Eur. J. Biochem. *250*, 403−410 (1997)

Dof DNA-binding domains of plant transcription factors contribute to multiple protein-protein interactions

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(Received 1 July/16 September 1997) − EJB 97 0929/2

Dof proteins are a family of plant transcription factors that have a strongly conserved DNA-binding domain, designated the Dof domain. This domain has the potential to form a single zinc finger. This report describes the self-association of a maize Dof protein, Dof1 (previously designated MNB1a). Affinity chromatography revealed that Dof1 also interacted with another maize Dof protein, Dof2, as well as with high-mobility-group (HMG) protein 1. Results of mapping of the region required for the protein-protein interactions of Dof1 suggested that these interactions may be mediated by the Dof domain. When gel mobility shift assays were performed with purified recombinant Dof proteins, homomeric and heteromeric complexes of Dof proteins on DNA were detected. It seems possible that formation of complexes of different Dof proteins through direct protein-protein interactions might be involved in the regulation of transcription. Evidence is also presented that HMG1 has an effect on the binding of Dof1 to DNA. Therefore, it appears that the Dof domain is a multifunctional domain that is involved not merely in binding to DNA but also in multiple protein-protein interactions.

Keywords: plant DNA-binding protein; zinc finger; transcription factor; high-mobility-group (HMG) protein 1; protein-protein interaction.

Dof proteins are a recently identified group of plant DNA-binding proteins. Members of this family are defined as proteins that contain a highly conserved DNA-binding domain that has been designated the Dof (DNA-binding with one finger) domain [1, 2]. This DNA-binding domain contains a $CX_2CX_{21}CX_2C$ motif that was predicted to have the ability to form a single zinc finger from the results of experiments with chelators and mutational analysis [1]. The maize DNA-binding protein MNB1a (Dof1) was the first member of the Dof family to be characterized. It can bind *in vitro* to two copies of the AAAAGG sequence motif in a narrow region (−276 to −252 relative to the site of initiation of transcription) in the 35S promoter of the cauliflower mosaic virus (CaMV) [3]. The cDNA clones for Dof proteins, designated Dof2 and Dof3, were subsequently obtained as representatives of the maize MNB1a (Dof1) multigene family [1]. For consistency in the naming of Dof proteins, MNB1a is referred to as Dof1 in this study. The Dof domain, a stretch of 52 amino acids in the N-terminal region of each protein, was identified from a comparison of the amino acid sequences of these proteins. Obvious similarity was found only among Dof domains and not in other regions when the clones were compared. Recent data indicate that the N-terminal region of Dof1, which contains a Dof domain, and the C-terminal region of Dof1 function independently as DNA-binding and transcription activation domains *in vivo* (Yanagisawa, S. and Sheen, J., unpublished results). Therefore, Dof1 that is encoded by the intronless gene [4] seems to have separable domains.

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Abbreviations. CaMV, cauliflower mosaic virus; bZip, basic leucine zipper; HMG1, high-mobility-group protein 1; β-gal, β-galactosidase; GST, glutathione *S*-transferase; EtBr, ethidium bromide; EMSA, electrophoretic mobility shift assay.

A previous study suggested that Dof proteins might be conserved not only in monocots but also in dicots, implying a pivotal role for such proteins in plants [1]. In fact, cDNAs with similarity to cDNAs for maize Dof proteins were recently isolated from *Arabidopsis*, pumpkin, and tobacco [2, 5, 6]. A cDNA clone which was isolated from *Arabidopsis* encoded a protein, OBP1, that can interact with a basic leucine zipper (bZip) protein from *Arabidopsis*, OBF. OBF binds to the ocs element in the promoter of the gene for octopine synthase. OBP1 itself can bind to the Dof1-binding site and enhance the binding of OBF to DNA [5, 7]. Another recently identified Dof protein is NtBBF from tobacco, which binds to regulatory domain B of the promoter of the plant oncogene *rolB* gene [6]. This region is required for expression of the *rolB* gene in meristems. These findings suggest that Dof proteins might be involved in the expression of diverse genes, and a comparison of the amino acid sequences of the Dof domains of these proteins suggests that Dof proteins can be separated into subgroups [2]. Although Dof proteins have been found in several plant species, the Dof domain has not yet been identified in any mammalian protein.

There appear to be about ten genes for Dof proteins in maize (Yanagisawa, S., unpublished results) and three maize cDNAs for Dof proteins have already been isolated [1, 3]. Dof1 is expressed constitutively [3, 4], while Dof2, which is structurally the closest relative of Dof1 among all Dof proteins identified to date [2], is expressed mainly in stems and roots (Yanagisawa, S. and Sheen, J., unpublished results). Dof2 binds to DNA with identical or very similar sequence specificity to Dof1 (Yanagisawa, S., unpublished results). The DNA-binding activity of Dof3, which is expressed mainly in stems and roots, remains to be examined *in vitro* since expression of Dof3 seems to be toxic to *E. coli* cells [1]. Our recent unpublished studies with maize protoplasts have demonstrated that Dof1 acts as an activator of transcription. By contrast, Dof2 appears not to activate transcrip-

tion, but to block transactivation by Dof1. Thus, these Dof proteins with opposing activities might be involved in the control of expression of the same gene(s).

