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A Maize Protein Associated with the G-Box Binding Complex Has Homology to Brain Regulatory Proteins

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The G-box element is a moderately conserved component of the promoter of many inducible genes, including the alcohol dehydrogenase genes of Arabidopsis and maize. We used monoclonal antibodies generated against partially purified G-box binding factor (GBF) activity to characterize maize proteins that are part of the DNA binding complex. Antibodies interacted with partially purified maize GBF complexes to produce a slower migrating complex in the gel retardation assay. Immunoprecipitation experiments suggested that the protein recognized by the antibody is not a DNA binding protein in and of itself, but rather is associated with the DNA binding complex. These monoclonal antibodies were used to isolate cDNA clones encoding a protein that we have designated GF14. Maize GF14 contains a region resembling a leucine zipper and acidic carboxy and amino termini, of which the latter can form an amphipathic α-helix similar to known transcriptional activators such as VP16 and GAL4. Protein gel blot analysis of cell culture extract showed that a single, major protein of approximately 30 kD is recognized by anti-GF14; the protein is also present predominantly in the kernel and root. The deduced amino acid sequence of maize GF14 is more than 80% identical to Arabidopsis GF14 and Oenothera PHP-O, and is more than 60% identical to a class of mammalian brain proteins described as both protein kinase C inhibitors and activators of tyrosine and tryptophan hydroxylases. GF14 is found in a variety of monocotyledons and dicotyledons, gymnosperms, and yeast. This suggests a deep evolutionary conservation of a potential regulatory protein associated with a core sequence found in the promoter region of many genes.

INTRODUCTION

Plants respond to various environmental stimuli by a rapid change in the pattern of gene expression (Cherry, 1989). The mechanisms and biochemical pathways by which cells receive these signals and bring about the appropriate transcriptional changes are largely unknown. However, it is clear that the initiation of mRNA synthesis ultimately depends on protein factors that bind to regulatory elements in the promoter of the stimuli-responsive genes (for a review, see Mitchell and Tjian, 1989). Several of these DNA sequence-specific transcription factors have been identified, purified, and subsequently cloned; these are largely of mammalian origin (Johnson and McKnight, 1989). Recently, notable progress has been made toward understanding the molecular basis of transcriptional stimulation in plant systems. For several genes, nuclear proteins have been identified that bind to regulatory DNA sequences in the promoter regions of stimuli-responsive genes (Giuliano et al., 1988; Staiger et al., 1989; McKendree et al., 1990; Schindler and Cashmore, 1990). Further characterization of these factors has been accomplished by using the DNA binding properties of the protein as a means to screen expression libraries (Mitchell and Tjian, 1989).

We are studying the transcriptional regulation of the alcohol dehydrogenase (*adh*) genes in higher plants. Their transcription is markedly induced by oxygen deprivation in a tissue-type–specific and development-specific manner (Bailey-Serres et al., 1988). The Arabidopsis *adh* and the maize *adh1* genes have been well studied both genetically and biochemically. In both genes, transient expression studies have identified a complex array of positive regulatory elements in the upstream sequences, and, in maize *adh1*, enhancer-like elements able to confer anaerobic inducibility in either orientation have been found (Walker et al., 1987; Olive et al., 1990; McKendree and Ferl, 1992).

In vivo detection of protein–DNA interactions by dimethyl sulfate footprinting revealed analogy among many of the binding sites of both the Arabidopsis *adh* and maize *adh1* 5′ flanking regions (Ferl and Nick, 1987; Ferl and Laughner, 1989). One such motif, 5′-GTGG-3′, is present in three binding sites of the maize *adh1* promoter and in four sites of the Arabidopsis *adh* promoter. One of the Arabidopsis *adh* sites is enlarged into an inverted dyad repeat (5′-CCACGTGG-3′) known as the G-box. The G-box is a moderately conserved DNA element present in the promoters of a diverse array of genes, including the UV light–regulated chalcone synthase (*chs*) (Schulze-Lefert et al., 1989; Staiger et al., 1989) and the light-regulated ribulose-1,5-

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bisphosphate-carboxylase/oxygenase small subunit (*rbcS*) gene family (Giuliano et al., 1988). Near-palindromic G-box sequences are also found to be associated with the anaerobic response element of the maize *adh1* gene (Walker et al., 1987) and are important in the abscisic acid–regulated, early-methionine–labeled polypeptide (*em*) gene (Marcotte et al., 1989) and the light-regulated chlorophyll *a/b* binding (*cabE*) gene (Schindler and Cashmore, 1990). These data suggest that the G-box is a *cis*-acting element with a generalized function.

Nuclear proteins interacting with the G-box motif have been described previously (Giuliano et al., 1988; DeLisle and Ferl, 1990; McKendree et al., 1990). In vitro binding experiments with the G-box motif from the Arabidopsis *rbcS* gene led to the identification of a nucleus-localized binding protein from tomato and Arabidopsis, which has been designated G-box binding factor (GBF) (Giuliano et al., 1988). Similar experiments by Staiger et al. (1989) using the homologous element from the *chs* gene of snapdragon demonstrated the presence of a nuclear protein, designated CG1, with a binding specificity similar to GBF. However, whether GBF and CG1 are identical or related proteins is currently unknown.

Recently, several laboratories have isolated cDNA clones encoding a DNA binding protein specific for a particular G-box element or G-box–like element by screening cDNA expression libraries with the promoter element (Tabata et al., 1989; Guiltinan et al., 1990; Oeda et al., 1991; Weisshaar et al., 1991; Schindler et al., 1992a). The deduced amino acid sequences of these clones revealed that they possess the characteristic features of the basic leucine zipper (bZIP) class of *trans*-acting factors. Striking conservation in the basic domain, involved in DNA recognition, has been observed among the GBFs, suggesting that they have similar binding site specificity. Thus far, two of the cloned GBFs, TAF-1 and GBF1, have been directly implicated in regulating transcription, based on transient expression studies (Oeda et al., 1991; Schindler et al., 1992b).

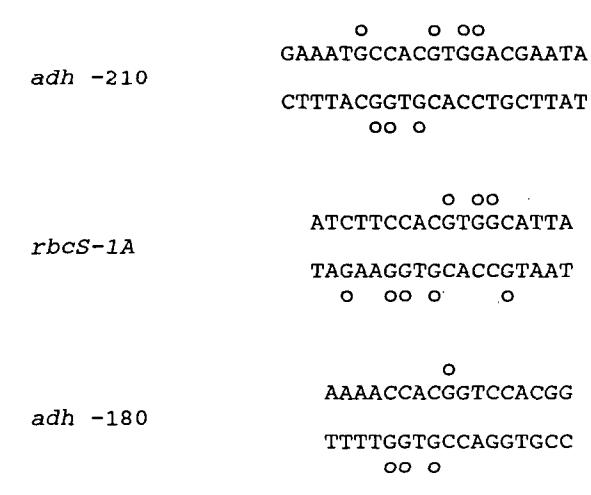

Figure 1. DNA Probes and Competitors.

Comparison of the Arabidopsis *adh* −210, Arabidopsis *rbcS-1A*, and maize *adh* −180 G-box motifs and their methylation patterns (Ferl and Nick, 1987; Ferl and Laughner, 1989; Schindler and Cashmore, 1990). DNA–protein interactions are designated by circles. The *adh* −210 oligonucleotide extends from position −203 to position −223 in the Arabidopsis *adh* promoter. The *rbcS-1A* oligonucleotide extends from position −227 to position −244 in the Arabidopsis *rbcS-1A* promoter. The *adh* −180 oligonucleotide extends from position −171 to position −187 in the maize *adh1* promoter.

We have taken a different approach to cloning the components of the G-box binding complex, that of generating monoclonal antibodies to the DNA binding complex. We have recently described a monoclonal antibody to GF14, a protein that is part of the G-box binding complex in Arabidopsis (Lu et al., 1992). In the present study, we demonstrate that anti-GF14 monoclonal antibodies cross-react with maize GBF complexes. We describe a near full-length cDNA clone and the structural features of maize GF14, and discuss its participation in the G-box binding complex as well as its homology to a class of protein kinase–dependent regulatory proteins found in mammalian brains.

RESULTS

G-Box Binding Activity Is Present in Maize Cell Suspension and Is Recognized by Anti-GF14

The 5′ flanking sequence of the maize *adh1* gene contains several near- and half-palindromic G-box elements, as shown in Figure 1 (Ferl and Nick, 1987; Ferl and Laughner, 1989), previously shown to have an in vivo footprint pattern similar to the Arabidopsis *adh* G-box (5′-CCACGTGG-3′) (McKendree et al., 1990). These data suggest that GBF also interacts with the maize *adh1* promoter. To investigate the interaction of proteins with maize G-box elements, we started with a palindromic type I G-box sequence, as described by Williams et al. (1992). This sequence is presented in Figure 1. Gel retardation assays (Fried and Crothers, 1981) were used to detect GBF activity in whole cell extracts. Initial experiments revealed that crude extracts exhibited several distinct shifted complexes of the G-box probe. The complexes were stable to competition by greater than 50 ng (100-fold) excess poly(dI-dC), but were obliterated in the presence of 50 ng of G-box competitor DNA (data not shown).

