DEVELOPMENTAL BIOLOGY 158, 475-486 (1993)

Developmental Localization of the Splicing Alternatives of Fibroblast Growth Factor Receptor-2 (FGFR2)

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Accepted February 25, 1993

The gene for fibroblast growth factor receptor-2 (FGFR2) encodes two splice variants designated here as keratinocyte growth factor (KGFR) and bek. Their ligand-binding specificity is markedly different due to mutually exclusive alternative splicing. We asked whether alternative exon usage, in addition to influencing receptor specificity, could be correlated with transcriptional localization. This problem was studied by *in situ* hybridization and PCR, using probes and primers specific for the alternative exons of FGFR2. Transcripts of both variants were detected in all three germ layers within the embryonic and the extraembryonic areas of the primitive-streak embryo. The overall level of KGFR expression surpassed that of bek. The localized expression of both variant receptors was, however, more diffuse in the gastrula than later during organogenesis, when KGFR transcripts were evident mainly in epithelia, whereas bek was present in the corresponding mesenchymes. Our findings show the following: (1) Expression of both FGFR2 variants is concordant with their involvement in murine gastrulation. They may endow competence to multiple areas, which may be restricted by their more confined ligands. (2) KGFR and bek seem to have unique roles in the development of the skin and its derivatives, whereas bek is preferentially expressed during osteogenesis. The two variants share potential regions of trans regulation in the genome; hence, we suggest that alternative splicing is jointly responsible for ligand binding and spatial specificity. (3) Finally, we defined the binding specificity of KGFR and bek to various FGF. The possibility of identifying specific functional areas for certain ligand–receptor pairs is discussed.

INTRODUCTION

Fibroblast growth factor receptors (FGFRs) consist of an extracellular ligand-binding region, composed of three (or two) immunoglobulin-like domains, a transmembrane domain, and an intracellular tyrosine kinase domain (for review, see Givol and Yayon, 1992). They are expressed as various isoforms at multiple sites in the adult (Houssaint *et al.*, 1990; Johnson *et al.*, 1990; Hou *et al.*, 1991; Eiseman *et al.*, 1991) and in the embryo (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1993; Yamaguchi *et al.*, 1992). One type of transcriptional variant maybe generated in various FGFR loci by differential use of exons encoding an approximately 50 amino acid long variable region in the C-terminal half of the third Ig-like domain (Johnson *et al.*, 1991; Champion-Arnaud, 1991). At the FGFR2 locus, two variants, KGFR and bek, are generated in this manner (Fig. 1) and they display a markedly different affinity for KGF/FGF-7 and bFGF/FGF-2 (Miki *et al.*, 1992; Yayon *et al.*, 1992).

In the face of these results the question arose whether the two variant receptors, distinguished by different binding specificity, are expressed at different locations. This question is relevant because in addition to ligand binding, compartmentalization can define the function of a cell membrane receptor. In fact the developmental localization of various FGFRs, as detected by probes, which do not distinguish the splice variants, has already been shown to be different. Both FGFR1 and FGFR2 were detected in the primitive ectoderm of the postimplantation embryo. Later, during organogenesis, FGFR1 (*flg*) was found to be expressed in various mesenchymes, FGFR2 (*bek*) in diverse epithelia (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992; Yamaguchi *et al.*, 1992), FGFR3 in the central nervous system and the vestibular organ (Peters *et al.*, 1993), and FGFR4 in the definitive endoderm and skeletal muscle (Stark *et al.*, 1991). A recent report by Patstone *et al.* (1993) compares the expression of three FGFRs in the late chicken embryo, and another report by Sato *et al.* (1992) suggests that avian variant FGF receptors, bek and Cek3, are differentially expressed in chicks. Here we present a detailed developmental analysis of FGFR variants in the mouse embryo. Our data suggest that various FGFR loci and their splicing alternatives may fulfill unique roles during organogenesis. Accordingly the FGF system is under mul-

[1] Contributions by the first three authors were equal.

0012-1606/93 $5.00

tiple regulation and variant receptors encoded at one locus not only may differ in binding specificity but also may be localized differentially. We assume that alternative splicing could be one such regulatory mechanism. In experiments reported here, this problem is analyzed by *in situ* hybridization using exon-specific probes and the localized expression of the two variant receptors encoded by the FGFR2 gene is compared.

We report that KGFR and bek display markedly different expression patterns. In the primitive-streak embryo both FGFR2 transcripts are present, but considerably more KGFR than bek transcripts are detectable and their expression pattern is rather diffuse. Later, during organogenesis, KGFR transcripts selectively accumulate in the surface ectoderm and in the epithelial lining of the inner spaces, whereas bek transcription localizes to the corresponding mesenchyme and to the developing skeleton. Their relative role in pattern formation is discussed on the basis of binding experiments, which demonstrated that both KGFR and bek bind FGF4, a growth factor localized to the posterior primitive streak during gastrulation (Niswander and Martin, 1992).

MATERIALS AND METHODS

Animals and Embryos

C57BL/6J mice were used. Pregnancy was established by vaginal plugs the morning following mating (pc), which was taken as Day 0.5. Ovarian oocytes and 6.5-, 7.5-, 8.5-, 10.5-, 12.5-, 13.5-, and 14.5-day-old (pc) embryos were investigated. Between Gestational Days 6 and 7.5 five experiments were performed approximately at 6.3, 6.5, 7.0, 7.25 and 7.5 days pc.

DNA Probes

Exon-specific cDNA probes specific for the two FGFR2 variants were prepared (Raz *et al.*, 1991; Yayon *et al.*, 1992) as shown in Fig. 1. A common *Ppu*MI restriction enzyme site at the 3′ end of the constant portion of the third Ig-like loop and either a *Hae*II site at the junction of the third Ig-like domain and the transmembrane exon of KGFR or an *Eco*RV site in the alternative region of the bek cDNA were used to isolate probes for *in situ* hybridization, as shown in Fig. 1. A 150-bp-long *Ppu*MI–*Hae*II fragment specific for KGFR and a 120-bp-long *Ppu*MI–*Eco*RV fragment specific for bek were subcloned into Bluescript. The so-called "common" 5′ FGFR2 probe (a 281-bp *Nla*IV–*Sma*I fragment) has been described previously (Orr-Urtreger *et al.*, 1991).

