Inhibition of Collagen α1(I) Expression by the 5′ Stem-Loop as a Molecular Decoy*

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Collagen α1(I) mRNA is posttranscriptionally regulated in hepatic stellate cells (HSCs). Binding of protein factors to the evolutionary conserved stem-loop in the 5′-untranslated region (5′ stem-loop) is required for a high level of expression in activated HSCs. The 5′ stem-loop is also found in α2(I) and α1(III) mRNAs. Titration of the 5′ stem-loop binding factors by a stably expressed RNA containing the 5′ stem-loop (molecular decoy) may decrease the expression of these collagen mRNAs. We designed a 108-nt RNA that is transcribed from the optimized mouse U7 small nuclear RNA gene and contains the 5′ stem-loop (p74WT decoy). This decoy accumulates in the nucleus and in the cytoplasm. When expressed in NIH 3T3 fibroblasts, the p74WT decoy decreased collagen α1(I) mRNA level by 60% and decreased collagen type I secreted into the cellular medium by 50%. We also expressed this decoy in quiescent rat HSCs by adenoviral gene transfer. Quiescent HSCs undergo activation in culture, resulting in a 60–70-fold increase in collagen α1(I) mRNA. The decoy decreases collagen α1(I) mRNA expression by 50–60% during activation of HSCs. It also decreases collagen α2(I) mRNA expression and collagen α1(III) mRNA expression. The cellular levels of collagen α1(I) propeptide and of disulfide-bonded collagen type I trimer are reduced by 70%. However, the p74WT decoy did not decrease α smooth muscle actin protein or the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase and interleukin-6. The p74WT decoy was also introduced into activated human HSCs. In these cells, the decoy decreased collagen α1(I) propeptide and disulfide-bonded collagen type I trimer by 50–60%. These results indicate that the 5′ stem-loop specifically regulates fibrillar collagen synthesis and represents a novel target for antifibrotic therapy. The molecular decoys provide a generalized method of assessing the functional significance of blocking the interactions of mRNA and proteins.

Cirrhosis is characterized by the accumulation of extracellular matrix proteins in the liver, including type I collagen (1, 2). Hepatic stellate cells (HSCs); also named Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the cirrhotic liver (3, 4). In normal liver, quiescent HSCs store vitamin A (5) but only express trace amounts of type I collagen. Upon a fibrogenic stimulus, HSCs become activated, a process in which they lose retinoid droplets, proliferate, change morphologically into myofibroblasts, and increase their synthesis of extracellular matrix proteins (6, 7). Culturing quiescent HSCs on plastic causes activation similar to that seen in liver fibrosis in vivo, including the accumulation of collagen α1(I) mRNA (7, 8). This provides a simple model system to study HSC activation and collagen gene regulation.

Expression of the collagen α1(I) gene is regulated at the transcriptional and posttranscriptional level, producing an increase in the mRNA steady-state levels by 60–70-fold in activated HSCs compared with quiescent HSCs (9). This results from a 3-fold increase in the transcription rate and a 16-fold increase in the half-life of the α1(I) mRNA (9), suggesting a predominantly post-transcriptional regulation. Two cis-acting elements in collagen α1(I) mRNA, the 5′ stem-loop and the C-rich sequence in the 3′-UTR, regulate the turnover of collagen α1(I) mRNA (9, 10). αCP is the protein that binds the C-rich sequence in the 3′-UTR of collagen α1(I) mRNA (11). Since this binding activity is only in activated HSCs, αCP is postulated to be involved in stabilization of collagen α1(I) mRNA.

