Collagenase-3 mRNA is initially detectable when osteoblasts cease proliferation, increasing during differentiation and mineralization. We showed that this developmental expression is due to an increase in collagenase-3 gene transcription. Mutation of either the activator protein-1 or the runt domain binding site decreased collagenase-3 promoter activity, demonstrating that these sites are responsible for collagenase-3 gene transcription. The activator protein-1 and runt domain binding sites bind members of the activator protein-1 and core-binding factor family of transcription factors, respectively. We identified core-binding factor a1 binding to the runt domain binding site and JunD in addition to a Fos-related antigen binding to the activator protein-1 site. Overexpression of both c-Fos and c-Jun in osteoblasts or core-binding factor a1 increased collagenase-3 promoter activity. Furthermore, overexpression of c-Fos, c-Jun, and core-binding factor a1 synergistically increased collagenase-3 promoter activity. Mutation of either the activator protein-1 or the runt domain binding site resulted in the inability of c-Fos and c-Jun or core-binding factor a1 to increase collagenase-3 promoter activity, suggesting that there is cooperative interaction between the sites and the proteins. Overexpression of Fra-2 and JunD repressed core-binding factor a1-induced collagenase-3 promoter activity. Our results suggest that members of the activator protein-1 and core-binding factor families, binding to the activator protein-1 and runt domain binding sites are responsible for the developmental regulation of collagenase-3 gene expression in osteoblasts.

Matrix metalloproteinases play an essential role in physiological processes of tissue remodeling, including embryonic development, bone remodeling, ovulation, uterine involution, and wound healing (1–2), and in pathological states such as rheumatoid arthritis and tumor invasion and metastasis (3–5). Recent studies have identified a novel matrix metalloproteinase from human breast carcinoma cells, collagenase-3 (matrix metalloproteinase-13) as an important metalloproteinase (6). Studies demonstrating a diminished response to PTH-induced bone resorption in collagenase-resistant mice implicate a role for collagenase-3 in the bone remodeling process (7). Collagenase-3 is a neutral metalloproteinase that can degrade types I, II and III fibrillar collagens and has been implicated in several disease states requiring the remodeling of extracellular matrices. Collagenase-3 has been detected in vivo in degenerative bone diseases including osteoarthritis and rheumatoid arthritis (4, 8, 9) as well as in several metastatic tumors including breast carcinomas (6), chondrosarcomas (10), and head and neck carcinomas (11). In addition, collagenase-3 has been detected during human fetal ossification (12, 13) and during murine fetal bone development (14), where it is likely to play an important role in bone development.

Humans express three collagenases, fibroblast collagenase (collagenase-1 or matrix metalloproteinase-1), neutrophil collagenase (collagenase-2 or matrix metalloproteinase-8), and collagenase-3, with each collagenase having preferential activity toward a specific collagen subtype. Currently, there has been one identified collagenase in rat and mouse (15, 16) that was found to be homologous to human collagenase-3 (17). The expression of collagenase-3 is regulated by both bone-resorbing and bone-forming agents. Parathyroid hormone (PTH) has been shown to increase both collagenase-3 mRNA (18) and protein secretion in the UMR 106-01 rat osteoblastic osteosarcoma cell line (19). Interleukin-1 and interleukin-6 up-regulated collagenase-3 expression in mouse calvariae (20), while interleukin-1 and transforming growth factor β were shown to increase expression in human fibroblasts (21, 22). In contrast, agents that promote bone formation, such as bone morphogenetic proteins and insulin-like growth factors decrease collagenase-3 mRNA expression in rat osteoblast cultures (23–26). Although collagenase-3 has been implicated in processes involving both normal and pathological remodeling of bone, little is known about the mechanisms involved in the regulation of this gene during development.

