

# Triiodothyronine Stimulates Maturation of Porcine Growth-plate Cartilage In Vitro

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**ABSTRACT** We studied the effect of triiodothyronine ( $T_3$ ) on mammalian growth-plate cartilage in vitro. Growth-plate cartilages from fetal pigs scapulae were incubated for 3 or 7 d in serum-free medium alone or medium containing  $T_3$ . Alkaline phosphatase activity, a marker of hypertrophied chondrocytes, was increased in  $T_3$  (10 nM)-treated growth-plate cartilage 152±36% above that of cartilage incubated in medium alone after 3 d of incubation, and 324±47% after 7 d of incubation. There was a dose-response increase in alkaline phosphatase activity to  $T_3$  over the range of 0.01–10 nM. The rise in alkaline phosphatase activity was specific for  $T_3$  since growth-plate cartilage alkaline phosphatase activity was not increased by cortisol, insulin, parathyroid hormone, or 5% fetal calf serum. Histological studies of growth-plate cartilage showed that  $T_3$  in a concentration-dependent manner increased the width of the zone of maturation (hypertrophied chondrocytes). Histochemical staining for alkaline phosphatase activity demonstrated that  $T_3$  caused the recruitment of new cells into the zone of maturation.  $T_3$  also stimulated incorporation of L-[ $^3H$ ]leucine into protein and  $^{35}SO_4$  into proteoglycan in growth-plate cartilage. In contrast,  $T_3$  did not increase alkaline phosphatase activity or radiolabeled precursor incorporation into nongrowth-plate scapular cartilage. These studies demonstrate that  $T_3$  directly stimulates maturation and, to a lesser degree, growth-related processes in fetal mammalian growth-plate cartilage.

## INTRODUCTION

Alterations in thyroid hormone secretion leading to hypothyroidism or hyperthyroidism in humans or laboratory animals are associated with dramatic changes in skeletal growth and osseous maturation (1–3). These observations indicate that thyroid hormones stimulate

the growth and maturation of the skeleton. It is unclear how thyroid hormones mediate these effects (4). Changes in thyroid function in vivo lead to alterations in the secretion of many other hormones and some investigators have proposed that those alterations are responsible for the changes in osseous development (5, 6). Although thyroid hormones have been shown to stimulate proteoglycan synthesis in vitro, in embryonic chick cartilage (7, 8), no in vitro effects of thyroid hormones on mammalian cartilage have been demonstrated (9).

We have recently used a 3–5-d serum-free organ culture system to study the growth and development of chick embryo pelvic cartilage (10). In this system, the addition of triiodothyronine ( $T_3$ )<sup>1</sup> (0.0015–0.15 nM) to the incubation medium increases growth and accelerates maturation. Since embryonic chick pelvic cartilage is rapidly maturing cartilage, it seemed reasonable to hypothesize that rapidly maturing mammalian cartilage, such as growth-plate cartilage, might respond to  $T_3$  in a similar manner. The present study shows that  $T_3$  promotes in vitro maturation of mammalian growth-plate cartilage, and that this direct effect of  $T_3$  on cartilage is one mechanism for the in vivo effect of thyroid hormones on mammalian skeletal growth and maturation.

## METHODS

*Experimental procedures.* Fetal pigs were obtained within 2 h of slaughter from a local abattoir. The fetuses weighed from 100 to 150 g, corresponding to a gestational age of 60–70 d. Each experiment was done with fetuses from a single sow and the weights of the fetuses were within a 5-g range. The cartilages from the scapular wings were removed, cleaned aseptically, and sectioned with a sterile razor blade into three parts: scapular bone, the adjacent growth-plate cartilage (width 2 mm), and the remaining scapular cartilage designated nongrowth-plate cartilage (Fig. 1). In some experiments (Tables I and IV), growth-plate cartilage from one scapula of each fetal pig was weighed, placed in

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Received for publication 22 February 1982 and in revised form 28 April 1982.

<sup>1</sup> Abbreviations used in this paper: bPTH, bovine parathyroid hormone;  $T_3$ , triiodothyronine.

## FETAL PIG SCAPULA

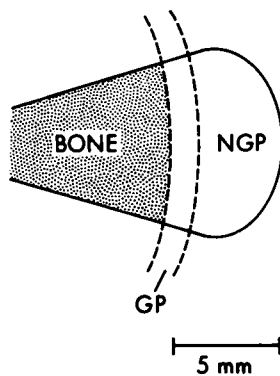


FIGURE 1 Diagrammatic representation of the zones of the scapula from a 100-g fetal pig. Growth-plate (GP) cartilage segments were prepared by sectioning with a razor blade along the hatched lines. The adjacent cartilage remaining is designated nongrowth-plate (NGP) cartilage, and segments of comparable width were prepared similarly.

