

Immunohistochemical Localization of TGF β 1, TGF β 2, and TGF β 3 in the Mouse Embryo: Expression Patterns Suggest Multiple Roles during Embryonic Development

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Abstract. Isoform-specific antibodies to TGF β 1, TGF β 2, and TGF β 3 proteins were generated and have been used to examine the expression of these factors in the developing mouse embryo from 12.5–18.5 d post coitum (d.p.c.). These studies demonstrate the initial characterization of both TGF β 2 and β 3 in mammalian embryogenesis and are compared with TGF β 1. Expression of one or all three TGF β proteins was observed in many tissues, e.g., cartilage, bone, teeth, muscle, heart, blood vessels, lung, kidney, gut, liver, eye, ear, skin, and nervous tissue. Furthermore, all three TGF β proteins demonstrated discrete cell-specific patterns of expression at various stages of development and the wide variety of tissues expressing TGF β proteins represent all three primary embryonic germ lay-

ers. For example, specific localization of TGF β 1 was observed in the lens fibers of the eye (ectoderm), TGF β 2 in the cortex of the adrenal gland (mesoderm), and TGF β 3 in the cochlear epithelium of the inner ear (endoderm). Compared to the expression of TGF β mRNA transcripts in a given embryonic tissue, TGF β proteins were frequently colocalized within the same cell type as the mRNA, but in some cases were observed to localize to different cells than the mRNA, thereby indicating that a complex pattern of transcription, translation, and secretion for TGF β s 1–3 exists in the mouse embryo. This also indicates that TGF β 1, β 2, and β 3 act through both paracrine and autocrine mechanisms during mammalian embryogenesis.

POLYPEPTIDE growth factors such as the β -type transforming growth factors (TGF β s) mediate many cell-cell interactions that occur during embryonic development (reviewed in Mercola and Stiles, 1988; Whitman and Melton, 1989; Nilsen-Hamilton, 1990). Complementary DNA clones have been isolated for five TGF β species (TGF β s 1–5); however, purified or recombinant protein has been obtained only for TGF β s 1–3 (Derynck et al., 1985, 1988; Madisen et al., 1988; ten Dijke et al., 1988; Jakowlew et al., 1988). Moreover, only TGF β s 1–3 have been found in mammals. The molecular structures of TGF β 1, β 2, and β 3 are very similar in that each polypeptide is synthesized as a pre-pro monomeric protein and is cleaved to yield a 112-amino acid polypeptide that remains associated with the latent (pro) portion of the molecule (reviewed in Lyons and Moses, 1990 or Miller et al., 1990). Biologically active TGF β protein results from dimerization of the monomers (usually homodimers) and release of the latent peptide portion. Overall, the mature region of the TGF β 3 protein has ~80% identity to the mature regions of both TGF β 1 and TGF β 2, however, the NH₂-terminal or precursor regions of these three molecules

share only 27% sequence identity (ten Dijke et al., 1988; Derynck et al., 1988). Some differences in the *in vitro* biological activities of TGF β 1 and TGF β 2 have been reported (Ohta et al., 1987; Jennings et al., 1988; Rosa et al., 1988; Merwin et al., 1991), however, in general, TGF β s 1, 2, and 3 have qualitatively similar activities when added to cells in culture (Graycar et al., 1989). In addition, competition studies with TGF β 1, TGF β 2, and TGF β 3 suggest that these proteins interact with the same cell-surface binding molecules (Graycar et al., 1989), although in some cell types, a given TGF β isoform may preferentially bind to an individual subset of these presumptive receptors (Segarini, 1990).

In recent years, several converging lines of evidence have indicated that members of the TGF β family play important roles in many different embryonic processes (reviewed in Akhurst et al., 1991 or in Pelton and Moses, 1990a). For example, *in vitro* TGF β s are mitogenic for cells derived from supporting tissues such as bone and cartilage but are inhibitory for many other cell types. The TGF β s also regulate differentiation (which may be stimulatory or inhibitory depending on the cell type), stimulate extracellular matrix deposition, are chemotactic for certain cells, and induce mesoderm formation during early embryogenesis. In an attempt to elucidate the *in vivo* activities of the TGF β s, we and

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others have investigated the expression patterns of TGF β genes during embryonic development and a number of studies have now described the *in vivo* localization of mRNAs for TGF β s 1–3 during murine embryogenesis (Lehnert and Akhurst, 1988; Pelton et al., 1989, 1990*a,b*; Akhurst et al., 1990; Miller et al., 1990; Fitzpatrick et al., 1990; Millan et al., 1991; Schmid et al., 1991). Recently we used *in situ* hybridization to directly compare the localization of TGF β 1, β 2, and β 3 mRNA transcripts in the mouse embryo (Pelton et al., 1990*a,b*). During mid to late embryogenesis, the murine TGF β s 1–3 were shown to have overlapping but distinct patterns of mRNA expression. These patterns changed as development progressed and were often found in tissues undergoing morphogenetic alterations. For example, during the development of the whisker follicles of the mouse, the mRNA expression patterns for TGF β s 1–3 were all different in the immature follicle but were very similar in the mature follicle (Lyons et al., 1990). *In situ* hybridization studies with human embryonic tissue have produced results similar to those obtained with mouse embryos (Sandberg et al., 1988*a,b*; Gatherer et al., 1990).

Although *in situ* hybridization experiments designate which cells and tissues synthesize mRNA transcripts for a given gene, these studies cannot determine if the mRNAs are translated into proteins; hence the relative amount of mRNA may not reflect similar amounts of protein. This phenomenon may have particular relevance in the study of TGF β expression. For example, Madisen et al. (1988) demonstrated that a human prostatic adenocarcinoma cell line produces higher levels of TGF β 1 and TGF β 2 mRNA but significantly higher levels of TGF β 2 than TGF β 1 protein. In addition, because the TGF β s 1–3 are secreted proteins, TGF β mRNAs may not colocalize with TGF β proteins. Indeed, by comparing their *in situ* hybridization studies with published immunohistochemistry data (Heine et al., 1987), Akhurst and co-workers have already shown this to be true for TGF β 1 mRNA and protein (Lehnert and Akhurst, 1988; Akhurst et al., 1990, 1991).

Using isoform-specific antibodies for TGF β 1, β 2, and β 3, we have investigated the expression of TGF β proteins in the mouse embryo and compared the distribution of these proteins with their respective mRNAs. Our results show that TGF β s 1–3 are expressed in unique temporal and spatial patterns in a wide range of embryonic tissues, suggesting that these factors play multiple roles during morphogenesis and organogenesis. Moreover, when compared with the TGF β *in situ* hybridization studies, the data presented here indicate that all three mammalian forms of TGF β (β 1, β 2, and β 3) act through both paracrine and autocrine mechanisms during murine embryonic development.

Materials and Methods

Mouse Tissues

Staged embryos (Theiler, 1989) were obtained from matings of ICR outbred female (Harland Sprague Dawley) and Swiss-Webster male mice (Taconic Farms, Germantown, NY). Noon on the day of vaginal plug was considered 0.5 days p.c. Pregnant females were killed via cervical dislocation and the 12.5–18.5 d.p.c. embryos were placed immediately in ice-cold 4% paraformaldehyde/PBS.

Antibody Preparation

Peptides of each TGF β isoform were synthesized using a 430A peptide syn-

thesizer (Applied Biosystems, Inc., Foster City, CA), incorporating the t-boc solid phase synthesis method followed by hydrofluoride cleavage. The following amino acid residues were used: TGF β 1 and TGF β 2, residues 4–19; TGF β 3, residues 9–20. The peptides were purified by high pressure liquid chromatography using a gradient composed of 0.1% trifluoroacetic acid and 100% acetonitrile. Each peptide (5.0 mg) was dissolved in 0.1 M NaHCO₃ and coupled to KLH at a 1:1 ratio (wt/wt), by adding 3– μ l vol of 1.25% glutaraldehyde 10 times over a period of 3 h. Rabbits were initially immunized with 500 μ g of each peptide and subsequently boosted with 250 μ g every 2.5 wk. Antibody titer was determined by ELISA using the appropriate corresponding uncoupled peptide and alkaline phosphatase goat anti-rabbit IgG as the second antibody (Promega Biotec, Madison, WI). The antisera did not cross-react with the TGF β peptides that were not used as immunogen. Each antiserum was purified by ammonium sulfate precipitation (31.3%), followed by affinity chromatography using the respective immunogenic peptide. The peptide (8.0 mg) was coupled to 2 ml of Tressyl-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) overnight according to the manufacturer's instructions. The purified IgG was eluted with 50 mM glycine (pH 2.5) into Tris buffer (pH 7.2) for neutralization, dialyzed against TBS, aliquoted, and stored frozen. Each antipeptide antiserum was tested for both immunoreactivity with the corresponding mature isoform of the TGF β molecule and for cross-reactivity with each other TGF β isoform by Western blot analysis.

Western Blot Analysis

Recombinant human TGF β 1 and TGF β 3 were generously provided by Dr. Michael Palladino and Dr. Rik Derynck, respectively, of Genentech, Inc. (Palo Alto, CA). Native porcine TGF β 2 was purchased from R & D Systems (Minneapolis, MN). Human and porcine TGF β amino acid sequences are identical. Each TGF β molecule was reduced with a final concentration of 0.1 M DTT, subjected to SDS-PAGE using a gradient polyacrylamide gel of 10–20% and subsequently transferred to a nitrocellulose membrane for 1 h at 100 V using the Biorad Miniblot System (Bio-Rad Laboratories, Cambridge, MA). The membranes were blocked with 3% non-fat dry milk in TBS (0.01 M Tris, 0.15 M NaCl, pH 8.0) for 1 h and directly incubated overnight in purified anti-peptide IgG in TBS containing 0.1% Tween 20 (TBST) at the following dilutions: TGF β 1, 1:50; TGF β 2 and TGF β 3, 1:25. The membranes were washed with TBST and incubated with alkaline phosphatase-labeled goat anti-rabbit IgG (Promega Biotec) at a dilution of 1:3,000 for 1 h. The blot was developed with NBT/BCIP (Promega Biotec).

Immunohistochemistry

The protocol used for these studies was a modified version of the method reported in Heine et al. (1987). Tissues were fixed overnight in 4% paraformaldehyde/PBS, dehydrated in increasing concentrations of ethanol, and embedded in paraffin wax (Fischer Scientific Co., Pittsburgh, PA). Sections of 5–7 μ m were cut and floated onto slides coated with 3-triethoxysilylpropylamide (Sigma Chemical Co.). The sections were submerged in TBS/0.1% (vol/vol) Triton X-100 at room temperature for 15 min followed by TBS for 5 min, methanol for 2 min, and methanol/0.6% (vol/vol) hydrogen peroxide for 30 min. Slides were subsequently washed at room temperature in methanol for 2 min, TBS for 5 min, and three times in TBS/0.1% (wt/vol) BSA for 3 min. After treatment with hyaluronidase (1 mg/ml in 100 mM sodium acetate, 0.85% (wt/vol) NaCl) and three washes in TBS/0.1% BSA, excess protein was blocked with 5% normal swine serum in TBS/0.5% BSA for 15 min at room temperature. Tissue sections were incubated with primary antibodies at a concentration of 5 μ g/ml (diluted in TBS containing 5% swine serum and 0.1% BSA) overnight at 4°C. Control slides were incubated with either an IgG fraction of normal rabbit serum at 5 μ g/ml (diluted in TBS containing 5% swine serum and 0.1% BSA) or without primary antibodies. Tissues were then washed in TBS/0.1% BSA and incubated for 60 min at room temperature with biotinylated swine antirabbit secondary antibody in TBS/0.1% BSA. After washes in TBS/0.1% BSA, the sections were exposed to avidin-biotin complex for 60 min at room temperature and again washed in TBS/0.1% BSA. Slides were reacted with 0.05% (wt/vol) DAB in 50 mM Tris-HCl (pH 7.4) with 0.1% (vol/vol) hydrogen peroxide for 5 min and counterstained in haematoxylin.