Earlier studies in rat osteoblasts showed that collagenase-3 gene expression is minimal in proliferating osteoblasts but continues to increase in basal expression as matrix maturation and mineralization of the extracellular matrix progresses (27, 28). In order to analyze the developmental expression of the collagenase-3 gene, we chose to use normal, differentiating osteoblasts. Our laboratory and others have shown that normal osteoblasts in culture follow a similar pattern of development as osteoblasts in vivo (27). In the present study, we demonstrated that the developmental increase in collagenase-3 gene expression is the result of an increase in collagenase-3 gene transcription, showing that the collagenase-3 gene is not transcribed until osteoblasts have ceased proliferation. Similar to...
Collagenase-3 Promoter Regulation in Differentiating Osteoblasts

studies in other systems (22, 29–31), our studies indicated that members of the activator protein-1 (AP-1)1 and core-binding factor (Cbfa) family of transcription factors working through the AP-1 and runt domain (RD) binding sites regulate collagenase-3 promoter activity in normal rat osteoblasts. However, our studies suggest a new and different role in the regulation of collagenase-3 promoter activity by AP-1 transcription factors. Through overexpression studies, we show that although c-Fos and c-Jun can synergistically increase collagenase-3 promoter activity in the presence of Cbfa1, Fra-2 and JunD repress Cbfa1-induced collagenase-3 promoter activity. This suggests that AP-1 proteins can have both inductive and repressive effects on collagenase-3 promoter activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Parathyroid hormone (rat PTH 1–34) was purchased from Sigma. Radiolabeled [3H]chlorpromazine was purchased from Amersham Pharmacia Biotech, and other radionuclides were obtained from NEN Life Science Products. Synthetic oligonucleotides were synthesized by Midland Certified Reagent Company (Midland, TX). Tissue culture media and reagents were obtained from Washington University Tissue Culture Center (St. Louis, MO).

**Antibodies**—Anti-Fos, pan-Fos, anti-Fra-1, anti-Fra-2, anti-FosB, anti-Jun, anti-JunB, and anti-JunD antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Cbfa1 was kindly provided by Dr. S. Hiebert (Vanderbilt University, Nashville, TN).

**Cell Culture**—Osteoblasts were isolated by the method of Shalhoub et al. (27). Osteoblasts were derived from postnatal day 1 rat calvariae by sequential digestions of 20, 40, and 90 min at 37 °C in 2 mg/ml collagenase A, 0.25% trypsin. Cells from digests one and two were discarded. Cells from the third digest were plated at 6.4 × 10⁶ cells/cm² and grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). After reaching confluence (day 7), the medium was switched to BGM, with 10% FBS containing 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate to allow for initiation of mineralization. Medium changes were performed every 2 days.

**RNA Isolation and Northern Blot Analysis**—Osteoblasts at various stages of development were treated with either 2% FBS/MEM or 10 mM PTH for 2 h. Total RNA was isolated using the QIAGEN RNaseasy Mini kit. Ten µg of each sample was electrophoresed on a 1.0% agarose, 2.2 M formaldehyde gel in 40 mM MOPS, pH 7.0, 10 mM formalde-3-phosphate dehydrogenase cDNA was purchased from ATCC and subcloned into pBluescript. Collagenase-3 cDNA is the rat collagenase cDNA clone UMRCase54 in pBluescript (Stragatafani) previously described (16). The LS2 plasmid contains cDNA for 18 S ribosomal RNA (33). Glyceraldehyde-3-phosphate dehydrogenase transcript levels. Filters were analyzed by autoradiography and quantitated using a PhosphorImager. Results are normalized to glyceraldehyde-3-phosphate dehydrogenase transcript levels.

