Expression of chicken fibroblast growth factor homologous factor (FHF)-1 and of differentially spliced isoforms of FHF-2 during development and involvement of FHF-2 in chicken limb development

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SUMMARY

Members of the fibroblast growth factor (FGF) family have been identified as signaling molecules in a variety of developmental processes, including important roles in limb bud initiation, growth and patterning. This paper reports the cloning and characterization of the chicken orthologues of fibroblast growth factor homologous factors-1 and -2 (cFHF-1/cFGF-12 and cFHF-2/cFGF-13, respectively). We also describe the identification of a novel, conserved isoform of FHF-2 in chickens and mammals. This isoform arises by alternative splicing of the first exon of the FHF-2 gene and is predicted to encode a polypeptide with a distinct amino-terminus.

Whole-mount in situ hybridization reveals restricted domains of expression of cFHF-1 and cFHF-2 in the developing neural tube, peripheral sensory ganglia and limb buds, and shows that the two cFHF-2 transcript isoforms are present in non-overlapping spatial distributions in the neural tube and adjacent structures. In the developing limbs, cFHF-1 is confined to the posterior mesoderm in an area that encompasses the zone of polarizing activity and cFHF-2 is confined to the distal anterior mesoderm in a region that largely overlaps the progress zone. Ectopic cFHF-2 expression is induced adjacent to grafts of cells expressing Sonic Hedgehog and the zone of cFHF-2 expression is expanded in talpid² embryos. In the absence of the apical ectodermal ridge or in wingless or limbless mutant embryos, expression of cFHF-1 and cFHF-2 is lost from the limb bud. A role for cFHF-2 in the patterning and growth of skeletal elements is implied by the observation that engraftment of developing limb buds with QT6 cells expressing a cFHF-2 isoform that is normally expressed in the limb leads to a variety of morphological defects. Finally, we show that a secreted version of cFHF-2 activates the expression of HoxD13, HoxD11, Fgf-4 and BMP-2 ectopically, consistent with cFHF-2 playing a role in anterior-posterior patterning of the limb.

Key words: FGFs, Limb morphogenesis, Anteroposterior axis, Pattern formation, Alternative splicing, Chick.

INTRODUCTION

The fibroblast growth factor (FGF) family comprises a group of at least 17 secreted polypeptides that act via transmembrane tyrosine kinase receptors. FGF family members have been implicated in a variety of biological processes, serving as regulators of cell proliferation, differentiation and survival in a variety of systems (reviewed in Brook et al., 1996; Burgess and Maciag, 1989; Gosporadowicz, 1990; Szelenyi and Fallon, 1999; Martin, 1998). During development, FGFs have been shown to play a central role in the genesis of various tissues and structures. For example, FGF-8 is involved in midbrain formation (Crossley et al., 1996a), FGF-4 and FGF-8 are involved in gastrulation (Feldman et al., 1995; Meyers et al., 1998), and FGF-1 and FGF-2 are involved in neurogenesis (Vicario-Abejon et al., 1995; Qian et al., 1997; Vescovi et al., 1993). The role of FGFs in limb development has been extensively studied. FGFs released from a bead grafted in the trunk between the limb-forming areas will induce ectopic limbs, indicating a role for one or more of these factors early in the limb initiation process (Cohn et al., 1995; Ohuchi et al., 1995, 1997a; Crossley et al., 1996b; Vogel et al., 1996; Martin, 1998).

FGF-2, FGF-4 and FGF-8 are expressed in the apical ectodermal ridge (AER; Niswander and Martin, 1992; Savage et al., 1993; Heikinheimo et al., 1994; Crossley and Martin, 1995; Savage and Fallon, 1995), and each of these three FGFs has been shown to replace the AER after its removal and to permit nearly normal determination of the limb skeleton (Niswander et al., 1993; Fallon et al., 1994; Mahmood et al., 1995; Crossley et al., 1996b; Vogel et al., 1996). FGF-2 and FGF-10 are expressed in the distal region of the limb bud mesoderm called the progress zone (Savage and Fallon, 1995; Ohuchi et al., 1997a). It is proposed that this region is under...
AER influence, maintaining the cells in an undifferentiated state (Summerbell et al., 1973). FGF-10 expression may be involved in limb initiation as well as AER initiation and maintenance based on the spatial and temporal pattern of FGF-10 expression and on experimental manipulations (Ohuchi et al., 1997a).

FGFs from the AER are probably required for the initiation and maintenance of Sonic Hedgehog (Shh) by ZPA cells, and these cells in turn influence FGF expression by the AER, thus forming a feedback loop (Laufc et al., 1994; Niswander et al., 1994a,b). The intermediaries in these signalling processes, the order of their appearance and their functional roles are currently the subjects of intense investigation.

We previously reported the identification of four novel members of the FGF family, which we named fibroblast growth factor homologous factors (FHFs; Smallwood et al., 1996). FHF-1, FHF-2, FHF-3 and FHF-4 are now equivalently referred to as FGF-12, FGF-13, FGF-11 and FGF-14, respectively. Based on sequence homology, the FHFs constitute a distinct branch of the FGF family. They share significant homology with other FGFs in the core structural domain common to all members of this family, but their amino- and carboxy-termini are highly divergent. We and others have shown that all of the FHFs show widespread expression in the developing and adult nervous system in mice, with some of the FHFs showing expression in a few other tissues (Smallwood et al. 1996; Hartung et al., 1997). Like FGF-1 and FGF-2, the FHFs lack a classical signal sequence for secretion. A nuclear localization signal (NLS) present in the first exon of FHF-1 shows that all of the FHFs share significant homology with other FGFs in the core structural domain common to all members of this family, but their amino- and carboxy-termini are highly divergent. We and others have shown that all of the FHFs show widespread expression in the developing and adult nervous system in mice, with some of the FHFs showing expression in a few other tissues (Smallwood et al. 1996; Hartung et al., 1997). Like FGF-1 and FGF-2, the FHFs lack a classical signal sequence for secretion. A nuclear localization signal (NLS) present in the first exon of FHF-1 constituting a distinct branch of the FGF family. They share significant homology with other FGFs in the core structural domain common to all members of this family, but their amino- and carboxy-termini are highly divergent.

