

4-29-1994

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Jimenez, Sergio A.; Varga, John A.; Olsen, Anne; Li, Liye; Diaz, Arturo; Herhal, Janet; and Koch, Julie, "Functional analysis of human alpha 1 (I) procollagen gene promoter. Differential activity in collagen-producing and -nonproducing cells and response to transforming growth factor beta 1." (1994). *Department of Medicine Faculty Papers*. Paper 186.
<https://jdc.jefferson.edu/medfp/186>

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Functional Analysis of Human $\alpha 1(I)$ Procollagen Gene Promoter

DIFFERENTIAL ACTIVITY IN COLLAGEN-PRODUCING AND -NONPRODUCING CELLS AND RESPONSE TO TRANSFORMING GROWTH FACTOR β_1 *

(Received for publication, December 30, 1993)

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To gain a further understanding of the regulation of human type I collagen gene expression under physiologic and pathologic conditions, we characterized 5.3 kilobase pairs (kb) of the human $\alpha 1(I)$ procollagen gene promoter. A series of deletion constructs containing portions of the $\alpha 1(I)$ procollagen 5'-flanking region (with end points from -5.3 kb to -84 base pairs (bp)) ligated to the chloramphenicol acetyltransferase (CAT) reporter gene were transiently transfected into NIH/3T3 cells. Maximal CAT activity was observed with constructs having 5' end points from -804 to -174 bp. A further 5' deletion to -84 bp caused a marked reduction in CAT activity. Cells transfected with plasmids containing longer 5'-flanking fragments of the $\alpha 1(I)$ procollagen gene (-2.3 or -5.3 kb) showed reduced CAT activity compared with the -804 bp construct. The activity of the $\alpha 1(I)$ procollagen promoter was much lower in cells that do not normally express type I collagen (HeLa cells) compared with collagen-producing NIH/3T3 cells. The CAT activity of deletion constructs containing longer 5' regions than -84 bp was increased by ≈ 2 -fold in NIH/3T3 cells treated with transforming growth factor β_1 (TGF β_1). Electrophoretic mobility shift assays indicated that protein-DNA complex formation with a probe corresponding to the -170 to -80 bp fragment of the $\alpha 1(I)$ procollagen promoter was markedly enhanced in nuclear extracts prepared from TGF β_1 -treated fibroblasts as compared with untreated fibroblasts. The DNA binding activity stimulated by TGF β_1 was specific for an Sp1-like sequence at positions -164 to -142 bp in the promoter. These results demonstrate that 1) there are both positive and negative cis-acting regulatory elements in the human $\alpha 1(I)$ procollagen promoter, 2) these regulatory regions function differently in collagen-producing and -nonproducing cells, 3) the $\alpha 1(I)$ procollagen promoter contains TGF β_1 -responsive sequences located between -174 and -84 bp from the transcription start site, and 4) TGF β_1 caused marked stimulation of the DNA binding activity of a nuclear factor interacting with an Sp1-like binding site located within a region encompassing -164 to -142 bp of the $\alpha 1(I)$ procollagen promoter.

Normal fibroblasts modulate their biosynthetic activity to maintain a precise balance between the synthesis and degradation of their products during dynamic events of tissue remodeling such as development, differentiation, and repair. It has been suggested that abnormalities in these regulatory mechanisms may be responsible for the excessive extracellular matrix accumulation in a variety of fibrotic diseases such as systemic sclerosis and idiopathic pulmonary fibrosis.

The collagens comprise a large family of widely distributed proteins that play a crucial role in the maintenance of the structural properties of the extracellular matrix. Despite the important structural and functional roles that the collagens play in normal tissues, the mechanisms that regulate their production are not completely understood. Variations in net collagen production occurring during growth and differentiation (1–3), viral (4–6) and chemical (7–9) transformation, cytokine and growth factor modulation (10–14), and spontaneous (15–17) and experimentally induced (18, 19) fibrotic processes have been ascribed to fluctuations in the steady-state collagen mRNA levels. The regulatory mechanisms responsible for the maintenance of normal procollagen mRNA levels have not been completely elucidated. Most of the available evidence suggests that the principal mechanisms operate at the level of transcription, although translational control and changes in mRNA processing and stability may also play a role (reviewed in Refs. 20 and 21). The broad spectrum of regulatory signals that can influence collagen gene transcription suggests that the collagen gene promoters are responsive to various trans-acting pathways. Several putative regulatory elements that may determine the transcriptional efficiency of procollagen genes have been identified in their corresponding promoters. These include the consensus TATA and CCAAT motifs as well as additional regulatory elements (22–25), which are the potential targets for the action of promoter-specific transcription factors (26–31). Furthermore, the transcriptional activity of some procollagen gene promoters appears to be modulated by enhancer and silencer elements located 3' from the transcription start site (32, 33).

Detailed characterization of the cis-acting elements involved in modulation of collagen gene expression is crucial for understanding the physiologic and pathologic regulation of tissue collagen deposition. The purpose of the work reported here was to analyze the human $\alpha 1(I)$ procollagen gene promoter in order to identify regulatory regions of the gene that may play a role in the modulation of its expression under normal and pathologic conditions.

MATERIALS AND METHODS

Construction of Plasmids—Several preliminary steps were necessary to prepare fragments with the appropriate restriction sites for ligations. The HindIII site in pSVO CAT¹ was changed to a KpnI site, and a 1.6 kb

* This work was supported by Grants AM 19106 and AR 42309 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U06669.

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¹ The abbreviations used are: CAT, chloramphenicol acetyltrans-

KpnI-*Bam*HI fragment was isolated. A 7 kb $\alpha 1(I)$ procollagen gene *KpnI* fragment extending from -5.3 to +1.7 kb was isolated from a human cosmid clone² and subcloned into pUC19, and the *KpnI* site at -5.3 kb was changed to a *NotI* site to give pJH49. A *HindIII*-*ThaI* fragment, extending from -804 to +42 bp, was subcloned into the *HindIII* and *SmaI* sites of pUC19, excised as a *HindIII*-*KpnI* fragment, and subcloned into the *HindIII* and *KpnI* sites of Bluescript KS+ to give p804BS. The 4.5 kb *NotI*-*HindIII* fragment from pJH49 (containing $\alpha 1(I)$ procollagen gene sequences from -5.3 kb to -804 bp) was then subcloned into the *NotI* and *HindIII* sites of p804BS to give p5.3kBS.

