Journal of Orthopaedic Research
15:371-380 The Journal of Bone and Joint Surgery, Inc.

Recombinant Bone Morphogenetic Protein (BMP)-2 Regulates Costochondral Growth Plate Chondrocytes and Induces Expression of BMP-2 and BMP-4 in a Cell Maturation-Dependent Manner

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Summary: This study examined the effect of recombinant human bone morphogenetic protein-2 on several parameters of growth, differentiation, and matrix synthesis and on the endogenous production of mRNA of bone morphogenetic proteins 2 and 4 by growth plate chondrocytes in culture. Chondrocytes from resting and growth zones were obtained from rat costochondral cartilage and cultured for 24 or 48 hours in medium containing 0.05-100 ng/ml recombinant human bone morphogenetic protein-2 and 10% fetal bovine serum. Incorporation of [^{3}H]thymidine, cell number, alkaline phosphatase specific activity, incorporation of [^{3}H]proline into collagenase-digestible protein and noncollagenase-digestible protein, and incorporation of [^{35}S]sulfate were assayed as indicators of cell proliferation, differentiation, and extracellular matrix synthesis. mRNA levels for bone morphogenetic proteins 2 and 4 were determined by Northern blot analysis. Recombinant human bone morphogenetic protein-2 increased the incorporation of [^{3}H]thymidine by quiescent resting-zone and growth-zone cells in a similar manner, whereas it had a differential effect on nonquiescent cultures. At 24 and 48 hours, 12.5-100 ng/ml recombinant human bone morphogenetic protein-2 caused a dose-dependent increase in cell number and DNA synthesis in resting-zone chondrocytes. No effect was seen in growth-zone cells. Recombinant human bone morphogenetic protein-2 stimulated alkaline phosphatase specific activity in resting-zone chondrocytes in a bimodal manner, causing significant increases between 0.2 and 0.8 ng/ml and again between 25 and 100 ng/ml. In contrast, alkaline phosphatase specific activity in growth-zone chondrocytes was significantly increased only between 12.5 and 100 ng/ml. Recombinant human bone morphogenetic protein-2 increased the production of both collagenase-digestible protein and noncollagenase-digestible protein by resting-zone and growth-zone cells, but incorporation of [^{35}S]sulfate was unaffected. Administration of recombinant human bone morphogenetic protein-2 also increased incorporation of [^{3}H]uridine in both resting-zone and growth-zone chondrocytes; these cells produced mRNA for bone morphogenetic proteins 2 and 4. Bone morphogenetic protein-2 mRNA levels in both resting-zone and growth-zone chondrocytes increased in the presence of recombinant human bone morphogenetic protein-2; however, bone morphogenetic protein-4 mRNA levels in growth-zone cells decreased under its influence, and those in resting-zone cells were upregulated only with a dose of 10 ng/ml. This indicates that recombinant human bone morphogenetic protein-2 regulates chondrocyte proliferation, differentiation, and matrix production, and the effects are dependent on the stage of cell maturation. Resting-zone chondrocytes were more sensitive, suggesting that they are targeted by bone morphogenetic protein-2 and that this growth factor may have autocrine effects on these cells.

Received August 6, 1996; accepted March 3, 1997.

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Dr. Erickson is an officer in the United States Air Force; the opinions expressed in this paper are not necessarily those of the United States Air Force. The work presented is in partial fulfillment of the requirements of an M.S. degree for Dr. Erickson.

It has been known for a long time that bone has a remarkable capacity for regeneration and repair in response to injury. Through a combination of endochondral and direct ossification, fractures of long bones heal to effectively 100% of their natural form and strength. However, the underlying mechanisms controlling bone regeneration are largely unknown and are the subject of intense study.

In the 1960s, Marshall Urist discovered that demineralized bone could stimulate bone formation in soft tissue and named the putative active agent "bone morphogenetic protein" (BMP) (49). When implanted into heterotopic host sites, BMPs induce endochondral ossification that terminates in the formation of a complete ossicle containing lamellar bone and bone marrow (42). When implanted orthotopically (18,23, 26-28,35,47), BMPs stimulate both endochondral and direct bone formation, indicating that this family of factors can induce differentiation of mesenchymal cells into the endochondral pathway and regulate osteogenic cells that are already committed to this pathway. At present, a variety of BMPs have been described, all of which are members of the transforming growth factor-β (TGF-β) superfamily (9,11,14,33,41,53,56). Although it is believed that individual BMPs can induce bone formation when implanted *in vivo*, other BMPs may also be involved and act on the same or different target cell populations during the various phases of chondrogenesis and osteogenesis in endochondral ossification.

Several studies have shown that purified and recombinant BMPs can induce mesenchymal cells to express a chondrogenic phenotype *in vitro* (6,11,39,42, 52,54). Treatment of primary avian limb bud cells (11) and immortalized mammalian limb bud cell lines (39) with BMP-2 and BMP-4 induces the cells to acquire markers characteristic of chondrocytes. Although limb bud cells may already be committed to an osteochondral phenotype, other studies have shown that treatment of multipotential cells with BMP-2 or BMP-4 results in the expression of various extracellular markers characteristic of cartilage cells (52,54).

Articular chondrocytes, which are fully committed cartilage cells, also respond to BMPs. BMP-4 has been shown to increase the expression of type-II collagen and aggrecan mRNAs and the incorporation of [^{35}S]sulfate in articular chondrocytes (30). In other studies, treatment of serum-free cultures of bovine articular cartilage with BMP-2B was found to increase proteoglycan synthesis and decrease proteoglycan degradation (29), and treatment of dedifferentiated lapine articular chondrocytes with BMP-3 promoted reexpression of the cartilage phenotype in serum-free medium (22).

These studies demonstrated that several of the BMPs can regulate the gene expression and matrix synthesis processes in articular chondrocytes, but the effects are similar to those observed with serum alone. Interestingly, Chen et al. (12) showed that BMP-7 could not stimulate bovine articular chondrocytes to undergo hypertrophy — a transition characteristic of chondrocytes in the endochondral lineage pathway — whereas chick sternal or articular chondrocytes cultured under the same conditions were able to do so (13). These investigators concluded that species differences could account for this discrepancy and that primary mammalian articular chondrocytes are fully committed to their hyaline cartilage phenotype. Thus, the response of articular chondrocytes to specific BMPs may not reflect how chondrocytes in other developmental lineages would respond.

