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Role of Transforming Growth Factor-β in Chondrogenic Pattern Formation in the Embryonic Limb: Stimulation of Mesenchymal Condensation and Fibronectin Gene Expression by Exogenenous TGF-β and Evidence for Endogenous TGF-β-like Activity

CLAIRE M. LEONARD,* HOWARD M. FULD,*,[1] DOROTHY A. FRENZ,*,[2] SHERRY A. DOWNIE,* JOAN MASSAGUÉ,† AND STUART A. NEWMAN*,[3]

*Departments of Cell Biology and Anatomy, New York Medical College, Valhalla, New York 10595; and †Cell Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021

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The possible role of TGF-β-like molecules in skeletal pattern formation in the embryonic vertebrate limb was studied by analyzing the mechanism of enhancement of chondrogenesis in chick wing bud mesenchyme *in vitro* and testing for the presence and distribution of endogenous TGF-β-like activity in this tissue. Transient exposure (3-6 hr) to TGF-β1 (1-2 ng/ml) on the day after plating resulted in a 1.5- to 2-fold enhancement of accumulation of Alcian blue (pH 1.0)-stainable extracellular matrix 5 days later. The enhancement of differentiation was preceded by an acceleration and an increase in the extent of precartilage condensation formation, visualized by Hoffman Modulation Contrast microscopy a day after TGF-β treatment. In contrast, neither condensation nor subsequent chondrogenesis was stimulated by transient treatment with TGF-β1 on the day of plating. The effectiveness of a TGF-β treatment regimen in enhancing chondrogenesis was correlated with its effectiveness in stimulating condensation formation. Exposures to the factor for 3-6 hr on the day after plating, which most consistently stimulated both condensation formation and chondrogenesis, also corresponded to a peak in the enhancement of the steady-state level of fibronectin mRNA (fourfold to eightfold over control levels) measured at the end of the treatment period. The elevation in fibronectin mRNA levels brought about by this treatment persisted throughout the period of condensation. Endogenous TGF-β-like activity was detected in limb mesenchyme: extracts of freshly isolated and cultured limb tissues contained 6-25 pg TGF-β-like activity per 1×10^6 cells by the Mv1Lu cell proliferation inhibition assay, and indirect immunofluorescence using a polyclonal antibody directed against a TGF-β-related peptide indicated a patchy distribution of endogenous TGF-β-like reactivity within a day after culture. These findings are discussed in relation to the "fibronectin prepattern" hypothesis for limb pattern formation.

INTRODUCTION

The formation of the skeleton in the developing vertebrate limb involves a sequence of events that may constitute a causal chain (Newman, 1977, 1988). These events include the accumulation of the extracellular glycoprotein fibronectin in a temporally and spatially regulated fashion (Newman and Frisch, 1979; Tomasek *et al.*, 1982; Kosher *et al.*, 1982), the formation of tight aggregates of precartilage mesenchymal cells, known as "condensations" (Fell and Canti, 1934; Umansky, 1966; Thorogood and Hinchliffe, 1975; Ede, 1983), and the cytodifferentiation of cartilage at sites of condensation (reviewed in Kosher, 1983).

Recent studies *in vitro*, where condensation and differentiation occur according to a timetable similar to that in the limb (Newman, 1988), have supported the hypothesis of causal linkage between these processes. In particular, interaction between extracellular fibronectin and heparin-like molecules of the mesenchymal cell surface is crucial for the formation of precartilage condensations (Frenz *et al.*, 1989a,b). Furthermore, fibronectin gene expression is regulated in a spatiotemporal pattern corresponding to that of the condensations (Kulyk *et al.*, 1989b), consistent with a causal role for the glycoprotein as a mediator of the condensation process.

The question of how the fibronectin "prepattern" for the limb skeleton becomes established is currently unresolved. In principle, a diffusible molecule that is a positive effector of the synthesis of both fibronectin and itself could cause an initially uniform distribution of fibronectin to break into a series of concentration peaks and valleys (Newman and Frisch, 1979; Newman *et al.*, 1988). Transforming growth factor-β1 and β2, members

[1] Permanent address: Department of Biology, Bronx Community College, Bronx, NY 10453.

[2] Present address: Department of Developmental Otobiology, Albert Einstein College of Medicine, Bronx, NY 10461.

[3] To whom correspondence should be addressed.

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of the TGF-β family of growth and differentiation factors (Massagué, 1987), are potent inducers of chondrogenesis in appropriately responsive mesenchyme (Seyedin *et al.*, 1987; Kulyk *et al.*, 1989a). TGF-β also positively regulates the synthesis of both fibronectin (Ignotz and Massagué, 1986) and itself (Van Obberghen-Schilling *et al.*, 1988) in a number of systems. We were therefore interested in determining whether the stimulatory effect of TGF-β on limb chondrogenesis (Kulyk *et al.*, 1989a) is mediated by stimulation of mesenchymal condensation in association with enhanced fibronectin production at early stages of development. We also wondered whether limb mesenchyme contains endogenous TGF-β-like activity.

Here we report that transient exposure of high density cultures of limb mesenchymal cells to TGF-β early during the culture period causes a marked enhancement of chondrogenesis according to a pattern of response that directly corresponds to the stimulation of the condensation process. These responses are also accompanied by an up to eightfold enhancement in the steady-state levels of fibronectin mRNA that is detectable immediately after TGF-β treatment, and persists throughout the condensation period. We also show that limb mesenchyme contains endogenous TGF-β-like activity that is nonuniformly distributed in the tissue at least a day earlier than condensations begin to form. These results are consistent with the hypothesis that a member of the TGF-β superfamily is an endogenous regulator of condensation formation in the developing limb.

MATERIALS AND METHODS

Cell culture. Fertile eggs were obtained from Avian Services, Inc. (Frenchtown, NJ). Chick limb mesenchymal cells were prepared from stage 22/23 embryonic wing buds (Hamburger and Hamilton, 1951) and cultured as 10-μl spots (2.75×10^5 cells) in 24-well plates (Falcon 3047 or Costar 3024) in Ham's F-12 medium containing 10% fetal bovine serum, as described (Frenz *et al.*, 1989a). Cells were allowed to adhere to the substratum for 30 min before adding 1 ml of medium to each well. The distal halves of the wing buds were used. In contrast to cultures prepared from whole stage 22/23 wing buds, which exhibited numerous myotubes under our culture conditions, myotubes were rare in cultures derived from distal tips. The extent of chondrogenesis was found to be more reproducible in cultures grown on glass coverslips (Fisher No. 12-545-80) than in cultures grown directly on plastic; coverslips were therefore routinely employed for all the studies reported below. Bovine transforming growth factor β1 (TGF-β1, Cheifetz *et al.*, 1987) or porcine TGF-β1 from R&D Systems (Minneapolis, MN) was added to cultures immediately after cell attachment on Day 0, or 24 hr later on Day 1, for different durations, as indicated in the text. The standard concentration of TGF-β in the cultures was 2 ng/ml, but amounts ranging from 0.1 to 5 ng/ml were used in some experiments (see text). Media were completely changed on each day and at the time of addition and removal of TGF-β.