For the -5.3 and -804 bp constructs, the *NotI*-*KpnI* fragments from p5.3kBS and p804BS were ligated with the 1.6 kb *KpnI*-*Bam*HI CAT coding fragment into the *NotI* and *Bam*HI sites of Bluescript KS+ to give p5.3kcal CAT and p804 $\alpha 1$ CAT, respectively. For the 2.3 kb construct, a 1.5 kb *Bam*HI-*HindIII* fragment containing sequences from -2.3 kb to -804 bp was converted to a *NotI*-*HindIII* fragment and inserted into the *NotI* and *HindIII* sites of p804 $\alpha 1$ CAT to give p2.3k $\alpha 1$ CAT. A series of deletions (from the *HindIII* site at -804 bp in p804 $\alpha 1$ CAT toward the start of transcription site) was made following the exonuclease III digestion procedure of Henikoff (34). Exact deletion end points were determined by sequencing, and clones ending at -675, -463, -369, -174, and -84 bp were selected for analysis. A promoterless CAT plasmid, p0CAT, was prepared by removing the 846 bp *HindIII*-*KpnI* $\alpha 1$ procollagen promoter fragment from p804 $\alpha 1$ CAT and religating.

The sequence from -2.3 kb to -804 bp of the $\alpha 1(I)$ procollagen gene was obtained from deletions made from the *NotI* site in p2.3k $\alpha 1$ CAT toward the initiation of transcription site (also following the exonuclease III digestion procedure). DNA sequencing of both strands was performed using the dideoxy chain termination procedure (35) with T7 polymerase (Sequenase, U. S. Biochemical Corp.) following the instructions provided by the supplier.

Cell Transfections—NIH/3T3 cells (obtained from the ATCC) from subconfluent cultures were plated at a density of 3×10^5 cells/60-mm dish and cultured in Dulbecco's modified Eagle's medium containing 10% calf serum. Transfections were performed 24 h later employing the calcium phosphate co-precipitation method, as described (36). The NIH/3T3 cells were transfected with 4 μ g of $\alpha 1(I)$ procollagen promoter-CAT plasmid DNA and 0.2 μ g of the pSV2AP plasmid DNA containing the SV40 early promoter and enhancer fused to a rat alkaline phosphatase cDNA (kindly provided by Dr. Kyong Yoon) (37) as an internal standard. After 4 h, the cultures were subjected to a 15% glycerol shock for 3 min at room temperature and then grown in fresh medium for 48 h before harvesting. HeLa cells (obtained from the ATCC) were plated in 60-mm plastic culture dishes (3×10^5 cells/dish), and 16 h later they were transfected with the plasmids with the same procedure as that used for NIH/3T3 cells, except that HeLa cells were cultured in minimum Eagle's medium containing 10% fetal calf serum instead of calf serum following the glycerol shock.

Effects of TGF β 1—To investigate the effects of TGF β 1 on the expression of the various $\alpha 1(I)$ procollagen promoter constructs, NIH/3T3 cells were transfected with the deletion construct plasmids and the pSV2AP control plasmid as described above. Six hours after the transfections, the culture media were removed, and a fresh medium containing 10% of the serum substitute Serum Plus (Hazelton Biologics) and 12.5 ng/ml human recombinant TGF β 1 (NIH Bureau of Standards, or R & D Systems) was added to duplicate cultures. Control cells incubated in parallel received culture medium without TGF β 1. The control and TGF β 1-treated cells were incubated for an additional 48 h and then harvested for assays of CAT and alkaline phosphatase activities as described below.

Assays of CAT and Alkaline Phosphatase Activities—Cell extracts were prepared by mechanically detaching the cells in 1.5 ml of phosphate-buffered saline and by centrifugation at 5000 $\times g$ for 3 min. The cell pellets were resuspended in 100 μ l of 0.25 M Tris-HCl, pH 7.8, 0.1% Triton X-100 and sonicated for 15 s. CAT activity was measured on 20- μ l aliquots of supernatants following centrifugation of the extracts for 5 min in a Microfuge. The supernatants were heated for 10 min at 60 °C prior to the assay of CAT activity by a liquid scintillation assay using 1 μ Ci of [³H]acetyl-CoA/sample as described (38). In some experiments, CAT activity in the cell extracts was determined employing [¹⁴C]chloramphenicol according to the method described by Gorman *et al.* (39). The conversion of chloramphenicol to acetylated forms was quantified by scraping the corresponding areas from the thin layer chromatography plates and measuring radioactivity by liquid scintillation spectroscopy. The two methods of assaying CAT activity yielded comparable

results. Alkaline phosphatase activity was assayed on 5 μ l of the extracts as described by Yoon *et al.* (37). CAT activity in each sample was normalized to alkaline phosphatase activity to correct for differences in transfection efficiency.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from confluent cultures of normal human skin fibroblasts that had been incubated with or without TGF β 1 (1 or 10 ng/ml) in the presence of 5% fetal calf serum for 48 h according to the method of Dignam *et al.* (40). Protein concentrations were determined by a dye binding assay (Bio-Rad), and the nuclear extracts were stored in 50- μ l aliquots at -70 °C until use. In order to ensure that fibroblast protein biosynthesis was stimulated by TGF β 1, cultures were labeled with [¹⁴C]proline (1.5 μ Ci/ml) for 24 h prior to harvesting. Only nuclear extracts prepared from cultures exhibiting a greater than 2-fold increase in [¹⁴C]proline-labeled protein production over untreated cultures were used. In a typical experiment, a confluent 175-cm flask (10⁷ cells) yielded ~100 μ g of crude nuclear protein.

Probes for electrophoretic mobility shift assays were prepared by enzymatic digestion of the p804 $\alpha 1$ CAT plasmid with *NotI* and *KpnI*. The resulting 431 bp fragment was gel-purified and further digested with *NaeI* and *HinfI*. The resulting 219 bp *NotI*-*HinfI* (200-3) and 90 bp *HinfI*-*NaeI* (200-2) fragments were purified and 5' end-labeled with [α -³²P]dCTP using the Klenow fragment of DNA polymerase I according to conventional procedures (41). Additional oligonucleotide probes were prepared by automated DNA synthesis (Applied Biosystems), and oligonucleotide Sp1 was obtained from Stratagene. Each oligonucleotide was annealed to its complementary strand and end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Boehringer Mannheim). Competition studies were performed with molar excesses of unlabeled DNA fragments or double-stranded oligonucleotides. Electrophoretic mobility assays were performed using low ionic strength buffers as described (42). Binding reactions contained 5–10 μ g of the nuclear extracts, 1–2 μ g of double-stranded poly[d(I-C)] (Pharmacia LKB Biotechnology Inc.), and radiolabeled probes containing 50,000 cpm for a total of approximately 0.5 ng. The reaction mixtures were incubated for 30 min on ice in a buffer containing 60 mM KCl, 10 mM HEPES pH 7.9, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol in a total volume of 10 μ l. Protein-DNA complexes were resolved from the free probes in non-denaturing 5% polyacrylamide gels. Electrophoresis was performed in Tris/glycine buffer (50 mM Tris-HCl, 260 mM glycine) at 120 V for 120 min. The gels were dried under vacuum and exposed to x-ray film with intensifying screens at -70 °C for 16–48 h.

