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by

Jean Hebert

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This work is dedicated to Lisa

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ABSTRACT

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by

Jean Hébert

My thesis research is focused first around the question of what role the *Fgf5* gene plays in mouse gastrulation and then around the role this gene plays in controlling hair growth. Initially, several mouse FGF genes were cloned and expression studies were used to determine which ones, if any, are likely to play a role in gastrulation. The timing and location of *Fgf5* expression, based on RNA in situ hybridizations, strongly suggests that this gene plays a role in gastrulation. A variety of molecular genetic approaches were undertaken to test this hypothesis, using as experimental systems both embryos and cultured embryoid bodies. Embryoid bodies which are homozygous null for *Fgf5* or overexpress *Fgf5* develop without any detectable phenotype. In order to better understand at a molecular level how cultured cells change in response to different factors, a generally applicable method for identifying sets of factor-responsive genes using a gene-trap strategy was elaborated and tested. Finally, to investigate in a general way the role *Fgf5* plays in mice, FGF5-deficient mice were generated via gene targeting. The only phenotype of the mutant mice is abnormally long hair. A genetic complementation test between FGF5-deficient mice and mice homozygous for the classical mutation *angora*, and analysis of the *Fgf5* gene in *angora* genomic DNA revealed that *angora* is a mutant allele of *Fgf5*. Furthermore, analyses of *Fgf5* expression in the hair follicle allowed a model for how *Fgf5* controls hair growth to be proposed.

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INTRODUCTION

My research interests are aimed at understanding at a molecular level how cells become specialized. As an example of this, the thesis research presented here has been focused on understanding what role one molecule, the signalling molecule FGF5, plays in the developing mammalian embryo and in an adult tissue that is cyclically regenerated. By learning about FGF5's function, insight might be gained into how some cells become specialized.

The mechanisms that determine cell fates during mammalian embryogenesis are not well understood. This lack of understanding stems at least in part from certain inherent difficulties in using mammals as experimental systems to study development. First, prior to and during gastrulation, when the fates of the major cell lineages are being decided, the small size of mammalian embryos (compared to that of other vertebrates) and the special culture conditions required to keep them alive *in vitro* makes dissections and physical manipulations of them technically difficult. Second, also during this early developmental period, the embryos that are implanted in the uterine walls are difficult to extract in large numbers, rendering biochemical analyses that require sizable quantities of tissue very tedious. And finally, due to the limited (although quickly growing) number of developmental mutants, the use of genetics to study mammalian development is still in its infancy.

However, there are several advantages to using the mouse as a system of study. First, mouse development closely reflects human development and is a good model for it. Second, the generation time of mice is short when compared to other mammals. And finally, the technology exists for creating just about any type of genetic alteration or mutation in mice, an advantage

not currently found with any other multicellular organism. These genetic alterations can be accomplished using traditional transgenic technology, homologous recombination in embryonic stem cells, or other emerging strategies for making tissue specific or other selected mutations (Kilby et al. 1993; Gossler et al. 1989).

A common approach taken when attempting to unravel the mysteries of mammalian development is to guess at what genes might be involved in particular developmental processes based on what is known about the function of similar genes in other systems. The expression patterns of these candidate genes are then studied to determine if expression is present in the developing tissue of interest. Examples include the study of homeobox-containing genes, which are suspected of specifying positional values to cells during mammalian development because similar genes were shown to do so in fly development; the study of oncogenes, which because of their roles in controlling cell proliferation in adult cells are suspected of regulating proliferation in the growing embryo as well (Adamson, 1987).

Mammalian embryogenesis is highly regulative; a cell's fate is primarily determined by its position in the embryo or developing tissue. This implies that cell-cell interactions play a major role in determining cell fates and that molecules known to be involved in cell-cell signalling in adult tissues are likely to play roles in development as well. Growth factors (perhaps better termed signalling factors since many of them affect differentiation and migration as well as growth) are therefore good candidates for mediating cell-cell interactions during development. We chose to focus our studies on the function in embryogenesis of members of the fibroblast growth factor (FGF) family of signalling molecules, because at the time this research was initiated, FGFs were known to have multiple effects on

controlling the states of adult cells (reviewed by Gospodarowicz et al. 1987) and had also been shown to induce mesoderm in amphibian embryonic cells (reviewed by Smith 1989).

The FGF gene family is comprised of at least nine members. It is considered a gene family solely on the basis of sequence similarity among its members, without much consideration given to their functions. Thus calling them "Fibroblast Growth" factor genes is somewhat of a misnomer, but the name has been officially adopted for all the factors for historical and practical reasons. FGF2, also known as basic FGF or bFGF, was the first of the factors to be identified and purified due to its mitogenic effect on fibroblast cells in vitro (Gospodarowicz 1974). Shortly thereafter, FGF1 (acidic FGF or aFGF) was identified due to its stimulatory effect on myoblast proliferation (Gospodarowicz et al. 1975). It was found to be biochemically and functionally similar to FGF2. Cloning of the human and bovine genes for these factors and sequence analyses then demonstrated that the *Fgf1* and *Fgf2* genes are related (Esch et al., 1985; Gimenez-Gallego et al. 1985; Abraham et al. 1986; Jaye et al. 1986).

Since then, identification of the seven other FGF family members occurred through a variety of experimental routes. The third family member, *Fgf3* (*int-2*), was identified as a mouse mammary tumor virus integration site commonly associated with tumor formation (Dickson et al. 1984). Cloning of this oncogene later led to the realization that it was related to *Fgf1* and *Fgf2* (Moore et al. 1986; Smith et al. 1988; Mansour and Martin 1988). The fourth FGF family member, *Fgf4* (*kFgf* or *hst1*) was also initially identified as an oncogene in transformation assays using DNA from Kaposi sarcoma and stomach tumors (Delli Bovi et al. 1987, Taira et al. 1987). *Fgf5* was identified as a fragment of genomic DNA that when transfected into cultured cells

transformed them (Zhan et al. 1987, 1988). A sixth family member, *Fgf6*, was identified by virtue of its sequence similarity to *Fgf4* (Marics et al. 1989). FGF7 (KGF) was purified on the basis of its ability to specifically stimulate proliferation of epithelial cells (Rubin et al. 1989, Finch et al. 1989). FGF8 (AIGF) was identified as an androgen-induced growth factor in a mammary carcinoma cell line (Tanaka et al. 1992). And finally, FGF9 was purified as a glia-activating factor (Miyamoto et al. 1993). Genomic sequence analysis of some of these genes indicates that they are all structurally similar. Each contains three coding exons with highly conserved exon-intron boundaries. Although their protein products are of different lengths, they all conserve an ~120 amino acid residue core.

Since the identification of FGFs, the number of cell types on which they have an effect, as well as the diversity of effects they have, are continually increasing. In particular, FGF1 and FGF2 are known to produce a wide range of effects on different adult cell types in vitro and in vivo (reviewed by Gospodarowicz et al. 1987). Notable examples include effects on cell proliferation, differentiation, survival, and migration: they promote myoblast proliferation, induce nerve cell differentiation, maintain nerve cell survival, and stimulate endothelial cell migration. It should be noted, however, that none of these functions have been shown to be the normal in vivo functions of these factors.

Six years ago, when I began my thesis research, little direct evidence existed for the involvement of FGFs in developmental processes. The one notable exception was the demonstration that in amphibians FGFs could induce primitive ectodermal cells to become mesoderm, one of the three primary germ layers (reviewed by Smith 1989). More recent experiments, in which a dominant negative form of an FGF receptor was used to interfere

with FGF signalling, have demonstrated the requirement for FGF function in normal mesoderm formation in Xenopus (Amaya et al. 1991).

Together, these two findings, that FGFs could promote changes in adult cells which normally occur throughout development and that an FGF might be involved in determining a major developmental lineage, mesoderm, in early vertebrate development, prompted us to investigate the role of FGFs in early mammalian development. To get an idea of what particular developmental processes each FGF family member might be involved in, an ongoing effort has been to determine when and where during mouse development the different family members are expressed. In order to get probes to do this, cDNAs for the FGFs that were known to exist at the onset of this project were cloned (Chapter 1). cDNAs for these family members, which included *Fgf1, 2, 4,* and *5,* had previously only been cloned in humans or cows (*Fgf3* had already been cloned in the mouse and its expression pattern in development had been characterized to some extent, Wilkinson et al. 1988, 1989). An initial expression study by Northern blot analyses of RNA isolated from tissues of embryos at various stages revealed a complex pattern of expression with different tissues and different aged embryos expressing varying levels of the different FGF genes (Chapter 1). The most striking finding to come out of this analysis was that *Fgf5* expression was induced to a high level just prior to the start of mesoderm formation and then decreased dramatically once mesoderm formation was essentially complete. This result lead us to focus our studies on *Fgf5*.

Fgf5 was first isolated as a human oncogene that had acquired transforming potential by being juxtaposed to a retrovirus transcriptional enhancer during transfection of NIH 3T3 cells with human tumor DNA (Zhan et al. 1987, 1988). The deduced amino acid sequence of FGF5 shows

approximately 50% homology with the other family members and the gene structure is comprised of three coding exons as is typical for this family. FGF5 was shown to be a secreted protein (Bates et al. 1991), as expected from its deduced amino acid sequence. No function for *Fgf5* was known at the time we undertook our studies.

Our finding that the period of *Fgf5* expression correlated with the time of mesoderm formation during embryogenesis lead us to investigate the role of this factor in gastrulation. Gastrulation in the mouse is defined as the transformation of a primitive embryonic ectoderm layer into a three-layered structure consisting of ectoderm, endoderm, and a middle layer of mesoderm. This process occurs between embryonic days 6.5 and 7.75 (E6.5-E7.75). Little is known about what regulates the movement of cells during this process or how cells are assigned to become ectoderm, mesoderm, or endoderm.

To investigate the possibility that *Fgf5* plays a role in gastrulation, we carried out RNA in situ hybridization analyses of embryos to determine in which cell type(s) *Fgf5* was expressed (Chapter 2). These analyses revealed that *Fgf5* expression was induced to a high level in the primitive ectoderm approximately half a day prior to the start of gastrulation (E6), remained high during gastrulation with expression also found in the newly formed mesoderm and definitive endoderm, and began to decrease in a gradient (with the strongest point of expression in a structure called the node), and then ceased once gastrulation was essentially over (E7.75). This tight regulation in the timing of *Fgf5* expression, which correlates with the time during which the embryo is gastrulating, raised the possibility that *Fgf5* is involved in this process. Although the expression pattern was not inconsistent with *Fgf5* playing a role in mesoderm formation, it was unlikely to be a mesoderm inducer of the type being studied in Xenopus because it was

expressed in the induced cells rather than in neighboring cells. *Fgf5* was instead hypothesized to play a role in promoting cell movements in gastrulation or in maintaining the pluripotency of cells until they become committed to one of the three germ layers. RNA in situ hybridization analyses of aggregated embryonal carcinoma (EC) cells differentiated in culture revealed that the regulation of *Fgf5* expression in cultured cells was faithfully maintained (Chapter 2), opening up the possibility for the study of *Fgf5*'s function using a culture system, in which experimental manipulations are easier than in embryos.

Fgf5 was also shown to be expressed in other tissues later in development (Haub and Goldfarb 1991). Notably, *Fgf5* mRNA was detected in lateral splanchic mesoderm at E10, in lateral somitic mesoderm at E11.5, in myotome at E11.5, in mastication muscle at ~E13, in limb mesenchyme at ~E13.5, and in the acoustic ganglion at ~E13.5. The only adult tissues shown to express *Fgf5* were portions of the cerebral cortex, the hippocampus, and the thalamus.

To investigate *Fgf5*'s role(s) during development and in particular during gastrulation, we used genetic approaches in which we perturbed its normal expression pattern with the goal of obtaining a phenotype that would lend insight into its function. Of some surprise was the finding that mice lacking FGF5 developed normally and became healthy and fertile adults (Chapter 3). The only observed phenotype was that they had abnormally long hair. The similarity of this phenotype to that of the classical mutation *angora* and the genomic proximity of *Fgf5* and *angora* suggested that *angora* was a mutant allele of *Fgf5*. DNA analyses of *Fgf5* from *angora* mice revealed that in fact this was the case (Chapter 3). The finding that *Fgf5* inhibits hair growth

by regulating a key step in the hair growth cycle has led to some insight into the biology of hair growth.

For reasons discussed in Chapter 4, a genetic approach, such as the gene targeting of *Fgf5*, is not always the best strategy for identifying the function of a molecule in a particular developmental process. For this reason, an alternative approach was elaborated for identifying *Fgf5*-responsive genes in EC or embryonic stem (ES) cells which serve as a model system for the embryonic ectoderm (Chapter 4). This approach involves randomly inserting a marker gene, which lacks any promoter element, in the genome of ES cells and identifying the genetic loci in which the marker is transcriptionally up or down regulated in response to FGF5. Only preliminary results were obtained. So, although *Fgf5*'s function(s) in gastrulation and development remains a mystery, a fortuitous step forward has been made in understanding what regulates hair growth in adult mammals.

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CHAPTER 1

Isolation of cDNAs Encoding Four Mouse FGF Family Members and Characterization of Their Expression Patterns during Embryogenesis

JEAN M. HÉBERT,* CLAUDIO BASILICO,† MITCHELL GOLDFARB,‡ OLIVIA HAUB,‡ AND GAIL R. MARTIN*,[1]

*Department of Anatomy and Genetics Program, University of California, San Francisco, California 94134; †Department of Pathology, New York University School of Medicine, New York, New York 10016; and ‡Departments of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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To initiate a study of the role of the fibroblast growth factor (FGF) family in mammalian development, we have isolated cDNAs encoding four mouse FGF family members, aFGF, bFGF, kFGF, and FGF-5. This was achieved by a process that circumvents the use of cDNA libraries: for each family member, a cDNA fragment containing the conserved portion of the coding region was amplified from a pool of embryonic and teratocarcinoma cell cDNAs using the polymerase chain reaction (PCR) and cloned; the remaining coding sequences 5′ and 3′ to the conserved region were cloned using the RACE method. The cDNA clones obtained were used as probes to analyze the expression of these genes at the RNA level in teratocarcinoma cells and embryos at 10.5 to 17.5 days of gestation. *Fgfk* appears to be specific to undifferentiated teratocarcinoma stem cells. *Fgf5* transcripts were detected at every stage and in every tissue tested, but showed a dramatic 15-fold increase in abundance as teratocarcinoma stem cells differentiated to simple embryoid bodies. *Fgfb* expression showed the greatest tissue-specific variability in abundance, with the highest levels detected in the developing limbs and tail. *Fgfa* showed the least variable pattern of expression, with transcripts detected at roughly equivalent levels in almost all samples analyzed. On the basis of these data we speculate on some possible roles that the different FGF family members may play in the developing embryo.

INTRODUCTION

Many mammalian genes encoding signaling factors have been identified as a consequence of their mitogenic or transforming activities. On the basis of amino acid sequence similarities and in some cases of functional similarities, most of these factors can be grouped into families. Among these is the family of heparin-binding factors related to basic fibroblast growth factor (bFGF),[2] a protein first identified by its ability to stimulate fibroblast proliferation *in vitro* (Gospodarowicz, 1974). Sequence analysis demonstrated that acidic FGF, the first molecule found to be biochemically and functionally similar to bFGF, is closely related to bFGF (Esch *et al.*, 1985; Gimenez-Gallego *et al.*, 1985; Abraham *et al.*, 1986b; Jaye *et al.*, 1986). Three other genes, mouse *int-2* and human FGFK and FGF5, initially identified as oncogenes, were found to be related to FGFB and FGFA on the basis of deduced amino acid sequence similarities (Delli Bovi *et al.*, 1987; Dickson and Peters, 1987; Taira *et al.*, 1987; Zhan *et al.*, 1988). Recently, a sixth family member, FGF6, was identified by virtue of its sequence similarity to FGFK (Marics *et al.*, 1989). All these genes appear structurally similar. Each contains three coding exons with highly conserved exon-intron boundaries. Although their protein products are of different lengths, they all conserve an ~120 amino acid residue core. In addition, a seventh family member containing only part of this core region has been identified (Finch *et al.*, 1989).

Evidence that the products of the FGF gene family play an important role as signaling molecules during vertebrate embryogenesis is rapidly accumulating. The most compelling data come from studies showing that members of the FGF family have mesoderm-inducing activity when tested on explants of *Xenopus* animal pole tissue and that bFGF protein is present in the *Xenopus* embryo during the time of mesoderm induction (reviewed by Smith 1989; Paterno *et al.*, 1989). Although

[1] To whom correspondence should be addressed.

[2] A variety of designations have been used previously for each of the FGF family members discussed in this paper. We have chosen to use the official gene designations according to the Human Gene Nomenclature Committee and the International Committee on Standardized Genetic Nomenclature for Mice. Therefore, throughout the text we have referred to the human and mouse genes encoding basic FGF (bFGF) protein as FGFB and *Fgfb*, respectively; similarly the human and mouse genes encoding acidic FGF (aFGF) protein are designated FGFA and *Fgfa*, respectively, and the genes encoding FGF-5 protein are designated FGF5 and *Fgf5*. Another family member has previously been termed hst (Taira *et al.*, 1987), HST-1/HSTF1 (Yoshida *et al.*, 1988), KS3 (Delli Bovi *et al.*, 1987), and K-fgf (Velcich *et al.*, 1989); in this paper for consistency we will refer to that protein as kFGF and the human and mouse genes encoding it as FGFK and *Fgfk*, respectively; these designations have been proposed to the appropriate nomenclature committees.

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the mechanism by which these signaling molecules function to induce mesoderm is unknown, it is intriguing to note that exposure of animal pole explants to bFGF rapidly induces the expression of the homeobox-containing gene Xhox-3 (Ruiz i Altaba and Melton, 1989b) and exposure to bFGF in conjunction with TGFβ-1 or -2 induces expression of another homeobox-containing gene, Mix.1 (Rosa, 1989). These genes are putative transcription factors, and there is evidence to suggest that Xhox-3 may be involved in anterior-posterior axial specification (Ruiz i Altaba and Melton, 1989a).

The evidence that members of the FGF family play roles in mammalian embryogenesis is less direct. It is known that two of the family members can promote processes in adult tissues that also occur during embryogenesis: acidic and basic FGFs produce a wide range of effects on different adult cell types *in vitro* and *in vivo*, including stimulation of endothelial cell migration and proliferation, neurite outgrowth, and nerve cell survival and differentiation (reviewed by Gospodarowicz *et al.*, 1987). Another family member, *int-2*, has been shown by *in situ* RNA hybridization analyses to display highly tissue-specific expression patterns from the time of early gastrulation to the mid-somite stage, as well as later in fetal development (Wilkinson *et al.*, 1988, 1989).

Studies of the roles of the different FGF family members in mammalian development are best carried out in the mouse. Several investigators have taken advantage of the model system provided by teratocarcinoma stem cells (Martin, 1980) to study expression of heparin-binding growth factors in early mouse development (Heath and Isacke, 1984; van Veggel *et al.*, 1987; Rizzino *et al.*, 1988; Heath *et al.*, 1989; van Zoelen *et al.*, 1989). However, for the most part these experiments have been difficult to interpret because the molecules under investigation have not been precisely identified. Moreover, since hybridization probes specific for the different mouse FGF family genes were not available, it was difficult to study their expression during development. We therefore undertook the cloning of the mouse cognates of the human FGFB, FGFA, FGFK, and FGF5 cDNAs. The cDNA clones isolated were then used as probes to study expression of these different FGF family members in mouse teratocarcinoma cells, whole embryos, and embryonic tissues at various stages of development by Northern blot analyses. The temporal and tissue-specific expression patterns observed suggest that kFGF and FGF-5 may play important roles early in development and that all of the family members, with the possible exception of kFGF, may have multiple roles at different times during development.

MATERIALS AND METHODS

Cell Cultures and Embryos

The origin of the PSA-1 teratocarcinoma stem cell line, the conditions under which it was maintained in the undifferentiated state by frequent subculture to STO feeder cell layers, and the methods used to obtain differentiation (embryoid body formation) of the PSA-1 cells were exactly as described by Martin *et al.* (1977). PSA-1 cell aggregates that had developed into simple embryoid bodies (approximately 3–6 days of culture in bacteriological petri dishes) were considered to be at stage 1 of differentiation; embryoid bodies that had developed into fully expanded cystic embryoid bodies (approximately 12–15 days of culture in bacteriological petri dishes) were considered to be at stage 2 of differentiation. Fully expanded cystic embryoid bodies that had been replated in tissue culture dishes and cultured for an additional 10 or more days (Martin and Evans, 1975) were considered to be at stage 3 of differentiation.

