Wheat nuclear protein HBP-1 binds to the hexameric sequence in the promoter of various plant genes

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ABSTRACT

HBP-1 is a sequence-specific DNA-binding protein that interacts with the hexameric sequence ACGTCA, the putative *cis*-acting element of the wheat histone H3 gene. Gel mobility shift and DNase I footprint analyses showed that this protein interacts with homologous sequences in the regulatory regions for the transcription of the cauliflower mosaic virus (CaMV) 35S RNA and nopaline synthase (NOS) genes, evidence that HBP-1 may bind to hexameric sequences in the regulatory regions of various genes. An HBP-1-like protein, indistinguishable from wheat HBP-1 in its the DNA-binding specificity, is present in sunflower nuclear extract, an indication that HBP-1-like DNA-binding proteins also exist in dicots.

INTRODUCTION

As for the transcriptional regulation of specific gene expression, much attention has been paid to interactions between *cis*-regulatory elements and *trans*-acting factors (1). In the higher plants, the CaMV 35S RNA and NOS gene promoters have been relatively well studied for the dissection of *cis*-acting elements (2−9). The 35S promoter is known to be very active in a wide range of both monocot and dicot cells (2−7). The NOS promoter in the Ti-plasmid of *Agrobacterium* is a strong one (8) although its activity is 30-fold less than the 35S promoter activity in transformed plant cells (9). The *cis*-elements in the promoters have been defined by examinations of the transcription activities of the various 5′ and 3′-deletion mutants of the genes (3−8); but, there is little detailed information on the interaction of the *cis*-sequences and *trans*-acting factors.

We previously identified the wheat nuclear protein HBP-1 that binds specifically to the hexameric sequence, ACGTCA, which is present in the upstream regions of a wide range of plant histone H3 and H4 genes (10,11) and which functions as a *cis*-element in the wheat H3 gene (12).

We here report that HBP-1 also binds to hexameric sequences located in the CaMV 35S RNA and NOS promoters that are very similar to the ACGTCA motif. The significance of the interaction of HBP-1 with homologous hexameric sequences in the transcription of these genes is discussed.

MATERIALS AND METHODS

Preparation of nuclear extracts

Wheat (*Triticum aestivum*) nuclear extract was prepared as described elsewhere (10,11). Sunflower (*Helianthus annuus*) nuclear extract was obtained from nuclei isolated from 1-day-old germinated seeds that had homogenized in liquid N_2. The procedure for the preparation of the sunflower extract was the same as that used for the wheat extract (10,11),

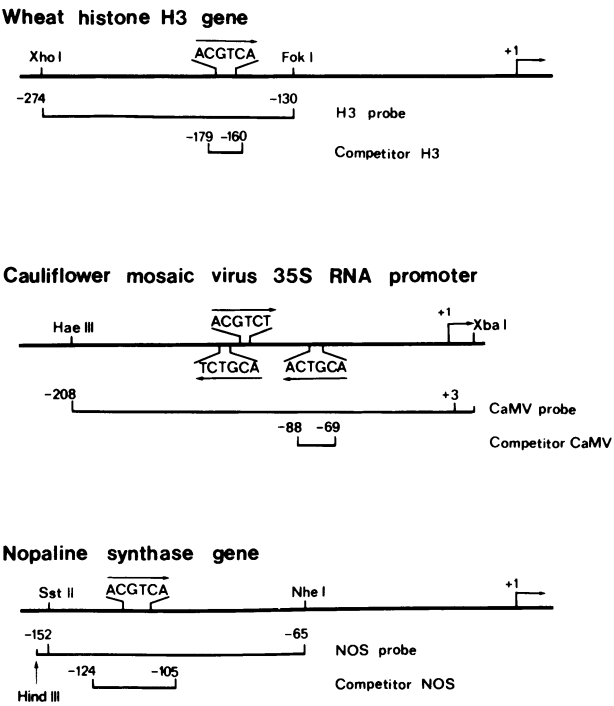


Fig 1 *Disposition of the hexameric sequence and its analogous sequence in promoter regions of wheat H3, CaMV 35S RNA and NOS genes.* Arrows above a sequence indicate the 5′ to 3′ direction of the hexameric sequence on the upper strand, and beneath the direction on the lower strand. The H3, CaMV and NOS probes and their competitors used in the mobility shift and DNase I footprinting experiments are indicated below the partial restriction map given for each gene. Negative numbers indicate the positions in the nucleotides relative to the cap site (+1). For competitors H3(m) and H3(O), see Table 1.

