A Splice Variant of E2–2 Basic Helix-Loop-Helix Protein Represses the Brain-specific Fibroblast Growth Factor 1 Promoter through the Binding to an Imperfect E-box*

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We previously demonstrated that a cis-element (–489 to –467) in the brain-specific fibroblast growth factor (FGF)-1 promoter (FGF-1.B) binds multiple nuclear factors, and this binding enhances transcriptional activity of this promoter. Here we report the isolation of three cDNA clones, VL1, VL2, and VL3, from a human brain stem cDNA expression library using four tandem repeats of the 26-base pair sequence (–492 to –467) as the probe. These cDNA clones represent the variant of bHLH protein E2–2/SEF2–1 in having 12 additional nucleotides encoding the amino acids RSRS. The glutathione S-transferase (GST) fusion proteins of VL1, VL2, and VL3 immunologically react with anti-E2–2 antibody and anti-GST–VL2 antibody. Electrophoretic mobility shift assay and methylation interference assay revealed that the GST fusion proteins specifically bind to an imperfect E-box sequence (GACCTG) present in the 26-base pair sequence. Transient expression of the full-length E2–2 without RSRS in U1240MG glioblastoma cells resulted in repression of FGF-1.B promoter activity. We further showed a significant repression of promoter activity (>40 fold) by E2–2 (lacking the amino acid sequence RSRS) when the E47 reporter construct, containing a hexameric E-box site, was used. In contrast, the E2–2 variant containing the RSRS sequence has no significant effect on either the FGF-1 promoter or E47 promoter. These results suggest that the relative abundance of the two splice variants of E2–2 in brain could be an important determinant for the expression of FGF-1.

A family of proteins that bind to a consensus DNA sequence CANNTG, also known as E-box, has been identified. E-box-binding proteins share a common amino acid sequence motif that is proposed to form two amphipathic helices interrupted by a loop, designated the helix-loop-helix (HLH)1 motif. The HLH domain of these proteins mediates the dimerization, and a basic region located N-terminal of the HLH domain is responsible for DNA binding (1). HLH proteins are mainly of two types, ubiquitous and tissue-specific. The interplay of these HLH proteins is particularly interesting because it plays a significant role in the regulation of tissue-specific gene expression (1).

Fibroblast growth factor (FGF)-1 is a prototype member of the structurally related FGF family, which comprises 14 proteins (2, 3). It is found primarily in brain and retina, although low levels of its mRNA have been demonstrated in other tissues (4–6). However, the role of FGF-1 in brain is not yet well understood. In situ hybridization and immunohistochemical analysis, it has been shown that the expression of this growth factor in brain is exclusively in neuronal cells but not in glial cells (7–9). It was also shown that the expression of FGF-1 mRNA in neurons is correlated with specific developmental events (10) and that FGF-1 coexists with tyrosine hydroxylase in neuronal cells (11). Importantly, transcription of tyrosine hydroxylase, a key enzyme for catecholamine synthesis, requires both FGF-1 and an activator (12). FGF-1 mRNA and protein levels were also reported to elevate in lesioned rat brain (13, 14). Interestingly, FGF-1 mRNA expression was reported to increase significantly in glioblastoma, the major human intracranial tumor (5, 15). However, the regulatory mechanism of FGF-1 gene expression is only beginning to be understood.

The study of tissue- and cell-specific distribution of FGF-1 transcripts has led to the identification of four different transcripts having the same protein coding exons but different 5′-untranslated exons (16–20). FGF-1.B is the predominant transcript in brain, gliomas, and some glioblastoma cell lines (e.g. U1240MG and U251MG) (5, 21). We have identified a 23-bp cis-element (–489 to –467) in FGF-1.B promoter that binds to a 37-kDa protein, p37***, and this binding is linked to the enhanced functional activity of the promoter (22). Here we screened a human brain stem cDNA expression library for DNA-binding proteins using a labeled oligonucleotide probe made of four tandem repeats of the sequence –492 to –467. We have isolated and expressed three overlapping cDNA clones. All three clones represent a splice variant of the E2–2 gene product (23, 24), and the encoded protein binds to an imperfect E-box present in the probe. We further showed that overexpression of an E2–2 variant lacking RSRS represses the transcriptional activities of both the FGF-1.B promoter and the E47 reporter containing a hexameric E-box site.