In Situ Hybridization

In situ hybridization of 14.5-day-old embryos was performed in frozen sectioned material, as described previously (Orr-Urtreger *et al.*, 1990), with the difference that the posthybridization wash was 60°C for the bek probe and 63°C for the KGFR probe. In the other experiments paraffin embedding was used (Wilkinson and Green, 1990). In each experiment and with each probe radiolabeled sense transcripts were also hybridized as control.

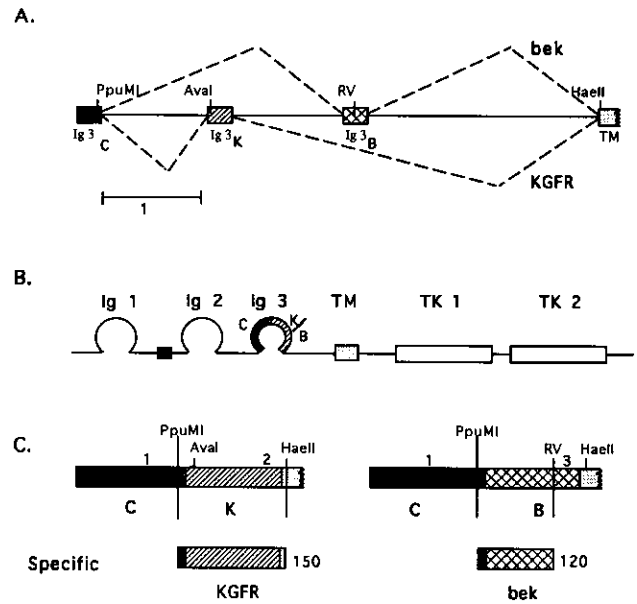


FIG. 1. Splicing alternatives of FGFR2 and probes and primers used for their detection. (A) Mutually exclusive splicing responsible for the alternatives of the 3[d] Ig-like loop. Exons: C, constant region of the 3[d] Ig-like loop; K, KGFR variant; B, the bek variant of the 3[d] Ig-like loop; TM, transmembrane exon. Note that the same shading is used to represent the different exons also in B and C. (B) The FGFR2 protein. Ig 1, Ig 2, and Ig 3, immunoglobulin-like loops; TM, transmembrane domain; TK1 and TK2, kinase domains. (C) Preparation of RNA probes. C, constant portion of the 3[d] Ig-like loop; K, the KGFR-specific; B, the bek-specific variant of the 3[d] Ig-like loop. 1, 2, and 3 show the approximate positions of the PCR primers (see Materials and Methods).

PCR Analysis

Embryos 7.25 days old were cleaned from the Reichert membrane and the ectoplacental cone and they were separated into extraembryonic and embryonic fragments, using tungsten needles, as described in Fig. 3 and the text. Fragments derived from two embryos were used for RNA extraction according to Chomczynski and Sacchi (1987). First-strand cDNA was isolated, using a primer derived from the transmembrane region (5′-CAGGCGATTAAAAGACCCCTATGCAG-3′). For PCR amplification of the first-strand cDNA, the following oligonucleotide primers were used: (1) from the constant exon, 5′-AACGGTCACCACACCGGC-3′; (2) from the KGFR-specific exon, 5′-AGGCAGACTGGTTGGCCTG-3′; and (3) from the bek-specific exon, 5′-CTGCA-

GAACTGTCAAC-3′ (see also Fig. 1). Thus, primers 1 and 2 gave KGFR-specific amplification, whereas primers 1 and 3 gave bek-specific amplification. The amplified DNA was separated by agarose gel electrophoresis. Specific bands were identified by Southern blot hybridization using exon-specific oligonucleotide probes (KGFR, 5′-CCCCAGCATCCATCTCCGTC-3′; bek, 5′-GGTGTTAACACCGGCGGC-3′).

Ligand Binding

The binding profile of secreted soluble FGF receptors was determined from the media of cells secreting the respective receptor–alkaline phosphatase fusion protein (Flanagan and Leder, 1990). The amount of receptor bound to heparin–Sepharose-bound FGF was monitored by measuring alkaline phosphatase activity, as described previously (Yayon *et al.*, 1992).

RESULTS

Differential Expression of the Splice Variants in Primitive-Streak Embryos

Previously, using a probe which did not distinguish the splice variants, expression of both FGFR1 and FGFR2 was detected in presomitic and early somitic embryos. They were present in the embryonic (primitive) ectoderm and mesoderm and also in the extraembryonic (chorionic) ectoderm (Orr-Urtreger *et al.*, 1991). This suggested that FGFR1 and FGFR2 could play roles in mammalian gastrulation. Here we determine whether the splice variants of FGFR2, KGFR, and bek are transcribed during early development.

We first investigated their expression in ovarian oocytes. Figures 2A and 2B show an experiment with the 5′, or common, FGRFR2 probe. In this and other experiments no FGFR2 (bek or KGFR) expression was detectable either in primary or in mature oocytes. This suggests that little or no FGFR2 transcription takes place during oogenesis and in the nonfertilized ovum of the mouse.

Next, egg-cylinder (6.3 days pc; Figs. 2C and 2D) and early primitive-streak embryos (7.0 days pc; Figs. 2E and 2F) were investigated. Weak signals were detected with both probes. Figures 2C–2F demonstrate the more abundant KGFR transcripts. Figure 2D shows a faint signal distinguishable in the extraembryonic area. Fewer autoradiographic grains were detectable above the embryonic than above the extraembryonic area and the decidua, suggesting that most of the embryo failed to express KGFR at this stage. In more mature (Day 7.0 pc) primitive-streak embryos stronger signals were detected. They were visible at the junction of the extraembryonic and embryonic areas (Fig. 2F). Inspection of serial sections revealed that KGFR transcription at the early primitive-streak stage localizes to the junction of the embryonic and extraembryonic areas, as shown in Figs. 2E and 2F, and includes all three germ layers. This signal was reminiscent of but weaker than what was observed with a common FGFR2 probe previously (Orr-Urtreger *et al.*, 1991).

Later, just before or after the formation of the amniotic cavity and the exocoelome, transcription increased significantly (Figs. 2G–2J) and both KGFR (Fig. 2H) and bek (Fig. 2J) transcripts were most clearly detectable above the extraembryonic area. They were present in all three germ layers. Diffusely distributed transcripts were, however, evident in most of the embryo. Comparison of the two transcriptional variants showed that the amount of KGFR expression surpassed that of bek, although the two patterns were not different (Figs. 2H and 2J).

