Type I and III fibrillar collagens are the major structural proteins of the extracellular matrix found in various organs including the myocardium. Abnormal and progressive accumulation of fibrillar type I collagen in the interstitial spaces compromises organ function and therefore, the study of transcriptional regulation of this gene and specific targeting of its expression is of major interest. Transient transfection of adult cardiac fibroblasts indicates that the polyurine-polypyrimidine sequence of \( \alpha(1)(I) \) collagen promoter between nucleotides −200 and −140 represents an overall positive regulatory element. DNase I footprinting and electrophoretic mobility shift assays suggest that multiple factors bind to different elements of this promoter region. We further demonstrate that the unique polyuridylate sequence between −172 and −138 of the promoter represents a suitable target for a single-stranded polyurine oligonucleotide (TFO) to form a triple helix DNA structure. Modified electrophoretic mobility shift assays show that this TFO specifically inhibits the protein-DNA interaction within the target region. In vitro transcription assays and transient transfection experiments demonstrate that the transcriptional activity of the promoter is inhibited by this oligonucleotide. We propose that TFOs represent a therapeutic potential to specifically influence the expression of \( \alpha(1)(I) \) collagen gene in various disease states where abnormal type I collagen accumulation is known to occur.

In response to tissue injury or invasion, a healing response is invoked that ultimately leads to an accumulation of fibrillar type I collagen. This is true for many systemic organs and the heart. Such a healing response, when unabated and invoked in the absence of injury, leads to a progressive interstitial fibrosis that proves pathologic. Parenchymal cell function is compromised by a disproportionate concentration of type I collagen, a characteristic feature of interstitial fibrosis in different organs (1–9). Various stages of organ dysfunction are marked by the absence of injury, leading to a progressive interstitial fibrosis that proves pathologic. Parenchymal cell function is compromised by a disproportionate concentration of type I collagen, a characteristic feature of interstitial fibrosis in different organs (1–9). Various stages of organ dysfunction are marked by the absence of injury, leading to a progressive interstitial fibrosis that proves pathologic. Parenchymal cell function is compromised by a disproportionate concentration of type I collagen, a characteristic feature of interstitial fibrosis in different organs (1–9).

A wide array of hormones, cytokines, and growth factors have been implicated in the mediation of fibrous tissue formation (10–20). Many of these factors mediate their action through transcriptional mechanisms. Therefore, the study of transcriptional regulatory elements within the \( \alpha(1)(I) \) and \( \alpha(2)(I) \) collagen gene promoters and their trans-acting protein factors is of major interest. Effector cells that bring about fibrosis include interstitial fibroblasts and phenotypically transformed fibroblast-like cells termed myofibroblasts (21).

Several cis-acting elements in the \( \alpha(1)(I) \) and \( \alpha(2)(I) \) collagen genes located on both sides of the transcription start site as well as their trans-acting factors have been identified (see recent reviews, see Refs. 22–24). Very little is known about the factor(s) binding to the −200 to −140 region of the \( \alpha(1)(I) \) collagen promoter that includes the 35-bp \( 1(I) \) collagen promoter could potentially serve as a target for a novel antisense strategy, namely the triplex strategy, which employs single-stranded DNA oligonucleotides that bind to the major groove of a double-stranded DNA to form a triple helix in a sequence-specific manner. These complexes have been shown to inhibit sequence-specific DNA-binding proteins, thereby affecting the transcriptional activity of various promoters in both in vitro and in vivo experiments (29–32).

In the present study, we provide evidence for the presence of...
multiple factors in rat cardiac fibroblasts that specifically interact with the -198 to -138 sequence of the rat a1(I) collagen promoter. We further show that a single-stranded polyuridine oligonucleotide can form a triple-helix structure with the long polypyrinmide segment of the promoter, inhibits the protein DNA interaction in this region and in an in vitro transcription system specifically blocks the transcriptional activity of the long a1(I) promoter. In addition, this triple-helix-forming oligonucleotide significantly inhibits the expression of a reporter gene, CAT, driven by the rat a1(I) promoter in rat cardiac fibroblasts.

**MATERIALS AND METHODS**

Oligonucleotide Synthesis and Preparation—All oligonucleotides used were synthesized on an Applied Biosystems 381A DNA synthesizer at the DNA Core Laboratory of the University of Missouri, Columbia, MO. Double-stranded oligonucleotides were prepared by mixing equal amounts of complementary single strands in the presence of 0.25 M NaCl. The mixture was heated to 80 °C for 5 min, incubated at 55 °C for 30 min, and then at 42 °C for 30 min. The resulting double-stranded oligonucleotides were gel-purified on a 10% polyacrylamide gel, eluted, and concentrated by ethanol precipitation.