Results

Antibody Specificity

Western Blot Analysis. The specific immunoreactivity of

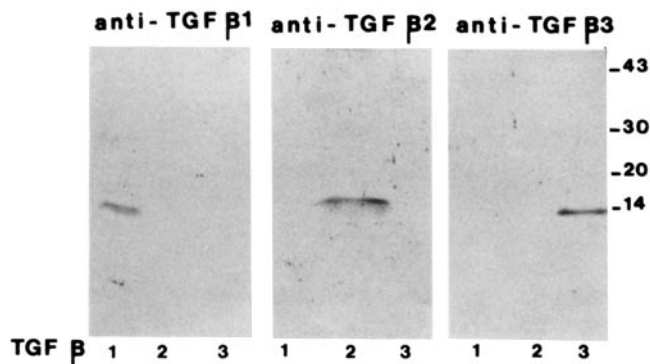


Figure 1. Western blot analysis demonstrating the specificity of TGF β antipeptide antibodies. Recombinant TGF β 1 and TGF β 3 proteins and porcine platelet TGF β 2 (50 ng each) were electrophoresed and transferred to nitrocellulose. Each TGF β protein was reacted with each antiserum to TGF β overnight. Dilution for antisera/anti-TGF β 1 was 1:50; and 1:25 for anti-TGF β 2 and anti-TGF β 3. Molecular weight markers are shown on the right.

each antipeptide antiserum with its respective TGF β isoform was demonstrated by Western blot analysis as depicted in Fig. 1. Each purified IgG to TGF β 1, β 2, or β 3 reacted only with the appropriate and corresponding native or recombinant molecules—(50 ng) of rTGF β 1 (Fig. 1, lane 1), TGF β 2

(Fig. 1, lane 2), or rTGF β 3 (Fig. 1, lane 3). No cross-reactivity was observed among any of the TGF β isoforms. The specificity of the individual antisera was further demonstrated after complete absorption of the immunoreactivity with 10 M excess of the corresponding peptide (data not shown). Lastly, the ability of the antipeptide antibodies to react (but not cross react) on a Western blot with the appropriate native TGF β 1, TGF β 2, and TGF β 3 molecules purified from human umbilical cord tissue further suggests that these antibodies are specific for their respective proteins (L. I. Gold, manuscript in preparation).

Immunohistochemistry. To ensure the specificity of the anti-TGF β antisera for immunohistochemical studies, serial tissue sections were used for control and experimental slides. Control slides were reacted with either (a) the IgG fraction of normal rabbit serum (at the same concentration as the primary antibody) in place of the peptide antibodies; or (b) no primary antibody. Fig. 2 shows examples of control sections directly compared against experimental sections. The epidermis and hair follicles in the skin of a 17.5 d.p.c. mouse showed strong immunoreactivity with TGF β 1 antibodies (Fig. 2 B), while absolutely no staining was observed with normal rabbit IgGs (Fig. 2 A). Similarly, while the keratinized epithelium of the palate and tongue of a 17.5 d.p.c. embryo showed intense staining with TGF β 1 antibodies, no staining was observed using the normal rabbit IgGs (com-

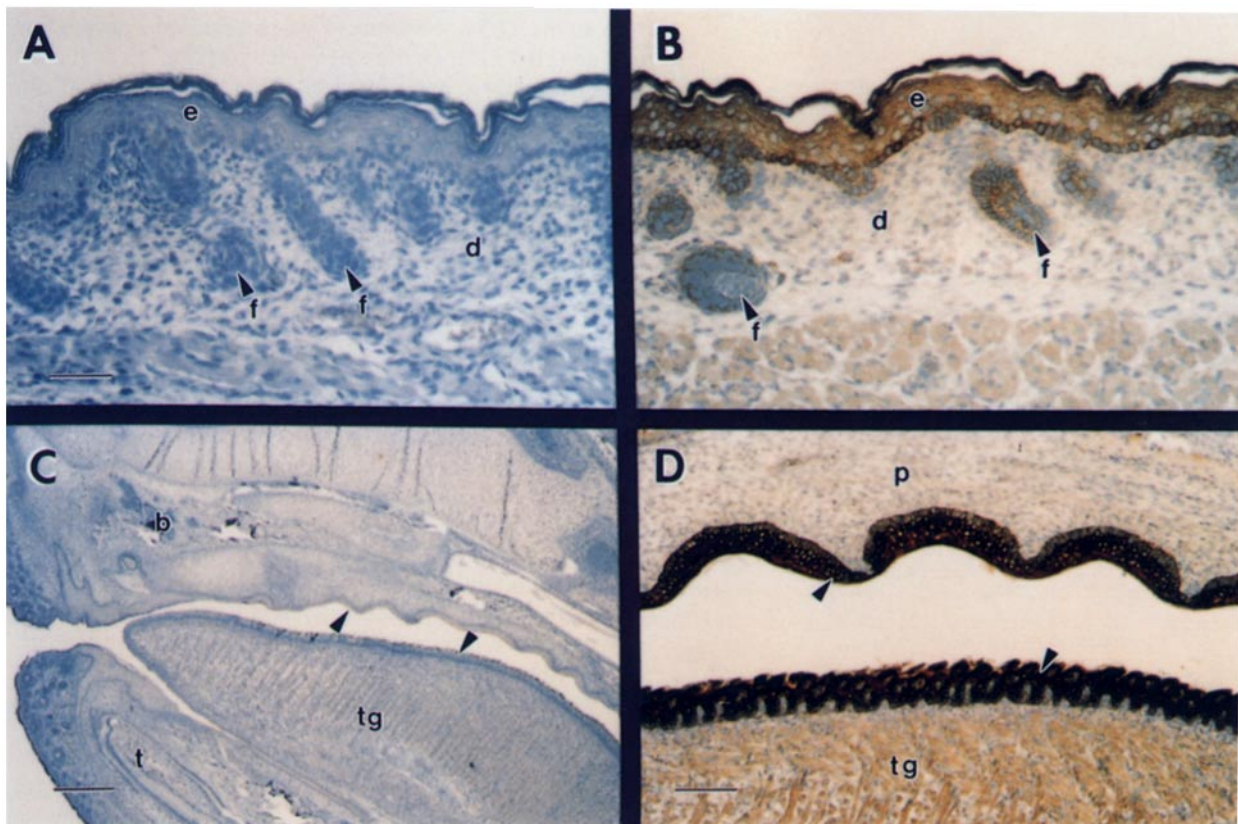


Figure 2. Comparison of embryo sections incubated with either normal rabbit IgGs or with antibodies to TGF β 1. (A and B) Section of 17.5 d.p.c. mouse skin incubated with normal rabbit IgGs (A) or with TGF β 1 antibody (B). Staining is seen only in the epidermis and hair follicles of B. (C and D) Section through the oral region of a 17.5 d.p.c. mouse embryo incubated with normal rabbit IgGs (C) or with TGF β 1 antibody (D). Staining is seen only in the keratinized epithelium of the palate and tongue of D. e, epidermis; d, dermis; f, follicle; b, bone; t, tooth; tg, tongue; p, palate. Bars: (A) 35 μ m; (C) 175 μ m; (D) 55 μ m.

Table I. Embryonic Cell Types Expressing TGF β Proteins

Cell type	TGF β 1	TGF β 2	TGF β 3
Cartilage			
perichondrium	+++	+	++
chondrocytes	+	++	++
Bone			
periosteum	++	-	+
osteocytes	++	++	++
Tooth			
ameloblasts	++	-	+
odontoblasts	-	++	-
pulp	+	+++	+
Muscle			
smooth	+	+	++
cardiac	+	+	+++
skeletal	+	+	++
Heart			
endothelium	-	-	-
myocytes	+	+	+++
Lung			
bronchi	++	++	++
alveoli	-	-	-
Blood vessels			
endothelium	-	-	++
smooth muscle	+	+	+++
Kidney			
tubules	++	++	++
basement membrane	-	+++	-
Adrenal			
cortex	+++	+++	-
medulla	-	-	-
Gut			
esophageal epithelium	+++	+	+
stomach epithelium	+++	+	+
intestinal epithelium	++	+	+
basement membrane	-	+++	-
muscularis	+	+	++
Liver			
capsule	-	-	++
parenchyma	-	-	-
megakaryocytes	+	-	++
Eye			
lens epithelium	-	-	-
lens fibers	+++	+	+
Ear			
cochlear epithelium	-	+	+++
basement membrane	-	+++	-
CNS			
meninges	+	+++	+
glia	-	++	++
choroid plexus	-	-	++
Skin			
periderm	++	+	++
epidermis	+++	+++	+++
dermis	+	+++	+
hair follicles	++	++	+

Immunolocalization of TGF β 1, β 2, and β 3 proteins in the 12.5–18.5 d.p.c. mouse embryo. A + indicates that positive staining was seen in this tissue at some stage between 12.5 and 18.5 d.p.c., while a - means that no staining was detected. The number of + signs denotes relative intensity of staining when compared against the other TGF β antibodies in that particular tissue and should not be compared against the staining intensity in other tissues. See text for a more detailed description of the temporal and spatial expression patterns. To compare the immunohistochemical expression of TGF β proteins with TGF β RNAs, the reader is referred to Table I of Millan et al. (1991).

pare Fig. 2, D with C). Although the control data for each tissue is not shown, in all cases examined the control slides were negative.