The 5′-UTRs of three fibrillar collagen mRNAs, α1(I), α2(I), and α1(III), have a stem-loop structure encompassing the translation initiation codon (12). These three mRNAs are coordinately induced in fibrotic processes of various organs (2, 13, 14). The 5′ stem-loop structure is located about 75 nt from the cap and has a stability of ΔG = 25–30 kcal/mol in different collagen mRNAs. Enzymatic probing of a synthetic 5′ stem-loop RNA demonstrated folding into a higher order structure with a bulged A nucleotide (10). The 5′ stem-loop is well conserved in evolution (15, 16), but the sequence flanking the stem-loop is not conserved, suggesting an important function. When the 5′ stem-loop is placed in the 5′-UTR of reporter genes, the expression is inhibited in quiescent HSCs but high in activated HSCs. Reporter genes with the mutated stem-loop are constitutively expressed to a high level in both cell types. Therefore, expression of the reporter mRNA with the 5′ stem-loop resembles expression of endogenous collagen α1(I) mRNA in HSCs; it is low in quiescent HSCs and elevated in activated HSCs and regulated by a posttranscriptional mechanism (10). The 5′ stem-loop is also necessary for down-regulating collagen α1(I) expression in a process where cells revert from an activated phenotype to a more quiescent phenotype. This was achieved by culturing mouse fibroblasts within a three-dimensional matrix composed of type I collagen (17). Similarly, activated HSCs reverse to a more quiescent phenotype when cultured in matrix.
trigel (18). When the 5’ stem-loop was mutated, the full size collagen α1(I) mRNA was more stable in cells grown in the matrix than when this stem-loop was intact (17). Thus, the 5’ stem-loop was required for accelerated decay of collagen α1(I) in this experimental system.

The collagen 5’ stem-loop binds nuclear and cytoplasmic proteins (10, 17). In quiescent HSCs, no protein binding to the 5’ stem-loop was detected. In activated HSCs, a cytosolic protein factor(s) of unknown identity binds to the stem-loop and requires a 7mG cap for RNA binding. A protein of 120 kDa was cross-linked to the 5’ stem-loop of collagen α1(I) mRNA in extracts of activated HSCs (10). The complex is also found in fibroblasts in the postpolysemal cytoplasmic fraction. Its binding is greatly reduced if the cells are cultured in three-dimensional matrix (17). Protein binding to the 5’ stem-loop may increase the steady state level of collagen mRNAs by diverting them from the degradative pathway. Extracts prepared from fibroblasts contain a nuclear protein that binds the collagen 5’ stem-loop. This activity is different from the cytoplasmic binding activity, because it is detected only in nuclear extracts, it does not require the presence of 7mG cap for binding, and it has a different electrophoretic mobility in native gels. The nuclear binding inversely correlates with the accumulation of collagen α1(I) mRNA (17). It is possible that after synthesis, the collagen 5’ stem-loop binds this nuclear factor, which may be required for nuclear export of collagen α1(I) mRNA and may accumulate in the nucleus if the cytoplasmic levels of collagen α1(I) mRNA are decreased. Binding of protein factors to the 5’ stem-loop is necessary for high level of expression of collagen α1(I) mRNA in activated HSCs and fibroblasts. Therefore, titration of these proteins by a highly expressed short RNA containing the 5’ stem-loop (a molecular decoy) may decrease the level of α1(I) mRNA and consequently collagen protein synthesis. Here we describe the design and expression of a short stable RNA containing the collagen α1(I) 5’ stem-loop and its effect on collagen type I synthesis in fibroblasts and HSCs.

**MATERIALS AND METHODS**

**Plasmid and Adenovirus Constructs**—The mouse U7 small nuclear RNA (U7 snRNA) gene with the optimal Sm binding site (p74) was constructed by Dr. C. Grimm (19) and was a kind gift of Prof. Dr. D. Schumperli (University of Bern, Bern, Switzerland). The EcoRI-BamHI fragment of this gene was recloned into the EcoRI-BamHI sites of the Bluescript SK+ vector. A double-stranded oligonucleotide with the sequence of mouse collagen α1(I) 5’ stem-loop was cloned in Stul and BspMI sites of the Bluescript construct, creating the p74WT decoy. The nucleotides flanking the 5’ stem-loop were designed to preserve the transcription start site of the gene, the first 5 nt of the RNA, and the optimal Sm binding site. A oligonucleotide with a mutation in the 5’ stem-loop was cloned by an identical procedure to create the p74MUT decoy. The sequences of WT and MUT stem-loop are published (10). Recombinant adenosviruses that express p74WT and p74MUT decoys were constructed using the simplified system for generation of adenosviruses (20). The HindIII-NotI fragments of the p74WT decoy and the p74MUT decoy constructs were recloned into the HindIII-NotI sites of the pADTRACK vector. The pADTRACK vector contains green fluorescent protein (GFP) expression cassette under control of cytomegalovirus promoter as a transcription unit that is independent of the cloning of the molecular decoys. The viral genomes were reconstituted by recombination in Escherichia coli between the pADTRACK constructs and pAdEasy-1 plasmid. Viral genomes were packed into infectious particles after transfection into 293 cells. The resulting viruses express both the p74WT or p74MUT decoy and GFP, which serves as a control of infection. Viruses were tested for expression and amplified by the UNC Viral Core facility.

