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Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse

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Summary

***Fgf-4*, initially isolated as a transforming gene from human tumors, is a member of the Fibroblast Growth Factor (FGF) family. It has previously been shown by northern blot hybridization analysis to be expressed in teratocarcinoma and embryonic stem cells, suggesting that it plays a role in embryonic development. We have carried out an RNA in situ hybridization analysis of *Fgf-4* expression in the developing mouse embryo, from fertilization through the 14th day of gestation (E14.5). Our results show that *Fgf-4* RNA is first detected at the late blastocyst stage in cells that give rise to all of the embryonic lineages (inner cell mass cells). During the early stages of gastrulation, expression becomes restricted to the primitive streak where mesoderm and definitive endoderm are formed. Expression continues in the distal (rostral) two-thirds of the streak through approx. E10, and then is detected in the tail bud, which replaces the streak as the primary source of mesoderm. Additional sites of expression are found after the three primary germ layers are established and organogenesis begins. *Fgf-4* RNA is detected transiently in the branchial arch units, the somitic myotome, the apical ectodermal ridge of the developing limb bud and the tooth bud, suggesting that the gene has multiple roles during embryogenesis. These results are compared with the expression patterns of other FGF genes. Taken together, the data suggest that individual members of the gene family are expressed sequentially in developmental pathways such as mesoderm formation and myogenesis, and play a role in specific epithelial-mesenchymal interactions.**

Key words: fibroblast growth factor, *Fgf-4*, *kFGF*, *hst-1*, mouse embryogenesis, gastrulation, limb bud, myogenesis, tooth bud.

Introduction

In mammals, there are presently seven genes that have been classified as members of the FGF family on the basis of conserved coding sequence (reviewed by Goldfarb, 1990). These are being renamed with the numerical designations *Fgf-1* through *Fgf-7* (Baird and Klagsbrun, 1991), and that nomenclature will be used here. The observation that the proteins encoded by at least four members of this gene family can induce *Xenopus* animal caps to form mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1987, 1988; Paterno et al., 1989) has raised considerable interest in the prospect that the FGF proteins, alone or in combination, could play a role in mesoderm formation in vivo. The recent demonstration that inhibition of *Xenopus* FGF-receptor function results in abnormal development during gastrulation and a loss of posterior structures (Amaya et al., 1991) strongly supports the view that an FGF signaling pathway is important for gastrulation and normal embryonic pattern formation in vertebrates. However, since those experiments presumably result in perturbation of all FGF ligand/receptor interactions, that study does not provide information on which specific member(s) of the family are involved. In addition, considerable evidence suggests that FGF family members have diverse functions later in embryonic and post-natal development, which include influences on cell survival, proliferation and migration, and depending on the context in which they are expressed, as inducers or repressors of cell differentiation (reviewed by Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). To begin to determine the function of these signaling molecules at various stages of development it is important to identify the tissues and cell types in which each family member is expressed.

RNA expression patterns in the mouse embryo have been examined for two FGF family members, *Fgf-3* (*int-2*; Wilkinson et al., 1988, 1989) and *Fgf-5* (Haub and Goldfarb, 1991; Hébert et al., 1991). These genes are expressed and presumably function during gastru-

lation and also later in embryogenesis. We report here studies on the expression of the *Fgf-4* gene, which was isolated as a transforming gene from human stomach tumors and Kaposi sarcomas and hence was known as the oncogene *hst*, *hst-1*, *kFgf* or KS3 (Delli Bovi et al., 1987; Taira et al., 1987). Subsequently, *Fgf-4* RNA was detected in male germ cell tumors (Yoshida et al., 1988), as well as in murine embryonic stem (ES) cells and embryonal carcinoma (EC) cells (Heath et al., 1989; Velcich et al., 1989; Hébert et al., 1990), which are capable of differentiating in vitro in a manner that mimics the behavior of early embryonic cells. The latter data suggested that *Fgf-4* expression is important during early embryonic development. As a step toward defining the role of *Fgf-4* during embryogenesis, we have carried out RNA in situ hybridization experiments that identify the developmental periods during which *Fgf-4* is expressed and the cell types in which the gene is transcribed.

Materials and methods

Embryos were obtained from random bred Swiss Webster mice (Simenson Laboratories, Gilroy, CA). Noon of the day on which the copulation plug was detected was considered to be 0.5 days of gestation (E0.5), although some variation was observed in developmental stage both between and within litters at the given embryonic ages. Gastrulating embryos were staged according to Lawson and Pedersen (1987). In experiments at the preimplantation stages, the embryos (8-cell through blastocyst) were flushed from the reproductive tracts of appropriately staged pregnant females, pooled, transferred to a single uterus and fixed in 4% buffered paraformaldehyde. Embryos at later stages were either fixed within the decidua (early postimplantation stage embryos) or dissected from the implantation site and then fixed. All samples were dehydrated, embedded in paraffin wax and sectioned (6 μm).

Two regions of the *Fgf-4* gene were used as probes: the full length coding sequence (Hébert et al., 1990) cloned into Bluescript (Stratagene, La Jolla, CA), and the 3′-untranslated (UT) portion from nucleotides 3630-3819 (Brookes et al., 1989), kindly provided by Dr. C. Basilico and cloned into pGEM-3 (Promega, Madison, WI). In every experiment, alternating slides were hybridized with one or the other of these probes and identical results were obtained, making it very unlikely that we were detecting transcripts of other FGF gene family members, in particular that of the *Fgf-6* gene, which is the most similar to *Fgf-4* (de Lapeyriere et al., 1990). Furthermore, both probes detect a single band on northern blots of ES cell RNA (data not shown).

Other probes used in this study included a *Fgf-3* cDNA (pInt-2 C.28) that spans the entire coding region (Mansour and Martin, 1988), a mouse α-cardiac actin (*Actc-1*) cDNA (Garner et al., 1986) kindly provided by J. Hébert, and a *MyoD1* cDNA (pVZC11; Davis et al., 1987) kindly provided by Dr. C. Ordahl.

Antisense probes were generated after linearization of plasmid DNA by restriction enzyme digestion and *in vitro* transcription by T7, T3 or SP6 RNA polymerase in the presence of [^{35}S]-UTP. High stringency hybridizations were performed as described by Frohman et al. (1990). RNA probes were routinely used at a concentration of 1×10^4 cts per min/μl. The slides were developed and stained after an exposure of approximately 3 weeks for *Fgf-4*, 1 week for *Actc-1* and 2 weeks for *Fgf-3* and *MyoD1*.

Results

Fgf-4 *expression before and during gastrulation*

In situ hybridization using *Fgf-4* probes was carried out on sections of ovaries containing developing oocytes, and on preimplantation embryos at the 8-cell, morula and blastocyst stages. *Fgf-4* transcripts were first detected in late blastocysts (E4.5). At that stage, *Fgf-4* RNA was observed at high levels within the embryonic ectoderm, but was not detected in the extraembryonic tissues, trophectoderm and primitive endoderm (Fig. 1C, D). No *Fgf-4* RNA was detected above background levels in blastocysts prior to primitive endoderm formation, in embryos at earlier stages of development, or in any cells within the ovary (Fig. 1A, B and data not shown). *Fgf-4* RNA was also not detected in testes or male germ cells (data not shown).

