An AP-1 Binding Sequence Is Essential for Regulation of the Human \(\alpha2(I)\) Collagen (COL1A2) Promoter Activity by Transforming Growth Factor-\(\beta\)*

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Kee-Yang Chungt, Akhilesh Agarwalt, Jouni Uittoš, and Alain Mauvielt

From the Departments of Dermatology and Cutaneous Biology and Biochemistry and Molecular Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Previous studies have shown that transforming growth factor-\(\beta\) (TGF-\(\beta\)) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) modulate type I collagen gene expression in fibroblasts. To fine-map the corresponding response elements in the human \(\alpha2(I)\) collagen (COL1A2) promoter, we have generated a series of 5' deletion promoter/chloramphenicol acetyltransferase (CAT) reporter gene constructs. Transient cell transfection assays using human dermal fibroblasts and stable transfection experiments using NIH 3T3 fibroblasts identified the region located between residues 265 and 241, as critical for TGF-\(\beta\) response. Specifically, we demonstrate that this 25-base pair region mediates the up-regulatory effect of TGF-\(\beta\) on COL1A2 promoter activity and allows antagonistic activity of TNF-\(\alpha\) on the TGF-\(\beta\) effect. Gel mobility shift assays indicate that nuclear factor binding to this 25-base pair region of COL1A2 promoter is competed by AP-1, but not NF-1 or NF-\(\kappa\)B, oligonucleotides. Transient cell transfection experiments with plasmid constructs in which the potential AP-1-binding site located within this short region of promoter was modified by site-directed mutagenesis indicated that this element plays a significant role in the basal activity of the promoter. Furthermore, this sequence is essential for TGF-\(\beta\) response and does not require the presence of the three Sp-1-binding sites located further upstream, between nucleotides 273 and 304. In addition, overexpression of c-jun in co-transfection experiments with COL1A2 promoter/CAT constructs blocks the TGF-\(\beta\) response, further implicating AP-1 in the regulation of COL1A2 gene expression. Our results clarify the molecular mechanisms involved in the regulation of type I collagen gene expression and further emphasize the importance of AP-1 in mediating some of the TGF-\(\beta\) effects on gene transcription.

Recently, significant progress has been made in understanding the expression of the human \(\alpha2(I)\) collagen (COL1A2) gene and its transcriptional regulation by cytokines and growth factors. In particular, it has been shown that a GC-rich region located between residues 303 and 271, containing Sp-1-binding sites, is important for high basal promoter activity (Tamaki et al., 1995). This region is comprised within a larger segment of the COL1A2 promoter which has been shown to confer both TGF-\(\beta\) and TNF-\(\alpha\) responsiveness (Inagaki et al., 1994). However, despite extensive analyses, these studies did not allow precise characterization of TGF-\(\beta\) or TNF-\(\alpha\)-response element(s) within the COL1A2 promoter. It was suggested that the TGF-\(\beta\)-responsive element (TbRE) is located within a 313-bp region, between nucleotides 378 and 255, and consists of at least two cis-elements which act in a concerted manner to mediate the effect of TGF-\(\beta\). Once inserted upstream of the thymidine kinase promoter, the TbRE confers TGF-\(\beta\) inducibility to this heterologous promoter. Two protein binding sequences within the TbRE, box 3A between residues 313 and 286 which contains Sp-1 binding sites, and box B between residues 271 and 255, were shown to interact to confer both nuclear protein binding and promoter inducibility, otherwise not observed with either box alone. In addition, these authors suggested that TNF-\(\alpha\) inhibitory effect requires the contribution of both the Sp-1 binding sequence of the TbRE and an inhibitory element, box 5A, immediately upstream of the TbRE, but excluded, using an antibody interference experiment, participation of both AP-1 and NF-\(\kappa\)B transcription factors in this phenomenon (Inagaki et al., 1995).

