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Suppression of Type I Collagen Gene Expression by Prostaglandins in Fibroblasts Is Mediated at the Transcriptional Level

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Abstract

Background: Tissues undergoing a chronic inflammatory process, such as the synovium in rheumatoid arthritis, are characterized by the infiltration of lymphocytes of different subsets and activation of monocyte/macrophages. Interleukin-1 (IL-1), a monocyte/macrophage product that stimulates synovial fibroblasts to produce matrix metalloproteinases (MMPs), prostaglandins, and other cytokines, also has profound effects on the synthesis of extracellular matrix components such as type I collagen. In previous studies, we have shown that synovial fibroblasts and chondrocytes isolated from human joint tissues are particularly sensitive to prostaglandins, which modulate the effects of IL-1 on collagen gene expression in an autocrine manner.

Materials and Methods: BALBc/3T3 fibroblasts were treated with IL-1 and prostaglandins in the absence and presence of indomethacin to inhibit endogenous prostaglandin biosynthesis. Collagen synthesis was analyzed by SDS-PAGE as [^{3}H]proline-labeled, secreted proteins, and prostaglandin production and cyclic adenosine 3′,5′-cyclic monophosphate (camp) content were assayed. The expression of type I collagen gene (Col1a1) promoter-reporter gene constructs was examined in transient transfection experiments, and the binding of nuclear factors to the Col1a1 promoter region spanning −222 bp/+116 bp was analyzed by DNase I footprinting and electrophoretic mobility shift (EMSA) assays.

Results: IL-1 increased the synthesis of type I and type III collagens in BALBc/3T3 fibroblasts; greater increases were observed when IL-1-stimulated synthesis of PGE_2 was blocked by indomethacin. Transient transfection experiments demonstrated dose-dependent inhibition of the −222 bp Col1a1 promoter by exogenously added prostaglandins with the order of potency of $PGF_{2\alpha} > PGE_2 > PGE_1$. DNase I footprinting showed increased protection, which extended from the region immediately upstream of the TATA box, owing to the binding of nuclear factors from PGE_2- or PGE_1-treated BALBc/3T3 cells. EMSA analysis showed zinc-dependent differences in the binding of nuclear factors from untreated and prostaglandin-treated cells to the −84 bp/−29 bp region of the Col1a1 promoter.

Conclusions: These results show that the inhibition of Col1a1 expression by IL-1 in fibroblasts is mediated by prostaglandins at the transcriptional level and suggest that PGE-responsive factors may interact directly or indirectly with basal regulatory elements in the proximal promoter region of the Col1a1 gene.

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Introduction

Collagen is one of the most abundant and ubiquitous proteins in mammals, accounting for about 30% of all proteins. As major compo-

nents of the extracellular matrix, collagens play a dominant role during early development and organogenesis, and are involved in maintaining the structural integrity of tissues and organs in the adult (1). In fibrotic diseases, increased deposition of extracellular matrix (ECM) proteins is the final outcome of an inflammatory process that is the earliest response triggered by various stimuli (2). Macrophages and lymphocytes often infiltrate tissues undergoing a chronic inflammatory process, such as the synovium in rheumatoid arthritis. These are the main cell types responsible for the production of various cytokines, which are involved in the initiation, progression, and eventual modulation of inflammatory and immunological responses (3). In articular joint tissues such as synovium and cartilage, the balance between synthesis and degradation must be accurately controlled to ensure the preservation of normal structure and function (4). Fibroblasts and chondrocytes respond to a wide range of cytokines that modulate migration and proliferation, as well as ECM production and degradation (5,6). For example, interleukin-1 (IL-1), a monocyte/macrophage product, stimulates fibroblasts to produce matrix metalloproteinases (MMPs), prostaglandins, and other cytokines, but it also has profound effects on the synthesis of ECM components.

Prostaglandin E_2 (PGE_2), the major product of the cyclooxygenase pathway in cultured fibroblasts, is a pleiotropic bioregulator with the ability to alter the expression of target genes involved in the pathophysiology of inflammatory and fibrotic diseases. PGE_2 can exert anticatabolic and anti-inflammatory effects by down-regulating the expression and synthesis of the inflammatory cytokines, IL-1 and tumor necrosis factor alpha (TNF-α) by monocyte/macrophages and other cell types (7). In addition, these same cytokines stimulate PGE_2 production in several cell types including macrophages (8) and synovial fibroblasts (9–13) by increasing COX-2 mRNA levels and gene transcription (14–16).

The effects of IL-1 on fibroblast collagen production are controversial, because this cytokine has been shown to either increase or decrease collagen production depending on the cell type. In most studies, these effects were accompanied by parallel changes in the steady-state levels of the corresponding mRNAs (12,13,17–22). In other studies, however, an inhibition of collagen production with a paradoxical increase in collagen mRNA levels was found in both synovial and dermal fibroblasts (23,24). In previous work from our laboratory, IL-1 increased the synthesis of type I and type III collagens and levels of α1(I), α2(I), and α1(III) procollagen mRNAs by dermal fibroblasts (12,25). Synovial cells treated with IL-1 for 48 hr or longer also showed increased collagen synthesis, which was enhanced in the presence of indomethacin, an inhibitor of prostaglandin biosynthesis (25). In contrast, when synovial fibroblasts were treated with IL-1 for 24 hr, stimulation of collagen synthesis was observed only when the synthesis of PGE_2, which inhibits type I collagen synthesis, was blocked (12). These findings suggested that the duration of treatment with IL-1 and consequent exposure to prostaglandins might influence the ability of the synovial fibroblast to synthesize collagens.

Prostaglandins are potent inhibitors of type I collagen synthesis in several connective tissue cell types (12,18,25–28). PGE_2 has been shown to inhibit collagen production by reducing proline uptake (29) and increasing the intracellular degradation of protein (28). However, observations that PGE_2 decreased steady-state mRNA levels of α1(I) procollagen in fibroblasts and chondrocytes also suggested a pre-translational mechanism (18,27). In more studies in lung fibroblasts, inhibition of α1(I) procollagen by IL-1 was found to occur at the transcriptional level by both PGE_2-dependent and -independent mechanisms (22). Furthermore, direct down-regulation of α1(I) procollagen gene transcription and promoter activity by PGE_2 and other prostaglandins was observed in osteoblasts (30,31).

In the studies reported here, we examined the molecular basis of the feedback modulatory effects of prostaglandins on the collagen response to IL-1 and found that prostaglandins inhibited directly the expression of the Col1a1 promoter in mouse fibroblasts. We also show functional evidence that the PGE response is mediated through the proximal promoter region of the Col1a1 gene.

