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Regulation of the Rat Interstitial Collagenase Promoter by IL-1β, c-Jun, and Ras-Dependent Signaling in Growth Plate Chondrocytes

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Abstract In an attempt to better define molecular influences on rat interstitial collagenase gene expression in cartilage, the promoter function was characterized using transient transfection assay, electrophoresis mobility shift assay, and genetic analysis in isolated growth plate chondrocytes. Data from 5′-flanking deletion and selected mutations suggest that multiple cis elements in both the proximal and distal regions of the promoter were important in the regulation of promoter activity. A proximal tumor response element (TRE) was shown to be necessary for basal and interleukin (IL)-1β–inducible reporter gene activity. Cells stimulated by IL-1β (1 ng/ml; 18 h) had elevated TRE binding activity, and one of the factors involved was identified as the nuclear protein, c-Jun. Indeed, c-Jun directed antisense oligonucleotides reduced rat interstitial collagenase mRNA. A sense oligonucleotide was ineffective. Regulation of promoter activity was susceptible to Ras-dependent signaling as expression of dominant negative mutant of Ras kinase (pZIP-RasN17) reduced reporter gene activity. In a comparison of proximal promoter reporter plasmid activity between proliferative and hypertrophic cells, inhibition of Ras-dependent signaling was less effective in the later cell type. This study suggests that the activation of nuclear binding proteins that bind TRE may be a common event with IL-1β regulation. Moreover, these data suggest that the regulation of rat interstitial collagenase gene expression is a combinatorial process and multiple cis-acting regulatory sites may interact to exert different effects dependent on the stage of chondrocyte differentiation. J. Cell. Biochem. 67:92–102, 1997.

Key words: collagenase promoter; TRE; chondrocyte; interleukin-1β; DNA binding

Interstitial collagenase initiates the turnover of connective tissue by solubilizing matrix collagen. Rat interstitial collagenase is a Zn^{2+}-dependent metalloendopeptidase of the matrix metalloproteinase family [Jeffrey, 1986; Quinn et al., 1990; Woessner, 1994]. The matrix metalloproteinase are widely expressed in several tissues and are part of normal tissue resorption and degradation [Salamonsen, 1996]. Matrix metalloproteinase are elevated in human osteoarthritic (OA) articular cartilage and degrade collagen and proteoglycan at physiological neutral pH [Sapolsky et al., 1976; Dean et al., 1989a], contributing to matrix fibrillation and the compromise of biomechanical function [Mow et al., 1990]. Molecular analysis of diseased cartilage shows discordant regulation between metalloproteinases [Wolfe et al., 1993; Mehraban et al., 1994], suggesting possibly unique regulatory mechanisms controlling their cell-specific expression.

Given these findings, it seems useful to delineate the mechanism that regulates matrix-degrading enzymes. To better understand the intrinsic regulatory patterns of normal and pathophysiological chondrocytes, this laboratory has identified interstitial collagenase gene expression in growth plate chondrocytes as a model to study. Chondrocytes arise from mesenchymal cells, and there is some similarity in diseased articular chondrocytes and normal growth plate chondrocytes. In the growth plate, chondrocytes undergo a rest period followed by a rapid proliferation stage which includes the

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initiation of massive matrix synthesis and establishment of columns for proliferative cells [Howell and Dean, 1992]. A hypertrophic cell stage soon begins with matrix remodeling and cell enlargement followed by mineralization and vascular capillary complex invasion. It is at this junction of cartilage and vascular tissue that bone is formed. In articular cartilage, a similar remodeling of the cartilage-bone junction is activated in the diseased joint. This growth plate–like activity results in chondrocyte hypertrophy and calcification in the deep radial zone of articular cartilage and bony spurs in the joint margins as well [Bullough and Jaganath, 1983; Lane and Bullough, 1980]. Collagen type X expression is apparent in the deep layer of chondrocytes within OA cartilage [Thomas et al., 1991; von der Mark et al., 1992], a phenotypic marker of growth plate hypertrophic chondrocytes [Sandell et al., 1994]. These observations emphasize the necessity to gather data about the basic mechanism of chondrocyte differentiation. Several cytokines are proposed to be important in the regulation of chondrocyte physiology and differentiation [Goldring, 1993; Poole, 1991]. Interleukin-1β (IL-1β) is elevated in arthritis [Ollivierre et al., 1986] and in hypertrophic cartilage [Grumbles et al., 1997] and induces degradation of proteoglycans and collagen type II in cartilage [Dodge and Poole, 1989]. Recently, we showed IL-1β elevated rat interstitial collagenase in growth plate chondrocytes [Grumbles et al., 1996]. Certainly, the chondrocyte activities of the growth plate are dominated by the activity of the hypertrophic cells which determine the extent of long bone growth and induce mineralization, and thus is an important cell type to elucidate mechanisms involved with gene regulatory networks [Hunziker and Schenk, 1989].