except that the crude nuclear phase was suspended in extraction buffer at 1:20, weight to volume. In some cases, the wheat nuclear extract was fractionated by phosphocelluluse column chromatography (10,11).

Mobility shift and competition assays

The *Xho*I-*Fok*I fragment (−274 ~ −130) of the wheat histone H3 gene (prepared as described previously) was used as the H3 probe (11). To prepare the CaMV probe, we first cloned a DNA fragment (−317 ~ +3) containing the 35S promoter between the *Hin*dIII and *Bam*HI sites of the polylinker region of pUC19. The recombinant pUC19 was cleaved at the *Xba*I site of the polylinker region, then ^{32}P-labeled and digested with *Hae*III (−209) to form a ^{32}P-labeled probe about 220 bp long. The NOS probe was a *Hin*dIII-*Nhe*I fragment 93 bp long from the NOS gene with a *Hin*dIII linker attached at the *Sst*II site of the *Sst*II-*Nhe*I fragment (−152 ~ −65). These probes were ^{32}P-end-labeled with [α-^{32}P]dCTP and Klenow enzyme or [γ-^{32}P]ATP and T4 polynucleotide kinase. Mobility shift and competition assays were done as described elsewhere (10,11). The double-stranded oligonucleotides used as the competitors (Table 1) were synthesized in an automatic DNA synthesizer (Milligen 75000).

Table 1 Sequence alignment of the synthetic oligonucleotides used as competitors in the cross-competition experiments

	Oligomer	Sequence alignment	Position [a]
Group A	competitor H3 [b]	5'-TCGGCCACGTCACCAATCCG-3' AGCCGGTGCAGTGGTTAGGC	-179/-160
	competitor CaMV	5'-TCTCCACTGACGTAAGGGAT-3' AGAGGTGACTGCATTCCCTA	-88/-69
	competitor NOS	5'-AGCACATACGTCAGAAACCA-3' TCGTGTATGCAGTCTTTGGT	-124/-105
Group B	competitor H3(m) [c]	5'-TCGGCCAAGTAACCAATCCG-3' AGCCGGTTCATTGGTTAGGC	-179/-160
	competitor H3(O) [d]	5'-CCAATCCGCGGCATTCCTAC-3' GGTTAGGCGCCGTAAGGATG	-167/-148

[a]Negative numbers are related to the transcriptional initiation site.
[b]Competitor H3 is the same as "competitor 1" in ref. (11).
[c]Competitor H3(m) is the same as "competitor 1(m)" in ref. (11).
[d]Competitor H3(O) is the same as "competitor 2" in ref. (11).

DNase I footprinting

DNase I footprinting was performed as described by Carthew et al. (13). The binding reaction took place under the conditions used for the mobility shift assay, except that the concentration of $MgCl_2$ was 5mM. After incubation, the reaction mixtures were treated

