INTRODUCTION

The FGFs constitute a family of related signaling molecules that act to promote the growth and differentiation of cells of diverse origins. In mammals, nine members of the FGF gene family (Fgf1–Fgf9) have been described thus far (reviewed by Basilico and Moscatelli, 1992; Baird, 1994). All FGF family members encode polypeptides that share a conserved ~120-amino acid ‘core’ but have different N and C termini. Most FGFs are secreted, and act primarily through an evolutionarily conserved ras-dependent intracellular signal transduction pathway. Four closely related mammalian genes (Fgfr1–Fgfr4) encoding members of the FGF receptor family of RTKs, and several low-affinity receptors have been identified. Although individual FGFs appear to signal most efficiently through specific FGF receptors, there is evidence to suggest that most of the ligands are capable of interacting with more than one FGF receptor (reviewed by Johnson and Williams, 1993).

Studies of the expression patterns, primarily in the mouse, of the Fgf1–Fgf7 genes (Wilkinson et al., 1988, 1989; Gonzalez et al., 1990; Fu et al., 1991; Haub and Goldfarb, 1991; Hébert et al., 1991; Niswander and Martin, 1992, de Lapeyrière et al., 1993; Han and Martin, 1993; Mason et al., 1994), as well as the four Fgfr genes (Orr-Urtreger et al., 1991, 1993; Stark et al., 1991; Yamaguchi et al., 1992; Peters et al., 1992, 1993) have shown that all of these genes are active during embryogenesis. A variety of experimental approaches have provided compelling evidence that the FGF signal transduction pathway plays key roles in the regulation of growth and patterning in the vertebrate embryo. For example, experiments in Xenopus embryos have shown that overexpression of a dominant-negative mutant form of a Fgfr gene results in a lack of posterior structures, suggesting that FGF signaling is required for normal gastrulation and differentiation of mesoderm (Amaya et al., 1991, 1993). A similar conclusion was drawn from analyses of mouse embryos homozygous for a null allele of the Fgfr1 gene (Yamaguchi et al., in press). Moreover, mice homozygous for a null mutation in Fgf3 have shortened tails (Mansouret al., 1993). In addition, experimental manipulations on embryonic chick wing buds have provided evidence that FGF-dependent signaling is required for normal development of the vertebrate limb (Niswander et al., 1993, 1994; Fallon et al., 1994).

In the present study, we have analyzed the mouse Fgfr8 gene, with particular emphasis on its pattern of expression in the developing embryo. This FGF family member was originally identified as the gene encoding a secreted, androgen-induced growth factor (AIGF) that mediates the androgen-dependent growth of a mouse mammary tumor cell line, SC-3 (Tanaka et al., 1992). Molecular analysis showed that Fgfr8 encodes two protein isoforms, which differ from each other only in the region N terminal to the conserved FGF core. Analysis of FGF8 signal transduction in SC-3 cells indicated that FGF8 binds to and activates an isoform of FGFR1 (Sato et al., 1993).

SUMMARY

Evidence is accumulating that members of the FGF gene family provide signals that act locally to regulate growth and patterning in vertebrate embryos. In this report, we provide a detailed analysis of the mouse Fgf8 gene. We have mapped the Fgf8 locus to the distal region of mouse chromosome 19, and sequenced the 5′ coding region of the gene. Our data identify a new coding exon, and locate multiple splice donor and splice acceptor sites that can be used to produce at least seven transcripts encoding a family of secreted FGF8 proteins with different N termini. From these results, it appears that Fgf8 is structurally the most complex member of the FGF family described to date. In the embryo, many of the regions in which Fgf8 RNA is localized are known to direct outgrowth and patterning, including the apical ectodermal ridge of the limb bud, the primitive streak and tail bud, the surface ectoderm overlying the facial primordia and the midbrain-hindbrain junction, suggesting that FGF8 may be a component of the regulatory signals that emanate from these regions.

Key words: AIGF, brain development, differential splicing, Fgf8, Fibroblast Growth Factor, gastrulation, limb development, mouse embryogenesis, pharyngeal arches

The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo

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INTRODUCTION

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In the present study, we have analyzed the mouse Fgfr8 gene, with particular emphasis on its pattern of expression in the developing embryo. This FGF family member was originally identified as the gene encoding a secreted, androgen-induced growth factor (AIGF) that mediates the androgen-dependent growth of a mouse mammary tumor cell line, SC-3 (Tanaka et al., 1992). Molecular analysis showed that Fgfr8 encodes two protein isoforms, which differ from each other only in the region N terminal to the conserved FGF core. Analysis of FGF8 signal transduction in SC-3 cells indicated that FGF8 binds to and activates an isoform of FGFR1 (Sato et al., 1993).
In addition, it has been shown that NIH3T3 cells expressing Fgf8 form tumors in nude mice, display focus forming activity in monolayer culture, and colony formation in soft agar, indicating that Fgf8 might act as an oncogene (Kouhara et al., 1994). Here, we provide evidence that Fgf8 is structurally the most complex FGF gene described to date, in that it can encode at least seven secreted protein isoforms including the two previously identified proteins AIGF1 and AIGF2, we map Fgf8 to the distal region of mouse chromosome 19, and we provide a detailed analysis of Fgf8 expression in the mouse embryo. Recently, Heikinheimo et al. (1994) have also described some aspects of the Fgf8 expression pattern in the embryo. The data show that Fgf8 RNA is localized in known signaling centers that direct the outgrowth and patterning of different regions of the embryo including the limb, the elongating body axis, the face and the midbrain/hindbrain region. This suggests that the FGF signal transduction pathway, activated by FGF8, may play a role in the development of these regions. Moreover, the observation that Fgf8 RNA is detected in tissues not previously thought to constitute such signaling centers, including the telencephalic commissural plate and the pharyngeal pouches and grooves separating the pharyngeal arches, leads us to speculate that these tissues may also be sources of regulatory signals, and that their activity may depend on FGF8.