To characterize the TGF-\(\beta\) and TNF-\(\alpha\) response elements within the human COL1A2 promoter in further detail, our experimental approach consisted of (a) development of a repertoire of 5' deletion constructs of the COL1A2 promoter and (b) site-directed mutagenesis of specific sequences characterized as essential for growth factor response. This approach allowed us to map the growth factor response elements, in the presence of homologous downstream sequences, reaching the position +58 of the COL1A2 gene. Specifically, we have narrowed the TGF-\(\beta\)-response element(s) to a 25-bp segment of the promoter, located between residues 265 and 241. In addition, we show that this fragment is sufficient to allow inhibition of the promoter activity by TNF-\(\alpha\). Furthermore, using site-directed mutagenesis, we have established that the potential AP-1-binding site, CGAGTCA, located within this short region of promoter, is essential for TGF-\(\beta\) response.

MATERIALS AND METHODS

Cell Cultures—Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskins, were utilized in passages 3–8. The cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 50 \(\mu\)g/ml of streptomycin.

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†To whom correspondence should be addressed: Dept. of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th St., BLSB Rm. 430, Philadelphia, PA 19107. Tel.: 215-955-5775; Fax: 215-955-5788.

‡The abbreviations used are: TGF-\(\beta\), transforming growth factor-\(\beta\); TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); TbRE, TGF-\(\beta\)-responsive element; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; bp, base pair(s).
Cytokines/Growth Factors—Human recombinant TGF-β2, was a generous gift from Dr. David R. Olsen, Celtrix Laboratories, Santa Clara, CA. Human recombinant TNF-α was purchased from Boehringer Mannheim.

Plasmid Constructs—To study the transcriptional regulation of COL1A2 gene expression, transient transfection experiments were performed with various deletion constructs of the COL1A2 promoter linked to the CAT reporter gene in the expression vector p8-CAT, a derivative of pEMBl plasmid (Boast et al., 1990). The promoter fragments were generated by polymerase chain reaction (PCR) and gel-purified. Smal fragments were similarly digested by expression vector. All deletion constructs were sequenced by automated sequencing (ABI) to verify the accuracy of the PCR. pRSV-c-jun (Chiu et al., 1989) was used to overexpress c-jun in co-transfection experiments with COL1A2 promoter/CAT constructs. Empty pRSVcVe was used as a control.

Site-directed Mutagenesis of the Putative AP-1-binding Site—Two point mutations were introduced into the putative AP-1-binding site using asymmetric PCR and a single mutant primer (Perrin and Gilliland, 1990). Specifically, the first PCR was prepared using a mutagenic primer containing two point mutations (bold) in the putative AP-1-binding site (CGAGCTCA → CCAGTGA) and a flanking primer closer to the transcription initiation site. The PCR product was gel-purified on a 2% agarose gel and used as a template for the second PCR to generate another flanking primer in the opposite direction and the −342/CAT and −265/CAT deletion constructs as templates. The promoter sequences were excised from the −342/CAT and −265/CAT constructs by digestion with BamHI and Xmal restriction enzymes and the final PCR products, digested with the same restriction enzymes, were ligated together, generating −342/CAT and −265/CAT constructs containing a mutated AP-1-binding site. Fidelity of the new plasmid constructs was checked by automated sequencing as described above.

Transient Transfections and CAT Assays—Transient transfections of human foreskin fibroblasts were performed by the calcium phosphate/DNA co-precipitation method, as described previously (Mauviel et al., 1990). Cells were transfected with 10 or 20 μg of DNA mixed with 5 μg of the pRSV-β-galactosidase plasmid DNA in order to monitor transfection efficiencies. After glycerol shock, the cells were plated in DMEM containing 1% fetal calf serum 4 h prior to the addition of growth factors and cytokines. In experiments without growth factors and cytokines, the cells were plated in DMEM containing 10% fetal calf serum. After an additional 40 h of incubation, the cells were rinsed twice with phosphate-buffered saline, harvested by scraping, and harvested in reporter lysis buffer (Promega, Madison, WI). The β-galactosidase activities were measured according to standard protocols (Sambrook et al., 1989). Aliquots corresponding to identical β-galactosidase activity were used for each CAT assay with [35S]chloramphenicol as substrate (Garman et al., 1982) using thin layer chromatography. Following autoradiography, bands were cut and quantified by liquid scintillation to quantify the acetylated [35S]chloramphenicol.

