# Interleukin-1β-modulated Gene Expression in Immortalized Human Chondrocytes

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## Abstract

Immortalized human chondrocytes were established by transfection of primary cultures of juvenile costal chondrocytes with vectors encoding simian virus 40 large T antigen and selection in suspension culture over agarose. Stable cell lines were generated that exhibited chondrocyte morphology, continuous proliferative capacity (> 80 passages) in monolayer culture in serum-containing medium, and expression of mRNAs encoding chondrocyte-specific collagens II, IX, and XI and proteoglycans in an insulin-containing serum substitute. They did not express type X collagen or versican mRNA. These cells synthesized and secreted extracellular matrix molecules that were reactive with monoclonal antibodies against type II collagen, large proteoglycan (PG-H, aggrecan), and chondroitin-4- and chondroitin-6-sulfate. Interleukin-1β (IL-1β) decreased the levels of type II collagen mRNA and increased the levels of mRNAs for collagenase, stromelysin, and immediate early genes (egr-1, c-fos, c-jun, and jun-B). These cell lines also expressed reporter gene constructs containing regulatory sequences (−577/+3,428 bp) of the type II collagen gene (COL2A1) in transient transfection experiments, and IL-1β suppressed this expression by 50–80%. These results show that immortalized human chondrocytes displaying cartilage-specific modulation by IL-1β can be used as a model for studying normal and pathological repair mechanisms. (J. Clin. Invest. 1994. 94:2307–2316.) Key words: simian virus 40 large T antigen • collagen • proteoglycan • cartilage • cytokine

## Introduction

The chondrocyte, the only cell type in mature articular cartilage, is regarded as a terminally differentiated cell that maintains the cartilage-specific matrix phenotype under normal conditions of low turnover. The collagen types II, IX, and XI are unique to this tissue and interact with specific proteoglycans to form the highly hydrated extracellular matrix of hyaline cartilage. Freshly isolated human chondrocytes express cartilage-specific type II collagen and continue to do so for several days to weeks in primary culture (1, 2). During prolonged culture and serial subculture in monolayer, these cells begin to express type I and type III collagens. This "switch" can be accelerated by plating the cells at low densities or by treating them with agents such as interleukin-1 (IL-1) (2, 3). The reexpression of the chondrocyte phenotype in subcultured cells can be induced by suspension culture on or within agarose or by use of serum-free defined medium (4–6). Studies in embryonic quail and chick chondrocytes demonstrated the potential of v-src and v-myc oncogenes to generate cell lines of high proliferative capacities that retained at least some differentiated chondrocyte properties (7, 8). Attempts to establish immortalized chondrocyte lines from murine sarcoma virus–transfected rat chondrocytes (9), simian virus 40 (SV40)[1]-transfected rabbit chondrocytes (10), and human chondrosarcomas (11, 12) have resulted in cells that express cartilage-specific proteoglycans but little or no type II collagen. Chondrocyte lines have also arisen spontaneously from fetal rat calvaria (13, 14).

Primary cultures of chondrocytes isolated from young animals that maintain the cartilage-specific phenotype are easily obtained and have been used widely to assess differentiated chondrocyte functions. The use of chondrocytes of human origin has been more problematic, because the source of the cartilage cannot be controlled, sufficient numbers of cells are not readily obtained from random operative procedures, and the phenotypic stability of adult human chondrocytes is lost more quickly upon expansion in serial monolayer cultures than that of cells of juvenile human (1) or young or embryonic animal origin (15, 16). The lack of a reproducible source of chondrocytes of human origin has hampered progress in studies of cartilage function relevant to human disease.

We have sought to develop chondrocyte lines that express the differentiated chondrocyte phenotype and display characteristic responses to cytokines that modulate this phenotype. IL-1 is thought to play a major role in inflammatory and destructive processes associated with the breakdown of cartilage matrix in rheumatoid arthritis and osteoarthritis (17). This cytokine suppresses the expression of cartilage-specific collagens (2, 18) and proteoglycans (19–21) in cultures of chondrocytes and intact cartilage. IL-1 may also have a role in the inappropriate repair of cartilage matrix that accompanies inflammatory joint disorders by stimulating the synthesis of noncartilage collagens (type I and type III) by chondrocytes (2, 3).

To obtain immortalized chondrocyte lines of human origin, we used a combination of strategies involving transfection with

1. Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40.

vectors containing SV40 DNA sequences, selection on agarose, expansion in serum-containing medium, and modulation of phenotype in serum-free defined medium. Because type II collagen expression is the most highly labile function of chondrocyte cultures, we used this marker as the primary indicator of the differentiated phenotype. We established immortalized human chondrocyte lines that express mRNAs encoding cartilage-specific matrix proteins, including type II, type IX, and type XI collagens and aggrecan, and that deposit an extracellular matrix with reactivity to antibodies against type II collagen, large proteoglycan, and sulfated glycosaminoglycans. They also exhibit the expected chondrocyte responses to IL-1$\beta$, including decreased expression of the endogenous or transiently transfected type II collagen gene and increased expression of metalloproteinase and immediate early genes.

## Methods

*Materials.* Recombinant human IL-1$\beta$ (provided by Dr. J.-M. Dayer, Division of Immunology and Allergy, University Hospital, Geneva, Switzerland), thawed from frozen stock (2.5 $\mu$M 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. The $\alpha$1(II) procollagen cDNA (KTh1330), the $\alpha$1(IX) procollagen cDNA (KTh123), and the $\alpha$2(XI) procollagen cDNA (KTh181) probes were cloned using a cDNA library prepared from our human juvenile costal chondrocyte cultures (22, 23). The type I cDNA probe, Hf677, a 1,500-bp cDNA encoding part of the $\alpha$1(I) procollagen subunit, was provided by Dr. F. Ramirez (Mt. Sinai School of Medicine, New York) and Dr. D. Prockop (Thomas Jefferson University, Philadelphia, PA) (24). A human aggrecan cDNA was provided by Dr. K. J. Doege (Shriner's Hospital, Portland, OR) (25). Probes encoding the small proteoglycans biglycan/PG-I and decorin/PG-II were provided by Dr. L. W. Fisher and Dr. M. F. Young (National Institute of Dental Research, Bethesda, MD) (26, 27). The cDNA probe for human procollagenase (XHF$_1$) was provided by Dr. H. J. Rahmsdorf (Kemforschungszentrum Karlsruhe, Karlsruhe, Germany) (28). An additional collagenase probe (pCOLL-4), a 1.5-kb XbaI cDNA fragment (29) that gave identical results and probes for stromelysin (pSTR-33), a 1.8-kb PstI cDNA fragment, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were provided by Dr. N. I. Hutchinson (Merck Sharp & Dohme Research Laboratories, Rahway, NJ) (29). The c-*jun* (30) and *jun*-B (31) cDNA probes were provided by Dr. D. Nathans (Johns Hopkins University School of Medicine, Baltimore, MD), and the v-*fos* cDNA (32) was provided by Dr. R. Bernards (Massachusetts General Hospital, Charlestown, MA). The *egr*-1 cDNA (OC3.1) is the 3.1-kb EcoRI fragment from Dr. V. P. Sukhatme (Beth Israel Hospital, Boston, MA) (33).