Immunohistological and functional collagenase assays suggest cell-specific regulation of interstitial collagenase with some expression in the transition from resting to proliferative cells but very elevated levels as proliferative cells progress to the terminal hypertrophic cells [Brown et al., 1989; Blair et al., 1989; Dean et al., 1989b]. It is believed that this elevation of collagenase activity creates lacunar around chondrocytes to allow cell expansion. Immunological techniques have identified this rat interstitial collagenase in the growth plate and articular cartilage [Blair et al., 1989]. Previous data shows that rat interstitial collagenase gene regulation occurs predominantly at the transcription level in chondrocytes [Grumbles et al., 1996]. Many inflammatory and phorbol ester induction mechanisms are mediated by a unique DNA sequence, ATGAGTCAG, the tumor response element (TRE) [Angel et al., 1987]. Sequence analysis for the rat interstitial collagenase 5′-flanking region has identified a number of potential DNA regulatory elements including a typical TATAA box (−32), a TRE, GTGACTCAT (−51), and a polyomavirus enhanced A-binding protein (PEA3) consensus element (−80) [Rajakumar and Quinn, 1996]. The factors most likely to bind TRE sequences are principally members of the Jun/Fos family, whose cellular effectiveness is affected by extracellular signals and is often Ras signaling–dependent [Karin, 1996]. Several AP-1 proteins have been found in cartilage, and, for example, elevated expression of c-Fos protein leads to chondrosarcomas [Wang et al., 1991]. Binding of transcription factors to DNA recognition elements ultimately triggers the expression of specific genes which together constitute the program for differentiation or pathophysiological expression of interstitial collagenase in chondrocytes.

Our knowledge about specialized processes with chondrocytes is incomplete, and we can anticipate further insight into the biology of chondrocytes through molecular analyses of the regulation of target genes in chondrocytes. In this communication we provide evidence that a proximal cis-regulatory element has a role in collagenase promoter function.

MATERIALS AND METHODS

Cell Culture

Chondrocytes were isolated from growth plate cartilage proximal tibias of both legs from rachitic male Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA) [Grumbles et al., 1996]. The cartilage was digested with collagenase type II (Worthington Biochemical Corp., Freehold, NJ) for 4 h at 37°C in Hank's balanced salts, washed several times in Hank's, filtered, centrifuged, and suspended in modified Eagle's medium (MEM) (GIBCO, Grand Island, NY) with 10% fetal calf serum, glutamine, and antibiotics [Grumbles et al., 1996]. Chondrocytes were plated a density of 5,000 cells/cm^2 and the medium changed every other

day. The chondrocytes were passaged once before protein and RNA experiments were conducted. Chondrocytes were grown 2 days postconfluence and then treated 48 h in serum-free medium with fresh medium added for the requisite factor additions. Phenotypically, these chondrocytes behave as expected for epiphyseal chondrocytes as they express collagen type II mRNA and do not synthesize collagen type I, and 1.25-$(OH)_2D_3$ increases alkaline phosphatase activity (data not shown).

RNA Isolation and Northern Analysis

Total RNA was isolated from cells using RNAzol reagent (Tel-Test, Inc., Friendswood, TX), a kit based on the Chomczynski and Sacchi [1987] method. The RNA pellet was dissolved in 200 µl of 20 mM TRIS, pH 7.4, 1 mM EDTA, 0.2% SDS, and the RNA was precipitated with sodium acetate and ethanol. The pellet was washed twice in 70% ethanol. The RNA sample (15 µg) was electrophoresed in a glyoxal-DMSO gel with 1 M sodium phosphate (pH 7.0) electrophoresis buffer. After overnight electrophoresis, the gel was prepared for transfer to a Biotrans (ICN Biochemicals, Inc., Costa Mesa, CA) nylon membrane by two washes in 20× SCP (1× = 130 mM NaCl, 37 mM Na_2HPO_4). The RNA was transferred in 20× SCP buffer by capillary action using a sponge. After transfer, the RNA was fixed to the nylon membrane by ultraviolet light exposure with a Stratagene UV linker (Stratagene, La Jolla, CA). After irradiation, the membrane was washed briefly in 2× SCP. The membrane was prehybridized 3–4 h in 2× SCP, 1.8% N-lauryl-sarcosine, heparin (1 mg/ml), and 0.5% Boehringer Mannheim (Indianapolis, IN) blocking reagent. The filter was hybridized with a random-primed [^{32}P]-labeled rat cDNA. Typical labeling reactions yielded probe-specific activities of 10^8 cpm/µg DNA (Prime-It! II Random Primer Kit; Stratagene). After overnight hybridization at 68°C, the membrane was washed twice in 2× SCP with 0.1% sodium dodecyl-sulfate (SDS) at room temperature and then stringently washed at 55°C using 0.1× SCP and 0.1% SDS. The filters were autoradiographed with Kodak X-OMAT-AR film for 24–72 h at −70°C. The intensity of the signals was analyzed by laser densitometry (Microtex Scan Maker IIHR). The autoradiograms were quantified using the program Scan Analysis from BioSoft (Cambridge, UK).

