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Thyroid Hormone Acts Directly on Growth Plate Chondrocytes to Promote Hypertrophic Differentiation and Inhibit Clonal Expansion and Cell Proliferation*

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ABSTRACT

T_3 is an important regulator of endochondral bone formation in epiphyseal growth plates. Growth arrest in juvenile hypothyroidism results from disorganization of growth plate chondrocytes and their failure to undergo hypertrophic differentiation, but it is unclear how T_3 acts directly on chondrocytes or whether its actions involve other pathways. To address this issue, we investigated whether thyroid hormone receptors (TR) were localized to discrete regions of the unfused epiphysis by immunohistochemistry performed in tibial growth plates from 21-day-old rats and examined the effects of T_3 on growth plate chondrocytes in agarose suspension cultures *in vitro*. TRα1, -α2, and -β1 were expressed in reserve and proliferating zone chondrocytes, but not in hypertrophic cells, suggesting that progenitor cells and immature chondrocytes are the major T_3 target cells in the growth plate. Chondrocytes in suspension culture expressed TRα1, -α2, and -β1 messenger RNAs and matured by an ordered process of clonal expansion, colony formation, and terminal hypertrophic differentiation. Clonal expansion and proliferation of chondrocytes were inhibited by T_3, which also induced alkaline phosphatase activity, expression of collagen X messenger RNA, and secretion of an alcian blue-positive matrix as early as 7 days after hormone stimulation. Thus, T_3 inhibited chondrocyte clonal expansion and cell proliferation while simultaneously promoting hypertrophic chondrocyte differentiation. These data indicate that thyroid hormones concurrently and reciprocally regulate chondrocyte cell growth and differentiation in the endochondral growth plate. (*Endocrinology* **141:** 3887–3897, 2000)

GROWTH ARREST, delayed bone age, and epiphyseal dysgenesis occur in juvenile hypothyroidism and in some patients with resistance to thyroid hormone, whereas accelerated growth and skeletal maturation are evident in childhood thyrotoxicosis (1–5). Studies in rats suggest that hypothyroid growth arrest results from disorganization of epiphyseal growth plate chondrocytes and their relative failure to undergo hypertrophic differentiation (6, 7). Although T_3 is well established as a key regulator of endochondral ossification *in vivo*, it has remained controversial whether its actions in cartilage are primary thyroid hormone receptor (TR)-mediated responses or whether they are indirect and involve other factors such as the GH signaling pathway (6–9).

The actions of T_3 are mediated by nuclear receptors that act as hormone-inducible transcription factors. Two TR genes, α and β, are alternatively spliced to produce several protein variants (10). Recently, expression of TRα1, -α2, and -β1 messenger RNAs (mRNAs) together with α1 and β1 proteins has been demonstrated by RT-PCR and Western blotting in whole rat growth plate RNA and nuclear extracts (11). High affinity nuclear T_3-binding sites have been documented in human fetal epiphyseal chondrocytes (12) and TRα1, -α2, and -β1 proteins have been identified by immunohistochemistry in all types of chondrocytes at sites of endochondral ossification in pathological human osteophytes (13). Thus, although TRs are likely to be expressed in chondrocytes, the locations of individual T_3 target cells within specific zones of the epiphyseal growth plate are unknown.

Preliminary descriptions of the skeletal phenotypes in TR knockout mice (14–17) reinforce the view that the actions of T_3 in growth plate cartilage are probably direct and mediated by TRs. In TRα knockout mice (14) there is complete growth arrest, with disorganization of epiphyseal growth plate chondrocytes and delayed cartilage mineralization and bone formation. These abnormalities result from severe hypothyroidism due to impaired thyroid hormone production at weaning, as the skeletal phenotype can be rescued by T_4 replacement (14). This finding suggests that TRβ can compensate for the loss of TRα in the growth plate of euthyroid animals. Despite this, TRβ is not essential for bone development, as TRβ null mice (15, 16) show no evidence of growth retardation or developmental abnormalities in bone and cartilage, although feedback control of the pituitary-

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thyroid axis and development of the auditory pathways are impaired. Furthermore, double knockout of both TRα and -β genes fails to modify the skeletal phenotype seen in TRα null mice (17). In addition, conclusions from several *in vitro* studies have highlighted the role of T_3 in the columnar organization of proliferating chondrocytes and in the differentiation and expression of a mature hypertrophic chondrocyte phenotype (18–21). However, there is little or no information regarding the mechanisms of T_3 action in epiphyseal growth plate cartilage.

Endochondral bone formation is a highly complex process that requires coordinated maturation, proliferation, and differentiation of epiphyseal growth plate chondrocytes to produce hypertrophic cells. Mature hypertrophic chondrocytes secrete a collagen X-rich matrix and eventually undergo apoptosis to leave a cartilage scaffold that is mineralized before deposition of new bone (22). These processes have been widely studied by differing cell culture methods (18, 21, 23–30), but they are difficult to model *in vitro*. Accordingly, it is unclear how the onset and progression of chondrocyte differentiation programs are modulated by thyroid hormones or whether they are specifically dependent on expression of TRs within chondrocytes.

Thus, the aims of this study were 1) to determine the location of T_3 target cells in the tibial epiphyseal growth plate of immature rats undergoing active linear growth, and 2) to establish primary cultures of growth plate chondrocytes in which TR expression is maintained during chondrogenesis *in vitro*. Such a model will enable the molecular mechanisms of T_3 action during endochondral bone formation to be analyzed more faithfully.

Materials and Methods

Immunohistochemistry

TR expression was determined in growth plates of 21-day-old male Sprague Dawley rats. Tibiae were excised and fixed overnight at 4 C in 3.7% formalin before decalcification in 12.5% EDTA. Three-micron paraffin sections were cut onto 3-aminopropyltriethoxysilane-coated slides, deparaffinized, and rehydrated. TRα1, -α2, and -β1 expression was determined using specific polyclonal antibodies (Affinity BioReagents, Inc., Golden, CO). Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol, and nonspecific binding was blocked with 10% normal goat serum (Vector Laboratories, Inc., Peterborough, UK) in PBS, 1% BSA, 1% Triton X-100, and 0.5% casein. Sections were incubated for 1 h at room temperature with primary antibody, diluted 1:200 for TRα1, 1:100 for α2, and 1:100 for β1, in PBS, 0.1% BSA, and 1% normal goat serum. Control sections were treated with rabbit IgG (DAKO Corp., Cambridge, UK). Bound antibody was detected by a biotinylated goat antirabbit antibody (Vector Laboratories, Inc.) diluted 1:400 in PBS for TRα1 and 1:200 for α2 or β1 for 30 min at room temperature followed by exposure to preformed avidin-biotin peroxidase complex (Vector) for 30 min. Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride plus 0.2% H_2O_2, and sections were counterstained with 5% hematoxylin. The specificity of antibodies for each TR isoform was determined previously by Western blotting in primary cultured and immortalized rat osteoblastic cells (31, 32). TR expression was semiquantitatively determined by counting the percentage of positively stained chondrocyte nuclei in 50-μm fractions throughout at least 50 growth plate chondrocyte columns. The percentage of TR-positive cells in each fraction was related to the zone of the growth plate, assessed as a function of distance from the epiphysis (Fig. 2). This assessment was based on similar methods used to quantify cell proliferation in the rat epiphyseal growth plate (33).