Quantitative Alcian blue staining of spot cultures. A modification of the procedure of Hassell and Horigan (1982) was used (Frenz *et al.*, 1989b; Leonard *et al.*, 1989). Briefly, cultures were fixed and stained with Alcian blue 8GX at pH 1.0 6 days after plating. Bound stain, which was proportionate to accumulated highly sulfated proteoglycan (Leonard *et al.*, 1989), was extracted with guanidinium chloride (8 *M*) and quantitated spectrophotometrically using an EIA reader with a 600-nm filter.

Identification and quantitation of condensations in living cultures. Cultures were viewed on a Zeiss IM35 inverted microscope, using low magnification Hoffman Modulation Contrast microscopy (Frenz *et al.*, 1989b), between Days 1 and 3. Condensation counts were recorded every 2–3 hr between 50 and 60 hr after plating. The maximum numbers of condensations measured prior to the onset of fusion between adjacent foci were reported for treated cultures in Tables 3 and 4. Contemporaneous values are given for the controls.

RNA isolation. RNA was isolated from spot cultures according to the procedure of White and Bancroft (1982) with modifications as described (Leonard *et al.*, 1989). Briefly, cells were scraped from culture wells, suspended in 10 m*M* Tris–HCl, pH 7.0, 1 m*M* EDTA on ice, and lysed by addition of two successive 0.1-vol aliquots of 5% Nonidet-P40 with a 5-min interval. After removal of nuclei by centrifugation (13,500*g*, 3 min), the cytoplasmic supernatant was extracted with 1 vol buffered phenol/chloroform (1:1 vol/vol), followed by 1 vol of chloroform/isoamyl alcohol (24:1 vol/vol). The aqueous supernatant was brought to 7.4% formaldehyde and 25 m*M* sodium phosphate, pH 6.5. The final mixture was incubated at 60°C for 15 min and stored at −70°C. Replicate cultures were homogenized in high salt buffer and analyzed for DNA content by the Hoechst 33258 dye binding method (Brunk *et al.*, 1979; Labarca and Paigen, 1980), using a Hoefer PK 100 spectrofluorometer. Volumes of cytoplasmic extract corresponding to equivalent amounts of DNA were applied with suction to GeneScreen (DuPont) presoaked in 25 m*M* sodium phosphate (pH 6.5), using a slot-blot manifold. The RNA was crosslinked to the membrane by exposure to shortwave uv light for 2 min.

Hybridization analysis. Prehybridization of filters was carried out overnight at 42°C in a solution containing 50% formamide, 0.2% polyvinylpyrollidone (M_r

40,000), 0.2% bovine serum albumin, 0.2% Ficoll (M_r, 400,000), 0.05 *M* Tris-HCl (pH 7.5), 1.0 *M* NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate (M_r 500,000), and 200 μg/ml salmon sperm DNA. Hybridization was performed for an additional 24 hr in the same buffer along with 10^7 cpm/ml denatured labeled probe. The probe consisted of chicken fibronectin cDNA (pFN600; Fagan *et al.*, 1980) labeled with [^{32}P]dCTP to a specific activity of $\sim 5 \times 10^8$ cpm per microgram using a multiprime DNA labeling system. The filters were washed twice for 5 min in 2× SSC (1× SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate) at room temperature, followed by two 45-min washes in 2× SSC, 1% SDS at 65°C, and one 10-min wash in 0.1× SSC at room temperature. Kodak X-Omat AR film was exposed to the filters for various lengths of time at −70°C, using a DuPont Cronex Lighting Plus intensifying screen. The relative amounts of hybridizable RNA sequences were quantified by scanning the autoradiograms with a Hoefer GS-300 scanning densitometer interfaced to a computer programmed to provide peak integration.

Isolation and assay of TGF-β-like activity from embryonic limb buds. Distal tips from wing buds of 5-day embryos (Newman, 1977), or micromass cultures of distal wing tips after 1 day of incubation were homogenized in 0.1 *M* acetic acid ($2\text{–}5 \times 10^6$ cells/ml) and stirred overnight at 4°C. The extract was dialyzed using a Spectropor No. 3 membrane (Spectrum Medical Industries, Los Angeles, CA) and lyophilized. Corresponding numbers of limb buds, wing tips, or cultures were homogenized in high salt buffer and assayed for DNA as described above. TGF-β-like activity was assayed by its inhibitory effect on proliferation of Mv1Lu mink lung epithelial cells, as previously described (Cheifetz *et al.*, 1987; Boyd and Massagué, 1989). Briefly, Mv1Lu cells (CCL64, American Type Culture Collection) grown in 24-well plates were labeled with 0.25 μCi/ml of [^{125}I]-iododeoxyuridine ([^{125}I]IdU, sp act 2200 Ci/mmole; DuPont-New England Nuclear) for the last 2 hr of incubation. Cells were washed three times with cold phosphate-buffered saline (PBS, 0.02 *M* sodium phosphate, pH 7.2, 0.15 *M* NaCl) and fixed with 95% methanol for 1.5 hr at 4°C. The cells were then washed once with PBS and extracted with 0.2 *N* NaOH for 0.5 hr at 4°C. The extracts were collected and counted in a gamma counter. Each assay was carried out in triplicate, and values varied by less than 5%. The percentage growth inhibition refers to the percentage decrease in [^{125}I]IdU incorporation relative to that in controls not treated with TGF-β.

Immunofluorescent localization of TGF-β-like molecules. Limb mesenchymal cultures were fixed for 10 min at 4°C with 4% paraformaldehyde in PBS, washed three times for 5 min with PBS, and incubated for 30 min with 0.3 mg/ml polyclonal antibody raised against synthetic peptide B1B containing residues 64–91 of human/porcine TGF-β1. This antibody is an IgG raised in a rabbit inoculated with the peptide coupled to keyhole limpet hemacyanin (Cheifetz *et al.*, 1987). The secondary antibody was FITC-conjugated goat anti-rabbit IgG. Stained cultures were viewed and photographed on a Leitz Dialux epifluorescence microscope using the FITC filter set. Controls were incubated in 10 μg/ml of the B1B peptide along with the primary antibody, a treatment that eliminated specific staining with this antiserum in Western blots (Cheifetz *et al.*, 1987).

