in promoting Dof2−DNA binding (cf. Figure 5B). When both terminal domains of HMGB5 were fused to the HMG-box domain of HMGB1 (B5N-HMGB1box-B5C), the stimulation of Dof2−DNA binding was reduced compared to the stimulation with HMGB1box-B5C. Consistent with these results, deletion of the basic N-terminal domain of HMGB1 (HMGB1ΔN) displays (compared to full-length HMGB1) an enhanced stimulation of Dof2−DNA binding (cf. Figures 5B and 6). Therefore, in the presence of the acidic tail, the N-terminal basic domain reduces the efficiency of the HMGB proteins in promoting the interaction of Dof2 with DNA. Deletion of the acidic C-terminal domain of HMGB1 resulted in a protein (HMGB1ΔC) whose ability to promote Dof2−DNA binding is comparable to that of full-length HMGB1 and the individual HMG-box domain (cf. Figure 5B). The experiments using chimeric and truncated HMGB proteins demonstrate that the acidic C-terminal domains of the plant HMGB proteins positively influence the stimulation of Dof2−DNA binding and that the C-terminal domain of HMGB5 displays a particularly positive effect.

DISCUSSION

Proteins of the HMGB family can act as architectural factors in a variety of nucleoprotein structures promoting the correct three-dimensional assembly of these complexes (*2*−*4*). Since HMG proteins bind DNA with little or no sequence specificity (*21*, *42*), the question regarding the recruitment of the HMGB proteins to their sites of action in the genome was puzzling. In principle, it appears that the HMGB proteins can be recruited (i) by structural trapping at (protein-induced) DNA structures or (ii) by interaction of the protein with certain transcription factors (*2*, *14*). Examples for the structural trapping mechanism of HMGB proteins (without requirement for protein−protein interactions) are site-specific recombination reactions (*13*, *43*) and the HMGB1-mediated stimulation of the DNA binding of the ZEBRA transcription factor (*44*). There are several examples of the recruitment of HMGB proteins by direct interaction of the protein with certain transcription factors (*5*−*12*). In this study, we have taken advantage of the variability of plant HMGB proteins to examine biochemically in more detail the specificity of the interaction between maize HMGB proteins and transcription factor Dof2.

For various HMGB−transcription factor interactions, the HMG-box DNA-binding domain of the HMGB proteins was identified as the domain that mediates the transcription factor interaction (*6*, *8*, *10*−*12*, *45*). In line with these findings, the Dof domain of Dof2 was found to interact with the HMG-box domain of maize HMGB1 (*35*, *36*). Therefore, we suspected that a differential interaction with the HMG-box domains of HMGB1 and HMGB5 may explain the markedly different stimulation of Dof2−DNA binding seen with full-length HMGB1 and HMGB5 (*36*). However, despite differences in the amino acid sequences of the two HMG-box domains, the CD wavelength spectra of the two domains are virtually identical. Moreover, the two domains interacted similarly with the Dof domain in the protein cross-linking and GST pulldown experiments as well as in the EMSAs, resulting in comparable stimulation of Dof2−DNA binding. Accordingly, differences within the HMG-box domains do not account for the very different functional interaction of HMGB1 and HMGB5 with Dof2.

To examine the role of the basic N-terminal and acidic C-terminal domains, chimeric and truncated HMGB proteins were analyzed. All tested HMGB protein variants interacted with Dof2, as they contain the HMG-box domain of HMGB1. However, the different recombinant HMGB proteins exhibited very different efficiencies in promoting the binding of Dof2 to its DNA target site. Fusing the N-terminal domain of HMGB5 to the HMG-box domain of HMGB1 (B5N-HMGB1box) hardly influenced the stimulatory effect on Dof2−DNA binding, compared to that of the individual HMG-box domain. By contrast, fusing the C-terminal domain of HMGB5 to HMGB1box (HMGB1box-B5C) resulted in an ∼3-fold enhancement of the stimulatory effect, and the stimulation seen with this protein is comparable to that of full-length HMGB5. Therefore, the acidic tail of HMGB5 has a clear positive effect on the functional interaction of the HMGB protein and Dof2. When both the basic N-terminal and acidic C-terminal domain were fused to HMGB1box (B5N-HMGB1box-B5C), the stimulatory effect was reduced compared to that of HMGB1box-B5C. Still the B5N-HMGB1box-B5C protein promotes Dof2−DNA binding efficiently, indicating that the acidic tail of HMGB5 is the main cause for the particularly strong stimulation of Dof2−DNA binding observed with full-length HMGB5 relative to that of other maize HMGB proteins (*36*). The intramolecular interaction of the acidic tail with the basic N-terminal domain that is seen with plant HMGB proteins (*40*) may limit the positive effect of the acidic tail on Dof2−DNA binding in full-length HMGB proteins and in the chimeric B5N-HMGB1box-B5C protein. Due to the length and number of positive charges within the basic N-terminal domain of HMGB1 (compared to the basic N-terminal domain of HMGB5; cf. Figure 1), the intramolecular interaction between the terminal domains might be stronger in HMGB1 than in HMGB5. In line with that, the truncated HMGB1 protein HMGB1ΔN consisting of HMGB1box and the acidic C-terminal domain is significantly more potent in promoting Dof2−DNA binding than full-length HMGB1 or truncated HMGB1 proteins lacking the acidic tail. The greater stimulation seen with HMGB1box-B5C relative to HMGB1ΔN (differing in the origin of the acidic tail) underscores the particular positive effect of the HMGB5 C-terminal domain on the functional interaction with Dof2.

