

# UC Irvine

## UC Irvine Previously Published Works

### Title

Effects of localized application of transforming growth factor  $\beta$ 1 on developing chick limbs

### Permalink

<https://escholarship.org/uc/item/2bf7h3v4>

### Journal

Developmental Biology, 145(1)

### ISSN

0012-1606

### Authors

Hayamizu, Terry F  
Sessions, Stanley K  
Wanek, Nancy  
[et al.](#)

### Publication Date

1991-05-01

### DOI

10.1016/0012-1606(91)90222-o

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

# Effects of Localized Application of Transforming Growth Factor $\beta$ 1 on Developing Chick Limbs

TERRY F. HAYAMIZU, STANLEY K. SESSIONS,<sup>1</sup> NANCY WANER,<sup>2</sup> AND SUSAN V. BRYANT<sup>3</sup>

*Developmental Biology Center, University of California Irvine, Irvine, California 92717*

*Accepted January 28, 1991*

The effects of exogenous transforming growth factor  $\beta$  (TGF- $\beta$ ) on chick limb development *in vivo* were studied by implanting carriers of TGF- $\beta$ 1 into developing wing buds. Agarose beads were soaked in solutions containing TGF- $\beta$ 1 and implanted into wing buds at stages 18 to 27. Localized application of TGF- $\beta$ 1 to distal regions of the wing bud caused specific skeletal elements in the limb to be reduced or absent. The particular proximal-distal limb element affected depended on the stage at which the bead was implanted. Position of the bead in the anterior-posterior axis also influenced the pattern of affected structures. Experiments in which TGF- $\beta$ 1 beads were implanted and then removed at 24- and 48-hr intervals indicate that there are specific periods during which a skeletal element appears to be sensitive to the effects of exogenous TGF- $\beta$ 1. In a few cases, beads placed in proximal positions in later staged limbs resulted in formation of ectopic cartilage near the bead. These results suggest that exposure to exogenous TGF- $\beta$ 1 *in vivo* influences the development of skeletal elements in the chick limb in a stage- and position-dependent manner. © 1991 Academic Press, Inc.

## INTRODUCTION

The transforming growth factor  $\beta$ s (TGF- $\beta$ s) are multifunctional peptides that have been shown to regulate cellular activities, including cell proliferation and cyto-differentiation, in a wide spectrum of normal and neoplastic cell types *in vitro* (Roberts and Sporn, 1988; Rizzino, 1988; Roberts *et al.*, 1988). Several distinct forms have so far been identified including TGF- $\beta$ 1 (Derynck *et al.*, 1985), TGF- $\beta$ 2 (Madisen *et al.*, 1988), TGF- $\beta$ 3 (ten Dijke *et al.*, 1988; Derynck *et al.*, 1988), TGF- $\beta$ 4 (Jakowlew *et al.*, 1988), and TGF- $\beta$ 5 (Kondaiah *et al.*, 1990). The effects of TGF- $\beta$ 1 and TGF- $\beta$ 2 have been most extensively studied. TGF- $\beta$  effects can be stimulatory or inhibitory and appear to be dependent both on cell type and on culture conditions, especially the "milieu" of other peptide growth factors present. TGF- $\beta$ s have also been shown to have dramatic effects on extracellular matrix components (Rizzino, 1988; Roberts *et al.*, 1990).

The TGF- $\beta$ s belong to a superfamily of peptide factors that includes the inhibins (Mason *et al.*, 1985) and activins (Ling *et al.*, 1986; Vale *et al.*, 1986); Müllerian inhibitory substance (MIS) (Cate *et al.*, 1986); the bone morphogenetic proteins BMP-2A, BMP-2B, and BMP-3 (Wozney *et al.*, 1988; Rosen *et al.*, 1989); and osteogenic

protein 1 (OP-1) (Özkaynak *et al.*, 1990; Sampath *et al.*, 1990). The TGF- $\beta$ s have also been shown to be related to the predicted protein products of the *Drosophila* decapentaplegic (dpp) gene complex (Padgett *et al.*, 1987) as well as that of Vg1 mRNA expressed in *Xenopus* oocytes (Weeks and Melton, 1987). In addition, Lyons *et al.* (1989a) have isolated a mouse cDNA, Vgr-1, that encodes a polypeptide structurally related to Vg-1. Finally, the mesoderm-inducing factor produced by *Xenopus* XTC cells (XTC-MIF) has recently been shown to be a homolog of mammalian activin A (Smith *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990).

It has been proposed that members of the TGF- $\beta$  superfamily may have a role in vertebrate limb development (Newman, 1988; Kulyk *et al.*, 1989a; Lyons *et al.*, 1989b). Temporally and spatially distinct patterns of expression of TGF- $\beta$ -like molecules have been seen in the developing mouse limb (Heine *et al.*, 1987; Sandberg *et al.*, 1988; Lehnert and Akhurst, 1988; Flanders *et al.*, 1989; Pelton *et al.*, 1989; Lyons *et al.*, 1989b; Lyons *et al.*, 1990; Pelton *et al.*, 1990). *In vitro*, TGF- $\beta$ 1 has been shown to have effects on proliferation and differentiation of cells within the chondrocyte lineage (Rosen *et al.*, 1988; Seyedin *et al.*, 1988; Kulyk *et al.*, 1989a; Solursh and Reiter, 1989; Carrington and Reddi, 1990). TGF- $\beta$ -like molecules may exert their effects either by directly affecting growth and differentiation, in concert with other regulators, or through effects on extracellular matrices (Newman, 1988; Kulyk *et al.*, 1989b). BMP-2A has been shown to induce ectopic bone formation in rats (Wozney *et al.*, 1988; Wang *et al.*, 1990), but so far there

<sup>1</sup> Present address: Hartwick College, Department of Biology, Oneonta, NY 13820.

<sup>2</sup> Present address: Rochester Institute of Technology, Department of Biology, Rochester, NY 14623.

<sup>3</sup> To whom correspondence should be addressed.

have been no studies looking at the TGF- $\beta$  effects in the developing limb bud *in vivo*.

The goal of this study was to evaluate the response of developing chick limbs to locally applied TGF- $\beta$ 1. We found that localized application of TGF- $\beta$ 1 to chick wing buds via agarose beads alters limb pattern in a concentration-, stage-, and position-dependent manner.

#### MATERIALS AND METHODS

##### *Preparation of Eggs*

Fertile White Leghorn chicken eggs (K&R Enterprises, Westminster, CA) were incubated at 38°C in a standard unit providing the necessary humidity and egg rotation. On the fourth day of incubation, eggs were prepared by withdrawing some of the albumen and creating a window in the shell overlying the embryo. Embryos were staged according to the criteria of Hamburger and Hamilton (1951) and then used immediately or reincubated at 38°C without rotation until the appropriate stages were achieved.

##### *Preparation of TGF- $\beta$ -Containing Beads*

The Affi-Gel Blue beads (Bio-Rad, 200–250  $\mu$ m diameter) used in this study are routinely used for protein purification and are composed of crosslinked agarose mesh with covalently attached Cibachrome blue F3GA dye. These carriers have the ability to absorb peptides in solution, to concentrate them within the matrices, and to slowly release them. Agarose beads have been used in previous studies (Schreiber *et al.*, 1986; Hayek *et al.*, 1987) as a system for the slow release of peptide growth factors *in vivo* and *in vitro*.

