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An RNA Oligonucleotide Corresponding to the Polypyrimidine Region of the Rat α1(I) Procollagen Promoter Forms a Stable Triplex and Inhibits Transcription

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In this report we demonstrate formation of a triplex structure by an antiparallel RNA oligonucleotide corresponding to the 21 bp polypurine-pyrimidine stretch from −141 to −162 of the rat α1(I) procollagen promoter with a K_d of 0.1-0.2 μM. The formation of triplexes by the triplex forming oligoribonucleotide (ORN) was also observed under physiological conditions. *In vitro* transcription run-off experiments showed that triplex formation results in inhibited transcription from the rat α1(I) procollagen gene. Our results demonstrate a novel approach for down-regulation of procollagen gene transcription *in vivo*.

Abnormal expression of the genes for types I and III collagen and subsequent deposition of collagen in various tissues under diverse pathologic conditions results in fibrosis and organ dysfunction (1-3). Specific inhibition of collagen gene expression by oligonucleotide based strategies has attractive therapeutic potential. Various cellular genes contain potential triple helix (triplex)-forming homopurine-pyrimidine sequences that can act as targets for triplex-forming oligonucleotides (4). The α1(I) procollagen gene contains two highly conserved polypurine-polypyrimidine sequences upstream of the transcriptional start site from −141 to −200, which have been shown to positively or negatively regulate transcription (5-6). We have previously shown that the polypurine sequence from −141 to −170 (C1) is capable of binding a polypurine DNA TFO within the major groove of the duplex C1 DNA, forming a purine motif (A:AT or G:GC) triplex structure (7-8). This binding occurred in antiparallel orientation to the native C1 sequence and formed triplexes more efficiently compared to parallel oligonucleotides (8). It was also shown that binding of this same antiparallel oligonucleotide correlated with significant inhibition of transcriptional activity from the rat α1(I) procollagen promoter *in vivo* (7-8).

In the present study we have examined the effects of an antiparallel RNA TFO corresponding to the polypurine C1 sequence. The results show formation of triplexes with the same C1 region, under artificial as well as physiological conditions. Furthermore, we show that this triplex formation effectively inhibits transcription from the rat α1(I) procollagen gene *in vitro* using HeLa cell-free nuclear extracts. The ability of an RNA oligonucleotide to bind to transcriptional regulatory *cis* sequences within the rat α1(I) procollagen promoter potentially allows novel therapeutic approaches to be used in controlling over-expression of α1(I) procollagen, and subsequent fibrosis, *in vivo*.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification. All oligoribonucleotides (ORNs) were synthesized by Oligos Etc. Inc. (Wilsonville, Or). Double-stranded oligodeoxynucleotides (ODNs) were prepared by mixing equal amounts of complementary single strands in 0.25 M NaCl, heating to 80°C, and subsequently cooling to room temperature. Annealed double-stranded oligonucleotides were gel-purified on a 6% polyacrylamide gel, eluted, and concentrated by ethanol precipitation.

Electrophoretic mobility shift assays for triple helix formation. Double-stranded ODNs were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified through a Sephadex G-50 column. Approximately 10,000 c.p.m. (≈2.0 nM) were incubated with increasing concentrations of TFO and a control ORN in a binding buffer consisting of 20 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 2.5 mM spermidine, 10% sucrose, 0.25 mg/ml bovine serum albumin, and incubated at 37°C for 60 min. The samples were analyzed on a 10% polyacryl-

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   -170          C1          -141
5'-CCTTTCCCTTCCTTTCCCTCCTCCCCCCTC-3'
3'-GGAAAGGGAAGGAAAGGGAGGAGGGGGGAG-5'

              5'-AAAGGGAGGAGGGGGGAG-3'  AP DNA
          5'-AGGAAAGGGAGGAGGGGGGAG-3'  AP RNA
          5'-GAGGGGGGAGGAGGGAAAGGA-3'  P  RNA
          5'-GAGCGGUGAGCAGGGUAAGGC-3'  NS RNA
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FIG. 1. Sequence of the rat α1(I) procollagen C1 region. The sequence of the antiparallel (AP) ODNs and ORNs as well as the parallel (P) and nonspecific (NS) ORNs are shown below.

amide-0.32% bisacrylamide gel in a buffer containing 89 mM Tris, 89 mM boric acid, pH 7.5, and 20 mM $MgCl_2$ for 5 h at 10 V/cm2 at 6°C. Gels were dried and autoradiographed at −70°C. K_d values were determined by calculating the concentration of TFO at which 50% of target duplex was converted to the triplex form (8).