MATERIALS AND METHODS

Screening of the cDNA Library—A Agt11 expression library, made from human brain stem as described previously (16), was screened for expression of DNA-binding proteins using the protocol described by Singh et al. (25). A SpeI–BamHI fragment with four tandem repeats of the sequence –492 to –467 (in the head-to-tail orientation) was labeled with [α-32P]dATP by the Klenow reaction and used as a probe. Three positive clones, VL1, VL2, and VL3, were plaque-purified using the same DNA probe.

Sequencing Analysis—Standard procedures (26) were used to purify the phage DNA, and the cDNA inserts from the phage clones were...
subcloned into the EcoRI site of pBluescript II KS(+) (+). The cDNA clones were sequenced using Sequenase (U.S. Biochemical Corp.). DNA sequence alignment was performed using DNASTAR software (Madison, WI).

Northern Blot Analysis—The VL2 cDNA was used as a probe to hybridize to human multiple-tissue Northern blot (BIOS Laboratories). The Northern blot contained 20 µg of total RNA extracted from different human tissues. The blot was subjected to Northern blot analysis using standard procedures (26). Hybridizations were performed in a buffer containing 50% formamide at 42 °C for 16 h. The final washes were done in 0.1 × SSC at 50 °C for 30 min.

Production and Purification of GST-fusion Proteins and Antibodies—The cDNA fragments of 1272, 1037, and 700 bp obtained by EcoRI digestion of VL2, VL3, and VL1 phage clones, respectively, were blunt ended and inserted, in frame, with the glutathione S-transferase gene, into blunt ended AscI site of pGEX-2T (Amersham Pharmacia Biotech). The proper orientation of the insert was confirmed by restriction digestion. The GST-fusion proteins were expressed and purified from Escherichia coli (BL21, pLysS) using glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described by Smith and Johnson (27). The purified GST-VL2 protein was used as the antigen for antibody production in rabbits (Cocalico Biologicals, Reamstown, PA). The antibodies were purified by protein A-Sepharose (Amersham Pharmacia Biotech) affinity column as described by Harlow and Lane (28). Anti-E2–2 and anti-VL1 antibodies were purchased from Promega (Madison, WI).

Western Blot Analysis—Purified GST fusion proteins of the spliced E2–2 gene products (GST-VL1, GST-VL2, and GST-VL3) and Nalm6 nuclear extract were subjected to electrophoresis in 10% SDS-polyacrylamide gel. For immunodetection, proteins were transferred to nitrocellulose membrane and blocked by 3% nonfat dry milk in phosphate-buffered saline (pH 7.2) containing 0.1% Nonidet 40. The membranes were probed with either monoclonal anti-E2–2 antibody or polyclonal anti-GST-VL2 antibody at 1:1000 dilution and washed three times (each for 15 min) with the blocking solution. The blots were then incubated with either horseradish peroxidase-conjugated anti-mouse IgG (for the monoclonal anti-E2–2 antibody) or horseradish peroxidase-conjugated anti-rabbit IgG (for the polyclonal anti-GST-VL2 antibody) at a dilution of 1:1000. Finally, the immuno detection was carried out by autoradiography using ECL kits (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay (EMSA)—The 26-bp (−492 to −467) double-stranded oligonucleotide was end-labeled with [α-32P]dATP using the Klenow enzyme. The sequence was described elsewhere (29). The binding reaction contained the binding buffer (25 mM Hepes, pH 7.9, 1 mM MgCl2, 0.5 mM dithiothreitol, 40 mM KCl, 5% glycerol, and 0.1% NP-40) with radiolabeled DNA probe (30,000 cpm, 3000 Bq) and an additional 2 µl of poly(dI-dC), the GST fusion protein (0.3 µg), and a cold competitor, when desired, in a total volume of 20 µl. The brain nuclear extract (2 µg) was also assayed in EMSA in the presence of 0.1 µg of GST or GST-VL2 under similar conditions except that 0.5 µg of poly(dI-dC) was used. Following 30 min of incubation at room temperature, the reaction mixture, when indicated, was further incubated with antibody for 1 h at 0 °C. A 12-µl aliquot of the reaction was analyzed in a 4% polyacrylamide gel in 0.25 × TBE (22.25 mM Tris borate, 0.5 mM EDTA, pH 8.0). Following electrophoresis, the gel was dried and autoradiographed. The human brain nuclear extract was made in accordance with the method described by Gorski et al. (30).