Recent studies of transcription factors in animal cells have revealed that protein-protein interactions between transcription factors are important in the regulation of gene expression [8–10]. It has also been reported that some plant transcription factors are involved in protein-protein interactions [11, 12]. Direct repeats of the AAAAGG motif can be found in the CaMV 35S promoter [3] and Dof1 binds to multiple binding sites in the promoter sequences of putative cellular target genes, such as the C4 photosynthetic phospho*enol*pyruvate carboxylase gene (Yanagisawa, S. and Sheen, J., unpublished results). Thus, multiple Dof1 proteins seem to be able to associate with a single target promoter. In the present study, the physical interactions of these proteins were investigated. This report describes the direct protein-protein interactions between Dof proteins and the formation of homomeric and heteromeric complexes of Dof proteins on DNA. Evidence is also presented that maize high-mobility-group (HMG) protein 1 affects the binding of Dof1 to DNA.

MATERIALS AND METHODS

Construction of plasmids for expression of fusion proteins. To obtain fusion proteins composed of *β*-galactosidase and a Dof protein or HMG1, several plasmids were constructed using the expression vector pAX4a (Mo Bi Tec). Construction of pAX-Dof1 (pAX-MNB1a) and its derivatives pWT(44–147), pCdel753, pCdel528, pCdel444, and pCdel278 for the expression of fusion proteins between *β*-galactosidase and Dof1 was described previously [1]. To obtain plasmid pAX-Dof2 for the expression of a fusion protein between *β*-galactosidase and Dof2, the cDNA for Dof2 was digested with *Eco*RI and *Sal*I, for which restriction sites were located in the linker sequence and the 3′ non-coding region, respectively. Then, the Dof2 cDNA was inserted into the multicloning site of pAX4a. A clone, designated *λ*MNB1b in a previous report [3], was chosen as a clone that encoded maize HMG1, since the clone was found to be identical to a cDNA clone that had been isolated with antibodies against the HMG1 protein of maize [13]. In general, HMG1 proteins are composed of an acidic C-terminus and two copies of a homologous domain, designated the HMG box, that mediate non-specific binding to DNA [14, 15]. However, maize HMG1 has only one copy of the HMG box [3, 13]. *λ*MNB1b was digested with *Nco*I and *Pst*I, for which restriction sites were located in the linker sequence and the coding region, respectively. The *Nco*I–*Pst*I fragment encoded a region (amino acids 1–116) containing an entire HMG box, but not the acidic C-terminus. This fragment was inserted between the *Nco*I and *Pst*I sites of pAX4a, which had stop codons in all three reading frames. The resulting plasmid, pAX-HMG1, was used for expression of a fusion protein that consisted of *β*-galactosidase and HMG1.

The pGEX bacterial expression vector system (Pharmacia) was used to produce fusion proteins with glutathione *S*-transferase (GST). A plasmid for expression of the entire Dof1-coding region, pGST-Dof1, was obtained by insertion of an *Eco*RI fragment of *λ*MNB1a cDNA [3] into the *Eco*RI site in the pGEX-5X-1 vector. The pGST-Dof2 construct was also obtained by insertion of an *Eco*RI–*Sal*I DNA fragment of pAX-Dof2 between the *Eco*RI and *Sal*I sites in the pGEX-5X-1 vector. The pGST-HMG1 construct was generated by insertion of the *Nco*I–*Pst*I DNA fragment of pAX-HMG1 between the *Nco*I and *Sal*I sites of the pGEX vector, using chemically synthesized DNAs with a termination codon (5′-TGAG-3′ and 5′-TCGACTCATGCA-3′).

Analysis of protein-protein interactions. After *Escherichia coli* cells had been transformed with an appropriate plasmid for expression of a GST fusion protein and grown in Luria-Bertani medium at 30 °C to mid-log phase, fusion proteins were induced by addition of 1 mM isopropyl thio-*β*-D-galactoside. Bacterial cells were harvested after 2 h, suspended in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3 (NaCl/P_i) that had been supplemented with 1 mM phenylmethylsulfonyl fluoride and sonicated. Insoluble materials were removed by centrifugation at 12000 *g* for 10 min and 1 ml supernatant, containing 5 mg protein, was incubated with 50 µl glutathione-Sepharose 4B resin (Pharmacia) for 30 min at 4 °C on a rotating wheel. The resin was washed with NaCl/P_i that contained 0.1 % nonidet P-40. The resin with bound GST or a GST fusion protein was mixed with 1 ml bacterial lysate (5 mg protein) that contained a fusion protein including *β*-galactosidase for 1 h at 4 °C on a rotating wheel. The bacterial lysates that contained fusion proteins with *β*-galactosidase were prepared similarly to those that contained fusion proteins with GST. These lysates were used after incubation with 0–100 µg/ml ethidium bromide (EtBr) for 30 min at 4 °C and removal of insoluble materials by centrifugation at 12000 *g* for 5 min. After incubation, the resin was washed four times with 1 ml NaCl/P_i plus 0.1 % nonidet P-40 that did or did not contain EtBr. When EtBr was added to binding and washing buffers, the same concentration was used in each case. The proteins trapped on the resin were eluted three times with 20 µl 10 mM glutathione. The eluates were subsequently subjected to western blotting analysis with antibodies against *β*-galactosidase as described previously [1].

