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Short Contribution

Expression of FGFR-4 mRNA in developing mouse tissues

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ABSTRACT We have previously reported the isolation of human FGFR-4 cDNA encoding a receptor for aFGF and shown that the expression patterns of FGFR-1 through FGFR-4 mRNAs are overlapping, but distinct in human fetal tissues (Partanen *et al.*, 1991). In order to define cells and tissues expressing FGFR-4 more accurately, we have cloned mouse FGFR-4 cDNA and used it in Northern and *in situ* hybridization of mouse embryonic tissues. Our results show that the 3.5 kb FGFR-4 mRNA is expressed in day 10 to full term embryos and newborn mice, with maximal expression levels during days 14-16 p.c. Adult mice express the FGFR-4 mRNA predominantly in the liver, kidney and lung. In *in situ* hybridization of 10-13 day p.c. mouse embryos, FGFR-4 mRNA is present primarily in tissues of mesodermal origin and in the developing lung and gut. Particularly the mesenchymal tissue in association with the first pharyngeal arch and along the prevertebrae (in developing myotomes) which will differentiate into muscle tissue express high amounts of FGFR-4 mRNA. In addition, the developing metanephros contains FGFR-4 transcripts. These results suggest that FGFR-4 may be particularly important for the differentiation of skeletal muscle and endodermally derived organs.

KEY WORDS: *fibroblast growth factor, fibroblast growth factor receptor, muscle differentiation, endodermal differentiation*

The recently revealed complexity of the fibroblast growth factor receptor (FGFR) system is a puzzle. Seven members of the FGF family have been cloned and only two of them, the acidic and basic FGFs (aFGF and bFGF, respectively) have been reasonably well characterized physiologically (Gospodarowicz, 1974, 1975; Thomas *et al.*, 1980; Gambarini and Armelin, 1982; Delli Bovi *et al.*, 1987; Dickson and Peters, 1987; Yoshida *et al.*, 1987; Zhan *et al.*, 1988; Finch *et al.*, 1989; Marics *et al.*, 1989). The complexity of the FGFRs is also great: five different FGFR polypeptides belonging to the receptor tyrosine kinase family have been cloned (Kornbluth *et al.*, 1988; Ruta *et al.*, 1988; Dionne *et al.*, 1990; Isacchi *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991; Wennström *et al.*, 1991). Besides these, there exists apparently a family of low-affinity receptors, which consist of various cell surface heparan sulfate proteoglycans (Kiefer *et al.*, 1990). Recent studies have shown that binding to the low-affinity receptor is required for interaction of FGFs with the high-affinity signal-transducing receptor (Ruoslahti and Yamaguchi, 1991; Yayon *et al.*, 1991), which may exist in various forms generated by differential splicing (Johnson *et al.*, 1990, 1991; Hou *et al.*, 1991).

We have recently cloned two novel members to the FGFR family from cultured human leukemia cells (Partanen *et al.*, 1990, 1991). Of these we have characterized in detail FGFR-4, which seems to bind preferentially aFGF over bFGF. Our studies suggested a differential expression pattern for FGFR-1 through FGFR-4 in human fetal tissues. In order to further define cells and tissues expressing the FGFR-4 mRNA we have cloned the mouse homologue of the human cDNA and used it in *in situ* hybridization of mouse embryonal tissues. During the preparation of our report on these findings, another group published their *in situ* hybridization experiments using the mouse FGFR-4 probe in 8.5 day and 14.5 day embryos (Stark *et al.*, 1991). We have therefore restricted our report to days 12 and 13 of mouse development.

Total RNA was isolated from 8 day p.c. to 18 day p.c. embryos and newborn as well as 2 day postnatal mice and analyzed by Northern blotting and hybridization with the d11Ga2 probe corresponding to the portion of the cDNA coding for the extracellular domain of FGFR-4. Fig. 1A shows that a 3.5 kb FGFR-4 mRNA signal is first weakly visible in a 10 day p.c. embryo, and increases about five- to tenfold thereafter, being maximal in a 14 day p.c. embryo and declining again in the 17 day sample, remaining barely detectable in the 18 day sample. Interestingly, small amounts of FGFR-4 mRNA are seen in the newborn and 2 day old mice.