Linbro plastic wells containing 2 ml of BGJ<sub>b</sub> (Fitton-Jackson modification) medium, and incubated at 37°C in 10% CO<sub>2</sub>-90% air for 3 or 7 d. No serum or antibiotics were added to the medium. The growth-plate cartilage isolated from the other scapula of the same fetal pig was incubated under similar conditions except that at the initiation of the incubation T<sub>3</sub> was added to the medium. In the remainder of the experiments the growth-plate cartilages were removed, pooled, and randomized among the various treatment incubations. The medium was changed on day 3 in experiments of 7-d incubation. Nongrowth-plate cartilages were removed, sectioned, and incubated in a similar manner.

At the end of 3 or 7 d of incubation, the cartilages were weighed and homogenized in 1 ml of 0.9% NaCl containing

0.2% Triton X-100. The homogenate was centrifuged at 10,000 g for 15 min and the supernate was assayed for alkaline phosphatase activity using *p*-nitrophenyl phosphate as substrate (11). Enzyme activity is expressed as micromoles *p*-nitrophenol released per milligram protein per hour. Protein was measured by the method of Lowry et al. (12), using bovine serum albumin as standard.

The effect of T<sub>3</sub> on cartilage alkaline phosphatase activity *in vitro* was initially determined by incubation of growth-plate and nongrowth-plate cartilage in medium containing 10 nM T<sub>3</sub> for 3 d. Subsequently, dose-response effects of T<sub>3</sub> on cartilage alkaline phosphatase activity were observed utilizing T<sub>3</sub> concentrations of 0, 0.01, 0.1, 1.0, and 10 nM. The effect of 3-d incubation of growth-plate cartilage in medium containing porcine insulin 250 ng/ml, hydrocortisone 25 ng/ml, bovine parathyroid hormone (bPTH) (1-34) 40 ng/ml, or fetal calf serum 5% (vol/vol) on alkaline phosphatase activity was assessed.

Histological studies were performed on growth-plate cartilage that had been incubated in organ culture. After 3-d incubation in medium alone or containing T<sub>3</sub> (0.1 to 10 nM), growth-plate cartilages were fixed in 3.7% buffered formaldehyde, embedded in paraffin, and tissue sections prepared and stained with hematoxylin and eosin. Histochemical staining for alkaline phosphatase activity was done on growth-plate cartilage that had been incubated in medium alone or containing 10 nM T<sub>3</sub> for 3 d. Cartilages from both groups were frozen in separate areas in the same block to insure uniform processing and staining. The tissue block was sectioned across the growth plate and the tissue sections were stained for alkaline phosphatase activity using the Sigma histochemical kit 85.

Protein and proteoglycan synthetic rates in the incubated cartilages were estimated by determining radiolabeled precursor incorporation into products. Growth-plate and nongrowth-plate cartilages were incubated for 3 d in medium alone, in medium containing 10 nM T<sub>3</sub>, or in medium containing 5 or 10% fetal or adult pig serum and pulsed during the final four hours of incubation with 2.0 μCi L-[<sup>3</sup>H]leucine and 1.25 μCi H<sub>2</sub><sup>35</sup>SO<sub>4</sub>. The cartilages were removed, rinsed, weighed, and homogenized in 5% trichloroacetic acid (TCA).

TABLE I  
Effect of T<sub>3</sub> on Alkaline Phosphatase Activity in Growth-plate Scapular Cartilage after 3 and 7 d of *In Vitro* Incubation

	0 d	3 d		7 d	
		Medium	Medium + T <sub>3</sub>	Medium	Medium + T <sub>3</sub>
Exp. 1	22±2 (6)	32±2 (6)	108±5 (6)*	32±3 (4)	154±24 (4)*
Exp. 2	20±1 (6)	24±1 (6)	52±7 (6)†	35±4 (3)	164±15 (3)*
Exp. 3	55±7 (6)	57±8 (3)	163±15 (3)†		
Exp. 4	60±3 (3)	63±2 (6)	109±5 (8)†	56±5 (3)	182±16 (3)*

Alkaline phosphatase activity in growth-plate scapular cartilage from 100 to 150-g fetal pigs incubated in medium alone and in medium containing T<sub>3</sub> 10 nM for 3 and 7 d. Activity is given as micromoles *p*-nitrophenol released per milligram protein per hour and the values are expressed as mean±SEM. The number of growth plates per group is in parentheses. The mean percent increase in alkaline phosphatase activity in cartilage incubated in T<sub>3</sub> in the four experiments was 152±36% above cartilage incubated in medium alone after 3 d and 324±47% after 7 d of incubation. The average fetal pig weights in experiments 1 and 2 were 100±10 g; in experiments 3 and 4, 150±15 g.

\* *P* < 0.01.

† *P* < 0.01 vs. incubation in medium alone.