**Fluorescent DNA Binding Assay**—Fluorescent DNA binding assay was performed as described in detail by Dignam et al. (38). Osteoblasts were plated at 6.4 × 10⁵ cells/cm² in six-well plates. After 4 days of culture, proliferating osteoblasts were transfected with collagenase-3 promoter deletion and mutation constructs (see Fig. 3) using the Superfect transfection reagent (QIAGEN). DNA solutions were prepared maintaining a ratio of 5:1 Superfect:DNA. One µg of DNA/5 µl of Superfect was diluted into 100 µl of serum-free MEM. These solutions were incubated at room temperature for 10 min. Cells were washed with PBS, pH 7.4, and 600 µl of 10% FBS/MEM was added to each well of a six-well plate. DNA solutions were added to each plate and incubated at 37 °C for 2 h. Cells were then washed with PBS, pH 7.4, fresh medium was added, and cells were allowed to recover overnight. Cells were treated for 24 h with control medium or medium containing 20% FBS. Cells were lysed in 100 µl of Reporter lysis buffer (Promega), and cellular debris was removed by centrifugation (12,000 × g, 1 min) and assayed for CAT activity.

**CAT Assays**—CAT activity was measured by the method of Seed and Shen (36). Fifty µl of lysate was measured in a reaction mixture containing 250 µl reporter vector containing Cbfa1, Fra-2 and JunD antibodies. The fractions were separated and the spent medium was added to 200 µl of Reporter lysis buffer (Promega), and cellular debris was removed by centrifugation (12,000 × g, 1 min) and assayed for CAT activity.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared by a modification of the method of Dignam et al. (38). Osteoblasts were treated with 1–2 h with control or 10 µM PTH-containing medium. Cells were scraped in PBS and pelleted by centrifugation at 200 × g for 10 min at 4 °C. Pelleted cells were then resuspended in 300 µl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol) and incubated on ice for 10 min. Cells were lysed by 30 strokes in a glass Dounce homogenizer. The homogenate was checked to ensure complete lysis and centrifuged at 500 × g for 10 min to pellet nuclei. The pellet was resuspended in 100 µl of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.20 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol) and homogenized with 10 strokes with a microhomogenizer. The resulting suspension was incubated in a shaking ice bath for 10 min. The reaction was briefly centrifuged to remove cellular debris, and the supernatant was used in gel mobility shift assays.

**Gel Mobility Shift Assays**—Approximately 5–10 µg of nuclear protein was incubated in a volume of 20 µl containing binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM dithiothreitol, 50 mM KCl, 10 mM Tris-HCl, pH 7.4), 100 ng/µl poly(dI-dC), and antisera or competitor DNA at

1 The abbreviations used are: AP, activator protein; PTH, parathyroid hormone; RD, runt domain; MEM, minimum essential medium; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; MOPS, 4-morpholinepropanesulfonic acid; CMV, cytomegalovirus; TTBS, Tween-Tris-buffered saline.
Collagenase-3 Promoter Regulation in Differentiating Osteoblasts

Osteoblasts derived from postnatal day 1 rat calvariae were grown in 100-mm dishes in MEM, 10% FBS to confluence (day 7), after which the cells were switched to mineralizing medium (BGL, 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate). Normal, differentiating osteoblasts were treated for 4 h in the presence or absence of 10−8 M PTH-containing medium at days 5, 7, 14, 21, and 28 of culture. Collagenase-3 mRNA was detected by Northern analysis of total cellular RNA (10 μg/lane). Samples were normalized with an 18 S ribosomal RNA probe. The autoradiogram is representative of several experiments with similar results.

Western Blot Analysis—Osteoblasts at various stages of development were washed twice in PBS, pH 7.4, and pelleted by centrifugation at 200 × g for 10 min at room temperature. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM Triton X-100, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 mg/ml protease mixture). Cell lysates were briefly centrifuged to remove cellular debris, and then equal amounts of total protein were determined by the Bradford (37) dye binding (Bio-Rad reagent) method. SDS sample buffer was added, and the samples were boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane and blocked for 4 h in 0.1% Tween-Tris-buffered saline (TTBS) (0.1% Tween 80, 138 mM NaCl, 5 mM KCl, 25 mM Tris base) containing 5% nonfat dry milk. Exposure to primary antibody diluted 1:1000 in TTBS for 2 h at room temperature. Membranes were washed three times in 0.1% TTBS for 15 min each. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RESULTS