During early postimplantation stages (E4.5 - E6.0), uniform expression of *Fgf-4* continues in the embryonic ectoderm (epiblast; Fig. 2A, B). As development

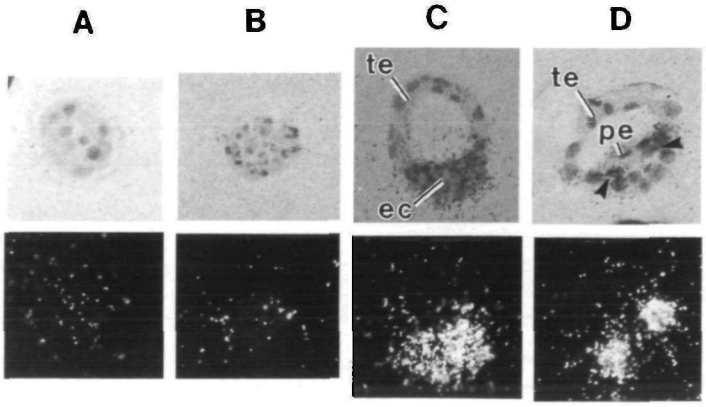


Fig. 1. Expression of *Fgf-4* in the pre-implantation embryo. In situ hybridization of antisense *Fgf-4* RNA to sections through: (A) an early morula; (B) a late morula; (C, D) late blastocysts. The top row shows sections viewed in bright-field illumination, the bottom row shows the corresponding dark-field images. Arrowheads in D point to the two embryonic ectoderm cells present in this section. ec, embryonic ectoderm; pe, primitive endoderm; te, trophectoderm. Magnification, 291 ×.

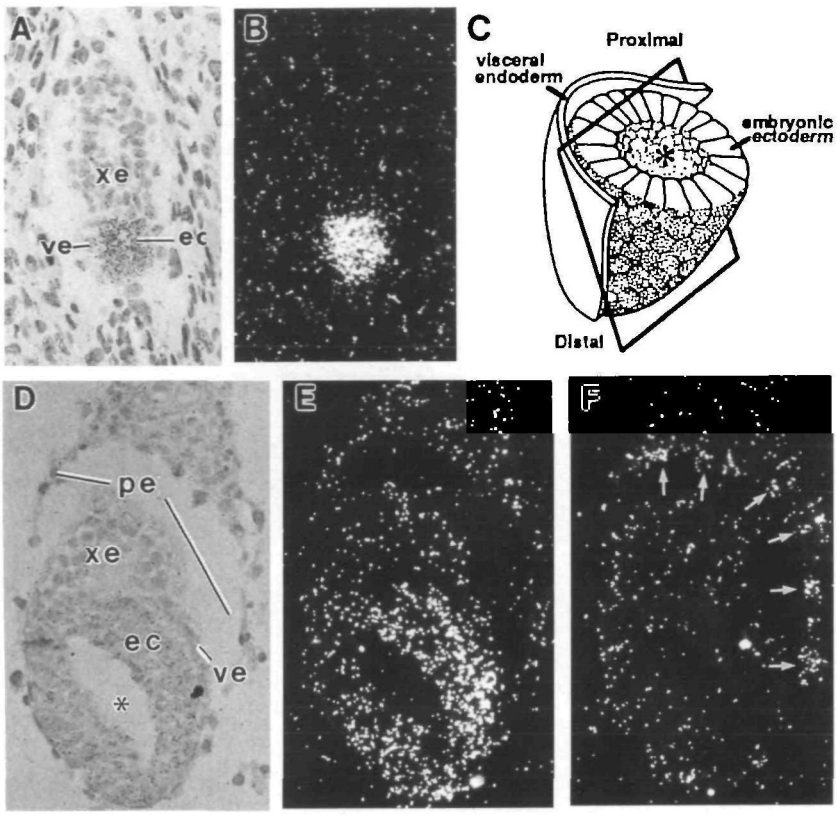


Fig. 2. Localization of *Fgf-4* transcripts in the early postimplantation embryo, prior to the start of gastrulation. (A) Hybridization of antisense *Fgf-4* RNA to a longitudinal section through an implantation site containing an embryo that has not yet cavitated (~E5.5), viewed in bright-field illumination. (B) Same, dark-field image. (C) Schematic representation of a cavitated pre-streak embryo (asterisk [*] indicates the proamniotic cavity), cut transversely at the embryonic-extraembryonic border. The visceral endoderm has been partially removed to reveal the underlying embryonic ectoderm (epiblast). The plane of section for D - F is indicated. (D) Section through an ~E6.25 embryo hybridized with an antisense *Fgf-4* probe, viewed in bright-field illumination. As shown in C, the plane of section is slanted at an ~80° angle to the proximodistal axis and thus passes very obliquely through the extraembryonic ectoderm. (E) Same, dark-field image. (F) Hybridization with an antisense *Fgf-3* (*int-2*) probe to a section adjacent (6 μm) to the one shown in D, E, viewed in dark-field. Arrows point to parietal endodermal cells in which *Fgf-3* RNA is detected. ec, embryonic ectoderm; pe, parietal endoderm; ve, visceral endoderm; xe, extraembryonic ectoderm; *, proamniotic cavity. Magnification, 200 ×.

proceeds, the epiblast is transformed from a solid ball of rounded cells into a cup-shaped columnar epithelium surrounding the proamniotic cavity (Fig. 2C) and, as gastrulation begins, cells within the epiblast move towards and begin to accumulate in the region known as the primitive streak (Fig. 3A). Two changes in the pattern of expression are detected in the epiblast shortly before the start of gastrulation (approx. E6.25). First, the level of expression throughout the epiblast appears to decrease. It is not known if this reflects a uniform decrease in the level of mRNA in the epiblast or if only a sub-population of cells is affected. Second, *Fgf-4* RNA is no longer detected above background levels in cells on one side of the epiblast at the embryonic/extraembryonic border (Fig. 2D, E). Throughout this period of development, *Fgf-4* expression is not detected in either the extraembryonic endodermal tissues (visceral or parietal endoderm) derived from the primitive endoderm of the late blastocyst or in the trophectoderm-derived extraembryonic ectoderm (Fig. 2A, B, D, E and data not shown).

A short time after the primitive streak first appears, *Fgf-4* expression becomes even more restricted. Reconstructions of transverse and sagittal serial sections of embryos at E6.75 - E7.0 (not shown) demonstrated that *Fgf-4* RNA could only be detected in the distal (rostral) two-thirds of the primitive streak and in ectodermal cells immediately adjacent to it. This pattern of expression continues as the primitive streak extends to the distal tip of the embryo and the lateral wings of mesoderm emerging from it meet in the midline at E7.5. Sagittal sections of an approx. E7.25 embryo (Fig. 3B, C) illustrate the observation that *Fgf-4* expression is detected throughout most of the primitive streak, except near the embryonic/extraembryonic border. Transverse sections further illustrate that in the ectoderm, *Fgf-4* RNA is limited to cells adjacent to positive regions of the primitive streak (Fig. 3D, E).

Cells exiting the primitive streak form either definitive endoderm, which incorporates into and replaces the outer visceral endoderm cell layer, or mesoderm. In embryonic mesoderm, *Fgf-4* RNA is not observed as the cells move laterally around the embryo (Fig. 3A, D, E), except in those mesodermal cells emerging from the distal (rostral) regions of the primitive streak where expression continues within 4-6 cell diameters of the streak (data not shown). Fate-mapping experiments (Tam and Beddington, 1987) have shown that cells emerging from different parts of the streak are fated to form different types of mesoderm. Taken in conjunction with those studies, the data reported here indicate that *Fgf-4* is expressed in the region of the streak fated to give rise to axial, paraxial, and lateral plate mesoderm, but is absent in the region that contains primarily prospective extraembryonic mesoderm. *Fgf-4* expression was never detected in any extraembryonic cells, in definitive endoderm, or in the head process, which contributes cells to the notochord as well as to the definitive endoderm (data not shown).