Recombinant TGF- $\beta$ 1 (kindly provided by A. F. Purchio, Oncogen, Seattle, WA), with bovine serum albumen (BSA) as a carrier, was lyophilized and stored at -20°C, then reconstituted in 4 mM HCl to achieve concentrations of 0.004 to 4.0  $\mu$ g/ $\mu$ l, aliquoted, and stored at 4°C. For these studies, 5  $\mu$ l of agarose beads (100–200 beads) in phosphate-buffered saline (PBS) were added to a 5- $\mu$ l aliquot of the TGF- $\beta$ 1 solution (final TGF- $\beta$ 1 concentrations of 0.002 to 2.0  $\mu$ g/ $\mu$ l). These were then incubated at 38°C for 30 min. Beads used as controls were incubated in a solution of BSA which had been lyophilized, reconstituted in 4 mM HCl, and then mixed with the beads in PBS.

A soaking solution of 0.02  $\mu$ g/ $\mu$ l is equivalent to 0.2  $\mu$ g of TGF- $\beta$  adsorbed onto 100–200 beads, or 1–2 ng per bead, assuming 100% adsorption. However, in a previous experiment, it was estimated that only 75% of peptide was adsorbed onto beads contained in 10  $\mu$ l of buffer solution (Hayek *et al.*, 1987). In addition, the soaking solutions used in our experiments contained

BSA which would also be bound, thereby reducing the amount of growth factor adsorbed onto the bead.

##### *Implantation of Beads*

Working through the window in the shell, we incised the vitelline and extraembryonic membranes overlying the right wing bud. Manipulations were performed using microsurgical instruments and fine glass probes. Individual agarose beads were inserted through incisions in the dorsal ectoderm of right wing buds. Following experimental manipulation, windows were sealed with removable adhesive tape (Scotch brand) and the eggs were returned to the incubator for reincubation at 38°C without rotation. Embryos were examined daily for at least 2 days following the operations to assess viability, bead position, and responses of limbs. Dead embryos were discarded.

##### *Skeletal Analysis*

On the 10th day of incubation, embryos were removed from the shells, rinsed in saline, and eviscerated. The upper torsos with attached wings were fixed in alcoholic Bouin's solution for at least 2 days, then rinsed, and stored in 70% ethanol. The pattern of cartilage structures in the limbs were determined in Victoria blue-stained whole mounts (Bryant and Iten, 1974). Left wings were used as controls for assessing the effects on the treated right wings. Some specimens were harvested 24 and 48 hr following bead implantation and were prepared by paraffin embedding, sectioning, and staining with hematoxylin and eosin for histological examination.

#### RESULTS

##### *Effects of TGF- $\beta$ 1 Concentration on Survival*

To evaluate the effects of different doses of exogenous TGF- $\beta$ 1 on developing chick limbs, agarose beads that had been soaked in solutions of TGF- $\beta$ 1 at concentrations ranging from 2.0 to 0.002  $\mu$ g/ $\mu$ l were implanted into chick wing buds at stages 18 to 27. The survival of embryos following TGF- $\beta$  bead implantation is shown in Table 1. Beads soaked in a 2.0  $\mu$ g/ $\mu$ l solution gave no surviving embryos. Decreasing the TGF- $\beta$ 1 concentration resulted in significant increases in embryo survival. Survival following implantation of beads soaked in 0.02 and 0.002  $\mu$ g/ $\mu$ l TGF- $\beta$ 1 was similar to survival following implantation of beads without TGF- $\beta$ 1.

The experiments reported below were all performed with beads soaked in solutions containing 0.02 and 0.002  $\mu$ g/ $\mu$ l TGF- $\beta$ 1. The maximum amounts of TGF- $\beta$ 1 that could be released from single beads soaked in these concentrations are calculated to be in the range of 1–2 ng

TABLE 1  
EFFECTS OF TGF- $\beta$  CONCENTRATION ON EMBRYO SURVIVAL AND ON  
FREQUENCY OF LIMB ABNORMALITIES

TGF- $\beta$ ( $\mu\text{g}/\mu\text{l}$ ) in soaking solution	<i>N</i>	No. of surviving embryos	No. of survivors with beads retained	No. of limbs with affected elements
2.0	18	0 (0%)	—	—
0.1-0.2	99	17 (17%)	15	15 (100%)
0.02	118	55 (47%)	45	45 (100%)
0.002	70	54 (77%)	40	18 (45%)
0.0	49	31 (63%)	27	0 (0%)

and 0.1-0.2 ng of growth factor per bead, respectively (see Materials and Methods).

#### *Effects of TGF- $\beta$ 1 on Limb Cartilage Formation*

TGF- $\beta$ 1-containing beads implanted distally at all stages resulted in limbs in which one or more of the skeletal elements either failed to form or were extremely reduced (Fig. 1). The frequency with which limbs were affected correlated with the concentration of TGF- $\beta$ 1 in the bead soaking solution (Table 1). Beads soaked in 0.02  $\mu\text{g}/\mu\text{l}$  TGF- $\beta$ 1 resulted in abnormal limbs in all embryos in which the bead was retained, while about half of the limbs implanted with beads soaked in 0.002  $\mu\text{g}/\mu\text{l}$  TGF- $\beta$ 1 were affected. In all cases, implantation of beads without TGF- $\beta$ 1 resulted in the development of normal limbs.

In each case, the entire segment of the limb containing the affected skeletal element was extremely reduced, indicating complete absence of part or all of an element, rather than its failure to react with the cartilage-specific stain. For example, in the specimen shown in Fig. 1B, extreme reduction of the ulna has led to gross distortion of the radius.

#### *Effects of TGF- $\beta$ 1 at Different Stages of Limb Development*

Implantation of TGF- $\beta$ 1 beads along the distal margin of chick wing buds resulted in localized reductions or deletions of skeletal structures within the limb. The particular proximal-distal (PD) limb segment affected by the TGF- $\beta$ 1 treatment depended on the stage at which the bead was implanted (Table 2). In wing buds treated at stages 19-21, the element most frequently affected was the humerus (Fig. 1A), and among limbs with affected humeri, the majority had normal elements distal to the humerus. At stages 22-24, the majority of limbs showed defects in the forearm (radius/ulna) (Fig. 1B). At stages 25-27, the majority of limbs showed defective

elements in the autopod (Fig. 1C), with no instances of defective humeri. By stage 27, all defects were localized to the autopod. Hence, the TGF- $\beta$ 1-sensitive target is shifted distally as limb outgrowth progresses.

In addition to the correlation between the stage of bead implantation and the PD position of the affected skeletal element, there was also a correlation between stage and final location of the bead. As limb outgrowth progresses, implanted beads are displaced proximally away from the distal margin of the elongating limb bud. Hence, beads implanted distally at stages 19-21 were eventually located in the humerus region, beads implanted at stages 22-24 in the radius/ulna region, and those implanted at stages 25-26 in the autopod (data not shown).

#### *Effects of Anterior-Posterior TGF- $\beta$ 1 Bead Position*

To evaluate whether the position of the bead in the anterior-posterior (AP) axis would also influence the pattern of affected structures, TGF- $\beta$ 1-containing beads were implanted along the distal margin of the limb bud in either anterior (A), apical or middle (M), or posterior (P) locations. As shown in Table 3, the position of TGF- $\beta$ 1 bead implantation along the AP limb bud axis also affected the position of the abnormal elements, regardless of stage. Anterior beads usually resulted in defects localized to the anterior elements of the limb (radius, anterior digits). When beads were placed in a posterior position, posterior elements (ulna, posterior digits) were affected to a greater extent (Fig. 1B). In the majority of cases, beads placed at the apex affected both sides of the limb to a similar extent.