Materials and Methods

BALBc/3T3 Fibroblast Cultures and Bioassays

BALBc/3T3 fibroblasts were plated in 12-well plates (22-mm diameter wells) at 50,000 cells/well and grown for 5 days in DMEM containing 10% calf serum. The medium was then

changed, hIL-1α (a gift from Dr. Peter Lomedico, Hoffmann-LaRoche, Nutley, NJ) was added at increasing concentrations in the absence or presence of 10^{-6} M indomethacin (Sigma, St. Louis, MO) to inhibit endogenous prostaglandin synthesis, and incubation was continued for 48 hr. The cells were then labeled for 24 hr with L-[5-^{3}H] proline (25 μCi/ml; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in serum-free DMEM supplemented with 50 μg/ml ascorbate and 50 μg/ml β-aminoproprionitrile fumerate (β-APN). Procollagens and fibronectin (FN) were analyzed by SDS-PAGE (5% acrylamide) of total medium proteins after reduction with 0.1% β-mercaptoethanol (β-ME). The labeled collagen chains in the culture medium were further characterized after limited digestion of nonhelical portions with pepsin in acetic acid for 18 hr at 4°C, as described previously (12,18). PGE_2 was measured in unlabeled culture medium using the Prostaglandin E_2 [^{125}I] Radioimmunoassay Kit, as described previously (18). The adenosine 3′,5′-cyclic monophosphate (cAMP) content was measured in lysed cell monolayers using the RIANEN cAMP [^{125}I] Radioimmunoassay Kit as described elsewhere (32). Both kits were purchased from NEN-Dupont (Boston, MA).

Transient Transfections of Col1a1-CAT Constructs

To analyze type I collagen gene expression, BALB/c3T3 fibroblasts were transfected with constructs of the Col1a1 promoter gene inserted upstream of the chloramphenicol acetyl transferase (CAT) reporter gene. These constructs contained Col1a1 sequences spanning −976 to +116 bp (pG100) and −222 to +116 bp (pG60), as described (33). The cells were plated at 0.6×10^6 cells/100-mm dish in DMEM with 10% calf serum and allowed to settle for 18–24 hr. The medium was then changed, and the cells were transfected 2–4 hr later, with 10 μg of plasmid DNA by the calcium-phosphate method using a Calcium Phosphate Mammalian Cell Transfection Kit (5 Prime → 3 Prime, Inc., Boulder, CO) (34). The plates were then incubated for 4 hr in a 37°C incubator and a 15% glycerol shock was carried out for 3 min. After washing with serum-free DMEM, the cells were allowed to recover 1–3 h in DMEM containing 1% calf serum. Recombinant hIL-1α (12.5 pM), indomethacin (10^{-6} M), and prostaglandins (25– 250 nM) were then added directly and incubations were continued for a further 36–48 hr. Cell lysates were analyzed for protein with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) (35). Portions of cell extracts containing 50 μg of protein were assayed for CAT activity by the fluor diffusion method using [^{3}H]acetyl-CoA (36,37). The reactions were allowed to proceed for 2–6 hr. First order slopes (Δcpm/min) were derived from plots of activity versus reaction time. Each transfection experiment was repeated at least three times, and each data point represents the mean of 3–10 experiments ±SEM. PGE_2, PGE_1, and $PGF_{2\alpha}$ were purchased from ICN Biomedical Research Products (Costa Mesa, CA), and misoprostol, a stable analog of PGE_1, was provided as free acid by G.D. Searle (Skokie, IL).

Nuclear Extract Preparation

BALBc/3T3 cells were plated at 0.4×10^7 cells per 24.5 cm^2 culture dish and incubated for 3 days to subconfluence. The medium was then changed to DMEM containing 1% calf serum, and 10^{-6} M indomethacin was added to some cultures to inhibit endogenous prostaglandin synthesis. Subsequently, prostaglandins were added at 250 nM and incubations were continued for 18 hr. Nuclear extracts were prepared by a modified method of Dignam et al. (38). Cell layers were harvested, centrifuged, and cell pellets were resuspended in buffer A (10 mM Hepes, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and protease inhibitors) and incubated on ice for 10 min. After centrifugation, pellets were homogenized in buffer A using a Wheaton Dounce tissue grinder. After a centrifugation step, pellets were resuspended in buffer B–low salt (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT, and protease inhibitors) and homogenized again. Then, buffer B–high salt (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.9 M NaCl, 0.5 mM DTT, and protease inhibitors) was added at 1:1 ratio and incubated for 30 min at 4°C. The homogenates were centrifuged, supernatants were assayed for protein concentration using the Bio-Rad protein assay system, and nuclear extracts were stored at −80°C in small volumes at 1 μg/μl.

DNase I Footprinting

The EcoRI-HindIII (−222/+116bp) fragment contained in the pG60 plasmid was end-labeled and binding reactions and treatment with DNAse I were carried out as described

(33). The same end-labeled DNA fragment was subjected to the G + A sequencing reaction (39). After electrophoresis on a 6% polyacrylamide denaturing gel, autoradiography was performed at −80°C.

Electrophoretic Mobility Shift Assays

Binding reactions were carried out in 20 μl containing 15,000 cpm of an end-labeled double-stranded 56-bp oligonucleotide fragment, 1.5 μg of poly (dI–dC), 4 μl of 5× binding buffer (4.7 mM EDTA, 4.25 mM DTT, 1 mM $ZnCl_2$, 400 mM KCl, 12.5% glycerol, 50 mM Tris) and 5 μg of nuclear extract. Reactions were incubated for 30 min at room temperature. The protein-DNA complexes were separated on low ionic strength 4% polyacrylamide gels and autoradiographed. The oligonucleotides of the Col1a1 promoter sequence from −84 to −29 bp (Col1/56) were 5′-CTGCTGGCTCCCCCTCTCCGAGAGGCAGGGTTCCTCCCAGCTCTCCATCAAGATGG-3′; and 5′-CCATCTTGATGGAGAGCTGGGAGGAACCCTGCCTCTCGGAGAGGGGGAGCCAGCAG-3′ (purchased from Integrated DNA Technologies, Inc., Coralville, IA) and were used to make the labeled probe and the unlabeled competitor.