During the period between E7.5 and E11.5, when the majority of the trunk is generated from a small population of embryonic ectodermal cells and from the primitive streak, *Fgf-4* RNA continues to be expressed within the primitive streak and within the tail bud, when

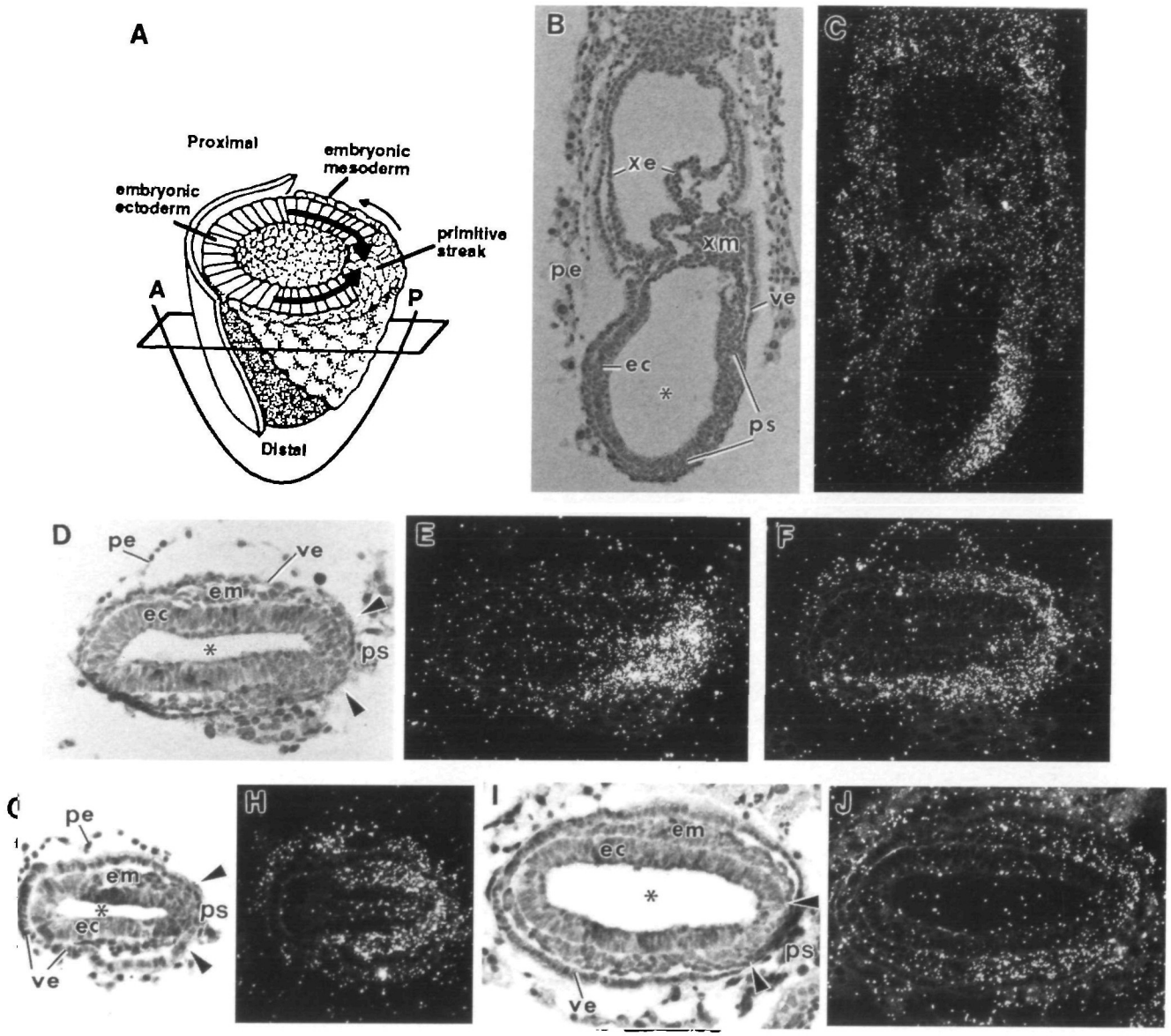


Fig. 3. *Fgf-4* and *Fgf-3* expression during gastrulation. (A) Schematic representation of a mid-streak (~E7.25) embryo. The visceral endoderm has been partially removed to reveal the underlying epiblast and mesoderm. The curved, thick arrows indicate the movement of embryonic ectoderm cells toward the primitive streak; the thin arrow represents the direction of movement of one of the lateral wings of embryonic mesoderm. The curved line represents the A (Anterior) - P (Posterior) axis. The plane of section for D - F, I, J is indicated. (Adapted from Hébert et al., 1991.) (B) Hybridization of antisense *Fgf-4* RNA to a slightly oblique sagittal section through the primitive streak of an embryo at ~E7.25, viewed in bright-field illumination. (C) Same, dark-field image. (D) Hybridization of an antisense *Fgf-4* probe to a transverse section through a mid-streak embryo (~E7.25), bright-field view. The plane and level of section are approximately those shown in A. (E) Same, dark-field image. (F) Hybridization of an antisense *Fgf-3* (*int-2*) probe to a section adjacent (6 μm) to the one shown in D, E, viewed in dark-field illumination. (G) Transverse section through an early-streak embryo (~E6.75). The section is cut roughly midway along the primitive streak. Hybridization with antisense *Fgf-3* RNA, viewed in bright-field illumination. (H) Same, dark-field image. (I) Hybridization with antisense *Fgf-3* RNA to a transverse section through a late-streak embryo (~E7.5) embryo viewed in bright-field illumination; the plane and level of section are approximately those shown in A. (J) Same, dark-field image. ec, embryonic ectoderm; em, embryonic mesoderm; pe, parietal endoderm; ps, primitive streak; ve, visceral endoderm; xe, extraembryonic ectoderm; xm, extraembryonic mesoderm; *, proamniotic cavity; arrowheads in D, G, and I indicate the approximate lateral limits of the primitive streak. Magnification, B, C: 171 ×; D - J: 190 ×.

it replaces the primitive streak as the primary source of mesoderm (data not shown). In the tail bud, *Fgf-4* RNA is detected at moderate levels overall and is additionally observed at very high levels within scattered individual cells (data not shown). The identity of the highly-expressing cells is not known.

Complementarity of Fgf-3 *and* Fgf-4 *RNA expression during gastrulation*

The previously published data on *Fgf-3* (*int-2*) RNA localization in the early embryo (Wilkinson et al., 1988), suggest that this gene may be expressed in a pattern that is complementary to what we observed for *Fgf-4*, with each gene identifying different regions or cell types in the embryo. To determine whether this is indeed the case, we undertook a detailed analysis of *Fgf-3* and *Fgf-4* gene expression during gastrulation. Comparison of serial sections cut in various orientations and alternately hybridized with probes for *Fgf-3* or *Fgf-4*, revealed that there was minimal overlap in the domains of expression of these two genes.

In the pre-streak embryo (E6.0 - E6.5), *Fgf-3* RNA is detected above background levels only in parietal endoderm cells, which are primitive endoderm-derived cells that line the inner surface of the trophectoderm (Fig. 2F). During early-streak stages (E6.5 - E6.75) *Fgf-3* transcripts are detected in mesoderm that has migrated into the extraembryonic region, as well as in embryonic mesoderm (data not shown). Towards the end of this period, *Fgf-3* RNA is detected in embryonic mesoderm as it emerges along the length of the streak and spreads laterally around the epiblast (Fig. 3G, H). As development proceeds and the mesoderm continues its migration around the epiblast, transcripts continue to be detected in cells exiting the streak; however, the level of expression decreases in cells near the leading edge of each mesodermal wing (Fig. 3F, and data not shown). By the late-streak stage (approx. E7.5), *Fgf-3* RNA is detected in the embryo proper only in mesoderm that extends out from the primitive streak 1/2-3/4 of the distance around the epiblast, and is not detected in the anterolateral mesoderm (Fig. 3I, J). In contrast, in the extraembryonic region, *Fgf-3* RNA remains detectable in mesoderm around the entire circumference of the exocoelomic cavity (data not shown).

A comparison of our observations on the *Fgf-3* and *Fgf-4* genes reveals the complementarity of their RNA expression patterns. *Fgf-4* RNA is expressed in ectodermal cells near and in the streak itself, whereas *Fgf-3* RNA is expressed at high levels in mesodermal cells as they exit the streak and move laterally. The only region in which both genes are clearly expressed is at the distal (rostral) end of the streak, in the mesoderm cell population exiting the streak and anterolateral to it. However, we cannot rule out the possibility that there are other regions, in or very near the streak, in which both genes are also expressed.