MATERIALS AND METHODS

Analysis of Fgf8 cDNA clones and genomic sequences

Fgf8 cDNA clones were isolated from an E6.5 and two independent E7.5 embryonic cDNA libraries (Lawler et al., 1993 and P. Crossley, K. Steiner and P. Tam, unpublished results) constructed in Lambda ZapII (Stratagene) using as a probe either Fgf8 3′ (nt 544-762 in AIGF1) or 5′ sequences (nt 160-598, but excluding nt 243-275, in AIGF1; Tanaka et al., 1992). High stringency hybridization and washing was performed according to standard methods (Church and Gilbert, 1984). A 3′ primer pair (5′GGCAAGGACCTGCTGATTCTACACAGC3′ and 5′GGTAAGGTGAGGAACACTCAAGGCG3′) was used in the initial screen of one of the E7.5 embryonic cDNA libraries. To determine the sequence of the 5′ ends of independent cDNA clones, plaque purified phage isolates were amplified by PCR using a 5′ primer pair (5′TGGTACACTGTCGCTTCTC3′ and 5′CTTCGCGATGCTGTA3′) designed to amplify sequences between the signal sequence and the conserved FGF core (see Fig. 1). The PCR products were gel purified and sequenced. The same primer pair was also used to amplify a ~1 kb fragment from C57BL/6J genomic DNA (purchased from The Jackson Laboratory, Bar Harbor ME) which was sequenced on both strands. To estimate the size of introns in the Fgf8 gene, we took advantage of the highly conserved position in FGF family members of intron/exon boundaries within the region encoding the conserved FGF core to design primers pairs that would amplify sequences between exon 1c and exon 2 (5′CCAACAAAACCACCTAGCAACAGCC3′ and 5′CTCTCCTGCTCAAAAGATCG3′) and between exon 2 and exon 3 (5′TGGAAGCAGAGTCCGAGTTC3′ and 5′GAACAGCCCCTGATAGCCG3′). From the sizes of the PCR products obtained with these primer pairs we estimate that the introns are ~3.2kb and ~800 bp, respectively.

Chromosome mapping

DNAs from N2 animals of an interspecific backcross (C57BL/6J × SPRET/Ei) F1 females × SPRET/Ei males (purchased from The Jackson Laboratory, Bar Harbour, ME) were digested with PstI, fragments were separated by gel electrophoresis, blotted and hybridized according to standard protocols (Church and Gilbert, 1984) with a 600 bp probe corresponding to the AIGF1 coding sequence (but excluding nt 243-275; Tanaka et al., 1992). This probe detected a ~1.4 kb fragment in C57BL/6J DNA and an ~1 kb fragment in SPRET/Ei DNA.

RNA in situ hybridization

Embryos were isolated from random bred Swiss Webster mice (Simenson Laboratories, Gilroy, CA). Noon of the day on which the vaginal plug was detected was considered to be 0.5 days of gestation (E0.5), although some variation was observed in developmental stage both between and within litters at the given embryonic ages. Embryos were dissected free of maternal decidual tissue and fixed overnight in 4% paraformaldehyde dissolved in PBS. Embryonic tissue was processed, sectioned and hybridized with 35S-labelled riboprobes according to published protocols (Vrohman et al., 1990). Whole-mount in situ hybridizations were performed as described by Wilkinson (1992) using digoxigenin-labelled antisense riboprobes detected with anti-digoxigenin antibodies coupled to alkaline phosphatase. In some experiments, the length of the proteinase K treatment was reduced to 2 minutes, in order to prevent damage to embryos at early stages of development or to superficial ectodermal structures such as the apical ectodermal ridge in older embryos.

The antisense riboprobes used in this study were as follows: Fgf8, a 400 nt cDNA probe containing the 3′ UTR and C-terminal coding sequences up to the PstI site at nt position 598 in AIGF1 (Tanaka et al., 1992); Wnt1, a 1900 nt cDNA probe containing the entire coding sequence and 3′ and 5′ UTRs (pBSK+Wnt1, a gift from J. KitaJenski, UCSF); Gsc: a 580 nt cDNA probe containing the 3′ UTR and C-terminal coding sequences and ending at the PstI site at nt 1253 in the published genomic sequence (Blum et al., 1992); Evx1, a 700 nt 3′ UTR probe described previously (probe 3, Dush and Martin, 1992).

In some cases, following the in situ hybridization procedure the whole-mount embryos were refixed for 20 minutes in 4% paraformaldehyde, incubated overnight in 30% sucrose, embedded in OTC compound (Tissue-Tek, Miles Inc., Elkhart, IN), frozen and sectioned (8 µm).

RESULTS

Multiple Fgf8 RNA isoforms generated by alternative splicing are expressed in the developing embryo

As a first step in determining whether the Fgf8 gene is expressed in the early mouse embryo, we screened a mouse E7.5 cDNA library by PCR amplification using two primer pairs corresponding to previously published Fgf8 sequences (Tanaka et al., 1992). Using a 3′ primer pair (see Materials and Methods) a single amplified product was obtained, indicating that the gene is active early in mouse embryogenesis. However, when a 5′ primer pair (see Fig. 1A and Materials and Methods) was used, considerable heterogeneity in the size of the amplified product was observed. These data suggest that early mouse embryos express multiple Fgf8 mRNA isoforms, which differ at their 5′ ends.

To explore the nature of this variability we isolated Fgf8 cDNA clones from three different cDNA libraries constructed from poly(A)+ RNA isolated from mouse embryos at E6.5 or E7.5 (see Materials and Methods). Using the 5′ primer pair, we amplified the N-terminal coding region of each cDNA clone and then sequenced it. The initial description of Fgf8 identified two RNA isoforms encoding secreted FGF8 proteins, AIGF1 and AIGF2 (Tanaka et al., 1992), which differ in their N-terminal regions (variants 1 and 2, respectively, Fig. 1A). Among the cDNA fragments that we amplified, several were
found to encode a N-terminal region identical to that of AIGF1, but none encoded that of AIGF2. The remaining amplified products fell into five classes (variants 3-7; Fig. 1A): two encode novel arrangements of the N-terminal sequences found in AIGF1 and AIGF2 (variants 3 and 4), and three contain a sequence not previously identified (variants 5, 6 and 7).

