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Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system

(DNA sequence/RNA blot hybridization/*in situ* hybridization)

OLIVIA HAUB, BEVERLY DRUCKER, AND MITCHELL GOLDFARB

Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, 630 West 168 Street, New York, NY 10032

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ABSTRACT **The murine homolog of the fibroblast growth factor-5 (FGF-5) gene has been cloned, and the sequence of the gene's three exons has been determined. The murine gene and the previously isolated human FGF-5 gene are substantially homologous within the coding sequences and in upstream sequences, which contain an additional open reading frame. We have used a portion of the murine gene as probe to detect FGF-5 RNA in adult mouse tissues by both Northern blot and *in situ* hybridization methods. FGF-5 RNA is present at low levels in widely distributed areas of the central nervous system. Several loci of FGF-5 expression could be localized by *in situ* hybridization and include portions of the cerebral cortex, hippocampus, and thalamus. Neuronal expression accounts for at least some of the FGF-5 RNA synthesized in the central nervous system.**

Fibroblast growth factors (FGFs) constitute a family of mitogenic proteins with related primary structures. Each mammalian factor, of which seven are now known, is encoded by a distinct gene (1–8). FGFs are mitogenic towards a broad spectrum of mesodermal and ectodermal cells (9) and can act also as inducers (10–13) and inhibitors (14) of developmental pathways. Fibroblasts and endothelial cells can respond to several different FGFs (9). By contrast, keratinocyte growth factor, the most recently characterized FGF, does not stimulate fibroblast growth (15). Hence, FGFs may have overlapping but distinct spectra of activities.

FGF-5 is a growth factor discovered in our laboratory as the product of a human oncogene detected by DNA transfection assays (6, 16). The protein is mitogenic towards fibroblasts and endothelial cells *in vitro* (6), but the natural targets for FGF-5 action *in vivo* have yet to be ascertained. As a first step towards an understanding of FGF-5 function, we are determining the factor's profile of expression in developing and adult tissues. The data we present here show that adult expression of FGF-5 RNA is restricted to regions of the central nervous system. These experiments required the molecular cloning and characterization of the murine FGF-5 gene.*

MATERIALS AND METHODS

Enzymes, Radionucleotides, and Vectors. Sequenase DNA polymerase was purchased from United States Biochemical; RNase inhibitor and DNase I, from Boehringer Mannheim; and all other enzymes, from New England Biolabs. Radiolabeled nucleotides were purchased from New England Nuclear. pUC18, pSP64, pSP65, and M13mp19 were obtained from New England Biolabs. pBS(KS+) was purchased from Stratagene.

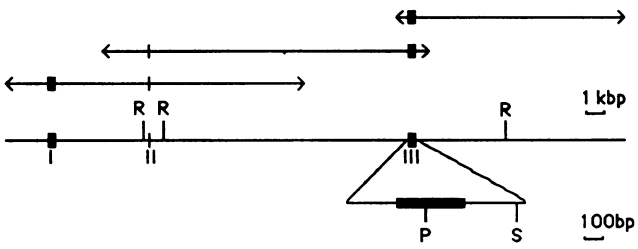

FIG. 1. Molecular clones spanning the murine FGF-5 gene. Lines bracketed by arrowheads indicate positions of inserts in genomic clones relative to the murine FGF-5 gene shown below. The three exons (thickened line) are labeled with Roman numerals, *Eco*RI cleavage sites are shown (R), and the *Pst* I (P)–*Sac* I (S) 450-base-pair (bp) segment used as hybridization probe is indicated.

Genomic Library Screening. Murine NIH 3T3 DNA partially digested with *Sau*3aI was cloned into EMBL4 λ phage DNA to generate one library, while another consisting of mouse spleen DNA cloned into λ phage EMBL3 was obtained from F. Costantini. Libraries were screened by a standard procedure (17) using as probe human FGF-5 cDNA (6) labeled with ^{32}P by random hexamer priming (18). Posthybridization washes were performed at 65°C in 0.30 M NaCl/0.030 M sodium citrate, pH 7.

Southern Blotting and Subcloning. Exons in genomic clones were localized by Southern blot hybridization (19) and subcloned into plasmid and λ phage M13 vectors for DNA sequencing and RNA probe preparation.

