Cloning and Characterization of Murine Glial Cell-Derived Neurotrophic Factor Inducible Transcription Factor (MGIF)

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The potent neurotrophic factor glial cell-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-β (TGF-β) superfamily of proteins. We report a transcription factor that is the first nuclear protein known to be induced by GDNF, thus designated murine GDNF inducible factor (mGIF). The cDNA was cloned in the course of investigating transcription factors that bind to Sp1 consensus sequences, using the *in situ* filter detection method, and it was found to encode a protein having the same C_2–H_2 zinc finger motif as Sp1. Sequence analysis indicated that mGIF is homologous to the human TGF-β inducible early gene (TIEG) and human early growth response gene-α (EGR-α). mGIF is widely distributed in the adult mouse with high mRNA levels in kidney, lung, brain, liver, heart, and testis. In the adult brain, mGIF is abundantly expressed in hippocampus, cerebral cortex, cerebellum, and amygdala with lower amounts in striatum, nucleus accumbens, olfactory tubercle, thalamus, and substantia nigra. During development, mGIF mRNA also has a wide distribution, including in cerebral cortex, cerebellar primordium, kidney, intestine, liver, and lung. GDNF induces the expression of mGIF rapidly and transiently both in a neuroblastoma cell line and in primary cultures of rat embryonic cortical neurons. Co-transfection of the *Drosophila* SL2 cells using mGIF expression plasmid and reporter constructs having Sp1 binding sites indicated that mGIF represses transcription from a TATA-containing as well as from a TATA-less promoter. These observations suggest that the zinc finger transcription factor mGIF could be important in mediating some of the biological effects of GDNF.

Key words: glial cell-derived neurotrophic factor (GDNF); zinc finger; Sp1; transcription; cloning; transforming growth factor-β (TGF-β)

Glial cell-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-β (TGF-β) superfamily, which comprises an expanding list of multifunctional proteins serving as regulators of cell proliferation and differentiation (Kingsley, 1994). GDNF has trophic effects on midbrain dopaminergic, spinal motor, cranial sensory, sympathetic, and hindbrain noradrenergic neurons (Lin et al., 1993; Henderson et al., 1994). It also protects and restores dopamine neurons from injuries *in vitro* as well as *in vivo* (Beck et al., 1995; Oppenheim et al., 1995; Tomac et al., 1995a; Yan et al., 1995; Gash et al., 1996). Thus, it is considered a potential therapeutic agent for neurodegenerative diseases such as Parkinson's disease, although its molecular signaling mechanisms remain to be elucidated. Although the receptors mediating the physiological actions of GDNF are being identified (Durbec et al., 1996; Jing et al., 1996; Trupp et al., 1996; Baloh et al., 1997), the transcriptional regulatory mechanisms that ensue activation of these receptors remain to be identified.

Transcription of genes in eukaryotes is controlled by a complex set of regulatory factors that bind to distinct DNA elements. Although many short DNA sequences are known to be specific transcription factor binding sites, three main motifs in the DNA binding domain of transcription factors have been identified including the zinc finger, helix-turn-helix, and leucine zipper motifs (Harrison, 1991). Among these, the zinc finger motif is composed of 25–30 amino acids containing cysteine and histidine residues that are involved in the tetrahedral coordination of a zinc atom. Considerable variations of this motif are known to occur depending on the number of fingers and the different combinations of cysteines or histidines in a finger.

A prototypical zinc finger protein is Sp1, which has a three-Cys_2–His_2 zinc finger motif. It was originally cloned as a transcription factor that binds to the simian virus 40 early promoter (Kadonaga et al., 1987) and is now known to activate a wide range of vertebrate genes. Several additional transcription factors having the same zinc finger motif as Sp1 have been cloned and found to bind to the GC box sequence. These include Sp2 (Kingsley and Winoto, 1992), Sp3 (Hagen et al., 1992; Kingsley and Winoto, 1992), Sp4 (Hagen et al., 1992), basic transcription element-binding protein (BTEB; Imataka et al., 1992), the *Drosophila* buttonhead gene product (btd; Wimmer et al., 1993), TGF-β inducible early gene (TIEG; Subramaniam et al., 1995), and early growth response gene-α (EGR-α; Blok et al., 1995).

In the present investigation, we cloned and characterized a murine Sp1-related zinc finger transcription factor cDNA, which is expressed in the adult and developing brain. We found that GDNF induces the mRNA expression of this nuclear protein, and, therefore, we named it murine GDNF inducible factor (mGIF).

MATERIALS AND METHODS

Expression cloning and rapid amplification of cDNA 5′ ends. Total RNA was prepared from the murine neuroblastoma cell line NB41A3 [American Type Culture Collection (ATCC), Rockville, MD] by the guanidinium/cesium chloride method (Sambrook et al., 1989), and poly(A)$^+$ RNA was obtained using a Quick poly(A) column (Stratagene, La Jolla, CA). A λgt11 cDNA library was constructed using oligo-dT primer and the SuperScript Choice System for cDNA Synthesis (Life Technologies, Gaithersburg, MD) and packaged with Gigapack II gold (Stratagene). The probe used was the Sp1(A) region in the rat D_2 dopamine receptor gene, which is one of two Sp1 binding sites within the NegB region of this gene (Minowa et al., 1994). Synthetic oligodeoxynucleotides 5′-*CTAG*CAAG<u>GGGCGG</u>GGTTC-3′ and 5′-*CTAG*GAACC<u>CCGCCC</u>CTTG-3′ were end-labeled with ^{32}P, annealed, and ligated to generate a concatenated probe. The Sp1 consensus sequence is underlined, and the bases added to the Sp1(A) sequence to generate the concatenated probe are in italic. Screening of the expression library followed published procedures (Vinson et al., 1988) with some modifications (Mizuno et al., 1990). One of the positive clones was subsequently characterized and designated mGIF.

To obtain the 5′ portion of mGIF cDNA, the rapid amplification of cDNA 5′ ends (5′-RACE) system (Life Technologies) was used with 1 μg of poly(A)$^+$ RNA from NB41A3 cells, 2 pmol of mGIF gene-specific primer (5′-CACCTGAGTGCCCATGAACAAC-3′, corresponding to nucleotides +933 to +912 relative to the translation initiation site), and a second mGIF gene-specific primer (5′-GACGAAGGAGCTGGCT-GAGAC-3′, +743 to +723). cDNA fragments of positive clones were subcloned in pUC19 and sequenced.

Plasmid constructions. Full-length mGIF cDNA was constructed by combining the original λgt11 clone and the 5′-RACE clone using the unique *Hae*II site within mGIF.