ES cell lines were derived from inner cell masses isolated from mouse blastocysts of the strains indicated in the text using the method described in detail by Martin *et al.* (1987). The ES cells were maintained in the undifferentiated state by subculture every 3–4 days to STO feeder cell layers.

Samples of undifferentiated PSA-1 and ES cells to be used for RNA isolation were separated from residual STO feeder cells by the "preplating" method, whereby freshly trypsinized cultures were seeded in tissue culture dishes and the cells that failed to attach after 20 min incubation at 37°C were collected. The majority of STO cells attach within 10 min of plating, and thus two or three such sequential preplatings yield cultures that contain <1% STO cells.

Mouse embryos at various stages of gestation were obtained by mating randomly bred ICR animals (Simenson Laboratories, Gilroy, CA). The day on which the vaginal plug was detected was considered 0.5 day of gestation. Embryos at the designated stages were dissected from the implantation site and separated from the extraembryonic membranes. RNA was isolated from pools of whole mouse embryos at various times of gestation or from tissues dissected from conceptuses at various stages of gestation (see text).

Isolation and Characterization of Fgf cDNAs

The substrates for all polymerase chain reaction (PCR) amplifications were prepared by reverse transcribing (as described by Frohman *et al.*, 1988) individual samples (1 μg each) of poly(A)$^+$ RNA from mouse embryos at various stages of gestation (E10.5, E11.5, E12.5, E14.5, and E17.5) and from teratocarcinoma cells

maintained in the undifferentiated state or cultured to stage 1 or 2 of differentiation. Reaction mixtures were diluted to 1 ml with TE (10 m*M* Tris-HCl, pH 7.5/1 m*M* EDTA) and a pool of these cDNAs (100 μl of each sample) was precipitated and resuspended in 10 μl of TE.

Fgf cDNA fragments in 1 μl of the cDNA pool were amplified using a DNA thermal cycler (Perkin-Elmer-Cetus; 50 cycles: 94°C, 1 min; 40°C, 1 min; 72°C, 1 min) as described by Frohman *et al.* (1988). A single pair of oligonucleotide primer sets was used for amplification of mouse *Fgfb* and *Fgfa*. The upstream primer set (5'-CTZTAYTGYAXZAAYGG; X: A/G, Y: C/T, Z: any base, synthesized by Operon, San Pablo, CA) contained most nucleotide sequences that encode the six amino acids [LYC(K or S)NG] at the N-terminal end of the conserved core of human and bovine basic and acidic FGF (Abraham *et al.*, 1986a,b; Gimenez-Gallego *et al.*, 1985; Jaye *et al.*, 1986), and the downstream primer set (5'-AAZAXZATZGCYTTYTG) contained the reverse complements (rc) of all possible nucleotide sequences that encode the six amino acids (QKAILF) near the C-terminal ends. Similarly, a second pair, for amplification of mouse *Fgfk* and *Fgf5*, consisted of an upstream primer set containing sequences (5'-CTZTAYTGYAXZGT-ZGG) that encode LYC(N or R)VG and a downstream primer set containing sequences (5'-CTZGGZAGX-AAXTGZGT) that encode THFLPR-rc, found at the N- and C-terminal ends, respectively, of the conserved core of human kFGF and FGF-5 (Delli Bovi *et al.*, 1987; Taira *et al.*, 1987; Zhan *et al.*, 1988).

Fgf amplification products were expected to contain ~340 base pairs (bp) of *Fgf* sequence between the primers, a stretch sufficiently long to allow for identification of the different genes by cross-hybridization with human or bovine probes. To confirm that the mouse *Fgf* cDNAs of interest had been amplified, the PCR products were assayed by Southern blot hybridization (as described by Frohman *et al.*, 1988) using probes for bovine FGFB (Abraham *et al.*, 1986a), human FGFA (Jaye *et al.*, 1986), human FGFK (Delli Bovi *et al.*, 1987), and a mouse *Fgf5* genomic clone (O. Haub and M. Goldfarb, unpublished data), at reduced stringency. Under our conditions [hybridization at 65°C in 0.5 *M* sodium phosphate, pH 7, 1 m*M* EDTA, 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin (BSA); washing at 50°C in 0.2× SSC, 0.1% SDS] each of the probes hybridized to its cognate genes but not to other family members. To enrich for the fragments of interest, the products of positive amplification reactions were separated by electrophoresis, and DNA of the appropriate size was extracted with Glassmilk (Bio 101, San Diego, CA) and cloned into a bluescript vector (Stratagene, San Diego, CA). Plasmids with *Fgf* cDNA inserts were identified by colony lift hybridization (Maniatis *et al.*, 1982, pp. 318, 319, 324-328) using bovine, human, or mouse probes. Plasmid DNA prepared by the alkaline hydrolysis method (Maniatis *et al.*, 1982, p. 368) was sequenced using Sequenase (United States Biochemicals), according to the supplier's recommendations.

Clones containing sequences upstream and downstream of the conserved core were obtained by means of the RACE protocol as described by Frohman *et al.* (1988) and sequenced as described above. Clones containing presumed full-length *Fgf* coding sequences were obtained by standard PCR amplification using presumed 5' and 3' untranslated sequences as primers.

Nucleotide sequences were obtained by analysis of both strands of at least three independently isolated clones. Mutations attributed to the use of PCR were found at a frequency of no more than 1 per 1000 bp. The nucleotide sequences of the four mouse *Fgf* cDNAs determined in this study have been deposited in GenBank.[3]

Northern Blot Analysis

Total cellular and poly(A)$^+$ RNAs were isolated as described by Shackleford and Varmus (1987) and by Maniatis *et al.* (1982, pp. 197-198), respectively. Northern blot analysis was carried out as described by Joyner *et al.* (1985). Blots were hybridized in 0.2 *M* sodium phosphate, pH 7.0, 35% formamide, 1 m*M* EDTA, 7% SDS, and 1% bovine serum albumin using *Fgf* cDNA probes or a rat actin probe (Nudel *et al.*, 1983) radiolabeled by nick translation or random priming (Bethesda Research Labs). Blots were washed at moderate (65°C in 30 m*M* sodium phosphate, 0.1% SDS) or high (65°C in 30 m*M* sodium phosphate, 0.1% SDS, 15% formamide) stringency. Hybridization signals on X-ray films were quantified using a Bio-Rad Model 620 video densitometer.

RESULTS

cDNA Cloning

cDNAs containing presumed full-length coding sequences of four of the mouse FGF family members were obtained by a three-step strategy using the polymerase chain reaction. For each step, the substrate for the amplification reaction was a pool of cDNAs obtained by reverse transcribing poly(A)$^+$ RNA from whole embryos at various stages of gestation between E10.5 and E17.5 and from cultures of teratocarcinoma cells, which represent embryonic cells at earlier stages of development (Martin *et al.*, 1977; Martin 1980).

[3] EMBL/GenBank Data Library Accession Nos. *Fgfa*: M30641; *Fgfb*: M30644; *Fgfk*: M30642; *Fgf5*: M30643.

(1) For each family member a cDNA fragment containing the conserved portion of the coding region was amplified by PCR and cloned. Two sets of "degenerate" oligonucleotide primers were used for each PCR: an upstream primer set containing most nucleotide sequences that encode six amino acids found at the N-terminal end and a downstream primer set containing the reverse complements of most nucleotide sequences that encode six amino acids near the C-terminal end of the conserved region in the cognate FGF from other species (see Materials and Methods and Fig. 1).

(2) Sequences 5′ and 3′ to the conserved region were

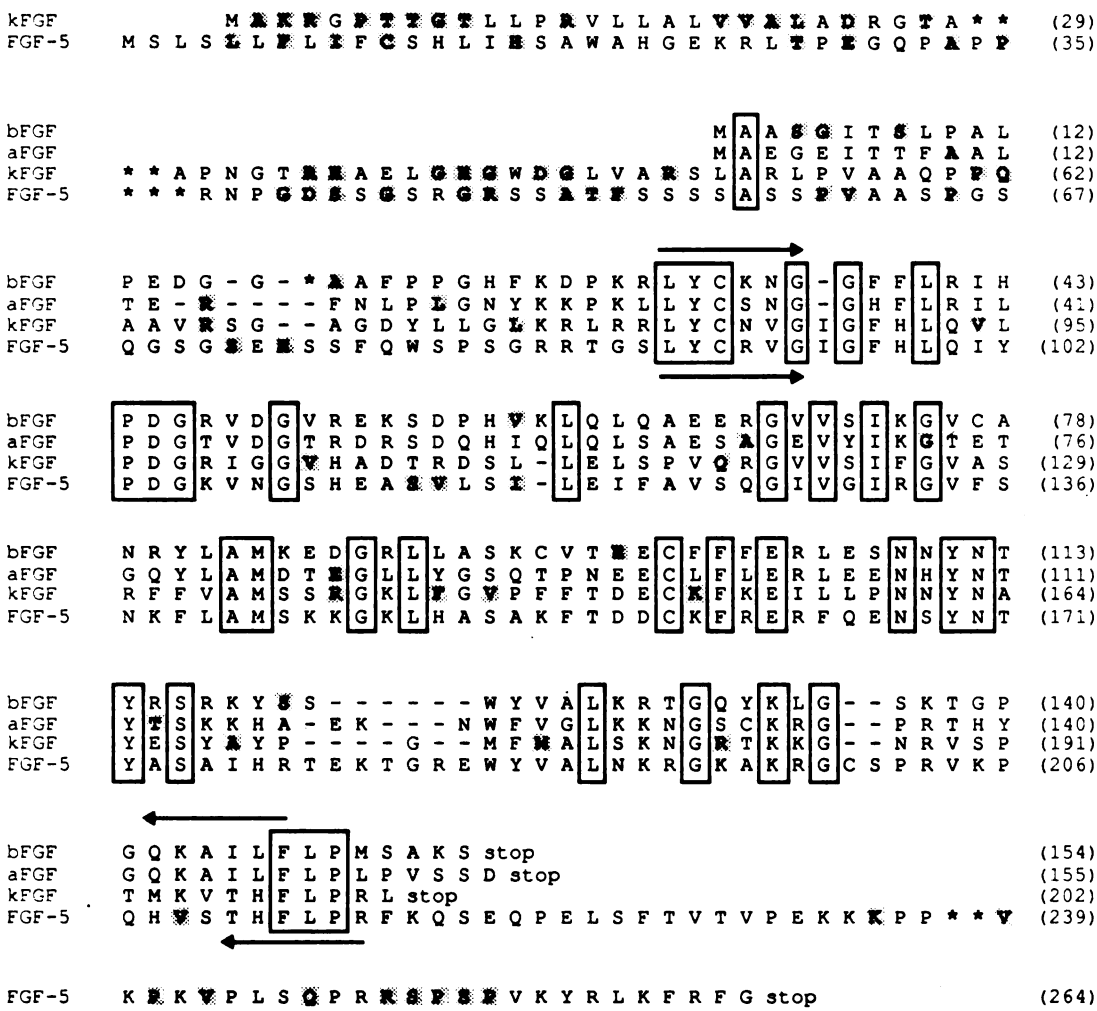

FIG. 1. Comparison of the amino acid sequences encoded by the mouse *Fgfb*, *Fgfa*, *Fgfk*, and *Fgf5* genes. The amino acid sequences of the mouse FGF genes isolated in this study are displayed using the single-letter code. In order to obtain maximum alignment of similar sequences it was occasionally necessary to insert gaps (—). Amino acid residues that differ (substitutions or additions) from those of the human cognate gene are highlighted in bold. Positions at which there is an amino acid residue in the human cognate but not in the mouse sequence are indicated by an asterisk (*). Amino acid residues that are identical in all four mouse sequences are boxed; all but one of these are found in the core region of sequence conservation which lies between the amino acids LYC and FLP (positions 31-33 and 147-149, respectively, in mouse bFGF). The arrows indicate the positions of the sequences used to design oligonucleotide primers for step 1 PCR amplification (upper arrows for bFGF and aFGF; lower arrows for kFGF and FGF-5). The sequence of mouse kFGF reported here differs from that described by Brookes *et al.* (1989) at residue 167. Murine *Fgf5* residues 79 to 85 (W to R) are indicated as being identical to those found in their human cognate; this reflects recent correction of the human FGF5 cDNA sequence (submitted to GenBank).

cloned using the RACE method (Frohman *et al.*, 1988), which achieves amplification of the region between a single known sequence in a cDNA molecule and its unknown 5′ or 3′ end. Primer sequences for the RACE cloning were determined by analysis of the cDNA fragments isolated in the first step. [In the case of *Fgfk*, however, the RACE protocol was not used; instead, the sequences of interest were obtained by analysis of a mouse genomic clone (B. Rogers, A. Mansukhani, and C. Basilico, unpublished data); similarly, in the case of *Fgf5*, sequences 3′ to the conserved core were obtained by analysis of a mouse genomic clone (O. Haub and M. Goldfarb, unpublished data)]. Although the RACE protocol was designed to obtain clones that extend full-length to the 5′ or 3′ cDNA end, in this study efforts were directed toward sequencing only the coding regions and the regions in each gene immediately upstream and downstream of the translation initiation and stop sites, respectively. In view of the extensive homology between the mouse genes and their human cognates (see Fig. 1), the translation start site for each mouse gene was presumed to be similar to that of its human cognate. However, since no in-frame stop codon was found upstream of the putative start site in the cDNAs for any family member (although in each case a maximum of only 100 bp were sequenced), the possibility remains that the open reading frame extends to a more 5′ ATG or to an unusual start codon, as has been reported for human hepatoma FGFB (Prats *et al.*, 1989).

(3) Individual cDNAs containing presumed full-length coding sequences for each of the mouse FGFs were amplified by PCR and cloned. The primer sequences for these amplification reactions were based on the 5′ and 3′ untranslated sequences determined by analysis of clones obtained in the second step.

A comparison of the deduced amino acid sequences of the mouse *Fgf* cDNAs isolated is shown in Fig. 1, with the residues that differ in the human cognates highlighted. Both basic and acidic FGFs are highly conserved among mammalian species, with >92% amino acid identity among the mouse, human, and bovine cognates (Gimenez-Gallego *et al.*, 1985; Abraham *et al.*, 1986a,b; Jaye *et al.*, 1986). *Xenopus* bFGF gene is almost as well-conserved, with 81% of the deduced amino acid residues identical to those of mouse bFGF (data from Kimelman *et al.*, 1988). Although overall there is less sequence conservation between the mouse and the human cognates of kFGF and FGF-5 (80 and 86%, respectively), most of the sequence differences occur in the long regions N-terminal and C-terminal to the conserved LYC to FLP core (see Fig. 1). When only the ~120 residues in the core region are considered, only 10 differences are found between the mouse and the human kFGF cognates, and only 4 between the FGF-5 cognates. Of the ~120 residues in the core, 34 are identical in all four mouse family members (see Fig. 1).

Expression in Developing Embryos

The availability of the cDNA clones described above made it possible for us to begin a comparison of the stage and tissue specificities of expression of the *Fgfb*, *Fgfa*, *Fgfk*, and *Fgf5* genes during mouse embryogenesis. The patterns of expression of the four genes at different stages of embryonic development are illustrated in the Northern blot shown in Fig. 2. In these experiments teratocarcinoma cells were used as a source of poly(A)$^+$ RNAs that are likely to be found in peri-implantation embryos, since it is difficult to isolate sufficient RNA for a Northern blot analysis from embryos at those stages: undifferentiated PSA-1 teratocarcinoma stem cells closely resemble the cells of the inner cell mass of the preimplantation embryo (~E3.5) and cells at stages 1, 2, and 3 of differentiation *in vitro* (see Materials and Methods) have features in common with mouse embryos at stages prior to (E4.5-E6.5), during (E6.5-E8.5), and after (E8.5-E10.5) gastrulation, respectively (Martin *et al.*, 1977). mRNAs expressed at later stages were isolated from whole embryos at E10.5 through E15.5.

Of the four family members studied, *Fgfk* appears to have the most restricted pattern of expression. Transcripts were undetectable by Northern blot analysis in RNA from whole embryos or various embryonic tissues, even after extended exposure times (Fig. 2 and data not shown). In contrast, *Fgfk* transcripts, predominantly 3.3 kb in length but in some cases minor amounts of several larger species, possibly representing unprocessed transcripts, were readily detected in RNA from undifferentiated teratocarcinoma stem cells as well as from embryonic stem cells isolated directly from mouse inner cell masses (Figs. 2 and 3). These data suggest that *Fgfk* is expressed in the inner cell mass of the embryo and thus plays a role in preimplantation development.

Fgfk mRNA was also detected, albeit at lower levels, in differentiating PSA-1 cultures (Fig. 2). To explore the cell type specificity of this expression, we assayed for *Fgfk* mRNA in different cell types found in PSA-1 cultures at stage 1 of differentiation. At this stage the cells are in aggregates known as simple embryoid bodies. These consist of an outer cell layer, containing a mixture of primitive, visceral, and parietal endodermal cell types, surrounding an inner cell core. The cell types found in the core are less well-defined, but presumably include undifferentiated stem cells as well as cells beginning to differentiate to mesoderm and possibly other

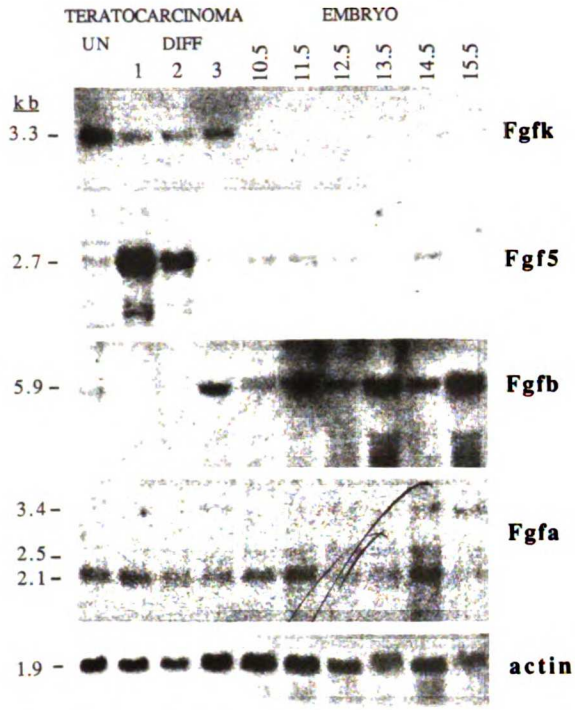

FIG. 2. *Fgfk*, *Fgf5*, *Fgfb*, and *Fgfa* expression at various stages of mouse embryogenesis. A Northern blot of poly(A)$^+$ RNA (~5 μg per lane) isolated from PSA-1 teratocarcinoma stem cells maintained in the undifferentiated state (un) or collected at various stages of differentiation induced by aggregation (see Materials and Methods) and from whole embryos at the days of gestation indicated was analyzed by reiterative hybridization under conditions of moderate stringency (see Materials and Methods) and stripping using cDNA probes for the FGF family genes indicated on the right. Hybridization with a probe for β-actin was included to demonstrate that all lanes contain roughly equivalent amounts of RNA. The sizes of the transcripts were estimated by comparison with RNA standards (RNA ladder, BRL). Owing to the differences in specific activities of the probes and autoradiograph exposure times, no direct comparisons can be made of the relative abundance of the different family members at the different stages of development. The larger of the two hybridizing transcripts detected with the *Fgf5* probe in the undifferentiated teratocarcinoma cell sample is identical in size to the 3.3-kb *Fgfk* mRNA; since its appearance is not due to previous hybridization of the blot with the probe for *Fgfk*, it may result from cross-hybridization of the *Fgf5* probe with the *Fgfk* transcript under the conditions of "moderate" stringency used here. It was not observed in other experiments under similar conditions.

cell types. To determine whether *Fgfk* is expressed in endodermal cells, we assayed for *Fgfk* transcripts in poly(A)$^+$ RNA from endoderm isolated from simple embryoid bodies, from PYS cells, a teratocarcinoma-derived parietal endodermal cell line, and from F9-derived endodermal cells obtained by treating F9 cell monolayers (for parietal endoderm) or aggregates (for visceral endoderm) with retinoic acid. *Fgfk* transcripts were detected only in the F9 visceral endoderm sample (Fig. 3). However, those cultures contain a substantial number of undifferentiated stem cells. Thus, it seems likely that endodermal cells do not express *Fgfk* and that the transcripts detected in differentiating PSA-1 samples are specific to the cells of the embryoid body cores, raising the possibility that the gene is also expressed in the early postimplantation embryo.