Two observations confirm the significance of the signals described above. Hybridization with the sense transcript of KGFR (Fig. 2L) resulted in a much lower density of autoradiographic grains than the antisense transcript, as can be seen in an adjacent section of the same embryo (Fig. 2J). A similar observation was made with the antisense KGFR probe above the embryonic part of a younger embryo, as shown in Fig. 2D. Hence we conclude that the two variants of FGFR2 are indeed expressed during gastrulation. They were first detectable at the junction of the embryonic and extraembryonic area at early primitive-streak stage. Later the strongest expression was above the extraembryonic part, whereas in the embryonic area the label was weaker and diffuse, including all three germ layers. The signals obtained in five experiments performed with gastrulating embryos gave similar results; nevertheless, the signal was weaker than what was previously observed with an FGFR2 probe, which did not discriminate the transcriptional alternatives (Orr-Urtreger *et al.*, 1991). The reason for this may be that the KGFR- and bek-specific probes were only 150 and 120 bp long, whereas the common FGFR2 probe was significantly longer (281 bp).

To add independent support to our conclusions a PCR assay was performed. First-strand cDNA was synthesized using RNA from 7.25-day-old embryos. Before RNA extraction the embryos were stripped from the parietal endoderm and ectoplacental cone, and rinsed to remove residual maternal cells, and separated into embryonic (EMP) and extraembryonic halves (EXP, see Fig. 3A). Complementary DNA prepared from these fractions was amplified with exon-specific oligonucleotides (see Fig. 1 and Materials and Methods). Electro-

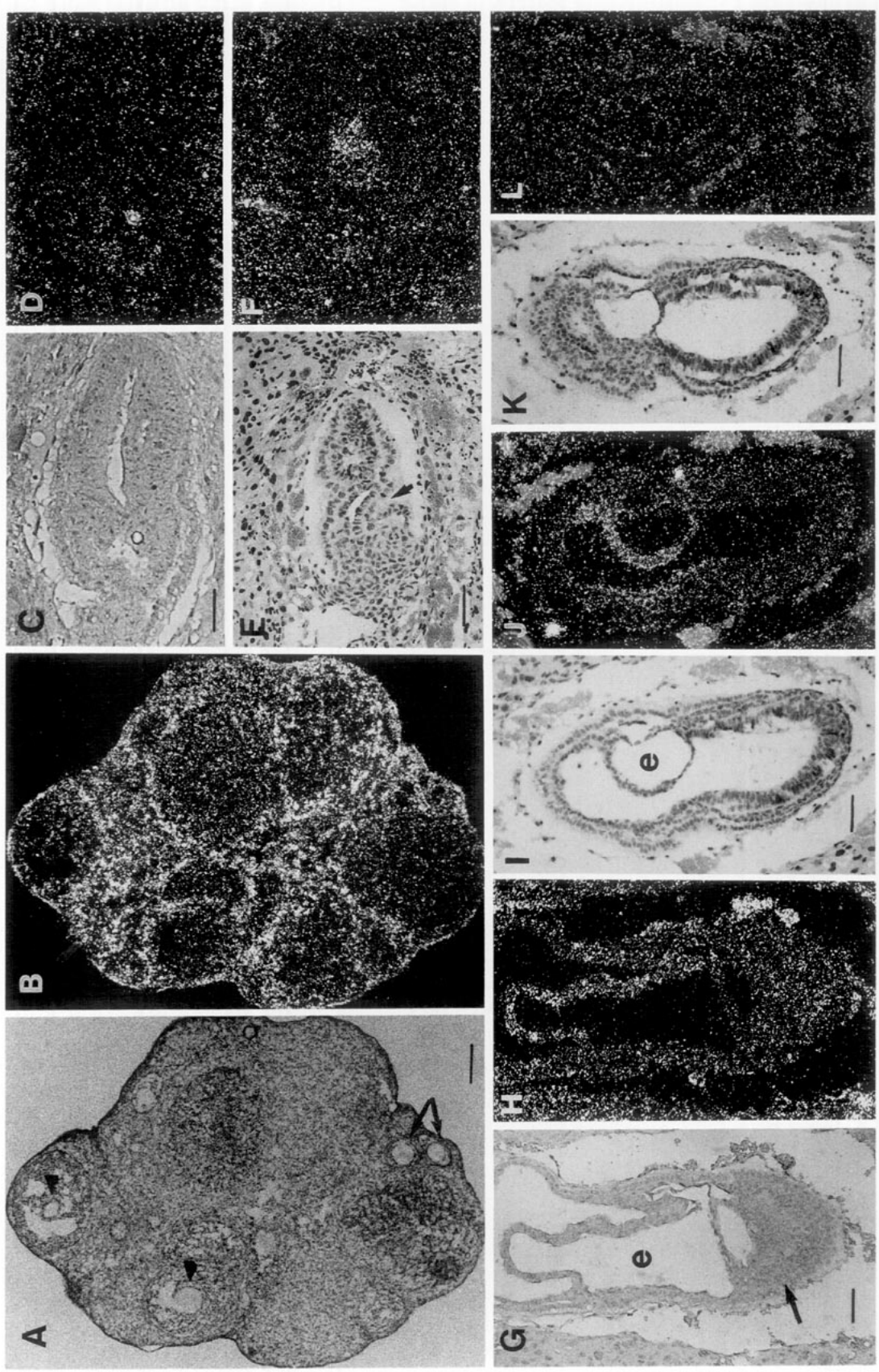


FIG. 2. Expression of the KGFR and bek during early development. Hybridization: (B) FGFR2 "common" antisense probe, (D, F, H) KGFR antisense probe, (J) bek antisense probe; (L) KGFR, sense probe. (A, C, E, G, I, K) Bright-field and (B, D, F, H, J, L) dark-field illumination. (A and B) adult ovary. Arrowheads, mature oocytes; arrows, immature oocytes. (C and D) 6.3-day egg-cylinder embryo. (E and F) 7.0-day-old embryo, parasagittal section; arrow shows the posterior amniotic fold. (G and H) 7.5-day-old embryo, parasagittal section. e, prospective exocoelome; arrow, posterior mesoderm. (I–L) 7.3-day-old embryos; e, future exocoelome, within the posterior amniotic fold. Bars: (A) 200 μm; (C, G, I, and K) 50 μm; (E) 100 μm.

