The TATA-containing core promoter of the type II collagen gene (*COL2A1*) is the target of interferon-γ-mediated inhibition in human chondrocytes: requirement for Stat1α, Jak1 and Jak2

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Interferon-γ (IFN-γ) inhibits the synthesis of the cartilage-specific extracellular matrix protein type II collagen, and suppresses the expression of the type II collagen gene (*COL2A1*) at the transcriptional level. To further examine this mechanism, the responses of *COL2A1* regulatory sequences to IFN-γ and the role of components of the Janus kinase/signal transducer and activators of transcription (JAK/STAT) pathway were examined in the immortalized human chondrocyte cell line, C-28/I2. IFN-γ inhibited the mRNA levels of *COL2A1* and aggrecan, but not Sox9, L-Sox5 and Sox6, all of which were expressed by these cells as markers of the differentiated phenotype. IFN-γ suppressed the expression of luciferase reporter constructs containing sequences of the *COL2A1* promoter spanning -6368 to $+125$ bp in the absence and presence of the intronic enhancer and stimulated activity of the γ-interferon-activated site (GAS) luciferase reporter vector, associated with induction of Stat1α-binding activity in nuclear extracts. These responses to IFN-γ were blocked by overexpression of the JAK inhibitor, JAK-binding protein (JAB), or reversed by dominant-negative Stat1α Y701F containing a mutation at Tyr-701, the JAK phosphorylation site. IFN-γ had no effect on *COL2A1* promoter expression in Jak1 (U4A)-, Jak2 (γ2A)- and Stat1α (U3A)-deficient cell lines. In the U3A cell line, the response to IFN-γ was rescued by overexpression of Stat1α, but not by either Stat1α Y701F or Stat1β. Functional analysis using deletion constructs showed that the IFN-γ response was retained in the *COL2A1* core promoter region spanning -45 to $+11$ bp, containing the TATA-box and GC-rich sequences but no Stat1-binding elements. Inhibition of *COL2A1* promoter activity by IFN-γ persisted in the presence of multiple deletions within the $-45/+11$ bp region. Our results indicate that repression of *COL2A1* gene transcription by IFN-γ requires Jak1, Jak2 and Stat1α and suggest that this response involves indirect interaction of activated Stat1α with the general transcriptional machinery that drives constitutive *COL2A1* expression.

Key words: DNA-binding protein, dominant-negative Stat1α, Janus kinase (JAK), Janus kinase inhibitor, transcription.

INTRODUCTION

Type II collagen is the major collagen synthesized by chondrocytes in mature articular cartilage. Together with other cartilage-specific collagens IX and XI, it forms a fibrillar collagen network that is responsible for the tensile strength of articular surfaces [1]. Each α1(II) procollagen chain of the triple helix is encoded by the *COL2A1* gene, whose transcription is regulated by DNA elements within both the promoter and first intron regions [2]. These regions may interact with positive or negative transcription factors that determine developmental-stage-specific and tissue-specific expression during chondrogenesis. Studies in transgenic mice have shown that a 309 bp intronic enhancer, which binds Sox9, L-Sox5 and Sox6 [3], is required for cartilage-specific expression of type II collagen during chondrogenesis *in vivo* [4]. The proximal promoter region is highly conserved among the human, mouse and rat genes [5], contains a TATA box and sites for binding Sp1, basic helix-loop-helix/E-box-binding proteins and cKrox, and expresses *in vitro* in the absence of the intronic enhancer [3,4,6–9].

Once the matrix has formed in mature cartilage, chondrocytes maintain steady-state expression of collagens and proteoglycans and may respond to a number of growth factors and cytokines that either stimulate or inhibit type II collagen synthesis. Certain cytokines that are involved in immune cell function play an important role in inflammatory arthritis and other forms of joint disease where degradation of cartilage matrix components is a prominent feature [10]. Among these, interleukin-1 and tumour necrosis factor-α stimulate metalloproteinases and other enzymes that degrade cartilage matrix, while interferon (IFN)-γ inhibits the synthesis of proteinases induced by interleukin-1 and tumour necrosis factor-α. However, all three cytokines have been shown to inhibit, both independently and co-operatively, the synthesis of type II collagen in chondrocytes [11–14] and may thereby play a role in further depletion of cartilage matrix components by preventing repair.

IFN-γ is among the class of cytokines that exert their biological effects through binding to cognate cell-surface receptors that are associated with subsets of the Janus kinases (JAKs). IFN-γ binding to its receptor leads to the activation of Jak1 and Jak2, rapid tyrosine phosphorylation of the cytoplasmic domains, and subsequent recruitment of various signalling proteins to the receptor complex, including Stat1, a member of the STAT (signal transducer and activators of transcription) family of transcription

Abbreviations used: BRE, TFIIB-recognition element; CBP, cAMP-response-element-binding protein-binding protein; CIITA, class II transactivator; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GAS, γ-interferon-activated site; IFN, interferon; JAB, JAK-binding protein; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; RLU, relative light units; STAT, signal transducer and activators of transcription; TAD, transactivation domain.

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factors [15,16]. The tyrosine-phosphorylated Stat1 homodimer translocates into the nucleus, where it binds to specific target sequences and regulates the expression of genes containing a DNA element called the γ-IFN-activated site (GAS) [15,16]. Cell lines deficient for Jak1, Jak2 or Stat1α are unable to respond to IFN-γ [17]. Between 200 and 500 genes may be activated by IFN-γ, largely in association with immune responses or anti-viral activity [18]. On the other hand, IFN-γ is also capable of repressing gene transcription. Genes encoding extracellular matrix proteins are prominent among the targets of negative regulation by IFN-γ [11,14,19–23].

We showed previously that IFN-γ decreases the synthesis of type I, type II and type III collagens, associated with suppression of α1(I), α2(I), α1(II) and α1(III) procollagen mRNAs, while stimulating the expression of histocompatibility lymphocyte (DR locus) antigens in human chondrocytes [11]. Although IFN-γ suppresses expression of the α1(II) procollagen gene (*COL2A1*) at the transcriptional level [14,24], the signalling pathways and transcription factors involved have not yet been identified. In the present study, we show that IFN-γ suppresses activity of the proximal *COL2A1* promoter and that Stat1α, Jak1 and Jak2 are required for this response. Results from functional analysis of *COL2A1* regulatory sequences demonstrate that inhibition of this constitutively expressed, chondrocyte-specific gene occurs through the JAK/STAT pathway and targets the TATA-containing core promoter in the absence of binding of IFN-γ-activated Stat1α to DNA elements.

EXPERIMENTAL

Cell cultures

The immortalized human chondrocyte cell line C-28/I2 [25] was cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1/1, v/v) containing 10% fetal calf serum (FCS) and passaged at $>95\%$ confluency every 5–6 days. The fibrosarcoma cell lines U3A, U6A, U4A, 2fTGH, γ2A and 2CA, kind gifts from Dr George R. Stark (The Cleveland Clinic Foundation, Cleveland, OH, U.S.A.), were cultured in DMEM containing 10% FCS and passaged at $>95\%$ confluency every 3–4 days. U3A, U6A and U4A were derived from 2fTGH and lacked Stat1, Stat2 and Jak1, respectively. The γ2A cell line was Jak2-deficient and derived from the fibrosarcoma cell line, 2CA [17].

RNA extraction and analysis

For experiments, confluent cultures of the C-28/I2 chondrocytes were changed to medium containing 1% Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN, U.S.A.), incubated overnight and treated with IFN-γ at 0.01–100 units/ml for a further 24 h. In some cases, cells were preincubated without or with cycloheximide (Sigma, St. Louis, MO, U.S.A.) for 30 min before addition of IFN-γ. Total RNA was isolated by a one-step extraction procedure using Trizol reagent (Life Technologies, Rockville, MD, U.S.A.) and 0.9 μg was reverse transcribed in 20 μl containing final concentrations of 2.4 i.u./μl Moloney murine leukaemia virus reverse transcriptase, 2.5 μM of oligo $d(T)_{16}$ and 1 unit/μl RNase inhibitor (all obtained from Perkin-Elmer), and amplified using primers specific for *COL2A1*, aggrecan and glyceraldehyde-3-phosphate dehydrogenase, as described previously [26]. Other primers were: L-Sox5, 5′-CAACTCCGACGGGAACAACA-3′ and 5′-AGGATCTT-TCTCCGTTCATC-3′ (300 bp; GenBank accession no. NM006940); Sox6, 5′-GGCAATTTACCAGTGATTTC-3′ and 5′-TTGAGGTTAAATCCTGGGTC-3′ (137 bp; GenBank accession no. NM033326), and Sox9, 5′-CCAACGCCATC-TTCAAGGC-3′ and 5′-CGTCGCGGAAGTCGATAGG-3′ (218 bp; GenBank accession no. XM039094). PCR amplification was performed in a 50 μl reaction volume containing 1 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer and 2.5 units of *Taq* DNA polymerase (Promega) using the Perkin-Elmer Gene Amp PCR System 9600. Following an initial denaturation at 95 °C for 2 min, amplification was performed at 95 °C for 30 s and 70 °C for 1 min for 35 cycles, with a final extension at 72 °C for 7 min. The annealing temperature was 55 °C for L-Sox5, Sox6 and Sox9. The PCR products, 30 μl of PCR/well, were separated on 1.5% agarose gels.