Fractionation of the maize crude cell extract by anion exchange chromatography on a fast protein liquid chromatography Mono Q column resulted in one peak of GBF activity eluting between 0.55 and 0.65 M KCl. The specificity of this G-box binding activity was determined by competition analysis, as shown in Figure 2A. Two retarded bands of slightly different mobility were detected (Figure 2A, lane 1). Both forms persisted in the presence of 1.0 μg of poly(dI-dC) (lane 2) or maize *adh1* −180 (lane 4), yet both were abolished in the presence of 50 ng of G-box competitor DNA (lane 3). These results support the binding specificity of the maize GBF for the G-box and suggest that the maize *adh1* −180 is unable to compete

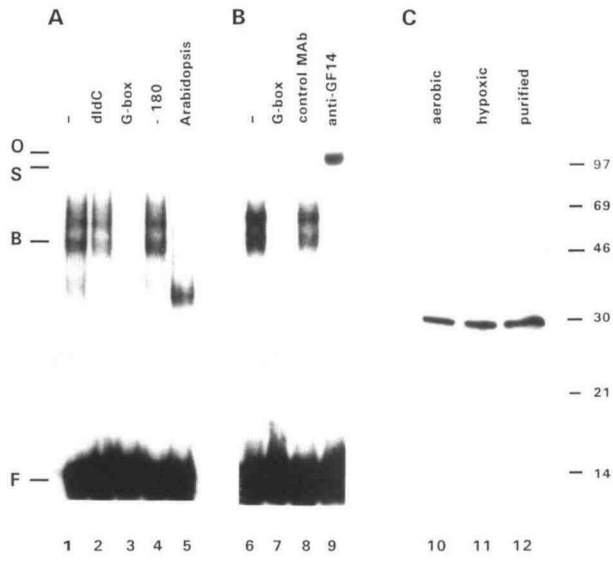

Figure 2. Maize Whole-Cell Extract Containing a Binding Factor Specific for the G-Box DNA Sequence That Is Recognized by Anti-GF14.

(A) Gel retardation assays of Mono Q–fractionated maize GBF. Binding reactions contained 50 ng of poly(dI-dC) (lane 1), 1 μg of poly(dI-dC) (lane 2), 50 ng of *rbcS* G-box (lane 3), or 50 ng of *adh* −180 (lane 4). The mobility of partially purified Arabidopsis GBF is shown in lane 5. O, S, B, and F denote the origin, antibody–protein–DNA complex, protein–DNA complex, and free DNA, respectively.

(B) Recognition of G-box–protein complexes by anti-GF14 IgG. Fractionated GBF extract was incubated with the G-box probe in the presence of 50 ng of poly(dI-dC) (lane 6), 50 ng of *rbcS* G-box (lane 7), nonspecific monoclonal antibody supernatant (lane 8), or anti-GF14 supernatant (lane 9).

(C) Immunodetection of the GF14 protein. Protein gel blot analyses of protein extracted from aerobically maintained cells (lane 10), hypoxically treated cells (lane 11), and Mono Q–fractionated GBF extract (lane 12) incubated with anti-GF14 monoclonal antibody supernatant. The migration of protein size markers is indicated to the right in kilodaltons.

with the dyad G-box for protein binding. Lane 5 of Figure 2A demonstrates that similar extracts from Arabidopsis form a faster migrating DNA–protein complex than do maize extracts.

To test whether anti-GF14 monoclonal antibodies (Lu et al., 1992) are able to interact with maize GBF complexes, gel retardation assays were performed in the presence of anti-GF14. Incubation of the protein–G-box complex with anti-GF14 monoclonal antibody supernatant shifted the complex to a slower mobility (Figure 2B, lane 9), whereas nonspecific monoclonal antibody supernatant had no effect (lane 8). It should be noted that fractionated whole-cell extract from both aerobically maintained and hypoxically induced cells were able to "supershift" upon incubation with anti-GF14 (data not shown). Protein blot analysis of aerobically maintained (lane 10) and hypoxically induced (lane 11) cell suspension crude extracts showed the presence of a single protein band of 30 kD upon incubation with anti-GF14 supernatant (Figure 2C). This protein, which we have designated GF14, was also detected in Mono Q–fractionated GBF complexes (lane 12).

If there is a functional interaction of GF14 and GBF in a DNA binding complex, GF14 should be detectable in nuclear extracts that are devoid of any contaminating cytoplasmic proteins. Nuclei were prepared from maize cell suspension by a Percoll gradient method (Green et al., 1987). Proteins from cytosolic and nuclear fractions, as shown in Figure 3A, were blotted and probed sequentially with different antibodies. Incubation with anti-GF14 antibodies showed that the 30-kD GF14 protein was found in both the cytosolic and nuclear fractions (Figure 3B). To confirm that the method produced highly purified nuclei, we subsequently used the same blot to probe with anti-high-mobility group (HMG) protein antiserum (detects a nuclear protein) and anti-sucrose synthase antiserum (detects a cytosolic protein). Anti-HMG antibodies recognized a 26-kD protein predominantly in the nuclear fraction (Figure 3C). The cytosolic fraction also contained a slight amount of HMGa,

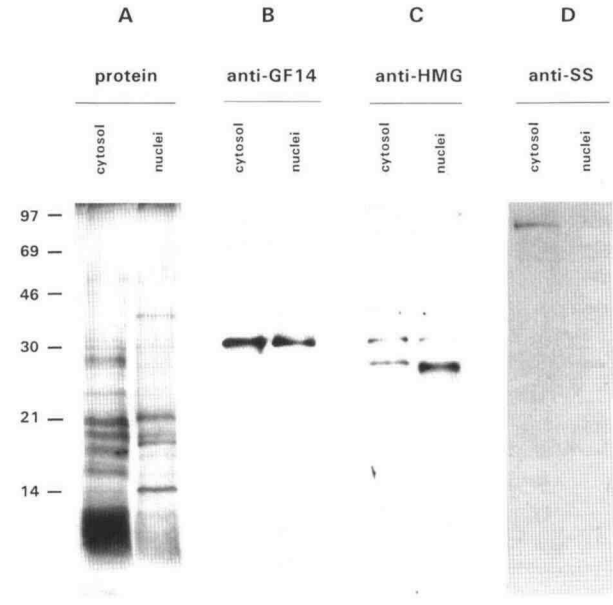

Figure 3. Subcellular Localization of GF14.

Proteins from the nuclear and cytosolic fractions were separated on SDS–15% polyacrylamide gels and visualized by silver staining for total protein and subjected to protein gel blot analyses.

(A) Total proteins subjected to gel blot analysis.

(B) Protein blot incubated with anti-GF14 monoclonal antibodies.

(C) The same protein blot subsequently incubated with anti-HMG antiserum.

(D) Incubation of the same blot with anti-sucrose synthase (anti-SS) antiserum and visualized using alkaline phosphatase–conjugated anti-rabbit IgG.

Both GF14 and HMGa proteins were detected in **(C)** because the same chemiluminescence detection method was used in **(B)** and **(C)**. Migration of protein mass markers is indicated to the left in kilodaltons.

which might be due to the possibility of nuclear rupture or the leakage of nuclear contents into the cytoplasm during isolation of the nuclei. (The 30-kD GF14 protein was also detected in the anti-HMG blot due to incomplete stripping of the membrane and usage of the same chemiluminescence immunodetection system for both blots.) Incubation of the same blot with anti-sucrose synthase antibodies recognized the 87-kD sucrose synthase protein only in the cytosolic fraction (Figure 3D). Thus, the substantial amount of GF14 observed in the nuclear fraction is not likely to be a cytosolic contaminant. The presence of GF14 in nuclei further substantiates the potential interaction of GF14 with GBF DNA binding complexes in maize.

These results suggest that GF14 is a physical part of the G-box binding complex. This conclusion is supported by the partial cofractionation of G-box binding activity and GF14, gel retardation experiments demonstrating interaction of GF14 and GBF, and the localization of both proteins to the nucleus. The presence of G-box binding activity in both hypoxically induced and aerobically maintained cells apparently precludes an obvious role for GF14 in the hypoxic induction of *adh*.

