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Expression of transforming growth factor β2 RNA during murine embryogenesis

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Summary

We have studied the temporal and spatial expression of transforming growth factor β2 (TGFβ2) RNA in mouse embryos from 10·5 days *post coitum* (*p.c.*) to 3 days *post partum* (*p.p.*) by *in situ* hybridization analysis. TGFβ2 RNA is expressed in a variety of tissues including bone, cartilage, tendon, gut, blood vessels, skin and fetal placenta, and is in general found in the mesenchymal component of these tissues. The expression of TGFβ2 RNA changes during development in a manner consistent with a role for the gene product in mediating mesenchymal–epithelial interactions.

Key words: TGFβ family, TGFβ2, *in situ* hybridization, mouse development, mesenchyme.

Introduction

The participation of polypeptide growth factors in mammalian embryonic development is well documented. For example, a variety of growth factors such as EGF, IGF, TGFα, and TGFβ as well as their receptors have been detected in embryonic cell lines and embryonic tissues (Proper *et al.* 1982; Twardzik, 1985; Nexo *et al.* 1980; Adamson *et al.* 1981; Moses *et al.* 1980; D'Ercole *et al.* 1980; Heath & Shi, 1986; Smith *et al.* 1987; Pfeilshifter & Mundy, 1987; Twardzik *et al.* 1982). Moreover, sequence and structural similarities have been found between these growth-related molecules and proteins such as Müllerian inhibiting substance, the gene products of *lin-12*, *Notch*, *decapentaplegic*, *Vg-1* and others which had been independently identified by virtue of their effects during embryonic development (Greenwald, 1985; Jakobovits *et al.* 1986; Knust *et al.* 1987; Mason *et al.* 1985; Padgett *et al.* 1987; Wharton *et al.* 1985; Weeks & Melton, 1987; Cate *et al.* 1986). Although it is not surprising to find growth factors present in an embryonic organism which is undergoing rapid cellular proliferation, evidence is now mounting that these peptide growth factors may be important in the regulation of cell differentiation and tissue morphogenesis as well as the control of cell division. This is perhaps best illustrated by transforming growth factor-β1 (TGFβ1) and its related family of proteins which have a multiplicity of cellular effects. In this paper, we begin to address the question of function in mouse development of one of these TGFβ-related peptides, TGFβ2.

The beta type transforming growth factors (TGFβs) are a family of polypeptides with a wide range of regulatory activities. Members include TGFβ1 (Derynck *et al.* 1985), TGFβ2 (Madisen *et al.* 1988), TGFβ3 (ten Dijke *et al.* 1988; Derynck *et al.* 1988) and TGFβ4 (Jakowlew *et al.* 1988), as well as more distantly related proteins such as Müllerian inhibiting substance (Cate *et al.* 1986), the inhibins and activins (Mason *et al.* 1985), the *Drosophila decapentaplegic* gene product (Padgett *et al.* 1987), the *Xenopus Vg-1* gene product (Weeks & Melton, 1987), the bone morphogenetic proteins BMP-2A, BMP-2B, and BMP-3 (Wozney *et al.* 1988) and the gene product of the recently described *vgr-1* (Lyons, K. *et al.* 1989). The protein now called TGFβ1 was the first member of the TGFβ superfamily to be isolated and characterized. Therefore, as subsequent members were discovered, their sequence similarity was reported relative to TGFβ1. Sequence analysis shows that, within the TGFβ superfamily, the amino acid identity of the distantly related group relative to TGFβ1 ranges from 28% to 38%, while the closely related group shows a range of 65%–80%. At least 7 of 9 cysteine residues are conserved in all members of the TGFβ superfamily and the highest level of amino acid sequence identity is, in general, found at the *C*-terminal end of the molecules (for a review on the structure of the TGFβ family proteins see Massagué, 1987; Derynck *et al.* 1988). TGFβ1 is synthesized in an inactive precursor form and is proteolytically cleaved to release the mature (*C*-terminal) peptide (Gentry *et al.* 1988) which remains noncovalently associated with the amino-terminal peptide in an inactive complex. This

latent peptide is then activated to give mature biologically active protein (Lyons, R. *et al.* 1988; 1989). Sequence data and other experiments suggest that TGFβ2 is processed in the same manner (Madisen *et al.* 1988). The activins and inhibins are also cleaved from a larger precursor but it is not yet known if cleavage and activation occurs in the other members of the TGFβ family.

TGFβ1 is synthesized and secreted by many different cells in culture and, although it is found in varying amounts in almost every tissue, highest levels are found in platelets and bone (for a more comprehensive review of TGFβ1's distribution and biological activities see Barnard *et al.* 1988; Rizzino, 1988; Sporn *et al.* 1987). Its effects *in vitro* include many actions that are thought to play an important role in embryogenesis. These include: 1) mitogenic effects on supporting tissues such as bone, cartilage and connective tissue fibroblasts, 2) inhibition of cell proliferation, in particular of epithelial cells, 3) regulation of differentiation (positive or negative depending on the cell type) and 4) stimulation of expression of extracellular matrix genes and of the integrin class of matrix protein receptors. TGFβ1 has been reported to induce mesoderm formation either alone on isolated *Triturus* ectoderm or synergistically with bFGF in excised *Xenopus* animal hemisphere cells (Knöchel *et al.* 1987; Kimelman & Kirschner, 1987). In addition, TGFβ1 has been isolated from mouse cells of embryonic origin both *in vitro* and *in vivo* (Proper *et al.* 1982; Twardzik *et al.* 1982). Consistent with these findings is the localization of TGFβ1 receptors on differentiated, but not undifferentiated, murine embryonal carcinoma cells (Rizzino, 1987). Lastly, recent reports using immunohistochemical techniques as well as *in situ* hybridization have localized TGFβ1 protein and mRNA to specific areas of the developing mouse embryo (Heine *et al.* 1987; Lehnert & Akhurst, 1988; Wilcox & Derynck, 1988; Sandberg *et al.* 1988*a*,*b*).