Electrophoretic mobility shift assay (EMSA) and supershift analysis

For preparation of nuclear extracts, C-28/I2 cells were passaged and grown to confluence, changed to medium containing 1% Nutridoma-SP overnight, and treated with IFN-γ (100 units/ml) for 0.25, 0.5, 1 and 3 h. The cells were lysed in hypotonic buffer with Nonidet P-40 at a final concentration of 0.5%, as described in [27]. Nuclear proteins were extracted in buffer C [20 mM Hepes, pH 7.4, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol, containing proteinase inhibitors (0.5 mM dithiothreitol, 1 mM PMSF, 20 μM leupeptin and 10 μM aprotinin)] according to the modified method of Dignam et al. [28], diluted with low-salt buffer D (20 mM Hepes, pH 7.4, 50 mM KCl, 0.2 mM EDTA and 20% glycerol, containing the same inhibitors as buffer C), and used directly for analysis of binding to DNA. The wild-type Stat1α consensus sequence (CAT GTT ATG CAT ATT CCT GTA AGT G) and mutant sequence (CAT GTT ATG CAT ATT gga GTA AGT G; where the lowercase sequence indicates the mutation; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used both as labelled probes and as unlabelled competitors. Double-stranded DNA oligonucleotide probes were end-labelled using T4 polynucleotide kinase and [γ-^{32}P]dATP. Binding reactions were carried out for 30 min at room temperature, using 5 μg of nuclear extract and 0.8 pmol of labelled probe in a final volume of 20 μl containing 10 mM Tris (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 80 mM KCl, 0.8 mM dithiothreitol, 2.5% glycerol and 1.8 μg of poly(dI-dC). The protein–DNA complexes were separated in low-ionic-strength 4% polyacrylamide gels using Tris/borate/EDTA buffer. For supershift analysis, a specific antibody against Stat1α (sc-346X; Santa Cruz Biotechnology) was incubated with the binding reaction mixture for 15 min at 4 °C before adding probe to nuclear extract.

Plasmid construction and site-directed mutagenesis

For construction of *COL2A1* reporter vectors, the regions spanning $-6368/+125$ bp (6.5) and $+2388/+2696$ bp (E309) were obtained by digestion from pKL7 and pAA2 [4,29]. The *COL2A1* fragments were then cloned into the pGL2-Basic luciferase vector (pGL2-B; Promega). Deletion constructs containing $-3373/+125$ bp, $-1880/+125$ bp, $-577/+125$ bp, $-525/+125$ bp, $-131/+125$ bp and $-90/+125$ bp were prepared by enzyme digestion of pGL2B6.5E309 using multiple cloning sites in the vector and *Bst*XI, *Nde*I, *Pst*I, *Nhe*I, *Apa*I and *Pvu*II, respectively, followed by blunting, if necessary, and ligation. The PCR products, including $-45/+125$ bp, $-90/+11$ bp and $-45/+11$ bp, were made using primers (Operon Technologies, Alameda, CA, U.S.A.) at positions indicated in Figure 8(A) (see below). The deletions and mutation of pGL2B$-577/+125$ (Figure 8B, see below) were generated using the QuikChange™ Site-Directed Mutagenesis Kit

(Stratagene, La Jolla, CA, U.S.A.). DNA sequences were assessed at the Beth Israel Deaconess Medical Center DNA sequencing facility using the ABI PRISM® BigDye™ primer cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and a model 373A Automatic DNA Sequencer (Applied Biosystems). The sequencing results were compared with the GenBank Nucleotide Sequence Database, including accession numbers M60299 [30], X16468, X58709 [5] and AC004801.

The wild-type and mutant Stat1 expression vectors, including pRc/CMV-Stat1α, pRc/CMV-Stat1α S727A, pRc/CMV-Stat1α Y701F and pRc/CMV-Stat1β [31,32], were kindly provided by Dr James E. Darnell (The Rockefeller University, New York, NY, U.S.A.). The pCAGGS-Neo-HA-Stat1α and pCAGGS-Neo-HA-Stat1α Y701F were gifts from Dr Toshio Hirano (Osaka University Graduate School of Medicine, Osaka, Japan) [33]. The wild-type and mutant JAK-binding protein (JAB) expression vectors, including pcDNA3-JAB and pcDNA3-dN75JAB, were donated by Dr Akihiko Yoshimura (Kurume University, Fukuoka, Japan) [34]. pRc/CMV, pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) and pCAGGS [35] (kindly provided by Dr Jun-ichi Miyazaki, Osaka University Medical School, Osaka, Japan) were used as empty vector controls. The reporter construct pGAS-Luc, driven by four repeats of the GAS from the glutamine-binding protein 1 promoter, AGT TTC ATA TTA CTC TAA ATC, was purchased from Stratagene.

Transient transfections and luciferase assays

Transient transfection experiments were carried out in C-28/I2 cells using the lipid-based transfection reagent Fugene 6 (Roche Molecular Biochemical, Indianapolis, IN, U.S.A.). Cells were seeded in six-well tissue-culture plates at 3.5×10^5 cells/well in DMEM/Ham's F12 containing 10% FCS 24 h prior to transfection. For each well, 1 μg of *COL2A1*-luciferase construct, 3 μl of Fugene 6 reagent and 100 μl of Opti-MEM (Life Technologies) were mixed and incubated for 15 min at room temperature. The transfection mixture was transferred to the cell monolayer in each well. After incubation for 4 h at 37 °C, the cells were treated for 20 h in the absence or presence of IFN-γ. For co-transfections, the expression vectors and the empty vectors were first titrated at amounts ranging from 10 to 200 ng/well; 100 ng was found to be optimal, whereas 200 ng often produced non-specific effects due to the cytomegalovirus (CMV) promoter. After transfection using 50 or 100 ng of expression vector/well, the cells were incubated for 24 h to permit expression of recombinant proteins prior to treatment with IFN-γ for a further 20 h. Cell lysates were prepared by extraction with 400 μl of Passive Lysis Buffer (Promega) and the protein content was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.).

Luciferase activities were determined by chemiluminescence assay using the Luciferase Assay Reagent (Promega) and the Autolumat LB953 luminometer (EG&G Berthold, Oak Ridge, TN, U.S.A.). Luciferase activities were normalized to the amount of protein and expressed as relative activities against that of untreated pGL2B$-$577/$+$125 or pGAS-Luc in each experiment. Each value was calculated as the mean $\pm$ S.D. of results from 3–6 wells, and each experiment was repeated at least three times. The levels of luciferase activity expressed as relative light units (RLU)/μg of protein ranged from approx. 1×10^3 to 1×10^5 for *COL2A1* promoter activity in untreated C-28/I2 cells and from 12.9×10^3 to 3.4×10^3 for pGAS activity in IFN-γ-treated cells. In wild-type fibrosarcoma cell lines, the values ranged from 1.8×10^4 to 2×10^5 for activity of *COL2A1* promoter and from 1.5×10^4 to 4.7×10^5 for pGAS activity in IFN-γ-treated cells.

RESULTS

Inhibition of *COL2A1* mRNA levels and promoter activity by IFN-γ in human chondrocytes

The immortalized human chondrocyte cell line, C-28/I2, selected for use in this study has been characterized previously and used

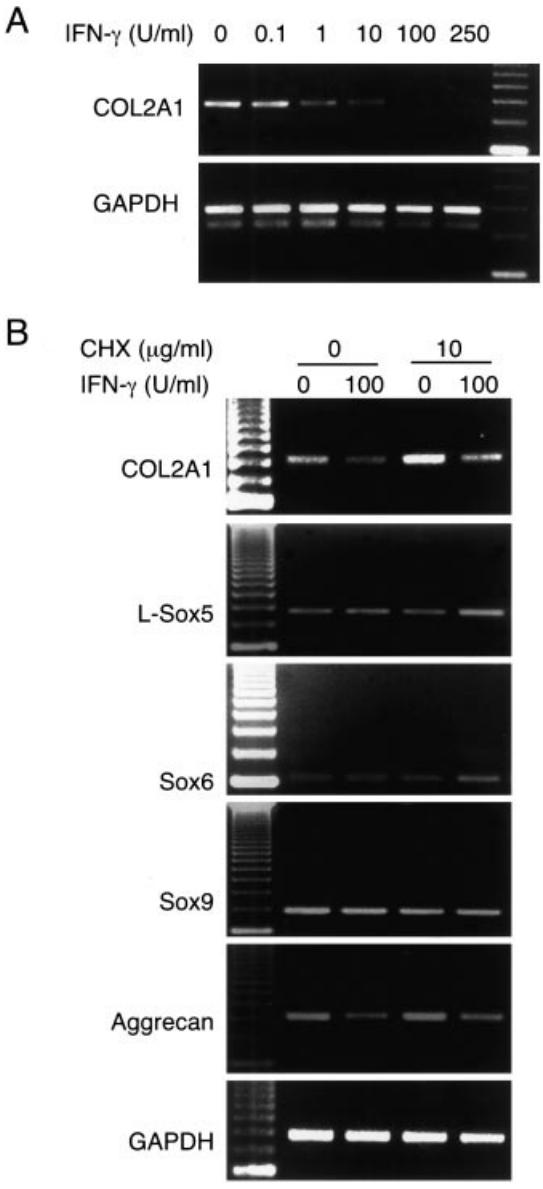

Figure 1 Regulation of *COL2A1* mRNA levels by IFN-γ in human chondrocytes

The C-28/I2 immortalized chondrocytes were plated at 0.5×10^6 cells/10 cm dish in DMEM/Ham's F12 with 10% FCS and cultured for 5 days until confluent. The medium was then changed to serum-free medium containing 1% Nutridoma-SP overnight, and IFN-γ was added at concentrations ranging from 0.1 to 250 units/ml (**A**) or at 100 units/ml in the absence or presence of 10 μg/ml cycloheximide (CHX) added 15 min before IFN-γ (**B**). After 24 h of incubation with IFN-γ, total RNA was extracted and *COL2A1*, L-Sox5, Sox6, Sox9, aggrecan and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were analysed by semi-quantitative reverse transcriptase PCR.