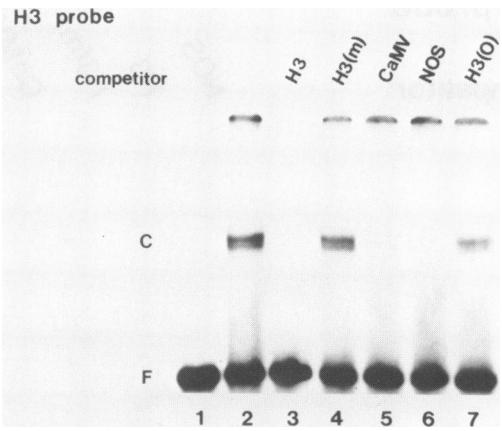
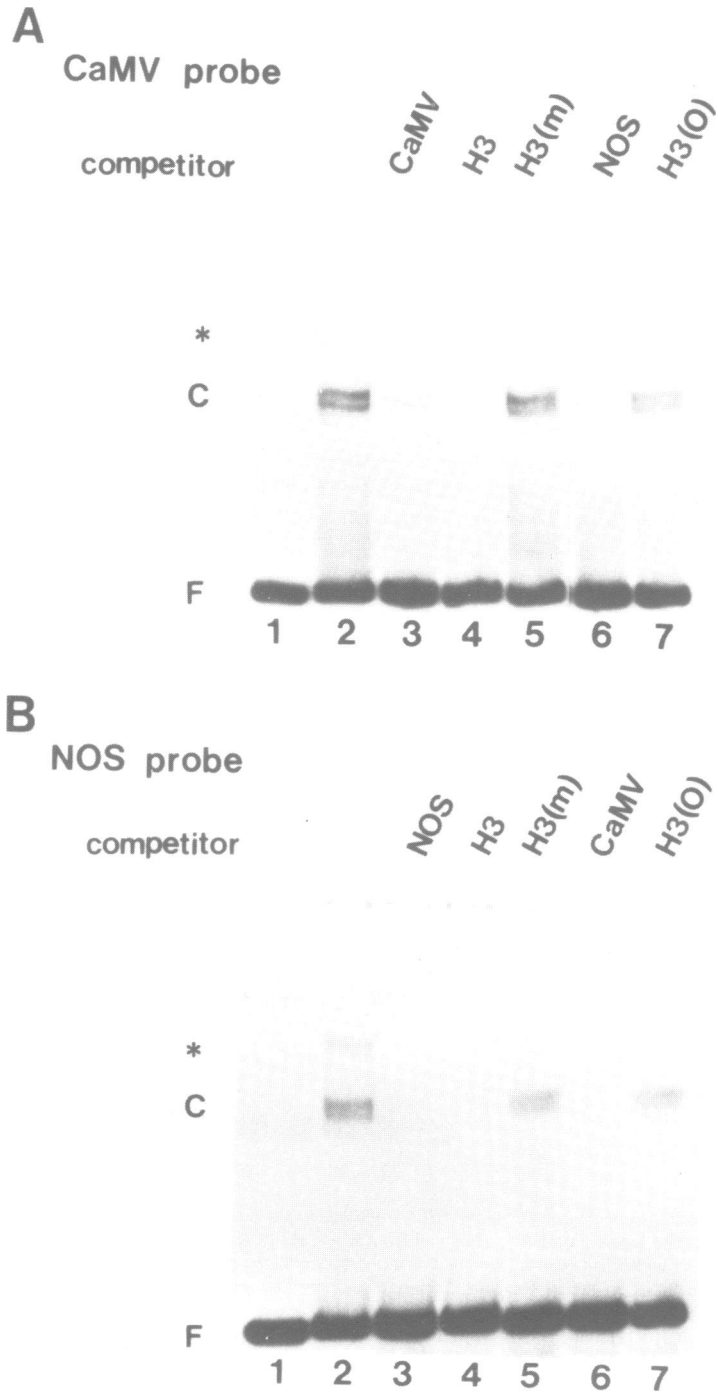


Fig 2 *Effects of various competitor DNAs on HBP-1 binding to the hexameric sequence in wheat histone H3 promoter.* The ^{32}P-end-labeled *Xho*I-*Fok*I fragment from wheat H3 gene (see Fig. 1) was incubated with wheat germ nuclear extract which had been fractionated on a phosphocellulose column as described elsewhere (10,11). All the binding reactions contained poly(dI-dC)·poly(dI-dC) without a competitor (lane 2) or a 50-fold molar excess of the unlabelled oligonucleotide competitor indicated above each lane (lanes 3 to 7). A low-ionic strength, 0.7% agarose-4% polyacrylamide gel was used for electrophoresis (10,11). Lane 1, reaction without the nuclear extract. C represents HBP-1-bound and F, HBP-1-unbound DNA.