During the period from E7.5 - E8.5, *Fgf-4* and *Fgf-3* mRNA expression patterns in the region of the primitive streak are similar to those described above (data not shown). These complementary patterns of expression may continue to even later stages, since Wilkinson et al. (1988) have reported high levels of *Fgf-3* expression in the primitive streak region to at least E9.5, and we have observed *Fgf-4* expression in the streak and tail bud through E11.5.

Fgf-4 *expression later in development*

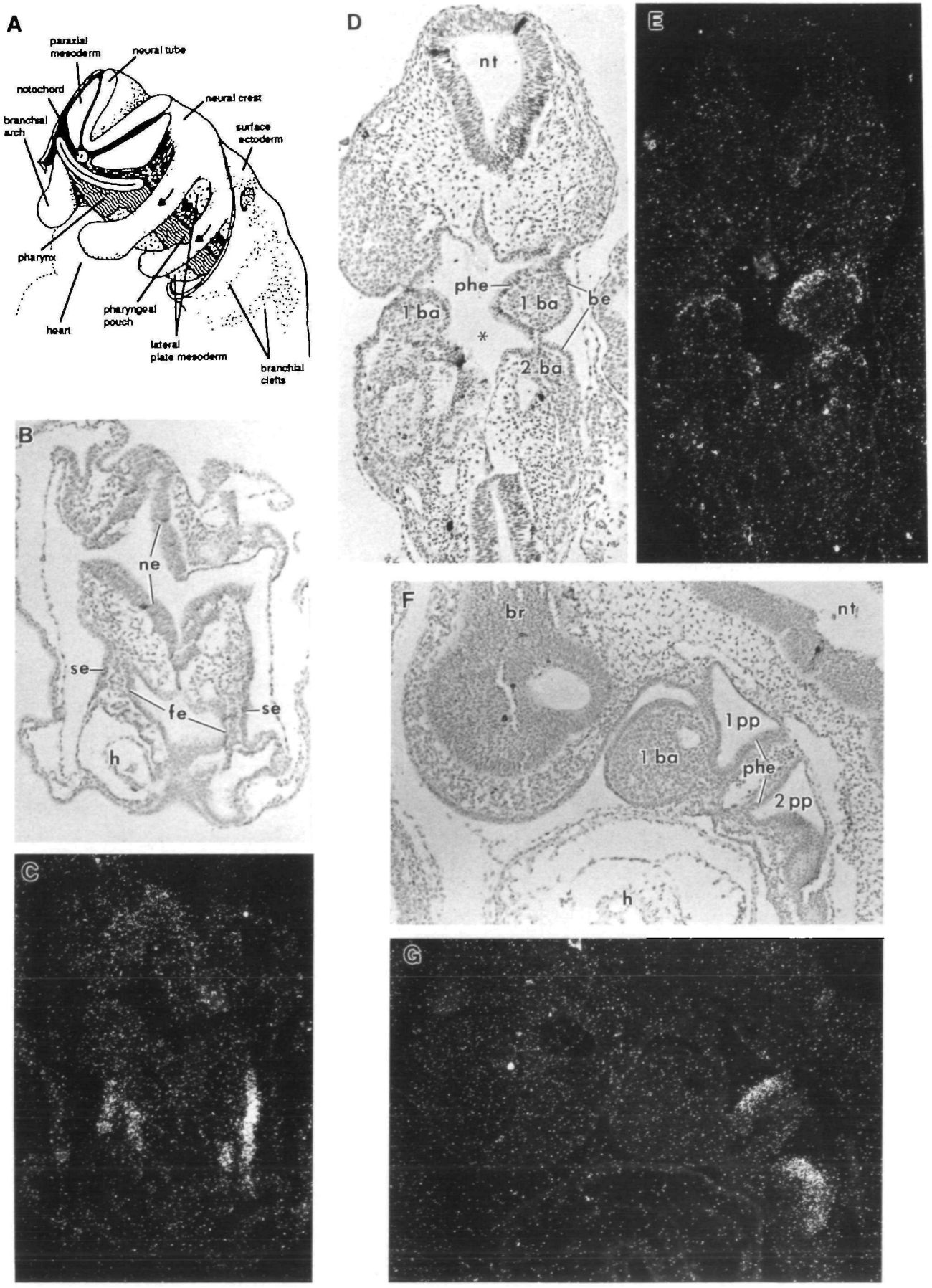
Once the three primary germ layers have been established, a series of complex morphogenetic movements, tissue interactions and cell differentiations quickly brings about the formation of a recognizable head, body and tail. During this period, *Fgf-4* RNA additionally becomes expressed at several discrete sites in the developing embryo.

Expression in the branchial arches and pharyngeal pouches

Beginning at approx. E9, the branchial arches are formed by neural crest cells migrating between the surface ectoderm and the outpouchings of the foregut known as the pharyngeal pouches (Fig. 4A). The branchial arch units thus formed (composed of surface ectoderm, pharyngeal endoderm and neural crest-derived mesenchyme) will give rise to components of the head and throat. By E8.5, high levels of *Fgf-4* RNA are detected in surface ectoderm that will presumably give rise to surface ectoderm covering the branchial arches and in foregut endoderm that will give rise to pharyngeal pouch endoderm (Fig. 4B, C). At E9.5, *Fgf-4* RNA is found in surface ectoderm covering the anterior half of the 1st (mandibular) and 2nd (hyoid) branchial arches (Fig. 4D, E). In the pharyngeal pouch endoderm, expression is limited to the lower surface of the 1st and 2nd pouch, and to the most lateral aspects of these outpocketings, and is not expressed in the medial aspects of the pouch or in the pharnyx (Fig. 4D-G and data not shown). As development progresses (E10.0) and the third pharyngeal pouch forms caudal to pouches 1 and 2, *Fgf-4* RNA is expressed in a similar pattern in the endoderm; however, expression is not detected in surface ectoderm covering any of the branchial arches (data not shown). By E10.5, *Fgf-4* RNA is barely detectable in the endoderm of any of the pharyngeal pouches. Throughout the embryonic stages analyzed, *Fgf-4* and *Fgf-3* are expressed coordinately only in the pharyngeal endoderm; however transcripts of *Fgf-4* are detected a day earlier (E8.5) than those of *Fgf-3* (E9.5; our observations, and Wilkinson et al., 1988). *Fgf-4* RNA was not detected in the brain throughout the periods examined (E8.0-E14.5), whereas *Fgf-3* is expressed at high levels in the hindbrain (E8.5-E9.5) and in cells of the cerebellum (Wilkinson et al., 1988, 1989).

Expression during myogenesis

Around E8.0, the paraxial mesoderm that emerged from the rostral regions of the primitive streak begins to condense into somites. Shortly after condensation, somite differentiation begins, leading to formation of sclerotome, dermatome and myotome (Fig. 5A, B). *Fgf-4* RNA could not be detected in condensed somites at E8.5 (data not shown). By E9.25 (23-24 somite stage), *Fgf-4* transcripts can be detected in myotome cells in mature somites that have undergone differentiation to form all three cell types (Fig. 5C, D). Alternate serial sections were also hybridized with a probe for α-cardiac actin (*Actc-1*) RNA, expression of