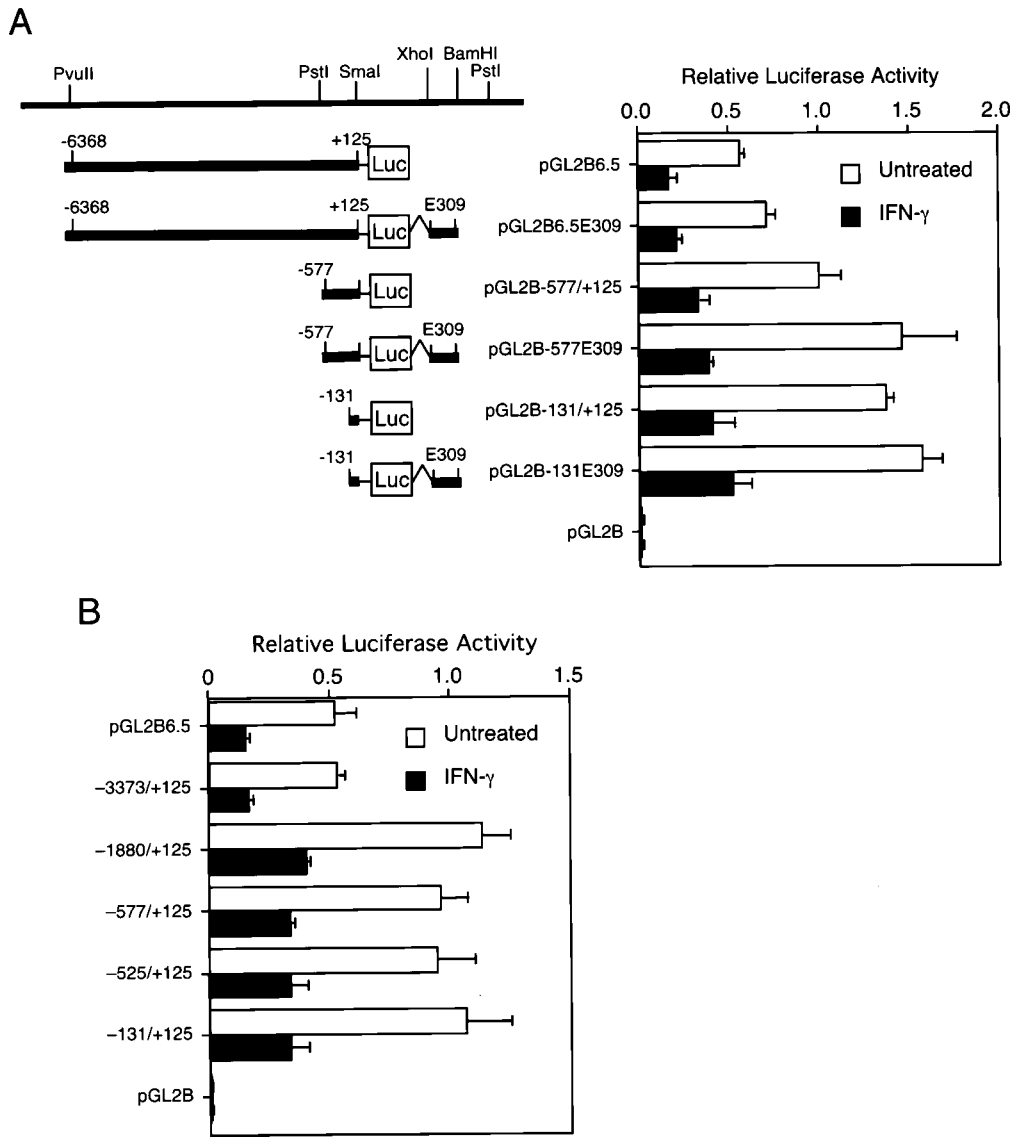

Figure 2 Regulation of *COL2A1* promoter activity by IFN-γ in human chondrocytes

Luciferase reporter constructs containing *COL2A1* sequences (**A**) were transfected into C-28/I2 cells by lipofection using Fugene 6, and IFN-γ was added for 20 h prior to harvest for luciferase assay. The promoter constructs pGL2B6.5, pGL2B $-577/+125$ and pGL2B $-131/+125$, without and with the E309 enhancer region ($+2388/+2696$ bp) from intron 1, and (**B**) deletion constructs of the $-6368/+125$ bp promoter were compared. Luciferase activity was normalized to the amount of protein and expressed as relative activity to that of untreated cells transfected with pGL2B $-577/+125$. Each value is calculated as the mean $\pm$ S.D. from 3–6 wells and results are representative of at least three experiments. Note that the empty vector, pGL2-Basic, was expressed at 1–2% of the levels of the *COL2A1* promoter constructs and did not respond to IFN-γ.

as a model to evaluate various chondrocyte functions [25,36]. We first verified that IFN-γ suppresses *COL2A1* mRNA levels in the C-28/I2 cells, as we had reported previously in primary chondrocyte cultures [11]. IFN-γ treatment of subconfluent cultures for 24 h suppressed the levels of *COL2A1* mRNA in a dose-dependent manner at concentrations between 1 and 250 units/ml (Figure 1A). Preincubation with cycloheximide (10 μg/ml) stabilized the levels of *COL2A1* mRNA but did not affect the extent of inhibition by IFN-γ (Figure 1B). L-Sox5, Sox6 and Sox9 mRNAs were also expressed in the C-28/I2 cells, indicating that these markers of differentiated chondrocytes are present at sufficient levels to permit endogenous *COL2A1* expression, as shown in Figure 1(B). The levels of Sox5, Sox6 and Sox9 mRNAs were unaffected by IFN-γ. Cycloheximide had no effect on Sox9 mRNA, but increased Sox5 and Sox6 mRNA levels in the presence but not in the absence of IFN-γ. Aggrecan mRNA levels were also decreased by IFN-γ in this experiment and cycloheximide did not block this inhibition. These results indicate that new protein synthesis is not required for inhibition of *COL2A1* mRNA levels by IFN-γ and that changes in the levels of Sox9 and related proteins cannot account for the IFN-γ-induced inhibition of *COL2A1* expression. Thus the C-28/I2 chondrocyte cell line was found to serve as a useful model for further examination of *COL2A1* regulatory elements responsive to IFN-γ.

To determine the *COL2A1* sequences that were required for expression and responsiveness to IFN-γ, transient transfections were performed using the C-28/I2 cells, as shown in Figure 2. We

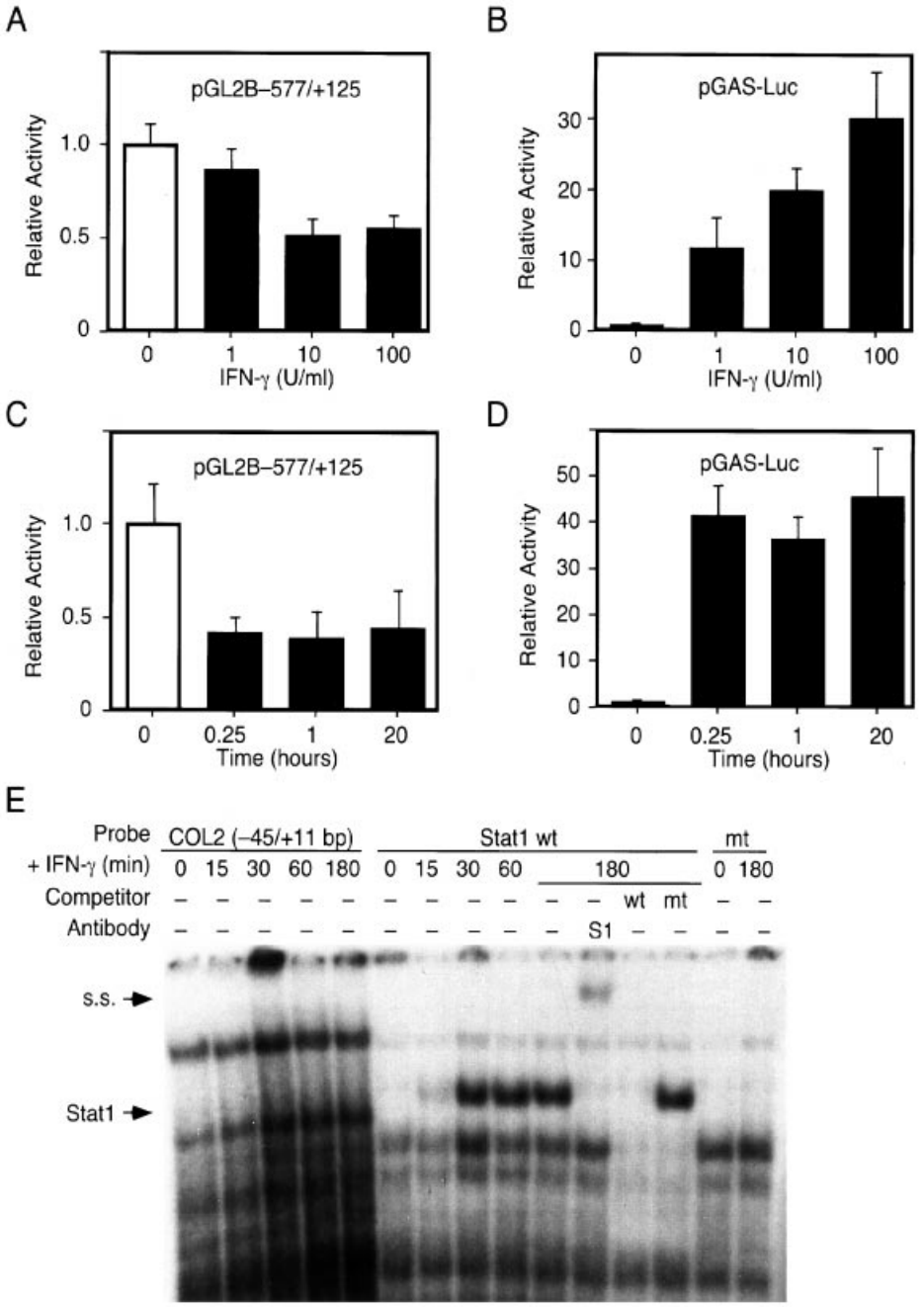

Figure 3 IFN-γ inhibits *COL2A1* promoter activity and stimulates GAS activity and Stat1α binding

The pGL2B $-577/+125$ (**A** and **C**) and pGAS-Luc (**B** and **D**) constructs were transfected into C-28/I2 cells. (**A** and **B**) The cells were incubated with increasing concentrations of IFN-γ (1–100 units/ml) for 20 h. (**C** and **D**) IFN-γ (100 units/ml) was added to the cultures for 0.25, 1 or 20 h, at which time the medium was changed, and incubation was continued in fresh medium without IFN-γ until cell harvest at 20 h. Relative activities to untreated pGL2B $-577/+125$ or pGAS-Luc activity are shown. (**E**) Nuclear extracts were prepared from C-28/I2 cells after treatment with IFN-γ (100 units/ml) for 0.25, 0.5, 1 or 3 h and incubated with the labelled $-45/+11$ bp fragment of the *COL2A1* promoter. The wild-type (wt) and mutant (mt) Stat1α oligonucleotides were used both as labelled probes and as unlabelled competitors in EMSAs. For supershift analysis, the Stat1α antibody was incubated with the binding reaction mixture for 15 min at 4 °C before adding probe to nuclear extract.