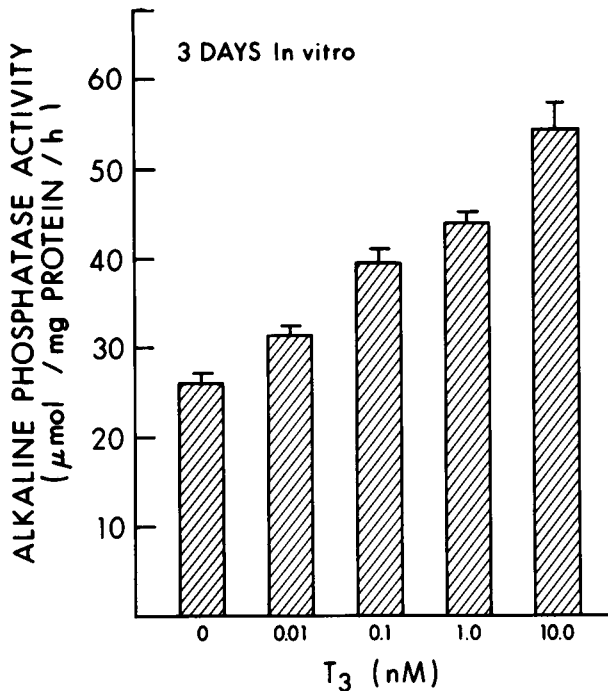


FIGURE 2 Effect of  $T_3$  concentration on alkaline phosphatase activity of growth-plate cartilage after 3 d of incubation. Three growth plates were used for each concentration of  $T_3$ . Significant increases in alkaline phosphatase activity occurred at  $T_3$  concentrations of 10 pM ( $P < 0.05$ ). Maximal response was with 10 nM  $T_3$ . These data are representative of two such dose-response experiments.

The homogenate was centrifuged at 10,000  $g$  for 15 min, and the supernate was discarded. The pellet was washed with 5% TCA, solubilized in Protosol (New England Nuclear, Boston, MA) and the acid insoluble counts representing [ $^3H$ ]leucine incorporation into protein and  $^{35}SO_4$  incorporation into proteoglycan were determined by liquid scintillation spectrophotometry (13).

**Materials.** BCG<sub>6</sub> (Fitton-Jackson modification) medium was obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). 3,3',5' Triiodo-L-thyronine, *p*-nitrophenol phosphate, hydrocortisone, and histochemical kit 85 were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue culture plates (76-045-55) were obtained from Linbro Chemical Company (Hamden, CT). L-[ $^3H$ ]Leucine (42 Ci/mmol) and Protosol were purchased from New England Nuclear (Boston, MA). Carrier-free  $H_2^{35}SO_4$  (27 Ci/mmol) was obtained from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY). Porcine insulin was a gift of Eli Lilly & Co. (Indianapolis, IN). Synthetic (1-34) bPTH (6,000 U/mg) was purchased from Beckman Instruments, Inc. (Palo

Alto, CA). Fetal calf serum was purchased from Flow Laboratories, Inc. (McLean, VA). Fetal pigs and fetal and adult porcine serum were obtained from Garrard Packing Co. (Durham, NC).

## RESULTS

*T<sub>3</sub> stimulation of alkaline phosphatase activity in growth-plate cartilage.* The activity of alkaline phosphatase in the growth-plate cartilage before in vitro incubation ranged from 22 to 60  $\mu\text{mol}/\text{mg}$  protein per h (0 d in Table I). The variation in basal alkaline phosphatase activity was related to the age of the fetal pig, as greater activity was seen in growth-plate cartilage from 150-g fetuses than from 100-g fetuses. Incubation of growth-plate cartilage in medium alone for 3 or 7 d resulted in no significant change in alkaline phosphatase activity, indicating that the cartilage neither matured spontaneously nor died over this period of incubation (Table I).

Growth-plate cartilage incubated in vitro with medium containing  $T_3$  had a marked increase in alkaline phosphatase activity as compared with appropriate growth-plate cartilage controls incubated in medium alone. As shown by the data in Table I, 10 nM  $T_3$  significantly increased alkaline phosphatase activity  $152 \pm 36\%$  after 3 d of incubation ( $P < 0.01$ ), and  $324 \pm 47\%$  after 7 d of incubation ( $P < 0.001$ ). The rise in alkaline phosphatase activity was significant from day 3 to day 7 in the  $T_3$ -treated cartilages ( $P < 0.05$ ).

The effect of  $T_3$  in stimulating growth-plate alkaline phosphatase activity is dependent on the concentration of  $T_3$  present in the medium. Fig. 2 shows representative data of the dose-response increase in alkaline phosphatase activity seen after 3 d incubation with  $T_3$ . A  $T_3$  concentration as low as 10 pM caused a significant increase in alkaline phosphatase activity. Maximal stimulation occurred with 10 nM  $T_3$  (higher concentrations caused no greater effect). Similar results were obtained with thyroxine (data not shown).

The specificity of the  $T_3$  stimulation of growth-plate cartilage alkaline phosphatase activity was assessed by evaluating the effect of factors known to influence other aspects of cartilage metabolism on alkaline phosphatase activity in the fetal pig growth-plate cartilage organ culture system. As shown in Table II, cortisol, 5% fetal calf serum, insulin, and bPTH had no effect on alkaline phosphatase activity after 3-d incubation. In contrast, 10 nM  $T_3$  consistently increased alkaline phosphatase activity (221%).

