Role of Transforming Growth Factor-β in the Development of the Mouse Embryo

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Abstract. Using immunohistochemical methods, we have investigated the role of transforming growth factor-β (TGF-β) in the development of the mouse embryo. For detection of TGF-β in 11–18-d-old embryos, we have used a polyclonal antibody specific for TGF-β type 1 and the peroxidase–antiperoxidase technique. Staining of TGF-β is closely associated with mesenchyme per se or with tissues derived from mesenchyme, such as connective tissue, cartilage, and bone. TGF-β is conspicuous in tissues derived from neural crest mesenchyme, such as the palate, larynx, facial mesenchyme, nasal sinuses, meninges, and teeth. Staining of all of these tissues is greatest during periods of morphogenesis. In many instances, intense staining is seen in mesenchyme when critical interactions with adjacent epithelium occur, as in the development of hair follicles, teeth, and the submandibular gland. Marked staining is also seen when remodeling of mesenchyme or mesoderm occurs, as during formation of digits from limb buds, formation of the palate, and formation of the heart valves. The presence of TGF-β is often coupled with pronounced angiogenic activity. The histochemical results are discussed in terms of the known biochemical actions of TGF-β, especially its ability to control both synthesis and degradation of both structural and adhesion molecules of the extracellular matrix.

TRANSFORMING growth factor-β (TGF-β),[1] first discovered in an assay based on its ability to transform fibroblasts phenotypically in culture, by now has been shown to have profound effects on nearly all cell types, influencing either their proliferation, differentiation, or other aspects of their function (for reviews, see Roberts and Sporn, 1987; Sporn et al., 1986, 1987). The amino acid sequence of TGF-β is totally conserved among human, bovine, and porcine species (Derynck et al., 1985; Derynck and Rhee, 1987; Van Obberghen et al., 1987), indicating that the functions it controls may be critical for the organism. Recently, a second form of TGF-β, with identical biological activity in many cells, has been described (for a review, see Massagué, 1987); the partial amino acid sequence of this peptide, called TGF-β2, is also highly conserved in humans, cows, and pigs (Wrann et al., 1987; Ikeda et al., 1987; Seyedin et al., 1987; Cheifetz et al., 1987).

The high concentrations of TGF-β found in platelets (Assoian et al., 1983) and in bone (Seyedin et al., 1985, 1986) suggest that it might have a significant role in repair of tissue damage (Sporn et al., 1983) and in bone formation and remodeling (Centrella et al., 1987; Robey et al., 1987; Pfeilschifter and Mundy, 1987). Indeed, subcutaneous injection of TGF-β into newborn mice produces a granulation response (formation of new blood vessels and connective tissue) similar to that seen in a healing wound (Roberts et al., 1986). Studies in vitro have shown that TGF-β controls formation of connective tissue proteins such as collagen and fibronectin in various mesenchymal cells (Ignotz and Massagué, 1986; Roberts et al., 1986; Varga and Jimenez, 1986; Fine and Goldstein, 1987; Ignotz et al., 1987). In this respect, TGF-β controls two separate processes: (*a*) synthesis, which TGF-β increases by enhancing the activity of the promoters for the fibronectin (Bourgeois, S., personal communication) and type I collagen genes (Rossi et al., 1988), and (*b*) degradation, which TGF-β blocks, both by decreasing secretion of proteases and by increasing secretion of protease inhibitors (Chiang and Nilsen-Hamilton, 1986; Laiho et al., 1986; Edwards et al., 1987; Lund et al., 1987). The net result of these effects of TGF-β on the synthesis and degradation of matrix proteins is a marked enhancement of their accumulation.

It has often been proposed that cellular and biochemical

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1. *Abbreviations used in this paper*: TGF-β, transforming growth factor-β; SPARC, secreted protein, acidic, rich in cysteine.

mechanisms involved in embryogenesis may be reiterated during tissue repair in the adult; angiogenesis, and the synthesis and degradation of matrix proteins such as collagen and fibronectin, are all critical processes in embryogenesis (Newgreen and Thiery, 1980; Hay, 1981; Rovasio et al., 1983; Icardo and Manasek, 1984). The remarkable conservation of TGF-β sequences between species, its ability to modulate cellular differentiation and cellular function, together with its specific effects on matrix protein formation, on chemotaxis of cells (Postlethwaite et al., 1987; Wahl et al., 1987), and on angiogenesis (Roberts et al., 1986; Madri et al., 1988), all strongly suggest that this peptide plays a fundamental role in embryogenesis. Two other members of the TGF-β family of peptides, Müllerian inhibitory substance (Cate et al., 1986) and the product of the decapentaplegic gene complex in *Drosophila* (Padgett et al., 1987) have already been implicated in embryonic remodeling of the developing male reproductive system of mammals and in dorsal-ventral patterning in the fly embryo, respectively. TGF-β–like activity has been reported in extracts of mouse embryos (Proper et al., 1982; Hill et al., 1986), and immunohistochemical staining has demonstrated the presence of TGF-β in bone, liver, and thymus of 6-mo bovine fetuses (Ellingsworth et al., 1986). However, the intrinsic physiological role of this peptide in the developing embryo is still unknown.

The present studies were therefore undertaken to obtain further information about the role of TGF-β at different stages of the development of the mouse embryo, beginning at 11 d of gestation; analysis of earlier embryos is clearly also required and will be the subject of future studies, focusing not only on rodent but also on early chick embryos. Here, we present data that suggest that TGF-β has an important role in many embryonic tissues, particularly in those of mesodermal and neural crest origin, at specific times when critical morphogenetic and histogenetic events occur. We have used Northern RNA blot and immunohistochemical methods for these studies and have developed improved methods for demonstrating the specificity of the immunohistochemical staining of TGF-β. These studies provide new insights into tissue and organ-specific sites of TGF-β action; both the spatial and temporal patterns of TGF-β expression provide clues to its specific effects on the developmental programs in target tissues. In this article, we present an overview of our results in the embryo as a whole; more detailed investigations of the specific immunohistochemical staining patterns of TGF-β in specialized tissues will be the subject of future communications.

Materials and Methods

Extraction of RNA from Mouse Embryos

RNA was prepared from Swiss-Webster mouse embryos at different stages of development; at early stages (days 8–10) the entire conceptus including both embryonic and extraembryonic tissue was removed from the uterine wall, whereas at later stages, the embryo proper was dissected free of placenta and extraembryonic tissues. Embryos were quick frozen in liquid nitrogen. Total RNA was isolated from ~1 g of embryos at each stage by the method of Auffray and Rougeon, 1980 (extraction in 3 M LiCl, 6 M urea, 0.1% SDS, 0.01 M NaOAc, pH 5, 100 μg/ml heparin); poly(A)$^+$ RNA was then prepared by one cycle of chromatography on oligo(dT)-cellulose. 1 μg of poly(A)$^+$ RNA was electrophoresed in 1.2% agarose gels and electroblotted onto nitrocellulose. Blots were hybridized with a nick-translated murine cDNA probe (Derynck et al., 1986). Blots were washed at 60°C in 0.1 × SSC, 0.1% SDS.

Antibodies

Polyclonal antibodies to TGF-β1 were made in rabbits to a synthetic peptide corresponding to the amino-terminal 30 amino acids of TGF-β1 as described by Ellingsworth et al. (1986). These antibodies specifically detect TGF-β1, and do not recognize TGF-β2. IgG fractions, prepared from these antisera by passage over protein A-Sepharose (Ellingsworth et al., 1986), were used for all experiments reported here. For control experiments, we removed TGF-β–specific antibodies by incubating aliquots of IgG (250 μl) with ~200 μl of Sepharose (agarose gel, Pharmacia Fine Chemicals, Piscataway, NJ), to which TGF-β1 had been coupled as described previously (Florini et al., 1986); an equivalent aliquot of the IgG was also incubated with uncoupled Affi-gel 10 (a derivatized cross-linked agarose gel, Bio-Rad Laboratories, Richmond, CA) as a positive control. After an overnight incubation at 4°C, the coupled Sepharose or the Affi-gel was removed by centrifugation. The supernatants were tested for their abilities to recognize TGF-β in an enzyme-linked immunosorbent assay (Flanders et al., 1987) to evaluate the completeness of removal of TGF-β–specific antibodies; the IgG treated with uncoupled agarose had a titer of 1:750 in the assay, whereas there was no detectable reactivity of the IgG that had been treated with agarose coupled to TGF-β.

Immunohistochemical Staining

The distribution of TGF-β was evaluated by immunohistochemical staining of sagittal sections prepared from whole mouse embryos of 11, 13, 15, and 18 d of gestation. Embryos were fixed in Bouin solution (0.9% picric acid, 9% [vol/vol] formaldehyde, 5% acetic acid). After dehydration through a graded series of ethanol solutions, the embryos were embedded in paraffin. Sections 5 μm thick were deparaffinized, stained with Harris hematoxylin, and subjected to immunohistochemical staining using the following schedule: (*a*) blocking of endogenous peroxidase with 0.3% hydrogen peroxide in methanol (30 min); (*b*) treatment with 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO), buffered to pH 5.5 with 0.1 M sodium acetate in 0.15 M NaCl (30 min); (*c*) blocking of nonspecific protein binding with 10% goat serum (45 min); (*d*) incubation with 0.1 mg/ml anti-TGF-β (at 4°C overnight); (*e*) incubation with affinity-purified goat anti–rabbit IgG diluted 1:40 in buffer (1 h); (*f*) incubation with rabbit peroxidase–antiperoxidase diluted 1:100 in buffer (1 h); and (*g*) treatment with 0.5 mg/ml diaminobenzidine (Sigma Chemical Co.) in 0.05 M Tris-buffered saline (0.05 M Tris buffer, pH 7.2, 0.15 M NaCl) containing 0.1% H_2O_2 (5 min). The buffer solution used for rinsing between each step consisted of 0.01 M Tris-buffered saline, 0.1% normal goat serum, and merthiolate in a dilution of 1:10,000. For antibody dilution, the following solution was used: 0.01 M Tris-buffered saline, 1% normal goat serum, and merthiolate diluted as above. Controls were done either by (*a*) replacing the anti–TGF-β rabbit IgG by normal rabbit IgG or bovine serum albumin or (*b*) using the antigen-depleted IgG preparation described above. Normal goat serum, affinity-purified goat anti–rabbit IgG, and peroxidase–antiperoxidase were obtained from Cooper Biomedical, Inc., Malvern, PA.

