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Two forms of the basic fibroblast growth factor receptor-like mRNA are expressed in the developing mouse brain

(protein-tyrosine kinase/gene family/brain development)

HUGH H. REID*, ANDREW F. WILKS†, AND ORA BERNARD*‡

*The Walter and Eliza Hall Institute of Medical Research and †The Ludwig Institute for Cancer Research, Melbourne Tumor Biology Branch, Post Office, Royal Melbourne Hospital, Victoria, 3050, Australia

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ABSTRACT **The embryonic neuroepithelium gives rise to the components of the central nervous system in the mature animal. To study the early development of the murine central nervous system we have sought to isolate growth factor receptors from the neuroepithelium of the neural tube of 10-day-old mouse embryos. Because many growth factor receptors are members of the protein-tyrosine kinase family, we have used the polymerase chain reaction to amplify mRNA sequences from 10-day-old mouse embryo neuroepithelium; these sequences lie between the nucleotide sequences of two highly conserved amino acid motifs from the catalytic domain of protein-tyrosine kinases. By using this technique we have isolated a clone encoding the murine basic fibroblast growth factor receptor (bFGF-R), as well as a shorter form of this mRNA. This latter cDNA comprised 75% of the bFGF-R cDNA clones isolated from the immortalized neuroepithelial cell lines. This variant mRNA, designated here as N-bFGF-R, appears to be expressed at higher levels in neuronal cells in early stages of development. The bFGF-R is a member of a multigene family, as demonstrated by Southern blot analysis and the cloning of two other members of this family.**

It is now well established that protein-tyrosine kinases (PTKs) play an important role in the regulation of cellular growth (1). This family of proteins comprises two broad groups. The first group includes cell-surface receptors that mediate the activity of known growth factors, such as epidermal growth factor (2), platelet-derived growth factor (3), and colony stimulating factor 1 (4). The extracellular domain of each member of this class of PTKs interacts with its respective ligand, activating the PTK activity of the intracellular part of the molecule, which in turn initiates the mitogenic response of the cell. The second group of PTKs is comprised of proteins that lack the extracellular domain of the growth factor receptor family and are frequently associated with the inner surface, the plasma membrane, or other intracellular structures. This latter class is typified by the c-*src*, c-*abl*, and c-*fes* protooncogenes (5–7). The regulation of these PTKs is not yet understood. Many of these proteins were first identified as the transforming proteins of retroviruses and have also been implicated in some human and murine cancers (8, 9).

In studying early development of the central nervous system (CNS), we have attempted to isolate growth factors and their receptors that are involved in the growth and differentiation of neuroepithelial cells of the neural tube of 10-day-old mouse embryos (E10) into neuronal and glial cells. That basic and acidic fibroblast growth factors (FGFs) stimulate the growth of mesoderm- and neuroectoderm-derived cells has been demonstrated (10–14). These growth factors support the growth and differentiation of E10 neuroepithelial cells, as well as survival of some neurons from different regions of the central and peripheral nervous systems.

The FGFs may, therefore, be involved in the embryonic development of the CNS. The FGF family consists of at least seven members (15–20), all of which are structurally related. Further, most of them bind heparin and stimulate the growth of a variety of different cell types. Demonstration of expression of receptors for various FGFs may help elucidate the involvement of FGFs in CNS development. Because a significant portion of growth factor receptors isolated to date are PTKs, it seemed reasonable to assume that many unidentified PTKs may be involved in the pivotal events controlling growth and differentiation of the developing CNS. In addition, the receptors for the FGF family of ligands may belong to this receptor group.

Several approaches have been used to clone members of the protein kinase family, including the screening of cDNA libraries with DNA probes derived from sequences of conserved regions of the protein kinase catalytic domain (21) and the use of antiphosphotyrosine antibodies to screen cDNA expression libraries (22–24). The polymerase chain reaction (PCR) technique was used to amplify a 200-base-pair (bp) region that lies between two highly conserved motifs within the catalytic domain of the PTKs (25). We have detected among 200 independent PTK clones isolated in this way, five previously undescribed and 10 previously described PTK-related sequences in cDNA made from E10 neuroepithelial cells.

