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Transcriptional Suppression by Interleukin-1 and Interferon-γ of Type II Collagen Gene Expression in Human Chondrocytes

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Abstract Type II collagen is one of the predominant extracellular matrix macromolecules in cartilage responsible for maintenance of integrity of this specialized tissue. We showed previously that interleukin-1 (IL-1) and interferon-γ (IFN-γ) are capable of decreasing the levels of α1(II) procollagen mRNA and suppressing the synthesis of type II collagen in cultured human chondrocytes. Data reported here show that these effects of IL-1 and IFN-γ on the expression of the human type II collagen gene (COL2A1) are mediated primarily at the transcriptional level. This conclusion is based on three types of experimental evidence: (1) in nuclear run-off assays, preincubation of chondrocytes with either IL-1 or IFN-γ decreased COL2A1 transcription; (2) experiments with the protein synthesis inhibitor cycloheximide and the transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) indicated that the suppression of α1(II) procollagen mRNA by IL-1 could not be ascribed to decreased mRNA stability; and (3) a plasmid (pCAT-B/4.0) containing 4.0 kb of 5′-flanking sequences of COL2A1 (−577/+3428), encompassing the promoter, exon 1 and the putative enhancer sequence in the first intron, linked to the chloramphenicol acetyltransferase (CAT) reporter gene, was transfected in human chondrocytes. A high level of expression of pCAT-B/4.0 was observed in human chondrocytes incubated with an insulin-containing serum substitute that is permissive for expression of the COL2A1 gene. Expression of pCAT-B/4.0 in these cells was inhibited by either IL-1 or IFN-γ. Furthermore, expression of pCAT-B/4.0 was not detected in human dermal fibroblasts. When the putative enhancer fragment in the first intron was removed, the expression in chondrocytes was greatly reduced. These studies demonstrate that expression of COL2A1 is tissue specific and that suppression by either IL-1 or IFN-γ is mediated primarily at the transcriptional level.

Key words: chondrocyte, mRNA stability, transient transfection, promoter, enhancer

The major constituents of the extracellular matrix of mature hyaline cartilage found in diarthrodial joints are type II (with minor amounts of types IX and XI) collagen and the cartilage-specific, large aggregating proteoglycan [Mayne and Irwin, 1986; Heinegard and Oldberg, 1989] called aggrecan [Doege et al., 1990]. Expression of the type II collagen gene must be stringently regulated during chondrogenesis and growth plate maturation but repressed in noncartilaginous tissue [Gerstenfeld et al., 1990; Sandell et al., 1991]. The importance of transcriptional regulation of this gene has been implicated in studies of responses of cultured chondrocytes to growth factors, cytokines and other cell response modifiers [Finer et al., 1985; Goldring et al., 1986, 1988; Sandell and Daniel, 1988; Horton et al., 1989; Askew et al., 1991; Sandell et al., 1992].

The only cell type in adult hyaline cartilage is the chondrocyte, a terminally differentiated cell that functions to maintain the cartilage-specific matrix phenotype under normal conditions when turnover is low. Under pathological conditions chondrocyte function is altered and there are changes in the composition of the extracellular cartilage matrix [Goldring, 1992]. The array of the factors responsible for the altered function of the chondrocyte that gives rise to defective cartilage matrix has not been defined fully. There is, however, evidence that the increased produc-

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tion of "inappropriate" cytokines or alterations in the temporal sequence of their release contribute to the abnormalities of cartilage matrix remodeling [Dodge and Poole, 1989; Krane et al., 1990; Goldring, 1992].

Interleukin-1 (IL-1), a monocyte–macrophage product first described as an immune modulator, has specific effects on the biosynthesis of cartilage-specific matrix macromolecules. We reported previously that IL-1 suppresses the expression of cartilage-specific type II and type IX collagens in human chondrocyte cultures and this suppression is potentiated by inhibitors of prostaglandin synthesis [Goldring et al., 1988, 1990]. In contrast, the synthesis of type I and type III collagens by chondrocytes is increased by IL-1, particularly when IL-1-stimulated synthesis of prostaglandin E_2 (PGE_2), which inhibits the synthesis of type I and type III collagens, is blocked by prostaglandin synthetase inhibitors [Goldring and Krane, 1987; Goldring et al., 1988]. IL-1 also suppresses the levels of cartilage-specific collagen mRNAs in intact cartilage [Tyler and Benton, 1988] and proteoglycan synthesis in cartilage or chondrocytes [Tyler, 1985; Ikebe et al., 1988].

Interferon-γ (IFN-γ), a product predominantly of T lymphocytes, also inhibits type II collagen synthesis in chondrocytes [Goldring et al., 1986]. In contrast to IL-1, IFN-γ inhibits the synthesis of type I and type III collagens by fibroblasts and dedifferentiated chondrocytes by a mechanism that is prostaglandin independent. These effects are associated with decreases in steady-state levels of procollagen mRNAs [Jimenez et al., 1984; Amento et al., 1985; Stephenson et al., 1985; Goldring et al., 1986]. IL-1 and IFN-γ may inhibit type II collagen gene expression by distinct mechanisms, since they appear to have opposite effects on the expression of other genes. For example, while IL-1 promotes cartilage degradation by stimulating metalloproteinase synthesis by chondrocytes, IFN-γ inhibits the production of collagenase and stromelysin induced by IL-1 [Andrews et al., 1989]. Although little attention has been paid to the potential roles for these cytokines during development, IL-1 mRNA has been localized in the cartilage resorption zone during endochondral ossification in immature mouse bone [Takacs et al., 1988].

Regulatory sequences responsible for constitutive and tissue-specific transcription have been identified in several collagen genes [see Sandell and Boyd, 1990; Sandell et al., 1992, for review]. The levels of expression of reporter genes directed by different regulatory domains in the promoter and first intron regions of several collagen genes, including type I [Bornstein et al., 1988; Karsenty and deCrombrugghe, 1990; Ravazzolo et al., 1991; Simkevich et al., 1992], type II [Horton et al., 1987; Wang et al., 1991; Wu et al., 1993], and type X [LuValle et al., 1993], have been compared by transient transfection of cultured cells that express these genes constitutively. Little is known concerning the mechanisms by which cytokines and growth factors regulate collagen gene transcription. The stimulation of expression of the α2(I) collagen promoter by transforming growth factor-β was shown to be mediated by a nuclear factor-1 (NF-1) binding sequence [Rossi et al., 1988]. A functional AP-1 binding site is present in the first intron of the human α1(I) collagen gene, but the direct involvement of this element in regulation by phorbol ester of transcription of this gene has not been established [Liska et al., 1990]. Inhibition of type II collagen gene transcription by IL-1 was demonstrated in rat and rabbit chondrocytes [Chandrasekhar et al., 1990], but transcriptional effects of IFN-γ on type II collagen gene expression have not been reported. Precise functional localization of regulatory sequences responsive to extracellular signals has not been done extensively in collagen genes.

In order to determine whether the suppression of type II collagen gene expression by IL-1 or IFN-γ that we observed previously in human chondrocytes occurs at the transcriptional or post-transcriptional level, we carried out both nuclear run-on and transient expression experiments and found that both cytokines suppressed transcription of COL2A1 to an extent comparable to their effects on levels of α1(II) procollagen mRNA. Furthermore, an analysis of mRNA stability using inhibitors of protein synthesis and transcription indicated that the effect of IL-1 could not be accounted for by decreased stability of α1(II) procollagen mRNA. Our results demonstrate that IL-1 and IFN-γ are negative signals for COL2A1 transcription. These cytokines may have specific roles in preventing appropriate repair of the extracellular matrix after cartilage destruction in inflammatory joint

disease or in mediating the developmental program preceding chondrogenesis.

