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Distinct developmental expression of a new avian fibroblast growth factor receptor

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SUMMARY

We have cloned a new member of the fibroblast growth factor receptor family from avian embryonic RNA. The FREK (for fibroblast growth factor receptor-like embryonic kinase) primary transcript can be alternatively spliced in a tissue- and stage-specific manner to give rise to molecules containing either two or three Ig-like domains. During elongating primitive streak stages, FREK is expressed in the rostral and lateral epiblast and in the Hensen's node. From 2.5 days of development (E 2.5) on, it is expressed in various ectoderm- and mesoderm-derived structures. Most striking is FREK expression in the skeletal muscle lineage. It is highly expressed in the early myotome and, at later stages, in all skeletal muscles of the embryo. From E9 to hatching, FREK expression in the muscles decreases dramatically but is maintained in satellite cells of adult muscles. FREK transcript is elevated upon addition of basic fibroblast growth factor to serum-starved satellite cells. From this sudy, we conclude: (1) that the structure and pattern of expression of FREK set it apart from other cloned fibroblast growth factor receptors (FGFR) and suggest that FREK is a new member of that family; (2) that FREK may play multiple roles in early avian development, including a specialized role in the early differentiation of skeletal muscle.

Key words: fibroblast growth factor receptor, skeletal muscle, embryonic development, gastrulation, satellite cells

INTRODUCTION

Experimental evidence, mostly acquired from amphibian embryos, has shown that fibroblast growth factors (FGFs) and their receptors are likely to play decisive roles in early vertebrate development. Basic FGF (FGF-2) as well as related molecules were found able to induce presumptive ectodermal cells to differentiate into ventral mesoderm (Kimelman and Kirschner, 1987; Kimelman et al., 1988; Paterno et al., 1989; Isaacs et al., 1992). Moreover, the recent demonstration that inhibition of FGF receptor function in *Xenopus* embryos leads to abnormal development of posterior and lateral mesoderm (Amaya et al., 1991) supports the view that FGF receptors and their ligands are important in the normal process of mesoderm formation. The presence of FGF receptors at the time of mesoderm induction was demonstrated in the mouse embryo, thus suggesting that the FGF signaling pathway might play similar roles in higher vertebrates (Orr-Urtreger, 1991; Yamaguchi et al., 1992). In addition, the data available so far on the tissue distribution of FGFs and FGF receptor transcripts throughout the embryo has revealed complex spatiotemporal patterns of gene expression, suggesting that these molecules could have multiple functions during development. Identification of all the members of the FGF signaling pathway, as well as of the tissues and cell types expressing them is, therefore, important to understand the functions of these molecules during embryogenesis.

To date, four high affinity receptors for FGF have been identified. The sequence analysis of the first isolated FGF receptor (named FGFR1) has shown that it belongs to the tyrosine kinase receptor family (Lee et al., 1989, Pasquale and Singer, 1989). The prototypic FGFR is a transmembrane protein with 3 immunoglobulin (Ig)-like domains in the extracellular part of the molecule. An 'acid box' of 4-8 acidic residues is located between the first and the second Ig-like domain. The hydrophobic transmembrane domain is followed by a long juxtamembrane region, a tyrosine kinase domain, which is divided into two by a 14 amino acid insertion (the kinase insert) and a C-terminal 'tail'. The kinase insert has been shown to play an important role in the receptor to second messenger interactions (Jaye et al., 1992). The acid box as well as the short kinase insert are found only in the FGF receptors and are therefore considered to be the hallmarks of the members of this family. In addition to the prototypic molecule described above, multiple cDNA variants have been isolated. These include cDNAs that code for molecules with two Ig-like domains, variants with insertions/deletions in their extracellular or cytoplasmic portion, cDNAs encoding secreted forms of the

receptor and intracellular variants. They are produced by alternative splicing of the primary transcript (reviewed in Jaye et al., 1992). Although the function of these variant molecules has not been clearly established in vivo, some of them have been shown to generate receptors with different ligand-binding capacities in vitro (Werner et al., 1992). Binding studies have shown that the prototypic forms of FGF receptors 1, 2 and 3 bind with similar affinities FGF-1, FGF-2 and FGF-3 (Dionne et al., 1990; Keegan et al., 1991; Olwin et al., 1991). FGFR4 is the only receptor displaying different binding affinities to the FGF molecules (Vainikka et al., 1992). Finally, recent studies have shown that binding of FGFs to low affinity receptors, which are cell surface heparan sulfate proteoglycans, is required for their interaction with high affinity receptors (Yayon et al., 1991). To understand the complexity of the FGF/FGFR system and to establish the role of each of its components during embryonic development, careful biochemical and biological studies are therefore needed.

Recently, we have isolated, by PCR, a family of protein kinase gene fragments from quail and chick blastula, neural crest and neural tube mRNA (Marcelle and Eichmann, 1992). Sequence comparison of one of the clones, named H7, to the other members of the protein kinase family suggested that this molecule, isolated from chick blastula mRNA, is a new member of the FGFR family. Here, we report the cloning of a cDNA coding for H7 (FREK) and the analysis of its pattern of expression during embryonic development. We found that during gastrulation high levels of FREK expression are found in the epiblast, both in the embryonic and extraembryonic regions of the embryo. At later stages of development, FREK is expressed in various tissues. Most striking is its expression during muscle development: high FREK expression was found in the myotome and in the undifferentiated muscles of the embryo whereas almost no expression was found in the adult muscle. In post-hatched chick muscle, FREK was found to be expressed mainly in the satellite cells. In a satellite cell culture system, we found that FREK is regulated at the transcriptional level by FGF-2.

MATERIALS AND METHODS

Embryonic material

Experiments were performed on embryos of quail (*Coturnix coturnix japonica*) or White Leghorn chicken. Fertilized eggs were purchased from a commercial breeder and incubated at 38°C in a humid atmosphere. Chick embryos were used for northern blot analyses and for in situ hybridization at presomitic stages; embryonic stages were determined according to Hamburger and Hamilton (1951). For later stages, quail or chick embryos were used and staged according to the number of somites formed.

Isolation of cDNA clones, sequencing

We used two oligo (dT)-primed amplified cDNA libraries (Invitrogen) available in our laboratory. One library was prepared from poly(A)$^+$RNA isolated from E4 quail embryos and cloned into a pCDNA1 vector. The second library was prepared from poly(A)$^+$RNA isolated from E16 quail spinal cord and cloned into a pCDM8 vector. Approximately 1×10^6 recombinants from each library were screened on duplicate replica filters with the 200 bp H7 PCR fragment (Marcelle and Eichmann, 1992). Hybridization was performed at 42°C in buffer containing 50% formamide, followed by washing at 65°C in 0.5× SSC/0.1% SDS. Nucleic acid sequences were determined on both strands by the dideoxynucleotide chain termination method. Sequence comparison and calculation of amino acid homologies were performed with the GAP sequence alignment program available from Genetics Computer Group, Inc. (GCG).

RT-PCR

To isolate a probe specific for FREK first Ig domain, oligonucleotide A, located 3′ of the acid box (primer A, see below) was used to RT PCR amplify adult chicken liver total RNA, in combination with primer B, located in the putative leader sequences. A second round of amplification was done on the first PCR product. In this reaction, primer A was replaced by a primer located in the first Ig-like domain (primer C, see below). A single 250 bp band was amplified, cloned and sequenced. The sequence is shown in Fig. 2B. RT-PCR was performed essentially as described (Marcelle and Eichmann, 1992).