*Chondrocyte isolation and transfection.* Human costal cartilage was obtained from tissue discarded after surgical repair of pectus excavatum. Chondrocytes were isolated by dispersion with proteases and cultured in Dulbecco's modified Eagle's medium (DME; GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS), as described previously (1–3). The immortalized human chondrocyte line, designated C-20/A4, was derived from juvenile costal chondrocytes isolated from a specimen of rib cartilage from a 5-yr-old male. At day 10 of primary culture, 10 confluent 75-cm$^2$ flasks were transfected with origin-defective SV40 (20 $\mu$g/flask of pMK/SV40 ori$^-$ plasmid DNA) (34) using the polybrene/DMSO method (35, 36), and two flasks were "mock" transfected. The cells were incubated at 37°C for 6 h with the plasmid DNA in DME containing 5% FCS and 10 $\mu$g/ml polybrene. The medium was then removed and 30% DMSO in DME/5% FCS added for 3 min. The cells were washed once and incubated for 2 d in DME containing 10% FCS. The medium was changed weekly, and foci appeared after 2–3 wk in the SV40-transfected but not in the mock-transfected flasks. After 6–8 wk, foci (10–20 per flask) were removed with the tip of a pasteur pipette, and each was placed in a 16-mm diameter well (24-well plate; Costar Corp., Cambridge, MA) containing 1 ml of DME

with 10% FCS. Confluent wells were obtained within 1 wk, and the cells from each well were passaged to 10-cm plates using trypsin-EDTA (GIBCO BRL). The cells were then passaged every week at a split ratio of 1:5 in DME containing 10% FCS. Of the 140 foci obtained originally, 20 of the resulting cultures were subcultured more than 20 passages to permit senescence of the parental cells.

The T/C-28 line was derived from primary cultures (day 5) isolated from costal cartilage from a 15-yr-old female. We used the SV40 large T antigen inserted in the retroviral neomycin-resistant pZipNeoSV(X) vector (37) and transfected using polybrene, as described previously (38). After selection of transfected cells in G418 (500 $\mu$g/ml; GIBCO BRL), the uncloned cell populations were established through several subcultures in DME containing 10% FCS. They were then further selected by suspension culture above agarose (2–4 wk) (6) followed by monolayer culture in DME/Ham's F12 (1/1, vol/vol) containing 10% FCS and cloned by limiting dilution. Over 40 clonal lines were established, most of which had the polygonal cobblestone morphology characteristic of chondrocytes.

*Collagen synthesis.* Subconfluent immortalized chondrocytes were trypsinized and plated at a split ratio of 1:10 in DME with 10% FCS and incubated at 37°C for 3–5 d. Proteins were labeled with [$^3$H]proline ($>$ 20 Ci/mmol; Amersham Corp., Arlington Heights, IL) at 25 $\mu$Ci/ml for a further 24 h in serum-free DME supplemented with 50 $\mu$g/ml ascorbate and 50 $\mu$g/ml $\beta$-aminoproprionitrile fumarate. Pepsin-resistant collagen chains secreted into the culture medium were analyzed by SDS-PAGE (5% acrylamide) with and without delayed reduction in 0.1% $\beta$-mercaptoethanol to distinguish $\alpha$1(III) from $\alpha$1(I or II) collagens, as described previously (1–3).

*Immunocytochemistry.* Cells were plated in plastic Lab-Tech 4-chamber slides (Nunc, Inc., Naperville, IL) at 6 $\times$ 10$^4$ cells/chamber in DME/Ham's F12 containing 10% FCS and grown for 3–5 d to subconfluence. The monolayer was rinsed with Dulbecco's Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline (PBS) and incubated with culture medium containing 1% Nutridoma-SP (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 25 $\mu$g/ml ascorbic acid. At the end of incubation periods of 1–8 d, the chambers were rinsed three times with PBS, and the cells were fixed with 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. After two rinses with 0.1 M cacodylate buffer, the following monoclonal antibodies were added to chambers on different slides: MAB1330 anti–human type II collagen (Chemicon International, Inc., Temecula, CA) and 2-B-1 anti–human large proteoglycan (Seikagaku America, Inc., Rockville, MD), which has been used to identify the protein core of proteoglycan in chondrocytes and extracellular matrix in articular cartilage (39). Other chamber slides were incubated with chondroitinase ABC for 30 min at 37°C before addition of monoclonal antibodies against chondroitin-4-sulfate ($\Delta$Di-4S), chondroitin-6-sulfate ($\Delta$Di-6S), or chondroitin-0-sulfate ($\Delta$Di-0S) (Seikagaku America, Inc.). The staining was visualized by incubation with a gold-conjugated secondary antibody using Auroprobe LM (Amersham Corp.) followed by silver enhancement with the IntenSE Kit (Amersham Corp.). The monoclonal antibody pAb 416 (purchased as SV40 T Ag from Oncogene Science, Inc., Uniondale, NY) and fluorescein-labeled anti–mouse Ig (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used to detect the expression of the SV40 large T antigen in the immortalized cells.

*Analysis of RNA.* Total RNA for Northern blots was extracted by the acid guanidinium thiocyanate-phenol-chloroform method adapted from Chomczynski and Sacchi (40) or a lithium chloride method adapted from Cathala et al. (41). The final preparations gave yields of ~ 10 $\mu$g of RNA per 1 $\times$ 10$^6$ cells with the appropriate A$_{260}$:A$_{280}$ ratio of ~ 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 & Schuell, Inc., Keene, NH) as described previously (2, 42).

The cDNA inserts were excised from the plasmids using appropriate restriction enzymes and labeled with [$\alpha$-$^{32}$P]dCTP (Dupont/New England Nuclear Research Products, Boston, MA) by random priming using an oligolabeling kit (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Blots were prehybridized in 50% formamide, 5$\times$ SSC, 5$\times$
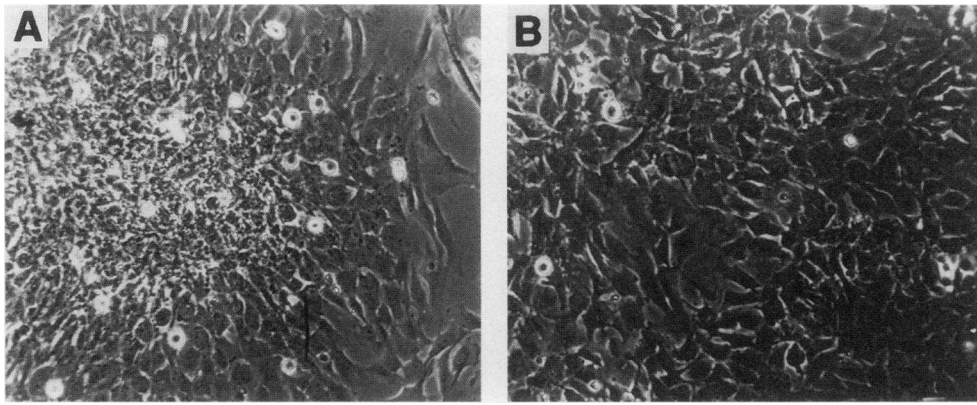
*Figure 1.* (*A*) Phase-contrast micrograph of juvenile human costal chondrocytes expanded from a focus selected after infection with origin-defective SV40. (*B*) After > 60 passages, the C-20/A4 line continued to grow well and maintain chondrocyte morphology.

