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OCSBF-1, a Maize Ocs Enhancer Binding Factor: Isolation and Expression during Development

Karambir Singh,[1] Elizabeth S. Dennis, Jeff G. Ellis, Danny J. Llewellyn, James G. Tokuhisa, Jill A. Wahleithner,[2] and W. James Peacock

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia

The ocs-elements comprise a family of related 20-base pair DNA sequences with dyad symmetry that are functional components of the promoters of several genes introduced into the plant nucleus by *Agrobacterium* transformation or infection by DNA viruses. We describe the isolation and characterization of a maize cDNA that encodes a protein, OCSBF-1, that binds specifically to ocs-element sequences. The 21-kilodalton OCSBF-1 protein was encoded by a single copy, intron-less gene. The gene was differentially expressed in maize plants. Developing leaves had a gradient of OCSBF-1 mRNA with the basal portion of the leaves, which contain dividing and differentiating cells, having 40-fold to 50-fold higher levels of OCSBF-1 transcripts than the apical portion of the leaves, where the cells are fully differentiated. Roots and shoots of young plants had levels of OCSBF-1 mRNA similar to the basal portions of developing leaves. OCSBF-1 contained a small basic amino acid region and a potential leucine zipper motif homologous to the DNA-binding domains of the basic region-leucine zipper family of transcription factors such as Jun and GCN4. A truncated protein with the amino-terminal 76 amino acids of OCSBF-1, encompassing the basic domain and leucine zipper motif, still bound to ocs-element sequences in vitro. OCSBF-1 was able to bind to a site within each half of the ocs-element as well as to animal AP-1 and CREB sites.

INTRODUCTION

The ocs-element is a transcriptional enhancer that has been shown to be a functional component of a number of promoters that are active in plants (Bouchez et al., 1989). The ocs-element was first characterized as a 16-bp palindrome present in the upstream region of the promoter of the octopine synthase (*ocs*) gene of the *Agrobacterium tumefaciens* T-DNA (Ellis et al., 1987). Ocs-element sequences have also been found in other T-DNA encoded genes including nopaline synthase (*nos*), agropine synthase, and mannopine synthase, and in the promoters of three plant DNA viruses of the caulimovirus group, including the type member cauliflower mosaic virus (CaMV) (Bouchez et al., 1989). Comparison of the sequences of the ocs-elements in these genes produces a 20-bp consensus sequence, which includes the 16-bp palindrome (Bouchez et al., 1989). A plant protein factor, ocs transcription factor (OCSTF), that interacts with the various ocs-element sequences has been identified in both monocot and dicot plants (Singh et al., 1989; Tokuhisa et al., 1990). OCSTF binds to either half of the ocs-element with binding to both halves being a prerequisite for transcriptional activation in vivo (Bouchez et al., 1989; Singh et al., 1989; Tokuhisa et al., 1990).

Although ocs-elements are important in regulating the transcription of genes introduced into plants by bacterial or viral infection, cellular genes regulated by such elements have not yet been identified. One approach to understanding the role of ocs-elements in normal plants would be to isolate such cellular genes. It seems likely that such cellular genes exist because the OCSTF binding activity is present in uninfected plants (Tokuhisa et al., 1990), and the ocs-element is active in transgenic plants where it directs tissue-specific expression patterns that are developmentally regulated (DeGreve et al., 1982; Benfey et al., 1989; Fromm et al., 1989; Leisner and Gelvin, 1989). In young tobacco seedlings, expression is primarily in the root tips, whereas in older seedlings, expression also occurs in the shoot apex (Benfey et al., 1989; Fromm et al., 1989).

We have taken a complementary approach to understand how ocs-elements function in the regulation of ocs-element containing promoters. By screening an expression library made from maize root-tip mRNA with an ocs-element sequence, we have isolated cDNA clones encoding specific ocs-element binding proteins. In this report, we present the molecular characterization of one such clone

[1] To whom correspondence should be addressed.
[2] Current address: Department of Biochemistry and Biophysics, University of California-Davis, Davis, CA 95616.

called OCSBF-1 (ocs-element binding factor). The 21-kD OCSBF-1 protein has homology to members of the basic region-leucine zipper (bZIP) family of transcription factors (Vinson et al., 1989), such as the animal proteins Jun (Bohmann et al., 1987; Angel et al., 1988), Fos (Van Beveren et al., 1983), and CREB (Hoeffler et al., 1988; Gonzales et al., 1989); the yeast protein GCN4 (Hope and Struhl, 1986); and the plant proteins O2 (Hartings et al., 1989; Schmidt et al., 1990), HBP-1 (Tabata et al., 1989), EmBP-1 (Guiltinan and Quatrano, 1990), and TGA1a/TGA1b (Katagiri et al., 1989). OCSBF-1 is transcribed from a single copy gene that is differentially expressed during maize leaf development. Although the precise relationship between OCSBF-1 and the maize factor OCSTF remains to be determined, our data indicate that OCSBF-1 has in vitro binding properties similar but not identical to those of OCSTF.

RESULTS

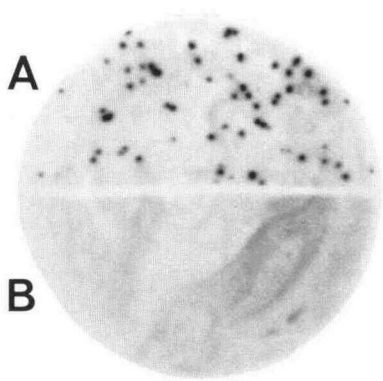
Isolation of a Gene from Maize Coding for an ocs-Element-Binding Protein

A cDNA expression library derived from maize root mRNA was screened for ocs-element specific binding proteins using a multimerized ocs-element sequence shown in Figure 1 (wild-type probe). The probe, which contained the 16-bp palindrome that occurs in the *ocs* gene promoter flanked by TG on the 5′ and CA on the 3′ side, was chosen because it best fitted the ocs-element consensus sequence (Bouchez et al., 1989). A total of 560,000 phage were screened and four cDNA clones were isolated. The specificity of binding was demonstrated using a mutant probe that differed in 8 out of 20 bases from the wild-type probe. The mutant probe, which does not bind OCSTF from maize nuclear extracts, also did not bind the four cDNA clones (Figure 1). The cDNA clones fell into two groups on the basis of DNA cross-hybridization. In this report we have focused on the characterization of the group 1 cDNA clones that appeared to be identical with respect to size and restriction map. One clone from the group, called OCSBF-1, was chosen for further analysis.

OCSBF-1 Is Differentially Expressed in Maize Plants

The tissue and developmental expression patterns of the OCSBF-1 transcript were analyzed during maize development by isolating total RNA from various organs and tissues, as shown in Figure 2. Significant levels of the 1450-base OCSBF-1 transcript were found in the primary root tip, the remainder of the primary root, and the coleoptile of 4-day-old to 5-day-old plants (Figure 2A, lanes 1 to 3). Twelve-day-old plants contained similar levels of OCSBF-1 transcripts in the primary root tip, the remainder of the primary root, and the shoot meristem (Figure 2B, lanes 1 to 3). Differences in the levels of OCSBF-1 transcripts were found in particular sections of the third leaf of 12-day-old plants. In the first 1 cm from the base of the leaf, the levels of OCSBF-1 transcripts were similar to those in other parts of the 12-day-old plants, such as the root tip (Figure 2B, lane 1 versus lane 4). The level of OCSBF-1 transcripts was reduced in the next 2 cm of the leaf and further reduced in the remaining portion of the leaf (Figure 2B, lanes 5 and 6).