At present, relatively little is known concerning the response of cells in the endochondral pathway to BMPs. Recent evidence from a study on the developing limb bud indicates a differential distribution of BMP-2 and BMP-13 transcripts (31) and suggests that embryonic cartilage cells can produce BMPs in a manner dependent on the degree of cell maturation. This hypothesis has been supported by the work of Chang et al. (10) showing that two new members of the TGF-β superfamily, cartilage-derived morphogenetic proteins 1 and 2, are also differentially expressed in the cartilage of embryonic bone. Less is known concerning chondrocytes from growth plates in postfetal development. The purpose of the present study was to determine whether mammalian chondrocytes in the endochondral lineage not only produce BMPs in a cell maturation-dependent manner but also respond to them differentially. To accomplish this goal, we used a model of cartilage cell culture in which chondrocytes are derived from two distinct zones of the rat costochondral junction, enabling us to compare cells at two maturation states in the endochondral lineage. Chondrocytes from the resting zone (reserve zone) and growth zone (prehypertrophic and upper hypertrophic zones) were exposed to recombinant human BMP-2 (rhBMP-2), and changes in cell proliferation, differentiation, and matrix protein synthesis were examined. Moreover, the ability of these chondrocytes to produce mRNA for BMP-2 and BMP-4 was also evaluated.

MATERIALS AND METHODS

Chondrocyte Cultures

The culture system used in the present study was previously described in detail (3) and is briefly outlined here. Chondrocytes were derived from the resting zone and growth zone of the costochondral cartilage of adult (125 g) male Sprague-Dawley rats. Intervening tissue was discarded to limit cross-contamination of the two populations of cells. Perichondrial tissue and calcified cartilage were discarded to limit contamination by fibroblasts and osteoblasts. After dissection, the cartilage was sliced and then incubated overnight in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, U.S.A.) at 37°C in a 5% CO_2 atmosphere. Chondrocytes were released from the tissue by sequential incubations in 1% trypsin (Gibco) for 1 hour and in 0.02% collagenase (type II; Worthington Biochemical, Freehold, NJ, U.S.A.) and Hanks' balanced salt solution (Gibco) for 3 hours. The chondrocytes were plated at an initial density of 10,000 cells/cm^2 for resting-zone cells and 25,000 cells/cm^2 for growth-zone cells. The cells were incubated in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 50 μg/ml ascorbic acid at 37°C in an atmosphere of 5% CO_2 and 100% humidity. The culture media were replaced

after 24 hours and then at 72-hour intervals until the cells reached confluence. Passaged cells were replated at the same seeding densities as before. Fourth-passage cells were used for all experiments because previous studies demonstrated that they retain their chondrogenic phenotype, including the ability to form cartilage nodules when implanted in nude mouse thigh muscle, differential responsiveness to vitamin D metabolites, and a number of other factors (2-5,7,43-46).

Incubation with rhBMP-2

rhBMP-2 (Genetics Institute, Cambridge, MA, U.S.A.) was dissolved at a concentration of 2 mg/ml in 10 m*M* histidine buffer containing 0.5 *M* arginine HCl (pH 6.5) and diluted 1:10 with 1% bovine serum albumin for storage at −70°C. Immediately prior to use, the rhBMP-2 was diluted to the appropriate concentration in complete medium and was then added to the cells. The rhBMP-2 was tested at concentrations ranging from 0.05 to 100 ng/ml. Each experiment included untreated controls that contained vehicle at the highest concentration used in the treated groups.

Incorporation of [^{3}H]thymidine

To determine the effect of rhBMP-2 on DNA synthesis by the cells, incorporation of [^{3}H]thymidine was measured. For the quiescent cells, confluent chondrocytes were incubated for 48 hours in Dulbecco's modified Eagle medium containing 1% fetal bovine serum. At the end of this time, the medium was replaced with Dulbecco's modified Eagle medium containing 1% fetal bovine serum and varying concentrations of rhBMP-2 for 24 hours. Two hours prior to harvest, 50 μl of a 5 μCi/ml (1.85×10^5 Bq/ml) stock of [^{3}H]thymidine was added. To examine the effect of rhBMP-2 on the incorporation of [^{3}H]thymidine in nonquiescent cells, chondrocytes were incubated for 24 or 48 hours in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and varying concentrations of rhBMP-2. Four hours prior to harvest, 50 μl of a 5 μCi/ml stock of [^{3}H]thymidine was added. At harvest, the cell layers were washed twice with cold phosphate buffered saline and twice with 5% trichloroacetic acid and the DNA precipitated by means of addition of saturated trichloroacetic acid for 30 minutes. The trichloroacetic acid-precipitable material was dissolved in 0.2 ml of 1% sodium dodecyl sulfate (SDS), and radioactivity in the resuspended pellet was measured by liquid scintillation spectroscopy.

Alkaline Phosphatase Specific Activity

Alkaline phosphatase specific activity was measured to assess the effect of rhBMP-2 on chondrocyte differentiation. Cell layers were prepared according to the method of Hale et al. (19), as previously described (3). The cells were cultured in 24-well culture dishes (Corning Glass Works, Corning, NY, U.S.A.); at harvest, the medium was decanted and the cell layer was washed twice with phosphate buffered saline and then removed with a cell scraper. After centrifugation, the cell layer pellet was washed two more times with phosphate buffered saline and resuspended by vortexing in 500 μl of deionized water containing 25 μl of 1% Triton X-100. Enzyme assays were performed on lysates of the cell layers. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase alkaline; EC 3.1.3.1) specific activity was assayed in the cell layer as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2 (8).