Expression of Collagenase-3 in Differentiating Osteoblasts—In order to establish the expression of collagenase-3 in normal, differentiating osteoblasts, total RNA was collected from cultured osteoblasts at various stages through differentiation and examined for collagenase-3 expression. Previous work had shown that collagenase-3 was expressed as a late stage differentiation gene (27). Our results confirm these findings (Fig. 1), demonstrating that collagenase-3 expression is low in proliferating osteoblasts (days 5 and 7) but begins to be expressed in differentiating cultures (day 14), reaching maximal levels of expression in mineralizing cultures (days 21 and 28). Collagenase-3 mRNA and protein expression had also been shown to be induced by treatment with PTH in osteoblastic cells (16, 18, 19, 39). In our studies in normal, differentiating osteoblasts, the results indicate that collagenase-3 mRNA expression was inducible by PTH with a slight induction in collagenase-3 levels at days 5 and 7 but with the greatest PTH-induced increase seen after collagenase-3 is expressed at basal levels from day 14 onward.

Collagenase-3 Transcription—Analysis of collagenase-3 mRNA abundance showed that there is a developmental increase in collagenase-3 gene expression but does not indicate whether this is a result of a developmental increase in transcriptional activation of the gene. In order to determine if the increase in collagenase-3 gene expression is due to an increase in rate of transcription of the collagenase-3 gene, nuclear run-on analysis was performed in both proliferating (day 5) and differentiating and mineralizing (day 21) osteoblasts, and results were compared. There is minimal transcription of the collagenase-3 gene occurring in proliferating osteoblasts; however, as osteoblasts differentiate and begin to mineralize the extracellular matrix there is a substantial increase in transcription (Fig. 2A). PhosphorImager analysis of PTH-treated osteoblasts indicated that the increase in collagenase-3 mRNA results from an increase in collagenase-3 transcription (Fig. 2B). These results demonstrate that the developmental increase in collagenase-3 mRNA is due to an increase in the rate of transcription of the collagenase-3 gene.

Identification of the Collagenase-3 Promoter Elements Involved in the Developmental Regulation of Collagenase-3 Gene—Since results show that the increase in collagenase-3 mRNA is due to an increase in transcription of the gene, we next determined the upstream collagenase-3 promoter elements that are involved in developmental regulation (Fig. 3). The collagenase-3 promoter region contains consensus binding sites for transcription factors such as AP-1, Oct1, Oct2, and Oct4, which are all involved in the regulation of collagenase-3 expression in osteoblasts. Previous studies had shown that collagenase-3 was expressed as a late stage differentiation gene (27). Our results confirm these findings (Fig. 1), demonstrating that collagenase-3 expression is low in proliferating osteoblasts (days 5 and 7) but begins to be expressed in differentiating cultures (day 14), reaching maximal levels of expression in mineralizing cultures (days 21 and 28).

FIG. 1. Collagenase-3 expression in control and PTH-treated cultures of differentiating osteoblasts. Osteoblasts derived from postnatal day 1 rat calvariae were grown in 100-mm dishes in MEM, 10% FBS to confluence (day 7), after which the cells were switched to mineralizing medium (BGL, 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate). Normal, differentiating osteoblasts were treated for 4 h in the presence or absence of 10−8 M PTH-containing medium at days 5, 7, 14, 21, and 28 of culture. Collagenase-3 mRNA was detected by Northern analysis of total cellular RNA (10 μg/lane). Samples were normalized with an 18 S ribosomal RNA probe. The autoradiogram is representative of several experiments with similar results.