Coimmunoprecipitation of GF14 and GBF

To provide further evidence for the mode of interaction of GF14 with the G-box DNA element, we performed immunoprecipitation and subsequent protein blotting experiments on fractionated maize GBF complexes. Initial experiments using λgt11-expressed protein revealed that GF14 is unable to directly bind DNA (data not shown). However, we could not exclude the possibility that GF14 would require some modification before being an active DNA binding protein. Therefore, native maize GBF complexes were subjected to immunoprecipitation with anti-GF14 monoclonal antibodies. The immunoprecipitate was resolved on an SDS–polyacrylamide gel and blotted to nitrocellulose filters, as shown in Figure 4, lanes 2 and 5. As a control for these experiments, we also ran on the same gel nonimmunoprecipitated maize GBF complexes (lanes 1 and 4) and immunoprecipitated the anti-GF14 antibody itself (lanes 3 and 6).

Anti-GF14 antibody analyses in Figure 4 show that a 30-kD GF14 protein was present in the fractionated maize GBF complexes (lane 1) and was immunoprecipitated with anti-GF14 antibodies (lane 2). (Two additional proteins of 25 and 55 kD in Figure 4A, lanes 2 and 3, represent the light and heavy IgG chains recognized by the secondary antibody.) Lanes from the same gel were transferred to nitrocellulose filters and incubated with ^{32}P-labeled G-box DNA element, as shown in Figure 4B. The DNA–protein gel blot analysis demonstrated that two DNA binding proteins with molecular masses of approximately 46 kD reacted with the labeled G-box probe in both the fractionated (lane 4) and immunoprecipitated (lane 5) maize GBF complexes. It is possible that any DNA binding activity of GF14 was lost during SDS-PAGE and blotting. It appears more likely, however, that GF14 is not a DNA binding protein in and of itself,

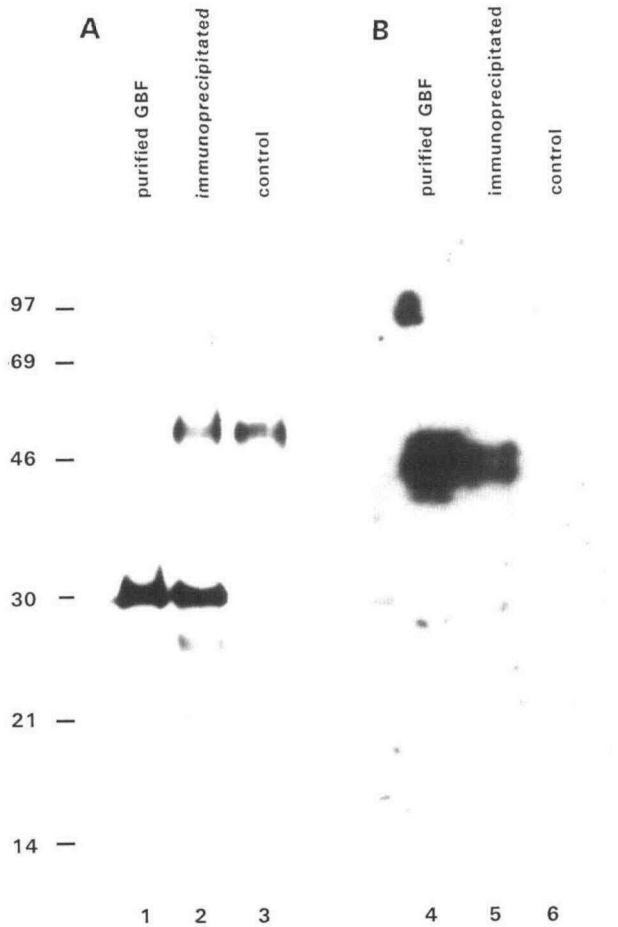

Figure 4. Anti-GF14 Coimmunoprecipitation of GBF.

(A) Protein gel blot analysis of immunoprecipitated GBF complexes. Lanes 1 and 2 contain 60 μL of fractionated maize GBF extract and immunoprecipitated GBF complexes, respectively. Lane 3 contains immunoprecipitated anti-GF14 monoclonal antibody. Blots were incubated with anti-GF14 supernatant. Migration of protein mass markers is indicated to the left in kilodaltons.

(B) DNA–protein gel blot analysis of immunoprecipitated GBF complexes. Blots of partially purified maize GBF extract (lane 4), immunoprecipitated maize GBF complexes (lane 5), and immunoprecipitated anti-GF14 (lane 6) were probed with the ^{32}P-labeled G-box oligonucleotide.

but rather is associated in the GBF complexes with G-box binding proteins of approximately 46 kD.

Isolation of cDNA Clones for Maize GF14

A cDNA expression library derived from the same maize cell suspension culture that showed a single reacting GF14 protein band was screened with anti-GF14 monoclonal antibody

supernatant. A total of 400,000 phages were screened, and six cDNA clones were isolated and subcloned into pUC18. The longest clone, which we designated pGF14-12, was chosen for further analysis. Preliminary analysis of the remaining clones indicated that four of the remaining clones represent the same sequence, and the fifth is very closely related.

Sequence analysis given in Figure 5 showed that pGF14-12 contains an insert of 1082 bp with an open reading frame that encodes 248 amino acids with a predicted molecular mass of 28.6 kD. The insert contains a 3′ untranslated region of 322 bp with a short poly(A) tract. The deduced protein sequence contains a motif resembling a leucine zipper, containing four leucine–isoleucine heptad repeats. However, this conserved repeat lacks the adjacent basic domain, characteristic of transcription factors of the bZIP family (Vinson et al., 1989).

Figure 6A shows the distribution of hydrophobic moment, charge, and predicted α-helix–forming regions of GF14 (Garnier et al., 1978; Eisenberg et al., 1984). Both the N terminus as

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G GAA AAT GTG TAC ATG GCG AAG CTC GCC GAG CAG GCA GAG AGG TAC GAG GAA ATG GTT    58
                  M  A  K  L  A  E  Q  A  E  R  Y  E  E  M  V                 (15)

GAG TTC ATG GAG AAG GTA GCG AAA ACT GTT GAC TCG GAG GAG CTC ACT GTG GAG GAG CGC   118
E  F  M  E  K  V  A  K  T  V  D  S  E  E  L  T  V  E  E  R                    (35)

AAC CTC CTG TCT GTT GCA TAC AAG AAC GTC ATT GGA GCC CGC CGT GCC TCA TGG CGC ATC   178
N  L  L  S  V  A  Y  K  N  V  I  G  A  R  R  A  S  W  R  I                    (55)

ATC TCC TCC ATC GAG CAG AAG GAG GAG GGT CGA GGC AAT GAG GAC CGT GTA ACA CTC ATC   238
I  S  S  I  E  Q  K  E  E  G  R  G  N  E  D  R  V  T  L  I                    (75)

AAG GAC TAC CGT GGC AAG ATT GAA ACT GAG CTC ACC AAG ATC TGT GAT GGC ATC CTC AAG   298
K  D  Y  R  G  K  I  E  T  E  L  T  K  I  C  D  G  I  L  K                    (95)

CTT CTC GAA TCT CAC CTT GTG CCG TCT TCC ACC GCT CCC GAG TCC AAG GTC TTC TAT CTC   358
I  L  E  S  H  L  V  P  S  S  T  A  P  E  S  K  V  F  Y  L                   (115)

AAG ATG AAG GGT GAC TAC TAC AGA TAC CTT GCT GAG TTC AAG ACT GGA GCT GAG AGA AAG   418
K  M  K  G  D  Y  Y  R  Y  L  A  E  F  K  T  G  A  E  R  K                   (135)

GAC GCT GCT GAG AAC ACC ATG GTG GCA TAC AAG GCT GCC CAG GAC ATT GCT TTG GCT GAG   478
D  A  A  E  N  T  M  V  A  Y  K  A  A  Q  D  I  A  L  A  E                   (155)

CTT GCT CCA ACT CAC CTT ATT AGG CTT GGA CTG GCA CTC AAC TTC TCA GTG TTC TAC TAT   538
L  A  P  T  H  P  I  R  L  G  L  A  L  N  F  S  V  F  Y  Y                   (175)

GAG ATT CTC AAC TCA CCC GAT CGC GCC TGC AGT CTT GCA AAG CAG GCT TTT GAC GAG GCC   598
E  I  L  N  S  P  D  R  A  C  S  L  A  K  Q  A  F  D  E  A                   (195)

ATC TCG GAA CTG GAC ACC CTG AGC GAG GAG TCC TAC AAG GAC AGC ACT TTG ATC ATG CAG   658
I  S  E  L  D  T  L  S  E  E  S  Y  K  D  S  T  L  I  M  Q                   (215)