DNA Transient Transfection and Determination of Reporter Activity

A 2.2 kb 5′-flanking DNA of the rat interstitial collagenase gene has been cloned [Jeffrey, in preparation], and the sequence is identical to that one submitted to GenBank [Rajakumar and Quinn, 1996]. The clone was inserted into a vector upstream of a recombinant clone that encodes the enzyme chloramphenicol acetyltransferase (CAT) using the Not1 and Xho1 sites of pSVOCAT. The −220 to +27 fragment was excised from the 2.2 promoter and subcloned into pSVOCAT using PCR amplification and the Pfu DNA polymerase using oligonucleotides 5′-GGCTGTTTATTTTGCC-3′ and 5′-GGTGCCCAGCAGTGCC-3′. The fragment −52 to +27 was cloned by restriction and modification into the pSVOCAT. A mutant, scrambled (SCR) in the proximal TRE (−51), was converted into GTTCCAAG in a fragment of −52 to +27. A second mutant, −220 to −40 vector was PCR-amplified using 5′-GGCTGTTTATTTTGCC-3′ as the 3′ primer AAGCTTGATGAGTTACCACTTGG. This mutates the wild-type TRE sequence from GTGACTCA to GTGAACTCA. Other constructs utilized a convenient restriction site or deletion for cloning. All clones were grown in XL1-Blue MRF′ cells, and sequence fidelity was verified by DNA sequencing. The expression plasmids pZIP-rasH (17N), a dominant negative mutation [Quilliam et al., 1994], and pCGN-RafN4, a dominant negative mutant plasmid [Brtva et al., 1995], were kindly provided by Dr. Channing Der (University of North Carolina, Chapel Hill, NC). Transfection of plasmid DNA into primary cells was performed using the lipofectamine technique [Lucas et al., 1995]. A six well plate was seeded with exponentially growing cells at 1–5 × 10^5 cells per well in 2 ml of complete medium and grown until 80–100% confluent (~36 h). For the 2.2 kb collagenase-CAT, 20 µg DNA plus 2 µg RSV-β-galactosidase plasmid (Promega Corporation, Madison, WI) and 2 µl of Lipofectamine (GIBCO) were mixed into 100 µl of phosphate buffered saline (PBS) and distributed into each well. After 18 h, the cells were gently washed twice with PBS and fresh MEM added to the cells ± treatment factors in 0.5% serum (phenol red minus). The cells were harvested 48 h later by lysis in 250 mM TRIS, pH 7.8, 0.5% (v/v) Triton X-100, and 5 mM dithiothreitol. After centrifugation, the supernatant was saved and

aliquoted for protein determination and assay of β-galactosidase activity. The β-galactosidase assay measured the catalysis of 2-nitro-β-D-galactopyranoside at 420 nm and 550 nm [Miller, 1972]. Protein measurements were made by the BCA protein assay kit (Pierce, Rockford, IL). Chloramphenicol acetyl transferase activity was determined using a phase-extraction assay and [^{3}H] chloramphenicol [Ausubel et al., 1992]. The experiments were repeated at least three times using at least two different CsCl-purified plasmid preparations.

Electrophoretic Mobility Shift Assay (EMSA)

Cells were washed once in ice-cold physiological saline and washed once in ice-cold physiological saline containing 1 mM Na_3VO_4 and 5 mM NaF. A hypotonic buffer was added, 20 mM HEPES-HCl (pH 8.0), 500 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF (1.74 mg/ml isopropanol), 5 mM NaF, 0.4 M Na_3VO_4, 5 μM microcystin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 mM dithiothreitol [Sadowski and Gilman, 1993]. Nonidet P-40 was added to 0.5% to lyse the cells. The cells were scraped into a 1.5 ml microcentrifuge tube, and the nuclei collected by centrifugation. The pellet was suspended in the buffer above but with 420 mM NaCl and 20% glycerol. The nuclei were collected by centrifugation at 1,000*g* for 5 min at 4°C. The nuclear pellet was suspended in 20 mM Tris, 20% glycerol, 1.5 mM $MgCl_2$, 500 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin, 5 mM NaF, 0.5 mM Na_3VO_4. This was rotated at 4°C for 45 min and centrifuged at 12,000*g* for 30 min at 4°C. The supernatant was dialyzed against already cold (4°C) dialysis buffer: 20 mM Tris, 20% glycerol, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA, 5 mM NaF, 0.5 mM Na_3VO_4. After 2 h at 4°C the extract was centrifuged at 15,000*g* for 15 min. The protein content was determined, and the extract was either used immediately or aliquoted at 1.5–4.0 mg/ml protein and stored at −70°C. Double-stranded oligonucleotides were end-labeled with ^{32}P-dATP (DuPont NEN Research Products, Wilmington, DE) using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA) following a standard protocol [Ausubel et al., 1992]. The rat collagenase interstitial collagenase promoter contains a nonconsensus TRE sequence, 5′-TGACTCA-3′, vs. the original TRE sequence, 5′-TGAGTCA-3′, reported in the human collagenase gene [Angel et al., 1987]. The sense oligonucleotide used to detect proteins that bind the collagenase promoter containing the TRE site was CCAAGTGGTGACTCATCAC-TAT. For the EMSA, 6 μg of protein was incubated in 2 μg poly (dl-dC) for 5 min at room temperature and then incubated for 30 min at room temperature with 20,000 cpm of the appropriate ^{32}P-labeled oligonucleotide probe in nuclear binding buffer (20 mM Tris-HCl, pH 7.8, 50 mM KCl, and 2 mM DTT). The DNA-protein complex was resolved in a 5% polyacrylamide (acrylamide/bis 75/20) gel using 0.4× TBE (1× TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). The gel was dried and exposed to film at −70°C. An immunodetection experiment for transcription factors bound to the TRE oligonucleotide was done with polyclonal antibodies against c-Fos, FosB, Jun D, and Fra-2 and on monoclonal antibodies against c-Jun (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Antisense Oligodeoxynucleotides Experiment

Oligodeoxynucleotides were synthesized either complementary (antisense) or similar (sense) to the published cDNA. The oligodeoxynucleotides were the c-Jun (18 mer, codons 2–7) and c-Fos (18 mer, codons 3–8) as described by Cosenza et al., [1994]. The DNAs were added in 20 μl of water (final concentration at 10 μM) to the chondrocytes in serum-free medium. RNA was isolated at 6 h. In the one preliminary experiment performed with the oligodeoxynucleotides, c-jun and c-fos mRNA levels were decreased (not shown), and these data supported the published work [Cosenza et al., 1994].