In vitro transcription assays. Rat a1(I) procollagen triplxes were formed by mixing 2.5 μl TFO binding buffer [20 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 2.5 mM spermidine, 10% sucrose, and 0.25mg/ml bovine serum albumin] with approximately 100 ng of a 0.7 kb collagen template (330 bp of the promoter followed by 360 bp downstream sequence) or CMV control template with increasing concentrations of antiparallel ORN or ODN TFO and a nonspecific RSV Ψ RNA 20-mer control ORN added to make a final concentration of 50 ng/μl. After addition of 10 units of RNase Inhibitor (Boehringer), the resulting 10 μl reaction was incubated at 37°C for 1 h. Templates were then combined with 8 μl nuclear extract buffer [20 mM HEPES (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, and 1 mM each of DTT, PMSF, and sodium bisulphite] followed by addition of 0.4 mM each of ATP, CTP, UTP, and 10 μM cold GTP plus 20 μCi [α-^{32}P]GTP. Four μl HeLa nuclear extract (41 μg of protein) was added and the 25 μl reactions were incubated for 1 h at 30°C. The reaction was stopped by adding 175 μl of stop mix [0.3M Tris-HCl (pH 7.4), 0.3M sodium acetate, 0.5% sodium dodecyl sulfate, 2 mM EDTA, 6 μg/ml Yeast tRNA]. Samples were extracted once with water saturated phenol, once with phenol/chloroform (1:1), then ethanol-precipitated, dried, and RNA resuspended in 20 μl 50% formamide loading dye. After heat-denaturation, samples were loaded on a 6% acrylamide, 7M urea gel, and run at 20 V/cm for 3 h. Gels were directly autoradiographed at −70°C.

RESULTS AND DISCUSSION

The polypurine-pyrimidine sequence from −141 to −170 (C1) is a unique structure that is highly conserved in mammalian α1(I) procollagen promoters. The importance of this and similar sequences in transcriptional regulation have been previously demonstrated (7-9). Earlier, we showed an 18 nt antiparallel DNA TFO exhibited triplex formation with the polypurine C1 target sequence, present on the non-coding strand of the rat α1(I) procollagen gene (8). This binding was subsequently shown to inhibit rat α1(I) procollagen transcription *in vivo* (8). We wanted to test whether antiparallel ORNs would also form triple helix DNA structures with the same C1 region. Therefore, electrophoretic mobility shift assays using 21 nt ORNs (Fig. 1) were performed to demonstrate the triplex formation. The results shown indicated that this antiparallel RNA TFO was able to form a stable triplex with a K_d of 0.1-0.2 μM (Fig.2A), whereas an identical DNA TFO was able to form the same structure with a K_d of 0.08 - 0.1 μM (Fig.2B). In contrast, a parallel ORN (sequence shown in Fig. 1) showed only minimal binding even at 20 μM (Fig.2C).

Previous reports had indicated that normal physiologic conditions are unfavorable for purine motif triplex formation (10). In particular, the high potassium concentration (140 mM) was shown to readily promote aggregation of guanine-rich oligonucleotides into guanine quartets. In addition, triplex formation requires relatively high concentrations (5-20 mM) of multivalent cations such as magnesium for stabilization (11). Earlier we had reported the ability of a DNA antiparallel TFO to form triplex structures even under physiological conditions (8). Here, we tested the ability of our RNA TFO to do the same and the results shown in Fig. 3 indicate binding in the presence of 140 mM KCl, 1 - 5 mM $MgCl_2$, and 1 mM spermidine, with a K_d of about 0.3 μM.

We next wanted to study the effect of RNA triplex formation on expression of the rat α1(I) collagen gene.