To determine the organization of the genomic interval that encodes this N-terminal variable region, we used the 5′ primer pair to amplify genomic DNA and sequenced the resulting ~1 kb PCR product (Fig. 1B). Comparison of genomic and cDNA sequences revealed that the coding region that we amplified is derived from three exons. Since the equivalent coding sequences in other FGF family members are derived from a single exon (exon 1), we have designated these three exons 1a, 1b and 1c. However, this nomenclature must be considered provisional until the organization of the genomic region 5′ of the segment that we amplified and sequenced is determined. Our analysis indicated that exon 1a contains an alternative splice donor site, and exon 1c contains an alternative splice acceptor site (Fig. 1A,B). Thus, the various cDNA isoforms that we isolated are generated by differential use of exon 1b and the splice donor and splice acceptor sites located within exons 1a and 1c, respectively.

Since all the N-terminal variant regions that we sequenced are in frame with the upstream signal sequence and the downstream conserved FGF core, it seems likely that the Fgf8 gene encodes a family of secreted proteins that differ from one another only in the variable region. Support for this idea comes from a complete analysis of the coding region of a cDNA containing variant 4 sequences (data not shown) and of cDNAs containing variants 1 (AIGF1) and 2 (AIGF2) (Tanaka et al., 1992). The sizes of the introns that separate exon 1c from exon 2, and exon 2 from exon 3, which are ~3.2 kb and ~800 bp, respectively, were estimated from additional PCR amplification and sequence analyses (Fig. 1 and data not shown).

In a preliminary RT-PCR analysis of four different Fgf8 expression domains in the E9.5 embryo (forebrain, midbrain-hindbrain region, limb bud and tail bud, see below), we found that the amplified product appeared to consist mainly of a fragment representing RNA isoform 1, but larger fragments presumably representing other spliced variants were also detected (data not shown). These data suggest that isoform 1 is expressed in all four Fgf8 expression domains tested, and may be the predominant form throughout embryogenesis. However, further analysis will be required to confirm this sug-
gestion, and also to determine whether any of the other isoforms are specific to a given region of the embryo.

**Fgf8 maps to mouse chromosome 19**

The chromosomal location of mouse *Fgf8* was determined by interspecific backcross analysis using progeny from matings of \((C57BL/6J \times SPRET/Ei)F_1 \times SPRET/Ei\) mice. This interspecific backcross mapping panel has been typed for 451 loci that are well distributed among all the autosomes as well as the X chromosome (Rowe et al., 1994). C57BL/6J and SPRET/Ei DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using an *Fgf8* probe (see Materials and Methods). A 1.4 kb C57BL/66 *Pst* fragment was used to follow the segregation of the *Fgf8* locus in 88 N2 progeny. The mapping results indicated that *Fgf8* is located in the distal region of mouse chromosome 19 and is flanked by the lipocortin 1 (*Lpc1*) and insulin 1 (*Ins1*) genes (Fig. 2). The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere – *Lpc1* (29/88) – *Fgf8* (3/88) – *Ins1* (2/88) – *D19Mit1*. The recombination frequencies, expressed as genetic distances in centi-Morgans (cM) (± the standard error), are: *Lpc1* (33.0±5.0) – *Fgf8* (3.4±1.9) – *Ins1* (2.3±1.6) – *D19Mit1*.

A comparison of our interspecific map of chromosome 19

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**Fig. 2.** *Fgf8* maps in the distal region of mouse chromosome 19. (Top) Haplotypes of the 88 N2 animals analyzed for each of three markers (*Lpc1*, *Ins1*, and *D19Mit1*) in addition to *Fgf8*. Black boxes represent the C57BL/6J allele, white boxes the SPRET/Ei allele. (Bottom left) The chromosome map shows the linkage relationships of *Fgf8* and flanking markers on chromosome 19, based on data from the interspecific backcross analysis described here. Numbers to the left represent the calculated map distances in cM with standard errors. (Bottom right) A partial consensus linkage map of the distal region of mouse chromosome 19 (Davies et al., 1994; Maltais et al., 1994), showing the location of marker genes used in this study and closely linked mutations (ep, ak, ab, ru). The genetic distances in cM are shown to the left.

**Fig. 3.** Localization of *Fgf8* RNA in the gastrulating mouse embryo. (A) Schematic diagram showing the fate map of the mouse epiblast at the prestreak stage of development (based on the work of Lawson and her colleagues, 1991, 1992), showing the approximate locations within the epiblast of the precursor cells of each of the germ layers, ectoderm (ecto), mesoderm (meso), and endoderm (endo). (B,C) *Fgf8* RNA was localized in the prestreak embryo by in situ hybridization analysis of a whole-mount embryo at E6.0 (B) and of an oblique sagittal section of an embryo at E6.25 (C). Magnification = ~160×. (D-F) Comparison of the domain of *Fgf8* expression at E6.5 (D) with those of *Evx1* at E6.25 (E) and *Gsc* at E6.5 (F). Magnification = ~160×. The horizontal line to the left of each embryo indicates the position of the junction between the embryonic and extraembryonic ectoderm. Abbreviations: ee, extraembryonic ectoderm; ep, epiblast (embryonic ectoderm); ve, visceral endoderm.
Fgf8 expression in the mouse embryo

with a consensus mouse linkage map (Fig. 2) shows that Fgf8 maps to a region in the vicinity of four recessive mouse mutations that affect hair pigmentation (pale ear, epi), hair and skin differentiation (asebia, ab), eye-pigmentation (ruby-eye, ru) and eye development (aphakia, ak), respectively.