Stable Transfections—To investigate the expression of promoter constructs with low activity, NIH 3T3 fibroblast cultures were stably transfected with various deletion constructs of the COL1A2 promoter (−342, −285, −265, and −241/CAT). For this purpose, each construct was co-transfected with pRC/CMV (InVitrogen, Portland, OR) in a 10:1 ratio by the calcium phosphate/DNA co-precipitation method. Four days after transfection, Geneticin® (Life Technologies, Inc.), 0.8 mg/ml, was added to the culture medium to allow selection of transfected cells. Medium was changed every other day and fresh Geneticin® (0.8 mg/ml) was added. After 18 days, all colonies (−100–150/construct) were picked in order to eliminate any potential influence of the integration site of the constructs within the cell genome on the promoter activity. The stably transfected cultures generated were maintained in DMEM containing 10% newborn calf serum and 0.4 mg/ml of Geneticin® and utilized at confluence to study the regulation of the COL1A2 promoter. Four hours prior to addition of cytokines and growth factors, the cultures were washed with phosphate-buffered saline and incubated in medium containing 1% newborn calf serum. Forty hours later, cells were lysed in lysis buffer (see above). The protein concentration of each extract was determined with a commercial assay kit (Bio-Rad), and CAT activity was measured as described above, using identical amounts of protein in each sample (50 μg).

Gal Mobility Shift Assays—Nuclear extracts were prepared according to the method of Andrews and Fallar (1991). For gel mobility shift assays, a 37-bp double-stranded DNA oligomer corresponding to the region −271 to −235 of the human COL1A2 promoter, overlapping the element(s) between residues −265 and −241 responsible for TGF-β response (see “Results”), was generated: 5′-GAGGATTCGACCACAAGTGCAGGTACCCCGGGCATG-3′. The end-labeled oligomer (7 × 10^6 cpm) was incubated with 10 μg of protein extracts for 30 min on ice in 20 μl of binding reaction buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 1 mM EDTA, 12% glycerol), in the presence of 2 μg of poly(dI-dC), as described previously (Dignam et al., 1983). For competition experiments, 20–60 fold molar excess of DNA was added to the binding reaction. Details of the competition assays are provided in the legend to Fig. 3 and the corresponding text under “Results.” DNA-protein complexes were separated from unbound oligomers on 4 or 6% polyacrylamide gels in 0.4 × TBE. The gels were fixed for 3 × 10 min in 30% methanol, 10% acetic acid, vacuum-dried, and exposed to x-ray films at −70°C. In some experiments, nuclear extracts were preincubated with antisera against c-jun (Santa Cruz Biotecnology, Santa Cruz, CA) or j-un-B (a kind gift from Dr. R. Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.).

RESULTS AND DISCUSSION

Deletion Analysis of the Human COL1A2 Promoter—We have constructed by a PCR-based methodology a large battery of 5’ deletions of the human COL1A2 promoter linked to the CAT gene. A schematic diagram of the promoter is shown in Fig. 1A, indicating the 5’ end position of each of the CAT constructs, relative to the transcription start site +1. The relative activity of the different constructs in transiently transfected human neonatal fibroblast cultures was compared with that of the longest one, the 3.5 kilobases promoter construct, set arbitrarily at 100. Deletion from position −3500 to −772 led to a dramatic reduction of promoter activity, which was partially recovered with further deletion to position −376 (Fig. 1B). These data are in agreement with the data published by Boast et al. (1990). Examination of the region comprised between residues −376 and −108 indicated the presence of several cis-elements potentially important for regulation of the basal expression of the COL1A2 promoter. Specifically, three potential Sp-1-binding sites, one potential AP-1 and one potential NF-κB-binding site were noted (Fig. 1A). Deletion of promoter sequences between residues −376 and −287 led to a ~50% reduction of promoter activity, whereas further deletion to position −265 did not reduce the activity further, as compared with the −287 fragment (Fig. 1B). Thus, our data indicate that the two upstream Sp-1 sites between nucleotides −432 and −287 play an important role in providing high basal activity to the promoter constructs, while the Sp-1 site further downstream (between residues −287 and −265) has little, if any, effect. These data contrast recent observations by Tamaki et al. (1995) who showed that deletion of all three Sp-1-binding sites located within this region of promoter is necessary to significantly alter the expression of the promoter. The reasons for this discordance are unknown, but both studies emphasize the importance of Sp-1 sites in maintaining high activity of the COL1A2 promoter.