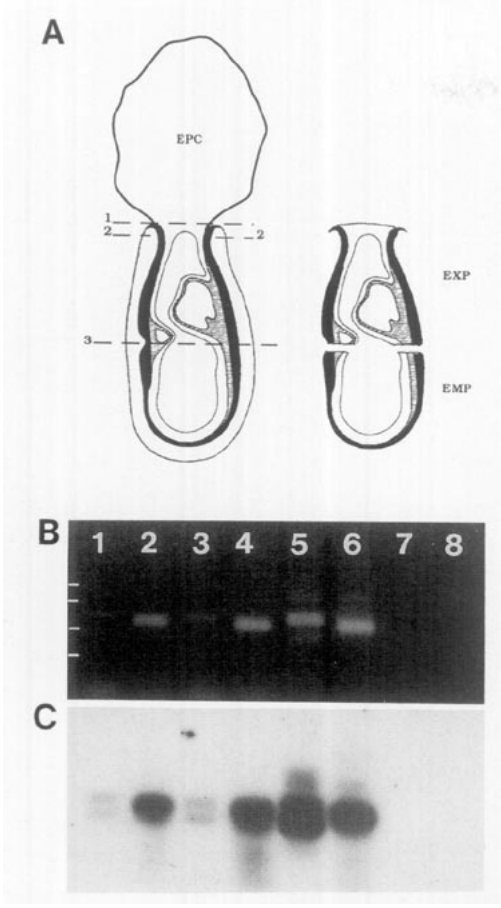


FIG. 3. Expression of FGFR2 variants in the presomitic embryo: PCR analysis. (A) Preparation of the 7.25-day (pc) embryo for RNA extraction. EPC, ectoplacental cone; EXP, extraembryonic portion; EMP, embryonic portion. 1–3 show lines of dissection. (B) PCR assay, ethidium bromide staining. Lanes 1–4, amplification from EMP (1, 2) and EXP (3, 4) parts of the embryo (approximately 1 embryo equivalent/reaction); lanes 5 and 6, amplification of cDNA from total RNA of 12.5-day-old embryos (control); lanes 7 and 8, like lanes 5 and 6, but no template added (control). Odd numbers, bek-specific amplification; even numbers, KGFR-specific amplification. Marker bands show 517, 394, 298, and 234 bp. (C) Southern blot hybridization with bek (lanes 1, 3, 5, and 7) or KGFR-specific (lanes 2, 4, 6, and 8) oligonucleotide probes. In lanes 1, 3, and 5 the FGFR2-specific oligonucleotide probe (3) detects a doublet. The larger of the two bands is that of the predicted size (336 bp). There is, however, an additional bek-specific band of 280 bp, which is at present being characterized.

phoretic analysis revealed abundant KGFR-specific product, but much less bek-specific product (Figs. 3B and 3C). After blotting to nylon membranes, the amplified cDNA was detected with KGFR- or bek-specific probes. The results confirmed that both KGFR and bek are expressed in the embryonic and extraembryonic parts of the presomitic embryo. Densitometric analysis (using PCR data from 12.5-day-old embryos as standard) revealed 70-fold more KGFR-specific than bek-specific hybridization.

Differential Expression of the Splice Variants during Organogenesis

Early organogenesis. To investigate the localized expression of KGFR and bek in the specification of the early mesoderm and definitive endoderm, 8.5- and 10.5-day-old embryos were studied. At 8.5 days (Figs. 4A–4D) the expression of both KGFR and bek was detectable in the neuroectoderm, in the somites, and also in the lateral mesoderm, with much of the label distributed diffusely above both the embryonic and extraembryonic parts. At 10.5 days pc bek still showed diffuse labeling, whereas selective transcription of KGFR could already be detected in the surface ectoderm, in the hindgut endoderm, in the nephrogenic cords, and in the spinal cord (Figs. 4E and 4F).

Advanced organogenesis. The expression of the KGFR and FGFR2 variants in 12.5- and 14.5-day-old mouse embryos was studied in detail. The first observation to be made was that in the more mature, 14.5-day-old embryos (Figs. 5A–5C), expression of the two FGFR2 variants showed distinct and different patterns, which was much more definite and less diffuse than that during the earlier stages.

Skin and its derivatives. KGFR was distinguished by abundant transcription in the surface ectoderm as early as Day 10 (Fig. 4F) and Day 12 of gestation (Fig. 4H) and became even more apparent at 14.5 days pc (Fig. 5B). The figures show a continuous contour of KGFR expression around the 12.5- and 14.5-day-old embryos. It also includes the epithelium of the internal spaces, like the oral cavity, pharynx, nasal cavity, and others. In the second third of gestation, as can be seen in higher magnification of the skin from the thoracic (Fig. 5E) and nasal areas (Fig. 5U), KGFR transcripts accumulated in the periderm, whereas in the dermis the signals did not exceed background level. This specific localization suggested that KGFR could indeed contribute to the mesenchymal stimulation of epithelial cell growth by its ligand, KGF (which is transcribed in the dermis), as suggested previously by others (Miki *et al.*, 1992; Rubin *et al.*, 1989). The expression domain of the other splice variant, bek, overlapped only partially with that of KGFR in the integument. It can be seen in the skin of the nasal (Fig. 5V) or thoracic (Fig. 5F) area that bek is not expressed in the most external layers, which are occupied by abundant KGFR transcripts (Figs. 5U and 5E). The bek domain also differs by extending into the dermis and beyond into the loose mesenchyme (Figs. 5F and 5V).