**Fgf8 is expressed prior to and during gastrulation**
The appearance of the primitive streak marks the start of gastrulation in the mouse embryo. Although morphologically homogeneous, the epiblast of the prestreak embryo is regionalized with respect to the fate of the cells within it (see Fig. 3A; Lawson et al., 1991; Lawson and Pedersen, 1992). During the prestreak period from E5.75 to E6.25, Fgf8 RNA is detected in embryonic ectoderm cells located on one side of the embryo (Fig. 3B and data not shown). The proximal boundary of expression coincides with the embryonic-extraembryonic junction and extends around approximately half of the circumference of the epiblast. The expression domain extends distally along approximately two-thirds the length of the embryo (Fig. 3B and data not shown). Expression

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**Fig. 4.** Localization of Fgf8 RNA in the primitive streak, the cardiogenic region and the somites. (A) Whole-mount preparation of an E7.5 embryo showing a proximal (posterior) to distal (anterior) gradient of Fgf8 RNA in the primitive streak, and a strongly labeled population of cells at the base of the allantois (arrowhead). (Magnification = ~100×). (B,G,H,I) Whole-mount preparations of an E7.75 (early neurula stage) embryo viewed from the side (B), the ventral surface (F) and in transverse section (H), and of an E8.0 embryo viewed from the side (C), the front (oblique view; G), and in transverse section (I). At these stages, Fgf8 RNA is detected at a uniformly high level throughout the primitive streak, with a sharp anterior boundary at the node (asterisk). RNA is also detected in cells located in the wall of the intraembryonic coelom (arrowheads in F and G). (Magnification = ~60× for B,C,F,G; ~90× for H and I). (D,E) Transverse section through a E9.25 embryo (D) and frontal section through a E9.5 embryo (E) showing Fgf8 RNA localized in the lateral region of the myotome at the anterior and posterior margins of the somites. Arrowheads in E indicate intersomitic regions. (Magnification = ~180×). Abbreviations: al, allantois; d, dermatome; fg, foregut; hf, headfold; ic, intraembryonic coelom; m, myotome; me, mesoderm; nt, neural tube; ps, primitive streak; s, sclerotome; se, surface ectoderm.
was also detected in cells of the embryonic visceral endoderm (Fig. 3C). No hybridization signal above background level was detected in the maternal decidua tissue or in any extraembryonic tissue.

The Fgf8 expression pattern is similar at E6.5, the stage at which the primitive streak first becomes apparent (Fig. 3D). From analysis of sectioned embryos, it was evident that the Fgf8 expression domain is localized on the side of the embryo where the streak forms (data not shown), suggesting that, in the prestreak embryo, expression of Fgf8 is localized in the cells that are the first to pass through the streak and to form mesoderm and definitive endoderm (see Fig. 3A; Lawson et al., 1991; Lawson and Pedersen, 1992). To analyze further the domain of Fgf8 expression in the early streak embryo, we compared it with the expression domains of Evx1 (Bastian and Gruss, 1990; Dush and Martin, 1992) and Gsc (Blum et al., 1992), homeobox-containing genes that are expressed in the proximal and distal ends of the streak, respectively. Our results clearly indicated that the domain of Evx1 expression (Fig. 3E) is contained within the proximal portion of the Fgf8 expression domain, in the region adjacent to the embryonic-extraembryonic border (compare Fig. 3D,E). In contrast, the Gsc expression domain (Fig. 3F) appears to be contained within the distal portion of the Fgf8 expression domain (compare Fig. 3D and F). Thus it appears that Evx1 and Gsc are expressed in non-overlapping domains that are both included within the domain of Fgf8 expression. Based on data obtained in this and previous studies (Blum et al., 1992; Dush and Martin, 1992), it appears that Fgf8 expression precedes that of both Evx1 and Gsc, making it the earliest marker to date of regionalization within the mouse epiblast.

At the late streak stage (E7.5), Fgf8 expression is restricted to the epithelial component of the primitive streak and the embryonic ectoderm cells immediately lateral to the streak. Analysis of transverse and sagittal sections indicated that Fgf8 expression is dramatically reduced as the cells pass through and emerge from the streak (Fig. 4A and data not shown). Moreover, at this stage the level of Fgf8 RNA appears to be substantially higher in the proximal part of the streak than at its distal end. High levels of Fgf8 RNA are also detected in a population of cells at the base of the allantois (Fig. 4A), but by E8.0 the number of Fgf8-expressing cells in this region is greatly reduced.

As the streak regresses and the embryo forms progressively more caudal structures, Fgf8 RNA continues to be detected in the posterior half of the embryo, in the streak and the ectoderm cells adjacent to it, with a sharp anterior boundary of expression in the vicinity of the node (Fig. 4B,C, and data not shown). However, two changes occur in the expression pattern in the streak region between E7.5 and E8.0. First, the proximodistal gradient of expression is no longer evident; instead the level of Fgf8 expression appears uniform along the entire length of the streak (Fig. 4B,C, and data not shown). Second, Fgf8 RNA becomes detectable in mesodermal cells emerging from the streak (Fig. 4C,I). This pattern of Fgf8 expression persists for as long as the streak exists (Fig. 5A,B, and data not shown) and, subsequently, Fgf8 RNA is detected in the tail bud through at least E10.5 (Fig. 5C and data not shown). Although by E8.0, expression is detected in the paraxial mesoderm exiting the streak, it is not detectable in the presomitic mesoderm or in the newly condensed somites (Fig. 4C and data not shown). However, by the time the somites have differentiated into dermatome, myotome and sclerotome Fgf8 RNA is detected in a subpopulation of lateral myotome cells located at the rostral and caudal margins of the somite (Fig. 4D,E).