Results

Expression of mRNA for TGF-β in Mouse Embryos

For our initial investigation of TGF-β during development, expression of TGF-β1 mRNA in mouse embryos of different gestational age was examined by Northern blot analysis. As shown in Fig. 1, relatively high levels of TGF-β mRNA were detected in poly(A)$^+$ RNA prepared from embryos ranging from 8 to 18 d of age. This continuous high expression is in marked contrast to that found for TGF-α, which is expressed primarily in early embryos (Lee et al., 1985). To address the roles of TGF-β in embryogenesis, and to identify tissue-specific and cell-specific developmentally regulated sites of TGF-β action, an immunohistochemical analysis of TGF-β localization in sections of whole, fixed mouse embryos was undertaken, using an antibody raised against the NH_2-ter-

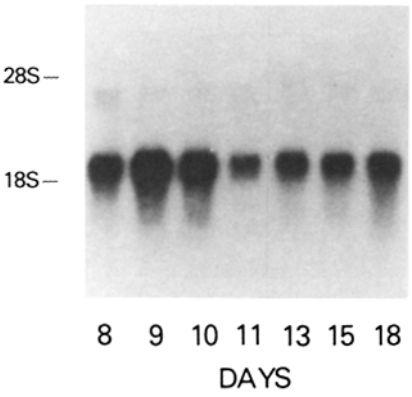


Figure 1. Poly(A)$^+$ RNA (1 μg) from mouse embryos of different ages was electrophoresed on agarose gels, blotted, and hybridized to a murine TGF-β1 cDNA probe. mRNA at days 8–10 of development was prepared from the entire conceptus, whereas mRNA from all other embryos was prepared from the embryo proper.

minal 30 amino acids of the mature TGF-β1 molecule (Ellingsworth et al., 1986).

Specificity of Staining

An IgG fraction from normal rabbit serum was used as the primary control for most studies. For more complete demonstration of the specificity of the staining, we first attempted to block the staining by preincubating the anti-TGF-β IgG with a solution of the peptide against which it was raised. While this procedure generally resulted in a substantial decrease in the intensity of the staining, it did not completely abolish the staining. Inasmuch as the peptide 1-30 of TGF-β has limited solubility at neutral pH, it is possible that the antibody was not completely saturated with peptide. Moreover, the ligand (either peptide 1-30 or TGF-β itself) bound to a solid support has higher affinity for the antibody than ligand in solution (not shown). For these reasons we adopted an alternative approach to demonstrate specificity: we removed the TGF-β-specific antibodies from the IgG by preincubating it with Sepharose (agarose gel) to which TGF-β had been coupled. This preincubated, depleted IgG preparation showed no reactivity toward TGF-β or peptide 1-30 in an ELISA assay (see Materials and Methods). In these experiments, anti-TGF-β IgG preincubated with agarose to which no ligand had been coupled was used as the positive control. Fig. 2 shows an area of the tail of a 15-d embryo stained with anti-TGF-β IgG that was preincubated with either control agarose (Fig. 2 *A*) or with agarose to which TGF-β had been coupled (Fig. 2 *B*). The TGF-β-specific staining is localized in the connective tissue sheath separating developing muscles from the subdermal mesenchyme and also in fibroblasts separating individual groups of myotubes. No staining was observed when the depleted IgG was used (Fig. 2 *B*), thus demonstrat-

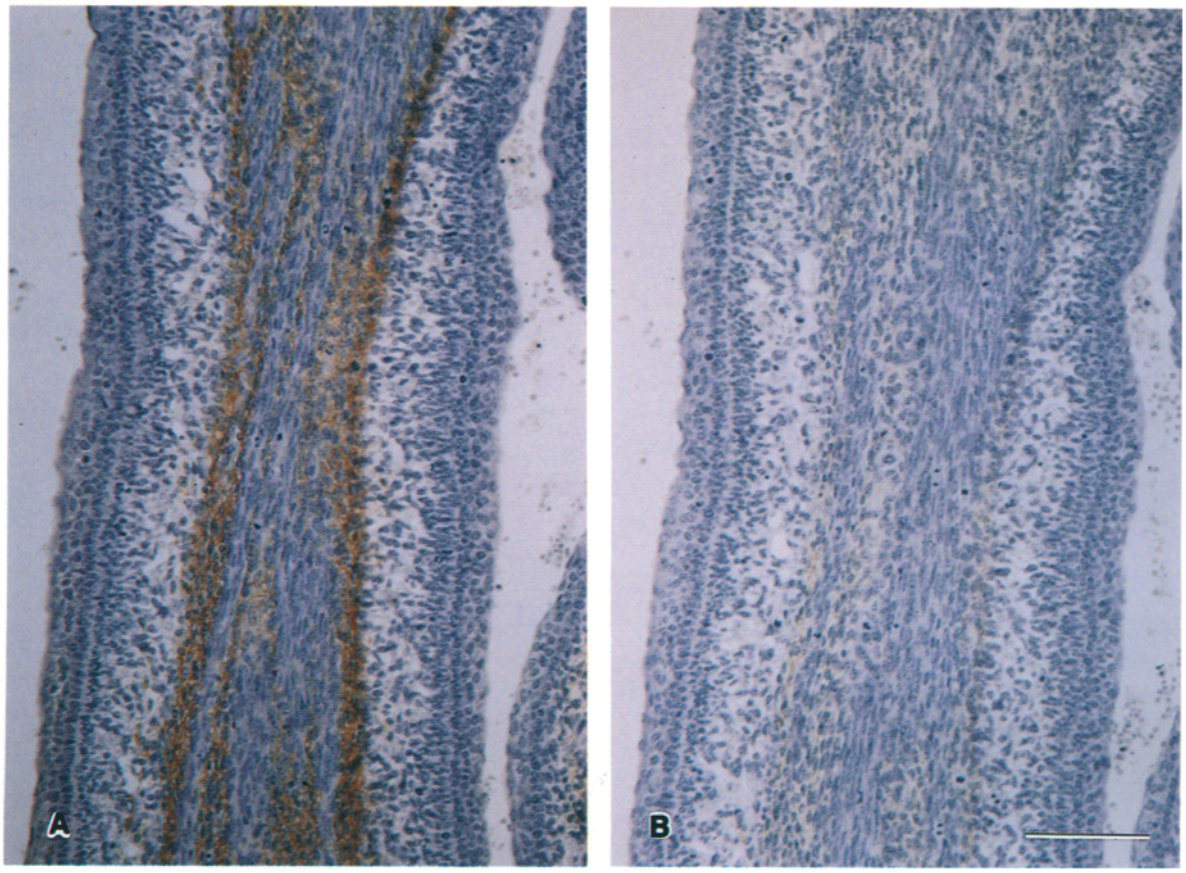


Figure 2. Parasagittal section through the tail of a 15-d embryo. (*A*) Antibodies to TGF-β incubated with uncoupled agarose (see Materials and Methods) stain connective tissue sheaths of muscle; (*B*) an equivalent aliquot of antibody incubated with agarose to which TGF-β had been coupled gave no staining. Dilution of the anti-TGF-β IgG preparation was 1:390. Bar, 100 μm.