A
CaMV probe
competitor
CaMV
H3
H3(m)
NOS
H3(O)
*
C
F
1 2 3 4 5 6 7
B
NOS probe
competitor
NOS
H3
H3(m)
CaMV
H3(O)
*
C
F
1 2 3 4 5 6 7

with freshly diluted DNase I (1 μg/ml) for 60 sec at 25°C. Digestion was stopped by the addition of 10 mM EDTA, after which the mixtures were electrophoresed on low-ionic 5% polyacrylamide gels. Wet gels were autoradiographed, and the portions corresponding to the DNA-protein complexes and free probes excised. Labeled DNA fragments were eluted from the gel pieces, then electrophoresed on denaturing gels, as described elsewhere (10,11).

RESULTS

Specific binding of HBP-1 to the regulatory regions of various genes

The hexameric sequence, related sequences, or both which are present in the promoter regions of wheat histone H3, CaMV 35S RNA and NOS genes are shown schematically in Fig. 1. To determine whether the hexameric sequences in the 35S and NOS promoters interact with HBP-1, we did cross-competition experiments using the mobility shift assay. The competitors were double-stranded synthetic oligonucleotides that contain the hexameric sequences of the wheat H3, CaMV 35S RNA or NOS genes (group A, Table 1), the point-mutated hexameric sequence, and the normal octameric sequence (14) which often is located near the hexameric sequence in plant histone genes (group B, Table 1).

The effects of synthetic oligomers on the binding of HBP-1 to the H3 probe are shown in Fig. 2. When the H3 probe was incubated with wheat nuclear extract, a specific protein-DNA complex was formed which frequently was detected as doublet bands (lane 2). The nuclear protein(s) that acts in the formation of the complex has been designated HBP-1 (10,11). Competition experiments showed that all the oligonucleotides in group A competed with the H3 probe in the formation of this complex (lanes 3, 5 and 6); whereas neither of the group B oligomers did (lanes 4 and 7). Because group A oligomers all have the normal hexameric sequence but group B ones do not, HBP-1 probably also interacted with the hexamer-containing sequences in the 35S and NOS promoters. This was confirmed by the results of mobility shift assays with the CaMV and NOS probes (Fig. 3). The appearance of shifted doublet bands was prevented by group A oligomers but not by group B oligomers (Fig. 3). The strengths of the binding affinities for HBP-1 were about equal for the three probes (data not shown).

Identification of HBP-1 binding sites in the CaMV 35S RNA and NOS promoter regions by DNase I footprinting

To confirm the results of the cross-competition experiments, we used DNase I footprinting with partially purified HBP-1 that has been obtained by butyl-Sepharose column chromatography (Takase et al., unpublished) to show the binding regions of HBP-1 to the CaMV and NOS probes.

With the CaMV probe, the DNase I-protected regions were defined from position −141 to −117 (region I) and −88 to −64 (region II) on the noncoding strand and from −87 to −60 (region II) on the coding strand (Fig. 4A). HBP-1 tends to interact with two copies of the three hexameric sequences in the 35S promoter (see Fig. 1). The footprint pattern

Fig 3 *Specific binding of HBP-1 to the hexameric sequences in the CaMV and NOS promoters.* The ^{32}P-end-labeled *Hae*III-*Xba*I and *Hin*dIII-*Nhe*I fragments, respectively prepared from the CaMV (A) and NOS (B) genes (see Fig. 1), were incubated with the nuclear extract in the absence (lane 2) or the presence (lanes 3 to 7) of a 50-fold molar excess of the competitor DNA indicated above each lane. These reaction mixtures were electrophoresed on low-ionic strength 0.7% agarose-4% poly acrylamide gels. The asterisk indicates a weak non-specific band (data not shown). (The competition profile for this band is the same as that for the specific complex indicated by [C].) Lane 1, reaction without the nuclear extract.