MATERIALS AND METHODS

Cytokines

Recombinant human IL-1β (provided by Dr. J.-M. Dayer, Geneva, Switzerland), thawed from frozen stock (2.5 μM in PBS containing 1 mg/ml BSA), remained stable for several months when kept at 4°C and was used at 5 pM unless stated otherwise. Recombinant preparations of human (3×10^8 U/mg) and mouse IL-1α (1.3×10^8 units/mg) were from Dr. A. Stern and Dr. P. Lomedico, Hoffmann-LaRoche, Nutley, NJ. Recombinant preparations of human IFN-γ (2×10^6 or 2.65×10^7 U/mg) or mouse IFN-γ (17.4×10^6 or 5.2×10^7 U/mg) were obtained from Dr. E. Amento at Genentech, Inc., South San Francisco, CA or they were purchased from AMGEN, Thousand Oaks, CA (hIFN-γ, $>1 \times 10^7$ units/mg) or from Genzyme, Boston, MA (mIFN-γ, 9×10^6 U/mg).

Probes

The 3.8-kb genomic fragment of human α1(II) procollagen (hgColII) encodes amino acid 892 to the end of the C-propeptide (~1,150 nucleotides of exon material) (Sandell, 1984; Sandell and Daniel, 1988). An additional α1(II) procollagen cDNA probe (pkTh1330), a ~450-bp fragment that encodes a helical portion of the type II collagen chain near the C-propeptide, was cloned using our human costal chondrocyte library (Kimura et al., 1989) in collaboration with Dr. T. Kimura, Osaka, Japan. The type I cDNA probes are Hf677, a 1,500-bp cDNA encoding part of the α1(I) procollagen subunit, and Hf1131, a 1500 bp cDNA encoding part of the α2(I) procollagen subunit (provided by Dr. F. Ramirez and Dr. D. Prockop) [Chu et al., 1982; Bernard et al., 1983]. The type III collagen probe is pHC III-1, a 1885 bp cDNA encoding the C-propeptide and portions of the helical and untranslated regions of α1(III) procollagen (provided by Dr. R.J. Crystal and Dr. M. Brantly, NIH) [Miskulin et al., 1986]. The cDNA probe for human procollagenase is XHF_1 (from Dr. H.-J. Rahmsdorf, Kemforschungszentrum Karlsruhe, Karlsruhe, Germany) [Stephenson et al., 1987].

Cell Cultures

Human costal cartilage was obtained from ribs removed during pectus excavatum repair. Chondrocytes were isolated by dispersion with proteases. Cartilage slices were incubated at 37°C with hyaluronidase (1 mg/ml in PBS; bovine testicular from Sigma) for 10 min, followed by 0.25% trypsin (GIBCO/BRL, Gaithersburg, MD) for 45 min. The cartilage was then chopped in small fragments and incubated at 37°C with collagenase (3 mg/ml in serum-free culture medium; clostridial peptidase from Worthington Biochemical Corp., Freehold, NJ) for 48 h. The dispersed cells were then washed with Ca^{++}- and Mg^{++}-free PBS and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) with medium changes every 3–4 days, as described previously [Goldring and Krane, 1987; Goldring et al., 1988]. Costal chondrocytes from 6 different individuals were used for the experiments described in this study. In some cases, monolayer cultures of chondrocytes were plated on agarose, as described by Castagnola et al. [1986], in Ham's F12/DMEM containing 10% FCS and cultured for 2–3 weeks with weekly medium changes. Human dermal fibroblasts were isolated from surgically removed foreskins by dispersion with proteases and used at passages 8 to 12. Rat chondroblasts (the RCJ 3.1 C5.18 subclone) were provided to us by Dr. Jane Aubin [Grigoriadis et al., 1988].

Nuclear Transcription Assay

Human costal chondrocytes were incubated for 24 h in the absence or presence of IL-1β or IFN-γ in medium containing 10% FCS. The cells were washed in cold hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol) containing 0.2 M sucrose, resuspended in hypotonic buffer containing no sucrose, placed on ice for 10 min and lysed with 5–8 strokes in a Dounce homogenizer. The resulting nuclear pellet after centrifugation at 500 *g* for 5 min was resuspended in storage buffer containing 40% glycerol, 50 mM Tris–HCl, pH 8.0, 5 mM Mg acetate, and 0.1 mM EDTA and stored in 100 μl aliquots containing 1 to 5×10^7 nuclei at −70°C. The nuclei were than labeled with α-^{32}P-UTP for 30 min at 30°C. The labeled RNA was extracted once with water-saturated phenol–chloroform (2:1) and unincorporated nucleotides were removed from the RNA by 2 sequential precipitations with 70% ethanol. The labeled RNA (1×10^6 cpm) was hybridized at 42°C for 48 h with 2 μg of each respective

linearized plasmid DNA immobilized on a single strip of nitrocellulose for each condition.

Analysis of RNA

Cytoplasmic RNA for dot blots was prepared by the method of White and Bancroft [1982] as described previously [Goldring et al., 1986, 1988; Goldring and Krane, 1987]. Total RNA for Northern blots was extracted according to a method adapted from Chomczynski and Sacchi [1987]. Chondrocytes ($>1 \times 10^6$) from 6- or 10-cm plates were lyzed directly in 500 μl (final volume) of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and sheared with a 27-gauge needle either before or after freezing at −80°C. The RNA was then extracted at 4°C by sequential addition of 2 M sodium acetate (50 μl), pH 4.0, water-saturated phenol (500 μl), and chloroform/isoamyl alcohol (49:1; 100 μl) and precipitation of the aqueous phase with an equal volume of isopropanol. The RNA pellets were then redissolved in Solution D and reprecipitated with isopropanol. The final pellets were washed twice with 75% ethanol in 0.01 M HEPES and once in absolute ethanol, dried in a Savant speed vac apparatus and dissolved in 50 μl of 0.01 M HEPES. Alternatively, total RNA from $>10 \times 10^6$ cells was extracted as described by Sandell et al. [1988]. The final preparations gave yields of approximately 10 μg of RNA per 1×10^6 cells with the appropriate A_{260}:A_{280} ratio of approximately 2.0. Total RNAs were separated on 0.8% agarose gels in the presence of 2% formaldehyde. Northern blots were prepared on either nitrocellulose or BAS-85 membranes (Schleicher and Schuell, Keene, NH) as described previously [Goldring et al., 1986, 1988; Goldring and Krane, 1987].

DNA probes were labeled with ^{32}P-dCTP and ^{32}P-dGTP by nick translation. Blots were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's solution and 0.3% SDS at 54°C, 100 ng/ml DNA probe added, and hybridization carried out for at least 16 h at 54°C. Staining with ethidium bromide and hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe were employed to monitor uniform loading of RNA on Northern blots.

Preparation of pCAT-B/4.0 and pCAT-B/2.9 Constructs

The pCAT-B/4.0 construct containing 4.0 kb of DNA sequences of COL2A1, including −577 bp of upstream promoter sequence and 3428 bp downstream of the mRNA start site (exon 1 and approximately ⅔ of the first intron), was constructed using the pCAT-Basic plasmid (Promega, Madison, WI). This 4.0 kb *PstI* fragment was cloned into the Pst I site located immediately upstream of the CAT reporter gene. The pCAT-B/2.9 construct was prepared by deletion of the Xho I/Pst I fragment (+2370 to +3428) of the first intron from the pCAT–B/4.0 plasmid. These plasmids were transfected in the JM109 bacterial strain (Promega) and grown in the presence of ampicillin.