Cloning of the Rat a1(I) Promoter Segment—A 1.1-kb fragment of the rat collagen I promoter was isolated by the polymerase chain reaction technique. Based on published sequence data (33), specific 20–30-mer oligonucleotides were synthesized corresponding to the rat cII site of pGEM-3Z vector.(Promega). Plasmid pColCAT140 was constructed as follows. pCol 1.1 (~ 100 to + 113) was digested with XbaI and BglII, which release a 338-bp fragment of the rat a1(I) gene (~ 225 to + 113). It should be noted that the XbaI site is in the vector 3’ to the collagen sequence. This fragment was cloned into the XbaI-BamHI site of the pCAT Basic vector (Promega, WI). To construct plasmid pCol140, the pColCAT220 plasmid was digested with EcoRI, the ends of the fragments were filled-in and were digested with XbaI. The resulting 249-bp fragment (~ 136 to + 113) was isolated and inserted into the XbaI-HindIII site of pGEM 4Z vector (Promega). Plasmid pColCAT140 was constructed by isolating the XbaI-HindIII fragment of plasmid pCol140, which contains the ~ 136 to + 113 sequence of rat a1(I) gene, and cloning it into the HindIII site of pCAT Basic vector. Plasmid DNA for transfection was prepared by the alkaline lysis method followed by CsCl-ethidium bromide gradient centrifugation.

Gel Mobility Shift Analysis of Triple Helix Formation—Target oligonucleotide Oligo C-1 was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase and was purified through Sephadex G50 column. Approximately 5000 cpm (0.6 ng) was incubated with increasing concentrations of trivalent helix-forming Oligo Col TFO and Oligo Control (final concentrations from 0 to 250 μM in a binding buffer (TFO binding buffer) consisting of 20 mM Tris-HCl (pH 7.4), 20 mM MgCl2, 2.5 mM spermidine, 10% sucrose, 0.25 mg/ml bovine serum albumin, and incubated at 22 °C for 60 min. Samples were electrophoresed through a 8% polyacrylamide gel (0.5 μM Oligo C-1, 0.125 μM hemin, 11 μmol of Hela nuclear extract containing 15% poly(dI-dC)). Twenty ng (30 fmol) of the 1.1-kb fragment was incubated in a volume of 10 μl containing 25 mM Tris and 10 μl of 250 μg/ml yeast RNA, 5% glycerol, 1 mM dithiothreitol, 0.1 M phenylmethylsulfonyl fluoride, 0.1 M sodium dodecyl sulfate, and 0.1% sodium dodecyl sulfate, 5% glycerol, 0.5 M sodium acetate, 10% glycerol, 0.5 mM dithiothreitol, 10% sucrose, and 5% glycerol, and 2 μg of poly(dI-dC). Following electrophoresis in a 6% polyacrylamide gel (acrylamide/bis at 40:1) in Tris-glycine-EDTA buffer (37 mM Tris-glycine-EDTA, pH 8.0) and 10 μl of 250 μg/ml yeast RNA, 5% glycerol, 0.5 M sodium acetate, 10% glycerol, and 2 μg of poly(dI-dC) resulting in a final volume of 12 μl. The reaction mixture was then loaded on the same gel as described above.

In Vitro Transfection Assays—Twenty ng (30 fmol) of the 1.1-kb collagen fragment, 5 ng (30 fmol) of the 250-bp fragment, isolated from pCol 1.1 and pCol140, respectively (final preincubation concentration 7.5 μM), and approximately the same concentration of 0.9-kb CMV IE fragment were preincubated in separate reactions with increasing concentrations of Col TFO and Oligo Control (0–25 μM) in 4 μl of TFO binding buffer as described above. After 1 h of preincubation at 22 °C, 10 μg of RCF nuclear extract was added in a volume of 8 μl containing 25% Tris-HCl (pH 6.8), 0.5 μM Oligo C-1, 0.1 mg/ml yeast RNA, 5% glycerol, 0.5 M sodium acetate, 10% sodium dodecyl sulfate, 0.1 M dithiothreitol, 10% glycerol, and 2 μg of poly(dI-dC) resulting in a final volume of 12 μl. Binding reaction was allowed to proceed for 25 min on ice, and the reaction mixture was then loaded on the same gel as described above.
the Lipofectamine method (Life Technologies, Inc.) according to the manufacturer’s recommendation. Four μg of ColCAT plasmids and 2 μg of PSV2Gal plasmid (Promega, WI) were preincubated with 20 μl of Lipofectamine in 300 μl of serum-free DMEM at room temperature for 30 min. Cells were washed twice with prewarmed DMEM. Then, the preincubated mixture was diluted in DMEM to a final volume of 3 ml and added to plates. Cells were incubated for 2 h at 37 °C. Oligonucleotides were preincubated with Lipofectamine under the same conditions as the plasmid DNAs, except that the final dilution to 3 ml was done in DMEM supplemented with 3% fetal calf serum. Cells were washed twice, and were incubated in the oligonucleotide containing mixture for 4 h at 37 °C following which the medium was changed to DMEM containing 10% fetal calf serum. Cells were harvested by scraping 24 h after start of transfection into a 1-ml solution of 40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl. Cells were centrifuged for 1 min in a microcentrifuge, and resuspended in 60 μl of 0.25 M Tris-HCl (pH 7.5). Cell extracts were made by three cycles of freezing and thawing in dry ice/ethanol and 37 °C water bath for 5 min each. Supernatant was removed, and protein concentration was measured by the Bradford method (42).