FIGURE 3 Photomicrographs ( $\times 100$ ) of growth-plate cartilage incubated for 3 d in medium alone (control) and in medium containing increasing concentrations of  $T_3$  (0.1-10 nM). The bracketed lines were inserted to help visually delineate the zone of maturation (hypertrophied chondrocytes) from the zone of proliferation (flattened chondrocytes). Hematoxylin and eosin staining.

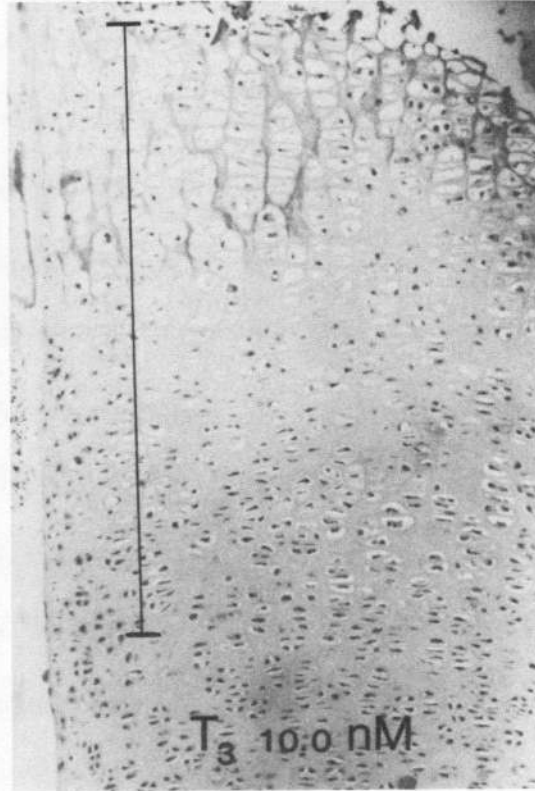
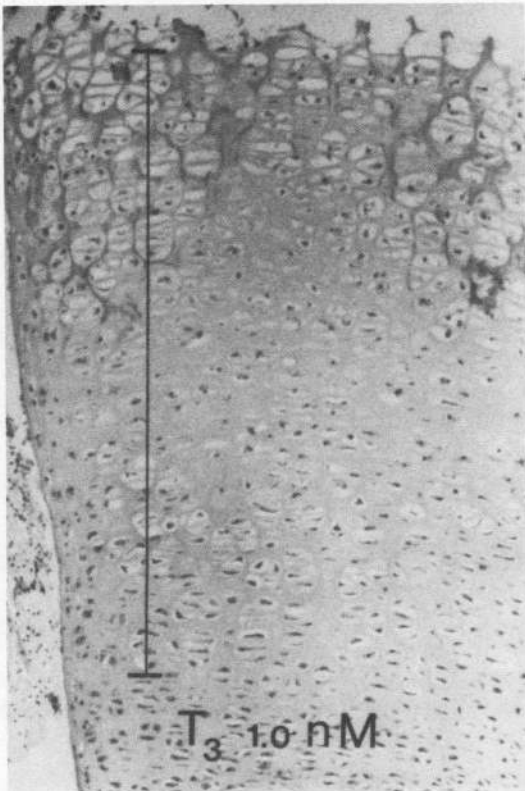
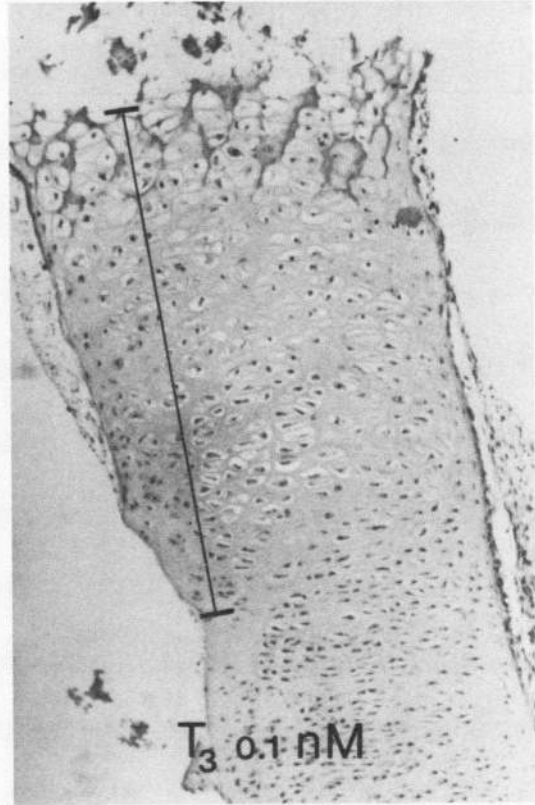
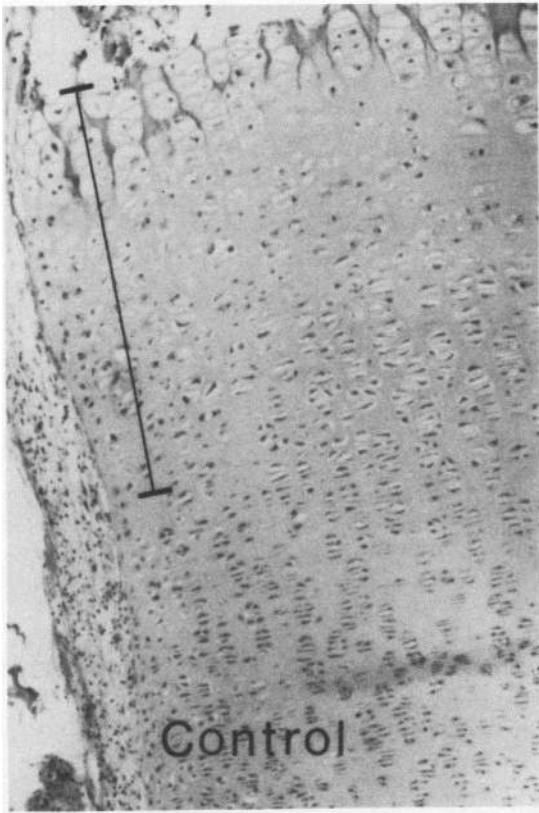