We report the structure of two cDNAs clones§ isolated from murine E10 neuroepithelial cells and immortalized neuroepithelium cell lines (14). We show that these clones represent murine mRNAs similar to the putative chicken basic FGF receptors (bFGF-R) (26). These cDNA clones are members of a family of genes that has at least four members.

MATERIALS AND METHODS

Analysis of RNA and DNA. Poly(A)$^+$ RNA was prepared as described by Gonda *et al.* (27). Samples of 2 μg were fractionated on a 1% agarose/formaldehyde gel and transferred onto nitrocellulose Hybond C-extra (Amersham) as described (28). DNA was prepared from 5×10^6 cells by lysis with guanidine hydrochloride (29) and analyzed by Southern blot hybridization (30).

DNA Amplification. DNA amplification was essentially as described by Wilks (25). Poly(A)$^+$ RNA from E10 mouse neuroepithelial cells (5 μg) was used to prepare cDNA primed with oligo(dT). PCR was performed on 1/16th of the cDNA

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Abbreviations: bFGF-R, basic fibroblast growth factor receptor; PTK, protein-tyrosine kinase; E10, 10-day-old mouse embryos; CNS, central nervous system; PCR, polymerase chain reaction.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M28998).

product by using *Thermus aquaticus* (*Taq*) DNA polymerase (New England Biolabs) with the manufacturer's recommended buffers and the PTK-I and PTK-II oligonucleotide sequences (25). Conditions for amplification were as follows: 35 cycles at 95°C × 1 min (denaturation), at 42°C × 2 min (hybridizing), and at 63°C × 3 min (elongation). The amplified DNA was purified on an Elutip-D column (Schleicher & Schüll), precipitated with ethanol, and resuspended in Tris/EDTA buffer before digestion with *Eco*RI and *Bam*HI. The restricted DNA was then ligated into *Eco*RI/*Bam*HI-digested M13mp19. Sequencing was done by the dideoxy chain-termination method (31) with a Sequenase kit (United States Biochemical) and deoxyadenosine [α-[^{35}S]thio]triphosphate (Amersham).

cDNA Cloning. cDNA libraries were prepared from 5 μg each of E10 neuroepithelium mRNA and the neuroepithelium-derived cell line NZen37 mRNA (14). The E10 neuroepithelial cell library was prepared using an oligo(dT)–*Xba*-primed cDNA kit with λGEM4 vector (Promega). The NZen37 library was constructed using a random-primer cDNA cloning kit (Amersham) together with a specific bFGF-R oligonucleotide 5′-GGTCGCCCCTAGGAGTCCC-TCGAG-3′ corresponding to positions 1447–1470 (Fig. 1) and cloned into λgt10. Initially, $\approx 7 \times 10^5$ clones of the E10 cDNA library were screened with the 200-bp PTK fragment amplified by PCR from the M13 clone containing the PTK of interest. Inserts from recombinant clones were subcloned after removing the pGEM-1 plasmid of λGEM4 by digestion with *Spe* I and religation. For isolation of the 5′ end of the cDNA clones, a 400-bp *Eco*RI–*Acc* I fragment from close to the 5′ end of the E10 cDNA clone R2.1 (nucleotides 1414–3426) was used to screen $\approx 3 \times 10^5$ clones of the random-primed NZen37 cDNA library. Inserts from recombinant clones were first subcloned into *Eco*RI-digested pGEM 3Zf(+) (Promega) and subsequently cloned into *Eco*RI-cleaved M13mp18 for sequencing. To obtain longer cDNA clones we also applied the "RACE" modification of the PCR technique (33). Full-length cDNA clones were constructed by ligating the *Eco*RI–*Bam*HI fragment comprising the 5′ end of the cDNA to a *Bam*HI–*Xba* I fragment containing the 3′ end of the cDNA including the poly(A) tail. DNA fragments were labeled with deoxyadenosine [α-^{32}P]triphosphate by using a random-hexamer-priming kit (Bresatec, Adelaide, South Australia).