A similar relationship can also be detected between the expression patterns of the two FGFR2 variants in various skin derivatives. This can be seen in the developing mammary gland (Figs. 5D–5F), in the hair roots of

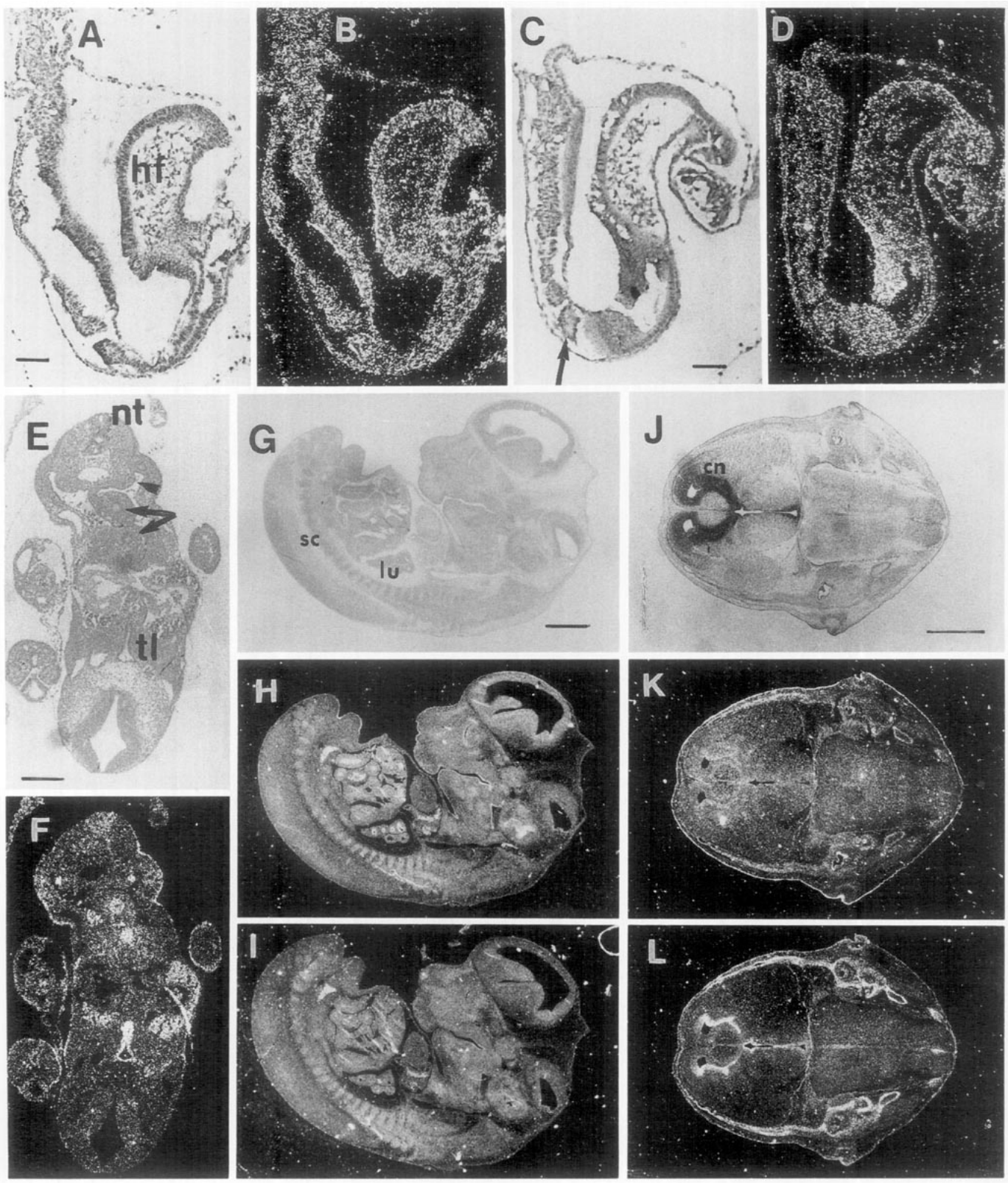


FIG. 4. Localized expression of KGFR and bek during early organogenesis. Hybridization: (B, F, H, and K) and KGFR; (D, I, and L) bek. (A–D) 8.5-day-old embryo sagittal sections. hf, headfold; arrow, somite. (E and F) 10.5-day-old embryo, transverse section. nt, neural tube; tl, trachea and lung bud; arrowhead, mesonephric tubule; arrows, hindgut. (G and H) 12.5-day-old embryo, sagittal section. sc, spinal cord; lu, lung. J–L, coronal section of the head of a 14.5-day-old embryo. The plane of the section is shown in Fig. 5A. cn, cortical neuroepithelium. Bars: (A and C) 50 μm; (E) 200 μm; (G and J) 1 mm.

the vibrissae (Figs. 5T–5V), in the enamel organ of a developing incisor tooth (Figs. 5M–5O), and in the developing cornea (Figs. 5P–5S). In all of these different localizations KGFR transcripts occupy the superficial cell layer, on the surface, as in the cornea (Fig. 5R), in the developing tooth (Fig. 5N), close to the lumen, as in the salivary gland (Fig. 5N), or in the hair root sheath (Fig. 5U). Transcription of bek in contrast is concentrated to a layer next to the one occupied by KGFR transcripts, as in the corneal mesenchyme (Fig. 5S) or in the outer hair root sheath of the vibrissae (Fig. 5V). In an earlier derivative of the surface ectoderm, the lens epithelium, bek was expressed more strongly than KGFR (Figs. 5R and 5S).

From these examples we can conclude that KGFR and bek may have major roles in the development and specification of the epithelial and mesenchymal components of the skin and its derivatives. Interestingly the spatial specificity of KGFR and bek expression is maintained once their expression patterns are established in the embryonic integument. This is first achieved in 9- to 10-day old embryos and it persists throughout the differentiation of the embryonic integument into skin and also throughout the formation of its highly specialized derivatives.

Bones and skeleton. Bones of the vertebral column, the ilium (Figs. 5A–5C), the long bones of the limbs, as well as the ribs (Figs. 5W–5X), the skull (Figs. 4K and 4L), the bones of the face and nasal cavity (Figs. 5A–5C; Figs. 5P–5S), were all found to express both KGFR and bek. Bek expression significantly surpassed that of KGFR at these localizations. This relationship could also be observed in the bony part of the inner ear and in the auditory ossicles (Figs. 5J–5L). It appears that bek is associated with osteogenesis in general.

The gut and its derivatives. These develop from the definitive endoderm. KGFR is expressed in the endothelium of the gut and the stomach (Figs. 5A and 5B; 5W and 5X). It is also expressed in the alveolar epithelium of the lung (Fig. 5Z), which develops from the foregut. Diffuse expression of bek and KGFR could be also seen in the liver (Fig. 5B), a midgut derivative.