#### *Effects of 24- and 48-hr TGF- $\beta$ 1 Exposure Intervals*

To learn more about the TGF- $\beta$ 1-sensitive step in the development of cartilage elements, we implanted TGF- $\beta$ 1-containing beads into limb buds and then removed them at 24- and 48-hr intervals. Beads implanted distally at stages 19-21 and removed 24 hr later at stages 22-24 did not cause limb abnormalities (Fig. 2a). However, beads left in place for 48 hr prior to removal at stages 25-26 resulted in typical humeral abnormalities in 88% of cases (Fig. 2b). These data suggest either that a prolonged exposure to TGF- $\beta$ 1 is necessary for an effect or that there is a sensitive period for humerus development that is between 24 and 48 hr after implantation at stages 19-21.

To distinguish between these possibilities, we performed an additional experiment in which beads were placed directly into a proximal (humerus-forming) position at stages 22-24. This is the position in which a bead implanted distally at stages 19-21 would be found 24 hr later. When these proximally implanted beads were re-

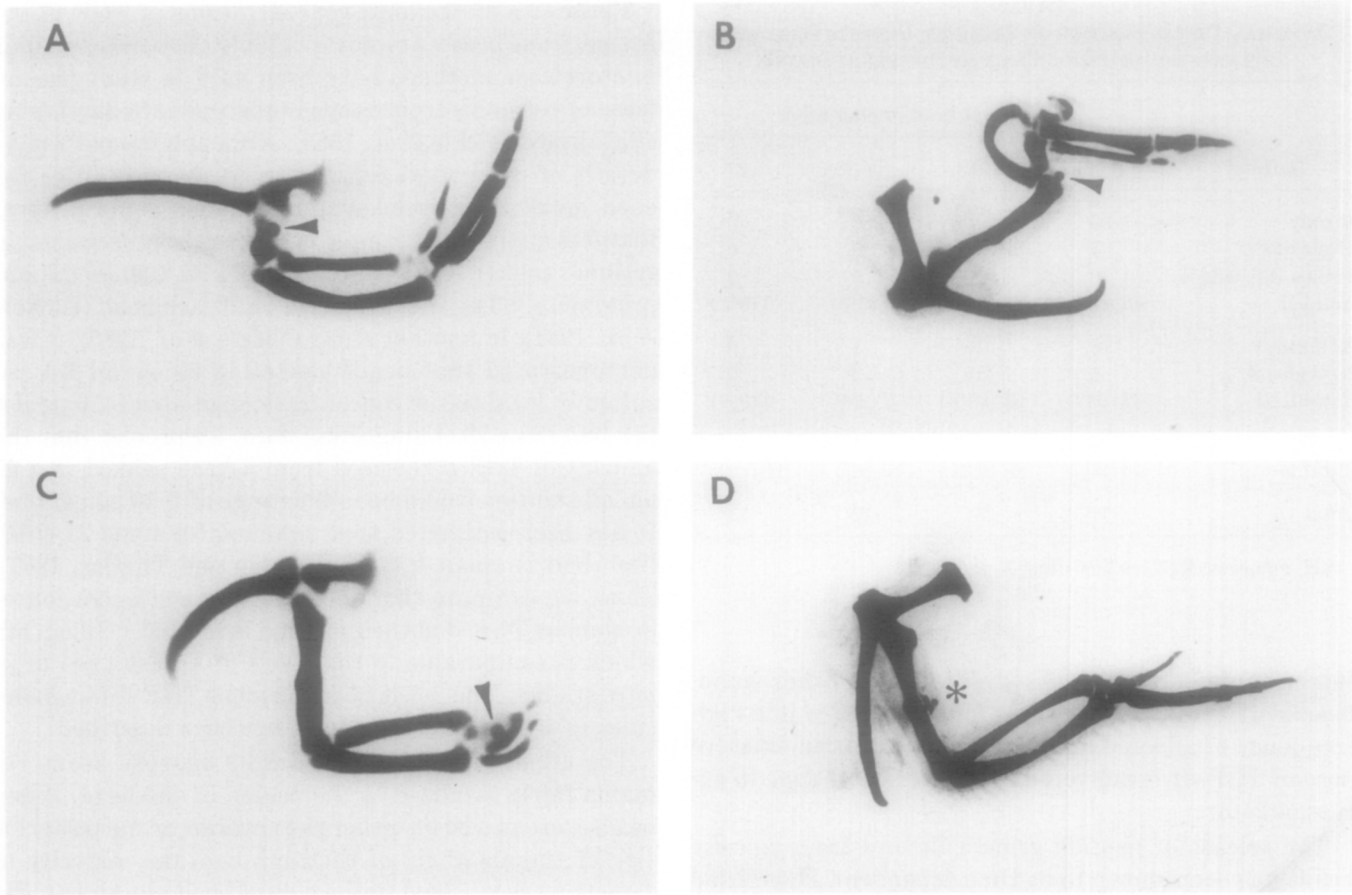


FIG. 1. Victoria blue-stained whole-mount preparations of limbs at Day 10 of incubation, after receiving a TGF- $\beta$ 1 bead implant at an earlier stage. Agarose beads within affected skeletal elements are indicated with arrows. (A) Bead implanted into the anterior distal margin of a stage 20 wing bud. The humerus is greatly reduced; distal elements are normal. (B) Bead implanted in the posterior distal margin of a stage 24 wing bud. The ulna is reduced in length. The radius, which does not appear shortened, is deformed. (C) Bead implanted into the anterior distal margin of a stage 26 wing bud. Defects are confined to autopodial skeletal elements. (D) TGF- $\beta$ 1 bead was implanted proximally at stages 25-26. An extra cartilage structure (\*) formed near the bead.

moved after 24 hr, 73% resulted in defective humeri (Fig. 2d). These results indicate that proximal limb cells exposed to TGF- $\beta$ 1 between stages 22-24 and stages 25-26 are sensitive to the inhibitory effects of the growth factor. Furthermore, limbs in which beads were implanted in proximal positions at stages 25-26 show a greatly reduced incidence of affected skeletal elements (Fig. 2e).

Finally, in a small number of limbs (7.3%) in which beads were implanted proximally at stages 25-26 (i.e., after the period during which cells of the presumptive humerus are sensitive to the inhibitory effects of TGF- $\beta$ 1), extra cartilage was formed next to the bead (Fig. 1D).

#### *Effects of TGF- $\beta$ 1 Beads on Cell Viability*

To evaluate the possibility that TGF- $\beta$ 1 might be having its effect by causing cell death in regions of the limb

bud near the bead, we examined histological sections of wing buds which had been fixed during the TGF- $\beta$ 1 sensitive period (i.e., between 24 and 48 hr following distal implantation of TGF- $\beta$ -containing beads at stages 19-21). As demonstrated in Fig. 3, there was no evidence of cell death in the tissue surrounding the bead releasing TGF- $\beta$ 1 when examined 24 (Figs. 3A and 3C) and 48 hr (Figs. 3B and 3D) after implantation. Rather, the limb cells are healthy and many are actively dividing.