RESULTS

Nucleotide Sequence of -2.3 kb to -804 bp Region of $\alpha 1(I)$ Procollagen Gene—The nucleotide sequences of the human $\alpha 1(I)$ procollagen promoter and 5'-flanking region to -804 bp have been previously reported (23). Our results extend the upstream sequence of the gene to the *Bam*HI site at -2.3 kb (Fig. 1). The only species for which the $\alpha 1(I)$ procollagen sequence in this area has been reported to date is rat (43). A comparison of the nucleotide sequences of human and rat $\alpha 1(I)$ procollagen gene 5'-flanking regions indicates a relatively low overall nucleotide identity (62%). However, there are several regions within the human and rat sequences (underlined in Fig. 1) that are greater than 90% identical. A search of all mammalian DNA sequences in GenBank (release 80.0) revealed no additional sequences of identity or high homology to either strand of the new upstream sequence reported here.

Deletion Analysis of 5.3 kb Upstream Sequence of the $\alpha 1(I)$ Procollagen Gene—To analyze sequences that may be important for regulation of collagen gene expression, a series of CAT constructs with 5' end points from -5.3 kb to -84 bp were prepared (Fig. 2) and examined in transient transfection assays of functional activity. To eliminate the $\alpha 1(I)$ procollagen gene ATG initiation codon at +120 bp and the two additional upstream ATG codons at +103 and +61 bp, the 3' end point of all fragments was +42 bp. Therefore, translation should initiate only at the ATG codon of the CAT gene. The plasmids were transfected into NIH/3T3 cells, and cell extracts were analyzed for CAT activity. To control for differences in transfection efficiency between samples, all results were normalized to a co-transfected reference plasmid (pSV2AP). A summary of these

ferase; kb, kilobase pair(s); bp, base pair(s).

² J. Hyland, unpublished observations.

FIG. 1. Nucleotide sequence of the 5'-flanking region of the human $\alpha 1(I)$ procollagen gene. The sequence of the *Bam*HI-*Hind*III fragment encompassing positions -2292 to -798 bp is shown. Regions with greater than 90% identity to the corresponding rat sequences (43) are underlined.

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-2292  GATCCCCAAATATCCTTCAGAACCCCAAGCCCATGATGTAGCAACCCCAATTCACACCTTGGAGGTTTC
-2222  AACTCTTCTTTAAGATGGGCGTGGGAAAGCCTGGATGGGAAACATATGGGGAGGGGGGGGAGCTGCAAG
-2152  CAGGAGCCCTTCTTACTACGAAAAACCAAGAAAGCAAGGAAGTGGACAGGTCACFAACCCCTCACTACCA
-2082  AGCCCTGCGGCACCCCTGCCTTAGACCACCCTCTAAATGTCTGTCCCTCCAAAAACAGGACCCCTGTCC
-2012  CCTATTAGGGAGGGGGTCTCTTGGAACTGACCCACAGTAGGGGGCAGGACTTTGGTGGGTTCAAGAACTG
-1942  CCATCTCAGCACCTCAGCCCCCTAGTCTGCCCCGCACTGCGTGGCACTAGGGGGGGCAGACCCCTGGGC
-1872  CACAAGTTGCTGCCACATGGTCCGGATAAATTGATGAAGGTCCATCCCTCCATTGCTGTCTCCAGCCCTGC
-1802  CTCTCTGGAAGCTCTATATTTTCCCTTAATTATAGCCCTGCACTCTCCCTCTGCTGCCCCACCCGGCAC
-1732  CGCTCATCCTGGCTGCCACCGCCAGCCGGCCAGCCGACGCTGGCTCCCTCCCTTCTGTTCTTTTTTTT
-1662  CCCCTTTGCTTCCGTTGCACAAAACCAAGCTGGGGGAGGGCTGGAGAGGGGGGGGGAGGCAATGGAAAT
-1592  CTTGGATGGTTTTGGGGGAGCCGGGACTCCCGCTCCACGTTTGCAGCTCTGGAGCACCCCGGGTGGGA
-1522  GCTGCACAGGAGGGAGAGAAATGAACAGGGCACTGCAAGGAGACCCCAAGCCCTTCTCTCAGCCCTACAG
-1452  AGTTTCTCAGGACGAGGTAGATTGGGGTTGAGGCAGAGCCCTGTTGGGGGAATGGACATGGAGGAAAGAA
-1382  AGGACGTGGAGTTCTAGAGCCATCTTCCTTAGATATAGCCTGCTGTCTTCGGGTCCCAAGACCCCTTCA
-1312  GAGTGTACAGATGATTCTCTCTGGTTCCTAAGGCATAGAGCAATGACCCGGGATTTTCAAGAAAGAGATGA
-1242  GGCAGTGGGAAGTAGCCCTAAAACAAAGTCAATCATCCTCTGCAGCCATCCCAACCCCAAGAGAAA
-1172  GTTTCACCCAGACACCCAAAATATCCCATACATCCCAACACTGAGTCCAGGTCAACTGGAGAAAGGGGC
-1102  TTTATGCAGCTCCCAAGAAAGACACCCCTTTAGCTAAGTGCCTCCCTCCACCCAGGTTCTCTCTGGTTG
-1032  ACTGTGCTGGGAAGGAGGGTCTCTAAGCAGCCCTGGCCACAGCCATGGCAAAACAAAACCTTCTCTAAG
-962   TCACCAATGATCACAGGCCTCCCACTAAAATACTTCCCAACTCTGGGGTGGAAAGATTTGGGGGATGAA
-892   TTTTATGGGATTGCAAGCCCAATCCCACTCTGTGTCCCTAGAATCCCAACCCCTACCTTGGCTGC
-822   TCCATCACCCCAACCAAAAGCTT