TABLE 1
ACCUMULATION OF HIGHLY SULFATED PROTEOGLYCAN BY WING BUD PRECARTILAGE MESENCHYME

Time of staining (hr)	A_{600} (range)[a]	Mean percentage of maximum
24	0.014 (0.003–0.025)	0.4
47.5[b]	0.265 (0.214–0.362)	7
72	1.223 (1.187–1.289)	34
144	3.552 (3.257–3.697)	100

[a] Alcian blue (pH 1.0)-stainable matrix extracted by GuHCl from cultures of stage 22/23 wing buds. Data for each time point represent five to six cultures.

[b] Staining was done when earliest condensations became identifiable.

RESULTS

Effect of Day and Duration of Exposure on Stimulation of Proteoglycan Accumulation by TGF-β

Previous studies showed that transient or continuous exposure of limb mesenchyme cultures to TGF-β stimulated the accumulation by these cells of sulfated proteoglycans, and of mRNA for the corresponding cartilage-specific proteoglycan core protein (Kulyk *et al.*, 1989a). We reasoned that if a TGF-β-like molecule is part of the endogenous mechanism of skeletal development, exogenous factor should be capable of exerting significant effects on chondrogenic differentiation and pattern formation with exposures of only a few hours (Newman and Frisch, 1979). We therefore first examined the effect of duration of exposure on the chondrogenic response to TGF-β treatment. Treatments were done on the day of plating (Day 0) or 24 hr after plating (Day 1), when the extent of chondrogenesis by these cultures, measured by the accumulation of Alcian blue-stainable matrix at pH 1.0, was less than 1% of its value at 6 days (Table 1). There was a general enhancement of proteoglycan accumulation as a result of treatment with TGF-

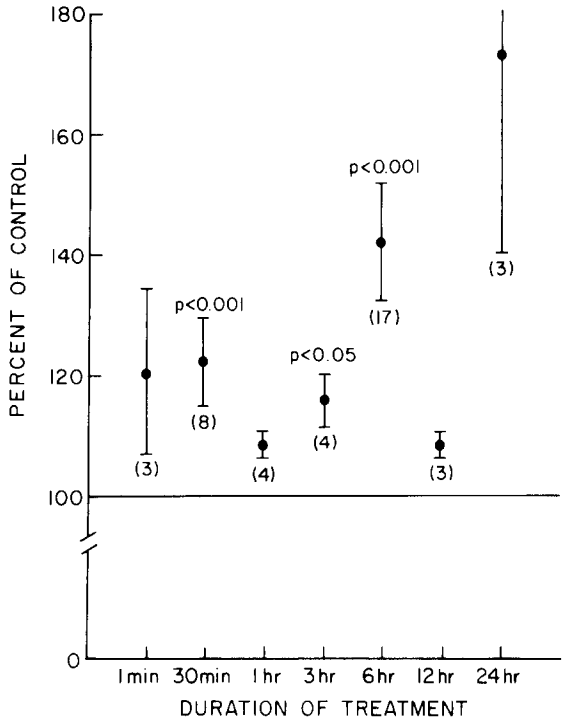

FIG. 1. Effect of duration of exposure to TGF-β1 on accumulation of proteoglycans during chondrogenesis of stage 22/23 chick wing mesenchyme. Exposures (2 ng/ml) were for the designated times, 1 day after cultures were established. At the end of the exposure period cultures were rinsed in F-12 medium, and medium was replaced. Cultures were stained with Alcian blue, pH 1.0, on Day 6 after plating. Bound stain was extracted with 8 *M* guanidinium chloride and quantitated spectrophotometrically (see Materials and Methods). Filled circles represent mean for (*N*) experiments and bars represent SEM. Each experiment comprised three to six treated and three to six control wells. Treatment durations for which experimental cultures differed significantly from their matched controls by Student's matched pair *t* test are indicated with the *P* value above the corresponding bar.

β1 (2 ng/ml) on the day after plating, which was more marked for some durations of treatment (e.g., 3-6 hr) than for others (e.g., 1 and 12 hr) (Fig. 1). Exposure of cultures to concentrations of TGF-β1 as low as 0.1 ng/ml for 6 hr caused comparable (~1.5-fold) enhancement of proteoglycan accumulation (data not shown).

Surprisingly, the effect on chondrogenesis of TGF-β1 (2 ng/ml) exposure for various durations on Day 0 differed from that of exposure on Day 1. Seven experiments, comprising three to six cultures each, representing exposure on Day 0 for durations of 1 min to 6 hr were compared with the corresponding data for Day 1 exposures (Table 2). The Day 0-treated cultures did not differ to a statistically significant extent from their matched controls in proteoglycan accumulation, but the difference between the Day 1- and Day 0-treated groups was highly significant.

Effect of TGF-β on Precartilage Mesenchymal Condensation

Between the second and the third days after plating, cultures of stage 22/23 limb mesenchymal cells undergo a process of cell condensation, the disruption of which is inhibitory to chondrogenesis (Frenz *et al.*, 1989b). On the basis of the hypothesis that TGF-β regulates skeletal development through its effect on precartilage condensation formation (Newman, 1988; Newman *et al.*, 1988) it was of interest to determine whether the effects of the factor on chondrogenesis described above were correlated with earlier effects on mesenchymal condensation.

In cultures prepared from the distal halves of stage 22/23 wing buds, condensations initially appeared on the second day after plating and grew larger with time, reaching a peak number before becoming confluent (Fig. 2). The dark patches visualized by Hoffman Modulation Contrast microscopy comprised slightly rounded, concentrically organized mesenchymal cells when viewed by phase-contrast microscopy (Frenz *et al.*, 1989b). Transient exposure to TGF-β1 on Day 1 after plating caused the condensation process to begin several hours precociously on the following day (Fig. 3). The number of condensations present in the treated cultures (measured on Day 2, before the expanding foci became confluent) was up to 1.6-fold the number that had formed by the same time in contemporaneous controls (Table 3). Condensations in the treated cultures also generally appeared larger than those in the controls at all times examined (e.g., Fig. 3). Because condensations continued to emerge at the periphery of the cultures after the central ones had fused, an ultimate number of condensations could not be determined.