For *in vitro* transcription to generate an *in situ* hybridization probe, the 578 bp *Hin*dIII–*Pst*I fragment of mGIF (nucleotides +31 to +609 relative to the first ATG) was subcloned into the same sites of pGEM3Zf(−) (Promega, Madison, WI).

For functional analysis, an expression plasmid of mGIF in the *Drosophila* Schneider's cell line SL2 was constructed in pRmHa3 (a kind gift from Carl Wu, National Cancer Institute, Bethesda, MD) (Rabindran et al., 1993), which has a multiple cloning site between the *Drosophila* metallothionein promoter and the 3′-end sequence of the *Drosophila* alcohol dehydrogenase gene in pUC18. The 2.6 kb *Hin*dIII–*Sac*I fragment of mGIF was inserted into the *Sac*I site of pRmHa3 to generate pRmGIF. This construct lacks nine amino acids from the N terminus of mGIF. As reporter plasmids, BCAT-2 (a kind gift from Robert Tjian, University of California, Berkeley, CA) (Pascal and Tjian, 1991) and pCATD2-116 (Minowa et al., 1994) were used.

Southern blot analysis. Mouse genomic DNA was digested with *Eco*RI or *Bam*HI, electrophoresed in a 0.7% agarose gel (7 μg/lane), and transferred to nitrocellulose filters. Hybridization was performed using the random-primed, ^{32}P-labeled, 578 bp *Hin*dIII–*Pst*I fragment of mGIF cDNA as probe in 4× SSC, 40% formamide, 0.1% SDS, 1× Denhardt's solution and 25 μg/ml sheared salmon DNA at 42°C overnight. Filters were washed three times in 1× SSC and 0.1% SDS at 25°C and four times in 0.1× SSC and 0.1% SDS at 40°C as described previously (Hara et al., 1992). Filters were then exposed to a phosphor screen and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell culture and transfection. For mGIF mRNA induction experiments in the neuroblastoma cell line NB41A3, cells were cultured in 100 mm dishes containing DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 10% CO_2 in the presence of 10% (for GDNF) or 0.2% (for TGF-β) serum for 36 hr. Subsequently, 800 ng of human recombinant GDNF (R&D Systems, Minneapolis, MN) or 50 ng of human platelet TGF-β (Calbiochem, San Diego, CA) was added per dish. Cells were harvested at different time points, and total RNA was prepared using RNAsolB (Tel-Test, Friendswood, TX).

For mGIF induction in primary neuronal cultures, whole neocortices of embryonic day 18 (E18) rats were dissociated mechanically and plated onto poly-D-lysine- and laminin-coated dishes at a density of 1×10^3 cells/mm^2. After growing the cells in neurobasal medium (Life Technologies), 0.5 mM glutamine, 2% fetal bovine serum, and nutrient mixture N2 (Life Technologies) for 80 hr, the medium was replaced with fresh medium lacking serum with or without 80 ng/ml GDNF or 5 ng/ml TGF-β. Total RNA was extracted at the indicated time points by an acid–phenol method (Sambrook et al., 1989).

To study the transcriptional activity of mGIF, SL2 cells (ATCC) were

maintained in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) in ambient air. Transfections were performed using Lipofectin (Life Technologies) with HyQ-CCM 3 serum-free medium (HyClone, Logan, UT) in 60 mm dishes. To study plasmid dosage effect, various amounts of BCAT-2 or pCATD2-116 were initially used individually to co-transfect SL2 cells. Subsequently, 2 μg of BCAT-2 or 1 μg of pCATD2-116 and varying amounts of pRmGIF were used to co-transfect SL2 cells. The vector pRmHa3 was added to keep the total plasmid content equal for all dishes. After a 16 hr incubation, mGIF expression was induced with 0.7 mM $CuSO_4$, and the cells were harvested 24 hr after induction and lysed by repeated cycles of freezing and thawing. Chloramphenicol acetyltransferase (CAT) activity was measured using a CAT ELISA kit (Boehringer Mannheim, Indianapolis, IN).

Northern blot analysis. To study the distribution of mGIF, a mouse multiple tissue northern (MTN) blot membrane (Clontech, Palo Alto, CA) was hybridized with a 36 mer mGIF probe (5′-GCAAGCTGT-CATCCTCGGAGGTATCAGACACTGGTG-3′, bases +229 to +194) using the manufacturer's protocol.

Induction of mGIF in NB41A3 cells was studied by electrophoresing 20 μg of total RNA in a 1.8% agarose–formaldehyde gel in 3-[*N*-morpholino]propane-sulfonic acid buffer, transferred to a Zeta membrane (Bio-Rad Laboratories, Hercules, CA), and cross-linked by UV light. The 578 bp *Hin*dIII–*Pst*I fragment of mGIF was ^{32}P-labeled with the random-priming method and used as probe in ExpressHyb (Clontech) at 68°C for 1 hr. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Minowa et al., 1996) was used as a control under the same hybridization conditions. Band intensities were measured by a PhosphorImager.

To study mGIF induction in primary neuronal cultures, 40 μg of total RNA was electrophoresed and subjected to Northern hybridization as described above using the 578 bp mGIF probe and the control GAPDH probe as described above. Filters were then exposed to a phosphor screen.

In situ hybridization. An adult mouse brain was sectioned at 12 μm thickness, sections were placed on silanized slides, and *in situ* hybridization was performed using a previously described procedure (Bradley et al., 1992; Young et al., 1993). The 578 bp mGIF riboprobe was labeled with ^{35}S-uridine triphosphate (UTP) by *in vitro* transcription using MAXIscript (Ambion, Austin, TX), and 1×10^6 cpm were applied to each slide. Finally, hybridized slides were dipped in NTB3 emulsion (Eastman Kodak, Rochester, NY). Sense riboprobe was used as a control.