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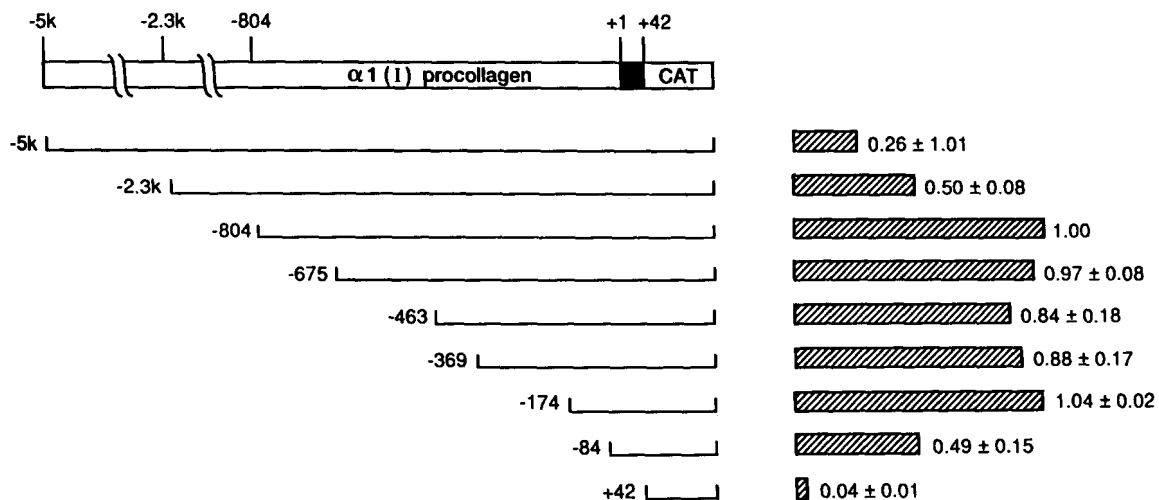


FIG. 2. Summary of CAT activity driven by various lengths of the 5'-flanking sequence of the human $\alpha 1(I)$ procollagen gene. A schematic linear map of p5.3K $\alpha 1$ CAT and 5' end points of the deletion constructs are shown on the left. NIH/3T3 cells were transiently co-transfected with the $\alpha 1(I)$ procollagen-CAT constructs containing various lengths of promoter sequences and the alkaline phosphatase-CAT construct; CAT and alkaline phosphatase activities were determined as described under "Materials and Methods." The hatched bars on the right represent CAT activity driven by each construct corrected for alkaline phosphatase activity in the same extracts relative to the CAT activity of the -804 bp construct. Plasmids were assayed in at least two separate transfection experiments with duplicate or triplicate determinations in each, except for the -675 bp construct, which was used in duplicate in a single experiment. Values shown represent the average \pm S.E.

experiments is shown in Fig. 2. The results indicate that CAT expression was maximal and constant in cells transfected with promoter constructs having 5' end points from -804 to -174 bp. A further 3' deletion to -84 bp caused a significant reduction in CAT activity, although even this short promoter was able to drive 10-fold higher CAT activity than the promoterless CAT plasmid pOCAT. Plasmids containing longer 5' fragments (extending to -2.3 or -5.3 kb) showed lower CAT activity than the -804 bp construct (50 and 20%, respectively).

Comparison of Activity of $\alpha 1(I)$ Procollagen Promoter in Collagen-producing and -nonproducing Cells—In order to assess the activity of $\alpha 1(I)$ procollagen promoter sequences in a cell line that does not normally exhibit high levels of type I collagen gene production, constructs with end points at -5.3 kb, -2.3 kb, and -804 bp were transfected into HeLa cells. As shown in Fig. 3, the CAT activity driven by these three constructs was markedly lower in HeLa cells than in NIH/3T3 cells. In NIH/3T3 cells, CAT activity driven by the -804 bp construct was about 26-fold higher than the CAT activity observed with the promot-

erless construct pOCAT, whereas in HeLa cells the relative activity of the -804 bp construct was 10-fold lower. In contrast to NIH/3T3 cells, transfection of constructs with longer 5' sequences into HeLa cells resulted in about 2-fold greater CAT activity than that obtained with the -804 bp plasmid. These results suggest that the positive and negative transcriptional regulatory regions of the $\alpha 1(I)$ procollagen promoter function differently in collagen-producing and -nonproducing cells.

Localization of $\alpha 1(I)$ Procollagen Promoter Regions Responsive to TGF $\beta 1$ —In order to localize regions within the $\alpha 1(I)$ procollagen promoter that are responsive to stimulation by TGF $\beta 1$ in the NIH/3T3 cells, the effects of TGF $\beta 1$ on the CAT activity in cells transfected with promoter deletion constructs with 5' end points at -5.3 kb, -2.3 kb, and -804 bp were examined. Incubation with TGF $\beta 1$ for 48 h resulted in an approximately 2-fold stimulation of CAT activity in cells transfected with each of the three constructs (Fig. 4), suggesting that TGF $\beta 1$ -responsive elements were located within the proximal region of the promoter 3' to the -804 bp end point. To further

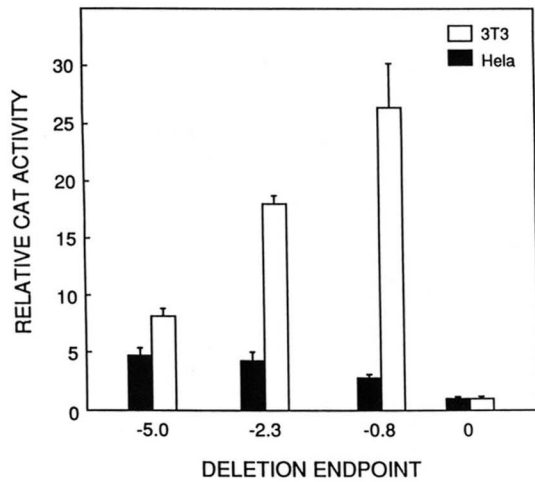


FIG. 3. Activity of the human $\alpha 1(I)$ procollagen gene promoter in collagen-producing (NIH/3T3) and -nonproducing (HeLa) cells. Cultured NIH/3T3 and HeLa cells were transiently transfected with various deletion constructs (described in the legend to Fig. 2) under identical conditions. CAT activity in the cell extracts was determined following a 48-h incubation, as described under "Materials and Methods." CAT activity driven by the individual constructs corrected for transfection efficiency, as determined by alkaline phosphatase activity in the same samples, is shown relative to the CAT activity of the promoterless plasmid pOCAT. The values shown represent the average \pm S.E. of three separate transfections with each plasmid, each assayed in duplicate.