#### DISCUSSION

Our results show that exogenous TGF- $\beta$ 1 released from agarose beads causes loss or extreme reduction of specific skeletal elements in the developing chick limb bud. When beads soaked in 0.02  $\mu\text{g}/\mu\text{l}$  TGF- $\beta$ 1 (with maximal loading of 1-2 ng of growth factor per bead; see Materials and Methods) were implanted distally in chick wing buds at stages 19-21 and then removed 24 hr

TABLE 2  
PROXIMAL-DISTAL POSITION OF SKELETAL DEFECTS FOLLOWING  
IMPLANTATION OF TGF- $\beta$  BEADS AT DIFFERENT STAGES

Defects <sup>a</sup>	Stages at bead implantation			
	19-21	22-24	25-26	27
H only	33	2	0	0
H plus R/U	3	0	0	0
H plus R/U and A	10	4	0	0
Total H	46 (66%)	6 (16%)	0 (0%)	0 (0%)
R/U only	18	9	1	0
R/U plus A	6	20	2	0
Total R/U	37 (53%)	33 (89%)	3 (25%)	0 (0%)
A only	0	2	9	11
Total A	16 (9%)	26 (59%)	11 (92%)	11 (100%)
Total	70	37	12	11

<sup>a</sup> H, humerus; R/U, radius/ulna; A, autopod.

later, no defects were observed. On the other hand, beads left in place for 48 hr or longer resulted in a high frequency of affected limbs, indicating that an effective dose of TGF- $\beta$ 1 is delivered over a period of time in excess of 24 hr.

The release of peptide growth factors from agarose beads has been shown to be time dependent. Hayek and colleagues (1987) reported that 50% of basic fibroblast growth factor (bFGF) adsorbed onto beads is released into solution over the first 24 hr, with the remaining growth factor slowly released over the next 3 days. Similar kinetics have been described for the release of epidermal growth factor (EGF) and transforming growth factor  $\alpha$  (TGF- $\alpha$ ) from agarose beads *in vivo* (Schreiber *et al.*, 1986). In experiments similar to those described above using TGF- $\beta$ 1, we have also looked at the effects of other peptide growth factors, including nerve growth factor (NGF), EGF, TGF- $\alpha$ , platelet-derived growth factor (PDGF), acidic and basic FGF, and tumor necrosis factor (TNF) on developing chick wing buds. At the levels used in these studies, so far only TGF- $\beta$ 1 has caused alterations in the limb pattern.

The release kinetics for TGF- $\beta$ 1 are expected to be comparable to those shown for other growth factors. Based on the amount loaded (see Materials and Methods), we conclude that less than 1 ng of TGF- $\beta$ 1 is slowly released into the limb bud over the course of 24 hr following bead implantation, with the remainder subsequently coming off the bead over the next few days. We also expect that diffusion of TGF- $\beta$  through tissue, as well as removal via blood perfusion, further reduces the local concentrations of TGF- $\beta$  to which the cells were exposed.

Estimates of the local concentrations of TGF- $\beta$ 1 released from beads are not available. However, similar microrelease methods have been used to study the release of retinoids from beads implanted into developing chick limbs (Tickle *et al.*, 1985). Although the particular details of compound delivery in tissue will differ for each molecule/carrier system studied, some general features apply. For example, when beads were soaked in retinoic acid (RA) at concentrations of 100  $\mu$ g/ml, approximately 3 ng was adsorbed onto each bead (Eichele *et al.*, 1984). In another study (Tickle *et al.*, 1985), it was demonstrated that beads soaked in 50  $\mu$ g/ml RA resulted in local tissue concentrations of about 7.6 pg RA per bud (25 nM). Using these data, we predict that the amount of TGF- $\beta$  released from a bead soaked in a 20  $\mu$ g/ml solution would be in the range of 1–10 pg per bud. It has been estimated that volume of a stage 21 chick limb bud is about 0.87  $\mu$ l (Eichele and Thaller, 1987). Thus, we estimate that the resulting concentration of exogenous TGF- $\beta$  in the limb bud is around 1–10 ng/ml, which is comparable to the levels of TGF- $\beta$  used in *in vitro* studies. The levels of endogenous TGF- $\beta$ -like molecules in developing limbs have not been described.

The effect of TGF- $\beta$  released by agarose beads remains fairly localized to the region of the bead. When beads were placed on either the anterior or the posterior side of the distal tip of the limb bud, the majority of defects were confined to the side of bead placement. We conclude that TGF- $\beta$ 1 is present in effective amounts only in the vicinity of the bead and that it does not spread to all regions of the limb bud. In addition, since the effect of localized application of TGF- $\beta$ 1 is to cause limb defects, we considered the possibility that the exogenous TGF- $\beta$ 1 was causing localized cell death. We have looked at numerous histological sections of limbs fixed 24 and 48 hr after distal implantation of TGF- $\beta$  beads (Figs. 3A–3D) and have detected no evidence of cell death. Cells located in the immediate vicinity of the

TABLE 3  
RELATIONSHIP OF ANTERIOR-POSTERIOR BEAD POSITION  
TO POSITION OF SKELETAL DEFECTS

Position of bead <sup>a</sup>	Position of defects <sup>b</sup>			Total
	Anterior	Posterior	Both	
Anterior	12 (75%)	0	4 (25%)	16
Posterior	0	24 (96%)	1 (4%)	25
Middle (apex)	6 (18%)	8 (24%)	19 (58%)	33

<sup>a</sup> Agarose beads soaked in 0.02  $\mu$ g/ $\mu$ l TGF- $\beta$  implanted along the distal margin of wing buds at stages 19–27.

<sup>b</sup> Only limbs with defects in the radius, ulna, and/or autopod were used; defects in the humerus alone were not included.

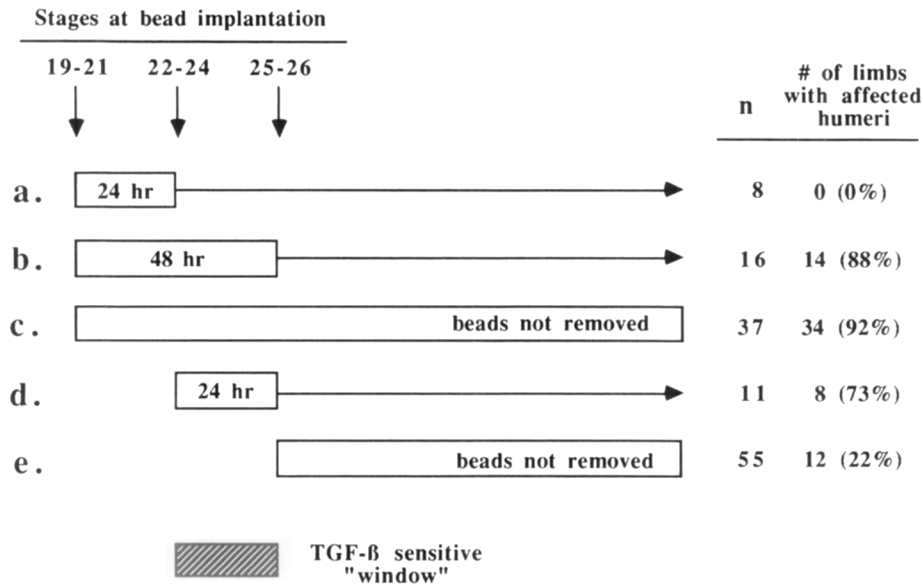


FIG. 2. The effects of different TGF- $\beta$ 1 exposure intervals on the incidence of humeral defects are shown. (a-c) TGF- $\beta$ 1-containing beads were implanted distally at stages 19-21 and removed after 24 or 48 hr or not removed. (d) TGF- $\beta$ 1 beads were implanted midway along the PD axis of limb buds at stages 22-24 and removed 24 hr later. (e) TGF- $\beta$ 1 beads implanted proximally at stages 25-26 were not removed.