To study the expression pattern of mGIF during development, E14 and E16 embryos of NIH Swiss mice were fixed in buffered 4% paraformaldehyde for 24 hr at 4°C, embedded in paraffin, and sectioned at 7 μm thickness followed by mounting onto Vectabond (Vector Laboratories, Burlingame, CA)-treated slides. Sections were cleared of paraffin with three 5 min washes in xylene and two washes in 100% ethanol. *In situ* hybridization was then performed as described above. Sections were counterstained with hematoxylin and eosin.
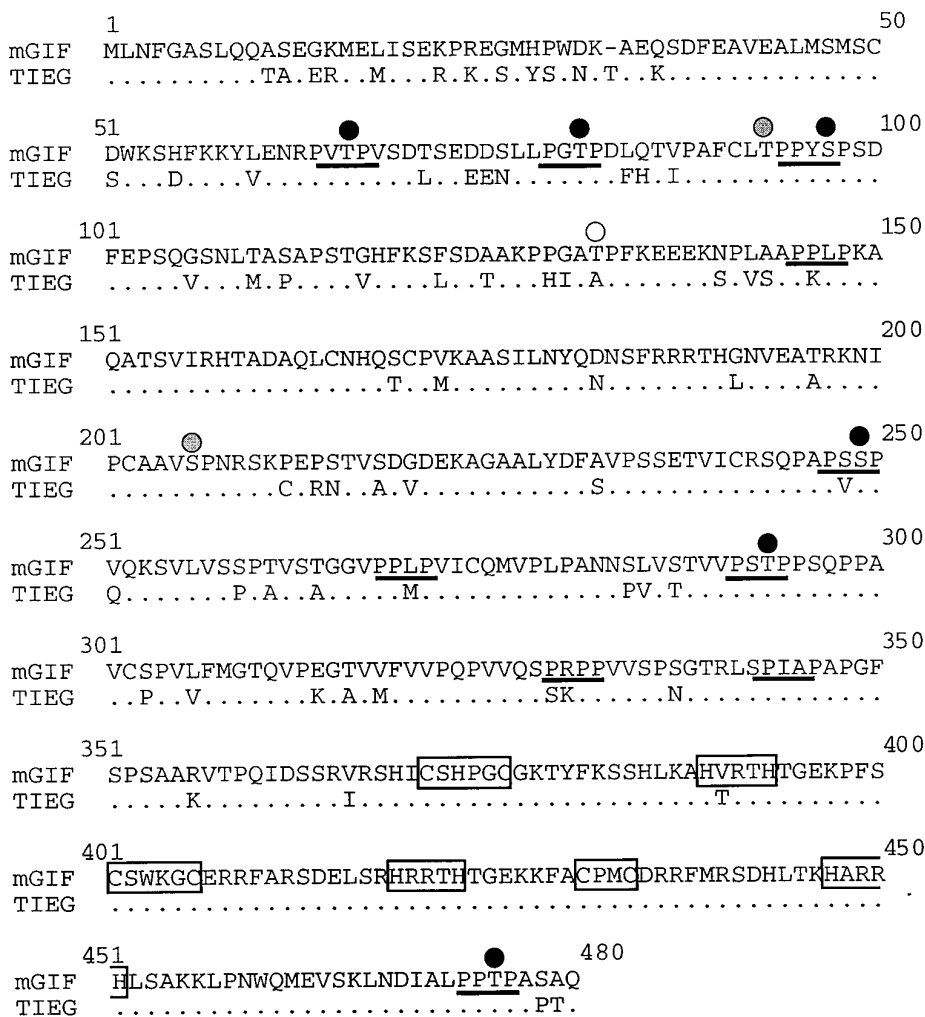
RESULTS

Cloning the cDNA for the zinc finger protein mGIF

In our efforts to clone transcription factors that bind to Sp1 consensus sequences, we used the NegB sequence of the rat D_2 dopamine receptor gene (Minowa et al., 1994) as a probe with an NB41A3 cell expression library in the *in situ* filter detection method. The NegB region consists of three TGGG repeats and one Sp1 consensus sequence [GGGCGG; Sp1(A)], both of which bind to the same proteins (Minowa et al., 1994). Initially, we used a concatenated, double-stranded oligonucleotide probe containing Sp1(A) sequence for library screening. After establishing positive clones, we confirmed their DNA-binding profile using a probe having the TGGG repeat sequence. Among ~500,000 plaques screened, six positive clones were identified.

DNA sequencing revealed that one of the clones, mGIF, codes for a three-Cys_2–His_2 zinc finger motif similar to Sp1. Because this clone did not include the entire open reading frame, we performed the 5′-RACE procedure using a gene-specific primer located in the 5′ end of the available sequence. A single band, about 900 bp long, was obtained and PCR products were sub-

A.

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        1                                                  50
mGIF  MLNFGASLQQASEGKMELISEKPREGMHPWDK-AEQSDFEAVEALMSMSC
TIEG  ..........TA.ER..M...R.K.S.YS.N.T..K..............

       51                 ●           ●            ◉  ●  100
mGIF  DWKSHFKKYLENRPVTPVSDTSEDDSLLPGTPDLQTVPAFCLTPPYSPSD
TIEG  S...D....V..........L..EEN.......FH.I.............

      101                             ○                 150
mGIF  FEPSQGSNLTASAPSTGHFKSFSDAAKPPGATPFKEEEKNPLAAPPLPKA
TIEG  .....V...M.P....V....L..T...HI.A.......S.VS..K....

      151                                             200
mGIF  QATSVIRHTADAQLCNHQSCPVKAASILNYQDNSFRRRTHGNVEATRKNI
TIEG  ...................T..M.........N........L....A....

       201      ◉                                    ● 250
mGIF  PCAAVSPNRSKPEPSTVSDGDEKAGAALYDFAVPSSETVICRSQPAPSSP
TIEG  ...........C.RN..A.V...........S..............V..

      251                                    ●        300
mGIF  VQKSVLVSSPTVSTGGVPPLPVICQMVPLPANNSLVSTVVPSTPPSQPPA
TIEG  Q.......P.A..A.....M............PV.T.............

      301                                             350
mGIF  VCSPVLFMGTQVPEGTVVFVVPQPVVQSPRPPVVSPSGTRLSPIAPAPGF
TIEG  ..P..V.......K.A.M.........SK......N.............

      351                                             400
mGIF  SPSAARVTPQIDSSRVRSHICSHPGCGKTYFKSSHLKAHVRTHTGEKPFS
TIEG  .....K.........I.........................T........

      401                                             450
mGIF  CSWKGCERRFARSDELSRHRRTHTGEKKFACPMCDRRFMRSDHLTKHARR
TIEG  .................................................

      451                       ●      480
mGIF  HLSAKKLPNWQMEVSKLNDIALPPTPASAQ
TIEG  ...........................PT.
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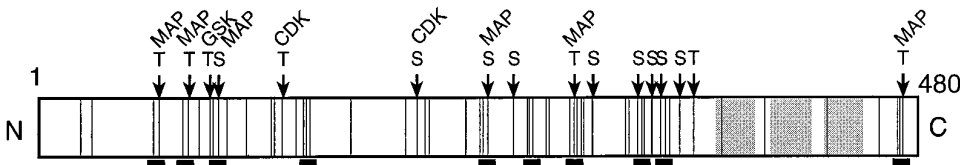
B.