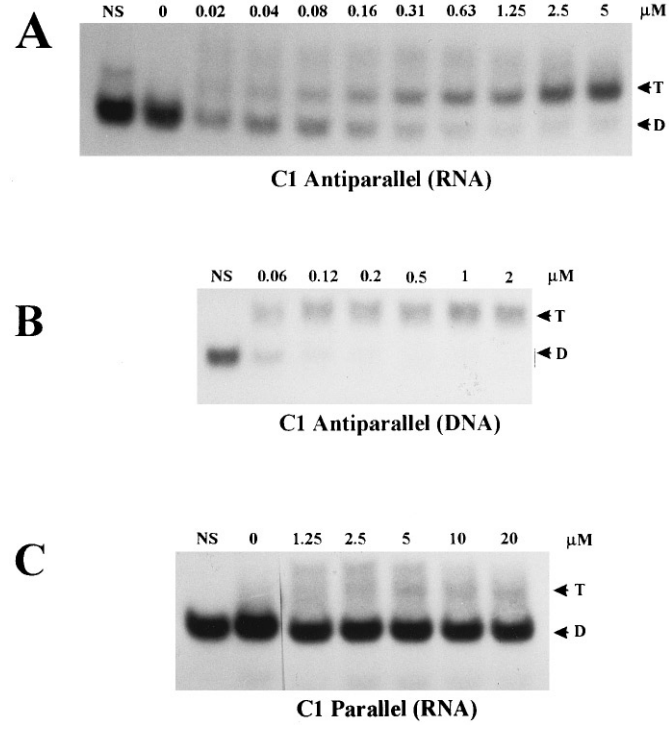

FIG. 2. Triplex formation with antiparallel ORN. Electrophoretic mobility shift assays showing triplex formation of ORN and ODN TFOs with C1 duplex DNA. Duplex DNA concentrations used in each reaction were 2 nM and TFO concentrations are shown above the corresponding lane. All reactions were run and carried out in a TFO binding buffer as described in Materials and Methods. (A) Effect of increasing concentrations of C1 21-mer antiparallel ORN TFO, (B) corresponding C1 18-mer antiparallel DNA TFO, and C1 RNA TFO in parallel orientation (C). **T,** triplex; **D,** duplex.

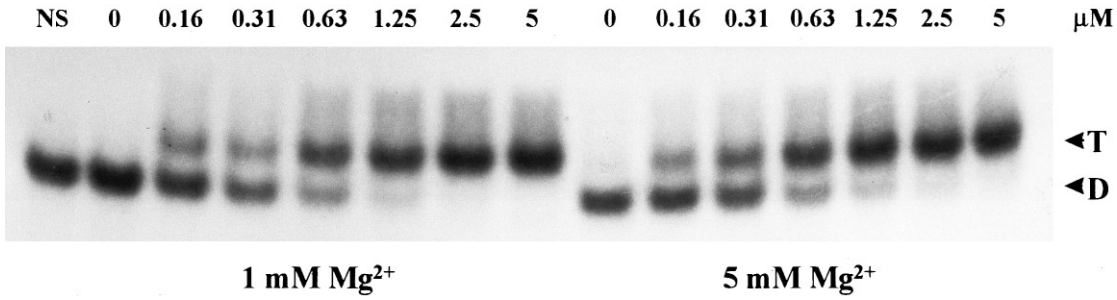

FIG. 3. Effect of physiological concentrations of 1 and 5 mM $MgCl_2$ on increasing concentrations of C1 21-mer antiparallel RNA with C1 duplex DNA. Duplex DNA concentrations used in each reaction were 2 nM and TFO concentrations are shown above the corresponding lane. All reactions were carried out in a physiological buffer containing 20 mM Tris-HCl pH 7.5, 140 mM K^+, 1 mM spermidine, and 1 mM Mg^{2+}. **T,** triplex; **D,** duplex.