Denhardt's solution, and 0.3% SDS at the hybridization temperature, 100 ng/ml DNA probe was added, and hybridization was carried out for at least 16 h at 54°C with the collagen probes and 45°C with the noncollagen probes. Staining with ethidium bromide and hybridization with the GAPDH cDNA probe were used to monitor uniform loading of RNA on Northern blots.

*Transient expression assay of type II collagen gene regulatory sequences.* The pCAT-B/4.0 construct contained a 4.0-kb PstI fragment of the human type II collagen gene (COL2A1) (43), spanning −577 bp of upstream promoter sequence to +3,428 bp downstream of the mRNA start site, cloned into the PstI site of the pCAT™-Basic plasmid (Promega Corp., Madison, WI) immediately upstream of the chloramphenicol acetyl transferase (CAT) reporter gene (42). Immortalized human chondrocytes were seeded at $0.5 \times 10^6$ cells per 10-cm plate and incubated overnight in DME/Ham's F12 containing 10% FCS. Fresh culture medium containing 10% FCS was added 2 to 3 h before transfection. The cells were transfected with 10 μg of plasmid DNA per 100-mm dish by the calcium-phosphate method (4 h) followed by glycerol shock (2 min) using the calcium phosphate mammalian cell transfection kit (5 Prime → 3 Prime, Inc., Boulder, CO). After washing twice with serum-free medium, DME/Ham's F12 containing 1% Nutridoma-SP and IL-1β was added. The cultures were incubated for a further 42–48 h and harvested for CAT assay. Aliquots of cell extracts containing 25 μg of protein (assayed with protein assay kit; Bio-Rad Laboratories, Richmond, CA) were analyzed for CAT activity by the fluor diffusion method (44, 45). First order slopes (Δcpm per min) were derived from plots of activity versus reaction time.

## Results

*Immortalization of human chondrocytes.* Juvenile human chondrocytes in early primary culture were used in these studies in an attempt to preserve the differentiated chondrocyte phenotype. We showed previously that these cells exhibit high levels of type II collagen mRNA and synthesis of type II collagen with little or no detectable type I collagen expression at least through the first 2–4 wk of culture (1, 2). Therefore, chondrocytes isolated from two donors were transfected within 4–10 d of primary culture with different vectors containing DNA encoding SV40 large T antigen.

The plasmid pSVori⁻, containing origin-defective SV40 sequences inserted into the pMK16 vector, had been used previously to establish permanent human fibroblast lines (34, 46). Primary human chondrocytes at day 10 of culture were transfected with pSVori⁻ DNA. Transformed foci appeared in all flasks within 2 wk as densely packed clusters. The immortalized cells were obtained by selection of foci 4–8 wk after transfection and weekly subculture in medium containing 10% FCS to produce senescence of the parental cells (Fig. 1 *A*).

After ~ 20 wk of continuous culture, the pSVori⁻-transformed chondrocytes entered a "crisis" phase corresponding to senescence in the nontransformed cells. Of the cultures that recovered, one of these, designated C-20/A4, continued to grow well and maintain a homogeneous polygonal morphology characteristic of chondrocytes rather than fibroblasts (Fig. 1 *B*). These cells were carried in culture medium containing 10% FCS and subcultured weekly in 10-cm tissue culture plates; it was not necessary to further select these cells by agarose suspension culture. The C-20/A4 line maintained stable growth and morphology for > 80 passages.

Another set of immortalized human chondrocytes, designated T/C-28, was derived by transfection of 5-d primary cultures with the retroviral vector pZipNeoSV(X) containing the SV40 large T antigen and the neomycin resistance gene (37). 1 wk after the transfection, neomycin-resistant cells were selected by treatment with G418. In initial attempts, resistant colonies that were picked randomly after 1 wk of treatment with G418 did not expand readily and generate stable cell lines, but underwent cell crisis within 4 mo. Successful establishment of clonal cell lines derived from the T/C-28 cultures required that they be established through several passages after G418 selection. Several cell lines were established after direct cloning of neomycin-resistant cells by limiting dilution and cultured continuously on tissue culture plastic in medium containing 10% FCS.