Chondrocyte cultures

Chondrocytes were isolated from tibial growth plates of 21-day-old male rats (34). After collagenase digestion, cells were washed in Ham's F-12 serum-free medium and counted. Cell viability was greater than 95%, as determined by trypan blue exclusion. Cells were resuspended in Ham's F-12 supplemented with 10% newborn calf serum (NCS; Life Technologies, Inc., Paisley, UK), 2 mM L-glutamine, and antibiotics.

Monolayer cultures

Chondrocytes were seeded in 96-well plates at a density of 5000 cells/100 μl/well in either F-12 plus 1% NCS or F-12 plus 10% NCS. After 24 h, T_3 (1–1000 ng/ml) or vehicle was added, and cells were cultured for an additional 10 days. At various time points after the start of treatment, cell number and alkaline phosphatase (ALP) activity were determined. To determine cell numbers, chondrocytes were fixed in 10% trichloroacetic acid for 1 h at 4 C and processed according to the method of Skehan *et al.* (35). Plates were washed in water and air-dried before the addition of 0.4% sulforhodamine B (Sigma, Dorset, UK) in 0.1% acetic acid for 1 h at room temperature. Wells were then washed with 0.1% acetic acid to remove unbound dye before solubilization of contents in 100 μl 10 mM Tris. Sulforhodamine B fluorescence was determined at 540 nm on a microplate reader (Molecular Devices, Menlo Park, CA) within 30 min of solubilization. For quantitative analysis of ALP activity, cells in a 96-well plate were washed and extracted with 20 μl 0.1% Triton X-100 in PBS and 0.1% BSA. ALP activity was determined using 200 μl *p*-nitrophenyl phosphate (Sigma) as a substrate (36) and incubation for an additional 30 min at room temperature in the dark. ALP was measured at 405 nm and expressed as units of enzyme activity per cell number determined in parallel.

Agarose suspension cultures

Chondrocytes were cultured in agarose-stabilized suspension using a modified method of Benya and Shaffer (37). Sixty-millimeter petri dishes were coated with 1% standard low agarose and autoclaved at 112 C for 45 min (Bio-Rad Laboratories, Inc., Richmond, CA). Low melting point agarose (Bio-Rad Laboratories, Inc.) was autoclaved and mixed with F-12 to a concentration of 1%. Chondrocytes were mixed with F-12 and agarose to a concentration of 0.5% agarose containing 10,000 cells/ml. Three milliliters were added to precoated dishes (30,000 cells/dish), and the gel was allowed to solidify at 4 C before the addition of T_3 (0.1–1,000 ng/ml) in DMEM/F-12 plus 10% FBS (BioWhittaker, Inc., Wokingham, UK) to the solidified cell suspension. Cultures were screened for clusters of more than 3 cells, and none was seen at the beginning of any experiment reported. Suspension cultures were maintained for 21 days, and medium with or without T_3 was replaced after 7 and 14 days. Chondrocytes were fixed in formalin and stained with alcian blue (0.5% in 0.04 M HCl) to identify colonies producing glycosaminoglycans. Colonies were defined as a cluster of cells with a diameter greater than 50 μm. ALP activity was determined in parallel by conversion of 5-bromo-4-chloro-3-indolyl phosphate substrate to insoluble blue product before fixation (Sigma, Kit B5655) (38).

RT-PCR

RNA was isolated from chondrocyte colonies in parallel. Cells were lysed with 1 ml Trizol (Life Technologies, Inc.)/60-mm dish for 20 min, and RNA was prepared by standard methods. Collagen X and TRα1, -α2, and -β1 mRNA expression was determined by RT-PCR. In cases where primers did not bridge intron/exon boundaries because of homology between TR isoforms, extracted RNA was incubated with RQ1 DNase (Promega Corp., Southampton, UK) to digest and remove any contaminating genomic DNA. One microgram of total RNA was incubated in a 25-μl reaction containing 10 μg/ml random hexamers, 16 U/ml Moloney murine leukemia virus RT and 1 mmol deoxy-NTPs (Promega Corp.) at 42 C for 1 h. Hot start PCR of specific complementary DNAs (cDNAs) was performed in 50-μl reactions containing 1 μl cDNA, 2 ng/ml of the appropriate forward and reverse primers, 20 mU *Taq* polymerase in 1 $\times$ polymerase buffer [Roche Molecular Biochemicals (Lewes, UK) or Promega Corp.] and 250 μmol deoxy-NTPs. H_2O blank, genomic DNA, and RNA lacking the reverse transcriptase step were

included as controls for each amplification. PCR conditions were as follows: TRα1 and -α2, 35 cycles of 94 C, 30-sec denaturation; 58 C, 30-sec annealing; 72 C, 60-sec extension. For TRβ1, 40 identical cycles were used, but annealing was modified to 57.5 C, and for collagen X and β-actin, 40 cycles were used with an annealing step at 56 C. TRβ2 mRNA was detected in rat pituitary positive control tissue, but not in growth plate chondrocytes, even when nested amplification and various PCR conditions were tested. The following primers were used in PCR amplifications: rat TRα1 (GenBank M18028): forward primer, nucleotides 1365–1384; reverse, 1590–1571; rat TRα2 (GenBank X07409): forward, 1081–1100; reverse, 1468–1449; rat TRβ1 (GenBank J03819): forward, 374–395; reverse, 633–604; and rat TRβ2 (GenBank M25071 for β2-specific forward primers and J03819 for common β1 and β2 reverse primers): forward, 150–171; reverse, 633–604; nested forward, 405–426; nested reverse, 560–539. Amplification of rat type X collagen and rat β-actin cDNAs was achieved using published primer sequences (39, 40).

Statistical analysis

Data were compared by ANOVA, and comparisons between means were made using unpaired Student's *t* tests. Differences were considered significant at $P \leq 0.05$.

Results

Localization of TR proteins in the epiphyseal growth plate

The tibial growth plates of 21-day-old rats were organized into zones containing chondrocytes at distinct stages of maturation. Reserve zone undifferentiated progenitor cells were located toward the epiphysis, proliferating chondrocytes were organized into discrete columns, and enlarging and differentiating hypertrophic chondrocytes were positioned proximal to the resorption front and primary spongiosum, toward the metaphysis. These features are characteristic of mammalian long bones undergoing longitudinal growth by endochondral ossification in unfused epiphyses.