FIG. 2. Nuclear run-on analysis of proliferating and mineralizing osteoblasts. A, proliferating (day 5) and mineralizing (day 21) osteoblasts were treated with or without 10−8 M PTH for 2 h (only the untreated signal is shown in A), and nuclei were isolated and subjected to nuclear run-on analysis. B, data were quantitated by PhosphorImager analysis and are presented as the ratio of collagenase-3 to glyceraldehyde-3-phosphate dehydrogenase

TABLE 1

| Sequences 1 and 2 |
|-------------------|
| Wild type AP-1 site | CCAAGTGCTCAGCACTCATCTAT |
| Wild type RD site  | GGGTGCACTCTAGTGATAGTA |
sites for several transcription factors including CCAAT enhancer-binding protein, RD, p53, polyomavirus enhancer activator-3, AP-2, and AP-1 (17, 31, 40). There are four consensus sites that are conserved between rat, mouse, and human collagenase-3 promoters, acute myelogenous leukemia-1/polyomavirus enhancer-binding protein-2/Cbfa/runt domain (or osteoblast-specific element-2 or RD), p53, polyomavirus enhancer activator-3, and AP-1. Analysis of the collagenase-3 promoter in the rat osteoblastic osteosarcoma cell line, UMR 106-01, had previously determined that the minimal PTH-responsive region in the collagenase-3 promoter was located within the first 148 base pairs of the transcriptional start site without a significant loss in collagenase-3 promoter activity (31). Transient transfection studies in normal osteoblasts (day 5) using rat collagenase-3 promoter deletion constructs ranging from -6500 down to -148 indicated that as constructs were further deleted basal CAT activity remained similar to that of the -6500 construct (data not shown). These results suggest that 148 base pairs of upstream sequence is the minimal region required for transcriptional activity in normal, differentiating osteoblasts, and since this region contains the major known regulatory elements we focused on it.

In order to further define the minimal region required for basal promoter activity in normal osteoblasts, the WT(-148) construct was further deleted (31) and transiently transfected into proliferating osteoblasts. The WT(-125) and WT(-104) constructs contain all of the above binding sites, excluding the RD binding site, while the WT(-54) construct contains only the AP-1 and CCAAT enhancer-binding protein binding sites. Further deletion of the WT(-148) resulted in a loss of basal promoter activity and PTH response (Fig. 4). These results suggest that the basal and PTH-responsive regions of the collagenase-3 promoter in proliferating osteoblasts lie within the first 148 base pairs of the collagenase-3 promoter.

In order to analyze specific response elements involved in collagenase-3 regulation, mutation constructs of the AP-1 and RD binding sites in the collagenase-3 promoter were made (Fig. 3), and the effect on CAT activity was assessed. As seen in Fig. 5, mutation of the AP-1 site resulted in a substantial loss of basal CAT activity and eliminated the PTH response. Mutation of the RD binding site also eliminated the PTH response but...
only reduced CAT activity, whereas the basal activity was almost completely abolished with the AP-1 mutation. Comparison of the M(−148R3) and WT(−125) construct, which lacks only the RD site, shows a greater loss in CAT activity with the deletion construct, but the loss is still not comparable with the loss with the M(−148A3) construct. These results suggest that the AP-1 site may play a greater role in basal expression than the RD site and that the RD site may still be partially functional in the M(−148R3) construct.

Identification of Proteins Binding to the AP-1 Site—The reduction observed in collagenase-3 promoter activity when the AP-1 and RD sites are mutated suggests that these sites are important in the regulation of collagenase-3 transcription and prompted us to investigate the proteins binding to these sites in normal, differentiating osteoblasts. Extracts prepared from both proliferating and mineralizing osteoblasts showed that there is a similar pattern of binding that can be competed out with cold competitor in both proliferating and mineralizing osteoblasts (data not shown).