**Isolation and Culture of HSCs**—HSCs were isolated by perfusion of the livers of adult Sprague-Dawley male rats with collagenase and pronase. The resultant cell suspension was washed and centrifuged over a Stractan gradient as described (8). HSCs were collected from the 5.5 and 11% stractan interphases and cultured on uncoated plastic tissue culture dishes in Dulbecco's modified Eagle's medium supple-

mented with 10% fetal calf serum in a 5% CO2, humidified atmosphere for 2 days. After 2 days, the medium was changed, and the cells were infected with adenoviruses expressing the molecular decoys with a multiplicity of infection of 500. Infection of HSCs with a multiplicity of infection of 500 resulted in GFP expression in 95–100% of the HSCs (not shown). After infection, the cells were harvested, RNA was isolated, and analyzed for expression of collagen mRNA and protein. Fully activated HSCs were obtained by culturing the cells for 7–14 days. When cellular medium was analyzed, the cells were cultured in 10 mM ascorbic acid 2-phosphate (unhydrolyzable derivative of ascorbic acid; Wako) in 0% serum 24 h before collection of the medium. Human HSCs were isolated by perfusion of surgically removed parts of human liver, as above. These cells were cultured for 7 days before they were infected with adenoviruses.

**Transfection of NIH 3T3 Fibroblasts**—NIH 3T3 fibroblasts were transfected by the calcium phosphate technique, using 10 μg of decoy plasmids and 1 μg of luciferase plasmid (pGL3; Promega) per 100-mm dish as a control for transfection efficiency. After 12 h, the medium was changed, and the cells were harvested after an additional 24 h of culturing. To make stable cell lines, 1 μg of pCDNA3 vector was co-transfected with 10 μg of decoy plasmids into NIH 3T3 fibroblasts, and stable transformants were selected with G418 for 3 weeks. Well isolated clones were expanded and analyzed for decoy expression by RNase protection assays (RPA).

**Isolation and Analysis**—Total cellular RNA was isolated by the standard procedure (21). Cytoplasmic and nuclear RNAs were isolated as described by Grimm (19). Expression of collagen α1(I) mRNAs was measured by RPA, according to our published procedure (9). Collagen-specific probes were hybridized together with a GAPDH probe, as an internal standard, to account for recovery of the RNA. Riboprobes specific for decoy mRNAs were derived by transcribing the antisense strand of the p74WT decoy and p74MUT decoy plasmids with T7 RNA polymerase, after linearizing the plasmids with HpaI. Typically, 5–20 μg of total or cytoplasmic RNA were analyzed, and nuclear RNA was analyzed in one-fourth of the amount of its corresponding cytoplasmic sample. This ratio represents the equivalent amounts of RNA that we consistently extracted from the two compartments from 3T3 fibroblasts and HSCs. RT-PCRs for collagen α1(I) mRNA and GAPDH mRNA were done as described (9). For collagen α2(I) mRNA, the primers were TGAATACACGGCAAGGGGTT (5’ primer) and TTTGGAAACAGA-CAGGCGCA (3’ primer), which amplify RT-PCR product of 385 nt. For α1(III) mRNA, the primers were GATCGGCGCAATGGCGAATG (5’ primer) and AAAAGCAAACAGGCAG (3’ primer), which amplify RT-PCR product of 259 nt. For interleukin-6 mRNA, the primers were designed. RT-PCRs were done with 100 ng of total RNA using the rTth reverse transcriptase RNA PCR kit (PerkinElmer Life Sciences) in the presence of 10 μCi of [32P]dCTP, according to the recommended protocol. Gene-specific primers were used together with GAPDH-specific primers, as an internal control, in the same reverse transcription and amplification reaction, which consisted of 20 cycles (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). The PCR step of 20 cycles is in the linear range of the reaction, and coamplification of GAPDH assures equal loading of RNA. For α1(III) mRNA, 30 cycles were used (1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C), and no coamplification with GAPDH primers was done, because 30 cycles is outside the linear range for this mRNA. PCR products were resolved on 6% denaturing acrylamide gels. All gels were quantified by phosphorimaging.