TABLE 2
EFFECT OF DAY OF EXPOSURE ON CHANGE IN PROTEOGLYCAN ACCUMULATION IN RESPONSE TO BRIEF EXPOSURE TO TGF-β

Day of exposure	Proteoglycan accumulation as a percentage of control
0[a]	0.85 ± 0.08* (*N* = 7)
1[b]	1.28 ± 0.06* (*N* = 36)

[a] Sample consisted of seven experiments, each comprising three to six cultures, treated with 2 ng/ml TGF-β1 for 1 min, 30 min, 1 hr, 3 hr, or 6 hr.

[b] Sample consisted of all experiments with treatments of 1 min to 6 hr shown in Fig. 1.

* Cultures treated on Day 1 differed significantly in proteoglycan accumulation from their matched controls by Student's matched pair *t* test, $P < 0.001$, while the cultures treated on Day 0 did not differ significantly from their matched controls. When the mean percentage of control for the Day 0 experiments was compared with that for the Day 1 experiments the difference was highly significant ($P < 0.003$).

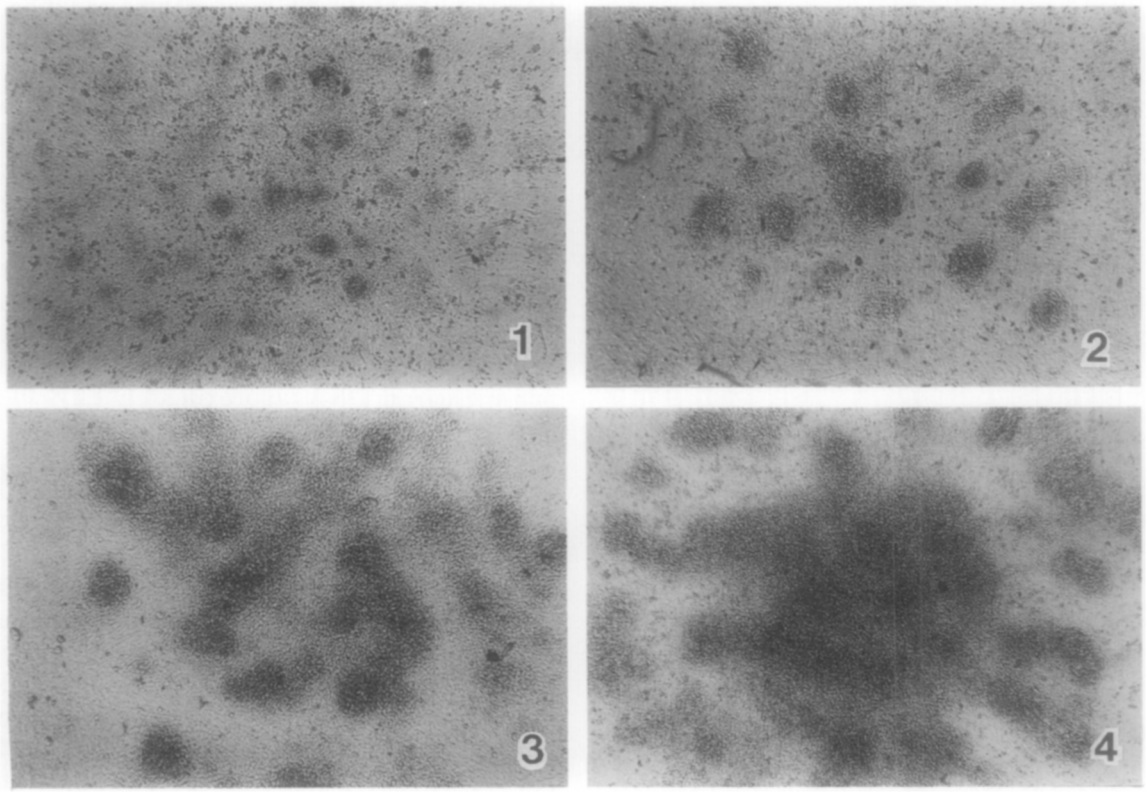

FIG. 2. Development of precartilage condensations (dark masses) in representative stage 22/23 limb mesenchyme cultures between the second and third day after plating (approximately 48–72 hr) as visualized by Hoffman Modulation Contrast microscopy. The time intervals between the panels are 4–6 hr.

Increasing the TGF-β1 concentration to as much as 5 ng/ml on Day 1 had no additional stimulatory effect on the condensation process (not shown). Moreover, as little as 0.1 ng/ml of the factor had effects comparable to the 2 ng/ml treatment when administered for the optimal duration of 6 hr on Day 1 (Table 4).

Interestingly, little or no stimulation of the condensation process occurred for treatment durations of 1 or 12 hr, exposures that also failed to significantly enhance proteoglycan accumulation by Day 6 (Fig. 1). Treatment with TGF-β on Day 0 also failed to stimulate condensation formation (Fig. 4).

Effect of TGF-β on Fibronectin Gene Expression

Cultures of stage 22/23 wing tip mesenchymal cells were exposed to 2 ng/ml TGF-β1 for various periods on

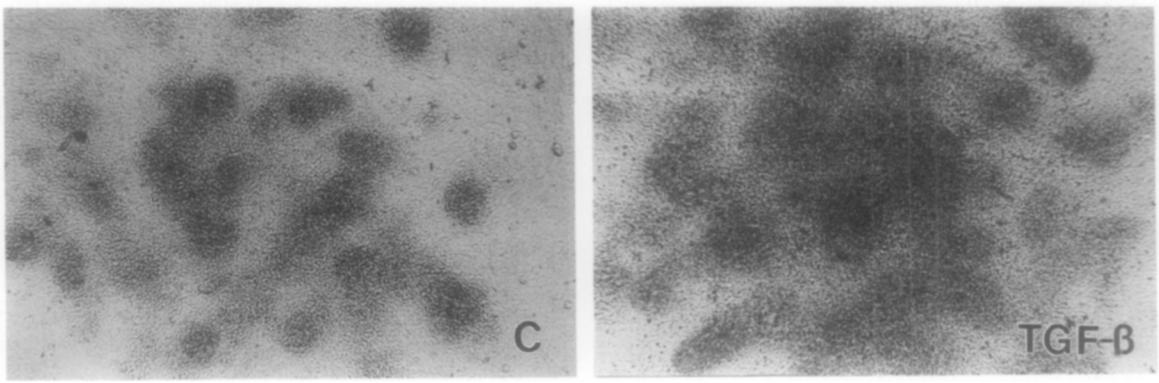

FIG. 3. Effect of 5-hr treatment with TGF-β1 (2 ng/ml) on day after plating on development of precartilage condensations 1 day later. Control (left) and treated (right) cultures were photographed within 1 min of one another. The control culture is same as that shown in Fig. 2, panel 3.