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primer A: 5′ CGGTGCTCTGTGCATATAGAC 3′
primer B: 5′ CGGCTGCTGCTGGCGGTC 3′.
primer C: 5′ GTGAAGTTGTGCAGGACT 3′.
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Northern blot analysis

Total RNA was isolated by the guanidinium-thiocyanate/acid phenol extraction method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ selection was done on oligo (dT)-cellulose prepacked columns (Pharmacia). 5 μg poly(A)$^+$RNA per lane were loaded on a 1% formaldehyde agarose gel, electrophoresed and blotted onto nitrocellulose membrane filters (Schleicher and Schuell) or onto nylon membrane filters (Genescreen Plus, New England Nuclear, Boston, Mass) as described (Maniatis et al., 1989). The filters were hybridized to a 1265 bp *Pst*I-*Pst*I ^{32}P-labeled probe encompassing a portion of the extracellular and most of the intracellular domains of the molecule (see Fig. 1A).

In situ hybridization

Paraffin sections (5 μm) of quail or chick embryos, fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) were prepared. The procedures applied for section treatment, hybridization and washing were those of Wakamatsu and Kondoh (1990), modified as already described (Eichmann et al., 1993). A 1265 bp *Pst*I-*Pst*I FREK cDNA fragment (see Fig. 1) cloned into pGEM3z vector (Promega), was used to prepare antisense and sense RNA probes.

Antibody staining

For antibody staining, the sections were deparaffined, rehydrated and transferred sequentially to PBS containing 0.03% H_2O_2 and PBS containing 2% new-born calf serum for 20 minutes each. Monoclonal IgM 13F4 ascites fluid (Rong et al., 1987) was diluted 500-fold in PBS and applied to sections overnight at 4°C. After washing in PBS, a horseradish peroxidase coupled secondary anti-mouse Ig antibody (Nordic Immunological Laboratories) at a 1:200 dilution in PBS was applied to the sections for 1 hour at room temperature. Staining was revealed with diaminobenzidinetetrahydrochloride (Sigma) at 1% in PBS. The reaction was started by the addition of 0.01% H_2O_2. The sections were then counterstained with hematoxylin, dehydrated and mounted.

Muscle satellite cell preparation

Chicken skeletal muscle satellite cells were isolated from the pectoral muscle of 5- to 7-day-old chicks as already described (Allen et al.,1984; Duclos et al., 1991), with some modifications (Halevy and Lerman, 1993). For FGF-2 induction experiments, cells were plated on 0.1% gelatin-coated plates and maintained for 24 hours in DMEM containing 10% fetal calf serum, 10% horse serum and 0.5% chicken embryo extract. The medium was switched to medium containing 0.02% FCS for 4 hours. 1 ng/ml FGF-2 (provided by G. Neufeld, Technion, Haifa, Israel) was then added to the cultures.

RESULTS

Cloning of a FREK cDNA

We have previously shown by northern blot analysis that a 200 bp H7 PCR clone specific for a portion of the tyrosine kinase domain hybridizes to a 3.1 kb transcript present in various chicken adult tissue mRNAs (Marcelle and Eichmann, 1992). To isolate a full-size FREK cDNA clone, we screened an E4 quail embryo cDNA library with the H7 probe (see Fig. 1B). We obtained multiple cDNA clones, which were further characterized. Fragments of one of the longest clones were subcloned into the pGem3Z vector and sequenced on both strands (Fig. 1A). The 2.5 kb cDNA of FREK and its deduced amino acid sequence are presented in Fig. 1A. We identified along the sequence the prototypic features of FGF receptors containing two Ig-like domains. The sequence starts with a stretch of hydrophobic residues, corresponding to the leader peptide. This is followed by five acidic residues, which correspond to the acid box. The extracellular portion of the molecule contains the consensus sequences for two Ig-like domains (Williams and Barclay, 1988). Five potential N-linked glycosylation sites are found in the extracellular domain of the molecule. The transmembrane domain is 21 amino acids long. The cytoplasmic domain starts with a 74 amino acid long juxtamembranal domain followed by the tyrosine kinase domain, split into two by a 14 amino acid kinase insert. The tyrosine kinase domain contains the consensus sequences found in all the molecules of this family, in particular the GXGXXG consensus ATP binding site. The H7 PCR sequences used as a probe to isolate the cDNA clone are present within tyrosine kinase domain II (see Fig. 1B). The molecule ends with a 56 amino-acid long C-terminal tail. We did not find a poly(A) signal or a poly(A) tail in the 408 bp 3′ untranslated region indicating that this cDNA clone is not complete at the 3′ end.

Isolation of sequences encoding the first Ig-like domain

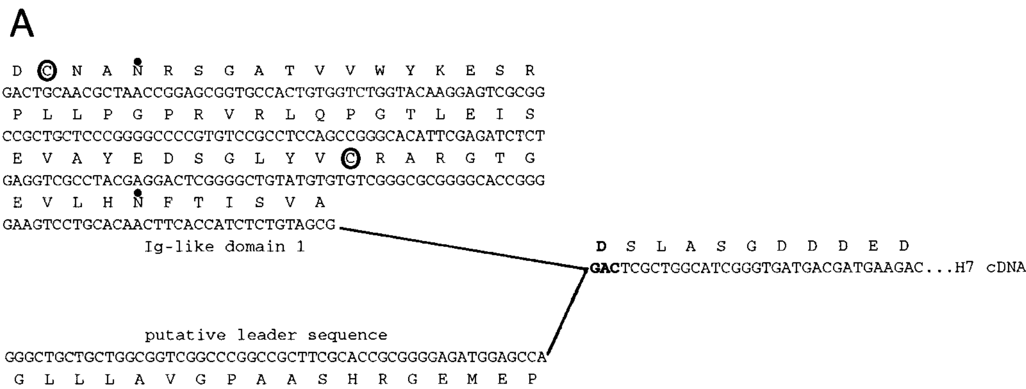
To isolate sequences specific for the first Ig-like domain, we screened an E16 quail spinal cord cDNA library with a 100 bp fragment corresponding to the most 5′ sequences of FREK cDNA described above (probe B, Fig. 1B). Two clones were isolated; sequence analysis showed

A

B

PCR
B A PROBES
Ig-2 Ig-3 TM TK I KI TK II
R P P P

Fig. 1. (A) Nucleic acid and derived amino acid sequences of the FREK quail cDNA clone. Open box, 5 residue-long acid box; shaded box, transmembrane domain. Cysteines of the extracellular domain are circled. Potential N-glycosylation sites are indicated by a dot. Arrows indicate the beginning and the end of the tyrosine kinase domain. Asterisks show the ATP-binding site. The 14 residue-long kinase insert is indicated by ~.
(B) Schematic representation of the FREK cDNA clone. The cDNA probes A and B used in these study (see text) are indicated.The H7 PCR clone used to screen the library is shown (PCR). Shaded box indicates the signal peptide. Ig-2 and Ig-3, immunoglobulin-like domains 2 and 3; TM, transmembrane domain; TKI and TKII, tyrosine kinase domains 1 and 2; KI, Kinase insert; R, *Rsa*I restriction site; P, *Pst*I restriction site. Stop codon is indicated by an arrow.



B

Ig-like domain 1

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Chicken    EEEHLLLDPGNALKLYCDVNQSGSASVVWYKESRPLLP
Quail      ----------------.NA.R...T............