Expression and purification of recombinant proteins. Several plasmids were constructed for preparation of recombinant Dof1 and Dof2 for electrophoretic mobility shift assays (EMSAs). Since the expression of the entire coding regions of Dof proteins in *E. coli* cells produced degraded forms of Dof proteins, plasmids that allowed expression of only the DNA-binding domains were constructed. The expression vector pGST-WT was obtained by insertion of an *Nco*I–*Bgl*II fragment of pWT(44–147), which encoded a region (amino acids 44–147) of Dof1, into *Nco*I/*Sal*I sites of pGEX-Dof1 using two oligonucleotides. The sequence of oligonucleotides were as follows: 5′-GATCTTGAG-3′ and 5′-TCGACTCAA-3′. The expression vector pGST-Dof1*Δ*C-HT was also created with an *Eco*RI–*Sac*I fragment of pGST-WT and the *Eco*RI–*Sal*I fragment of pGEX-5X-1, using two chemically synthesized oligonucleotides, as shown in Fig. 3 A. The pGST-Dof2*Δ*C-HT expression vector was generated by insertion of an *Eco*RI–*Dde*I fragment of pGST-Dof2 that encoded the complete Dof domain into the *Eco*RI and *Sal*I sites of pGST-5X-1 using the two synthetic oligonucleotides shown in Fig. 3 A. *E. coli* cells that were transformed with each expression vector and grown in Luria-Bertani medium to mid-log phase at 37 °C. Synthesis of proteins was induced by 1 mM isopropyl thio-*β*-D-galactoside at 30 °C and cells were harvested after 2 h. Cells were suspended in NaCl/P_i supplemented with 1 mM phenylmethysulfonyl fluoride and sonicated. GST fusion proteins were prepared by a standard method [16]. Attachment of a His_6 tag to each product was confirmed with Talon metal affinity resin (Clontech). Recombinant HMG1 protein was prepared similarly, using pGST-HMG1.

Electrophoretic mobility shift assays (EMSAs). Purified proteins were incubated in a solution of 17.5 mM Tris/HCl, pH 7.5, 60 mM NaCl, 10 % glycerol, 100 ng poly(dA) · (dT), 50 ng poly(dI-dC) and 0.5 ng (10000 cpm) probe DNA at room temperature for 20 min. Amounts of recombinant proteins used are indicated in the legends of the figures. Electrophoresis was carried out as described previously [17], with the exception that

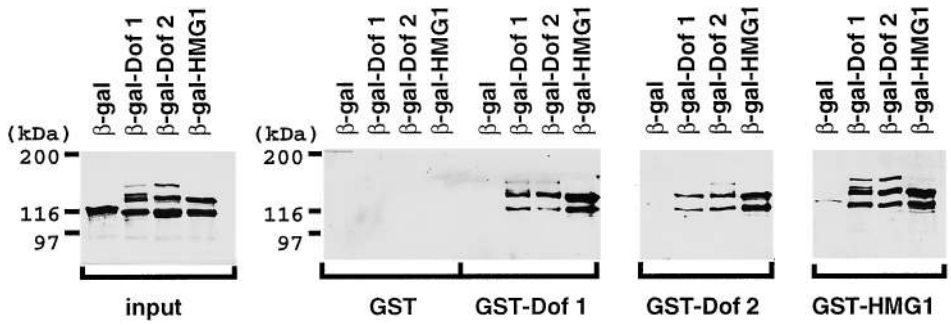

Fig. 1. Physical interactions between Dof proteins. A bacterial lysate that contained either *β*-galactosidase or a *β*-galactosidase (*β*-gal) fusion protein with Dof1, Dof 2, or HMG1, as indicated, was incubated with either GST or the indicated GST fusion protein that had been immobilized on glutathione-Sepharose 4B resin. The *β*-gal fusion proteins trapped on the resin were analyzed on SDS/polyacrylamide gels and visualized by western blotting with polyclonal antibodies against *β*-galactosidase. The *β*-galactosidase and fusion proteins in bacterial lysates were also analyzed by western blotting (panel labeled input).

5% acrylamide gels (50:1 acrylamide/bisacrylamide) were used. The sequences of three synthetic probe DNAs are shown in Table 1. The sequences were taken from the sequence of the Dof1-binding sites in the 35S promoter of CaMV [3].

RESULTS

Multiple protein-protein interactions of Dof1. Affinity chromatography was performed to examine the possibility that Dof1 might interact with Dof1 itself and/or with other Dof proteins. A fusion protein containing Dof1 and GST was expressed in *E. coli* and immobilized on glutathione-Sepharose resin. Proteins in a bacterial lysate that contained either *β*-galactosidase or a fusion protein that contained *β*-galactosidase and Dof1, Dof2 or the HMG box of maize HMG1 were allowed to absorb to the resin with the bound GST or GST fusion protein. Finally, proteins trapped on the resin were analyzed by western blotting with polyclonal antibodies against *β*-galactosidase. As shown in the panel labeled input in Fig. 1, detection of proteins with molecular masses higher than that of endogenous *β*-galactosidase (116 kDa) suggested the expression of each fusion protein in *E. coli* cells. The pairs of bands that were detected in the lanes labeled Dof1 and Dof2 presumably reflected the intact and proteolytically cleaved forms of two fusion proteins. Western blotting analysis of GST-Dof1 and GST-Dof2 fusion proteins with antibodies against GST, which allowed analysis of fusion proteins with better resolution, also suggested that proteolytically cleaved forms of these proteins had been produced (data not shown). The estimated positions of the cleavage sites in Dof1 are shown in Fig. 2A.

