Isoform Diversity among Fibroblast Growth Factor Homologous Factors Is Generated by Alternative Promoter Usage and Differential Splicing

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Fibroblast growth factor (FGF) homologous factors-1, -2, -3, and -4 (FHF-1–4; also referred to as FGFs 11–14) comprise a separate branch of the FGF family and have been implicated in the development of the nervous system and limbs. We report here the characterization of multiple isoforms of FHF-1, -2, -3, and -4 which are generated through the use of alternative start sites of transcription and splicing of one or more of a series of alternative 5′-exons. Several isoforms show different subcellular distributions when expressed in transfected tissue culture cells, and the corresponding differentially spliced transcripts show distinct expression patterns in developing and adult mouse tissues. Together with the evolutionary conservation of the FHF isoforms among human, mouse, and chicken, these data indicate that alternative promoter use and differential splicing are important regulatory processes in controlling the activities of this subfamily of FGFs.

Members of the fibroblast growth factor (FGF) family of signaling proteins play diverse roles in numerous developmental processes (for reviews, see Refs. 1–3). Previously we described the identification of four novel FGFs, referred to as fibroblast growth factor homologous factors (FHF-1, -2, -3, and -4; now equivalently called FGF-12, -13, -11, and -14, respectively) which are prominently expressed in the nervous system in mice and chickens (4, 5). The FHFs form a distinct branch of the FGF family and show 30–50% amino acid sequence identity with other FGFs. Within the FHF subfamily, different members share 60–70% identity. We and others have reported the identification of several alternatively spliced FHF transcripts in mice and chickens which code for FHF proteins with distinct amino termini (5–7).

In chicken embryos the two previously identified FHF-2 transcripts have distinct and nonoverlapping spatial distributions, suggesting that the encoded proteins may have distinct roles during development (5).

To explore the extent to which the diversity and expression of the FHFs are regulated by differential promoter use and/or splicing of 5′-exons, we searched for novel FHF transcripts that differ at their 5′-ends. This paper reports the identification and characterization of a large variety of FHF isoforms that are conserved among human, mouse, and chicken. These isoforms (three in the FHF-1 gene, nine in the FHF-2 gene, and two each in the FHF-3 and FHF-4 genes) code for proteins with highly divergent amino termini. When expressed in cultured cells, several of the isoforms have different subcellular distributions. Analysis of the expression of the differentially spliced coding exons of each of the FHFs by RNase protection and in situ hybridization in the developing and adult mouse shows that many isoforms have distinctive patterns of expression. Taken together, these observations imply that alternative promoter use and alternative splicing of 5′-exons are important for the regulation of FHF gene expression and for the generation of FHF proteins with distinct biochemical properties.

MATERIALS AND METHODS

Identification of FHF Splice Isoforms—Double-stranded e15 mouse embryo cDNA (CLONTECH) was used as template for 5′-rapid amplification of cDNA ends (RACE; 8). Two sequential PCRs were performed with two pairs of nested adaptor primers and gene-specific primers located in exon 2, and the resulting PCR products were resolved by agarose gel electrophoresis, cloned into pBS-KS or pBS-KS-α (Stratagene) and sequenced. An e10 chicken brain cDNA library (a gift of Dr. M. Tessier-Lavigne) was screened with probes derived from: (a) the alternatively spliced isoforms obtained in the mFHF-2 5′-RACE PCR; (b) exon 1S of hFHF-2; and (c) exons 2–5 of mFHF-4. A chicken e6 retina cDNA library (a gift of Dr. L. Gan) and a chicken stage 21–22 limb cDNA library (a gift of Dr. J. F. Fallon) were screened with probes derived from exons 2–5 of mFHF-1. These screens yielded cDNAs encoding the complete coding regions of cFHF-1(1B), cFHF-2(1Q + 1V), cFHF-2(1Q + 1V′ + 1V), and cFHF-4(1B), and a cDNA encoding cFHF-4(1A) lacking the first 10 codons. Filters were hybridized in 30–40% formamide, 5 × SSC at 42 °C, and washed in 2 × SSC at 50 °C–55 °C.