RNase protection was used to confirm and extend these findings. As shown in Fig. 1B, this more sensitive technique also detects a protected band of 337 nucleotides in a 10 day embryo, maximal levels again occurring in 14-16 day embryos, with a significant decline, but persistence of the protected band in later samples.

In isolated organs of adult mice, highest amounts of FGFR-4 mRNA were detected in liver and kidney and a somewhat lower level in lung. No expression was present in the heart or brain (Table 1). Also, RNA from mouse NIH 3T3 fibroblasts and MK keratinocytes as

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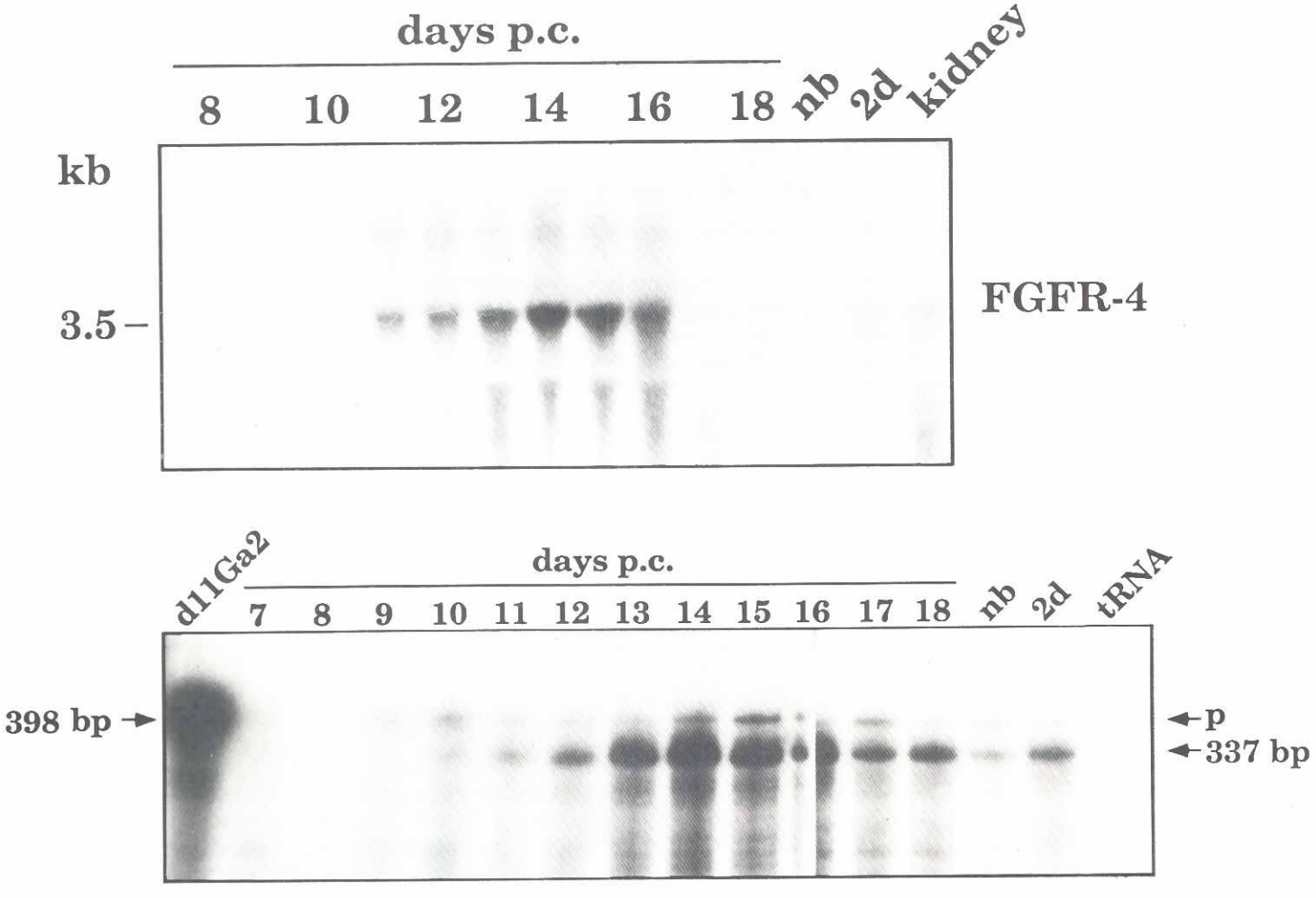

Fig. 1. Expression of FGFR-4 mRNA in 8-18 day p.c. mouse embryos, newborn and 2 days postnatal mice. *Total RNA from whole embryos was extracted and equal amounts were analyzed by Northern blotting and hybridization as described in Materials and Methods* **(A)**. *Kidney RNA from adult mice was used as a positive control. The same samples were also subjected to RNAse protection analysis* **(B)**. *The protected band of 337 nucleotides shows the specific FGFR-4 signal. Note the presence of some remaining undigested probe (p) in several of the lanes.*

well as from various endothelial cells was negative for FGFR-4 mRNA.