TABLE II  
Alkaline Phosphatase Activity after 3 d of In Vitro Incubation of Growth-plate Cartilage

	Exp. 1	Exp. 2	Exp. 3
Control	23±3 (6)	25±2 (3)	44±4 (3)
Cortisol	29±3 (3)	21±4 (3)	43±2 (3)
Fetal calf serum	24±4 (3)	34±4 (3)	44±4 (3)
Insulin	26±3 (3)	23±2 (3)	
bPTH	29±5 (3)	27±5 (3)	
T <sub>3</sub>	82±4 (4)*	92±10 (3)*	106±5 (3)*

Alkaline phosphatase activity (micromoles per milligram protein per hour) was assayed in growth-plate cartilage after 3 d of incubation in medium alone (control) and medium containing each of the following: cortisol 25 ng/ml; fetal calf serum 5% (vol/vol), porcine insulin 250 ng/ml, bPTH (1-34) 40 ng/ml, and T<sub>3</sub> 10 nM. Activity is expressed as mean±SEM. The number of growth plates per group is in parentheses.

\*  $P < 0.01$  vs. incubation in medium alone.

Histological and histochemical studies were done to determine whether the T<sub>3</sub> effect on alkaline phosphatase activity involved increased activity of hypertrophied chondrocytes already present in the growth plate, or was due to increased conversion of immature chondrocytes into hypertrophied chondrocytes, thus hastening the maturation process. Fig. 3 shows histological sections of growth-plate cartilage incubated in medium alone or containing increasing concentrations of T<sub>3</sub> (0.1-10 nM) for 3 d. Although the demarcation between the zone of proliferation (flattened chondrocytes) and zone of maturation (hypertrophied chondrocytes) is somewhat arbitrarily defined by the lower bracket on the line, it is obvious that the zone of maturation of growth-plate cartilage incubated with T<sub>3</sub> is increased in a dose-dependent manner. Thus, hematoxylin and eosin staining of the incubated growth-plate cartilage shows that T<sub>3</sub> increases the size of the zone of hypertrophied chondrocytes. Histochemical staining for alkaline phosphatase activity in growth-plate cartilage incubated in vitro in medium alone or containing 10 nM T<sub>3</sub> for 3 d gives even more striking evidence of the recruitment of new cells into the zone of maturation (Fig. 4). The demonstration of increased alkaline phosphatase activity in chondrocytes that were initially in the zone of proliferation correlates well with the increase width of the zone of maturation shown by hematoxylin and eosin staining.

T<sub>3</sub> stimulation of radiolabeled precursor incorporation into protein and proteoglycan in growth-plate cartilage. Growth-plate cartilage incubated in organ culture for 3 d in medium alone or containing 10 nM T<sub>3</sub> were pulsed from 68 to 72 h with L-[<sup>3</sup>H]leucine and

<sup>35</sup>SO<sub>4</sub> to obtain an estimate of protein and proteoglycan synthetic rates. Table III gives the results of the TCA precipitable counts in four such experiments. In each of the four individual experiments [<sup>3</sup>H]leucine incorporation was significantly increased by T<sub>3</sub>. <sup>35</sup>SO<sub>4</sub> incorporation was significantly increased by T<sub>3</sub> in two of the four experiments. When the data from all the experiments were expressed as percent increase above appropriate medium controls and pooled (17 cartilages incubated in medium alone and 27 in medium plus 10 nM T<sub>3</sub>), it was noted that T<sub>3</sub> stimulated [<sup>3</sup>H]leucine incorporation into protein 38.4±8.9% ( $P < 0.001$ ) and <sup>35</sup>SO<sub>4</sub> incorporation into proteoglycan 45.0±12.6% ( $P < 0.01$ ). Thus, T<sub>3</sub> stimulates about equally precursor incorporation into growth-plate cartilage protein and proteoglycan.