DNA Sequencing. Sequencing both strands of DNA was carried out by the dideoxynucleotide chain-termination method (20) using plasmid DNA template and Sequenase polymerase according to the manufacturer's instructions. Accurate sequencing in some regions required the use of M13 single-stranded DNA template or the substitution of dITP for dGTP in sequencing reactions.

RNA Filter Blot Hybridization. Tissues were dissected from 8-week-old C57BL/6 mice, and the RNA was isolated after solubilization in guanidinium isothiocyanate (21). RNA was also isolated from seven components of brain dissected by standard procedure (22). RNA filter blot hybridization after agarose gel electrophoresis followed a standard protocol (23) using a ^{32}P-labeled *Pst* I/*Sac* I 450-bp DNA fragment containing exon 3 of the murine FGF-5 gene (see Fig. 1).

***In Situ* Hybridization.** The exon 3 DNA fragment was cloned into pSP64 and pSP65 to allow *in vitro* RNA synthesis of sense and antisense strands, respectively. RNA was synthesized from linearized templates by using SP6 phage RNA polymerase (24) and uridine 5′-[α-(^{35}S)thio]triphosphate to yield probes labeled to ≈7 × 10^8 cpm/μg. Tissues

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Abbreviations: FGF, fibroblast growth factor; ORF, open reading frame.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M37821-4 for the murine FGF-5 gene and M37825 for the corrected human FGF-5 cDNA).

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                                                                                                                              20
Murine ORF-1                                                        M  S  T  L  C  G  E  G  G  S  A  R  G  T  Q  P  R  G  G  Y

Murine FGF5 DNA  GCTCGGGTGGCCTCTCTCTCTCCCCCTCTCCCTCTCCCTTCCCCGAGGCTATGTCCACCCTGTGCGGCGAGGGAGGCAGCGCCAGAGGCACGCAGCCGCGCGGGGGCTAC
Human FGF5 DNA                      -     -      CT  T                       G           CG   CAGAG                 A A

