*Proc. Natl. Acad. Sci. USA*
Vol. 89, pp. 11490–11494, December 1992
Plant Biology

# Brain proteins in plants: An *Arabidopsis* homolog to neurotransmitter pathway activators is part of a DNA binding complex

(alcohol dehydrogenase/G box/14-3-3 protein/transcription factor)

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*Communicated by C. S. Levings III, August 13, 1992 (received for review March 9, 1992)*

ABSTRACT **The G box is a well-characterized cis-acting DNA regulatory element found in the promoters of several seemingly unrelated plant genes, including the alcohol dehydrogenase (*Adh*) gene of *Arabidopsis thaliana*. Using a monoclonal antibody screening approach coupled with electrophoretic mobility shift assays, we have isolated a cDNA clone encoding a protein that is part of the *in vitro* protein/G box complex. The derived amino acid sequence is homologous to a class of proteins in mammalian brains described as protein kinase C inhibitors and as activators of tyrosine and tryptophan hydroxylases, the rate-limiting enzymes in the pathways leading to the catecholamines and serotonin. The fact that a homologous member of this regulatory protein family is found in plants and is associated with binding to transcriptional regulatory elements suggests a much wider role for these proteins.**

The expression of alcohol dehydrogenase (*Adh*) genes in plants is organ-specific and environmentally inducible by hypoxic conditions (1–3). Transient expression studies (3, 4), *in vivo* footprinting (5, 6), and *in vitro* DNA binding studies (5, 7) have identified several cis regulatory elements within the *Arabidopsis Adh* promoter, among them the G box (5′-CCACGTGG-3′).

The G box motif is moderately conserved in *Adh* genes from *Arabidopsis* (6) and maize (8) and is also found in the promoters of several diverse plant genes, such as the ribulose-1,5-bisphosphate carboxylase small subunit (*RbcS*) genes of several plants (9), the parsley chalcone synthase gene (10), and the wheat early-methionine-labeled polypeptide (Em) gene (11). Although the *Adh*, *RbcS*, chalcone synthase, and Em genes are all cell-type-specific and inducible, the tissues in which these genes are expressed, their modes of induction, and their protein functions are distinct. For example, *Adh* is expressed in non-green tissues, induced by anaerobiosis, and repressed in leaves (1–3), whereas *Rbcs* is only expressed in green tissues and induced by light (9). The Em gene is induced by abscisic acid (11), whereas *Adh* can be induced by 2,4-dichlorophenoxyacetic acid (3, 6).

Various versions of the G box are also found in animal gene promoters, such as the sequence within the simian virus 40 major late promoter (12). G box binding factors (GBFs) are widely distributed in various organs of plants (5, 9) and are also found in yeast (13). These facts imply that the G box is a general cis-acting element that is involved in the regulation of many types of genes. Further characterization of the G box binding complexes is necessary to determine whether multiple GBFs are present in different tissues and if different factor complexes are involved in specific expression of various genes.

Using a monoclonal antibody screening approach (14), we have isolated a cDNA clone encoding a protein that is apparently part of the protein/G box complex.§ The protein is unlike any G box factors cloned by direct binding of G box oligonucleotides. Interestingly, the amino acid sequence is homologous to a family of mammalian brain proteins involved in regulating protein kinase C activity and neurotransmitter biosynthesis.

## MATERIALS AND METHODS

**Preparation of G Box Binding Activity (GBF) and Production of Monoclonal Antibodies.** GBF was partially purified from whole-cell extract (5) of *Arabidopsis thaliana* suspension cells by minor modifications of previous protocols (7). Monoclonal anti-GBF hybridoma cell supernatants were produced according to standard methods (15). Hybridoma cell supernatants were individually screened to identify supernatants with the ability to alter the mobility of the protein/G box complex in a modification of the electrophoretic mobility shift assay (see below).