CTC CTT CAT GAT AAC TTG ACC CTA TGG ACT TCT GAC ATC TCG GAG GAC CCT GCG GAA GAG   718
L  L  H  D  N  L  T  L  W  T  S  D  I  S  E  D  P  A  E  E                   (235)

ATC AGG GAG GCG CCC AAG CAC GAC TTG AGC GAG GGG CAG TAA AGC CGG CTT TAC ATG CCT   778
I  R  E  A  P  K  H  D  L  S  E  G  Q  .                                      (248)

AGA AGC TAG TAC CTA GTG TTT GCT GCT GTC TAA TGA CAT TAT GCA ATG TGA TCT GGA CCC   838

TGG TTT CTT GGG TGC GAC GCT TGT AGC AGC CGT CTT ATG ATT ATT TGT CGG GAG AAT TTG   898

GGG GCT CCC CAT ATG AGA TGC TGC CGG GCA AGA CCT CGC CAT CCC CCA CCT CGT TTC AGG   958

CGG GGG ACG CTG GAG CCT GGT CCG GTT GGG TTG GGA AGC CCC TTA AAC TGT TGT CGC TTG   1018

CAT TTT AAC CTT TTC ATC GCC GTT CAG TGG TCC TAA ATC TAT GGC TCG TCT CAG TGG TCT   1078

AAA A                                                                         1082
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Figure 5. Nucleotide Sequence of Clone pGF14-12 and Deduced Amino Acid Sequence of the Encoded Open Reading Frame.

Underlined amino acids indicate the putative amphipathic α-helix (Figure 6). The boxed amino acids correspond to potential protein kinase A and C phosphorylation sites. The circles indicate three isoleucine residues and one leucine residue potentially involved in protein–protein interactions. The GenBank accession number for the nucleotide sequence of maize GF14-12 is M96856.

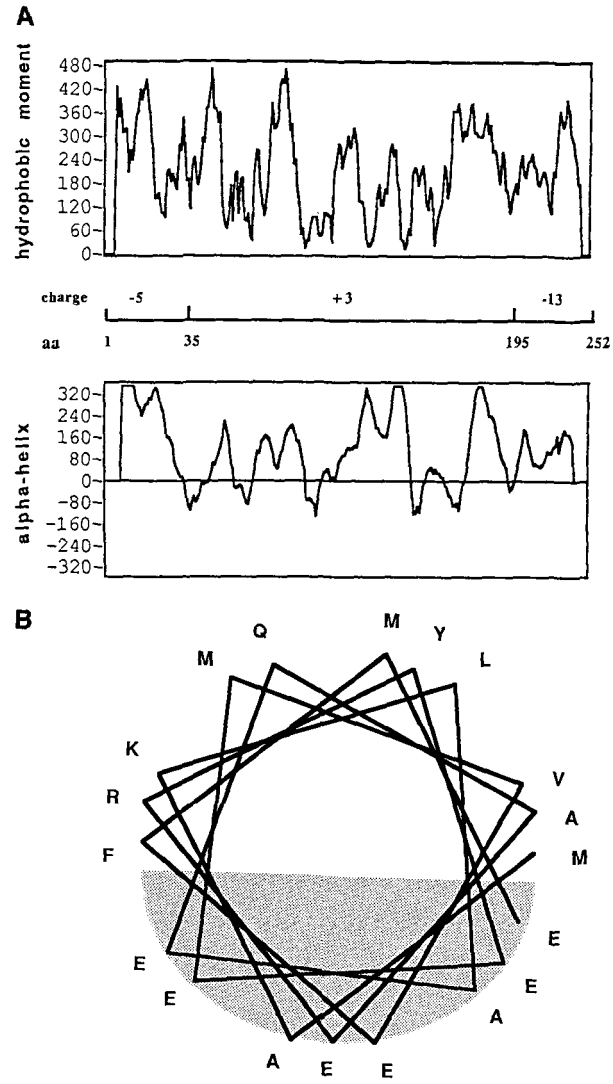

Figure 6. Predicted Secondary Structure of Maize GF14.

(A) Hydropathy plot, charge distribution, and α-helix structure prediction of GF14. aa, amino acid.

(B) Helical wheel representation of the acidic amphipathic α-helix indicated by the underlined N-terminal amino acids, as shown in Figure 5. The negatively charged face is shaded.

well as the C terminus have a strong negative charge, separated by a region of relatively neutral charge. Highly acidic regions are a feature common to various eukaryotic transcription activators, including VP16 (Cress and Triezenberg, 1991) and GAL4 (Giniger and Ptashne, 1987). A second characteristic of acidic activation domains is that they can form amphipathic α-helices based on secondary structure predictions. Indeed, the N-terminal region of GF14 can be modeled as an amphipathic helix with distinct negatively charged and hydrophobic faces (Figure 6B).

In addition, sequence analysis of GF14 predicts a cluster of protein kinase A and protein kinase C recognition sites in the N-terminal portion of the protein (Kennelly and Krebs, 1991). A similar motif appears to be important for the dimerization and the transcriptional efficacy of the cAMP-regulated nuclear factor CREB (Gonzalez et al., 1989).

The deduced protein sequence of maize pGF14-12 shares more than 80% homology with Arabidopsis GF14 (Lu et al., 1992) and Oenothera PHP-O (Hirsch et al., 1992), as shown in Figure 7. Furthermore, a computer search for related protein sequences revealed definite similarity between GF14 and a class of proteins isolated from mammalian brain described as protein kinase–dependent activators of tryptophan and tyrosine hydroxylases (14-3-3 proteins) and inhibitors of protein kinase C (KCIP), as shown in Figure 7 (Ichimura et al., 1988; Aitken et al., 1991).

Genomic Organization and Expression of GF14

DNA gel blots of restriction enzyme–digested genomic maize DNA isolated from the cell suspension were probed with

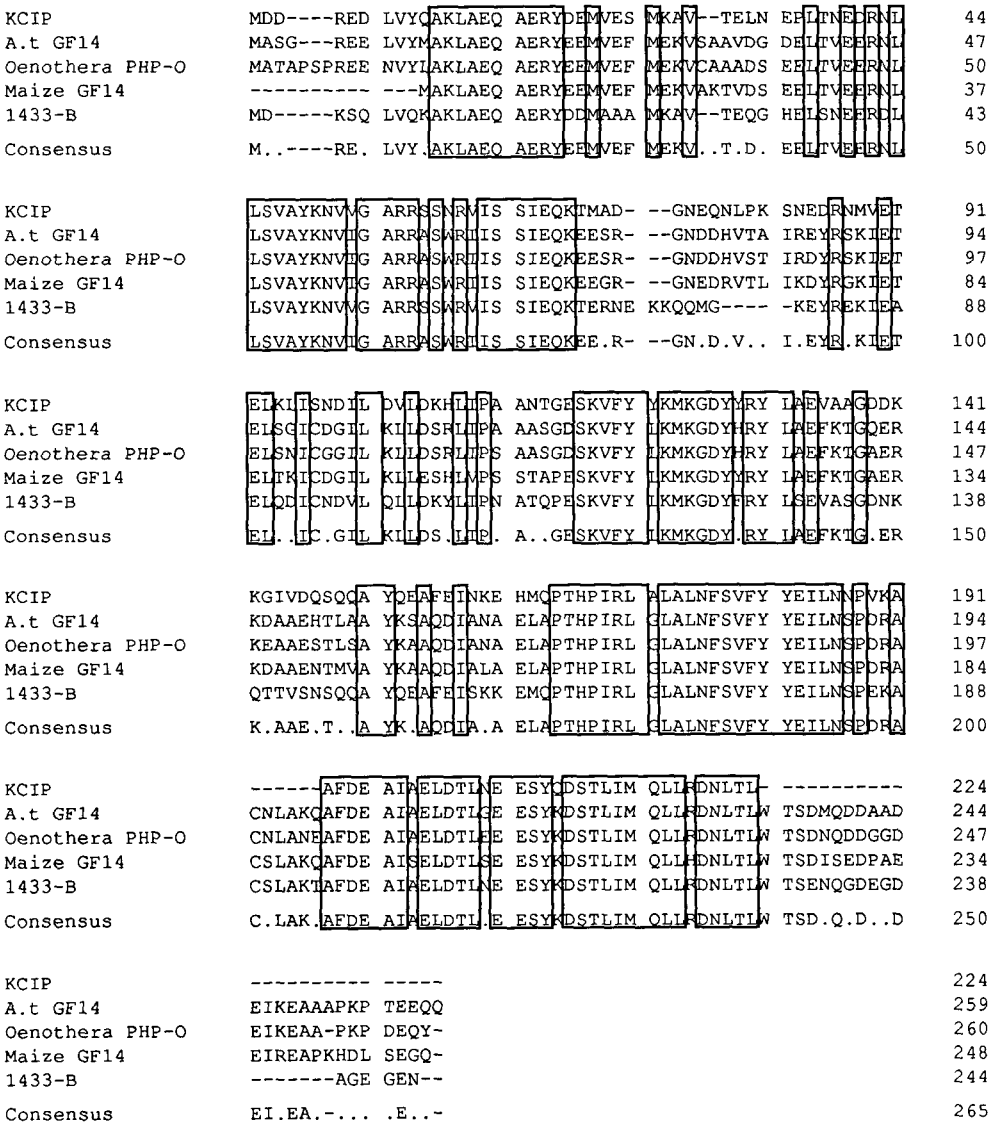

Figure 7. Sequence Comparison of Maize GF14.