***In Vitro* Translation.** Full-length cDNA clones of both N-bFGF-R and bFGF-R were subcloned into the *Eco*RI and *Xba* I sites of the DAMP56 expression vector that is a modification of CDM8 (34). The DAMP56 vector includes the ampicillin-resistant gene and a modified polylinker while retaining the elements of CDM8 necessary for transient expression. *In vitro* transcripts of each clone were obtained using T7 DNA polymerase and the Riboprobe (Promega) *in vitro* transcription system. The RNA transcripts (≈ 1 μg) of each bFGF-R construct were translated in rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine (Amersham), and samples were run on 7.5% SDS/PAGE. The gels were stained, fixed, and treated with Amplify (Amersham) before being dried for autoradiography with Kodak XAR-5 x-ray film.

RESULTS

Identification of PTK-Related Sequences. We have used PCR to amplify PTK-related sequences expressed in E10 neuroepithelium. The oligonucleotides were based on the protein sequence of two of the most highly conserved regions of the PTK catalytic domain, Ile-His-Arg-Asp-Leu (PTK-I) at the N terminus and at the C-terminal end, the sequence Asp-Val-Trp-Ser-Phe-Gly (PTK-II) (domain VI and domain IX, respectively) (25, 35). cDNA generated from 5 μg of E10 neuroepithelium mRNA was subjected to PCR using PTK-I and PTK-II oligonucleotides as primers. The 210-bp fragments were then cloned into M13mp19 as described above. About 200 clones were examined by comparison of a single-base (guanine) sequencing reaction and divided accordingly into 30 groups with identical patterns. A single clone from each group was fully sequenced. Of the 200 clones, 140 contained PTK-related sequences, whereas the other clones contained sequences unrelated to PTKs. The clones containing known PTK sequences were as follows: insulin-like growth factor receptor (36), the murine hemopoietic cell kinase (37), c-*abl* (7) and c-*src* (6). Two unusual PTK-related sequences were specific to E10 neuroepithelial cells; we designate these PTKs as NE2 and NE3. These cDNA clones are coding for closely related proteins. Of the 65 amino acids in the conserved PTK domain, only 5 amino acids differ between the two proteins (92% homology). These PTKs are closely related to *FLG* (32) and *bek* (38). NE3 has an identical amino acid sequence to that of *bek* (38). Having completed this work we have learned that NE2 and *FLG* (32) sequences are closely related to the chicken bFGF-R and are presumed to be the mouse and human bFGF receptors, respectively.

Two Different bFGF-R-like mRNAs. Intriguingly, two species of closely related cDNA clones were isolated by using the procedures described above. The longest clone has an open reading frame of 822 amino acids and is closely related to the previously published chicken sequence of bFGF-R (26). This clone will henceforth be referred to as the murine bFGF-R. The second species of cDNA isolated, comprising 75% of the bFGF-R clones, has an identical sequence to that of the bFGF-R except that it has a deletion of 267 bp corresponding to 89 amino acids in the extracellular domain (shaded region in Fig. 1). These shorter cDNA clones were isolated from the NZen37 library and by the RACE technique.

Sequence Analysis of bFGF-R. Comparison of the putative mouse bFGF-R with the chicken bFGF-R sequences revealed an overall identity of 91% at the amino acid level. A likely initiation methionine is located at position 58 followed by a stretch of 20 amino acids that is hydrophobic in character and probably represents a leader sequence (Fig. 1). No in-frame ATG sequences were found in the 500 nucleotides 5′ to the methionine of position 58. The region absent in the shorter bFGF-R clone has a much less identity (80%) with the chicken mRNA. In contrast, the region downstream from the deletion and the beginning of the transmembrane segment has 93% homology with chicken bFGF-R. If the two forms of the receptor predicted from these sequences have a common binding site, this site must lie on the carboxyl side of amino acid 118. A high level of identity is also seen within the tyrosine-kinase domain, where the homology is 98%.