In contrast to the abundant evidence for a role for TGFβ1 in embryonic development, relatively few data are available linking the other closely related members of this gene family to developmental processes. TGFβ3 and TGFβ4 mRNAs are abundant in embryonic chicken chondrocytes suggesting a role for these molecules in chicken skeletal development (Jakowlew *et al.* 1988*a*,*b*). TGFβ2 (originally called BSC-1 growth inhibitor) was first isolated from the simian kidney epithelial cell line, BSC-1, by virtue of its growth inhibitory effects on these same cells (Holley *et al.* 1980, 1983). It was subsequently shown to have biological activity similar to TGFβ1 (Tucker *et al.* 1984). Molecular cloning of the growth inhibitor mRNA revealed that the putative translation product has very high amino acid sequence identity to TGFβ1 (Hanks *et al.* 1988) and it appears from sequence analysis that TGFβ2 protein is processed to a mature form very much like TGFβ1 (Madisen *et al.* 1988). TGFβ2 has also been isolated from other cell types including a human prostatic adenocarcinoma, a human glioblastoma, porcine platelets, and bovine bone (Madisen *et al.* 1988; Ikeda *et al.* 1987; Marquardt *et al.* 1987; Wrann *et al.* 1987; de Martin *et al.* 1987; Seyedin *et al.* 1987). When TGFβ2 was first isolated from bovine bone, it was named cartilage-inducing factor-B (CIF-B), and was shown to have the ability to induce fetal rat muscle mesenchymal cells to express a cartilage phenotype *in vitro* (Seyedin *et al.* 1987). Although TGFβ1 and TGFβ2 have similar functions in most *in vitro* systems, TGFβ2 appears to be much more effective in the induction of mesoderm in isolated animal pole explants of the *Xenopus* blastula. At levels of 3–12 ng ml^{-1}, TGFβ2 induces the expression of α-actin (a marker for muscle, a dorsal mesoderm-derived tissue) and induces the animal caps to form elongated structures similar to those arising from the marginal zone (Rosa *et al.* 1988). In addition, medium conditioned by *Xenopus* XTC cells contains a mesoderm-inducing factor (MIF) (Smith, 1987), the activity of which can be blocked by antibodies to porcine TGFβ2 (Rosa *et al.* 1988). The specific relationship of the XTC MIF to TGFβ2, however, remains to be determined (Smith, 1989).

Preliminary to defining a more precise role of TGFβ2 in mouse embryogenesis, we have determined the spatial and temporal localization of TGFβ2 transcripts in the developing mouse embryo. In this paper, we report studies on the expression of TGFβ2 mRNA in later stage embryos as revealed by *in situ* hybridization analysis. The pattern of gene expression observed is consistent with a role for TGFβ2 in tissue morphogenesis, in particular in regions of the mesenchyme where extracellular matrix production and angiogenesis are taking place. The pattern is also consistent with a role for TGFβ2 in mesenchymal–epithelial tissue interactions during development.

Materials and methods

Mouse embryos

All embryos were obtained from ICR outbred females mated with (C57Bl × DBA) F_1 males (Harlan Sprague Dawley). Noon on the day of vaginal plug is 0·5 day *post coitum*.

Probe construction

A 2·6 kb cDNA clone (pPC-21) covering the entire coding region as well as both 5′ and 3′ regions of the human TGFβ2 gene was kindly provided by Dr A. F. Purchio of Oncogene (Madisen *et al.* 1988). In order to minimize the possibility of cross-hybridization of our hTGFβ2 probe with any of the other TGFβs, we chose a 929 bp *Eco*RI–*Hin*dIII fragment which eliminates the 3′ terminal (mature) region of the cDNA (the region of highest sequence conservation in the TGFβ family). This fragment contains 179 b.p. of 5′ noncoding as well as 750 b.p. of precursor coding region. Our cDNA probe shows 85% nucleotide sequence identity to the murine TGFβ2 gene (isolated since the completion of these studies; Miller *et al.* 1989), but only 42% sequence identity to the murine TGFβ1 gene (Derynck *et al.* 1986), and 53% sequence identity to the murine TGFβ3 gene (D. Miller, personal communication). The 929 b.p. fragment was subcloned into a Bluescript vector (Stratagene, La Jolla, CA) and [α-^{35}S] UTP (1400 Ci mM^{-1}, New England Nuclear)-labeled single-stranded sense and antisense RNA probes (specific activity $2{\cdot}2\times10^9$ disints min^{-1} μg^{-1} RNA) were prepared using either