Results

Regulation of Collagen Synthesis and PGE_2 Production in BALBc/3T3 Fibroblasts by IL-1 and Indomethacin

In previous studies using human dermal and synovial fibroblasts, we showed that IL-1 increased the synthesis of types I and III collagens and levels of α1(I), α2(I), and α(III) procollagen mRNAs, particularly when IL-1–stimulated biosynthesis was blocked by indomethacin (12). In the present studies, we used BALBc/3T3 fibroblasts as a model to further examine the mechanism of action of endogenously produced prostaglandins. SDS-PAGE analyses of [^{3}H]proline-labeled total medium proteins and pepsin-resistant collagen chains, which are secreted in the presence of β-APN, are shown in Figs. 1A and B, respectively. Untreated cells synthesized primarily type I collagen, which was visualized after reduction as unprocessed procollagen chains migrating between the reduced fibronectin monomer and the α1(I) collagen marker (Fig. 1A, lane 1). Digestion of the procollagens with pepsin under nondenaturing conditions revealed the predominance of α1(I) and α2(I) collagen chains at the expected ratio of approximately 2:1 (Fig. 1B, lane 1). The band at the top of the gel was identified as type III collagen after delayed reduction (not shown), which disrupts the interchain disulfide bonds. A small amount of type V collagen was also produced which was visualized as the α1(V) collagen chain migrating just above of the α1(I) collagen band.

Treatment with low concentrations of hIL-1α (0.01 and 0.05 nM) increased the synthesis of type I and type III collagens and FN both in the absence and presence of indomethacin (Figs. 1A and B). Note that the synthesis of types I and III collagens was slightly higher in the presence of indomethacin and the two lowest concentrations of hIL-1α. Treatment with 0.1 nM of IL-1 had less effect on synthesis of some of the procollagen and collagen chains. However, the highest concentrations of hIL-1α (0.5 and 1.0 nM) added alone decreased collagen and FN synthesis, while indomethacin completely or partially reversed the inhibition. These results suggested that downstream products of cyclooxygenase were at least partially responsible for the inhibitory effects of IL-1 on type I collagen synthesis in mouse fibroblasts and were consistent with our previous findings using synovial fibroblasts (12). Analysis of PGE_2 production by BALBc/3T3 cells showed that incubation with IL-1 at the concentrations used here produced a dose-dependent stimulation between 2.5- and 50-fold compared with ~1 ng/ml PGE_2 in control cultures. Indomethacin blocked this stimulation, but only partially at concentrations of IL-1 of 0.1 nM and above (data not shown).

Regulation of Col1a1 Promoter Expression by hIL-1α, Indomethacin, and PGE_2

To determine whether the molecular mechanisms underlying the effects of prostaglandins on type I collagen synthesis were mediated at the transcriptional level, we carried out transient transfection experiments with deletion constructs of the Col1a1 promoter in the CAT reporter gene vector. Preliminary work showed that a construct containing 3.5 kb of the Col1a1 promoter responded to hIL-1α in the presence but not in the absence of indomethacin with 3- to 4-fold increased activity compared with untreated or indomethacin-treated controls (not shown). When the promoter was further deleted to −976 bp, this promoter construct was found

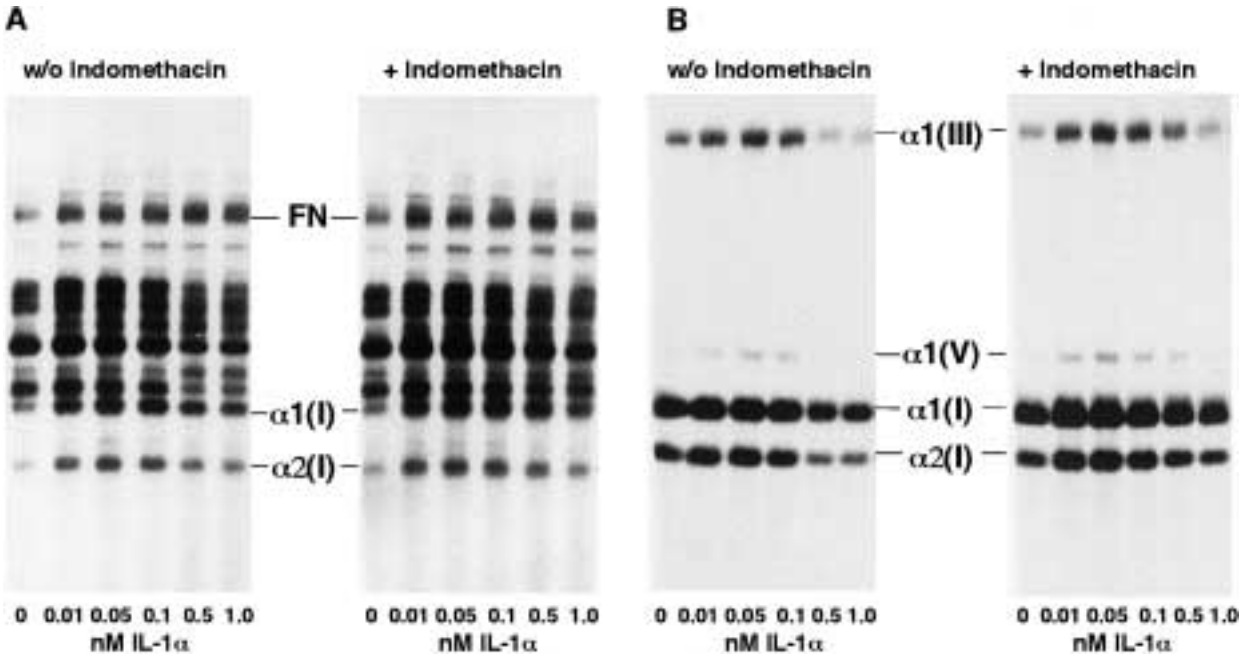

Fig. 1. Regulation of type I and type III collagen synthesis in BALBc/3T3 fibroblasts by hIL-1α and indomethacin. Cells were preincubated for 48 hr without (0) or with increasing concentrations of IL-1α between 0.01 and 1.0 nM in the absence (w/o) or presence (+) of indomethacin at 10^{-6}, as indicated. Proteins were then biosynthetically labeled with [^{3}H]proline in the presence of ascorbate and β-APN for 24 hr and electrophoresed on SDS-5% polyacrylamide gels (A) with reduction to separate procollagen chains and FN or (B) without reduction to separate pepsin-resistant collagen chains. Migration positions of collagen chains were identified using [^{14}C]-labeled rat tail tendon collagen as a standard on each gel.

to retain the DNA elements sufficient for responsiveness to both IL-1 and PGE_2. In the presence of indomethacin, but not in its absence, hIL-1α, at concentrations of up to 12.5 pM, stimulated expression of the −976 bp promoter in a dose-dependent manner with maximal effects at 5 pM in this representative experiment (Fig. 2). In other similar experiments, concentrations of IL-1 between 5 and 50 pM stimulated expression to a similar extent, while at higher concentrations the stimulatory effect declined or disappeared, thereby reflecting the changes observed in Fig. 1. A concentration of 10 pM was chosen for all subsequent experiments.