In contrast to the temporal restriction of *Fgfk* expression observed by Northern blot hybridization, expression of the other three FGF family members was detected at all stages of development tested (Fig. 2). However, there were significant differences in the levels of expression at different stages for two of the genes (*Fgf5* and *Fgfb*), suggesting differences in the tissue specificity of their expression. In particular, a dramatic (15-fold) increase in steady-state levels of the

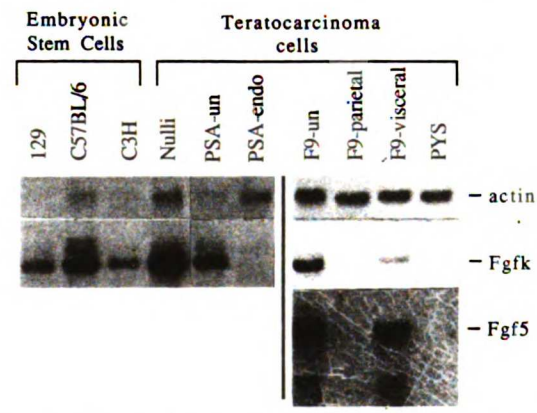

FIG. 3. *Fgfk* and *Fgf5* expression in undifferentiated embryonic stem cells and teratocarcinoma stem cells and in their differentiated derivatives. Poly(A)$^+$ RNA was isolated from embryonic stem cells derived from three different strains of mice [129, C57BL/6 and C3H (Martin, unpublished; see Materials and Methods)] and from three different teratocarcinoma stem cell lines (Nulli, PSA-1, and F9), all maintained in the undifferentiated state (un). In addition, Poly(A)$^+$ RNA was isolated from the endodermal cells found in the embryoid bodies formed by PSA-1 cells (PSA-endo; Grabel and Martin, 1983) from the endodermal cells formed when F9 cells are induced to differentiate to either parietal (Strickland *et al.*, 1980) or visceral endoderm (Hogan *et al.*, 1981) following treatment with retinoic acid, and from PYS cells, an established endodermal cell line with many of the characteristics of parietal endoderm (Lehman *et al.*, 1974). Separate Northern blots (left and right of vertical line) of these samples (~5 μg per lane) were analyzed by reiterative hybridization under conditions of high stringency and stripping using cDNA probes for the genes indicated on the right. The sizes of the transcripts detected with the *Fgfk* and *Fgf5* probes were the same as those in Fig. 2. Rehybridization of these and other Northern blots of ES cell mRNA with probes for *Fgf5*, *Fgfb*, and *Fgfa* indicated that the expression of these genes in all the ES cell lines tested was essentially the same as that in PSA-1 cells (data not shown).

2.7-kb *Fgf5* transcript was observed and a smaller transcript (~1.6 kb) became readily detectable when PSA-1 cells differentiated to stage 1 simple embryoid bodies (Fig. 2). To determine which cell type is producing the increased levels of *Fgf5* mRNA, we tested for expression in various endodermal cell types (Fig. 3). *Fgf5* transcripts were undetectable in the RNA from parietal endodermal cells (F9-derived parietal and PYS cells) and were detected at slightly reduced levels in F9 cultures that had been stimulated to form visceral endoderm. Since the level of *Fgf5* mRNA did not increase when F9 cells differentiated to visceral endoderm, the dramatic increase in *Fgf5* mRNA levels as PSA-1 cells differentiate is likely due to changes in the inner core cells of the embryoid bodies rather than to formation of endodermal cells. Interestingly, as the cultures progressed to stage 3 of differentiation the levels of *Fgf5* mRNA decreased to low levels similar to those detected in whole embryos at later stages of development (E10.5 to E15.5; Fig. 2). The results of Northern blot assays of total RNA from a variety of tissues dissected from embryos at E12.5, E13.5, E15.5, and E17.5 showed that *Fgf5* transcripts could be detected at approximately equivalent, relatively low levels in all tissues at all stages (Fig. 4 and data not shown). Taken together, these data suggest that *Fgf5* plays a role at many different times in development, but may have an especially important one in the peri-implantation period of development.

The pattern of *Fgfb* expression in differentiating PSA-1 teratocarcinoma cells differs markedly from that of *Fgf5*. *Fgfb* transcripts were barely detectable in embryoid bodies at stages 1 and 2. However, by stage 3 of differentiation, a predominant 5.9-kb *Fgfb* transcript became relatively abundant. The levels of these transcripts were roughly equivalent in stage 3 PSA-1 cells and whole embryos from E10.5 to E15.5 (Fig. 2). In embryos, too, the pattern of *Fgfb* expression was distinctly different from that of *Fgf5*. Although *Fgfb* transcripts were detectable in all samples assayed, there was a striking tissue-specific variability in abundance. At E13.5 *Fgfb* transcripts were most abundant in the developing limbs, relatively abundant in face, heart and lung, tail, and carcass, and barely detectable in the brain, liver and gut, yolk sac, and placenta (Fig. 4). In older embryos the levels of *Fgfb* transcripts remained high in the limbs and low in the brain, but there was a dramatic increase in abundance in the tail between E13.5 and E15.5, with levels remaining high until at least E17.5 (data not shown).

Among the FGF family genes *Fgfa* showed the least variable pattern of expression. It was detected at all stages of development and in all tissues analyzed, and in most tissues the three *Fgfa* transcripts, 2.1, 2.5, and 3.4 kb in length, respectively, were each detected at roughly equivalent levels (Figs. 2 and 4). There were, however, some tissues in which their relative abundance varied. For example, the levels of the 2.5- and 3.4-kb transcripts are elevated in 13.5-day yolk sac (Fig. 4), and these species are 2- to 10-fold more abundant in lungs than in other tissues from embryos at 15.5 and E17.5 (data not shown). All three transcripts are detectable under high-stringency hybridization conditions (see Materials and Methods), indicating that none of the three bands detected by Northern blot analysis is due to cross-hybridization of the *Fgfa* probe with other FGF family members.

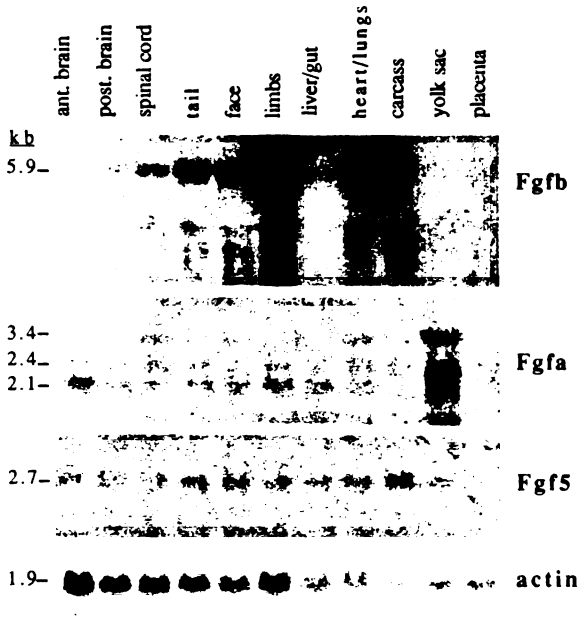

FIG. 4. Tissue specificity of *Fgfb*, *Fgfa*, and *Fgf5* expression at E13.5. A Northern blot of total RNA (~20 μg per lane) isolated from the tissues indicated, dissected from mouse embryos at 13.5 days of gestation, was analyzed by reiterative hybridization under conditions of moderate stringency and stripping using cDNA probes for the genes indicated on the right.

DISCUSSION

Using a three-step cDNA cloning protocol that circumvents the need for constructing and screening libraries, we have succeeded in isolating cDNAs containing presumed full-length protein coding sequences for the mouse *Fgfb*, *Fgfa*, *Fgfk*, and *Fgf5* genes. Although no attempt was made to isolate cDNAs representing *Fgf6*, or any currently unknown family members, the protocol described here could easily be used for that

purpose. Analysis of the cDNAs obtained shows extensive sequence identity between the deduced mouse protein sequences and their human cognates, indicating that the genes have been highly conserved during mammalian evolution. This presumably signifies that the structure and functions of the individual gene products have been equally well-conserved in mammals.

Using the cDNA clones described we began to explore the question of when and where these FGF genes are expressed in the developing mouse embryo. Previous studies of the FGF family member *int-2* have shown that its expression in the embryo is restricted to a very small number of cell types at only a few stages of development (Wilkinson *et al.*, 1988, 1989). We therefore sought to determine whether other family members might also have highly restricted patterns of expression. The data presented here suggest that the expression of *Fgfk* may be limited to early stages of embryonic development. In contrast, *Fgfb*, *Fgfa*, and *Fgf5* transcripts were detected at all stages of development in virtually all tissues analyzed. Despite this apparent ubiquity, there were some intriguing differences in expression patterns that have provided us with clues as to when and where the different genes might be particularly active or have special functions during development.

Data on expression in teratocarcinoma cells, which in the past have provided a reliable indicator of stage- and tissue-specific expression in the peri-implantation embryo (see, for example, Jakobovits *et al.*, 1986), suggest that *Fgfk* is expressed early in development and may possibly be restricted to pluripotent cells in the pre- or very early postimplantation embryo. Results similar to those described here have also been reported recently by Velcich *et al.* (1989) and Peters *et al.* (1989). What remains unclear, however, is whether *Fgfk* is also expressed in the mid- to late-gestation fetus. Yoshida *et al.* (1988) have reported that a mouse *Fgfk* genomic clone hybridizes to transcripts in Northern blots of RNA from mouse embryos at various postimplantation stages of development. Under our conditions of Northern blot hybridization we have been unable to detect any *Fgfk* transcripts in RNA from mid- to late-gestation embryos, although we did detect a hybridizing band when a probe for human *Fgfk* was used at reduced stringency (G. Martin and J. Scott, unpublished data). This raises the possibility that the transcripts detected by Yoshida *et al.* may have been cross-hybridizing species. On the other hand, when we assayed for *Fgfk* expression in whole embryos at E12.5 by reverse transcribing poly(A)$^+$ RNA and using it as a substrate in a PCR amplification reaction with mouse *Fgfk*-specific primers, a product representing *Fgfk* mRNA was obtained (Hébert and Martin, unpublished data). In view of the extreme sensitivity of such an assay the significance of this observation is unclear, but it might indicate that *Fgfk* is expressed later in development, possibly in few specific cell types. This, as well as the question of what cell types express *Fgfk* in the peri-implantation embryo, can best be addressed by RNA *in situ* analyses. Irrespective of whether *Fgfk* is expressed in older embryos, the data described here point to a role for kFGF in the peri-implantation embryo.

Data on expression in teratocarcinoma cells also point to *Fgf5* as a gene that may play a significant role in early postimplantation development. We observed a dramatic increase in the levels of *Fgf5* mRNA as PSA-1 teratocarcinoma stem cells form embryoid bodies. Similar results were obtained with a probe for *int-2*, which was found to be expressed at relatively high levels in endodermal cells (Jakobovits *et al.*, 1986; Wilkinson *et al.*, 1988). In contrast, our data suggest that the high level of *Fgf5* expression in differentiating PSA-1 cultures is not attributable to expression in newly formed endodermal cells, but rather to expression in cells in the embryoid body cores. This observation is particularly intriguing because the peak of *Fgf5* expression precedes or is coincident with the appearance of overtly differentiated mesodermal cells in the embryoid body cores. This raises the possibility that *Fgf5* may play a role in the induction of mesodermal differentiation in the mouse embryo, similar to the role proposed for basic FGF in *Xenopus* embryos (Kimelman *et al.*, 1988).

Our observations show that *Fgfb* is not expressed at high levels in teratocarcinoma cells until stage 3 of their differentiation, long after mesodermal induction has occurred. Although this does not preclude a role for bFGF in mesoderm formation, it focuses attention on other functions of bFGF in the mid- and late-gestation embryo. *Fgfb* expression is detected, albeit at relatively low levels, in the central nervous system, consistent with its known functions as a neurotrophic and neurotropic factor (Togari *et al.*, 1985; Morrison *et al.*, 1986; Walicke *et al.*, 1986; Hatten *et al.*, 1988). More remarkable, however, are the findings that *Fgfb* transcripts are most abundant in developing limbs at E13.5, that expression persists at high levels to at least E17.5, and that expression levels increase dramatically in the developing tail between E13.5 and E15.5. Such expression is subsequent to limb or tail pattern formation, and thus at these stages bFGF is most likely being used for one or more of its many well-characterized functions such as promoting angiogenesis or neurite outgrowth from neuronal cells (for a review see Gospodarowicz, 1987).

Braunhut *et al.* (1989) have reported that bFGF- and aFGF-like proteins are expressed by F9 cells only after differentiation, whereas *Fgfb* and *Fgfa* transcripts are detected in both undifferentiated and differentiated cells. However, the sizes of the mRNAs detected in their study were completely different from the sizes of the transcripts we detected using mouse *Fgfb* and *Fgfa* cDNA probes. A likely explanation for this discrepancy is that the bovine probes they used hybridized to mRNA species other than the mouse *Fgfb* or *Fgfa* transcript.

Although studies such as the one described here, as well as more detailed *in situ* analyses of RNA and protein expression patterns, can provide some clues, additional types of information will be required to achieve an understanding of the many different functions of the FGF family members in development. In particular, it will be necessary to identify the cells that may be competent to respond to FGF signals. The recent cloning of the bFGF receptor gene by Lee *et al.* (1989) will facilitate such studies. Moreover, it will be necessary to learn more about how FGFs affect different cell types and how other signaling molecules modulate their effects. For example, bFGF promotes differentiation of neuronal cells, whereas it stimulates proliferation of vascular endothelial cells (Gospodarowicz *et al.*, 1985; Togari *et al.*, 1985); the latter effect, however, is inhibited by TGFβ (Baird and Durkin, 1986; Frater-Schroder *et al.*, 1986). Despite this potential complexity, advances in molecular genetic techniques in the mouse, in conjunction with biochemical and genetic approaches using *in vitro* model systems such as the teratocarcinoma cell system described here, should ultimately help us to understand how and in what circumstances FGFs affect cell fate and behavior during development.

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mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation

JEAN M. HÉBERT, MONICA BOYLE and GAIL R. MARTIN

Department of Anatomy and Programs in Genetics and in Developmental Biology, School of Medicine, University of California at San Francisco, San Francisco, CA 94143, USA

Summary

During gastrulation in the mouse, the pluripotent embryonic ectoderm cells form the three primary germ layers, ectoderm, mesoderm and endoderm. Little is known about the mechanisms responsible for these processes, but evidence from previous studies in amphibians, as well as expression studies in mammals, suggest that signalling molecules of the Fibroblast Growth Factor (FGF) family may play a role in gastrulation. To determine whether this might be the case for FGF-5 in the mouse embryo, we carried out RNA *in situ* hybridization studies to determine when and where in the early postimplantation embryo the *Fgf-5* gene is expressed. We chose to study this particular member of the FGF gene family because we had previously observed that its pattern of expression in cultures of teratocarcinoma cell aggregates is consistent with the proposal that *Fgf-5* plays a role in gastrulation *in vivo*. The results reported here show that *Fgf-5* expression increases dramatically in the pluripotent embryonic ectoderm just prior to gastrulation, is restricted to the cells forming the three primary germ layers during gastrulation, and is not detectable in any cells in the embryo once formation of the primary germ layers is virtually complete. Based on this provocative expression pattern and in light of what is known about the functions *in vitro* of other members of the FGF family, we hypothesize that in the mouse embryo *Fgf-5* functions in an autocrine manner to stimulate the mobility of the cells that contribute to the embryonic germ layers or to render them competent to respond to other inductive or positional signals.

Key words: gastrulation, mouse embryogenesis, *Fgf-5*, fibroblast growth factor-5.

Introduction

Gastrulation is generally defined as the process whereby a population of embryonic cells is transformed into a three-layered structure, consisting of ectoderm, endoderm and a middle layer of mesoderm. In mammals, the starting cell population, known as the embryonic ectoderm or epiblast, can be thought of as a flat, roughly circular, pseudostratified, columnar epithelial sheet. At some time prior to the onset of gastrulation, a particular region on the sheet's circumference becomes determined as the posterior end of the embryo, and a corresponding position ~180° around the circumference becomes defined as the anterior end. Gastrulation begins when cells within the sheet move towards the posterior end of the embryo. This presumably occurs by a process of movement within the epiblast, rather than by cells exiting from the sheet and moving across its surface (Lawson *et al.* in preparation). The net effect of this movement is observed as an accumulation of cells at the posterior end of the embryo, initially only near the circumference of the cell sheet. This thickening in the cell sheet is known as the primitive streak. As gastrulation proceeds, cell movement within the sheet results in progressive accumulation of cells (lengthening of the primitive streak) along a line from the circumference of the sheet towards its center. Following their accumulation in the streak, cells exit from it and go on to form the mesodermal and endodermal cell layers.

Little is known about the key steps in this process, such as what regulates the movement of cells within the sheet, how this movement results in cell accumulation at the posterior end, or how cells acquire either a mesodermal or an endodermal cell fate. Similarly, there is little information about the molecules that regulate and mediate these processes. Our goal is to identify such molecules. Our approach is based on the widely accepted hypothesis that basic mechanisms of vertebrate embryogenesis have been evolutionarily conserved, and therefore that specific molecules that play a key role in gastrulation in other organisms also do so in mammals.

Based on this hypothesis, members of the FGF gene family are good candidates for molecules that play some role in gastrulation. They are known to induce gastrulation-like movements in isolated animal caps from *Xenopus* blastulae (Paterno *et al.* 1989; reviewed

by Smith, 1989). In addition, of the seven known members of this family (Goldfarb, 1990), at least four have the capacity to induce amphibian embryonic cells that would otherwise assume an ectodermal fate to differentiate as mesoderm (Kimelman and Kirschner, 1987; Slack *et al.* 1987; Slack *et al.* 1988; Paterno *et al.* 1989). We previously cloned the mouse cognates of four of these genes and provided a preliminary description of their patterns of expression in the developing mouse embryo (Hébert *et al.* 1990). Based on those data, we proposed that *Fgf-5* is likely to play a role during early postimplantation development. To explore the possibility that it has a function in gastrulation, we have carried out RNA *in situ* hybridization analyses of gastrulating embryos. The results obtained demonstrate that although *Fgf-5* mRNA is not detectable in the mouse embryo shortly after implantation, expression increases dramatically just prior to the onset of gastrulation in the epithelial cells that give rise to the three germ layers. Throughout gastrulation *Fgf-5* expression appears to be restricted to the cells that are forming the three primary germ layers, and by the time germ layer formation is virtually complete, *Fgf-5* mRNA is undetectable. This unique pattern of expression, especially its temporal restriction to the period when cells within the epithelium are mobile, suggests to us that the FGF-5 protein may participate in the maintenance of the mobile state. Alternatively, since *Fgf-5* expression ceases shortly after cells become allocated to particular lineages, another possibility is that FGF-5 may play a role in sustaining the pluripotency of the cells that form the germ layers, and thus renders them competent to respond to inductive or positional signals.

Materials and methods

Embryos and teratocarcinoma cell cultures

Mouse embryos at various stages of development were obtained by mating random bred ICR animals (Simenson Laboratories, Gilroy, CA). Embryonic age was estimated by taking the afternoon of the day on which the copulation plug was detected as 0.5 days of gestation (E0.5). There was, however, considerable variation in developmental stage both between and within litters at a given embryonic age. Embryos at the designated stages were dissected from the implantation site and fixed in 4% paraformaldehyde.

The origin of the PSA-1 teratocarcinoma stem cell line and the methods used to obtain differentiation (embryoid body formation) were exactly as described by Martin *et al.* (1977). At various times after differentiation had begun, PSA-1 cell aggregates were collected from suspension culture and fixed in 4% paraformaldehyde.

RNA probes

A plasmid clone containing the full-length *Fgf-5* coding sequence (Hébert *et al.* 1990) was linearized with *Eco*RV before being transcribed with T7 polymerase to generate a α^{35}S-UTP (1400 Ci mmol^{-1}, New England Nuclear)-labeled single-stranded antisense RNA probe. Alkaline hydrolysis was used to reduce probes to an average size of 50–150 nt (Cox *et al.* 1984). The specificity of this probe for *Fgf-5* mRNA is evidenced by the fact that the *in situ* hybridization pattern observed does not coincide with that obtained using probes for two other members of the FGF gene family, *Fgf-k* (Niswander and Martin, unpublished data) and *int-2* (Wilkinson *et al.* 1988). Furthermore, a DNA probe composed of the same *Fgf-5* sequence does not cross-hybridize with mRNA from any of the other known FGF family members in northern blot analyses at moderate stringency; the only exception to this was in one unusual experiment in which faint cross-hybridization with *Fgf-k* mRNA was observed (Hébert *et al.* 1990). When a single-stranded sense RNA probe was used as a control, no specific hybridization was observed (data not shown).