As shown in the panel labeled GST in Fig. 1, neither *β*-galactosidase nor the fusion proteins were able to bind to GST that had been immobilized on the resin. *β*-Galactosidase (*β*-gal) also did not interact with the GST-Dof1 fusion protein. However, two fusion proteins, *β*-gal−Dof1 and *β*-gal−Dof2, clearly bound to the resin that had been loaded with GST-Dof1 (panel labeled GST-Dof1). The detection of endogenous *β*-galactosidase in these lanes was not unexpected, since *β*-galactosidase is a tetramer [18]. In addition, *β*-gal−HMG1, which was used with the expectation that it would behave as a negative control, also bound to resin loaded with GST-Dof1. It was confirmed by staining of proteins that other proteins in bacterial lysates did not bind to resin loaded with GST-Dof1 in this experiment (data not shown). To confirm these observations, reciprocal experiments with GST-Dof2 and GST-HMG1 were performed. As shown in the panels labeled GST-Dof2 and GST-HMG1 in Fig. 1, interactions between Dof1 and Dof2 and between Dof1 and HMG1 were again observed. Thus, the apparent interactions between Dof proteins and the interaction between HMG1 and Dof proteins seemed not to be artifacts but to reflect true affinity. Since an interaction between Dof2 and HMG1 was also observed, the ability to interact with HMG1 seemed to be a common feature of Dof proteins. The detection of an interaction between GST-HMG1 and *β*-gal−HMG1 was not unexpected, since it had already been reported that the HMG boxes of mammalian HMG1 protein form a dimer [19].

The interactions that were observed might possibly have been dependent on contaminating DNA from the *E. coli* chromosome. To exclude this possibility, the experiments were repeated in the absence and in the presence of various concentrations of ethidium bromide (EtBr, 10−100 μg/ml), since it has been suggested that use of EtBr provides a general indication of whether associations between proteins are DNA dependent or DNA independent [20]. The presence of EtBr influenced the amounts of GST fusion proteins that were retained on the resin to some extent, but did not affect protein-protein interactions (data not shown). The results suggested that the interactions that had been observed, including interactions with HMG1, were direct, physical, protein-protein interactions and not interactions that were dependent on binding to the contaminated chromosomal DNA. An experiment using mutant Dof1 protein without any DNA-binding activity also supported the finding that the interactions were not dependent on contaminating DNA, as described in the next section. In addition, since the GST-HMG1 fusion protein used in these experiments contained an HMG-box but lacked the C-terminal acidic region (see Materials and Methods section), it seems likely that the Dof proteins interacted with the HMG box.

The Dof domain as a multifunctional domain. Even degraded forms of Dof proteins seemed to be able to participate in protein-protein interactions (Fig. 1). Therefore, it seemed possible that the N-terminal region that contained the Dof domain might be sufficient for such interactions. Several plasmids that encoded fusion proteins between *β*-galactosidase and several deletion-mutant derivatives of Dof1 were then used to identify the region required for the interactions (Fig. 2A). As shown in Fig. 2B (panel input), each lysate seemed to contain similar amounts of the proteins of interest; two forms were produced in cells transformed with pCdel753 and pCdel528, whereas only one form was produced in cells transformed with pCdel444 and pCdel278. The products obtained with pCdel753, pCdel528, and pCdel444, which lacked various segments of the C-terminal region, were able to interact with GST-Dof1, while the product obtained with pCdel278, which included only the N-terminal

