The Interaction of DOF Transcription Factors with Nucleosomes Depends on the Positioning of the Binding Site and Is Facilitated by Maize HMGB5†

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ABSTRACT: The expression of genes involved in C_4 photosynthesis in maize is under tight tissue-specific and light-dependent control. There is strong evidence that this control is at least in part brought about by DOF transcription factors binding to the respective promoters. We analyzed the interaction of DOF1 and DOF2 proteins with a functional and a cryptic endogenous binding site derived from the maize phosphoenolpyruvate carboxylase promoter (−300 bp region) in the nucleosomal context. Various DNA fragments comprising this promoter region were reconstituted into mononucleosomes from purified components, resulting in different positions of the DOF binding sites on the nucleosome surface. Binding of recombinant transcription factors to the different types of nucleosomes was examined using electrophoretic mobility shift assays. Changing the translational position of the binding site on the nucleosome surface strongly affected the efficiency of the interaction with the DOF factors. Deletion of individual recognition motifs revealed a positive impact of DOF protein binding to the main binding site on interactions with the cryptic binding site. The addition of the chromosomal high-mobility group (HMG) protein HMGB5 to the binding reaction mixture facilitated nucleosome binding of the transcription factor independent from the position of the recognition sites. The relevance of the data for the activation of the promoter in vivo is discussed.

Transcription in eukaryotes is often modulated by the activity of specific transcription factors that recognize defined sequences in promoters and interact with multiple proteins to influence directly or indirectly the efficiency of initiation complex assembly. DOF[1] proteins are transcription factors from plants with a unique DNA binding domain comprising a single zinc finger (*1*). The first DOF proteins have been identified in maize as interactors of the phosphoenolpyruvate carboxylase (PEPC) promoter (*2*) and have been shown to be involved in the regulation of several photosynthetic genes in maize (*3*). Transient overexpression in protoplasts together with Northern analyses revealed that DOF1 might serve as a ubiquitously expressed activator of transcription, whereas DOF2 may act as a tissue-specific repressor (*4*). Both factors recognize the same binding sites and seemingly compete for binding. Although the N-terminal DNA binding domain is highly conserved, the C-terminal domains are unrelated (*5*). The maize DOF1 protein served as the founder of a plant-specific family of transcription factors with multiple roles in light induction, stress response, or tissue specificity of gene expression (*3*, *6*−*11*).

The interaction of maize DOF proteins with naked DNA has been investigated in detail (*3*, *6*, *12*); however, transcription factors are compelled to recognize their binding sites in the chromatin context inside the cell nucleus. The basic repeat unit of chromatin is the nucleosome, which itself is made up of two molecules each of the core histones H2A, H2B, H3, and H4, and approximately 146 bp of DNA wound around the histone octamer. This structure actively participates in the regulation of gene expression. The specific positioning of nucleosomes can either facilitate or interfere with the interaction of transcription factors and their cognate binding site (*13*−*15*). Besides the histones, chromatin contains several non-histone proteins such as the relatively abundant high-mobility group (HMG) proteins of the HMGB class [previously named HMG1/2 (*16*)]. HMGB proteins contain one or two copies of the HMG box DNA-binding domain and interact with DNA in a non-sequence-specific manner (*17*−*19*). Specificity might be brought about by the interaction with transcription factors recruiting these factors to defined chromosomal locations (*18*−*20*). In maize, five different HMGB proteins (HMGB1−HMGB5) have been identified (*21*). Recently, it has been shown that these factors specifically interact with DOF2 and support the binding to its recognition site on naked DNA. The individual proteins differed in activity, and HMGB5 was most efficient in stimulating the DNA binding of DOF2 (*22*).

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[1] Abbreviations: DOF, DNA binding with one finger; EMSA, electromobility shift assay; HMG, high-mobility group; HMG B, high-mobility group domain protein class B; PAA, polyacrylamide; PEPC, phosphoenolpyruvate carboxylase; SOE, gene splicing by overlap extension.

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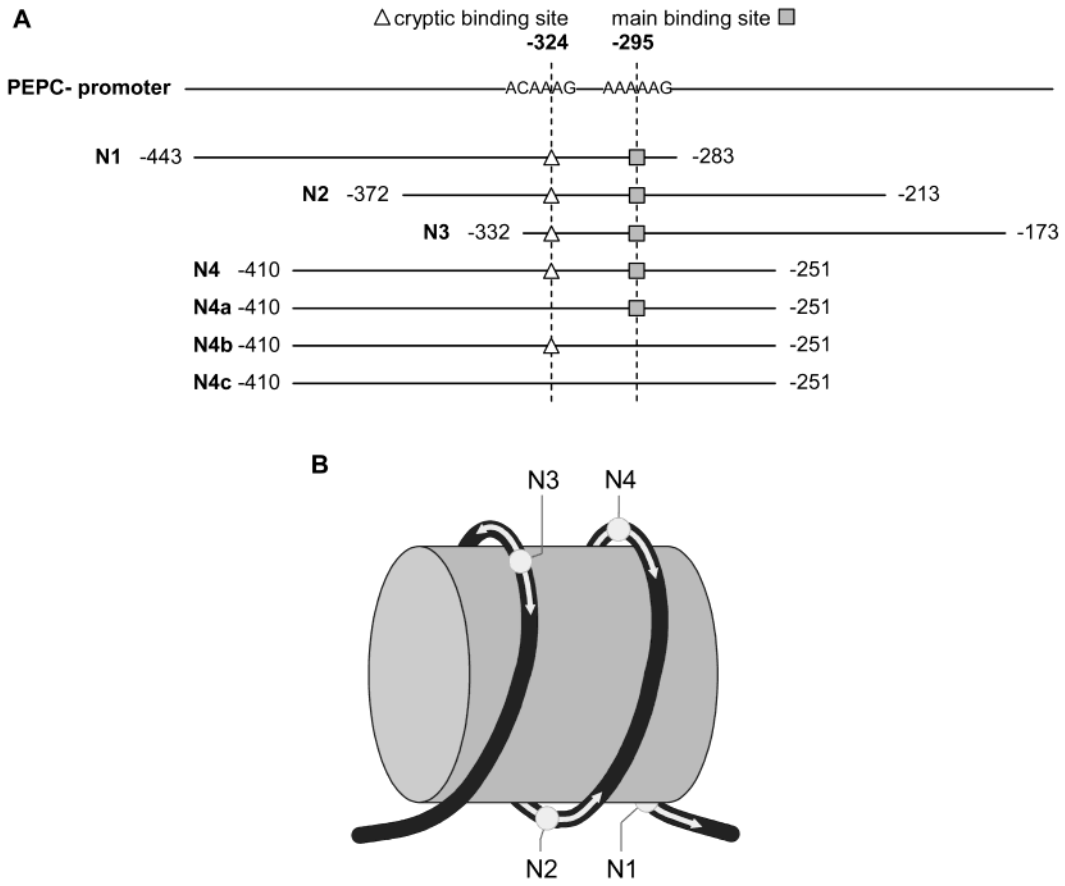