Generation of stable chondrocyte lines was accomplished after further selection of neomycin-resistant cells by suspension culture on agarose-coated plates for 2–4 wk, as described for chick embryo chondrocytes by Castagnola et al. (6). The noncloned T/C-28 chondrocytes at passage 20 (Fig. 2 *A*), 6 mo after transfection, were incubated with trypsin-EDTA, and the resulting cell suspension was washed, transferred to plates that had been coated with 1% agarose, and incubated in culture medium containing 10% FCS. The chondrocytes aggregated and within 24 h began to form clumps that increased in size and number through the first week, as we have observed in agarose suspension cultures of normal human juvenile costal chondrocytes (our unpublished data). After 10–14 d of agarose culture, the clumps began to disaggregate, become smaller, and form a single cell suspension. When this suspension was replated in tissue culture dishes (Fig. 2 *B*), a more homogeneous monolayer of polygonal cells was generated that proliferated readily in culture medium containing 10% FCS and reached confluence within 1 wk. Confluent cultures formed nodules reminiscent of the original transformed foci (Fig. 2 *C*). These agar-
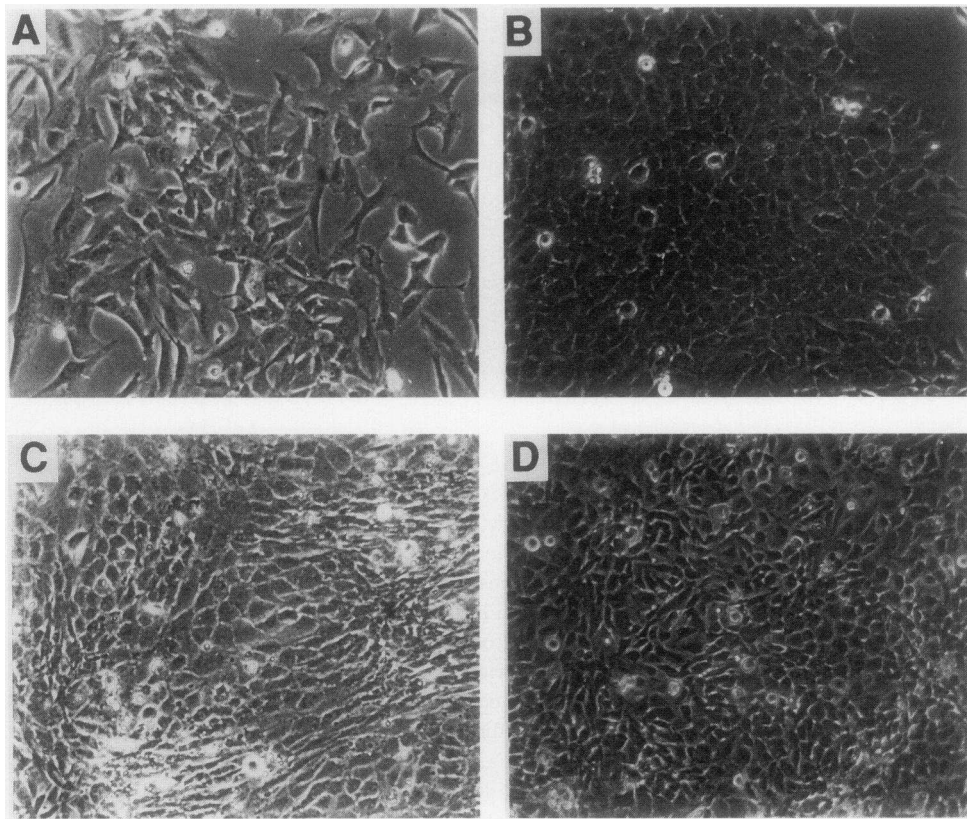
Figure 2. Phase-contrast micrographs of juvenile human costal chondrocytes after immortalization with pZipNeoSV40. (A) Neomycin-resistant T/C-28 cultures maintained stable morphology and proliferative capacity after 20 passages (1:5 split ratio). (B) 6 mo after transfection, T/C-28 chondrocytes were transferred to suspension culture in dishes coated with 1% agarose for 10 d and replated in tissue culture dishes. (C) A homogeneous monolayer of polygonal cells, designated T/C-28a, was generated that reached confluence within 1 wk. (D) After > 40 passages, the T/C-28a line was reselected on agarose to generate the T/C-28a2 line and further subcultured > 20 passages.

ose-selected chondrocytes, designated T/C-28a, were passaged weekly at subconfluence in medium containing 10% FCS, and they maintained stable morphology and proliferative capacity throughout > 40 passages in monolayer culture. The doubling time during 4 d of culture was 18–20 h in 10% FCS and 32 h in cells plated in 10% FCS but changed to 1% Nutridoma after 24 h. Further cloning by limiting dilution of the agarose-selected cells was accomplished easily after replating the cells on tissue culture plastic. After > 40 additional passages, the T/C-28a cells were reselected on agarose to generate the T/C-28a2 line (Fig. 2 D). These immortalized chondrocyte lines retained the capacity to produce immunoreactive T antigen throughout the various selection procedures (Fig. 3).

*Expression of cartilage-specific phenotype by immortalized human chondrocytes.* These stable proliferating lines of immortalized human chondrocytes (C-20/A4 and T/C-28) expressed differentiated cartilage-specific phenotype under defined conditions. In previous studies we showed that 10% FCS suppressed the capacity of the nonimmortalized human costal chondrocytes to express regulatory sequences of the type II collagen gene in transient transfection experiments and that removal of serum and addition of an insulin-containing serum substitute was permissive for expression of either endogenous type II collagen or the transfected type II collagen gene regulatory sequences even in subcultured chondrocytes (42). The levels of cartilage-specific matrix mRNAs were compared in our immortalized chondrocyte lines. The C-20/A4 and T/C-28a2 chondrocyte lines, as well as the C-28/I2 and C-28/D8 clonal lines, could be grown and subcultured continuously in culture medium containing 10% FCS, but they exhibited optimal expression of type II collagen mRNA only after one to several days of culture in serum-free defined medium containing 1% Nutridoma-SP (Fig. 4 A). The cartilage-specific mRNAs encoding aggrecan and

collagen types IX and XI were also detected under these conditions (Fig. 4 B). These cells also expressed the mRNAs encoding link protein and the small proteoglycans biglycan/PG-I and decorin/PG-II (Fig. 4 B). Expression of mRNA encoding versican, a proteoglycan not found in cartilage, and type X collagen, a marker of the hypertrophic chondrocyte, was not observed on blots exposed for 4 wk under any of the conditions of culture tested. Type I collagen mRNA was not detected in cells incubated in Nutridoma unless they were treated with IL-1$\beta$ (data not shown).

Type II collagen synthesis, analyzed by SDS-PAGE as the $\alpha 1$ band comigrating with the $\alpha 1$(I) collagen band of the rat tail tendon collagen standard, was observed in the both the C-20/A4 and T/C-28a immortalized chondrocyte lines after culture in 10% FCS and transfer to either 0.5% FCS (Fig. 5) or Nutridoma-SP (not shown) before labeling with [$^3$H]proline in the absence of serum. The absence of the $\alpha 2$(I) band in medium from the T/C-28a chondrocytes indicated that the single band comigrating with the standard $\alpha 1$(I) collagen band consisted primarily of $\alpha 1$ collagen chains of type II collagen. The C-20/A4 chondrocytes synthesized very low levels of type I collagen, analyzed as the $\alpha 2$(I) band and quantitated as less than 2% of the $\alpha 1$(I + II) collagen band by densitometric scanning of multiple gels. Furthermore, serum appeared to be permissive for type I collagen synthesis, whereas the serum substitute and radiolabeling in the absence of serum permitted type II collagen synthesis. Cell layers from the T/C-28a2 chondrocytes also incorporated [$^{35}$S]sulfate into high molecular weight bands that comigrated with toluidine blue–stainable material on agarose/polyacrylamide gels indicative of large aggregating proteoglycan (Green, G., S. Chipman, J. Birkhead, O. Troubetskoy, and M. Goldring, manuscript in preparation).