**Fgf8 is expressed during the very early stages of cardiogenesis**

By the early headfold stage (E7.75), Fgf8 expression was detected for the first time in the anterior of the embryo (Fig. 4B and data not shown) in two bilaterally symmetrical crescents of cells that flank the invaginating foregut (Fig. 4F and data not shown). Analysis of serial sections revealed that the Fgf8-expressing cells are localized in the mesoderm adjacent to and within the medial half of the ventral wall (splanchnic mesoderm) of the intraembryonic coelom (Fig. 4H). A similar domain of expression was also detected at a slightly later stage (E8.0, Fig. 4G,I). The Fgf8-expressing cell population at E7.75 and E8.0 includes precursors of the heart and associated vessels (Kaufman and Navaratnam, 1981; DeRuiter et al., 1992). By E9.25, however, Fgf8 RNA was not detected above background levels in the developing heart and vasculature (Fig. 5A-C, and data not shown). These observations suggest that Fgf8 may play a role in the very early stages of cardiogenesis.

**Fgf8 is expressed in the developing brain**

Between E8.0 and E8.5, Fgf8 expression was detected in two distinct domains in the developing neural plate, one at its rostral end, and the other in a more caudal position in the region between the prospective midbrain and hindbrain (Fig. 5A). By E9.0 to E9.5, the caudal Fgf8 expression domain is restricted to a sharp, narrow band of neuroepithelial cells that extends from the dorsal midline around the lateral walls of the neural tube in the region of the constriction (isthmus) that lies at the midbrain-hindbrain junction (Fig. 5C,D). Analysis of serial sections indicates that this expression domain does not include cells in the ventral midline (Fig. 5D and data not shown). Expression of Fgf8 in the isthmus region was detected until at least E 12.5 (Fig. 5H,I).

The Wnt1 gene, which encodes a secreted signaling molecule, is also expressed in a sharp band of cells in the vicinity of the isthmus (Wilkinson et al., 1987; Parr et al., 1993). To define the relative positions of the two expression domains, we performed a comparative in situ hybridization analysis using probes for Fgf8 or Wnt1 RNA on serial sections of embryos at E9.5 (Fig. 5D,E and data not shown). Our data indicate that the band of Fgf8-expressing cells is located immediately caudal to the band of Wnt1-expressing cells. However, we cannot rule out the possibility that there is a small degree of overlap in the two expression domains. These data are summarized in Fig. 5G.

The second domain of Fgf8 expression is localized to cells at the rostral limit of the neural plate (Fig. 5A and data not shown). As the neural plate folds and its lateral edges fuse to form the dorsal midline (roof plate), this rostral edge likewise folds and eventually fuses to form the telenchephalic commissural plate. Cells on either side of this midline develop into the paired telenchephalic vesicles. As the rostral edges of the neural plate become more closely apposed (E8.75), there is an expansion of the Fgf8 expression domain in its most lateral aspects, resulting in the appearance of bilaterally symmetrical groups of Fgf8-expressing cells (Fig. 5B). Following fusion (~E9.0-E 9.5) Fgf8
expression is localized in the commissural plate (Figs 5C,D, 6D), and continues to be expressed at high levels in this region through at least E12.5. However, by that stage this expression domain has extended caudally to include the roofplate of the diencephalon in the region rostral of the developing pineal gland (Fig. 5H and data not shown). Expression in this domain persists until at least E14.5 (data not shown).

By E9.5 three additional domains of Fgf8 expression were detected in the developing forebrain. First, Fgf8-expressing cells are observed in the ventral midline, in an area that includes the infundibular region of the hypothalamus as well as the anlage of the posterior pituitary, with a sharp caudal boundary at the mamillary area (Fig. 5D and data not shown). Expression persists in the infundibular ventral midline and the posterior pituitary until at least E14.5 (Fig. 5H and data not shown). Second, Fgf8 RNA was detected in a ventral zone of the optic recess and optic stalk (Fig. 5F) and was found to persist in the optic stalks, optic nerves and chiasmatic area until at least E14.5 (Fig. 5I and data not shown). Finally, Fgf8 expression was detected in the alar plate of the ventral thalamus, in a small domain spanning and extending lateral to the dorsal midline in the region just rostral to the zona limitans, and this expression was found to persist until at least E12.5 (Fig. 5D and data not shown).

**Fgf8 is expressed in the developing pharyngeal region and facial primordia**

In addition to the expression domains in the brain described above, Fgf8 RNA was also detected in other tissues in the developing head. Specifically, Fgf8 is expressed in the pharyngeal region, which contains a series of pharyngeal pouches, arches and grooves that give rise to many of the components of the face, ears and glands of the head and neck (Fig. 6A). At the early stages of development in this region (E8.0), Fgf8 RNA was first detected in bilaterally symmetrical bands along the length of the lateral walls of foregut endoderm, and also in the overlying surface ectoderm and intervening lateral mesoderm (Figs 4G,I, 6B). By E9.5, Fgf8 expression has become localized to the extreme lateral regions of each pharyngeal pouch and the overlying surface ectoderm of the pharyngeal groove (Fig. 6C; see also Fig. 5C). In addition, high levels of Fgf8 RNA are present in the surface ectoderm that covers the mandibular and maxillary prominences derived from the first pharyngeal arch (Figs 5C,F, 6D). In the mouse, these discrete patches of ectoderm are thought to provide an inductive signal required for initiation of tooth formation (Lumsden, 1988).

Fgf8 expression was also detected in the region overlying the rostral forebrain following closure of the anterior neuropore (~E9.5), in bilaterally symmetric patches of surface ectoderm ventral and lateral to the telencephalic commissural plate (Fig. 6D). These patches are localized in the region of the prospective nasal placodes. During the period from E 9.0 to E10.5, the portion of the embryonic head overlying the forebrain vesicle undergoes rapid and extensive morphogenesis to give rise to the frontal nasal prominence from which the midline structures of the face are generated. By ~E10.5, Fgf8 expression in this region is localized in the surface ectoderm surrounding the nasal pit (Fig. 6E). At E12.5, Fgf8 RNA was detected in the olfactory epithelium (data not shown). These aspects of Fgf8 expression suggest that it may play a role in outgrowth and patterning of the facial primordia.