In the reserve and proliferative zones, progenitor cells and proliferating chondrocytes clearly exhibited specific nuclear staining for TRα1, -α2, and -β1 proteins. In these regions approximately 30% of chondrocytes expressed TRα1, 45% expressed TRα2, and 45–50% expressed TRβ1 (Figs. 1 and 2). The number of TRα1- or -α2-positive nuclei in columns of growth plate chondrocytes decreased with increasing distance from the epiphyseal border (Fig. 2), such that cells expressing these TR isoforms were completely absent from the hypertrophic zone. TRβ1-expressing chondrocytes also decreased with increasing distance from the epiphyseal border, but in contrast to the α isoforms, positive nuclei were observed in prehypertrophic chondrocytes, although they were completely absent from other areas of the hypertrophic zone. All TR isoforms were absent from the region of provisional cartilage calcification and at the resorption front (Figs. 1 and 2), indicating that TR expression diminishes with the progression of growth plate chondrocyte differentiation. In the metaphyseal primary spongiosum, the nuclei of invading osteoblasts surrounding cartilaginous trabeculae stained intensely for all TR variants. In addition, nuclei of osteoblasts and osteocytes in adjacent metaphyseal and epiphyseal sites of active bone turnover stained strongly for TRα1, -α2, and -β1 proteins, but osteoclasts in these regions were negative (data not shown). No differences in the distribution of the three TR isoforms occurred in any region of the growth plate or primary spongiosum.

Effects of T_3 on monolayer cultures of growth plate chondrocytes

Monolayer cultures of growth plate chondrocytes were examined for T_3 responsiveness at various plating densities and time points in the presence of two concentrations of serum-containing medium and increasing concentrations of T_3. Treatment of cells with T_3 had no significant effect on chondrocyte number at any T_3 concentration used (1–1000 ng/ml), compared with that in untreated controls, in cultures grown for 7 days in medium supplemented with either 1% or 10% serum (Fig. 3). Chondrocytes cultured in the presence of 1% serum proliferated at a much slower rate than those cultured in 10% serum, as indicated by the lower cell numbers. This finding suggests that neither cell density nor growth kinetics influenced chondrocyte responsiveness to T_3 in monolayer culture. In support of this hypothesis, addition of T_3 (100 ng/ml) for 7 days did not affect chondrocyte number when cultures were plated initially at either 5,000 or 10,000 cells/well in medium containing either 1% or 10% serum (data not shown). Chondrocytes cultured in the presence of 10% serum were also analyzed after 4 and 10 days within the same experiment, and T_3 had no significant effect on cell number at either time point. Replacement of the incubation medium containing T_3 every 2 days during the 10-day incubation period also failed to influence chondrocyte number. Furthermore, T_3 did not influence the proliferation of chondrocytes cultured in medium supplemented with 10% charcoal-stripped (41) FBS that lacks endogenous hormones (data not shown).

Chondrogenic maturation was assessed in parallel experiments using ALP activity as a marker of hypertrophic differentiation. T_3 (1–1000 ng/ml) had no significant effect on chondrocyte ALP activity in cultures containing either 1% or 10% serum (Fig. 3). Normalized basal ALP activity, however, was 4-fold higher in 7-day cultures supplemented with 1% serum than in those given 10% serum (Fig. 3). These data are in keeping with the observation that the cell proliferation rate was increased in the presence of 10% serum during the initial 7-day culture period compared with cultures supplemented with 1% serum. The findings indicate that chondrocyte maturation during this period of increased cell proliferation was reduced in cultures containing 10% serum. These data suggest the presence of additional factors in newborn calf serum that increase chondrocyte proliferation and inhibit hypertrophic differentiation in monolayer cultures.

Effects of T_3 on chondrocyte growth and maturation in agarose suspension culture

Although thyroid hormones are essential for linear growth and epiphyseal growth plate chondrocyte maturation *in vivo* (1–7, 14–17), primary monolayer cultures of growth plate chondrocytes were apparently unresponsive to T_3 *in vitro*. We considered, therefore, whether this lack of T_3 responsiveness may be due to the constraints of monolayer culture, in which the organized spatial relationship seen between maturing and differentiating chondrocytes in the normal growth plate would be lost. To test this hypothesis, we examined the T_3 responsiveness of chondrocytes in agarose-supported suspension cultures in which cell surface attach-