Figure 1. Sequence alignment, homology analysis, and primary structure of mGIF. *A*, Amino acid sequence alignment between mGIF and human TIEG (Subramaniam et al., 1995). Only amino acids that are different from mGIF are shown in the TIEG sequence. Human EGRα has the same amino acid sequence as TIEG except for 12 residues absent from its N terminus (Blok et al., 1995). C-terminal *boxed* residues indicate the zinc finger motif. *Underlined* sequences are putative SH-3 binding sites (Pro-*X*-*X*-Pro). *Filled*, *open*, and *shaded circles* show putative phosphorylation sites by MAP kinase, GSK, and CDK, respectively. *B*, Diagram of the primary structure of mGIF protein. *Vertical lines* represent proline residues, and *T* or *S* shows Thr or Ser followed by proline. Putative phosphorylated residues are indicated by the name of kinases on the *T* or *S*. *Shadowed boxes* show the zinc finger regions. *Dashes* indicate the positions of putative SH-3 binding sites.

cloned in pUC19. Two independent clones were sequenced for confirmation and found to have identical sequence. Subsequently, the 5′ fragment from the 5′-RACE clone and the 3′ fragment obtained from the original library screening were ligated using the unique *Hae*II site to yield the full-length expression clone pRmGIF. Both strands of the 3036 bp insert were sequenced. The deduced amino acid sequence and other structural features are shown in Figure 1. Sequence comparison with the GenBank database showed that mGIF had 85% identity in its amino acid sequence to human TIEG (Subramaniam et al., 1995) and human EGR-α (Blok et al., 1995).

Southern blot analysis with mGIF probe

To assess the possible presence of other genes homologous to mGIF in the mouse genome, we performed Southern blot anal-

ysis followed by washing at low-stringency conditions (Fig. 2). This experiment revealed a single intense band as well as at least two additional bands, suggesting the presence of other genes having various degrees of sequence homology to mGIF.

Tissue distribution of mGIF

To establish the tissue distribution of mGIF mRNA in the adult mouse, we performed Northern blot analysis using a mouse MTN blot membrane (Fig. 3). A 3.6-kb-long mRNA was detected in various tissues at different abundance levels. Highest amounts of mGIF mRNA were found in kidney and lung, followed by brain, liver, and heart. Minimal levels were seen in testis, and none was seen in skeletal muscle or spleen. Thus, mGIF has a relatively wide tissue distribution.
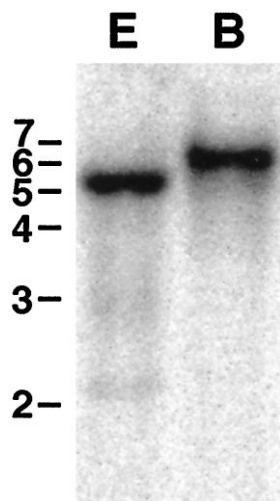
Figure 2. Genomic DNA Blot Analysis with mGIF probe. Southern hybridization was performed on mouse genomic DNA followed by washing at low-stringency conditions. Each lane contains 7 μg of genomic DNA. The probe used is the 578 bp *Hin*dIII–*Pst*I mGIF fragment labeled with ^{32}P by random priming. *E, Eco*RI; *B, Bam*HI.
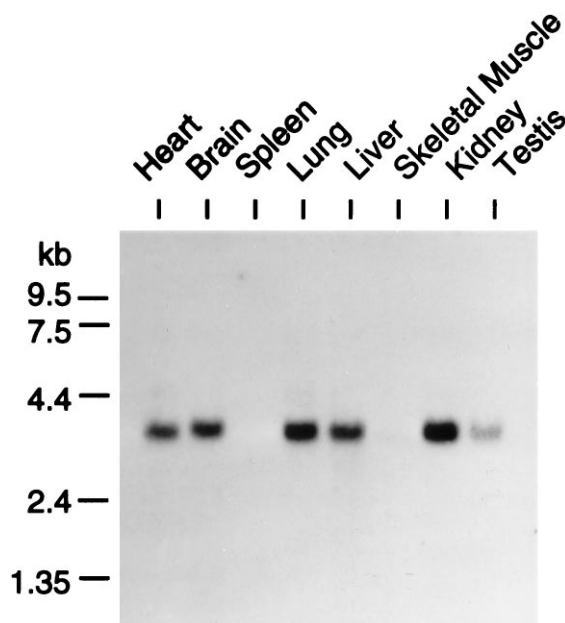

Figure 3. Multiple-tissue Northern blot analysis of mGIF. Each lane contains 2 μg of poly(A)$^+$ RNA from mouse tissues. The probe used is a 5′ end-labeled 36 mer mGIF oligonucleotide.

Brain regional expression of mGIF

To determine the brain regional distribution of mGIF, *in situ* hybridization was performed using adult mouse brain sections and a probe generated by *in vitro* transcription with ^{35}S-UTP (Fig. 4). Specific hybridization was observed throughout the cerebral cortex, including neocortex, archicortex, and paleocortex. In the neocortex, mGIF was expressed most prominently in layer 6 and to a lesser extent in other cortical layers. In the hippocampus, strong expression was present in pyramidal cells of CA1, CA2, and CA3, as well as in granule cells of the dentate gyrus and in tenia tecta. Intense signal was also detected in pyriform cortex. Caudate–putamen, accumbens, and olfactory tubercle had signif-

icant but weaker signals. In the amygdala, the most intense hybridization was detected in the basolateral and lateral nuclei with weaker signals in other areas. In the thalamus, the parafascicular nucleus had a detectable signal. In the substantia nigra, weak but significant signal was detected in pars compacta with scattered positive cells in pars reticulata. Prominent hybridization was also seen in the granule cell layer of cerebellum. Specific hybridization was also present in the trigeminal motor nucleus of the brainstem.

Expression of mGIF during development

Whole mouse embryos were also analyzed for mGIF mRNA expression by *in situ* hybridization histochemistry. Using embryos at E14 and E16, we found that mGIF is widely expressed (Fig. 5). In the nervous system, it is most prominent in cerebral cortex, cerebellar primordium, and the dorsal edge of the spinal cord. Although the level of mGIF expression in the embryonic brain is not as high as in the adult brain, clearly some similarities are noted, especially in the cerebral cortex and cerebellar primordium. mGIF is also expressed in the body surface, most clearly in the vibrissa of the nose and to a lesser extent in other areas of skin. Bones and differentiating mesenchyme surrounding the nasal cavity, jaw, basisphenoid bone of the skull, as well as back bones and ribs also have this transcript. In the respiratory and digestive systems, significant levels of mGIF mRNA are present in lung, liver, and intestine, including myenteric ganglia. In the excretory system, mGIF is prominently expressed in the kidney at E16.