In order to analyze the tissue distribution of the FGFR-4 mRNA during embryogenesis, 12 day p.c. mouse embryos undergoing active organogenesis were used. Sagittal and transverse sections were cut and hybridized with the antisense and sense RNA transcribed from linearized d11Ga2 plasmid. Fig. 2A shows the light-field image of a parasagittal section probed with the antisense RNA. The FGFR-4 mRNA signals were visualized in the dark-field microscopy of the same section (Fig. 2B). FGFR-4 transcripts were detected in the mesenchyme along the 1st pharyngeal arch (pa), the developing neck muscles (nm) and in the developing mesenchyme of prevertebrae (pv). Fig. 3 shows that besides the intervertebral mesenchyme (m), the chondrocytes (c) in prevertebra also express FGFR-4, albeit weakly (Fig. 3). The red blood cells in the liver and the pigment granules of the retina have some unspecific refraction which does not correspond to the FGFR-4 signal. Despite this background in liver, weak specific hybridization was also detected at higher magnifications (data not shown). The brain or the spinal cord do not appear to express FGFR-4 in 12 day embryos.

When viewed with higher magnification, the abdominal wall of 13 day p.c. embryo clearly shows the organized structure of three muscle layers (Fig. 4A). All of these layers, external, transverse and internal, have prominent FGFR-4 expression (B and C). As shown in Fig. 5, the epithelial cells of the gut of 13 day p.c. embryo also express FGFR-4.

We have used the mouse FGFR-4 cDNA as a probe for the *in situ* hybridization analysis of FGFR-4 mRNA expression in developing mouse tissues. The mouse cDNA shows a high degree of homology to the corresponding region of the human FGFR-4 cDNA encoding the three extracellular immunoglobulin-like loops of the receptor (Stark *et al.*, 1991 and our unpublished observations). The probe also corresponds to the region we used in our earlier studies on FGFR-4 expression in human fetuses (Partanen *et al.*, 1991). According to our data on 12 day p.c. embryos, a predominant site of FGFR-4 expression is the embryonic mesenchyme differentiating into muscle. Furthermore, the lung, liver and gut epithelia are positive for the FGFR-4 mRNA. These results are consistent with our