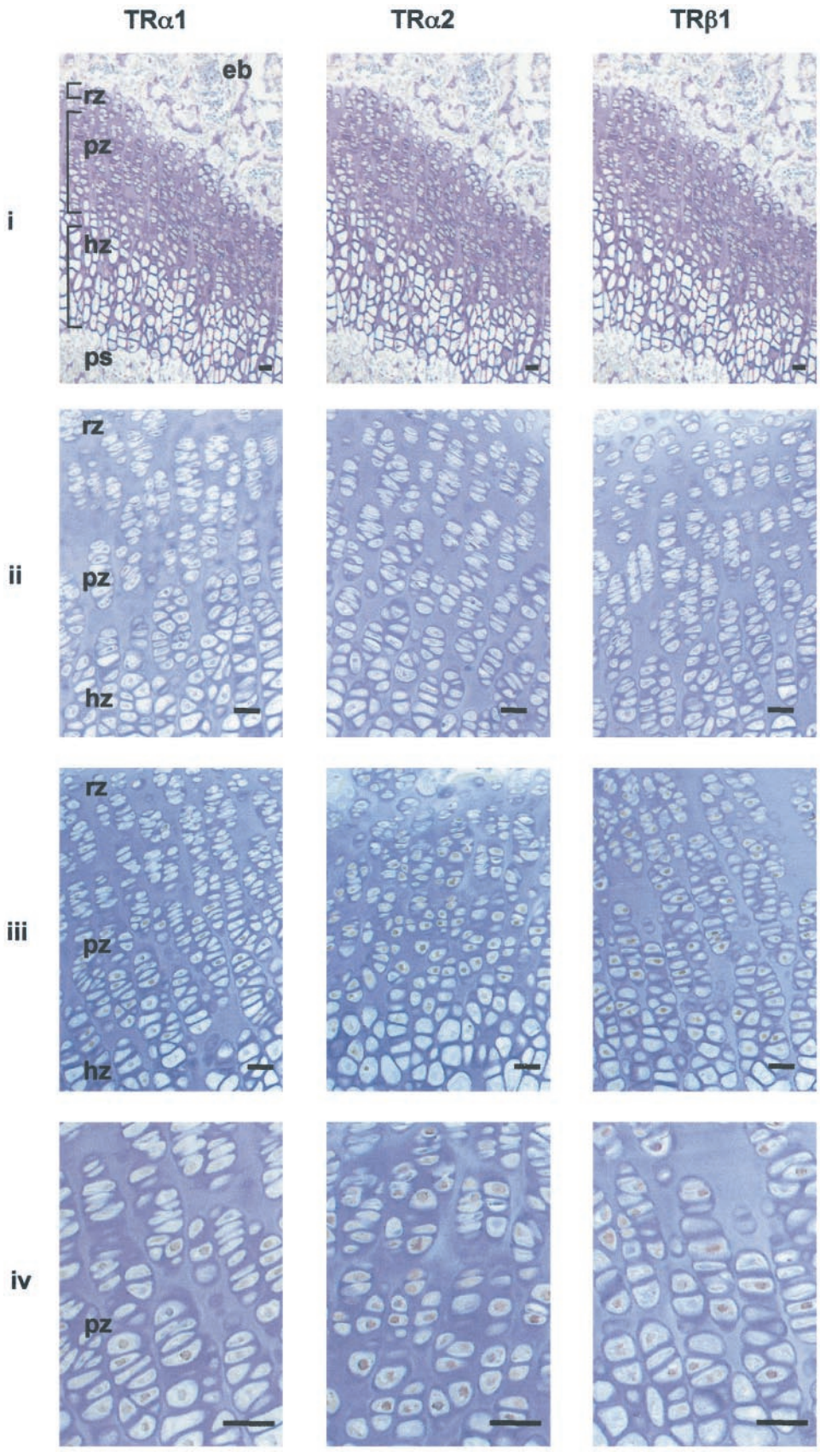

FIG. 1. Immunohistochemical localization of TRα1, -α2, and -β1 proteins in rat tibial growth plates, counterstained with 5% hematoxylin. i, Low power view of serial sections to show regions of epiphyseal bone (eb): the reserve zone (rz), the proliferative zone (pz), and the hypertrophic zone (hz) chondrocytes of the growth plate and primary spongiosum (ps) within the metaphysis. ii, Intermediate power view showing TRα1, -α2, and -β1 negative controls. iii, Intermediate power view showing TRα1, -α2, and -β1 positive nuclear staining in rz and pz chondrocytes and absence of staining in TR-negative hz cells. iv, High power view showing specific nuclear staining for TRα1, -α2, and -β1 in proliferating chondrocytes. *Scale bar*, 50 μm in each panel.

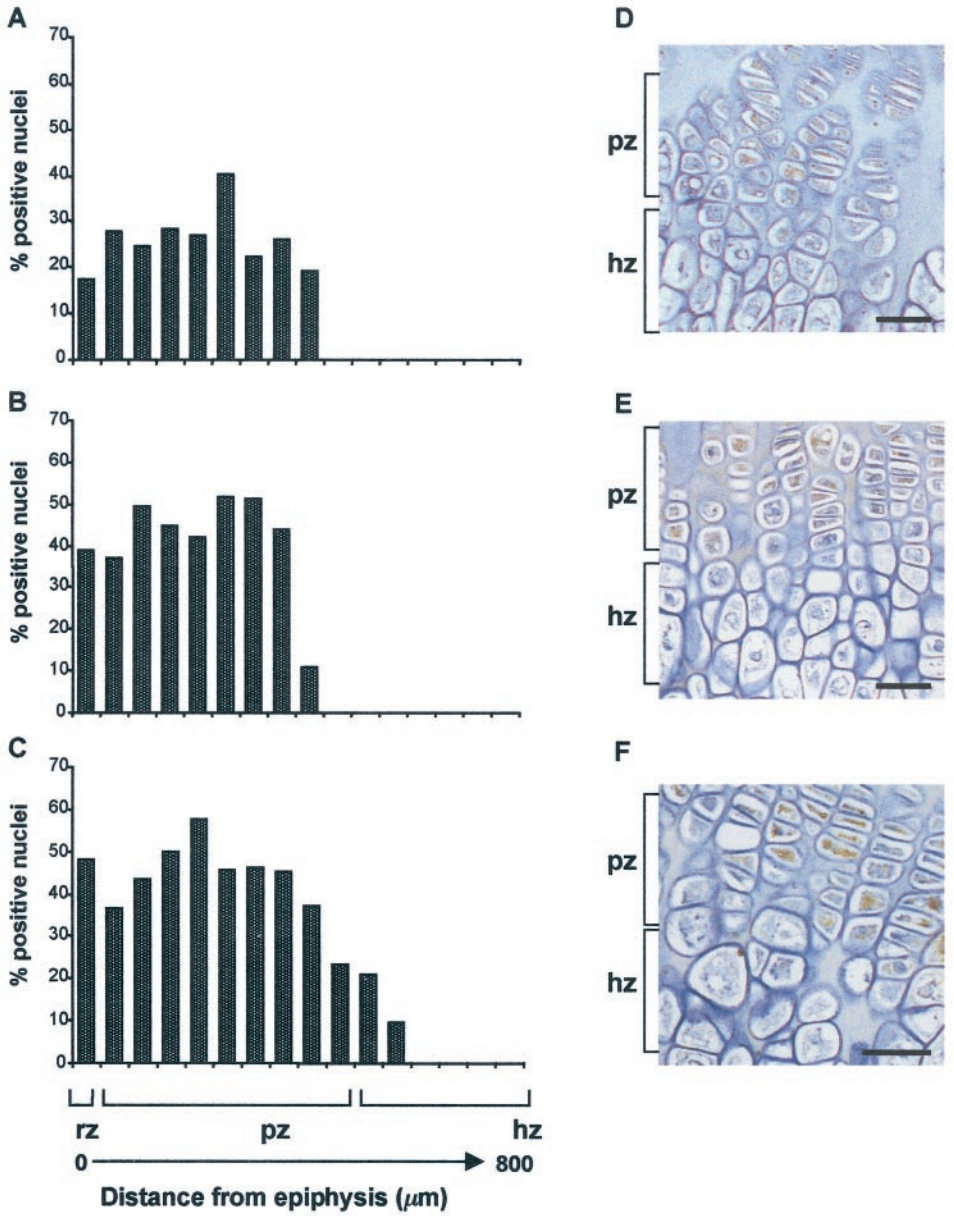

FIG. 2. Distribution of cells expressing TRα1 (A), TRα2 (B), and TRβ1 (C) in rat tibial growth plate chondrocyte columns with increasing distance from the epiphysis. The location of positively stained nuclei was determined in at least 50 chondrocyte columns. Data are expressed as the percentage of positive chondrocyte nuclei counted at 50-μm intervals from the epiphyseal to metaphyseal borders. Regions of the growth plate are defined as follows: reserve zone (rz) extends over the first 50 μm, proliferative zone (pz) extends over 50–500 μm, and hypertrophic zone (hz) extends over 500–800 μm from the epiphyseal border. Photomicrographs show high power immunohistochemical localization of TRα1 (D), TRα2 (E), and TRβ1 (F) in nuclei of pz chondrocytes and the absence of receptor expression in hz chondrocytes that retain unstained receptor negative nuclei and cytoplasm. *Scale bar*, 50 μm in each panel.

ments are prevented, the spherical chondrocyte phenotype is maintained, and colonies of differentiating chondrocytes form without spatial constraint (37).