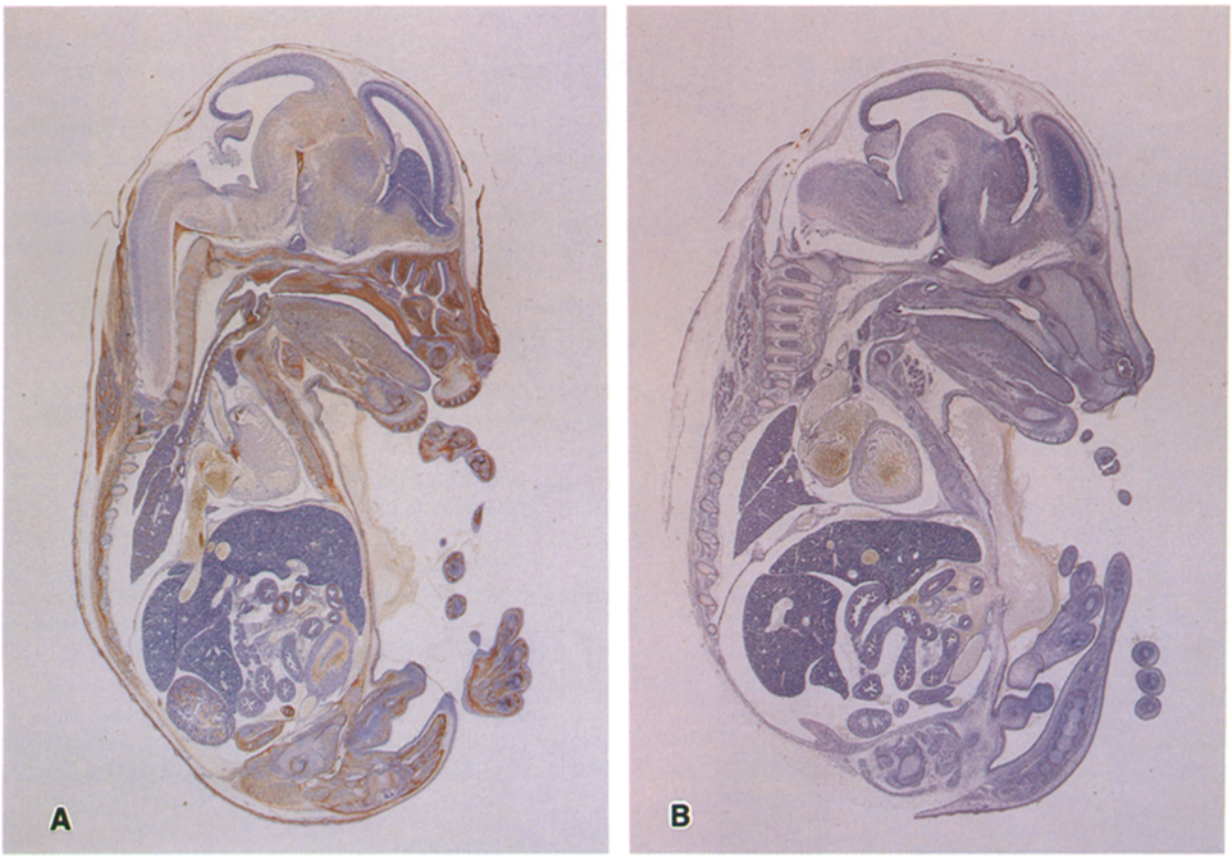


Figure 3. Sagittal section through 15-d embryo stained with (*A*) anti-TGF-β IgG or (*B*) with normal rabbit control IgG. TGF-β staining is seen in many tissues in *A*, and essentially nowhere in *B*. Erythrocytes within the heart and liver are peroxidase-positive in both figures. ×8.5. Bar, 100 μm.

ing specific TGF-β immunoreactivity. Although we have shown that immunoreactive staining is abolished by preadsorbing immune IgG with TGF-β, at present we do not know which specific epitope or set of epitopes on TGF-β is recognized by this IgG preparation, nor do we know the molecular form or physical state of the epitope. Thus, it is possible that some of the staining we have observed is the result of the antibody interacting with TGF-β precursor molecules, rather than the mature molecular species, although we can exclude the possibility of staining of type 2 TGF-β (Ellingsworth et al., 1986).

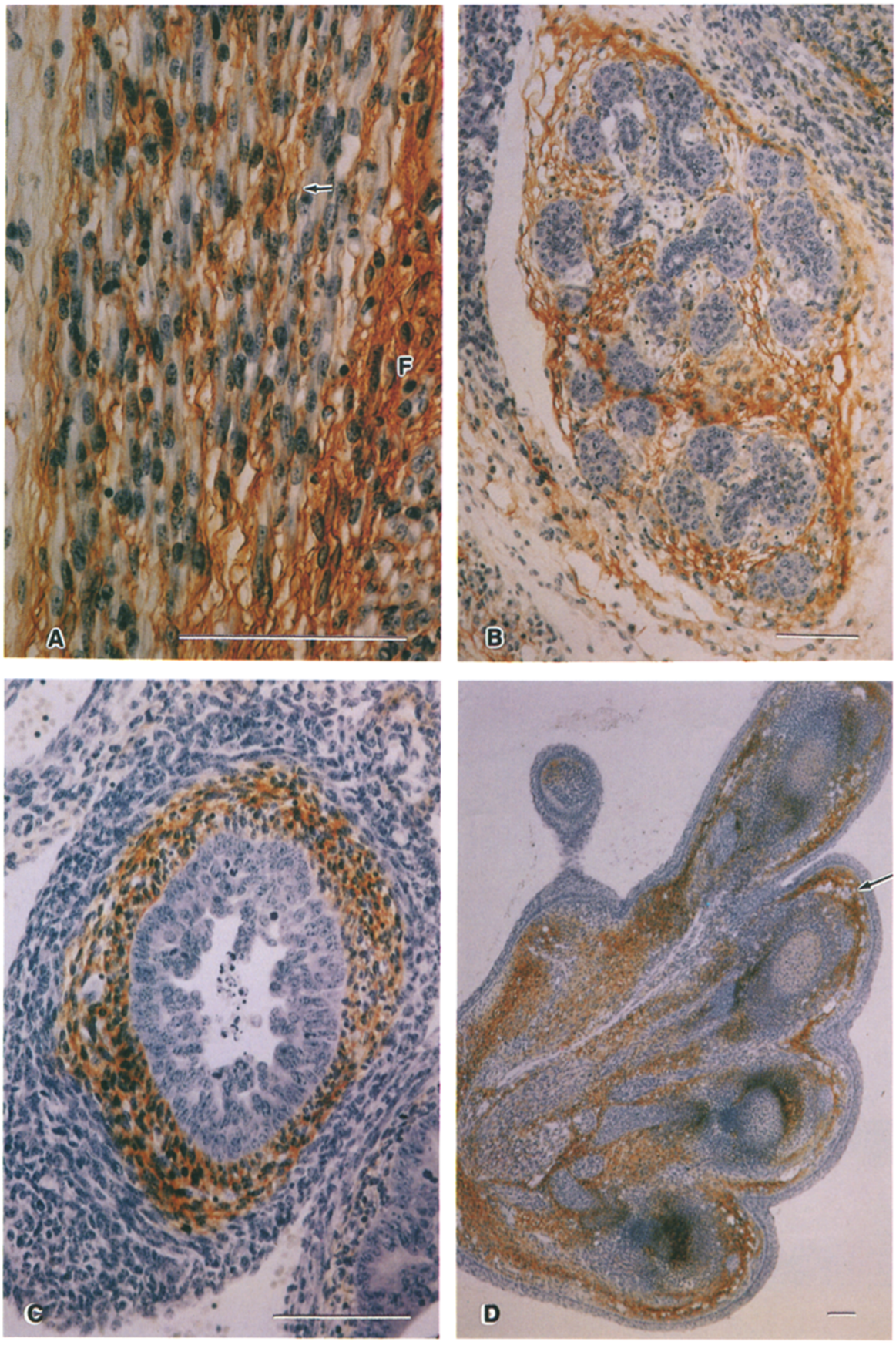
Effect of Fixative

The distribution pattern of TGF-β observed in the developing embryo was found to be dependent on the fixative used. Fixation with *p*-formaldehyde at neutral pH resulted in staining of cartilage only. This pattern was most markedly seen in embryos of days 13 and 15, after the cartilaginous skeleton has been formed (not shown). In contrast, Bouin fixation, at pH 2, also resulted in staining of cartilage, but, in addition, resulted in strong staining of the mesenchyme in many areas of the embryo (Fig. 3 *A*); TGF-β staining was especially intense in regions of active histogenesis and organogenesis. All of these areas were devoid of reaction in sections stained with normal rabbit IgG (Fig. 3 *B*). Because Bouin fixation was more useful, it was used exclusively for the rest of the study of the developmental patterns of TGF-β expression reported here.

Immunohistochemical Localization of TGF-β

A general conclusion is that TGF-β is often closely associated with mesenchyme per se or with tissues that are derived from mesenchyme, such as connective tissue, cartilage, and bone. TGF-β is particularly conspicuous in tissues derived from the neural crest mesenchyme as seen, for example, in the developing palate, larynx, facial mesenchyme, nasal sinuses, meninges, and teeth (Figs. 3 *A*, 5, 8, 10, and 11). The expression of TGF-β in all of these tissues is greatest during periods of morphogenesis. Often, the staining pattern of TGF-β in connective tissue outlines demarcations both within and between individual tissues, such as in the connective tissue sheaths separating groups of myotubes in develop-

Figure 4. TGF-β in mesenchymal connective tissue of a 15-d embryo. (*A*) Muscle. TGF-β is present in the cytoplasm of fibroblasts (*arrows*) that separate individual myotubes. Fascia (*F*) between two muscles shows intense staining for TGF-β. ×400. (*B*) Submandibular gland. TGF-β is stained in the connective tissue surrounding the glandular epithelium. ×150. (*C*) Intestine. TGF-β is present in the submucosa. ×250. (*D*) Hindfoot. TGF-β staining is localized preferentially in the capillary-rich, subdermal mesenchyme (*arrow*), in fascia surrounding developing muscles, and in joints between the cartilaginous bones of the digits. ×60. Bar, 100 μm.


A
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ing muscle (Figs. 2 *A* and 4 *A*) and in the fascial planes between different tissues, such as between muscle and dermis (Fig. 2 *A*). In many instances, such as in the development of hair follicles (see Fig. 10), and teeth (see Fig. 11), expression of TGF-β is found in tissues in which critical mesenchymal–epithelial interactions occur (see Hay, 1981). Further examples are seen in the submandibular gland, where intense TGF-β staining was seen in underlying mesenchyme (also of neural crest origin) but not in glandular epithelial cells (Fig. 4 *B*) and in the developing intestine, where staining was seen only in the submucosa, but not in mucosal epithelial cells (Fig. 4 *C*). Particularly intense TGF-β staining also can be seen when remodelling of mesenchyme or mesoderm occurs, as during formation of digits from limb buds (Fig. 4 *D*), formation of the palate (Fig. 5 *A*), and formation of the heart valves (see Fig. 9). In many of these situations, the presence of TGF-β was coupled with marked angiogenic activity, as seen, for example, in the marginal blood sinuses of the limbs between days 13 and 15 (Fig. 4 *D*). Specific features of the staining patterns of TGF-β in a variety of tissues and their developmental sequences will be discussed in greater detail below.

Notochord. Between days 11 and 15, the notochord (not illustrated) shows two distinct areas of staining for TGF-β: (*a*) the cell-free sheet of collagen fibers surrounding the notochord and (*b*) single cells or small groups of cells in the central part of the notochord. The distribution of these cells coincided with the future localization of intervertebral disks.