To identify the proteins binding to this site during osteoblast differentiation, gel shift analysis was performed using antisera for various members of the AP-1 family of transcription factors. As indicated in Fig. 6A, JunD bound to the AP-1 site in proliferating osteoblasts resulting in a supershifted band. There also appears to be a decrease in the binding intensity following the addition of c-Fos or c-Jun antibody showing the presence of these proteins. Preincubation with a Fos antiserum that recognizes all Fos family members gives a distinct supershift that clearly demonstrates that a Fos family member is binding to this site. Gel shift analysis of differentiating (day 14) and mineralizing (day 21) osteoblasts also indicated that JunD in addition to a Fos family member was binding to the AP-1 site at all stages of differentiation (data not shown).

We next chose to directly compare Fos family members binding to the AP-1 site during osteoblast differentiation. Fig. 6B indicates that Fos family members able to bind to the AP-1 site decrease in abundance during osteoblast differentiation. Binding to the AP-1 site is high in proliferating cells, declining in differentiating and mineralizing cells. Although we obtain a supershift with the nonspecific Fos antiserum at all stages of osteoblast differentiation (data not shown), Fig. 6B demonstrates that the amount of supershift decreases dramatically with differentiation and is only just detectable compared with proliferating osteoblasts (day 7) with the same decrease in supershift observed with the JunD antiserum. These results suggest that there is a decrease in the amount of Fos family proteins able to bind to the AP-1 site of the collagenase-3 gene during osteoblast differentiation.

Developmental Expression of c-Fos and JunD Proteins—The decrease in proteins able to bind to the collagenase-3 AP-1 site during osteoblast differentiation led us to investigate the developmental expression of AP-1 factors. Since prior studies in UMR 106-01 cells indicated that c-Fos was involved in the expression of collagenase-3, we speculated that this protein
may be developmentally regulated. Through Western blot analysis, we found that c-Fos is predominantly expressed in proliferating osteoblasts (Fig. 6C). After osteoblasts cease proliferation, levels of c-Fos decline but are still detectable. These results were confirmed by Northern blot analysis (data not shown). The results suggest that c-Fos expression reaches its peak levels when collagenase-3 message first begins to be detectable.

Identification of Proteins Binding to the RD Site—Analysis of proteins binding to the RD site using nuclear extracts prepared from both proliferating and mineralizing osteoblasts showed a similar pattern in binding with one major shifted band and occasionally the appearance of an upper band that was generally more prevalent in mineralizing osteoblasts. In proliferating osteoblasts, only one shifted band was observed that could be competed out with excess unlabeled probe; however, in mineralizing osteoblasts, there were two shifted bands that could be competed out with excess unlabeled probe (data not shown).

Evidence suggests that Cbfa1 is a candidate for binding to the collagenase-3 RD site in osteoblasts. Cbfa1 has previously been shown to be involved in the regulation of osteocalcin gene expression (44, 45) and in the PTH regulation of collagenase-3 in UMR 106-01 cells (31). To test for the possibility of Cbfa1 binding to the RD binding site in the collagenase-3 promoter in normal osteoblasts, gel mobility shift analysis using antisera specific for Cbfa1 indicated that Cbfa1 was binding to the RD site in these cells (Fig. 7A). The addition of Cbfa1 antisera resulted in a supershifted band at all stages of osteoblast differentiation.

Developmental Expression of Cbfa1 Protein—Western blot analysis of Cbfa1 demonstrated that there is no change in the abundance of this protein during osteoblast differentiation (Fig. 7B). There appear to be two specific bands for Cbfa1, approximately at 32 and 47 kDa. These bands appear to be expressed at comparable levels throughout osteoblast differentiation. These data suggest that the developmental expression of the collagenase-3 gene is not occurring through a change in the abundance of the Cbfa1 protein.

