The high mobility group (HMG) proteins of the HMG family are chromatin-associated proteins that can contribute to transcriptional control by interaction with certain transcription factors. Using the transcription factor Dof2 and five different maize HMGB proteins, we have examined the specificity of the HMGB-transcription factor interaction. The HMGB-box DNA binding domain of HMGB1 is sufficient for the interaction with Dof2. Although all tested HMGB proteins can interact with Dof2, the various HMGB proteins stimulate the binding of Dof2 to its DNA target site with different efficiencies. The HMGB5 protein is clearly the most potent facilitator of Dof2 DNA binding. Maximal stimulation of the DNA binding by the HMGB proteins requires association of HMGB and Dof2 prior to DNA binding. HMGB5 and Dof2 form a ternary complex with the DNA, but within the protein-DNA complex the interaction of HMGB5 and Dof2 is different from that in solution, as in contrast to the proteins in solution, they cannot be cross-linked with glutaraldehyde when bound to DNA. Phosphorylation of HMGB1 by protein kinase CK2 abolishes the interaction with Dof2 and the stimulation of Dof2 DNA binding. These findings indicate that transcription factors may recruit certain members of the HMGB family as assistant factors.

Eukaryotic gene expression in general is controlled by assembly of multicomponent nucleoprotein complexes on regulatory DNA sequences. The formation of these complexes (also termed enhancosomes) is primarily driven by the sequence-specific binding of transcription factors to their cis-acting DNA target sites. Post-translational modifications and various protein/protein interactions modulate the assembly of the regulatory nucleoprotein structures (1). Because of the structural inflexibility of the DNA, the interaction of transcriptional regulators tethered by DNA often requires the assistance of architectural factors. Due to their DNA bending activity, they can facilitate the coordinated and stereo-specific assembly of the nucleoprotein complexes (2–6). Among these architectural proteins are the chromatin-associated high mobility group (HMG) proteins of the HMGB family (previously termed HMG1/2 proteins (7)). They contain one or two copies of the HMG-box DNA binding domain, which consists mainly of three α-helices, arranged in an L-shaped structure (6, 8, 9). HMGB proteins share many properties with the structurally unrelated prokaryotic HU proteins, such as the interaction with the minor groove of DNA, bending the DNA by over 90°, and the recognition of distorted DNA sequences (5, 10). The HMGB proteins are non-classical transcriptional activators (11), but nevertheless they have been implicated in the regulation of transcription (12, 13), although the mechanism of action has still not been completely elucidated. In many cases, the non-sequence-specific HMGB proteins are recruited to particular DNA sites by direct interactions with certain transcription factors. HMGB proteins stimulate the binding of transcription factors of the MLTF (14), Oct (15), Hox (16), p53 (17, 18), and Rel (19, 20) families and of steroid hormone receptors (21, 22) to their cognate DNA sites. Furthermore, HMGB proteins interfere with the formation of the RNA polymerase II preinitiation complex by interactions with the basal transcription machinery (23–27). In line with these findings, the HMGB-type proteins NHP6A/B play an important regulatory role, repressing as well as potentiating the expression of various genes in yeast (28, 29).

Both monocotyledonous and dicotyledonous plants contain several relatively abundant chromosomal HMGB proteins (30, 31). Thus, five HMGB proteins have been identified and characterized from maize and Arabidopsis (30). They differ from each other in their chromatin association, in their post-translational modifications and in some of their DNA interactions (32–34). Plant HMGB proteins typically have a single HMG-box DNA binding domain, which is flanked by a basic N-terminal and an acidic C-terminal domain. While the HMGB-box domains of the different HMGB proteins are relatively conserved, the basic and acidic flanking regions are more variable in length and sequence (35). An HMGB protein from wheat has been shown to stimulate the binding of the bZIP factor EmBP-1 to its DNA recognition site (36). Furthermore, the maize HMGB1 protein can physically interact with the transcription factors DoF1 and DoF2 and facilitates the DNA binding of DoF1 (37). The DoF factors represent a plant-specific family of transcription factors that contain a transcriptional activation domain and a highly conserved amino acid sequence termed the DoF domain, which may form a single zinc finger critical for DNA binding (38, 39). DoF proteins recognize specifically the AAG core motif occurring in different promoter regions (40) and have been implicated in the regulation of various genes, including tissue specifically expressed, light-regulated, and stress/phytohormone-responsive genes (41–48).