RNA and Protein Synthesis

[^{3}H]uridine incorporation: To determine the effect of rhBMP-2 on RNA synthesis by the cells, incorporation of [^{3}H]uridine was measured (44). Fourth-passage growth-zone and resting-zone chondrocytes were grown to confluence in 96-well culture plates (Corning Glass Works). The cells were incubated for either 5 or 24 hours in medium containing 10% fetal bovine serum and varying concentrations of growth factor. [^{3}H]uridine (14 μCi/ml [5.18×10^5 Bq/ml]) was added 2 hours prior to harvest. At harvest, the cell layers were washed twice with phosphate buffered saline and twice with 5% trichloroacetic acid; they were then treated with saturated trichloroacetic acid for 30 minutes. The trichloroacetic acid-precipitable material was dissolved in 0.2 ml of 1% SDS, and the radioactivity was measured by liquid scintillation spectrometry.

Synthesis of collagen and NCP (noncollagen protein): To determine the effect of rhBMP-2 on the synthesis of collagen and NCP by the cells, incorporation of [^{3}H]proline into collagenase-digestible and noncollagenase-digestible protein was measured (38,44). At confluence, the medium was replaced with fresh Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 1% antibiotics, 50 μg/ml ascorbic acid, 5 μCi/ml L-[G^{3}H]proline (New England Nuclear, Boston, MA, U.S.A.), and varying amounts of rhBMP-2, and the incubation was continued for 24 hours. At harvest, the medium was decanted and the cell layer (cell and matrix) was collected in two 0.2 ml portions of 0.2 N NaOH. Proteins in the medium or cell layer were precipitated with 0.1 ml of 100% trichloroacetic acid containing 15% tannic acid and were washed three times with a mixture of 10% trichloroacetic

TABLE 1. *The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the cell number in cultures of resting-zone and growth-zone chondrocytes*

Treatment (ng/ml rhBMP-2)	Cell number ($\times 10^5$)	
	Growth-zone chondrocytes	Resting-zone chondrocytes
0.0	2.55 ± 0.12	1.65 ± 0.10
0.2	2.63 ± 0.19	1.69 ± 0.23
0.8	2.48 ± 0.14	1.54 ± 0.26
3.1	2.42 ± 0.22	1.60 ± 0.24
12.5	2.55 ± 0.18	1.66 ± 0.23
50.0	2.60 ± 0.21	2.23 ± 0.14[a]
100.0	2.63 ± 0.13	2.34 ± 0.11[a]

Values are given as the mean ± SEM of six cultures.
[a] $p < 0.05$ compared with untreated control.

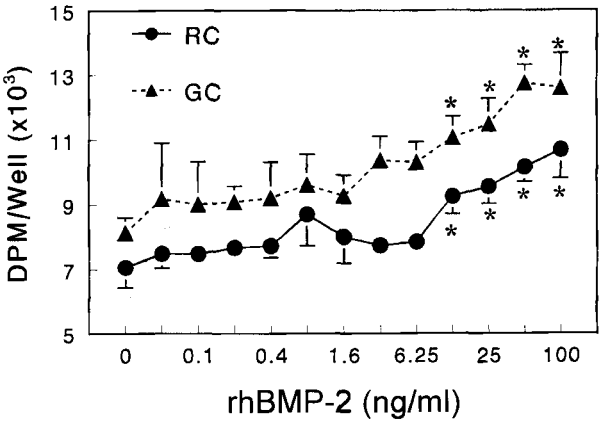

FIG. 1. Incorporation of [^{3}H]thymidine by quiescent resting-zone (RC) and growth-zone (GC) chondrocytes after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). Fourth-passage growth-zone and resting-zone cells were placed into quiescence by incubation in medium containing 1% fetal bovine serum and were then treated with rhBMP-2 for 24 hours. Data are from one of three representative experiments and are given as the mean ± SEM; n = 6. *$p < 0.05$, treated cells compared with untreated controls.

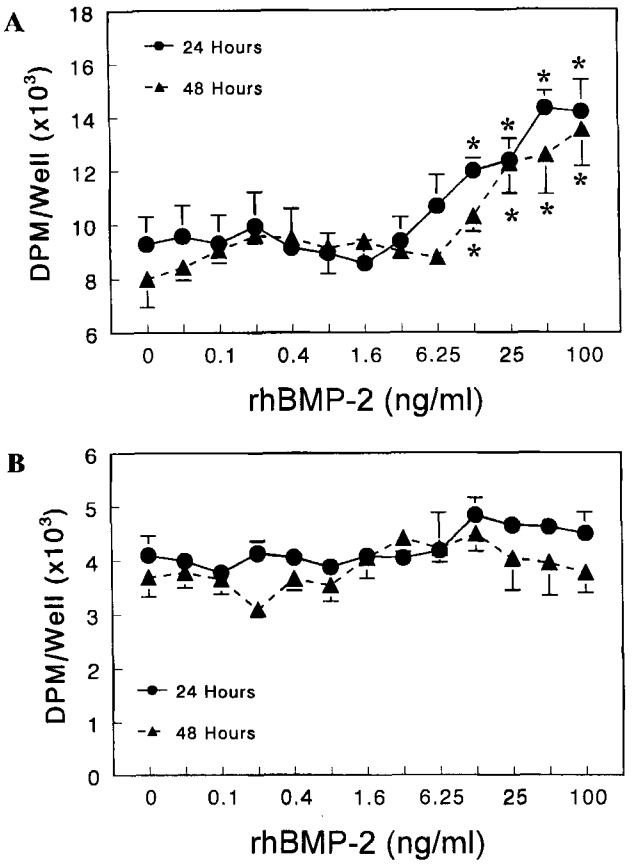

FIG. 2. Incorporation of [^{3}H]thymidine by nonquiescent resting-zone and growth-zone chondrocytes after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). Confluent, fourth-passage **A:** resting-zone cells and **B:** growth-zone cells were treated with rhBMP-2 for 24 or 48 hours, and incorporation of [^{3}H]thymidine was measured. Data are from one of three representative experiments and are given as the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls.

acid and 1% tannic acid and twice with ice-cold acetone. The final pellet was dissolved in 500 μl of 0.05 N NaOH. The amount of radiolabeled proline that had been incorporated into collagenase-digestible protein and noncollagenase-digestible protein was determined only for the cell layer (37) and not for the medium, because more than 80% of the total was incorporated into the cell layer under control conditions (44).