The chicken FHF-2(1U) isoform was amplified by degenerate PCR using chicken genomic DNA as template and the following primers (containing BamHI and EcoRI sites at the 5′- and 3′-ends): 5′-GCTCTATTGATCCACCTGAAT/CTGGAAGGCTTGGATGTTCTGCGC-3′ (sense primer) and 5′-GCTCATGATATCTGCTGAATA/CTGAGCTCTCTTAAA-CAAAAACCC-3′ (antisense primer). PCR conditions were as follows: (1) 94 °C, 7 min; (10) 94 °C, 30 s; 95 °C, 15 s; 45 °C, 2 min; 72 °C, 30 s; (20) 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; (1) 72 °C, 10 min. The PCR product was digested and subcloned into pBS-KS (Stratagene) and sequenced.

Production of Anti-FHF-2 Antibodies—Rabbit polyclonal anti-FHF-2 antibodies were raised against a bacterial fusion protein consisting of the entire FHF-2 open reading frame fused to the T7 gene 10 protein (9). Anti-FHF-2 antibodies were affinity purified using the fusion protein.
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immobilized onto nitrocellulose; those antibodies directed against the fusion partner were removed by absorption to immobilized T7 gene 10 protein. By Western blotting and by staining of transfected cells, the antibodies purified in this fashion did not cross-react with FHF-1 or FHF-4 proteins. Anti-mFHF-1 antibodies are described in Ref. 4.

Production of FHF Isoforms in Transfected Cells—In Situ Hybridization to Tissue Sections—In Situ hybridization was performed as described in Ref. 4. The sections were also hybridized with probes derived from exons 2–5 of mFHF-1, mFHF-2, mFHF-3, and mFHF-4. The sections were hybridized with probes derived from the following alternate mouse exons: mFHF-1, exon 1A; mFHF-2, exons 1S, 1Y, 1V, and 1U; mFHF-3, exons 1A and 1B; mFHF-4, exons 1A and 1B. 32P-Labeled probes were prepared by in vitro transcription of PCR-amplified templates containing the T3 promoter in the antisense primer. In situ hybridization was performed essentially as described in Ref. 4, with the addition of 10 mM Na2EDTA to all buffers. Autoradiographic images and cresyl violet-stained tissue sections were scanned, and the images were merged and manipulated using Photoshop 5.0 software for the Macintosh.

RESULTS

Identification of Multiple Spliced Isoforms of FHF-1, -2, -3, and -4—We and others (5–7) have reported the identification of several isoforms of FHF-1, FHF-2, and FHF-4 in which distinct amino termini are generated by differential splicing. Our initial characterization of transcripts encoding the cFHF-2(1V) and cFHF-2(1S) isoforms in chicken embryos showed that they are expressed differentially in the developing neural tube (5). Differential expression of two mFHF-4 splice isoforms was reported in the adult rat brain (7). These observations, together with the high degree of conservation of the two alternate FHF-2 exons between humans and chickens, suggested that alternative splicing of the FHF genes may be a general and conserved mechanism for regulating FHF gene expression.

To investigate whether there are other splice isoforms of the FHF genes with divergent amino termini, we screened cDNA libraries from mouse, chicken, brain, and retina and performed 5'-RACE on cDNA libraries from mouse brains, retina, and testis. We also examined human brain and retinal cDNA clones from earlier screens for evidence of alternative splicing. This search identified three isoforms of FHF-1, nine isoforms of FHF-2, and two isoforms each of FHF-3 and FHF-4 (Table I). Results from 5'-RACE suggest that in the mouse embryo, FHF-2 and FHF-3 display the largest number of distinct transcripts. In contrast, FHF-1 and FHF-4 5'-RACE reactions with mouse embryo RNA revealed only a single product in each case, and these were identified as the mFHF-1(1A) and mFHF-4(1B) isoforms. Thus far, one novel mFHF-3 isoform has been identified from the 5'-RACE reaction, mFHF-3(1B).

One cFHF-1 cDNA clone isolated from a chicken limb cDNA library (isoform 1D; Table I) does not appear to encode a functional open reading frame unless translation is initiated from a non-AUG codon, as is the case for some FGFs (12–14). The existence of this isoform was verified independently by reverse transcriptase PCR from stage 23–24 chicken embryo RNA.