Sequence alignment of the derived maize GF14 protein with Arabidopsis (A.t.) GF14 (Lu et al., 1992), Oenothera PHP-O (Hirsch et al., 1992), protein kinase C inhibitor protein (KCIP) (Aitken et al., 1991), and the 14-3-3 protein (β-chain) (Ichimura et al., 1988) in single-letter amino acid code. Residues identical to GF14 are boxed.

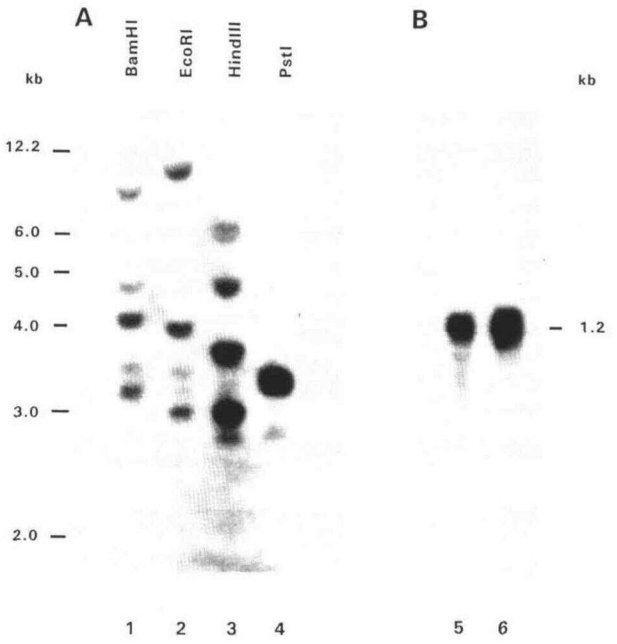

Figure 8. DNA and RNA Gel Blot Analyses of the Maize GF14-12 Gene.

(A) Genomic DNA of maize line P3377 cell suspension (10 μg) was digested with the indicated restriction enzymes. The membranes were hybridized at 65°C to the pGF14-12 cDNA insert. The position of molecular length markers are indicated at left.

(B) RNA gel blot analysis of the pGF14-12 transcript. Total RNA (10 μg, lane 5, or 20 μg, lane 6) was electrophoresed through agarose, transferred to a nylon membrane, and probed with a ^{32}P-labeled pGF14-12 cDNA insert. The RNA molecular length marker is indicated at right.

pGF14-12, as shown in Figure 8A. Hybridization at high stringency revealed several strongly hybridizing bands, demonstrating a very limited potential number of genes. In addition, the presence of weakly hybridizing bands suggests that there are related genomic sequences that may encode proteins related to GF14.

Figure 8B shows RNA gel blot analysis of total RNA extracted from a maize cell suspension. pGF14-12 hybridized to a single 1.2-kb RNA present in aerobically maintained cells. This is approximately 100 nucleotides longer than the pGF14-12 cDNA and indicates that the mature mRNA most likely contains additional 5′ sequences.

Expression of GF14 in Various Maize Organs

The tissue and developmental expression patterns of GF14 during maize development were analyzed by isolating total protein from various organs, as shown in Figure 9. Although the cell suspension culture used for the cDNA cloning and GBF activity studies contained a single major GF14 protein band, additional GF14 protein bands were noted in some intact maize organs. Anti-GF14 again detected the same, major protein of 30 kD in all tissues. An additional weaker band corresponding to a 26-kD protein was also detected in the tassels, silks, kernels, cobs, and roots of mature plants. In 7- to 8-day-old seedlings, GF14 is present at a level threefold higher in stems and roots than in leaves (Figure 9A, lanes 1 to 3). Figure 9B shows that GF14 in mature leaves decreased 10-fold to an almost undetectable level, suggesting some degree of developmental regulation of GF14 (lane 4). GF14 protein in stems and nodes remained fairly constant during development (compare Figure 9A, lane 2, and Figure 9B, lanes 2 and 3). Relatively high levels of GF14 protein were found in silk, tassel, kernel, and cob tissue. Interestingly, relative levels of GF14 in root tissue increased approximately fourfold from seedling to maturation. Roots and kernels (lanes 6 and 8), which have a similarly high level of GF14 protein, are organ types known to respond to anaerobic stress (Freeling and Bennett, 1985).

The presence of GF14 protein in many different organ types raises the question of whether or not GF14 is associated with GBF complexes in all these tissues. Gel retardation assays of protein extracted from cell culture and seedling leaves in the presence of anti-GF14 supernatant are shown in Figure 10. As was seen in previous experiments (Figure 2), cell culture protein extracts form DNA–protein complexes that were recognized by anti-GF14 antibodies; the complexes exhibited

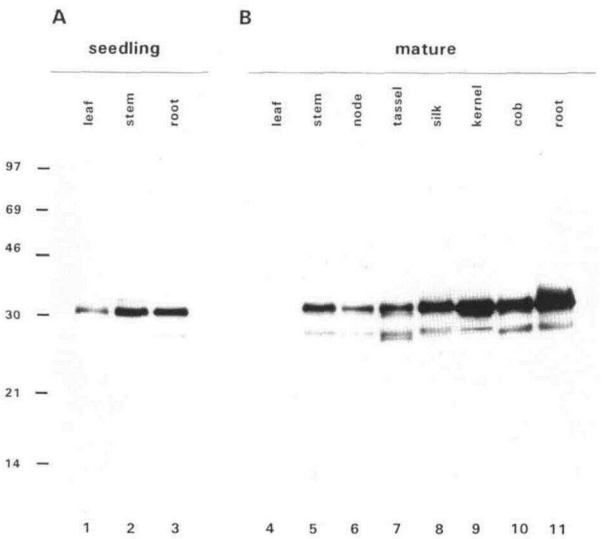

Figure 9. Tissue Specificity and Developmental Regulation of the GF14 Protein in Maize.

Total SDS-extracted protein from different organs was isolated from maize plants of various ages, fractionated on SDS–polyacrylamide gels, transferred to nitrocellulose filters, and incubated with anti-GF14 monoclonal antibody supernatant. Protein molecular mass markers are indicated in kilodaltons at left.

(A) Fifty micrograms of protein isolated from seedlings. Lane 1, leaf; lane 2, stem; lane 3, root.

(B) Fifty micrograms of protein extracted from mature maize plants. Lane 4, leaf; lane 5, stem; lane 6, node; lane 7, tassel; lane 8, silk; lane 9, kernel; lane 10, cob; lane 11, root.

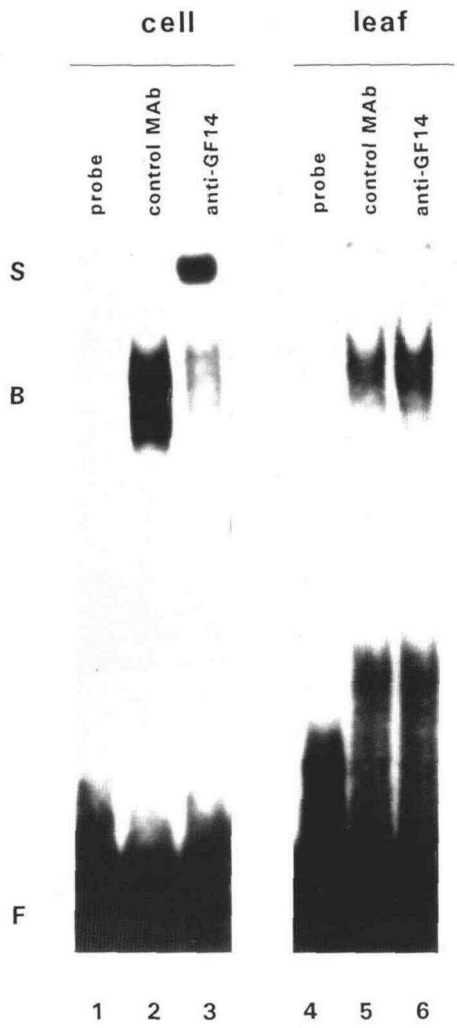

Figure 10. GF14 Association with the GBF Complexes in Cell Culture, but Not in Seedling Leaves.