showed previously that IFN-γ inhibits *COL2A1* gene transcription and activity of the region spanning -577 to $+3426$ bp, which is expressed in both normal and immortalized chondrocytes [24–26]. To determine the contribution of upstream sequences to promoter activity and the minimal promoter region required for the IFN-γ response, deletion analysis of the $-6368/+125$ bp promoter region was performed using luciferase reporter constructs containing *COL2A1* promoter regions spanning bp -6368, -3373, -1880, -577, -525 and -131 to bp $+125$. Addition of the E309 enhancer region increased by 10–30 % the constitutive activities of the promoter constructs but not the extent of inhibition by IFN-γ (Figure 2A). Compared

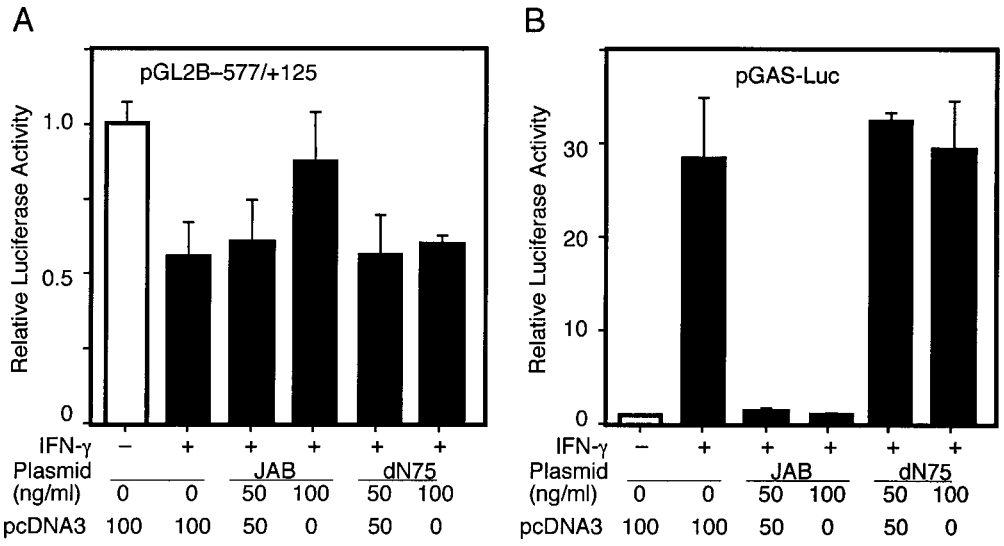

Figure 4 Overexpression of JAB reverses both inhibition and activation by IFN-γ in chondrocytes

The C-28/I2 cells were co-transfected with pGL2B — 577/ + 125 (**A**) or pGAS-Luc (**B**) and 50 or 100 ng of pcDNA3 (empty vector), pcDNA3-JAB (JAB) or pcDNA3-dN75JAB (dN75), as indicated. After transfection, the cells were incubated for 24 h prior to the addition of IFN-γ for a further 20 h. Relative activities to untreated pGL2B — 577/ + 125 or pGAS-Luc activity are shown.

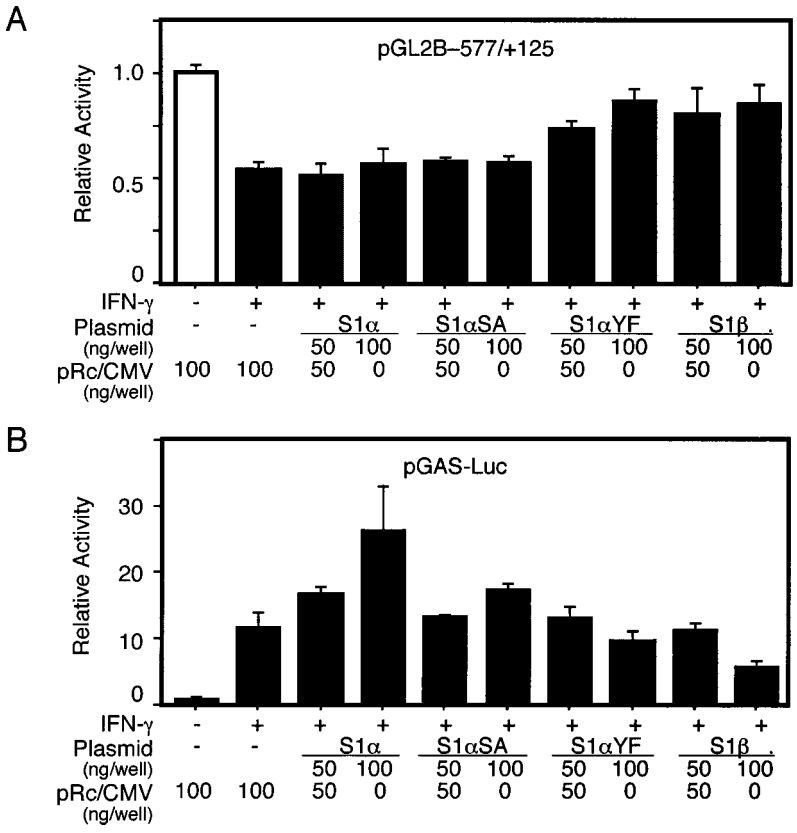

Figure 5 Effects of Stat1α expression vectors on IFN-γ-mediated effects in chondrocytes

The C-28/I2 cells were co-transfected with pRc/CMV (empty vector), pGL2B — 577/ + 125 (**A**) or pGAS-Luc (**B**) and pRc/CMV-Stat1α (S1α), pRc/CMV-Stat1α S727A (S1αSA), pRc/CMV-Stat1α Y701F (S1αYF) or pRc/CMV-Stat1α (S1β) at 100 ng of total DNA. After transfection, the cells were incubated for 24 h prior to the addition of IFN-γ for a further 20 h. Relative activities to untreated pGL2B — 577/ + 125 or pGAS-Luc activity are shown.

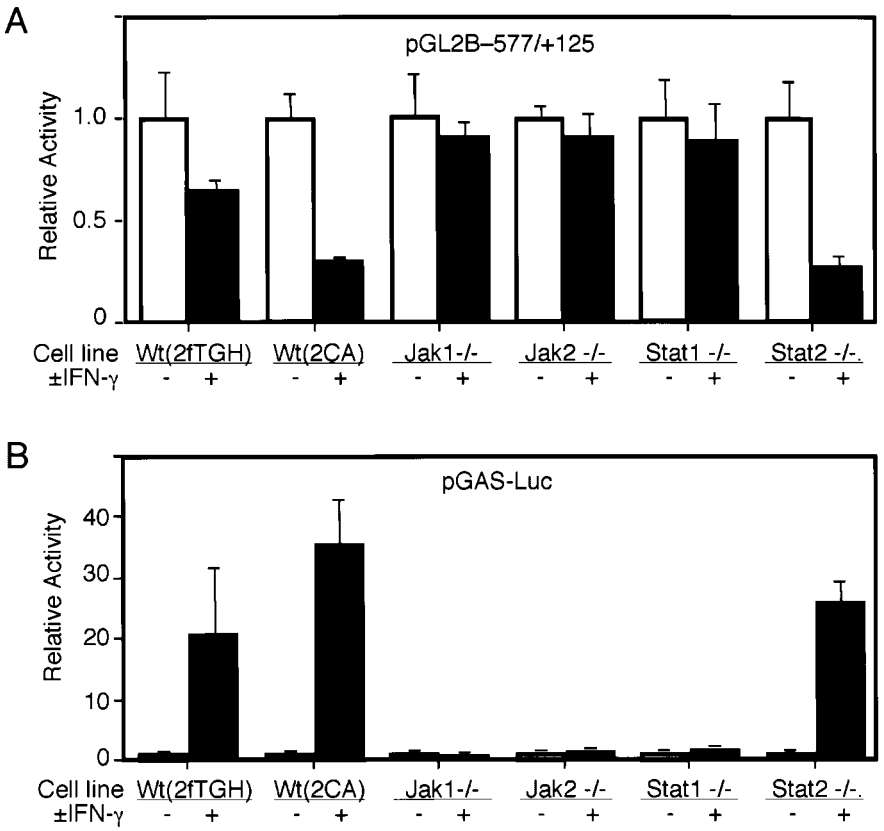

Figure 6 Loss of inhibitory and stimulatory effects of IFN-γ in Jak1-, Jak2- and Stat1-deficient cell lines

The fibrosarcoma cell lines U4A (Jak1$^{-/-}$), U3A (Stat1$^{-/-}$) and U6A (Stat2$^{-/-}$) are derived from the wild-type (Wt) 2fTGH cell line. The γ2A (Jak2$^{-/-}$) cell line is derived from wild-type 2CA. These cell lines were transfected with pGL2B$-577/+125$ (**A**) or pGAS-Luc (**B**) and incubated without or with IFN-γ (100 units/ml) for 20 h. Relative activities to untreated pGL2B$-577/+125$ or pGAS-Luc activity in each cell line are shown.

with the activity of the full-length -6368 bp promoter, deletion to -3373 bp did not affect promoter activity, but deletions to -1880 bp and greater increased activity by 1.5–2.5-fold. Nevertheless, IFN-γ suppressed expression of these promoter constructs to a similar extent, or to 20–40 % of the constitutive activity of each construct (Figure 2B). These results indicate that the proximal *COL2A1* promoter contains elements sufficient for the response to IFN-γ.