In this paper, we report the cloning and characterization of the chicken orthologues of FHF-1 and FHF-2, referred to below as cFHF-1 and cFHF-2, respectively. Whole-mount in situ hybridization reveals a complex and dynamic pattern of expression of cFHF-1 and cFHF-2 in the developing spinal cord and sensory ganglia and in limb buds during active periods in the morphogenesis of these structures. We have also identified a splice variant of the chicken FHF-2 gene that encodes a protein isoform with a distinct amino-terminus. The two cFHF-2 isoforms have distinct, and non-overlapping, spatial and temporal expression patterns in the chicken embryo. Finally, we show that known regulators of limb morphogenesis also regulate the pattern of expression of cFHF-1 and cFHF-2 in the limb, and that engrafment of cells expressing cFHF-2 into developing limb buds leads to morphological abnormalities consistent with a role for cFHF-2 in the patterning and growth of the limbs. The developmental abnormalities observed as a result of cFHF-2 misexpression appear to be mediated through the ectopic activation of several genes involved in anterior posterior patterning of the limb, such as HoxD13, HoxD11, Fgf-4 and BMP-2.

MATERIALS AND METHODS
Cloning of chicken FHFs
A fully degenerate sense primer encoding the amino acid sequence VFENYYV with a 5’ EcoRI site and three partially degenerate antisense primers encoding the amino acid sequence MKGN(H/R)VK with a 5’ BamHI site were used to amplify chicken genomic DNA. After 35 cycles of PCR with an annealing temperature of 45°C, the PCR products were digested with EcoRI and BamHI, separated by agarose gel electrophoresis, subcloned into pBS-KS (Stratagene) and sequenced. The degenerate PCR products derived from cFHF-1 and cFHF-2 were used to isolate cDNA clones encoding full-length cFHF-1 and the cFHF-2 (1y+1v) splice isoforms from an e6 chick retina cDNA library (a gift of Dr R. Adler). cDNA clones encoding the FHF-2(1s) isoform were isolated from an e10 chick brain cDNA library (a gift of Dr M. Tessier-Lavigne) by screening with exon 1s of FHHF-2. Filters were hybridized in 30% formamide, 5x SSC at 42°C, and washed in 2x SSC at 50°C.

Whole-mount in situ hybridization
Riboprobes were synthesized from templates prepared by PCR amplification with an antisense primer that contained a T3 promoter sequence at its 5’ end. 100 ng of each PCR product was used for in vitro transcription with T3 RNA Polymerase in the presence of digoxigenin UTP. The following probes were used: 0.55 kb containing cFHF-1 exons 2-5; 0.6 kb containing cFHF-2 exons 2-5; 0.3 kb containing cFHF-2 exons 1y and 1v; and 0.25 kb containing cFHF-2 exon 1s. The whole-mount in situ hybridization was performed essentially as described in Li et al. (1994).

Plasmids containing probes for cHoxD11a, cHoxD13 and cBMP-2 were a gift of C. Tabin, cSHH plasmid was a gift of P. A. Beachy and cFgf-4 plasmid was a gift of L. Niswander.

QT6 cell grafting
Shh-N
QT6 cells were transiently cotransfected with a pCIS expression plasmid encoding the N-terminal product of the Shh gene (Roelink et al., 1995; Fan et al., 1995; Lopez-Martinez et al., 1995; a gift of P. Beachy) and a pCIS expression plasmid encoding β-galactosidase (Chang et al., 1994). For control grafts, QT6 cells were transfected with the β-galactosidase plasmid alone. Transfections were performed with a low pH, calcium-phosphate method (Chen and Okayama, 1987). Secretion of Shh-N was assessed by western blotting of conditioned media with antibodies against Shh (a gift of Dr P. Beachy). Expression of β-galactosidase, monitored by X-gal staining, showed that greater than 50% of the cells were transfected. 24-36 hours after transfection, the cells were resuspended in L-15 media (GIBCO) and poly-D-lysine added to 33 µg/ml (Chang et al., 1994).

Suspended cells were incubated at 37°C for 20 minutes with shaking and a pellet of cells was grafted into the anterior mesoderm of limb buds in stage 19-21 embryos.

cFHF-1 and cFHF-2
QT6 cells were transiently cotransfected with a pCIS construct encoding cFHF-1A or cFHF-2(1y+1v) and a pCIS construct encoding β-galactosidase, or, for control grafts, were transfected with the β-galactosidase plasmid alone. Transfection efficiency (typically greater than 50% of cells) and protein production were monitored by immunostaining and western blotting using affinity-purified anti-FHF1 and anti-FHF-2 antibodies. Cells were grafted into either the anterior mesoderm or the posterior mesoderm as described above and embryos were allowed to develop to day 10. X-gal staining 2 days after grafting revealed minimal migration of the engrafted cells from the original site.