In situ *hybridization*

Embryo and teratocarcinoma cell samples were dehydrated, embedded in paraffin wax and sectioned (6 μm). *In situ* hybridizations were performed at high stringency as described by Frohman *et al.* (1990). The probe concentrations used in the hybridization solution ranged from 1 to 5×10^4 cts min^{-1} μl^{-1}. Slides were exposed for one to two weeks before being developed and stained.

Results

Expression of Fgf-5 *in the gastrulating mouse embryo*

To determine the pattern of *Fgf-5* mRNA expression during gastrulation, we carried out RNA *in situ* hybridization studies of mouse embryos at various stages of early postimplantation development. In the period between implantation and the start of gastrulation (~E4.5–E6.5; Fig. 1A, D), the embryonic portion of the conceptus develops from a solid ball of rounded cells into a cup-shaped epithelial cell sheet, the epiblast, surrounding a space known as the proamniotic cavity. The apical surface of the epiblast faces the cavity. Its basal (outer) surface is covered by a single layer of cells known as the visceral endoderm. These cells are not destined to form part of the fetus, but rather will contribute to extraembryonic membranes (Gardner, 1982; Gardner, 1984). The 'base' of the cup is the distal end of the embryo, and the 'rim' is its proximal end. Abutting the proximal end of the embryo is extraembryonic tissue, through which the embryo is connected to the uterine wall (Fig. 1A, D).

In one of the embryos removed from the uterus during this period (~E5.5), it appeared that the proamniotic cavity was just forming and the embryonic ectoderm had not yet become organized into an epithelial sheet. In that embryo, *Fgf-5* mRNA was not detectable (Fig. 1B, C). In embryos at a slightly more advanced stage of development (E6.0–E6.5), *Fgf-5* mRNA was readily detectable at uniformly high levels throughout the embryonic ectoderm (Fig. 1E, F, and data not shown). It was clear from analysis of serial sections that in some of these embryos the primitive streak had not yet formed (data not shown). In contrast, *Fgf-5* mRNA was undetectable in the proximal extraembryonic tissue. The boundary between the *Fgf-5*-expressing and non-expressing tissues was very sharp. With respect to the visceral endoderm cells, which surround the embryonic ectoderm but will

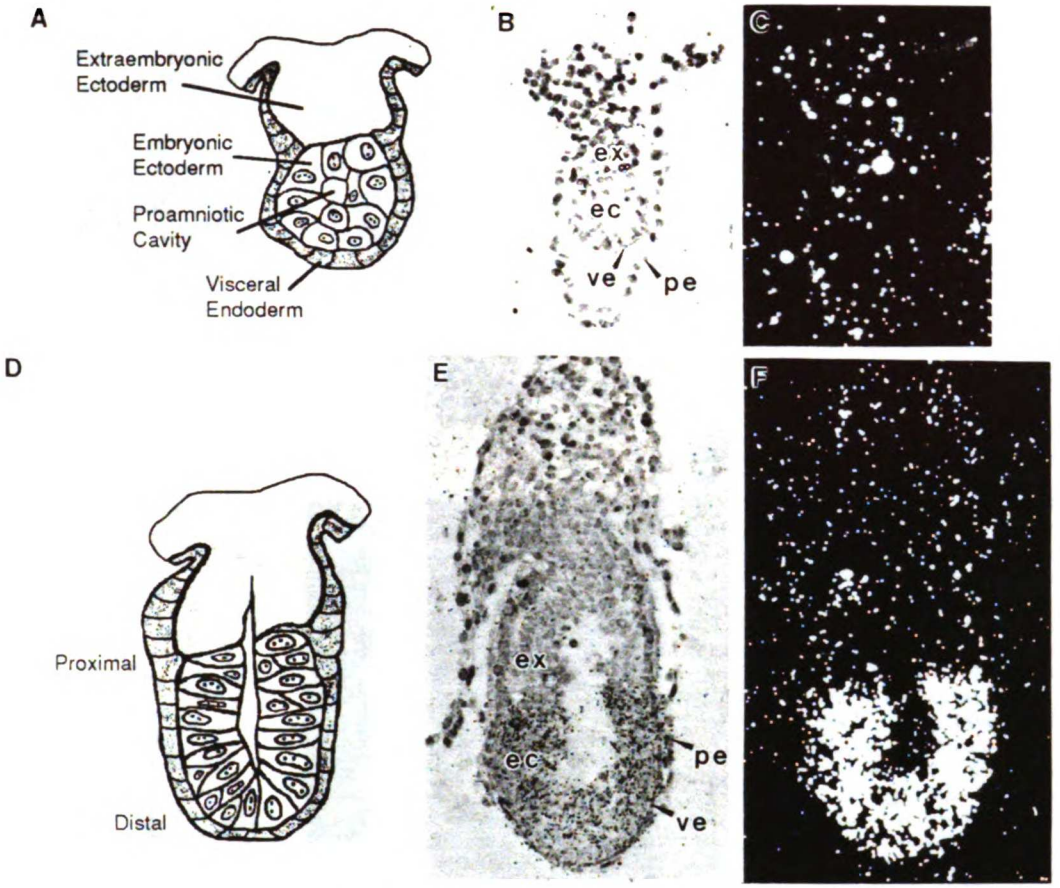

Fig. 1. Expression of *Fgf-5* in the mouse embryo at the pre-streak stages of development. (A) Diagram of a section through the early postimplantation mouse embryo (~E5.5), showing the beginning of proamniotic cavity formation. (B) *In situ* hybridization of antisense *Fgf-5* RNA to a section through an embryo at ~E5.5, viewed in bright field. (C) Same, dark field. (D) Diagram of a section through an embryo at the onset of primitive streak formation (~E6.5). (E) Hybridization of the antisense *Fgf-5* probe to a section through an embryo at ~E6.5 viewed in bright field. (F) Same, dark field. Abbreviations: ec, embryonic ectoderm; ex, extraembryonic ectoderm; pe, parietal endoderm; ve, visceral endoderm. Magnification=~200×.

ultimately form extraembryonic tissue, it was difficult to determine whether *Fgf-5* mRNA was expressed in them since they are extremely flat and closely apposed to the strongly positive embryonic ectoderm (Fig. 1E, F).

A few hours later (the early-streak stage, ~E6.5), gastrulation begins as cells within the epiblast begin to move towards and accumulate in the primitive streak (Fig. 2A). As gastrulation proceeds (early- to mid-streak stage, ~E6.5–E7.0), cells that have accumulated in the streak begin to exit from it. Some of the cells that become mesoderm move proximally and cross the boundary between the epiblast and the extraembryonic region, where they develop into the extraembryonic mesoderm. The remainder of the mesodermal cells spread laterally towards the anterior end of the embryo, between the epiblast basal surface and the visceral endoderm (see Fig. 2B). Cells that become embryonic endoderm incorporate into and replace the outer endodermal cell layer. Finally, cells that remain in the epiblast are the precursors of the neuroectoderm and epidermis (Lawson and Pedersen, 1987; Tam, 1989; Carey *et al.* in preparation; Lawson *et al.* in preparation).

At this stage (~E7.0), expression of *Fgf-5* remained restricted to the embryonic portion of the conceptus (Fig. 2C, D, and data not shown). Levels of *Fgf-5* mRNA appeared to be relatively high throughout the embryonic ectoderm and primitive streak. There was, however, some evidence of differential expression along the proximodistal axis in the embryonic ectoderm: based on estimates of grain counts, it appeared that the signal intensity in the most proximal region was 2- to 3-fold lower than in the most distal region.

Fgf-5 mRNA was not detected in the mesodermal cells that had migrated proximally into the extraembryonic region, nor was it observed in those mesodermal cells that had migrated proximolaterally between the embryonic ectoderm and visceral endoderm (lateral

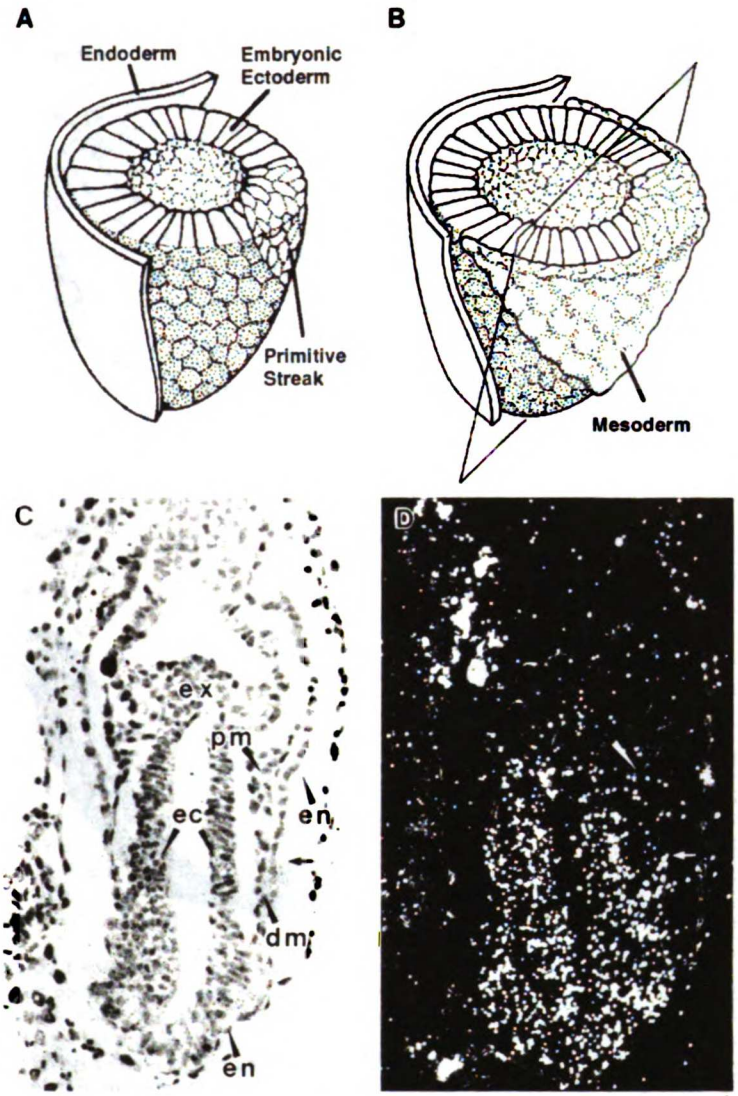

Fig. 2. Expression of *Fgf-5* in the mouse embryo at the mid-streak stage of development. (A) Diagram of the embryo showing the early stage of primitive streak formation. In this drawing, the endoderm is peeled from the posterior half of the embryo to reveal the underlying epiblast. (B) A similar diagram at the mid-streak stage, showing anterolateral migration of the embryonic mesoderm emerging from the primitive streak. The plane through which the embryo in C and D was sectioned is shown. (C) Hybridization of the antisense *Fgf-5* probe to a frontal section of an embryo at ~E7.0, viewed in bright field. The endoderm covering the epiblast at this stage is a mixture of visceral (extraembryonic) and definitive (embryonic) endoderm. (D) Same, dark field. The arrowhead points to proximal embryonic mesoderm, in which no *Fgf-5* mRNA was detected. The horizontal arrow in C and D marks the boundary between *Fgf-5*-positive and negative endodermal cells. Abbreviations: dm, distal mesoderm; ec, embryonic ectoderm; en, endoderm; ex, extraembryonic ectoderm; pm, proximal embryonic mesoderm. Magnification = ~200×.

mesoderm). It is possible that these mesodermal cells are derived from embryonic ectoderm cells that never expressed *Fgf-5*. However, we consider this possibility unlikely, since the fate of the majority of the cells in the proximal posterior region of the embryonic ectoderm at the pre-streak stage is to form extraembryonic mesoderm (Lawson *et al.* in preparation), and we observed high levels of *Fgf-5* expression in those cells. By contrast, embryonic mesodermal cells that have exited the streak in the more distal region of the embryo (paraxial mesoderm) did appear to contain *Fgf-5* mRNA at levels similar to those observed in the distal embryonic ectoderm.

Some of the cells that exit the primitive streak at the early- to mid-streak stage assume a definitive endodermal cell fate. They insert into the endodermal cell layer and mix with the visceral endodermal cells of which it was initially composed (Lawson and Pedersen, 1987). We observed both *Fgf-5*-expressing and non-expressing cells in the endodermal cell layer (Fig. 2C, D). In cells covering the distal portion of the embryo, *Fgf-5* mRNA was detected at levels equal to or higher than those in the adjacent embryonic ectoderm whereas, in cells covering the more proximal region of the embryo and in the extraembryonic endoderm, no *Fgf-5* mRNA was detectable. The boundary between the positive and negative cells was extremely sharp, and the change in expression pattern appeared to correlate with a change in endodermal cell morphology. Thus, it seems likely that the *Fgf-5*-positive cells in the endodermal cell layer are definitive endodermal cells that are continuing to express *Fgf-5* after they have exited from the primitive streak, rather than visceral endoderm cells.

During the mid- to late-streak stage (Fig. 3A), the primitive streak increases in length until it stretches from the proximal boundary (i.e. the rim of the cup) to the distal tip of the embryo. Just anterior to the distal end of the primitive streak, cells insert into the outer

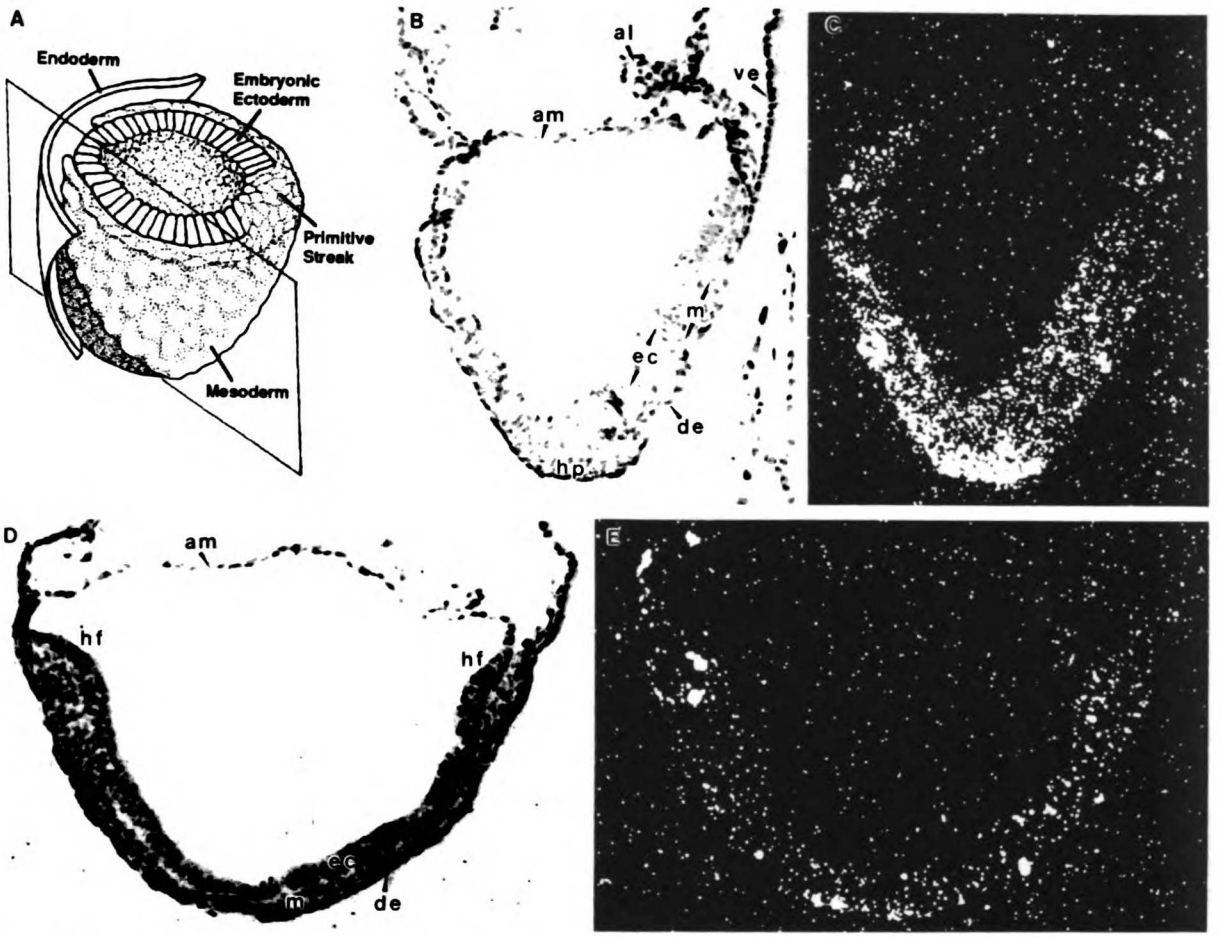

Fig. 3. Expression of *Fgf-5* in the mouse embryo at the late-streak and early neurula stages of development. (A) Diagram of the embryo at the late-streak stage, showing almost complete anterior migration of the embryonic mesoderm. The plane through which the embryo shown in B and C was sectioned is shown. (B) Hybridization of the antisense *Fgf-5* probe to a near-sagittal section of an embryo (~E7.5) at the late-streak stage, showing the head process. (C) Same, dark field. (D) Hybridization of the antisense *Fgf-5* probe to a near-frontal section of an embryo (~E7.75) in which the mesoderm completely surrounds the embryonic ectoderm and neurulation has begun, as evidenced by the appearance of the head folds. This embryo was a littermate of the one shown in B and C, and was embedded adjacent to it in the paraffin block; both embryos were therefore subjected to the *in situ* hybridization protocol under identical conditions. (E) Same, dark field. Abbreviations: Extraembryonic tissues: al, allantois; am, amnion; ve, visceral endoderm. Embryonic tissues: ec, embryonic ectoderm; de, definitive endoderm; hf, head folds; hp, head process; m, embryonic mesoderm. Magnification = ~200×.

endodermal layer and thus form the exposed 'head process,' which subsequently extends anteriorly in the midline and contributes to the notochord, trunk endoderm and other structures (Poelmann, 1981; Lawson *et al.* 1986; Lamers *et al.* 1987).

At this stage (~E7.5), embryonic ectoderm continues to express *Fgf-5* mRNA, with increasingly higher levels detectable towards the distal end. No expression was detected in the extraembryonic or proximolateral embryonic mesoderm, whereas the distal embryonic mesoderm was positive and contained roughly the same levels of mRNA as were observed in the adjacent ectoderm. The endoderm surrounding the embryo, which is still a mixed population of epiblast-derived and primitive endoderm-derived cells (Lawson *et al.* 1986), contains levels of *Fgf-5* mRNA similar to those in adjacent mesoderm. The head process cells are also positive, containing the highest levels of *Fgf-5* mRNA detected in the embryo at this stage (Fig. 3B, C, and data not shown).

Shortly after the late-streak stage (E7.5–7.75), the embryo expands most markedly in the posterior–anterior direction, and thus is transformed into a more open bowl-shaped structure. Neurulation begins, as evidenced by the appearance of the head folds of the neuroectoderm (Fig. 3D). Our most striking observation was that *Fgf-5* mRNA was no longer detectable in embryos that had reached this stage (~E7.75), as

determined by analysis of serial sections through the embryos (Fig. 3D, E, and data not shown), although it was abundant in embryos from the same litter that were not as advanced in development (Fig. 3B, C, and data not shown). Thus it appears that expression abruptly ceases when mesoderm has completely covered the basal surface of the embryonic ectoderm and formation of the three primary germ layers is virtually complete. *Fgf-5* transcripts were also not detected in embryos at ~E8.0 (data not shown), although they are detectable in specific tissues at later stages of development (Niswander and Martin, unpublished data) as well as in the adult (Haub *et al.* 1990).

Expression of Fgf-5 *in teratocarcinoma cell cultures*

Certain teratocarcinoma stem cell lines, including the PSA-1 cell line, differentiate *in vitro* in a manner that is remarkably similar to the development of the early postimplantation mouse embryo (Martin, 1980). The steps in this process, which is known as embryoid body formation, include: (1) the formation of a layer of extraembryonic endoderm surrounding a pluripotent cell core that is analogous to the embryonic ectoderm; (2) the formation of a cavity within the core, which is analogous to the proamniotic cavity; (3) the reorganization of core cells into a columnar epithelium resembling the pre-streak epiblast; (4) the expansion of the embryoid bodies with concomitant transformation of the pluripotent core cells into mesoderm-like cells similar to extraembryonic mesoderm (Martin *et al.* 1977). Since these embryoid bodies can be produced in large quantities under controlled conditions *in vitro*, they provide a more readily accessible source of material for genetic and biochemical manipulations than do embryos at the equivalent stages of gestation. We had previously found by northern blot analysis that there is a dramatic (~15-fold) increase in steady-state levels of *Fgf-5* mRNA when PSA-1 cells form embryoid bodies (Hébert *et al.* 1990). We were therefore interested in determining which cells in the embryoid bodies express *Fgf-5*, and whether the pattern of expression is analogous to that in the gastrulating embryo.