Transient Expression Assay

Normal human chondrocytes in primary culture or first or second subculture were plated at 10^6 cells per 100-mm dish in Ham's F12/DMEM (1:1) containing 10% FCS and allowed to settle down for 24 h. The medium was then changed and the cells transfected with 10 μg of plasmid DNA by the calcium-phosphate method (4h) followed by glycerol shock (2 min). After washing, the cells were allowed to recover overnight in culture medium containing 10% FCS. The medium was then changed to Ham's F12/DMEM (1/1, v/v) containing 1% Nutridoma-SP (Boehringer-Mannheim, Indianapolis, IN) or 1% ITS+ (Collaborative Research, Bedford, MA) and hIL-1β, hIL-1α or hIFN-γ added, and the cultures were incubated for 42–48 h and harvested for CAT assay. Rat chondroblasts, the RCJ 3.1 C5.18 subclone [Grigoriadis et al., 1988], were also used for some transient expression experiments after plating at 0.5×10^6 cells per 100-mm dish, and hIL-1β, mIL-1α or mIFN-γ was added 2–3 h after glycerol shock. Cell extracts were assayed for CAT activity by the fluor diffusion method (Eastman, 1987; Neumann et al., 1987). First order slopes (Δcpm/min) were derived from plots of activity versus reaction time. Based on these data, the optimal time of incubation was selected and CAT activity determined in some samples using the thin layer chromatography method [Gorman et al., 1982].

RESULTS

Inhibition by IL-1β and IFN-γ of Nuclear Transcription of the Type II Collagen Gene in Human Chondrocytes

We reported previously that human recombinant preparations of either IL-1β or IFN-γ could suppress the synthesis of type II collagen and the level of α1(II) procollagen mRNA in cultured

human adult articular or juvenile costal chondrocytes [Goldring et al., 1986, 1988]. To determine whether this reduction in the level of type II collagen mRNA was due to decreased transcription of the type II collagen gene, experiments were carried out using nuclei isolated from primary cultures of human costal chondrocytes after treatment with either IL-1β or IFN-γ for 24 h. Labeled nascent transcripts from each incubation condition were hybridized to separate strips of nitrocellulose containing 2 μg of each unlabeled DNA encoding α1(II) procollagen, α1(III) procollagen, pBR325 plasmid and β-actin. The signal for transcription of the α1(II) procollagen gene in these freshly isolated chondrocytes at day 11 of culture was very strong compared with the other noncartilage collagen genes consistent with the relative levels of procollagen mRNAs that we reported previously [Goldring et al., 1986, 1988]. IL-1β treatment of the chondrocytes resulted in a significant reduction in transcription of the type II collagen gene (Fig. 1A,B). Preincubation of chondrocytes with IL-1β in the presence of indomethacin slightly potentiated this suppression consistent with effects we had shown previously on type II collagen synthesis and mRNA levels [Goldring et al., 1988, 1990]. The signals for transcription of the α1(I) and α2(I) procollagen genes were weak such that effects of IL-1β could not be assessed in this experiment. Interestingly, the signal for α1(III) procollagen gene transcription was strong and a stimulatory effect of IL-1 was observed (Fig. 1A). IFN-γ also decreased transcription of the type II collagen gene (Fig. 2A–C). This effect was somewhat dose dependent with maximal (> 95%) suppression at a relatively high concentration of 500 units per ml of IFN-γ (Figure 2A). Transcription of the α2(I) and α1(III) procollagen genes was also inhibited by IFN-γ (Fig. 2A). Hybridization against PBR325 plasmid DNA or a β-actin cDNA showed that these effects of IFN-γ were specific (Fig. 2A,B). Suppression by IFN-γ of transcription of the α1(I) and α1(II) procollagen genes was compared when the labeled transcripts were hybridized to the respective DNAs which had been run on agarose gels prior to blotting on nitrocellulose (Fig. 2C). Dot hybridization analysis of cytoplasmic RNAs extracted from parallel cultures demonstrated similar inhibitory effects of IL-1β and IFN-γ on the levels of α1(II) procollagen mRNA, as well as the effects of these cytokines on levels of α1(I), α2(I) and α1(III) procollagen mRNAs that we had

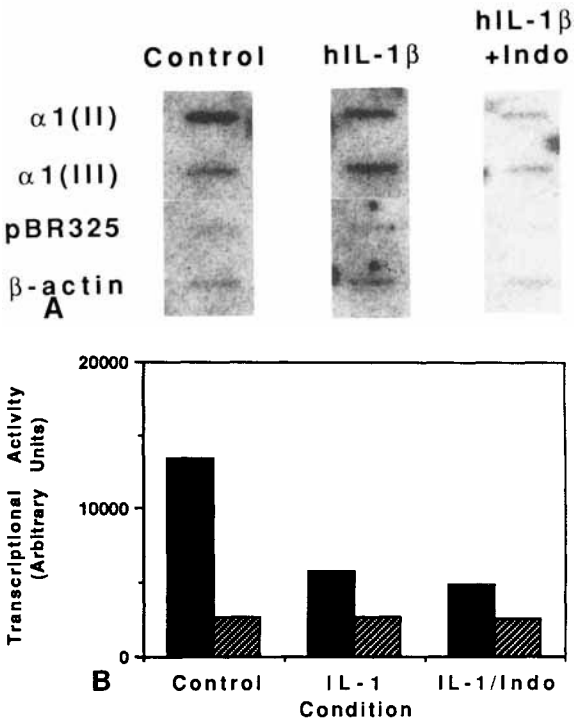

Fig. 1. Effects of hIL-1β on nuclear transcription of collagen genes in human chondrocytes. Costal chondrocytes (day 11 of primary culture) were preincubated for 24 h alone (control) or with hIL-1β (5 pM) without or with indomethacin (1 μM). The nuclei were isolated as described in the methods and labeled using [^{32}P]UTP. **A:** The labeled transcripts were hybridized to linearized human α1(II) procollagen, α1(III) procollagen, pBR325 plasmid and β-actin DNAs that had been immobilized on nitrocellulose strips using a slot blot apparatus. **B:** Values determined by densitometric analysis of the autoradiographs are plotted for α1(II) procollagen (■) and β-actin (▨) transcripts.

reported previously [Goldring et al., 1986, 1988] (Fig. 3).

Effects of IL-1β on the Kinetics and Stability of α1(II) and α1(I) Procollagen mRNAs in Human Chondrocytes

The kinetics of the effects of IL-1β on type II and type I procollagen mRNA levels were determined in the absence and presence of the protein synthesis inhibitor cycloheximide (CHX). For these experiments, subconfluent chondrocytes were made quiescent by preincubation in the presence of 0.5% FCS for 16–24h. To determine whether protein synthesis was required for the effects of IL-1β on the levels of type II and type I collagen mRNAs, CHX (10 μg/ml) was then added in the absence or presence of IL-1β and cells were harvested for extraction of total RNA after 3, 6, and 24 h of incubation. Duplicate blots were prepared and probed at the same time with the ^{32}P-labeled α1(II) and α1(I)