To determine whether the differential pattern of OCSBF-1 expression also occurred in older maize leaves,



TGACGTAAGCGCTTACGTCA WILD TYPE
*** * * ***
TGCAATATGCGCCTTATTCA MUTANT

Figure 1. Binding Specificity of OCSBF-1 to the ocs-Element.

(A) Recombinant λgt11 plaques containing OCSBF-1 were hybridized with a DNA probe made from the wild-type ocs-element probe that contained a 20-bp perfect palindrome that fitted the consensus sequence derived from all the characterized ocs-element sequences (Figure 5).

(B) Recombinant λgt11 plaques containing OCSBF-1 were hybridized with a mutant probe in which 8 out of 20 bases had been altered.

RNA was isolated from the leaves of 28-day-old plants. Leaves 5 and 6 were the largest of the leaves that lacked a ligule, signifying that the leaves had not completed their development. With both leaves 5 and 6, the levels of OCSBF-1 transcripts were high in the first 8 cm to 10 cm from the base of the leaf and comparable with the levels found in the base of the third leaf of 12-day-old plants (Figure 2C, lanes 1, 2, and 5). In the next 8 cm to 10 cm, the levels of OCSBF-1 transcripts were fivefold to sixfold lower (lanes 3 and 6) and 40-fold to 50-fold lower in the remaining part of the leaves (Figure 2C, lanes 4 and 7). Thus, in the developing leaves of maize, expression of OCSBF-1 appears to be highest in regions that contain dividing and differentiating cells and very low in regions where the cells have fully differentiated.

Structure of the OCSBF-1 Gene and Gene Product

DNA gel blot analysis of maize genomic DNA, as shown in Figure 3, revealed single bands with a number of restriction enzyme digests, suggesting that OCSBF-1 is a member of a single or low copy gene family.

The size of the initial OCSBF-1 cDNA (OCSBF-1a) clone was 1050 bp. Because this clone was smaller than the 1450-base OCSBF-1 transcript, additional OCSBF-1 cDNA clones were isolated from the library by DNA hybridization, the largest of which, OCSBF-1b, was 1408 bp. Sequence analysis and restriction mapping demonstrated that the original OCSBF-1a cDNA clone was contained within the larger OCSBF-1b clone. The sequence of OCSBF-1b is presented in Figure 4.

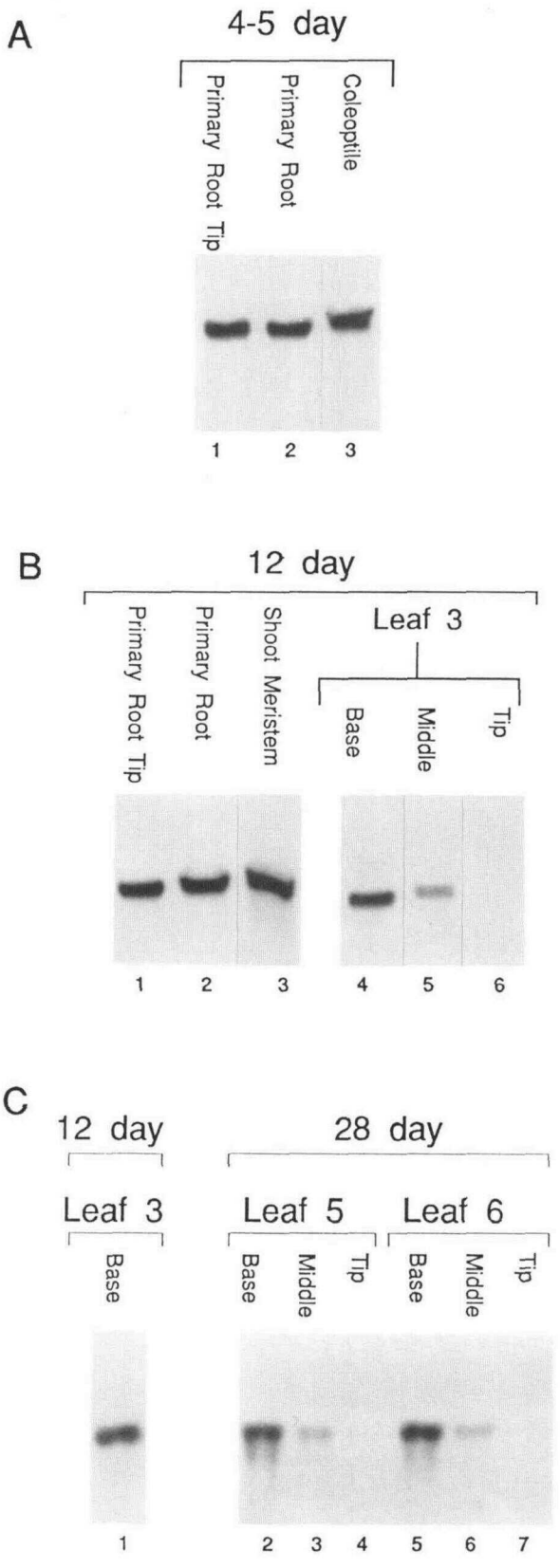


Figure 2. RNA Gel Blot Analysis of OCSBF-1 Expression in Maize.

Total RNA from different tissues and organs was isolated from maize plants of various ages, fractionated by electrophoresis on formaldehyde gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled single-stranded RNA probes made from OCSBF-1. The transcript size is indicated in bases.*

(A) Fifteen micrograms of RNA from 4-day-old to 5-day-old plants. Lane 1, primary root tip; lane 2, remainder of primary root; lane 3, coleoptile.

(B) Fifteen micrograms of RNA from 12-day-old plants. Lane 1, primary root tip; lane 2, remainder of primary root; lane 3, shoot meristem; lane 4, basal 0 cm to 1 cm of leaf 3; lane 5, basal 1 cm to 3 cm of leaf 3; lane 6, remaining distal portion of leaf 3 (3 cm to 6 cm).

(C) Twenty-five micrograms of RNA from 12-day-old plants (lane 1) and 28-day-old plants (lanes 2 to 7). Lane 1, basal 0 cm to 1 cm of leaf 3; lane 2, basal 0 cm to 10 cm of leaf 5; lane 3, basal 11 cm to 20 cm of leaf 5; lane 4, remainder of leaf 5 (21 cm to 45 cm); lane 5, basal 0 cm to 8 cm of leaf 6; lane 6, basal 9 cm to 16 cm of leaf 6; lane 7, remainder of leaf 6 (17 cm to 28 cm).