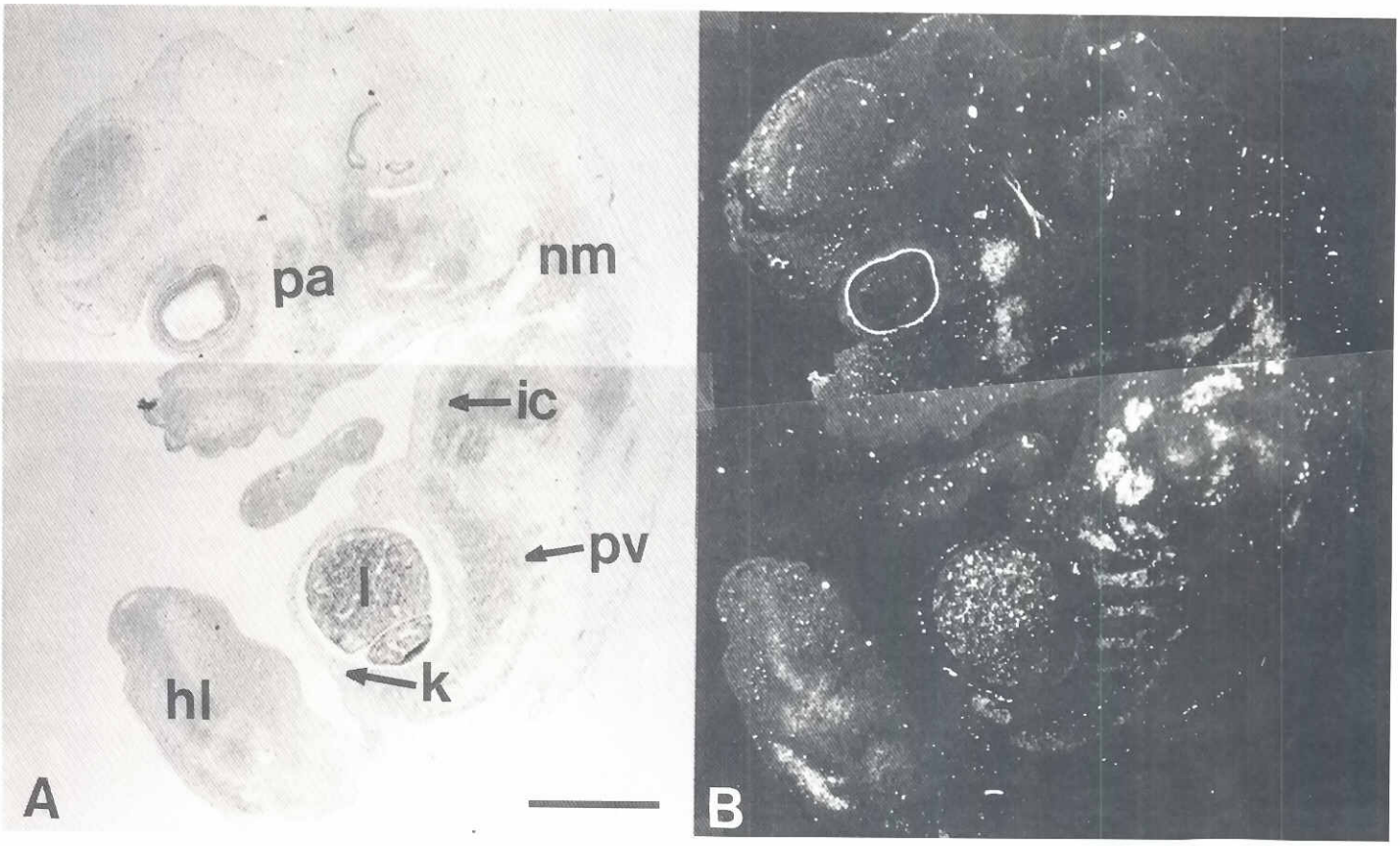

Fig. 2. Light-field (A) and dark-field (B) photomicrographs of a parasagittal section of a 12 day p.c. mouse embryo following *in situ* hybridization with antisense probe. *As can be seen from the figure, FGFR-4 mRNA is present in muscle tissue of first pharyngeal arch (pa), neck muscles (nm), intercostal muscles (ic), myotomes of prevertebra (pv), liver (l), kidney (k) and hindlimb (hl). Weak expression is also detected in cartilage of the ribs and hindlimb and in prevertebrae (see also Fig. 3). Comparison with a similar section hybridized with the sense probe shows that the signal from the eye is nonspecific (resulting from the retinal pigment granules). Also, the signal from the liver is noninformative, because of high background (apparently due to the presence of excessive amounts of red blood cells). Scale bar, 1 mm.*

previous data on FGFR-4 expression in the 17-18 week human fetus, where highest levels of mRNA were seen in the adrenal, lung, striated muscle, intestine, pancreas, kidney and spleen (Partanen *et al.*, 1991). The cDNA fragment used as the FGFR-4 probe covers regions which may be subject to differential splicing. Details of FGFR mRNA splicing have so far been described only for FGFR-1 (Johnson *et al.*, 1990, 1991; Eisemann *et al.*, 1991; Hou *et al.*, 1991). It should be noted that our probe covers the region encoding the extracellular domain of FGFR-4 while Stark *et al.* (1991) used the 3´untranslated region as their probe. Although we have also used embryos of slightly different developmental stage, our results are consistent with their findings.

Our studies and those of Orr-Urtreger *et al.* (1991) show that FGFR-4, FGFR-1 and FGFR-2 have clearly distinguishable spatial patterns of expression during development. FGFR-1 is typically expressed in the undifferentiated mesenchyme, whereas FGFR-2 is preferentially expressed in ectodermally derived epithelial tissues.