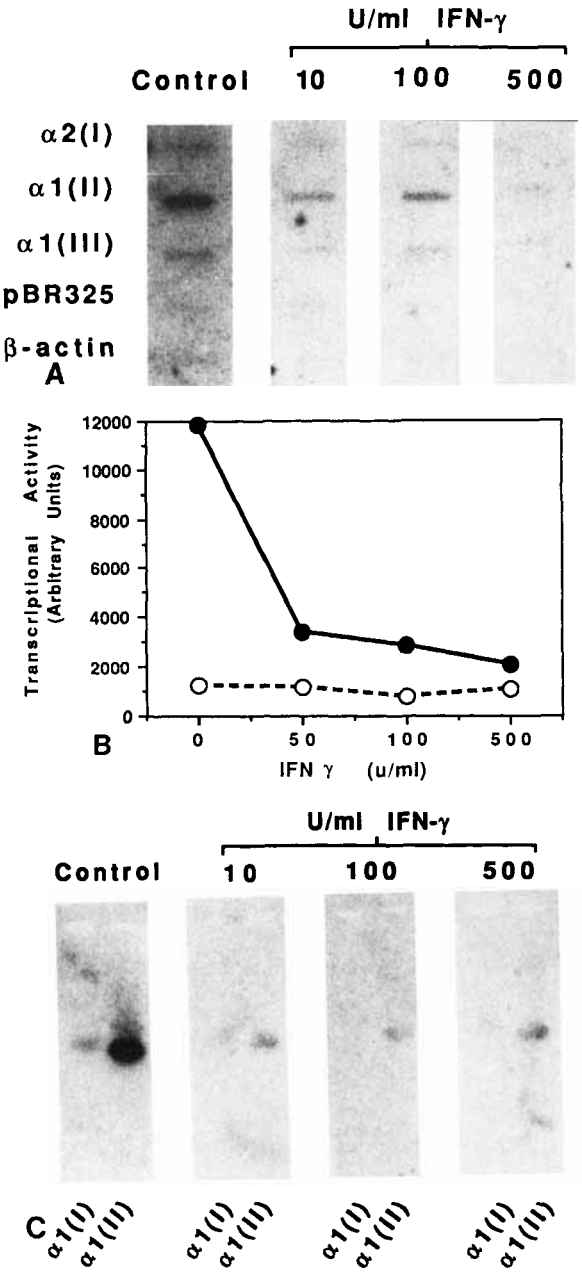

Fig. 2. Effects of IFN-γ on nuclear transcription of collagen genes in human chondrocytes. Costal chondrocytes (see Fig. 1) were preincubated for 24 h alone (control) or with IFN-γ (10, 100, and 500 U/ml). The nuclei were isolated as described in the methods and labeled using [^{32}P]UTP. **A:** The labeled transcripts were hybridized to linearized α2 (I) procollagen, α1(II) procollagen, α1(III) procollagen, pBR325 plasmid and β-actin DNAs that had been immobilized on nitrocellulose strips using a slot blot apparatus. **B:** Values determined by densitometric analysis of the autoradiographs are plotted for α1(II) procollagen (●) and β-actin (○) transcripts. **C:** The labeled nuclei were also hybridized to α1(II) and procollagen DNAs that had been run on agarose gels prior to blotting on nitrocellulose.

procollagen cDNAs. Northern blot analysis demonstrated that CHX reversed the IL-1β-induced suppression of levels of α1(II) procollagen mRNA at 3 and 6 h, but not at 24 h (Fig. 4, left). Interestingly, CHX in the presence of IL-1 caused a transient increase above control type II collagen mRNA levels at the early time points.

CHX had no significant effect on the level of α1(I) procollagen mRNA in the absence of IL-1β at any of the time points (Fig. 4, right; results are plotted only for incubations in the presence of indomethacin). In the presence of IL-1β, CHX also had no significant effect on type I collagen mRNA after 3 and 6 h of incubation. In contrast, the increase in the level of α1(I) procollagen mRNA observed after 24 h of treatment with IL-1β was inhibited by CHX.

The effect of IL-1β on procollagen mRNA stability was examined by inhibition of RNA synthesis with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) [Zandomeni et al., 1986]. Indomethacin was added in this experiment because we showed previously that this prostaglandin synthetase inhibitor prevents the synthesis of PGE_2 and its inhibitory effect on type I collagen gene expression, thereby unmasking the stimulatory effect of IL-1β [Goldring and Krane, 1987; Goldring et al., 1988]. Quiescent chondrocytes were incubated with IL-1β, indomethacin and/or CHX for 24 h prior to the addition of DRB at time 0. Northern blots of total RNAs harvested from cells 5, 10, and 24 h after addition of DRB, shown in Figure 5A, were analyzed by lazer scanning densitometry, as shown in Figure 5B. No significant change in the relative stability of the 5.5 kb α1(II) procollagen gene transcript was found in any of the preincubation conditions (Fig. 5A,B). Linear regression analysis of the densitometric data shown in Figure 5B (left panel) demonstrated that the slopes of the decay curves for α1(II) procollagen mRNA were similar in the absence and presence of IL-1β with an apparent half-life of ~ 18 h. The levels of GAPDH mRNA did not change in response to any of the culture conditions (Fig. 5A).

In the Northern blots shown in Figure 5A, the levels of α1(I) procollagen mRNA were decreased after incubation for 24 h (time 0) with IL-1β alone, but coincubation with indomethacin reversed this suppression and unmasked the direct stimulatory effect of IL-1β. In the absence of indomethacin, the rate of decay (~ 18 h) was similar in either the absence or presence of

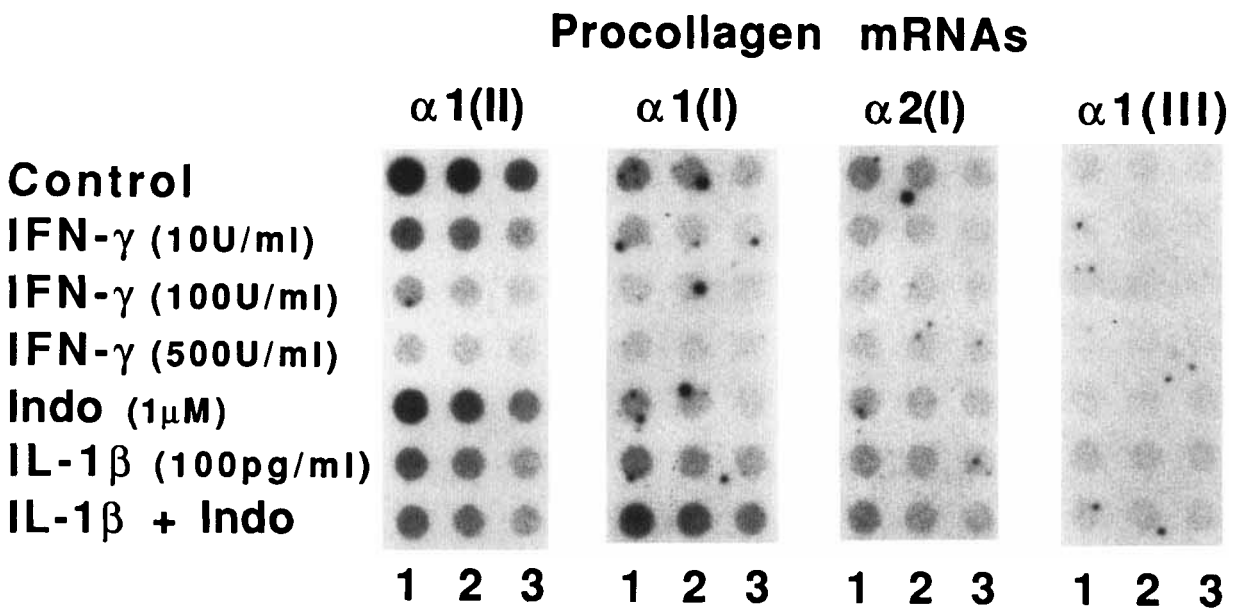

Fig. 3. Dot hybridization analysis of procollagen mRNA levels in costal chondrocytes treated with hIFN-γ and hIL-1β. Chondrocytes from the experiments described in Figs. 1 and 2 were incubated in parallel cultures for 24 h alone (control) or in the presence of either IFN-γ (50, 100, 500 U/ml) or IL-1β (5 pM) without or with indomethacin (1 μM) in culture medium containing 10% FCS. Cytoplasmic RNA was then extracted from the cell layers and applied to nitrocellulose at a range of concentrations (1.0, 0.5, and 0.25 μg of DNA equivalents in lanes 1, 2 and 3, respectively), and replicate blots were hybridized at the same time against a ^{32}P-labeled DNA probes encoding α1(II), α1(I), α2(I), and α1(III) procollagens. Films were exposed to blots for 48 h.