Shortly after formation of the outer endodermal cell layer, relatively low levels of *Fgf-5* mRNA were detected in most or all cells in the cultures. It is difficult to determine whether this is due to non-specific hybridization, or if it reflects a low level of expression in the embryoid bodies. We think that the latter is more likely, since our previous studies showed that *Fgf-5* mRNA was detectable by northern blot hybridization in cells at that stage (Hébert *et al.* 1990). Once cavitation had begun, high levels of *Fgf-5* mRNA were detected in ~20% of the embryoid bodies, and these levels appeared to be higher when the embryoid bodies had been kept in suspension culture for a longer period of time. In all cases, this high level of expression was observed only in the core cells of cavitated embryoid bodies, but not in their endodermal or mesodermal derivatives (Fig. 4), which are analogous to extraembryonic cells in the normal embryo.

These data suggest to us that *Fgf-5* expression is regulated in embryoid bodies in the same way as it is in the early gastrulating embryo: it is expressed at high levels as embryonic ectoderm cells progress beyond the cavitation stage and is down-regulated when the cells form mesoderm. We presume that the reason such abundant *Fgf-5* mRNA expression is detected in the core cells of only ~20% of embryoid bodies examined at any particular time, is that in an asynchronously developing population of embryoid bodies only a fraction of them contains cells that have reached a stage appropriate for *Fgf-5* expression and have not yet progressed beyond it. Moreover, these results suggest to us that cultures of PSA-1 teratocarcinoma cells can serve as an *in vitro* model system for studying the regulation of *Fgf-5* gene expression as well as its function in the embryo.

Discussion

The data reported here describe the pattern of *Fgf-5* expression in the early postimplantation mouse embryo. What is most striking about this expression is that it commences shortly before the onset of gastrulation and abruptly ceases when formation of the three primary germ layers is virtually complete; moreover, it is limited to the cells that are giving rise to the three primary germ layers. This suggests that *Fgf-5* has some function in gastrulation. In trying to formulate a hypothesis about what this function might be, we have taken into consideration what is known about the specific processes occuring in the embryo during gastrulation and about the biological activities of the different members of the FGF family.

As previously described, cell mobility within the epiblast is a key feature of gastrulation. Although there is little direct evidence concerning patterns of cell movement in the mammalian epiblast (Daniel and Olson, 1966), it has recently been shown that the daughters of epiblast cells labelled at the pre- and early-streak stages are later found widely separated from one another (Lawson *et al.* in preparation), whereas the daughters of cells labelled at the late-streak stage are subsequently found adjacent to one another (Carey *et al.* in preparation). This suggests that there is considerable cell mobility within the epiblast early in gastrulation and little if any by the late-streak stage. Thus, there is a good correlation between the period of cell mobility within the epiblast and the time during which *Fgf-5* is expressed in that tissue.

One possibility is that FGF-5 is required to sustain cell mobility within the embryonic ectoderm during gastrulation. Since FGF-5 is known to be secreted (Haub *et al.* 1990), its activity in this case could be autocrine. The observation that the level of *Fgf-5* mRNA is reduced in the proximal region of the embryonic ectoderm by the mid-streak stage would be consistent with this hypothesis if cell mobility decreases earlier in that region than in the more distal part of the embryo. Such a function for FGF-5 is consistent with

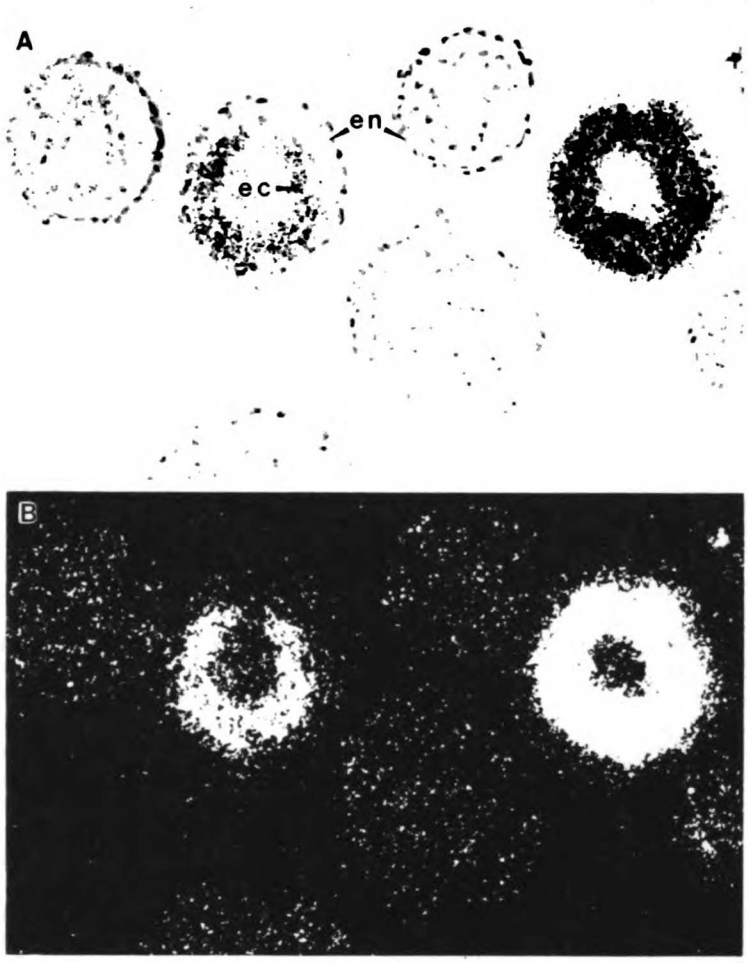

Fig. 4. Expression of *Fgf-5* in teratocarcinoma embryoid bodies. (A) Hybridization of the antisense *Fgf-5* probe to a section of PSA-1 embryoid bodies cultured for 12 days in suspension (Martin *et al.* 1977). (B) Same, dark field. Abbreviations: ec; embryonic ectoderm-like cells; en, extraembryonic endoderm-like cells. Magnification = ~200×.

the observation that the release of a related protein, basic FGF, from bovine aortic endothelial cells is required for their migration (Sato and Rifkin, 1988; Tsuboi *et al.* 1990).

After cells exit the streak, they appear to cease expressing *Fgf-5* mRNA. There are two possible explanations for the observed differences in levels of *Fgf-5* expression in cells that have passed through the streak (high in definitive endodermal cells and embryonic mesodermal cells exiting from the more anterior [distal] portions of the streak and undetectable in mesodermal cells that have exited from the posterior end). One is that *Fgf-5* expression persists for different lengths of time in different cell populations; alternatively, it may be down-regulated at a particular time after exit from the steak and the cells that we found to contain *Fgf-5* mRNA are those that have most recently emerged from the streak. The finding that the highest levels of *Fgf-5* mRNA at the late-streak stage are in the emerging head process is consistent with the latter idea.

The expression of one other member of the FGF gene family, *int-2*, has been studied in the gastrulating mouse embryo (Wilkinson *et al.* 1988). It is evident from a comparison of the data from that study and the results described here that there is some overlap in the domains of expression of *int-2* and *Fgf-5*, since both are expressed in the primitive streak. However, *int-2* continues to be expressed in mesodermal cells migrating proximally to the extraembryonic region, whereas *Fgf-5* expression appears to be down-regulated as cells leave the primitive streak and is never detected in the extraembryonic mesoderm. In addition, *Fgf-5* mRNA is detected throughout the embryonic ectoderm, whereas *int-2* mRNA is not. Furthermore, whereas *int-2* expression persists in the primitive streak until E9.5, *Fgf-5* expression abruptly ceases at the start of neurulation. Based on their observations, Wilkinson *et al.* (1988) suggested that *int-2* functions to stimulate the migration of proximal embryonic and extraembryonic mesodermal cells. Thus it is intriguing to speculate that INT-2 and FGF-5 may have related functions in different cell types in the gastrulating embryo.

Another key feature of gastrulation is that during this process lineage decisions are made. By the late streak/head fold stages (~E7.75), most of the cells that are destined to form mesoderm or endoderm have already entered the primitive streak, whereas those that will become neuroectoderm or epidermis remain in the embryonic ectoderm (Tam, 1989; Carey *et al.* in preparation). It is possible that cells that are allocated

in this way have become committed to their fates. In that case, the temporal pattern of *Fgf-5* expression would correlate with the period of pluripotency in the epiblast. Thus, another possible role of FGF-5 may be to render cells in the embryonic ectoderm competent to respond to signals that specify positional values or cell lineages. Basic FGF is thought to have such a function in the development of a neural crest cell lineage: it appears to render chromaffin cells competent to respond to nerve growth factor, which is needed for their differentiation into sympathetic neurons (Stemple *et al.* 1988; Birren and Anderson, 1990).

The two hypotheses about the possible function of FGF-5 proposed here are not inconsistent with the notion that FGFs play a role in mesoderm formation in *Xenopus* (reviewed by Smith, 1989). FGF-5 could have an indirect role in this process by stimulating the mobility of epiblast cells or by rendering them competent to respond to inductive or positional signals. Alternatively, in view of its known activity as a mitogen for cultured fibroblasts (Zhan *et al.* 1988), FGF-5 may function to stimulate the proliferation of cells during gastrulation. However, if this were the case, it is unclear why *Fgf-5* expression ceases abruptly at the beginning of neurulation whereas cell proliferation continues.

It is obvious that considerably more information is required before we can go beyond mere speculation about the possible function(s) of *Fgf-5* during gastrulation. Experiments are in progress to determine the temporal and spatial patterns of expression of the FGF-5 protein, as well as the cell surface receptor(s) through which it presumably acts, and to determine the effects on development of eliminating *Fgf-5* gene expression. The results of such studies should provide us with a clearer picture of what the function of *Fgf-5* might be, as well as the mechanism underlying a process in gastrulation.

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FGF5 as a Regulator of the Hair Growth Cycle: Evidence from Targeted and Spontaneous Mutations

Jean M. Hébert, Thomas Rosenquist, Jürgen Götz,* and Gail R. Martin
Department of Anatomy and Programs in Genetics and in Developmental Biology
School of Medicine
University of California, San Francisco
San Francisco, California 94143

Summary

Fibroblast growth factor 5 (FGF5) is a secreted signaling protein. Mice homozygous for a predicted null allele of the *Fgf5* gene, *fgf5^{neo}*, produced by gene targeting in embryonic stem cells, have abnormally long hair. This phenotype appears identical to that of mice homozygous for the spontaneous mutation angora (*go*). The *fgf5^{neo}* and *go* mutations fail to complement one another, and exon 1 of *Fgf5* is deleted in DNA from *go* homozygotes, demonstrating that *go* is a mutant allele of *Fgf5*. Expression of *Fgf5* is detected in hair follicles from wild-type mice and is localized to the outer root sheath during the anagen VI phase of the hair growth cycle. These findings provide evidence that FGF5 functions as an inhibitor of hair elongation, thus identifying a molecule whose normal function is apparently to regulate one step in the progression of the follicle through the hair growth cycle.

Introduction

In mammals, hair is produced in a cycle of tissue growth, degeneration, and renewal (reviewed by Hardy, 1993; Messenger, 1993). The hair growth cycle is typically defined as having three phases: anagen, the stage during which the follicle is regenerated and a new hair is produced; catagen, the stage during which hair elongation ceases and the hair follicle regresses; and telogen, the stage during which the follicle is at rest. As yet, little is known about the molecular nature of the signals that regulate progression through this cycle. Numerous mutations that affect hair production in mice have been identified. However, in most cases the basis of abnormal hair formation or loss of hair observed in the mutant animals either has not been elucidated or has been attributed to structural abnormalities in the hair follicles or in the hairs that are produced (Green, 1990). One gene that does appear to have a regulatory function is angora (*go*), which has been defined by two recessive mutations that cause the production of abnormally long hair (Dickie, 1963; Konyukhov and Berdaliev, 1990). The hair follicles in *go/go* animals have no obvious structural abnormalities, and the observed increase in hair length appears to be due to an increase in the length of time that follicles remain in the phase of active hair production (Pennycuik and Raphael, 1984). In this report, we demonstrate that *go* is a mutant allele of the fibroblast growth factor 5 gene (*Fgf5*), thus providing evidence that FGF5 is a molecule that plays a role in regulating the hair growth cycle.

Fgf5 is a member of the *Fgf* gene family, which is known to contain at least nine members (reviewed by Goldfarb, 1990; see also Tanaka et al., 1992; Miyamoto et al., 1993). The first two family members to be identified, acidic FGF (FGF1) and basic FGF (FGF2), were named for their ability to stimulate the proliferation of fibroblasts. However, it is now known that these and other FGFs are signaling molecules that can stimulate or inhibit proliferation or that can influence survival or differentiation of a variety of cell types, including muscle cells (Clegg et al., 1987) and neurons (reviewed by Wagner, 1991). On the basis of their expression patterns in the embryo, it has been suggested that FGFs play roles in a wide range of developmental processes (Wilkinson et al., 1988, 1989; Gonzalez, 1990; Haub and Goldfarb, 1991; Hébert et al., 1991; Niswander and Martin, 1992; de Lapeyrière et al., 1993; Han and Martin, 1993). Gene targeting experiments in mice have revealed that FGF3 is required for normal development of the ear and the tail (Mansour et al., 1993) and that FGF4 is required for survival during the early postimplantation period of development (B. Feldman, W. T. Poueymirou, T. N. DeChiara, and M. Goldfarb, personal communication).

In the mouse embryo, *Fgf5* RNA is detected just prior to the start of gastrulation and appears to be restricted to the embryonic ectoderm (Haub and Goldfarb, 1991; Hébert et al., 1991). Later in development, *Fgf5* is expressed in the somitic myotome and the precursors of specific skeletal muscles (Haub and Goldfarb, 1991). In the adult, *Fgf5* RNA is detected in the spinal cord and hippocampus (Haub et al., 1990). To help determine the function of FGF5 in vivo, we used the gene targeting approach in embryonic stem (ES) cells (reviewed by Capecchi, 1989; Koller and Smithies, 1992) to create mice deficient for FGF5. We describe here the phenotype of such mice, which indicates that FGF5 is an inhibitor of hair growth. Moreover, our data provide genetic and molecular evidence that *go* is a mutant allele of *Fgf5*. On the basis of the mutant phenotype and data demonstrating *Fgf5* expression in the outer root sheath of the hair follicle, we suggest that FGF5 regulates the onset of the catagen phase of the hair growth cycle.

Results

The *Fgf5* Gene Was Disrupted by Gene Targeting

A replacement-type targeting vector (Thomas and Capecchi, 1987) was constructed in which a neomycin resistance gene (*neo*) under the control of the phosphoglycerate kinase 1 (*Pgk1*) gene promoter was inserted in the opposite transcriptional orientation to the *Fgf5* gene at a SmaI site in exon 1. The *neo* expression cassette was flanked on both sides by ~2 kb of *Fgf5* genomic DNA (Figure 1). The

*Present address: Institute for Molecular Biology I, University of Zurich, Hoenggerberg, CH-8093 Zurich, Switzerland.

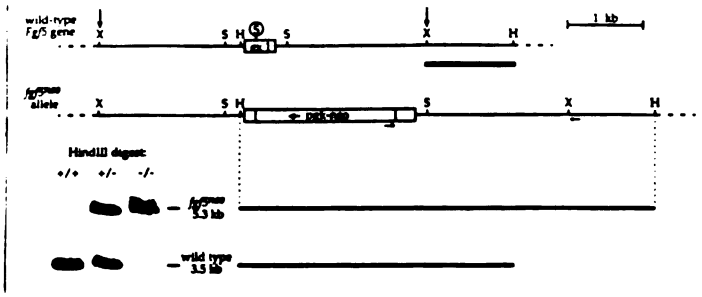

Figure 1. Disruption of the *Fgf5* Gene in Mice

The uppermost line represents a restriction map of wild-type *Fgf5* genomic DNA with only the coding portion of exon 1 (ex 1) illustrated (stippled box). The vertical arrows indicate the ends of the fragment used to construct the targeting vector. The SmaI site into which the *neo* expression cassette was inserted to disrupt the gene is designated by a circle. The XhoI–HindIII fragment used as a probe in this study is represented by a thick horizontal bar. Beneath is shown a restriction map of the mutant *fgf5*^{neo} allele, illustrating the *neo* expression cassette (open box) inserted into the exon 1 coding sequence. The arrow within the box indicates that the *neo* cassette is present in the opposite orientation to the direction of transcription of the *Fgf5* gene. The horizontal arrows below this map illustrate the location of sequences included in primers used for PCR analysis of potential homologous recombinant ES cell clones. Restriction enzymes: X, XhoI; S, SmaI; H, HindIII. The autoradiograph shows a Southern blot of HindIII-digested DNA isolated from tails of the offspring of a cross between animals heterozygous for the *fgf5*^{neo} mutant allele. The mutant and wild-type fragments detected in this analysis are illustrated by the horizontal lines to the right, which can be related to the maps shown above.

neo insertion interrupts the *Fgf5* coding sequence at a site 113 bp downstream of the translational start site (Haub et al., 1990; Hébert et al., 1990) and introduces multiple stop codons in all three reading frames. This *fgf5*^{neo} targeting construct was transfected by electroporation into ES cells, and G418-resistant clones were isolated. The clones were screened by polymerase chain reaction (PCR) analysis (Figure 1; see Experimental Procedures) to identify those in which homologous recombination had occurred. Their identity as homologous recombinants was confirmed by Southern blot analysis of ES cell genomic DNA digested with HindIII and probed with an *Fgf5* intron 1 DNA fragment that lies just downstream of the *Fgf5* sequences present in the targeting vector (Figure 1; data not shown).

Homologous recombinant cells were injected into C57BL/6 host blastocysts to generate chimeric mice. Offspring of these chimeras heterozygous for the *fgf5*^{neo} allele were identified by genomic Southern blot analysis of tail DNA digested with HindIII and hybridized to the intron 1 probe (data not shown). Heterozygous mice were crossed inter se to generate homozygous animals. Analysis of mutant homozygote DNA digested with a number of different enzymes demonstrated the absence of a wild-type *Fgf5* exon 1 and its replacement by *fgf5*^{neo} sequences (Figure 1; data not shown).

Mice Homozygous for the *fgf5*^{neo} Allele Have Abnormally Long Hair

Mice homozygous for the *fgf5*^{neo} mutation are born at a frequency consistent with Mendelian patterns of inheritance. The homozygotes were found to be healthy and fertile and appeared phenotypically normal until ~3 weeks after birth, when their hair grew noticeably longer than that of their heterozygous and wild-type littermates (Figure 2). This shaggy appearance has persisted for as long as the animals have been observed (~1 year).

Upon inspection, it was evident that all types of hairs examined from *fgf5*^{neo} homozygotes were longer than those from their heterozygous or wild-type littermates. To quantitate this effect, guard hairs, which are the long and straight hairs whose tips project above the other coat hairs, were plucked from a 0.5 cm^2 middorsal patch. Those plucked from homozygotes were found to be 1.1 cm ± 0.1 in length (n = 28), whereas those from their heterozygous littermates were 0.74 cm ± 0.1 (n = 30). Other than this 50% increase in length, the hairs appear normal. Since the *fgf5*^{neo} mutation is presumably a null allele (see below), these results indicate that FGF5 either directly or indirectly provides the normal signal for limiting hair elongation.

go Is a Mutant Allele of *Fgf5*

The *Fgf5* gene has been mapped by in situ hybridization to the E1-F region of mouse chromosome 5 (Mattei et al., 1992), and analysis of the offspring of an interspecific backcross localized it to a map position 3.6 ± 1.4 cM distal to *Alb-1* (Benovic et al., 1991). This places it ~3 cM distal to *go*, the only previously known mutation that causes abnormally long hair. All hair types of *go* homozygotes are 55% longer than those of their heterozygous littermates, but the mice appear otherwise normal (Dickie, 1963; Pennycuik and Raphael, 1984). The relatively close map positions of *Fgf5* and *go* and the similarity in the phenotypes of *fgf5*^{neo}/*fgf5*^{neo} and *go/go* mice suggested that *go* might be a mutant allele of *Fgf5*. To test this hypothesis directly, we performed a genetic complementation test. Male or female *go/go* mice were mated to *fgf5*^{neo}/+ heterozygotes. If *go* is a mutant allele of *Fgf5*, we would expect the *go*/*fgf5*^{neo} offspring of this cross to have abnormally long hair. Of 10 progeny from this cross, 6 had the long hair phenotype. Analysis of the DNA of these mice demonstrated that those with long hair were of the *go*/*fgf5*^{neo} genotype, whereas those with hair of normal length were of the *go*/+ genotype (data not shown), showing that *Fgf5* and *go* are allelic.