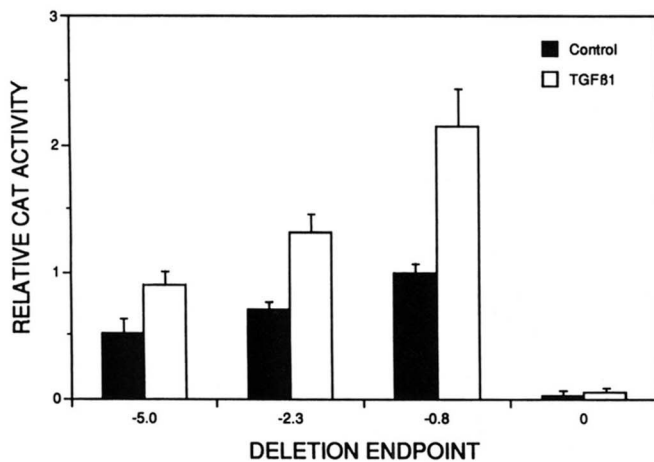


FIG. 4. Effects of TGF β 1 on CAT activity driven by various lengths of the 5'-flanking sequence of the human $\alpha 1(I)$ procollagen gene. NIH/3T3 cells were transiently transfected with the progressive deletion constructs derived from the p5.3KBS plasmid, as described under "Materials and Methods," and incubated in Dulbecco's modified Eagle's medium containing 10% Serum Plus with or without TGF β 1 (12.5 ng/ml) for 48 h. CAT activity of the cell extracts, corrected for alkaline phosphatase activity, is shown relative to the -804 bp construct.

identify TGF β 1-responsive sequences within the proximal region of the $\alpha 1(I)$ procollagen promoter, NIH/3T3 cells were transfected with constructs with 5' end points at -675, -463, -369, -174, and -84 bp or with the promoterless construct pOCAT and incubated with TGF β 1. The results indicate that TGF β 1 caused a greater than 2-fold increase in CAT activity driven by the constructs with 5'-flanking regions longer than -84 bp. In contrast, TGF β 1 did not stimulate the CAT activity driven by the -84 bp promoter. A representative transfection experiment is shown in Fig. 5. These results indicated that TGF β 1-responsive sequences are located between -174 and -84 bp of the $\alpha 1(I)$ procollagen promoter.

Nuclear Protein Binding to $\alpha 1(I)$ Procollagen Promoter in Control and TGF β 1-treated Fibroblasts—In order to elucidate

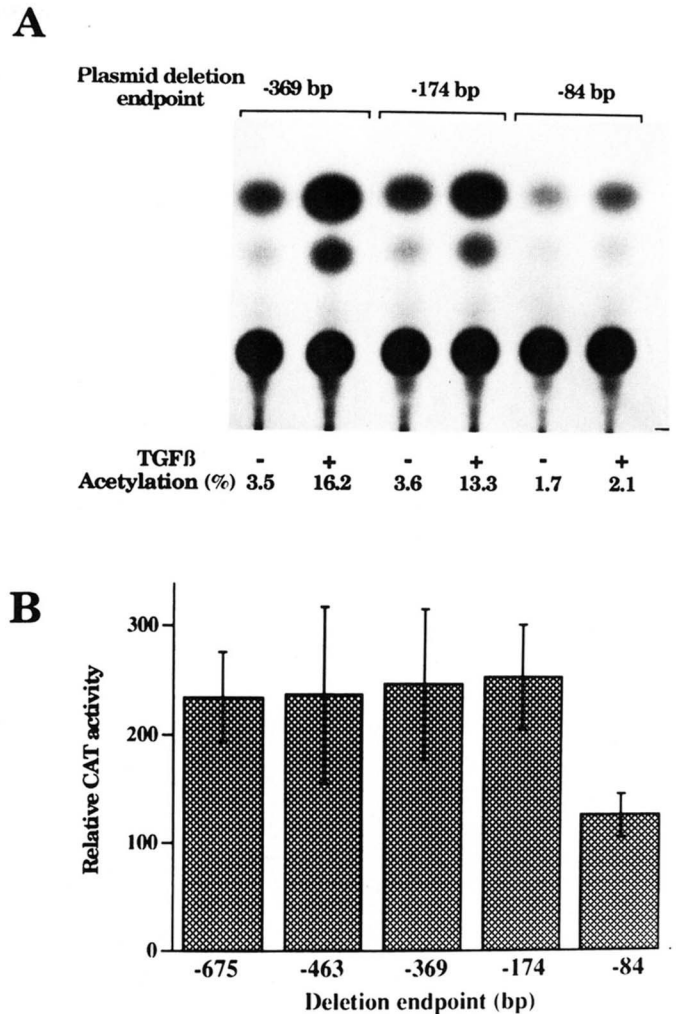


FIG. 5. Regulation of the $\alpha 1(I)$ procollagen promoter activity by TGF β 1. A, representative CAT assay demonstrating the effect of TGF β 1 on three deletion constructs of plasmid P804 α 1 CAT. NIH/3T3 cells were transfected with the -369, -174, and -84 bp end point constructs. Following a 48-h incubation with or without TGF β 1, CAT activity in the cell extracts was determined as described under "Materials and Methods." Values are expressed as percentage of [14 C]chloramphenicol that was acetylated. B, histogram showing results of transient transfections of deletion constructs from four separate experiments. CAT activity in TGF β 1-treated cells (mean counts/min \pm S.E. from duplicate determinations) is expressed relative to the CAT activity in untreated cells (100%).

changes in trans-acting protein-DNA interactions involving the $\alpha 1(I)$ procollagen promoter that were associated with TGF β 1-induced stimulation of collagen production, electrophoretic mobility shift assays were performed. For this purpose, nuclear extracts were prepared from untreated and TGF β 1-treated fibroblasts. Two fragments (200-2 and 200-3) of the $\alpha 1(I)$ procollagen promoter region spanning the sequences from -389 to -80 bp relative to the transcription start site were used as probes (Fig. 6A). Incubation of the 200-2 probe (-170 to -80 bp) with nuclear extracts from untreated or TGF β 1-treated cells resulted in the formation of two complexes with retarded electrophoretic mobility (labeled R1 and R2), indicating the presence of nuclear DNA binding factor(s) recognizing sequences within the probe (Fig. 7). The intensity of the R1 and R2 complexes determined by laser densitometry of the autoradiograms was increased 18- and 8-fold, respectively, when nuclear extracts prepared from fibroblasts that had been treated with TGF β 1 (10 ng/ml) were used (Fig. 7). Competition experiments indicated that a 4-fold molar excess of unlabeled 200-2 probe completely prevented the formation of the R1 complex and re-

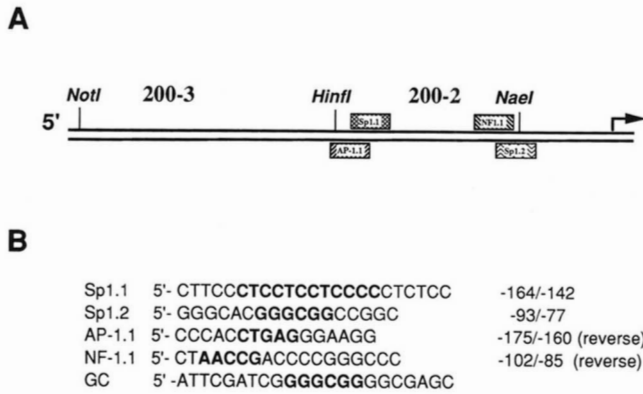


FIG. 6. Locations and sequences of the probes used in electrophoretic mobility shift assays. A, 200-2 and 200-3 fragments and the Sp1-, AP-1-, and NF-1-like elements in the proximal region of the $\alpha 1(I)$ procollagen promoter are shown. The arrow indicates the transcription start site. B, nucleotide sequences of the sense (Sp1.1 and Sp1.2) or antisense (AP-1.1 and NF-1.1) strands of the binding site oligonucleotides used. The core motifs are shown in *boldface*. The GC-box oligonucleotide contains the Sp1 consensus binding sequence.