TABLE 3
EFFECT OF DURATION OF TGF-β EXPOSURE ON CONDENSATION NUMBER

Duration of exposure	Condensation number	
	Treated	Control
30 min	27 ± 4*	19 ± 4 (*N*=8)
1 hr	36 ± 11	35 ± 2 (*N*=3)
3 hr	34 ± 3*	25 ± 2 (*N*=6)
6 hr	50 ± 8*	30 ± 7 (*N*=6)
12 hr	24 ± 6	20 ± 5 (*N*=4)
24 hr	23 ± 4*	17 ± 3 (*N*=3)

Note. All exposures were initiated 24 hr after plating and were at a concentration of 2 ng/ml TGF-β. Condensations in treated cultures were counted at 2- to 3-hr intervals beginning at 50 hr after plating; numbers shown for treated cultures represent last reading prior to the onset of fusion of foci. Condensation numbers shown for controls were determined contemporaneously with those of treated cultures. Each individual experiment represents the mean of three to six treated cultures and three to six controls.

* Significantly different from control ($P < 0.05$) by Student's matched pair *t* test.

the day after plating, and cytoplasmic RNA was prepared from cultures at the end of each exposure period. RNA was immobilized on filters and assayed for fibronectin mRNA sequences by hybridization. Exposure to TGF-β for between 2 and 5 hr elicited a four- to eightfold increase in accumulation of fibronectin mRNA (Figs. 5, 6). Exposure for as little as 1 hr also led to modest increases in this mRNA. No cultures that were exposed to TGF-β for 10 hr or less contained less than control levels of fibronectin mRNA. However, for exposures longer than 15 hr the response was erratic, with cultures exposed for 24 hr generally containing less than control levels of fibronectin mRNA at the end of the exposure period (Fig. 6).

Levels of fibronectin mRNA in cultures exposed to TGF-β for 5 hr on the day after plating continued to be severalfold higher than controls a full day after exposure to the factor was discontinued (i.e., during the period when condensations were beginning to form), (Fig. 5), but no attempt was made to systematically follow the fate of fibronectin mRNA in cultures exposed for other durations. Durations of TGF-β exposure that most effectively elevated fibronectin mRNA accumulation corresponded in general to those that most effectively increased condensation number on the following day (Fig. 6; Table 3).

Endogenous TGF-β-like Activity in Precartilage Mesenchyme

The foregoing results raised the possibility that fibronectin production and subsequent condensation formation in *untreated* limb mesenchyme could be regulated by endogenous TGF-β-like activity. We therefore assayed acetic acid extracts of freshly isolated precartilage mesenchyme from 5-day wing tips (Newman, 1977), and of this tissue after 1 day in micromass culture, for inhibition of proliferation of Mv1Lu mink lung epithelial cells, an index of TFG-β-like activity (Tucker *et al.*, 1984; Cheifetz *et al.*, 1987; Boyd and Massagué, 1989). A standard preparation of TGF-β1 had an ED_{50} of 0.35 p*M* (8.8 pg/ml) in this assay (Fig. 7b). In the assays shown in Fig. 7a, freshly isolated limb mesenchyme and Day 1 cultures contained 3.3 and 1.4 pg TGF-β1-like activity per microgram DNA (approximately 1.6×10^5 cells), respectively.

Spatial Pattern of Endogenous TGF-β-like Activity

Immunofluorescent staining of cultures prepared from stage 22/23 limb buds using a polyclonal antibody directed against a TGF-β1-related peptide indicated the presence of extracellular TGF-β-like immunoreactivity as early as 18 hr after plating (Fig. 8, upper left). Although this was more than 24 hr before condensations and the corresponding accumulations of extracellular fibronectin first appeared (Frenz *et al.*, 1989a,b), the pattern of TGF-β-like immunoreactivity was distributed in a patchy fashion. Controls that were incubated in the same primary antibody along with the cognate peptide showed no fluorescence (upper right), indicating that staining was specific for a TGF-β-like protein. Although we were not able to determine whether the patches of TGF-β-like immunoreactivity eventually became sites of enhanced fibronectin biosynthesis in any one culture, the size and spatial distribution of the "TGF-β" patches resemble the subsequent pattern of fibronectin accumulation and condensation (Frenz *et al.*, 1989b).

DISCUSSION

We have shown that the enhanced chondrogenesis in embryonic limb mesenchyme caused by transient expo-

TABLE 4
EFFECT OF TGF-β DOSE ON CONDENSATION NUMBER

	Dose (ng/ml)			
	0	0.1	0.25	0.5
Expt 1	45	59	68	68
Expt 2	45	64	65	78

Note. All exposures had a duration of 6 hr and were initiated 24 hr after plating. Cell densities were 2.75×10^7 per milliliter. The mean number of condensations is listed for each treatment group, which consisted of three to five cultures in each experiment. Values for each culture were within 10% of mean value in all cases.

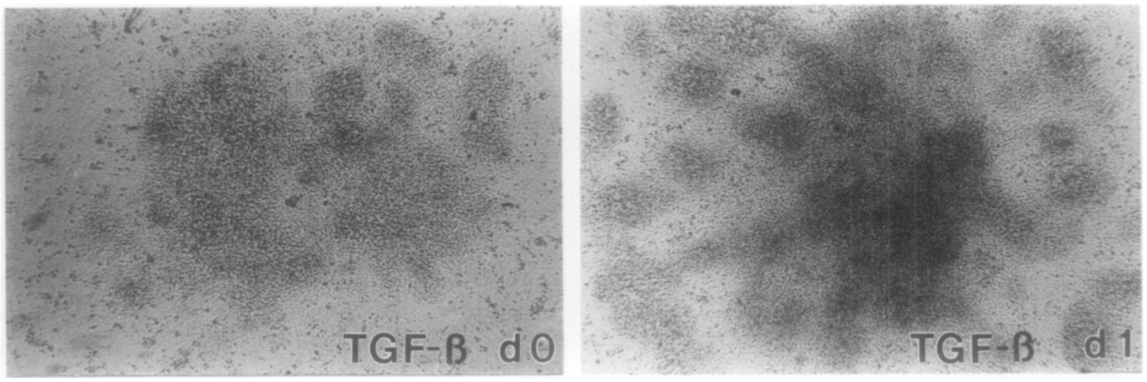

FIG. 4. Comparison of effect of 5-hr treatment with TGF-β1 (2 ng/ml) on day of plating (left) and day after plating (right) on formation of precartilage condensations during the second day after plating. Cultures were photographed within 1 min of one another.

sure to TGF-β (Kulyk *et al.*, 1989a) is preceded by a stimulation of mesenchymal condensation. The effect on condensation formation is elicited by treatments that occur a full day before condensations themselves are evident, with exposures as low as 0.1 ng/ml TGF-β in the surrounding medium. Our results thus suggest that TGF-β stimulates the chondrogenic pathway at a step that is critical in cartilage pattern formation (Fell and Canti, 1934; Newman, 1977; Ede, 1983). Together with the evidence for endogenous TGF-β-like activity in these cells (Figs. 7, 8), these studies support the hypothesis that a member of this family of factors is involved in determining the pattern of skeletal elements during limb development (Newman *et al.*, 1988; Newman, 1988).