Lack of effect of T<sub>3</sub> on nongrowth-plate cartilage in vitro. In contrast to the increase in alkaline phosphatase activity consistently seen in growth-plate cartilage incubated with T<sub>3</sub>, no change in alkaline phosphatase activity was repeatedly observed in nongrowth-plate cartilage incubated with T<sub>3</sub> (Table IV). As expected, nongrowth-plate cartilage had alkaline phosphatase activity that was 20- to 40-fold lower than that of growth-plate cartilage (Table IV). Similarly, nongrowth-plate cartilage incubated in medium containing 10 nM T<sub>3</sub> had no increase in [<sup>3</sup>H]leucine incorporated into protein or <sup>35</sup>SO<sub>4</sub> incorporated into proteoglycan as compared with that incubated in medium alone (Table V). Thus, incubation of nongrowth-plate cartilage with T<sub>3</sub> for 3 d was without effect on either alkaline phosphatase activity or radiolabeled precursor incorporation into protein or proteoglycan.

Effect of serum growth factors on radiolabeled precursor incorporation into fetal pig growth plate and nongrowth-plate cartilage. Table V shows that the fetal pig cartilage system, which we have used, responds to serum growth factors. Both fetal and adult pig serum cause a dose-related increase in [<sup>3</sup>H]leucine incorporation into protein and <sup>35</sup>SO<sub>4</sub> incorporation into proteoglycan in fetal pig growth-plate cartilage. Nongrowth-plate cartilage is equally responsive to the serum growth factors.

## DISCUSSION

Many clinical and experimental observations have shown that thyroid hormones stimulate in vivo growth and maturation of the skeleton. Experimental or spontaneous hypothyroidism in man or other mammals is associated with decreased rates of linear growth and delayed ossification of the skeleton. Appropriate treatment with thyroid hormones restores these processes

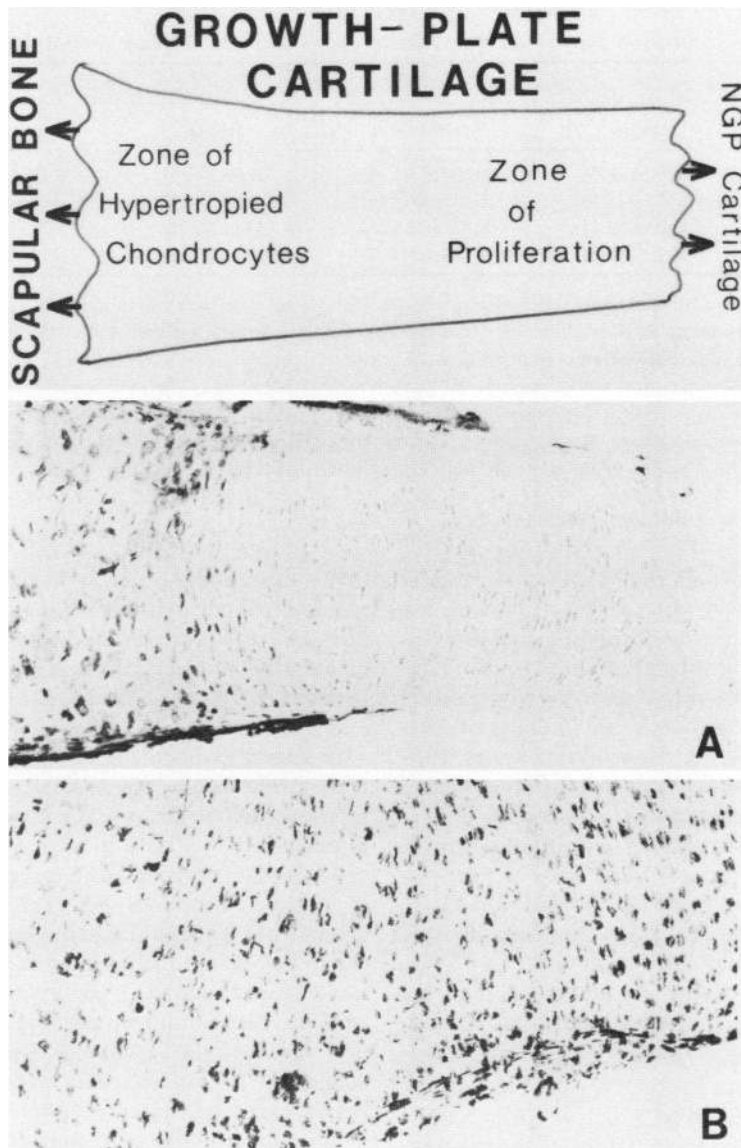


FIGURE 4 Photomicrograph ( $\times 80$ ) of growth-plate cartilage incubated in medium alone (A) and in medium containing  $T_3$  10 nM (B) for 3 d. The cartilages were frozen, sectioned, and histochemically stained for alkaline phosphatase activity (Methods). Comparable areas of the growth-plates were photographed. The upper panel gives the orientation in relationship to the scapular bone and the nongrowth-plate cartilage.

to normal (3). Treatment of chick embryos with thiourea results in decreased rate of maturation of chondroblasts into chondrocytes, reduction of chondrocyte hypertrophy, and defective deposition of acid mucopolysaccharide into cartilage matrix (14). Experimental hyperthyroidism in puppies causes increased cartilage cell proliferation but an even disproportionately greater acceleration of osseous maturation, so that an initial increased growth rate ends in subnormal statural

growth (1). Thyroxine administration to rats leads to premature acceleration of the differentiation of long bones (2). Human fetal thyrotoxicosis as well as thyrotoxicosis and thyroid overdosage in children are accompanied by advanced skeletal maturation (15, 16).