FIGURE 1: Schematic illustration of the experimental system. Panel A shows the position of the main DOF binding site and the cryptic binding site on the different PCR products used in this study. Positions are calculated in base pairs relative to the transcription initiation site on the C_4-specific PEPC promoter. The main DOF binding site is represented by a gray square and the cryptic binding site by a white triangle. In fragments N4a, N4b, and N4c, binding sites have been modified. (B) Overview of the positions of the main DOF binding site on the four types of nucleosomes used in the EMSA. The black line represents DNA wound around the histone octamer that is itself represented by the gray barrel. Four different 160 bp PCR products (N1−N4) amplified from maize genomic DNA containing a single DOF binding site from the C_4-PEPC promoter were used to reconstitute nucleosomes by salt dialysis. Points indicate the calculated position of the DOF binding site on the nucleosome, and arrows indicate the potential variability in the position of the binding site.

In this study, we show that DOF1 and DOF2 are capable of recognizing their binding site on reconstituted nucleosomes. Both the positioning of the binding site on the nucleosome and the presence of the HMGB5 protein in the binding reaction markedly affect the efficiency of binding.

RESULTS

Establishment of a Nucleosomal Binding Assay for DOF Proteins. We selected endogenous DOF binding sites on the promoter of the C_4-specific PEPC gene from maize (approximately 300 bp upstream of the transcription initiation site) to analyze DOF protein binding in the nucleosomal context. As shown in Figure 1A, the amplified region contains the core recognition motif AAAG (*23*) twice separated by 29 bp. However, EMSA analyses with labeled oligonucleotides revealed that the upstream motif (cryptic binding site) is not recognized by recombinant transcription factors in vitro (*4*). PCR products 160 bp in length containing the binding sites were amplified from genomic DNA. Primers were designed in such a manner that DOF binding sites were located at different positions on the nucleosomal surface. Since only 146 bp of DNA are assembled into a core particle, some variation of the exact position is expected assuming a random integration of DNA into the nucleosome. If the core particle is situated at one end of the PCR product, a maximum of 14 bp of DNA at the other end are not assembled into the nucleosome. If the core particle is centered on the DNA product, each 7 bp segment of free DNA on both ends is not assembled. Figure 1B shows the calculated positions of the main binding site. Whereas the DOF binding site in nucleosome N1 is proximal to the end of the assembled region, this sequence is located near the dyad axis of the nucleosome in nucleosome N2. On both nucleosomes N3 and N4, the binding site is exposed on top of the core particle. The additional variants of nucleosome N4 are described below.

We analyzed whether DOF proteins are in general capable of binding to their endogenous binding site either on naked DNA or assembled into a nucleosome. As shown in Figure 2, the recombinant purified DOF proteins bind to the PCR product N3. Up to three different shift positions were observed (in lanes 2 and 9, empty arrows indicate retarded complexes derived from naked DNA), depending on the amount of protein added to the assay. The same fragment was assembled into a nucleosome by salt dialysis, leading to a reduced electrophoretic mobility compared to that of the naked DNA fragment (lanes 3 and 10). Addition of DOF1 or DOF2 to this nucleosome induced the formation of additional novel complexes with lower electrophoretic mobility in this assay (in lanes 5 and 12, filled arrows indicate

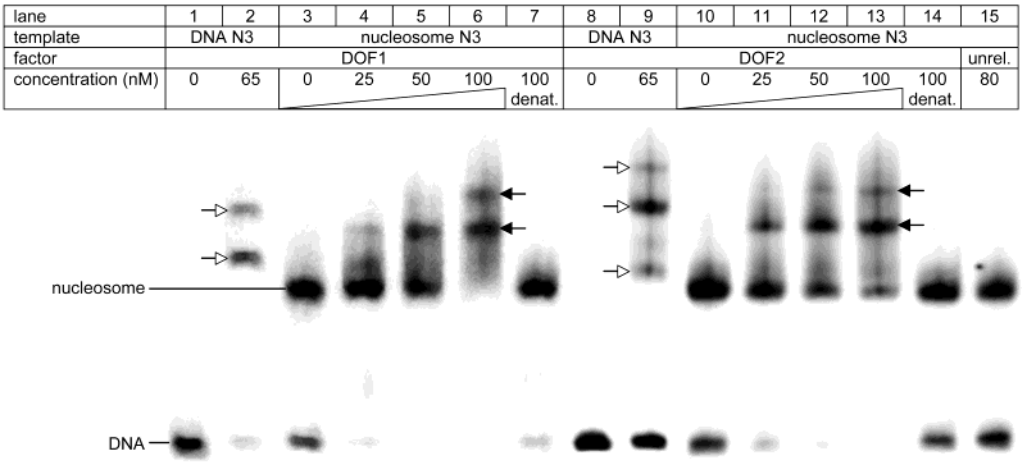

FIGURE 2: Characterization of the electromobility shift assay (EMSA). Shown is the autoradiography of an EMSA. The indicated amounts of DOF1, DOF2, or an unrelated protein (*E. coli* glyoxylate carboligase, unrel.) were incubated with either naked DNA or nucleosome N3, and complexes were separated on a 6% PAA gel. Filled arrows denote nucleosomal shifts and empty arrows DNA shifts. The increase in the DOF protein concentration is indicated as a triangle. Empty arrows mark complexes of naked DNA and DOF proteins, and filled arrows mark the positions of DOF−nucleosome complexes. denat. means the compound was denatured for 15 min at 65 °C.