Immunocytochemical analyses revealed that the T/C-28a2
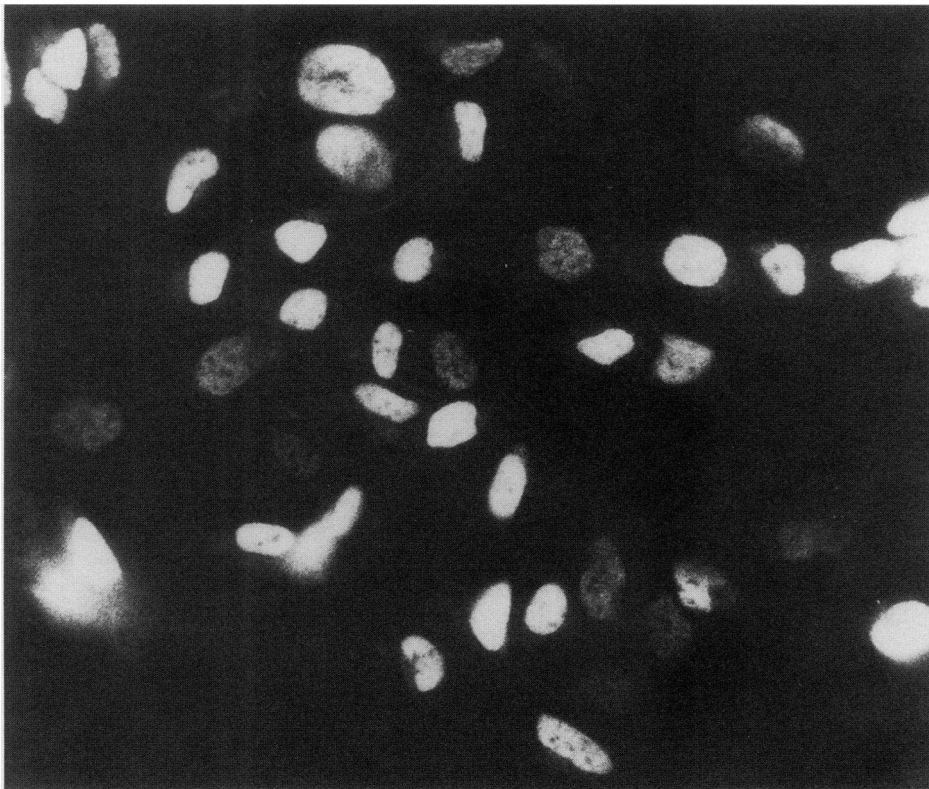
*Figure 3.* Immunoreactive SV40 large T antigen in immortalized human chondrocytes. The T/C-28a2 chondrocytes were plated on 4-chamber plastic slides in culture medium containing 10% FCS and grown to confluence for 4 d. After fixation, the cells were permeabilized in 0.1% Triton X-100 for 5 min and stained with the PAb 416 monoclonal antibody that recognizes specific antigenic determinants unique to the SV40 large T antigen.

chondrocytes synthesized and deposited extracellular matrix molecules immunoreactive with monoclonal antibodies against type II collagen, large proteoglycan, and chondroitin-4- and -6-sulfate. The data from three separate experiments are summarized in Table I. Pericellular and extracellular reactivity with these antibodies was observed as early as 1 d after transfer to the serum-free defined medium with maximal expression after 5 d, as shown for the type II collagen antibody in Fig. 6. Cells maintained continuously in 10% FCS demonstrated no immunoreactivity with these cartilage-specific monoclonal antibodies. In the absence of ascorbate, type II collagen immunoreactivity remained within the cells, and the staining for proteoglycan was punctate rather than diffuse in the extracellular matrix. Staining for the nonspecific chondroitin-0-sulfate was not observed under any condition. The C-20/A4 line, which maintained a proliferative state even in the presence of the serum substitute, did not deposit significant amounts of immunostainable matrix.

*Chondrocyte-specific responses to IL-1β.* Both the C-20/A4 and T/C-28a2 immortalized chondrocyte lines demonstrated responses to IL-1β similar to those described previously in normal human chondrocytes, including decreased expression of cartilage-specific matrix genes and increased expression of matrix metalloproteinase and immediate early genes (2, 42, 47). The time courses of expression of the α1(II) procollagen gene, the matrix metalloproteinase gene, collagenase, and the immediate early genes, *egr*-1 and *jun*-B, and the kinetics of their stimulation or suppression by IL-1β in the absence and presence of cycloheximide (CHX) were compared in the C-20/A4 (Fig. 7 *A*) or the T/C-28a2 (Fig. 7 *B*) chondrocytes in parallel incubations up to 24 h. Type II collagen mRNA was detected in both immortalized chondrocyte lines and increased with time throughout the 24 h of incubation in 1% Nutridoma. At 1 h, IL-1β had no effect on type II collagen mRNA levels

in the C-20/A4 chondrocytes (Fig. 7 *A*), but a slight suppression by IL-1β was evident in the T/C-28a2 chondrocytes at both 1 and 6 h (Fig. 7 *B*). A > 60% suppression was achieved by 24 h in both chondrocyte lines. CHX suppressed type II collagen mRNA levels, particularly at 24 h, but it did not have a further suppressive effect in the presence of IL-1β. The partial reversal by CHX of the suppression by IL-1β at 24 h was observed more consistently in the T/C-28a2 than in the C-20/A4 chondrocytes where type II collagen mRNA levels were comparatively lower. These results are consistent with previous findings in normal human chondrocytes that indicated that the inhibitory effect of IL-1β on type II collagen synthesis was not a result of decreased α1(II) procollagen mRNA stability but suggested that CHX may block the synthesis of an IL-1–induced repressor factor for α1(II) procollagen gene transcription (42).