Methylation Interference Assay—The HindIII–EcoRI fragment from plasmid pGFP-1B (−492 to −467) was S-ended at HindIII II (antisense strand), partially methylated with dimethyl sulfate, and used as a probe in binding reactions containing GST-VL2. DNA-protein complexes were separated from the free DNA by EMSA, eluted from the gel, cleaved with piperidine, and then analyzed in 5% sequencing gel as described (22).

Plasmid Constructs—VL3-FL is a full-length E2–2 expression plasmid that was constructed by replacing, in frame, the EcoRV–EcoRI fragment of pCMV2961 (SEF2–1B) (24) with the EcoRV-EcoRI fragment from VL3. This replacement allows the resultant construct to encode full-length E2–2/SEF2–1 with four additional amino acids (RSRS) as shown in Fig. 1. The constructs SEF2–1B, pCMVβCAT, and E47 luciferase reporter (31) were kindly provided by Drs. Brit Cornelius and Thomas Grundstrom. SEF2–1B, previously described in pCMV2961, was cloned in the expression vector pCMVβCAT (24). E47/ pCSA, an expression vector for the bHLH protein E47 (32), was provided by Dr. Andrew Lassar (Harvard Medical School, Boston). Plasmid pRL-tk (Promega) contains the sea pansy Renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter.

Transfection Assay—The transfection protocols were as described previously (22). Briefly, U1240MG cells were plated in 60-mm Falcon tissue culture dishes (Becton Dickinson Labware) to achieve 60–80% confluence by day 2. On day 2, cells were transfected with the reporter expression plasmid DNA using the cationic lipid transfection reagent, DOTAP (Boehringer Mannheim). Plasmid DNA was prepared using the Qiagen plasmid kit (Qiagen Inc., Chatsworth, CA). In each experiment, two plasmid constructions were transfected in triplicate. The results show the mean of at least two separate experiments. The luciferase activity was normalized by the protein contents as described (22) or by the Renilla luciferase activity using the Dual-luciferase Reporter Assay system (Promega) as described by the manufacturer’s protocol.

RESULTS

Cloning of VL1, VL2, and VL3 from Human Brain Stem cDNA Library—Using both functional and biochemical analyses, we previously identified a 26-bp region (−492 to −467) within RR2 of the brain-specific FGF-1.B promoter (22, 29). The 26-bp sequence was concatenated, and a probe with four tandem repeats was used to screen a human brain stem cDNA expression library constructed in λgt11 (16). Two positive clones (VL1 and VL2) were isolated during the first screening, and an additional clone (VL3) was isolated during the second screening. The cDNA inserts from three plage clones were subcloned into pBluescript II KS(+) (+). Sequence analysis showed that the three clones have an identical sequence with E2–2, also known as SEF2–2 and ITF-2, a basic HLH (bHLH) protein which was previously isolated using E-box probes (23, 24). Both E2–2 (23) and one of the four SEF2–2 (24) cDNAs lack four amino acids (RSRS) just upstream of the bHLH region. Using sequence analysis and restriction digestion, we showed that all three of our cDNA clones contain the RSRS sequence (Fig. 1). The different most likely resulted from alternative splicing of the same E2–2/SEF2–1 gene (23, 24).

E2–2/SEF2–1 Is Abundantly Expressed in Human Brain—To understand the tissue distribution of E2–2 mRNA, we hybridized Northern blot containing an equal amount of RNA from different human tissues with VL2 cDNA probe. As shown in Fig. 2, the 7.5-kilobase pair human E2–2 transcript is detected in brain (lane 1), lung (lane 4), and to a lesser extent spleen (lane 6). The expression of the 7.5-kilobase transcript in other tissues (e.g. liver, heart, ovary, skeletal muscle, and duodenum) was below the detectable level. It also noted that the expression of E2–2 mRNA is relatively higher in the brain.