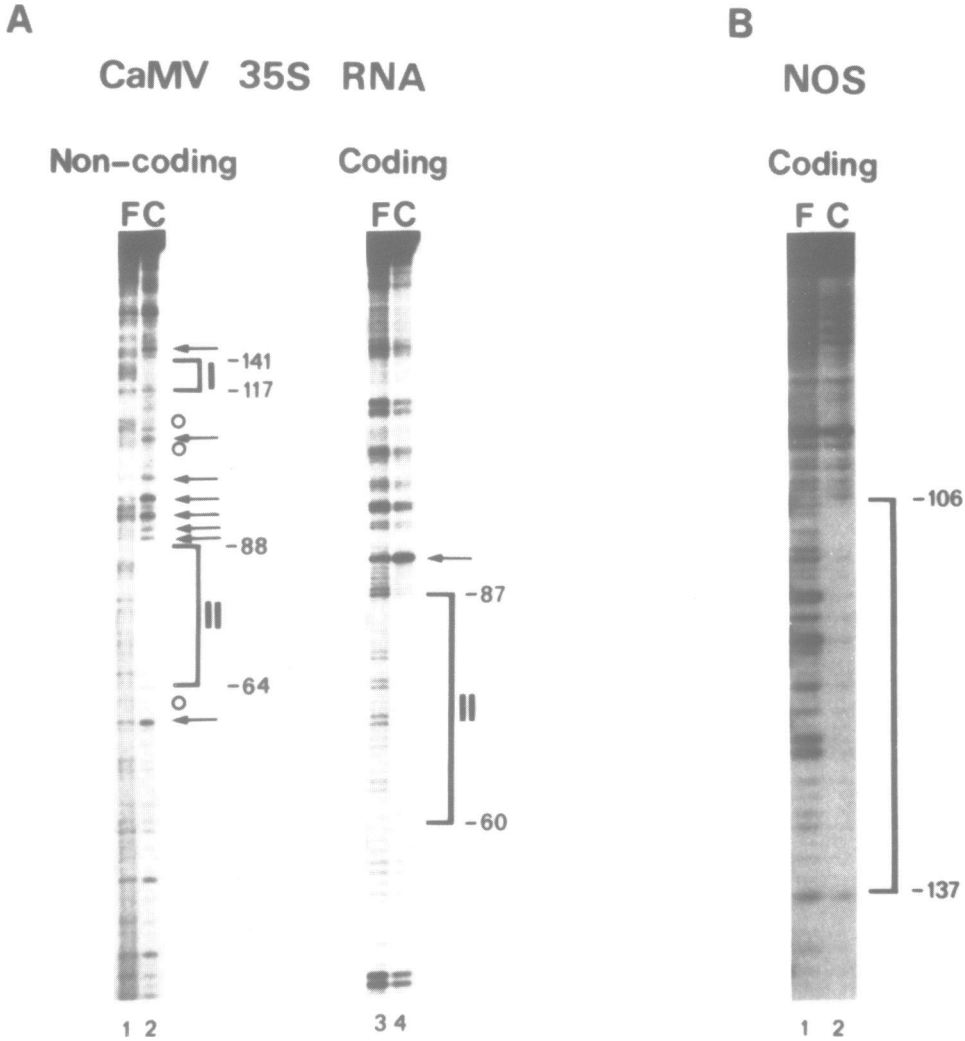


Fig 4 *DNase I footprinting of HBP-1 binding regions.* ^{32}P-end-labeled CaMV (A) and NOS (B) probes were incubated with wheat germ nuclear extract then digested with DNase I as described in Materials and Methods. To map the binding regions, chemical cleavage ladders of the CaMV and NOS probes were co-electrophoresed with DNase I-digested probes in the absence of the nuclear extract (data not shown). F represents the cleavage pattern of the free probe, and C of the bound probe. Regions protected from DNase I digestion are shown in brackets for each sample. Enhanced DNase I-cutting sites are indicated by arrows and reduced ones by open circles.

also showed that in region I HBP-1 bound only to the non-coding strand; whereas, in region II binding took place on both strands. In addition, several DNase I-hypersensitive sites were present between regions I and II (Fig. 4A), which indicates that HBP-1 binding might cause conformational changes in the DNA, thereby producing the hypersensitive sites.