Spliced Isoforms of the FHF-2 Gene—FHF-2 shows the greatest diversity of spliced isoforms, with nine different transcripts identified in various species (Table I). The majority of the FHF-2 transcripts were identified either from mouse or chicken cDNAs, but most of the alternate exons also have counterparts in human genomic DNA. All of the homologous human sequences are present at the FHF-2 locus 5' of the invariant exons (exons 2–5) and in the same orientation as those exons. For example, exon 1U was identified initially as part of a mouse 5'-RACE product and was found subsequently in the chicken genome by PCR, in human genomic DNA sequences from the FHF-2 locus, and in an expressed sequence tag from developing human fetal heart. One cDNA segment from the mouse 5'-RACE analysis, exon 1R, is highly homologous to a small segment of human genomic DNA between exons 1S and 2 of the human FHF-2 gene. Although this exon has not been found in any human cDNA, its location within the human FHF-2 gene suggests that it forms a part of that gene. All of the alternate 5'-exons that contain coding sequences (1S, 1U', 1V, and 1Y) have been identified in human, mouse, and chicken and are highly conserved (Table I).

Fig. 1 shows the complex structures of the differentially spliced 5'-ends of the FHF-2 transcripts. In several cases, the 5'-coding exons are spliced together with different noncoding exons. Within exons 1Y and 1S, some splicing events retain only the 3' regions of these exons (termed 1Y' and 1S', respectively) as parts of longer transcripts. Several of the FHF-2 5'-exons reported here are noncoding because there are stop codons in all three reading frames (exons 1P, 1Q, 1T, 1W, 1X, and 1Z). Most of the putative 5'-coding exons contain only one potential initiator methionine codon, and, except for exon 1U, the sequence around that methionine is not a close match to the optimal consensus for the initiation of translation (15). In one instance (exon 1V) there is a potential initiator methionine codon in chicken cDNA which is absent from the human and mouse cDNAs (AUG in chicken versus CUG in mouse and human; see Table II). In this regard, it may be relevant that some FGF-2 and FGF-3 translation products are initiated at CUG codons (12–14).

Structure of the hFHF-2 Gene—Identification of homologous spliced isoforms in the chicken and mouse FHF-2 genes suggests that the alternative 5'-exons have been conserved through evolution. The human FHF-2 gene maps to Xq26 (4), and this region has been sequenced extensively, allowing the unambiguous identification of human counterparts for most of the exons identified in other species. Fig. 2 shows the current map of the hFHF-2 gene which encompasses at least 400 kilobases of genomic DNA. Many of the alternate 5'-exons cluster within the gene, and several predicted introns are more than 100 kilobases in length. Noncoding 5'-exons 1T and 1Q have not yet been identified in humans. The nucleotide sequences of both coding and noncoding exons are highly conserved across species. For example, 5'-untranslated regions within the noncoding portions of the alternative coding exons share >90% nucleotide sequence identity between human and chicken.
Each class of FHF transcript is listed on a separate line, together with the species from which the corresponding cDNA and/or RACE PCR product was derived. Some transcript classes include more than one exon upstream of exon 2; these are listed in the table in the order in which they are spliced together (5' to 3'). In each class, the 3'-most exon listed is spliced to the second exon of the indicated FHF. References are listed for those exons that have been reported previously.

### Table I

| Gene  | 5'-exons | Species | Source |
|-------|----------|---------|--------|
| FHF-1 | 1A       | Human   | Smallwood et al. (4). |
|       |          | Mouse   | e15 mouse embryo RACE; Smallwood et al. (4); Hartung et al. (6). |
|       |          | Chicken | e10 brain, e6 retina chick cDNA libraries; Munoz-Sanjuan et al. (5). |
| FHF-1 | 1B       | Human   | Human brain cDNA library. |
|       |          | Mouse   | Hartung et al. (6). |
|       |          | Chicken | e10 chick brain cDNA library. |
| FHF-1 | 1C       | Mouse   | Hartung et al. (6); incomplete cDNA. |
| FHF-1 | 1D       | Chicken | Stage 21–22 limb library; no initiator Met found. Reverse transcriptase PCR from stage 23–24 chick embryo. |
| FHF-2 | 1S       | Human   | Smallwood et al. (4); Hartung et al. (6). |
|       |          | Mouse   | Smallwood et al. (4); Hartung et al. (6). |
| FHF-2 | 1T+ 1S'  | Chicken | e10 chick brain cDNA library. |
| FHF-2 | 1P+ 1Y+1V | Mouse   | e15 mouse embryo RACE. |
| FHF-2 | 1Q+1Y+1V | Chicken | e6 retina and e10 brain chick cDNA libraries; Munoz-Sanjuan et al. (5). |
| FHF-2 | 1Q+1Y'   | Chicken | e6 retina and e10 brain chick cDNA libraries. |
| FHF-2 | 1R       | Mouse   | e15 mouse embryo RACE; incomplete cDNA. |
| FHF-2 | 1U       | Human   | Human fetal heart, ESTs AA449030 and AA427960; Gecz et al. (16). |
|       |          | Mouse   | e15 mouse embryo RACE. |
|       |          | Chicken | Degenerate genomic PCR. |
| FHF-2 | 1X+1W+1V | Human   | Human brain cDNA library. |
| FHF-2 | 1Z+1Y   | Human   | Human brain cDNA library. |
| FHF-3 | 1A       | Human   | Smallwood et al. (4). |
|       |          | Mouse   | Smallwood et al. (4). |
| FHF-3 | 1B       | Mouse   | e15 mouse embryo RACE. |
| FHF-4 | 1A       | Human   | Smallwood et al. (4). |
|       |          | Mouse   | e15 mouse embryo RACE; Smallwood et al. (4). |
|       |          | Chicken | e10 chick brain cDNA library. |
| FHF-4 | 1B       | Mouse   | e15 mouse embryo RACE; Yamamoto et al. (17). |
|       |          | Chicken | e10 chick brain cDNA library. |