**Recovery of a cDNA Clone to Part of the DNA Binding Complex.** One of the supernatants that tested positive in the electrophoretic mobility shift assay was used to screen $2 \times 10^6$ bacteriophage plaques of a λgt11 library commercially prepared (Clontech) from an *Arabidopsis* suspension cell culture (16). This cell culture constitutively expressed *Adh* (6). Positive clones were plaque purified and the *Eco*RI inserts were amplified by PCR using λgt11 primers and subcloned into pUC18. The longest cDNA clone, pLU14, was sequenced on both strands by automated dideoxy methods on an Applied Biosystems Incorporated (ABI) 373 and a DuPont Genesis 2000 according to manufacturers' protocols.

**Expression in *Escherichia coli*.** The *E. coli* expression plasmid was constructed by moving the insert from pLU14 into *Eco*RI-digested pETH3C (17). The subsequent recombinant protein contains an additional 21 amino acids contributed by the vector plus 22 amino acids encoded by what was previously the untranslated 5′ leader sequence of the cDNA clone.

**Electrophoretic Mobility Shift Assays.** The binding reactions and mobility shift assay were essentially as described (5, 14) and consisted of partially purified *Arabidopsis* cell

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Abbreviations: 14-3-3 protein, activator of tyrosine and tryptophan hydroxylases; *Adh*, alcohol dehydrogenase; GBF, G box binding factor; KCIP, kinase C inhibitor protein; BSA, bovine serum albumin.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96855).

extracts incubated for 1.5 hr on ice with 2 μl of hybridoma cell supernatant or control supernatants as indicated in the legends of Figs. 1 and 4. After addition of 1 μg of poly(dI-dC), 2 μg of tRNA, and 2 pmol of $^{32}$P-labeled G box oligonucleotide (7), the samples were further incubated for 10 min at room temperature before electrophoresis. When used, unlabeled G box or mutant G box oligonucleotides [−310 (4)] were added as competitor.

**DNA, RNA, and Protein Blot Analyses.** Genomic DNA was prepared from the *Arabidopsis* suspension cells as described (8) and was digested with the indicated restriction enzymes, subjected to 1% agarose gel electrophoresis, and transferred to GeneScreen (NEN/Dupont) (16, 18). Total RNA from *Arabidopsis* suspension cells or plants was prepared (2) and electrophoresed in a 2.2 M formamide/1.5% agarose gel. Samples were blotted onto GeneScreen (16). The blots were hybridized with $^{32}$P-labeled pLU14 insert and washed at a high stringency that is capable of differentiating maize *Adh1* from *Adh2* (2). Bands were visualized by autoradiography.

Total protein was extracted using SDS sample buffer (19). Cell extracts were prepared as described (5, 7). Three micrograms of *E. coli* expressed fusion protein and 50 μg of cell extract or 5 μg of partially purified GBF complex or 50 μg of total proteins from different seedling parts were separated by 12.5% SDS/PAGE (19) and electrotransferred to nitrocellulose membrane (S&S NC, Schleicher & Schuell). The blots were probed with the hybridoma supernatant and the cross-reactions were visualized by ECL Western blotting detection reagents (Amersham).

**Southwestern Blot Assay.** Nuclei were isolated as described (20). After washing the nuclei three times with extract buffer (15 mM Hepes, pH 7.6/110 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride), the nuclear proteins were prepared (5). Fifty micrograms of nuclear protein was incubated with 5 μl of anti-GF14 ascitic fluid in NEBD buffer (7) without 2-mercaptoethanol for 4 hr on ice. The immunoprecipitin (BRL) was then added, and the incubation was continued for another 3 hr. After washing the immunoprecipitated pellet two times with NEBD buffer, 1× SDS buffer was added for loading on 12.5% SDS/PAGE gels. The Southwestern blot assay was done as described (21). The same blot was then used for the Western analysis.