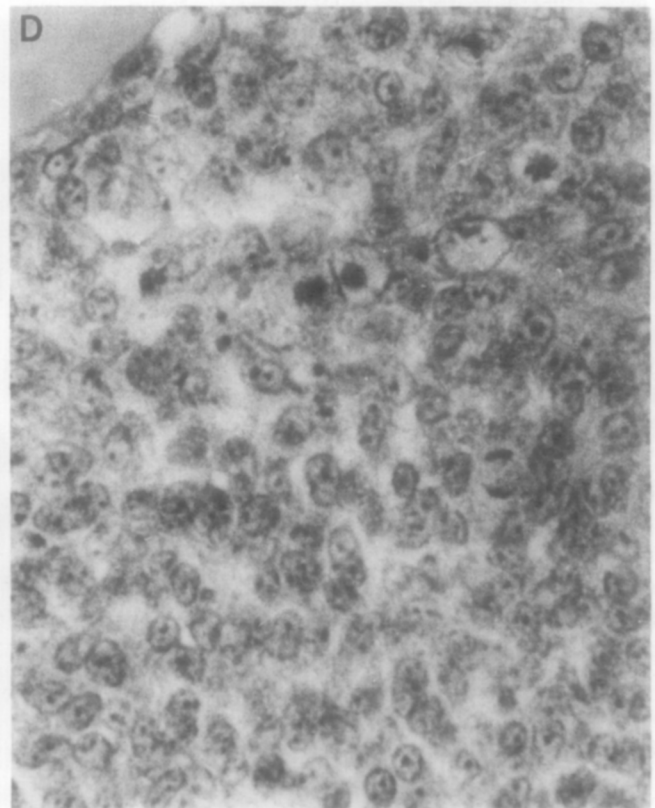
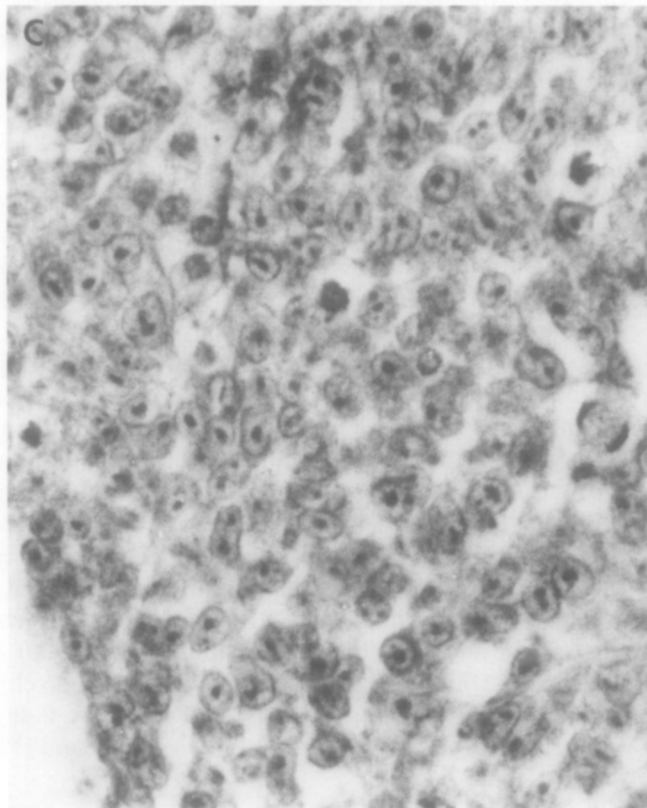
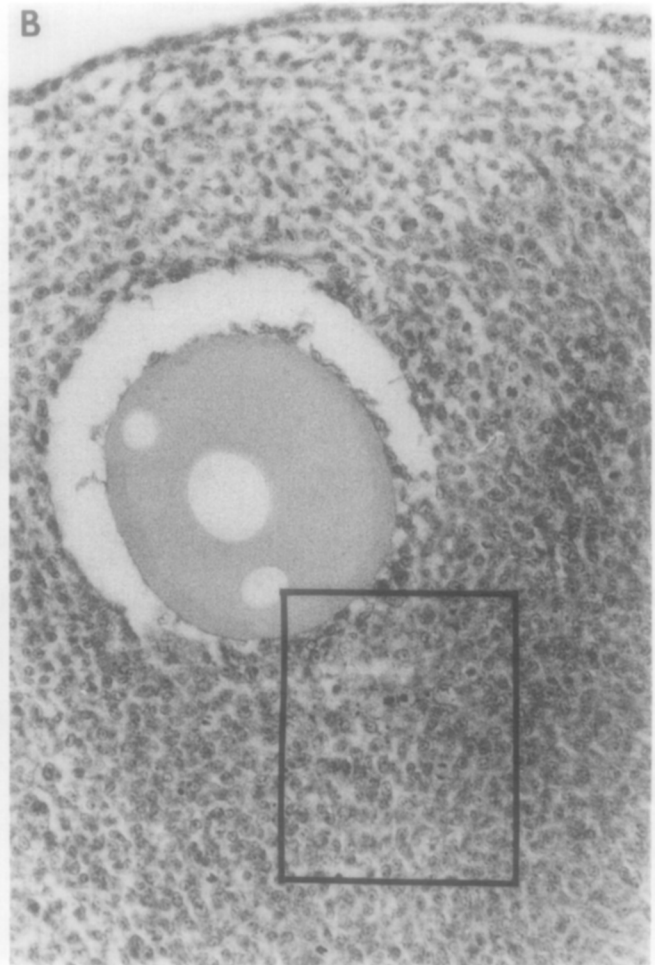
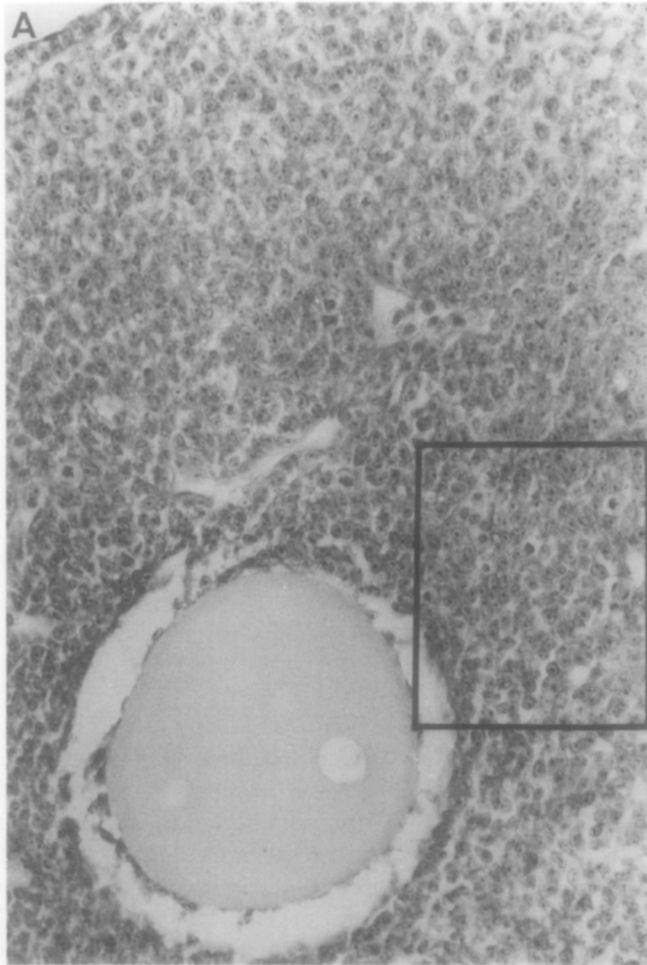
bead exhibit no apparent morphological changes, and mitotic figures are evident in many of the sections.