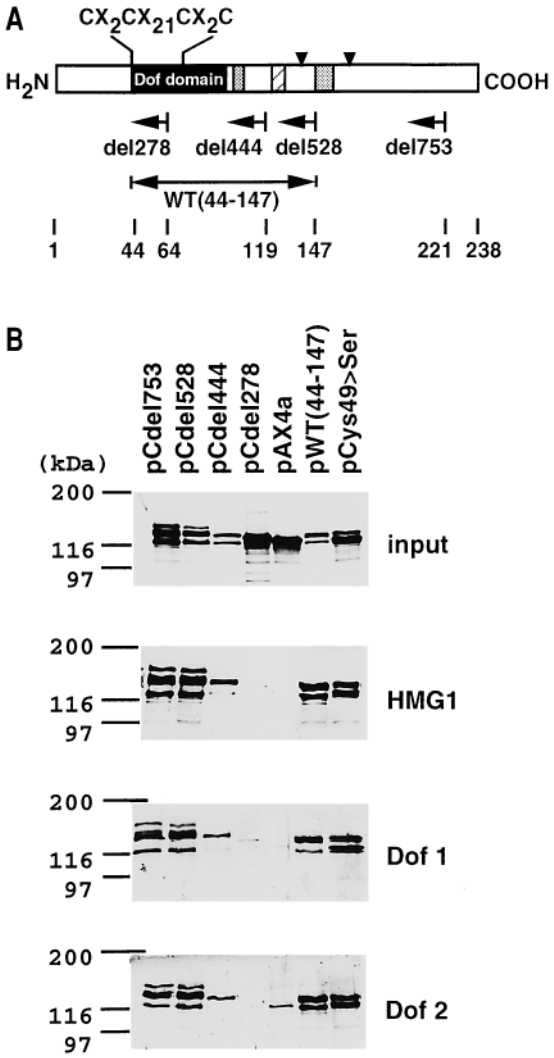

Fig. 2. Identification of the region required for protein-protein interactions. (A) Structure of Dof1. The locations of the Dof domain and a $CX_2CX_{21}CX_2C$ motif are indicated. A region rich in basic amino acid residues, and stretches of serine and threonine residues are also indicated by shaded and striped boxes, respectively. The sites in Dof1 that were hypersensitive to endogenous proteases in *E. coli* cells are also marked by arrowheads. The positions of the C-termini of the mutant Dof1 proteins and the region encoded by plasmid pWT(44−147) are indicated. (B) After *E. coli* cells had been transformed with an expression vector (pAX4a) or its derivatives for expression of Dof1 with deletions [pCdel753, pCdel528, pCdel444, pCdel278, and pWT(44−147)] or with both a deletion and a point mutation (pCys49 > Ser), the products derived from these plasmids were analyzed (panel labeled input). Each bacterial lysate contained either *β*-gal or a *β*-galactosidase (*β*-gal) fusion protein and was incubated with the indicated GST fusion protein that had been immobilized on glutathione-Sepharose 4B resin. Bound proteins were eluted with 10 mM glutathione, analyzed on SDS/polyacrylamide gels and visualized by western blotting with polyclonal antibodies against *β*-galactosidase.

half of the Dof domain, did not bind to GST-Dof1. Therefore, the Dof domain seemed to be important for the self-association of Dof1. The product obtained with pWT(44−147), which contained the region from amino acid 44 to the amino acid 147, also bound to GST-Dof1. The product of pCys49 > Ser encoded the same region as pWT(44−147), but the former product was unable to form a zinc finger structure because of the point mutation that eliminated the residue 49 (cysteine) that is required for coordination of a zinc ion [1]. This protein did interact with GST-Dof1. These results indicated that the zinc finger was not essential for the self-association of Dof1. The result of the experiment with pCys49 > Ser also supports the hypothesis that the self-association of Dof1 was caused by direct protein-protein interaction and did not involve formation of a ternary complex with DNA, since the protein encoded by this plasmid was unable to bind to DNA [1]. A similar experiment was performed with GST-Dof2 and a similar result was obtained (Fig. 2B, panel Dof2). Thus, both the self-association of Dof1 and the interaction of Dof1 with Dof2 seemed to be mediated by the Dof domain. Moreover, an experiment with GST-HMG1 also gave a similar result, suggesting that the interactions of Dof proteins with HMG1 might also be mediated by the Dof domain. These results together suggested that the Dof domain is a multifunctional domain that is involved in binding to DNA and in multiple protein-protein interactions.

Production of Dof proteins in *E. coli*. Recombinant forms of Dof1 and Dof2 were prepared for an examination of the effects of various protein-protein interactions on the binding of Dof proteins to DNA. Intact Dof proteins were not produced efficiently in *E. coli* cells. Therefore, attempts were made to produce a region (amino acids 44−136) of Dof1 and a region (amino acids 1−115) of Dof2 that contained the entire respective Dof domains. These regions should be sufficient for DNA-binding and protein-protein interactions. Two expression vectors (pGST-Dof1*Δ*C-HT and pGST-Dof2*Δ*C-HT) were constructed for expression of GST-Dof fusion proteins with a His_6 tag at each C-terminus using the synthetic oligonucleotides shown in Fig. 3A (see Materials and Methods section and Fig. 3B). These proteins were expressed in *E. coli* and purified by affinity chromatography on columns of glutathione-Sepharose 4B. The segments of Dof proteins (Dof1*Δ*C and Dof2*Δ*C) were released from GST by treatment with factor Xa. As shown in Fig. 3C, the analysis of products by SDS/PAGE suggested that the purified preparations were of high purity, even though the apparent molecular masses were higher than the calculated values. The proteins that are visible in lanes 2 and 3 in Fig. 3C also interacted selectively with a metal affinity resin (data not shown), suggesting that these proteins each had a His_6 tag. Thus, in spite of the abnormal mobility on a polyacrylamide gel that contained SDS, the samples seemed to have the expected amino acid sequences. Such abnormal migration during SDS/PAGE has also been observed with other plant transcription factors [20, 21].

Formation of homomeric and heteromeric complexes of Dof proteins on DNA. To investigate whether Dof1 can form a complex when it binds to DNA, EMSAs were performed with purified GST-Dof1*Δ*C and Dof1*Δ*C proteins using a synthetic DNA that contained a Dof1-binding site (the probe designated one site in Table 1). As shown in Fig. 4A, incubation of this DNA probe with GST-Dof1*Δ*C resulted in a retarded band (lane 1). This band seemed to have originated from a specific interaction of the fusion protein with the probe since (a) addition of antibodies against GST shifted the positions of the bands; (b) the intensity of this band was reduced by addition of non-labeled probe DNA; and (c) such a band was not observed using a mutated probe (data not shown). An additional band due to a specific DNA-protein interaction was observed using Dof1*Δ*C protein (lane 4). When Dof1*Δ*C was present together with GST-Dof1*Δ*C in binding reaction mixtures, a new, more slowly migrating band was recognized (lane 3), suggesting formation of a higher-order complex. A similar result was obtained when EMSA was performed with GST-Dof2*Δ*C (lanes 5−8). Thus, Dof1 seemed able to form not only a homomeric complex, but also a heteromeric complex with Dof2 on DNA. As shown in Fig. 4B, a higher-