Human ORF-1                                                                              R           A     Q  S                 H  R
                                                                     38
Murine ORF-1      G  A  R  S  Q  P  C  K  M  H  L  G  P  P  R  P  E  E ***                                                     20
Murine FGF5 Pro                                                          M  S  L  S  L  L  F  L  I  F  C  S  H  L  I  H  S  A  W  A
Murine FGF5 DNA  GGAGCCCGGAGCCAGCCCTGCAAGATGCACTTAGGACCCCCGCGGCCGGAAGAATGAGCCTGTCCTTGCTCTTCCTCATCTTCTGCAGCCACCTGATCCACAGCGCTTGGGCT
Human FGF5 DNA   A      A  AT       A                          T            T       C   C     C      T              T      C
Human FGF5 Pro                                                                    F     L     L     F              L
Human ORF-1       R     Q  N        Y                          L
                                                                                                                                55
Murine FGF5 Pro   H  G  E  K  R  L  T  P  E  G  Q  P  A  P  P  -  -  -  R  N  P  G  D  S  S  G  S  R  G  R  S  S  A  T  F  S  S  S
Murine FGF5 DNA  CACGGGGAGAAGCGTCTCACTCCCGAAGGGCAACCCGCGCCTCCT---------AGGAACCCGGGAGACTCCAGCGGCAGCCGGGGCAGAAGTAGCGCGACGTTTTCTTCGTCT
Human FGF5 DNA                     G C   A            GA  CG  GCCACTGAT         TA  G       A    A A A    C        T T ---     C
Human FGF5 Pro                      A     K           G     A  A  T  D           R  G        S  R  Q  S  S           M  -
                                                                                                                                93
Murine FGF5 Pro   S  A  S  S  P  V  A  A  S  P  G  S  Q  G  S  G  S  E  H  S  S  F  Q  W  S  P  S  G  R  R  T  G  S  L  Y  C  R  V
Murine FGF5 DNA  TCTGCCTCCTCACCAGTCGCAGCTTCTCCGGGCAGCCAAGGAAGCGGCTCGGAACATAGCAGTTTCCAGTGGAGCCCTTCGGGGCGCCGGACCGGCAGCCTGTACTGCAGAGTG
Human FGF5 DNA              CT CCC           T               T    T   G  G                 [  C          ]            C
Human FGF5 Pro                S  P           L                    L     Q
                                                                                                                               125
Murine FGF5 Pro   G  I  G  F  H  L  Q  I  Y  P  D  G  K  V  N  G  S  H  E  A  S  V  L                    S  I  L  E  I  F  A  V  S
Murine FGF5 DNA  GGCATCGGTTTCCATCTGCAGATCTACCCGGATGGCAAAGTCAATGGCTCCCACGAAGCCAGTGTGTTAAgtaagt~4.5Kb~cctagGTATTTGGAAATATTTGCTGTGTCT
Human FGF5 DNA                                                  A            A A                          G
Human FGF5 Pro                                                               N  M                          V
                                                                                                                               157
Murine FGF5 Pro   Q  G  I  V  G  I  R  G  V  F  S  N  K  F  L  A  M  S  K  K  G  K  L  H  A  S                    A  K  F  T  D  D
Murine FGF5 DNA  CAGGGGATTGTAGGAATACGAGGAGTTTTCAGCAACAAATTTTTAGCGATGTCAAAAAAAGGAAAACTCCATGCAAGTgtaag~12.5Kb~cccagGCCAAATTTACGGATGAC
Human FGF5 DNA                                                                                                        G  C  A
Human FGF5 Pro
                                                                                                                               195
Murine FGF5 Pro   C  K  F  R  E  R  F  Q  E  N  S  Y  N  T  Y  A  S  A  I  H  R  T  E  K  T  G  R  E  W  Y  V  A  L  N  K  R  G  K
Murine FGF5 DNA  TGTAAGTTCAGGGAGAGATTCCAAGAAAACAGCTATAATACCTATGCGTCCGCGATCCACAGAACTGAAAAGACAGGCCGAGAGTGGTACGTGGCCCTGAACAAGAGAGGGAAA
Human FGF5 DNA     C             C T T        T                 C  A  A  A  T           A     G  G        T  T        T  A     A
Human FGF5 Pro
                                                                                                                               233
Murine FGF5 Pro   A  K  R  G  C  S  P  R  V  K  P  Q  H  V  S  T  H  F  L  P  R  F  K  Q  S  E  Q  P  E  L  S  F  T  V  T  V  P  E
Murine FGF5 DNA  GCCAAGAGAGGCTGCAGCCCACGAGTCAAACCCCAACACGTCTCCACCCACTTCCTACCCAGGTTCAAGCAGTCCGAGCAACCGGAACTTTCCTTCACCGTCACTGTTCCAGAA
Human FGF5 DNA        AC    G        C  G  T        G  TA    T     T  T  T  A  A           G     G  A        T     G  T        T
Human FGF5 Pro                                           I
                                                                                                                 264
Murine FGF5 Pro   K  K  K  P  -  -  P  V  K  P  K  V  P  L  S  Q  P  R  R  S  P  S  P  V  K  Y  R  L  K  F  R  F  G ***
Murine FGF5 DNA  AAGAAAAAGCCA------CCGGTGAAACCAAAGGTGCCCCTGTCGCAGCCTCGCAGAAGTCCCAGCCCAGTGAAGTACAGACTGAAGTTTCGCTTTGGATGATGCTCATCGTCG
Human FGF5 DNA           T   CCTAGC  TA C  GT     A T     T  TGCA     G A  A     A T       A        C                A AT  A   T G
Human FGF5 Pro          N     P  S     I     S     I           A        K  N  T  N  S
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FIG. 2. Comparison of murine and human FGF-5 nucleotide and predicted amino acid sequences. The sequence of the murine FGF-5 gene's three exons is shown in capital lettering, while edges of introns are in small lettering. Exon/intron boundaries were determined by comparison to the murine FGF-5 cDNA sequence (27). The sequence begins with the presumptive cap site, based upon sequence comparison to the cap site in the human gene. Predicted amino acid sequences for murine FGF-5 and the hypothetical translation product of ORF-1 are shown in single letter code. The nucleotide sequence of human FGF-5 along with predicted ORF-1 and FGF-5 protein sequences are shown only for residues that differ from the murine homology. – signifies a gap in nucleotide or amino acid sequence; *** signifies a termination codon. The boxed area in the human sequence incorporates corrections from the originally reported sequence (6). Both murine and corrected human FGF-5 sequences have been submitted to GenBank.