RCAS-cFHF-2(1y+1v)
The cFHF-2(1y+1v) coding region was subcloned into the Cla-12 adaptor vector (Hughes et al., 1987). The cFHF2(1y+1v) was then subcloned into the RCAS-BP (type A) vector in the 5′ site (Mason and Fekete, 1995). Expression of cFHF-2 was assayed by immuno-staining of transfected QT6 cells with anti-FHF-2 antibodies.
To construct a secreted version of cFHF-2(1y+1v), the coding region, beginning at the initiator methionine, was fused to the signal sequence of the heavy chain of human IgG (codons 1-24) (Aruffo et al., 1990). The efficiency of secretion was assessed by western blotting of conditioned media from 293 and QT6 cells with anti-FHF-2 antibodies. Immunostaining of transfected cells revealed an ER-Golgi pattern (not shown).

**Expression of cFHF-1 and cFHF-2 in the developing nervous system**

As determined by in situ hybridization, the expression of cFHF-1 and cFHF-2 in the chicken embryo is complex and dynamic (Figs 2-5). In this paper, we focus our analysis on the developing spinal cord, dorsal root ganglia (DRG), and limbs, although there are other sites of cFHF-1 and cFHF-2 expression, such as the heart, brain and retina. Unless indicated otherwise, the in situ hybridization probes used in the analyses described below encompass exons 2-5 of cFHF-1 or cFHF-2; these exons are present in all of the splice forms identified thus far (I. M. S. and J. N., unpublished observations).

A probe encompassing exons 2-5 of cFHF-1 detects transcripts in the neural tube beginning at stage 18 (Fig. 2). (Throughout this paper the Hamburger and Hamilton staging system is used; Hamburger and Hamilton, 1951.) At this stage, expression is confined to the roof and floor plates. At stage 20-22, cFHF-1 expression expands ventrolaterally, increasing in intensity in the roof plate and decreasing in the floor plate. By stage 24-25, cFHF-1 expression in the floor plate has disappeared, and the highest levels of expression are seen in the roof plate and dorsolateral neural tube (Fig. 2D). A discrete zone of expression in the ventral neural tube is seen between stages 20-21 and 26 (Fig. 2D).

The cranial ganglia express cFHF-1 between stages 22 and 28, and by stage 23-24, the DRG also express cFHF-1 (Fig. 2B,D). At stage 25-26, cFHF-1 expression is highest in those DRG that will innervate the limbs as well as in the most caudal ganglia (Fig. 2C). After stage 28, the expression of cFHF-1 in the DRG falls below detectable levels. We tested whether expression of cFHF-1 in the DRG was target-dependent by removing one of the wing buds in stage 20 embryos; at this stage, the axons have not yet entered the limbs (Tosney and Landmesser, 1984). Embryos were allowed to develop until stage 25-26 and then examined by in situ hybridization (not shown). No differences in cFHF-1 expression were observed between the operated and control sides of the embryos, suggesting that the distinctive rostrocaudal pattern of cFHF-1 expression in the DRG is not regulated by target availability.

A probe encompassing exons 2-5 of cFHF-2 reveals expression in the floor plate between stages 17 and 19-20 (Fig. 3D). Beginning at stage 19, there is prominent cFHF-2 expression in the lateral regions of the neural tube. At stage 21, expression is also seen in the ventrolateral neural tube in the region populated by motor neurons, and in the DRG (Fig. 3E). Beginning at stage 17-18, there is strong expression in the most
rostral cranial ganglia, in particular the trigeminal ganglia, and expression subsequently appears in more caudal ganglia, where it persists at least through stage 28 (Fig. 3A). cFHF-2 is expressed beginning at stage 17-18 in migrating neural crest cells that subsequently coalesce to form the DRG (Fig. 3B,C). High level expression in the DRG (Fig. 3F) continues at least through stage 31. The tail bud shows strong expression beginning at stage 18.

Fig. 1. Comparisons of the deduced amino acid sequences of human and chicken FHF-1 (A) and FHF-2 (B). The full sequences show the isoforms reported by Smallwood et al. (1996): FHF-1A and FHF-2(1s). Amino acid identities are shown by those letters on black. (B, lower) Deduced amino acid sequence of the divergent amino terminus of the human and chicken FHF-2 splice isoform encoded by exons 1y+1v; the next amino acids in both proteins are EPQL..., encoded by exon 2 as defined in Smallwood et al. (1996). The corresponding DNA sequences have been deposited in the GenBank database (accession numbers AF108754-AF108757).
Differential tissue distribution of cFHF-2 isoforms in the chicken embryo

To begin to examine the functional significance of differential splicing of cFHF-2, we determined the expression patterns of isoforms cFHF-2(1y+1v) and cFHF-2(1s) during development (Fig. 4). Whole-mount in situ hybridization with a riboprobe from exons (1y+1v) reveals that this isoform is expressed in the limbs, neural tube, and presumptive myoblasts beginning at stage 21 (Fig. 4A, right, and 4B). A probe from exon 1s reveals expression only in the neural tube at this stage (data not shown). Cross sections of stage 21 embryos show that transcripts containing exons (1y+1v) are present in the dorsolateral neural tube, a subset of cells within the DRG and what appears to be myoblasts (Fig. 4B), whereas transcripts containing exon (1s) are expressed exclusively in the ventrolateral neural tube, in a subset of motor neurons (Fig. 4C). Thus, these two splice variants of cFHF-2 are expressed in distinct and non-overlapping cell populations, suggesting the general possibility that alternative splicing at the 5’ end may reflect a requirement for spatial regulation of gene expression.