Somites, Sclerotome, and Early Vertebrae. Because the anterior axial skeleton develops earlier than the caudal skeleton, it was possible to evaluate the entire developmental pattern of TGF-β expression by examination of different regions of the skeleton in single embryos. At day 11, the caudal part of the axial skeleton is still composed of somites. The distribution of TGF-β was homogeneous throughout each caudal somite; numerous individual cells with cytoplasmic localization of the peptide were scattered throughout each segment (Fig. 6 *A*). Between days 11 and 13, after the somites have differentiated into sclerotome, myotome, and dermatome, only the sclerotome and dermatome stained positively for

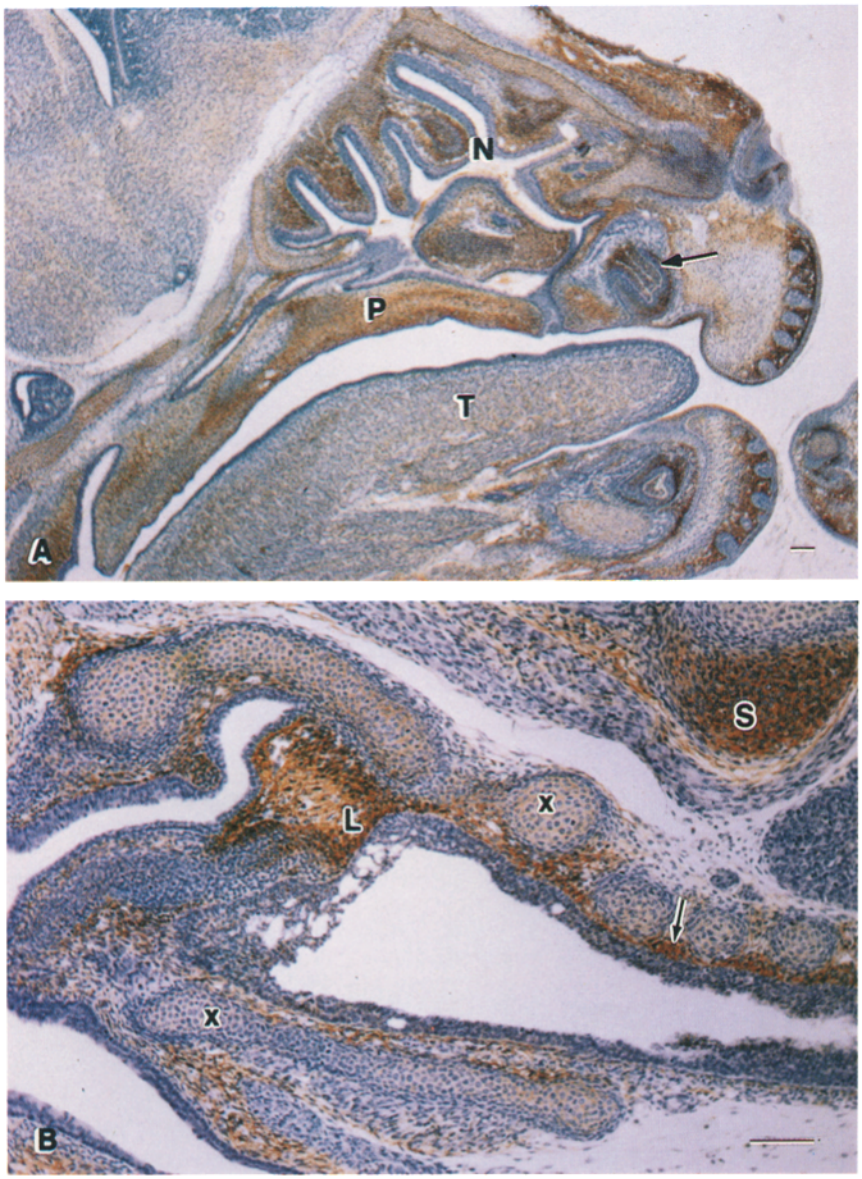

Figure 5. Staining of TGF-β in craniofacial and cervical structures. (*A*) Parasagittal section through the head of a 15-d embryo. TGF-β is found in the dermis, connective tissue of the tongue (*T*), and the stellate reticulum of the developing incisor (*arrow*). Intense staining is present in various tissues of neural crest origin, such as the cartilage of facial bones, mesenchyme of developing nasal conchae (*N*), palate (*P*), and papilla of incisor. ×24. (*B*) Larynx. Section through the larynx of a 15-d embryo. Laryngeal and tracheal cartilages (*x*) as well as the laryngeal ventricles are forming at this time. Intense staining for TGF-β is seen in mesenchyme between tracheal cartilage and epithelium (*arrow*), in the sternum (*S*), and in the larynx (*L*). Bar, 100 μm.

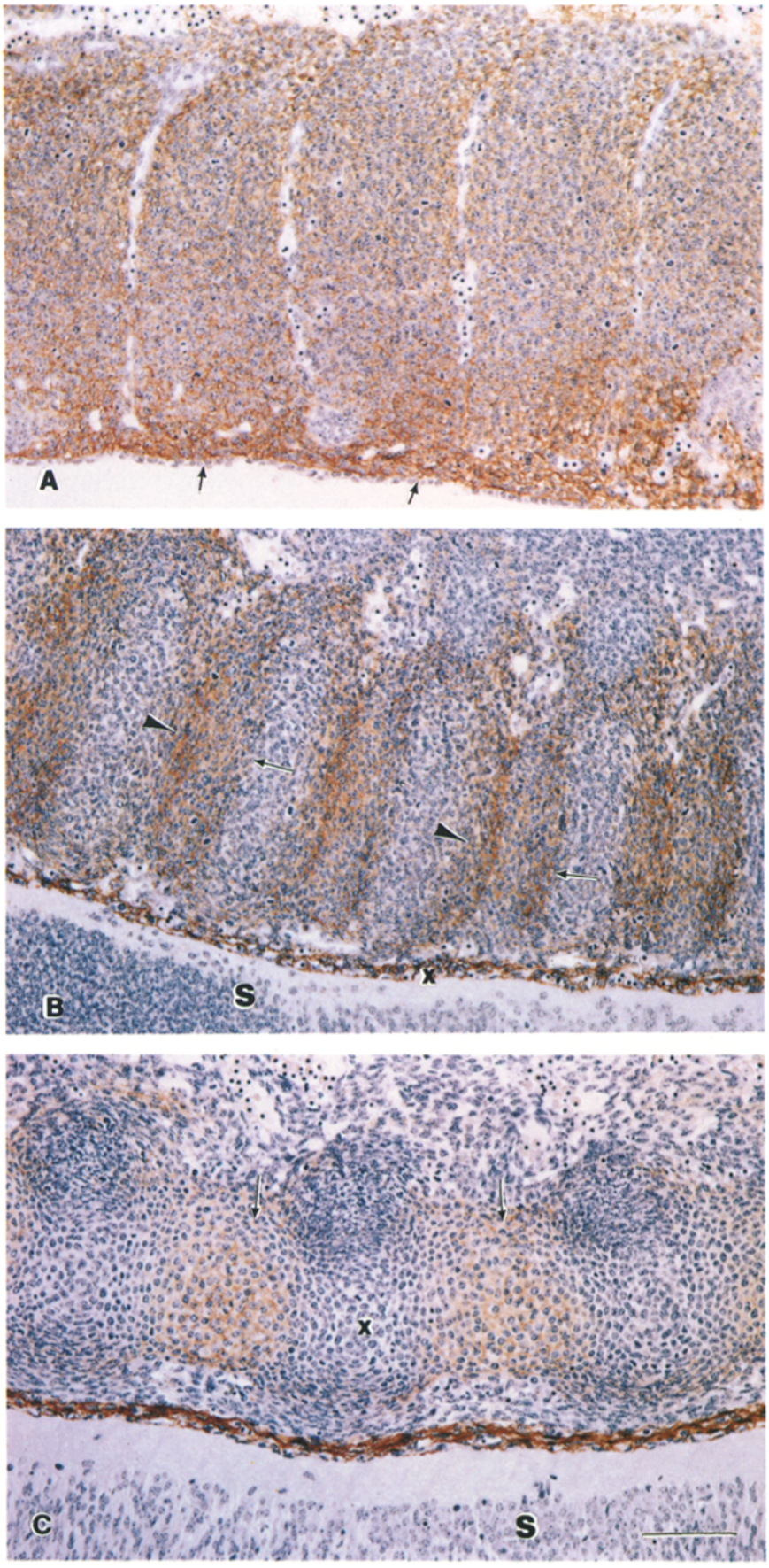

Figure 6. Staining of TGF-β during morphogenesis of vertebrae. (*A*) Somites in the tail region of an 11–12-d embryo. TGF-β is present in the cytoplasm of individual cells that are distributed evenly throughout each somite. Epidermis consisting of a single cell layer is marked by arrows. (*B*) Sclerotomes in the caudal spinal column of a 13-d embryo. TGF-β is localized in the anterior (*arrows*) and posterior (*arrowheads*) regions of individual sclerotomes. The staining pattern demarcates the formation of future vertebrae. Meninges (*x*) covering the spinal cord (*S*) are intensely stained. (*C*) Definitive vertebrae in the rostral spinal column of a 13-d embryo. Areas of vertebra formation (*arrows*), distinguished by low cell density (indicating the beginning of chondrogenesis), show staining for TGF-β. In contrast, the areas representing the perichordal discs (*x*) do not stain. The meninges covering the spinal cord (*S*) are strongly positive for TGF-β, as seen in *B*. Bar, 100 μm.