By implanting TGF- $\beta$ 1 beads at different stages, we have shown a stage-dependent sequence of defects along the proximal-distal (PD) axis. Beads implanted distally at stages 19-21 lead primarily to defects in the humerus, at stage 22-24 to defects in the radius and/or ulna, and at stages 25-26 to defects of the hand skeleton. The pattern of defects along the PD axis could also be correlated with the eventual PD position of the bead at later stages in development. Beads implanted at stages 19-21 are found in presumptive upper arm regions 24 hr later at stage 22-24; beads implanted distally at stages 22-24 are later found in forearm regions. Hence, beads are found in the affected segment by 24 hr after distal implantation. Taken together with the implantation/removal experiments, which show that the TGF- $\beta$ 1-sensitive window for the humerus begins at 24 hr and ends at 48 hr after stages 19-21, these data suggest that the humerus anlagen is sensitive to the effects of TGF- $\beta$ 1 at stages 22-24.

The timing of the TGF- $\beta$ 1 effect is consistent with the idea that exogenous TGF- $\beta$ 1 is influencing the early events of chondrogenesis. Prior to stage 22, the start of the TGF- $\beta$ 1-sensitive period for the humerus, the limb mesenchyme appears homogeneous and extracellular matrix components are thought to be evenly distributed. Starting at this stage, fibronectin (FN) and type I collagen expression increase in prechondrogenic areas (Dessau *et al.*, 1980; Melnick *et al.*, 1981). Newman and colleagues (Newman and Frisch, 1979; Tomasek *et al.*, 1982)

have suggested that FN is involved in promoting the formation of prechondrogenic aggregates at the onset of chondrogenesis. It is during this time period that we obtain our TGF- $\beta$  effects. At about stages 24-25, at the end of the sensitive period, distinct cellular condensations within the humeral region can be observed. As chondrogenesis begins, levels of FN and type I collagen start to decline (Dessau *et al.*, 1980), and expression of cartilage-specific type II collagen and sulfated cartilage proteoglycan core protein increase (Kosher *et al.*, 1986a,b).

From *in vitro* studies, TGF- $\beta$ -like molecules are known to be able to affect many of the processes involved in cartilage formation. However, the reported effects of TGF- $\beta$  are variable and cover the range from stimulation to inhibition of chondrogenesis. Studies showing stimulation include the report by Seyedin and colleagues (1985) that rat mesenchymal cells can be stimulated by TGF- $\beta$ 1 to synthesize collagen type II and cartilage proteoglycan core protein. Kulyk *et al.* (1989a) demonstrated that TGF- $\beta$ 1 promotes chondrogenic differentiation in both high and low density micromass cultures of stage 23-24 whole bud and stage 25 distal limb bud cells. These investigators also reported that TGF- $\beta$  did not appear to affect cell proliferation or cell viability *in vitro*, as they observed little difference in total DNA content between TGF- $\beta$ -treated and control cultures. In addition, Carrington and Reddi (1990) reported that, when micromass cultured stage 24-25 chick wing bud cells were treated with TGF- $\beta$ 1 for 1-2 days, an increase in alkaline phosphatase activity and [ $^{35}$ S]-





sulfate incorporation into proteoglycans was observed. In our lab, we have also found that TGF- $\beta$ 1 (10 ng/ml) added to the culture medium during the first 24 hr of culture promotes accumulation of Alcian blue-stainable matrix in micromass cultures of stage 23–24 chick wing bud cells (unpublished results).

In contrast, there are several reports in the literature of inhibition of chondrogenesis in response to TGF- $\beta$  exposure. In monolayer cultures grown on plastic, TGF- $\beta$ 1 has been shown to inhibit expression of type II collagen and cartilage proteoglycan by chondroblasts in a dose-dependent manner (Rosen *et al.*, 1988). Solursh and Reiter (1989) also reported that TGF- $\beta$  had an inhibitory effect on chondrogenesis (evaluated by Alcian blue staining, as well as collagen type II and cartilage proteoglycan expression) in micromass cultures of stage 23–24 chick wing bud cells. Furthermore, in addition to the results mentioned above, Carrington and Reddi (1990) also showed that the effects of TGF- $\beta$ 1 on limb bud cells in micromass culture depended on the timing and duration of treatment. Exposure of stage 24–25 chick wing bud cells to TGF- $\beta$ 1 over the course of 4–7 days in culture, or during days 3–4, resulted in a reduction of both alkaline phosphatase activity and [<sup>35</sup>S]sulfate incorporation into proteoglycans. It has also been reported that TGF- $\beta$  inhibited chondrogenesis by mesenchymal cells grown on collagen gels (Solursh and Reiter, 1989). Preliminary experiments in our lab have shown that TGF- $\beta$ 1 delivered via agarose beads to micromass cultured limb bud cells in collagen gels, at the same concentrations used in our *in vivo* studies, neither stimulated nor inhibited cartilage differentiation (unpublished observations).

One possible explanation for the variety of *in vitro* results is the diversity of culture conditions, limb bud stages, and limb bud regions used in the various studies. Carrington and Reddi (1990) have suggested that the effects of TGF- $\beta$ 1 on chick limb bud cells *in vitro* may also depend on the state of differentiation of the cells at the time of exposure to the growth factor. Our results support this idea. Whereas implants at early stages lead to inhibition of cartilage formation, proximal implants at later stages (when cells are in a different developmental state) stimulate the formation of extra cartilage at low frequency (4 of 55 cases).

The demonstration that TGF- $\beta$ 1 has effects on chick limb bud cells at comparable stages both *in vitro* and *in vivo* appears to indicate that these cells have receptors

that can be influenced by TGF- $\beta$ 1 binding. Hence, exogenous TGF- $\beta$ 1 can be viewed as interfering with the normal function of an endogenous TGF- $\beta$ -like molecule(s). At present, there are no reports of the expression of TGF- $\beta$ -like gene products in early chick limb buds. However, several TGF- $\beta$ s (1–4) have been identified in developing chicks (Jakowlew *et al.*, 1991), and several members of the TGF- $\beta$  superfamily (BMP-2A and Vgr-1, as well as TGF- $\beta$ 2 and another “TGF- $\beta$ -like” molecule) have been shown to be expressed in spatially and temporally distinct patterns in the developing mouse limb (Lyons *et al.*, 1989b).

In conclusion, both the present *in vivo* study and previous *in vitro* studies suggest that TGF- $\beta$ -like molecules are involved in the development of the limb skeleton and that the variable responses of cells to exogenous TGF- $\beta$  can be attributed to the state of differentiation of the cells at the time of exposure. Clarification of the way in which molecules like TGF- $\beta$  affect limb bud cells *in vivo* as well as *in vitro* will be an important part of understanding the way in which these factors might function in normal limb outgrowth.