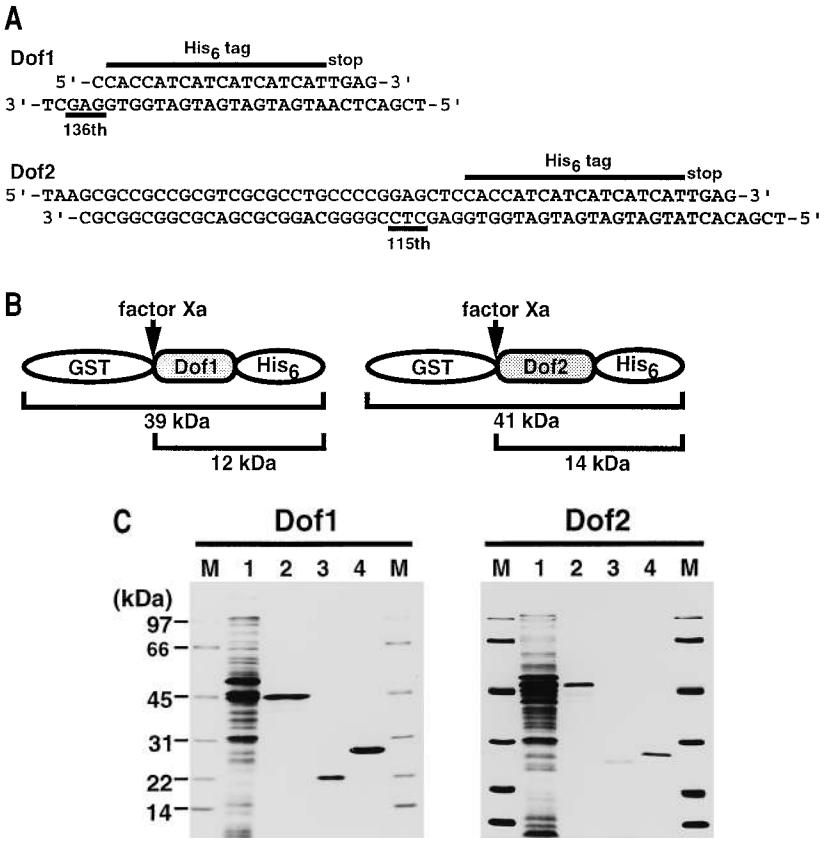

Fig. 3. Purification of recombinant Dof proteins. (A) Synthetic oligonucleotides that were used for construction of expression vectors. The ends of nucleotide sequences that encoded amino acid sequences of Dof proteins are indicated. (B) Schematic representation of products from two expression vectors. The molecular masses that were calculated from amino acid sequences were also shown. (C) After Dof1 and Dof2 had been expressed as fusion proteins in *E. coli*, each protein was purified on a GST affinity column. The proteins at different steps of the purification were analyzed on a 15% polyacrylamide gel that contained SDS and stained with Coomassie brilliant blue. Lanes M, molecular mass markers; lanes 1, crude extracts of *E. coli* after lysis of cells; lanes 2, eluates (GST-Dof fusion proteins) from affinity columns; lanes 3, purified Dof proteins that had been released from the respective fusion proteins by treatment with factor Xa; lanes 4, eluates (GST) from the affinity columns after treatment with factor Xa.

Table 1. Synthetic DNA probes used for EMSAs. Bold-face letters indicate Dof1-recognition sites and small letters indicate altered nucleotides.

Probe name	Sequence
One site	5′–CCGGACAGTAG**AAAAGG**TGGCACTA–3′ 3′–TGTCATC**TTTTCC**ACCGTGATGGCC–5′
Two sites	5′–CCGGTTCATC**AAAAGG**ACAGTAG**AAAAGG**–3′ 3′–AAGTAG**TTTTCC**TGTCATC**TTTTCC**GGCC–5′
Mutated	5′–CCGGTTCATC**AAcAtG**ACAGTAG**AAcAtG**–3′ 3′–AAGTAG**TTgTaC**TGTCATC**TTgTaC**GGCC–5′

order complex (complex II) was also observed in an experiment using increasing amounts of alternative Dof1*Δ*C protein without a His_6 tag that was obtained with pGST-WT (lanes 1−5). Thus, the formation of complexes seemed to be derived from the nature of Dof1, but not from those of GST and the His_6 tag. In agreement with this idea, formation of homomeric and heteromeric complexes of Dof2 protein without a His_6 tag was also observed (data not shown). In addition, the use of DNA probe containing two Dof1-binding sites (the probe designated two sites in Table 1) produced complex II more effectively, in spite of the abundant presence of free DNA (lanes 6−10). A more higher-order complex (complex III) was also observed. Thus, multiple binding sites seemed to be important for Dof1 to form higher-order complexes on DNA. At a low concentration of Dof1 protein, the protein-protein interaction of Dof1 may cause only oligomerization that is dependent on the number of binding sites on the DNA. The oligomers might be stabilized by multiple binding sites. When the concentration of Dof1 protein is high, the interaction may cause oligomerization that is independent of the number of binding sites.