We first demonstrated that chondrocytes in suspension culture had the potential to respond to T_3 by showing that mRNAs encoding T_3 receptors α1, α2, and β1 were expressed in both unstimulated and T_3-treated chondrocytes cultured for 7- and 21-day periods. Expression of TRβ2 mRNA, which is predominantly expressed in pituitary and hypothalamus (10), was not detected in any culture despite nested RT-PCR amplification (Fig. 4).

The suspension cultures of isolated chondrocytes initially proliferated and formed colonies by clonal expansion. Subsequently, cells differentiated to produce clusters of mature hypertrophic chondrocytes. These changes occurred reproducibly over a 21-day period to allow analysis of the process of chondrogenesis *in vitro* and investigation of the effects of T_3 on its progression. Thus, chondrocytes that were plated as single isolated cells formed small colonies containing only a few cells after 7 days in culture, but by 14 days, clonal expansion resulted in the formation of two distinct colony populations that displayed phenotypes similar to those reported previously by Ohlsson *et al.* (42). The predominant population consisted of colonies of small chondrocytes from which surrounding matrix was absent. The minor population consisted of colonies with fewer, enlarged chondrocytes that were surrounded by an acidic mucopolysaccharide-containing matrix, which stained positively with alcian blue (43). By 21 days, the majority of colonies displayed the latter phenotype.

Treatment of suspension cultures with T_3 (0.1–1000 ng/ml) resulted in a dose-dependent reduction in chondrocyte colony formation (Fig. 5), as determined after 21 days in culture. Colony formation reached minimums of 28.6 $\pm$ 5.4%

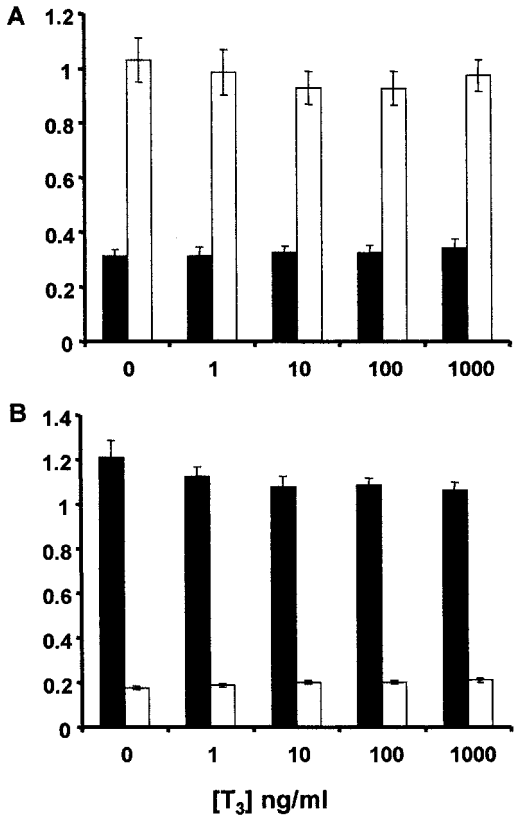

FIG. 3. Effect of T_3 (1–1000 ng/ml) on cell numbers (■) and ALP activity (□) in primary monolayer cultures of rat tibial growth plate chondrocytes. Cells were cultured for 7 days in medium supplemented with 1% (A) or 10% (B) NCS. Cell numbers are expressed as the OD_{540nm}, and ALP activity, determined as the OD_{405nm}, was normalized for differences in cell number. All values are expressed as the mean ± SEM (n = 6).

($P < 0.005$), 36.4 ± 8.4% ($P < 0.01$), and 27.8 ± 5.7% ($P < 0.005$) relative to control colony numbers after 7, 14, and 21 days of treatment, respectively, with 100 ng/ml T_3 (Table 1). The response to T_3 did not differ significantly among the time points, although variation in the degree of T_3-mediated reduction in chondrocyte colony formation was seen when cells were isolated from separate batches of rats (compare Table 1 and Fig. 5). This variation was probably due to inherent differences in the heterogeneity of isolated chondrocytes between animals that resulted in differing proportions of clonogenic cells between separate experiments. Nevertheless, the inhibitory effect of T_3 was consistent, and after 21 days, T_3 treatment resulted in an increased percentage of colonies containing chondrocytes with an enlarged cytoplasmic volume that were surrounded by an alcian blue-positive matrix, but T_3 also reduced the total colony size (Table 2). The smaller T_3-treated colonies resulted from the presence of fewer cells per colony compared with controls. Taken together with the finding of reduced colony formation in T_3-treated cultures, this suggests that T_3 treatment results in inhibition of chondrocyte clonal expansion and cell proliferation. These effects were modest relative to the apparent effects of serum on cells grown in monolayers, when chondrocyte responses were compared between cultures containing 1% or 10% added serum (Fig. 3). The clear inhibition of clonal expansion and proliferation by T_3 in suspension cultures (Tables 1–3), however, was in contrast to the effect of serum (which promoted chondrocyte proliferation) and oc-