### Subcellular Localization and Biochemical Implications of the FHF Isoforms

In earlier work, we identified a nuclear localization signal (NLS) in FHF-1 (now FHF-1(1A)), which is sufficient to confer nuclear localization in a variety of tissue culture cell lines (4). The NLS sequence is conserved among those isoforms of FHF-2, -3, and -4 which differ in their amino-terminal sequences (Table I).

To analyze the subcellular distribution of the different FHFs and their various isoforms, chicken, mouse, and human FHFs were produced in transiently transfected 293, COS, and QT6 cells. Similar results were obtained with the different cell lines. As expected, FHF-2(1S), the isoform most similar to FHF-1(1A), localizes to the nucleus in transfected cells (Fig. 3A). The FHF-2 isoforms beginning with exons 1U, 1V, 1Y, or (1Y+1V) lack a consensus NLS, and the encoded proteins show a diffuse cytosolic and nuclear distribution when transiently transfected into 293 or QT6 cells (Fig. 3B; Table I). Double immunofluorescence staining of these FHF-2 isoforms and BiP, an ER-resident protein, reveals that they are not localized to the ER (Fig. 3B). Their localization in the nucleoplasm may reflect passive diffusion of the FHFs through nuclear pores. In contrast, a myc-tagged version of cFHF-4(1B) is localized exclusively in the cytosol in COS and 293 cells (Fig. 3, D and E), whereas mFHF-4(1A), which has a consensus NLS, shows nuclear localization, although it is excluded from the nucleoli (Fig. 3C). Hydropathy analysis of the FHF-4(1B) splice variant shows a hydrophobic stretch in the first 25 residues of this isoform which could act as an ER-targeting signal sequence, the only such sequence identified thus far among the FHFs. However, we have been unable to detect significant quantities of myc-tagged FHF-4(1B) or any other FHF in conditioned media from transfected cells.

The FHF-2(1S), FHF-2(1V), and FHF-2(1Y+1V) isoforms were tagged at their carboxy termini with six histidines and purified from 293 cell lysates by affinity chromatography using nickel resin and heparin agarose. The FHF-2(1S) isoform binds heparin more tightly than the other two isoforms, eluting at 0.7 M NaCl rather than at 0.4 M NaCl as seen for FHF-2(1V) and FHF-2(1Y+1V). This observation suggests that different FHF isoforms may differ in their interactions with polyanionic extracellular matrix components.

### Differential Expression of mFHF-1 and mFHF-2 Iso-
### Table II

**FGF Isoforms generated by 5′ Alternative Splicing**

Amino acid sequences of the alternative amino termini for the various FHF isoforms derived from human, mouse, and chicken. A slash indicates the junction between exons. Subcellular localization was determined by immunostaining of protein produced in transfected cells as described in the text. ND, not determined. Underlined residues in the FHF-1(1A) isoform indicate the bipartite (left) and secondary (right) nuclear localization sequences as described in Ref. 4.