Induction of mGIF mRNA expression by GDNF

Because mGIF is homolgous to a human gene that was cloned based on its inducibility by TGF-β, we sought to determine whether GDNF, which belongs to the TGF-β superfamily, induces mGIF. Thus, GDNF was added to NB41A3 cells, and the expression level of endogenous mGIF mRNA was examined by Northern blot analysis. We consistently found at least a twofold to threefold increase in mGIF mRNA levels attaining a peak at 1.5 hr after the addition of GDNF (Fig. 6*A*). This induction was brief, with a rapid decline as early as 2 hr. Northern blot analysis using the control probe GAPDH indicated uniformity of the amount of mRNA loaded per lane as well as specificity of mGIF induction. Similar to human TIEG, mGIF could also be induced at least 2- to 2.5-fold by TGF-β peaking at 1.5 hr and persisting for at least 4 hr (Fig. 6*B*).

The ability of GDNF and TGF-β to induce mGIF mRNA expression was also evaluated in primary cultures of rat embryonic cortical neurons (Fig. 6*C*). RNA was extracted after different periods of treatment, and Northern blot analysis was performed using the mouse mGIF riboprobe. A rapid and transient induction of mGIF expression was found peaking at 30 min after incubation with GDNF or TGF-β (Fig. 6*C*).

Functional characterization of mGIF

Based on the deduced amino acid sequence of mGIF, which has an Sp1-like zinc finger motif, as well as its DNA-binding activity, we expected it to function as a transcription factor. To examine this possibility, we performed transient co-transfection assays in Schneider's *Drosophila* cells using the mGIF expression plasmid and two different CAT reporter plasmids. These cells were chosen to allow analysis of mGIF function under controlled conditions, because they do not express Sp family proteins, which bind to the same DNA sequences as mGIF (Santoro et al., 1988).

The BCAT-2 reporter plasmid has two Sp1 binding sites and a TATA box followed by the CAT gene. SL2 cells were transfected
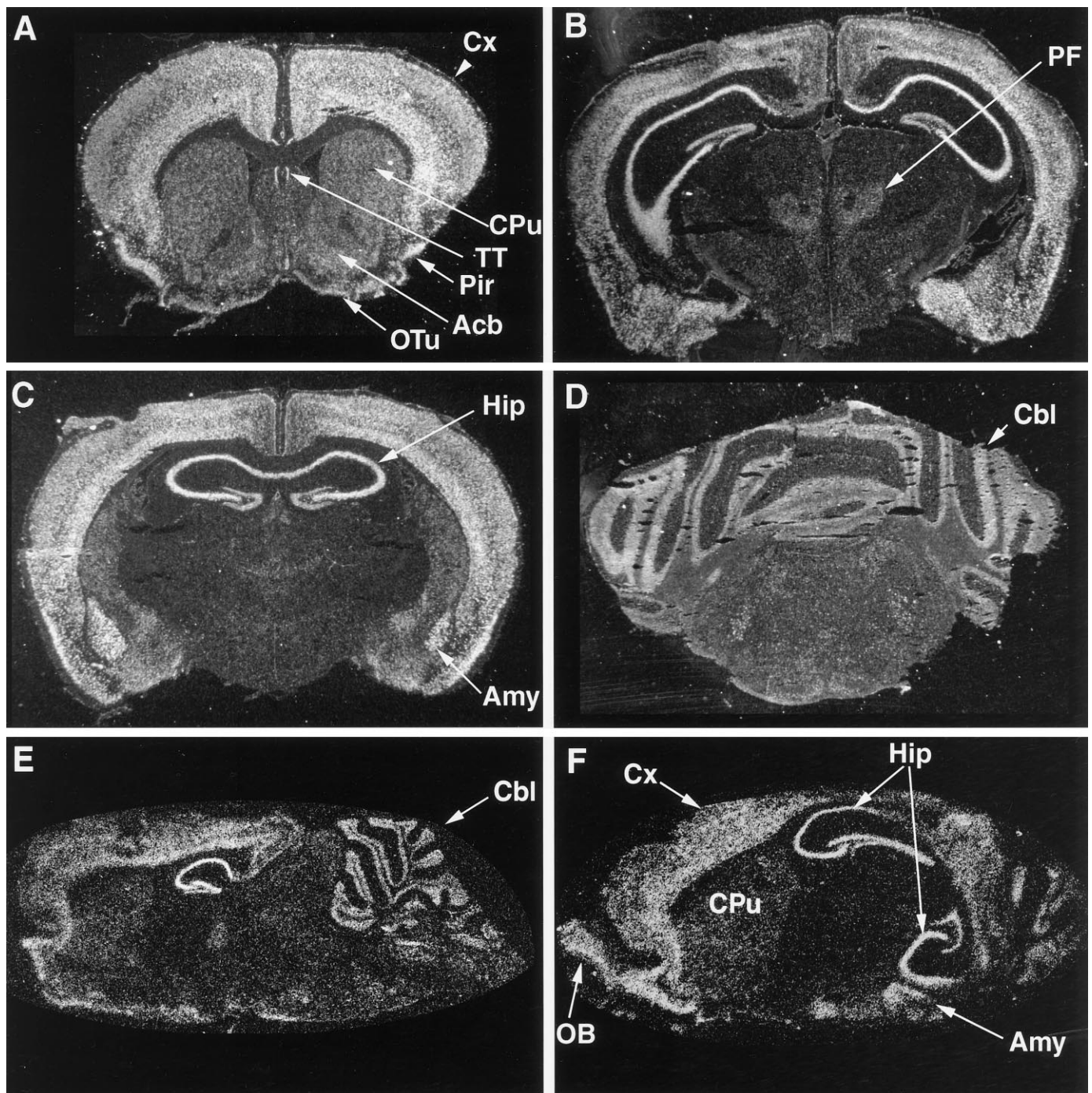
Figure 4. mGIF mRNA localization in the adult mouse brain. Dark-field photomicrographs from liquid emulsion autoradiograms of coronal sections (*A–D*; anterior to posterior) and x-ray film autoradiograms of sagittal sections (*E, F*; medial and lateral, respectively). *Acb*, Accumbens; *Amy*, amygdala; *Cbl*, cerebellum; *CPu*, caudate putamen; *Cx*, cerebral cortex; *OB*, olfactory bulb; *OTu*, olfactory tubercle; *PF*, parafascicular thalamic nucleus; *Pir*, pyriform cortex; *TT*, tenia tecta. Twelve-micrometer-thick sections were hybridized with the 578 bp *Hin*dIII–*Pst*I mGIF riboprobe.

with varying amounts of BCAT-2 alone to establish the linear portion of plasmid concentration–CAT level curve (Fig. 7*A*). Subsequently, when SL2 cells were co-transfected with a fixed "non-saturated" amount of BCAT-2 and the mGIF expression plasmid pRmGIF, CAT activity decreased significantly in a dosage-dependent manner with increasing amounts of pRmGIF (Fig. 7*C*). We also investigated the activity of mGIF on the NegB sequence in the TATA-less D_2 dopamine receptor promoter (Minowa et al., 1994), which was originally used as the screening probe. We used

the reporter plasmid pCATD2-116, which has the NegB sequence. After performing titration experiments with pCATD2-116 (Fig. 7*B*), the results of co-transfection with pRmGIF were similar to those obtained with BCAT-2 (Fig. 7*D*). Thus, mGIF could repress promoter activity in two different reporter constructs.