Assays for CAT and β-Galactosidase Activity—CAT activity was measured according to Gorman et al. (37). Samples were heated for 10 min at 68 °C prior to assay of CAT activity. Radiolabeled chloramphenicol (0.1 μCi of d-threo-[1-14C]chloramphenicol) and 20 μl of 4 mM acetyl coenzyme A were added to samples containing 10 μg of protein and 50 μl of 1 M Tris-HCl (pH 7.8) in a final volume of 100 μl. The samples were incubated for 1.5 h at 37 °C, which was within the linear range for these reactions. After ethyl acetate extraction, the chloramphenicol and the acetylated products were separated by thin layer chromatography for 1.5 h in a 95:5 ratio of chloroform to methanol and 37 °C water bath for 5 min each. Supernatant was removed, and protein concentration was measured by the Bradford method (42).

RESULTS

The −220 to −120 Segment of the α1(I) Collagen Promoter Contains Multiple Binding Sites for Factors Present in RCF Nuclear Extracts—in order to identify specific binding proteins in rat cardiac fibroblasts, nuclear extracts were prepared from cultured adult rat cardiac fibroblasts and DNase I footprint analysis was carried out as described using a BglII-HaeIII restriction fragment of the α1(I) collagen promoter (−225 to −123), labeled at the −223 position on the noncoding strand (Fig. 1, asterisk). Five areas of protection designated a through f were seen (Fig. 2, lanes 3 and 4). The corresponding nucleotide sequences are underlined in Fig. 1. The protected areas a and b are part of the 35-bp-long purine-rich region, areas d, e, and f correspond to the purine-rich sequence distal to the purine-rich region, and the protected area c is an interconnecting sequence between the two. Examination of the sequences protected by nuclear factors revealed that among the different areas, the complementary sequence to AAAGGG of area f can be found in areas b and a; furthermore, the complementary sequence to GGAGG of area e is present in area a. These results indicate that multiple binding sites are present within the −200 to −120 region of the α1(I) collagen promoter that form complexes with protein factor(s) present in the RCF as well as HeLa cell nuclear extracts. It is important to note that many of these binding sequences, especially binding sites a, b, and c, are highly conserved among species (25), indicating the functional importance of these elements in the regulation of collagen gene expression.

The Purine-rich Elements Bind More than One Factor—In order to better characterize the protein-DNA interactions within these footprinted segments of the α1(I) promoter, double-stranded oligonucleotides corresponding to the −170 to −141 purine-rich sequence (designated Oligo C-1), and to the −197 to −173 purine-rich sequence (designated Oligo C-2) were synthesized and used in electrophoretic mobility shift assays. A sequence farther upstream (designated Oligo C-3) was also used as a control in competition experiments. The location and the sequences of these oligonucleotides are shown in Fig. 1. In earlier work by Karsenty and de Crombrugghe (27), oligonucleotide sequences contained within the mouse α1(I) collagen promoter corresponding to Oligo C-1 and Oligo C-2 were used in electrophoretic mobility shift assays and were found to bind the same factor present in NIH 3T3 cell nuclear extracts. Transient transfection experiments using substitution mutations of the nucleotides labeled with vertical arrows in Fig. 1 indicated that this factor acted as a transcriptional inhibitor. It is of note that while the sequence of Oligo C-1 is identical in rat and mouse, the purine-rich Oligo C-2 shows only about 80% homology between these two species. The results of the EMSAs in this study are shown in Fig. 3. Oligonucleotides C-1 and C-2 were end-labeled and incubated
with RCF nuclear extracts as described. Using Oligo C-1 as a probe, two distinct slow migrating bands, indicating the formation of DNA-protein complexes, are seen (Fig. 3, lane 2, see arrows on left). The incubation of Oligo C-2 probe with nuclear extract resulted in three slow migrating bands (Fig. 3, lane 9, arrows on right). Competition experiments showing inhibition of complex formation by the use of 10- and 100-fold molar excess of unlabeled C-1 and C-2 oligonucleotides with their respective probes demonstrate the specificity of these complexes (Fig. 3, lanes 3 and 4 and lanes 10 and 11). This is further supported by the fact that the Oligo C-3 was unable to inhibit the formation of any of these complexes (Fig. 3, lanes 7 and 14). Looking at the mobility and the pattern of the complexes, it is interesting to note that the sharper top bands in lanes 2 and 9 migrate at the same position, while the mobility of the more diffuse lower bands is different. Furthermore, the pattern of competition indicates that the affinity of protein-DNA binding may be different within the various complexes, as evidenced by complete inhibition of the top complexes at 10 times lower molar excess of the respective competitors than is seen with the lower complexes.