were freshly dissected from 8-week-old mice, frozen in liquid nitrogen, and sectioned at 12-μm thickness (25). Sections were stored at −80°C and were paraformaldehyde-fixed and acetylated prior to hybridization. Hybridization and washing of sections were carried out as described (25) with some modifications. Three nanograms of probe in 200 μl of hybridization buffer were applied to each slide. The first posthybridization wash was in 0.15 M NaCl/0.015 sodium citrate, pH 7/50% formamide for 30 min at 37°C, and high- and low-salt washes after RNase treatment were performed at 56°C and contained 14 mM 2-mercaptoethanol. Slides were autoradiographed for 90 days at 4°C after they were dipped in undiluted NTB2 emulsion (Eastman Kodak) and dried for 2 hr at room temperature in the presence of dessicant. Exposed slides were developed, stained with hematoxylin, and mounted with Permount (Fisher). Light- and dark-field photomicroscopy were performed with a Nikon Microphot-FXA microscope (courtesy of F. Costantini). Positions of sections along the anterior–posterior axis and structures within sections were identified by using an atlas of the rat brain (26).

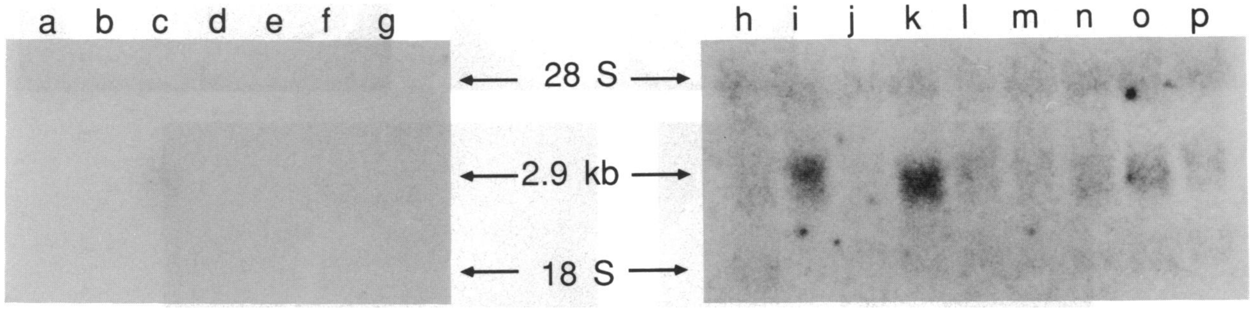

FIG. 3. Detection of FGF-5 RNA in adult mouse tissues by filter blot hybridization. Twenty micrograms of total RNA from dissected tissues of 8-week-old mice were electrophoresed through formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled *Pst* I–*Sac* I 450-bp fragment from the murine FGF-5 gene. RNA was prepared from heart (lane a), skeletal muscle (lane b), whole brain (lane c), lung (lane d), liver (lane e), kidney and adrenal gland (lane f), thymus and spleen (lane g), whole brain (lane h), spinal cord (lane i), hypothalamus (lane j), hippocampus (lane k), hindbrain (lane l), basal ganglia (lane m), cerebellum (lane n), cerebral cortex (lane o), and midbrain (lane p). The probe sometimes binds weakly to 28S and 18S ribosomal RNAs, as seen in *Right*.