Another region with less identity to the chicken sequences is the most-3′ region adjacent to the termination codon. Of 15 amino acids, 8 differed between the mouse and the chicken, with 1 amino acid being deleted in the chicken bFGF-R. When this region is compared with the human bFGF-R (*FLG*), only a difference in two amino acids is seen. Threonine is changed to alanine at position 812 from the change in one nucleotide from adenine to guanine (Fig. 1). Ser-817 is replaced by glycine, also as a result of a one-nucleotide change (from an adenine to a guanine). Amino acids 148 and 149 are sometimes deleted from the shorter mouse bFGF-R clones. Of 8 clones sequenced, 2 lack these amino acids. Possibly this region is a miniexon. Alternatively, there may be exons containing two splice acceptor sites or two splice donor sites close to each other such that the region is sometimes spliced out in the shorter transcripts. Interestingly, in the chicken bFGF-R gene a codon for one of these amino acids is missing.

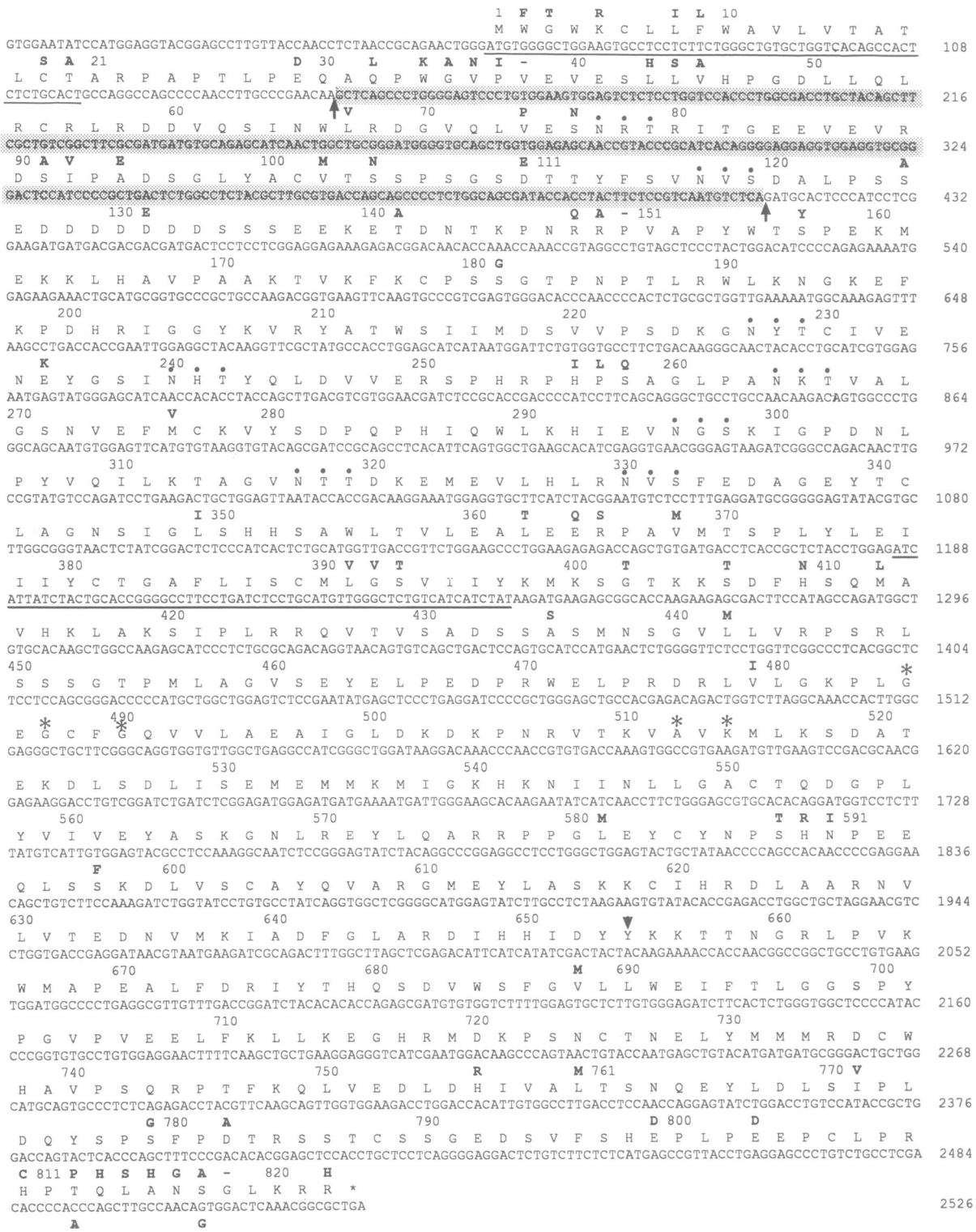

FIG. 1. Nucleotide sequence and deduced amino acid sequence of bFGF-R. Numbers at right indicate positions of nucleotides, and numbers above amino acids refer to the amino acid sequence. Underlined regions indicate the presumed signal peptide and transmembrane regions, respectively. Underlined amino acids (126–133) indicate a stretch of acidic residues. The shaded region between the two arrows represents the region deleted in N-bFGF-R mRNA. Dots indicate possible sites of N-glycosylation (N-X-S/T), asterisks indicate the ATP-binding site, and the arrow above tyrosine identifies the putative site of autophosphorylation. Bold letters above the amino acid sequence indicate amino acids that differ between chicken and mouse bFGF-R, whereas bold letters below the nucleotide sequence (amino acids 812 and 817) are amino acids from human bFGF-R (*FLG*) (32) that differ from mouse bFGF-R in this region.