retarded complexes derived from nucleosomal DNA). The position of the DOF−nucleosome complex in the gel was different from the complexes formed by DOF proteins with naked DNA, excluding the possibility that addition of the recombinant protein disrupts the nucleosome. At least one additional shift complex was formed, depending on the concentration of the DOF protein in the reaction mixture. However, when the transcription factor was denatured before addition of the nucleosome or when an unrelated protein (bacterial glyoxylate carboligase; see Materials and Methods) was used instead of the transcription factors in this assay, no shift complexes were formed (lanes 7, 14, and 15). These controls were performed with all nucleosomes applied in this study (data not shown). No major differences were observed when the efficiency of DOF1 or DOF2 in binding to the individual nucleosomes was compared. Therefore, DOF proteins in general recognize their binding site in the nucleosomal context and form defined complexes with nucleosomes in electromobility shift assays.

The Positioning of the Binding Site on the Nucleosome Affects the Binding of DOF Proteins. To analyze the efficiency of the binding of DOF proteins to the different nucleosomes as described in Figure 1, we compared the formation of retarded complexes in EMSA after addition of limited amounts of the recombinant proteins. The results obtained with DOF1 are shown in Figure 3A. For the N1 nucleosome, a nucleosomal shift complex (marked with a filled arrow) is already observable with the smallest amount of DOF1 added. The intensity of this signal increases with higher DOF1 concentrations, whereas the intensity of the free nucleosome signal decreases (lanes 3−6). In contrast, a weak binding to nucleosome N2 is only detectable with the highest concentration of DOF1 (lane 12). For nucleosomes N3 (lanes 15−18) and N4 (lanes 21−24), results are intermediate with a tendency for a higher efficiency of binding of DOF1 to N3 than to N4 (for a quantification, see below). However, the binding affinity of DOF1 for naked DNA is similar for molecules N1−N4 (lanes 2, 8, 14, and 20).

As more than one retarded complex was observed in the EMSA dependening on the amount of recombinant protein added, we constructed additional variants of the N4 fragment that contained only the main binding site (N4a), only the cryptic binding site (N4b), or none of the DOF binding sites (N4c) (see also Figure 1A). The fragments were used in the EMSA as shown in Figure 3B. For wild-type DNA incubated with recombinant DOF1, two retarded complexes are observed as expected (lane 1, empty arrows). When the cryptic binding site is deleted, the complex exhibiting the lower mobility disappears and only one shift band derived from the main binding site is visible (lane 2). In contrast, deletion of the main binding site leads to the formation of a new DNA−DOF complex with a lower intensity, but a different electrophoretic mobility (lane 3). When both binding sites are deleted, no binding of recombinant DOF1 to the PCR product can be observed (lane 4). Repetition of the experiments with the different DNA fragments assembled into a nucleosome led to comparable results. Besides the complexes formed with the remaining free DNA, a strong and a weak complex are formed with the nucleosome containing both binding sites (lane 5, filled arrows). When the cryptic binding site is deleted, only the stronger DOF1−nucleosome complex is observed (lane 6). However, no binding of DOF1 to a nucleosome containing only the cryptic binding site or containing no binding site is detectable (lanes 7 and 8). Thus, in both the DNA and the nucleosome context, DOF1 binds preferentially to the main binding site. Formation of this complex might assist binding of the transcription factor to the cryptic binding site.

The impact of the positioning of the binding site was also investigated for DOF2 interacting with nucleosomes (Figure 4A). Formation of the most prominent DOF2−nucleosome complex is clearly most efficient with nucleosome N1 and weakest with nucleosome N2. Because a higher concentration of recombinant protein was used (60−120 nM compared to 40−60 nM for DOF1), additional shift complexes are formed in part. To quantify the efficiency of binding, these assays were repeated three times independently and analyzed on a phosphorimager. Figure 4B indicates the percentage of retarded complexes formed at an intermediate concentration of DOF2 (90 nM). For nucleosome N1, more than 60% of the core particles are bound at the given concentration