Chicken    GPRVRLQQMRLEIAEVATEDSGLYVCRARGTGEVLHNF
Quail      .......PGT...S........................
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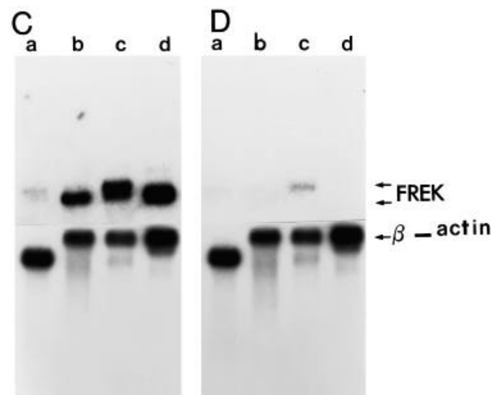


Fig. 2. (A) Alternative splicing of FREK signal peptide to the second Immunoglobulin-like domain. Shown are the nucleic acid and derived amino acid sequences of a FREK cDNA clone coding for the first Ig-like domain, isolated from a quail spinal cord cDNA library. An aspartic acid (in bold) indicates the splicing site of the putative leader sequences to the rest of the molecule. (B) Comparison of the amino acid sequences of the first Ig-like domain amplified by RT PCR from chicken liver RNA to the corresponding quail sequences. (C,D) Stage- and tissue-specific expression of the FREK transcript coding for two and three Ig-like domains. Poly(A)$^+$ RNA from chick adult muscle (a), ovary (b), whole E13 (c) and E4 embryo (d) were hybridized to a FREK probe encompassing most of the FREK sequences (C) or to a probe specific of the first Ig-like domain (D).

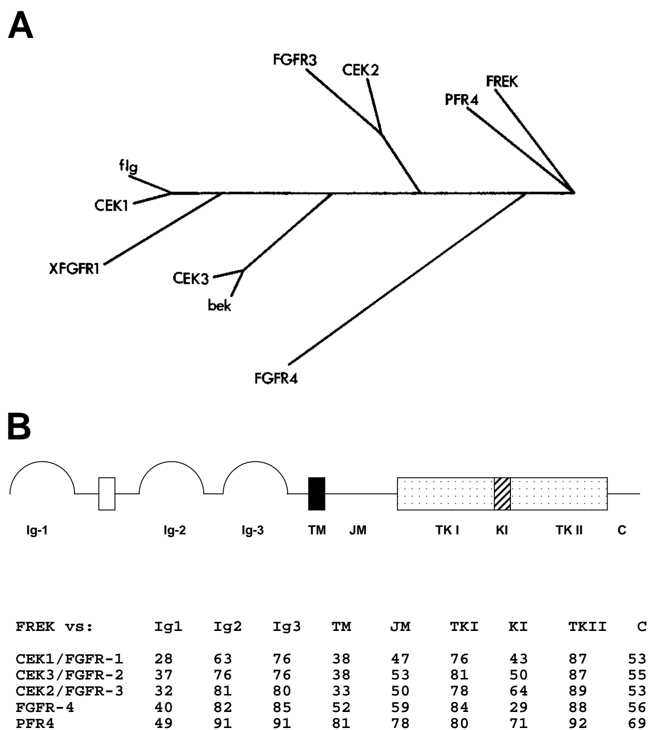
that one of the two contained at its 5′ end 200 bp of new sequences. The nucleic and deduced amino acid sequences of these additional 200 bp sequences are shown in Fig. 2A. They correspond to a sequence encoding the entire first Ig-like domain. Two potential N-linked glycosylation sites are found along the amino acid sequence. Fig. 2A shows that the sequence divergence between the two clones is located six amino acids upstream of the acid box at a position (an aspartic acid) which has already been identified in other FGF receptors as the splice site for the signal peptide sequence to the second Ig-like domain (Hou et al., 1991; Johnson et al., 1990; Eisemann et al., 1991). Together, these results indicate that the sequences corresponding to the first Ig-like domain contained in the FREK primary transcript can be spliced out to give rise to a molecule containing two Ig-like domains.

Distribution of FREK transcripts coding for two and three Ig-like domains

To identify transcripts coding for molecules containing two and three Ig-like domains, we prepared two cDNA probes. The first probe is a 1265 bp *Pst*I-*Pst*I fragment encompassing the third Ig-like domain, the transmembrane domain and the tyrosine kinase domain (probe A, see Fig. 1B). This probe will therefore hybridize to both forms of the molecule (and similarly to chicken and quail mRNA, unpublished observation). A second probe specific for the first Ig-like domain was isolated by RT PCR amplification of chicken liver RNA (see Material and Methods). The amplified sequence displays high homology to that obtained from the quail cDNA library (81% at the nucleic acid level in the overlapping region). We used this clone as a probe specific for the first Ig-like domain. We prepared poly(A)$^+$ RNAs from whole chick embryos at E4 and E13 and from adult ovary and muscle tissues. With probe A, a transcript of around 3.1 kb was found in all tissues. In E13 and adult muscle mRNAs, we detect an additional, slightly larger transcript of around 3.3 kb (Fig. 2C). With the 250 bp PCR probe specific for the first Ig-like domain, only the 3.3 kb transcript is detected in E13 mRNA (Fig. 2D) and in adult muscle mRNA after a longer exposure (not shown). This result provides direct evidence for a stage- and tissue-specific expression of a longer and a shorter form of the FREK transcript.

Sequence comparison of FREK to other FGF receptors

Sequence comparison of FREK to other members of the FGFR family in the NBRF data bank showed that FREK is closely related (80% homology at the amino acid level) to a FGF receptor recently isolated from a *Pleurodeles* tail bud cDNA library, PFR4 (Shi et al., 1992). A phylogenic tree was constructed with the amino acid sequences of the three Ig-like containing form of FREK and the corresponding regions of some of the members of the FGFR family isolated from various species (Fig. 3A). The tree illustrates the close relationship between FREK and the *Pleurodeles* cDNA clone PFR4. The overall homology observed between FREK and PFR4 is similar to that of chicken FGFR1 (CEK1) to its *Xenopus* homologue, XFGFR1 (80%. Lee et al., 1989; Pasquale and



FREK vs:	Ig1	Ig2	Ig3	TM	JM	TKI	KI	TKII	C
CEK1/FGFR-1	28	63	76	38	47	76	43	87	53
CEK3/FGFR-2	37	76	76	38	53	81	50	87	55
CEK2/FGFR-3	32	81	80	33	50	78	64	89	53
FGFR-4	40	82	85	52	59	84	29	88	56
PFR4	49	91	91	81	78	80	71	92	69

Fig. 3. (A) Phylogenic tree illustrating the relationship of the FREK cDNA clone to the other members of the FGF receptor family. The amino sequences of the molecules shown in this tree were aligned using a CLUSTAL V multiple alignment program. Based on this alignment, a similarity score matrix (mutation matrix of Dayhoff) was calculated. The phylogenic tree was then constructed according to the least squares method of Fitch and Margoliash. References of the sequences used to build the tree: FGFR1, murine/flg: Safran et al., 1990; chicken/CEK1: Pasquale and Singer, 1989; Xenopus/XFGFR1: Friesel and Dawid, 1991. FGFR2: human/bek: Dionne et al., 1990; chicken/CEK3: Pasquale, 1990. FGFR3: human/FGFR3: Keegan et al., 1991; chicken/CEK2: Pasquale, 1990. FGFR4: human/FGFR4: Partanen et al., 1991. PFR4: Shi et al., 1992. (B) Comparison, domain by domain, of the FREK amino acid sequences to the corresponding sequences of chicken FGFR1, 2 and 3, human FGFR4 and the PFR4 cDNA clone isolated from *Pleurodeles* (references in text). Numbers indicate the percentage of identical amino acids. Domains are indicated in a schematic representation of a prototypic FGF receptor molecule. Ig-1,Ig-2,Ig-3, immunoglobulin-like domains 1 to 3; TM, transmembrane domain; JM, Juxtamembrane domain; TKI and TKII, tyrosine kinase domain 1 and 2; KI, kinase insert; C, carboxy terminus. Open box: acid box.