Whether thyroid hormones have direct effects on cartilage growth and maturation, or act through altering the in vivo secretion or action of other factors, has been the subject of considerable debate. Several

TABLE III  
Radiolabeled Precursor Incorporation into Growth-plate Scapular Cartilage after 3 d In Vitro

	$^3\text{H}$ Leucine		$^{35}\text{SO}_4$	
	Medium	Medium + $\text{T}_3$	Medium	Medium + $\text{T}_3$
Exp. 1	946±121 (4)	1,256±58 (6)*	106±24 (4)	141±9 (6)
Exp. 2	805±94 (3)	1,135±56 (8)*	318±35 (3)	448±49 (8)
Exp. 3	1,810±239 (4)	2,728±225 (8)*	844±33 (4)	1,403±155 (8)*
Exp. 4	2,791±126 (6)	3,609±289 (5)†	853±55 (6)	1,187±72 (5)†

Growth-plate cartilages from the scapulae of 100–150-g fetal pigs were incubated in medium or medium containing  $\text{T}_3$  10 nM for 3 d. Cartilages were pulsed during the final 4 h of the incubation with each of the radiolabeled precursors and acid insoluble counts determined (Methods). Values are expressed as mean counts per minute per milligram wet weight±SEM. The number of cartilages for each observation is recorded in parentheses. When the data from all the experiments are expressed as percent increase above medium alone,  $\text{T}_3$ -stimulated  $^3\text{H}$ leucine incorporation into protein was 38.4±8.9% and  $^{35}\text{SO}_4$  incorporation into proteoglycan was 45.0±12.6%.

\*  $P < 0.05$ .

†  $P < 0.01$  vs. incubation in medium alone.

investigators have suggested that thyroid hormones influence skeletal growth and maturation through stimulating production of somatomedins and perhaps other cartilage-regulating substances in vivo (6). Thyroid hormones have been reported to have no effect on mammalian cartilage in vitro (9). In contrast, several in vitro studies show that thyroid hormones stimulate proteoglycan synthesis and potentiate somatomedin action in embryonic chick cartilage (7, 8).

We have recently shown that  $\text{T}_3$  stimulates embryonic chick pelvic cartilage growth in vitro in a serum-free organ culture system (10). Over a 3-d period,  $\text{T}_3$  increased cartilage wet weight 100%, dry weight 77%, length 37%, and total soluble protein content 67% as compared with chick cartilage incubated in medium alone.  $\text{T}_3$  stimulated growth predominantly by cellular hypertrophy rather than cellular hyperplasia. In ad-

dition to its effect on growth,  $\text{T}_3$  stimulated embryonic chick cartilage maturation, as reflected by increased numbers of hypertrophied chondrocytes and elevation of alkaline phosphatase activity. Thus, in avian embryonic cartilage,  $\text{T}_3$  stimulates both in vitro growth and maturation.

In this study, we reasoned that growth-plate cartilage might be an appropriate mammalian cartilage model in which to look for in vitro effects of thyroid hormones. All of the in vivo studies have identified growth-plate cartilage as a target organ for the effects of thyroid hormones. Several in vitro studies have shown that rat costal cartilage and growth-plate cartilage have clear differences in their responses to hormone stimulation (17), their morphological characteristics in tissue culture, and their osteogenic potential (18). Growth-plate cartilage is a rapidly maturing cartilage and, in this regard, is more like embryonic chick pelvic cartilage than any other mammalian cartilage. The scapula of the fetal pig was chosen as the mammalian cartilage model because the flatness of the scapular wing and its clear demarcation of bone and cartilage that make isolation of the growth-plate technically easy. The relative thinness of the growth-plate allows for easy diffusion of nutrients during prolonged periods of organ culture.