When individual organs were further inspected it became apparent that both FGFR2 variants are expressed in the vestibular system. Figures 5J–5L demonstrate their expression in the cochlear duct. Very recently Peters *et al.* (1993) have reported the expression of another FGF receptor, FGFR3, in the differentiating sensory hair cells and their underlying support cells in this organ. It appears, from Figs. 5J–5L, that the two FGFR2 variants, in contrast to FGFR3, are expressed by cells of the cochlear endothelium. Hence FGFR2 and FGFR3 may have different, but complementary, roles in the development of this highly specialized organ. It maybe of importance that FGF3/Int2 is also expressed in the developing inner ear, where it is restricted to the sensory areas (Wilkinson *et al.*, 1989). Whether FGF3 is the ligand for FGFR3 and FGFR2 in this organ is not clear, because in the absence of soluble protein the binding characteristics of FGF3 are not sufficiently understood.

Strong prevalence of bek transcripts could be detected in the cortical neuroepithelium of the brain (caudate putamen) (Figs. 4K and 4L), in the adrenal gland (Fig. 5C), and in the atrioventricular heart valve (Figs. 2V and 2W). In contrast, KGFR expression was preponderant in the collecting ducts of the kidney, in the thymus, in Rathke's pouch, and in the epithelium of the pancreas and stomach (Fig. 5B).

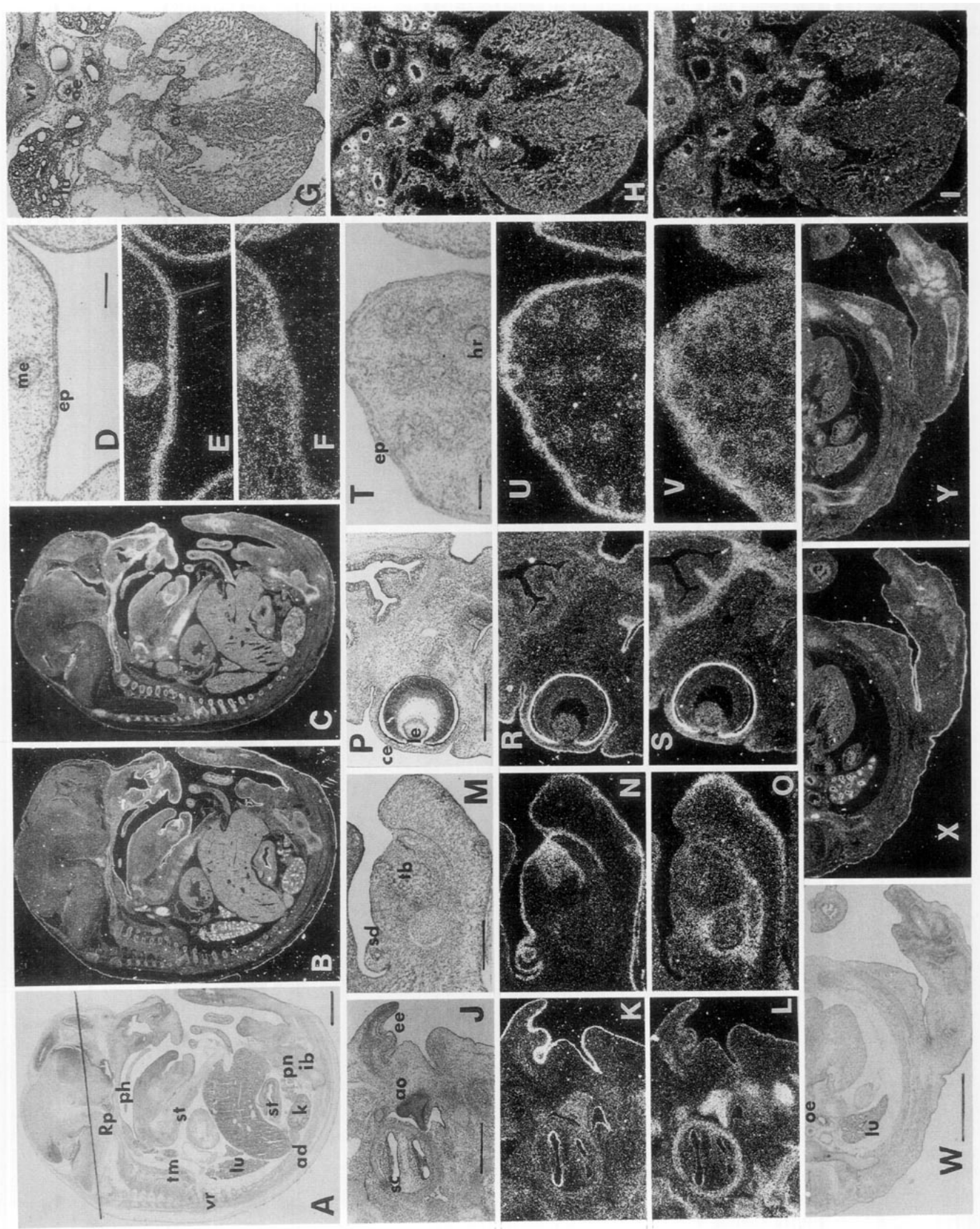
Ligand-Binding Specificity of KGFR and bek

The data presented demonstrate that the two splice variants of FGFR2 display significantly different expression patterns during various stages of development. To broaden the functional relevance of these observations we will have to know which FGF is the potential ligand of which variant and whether the receptor and the ligand are expressed within the same spatiotemporal framework.

It has been already reported that bek binds selectively FGF2, whereas KGFR is specific for FGF7 (KGF) and that both bind FGF1 (Johnson *et al.*, 1991; Miki *et al.*, 1992; Yayon *et al.*, 1992). Here we compare these with the specificity of KGFR and bek to two additional growth factors, FGF4 and FGF5, which are known to be expressed in the gastrulating mouse embryo (Niswander and Martin, 1992; Haub and Goldfarb, 1991; Hebert *et al.*, 1991). Figure 6 demonstrates that both bek and KGFR bind FGF4, whereas neither binds FGF5. Moreover, in agreement with previous results, FGF7 was bound by KGFR, but not by bek, whereas bek, but not KGFR, bound FGF2, and both had high affinity for FGF1. This suggests that bek- and/or KGFR-expressing cells may be activated by FGF4 in the gastrulating mouse embryo.