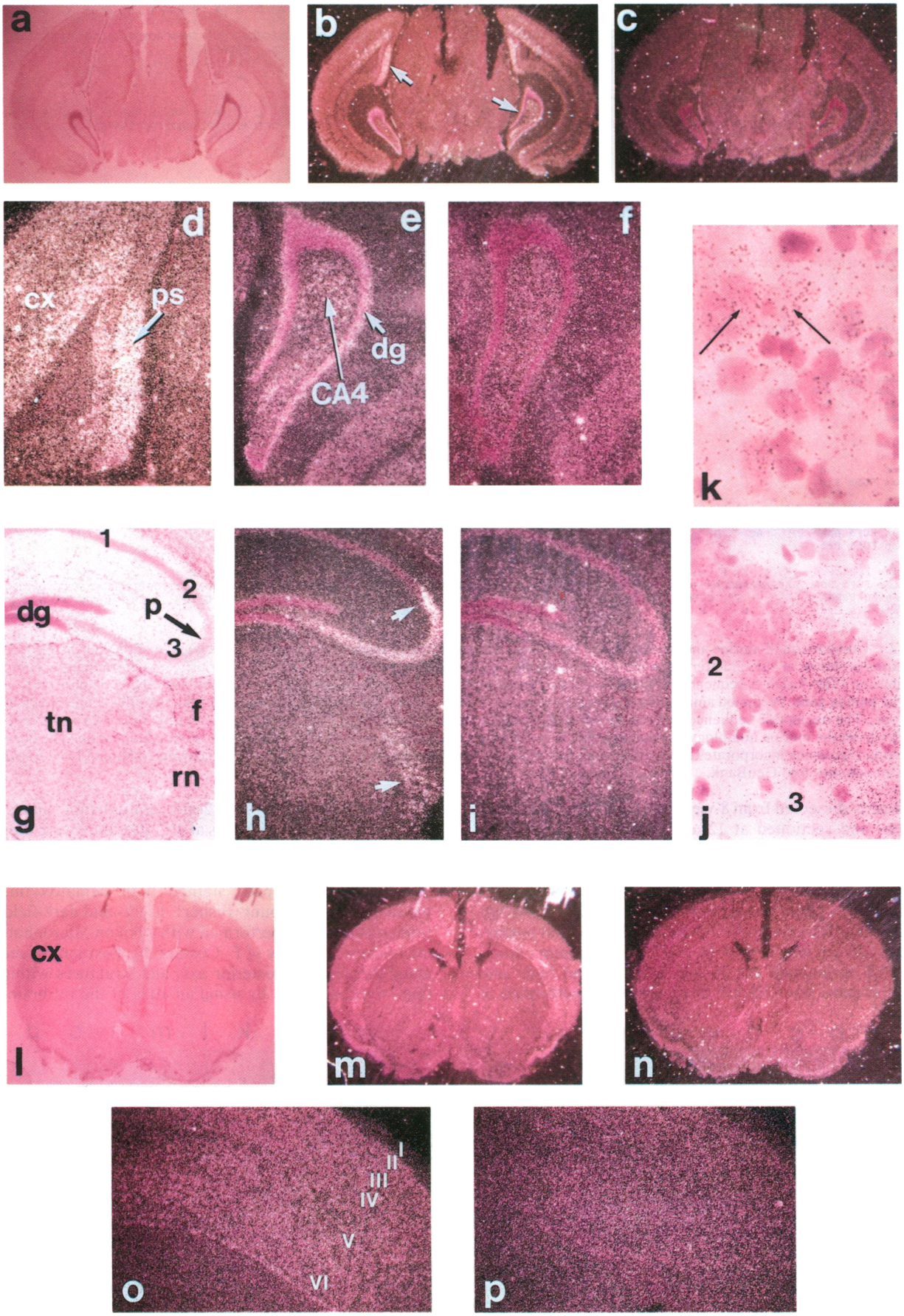

FIG. 4. (*Legend appears at the bottom of the opposite page.*)

RESULTS

Murine FGF-5 Genomic Sequence. Human FGF-5 cDNA was used as probe to screen a murine genomic library. Three clones were isolated that span the three exons of the FGF-5 gene (Fig. 1). The exons were subcloned, and their sequences were determined.

Fig. 2 shows the murine genomic sequence and its comparison to the human FGF-5 gene. The coding sequence in the murine genomic clone is identical to that determined from a murine embryonic cDNA clone (27). Homology between the murine and human genes spans the FGF-5 coding sequences as well as upstream sequences, which include an additional open reading frame, termed ORF-1. The predicted amino acid sequences for murine and human FGF-5 are 84% homologous, with most substitutions clustered near the N and C termini of the proteins. Features shared by the two proteins include a hydrophobic N terminus and a consensus sequence for N-linked glycosylation. These elements enable human FGF-5 to be secreted from cells as a glycoprotein (B. Bates and M.G., unpublished data).