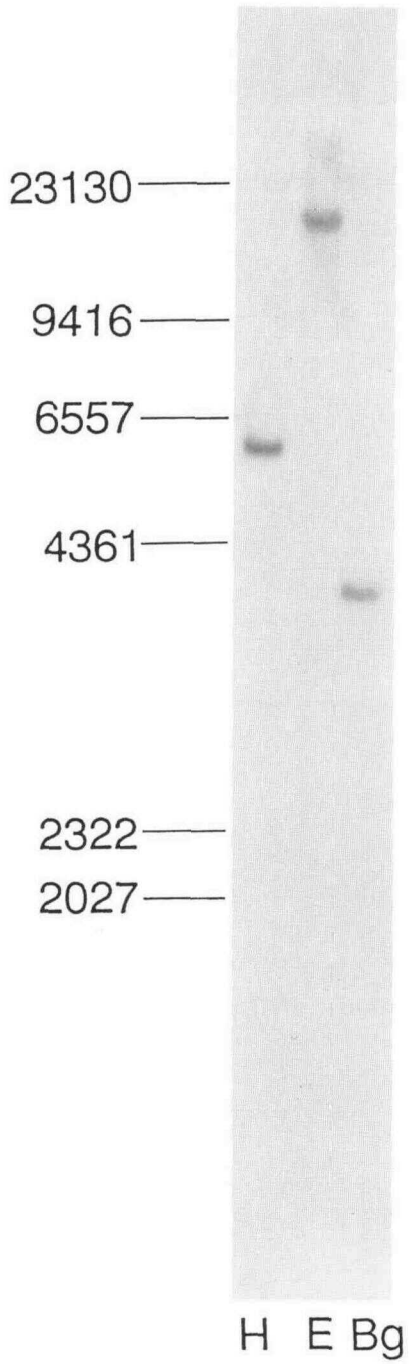


Figure 3. DNA Gel Blot Analysis of Maize Genomic DNA Using OCSBF-1 as a Probe.

Lanes contained 10 μg of maize DNA digested with HindIII (H), EcoR1 (E), and BglII (Bg) and probed with a 450-bp OCSBF-1 fragment from base pairs 350 to 875 that had been labeled with ^{32}P by the random primer method. Molecular weight markers (in base pairs) are λ-DNA digested with HindIII.

A genomic clone of OCSBF-1 was isolated from an EMBL4 library of maize DNA. The region corresponding to the large OCSBF-1b cDNA clone and approximately 250 bp upstream was sequenced. Alignment of the cDNA and genomic sequences indicated that the gene did not contain any introns. Major and minor transcription initiation sites were located by S1 mapping to be approximately 601 bp and 607 bp, respectively, upstream of the start of translation. There is no obvious TATA box located in the region 25 bp to 35 bp upstream from the transcription start (Figure 4). The 5′ end of the OCSBF-1b cDNA clone was 6 bases downstream from the major start of transcription. OCSBF-1b has an open reading frame extending from a methionine residue at nucleotide position 601 to a stop codon at 1200, yielding a predicted protein of 193 amino acids with a computed molecular size of 21 kD (Figure 4). The 601-bp-long 5′-untranslated leader sequence contains three short open reading frames, one of 11 amino acids in the correct reading frame and two of 4 and 28 amino acids, respectively, in different reading frames. There are five As at the end of the 220-bp, 3′-nontranslated region of the cDNA. Because the genomic and cDNA sequences diverge after the first A in the cDNA, this is the beginning of the poly A tail of the OCSBF-1 message. A putative poly A addition sequence, AATAAT, is present 17 bp upstream of the poly A tail (Figure 4).

OCSBF-1 Has Properties of a bZIP Transcription Factor

Translation of the open reading frame of OCSBF-1 revealed a small, basic region (Figure 4) that, as shown in Figure 5, is homologous to the DNA binding domains of animal and yeast bZIP transcription factors, such as Jun (Bohmann et al., 1987; Angel et al., 1988), Fos (Van Beveren et al., 1983), GCN4 (Hope and Struhl, 1986), and the related factor CREB (Hoeffler et al., 1988; Gonzales et al., 1989) (Figure 5A). Two leucines and a valine, each separated by 6 amino acids, occur adjacent to the basic domain in OCSBF-1. There are acidic regions near the C terminus (residues 150 to 180) and N terminus (residues 15 to 25) of OCSBF-1 (Figure 4) that may correspond to the acidic domains thought to be important for transcriptional activation by GCN4 (Hope and Struhl, 1986) and Jun (Bohmann et al., 1987; Angel et al., 1988). The C-terminal region is also proline rich (13 residues out of 75), suggesting that it may also serve as an activation domain, as has been found with Jun (Bohmann and Tjian, 1989) and mammalian CTF/NF-1 (Mermod et al., 1989).

OCSBF-1 also shares homology in the binding domain with several other bZIP proteins recently identified in plants (Figure 5A). The greatest homology is shared with the wheat proteins HBP-1 and EmBP-1 (Tabata et al., 1989; Guiltinan and Quatrano, 1990) and the maize protein O2 (Hartings et al., 1989; Schmidt et al., 1990). We have isolated another maize DNA-binding protein, called OCSBF-2, which also binds to the ocs-element probe.

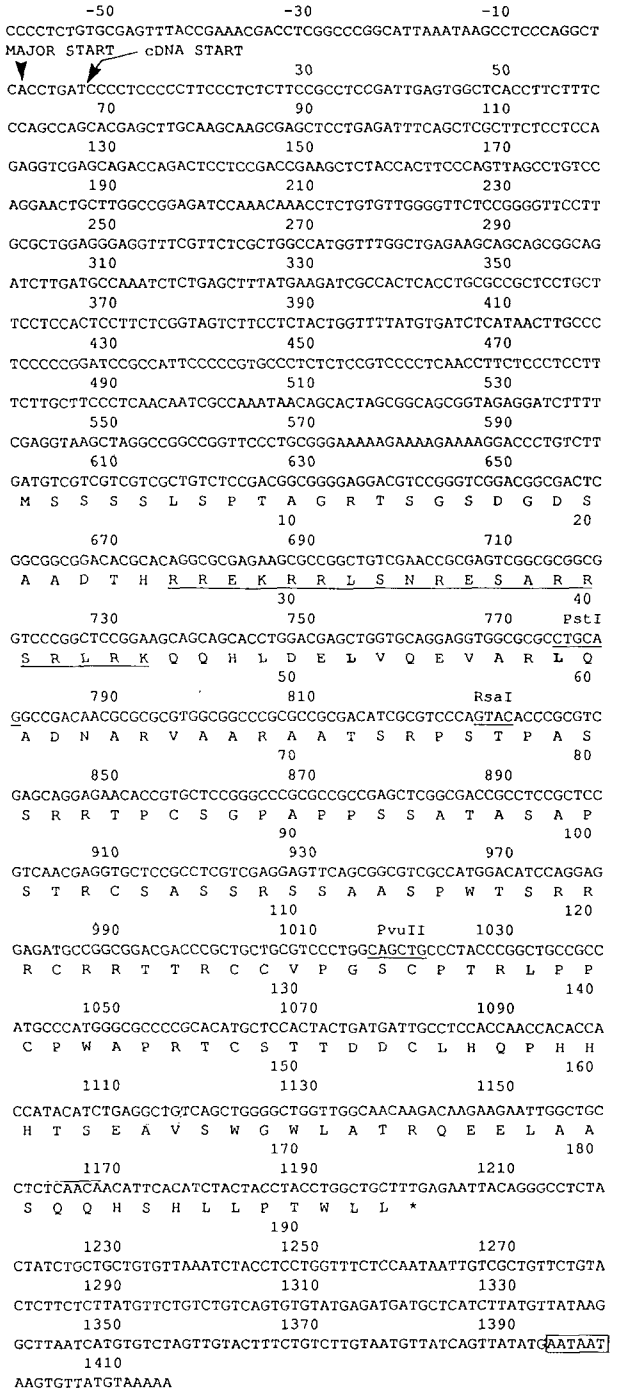


Figure 4. Sequence of the Gene for OCSBF-1 and Predicted Amino Acid Sequence for the Longest Open Reading Frame of the OCSBF-1 Clone.