Singer, 1989; Friesel and Dawid, 1991) or that of chicken FGFR2 (CEK3) to its *Xenopus* homologue, XFGFR2 (78%. Pasquale, 1990; Friesel and Brown, 1992). The homologies of FREK and PFR4 to human FGFR4 are 72% and 68%, respectively; to human FGFR3: 68% and 66%, respectively; to human FGFR2; 67% and 61%, respectively; to human FGFR1: 64% and 62%, respectively.

A comparison of FREK was made, domain by domain, with the corresponding sequences of the three cloned chicken FGFRs, the human FGFR4 and the amphibian PFR4 molecule. Fig. 3B shows that, in six out of seven portions of the molecule, FREK is most closely related to PFR4. Moreover, the homology of FREK to PFR4 is much higher than to the other FGFRs in the regions of the molecule which are the least conserved between members of the FGFR family (i.e. the first Ig-like domain, the transmembrane and juxtamembrane domains, the kinase insert and the C-terminus). The juxtamembrane domain, the kinase insert and the C terminus have been shown in other growth factor receptors to define substrate specificity (Schlessinger and Ullrich, 1992; Cantley et al., 1991 for reviews). The high degree of homology detected in these regions between PFR4 and FREK might reflect similar receptor interactions with second messenger molecules. Together, the sequence comparisons described above suggest that FREK and PFR4 are homologous molecules.

Expression pattern of FREK in the developing embryo

Early developmental stages (HH2-HH6)

The in situ hybridizations described in the following sections were performed with a 1265 bp antisense riboprobe (probe A, see Fig. 1B), which encompasses a portion of the third Ig-like domain, the transmembrane domain and most of the cytoplasmic region of the molecule. Alternate sections hybridized to a FREK sense probe did not show any significant signal (not shown).

During gastrulation, cells of the epiblast are invaginating through the primitive streak and the Hensen's node to form the mesoderm and the endoderm. At the extending primitive streak stages (HH2-HH4), high levels of FREK expression are seen in the entire epiblast anterior to Hensen's node (Fig. 4A). At the level of the node itself, FREK transcription is restricted to the lateral regions of the epiblast and the ectoderm of the area opaca. High levels of FREK transcription are detected in the node, except for its superficial layer (Fig. 4B). More caudally, expression of FREK is detected neither in mesodermal cells, nor in the endoderm (Fig. 4C). From stage HH4 on, the primitive streak is fully extended while the notocord and neural plate are being formed. At this stage, the cells in the Hensen's node do not express FREK any more (not shown).

In a stage HH6 embryo, the overall level of FREK transcription has decreased. A significant level of transcripts is however still present in the ectoderm of the area opaca (Fig. 4D) and at the level of the head process (not shown). No FREK expression is found in the extraembryonic and intraembryonic mesoderm (Fig. 4D). From the one somite stage (HH7), (about E1), until around E2.5, no FREK transcript could be detected in the developing embryo (not shown).

FREK expression in E2.5 days to E9 embryos

Ectoderm-derived structures

After about 60 hours of incubation, when the embryo reaches the stage HH17, a strong labelling is observed in two ectoderm-derived structures, the lens and the developing trigeminal ganglion. Labeling of the lens is restricted to the internal part of the early lens vesicle (Fig. 5A,B). At later stages (E4 and E6), it persists in the germinative zone, where cell proliferation mostly occurs and in the transition zone where cells elongate and differentiate into fibers (McAvoy et al., 1991. Fig. 5C,D).

During formation of the trigeminal ganglion, cells emigrating from the trigeminal placode yield the neurons of the distal portion of the trigeminal sensory ganglion. The neural crest provides the neurons of the proximal region and the glial cells of the whole ganglion (Ayer-Lelièvre and Le Douarin, 1983; D'Amico-Martel and Noden, 1983). Fig. 5E and F show that both the placode and the placode-derived neuroblasts are

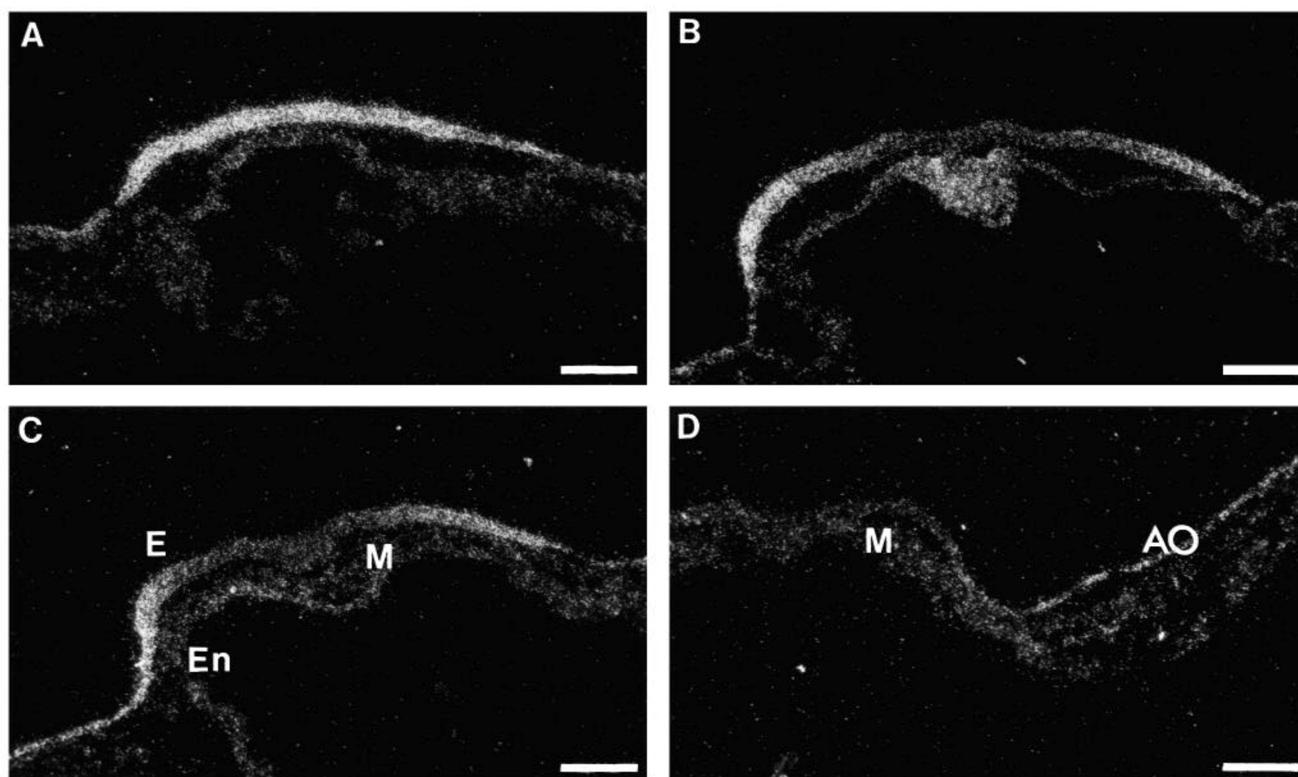


Fig. 4. Expression of the FREK mRNA in gastrulating chick embryos. Transverse sections of chick embryos at the extending primitive streak stage (stage 3+ of Hamburger and Hamilton, A-C) and at E1 (stage HH6, D) were hybridized to a FREK antisense RNA probe. (A) Transverse section anterior to Hensen's node; (B) section through Hensen's node; (C,D) sections at the mid-primitive streak level. Scale bars: 100 μm. E, ectoderm; M, mesoderm; En, endoderm; AO, area opaca.

expressing FREK RNA, while the crest-derived proximal portion of the ganglion is not labeled. We did not detect labeling of any other placode of the cranial peripheral ganglia. Transcription of FREK in the distal part of the trigeminal ganglion is transient: at E4 labeling of this structure is no longer observed (not shown).