Gel retardation assays of partially purified maize GBF from cell culture (lanes 2 and 3) and seedling leaves (lanes 5 and 6). Protein extract was incubated with the G-box probe (5000 cpm) and 1 μg of poly(dI-dC) in the presence of nonspecific antibody supernatant (lanes 2 and 5) or anti-GF14 supernatant (lanes 3 and 6). Lanes 1 and 4 contain the labeled G-box probe only. S, B, and F denote antibody–protein–DNA complex, protein–DNA complex, and free DNA, respectively.

a shift to a slower mobility (Figure 10, lane 2). The G-box binding activity from seedling leaf extract has a comparable migration rate relative to that observed in cell culture extract (Figure 10, lanes 1 and 3). However, the leaf-derived DNA–protein complex is not influenced by the anti-GF14 antibodies. Therefore, we concluded that the association of GF14 in the GBF complexes is tissue specific in that leaf GBF complexes apparently contain no GF14. Because GF14 was immunologically detected in seedling leaves (Figure 9A), the possibility remains that GF14 has additional functions other than its participation in DNA–protein complexes.

GF14 Is Conserved among a Variety of Plant Species

Previous experiments from this and other laboratories have shown that GBF activity or the related CG1 protein is present in a number of plant species including tomato, Arabidopsis, snapdragon, tobacco, petunia, soybean, and maize (Giuliano et al., 1988; Staiger et al., 1989; McKendree et al., 1990; this study). In addition, GBF activity was detected in nuclear extract prepared from yeast (Donald et al., 1990). This suggests that GBF is conserved over a wide evolutionary range and further suggests that GF14, as a part of this complex, might be detected in other species. This suggestion was also tendered based on the percent similarity noted between mammalian and plant 14-3-3 homologs (Hirsch et al., 1992).

To test this proposal, total protein was prepared from selected plants, electrophoretically separated, and immunoblotted. Incubation with anti-GF14 antibodies identified proteins of 30 kD in each of the tested plant species, as shown in Figure 11A, although the number of proteins detected was different among species (from three in Arabidopsis to one in cotton). Furthermore, a 30-kD protein from fern cross-reacted with anti-GF14, as did protein extracted from the lichen reindeer moss (Figure 11B). In yeast, a number of proteins varying in molecular weight were identified upon incubation with anti-GF14. These results

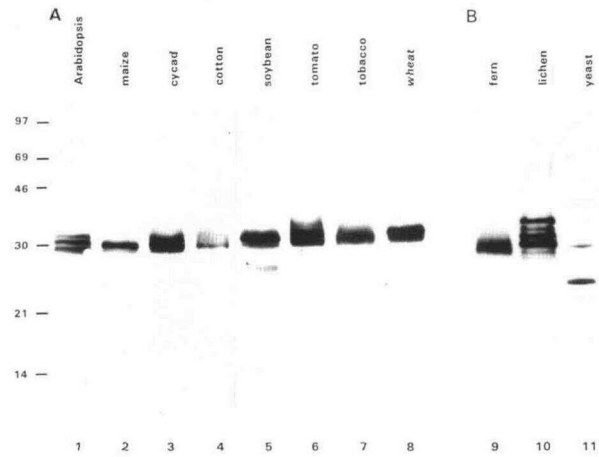

Figure 11. The Presence of GF14 Homologs in Different Plant Species, Lichen, and Yeast.

(A) Fifty micrograms of SDS-extractable protein from leaf tissue of Arabidopsis (lane 1), maize (lane 2), cycad (lane 3), cotton (lane 4), soybean (lane 5), tomato (lane 6), tobacco (lane 7), and wheat (lane 8) was electrophoresed and immunoblotted to nitrocellulose filters; GF14 homologs were detected after incubation with anti-GF14 IgG.
(B) Protein gel blot analyses of protein extracted from fern (lane 9), lichen (lane 10), and yeast (lane 11).
Molecular mass markers in kilodaltons are given at left.

correlate with the degree of amino acid similarity observed between plants and animals and strengthen the suggestion that GF14 is an evolutionarily conserved protein.

DISCUSSION

Maize GF14 Is Part of the G-Box-Protein Complex

In this report, we have shown that maize crude whole-cell extract contains a factor or factors that specifically interacts with the G-box DNA motif. A monoclonal antibody generated against the purified Arabidopsis GBF extract recognizes maize GBF complexes. This recognition is detected as a new complex of higher relative molecular mass in the gel retardation assay (Figure 2B) and by coimmunoprecipitation experiments (Figure 4). After SDS-PAGE and protein gel blotting, we observed a single immunoreactive product of 30 kD in the maize cell suspension line. A cDNA expression library prepared from the same cell suspension was screened to isolate a cDNA encoding the GF14 protein.

The monoclonal antibodies used in this study were selected specifically for their ability to interact with the GBF complexes (Lu et al., 1992). Therefore, it is the antibodies that constitute the functional probe of GBF complexes in extracts as well as the discriminating probe for screening cDNA expression libraries. The present results do not address the nature of the interaction between GF14 and the GBF–DNA complex. They only demonstrate the fact that in two separate assays involving the monoclonal antibodies, the gel retardation supershift and the coimmunoprecipitation assays, GF14 is physically associated with the DNA binding complex.

Even though the cDNA clone was derived from a cell suspension that appears to express only a single GF14 protein recognized by anti-GF14 antibodies, the results presented here may well apply to all members of the family of GF14-like proteins described in other systems. Until such time as nucleic acid and protein probes are available to discriminate among the members of the family, these and other results must be considered to represent activities of the entire family.

Structural Features and Functional Implications of GF14

Analysis of the deduced amino acid sequence of GF14 shows that this protein is distinct from previously isolated G-box binding factors that were cloned by direct DNA binding. It contains an isoleucine-leucine repeat, implicated in protein–protein interactions (O'Shea et al., 1989), but lacks a basic region, characteristic of the bZIP class of DNA binding proteins (Vinson et al., 1989). So far, at least five distinct cDNA clones encoding GBF or related factors have been isolated from different plant species, each encoding a bZIP-type of DNA binding protein (Tabata et al., 1989; Guiltinan et al., 1990; Oeda et al., 1991; Weisshaar et al., 1991; Schindler et al., 1992a). Amino acid comparisons among these isolated GBFs show a high degree of similarity (90–95%) only in the basic domain, which is involved in DNA recognition.

The lack of this region in GF14 suggests that this protein might not be a DNA binding protein in and of itself. This is substantiated by protein expression studies, showing that the GF14 protein produced in *Escherichia coli* or λgt11 fails to directly bind DNA (N.C. de Vetten, G. Lu, and R.J. Ferl, unpublished data), and protein–DNA gel blot analysis, demonstrating a substantial molecular weight difference between GF14 and bona fide DNA binding proteins within the complex (Figure 4). It remains possible, however, that GF14 is able to bind DNA only in the presence of a heterologous partner protein by means of its dimerization motif. In this regard, GF14 may be operationally similar to FOS (Chiu et al., 1988).

Alternatively, GF14 could be recruited to the complex through protein–protein interactions with the G-box–specific DNA binding protein. Several transcription factors have been described in mammals, in yeast, and recently in plants that bear an activating region and are unable to bind DNA directly. The most well studied of these include the viral protein components VP16 (Cress and Triezenberg, 1991), E1A (reviewed in Berk, 1986), and VP1 (McCarty et al., 1991). The mechanism by which these proteins interact with DNA binding proteins is thus far not well defined. Because there appear to be multiple GBFs, a clear assessment of the interactions within the G-box complex awaits the cloning of the particular maize G-box binding factor that immunoprecipitates with GF14.

GF14 is not an obligate member of the G-box binding complex. GBF activity is abundant in leaves, correlating with the presence of the G-box in light-regulated genes. However, leaf-derived GBF complexes appear to lack GF14, because anti-GF14 antibodies have no effect on the electrophoretic mobility of the complex (Figure 10).

An interesting feature of GF14 is the amphipathic α-helical N terminus (Figure 6B). Short acidic domains have been shown to be critical components of proteins involved in activation of transcription in yeast, mammals, and plants (Ma and Ptashne, 1987; Cress and Triezenberg, 1991; McCarty et al., 1991). Mutational analysis of the activating region I of GAL4 (Gill and Ptashne, 1987) and VP16 (Cress and Triezenberg, 1991) revealed a strong correlation between the relative transcriptional activity and the acidity of the region, although net charge was not the sole determinant for activation. To determine the potential function of GF14 as a *trans* activator, we are currently testing whether overexpression of GF14 will result in transcriptional activation of a reporter gene fused to the G-box promoter element.