The 5′ end of the OCSBF-1b cDNA clone is marked. The basic region is underlined, and the residues forming the leucine zipper motif are in bold-face type. The poly A addition sequence is boxed, and the major transcription initiation site is marked. The positions of various restriction sites referred to in other parts of the paper are indicated.

Although the cDNA encoding this protein is not full length, the OCSBF-2 protein sequence contains a basic region and leucine zipper motif (Figure 5A). The homology in the basic region between OCSBF-1 and OCSBF-2 is less and of the same order as that between OCSBF-1 and two tobacco DNA-binding proteins TGA1a and TGA1b (Katagiri et al., 1989).

OCSBF-1 Has Binding Properties Similar to but Distinct from OCSTF

Coupled in vitro transcription/translation of the OCSBF-1 cDNA sequence in pGEM3Z$^+$ using T7 polymerase and rabbit reticulocyte lysates produces a 21-kD protein (data not shown). The binding characteristics of the OCSBF-1

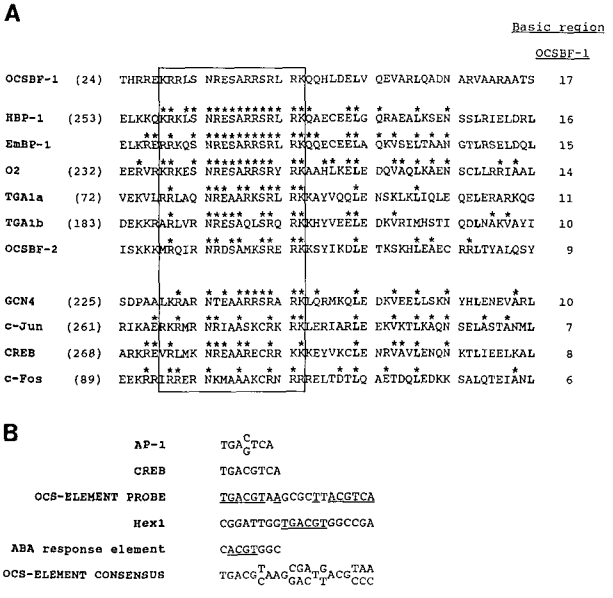
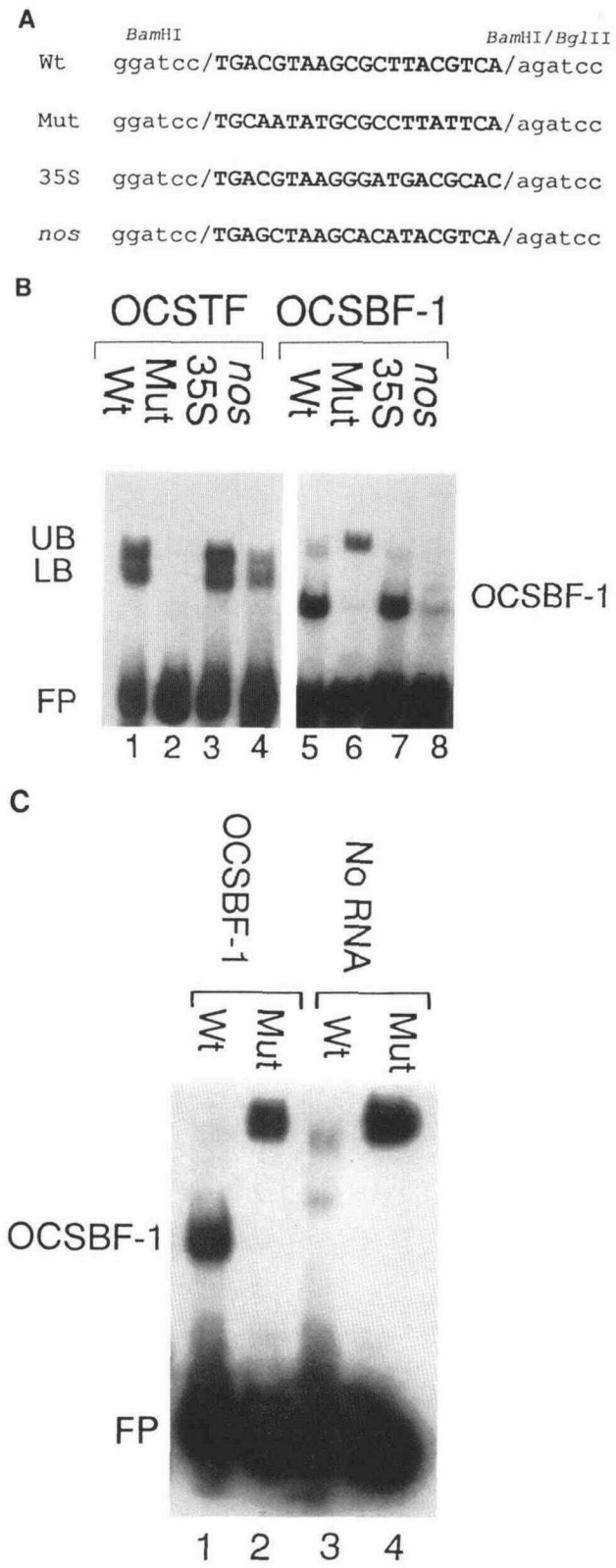


Figure 5. Comparison of the DNA Binding Domains of OCSBF-1 and Other Animal, Yeast, and Plant Transcription Factors and Their Respective DNA Recognition Sequences.

(A) Amino acid sequence homologies in the basic region and part of the leucine zipper motif of the OCSBF-1 cDNA clone, the wheat factors HBP-1 and EmBP-1, the maize factors O2 and OCSBF-2, the tobacco factors TGA1a and TGA1b, the yeast factor GCN4, and the mammalian factors c-Jun, CREB, and c-Fos. The position of the first amino acid used in each protein is shown in parentheses. Residues conserved with OCSBF-1 are marked with an asterisk. A 17-amino acid basic region is boxed and the residues forming the leucine zipper motif are in bold-face type. The number of amino acids within the boxed basic region that are identical between OCSBF-1 and the other proteins is indicated.

(B) The AP-1 and CREB DNA recognition sequences, the ocs-element probe used to select OCSBF-1, the Hex1 sequence and ABA response element, and the consensus OCSTF binding site. The CREB-like binding sites in the ocs-element and the Hex1 sequence are underlined.



protein produced in vitro were compared with OCSTF isolated from maize nuclear extracts, as shown in Figure 6. Both proteins were able to bind well to the consensus ocs-element probe (Figure 6B, lane 1 versus lane 5) and were unable to bind to a mutant ocs-element probe (Figure 6B, lanes 2 and 6). Binding of OCSTF to the consensus probe gave two retarded bands, the lower retarded band having previously been shown to be due to binding of OCSTF to either half of the ocs-element and the upper band resulting from OCSTF binding to both halves of the ocs-element (Tokuhisa et al., 1990). In contrast, binding of OCSBF-1 to the consensus probe gave only a single retarded band, and the mobility of this band was faster than the lower retarded band obtained with OCSTF.