Mesoderm-derived structures

Three mesoderm-derived structures express FREK transcripts during early embryonic development: kidney, cartilage and striated muscles. From E4, and at least up to E6, the mesonephritic tubules express high levels of FREK transcript, whereas the proximal nephrogenous region and the gonadal ridge do not display any labeling (Fig. 6C).

At E5, chondroblasts differentiating within the condensed sclerotome express low levels of FREK transcript. Fig. 6C shows FREK expression in the cartilage primordia forming around the notocord. Staining of the chondromucoids of the hyaline matrix with alcian blue confirmed that FREK message is within the cartilage primordia (not shown). With time, all the differentiating cartilaginous cells start expressing high levels of FREK transcript. At E9, longitudinal sections of long bones display successive stages of cartilage formation. Fig. 6D,E shows that FREK expression decreases with chondroblast differentiation: high expression is seen in the early chondroblast, while it is no longer detected in hypertrophied cartilage. It should be noted that the membrane bones of the skull do not express FREK (not shown).

From E2.5 onward, FREK is expressed in the skeletal muscle lineage of the developing embryo. In a 30-somite embryo, the myotomes express low levels of FREK transcript in the cervicotruncal region (not shown). Expression rapidly increases with age. At E4, high transcription levels of FREK are observed in the myotomal cells extending ventrally to form the future body wall muscles (Fig. 6A-C), in the mesodermal cells that condense to form the premuscle masses of the limb, as well as in the mesodermal cells that will form the oculomotor muscles in the head (not shown). Some labelling can be detected in the neural tube and the dorsal root ganglion at this stage (Fig. 6A,B). At these stages of development, FREK labeling in the somites and in the limb parallels that of 13F4, a monoclonal antibody prepared in our laboratory specific for early avian myogenic cells (Rong et al., 1987). 13F4 has been shown to stain cardiac, smooth and skeletal muscles from early developmental stages on, as well as adult muscles (Rong et al., 1987). Alternate sections through the truncal region of a E9 embryo were labeled with the FREK probe and the 13F4 antibody (Fig. 6D,E). The 13F4 antibody stains cardiac muscle, smooth muscle of the oesophagus and myotome-derived skeletal muscle. In contrast, FREK is expressed exclusively in myotome-derived skeletal muscles (i.e. pectoral, vertebral and limb musculature).

FREK expression in differentiating and adult striated muscles

We analyzed FREK expression during muscle differentiation

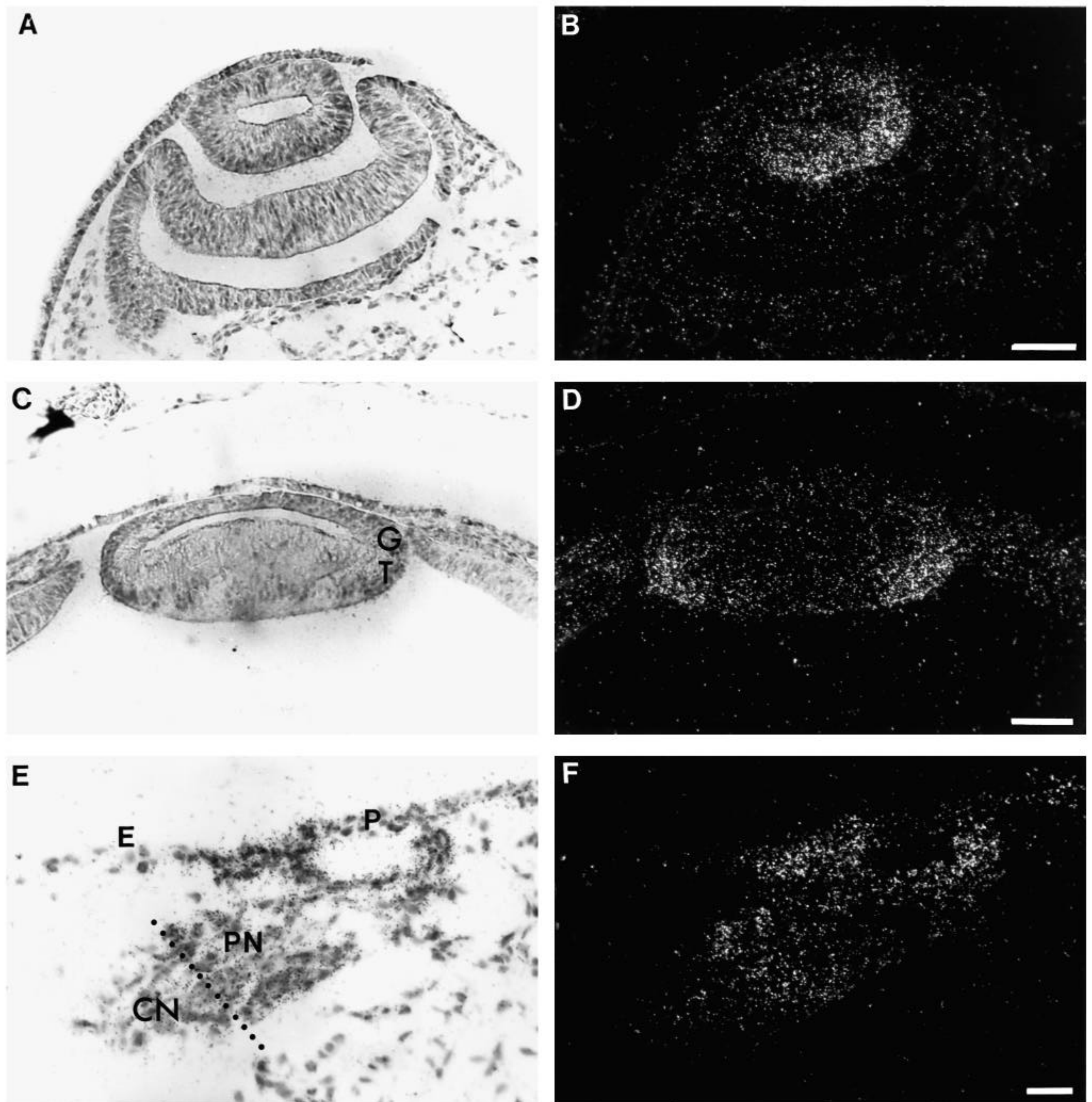


Fig. 5. Expression of the FREK mRNA in ectoderm-derived structures of the chick embryo. (A,B) Developing lens of an E2.5 embryo; (C,D) lens of an E4 embryo; (E,F) expression in the placode and the placode-derived neuroblasts of the trigeminal ganglion. Scale bars, 50 μm in A-D and 20 μm in E and F. E, ectoderm; P, placode; PN, placode-derived neuroblasts; CN, crest-derived neuroblasts; G, germinative zone; T, transition zone.

by in situ hybridization on E9, E12 and E15 quail limbs. Fig. 7AC shows a dramatic decrease in FREK expression with the differentiation of myoblasts and their fusion into myotubes. In E15 embryonic muscle, FREK hybridization seems to become restricted to a few positive cells, while multinucleated myotubes are negative (Fig. 7C). We further documented the decrease of FREK expression in muscle tissues by northern blot analysis. Total RNA of E9, E12, E15 chicken pectoral muscles and new-born quail leg muscle were hybridized to the 1265 bp probe described above. Fig. 7D shows that FREK expression in the muscle decreases from a high to an undetectable level of expression between E9 and hatching, thereby confirming the in situ hybridization data.