To determine whether treatment with PGE_2 could duplicate the effects of endogenously produced prostaglandins, we performed the transfection experiment shown in Fig. 3. The −976/+116 bp construct (lane 1) exhibited lower basal expression than the −222/+116 bp construct (lane 6) consistent with previous findings (33). Indomethacin consistently increased the expression of the −976/+116 bp construct; significant effects were not observed on the high levels of expression of the −222/+116 bp construct. The expression of both constructs was inhibited by incubation with hIL-1α alone (lanes 3 and 8) compared to indomethacin-treated controls (lanes 2 and 7). The variability in the response to IL-1-induced prostaglandins compared to untreated controls (compare results in Fig. 2 in which IL-1 alone stimulated expression) is probably due to differences in basal PGE concentrations at the beginning of the incubations and subsequent cell responsiveness. Incubation with hIL-1α in the presence of indomethacin reversed the IL-1-induced inhibition and unmasked the stimulatory effect of hIL-1α on the expression of the −976 bp construct (lane 4). However, there was no significant stimulatory effect of hIL-1α in the presence of indomethacin on the expression of the −222 bp construct (lane 9) compared with untreated or indomethacin-treated cells (lanes 6 and 7). In contrast, treatment with exogenous PGE_2 (250 nM) in the presence of hIL-1α and indomethacin inhibited the expression of both constructs (lanes 5 and 10). Furthermore, PGE_2 duplicated the effect of hIL-1α alone on the −976 bp construct (lane

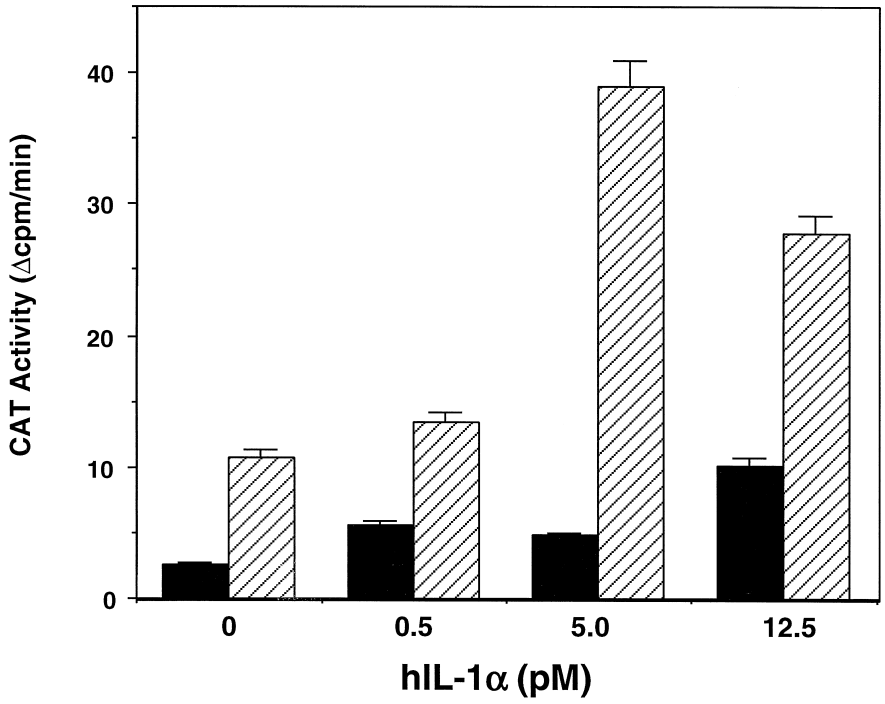

Fig. 2. Regulation of the −976 bp Col1a1 promoter-CAT construct by hIL-1α and indomethacin in BALBc/3T3 fibroblasts. BALBc/3T3 fibroblasts were transfected with the Col1a1-CAT construct containing the sequence spanning −976/+116 bp. The cells were then treated with increasing concentrations of hIL-1α, as indicated, in the absence (solid bars) or presence (cross-hatched bars) of 10^{-6} M indomethacin and CAT activity was determined after 48 hr by the fluor diffusion method. Values are means ± SEM of triplicate plates.

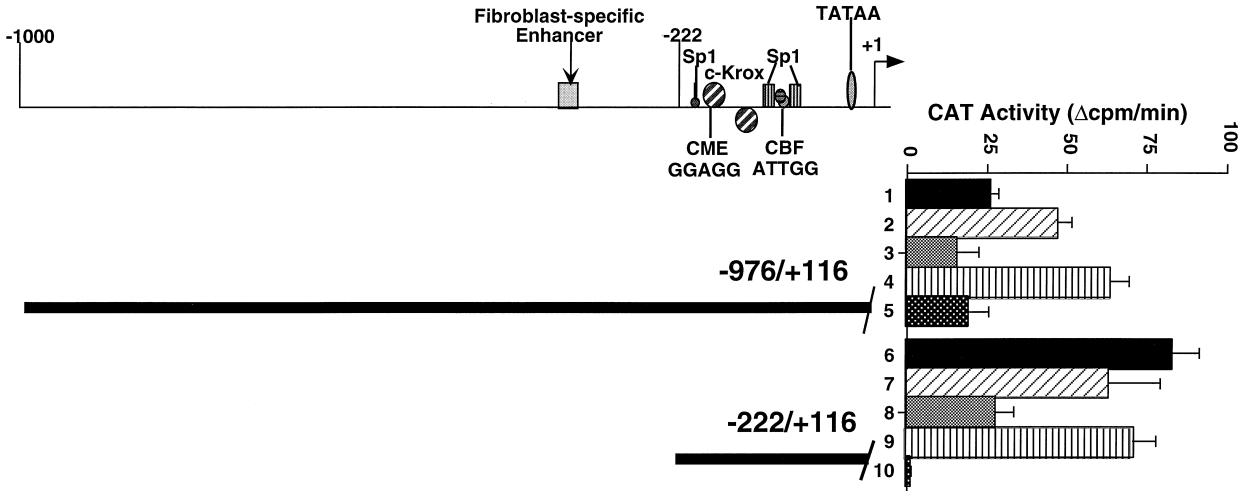

Fig. 3. Regulation of Col1a1 promoter-CAT constructs by hIL-1α, indomethacin, and PGE_2. BALBc/3T3 fibroblasts were transfected with CAT constructs containing the sequences spanning −976/+116 bp (lanes 1–5) or −222/+116 bp (lanes 6–10) of the Col1a1 promoter. CAT activity was determined after 48 hr of treatment without (lanes 1 and 6) or with 10^{-6} M indomethacin (lanes 2 and 7), 10 pM hIL-1α, (lanes 3 and 8), IL-1 plus indomethacin (lanes 4 and 9), or IL-1, indomethacin, and 250 nM PGE_2 (lanes 5 and 10). The values are means ± SEM from two experiments with duplicate plates. The scheme represents positions of DNA elements defined previously (33,40,67).