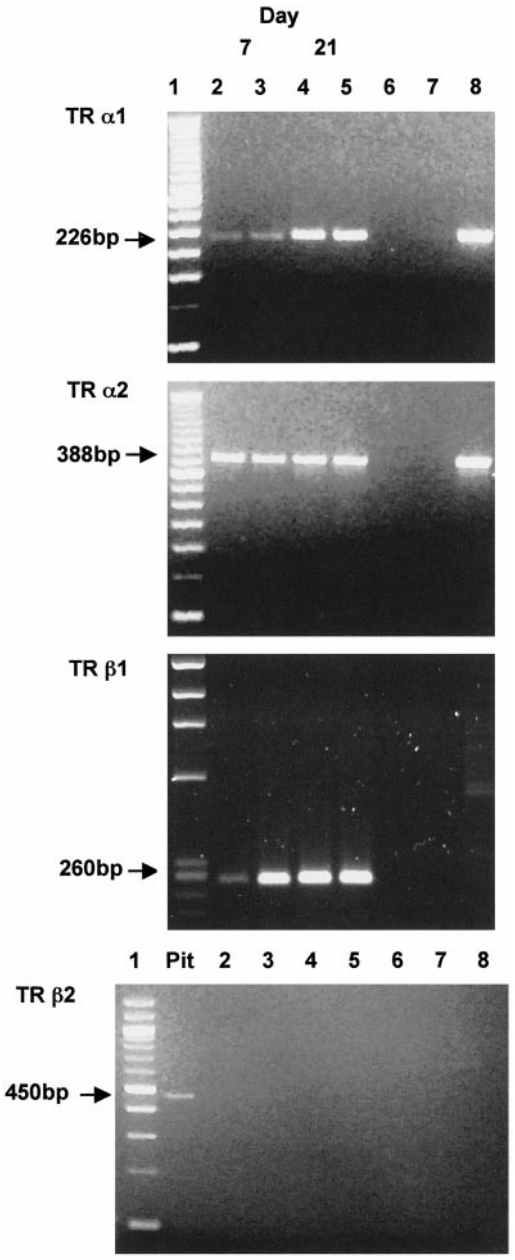

FIG. 4. Expression of TRα1, -α2, and -β1 mRNAs, analyzed by RT-PCR, in primary suspension cultures of unstimulated control (lanes 2 and 4) or T_3-treated (lanes 3 and 5) growth plate chondrocytes cultured for 7 (lanes 2 and 3) and 21 (lanes 4 and 5) days. Lane 1 contains DNA size marker, and lanes 6–8 contain H_2O blank, input RNA lacking RT, and genomic DNA controls, respectively. All products were absent from negative control lanes. TRα1 and -α2 products were present in genomic DNA reactions, because amplification primers were located within a single isoform-specific exon in each case. In contrast, TRβ1 and -β2 primers were in separate exons, and no specific product was detectable in the genomic DNA lanes. TRβ2 product was only present in RNA extracted from rat pituitary (Pit). *Arrows* indicate the sizes of the PCR products. Expression of β-actin mRNA, as a control for RNA loading and integrity in these samples, is shown in Fig. 6.

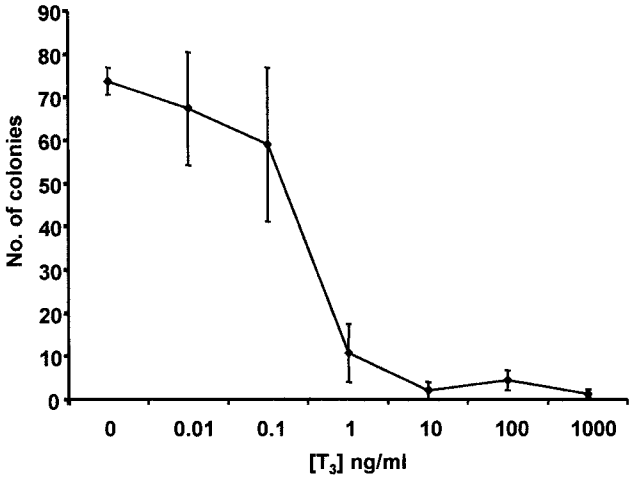

FIG. 5. Effect of 21 days of T_3 treatment (0.1–1000 ng/ml) on rat tibial growth plate chondrocyte colony formation in suspension culture. All colonies 50 μm or larger were counted and included populations containing both chondrocytes and surrounding matrix and colonies that lacked surrounding matrix (see text). Values are expressed as the mean colony number ± SEM (n = 12).

TABLE 1. Effects of T_3 (100 ng/ml) on absolute numbers of colonies formed in suspension cultures of rat tibial growth plate chondrocytes seeded at identical initial plating densities

Days in culture	Total colonies >50 μm		
	Control	T_3	% of control
7	22.4 ± 2.2	6.4 ± 1.2[a]	28.6 ± 5.4
14	68.8 ± 6.1	25.0 ± 5.8[b]	36.3 ± 8.4
21	120.6 ± 6.6	33.5 ± 6.8[a]	27.8 ± 5.7

Values are the mean ± SEM (n = 12).
[a] $P < 0.005$, T_3 treated *vs.* control.
[b] $P < 0.01$, T_3 treated *vs.* control.

curred despite the fact that suspension cultures were maintained in the presence of 10% serum. These findings indicate that T_3 exerts a potent direct action on chondrocyte differentiation over and above any effect of serum alone, but emphasize that the physiological actions of T_3 should be considered in the context of other circulating physiological factors.

To investigate the effects of T_3 further, we examined the morphological and phenotypic characteristics of chondrocytes in suspension culture (Fig. 6 and Table 3). In addition to the diameter and number of cells within individual colonies, chondrocytes were examined for hypertrophic cell differentiation. Thus, after 21 days of culture, chondrocytes expressed clear evidence of hypertrophy, as characterized by the production of an alcian blue-positive acid mucopolysaccharide-rich matrix, the presence of ALP enzyme activity, and the expression of the hypertrophic chondrocyte-specific marker, collagen X (43–45). These features were present in both untreated control and T_3-treated cultures on day 21 (Fig. 6) and are consistent with previous reports that chondrocytes in suspension culture are able to undergo terminal differentiation to the hypertrophic stage (44, 45). In contrast, small colonies of enlarged cells or isolated large chondrocytes that possessed ALP activity and secreted an alcian blue-positive matrix were present in T_3-treated cultures as early as 7 days,

TABLE 2. Effects of 21 days T_3 treatment on the maturation and colony size of rat tibial growth plate chondrocytes in suspension culture

T_3 (ng/ml)	% Total colonies/dish	
	With enlarged cells + surrounding matrix	>100-μm diameter
0	75.1 ± 4.1	31.1 ± 6.7
1	87.2 ± 0.2[a]	15.2 ± 7.1[b]
10	92.3 ± 6.3[b]	17.3 ± 2.7[a]
100	92.6 ± 6.4[b]	15.5 ± 4.2[a]

Values are the mean ± SEM (n = 12).
[a] $P < 0.01$, T_3 treated *vs.* control.
[b] $P < 0.05$, T_3 treated *vs.* control.

TABLE 3. Effects of T_3 (100 ng/ml) on the number of single chondrocytes and chondrocyte colonies that stain positively for ALP activity and matrix after 7 days in suspension culture

T_3 (ng/ml)	No. of positive cells/colonies	
	ALP	Matrix
0	40.6 ± 5.4	58.2 ± 3.7
100	129.3 ± 6.1[a]	135.2 ± 6.3[a]

Values are the mean ± SEM (n = 6).
[a] $P < 0.005$, T_3 treated *vs.* control.

but were largely absent from control cultures (Fig. 6 and Table 3). Furthermore, collagen X mRNA expression was readily detectable by RT-PCR in T_3-treated chondrocytes after 7 days, but was only present in trace amounts in control untreated cells (Fig. 6). Thus, the presence of mature differentiated hypertrophic chondrocytes occurred much earlier in T_3-treated cultures, and the emergence of the hypertrophic phenotype was concomitant with the inhibition of chondrocyte clonal expansion and cell proliferation by T_3.