Binding to the ocs-element sequences of the promoters of the 35S transcript of CaMV and the *nos* gene (Bouchez et al., 1989) was also tested. The 35S probe differed at six out of 20 positions from the consensus ocs-element probe, whereas the *nos* probe differed in four positions from the consensus ocs-element probe (Figure 6A). The sequences flanking the consensus, mutant, 35S, and *nos* probes were identical. Both the OCSBF-1 and OCSTF proteins bound well to the 35S probe at levels similar to that seen with the consensus probe (Figure 6B, lanes 3 and 7). OCSTF also bound the *nos* probe, although the amount of binding was less than to either the consensus or the 35S probes (Figure 6B, lane 4). The binding of OCSBF-1 to the *nos* probe was very low (Figure 6B, lane 8).

Figure 6. Comparison of OCSTF and OCSBF-1 Binding to Various ocs-Element Sequences.

(A) The sequences of the wild type (Wt), mutant (Mut), 35S, and *nos* ocs-elements are shown.

(B) Binding reactions contained in vitro translation products or maize nuclear extracts incubated with 10^4 cpm of ^{32}P-labeled fragment, 0.5 μg of poly d(I-C) (unless otherwise indicated) in 20 μL of binding buffer. After incubation at 30°C for 30 min, the free and bound DNAs were resolved on a 4% polyacrylamide gel and visualized by autoradiography. Lanes 1 to 4, binding of OCSTF isolated from maize nuclear extracts; lanes 5 to 8, binding of full-length OCSBF-1 produced by coupled in vitro transcription and translation reactions. The positions of the free probe (FP), lower retarded band (LB), and the upper retarded band (UB) produced with OCSTF and the single retarded band produced with OCSBF-1 (OCSBF-1) are shown.

(C) Comparison of the OCSBF-1 reticulocyte extract and a "No RNA" control reticulocyte extract with the Wt and Mut probes. This demonstrates that the retarded band obtained with the Wt probe and the OCSBF-1 reticulocyte extract is due to OCSBF-1, and that the slow mobility band present at the top of Figure 6B, lane 6 and Figure 6C, lanes 2 and 4 is not derived from OCSBF-1 but is due to an activity present in the reticulocyte extract. "No RNA" control experiments were also performed on the 35S and *nos* probes (data not shown). The positions of the free probe (FP) and the single retarded band produced with OCSBF-1 (OCSBF-1) are shown.

OCSBF-1 Recognizes a Site within a Half ocs-Element

Mutant derivatives of the ocs-element were used to further characterize the binding properties of OCSBF-1, as shown in Figure 7. The aim was to determine whether the single retarded band that results from OCSBF-1 binding to wild-type ocs-element sequences is due to OCSBF-1 binding to the entire ocs-element or to one or other half of the ocs-element. Mutants 3.3 and 6.1 contain base substitutions confined to the 5′ or 3′ half of the ocs-element, respectively (Figure 7A) (Singh et al., 1989). We had previously shown that OCSTF from maize will bind to both of these mutants but will form only the lower retarded band under reaction conditions in which both the lower and upper retarded band are formed with a wild-type probe (Tokuhisa et al., 1990). OCSBF-1 bound to the mutant probes 3.3 and 6.1, and the resulting single retarded bands had the same mobility as the single retarded band that results from binding to the wild-type probe (Figure 7B, lanes 1 to 3). However, OCSBF-1 was unable to bind to the mutant probe 5.1, where both halves of the element had been mutagenized (Figure 7B, lane 4), again a result similar to that obtained with OCSTF (Tokuhisa et al., 1990). These results demonstrate that OCSBF-1 recognizes a binding site in either half of the ocs-element.

OCSBF-1 Is Able To Bind to Animal AP-1 and CREB Sites

Each half of the ocs-element contains a sequence that resembles the binding sites for the animal transcription factors Jun and CREB (Figure 5B). The CREB site has the greater homology to the ocs-element with each half of the ocs-element containing seven out of a possible eight matches with the CREB consensus sequence. Because OCSBF-1 is able to bind to a half site within the ocs-element, we determined whether OCSBF-1 could bind to either an AP-1 or a CREB site, as shown in Figure 8. DNA probes containing an AP-1 site, a CREB site, and an ocs-element were used in gel retardation experiments with OCSBF-1 (Figure 8A). OCSBF-1 bound at comparable levels to all three probes to give a single retarded band (Figure 8B, lanes 1 to 3). The maize factor OCSTF was also able to bind to the AP-1 and CREB probes (data not shown).

The Region of OCSBF-1 Encompassing the Putative Leucine Zipper Motif Is Required for DNA Binding

To further characterize the DNA-binding properties of OCSBF-1, C-terminal deletions of the OCSBF-1 protein were generated using suitable restriction sites within the OCSBF-1 coding sequence and coupled in vitro transcription/translation reactions, as shown in Figure 9. When the pGEM3Z$^+$/OCSBF-1 DNA template was linearized with a restriction site (BamHI) 3′ of the OCSBF-1 insert, before the in vitro transcription reaction, a 1056-base RNA was produced. Translation of this RNA in the presence of ^{35}S-methionine produced a 193-amino acid polypeptide that could be detected by SDS-polyacrylamide gel electrophoresis (data not shown). If the DNA template was linearized at the PvuII site at nucleotide position 1116, the RsaI site at nucleotide position 828, or the PstI site at nucleotide position 776 before the in vitro transcription reaction, the correspondingly smaller RNAs were produced which,

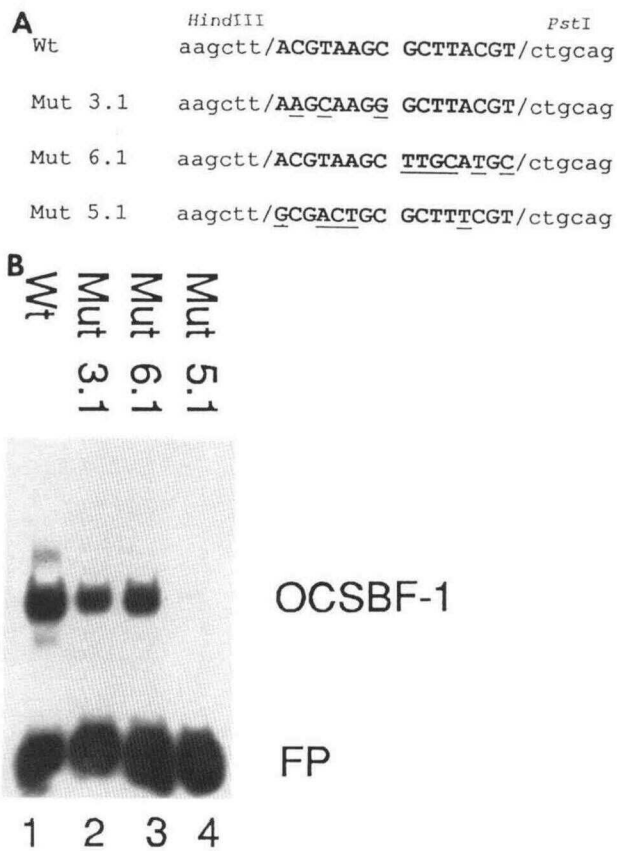


Figure 7. Binding of OCSBF-1 to Mutant ocs-Elements.