Since little is known about the specificity of the interactions...
between HMGB proteins and transcription factors, we have taken advantage of the variability of HMGB proteins in plants (30, 35) to examine biochemically the specificity of the interaction between Dof2 and the various maize HMGB proteins HMGB1, HMGB2/3, HMGB4, and HMGB5 (previously termed HMGa, HMGc1/2, HMGd, HMGe). It is shown that the HMGB-box domain is sufficient for the interaction with Dof2 and that HMGB5 is markedly more efficient than HMGB1, HMGB2/3, and HMGB4 in stimulating the binding of Dof2 to its DNA recognition sequence. Moreover, the phosphorylation of HMGB1 by protein kinase CK2 abolishes the interaction with Dof2.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Proteins**—The various maize HMGB proteins and the truncated versions of HMGB1 were expressed as His6-tagged fusion proteins in *E. coli* and purified by three-step column chromatography as described previously (49, 50). The fusion protein of the Dof domain of maize Dof2 with an N-terminal GST and a C-terminal His tag, here termed GST-ΔDof2, was expressed in *Escherichia coli* and purified as described previously (37). For control experiments, the GST protein (without ΔDof2 fusion) was expressed in *E. coli*, using the original plasmid pGEX-5X-1 and purified as described by the manufacturer (Amersham Biosciences). The DNA sequence encoding the Dof domain of Dof2 (K1-E115) was amplified by PCR with Deep Vent DNA Polymerase (New England Biolabs) and primers P1 (5′-AAGGATCCAAGGGCTACCCGT) and P2 (5′-AATGCGACTC-CCGGCGAGCCTG) using the plasmid pGST-Dof2(37) as template. The PCR product was digested with BamHI and PstI and cloned into the expression vector pQE9 cm (49) giving pQE9 cm-ΔDof2, which was checked by DNA sequencing. The His-tagged ΔDof2 was expressed in *E. coli* and purified by metal-chelate chromatography as described previously (37, 49). Maize protein kinase CK2 was expressed in *E. coli* and purified by three-step column chromatography. The enzyme was used to phosphorylate HMGB1 and HMGB3 within their acidic C-terminal domains as described previously (34).

**GST Binding Assay**—GST-tagged ΔDof2 protein (500 ng) was incubated for 10 min in a total volume of 100 μl with 50 μl of a 50% glutathione-Sepharose bead slurry prepared and equilibrated as specified by the supplier (Amersham Biosciences). After centrifugation the supernatant was discarded, and the proteins tested for interaction with ΔDof2 were added (~500 ng) in a final volume of 150 μl. After incubation at 20 °C for 1 h, the Sepharose beads were washed four times with 100 μl of NaCUP, (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 0.1% Nonidet P40). Bound proteins were eluted with 10 μl glutathione, precipitated with 25% trichloroacetic acid, washed twice with acetone, dried, and resuspended in SDS loading buffer and heating the samples for 2 min at 95 °C. Some cross-linking reactions contained also DNA, either 200 ng of the 21-bp Dof2 site oligonucleotide containing no sequence that matches Dof binding sites (40) or 200 ng of an unrelated 21-bp oligonucleotide containing a sequence that matches Dof2 binding sites (40). The cross-linking reaction was started by adding glutaraldehyde to a final concentration of 0.0125%. The reaction was stopped by adding SDS-loading buffer and heating the samples for 2 min at 95 °C.

