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Developmental Expression and Hormonal Regulation of the Rat Matrix GLA Protein (MGP) Gene in Chondrogenesis and Osteogenesis

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Abstract Matrix Gla protein (MGP), a vitamin K dependent protein, has recently been identified in many tissues. However, it is accumulated only in bone and cartilage suggesting that the expression of MGP may be related to the development and/or maintance of the phenotypic properties of these tissues. We systematically evaluated MGP mRNA expression as a function of bone and cartilage development and also as regulated by vitamin D during growth and cellular differentiation. Three experimental models of cartilage and bone development were employed: an in vivo model for endochondral bone formation, as well as in primary cells of normal diploid rat chondrocyte and osteoblast cultures. MGP was expressed at the highest level during cartilage formation and calcification in vivo during endochondral bone formation. In chondrocyte cultures, MGP mRNA was present throughout the culture period but increased only after 3 weeks concomitantly with type I collagen mRNA. In osteoblast cultures, MGP mRNA was expressed during the proliferative period and exhibited increased expression during the period of matrix development. In contrast to osteocalcin (bone Gla protein), this increase was not dependent on mineralization but was related to the extent of differentiation associated with and potentially induced by extracellular matrix formation. During the proliferative period, type I collagen mRNA peaked and thereafter declined, while type I collagen protein steadily accumulated in the extracellular matrix. Constant MGP levels were maintained in the mineralization period of osteoblast differentiation in vitro which is consistent with the constant levels found during the osteogenic period of the in vivo system. MGP mRNA levels in both osteoblasts and chondrocytes in culture were significantly elevated by 1,25-$(OH)_2D_3$ (10^{-8} M, 48 h) throughout the time course of cellular growth and differentiation. Interestingly, when MGP mRNA transcripts from vitamin D treated and untreated chondrocytes and osteoblasts were analyzed by high resolution Northern blot analysis, we observed two distinct species of MGP mRNA in the vitamin D treated chondrocyte cultures while all other cultures examined exhibited only a single MGP mRNA transcript. Primer extension analysis indicated a single transcription start site in both osteoblasts and chondrocytes with or without vitamin D treatment, suggesting that the lower molecular weight MGP message in vitamin D treated chondrocytes may be related to a modification in post-transcriptional processing. In conclusion, these results show that the selective accumulation of MGP in bone and cartilage tissues in vitro may be related to the development and/or maintance of a collagenous matrix as reflected by increases in MGP mRNA during these periods. Moreover, our data suggest that cartilage and bone MGP mRNA may in part be selectively regulated by 1,25-$(OH)_2D_3$ at the post-transcriptional level.

Key words: MGP, chondrogenesis, osteogenesis, gene expression, vitamin D

Two structurally related vitamin K dependent γ-carboxyglutamic acid (Gla) proteins have been identified in bone, osteocalcin (OC), and matrix Gla protein (MGP) [1–5]. MGP is an 84-residue noncollagenous protein that contains 5 residues of the Ca^{2+} binding amino acid Gla, while OC, a 49-residue protein, contains only 3 Gla residues. Although the degree of sequence identity between these two proteins is sufficient to indicate that they evolved from a common ancestor by gene duplication, and subsequent divergent evolution [5], the level of sequence identity is too low (20%) to suggest that they are functionally related. Other studies have indicated that MGP is quite different from OC. Osteocalcin is primarily found in bone while MGP has a broad tissue distribution. High levels of MGP have been detected in lung, heart, and kidney; however, its

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accumulation in the extracellular matrix of tissues has been found only in bone, cartilage, and dentin [6]. Furthermore, it also has been observed that MGP is represented at higher levels in post-natal bone (birth–2 days) earlier than OC [7]. MGP remains at a constant level from birth to adulthood and is associated with the guanidine soluble extracellular matrix while nearly all of the OC in bone is bound to the mineral phase and continues to accumulate exceeding MGP levels beginning 4 days after birth increasing to sixfold in adult bone.

One feature of MGP synthesis similar to several cartilage and bone proteins is its modulation of synthesis by vitamin D. The effect of vitamin D on MGP synthesis has been examined in osteosarcoma cells [8,9]. MGP is expressed, secreted, and stimulated by vitamin D in UMR 106-1, Ros 25/1, and Ros 25/4. In contrast, Ros 17/2.8 cells which do not synthesize MGP were induced to synthesize MGP only after prolonged treatment with vitamin D.

In the present study, we evaluated MGP gene expression first as a function of the development of bone and cartilage and secondly as regulated by 1,25-$(OH)_2D_3$ during growth and cellular differentiation of normal diploid osteoblasts and chondrocytes. MGP mRNA expression, as well as that of OC, type I collagen, and type II collagen, was systematically evaluated in three experimental models of cartilage and bone development: (1) an in vivo model for endochondral bone formation (subcutaneous implantation of demineralized bone particles in rats); (2) an in vitro normal diploid rat chondrocyte culture system; (3) an in vitro normal diploid rat osteoblast culture system. From these studies, we report that MGP expression in the in vivo endochondral bone formation model system is expressed at the highest level during the phase of cartilage formation and calcification. In vitro MGP mRNA levels increase after the development of the extracellular collagenous matrix in both osteoblast and chondrocyte cultures.

The present studies also show that while 1,25-$(OH)_2D_3$ increases MGP mRNA in both cell types, high resolution Northern blot analysis revealed that vitamin D treated chondrocytes expressed two MGP mRNA transcripts while vitamin D treated osteoblasts exhibited one MGP mRNA transcript. Primer extension analysis indicated a single transcription start site in both osteoblast and chondrocyte cultures. While the precise function(s) for MGP in cartilage, bone, and other tissues remains to be established, its selective accumulation in bone and cartilage tissues may be related to the development of a collagenous matrix in these tissues as reflected by increases in MGP mRNA during these periods in the in vitro culture system.

EXPERIMENTAL PROCEDURES

Animals and Implantation Procedure

Male day 28 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA), were implanted with 50 mg of demineralized bone particle (DBP) prepared as described below. The DBP was implanted in bilateral subcutaneous pockets on the thoracic region in the rats [10]. Within 24 h, the DBP became encapsulated forming a discrete implant. Groups of 4 rats per experimental time point were evaluated. Explants were collected on days 7, 9, 11, and 15 post-implantation for total cellular RNA isolation. These time points were selected to demonstrate specific stages of the endochondral bone sequence.

Preparation of Demineralized Bone Particles (DBP)

Bone powder was prepared from the diaphyses of tibia and femurs of 6–10 week old rats which had been split and cleaned of marrow. The clean diaphyses were extracted with absolute ethanol followed by anhydrous ether, dried, then pulverized in a Spex liquid nitrogen mill and sieved to particle sizes of 75 to 250 microns. Demineralization of this bone powder was accomplished by extracting with 0.5 M HCl (25 meq/g bone) for 3 h at room temperature followed by washes in distilled water to remove all acid following the procedure described by Glowacki and Mulliken [11]. The DBP was then dried in absolute ethanol and ether.

Rat Chondrocyte Cultures

Chondrocytes were isolated from cartilage tissue dissected from the ends (above the growth plate) of the tibia and femur from 21 day fetal rats. Minced tissue was subjected to digestions of 30 and 120 minutes at 37°C in 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) 0.25% trypsin (Gibco, Grand Island, NY). The cells of the first digest were discarded and those released from the second digestion

were plated in Dulbecco's modified Eagles medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS) and 25 μg/ml ascorbic acid in 100 mm dishes (Corning, Corning, NY) at a density of 2.2×10^6 cells/dish. On day 7, the cells were subcultivated and again plated at 2.2×10^6 cells/100 mm dish and fed with DMEM, 10% FCS, and 25 μg/ml ascorbic acid. 1,25-$(OH)_2D_3$ (Hoffman LaRoche, Nutley, NJ; gift of M. Uskokovic) was included in the media at 10^{-8} M for 48 h when indicated. In some instances, primary cells were grown in the presence or absence of ascorbic acid and taken for RNA on day 6. In addition, some primary cells were grown, subcultivated, and plated with or without ascorbic acid and extracted for RNA on days 7, 15, and 21.