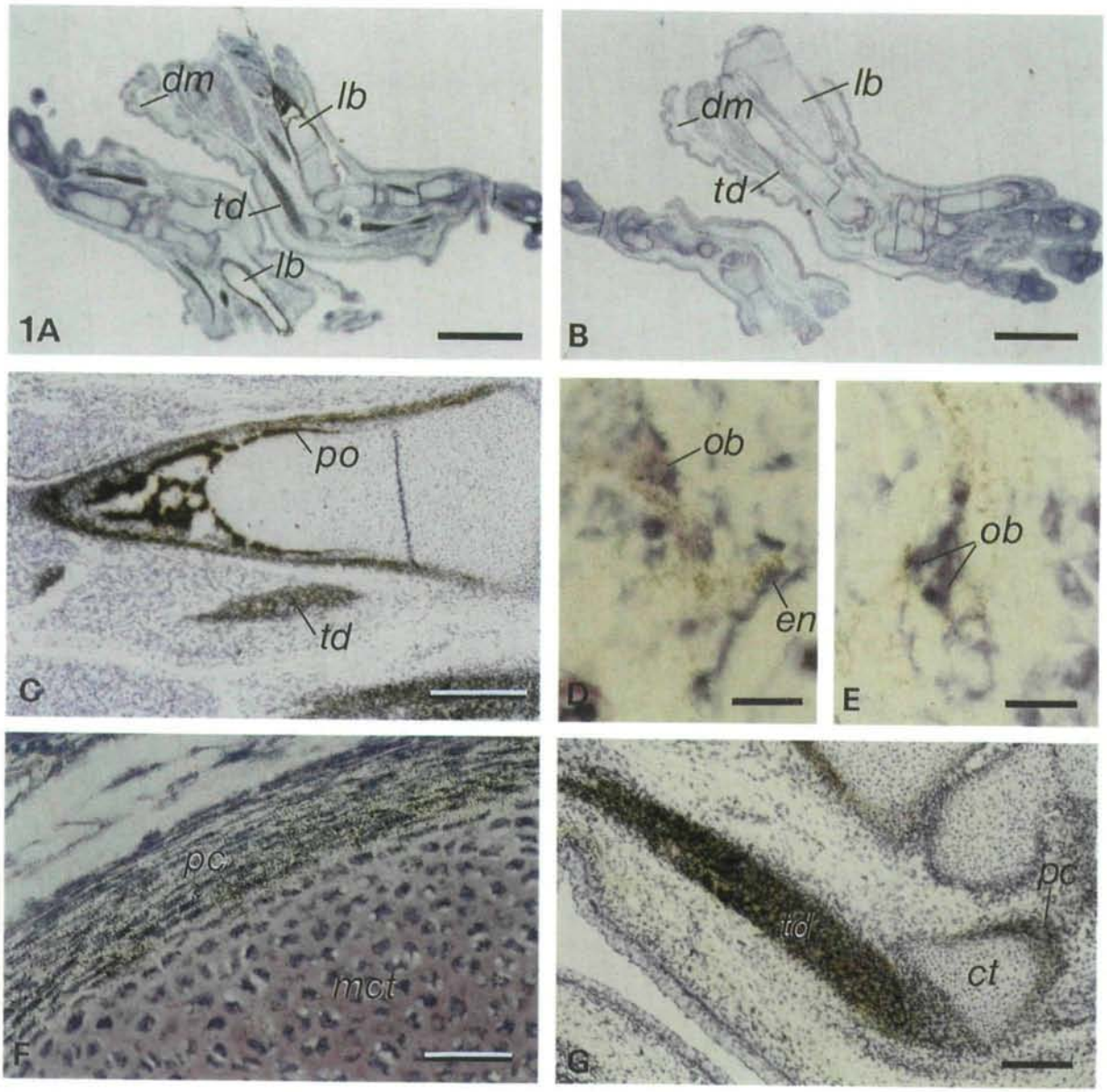

Fig. 1. Expression of TGFβ2 RNA in the embryonic skeletal system. (A) Longitudinal section through limbs of a 15·5 days *p.c.* embryo hybridized with antisense probe. Strong hybridization is seen in the long bones, perichondrium, and tendon. Hybridization can also be seen in the dermis of the skin. Bar = 1 mm. (B) Adjacent section to A, hybridized with sense strand (control) probe. Bar = 1 mm. (C) Higher magnification of the long bone seen in A, showing the TGFβ2 signal in the periosteum and in the central region of the bone. Strong hybridization is also seen in tendon. Bar = 250 μm. (D) High magnification of the hybridizing cells in the central region of the long bone seen in A and C, showing highest grain density over osteoblasts and endothelial-like cells. Bar = 2·5 μm. (E) High magnification of the strongly hybridizing cells in the periosteum of the long bone seen in A and C, showing highest grain density over the presumptive osteoblasts. Bar = 2·5 μm. (F) Section through embryonic cartilage of a 16·5 days *p.c.* embryo showing strong hybridization in the cells of the perichondrium but not in the mature cartilage. Bar = 100 μm. (G) High magnification of the tendon seen in A showing high grain density in the fibroblast population. This level of hybridization abruptly stops as the tendon inserts into the cartilage. Hybridization is also seen in the perichondrium of several cartilage elements. Bar = 150 μm. Sections were exposed for 5–7 days. *lb*, long bone; *td*, tendon; *ob*, osteoblast; *en*, endothelial-like cell; *po*, periosteum; *pc*, perichondrium; *mct*, mature cartilage; *ct*, cartilage; *dm*, dermis.

T3 or T7 polymerase, essentially as described by Hogan *et al.* (1986). Northern blot analysis with this probe revealed that it recognizes the same transcripts as the murine TGFβ2 (Miller *et al.* 1989) and does not cross-hybridize with mTGFβ1 or mTGFβ3 (data not shown). In addition, comparison of our hTGFβ2 probe against the mTGFβ2 as revealed by *in situ* hybridization analysis demonstrates that these probes recognize the same RNA species (data not shown). For *in situ* analysis, these probes were reduced to an average size of 100–150 bases by limited alkaline hydrolysis (Cox *et al.* 1984) and were used for hybridization at a final concentration of 2×10^5 disints min^{-1} μl^{-1}.

In situ *hybridization analysis*

In situ hybridizations were performed essentially as described by Nomura *et al.* (1988). Briefly, embryos were fixed in 4% paraformaldehyde, dehydrated through alcohol and embedded in paraffin wax. Sections of 5–7 μm were cut and floated onto polylysine-coated slides. They were then rehydrated, fixed in 4% paraformaldehyde, acetylated and dehydrated through alcohol. The slides were hybridized at 50°C for ~18 h in 50% formamide, 10% dextran sulphate, 8 mM-dithiothreitol (DTT), 300 mM-NaCl, 10 mM-Tris–HCl (pH 7·4), 5 mM-EDTA, 10 mM-Na_2HPO_4, 1 × Denhardt's. After hybridization, coverslips were removed in 5 × SSC, 10 mM-DTT at 50°C. The slides were then washed at either 55°C or 65°C, in 2 × SSC, 100 mM-DTT, 50% formamide and, unless otherwise stated, were treated with RNase A (10–20 μg ml^{-1}, 37°C, 30 min), followed by washes in 2 × SSC, and 0·1 × SSC at 65°C. Slides were then dehydrated, dipped in emulsion (Ilford K5, Knutsford, Cheshire, England) diluted 1:1 with 2% glycerol/water and exposed for 5–7 days at 4°C in the presence of desiccant. After standard development (Kodak D-19), the slides were counterstained with 0·2% toludine blue and analyzed on a Zeiss Axiophot microscope using bright-field and dark-ground optics.