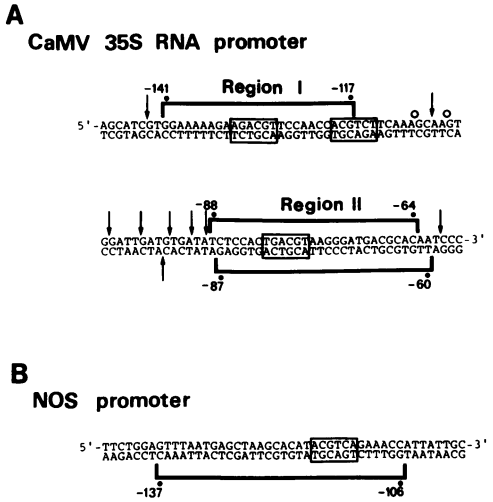


Fig 5 *DNase I-protected regions in the CaMV 35S RNA and NOS promoters.* A, part of the CaMV 35S promoter; B, part of the NOS promoter. The DNase I-protected sequences are indicated in brackets. Negative numbers represent the position in the nucleotide relative to the transcriptional initiation site. Vertical arrows indicate DNase I-hypersensitive sites, and open circles the positions of nucleotides resistant to DNase I digestion. Hexameric sequences are boxed.

With the NOS probe, a DNase I-protected region was defined from position −137 to −106 on the coding strand that contains the hexameric motif (Fig. 4B). Analysis of the HBP-1 binding site on the opposite strand indicated poorly defined footprint spanning from approximately −130 to −103 (data not shown), which also contains the hexameric motif.

As shown in Fig. 5, all the DNase I-protected regions fully cover the hexameric motif of these promoters. The DNase I footprinting experiment demonstrated that HBP-1 also recognizes hexameric sequences in the CaMV 35S RNA and NOS promoters, which agrees with the results of the cross-competition experiments done with the mobility shift assay (Figs. 2 and 3).

Existence of an HBP-1 homologue in sunflower cells

Does a DNA-binding nuclear protein that resembles HBP-1 exist in sunflower cells? The CaMV 35S RNA and NOS genes are efficiently transcribed in sunflower cells (12,15). To answer this question, we examined nuclear extract prepared from sunflower seedlings by the procedure used for wheat germ extract (10,11) using the mobility shift assay with the NOS probe. The results indicate that sunflower nuclear extract does contain a protein(s) capable of interacting with the NOS probe (Fig. 6, lane 5). Formation of the DNA-protein complex was inhibited by group A oligomers but not by group B ones (Fig. 6, lanes 6 to 10), evidence that there is an HBP-1-like protein which is able to bind to the hexameric sequence of the NOS promoter. Difference in mobility of the HBP-1-probe and HBP-1-like protein-probe complexes suggests that the two proteins may be different from each other in the molecular weight and/or charge. As with wheat nuclear extract, the specific DNA-protein complex appeared as doublet bands (compare lanes 2 and 5 in Fig. 6). The same results were obtained for the wheat H3 and CaMV probes (data not shown). We therefore