Using our previous organ culture techniques and the fetal pig growth-plate cartilage, we have shown in this study that  $\text{T}_3$  has direct in vitro effects on mammalian growth-plate cartilage. Specifically,  $\text{T}_3$  promotes the conversion of immature dividing chondrocytes into mature hypertrophied chondrocytes. The pieces of evidence that support this conclusion are the  $\text{T}_3$ -induced in vitro increase in the zone of hypertrophy seen on hematoxylin and eosin stained sections (Fig. 3), recruitment of immature chondrocytes (slight alkaline

TABLE IV  
Effect of  $\text{T}_3$  on Alkaline Phosphatase Activity in the Growth-plate Scapular Cartilage and in the Nongrowth-plate Cartilage after 3 d of In Vitro Incubation

		Medium	Medium + $\text{T}_3$
Experiment 1	GP	62±10	181±12*
	NGP	0.94±0.21	0.96±0.3
Experiment 2	GP	37±2	164±7*
	NGP	1.1±0.1	1.4±0.7

Scapular cartilages from 100 to 150-g fetal pigs were separated into growth plate (GP)- and nongrowth plate (NGP)-cartilage and incubated with medium alone or with medium containing  $\text{T}_3$  10 nM for 3 d. Alkaline phosphatase activity is expressed as micromoles *p*-nitrophenol released per milligram protein per hour with values given as mean±SEM. Three cartilages were in each group.

\*  $P < 0.001$  vs. incubation in medium alone.

TABLE V  
*Effect of Fetal and Adult Pig Serum on Radiolabeled Precursor Incorporation after 3 d of In Vitro Incubation of Growth-plate and Nongrowth-plate Fetal Scapular Cartilage*

	Growth-plate cartilage		Nongrowth-plate cartilage	
	[ <sup>3</sup> H]Leucine	<sup>35</sup> SO <sub>4</sub>	[ <sup>3</sup> H]Leucine	<sup>35</sup> SO <sub>4</sub>
Control	1,136±126	190±23	1,075±79	182±16
T <sub>3</sub> 10 nM	1,638±96†	277±25*	1,297±93	216±24
5% fetal pig serum	1,368±61	244±18	1,393±114*	231±25
10% fetal pig serum	2,028±182†	342±32†	2,292±201†	255±10†
5% adult pig serum	1,698±102§	323±23†	2,011±104§	361±17§
10% adult pig serum	2,493±175§	463±46§	2,648±262§	466±40§

Scapular cartilage from 100-g fetal pigs of the same litter were isolated, pooled, and randomized into various treatment groups ( $n = 6$  for each group). Cartilages were incubated in medium alone (control), medium containing T<sub>3</sub> 10 nM, medium containing 5 and 10% fetal or adult pig serum for 3 d. Each cartilage was pulsed during the final 4 h of the incubation with each of the radiolabeled precursors and acid insoluble counts determined (Methods). Values are expressed as mean counts per minute per milligram wet weight±SEM.

\*  $P < 0.05$ .

†  $P < 0.01$ .

§  $P < 0.001$  vs. incubation medium alone.

phosphatase activity) to mature hypertrophied chondrocytes (heavy alkaline phosphatase activity) seen on histochemical staining (Fig. 4), and increase in total alkaline phosphatase activity of the growth-plate cartilage (Table I). The histological changes seen in the fetal pig growth-plate cartilage incubated in vitro with T<sub>3</sub> are similar to the increased width of the rat tibial epiphyseal plate seen following in vivo thyroid feeding (19). Since alkaline phosphatase activity is found in high concentrations in hypertrophied chondrocytes and surrounding matrix vesicles and is only minimally if at all present in immature cartilage, it is a reasonable biochemical marker of cartilage maturation (20, 21).

The effect of T<sub>3</sub> in stimulating fetal pig growth-plate cartilage maturation in vitro is concentration and time dependent. Additionally, it is relatively specific, since insulin, parathyroid hormone, cortisol, and fetal calf serum added to the organ culture system were without effect on the growth-plate cartilage alkaline phosphatase activity.

T<sub>3</sub> increased the growth-plate cartilage incorporation of radiolabeled leucine and sulfate into protein and proteoglycan, respectively. The percent increase in incorporation of both radiolabels was approximately the same, suggesting that proteoglycan synthesis might be preferentially stimulated. Additional studies are needed to validate this hypothesis. In spite of the increase in radiolabeled precursor incorporation observed during day 3 of the organ culture, no increase in wet weight or dry weight of the fetal pig growth-plate cartilage was observed even after 7 d of incubation. This is in contrast to the results of similar stud-

ies with embryonic chick pelvic cartilage. Perhaps the protein synthetic rate is so slow in fetal pig growth-plate cartilage that 7 d is insufficient to observe gross effects on growth.

Of considerable importance are our observations that T<sub>3</sub> had no effect on nongrowth-plate cartilage incubated under identical conditions as the growth-plate cartilage. The lack of change of alkaline phosphatase activity, histological appearance, and radiolabeled precursor incorporation into products confirms previous studies of Salmon and Daughaday (9) reporting the lack of effect of T<sub>3</sub> on radiolabeled sulfate incorporation into rat costal cartilage.

We can conclude from our studies that the maturation of mammalian growth-plate cartilage can be directly regulated by T<sub>3</sub>, and that a direct effect of thyroid hormones on cartilage is one mechanism by which thyroid hormones regulate skeletal growth and maturation in vivo.

#### ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mr. Jamie Wisser for his technical assistance.

This study was supported by grants AM 0132 and 1K08 AM 01021-01 from the National Institutes of Arthritis, Metabolism, Diabetes, and Kidney Diseases.

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