Results

Analysis of TGFβ2 expression by in situ *hybridization*

Northern analysis of mouse embryo RNA has shown that TGFβ2 is expressed during embryogenesis in a range of tissues (Miller *et al.* 1989). In order to more precisely define the cell types expressing TGFβ2 mRNA, we hybridized sections of 13·5 days *p.c.* embryos to 3 days *p.p.* pups with a ^{35}S-labelled single-stranded antisense riboprobe synthesized from a 929 b.p. human cDNA coding for a region of the precursor portion of TGFβ2. (see Materials and methods). As a negative control, we used ^{35}S-labelled sense strand riboprobe from the same TGFβ2 cDNA. A radiolabelled antisense riboprobe for the 2ar (osteopontin or secreted phosphoprotein I) gene (Nomura *et al.* 1988) was used as a positive control in bone and as a negative control elsewhere. *In situ* hybridization demonstrates that TGFβ2 is expressed in several different tissues including bone, cartilage, tendon, gut, placenta and blood vessels, as well as in both dermis and epidermis of the skin. Except for the epithelial localization in the skin, TGFβ2 transcripts are found predominantly in cells of mesenchymal origin.

TGFβ2 RNA in the embryonic skeletal system

Bone is formed through either endochondral ossification (forming cartilage bone) or intramembranous ossification (forming membrane bone). Endochondral bone formation gives rise to the long bones of the limbs, the vertebral column, pelvis and base of the skull, while intramembranous ossification forms the bones of the skull, mandible and maxilla. Figs 1 and 2 suggest that TGFβ2 is involved in both of these processes.

Sections of limbs of 13·5 days *p.c.* embryos through to 3 days p.p. pups were hybridized with radiolabelled antisense TGFβ2 RNA. Although a positive signal could be detected at day 13·5 *p.c.* around the cartilage model of the bones (data not shown), highest levels were not seen until periosteal ossification was underway at about 15·5 days *p.c.* The high levels of TGFβ2 transcripts in the long bones were maintained through 3 days *p.p.* Fig. 1A is a longitudinal section through a limb of a 15·5 days *p.c.* embryo. This demonstrates that TGFβ2 RNA is found in both the central region of the developing bone as well as in the surrounding periosteal layer. Higher magnification (Fig. 1C,D) reveals that TGFβ2 RNA is seen primarily in two cell types in the bones of the limb. In the central region of the diaphysis evacuated by the vascular bud, TGFβ2 RNA is localized in large dark-staining cells adjacent to bone extracellular matrix as well as in long thin cells resembling endothelial cells (Fig. 1D). Due to their morphology and close association to the developing trabeculae, we presume the larger, dark-staining cells to be osteoblasts. Although red blood cells are seen near the longer thin cells, we cannot at this time definitively identify them as endothelial cells. The periosteum of developing bone consists of an outer layer of loose fibrous connective tissue and an inner layer of osteoblasts two to three cells thick. Fig. 1E shows that in the periosteum, TGFβ2 RNA is found in the osteoblastic layer.

Fig. 2 is a sagittal section through the head of a 17·5 days *p.c.* embryo. The three areas indicated by the arrows demonstrate that TGFβ2 expression occurs at the site of intramembranous ossification around the developing mandible, maxilla, and calvarium. Higher magnification of the cells in the maxilla expressing TGFβ2 RNA reveals that they have the same morphology as the presumptive osteoblasts and endothelial-like cells that are seen in the central region of the bones of the forelimb (data not shown). In addition, TGFβ2 RNA was localized to multinucleate osteoclasts in the central region of the bone near areas of matrix deposition (data not shown).

In embryonic cartilage, TGFβ2 RNA transcripts are localized in the chondroblasts of the surrounding perichondrium and not in the hypertrophic, proliferating, or mature chondrocytes (Fig. 1F). A similar pattern of expression is also seen in the cartilage of the trachea, nasal turbinates and calvarium (data not shown).

TGFβ2 RNA is also clearly expressed in the tendons of the embryonic limb. This is illustrated in Fig. 1G, which shows a tendon inserting into cartilage in the 15·5 days *p.c.* limb. TGFβ2 RNA is localized throughout the