The stimulation of the rate of condensation by TGF-β is reflected in the greater number of foci present in treated cultures relative to that in contemporaneous controls by the time the central condensations in the treated cultures had begun to fuse (Fig. 3; Table 3). Because the condensations in the TGF-β-treated cultures tended to be larger than those in controls (Fig. 3), the enhancement of condensation formation in the treated cultures was even more extensive than indicated by the condensation counts.

The correspondence between stimulation of condensation formation and enhancement of limb bud chon-

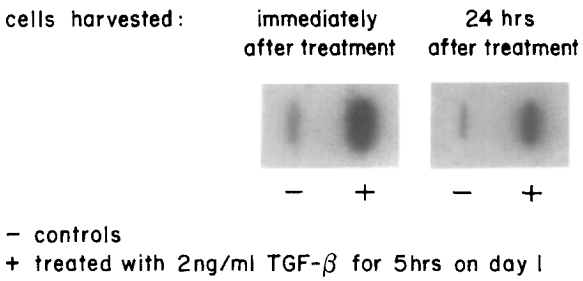

FIG. 5. Autoradiogram of slot-blot hybridization of cytoplasmic RNA isolated from equivalent numbers of cells from control and TGF-β-treated limb mesenchyme cultures. Cells were harvested immediately after the 5-hr treatment, or 24 hr later. The blot was probed with [^{32}P]-labeled pFN600 (fibronectin) cDNA (Fagan *et al.*, 1980).

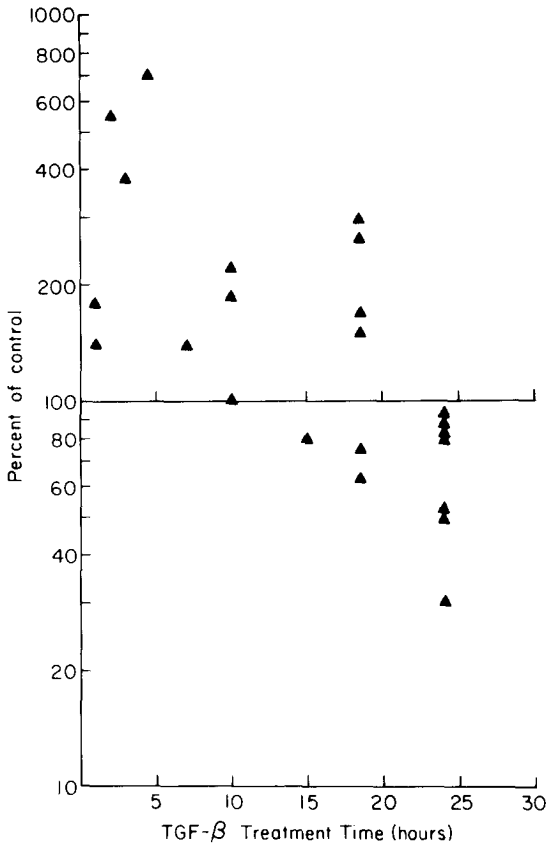

FIG. 6. Graphical representation of 23 experiments similar to that in Fig. 1, but with TGF-β treatments of varying duration. Cells were harvested immediately after treatment in all cases. Autoradiograms were densitometrically scanned and the resulting peaks numerically integrated. The data are expressed as 100× the ratio of the integrated signal from each treated culture to that of its corresponding control. Note that the ordinate scale is logarithmic.

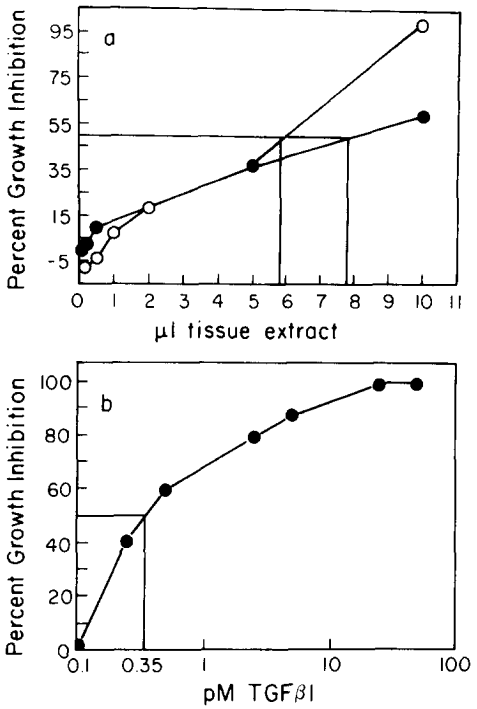

FIG. 7. (a) Inhibition of proliferation of Mv1Lu cells by varying amounts of aqueous solutions of lyophylized, dialyzed acetic acid extracts of freshly isolated 5-day chick wing bud tips (open circles) and micromass cultures of the same cell population 1 day after plating (closed circles). Extract volumes giving 50% inhibition of growth are indicated. (b) Mv1Lu proliferation assay as in (a), using known concentrations of TGF-β1.

drogenesis (as measured by accumulation of Alcian blue-staining matrix) by TGF-β was also seen in the parallel responses of these two indices to duration of treatment with the factor. Treatments of durations of 30 min, 3 hr, 6 hr, and 24 hr on Day 1 all significantly stimulated condensation formation by the following day (Table 3), and, for all but the 24-hr treatment, led to significant increases in proteoglycan accumulation 4 days later (Fig. 1), whereas 1 or 12 hr treatments on Day 1 led to little, if any, increase in the mean number of condensations and nonsignificant increases in proteoglycan accumulation.