DISCUSSION

We have cloned the cDNA encoding a murine GDNF inducible transcription factor designated mGIF. It is homolgous to two
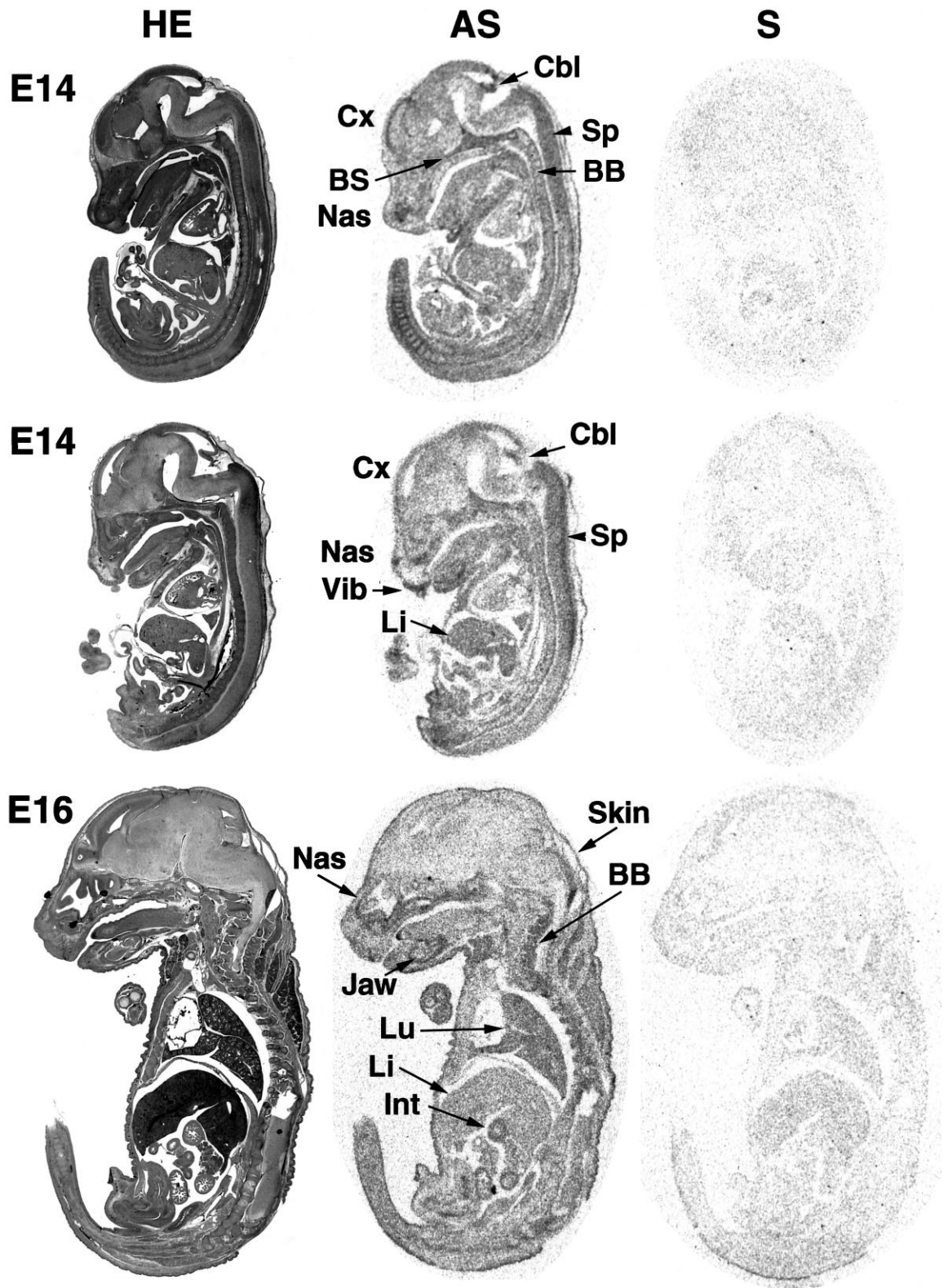
Figure 5. Expression pattern of mGIF mRNA during embryogenesis. Expression of mGIF mRNA was analyzed by *in situ* hybridization histochemistry in the whole mouse embryo on embryonic E14 (*first* and *second rows*) and E16 (*third row*). Photomicrographs of sagittal sections counter-stained by hematoxylin and eosin (*HE*) and x-ray film autoradiograms hybridized with antisense (*AS*) and sense (*S*) mGIF probes are shown in the *first, second,* and *third columns*, respectively. *BS*, Basisphenoid bone; *BB*, back bone; *Cbl*, cerebellum; *Cx*, cerebral cortex; *Int*, intestine; *Li*, liver; *Lu*, lung; *Nas*, nasal cavity; *Sp*, spinal cord; *Vib*; Vibrissa. Seven-micrometer-thick sections were hybridized with the 578 bp *Hin*dIII–*Pst*I mGIF riboprobe.