DISCUSSION

We have demonstrated here that the two mutually exclusive splice variants of FGFR2 are differentially expressed during development. Two kinds of regulation have been distinguished. From early primitive-streak stage to early organogenesis, quantitative rather than positional differences were observed between the expression pattern of KGFR and bek. With the progress of organogenesis, however, their localization became more and more specific. The two periods are discussed separately.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Transcriptional Localization of the FGF System during Gastrulation

Members of the FGF and TGFβ polypeptide growth factor families are the main mesoderm inducers in amphibia (for review, see Smith, 1989). Recent experiments demonstrate that biologically active FGF (Isaacs *et al.*, 1992) and FGFR are indeed synthesized in amphibian embryos (Amaya *et al.*, 1991) and that they are responsible for ventral and posterior mesoderm development. There is, however, no direct evidence for their involvement in mammalian development, although the biochemistry and molecular genetics of FGF and FGFR are considerably better known in mammals than in amphibia. Therefore it is of interest to establish which of the numerous murine or human FGF and FGFR isotypes are expressed in the spatiotemporal framework of gastrulation and which in various aspects of organogenesis. The results presented here contribute to this undertaking.

Our assay could not detect FGFR2 transcription during oogenesis or in the unfertilized egg. This was somewhat surprising because *Xenopus* FGFR was found to be under maternal regulation (Musci *et al.*, 1990; Friesel and Dawid, 1991) and similar observations were reported also for XeFGF, an FGF4-like growth factor of *Xenopus* (Isaacs *et al.*, 1992). It is possible that mammalian FGFR genes other than FGFR2 are indeed maternally regulated. Future experiments with higher resolution, including additional FGFR loci, are required to resolve this problem.

During gastrulation both FGFR2 variants were expressed. The first faint signals were detected in early primitive-streak embryos and they became amplified considerably by early headfold stage. The pattern was rather diffuse, with some highlights in the extraembryonic area and around the root of the allantois. According to our earlier study the expression of FGFR1 is also rather diffuse. FGFR1 expression was, however, somewhat weaker than that of FGFR2 in egg-cylinder embryos (Orr-Urtreger *et al.*, 1991). Newer evidence argues that the expression patterns of FGFR1 at later gastrulation may be somewhat different from those of FGFR2. Yamaguchi *et al.* (1992) have shown that FGFR1 transcripts concentrate in the posterior region of the headfold embryo and in the unsegmented paraxial mesoderm plus somites at the early somitic stage. Nevertheless the rather undistinguished expression pattern displayed by of all three FGFR during early gastrulation differs strikingly from the diversity displayed by them and by other FGFR (Peters *et al.*, 1993; Stark *et al.*, 1991) during organogenesis. Hence it is possible that during early embryogenesis the various FGFR may be redundant. Functional experiments are required to clarify this problem. The present data, however, demonstrate that with advancing embryogenesis these receptors acquire selective expression patterns and that they may have specific roles.

Insights into the biological significance the transcriptional localization of these receptors may be obtained by comparing their expression patterns with those of their ligands. Of the seven FGF known in amniotes, five, FGF3, 4, 5, 6, and 7, are secreted. Overexpression of various FGF in *Xenopus* embryos revealed that the nonsecreted FGF1 (aFGF) and FGF2 (bFGF) isotypes display less activity during gastrulation than the secreted five (Thompson and Slack, 1992). Among murine FGFs the transcriptional localizations of FGF3 (Wilkinson *et al.*, 1988), FGF4 (Niswander and Martin, 1992), and FGF5 (Haub and Goldfarb, 1991; Hebert *et al.*, 1991) have been established during gastrulation. Although they are secreted proteins, their affinity for the proteoglycans of the intercellular matrix is likely to localize them to the vicinity of their transcription (for review, see: Klagsbrun and Baird, 1991). Hence the specific transcriptional localizations of FGF3, 4, and 5 should reveal aspects of their activity.

The expression patterns of FGF3, 4, and 5 were more defined than these of their receptors, and in contrast to FGFR1 and FGFR2, they are restricted to the embryonic part of the egg cylinder. In particular, FGF4 is expressed in the posterior part of the primitive streak and FGF5 in the epiblast (primitive ectoderm), with a tendency to decrease as cells pass through the streak, whereas FGF3 becomes expressed as cells leave the streak and differentiate into mesoderm (Niswander and Martin, 1992). Ligand-binding experiments presented here (Fig. 6) and elsewhere (Mansukhani *et al.*, 1992; Ornitz and Leder, 1992; Yayon *et al.*, 1992) revealed that

FIG. 5. KGFR and bek expression during advanced organogenesis (14.5 days pc). Hybridization: (B, E, H, K, N, R, U, and X) KGFR; (C, F, I, L, O, S, V, and Y) bek. (A–C) Sagittal section, whole embryo. (D–F) Abdominal area with mammary gland, transverse section. (G–I) Thoracic cavity, heart, lung, etc. (J–L) External, middle, and inner ear, transverse section. (M–O) Lower jaw with incisor tooth. (P–S) Eye and nasal cavity, transverse section. Note that the strong signal following the internal outline of the eye is light scattering from the pigmented choroid layer. (T–V) Nasal area with vibrissal hair roots. (W–Y) Thorax and forelimb, transverse section. Abbreviations: ad, adrenal gland; ao, auditory ossicle; av, atrioventricular valve; ce, corneal epithelium; ee, external ear; ep, epidermis; hr, hair root; ib, ileum bone; k, kidney; le, lens epithelium; lu, lung; me, mammary epithelium; oe, esophagus; ph, pharynx; pn, pancreas; Rp, Rathke's pouch (prospective hypophysis); sd, salivary duct; st, sternum; tb, toothbud; tm, thymus; vr, vertebrae. Bars: A, D, and W, 1 mm; M and P, 250 μm; J and G, 500 μm; T, 100 μm.