(A) The sequences of the wild-type 16-bp ocs-element and mutant 16-bp ocs-elements 3.3, 6.1, and 5.1 are shown.

(B) The binding reactions contained OCSBF-1, produced by coupled in vitro transcription and translation reactions, incubated with 10^4 cpm of the ^{32}P-labeled PvuII-SalI fragments containing various ocs-element probes, 0.5 μg of poly d(I-C) in 20 μL of binding buffer. Gel retardation conditions were as described for Figure 6. The positions of the free probe (FP) and the OCSBF-1 protein/DNA complexes (OCSBF-1) are shown. The OCSBF-1 protein/DNA complexes in lanes 1 to 3 were not present with "no RNA" control reticulocyte extracts (data not shown).

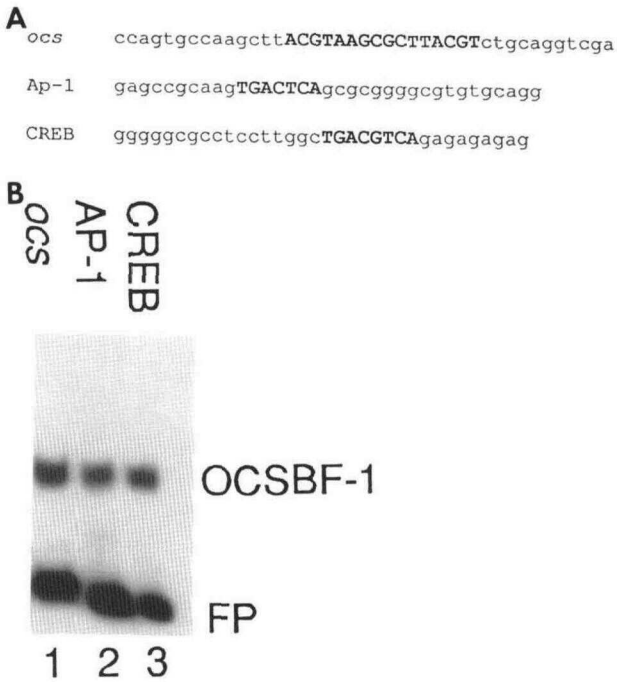


Figure 8. Binding of OCSBF-1 to Animal AP-1 and CREB Sites.

(A) The sequences of the *ocs*, AP-1, and CREB probes are shown.
(B) The binding reactions contained OCSBF-1, produced by coupled *in vitro transcription and translation* reactions, incubated with 10^4 cpm of the ^{32}P-labeled ocs, AP-1, and CREB fragment probes, 0.5 μg poly d(I-C) in 20 μL of binding buffer. Gel retardation conditions were as described for Figure 6. The positions of the free probe (FP) and the OCSBF-1 protein/DNA complexes (OCSBF-1) are shown. Control experiments using C-terminal deletions of OCSBF-1 (as shown in Figure 9 for the CREB probe) were performed with all three probes to demonstrate that the retarded band was due to OCSBF-1 binding.

when translated, produced N-terminal OCSBF-1 polypeptides of 133, 76, and 58 amino acids, respectively. Although it was possible to resolve the 133 amino acid polypeptide on SDS-polyacrylamide gels, we have not been able to resolve clearly the 76- or 58-amino acid polypeptides.

The DNA-binding properties of the N-terminal 133, 76, and 58 amino acids of OCSBF-1 were compared with the full-length OCSBF-1 protein. Both the 133 and 76 N-terminal amino acid polypeptides gave correspondingly faster migrating DNA/protein complexes compared with the full-length OCSBF-1 protein in binding studies with CREB (Figure 9B, lane 1 versus lanes 2 and 3) or ocs-element probes, demonstrating conclusively that the binding activity is derived from the in vitro expression of the OCSBF-1 cDNA clone. However, the N-terminal 58-amino acid OCSBF-1 polypeptide was no longer able to bind (Figure 9B, lane 4). Although the N-terminal 76-amino acid polypeptide still contained the basic region and putative leucine zipper motif, in the N-terminal 58-amino acid polypeptide part of the leucine zipper motif had been deleted. These results demonstrate that a small N-terminal OCSBF-1 polypeptide that still encompasses the basic region and leucine zipper motif retains DNA binding but that deletions of part of the leucine zipper motif abolish DNA binding.

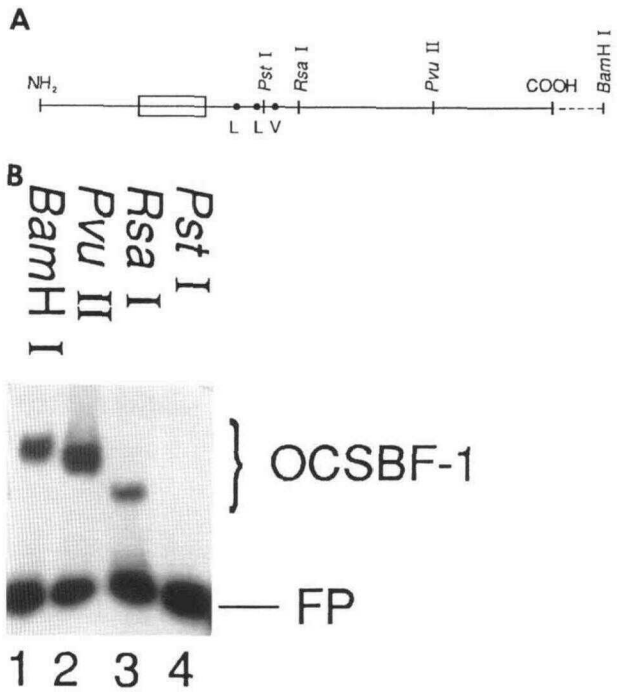


Figure 9. Binding Properties of C-Terminal Deletions of OCSBF-1.

(A) A schematic representation of the OCSBF-1 cDNA insert on pGEM3Z$^+$ showing the positions of the restriction sites used to generate the C-terminal deletions.
(B) Full-length and C-terminal deletions of the OCSBF-1 protein were generated using suitable restriction sites (shown above the lanes) in the OCSBF-1 cDNA clone and coupled in vitro transcription and translation reactions. The binding reactions contained the different OCSBF-1 polypeptides, incubated with 10^4 cpm of the ^{32}P-labeled CREB probe (similar results were obtained with ocs-element probes), 0.25 μg of poly d(I-C) in 20 μL of binding buffer. Gel retardation conditions were as described for Figure 6. The position of the free probe (FP) and the OCSBF-1 protein/DNA complexes (OCSBF-1) are shown. Lane 1, full-length (193 amino amino acids) OCSBF-1 protein using the BamH1 site 3′ of the OCSBF-1a insert in pGEM3. Lane 2, N-terminal 133 amino acids of OCSBF-1. Lane 3, N-terminal 76 amino acids of OCSBF-1. Lane 4, N-terminal 58 amino acids of OCSBF-1.