Detection of FGF-5 RNA by Blot Hybridization. Samples of total RNA prepared from tissues of adult mice (8 weeks old) were electrophoresed through a formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled DNA probe derived from the murine FGF-5 gene. The probe contained sequences 3′ to FGF-5 codon 201 and thereby lacked homology to other FGF genes. Low levels of a 2.9-kilobase (kb) FGF-5 transcript were detected solely in RNA from brain (Fig. 3, lanes a–g). By comparison of this signal with that obtained with an FGF-5 genomic DNA fragment (data not shown), we estimate that FGF-5 RNA constitutes only 0.0001% of brain mRNA. As this abundance corresponds to an average of only 0.3 transcript per cell, FGF-5 expression must be restricted to a subset of brain cells.

To assess the distribution of FGF-5 RNA in the central nervous system, brains were subjected to a standard seven-part dissection (22) prior to RNA isolation. RNA was also prepared from spinal cord. Analysis of these samples for FGF-5 RNA revealed a broad distribution of expression (Fig. 3, lanes h–p). Hippocampus, spinal cord, and cerebral cortex had somewhat higher levels of FGF-5 message than did basal ganglia, cerebellum, midbrain, and hindbrain. Only in the hypothalamus was FGF-5 RNA undetectable.

***In Situ* Detection of FGF-5 RNA.** To further localize FGF-5 gene expression, coronal sections of brain were hybridized *in situ* with radiolabeled antisense- and sense-strand RNA probes. Initial experiments using sections of paraformaldehyde-fixed tissues failed to detect any specific signal. Only through the use of sections from freshly frozen tissue did we detect reproducible, though often faint, signals of expression over the background observed with the sense-strand control probe. The overall background in brain sections was the same as that observed on liver, kidney, and spleen sections with either sense or antisense probes; the levels of background observed reflected the 90-day exposure required to detect the specific FGF-5 signal.

Fig. 4 illustrates the detection of FGF-5 RNA in regions of the hippocampus, thalamus, and cerebral cortex. At one level along the anterior–posterior axis (Fig. 4*A*), a comparison of dark-field micrographs following hybridization with experimental and control probes (Fig. 4 *B* and *C*) reveals FGF-5 RNA in several regions of the section. Viewed at higher magnification, these regions include the presubiculum (Fig. 4*D*) and the dentate gyrus and the CA4 field of the hippocampus (Fig. 4*E*). A more rostral section (Fig. 4*G*) shows significant hybridization in the dentate gyrus and the pyramidal layer within the CA3 field of the hippocampus, but hybridization is not above background in the adjacent CA2 and CA1 hippocampus fields (Fig. 4 *H* vs. *I*). This section also shows mottled expression within the reticular nucleus of the thalamus. Higher magnification shows expression by CA3 pyramidal neurons (Fig. 4*J*) and some thalamic lateral reticular neurons (Fig. 4*K*). The possibility of additional expression by glial cells cannot be excluded. More rostral sections, such as those shown in Fig. 4*L*, display stratified expression throughout the cerebral cortex (Fig. 4*M*). Expression is highest in the basal-most cells of cortical layer VI (Fig. 4*O*). Those structures in the brain that express FGF-5 do so at all sampled planes along the anterior–posterior axis that the structures span, and the same overall profile of detection has been obtained by using a hybridization probe corresponding to different and nonoverlapping portions of the FGF-5 coding sequence (data not shown).

DISCUSSION

We have detected low levels of FGF-5 gene expression in the brain and spinal cord of the adult mouse. Other adult tissues that scored negative upon Northern blot analysis must have less than 0.05 FGF-5 mRNA molecules per cell on average. Of course, this survey of adult expression is not exhaustive, leaving open the possibility for FGF-5 production by cells outside the central nervous system. FGF-5 is also expressed during embryogenesis (27) by cells that are precursors to the nervous system and to other tissues (O.H. and M.G., unpublished data).