The Wider Role of the GF14 in Plants?

The finding of homology between GF14 and a class of brain proteins regulating tyrosine and tryptophan hydroxylase (14-3-3 proteins) and KCIP (Figure 7) suggests that GF14 may be

more than a participant in G-box binding complexes. GF14, together with the 14-3-3 proteins and KCIP proteins of mammalian brain tissue, would appear to be multifunctional regulators. The 14-3-3 proteins, isolated from bovine brain tissue, were initially characterized as activators of tyrosine and tryptophan hydroxylase in the presence of Ca^{2+}/calmodulin-dependent protein kinase II (Ichimura et al., 1987, 1988). The activation of these hydroxylases leads to the synthesis of the neurotransmitters catecholamines and serotonin. However, a subsequent study of Aitken et al. (1991) demonstrated a strong homology (~70% identical residues) of the 14-3-3 proteins to an inhibitor of Ca^{2+}/phospholipid-dependent protein kinase C (PKC). These results raise the possibility that the activity of PKC and other kinase-dependent functions, such as DNA binding and hydroxylase activation, are regulated by members of the GF14/14-3-3 protein family.

Immunoassays using specific antiserum demonstrated the presence of 14-3-3 proteins in various vertebrate species (Ichimura et al., 1991). This observation is paralleled with the presence of GF14 protein in a wide variety of plant species and yeast (Figure 11) and indicates further evolutionary conservation of this protein family. The distribution of 14-3-3 protein in various bovine tissues was found to be closely related to the known distribution of PKC and Ca^{2+}-dependent protein kinase II, i.e., high levels in brain tissue and low levels in most other tissues (Ichimura et al., 1991). The presence of a plant protein with PKC activity has not yet been described, although PKC homologs with limited conservation to their mammalian counterparts have been isolated (Lawton et al., 1989). Therefore, the potential roles of GF14 relative to phosphorylation and signal transduction in plants remain to be elucidated. The great similarity between GF14 and 14-3-3/KCIP, however, indicates strong conservation of the structure and highlights the likely central importance of these proteins in both plants and mammals. All evidence gathered thus far suggests an important function in the regulation of kinase-mediated signal transduction pathways.

We have demonstrated here the cloning of a maize protein associated with the GBF complexes using a monoclonal antibody strategy. The cloned factor GF14 has some characteristics of a transcriptional activator. However, its homology with a class of proteins able to modulate kinase-dependent activities suggests a regulatory role for GF14 in the DNA binding complex.

METHODS

Isolation of GF14 Recombinant Phage

A random-primed cDNA library (Clontech, Palo Alto, CA) was constructed in λgt11 vectors using poly(A)RNA prepared from maize cell suspension culture (*Zea mays* line P3377). The amplified library was screened with anti-GF14 monoclonal cell supernatant (Lu et al., 1992) essentially according to Sambrook et al. (1989). Approximately 400,000 recombinant phage were grown on *Escherichia coli* Y1090 at a density of 50,000 per 150-mm-diameter Petri plate, and the expressed fusion proteins were immobilized onto isopropyl β-D-thiogalactopyranoside-impregnated nitrocellulose filters. The filters were washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and blocked overnight with 10% nonfat dry milk suspended in TBST. After incubation with the primary antibody cell supernatant, filters were washed and incubated with 1:8000 alkaline phosphatase–conjugated anti–mouse IgG for 1 hr. Filters were washed, and this was followed by BCIP/NBT color development according to the procedure supplied by GIBCO–Bethesda Research Laboratories. Positive phage were plaque purified to homogeneity.

Sequence Analysis

cDNA inserts of the λ clones were subcloned into pUC18. Both strands of the cDNA clone were sequenced from plasmid DNA by automated dideoxy chain termination on an ABI 373 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols.

Nucleotide and amino acid sequence analysis was performed using either the MacMolly or SeqEd program (Applied Biosystems). The data base search was done using the FASTA program (Pearson and Lipman, 1988). Amino acid alignments were obtained using GeneWorks (IntelliGenetics, Mountain View, CA).

Gel Retardation Assays

Gel retardation assays were performed according to McKendree et al. (1990). The standard assay mixture consisted of fast protein liquid chromatography, Mono Q–fractionated whole-cell protein extract (Mono Q column; Pharmacia) (DeLisle and Ferl, 1990; McKendree et al., 1990), 2 μg tRNA, competitor DNA at the concentration indicated in the legend of Figure 2, and 2 μL of monoclonal antibody cell supernatant. After incubation for 1 hr at 4°C, 0.5 ng of end-labeled alcohol dehydrogenase (*adh*) G-box oligonucleotide was added, and the samples were further incubated for 10 min at 4°C before electrophoresis.

Immunoprecipitation

Fractionated maize GBF complexes (60 μL) were incubated with 1 μL of anti-GF14 monoclonal antibody ascites for 2 hr on ice. After incubation for 30 min with 15 μL (10% wet w/v) *Staphylococcus aureus* crude cell suspension (protein A), the antigen–antibody–protein A complex was centrifuged for 5 min. The pellet was washed with phosphate-buffered saline and centrifuged; the precipitates were analyzed by protein and DNA–protein blotting.

Genomic DNA and RNA Gel Blot Analyses

Total RNA from maize suspension cells was prepared according to McCarty (1986), separated on formaldehyde-agarose gels, and capillary blotted onto GeneScreen (Du Pont–New England Nuclear). The filters were hybridized to a random prime-labeled pGF14-12 (1.1 kb) and washed as described by Ferl et al. (1987).

High molecular weight DNA was prepared from cells and recovered from a $CsCl_2$ gradient (Paul and Ferl, 1991). DNA (10 μg) was restricted, subjected to electrophoresis on 0.8% agarose gels, and transferred to a GeneScreen membrane.

Protein Gel Blot Analysis

Plant tissue (2 to 5 g) was homogenized in 2 × SDS-PAGE loading buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 0.7 M β-mercaptoethanol, 20% glycerol), after which samples were boiled for 5 min and insoluble debris were removed by centrifugation at 11,000*g*. Proteins of an aliquot of the supernatant were precipitated with five volumes of acetone (−20°C) and resuspended in 100 mM NaOH. Protein concentration of these aliquots was determined by the method of Bradford (1976), using BSA as a standard. Fifty micrograms of protein was separated on a 15% SDS–polyacrylamide gel, and proteins were transferred to nitrocellulose filters (Schleicher & Schuell), according to Towbin et al. (1979). Filters were blocked overnight in 10% nonfat dry milk, incubated for 1 hr in anti-GF14 monoclonal antibody cell supernatant, washed, and incubated for an additional 1 hr with 1:2000 dilution of sheep anti–mouse horseradish peroxidase–conjugated IgG (Amersham Corp.). Immunodetection was performed with an ECL Western Blotting Detection reagent (Amersham Corp.). Autoradiograms were scanned in a computing densitometer (Molecular Dynamics, Mountain View, CA).

DNA-Protein Gel Blot Analyses

Fractionated maize GBF extract (60 μL) or immunoprecipitated extract was separated on a 15% SDS–polyacrylamide gel and transferred to a nitrocellulose filter as described above. DNA–protein gel blot analyses were conducted essentially as described by Miskimins et al. (1985). The filters were blocked in a solution containing 10% (w/v) nonfat dry milk in 10 mM Hepes, pH 8.0, overnight at 4°C. The filters were subsequently incubated for 2 hr at room temperature in binding buffer (10 mM Hepes, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.25% nonfat dry milk), containing 10^6 cpm of ligated ^{32}P-labeled G-box oligonucleotide per mL. The filters were washed in three changes of binding buffer containing 100 mM NaCl over a period of 1 hr and then exposed to x-ray film.

Isolation of Nuclei

Nuclei from maize cell suspension were prepared by the method of Luthe and Quatrano (1980). The nuclear suspension was more extensively purified by centrifugation in a discontinuous 30%/60% Percoll/85% sucrose gradient, as described by Green et al. (1987). The cytosolic fraction was obtained by centrifuging the postnuclei extract for 1 hr at 150,000*g*. Proteins from the nuclear and cytosolic fractions were prepared for SDS-PAGE, and protein gel blot analyses were conducted as described previously.

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REFERENCES

Aitken, A., Ellis, C.A., Sellers, L.A., and Toker, A. (1991). Kinase and neurotransmitters. Nature **344,** 594.

Bailey-Serres, J., Kloeckner-Gruissem, B., and Freeling, M. (1988). Genetic and molecular approaches to the study of the anaerobic response and tissue specific gene expression in maize. Plant Cell Environ. **11,** 351–357.