Statistical Analysis

Differences were determined by analysis of variance or the Student's paired *t*-test or one-way analysis of variance. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Transient Transfection Analysis of Rat Interstitial Collagenase Promoter

The current experiments were designed to show what part of the 5′-flanking DNA mediates changes in gene expression by IL-1β. In order to identify the sequences essential for transcription of the rat interstitial collagenase gene in epiphyseal chondrocytes, we inserted processively shorter fragments of the 5′-flank-

ing DNA in front of the coding region of the CAT gene and transfected them into chondrocytes. Cotransfection with RSV-β-galactosidase gene reporter plasmids was routinely carried out to compensate for transfection efficiency for subsequent calculation of reporter activities from the CAT constructs. Figure 1 shows reporter activity in the presence and absence of IL-1β. In the presence of IL-1β, transcription was higher in the larger 5′-flanking DNA construct vs. the smallest one of −52 to +27 nucleotides of the proximal promoter DNA. Deletion of −2,200 bp to −77 reduced transcription 6.8-fold. The data show that a possible negative element may be present in the −464 to −220 5′-flanking DNA. The data also show relatively high reporter activity in the absence of IL-1β, as, for instance, control activity for the −2,200 construct was 6,195 ± 155 cpm vs the IL-1β–induced transcription of the −52 to +27 5'-flanking region of 4,395 ± 790 cpm (Fig. 1).

The functional importance of the proximal TRE sequence (−51) was ascertained in promoter constructs that mutated the sequence. First, in the minimal construct (−52 to +27), there was very little transcription when the TRE was mutated and no stimulation with IL-1β (Fig. 2). However, with a larger fragment of −220 to −40, mutation of the TRE reduced transcription from a mean of 9,856 ± 567 cpm to 4,256 ± 89 cpm in the presence of IL-1β, but there was still an elevation in CAT activity with IL-1β in the mutant.

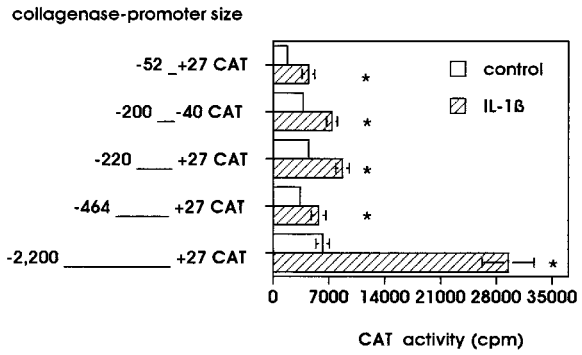

Fig. 1. Effect of IL-1β on the activity of the rat interstitial collagenase gene promoter. Plasmids containing various lengths of collagenase promoter sequences were ligated upstream of the chloramphenicol acetyl transferase (CAT) reporter gene, and these plasmids were transiently transfected into chondrocytes. For the longest construct, 20 μg of collagenase-CAT were transfected, and corresponding equimolar amounts of DNA were used for shorter collagenase-CAT constructs. Data are present as the fold activation relative to the value of a promoterless reporter vector, in control cells (open bars), which was set at an arbitrary value of 1.0, in each experiment. Cells stimulated with IL-1β (1 ng/ml; 48 h) are represented by cross-hatched bars. The data are from three experiments. *Statistically significant difference from the control level at $P < 0.05$.

EMSA

In order to detect what cis element may be responsible for the positive response to IL-1β, we made a number of oligonucleotides to a DNA sequence in the proximal 5′-flanking region. Of the several tested, only one containing a TRE sequence showed increased binding of nuclear proteins when comparisons were made between untreated cells and those exposed to 1 ng/ml IL-1β for 6 or 18 h (not shown). Thus, this study concentrated on the regulation of nuclear proteins binding to an oligonucleotide containing only the TRE sequence. Control first passage proliferative cells showed little binding to the TRE; however, 18 h after IL-1β application this activity was increased (Fig. 3). Competitive oligonucleotides, TRE or a 77 bp fragment of the first part of the promoter (−52 to +27), blocked nuclear binding to the radiolabeled TRE oligonucleotide (Fig. 3).