Substantial homology exists in the sequences of the different binding sites as we discussed above. The experiments of Karsenty and de Crombrugghe (27) suggested the presence of negative regulatory factors in NIH 3T3 cell nuclear extracts that bind to both oligonucleotides of the mouse promoter corresponding to Oligo C-1 and C-2. Therefore, we performed cross-competition experiments using unlabeled Oligo C-1 to compete the binding of labeled Oligo C-2 and vice versa (Fig. 3, lanes 5 and 6 and lanes 12 and 13). The results show that Oligo C-1 was able to completely compete with the binding of all three complexes to Oligo C-2 probe (Fig. 3, lanes 12 and 13) indicating that Oligo C-1 contains all the elements required for binding of nuclear factors to Oligo C-2. Once again, a differential competition pattern among the three complexes can be seen (the middle complex is competed at lower molar excess of Oligo C-1 than the top and lower ones). By contrast, when unlabeled Oligo C-2 was used to compete with the binding of nuclear factors to Oligo C-1 probe, complete competition was not seen (Fig. 3, lanes 5 and 6). Only the slower moving complex was competed, and the lower complex showed no inhibition. In fact, it would appear that in the presence of unlabeled Oligo C-2, the binding of Oligo C-1 to nuclear factor(s) becomes more compact and slightly shifted. The results suggest that Oligo C-2 does not contain all elements necessary for the formation of complexes like Oligo C-1. It is also possible that Oligos C-1 and C-2 may contain some closely related elements that have different binding affinities to the same protein factor. The fact that the slower moving complex (Fig. 3, top arrow) is formed with both C1 and C2, it is very likely that this factor may be similar to 1F-1 (27). The other complex may be more specific to rat cardiac fibroblasts. Further analyses with mutant oligonucleotides and both mouse and rat nuclear extracts are required to identify the nature of these factors.

Oligonucleotide Col TFO Forms Triple Helix Structure with Oligo C-1—The 35-bp long polypuridine sequence from -172 to -138 represents a unique structure in the promoter of the rat α1(I) collagen gene. Such long stretches of all C and T nucleotides do occur in other genes, but only rarely. We have hypothesized that a single-stranded oligonucleotide with complementary sequence could be able to form a triple helix DNA structure with the polypyrmidine portion of the collagen promoter as target sequence. In order to demonstrate the formation of triple helix on target sequence, gel mobility shift assays (39) have been performed. The detection of triple structure in this electrophoresis system is based on the observation that the triple helix migrates slower as compared to double-stranded oligonucleotide in acrylamide gel due to the reduction of DNA charge that is likely to accompany triplex formation (31). Initial testing of triple helix formation was performed with the use of a single-stranded oligonucleotide with identical sequence to the polypurine strand of Oligo C-1. The sequence and orientation of this oligonucleotide, designated Oligo Col TFO is shown in Fig. 1. Radiolabeled double-stranded DNA target (Oligo C-1) was incubated with increasing amounts of specific Oligo Col TFO
and Oligo Control. The results are shown in Fig. 4. The addition of increasing concentrations of Oligo Col TFO relative to target results in a gradual shift from duplex (D) to a distinct higher migrating band (T), indicating the formation of triple helix. This concentration-dependent shift reaches 50% at approximately 1 μM. This corresponds to a 1000-fold molar excess of oligonucleotide to duplex, yielding an approximate dissociation constant $K_d$ of $10^{-6}$ M, based on the equation $D/T = K_d \times [\text{Pur}]$, where $[\text{Pur}]$ is the final concentration of purine oligonucleotide (31). By contrast Oligo Control failed to form triple helix as evidenced by the absence of retarded band even at higher concentrations (Fig. 4, lanes 6–10). In summary, the gel mobility shift analysis showed that the single-stranded Oligo Col TFO forms triplex with target Oligo C-1 in a sequence-specific manner within the expected $K_d$ range for triple helix structures.