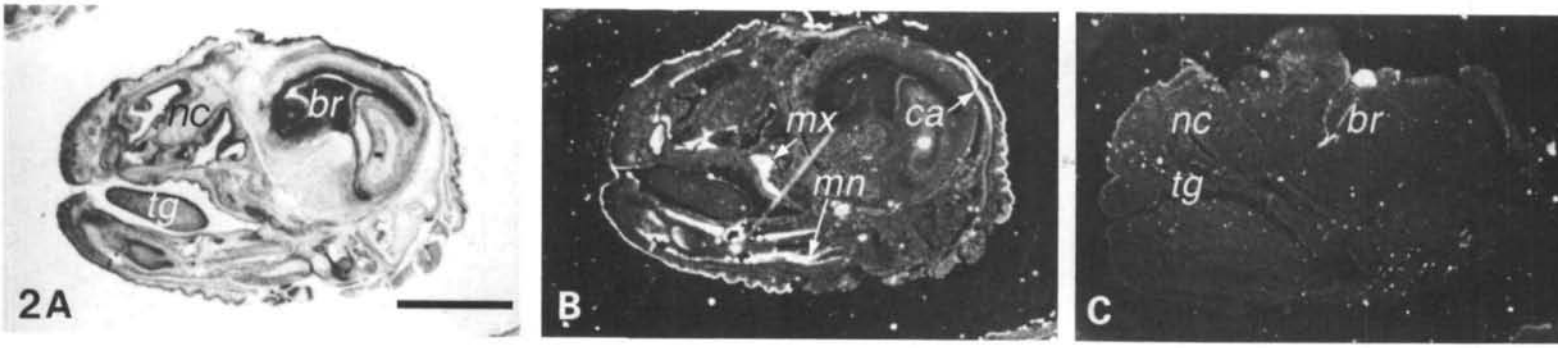

Fig. 2. Expression of TGFβ2 RNA in the head of a 17·5 day *p.c.* mouse embryo as revealed by *in situ* hybridization. (A) Midsagittal section of a head, hybridized with antisense probe and photographed under bright-field illumination. Bar = 2 mm. (B) Same section as in A photographed under dark-ground illumination. Strong hybridization is seen in the intramembranous bone (arrows). The signal outlining the head is an edge artifact. (C) Control section through the same head, hybridized with the sense strand probe. Sections were exposed for 7 days. *br*, brain; *tg*, tongue; *nc*, nasal cavity; *mx*, maxilla; *mn*, mandible; *ca*, calvarium.

length of the tendon, in which the principal cell type is the fibroblast, but the expression abruptly declines upon the insertion of the tendon into the cartilage.

TGFβ2 RNA in the embryonic digestive tract

The digestive tract is composed of many different cell types and contains four functionally distinct layers; the mucosa, submucosa, muscularis, and adventitia. While the mucosa is epithelial in origin, the submucosa is composed of mesenchymal cells which form a type of loose connective tissue. TGFβ2 RNA is found in the submucosal layer of the embryonic esophagus, stomach, small intestine, and colon. Fig. 3B is a section through the small intestine of a 16·5 days *p.c.* embryo showing that, while strong hybridization is seen in the submucosa, no TGFβ2 RNA was found in the other layers. The exact cell type expressing TGFβ2 in the submucosa could not be positively identified. However, neither the small capillaries nor the lymphatic vessels that leave this layer and continue on through into the lamina propria of the villi express TGFβ2 (Fig. 3A,B, arrows).

In addition to the expression seen in the submucosal layer, TGFβ2 is localized to other tissues of the digestive tract (data not shown). The mesentery, which supports the gut, shows relatively high levels of TGFβ2 RNA in cells of fibroblastic morphology. No TGFβ2 RNA was detected in the embryonic liver parenchyma.

TGFβ2 RNA in the embryonic respiratory tract

The lower respiratory tract consists of the trachea, the bronchiolar tree, and the alveoli. TGFβ2 antisense probe hybridizes to the submucosal layer underlying the epithelial layer of the trachea, bronchi and larger bronchioles, but hybridization diminishes as the bronchioles grow smaller and finally disappears in the terminal bronchioles (Fig. 3D). In the alveoli, no specific TGFβ2 hybridization is observed. Again, the small capillaries surrounding the alveoli are negative for TGFβ2 RNA, indicating that these vessels do not express the gene.

TGFβ2 RNA in the peripheral embryonic and extraembryonic circulatory system

The peripheral circulatory system consists of arteries, arterioles, capillaries, venules, and veins. Antisense TGFβ2 probe hybridizes specifically to the cells surrounding larger rather than smaller blood vessels in several different organs including lung, liver, and kidney. Fig. 3D shows TGFβ2 antisense probe hybridizing in the wall of a representative large, multilayered vessel in a 16·5 days *p.c.* embryo. Expression is seen in all layers of the vessel wall but are highest in the adventitial layer in the outermost segment of the wall and lowest in the strata containing smooth muscle. Once these vessels reach a size of about 2–3 RBC's in diameter, TGFβ2 RNA is no longer detected. In the placenta, high levels of hybridization are seen in the extraembryonic mesoderm, which contains fetal blood vessels surrounded by fibroblastic tissue (Fig. 3E,F).

TGFβ2 RNA in the skin

The two principal components of the skin are the epidermis, which is composed of epithelial cells, and the dermis, which is composed of cells of mesenchymal origin. TGFβ2 RNA is expressed in both of these layers of the skin as illustrated in a series of sections of the limb at different stages of development. From days 13·5 to 14·5 *p.c.*, no TGFβ2 expression was detected. On day 15·5 *p.c.*, the dermis begins to show strong hybridization with the TGFβ2 antisense probe (Fig. 4B), but neither the hair follicles nor the epidermis show any hybridization at this time. This level of hybridization remains through day 16·5 *p.c.* On day 17·5 *p.c.*, TGFβ2 expression in the dermis begins to dissipate but by day 18·5 *p.c.* expression appears in the suprabasal layer of the epidermis and in the hair follicles and remains at 3 days *p.p.* (Fig. 4E).

Discussion

This study was undertaken to explore the possibility that TGFβ2, a member of the TGFβ family, is involved