TGF-β. The development of the centra of the future definitive vertebrae from individual sclerotomal segments was preceded by a change in TGF-β distribution, namely, the staining became intensified in the posterior half of one segment and in the anterior half of the following segment, thus defining the future vertebral centrum (Fig. 6 *B*). Soon after these two areas fused to form the body of a single vertebra (as first described by Remak, 1855), they became characterized by low tissue density, indicating the beginning of chondrification (Fig. 6 *C*). Areas of chondrification, as well as the resulting cartilage and, later on, calcified cartilage and ossification centers of individual bones, stained positive for TGF-β (Fig. 7 *A*).

Bones. In long bones, endochondral ossification starts by invasion of capillaries into the perichondrium, thus triggering the transformation of the perichondrium into a periosteum. In these areas, narrow bands of osteoblasts adjacent to the cartilaginous bone form the primary ossification centers. In such areas of active capillary growth and bone matrix formation, as well as in the cytoplasm of the osteoblasts per se (days 15 and 18), intense staining for TGF-β was observed (Fig. 7 *A*). Areas of joint formation, such as the articulations between vertebrae and between long bones, also stained positive for TGF-β (Fig. 4 *D*). At day 15, strong TGF-β staining was found in areas of intramembranous ossification of flat bones, as in the calvarium (Fig. 7 *B*).

Meninges. A transverse section of the head at day 13 clearly outlines the presence of TGF-β in the meninges of the brain (Fig. 8 *A*). In the mouse, the three structures (dura mater, arachnoid, and pia mater) comprising the meninges differentiate between days 11 and 15 of gestation. During development of the meninges, two zones may be distinguished: an outer one of condensation of mesenchyme, which gives rise to the periosteum, dura mater, and membranous arachnoid, and an inner zone, which becomes the pia mater. The developmental pattern of TGF-β staining reflects sequential differences in the development of these two zones. At day 11, TGF-β staining was seen mainly in a single layer of elongated fibroblasts overlying neural tissue (Fig. 8 *B*). These were the first recognizable cells of the developing meninges. As dif-

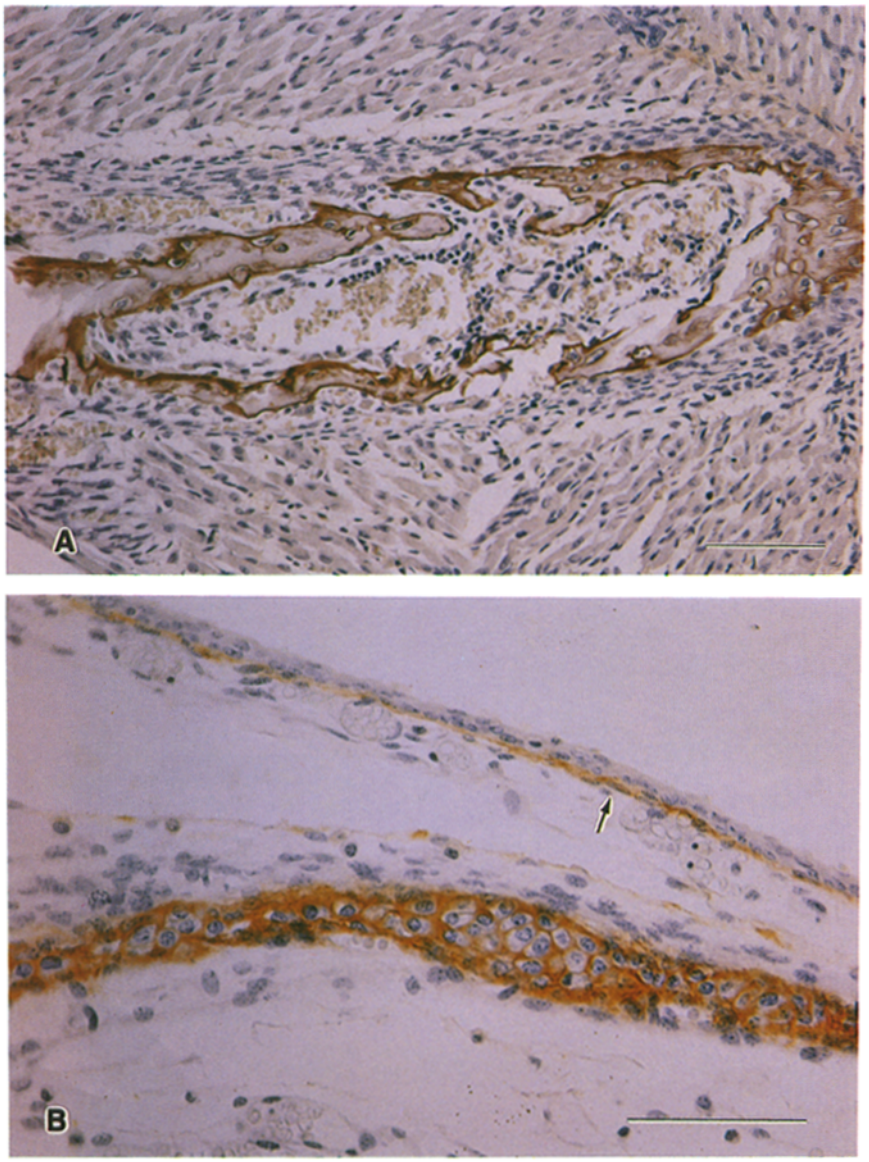

Figure 7. Staining of TGF-β during both endochondral and intramembraneous ossification. (*A*) Section through the rib of an 18-d embryo with areas of calcification that are positive for TGF-β. ×150. (*B*) Calvarium of a 15-d embryo with intense staining of membranous bone. Note also the narrow band of staining in the dermis (*arrow*). Bar, 100 μm.

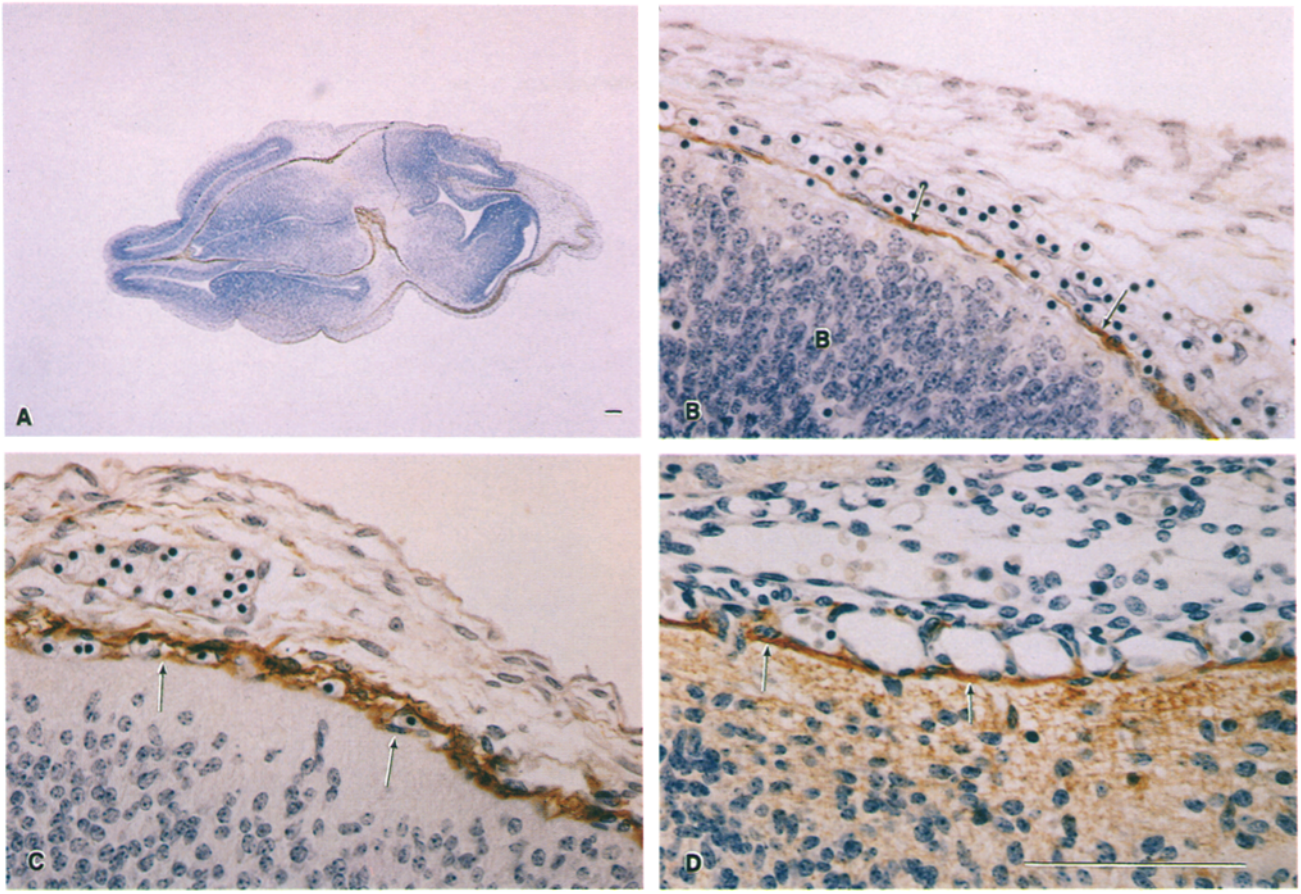

Figure 8. Staining of TGF-β in developing meninges. (*A*) Low-power transverse section through the head of a 13-d embryo. The meninges stain strongly. ×22. (*B*) Parasagittal section through the parietal lobe of a 11-d embryo. A single layer of elongated TGF-β–positive cells (*arrows*) indicates the earliest stage in the development of the meninges of the brain (*B*). ×400. (*C*) Parasagittal section through the frontal lobe of an 11-d embryo. In early stages of development of the arachnoidal spaces, staining is localized in the cytoplasm of the arachnoid endothelium and in cells of the pia mater (*arrows*). Arachnoidal spaces are filled with immature, nucleated red blood cells. ×400. (*D*) Section through the meninges of a 15-d embryo. Late stages of arachnoidal development are characterized by reduced TGF-β staining in the arachnoidal endothelium and enhanced staining in cells of the pia mater (*arrows*). Neural tissue in the adjacent brain also shows diffuse staining for TGF-β at this time. ×400. Bar, 100 μm.

ferentiation of the netlike structure of the arachnoid proceeded from this cell layer, staining for TGF-β in this area remained intense. At this same time, numerous cells of the underlying pia mater were still negative for TGF-β but began to stain positively between days 11 and 13 of gestation (Fig. 8 *C*). By day 15, differentiation of the arachnoid was complete in many areas, and arachnoidal staining was very weak, whereas in contrast, the pia mater now stained strongly positive for TGF-β (Fig. 8 *D*). In addition, marked staining of the meninges surrounding the spinal cord was seen between days 11 and 15 of gestation (Fig. 6, *B* and *C*).