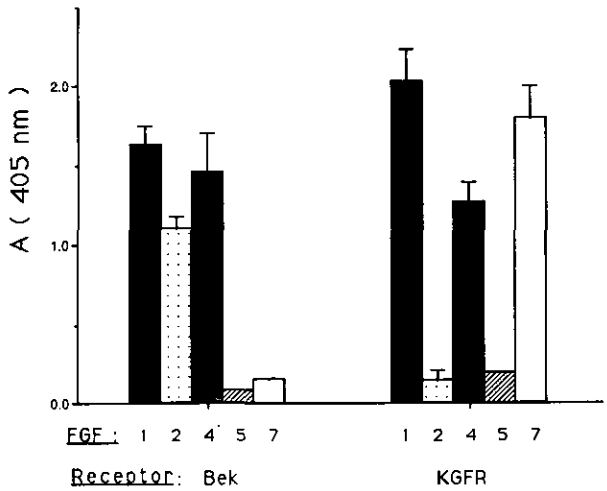

FIG. 6. Ligand-binding profile of bek and KGFR. The assay uses heparin–Sepharose-bound ligands to bind receptor–alkaline phosphatase fusion proteins from tissue culture medium. The medium was normalized by determining its alkaline phosphatase activity. Routinely 25–100 μl medium, containing approximately 0.1 μg of the respective receptor, was used in the assay. Conditioned medium from the indicated cell lines was incubated with 30 μl Sepharose-bound ligand (30 ng/ml) in a total volume of 1 ml in phosphate-buffered saline. After washing, the beads were suspended in 100 μl of the substrate and they were incubated for 30 min. The extent of receptor activity was determined by measuring alkaline phosphatase activity (absorbance 405 nm). 1, 2, 4, 5, and 7 designate heparin–Sepharose bound aFGF, bFGF, FGF4 (hst/kfgf), FGF5, and FGF7 (KGF), respectively. The results represent the mean and standard deviation of three experiments.

these receptors display affinity for FGF4. None of them binds, however FGF5, and KGFR has high affinity also for FGF7 (KGF). Because FGF7 is not expressed during gastrulation (Bedford *et al.*, in preparation), the most likely common ligand of the FGFR2 variants, KGFR, bek, and at least one variant of FGFR1, could be FGF4. It follows from the restricted expression of FGF4 that this growth factor rather than its receptors could be responsible for pattern formation. Similar observations were made in *Drosophila*, where the receptor tyrosine kinase-like *torso* gene is transcribed uniformly, whereas the spatial restriction of its pattern-forming activity in terminal structures is mediated by the local availability of its ligand (Casanova and Struhl, 1989).

This reasoning is relevant to FGF4 and its possible receptors. Without knowing the binding specificity of two additional fibroblast growth factors, FGF3 and FGF5, which are also expressed during gastrulation (Wilkinson *et al.*, 1988; Haub and Goldfarb, 1991; Hebert *et al.*, 1991), and without more information on the localization of two additional receptors, FGFR3 and FGFR4, in the early mouse embryo, our understanding regarding the functional relevance of FGF and FGFR during mammalian gastrulation cannot be complete.

Alternative Splicing and the Positional Control of FGFR during Organogenesis

A main finding of this study was that the two splice variants of FGFR2 display significantly different expression patterns during organogenesis. Central among our results was the observation that each of the two splice variants occupied characteristic expression domains in different developing organs. Thus KGFR transcripts were present mostly in the surface ectoderm and in the epithelia of the internal spaces, whereas bek transcripts occupied the superficial mesenchyme immediately beneath the area of KGFR expression. This is in agreement with previous data on KGFR and KGF (Rubin *et al.*, 1989; Miki *et al.*, 1992) and indicates that they may be important in the development and function of the skin. Our results also emphasize the involvement of KGFR and bek in the development of various skin derivatives. It is noteworthy that in all organs deriving from the embryonic integument the relative pattern of their spatial regulation, established during early development, is preserved throughout organogenesis.

FGFR1 is transcribed mostly in the deeper mesenchyme, a domain different from that of bek, KGFR (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992) or FGFR4 in the developing muscles (Stark *et al.*, 1991) and of FGFR3 in the central nervous system, the vestibular organ, and during osteogenesis (Peters *et al.*, 1993). It can be assumed that as yet uninvestigated splice variants of these receptors will also exhibit unique localization. It is therefore possible that each FGFR may occupy a unique domain during organogenesis. From this it follows that the multiple FGF–FGFR system may fulfill a multitude of specific roles in the molecular control of organogenesis.

The question arises what mechanism may be responsible for the positional control of FGFR and its variants. We found that the unique spatial regulation of FGFR is preceded during organogenesis by less discriminating patterns in the gastrulating embryo. In the study of the developmental expression of various receptor tyrosine kinases, increasingly specific expression from postimplantation toward advanced organogenesis was a recurrent observation (Orr-Urtreger *et al.*, 1990, 1991, 1992). We think therefore that there may be concrete differences between the regulation of these receptors in early embryos and that in more differentiated embryos and the adult.

Current thinking in developmental biology tends to emphasize the role of transacting transcriptional activators in cell- and tissue-specific gene expression. The variants of FGFR2 studied in this work, however, derive from the same locus and share most potential trans-regulatory elements. Hence their mutually exclusive spa-

tiotemporal specificity, displayed during organogenesis, is most likely controlled by the splicing mechanism (for review, see Smith *et al.*, 1989). The role of alternative splicing in cell- and tissue-specific gene expression has been analyzed in the muscle- and fibroblast-specific tropomyosin variants of the rat (Helfman *et al.*, 1990) and in the sex determination of *Drosophila* (Hodgkin, 1989). It is suggested that intronic splice signals, as well as the cell and tissue specificity and concentration of splicing factors, are responsible for alternative splicing. Our observation emphasizes that ligand binding and spatial specificity can be generated in a receptor gene by the same mechanism, alternative splicing. Because FGFRs are structurally related (Champion-Arnaud *et al.*, 1991; Johnson *et al.*, 1992; Yayon *et al.*, 1992), it is possible that this mechanism should contribute to the spatial and receptor specificity of FGFRs in general.

In summary we conclude that the splice variants of FGFR2 have similar, diffuse expression patterns in the gastrulating mouse embryo, and that the positional control of their most likely ligand, FGF4, may be responsible for their possible pattern-forming activity during this stage. We also show that toward advanced organogenesis the expression of KGFR and bek becomes clearly defined and highly specific. This observation is interpreted to suggest that the positional regulation of FGFR progresses with advanced embryogenesis and that during organogenesis alternative splicing is responsible for the positional regulation of these FGFR variants.

Our thanks to Professor L. T. Williams for showing us his manuscript (Peters *et al.*, 1993) before publication. This work was supported by the Raphael and Regina Levy Fund, by the Pasteur-Weizmann Joint Research Program, by the Center for Molecular Genetics of the Weizmann Institute (to P.L.), and by the Israel Academy of Sciences (to D.G. and A.Y.).

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