Oligonucleotide Col TFO Inhibits the DNA-Protein Interaction between Oligo C-1 and Protein Factors Present in RCF Nuclear Extracts—There is a growing body of evidence that specific triple helix-forming oligonucleotides can effectively inhibit the binding of trans-acting factors to their cis-acting element in a variety of gene promoters both in vitro and in vivo. In order to evaluate the effect of Oligo Col TFO on the binding of nuclear factors contained in RCF nuclear extracts to Oligo C-1, we performed electrophoretic mobility shift assays as described. The conditions were slightly altered to optimize both Col TFO and protein binding. Fig. 5 shows the results of competition experiments using specific Col TFO and control oligonucleotides (Oligo Ctr) as competitors for the RCF nuclear protein binding to target oligonucleotide. Increasing amounts of Col TFO completely eliminated the formation of protein DNA complexes (Fig. 5, lanes 4–8), whereas the control oligonucleotides had no effect on the binding of these factors (Fig. 5, lanes 9–12). To exclude the possibility of protein factors binding to single-stranded oligonucleotide, Oligo Col TFO was end-labeled and incubated with RCF nuclear extract at the highest concentration used in competition experiments. The results (Fig. 5, lane 2) clearly show that inhibition of protein binding in the target region is not due to the ability of Oligo Col TFO to form complexes with the same protein factors. It is interesting to note that specific Col TFO was able to significantly compete at 30 times less molar excess than that required to convert duplex DNA into a triplex. Moreover, the two complexes seen in Fig. 5 (lane 4) are differentially competed by the specific TFO. For instance, approximately 100 times more molar excess of Oligo Col TFO is required to achieve the same level of competition of the upper complex than the lower one. This, once again, supports the notion that factors forming complexes within the target region (Oligo C-1) have different binding affinities to their cognate sequences.

Effect of Triplex Formation on $\alpha 1(I)$ Collagen Transcription—The effect of triplex formation and inhibition of protein factor binding to template in vitro transcription of $\alpha 1(I)$ collagen gene was studied using a HeLa nuclear extract transcription system. The 1.1-kb fragment of the rat $\alpha 1(I)$ collagen gene used as template DNA contains 1000 bp of the promoter and 100 bp 3′ to the transcription start site. Promoter-dependent run-off transcription generates a 100-nucleotide native $\alpha 1(I)$ collagen transcript (Fig. 6, lane 1). Incubation of the collagen template with increasing amounts of triple helix-forming Oligo Col TFO resulted in a concentration-dependent inhibition of transcription (Fig. 6, lanes 2–5), whereas the non-triplex-forming Oligo control had no effect even at the highest concentration used (Fig. 6, lane 6). Additionally, $\alpha 1(I)$ collagen transcription in the presence of Oligo Col TFO does not appear to give rise to increased amounts of RNA transcripts of smaller sizes, suggesting that inhibition of transcription occurs at the level of initiation rather than elongation. To determine the specificity of Oligo Col TFO inhibition of collagen gene transcription, template DNA containing the −136 to +113 sequence, which is downstream from the Oligo Col TFO target site, was used in vitro transcription under the same conditions. This deleted promoter is sufficient to drive the production of a primary transcript of the same size (approximately 100 nucleotides) (Fig. 6, lane 7). Preincubation of this template with Oligo Col TFO in the same concentration increments resulted in no significant change in the amount of transcript produced (Fig. 6, lanes 8–10). These results suggest that the presence of the poly pyrimidine target sequence is required for Oligo Col TFO to inhibit promoter activity. The effect of oligonucleotides on the transcription process as well as RNA stability was assessed by using the cytomegalovirus immediate early gene used as template DNA contains 1000 bp of the promoter and 100 bp 3′ to the transcription start site. Promoter-dependent run-off transcription generates a 100-nucleotide native $\alpha 1(I)$ collagen transcript (Fig. 6, lane 1). Incubation of the collagen template with increasing amounts of triple helix-forming Oligo Col TFO resulted in a concentration-dependent inhibition of transcription (Fig. 6, lanes 2–5), whereas the non-triplex-forming Oligo control had no effect even at the highest concentration used (Fig. 6, lane 6). Additionally, $\alpha 1(I)$ collagen transcription in the presence of Oligo Col TFO does not appear to give rise to increased amounts of RNA transcripts of smaller sizes, suggesting that inhibition of transcription occurs at the level of initiation rather than elongation. To determine the specificity of Oligo Col TFO inhibition of collagen gene transcription, template DNA containing the −136 to +113 sequence, which is downstream from the Oligo Col TFO target site, was used in vitro transcription under the same conditions. This deleted promoter is sufficient to drive the production of a primary transcript of the same size (approximately 100 nucleotides) (Fig. 6, lane 7). Preincubation of this template with Oligo Col TFO in the same concentration increments resulted in no significant change in the amount of transcript produced (Fig. 6, lanes 8–10). These results suggest that the presence of the poly pyrimidine target sequence is required for Oligo Col TFO to inhibit promoter activity. The effect of oligonucleotides on the transcription process as well as RNA stability was assessed by using the cytomegalovirus immediate early
gene as template. Run-off transcription from the CMV IE promoter generates a 300-nucleotide RNA transcript (Fig. 6, lane 11). As shown in lanes 12–16, at the same concentrations that inhibit collagen transcription, Oligo Col TFO has no effect on transcription from the CMV IE promoter under the same assay conditions. The above results, taken together, strongly suggest that inhibition of \( \alpha_1(I) \) collagen transcription by Oligo Col TFO is due to promoter-targeted triple helix formation.