Immunohistochemical Localization of TGF β Proteins

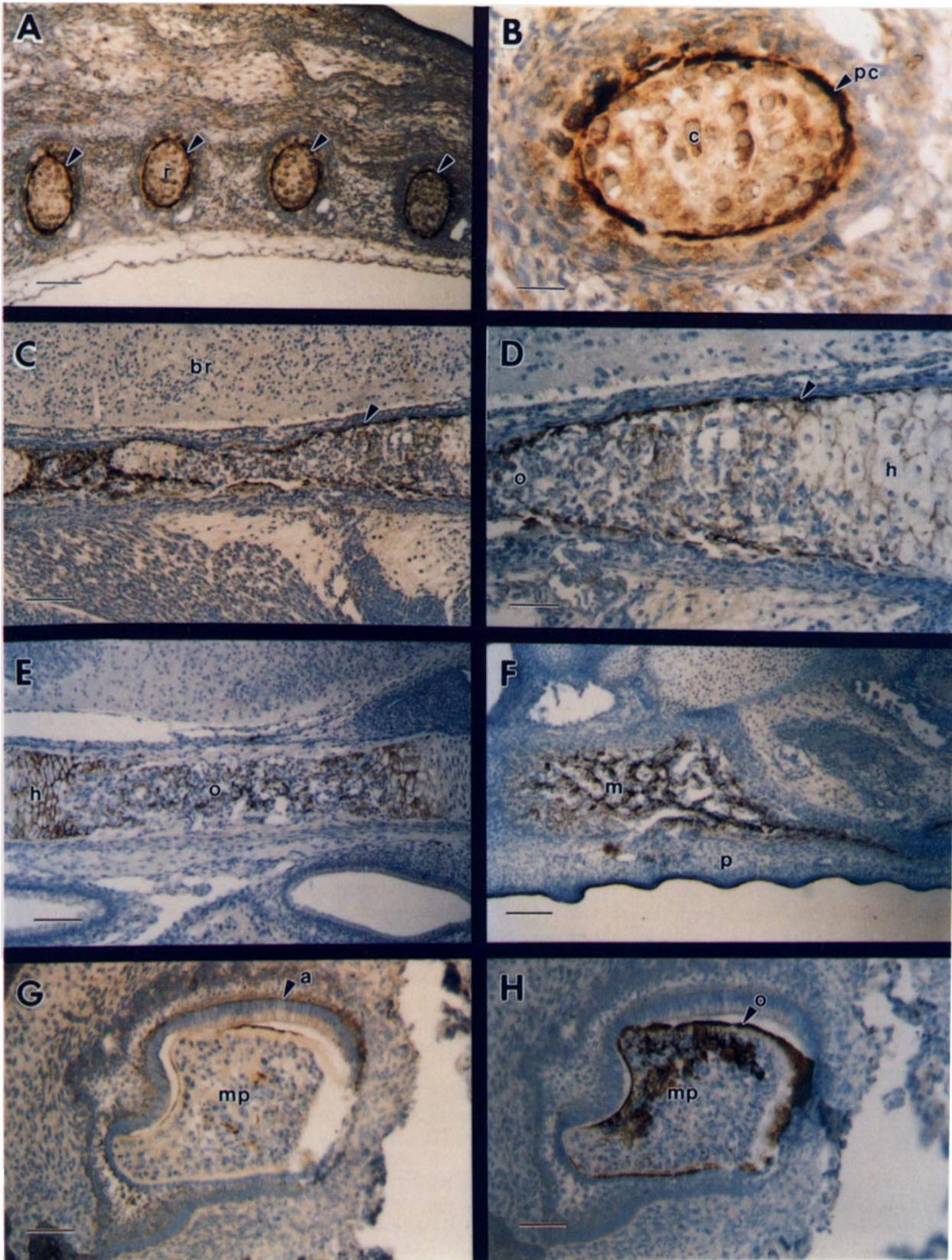
The results of the immunohistochemical localization of TGF β s 1–3 are summarized in Table I. Data not shown in Figs. 2–11 are referred to in Table I.

The Embryonic Skeletal System. Chondrification centers for most bones are first seen in the embryo at ~13–14 d.p.c. with centers of ossification usually appearing 2–3 d later (Rugh, 1990). At 12.5 d.p.c., chondrocytes in the ribs and vertebrae showed strong TGF β 3 staining but only very weak staining for TGF β 1 or TGF β 2. The cartilage rudiments in other bones at 12.5 d.p.c. showed little TGF β 3 staining. From 13.5 to 15.5 d.p.c., TGF β 3 staining in the chondrocytes remained high and as the maturing chondrocytes became hypertrophic, TGF β 1 (Fig. 3, A and B) and TGF β 2 expression was seen. TGF β 1 levels were especially high in the perichondrium (Fig. 3, A and B). By 16.5 d.p.c., high levels of TGF β 1 were localized to the periosteum of several bones (Fig. 3, C and D) as well as in the maturing chondrocytes of various cartilage rudiments. Although little TGF β 2 or TGF β 3 was detected in the periosteum, both were found in osteocytes of bone and maturing chondrocytes such as those of the tracheal cartilage. The TGF β 3 staining in bone was found in the cytoplasm of osteocytes, while the TGF β 2 staining was found predominantly in the extracellular matrix surrounding the osteocytes (Fig. 3 E). TGF β 1 immunoreactivity in the 17.5 d.p.c. embryo was found in the developing maxilla (Fig. 3 F), an intramembranous bone, as well as in the periosteum of endochondral bones (Fig. 3, C and D). Whereas TGF β 1 and TGF β 2 were both still present at high levels in osteogenic bone and hypertrophic cartilage at this stage, TGF β 3 staining had diminished (Fig. 3, C–F).

It has been previously demonstrated that at 16.5 d.p.c., very high levels of TGF β mRNAs are found in the embryonic tooth, primarily in the odontoblast and mesenchymal pulp cells (Pelton et al., 1990a). However, TGF β proteins in the 17.5 d.p.c. tooth were localized to additional structures. For example, although TGF β 1 mRNA was very abundant in the pulp cells, TGF β 1 antibody staining was seen at highest levels in the ameloblast layer (Fig. 3 G). In addition, while TGF β 2 mRNA was found at high levels in the odontoblast layer and to a lesser extent in the pulp cells, TGF β 2 protein was observed at high levels in both the odontoblast and pulp cells (Fig. 3 H). TGF β 3 immunostaining was found at low levels in both the pulp cells and the ameloblasts but was not seen in the odontoblast layer.

The Embryonic Circulatory System. Previous reports have shown that at 7 d.p.c., TGF β 1 mRNA was observed in cardiac mesoderm cells; however, TGF β 1 protein could not be detected (Akhurst et al., 1990). Transcripts for TGF β 3 are restricted to the atrioventricular cushions of the 11.5 d.p.c. heart and are not found in the ventricles (Pelton et al., 1990a). Although immunostaining for TGF β 3 protein, and to a lesser extent for TGF β 1 and TGF β 2, was also present in the embryonic heart from 12.5 through 16.5 d.p.c., as seen in the tooth, there were differences in the TGF β mRNA and

Figure 3. Localization of TGF β proteins in the murine embryonic skeletal system. (A) Cross section through the ribs of a 14.5 d.p.c. embryo stained with TGF β 1 antibodies. Staining is seen primarily in the perichondrium surrounding the hypertrophic cartilage in the ribs. (B) Higher power magnification of A, illustrating high TGF β 1 expression in the perichondrium of a rib. Lower levels were seen in the hypertrophic cartilage. (C) Longitudinal section of a bone at the base of the skull in a 17.5 d.p.c. embryo stained with TGF β 1 antibodies. TGF β 1



is seen in the periosteum (*arrow*) and osteogenic zone of the bone. (*D*) Higher power of *C* showing the TGFβ1 expression in the periosteum of the bone (*arrow*). (*E*) Same bone as in *C* stained with TGFβ2 antibodies. Staining is seen in the hypertrophic cartilage and osteogenic zone of the bone. (*F*) Staining with TGFβ1 antibodies in osteoblasts in the maxilla of a 17.5 d.p.c. embryo. (*G*) Section through a tooth in a 17.5 d.p.c. embryo incubated with TGFβ1 antibodies. Staining is seen in the basal region of the ameloblasts (*arrow*). (*H*) Same section as in *G* incubated with TGFβ2 antibodies. Staining is seen in the odontoblasts (*arrow*) and in the mesenchymal primordium of the pulp. r, ribs; c, cartilage; pc, perichondrium; br, brain; o, osteogenic zone (*D* and *E*); h, hypertrophic cartilage; m, maxilla; p, palate; a, ameloblasts; mp, mesenchymal primordium of the pulp; o, odontoblasts (*H*). Bars: (*A*) 110 μm; (*B*) 30 μm; (*C*) 120 μm; (*D*) 65 μm; (*E*) 115 μm; (*F*) 145 μm; (*G*) 80 μm; (*H*) 80 μm.

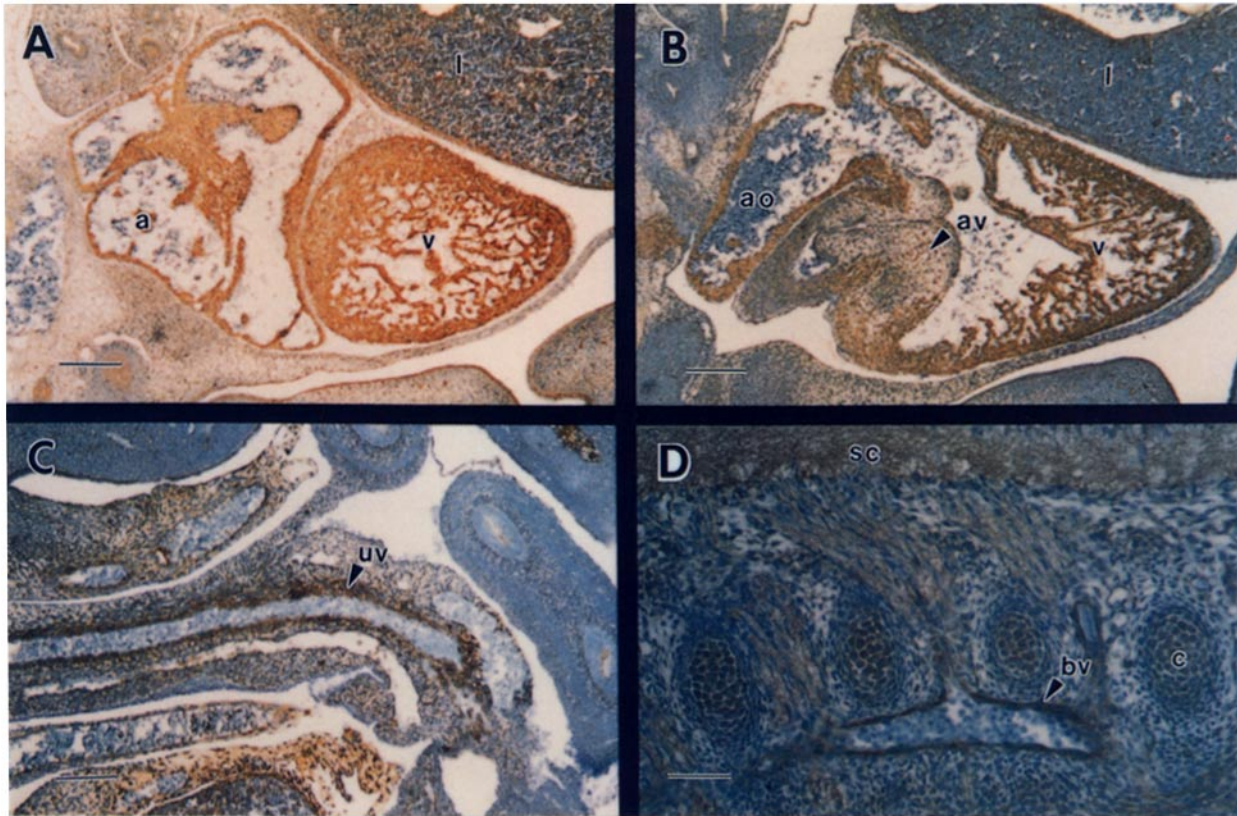


Figure 4. Expression of TGF β 3 in the 12.5 d.p.c. embryonic circulatory system. (A) Parasagittal section through a 12.5 d.p.c. heart stained with TGF β 3 antibodies. Staining is seen in the atria and ventricles. (B) Sagittal section through same heart as in A illustrating TGF β 3 in the walls of the aorta and at low levels in the atrioventricular cushions (arrow). (C) Section showing TGF β 3 localization in an umbilical vessel (arrow). (D) TGF β 3 staining in an embryonic blood vessel (arrow) near the spinal cord. a, atria; v, ventricle; l, liver; ao, aorta; av, atrioventricular valve; uv, umbilical vessel; sc, spinal cord; bv, blood vessel; c, cartilage. Bars (A) 175 μ m; (B) 175 μ m; (C) 115 μ m; (D) 60 μ m.