## RESULTS

**Recovery of a Monoclonal Antibody to a Part of the Protein/G Box Complex.** A panel of monoclonal antibodies was prepared against partially purified GBF to recover antibodies to the DNA binding proteins as well as any other proteins associated with the protein/G box complex. As an initial screen, all hybridoma cell supernatants were tested in an adaptation of the electrophoretic mobility shift assay (14, 22, 23). In the assay, partially purified GBF was used to form a protein/G box complex (Fig. 1, lane 4) and incubation of the complex with a positively reacting supernatant caused a shift of this complex to a slower electrophoretic mobility (Fig. 1, lane 11).

The phenomenon of antibody shifting of protein/DNA complexes (14, 23) is referred to as "supershifting." Control incubations with the medium used to grow the hybridoma cells (Fig. 1, lane 9) and antibody supernatant from an unrelated experiment (Fig. 1, lane 10) failed to demonstrate any supershifting ability. The anti-GF14 supernatant had no G box binding activity (Fig. 1, lane 3). Furthermore, the bound G box complex (B) and slower mobility complex (S) were competitively inhibited by unlabeled G box (Fig. 1, lanes 7 and 12) but not by the mutant (Fig. 1, lanes 8 and 13). Four hybridoma cell lines testing positive in the supershift assay were recovered out of several hundred tested. All four antibody supernatants recognized a small family of proteins of ≈30 kDa in a protein blot analysis (data not shown).

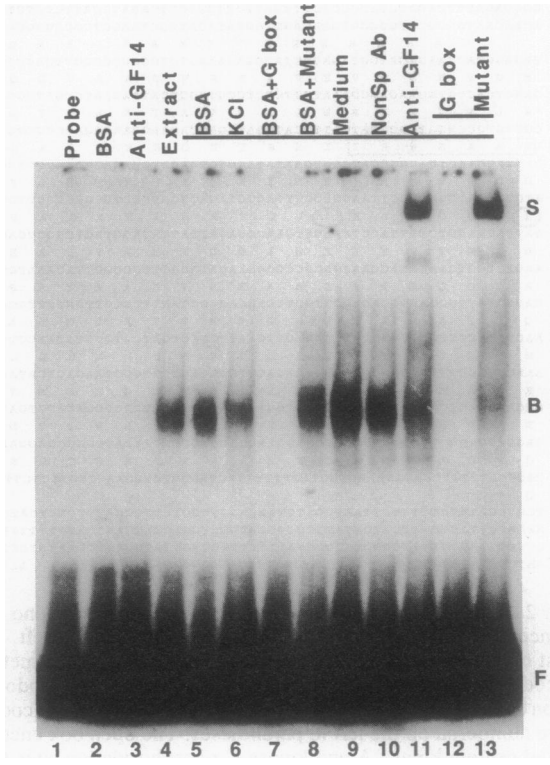


FIG. 1. Electrophoretic mobility shift and competition analyses using control medium and nonspecific and specific antibodies. Lane 1, radiolabeled G box oligonucleotide from the −210 region of the *Arabidopsis Adh* promoter (6); lanes 2 and 3, labeled G box with 15 μg bovine serum albumin (BSA) or 2 μl of anti-GF14 supernatant, demonstrating the lack of G box binding activity in protein or anti-GF14; lane 4, partially purified *Arabidopsis* GBF extract; lanes 5 and 6, GBF in the presence of an additional 15 μg of BSA or 100 mM KCl, demonstrating that additional protein or salt has little effect on GBF activity; lanes 7 and 8, competition of GBF activity by 100-fold excess unlabeled G box but not by 100-fold excess of mutant G box; lanes 9–11, GBF extract in the presence of hybridoma medium, an unrelated antibody supernatant, and anti-GF14, showing that a supershifted band (S) occurs only in the presence of anti-GF14; lanes 12 and 13, competition of the supershifted complex by 100-fold excess of G box but not by 100-fold excess of mutant G box. S, B, and F denote antibody/protein/G box complex, protein/G box complex, and free G box, respectively. The medium, nonspecific antibody, and anti-GF14 were dialyzed for 4 hr against 4000-fold excess of NEBD buffer without 2-mercaptoethanol.