Specifically, FGFR-1 was present in the sclerotomal tissue condensating into intervertebral disks, in the facial area in the nasal and maxillar area, tongue and jaws (Safran *et al.*, 1990). Also, FGFR-2 transcripts are present in the vertebral bodies on day 12.5 p.c., whereas FGFR-4 resides in between the vertebrae during this

TABLE 1

FGFR-4 mRNA IN VARIOUS ADULT TISSUES

Tissues	
liver	++
kidney	++
lung	+
heart	-
brain	-
spleen	-
thymus	-
pancreas	-
striated muscle	-
adipose tissue	-
Cell lines	
MK keratinocytes	-
NIH 3T3 fibroblasts	-

Hybridization signals on Northern blots were quantitated by densitometric scanning and normalized for the exact amount of RNA loaded using the ß-actin signal as a standard.

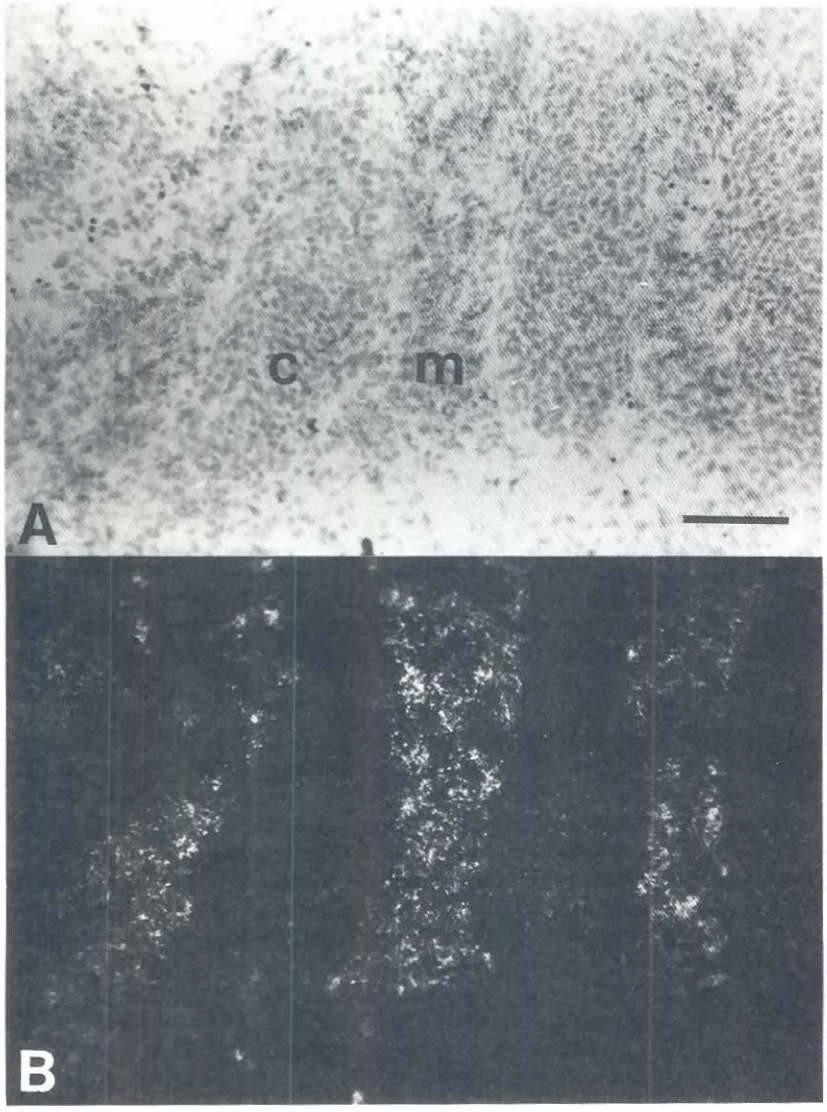

Fig. 3. Light-field (A) and dark-field (B) photomicrographs of sagittally sectioned prevertebrae of a 12 day p.c. mouse embryo. *High expression of FGFR-4 is detected in myotomes (m). Chondrocytes (c) also express FGFR-4, but very faintly. Scale bar, 0.1 mm.*

developmental phase. The most striking feature, however, is the absence of FGFR-4 from the nervous system, where aFGF and both FGFR-1 and FGFR-2 have been detected (Safran *et al.*, 1990; Elde *et al.*, 1991; Orr-Urtreger *et al.*, 1991). For example, FGFR-1 was present in the lateral and fourth chambers of the prospective brain (Safran *et al.*, 1990; Orr-Urtreger *et al.*, 1991). However, we observed FGFR-4 in the developing human cerebellum (Partanen *et al.*, 1991) and Stark *et al.* cloned their probe from a 17.5 day mouse cerebellar cDNA library. Yet, neither we nor Stark *et al.* could detect FGFR-4 mRNA in the developing cerebellum in 8.5 to 14.5 day p.c. mouse embryos. We are analyzing embryos of later developmental stages to clarify this discrepancy.