**DNA Binding Analysis by Electrophoretic Mobility Shift Assays (EMSAs)**—Protein binding to a double-stranded 32P-labeled 21-bp Dof site oligonucleotide containing the Dof binding site (37) was examined using EMSAs. Binding reactions contained binding buffer (10 mM Tris/ HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol) and 40-fold excess of poly(dI-dC) as non-specific competitor DNA. In the standard binding reactions, GST-ΔDof2 (10 nm) and the respective HMGB proteins (0–1 μM) were preincubated for 5 min prior to the addition of the oligonucleotide (3.5 nM). In some assays, GST-ΔDof2 and the oligonucleotide were preincubated for 5 min before the HMGB protein was added. After a final incubation for 5 min, the samples were applied onto 7% polyacrylamide gels in 1× TBE (90 mM Tris borate, 2 mM EDTA). When electrophoresis was completed, the gels were dried under vacuum and autoradiographed and/or analyzed with a phosphorimager (Bio-Rad).

**RESULTS**

**The Interaction with Dof2 Is Mediated by the HMGB-box DNA Binding Domain**—It has been shown previously that the maize HMGB1 protein interacts with the maize transcription factors Dof1 and Dof2 and that HMGB1 can stimulate the DNA binding of Dof1. The interaction with HMGB1 is mediated by the HMG-box DNA binding domain and involves the acidic C-terminus of the protein (40). It has been shown that the maize HMGB1 by protein kinase CK2 abolishes the interaction with Dof2. The HMGB1 protein or truncated derivatives of HMGB1 (as indicated in Fig. 1A) were incubated with acrylamide gels in 1× TBE (90 mM Tris borate, 2 mM EDTA). When electrophoresis was completed, the gels were dried under vacuum and autoradiographed and/or analyzed with a phosphorimager (Bio-Rad).

In the experiments, which were performed to determine the composition of the protein-DNA complex, the analytical ΔDof2/HMGB5/Dof site oligonucleotide binding reaction used in EMSAs (described above) was scaled up as described in the cross-linking experiments and analyzed by preparative PAGE. The gels were stained with SYBR gold (Molecular Probes), and the band corresponding to the protein-DNA complex was cut from the gel. The excised polyacrylamide gel slice was soaked in SDS-loading buffer, inserted in the well of a 18% polyacrylamide gel, and the proteins were separated by SDS-PAGE.