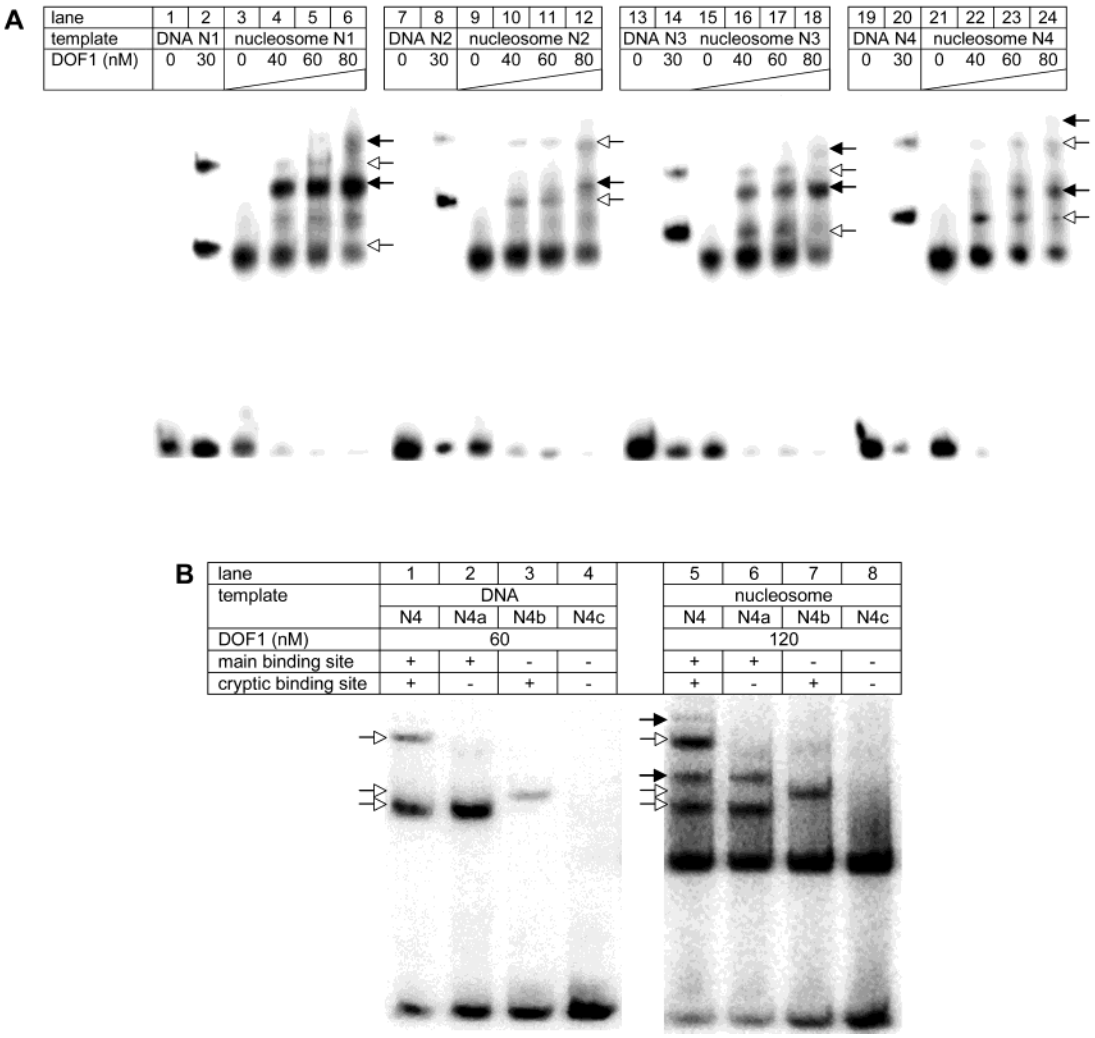

FIGURE 3: Binding of DOF1 to nucleosomes. (A and B) Shown are autoradiographies of EMSA analyses with DOF1. Naked DNA or DNA assembled into a nucleosome was incubated with the indicated concentrations of the DOF1 protein, and complexes were separated on a 6% PAA gel. The increase in DOF1 concentration is represented by a triangle. Empty arrows denote complexes of naked DNA and DOF1, and filled arrows mark the positions of DOF1−nucleosome complexes. DNA and nucleosome templates are as indicated in the legend.

whereas only ~10% of the N2 nucleosomes form complexes. For nucleosome N3, an affinity of ~50% is observed, and this value is clearly higher compared to that for nucleosome N4 (30%). All values that were measured differ significantly from each other with a p of <0.05 according to a t-test. Taken together, the data indicate clear differences in the efficiency of DOF protein binding to nucleosomes depending on the positioning of the binding site on the surface of the core particle.

Maize HMGB5 Facilitates the Binding of DOF2 to Nucleosomes. HMGB proteins are non-histone components of chromatin that are known to modify binding of transcription factors to their binding sites. We applied our binding assay to analyze whether this also holds true for DOF proteins and maize HMGB5 in the nucleosomal context (Figure 5). At the concentrations that were used, HMGB5 did not form any retarded complexes with either naked DNA or nucleosomes (lanes 2 and 5). For the complex of naked DNA and DOF2, an increase in intensity was observed when HMGB5 was added to the assay (compare lanes 1 and 3). These results were similar for all DNA fragments (data not shown), and the same effect has recently been reported by Krohn et al. (*22*). In the nucleosomal shift assays, a concentration of DOF2 was used that itself is not sufficient to induce the formation of retarded complexes. In the presence of HMGB5, the efficiency of complex formation was clearly enhanced in a concentration-dependent manner. However, the electrophoretic mobility of the complex in the EMSA was not altered relative to that in assays with higher concentrations of DOF2 in the absence of HMGB5 (lanes 6, 11, 16, and 21). When the amounts of retarded complexes formed were compared, again clear differences were found between the four different nucleosomes. Whereas a significant part of the N1 and N3 nucleosomes are bound by DOF2 in the presence of HMGB5, this effect is clearly weaker for the N4 nucleosome and virtually absent for the N2 nucleosome. However, when four times higher concentrations of DOF2 were used in the assay, a positive impact of HMGB5 on the binding of DOF2 to the N2 nucleosome could also be observed (Figure 5B). Thus, HMGB5 facilitates the interaction of DOF2 with its binding site assembled into a nucleosome, but does not relieve the impact of the positioning of the binding site on the nucleosomal surface.

DISCUSSION

We have analyzed the interaction of DOF transcription factors with their binding sites in the nucleosomal context. Most in vitro analyses of DOF−DNA interactions have been