protein expression patterns in this tissue. For example, while TGF β 3 mRNA signal was high in the atrioventricular cushions and low in the ventricles of the 11.5 d.p.c. mouse heart, TGF β 3 protein in the 12.5 d.p.c. heart was high in the ventricles and atria and low in the atrioventricular cushions (Fig. 4, A and B). By 17.5 d.p.c., the staining for TGF β 1 and TGF β 2 proteins was barely detectable above background while TGF β 3 staining remained strong. TGF β 1–3 staining was also found in blood vessels throughout the body. For example, TGF β 3 immunoreactivity was found in small vessels such as those entering the spinal cord (Fig. 4 D) as well as in large arteries and veins, such as the umbilical and pulmonary vessels (Fig. 4 C and 8, A and B).

The Embryonic Internal Organs. Thompson et al. (1989) have shown that TGF β 1 is localized to a subset of tubule cells in the cortex of the adult kidney and in the present study, TGF β proteins were also found in the embryonic kidney (Fig. 5, A–D). In the 12.5 d.p.c. embryo, the developing kidney consists of metanephric tubules lined by a cuboidal epithelium (Rugh, 1990). At 12.5 d.p.c., TGF β 2 staining was present in a very restricted pattern in the basement membrane of the cuboidal epithelium. Neither TGF β 1 nor TGF β 3 was detected in the kidney at this stage. From 13.5 to 14.5 d.p.c., the TGF β 2 expression in the basement membrane of the cuboidal epithelium increased in intensity and became more extensive (compare Fig. 5, A and B). In contrast, dur-

ing this stage TGF β 1 and TGF β 3 were found in the cuboidal epithelial lining of the tubules, but not in the basement membranes. The expression of TGF β 1 and β 3 remained high in the tubule epithelium throughout development. TGF β 2 staining was found in the basement membrane surrounding the tubules throughout development, but was also detectable in the tubule epithelium beginning at 15.5 d.p.c. (Fig. 5, C and D) in a pattern similar to TGF β 1 and TGF β 3. Although the staining intensity for all three TGF β s in the tubular epithelium of the kidney began to decrease at 17.5 d.p.c., immunoreactivity for TGF β 1s 1–3 in the mesenchymal cells that support the tubules could be seen at this stage.

TGF β 1 has been previously localized to the zona fasciculata and zona reticularis in the cortex of the adult murine adrenal gland (Thompson et al., 1989). Similarly, in our studies, the embryonic adrenal gland also shows very strong TGF β immunoreactivity. Adrenal blastemas are present in the 11 d.p.c. embryo, but these do not consolidate into cortex and medulla until 14 d.p.c. (Rugh, 1990). Antibodies to TGF β 1 and TGF β 2 showed only slight reactivity in the 13.5 d.p.c. embryos; however by 15.5 d.p.c., very strong staining for TGF β 2 (Fig. 5, C, E, and F) and TGF β 1 was present in the cortex (presumptive zona fasciculata and zona reticularis) of the adrenal gland. This high level of expression for TGF β 1 and TGF β 2 persisted through 17.5 d.p.c.. Antibody staining was confined to the cortex since the medulla was negative for

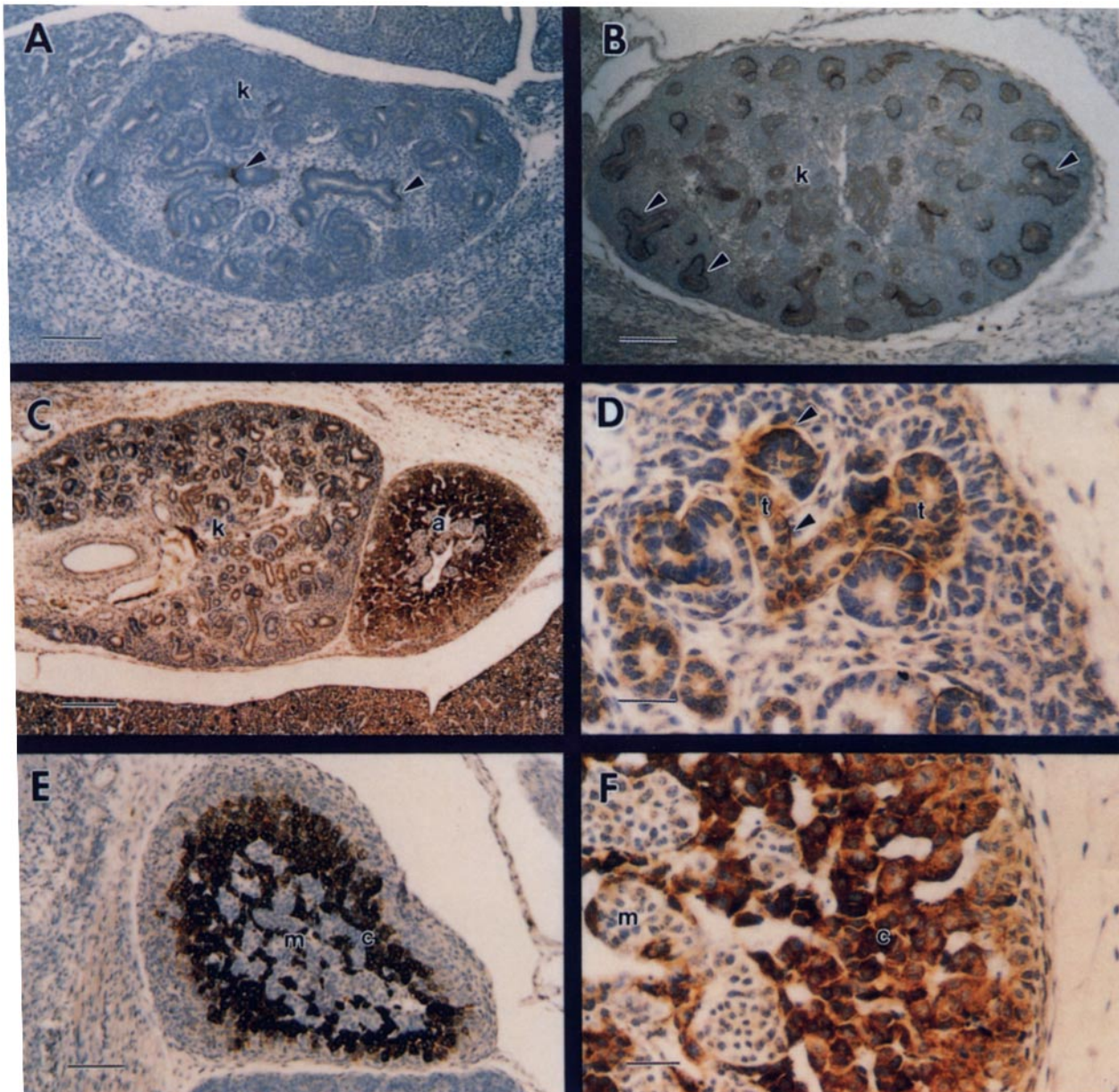


Figure 5. TGF β 2 localization in the embryonic kidney and adrenal gland. (A) 13.5 d.p.c. kidney showing TGF β 2 at the base of the epithelium in a subpopulation of tubules (arrows). (B) 14.5 d.p.c. kidney demonstrating TGF β 2 staining similar to that in (A). At 14.5 d.p.c., the staining is stronger and in almost all tubules of the kidney cortex (arrows). (C) Section through a 15.5 d.p.c. kidney and adrenal gland showing TGF β 2 localization in kidney tubules and cells of the adrenal cortex. (D) Higher power of the kidney in C showing TGF β 2 staining in the epithelium of the kidney tubules as well as in the supporting cells at the base of the epithelium (arrows). (E) TGF β 2 expression in the cortex of a 15.5 d.p.c. adrenal gland. The medullary cells show no TGF β 2 staining. (F) Higher power of the section in E showing TGF β 2 staining in the cells of the adrenal cortex but not the adrenal medulla. k, kidney; a, adrenal gland; t, kidney tubule; m, adrenal medulla; c, adrenal cortex. Bars: (A) 120 μ m; (B) 125 μ m; (C) 200 μ m; (D) 35 μ m; (E) 125 μ m; (F) 40 μ m.

TGF β 1 and β 2 immunoreactivity (Fig. 5, E and F). TGF β 3 did not appear to be present in the adrenal gland during these stages of development (13.5–17.5 d.p.c.).