The acidic tail of mammalian HMGB1 plays a critical role in the functional interaction with the TATA-binding protein (TBP), but in this case, the acidic domain of HMGB1 is required for formation of the complex with TBP (*46*). The interaction of maize HMGB proteins with Dof2 rather resembles the functional interaction of p53 family members with mammalian HMGB1. The HMG-box domain(s) of HMGB1 (and HMGB2) mediates formation of the complex with p53 (*10*, *45*), but the acidic tail plays a critical role in the interaction (*47*).

Various studies demonstrated that the acidic tails of vertebrate and insect HMGB proteins reduce the affinity of the proteins for linear DNA (*48*−*52*). Consistent with these findings, the acidic C-terminal domain of maize and rice HMGB1 negatively influences the binding to linear DNA (*15*, *16*). Moreover, removal of the acidic tail of *Arabidopsis*

HMGB1 and HMGB5 stabilizes DNA/chromatin binding in vivo, resulting in decreased dynamics of the proteins within the nucleus (*53*). Generally, it appears that the length (and possibly sequence) of the acidic tails determines the affinity of HMGB proteins for linear DNA (*15*, *48*, *52*). On the other hand, the basic N-terminal domain typical of plant (and certain yeast) HMGB proteins positively influences the DNA binding of the proteins. Accordingly, the maize and rice HMGB1 proteins lacking the basic N-terminal domain display a significantly reduced affinity for linear DNA relative to that of the full-length protein (*15*, *16*). In our EMSA experiments, the maize HMGB proteins lacking the basic N-terminal domain (HMGB1box-B5C, HMGB1ΔN) are markedly more potent in promoting Dof2−DNA binding than the corresponding proteins containing both terminal domains (B5N-HMGB1box-B5C, HMGB1), and the proteins lacking the acidic tail (B5N-HMGB1box, HMGB1ΔN). Therefore, the DNA binding affinity of the HMGB proteins does not correlate with the efficiency in assisting Dof2−DNA binding. This finding resembles the mammalian HMGB1-facilitated ACF/CHRAC-dependent nucleosome sliding. HMGB1 lacking its acidic tail (displaying an enhanced affinity for DNA and nucleosomes) is less efficient in stimulating nucleosome sliding (*54*). In this process, HMGB1 acts as a DNA chaperone, which requires reversible and transient DNA binding. A chaperone-like mechanism has been also suggested for the HMGB-assisted Dof2−DNA binding (*36*), and as shown here, a relatively low affinity of the HMGB protein for DNA appears to be favorable for the stimulation of binding of Dof2 to its DNA target site. Another set of experiments has demonstrated that mammalian HMGB1 can facilitate the DNA binding of steroid hormone receptors (*5*, *41*). Using this system, intriguing novel perspectives of HMGB−transcription factor interactions have been uncovered recently. HMGB1 and the glucocorticoid receptor are mobile molecules, but by means of "kinetic cooperativity", they increase each other's residence time at DNA target sites, forming a rather stable functional complex at promoter response elements in vivo (*55*).

In summary, our experiments have shown that (i) sequence differences within the HMG-box domains do not account for the very different efficiency of HMGB1 and HMGB5 in stimulating the DNA binding of the transcription factor Dof2 and (ii) the acidic C-terminal domains of the HMGB proteins have a clear positive effect on the functional interaction with Dof2. This effect is particularly prominent with the acidic tail of HMGB5. On the basis of the results reported here, the exact mechanism of the HMGB−Dof2 interaction may be elucidated by future structural studies and analysis of the dynamic binding of the interaction partners.

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