In order to determine the composition of the protein complex binding to the TRE, we added oligonucleotide-specific antibodies directed against known TRE binding proteins to the incubation with the nuclear proteins. Antibodies against c-Fos, FOSB, JunB, and Fra-2 did not alter the EMSA pattern (Fig. 4). Figure 4 shows the inhibition of the TRE binding activity by c-Jun antibodies, indicating the presence

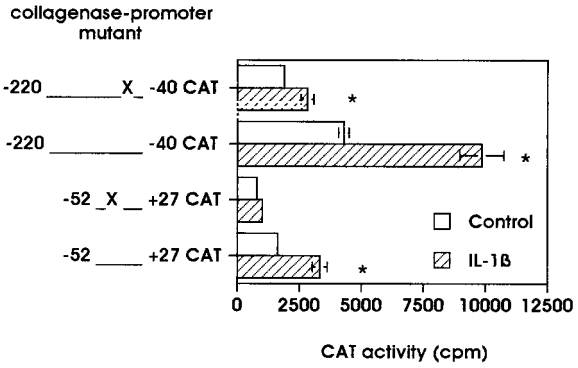

Fig. 2. Functional importance of the proximal TRE of the rat interstitial collagenase promoter in chondrocytes. Plasmids containing a mutation (indicated by an X in the promoter schematic) in the TRE were transiently transfected into chondrocytes. The experimental conditions were as in Figure 1, except these experiments were done independently. *Statistically significant difference from the control level at $P < 0.05$.

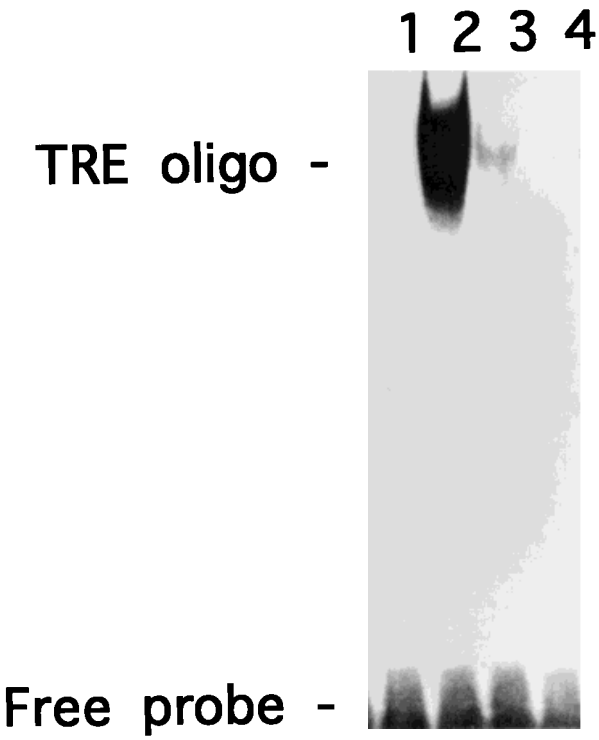

Fig. 3. Binding of nuclear proteins to the TRE oligonucleotide using proliferative cell nuclear extract. Extracts were prepared from control cells (**lane 1**), cells stimulated 18 h with IL-1β (**lane 2**), cells stimulated with IL-1β but with EMSA performed with expression of unlabeled TRE oligonucleotide (fiftyfold molar excess) (**lane 3**), and cells stimulate with IL-1β but with the EMSA performed with an unlabeled DNA fragment of the first 77 nucleotides (−52 to +27; fiftyfold molar excess) (**lane 4**).

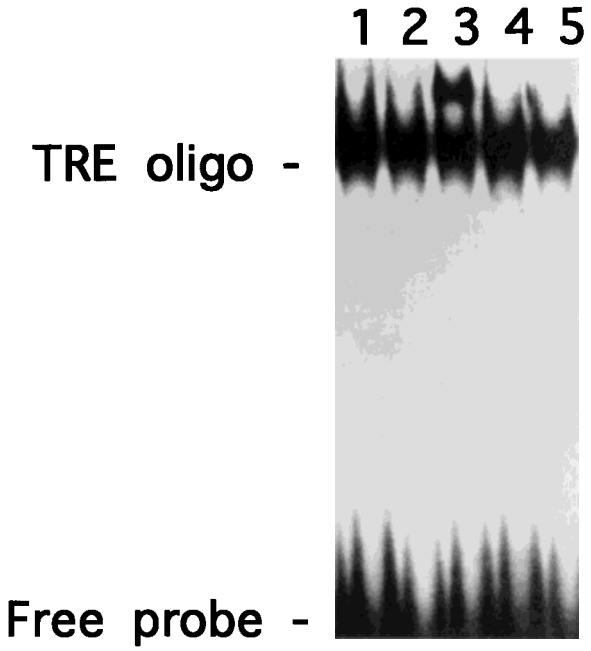

Fig. 4. Immunodetection of the c-Jun using EMSA. First-passage hypertrophic chondrocytes were treated for 18 h with 1 ng/ml of IL-1β and nuclear protein fractions prepared. The fractions and labeled TRE oligonucleotides were treated with the sera against c-Fos (**lane 1**), JunB (**lane 2**), c-Jun (**lane 3**), Fra-2 (**lane 4**), and FosB (**lane 5**).

of a c-Jun–specific antigen in the TRE complex. Other time points of 30 and 60 min were used for supershift EMSA, but the data were no different from that shown in Figure 4 (not shown). Other antibodies directed to transcription factors known to interact with TRE-like sequences in other cell types, cAMP response element binding-1, and ATF-2 did not alter the EMSA pattern (not shown). Although fetal calf serum can elevate the level of collagenase mRNA in these chondrocytes [Grumbles et al., 1996], we found no difference from our results with IL-1β (not shown). The antibodies were tested in EMSA supershift assays using Chinese hamster ovary (CHO) and UMR 106 cells and did show supershifts for c-fos, FOSB, and Fra-2 (not shown), supporting their ability to create supershifts. The mRNA for all of the transcription factors tested was present in these chondrocytes (data not shown).