Using sequential elution of intestinal villus cells and Northern blot analysis, Barnard et al. (1989) have demonstrated that TGF β 1 mRNA is present in a gradient along the villi epithelium in the adult rat intestine with highest levels evident at the tip of the intestinal villus. Immunohistochemistry reflects a similar gradient of TGF β proteins in the embryonic gut. Slight reactivity was seen at 14.5 d.p.c. in the

intestinal epithelium with all three TGF β antibodies, but by 15.5 d.p.c. significantly stronger staining in these cells was observed (Fig. 6 A). Similar to the TGF β 1 mRNA localization, staining appeared in a gradient fashion with the highest levels of TGF β proteins at the villus tip; lowest levels were in the intestinal crypts. Although TGF β 1 staining remained high at 17.5 d.p.c. (Fig. 6 B), TGF β 2 and TGF β 3 staining was not as intense as seen during earlier stages. In addition, the stratified squamous epithelium of the esophagus and fore-stomach in the 17.5 d.p.c. embryo showed intense TGF β 1

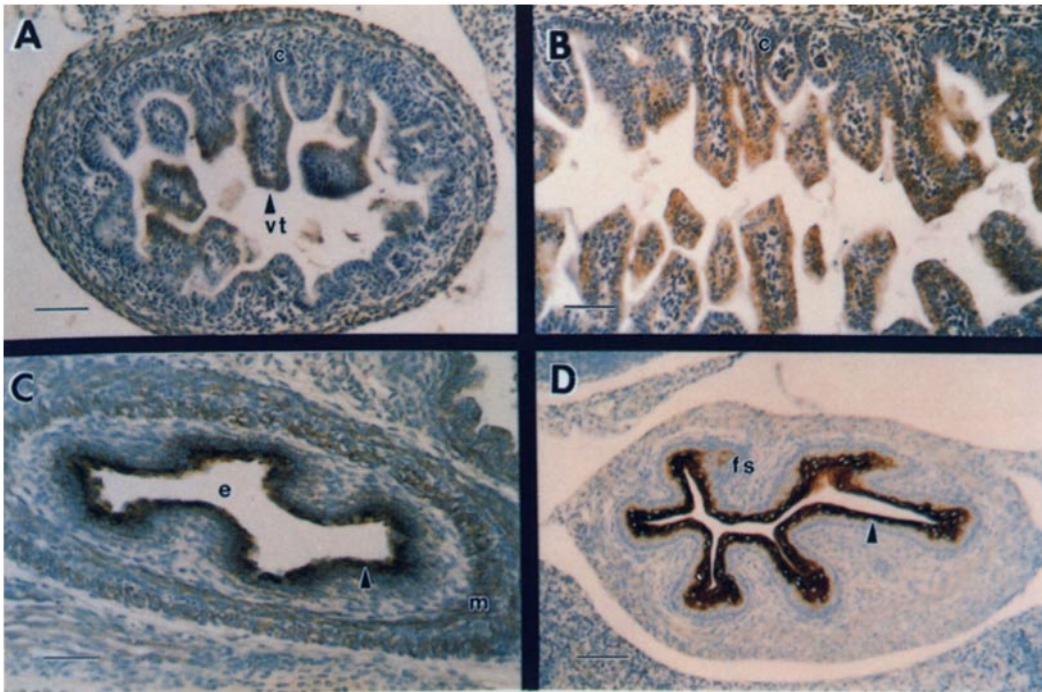


Figure 6. Expression of TGF β 1 and TGF β 2 in epithelia of the embryonic digestive tract. (A) TGF β 1 expression in the villi of the 15.5 d.p.c. intestine. Staining is highest at the villus tip (arrow) and lowest in the crypts. (B) Section through the 17.5 d.p.c. intestine showing TGF β 1 staining in the villi. Staining is similar to that in A. (C) Localization of TGF β 1 in the squamous epithelium (arrow) of the 17.5 d.p.c. esophagus. (D) TGF β 1 expression in the squamous epithelium of the fore stomach of a 17.5 d.p.c. embryo. g, gut; vt, villus tip; c, crypt; e, esophagus; m, muscle; fs, fore stomach. Bars: (A) 80 μ m; (B) 75 μ m; (C) 50 μ m; (D) 95 μ m.

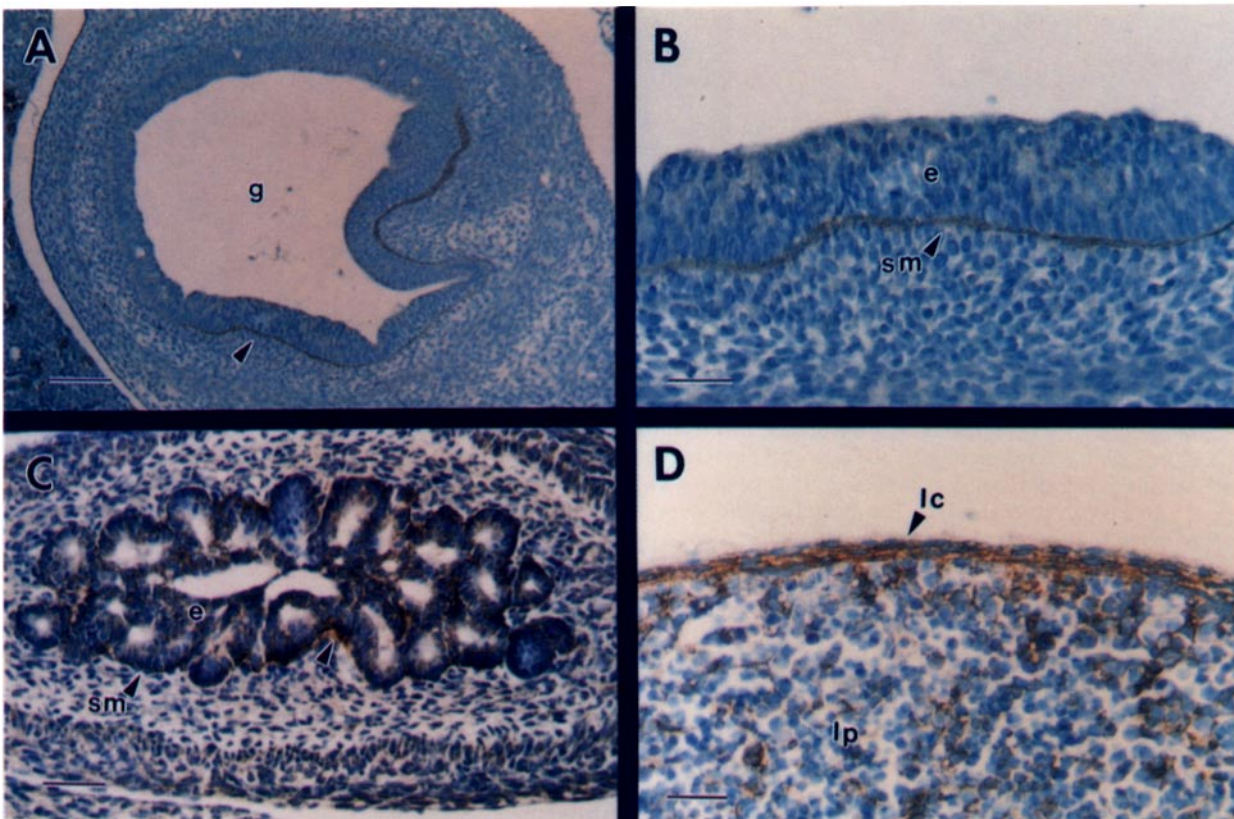


Figure 7. Localization of TGF β proteins in the submucosa of the embryonic stomach, intestines and in the liver capsule. (A) Section through a 13.5 stomach showing TGF β 2 expression in the submucosal region just basal to the epithelium (arrow). (B) Higher power of the section in A showing TGF β 2 staining in the submucosa (arrow). (C) Section demonstrating TGF β 2 localization in the submucosa of the 15.5 d.p.c. intestine (arrows). (D) Expression of TGF β 3 in the capsule (arrow) of a 13.5 d.p.c. liver. Low levels of TGF β 3 staining were also seen in the mesenchymal cells supporting the liver parenchyma. g, gut; e, epithelium; sm, submucosa; lc, liver capsule; lp, liver parenchyma. Bars: (A) 90 μ m; (B) 25 μ m; (C) 35 μ m; (D) 35 μ m.

staining (Fig. 6, C and D), but immunoreactivity for TGF β 2 or β 3 was not observed. Moderate staining for TGF β 1 (Fig. 6, A and C), β 2, and β 3 was also seen in the smooth muscle of the esophagus and intestine.

Earlier studies have reported that TGF β 2 mRNA is localized to the submucosal layer of the embryonic gut (Pelton et al., 1989). TGF β 2 protein staining was also found in the basement membrane of this layer (Fig. 7, A and B). At 13.5 d.p.c., a well demarcated line of staining for TGF β 2 was evident just basal to the mucosal (epithelial) layer of the developing stomach and intestines (Fig. 7, A-C). TGF β 2 immunoreactivity was not seen in the mucosal layer of the stomach nor in the surrounding mesenchymal layers, although staining was observed in the epithelial cells of the intestine (Fig. 7 C).

Previous reports have demonstrated the presence of TGF β 1 mRNA in megakaryocytes in the embryonic liver (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988) and although no TGF β mRNA has been found in liver hepatocytes, transcripts for TGF β 2 and β 3 have been localized to the capsule surrounding the liver of the 11.5 d.p.c. embryo (Pelton et al., 1990a). Similarly, light staining for TGF β 1 and β 3 was evident in megakaryocytes of the 12.5 d.p.c. liver and TGF β 3 immunostaining was observed in the liver capsule at 13.5 d.p.c. (Fig. 7 D). Although light staining for TGF β 3 was observed in the mesenchymal cells supporting the liver

parenchyma (Fig. 7 D), TGF β 1-3 staining of the hepatocytes was not detected.

Heine et al. (1990) have used immunohistochemical analysis to demonstrate that TGF β 1 colocalizes in the embryonic lung with a number of important ECM proteins. In addition, earlier reports have shown that TGF β 1, β 2, and β 3 mRNAs can be detected in the embryonic lung (Pelton et al., 1989, 1990a; Lehnert and Akhurst, 1988; Millan et al., 1991; Schmid et al., 1991). In the adult murine lung, the protein localization patterns in the proximal airways for all three TGF β s are essentially identical (Pelton et al., 1991). Correspondingly, TGF β proteins in the embryonic mouse lung, showed strikingly similar patterns of expression (Fig. 8, A-D). Although slight TGF β 1-3 immunoreactivity was seen in the epithelium of 17.5 d.p.c. bronchioles, staining for the TGF β s in the airways was seen primarily just basal to the respiratory epithelium (Fig. 8, A-D). Moreover, staining for TGF β 1-3 proteins was present in the smooth muscle cells in the walls of large blood vessels of the lung in a pattern similar to that seen in other large vessels of the embryo (compare Figs. 8, B and C and 4, C and D). The highest levels of TGF β expression in the embryonic lung were seen in late gestation at 17.5-18.5 d.p.c..