**FIG. 1.** The HMGB-box DNA binding domain is sufficient for the interaction with Dof2. A, schematic representation of the HMGB1 protein indicating the relevant amino acid residues that delineate the different (truncated) versions of the protein used in this study. The HMGB-box domain is indicated by a hatched box, while the acidic C-terminal domain is indicated by a black box. B, GST binding assay demonstrating that full-length HMGB1(Met₁-Glu¹⁵⁷) and the truncated derivatives HMGB1(Met₁-Tyr¹⁰⁹) and HMGB1(Gly³⁵-Tyr¹⁰⁹) bind to the GST-ΔDof2 fusion protein. GST-ΔDof2 (or as control GST) were bound to glutathione-Sepharose beads and incubated with HMGB1, HMGB1(Met₁-Tyr¹⁰⁹), and HMGB1(Gly³⁵-Tyr¹⁰⁹). After extensive washing of the beads, proteins were eluted with glutathione and analyzed by SDS-PAGE and Coomassie Blue staining. The input proteins (load) and the eluted HMGB proteins that were bound to GST-ΔDof2 are shown; no interaction was observed with GST. C, the HMGB-box domain HMGB1(Gly³⁵-Tyr¹⁰⁹) interacts with ΔDof2 in protein cross-linking experiments. ΔDof2 and HMGB1(Gly³⁵-Tyr¹⁰⁹) were either individually or as a mixture reacted with glutaraldehyde. Aliquots of the reaction were taken at the indicated times and analyzed by SDS-PAGE and Coomassie Blue staining. The migration positions of the individual proteins and of the ΔDof2/HMGB1(Gly³⁵-Tyr¹⁰⁹) complex are indicated.
GST-ΔDof2 bound to glutathione-Sepharose. The glutathione-Sepharose was thoroughly washed and the proteins bound to the matrix through the GST fusion portion were eluted with glutathione. In control experiments, no HMGB1 protein binding was detected when the GST protein (without ΔDof2 fusion) was used. The GST-ΔDof2 fusion protein was bound by full-length HMGB1(Met1-Glu157) by the protein consisting only of the N-terminal portion HMGB1(Met1-Tyr109) and by the individual HMG-box DNA binding domain HMGB1(Gly35-Tyr109) (Fig. 1B). Therefore, the HMG-box domain is sufficient to mediate the interaction with the Dof domain of Dof2. The interaction between the HMG-box domain and ΔDof2 was further analyzed by chemical protein cross-linking. ΔDof2 was mixed in equimolar amounts with the individual HMG-box domain of HMGB1 and treated for various times with glutaraldehyde, before the proteins were separated by SDS-PAGE. Due to the covalent cross-linking, the amount of the original protein bands was reduced and a new protein band appeared, which corresponds to the ΔDof2-HMGB1(Gly35-Tyr109) complex (Fig. 1C). Immunoblot analyses of cross-linked complexes with antisera against HMGB and Dof confirm the presence of both proteins in the complex band. Comparison of the migration position of the ΔDof2-HMGB1(Gly35-Tyr109) complex with that of marker proteins suggests that the two proteins most likely form a 1:1 complex. Treatment of either HMGB1(Gly35-Tyr109) or ΔDof2 individually with glutaraldehyde did not result in any cross-linked products (Fig. 1C).

To examine whether the HMG-box DNA binding domain is sufficient for stimulating the binding of ΔDof2 to its DNA recognition sequence, EMSAs were performed. In pilot shift experiments, the concentration of ΔDof2 (10 nM) was determined that resulted only in a small amount of protein-DNA complex (of low electrophoretic mobility) with the 32P-labeled Dof site oligonucleotide (Fig. 2A, lanes 3 and 8), while HMGB proteins form only at higher concentrations a complex with the oligonucleotide that has a higher mobility than the ΔDof2 complex (lane 2). To the fixed amount of ΔDof2, increasing concentrations of HMGB1 and of the individual HMG-box domain of HMGB1 were added and analyzed by EMSA (Fig. 2A). Addition of the HMGB1 proteins to the ΔDof2 protein resulted in an enhanced formation of the ΔDof2-DNA complex (without altering the electrophoretic mobility of the complex relative to the complex formed in the absence of HMGB1), demonstrating that HMGB1 facilitates the binding of ΔDof2 to its target sequence. The individual HMG-box DNA binding domain is 2-fold more efficient than full-length HMGB1 (and HMGB1(Met1-Tyr109)) in stimulating the DNA binding of ΔDof2 (Fig. 2B). Therefore, the stimulatory effect appears to depend not directly on the DNA binding affinity of the HMGB1 proteins, since the relative affinity of the HMGB1 derivatives for DNA is HMGB1(Met1-Tyr109) > HMGB1(Gly35-Tyr109) (50), whereas the relative stimulation of Dof2 DNA-binding is HMGB1(Gly35-Tyr109) > HMGB1(Met1-Tyr109) > HMGB1.