5), while producing much stronger inhibition on the −222 bp promoter (lane 10). These results strongly suggest that the PGE_2 response was retained within the −222 bp promoter fragment. Further work localized the PGE response to the proximal promoter region, because the already low expression of a −86 bp construct was strongly inhibited by hIL-1α-induced PGE_2 (data not shown).

PGE_2, $PGF_{2\alpha}$, PGE_1, and Misoprostol Inhibit Expression of the Col1a1 Proximal Promoter

To determine whether the inhibitory effect of PGE_2 on Col1a1 expression was unique and to explore whether other prostaglandins might have similar effects, we treated the BALBc/3T3 cells with PGE_2, $PGF_{2\alpha}$, PGE_1, and misoprostol at 25, 125, and 250 nM in the presence of indomethacin to inhibit endogenous prostaglandin production. As shown in Fig. 4A, all four prostaglandins inhibited expression of the −222 bp promoter construct both in the absence and presence of hIL-1α. PGE_2 and $PGF_{2\alpha}$, both products of cyclooxygenase conversion of arachidonic acid, were more potent inhibitors than PGE_1, a product of conversion of dihomogammalinolenic acid, or the stable PGE_1 analog misoprostol. $PGF_{2\alpha}$ produced maximal inhibition at 25 nM, the lowest concentration studied, while PGE_1 and misoprostol produced dose-dependent inhibition both in the absence and presence of hIL-1α. $PGF_{2\alpha}$, PGE_1, and misoprostol were equally effective in the absence and presence of hIL-1α, whereas PGE_2 was a more effective inhibitor in the absence than in the presence of the cytokine. Because PGE_1 and misoprostol were equipotent, we concluded that chemical instability of PGE_1 did not account for the less potent inhibition by this prostaglandin. However, the pattern of cAMP responses to the different prostaglandins was not the same as that observed in the transfection experiments, as shown in Fig. 4B. After a 20-min challenge with the different prostaglandins, PGE_1 produced the greatest increase in cAMP content, while PGE_2 and misoprostol stimulated moderate increases, and $PGF_{2\alpha}$ had no effect.

DNase I Footprinting Analysis of the −2222 bp Col1a1 Promoter

To localize the PGE-responsive region, DNase I footprinting was performed using the end-labeled −222/+116 bp probe and nuclear extracts from BALBc/3T3 fibroblasts incubated without or with the prostaglandins PGE_1, PGE_2, and $PGF_{2\alpha}$ at 250 nM in the presence of indomethacin to prevent endogenous prostaglandin biosynthesis (Fig. 5). Nuclear extracts from untreated cells (lane 3) showed a pattern of protection of this fragment by known constitutive transcription factors, including CBF, Sp-1, and c-Krox, similar to that reported previously (33,40). After treatment of the cells with PGE_1 (lane 4) and PGE_2 (lane 5), but not with $PGF_{2\alpha}$ (lane 6), a protected region appeared in the −84 bp to −29 bp promoter region between the TATA box and Sp1/CBF-binding region, which extended throughout the −222 bp region. Similar results were found using three different sets of nuclear extracts in several experiments in which DNase I concentrations, incubation times, and nuclear extract concentrations were varied.

Electrophoretic Mobility Shift Assays Analysis of the Proximal Col1a1 Promoter

To determine whether DNA sequences upstream of the TATA box in the Col1a1 promoter exhibited specific binding to nuclear protein factors extracted from BALBc/3T3 cells, we carried out electrophoretic mobility shift assays (EMSA) experiments. An end-labeled double-stranded oligonucleotide (col1/56) corresponding to the −84/−29 bp Col1a1 promoter region was incubated with the same nuclear extracts as those used in the DNase I footprinting experiments. The experiment presented in Fig. 6 showed the formation of several protein–DNA complexes that did not vary with treatment conditions. Using nuclear extracts from prostaglandin-treated cells, we observed 2- to 3-fold increases in the intensity of the major shifted protein–DNA complex in region A, with the appearance of two less intense bands (Fig. 6). The binding activities in region A were more intense in the presence of extracts from the PGE_2-treated than from the PGE_1- or $PGF_{2\alpha}$-treated cells. Furthermore, the PGE_2-treated extracts produced an additional band in the E region with lower molecular weight, or increased mobility, while $PGF_{2\alpha}$ increased the intensity of the D band. The intensities of the protein-DNA complex C and the lower D band were not notably changed. The A, B, and E complexes were competed by excess unlabeled col1/56 probe, but not by an unrelated probe of the same size, while bands C and D were nonspecific (data not shown). The formation of the complexes A, B, and E was found to be zinc dependent, because binding increased in a dose-dependent manner when the $ZnCl_2$ concen-