Expression patterns of cFHF-1 and cFHF-2 in the developing limbs

Both cFHF-1 and cFHF-2 are expressed in the limb bud mesoderm in patterns suggestive of a role for these factors in limb patterning and growth (Fig. 5); they are not expressed in the early limb bud ectoderm. cFHF-1 expression in the leg and wing buds starts at stage 19-20. Between stages 19 and 21, the expression of cFHF-1 is confined to the posterior mesoderm in a pattern similar to that of BMP-2 in the mesoderm (Francis et al., 1994), and including the zone of polarizing activity (ZPA) (Figs 2A, 5A,F). Between stages 22 and 25, while expression remains most prominent in the posterior region, there is also low level expression throughout the remaining distal mesoderm (Figs 2B, 5B,G). Beginning at stage 26, the anterior mesoderm also expresses cFHF-1 (Figs 2C, 5C,H). After stage 28, both the anterior and posterior domains of cFHF-1 expression decline rapidly. At stage 30, expression is detected weakly in the areas of interdigital cell death and in the exterior of the digital condensations. This pattern continues through stage 31-32, at which point the tendons also express cFHF-1. These later developmental patterns of expression are roughly consistent with those reported by Hartung et al. (1997), who have shown that, in the e10.5-e12.5 mouse embryo, mFHF-1 is expressed in connective tissue around regions of bone condensation.

cFHF-2 expression in the limb buds begins at stage 20-21 in the hindlimbs and at late stage 21 in the forelimbs (Figs 3A, 4A, 5D,I). This temporally distinct expression in the forelimbs and hindlimbs is maintained throughout the stages analyzed. The domain of cFHF-2 expression is initially limited to the most distal mesoderm, immediately under the AER. During stages 22-25, this domain expands anteriorly, encompassing the progress zone and immediately adjacent mesoderm, but excluding the posterior mesoderm where cFHF-1 is expressed (Fig. 5E,J). We note that cFHF-2 is symmetrically expressed along the dorsoventral axis and that it is not expressed in the...
ectoderm (Fig. 5K, inset). At stage 28, cFHF-2 expression is high in areas of condensing cartilage, but it is expressed very weakly in the distal mesoderm. By stage 30, the distal mesoderm expression falls below detectable levels, but the AER expresses cFHF-2. In addition, both the dorsal and ventral muscles express cFHF-2, as revealed by cross-section of stage 30 limbs (not shown).

The predominant cFHF-2 isoform in the limbs appears to be cFHF-2(1y+1v) as judged by the nearly equal intensities of hybridization in the limbs produced by probes derived from exons (1y+1v) and from exons 2-5 (Fig. 4A). By contrast the cFHF-2(1s) isoform is not expressed in the limb.

Expression of cFHF-1 and cFHF-2 in the limb: induction by Shh and BMP-2

The expression patterns of cFHF-1 and cFHF-2 in the developing limbs differ from those of other FGF family members, suggesting that the FHFs may play a role in this process that is distinct from those of other FGFs. As an initial step in examining this possibility, we asked whether the patterns of cFHF-1 and cFHF-2 expression are regulated by sonic hedgehog (Shh) and bone morphogenetic protein-2 (BMP-2), two known components of the anterior posterior (A/P) axis pathway. Shh acts from the ZPA to polarize the limb bud along the A/P axis, and also acts to maintain the expression of FGF-4 in the AER, hence also playing a role in the proximodistal growth of the limbs (Riddle et al., 1993; Lauffer et al., 1994; Niswander et al., 1994b). In the absence of Shh, the limbs buds are truncated (Chiang et al., 1996). BMP-2 is regulated by Shh in the limb (Francis et al., 1994), and its expression has been linked to the regulation of programmed cell death during skeletogenesis of the limb bud (Gañán et al., 1996, Macias et al., 1997). BMP-2 expression in the earliest stages of limb bud morphogenesis is restricted to the posterior distal mesoderm and to the AER.

Quail fibroblasts (QT6 cells) transiently transfected with a plasmid encoding the N-terminal domain of the Shh gene were grafted into the anterior mesoderm of stage 19-20 wing and leg buds, and then analyzed for cFHF-1 and cFHF-2 expression 20-24 hours later by whole-mount in situ hybridization (Fig. 6A). To assess the efficacy of the Shh grafts, several Shh-grafted embryos were allowed to develop to day 10. These specimens showed a range of Shh effects, from a duplication of digit 2 to full wing duplications, a result indicative of varying levels of Shh activity under the conditions of this experiment. In situ hybridization experiments revealed an induction of cFHF-2 expression in the mesoderm immediately adjacent to the graft in 14 of 24 grafted limbs. None of the 14 control grafts with β-galactosidase-transfected QT6 cells showed ectopic cFHF-2 transcripts (Fig. 6). Ectopic expression of digit 2 to full wing duplications, a result indicative of varying levels of Shh activity under the conditions of this experiment. In situ hybridization experiments revealed an induction of cFHF-2 expression in the mesoderm immediately adjacent to the graft in 14 of 24 grafted limbs. None of the 14 control grafts with β-galactosidase-transfected QT6 cells showed ectopic cFHF-2 transcripts (Fig. 6). Ectopic expression...
was seen as early as 20 hours after grafting and was only observed in cases where the grafts were placed at a distal location within the limb and adjacent to the AER; more proximal grafts did not induce ectopic cFHF-2 expression. The abundance of ectopic cFHF-2 transcripts was comparable to that of the endogeneous cFHF-2 transcripts within the normal zone of expression. By contrast, in a similar set of grafts, very weak ectopic cFHF-1 expression was observed in 2 of 10 limbs. The level of the cFHF-1 expression was significantly lower than that seen within the normal zone of cFHF-1 expression. In a second set of Shh grafts in which complete wing duplications were observed in each of 2 embryos that were allowed to develop for 10 days, 0 of 9 limbs examined by in situ hybridization showed ectopic cFHF-1 expression. These results indicate that Shh is not sufficient to induce cFHF-1 expression under the conditions assayed. However, it is possible that Shh needs to act in conjunction with another signal derived from the posterior ectoderm, such as FGF-4, in order to activate cFHF-1 expression.