Berk, A.J. (1986). Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20,** 45–79.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Cherry, J.H. (ed) (1989). Environmental Stress in Plants (Berlin: Springer-Verlag).

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-FOS protein interacts with c-JUN/AP-1 to stimulate transcription of AP-1 responsive genes. Cell **54,** 541–552.

Cress, W.D., and Triezenberg, S.J. (1991). Critical structural elements of the VP16 transcriptional activation domain. Science **251,** 87–90.

DeLisle, A.J., and Ferl, R.J. (1990). Characterization of the *Arabidopsis Adh* G-box binding factor. Plant Cell **2,** 547–557.

Donald, R.G.K., Schindler, U., Batschauer, A., and Cashmore, A.R. (1990). The plant G box promoter sequence activates transcription in *Saccharomyces cerevisiae* and is bound *in vitro* by a yeast activity similar to GBF, the plant G box binding factor. EMBO J. **9,** 1727–1735.

Eisenberg, D., Weiss, R., and Terwilliger, T. (1984). The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. USA **81,** 140–144.

Ferl, R.J., and Laughner, B.H. (1989). *In vivo* detection of regulatory factor binding sites of *Arabidopsis thaliana Adh.* Plant Mol. Biol. **12,** 357–366.

Ferl, R.J., and Nick, H.S. (1987). *In vivo* detection of regulatory factor binding sites in the 5′ flanking region of the maize *Adh1.* J. Biol. Chem. **262,** 7947–7950.

Ferl, R.J., Nick, H.S., and Laughner, B.H. (1987). Architecture of a plant promoter: S1 nuclease hypersensitive features of maize *Adh1.* Plant Mol. Biol. **8,** 299–307.

Freeling, M., and Bennett, D. (1985). Maize *Adh1.* Annu. Rev. Genet. **19,** 297–323.

Fried, M., and Crothers, D.M. (1981). Equilibria and kinetics of lac repressor–operator interaction by polyacrylamide gel electrophoresis. Nucl. Acids Res. **23,** 6505–6525.

Garnier, J., Osguthorpe, D., and Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the

secondary structure of globular proteins. A simple model. J. Mol. Biol. **120,** 97–120.

Gill, G., and Ptashne, M. (1987). Mutants of GAL4 protein altered in an activating function. Cell **51,** 121–126.

Giniger, E., and Ptashne, M. (1987). Transcription in yeast activated by a putative amphipathic α-helix linked to a DNA binding unit. Nature **330,** 670–672.

Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P.A., and Cashmore, A.R. (1988). An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc. Natl. Acad. Sci. USA **85,** 7089–7093.

Gonzalez, G.A., Yamamato, K.K., Fischer, W.H., Karr, D., Menzel, P., Biggs, W., III, Vale, W.W., and Montminy, M.R. (1989). A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature **337,** 749–752.

Green, P.J., Kay, S.K., and Chua, N.-H. (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rcbS*-3A gene. EMBO J. **6,** 2543–2549.

Guiltinan, M.J., Marcotte, W.M., and Quatrano, R.S. (1990). A leucine zipper protein that recognizes an abscisic acid response element. Science **250,** 267–271.

Hirsch, S., Aitken, A., Bertsch, U., and Soll, J. (1992). A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor. FEBS Lett. **296,** 222–224.

Ichimura, T., Isobe, T., Okuyama, T., Yamauchi, T., and Fujisawa, H. (1987). Brain 14-3-3 protein is an activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca^{2+}, calmodulin-dependent protein kinase II. FEBS Lett. **219,** 79–82.

Ichimura, T., Isobe, T., Okuyama, T., Takahashi, N., Araki, K., Kuwano, R., and Takahashi, Y. (1988). Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein-kinase–dependent activator of tyrosine and tryptophan hydroxylases. Proc. Natl. Acad. Sci. USA **85,** 7084–7088.

Ichimura, T., Sugano, H., Kuwano, R., Sunaya, T., Okuyama, T., and Isobe, T. (1991). Widespread distribution of the 14-3-3 protein in vertebrate brains and bovine tissues: Correlation with the distributions of calcium-dependent protein kinases. J. Neurochem. **56,** 1449–1451.

Johnson, P.F., and McKnight, S.L. (1989). Eukaryotic transcriptional regulation regulatory proteins. Annu. Rev. Biochem. **58,** 799–839.

Kennelly, P.J., and Krebs, E.G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. **266,** 15555–15558.

Lawton, M.A., Yamamoto, R.T., Hanks, S.K., and Lamb, C.J. (1989). Molecular cloning of plant transcripts encoding protein kinase homologs. Proc. Natl. Acad. Sci. USA **86,** 3140–3144.

Lu, G., DeLisle, A.J., de Vetten, N.C., and Ferl, R.J. (1992). An *Arabidopsis* homolog to neurotransmitter pathway activators is part of a DNA binding complex. Proc. Natl. Acad. Sci. USA (in press).

Luthe, D.S., and Quatrano, R.S. (1980). Transcription in isolated wheat nuclei. I. Isolation of nuclei and elimination of endogenous ribonuclease activity. Plant Physiol. **65,** 305–308.

Ma, J., and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48,** 847–853.

Marcotte, W.R., Jr., Russell, S.H., and Quatrano, R.S. (1989). Abscisic acid–responsive sequences from the Em gene of wheat. Plant Cell **1,** 969–976.

McCarty, D.R. (1986). A simple method for extraction of RNA from maize tissues. Maize Genet. Coop. Newslett. **60,** 61.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., and Vasil, I.K. (1991). The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell **66,** 895–905.

McKendree, W.L., Paul, A.-L., DeLisle, A.J., and Ferl, R.J. (1990). In vivo and in vitro characterization of protein interactions with the dyad G-box of the *Arabidopsis Adh* gene. Plant Cell **2,** 207–214.

McKendree, W.L., and Ferl, R.J. (1992). Functional elements of the *Arabidopsis Adh* promoter include the G-box. Plant Mol. Biol. **19,** 859–862.

Miskimins, W.K., Roberts, M.P., McClelland, A., and Ruddle, F.H. (1985). Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the protein region of the transferrin receptor gene. Proc. Natl. Acad. Sci. USA **82,** 6741–6744.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245,** 371–378.

Oeda, K., Salinas, J., and Chua, N.-H. (1991). A tobacco bZip transcription activator (TAF-1) binds to a G-box–like motif conserved in plant genes. EMBO J. **10,** 1793–1802.

Olive, M.R., Walker, J.C., Singh, K., Dennis, E.S., and Peacock, W.J. (1990). Functional properties of the anaerobic response element of the maize *Adh1* gene. Plant Mol. Biol. **15,** 593–604.

O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. Science **243,** 538–542.

Paul, A.-L., and Ferl, R.J. (1991). In vivo footprinting reveals unique *cis*-elements and different modes of hypoxic induction in maize *Adh1* and *Adh2*. Plant Cell **3,** 159–168.

Pearson, W.R., and Lipman, D.J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85,** 2444–2448.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schindler, U., and Cashmore, A.R. (1990). Photoregulated gene expression may involve ubiquitous DNA binding proteins. EMBO J. **11,** 3415–3427.

Schindler, U., Menkens, A.E., Beckmann, H., Ecker, J.R., and Cashmore, A.R. (1992a). Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis thaliana* GBF bZIP proteins. EMBO J. **4,** 1261–1273.

Schindler, U., Terzaghi, W., Beckmann, H., Kadesch, T., and Cashmore, A.R. (1992b). DNA binding site preference and transcription activation properties of the *Arabidopsis* transcription factor GBF1. EMBO J. **11,** 1275–1289.

Schulze-Lefert, P., Dangl, J.L., Becker-Andre, M., Hahlbrock, K., and Schulz, W. (1989). Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. EMBO J. **8,** 651–656.

Staiger, D., Kaulen, H., and Schell, J. (1989). A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an evolutionarily conserved nuclear protein. Proc. Natl. Acad. Sci. USA **86,** 6930–6934.

Tabata, T., Takase, H., Takayama, S., Mikami, K., Nakatsuka, A., Kawata, T., Nakayama, T., and Iwabuchi, M. (1989). A protein that

binds to a *cis*-acting element of wheat histone genes has a leucine zipper motif. Science **245,** 965–967.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

Vinson, C.R., Sigler, P.B., and McKnight, S.L. (1989). Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science **246,** 911–916.

Walker, J., Howard, E., Dennis, E., and Peacock, W. (1987). DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. Proc. Acad. Natl. Sci. USA **84,** 6624–6628.

Weisshaar, B., Armstrong, G.A., Block, A., Da Costa e Silva, O., and Hahlbrock, K. (1991). Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J. **10,** 1777–1786.

Williams, M.E., Foster, R., and Chua, N.-H. (1992). Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell **4,** 485–496.