To determine the molecular nature of the *go* mutation, *go/go* DNA was compared with wild-type DNA. Since *go* arose spontaneously in a BALB/c mouse and was then transferred to C57BL/6 by repeated backcrossing and selection, we used both BALB/c and C57BL/6 genomic DNAs in this comparison. PCR analysis suggested that exon 1, but not exons 2 or 3, was deleted in *go/go* DNA (data not shown). This was confirmed by Southern blot analysis of *go/go* and (+/+) genomic DNA. An exon 1–specific probe

Figure 2. Phenotype of *fgf5*^{neo}/*fgf5*^{neo} Mice

The animal on the right (front) is a mutant homozygote at ~6 weeks of age. The animal on the left (behind) is its heterozygous littermate. The mutant homozygotes have no obvious phenotypic abnormalities other than their exceptionally long hair.

failed to hybridize to *go/go* DNA, whereas a cDNA probe containing only exons 2 and 3 hybridized to the same size fragments in *go/go* and (+/+) DNA (Figure 3). To map the 3′ deletion breakpoint, we carried out further analysis with several probes containing sequences in intron 1. The results indicated that the 3′ breakpoint lies within the SmaI fragment that contains the 3′ part of exon 1 (Figure 3; data not shown). Information on the 5′ deletion breakpoint was obtained using an intron 1 probe. The HindIII and XhoI fragments detected with this probe are larger in *go/go* DNA than in (+/+) DNA (Figure 3), indicating that the deletion includes the HindIII and XhoI sites 5′ of exon 1. Thus, the deletion extends at least 2 kb 5′ of the translation start site in exon 1 (Figure 3).

These observations raised the possibility that the promoter of the *Fgf5* gene is deleted in the *go* allele. To explore this, we carried out a Northern blot analysis of RNA from hippocampi of *go/go* mice, as well as from *fgf5*^{neo} homozygotes and wild-type animals. This tissue was chosen because it is a site of abundant *Fgf5* expression (Haub et al., 1990). When the blot was hybridized with a cDNA probe containing only exons 2 and 3, no signal was detected in the lane containing RNA from *go/go* mice, whereas signal was readily detected in the other two lanes (Figure 4). All three lanes contained roughly equivalent amounts of intact RNA as assessed by rehybridizing the blot with a probe for β-actin (Figure 4). The observation that exon 1 is deleted and that no RNA containing exons 2 or 3 of *Fgf5* was detected in this analysis led us to conclude that *go* is a null allele of *Fgf5*.

The reduced length of the transcript in the *fgf5*^{neo} homozygotes (Figure 4) could be explained either by initiation of transcription within the inserted sequences as a consequence of the bidirectional activity of the *Pgk1* promoter (Johnson and Friedmann, 1990) or by aberrant splicing. Whatever the structure of this abnormal RNA may be, it is very unlikely that it encodes a functional FGF5 protein, since the phenotype of the *fgf5*^{neo} homozygotes appears to be identical to that of mice homozygous for *go*.

Fgf5 Is Expressed in the Outer Root Sheath of the Hair Follicle

The long hair phenotype of *Fgf5*-deficient mice was unexpected because there had been a lack of reports describing *Fgf5* expression in the hair follicle. To determine whether hair follicle cells express *Fgf5*, we carried out an in situ hybridization analysis of *Fgf5* RNA in skin taken from the dorsal midline of wild-type mice at birth and at frequent intervals through postnatal day 24 (P0–P24) when the follicles are all in the first round of the hair cycle. During the period examined, the follicles progress through the three stages of the hair cycle, anagen (follicle generation and hair production), catagen (follicle regression), and telogen (resting phase), as illustrated in Figure 5A. We chose to perform our analysis of *Fgf5* expression from P0 to P24 because all of the follicles are progressing relatively

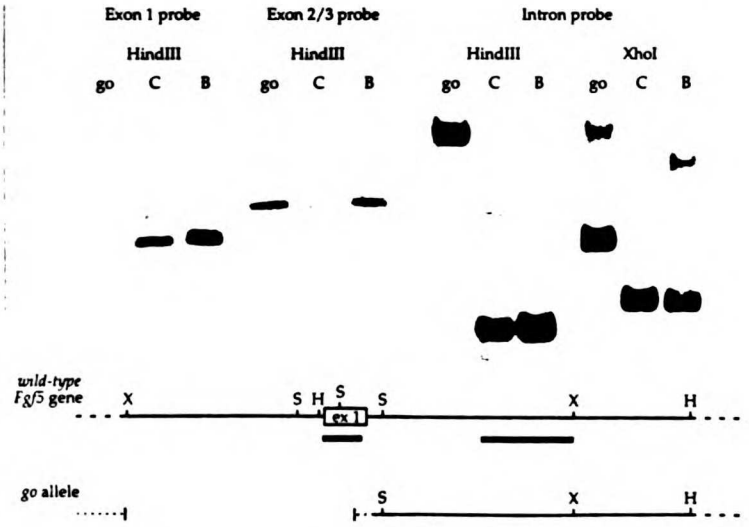

Figure 3. Analysis of the *Fgf5* Gene in *go* Mice

The autoradiographs show Southern blots of DNA isolated from *go/go* and (+/+) (BALB/c [B] and C57BL/6 [C]) mice, digested with HindIII or XhoI, and hybridized with three different probes as indicated. Below the photographs is a restriction map of the wild-type *Fgf5* gene with the coding portion only of exon 1 (ex 1), illustrated as a stippled box. Exon 1 and intron 1 sequences used as probes are represented by horizontal bars; the exon 2/3 probe also used in this study was derived from a cDNA clone containing the full-length *Fgf5* coding sequence (Hébert et al., 1990). The restriction map of the *go* allele illustrates the known limits of the deletion (broken line). The vertical lines on the left and right mark the known 5′ and 3′ limits, respectively, of the deletion, and the dotted lines represent sequences that may be deleted in *go* DNA.

Figure 4. Northern Blot Hybridization Analysis of *Fgf5* RNA in *go/go*, *fgf5*^{tm}/*fgf5*^{tm}, and (+/+) Mice

Each lane contains 20 μg of total RNA isolated from hippocampi of adult mice of the genotype indicated. The upper autoradiograph shows hybridization with an *Fgf5* cDNA probe containing only exons 2 and 3. The lower autoradiograph shows the same blot, rehybridized with a probe for β-actin (Nudel et al., 1983).

synchronously through the hair cycle during that period. Later in life, the follicles cycle asynchronously.

Fgf5 RNA was not detected in hair follicles from P0 to P6 (Figure 5B; data not shown). At P6, the follicles are in anagen VI, the final stage of anagen. During this phase, the follicle is a flask-shaped structure containing a bulbous base, a long neck, and the elongating hair, which protrudes above the surface of the skin (Figure 5A). The follicle consists of a mesoderm-derived structure, the dermal papilla, which is located in the center of the follicle bulb and is surrounded by an epidermally derived germinative epithelium, the hair matrix. The descendants of the hair matrix cells are gradually being pushed upward and are differentiating into at least six different cell types, which are organized in concentric rings. The three innermost cell layers form the medullary, cortical, and cuticular layers of the emerging hair. The three sequentially more peripheral cell layers form the inner root sheath, which extends part way up and is shed into the neck of the follicle. The outermost layer of the follicle is the outer root sheath. Near the base of the follicle, it is a single layer of cells, whereas further up in the follicle, it is multilayered (see Figure 5A). By P9, *Fgf5* RNA was readily detectable in all follicles (data not shown), and expression persisted through at least P12, while the follicles remain in anagen VI (Figures 5C and 5E; data not shown). Expression was localized in the cells of the outer root sheath. Although the outer root sheath surrounds the entire follicle and is continuous with the basal layer of the interfollicular epidermis (see Figure 5A), *Fgf5* RNA was restricted to outer root sheath cells in the lower one third of the follicle (Figure 5E; data not shown). In those follicles that were sectioned longitudinally through the center of the base (as evidenced by the presence of the dermal papilla), *Fgf5* RNA was also detected in a small number of cells at the very base of the follicle, some of which may be inner root sheath cell precursors (Figure 5C). No *Fgf5* RNA was detected above background levels in other components of the follicle, including the dermal papilla.

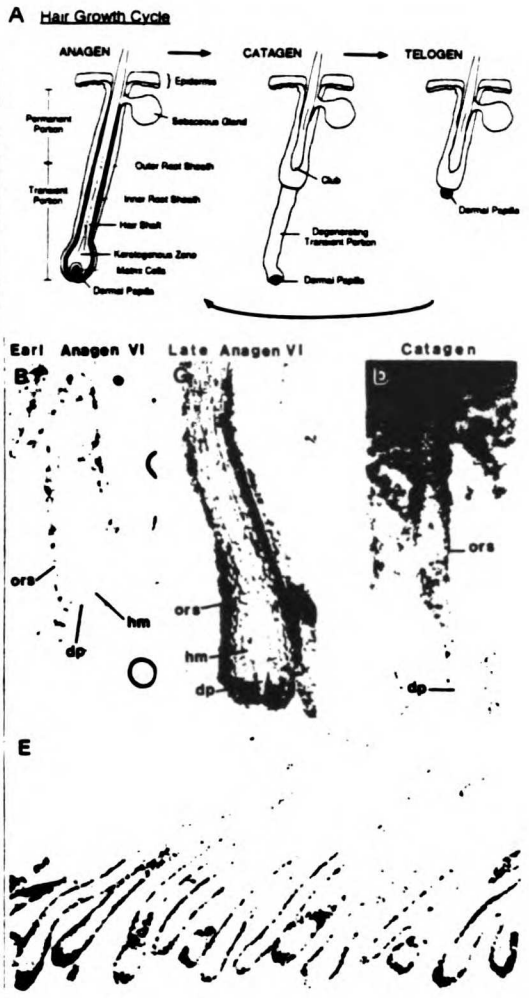

Figure 5. RNA In Situ Hybridyzation Analysis of *Fgf5* Expression during the Hair Growth Cycle

(A) The schematic diagram illustrates the morphology of the hair follicle at the three stages of the mammalian hair growth cycle: anagen, the stage during which the transient portion of the follicle is regenerated and descendants of the matrix cells produce a new hair; catagen, the stage during which matrix cell proliferation and thus hair elongation ceases and many of the structures of the anagen follicle are eliminated (by programmed cell death) as new structures of the resting follicle are formed; and telogen, the stage during which the follicle is at rest. During telogen, the hair that was produced during the preceding anagen phase is held in place by means of a structure known as the club. The arrow at the bottom indicates the beginning of a new cycle, during which the hair that is produced replaces the old one. Diagram adapted from Rook and Dawber (1982).

(B–E) Longitudinal sections of dorsal skin taken from Swiss–Webster (albino) mice at various times after birth: P6 (B), P12 (C and E), and P18 (D). Staining for alkaline phosphatase activity (brownish purple) identifies cells expressing *Fgf5* RNA. Early in anagen VI (P6), the phase during which the hair protrudes above the skin and is elongating, no *Fgf5* RNA is detected above background. Late in anagen VI (P12), staining is detected in the lower third of the outer root sheath of all follicles. In catagen (P18), no *Fgf5* RNA is detected. Abbreviations: dp, dermal papilla; hm, hair matrix cells; ors, outer root sheath. Magnification for (B)–(D), ~160×; for (E), ~80×.

During the period from P14 to P16, the follicles progress from anagen VI to a stage at which they clearly display the morphological changes characteristic of catagen (Straile et al., 1961; Parakkal and Alexander, 1972). These include cessation of matrix cell proliferation and degeneration of the transient portion (lower one third to lower two thirds) of the follicle, presumably as a consequence of programmed cell death. Daughters of the matrix cells produced prior to the onset of catagen continue to differentiate and move upward to form the last part of the hair shaft, which is attached to a structure known as the anchoring club, formed by modified cortical cells that are filled with nonoriented filaments. Surrounding this club are modified outer root sheath cells. During this period, *Fgf5* expression is extinguished (data not shown). From P17 through P24, the follicles progress through catagen to telogen (the resting phase of the cycle) and then begin the regeneration process (thereby entering the anagen phase of the second hair cycle). During this period, *Fgf5* RNA is also undetectable (Figure 5D; data not shown). As expected, no *Fgf5* RNA was detected in late anagen VI hair follicles from *go/go* mice (data not shown).

Discussion

FGF5-Deficient Mice Have Abnormally Long Hair

We describe here the production via gene targeting in ES cells of mice homozygous for a mutation in the *Fgf5* gene, *fgf5*^{neo}. The only obvious abnormality in these mice is the growth of hair that is ~50% longer than that of their heterozygous or wild-type littermates. This phenotype is apparently identical to that of mice homozygous for the *go* mutation, which maps very near the *Fgf5* locus. A genetic complementation test between *fgf5*^{neo} and *go* showed that the two alleles fail to complement one another, demonstrating that *go* is a mutant allele of the *Fgf5* gene. We have found that the *go* mutation is a deletion that removes all or most of *Fgf5* exon 1 and at least 2 kb of upstream sequences, which may include the promoter of the *Fgf5* gene. Consistent with this idea is the finding that no RNA containing exons 2 and 3 of *Fgf5* could be detected in tissue from *go/go* mice. Thus, *go* appears to be a null allele of *Fgf5*. Since *fgf5*^{neo} and *go* homozygotes appear to have identical phenotypes, it is likely that the *fgf5*^{neo} mutation we created is also a null allele. It is formally possible that the phenotype of these two distinct mutations is due to altered expression of some gene other than *Fgf5*. However, if that were the case, then both mutations must cause a recessive effect on that other gene. Although this is possible in the case of the *go* deletion, it is extremely unlikely in the case of the *neo* insertion into the *Fgf5* gene. Moreover, *Fgf5* is expressed in the hair follicle in a pattern consistent with a function as a regulator of hair length. Thus, we conclude that loss of *Fgf5* expression results in the long hair phenotype.

Recessive mutations that cause the production of abnormally long hair are known in several other species, including dogs (Burns and Fraser, 1966), cats (Robinson, 1977), and rabbits (Crary and Sawin, 1953; Fraser, 1953). As in mice, the *go* mutation in rabbits causes an increase in the length of anagen VI. In view of the data reported here, it would be interesting to determine whether the mutations in those species reside in the *Fgf5* gene or affect its expression.

Previous studies of *Fgf5* RNA expression in vivo (Haub et al., 1990; Haub and Goldfarb, 1991; Hébert et al., 1991) and protein function in vitro (Hughes et al., 1993) have suggested that during development *Fgf5* might play a role in gastrulation, myogenesis, motor neuron survival, or some combination of those, and in the adult it might be involved in hippocampal function. The data described here demonstrate that the *Fgf5* gene is not essential for viability and fertility. However, this does not preclude the possibility that the gene product functions in the developing embryo or adult, but that other genes compensate for the absence of a functional *Fgf5* allele. For example, the products of two other members of the *Fgf* gene family, *Fgf4* and *Fgf6*, that are coexpressed with *Fgf5* in the somitic myotome (Haub and Goldfarb, 1991; Niswander and Martin, 1992; de Lapeyrière et al., 1993; Han and Martin, 1993) might perform the normal function of FGF5 in its absence. Although the only abnormality detected in FGF5-deficient mice thus far is the production of long hair, it is possible that less-readily detectable abnormalites also exist in tissues other than the hair follicle. However, preliminary studies of neural structures suggest that the number of motor neurons in the spinal cord is normal in *go/go* mice (R. Oppenheim, L. Houenou, and L.-X. Li, personal communication), and the hippocampus appears morphologically normal (D. Lowenstein, personal communication).

FGF5 as a Regulator of the Hair Growth Cycle

After birth, the primary hair follicles that are formed late in embryogenesis progress through the first phase (anagen) of a cycle that continues throughout the life of the animal (see Figure 5A). During anagen, the follicle lengthens and penetrates deep into the dermis, and the descendants of the hair matrix cells at the base of the follicle bulb are gradually being pushed upward, differentiating into the hair that emerges from the mouth of the follicle. In the second phase of the cycle (catagen), proliferation of hair matrix cells stops, leading to a cessation of hair elongation. During catagen, the transient portion of the follicle degenerates and an anchoring club is formed. During the third phase (telogen), the follicle is at rest, and no morphological changes are detectable. A new round of the hair cycle begins with a downgrowth of epithelial cells, apparently derived from a stem cell population that permanently resides in the upper region of the follicle that does not regress (Cotsarelis et al., 1990; Kobayashi et al., 1993; Rochat et al., 1994). These dividing cells regenerate the transient portion of the follicle, which gives rise to a new hair that replaces the old one.

A detailed analysis of hair follicle morphology throughout the hair growth cycle in angora mice was reported by Pennycuik and Raphael (1984). They found that follicle morphology and hair growth rate are normal in *go* mice. The only detectable abnormality in the angora hair cycle is that anagen VI, the phase of active hair growth during which the hair matrix cells are proliferating, is extended

by 3 days; the lengths of the other phases of the cycle are normal. These observations, taken together with our data defining the molecular basis of the *go* mutation and demonstrating that it is a null allele of *Fgf5*, suggest that in the normal hair follicle the transition from anagen VI to the phase of follicle regression (catagen) is induced by FGF5 and that in its absence this key step in progression through the hair growth cycle is substantially delayed.

Tissue recombination experiments have shown that the *go* gene product is produced in a tissue derived from embryonic epidermis (Pennycuik and Raphael, 1984). Consistent with this, our in situ hybridization analysis shows that *Fgf5* RNA is expressed in epidermally derived cells, the outer root sheath cells. Moreover, *Fgf5* RNA is restricted to cells in the lower third of the outer root sheath that surrounds the hair bulb in which the hair matrix cells responsible for hair elongation are situated. It is in this part of the follicle that the degenerative changes characteristic of the onset of catagen are first detected (Straile et al., 1961). *Fgf5* expression in the outer root sheath appears to be initiated after the follicles have entered anagen VI and down-regulated just prior to the onset of catagen. Thus, the pattern of gene expression is consistent with the conclusion that FGF5, a secreted protein (Bates et al., 1991), either directly or indirectly induces the onset of catagen. In *go* or *fgf5*^{go} homozygotes, hair matrix cell proliferation and hair elongation presumably continue because FGF5 is absent. There are a number of ways in which FGF5 could possibly induce catagen. It might act directly on hair matrix cells to inhibit their proliferation, induce terminal differentiation, or induce apoptosis. Another possibility is that FGF5 affects hair matrix cells indirectly via the dermal papilla, the mesoderm-derived component of the follicle that is thought to provide signals important in various phases of the life cycle of the hair follicle (reviewed by Hardy, 1993).

An important point that must be borne in mind is that hair production in FGF5-deficient mice remains cyclical. Although anagen VI continues for longer than normal (Pennycuik and Raphael, 1984), eventually it does end and catagen ensues. It remains to be determined how catagen is induced in the absence of FGF5. One possibility is that hair matrix cells have a finite capacity for proliferation (Cotsarelis et al., 1990; Lavker et al., 1993), and in the absence of FGF5, they continue to proliferate only until the maximum number of divisions has occurred. Another possibility is that some other signaling molecule capable of inducing catagen acts in the follicle when anagen VI is prolonged owing to a lack of FGF5.

As yet, the roles played by signaling molecules other than FGF5 in the hair growth cycle have not been precisely defined. Studies in transgenic mice have shown that ectopic expression of bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor α (TGFα) superfamily, in the outer root sheath appears to inhibit proliferation of hair matrix cells (Blessing et al., 1993). Based on this observation, in conjunction with the finding that a closely related member of the BMP family, BMP2, is expressed in cells at the base of the hair follicle that will give rise to the cortex of the hair (Lyons et al., 1990), it has been suggested that BMP2 functions to inhibit cell proliferation and trigger differentiation of precortex cells during anagen VI (Blessing et al., 1993).

FGFs and TGFα Can Influence the Formation of the Hair Follicle

In addition to regulating progression of the hair follicle through the phases of the hair growth cycle, signaling molecules clearly play an important role in the formation of the hair follicle. Development of the primary hair follicle is initiated late in embryogenesis when a signal produced by the mesenchyme (dermis) stimulates the overlying surface epithelium to form a thickening and then a downgrowth (the hair plug or follicle bud). This first dermal signal appears to be conserved among different classes of vertebrates, as mouse dermis is able to induce the development of feather buds in chick epidermis and scale follicle buds in lizard epidermis. The hair plug is then believed to send a signal to a cluster of mesenchymal cells that is enveloped as the base of the follicle expands, instructing them to form the dermal papilla. In turn, the dermal papilla is presumed to send a second dermal signal, which stimulates rapid proliferation of adjacent epithelial cells in the hair plug (hair matrix cells) (reviewed by Hardy, 1993).