The HMGB5 Protein Is Most Efficient in Stimulating Dof2 DNA Binding—Using the GST binding assay with GST-ΔDof2, we have analyzed whether ΔDof2 can interact with maize HMGB proteins other than HMGB1. Therefore, HMGB1, HMGB2, HMGB4, and HMGB5 were examined for their ability to bind GST-ΔDof2. All five maize HMGB proteins can interact with GST-ΔDof2 as they are retained on the matrix by GST-ΔDof2 bound to glutathione-Sepharose, but not by GST without ΔDof2 fusion (Fig. 3A). The interaction of ΔDof2 with HMGB1 and HMGB5 was further examined by chemical protein cross-linking experiments. Equimolar mixtures of ΔDof2 and HMGB1 or HMGB5 proteins were treated with glutaraldehyde for various times before the proteins were analyzed by SDS-PAGE (Fig. 3B). Both HMGB1 and HMGB5 formed a specific, cross-linked complex with ΔDof2, whereas no cross-linked products were obtained, when the proteins were individually treated with glutaraldehyde. Quantification of the bands corresponding to the cross-linked complex revealed that compared with HMGB1, HMGB5 was 3-fold more readily cross-linked to ΔDof2 (as also evident from more intense bands of the cross-linked complex with HMGB5), suggesting that ΔDof2 has a higher affinity for HMGB5. The migration of the cross-linked complexes relative to ΔDof2 reflects the difference.

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\[ * N. M. Krohn and K. D. Grassner, unpublished results.\]
We then changed the order of addition of the HMGB4 were significantly less effective (5-fold stimulation of Dof site binding by HMGB proteins) than HMGB1 and HMGB5 when the preincubation of Dof2 and HMGB5 is omitted. The observed reduction of the stimulatory effect was most striking with HMGB5, which still displayed the largest effect on Dof2 DNA binding, however, only ~20% compared with the experiment including a preincubation of Dof2 and HMGB5 (cf. Fig. 4A). The marked positive effect of the preincubation of the two proteins on the DNA binding of Dof2 suggests that the protein/protein interaction between Dof2 and the HMGB proteins (which is mediated by the DNA binding domains of both proteins) is essential for maximal HMGB-mediated stimulation of the DNA target site binding by Dof2. Analysis of the time course of the binding of Dof2 and HMGB to the DNA by EMSAs revealed that the preincubation results in more rapid assembly of the protein-DNA complex. 2

**Dof2 and HMGB5 Form a Ternary Complex with the Dof DNA Binding Site**—As the HMGB-stimulated Dof2-DNA complex formed on the Dof site oligonucleotide co-migrates with the Dof2 protein complex formed in the absence of HMGB proteins (Fig. 2A), the question arises whether the HMGB protein is present in the final protein-DNA complex. To investigate this, the analytical binding reaction of preincubated Dof2 and HMGB5 to the Dof site oligonucleotide used in EMSAs was scaled up (in quantities as described for the crosslinking experiment) and separated by preparative polyacrylamide gel electrophoresis. The DNA was detected in the EMSA.
Plant HMGB Proteins Interacting with Transcription Factor Dof2