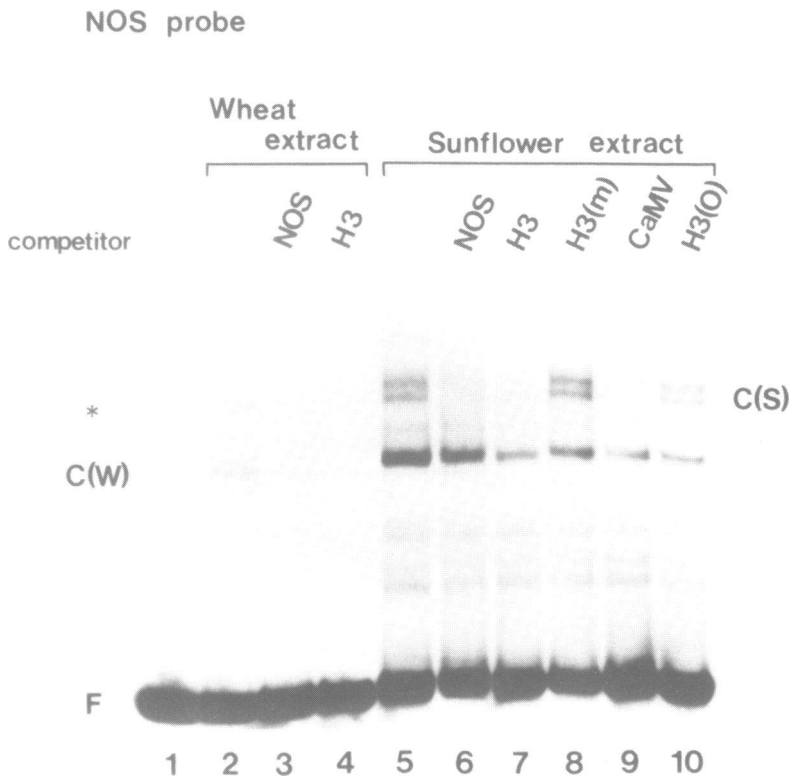


Fig 6 *Comparison of the DNA-binding proteins in wheat and sunflower nuclear extracts which specifically recognize the hexameric sequence of the NOS promoter.* The ^{32}P-end-labeled *Hin*dIII-*Nhe*I fragment (see Fig. 1) from the NOS gene was incubated with nuclear extract from wheat germ (lanes 2 to 4) or from sunflower seedlings (lanes 5 to 10), after which the mixtures were electrophoresed on low-ionic strength gels. The unlabelled oligonucleotides used as competitors (50-fold molar excess) are shown at the tops of the lanes. C(W) indicates the specific complex formed between the probe and wheat HBP-1 and C(S) the complex with the sunflower HBP-1 homologue. Lane 1, reaction without the nuclear extract.

conclude that sunflower has a hexameric sequence-specific DNA-binding protein(s) homologous to wheat HBP-1.

DISCUSSION

Using cross-competition and DNase I footprinting techniques combined with the mobility shift assay, we have shown that the wheat DNA-binding protein, HBP-1, binds to the hexameric sequence ACGTCA or an analogous sequence located in the promoter region of various plant genes.

Ow et al. (3) investigated the structure and function of the CaMV 35S promoter in carrot cells using a transient expression system and showed that the regulatory sequence for maximum promoter activity is located in the regions between −148 and −89 and −89 and −73. We here have shown that both regions contain a sequence which is highly

analogous to the hexameric sequence in the wheat H3 promoter and that wheat HBP-1 can bind specifically to these hexamer-like sequences in the 35S promoter. We also found a similar hexameric sequence in the upstream regulatory region (−115 ~ −101) of the NOS promoter first reported by An et al.(8). The DNase I footprinting analyses we used indicated that HBP-1 also binds specifically to this regulatory region. The results suggest that the specific binding of HBP-1 to the hexamer, or its analogous sequence, is correlated with the function of the hexameric motif in transcription.

Lam et al. (16,17) identified a nuclear protein, ASF-1, from pea and tobacco nuclear extracts which bind to the two TGACG-motifs (TGACGT recognized by HBP-1 and TGACGC) of the 35S promoter. Singh et al. (18) found other tobacco nuclear protein that may bind to a 16 bp palindromic enhancer sequence of *Agrobacterium* octopine synthase (OCS) gene (19) which contains two copies of the hexamer-like sequence ACGTAA. We previously investigated the role of individual nucleotides of the ACGTCA motif in HBP-1 binding by the competition assays using a series of the point-mutated hexameric sequences (20), and obtained evidence that all the residues except the last A of this motif are essential for the HBP-1 binding. Thus, it is conceivable now that HBP-1 can not bind to the TGACGC sequence (−72 ~ −67) in the region II of the 35S promoter and the ACGTAA sequence of the OCS enhancer. Likely, HBP-1 may not bind to the TGAGCT sequence (−131 ~ −126) in the HBP-1 binding region of the NOS promoter (Figs. 4 and 5). It is, however, unknown why the HBP-1 binding did not occur in the ACGTCT sequence downstream the 35S region I (Figs. 4 and 5). Judging from the rigidity in the sequence recognition of HBP-1, as mentioned above, we conclude that HBP-1 can bind to only the real hexameric motif (ACGTCA or ACGTCT) existing in the 35S and NOS promoters, although the DNase I-protected regions were spanning from 25 to over 30 base paires.