Rat Osteoblast Cultures

Calvaria from 21 day fetal rats were isolated and subjected to sequential digestions of 20, 40, and 90 minutes at 37°C in 2 mg/ml collagenase A/0.25% trypsin [12,13]. The cells of the first two digests were discarded and those released from the third digestion were plated in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS) in 100 mm dishes or 6 well dishes (Corning) at a density of 5×10^5 or 3.6×10^5 cells/dish, respectively. At confluence (day 7), the time course of mineralization was accelerated by the addition of BGJb medium (Gibco) supplemented with 10% FCS, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate, or in some experiments in the absence of 50 μg/ml ascorbic acid, or 10 mM β-glycerol phosphate. When calvarial osteoblasts were isolated from 21 day fetal rats, the time of maximal expression of the principal phenotype markers of the 35 day developmental sequence did not vary by more than 48 h in independent experiments using a standardized lot of fetal calf serum [13]. 1,25-$(OH)_2D_3$ was included in the media at 10^{-8} M for 48 h when indicated.

RNA Isolation and Analysis

For each time point from the in vitro cell culture systems, cells were scraped and pooled from 3 × 100 mm dishes in phosphate buffered saline (PBS) and pelleted by centrifugation. The PBS was decanted and the cell pellet immediately frozen in liquid nitrogen and stored at −70°C until the experiment was completed. For the DBP implant study, the bone implant was excised from the rats and immediately immersed in liquid nitrogen. The specimen was then pulverized under liquid nitrogen into a powder. Total cellular RNA was isolated from each cell pellet and DBP implant powder by the SDS-proteinase K method as described by Plumb et al. [14]. RNA preparations were quantitated by absorbance at 260 nm and intactness assessed by ethidium bromide staining following separation in 6.6% formaldehyde–1% agarose gels and when indicated 6.6% formaldehyde–1.5% agarose gels were used. RNA fractionated in such gels was transferred to Zeta-Probe membrane (BioRad, Richmond, CA) in 20× SSC by the capillary method of Thomas [15]. In other cases, RNA samples were bound to Zeta-Probe blotting membrane by slot blot under conditions described by the apparatus manufacturer (Schleicher and Schuell, Keene, NH). DNA probes used for hybridization were the following: rat H4 histone [16]; rat Type I collagen [17]; rat Type II collagen [18]; rat osteopontin [19]; rat osteocalcin [20]. All DNA probes were labeled with [^{32}P]dCTP by the random primer method [21]. For MGP, a synthetic oligonucleotide probe spanning nucleotides 256 to 288 [22] was labeled with [γ^{32}P]dATP at the 5′ end using T4 polynucleotide kinase. All prehybridizations and hybridizations were performed at 42°C in 50% formamide; 5× SSC; 5× Denhardts; 50 mM PO_4 buffer (pH 6.5); 1% SDS; 250 μg/ml salmon sperm DNA at 42°C for 4 h. For hybridizations using random labeled probes, 10^6 cpm/ml probe and 10% dextran sulfate were added [23]. For hybridizations with MGP, the entire probe was added in addition to 10% dextran sulfate. Following hybridization, blots were washed twice at room temperature and once at 65°C in 2× SSC/0.1% SDS (15 minutes per wash) and then twice at 65°C in 0.1× SSC/0.1% SDS (30 minutes per wash). The resulting autoradiographs were quantitated by scanning laser densitometry. Each time point represents the average of triplicate assays from at least two independent experiments.

Primer Extension Analysis

Primer extension analysis was carried out using a 33 nucleotide synthetic primer spanning nucleotides 256 to 288 [22]. Primer (10 ng, [^{32}P]5′ end labeled to a specific activity of 1.5–3×10^8 cpm/μg) was mixed with 10–15 μg total cellular RNA in 10 μl of 0.4 M NaCl and 10 mM

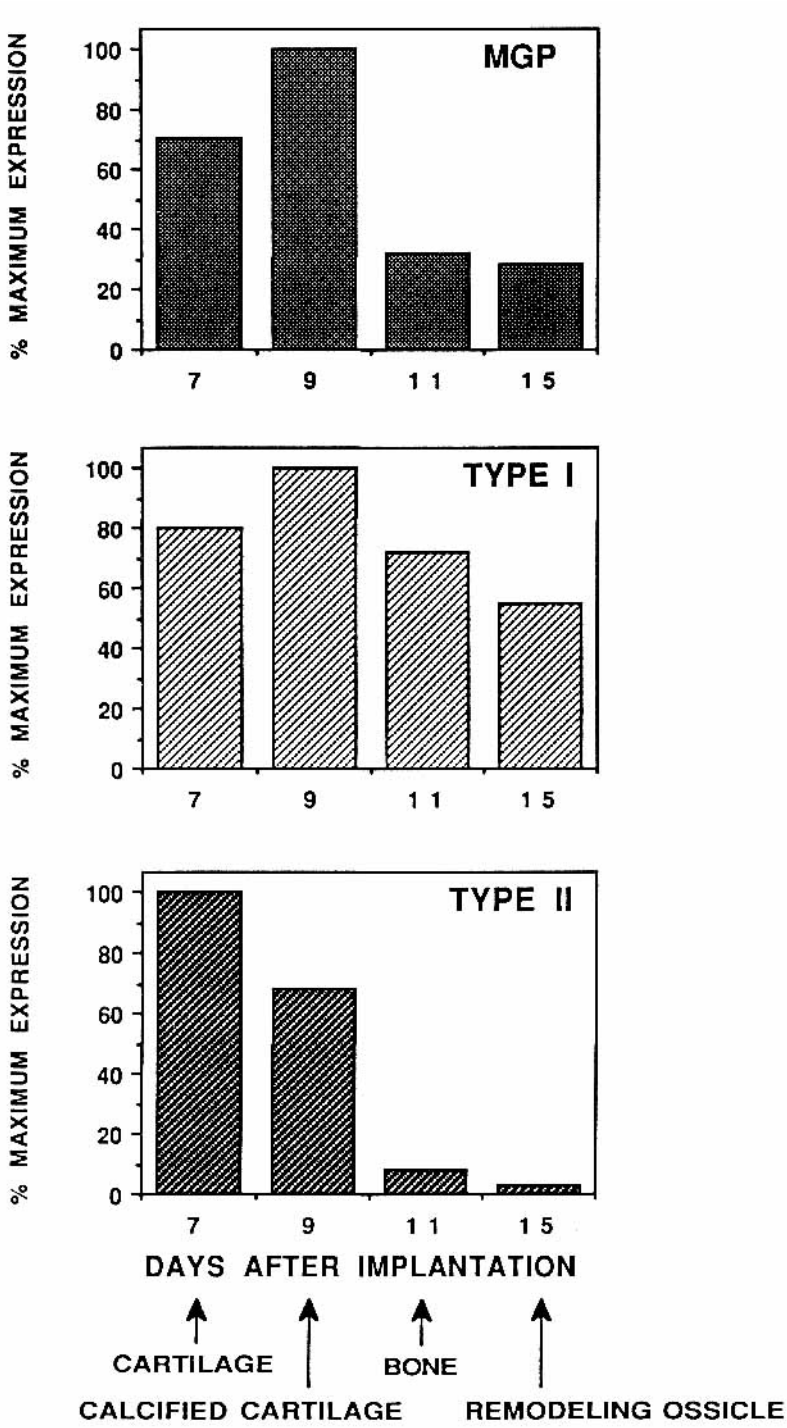

Fig. 1. MGP, type I collagen (Type I), and type II collagen (Type II) mRNA expression during endochondral bone formation. RNA was extracted from demineralized bone particle implants on days 7, 9, 11, and 15. The time points were selected to encompass the transition from cartilage to bone. Results are the mean of the data from three animals per time point. Values were normalized to 28S ribosomal RNA and are expressed for each parameter as % maximum average densitometric value from triplicate slot blot analyses of total cellular RNA. Standard deviation for all data shown is within 5%.