Antisense Inhibition of Rat Interstitial Collagenase mRNA Levels

To substantiate a role for AP-1 factors in regulating collagenase, we used antisense oligonucleotides directed to c-Fos and c-Jun near the initiation mRNA codons. The antisense, sense, and nonsense oligonucleotides were added to serum-free medium, and RNA was isolated 6 h later. There was no effect on any of the oligonucleotides on the level of rat elongation factor 1a mRNA in these cells (not shown). A c-Jun antisense oligonucleotide reduced collagenase mRNA levels by 32%. (Fig. 5). Despite the lack of a c-Fos protein in the proximal TRE EMSA, c-Fos antisense oligonucleotide did reduce collagenase mRNA by 18%. There was little change in mRNA level with the sense or nonsense oligonucleotides. Antisense oligonucleotides directed against c-Jun did not produce any dramatic change in nuclear protein binding to the proximal TRE (not shown). Thus, these EMSA data, coupled to the c-Fos antisense inhibition of collagenase mRNA, suggest that one or more distal TRE elements are integrated into the regulation of collagenase gene expression in proliferative chondrocytes.

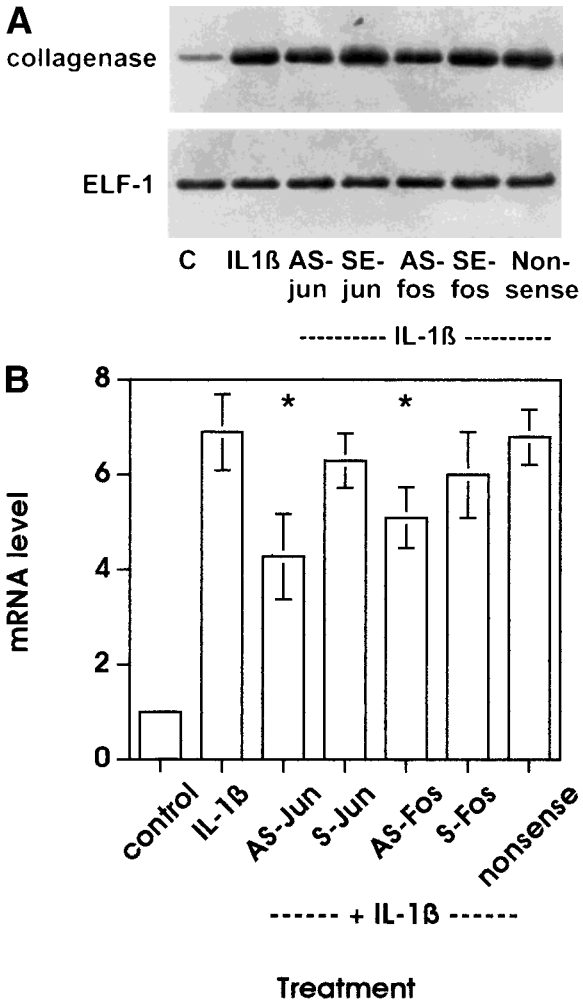

Fig. 5. The effects of antisense oligodeoxynucleotides on the IL-1β induction of rat interstitial collagenase mRNA in first-passage proliferative chondrocytes. **A:** Picture of a typical Northern hybridization of total RNA with probes against rat interstitial collagenase and elongation factor 1 (ELF-1). **B:** Summation of relative hybridization signals from three Northern blot experiments with independent chondrocytes after exposure to antisense oligodeoxynucleotides. Data are presented as relative mRNA levels compared to the control mRNA level, which was given an arbitrary value of 1.0. The control is the level of collagenase mRNA in the presence of IL-1β (1 ng/ml; 6 h). Antisense oligodeoxynucleotides against c-Fos and c-Jun significantly reduced rat interstitial collagenase mRNA. $*P < 0.05$.

Requirement for Ras in the Regulation of Rat Interstitial Collagenase Promoter

The c-Jun monoclonal used in this study is directed against the c-Jun p39 phosphorylation on serine-63 and does not recognize the c-Jun nonphosphorylated at serine-63 (data from Santa Cruz Biotechnology, Inc.). Ras has been established as a crucial component in the activation of various promoter elements including the AP-1 and Ets transcription proteins [Quilliam et al., 1994]. Transient cotransfection assays in which one plasmid contains a oncogene/signaling factor with a second reporter plasmid is an effective method to assay the dependence of *trans*-activation with an oncogene function [Quilliam et al., 1994]. Thus, one gains information on effecters upstream of Ras and their downstream ability to alter gene expression. This experiment was done with the additional intent of comparing the TRE-mediated reporter activity between proliferative and hypertrophic cells. Typically, a CAT expression vector driven by a −220 to +27 promoter fragment was active in both hypertrophic and proliferative chondrocytes, with greater activity in hypertrophic chondrocytes (Fig. 6). Part of the difference between proliferative vs. hypertrophic chondrocytes might be a result of a difference in transfection efficiency. However, we used a control plasmid encoding β-galactosidase to monitor plasmid uptake, and in controlled pilot experiments we isolated RNA from transient transfected cells and probed for either β-galactosidase or Ras mRNAs. We found no difference in mRNA levels between proliferative and hypertrophic chondrocytes (not shown). Transient cotransfection with a plasmid encoding altered Ras protein, pZIP-RasH(17N) [Quilliam et al., 1994], showed that this dominant negative mutant yielded a 40% reduction of −220 to +27 driven reporter activity in proliferative cells and a 22% reduction in hypertrophic cells (Fig. 6). The reporter gene activity from the −52 to +27 plasmid was active in hypertrophic vs. proliferative chondrocytes but was less sensitive to inhibition of Ras function (Fig. 6).