**Western Blots**—20 μg of cellular proteins were resolved on a 7.5% SDS-PAGE gel under reducing or nonreducing conditions, as indicated. After blotting, the membrane was probed with an antibody raised against human collagen type I (600–401–103; Rockland, Inc., Rockland, PA). This antibody recognizes the pro-α1(I) chain, the mature α1(I) chain, and the heterotrimer of type I collagen. It does not recognize α1(I) chain2 and does not cross-react with other collagens. Prior to electrophoresis, some samples were digested at room temperature for 30 min with pepsin (1000 units; Sigma) at pH 2.5 or with 7.5 units of bacterial collagenase (Roche Molecular Biochemicals), as controls for antibody specificity. Treatment with collagenase resulted in complete loss of signal, whereas treatment with pepsin resulted in reduction of the molecular mass from 170 to 120 kDa (not shown). Collagen in the medium was as above, after the medium by ultrafiltration (Amicon; cut-off limit 100 kDa). To optimize collagen secretion, the cells were grown in the presence of 10 mM ascorbic acid 2-phosphate, in 0% serum for 24 h before medium collec-

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tion. The amount of medium that was analyzed on the gel was proportional to the number of cells in a given plate. Western blots were quantified by densitometry.

RESULTS

Construction and Expression of Molecular Decoys—A molecular decoy containing the collagen 5’ stem-loop was based on the modified mouse U7 small nuclear RNA (snRNA) gene. The wild type Sm binding site of this gene was replaced by the optimal Sm binding site (construct p74). This modification rendered this U7 snRNA nonfunctional in histone processing (the normal function of U7 snRNA) but produced a higher accumulation, predominantly in the cell nucleus (19). The metabolic pathway of snRNAs is shown at the bottom of Fig. 1A. The 5’-end of U7 RNA is free of proteins (23) and can accommodate various sequences unrelated to U7 function (24). The p74WT decoy was constructed by introducing the 5’-end of the optimal U7 snRNA gene. This insertion increased the total length of the transcript to 108 nt and did not affect the transcription start site or the sequence of the optimal Sm binding site (Fig. 1A).

Fig. 1. Expression of molecular decoys in NIH 3T3 cells. A, top panel, schematic representation of the molecular decoys. The 108-nt RNA is shown as a line, eight core Sm proteins are indicated as circles, and the 5’ stem-loop of collagen α1(I) mRNA is indicated. p74MUT decoy has a substitution of 15 nt, which abolishes formation of the 5’ stem-loop. 7mG represents the monomethylated cap structure that the decoys initially acquire. Bottom panel, metabolism of snRNAs. Nucleocytoplasmic trafficking and modifications to snRNAs in the cytoplasm are indicated. B, expression of molecular decoys in transiently transfected NIH 3T3 fibroblasts. 48 h after transfection of the p74WT plasmid (lanes 1 and 2) or the p74MUT plasmid (lanes 3 and 4), nuclear (N) and cytoplasmic (C) RNA was extracted and analyzed by RNase protection assays with riboprobes specific for the p74WT decoy (WT) and p74MUT decoy (MUT). Lane 5, total RNA (T) from nontransfected cells (−) probed with the p74WT riboprobe as a control. The arrows indicate migration of the bands corresponding to the longer isoform of decoys (pre-decoy) and the mature size decoy (decoy). C, the same RNA as in B was probed with riboprobe specific for mouse GAPDH mRNA. The expression of this mRNA is indicated by GAPDH.
The p74MUT decoy is identical except that 20 nt of the 5' stem-loop were mutated to abolish formation of the 5' stem-loop. This control decoy also has a length of 108 nt (Fig. 1A).