These studies show that FGFR-4 may be a major FGF receptor of the developing muscle. FGFs may have an important role during myoblast differentiation as they have been shown to prevent the differentiation of cultured myoblasts (Lathrop *et al.*, 1985; Clegg *et al.*, 1987). Furthermore, cell surface heparan sulfate is essential for this effect of bFGF (Rapraeger *et al.*, 1991). It is also of interest that cardiac muscle is negative for FGFR-4 mRNA. Yet, cardiac muscle cells have been shown to respond to aFGF and bFGF (Parker *et al.*, 1990a). In fact, the expression of the fetal/mature cardiac muscle phenotype can be manipulated by FGFs (Parker *et al.*, 1990b).

All FGFRs bind more than one polypeptide growth factor with high affinity. Of the described patterns of FGF expression, FGF-5 and FGF-6 overlap with that of FGFR-4 (Haub and Goldfarb, 1991; Daniel Birnbaum, personal communication). On the other hand, there is no significant correlation with the expression of aFGF (Elde *et al.*, 1991), which is preferentially bound by FGFR-4 (Partanen *et al.*, 1991). Determination of the role of FGFR-4 in these tissues must wait for experiments specifying the more complete ligand-binding spectrum of this receptor.

Experimental Procedures

Cloning of partial cDNA for mouse FGFR-4

Approximately 2 x 10^6 plaques from two different λgt10 libraries (a kind gift of Dr. Brigitte Galliot, Zentrum für Molekülarbiologie Heidelberg, Germany) prepared from 10 and 11 day post coitum (p.c.) mouse embryonic mRNA