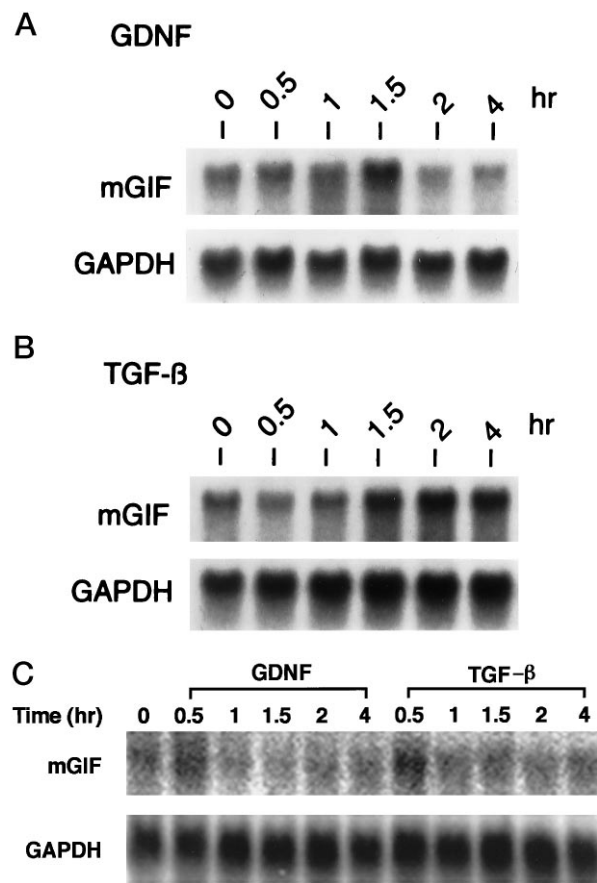*Figure 6.* mGIF mRNA induction by GDNF and TGF-β. Northern blot analysis of mGIF mRNA levels after GDNF (*A*) or TGF-β (*B*) induction in NB41A3 cells. Indicated times show incubation periods. The probe was the 578 bp *Hind*III–*Pst*I mGIF fragment labeled with ^{32}P by the random-primed method. *C*, Induction of mGIF mRNA in primary rat embryonic neuronal cultures after treatment with 80 ng/ml GDNF or 5 ng/ml TGF-β. Northern analysis for GAPDH mRNA was performed as a control.

Figure 7. Functional analysis of mGIF by co-transfection CAT assay. SL2 cells were transfected with increasing amounts of BCAT-2 (*A*) or pCATD2-116 (*B*) reporter plasmids. SL2 cells were co-transfected with various amounts of mGIF expression plasmid pRmGIF and either 2 μg of BCAT-2 (*C*) or 1 μg of pCATD2-116 (*D*) reporter plasmids. Means ± SE for triplicates are shown as percentage of basal activity for each reporter plasmid. *ANOVA, $p < 0.01$; **$p < 0.05$ compared with no pRmGIF. Similar experiments repeated two to three times yielded reproducible results.

human genes, TIEG (Subramaniam et al., 1995) and EGR-α (Blok et al., 1995). TIEG was cloned from fetal osteoblastic cells and found to be induced by TGF-β and by epidermal growth factor (EGF), whereas EGR-α was cloned from prostate carcinoma cells and found to be induced by EGF and repressed by androgens. TIEG and EGRα have identical amino acid sequences except for 12 residues absent in the N terminus of EGRα. Thus, TIEG and EGRα appear to be encoded by the same gene. Sequence comparison between murine mGIF and these two human proteins indicates 85% amino acid identity. Comparison of their nucleotide sequences revealed that although these cDNAs are homologous within their open reading frame, more diversity exists in their 3′ untranslated regions. Both the human and murine proteins are rich in proline. mGIF has two proline-rich regions; one contains 17 prolines of 90 residues, and the other contains 31 prolines of 160 residues. Although mGIF does not show any sequence homology to the TGF-β-responsive domain located in the proline-rich region of CTF/NF-1 (Alevizopoulos et al., 1995), it has 10 Pro-*X-X*-Pro sequences, which are putative Src homology 3 (SH-3) binding motifs (Yu et al., 1994) and 16 Ser/Thr-Pro sequences, which may be targets for proline-directed kinases. Among these Ser/Thr-Pro sequences, six are putative target sites for mitogen-activated protein (MAP) kinase (Pro-
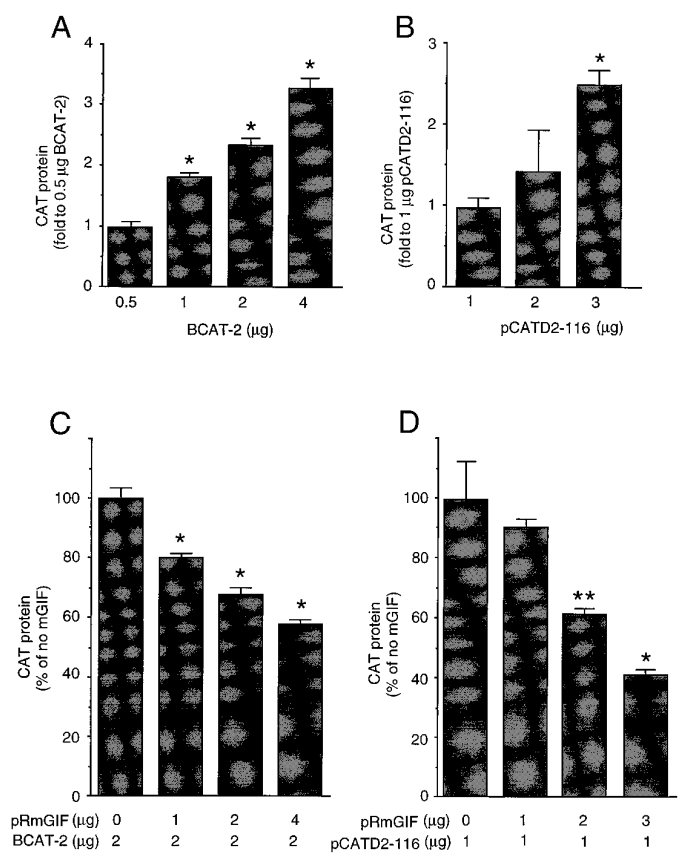
basic/neutral-Ser/Thr-Pro) (Alvarez et al., 1991; Clark-Lewis et al., 1991; Mukhopadhyay et al., 1992; Sanghera et al., 1992), two are for cyclin-dependent protein kinase (CDK) (Ser/Thr-Pro-*X*-Lys/Arg) (Moodie et al., 1993), and one is for glycogen synthase kinase (GSK) (Ser/Thr-Pro-*X-X*-phospho-Ser/phospho-Thr) (Pelech, 1995) (Fig. 1*A,B*). These features suggest that mGIF could be regulated post-translationally and could be involved in signal transduction pathways modulating the expression of various genes.

Although the similarity between mGIF and TIEG/EGR-α is 85% at the amino acid level and 81% at the nucleotide level, substantial differences exist in both their sequence and tissue distribution. mGIF mRNA in the adult mouse is present in heart, lung, brain, liver, kidney, and testis but not in spleen or skeletal muscle. On the other hand, TIEG is strongly expressed in skeletal muscle but not in brain, lung, or kidney (Subramaniam et al., 1995). Furthermore, Southern blot analysis revealed the presence of at least three genes in the mouse genome with various degrees of sequence homology to mGIF. Thus, these observations suggest a new class of TGF-β family inducible transcription factors, which, to date, include mGIF and TIEG/EGR-α. Whether mGIF and human TIEG represent homologs of the same gene in two different species or distinct members of a group of proteins remains to be determined by isolating additional homologs.