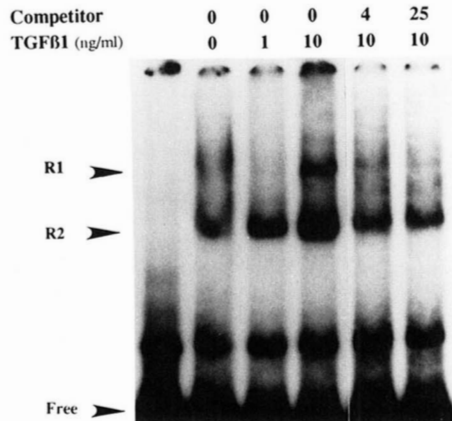


FIG. 7. Electrophoretic mobility shift assay with competing unlabeled DNA. A 90 bp double-stranded fragment of the human $\alpha 1(I)$ procollagen gene promoter encompassing the sequence from positions -170 to -80 bp (200-2) was end-labeled and used as a probe in electrophoretic mobility shift assays. Labeled probes were incubated with 1 μ g of poly[d(I-C)] and 5 μ g of nuclear extracts prepared from normal human dermal fibroblasts that had been treated with TGF $\beta 1$ (1 or 10 ng/ml) or left untreated for 48 h. Reactions were carried out in the absence or presence of 4- and 25-fold molar excesses of unlabeled 200-2 DNA probe, and the formation of DNA-protein complexes was analyzed by electrophoresis in 5% polyacrylamide gels under nondenaturing conditions. The two slowly migrating DNA-protein complexes are labeled R1 and R2. Lane 1, control containing the labeled probe but no protein.

duced by about 60% the formation of the R2 complex with the labeled 200-2 probe. A 25-fold molar excess of unlabeled 200-2 probe did not completely prevent the formation of the R2 complex (Fig. 7). These results suggest substantial differences in the DNA binding affinities of these nuclear proteins. Several DNA-protein complexes were detected when nuclear extracts were incubated with probe 200-3, spanning the sequences from -389 to -170 bp (results not shown). However, in contrast to the results obtained with the 200-2 probe, no qualitative or quantitative differences in DNA-protein complex formation were noted when the binding of nuclear proteins prepared from untreated or TGF $\beta 1$ -treated cells to the 200-3 probe was examined. The increase in nuclear binding activity specific for the 200-2 probe in extracts prepared from TGF $\beta 1$ -treated cells suggested the enhancement of either the synthesis or the DNA binding activity of trans-acting factors recognizing an element(s) located within the -170 to -80 bp region of the $\alpha 1(I)$ procollagen promoter.

Binding of Nuclear Proteins from Control or TGF $\beta 1$ -treated Fibroblasts to Oligonucleotides with Sp1-like Binding Sequences—A detailed analysis of the nucleotide sequence of the -170 to -80 bp region of the human $\alpha 1(I)$ procollagen promoter revealed two regions of homology with consensus sequences recognized by the transcription factor Sp1, here designated Sp1.1 and Sp1.2 (44, 45). To determine whether nuclear proteins from human fibroblasts may interact with these Sp-1-like elements in the $\alpha 1(I)$ procollagen promoter, electrophoretic mobility shift assays were performed with synthetic double-stranded oligonucleotides corresponding to the Sp1.1 and Sp1.2 sequences as well as with oligonucleotides corresponding to promoter sequences that display homology with the AP-1 and NF-1 consensus binding elements. The location and nucleotide sequences of these potential regulatory elements in the $\alpha 1(I)$ procollagen promoter are shown in Fig. 6. Upon incubation of the Sp1.1 probe with nuclear extracts prepared from untreated fibroblasts, two distinct DNA-protein complexes, designated R1 (*upper*, a duplex) and R2 (*lower*), were detected (Fig. 8A). In nuclear extracts prepared from fibroblasts treated with TGF $\beta 1$, a reproducible 4-fold increase in the intensity of the R1 complex and a less consistent 30% increase in the intensity of the R2 complex were noted compared with the intensity of the corresponding complexes formed with nuclear extracts from untreated fibroblasts. Essentially no complex formation was detected when Sp1.2 was used as the probe (Fig. 8A). To determine whether the formation of the R1 and R2 complexes resulted from specific protein interactions with the Sp1.1 probe, competition experiments with increasing amounts of unlabeled oligonucleotides containing the Sp1.1 or Sp1.2 sequences or the Sp1 consensus binding site were performed. As shown in Fig. 8B, the formation of the R2 complex was completely prevented by the addition of excess unlabeled Sp1.1 oligonucleotide, whereas excess Sp1.2 or consensus Sp1 oligonucleotides failed to compete for binding. In contrast, the formation of the R1 duplex was markedly reduced by each of the three competitor oligonucleotides. Incubation of the nuclear extracts with the AP-1.1 or NF-1.1 oligonucleotide probes resulted in the formation of single DNA-protein complexes (Fig. 8C). However, in contrast to results obtained with the Sp1.1 probe, there were no differences in the intensities of protein-DNA complexes when nuclear extracts prepared from untreated or from TGF $\beta 1$ -treated fibroblasts were used. These results indicate that a nuclear trans-acting protein binds to the Sp1.1 element at -164 to -142 bp on the antisense strand of the $\alpha 1(I)$ procollagen promoter and that this DNA binding activity is increased by treatment of human fibroblasts with TGF $\beta 1$. An additional DNA-protein complex (R2) is formed with this Sp-1-like element, but this binding activity is less consistently increased by TGF $\beta 1$.