Expression of the decoys and their subcellular accumulation was analyzed by transient transfections into NIH 3T3 fibroblasts. Luciferase control gene was cotransfected with the decoy genes to assess transfection efficiency. Cytoplasmic and nuclear RNA were extracted and analyzed by RNase protection assay using decoy-specific riboprobes (Fig. 1B) and GAPDH probe (Fig. 1C) as a control for nuclear and cytoplasmic separation. In several experiments, we consistently extracted about 4-fold more RNA from the cytoplasmic fraction than from the nuclear fraction, so in subsequent experiments we used this ratio as a representative abundance of the RNA in these compartments. Total expression (nuclear + cytoplasmic) of p74MUT decoy was comparable with the expression of p74WT decoy (Fig. 1B). Luciferase activity was also similar in these samples, suggesting similar transfection efficiencies (not shown). The p74MUT decoy accumulated mostly as the RNA of expected size of 108 nt (decoy) and to about the same levels in the nucleus and in the cytoplasm (lanes 3 and 4). p74WT decoy RNA accumulated as a longer species of about 115–120 nt (pre-decoy) as well as the mature size species (decoy). In the cytoplasm, both of these isoforms were found (lane 1), whereas the mature size decoy mostly accumulated in the nucleus (lane 2). The longer isoform of the p74WT decoy presumably contains additional nucleotides at its 3’ end (see “Discussion”). Similar to the p74MUT decoy, 50% of total p74WT decoy was found in the cytoplasm and 50% in the nucleus. GAPDH mRNA showed predominant cytoplasmic accumulation (Fig. 1C), as expected for this mRNA, which suggests minimal cross-contamination of the nuclear and cytoplasmic fractions. From these experiments, we conclude that the collagen 5’ stem-loop can be expressed as a short stable RNA using the optimal U7 snRNA stem-loop-binding proteins.

**p74WT Decoy Decreases Collagen mRNA in NIH 3T3 Fibroblasts**—To assess how expression of the p74WT decoy affects the expression of endogenous collagen α1(I) mRNA, we developed NIH 3T3 cell lines that stably express the p74WT decoy and p74MUT decoy. Fig. 2A shows an experiment where we characterized one cell line expressing a high level of the p74WT decoy (lane 1, WT#1), a control cell line not expressing a decoy (lane 2, -), and two cell lines expressing the p74MUT decoy (lanes 3 and 4, MUT#1 and MUT#2). Decoy expression was analyzed in total RNA, and a longer isoform of the p74WT decoy was detected (Fig. 2A, pre-DECOY). For analysis of endogenous collagen α1(I) mRNA, nuclear and cytoplasmic RNAs were extracted from the same cell lines and probed with riboprobes specific for mouse collagen α1(I) mRNA and GAPDH mRNA, as a control for loading. Fig. 2B demonstrates that the expression of p74WT decoy decreased the cytoplasmic levels of endogenous collagen α1(I) mRNA (Fig. 2B, lane 1), compared with the level in cells without decoy (lane 3) and in cells expressing the p74MUT decoy (lanes 5 and 7). Collagen α1(I) mRNA in the nucleus is not affected by the decoy (lanes 2, 4, 6, and 8). The band indicated with an asterisk is a nonspecific band present when tRNA is probed with the same riboprobes and compared with total RNA from NIH 3T3 fibroblasts (compare lanes 9 and 10). Additional cell lines of NIH 3T3 fibroblasts expressing the p74WT and p74MUT decoy were developed, and cytoplasmic RNA was analyzed for expression of endogenous collagen α1(I) mRNA (Fig. 2C). All cell lines expressing the p74WT decoy (WT#2 to WT#6) had a decreased level of collagen α1(I) mRNA to about 40% of the level seen in the cell lines expressing the p74MUT decoy (MUT#3 and MUT#4). From these experiments, we conclude that a high level of p74WT decoy down-regulates the cytoplasmic level of collagen α1(I) mRNA.

Decreased cytoplasmic levels of collagen α1(I) mRNA may result in diminished synthesis of collagen protein. Therefore, we analyzed cellular medium of the cell lines WT#1 and MUT#1 for accumulation of procollagen by Western blot. Prior to harvesting the medium, the cells were grown for 24 h in presence of ascorbic acid 2-phosphate, to maximize the synthesis and secretion of triple-helical collagen (25). Equivalent amounts of cellular medium are resolved under reducing conditions (Fig. 2D). Cells expressing the p74WT decoy secreted 50% less procollagen than cells expressing the p74MUT decoy (compare lane 1 with lane 2). We concluded that p74WT decoy can inhibit collagen α1(I) mRNA and collagen protein secretion by NIH 3T3 fibroblasts.