The induction of mGIF by GDNF was hypothesized based on its homology to TIEG and based on its high expression level in brain and other GDNF-responsive tissues. mGIF expression in NB41A3 cells, from which it was originally cloned and which express Ret receptor mRNA determined by reverse transcription-PCR (data not shown), increased transiently after GDNF treatment. In addition, mGIF mRNA levels increased briefly in primary cultures of rat embryonic cortical neurons after GDNF treatment. Thus, mGIF is the first recognized transcription factor to be induced by GDNF. mGIF is also inducible by TGF-β. However, in contrast to the 10-fold induction of TIEG (Subramaniam et al., 1995), mGIF mRNA increased to a much lesser extent after TGF-β treatment.

The distribution of mGIF mRNA was examined in the adult mouse brain by *in situ* hybridization, because we found it to be inducible by GDNF. The results showed a specific expression pattern with strong hybridization in several regions particularly in hippocampus, cerebral cortex, amygdala, and cerebellum, with weaker signals in striatum, substantia nigra, nucleus accumbens, olfactory tubercle, and thalamus, supporting an important role for mGIF in various brain functions.

Comparison of the brain expression pattern of mGIF with that of GDNF and its cell surface receptors revealed both similarities and differences. The mRNA distribution of GDNF and its currently known receptors is complex and only partially overlapping. In the developing brain, GDNF mRNA is expressed mainly in the striatum and olfactory tubercle but barely detectable in substantia nigra, where this factor exerts its trophic actions after retrograde transport from the striatum (Tomac et al., 1995b). On the other hand, the basal expression of GDNF in the adult brain is very low (Nosrat et al., 1996). Three GDNF receptor molecules, Ret, GDNFR-α (TrnR1), and TrnR2, have thus far been identified (Durbec et al., 1996; Jing et al., 1996; Trupp et al., 1996; Baloh et al., 1997). Ret is highly expressed in the adult brain in substantia nigra, pons, medulla, thalamus, and cerebellum but not in cerebral cortex, hippocampus, or striatum (Trupp et al., 1996). On the other hand, GDNFR-α mRNA is clearly expressed in cerebral cortex and hippocampus, and it partially overlaps with Ret mRNA in substantia nigra, pons, and thalamus. Furthermore, in several areas of known GDNF action, including nigral and motor neurons, TrnR2 expression is low or undetectable (Baloh et al., 1997). These variations in mRNA expression pattern among GDNF and its receptors are considered indications that such trophic circuits use multiple mechanisms for different neuronal populations (Trupp et al., 1997). The expression pattern of mGIF overlaps with that of GDNF receptors, including in thalamus, cerebellum, and substantia nigra with Ret and in cerebral cortex, hippocampus, cerebellum, and nigra with GDNFR-α. Although GDNF mRNA is barely expressed at basal conditions in the adult brain, treatment with kainic acid or pilocarpine increases its expression in striatum, hippocampus, and cerebral cortex (Humpel et al., 1994; Schmidt-Kastner et al., 1994). Thus, mGIF could transduce the GDNF signal after stimulation of one of its receptors in response to certain physiological or pathological conditions. mGIF is also induced by TGF-β, which transduces its signals across the cell membrane via serine and threonine kinase receptors (Massague, 1996). Although the mRNA expression of Habrec-1, a recently identified TGF-β type I-like receptor, overlaps with that of mGIF (Lorentzon et al., 1996), such as in striatum, olfactory tubercle, and cerebral cortex, the expression patterns of mGIF

and Habrec-1 are not identical. Nevertheless, mGIF could transduce TGF-β signals in certain cell populations. These observations taken collectively could suggest that mGIF is involved in signal transduction from multiple TGF-β-like signals including GDNF.

The distribution of mGIF mRNA in the mouse during embryogenesis also supports its role in transducing the GDNF signal. mGIF is widely expressed in the embryo but most prominently in kidney and intestine, including the enteric nervous system, consistent with the fact that GDNF-null mice lack kidneys and enteric neurons (Schuchardt et al., 1994, 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), and that GDNF mutations are associated with the human intestinal disorder Hirschsprung disease (Angrist et al., 1996; Ivanchuk et al., 1996). Additional overlap is noted in the distribution of mGIF, GDNF, and its receptors in the embryo. It would, thus, be interesting to compare the phenotype of mGIF-null mice with strains lacking the gene for GDNF or its receptors in future studies.

The conserved Sp1-like zinc finger motif in mGIF as well as its ability to bind to Sp1 consensus sequences suggested that it functions as a transcription factor. Among the Sp family of proteins, Sp1 is generally known to be a strong activator, whereas Sp3 can either activate or repress transcription, depending on promoter context (Hagen et al., 1994; Liang et al., 1996). Functional analysis of mGIF by co-transfection CAT assays in SL2 cells, which lack Sp family proteins, allowing interpretation of these reconstitution experiments, revealed that mGIF represses promoter activity both from TATA-containing and TATA-less promoters. Thus, it appears that GDNF-induced increase in mGIF expression results in decreased transcription rate of its target gene(s). It is conceivable, however, that mGIF could also act as an activator in certain promoters.

Several growth factors have been demonstrated to influence the expression of specific genes, mostly activating them. For examples, nerve growth factor activates the ornithine decarboxylase gene (Feinstein et al., 1985); brain-derived neurotrophic factor, neurotrophin-3 (NT-3), and NT-4 induce c-fos (Ip et al., 1993); and TGF-β activates cyclin-dependent kinase inhibitor p15^{INK4B} (Li et al., 1995; Collazo et al., 1992) and α2(I) collagen gene (Inagaki et al., 1994) both through Sp1 consensus sites in the respective promoters. On the other hand, nerve growth factor suppresses the epidermal growth factor receptor gene (Huff et al., 1981; Lazarovici et al., 1987), and basic fibroblast growth factor represses the NMDA receptor gene (Mattson et al., 1993). Although the physiological target gene(s) for mGIF remain(s) to be determined, our results suggest that one of the functions of mGIF is negative transcriptional regulation in the GDNF- and TGF-β-initiated cascades *in vivo*. At present, we have no evidence that mGIF is involved in the *in vivo* regulation of the D_2 dopamine receptor gene, although its consensus Sp1 binding sequences were initially used to clone mGIF.

These observations taken together suggest that mGIF induction could be a key step downstream from GDNF- and TGF-β-related factors. The overlap as well as the diversity in the expression of mGIF with that of TGF-β superfamily members and their respective receptors suggest that the intracellular cascades after TGF-β-related signals might converge on mGIF. The latter could, thus, operate as one of the final common pathways in these signal–transcription coupling events in certain neuronal populations.

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