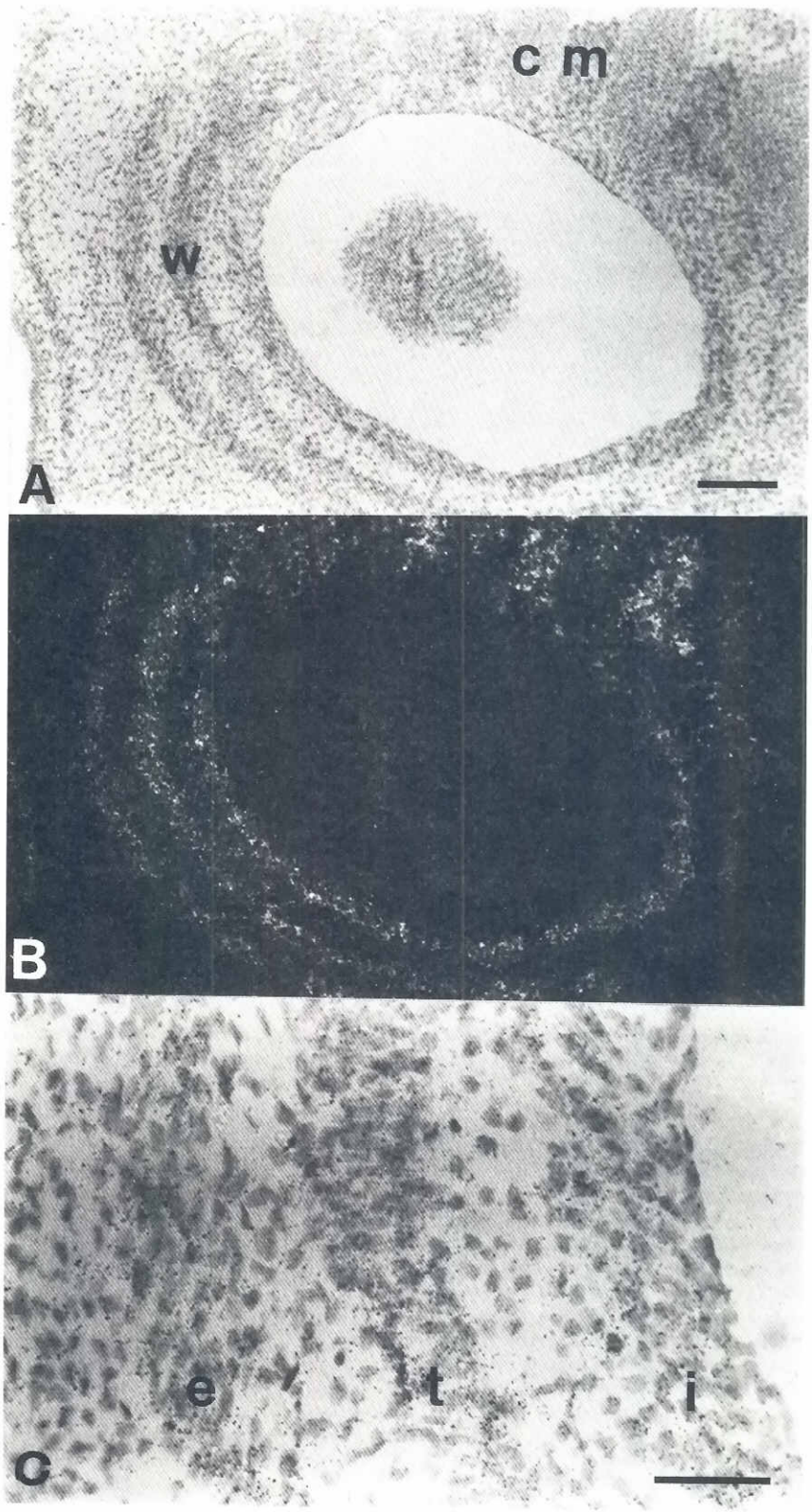

Fig. 4. Expression of FGFR-4 mRNA in prevertebral myotomes (m) and chondrocytes (c) and in three separate muscle layers of the abdominal wall (w). *The tissue in the center is liver. Scale bar, 0.1 mm. (C) shows a high power light-field view of muscles of the abdominal wall. The autoradiographic grains can clearly be seen over the elongated cells developing into muscle (m). Marked are the internal (i), transverse (t) and external (e) muscles. Scale bar, 50 μm.*