Previous *in vitro* transcription results from our lab clearly showed that triplex formation with a DNA TFO to the C1 promoter region resulted in a concentration-dependent inhibition of transcription, which was further supported by subsequent *in vivo* data (7). Here, a 0.7 kb α1(I) collagen fragment (−330 to +360) was used as a template and run-off transcription generated a 360 nt transcript. Incubation of the collagen template with increasing amounts of the previously used polypurine antiparallel ORN or ODN TFO resulted in transcriptional inhibition of the α1(I) collagen gene (Fig.4); whereas no inhibition was seen with a 20-mer nonspecific RSV Ψ RNA oligonucleotide (NS), or TFO in parallel orientation (not shown). The same antiparallel ORN did not affect transcription from a heterologous CMV promoter. These results strongly indicate that the inhibition of transcription is mainly due to the sequence specific triplex binding of the ORN to the C1 promoter region. However, it should be pointed out that although these results are highly reproducible, the K_d for inhibition of transcription varied, probably due to ribonuclease degradation. Even with the addition of RNase inhibitors during triplex formation, the inhibitory effects of the RNA TFO varied to some extent between experiments; and higher concentrations of TFO even abolished transcriptional inhibition (not shown), which may be due to aggregation of ORNs.

The RNA TFO data described here, and our previous *in vivo* results with a comparable DNA TFO, suggest the feasibility of utilizing RNA transcripts in the application of novel therapeutic strategies to down-regulate α1(I) collagen expression *in vivo*. It's conceivable that a retrovirally integrated TFO sequence could be expressed in the nucleus by endogenous RNA polymerases, placing the RNA TFO transcript within proximal vicinity of the target cell chromatin, thereby allowing it to mediate its inhibitory triplex binding effects. Furthermore, it is tempting to speculate that if the TFO sequence were coded into the retroviral vector as an intronic sequence flanked by splice donor, acceptor, and branch sequences, the endogenous splicing machinery of the cell could then be taken advantage of to generate the antiparallel RNA TFO as the overhanging tail of a spliced intron lariat structure, which should then be free to bind to the chromosomal α1(I) collagen gene. Alternatively, taking advantage of catalytic RNAs might offer an additional therapeutic approach to intranuclear RNA TFO production in that an α1(I) collagen binding ribozyme could be coded into a retroviral vector which would cleave the α1(I) mRNA product while still in the nucleus.

We believe that these types of strategies offer a superior approach to conventional antisense strategies which rely heavily on efficient delivery of oligonucleotides into the target cell, their stability within the cell, and specific affinity to the target mRNA message once within the cytosol or nucleus (12-14). In contrast, triplex DNA strategies have the benefit of acting at the gene level within the chromatin itself, but still retain the problem of effective delivery and stability within a target cell (11,15). The use of an ORN TFO delivered retrovirally offers high infectivity and integration rates (16), allowing the TFO to be produced endogenously and in immediate vicinity to the collagen chromatin within the nucleus. Potential nuclear degradation of the RNA transcript in the nucleus would remain a chal-

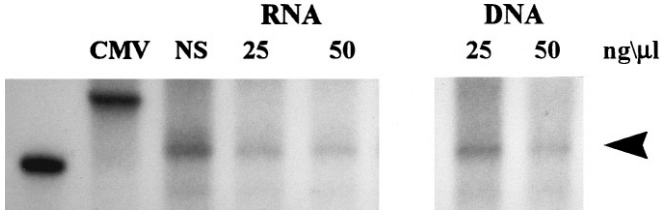

FIG. 4. *In vitro* transcription assays showing the inhibitory effect of both ORN and ODN antiparallel TFOs on the expression of a rat α1(I) procollagen template. ORN and ODN TFO concentrations used are shown above the corresponding lane. Lane 1, 300nt RNA Marker; CMV, cytomegalovirus control template; NS, non-specific RNA oligonucleotide. All reactions were carried out as described in Materials and Methods.

lenge, but the RNA:DNA:DNA hybrid may be more stable *in vivo* than conventional DNA triplexes (17), thereby strengthening the overall lifetime of the message and its ability to bind and inhibit gene expression. The results obtained in this study are encouraging in the effort to design effective, non-toxic delivery systems that offer therapeutic alternatives to control fibrosis-related disorders.

ACKNOWLEDGMENT

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