There is some evidence that members of the FGF family may play a role in hair follicle formation. Ectopic expression of *Fgf7* (also called keratinocyte growth factor) at high levels in the suprabasal epithelial layer inhibits the formation of primary hair follicles (Guo et al., 1993). Similar results were obtained following repeated subcutaneous injection of newborn mice with large amounts of FGF2, and such injections also inhibit maturation of existing follicles (Du Cros, 1993). One possible explanation of these results is that ectopic FGF proteins interfere with receipt of the dermal signal for follicle formation (Guo et al., 1993). However, as yet it is not clear which FGF family member, if any, plays a role in hair follicle formation.

One signaling molecule that has been shown by genetic analysis to play a role in hair follicle formation is TGFα. In studies analogous to the one described here, mice deficient in TGFα were created by gene targeting (Luetteke et al., 1993; Mann et al., 1993). The observation that the phenotype of the mice resembled that of animals homozygous for the waved-1 mutation led to the discovery that waved-1 is a mutant allele of TGFα. The mutant mice have irregularly distributed and variably angled follicles. This phenotype suggests that TGFα in some way influences the proper positioning of follicles. Moreover, the finding that TGFα RNA localizes primarily to the inner root sheath (Luetteke et al., 1993) and its receptor to the outer root sheath (Green et al., 1984) suggests that one normal function of TGFα may be to regulate interactions between these cell layers, leading to proper elongation of the hair (Luetteke et al., 1993). Recently, it has been shown that waved-2, a mutation that causes a phenotype strikingly similar to that of TGFα-deficient mice, is a mutant allele of the epidermal growth factor/TGFα receptor (Luetteke et al., 1994).

In summary, we have identified FGF5 as a molecule whose normal function appears to be the regulation of

one step in the progression of the follicle through the hair growth cycle. In FGF5-deficient mice, the structure of the follicle is normal and only the length of one phase, anagen VI, is affected (Pennycuik and Raphael, 1984). The data presented here indicate that FGF5 is an essential component of the normal signal to end anagen VI and initiate catagen and point to a role for intercellular signaling in this process. This information provides an entry point for studies aimed at elucidating this key regulatory mechanism.

Experimental Procedures

Construction of the *Fgf5* Targeting Vector

The targeting vector containing the disrupted *Fgf5* allele for homologous recombination was constructed as follows. Using an *Fgf5* cDNA clone as a probe (Hébert et al., 1990), an 18.5 kb *Fgf5* genomic DNA fragment was cloned from an EMBL3 phage library made from a partial Sau3A digest of BALB/c genomic DNA (Clontech, Palo Alto, CA). From this clone, a 4.2 kb XhoI fragment containing exon 1 (see Figure 2) was isolated and cloned into the XhoI site of the pBluescript SK vector (Stratagene, La Jolla, CA). To create a mutation in *Fgf5*, a 1.8 kb DNA fragment containing the *neo* gene driven by the *Pgk1* promoter (isolated from pKJ1; Tybulewicz et al., 1991) was inserted into the SmaI site in the coding portion of exon 1 (see Figure 1). The mutated fragment was then cloned into the XhoI site of the pBltk4 plasmid (Johnson et al., 1989) containing the *HSV-tk* gene, to allow use of the positive/negative selection method for ES cell clones in which homologous recombination had occurred (Mansour et al., 1988). The construct was linearized with NotI before electroporation into ES cells.

Identification of Homologous Recombinant Cell Clones and Production of Mutant Mice

ES cells of several different established lines were maintained in the undifferentiated state by coculture with G418-resistant STO cells as previously described (Martin et al., 1987). In a typical experiment, 10^7 ES cells were electroporated with 10 μg of the targeting vector DNA and plated in media containing 150 μg/ml G418 (GIBCO BRL, Grand Island, NY) to select for clones that had stably integrated the construct. In initial experiments, FIAU was also present in the medium to allow negative selection against nonhomologous recombinants. However, enrichment was negligible, and this selection was omitted in subsequent experiments.

Homologous recombinant cell clones were identified by a PCR analysis in which two primers, one (5′CCAGACTGCCTTGGGAAAAG3′) that hybridizes to a sequence in the *Pgk1* promoter and the other (5′GTGCCCACCTTTCAATGAGG3′) to an *Fgf5* intron sequence immediately downstream of the XhoI site at the 3′ end of the targeting vector (see Figure 1), were used to amplify a DNA fragment that includes the 3′ recombination junction from pooled DNA isolated from six to ten G418-resistant clones. DNA from individual clones in the PCR-positive pools was then amplified to identify the homologous recombinants, and this was confirmed by Southern blot analysis using an intron 1 probe (Figure 1). Surprisingly, the frequency of homologous recombination was found to vary with the parental ES cell line used, even though all of the cell lines were derived from strain 129 mice. The highest frequency (1 in 20) was obtained with R1 parental ES cells (Nagy et al., 1993) and was 10-fold higher than the frequency obtained with J1 (Li et al., 1992), AB1 (McMahon and Bradley, 1990), or D3 (Doetschman et al., 1985) ES cells.

Homologous recombinant cells were introduced into C57BL/6 blastocysts by injection using a microinjection apparatus as described (Bradley, 1987). Blastocysts were then transferred to foster mothers and allowed to develop to term. Chimeric pups carrying the mutant allele were identified on the basis of agouti coat color. Once they reached adulthood, they were bred to B6D2 or 129 mice. The offspring of these crosses were analyzed for heterozygosity at the *Fgf5* locus by Southern blot analysis of tail DNA probed with the exon 1 or intron 1 probe (Figure 1).

Southern and Northern Blot Analyses

Genomic DNA restriction fragments were separated by electrophoresis, transferred to GeneScreen (New England Nuclear, Boston; see Church and Gilbert, 1984), and hybridized (Joyner et al., 1985) with a ^{32}P-labeled probe (random priming kit, Boehringer Mannheim, Indianapolis, IN). *go/go*, BALB/c, and C57BL/6 mouse DNAs were purchased from Jackson Laboratories (Bar Harbor, ME). Total RNA was isolated from hippocampi dissected from adult mice, and Northern blot analysis of this RNA was carried out as previously described (Joyner et al., 1985).

RNA in Situ Hybridization Analyses

RNA in situ hybridization analyses were performed using a digoxigenin-labeled antisense RNA probe essentially as described by Schaeren-Wiemers and Gerfin-Moser (1993), but with two modifications to their protocol, the addition of a proteinase K treatment step and a decrease in the hybridization temperature. In brief, dorsal skin samples from Swiss–Webster mice (Simonson Laboratories, Gilroy, CA) were fixed in 4% paraformaldehyde and frozen in OCT compound (Miles Corporation, Elkhart, IN). Sections (8 μm) were collected on SuperFrost Plus slides (Fisher Scientific, Pittsburgh) and then treated with proteinase K (10 μg/ml for 10 min at room temperature). The sections were then hybridized at 65°C to an RNA probe prepared as previously described (Hébert et al., 1991), except the labeling reaction included digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, IN) instead of ^{35}S-labeled UTP. The slides were then treated with an anti-digoxigenin antibody coupled to alkaline phosphatase and were stained for alkaline phosphatase activity.

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CHAPTER 4

New strategies for studying a gene's function

Introduction

The most common genetic approaches used to investigate the function of a gene in a particular developmental process involve perturbing the gene's normal expression pattern. This is usually accomplished by either abolishing the gene's expression or ectopically expressing it. The hope is that the phenotype will reveal something about the gene's role. However, several problems are often encountered when using these approaches.

Gene targeting by homologous recombination to abolish a gene's expression has been used to generate many interesting and informative phenotypes. However, a common problem with this approach is that the specific developmental process that one wished to perturb initially is not detectably affected. The only thing learned in such a case is that the gene's function, which remains unknown, is redundantly carried out by other factors, or, that it has no function in that specific process. An illustration of this can be found in Chapter 3 where the targeting of *Fgf5*, which was suspected of playing a role in gastrulation due to its striking expression pattern at that stage (Chapter 2), did not lead to an embryonic phenotype, but instead to a hair phenotype in adults. Another example is the targeting of *Fgf3* which leads to a rather subtle ear phenotype despite expression of this gene in the gastrulating embryo (Wilkinson et al. 1988; Mansour et al. 1993).

Another kind of problem can be encountered when using gene targeting. This approach sometimes leads to a phenotype that prevents the study of the developmental process of interest. If the phenotype occurs at a

stage earlier than the one of interest and is disruptive enough, development never reaches the stage of interest, thus precluding the study of any effect that the ablation of the gene's function might have had at that later stage. An example of this can be found in the targeting of *Fgf4*. Disruption of this gene leads to a lethal phenotype shortly after implantation (M. Goldfarb, personal communication), precluding any potential phenotype that might have resulted at a later stage, such as when limb development occurs, a tissue in which *Fgf4* is believed to play an important role (Niswander et al. 1993).

Another common result of the gene targeting approach is a phenotype that disrupts the process of interest, but does not readily reveal anything about what the function of the gene is in that process. An example of this is found in the targeting of *TGFa* which leads to a complicated hair follicle phenotype denoting a role for this factor in this tissue without revealing what the role is (Luetteke et al. 1993; Mann et al. 1993).

The second type of genetic approach commonly used to study a factor's function is to ectopically express it or to overexpress it. Although these strategies have been very successful in some cases, they are even more likely than the gene targeting approach to lead to uninterpretable phenotypes. Artificially high levels of gene expression, or expression in a cell type that normally never encounters the gene's product, can generate phenotypes that are irrelevant to understanding the normal function of the gene in the process of interest, even if that process is disrupted. Moreover, this approach is currently very limited in scope due to the small number of well characterized tissue-specific promoters available for driving expression of genes at appropriate levels in well defined locations.

In this chapter, an alternative approach to understanding a gene's function is presented. The approach involves identifying genes whose

expression are responsive to a factor, for example FGF5, without any preconceptions as to what these genes might be. The strategy is based on the premise that if a cell changes its differentiation state or its developmental fate in response to FGF5, changes in the transcriptional state of a number of "downstream" genes will occur in that cell. Both identification and sequence analysis of these genes can then directly inform us as to how the cells are changing in response to FGF5. As an example, let us postulate that FGF5 added to embryonic stem cells causes certain cell surface adhesion genes to turn off and others to turn on. From this we learn that FGF5 changes the affinity of the cells for their environment and furthermore in what way it does so. In fact, regardless of what types of genes are found to be responsive to FGF5, as long as these genes can be recognized as encoding proteins of known functional classes (transcription factors, cell surface receptors, structural components, etc.), pertinent information is gained on how the cells have changed in response to FGF5. This approach is unlike most others because it does not depend on any preconceptions as to what kind of effect FGF5 should have on the cells, and hence the search for an effect is not limited to only the suspected morphological or molecular responses. Such information can currently only be obtained using relatively tedious approaches such as subtractive hybridization, in which the abundance of mRNA for a gene is often a main determinant in whether or not it can be identified as responsive.

The feasibility of two new experimental strategies for identifying responsive genes have been evaluated and are presented here. Both strategies involve trapping the genes of interest using a marker. Genetraps have successfully been used in mice by multiple groups to identify and characterize genes (Skarnes et al. 1992; von Melchner et al. 1992). The genetrap marker used does not have any promoter or regulatory elements, so it must be

inserted into an active gene in order to be expressed. The approach used here is as follows: the promoterless marker is introduced into a population of cells so that each cell that has stably taken up the construct has only incorporated it into one genomic locus. In such a way, a library of clones is made in which each clone has the construct incorporated somewhere in its genome and there are enough clones to ensure that each genetic locus has been targeted by the construct in the right orientation and reading frame. An aliquot of the library is then challenged with a factor, FGF5 for example, and clones in which expression of the genetrap marker is upregulated or downregulated are identified. From these responsive clones, cDNAs containing marker sequences are cloned and their 5' ends used to identify the endogenous FGF5-responsive genes.

A major challenge in elaborating an experimental strategy for identifying responsive genes has been to come up with a genetrap marker that can be selected for and against, or screened, in a large number of clones. The first approach presented uses lacZ as the genetrap marker and fluorescence activated cell sorting (FACS) as a means of selecting both lacZ expressing and non-expressing cells. The second approach uses a lacZ-neomycin^R fusion gene (βgeo) as the marker, neomycin resistance to select for genetraps and a fluorescent βgal substrate to screen for lacZ expressing and non-expressing cells.

Materials and Methods

Tissue culture techniques

Embryonic carcinoma (EC) and embryonic stem (ES) cells were grown as previously described (Martin et al. 1977). In the experiments described,

addition of LIF is the addition of a 1:50 dilution of conditioned medium from CHO cells transfected with a gene encoding LIF under the control of a strong promoter. Transfection of constructs was done by electroporation. A transfection efficiency of 1:1000 was obtained for the lacZ genetrap construct (generously provided by W. Skarnes and A. Joyner) and 1:400,000 for the βgeo construct (generously provided by G. Friedrich and P. Soriano).

FACS

Cells were exposed to 0.25% trypsin for 5 minutes, resuspended in DME with 15% fetal calf serum, and passed through an autoclaved nylon filter (37 microns mesh opening, 30 micron thread diameter, Cat# J-CMN-37, Small Parts Inc., Miami FL) to eliminate clumps of cells. Cells were then counted, pelleted, and resuspended at a concentration of $4X10^7$ cells/ml in cold DME medium with 4% serum. Cells were then loaded with fluoroscein di-β-D-galactopyranoside (FDG) by hypotonic shock as described (Nolan et al. 1988) and incubated on ice for approximately 1 hour, during which time βgal cleaves FDG to release fluoroscein. FACS was performed on a prototype cell sorter (HHMI, UCSF) with the invaluable help of Paul Dazin. Several controls were included in each run: a PETG control (PETG is an inhibitor of E. coli βgal); a propidium iodide (PI) control (PI stains the DNA of dead or dying cells); a calcein blue (CaBl) control (CaBl is a metabolic marker that stains metabolically active cells); and a 'no FDG' control to measure background fluorescence. FDG, CaBl, and PI are all vital dyes obtained from Molecular Probes Inc., Eugene OR.

MUG assay

4-methylumbelliferyl β-D-galactopyranoside (MUG), a βgal substrate that fluoresces once cleaved, was used as described (Fiering et al. 1991) to assay levels of lacZ expression in ES cells. Reactions times were of 10 minutes. Fluorescence was quantified on a Labsystems Fluoroskan II 96-well plate reader (Lab Products International LTD, North Carolina).

Results

Using lacZ *as the genetrap marker and FACS as a selection*

The lacZ open reading frame has efficiently been used as a genetrap marker in mouse embryonic stem (ES) cells (Gossler *et al.* 1989). It lacks any promoter element and has at its 5'end a splice acceptor site so that it has to integrate in an exon or in an intron downstream from an exon in order to be expressed. The idea here is to make a library of clones, each clone having a copy of the promoter-less lacZ integrated randomly in its genome, sometimes in active genes, rendering those clones βgal(+), and sometimes in inactive genes or junk DNA to give βgal(-) clones. Cells expressing lacZ and those not expressing lacZ are collected as two separate populations using the fluorescent vital βgal substrate FDG and fluorescence activated cell sorting (FACS) (Nolan et al. 1988). Both populations would then be challenged with the factor, e.g. FGF5, and then βgal-positive cells that had become negative and βgal-negative cells that had become positive would be selected in a second round of sorting, thus identifying FGF5 down- and up-regulated genes, respectively.

The number of clones needed in the library in order to have a 95% chance of the construct integrating into any one particular locus in the right orientation and frame is $5.5X10^6$ (ln [1 - probability] / ln [1 - size of locus/size

of genome] X reading frames X orientations = ln[1-0.95]/ln[1-$10^4/10^9$] X 3 X 2), assuming the average target size for a gene, including coding sequences and their intervening sequences, is 10^4 base pairs.

A library of $7X10^6$ clones was generated by a total of 25 electroporations each utilizing 40 μg of the genetrap construct and $5X10^7$ PSA EC cells. In the lacZ genetrap construct, there is a neomycin resistance gene linked to a strong promoter enabling one to select, in the presence of G418, for cells that have stably integrated the construct in their genomes. Each clone was represented by approximately 900 cells. All the clones were mixed together and aliquoted into 42 samples, which were stored at -80°C.

To determine how well the cells survive the FACS procedure, one aliquot of the library was thawed and grown for one passage before trypsinization and cell sorting. Two factors were found to decrease the viability of the cells: the amount of trypsinization before sorting can reduce viability by ~50%, and the sorting itself by another ~50%. It is essential that the cells be well trypsinized before sorting. Ideally, the sample to be sorted should be a suspension of single cells. Any aggregate of cells will be discarded in the sort to avoid sorting βgal(+) cells with βgal(-) cells and vice versa (also clumps of cells can clog the cell sorter). On the other hand, it is also important to minimize the trypsinization time to avoid affecting gene expression and the βgal levels in the cells. To maximize viability of the cells, the concentration of trypsin used to dissociate them was minimized and the sample was put through a sterile filter to remove the multicellular clumps. The second factor affecting viability of the cells is the sort itself, probably due to the hypotonic shock the cells must undergo to become loaded with the βgal substrate FDG. With milder trypsinization of the cells, the hypotonic shock was found to be

less detrimental, and in some experiments cell survival was as high as 40% after sorting and plating.

During FACS, several factors other than loss of viability contribute to the overall decrease in cell numbers (see Figure 1). The sorting parameters are set so that the βgal(-) cell population sorted is not contaminated with βgal(+) cells and the βgal(+) population contains a minimal number βgal(-) cells. Based on particle or cell size and on FITC fluorescence, which reflects levels of βgal, two windows are established for collecting the (+) and (-) cells. 23% of the cells fall between the levels of fluorescence considered distinctly (+) or (-) and are not collected. 70% of the remainder of the cells are discarded on the basis of size, presumably because they are in small aggregates of two or more cells. After plating the (-) cells, at best 10% of the cells in the initial sample, i.e. $\sim 10^7$ out of a starting population of 10^8 cells, survive and grow to give βgal(-) colonies. After plating the (+) cells, at best $\sim 10^6$ cells are recovered as colonies, and only 15% of these colonies are actually βgal(+) by X-gal staining (Figure 1).

This low percentage of βgal(+) colonies by X-gal staining is not completely unexpected. For some unknown reason, approximately one in a hundred EC cells fluorescences in the absence of any fluorescent substrate at the same wavelength as fluoroscein. Since βgal(+) cells are present only at a frequency of approximately one in a thousand, the background of false positives is very high (9 out of 10 fluorescing cells is a false positive before sorting). A second round of cell sorting would suffice to reduce the frequency of false positives to sufficiently low levels, as has been reported elsewhere (Reddy et al. 1992). The purified (+) and (-) cell populations could then be challenged with factor and sorted once again to identify clones in which the levels of expression of the lacZ gene has changed in response to the factor. However, the tremendous loss in cell numbers during each sort, which

represents a loss in clone numbers, make this strategy tedious at best for identifying sets of factor-responsive genes.