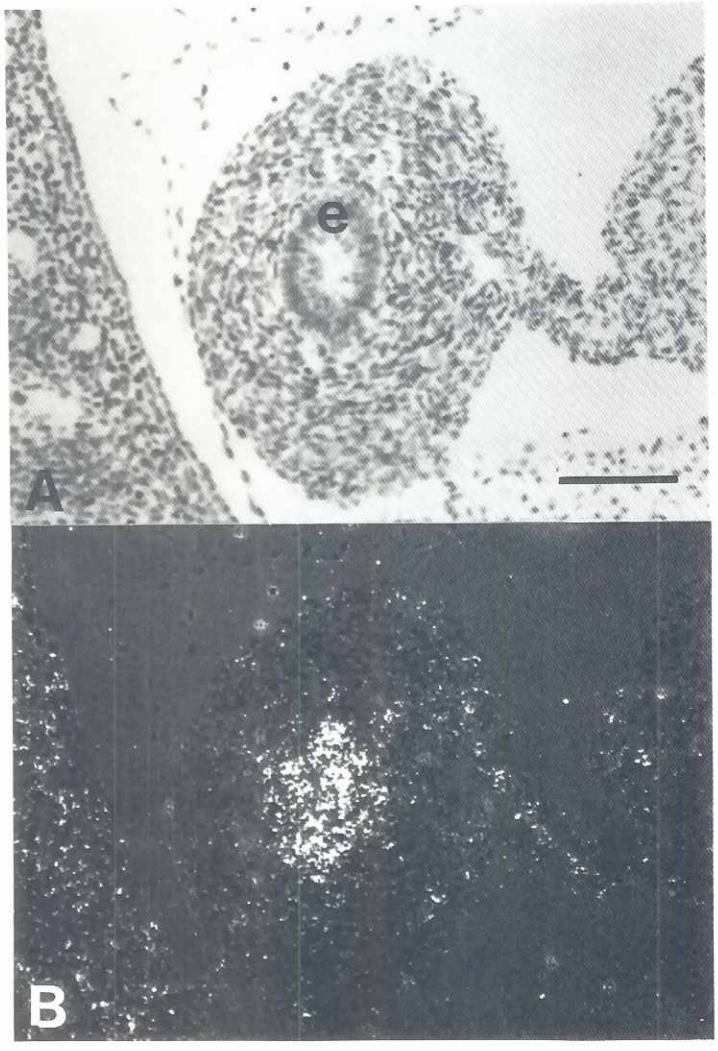

Fig. 5. FGFR-4 expression in epithelial cells of the gut of a 13 day p.c. mouse embryo. *Only the epithelial cells (e) are positive for FGFR-4 mRNA. Scale bar, 50 μm.*

were screened with a human HE 6-2 fragment (this cDNA encodes the immunoglobulin-like domains I-III of the extracellular part of the FGFR-4; Partanen *et al.*, 1991). These cDNA segments have only little homology with other known genes. The probe was labeled with [α^{35}P]dCTP by the random priming method. The nitrocellulose replicas of each phage-infected plate were hybridized in 50% deionized formamide, 5xDenhards´ solution, 5xSSPE, 0.1% SDS and 100 μg/ml ssDNA. One positive clone d11G2 was purified and subcloned into pGEM 3Zf(+) (Promega) for use as a probe. Nucleic acid sequence of this clone showed that it is about 90% identical with the corresponding human cDNA and corresponds to nucleotides 165-1002 of the human FGFR-4 cDNA sequence reported by Partanen *et al.* (1991).

Isolation and analysis of RNA

Total RNA was isolated from adult mouse organs and developing embryos (8-18 days p.c. and newborn) according to Chirgwin *et al.* (1979). Poly(A)+ RNA (5μg) and total RNA (10μg) were electrophoresed in 0.8% agarose gels containing formaldehyde and blotted into Hybond-N (Amersham) filters using standard conditions. After transfer the filters were exposed to ultraviolet radiation for 4 min, hybridized and washed in stringent conditions (Sambrook *et al.*, 1989).

RNAse protection analysis

A cRNA probe of 398 bp was generated using linearized plasmid, SP6 polymerase and [^{32}P]-UTP (Melton *et al.*, 1984). The labelled cRNA was purified in a 4% sequencing gel. 2 μl of labeled probe was added to each RNA sample and hybridized at 53°C overnight. Unhybridized RNA was digested with RNAse U2 (10 U/μl) and T1 (1 μg/ml) at 30°C and at pH 4.8 for 1 h. The RNAses were inactivated by Proteinase K digestion at 37°C for 15 min and phenol/chloroform extraction, precipitated with ethanol and dissolved in 5 μl of stop solution (from the Sequenase kit, USB). The samples were analyzed in 8% sequencing gels.

Mouse embryos and organs

Mouse embryos of 10-13 day p.c. were derived from matings of CBA and NMR mice. Embryonic age was calculated from the day on which the copulation plug was detected as day 0 (estimated copulation time 2 a.m.). Pregnant mice were killed by cervical dislocation, the embryos were removed and transferred immediately via phosphate buffered saline (PBS) into 4% paraformaldehyde in PBS, pH 7.2. The embryos were fixed for 18 h at 4°C, dehydrated, embedded in wax (Fisher Scientific Co) and cut into 6 μm sections. Isolated mouse organs were treated similarly.

In situ *hybridization*

The cRNA probes of 398 and 808 bp (antisense and sense) were generated by using linearized plasmid and SP6 and T7 polymerases and (^{35}S)-UTP. *In situ* hybridization of sections was performed according to Wilkinson (1987a,b) with following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax, 2) 6 μm sections were cut and placed on a layer of diethyl pyrocarbonate-treated (DEPC) water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine (TESPA) (Sigma), 3) alkaline hydrolysis of the probes was omitted 4) the hybridization mixture contained 60% deionized formamide, 5) the high stringency wash was for 80 min at 65°C in solution containing 50 mM DTT and 1 x SSC, 6) the sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. After an exposure time of 14 days the slides were developed for 2.5 min in Kodak D-19 developer and fixed for 5 min with Unifix (Kodak). The sections were stained with 0.2% toluidine blue in water. Control hybridizations with sense strand and RNAse A-treated sections did not give a specific signal above background.

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