A further analysis by chemical cross-linking experiments, the protein-DNA complex was cut from the gel, and the proteins contained in the polyacrylamide slice were examined by SDS-PAGE. The migration positions of the individual proteins and of the Dof site oligonucleotide were analyzed by the GST binding assay. Non-phosphorylated HMGB1, but not the CK2-phosphorylated HMGB1, was specifically retained on the glutathione affinity matrix by the GST-Dof2 protein (Fig. 6A). Analysis of the Dof2-HMGB1 interaction by the more sensitive chemical cross-linking approach using glutaraldehyde (Fig. 6B) demonstrated that the interaction is markedly reduced, but not completely abolished, by CK2-mediated phosphorylation of HMGB1, since after 2 min of cross-linking some residual Dof2-HMGB1 complex could be detected with phosphorylated HMGB1. This may be due to the fact that the HMGB1 protein was not completely phosphorylated by CK2, as determined by acetic acid urea PAGE and mass spectrometry. To examine the effect of the HMGB1 phosphorylation status on the stimulatory effect on Dof2 DNA binding, EMSAs with Dof2, HMGB1, and the Dof site oligonucleotide were performed. A fixed amount of Dof2 was preincubated without HMGB1 or in the presence of various concentrations of non-phosphorylated or CK2-phosphorylated HMGB1 before the 32P-labeled oligonucleotide was added. The formation of protein-DNA complexes was analyzed by EMSA and quantified. The dose-dependent, enhanced DNA binding of Dof2 seen in the presence of non-phosphorylated HMGB1 was not observed with the CK2-phosphorylated HMGB1 (Fig. 6C), indicating that the phosphorylation within the acidic C-terminal domain of HMGB1 by CK2 abolishes the stimulation of Dof2 binding to the Dof site oligonucleotide. A comparable effect of CK2-mediated phosphorylation was observed with HMGB3 (which is also phosphorylated by CK2), whereas there was no influence of the CK2 treatment on HMGB5 (which is no substrate for CK2) concerning the stimulation of Dof2 DNA binding. Since the majority of the HMGB1 and HMGB2/3 proteins is phosphorylated by CK2 in maize (34), only HMGB5 (and
HMGB1, HMGB2, HMGB3, HMGB4, and HMGB5 proteins can interact with Dof2 as examined by GST binding assays and protein cross-linking. All five HMGB proteins facilitated the binding of Dof2 to DNA; however, HMGB5 was remarkably more effective than the other HMGB proteins. While HMGB1 to HMGB4 stimulated the binding reaction 5–10-fold, HMGB5 enhanced the binding of Dof2 to the Dof site oligonucleotide >30-fold at a concentration of 400 nM. Since the amino acid sequence of HMGB5 is similar to those of the other maize HMGB proteins (38–48% amino acid sequence identity (55)), the significantly greater ability of HMGB5 to assist the DNA binding of Dof2 was surprising. Considering the conservation of the global fold of the HMGB-box DNA binding domain (6, 9), which represents the interaction surface for Dof2, it may be critical that in HMGB5 the HMGB-box domain is more readily accessible, as the domains flanking the HMGB-box domain in HMGB5 are smaller than in the other maize HMGB proteins (55). In line with this assumption, HMGB1 and HMGB1(Met1-Tyr109) are less effective in assisting Dof2 DNA binding than the individual HMGB-box domain HMGB5(Gly35-Tyr109). Alternatively, there could be a specific amino acid sequence motif in the HMG-box domain of HMGB5 (which does not occur in the other HMGB proteins) that favors the interaction with Dof2, since the rat HMGB1 protein was recently found to interact with short amino acid sequences (56). The marked reduction of the interaction of HMGB1 and Dof2 upon CK2-mediated phosphorylation of HMGB1 may be explained similarly. CK2 phosphorylates up to three serine residues within the acidic C-terminal domain of HMGB1 (34), which is not directly involved in the interaction with Dof2. However, the phosphorylation by CK2 alters intramolecular interactions within the HMGB1 protein, resulting in an increased thermostability of the protein (34). Therefore, the phosphorylation-induced altered intramolecular interaction of the acidic tail of HMGB1 may hide the surface of the HMGB-box domain, which is required for interaction with Dof2 and which is accessible in the non-phosphorylated protein.

In addition to HMGB1 (37), the HMGB2/3, HMGB4, and HMGB5 proteins can interact with Dof2 as examined by GST binding assays and protein cross-linking. All five HMGB proteins facilitated the binding of Dof2 to DNA; however, HMGB5 was remarkably more effective than the other HMGB proteins. While HMGB1 to HMGB4 stimulated the binding reaction 5–10-fold, HMGB5 enhanced the binding of Dof2 to the Dof site oligonucleotide >30-fold at a concentration of 400 nM. Since the amino acid sequence of HMGB5 is similar to those of the other maize HMGB proteins (38–48% amino acid sequence identity (55)), the significantly greater ability of HMGB5 to assist the DNA binding of Dof2 was surprising. Considering the conservation of the global fold of the HMGB-box DNA binding domain (6, 9), which represents the interaction surface for Dof2, it may be critical that in HMGB5 the HMGB-box domain is more readily accessible, as the domains flanking the HMGB-box domain in HMGB5 are smaller than in the other maize HMGB proteins (55). In line with this assumption, HMGB1 and HMGB1(Met1-Tyr109) are less effective in assisting Dof2 DNA binding than the individual HMGB-box domain HMGB5(Gly35-Tyr109). Alternatively, there could be a specific amino acid sequence motif in the HMG-box domain of HMGB5 (which does not occur in the other HMGB proteins) that favors