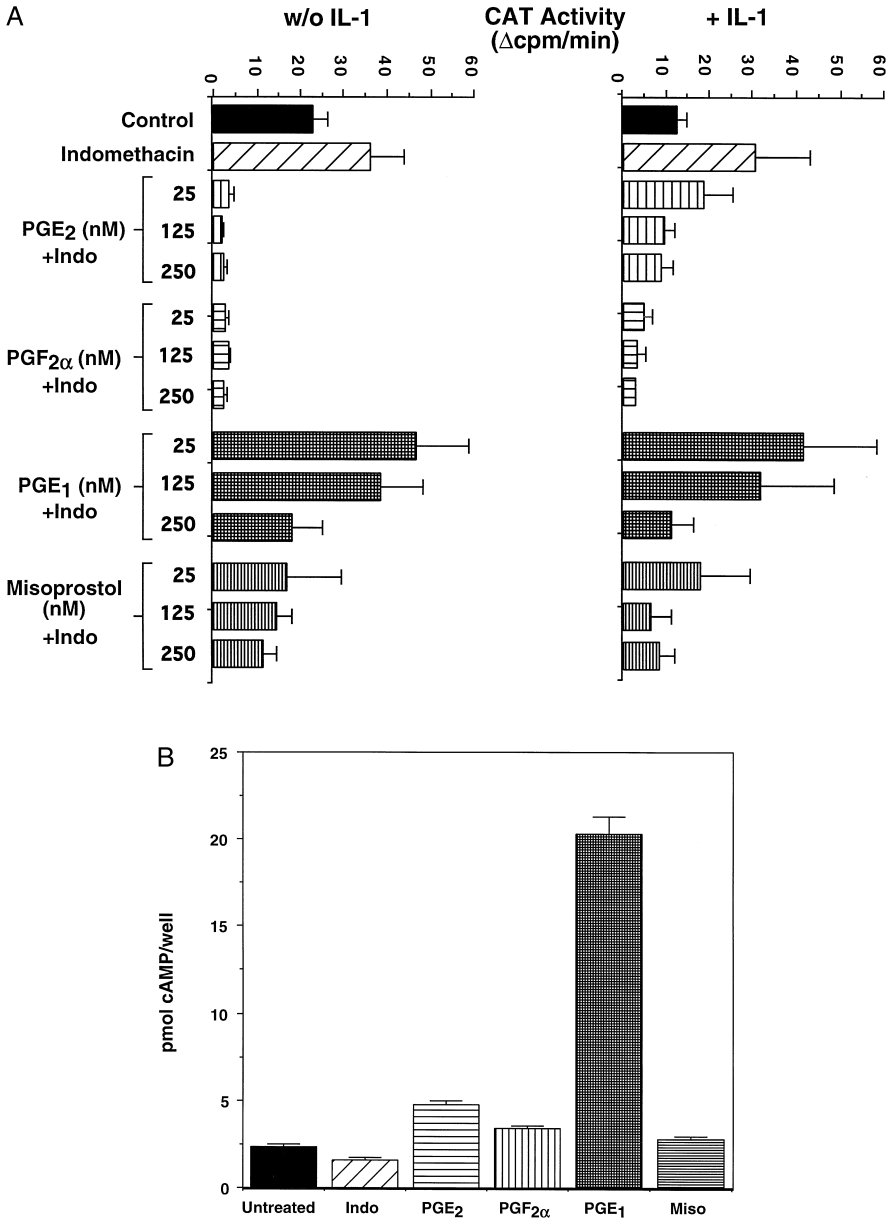

Fig. 4. Comparison of the effects of PGE_2, $PGF_{2\alpha}$, PGE_1, and misoprostol on expression of the Col1a1 proximal promoter and cAMP response. (A) BALBc/3T3 fibroblasts were transfected with the −222/+116 bp Col1a1-CAT construct and incubated in the absence (left panel) or presence (right panel) of hIL-1α (12.5 pM) with increasing concentrations (25, 125, and 250 nM) of PGE_2, $PGF_{2\alpha}$, PGE_1, and misoprostol, as indicated on the left. Indomethacin (Indo) was also added to all cultures, except to controls (solid bars), to inhibit endogenous prostaglandin synthesis. Values are means ± SEM from at least six independent transfections each performed in duplicate. (B) The cells were preincubated for 24 hr in the presence of indomethacin or left untreated (solid bar). The cell layers were then washed and challenged for 20 min with PGE_2, PGE_1, $PGF_{2\alpha}$, or misoprostol at 250 nM. cAMP content was measured in the cell lysates by radioimmunoassay. Values are means ± SEM of triplicate wells.

tration was varied between 50 and 400 μM. Furthermore, the zinc-dependent binding was ablated with increasing concentrations of EDTA (data not shown).

Discussion

In this study, we demonstrated that IL-1 is capable of inhibiting type I collagen synthesis by fibroblasts under the appropriate experimental

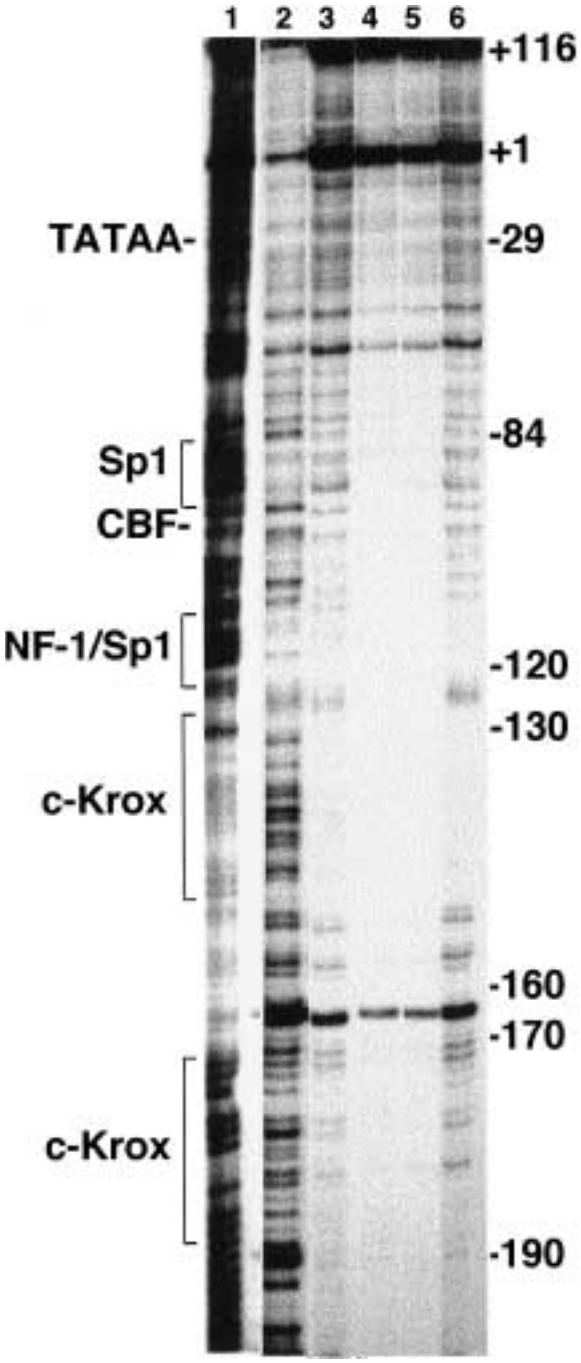

Fig. 5. DNAse I footprinting analysis of the Col1a1 promoter using nuclear extracts from prostaglandin-treated BALBc/3T3 fibroblasts. The EcoR1-HindIII fragment of plasmid pG60 was labeled at the EcoR1–5′ end. Lane 1 shows the G + A sequencing reaction of the unlabeled fragment. The end-labeled fragment was incubated without (lane 2, naked DNA) or with nuclear extracts from BALBc/3T3 cells cultured alone (lane 3) or with 250 nM PGE_1 (lane 4), PGE_2 (lane 5), or $PGF_{2\alpha}$ (lane 6), and treated with DNase I. The different areas of protection by known nuclear factors, as described previously (33,68), are marked by brackets. The numbers at the right correspond to base pairs relative to the start of transcription.