**cDNA Cloning.** One supernatant causing a supershift of the protein/G box complex (Fig. 1, lane 11) was subsequently used to screen a λgt11 cDNA expression library made from the poly(A)$^+$ RNA of an *Arabidopsis* cell suspension culture.

Sequence analysis of the longest positive cDNA clone, pLU14, reveals one significant open reading frame encoding a protein of 259 amino acids (Fig. 2) that we have designated GF14. The insert is 1133 bases long and contains 66 nucleotides of 5′ untranslated region and 265 bases of 3′ trailer with a poly(A) tract.

The derived amino acid sequence of GF14 reveals motifs for potential phosphorylation by protein kinase A and protein kinase C (23) (Fig. 2, residues 59–65). The carboxyl and amino termini are acidic, similar to the activation domain of transcription factors such as *Vp1* (17), *FOS* (24), GAL4 (25), and GCN4 (26). There is a sequence resembling a leucine zipper, but it consists mainly of isoleucines and is not preceded by a basic domain homologous to known bZIP proteins (27).

The cDNA is very nearly full length, as mRNA blot analysis indicates a single mRNA of ≈1200 bases even when heavily loaded (Fig. 3*A*). Genomic Southern blot (16, 18)

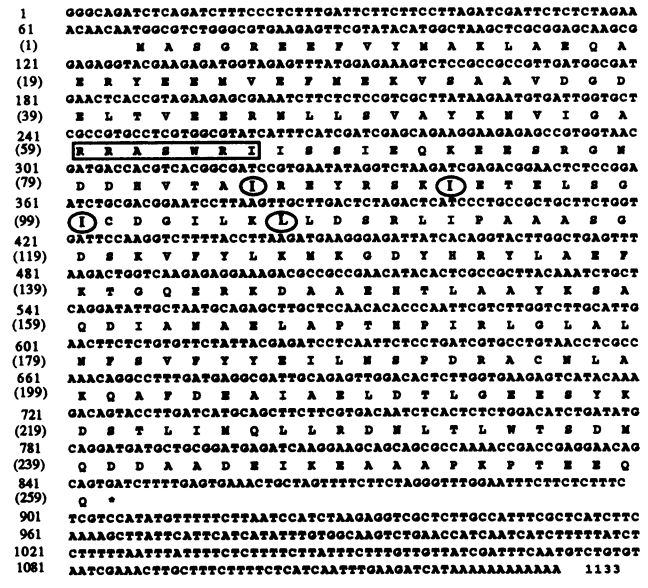

FIG. 2. Nucleotide sequence of pLU14 and derived amino acid sequence of GF14. DNA sequence is numbered on the left. The longest open reading frame, 777 nucleotides, begins with a methionine codon at position 67 and ends with a termination codon at position 844. The derived amino acids are indicated below the codons and are numbered on the left in parentheses. The open box encodes potential protein kinase A and kinase C phosphorylation sites (23). The circles indicate three isoleucine and one leucine residue resembling a potential leucine zipper-type repeat.

analysis indicates that GF14 is likely to be a member of a small family of closely related genes (Fig. 3*B*). This conclusion is strengthened by Western blot analysis of crude cell extracts as well as partially purified GBF complex (Fig. 3*C*) in which four or five proteins of 27–32 kDa molecular mass are recognized by the monoclonal antibody.