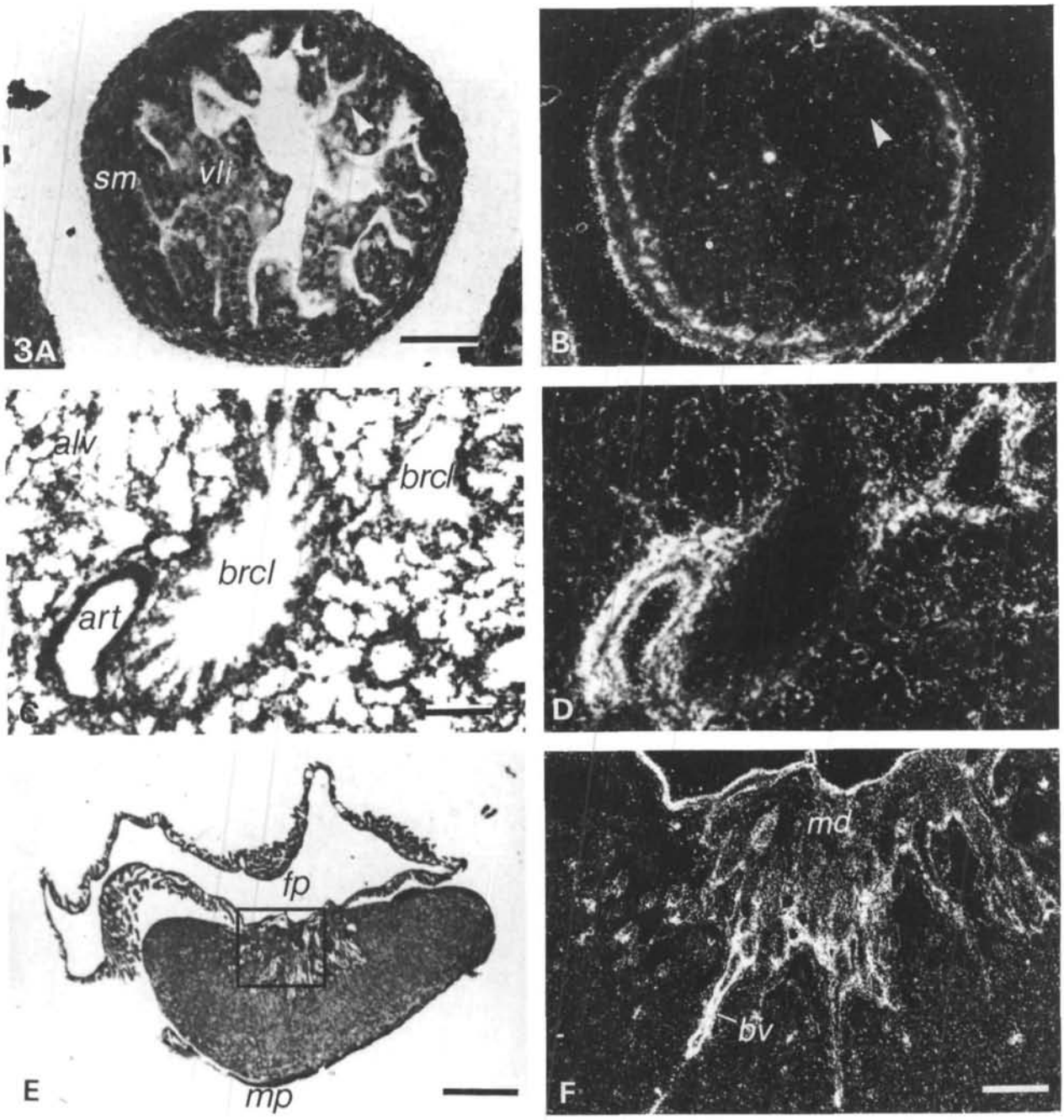

Fig. 3. Expression of TGFβ2 RNA in the embryonic gut, and lung and 18·5 days *p.c.* placenta. (A) Section through the small intestine of a 16·5 days *p.c.* embryo hybridized with antisense probe and photographed under bright-field illumination. Bar = 100 μm. (B) Same section as in A, photographed under dark-ground illumination. Strong hybridization is seen as a sharp band in the submucosal layer. The arrow demonstrates that the villi do not show hybridization. The signal on the periphery of the section is an edge artifact. (C) Section through the lung of a 16·5 days *p.c.* embryo hybridized with antisense probe and photographed under bright-field illumination. Bar = 100 μm. (D) Same section as in C, photographed under dark-ground illumination. Strong TGFβ2 signal is seen in the bronchioles and arterial wall but is absent from the alveoli. (Although it appears that only one bronchiole is expressing TGFβ2 RNA, the left-most (larger) vessel also shows signal. However, due to the very high expression by the artery and other bronchiole, we were not able to obtain an appropriate exposure to illustrate this without overexposing these tissues). (E) Midsagittal section through the 18·5 days *p.c.* placenta probed with antisense probe and photographed under bright-field illumination. Bar = 1 mm. (F) Higher magnification of the same section (see box) as in E, photographed under dark-ground illumination. Intense signal is seen in blood vessels in the fetal placenta as well as in embryonic mesoderm. Bar = 250 μm. Sections were exposed for 5–7 days. *vli*, villi; *sm*, submucosa; *art*, artery; *brcl*, bronchiole; *alv*, alveoli; *fp*, fetal placenta; *mp*, maternal placenta; *md*, mesoderm; *bv*, blood vessel.