Southern Blot Analysis. It is apparent from Southern blot analysis (Fig. 2) that the bFGF-R belongs to a family of receptors. Hybridization of genomic DNA to a probe containing the tyrosine kinase domain revealed at least four additional bands not detected with a probe containing only the extracellular domain. Consistent with these data, two other FGF-R-like molecules were isolated using the PCR reaction (data not shown). One molecule has an identical sequence to that of *bek* (38) and the other, an as-yet-unpublished molecule, is very similar (98%) to both *bek* and bFGF-R (data not shown).

Expression Pattern of bFGF-R. Expression of the putative bFGF-R mRNA was examined by Northern (RNA) blot

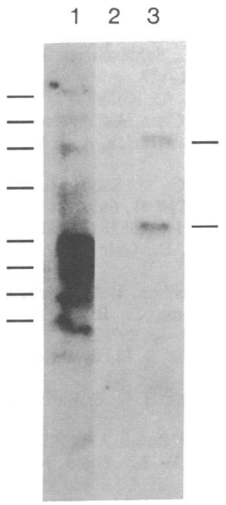

FIG. 2. Southern blot analysis of bFGF-R sequences. 3T3 cell DNA (10 μg) was digested with *Eco*RI (lanes 1 and 2) and *Xba* I (lane 3), fractionated on a 0.7% agarose gel, and transferred onto Hybond C-extra membrane. Hybridization was to the *Eco*RI–*Xba* I fragment for lane 1 and the *Eco*RI–*Bam*HI fragment for lanes 2 and 3.

hybridization using poly(A)$^+$ RNA from different mouse tissues, from mouse brain during embryonic development, and from several murine cell lines (Fig. 3). The highest expression was seen in mouse brain and in cell lines derived from mouse E10 neuroepithelium, NZen37 (14) and 2.3D (39). Only very low levels of bFGF-R mRNA are expressed in thymus, heart, and spleen. Expression of bFGF-R is seen in E10 mouse neuroepithelium (data not shown) and continues to be expressed in adult mouse brain. Although the two forms of the bFGF-R differ only in 267 bp and are not well resolved on the RNA blot, the data are consistent with the idea that the two forms of the receptor are expressed by these cells (Fig. 3). Addition of bFGF to 2.3D cells 24 and 72 hr before preparation of the poly(A)$^+$ RNA did not alter the expression of bFGF-R mRNA. bFGF-R is also expressed in a neural crest cell line, the neuroblastoma N2A, and a teratocarcinoma (tera-2), Ψ2 NZen6, a Ψ2 cell line producing N-*myc* retrovirus (14), also expressed low levels of bFGF-R.