**Fgf8 is expressed in the developing limbs**

In the mouse, forelimb bud formation initiates at approximately E9.0-E9.5, with the proliferation of cells in the lateral plate mesoderm (stage 1), resulting in a substantial outgrowth by E10.0-10.5. At this time (stage 2), the ectoderm overlying the distal tip of the limb begins to thicken under the influence of signals from the mesenchyme to form a ridge, termed the apical ectodermal ridge (AER), that runs parallel to the A-P body axis (stage 3; Wanek et al., 1989). Classical experiments in the chick have established the importance of the AER as a source of signals required for outgrowth of the limb (reviewed by Hinchliffe and Johnson, 1980). Our in situ hybridization data showed that Fgf8 RNA is present in stage 1 limb buds, and is localized to the ventral ectoderm (Fig. 7A). By stage 2-3, when the ectoderm at the distal tip of the limb bud is thickening, Fgf8 RNA is restricted to the developing AER and is no longer detectable in the ventral ectoderm (Fig. 7B). Subsequently, when the AER is fully formed, Fgf8 RNA is expressed at high levels in cells along its entire length (Fig. 7C). Expression in the AER persists until stage 8 (~E12.5; data not shown), by which time the AER is regressing.

**Other sites of Fgf8 expression**

In addition to the expression domains described above, Fgf8 RNA was also detected in a number of other tissues in the developing embryo, including the urogenital system and the inner ear (data not shown). In the urogenital system, Fgf8 RNA was detected at E10.5 in the surface ectoderm overlying the genital tubercle, at E9.5-E10.0 in the nephrogenic cords, and at E14.5 and E15.5 in Bowman’s capsule/renal tubule complexes. In the inner ear, Fgf8 RNA was detected at high levels in discrete locations in the developing labyrinth at E14.5. These observations suggest a possible role for FGF8 in the inductive interactions associated both with kidney and inner ear development.

**DISCUSSION**

In this report, we describe the genomic organization, chromosomal location and embryonic expression pattern of the mouse Fgf8 gene. Below, we discuss the unique aspects of the Fgf8 gene structure that make possible the production of multiple secreted FGF8 isoforms with potentially different activities in vivo. In addition, we discuss the striking observation that many of the sites of Fgf8 expression are signaling centers that direct outgrowth and patterning at specific sites in the embryo, and relate our findings to what is known about the expression of other FGF family members.

**The Fgf8 gene can encode a family of secreted FGF8 polypeptides**

Most members of the FGF family appear to have a relatively simple genomic organization: they contain three exons that encode a single gene product (Basilico and Moscatelli, 1992; Baird, 1994). However, in the case of Fgf2 and Fgf3, the use of several different protein translation start sites results in the production of protein isoforms that are localized in the nucleus (Basilico and Moscatelli, 1992; Baird, 1994). The function of such nuclear FGF proteins is not yet known. In addition, there is evidence for some alternative splicing of FGF genes. Fgf1 transcripts lacking exon 2 have been identified (Yu et al.,
The chick Fgf2 gene has been found to contain an alternative first exon ('alt'), and therefore produces RNA isoforms that encode proteins with unrelated N termini (Zúñiga-Mejía-Borja et al., 1993). Interestingly, alt-Fgf2 transcripts are much more prevalent in embryos than in adults, suggesting alt-Fgf2 may play a specific role in development. Here, we present evidence that the Fgf8 gene has the most complex structure of any FGF family member described thus far.

In their initial description of Fgf8, Tanaka et al. (1992) reported the isolation of cDNAs representing two Fgf8 RNAs each encoding a different protein isoform, which were evidently produced by alternative splicing in the 5' region of the gene. We have isolated cDNAs representing five additional Fgf8 RNAs that encode different protein isoforms, and have sequenced the region of the Fgf8 gene in which this isoform diversity arises. Our data show that the N-terminal region of
the FGF8 protein is encoded in at least three separate exons (here termed exons 1a, b, and c) rather than the single exon found in other FGF family members. Differential exon usage and the differential use of splice donor and splice acceptor sites within these three exons makes possible the production of a family of secreted FGF8 polypeptides, apparently differing only in a short domain that lies between the signal sequence and the start of the conserved FGF core. It is not yet known whether particular isoforms are uniquely expressed in specific tissues during embryogenesis or in the adult.

The data show that the Fgf8 gene can produce a remarkable range of secreted protein isoforms. The functional significance of this isoform diversity remains to be determined, but could be related to the fact that the different predicted proteins may be glycosylated to different extents. N-linked glycosylation may be important in regulating FGF activity, as suggested...
by the recent finding that proteolytic cleavage causes an increase in the mitogenic activity of the FGF4 protein, and that glycosylation at a site in the N-terminal region of FGF4 appears to inhibit such cleavage (Bellosta et al., 1993). The N-linked glycosylation site that regulates susceptibility of FGF4 to proteolysis, and hence its potential activity, is in the region between the signal sequence and the conserved FGF core, as is the Fgf8 variable region that contains two potential sites for N-linked glycosylation. The different FGF8 isoforms contain 0, 1, or 2 sites for N-linked glycosylation, and therefore may have different activities in vivo.

**Fgf8 function in the developing limb**

Classical studies have established the limb as an excellent experimental model system for studying the signals that regulate growth and patterning in the vertebrate embryo. The limb elongates by the addition of progressively more distal elements, derived from a small population of undifferentiated mesenchymal cells (the progress zone) at the distal tip of the limb bud. Experimental manipulations in chick embryos have identified the AER as the source of the signals required for the proliferation and maintenance of the progress zone. The AER is also required to maintain the zone of polarizing activity (ZPA), a region at the posterior margin of the limb bud that plays a critical role in patterning the developing skeletal elements (reviewed by Hinchliffe and Johnson, 1980; Tabin, 1991; Tickle and Eichele, 1994).