DISCUSSION

The mechanisms involved in the regulation of collagen production under normal or pathologic conditions are not completely understood (reviewed in Refs. 20 and 21). Although the synthesis of most proteins in eukaryotic cells appears to be regulated at a transcriptional level, post-transcriptional events, such as the regulation of the stability of newly synthesized mRNA, may play an important role under certain conditions (46, 47). Studies of the transcriptional regulation of various collagen genes in human and rodent cells *in vitro* have demonstrated the presence of regulatory elements located immediately 5' upstream of the transcription initiation site (22-26). In addition, an enhancer element located within the first intron has been identified in the type I collagen genes (32-34). Sequences located far upstream of the initiation of the transcription site may also be involved in the regulation of expres-

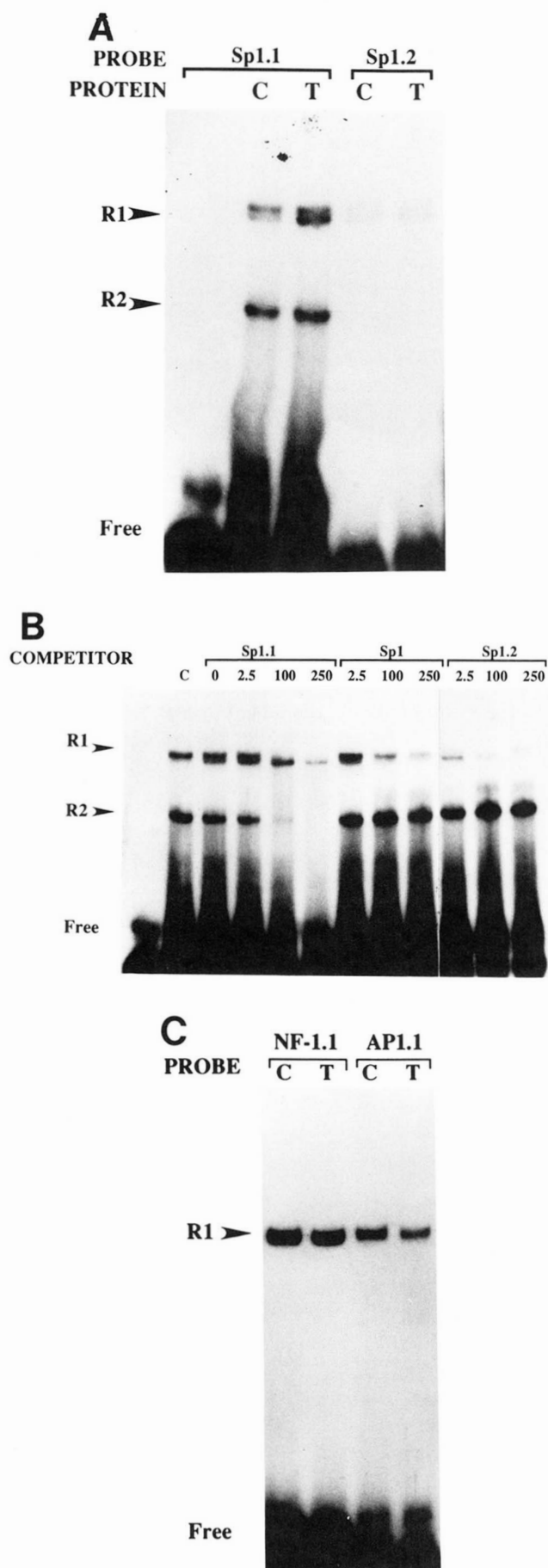


FIG. 8. Electrophoretic mobility shift assay with Sp1.1 and Sp1.2 probes and competitors. Panel A, the 23 bp Sp1.1 and

Sp1.2 probes and competitors. Panel B, the 23 bp Sp1.1 and Sp1.2 probes and competitors. Panel C, the 23 bp Sp1.1 and Sp1.2 probes and competitors.

sion of the collagen genes (48, 49). Rippe *et al.* (49) analyzed murine $\alpha 1(I)$ procollagen gene sequences encompassing -3700 to $+1400$ bp and found that a short segment of the promoter (220 bp upstream from the start of the transcription site) was sufficient for expression of the gene, whereas further upstream flanking sequences had a negative effect on transcription. In order to identify upstream elements that may be involved in the regulation of transcription of the human $\alpha 1(I)$ procollagen gene, we determined the nucleotide sequence of the promoter region encompassing from -804 to -2292 bp. Comparison of the newly obtained sequence with that of the promoter region in the rat $\alpha 1(I)$ procollagen gene (43) indicated less than 65% overall nucleotide identity. However, several regions with highly conserved sequences in the two species were found between -1900 and -1540 bp (*underlined* in Fig. 1). The high degree of nucleotide sequence conservation between the two species in this region suggests that these sequences may have important regulatory functions. In the rat gene, this region contains two repeats that are variations of the palindromic sequence CCCTCCC. Analysis of the newly obtained sequences of the human gene reported here demonstrated two identical palindromes localized at -1687 and -1063 bp. Other putative regulatory sequences were also identified in the newly obtained sequence. These included two Sp1 binding sites (GGGCGG) at positions -2168 and -1614 bp and one NF-1 (half-site) binding sequence (GCCAA) at position -830 bp (reverse strand).

To perform a functional analysis of the upstream sequences, several chimeric constructs containing DNA fragments spanning up to -5.3 kb of the $\alpha 1(I)$ procollagen promoter ligated to the CAT gene were prepared, and their expression was examined following transient transfection into NIH/3T3 cells. Maximal transcriptional activity was noted when promoter sequences with 5' end points from -176 to -804 bp were included. Constructs containing further upstream sequences showed progressively decreasing activity (Fig. 2). These results are similar to those reported by Rippe *et al.* (49) for the murine $\alpha 1(I)$ procollagen gene.

In order to examine if there were differences in the regulation of $\alpha 1(I)$ procollagen gene transcription in cells that constitutively produce high levels of collagen and cells that normally do not display expression of interstitial collagen genes, we compared the expression of the promoter-CAT constructs following their transfection into NIH/3T3 cells or into HeLa cells. Marked differences in the expression of the promoter were observed between these two types of cells. Transfection of non-collagen-producing HeLa cells resulted in relatively low levels of CAT activity, which, in marked contrast to NIH/3T3 cells, was increased when promoter constructs containing -2.3 and -5.3 kb upstream sequences were examined (Fig. 3). The dif-

17 bp Sp1.2 double-stranded oligonucleotides encompassing the human $\alpha 1(I)$ procollagen promoter sequences -164 to -142 bp or -93 to -77 bp, respectively, were end-labeled and used as probes in electrophoretic mobility shift assays. Nuclear extracts were prepared from normal human skin fibroblasts that had been treated with TGF β 1 (10 ng/ml) or left untreated for 48 h. Labeled probes were incubated with nuclear extracts under identical conditions, as described in the legend to Fig. 7. The positions of the shifted complexes (R1 and R2) are indicated, along with the position of the unbound DNA. Lane 1, control containing labeled Sp1.1 probe but no protein. C, nuclear extract prepared from untreated fibroblasts; T, nuclear extract prepared from TGF β 1-treated fibroblasts. Panel B, same as panel A, except that only the Sp1.1 oligonucleotide probe and increasing concentrations (2.5-, 100-, and 250-fold molar excesses) of unlabeled double-stranded competitor oligonucleotides were used. Nuclear extracts were prepared from TGF β 1 (10 ng/ml)-treated fibroblasts, except in lane 2 (C, untreated fibroblasts). Panel C, same as panel A, except the AP-1.1 and NF-1.1 oligonucleotides, corresponding to the $\alpha 1(I)$ procollagen gene sequence from positions -175 to -160 bp and -102 to -85 bp, respectively, were used. The arrowhead denotes the retarded DNA-protein complex (R1).

ferences in expression of the various constructs when transfected into collagen-producing and -nonproducing cell types suggest that the intracellular milieu plays an important role in the regulation of the rates at which the collagen genes are transcribed. Furthermore, these results suggest that cell-specific transcriptional factors that act on the upstream sequences of the $\alpha 1(I)$ procollagen gene may influence the expression of this gene.