DISCUSSION

A maize gene coding for an ocs-element DNA-binding protein has been isolated by expression library screening with an ocs-element probe. The single copy gene codes for a 21-kD protein called OCSBF-1 that has characteristics of the bZIP family of transcription factors (Vinson et al., 1989). The function of OCSBF-1 remains to be determined, but the observation that OCSBF-1 is differentially expressed during maize leaf development suggests that it may contribute to developmentally specific patterns of gene expression. Cellular differentiation in monocot leaves such as maize proceeds in a basipetal direction (reviewed in Nelson and Langdale, 1989). The basal meristem generates files of cells that remain aligned from the base to the tip of the leaf. The oldest cells are at the tip of the leaf and are the most photosynthetically developed, whereas basal leaf segments contain cells at progressively earlier stages of development. This basipetal gradient of cell maturation makes monocot leaves a good system for studying aspects of leaf development such as plastid development, photosynthetic development, and morphological development (reviewed in Leech, 1985). In this context, the observation that the expression of the putative plant transcription factor OCSBF-1 is down-regulated 40-fold to 50-fold along this basipetal gradient opens up the possibility that OCSBF-1 plays a role in one or more aspects of leaf development.

Relationship of OCSBF-1 to Other Animal and Plant bZIP Proteins

The high degree of sequence conservation of the DNA binding domains between transcription factors from the plant and animal kingdoms suggests that the same DNA-protein interaction has evolved to perform diverse regulatory roles in eukaryotic transcription. The mammalian and yeast transcription factors Jun (Bohmann et al., 1987; Angel et al., 1988), Fos (Van Beveren et al., 1983), CREB (Hoeffler et al., 1988; Gonzales et al., 1989), and GCN4 (Hope and Struhl, 1986) contain a conserved basic region, which precedes a leucine zipper motif. Mutagenesis experiments have demonstrated that these regions are required for dimerization and DNA binding of these proteins (see Abel and Maniatis, 1989, for review). OCSBF-1 contains an analogous basic region and leucine zipper motif. A deletion derivative of OCSBF-1 that contains only the 76 N-terminal amino acids and retains the basic region and the leucine zipper motif is able to bind to the ocs-element. A C-terminal deletion of OCSBF-1 that removes part of the leucine zipper motif is no longer capable of DNA binding.

Sequences homologous to the Jun and CREB binding sites are contained within each half of the ocs-element sequence used to select OCSBF-1 (Figure 5B). Although the ocs-element sequence has greatest homology to a consensus CREB binding site, the DNA binding domains of OCSBF-1 show a similar degree of homology to both Jun and CREB (Figure 5A). OCSBF-1 can bind to both AP-1 and CREB sites and in this regard is like Jun (R. Turner and R. Tjian, personal communication) and unlike CREB, which can bind only to a CREB site (Hardy and Shenk, 1988).

The proposed binding domain of OCSBF-1 is homologous to those of several DNA-binding proteins recently isolated from plants (Figure 5A), although outside this domain there is little homology to the other proteins. The greatest homology is among OCSBF-1, the wheat HBP-1 and EmBP-1 proteins, and the maize O2 protein. HBP-1 is a DNA-binding protein that interacts with the Hex1 site in the promoter of the wheat histone 3 gene (Tabata et al., 1989) and has 17 of 18 contiguous amino acids that are identical to those in OCSBF-1. The plant Hex1 binding site has a 6-base core sequence, TGACGT, that is contained within the CREB binding site and is also present in both halves of the ocs-element consensus sequence (Figure 5B). EmBP-1 is a DNA-binding protein that interacts with the abscisic acid response element (Figure 5B) present in the abscisic acid-regulated Em gene from wheat (Guiltinan and Quatrano, 1990). O2 is the gene product of the Opaque 2 locus (Hartings et al., 1989; Schmidt et al., 1990) and controls the highly localized expression of the 22-kD class of the zein seed storage protein genes in the endosperm of maize seedlings 15 days to 40 days after pollination (Jones et al., 1977). O2 has been found to bind specifically to the upstream region of a zein genomic clone (Schmidt et al., 1990), although the precise DNA recognition site for the O2 protein has not been identified. The homology between OCSBF-1 and O2 is not as striking in the basic region as between OCSBF-1 and HBP-1 but does extend into the leucine zipper. OCSBF-1 also shares homology with OCSBF-2, a second maize ocs-element binding protein, and with two tobacco DNA-binding proteins, TGA1a and TGA1b (Katagiri et al., 1989), that were isolated by their ability to bind to the Hex1 sequence. Again, the homology is confined to the basic DNA binding domains of the various proteins. The 40-kD TGA1a protein (Katagiri et al., 1989), in contrast to TGA1b (Katagiri et al., 1989), is also able to bind to the ocs-elements present in the 35S and *nos* promoters and has been proposed as a likely candidate for the OCSTF protein characterized in tobacco, which has also been called ASF-1 (Lam et al., 1989).

The 21-kD OCSBF-1 protein is significantly smaller than other animal and plant bZIP proteins, which range in size from 35 kD to 50 kD. Like the c-Jun gene, the OCSBF-1 gene contains no intron and no obvious TATA box (Hattori et al., 1988). In plants, the only other genomic sequence

available is for the maize O2 gene,which contains six introns, including an intron within the beginning of the leucine zipper motif (Hartings et al., 1989). The homology between the binding domains of OCSBF-1 and the O2 proteins extends beyond the exon boundries of the O2 binding domain (Hartings et al., 1989). OCSBF-1, like GCN4 (Hinnebusch, 1984), Jun (Hattori et al., 1988), and O2 (Hartings et al., 1989; Schmidt et al., 1990), has a long, 5′-untranslated leader sequence that contains short open reading frames. It is not known whether the small open reading frames play a role in the expression of OCSBF-1, although such open reading frames have been found to regulate translation in a number of genes (Hinnebusch, 1988).

Relationship of OCSBF-1 and OCSTF

We have previously reported the identification of a plant factor from maize, OCSTF, that recognizes the various ocs-element sequences (Bouchez et al., 1989; Tokuhisa et al., 1990). Although the precise relationship between OCSBF-1 and OCSTF remains to be determined, a number of observations in the present study bear on this point and are discussed below.

Although both OCSTF and OCSBF-1 are able to bind to the consensus ocs-element probe, there are differences in the patterns of the resulting DNA/protein complexes obtained by gel electrophoresis. OCSTF binds to either half of the ocs-element as well as to both halves simultaneously, resulting in two retarded bands, a lower and upper band, respectively (Tokuhisa et al., 1990). OCSBF-1 synthesized in vitro also binds to the consensus ocs-element, but appears to produce only a single retarded band. The use of mutated ocs-elements has demonstrated that this band is due to binding of OCSBF-1 to a sequence within either half of the element. The mobility of the OCSBF-1 retarded band is faster than the lower retarded band generated by OCSTF. The difference in mobility could occur because of the way the OCSTF and OCSBF-1 proteins were isolated. OCSTF has been isolated from maize tissue or cell suspension extracts, whereas OCSBF-1 has been produced in vitro by coupled transcription/translation reactions using T7 polymerase and rabbit reticulocyte lysates. It is possible that differences in posttranslational modifications, such as phosphorylation or glycosylation, the absence of cofactors, or the different in vitro binding conditions may cause differences in mobility. Alternatively, the difference in mobility may reflect differences in molecular weight between OCSBF-1 and OCSTF.