Incorporation of [^{35}S]sulfate

To determine the effect of rhBMP-2 on proteoglycan synthesis by the cells, incorporation of [^{35}S]sulfate was measured, as described by O'Keefe et al. (36). In previous studies, we found that the amount of radiolabeled proteoglycan secreted by the chondrocytes into the medium was less than 15% of the total radiolabeled proteoglycan produced (medium and cell layer) (34). Because more than 85% of the radiolabeled proteoglycan was in the cell layer, we examined the effects of growth factor on the incorporation of [^{35}S]sulfate only in the cell layer.

Fourth-passage growth-zone and resting-zone chondrocytes were grown to confluence in 24-well culture plates with medium containing 10% fetal bovine serum and different concentrations of growth factor for 24 hours. Four hours prior to harvest, 50 μl of Dulbecco's modified Eagle medium containing 18 μCi/ml (6.66×10^5 Bq/ml) [^{35}S]sulfate and 0.814 m*M* carrier sulfate was added to each culture. At harvest, the conditioned medium was removed and the cell layer was collected in two 0.25 ml portions of 0.25 *M* NaOH. The total volume of the sample was adjusted to 0.75 ml by addition of 0.15 *M* NaCl, and the sample was dialyzed against buffer containing 0.15 *M* NaCl, 20 m*M* Na_2SO_4, and 20 m*M* Na_2HPO_4 (pH 7.4) at 4°C. The dialysis solution was changed until the radioactivity in the dialysate reached background levels. The amount of [^{35}S]sulfate incorporated was determined by liquid scintillation spectrometry.

Expression of BMP-2 and BMP-4 mRNA

Levels of mRNA for BMP-2 and BMP-4 were determined using Northern blot analysis. Total RNA was extracted by the RNasoyl B method (Cinna Biotex, Houston, TX, U.S.A.) (20,21). RNA was dissolved in 0.1 *M* Tris buffer (pH 7.2) containing 0.4% SDS and 2 m*M* EDTA. The RNA concentration was determined by absorbance at 260 nm. Poly(A) RNA was isolated using small oligo deoxythymidine cellulose push columns as described by the manufacturer (Stratagene, San Diego, CA, U.S.A.), except that 0.1% SDS was used in all of the buffers. Poly(A) RNA (5 μg) from each experimental condition was denatured in 2.2 *M* formaldehyde and 50% formamide (vol/vol) and separated on a 1% agarose gel containing 2.2 *M* formaldehyde (100 V for 3.5 hours). After electrophoresis, the gel was stained with ethidium bromide, photographed, and then transferred to a Nytran filter (Schleicher and Schuell, Keene, NH, U.S.A.) by capillary blotting with ×10

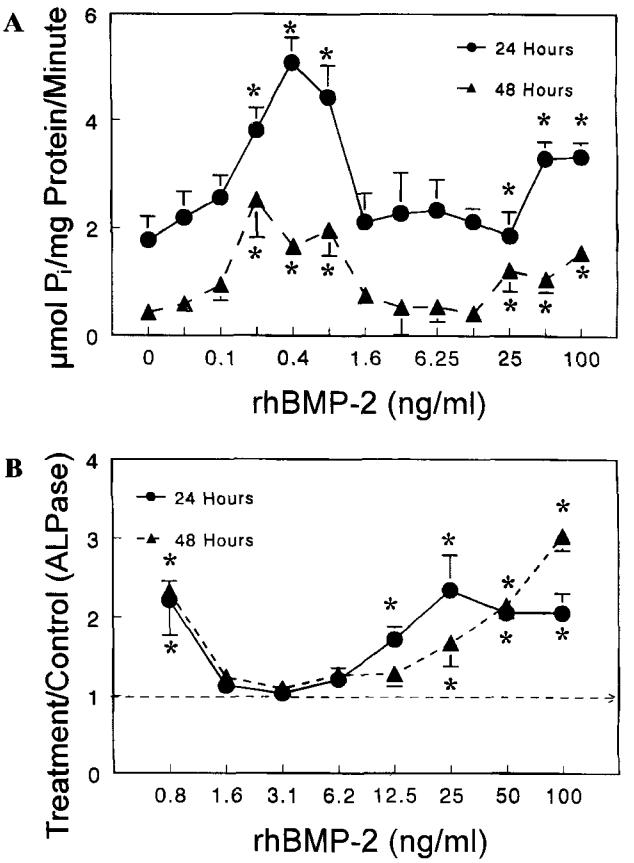

FIG. 3. Alkaline phosphatase (ALPase) specific activity in the cell layer of confluent, fourth-passage resting-zone chondrocytes after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). **A:** 24 or 48 hours of treatment with rhBMP-2. Data are from one representative experiment and are given as the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls. **B:** Treatment/control ratios for three additional experiments performed exactly as described in **A**. Data are given as the mean ± SEM. *p < 0.05 as compared with a treatment/control ratio of 1.0.