AP-1 and Cbfa Proteins Stimulate Collagenase-3 Promoter Activity and Require both the AP-1 and RD Sites—To determine if the AP-1 and Cbfa proteins were important in the regulation of collagenase-3 promoter activity, we transiently transfected c-Fos, c-Jun, and Cbfa1 expression vectors into proliferating osteoblasts (Fig. 8A). Overexpression of either Cbfa1 or both c-Fos and c-Jun resulted in a significant increase in collagenase-3 promoter activity. In addition, overexpression of all three transcription factors resulted in a synergistic increase in collagenase-3 promoter activity. Mutational analysis indicated that mutation of either the RD or the AP-1 binding sites resulted in the inability of these transcription factors to increase collagenase-3 promoter activity (Fig. 8B). Consistent with previous transfections, the WT(-148R3A3) mutant exhibited minimal promoter activity in the presence of overexpressed c-Fos, c-Jun, and Cbfa1. More importantly, mutation of the RD binding site prevented c-Fos and c-Jun from increasing collagenase-3 promoter activity above basal levels. Similarly, mutation of the AP-1 site indicated that Cbfa1 could not increase collagenase-3 promoter activity above basal levels (Fig. 8B). These findings suggest that both the AP-1 and the RD binding sites and proteins are required for regulation of collagenase-3 gene expression and probably interact cooperatively.

Since gel shift analysis indicated that JunD was binding to the AP-1 site during osteoblast differentiation, we also chose to examine its protein expression during osteoblast differentiation. Western blot analysis of JunD showed that JunD was uniformly expressed throughout osteoblast differentiation (Fig. 6D). JunD expression was seen as two specific bands approximately corresponding to 38 and 43 kDa (41–43) during osteoblast differentiation. The lower band was determined to be nonspecific, since this band could not be competed out with peptide-neutralized antiserum, as seen in the bottom panel of Fig. 6D. In addition, we observed an upper band that was competed out with peptide-neutralized antiserum but were unable to determine the identity of this band.

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in osteoblasts. As seen in Fig. 9, overexpression of Fra-2 alone or JunD alone resulted in a small increase in basal or PTH-stimulated collagenase-3 promoter activity. Overexpression of both Fra-2 and JunD, however, blocked the PTH-induced response while having little effect on basal promoter activity. Importantly, overexpression of Fra-2 and JunD decreased the stimulation of basal and PTH-induced collagenase-3 promoter activity by Cbfa1.

**DISCUSSION**

In this present work, through nuclear run-on analysis we demonstrate that the increase in collagenase-3 gene expression during osteoblast differentiation is due to an increase in the transcriptional activity of the collagenase-3 gene. Furthermore, we find that the increase in collagenase-3 mRNA following PTH induction is occurring primarily through an increase in transcription of the collagenase-3 gene. Subsequent analysis of the collagenase-3 promoter region demonstrated that the AP-1 and RD binding sites were responsible for the developmental regulation of collagenase-3 promoter activity.

The AP-1 site, TGA(C/G)TCA, is found in the promoters of several developmentally regulated genes including osteocalcin, type I collagen, and several of the matrix metalloproteinases. The AP-1 site was found to be an important regulator of transcriptional activation of matrix metalloproteinase genes (17, 47, 48). Previously, there have been conflicting reports regarding the role of c-Fos in the transcriptional activity of developmentally regulated genes. In ROS 17/2.8 cells, c-Fos was reported to repress osteocalcin gene expression (46, 49), although it enhanced collagenase-3 promoter activity in UMR 106-01 cells (31). Our studies found the AP-1 site to be critical for collagenase-3 promoter activity.

Previous studies have implicated JunD as a contributor to the regulation of collagenase-3 promoter activity. In human fibroblasts, JunD was found to bind to the AP-1 site following...
Cells were treated with control or 10
DNA transfected was kept equal with the addition of pCMV vector.
them together into proliferating osteoblasts (day 5). The total amount of
Fra-2 (100 ng), pCMV JunD (500 ng), pCMV-Cbfa1 (250 ng), or all of
collagenase-3 promoter construct (1000 ng) was co-transfected with pCMV



The wild-type, WT(-148), collagenase-3 promoter construct (1000 ng) was co-transfected with pCMV Fra-2 (100 ng), pCMV JunD (500 ng), pCMV-Cbfa1 (250 ng), or all of them together into proliferating osteoblasts (day 5). The total amount of DNA transfected was kept equal with the addition of pCMV vector.
Cells were treated with control or 10−8 M PTH-containing media for 24 h, and the effect on CAT activity was assessed. Data represent mean ± S.E. of three replicate plates.