The Protein Encoded by the VL cDNA Immunologically Reacts with Anti-E2–2 Antibody—In order to characterize the protein encoded by our cDNA clones (VL1, VL2, and VL3), we expressed these cDNAs as GST fusion proteins and raised antibody in rabbit against one of the fusion proteins (GST-VL2) (Fig. 3). The fusion proteins were then tested for the immuno reactivity with the commercially available E2–2 antibody or anti-GST-VL2 antibody. In the same assay, we also included nuclear extract from Nalm6 (pre-B) cell line, which is known to express E2–2 protein (32). It is evident from the results that anti-E2–2 antibody recognized GST-VL3 (lane 3) and GST-VL2 (lane 4), but it did not cross-react with GST (lane 1) nor with GST-VL1 (lane 2). Multiple protein bands were observed to cross-react with anti-E2–2 antibody in the case of GST-VL3 or GST-VL2. This could be due to degradation of the fusion proteins or its premature termination of translation. The sizes of the major GST fusion proteins that were recognized by anti-E2–2 antibody for GST-VL2 and for GST-VL3 are similar to the size predicted based on the nucleotide sequence information (Fig. 1). Two major proteins of 90 and 70 kDa (marked in Fig. 3 by the open arrowheads) in nuclear proteins from Nalm6 cells also cross-reacted with the same antibody (lane 5). The nucleotide sequence analyses showed that the three clones encoded in this paper are the splice variant of the E2–2 gene. The fact that GST-VL1 was not recognized by anti-E2–2 is probably due
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The Protein Product of VL1, VL2, and VL3 binds to an Imperfect E-box (μE5) Site Present in RR2 of the FGF-1.B Promoter—To confirm the sequence-specific binding, we performed EMSA using the GST fusion protein and labeled oligonucleotide probe containing the sequence −492 to −467 in the absence or presence of a 50-fold molar excess of cold competitor A (−492 to −467) or B (−484 to −467) (Fig. 4). It is evident that GST-VL1 (lane 1), GST-VL3 (lane 6), and GST-VL2 (lane 11) retarded the migration of the labeled oligonucleotide probe, and the degree of retardation was dependent on the size of the binding protein. It is also clear that these retarded DNA-protein complexes are specific, since they could be competed by the lack of epitope in this protein that can be recognized by the monoclonal anti-E2–2 antibody. Hence, we repeated the same experiment with anti-GST-VL2 antibody (lanes 6–10). This antibody recognized all the GST fusion proteins (lanes 7–9) and GST itself (lane 6). The same two major polypeptides (90 and 70 kDa) were found to be present in Nalm6 cells that could cross-react with both anti-E2–2 (lane 5, open arrowhead) and anti-GST-VL2 (lane 10, open arrowhead) antibodies. It is noteworthy to mention that Bain et al. (32) detected a 90-kDa protein in Nalm6 cells cross-reacting with anti-E2–2 antibody. Therefore, it is most likely that the antigenic determinant for anti-GST-VL2 antibody includes both the GST and E2–2 portions of the fusion proteins. We do not know whether the 70-kDa protein is a degradative E2–2 protein or a related protein having the common epitope. These results suggest that the epitope for the anti-E2–2 antibody lies in the 100-amino acid domain encoded by nucleotides 1396–1720, and that VL1, VL2, and VL3 represent the splice variant of E2–2 gene product.

The Protein Product of VL1, VL2, and VL3 contains an equal amount (20 μg) of total RNA from different human tissues as indicated at the top. The 28S and 18S RNA controls are shown by the arrowheads in the left. The arrow on the right indicates the 7.5-kilobase transcript of E2–2/SEF2–1.

Each human tissue contains an equal amount (20 μg) of total RNA from different human tissues as indicated at the top. The 28S and 18S RNA controls are shown by the arrowheads in the left. The arrow on the right indicates the 7.5-kilobase transcript of E2–2/SEF2–1.

FIG. 1. Cloning of VL1, VL2, and VL3 and alignment of each cDNA with human E2–2/SEF2–1 cDNA. A schematic diagram of human E2–2 cDNA clone, SEF2–1B (GenBank™/EMBL accession number M74719) is shown. The relative positions of VL1, VL2, and VL3 cDNA are delineated by the vertical bars. The common restriction sites, NcoI, EcoRV, and AvaI, in each cDNA are aligned and are indicated by the arrows. The open box represents the open reading frame, and the hatched box represents the bHLH domain. The four amino acids RSRS, which are absent in both E2–2 (23) and SEF2–1B (24) cDNA clones, are indicated by open triangles. The cDNA sizes and the predicted sizes of the cDNA-encoded and the GST fusion proteins for VL1, VL2, and VL3 are indicated next to the diagram.