DISCUSSION

The non-sequence-specific architectural HMGB proteins can facilitate the formation of certain nucleoprotein structures (2, 5, 6). In several instances, they are recruited to their sites of action by direct protein contacts with sequence-specific proteins, for instance, with certain transcription factors (15, 16, 21). In some other cases such as site-specific recombination reactions (51–53) or the binding of the ZEBRA transcription factor to its response element (54), the HMGB proteins are recruited independent of protein-protein interactions, presumably by a “DNA structural trapping mechanism.” In this study, we have used the variability of the HMGB proteins in plants (30, 35) to examine biochemically the specificity of the interaction between different HMGB proteins and a transcription factor. Based on the initial finding of a stimulatory interaction between maize HMGB1 and Dof transcription factors (37), the ability of the proteins HMGB1–HMGB5 to interact with the Dof2 protein was analyzed comparatively. Using the HMGB1-Dof2 interaction as a starting point, it was demonstrated that the HMGB-box DNA binding domain is sufficient for the interaction with the Dof domain of Dof2 (ΔDof2). Despite the remarkably basic theoretical pi of ΔDof2 (pi = 10.7), the acidic tail of HMGB1 is not involved in (electrostatic) interactions with the transcription factor. The HMGB-box domains of mammalian and Drosophila HMGB proteins are responsible for the interaction with several other transcription factors such as POU domain, Rel, p53, and Hox factors (15, 16, 18, 20), while the interaction with TBP is mediated by the acidic C-terminal domain (23). As measured by EMSAs, the individual HMGB-box domain can stimulate the binding of ΔDof2 to the DNA target site, and the individual domain is even more efficient in facilitating ΔDof2 DNA binding than full-length HMGB1 and HMGB1(Met1-Tyr109). This result indicates that the stimulation of ΔDof2 DNA binding is not directly correlated to the affinity of HMGB1 for DNA, because HMGB1 and the individual HMGB-box domain display a similar affinity for DNA, while HMGB1(Met1-Tyr109) binds significantly better to DNA (50), but still the individual HMGB-box domain is most efficient in assisting ΔDof2 DNA binding.

In addition to HMGB1 (37), the HMGB2/3, HMGB4, and HMGB5 proteins can interact with Dof2 as examined by GST binding reactions and protein cross-linking. All five HMGB proteins facilitated the binding of ΔDof2 to DNA; however, HMGB5 was remarkably more effective than the other HMGB proteins. While HMGB1 to HMGB4 stimulated the binding reaction 5–10-fold, HMGB5 enhanced the binding of ΔDof2 to the Dof site oligonucleotide >30-fold at a concentration of 400 nM. Since the amino acid sequence of HMGB5 is similar to those of the other maize HMGB proteins (38–48% amino acid sequence identity (55)), the significantly greater ability of HMGB5 to assist the DNA binding of ΔDof2 was surprising. Considering the conservation of the global fold of the HMGB-box DNA binding domain (6, 9), which represents the interaction surface for ΔDof2, it may be critical that in HMGB5 the HMGB-box domain is more readily accessible, as the domains flanking the HMGB-box domain in HMGB5 are smaller than in the other maize HMGB proteins (55). In line with this assumption, HMGB1 and HMGB1(Met1-Tyr109) are less effective in assisting ΔDof2 DNA binding than the individual HMGB-box domain HMGB5(Gly35-Tyr109). Alternatively, there could be a specific amino acid sequence motif in the HMG-box domain of HMGB5 (which does not occur in the other HMGB proteins) that favors