Cushion Tissue of the Heart. The mesenchymal cushion tissue of the heart stained strongly positive for TGF-β at days 11 and 13 of gestation (Fig. 9, *A* and *B*). Neither the overlying endocardium, thought to be the origin of the cushion tissue, nor the developing myocardium showed any staining for TGF-β. The period between days 10 and 14 of gestation is of major importance in the development of the heart inasmuch as both cytodifferentiation and remodeling occur, especially in cushion tissue and adjacent areas. The progressive changes that occur include fusion of the endocardial cushions (day 11), and development of the septum primum (day 11.5), the atrioventricular valves (day 13), and semilunar valves (day 14). TGF-β appears to be involved in all these events. At day 15, TGF-β still stained strongly in the mesenchyme of the valves (Fig. 9, *C* and *D*).

Hair Follicles. The developmentally dependent staining pattern of TGF-β during maturation of hair follicles indicates that it might play a role, either direct or indirect, in mesenchymal–epithelial interactions involved in their differentiation. This can be seen particularly during the development of the hair follicles of the snout, which form the vibrissae (sensory whiskers), as shown in Figs. 5 *A* and 10. The mesenchyme underlying the epidermis stained weakly for TGF-β before the actual appearance of the hair germ cells (early hair follicle). During this time, around day 11, numerous capillaries were present in the mesenchyme (Fig. 10 *A*). As development of the hair follicles continued, the staining of TGF-β in the mesenchyme increased in intensity (day 13, Fig. 10 *B*; day 15, Figs. 5 *A* and 10 *C*); however, this staining disappeared after completion of hair follicle formation (day 18, Fig. 10 *D*). The staining pattern of TGF-β in the mesenchyme surrounding the developing follicle was always correlated with hair development in the epithelial cells of the fol-

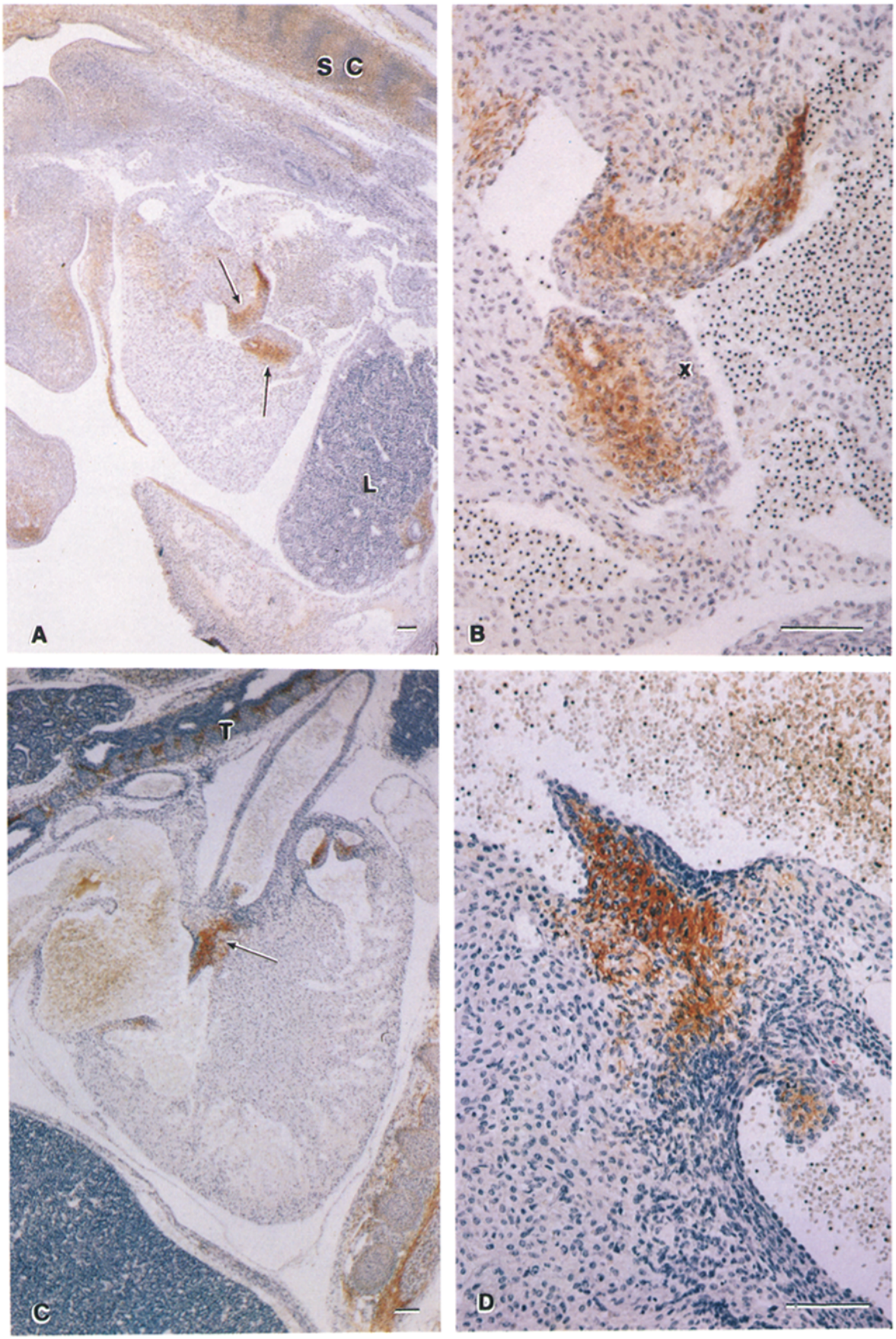

Figure 9. Staining of TGF-β in the cushion tissue of the heart. (*A*) Section through the heart of an 11–12-d embryo. TGF-β is localized in the cushion tissue (*arrows*) of the developing valves. Liver (*L*) and spinal column (*SC*) are recognizable. ×86. (*B*) Enlargement of Fig. 7 *A*. TGF-β is localized in the cytoplasm of cells of the cushion tissue. Adjacent multilayered endocardium (*X*) is negative for TGF-β. ×150. (*C*) Section through the heart of a 15-d embryo. TGF-β is present in the atrioventricular valve (*arrow*). Note also the staining for TGF-β in the mesenchyme of the trachea (*T*). ×36. (*D*) Enlargement of Fig. 7 *C*. Particularly intense TGF-β staining is present in the cytoplasm of cells comprising the connective tissue of the atrioventricular valve. ×150. Bar, 100 μm.