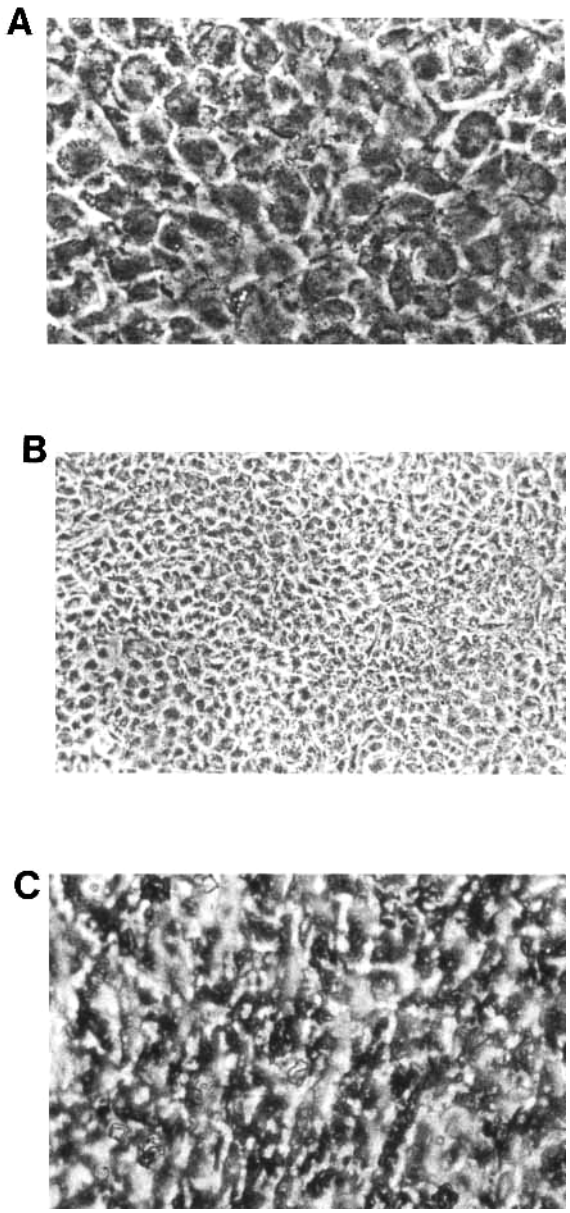

Fig. 2. Phase-contrast photomicrographs taken of subcultivated normal rat chondrocyte cultures. **A:** Articular chondrocytes derived from 21 day gestation fetal rats show typical chondrocyte morphology of large rounded cells with prominent nuclei and nucleoli 7 days after subcultivated (×320). **B:** Lower magnification (×100) showing initiation of multilayering which progresses as the cultures mature. **C:** Cultures stained with alcian blue (×100) revealing extensive proteoglycan-containing cartilage extracellular matrix.

PIPES (pH 6.4) and heated for 10 minutes at 85–90°C. The reaction tubes were then directly immersed in a 45°C water bath to hybridize for 2 h. The reactions were quick cooled in ice water and diluted by the addition of dNTPs (to 1.5 mM each) and 100 μl ice cold reverse transcriptase buffer (50 mM Tris-HCl (pH 8.3) 6 mM $MgCl_2$, 40 mM KCl). 50 U of AMV reverse transcriptase (Life Sciences) per 10 μg of RNA were added and the reaction mixture was incubated at 37°C for 45–60 minutes. The nucleic acids were purified by one phenol:chlorform:isoamyl alcohol (25:24:1) extraction and precipitated with ethanol in the presence of 3 μg of glycogen as a carrier. The reaction products were analyzed by electrophoresis through 0.5 mm thick × 40 cm long 7.0 M urea 10% polyacrylamide gels. Gels were run in 1× TBE buffer (1× = 50 mM Tris-HCl (pH 8.3), 50 mM boric acid, 1 mM EDTA) for 16 h at 50 W constant power. The gels were dried under vacuum and exposed to Kodak XAR-5 preflashed film overnight using a Cronex Lightening Plus intensifying screen.

Collagen Content Determination

Cell layers were hydrolyzed in 6 N HCl under vacuum at 110°C for 24 h [24]. Aliquots were assayed for total amino acid composition using a Beckman 121M autoanalyzer and collagen content was calculated from the hydroxyproline concentration. The value represents the mean of three independent samples.

Phase Contrast Microscopy

Phase contrast micrographs of the cell cultures were taken with a Zeiss Phase Contrast microscope equipped with a Zeiss 35 mm camera.

Histochemistry

Chondrocytes were stained with alcian blue (pH 2.8) throughout the time course.

RESULTS

Expression of MGP During Endochondral Bone Formation

The in vivo experimental model system of bone formation in which demineralized bone particles are implanted into subcutaneous pockets of growing rats exhibits a tightly controlled progression of events of the stages of matrix-induced endochondral bone development. The process of endochondral ossification (that is, the formation of bone following cartilage replacement) occurs normally in the development of limbs in the embryo and is the process by which elongation of bone occurs throughout development and growth. The sequential programmed events of endochondral bone formation which

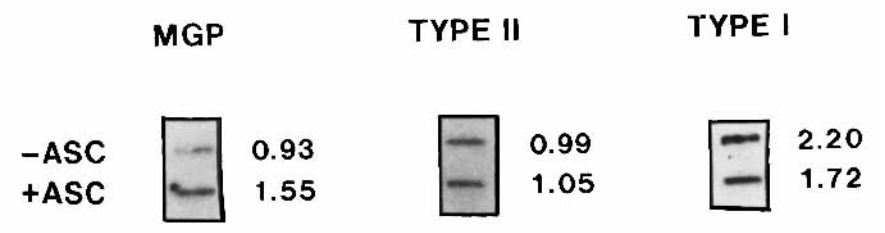

Fig. 3. MGP, type II collagen (Type II), and type I collagen (Type I) mRNA expression in primary chondrocytes grown for 6 days in the presence or absence of ascorbic acid. Articular chondrocytes were grown in DMEM, 10% FCS, and either in the presence or absence of 25 μg/ml ascorbic acid. Cellular RNA was isolated on day 6 and 6 μg of RNA was bound to a filter by the slot blot procedure and hybridized to MGP, type II collagen, and type I collagen–specific probes. The probes were stripped from the filter completely after each hybridization. The blots were quantitated by scanning densitometry and the densitometric units are indicated. This figure is representative of duplicate experiments. −Asc, cells grown in the absence of ascorbic acid; +Asc, cells grown in the presence of ascorbic acid.

occurs in the demineralized bone particle implant model have been well characterized [25–29]. Recently, mRNA levels in the implants were shown to reflect histologically and biochemically the defined periods of endochondral bone formation [29].

Examination of MGP mRNA levels in this model (Fig. 1) reveals that MGP is expressed throughout the periods of cartilage and bone formation. At early stages of the model involving the recruitment of fibroprogenitor cells (day 5), MGP mRNA was expressed at very low levels (data not shown). However, MGP mRNA was expressed at the highest levels during the chondrogenic period (days 7–9) and peaked at day 9, the period corresponding to cartilage calcification. The chondrogenic period was confirmed by the high type II collagen mRNA levels only during this period. Type I collagen mRNA was high throughout endochondral bone formation, exhibiting maximal levels at day 9 with the onset of the calcification period and remains high during bone formation. MGP levels however decreased threefold during bone development and remodeling.