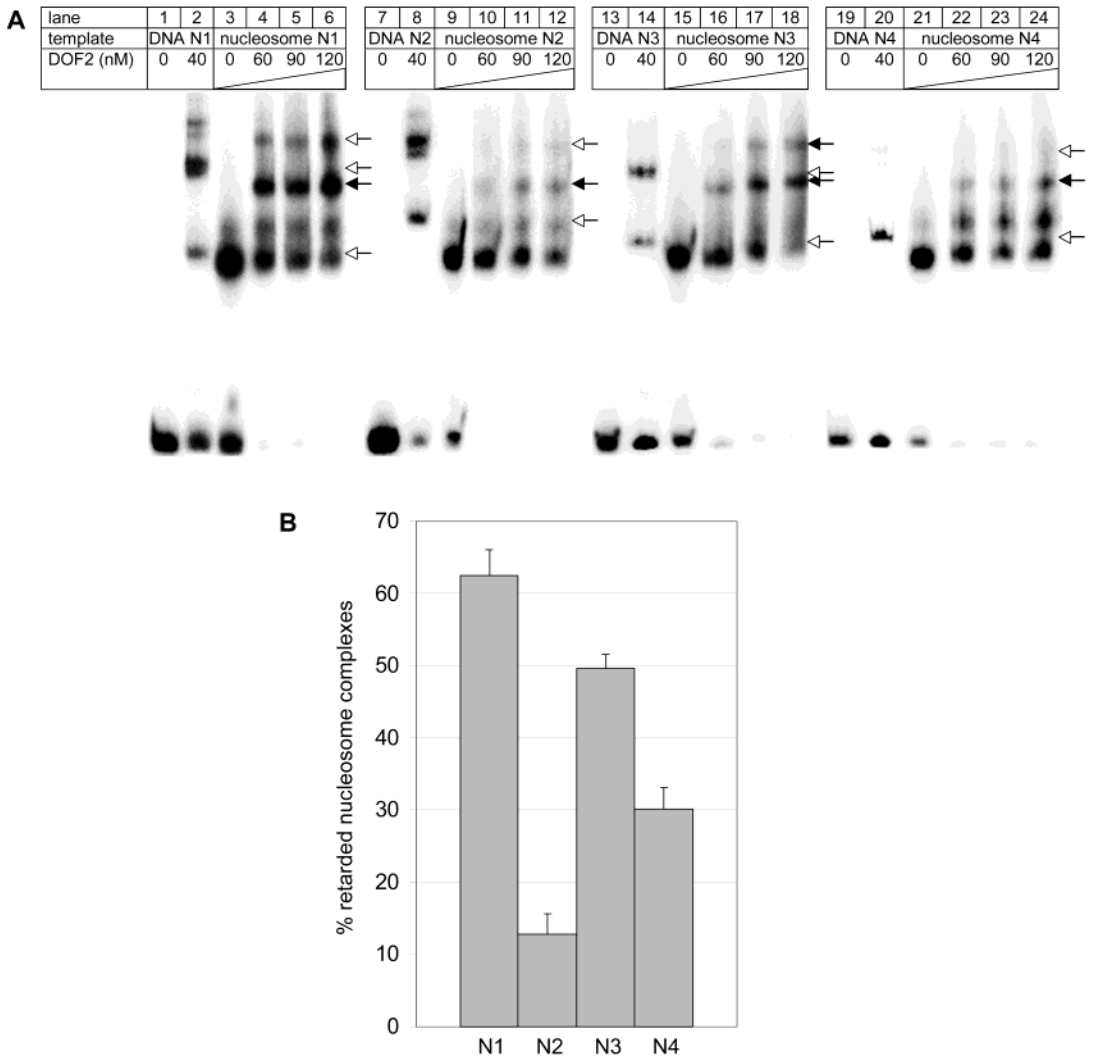

FIGURE 4: Binding of DOF2 to nucleosomes. (A) Shown is an autoradiography of EMSA analyses with DOF2. Naked DNA or DNA assembled into a nucleosome was incubated with the indicated concentrations of DOF2 protein, and complexes were separated on a 6% PAA gel. The increase in DOF2 concentration is represented by a triangle. Empty arrows mark complexes of naked DNA and DOF2, and filled arrows mark the positions of DOF2−nucleosome complexes. (B) Bar chart showing the percentage of nucleosomes forming complexes with DOF2 at a transcription factor concentration of 90 nM. Data were derived from three independent experiments. Vertical lines represent standard deviations.

worked out previously with the isolated DNA binding domain. Several independent experiments revealed that this domain is sufficient for DNA binding and that no major differences are found for DOF1 compared to DOF2 in this respect (*5*). Nevertheless, there are clear differences in the transcriptional activation brought about by both factors. Whereas a short amino acid stretch in the C-terminal part of the DOF1 protein acts as a strong activation domain in plants, yeasts, and animals (*24*), transcriptional activation by DOF2 is barely detectable (*4*). We wanted to test whether this partitioning of functions between the different domains also holds true in the chromatin context and thus expressed the complete available DOF sequences in *Escherichia coli*. Although previous experiments revealed that the proteins are degraded during production, an optimized expression and purification protocol (see Materials and Methods) allowed the isolation of sufficient amounts of the active recombinant protein for multiple analyses. As the proteins were tagged on the C-terminus, degradation products containing the N-terminal DNA binding domain would not bind to the column and would thus be separated. We can therefore assign all observed effects to the full-length proteins. However, our studies revealed no clear differences for DOF1 and DOF2 in binding to nucleosomes, underscoring the central role of the N-terminus.

Besides the main shift band, we can observe at least a second retarded complex that is formed when higher concentrations of DOF protein are added to both naked DNA and nucleosomes. As the natural sequence environment of the recognition sequence was used in the assays, it was inevitable to include additional sequence elements that show similarity to DOF binding sites. Indeed, a second AAAG motif is found only 29 bp upstream of the main recognition site as described. However, a detailed analysis of recognition sequences of DOF proteins revealed that an A or T nucleotide is highly preferred by both DOF1 and DOF2 at the −1 position relative to the core element (*23*). Whereas the main binding site studied here shows such a sequence, a C nucleotide is found at position −1 of the cryptic binding site. Deletion of one or both binding sites on the PCR product used for nucleosome assembly (Figure 3B) facilitated the assignment of the observed complexes to the different