Inhibition of \( \alpha_1(I) \) Promoter Activity by Oligo Col TFO in Cultured RCF—We next investigated whether Oligo Col TFO had any effect on transcription from the CMV IE promoter under the same assay conditions. The above results, taken together, strongly suggest that inhibition of \( \alpha_1(I) \) collagen transcription by Oligo Col TFO is due to promoter-targeted triple helix formation.

Inhibition of \( \alpha_1(I) \) Promoter Activity by Oligo Col TFO in Cultured RCF—We next investigated whether Oligo Col TFO had any effect on the transcriptional activity of the rat \( \alpha_1(I) \) collagen promoter. The \( -1000 \) to \( +100 \) sequence (lanes 1–6) and the \( -150 \) to \( +100 \) sequence (lanes 7–10) of the \( \alpha_1(I) \) collagen gene, and, the \( -600 \) to \( +300 \) fragment of the CMV IE gene (lanes 11–16) were used as templates in in vitro transcription assays. Oligonucleotides at the indicated concentrations were incubated with these templates in separate reactions, followed by HeLa nuclear extract-initiated transcription. Radiolabeled transcription products of expected sizes (100 and 300 nucleotides, respectively) are shown.

The first transfaction and then were retransfected with oligonucleotides complexed with Lipofectamine. As shown in Fig. 7, a dose-dependent inhibition of the CAT activity was observed with Oligo Col TFO on the pColCAT220 construct (Fig. 7, lanes 1–4), whereas the same doses the TFO have no effect on the deletion mutant pColCAT140 (Fig. 7, lanes 5–8). These results suggest that an intact target sequence is required for Oligo Col TFO to exert its inhibitory effect on the expression of the reporter gene. The specificity of this inhibition is further supported by the fact that Oligo Control had no effect on the expression of either of these plasmids (Fig. 7, lanes 9 and 10). These results parallel the results of the in vitro transcription assays and demonstrate that the interaction of Oligo Col TFO with its target sequence leads to inhibition of transcriptional activity of the rat \( \alpha_1(I) \) promoter.

**DISCUSSION**

Two previous studies on the regulatory elements of the \( \alpha_1(I) \) collagen gene have shed considerable light on the cis-acting elements, trans-acting factors and their functional properties in both in vitro and in vivo experiments. Data from Karsenty and de Crombrugghe (27) have shown two distinct binding sites (from \(-190\) to \(-170\), and from \(-160\) to \(-133\)) within the mouse

**FIG. 6.** In vitro run-off transcription assay showing the effect of promoter-targeted triplex-foaming Oligo Col TFO on the transcriptional activity of the \( \alpha_1(I) \) collagen promoter. The \( -1000 \) to \( +100 \) sequence (lanes 1–6) and the \( -150 \) to \( +100 \) sequence (lanes 7–10) of the \( \alpha_1(I) \) collagen gene, and, the \( -600 \) to \( +300 \) fragment of the CMV IE gene (lanes 11–16) were used as templates in in vitro transcription assays. Oligonucleotides at the indicated concentrations were incubated with these templates in separate reactions, followed by HeLa nuclear extract-initiated transcription. Radiolabeled transcription products of expected sizes (100 and 300 nucleotides, respectively) are shown.

**FIG. 7.** Inhibition of \( \alpha_1(I) \) collagen promoter directed transcription by Oligo Col TFO in adult rat cardiac fibroblasts. A, pColCAT220 (lanes 1–4) or pColCAT140 (lanes 5–8) reporter constructs were transfected into RCF cells. Two h later cells were retransfected with Oligo Col TFO or Oligo Control as indicated. Cell lysates were assayed for CAT activity 24 h later. These data are representative of three independent experiments. cpm/\( \mu g \) represents the acetylated counts/\( \mu g \) of protein. The percentage acetylation was calculated as (radioactivity in the acetylated areas)/total extracted from thin layer plates) \times 100. b, histogram showing the results of transient transfection. After adjusting for \( \beta \)-galactosidase activity to normalize transfection efficiency, the CAT assay counts from treated plates were divided by full activity counts to generate percent activity.
Collagen Gene Transcription