To test the effect of BMP-2 on cFHF-1 and cFHF-2 expression in the limb, heparin-acrylic beads incubated with 25 ng/ml of recombinant BMP-2 were implanted in the anterior mesoderm of the wing bud, under the AER. Three out of five embryos had characteristic BMP-2-induced defects in the wings, namely absence of the radius and second digit (Macias et al., 1997). Twelve embryos were examined 20-24 hours after bead implantation, and analyzed for alterations in cFHF-1 and cFHF-2 transcripts by in situ hybridization (data not shown). No effects were seen on the expression of the cFHFs. We conclude that, at the dose tested, BMP-2 is not able to induce cFHF-1 or cFHF-2 expression in the wing buds, and hence is not mediating Shh induction of cFHF-2 expression.

Expression of cFHF-1 and cFHF-2 in the limb: effect of AER removal

The AER is a thickened epithelium that forms at the dorsoventral boundary of the limb buds. Expression of FGF-4 and FGF-8 in the AER is responsible for inducing and maintaining the expression of Shh in the ZPA (Niswander et al., 1994b). The AER is also known to signal to the progress zone to maintain it in a proliferative state, thus promoting the proximodistal growth of the limbs. Removal of the ridge leads to programmed cell death (Rowe et al., 1982) in the mesoderm, and to truncated limbs (Saunders, 1948). Since the ZPA and the AER work as a coordinated system to maintain expression of genes involved in the establishment of the A/P axis, we tested whether AER removal from stage 19-20 embryos altered the expression of cFHF-1 and cFHF-2. 20 hours after AER removal, cFHF-1 and cFHF-2 transcripts were undetectable in the mesoderm in 7/7 limbs examined for each (Fig. 7A). In a second set of experiments, embryos were fixed and processed for in situ hybridization 7 hours after AER removal. In all cases observed (3/3), cFHF-2 expression was significantly downregulated (not shown).

The downregulation of cFHF-1 and cFHF-2 expression resulting from AER removal could be an indirect effect since removal of the AER produces multiple effects, including rapid Shh downregulation (Ros et al., 1996), and, as described above, Shh induces cFHF-2 expression. Alternatively, the loss of signals emanating from the AER might directly downregulate cFHF-1 and cFHF-2 expression. To distinguish these two possibilities, we removed only the anterior half of the AER from stage 20 embryos, while leaving the posterior half intact. Current evidence indicates that signals derived from the AER act locally; by leaving the posterior half of the AER intact, Shh...
expression is maintained in the ZPA (Vogel et al., 1995; Todt and Fallon, 1987). Following removal of the anterior AER, cFHF-2 transcripts were downregulated in the anterior region in 3/3 limb buds, while the expression of cFHF-1 in the posterior region remained unchanged in 3/3 similarly operated limbs. Taken together, these data suggest that the AER signals the underlying mesoderm to induce or maintain cFHF-1 and cFHF-2 expression and that the maintenance of cFHF-2 expression occurs via a local interaction.

Expression of cFHF-1 and cFHF-2 in the limb: alterations in limbless, wingless and talpid2 embryos

As a second step in analyzing the role of cFHF-1 and cFHF-2 in limb development, we examined their patterns of expression in mutant embryos that display various limb defects. talpid2 is a mutation affecting A/P patterning in the limbs, leading to weak anteroposterior patterning and polydactyly (Dvorak and Fallon, 1991; Krabbenhoft and Fallon, 1992). The expression of Shh in talpid2 mutants is unaffected, but there is a loss of A/P polarity among the downstream components, which are expressed uniformly throughout the limb buds. In stage 21-22 talpid2 embryos, we found that the zone of cFHF-2 expression in the distal mesoderm is greatly expanded (Fig. 7B). By contrast, cFHF-1 expression is unaffected (data not shown).

The wingless and limbless mutations affect the maintenance and formation of the AER, respectively (Ohuchi et al., 1997b; Carrington and Fallon, 1984; Ros et al., 1996; Noramly et al., 1996; Griesshammer et al., 1996). In the limbs of stage 21-22 wingless and stage 19 limbless mutant embryos, expression of both cFHF-1 and cFHF-2 is below the limit of detection (Fig. 7C and data not shown), whereas their expression appears normal in other tissues. In the case of limbless embryos, the limb bud initiates but fails to grow. The limbless buds undergo programmed cell death after stage 19, and hence cFHF-2 expression in the limbs, which begins at stage 21 in wild type embryos, could not be examined in limbless embryos. Wingless mutant embryos also show developmental defects in the most caudal regions of the embryo (J. Fallon, unpublished). cFHF-2 expression in the tail bud is lost in the wingless embryos, but its expression is maintained in the most caudal DRG, which appear disorganized (Fig. 7C). The expression pattern of cFHF-1 and cFHF-2 in all other tissues in wingless embryos appears indistinguishable from that of wild-type embryos. These observations are consistent with those described above for the effects of AER removal on cFHF-1 and cFHF-2 expression.