Expression of MGP in Normal Diploid Chondrocytes

Based on the preferential expression of MGP mRNA during chondrogenesis, the expression of MGP in relation to the development of the cartilage matrix was next examined. To ensure that the integrity of our cultures was maintained throughout the time course, phase contrast photomicrographs were taken of the cultures. Our cultures exhibited the polygonal shape characteristic of chondrocytes (Fig. 2) and organized into multilayered nodules throughout the culture, typical of cultured chondrocytes. Proteoglycan was abundant and uniformly represented throughout the extracellular matrix. Initially MGP, type I collagen, and type II collagen mRNA levels were established in primary cultures of articular chondrocytes grown in the presence or absence of 25 μg/ml ascorbic acid for 6 days (Fig. 3), at which time they were subcultivated. When these subcultivated cells were plated and grown in the presence or absence of ascorbic acid (Fig. 4), we observed that even in the absence of ascorbate, both type I and type II collagens are expressed, as they were in primary cells. These secondary chondrocytes express type II and type I collagens at constant levels throughout the time course, as well as constant MGP mRNA levels. But when the expression of these genes from cells grown in the presence of ascorbic acid were compared, a different pattern emerged. MGP mRNA levels continued to increase throughout the time course as did type I collagen while type II collagen mRNA levels decreased. We then used these conditions, the presence of ascorbate and subcultivation, to produce the synthesis of both type II and type I collagens, features of a more mature chondrocyte in the hypertrophic zone. Osteocalcin (OC) mRNA, a phenotypic marker for bone, was not detectable at any time in the chondrocyte cultures, indicating the absence of osteoblasts.

In primary cultures, MGP mRNA was present at a constant level throughout the culture through day 20. In subcultivated chondrocytes, a different pattern was produced (Fig. 5). At earlier times in culture, the levels of MGP mRNA remained fairly constant and thereafter increased three to four fold when type I collagen mRNA was maximally expressed, suggesting a relationship to the formation of type I collagen.

MGP mRNA was not related to the early proliferative period as reflected by expression of the cell growth–associated H4 histone gene

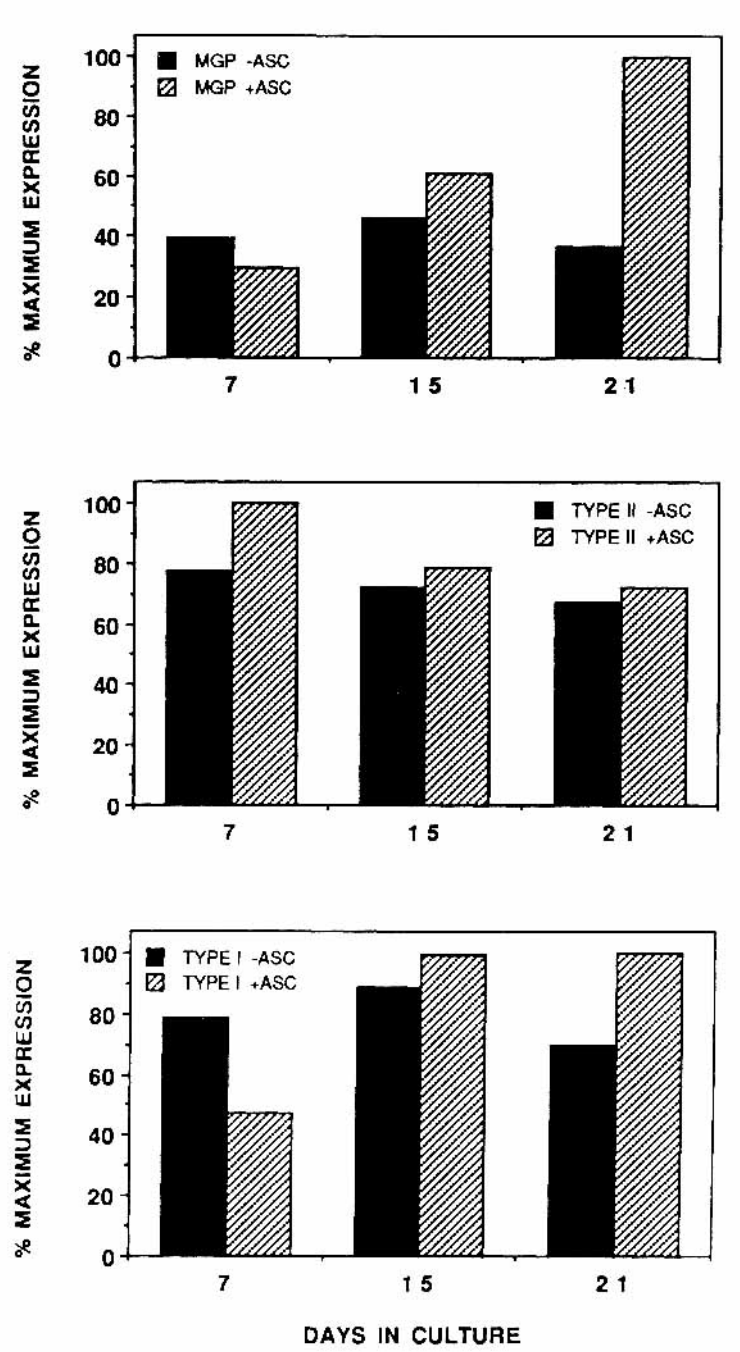

Fig. 4. Temporal expression of MGP, type II collagen (Type II), and type I collagen (Type I) genes in subcultivated diploid chondrocytes grown in the presence or absence of ascorbic acid. Chondrocytes were subcultivated and grown in DMEM, 10% FCS, and either in the presence or absence of 25 μg/ml ascorbic acid. RNA was isolated on days 7, 15, and 21 and assayed for MGP, type II collagen, and type I collagen transcripts by Northern blot or slot blot analysis. The resulting blots were quantitated by scanning densitometry and plotted. Values are expressed as % maximum average densitometric value for the experimental period for each parameter from duplicate slot blots. −Asc, grown in the absence of ascorbic acid; +Asc, grown in the presence of ascorbic acid.