**Confirmation of Participation in G Box/Protein Complexes.** To confirm that GF14 is part of the protein/G box complex, GF14 was expressed in *E. coli* (17) with a 43-residue amino-terminal addition resulting from the vector and the 5′ leader. The recombinant GF14 product (34 kDa, Fig. 3*C*) was soluble in the *E. coli* extracts but failed to bind DNA in a mobility shift assay (data not shown). Therefore, the partially purified recombinant GF14 was used in a supershift competition assay to verify that native GF14 is part of the protein/G box complex. Supershifted native protein/G box/antibody complexes (Fig. 4*A*, complex S) were titrated with increasing amounts of recombinant protein or with BSA as a control. Clearly, the recombinant protein competively inhibits the protein/G box/antibody complex (Fig. 4*A*, lanes 2–6), whereas BSA has no effect (lanes 7–11). These data provide confirmation that GF14 is a part of the protein/G box complex. However, the lack of detectable DNA binding activity from the recombinant protein suggests that GF14 is improperly processed in *E. coli* such that DNA binding abilities were lost or that GF14 is not a DNA binding protein in and of itself.

To characterize further the association between GF14 and the GBF DNA binding complex, we carried out Southwestern and Western assays on a blot of nuclear proteins precipitated with anti-GF14. Labeled G box incubated with the protein blot detects a group of DNA binding proteins near 69 kDa in crude nuclear extracts as well as in nuclear proteins precipitated with anti-GF14 (Fig. 4*B*, lanes 3 and 2). Probing the same blot with anti-GF14 (lanes 6 and 5) reveals the suite of four or five GF14 proteins at 30 kDa. These data indicate that GF14 associates with G box binding proteins of ≈69 kDa, that GF14 from nuclei cannot bind DNA in the Southwestern assay, and that bona fide G box binding proteins do not share antibody epitopes with GF14.

**Organ Distribution of Expression.** Significant amounts of GF14 transcript were found in the suspension cells (lane 6), roots (lane 1), flowers (lane 4), and developing siliques (lane 5) (Fig. 5*A*). However, the GF14 mRNA is undetectable in leaves (Fig. 5*A*, lane 3). As shown in Fig. 5*B*, a family of 27- to 32-kDa polypeptides was clearly present in flowers, siliques, and cells and barely detectable in leaves, consistent with the RNA blot assay (Fig. 5*A*). The GF14 protein pattern is qualitatively different among the various organs (Fig. 5*B*). This may indicate that individual members of this protein family exhibit organ-specific expression.

## DISCUSSION

Our experimental approach was designed to recover cDNAs for proteins involved in complexes with the G box DNA