| Exons | Sequence | Species | Subcellular localization |
|-------|----------|---------|--------------------------|
| FHF-1 |          |         |                          |
| 1A    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Human  | Nuclear                  |
|       |          |         |                          |
| 1B    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Mouse  | Absent in nucleoli       |
|       |          |         |                          |
| FHF-2 |          |         |                          |
| 1A    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Mouse  | Cytosolic + nuclear      |
|       |          |         |                          |
| 1B    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Human  | Cytosolic + nuclear      |
|       |          |         |                          |
| FHF-3 |          |         |                          |
| 1A    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Mouse  | ND                       |
|       |          |         |                          |
| 1B    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Human  | ND                       |
|       |          |         |                          |
| FHF-4 |          |         |                          |
| 1A    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Mouse  | ND                       |
|       |          |         |                          |
| 1B    | MAAAIASSLRKRQARRESNSDRTVAIASKRRSSPSFSKGRSLLERVLGLGFSKVRFCGRELKRPVR/EPLQ... | Mouse  | ND                       |

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forms—To investigate the tissue distribution of different FHF isoforms, riboprobes specific for various alternate exons were hybridized in situ to sections of e12.5 and e18.5 mouse embryos and adult mouse brain (Fig. 4). Expression levels in eight tissues in the adult mouse were determined for various isoforms by RNase protection.

The tissue distribution of mFHF-1 transcripts carrying exons 2–5, the exons common to the different isoforms, has been reported by us and others (4, 6). The expression pattern of the mFHF-1(1A) isoform accounts for most of the reported pattern of mFHF-1 expression, especially during embryonic development when its expression in the nervous system is widespread (data not shown). mFHF-1(1A) message is abundant in the dorsal root ganglia of e12.5 mouse embryos, as reported previously for cFHF-1 in chicken embryos (5). In e18.5 mouse embryos, mFHF-1(1A) is present in the brain, spinal cord, and retina. In the adult brain, mFHF-1(1A) shows high expression in cerebellum and olfactory bulb and weak expression in the retina. In the adult brain, mFHF-1(1A) is present in the brain, spinal cord, and enteric ganglia. RNase protection with adult mouse RNA reveals mFHF-1(1A) expression most strongly in the brain and at lower levels in the eye.

For mFHF-2, we mapped the expression patterns of the four alternate 5'–coding exons, 1Y, 1V, 1S, and 1U. Exons 1Y and 1V are found spliced together in a subset of transcripts in both chickens and mice and are also found separately (Table I and Fig. 1). In the e12.5 embryo both exons are present in the central nervous system, heart, and limbs (Fig. 5, B and C). However, exon 1Y is present in the dorsal root ganglia, whereas exon 1V is not. Both are present in the enteric ganglia, although the in situ hybridization signal for exon 1Y is stronger. In the e18.5 embryo, mFHF-2(1Y) and mFHF-2(1V) show essentially identical expression patterns. Both exons are present in the brain, spinal cord, retina, heart, enteric ganglia, tongue, condensing cartilage in the limbs, and in connective tissue around the vertebrae and ribs (Fig. 5, E and F). These two isoforms are found in virtually all of the expression domains of the mFHF-2 gene in the developing embryo. In e12.5 embryos, mFHF-2(1S) is present in the central nervous system and liver and at lower levels in developing vertebral bodies (Fig. 5A). In e18.5 embryos, exon 1S is present in the brain, spinal cord, and enteric ganglia and at lower levels in the heart (Fig. 5D).

In the adult brain, mFHF-2(1S) is present at highest levels in the cortex and hippocampus, and in a subset of midbrain nuclei (Fig. 6, C and D). By contrast, mFHF-2 exons 1Y and 1V show a pattern of central nervous system expression which is largely nonoverlapping with that of FHF-2(1S), including strong expression in the cerebellum (especially in the anterior lobe), thalamus, olfactory bulb, amygdala, facial nucleus, medial geniculate nuclei, and a subset of nuclei in the basal brainstem (Fig. 6, E–H). Lower expression of mFHF-2 exons 1Y and 1V is observed in some cortical areas. The in situ hybridization patterns of mFHF-2 exons 1S, 1V, and 1Y together account for most of the hybridization signal seen with a probe derived from mFHF-2 exons 2–5 (compare Fig. 6, A and B, with panels C–H).

The most abundant class of mFHF-2 cDNAs isolated in the 5'–RACE screen from e15 embryos contains exon 1U (Table I). However, the exon 1U probe hybridizes only weakly to e12.5 embryos (in the spinal cord), and there is no detectable in situ hybridization signal in e18.5 embryos. Gecz et al. (16) report that this isoform is detectable by reverse transcriptase PCR from human fetal muscle and infant brain. The low level of in situ hybridization may therefore reflect masking of this region in the transcript or a technical limitation related to secondary structure within the probe.