FIG. 2. Northern blot analysis of E2–2/SEF2–1 expression in human tissues. Each lane contains an equal amount (20 μg) of total RNA from different human tissues as indicated at the top. The 28S and 18S RNA controls are shown by the arrowheads in the left. The arrow on the right indicates the 7.5-kilobase transcript of E2–2/SEF2–1.

FIG. 3. Western blot analysis of recombinant GST, GST-VL1, GST-VL2, and GST-VL3 proteins. An equal amount (100 ng) of the purified GST (lanes 1 and 6), GST-VL1 (lanes 2 and 7), GST-VL3 (lanes 3 and 8), or GST-VL2 (lanes 4 and 9) recombinant protein or 10 μg of nuclear extract prepared from Nalm6 pre-B cells (lanes 5 and 10) was separated in 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and probed with anti-E2–2 antibody (lanes 1–5) or anti-GST-VL2 antibody (lanes 6–10). Two major proteins (90 and 70 kDa) in the Nalm6 nuclear extract that cross-react with anti-E2–2 antibody and anti-GST-VL2 are indicated by open and closed triangles, respectively.

A (-492 to -467) or B (-484 to -467) (Fig. 4). It is evident that GST-VL1 (lane 1), GST-VL3 (lane 6), and GST-VL2 (lane 11) retarded the migration of the labeled oligonucleotide probe, and the degree of retardation was dependent on the size of the binding protein. It is also clear that these retarded DNA-protein complexes are specific, since they could be competed by the cold competitor A (lanes 2, 7, and 12). The lack of competition by the same molar excess of cold competitor B (lanes 3, 8, and 13) suggests that the site where these proteins bind resides within the sequence −492 to −484. Interestingly, a closer look of this binding site revealed a sequence ACGACCTGC (29) that contains an imperfect E-box (μE5) site, GACCTG (23). To further confirm the identity of these proteins, we added anti-E2–2 or anti-E2A antibody following the DNA-protein complex formation. As shown in Fig. 4, anti-E2–2 antibody supershifted the DNA-protein complex formed either by GST-VL3 (lane 9) or GST-VL2 (lane 14). These supershifts are also specific for the E2–2 antibody, since anti-E2A was unable to supershift the retarded complex (lanes 9 and 15). However, the DNA-protein complex formed by GST-VL1 was not supershifted in the same
assay by anti-E2–2 antibody. This result conforms to the earlier data (Fig. 3), which showed that GST-VL1 lacked the epitope to be recognized by anti-E2–2 antibody. However, anti-GST-VL2 could supershift the DNA-protein complex formed with GST-VL1 (Fig. 5, lane 4). The same antibody was also shown to supershift GST-VL3-DNA complex (lane 9). The control antibody (IgG from the same rabbit used to generate anti-E2A antibody) failed to supershift GST-VL3-DNA complex (lane 10) or GST-VL2-DNA (lane 10) complex.

To determine the exact contact point in the DNA where these fusion proteins bind, we performed the methylation interference assay using GST-VL2 and the methylated probe as described under “Materials and Methods.” Fig. 6A shows that the guanine nucleotide in the antisense strand at −484 is important for the binding of this GST fusion protein with the DNA. It is noteworthy to mention that this guanine nucleotide at −484 is in close proximity to the imperfect E-box (μE5) site. Fig. 6B represents the quantitative levels by a comparison of the G-ladder radioactivity of free versus bound. It is also obvious from this analysis that the peak radioactivity at −484 in the bound is significantly reduced (42%) in comparison with the same in the free.

To confirm the methylation interference data, we performed EMSA using GST-VL2 and the 26-bp (−492 to −467) oligonucleotide probe having the wild type sequence or having mutation C to A at −484, −478, and −472 (Fig. 7). The binding of GST-VL2 with the mutant probe was reduced dramatically in comparison with that achieved by the wild type probe. Since the binding site of GST-VL2 was identified to be in the region −492 to −484 (Fig. 4), it is most likely that the mutation at −484 caused this diminution of the DNA-protein interaction.