Because of the differences in mobility and number of DNA/protein complexes formed by OCSBF-1 and OCSTF, it is unclear whether they are the same protein, although OCSTF could be a complex of polypeptides, one of which may be OCSBF-1. Antibodies to OCSBF-1 should determine the exact relationship between OCSBF-1 and OCSTF. What is clear is that OCSBF-1 and OCSTF recognize very similar DNA sequences; they both bind to ocs-element sequences present in the *ocs*, 35S, and *nos* promoters, although the binding of OCSBF-1 to the *nos* sequence is low. OCSBF-1 and OCSTF also bind in a similar manner to a number of mutant ocs-elements.

How Many bZIP Transcription Factors Are There in Maize?

A major finding of this and other work is that the protein factors that recognize ocs-element sequences (Katagiri et al., 1989) or sequences within an ocs-element half-site (Katagiri et al., 1989; Tabata et al., 1989) are bZIP transcription factors. In view of the similarities in the binding domains of the maize bZIP transcription factors, the OCSBF, and the O2 proteins, it seems likely that there are different classes of bZIP proteins in plants that recognize similar DNA sequences but regulate different genes and patterns of gene expression. It will be informative to determine the expression patterns of the various maize bZIP proteins during maize development as well as their precise DNA recognition sequences. A number of Jun and Fos proteins have now been identified in animals, and the ability to form various Jun/Fos heterodimers has been suggested to contribute significantly to tissue-specific patterns of gene expression (Abel and Maniatis, 1989). It will be interesting to determine whether the maize bZIP proteins can form heterodimers among themselves and whether these heterodimers modulate the binding specificities and patterns of gene expression of ocs-element-containing genes.

METHODS

Expression Library Screening

A maize λgt11 cDNA library made from root-tip mRNA was purchased from Clontech. Screening of the expression library with the wild-type probe was performed essentially as described by Singh et al. (1988). Five hundred sixty thousand phage were screened at a density of 40,000 per 11-cm filter. The 20-bp wild-type and mutant ocs-element sequences (Figure 1A) were flanked by BamHI and BglII sites that allowed the annealed oligonucleotides to be oligomerized with T4 ligase. Filters were screened in duplicate with either ^{32}P end-labeled, oligomerized (average size, 130 bp) wild-type or mutant ocs-element probes (10^6 cpm/mL) (Figure 1) using 20 μg/mL poly d(I-C) as a nonspecific DNA binding competitor. In secondary and subsequent screening, the denaturation/renaturation technique of Vinson et al. (1988) was used before the DNA binding step to enhance the specific binding of the probe to the filters.

RNA Analysis

Total RNA from different tissues and organs was isolated from maize plants as described by Nelson et al. (1984). Equal amounts of RNA were fractionated by electrophoresis on 1.5% formaldehyde agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled single-stranded RNA probes made from OCSBF-1 cloned in pGEM3Z$^+$. Poly A$^+$ RNA was isolated by standard procedures from seedling roots and used in S1 mapping experiments to determine the start of transcription and in RNA gel blot experiments to determine the size of the OCSBF-1 transcript using viral brome mosaic virus RNAs (Promega Biotec catalog no. D1541) as size markers.

DNA Gel Blot Analysis and Genomic Library Preparation and Screening

Genomic DNA from the maize line BKS (Sachs et al., 1980) was prepared and used for DNA gel blot analysis as previously described (Sutton et al., 1984; Dennis et al., 1985). A maize genomic library was prepared from the BKS genomic DNA. The DNA was digested with BglII and size fractioned on a glycerol gradient, and the size fraction 2.5 kb to 4.0 kb was isolated. The isolated DNA was ligated to BamHI cut arms of λ EMBL4 that had been treated with alkaline phosphatase. Packaging and screening of the library were carried out by standard procedures.

DNA Sequence Analysis

Sequence analysis was performed using the method of Sanger et al. (1977). The vectors used were mp18, mp19, pUC118, and pUC119; single-stranded templates were generated from the pUC vectors using the M13K07 helper phage. The single-stranded DNA templates were sequenced either on an ABI 370A sequencing machine with Taq polymerase or with conventional sequence apparatus, ^{32}P-labeled nucleotides, and T7 polymerase. The nucleotide sequence and the deduced amino acid sequence were compared with sequences in the GenBank and EMBL data bases.

Isolation of Binding Activities

Maize Extracts

OCSTF binding activity was isolated from nuclear extracts prepared from maize cell suspension cultures as previously described (Tokuhisa et al., 1990).

In Vitro Transcription and Translation

OCSBF-1 was produced by coupled in vitro transcription and translation reactions. The OCSBF-1a cDNA clone was cloned into the EcoRI site of pGEM3Z$^+$. The DNA was linearized either at the BamHI site, 3′ of the OCSBF-1 insert to generate a full-length OCSBF-1 polypeptide, or at restriction enzyme sites within the OCSBF-1 coding sequences to generate C-terminal deletions of the OCSBF-1 polypeptide. The 100-μL transcription reactions contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 100 units of RNasin (Promega Biotec), 500 μm each of ATP, CTP, GTP, and UTP, 5 μg of linearized DNA template, and 30 units of T7 RNA polymerase (Promega Biotec). The reactions were incubated at 40°C for 2 hr. The DNA template was removed by digestion with 5 units of RQ1 RNase-free DNase (Promega Biotec) for 15 min at 37°C, followed by phenol extraction. The synthetic mRNAs were precipitated with 2 M ammonium acetate and 3 volumes of ethanol. The reactions typically yielded 5 μg to 10 μg of RNA.

The 50-μL translation reactions contained 1 μg of RNA and 35 μL of rabbit reticulocyte extract and were performed as directed by the supplier (Promega Biotec). Translation products were stored at −80°C in 10% glycerol. ^{35}S-methionine-labeled translation products were analyzed by 15% SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970).

Isolation of ^{32}P-Labeled DNA Fragments

The consensus, mutant, *nos*, and 35S ocs-elements (Figure 6A) were synthesized such that the double-stranded oligonucleotides contained a 5′ BamHI site and a 3′ BglII site flanking the 20-bp ocs-element sequences. The oligonucleotides were subcloned into the BamHI site of pUC118, and clones that contained the oligonucleotides inserted in the same orientation in the polylinker (SmaI, BamHI-20-bp ocs-element-BamHI/BglII, XbaI) were isolated. These clones were digested with SalI and HindIII, and the resulting 60-bp fragments were isolated and end labeled as described previously (Singh et al., 1989). The SalI-PvuII end-labeled fragments containing the wild-type and mutant ocs-elements 3.1, 5.1, and 6.1 (Figure 7A) were described previously (Singh et al., 1989). The AP-1 and CREB oligonucleotides (Figure 8A) and their complements were end labeled with T4 kinase before annealing. The AP-1 sequence is from the human metallothionein IIa gene (Turner and Tjian, 1989) and the CREB sequence is from the human somatostatin gene (R. Turner and R. Tjian, personal communication).

Gel Retardation Assays

Gel retardation experiments were performed as previously described (Singh et al., 1989).

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As this article went to press, we received word that the reference Guiltinan and Quatrano (1990) has been superseded by: **Guiltinan, M. J., Marcotte, W. R., Jr., and Quatrano, R. S.** (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. Science, in press.

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