The residual FREK expression detected in adult poly(A)$^+$ mRNA (Marcelle and Eichmann, 1992), as well as its restricted expression to few cells at the periphery of myotubes suggested that the remaining FREK-positive cells could be the satellite cells. Satellite cells are the only embryonic-like cells in the adult muscle that can undergo proliferation and terminal differentiation towards myotubes. This occurs notably in the case of

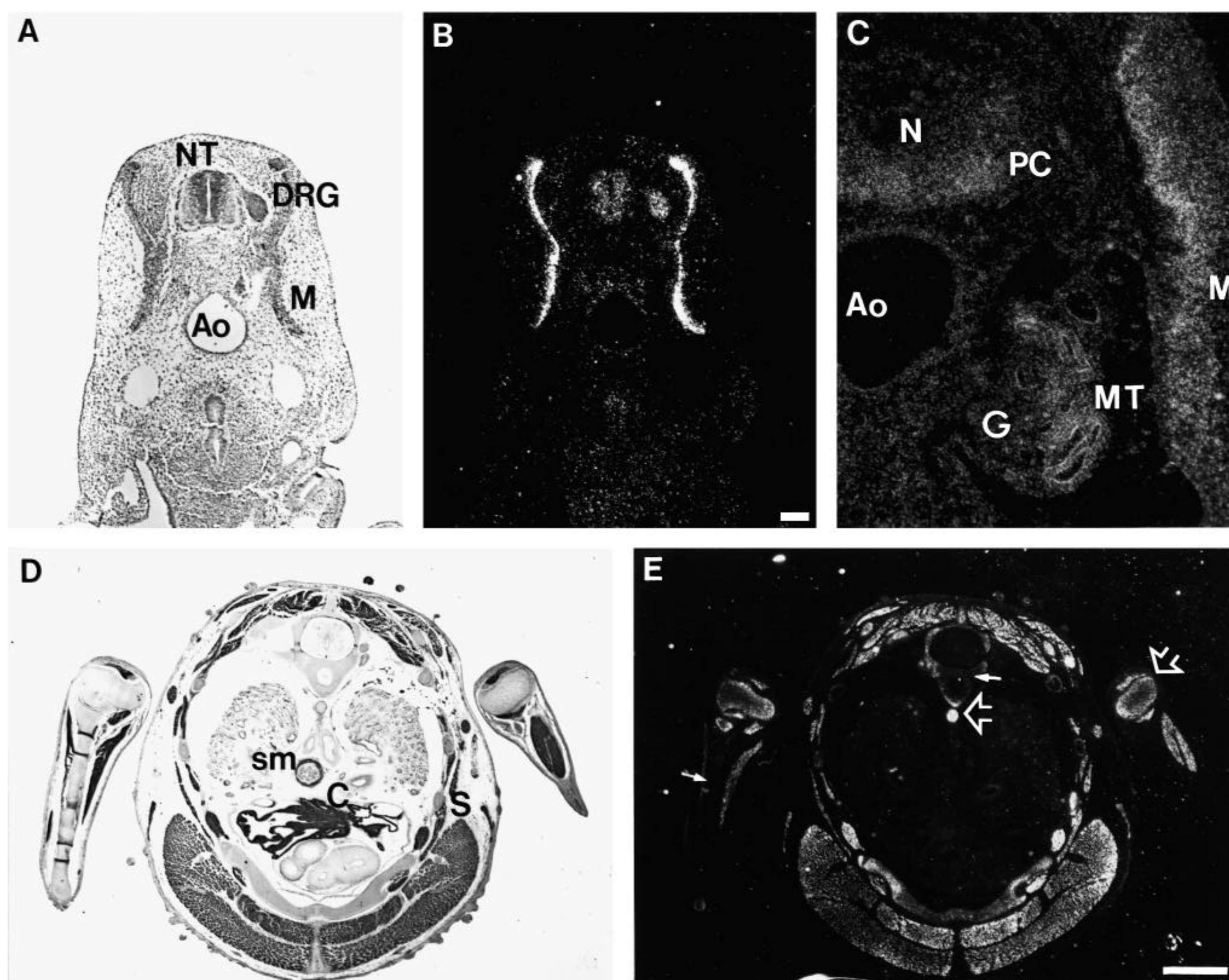


Fig. 6. Expression of the FREK mRNA in mesoderm-derived structures of the chick embryo. (A,B) FREK expression in an E4 chick embryo; (C) expression in an E5 embryo; (D) staining of an E9 embryo with the muscle-specific monoclonal antibody 13F4, which stains skeletal (S), cardiac (C) and smooth muscle (Sm) cells. (E) FREK expression in the skeletal musculature of the same embryo. FREK is expressed in all skeletal muscles and in the early chondroblasts of the forming bones (big arrows), while it is not expressed in hypertrophied cartilage (small arrows). Scale bars: 100 μm in A, B and C and 1 mm in D and E. NT, neural tube; N, notocord; PC, precartilagenous tissue; M, myotome; AO, aorta; MT, mesonephritic tubule; DRG, dorsal root ganglion; G, gonadal ridge.

muscle hypertrophy and in muscle repair following injury. FREK expression was analyzed in avian muscle satellite cells. A northern blot with total RNA isolated from satellite cells and from whole muscle of 5-day-old chick was hybridized to the 1265 bp *Pst*I-*Pst*I fragment described above. Fig. 8A shows that FREK mRNA expression is approximately tenfold higher in satellite cells as compared to muscle tissue, which contains predominantly differentiated myotubes.

Basic and acidic FGFs (FGF-2 and FGF-1) have been known to play a key role during proliferation and differentiation of primary cultures of presumptive skeletal cells as well as of muscle cell lines (Gospodarowicz et al., 1976; Allen et al., 1984; Clegg et al., 1987; Seed and Hauschka, 1988). We analyzed the effect of FGF-2 on FREK expression using a satellite cell culture system. FGF-2 at the concentration of 1 ng/ml was added to serum-starved satellite myoblast cultures and the time course of FREK mRNA expression was analyzed by northern blot analysis. FGF-2 induced FREK mRNA expression within 2 hours of incubation in the presence of this factor (Fig. 8B). FREK mRNA expression continues to increase as the incubation proceeds until after 24 hours of exposure to FGF-2.

DISCUSSION

In this report, we present the molecular cloning and the pattern of expression of a new avian FGF receptor-like cDNA clone. We have shown in a tissue culture system that FREK transcription level is quickly upregulated by the addition of FGF-2 to the culture medium. This supports the contention that FREK is active as a receptor for FGF-like molecules.