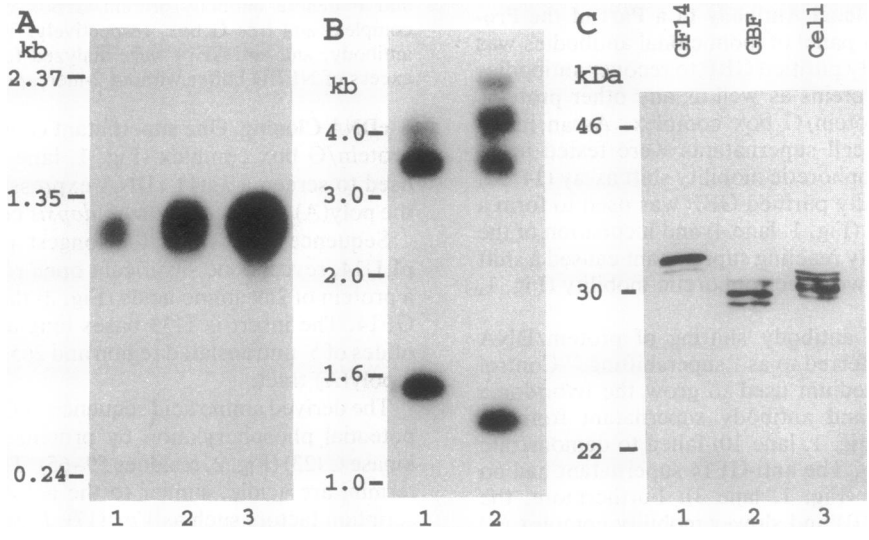


FIG. 3. Blot analyses of GF14 RNA, DNA, and protein. (*A*) Total RNA. Lanes 1–3, 7.5, 15, and 30 μg, respectively, of total RNA from *Arabidopsis* cell cultures (5). The positions of RNA size markers (BRL) are indicated (in kilobases). (*B*) Genomic DNA. *Arabidopsis* genomic DNA (10 μg) digested with *Eco*RI (lane 1) and *Hin*dIII (lane 2). The positions of DNA size markers (BRL) are indicated. (*C*) Immunodetection of GF14 protein by Western blot analysis of protein separated by 12.5% SDS/PAGE (19). Lane 1, partially purified recombinant GF14 as expressed in *E. coli*; lanes 2 and 3, partially purified GBF complex proteins and crude cell extracts, respectively. The migration of protein size markers (Amersham) is indicated.