Inhibition of *COL2A1* promoter activity and activation of a GAS element by IFN-γ

To further characterize the response to IFN-γ, the C-28/I2 cells were transfected with either pGL2B$-577/+125$ or the pGAS-Luc reporter vector and incubated with IFN-γ at 1, 10 and 100 units/ml. The effects of IFN-γ were concentration-dependent. The highest concentration used, 100 units/ml, suppressed pGL2B$-577/+125$ activity by approx. 50 % (Figure 3A) and increased pGAS-Luc activity by more than 30-fold (Figure 3B). To examine the time course of the IFN-γ response, the cells were incubated with 100 units/ml IFN-γ for between 15 min and 20 h. The medium was then changed, incubations were continued in the absence of IFN-γ, and all cultures were harvested at 20 h. The expression of pGL2B$-577/+125$ was inhibited by IFN-γ within 15 min, and exposure for 1 or 20 h did not produce any further suppression (Figure 3C). The expression of pGAS-Luc was almost fully activated by IFN-γ after 15 min of treatment (Figure 3D). These results indicate that IFN-γ produces a rapid activation of Stat1α in chondrocytes in parallel with suppression of *COL2A1* promoter activity and suggest that these positive and negative effects of IFN-γ may occur via the JAK/STAT pathway.

Activation of Stat1α DNA-binding activity in chondrocytes

To determine whether IFN-γ treatment of chondrocytes could induce activation of Stat1α, we compared the DNA-binding activities on *COL2A1* promoter elements and a Stat1 consensus sequence. Nuclear extracts were prepared from the C-28/I2 cells after incubation with IFN-γ for 0.25, 0.5, 1 and 3 h. Computer-assisted analysis of consensus sequences by MatInspector (http://www.gsf.de/biodv/matinspector.html) and the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess) did not detect any potential Stat1-binding site within the IFN-γ-responsive *COL2A1* promoter region. Nevertheless, we performed extensive EMSA analyses using overlapping oligonucleotides derived from the $-131/+125$ bp region. We did not, however, observe any IFN-γ-induced binding activity that we could attribute to Stat1 by competition or supershift assays, as shown in the representative EMSA using the $-45/+11$ bp element (Figure 3E). In contrast, when the Stat1 consensus was used as probe on the same gel, binding activity was induced within 15 min after addition of IFN-γ, and the induction peaked by 30 min and remained stable for 3 h (Figure 3E) and even up

to 24 h (results not shown). This IFN-γ-induced binding activity was competed with by excess unlabelled Stat1 consensus DNA, but not by the mutant oligonucleotide, and supershifted by a Stat1α antibody (Figure 3E). Antibody against either Stat2 or Stat3 did not alter the pattern of binding to this probe (results not shown). Furthermore, the mutant Stat1 oligonucleotide did not bind IFN-γ-induced Stat1 (Figure 3E). Exposure of the cells to IFN-γ for 1 h, followed by replacement with fresh medium containing no IFN-γ, was found to be sufficient for sustained activation of Stat1 binding for at least 24 h (results not shown).

Overexpression of JAB prevents *COL2A1* inhibition and GAS activation by IFN-γ in chondrocytes

To determine the involvement of JAK tyrosine kinases in negative regulation of *COL2A1* expression by IFN-γ in chondrocytes, we examined whether overexpression of the JAK inhibitor JAB1 could block the IFN-γ response. Co-transfection with 100 ng of wild-type JAB expression vector reversed the suppression by IFN-γ of *COL2A1* promoter activity, whereas overexpression of dN75JAB, containing a deletion of the N-terminal 75 amino acids of wild-type JAB, had no effect (Figure 4A). Similarly, the activation of pGAS-Luc induced by IFN-γ was completely blocked by overexpression of wild-type JAB, but not by dN75JAB overexpression (Figure 4B). These results indicate that JAK tyrosine kinase activity is necessary for the inhibitory effect of IFN-γ on *COL2A1* expression, as well as GAS activation, in chondrocytes.

Effects of Stat1α expression vectors on IFN-γ responses in chondrocytes

To determine whether Stat1α participates functionally in IFN-γ-induced inhibition of *COL2A1* promoter activity, pGL2B$-$577/$+$125 or pGAS-Luc reporter vectors were transfected in C-28/I2 chondrocytes together with 50 or 100 ng of each wild-type or mutant Stat1α expression vector or the empty vector, pRc/CMV (Figure 5), or pCAGGS (results not shown). Co-transfection of wild-type Stat1α (S1α) did not modify the inhibitory effect of IFN-γ on *COL2A1* promoter activity (Figure 5A), whereas the activation of pGAS-Luc by IFN-γ was potentiated (Figure 5B). The overexpression of Stat1α S727A (S1αSA), which is reported to have partial activity compared with wild-type Stat1α due to the mutation of the Ser-727 mitogen-activated protein kinase (MAPK) phosphorylation site [31], neither reversed nor potentiated the effects of IFN-γ (Figure 5). In contrast, overexpression of dominant-negative Stat1α Y701F (S1αYF), containing a mutated JAK kinase phosphorylation site at Tyr-701, partially reversed the IFN-γ-induced suppression of *COL2A1* activity (Figure 5A) and activation of pGAS-Luc (Figure 5B). The overexpression of Stat1β, the C-terminal transactivation domain (TAD)-truncated form of Stat1α, also partially reversed both of the IFN-γ-induced responses (Figure 5), thus suggesting that Stat1α requires the TAD for full activity. This result also suggests that endogenous Stat1α activation contributes to the observed responses, perhaps explaining the partial effects of Stat1α mutants in these experiments.

Inhibition and activation by IFN-γ are lost in Jak1-, Jak2- and Stat1-deficient cell lines

To confirm the involvement of Jak1, Jak2 and Stat1α in the suppression of *COL2A1* promoter activity by IFN-γ, we transfected pGL2B$-$577/$+$125 or pGAS-Luc into the Jak1-, Jak2-, Stat1- and Stat2-deficient fibrosarcoma cell lines U4A, γ2A, U3A and U6A. Although these cell lines do not express endogenous *COL2A1*, the proximal promoter sequences of type II collagen genes have been shown to be expressed non-specifically in fibroblasts *in vitro*, and therefore fibrosarcoma cell lines are convenient models for studying regulation of ectopic promoter expression. In this experiment, for example, the level of *COL2A1*-luciferase activity expressed as RLU/μg of protein was $(2\pm0.3)\times10^5$ in untreated 2CA cells compared with $(7\pm0.1)\times10^3$ RLU/μg of protein in C-28/I2 cells in the experiments shown in Figures 4(A) and 5(A). In the wild-type 2CA and 2fTGH cell lines, IFN-γ suppressed *COL2A1* promoter activity by 30–70% (Figure 6A) and activated pGAS-Luc expression by 20–40-fold (Figure 6B). The IFN-γ-induced suppression of pGL2B$-$577/$+$125 (Figure 6A) or activation of pGAS-Luc (Figure 6B) was not observed in U4A (Jak1-deficient), γ2A (Jak2-deficient) and U3A (Stat1-deficient) cell lines. In contrast, both responses to IFN-γ were retained in the U6A (Stat2-deficient) cell line (Figure 6). The absence of IFN-γ responses in Jak1-, Jak2- and Stat1α-deficient cell lines was consistent with the results shown in Figures 4 and 5, in which JAB and Stat1α mutants were overexpressed in chondrocytes. These data confirm that Jak1, Jak2 and Stat1α are required for the suppression of *COL2A1* promoter activity by IFN-γ.

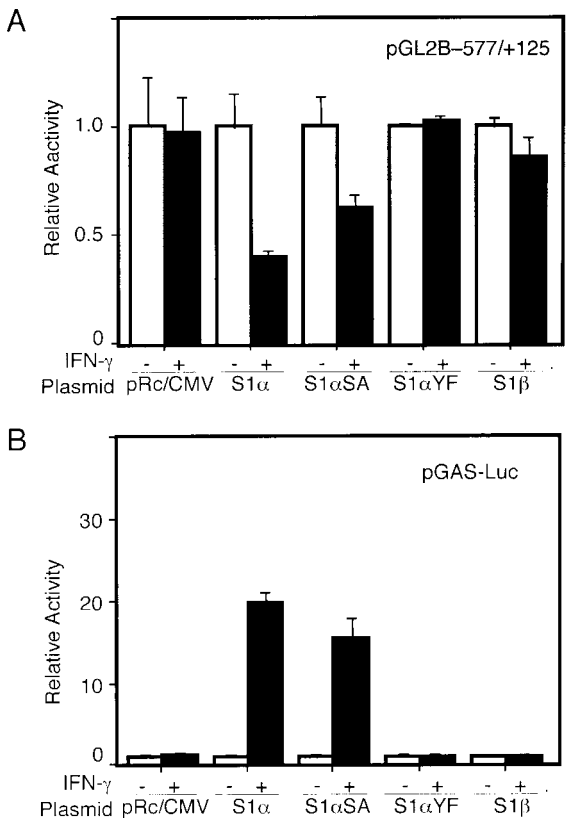

Figure 7 Stat1α rescues IFN-γ-dependent responses in Stat1α-deficient cells

The U3A cells were co-transfected with pGL2B$-$577/$+$125 (**A**) or pGAS-Luc (**B**) with the empty vector, pRc/CMV, or with expression vector for Stat1α (S1α), Stat1α S727A (S1αSA), Stat1α Y701F (S1αYF) or Stat1α (S1β) at 100 ng of total DNA. After transfection, the cells were incubated for 24 h prior to the addition of IFN-γ for a further 20 h. Relative activities to untreated pGL2B$-$577/$+$125 or pGAS-Luc activity are shown.