The sequence comparison of FREK to other cloned FGF receptors has shown that FREK displays a high degree of homology to PFR4, another FGF receptor-like cDNA recently cloned from amphibian tail bud stage embryos (Shi et al.,

1992). The extensive homologies between different domains of the two molecules suggests that FREK and PFR4 are the avian and amphibian homologues of the same FGF receptor molecule. Further studies on the comparison of the patterns of expression of these two molecules should clarify their identity. FREK is related to a lesser extent to the other FGF receptors. Chicken homologues of the mammalian FGF receptor 1, 2 and 3 have been cloned previously. A possibility remains as to whether FREK would correspond to the avian homologue of FGFR4. The overall homology of FREK and FGFR4 (72%) is lower than the homologies that have been observed between the chick and human FGFR1 (92%), the chicken and human FGFR2 (93%) and the chicken and human FGFR3 (81%). As a comparison, two unrelated FGFRs, the chicken FGFR1/CEK1 and the human FGFR1/flg are themselves 74% homologous. In addition to the structural differences outlined above, we have observed differences between the expression patterns of FREK and FGFR4 both in adult and embryonic life. In adults, FREK is mostly expressed in liver and kidney and not at all in lung (Marcelle and Eichmann, 1992), whereas FGFR4 is expressed in liver and lung and not at all in kidney (Stark et al., 1991). During embryonic life, FGFR4 is not expressed during gastrulation, but is highly expressed in the endodermal lineage from early embryonic stages on (Korhonen et al., 1992; Stark et al., 1991). In contrary, FREK is highly expressed during gastrulation, and no FREK expression is seen in the endoderm at stages equivalent to those where FGFR4 expression was observed. This indicates that FREK and FGFR4 play different roles during adult life and embryonic development and suggests therefore that they are distinct molecules. Unequivocal demonstration that FREK is a fifth member of the FGF receptor family will await the cloning of an avian FGFR4 and/or that of a mammalian FREK.

We have shown that the FREK gene gives rise to molecules containing two or three Ig-like domains by alternative splicing of the primary transcript. Similar variants have already been described for FGFR1 and 2. Although the role of these variants in vivo has not yet been established, in vitro binding studies have shown that they have similar ligand specificities (reviewed in Jaye et al., 1992). In the present study, we show by northern blot analysis that the two forms of FREK have distinct patterns of expression, which suggests that they might have different roles in vivo.

Expression of FREK during embryonic development is not restricted to a particular cell type or lineage. Rather, FREK displays a wide distribution in various ectoderm- and mesoderm-derived structures. In most of these structures (myoblasts, chondrocytes...), FREK is expressed transiently and at an early stage of cell differentiation. FREK expression therefore coincides with a proliferative and undifferentiated state of various ectomesodermal cells.

At early embryonic stages, very high levels of FREK expression are found in the ectoblast, rostral and lateral to the extending primitive streak. The region of the epiblast where FREK is expressed corresponds to an embryonic territory from

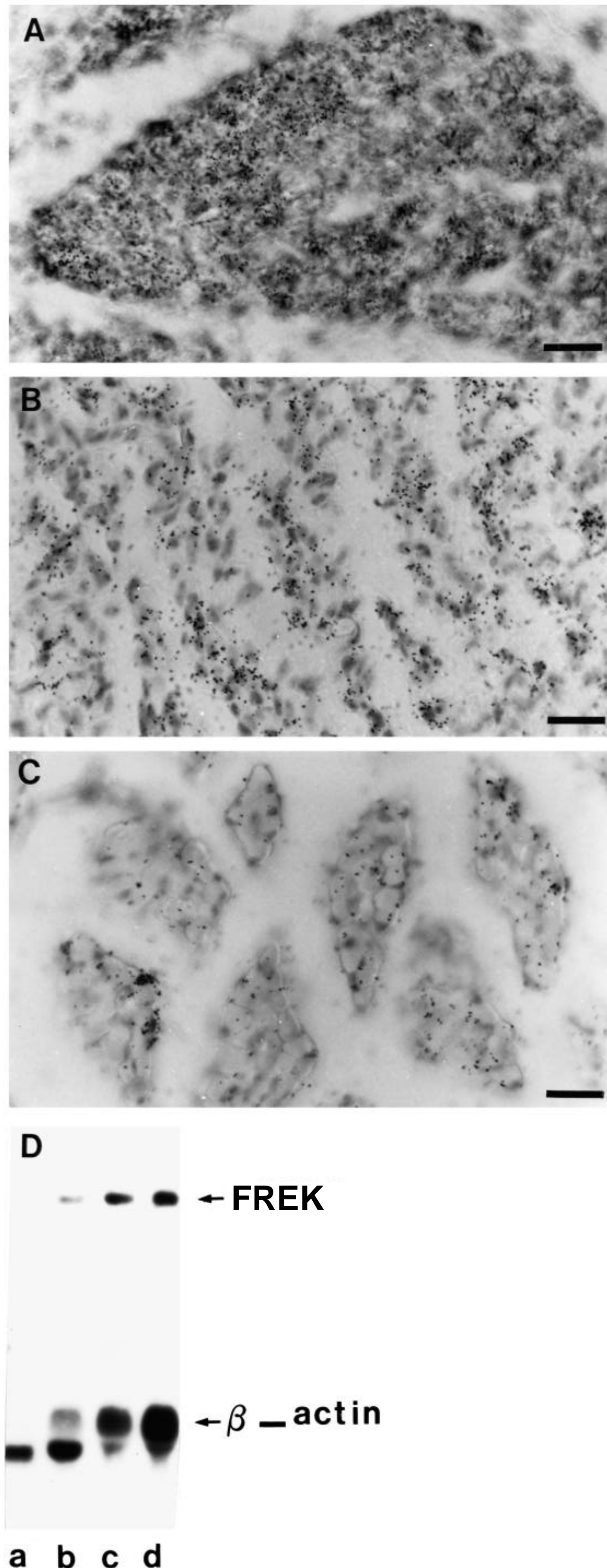


Fig. 7. Expression of FREK in the developing muscle. A FREK antisense riboprobe was hybridized to transverse sections of limb muscle of E9 (A), E12 (B) and E15 quail embryos (C). Scale bars: 20 μm in A-C. (D) Northern blot analysis of the FREK transcript in the developing muscle. Total limb muscle RNAs from new born (a), E15 (b), E12 (c), and E9 (d) quail embryos were hybridized to a FREK cDNA probe. The same blot was rehybridized to a β-actin cDNA probe to verify that equivalent quantities of RNA were loaded.

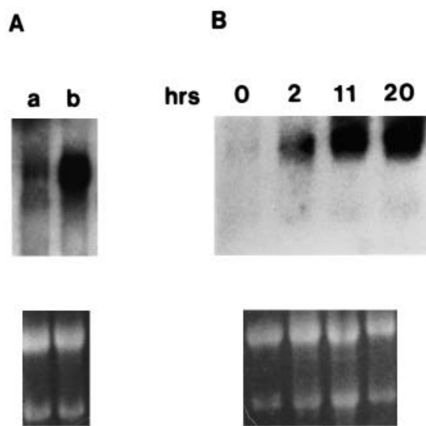


Fig. 8. (A) Expression of the FREK transcript in adult muscle satellite cells. Total RNA from post-hatch chick muscle (a) and from isolated satellite cells (b) was hybridized to a FREK cDNA probe. (B) FREK is regulated at the transcriptional level by FGF-2. 1 ng/ml of FGF-2 was added to serum-starved satellite cells. Their RNA was analyzed for FREK expression after the indicated times.

which the future head and extraembryonic ectoderm are derived (reviewed in Stern, 1992). During primitive streak extension, FREK expression in the deeper cell layers of the Hensen's node determines a distinct compartment within this apparently homogenous structure.