The reason for the apparent nonmonotonic variations in the response to TGF-β with respect to duration of treatment (Fig. 1; Table 3) is unclear, but may be related to the metabolic circuitry by which TGF-β is regulated. In particular, TGF-β1 positively regulates its own synthesis in a variety of systems (Van Obberghen-Schilling *et al.*, 1988). Because the concentrations of molecules whose regulation involves positive feedback can oscillate with time (e.g., Sinha and Ramaswamy, 1987), endogenous pools of TGF-β-like molecules may undergo periodic fluctuations. If the *phase* of such an oscillation in relation to other developmental events were critical, then resetting the phase to a new value could perturb development. For many biological oscillations the phase to which the oscillator is reset varies periodically with the duration of an external stimulus, and the response to some perturbations is much more variable than the response to others, as seen in Fig. 1 and Table 3 (Winfree, 1980).

Proteoglycan accumulation was not enhanced in cultures treated with TGF-β for brief periods (1 min–6 hr) on the day of plating (Table 2). This contrasts with the findings of Kulyk *et al.* (1989a) who observed a 50% enhancement of sulfate incorporation in cultures that had been treated with TGF-β2 for as little as 2 hr on the day of plating. The different results could have arisen from different efficacies of members of the TGF-β family (our 0-day experiments were performed with TGF-β1), different doses (our experiments used 2 ng/ml TGF-β, while those of Kulyk *et al.* used 5–10 ng/ml), or different cell densities (2.0×10^7 cells/ml in the experiments of Kulyk *et al.*, vs 2.75×10^7 cells/ml in our 0-day experiments). Another significant difference between these studies is that our experiments were performed with distal halves of stage 22/23 wing buds rather than the stage 25 wing tips used by Kulyk *et al.*.

One component of the complex pattern of the chondrogenic response to TGF-β may be the synthesis of tenascin by limb mesenchyme (Mackie *et al.*, 1987). This extracellular glycoprotein has been found to interfere with certain functions of fibronectin (Chiquet-Ehrismann *et al.*, 1988), which, in turn, plays an important role in mediating precartilage condensation (Frenz *et al.*, 1989a,b). TGF-β induces the synthesis of both of these matrix proteins in various systems (Ignotz and Massagúe, 1986; Pearson *et al.*, 1988). If they are correspondingly induced in limb mesenchyme with different efficacies at different stages of development, a complex pattern of response to the factor would be expected. Marked differences in biosynthetic response of cultured chick limb mesenchyme to TGF-β as a function of day of exposure have also been observed by other investigators (Carrington and Reddi, 1990).

With respect to the hypothesized role of fibronectin in the response to TGF-β, our results indicate that the stimulatory effect of the factor on limb mesenchymal condensation and subsequent cartilage differentiation is associated with an enhancement in fibronectin mRNA accumulation. It is striking that treatments of 3–6 hr on the day after plating, which were most consistently effective in increasing steady-state levels of fibronectin mRNA (Fig. 6), were also most consistently effective in increasing condensation number (Table 3). Moreover, proteoglycan accumulation measured at 6 days was consistently elevated by 30–60% with 6-hr treatments (Fig. 1). This period of responsiveness to TGF-β is