A
paraxial mesoderm
neural tube
notochord
neural crest
branchial arch
surface ectoderm
pharynx
heart
pharyngeal pouch
lateral plate mesoderm
branchial clefts
B
ne
se
fe
se
h
C
D
nt
phe
1 ba
1 ba
be
*
2 ba
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F
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Fig. 4. *Fgf-4* expression during branchial arch unit formation. (A) Schematic diagram of an embryo at ~E9.0, showing the branchial arches, which arise from neural crest cells migrating (small arrows) between the surface ectoderm and outpouchings of the foregut, the pharyngeal pouches. (Adapted from Frohman et al., 1990). (B) Transverse section through an embryo at ~E8.5 hybridized to an antisense *Fgf-4* probe, bright-field illumination. (C) Same, dark-field image. (D) Frontal section through an ~E9.5 embryo hybridized to an antisense *Fgf-4* probe, bright-field illumination. (E) Same, dark-field image. (F) Parasagittal section through the first and second pharyngeal pouches of an ~E9.5 embryo hybridized to an antisense *Fgf-4* probe, bright-field illumination. (G) Same, dark-field image. Abbreviations and symbols: br, brain; be, branchial arch ectoderm; fe, foregut endoderm; h, heart; ne, neuroectoderm; nt, neural tube; phe, pharyngeal endoderm; se, surface ectoderm; *, foregut; 1 ba, first branchial arch; 2 ba, second branchial arch; 1 pp, first pharyngeal pouch; 2 pp, second pharyngeal pouch. Magnification, 94 ×.

which is restricted to the myotome and cardiac and skeletal muscle. This probe clearly identified the population of cells in which *Fgf-4* is expressed as myotome cells (data not shown). From E10.0 (30-32 somite stage) to E11.5, expression continues in myotomal cells, but *Fgf-4* RNA becomes limited to a sub-population of the myotome, as illustrated by the observation that only a fraction of the cells that are positive for *Actc-1* RNA are also *Fgf-4*-positive (Fig. 5E, F and H, and data not shown). Sections cut in various orientations revealed a stripe of *Fgf-4*-positive myotome cells, which is centrally positioned with respect to the craniocaudal axis and runs the entire length of the dorsovental axis; myotome cells on both the cranial and caudal side of the stripe of *Fgf-4*-expressing cells are positive for *Actc-1* RNA (Fig. 5H). In addition, *Fgf-4* expression is found only in the myotomal compartment and not in muscle masses that derive from it. By E14.5, *Fgf-4* RNA cannot be detected in the somitic myotome or its derivatives, the skeletal muscle (data not shown).

Previous studies have demonstrated that *MyoD1*, one of the genes that regulates early myogenic differentiation (Davis et al., 1987), is first expressed at E10.5 in the myotome (Sassoon et al., 1989) and its transcription can be repressed by treatment with FGF-2 (bFGF; Vaidya et al., 1989). The finding that *Fgf-4* is expressed a day earlier than *MyoD1* and then becomes restricted within the myotome prior to the time *MyoD1* RNA is detected suggests that *Fgf-4* could regulate *MyoD1* expression in vivo. To extend these findings we compared the expression pattern for *Fgf-4* and *MyoD1* by in situ hybridization to alternate sections through the myotome at E10.5 and E11.5. The expression patterns at E10.5 indicate that these genes are expressed in apparently different regions of the myotome (Fig. 5F, G). By E11.5, high levels of *MyoD1* RNA are detected throughout the myotomal compartment and in muscle anlagen and its expression correlates with that of *Actc-1* (Sassoon et al., 1989; and data not shown). At this time, the regions in which *Fgf-4* and *MyoD1* are expressed appear to overlap. However, since both genes are expressed in a 'salt and pepper' pattern, it is possible that the two genes are not expressed in the same cells. Moreover, outside of the region where *Fgf-4* RNA is found, *MyoD1* RNA is present at uniformly high levels, again suggesting that *Fgf-4* and *MyoD1* transcription does not occur in the same cell (data not shown).

Expression in early limb buds

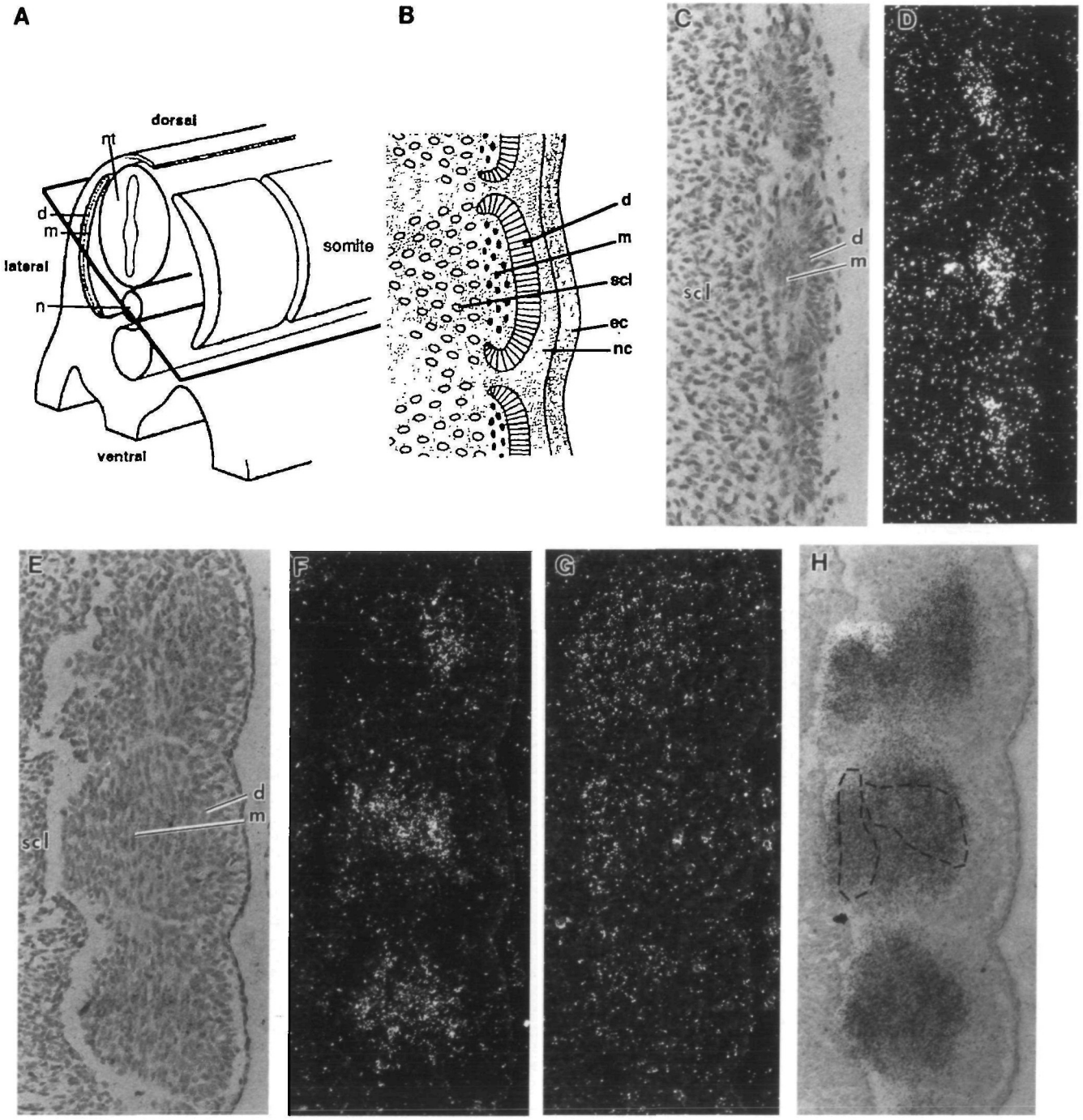
In the mouse, forelimb bud formation begins at approx. E9.5, through proliferation and outgrowth of lateral plate mesenchyme at the level of the 8th-12th somites. By E10.0 - E10.5, the undifferentiated mesenchyme induces overlying ectoderm along the apical, anteroposterior margin to thicken and form a morphologically distinct structure, the apical ectodermal ridge (AER). During limb bud formation, *Fgf-4* RNA is first detected in cells of the newly formed AER in E10.0 forelimbs (30-32 somite stage); no expression is detected in limb mesenchyme (data not shown). A half-day later, *Fgf-4* RNA is expressed at very high levels in the AER (Fig. 6A, B), and then the levels of *Fgf-4* RNA gradually decrease until E12.0, when it is no longer detected although the AER is still present. Of great interest (see Discussion) is the finding that expression is spatially restricted, and is localized to the posterior half of the AER (Fig. 6A, B and data not shown). A similar pattern of expression in the hindlimb AER is observed approx. 0.5-1.0 days later (data not shown).