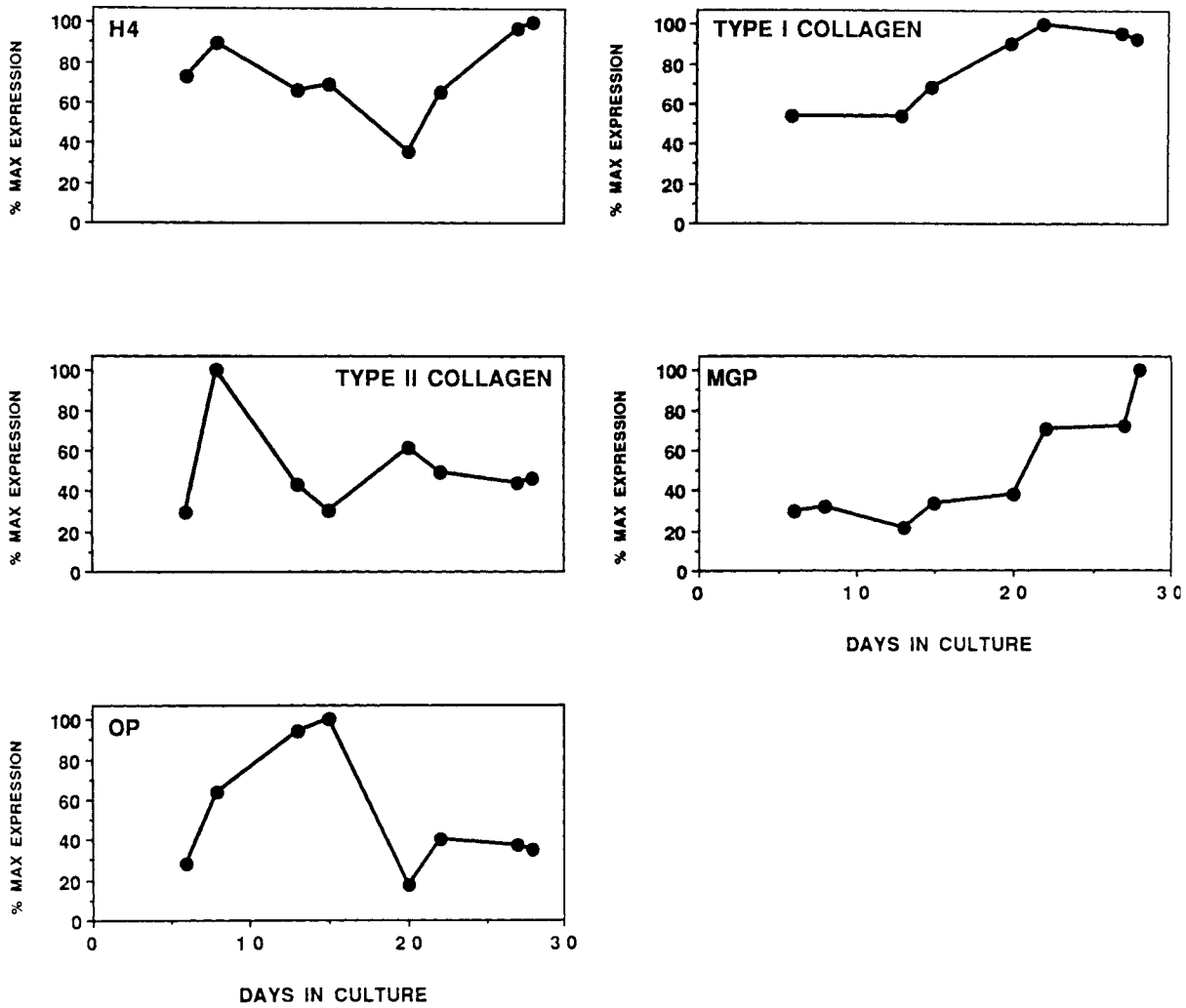

Fig. 5. Temporal expression of H4 histone (H4), type II collagen (Type II), osteopontin (OP), type I collagen (Type I), and MGP genes in normal subcultivated diploid chondrocyte cultures. Chondrocytes were subcultivated and grown in DMEM, 10% FCS, and 25 μg/ml ascorbic acid. Cellular RNA was isolated at different times during the 28 day culture period and assayed for the steady state levels for the various transcripts by Northern blot or slot blot analysis. The resulting blots were quantitated by scanning densitometry and the results plotted relative to the maximal expression of each transcript. Values are expressed for each parameter as densitometric averages from duplicate experiments.

which is coupled to DNA synthesis [14]. The second passage rat chondrocytes in the presence of ascorbate exhibit two proliferative stages: one early in the culture (day 8) characterized by high levels of type II collagen mRNA and osteopontin (OP) mRNA and another later in the culture (day 28) when OP levels have declined but MGP and type I collagen mRNA levels become maximally expressed.

MGP Gene Expression During the Development of the Osteoblast Phenotype

To further examine a relationship between MGP expression and the formation of the bone type I collagen matrix, MGP mRNA was evaluated in primary cells of normal diploid rat osteoblast cultures (Fig. 6). The progression of isolated normal osteoblasts through the developmental sequence of cellular differentiation is characterized by the temporal expression of genes which characterize three periods: proliferative period, matrix maturation period, and a mineralization period. The proliferative period is indicated by high levels of histone mRNA, reflecting the well-known coupling between histone gene expression and DNA synthesis. Also during this period, type I collagen mRNA peaks and thereafter declines, while type I collagen protein steadily accumulates in the extracellular

matrix. Examination of MGP gene expression reveals that MGP mRNA levels continue to increase with the formation of the collagen matrix. MGP mRNA was found to be expressed in the early proliferative period in contrast to OC expression but similar to OP expression. MGP mRNA levels like OC mRNA levels increased during the 30 day culture period to highest expression in mineralized cultures. This is in contrast to the decline in OP mRNA.

To further substantiate the finding that increased levels of MGP gene expression were related to the formation of a type I collagen matrix and not necessarily to the formation of a mineralized matrix, osteoblasts were grown in BGJb medium alone, or BGJb medium with 50 μg/ml ascorbic acid, or BGJb medium with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate. Ascorbic acid is a necessary cofactor in the formation of mature fully hydroxylated collagen fibers. We have previously demonstrated a dose dependent effect of ascorbic acid on collagen synthesis which is essential for bone tissue formation in vitro [12,13]. β-glycerol phosphate is a source of phosphate which promotes mineralization in osteoblasts. The temporal expression of MGP from osteoblasts between days 8 and 18 (Fig. 7) maintained in the three different medium conditions was examined and MGP mRNA was expressed in cells grown in all conditions. Cells grown in only BGJb medium expressed MGP at somewhat constant and lower levels than in the presence of ascorbic acid. Cells grown with the ascorbic acid addition had increasing amounts of MGP mRNA levels throughout this period. These two culture conditions never led to the expression of osteocalcin mRNA through day 18. However, when cells were grown under mineralizing conditions, MGP expression increased to a constant steady level between days 8 and 11, and osteocalcin was not expressed until day 15, the onset of mineralization. This further indicates that mineralization is not required for the induction of MGP expression.