Addition of IL-1β to quiescent chondrocytes increased the levels of *egr*-1, c-*jun*, c-*fos*, and *jun*-B mRNAs within 1 h which declined rapidly thereafter (Figs. 7 and 8). Incubation with CHX augmented the levels of these mRNAs and prolonged the IL-1β–induced expression past 6 h. Constitutive expression of collagenase mRNA was observed in both the C-20/A4 (Fig. 7 *A*) and T/C-28a2 chondrocytes (Fig. 7 *B*); this was relatively stable at least up to 6 h but declined within 24 h. Stimulation of collagenase mRNA by IL-1β was apparent by 6 h, and the magnitude of this stimulation relative to the constitutive expression increased after 24 h of incubation. The inhibitory effect of CHX on the IL-1β–stimulated level of collagenase mRNA was apparent after 24 h (Fig. 7). Lower constitutive expression of both collagenase and stromelysin mRNAs, as well as the early gene mRNAs, was observed when the medium was not changed after the initial 24 h in Nutridoma-containing medium, and CHX more effectively blocked the effects of IL-1β (Fig. 8). These
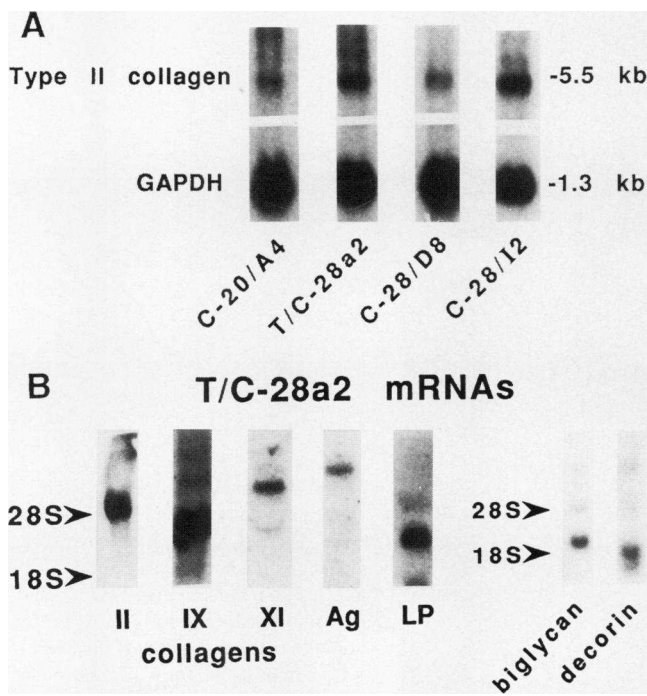
**Figure 4.** Expression of mRNAs encoding cartilage-specific collagens and other matrix proteins by immortalized chondrocytes cultured in serum-free defined medium. (*A*) The immortalized chondrocytes C-20/A4, T/C-28a2, C-28/D8, and C-28/I2 were passaged in medium containing 10% FCS, grown for 5 d, and changed to medium containing 1% Nutridoma-SP 24 h before harvest of the cells for RNA extraction. Total RNAs (10 μg/lane) were electrophoresed on a 0.8% agarose gel in the presence of 2% formaldehyde, blotted on nylon supported nitrocellulose membranes, and hybridized with the $^{32}$P-labeled cDNA probes encoding α1(II) procollagen and GAPDH. (*B*) Total RNAs from the T/C-28a2 chondrocytes incubated in 1% Nutridoma-SP for 24 h were blotted and hybridized with the $^{32}$P-labeled cDNA probes indicated at the bottom. The sizes of the mRNA transcripts in kilobases were: 5.5 kb, type II collagen; 4.0 kb, type IX collagen; 7.5 kb, type XI collagen; 9.5 kb, aggrecan (*Ag*); 2.5 kb, link protein (*LP*); 2.5 kb, biglycan; and 1.9 and 1.6, decorin. Overexposed blots are shown for type IX collagen and link protein mRNAs. The positions of the 28S and 18S ribosomal RNAs are shown on the left.
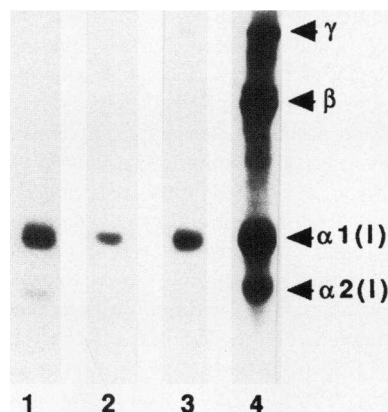


**Figure 5.** SDS-PAGE analysis of pepsin-resistant [$^3$H]proline-labeled collagen in monolayer cultures of chondrocytes. Lane *1*, C-20/A4 immortalized chondrocytes; lane *2*, T/C-28a immortalized chondrocytes; and lane *3*, C-28 primary nonimmortalized chondrocytes. The cells were grown to subconfluence in medium containing 10% FCS and transferred to serum-free culture medium containing ascorbic acid for radiolabeling. Positions of the α1(I), α2(I), β, and γ chains of the rat tail tendon collagen standard (lane *4*) are identified by arrowheads.

*Table I. Location and Time of Appearance of Immunoreactivities in Immortalized Human Chondrocyte Cultures*

| Antibody | Without ascorbic acid | | With ascorbic acid | |
| --- | --- | --- | --- | --- |
| | Pericellular | Extracellular | Pericellular | Extracellular |
| | | *d* | | *d* |
| Anti–large proteoglycan | 1 | — | 1 | 3 |
| Anti–collagen II | 3 | — | 2 | 8 |
| Anti–chondroitin sulfate | 7 | — | 2 | 8 |

The T/C-28a2 chondrocytes were grown to subconfluence for 4 d in culture medium containing 10% FCS and transferred to medium containing 50 μg/ml ascorbic acid and 1% Nutridoma-SP. The results of three separate experiments are summarized as the days of appearance of immunoreactivities after transfer from serum- to Nutridoma-containing medium. Note that in cultures incubated without ascorbic acid, no significant immunoreactivity was observed in the extracellular compartment.

results indicate that the medium change at 0 h in the experiment shown in Fig. 7 may have increased constitutive levels of the metalloproteinase mRNAs and prevented early inhibition by CHX. No change in GAPDH mRNA with time or in response to IL-1β was observed in these experiments.

*Expression of regulatory sequences of the human type II collagen gene in immortalized chondrocytes.* To determine whether the immortalized chondrocytes could express regulatory sequences of the type II collagen gene (COL2A1), we used a 4.0-kb fragment containing the promoter domain and a portion of intron 1 (spanning −577 to +3,428 bp) fused to the CAT reporter gene in transient expression experiments. Expression of this construct, designated pCAT-B/4.0, was observed in both T/C-28a2 and C-20/A4 (Fig. 9) in the presence of 1% Nutridoma-SP. The expression of pCAT-B/4.0 was generally higher in the T/C-28a2 than in the C-20/A4 chondrocytes. IL-1β inhibited the expression by 30–80% in more than six separate experiments in each chondrocyte line. These results are consistent with previous findings of expression of pCAT-B/4.0 in nonimmortalized human costal chondrocytes cultured under the same conditions but no expression in human fibroblasts (42). Nuclear extracts from these immortalized human chondrocytes contain factors that bind specifically to sequences within the COL2A1 promoter and are modulated by IL-1β (48).