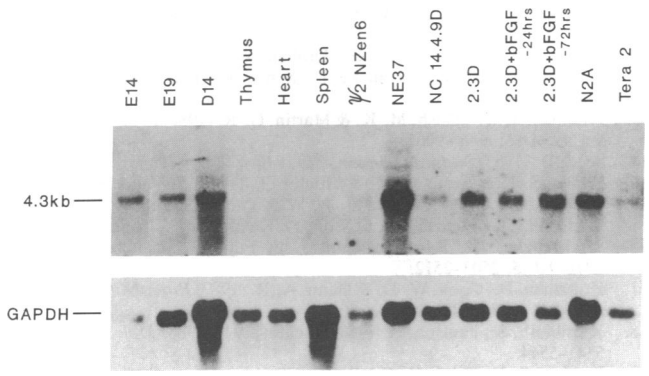

FIG. 3. Expression of bFGF-R and N-bFGF-R mRNA. Poly(A)$^+$ RNA from several cell lines and mouse tissues (indicated above lanes) was fractionated on an agarose/formaldehyde gel and transferred onto Hybond-C extra membrane. The filter was hybridized to the full-length bFGF-R fragment. E14, E19, and D14 indicate RNA isolated from brain at embryonic day 14, embryonic day 19, and 14-day-old mouse, respectively. Ψ2NZen6 is a Ψ2 cell line transfected with N-*myc* (14), NE37 (NZen37) and 2.3D are neuroepithelial cell lines (14, 39), and NC14.4.9D is a neural crest-derived cell line. N2A and Tera2 cell lines are neuroblastoma and teratocarcinoma, respectively. The same filter was hybridized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a quantitative control (40).

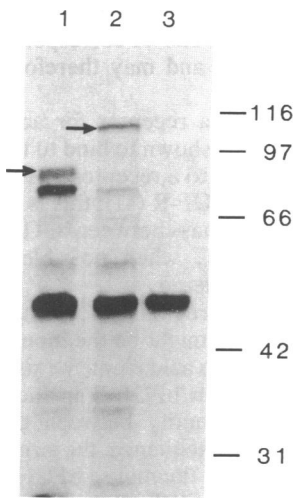

FIG. 4. *In vitro* translation of the two bFGF cDNA clones. The two cDNA clones were transcribed using T7 polymerase and then translated in rabbit reticulocyte lysates. Ten-microliter samples were run on 7.5% SDS/PAGE. Lanes: 1, N-bFGF-RNA; 2, bFGF-R RNA; 3, no RNA. Molecular mass standards used were β galactosidase (116 kDa), phosphorylase B (97.4 kDa), and bovine serum albumin (66.2 kDa) (Bio-Rad). Arrows indicate 100-kDa bFGF-R and 85-kDa N-bFGF-R.

Translation *in Vitro* of the Two bFGF-R cDNA Clones. Full-length cDNA clones of bFGF-R and its shorter version were subcloned into the DAMP 56 vector. The cDNA was transcribed using T7 polymerase and then translated in rabbit reticulocyte lysates. Fig. 4 demonstrates the translation products of the two cDNAs. The bFGF-R cDNA clone was translated into a protein of ≈100 kDa, whereas the shorter gene was translated into a protein of an approximate molecular mass of 85 kDa. The difference in molecular masses is consistent with the 89-amino acid deletion predicted from cDNA sequences. The molecular mass of the *in vitro* translation product is much smaller than the native molecule (145 kDa) (41), most probably because of lack of glycosylation.