We thank Tony Purchio (Oncogen) for the gift of TGF- $\beta$ 1. We also thank David Gardiner and the Muneoka lab at Tulane University for helpful discussions and for criticisms of the manuscript. This research was supported by PHS Grant HD20662 and by a gift from the Monsanto Co.

#### REFERENCES

- BRYANT, S. V., and ITEN, L. (1974). The regulative ability of the limb regeneration blastema of *Notophthalmus viridescens*: Experiments *in situ*. *Wilhelm Roux' Arch.* 174, 90–101.
- CARRINGTON, J. L., and REDDI, A. H. (1990). Temporal changes in the response of chick limb bud mesodermal cells to transforming growth factor  $\beta$ -type 1. *Exp. Cell Res.* 186, 368–373.
- CATE, R. L., MATTALIANO, R. J., HESSION, C., TIZARD, R., FARBER, N. M., CHEUNG, A., NINFA, E. G., FREY, A. Z., GASH, D. J., CHOW, E. P., FISHER, R. A., BERTONIS, J. M., TORRES, G., WALLNER, B. P., RAMACHANDRAN, K. L., RAGIN, R. C., MANGANARO, T. F., MACLAUGHLIN, D. T., and DONAHOE, P. K. (1986). Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45, 685–698.
- DERYCK, R., JARRET, J. A., CHEN, E. Y., and GOEDEL, D. V. (1985). Human transforming growth factor- $\beta$  complementary DNA sequence and expression in normal and transformed cells. *Nature* 316, 701–705.
- DERYCK, R., LINDQUIST, P. B., LEE, A., WEN, D., TAMM, J., GRAYCAR, J. L., RHEE, L., MASON, A. J., MILLER, D. A., COFFEY, R. J., JR., MOSES, H. L., and CHEN, E. Y. (1988). A new type of transforming growth factor- $\beta$ , TGF- $\beta$ 3. *EMBO. J.* 7, 3737–3743.
- DESSAU, W., VON DER MARK, K., and FISCHER, S. (1980). Changes in the

FIG. 3. Histological sections of chick wing buds following implantation of TGF- $\beta$ -containing beads. (A, C) Section of a stage 23 limb bud, 24 hr after bead implantation at stage 20. Cells surrounding the bead appear healthy, with no evidence of pyknotic nuclei or cell debris. The space around the bead is a processing artifact. (B, D) Section of a stage 25 limb bud, 48 hr after TGF- $\beta$  bead implantation. Bead is located proximally in the limb bud, in the region of the prospective humerus. At both time points all cells appear healthy, and numerous mitotic figures are evident.

- patterns of collagens and fibronectin during limb bud chondrogenesis. *J. Embryol. Exp. Morphol.* **57**, 51-60.
- EICHELE, G., and THALLER, C. (1987). Characterization of concentration gradients of a morphogenetically active retinoid in the chick limb bud. *J. Cell Biol.* **105**, 1917-1923.
- EICHELE, G., TICKLE, C., and ALBERTS, B. M. (1984). Microcontrolled release of biologically active compounds in chick embryos: Beads of 200- $\mu$ m diameter for the local release of retinoids. *Anal. Biochem.* **142**, 542-555.
- FLANDERS, K. C., THOMPSON, N. L., CISSEL, D. S., VAN OBERGHEN-SCHILLING, E., BAKER, C. C., KASS, M. E., ELLINGSWORTH, L. R., ROBERTS, A. B., and SPORN, M. B. (1989). Transforming growth factor- $\beta$ 1: Histochemical localization with antibodies to different epitopes. *J. Cell Biol.* **108**, 653-660.
- HAMBURGER, V., and HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
- HAYEK, A., CULLER, F. L., BEATTIE, G. M., LOPEZ, A. D., CUEVAS, P., and BAIRD, A. (1987). An *in vivo* model for the study of the angiogenic effects of basic fibroblast growth factor. *Biochem. Biophys. Res. Commun.* **147**, 876-880.
- HEINE, U. I., MUNOZ, E. F., FLANDERS, K. C., ELLINGSWORTH, L. R., LAM, H.-Y. P., THOMPSON, N. L., ROBERTS, A. B., and SPORN, M. B. (1987). Role of transforming growth factor- $\beta$  in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861-2876.
- JAKOWLEW, S. B., DILLARD, P. J., SPORN, M. B., and ROBERTS, A. B. (1988). Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor  $\beta$ 4 from chick embryo chondrocytes. *Mol. Endocrinol.* **2**, 1186-1195.
- JAKOWLEW, S. B., DILLARD, P. J., WINOKUR, T. S., FLANDERS, K. C., SPORN, M. B., and ROBERTS, A. B. (1991). Expression of transforming growth factor- $\beta$ s 1-4 in chicken embryo chondrocytes and myocytes. *Dev. Biol.* **143**, 135-148.
- KONDAIAH, P., SANDS, M. J., SMITH, J. M., FIELDS, A. F., ROBERTS, A. B., SPORN, M. B., and MELTON, D. A. (1990). Identification of a novel transforming growth factor- $\beta$  (TGF- $\beta$ 5) mRNA in *Xenopus laevis*. *J. Biol. Chem.* **265**, 1089-1093.
- KOSHER, R. A., GAY, S. W., KAMANITZ, J. R., KULYK, W. M., RODGERS, B. J., SAI, S., TANAKA, T., and TANZER, M. L. (1986a). Cartilage proteoglycan core protein gene expression during limb cartilage differentiation. *Dev. Biol.* **118**, 112-117.
- KOSHER, R. A., KULYK, W. M., and GAY, S. W. (1986b). Collagen gene expression during limb cartilage differentiation. *J. Cell Biol.* **102**, 1151-1156.
- KULYK, W. M., RODGERS, B. J., GREER, K., and KOSHER, R. A. (1989a). Promotion of embryonic chick cartilage differentiation by transforming growth factor- $\beta$ . *Dev. Biol.* **135**, 424-430.
- KULYK, W. M., UPHOLT, W. B., and KOSHER, R. A. (1989b). Fibronectin gene expression during limb cartilage differentiation. *Development* **106**, 449-455.
- LEHNERT, S. A., and AKHURST, R. J. (1988). Embryonic expression pattern of TGF- $\beta$  type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Genes Dev.* **3**, 1-15.
- LING, N., YING, S.-Y., UENO, N., SHIMASAKI, S., ESCH, F., HOTTA, M., and GUILLEMIN, R. (1986). Pituitary FSH is released by a heterodimer of the  $\beta$ -subunits from the two forms of inhibin. *Nature* **321**, 779-782.
- LYONS, K., GRAYCAR, J. L., LEE, A., HASHMI, S., LINDQUIST, P. B., CHEN, E. Y., HOGAN, B. L. M., and DERYNCK, R. (1989a). Vgr-1, a mammalian gene related to *Xenopus* Vg-1, is a member of the transforming growth factor  $\beta$  gene superfamily. *Proc. Natl. Acad. Sci. USA* **86**, 4554-4558.
- LYONS, K. M., PELTON, R. W., and HOGAN, B. L. M. (1989b). Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor- $\beta$ -like genes coordinately regulate aspects of embryonic development. *Genes Dev.* **3**, 1657-1668.
- LYONS, K. M., PELTON, R. W., and HOGAN, B. L. M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development* **109**, 833-844.
- MADISEN, L., WEBB, N. R., ROSE, T. M., MARQUARDT, H., IKEDA, T., TWARDZIK, D., SEYEDIN, S., and PURCHIO, A. F. (1988). Transforming growth factor- $\beta$ 2: cDNA cloning and sequence analysis. *DNA* **7**, 1-8.
- MASON, A. J., HAYFLICK, J. S., LING, N., ESCH, F., UENO, N., YING, S.-Y., GUILLEMIN, R., NIALL, H., and SEEBURG, P. H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor- $\beta$ . *Nature* **318**, 659-663.
- MELNICK, M., JASKOLL, T., BROWNELL, A. G., MACDOUGALL, M., BESSEM, C., and SLAVKIN, H. C. (1981). Spatiotemporal patterns of fibronectin distribution during embryonic development. I. Chick limbs. *J. Embryol. Exp. Morphol.* **63**, 193-206.
- NEWMAN, S. A. (1988). Lineage and pattern in the developing vertebrate limb. *Trends Genet.* **4**, 329-332.
- NEWMAN, S. A., and FRISCH, H. L. (1979). Dynamics of skeletal pattern formation in developing chick limb. *Science* **205**, 662-668.
- ÖZKAYNAK, E., RUEGER, D. C., DRIER, E. A., CORBETT, C., RIDGE, R. J., SAMPATH, T. D., and OPPERMANN, H. (1990). OP-1 cDNA encodes an osteogenic protein in the TGF- $\beta$  family. *EMBO J.* **9**, 2085-2093.
- PADGETT, R. W., ST. JOHNSTON, R. D., and GELBART, W. M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor- $\beta$  family. *Nature* **325**, 81-84.
- PELTON, R. W., NOMURA, S., MOSES, H. L., and HOGAN, B. L. M. (1989). Expression of transforming growth factor  $\beta$ 2 RNA during murine embryogenesis. *Development* **106**, 759-767.
- PELTON, R. W., DICKINSON, M. E., MOSES, H. L., and HOGAN, B. L. M. (1990). *In situ* hybridization analysis of TGF $\beta$ 3 RNA expression during mouse development: comparative studies with TGF $\beta$ 1 and  $\beta$ 2. *Development* **110**, 609-620.
- RIZZINO, A. (1988). Transforming growth factor- $\beta$ : Multiple effects on cell differentiation and extracellular matrices. *Dev. Biol.* **130**, 411-422.
- ROBERTS, A. B., FLANDERS, K. C., KONDAIAH, P., THOMPSON, N. L., VAN OBERGHEN-SCHILLING, E., WAKEFIELD, L., ROSSI, P., DE CROMBRUGGHE, B., HEINE, U., and SPORN, M. B. (1988). Transforming growth factor  $\beta$ : Biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. *Recent Prog. Horm. Res.* **44**, 157-197.
- ROBERTS, A. B., HEINE, U. I., FLANDERS, K. C., and SPORN, M. B. (1990). Transforming growth factor- $\beta$ : Major role in regulation of extracellular matrix. *Ann. N.Y. Acad. Sci.* **580**, 225-232.
- ROBERTS, A. B., and SPORN, M. B. (1988). Transforming growth factor  $\beta$ . *Adv. Cancer Res.* **51**, 107-145.
- ROSEN, D. M., STEMPIEN, S. A., THOMPSON, A. Y., and SEYEDIN, S. M. (1988). Transforming growth factor- $\beta$  modulates expression of osteoblast and chondroblast phenotypes *in vitro*. *J. Cell. Physiol.* **134**, 337-346.
- ROSEN, V., WANG, E. A., WOZNEY, J. M., CORDES, P., MCQUAID, D., and KURTZBERG, L. (1989). Purification and molecular cloning of a novel group of BMPs and localization of BMP mRNA in developing bone. *Connect. Tissue Res.* **20**, 313-319.
- SAMPATH, T. K., COUGHLIN, J. E., WHETSTONE, R. M., BANACH, D., CORBETT, C., RIDGE, R. J., ÖZKAYNAK, E., OPPERMANN, H., and RUEGER, D. C. (1990). Bovine osteogenic protein is composed of