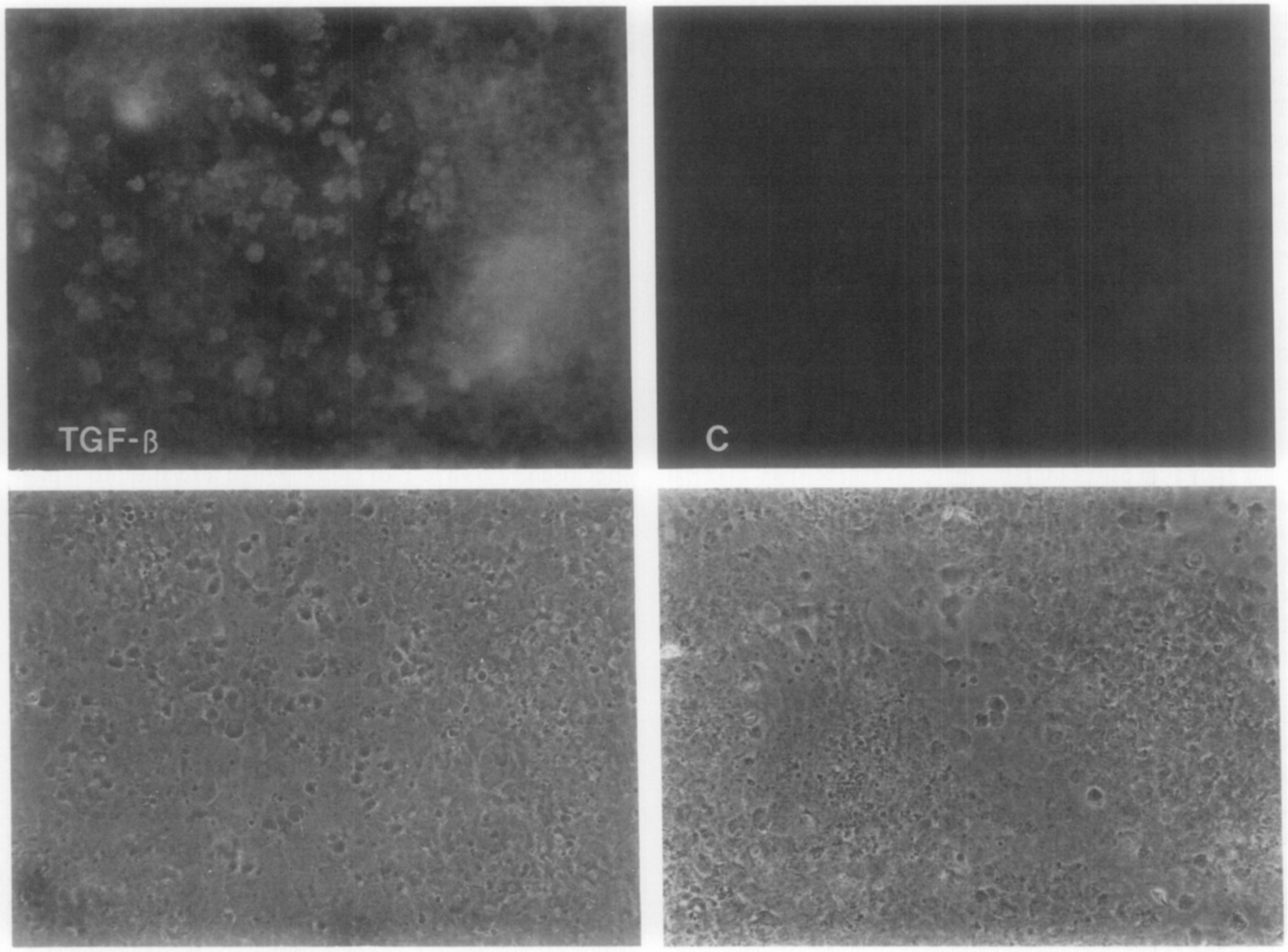

FIG. 8. (Top left) Indirect immunofluorescence of TGF-β-like immunoreactivity in fixed culture of stage 22/23 chick limb mesenchyme 18 hr after plating. Primary antiserum was anti-B1B, prepared against a TGF-β1-related peptide. (Bottom left) Phase-contrast micrograph of field shown above. (Top right) Control for specificity of immunofluorescence. Preparation and staining protocol was similar to that in top left panel, but 10 μg/ml B1B peptide was present during incubation with antiserum. (Bottom right) Phase-contrast micrograph of field shown above.

at a stage when accumulation of extracellular fibronectin is low compared to levels that will be achieved a day later (compare Frenz *et al.*, 1989a, Fig. 3 to Frenz *et al.*, 1989b, Fig. 1), and when the extent of sulfated proteoglycan accumulation is less than 1% of the 6-day levels (Table 1). It is reasonable to suppose that the immediate increase in fibronectin mRNA in the treated cultures is closely coupled with increased fibronectin synthesis, as it is in unstimulated cultures (Kulyk *et al.*, 1989b). The resulting enhanced accumulation of extracellular fibronectin over the following day would then correlate with enhanced condensation formation.

Treatments with TGF-β for 24 hr on the day after plating, which also stimulated condensation formation (Table 3), led to decreased levels of fibronectin mRNA (Fig. 6) by the end of the treatment period. Because significant changes in the pattern of precartilage condensation in the developing avian limb occur over the course of a few hours (Ede, 1983), response of limb mesenchyme to prolonged treatment with TGF-β is probably less relevant to the potential role of such a factor in skeletal pattern formation than is the response to brief, transient treatments. However the down-regulation of steady-state fibronectin mRNA levels with prolonged TGF-β treatment is an unexpected finding; in relation to the general hypothesis considered in this study it would be useful to know whether the levels of extracellular fibronectin in such cultures are elevated in correspondence to the enhanced condensation numbers, despite the depressed message levels seen at the end of the treatment period.

A TGF-β-like molecule is a plausible regulator of the skeletal pattern in the vertebrate limb. If, as suggested, the placement of cartilage elements is dependent on the

prior placement of condensations (Newman, 1977, 1988; Ede, 1983), and this, in turn, is dependent on the local accumulation of fibronectin (Newman and Frisch, 1979; Tomasek *et al.*, 1982; Frenz *et al.*, 1989b), then an extracellular effector like TGF-β which elicited the synthesis of fibronectin (Ignotz and Massagué, 1986; Ignotz *et al.*, 1987) could play a major role in pattern formation. This family of factors also provides a way of avoiding the "infinite regress" normally implicit in this sort of scheme. As Turing (1952) showed mathematically, and as Castets *et al.* (1990) have recently demonstrated experimentally, reaction-diffusion systems can spontaneously give rise to spatially precise patterns of peaks and valleys of chemical concentration if one of the diffusible components positively regulates its own synthesis (see also Newman and Comper, 1990). The fact that TGF-β1 enhances its own synthesis in a variety of mesenchymal systems (Van Obberghen-Schilling *et al.*, 1988) suggests that a molecule of this type could be a component of a reaction-diffusion-based pattern generating system in the developing limb (Newman and Frisch, 1979; Newman *et al.*, 1988; Newman, 1988).

The proposed role for a TGF-β-like molecule in chondrogenic pattern formation implies that such a molecule would be present endogenously in the developing limb, and that it would become nonuniformly distributed during the course of development. The Mv1Lu cell proliferation inhibition assay (Fig. 7) and immunostaining with a polyclonal antibody directed against a TGF-β-related peptide (Fig. 8) both indicated the presence of endogenous TGF-β-like molecules in developing limb tissues a day before any overt condensation is evident. The second of these assays showed, moreover, that at least a portion of this activity is present in a "condensation-like" pattern by this time. In previous studies, antibodies directed against TGF-β1 were shown to be reactive with limb bud precartilage mesenchyme in sections of mouse embryos (Heine *et al.*, 1987; Flanders *et al.*, 1989), but mRNA for this factor could not be detected in these cells under stringent *in situ* hybridization conditions (Lehnert and Akhurst, 1988). In contrast, hybridization probes to the RNA for bone morphogenetic protein 2a (BMP-2a), a member of the TGF-β superfamily (Wozney *et al.*, 1988) were highly reactive with condensing mouse precartilage mesenchyme (Lyons *et al.*, 1989). In light of these findings it is likely that the endogenous TGF-β-like factor(s) that we have detected in chick embryonic limb precartilage mesenchyme represents one or more members of the TGF-β superfamily other than TGF-β1 itself.

Drushel and Caplan (1988) have suggested that interstitial fluid dynamics in the limb mesenchyme may place limits on the ability of diffusible extracellular factors to direct skeletal pattern formation, since any prepatterns that are formed would tend to dissipate over relatively short times. The present results indicate how the developing mesenchyme may circumvent this problem. Transient exposures to TGF-β are capable of enhancing condensation formation and subsequent chondrogenesis (Table 2; Fig. 1). These exposures are reflected in increased steady-state levels of fibronectin mRNA (Figs. 5, 6) that are "remembered" by the tissue for at least a day after the factor is withdrawn (Fig. 5).

As in the foregoing example, some aspects of our interpretation of the role of endogenous TGF-β-like activity in skeletal pattern formation are based on analogy with results of studies of the administration of exogenous TGF-β. The relevance of these analogies is supported by our preliminary studies, which indicate that endogenous TGF-β-like factor, when added to limb mesenchyme in culture, greatly enhances the condensation process (C.M.L. and S.A.N., in preparation). It remains to be demonstrated that the endogenous activity, like the exogenous molecule, acts on limb mesenchyme through fibronectin gene expression. In addition, we would like to know whether the endogenous factor acts in an autoenhancing fashion on its own production, as shown for TGF-β1 in other systems. Finally, ectopic introduction of endogenous TGF-β-like activity into the limbs of developing embryos would help in further defining its role in skeletal pattern formation.

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