Discussion

Localization of T_3 receptor α1, α2, and β1 proteins to the reserve and proliferating zones of the unfused epiphysis is a novel finding that is consistent with data from TR knockout mice indicating that both TRα and -β genes are functionally active in the growth plate (14–17) and with a recent study showing the expression of TR isoforms in whole growth plate RNA and protein extracts (11). Our data suggest that progenitor cells and immature proliferating chondrocytes are the primary target cells for T_3 within cartilage, but that differentiated matrix secreting hypertrophic chondrocytes lose the ability to express TRs and are unresponsive to T_3. This view is further supported by the many reports in different systems, using various anatomical sources of chondrocytes from several species, which indicate that T_3 regulates chondrocyte proliferation and the organization of proliferating chondrocyte columns and is required for terminal differentiation of hypertrophic chondrocytes (12, 18, 21, 23–30). The expression of TRs in osteoblasts that invade the growth plate primary spongiosum and lay down new bone also suggests that T_3 acts directly on osteoblasts and may integrate the activities of growth plate chondrocytes and osteoblasts during endochondral ossification. The consistent finding of TR expression in osteoblasts located at adjacent metaphyseal and epiphyseal sites of bone turnover and the previous doc-

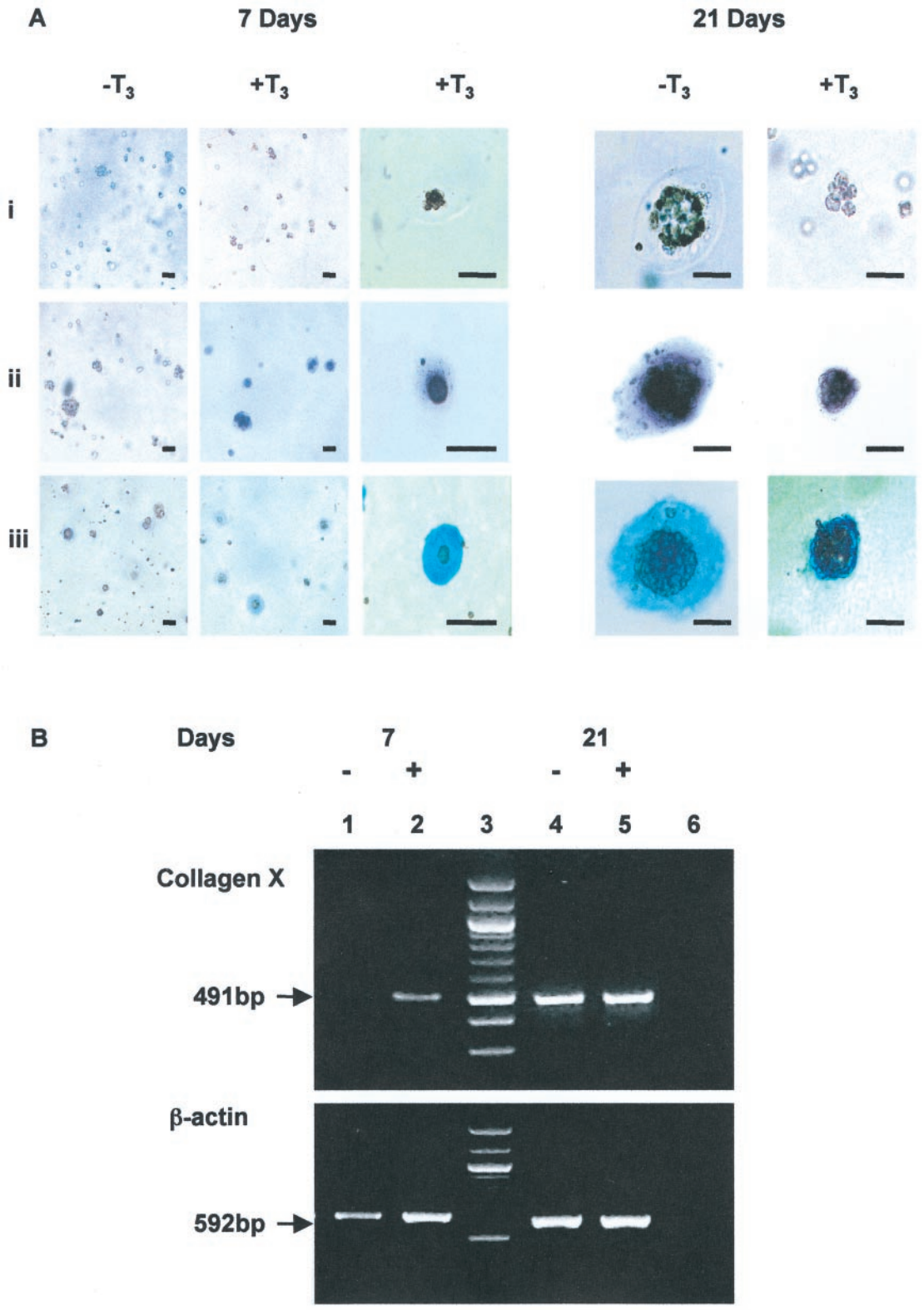

FIG. 6. Growth and maturation of control and T_3-treated (100 ng/ml) growth plate chondrocyte colonies after 7 and 21 days. A, Colonies are shown either unstained (i), stained *purple* for ALP activity (ii), or stained with alcian *blue* (iii). *Scale bar*, 50 μm in each panel (low and higher power images are shown at 7 days for T_3-treated chondrocytes). B, Expression of collagen X and β-actin mRNAs, determined by RT-PCR, after 7 (lanes 1 and 2) and 21 (lanes 4 and 5) days in culture in the absence (−) and presence (+) of T_3. Lane 3 contains DNA size markers, and lane 6 is a negative control H_2O blank. *Arrows* indicate the sizes of the PCR products. RT-PCR data were derived from RNA pooled from triplicate cultures and were reproducible in two independent experiments and in two independent RT duplicates for each experiment.

umentation of TR expression at pathological sites of endochondral ossification (13) support this conclusion.