E2–2 Forms a Heterodimer with Proteins Present in Brain

Nuclear Extract—Since GST-VL2 binds to the sequence from −492 to −484, we looked for the endogenous E2–2 protein in crude brain nuclear extract that can bind to that site (Fig. 8). Although we could detect one such complex (shown by the open arrowhead, lane 7), it was not recognized by anti-E2–2 antibody (lane 9) or by anti-E2A (lane 10). However, we could detect an additional complex (shown by the solid arrowhead, lane 1) having mobility faster than GST-VL2 (lane 12) when the crude brain nuclear extract was preincubated with GST-VL2. This complex was not formed when the nuclear extract was preincubated with GST under the same conditions (lanes 6–10). Importantly, this additional complex was supershifted by anti-E2–2 antibody (lane 4) but not by the anti-E2A antibody (lane 5). These results suggest that E2–2 forms a heterodimer with partner proteins present in brain and that the heterodimer binds to the site −492 to −484.

In Vivo Effect of E2–2 Proteins in U1240MG Cells on the Activity of Promoter Containing E-box (μE5) Site(s)—Two spliced forms of E2–2 proteins, with and without RSRS, are known to be expressed in different cells (23, 24, 33, 34). The three cDNA clones (VL1, VL2, and VL3) isolated from human brain stem cDNA library represent the splice variant of E2–2 containing RSRS (Fig. 1). However, an E2–2 cDNA that lacks the sequence encoding RSRS was isolated from a murine brain cDNA library (33). Hence, the potential presence of both spliced...
forms of E2–2 in brain is possible. Considering these facts, we tested the effect of transient overexpression of both spliced forms (with and without RSRS) on the FGF-1.B promoter activity in U1240MG cells (Fig. 9). We observed that SEF2–1B, which encodes full-length E2–2 protein without RSRS (24), significantly reduced the promoter activity, while the other splice variant with RSRS (VL3-FL) did not affect this promoter activity. Under the same conditions, the empty vector pCMVΔCAT and another bHLH protein NeuroD (35) did not alter the activity of the FGF-1.B promoter.

To determine whether repression of FGF-1.B promoter activity is specific, we tested E47 promoter reporter construct which contains a hexameric E-box (mE5–mE2) site. The reporter activity of this promoter construct was previously shown to increase upon expression of E47 effector cDNA (23, 31, 32). Fig. 10A shows that the expression plasmid E47/pCSA, encoding E47 protein (an E2A gene product), activated the E47 reporter, and this activation attained a peak level of 27-fold at a 0.5-μg dose in comparison with that achieved by the same dose of the empty vector, pCMVΔCAT. These results suggest that the activation of the promoter through the mE5–mE2 sites is also reproducible in U1240MG cells. On the other hand, at the same dose level and under the similar experimental conditions, SEF2–1B, which encodes the full-length E2–2 protein without RSRS exerted a dramatic inhibition (7-fold) of the promoter activity. This inhibition of the reporter activity was dependent on the dose of this E2–2 splice variant having the repression greater than 40-fold when 5 μg of the SEF2–1B effector plasmid was cotransfected (Fig. 10A). In contrast, VL3-FL (E2–2 with RSRS) did not confer any remarkable effect on this E47 reporter activity at any of the doses tested (Fig. 10A). Interestingly, the activity of the FGF-1.B promoter (containing an imperfect μE5 site at −490 to −485) was also repressed in SEF2–1B-co-transfected cells in a dose-dependent manner (Fig. 10B). Although the degree of reduction of FGF-1.B promoter activity was not as dramatic as that of E47 reporter, it was reproducible in all five experiments carried out in our laboratory. The transfection of E47/pCSA, on the other hand, did not affect the FGF-1.B promoter activity. These re-