**Expression of Molecular Decays in Rat HSCs—**Specific inhibition of fibrillar collagen expression in HSCs is one of the goals for therapy of liver fibrosis. To express molecular decoys in HSCs, we constructed adenoviruses expressing the p74WT decoy and the p74MUT mutant decoy. The viruses also express GFP from an independent transcription unit, so that GFP can serve as a marker for efficiency of infection. We infected rat HSCs at day 7 after isolation with viruses expressing the decoys and analyzed decoy RNA expression by RNase protection assays at 4 days postinfection (Fig. 3A). Control decoy (p74MUT, lane 2) was expressed at a 1.25-fold higher level than the decoy with the 5’ stem-loop (p74WT, lane 1). The p74WT decoy accumulated partly as a longer precursor RNA (predecoy). Infection efficiency of both decoys was similar as judged by expression of GFP from the same samples (Fig. 3B). Having established that the decoys can be expressed in HSCs, we assessed their effectiveness in inhibiting collagen type I expression during culture activation of rat HSCs.

**Molecular Decoy Decreases the Steady-state Level of Collagen α1(I) mRNA in HSCs—**Freshly isolated quiescent HSCs change to an activated phenotype when plated on plastic. During this process, collagen type I expression progressively increases from day 3 and reaches the maximum expression at day 7 after plating (8). To assess the inhibitory potential of molecular decoys during this activation, we infected HSCs at day 2 after plating with adenoviruses expressing the p74WT decoy and p74MUT decoy with a multiplicity of infection of 500. This resulted in 95% of cells expressing GFP at the time of analysis. The cells were further cultured until day 7 (5 days after infection with the viruses), when nuclear and cytoplasmic RNA was extracted and analyzed for expression of endogenous collagen α1(I) mRNA and GAPDH mRNA, as a control for loading. The results of one such experiment are shown in Fig. 4A. During culture activation of HSCs, the p74WT decoy significantly reduced the steady state level of collagen α1(I) mRNA in the cytoplasm of HSCs, as compared with the level seen in the presence of the p74MUT decoy (compare lanes 3 and 5). Accumulation of collagen α1(I) mRNA was barely detectable in the nucleus and was unaffected by the p74WT decoy (lanes 2 and 4). Expression of GFP, as a marker for viral delivery, was similar in both infections (Fig. 4B). Fig. 4C shows quantification of the results of three independent experiments. Expression of collagen α1(I) mRNA was reduced by 46 ± 16% by the p74WT decoy. From these results, we concluded that the p74WT decoy is effective in decreasing collagen α1(I) mRNA expression during *in vitro* activation of quiescent HSCs into myofibroblasts.
FIG. 2. Effect of molecular decoys on endogenous collagen α1(I) mRNA in stably transfected NIH 3T3 cells. A, expression of molecular decoys in clonal cell lines. Total RNA was extracted from a cell line expressing the p74WT decoy (lane 1, WT#1), a control cell line expressing no decoy (lane 2, −), and two cell lines expressing p74MUT decoy (lanes 3 and 4, MUT#1 and MUT#2) and probed with the decoy-specific riboprobes as in Fig. 1. The arrows indicate migration of the two decoy isoforms. B, expression of endogenous collagen α1(I) mRNA in the same cell lines as in A. Nuclear (N) and cytoplasmic (C) RNA was extracted from the cell lines: WT#1 (lanes 1 and 2), control (lanes 3 and 4), MUT#1 (lanes 5 and 6), and MUT#2 (lanes 7 and 8) and analyzed by RNase protection assays with riboprobes specific for mouse collagen α1(I) mRNA (COLL) and mouse GAPDH mRNA (GAPDH). Lane 9, tRNA analyzed with collagen and GAPDH riboprobes; lane 10, total RNA from NIH 3T3 cells analyzed with the same riboprobes. The migrations of the relevant bands are indicated by arrows. The band indicated with an asterisk is nonspecific, since it is seen in the tRNA lane. C, additional NIH 3T3 cell lines expressing the p74WT decoy (lanes 2–6) and p74MUT decoy (lanes 7 and 8) were analyzed for decoy expression as in Fig. 2A (top panel) and for collagen α1(I) mRNA expression as in Fig. 2B (bottom panel; only results with cytoplasmic RNA are shown). Lane 1, top panel, RNA from control cells; lane 9, bottom panel, tRNA control. Lane 9, top panel, and lane 1, bottom panel, size markers. Migration of the relevant bands is indicated by arrows, and migration of nonspecific bands is indicated by asterisks. D, accumulation of collagen protein in cellular medium from the above cell lines. Cells from cell line WT#1 (lane 1) and MUT#1 (lane 2) were seeded at equal density and incubated for 24 h with ascorbic acid 2-phosphate to stimulate collagen production. Cellular medium was collected and concentrated, and equivalent amounts were analyzed by Western blot. The samples were resolved under reducing conditions. The migration of pro-α1(I) chain (pro-alpha1(I)) is indicated. Migration of molecular weight markers is indicated to the right.
collagen α2(I) mRNA and α1(III) mRNA, we assessed if the p74WT decoy would decrease steady state level of these mRNAs. We analyzed α2(I) mRNA level by semiquantitative RT-PCR. Expression of collagen α1(I) mRNA by RT-PCR was similar to the result obtained by RPA analysis (Fig. 5), suggesting that our RT-PCR analysis is appropriate for assessment of steady-state levels of various collagen mRNAs. Collagen α2(I) mRNA was similarly decreased by the p74WT decoy compared with the p74MUT decoy (Fig. 5). Collagen α1(III) mRNA was decreased by the p74WT decoy as well (Fig. 5). In this reaction, we did not coamplify GAPDH mRNA, because the reaction required 30 cycles, which is out of the linear range for GAPDH. However, since the same RNA was used as for α1(I) and α2(I) analysis, we assumed that its integrity is comparable. Interleukin-6 mRNA was unaffected by the p74WT decoy. Since GAPDH mRNA also did not show a significant difference in multiple experiments, we concluded that inhibition by the p74WT decoy is specific for the three fibrillar collagen mRNAs.