The Embryonic Sense Organs. The lens of the eye is derived from the surface embryonic ectoderm and by 12.5 d.p.c. becomes separate from the epidermis. Distinct layers

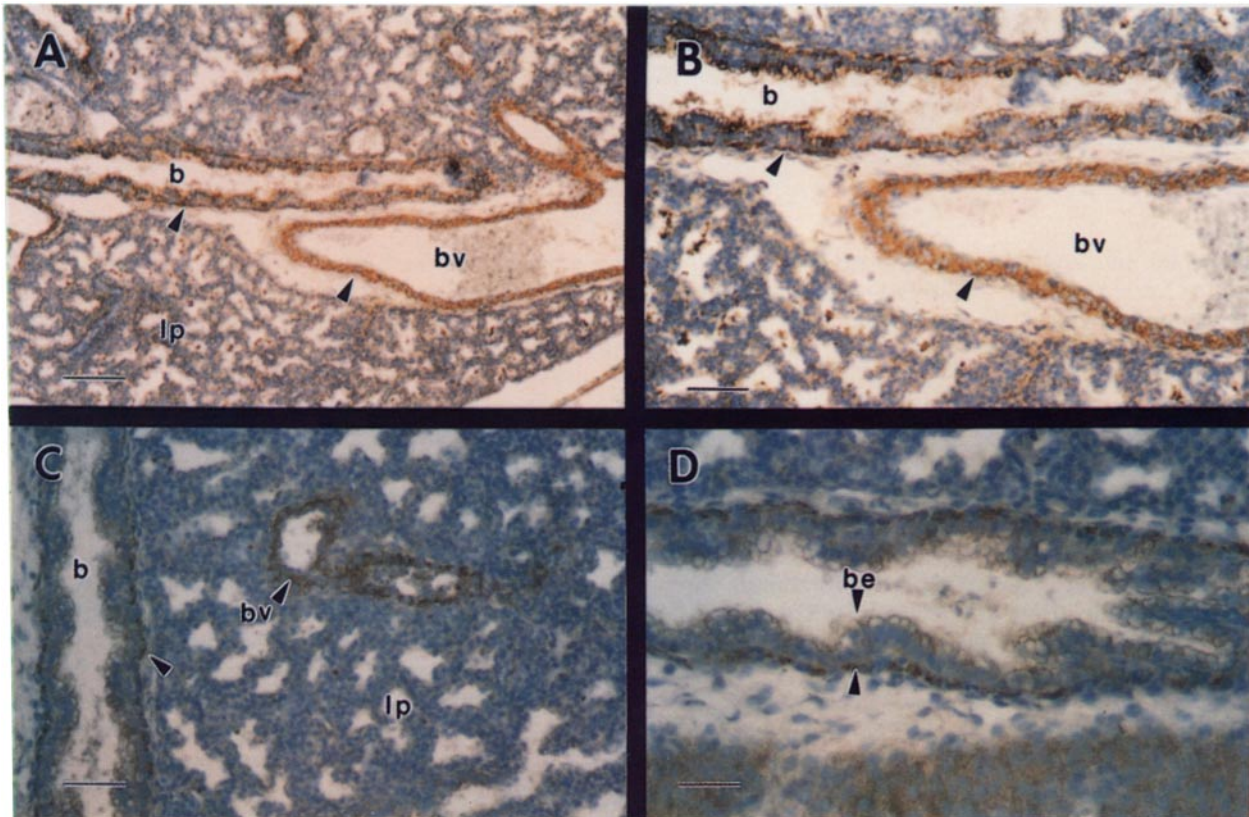


Figure 8. Expression of TGF β s in the 17.5 d.p.c. embryonic lung. (A) Section of a lung in a 17.5 d.p.c. embryo stained with TGF β 3 antibodies. Strong staining is seen in the bronchioles and large blood vessels (arrows). (B) Higher power of the section seen in A to show the TGF β 3 staining in the subepithelial region of the bronchioles and in the smooth muscle cells of the blood vessel. (C) Similar section to that in A incubated with TGF β 2 antibodies. Staining is seen just basal to the epithelium in the bronchiole as well as in the small blood vessel (arrows). (D) High power photomicrograph of a lung section stained with TGF β 1 antibodies. Low levels of TGF β 1 were localized to the epithelium but higher levels were seen in the subepithelium at the base of the epithelial cells (arrows). b, bronchiole; bv, blood vessel; lp, lung parenchyma; be, bronchiolar epithelium. Bars: (A) 115 μ m; (B) 60 μ m; (C) 55 μ m; (D) 35 μ m.

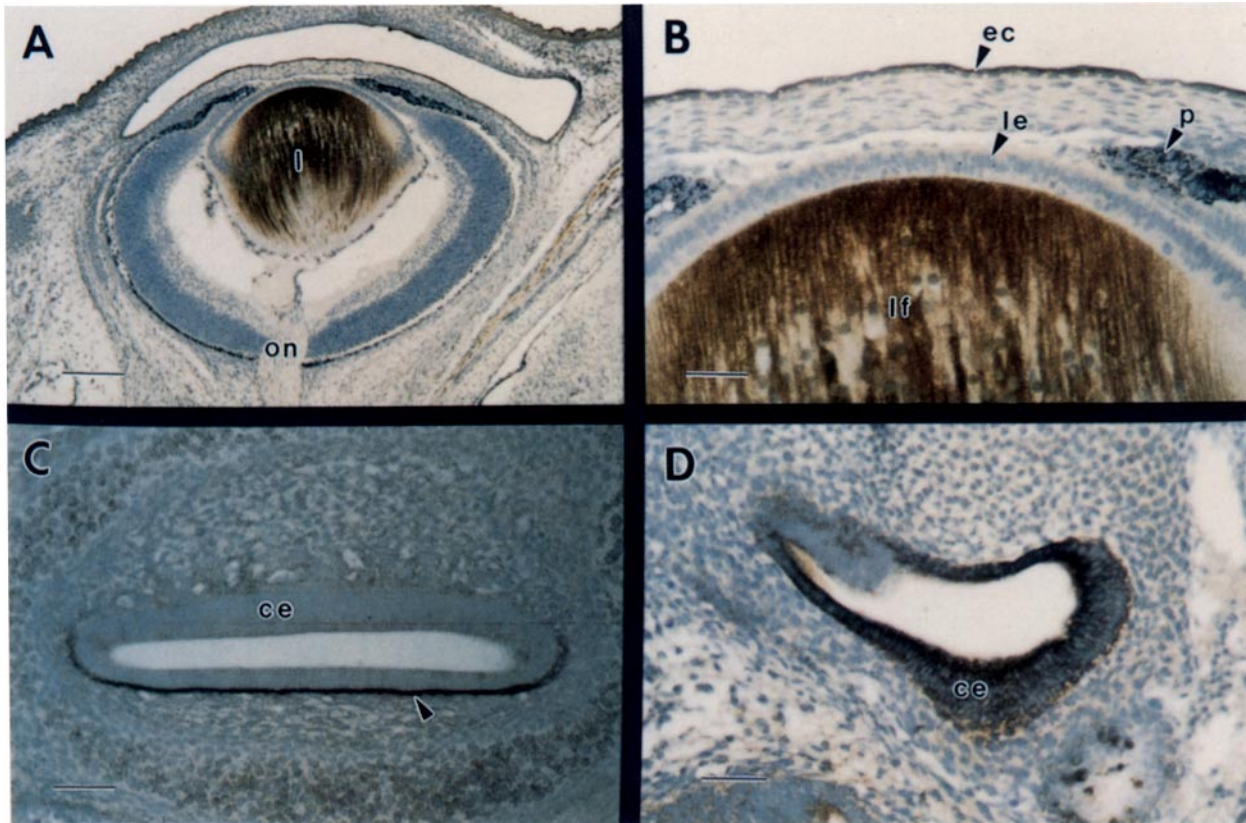


Figure 9. Localization of TGF β s in the sensory organs. (A) Expression of TGF β 1 in the lens fibers (but not the lens epithelium) of the 17.5 d.p.c. embryonic eye. (B) Higher power of A to illustrate the lack of staining at the border between the lens fibers and the lens epithelium. (C) Section through the 14.5 d.p.c. cochlea of the inner ear showing staining for TGF β 2 in the basement membrane just basal to the cochlear epithelium (arrow). (D) Section through the inner ear of a 13.5 d.p.c. embryo showing TGF β 3 staining throughout the cochlear epithelium. l, lens; on, optic nerve; ec, epithelium of cornea; p, retinal pigment; le, lens epithelium; lf, lens fibers; ce, cochlear epithelium. Bars: (A) 175 μ m; (B) 45 μ m; (C) 60 μ m; (D) 45 μ m.

of lens epithelium and lens fibers have formed by 13 d.p.c. (Rugh, 1990). Intense staining for TGF β 1 was seen in the lens fibers as early as 14.5 d.p.c. and remained strong through 17.5 d.p.c. (Fig. 9, A and B). However, neither the lens epithelium nor the equatorial region of lens fiber elongation showed TGF β 1 expression (Fig. 9, A and B). TGF β 2 and β 3 staining was also observed in the lens fibers but was much weaker than that for TGF β 1. Previous studies have found the presence of TGF β 2 mRNA in the embryonic eye although not in the lens fibers (Millan et al., 1991). Moreover, recent reports have found TGF β protein activity (predominantly TGF β 2) in the aqueous and vitreous humors of adult eyes (Connor et al., 1991; Jampel et al., 1991) indicating that TGF β expression in the eye continues from the embryo into adulthood.

TGF β 2 mRNA is present in the ciliated cochlear epithelium of the inner ear (Pelton et al., 1990a; Millan et al., 1991; Schmid et al., 1991). Immunostaining demonstrated that TGF β 2 protein was also found in the inner ear; however, in contrast to the TGF β 2 mRNA, it was localized in the basement membrane just basal to the cochlear epithelium (Fig. 9 C). This staining pattern was seen as early as 12.5 d.p.c. and was consistently high through 17.5 d.p.c.. Although TGF β 1 staining in the cochlea was not detected, TGF β 3 was localized throughout the cochlear epithelium in the 13.5

d.p.c. embryo with the highest levels of immunoreactivity found in the apical region (Fig. 9 D). Therefore, one of the most striking differences in immunostaining for TGF β isoforms was found in the ear. By 17.5 d.p.c., TGF β 3 staining was no longer present in this epithelium.

The Embryonic Central Nervous System. The central nervous system (CNS) is unique in that it is one of the first embryonic systems to arise, yet one of the last to complete development (Rugh, 1990). In general, the highest levels of TGF β immunoreactivity in the embryonic CNS were found with antibodies to TGF β 2 and TGF β 3; very little staining in the CNS was observed with TGF β 1 antibodies. By 12.5 d.p.c., the brain and spinal cord are well developed in the embryo and at this stage, TGF β 2 staining was already apparent in the meninges which envelop the CNS. The TGF β 2 staining in the meninges remained strong in the embryo from 13.5 until 17.5 d.p.c. (Fig. 10, A–E). This is in agreement with our in situ hybridization data which also shows TGF β 2 mRNA expression in the meninges of the embryonic brain (Pelton et al., 1989). In the 17.5 d.p.c. embryo, TGF β 2 staining was also found in glia surrounding neurons in the brain and in spinal cord astrocytes (Fig. 10, F and G). Although little TGF β 1 immunoreactivity was seen in the CNS, TGF β 3 staining was very strong in the brain, choroid plexus, and spinal cord (Fig. 10 H). Both TGF β 2 and β 3 were localized