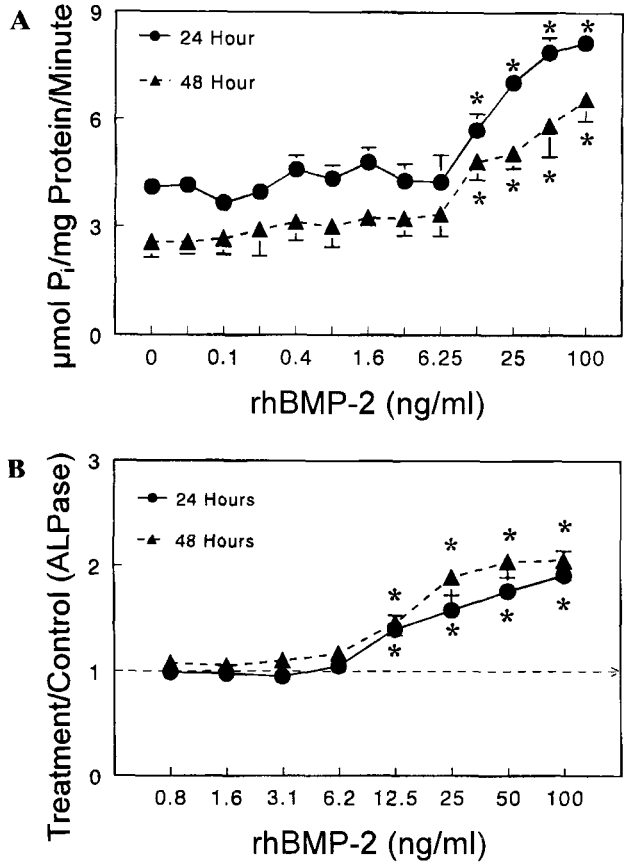

FIG. 4. Alkaline phosphatase (ALPase) specific activity in the cell layer of confluent, fourth-passage growth-zone chondrocytes after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). **A:** 24 or 48 hours of rhBMP-2 treatment. Data are from one representative experiment and are given as the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls. **B:** Treatment/control ratios for three additional experiments performed exactly as described in **A**. Data are given as the mean ± SEM. *p < 0.05 as compared with a treatment/control ratio of 1.0.

SSPE buffer (×1 SSPE = 0.15 *M* NaCl, 0.01 *M* $NaHPO_4$ buffer [pH 7.2], and 10 m*M* EDTA) (40). After the transfer was completed, the RNA was ultraviolet crosslinked to the filter using Stratalink (Stratagene) and dried. The BMP-2 probe was a 0.6 kb HindIII/EcoR1 fragment from exon 3 of the mouse BMP-2 gene (16). The mouse BMP-4 (1.6 kb) cDNA probe was obtained from B. Hogan (Vanderbilt University, Nashville, TN, U.S.A.) and was used as described previously (17).

DNA probes were labeled using a random-primer labeling kit from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Prehybridization was carried out at 42°C in ×5 SSPE containing 50% formamide, 150 µg/ml denatured salmon sperm DNA, and 50 m*M* $NaHPO_4$ buffer (pH 6.5). After 1 hour of prehybridization, the DNA probe was added at a concentration of 5×10^5 cpm/ml, and hybridization was conducted for 12-16 hours at 42°C. The filters were then washed two times with ×0.1 SSPE with 1% SDS for 20 minutes at 64°C. The filters were dried at room temperature, exposed for 24 hours at −20°C with an intensifying screen, and quantitated using a blot analyzer (Betascope 603; Betagen, Waltham, MA, U.S.A.). In most cases, the filters were stripped by a wash in ×0.05 SSC (0.15 *M* NaCl and 0.015 *M* sodium citrate) with 0.1% SDS at 90°C for 10 minutes. The same filters were then reprobed with the appropriate cDNA. Each blot was also probed for glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene. In our hands, BMP-2 runs as a 3.8 kb mRNA and BMP-4, as a 1.8 kb mRNA; these are easily distinguishable on Northern blots. Levels of BMP-2 and BMP-4 mRNA were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. The normalized values for the treated cultures were compared with those for the controls.

Statistical Analysis

The data presented here are from one of three or more replicate experiments. For any given experiment, each datum point represents the mean ± SEM for six individual cultures (cell layers). Treatment/control ratios were derived from five or more independent experiments, with untreated controls having a ratio of 1.0. The data were analyzed by analysis of variance, and statistical significance was determined by comparing each datum point with the untreated control using Bonferroni's modified *t* test. Treatment/control ratios were compared with use of the Wilcoxon paired rank sum test. $P < 0.05$ was considered significant.

RESULTS

Effect of rhBMP-2 on Chondrocyte Proliferation

rhBMP-2 caused a dose-dependent increase in the incorporation of [^{3}H]thymidine by quiescent (Fig. 1)

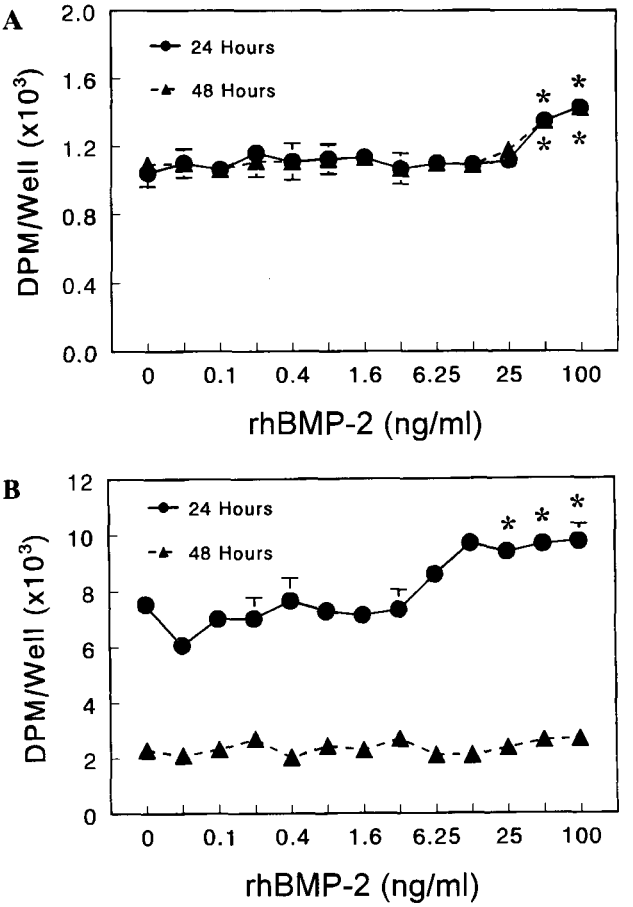

FIG. 5. Incorporation of [^{3}H]uridine by confluent, fourth-passage **A:** resting-zone chondrocytes and **B:** growth-zone chondrocytes after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). The cells were treated with rhBMP-2 for 24 or 48 hours, and incorporation of [^{3}H]uridine was measured. Data are from one of three representative experiments and are given as the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls.