**Molecular Decoy Inhibits Collagen Protein Synthesis in Rat HSCs**—Two independent experiments were performed to assess the effect of the decoys on collagen protein synthesis. First, we transduced quiescent rat HSCs at day 2 after isolation with the viruses expressing the p74WT and p74MUT decoys. Expression of collagen α1(I) protein was analyzed by Western blot at day 5 after isolation (3 days after infection with the viruses). The Western blot was performed with cellular proteins under reducing conditions (Fig. 6A). The decoy with the 5′ stem-loop (p74WT) inhibits procollagen synthesis by about 65%, compared with the decoy without the stem-loop (p74MUT) (compare lanes 1 and 2, pro-alpha 1(I)). Expression of GFP serves as a control for decoy delivery into HSCs. Second, rat HSCs were incubated for 5 days after viral infection (a total of 7 days after isolation), and collagen type I was analyzed under nonreducing conditions (Fig. 6B, pro-collagen). In this experiment, we wanted to see if the decoy inhibits synthesis of procollagen assembled into the disulfide-bonded multichain complex, and if this inhibition persisted in activated HSCs. Again, the wild type decoy decreased procollagen synthesis by 70%. Reprobing for αSMA reveals a slightly higher expression of this protein in...
the cells expressing p74WT decoy, further demonstrating that the wild-type decoy is a specific inhibitor of fibrillar collagen.

Molecular Decoy Is Effective in Decreasing Collagen Protein Synthesis in Human HSCs—Finally, we wanted to see if the p74WT decoy is effective in primary human HSCs. Human HSCs are efficiently infected with adenoviruses, and the mouse U7 promoter is active in human cells (19, 24). Also, the mouse 5’ stem-loop in the p74WT decoy is identical to the 5’ stem-loop of human collagen α1(I) mRNA. Therefore, we infected activated primary human HSCs and analyzed collagen α1(I) protein expression 5 days after infection (Fig. 7). Collagen expression was analyzed by Western blot of cellular proteins. Proteins were resolved under reducing conditions (Fig. 7A) or, in an independent experiment, under nonreducing conditions (Fig. 7B, pro-collagen). In both experiments, the p74WT decoy decreased procollagen α1(I) protein expression to 50–60%. Expression of the GFP was equal for both decoys (Fig. 7A, GFP), suggesting similar transduction efficiency. Thus, molecular decoys reduce excessive collagen production in activated primary human HSCs.