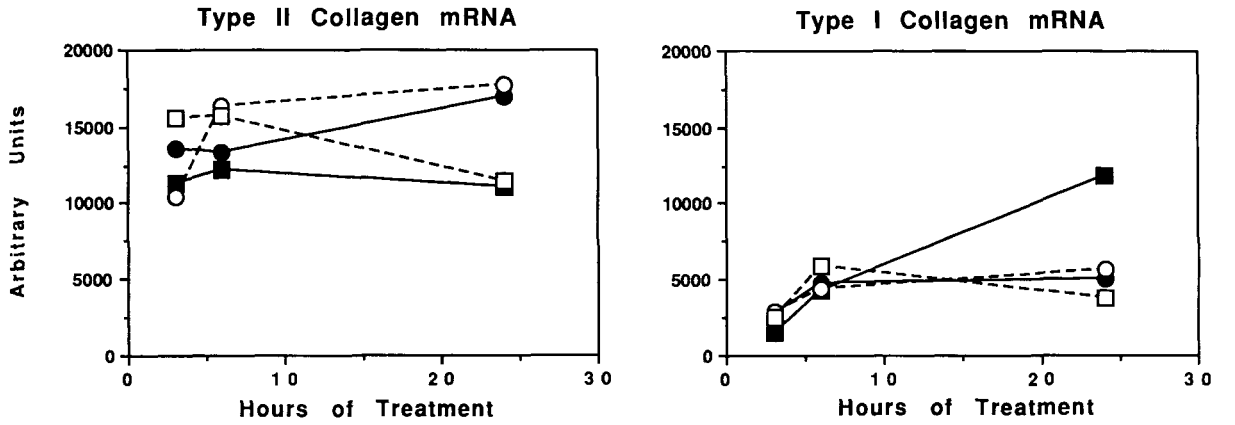

Fig. 4. Kinetics of α1(II) and α1(I) procollagen mRNA expression in costal chondrocytes treated with hIL-1β and indomethacin in the absence and presence of cycloheximide. Subconfluent costal chondrocytes (day 9 of primary culture) were made quiescent by preincubation in the presence of 0.5% FCS for 16 h. Cells were then treated without (●) or with IL-1β at 5 pM (■), CHX at 10 μg/ml (○), or IL-1β and CHX (□) and harvested for extraction of total RNA after 3, 6, and 24 h of incubation. Total RNA was extracted from the cell layers, separated on agarose gels (5 μg of total RNA per lane) and blotted on nitrocellulose. Replicate blots were hybridized against ^{32}P-labeled cDNAs encoding α1(II) (left panel) or α1(I) (right panel) procollagen. Films were exposed to blots for 2 and 14 days for α1(II) and α1(I) procollagen mRNAs, respectively. Values were determined by densitometric analyses of autoradiographs. The values for α1(I) procollagen mRNA represent the sums of the 5.9- and 7.2-kb transcripts.

IL-1β or cycloheximide. In contrast to the results with α1(II) procollagen mRNA, the rate of decay of α1(I) procollagen mRNA was greater in the presence than in the absence of IL-1β (Figure 5B, right panel; results are plotted only for incubations in the presence of indomethacin). Similar to the results shown in Figure 4, CHX prevented the stimulatory effect of IL-1β after 24 h of incubation (time 0). In the presence of indomethacin, CHX decreased the half-life of

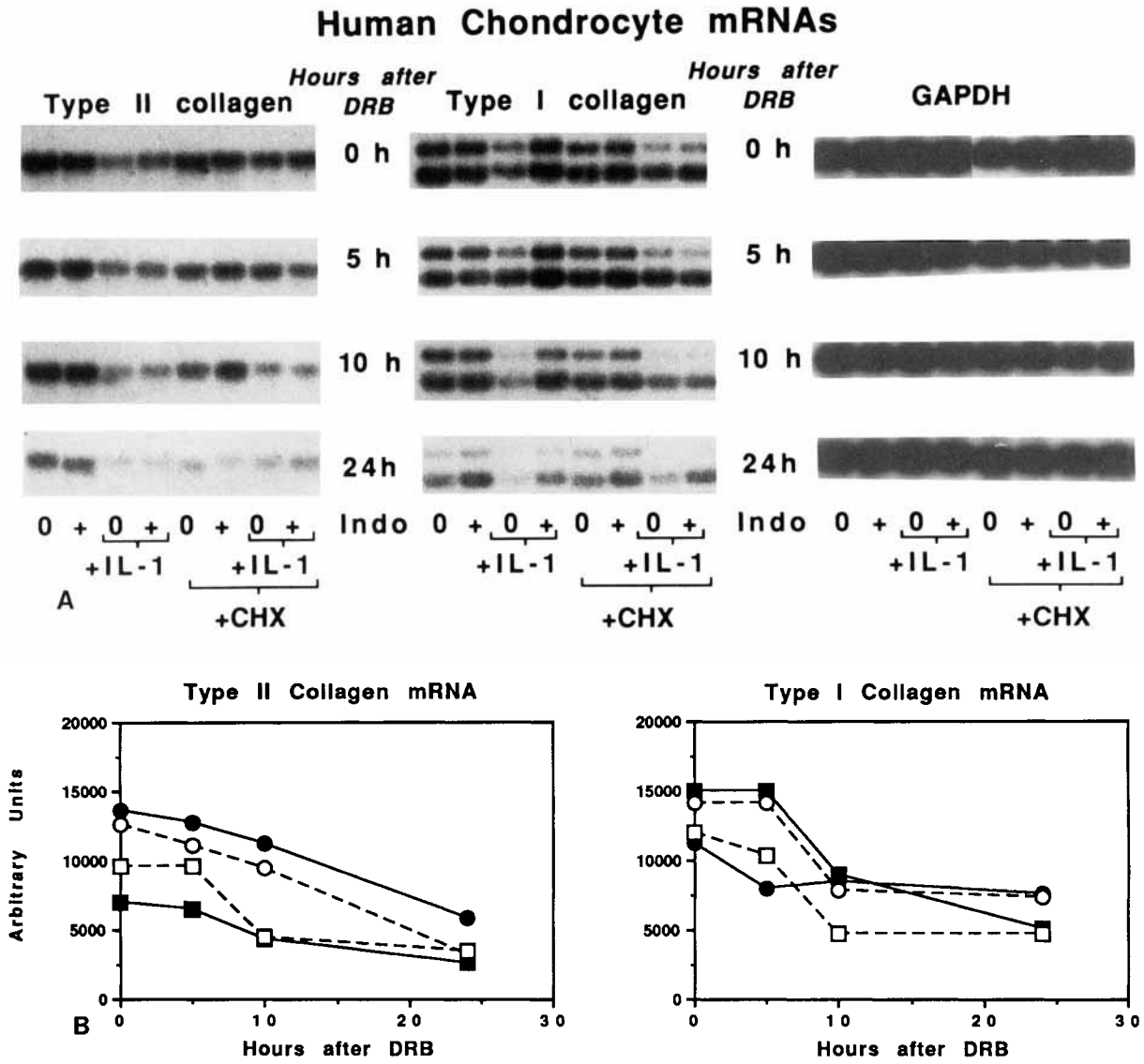

Fig. 5. Analysis of the stability of α1(II) and α1(I) procollagen mRNAs treated with IL-1β in the absence and presence of cycloheximide (CHX). Subconfluent costal chondrocytes (day 16 of primary culture) were made quiescent by preincubation in the presence of 0.5% FCS for 16 h. The medium was then changed (1% FCS) and CHX (10 μg/ml) was added in the absence or presence of hIL-1β (5 pM) and/or indomethacin (Indo; 1 μM) and the incubation continued for a further 24 h. DRB (10 μg/ml) was then added and cells were harvested after 0, 5, 10, and 24 h of incubation. Total RNA was analyzed on Northern blots as described in Fig. 4. (A) Films were exposed to blots for 3 days and 4 h for α1(II) and α1(I) procollagen mRNAs, respectively. (B) Values for α1(II) (left panel) or α1(I) (right panel) procollagen mRNAs from chondrocytes pretreated without (●) or with IL-1β (■), CHX (○), or IL-1β and CHX (□) were determined by densitometric analyses of autoradiographs. Values for α1(I) procollagen mRNA represent the sums of the 5.9- and 7.2-kb transcripts.

the control levels of α1(I) procollagen mRNA from 30 h to 23 h (Fig. 5B), while it had no significant effect in the presence of IL-1β. Consistent results have been observed in at least three separate experiments using CHX and DRB.