**Fig. 6.** Methylation interference showing binding of E2–2 to the guanine nucleotide at position −484 of the FGF-1.B promoter. A, the methylated HindIII–EcoRI fragment containing nucleotides −492 to −467 with the antisense strand radioactively labeled was incubated with GST-VL2 protein. The specific DNA-protein complex and free DNA, localized following EMSA, were eluted, treated with piperidine, and analyzed on an 8% sequencing gel. Lane G, partial chemical degradation specific for guanine residues; lane F, free DNA probe; lane B, bound DNA probe. The sequence of the antisense strand (−467 to −492) as well as the positions of the guanine nucleotides are depicted on the left. The asterisk represents the guanine nucleotide at position −484 critical for the specific DNA-protein complex formation. B, quantitation of methylation interference assay using a PhosphorImager (Molecular Dynamics, Inc.). The amount of radioactivity in the free (top) versus bound fraction (bottom) is depicted. The peak value of nucleotide −484 (∗) in the bound fraction is 42% of that in the free fraction. The dagger indicates that the cytosine nucleotide at position −485 is also the DNA-protein contact site.

**Fig. 7.** EMSA of the wild type (WT) and mutant (mut) sequence −492 to −467 of FGF-1.B promoter with E2–2. Equal counts (50,000 cpm) of wild type (lanes 1 and 2) or mutant (lanes 3 and 4) oligonucleotide were incubated with 300 ng of GST-VL2 protein in the absence (−) (lanes 1 and 3) or presence (+) (lanes 2 and 4) of a 50-fold molar excess of self-oligonucleotide. In the mutant oligonucleotide, cytosine residues in the sense strand at positions −484, −478, and −472 were replaced by adenine residues.
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FIG. 8. GST-VL2 forms a heterodimer with cellular protein(s) present in human brain nuclear extract. Two micrograms of human brain nuclear extract was assayed in the presence of 100 ng of GST-VL2 (lanes 1–5) or GST (lanes 6–10) in EMSA with the oligonucleotide probe A (−492 to −467) in the absence (lanes 1 and 6) or presence of a 100-fold molar excess of cold competitor A (lanes 2 and 8) or B (−484 to −467) (lanes 4, 5, 7, 9, and 10). An equal amount (100 ng) of GST-VL2 was also assayed in the presence of 2 μg of bovine serum albumin (lane 11). Lanes 4 and 9, the reaction carried out in the presence of anti-E2–2 antibody; lanes 5 and 10, the reaction carried out in the presence of anti-E2A antibody; lane 12, GST-VL2 assayed in the presence of a lower concentration of poly(dI-dC) (0.2 μg/assay). An equal amount (100 μg) of bovine serum albumin was added to lanes 11 and 12. An equal amount of each of the lane 12 samples was also assayed (Fig. 8). The closed arrowhead indicates the heterodimer complex formed in the presence of GST-VL2.

results suggest that the μE5 site present in both promoters may be the target of suppression by E2–2 protein lacking the RSRS domain.

DISCUSSION

The present study was undertaken to identify the transacting factor(s) that binds to RR2 of FGF-1.B promoter. Earlier studies (22, 29) showed that the specific DNA-protein interaction in a 23-bp sequence (−489 to −467) of RR2 is linked to the enhanced functional activity of the FGF-1.B promoter in the FGF-1.B-positive glioblastoma cell line, U1240MG. The present paper reports the identification of three E2–2/SEF2–1 cDNA clones (VL1, VL2, and VL3) from the brain stem cDNA library using four tandem repeats of the sequence −492 to −467. Comparison of VL1, VL2, and VL3 with the full-length SEF2–1B cDNA (24) showed that our clones encode four additional amino acids (RSRS) N-terminal to the bHLH domain (Fig. 1). The immunoreactivity of GST fusion proteins of VL1, VL2, and VL3 to the monoclonal anti-E2–2 antibody in Western blot and in EMSA further confirmed that these clones represent a splice variant of the E2–2 gene product.

The localization of the binding site by using competitor oligonucleotides of overlapping sequence as well as by methylation interference assay revealed that the protein product of these cDNA clones binds to an imperfect E-box (μE5) site within the sequence −492 to −487. Recently, we showed that the 18-bp sequence (−484 to −467) of FGF-1.B is sufficient to activate the promoter in U1240MG cells (29). Hence, the DNA-protein interaction at the μE5 site may not be essential in the context of FGF-1.B promoter activity in U1240MG cells. On the other hand, this study’s findings are important because (i) FGF-1.B is expressed in neuronal cells (7, 8, 10, 14); (ii) the HLH proteins, which bind to E-box as homo- or heterodimers (1), are also expressed in neuronal cells (35, 36); (iii) the expression of these HLH factors in neurons also regulates the development of the nervous system (35–40); and (iv) the expression of FGF-1 gene in brain is also developmentally regulated (7, 10).