TABLE 2. *The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the incorporation of [^{35}S]sulfate by cultures of growth-zone and resting-zone chondrocytes*

Treatment (ng/ml rhBMP-2)	[^{35}S]sulfate incorporation (dpm/μg protein)	
	Growth-zone chondrocytes	Resting-zone chondrocytes
0.0	35.88 ± 6.52	36.11 ± 6.52
0.8	27.71 ± 7.54	27.95 ± 7.48
1.6	22.67 ± 3.90	34.83 ± 3.56
3.1	26.19 ± 4.14	30.49 ± 4.52
6.2	32.09 ± 8.57	26.32 ± 9.73
12.5	37.77 ± 9.56	32.11 ± 9.85
25.0	28.04 ± 6.62	28.58 ± 0.66
50.0	28.67 ± 2.39	26.52 ± 2.39
100.0	29.03 ± 5.82	28.78 ± 5.59

Values are given as mean ± SEM of six cultures.

and nonquiescent (Fig. 2A) resting-zone chondrocytes. Maximal stimulation of DNA synthesis in nonquiescent cells was observed at BMP concentrations ranging from 12.5 to 100 ng/ml at both 24 and 48 hours. When the effect of rhBMP-2 on cell number was examined in parallel cultures (Table 1), only resting-zone cells treated with 50 or 100 ng/ml concentrations of growth factor showed significant increases.

Quiescent cultures of growth-zone cells exhibited a dose-dependent increase in incorporation of [^{3}H]thymidine after treatment with 12.5-100 ng/ml rhBMP-2 (Fig. 1). In contrast, incorporation of [^{3}H]thymidine by nonquiescent growth-zone chondrocytes was unaffected by rhBMP-2 (Fig. 2B). Similarly, there was no significant change in cell number (Table 1).

Effect of rhBMP-2 on Alkaline Phosphatase Specific Activity

rhBMP-2 caused a dose-dependent, bimodal stimulation of alkaline phosphatase specific activity in cultures of resting-zone chondrocytes (Fig. 3A) between 0.2 and 0.8 ng/ml and between 25 and 100 ng/ml. The bimodal nature of the response was consistently observed (n = 4) (Fig. 3B). Alkaline phosphatase specific activity in growth-zone chondrocyte cultures was significantly increased at concentrations of 12.5-100 ng/ml rhBMP-2 (Fig. 4A). This effect was also consistently observed (Fig. 4B).

Effect of rhBMP-2 on RNA and Protein Synthesis

rhBMP-2 appeared to have an overall anabolic effect on resting-zone and growth-zone chondrocytes. Incorporation of [^{3}H]uridine, a measure of general RNA synthesis, by resting-zone chondrocytes was significantly increased after treatment with 50-100 ng/ml rhBMP-2 for either 24 or 48 hours (Fig. 5A). Growth-zone cells showed a significant increase in the incorporation of [^{3}H]uridine after treatment with 25-100 ng/ml rhBMP-2 for 24 but not 48 hours (Fig. 5B).

The cultures of resting-zone chondrocytes contained increased amounts of both collagenase-digestible protein (Fig. 6) and noncollagenase-digestible protein (Fig. 7) after treatment with 3.1-25 ng/ml rhBMP-2 for 24 hours, indicating that rhBMP-2 stimulates synthesis of both collagen and NCP by the cells. Similar results were observed for cultures of growth-zone chondrocytes, with significant increases in the production of collagenase-digestible protein and noncollagenase-digestible protein occurring over the same range of rhBMP-2 concentrations (Figs. 6 and 7).

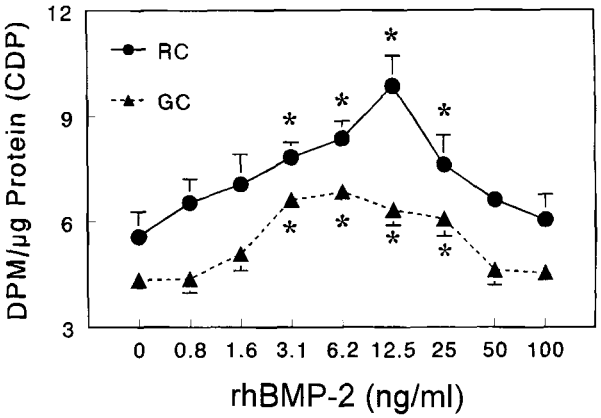

FIG. 6. Effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the incorporation of [^{3}H]proline into collagenase-digestible protein (CDP) by resting-zone (RC) and growth-zone (GC) chondrocytes. The cells were treated for 24 hours with rhBMP-2, and incorporation of [^{3}H]proline into collagenase-digestible protein was determined. Data are from one of three representative experiments and are given as the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls.

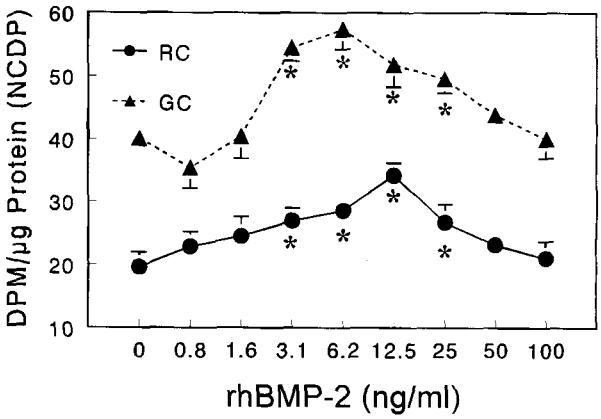

FIG. 7. Effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the incorporation of [^{3}H]proline into noncollagenase-digestible protein (NCDP) by resting-zone (RC) and growth-zone (GC) chondrocytes. The cells were treated for 24 hours with rhBMP-2, and incorporation of [^{3}H]proline into noncollagenase-digestible protein was measured. Data are from one of three representative experiments and are the mean ± SEM; n = 6. *p < 0.05, treated cells compared with untreated controls.