α(I) collagen promoter. Competition experiments coupled with substitution mutation analyses indicated that the same factor contained in NIH-3T3 nuclear extracts bound to both of these sites. DNA transfection experiments using 3-bp substitution mutants in these polypurine and polypurine-rich sites suggested that this factor acted as a transcriptional inhibitor (designated IF1) (27). Our data indicate the presence of multiple binding sites within the sequence −190 to −130. EMSAs identified two distinct complexes bound to Oligo C-1 and three slow migrating bands when Oligo C-2 was used as probe. It appears that at least one factor binding to C1 is unique as it is not competed out by C-2. While some of these proteins are similar to the ones described by Karsenty and de Crombrugghe (27), distinct differences are also evident. These multiple bands could be produced by the interaction of different size proteins with or by the formation of homo- or heterodimers. The possible presence of multiple factors and binding elements within these two regions may offer an alternative solution to the seemingly conflicting data shown in the two studies cited, regarding the opposite functional activity of these promoter elements. Karsenty and de Crombrugghe (27) introduced substitution mutations into both proximal and distal elements, while leaving other potentially positive binding elements intact. On the other hand, both we and Brenner et al. (28) used deletion mutations of longer segments of the promoter, thereby eliminating the binding of both negative and putative positive transacting factors. It should be pointed out that deletions, as opposed to point mutations, disrupt the normal organization of the promoter and enhancer and therefore may affect the interactions of different factors. Nevertheless, our studies clearly indicate the presence of some positive regulatory factors that interact with the triplex-forming sequence. The data of Karsenty and de Crombrugghe (27) indicated the binding of a single factor binding to both sites (−190 to −170 and −160 to −130). Since TFO used here is specific to −170 to −141, it is possible that the binding of the negative factor to both sites is essential to exert its maximal effect. Some of the differences between these studies may be due to inherent differences in the promoter sequences and also in the host cells used. For instance, while the proximal polypurine sequence is identical between rat and mouse α(I) collagen promoter, the distal polypurine sequence shows only 80% homology.

Oligonucleotides provide novel reagents for inhibition of gene expression because of their high affinity binding to specific nucleotide sequences. Two strategies for oligonucleotide reagents have been used. The best known involves antisense oligonucleotides, which bind mRNA to inhibit its processing or translation. The second is the triplex strategy, which employs single-standard DNA oligonucleotides that bind to the major groove of a double-standard target DNA (for review, see Refs. 40 and 46). The advantages of the triplex approach include fewer and less degenerative targets, thus offering the potential for low dose long-acting therapeutics. The major limitation of the application of oligonucleotide-directed triplex formation to naturally occurring sequences is the requirement for predominantly purine-pyrimidine tracts. The long pyrimidine sequence of the α(I) collagen promoter represents a unique structure that provides an attractive target for the design of sequence-specific DNA binding agents, which may influence transcription of this biologically important gene. Although most studies have employed pyrimidine-rich TFOS, we chose to use a 30-mer polypurine oligonucleotide corresponding to the noncoding strand of the promoter between −170 and −140 because of its binding stability at physiological pH. It has been suggested that triplex formation is based on the assembly of G-GC, T-AT, and A-AT triplets (39, 41, 46). The orientation of the purine type TFOS in the major groove of the double helical DNA has initially been a matter of controversy. In the first description of triplex formation in the promoter of the human c-myc gene, it was implied that the TFO was bound parallel to the purine strand (31). Later evidence suggested that the TFO in that study could potentially bind either parallel or antiparallel and make similar base contacts with the duplex (42). This is because the c-myc target is pseudopalindromic. We used a TFO that is in parallel orientation with the purine strand and similarly has pseudopalindromic sequences. The binding orientation therefore is likely to be antiparallel. This may explain why slightly higher Kd values were observed in gel mobility shift assays. The repression of the in vitro transcription of the collagen promoter by the specific TFO was complete even at lower concentration than was predicted by TFO titration experiments. This difference could be the result of stabilization of the template and the triplex by components of the nuclear extract (e.g. proteins and polyamines).

To evaluate the effect of triple helix formation on the transcriptional activity of the α(I) promoter, we employed an in vitro transcription system using HeLa nuclear extracts. The reason for this is that extracts from rat cardiac fibroblasts could not sustain transcription due to an apparent RNase activity, which we were unable to avoid even with the use of RNase inhibitors or different ways of preparing nuclear extracts. The usefulness of the HeLa system in the study of collagen gene expression is supported by the work of Furth et al. (43), who showed that type I collagen mRNAs are accurately initiated by HeLa cell RNA polymerase II. In HeLa cells, significant amount of collagen mRNA is synthesized. However, steady-state levels of mRNA are not detected, suggesting posttranscriptional regulation of collagen synthesis in HeLa cells. Furthermore, in the study of Brenner et al. (28), deoxyribonuclease I footprinting of the more proximal promoter from −103 to −82 showed the same pattern of protection for both HeLa and NIH 3T3 nuclear extracts. We also performed DNase I footprinting assays on the promoter fragment from −220 to −120 using HeLa nuclear extract. Fig. 2 shows that the protection pattern of HeLa (lanes 5 and 6) and RCF (lanes 3 and 4) nuclear extracts is identical, lending further support to the usefulness of the HeLa transcription system.