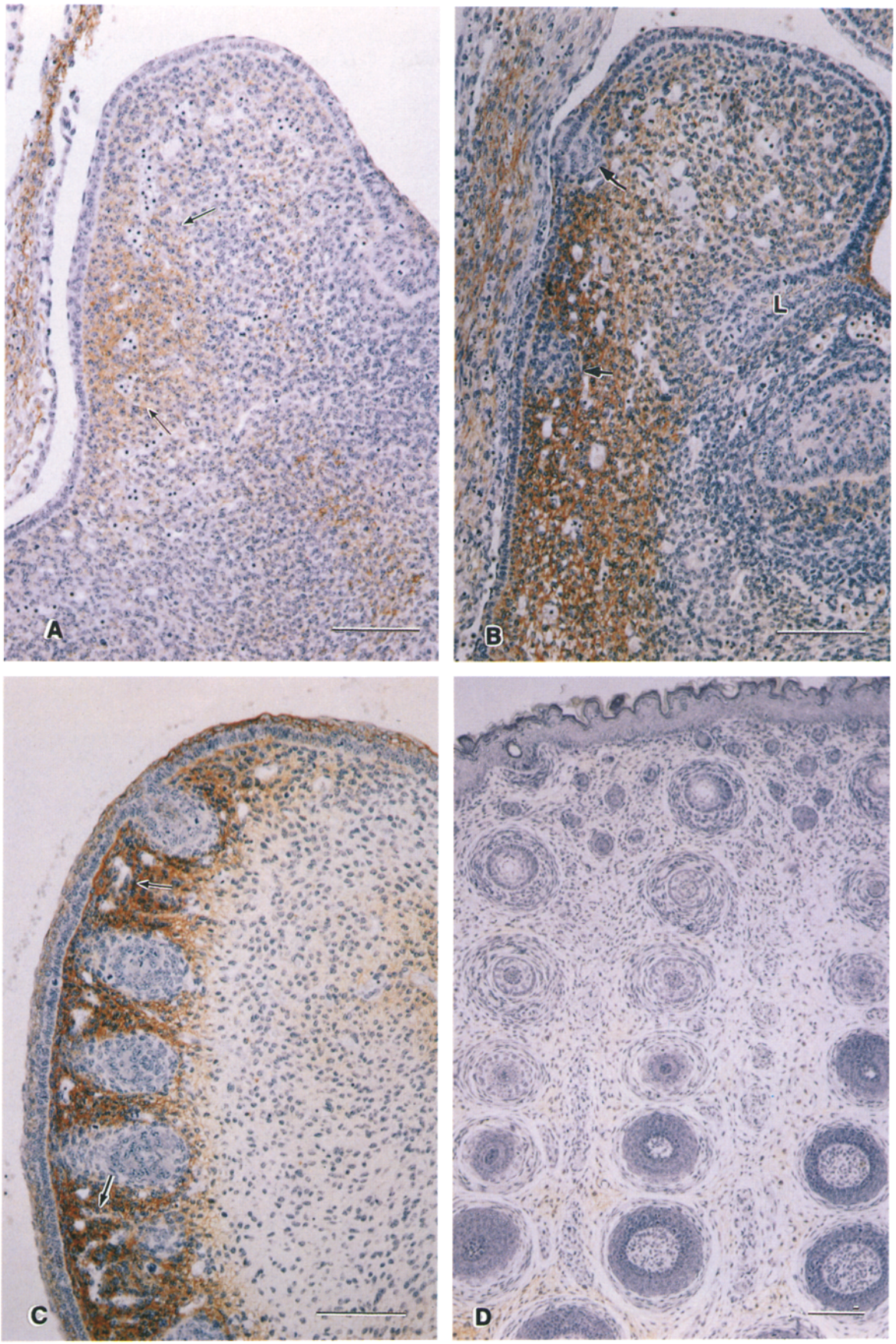

Figure 10. Staining of TGF-β in mesenchyme associated with development of hair follicles of the snout. (*A*) Parasagittal section through the mandible of an 11-d embryo. The earliest indication of the development of vibrissae is indicated by a light staining for TGF-β in the mesenchyme of the dermis and by the vascularization of the same area (*arrows*). ×150. (*B*) Parasagittal section through the mandible of a 13-d embryo. Intense staining for TGF-β in the mesenchyme underlying the epidermis. Local aggregation of epidermal cells (*arrows*) indicates early hair follicle formation. L-lip furrow band. ×150. (*C*) Parasagittal section through the mandible of a 15-d embryo. Intense staining of TGF-β is present in the mesenchyme of the dermis surrounding the developing hair follicles. The vascularization of this area is pronounced (*arrows*). ×150. (*D*) Parasagittal section through the mandible of an 18-d embryo showing the lack of TGF-β staining in mesenchyme at a time when the development of the vibrissae is near completion. ×90. Bar, 100 μm.

licle; thus, as formation of the vibrissae preceded that of the body hair, staining of TGF-β in the mesenchyme around the developing vibrissae preceded that in mesenchyme in regions of presumptive hair follicles of body hair (not shown). Little, if any, staining of any follicles themselves was seen at any time. Furthermore, in those areas of skin that do not participate in hair formation, such as the tail region, mesenchyme underlying epithelium did not stain for TGF-β.

Teeth. Development of the teeth, especially the incisors, like that of the vibrissae discussed above, is another instance in which TGF-β may influence mesenchymal–epithelial interactions and may also induce mesenchymal cells to synthesize a specific set of matrix proteins. At day 11, faint staining for TGF-β was observed in areas of neural crest-derived mesenchyme that underlie the epithelial tooth buds. During the cap stage of tooth development (day 13), faint staining was found in the mesenchyme adjacent to the forming tooth (Fig. 11 *A*). In the subsequent bell stage of development (day 15), TGF-β staining became locally pronounced in cells of neural crest origin, as they were incorporated into the primitive dental papilla. The deep staining of the mesenchyme of the papilla at this stage suggests a high concentration of TGF-β (see Figs. 5 *A* and 11 *B*).

During the course of epidermal invagination into the underlying mesenchyme (to form the bell-shaped tooth), an undifferentiated mesenchyme of epithelial origin, the stellate reticulum, accumulates between the outer and inner dental epithelium. This reticulum also reacted positively with antibodies to TGF-β (Fig. 11 *B*). Likewise, positive staining for TGF-β was observed in tissue surrounding the capillaries that infiltrate the developing tooth under the outer dental epithelium. At day 18 of gestation, TGF-β was still detectable in the less developed teeth (not shown). It is of interest that biochemical studies have recently shown high levels of expression of mRNA for TGF-β in cells derived from the ameloblastic layer of fetal bovine teeth (Robey et al., 1987).

Muscle. Sections of the developing muscle taken between days 13 and 18 did not show TGF-β–specific staining in either myogenic cells or myotubes, per se. However, during the same period, loose connective tissue sheaths surrounding the

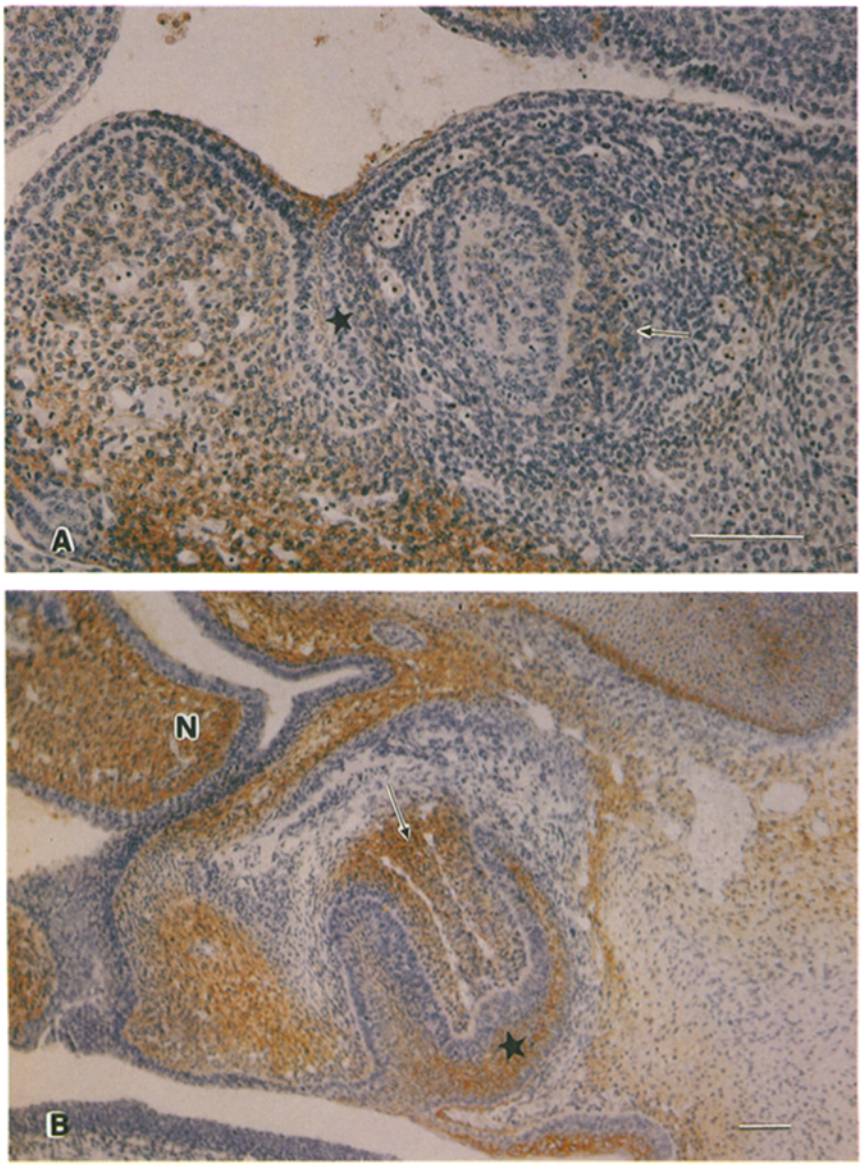

Figure 11. Staining of TGF-β in the developing incisors of the 13–15-d embryo. (*A*) Lower incisor (13 d) showing faint staining of the mesenchyme adjacent to the incisor primordium (*arrow*). (*) Lip furrow band. (*B*) Upper incisor (15 d) showing strong staining for TGF-β in the papilla (*arrow*) and stellate reticulum (*). Note also the intense staining in the nasal sinuses (*N*). Bar, 100 μm.

developing muscles, as well as such sheaths separating groups of myotubes within the muscle proper, stained positive for TGF-β (Figs. 2 *A* and 4 *A*). This pattern was especially pronounced in the tongue (musculus verticalis linguae), in which individual myotubes alternated with deeply stained narrow strands of connective tissue. At day 18, after striated muscle fibers have been formed, the fascia surrounding many muscles continued to stain positive for TGF-β.

Discussion

Results obtained in the present study clearly indicate that TGF-β is expressed in a unique pattern, both spatially and temporally, in the developing mouse embryo. This highly specific pattern of histochemical staining of TGF-β in the embryo appears to correlate with specific morphogenetic and histogenetic events, particularly those involving cells and tissues of mesenchymal or mesodermal origin. In accordance with our observations in the mouse, it has recently been shown that TGF-β can exert a potent inductive effect on the formation of mesoderm from ectoderm in amphibian embryos, prior to gastrulation (Kimelman and Kirschner, 1987). Because the bulk of the vertebrate organism is composed of mesodermal cells and tissues, it is apparent that TGF-β participates in some fundamental way in the basic architecture and organization of almost the entire developing embryo.