Additional deletions to position −241 and to position −161, which removed two potential regulatory elements, AP-1 and NF-κB-binding sites (see Fig. 1A), did not lead to further reduction in promoter activity (Fig. 1B), suggesting that the elements important for the basal expression of the COL1A2 promoter are mostly the Sp-1 sites described above and the sequences upstream of nucleotide −772. Further deletion to position −108 reduced the promoter activity to levels about 2–5% of the 3.5-kilobase promoter construct, suggesting that sequences between −161 and −108 are a prerequisite for the expression of COL1A2 at a significant level.

Definition of TGF-β-responsive Elements within the Human COL1A2 Promoter—The TGF-β responsiveness of the different deletion constructs described above was investigated. The stimulatory effect of TGF-β (5–10-fold) was observed with every construct containing at least 265 bp of COL1A2 promoter.
Together with the analysis of the promoter sequences present upstream of nucleotide –265, these data suggest that the Sp-1-binding sites located between nucleotides –330 and –265 (see Fig. 2A), important for basal expression of the promoter (Tamaki et al., 1995; our results above), are not required for TGF-β response. Further deletion of 5’ sequences to position –241 of the promoter led to complete loss of TGF-β responsiveness, suggesting that element(s) essential for TGF-β response are located within a relatively short stretch of DNA, the 25-bp segment between positions –265 and –241 of the COL1A2 promoter.
promoter. To verify the TGF-β responsiveness in a stable expression system, NIH 3T3 fibroblast cultures stably transfected with constructs −342, −287, −265, and −241/CAT were generated. Incubation of these transfectants with TGF-β confirmed the data obtained in transient transfections of human dermal fibroblasts, indicating that the segment of promoter located between residues −265 and −241 is indeed essential for TGF-β response. Specifically, all stably transfected constructs, except −241/CAT, responded to TGF-β by a 3–5-fold elevation of promoter activity (not shown). These data contrast those by Inagaki et al. (1994) which suggested that sequences extended to span nucleotides −330 to −286 had to be present, together with the sequences located between residues −271 and −255, to confer TGF-β responsiveness to a heterologous promoter, the thymidine kinase promoter, normally unresponsive to TGF-β.

The experimental approach taken by these authors consisted in analyzing the region of the COL1A2 promoter located between residues −378 and −183 by DNase footprinting. Two distinct areas protected from nuclease digestion were characterized, one between nucleotides −271 and −255, the other between nucleotides −330 and −286. Deletion of either one of the protected fragments led to significant reduction in the promoter activity, suggesting that these two regions of COL1A2 promoter are necessary for TGF-β effect. In our experiments, upstream Spi-1 sequences were found to be fundamental for high expression of the promoter, as demonstrated by Tamaki et al. (1995), but are not required for TGF-β response. Further evidence for the lack of involvement of Spi-1 in TGF-β response is provided below (see Figs. 3 and 5 and the corresponding text).

Additional 5′ deletion to position −161 of the promoter restored some of TGF-β responsiveness (−2.5-fold stimulation) (Fig. 28). This elevation of promoter activity was lost when the 5′ end of our construct was decreased to position −108 of the promoter. Since the activity of the shortest construct was extremely low (see Fig. 1 and related text under “Results”), we established stably transfected NIH 3T3 fibroblast cultures with the −108/CAT construct. No significant effect of TGF-β was detected on the promoter activity using this experimental approach (not shown). It appears, therefore, that an essential TGF-β-responsive element is located between residues −265 and −241 of the COL1A2 promoter, although additional, yet somewhat secondary, sequences allowing some TGF-β responsiveness may exist downstream from nucleotide −161.