## Discussion

Our results demonstrate the generation of stable lines of immortalized human chondrocytes that express the differentiated phenotype under defined conditions. Different vectors, both containing DNA encoding SV40 large T antigen, and different transformation and selection protocols were used in deriving the two chondrocyte lines that we have described in detail. Both lines proliferated readily in monolayer culture in the presence of 10% FCS and displayed a morphology similar to that in normal primary human chondrocyte cultures as described previously (2). A serum substitute provided conditions that permitted expression of either the endogenous or introduced type II collagen gene, as we had found previously in the nonimmortalized juvenile costal chondrocyte cultures (42).
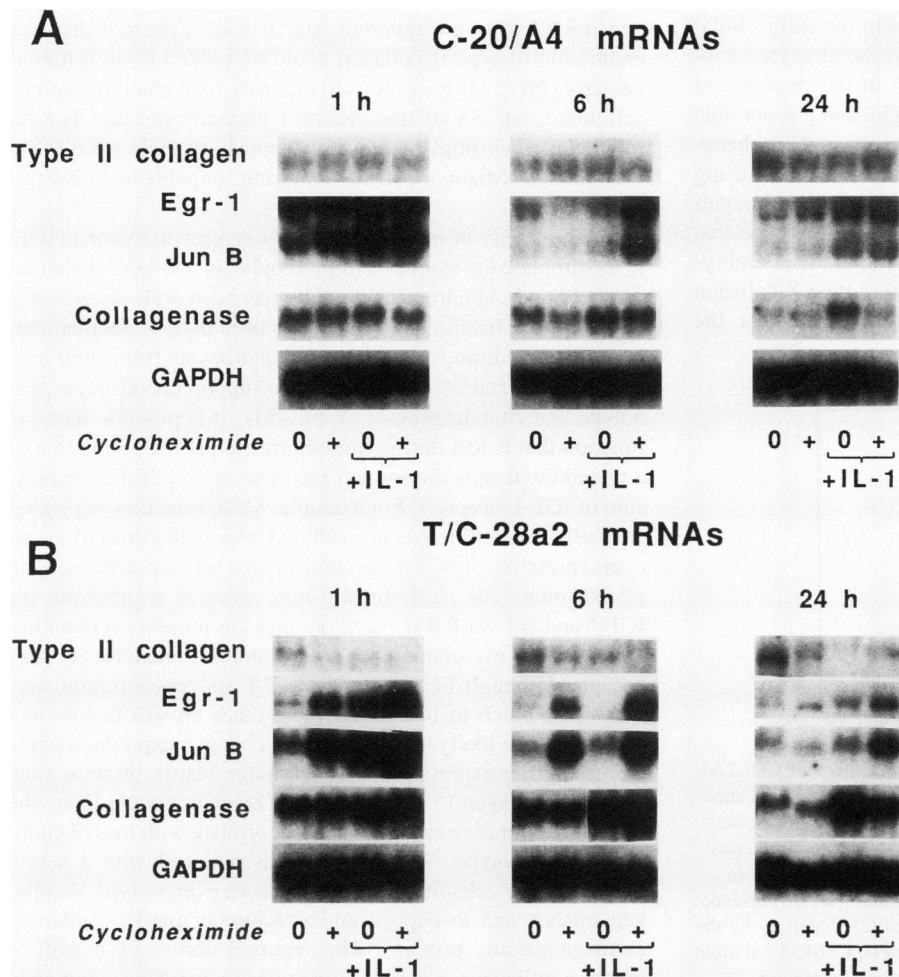
Immortalized chondrocyte lines were generated by others
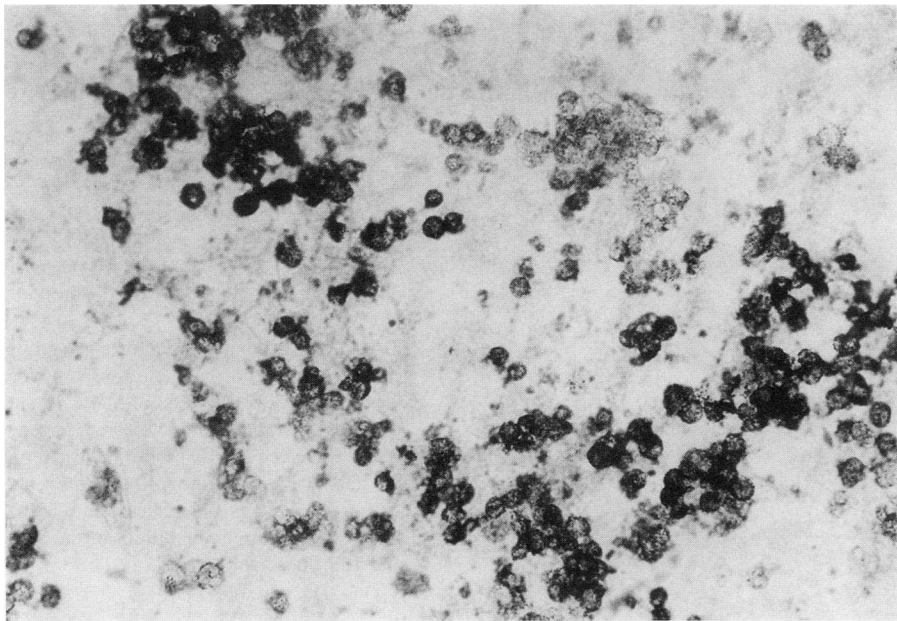
*Figure 6.* Immunocytochemical analysis of cartilage-specific matrix proteins in immortalized human chondrocytes. The T/C-28a2 chondrocytes were plated on 4-chamber plastic slides in culture medium containing 10% FCS and grown to subconfluence for 4 d. The serum-containing medium was then removed and changed to medium containing 50 $\mu$g/ml ascorbic acid and 1% Nutridoma-SP, and incubations continued for 5 d. Immunoreactivity with the monoclonal antibody against human type II collagen was visualized using a silver-enhanced immunogold detection system as described in Methods. Cells incubated without primary antibodies served as negative controls.

using either the *myc* or the SV40 large T oncogene transfected in embryonic or young animal chondrocytes (7–10, 49). Whereas those were highly proliferating cloned lines, all aspects of the differentiated phenotype and responses to modulatory signals

were not stabilized. Dissociation of proliferative and phenotypic stabilities has been found in other cell systems immortalized with SV40. Although cartilage-specific proteoglycan synthesis has been demonstrated in several immortalized chondrocyte



*Figure 7.* Kinetics of expression of type II collagen, collagenase, and early gene mRNAs by immortalized chondrocytes and effects of IL-1$\beta$. The C-20/A4 (*A*) or T/C-28a2 (*B*) chondrocytes were passaged in medium containing 10% FCS, grown for 5 d, and changed to medium containing 1% Nutridoma-SP 24 h before medium change and incubation for 1, 6, and 24 h in the absence or presence of IL-1$\beta$ (5 pM) and CHX (10 $\mu$g/ml) as indicated below each panel. Northern blots were prepared as described in Methods, and replicate or stripped blots were hybridized with $^{32}$P-labeled cDNA probes indicated on the left.
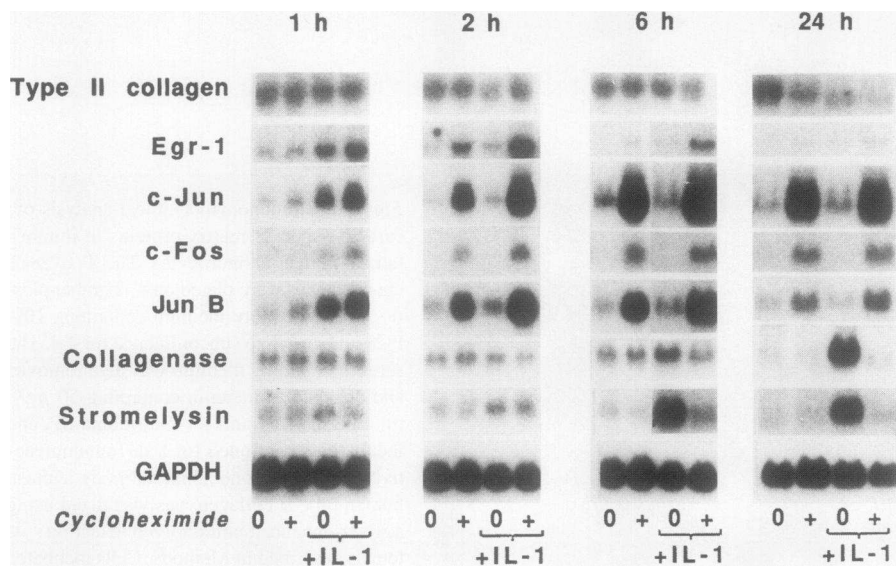
*Figure 8.* Effects of IL-1β and CHX on expression of type II collagen, early gene, and matrix metalloproteinase mRNAs by immortalized human chondrocytes. The T/C-28a2 chondrocytes were passaged in medium containing 10% FCS, grown for 5 d, and changed to medium containing 1% Nutridoma-SP 24 h before addition of IL-1β (5 pM) and CHX (10 μg/ml) without medium change and incubation for 1, 2, 6, and 24 h. Northern blots were prepared as described in Methods, and replicate or stripped blots were hybridized with $^{32}$P-labeled cDNA probes indicated on the left. The sizes of the mRNA transcripts shown in kilobases were calculated relative to the electrophoretic mobilities of the 28S and 18S ribosomal RNAs.