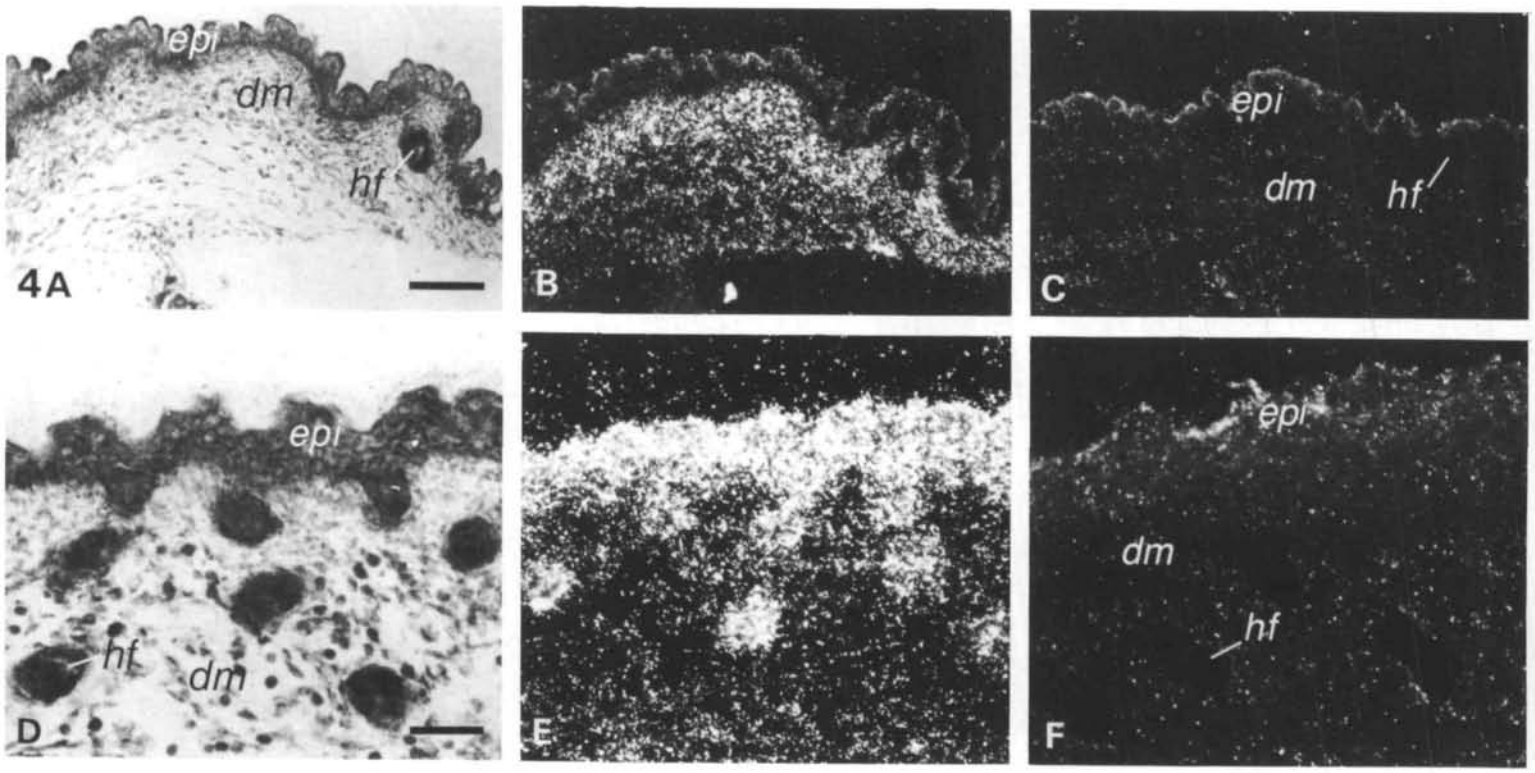

Fig. 4. Expression of TGFβ2 RNA in the skin at two different stages of mouse development. (A) Section through the skin of a limb of a 15·5 days *p.c.* embryo hybridized with antisense probe and photographed under bright-field illumination. Bar = 100 μm. (B) Same section as in A, photographed under dark-ground illumination. Strong hybridization is seen in the dermis but is absent in the epidermis and hair follicle. (C) Adjacent section to A, hybridized with the sense strand and photographed under dark-ground illumination. The apparent signal in the outermost region of the epidermis is an artifact due to light scattering by highly keratinized cells. (D) Section through the skin of a limb of an 18·5 days p.c. embryo hybridized with antisense probe and photographed under bright-field illumination. Bar = 50 μm. (E) Same section as in D, photographed under dark-ground illumination. Strong signal is evident in the epidermis and hair follicles but is not seen in the dermis. (F) Adjacent section to D, probed with sense strand probe and photographed under dark-ground illumination. Again, the apparent signal at the edge of the epidermis is due to light scattering by keratinized cells. Sections were exposed 5 days. *epi*, epidermis; *dm*, dermis; *hf*, hair follicle.

in mammalian development. We have used *in situ* hybridization to localize TGFβ2 RNA in mouse embryos ranging in age from 13·5 days *p.c.* to 3 days *p.p.* Our results clearly indicate that TGFβ2 is expressed in a variety of organ systems in the embryo and that this expression is regulated in a spatial as well as temporal manner. Although TGFβ2 RNA is seen in one epithelial cell type, the epidermis of the skin, the most common site of expression is in the cells of the mesenchyme. This is reminiscent of earlier observations by Lehnert and Akhurst in which TGFβ1 RNA was found by *in situ* hybridization to be expressed in the mesenchymal component of several tissues in the mouse embryo (Lehnert & Akhurst, 1988). Although these investigators used a full-length murine TGFβ1 cDNA probe, which included the mature region (which has a high level of sequence identity to the mature region of mTGFβ2), they washed their sections at high stringency, thus minimizing the likelihood of cross-hybridization between their probe and TGFβ2 mRNA. Our probe consisted of sequence in the precursor region of human TGFβ2, which is very dissimilar to TGFβ1 and TGFβ3, and, in addition to this, our washing conditions were stringent (see Materials and methods). Hence, the possibility of cross-hybridization of our probe with another RNA is also unlikely. The implication therefore is that TGFβ1 RNA and TGFβ2 RNA are expressed in many of the same or adjacent cells at the same time in development.

Comparison of TGFβ1 and TGFβ2 in the developing mouse

Previous studies by other investigators have shown that TGFβ1 is expressed in the developing calvaria and long bones in both human (16 week) and murine (14·5 days *p.c.*) embryos (Sandberg *et al.* 1988*a*,*b*; Lehnert & Akhurst, 1988). TGFβ1 mRNA has been localized to several different cell types in the embryonic bone including osteoblasts, osteocytes, and osteoclasts. Our data show that TGFβ2 is also expressed in these cell types and, in addition, is found in chondroblasts and the fibroblasts of embryonic tendon. TGFβ1 RNA has not been reported in tendon. Endochondral bone formation is a complex process in which a hyaline cartilage model of the bone is formed and then destroyed while being replaced by mineralized bone. The localization of

TGFβ2 mRNA in several of the cell types involved in endochondral bone formation suggests that the protein is synthesized by these cells. There are several possible functions for this protein in developing cartilage and bone. Like TGFβ1 it may 1) stimulate synthesis of matrix proteins by osteoblasts (Noda *et al.* 1988), 2) regulate differentiation of osteoblasts into osteocytes (Centrella *et al.* 1988) or 3) induce cartilage formation from mesenchymal precursors (Seyedin *et al.* 1986). There is some *in vitro* evidence supporting a role for TGFβ2 in cartilage formation (Seyedin *et al.* 1987; Noda *et al.* 1988).