By RNase protection, isoforms 1S, 1V, and 1U show different tissue distributions in the adult mouse (Fig. 7). mFHF-2 exon 1S shows highest expression in the brain and lower expression in the eye, spleen, and testis. Exon 1V shows highest expression in the brain and heart and low expression in the eye. Exon 1U is detected exclusively in the heart.

Differential Expression of mFHF-3 and mFHF-4 Isoforms—In e12.5 embryos, FHF-3 is expressed in the central nervous system and dermomyotome (4, and data not shown). In e18.5 embryos, the FHF-3 gene is expressed in numerous tissues, including the brain, spinal cord, tongue, kidney, thymus, lung, and heart (Fig. 4C). At this stage, the FHF-3(1B) isoform is expressed throughout the nervous system and in the tongue but at lower levels than the mFHF-3(1A) isoform.

In the adult brain, mFHF-3 is expressed in the caudate and putamen, cerebellum, layers 4 and 5 of the cortex, accumbens nuclei, superior colliculus, deep cerebellar nuclei, red nuclei, in a variety of brain stem regions, and in a subset of anterior thalamic nuclei (Fig. 4, D–F). mFHF-3(1A) appears to account for most of the in situ hybridization signal obtained with probes for the conserved exons 2–5. In situ hybridization with the FHF-1(1B) probe shows largely similar expression domains but at much lower levels. By RNase protection, the mFHF-3(1A) isoform is found in the adult brain, lung, and testis, and the mFHF-3(1B) isoform is found in the brain and eye and at lower levels in the lung and testis.

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**Fig. 1. Structures of alternatively spliced FHF-2 transcripts.** Each exon is represented by a box. Intron lengths are not to scale. The 5'-end of exon 2 is shown to the right. Exons 1S, 1V, 1Y, and 1U are coding exons. Other exons are noncoding. The sequence of exon 1R is incomplete at the 5'-end. Exons 1S' and 1Y' correspond to the 3'-ends of exons 1S and 1Y. The species from which the transcripts were identified are shown on the right: h, human; m, mouse; c, chicken.

**Fig. 2. Structure of the human FHF-2 gene at Xq26.** The map is shown at two scales to illustrate the clustering of exons along the different contigs. Gaps between contigs are of unknown size. Exons 1P, 1Z, 1Y, and 1X map to PAC 227P17. Exons 1W and 1V map to contig J362G171. Exons 1U, 1S, 1R, 2, and 3 map to PAC 260J9. Exons 4 and 5 map to nonoverlapping λ phage clones. Exons 1Q and 1T have been identified in chickens (Table I), but their orthologs have not been identified in currently available human genomic sequences and therefore are not included in the hFHF-2 gene map. kb, kilobases.
In e12.5 embryos, the expression of the two FHF-4 isoforms is limited to the brain and developing spinal cord. In e18.5 embryos, both isoforms are expressed in the brain and spinal cord, but exon 1A is also expressed in the thymus, whereas exon 1B is not (Fig. 8, A–C). Fig. 8, D–F, shows that in the adult brain mFHF-4(1B) is expressed at higher levels than mFHF-4(1A) in the cortex. FHF-4(1A) shows strong expression in the mamillary nuclei and weak expression in the hippocampus, whereas mFHF-4(1B) is not found in the mamillary nuclei and is present at high level throughout the hippocampus and dentate gyrus and in the cerebellum. The patterns of expression of these two isoforms appear to account for most of the hybridization signal obtained with the constant region probe (exons 2–5). By RNase protection, mFHF-4(1A) is found in the adult brain and at lower level in the eye.

**DISCUSSION**

Fibroblast growth factors comprise a large family of signaling molecules with diverse roles during embryonic and adult life. Both FGFs and their receptors show a complex pattern of gene regulation involving alternate splicing and, in some instances, translational control (13, 14, 17, 18). The cell biology of the FGFs is complex and poorly understood. Some FGFs are likely to be constitutively secreted, but others, such as FGF-1, FGF-2, and FGF-3, exhibit regulated secretion (19–21). FGF-1 and FGF-2 lack consensus secretion signal sequences and appear to be released from cells by a route that is independent of the ER-Golgi secretory pathway. In some instances, FGFs have been localized to the nuclei of cells that produce them, and both FGFs and FGF receptors have been localized to the nuclei of cells that receive their signals (12–14, 22, 23). One FGF receptor-like molecule of unknown function, MG160, localizes to the Golgi apparatus (24).