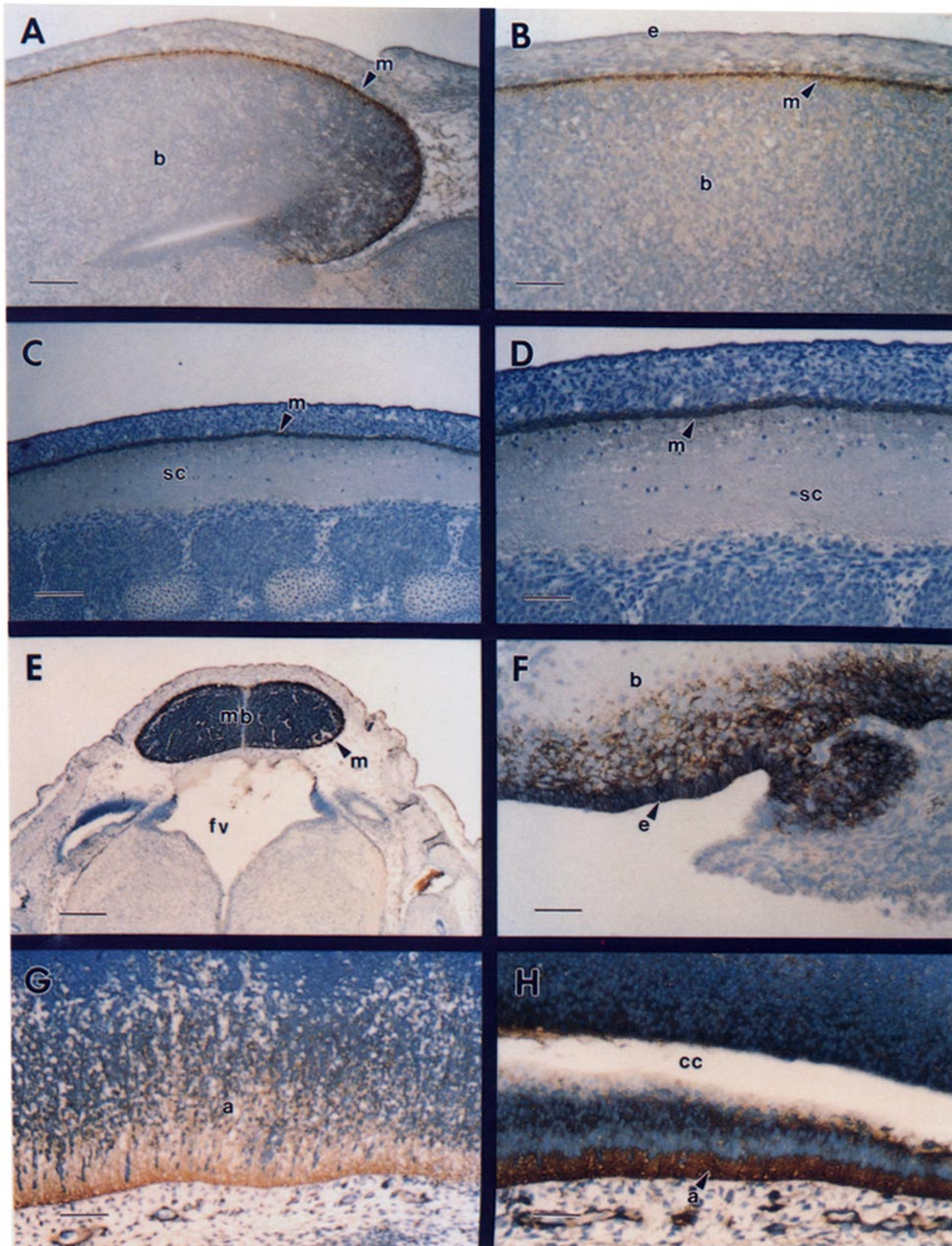


Figure 10. Expression of TGF β proteins in the embryonic central nervous system. Sagittal section through the midbrain region of a 14.5 d.p.c. embryo demonstrating TGF β 2 staining in the meninges surrounding the brain. (b) Higher power of the section in A to show staining in a thin layer of meningeal cells surrounding the brain. (C) Section showing TGF β 2 expression in the meninges surrounding the dorsal spinal cord in a 13.5 d.p.c. embryo. (D) Higher power of section in C to show strong TGF β 2 staining in the thin layered meninges and only weak staining in the neurons. (E) Frontal section through the midbrain region of a 14.5 d.p.c. embryo to demonstrate TGF β 2 staining in the meninges. (F) High power micrograph of a midbrain region of a 17.5 d.p.c. embryo to show TGF β 2 expression in the glial tissue surrounding the neurons. (G) Section through the 12.5 d.p.c. ventral spinal cord demonstrating TGF β 2 in astrocytes. (H) Section similar to G showing strong TGF β 3 staining in the astrocytes of the ventral spinal cord. b, brain; m, meninges; e, epidermis (B); sc, spinal cord; mb, midbrain; fv, fourth ventricle; a, astrocytes; cc, central canal of spinal cord. Bars: (A) 100 μ m; (B) 85 μ m; (C) 115 μ m; (D) 65 μ m; (E) 220 μ m; (F) 30 μ m; (G) 30 μ m; (H) 30 μ m.

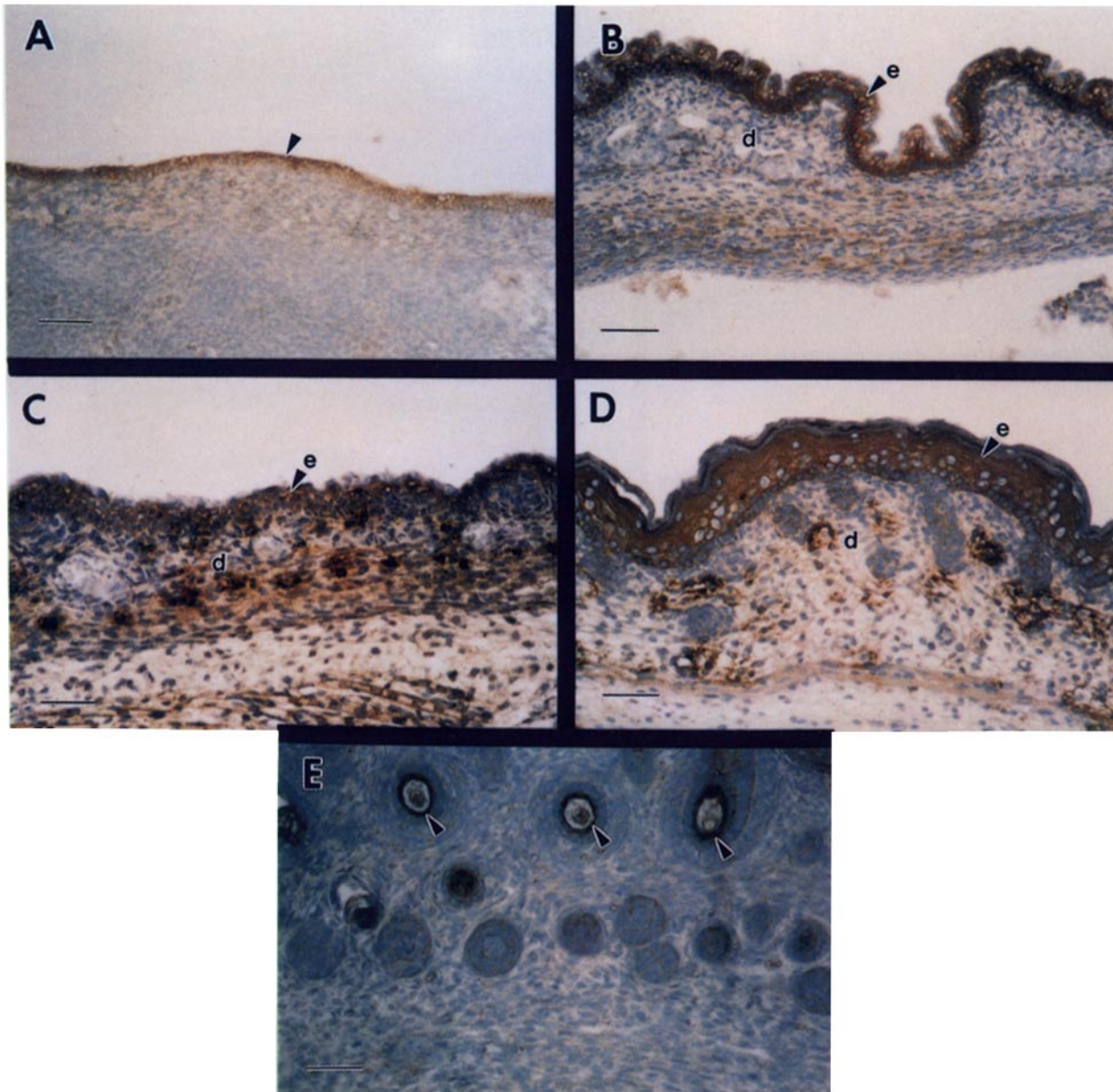


Figure 11. Localization of TGF β s in the embryonic skin. (A) TGF β 1 staining in epidermis (arrow) of the 14.5 d.p.c. skin but not the dermis. (B) TGF β 1 expression in the epidermis (arrow) of the 17.5 d.p.c. skin. The dermis is also negative at this stage. (C) Expression of TGF β 2 in the 15.5 d.p.c. embryonic skin. Staining is seen in both the dermis and epidermis. (D) Section through skin of a 17.5 d.p.c. embryo illustrating TGF β 2 staining primarily in the epidermis at this stage; only low levels were seen in the dermis. (E) Staining of 17.5 d.p.c. whisker follicles (arrows) with TGF β 1 antibodies. e, epidermis; d, dermis. Bars: (A) 80 μ m; (B) 50 μ m; (C) 35 μ m; (D) 35 μ m; (E) 50 μ m.

to spinal cord astrocytes as early as 12.5 d.p.c. (Fig. 10, G and H). Whereas, TGF β 2 staining in the astrocytes was absent after 13.5 d.p.c., TGF β 3 expression remained consistently high in these cells until late in gestation at 17.5–18.5 d.p.c.

The Embryonic Skin. The skin is formed first as a single cell layer, the periderm, and begins to differentiate into multiple layers at \sim 14 d.p.c. (Rugh, 1990). Significant staining for the TGF β s was first seen at this stage. At 14.5 d.p.c., intense staining for TGF β 1 and TGF β 3 was seen in the epithelial layer of the skin over the entire body, while only low levels of TGF β 2 could be detected at this stage (Fig. 11 A). TGF β 1 and TGF β 3 were still localized to the epidermis and

hair follicles through 17.5 d.p.c. (Fig. 2, A and B and 11, B and E). In the 17.5 d.p.c. embryo, TGF β 1 was also expressed at very high levels in the keratinized squamous epithelium of the mouth (Fig. 2, C and D). Earlier, we reported that TGF β 2 mRNA is localized to the dermis of 15.5 d.p.c. mouse embryo skin and showed that by 18.5 d.p.c., the expression had switched from the dermis to the epidermis (Pelton et al., 1989). In contrast, TGF β 2 protein in the 15.5 d.p.c. embryo was present in the dermis, epidermis and hair follicles of the skin (Fig. 11 C). By 17.5 d.p.c., most of the TGF β 2 staining in the dermis had faded but the staining in the epidermis remained strong (compare Fig. 11 C with D). The highest levels of TGF β 2 immunostaining at this stage were seen in the

epidermis of the skin, although the hair follicles were also positive for TGF β 2 staining.

Discussion

The data presented in these studies demonstrate that TGF β 1, TGF β 2, and TGF β 3 proteins are expressed during murine embryogenesis in a temporally and spatially regulated fashion. TGF β proteins are differentially expressed in the embryo and show isoform-specific and overlapping localization patterns. Moreover, in many tissues, the restriction of TGF β proteins corresponds with morphogenetic events in the embryo. Comparison of TGF β protein and mRNA localizations is consistent with an intricate pattern of gene transcription, translation, secretion, storage, and degradation of TGF β 1, β 2, and β 3 during embryogenesis of the mouse. The separate localization of TGF β mRNAs and proteins in some tissues and colocalization in others suggests that TGF β 1-3 act through both paracrine and autocrine mechanisms in the morphogenesis of many organs.