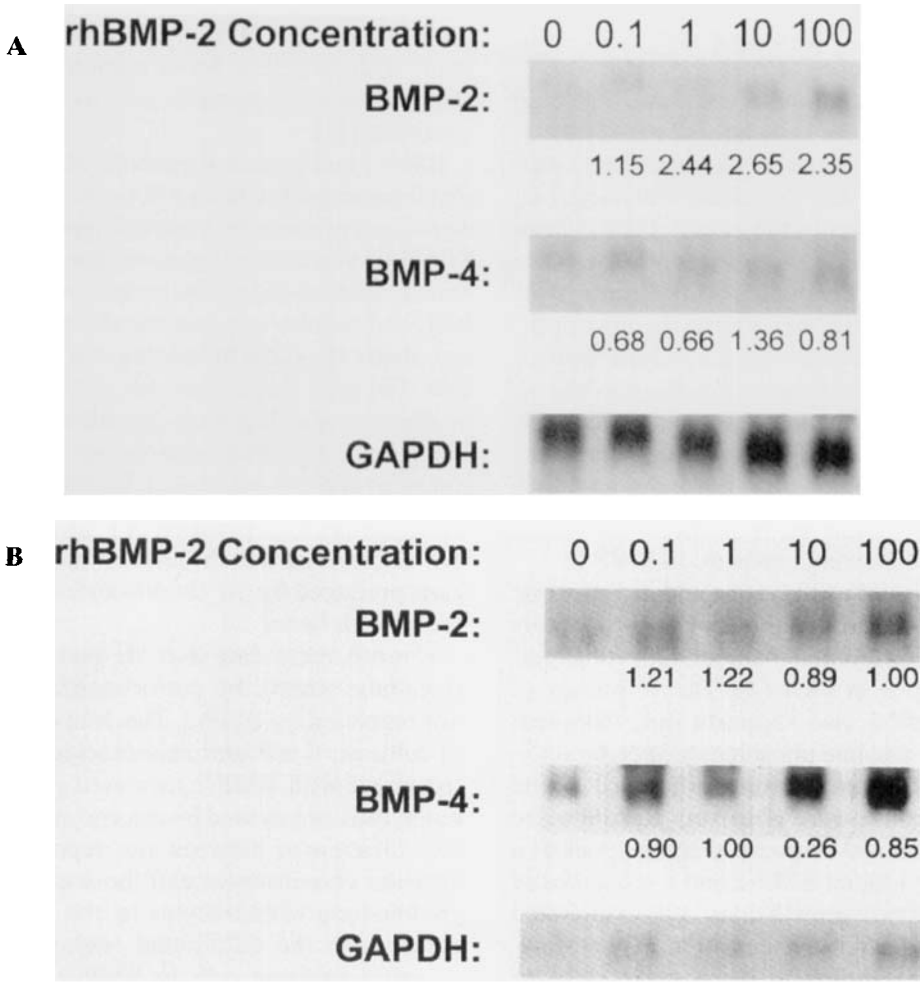

FIG. 8. Northern blot analysis of bone morphogenetic protein (BMP)-2 and BMP-4 mRNA in growth-zone and resting-zone chondrocytes. The cells were treated for 24 hours with the different concentrations (in nanograms per milliliter) of recombinant human BMP-2 (rhBMP-2) shown above the bands corresponding to the BMP-2 mRNA. Five micrograms of RNA was run per lane on the Northern blot. The numbers below the BMP-2 and BMP-4 bands in each lane are treatment/control ratios calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a blot analyzer. **A:** rhBMP-2 stimulated the production of BMP-2 mRNA in resting-zone cells. **B:** No effect was observed in similarly treated cultures of growth-zone cells. Production of BMP-4 mRNA was increased in cultures of resting-zone cells and inhibited in those of growth-zone cells by treatment with 10 ng/ml rhBMP-2.

Incorporation of [^{35}S]sulfate

rhBMP-2 had no effect on the incorporation of [^{35}S]sulfate by either cell type (Table 2).

Expression of BMP-2 and BMP-4 mRNA

Growth-zone and resting-zone chondrocytes were both found to constitutively express mRNA for BMP-2 and BMP-4 (Fig. 8). When cultures of resting-zone cells were treated with rhBMP-2, the level of BMP-2 mRNA increased 2-fold in those cultures receiving 1-100 ng/ml of growth factor (Fig. 8A), whereas the level of BMP-4 mRNA increased only in those cultures treated with 10 ng/ml rhBMP-2. When growth-zone cells were treated with rhBMP-2, no significant change in mRNA levels for BMP-2 was observed (Fig. 8B); however, a decrease in mRNA for BMP-4 was seen only after treatment with 10 ng/ml rhBMP-2. Related studies in our laboratory indicate that these effects are dose-dependent (unpublished data).

DISCUSSION

The results of the present study show that BMP-2 has a direct effect on chondrocytes in the endochondral lineage pathway. The growth factor regulated the proliferation, phenotypic expression, and protein synthesis of chondrocytes. The effect of BMP-2 was dependent on the degree of cell maturation; it regulated resting-zone cells and growth-zone cells differently depending on the parameter being examined. Finally, BMP-2 induced not only its own production but production of BMP-4 as well.

BMP-2 exerted its effects on cell proliferation in a differential manner, depending on the state of confluence of the culture. The growth factor stimulated incorporation of [^{3}H]thymidine by quiescent cultures of both resting-zone and growth-zone chondrocytes; however, in nonquiescent cultures, BMP-2 stimulated only the less mature resting-zone cells, regardless of how long the cultures were exposed to it. This increase

in the incorporation of [^{3}H]thymidine correlated with an increase in the number of resting-zone cells.

The difference in proliferative response between quiescent and nonquiescent cells treated with BMP-2 is more likely due to the presence of serum than to differences in cell density. The quiescent cell model is more sensitive with respect to the effect of BMP-2 on synchronized cells in the absence of serum. However, *in vivo* cells would not encounter BMP-2 in a low-serum background, nor do the cultures behave optimally in a low-serum medium. Under serum-replete conditions, the differential response of the two chondrocyte populations could be observed. Since cell density in confluent cultures of resting-zone and growth-zone cells was comparable, this parameter did not account for the difference in proliferation exhibited by these cells following exposure to BMP-2.