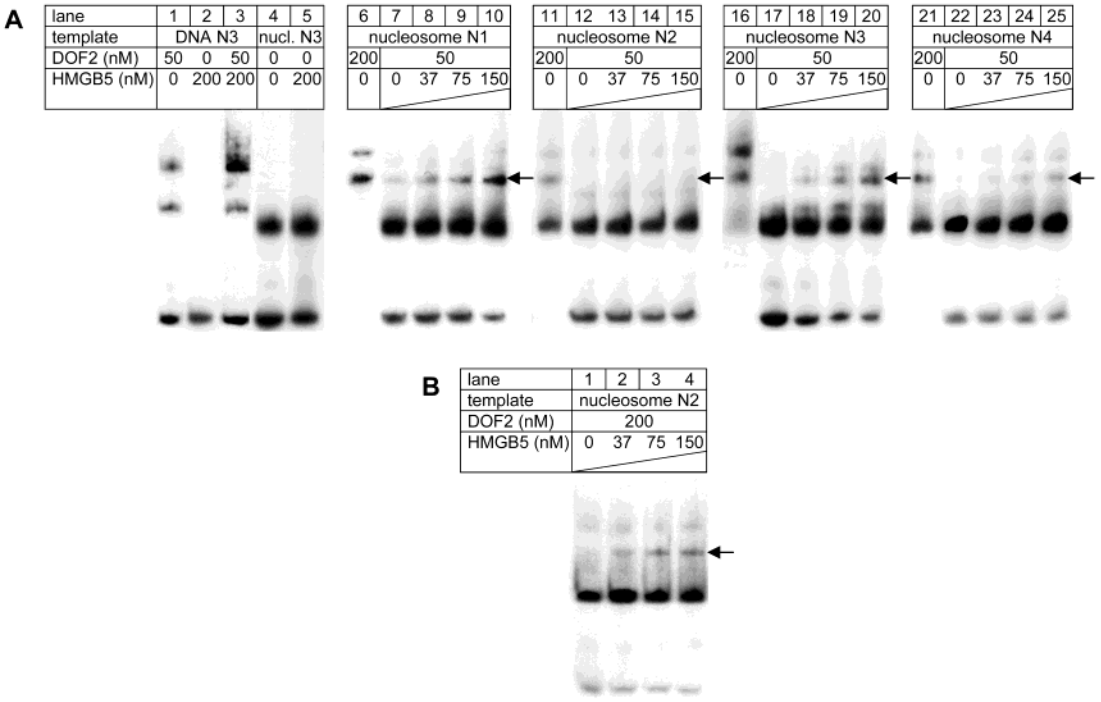

FIGURE 5: Binding of DOF2 to a nucleosome is facilitated by HMGB5. Shown are autoradiographies of EMSA analyses with DOF2 and HMGB5. Naked DNA or DNA assembled into a nucleosome was incubated with the indicated concentrations of DOF2, and HMGB5 proteins and complexes were separated on a 6% PAA gel. The increase in HMGB5 concentration is represented by a triangle. Filled arrows indicate the position of the DOF2−nucleosome complex. (A) Shift assays were performed at a DOF2 concentration of 50 nM. (B) Shift assays were performed at a DOF2 concentration of 200 nM. nucl. means nucleosome.

binding sites. Interestingly, both the main and cryptic binding sites alone are capable of interacting with DOF1 on naked DNA, albeit the cryptic binding site with a lower efficiency. In the presence of both binding sites, the complex of DOF1 with the cryptic binding site is not observed, but a third band appears presumably due to binding of DOF1 to both the cryptic and the endogenous binding site. A dimerization of the transcription factor on the main binding site can be ruled out because such an effect should also be observed with the PCR product with a mutation of the cryptic binding site. The data are consistent with a positive synergistic impact of DOF1 binding to the main binding site on interactions with the cryptic binding site. In the nucleosomal context, formation of the second more retarded complex is basically weaker, but again this complex is only formed when both binding sites are present on the nucleosome, arguing in the same direction. The small distance of the major and the cryptic binding site might allow such synergistic interaction between DOF proteins that have been shown before to dimerize via the DOF domain (*5*).

Comparing binding studies with nucleosomes bearing DOF recognition sequences at different spatial positions, we revealed striking differences in the efficiency of DOF nucleosome interactions. The length of the assembled fragment (160 bp) does not allow an absolute precise prediction of the exact positioning of the binding site, but the region can be pinpointed to a small sequence interval assuming always that 146 bp of DNA is assembled into a nucleosome. We have tested this by limited digestion of nucleosomes with micrococcal nuclease and found protection of a 146 bp fragment after assembly (data not shown). The exact winding of the DNA around the octamer can affect both the translational positioning of the binding site, i.e., the position relative to the dyad of the nucleosome, and the rotational positioning, i.e., whether the binding site is exposed toward solution or hidden in direction of the histone core on the surface of the nucleosome (*25*). Both factors have been shown to severely affect nucleosome−transcription factor interactions. For instance, translational positioning is a major determinant of the binding of TFIIIA to nucleosomes (*26*). Li et al. (*27*) reported that both rotational positioning and translational positioning of a glucocorticoid response element affect its accessibility and that the impact of the rotational position is modulated at different translational positions. For the thyroid response element, changes of only 3 bp in positioning relative to the histone surface are sufficient to reduce the binding efficiency of a receptor (*28*), and similar effects have been found for TBP recognizing the TATA box (*29*). However, other factors such as NF1 show a reduced level of binding to nucleosomes compared to DNA irrespective of the positioning of the binding site (*30*, *31*) or like Gal4-VP16 bind to chromatin and DNA with similar affinity (*32*). In plants, the binding of EMBP-1 to an abscisic acid response element has been shown to be dependent on the rotational and translational position of the recognition sequence (*33*). The relevance of such effects for promoter activity in vivo has recently been worked out for the β-phaseolin promoter from bean (*34*).