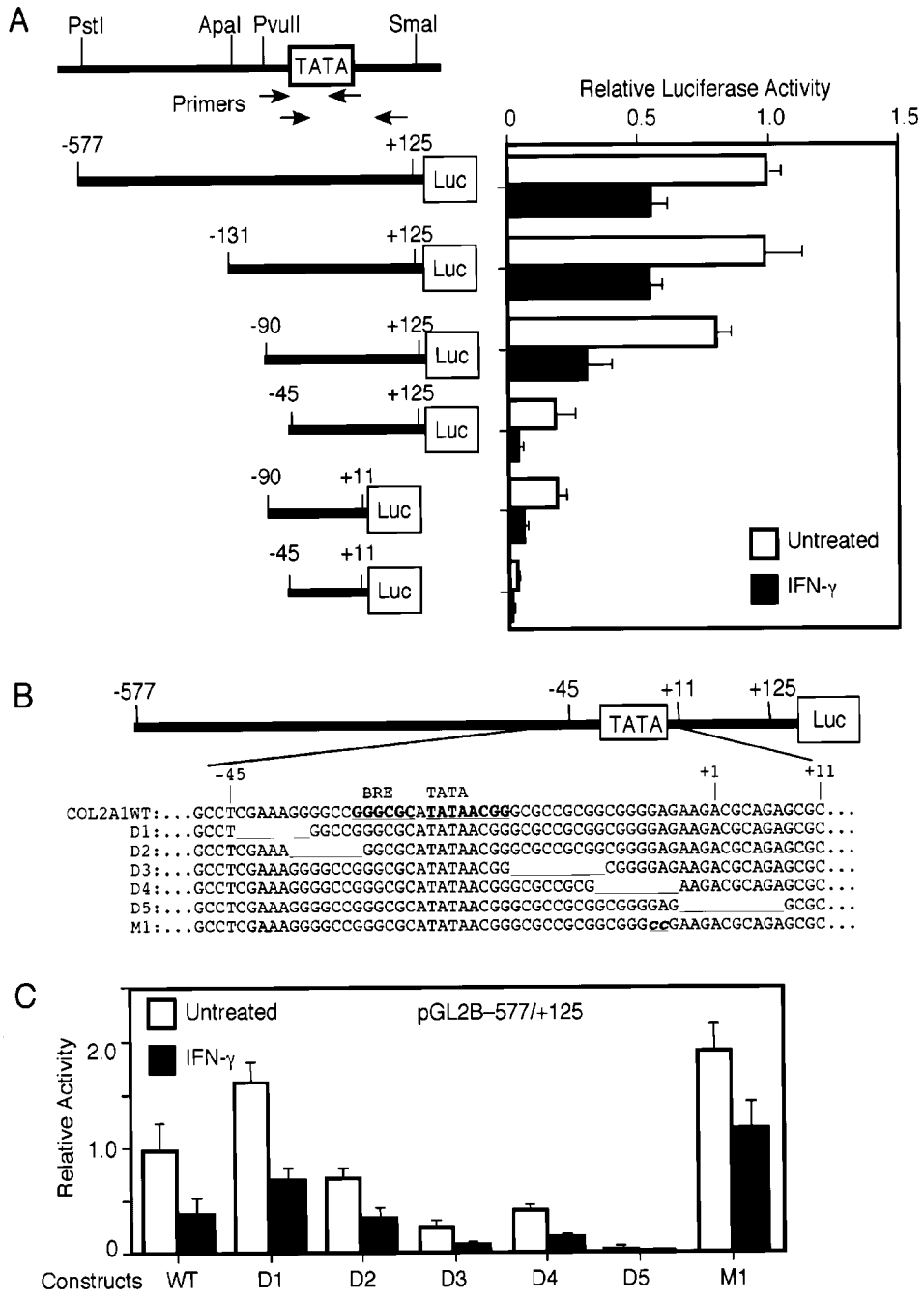

Figure 8 Functional analysis of the TATA-containing *COL2A1* promoter

(**A**) C-28/I2 cells were transfected with pGL2B reporter constructs containing *COL2A1* sequences −577/+125, −131/+125, −90/+125, −45/+125, −90/+11 or −45/+11 bp, and incubated without or with IFN-γ (100 units/ml) for 20 h. Positions of the primers used to generate these constructs are indicated by arrows. (**B**) Mutations and deletions within the −45/+11 bp region surrounding the TATA box were generated in the pGL2B−577/+125 construct. The positions of the BRE, TATA motif and transcription start site (+1) are indicated above the wild-type sequence. (**C**) The pGL2B−577/+125 construct without (WT) or with the mutations and deletions indicated in (**B**) were transfected into C-28/I2 cells and incubated for 20 h without or with IFN-γ (100 units/ml). Relative activities to untreated pGL2B−577/+125 are shown.

Stat1α rescues responses to IFN-γ in Stat1α-deficient cell lines

To further substantiate the importance of Stat1α in the IFN-γ-induced suppression of *COL2A1* promoter activity, we performed co-transfections using Stat1α expression vectors in the Stat1α-deficient cell line, U3A (Figure 7). As was also shown in Figure 6, IFN-γ was not able to regulate the activities of pGL2B−577/+125 and pGAS-Luc in Stat1α-deficient cells. The overexpression of wild-type Stat1α in U3A resulted in rescue of the suppression of *COL2A1* activity by IFN-γ, while overexpression of the S1αSA mutant produced only a partial rescue (Figure 7A). However, overexpression of Stat1β and S1α YF did not rescue the IFN-γ response. Similarly, the IFN-γ-induced activation of pGAS-Luc was recovered completely by co-

transfection with wild-type Stat1α or partially with S1αSA, but not when Stat1β or Stat1αYF were expressed (Figure 7B). These results further support the critical role of Stat1α activation via Tyr-701 in IFN-γ-induced suppression of the *COL2A1* promoter.

Deletion analysis of the *COL2A1* promoter region

In the transient transfection experiments performed thus far in C-28/I2 chondrocytes, the activity of the pGL2B$-131/+125$ was similar to or higher than that of pGL2$-577/+125$ and the response to IFN-γ was retained in the proximal promoter region (see Figure 2). To further target the DNA elements required for the IFN-γ response, we examined several deletion constructs of the proximal *COL2A1* promoter. The $-90/+125$ bp deletion construct, which contains one Sp1 site at $-81/-74$ bp, was expressed at a lower level than pGL2$-131/+125$ bp, which contains an additional Sp1 site at $-118/-112$ bp. Deletion to $-45/+125$ bp further reduced constitutive activity to 20% of the -131 bp construct (Figure 8A). Further deletion of the 3′-end produced activities that were 20% ($-90/+11$ bp) and 2% ($-45/+11$ bp) of the constitutive activity of pGL2B$-131/+125$. Nevertheless, the activities of all of the promoter deletion constructs were suppressed by IFN-γ by 40–75% (Figure 8A). These results indicate that the sequences required for full constitutive activity reside upstream of -90 bp. Nevertheless, the inhibitory response to IFN-γ was retained by all promoter deletion constructs containing the $-45/+11$ bp core promoter. The lack of an inhibitory effect of IFN-γ on the activity of pRL-TK driven by the TATA-containing thymidine kinase promoter (results not shown) indicated that the response of the *COL2A1* promoter was specific.

Functional analysis of the TATA-containing core promoter

Since the $-45/+11$ bp fragment did not bind Stat1 after IFN-γ treatment, as shown in Figure 3(E), we wished to determine the functional importance of sequences within this core promoter. Thus several deletions were made within the $-45/+11$ bp region, including two purine-rich sequences (D1 and D4) and two GC-rich sequences (D2 and D3) flanking the TATA box, as shown in Figure 8(B). The deletion D1, containing the most distal deletion at $-45/-39$ bp, was expressed at a higher level compared with the wild-type pGL2$-577/+125$ construct (Figure 8C). The D2 deletion, which partially removed the TFIIB-recognition element (BRE), and D3 and D4, which decreased the distance between the TATA box and the transcription start site, decreased activity by 50–70%. Not surprisingly, the D5 deletion, which removed the transcription start site, resulted in barely detectable activity. In contrast to the low level of expression of the D4 deletion construct, mutation within the D4 sequence (M1) produced a somewhat higher level of expression than the wild-type construct. Nevertheless, the extent of inhibition by IFN-γ remained significant on all constructs (Figure 8C). These results suggest that, although the IFN-γ response of the *COL2A1* promoter does not require direct binding of Stat1α to a classical *cis*-acting element, the $-45/+11$ bp core promoter region determines, at least partially, the extent of the response to IFN-γ.

DISCUSSION

We showed previously that IFN-γ decreases *COL2A1* mRNA expression and type II collagen synthesis [11], as well as the expression of *COL2A1* sequences spanning -577 to $+3426$ bp in a chloramphenicol acetyltransferase reporter vector [24] in human chondrocytes. However, the mechanism by which IFN-γ inhibits *COL2A1* gene transcription has not been characterized previously. The findings reported here indicate that the inhibitory effect of IFN-γ on *COL2A1* expression requires activation of the JAK/STAT pathway and targets the core promoter without direct binding of Stat1α to DNA elements.

Jak1 and Jak2 are cytoplasmic tyrosine kinases that are required for signalling by IFN-γ. The binding of IFN-γ to its receptor complex results in JAK activation via transphosphorylation of tyrosine within the activation loop of the kinase domain. The JAK activation leads to tyrosine phosphorylation of the cytoplasmic domain of the receptor and recruitment of Stat1 to the receptor complex. Stat1 then undergoes tyrosine phosphorylation, dimerization and translocation to the nucleus [15,16]. JAB (also known as SOCS1 and SSI-1) was identified as an Src homology 2 (SH2) domain-containing protein that interacts directly with Jak1 and Jak2 and thereby reduces tyrosine kinase activity and Stat1 activation [37]. As shown in Figure 4, we confirmed the involvement of Jak1 and Jak2 in IFN-γ-mediated suppression of *COL2A1* promoter activity by overexpressing JAB in chondrocytes. The importance of the N-terminal region, reported to be critical for JAB activity [34], was confirmed by the lack of effect of N-terminally deleted JAB, dN75JAB, on the IFN-γ responses in co-transfections in chondrocytes. Our observations in Jak1- and Jak2-deficient fibrosarcoma cell lines also support the involvement of both kinases in the IFN-γ-mediated pathway that suppresses *COL2A1* promoter activity. Although these cell lines do not express endogenous *COL2A1*, similar type II collagen gene promoter sequences have been shown to express highly in fibroblasts or dedifferentiated chondrocytes when transiently transfected in the absence of the intronic enhancer [7,8]. In fact, the actual levels of *COL2A1* luciferase activity were 10–100-fold higher in the fibrosarcoma cell lines than in the C-28/I2 cells, consistent with the differentiated phenotype of the chondrocyte cell line. Similarly, it was shown that 309 bp of the rat *COL2A1* proximal promoter was expressed in differentiating chick limb bud mesenchymal cells and sternal chondrocytes and responded to negative regulation by δEF1 in a manner independent of the intronic enhancer [7].