TGFβ2 shows a complex pattern of expression in the embryonic skin in which the localization of TGFβ2 RNA in the dermis precedes that of the epidermis. With the dissipation of the TGFβ2 signal in the dermis, there is a corresponding increase in signal in the epidermis which is maintained at high levels until at least 3 days *post partum*. The expression in the epidermis is also seen in the developing hair follicles but only as they reach a mature state. This pattern of expression is in marked contrast to what has been reported for TGFβ1 RNA by *in situ* hybridization analysis on mouse embryos (Lehnert & Akhurst, 1988). Although TGFβ1 RNA has also been localized in developing hair follicles, it apparently declines as the follicle reaches a mature state and, while TGFβ1 RNA is expressed in 'interfollicular' epidermis, it is seen only at low levels (Lehnert & Akhurst, 1988). Though no specific expression of TGFβ1 RNA has been reported in the dermis, TGFβ1 protein has been localized to the dermis by immunohistochemistry (Heine *et al.* 1987).

Throughout the embryo, TGFβ2 RNA is expressed in the large vessels of the peripheral circulatory system. Vessels in the lung, liver, kidney, limb, and fetal placenta show strong hybridization to the TGFβ2 antisense probe. Although embryonic vessels do not show all of the well-differentiated layers of adult vessels, the TGFβ2 RNA appears to be expressed at high levels in the tunica adventitia and tunica intima and at lower levels in the tunica media. While TGFβ1 RNA expression has been localized to the cardiac cushions of the embryonic heart, it has not been reported in any peripheral vessels, large or small (Lehnert & Akhurst, 1988).

TGFβ2 RNA is expressed in the submucosal layer throughout the developing digestive and respiratory tracts. Our data show that TGFβ2 transcripts are seen in the submucosa of the esophagus, stomach, small intestine, colon, trachea, bronchi, and bronchioles. TGFβ1 RNA has been localized in the submucosal layer of the gut but has not been reported in the trachea or bronchioles (Lehnert & Akhurst, 1988). In addition, Lehnert & Akhurst report that TGFβ1 is localized in the mesenchyme of the embryonic lung; however, we have seen TGFβ2 expression only in the arteries and bronchiolar tree in this organ.

TGFβ2 in mesenchymal–epithelial interactions

We report the localization of TGFβ2 RNA in several areas of mesenchyme which are bounded by an adjacent epithelium. The submucosa of the esophagus, gut, and respiratory tree, as well as the dermis of the skin all support an epithelial layer. Evidence exists that these two layers interact. This has been particularly well studied in the skin where the dermis (mesoderm) has profound effects on the development of the epithelium and can influence the growth and differentiation of cells in the epidermal layer (Wessels, 1977). In addition to being stimulatory to many mesodermally derived cell types, TGFβ2 is a powerful inhibitor of epithelial cell proliferation and the high levels of expression of TGFβ2 RNA by the cells of the mesenchyme raises the possibility that the submucosa may regulate the growth of the overlying epithelium in an autocrine or paracrine fashion through the production of this polypeptide. We have shown that TGFβ2 RNA is expressed in the dermis of 15·5 and 16·5 days *p.c.* embryos when the overlying epidermis is only 3–4 cell layers thick. As development proceeds, the epidermis continues to thicken and hair follicles mature and the TGFβ2 RNA expression in the dermis fades. Concomitant with the decrease of TGFβ2 RNA in the dermis, there is the increase in the level of hybridizing RNA in the epidermis. By day 18·5 *p.c.*, the epidermis is 8–10 cell layers thick and has the appearance of fully developed skin. Strong hybridization in the epidermis is present at this stage and is still seen at 3 days *p.p.* Although definitive evidence is not yet available for the biological function of TGFβ2 in this system, we propose a model in which this polypeptide is synthesized by the cells of the mesenchyme and stimulates their growth and differentiation in an autocrine fashion during periods when extracellular matrix production and angiogenesis are actively taking place within the dermis. Our model suggests that TGFβ2 produced locally in the dermis has direct stimulatory effects on the proliferation and differentiation of mesenchymal cells and that these cells in turn produce factors (perhaps TGFα) which directly stimulate proliferation and differentiation of the overlying epidermis. TGFβ2 would thereby indirectly effect the epithelial cells in the epidermis. Once the epidermis has reached its mature state, expression of TGFβ2 by the cells in the dermis wanes and expression in the epidermis is seen. The synthesis of this polypeptide by the cells in the epidermis would inhibit their further growth in an autocrine fashion (Shipley *et al.* 1986). This model for the mode of action of TGFβ2 differs from the mechanism of TGFβ1 action proposed by Lehnert and Akhurst who suggested that TGFβ1 is made by cells in the epithelial component of an organ, is transferred to the underlying mesenchymal layer and acts through a paracrine fashion on the mesodermal cells (Lehnert & Akhurst, 1988). Our model is also consistent with a similar role for TGFβ2 in the submucosa of the respiratory and digestive tracts as in the skin.

Although the use of Northern analysis and *in situ* hybridization reveal valuable information about the cells that synthesize TGFβ2 mRNA, several important questions remain unanswered. For example, 1) which of the multiple TGFβ2 transcripts (Webb *et al.* 1988;

Miller *et al.* 1989) are being synthesized, 2) do the cells which synthesize TGFβ2 mRNA also make the protein, 3) is the secreted protein activated locally and 4) which cells have receptors for TGFβ2? In future studies we hope to address some of the questions which we have now raised and, in addition, we would like to probe embryo sections of earlier developmental stages. In light of the reports that TGFβ2 can induce mesoderm formation in the *Xenopus* embryo (Rosa *et al.* 1988), it would be of interest to examine mouse sections before and during gastrulation. The notion that a mammalian growth factor can induce mesoderm formation as well as alter cell behavior during morphogenesis could be of importance in furthering our understanding of embryonic development.

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