Expression in the developing tooth bud

By E14.5, *Fgf-4* RNA is no longer detected at the sites of earlier expression. However, *Fgf-4* transcripts are found at very high levels within a small portion of the developing tooth bud ectoderm during the cap stage (Fig. 6C, D). This group of cells in the center of the inner enamel epithelium is termed the enamel knot, and its function may be to act as a reservoir of dividing cells (Sharawy and Bhussry, 1986). Such expression appears to be stage-specific, since transcripts were not detected at E12.5 when the tooth rudiment is already established (data not shown). At E14.5, *Fgf-4* RNA was also detected at very low levels within a few muscle fibers in the limb near chondrogenic sites (data not shown).

Discussion

Expression of the murine *Fgf-4* gene from the pre-implantation stages of development through mid-gestation has been examined in detail and found to be restricted both temporally and spatially to specific embryonic cell types. *Fgf-4* RNA is first detected at the late blastocyst stage in the epiblast which gives rise to all of the embryonic cell lineages. Expression in these cells continues until just prior to gastrulation, at which time *Fgf-4* RNA becomes progressively restricted such that by the mid-primitive streak stage it is detected only in cells very near and within the distal (rostral) two-thirds



of the streak. During subsequent development, *Fgf-4* expression is localized to specific cell populations in the branchial arch units, somitic myotome, the apical ectodermal ridge of the developing limb bud, and the tooth bud.

Expression of FGF gene family members prior to and during gastrulation

Fgf-4 is expressed uniformly in the pluripotent epiblast from the time it gives rise to the primitive endoderm (approx. E4.5) until just prior to the start of gastrulation. This raises the possibility that it may play a role in maintaining the pluripotency of this cell population. Interestingly, the two other FGF members whose expression has been localized in the embryo prior to gastrulation are expressed in cells that have undergone at least one additional step along the differentiative pathway. *Fgf-3 (int-2)* expression is restricted to parietal endoderm (our observations) and *Fgf-5* RNA is detected in visceral endoderm surrounding the embryo proper and also is initiated in the embryonic ectoderm after implantation, but prior to formation of the primitive streak (Haub and Goldfarb, 1991; Hébert et al., 1991). The finding that early in gastrulation *Fgf-4* expression becomes restricted to the primitive streak

Fig. 5. *Fgf-4* and *MyoD1* expression during myotome differentiation. (A) Diagrammatic representation of the somites and neural tube. Three-dimensional view of the somite, depicted as a rectangular plate of cells, which has undergone differentiation into three compartments, the dermatome, myotome and sclerotome. This figure shows the lateral or outer layer of dermatome cells (d) which generates the dermis and the medial or inner layer of myotome cells (m) from which the trunk skeletal muscles arise. Sclerotome cells (not depicted) move axially, eventually producing the vertebrae and ribs. The plane of section for E - H is indicated. (Adapted from Keynes and Stern, 1985). (B) Schematic representation of a parasagittal section through a mature somite showing the three differentiated components: d, dermatome; m, myotome; scl, sclerotome. (Adapted from de la Brousse and Emerson, 1990). (C) Parasagittal section through an ~E9.5 embryo at the level of the forelimb, hybridized to an antisense *Fgf-4* probe, bright-field illumination. (D) Same, dark-field image. (E) Hybridization of *Fgf-4* antisense RNA to an oblique frontal section (plane of section shown in A) through three E10.5 somites located approximately mid-way between the developing fore- and hindlimbs; bright-field illumination. Anterior is at the top. (F) Same, dark-field image. (G) Hybridization of an antisense *MyoD1* probe to a section near to the one shown in E, F. *MyoD1* is first expressed at this stage (Sassoon et al., 1989) albeit at relatively low levels. (H) Hybridization of an antisense a-cardiac actin (*Actc-1*) probe to a section near to the ones shown in E - G, demonstrating the extent of the myotome compartment. *Actc-1* RNA is present at considerably higher levels in the myotome than either *MyoD1* or *Fgf-4*. Camera lucida drawings of the regions in which *Fgf-4* and *MyoD1* are expressed are superimposed on one somite. d, dermatome; ec, ectoderm; m, myotome; nc, neural crest; n, notochord; nt, neural tube; scl, sclerotome. Magnification, 180 ×.