Inhibition of Expression of the Type II Collagen Gene by IL-1β and IFN-γ in Transient Expression Experiments

The 5′-flanking DNA sequence of the human type II procollagen gene (COL2A1) comprising the promoter, first exon and part of the first intron was cloned into a CAT gene expression vector. This construct, designated pCAT-B/4.0 (Fig. 6), spanning −577 to +3,428 bp, contains potential regulatory sequences that are homologous with sequences described in the rat [Horton et al., 1987] and mouse [Metsaranta et al., 1991] type II collagen genes, including silencer sequences responsive to fibroblast-specific nuclear factors in the upstream promoter region and a putative tissue-specific enhancer element

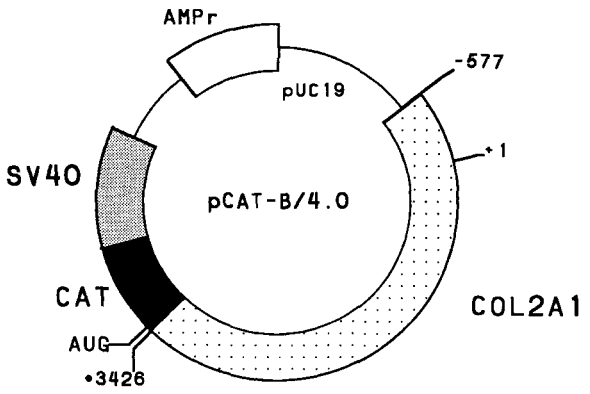

Fig. 6. Diagram of the pCAT-B/4.0 plasmid construct containing COL2A1 regulatory sequences. The 4.0-kb *PstI* fragment spanning −577 to +3426 of promoter, exon I and ⅔ of the intron I of COL2A1 was subcloned in the pCAT-Basic vector (Promega) containing the CAT reporter, SV40 small T antigen, and ampicillin resistance (AMPr) genes.

in the first intron [Ryan et al., 1990]. We used the pCAT-B/4.0 construct to determine the conditions required for its expression in human chondrocytes and to determine whether its expression could be inhibited by IL-1 and IFN-γ.

Monolayer cultures of chondrocytes isolated from two separate costal cartilage specimens were compared after passage through agarose suspension culture versus continuous monolayer culture. Low levels of expression of the pCAT-B/4.0 construct were observed in chondrocytes incubated in 10% FCS, whether they had been passaged over agarose or kept in monolayer culture (Fig. 7A,B). This construct was expressed in chondrocytes, after either agarose or monolayer culture, in the presence of an insulin-containing serum substitute, particularly in 1% Nutridoma-SP. Another insulin-containing serum substitute, ITS+, was also permissive for expression of pCAT-B/4.0 in chondrocytes but was more effective in cultures that had been redifferentiated on agarose than in those that had been subcultured only on tissue culture plastic. Similar results were obtained using subcultured chondrocytes derived from at least three additional specimens of costal cartilage and two of articular cartilage (data not shown). All further transient expression assays were performed in culture medium containing 1% Nutridoma.

The effects of recombinant human preparations of IL-1β and IL-1α at 2.5, 10, and 25 pM on transient expression of pCAT-B/4.0 were compared in chondrocytes (passage 2) that had been subcultured on tissue culture plastic. Both IL-1β

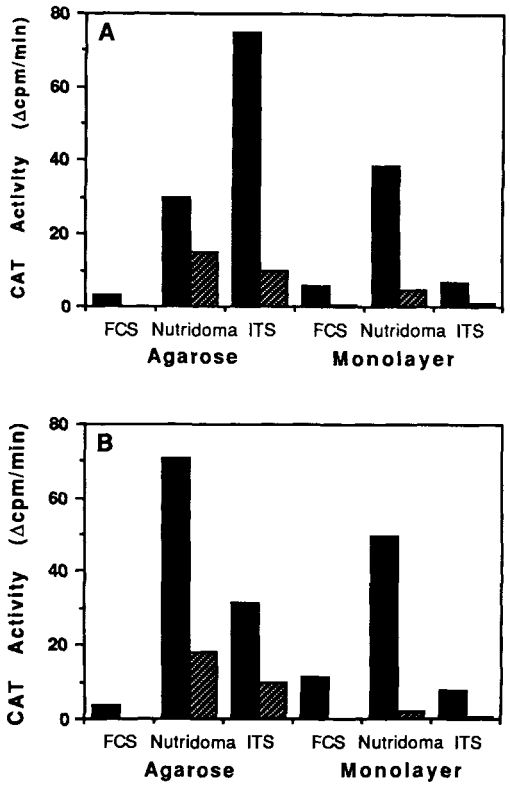

Fig. 7. Expression of 5'-flanking regulatory sequences of COL2A1 by human costal chondrocytes in different culture conditions. Monolayer cultures of chondrocytes isolated from two separate costal cartilage specimens at (**A**) passage 2 and (**B**) passage 1 were plated either on agarose or tissue culture plastic in Ham's F12/DMEM containing 10% FCS and cultured for 2 weeks with weekly medium changes. The cells on agarose were then transferred to tissue culture plastic and those on plastic were passaged to clean dishes at 10^6 cells per 100-mm dish and allowed to settle down for 24 h. The medium was then changed and the cells transfected with 10 μg of pCAT-B/4.0 DNA by the calcium-phosphate method (4 h), followed by glycerol shock (2 min). After washing, the cells were allowed to recover overnight in 10% FCS. Separate cultures were then incubated in culture medium containing 10% FCS, 1% Nutridoma-SP or 1% ITS+ without (■) or with (▨) hIL-1β (5 pM) for 24 h and harvested for CAT assay by the fluor diffusion method.

and IL-1α inhibited COL2A1 expression in a dose-dependent manner (Fig. 8). IL-1β was more potent than IL-1α, consistent with their relative effects on type II collagen synthesis and steady-state levels of α1(II) procollagen mRNA that we had observed previously. In addition, indomethacin potentiated the inhibitory effect of IL-1β added at 2.5, 5.0, and 25 pM (Fig. 9).