Fig. 9. Effect of overexpression of Cbfa1, Fra-2, and JunD on collagenase-3 promoter activity. The wild-type, WT(-148), collagenase-3 promoter construct was transiently transfected into rat osteoblasts. The total amount of DNA transfected was kept equal with the addition of pCMV vector. Cells were treated with control or 10−8 M PTH-containing media for 24 h, and the effect on CAT activity was assessed. Data represent mean ± S.E. of three replicate plates.

The goal of this study was to determine the factors responsible for the developmental regulation of the collagenase-3 gene. Based on the gel shift analysis, we concluded that there was no substantial change in the identity of the proteins binding to the AP-1 and RD binding sites during osteoblast differentiation. However, we did observe an overall binding activity decrease in the proteins binding to the AP-1 site. Direct comparison of the binding to the AP-1 site indicated that there was substantially more binding to the AP-1 site in proliferating osteoblasts than in mineralizing osteoblasts. This leads to further speculation that the AP-1 site may be the primary initiator of collagenase-3 gene transcription. Binding to the AP-1 site may be highest in proliferating osteoblasts when osteoblasts first begin to express collagenase-3. Once osteoblast proliferation has ceased, these factors may be down-regulated or post-translationally modified, culminating in an overall decrease in AP-1 site binding. Following initiation and the decrease of AP-1 factor binding to the AP-1 site, other transcription factors, such as Cbfa1, compensate for its loss as previously suggested (30). This may explain why Fos-null mice, while initially expressing low levels of collagenase-3, are able to express collagenase-3 comparable with wild type levels later in development (30).

The near absence of collagenase-3 gene expression in proliferating osteoblasts although Cbfa1, c-Fos, and c-Jun are present raises the possibility that another event is required for the activation of the collagenase-3 gene. One possibility is that the formation of an extracellular matrix may be required for expression of post-proliferative genes. Once the extracellular matrix begins to form, the binding of collagen to integrins may launch a signal transduction cascade that leads to an alteration of the osteoblast’s phenotype. This cascade may result in chromatin remodeling, thus allowing transcription factors access to previously unavailable sites. Another possibility is that colla-
Collagenase-3 Promoter Regulation in Differentiating Osteoblasts

...gene binding leads to a post-translational modification of transcription factors involved in collagenase-3 activation or of repressor proteins causing their inactivation. Alternatively, collagen binding may result in down-regulation of repressor proteins. A requirement for the presence of a collagen matrix has been demonstrated in MC3T3 cells, where inhibition of collagen synthesis resulted in a loss of osteocalcin promoter activity (56).

Another possibility to explain the absence of collagenase-3 mRNA in proliferating osteoblasts may be prevention of collagenase-3 mRNA basal levels are superinduced following cycloheximide treatment of osteoblast cells (28, 57). This effect may be the result of cycloheximide treatment blocking the synthesis of a transcriptional repressor. Alternatively, it is possible that cycloheximide may block the synthesis of a factor that destabilizes or degrades collagenase-3 mRNA. However, stabilization of mRNA does not appear to be plausible, since transcriptional activity is barely detectable in proliferating osteoblasts (Fig. 2). If cycloheximide treatment resulted in the stabilization of collagenase-3 mRNA, then some basal level of transcriptional activity would be expected in proliferating osteoblasts.

In summary, we can conclude that the regulation of the developmental expression of collagenase-3 gene expression in normal, differentiating osteoblasts occurs at the transcriptional level. Based on our results, it appears that the AP-1 and the RD binding sites are required for the transcriptional regulation of the collagenase-3 gene. Furthermore, members of the AP-1 and Cbfa family of transcription factors are involved in the developmental regulation of expression of this gene.

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