lines, expression of type II collagen seems an unstable phenotype. Rabbit articular chondrocytes immortalized with SV40 did not synthesize type II collagen even after culture in agarose (10), a procedure that readily induces reexpression in dedifferentiated normal chondrocytes (4). A similar origin-defective SV40 vector containing both large T and small t antigens was used to generate our C-20/A4 line that expressed type II collagen mRNA and radiolabeled protein but did not deposit immunoreactive type II collagen or proteoglycans in the extracellular matrix. Although SV40 immortalized mouse chondrocytes demonstrated type II collagen expression even in the presence of 10% FCS (49), they were of embryonic origin, and it is possible that the cloned line selected was of a more pluripotent phenotype. Our T/C-28a2 chondrocytes that were generated using the same SV40 vector as that used for the mouse chondrocytes exhibited all aspects of differentiated cartilage phenotype that we have tested thus far. Although v-*myc* immortalized embryonic quail chondrocytes were shown to express type X collagen (8), neither our immortalized human chondrocytes nor the

mouse chondrocytes (49) expressed this marker of the hypertrophic phenotype. However, fully differentiated chondrocytes that express type II, IX, and XI collagens and aggrecan would not be expected to express type X collagen.

Human chondrosarcoma cell lines have been established that synthesize cartilage-specific proteoglycans (11, 12, 50) and low levels of type II collagen detected by immunocytochemistry and SDS-PAGE (11, 50). Sustained expression of type II collagen mRNA was not reported, and it was suggested that low expression of type II collagen could be related to high tumorgenicity (50–52). In contrast to the malignant chondrosarcoma cell lines, our SV40 immortalized chondrocytes are not tumorgenic when implanted subcutaneously in nude mice (Glowacki, J., S. Mizuno, and M. Goldring, unpublished observations).

The capacity of insulin or insulin-like growth factor-I (IGF-I) to stimulate the synthesis of proteoglycan and DNA by chondrocytes and chondrosarcoma cells has been well documented (50, 53–55). Insulin and IGF-I have been used as supplements in defined medium for culture of chondrocytes from limb bud, growth plate, and articular cartilage to support chondrocyte phenotype and chondrogenesis (5, 56–60). It is possible that one function that is lost during immortalization is the production of a chondrocyte-specific growth factor such as IGF-I or expression of IGF-I receptors. For example, SV40 infection of pancreatic cells resulted in loss of insulin synthesis in vitro (61), and overexpression of IGF-I resulted in loss of tumorigenicity in gliobastoma cells (62). Insulin may serve as a substitute for IGF-I and act via IGF-I receptors that are present on chondrocytes and are associated with preservation of chondrocyte phenotype. Although FCS contains IGF-I, its concentration may vary from batch to batch, and most other growth factors that are present are likely to inhibit type II collagen expression while increasing the expression of noncartilage matrix proteins such as type I collagen (17). In our T-C/28 chondrocyte lines, the loss of proliferative capacity did not correlate with loss of chondrocyte phenotype. When serum was replaced with a serum substitute, the cells immediately began to express type II collagen mRNA and to deposit an extracellular matrix containing cartilage-specific proteins. The reported ability of insulin to increase collagenase and early response gene expression (63)
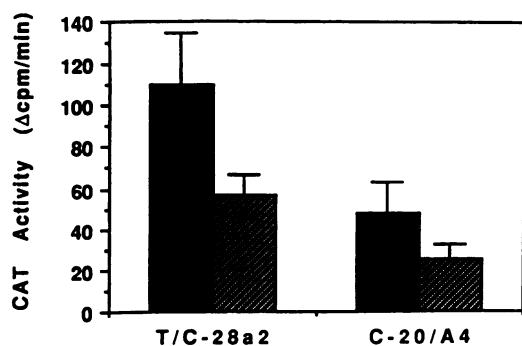


*Figure 9.* Expression of 5'-flanking regulatory sequences of COL2A1 by immortalized human chondrocytes. The T/C-28a2 or C-20/A4 chondrocytes were seeded at 0.5 × 10$^6$ cells/10-cm plate and transfected the following day with 10 μg/plate of pCAT-B/4.0 in medium containing 10% FCS. The cells were further incubated for 36–48 h in medium containing 1% Nutridoma-SP in the absence (■) or presence (■) of IL-1β (10 pM) and harvested for assay of CAT activity by the fluor diffusion method. Each bar represents the mean (±SEM) of eight (T/C-28a2) or six (C-20/A4) separate transfection experiments.

could also account for the constitutive levels of those mRNAs observed in the early stages of our time course experiments.

During development, the expression of c-*fos*, c-*jun*, *jun*-B, and *egr*-1 is found in hypertrophic chondrocytes in regions that are destined to be replaced by bone, in prechondrocytes that express an alternative type II collagen transcript (64), and in interstitial zones surrounding cartilage (65–67). The potential for involvement of IL-1 in regulation of chondrocyte hypertrophy and cartilage mineralization has been suggested by a study in vitro using rabbit growth plate chondrocytes in which type X collagen synthesis was inhibited by this cytokine (68). Availability of immortalized human chondrocytes that can be manipulated in culture will provide a basis for developing a human model of chondrogenesis in vitro. Our studies indicate that these cells will also serve as excellent host cells for transfection of chondrocyte-specific genes. Chondrocyte-specific responses to IL-1$\beta$, as reported here, and to IFN-$\gamma$ (48) were similar to those found previously in normal human chondrocyte cultures (12, 42, 47, 48). The mechanisms that control transcription of the late genes encoding collagens and metalloproteinases have not been defined precisely, but may involve some of the early genes studied here. Our studies demonstrate the utility of these immortalized human chondrocytes for examining the molecular events involved in responses of chondrocytes to inflammatory mediators.

## Acknowledgments

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