To explore the tissue specificity of COL2A1 expression, pCAT-B/4.0 was transfected in human fibroblasts and CAT activity assessed in the presence of either 1% FCS or 1% Nutridoma. Virtually no expression of pCAT-B/4.0 could be detected in fibroblasts cultured in any of the

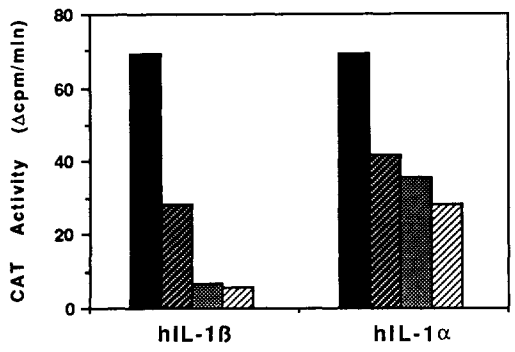

Fig. 8. Dose-dependent inhibition of expression of pCAT–B/4.0 by hIL-1β and hIL-1α. Subcultured human costal chondrocytes were cultured on tissue culture plastic and transfected with pCAT-B/4.0 as described in Fig. 6. After recovery overnight in 10% FCS, the medium was changed to 1% Nutridoma-SP. Increasing concentrations (2.5, 10, and 25 pM) of either hIL-1β or hIL-1α were added and the incubation continued for a further 42 h until extraction for CAT assay. The CAT activity was plotted for untreated control (■), 2.5 pM (▨), 10 pM (□), and 25 pM (⊡) for each IL-1 preparation.

conditions tested (Table I). Moderate expression of pCAT-B/4.0 could be observed in rat chondroblasts, RCJ 3.1 C5.18 subclone (Grigoriadis et al., 1988), incubated in 1% Nutridoma, although expression was 20 to 25% of that in human chondrocytes in at least three experiments performed thus far; this expression was inhibited by human IL-1β, or murine IL-1α and by murine IFN-γ (Table I). Human IL-1α was also an effective inhibitor of pCAT-B/4.0 expression in the rat chondroblasts (not shown). In these experiments, murine IFN-γ was used in the rat cultures, since IFN-γ responsiveness is known to be relatively species-specific and rat cells are known to respond to murine but not to human IFN-γ. The pCAT-Control plasmid, containing the SV40 promoter and enhancer expressed in both the human fibroblasts and the rat chondroblasts (Table I) and human chondrocytes (see Fig. 10); this expression was not inhibited by any of the IL-1 and IFN-γ preparations. These results indicate that IL-1- and IFN-γ-responsive

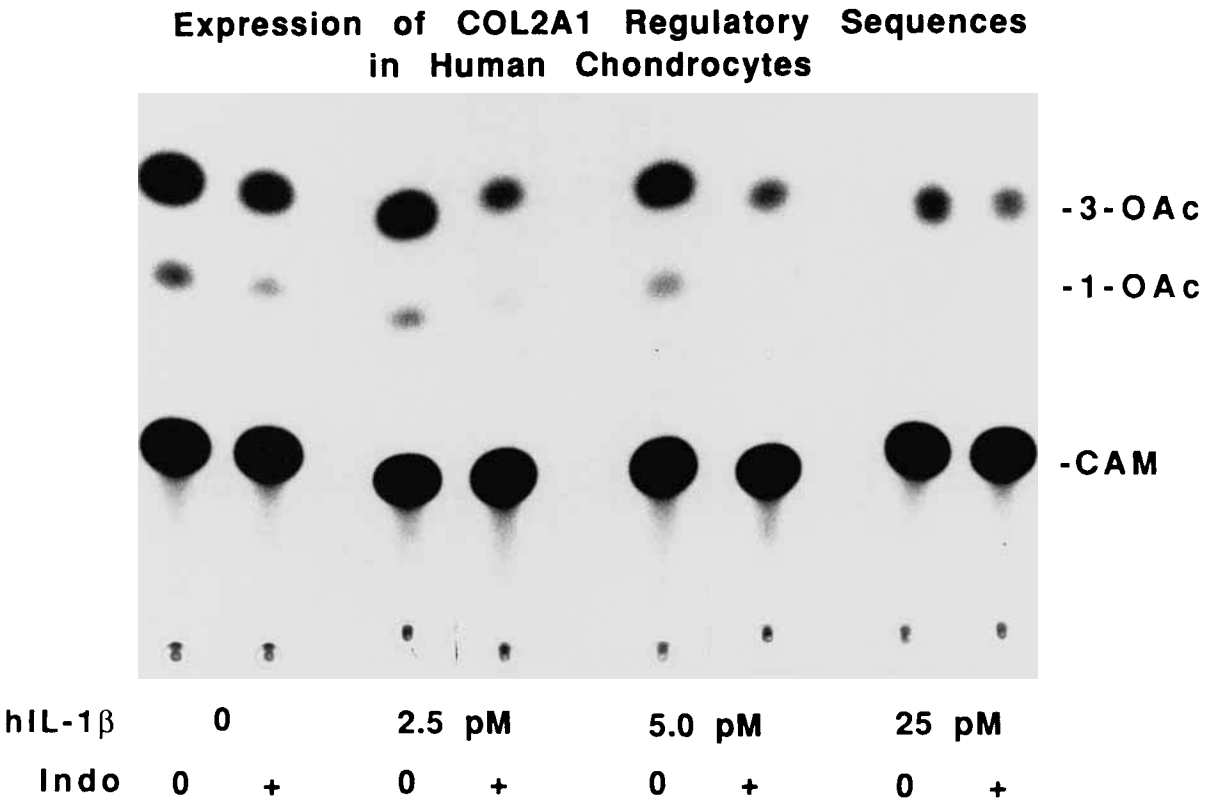

Fig. 9. Dose-dependent suppression of pCAT–B/4.0 expression by hIL-1β and potentiation of this suppression by indomethacin in human costal chondrocytes. Chondrocytes were cultured, transfected and allowed to recover as described in Fig. 6. Increasing concentrations of hIL-1β (2.5, 5.0, and 25 pM) were added in culture medium containing 1% Nutridoma-SP in the absence or presence of indomethacin (Indo; 1 μM) and incubated for 46 h until extraction for CAT assay by the thin layer chromatography method. CAT activity was assessed as the conversion of the substrate [^{14}C]chloramphenicol to the acetylated products, 3-OAc and 1-OAc, as indicated.

TABLE I. Tissue-Specific Expression of pCAT-B/4.0 in Rat Chondroblasts but Not in Human Fibroblasts*

Cell type	Condition	pCAT-B/4.0 Activity (Δcpm/min)				pCAT-control	
		Untreated	hIL-1β	mIL-1α	mIFN-γ	Untreated	hIL-1β
Rat chondroblasts	1% FCS	4.2	3.3	2.5	1.5	—	—
	1% Nut	9.7	4.1	2.5	3.2	—	—
	1% Nut	15.3	9.0	—	3.0	—	—
	1% Nut	9.7	—	—	2.5	21.7	—
Human fibroblasts	1% Nut	0.2	0.2	—	—	—	—
	1% FCS	0.5	0.5	—	—	—	—
	1% Nut	0.2	0.2	—	—	35.2	34.3

*Rat chondroblasts (RCJ 3.1) were plated at 0.5×10^6 cells per 100-mm dish in culture medium containing 15% FCS and 10^{-8}M dexamethasone. Subcultured human foreskin fibroblasts were plated at 1.0×10^6 cells per 100-mm dish in DMEM containing 10% FCS. The cells were allowed to settle down overnight and the culture medium was then changed. The cells were trarnsfected 2–4 h later with 10 μg/plate of pCAT–B/4.0 or pCAT-Control DNA as described in Figure 6. After washing, fresh culture medium containing either 1% FCS or 1% Nutridoma was added. The rat chondroblasts were allowed to recover for 2–3 h and the human fibroblasts for 16 h, hIL-1β (10 pM), mIL-1α (10 pM), or mIFN-γ (500 U/ml) was then added, and the incubation was continued for 42–48 h.

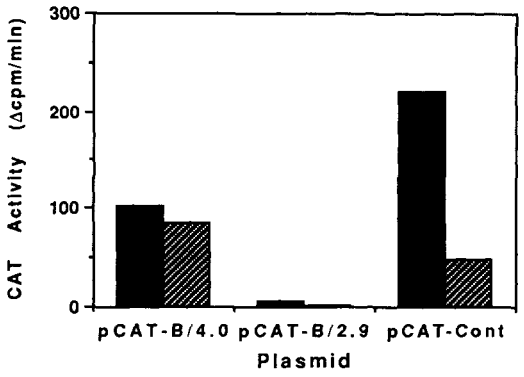

Fig. 10. Expression of pCAT-B/4.0 compared with pCAT–B/2.9 in human chondrocytes. Chondrocytes (passage 4) that had been passaged through agarose suspension culture for 3 weeks then replated on tissue culture plastic (■) were compared with cells that had been passaged in monolayer culture (□). The cells were transfected with pCAT–B/4.0 or pCAT–B/2.9 as described in Fig. 6 and incubated in DMEM/F12 containing 1% Nutridoma for 42 h.

elements reside in the 5′-flanking sequences of COL2A1 rather than in the SV40 or plasmid DNA sequences in the pCAT-B/4.0 construct.