In many tissues, TGF β proteins are found in the same cells as the TGF β mRNA. For example, in situ hybridization analysis demonstrates TGF β 1-3 mRNA expression in the periosteum and osteocytes of developing skeletal tissue (Pelton et al., 1990a) in a pattern very similar to the TGF β immunostaining presented here. All three TGF β mRNAs (Pelton et al., 1990a) and proteins colocalized in the osteocytes of the intramembranous bones of the head and TGF β 1 immunostaining in the perichondrium of developing cartilage was strikingly similar to the TGF β 1 mRNA pattern (Pelton et al., 1990a) in this tissue. TGF β 2 and β 3 also showed examples of colocalization of mRNA and protein including TGF β 2 in the submucosal layer of the gut, the pulp and odontoblasts of the tooth, the meninges of the CNS, and the skin; and TGF β 3 in the capsule of the liver, the choroid plexus, and the large blood vessels of the embryo. Thus, for the cells in these tissues, the TGF β s may be working in an autocrine fashion.

In contrast to this, in many instances, TGF β mRNA and proteins were expressed in the same organ and yet were localized to different, but adjacent, cell types. For example, in the inner ear, transcripts for TGF β 2 are found in the cochlear epithelium (Pelton et al., 1990a; Schmid et al., 1991; Millan et al., 1991), while TGF β 2 protein was restricted to the basement membrane just basal to the cochlear epithelium (Fig. 9 C). Other examples of differential localization of TGF β mRNAs and proteins were seen in the cartilage, brain, heart, teeth, and skin. It is well established that the TGF β s are secreted proteins, thus, certain cells may synthesize and release TGF β proteins into the extracellular matrix of adjacent cells which have the potential to respond to these factors. Thus, our TGF β immunolocalization data, in conjunction with previous TGF β in situ hybridization studies, suggest that in many tissues, the TGF β s act in a paracrine mode. In support of this model, it has recently been shown that TGF β 1 bound to type IV collagen remains biologically active (Vishwas et al., 1990) and the ability of latent TGF β 1 to bind to heparin-Sepharose suggests that it contains a specific heparin binding site (Wakefield et al., 1989). Moreover, the latent forms of all TGF β s (except TGF β 2) contain the RGD integrin cellular recognition sequence (Ruoslahti and Pierschbacher, 1986). Thus, the extracellular matrix may act as a

repository for TGF β proteins. Indeed, at least one study has demonstrated that both freshly isolated and commercially available fibronectins can be contaminated with TGF β activity (Fava and McClure, 1987).

Secretion and subsequent storage of TGF β s in the extracellular matrix may be a primary regulatory step in the production of mature, biologically active TGF β protein in vivo. Experiments by Sato et al. (1990) demonstrate that homotypic cultures of both endothelial cells and smooth muscle cells secrete TGF β only in a latent form. However, if these two cell types are cocultured, a portion of the total TGF β secreted into the culture medium is activated. This suggests that in vivo, latent TGF β s may be produced and secreted by one cell type and activated in conjunction with an adjacent cell type. Activation of latent TGF β may be through serine proteases (e.g., plasmin; Lyons et al., 1989) released locally by cells. This idea is supported by the observation that specific inhibitors of plasmin block the activation of TGF β in heterotypic cultures of endothelial cells and pericytes (Sato and Rifkin, 1989). Furthermore, Saksela and Rifkin (1990) have proposed a model whereby TGF β regulates the synthesis of plasminogen activator which in turn regulates the release of bFGF from extracellular matrix. Interestingly, a comparison of TGF β 2 and bFGF staining during embryogenesis demonstrates that both of these proteins are often found in the basement membrane next to an epithelial cell type (Figs. 5, A and B; 7, A-C; 8 C; 9 C; and Gonzalez et al., 1990), indicating that these molecules may interact in vivo.

In some of the tissues we examined that express high levels of TGF β mRNA, very little TGF β protein was observed; the converse is also true. Other groups have also observed this phenomenon of TGF β expression (Lehnert and Akhurst, 1988; Akhurst et al., 1991). This may suggest that some of the reported mRNA or protein localization is nonspecific; however, the controls on our experiments have consistently shown that the TGF β probes used for the in situ hybridization analysis as well as the TGF β antibodies used in the immunohistochemistry are specific for their respective molecules. An alternative explanation that is consistent with this data is suggested by the work of Madisen et al. (1988) who found that although a prostatic adenocarcinoma cell line produced more TGF β 1 mRNA than TGF β 2, there was significantly more TGF β 2 protein than TGF β 1 present in these cells. Hence, it is possible that some cells transcribe but never translate a given TGF β gene. It is known, for example, that TGF β 2 has at least five different transcripts in the mouse, but it is not known how many are translated into functional proteins (Miller et al., 1990).

To date there have been no reports concerning the embryonic expression of TGF β 2 and TGF β 3 proteins in mammals; however, several papers studying the distribution of TGF β 1 have been published (Heine et al., 1987, 1990; Thompson et al., 1989; Flanders et al., 1989). Comparison of our results of TGF β 1 staining with these previous data shows that in some tissues the staining patterns were very similar, while in others they differ. For example, immunostaining for TGF β 1 has been reported in adult murine adrenocortical and kidney tubule cells (Thompson et al., 1989) as well as in murine embryonic chondrocytes (Flanders et al., 1989) and osteocytes (Heine et al., 1988); these tissues showed very strong staining for TGF β 1 in our study as well.

Similarly, Jakowlew et al. (1991) observed staining for TGF β 2, β 3, and β 4 (and to a lesser extent β 1) in avian embryonic chondrocytes and cardiac myocytes in patterns much like those seen in the data presented here. Thus, in general, our results are consistent with the expression of TGF β 1 as reported by others (Thompson et al., 1989; Heine et al., 1988; Flanders, et al., 1988; Jakowlew et al., 1991), however, we do find some differences between our studies and those reported by Heine et al. (1988). For example, the intense TGF β 1 staining in the epidermis of the embryonic skin (Figs. 2 B and 11, A-E) and in the epithelium of the gut (Figs. 6, A-D) was not reported by Heine et al. (1988). In contrast, Heine and colleagues found very strong TGF β 1 staining in the atrioventricular valves of the heart which was not seen in our studies. There are several possible reasons for these discrepancies between our results and those of Heine et al. (1988). The TGF β 1 antipeptide antibodies used in their experiments were raised against the first 30 amino acids in the mature region of TGF β 1 (Heine et al., 1988), while those used in our studies were raised against residues 4-19 of the TGF β 1 amino acid sequence. Thus, while the anti-TGF β 1 antibodies used in both studies react with β 1, they may recognize different conformations of the protein. For example, Heine et al. (1988) found that embryos fixed in paraformaldehyde did not show immunoreactivity with their antibodies, while embryos fixed in Bouin's fixative reacted strongly. In contrast, all embryos used for our immunohistochemistry were paraformaldehyde fixed. Alternatively, the possibility that some antibodies may cross-react with epitopes on other proteins should also be entertained; however, Western blot analysis indicates that specific staining is achieved with each of the TGF β antibodies used in this study (Fig. 1).

Yang and Moses (1990) have demonstrated a range of in vivo cellular events (migration, proliferation, inhibition, angiogenesis, etc.) in the chick embryo chorioallantoic membrane in response to exogenously added TGF β 1. A large body of in vitro evidence also strongly suggests that many cellular activities critical to morphogenesis (cell proliferation, cell migration, cell differentiation, extracellular matrix production, etc.) are mediated by the TGF β s (reviewed in Pelton and Moses, 1990a). The expression of TGF β 1, β 2, and β 3 during mouse embryogenesis, as presented in our results, correlates well with these in vivo and in vitro observations. For example, TGF β 1 and β 2 are found in the adrenal cortex of the mouse embryo (Fig. 5, E and F) and human fetal adrenocortical cell differentiation is inhibited by TGF β 1 (Riopel et al., 1989). TGF β proteins also inhibit the growth of mesangial and proximal tubular cells in the kidney (Avner, 1990), regulate differentiation, proliferation and extracellular matrix deposition in bone and cartilage cells (Centrella et al., 1988) as well as in lung cells (Pelton and Moses, 1990b), mediate transdifferentiation of cells in the embryonic heart (Potts and Runyan, 1989), control the growth and differentiation of skin cells (Pittelkow et al., 1988), and appear to regulate morphogenesis, proliferation, and differentiation in astroglial cells of the central nervous system (Toru-Delbauffe et al., 1990). These are all cell types which we show to express TGF β s 1-3 during embryogenesis. A major challenge is to now provide evidence that the localization of TGF β 1, β 2, and β 3 proteins in specific tissues and cells in the embryo has biological relevance for murine embryonic development. The most direct in vivo method to achieve this

end may be to construct transgenic and/or chimeric animals which either mis-express or do not express TGF β genes. Nonetheless, the temporal and spatial expression of TGF β s 1-3 at crucial steps in the morphogenesis of specific organs in the mouse undoubtedly implicates a role for these factors in the development of the mammalian embryo.

In conclusion, we have generated isoform-specific antibodies which recognize TGF β 1, β 2, or β 3 and have used them to study the distribution of these three proteins during murine embryogenesis. We have observed that the expression patterns for each protein are both temporally and spatially unique. Similarly, these distinct but overlapping patterns of expression are seen for TGF β 1, β 2, and β 3 mRNAs as well (Pelton et al., 1990a). While the mRNA and protein expression patterns do not always strictly coincide, TGF β protein is often found in cells or extracellular matrix positioned next to a cell type in which the mRNA is found in many tissues. This is consistent with the premise that during embryonic development, TGF β 1, β 2, and β 3 may act through both autocrine and paracrine mechanisms. Currently, there is little evidence ascribing unique functions for these three closely related isoforms of TGF β . The expression patterns shown in this study of different isoforms of TGF β in dissimilar cell types, for example, in the eye and ear, may imply separate cell-specific functions for each of the TGF β s in vivo. Together with the known biological activities of the β -TGFs, these data support the hypothesis that TGF β 1, β 2, and β 3 are important mediators of cell-cell interaction during embryonic development and may offer insight into an explanation for the evolutionary persistence of these very closely related proteins.

The authors thank Dr. Jeanette Thorbecke for help with the preparation of antipeptide antibodies to TGF β 1 and β 2, and Dr. Mahlon Johnson for expert help with the immunohistochemistry. We are indebted to Dr. Kathleen Flanders for her help in designing the immunostaining protocol as well as for invaluable advice on the technique. We also thank Dr. Cathy Murphy and Dr. Chris Wright for helpful comments on the manuscript.

R. W. Pelton was supported by National Institutes of Health (NIH) grant T32-GMO7347 for the medical scientist training program, H. L. Moses by NIH grants CA 42572 and CA 48799; and L. I. Gold by NIH grant CA 49507.

Received for publication 17 May 1991 and in revised form 14 August 1990.

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