Delineation of TNF-α-responsive Elements within the Human COL1A2 Promoter—Using the same set of 5′ deletion constructs of the human COL1A2 promoter described above, we analyzed the potential regions of the promoter responsible for transcriptional inhibition by TNF-α. TNF-α had a strong inhibitory effect on the constructs containing at least 265 bp of COL1A2 promoter sequences (between 45 and 74% inhibition as compared with the basal activity of each individual CAT construct, Fig. 2C). Further deletion of the 5′ end of the promoter to either position −241 not only abolished the inhibitory effect but actually reversed the response to TNF-α (Fig. 2C). The extent of stimulation of the activity of the −241/CAT construct by TNF-α reached ∼3-fold above control. Similar results were obtained when using NIH 3T3 fibroblast cultures stably transfected with the various COL1A2 promoter/CAT constructs were examined, excluding any misleading result due to the transient transfections (not shown).

Sequences within the Proximal 265-bp Segment of COL1A2 Promoter Are Sufficient to Mediate Antagonism between TGF-β and TNF-α—Since our data indicate that the region located between nucleotides −265 and −241 is essential to mediate both TGF-β up-regulation and TNF-α down-regulation of COL1A2 promoter activity, we proceeded to fine-map the region of the promoter allowing the antagonist effect of these two cytokines. For this purpose, fibroblast cultures were transiently transfected with various deletion constructs and incubated with TGF-β, in the absence or presence of TNF-α. The results, presented in Table 1, clearly establish that 265 bp of promoter sequences are sufficient to mediate the antagonism between TGF-β and TNF-α. Specifically, when TNF-α was added concomitantly with TGF-β, the promoter activity was reduced to 68–17% of the activity observed in the presence of...
TABLE I
Interaction of TNF-α with TGF-β on the activity of various COL1A2 collagen promoter/CAT reporter gene constructs in transient cell transfected experiments

| Promoter construct (5' end) | Control | TGF-β | TGF-β + TNF-α |
|-----------------------------|---------|-------|---------------|
| −3500                       | Exp. 1  | 46.8 ± 15.6 | 94.0 ± 1.0 |
|                             |         | (100)       | (100)        |
| −772                        | Exp. 1  | 1.15 ± 0.1  | 3.5 ± 0.2    |
|                             |         | (100)       | (100)        |
| −376                        | Exp. 1  | 21.6 ± 7.5  | 94.5 ± 0.2   |
|                             |         | (100)       | (100)        |
| −342                        | Exp. 1  | 1.2 ± 0.1   | 19.3 ± 3.4   |
|                             |         | (100)       | (100)        |
| −287                        | Exp. 1  | 1.4 ± 0.3   | 6.5 ± 0.6    |
|                             |         | (100)       | (100)        |
| −265                        | Exp. 1  | 1.1 ± 0.3   | 13.1 ± 1.2   |
|                             |         | (100)       | (100)        |
| −241                        | Exp. 1  | 3.6 ± 2.0   | 5.6 ± 0.1    |
|                             |         | (100)       | (100)        |

TGF-β alone. When −241/CAT was used in transient transfection experiments, the promoter activity was not enhanced by TGF-β but TNF-α significantly elevated its activity, i.e., 1.7- to 5-fold above the promoter activity noticed in the presence of TGF-β alone.

AP-1 Binds to the −265/−241 Region of the COL1A2 Promoter—To determine whether the region of the COL1A2 promoter located between nucleotides −265 and −241 was a site for binding of transcription factors, we performed gel mobility shift assays first with a radiolabeled 37-bp oligonucleotide containing the sequence of the −271/−235 region of the human COL1A2 promoter. Our results indicate that this region binds nuclear proteins isolated from both control and TGF-β-treated fibroblasts (Fig. 3A, lanes 2 and 3). As predicted, the specific binding activity of nuclear extracts from control confluent fibroblast cultures (Fig. 3A, lane 2) could be competed in a dose-dependent manner by addition of 20- and 60-fold excess of homologous DNA (Fig. 3A, lanes 4 and 5). In addition, a 15-bp oligonucleotide containing the potential AP-1-binding site identified at position −250 (underlined), 5′-AACGAGTCA-GAGTTT-3′, also competed with the binding when added in a 20-fold molar excess to the reaction mixture (Fig. 3A, lane 6). Similarly, a 23-bp oligonucleotide containing the collagenase AP-1-binding site (underlined), 5′-CTAGTGAGTACGGGATC-3′, competed with the binding when added in a 20-fold molar excess to the reaction mixture (Fig. 3A, lane 7). To investigate further the specificity of the protein binding to this DNA sequence, additional competition experiments were performed, utilizing unlabeled oligonucleotides containing consensus sequences for either NF-1 or NF-κB (Fig. 3B). The binding activity of control nuclear extracts (Fig. 3B, lane 2) was competed by the addition of cold homologous competitor (Fig. 3B, lanes 4 and 5) but was not altered by the addition of either NF-1 or NF-κB oligonucleotides (Fig. 3B, lanes 6, 7, and 8, 9, respectively). Collectively, these data indicate that AP-1 binds to the segment of COL1A2 promoter 5′-CGAGTCA-3′ within the TGF-β-responsive region identified above.