When the putative enhancer fragment in the first intron of COL2A1 was removed by deleting the 3′ end to +2370 (pCAT-B/2.9), the expression in chondrocytes was reduced markedly in human chondrocytes that had been either passaged through agarose or maintained in monolayer culture (Fig. 10). Interestingly, IL-1β inhibited the residual activity of the pCAT-B/2.9 construct (data not shown). These results suggest that the negative regulatory element important for the response to IL-1 is not contained in the domain spanning +2370 to +3426, but is likely to be in the region spanning −577 to +2,370 of the COL2A1 gene.

DISCUSSION

Our results demonstrate that both IL-1 and IFN-γ inhibit type II collagen gene expression at the transcriptional level. Our human chondrocyte culture system has served as an excellent model for examining the effects of cytokines on cartilage-specific phenotypic expression. We showed previously that IL-1 accelerates the loss of type II collagen gene expression by chondrocytes in primary culture while it increases the expression of type I and type III collagens [Goldring et al., 1988]. Since IFN-γ inhibits the expression of all three types of collagens in chondrocytes [Goldring et al., 1986], it has been useful to compare its effects with those of IL-1. Inhibition of type II collagen gene transcription by IL-1 has been demonstrated in rat and rabbit chondrocytes [Chandrasekhar et al., 1990], but transcriptional effects of IFN-γ on type II collagen gene expression have not been reported previously. Our results also suggest that the suppressive effect of IL-1 on the levels of type II collagen mRNA cannot be accounted for by changes in mRNA stability, and indicate that cycloheximide may block the synthesis of an IL-1-induced repressor factor for α1(II) procollagen gene transcription.

The regulation of expression of the type I collagen gene by IL-1 may be more complex than that of the type II collagen gene. Our results suggest that CHX may block synthesis of a constitutive repressor factor which suppresses transcription of the α1(I) procollagen gene or, alternatively, inhibit synthesis of an IL-1-induced enhancer factor. Experiments with the transcriptional inhibitor DRB indicated that the stimulatory and inhibitory (via PGE_2) effects of IL-1 cannot be entirely accounted for by changes in the stability of α1(I) procollagen mRNA. Our results are somewhat analogous to those of Askew et al. [1991], who demonstrated transcriptional regulation of type II collagen gene expression in chick embryo chondrocytes treated with 5-bromo-2′-deoxyuridine, but complex transcriptional regulation of type I collagen gene expression involving nuclear stability of α1(I) RNA and cytoplasmic stability of alternatively spliced α2(I) mRNA.

In nuclear transcription experiments we also demonstrated clearly that IFN-γ inhibits type I collagen gene expression in human chondrocytes at the transcriptional level. Kahari et al. [1990] reported that expression of the α2(I) collagen gene was not inhibited by IFN-γ in transient transfection experiments in human dermal fibroblasts and mouse NIH/3T3 cells. However, the human recombinant IFN-γ used would not be expected to activate murine IFN-γ receptors, [Gray et al., 1989; Garotta et al., 1990]. In addition, we noted previously that the expression of α2(I) procollagen mRNA in human fibroblasts and chondrocytes is not stringently regulated by IFN-γ compared with α1(I) procollagen mRNA [Stephenson et al., 1985; Goldring et al., 1986].

In the experiments reported here we found, surprisingly, that subcultured chondrocytes expressed COL2A1 regulatory sequences in the pCAT-B/4.0 construct to the same extent as the chondrocytes that had been passed through agarose suspension culture. The major determining requirement appears to be that serum be removed after the transfection and an insulin-containing serum substitute added during the incubation. The insulin in these preparations may serve as a substitute for insulin-like growth factor-I (IGF-I) that has been shown to be a relatively specific factor for maintenance of cartilage-specific phenotype. We found that 1% Nutridoma was permissive for IL-1 responsiveness in subcultured human chondrocytes and a similar serum substitute, ITS+, maintained chondrocyte phenotype after transfer through agarose suspension culture. Our experiments demonstrated that (1) FCS may contain factors (e.g., PDGF) that prevent expression of COL2A1 in chondrocytes grown either in monolayer on plastic or in chondrocytes that have been redifferentiated by suspension culture on agarose; (2) insulin, possibly serving as a substitute for IGF-I, is permissive for expression of the cartilage-specific pCAT-B/4.0 construct, even in subcultured chondrocytes where endogenous expression is low; and (3) in all cases in which COL2A1 is expressed, this tissue-specific expression is suppressed by IL-1 by 50–80%. Our results agree with those of Chandrasekhar et al. [1990], who showed that transient expression of fusion constructs of the entire 5′-flanking regulatory region of the rat type II collagen gene containing the promoter and first intron could be suppressed by IL-1 in primary cultures of rabbit chondrocytes in serum-free conditions. In our study, primary cultures of human chondrocytes, as well as subcultured human chondrocytes, and rat chondroblasts also expressed pCAT-B/4.0, while expression by human fibroblasts was not detected. The inability of human fibroblasts to express COL2A1 may be due to the presence of sequences in the human promoter that are homologous to rat promoter sequences that may bind to fibroblast-specific nuclear factors termed "silencers" [Ryan et al., 1990; Savagner et al., 1990]. Our results indicate that subcultured chondrocytes have not "dedifferentiated" to fibroblasts, since they are able to express the transfected COL2A1 regulatory sequences and, therefore, presumably have not acquired fibroblast-specific silencer factors.

While IL-1 and IFN-γ both specifically and reproducibly suppress COL2A1 transcription, their effects are synergistic (unpublished data), and the signaling pathways involved are, therefore, likely to be distinct. These cytokines are known to exert their effects on a variety of cellular processes via different receptors and signaling pathways [see Goldring and Goldring, 1991 for review]. IL-1 has been shown to induce or activate a complex array of transcription factors in fibroblasts and other connective tissue cell types. In addition to the AP-1 (c-Fos/c-Jun) site [Schonthal et al., 1988; Quinones et al., 1989; Case et al., 1990; Conca et al., 1991], PEA3 binding sites in the promoters of IL-1-responsive genes may be necessary for full re-

sponsiveness [Gutman and Wasylyk, 1990; Sirum-Connolly and Brinckerhoff, 1991]. Activation of NF-κB has also been implicated in IL-1-induced responses [Ron et al., 1990; Zhang et al., 1990; Anisowicz et al., 1991; Kessler et al., 1992]. In some genes, multiple cytokine-responsive elements contain overlapping sites that recognize NF-κB- and C/EBP-related proteins and permit cooperative effects of cytokines [Ray et al., 1989; Isshiki et al., 1990; Ron et al., 1990; Won and Baumann, 1990]. Multiple overlapping sites may also be important for IFN-γ-induced genes, including the interferon-stimulated response element (ISRE) [Reid et al., 1989], the Complex A-binding site [Finn et al., 1990] and the IFN-γ activation site, or GAS [Lew et al., 1991]. IFN-γ also suppresses expression of pCAT-B/4.0 in human chondrocytes and its effect is more potent than that of IL-1. Further studies to determine the precise DNA elements and transcription factors involved indicate that both the IL-1- and IFN-γ-responsive elements lie in the promoter region of the COL2A1 gene [Yamin et al., 1992].

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