HMG1 affects the binding of Dof1 to DNA. To investigate the effects of interactions between Dof1 and HMG1, EMSA was performed in the presence of both these proteins together. No retarded bands were visualized initially when only the HMG1 protein was used (Fig. 5, lanes 1−3). Only exposure for a longer period allowed detection of such bands that were distinguishable from GST-Dof1*Δ*C protein-dependent complexes in terms of

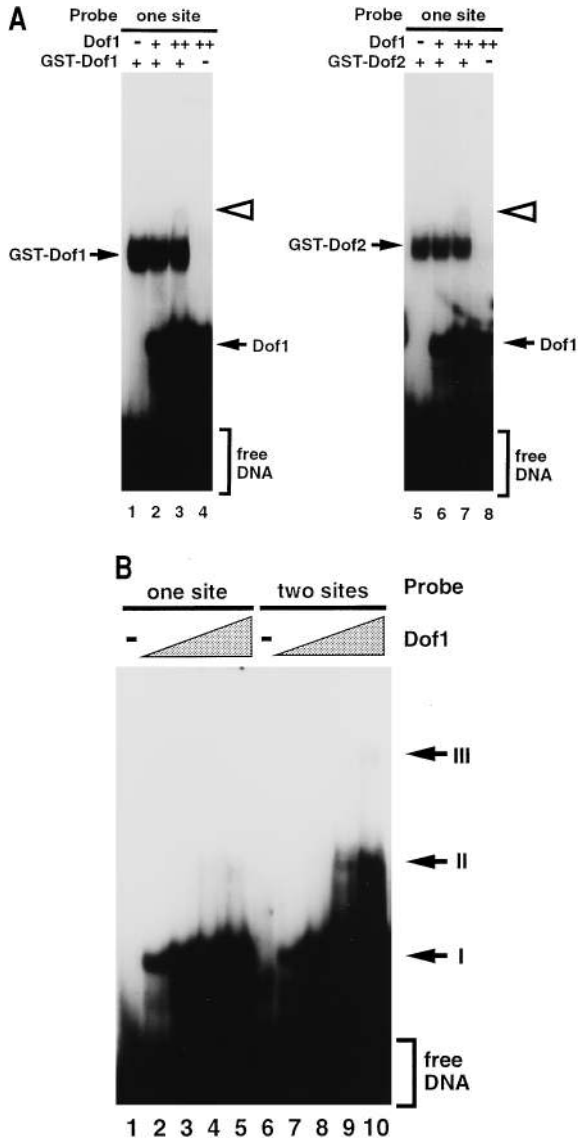

Fig. 4. EMSAs with purified recombinant Dof proteins. (A) The probe designated one site was incubated with GST-Dof1*Δ*C or GST-Dof2*Δ*C (800 ng, lanes 1−3 and 5−7) in the absence (lanes 1 and 5) or presence of 200 ng (lanes 2 and 6) or 600 ng (lanes 3 and 7) of Dof1*Δ*C protein. Only Dof1*Δ*C protein (600 ng) was also incubated by itself with the probe (lanes 4 and 8). The positions of complexes are indicated. The higher-order complexes indicated by open arrowheads were observed faintly. (B) EMSA was performed with two kinds of probe DNAs and various amounts of alternative Dof1*Δ*C protein without a His_6 tag that corresponded to a region (amino acids 44−147) of Dof1. Lanes 1 and 6, no protein; lanes 2 and 7, 100 ng alternative Dof1*Δ*C; lanes 3 and 8, 300 ng alternative Dof1*Δ*C; lanes 4 and 9, 600 ng alternative Dof1*Δ*C; lanes 5 and 10, 900 ng alternative Dof1*Δ*C. The positions of complexes observed are indicated by arrowheads. The higher-order complexes that were produced independent of binding sites (complex II in lane 5 and complex III in lane 10) were observed faintly. The alternative Dof1*Δ*C protein without a His_6 tag was prepared from *E. coli* cells that were transformed with pGST-WT. The comparison of this protein and the Dof1*Δ*C protein from pGST-Dof1*Δ*C-HT on a polyacrylamide gel containing SDS suggested that the alternative Dof1*Δ*C also had the expected amino acid sequence (data not shown).

their positions. Incubation of GST-Dof1*Δ*C protein and DNA produced the complex that is indicated by a in lane 4 of Fig. 5. When GST-Dof1*Δ*C was incubated with DNA in the presence of maize HMG1, a new, more slowly migrating complex (complex a* in Fig. 5) was observed, suggesting formation of a

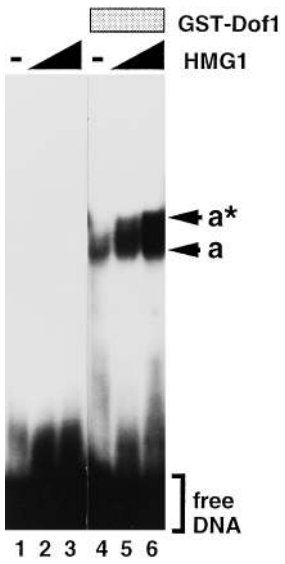

Fig. 5. Binding to DNA of Dof1 in the presence of HMG1. The ^{32}P-labeled one site probe was incubated with no protein (lane 1), with 300 ng (lane 2), or with 1 μg (lane 3) purified recombinant maize HMG1. The DNA probe was also incubated with 200 ng GST-Dof1*Δ*C protein in the absence (lane 4) or in the presence of 300 ng (lane 5) or 1 μg (lane 6) recombinant HMG1. The position of the DNA/GST-Dof1*Δ*C complex is indicated by a. The position of the complex that depended on the combined presence of both GST-Dof1*Δ*C and HMG1 is indicated by a*.

higher-order complex. Since addition of antibodies against GST to the binding reaction mixtures changed the mobility of both bands, complexes a and a* seemed to contain GST-Dof1*Δ*C protein (data not shown). The binding of GST-Dof1*Δ*C protein also seemed to be slightly enhanced by the presence of HMG1 protein. These results suggested that maize HMG1 affects the binding of Dof1 to DNA.