The results of the transient transfection experiments parallel the findings of the in vitro transcription assays. Nevertheless, there are important differences that deserve further discussion. First, the concentration of oligonucleotides relative to plasmid template was markedly different between these two assays. One possible explanation is that a much smaller proportion of plasmids are actually expressed in cells after transfection is established and that oligonucleotides are taken up by cells with much higher efficiency. The intracellular environment may also be more stabilizing for the formation of triple helix structure. Second, the maximum inhibition was about 50%, as opposed to almost complete inhibition of in vitro transcription by Oligo Col TFO. At higher than 1 μM oligonucleotide concentration, there was a decrease in the extractable protein concentration seen, and the expression of pSV2Gal was also affected (data not shown). These nonspecific effects likely represent toxicity and demonstrate the relatively narrow “therapeutic range” of TFOS. Chemical modification of oligonucleotides may improve their potency and will likely widen the margin of safety. Recently published data from Laptev et al. (44) showed the expression of human α(I) collagen gene can be effectively inhibited by modified antisense oligonucleotides at 0.2 μM concentration targeted at specific regions of the α(I) mRNA. Due to the differences in experimental conditions (cell line used; stable versus transient transfection; reporter con-
structure; chemical modification of oligonucleotides), it is difficult to extrapolate data for comparison of relative efficiencies. Therefore, it will be important to directly compare the efficacy of TFOs to antisense oligonucleotides under the same test conditions.

The mechanism by which Oligo Col TFO-directed triple helix formation inhibits the transcriptional activity of \(\alpha I(I)\) collagen promoter is not entirely clear from the data presented here. One likely possibility is the concentration-dependent interference of Oligo Col TFO with the formation of complexes between cis-acting elements within the target region and their cognate trans-acting factor(s). The ability of triple-forming oligonucleotides to compete with site-specific DNA-binding proteins for binding to target sites, as the mechanism accounting for transcriptional repression, has been demonstrated in a number of in vitro and in vivo experiments. The close correlation observed in our study between the ability of Oligo Col TFO to inhibit protein-DNA interaction (Fig. 5) and to repress promoter activity (Figs. 6 and 7) would support, but not conclusively prove, this mechanism. However, considering that the polypyrrimidine target site for Oligo Col TFO has previously been shown to contain elements for binding of a negative trans-acting factor (IF-1) (27), one would expect that inhibition of this factor to bind to its cis-element would result in transcriptional activation. To reconcile these seemingly contradictory findings, the presence of factor(s) with potential positive regulatory activity and an overall positive transcriptional net effect within this target region could once again be considered. Our DNase I footprinting and EMSA data supports this notion, and so does the previously cited result of Brenner et al. (28), confirmed by our transient transfection experiment, showing a 50% reduction of promoter activity upon complete deletion of sequences corresponding to our TFO target site. An alternative explanation for the inhibitory effect of Oligo Col TFO on \(\alpha I(I)\) collagen promoter activity could be adopted from the studies by Maher et al. (45), which showed that site-specific DNA triple helices can repress transcription even when the complexes do not overlap transcription factor binding sites. Their results suggested other possible repression mechanisms including effects on DNA flexibility, recruitment of inhibitory factors, or alteration of chromatin structure. The results of our in vitro transcription assays, showing complete elimination of promoter activity, as opposed to only partial inhibition that would be expected if protein factor binding inhibition was primarily operational, support these latter mechanisms. The only partial inhibition (around 50%) of reporter gene expression by Oligo Col TFO in transient transfection experiments demonstrates the differences and difficulties one encounters when using intact cells. Apparent toxicity prevented us from employing higher concentrations of oligonucleotides.

The identification and characterization of genes that play important roles in cellular processes leading to interstitial fibrosis have provided excellent targets for transcriptional modulation. Because of the ability of TFO’s to selectively inhibit transcription of their target genes in intact cells (29, 30, 32), these oligonucleotides appear to have considerable potential as therapeutic agents. We have identified the unique polypyrimidine of the \(\alpha I(I)\) collagen promoter as a suitable target for a single-stranded polypyrimidine oligonucleotide to form a triple helix structure that could effectively inhibit transcription in vitro and in cultured cells. Further experiments to explore the potential therapeutic applications of chemically modified TFOs in tissue culture and in animal model systems are in progress.

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