DISCUSSION

We have described the isolation of two bFGF-R-like genes from a cDNA library constructed with mouse E10 neuroepithelium mRNA and NZen37 mRNA. Two variant cDNA clones from the NZen37 cDNA library coding for two forms of bFGF-R, namely, the bFGF-R and the shorter version designated N-bFGF-R, were sequenced. The two cDNA clones are identical, except for a 269-bp insert near the 5′ terminus, and were most probably transcribed from the same gene. Because of the very high amino acid sequence identity with the chicken bFGF-R (26), we believe that these clones encode two forms of the mouse bFGF-R. The shorter bFGF-R-like mRNA is designated N-bFGF-R because it was isolated from neural tissue and is identical to bFGF-R, except for a deletion of 89 amino acids from amino acids 31–118. Probably this deletion has arisen by a differential splicing mechanism.

Lee *et al.* (26) suggested that chicken bFGF-R belongs to the immunoglobulin supergene family (42). The chicken bFGF-R has three of the characteristic immunoglobulin-like domains containing two cysteine residues, which are common to this superfamily. These cysteine residues are also conserved in the mouse bFGF-R. In many of the immunoglobulin superfamily genes, such as PolyIgR (43), CD4 (44), and myelin-associated glycoprotein (45), each immunoglob-

ulin-related domain is encoded by one exon. The deleted region in the N-bFGF-R cDNA corresponds to an immunoglobulin-related domain and may therefore be encoded by one exon.

N-bFGF-R could be a receptor for acidic FGF (aFGF) because aFGF has been shown to bind to the bFGF-R and to bind with higher affinity to a receptor of 125 kDa that is ≈20 kDa smaller than the bFGF-R (41); this is approximately the difference in molecular mass between bFGF-R and its shorter counterpart, N-bFGF-R, when possible posttranslational modifications are considered.

Lee *et al.* (26) have previously suggested that the mouse tyrosine kinase *bek* (32) might be the mouse bFGF-R. However, because 85% amino acid identity exists between mouse bFGF-R and the chicken bFGF-R in the catalytic domain compared with 98% identity between the gene we have isolated and the chicken sequence, the larger cDNA clone we have isolated is probably the mouse bFGF-R, whereas *bek* is another gene belonging to the same family. Our data also indicate that bFGF-R belongs to a multigene family (Fig. 2) that most probably encodes receptors binding to the other members of the FGF family, such as aFGF (15), *int-2* (17), *hst* (16), FGF-5 (18), keratinocyte growth factor (19), and FGF-6 (20). We have demonstrated here that the tyrosine kinase domain is highly conserved between the members of this family while the extracellular portion differs; hybridization with a tyrosine kinase domain probe yielded additional bands on the Southern blots that were not seen after hybridization with a probe from the extracellular domain of the bFGF-R cDNA (Fig. 2).

These Southern blot data are supported by the subsequent isolation of two additional FGF-like receptors by the PCR reaction with PTK-I and PTK-II oligonucleotides. One of these receptors has an identical tyrosine kinase domain sequence to *bek* (NE3) and the other, the NE5 cDNA (data not shown), has a very high level of homology with mouse bFGF-R and *bek*. Although a high level of identity is seen in the intracellular domain outside the tyrosine kinase domain, we do not yet know whether the homology also extends to the extracellular domain of the receptors. We have also isolated additional protein kinase sequences from the E10 neuroepithelium cDNA, including three members of the Thr/Ser kinase family also expressed in WEHI-3B cells (46) (D06, D14, and D16; data not shown). Two PTKs, JAK1 and JAK2 (25), and a putative growth factor receptor, BM13, are also expressed in hemopoietic and epithelial cells (data not shown). Two putative growth factor receptors (NE1 and NE5) and one *src*-like PTK were also identified only in E10 neuroepithelium cDNA (data not shown).

The isolation of different members of the FGF-R family from neuronal tissues of the developing mouse embryos suggests that FGF-like molecules other than bFGF may play an important role in development of the CNS. Isolation of the bFGF-R cDNA and the shorter N-bFGF-R cDNA from E10 neuroepithelium and from immortalized neuroepithelial cell lines is a first step in determining whether bFGF-R and N-bFGF-R, together with their ligands, are important in regulating growth and differentiation of E10 mouse neuroepithelium.

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