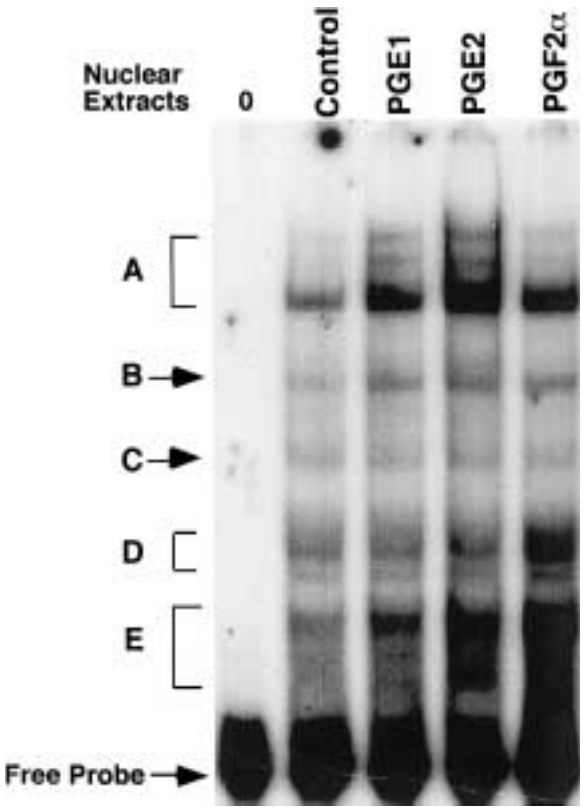

Fig. 6. EMSA analysis of protein-DNA complexes in nuclear extracts from prostaglandin-treated BALBc/3T3 fibroblasts. The double-stranded oligonucleotide probe (Col1/56) encompassing the sequence spanning −84 to −29 bp of the Col1a1 promoter was end-labeled with ^{32}P and incubated alone (first lane showing free probe only) or with 5 μg of nuclear extract from untreated (control) or PGE_1-, PGE_2-, or $PGF_{2\alpha}$-treated BALBc/3T3 cells. The protein-DNA complexes A–E were separated on a 4% polyacrylamide gel. The complexes A, B, and E were competed by excess unlabeled probe titrated at 10×, 50×, 100×, and 200×, and complexes C and D were nonspecific (not shown).

conditions and that this effect is mediated at the transcriptional level by endogenously produced prostaglandins. Using BALBc/3T3 fibroblasts, we found that the inhibition of collagen synthesis by IL-1 was ascribable to the increased ambient level of prostaglandins, primarily PGE_2, induced by this cytokine. Indeed, the exposure of the cells to the cyclooxygenase inhibitor indomethacin prevented endogenous prostaglandin biosynthesis and allowed the cells to maintain type I collagen synthesis at the basal level or revealed a direct stimulatory effect of IL-1. Furthermore, exogenously added prostaglandins inhibited the expression of the −222 bp Col1a1 promoter in a dose-related

manner via DNA elements within the proximal promoter region.

In previous studies, we found that human synovial fibroblasts, but not dermal fibroblasts, were particularly responsive to IL-1 with respect to stimulation of prostaglandin biosynthesis, and that they were more sensitive to PGE_2 (12). PGE_2 production stimulated by IL-1 in synovial fibroblasts was 20- to 50-fold greater than in dermal fibroblasts. Furthermore, inhibition of type I collagen synthesis by endogenously produced IL-1-stimulated PGE_2 was not observed in the dermal fibroblasts, although these levels were inhibitory in the synovial fibroblasts. In the present studies, we used the mouse BALBc/3T3 fibroblasts as a model for studying regulation of the mouse Col1a1 promoter because they were responsive to both IL-1 and PGE_2, unlike the NIH/3T3 fibroblasts, which we had tested in preliminary studies (unpublished).

Our present findings indicate that prostaglandins influence collagen gene expression directly and act as autocrine factors in modulating the effects of IL-1. Previous studies suggested that the regulation of type I collagen gene expression by IL-1-induced prostaglandins may occur, at least partially, at the transcriptional level (22,41, 42). Our transient transfection experiments using the Col1a1 promoter confirmed that theory and demonstrated dose-dependent inhibition by exogenously added PGE_2. PGE_1 and misoprostol, the stable PGE_1 analog, produced dose-dependent inhibition that was less potent than the effect of PGE_2. Similar to the findings of Fall et al. (31) in an osteoblastic cell line, $PGF_{2\alpha}$ was the most potent of the prostaglandins tested in the transfection assays. However, using DNase I footprinting, we found that only the E-series prostaglandins affected binding of nuclear proteins to the Col1a1 promoter, while in EMSAs the pattern of binding in response to $PGF_{2\alpha}$ was different from that induced by E-series prostaglandins.

Distinct patterns of responses to different prostaglandins have been found in different cell types. Generally, PGE_2 and PGE_1 are more potent stimulators of cAMP content than $PGF_{2\alpha}$, and $PGF_{2\alpha}$ is a more potent inhibitor of collagen synthesis (30,31,43,44). Differential sensitivity to prostaglandins may also be explained by differences in duration of exposure in previously reported studies. In previous studies in synovial fibroblasts and chondrocytes, exposure to IL-1 in the presence of indomethacin resulted in increased sensitivity to the effects of exogenous PGE_2, manifested by an increase in the magnitude of adenylate cyclase responses (43,45). Because up-regulation of type I collagen gene expression by IL-1 could be observed in the absence of indomethacin during incubations of several days or weeks, we proposed that long-term exposure to PGE_2 could desensitize responsive cells (18,25). In the present studies, we examined responses after relatively short incubation times of up to 46 hr. The biphasic effects of IL-1 on collagen synthesis, stimulation at low concentrations, and prostaglandin-dependent and -independent inhibition at high concentrations observed here are consistent with previous reports in bone cells (46) and could explain discrepancies in previous studies. While the E-series prostaglandins directly inhibited Col1a1 expression over the same range of doses, the magnitude of the response to PGE_2 was less potent if IL-1β was present even with indomethacin added at the same time. This result suggests that a small amount of IL-1-induced PGE_2 secreted initially may have prevented the full response, because PGE_2, with lower levels of $PGF_{2\alpha}$, is the major cyclooxygenase product of cultured fibroblasts (47). Indomethacin was not included in the preincubation medium, and previous studies have shown that even low basal levels of PGE_2 may be sufficient to desensitize responsive cells (12,32). Furthermore, PGE_2 biosynthesis is the combined result of constitutive COX-1 and inducible COX-2 activities and inhibition by indomethacin may be incomplete in some circumstances (48). Taken together, these findings have important implications with regard to nonsteroidal anti-inflammatory (NSAID) therapy, because withdrawal from cyclooxygenase inhibition may result in subsequent increased sensitivity to reexpressed prostaglandins.