The differentially exposed binding sites applied in our study clearly differ in translational positioning. Most efficient binding, comparable to the efficiency of naked DNA recognition, was observed when the binding site was positioned at the border of the nucleosome. Shifting the DOF site near the dyad of the nucleosome strongly affected binding and reduced the affinity approximately 6-fold as deduced from quantitative phosphorimager analyses. This

reduction is rather weak compared to most of the factors cited above, suggesting that DOF proteins show a clear affinity for their binding site also at unfavorable nucleosomal positions. Significant differences were also found when comparing nucleosomes N3 and N4, although the DOF site is located in both cases on top of the nucleosome. However, the recognition sequences show a distance of 78 bp and are thus located on opposite sites of the DNA molecule; i.e., if the one site is exposed to the solution, the other site will be oriented toward the octamer surface. This scenario implies that both nucleosomes are positioned similarly in the middle of the DNA stretch, leaving 7 bp of free DNA at each end. Nucleosome assembly by salt gradient dialysis is expected to lead to the equilibrium distribution of core particles on the DNA molecule, and these core particles are mobile on the DNA stretch under physiological conditions (*25*); on the other hand, rotational positioning is seemingly conserved during these movements (*35*). Thus, the differences in affinity may be explained by differences in the rotational positioning of the binding sites on nucleosomes N3 and N4, although we cannot predict the exact rotational positioning of the binding site on the nucleosome surface. It will be interesting to see whether these results are reproducible in nucleosomes where the rotational positioning is fixed by the integration of an artificial TG motif sequence stretch (*36*). However, in this case, assays cannot be performed in a natural sequence environment, and thus, a possible influence of the DNA sequences adjacent to the recognition site on the binding of DOF factors will not be observed.

Besides the positioning of the target sequence, we have shown a positive impact of the HMGB5 protein on the interaction of DOF proteins with nucleosomes. Interestingly, the stimulation of DOF binding by HMGB5 does not override the effects brought about by positioning, but HMGB5 rather reduces the threshold concentration of the DOF protein that is necessary for formation of nucleosome complexes. To our knowledge, this is the first report on a positive effect of HMGB proteins on transcription factor binding to nucleosomes. In contrast, the function of HMGB proteins in facilitating protein interactions with naked DNA is well-described in both animals and plants (*20*, *37*−*39*). In a recent study, Krohn et al. (*22*) have investigated the influence of different maize HMGB proteins on the binding of DOF2 to naked DNA. HMGB5 exerted the strongest effects in these analyses and was therefore chosen for our investigations with reconstituted nucleosomes. Many of the results obtained in this study are similar to those obtained with naked DNA. Particularly, the gel position of the DOF−DNA complex is identical in the presence or absence of HMGB proteins, and the same is observed for the DOF−nucleosome complex. Nevertheless, cross-linking analyses on naked DNA revealed that HMGB proteins interact with both the protein and the DNA component and are seemingly present in the DNA−protein complex without altering the gel retardation. The same complex formation might occur in the DOF−nucleosome interaction in the presence of HMGB proteins. However, we expect different domains of the HMG protein to be involved in the interaction. Whereas the individual HMG domain of HMGB1 is capable of facilitating the DOF−DNA interaction, specific nucleosome binding requires also the N- and C-terminal domains of full-length HMGB1 (*40*). Future analyses will reveal which HMG protein domains are necessary to assist in binding of DOF proteins to nucleosomes.

What do the results obtained in this study tell us about the activation of photosynthetic genes in maize? The DOF recognition site on the C_4-PEPC promoter under investigation lies within a region of known importance for promoter activation (*4*, *41*). As DOF1 is capable of interacting with its recognition sequence also on nucleosomes, it might initialize the formation of a transcription initiation complex. This can be facilitated by recruitment of HMGB proteins or by dimerization of factors binding to the multiple other potential binding sites on the promoter. Although these binding sites are separated by more than 100 bp in part, assembly of the promoter into nucleosomes might bring distant sites into spatial proximity as described previously (*42*−*44*). Alternatively, another so far unidentified factor induces repositioning of nucleosomes on the promoter in a way that the DOF binding site is exposed on top of the nucleosome and therefore more easily accessible. In vivo analyses of the accessibility of promoter chromatin for restriction endonucleases revealed clear evidence for a chromatin reorganization on the C_4-PEPC promoter upon activation (R. Kalamajka and C. Peterhänsel, unpublished data). However, it is still unclear whether chromatin activation follows DOF1 association with the promoter or vice versa. The temporal order of events can be analyzed by chromatin immunoprecipitation with specific antibodies as shown previously in yeast and animal systems (*15*, *45*). The demonstration of efficient nucleosome interactions of DOF proteins in this study is a valuable starting point for this purpose.

MATERIALS AND METHODS

DNA Probe Preparation. The four different DNA fragments applied in shift assays were amplified with four different primer systems (probe N1, primer N1F 5′-TTCCTCCAAATTCTTGCGAT-3′ and primer N1R 5′-CTCCTTGCTCCTTTTGGCT-3′; probe N2, primer N2F 5′-CCCAATTAGCCAACGGAAT-3′ and primer N2R 5′-GGGTGTCTGCTACTGCTGG-3′; probe N3, primer N3F 5′-AGTGACAAAGCACGTCAAC-3′ and primer N3R 5′-TTGGCTTGCTGCTGTTAGC-3′; and probe N4, primer N4F 5′-CATTAACTGCTAAGGGACGC-3′ and primer N4R 5′-TGGAGAGCTGCGGCTGCGG-3′). Sequences were amplified from the promoter of the C_4-specific PEPC gene from maize (gi 22396) using genomic maize DNA as a template and standard PCR conditions. All fragments cover a DOF binding site 300 bp upstream from the transcription initiation site as well as a cryptic binding site 20 bp upstream of the main binding site that was shown to be nonfunctional in shift assays with oligonucleotides (*4*). The PCR fragments were purified from 1% agarose gels according to standard protocols (*46*) followed by end labeling with [γ-^{32}P]ATP and another purification step with a commercial PCR purification kit. Typically, 200 ng of fragment was labeled, and 1.2×10^7 cpm was achieved.