DISCUSSION

The promoter of rat interstitial collagenase was investigated in proliferative and hypertrophic chondrocytes. Analysis of the 5′-flanking region of the gene by transient transfection showed that a proximal TRE site mediated basal and IL-1β gene induction (Fig. 1). In the TRE mutant of the −52 to +27 fragment, the much lower level of CAT activity suggests that the TATAA and transcription initiation sites were not sufficient to respond to IL-1β (Fig. 2). In EMSA assays, an oligonucleotide with this mutation does not bind any nuclear proteins in vitro (not shown), suggesting that this mutation is not effective in promoting transcription

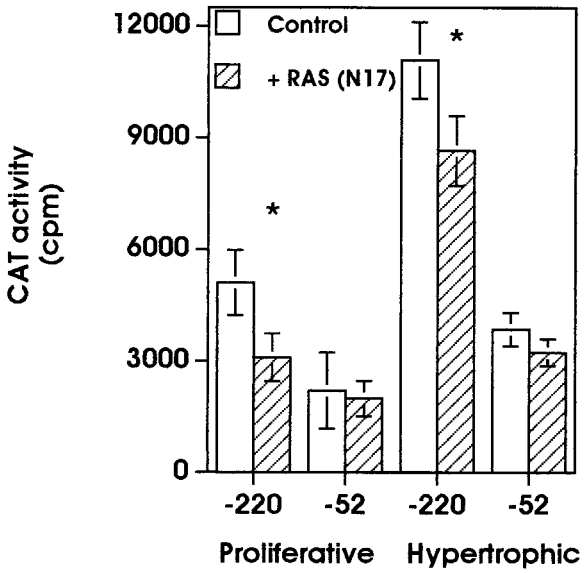

Fig. 6. The effect of interruption in the Ras-dependent signaling on functional −220 to +27 and −52 to +27 rat interstitial collagenase promoter fragments on CAT expression in rat proliferative and hypertrophic chondrocytes. Transient gene expression experiments were performed with chimeric plasmids. The data are expressed as the level of reporter activity for each construct in cells stimulated with IL-1β and the reporter CAT vector alone (cross-hatched bars) or cells cotransfected with pZIP-Ras(N17) (open bars). Each data bar is the mean ± standard error from at least four independent experiments. *Significant change in CAT activity between control vs. Ras inhibition of promoter activity ($P < 0.05$).

from this DNA sequence. However, the in vitro environment is different from that of the in vivo one, and possibly this site is still recognized by nuclear factors within the cell. However, the TRE mutant construct in −220 to −40 showed activity above that of the −52 to +27 one, suggesting to us that one or more additional sites in the proximal region are important in gene regulation. These data are similar to the findings for the stromelysin promoter where a TRE mutation in a 1,300 bp construct affected basal reporter activity, whereas IL-1β induction still occurs [Buttice et al., 1991]. In transient transfection assays, it is assumed that TRE transcriptional potency is coupled to the presence of nearby transcription-activating proteins [Buttice et al., 1991]. A single TRE is not stimulated by TPA in HepG2 cells [Buttice and Kurkinen, 1993]. In these chondrocytes, when a minimal promoter construct containing a single TRE copy was used, reporter activity was found and regulated by IL-1β. Thus, cell-specific differences in AP-1 activity occur. In the −220 to −40 construct, the PEA3 element is present, and although there is only one copy of the PEA3, it is likely that it does cooperate with any TRE bound protein to increase transcriptional activity. Other studies show that proximal PEA3 and TRE have a major role in phorbol induction of stromelysin and collagenase transcription [Buttice and Kurkinen, 1993; Gutman and Wasylyk, 1990]. In vitro DNAse I footprinting in osteoblast-like cells shows occupancy of the nearby PEA site in the rat interstitial collagenase promoter [Rajakumar and Quinn, 1996]. Preliminary data with rat hypertrophic chondrocytes revealed PEA3 binding activity as basally present but showed no elevated nuclear protein binding with IL-1β [in preparation]. Thus, possibly there was cooperative activation of transcription though these two DNA sites. Additional transient transfection experiments clearly showed a higher level of reporter activity when the 2.2 kb 5′-flanking protein of the promoter was used. There was also a difference in reporter gene activity with different chondrocyte types, as transient transfection analysis showed elevated activity in hypertrophic vs. proliferative chondrocytes. This corroborates the literature that collagenase is differentially regulated between these zones [Brown et al., 1989; Dean et al., 1989b]. Studies with osteocalcin expression in osteoblast-like cell lines have demonstrated that gene regulation in response to hormones and physiological simulators involves multiple basal promoter and enhancer elements [Heinrichs et al., 1995; Aslam et al., 1995]. Thus, one assumption is that a combinatorial mechanism lies at the foundation of rat interstitial collagenase gene expression.