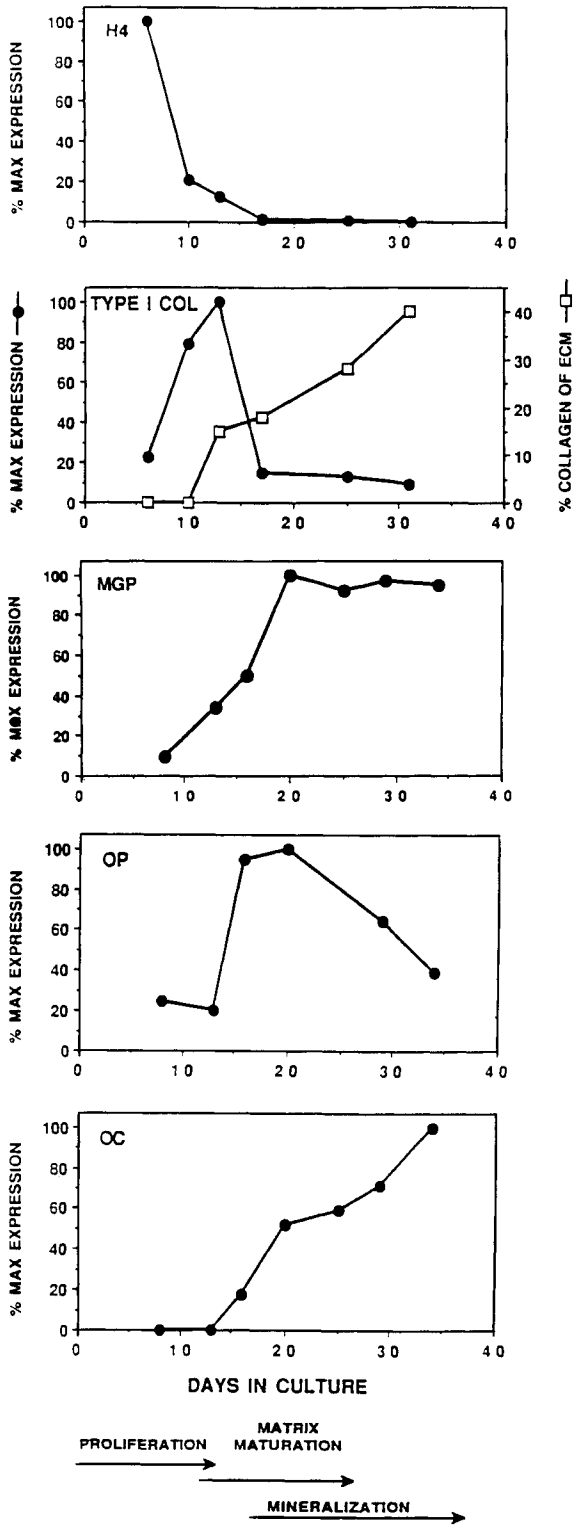

Fig. 6. Temporal expression of H4 histone (H4), type I collagen (Type I Col), MGP, osteopontin (OP), and osteocalcin (OC) genes during the development of the osteoblast phenotype in vitro. Primary isolated cells were cultured after confluence in BGJb medium supplemented with 10% FCS, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate. Cellular RNA was isolated throughout the time course and assayed for the steady state levels of the transcripts indicated by Northern blot or slot blot analysis. The resulting blots were quantitated by scanning densitometry and the results from triplicate experiments plotted as the maximal expression of each transcript. Percent collagen accumulated in the extracellular matrix (—□—) was determined from the hydroxyproline concentration in total amino acid analysis of the cell layers. The periods of activity of cells reflecting progressive expression of the osteoblast phenotype are indicated by arrows at the bottom of the figure.

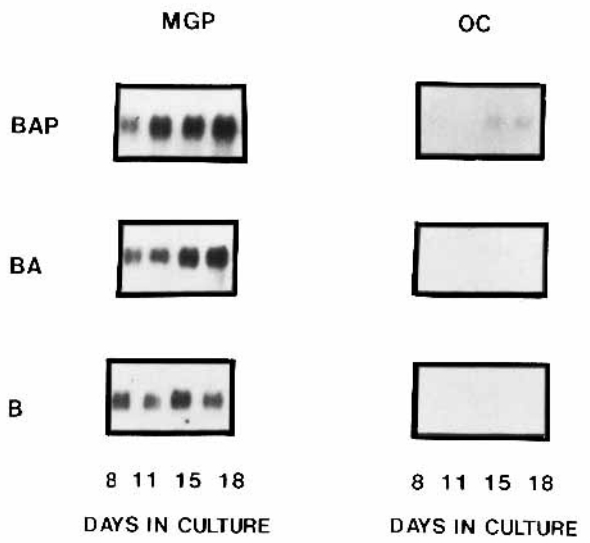

Fig. 7. Early temporal expression of MGP and osteocalcin (OC) genes in osteoblasts cultured under conditions where the extent of extracellular matrix biosynthesis and mineralization are modified. Primary osteoblasts were cultured after confluence in BGJb medium with 10% FCS (B; no accumulation of collagen), or BGJb medium with 10% FCS and 50 μg/ml ascorbic acid (BA; extracellular matrix formation, not mineralized on day 18), or BGJb medium with 10% FCS, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate (BAP; accelerated mineralization begins on day 15). Cellular RNA was isolated on days 8, 11, 15, and 18 and 10 μg of RNA assayed for steady state levels of the transcripts by Northern blot analysis. This is representative of duplicate experiments. MGP levels are constant in B, increase with formation of the matrix (BA), and are present at a higher constant level in BAP cultures. Note the appearance of OC mRNA in BAP cultures between days 15 and 18, the onset of mineralization, and its absence in BA and B groups.

To determine which of our cultures, subcultured chondrocytes, or primary osteoblasts expressed more MGP mRNA, RNA was extracted on day 6 and day 28 from these cells. Equal amounts were assayed by the slot blot procedure and examined for MGP gene expression (Fig. 8). On day 6, osteoblasts and chondrocytes expressed similar amounts of MGP mRNA. However, on day 28, osteoblasts expressed two times as much MGP message as chondrocytes. Both cultures expressed type I collagen and not surprisingly, osteoblasts expressed more type I collagen mRNA, thus supporting a relationship between MGP mRNA levels and production of type I collagen matrix.

The Effect of Vitamin D on MGP Gene Expression in Normal Osteoblast and Chondrocyte Development

The effect of acute (48 h exposure) treatment of 10^{-8} M 1,25-$(OH)_2D_3$ on MGP gene expression during osteoblast and chondrocyte growth and development were examined. Figure 9 illustrates the MGP mRNA levels in vitamin D treated and untreated osteoblast and chondrocyte cultures as well as the ratios of MGP mRNA levels of vitamin D treated cells over untreated cells. MGP mRNA levels were significantly elevated by hormone treatment throughout the time course in both osteoblast and chondrocyte cultures. Examination of the ratio of vitamin D/control MGP mRNA levels, reveals that MGP expression was stimulated by vitamin D to a greater extent during early culture times when endogenous basal levels were lower than in later culture times.

To begin addressing molecular mechanisms that may be associated with vitamin D effects on MGP gene expression, MGP mRNA transcripts were analyzed by Northern blot analysis followed by electrophoretic fractionation. However, in this instance, a high resolution agarose-formaldehyde gel was used, since a very broad band was observed for MGP mRNA transcripts from vitamin D treated chondrocytes run on 1% agarose-formaldehyde gel (Fig. 10). Vitamin D treatment of chondrocyte cultures revealed two

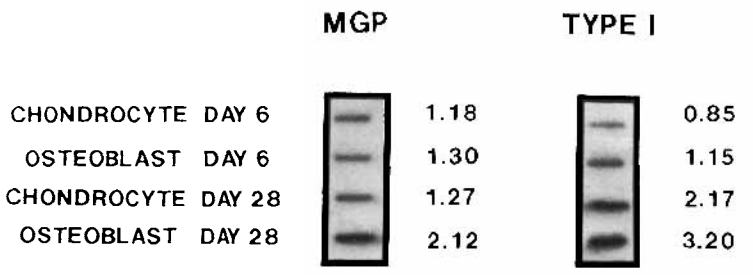

Fig. 8. Relative expression of MGP and type I collagen (Type I) genes in normal chondrocytes and osteoblasts on days 6 and 28. Cellular RNA was isolated from subcultivated chondrocytes and primary osteoblasts on days 6 and 28. Three μg of each were assayed by slot blot analysis. The resulting blots were quantitated by scanning densitometry and the densitometric units indicated. This figure is representative of duplicate experiments.