It should be noted that the binding activity, as determined by the intensity of the DNA/protein band, of extracts from TGF-β-treated cells was not significantly altered as compared with that of control extracts. This quantitative similarity may, however, be masking changes in both the content and the transcriptional activity of the bound complexes. Specifically, the AP-1 complex is a dimer of gene products of the Fos and Jun families of oncogenes, which have closely related recognition sites but different transcriptional activities and DNA binding affinities (reviewed in Vogt and Bos (1990)). For example, whereas c-jun is a potent activator of the c-jun and collagenase promoters, J un-B is not and inhibits their trans-activation by c-jun (reviewed in Mauviel (1993)). To characterize further the protein complex binding to the −265/−241 region of human COL1A2, nuclear extracts from TGF-β-treated fibroblast cultures were incubated with antibodies against c-jun or J un-B, prior to detection of DNA/protein interactions by gel mobility shift assay. As shown in Fig. 4, the antibody against J un-B (lane 2), but not that against c-jun (lane 1), induced a supershift of the labeled DNA probe, indicating that J un-B partici-
pates in the formation of the complex that binds to the −265/−241 region of COL1A2. This result is in agreement with our previous observations that TGF-β is a potent activator of jun-B expression in fibroblasts (Mauviel et al., 1993) and further indicates that certain components of AP-1 are part of the transcription factor binding to the COL1A2 promoter.

Mutation of the AP-1-binding Site within the Proximal COL1A2 Promoter Region Abolishes TGF-β Responsiveness—We have previously shown that TGF-β is capable of modifying AP-1-driven but not NF-κB-driven transcription of the genes encoding collagenase and IL-8, respectively (Mauviel et al., 1993). Together with the data presented above, these observations led us to investigate the role of the putative AP-1-binding site located between residues −265 and −241 in mediating TGF-β effect on COL1A2 promoter. To investigate this possibility, we employed site-directed mutagenesis to modify the AP-1 site from its original sequence 5′-CGAGTCA-3′ to 5′-CCAGTGA-3′ in two constructs, the −342/CAT and the −265/CAT. The first one contains three Sp-1 sites upstream of the AP-1 sequence whereas the latter construct has the AP-1 binding site located right at its 5′ end. The mutated constructs were then used in transient transfection experiments in parallel with their wild-type counterparts. Mutation of the AP-1 site in either construct led to a dramatic drop, −50%, of basal activity as compared with their unmutated counterparts and resulted in almost complete loss of TGF-β responsiveness (Fig.