The recent demonstration that exogenously applied FGF can substitute for the AER to promote virtually normal limb outgrowth and skeletal patterning (Niswander and Martin, 1993; Fallon et al., 1994) provides strong evidence that a normal function of the AER is to produce FGF activity. Previous studies of Fgf1-Fgf7 suggested that Fgf2 and Fgf4 were the only FGF family members expressed in the AER. RNA and protein localization experiments showed that Fgf2 is expressed widely in the chick limb ectoderm including the AER and also in the underlying mesenchyme (Dono and Zeller, 1994; Fallon et al., 1994). In contrast, within the limb ectoderm, Fgf4 mRNA is localized in the posterior half of the AER and it is not detected in limb mesenchyme (Niswander and Martin, 1992; Niswander et al., 1994). On the basis of these findings, it was proposed that FGF4 provides the AER-derived FGF signal that maintains limb outgrowth and patterning (Niswander et al., 1993, 1994). However, in this report, we show that Fgf8 RNA is detected at very high levels along the entire length of the AER from the time that it first becomes morphologically distinct (~E10.0) until it regresses (E12.5), suggesting that FGF8 constitutes part of the AER-derived FGF signal.

Since the AER is not formed until stage 3 of limb development, the initial outgrowth of the limb bud must occur independent of AER-derived signals. Recent studies have demonstrated that local application of FGF to flank mesenchyme is sufficient to induce ectopic limb formation in the chick (M. Cohn and C. Tickle, personal communication), suggesting that a local FGF signal is responsible for the initial outgrowth of the limb. Until now, the only FGF family member known to be expressed in the very early limb bud or its vicinity is FGF2, which has been found by immunohistochemical analysis of chick embryos to be widely distributed throughout the mesoderm and surface ectoderm (Savage et al., 1993; Dono and Zeller, 1994). Our data show that Fgf8 RNA is detectable in the stage 1 limb bud, and is localized in the ventral ectoderm of the bud. This leads us to suggest that the local expression of Fgf8 in the very early limb bud plays a key role in directing the initial outgrowth of the limb.

**Fgf8 expression during gastrulation**

In several respects, the process of body axis elongation resembles that of limb outgrowth. The body elongates by the addition of progressively more caudal structures derived from a population of progenitor cells located at the caudal end of the embryo, which can be considered analogous to the progress zone of the limb. At early stages of development, these progenitor cells are located in the primitive streak, and at later stages in the tail bud. Patterning along the body axis requires the activity of the node, a region at the anterior end of the streak, which appears to perform a function analogous to that of the ZPA of the limb (Hornbruch and Wolpert, 1986; Hogan et al., 1992). Whereas in the limb, the AER has been identified as the source of the signal required to stimulate proliferation of cells in the progress zone and to maintain polarizing activity, in the primitive streak and tailbud the source of the signal(s) required to continue extension of the body axis is not yet known. However, in both cases, it appears that FGF is a critical component of this signal. Evidence that FGF is important for outgrowth and patterning of the body axis is provided by studies designed to interfere with the FGF signal transduction pathway. Xenopus embryos expressing a dominant-negative mutant form of a Fgfr gene fail to develop caudal structures (Amaya et al., 1991, 1993), and mouse embryos homozygous for a null allele of the Fgfr1 gene display abnormalities in mesoderm patterning and die between E7.5 and E9.5 (Yamaguchi et al., in press).

The data described here show that Fgf8 is expressed in the primitive streak from the time at which it first appears (E6.5) and for as long as it persists, and subsequently Fgf8 RNA is detected in the tail bud. At least two other members of the FGF family, Fgf3 and Fgf4, are likewise expressed at the caudal end of the embryo (Wilkinson et al., 1988; Niswander and Martin, 1992). This coordinate expression of three secreted FGFs within the same domain is consistent with the hypothesis that FGF activity is required for the elongation of the body axis. Furthermore, it raises the possibility that there is a requirement for the combined activities of several FGFs to obtain normal axial development. If so, one would predict that whereas loss of function of one of these genes would have only a modest effect on the elaboration of the body axis (as is the case for FGF3-deficient mice, which have shortened tails [Mansour et al., 1993]), simultaneous loss of function of two or three would cause much more severe truncations of the body axis.

In addition to its potential function during extension of the body axis, our data raise the possibility that Fgf8 may also play a role in establishing the primitive streak. This is suggested by the observation that Fgf8 RNA is detected at least a half day prior to streak formation and appears to be localized in the region of the epiblast containing cells that are fated to move through the streak and form both the mesoderm and definitive endoderm. One mechanism by which Fgf8 might influence streak formation is by regulating the expression of genes that play a role in specifying mesendodermal cell fate. Possible downstream targets of Fgf8 activity include Evx1, a homolog of the *Drosophila* even-skipped gene (Bastian and Gruss, 1990;
Dush and Martin, 1992) and an ortholog of Xenopus Xhox-3 (Ruiz i Altaba et al., 1991), and Gsc, a vertebrate gene containing a homeobox related to those of Drosophila gooseberry and bicoid (Blumberg et al., 1991; Blum et al., 1992). These vertebrate homeobox-containing genes are thought to play a role in determining posterior/ventral and anterior/dorsal cell fate, respectively. Consistent with the idea that FGF8 may regulate their activity is our finding that Fgf8 expression in the early postimplantation embryo is localized in a domain that includes both Evx1- and Gsc-expressing cells, and that Fgf8 RNA is detected at least half a day before Evx1 and Gsc RNAs are observed. Furthermore, there is evidence that FGFR2 can induce the expression of Xhox3 in Xenopus animal caps (Ruiz i Altaba and Melton, 1989) and that FGF4 regulates Evx1 expression in limb mesenchyme (Niswander and Martin, 1993).