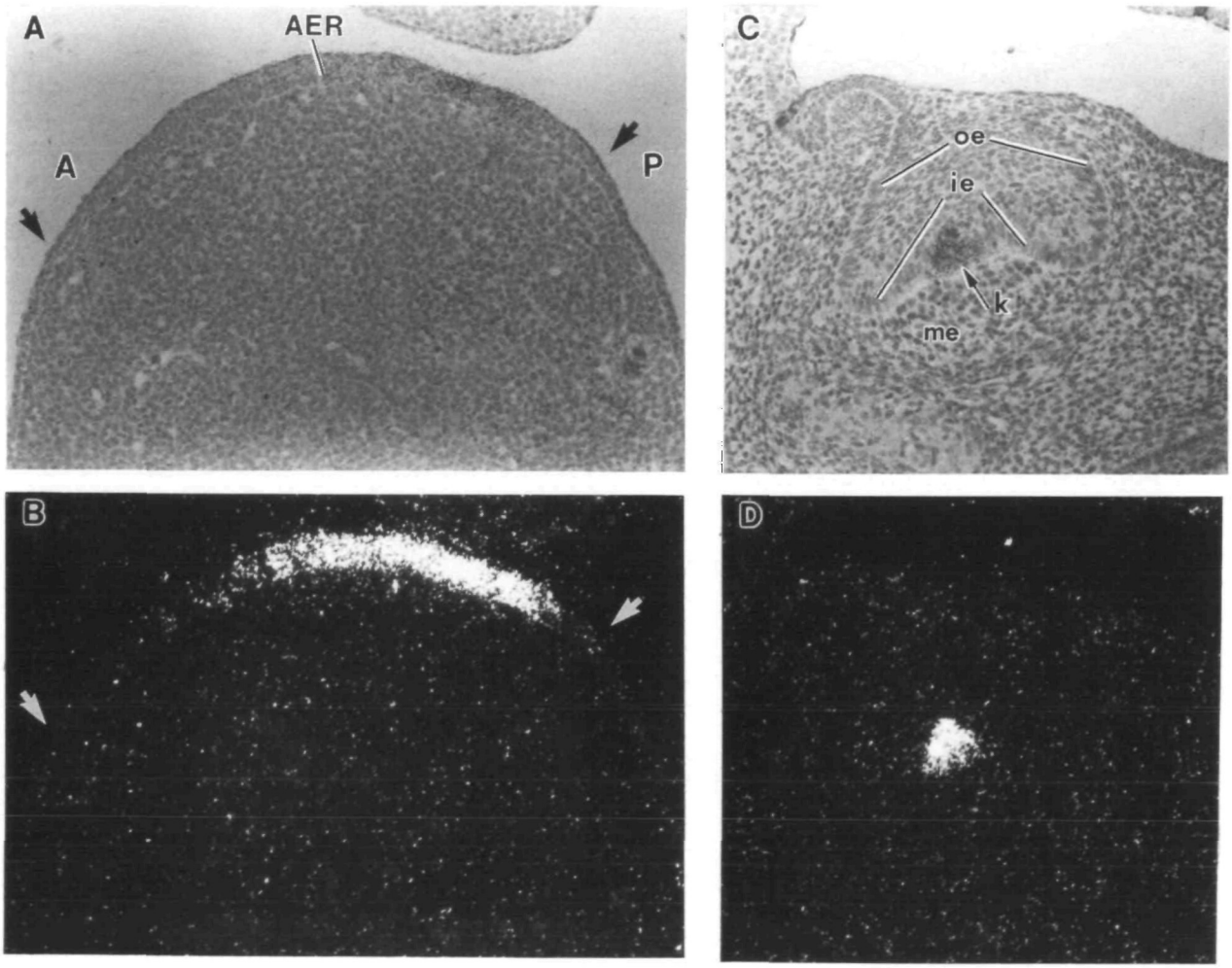


Fig. 6. Localization of *Fgf-4* RNA in the developing limb bud and tooth bud. (A) Hybridization of antisense *Fgf-4* RNA to a parasagittal section through an E10.5 forelimb, viewed in bright-field illumination. (B) Same, dark-field image. Arrows mark the approximate extent of the AER. (C) Hybridization of antisense *Fgf-4* RNA to a section through the mandible of an E14.5 fetus showing developing tooth bud, viewed in bright-field illumination. (D) Same, dark-field image. A, anterior; AER, apical ectodermal ridge; ie, inner epithelium; k, enamel knot; me, mesenchyme; oe, outer epithelium; P, posterior. Magnification, 100 ×.

distinguishes this gene from the other members of the FGF gene family that have been examined: *Fgf-5* RNA is detected throughout the epiblast but decreases as cells pass through the streak (Haub and Goldfarb, 1991; Hébert et al., 1991); *Fgf-4* is expressed in cells that are just about to enter or that are within the streak; *Fgf-3* becomes expressed as cells leave the streak and differentiate to mesoderm (Wilkinson et al., 1988; and the results reported here). These complementary patterns of expression are summarized in Fig. 7.

Although these three genes encode secreted proteins, it is likely that they act locally due to their avid interaction with cell surface and extracellular matrix molecules (reviewed by Rifkin and Moscatelli, 1989; Ruoslahti and Yamaguchi, 1991). Moreover, there is evidence that the use of an alternative translation initiation site leads to the production of a form of *Fgf-3* that is localized to the nucleus, suggesting that this gene product may act intracellularly (Acland et al., 1990). The RNA localization data suggest that each of these members of the FGF gene family may influence progressive steps in the process of mesoderm formation. The finding that *Fgf-4* expression is localized to the distal (rostral) 2/3 of the streak in cells that are fated to become axial, paraxial or lateral but not extraembryonic mesoderm, further suggests that it might play some role in the specification of cell types within the streak.

Little is known about the factors that control the expression of the FGF genes in the developing embryo. However, it has been suggested that *Fgf-4* expression is regulated by the product of the *Oct-4* (also termed *Oct-3* or NF-A3) gene, which is a transcription factor containing a POU-specific domain and a homeodomain. Three consensus binding sequences for OCT-4 have been identified within the *Fgf-4* gene (Curatola and Basilico, 1990) and based on the data previously available it was suggested that the two genes might be expressed in the same cell types during early embryonic development. However, comparison of *Oct-4* RNA localization data (Rosner et al., 1990) with our results reveals that few similarities exist between the patterns of expression of these genes, either temporally or spatially, and suggests that there are cell types in which OCT-4 is neither necessary nor sufficient for the regulation of *Fgf-4* expression.

Elucidating the function(s) of the different FGF genes will require not only additional information about the ligands themselves, but also about the high-affinity cell surface receptors with which they interact. The FGF receptors (FGFR), like the FGFs themselves, appear to be encoded by a family of evolutionarily related genes, four of which have been described to date (Lee et al., 1989; Dionne et al., 1990; Pasquale, 1990; Keegan et al., 1991; Partanen et al., 1991; Stark et al., 1991). There is evidence from in vitro studies for a potential complexity of interactions between the FGFs and their receptors. Individual FGFs can bind to and activate several different FGFR proteins, although recent studies have suggested that there may be a higher degree of specificity in this signaling pathway than previously thought. For example, Partanen et al. (1991) have shown that FGF-1 (aFGF) but not FGF-2 (bFGF) binds to the human FGFR-4. Furthermore, alternatively spliced forms of the receptors are generated from these genes (Johnson et al., 1991, and references therein), and it has been demonstrated that alternate forms of FGFR-1 differentially bind FGF-1 and FGF-2 (Werner et al., 1992). However, it remains to be determined what governs the specificities of the interactions between the ligands and high affinity receptors

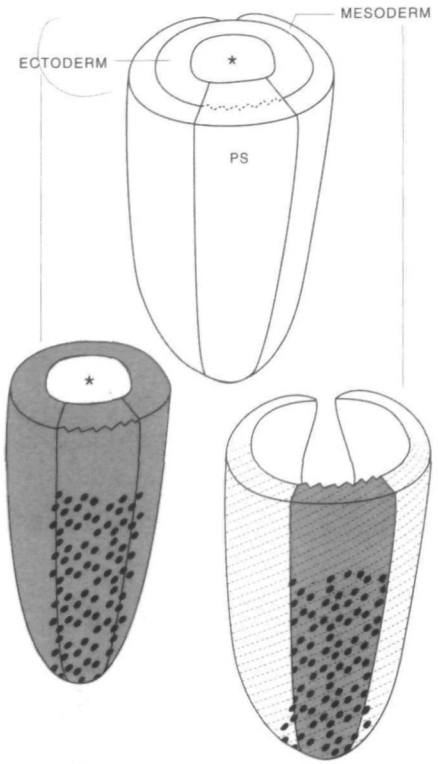


Fig. 7. Schematic representation of the regions of *Fgf-3, -4, -5* expression during gastrulation. (A) Diagrammatic representation of two layers of a late-streak embryo (the visceral endoderm layer is not depicted), viewed from the posterior side. The wavy dotted line represents an arbitrary division between the "deep" and "superficial" layers of the primitive streak (PS). An asterisk (*) indicates the proamniotic cavity. (B) The ectoderm (epiblast) and the deep layer of the primitive streak illustrated in A, are shown with large dots depicting *Fgf-4* expression and grey shading depicting the region in which *Fgf-5* RNA is found. Note that this shows a simplified representation of the *Fgf-5* expression pattern, and details such as the finding that *Fgf-5* RNA is detected at lower levels in the proximal than in the distal region of the epiblast (Hébert et al., 1991), are not illustrated. (C) The mesodermal wings and the superficial layer of the primitive streak illustrated in A, are shown with large dots and grey shading depicting *Fgf-4* and *Fgf-5* expression, respectively. Again, the *Fgf-5* expression pattern has been simplified, and *Fgf- 5* expression in mesoderm, which is limited to cells exiting from the more distal regions of the streak (Hébert et al., 1991), is not illustrated. Diagonal lines depict the region in which *Fgf-3* RNA is found. It should be noted that *Fgf-3* expression in the part of the primitive streak illustrated here is limited to only the most superficial cells.

that occur in vivo. Moreover, it is important to bear in mind that these interactions appear to be controlled by specific mechanisms, involving heparan sulfate proteoglycans (HSPGs), for sequestering the ligands in the extracellular matrix and presenting them to the high affinity receptors (reviewed by Klagsbrun and Baird, 1991).

These data raise the intriguing question of why at least three members of the FGF family of signaling molecules are expressed in a highly-regulated and complementary fashion in the gastrulating mouse embryo. One possibility is that they all elicit the same biological response, whatever it may be. The use of different genes to perform the same function, each expressed under the control of its own regulatory sequences, provides a means of independently regulating activity in different cell types and making very fine adjustments in ligand concentration and/or availability in any particular setting. Alternatively, each of the FGF ligands might mediate different cellular responses in the gastrulating embryo. In this case, however, the responding cells would require some mechanism for distinguishing among the different ligand signals, possibly by expression of different FGF receptors or combinations of receptors. Obviously, numerous other possibilities exist. Unfortunately, at this time our knowledge about the signaling pathway in general is limited, and as yet there have been few studies of FGFR or HSPG expression in the gastrulating embryo (Sutherland et al., 1991; Orr-Urtreger et al., 1991). Additional information on the expression of these molecules, as well as experiments to perturb the function of each element in the FGF signaling pathway, should lead to a greater understanding of its role in gastrulation, and development in general.