Activity of cFHF-2 in the limb

The experiments described thus far strongly suggest that both cFHF-1 and cFHF-2 play a role in limb bud morphogenesis. To directly test this hypothesis, QT6 cells cotransfected with plasmids encoding β-galactosidase and cFHF-2(1y+1v), the isoform that is expressed in the limb bud (Fig. 4A), were grafted into stage 18-23 limb buds. Control limb buds received grafts of QT6 cells transfected with the β-galactosidase plasmid alone. Embryos were analyzed for morphological and skeletal abnormalities at 9-10 days of development. Most grafts were placed in the proximal anterior region of the limb buds; in this location, both the cFHF-2(1y+1v) and control grafts remained at the anterior base of the limb and away from the AER throughout the incubation period as determined by X-gal staining (data not shown).

In a second set of experiments, QT6 cells were implanted as described above following either mock transfection, transfection with an RCAS retroviral vector expressing cFHF-2(1y+1v), or transfection with a plasmid encoding a derivative of cFHF-2(1y+1v) in which a signal sequence from human IgG was added in frame at the amino terminus (referred to as hIgG-cFHF-2). These grafting experiments were designed to reveal developmental effects that might be referable to larger numbers of FHF-2(1y+1v)-expressing cells in the case of the RCAS transfected cells, or to enhanced cFHF-2(1y+1v) secretion in the case of the signal sequence construct.

A variety of morphological and skeletal defects were observed at high frequency in both the wings and legs that received grafts of cells transfected with cFHF-2(1y+1v), RCAS/cFHF-2(1y+1v) or hIgG-cFHF-2(1y+1v). The severity of the grafts was, on average, greater with the latter two constructs. No defects were observed in limbs that received control grafts or grafts of QT6 cells transfected with cFHF-1A. Wing defects were only observed when stage 21 but not earlier stage embryos were engrafted. Hindlimb defects were observed following engraftment at all stages tested, beginning as early as stage 18. The defects can be grouped into several categories: (1) changes in digit number, either by duplication of digit 1 or by deletion of digits 1 or 2 in the legs, (2) changes in digit identity, (3) ectopic cartilage formation, (4) cartilage fusion and abnormal joint formation, and (5) bone shortening (hemimelia) in the zeugopods (Table 1; Figs 8, 9). When
The present, ectopic cartilage formation was located anterior to digit 2 in the wings and digit 1 in the legs, and toward the site of the graft (Figs 8C,E, 9B,D).

The most frequently observed phenotype of cFHF-2(1y+1v) engraftment in the wing was shortening of the radius, the most anterior forelimb bone (Fig. 8). In the most extreme cases, the radius was almost completely absent, with only the most distal portion remaining (Fig. 8D). The ulna was bent anteriorly in some embryos, perhaps due to the lack of comparable growth of the radius, but was otherwise unaffected by the graft. A similar phenomenon was observed in the leg, where the tibia was frequently reduced in size and thickened in diameter (Fig. 9F). In two embryos, placement of the graft in the posterior margin of the leg bud produced a posterior deviation of the grafted limb (Fig. 9A), analogous to the anterior deviation observed with anterior grafts. In six leg grafts, we observed changes in digit identity or morphology (Fig. 9E,F). In some instances, the digits showed fusions of the cartilage along the prephalangeal region or an absence of the phalangeal joints (Fig. 9C). No defects were observed in the pattern of feathers or any other ectodermal structure.

**Effects of anterior misexpression of cFHF-2 on gene expression in developing limbs**

To analyze the mechanistic basis for the cFHF-2(1y+1v) effects in limb development, we examined by in situ hybridization the patterns of expression of cBMP-2, cShh, cFgf-4, cHoxD13 and cHoxD11a in limbs engrafted with control QT6 cells, or with QT6 cells transfected with each of the three cFHF-2(1y+1v) constructs described above. When grafts of QT6 cells transfected with cFHF-2(1y+1v) or RCAS/cFHF-2(1y+1v) were placed in the anterior mesoderm of stage 20-21 leg buds and stage 21-23 wing buds and examined by in situ hybridization either 24 or 40 hours later, none of the markers showed evidence of an altered pattern of expression. By contrast, hIgG-cFHF-2(1y+1v) grafts induced upregulation of cHoxD13 (12/15 embryos; Fig. 10B), cBMP-2 (6/12 embryos; Fig. 10E), cHoxD11 (3/5 embryos; Fig. 10C) and cFgf-4 (4/4 embryos; Fig. 10D) in cells adjacent to the grafts. cShh expression was unchanged in response to the grafts (0/9 embryos; Fig. 10A). The higher activity of the hlgG-cFHF-2(1y+1v) construct relative to the unmodified cFHF-2(1y+1v) constructs is presumably related to the observation that the signal sequence derivative is efficiently secreted from transfected QT6 and 293 cells whereas the splice isoform cFHF-2(1y+1v) shows a diffuse distribution throughout the cell (not shown). Furthermore, these two splice variants of cFHF-2 differ from one another and from other FGF family members in their interaction with extracellular matrix-associated molecules such as heparin sulfate proteoglycans (J. M. S. and J. N., unpublished observations). These differences suggest that the different splice isoforms of the FHF-2 gene may have distinct activities/properties in vivo.