HMGB4) have the potential of acting as co-factors that facilitate the binding of Dof2 to DNA target sites.
considered interaction partners of DoF2. These experiments demonstrate that post-translational modifications of the HMGB proteins may be critical determinants of HMGB-protein interactions. The ability of mammalian HMGB1 and HMGB2 to facilitate the DNA binding of transcription factors has been analyzed comparatively in just a few cases (18, 21), and so far no significant differences in their transcription factor interactions have been reported.

The stimulation of the binding of ΔDoF2 to its DNA target site by HMGB proteins was reduced, when the preincubation of ΔDoF2 and HMGB was omitted. This difference in the order-of-addition experiment is seen best with HMGB5, which is most effective in assisting ΔDoF2 DNA binding (compare Fig. 4, A and B). The dependance of the stimulatory effect on the preincubation of the proteins implies that HMGB5 and ΔDoF2 to have associate first to achieve maximal stimulation of the ΔDoF2 DNA binding. Analysis of the composition of the HMGB5-stimulated ΔDoF2-DNA complex obtained in EMSA experiments by SDS-PAGE (Fig. 5A) revealed that the complex contained in addition to the DoF site oligonucleotide and ΔDoF2, the HMGB5 protein. Despite the presence of HMGB5 in the complex, the migration of the ternary protein-DNA complex was indistinguishable from that of the ΔDoF2-DNA complex (Fig. 2A), an observation that has been reported for other HMGB-transcription factor interactions (15–17). Nevertheless, in a few cases the existence of ternary DNA-HMGB-transcription factor complexes could be proven by antibody supershift experiments (21) by co-immunoprecipitation (22) or by affinity chromatography (16). Likewise, HMGB5 interacts with ΔDoF2 in solution and is also present in the final ternary protein-DNA complex. In contrast to the proteins in solution, in the protein-DNA complex, HMGB5 and ΔDoF2 could no longer be cross-linked by glutaraldehyde, which cross-links proteins that are in close contact. Unlike the experiment using glutaraldehyde, HMGB5 and ΔDoF2 could be crosslinked independent of the presence of DNA by the crosslinking reagent DSS, which has a 11.4-Å spacer arm, and accordingly can cross-link proteins that are in proximity to each other even if they are not in immediate contact. Therefore, HMGB5 and ΔDoF2 may not be in direct contact when bound to the DoF site oligonucleotide, or at least their interaction (relative to that occurring in solution), is different in the protein-DNA complex, so that it can be fixed by DSS, but not by glutaraldehyde cross-linking (Fig. 5, B and C). Since HMGB proteins interact predominantly with the minor groove of DNA (6, 9), it is possible that DoF2 binds to the major groove at an (partially) overlapping site. Accordingly, HMGB5 could act in a “chaperone-like” manner, delivering bound ΔDoF2 to its DNA binding site. Association of HMGB5 and ΔDoF2 prior to DNA binding may facilitate the proper orientation of the two proteins relative to each other on the DNA. The architectural DNA bending function of HMGB5 could thereby deform the DoF DNA binding site in a way that is favorable for the DNA binding of ΔDoF2. The involvement of proteins that facilitate the binding of transcription factors to their target sites allows specific transcriptional regulators to act at markedly lower cellular concentrations and offers the possibility of combinatorial co-regulation. In the case of the proteins studied here, HMGB and DoF2, the DNA binding domains of both proteins are involved in the protein interaction, but at least in the case of DoF2, the DNA binding surface must be accessible to specifically recognize the DNA target site. To further elucidate the mechanism of the functional interaction of HMGB proteins and transcription factors, detailed structural studies will be required.

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