We have shown here that the specific binding of these E2–2 proteins (GST-VL1, GST-VL2, and GST-VL3) with the oligonucleotide probe (−492 to −467) was through the imperfect E-box (μE5 site). However, we were unable to detect, by EMSA, cellular E2–2 homodimer either in U1240MG cells (data not shown) or in human brain nuclear extract (Fig. 8) that can bind to the imperfect μE5 site. The Northern blot analysis showed that the steady state levels of SEF2–1 mRNA is relatively higher in brain tissue than in other tissues studied. Therefore, it is unlikely that the level of this protein in the brain tissue is too low to be detectable. However, it is pertinent to mention that protein binding to the μE5 site has not been detected in crude nuclear extract (41, 42). We do not know at this point whether our failure to detect the endogenous DNA-protein complex is due to the low affinity of the E2–2 proteins for the imperfect μE5 site or to the fact that the concentration of this protein in crude nuclear extract in a suitable form to bind to this site is too low to be detectable. The former possibility is unlikely, because the fusion of VL1 cDNA with the GAL4 activation domain could activate, in the yeast one-hybrid system (43), the minimal histidine promoter containing four tandem repeats of the same DNA binding sequence (−492 to −467) (data not shown). Moreover, the addition of GST-VL2 protein in the brain nuclear extract generated a unique retarded complex (most likely a heterodimer), which can be recognized by the E2–2 antibody (Fig. 8).

Given the fact that the 18-bp (−484 to −467) cis-sequence is sufficient to activate the FGF-1.B promoter in normally growing U1240MG cells (29), the regulation of FGF-1.B promoter activity in other condition (e.g. in neuronal cells at different
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stages of development) awaits further exploration. In fact, the transfection experiment in U1240MG cells showed that the overexpression of the full-length E2–2 protein lacking the RSRS domain can reduce the activity of an heterologous promoter (without RSRS) has repressor activity.

The mechanism that could discriminate the two splice variants of E2–2 protein (i.e. with or without RSRS) in terms of their effect on the promoter activity through the μE5 site is not known. The possibility of squelching effect through overexpressed SEF2–1B (without RSRS) also has not been ruled out. However, it is interesting to note that RSRS is inside a highly negatively charged stretch of 50 amino acids. In fact, the activation domains of many transcriptional activators are highly negatively charged (44). Thus, the presence or absence of RSRS inside of the highly negatively charged domain in the E2–2/SEF2–1B protein (encoded by one of the splice variants) could be very important, contributing to the different transcriptional properties of these proteins. It is likely that the relative abundance and dynamic balance of the two splice variants in the brain could be an important determinant for the expression of FGF-1.B. Finally, we showed that in brain nuclear extract, a heterodimer comprising E2–2 binds to the imperfect μE5 site of the FGF-1.B promoter (Fig. 8). It would be of interest to identify the brain-specific bHLH transcription factor that partners with E2–2 or with other bHLH proteins to activate the FGF-1.B gene expression in the brain.

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FIG. 10. SEF2–1B (lacking RSRS) represses the luciferase reporter activity mediated by a hexameric E-box (μE5–μE2) linked to alkaline phosphatase TATA box (E47 reporter (A)) or of FGF-1.B promoter containing an imperfect E-box (μE5) site (B). Effector plasmids SEF2–1B (lacking RSRS) (closed circle), E47/pCSA (open circle), VL3-FL (with RSRS) (closed triangle), and pCMVAcAT (open triangle) (described under “Materials and Methods”) at different doses (0–5 μg) were cotransfected into U1240MG cells with 10 μg of E47 reporter plasmid (A) or FGF-1.B (–540) in pGL2Basic (B). Plasmid pRL-tk (0.5 μg) was also cotransfected in the same cell line in each experiment as an internal control. Sonicated herring sperm DNA was supplemented to make up 15 μg of total DNA in each transfection. Luciferase activity was normalized by the dual-luciferase reporter assay system (Promega). The results of a representative experiment are shown here. The standard errors of the triplicates carried out in each experiment are within 5–10%.
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