Expression of Fgf-4 *and myogenic determination genes during myotome development*

Evidence from studies of myogenic cell lines indicates that FGF-1 (aFGF) and FGF-2 (bFGF) stimulate the proliferation and inhibit differentiation of the myoblast in vitro (Lathrop et al., 1985; Clegg et al., 1987). Moreover, FGF-2 can repress transcription of at least two of the myogenic regulatory genes, myogenin (*Myog*) and *MyoD1* (Vaidya et al., 1989; Brunetti and Goldfine, 1990). These data suggest that *Fgf-2* or other FGF gene family members may play critical roles in the myogenic regulatory pathway. The results reported here demonstrate that *Fgf-4* RNA is present during early stages of myoblast formation in the embryo, thus raising the possibility that this particular member of the FGF gene family functions in the regulation of myogenesis.

Some evidence concerning this latter possibility can be gained by comparing the patterns of expression of *Fgf-4*, *Myog* and *MyoD1* in the developing myotome. In situ hybridization studies have demonstrated that in the mouse *Myog* can be detected as early as E8.5, after condensation of the somites but prior to formation of the myotomal component. By E9.25 it is expressed at high levels in the myotome, and subsequently in myotomal muscle masses (Sassoon et al., 1989). Our data show that *Fgf-4* expression is first detected approximately 18 hours later than *Myog,* in what appears to be the same cell population. Thus, our results do not lend support to the idea that *Fgf-4* regulates expression of *Myog* in vivo. In contrast, we find that *Fgf-4* expression precedes by more than 24 hours that of *MyoD1,* which is not detected until E10.5, relatively late in the sequence of myotomal cell development (Sassoon et al., 1989; and our observations). Moreover, our data show that *Fgf-4* RNA becomes restricted to a subpopulation of myotomal cells that lies in a stripe which is centrally positioned along the A-P axis, and this subpopulation is different from the one in which *MyoD1* is first expressed. These observations, taken in conjunction with the data on FGF repression of *MyoD1* transcription in cultured cells (Vaidya et al., 1989), lead us to speculate that, in vivo, *MyoD1* transcription may be repressed in cells that express *Fgf-4*.

Interestingly, expression of two other members of the FGF family has been localized to the somitic myotome. In the mouse, *Fgf-5* RNA has been found in the myotome, but it is first detected at E10.5 (Haub and Goldfarb, 1991), at least one day after *Fgf-4* expression is first observed in that region. Within the myotome, *Fgf-5* RNA is not preferentially localized and it can be detected in trunk muscle precursor cells during their migration. In addition, *Fgf-5* expression is never detected in tail region myotomes (Haub and Goldfarb, 1991). In the chick, FGF-2 protein is found throughout the somitic myotome at stage 19, which is approximately comparable to E10.5 in the mouse (Wanek et al., 1989), and later it is detected in multinucleated myotubes in the somite region (Joseph-Silverstein et al., 1989). The distinct temporal and spatial differences in the expression patterns of these FGF family members in the myotome are consistent with the suggestion that they may participate in the regulation of different, possibly sequential, steps along the pathway of myotomal muscle determination, differentiation or migration.

Fgf-4 *expression in the developing limb bud*

Our studies demonstrate that *Fgf-4* is expressed at high levels in cells of the AER as soon as it is morphologically detectable (E10.0-E10.5) and that expression levels gradually decrease during subsequent early development of the limb, so that by E12.0, it is no longer detected. In addition, throughout this period of expression, *Fgf-4* RNA is restricted to the posterior half of the AER. Studies in the chick have provided considerable evidence that interactions between cells of the AER and underlying mesenchyme are required for normal limb development. Signals from the AER affect the mesodermal cells underlying it, maintaining their viability and causing them to proliferate (Saunders, 1948; Summerbell, 1974; Reiter and Solursh, 1982). In addition, there is evidence to suggest that the AER may be regionalized with respect to the production of factors that are responsible for its effects on the underlying

mesenchyme. Analysis of the consequences of removing portions of the AER suggests that the anterior AER is necessary for development of anterior limb elements only, whereas the posterior AER is necessary for normal development of both posterior and anterior limb structures (Rowe and Fallon, 1981; Todt and Fallon, 1987). Several other genes have been found by RNA in situ hybridization to be expressed in the AER. These include CRABP (cellular retinoic acid-binding protein; Dollé et al., 1989), *BMP-2A* (Lyons et al., 1990), *Wnt-5a* (Gavin et al., 1990), *BMP-4* (Jones et al., 1991) and *Hox-8.1* (Davidson et al., 1991). Thus far, however, *Fgf-4* is the only gene whose expression has been localized within the AER to the posterior region.

Proteins encoded by members of the FGF gene family, particularly FGF-2 (bFGF), have been found to function as signals for cell survival or proliferation of limb mesenchyme. MacCabe et al. (1991) found that FGF-2 can rescue mesodermal cells that would otherwise die following removal of the AER, thus demonstrating that FGF-2 can substitute for the AER for maintenance of mesenchyme viability. The AER also acts as a mitogen for underlying mesoderm (Reiter and Solursh, 1982) and Aono and Ide (1988) have demonstrated that limb bud mesenchyme responds to the growth-promoting effects of FGF. However, despite its activity in these assays, it remains to be determined whether *Fgf-2* or some other member of the FGF gene family actually plays a role in limb development in vivo. Biochemical analysis of chick limb extracts indicates that FGF-2 is present in the limb (Munaim et al., 1988), although it has not been localized. Our data demonstrate that *Fgf-4* RNA is present in the mouse AER at the time when it functions to maintain the survival and affect the proliferation of the limb mesenchyme, suggesting FGF-4 is a family member that serves as an endogenous ligand in the FGF signaling pathway in the limb. Of the three FGF family members analyzed by RNA in situ hybridization (*Fgf-3, -4, -5*), only *Fgf-4* is expressed in the limb.

FGF gene expression during early tooth bud formation

Epithelial-mesenchymal interactions are also critical during different stages of tooth formation. Initially, in the branchial arch region, mandibular arch ectoderm and pharyngeal endoderm are implicated in the induction of tooth-specific properties in the as yet undetermined jaw mesenchyme that migrates between these two cell types (reviewed by Lumsden, 1987). We have found that *Fgf-4* is expressed in both the mandibular arch ectoderm and pharyngeal endoderm throughout the initial inductive phase, and *Fgf-3* is also expressed here, but one day later than *Fgf-4* and only in the pharyngeal endoderm (Wilkinson et al., 1988). These data suggest that this is another site where the FGF family plays a role in an epithelial-mesenchymal signaling process. Later in tooth development, when additional reciprocal and sequential interactions occur as jaw mesenchyme interacts with the overlying ectoderm and, in turn, the ectoderm influences the mesenchymal cells (Lumsden, 1987), we observed that *Fgf-4* RNA is detected in tooth bud ectoderm (enamel knot), whereas *Fgf-3* RNA is detected in the mesenchyme (Wilkinson et al., 1989). This raises the intriguing possibility that in this case one member of the FGF family is involved in sending a signal from the epithelium to the mesenchyme, and another family member plays a role in sending a reciprocal signal from the mesenchymal tissue.

In conclusion, as information about FGF gene expression during embryonic development accumulates, it becomes increasingly apparent that members of this gene family are differentially expressed in cells undergoing specific developmental processes, for example, in cells that are at different stages along a particular differentiative pathway, or in epithelial or mesenchymal cells participating in inductive interactions. As we learn what the specific functions of the FGF genes are in these developmental pathways, we should begin to understand the relationships among the participating cells and tissues and the mechanisms that regulate their progressive determination and differentiation.

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