**DISCUSSION**

**Cloning of chick FHF-1 and FHF-2 isoforms**

This paper reports the identification of the chicken orthologues of mammalian FHF-1 and FHF-2. We also report the identification of a cFHF-2 isoform generated by alternative splicing of the first exon. These isoforms are expressed in mutually exclusive patterns, suggesting that the presence of different first exons may reflect, at least in part, a requirement for different transcriptional control regions.

In earlier work, we showed that the human FHF-1A isoform (Smallwood et al., 1996, Hartung et al., 1997), possesses a bipartite nuclear localization signal. This sequence is conserved in cFHF-2(1s) and this isoform accumulates in the nuclei of transfected human embryonic kidney (293) cells and quail fibroblast (QT6) cells, whereas the splice isoform cFHF-2(1y+1v) shows a diffuse distribution throughout the cell (not shown). Furthermore, these two splice variants of cFHF-2 differ from one another and from other FGF family members in their interaction with extracellular matrix-associated molecules such as heparin sulfate proteoglycans (I. M. S. and J. N., unpublished observations). These differences suggest that the different splice isoforms of the FHF-2 gene may have distinct activities/properties in vivo.
Like FGF-1 and FGF-2, all of the FHF-1 and FHF-2 isoforms described to date lack a classical signal sequence, and none of the isoforms tested thus far are detectably secreted from transfected cells. At present, the question of when, how and to what extent the FHFs are released from cells remains open.

**Expression of cFHF-1 and cFHF-2 in the developing embryo**

The expression patterns of the cFHFs suggest that these factors play a role in the determination of axial polarity in the neural tube and in the limbs. In this report, we have focused on the role of the cFHFs in limb bud development. In the neural tube and adjacent structures, both cFHF-1 and cFHF-2 are expressed in a dynamic fashion in regions important for D/V axis specification. For instance, cFHF-1 is transiently expressed in the floor plate and shows strong expression in the roof plate. cFHF-2 also shows transient expression in the floor plate and in the ventrolateral spinal cord, in the region containing developing motor neurons. Both of these domains have been shown to respond to Shh signals for their specification, and hence it is possible that Shh may regulate, as it does in the limbs, cFHF-2 expression in these cell populations.

The expression patterns of FHf-1 and FHf-2 at several developmental stages in the mouse have been determined by in situ hybridization to tissue sections (Smallwood et al., 1996; Hartung et al., 1997). In the neural tube and peripheral nervous system, the expression patterns are similar to those reported here in chickens. The few differences in expression patterns reported thus far between mice and chickens may reflect differences in the times of tissue sampling, differences in the temporally dynamic patterns of FHf-1 and FHf-2 gene expression, or real species differences in the spatial domains

![Fig. 9](image-url) Bone and cartilage deformities in the leg following engraftment of QT6 cells transfected with cFHF-2(1y+1v). (A) Leg that received a cFHF-2(1y+1v) and β-galactosidase graft in the posterior margin of the leg bud. (B-F) Examples of legs from embryos that were engrafted at stages 18-22 in the anterior margin of the leg bud with cFHF-2(1y+1v) and β-galactosidase. Legs from day 10 embryos were stained with Alcian blue and clarified. (B) Grafted and control (contralateral) legs from one embryo. An ectopic digit 1 (arrow) is seen in the leg that received the graft. (C-F) Examples of engrafted legs. (D) Ectopic digit 1 (right arrow) and partially duplicated tibia (left arrow). (E) Digits 1 and 2 are missing. (F) Hypoplastic tibia (arrowhead). Ti, tibia; Fi, fibula; Fe, femur.

![Fig. 10](image-url) Changes in gene expression associated with engraftment of QT6 cells transfected with hIgG-cFHF-2 (1y +1v) in the wings. Embryos were grafted with hIgG-cFHF-2 (1y+1v) expressing QT6 cells at stages 21-23 and analyzed 24-30 hours later by in situ hybridization for the following genes: (A) cShh. Notice lack of induction in the graft area. (B) cHoxD13. Notice strong ectopic expression anteriorly. cHoxD13 showed the highest upregulation of all the genes tested. (C) cHoxD11. Notice few cells showing expression adjacent to the graft. (D) cFgf-4. Notice expression in the anterior AER, extending posteriorly from the graft. At this stage, there is extremely low expression of Fgf-4 in the posterior AER. (E) cBMP-2. In all images, anterior is up. Images (C-E) show the D/V boundary of the anterior part of the wing. Arrowheads point to the grafted QT6 cells, where visible. Arrows point to cells responding to the graft.
of expression. Although expression of FHF-1 or FHF-2 has not been reported in the early mammalian limb bud, we have observed by whole-mount in situ hybridization that the expression pattern of FHF-2 in the developing mouse limb bud is very similar to that in the chicken (I. M. S. and J. N., unpublished).

In the limb bud, cFHF-1 is expressed in the posterior mesoderm, in a region that includes the ZPA. cFHF-2 is expressed in more distal mesoderm, in a region that largely overlaps the progress zone. The expression domains of cFHF-1 and cFHF-2 appear non-overlapping, although we cannot exclude the possibility that some cells express both genes. Both the ZPA and the PZ are important centers in the specification of the anteroposterior axis in the limbs. The localization of cFHF-1 and cFHF-2 transcripts to these regions represents the first examples of FGF family members that are expressed in such restricted domains in the distal limb mesoderm. We have shown that cFHF-2 can be induced by the N-terminal product of Shh, and that the time course of this induction is similar to that observed for other known components of the A/P patterning process, such as FGF-4, BMP-2 and HoxD13 (RIDDLE et al., 1993; LAUFER et al., 1994; LOPEZ-MARTINEZ et al., 1995; YANG et al., 1997). Whether the induction of cFHF-2 is mediated directly by Shh remains unknown. It is possible that the Shh signal acts in conjunction with signals from the AER, since removal of the anterior AER leads to a rapid downregulation of cFHF-2 in the underlying mesoderm.