To further investigate the functional role that the $\alpha 1(I)$ procollagen promoter may play during dynamic events requiring remodeling of the extracellular matrix, we examined the effects of TGF $\beta 1$ on the expression of the various promoter constructs. It has been suggested that this growth factor acting in autocrine and paracrine fashion plays an important role in the regulation of extracellular matrix metabolism during development, differentiation, and repair (reviewed in Refs. 50 and 51). TGF β causes marked accumulation of collagen *in vivo* and *in vitro* (52, 53). In animal models of hepatic and pulmonary fibrosis, TGF β mRNA is expressed in high levels in tissues prior to the development of fibrosis (54–56). Furthermore, recent observations implicate TGF β in the pathogenesis of various human diseases characterized by exaggerated fibrosis (57–60). In the present study, we found that TGF $\beta 1$ stimulated CAT activity driven by $\alpha 1(I)$ procollagen promoter constructs with 5' end points distal to –84 bp in transiently transfected NIH/3T3 cells. These results indicate that TGF $\beta 1$ -responsive sequences in the human $\alpha 1(I)$ procollagen promoter are located between –174 and –84 bp from the transcription start site.

To determine if TGF $\beta 1$ induced alterations in DNA binding activity in fibroblasts, we examined DNA-protein complex formation in nuclear extracts prepared from TGF $\beta 1$ -treated and untreated cells by electrophoretic mobility shift analysis using DNA probes spanning the proximal region of the $\alpha 1(I)$ procollagen promoter. The results showed a marked increase in DNA binding activity that was specific for the probe encompassing nucleotides –170 to –80 bp in nuclear extracts from TGF $\beta 1$ -treated cells. This region of the human $\alpha 1(I)$ procollagen promoter contains a binding site for Sp1 at –87 bp and an element with complete identity to the 3' portion of the canonical NF-1 binding motif (GCCAA), located in reverse orientation at –95 bp. These overlapping potential binding sites have been shown to be important in the basal expression of the mouse $\alpha 1(I)$ procollagen gene (29). In addition, a sequence similar to the AP-1-binding site (5'-GAGTCC) is located in reverse orientation at –165 bp, and a sequence of perfect identity with a binding site for an Sp1-like factor from the GC2 cis-acting element of the rat growth hormone and the F2 element of the human growth hormone genes (GGGAGGAG) is found at –148 bp in reverse orientation (45). When sequences in the –170 to –80 bp region of the human $\alpha 1(I)$ procollagen promoter homologous to these consensus binding sites were examined in electrophoretic mobility shift assays, only the Sp1.1 probe (corresponding to the sequence identical with GC2 and F2) demonstrated increased DNA-protein complex formation with nuclear extracts from TGF $\beta 1$ -treated when compared with untreated fibroblasts. The formation of the R1 complex was abolished or decreased in competition assays with all three Sp1-related oligonucleotides, in contrast to the R2 complex, which was prevented only by the Sp1.1 oligonucleotide competitor. These observations suggest that at least one component of the R1 complex may be a member of the Sp1 family of transcription factors. Of interest in this regard is the previous demonstration that Sp1 interacted with cis-acting elements within the first intron (34) or the promoter (61) of the $\alpha 1(I)$ procollagen gene. Sp1, originally described as a factor required for SV40 transcription, binds to "GC boxes," which are found in the promoters of many mammalian genes (44). Although ubiquitously ex-

pressed, recent evidence points to transcriptional regulation as an important role for Sp1 (62). Overexpression of Sp1 was shown to inhibit mouse $\alpha 1(I)$ procollagen promoter activity in transiently transfected NIH/3T3 cells (29). However, when co-transfected into *Drosophila melanogaster* Schneider L2 cells, which are devoid of homologs of mammalian transcription factors, Sp1 overexpression caused potent trans-activation of the $\alpha 1(I)$ procollagen promoter (63).

In the rat $\alpha 1(I)$ and the mouse $\alpha 2(I)$ procollagen genes, TGF β -responsive elements resembling the NF-1 consensus sequence have been described (64, 65). In the rat $\alpha 1(I)$ procollagen gene, the putative "TGF β activating element" was reported to be located 1.6 kb upstream from the transcription start site (65). The element contains the 3' portion of the canonical NF-1 sequence GCCAAG also found in the mouse $\alpha 2(I)$ promoter. However, in contrast to the mouse $\alpha 2(I)$ procollagen gene, stimulation of rat $\alpha 1(I)$ procollagen gene expression by TGF $\beta 1$ does not appear to involve NF-1 binding to its cognate DNA element (66). Analysis of the nucleotide sequence of the human $\alpha 1(I)$ procollagen gene indicates that a NF-1-like sequence similar to the rat TGF β activating element is located at –1718 bp (5'-TGCCCACGCCAGC). However, our results indicate that deletion of a 1.5 kb fragment including this element did not prevent stimulation of promoter activity by TGF $\beta 1$ (Fig. 4), suggesting that in NIH/3T3 cells this NF-1-like element is not involved in TGF $\beta 1$ -induced transcriptional activation of the $\alpha 1(I)$ procollagen gene. The nature of trans-acting factors, which are implicated in the modulation of the expression of diverse TGF β -responsive genes, is not completely understood. Recent studies suggest that, depending on the cell type and the gene that is regulated, a variety of distinct nuclear proteins may be involved (67). Moreover, transcriptional regulation by TGF β is likely to be a complex process, associated with the combinatorial interactions of ubiquitous and inducible trans-acting factors. The identification of cell-specific DNA binding factors and of the stimulatory and inhibitory cis-acting elements in the promoter of the human $\alpha 1(I)$ procollagen gene that are targets for these factors will permit a better understanding of the complex mechanisms that modulate the transcriptional activity of collagen genes during physiologic processes of development and repair as well as in a variety of diseases characterized by excessive collagen production.

Acknowledgments—We thank Dr. Kyong Yoon for providing the pSV2AP plasmid and Dr. David Hall for thoughtful suggestions. The skilled secretarial assistance of O. Ma in the preparation of this manuscript is gratefully acknowledged.

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