The FHF s form a distinct branch of the FGF family and have been implicated in the development of the nervous system and other tissues, based on *in situ* hybridization studies (4–6). In chicken embryos, FHF-2 (FGF-13) has been implicated in the control of patterning and growth of the cartilagenous elements of the limb (5), although the signaling pathways that lead to this activity are largely unknown. We previously identified two spliced isoforms of the FHF-2 gene which showed differential and nonoverlapping distributions during chicken development (5). In the present report, analysis of 5′-RACE products from developing mouse embryo RNA and screening of cDNA libraries from a variety of human, mouse, and chicken tissues have yielded three 5′-spliced isoforms of the FHF-1 gene, nine of the FHF-2 gene, and two each of the FHF-3 and FHF-4 genes. Together with the identification of two alternatively spliced isoforms of FHF-1 by Hartung *et al.* (6) and one alternatively spliced isoform of FHF-4 by Yamamoto *et al.* (7), the present...
work brings to 17 the total number of alternatively spliced isoforms identified in 5′-exons of the FHF subfamily (Table I).

The nine isoforms of the FHF-2 gene encode five distinct amino termini (Table II). Among the FHF cDNA clones characterized in this work, alternative splicing appears to be confined to the region 5′ of exons 2–5, as we did not observe spliced isoforms of the FHFs that alter any other regions of the transcript. However, Gecz et al. (16) has reported an isoform of the FHF-2 gene which contains an alternatively spliced exon 3, which leads to a premature termination of the protein.

Because large parts of the human FHF-2 locus have been sequenced, it is possible to identify unambiguously the human genomic counterparts of exons identified in chicken and/or mouse cDNAs. Most of the exons reported here have counterparts in humans, mice, and chickens. The high degree of evolutionary conservation among orthologous alternative exon sequences suggests that there is strong selective pressure to maintain these complex gene structures.

The hFHF-2 gene resides in Xq26 (4), where a variety of human disorders, including several X-linked mental retardation syndromes and X-linked polydactyly/syndactyly, have been mapped (16, 25). The identification of multiple alternate exons in the hFHF-2 gene should facilitate the analysis of the hFHF-2 gene in these and other disorders.

Effect of Alternative Promoter Use and Splicing on the Expression Patterns of the FHFs—The expression patterns of the different isoforms of the FHFs reported here most likely reflect regulation by alternative promoter use. For each FHF, the different isoforms generally show distinct spatial distributions, both in the developing embryo and the adult, and all are expressed in neural tissues. For example, in comparing mFHF-2(1S) with mFHF-2(1Y) and mFHF-4(1A) with mFHF-4(1B), several nonoverlapping expression domains are observed within the adult mouse brain (Figs. 5, 6, and 8).

The general pattern of distinct distributions of isofrom expression, which is observed among the FHFs, is seen to varying extents in the expression patterns of other differentially spliced growth factors (26–29). For example, the eight spliced isoforms of FGF-8 show largely overlapping expression domains (27), whereas two of the three COOH-terminal splice variants of bone morphogenetic protein-1 show distinct expression patterns in the developing mouse but are coexpressed in the adult (30).

![Fig. 5. In situ localization of differentially spliced mFHF-2 transcripts in the developing mouse.](image)

![Fig. 6. In situ localization of differentially spliced mFHF-2 transcripts in the adult mouse.](image)
species-specific differences in the expression patterns of some of the alternative 5′-coding exons. We speculate that these differences may be caused by the differential association of these 5′-coding exons with different 5′-noncoding exons and therefore different start sites of transcription. For example, in mouse and human cDNAs, exon 1S of mFHF-2 contains a very long 5′-noncoding region. In chicken cDNA, we have found only the 3′-end of this exon (referred to as exon 1S′) which contains the coding region preceded by a small 5′-noncoding region. This exon is spliced to a novel, noncoding exon, exon 1T, which has not thus far been identified in human cDNA (Fig. 1 and Table I). In chicken embryos, exon 1S′ is expressed exclusively in the ventral region of the neural tube (5); in e12.5 mouse embryos, exon 1S is expressed in the neural tube, liver, and heart (Fig. 5). Similarly, exon 1Y or its 3′-end, which we have termed 1Y′, is found associated with any of three different noncoding exons (1Z, 1P, and 1Q; Fig. 1 and Table I). In mouse cDNA, exon 1Y has been found as part of a longer cDNA that also contains exon 1P; in human cDNA, exon 1Y has been found as part of a longer cDNA that also contains exon 1Z; and in chicken cDNA, exon 1Y′ has been as part of a longer cDNA that also contains exon 1Q.