The patterns of expression of cFHF-1 and cFHF-2 in several chick mutants also lends support to the idea that these factors are important in limb morphogenesis. In wingless and limbless embryos, expression of both cFHF-1 and cFHF-2 is absent from the limb buds, but is present in a normal pattern in other tissues. In talpid embryos, in which the expression of all known components downstream of Shh show a loss of anterior posterior asymmetry (DVORAK and FALLON, 1991), the zone of expression of cFHF-2 is also expanded. In this mutant, cFHF-2 is expressed at higher levels than in the wild type, and its expression broadens both anteriorly and posteriorly. In contrast, expression of cFHF-1 is unaltered. This apparently normal pattern of cFHF-1 expression in talpid embryos and the minimal effect of Shh engrafment on cFHF-1 expression suggests that, in the limb, cFHF-1 may form part of a pathway distinct from that of Shh. Although the expression pattern of cFHF-1 and its regulation by the AER are suggestive of this gene playing a role in limb development, the elucidation of its function in this system awaits further experiments.

Analysis of the activity of cFHF-2 in limb buds

Overexpression of cFHF-2(1y+1v), a splice variant expressed in the limb buds, leads to morphological abnormalities reflecting both transformations in digit identity and changes in cell growth and/or death rates during chondrogenesis. Similar results were obtained with cFHF-2(1y+1v) expressed from a plasmid or a retroviral vector, or produced with an amino terminal signal sequence from a plasmid vector. These morphological defects suggest that the cFHF-2 protein may be functioning during the initial patterning events, as well as in a more direct regulation of the growth of the skeletal elements. The effects observed in these studies resemble those induced by ectopic polarizing activity (RIDDLE et al., 1993; LAUFER et al., 1994; LOPEZ-MARTINEZ et al., 1995; MASUYA et al., 1995, 1997; KNEZEVIC et al., 1997; GOFF and TABIN, 1997; YANG et al., 1997). Bone shortening, ectopic digit and cartilage formation, and changes in digit identity are all observed following anterior misexpression of Shh or its downstream components. In the mouse, this is exemplified in the luxoid/luxate mutants (MASUYA et al., 1995, 1997). The hemimelia observed in the overexpression of cFHF-2(1y+1v) suggests that this protein may upregulate Shh, or the Shh pathway, adjacent to the site of the graft. The induction of partially duplicated cartilaginous elements and of ectopic cartilage condensations by cFHF-2(1y+1v) further suggests a direct affect on the proliferation of chondrogenic precursors. cFHF-2(1y+1v) induces a similar response when placed in the posterior mesoderm, implying that functional receptors for cFHF-2 are distributed throughout the mesoderm.

To test the hypothesis that cFHF-2(1y+1v) acts through known components of the A/P patterning process, we looked for ectopic expression of cBMP-2, cShh, cFGF-4, cHoxD13 and cHoxD11 after engrafment QT6 cells transfected with each of the three cFHF-2(1y+1v) constructs described above. Only the signal sequence construct showed any ectopic activation of these genes within the 24-40 hour time window between engrafment and killing. Interestingly, the signal sequence construct induced all of the markers except for cShh, a pattern reminiscent of that reported for local application of BMP-2, which activates Hox genes but not Shh (Duprez et al., 1996).

These data suggest that the effect of cFHF-2 on limb patterning is mediated through the Shh pathway but is not mediated by Shh itself. The failure of QT6 cells transfected with the cFHF-2(1y+1v) constructs lacking a signal sequence to induce any of the markers tested could reflect a requirement for high local concentrations of extracellular cFHF-2(1y+1v) to induce expression of these markers within the 24-40 hour time window of the experiment. These observations are consistent with the greater biological potency of the signal sequence construct as judged by the higher frequency and greater severity of limb defects induced by this construct relative to the parental construct which lacks a signal sequence. A similar increase in potency in a limb induction assay has been reported following the addition of an amino-terminal signal sequence to bFGF, which also lacks a classical signal sequence (RILEY et al., 1993).

The expression patterns of the FHFs in both the limbs and the neural tube differ from those of other FGF family members, implying a distinct and specialized role for the FHFs in the development of these tissues. The FHFs also differ biochemically, with affinities for heparin that are lower than those reported for other FGFS (SHING et al., 1984; SQUIRES et al., 1988; MIYAMOTO et al., 1993). This suggests that the FHFs may differ from other FGFs in their interactions with extracellular matrix components. These differences raise the question of whether FHFs signal through the known FGF receptors (FGFR). In the limb bud, FGFR-1 and FGFR-2c are expressed in the mesoderm and FGFR-2b is expressed in the ectoderm, including the AER (ORR-URTREGER et al., 1993; PETERS et al., 1992; SZEBENYI et al., 1995). In mice and humans, FGFR-1 and FGFR-2 are essential for normal limb development and FGFR-3 is essential for long-bone growth (COLVIN et al., 1996; DENG et al., 1996, 1997; XU et al., 1998; human data reviewed in DE MOERLOOZE and DICKSON, 1997). It will therefore be of
considerable interest to determine whether the FHF's interact with these or other known FGFR receptors in the developing limbs or nervous system.

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REFERENCES


FHF5s in limb development