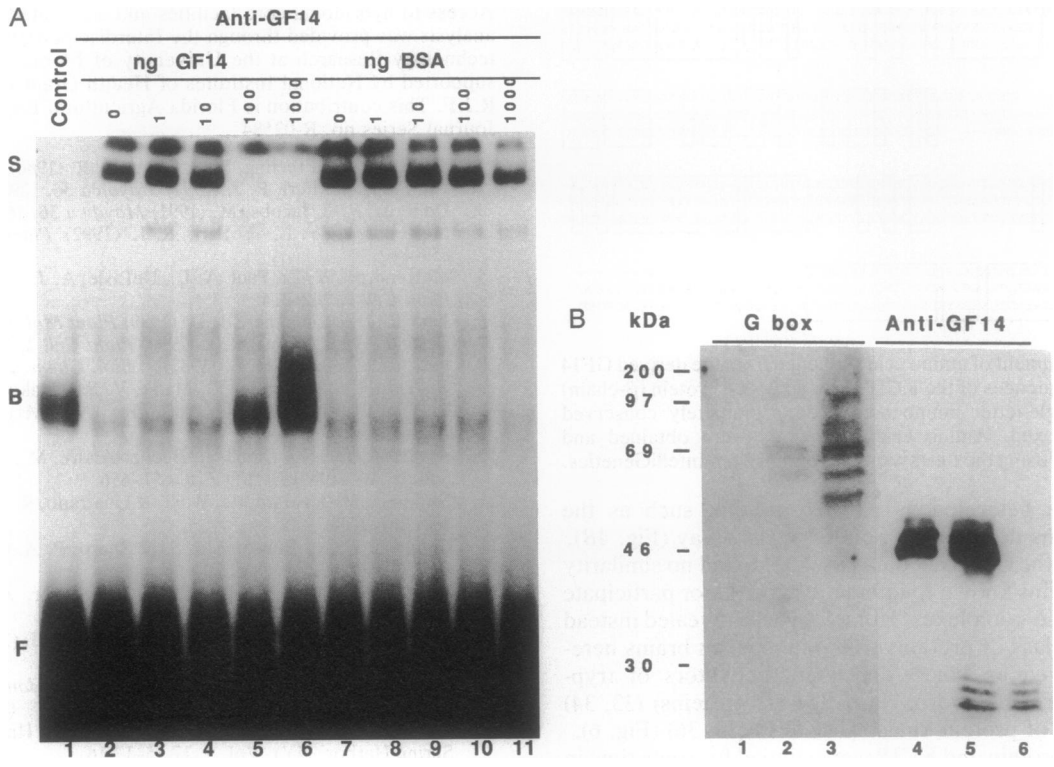


FIG. 4. Competition analysis of the impact of recombinant GF14 and BSA (Sigma) on the antibody/native GBF protein/G box complexes (*A*) and Southwestern and Western assays of nuclear proteins (*B*). (*A*) Lane 1, labeled G box and partially purified GBF (7) incubated with control medium; lanes 2–6, labeled G box and GBF incubated with the anti-GF14 monoclonal cell supernatant in the presence of the indicated concentration of recombinant GF14; lanes 7–11, labeled G box and GBF incubated with monoclonal cell supernatant in the presence of the indicated concentration of BSA. (*B*) Lanes 1 and 4, precipitated anti-GF14; lanes 2 and 5, nuclear proteins precipitated with anti-GF14; lanes 3 and 6, nuclear proteins without antibody treatment. The same membrane was used for DNA binding analysis (lanes 1–3) and detection of GF14 (lanes 4–6). The blot was incubated 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 milk) containing $10^6$ cpm of $^{32}$P-labeled G box per ml. The blot was washed in three changes of binding buffer over a period of 1 hr and then exposured to Kodak XAR-5 film (21).

element. An adaptation of the electrophoretic mobility shift assay was used as a screen of hybridoma supernatants to identify antibodies specific to a protein/DNA complex. The antibodies were subsequently used to recover a cDNA encoding a protein named GF14.

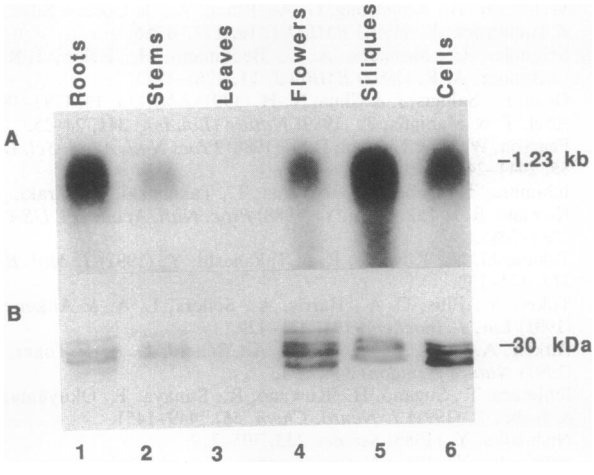


FIG. 5. Organ-specific expression. (*A*) RNA gel blot analysis of GF14 expression in *Arabidopsis*. Ten micrograms of RNA from different organs of 40-day-old *Arabidopsis* plants and RNA from the suspension cell culture was subjected to RNA blot analysis. (*B*) Western blot analysis. Fifty micrograms of total proteins was fractionated by electrophoresis on 12.5% SDS/PAGE and immunodetected by anti-GF14 monoclonal antibody.

Electrophoretic mobility shift and immunoprecipitation assays indicate that GF14 is physically associated with the protein/G box complex but does not bind DNA in and of itself. In the presence of monoclonal anti-GF14, the protein/G box complex is shifted to a slower migrating form in the electrophoretic assay, and the slower migrating complex (S) demonstrates the same competition specificity as does the bound G box complex (B) (Fig. 1). Immunoprecipitation of nuclear proteins with anti-GF14 reveals coprecipitation of GF14 and bona fide G box binding proteins of about 69 kDa, yet Western analysis shows no cross-reactivity between GF14 and the DNA binding proteins.

Although we present here the complete sequence of one GF14 cDNA, it appears that GF14-like proteins are a small family of very closely related sequences. Preliminary sequencing of other GF14 cDNAs reveals amino acid similarities of >90% among the members of the family, and several different monoclonal antibodies all behave like the anti-GF14 described in this report (unpublished observation). Thus our conclusions regarding potential GF14 structure and function are extended to the other members of the protein family until such time as functionally unique properties might be assigned to individual members.