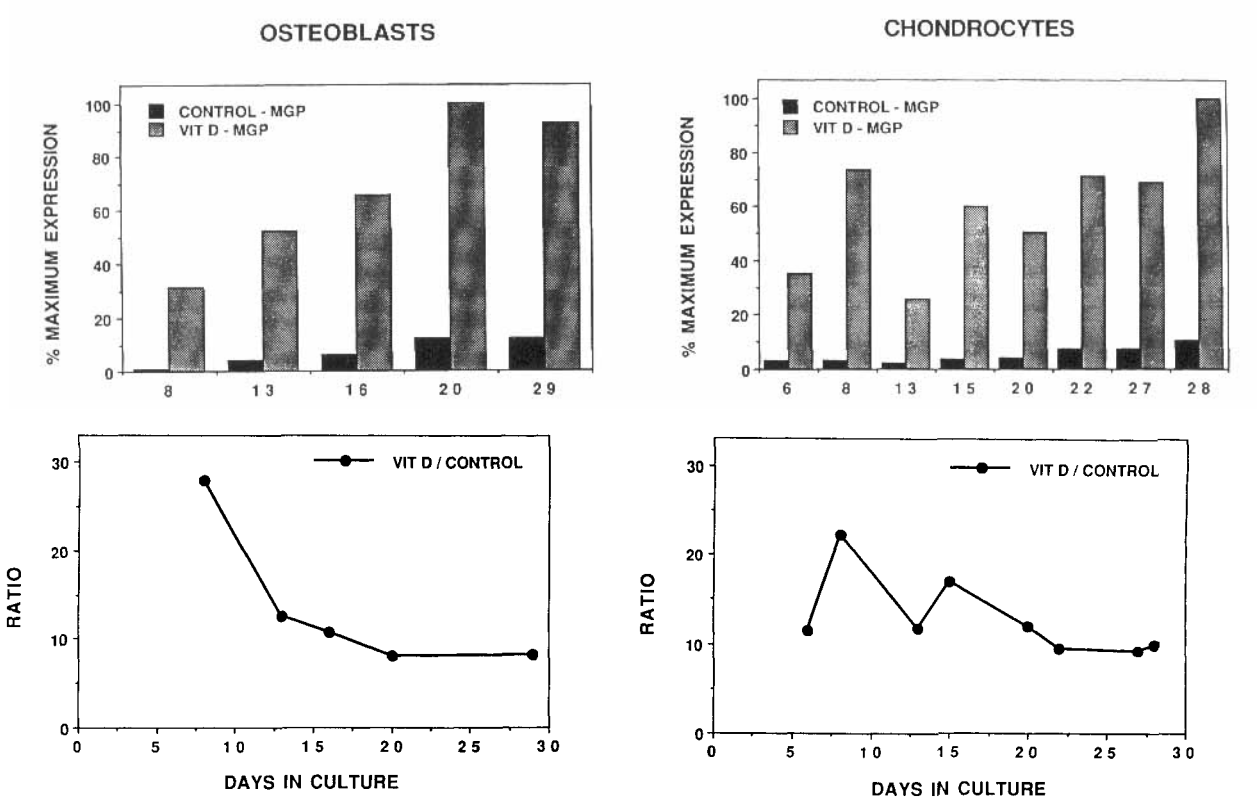

Fig. 9. Effect of 1,25-$(OH)_2D_3$ treatment on the expression of MGP gene in normal diploid osteoblast and chondrocyte cultures. Total RNA was extracted from osteoblast and chondrocyte cultures treated (vit D) or not treated (Control) with 10^{-8} M 1,25-$(OH)_2D_3$ for 48 h and then assayed for the steady state levels of MGP by Northern blot analysis or slot blot analysis. The blots were quantitated by scanning densitometry and the results plotted relative to the maximal expression of MGP transcript. Values are expressed as the densitometric average from duplicate experiments. Also shown are the ratios of MGP mRNA levels of vitamin D treated cells to mRNA levels of untreated cells (Vit D/Control).

distinct species of MGP mRNA at all time points throughout the culture. In contrast, only a single MGP mRNA transcript was detected in the vitamin D treated osteoblasts. We also observed a single MGP mRNA transcript in untreated chondrocyte and osteoblast cultures (data not shown). Primer extension analysis indicated a single transcription start site in both Vitamin D treated osteoblast and chondrocyte cultures (Fig. 10). This suggests that the lower molecular weight MGP message in the vitamin D treated chondrocyte cultures reflects a modification in post-transcriptional processing.

DISCUSSION

The present studies have shown that MGP expression in the in vivo endochondral bone formation model system was expressed at the highest level during cartilage formation and calcification. In vitro models of bone and cartilage development further showed that MGP mRNA levels increased after the development of the extracellular collagenous matrix. Such evidence suggests that the selective expression of MGP as quantitated by mRNA levels in cartilage and bone tissues may be related to the development of a collagenous matrix as supported by the observed increases in MGP mRNA during these periods in the in vitro culture systems and the in vivo model system.

It has been well documented that endochondral bone development in the DBP implant model system follows distinct stages [25–29]. The time points selected in this study were chosen to demonstrate the progressive stages of endochondral bone formation in the DBP implants [29]. Day 7 corresponds to the formation of cartilage which calcifies by day 9. On day 11, bone is formed and by day 15, bone formation is complete and the onset of bone remodeling is observed. High levels of MGP mRNA expression during the stage of cartilage calcification in this

DBP model system indicate that MGP may provide a developmental marker for chondrogenesis. The production of type II collagen mRNA was highest on days 7 and 9 while the levels of type I collagen mRNA were high on day 7 and elevated further at day 9, similar to MGP. This suggested that the expression of MGP may be related to the development of the extracellular collagenous matrix in cartilage and bone.

These results were further substantiated when MGP expression was examined in chondrocyte cultures. MGP mRNA levels increased with time in culture in these rat chondrocytes. The levels of MGP mRNA remained fairly constant at early times in culture and increased three- to fourfold when type I collagen mRNA was maximally expressed. These data further suggest that MGP mRNA expression increased with formation of a type I extracellular collagenous matrix.

Because these chondrocytes were derived from fetal cartilagenous tissue (epiphysis), the cellular origin of MGP in these chondrocyte cultures can be questioned. Although the chondrocytes exhibited typical polygonal morphology throughout the time course, it is likely that some of the cells derived from fetal articular cartilage could include undifferentiated progenitor cells. Organ cultures of mandibular condyles have demonstrated osteogenic differentiation of progenitor cells and hypertrophic chondrocytes to osteoblast-like cells [30,31]. Type I collagen mRNA as well as the presence of osteopontin mRNA suggests promotion of the osteogenic phenotype. However, the decrease of osteopontin mRNA while type I collagen mRNA increases is inconsistent with the pattern of osteopontin expression with osteogenesis (Fig. 5) [32]. Rather the profile of osteopontin mRNA more closely parallels type II collagen mRNA and H4 histone mRNA and may reflect other properties of osteopontin. Osteopontin (2ar) has been shown to be expressed by a number of osteogenic and nonosteogenic cell types [33]. Osteopontin has been shown to be related to oncogenic transformation [34–37] as well as processes related to osteoblast