**Fgf8 expression during morphogenesis of the head and neck**

Experimental studies in the chick suggest that there may be fundamental similarities between the development of the facial primordia (frontonasal mass and prospective upper [maxilla] and lower [mandible] jaws) and the development of the limb. As in the limb, proximodistal outgrowth and patterning of the facial mesenchyme is dependent on a signal from the overlying ectoderm (Wedden, 1987; Richman and Tickle, 1989). Moreover, reciprocal grafts between the facial primordia and the limb bud of either ectoderm or mesoderm suggest that the signals responsible for epithelial-mesenchymal interaction may be similar (Richman and Tickle, 1992). Our data show that Fgf8 is expressed in patches of surface ectoderm overlying the facial primordia: at E9.5, Fgf8 is detected in the surface epithelium that covers the mandibular and maxillary prominences and the region that will give rise to the frontonasal mass. Moreover, the gene encoding FGFR1, with which FGF8 is known to interact (Sato et al., 1993), is widely expressed in the mesenchyme (Orr-Urtreger et al., 1991; Peters et al., 1992; Yamaguchi et al., 1992). These results suggest that Fgf8 may be a component of the epithelium-derived signal involved in regulating the outgrowth and patterning of these facial primordia. At present there is no evidence to indicate that other secreted FGF family members are coordinately expressed in these regions of facial ectoderm.

Other skeletal elements in the head and neck arise from neural crest mesenchyme located in the pharyngeal arches. The pharyngeal arches are separated from one another by pharyngeal pouches (outpocketings of the lateral walls of the foregut) which are closely apposed to the overlying surface ectoderm of the pharyngeal arches (see Fig. 6A). Studies in a variety of species suggest that both pharyngeal endoderm and surface ectoderm can provide the inductive signals required for cartilage formation by pharyngeal arch mesenchyme (reviewed by Hall and Horstadius, 1988). Furthermore, analyses of mice homozygous for a null allele of Hoxa-2 suggest that a signal(s) for patterning of the first and second pharyngeal arch mesenchyme emanates from the region between them (Rijli et al., 1993). Our data show that Fgf8 is detected in the endoderm of all the pharyngeal pouches and the ectoderm of the all the pharyngeal grooves. At least two other FGF family members, Fgf3 (Wilkinson et al., 1988) and Fgf4 (Niswander and Martin, 1992), are coordinately expressed in these inter-arch regions. An intriguing possibility is that the FGF expression detected in the region separating the first and second pharyngeal arches contributes to the patterning signal that is apparently produced in this region and, furthermore, that a similar mechanism may operate to pattern the more caudal pharyngeal arch units.

**Fgf8 expression in the developing brain**

Although there is no direct evidence linking the FGF signal transduction pathway to the growth and patterning of the brain, the widespread expression of the mouse Fgfr1 and Fgfr2 genes throughout the brain beginning at the neural plate stage (Orr-Urtreger et al., 1991, 1993; Peters et al., 1992; Yamaguchi et al., 1992), suggest that FGFs may have a function early in normal brain development. However, to date, only one member of the FGF family encoding a secreted protein, Fgf3, is known to be expressed in the developing mouse brain prior to E13.5, and this expression is restricted to rhombomeres 5 and 6 of the hindbrain (Wilkinson et al., 1988). In this study, we demonstrate that Fgf8 is expressed early in brain development in both the forebrain and in the isthmus at or near the junction of the midbrain and hindbrain. A number of experiments have demonstrated that a signaling center important for patterning the midbrain is located in the isthmus at this time (E9.5) (Bally-Cuif et al., 1992; reviewed in Marin and Puelles, 1994). Thus our data raise the possibility that Fgf8 may contribute to the patterning activity emanating from this region.

As yet signaling centers responsible for patterning the embryonic forebrain have not been identified. Here we report that FGF8 RNA is expressed in discrete regions of the forebrain including the telencephalic commissural plate, the ventral midline of the hypothalamus and the optic stalks. When considered in conjunction with the striking correlations discussed above between sites of Fgf8 expression and signaling centers known to direct the outgrowth and patterning in other regions of the embryo, it is tempting to suggest that the Fgf8 expression domains in the forebrain identify signaling centers responsible for patterning of the forebrain.

FGFs and WNTs appear to act synergistically in developmental settings such as gastrulation in Xenopus (reviewed by Kimelman et al., 1992; Moon and Christian, 1992), as well as in PC12 cells in culture (Shackleford et al., 1993). A comparison of the Fgf8 expression pattern with that of the gene encoding the WNT1 signaling molecule reveals an interesting juxtaposition of their respective expression domains in the brain. In the isthmus, Fgf8 and Wnt1 are expressed in abutting but apparently non-overlapping rings around most of the neural tube. Likewise, in the dorsal and ventral regions of the diencephalon they are in adjacent domains that share common boundaries at the zona limitans and in the mammillary region, respectively. This striking juxtaposition of Fgf8 and Wnt1 expression domains in the brain suggests that cooperative action between the two signaling molecules might play a role in the development of the midbrain/hindbrain region and the dorsal and ventral diencephalon. Furthermore, the observation that their domains of expression appear to be mutually exclusive raises the possibility that each of the genes acts to regulate the expression of the other.

**Concluding remarks**

Above, we have discussed the evidence that a variety of signaling centers that regulate growth and patterning of the embryo are dependent on the FGF signal transduction pathway...
for normal function. Our data show that Fgf8 expression is associated with many of these centers. However, it is also evident that in many settings the available FGF activity is the product of several different FGF family members. It remains to be determined whether this reflects the requirement for a particular sum of FGF activities or if the different ligands have qualitatively different effects. Whatever role the different FGFs may play in controlling outgrowth and patterning in the embryo, it is clear that they do not act alone. Evidence from studies in a variety of experimental organisms suggests that developmentally processes are regulated by a set of common molecular interactions that involve the products of several evolutionarily conserved gene families (reviewed by Melton and Jessell, 1993), including the WNT (Dickinson and McMahon, 1992; Nusse and Varmus, 1992), Hedgehog (Echelard et al., 1993; Riddle et al., 1993), TGF-β/BMP (Kingsley, 1994) and FGF families. Thus, future studies must be aimed at understanding not only the function of individual FGF genes, such as Fgf8, but also how members of these different gene families interact to control embryonic patterning.

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Note added in proof: The EMBL database Accession number for the nucleic acid sequence of the Fgf8 genomic region described here is Z46883.