This study demonstrated a seminal role for c-Jun in the regulation of collagenase gene expression. IL-1β elevated TRE binding activity, immunoreactive analysis detected c-Jun in this protein-DNA complex (Fig. 4), and c-Jun antisense oligonucleotides reduced collagenase mRNA levels (Fig. 5). Antisense oligonucleotides directed against the c-Fos reduced collagenase mRNA levels (Fig. 5); however, we saw no changes in the EMSA profile if the nuclear extract was pretreated with a polyclonal antibody against c-Fos (Fig. 4). However, this antibody was effective in CHO cells in creating a supershift with a TRE oligonucleotide (data not shown). But the rat interstitial collagenase promoter contains several upstream TRE-like as well as CRE-like sequences in the promoter,

and possibly one of these distal sites binds c-Fos and thus has a role in determining the extent of rat interstitial collagenase gene expression. Several transcription factors can bind TRE, and it is likely more than just c-Jun binds as the addition of c-Jun antibody supershifted only a part of the DNA-protein complex, not the entire mixture. Some other factors which were not tested with antibodies include c-MAF and NRL, both known to bind TRE sites with heterodimer formations with either Fos or Jun [Kerppola and Curran, 1994]. Interestingly, c-Jun–deficient mice do not have cartilage defects [Hilberg et al., 1993]. There are explanations for these data: there is redundancy of AP-1 factors in developing cartilage to compensate for c-Jun absence, and/or, possibly in mice, the absence of c-Jun does not cause complete absence of functional interstitial collagenase activity and, for example, a 10–30% level of collagenase activity is present in c-Jun–deficient mice which is sufficient activity to allow remodeling of the growth plate and/or other matrix metalloproteinases can compensate for the lack of interstitial collagenase activity. Further approaches to better define the role of c-Jun and related ancillary factors will use transactivation experiments with transcription factor cDNA expression clones and a search for c-Jun interacting proteins by the yeast two-hybrid system in a chondrocyte cDNA library. While this work was in progress, a similar study with rat interstitial collagenase reported the proximal TRE important in parathyroid peptide induction of collagenase [Rajakumar and Quinn, 1996]. In contrast to the situation with chondrocytes, it is reported that osteoblast-like cells use a cAMP response element binding protein to recognize TRE. Certainly, the reported differences in TRE involvement in rat interstitial collagenase suggest an alternative mechanism of gene induction, possibly specific to the means of incubation and/or the physiological milieu between osteoblast-like cells and chondrocytes.

Functional promoter assays showed a contrast in the extent of Ras-functional dependency between proliferative and hypertrophic chondrocytes (Fig. 6). Biochemical experiments have described several genes to be differentially transcribed between hypertrophic and proliferative epiphyseal cells [Poole, 1991; Sandell et al., 1994]. It is assumed that proliferative and hypertrophic chondrocytes have certain intrinsic properties that are the result of the many types of receptor activities and intrinsic influences on autocrine factors and several regulatory molecules synthesized within the cells [Howell and Dean, 1992; Nilsson et al., 1994]. Intrinsic signaling transduction pathways that include growth factor– and stress-activated protein kinase would profoundly influence cartilage physiology, but a paucity of information exists regarding the specific roles of these signaling pathways in chondrocytes. The monoclonal antibody used in the experiment represented in Figure 4 is specific for phosphorylation of the serine residue at position 63 of c-Jun, an essential phosphorylation in the transactivation domain that enhances binding to DNA [Karin, 1996]. In other cell systems, this modification is made through several mechanisms including phorbol esters and expression of certain oncogenes. Ras activation is implicated in the regulation of TRE-mediated genes such as matrix metalloproteinases [Karin, 1996]. Here, utilization of dominant negative mutant against Ras-dependent signaling resulted in the reduction of rat interstitial collagenase promoter activity in these chondrocytes (Fig. 6). However, this dominant mutation was less effective in the hypertrophic vs. proliferative ones, suggesting the participation of more than one mechanism in rat interstitial collagenase regulation. Conceptually, either a Jun kinase is activated independently of Ras, possibly through a stress-activated kinase [Burgerling and Coffer, 1995], via a low molecular weight G-protein(s) in the Rho subfamily [Zhang et al., 1995], or through a mitogen-activated kinase that is independent of Ras signaling, as, for instance, with p54 mitogen activated kinase in fibroblasts and HepG2 cells [Quilliam et al., 1994]. The resulting use of parallel signal induction may stimulate other ancillary transcription factors that recognize the promoter DNA or produce a similar phosphorylation of c-Jun and, ultimately, enhance rat interstitial collagenase transcription activation.

Directing research efforts to elucidate the chondrocyte-specific signaling that leads to altered rat interstitial collagenase may yield new clues about the information flow from the cytosol into the nuclei. This information may be used to inhibit the c-Jun modifying protein kinase and limit hypertrophic/OA-induced collagenase expression and should inhibit matrix

loss and potentially enhance long-term repair mechanisms in cartilage.

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