To investigate how T_3 promotes chondrocyte differentiation, cell culture methods that model early phases of chondrogenesis and hypertrophic cell differentiation need to be established, and chondrocytes in such a system must retain the capacity to respond to T_3 by continuing to express the same repertoire of TRs as that seen in the intact growth plate. We initially studied primary monolayer cultures of isolated rat tibial growth plate chondrocytes, which comprise a heterogeneous population of resting, proliferating, and early hypertrophic cells. In this system, T_3 did not influence cell proliferation or ALP activity relative to that in control cultures, indicating that hypertrophic differentiation was not stimulated by T_3. The lack of T_3 effect was independent of cell plating density and proliferation rate, was not due to inactivation of T_3 during the culture period, and was not influenced by the presence of endogenous hormones in serum supplements to growth medium. It is likely, therefore, that the well established dedifferentiation and morphological change induced in chondrocytes dividing on a plastic surface in monolayer culture (21, 37) contributed to the loss of T_3 sensitivity. This problem can be exacerbated by plating cells at low density or allowing cells to undergo several doublings. However, the use of several plating densities and study of primary first passage cells failed to overcome the lack of T_3 response, which is thus likely to be an inherent problem with monolayer cultures. Indeed, studies of T_3 responsiveness of chondrocytes from several sources cultured in monolayers have yielded variable results. Studies have shown dose-dependent stimulatory effects on ALP activity and inhibitory effects on cell proliferation (27), no effect on proliferation and a weak, biphasic stimulation of ALP (12), or a 2- to 3-fold stimulation of ALP activity only (30). Furthermore, studies of chick growth plate chondrocytes in primary monolayer culture (23) demonstrated both a stimulatory and a biphasic, stimulatory and inhibitory, effect on ALP activity that was dependent on the confluence of cells in culture and on the absence or presence of β-glycerophosphate or serum in the culture medium. These studies also indicated that T_3 induces terminal differentiation of prehypertrophic chondrocytes (23). Thus, it appears likely that differing responses in these studies and in ours may result from the differing cell sources, species differences, and experimental methods that were employed. However, responses of growth plate chondrocytes in monolayer culture to other hormones, such as 1,25-dihydroxyvitamin D_3, are variable also. For example, ALP activity has been shown to be stimulated in proliferating growth plate chondrocytes, but not resting zone cells (46), or inhibited in whole growth plate chondrocyte cultures (47) by 1,25-dihydroxyvitamin D_3. Thus, we conclude that monolayer culture of growth plate chondrocytes is a poor system in which to study the effects of T_3 on chondrogenesis and should not be employed.

We, therefore, studied chondrocytes in agarose-supported suspension cultures that are capable of supporting clonal expansion, proliferation, and terminal differentiation of cells under appropriate conditions (25, 37, 44, 45, 48). Chondrocytes cultured in suspension over a 21-day period expressed TRα1, -α2, and -β1 mRNAs and are, therefore, likely to possess the capability to respond to T_3. Although TR mRNAs were expressed in chondrocytes cultured in suspension after 21 days, at which time most cells displayed a hypertrophic phenotype, TR proteins were absent from hypertrophic chondrocytes *in situ*. This discrepancy may reflect the high sensitivity of RT-PCR for detection of TR mRNAs relative to immunohistochemical methods for protein detection or may be due to the poor correlation between TR mRNA and protein expression (10). Importantly, it is probable that the inherent heterogeneity of cells in suspension culture results in the presence of a population of undifferentiated TR-expressing chondrocytes in 21-day cultures that results in the persistence of TR mRNA expression in these cultures, as detected by RT-PCR.

In our studies untreated chondrocytes in suspension matured via clonal expansion of single cells to form colonies that proliferated and ultimately underwent terminal differentiation. According to Corvol *et al.* (49), progenitor cells for colony formation in suspension culture are derived from chondrocytes of different maturation status. Thus, cells that originate from the growth plate at later stages of maturation undergo fewer cell divisions before terminal differentiation and form smaller colonies that display a mature differentiated phenotype over a shorter period of time. These features enabled us to study the T_3 responsiveness of chondrocytes at various stages of maturation and also provide an explanation for the two populations of colonies formed in unstimulated cultures after 14 days. The reduction in colony formation and size (due to reduced cell numbers) after T_3 stimulation indicates that T_3 inhibits clonal expansion and proliferation of immature chondrocytes at all time points during their maturation and confirms that reserve zone progenitor cells and proliferating chondrocytes, which express TR proteins *in vivo*, are directly responsive to T_3. Furthermore, T_3-induced stimulation of increased numbers of cells displaying phenotypic features of hypertrophy correlated with the inhibitory effects of T_3 on cell proliferation (Tables 1 and 3, effects of T_3 after 7 days). Emergence of hypertrophic chondrocytes occurred earlier in the presence of T_3 and included small colonies of enlarged cells or isolated single differentiated chondrocytes. These data indicate that T_3 can, in certain instances, promote hypertrophic differentiation of single immature chondrocytes directly without prior clonal expansion and proliferation, as single cells, by definition, have not undergone cell division.

Direct stimulation of chondrocyte hypertrophy without prior proliferation has been documented previously using embryonic chicken sternal chondrocytes (25), but it has been considered that proliferation is a prerequisite step that precedes terminal hypertrophic chondrocyte differentiation in the postnatal epiphyseal growth plate (21, 25, 45). Our data provide evidence that T_3 can inhibit clonal expansion and promote the hypertrophic differentiation of growth plate chondrocytes concurrently. This suggests that thyroid hormones may, under specific physiological or pathological conditions, recruit growth plate chondrocytes more rapidly into the hypertrophic differentiation pathway to enhance endochondral bone formation and linear growth. This hypothesis may explain in part the clinical observations of accelerated skeletal maturation in childhood thyrotoxicosis (1, 4) and the

promotion of a rapid initial period of catch-up growth in hypothyroid children treated with T_4 (5). The localization of TRs in reserve zone progenitor cells and proliferating chondrocytes is consistent with this view, although sustained growth during thyroid hormone therapy in childhood hypothyroidism probably requires both proliferation and hypertrophic maturation of chondrocytes and could result from differentiation of chondrocytes that had previously undergone proliferation.

In our agarose-supported suspension culture system, T_3 stimulated hypertrophic differentiation, but inhibited chondrocyte proliferation. In contrast, it has been suggested that thyroid hormones may stimulate growth plate chondrocyte proliferation in Snell dwarf mice *in vivo* (50), based on measurement of T_4 and GH-stimulated increases in [^{3}H]thymidine uptake by growth plate chondrocytes. Nevertheless, these researchers have also shown that T_4, unlike GH, does not influence the height of proliferating columns of growth plate chondrocytes (51), suggesting that increased chondrocyte [^{3}H]thymidine labeling indexes in response to T_4 may result from changes in the duration of the DNA synthesis phase of the cell cycle rather than from effects on cell proliferation directly. These data in GH-deficient Snell mice (50, 51) indicate, therefore, that the actions of thyroid hormones on growth plate chondrocytes *in vivo* are probably influenced by additional circulating factors. Other studies have shown that the growth plate actions of T_3 are also influenced by GH (6–9). Thus, apparently discrepant data derived from our cell culture studies and from animal studies *in vivo* suggest that our suspension culture system may not completely replicate *in vivo* responses of growth plate chondrocytes to thyroid hormones, presumably because of the complex growth plate architecture and cellular interactions that occur *in vivo*. Nevertheless, our suspension culture system provides a good model of T_3-regulated chondrogenesis *in vitro* and provides a novel method that will facilitate investigation of the molecular events that mediate direct T_3 induction of hypertrophic chondrocyte differentiation in the absence of the modifying effects of other signaling pathways.

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