Fig. 5. Effect of a mutation in the AP-1-binding site on COL1A2 promoter activity and its regulation by growth factors. Two 5′ deletion constructs (−342 and −265) were subjected to substitution mutations in the potential AP-1-binding site located at position −250, as described under "Materials and Methods." The mutated and the parent constructs were used in parallel transfections of fibroblasts, as described in the legend to Fig. 2. After glycerol shock, the cultures were incubated for 3 h in medium containing 1% fetal calf serum and incubated for another 40 h without or with TGF-β (10 ng/ml), in the absence or presence of TNF-α (25 ng/ml). Processing of samples was carried out as described in the legend to Fig. 2. The various promoter constructs used were as follows: panel 1, −342COL1A2 promoter/CAT; panel 2, mutated −342COL1A2 promoter/CAT; panel 3, −265COL1A2 promoter/CAT; panel 4, mutated −265COL1A2 promoter/CAT. A, autoradiograms of a representative experiment. B, the results, presented as relative promoter activity, are the mean ± S.D. of three independent experiments, each performed using duplicate samples, and are expressed as percent of acetylation of the [14C]chloramphenicol substrate.
Fig. 6. Effect of overexpression of c-jun blocks the effect of TGF-β-induced up-regulation of COL1A2 promoter activity. Confluent fibroblast cultures were transfected with 2 μg/plate of −342/CAT plasmid construct together with 18 μg/plate of either an empty expression vector pRSVe or a c-jun expression vector pRSVc-jun, as indicated. Following glycerol shock, the cells were placed in DMEM supplemented with 1% fetal calf serum. Three hours later, TGF-β (10 ng/ml) was added, and the incubation was continued for another 40 h. CAT activity, representing the activity of the COL1A2 promoter, was determined, and autoradiograms of a representative experiment are shown, together with their corresponding values of CAT acetylation.

5, A and B, panels 1 and 3 versus panels 2 and 4, respectively. Specifically, as observed in three separate experiments (Fig. 5B), the level of induction of the parent constructs by TGF-β, −10–13-fold induction above control levels for both −342/CAT and −265/CAT constructs, was reduced to 1.6–2.2-fold above control levels for both −342/CAT and −265/CAT constructs, was reduced to 1.6–2.2-fold above control for the mutated constructs. These results clearly demonstrate that the AP-1 binding site within this 25-bp promoter is fundamental for TGF-β response, independently of the upstream Sp-1 sites.

Overexpression of c-jun Blocks the Effect of TGF-β on COL1A2 Promoter Activity—We have previously established that TGF-β and TNF-α exert antagonistic activities on AP-1 driven promoters (Mauviel et al., 1993). This antagonism is due to differential induction of oncogenes of the J un family, namely jun-B and c-jun; their products have been shown to exert opposite transcriptional activities (Schütte et al., 1989; Deng and Karin, 1993; Mauviel et al., 1993). Specifically, TNF-α is a potent inhibitor of c-jun, whereas TGF-β induces jun-B in dermal fibroblasts, leading to opposite effects on collagenase gene expression through AP-1 controlled trans-activation of the promoter (reviewed in Mauviel (1993)). Since TNF-α is a potent inhibitor of c-jun (Brenner et al., 1989; Mauviel et al., 1993) and since mutations in the AP-1 site of the COL1A2 promoter abolish TGF-β response (see above), we investigated the effect of c-jun overexpression on TGF-β-induced activation of the COL1A2 promoter. For this purpose, confluent fibroblast cultures were co-transfected with pRSVc-jun and −342/CAT and treated with TGF-β. As shown in Fig. 6, overexpression of c-jun led to a dramatic reduction of −342/CAT activity and abolished the effect of TGF-β. Specifically, c-jun overexpression reduced the basal COL1A2 promoter activity by −60% and totally blocked the response to TGF-β. These data suggest that (a) AP-1/c-jun plays a significant role in the basal activity of the COL1A2 promoter, and (b) the antagonistic effect of TNF-α on TGF-β induced up-regulation of collagen gene expression may be mediated, at least in part, by c-jun overexpression upon TNF-α stimulation.

Conclusions—Using a series of 5′ deletion constructs of the COL1A2 promoter, we have excluded Sp-1-binding sites as being necessary for TGF-β response. In addition, we have characterized a short fragment of the COL1A2 promoter, spanning from nucleotides −265 to −241, as an AP-1-binding site. Using site-directed mutagenesis, we have demonstrated that this region plays a regulatory role in the basal activity of the promoter and is fundamental for TGF-β response. Furthermore, our data suggest that this AP-1 site may be sufficient to mediate TNF-α inhibitory effect on TGF-β driven up-regulation of COL1A2 gene expression. This study extends our understanding of the molecular mechanisms involved in the transcription of the COL1A2 gene and provides novel mechanistic information on growth factor and cytokine regulation.

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