Effect of Alternative Splicing on the Subcellular Localization and Bioactivities of the FHFs—The implications of alternative splicing of the FHFs on their bioactivities are yet to be fully elucidated, but our analysis of the effects of alternative splicing on the tissue distribution and subcellular localization of recombinant FHFs suggests that this process plays an important role in regulating FHF gene expression and may also affect the biochemical and secretory properties of the encoded proteins. Preliminary experiments in the chicken embryo have suggested that there are functional differences between the spliced isoforms of cFHF-2 during limb development (5). These differences may be caused by differences in the interaction between the cFHF-2 isoforms and their receptor(s) and/or the extracellular matrix, or they may reflect differences in the efficiencies of secretion and/or stabilities of the different isoforms.

In the chicken embryo, the expression and regulation of the cFHF-4 gene suggest that this gene plays a role during limb patterning and innervation and during the patterning of the frontonasal facial primordia. Only the nuclear localized cFHF-4(1A) isomer is expressed in these tissues or in response to a variety of experimental manipulations. These results, together with our previous functional data on the cFHF-2 gene (5), further suggest that the nuclear and cytosolic isoforms of the FHFs are not functionally redundant.

In a previous report (4) we described the pattern of FHF-1 immunoreactivity in macaque brain. Those results suggested that FHF-1 is present in both the nucleus and cytoplasm of expressing cells in the adult brain. However, because both the anti-FHF-1 and anti-FHF-2 antibodies bind to the shared core domain of the FHF used as an immunogen (exons 2–4), they are unable to distinguish among the different isoforms.

The amino termini of the various isoforms are diverse, both in size and sequence, and we show here that in transfected tissue culture cells different isoforms differ in subcellular distribution. The FHF-1(1A), FHF-2(1S), and FHF-4(1A) isoforms localize to the nucleus of transfected cells, whereas other FHF-2 isoforms are either cytosolic or both cytosolic and nu-

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**Fig. 7. Tissue distribution of FHF-2 transcripts in the adult mouse.** 10 μg of total RNA from the indicated tissues was used for RNase protection with the indicated exon probes of the FHF-2 gene. An RNA polymerase II probe was used as a control.

**Fig. 8. In situ localization of differentially spliced mFHF-4 transcripts in the developing and adult mouse.** 33P in situ hybridization is shown in red superimposed upon a cresyl violet stain shown in gray and white. Panels A–C, e18.5 embryos; panels D–F, adult brain. The probes used were as follows: mFHF-4 (exons 2–5; panels A and D); mFHF-4 (1A; panels B and E); mFHF-4 (1B; panels C and F). Sections in panels A–C are adjacent or separated by no more than 40 μm. For a description of the hybridization patterns, see “Results.”

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2 I. Munoz-Sanjuan, M. K. Cooper, P. A. Beachy, J. F. Fallon, and J. Nathans, unpublished data.
clear. The nuclear localization of FHF-1(1A) and FHF-4(1A) excludes the nucleoli, in contrast to the uniform nuclear localization of FHF-2(1S). We have shown previously that the nuclear localization of FHF-1(1A) is mediated by a bipartite nuclear localization signal (4). An additional basic region within that exon could play a secondary role in nuclear localization, because deletions of half of the bipartite NLS still permit nuclear localization in 293 cells as long as the presumptive secondary NLS is present. Both the bipartite NLS and the secondary NLS sequences are conserved in the FHF-4(1A) isoform. The second half of the bipartite NLS is missing in the FHF-2(1S) isoform. These observations suggest that the FHFs may act intracellularly, in the cells in which they are synthesized, in target cells, or in both. In vitro, different FHF-2 isoforms bind with different affinities to heparin as judged by the NaCl concentration required to dissociate the complex. Heparin modulates the range or potency of signaling by different FHF-2 isoforms.

In summary, the evolutionary conservation of multiple FHF isoforms, together with their different subcellular distributions, heparin binding affinities, and expression patterns, suggests that alternative promoter use and differential splicing are important regulatory processes for controlling the activities of the FHF family.

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