Construction of Mutated Probes. The construction of probes with mutations in DOF binding sites was performed using SOE-PCR (*47*). In each case, the nucleotide sequence AAAG from the recognition motif was replaced with a GCGG sequence. For mutation of the main binding site, the

primer DOFm3 (5′-CACGTCAACAGCACCGAGCCAAGCCAGCGGGAGCAAG-3′) was used, and for mutation of the cryptic binding site, the primer DOFm4 (5′-GGCTCGGTGCTGTTGACGTGCGCGGTCACTGCTC-3′) was used. Primers DOFm1 and DOFm2 contained the respective wild-type sequences instead. Fragments were amplified using primers N1F and DOFm3, N1F and DOFm1, N3R and DOFm4, and N3R and DOFm2. PCR products were diluted 1:1000 and combined in a second PCR mixture using primers N1F and N3R. The products were cloned, and mutation of the binding sites was verified by sequencing (data not shown). The resulting clones were used for the amplification of DNA probes as described above.

Nucleosome Reconstitution by Salt Gradient Dialysis. The nucleosome reconstitution was achieved by salt gradient dialysis according to the method of Stein (*48*). A trace amount (approximately 120 000 cpm corresponding to 2 ng) of radiolabeled PCR product was mixed with 0.4 μg of core histones [purified from Hybridoma C25-6 Ramos mouse B-lymphocytes as described previously (*49*)], 5 μg of BSA, and 90 ng of nonlabeled competitor (λ-DNA, *Pst*I- and *Hae*III-digested) in a 50 μL reaction volume containing 20 mM Tris-HCl (pH 7.6), 1 M NaCl, and 0.2 mM EDTA. This mixture was dialyzed in Pierce Slide-A-Lyzer Mini Dialysis Units (3500 molecular weight cutoff) for 1.5 h at room temperature against 1 L of 0.80 M NaCl, 20 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, and 1 mM 2-mercaptoethanol. The sample was next dialyzed for 1.5 h at room temperature against 1 L of 0.15 M NaCl, 20 mM Tris-HCl (pH 7.6), and 0.2 mM EDTA and finally against 1 L of 20 mM Tris-HCl (pH 7.6) and 0.2 mM EDTA for an additional 1.5 h.

Electromobility Shift Assay (EMSA). All binding reaction mixtures contained, in addition to the proteins denoted in the figures, radiolabeled DNA or nucleosomes corresponding to 1000 cpm, 100 ng of poly(dA-dT), 100 ng of BSA, 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 2.5% (w/v) glycerol, and 1 mM EDTA in a final volume of 20 μL. The reaction mix was incubated at room temperature for 30 min and afterward loaded onto a 6% (w/v) polyacrylamide [37.5:1 (w/w) acrylamide/bisacrylamide] gel that was run in 0.25× TBE at 110 V for 1.25 h at room temperature. Gels were dried, exposed on Fuji BAS-MS imaging plates, and analyzed with a Fuji FLA-3000 fluorescence scanner (Raytest, Straubenhardt, Germany). Alternatively, gels were exposed on Kodak BioMax MR films, and the films were developed using conventional techniques.

Expression and Purification of Recombinant Proteins. The coding sequences of the maize DOF1 (gi 517258, 25 kDa) and DOF2 (gi 1061306, 23 kDa) proteins were amplified by PCR from plasmids pGST-DOF1 and pGST-DOF2 (*5*) kindly provided by S. Yanagisawa. 5′-Primers contained an additional *Nco*I site and 3′-primers an additional *Xho*I site. Primer sequences were 5′-ACGTCCATGGCAATGCAGGAGGCGTCATC-3′ and 5′-ACCTCGAGCGGGAGGTTGAGGAAGATG-3′ for DOF1 and 5′-ACGTCCATGGCCAAGGGCTACCCCGTGC-3′ and 5′-ACCTCGAGCGGCAGATTGAGGAACAGCGCC-3′ for DOF2. PCR fragments were ligated into the plasmid pET22b(+) (Novagen), resulting in a C-terminal translational fusion containing six histidines. The constructs pET-DOF1 and pET-DOF2 were transformed into *E. coli* strain ER2566 (Novagen). For protein expression, a 2 L culture of LB medium was grown to an OD_{600} of 0.6. The expression of the proteins was induced by adding 1 mM IPTG, and culture growth was continued for 1 h at 37 °C. The cells were washed in TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)] and subsequently incubated for 10 min in lysis buffer containing 20 mM Tris-HCl (pH 7.6), 300 mM NaCl, and a trace amount of lysozyme. After the cells were lysed by sonification on ice, they were centrifuged for 40 min at 200000*g*. The proteins were purified at 4 °C by single-step metal chelate chromatography (Ni−NTA agarose, Qiagen, Hilden, Germany). After being loaded, the column was washed with 15 column volumes each of lysis buffer, wash buffer [20 mM Tris-HCl, 300 mM NaCl, and 0.5% (v/v) Igepal CA 630 (pH 7.6)] containing 5 and 30 mM imidazole, respectively, and wash buffer containing 30 mM imidazole but no Igepal CA 630. Elution was performed with 3.5 column volumes of 20 mM Tris-HCl, 300 mM NaCl, and 300 mM imidazole (pH 7.6). Typically, a yield of 25 μg of recombinant protein per 2 L of culture was obtained. The unrelated protein glyoxylate carboligase from *E. coli* was expressed and purified the same way, and a similar yield of recombinant protein per liter of culture was obtained. The maize HMGB5 protein was expressed as a six-His-tagged fusion protein in *E. coli* and purified by three-step column chromatography as described previously (*50*, *51*).

Quantification of EMSA Signals. The readout signals from the imaging plates were quantified using the Advanced Image Data Analyzer V3.11.002 software (Raytest). The term signal denotes the relative fluorescence strength in light absorption units for a particular band. The bound fraction of nucleosomes (Figure 4B) was calculated as

$$\text{bound fraction} = \frac{[100(\text{signal shift band for nucleosome DOF} - \text{signal background})]}{(\text{signal for nucleosome} - \text{signal background})}$$

The scanning area for the nucleosome and the area for the shift band as well as the background area were equal. The background signal was measured for each band directly below the shift signal.

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