Prostaglandins exert their actions on various cell types via specific cell-surface receptors, including EP_{1-4}, which are more responsive to E-series prostaglandins, and FP, which responds more strongly to $PGF_{2\alpha}$ than to PGE_2 (49,50). They all have seven transmembrane domains and are coupled to different G-proteins that activate different secondary messenger systems such as cAMP, Ca^{+2}, and phospholipase C. EP_2 and EP_4, the two receptors that use the cAMP signal transduction system via adenylate cyclase, appear to be the most important receptors for transducing the anabolic effects of PGE_2 on bone cells (51–53). Our results showing that

the order of potency of the cAMP response differed from that of the collagen inhibitory effects are consistent with the findings of Fall et al. (31). They suggested that a cAMP-independent pathway regulated by protein kinase C was involved, because forskolin and other cAMP inducers do not mimic the inhibitory effects of prostaglandins on collagen synthesis and Col1a1 promoter activity. Furthermore, FP receptor agonists produced the most potent inhibitory effects. However, the upstream promoter was required to observe osteoblast-specific expression and the DNA elements and nuclear factors involved were not identified (31).

Because the cAMP-responsive DNA binding protein CREB may be phosphorylated by either protein kinase A or Ca^{+2}-dependent kinases (54,55), it could be involved in signaling by any of the different EP and FP receptor subtypes. However, the Col1a1 proximal promoter does not contain a consensus sequence for binding CREB. Di Battista et al. (56) showed that Ca^{+2}, but not cAMP, was involved in mediating the stimulatory effects of PGE_2 on levels of insulin-like growth factor-I (IGF-I) and type II collagen mRNAs. Previous studies by Thomas et al. (57) and Umayahara et al. (58) demonstrated that the anabolic effects of PGE_2 in bone cells were due to stimulation of IGF-I transcription in osteoblasts via a novel cAMP-responsive element that bound C/EBPδ. However, the time course of inhibition in our experiments was not consistent with the involvement of a secreted intermediate such as IGF-I. Furthermore, the proximity of the putative PGE-responsive region to the TATA box suggested an indirect mechanism in which PGE-induced factors could interact with bound constitutive factors through protein–protein rather than protein–DNA binding.

We cannot yet explain the discrepancy that no change was observed in the DNase I footprint using nuclear extracts from $PGF_{2\alpha}$-treated cells, although $PGF_{2\alpha}$ was the most potent inhibitor of transient expression of the Col1a1 minimal promoter in transfection experiments and increased binding in EMSA assays. In some systems, $PGF_{2\alpha}$ does not stimulate cAMP and this prostaglandin is thought to increase intracellular [Ca^{+2}] through coupling of the FP receptor to a phospholipase C pool distinct from that coupled to EP receptors (59). It is possible, therefore, that the effects of $PGF_{2\alpha}$ are mediated through signaling pathways that affect different DNA binding activities than the E-series prostaglandins.

A search for potential *cis*-acting elements within the −84/−29 bp Col1a1 region was inconclusive. We found numerous GA-binding sites within the region as well as some potential TR-boxes and pyrimidine sandwich elements (PSEs, which may be involved in determining the DNA conformation and helical twist (60). These sequences could affect DNA–protein interactions and DNA loop formation, and thus affect transcriptional initiation. In the human α2(I) collagen gene (COL1A2), a sequence adjacent to the CCAAT box, termed collagen modulating element (CME), was reported to bind a novel DNA-protein complex (complex III) and to act cooperatively with the G/CBE binding protein (61). This study suggested the crucial importance of GGAGG sequences flanking the human α2(I) CCAAT box in promoter activity. The presence of a GGAGG element on the antisense strand of the Col1a1 −84 bp/−29 bp region suggests a function in DNA conformation via loop formation.

The regulation of collagen gene promoters by TGF-β has been studied extensively. However, the precise cytokine-responsive elements in collagen genes have been more difficult to localize, since consensus sequences have been defined in terms of immune responses in T or B lymphocytes or monocyte/macrophages. AP-1 and NF-κB have been shown to be involved in inhibition of type I collagen genes by TNF-α through regulatory regions upstream of its minimal promoter, which overlap those mediating stimulatory effects of TGF-β (62–65). However, involvement of cytokine-induced prostaglandins has been addressed infrequently. Mori et al. (66) reported that TNF-α inhibition of COL1A1 gene expression was partially prostaglandin dependent and was mediated via binding of unidentified nuclear factors to two elements, which they termed TNF-RE, immediately upstream of the TATA box. TNF-α and IL-1 share many effects on fibroblasts, including stimulation of synthesis of PGE_2, matrix metalloproteinases, and IL-6, where transcriptional mechanisms involving NF-κB, AP1, and ETS factors have been implicated. Although 50- to 100-fold higher concentrations of TNF-α are required to produce the same magnitude of stimulation as IL-1, these two cytokines can produce synergistic responses at submaximal concentrations (4) and coincubation with indomethacin may not completely inhibit a high level of prostaglandin biosynthesis.

Our results show that the proximal promoter region (−84 bp/−29 bp) has a surprisingly high capacity to bind nuclear factors, particularly in PGE_2-treated cells. In EMSA assays, the binding activities of at least three zinc-dependent complexes were enhanced after PGE_2 treatment of BALBc/3T3 cells. However, because this fragment did not contain sites for binding constitutive factors such as CBF/p300 or the TATA box, the DNase I footprinting may more accurately reflect the response in vivo. Discrete binding sites protected by nuclear proteins were not observed, but the generalized fading of the footprint upstream of the TATA box suggests the close interaction of PGE-modulated factors with the RNA polymerase machinery. In further work, we have identified a zinc-dependent factor that binds to a site proximal to the TATA box, which may be involved in modulation of Col1a1 expression by prostaglandins (Riquet et al., manuscript in preparation). Another zinc-dependent factor, c-Krox, has also been shown to bind weakly to this fragment, but to a site further upstream (40).

In conclusion, we have shown that the inhibition of Col1a1 gene expression by IL-1 in fibroblasts is mediated by prostaglandins and occurs at the transcriptional level. Our results further suggest that PGE-responsive factors may interact directly or indirectly with basal regulatory elements in the proximal promoter region of the Col1a1 gene. Elucidation of the specific signals mediating the different actions of prostaglandins may permit targeted therapies that could dissociate the antifibrotic from the inflammatory and anabolic actions of PGE_2.

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