- dimers of OP-1 and BMP-2A, two members of the transforming growth factor- $\beta$  superfamily. *J. Biol. Chem.* **22**, 13,198-13,206.
- SANDBERG, M., VUORIO, T., HIRVONEN, H., ALITALO, K., and VUORIO, E. (1988). Enhanced expression of TGF- $\beta$  and c-fos mRNAs in the growth plates of developing human long bones. *Development* **102**, 461-470.
- SCHREIBER, A. B., WINKLER, M. E., and DERYNCK, R. (1986). Transforming growth factor- $\alpha$ : A more potent angiogenic mediator than epidermal growth factor. *Science* **232**, 1250-1252.
- SEYEDIN, S. M., ROSEN, D. M., and SEGARINI, P. R. (1988). Modulation of chondroblast phenotype by transforming growth factor- $\beta$ . *Pathol. Immunopathol. Res.* **7**, 38-42.
- SEYEDIN, S. M., THOMAS, T. C., THOMPSON, A. Y., ROSEN, D. M., and PIEZ, K. A. (1985). Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc. Natl. Acad. Sci. USA* **82**, 2267-2271.
- SMITH, J. C., PRICE, B. M. J., VAN NIMMEN, K., and HUYLEBROECK, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* **345**, 729-731.
- SOLURSH, M., and REITER, R. S. (1989). Comparison of effects of ectoderm, EGF and TGF $\beta$  on cell differentiation by limb bud cell cultures. *Anat. Rec.* **223**, 109A.
- TEN DIJKE, P., HANSEN, P., IWATA, K. K., PIELER, C., and FOULKES, J. G. (1988). Identification of another member of the transforming growth factor type  $\beta$  family. *Proc. Natl. Acad. Sci. USA* **85**, 4715-4719.
- TICKLE, C., LEE, J., and EICHELE, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109**, 82-95.
- TOMASEK, J. J., MAZURKIEWICZ, J. E., and NEWMAN, S. A. (1982). Non-uniform distribution of fibronectin during avian limb development. *Dev. Biol.* **90**, 118-126.
- VALE, W., RIVIER, J., VAUGHAN, J., MCCLINTOCK, R., CORRIGAN, A., WOO, W., KARR, D., and SPIESS, J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776-779.
- VAN DEN EIJNDEN-VAN RAAIJ, A. J. M., VAN ZOELENT, E. J. J., VAN NIMMEN, K., KOSTER, C. H., SNOEK, G. T., DURSTON, A. J., and HUYLEBROECK, D. (1990). Activin-like factor from a *Xenopus laevis* cell line responsible for mesoderm induction. *Nature* **345**, 732-734.
- WANG, E. A., ROSEN, V., D'ALESSANDRO, S., BAUDUY, M., CORDES, P., HARADA, T., ISRAEL, D. I., HEWICK, R. M., KERNS, K. M., LAPAN, P., LUXENBERG, D. P., MCQUAID, D., MOUTSATSOS, J. K., NOVE, J., and WOZNEY, J. M. (1990). Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. USA* **87**, 2220-2224.
- WEEKS, D. L., and MELTON, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF- $\beta$ . *Cell* **51**, 861-867.
- WOZNEY, J. M., ROSEN, V., CELESTE, A. J., MITSOCK, L. M., WHITTERS, M. J., KRIZ, R. W., HEWICK, R. M., and WANG, E. A. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science* **242**, 1528-1534.