GF14 is not homologous to any of the putative G box binding proteins that have been isolated from plants. There are several examples of bZIP proteins that have been isolated from expression libraries by means of their ability to bind G box oligonucleotides (11, 28–30). None is related to GF14. However, the GF14 protein may be operationally similar to FOS (31), possessing a dimerization zipper and acidic activation domain but being able to bind to DNA only in the

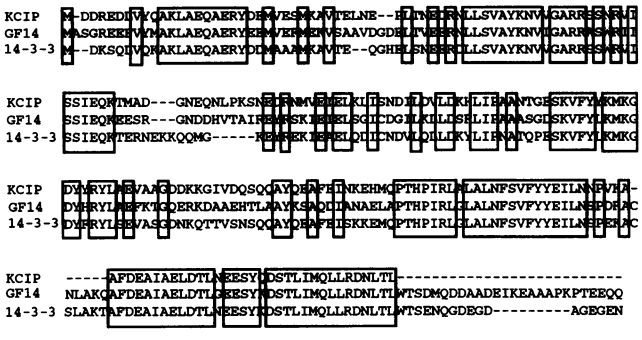

FIG. 6. Alignment of amino acid sequence from the derived GF14 protein with sequences of the KCIP (36) and 14-3-3 protein ($\beta$-chain) (33) in the single-letter amino acid code. Completely conserved residues are boxed. Amino acid alignments were obtained and manually edited using the GENEWORKS program from IntelliGenetics.

presence of a heterologous partner protein, such as the 69-kDa proteins detected on Southwestern assay (Fig. 4*B*).

A search of the GenBank data base (32) found no similarity with any proteins known to interact with DNA or participate in protein/DNA complexes. Homology was revealed instead with a small class of proteins from mammalian brains heretofore described as kinase-dependent activators of tryptophan and tyrosine hydroxylases (14-3-3 proteins) (33, 34) and inhibitors of protein kinase C (KCIP) (35, 36) (Fig. 6).

The 14-3-3 protein and KCIP were studied for some time in mammalian brains for their separate activities before the structural homology between the two was identified. Their activities are potentially interrelated in that both involve $Ca^{2+}$ and protein kinases. The 14-3-3 proteins were characterized as activators of tyrosine and tryptophan hydroxylases in the presence of $Ca^{2+}$/calmodulin-dependent protein kinase II (33, 34). The tyrosine and tryptophan hydroxylase activities lead to the production of the neurotransmitters dopamine and serotonin, respectively. However, recent results have shown that the 14-3-3 proteins are also found in tissues where the hydroxylase activities are absent (37), suggesting the potential for other roles for the 14-3-3 proteins. The KCIPs were characterized as potent inhibitors of protein kinase C (35). Protein kinase C activity is nearly ubiquitous in animals and mediates a wide range of physiological processes in a $Ca^{2+}$/phospholipid-dependent manner (38, 39). Currently, it would appear that the 14-3-3/KCIP family of proteins serves a broad set of perhaps interrelated functions involving kinases, $Ca^{2+}$, and other second messengers.

Based on the degree of similarity among the GF14, 14-3-3, and KCIP, especially in the regions of kinase recognition (Fig. 6, residues 57–72) and the potential activation domain for tyrosine and tryptophan hydroxylases (Fig. 6, residues 168–233), we predict that GF14 proteins may likewise function in the regulation of protein kinase activity and biosynthesis of neurotransmitter compounds in plants. There is a wide distribution of neurotransmitter compounds (40) and kinase C-like activities (41) in plants, though their physiological roles are poorly understood. The results presented here further indicate that GF14 is involved in binding to a general cis-acting element, the G box, that is present in the promoters of many classes of genes. These facts suggest central and evolutionarily conserved roles for these proteins in the regulation of diverse cellular processes.

**Note.** While this manuscript was under review, 14-3-3 homologs were reported from Oenothera and spinach (42).

We thank Linda Green, Beth Laughner, Anna-Lisa Paul, Ernie Almira, Curt Hannah, and Don McCarty for help and discussion during these experiments and the preparation of this manuscript. Access to hybridoma core facilities and automated DNA sequence analysis was provided through the Interdisciplinary Center for Biotechnology Research at the University of Florida. This work was supported by National Institutes of Health Grant R01 GM40061 to R.J.F. This contribution is Florida Agricultural Experiment Station Journal Series no. R-02584.

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