Particularly striking in this respect is the manner in which TGF-β contributes to the segmentation of the axial skeleton, a fundamental characteristic of all vertebrates. It has been known for more than a century (Remak, 1855) that each vertebral body is derived from sclerotomal mesenchyme originating from two somites (the posterior part of one somite and the anterior part of the somite immediately caudal to it), and the participation of TGF-β in this process can be seen in Fig. 6, *A–C*. Furthermore, not only does TGF-β appear to have some organizational role in the original morphogenesis of a vertebral body, but it then has some additional role in its subsequent chondrification and ossification. The ultimate formation of a definitive structure such as a vertebra involves a set of steps which, at the cellular level, consist of a series of chemotactic, proliferative, and differentiative events, and which, at the tissue level, consist of a sequence of modeling and remodeling events, until the final mature structure is formed (Bellairs et al., 1986).

Although one cannot prove that TGF-β is causally related to these various steps merely from its appropriate presence in a series of photomicrographs, the cellular specificity of the localization of TGF-β, particularly at times when critical morphogenetic and histogenetic steps occur, strongly argues that this might be the case. Furthermore, known biochemical actions of TGF-β also support this view. Thus, TGF-β is known to have very potent chemotactic (Postlethwaite et al., 1987), proliferative (Moses et al., 1985; Centrella et al., 1987; Hill, et al., 1986; Robey et al., 1987), and differentiative actions (Ignotz and Massagué, 1986; Seyedin et al., 1985, 1986; Florini et al., 1986; Massagué et al., 1986; Olson et al., 1986) on various cells of mesenchymal origin. It is well known that there is a direct role of extracellular matrix in promoting and stabilizing specific protein and polysaccharide synthesis during the chain of events involved in this entire sequence (Hay, 1981), including the formation of somites, the differentiation of somitic mesenchyme to form sclerotome, as well as the subsequent chondrification and ossification of the sclerotome to form vertebrae. TGF-β has recently been shown to be of major importance in controlling the formation and destruction of numerous components of the extracellular matrix, including various types of collagen, as well as fibronectin and proteoglycans; many of these actions appear to be a direct effect on gene expression (for a review, see Sporn et al., 1987). In some cells, it also controls the levels of receptors for molecules such as fibronectin (Ignotz and Massagué, 1987; J. McDonald, personal communication). In addition to its known action on the fibronectin system, it remains to be determined whether TGF-β might also be a regulator of the synthesis of various other adhesion molecules, such as cytotactin, neural cell adhesion molecule (N-CAM), and N-cadherin; these molecules have recently been shown to have an important role during somitogenesis and the subsequent remodeling of somites (Crossin et al., 1986; Duband et al., 1987). Of particular relevance to the role of TGF-β in chondrification and ossification is its known ability to induce the cartilaginous phenotype (especially type II collagen and cartilage-specific proteoglycans) in embryonic mesenchyme (Seyedin et al., 1985, 1986), as well as its proliferative and differentiative actions on osteoblasts (Centrella et al., 1987; Robey et al., 1987).

The participation of TGF-β in cartilage and bone formation is not limited to the axial skeleton, since intense staining is also found in craniofacial mesenchyme of neural crest origin, destined to become mandible, maxilla, palate, nose and nasal sinuses, and other important craniofacial structures (see Figs. 3 *A* and 5). The morphogenetic role of TGF-β in these structures of neural crest origin is not understood at present, although it should again be noted that extracellular matrix proteins, particularly fibronectin (Newgreen and Thiery, 1980; Rovasio et al., 1983), may have an important permissive role in the migration and subsequent differentiation of neural crest cells, in that they become craniofacial mesenchyme. However, recently it has been emphasized that matrix proteins other than fibronectin may also have an important role in guiding migration of neural crest cells (Rickmann et al., 1985). In the case of the neural crest-derived mesenchyme that forms the developing palate, it is known that these cells are highly sensitive to epidermal growth factor (EGF) (Yoneda and Pratt, 1981), a peptide whose receptor is controlled both by TGF-β (Assoian et al., 1984) and retinoic acid (Jetten, 1981; Roberts et al., 1984).

Although the particular antibody we have used in this study, directed against residues 1–30 of the TGF-β1 sequence, shows little staining of endothelial cells or smooth muscle cells of the developing vascular system, there is clearly some role (possibly indirect) of TGF-β in angiogenesis, in that extensive capillary networks are found in areas of remodeling and reorganization where TGF-β staining is associated with the mesenchyme. Furthermore, there appears to be a direct role for TGF-β in the formation of specialized structures in the vascular system, such as the primordia of the heart valves, which are derived from the mesenchyme of the endocardial cushion tissue. The endocardial cushions form in the early heart as massive swellings rich in collagen (types I and III), fibronectin, and proteoglycans and serve as prevalvular swellings in the atrioventricular canal and truncus arteriosus (Fitzharris and Markwald,

1982; Icardo and Manasek, 1984). The mesenchyme of this endocardial cushion tissue forms a plastic connective tissue which is involved in the modeling and remodeling of the heart, leading to the partitioning of the atrioventricular canal and the formation of the connective tissue framework of the cardiac valves.

Again, as in the case of the skeletal system, the known biochemistry and cell biology of TGF-β strongly suggest a significant role for TGF-β in early angiogenesis and then in subsequent development of cardiovascular structures. Effects of TGF-β on synthesis of matrix proteins and its ability to shift the relative activities of proteolytic enzymes and their corresponding inhibitors are clearly both involved. Thus, both the rate of growth and the phenotype of capillary endothelial cells are known to be a function of the type of extracellular matrix on which they are cultured (Madri et al., 1983; Madri and Pratt, 1986), and the relative activities of plasminogen activator and plasminogen activator inhibitor (PAI) are important for capillary sprouting and branching (Rifkin et al., 1984; Dano et al., 1985). Recently, it has also been shown that TGF-β has a direct organizational action on capillary endothelial cells in vitro, which can lead to their formation of tubular structures in three-dimensional collagen gels (Madri et al., 1988). Finally, it has been shown that TGF-β is a potent regulator of the plasminogen activator system, by inducing transcription of the gene (or by stabilizing the mRNA) for type 1 plasminogen activator inhibitor (PAI-1, an active site–directed serine protease inhibitor, which can irreversibly inactivate plasminogen activator; Laiho et al., 1986; Lund et al., 1987). Since plasminogen activator has long been implicated in embryonic modeling and remodeling steps (Strickland et al., 1976; Dano et al., 1985), particularly during angiogenesis (Rifkin et al., 1984), the induction of plasminogen activator inhibitor by TGF-β would appear to provide an important regulatory control on unlimited proteolysis. Similar considerations regarding proteolytic modeling and remodeling may also be germane to the histogenesis and organogenesis of the heart and its septa and valves.

A third major area in which TGF-β would appear to play an important role is in the differentiation of specialized structures in which there is a known interaction between mesenchyme and adjacent epithelial cells. In this study, we have shown two such examples, namely the development of the hair follicles on the upper and lower labial surfaces of the snout (Fig. 10) and the development of tooth buds (Fig. 11). In both situations, intense staining of mesenchyme, as well as absence of staining of epithelium, is seen. It is well known that embryonic mesenchyme may act in some way to "induce" adjacent epithelium to differentiate (Grobstein, 1967; Slack, 1983), although the simple notion of a mesenchymal inducer of differentiation has been refined to include the concept that the induced epithelial tissue may already be "predifferentiated" and that a tissue interaction, catalyzed by some product of mesenchyme, may somehow stabilize or enhance a "preinduced" state (see Hay, 1981).

Although at present we clearly cannot directly implicate TGF-β in such a mechanism, one might speculate that TGF-β, produced in underlying mesenchyme, induces the formation of another molecule, such as osteonectin/SPARC, which is believed to have a definite role in modeling and remodeling of embryonic tissues (Mason et al., 1986), and which is known to be localized in the outer cells of whisker follicles (Holland et al., 1987). The recent demonstration that TGF-β increases cellular levels of mRNA for osteonectin/SPARC provides some credence for such a mechanism (Noda and Rodan, 1987). Because a principal function for osteonectin/SPARC is to bind calcium and hydroxyapatite (Engel et al., 1987; Termine et al., 1981), and because the mRNA for this protein is found in abundance in osteoblasts and odontoblasts (Holland et al., 1987), the role of TGF-β in formation of bones and teeth may also be related to an inductive action on osteonectin/SPARC.

Although we have shown an important role for TGF-β in embryogenesis in the present study, it is readily apparent that a host of new problems remain to be investigated. These include the need to investigate the role of TGF-β in earlier embryos than the ones studied here, the need to study species other than the mouse (such as the chick, and even to extend these studies to amphibian embryogenesis), the need to localize TGF-β mRNA formation by in situ hybridization, the need to develop satisfactory antibodies for immunohistochemical localization of the other form of TGF-β (type 2), and the need to determine specific mediators of TGF-β action in specific tissues. We are currently pursuing investigations in all of these areas.

Mechanistically, TGF-β appears to have a unique role in controlling the function of many other substances of critical significance in embryogenesis, whether they be other peptide growth factors, receptors for these peptide growth factors, or structural or adhesion molecules of the extracellular matrix. It is clear from the survey of the present study that a wealth of new information will be forthcoming on the role of TGF-β in the embryo. It should be noted that many common congenital anomalies, such as those of craniofacial structures or structures derived from endocardial cushions, occur in tissues in which TGF-β appears to have a definite morphogenetic action. Conversely, studies on embryos or utilizing embryonic cells may provide further information relating to critical mechanistic problems in TGF-β physiology, particularly in relation to the problem of what signals, in turn, control TGF-β itself. In this regard, the recent identification of retinoic acid as a morphogen in the embryonic chick limb bud (Thaller and Eichele, 1987) is particularly intriguing and suggests that there may be a significant relationship between the mechanisms of action of retinoic acid and TGF-β (Sporn and Roberts, 1983).

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