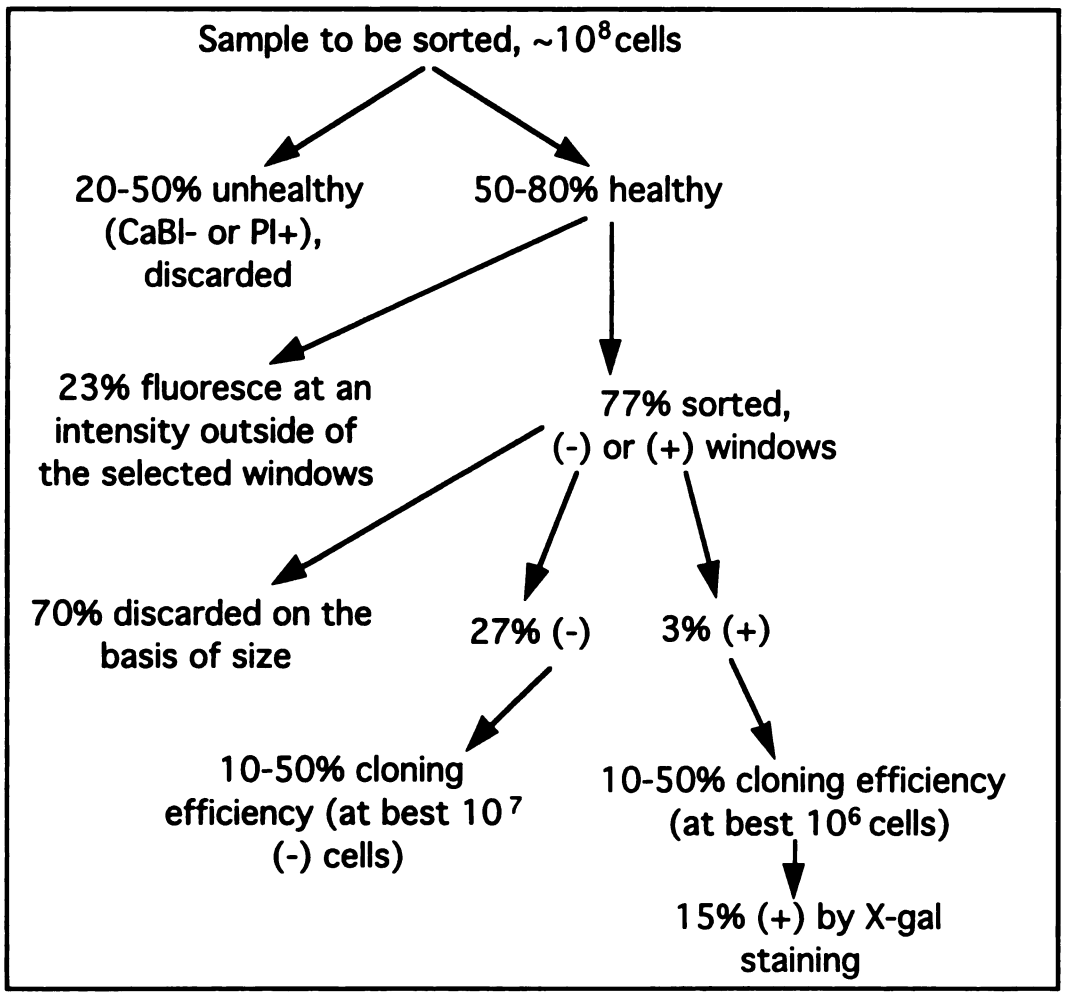

Figure 1. Loss of cells during FACS.

Using βgeo *as the genetrap marker*

The βgeo genetrap marker is similar to the lacZ marker described above in that it lacks any promoter element and has at its 5'end a splice acceptor site so that it will be expressed only when it integrates in an exon or in an intron

downstream from an exon (Friedrich and Soriano 1991). Unlike the lacZ construct, however, the βgeo construct does not include a second marker to select for stable integration. Instead, selection for integration employs βgeo itself, which when expressed confers neomycin resistance (as well as βgal activity). This difference is significant in several respects. First, the only clones selected are those in which βgeo has integrated into a gene that is at least minimally active and is therefore expressed. Using this approach, one cannot identify genes that are completely silent prior to exposure to factor, as one could using the lacZ construct. The first advantage of this approach is that each clone in the initial library represents an integration into a gene in the right orientation and frame, whereas with the lacZ construct the primary library consisted of random integrants. Thus the number of clones that need to be screened using the βgeo construct is much smaller. The second advantage is that there are no promoter elements in the βgeo construct that could potentially interfere with the promoter it integrates close to, whereas in the lacZ construct the accompanying neo^R gene has a promoter.

The level of expression of one gene can vary greatly from one cell type to another. Therefore, to detect changes in the level of expression of several genes in response to factor, the assay used must be capable of quantifying βgal activity over several orders of magnitude. The MUG assay was tested for this purpose (see Figure 2). Active βgal protein was diluted to different concentrations that reflect expected levels of expression in cells. Activity was measured using the MUG substrate in a 96-well fluorescence plate reader and was found to be linear over several orders of magnitude.

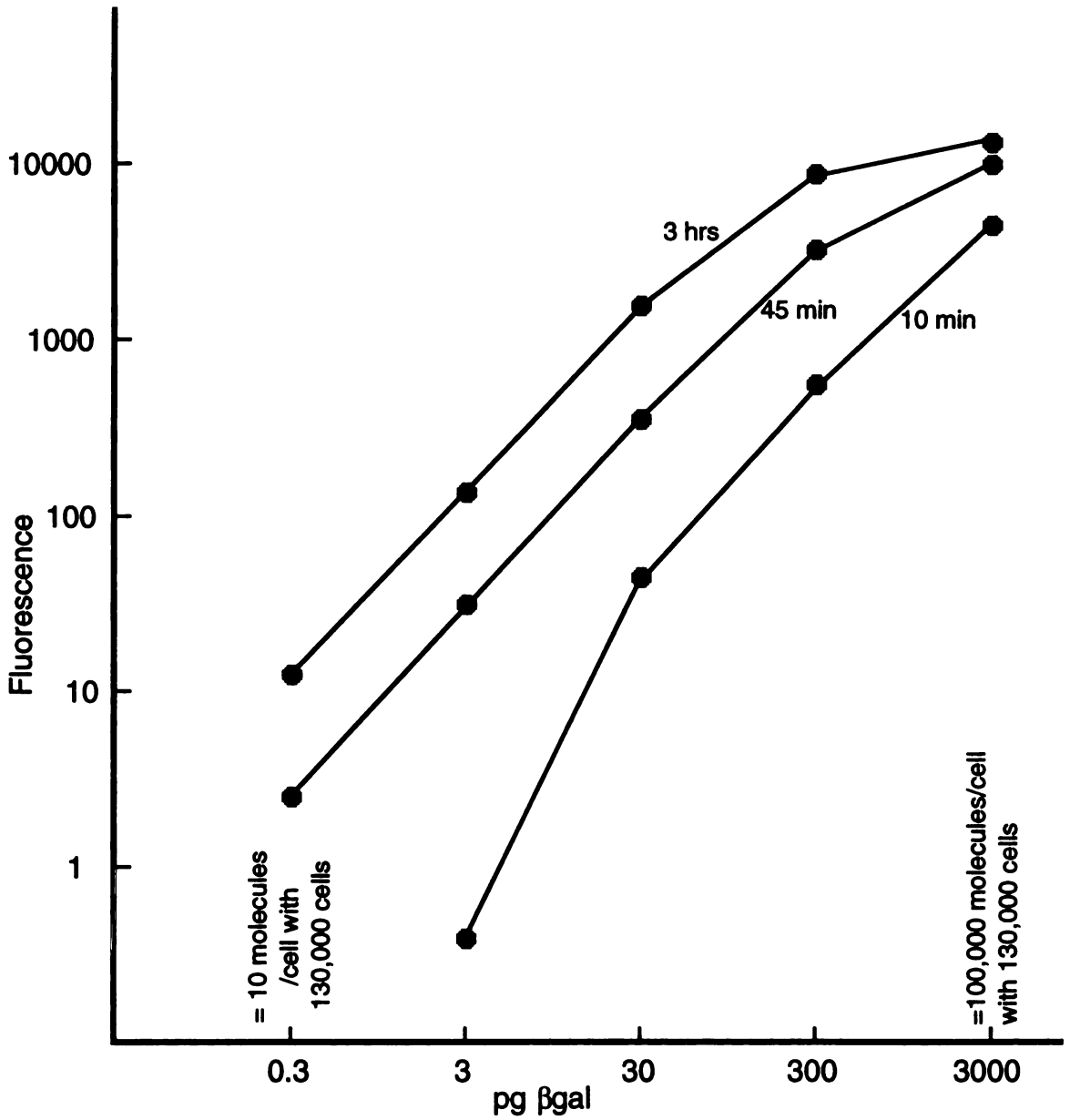

Figure 2. Test of the MUG assay after three given reaction times for concentrations of βgal that reflect possible cellular concentrations.

The βgeo construct was electroporated into J1 ES cells and 400 clones were isolated for analysis. After 2 days, the clones were passed in triplicate to 96-well dishes, two for the MUG assay (one with factor, one without) and the third for growth. The 'factor' used in these experiments was the removal of LIF, which is known to have an effect on ES cells - its removal promotes

differentiation. Four clones were identified that showed a greater than 10-fold decrease in the level of βgal activity in response to the removal of LIF. When retested, however, only two of the four were found to display a decrease in βgal activity in response to the removal of LIF. There are several possible reasons why two of the clones showed no response. ES cells will sometimes differentiate spontaneously in culture even in the presence of LIF. Differentiated cells would presumably no longer respond to the removal of LIF. Alternatively, the initial identification of the clones as responsive may have been incorrect due to a 10-fold difference in cell numbers between the untreated and treated samples. Such errors are possible in the primary screen since the samples are dealt with in large numbers and a pipetting error resulting in such a discrepancy would easily go undetected. Although this approach can be used to identify responsive genes, it would be greatly improved if each duplicate sample were normalized for total protein.

One of the two clones that showed a 10-fold decrease in βgal activity in response to the removal of LIF was further characterized. As anticipated, the decrease in βgal activity reflected a decrease in the level of βgeo encoding message, as determined by Northern blot analysis (Figure 3). The 5' region of this clone's βgal encoding cDNA, which contains sequences from the endogenous gene, was cloned using the RACE protocol and was sequenced (data not shown). The stretch of sequence from the endogenous gene did not show significant homology to any sequence in the Genbank database, and is probably 5' untranslated sequence. Expression of the wild type endogenous gene in response to the removal of LIF was examined by Northern blot analysis (Figure 3). Messages of several sizes were observed, at least two of which show a significant decrease in abundance upon removal of LIF, thus confirming that this gene is LIF-regulated.

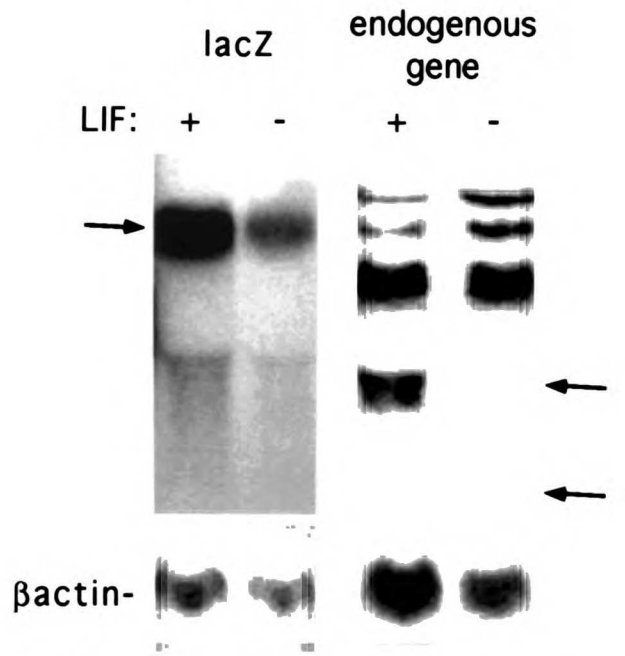

Figure 3. Northern Blot Analyses.

Discussion

In this chapter, two strategies have been assessed for identifying sets of genes that change their levels of expression in a cell type in response to a factor. Although both the FACS and the βgeo strategies, as well as very similar strategies used by other groups (Brenner et al. 1989; Kerr and Herzenberg 1991; Reddy et al. 1992), were shown to work for identifying very small numbers of factor-responsive genes, these approaches do not readily permit the identification of large numbers of such genes. Despite this current limitation, there are several advantages to these types of strategies over others such as subtraction hybridization or differential display PCR.

One advantage of a genetrap approach is that a library of genetrap clones can be of general use; the library can be challenged with not only one factor, but also with other factors in subsequent experiments. Furthermore, the factor does not have to be a secreted protein, it could be an intracellular protein (such as a transcription factor) introduced into the cells by transient transfection of the gene that encodes it. Once a clone is identified in which the genetrap marker's expression responds to a factor, this clone can then be challenged with other factors to determine how specific the response is or whether these other factors can inhibit the response. Moreover, if the cells can be introduced in vivo and are still competent to differentiate, expression of the marker in vivo can be used to confirm the relevance of the in vitro response (Reddy et al. 1992). Another potential advantage of a genetrap approach is the identification of genes expressed at low levels, since methods such as subtraction hybridization favors identification of abundantly expressed genes. Another advantage is that the genetrap marker, once

inserted into a responsive gene, provides the opportunity to study the mechanism by which the signal is transduced from the factor to the gene. This can be done using the genetrap marker by screening or selecting for mutant subclones that no longer transduce the signal. And finally, when using ES cells, it may be possible to determine the loss of function phenotype of the responsive genes in mice since they may have been inactivated by insertion of the marker (Friedrich and Soriano 1991; Reddy et al. 1992; Skarnes et al. 1992; von Melchner et al. 1992).

In principle, the only limiting factors that determine whether or not a responsive gene is identified by this approach are the sensitivity with which one can detect marker expression when the responsive gene is expressed at a low level, and how well changes in the level of expression of the marker can be quantified when these changes are small. In practice, however, several significant limitations exist. The first of these is the need for a cell line that is representative of the cells of interest in vivo. Although more and more cell lines are being generated, the cell line used must behave like the cells in the tissue from which it was derived in order to maximize the chances of identifying relevant responsive genes. Another problem concerning the identification of only those genes that are repressed by the factor is that although transcription of the marker gene may be turned off, the βgal protein (or fusion protein) may be stable for some time, making it more difficult to identify genes that are turned off.

Perhaps the greatest limitation to the strategies used so far is that only a small number of clones harboring trapped, responsive genes can be identified. To obtain a more complete picture of how a cell changes in response to a factor, many more responsive genes would need to be identified. Perhaps better selectable markers can be used to achieve this. Hprt and gpt are two

markers that can be both selected for and against. However, the use of these markers was avoided because of the "kiss of life/kiss of death" phenomena whereby cell contact can confer resistance or sensitivity to the drugs used in the selection. The use of fusion genes, such as herpes TK fused to neoR or other combinations, may be more successful. Alternatively, the use of plate imaging systems and fluorescent markers such as luciferase or the fluorescent green protein could be used to quantify expression directly in growing colonies.

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DISCUSSION

Several findings are presented in this thesis. The first of which is that *Fgf1, 2, 4,* and *5* appear to be expressed in a wide range of tissues throughout development, suggesting that the FGF gene family plays many roles in development (Chapter 1). This idea is further reinforced by more recent RNA in situ hybridization experiments showing the multitude of tissues in which all the different FGF genes are expressed (Niswander and Martin 1992; Han and Martin 1993; Mason et al. 1994). Furthermore, there are likely to be additional as yet unidentified family members. It should be noted, however, that despite the wealth of information on expression of these genes, and some clues as to their function in vivo from embryological manipulations, conclusive evidence for an in vivo function for an FGF gene in nearly all these cases is still lacking. The few exceptions include a role for *Fgf3* in ear development (Mansour et al. 1993), the involvement of FGF(s) in amphibian mesoderm formation (Amaya et al. 1991), and a role for *Fgf5* in the control of hair growth (Chapter 3).

The second finding reported here concerns the expression pattern of *Fgf5* in gastrulating embryos as well as in aggregated embryonic stem cells differentiated in culture (Chapter 2). The observation that *Fgf5* expression is induced hours before the start of gastrulation and ceases when gastrulation is largely complete, and that expression is restricted to the embryonic ectoderm and newly formed mesoderm and endoderm, clearly suggests a role for *Fgf5* in gastrulation. What that role might be, however, is not obvious. Speculations include a role in maintaining the mobility of the embryonic ectoderm cells since the timing of *Fgf5* expression correlates with the time when these cells are mobile (R. Pedersen, personal communication). In this

way, *Fgf5* could be indirectly involved in mesoderm formation by allowing the cells to move out of the ectoderm to become mesoderm. Alternatively, *Fgf5* may be needed to maintain the pluripotency of the embryonic ectoderm, since once *Fgf5* is turned off, the cells are committed to one of the three primary germ layers. Finally, because of its expression in a gradient late in gastrulation, *Fgf5* could be involved in assigning positional values to cells. The finding that expression was faithfully preserved in cultured ES cells differentiated in aggregates suggested that further study of *Fgf5* function could be undertaken using the advantages of a culture system.

The third main finding of this thesis is that mice lacking FGF5 develop into healthy, fertile adults with abnormally long hair. From this it can be concluded that *Fgf5* either has no function in gastrulation or development in general, or that its function(s) is(are) redundantly carried out by other factors. This latter possibility appears likely given that the expression of other FGF family members is known to overlap with that of *Fgf5* in at least some tissues. For instance, *Fgf4* and *Fgf6* are expressed in the somitic myotome at the same time as*Fgf5*. However, the overlap of expression of other FGF genes with that of *Fgf5* in gastrulation is not complete. *Fgf4* is expressed just prior to the start of gastrulation in the embryonic ectoderm as is *Fgf5*, but then their patterns diverge during gastrulation. Other FGF genes are also expressed during gastrulation, but their is little or no overlap with *Fgf5* in their pattern of expression. There may of course be other, as yet unidentified FGF genes (or even unrelated genes) whose expression would compensate for the absence of *Fgf5* expression. The question is also raised, and remains unanswered, as to whether or not there is any functional significance to the fact that several FGF genes are expressed during gastrulation, each with its own pattern of expression.

Other approaches have been used to investigate *Fgf5*'s roles in development. FGF5 was found to support the survival of a highly enriched population of embryonic motoneurons in culture and found to be present in embryonic muscle suggesting that it functions as a target-derived trophic factor for spinal motoneurons (Hughes et al. 1993). A possible function for *Fgf5* early in development in the embryonic ectoderm was studied using ES cells differentiated in culture (data not shown). However, neither overexpression of *Fgf5* under the control of the strong ubiquitous Elongation Factor 1 α promoter, nor addition of recombinant FGF5 protein (shown to be active in other FGF assays), nor the targeting of both alleles (using a *neo* insertion for one allele and a *hygro* one for the other) led to any readily detectable morphological phenotype in differentiating ES cells (data not shown). Levels of expression of cell lineage markers (T-gene and cardiac actin for mesoderm, and nestin for neuroectoderm) were also found to be unaffected by these manipulations (data not shown). However, in a collaboration with van der Kruijssen et al. (1994), addition of recombinant FGF5 protein to primitive ectoderm-like teratocarcinoma cells led to either a stimulation of growth or an inhibition of differentiation. Perhaps the expression of a dominant negative FGF receptor under the control of an appropriate tissue-specific promoter will be needed to obtain a phenotype in vivo that will provide insight into the role of FGF genes in gastrulation.

Despite efforts to raise anti-FGF5 mono- or polyclonal antibodies, no antibody was obtained that could detect FGF5 protein in vivo by immunohistochemistry. A polyclonal serum was obtained that could efficiently immunoprecipitate labeled FGF5 produced in COS cells, but the amounts of protein in differentiating ES cells is apparently too low for detection (data not shown). It thus remains formally possible that *Fgf5* has no

function in development (or in a subset of developmental tissues in which its mRNA is found) because its mRNA is not translated. Indirect evidence does exist for translational control of *Fgf5* expression (Bates et al. 1991). In these experiments, removal of two small open reading frames upstream of the *Fgf5* open reading frame lead to a considerable increase in translation in vitro.

One conclusion can be drawn with certainty about *Fgf5*'s function. Since mice deficient for *Fgf5* grow abnormally long hair throughout their lives, without any obvious morphological change in their hair follicles, a normal role for *Fgf5* is to regulate hair length by directly or indirectly inhibiting hair growth (Chapter 3). The classical mutation *angora*, which also leads to a long hair phenotype, was found to be a mutant allele of *Fgf5*. *Fgf5* was found to be expressed in the outer most layer of cells of the hair follicle, the outer root sheath, suggesting that it may carry out its function by signaling the progenitor cells of the hair itself, the hair matrix cells, to cease proliferating (Chapter 3).

The mechanism by which the FGF5 signal is transduced in its target cells is not entirely clear. Four genes have been identified that encode cell surface tyrosine kinase receptors for FGFs, indicating that at least part of the FGF signal is transduced through a kinase cascade (reviewed by Johnson and Williams 1992). FGF5 appears to bind to both FGFR1 and 2, although with a 10-fold lower affinity than does FGF2 (Clements et al. 1993); FGF5 does not appear to have any significant affinity for FGFR4 (Vainikka et al. 1992); and FGF5's affinity for FGFR3 is unknown. It should be noted, however, that each FGFR gene encodes multiple forms of receptors through alternative splicing, with different forms having different affinities for ligands (Johnson and Williams 1992), and that FGF5's affinity for each receptor has only been tested for a subset of forms. So it remains unknown which receptor(s) is(are) used by

FGF5 in vivo. FGF5 is known to bind heparin and is therefore thought to bind the low affinity heparan proteoglycan receptors in vivo. These low affinity receptors are thought to be needed to present the FGF ligand to its high affinity tyrosine kinase receptor. In addition, the FGF signal may yet be shown to be transduced through a pathway other than a kinase cascade. There is a growing body of literature demonstrating that at least some of the FGFs are translocated to the nucleus and bind DNA in a sequence specific manner (Imamura et al. 1990). No doubt the study of FGF signal transduction will lead to new insights into the biology of the cell. One thing has become clear: FGFs will be shown to play many more important roles in development.

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