FGF-5 expression is widespread within the central nervous system, as detected by filter blot hybridization. *In situ* hybridization experiments revealed portions of the hippocampus, thalamus, and cerebral cortex where the growth factor gene is expressed, and at least some of this expression is by neurons. Sites of FGF-5 transcription in the cerebellum, the midbrain, the basal ganglia, and the spinal cord could not be localized by *in situ* methods, presumably because of the technical problems of regionally high background and relatively uniform expression.

FIG. 4 (*on opposite page*). *In situ* detection of FGF-5 RNA in the brain. Coronal sections of adult mouse brain were hybridized with *in vitro* synthesized ^{35}S-labeled RNA corresponding to the antisense (test) and sense (control) strands of the *Pst* I–*Sac* I fragment containing exon 3 of the murine FGF-5 gene. (*A* and *B*) Bright-field (*A*) and dark-field (*B*) images of a section hybridized with the test probe; arrows denote regions magnified in other panels. (*C*) Dark-field image of an adjacent section (control probe). (*D*) Higher magnification of *B*, showing the signal in presubiculum (ps) and cerebral cortex (cx). (*E*) Higher magnification of *B*, showing the signal in dentate gyrus (dg) and the CA4 region of hippocampus (test probe). (*F*) Same area as *E* (control probe). (*G* and *H*) Bright-field (*G*) and dark-field (*H*) images of a section through hippocampus and thalamus (test probe), fields CA1–CA3 of hippocampus (1–3), pyramidal layer (p), fimbria (f), reticular thalamic nucleus (rn), and other thalamic nuclei (tn); arrows in *H* denote regions magnified in other panels. (*I*) Dark-field image of an adjacent section (control probe). (*J*) Higher magnification of hippocampal pyramidal layer at the CA3/CA2 boundary (test probe). (*K*) Higher magnification of reticular nucleus of thalamus (test probe), showing FGF-5-positive neurons (arrows). (*L* and *M*) Bright-field (*L*) and dark-field (*M*) images of a section through cerebral cortex and underlying forebrain (test probe). (*N*) Dark-field image of an adjacent section (control probe). (*O*) Higher magnification of *M*, showing layered expression in cerebral cortex—six layers of cortex (I–VI). (*P*) Adjacent section to *O* (control probe). Substantial nonspecific background of silver grains reflects 90-day exposure required to detect specific FGF-5 signals; background levels differ across some regions varying in neuronal cell density (*F*, *I*, and *N*). Dorsal is upward in all panels except *O* and *P*, where it is rightward.

Adult brain contains acidic and basic FGFs in addition to FGF-5 (9). Immunohistochemical methods have detected these other FGFs within the cytosol of neurons (28), although location at additional sites cannot be excluded. By contrast, we expect FGF-5 protein to reside extracellularly. The FGF-5 precursor contains an amino-terminal signal sequence that results in secretion of the factor as a glycoprotein from cultured cells (B. Bates and M.G., unpublished data).

We can speculate on the role of FGF-5 in brain function. The factor may serve as a continually released trophic factor for neurons or glia. In this regard, it should be noted that basic FGF acts as a glial growth factor (29, 30) and as a neurotrophic factor *in vitro* and *in vivo* (12, 13, 31). Alternatively, FGF-5 may be secreted at synapses and modulate neuronal communication. Experiments that define and localize FGF-5 receptors within the brain should provide clues as to functions of this growth factor.

Several features of the murine FGF-5 genomic sequence deserve comment. Both human and murine genes contain an ORF (ORF-1) in exon 1 upstream from and overlapping the FGF-5 coding sequence. The positions of the two methionine codons and the termination codon of ORF-1 relative to the FGF-5 coding sequence are conserved between species, although some sequence divergence exists within this region. The two methionine codons of ORF-1 in the human RNA inhibit FGF-5 translation (B. Bates and M.G., unpublished data). Murine FGF-5 synthesis is most likely under similar translational constraints. ORF-1 may allow FGF-5 synthesis to be regulated at the translational level, as has been documented for the yeast *GCN4* gene (32).

The amino acid sequences of murine and human FGF-5 are 84% identical overall; residues 75–235 of the murine protein are 97.5% identical to the corresponding region of human FGF-5. Other FGFs show a similar degree of interspecies homology (9, 26). Dramatic sequence conservation may be required to give each FGF the ability to interact with a distinct set of target molecules, which may include receptors and matrix components.

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