DISCUSSION

The studies demonstrated that various protein-protein interactions of Dof1, which included self-association, interaction with another maize Dof protein, and interaction with maize HMG1, seemed to be mediated by Dof domains. In addition, the formation of homomeric and heteromeric complexes of Dof proteins on DNA was observed. Thus, it appears that the Dof domain is a multifunctional domain that contributes not only to binding to DNA, but also to protein-protein interactions for the formation of complexes on DNA.

The DNA-binding activity of Dof1 requires the presence of a specific zinc finger structure in the Dof domain, since replacement of cysteine by serine at position 49 abolished the DNA-binding activity [1]. However, the mutated Dof protein was able to interact with other proteins, suggesting that the zinc finger structure is not essential for protein-protein interactions. It was reported recently that other zinc finger motifs, namely, the LIM domain and a zinc finger motif of GATA-1, are involved both in binding to DNA and in self-association [23, 24]. In the case of GATA-1, it was shown that the C-terminal half of the zinc finger, which is composed of 39 amino acids, rather than the zinc finger structure itself was essential for self-association [24]. Since a mutant protein consisting only of the amino-terminus of the Dof domain had lost the capacity for protein-protein interaction, the carboxy-terminus of the Dof domain also seemed to be important for protein-protein interactions.

It remains unknown whether or not all Dof proteins can self-associate. However, a Dof protein, AOBP1, from pumpkin interacts with an AT-rich direct-repeated sequence in the promoter of the gene for ascorbate oxidase (information from EMBL database; accession number D45066). The tobacco Dof protein NtBBF1 binds to the (A)CTTT(A) sequence in regulatory domain B of the plant oncogene *rolB*. Two copies of this sequence motif, which is similar to the AAAAGG motif in the reverse orientation [6], can be found in domain B. Recently, a maize cDNA that encoded an endosperm-specific Dof protein PBF was isolated [25]. PBF bound to the prolamin box which was regarded as a *cis*-element for endosperm-specific gene expression. In the analysis of the protein-protein interaction between PBF and a basic leucine zipper (bZip) transcriptional activator, Opague2, self-association of PBF was observed, although further analyses have not been performed [25]. Thus, it seems likely that self-association and formation of complexes on DNA are common features of Dof proteins. Since it has been demonstrated that Dof proteins can interact with promoters of diverse genes, Dof proteins seem likely to be involved in several different mechanisms in the control of gene expression in plants. Thus, even though the Dof domain is strongly conserved, it seems unlikely that all Dof proteins participate in cross-talk through their protein-protein interactions. The identification of the amino acid residues that are critical for protein-protein interactions in the Dof domain and analysis of the specificity of protein-protein interactions should provide clues to the mechanisms of regulation that are mediated by Dof proteins.

Our unpublished studies with maize protoplasts have revealed several aspects of the regulation by maize Dof proteins of transcription: (a) Dof1 works as a transcriptional activator with separable DNA-binding and activation domains; (b) Dof 2 cannot activate transcription, but blocks activation of transcription by Dof1, suggesting that Dof2 may be a repressor. It seems likely that these Dof proteins, which have opposing activities, might modulate transcription through competitive binding to target sequences and formation of homomeric and heteromeric complexes on DNA.

The Dof domain is also important for interactions with the HMG1 protein. HMG1 is an abundant, nuclear, non-histone, chromatin-associated protein. In spite of intensive studies of the HMG1 protein, its physiological function remains obscure (for reviews, see [14, 15, 26]). Although it has been suggested that HMG1 might be involved in the replication of DNA and might act as a nucleosome-assembly factor, recent evidence suggests that the major function of HMG1 might be related to transcription [14, 15, 26, 27]. Physical interactions between HMG1 and transcription factors were reported recently: Human HMG1 interacts with the TATA-binding protein (TBP) [28]; the mammalian transcription factor Oct1 interacts directly and functionally with HMG2, which is very closely related to HMG1 in terms of structure [29], and human Hox proteins also interact functionally with HMG1 [30]. These interactions have different effects. HMG1 enhanced the sequence-specific DNA-binding of Oct1 and HOXD9 protein [29, 30], while HMG1 competed with TFIIA for binding to TBP and inhibited transcription by RNA polymerase [28]. The interaction between Dof1 and maize HMG1 seemed to produce a higher-order complex and slightly enhance the binding of Dof1 to DNA. A positive effect of maize HMG1 on reporter gene expression in maize protoplasts has been reported [31]. The HMG-box but not the C-terminal region was essential for the stimulation on transcription [31]. The interaction with Dof proteins seemed to depend on the HMG-box in maize HMG1. Thus, it seems likely that maize HMG1 might be also involved in transcriptional regulation through interactions with transcription factors. Since the Dof domain is highly conserved in all Dof proteins, all Dof proteins might interact with HMG1. The POU domain in Oct1 and the homeodomain in HOXD9 also interact with the HMG box. Although these HMG boxes are very similar to each other, the Dof domain has not been recognized in Oct1 and Hox proteins or any other mammalian protein. Thus, the structural determinants of these interactions remain to be elucidated.

The author is indebted to Prof. H. Akanuma (University of Tokyo) for his continuous support. This work was supported in parts by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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