Similar to what we observed with the FREK probe in chicken and quail embryos, Flg/FGFR 1 and bek/FGFR 2 are expressed in the primitive ectoderm of mouse blastula embryo (Orr-Urtreger et al., 1991, Yamaguchi et al., 1992). Assuming that the patterns of expression of FGFR1 and FGFR 2 are similar in avian and mammalian species, the cells of the primitive ectoderm are expressing multiple FGF receptors. It would be interesting to determine at the cellular level whether the cells of the epiblast are coexpressing several FGF receptors or if their expression occurs in distinct subpopulations of cells endowed with different developmental potentials. Interestingly, the domain of FGFR1 expression in the gastrulating mouse embryo complements that of FREK (Yamaguchi et al., 1992). FGFR1 is expressed in the mesodermal cells of the posterior primitive streak and is not expressed in the regions where FREK is expressed, i.e. the anterior primitive streak and the ectoderm. The distinct pattern of expression of FREK during gastrulation therefore suggests that this molecule plays a specific role during gastrulation in birds.

The FGF-like molecules display very distinct patterns of expression: FGF-2 is evenly distributed in the chick gastrulating embryo (Mitrani et al., 1990); FGF-5 is expressed in the primitive ectoderm of the mouse (Haub and Goldfarb, 1991; Hébert et al., 1991). FGF-4 was found to be expressed in cells within the rostral part of the primitive streak of mouse embryos (Niswander and Martin, 1992), while int2/FGF-3 is expressed in the mesodermal cells as soon as they leave the primitive streak (Wilkinson et al., 1988, Niswander and Martin, 1992). Due to the high affinity of these factors for cell surface and extracellular matrix, it is likely that they act locally (reviewed by Rifkin and Moscatelli, 1989; Ruoslahti and Yamagushi, 1991). Therefore, the localization of FGF-5 and FGF-2 makes them likely candidates for the activation of FREK during gastrulation. We have shown in a satellite culture cell system that FREK expression can be activated by FGF-2. Ligand-binding studies should determine if FREK can bind with high affinity to FGF-2 and/or to other members of the FGF family.

Expression of FREK during gastrulation strongly supports the view that FGF receptors play important roles at the time of gastrulation in higher vertebrates, as was already demonstrated in amphibians. The role of FREK during mesoderm induction has nevertheless still to be determined.

At later stages of development, FREK expression has been detected in various ectoderm and mesoderm-derived structures. Expression of FREK in the trigeminal placode and later in the distal part of the ganglion suggests that this molecule has a role in the formation and differentiation of the trigeminal ganglion. This role would be specific for the Vth nerve ganglion, since FREK is not expressed during the formation of any of the other cranial sensory ganglia. Interestingly, FGF-5 expression restricted to the acoustic ganglion has been reported (Haub and Goldfarb, 1991). This might imply that specific FGF receptor/ligand interactions may take place during the formation of each of the cranial ganglia.

FREK is highly expressed in the forming lens. FGFR1, FGFR2 and FGFR3 expressions have already been reported during lens formation in the mouse (Orr-Urtreger et al., 1991; Peters et al., 1993). It has been shown that FGF-1 and FGF-2 induce either proliferation, migration or fiber differentiation in neonatal lens explants, according to the dose of growth factor administered to the cultures (McAvoy et al., 1991). A likely hypothesis would be that the dose-dependent effect of FGF on lens cells is due to the activation of different FGF receptors which, once activated, elicit different cell responses. The localization of large quantities of FGF-1 in rat embryonic lens in the band of cells that are expressing FREK in the chicken, suggests that FREK could be activated by FGF-1 (McAvoy et al., 1991).

The patterns of expression of the various FGF receptors in the developing cartilage are well documented and display interesting features. In the early cartilage blastema, FREK as well as FGF receptors 1, 2, 3 and 4 are expressed uniformly (Peters et al., 1992; Orr-Urtreger et al., 1991; Stark et al., 1991; Korhonen et al., 1992; Patstone et al., 1993; Peters et al., 1993). When cartilage differentiates into bone, expression of these receptors segregates into the various cell types that form the skeleton of the embryo: first FREK and FGFR3 expression becomes restricted to early chondrocytes, then FGFR1 is expressed in the hypertrophied cartilage cells and in the osteoblasts whereas FGFR2 remains expressed in the perichondrium and later in the periostium. Members of the FGF family are potent mitogens and morphogens for chondrocytes and osteocytes (McCarthy et al., 1989; Globus et al., 1988; Kasperk et al., 1990; Too et al., 1987; Hill et al., 1992). The presence of FREK and other FGF receptors during cartilage differentiation strongly supports the contention that they might have an important role during this morphogenetic process. Moreover, their differential expression during endochondral ossification suggests that each one of the FGF receptors performs one or several selective functions.

In the muscle lineage, FREK expression is strictly restricted to the skeletal muscle lineage derived from the myotome. With muscle differentiation, the level of FREK expression decreases dramatically. Its expression becomes restricted to the satellite cells of the adult muscles. Evidence from studies of myogenic

cell lines indicates that both FGF-1 and FGF-2 stimulate the proliferation and inhibit the differentiation of myoblasts (reviewed in Burgess and Maciag, 1989). Moreover, binding studies have shown that FGF receptors are permanently lost during skeletal muscle terminal differentiation (Olwin and Hauschka, 1988). The FREK expression pattern is consistent with a role of this molecule in muscle differentiation: its expression might maintain myoblasts in an undifferentiated and/or proliferative stage. Satellite cells behave in many ways as embryonic myoblasts, and can undergo proliferation and terminal differentiation in case of muscle repair after injury. In an adult mouse muscle, FGF-2 is located in the basement membrane at the periphery of the myofibers. Strong FGF-2 staining appears to be located on the satellite cells (Anderson et al., 1991). Addition of minute quantities of FGF-2 to cultured satellite cells induces them to proliferate. The concomitant rapid activation of FREK transcription suggests that FREK is implicated in this process. It is possible that FREK is transcriptionally upregulated by FGF-2, although we have no evidence that FGF-2 mediates its effect by direct binding to FREK or to another receptor.

Studies of vertebrate embryo myogenesis have supported the idea that the multiple types of muscle fibers that compose the adult muscle may be derived from distinct subpopulations of myoblasts (reviewed in Stockdale, 1992). In the developing embryo, FREK as well as FGFR1 and FGFR4, have been shown to be expressed in the myotome (Stark et al., 1991; Patstone et al., 1993). FGFR4 displays a pattern of expression similar to FREK, while FGFR1 is expressed also in the smooth and cardiac muscle lineages. In addition, a recent study has shown the expression of FGFR1 isoforms in MM14, a mouse myogenic cell line derived from adult muscle (Templeton and Hauschka, 1992). Various FGF-like molecules are expressed during muscle differentiation: FGF-2 is found in all the muscle lineages (Joseph-Silverstein et al., 1989), FGF-4 expression has been detected in the early myotome of the developing mouse (Niswander and Martin, 1992), FGF-5 was found in the myotomes of the anterior part of the mouse embryo (Haub and Goldfarb, 1991), and FGF-6 expression was found in the skeletal muscle lineage of the developping mouse embryo (Han and Martin, 1993). These data indicate that multiple receptor/ligand interactions very likely take place during myotome and muscle formation and could account for the differentiation of the various types of muscle fibers forming the adult organ.

In conclusion, as our knowledge of FGF and FGF receptor embryonic patterns of gene expression increases, it becomes apparent that members of these families are differentially expressed in cells undergoing specific developmental processes. It is therefore likely that the FGF receptors have diverse and specific roles during embryonic development. Likewise, the various and sometimes very different effects observed upon addition of FGFs to cells in culture is probably due to the activation of distinct receptors expressed in different cell types. In many instances, multiple receptors are coexpressed in a single tissue. It will be important in the future to analyze the distribution of these molecules at the cellular level to shed light on the mechanisms that regulate the progressive cell determination and differentiation during embryonic development.

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Note added in proof

FREK sequence has been entered in the EMBL data bank under the accession number X76885.