Our experiments also show that Stat1α, the target of Jak1 and Jak2, is critical for IFN-γ-mediated suppression of *COL2A1* promoter activity. Although IFN-γ increased the Stat1α-binding activity in chondrocyte nuclear extracts to a GAS sequence, we could not demonstrate binding to the *COL2A1* promoter, which contains no known Stat1α-binding element. Nevertheless, co-transfection of dominant-negative vectors, Stat1α Y701F and Stat1β, resulted in reversal of the inhibition of *COL2A1* promoter activity by IFN-γ in chondrocytes. Furthermore, in Stat1α-deficient cells, the absent IFN-γ response could be rescued by co-transfection with a vector expressing wild-type Stat1α. Parallel experiments showing expected effects of these vectors on IFN-γ-induced transcriptional activation of the pGAS-Luc reporter confirmed that JAK/STAT signalling pathways are intact in both chondrocytes and wild-type fibrosarcoma cell lines.

Our results indicate that the tyrosine residue at 701, which is absolutely required for Stat1 regulation of promoter activity directed by a GAS element, is also important for the negative response of the *COL2A1* promoter to IFN-γ. Nakajima et al. [33] reported that Stat1α Y701F in a pCAGGS expression vector functions in a dominant-negative manner in IFN-γ-induced signalling [33]. On the other hand, Bromberg et al. [32] reported that Stat1α Y701F in a pRc/CMV expression vector functioned in a dominant-negative manner on the epidermal growth factor-induced JAK/STAT pathway but not on IFN-γ-induced signalling. Nevertheless, Stat1α Y701F in both pCAGGS (results not shown) and pRc/CMV (Figure 5) produced similar

dominant-negative effects on both suppression and activation mediated by IFN-γ in chondrocytes. It is likely that the partial effects were due to insufficient expressed protein to counteract the endogenous Stat1α, since Stat1α Y701F was not able to rescue either of the responses to IFN-γ in the Stat1α-deficient cell line.

Stat1β, the naturally occurring C-terminal truncated form of Stat1α, also acted in a dominant-negative manner on IFN-γ-induced repression of *COL2A1* promoter activity and activation of pGAS-Luc, thus suggesting that Stat1β may function in the cell to limit the full response. Stat1β was not capable of IFN-γ-dependent regulation, as indicated by its inability to rescue the responses of pGL2B $-577/+125$ or pGAS-Luc in the Stat1α-deficient cells, thus arguing that the C-terminal TAD of Stat1α is critical for inhibition as well as activation of gene expression. In contrast, although it has been reported that the MAPK phosphorylation site at Ser-727 is required for maximal transcriptional activation [31], overexpression of Stat1α S727A had no dominant-negative effect on IFN-γ-mediated suppression of *COL2A1* promoter activity in chondrocytes and also rescued the responses to IFN-γ in Stat1α-deficient cells almost as well as Stat1α. These findings suggest that IFN-γ-induced transcriptional activation and repression share a similar signalling mechanism that depends upon tyrosine phosphorylation of Stat1α but not MAPK activation.

Many of the genes that are suppressed by IFN-γ are involved in growth and differentiation of connective tissue cells and studies on transcriptional regulation of these genes provide evidence of both direct and indirect regulation of promoter activities by Stat1α. The IFN-γ-mediated transcriptional suppression of the perlecan gene is Stat1-dependent and requires a distal promoter region containing multiple GAS elements [20]. However, a consensus GAS element in the c-*myc* promoter is necessary but insufficient for suppression by IFN-γ, although this response requires phosphorylation of both tyrosine and serine residues of Stat1α [38]. There are also reports of promoters that respond to IFN-γ in the absence of Stat1α-binding GAS elements [39,40]. In the *COL1A1* promoter, a region spanning -129 to -109 bp, which contains Sp1 and NF-1 sites, but no IFN-γ-response element such as GAS or interferon-stimulated response element, confers responsiveness to IFN-γ in transient transfection assays [23]. Higashi et al. [22] reported that a pyrimidine-rich sequence at $-161/-154$ bp in the promoter of the α2(I) procollagen gene (*COL1A2*), CTCCCTCC, termed IgRE, was involved in the IFN-γ response in fibroblasts. Similar to our results, IFN-γ treatment does not alter the DNA-binding activities in either type I collagen-encoding gene [22,23]. However, the *COL2A1* promoter has a somewhat distinct organization compared with the promoters of the type I collagen genes, which are ubiquitously expressed. Furthermore, our previous studies showing that interleukin-1, another cytokine that inhibits *COL2A1* expression in chondrocytes but stimulates type I collagen gene expression in fibroblasts and chondrocytes [12,24,41,42], suggest that there may exist multiple cytokine- and promoter-specific mechanisms for modifying the activities of constitutively expressed collagen gene promoters.

Our functional analysis suggests that the $-45/+11$ bp *COL2A1* core promoter region determines, at least partially, the extent of the inhibition by IFN-γ in the absence of direct Stat1α binding to a *cis* element. It is possible to explain those results by the proximity of a given deletion or mutation to the TATA box and its distance from the transcription start site. Thus deletions between the TATA box and the start site reduce constitutive activity, whereas a substitution mutation within this region does not decrease, and can even increase, the activity. In fact, this mutation, by introducing two pyrimidines, may produce a classical initiator element (Inr) that is able to promote more effective interaction with TATA-binding protein/TFIIB. The upstream deletion D1 does not decrease activity, whereas D2, which removes part of the BRE, reduces activity by 25%. Similar to our findings on the *Col1a1* promoter where the well-known TgTA mutation results in complete loss of function [27], the complete loss of activity of the $-22/+11$ bp deletion construct (results not shown) indicates that the TATA box is a requirement for constitutive activity of the *COL2A1* gene. The $-45/+125$ bp promoter responds to JAB and Stat1α expression vectors in a similar manner as the $-577/+125$ bp promoter in both C-28/I2 and U3A cell lines (results not shown), further confirming the functional importance of the core promoter in the IFN-γ response.

Several studies suggest that the proximal promoter of type II collagen genes may operate at specific times during development when negative regulation is required and via promoter regions distinct from those required for positive regulation [4,7]. Our findings indicate that the *COL2A1* core promoter is capable of being expressed in differentiated chondrocytes at a post-developmental stage of low-turnover cartilage matrix synthesis and responding to negative regulation by cytokines. The early cytokine-activated events that are usually associated with positive responses may produce a negative response in this promoter context by effecting the release of constitutive factors from DNA elements. In cytokine-activated genes, the assembly of higher-order nucleoprotein complexes orchestrated by high mobility group I(Y) may be important for integrating the responses to the induced signalling pathways [43]. Similarly, Sox9 and related high-mobility-group factors are architectural proteins that act to maintain the nucleosomes in an open configuration, thereby exposing the endogenous, chromatin-integrated *COL2A1* promoter to constitutive factors that interact directly with the promoter [44]. However, we found that the levels of L-Sox5, Sox6 and Sox9, which bind to the intronic enhancer [3], are not modified by IFN-γ and that the promoter response to IFN-γ occurs in the absence of the intronic enhancer. Thus it is likely that IFN-γ-induced Stat1α mediates repression, directly or indirectly, by interfering with the constitutive transcriptional machinery.

Stat1α is known to interact with co-activators, such as the cAMP-response-element-binding protein-binding protein (CBP)/p300 [45–47]. CBP/p300 interacts with Stat1α via the serine at 727 and may thereby promote chromatin remodelling through histone acetylase activity [45]. Although co-repressors that interact with Stat1α have not been identified, Stat1α may antagonize the activities of other transcription factors by competing for limiting amounts of CBP/p300 [47]. A recent report indicates that IFN-γ inhibits TGF-β-induced *COL1A2* promoter expression in fibroblasts by reducing the interaction of p300 with Smad3 [48]. Similarly, IFN-γ-induced class II transactivator (CIITA) mediates suppression of *Col1a2* transcription by squelching CBP activity [49]. Thus recruitment of CBP/p300 by Stat1α could block interactions with constitutive factors such as Sp1 and thereby explain IFN-γ-mediated suppression of *COL2A1* core promoter activity. The recent report that IFN-γ inactivates the binding of the regulatory factor for X-box proteins 1 (RFX1) to the *Col1a2* transcription start site suggests that a similar mechanism could account for the response of the *COL2A1* core promoter [50]. However, the lack of dependence on protein synthesis for the IFN-γ-induced suppression of *COL2A1* mRNA levels and the rapidity of the promoter response to IFN-γ argue against an indirect effect via synthesis of protein factors such as CIITA. Since the transcriptional

repression of *COL2A1* by IFN-γ requires sustained activation of Stat1α in the absence of direct binding of Stat1α to the core promoter region, this response likely involves disruption of protein–protein interactions among the co-activators and general transcription factors within the complex that drives constitutive *COL2A1* expression.