We also showed here that a hexameric sequence-specific binding protein similar to HBP-1 exists in sunflower cells. Recently we reported that the hexameric sequence of the wheat H3 gene functions as a *cis*-element for transcription in sunflower cells (12). From these observations, we infer that the transcription of the wheat H3 gene introduced into sunflower cells may be regulated through the specific binding of the HBP-1 homologue to the H3 hexameric motif. This is evidence that this transcriptional regulation takes place in both monocot and dicot cells, which is supported by the fact that the CaMV 35S promoter functions in carrot, tobacco, maize, soybean, rice, wheat, and sorghum cells (2−7) and the NOS promoter in tobacco and maize cells (4,8).

In general, the transcription of eukaryotic genes with a regulatory sequence such as the GC or CCAAT box is regulated by the specific binding of a single protein factor, or a member of a closely related protein family, to these sequences (21−24). We therefore speculate that HBP-1 and HBP-1 homologues belong to protein family members which function as *trans*-acting factors for the transcription of various plant genes that have a common hexameric sequence. In fact, the hexameric motif also existed, although in reverse direction, in the upstream region of an auxin-regulated DNA-binding protein gene of *Arabidopsis* (25).

Previously we reported the results of the structual analysis of a wheat cDNA encoding HBP-1 (26). Katagiri et al. (27) cloned two tobacco cDNAs encoding TGA1a and TGA1b which may bind to the TGACG-motif of the CaMV 35S promoter (16,17). In comparison with the nucleotide and deduced amino acid sequences of the above three cDNA clones, there were no homologies except for the basic and leucine repeat regions (data not shown). Although Katagiri et al. (27) who used the wheat H3 hexameric motif as a probe for TGA1a

and TGA1b cDNA cloning have not shown the critical binding sites on the DNA strands to interact with TGA1a and TGA1b, we can suppose that these nuclear proteins including OCS enhancer binding protein may be distinct members of a protein family which recognizes the hexameric and hexamer-like sequences.

The specific complexes comprised of HBP-1 and the probe DNAs often were detected as doublet bands in the mobility shift assay (Figs. 2,3 and 6). Whether these doublets are due to the existence of two closely related HBP-1s or to partial degradation of HBP-1 during preparation of the nuclear extracts is not clear. Since the relative amounts of the DNA-protein complexes were almost constant regardless of the amounts of the nuclear extract added to the reaction mixture (data not shown), it is unlikely that the doublet bands were due to the dimerization of protein(s) in the complex formation. If there are two species of HBP-1, the difference in the mobility of the complexes must be due to a difference in their molecular weights due to alternative splicing or post-translational modification(s). Post-translational modification of transcription factors by phosphorylation (28−30) or O-glycosylation (31,32) has, in fact, been reported.

The strength of the promoter activity of the three genes considered here is in order of CaMV 35S > NOS >> H3. The marked difference in promoter activity can not be explained by the binding affinity of HBP-1 for the promoters alone because the strength of the affinity was nearly the same for the three genes. A similar phenomenon has been reported by Cross et al. (33), who indicated that although the transcription factor NF-$\varkappa$B could bind with the same affinity *in vitro* to the immunogloblin (Ig) $\varkappa$ light chain gene enhancer and interleukin-2 receptor α chain gene promoter, only the Ig$\varkappa$ light chain enhancer was activated *in vivo*. We must therefore establish what the relation is between the *in vitro* strength of the HBP-1 binding affinity and the *in vivo* strength of the promoter activity.

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