Confluent cultures of resting-zone and growth-zone chondrocytes respond to a number of growth factors and hormones in a differential manner, reflecting their different phenotypes *in vivo* (4). The responses of these cells to BMP-2 also supports this. Although BMP-2 stimulated alkaline phosphatase specific activity in both types of cultures, distinct differences in the nature of the response were observed. Resting-zone cells exhibited a bimodal response, with one peak at a concentration of 0.4 ng/ml BMP-2 and a second peak at 50 ng/ml. In contrast, growth-zone cells exhibited a single peak at the higher concentration. The resting-zone cells therefore appear to be more sensitive to BMP-2, suggesting that the growth factor may target less mature cells in the endochondral developmental pathway. This is in agreement with studies that implicated some of the BMPs, like 2, 4, and 5, in early development, whereas other BMPs, like 3, 6, 7, and cartilage-derived morphogenetic protein-2, may be involved in later stages of skeletal formation (10,32,51).

Alkaline phosphatase activity is frequently used as an indicator of increased differentiation for bone and cartilage cells. As growth plate chondrocytes hypertrophy *in vitro*, this enzyme increases markedly (3). The bimodal effect of BMP-2 on resting-zone cells suggests that two responding subpopulations of cells may exist: one population that is sensitive to low concentrations of growth factor and a second population that has already acquired the phenotype of the growth-zone chondrocyte and responds only to the higher doses of growth factor. Regardless of the etiology of the bimodal response, there was no morphological evidence of hypertrophy in the cultures of resting-zone cells even after 24 hours of exposure to BMP-2. This is in concert with other studies that examined shifts in the maturation state of resting-zone cells. When resting-zone cells are incubated with 24,25-$(OH)_2D_3$, a minimum of 36 hours is needed before the cells acquire the responsiveness to 1,25-$(OH)_2D_3$ typical of growth-zone cells (45). In other related studies, treatment with rhTGF-β for a minimum of 5 days was required before responsiveness to 1,25-$(OH)_2D_3$ was observed (25).

BMP-2 had a general anabolic effect on the growth plate chondrocytes. Both RNA synthesis and total production of proteins by both cell types were enhanced. Synthesis rates of collagenase-digestible protein and noncollagenase-digestible protein were affected similarly, and cellular responses to the growth factor were essentially the same for resting-zone and growth-zone cells. Because there were no net differences in the production of collagenase-digestible protein and noncollagenase-digestible protein, no specific effect on collagen synthesis was noted. Similarly, BMP-2 had no effect on the incorporation of [^{35}S]sulfate in either cell type, suggesting that the amount of sulfated proteoglycans produced by the chondrocytes was insensitive to the growth factor.

Overall, these data indicate that production of extracellular matrix by costochondral chondrocytes is not regulated by BMP-2. This is in contrast to reports of cultures of articular chondrocytes (22,50) in which treatment with BMP-2 increased production of proteoglycans, as assessed by incorporation of [^{35}S]sulfate. The differences between the reported responses of articular chondrocytes and those of resting-zone and growth-zone chondrocytes in the present study, together with the differential responses of the costochondral cartilage cells to BMP-2, demonstrate that this growth factor can act at discrete stages of chondrogenic development.

It should be noted that, in a previous study, articular chondrocytes were exposed to BMP in serum-free medium and that the stimulatory effect of BMP on proteoglycan synthesis was to raise only the levels of [^{35}S]sulfate incorporation to those observed in the presence of serum (29). Thus, the lack of any observable stimulatory effect of rhBMP-2 in our study is in agreement with the previous findings because we cultured cells in the presence of 10% fetal bovine serum during treatment. It should also be noted that the highest concentration of rhBMP-2 used in our study was 100 ng/ml and that most of the effects induced by the growth factor were observed in cultures exposed to concentrations of 10-15 ng/ml; this is in contrast to earlier studies that used concentrations as high as 1,000 ng/ml (48,55). Finally, in the present study, we measured proteoglycan synthesis by means of incorporation of [^{35}S]sulfate. This is an indirect measure of aggrecan synthesis because it measures glycosaminoglycan sulfation rather than production of proteoglycan core protein. It is possible that BMP-2 regulates an enzyme or enzymes involved in glycosaminoglycan synthesis rather than aggrecan *per se*. However, even when aggrecan mRNA levels are measured (30), ab-

solute statements about the production and secretion of proteoglycan into the extracellular matrix cannot be made.

Although BMP-2 is a member of the TGF-β superfamily, its effects are distinct from those of TGF-β on the costochondral chondrocyte model used in this study. Whereas BMP-2 had a bimodal effect on the activity of alkaline phosphatase in resting-zone cells, TGF-β elicits a single biphasic response, evident only at the lowest concentrations of growth factor. In general, TGF-β appears to promote differentiation of mesenchymal cells and matrix production, but it blocks terminal differentiation of chondrocytes and osteoblasts to a calcifying phenotype (1,15). In contrast, BMP-2 enhances calcification (24). Thus, one of the major differences between TGF-β and BMP-2 may be in the regulation of the terminal differentiation of the cells to calcifying chondrocytes. It would be of interest to know if BMP-2 can promote the production of proteins like osteocalcin by the rat costochondral cartilage cells.

Some of the effects of BMPs on chondrocytes appear to be autocrine, since both resting-zone and growth-zone cells constitutively produce BMP-2 and BMP-4 mRNA. Why the cells would produce more than one species of BMP is unclear. One possibility is that BMP-4 augments the chondrogenic capability of BMP-2, as was suggested for osteogenesis by Celeste et al. (9). The presence of cartilage-derived morphogenetic proteins in cartilage in the endochondral developmental pathway (10) indicates that chondrocytes produce and respond to a number of autocrine regulatory factors. The differential localization of these factors, as well as of BMP-2 and BMP-13 in embryonic development (31), suggests that the cells may autoregulate the production of growth factors as they move from one maturation state to another in the lineage.

Levels of BMP-2 and BMP-4 mRNA in resting-zone cells are sensitive to regulation by BMP-2, whereas those in growth-zone cells are not. This supports the hypothesis that BMP-2 targets the less mature cells. In addition, the data suggest that increased levels of these BMPs may play a role in the transition of chondrocytes from a less mature stage to a more mature stage in the endochondral pathway.

Acknowledgment: This research was supported by Public Health Service Grants DE-05937 and DE-08603.

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