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REFERENCES

1 Poole, A. R. (1997) Cartilage in health and disease. In Arthritis and Allied Conditions: a Textbook of Rheumatology (Koopman, W. J., ed.), pp. 255–308, Williams and Wilkins, Baltimore
2 Sandell, L. J. (1996) Genes and gene regulation of extracellular matrix proteins: an introduction. Connect. Tiss. Res. **35**, 1–6
3 Lefebvre, V., Li, P. and de Crombrugghe, B. (1998) A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J. **17**, 5718–5733
4 Leung, K. K., Ng, L. J., Ho, K. K., Tam, P. P. and Cheah, K. S. (1998) Different *cis*-regulatory DNA elements mediate developmental stage- and tissue-specific expression of the human COL2A1 gene in transgenic mice. J. Cell Biol. **141**, 1291–1300
5 Vikkula, M., Metsaranta, M., Syvanen, A. C., Ala-Kokko, L., Vuorio, E. and Peltonen, L. (1992) Structural analysis of the regulatory elements of the type-II procollagen gene. Conservation of promoter and first intron sequences between human and mouse. Biochem. J. **285**, 287–294
6 Dharmavaram, R. M., Liu, G., Mowers, S. D. and Jimenez, S. A. (1997) Detection and characterization of Sp1 binding activity in human chondrocytes and its alterations during chondrocyte dedifferentiation. J. Biol. Chem. **272**, 26918–26925
7 Murray, D., Precht, P., Balakir, R. and Horton, W. E. J. (2001) The transcription factor δEF1 is inversely expressed with type II collagen mRNA and can repress Col2a1 promoter activity in transfected chondrocytes. J. Biol. Chem. **275**, 3610–3618
8 Ghayor, C., Herrouin, J.-F., Chadjichristos, C., Ala-Kokko, L., Takigawa, M., Pujol, J.-P. and Galéra, P. (2000) Regulation of human COL2A1 gene expression in chondrocytes. Identification of c-Krox-responsive elements and modulation by phenotype alteration. J. Biol. Chem. **275**, 27421–27438
9 Ghayor, C., Chadjichristos, C., Herrouin, J.-F., Ala-Kokko, L., Suske, G., Pujol, J.-P. and Galéra, P. (2001) Sp3 represses the Sp1-mediated transactivation of the human COL2A1 gene in primary and de-differentiated chondrocytes. J. Biol. Chem. **276**, 36881–36895
10 Goldring, M. B. (1999) The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. Connect. Tiss. Res. **40**, 1–11
11 Goldring, M. B., Sandell, L. J., Stephenson, M. L. and Krane, S. M. (1986) Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. J. Biol. Chem. **261**, 9049–9056
12 Goldring, M. B., Birkhead, J., Sandell, L. J., Kimura, T. and Krane, S. M. (1988) Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J. Clin. Invest. **82**, 2026–2037
13 Lefebvre, V., Peeters-Joris, C. and Vaes, G. (1990) Modulation by interleukin 1 and tumor necrosis factor α of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. Biochim. Biophys. Acta **1052**, 366–378
14 Reginato, A. M., Sanz-Rodriguez, C., Diaz, A., Dharmavaram, R. M. and Jimenez, S. A. (1993) Transcriptional modulation of cartilage-specific collagen gene expression by interferon γ and tumour necrosis factor α in cultured human chondrocytes. Biochem. J. **294**, 761–769
15 Chatterjee-Kishore, M., van den Akker, F. and Stark, G. R. (2001) Association of STATs with relatives and friends. Trends Cell Biol. **10**, 106–111
16 Ihle, J. N. (2001) The Stat family in cytokine signaling. Curr. Opin. Cell Biol. **13**, 211–217
17 Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improta, T., Darnell, Jr, J. E., Stark, G. R. and Kerr, I. M. (1993) Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways. EMBO J. **12**, 4221–4228
18 Der, S. D., Zhou, A., Williams, B. R. and Silverman, R. H. (1998) Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. Proc. Natl. Acad. Sci. U.S.A. **95**, 15623–15628
19 Amento, E. P., Bhan, A. K., McCullagh, K. G. and Krane, S. M. (1985) Influences of gamma interferon on synovial fibroblastlike cells. Ia induction and inhibition of collagen synthesis. J. Clin. Invest. **76**, 837–848
20 Sharma, B. and Iozzo, R. V. (1998) Transcriptional silencing of perlecan gene expression by interferon-γ. J. Biol. Chem. **273**, 4642–4646
21 Dodge, G. R., Diaz, A., Sanz-Rodriguez, C., Reginato, A. M. and Jimenez, S. A. (1998) Effects of interferon-γ and tumor necrosis factor α on the expression of the genes encoding aggrecan, biglycan, and decorin core proteins in cultured human chondrocytes. Arthritis Rheum. **41**, 274–283
22 Higashi, K., Kouba, D. J., Song, Y. J., Uitto, J. and Mauviel, A. (1998) A proximal element within the human α2(I) collagen (COL1A2) promoter, distinct from the tumor necrosis factor-α response element, mediates transcriptional repression by interferon-γ. Matrix Biol. **16**, 447–456
23 Yuan, W., Yufit, T., Li, L., Mori, Y., Chen, S. J. and Varga, J. (1999) Negative modulation of α(I) procollagen gene expression in human skin fibroblasts: transcriptional inhibition by interferon-γ. J. Cell. Physiol. **179**, 97–108
24 Goldring, M. B., Fukuo, K., Birkhead, J. R., Dudek, E. and Sandell, L. J. (1994) Transcriptional suppression by interleukin-1 and interferon-γ of type II collagen gene expression in human chondrocytes. J. Cell. Biochem. **54**, 85–99
25 Goldring, M. B., Birkhead, J. R., Suen, L.-F., Yamin, R., Mizuno, S., Glowacki, J., Arbiser, J. L. and Apperley, J. F. (1994) Interleukin-1β-modulated gene expression in immortalized human chondrocytes. J. Clin. Invest. **94**, 2307–2316
26 Robbins, J. R., Thomas, B., Tan, L., Choy, B., Arbiser, J. L., Berenbaum, F. and Goldring, M. B. (2000) Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1β. Arthritis Rheum. **43**, 2189–2201
27 Riquet, F. B., Tan, L., Choy, B. K., Osaki, M., Karsenty, G., Osborne, T. F., Auron, P. E. and Goldring, M. B. (2001) YY1 is a positive regulator of transcription of the Col1a1 gene. J. Biol. Chem. **20**, 38665–38672
28 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**, 1475–1489
29 Cheah, K. S., Levy, A., Trainor, P. A., Wai, A. W., Kuffner, T., So, C. L., Leung, K. K., Lovell-Badge, R. H. and Tam, P. P. (1995) Human COL2A1-directed SV40 T antigen expression in transgenic and chimeric mice results in abnormal skeletal development. J. Cell Biol. **128**, 223–237
30 Ryan, M. C., Sieraski, M. and Sandell, L. J. (1990) The human type II procollagen gene: identification of an additional protein-coding domain and location of potential regulatory sequences in the promoter and first intron. Genomics **8**, 41–48
31 Wen, Z., Zhong, Z. and Darnell, Jr, J. E. (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell **82**, 241–250
32 Bromberg, J. F., Fan, Z., Brown, C., Mendelsohn, J. and Darnell, Jr, J. E. (1998) Epidermal growth factor-induced growth inhibition requires Stat1 activation. Cell Growth Differ. **9**, 505–512
33 Nakajima, K., Yamanaka, Y., Nakae, K., Kojima, H., Ichiba, M., Kiuchi, N., Kitaoka, T., Fukada, T., Hibi, M. and Hirano, T. (1996) A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J. **15**, 3651–3658
34 Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N. and Yoshimura, A. (1999) The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. EMBO J. **18**, 1309–1320
35 Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**, 193–199
36 Loeser, R. F., Sadiev, S., Tan, L. and Goldring, M. B. (2000) Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for $\alpha1\beta1$ and $\alpha2\beta1$ integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage **8**, 96–105
37 Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H. et al. (1997) A new protein containing an SH2 domain that inhibits JAK kinases. Nature (London) **387**, 921–924
38 Ramana, C. V., Grammatikakis, N., Chernov, M., Nguyen, H., Goh, K. C., Williams, B. R. and Stark, G. R. (2000) Regulation of c-myc expression by IFN-gamma through Stat1-dependent and -independent pathways. EMBO J. **19**, 263–272
39 Tamai, K., Li, K., Silos, S., Rudnicka, L., Hashimoto, T., Nishikawa, T. and Uitto, J. (1995) Interferon-gamma-mediated inactivation of transcription of the 230-kDa bullous pemphigoid antigen gene (BPAG1) provides novel insight into keratinocyte differentiation. J. Biol. Chem. **270**, 392–396
40 Sibinga, N. E., Wang, H., Perrella, M. A., Endege, W. O., Patterson, C., Yoshizumi, M., Haber, E. and Lee, M. E. (1999) Interferon-gamma-mediated inhibition of cyclin A gene transcription is independent of individual cis-acting elements in the cyclin A promoter. J. Biol. Chem. **274**, 12139–12146

41 Goldring, M. B. and Krane, S. M. (1987) Modulation by recombinant interleukin 1 of synthesis of types I and III collagens and associated procollagen mRNA levels in cultured human cells. J. Biol. Chem. **262**, 16724–16729
42 Riquet, R. B., Lai, W.-F. T., Birkhead, J. R., Suen, L.-F., Karsenty, G. and Goldring, M. B. (2000) Suppression of type I collagen gene expression by prostaglandins in fibroblasts is mediated at the transcriptional level. Mol. Med. **6**, 705–719
43 Pellacani, A., Chin, M. T., Wiesel, P., Ibanez, M., Patel, A., Yet, S. F., Hsieh, C. M., Paulauskis, J. D., Reeves, R., Lee, M. E. and Perrella, M. A. (1999) Induction of high mobility group-I(Y) protein by endotoxin and interleukin-1β in vascular smooth muscle cells. Role in activation of inducible nitric oxide synthase. J. Biol. Chem. **274**, 1525–1532
44 Marshall, O. J. and Harley, V. R. (2000) Molecular mechanisms of SOX9 action. Mol. Genet. Metab. **71**, 455–462
45 Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M. and Darnell, Jr, J. E. (1996) Two contact regions between Stat1 and CBP/p300 in interferon γ signaling. Proc. Natl. Acad. Sci. U.S.A. **93**, 15092–15096
46 Zhang, J. J., Zhao, Y., Chait, B. T., Lathem, W. W., Ritzi, M., Knippers, R. and Darnell, Jr, J. E. (1998) Ser727-dependent recruitment of MCM5 by Stat1α in IFN-γ-induced transcriptional activation. EMBO J. **17**, 6963–6971
47 Horvai, A. E., Xu, L., Korzus, E., Brard, G., Kalafus, D., Mullen, T. M., Rose, D. W., Rosenfeld, M. G. and Glass, C. K. (1997) Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. Proc. Natl. Acad. Sci. U.S.A. **94**, 1074–1079
48 Ghosh, A. K., Yuan, W., Mori, Y., Chen, S. and Varga, J. (2001) Antagonistic regulation of type I collagen gene expression by interferon-γ and transforming growth factor-β. Integration at the level of p300/CBP transcriptional coactivators. J. Biol. Chem. **276**, 11041–11048
49 Zhu, X.-S. and Ting, J. P.-Y. (2001) A 36-amino-acid region of CIITA is an effective inhibitor of CBP: novel mechanism of gamma interferon-mediated suppression of collagen α_2(I) and other promoters. Mol. Cell. Biol. **21**, 7078–7088
50 Sengupta, P. K., Fargo, J. and Smith, B. D. (2002) RFX family interacts at the collagen (COL1A2) start site and represses transcription. J. Biol. Chem. **277**, 24926–24937

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