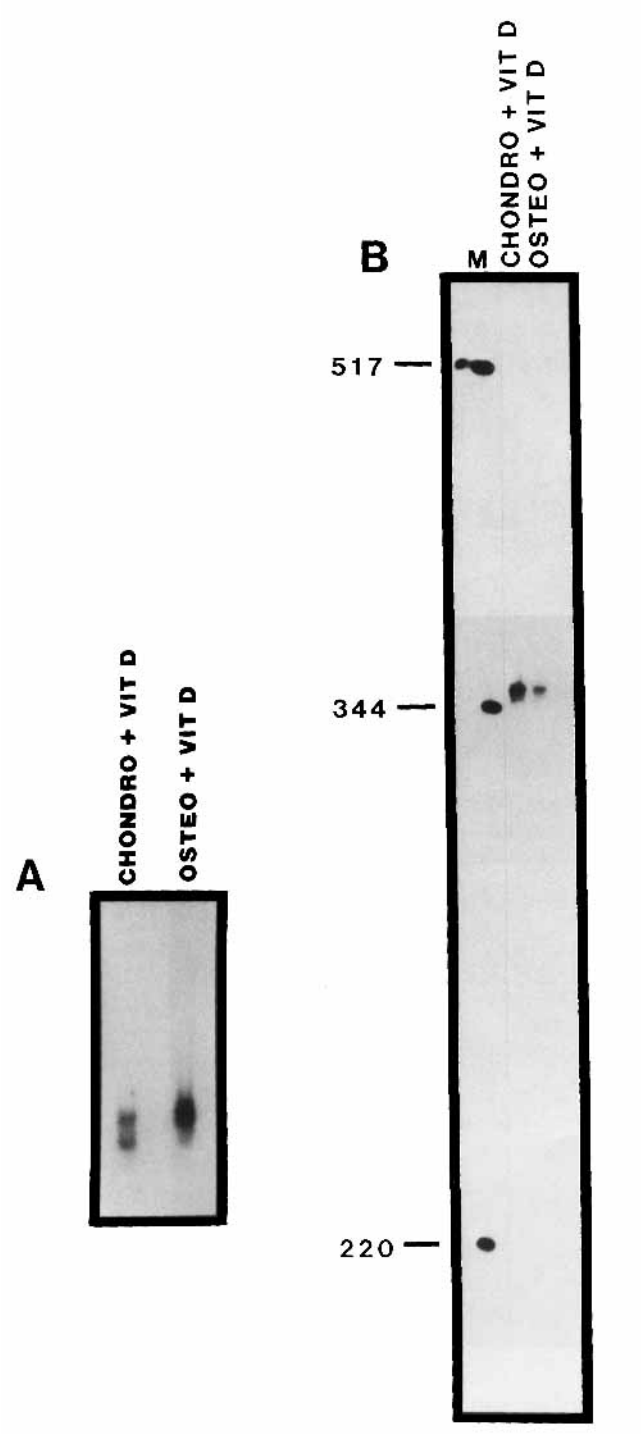

Fig. 10. High resolution Northern blot analysis (**A**) of MGP message and primer extension analysis (**B**) of RNA extracted from chondrocyte and osteoblast cultures treated with 10^{-8} M 1,25-$(OH)_2D_3$ for 48 h. Shown is a representative time point (day 20) for chondrocyte and osteoblast cultures. RNA was extracted and 10 μg of total RNA was run on a 1.5% formaldehyde-agarose gel and then blotted onto Zeta-Probe membrane. For primer extension analysis, 10 ng of a 33 nucleotide synthetic MGP primer was ^{32}P-5′ end labeled and mixed with 10 μg total cellular RNA. The reaction products were analyzed by electrophoresis through 7.0 M urea 10% polyacrylamide gels. Size markers were ^{32}P-labeled HinF1 of pBR322. This figure is representative of triplicate experiments. Lighter exposures confirmed only a single band for the vitamin D treated osteoblast mRNA hybridized to MGP in A.

adhesion during bone development [38–40]. Since osteopontin is found in our chondrocyte cultures, its presence may be related to an adaptation of growth in culture or to expression of the chondrocyte phenotype. Antibody localization has shown its presence in calcified cartilage [41]; however, in situ hybridization studies demonstrated nondetectable osteopontin mRNA in cartilage [33].

In further support that the majority of cells maintained the chondrocyte phenotype, none of the cells stained for alkaline phosphatase (data not shown), and moreover osteocalcin, a bone specific marker, was never expressed. It also has been shown that type I collagen, the major constituent of bone matrix, is expressed in a variety of other cell types, including chondroprogenitor cells [42,43]. It also has been demonstrated by in situ hybridization that type I collagen mRNA is expressed although at very low levels in resting, proliferating, and hypertrophic regions of chondrocytes in chick growth cartilage [44]. The presence of type I collagen mRNA in our rat chondrocytes simply may be due to the fact that the cells were grown in the presence of ascorbic acid or were subcultivated. It has been shown that when chondrocytes are grown in the presence of ascorbic acid and/or subcultivated they will express type I collagen [45–47]. The existence of type I collagen mRNA was probably not derived from a contamination of fibroblasts. Since no MGP mRNA is found in fibroblasts (unpublished observation), the MGP mRNA was probably derived from the chondrocytes and appears to be related to the development of the extracellular matrix.

The ordered developmental sequence of cellular differentiation and temporal expression of genes characterizing the osteoblast phenotype during the production of the extracellular bone tissue-like matrix and organized mineralized nodules has been reported [13]. The development of the osteoblast phenotype includes first, a period of proliferation in which high levels of histone genes and type I collagen are expressed, followed by periods of matrix maturation and mineralization. In this study, MGP mRNA was detected in rat osteoblast cultures during the proliferative period, increased during the period of matrix development and maturation, and then remained at these elevated levels during the mineralization period. These results are in contrast to the results observed when osteocalcin mRNA was investigated. Osteocalcin was not expressed during the period of osteoblast proliferation; however, its expression increased during osteoblast matrix maturation and peaked later than MGP during the matrix mineralization period.

The observed differences between MGP (expressed in proliferating cells) and osteocalcin (expressed post-proliferatively) mRNAs during the rat osteoblast development sequence as well as the expression of osteocalcin exclusively in bone as compared to the more widely distributed MGP may have to do with the organization of the promoter of the two genes [20,48–53]. AP1 sites within the vitamin D responsive element (VDRE) and surrounding the CCAAT box of the osteocalcin gene promoter have been proposed to suppress the expression of the osteocalcin gene in osteoblasts and possibly non-osseous cells [52,53]. Since this arrangement of AP1 sites is not observed in the hormone responsive and basal regulatory elements identified in the MGP gene promoter, the organization of this promoter may be compatible with MGP expression in proliferating osteoblasts as well as other cell types, in contrast to osteocalcin.

While MGP gene expression was maximally expressed during the mineralization period, its expression increased only with the formation of an extracellular matrix as observed when osteoblasts were in BGJb medium supplemented with ascorbic acid, compared to cells maintained in BGJb medium alone. When ascorbic acid, required for mature collagen matrix formation, was added to the medium, there was an induction of MGP mRNA expression throughout the time course. While the addition of β-glycerol phosphate, which promotes mineralization, accelerated the differentiation process, mineralization per se was not an absolute requirement for induced expression of MGP, as is osteocalcin. It was during the mineralization period that we observed constant but high levels of MGP mRNA. In addition, when osteoblasts and chondrocytes were analyzed on the same slot blot, the relationship between MGP expression and type I collagen expression was further substantiated. We observed that the increase in MGP expression in osteoblasts and chondrocytes was paralleled by the increase in type I collagen mRNA.

The steroid hormone, 1,25-$(OH)_2D_3$, dramatically increased MGP expression in both osteo-

blasts and chondrocytes in acute treatments (48 h) throughout the time course. Moreover, its stimulatory effect was more pronounced during earlier culture times than in later culture times in both the cultures. 1,25-$(OH)_2D_3$ has been shown to be a potent inducer of differentiation in several model systems [54–60]. Recently, our laboratory examined the effect of chronic treatment with 1,25-$(OH)_2D_3$ on osteoblast growth and differentiation [61]. The results demonstrated differential responses of osteocalcin and MGP expression to the hormone. When hormone treatment was initiated during the proliferation period (day 6), MGP expression was stimulated to the same extent throughout the time course of 30 days as was seen with acute vitamin D treatment in these studies. However, with chronic treatment, extracellular matrix mineralization was inhibited as well as the subsequent expression of osteocalcin, which is normally expressed during the period of extracellular matrix mineralization. Thus MGP induction by vitamin D occurs in cultured cells exposed to both acute and chronic treatments, supporting the concept that its expression is independent of mineralization.

The observation that there exist two distinct species of MGP mRNA in only the vitamin D treated chondrocytes remains puzzling. Primer extension analysis revealed that there was a single transcription start site for both the osteoblast and chondrocyte cultures treated or not treated with vitamin D. Therefore, the lower molecular weight MGP message in the vitamin D treated chondrocytes reflects a modification in post-transcriptional processing. Alternative splicing, termination, processing, or polyadenylation is possible.

The two mRNA species induced selectively in chondrocytes may also reflect different tissue specific functions in response to the hormone. While the precise functions for MGP in cartilage, bone, and other tissues remain to be established, its selective accumulation in bone and cartilage may be related to the development of a collagenous matrix. The expression of MGP during the early development of a collagenous matrix may provide a signal for subsequent events in the development of mineralized tissues.

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