DISCUSSION

The 5’ stem-loop of fibrillar collagen mRNAs is a critical cis-acting element that regulates expression. In quiescent HSCs, the 5’ stem-loop of collagen α1(I) mRNA in the absence of its cognate binding proteins inhibits expression of its mRNA (10). In activated HSCs, where the 5’ stem-loop binding proteins are present, there is a high level of expression of collagen α1(I) mRNA as well as reporter mRNAs with the 5’ stem-loop (10). Therefore, it should be possible to inhibit collagen α1(I) expression in activated HSCs by titrating out these proteins. This was achieved by expressing a short stable RNA containing the sequence of the 5’ stem-loop to act as a molecular decoy (Figs. 1A and 8).

The decoy described here was expressed from a modified mouse U7 snRNA gene (19). snRNA genes are active in all cell types and direct transcription by RNA polymerase II, so that all transcripts initially acquire a 7mG cap (26). The modified U7 gene encodes an snRNA that has an optimized Sm binding site, which increases stability of the RNA and targets it to the nucleus (19), but is nonfunctional in histone pre-mRNA processing (27, 28). The 5’-end of U7 snRNA is free of proteins and can accommodate any short RNA sequence (24). Thus, we placed the 5’ stem-loop sequence of the mouse collagen α1(I) mRNA at the 5’-end of the optimized U7 snRNA gene (p74WT decoy). The collagen 5’ stem-loop is mutated in the control decoy (p74MUT) (Fig. 1A). Both modifications increased the length of the RNA to 108 nt, without significantly affecting expression (Figs. 1B and 3A). Being synthesized by an snRNA gene, the decoy RNAs are exported from the nucleus into the cytoplasm, where assembly with the Sm proteins into a ribonucleoprotein particle takes place (29). Assembly with Sm proteins leads to hypermethylation of the cap (30) and maturation of the 3’ end by removal of several nt (31). These modifications provide stability and redirect the particle back into the nucleus (32) (Fig. 1A, bottom panel). Association with Sm proteins is an
absolute requirement for nuclear accumulation of snRNAs (32). Thus, the decoy may encounter both the nuclear and the cytoplasmic 5' stem-loop binding proteins. We believe that sequestration of these binding proteins by the p74WT decoy renders the three fibrillar collagen mRNAs unstable and reduces collagen expression in fibroblasts and HSCs (Fig. 8). Our preliminary results indicate that two proteins can be specifically UV-cross-linked to the p74WT decoy RNA (not shown). Binding of cytoplasmic proteins to the 5' stem-loop in vitro requires the presence of the 7mG cap. In the initial stage after its synthesis, the decoy acquires a 7mG cap, because it is transcribed by RNA polymerase II (26), and this may facilitate binding of the cytoplasmic proteins.

We have repeatedly detected a precursor form of the p74WT decoy (Figs. 1B, 2A, and 3A). This form is longer by 10–15 nt and presumably arises from inefficient 3'-end maturation. Why the 5' stem-loop inhibits the 3'-end formation is not clear. It may interfere with assembly of Sm proteins, which is required for 3'-end trimming (31), or change the RNA structure so that the 3'-end is inaccessible to the processing factors. The p74WT decoy accumulates to a similar level as the p74MUT decoy, which undergoes complete maturation, suggesting that both decoys have a similar stability (Figs. 1B and 3A). However, the precursor form of the p74WT decoy accumulates almost exclusively in the cytoplasm, while the mature form is found in the nucleus (Fig. 1B). Regardless of its length, both forms of the p74WT decoy should be effective in titration of the 5' stem-loop-binding proteins.

The inability to efficiently introduce genes into quiescent HSCs has previously limited studies of gene expression in this cell type. However, delivery of genes using adenoviral vectors is a receptor-mediated process, and infection with equal multiplicity of infection into a given population of cells results in uniform and reproducible gene transfer (10, 33). Using this technology, we successfully introduced molecular decoys in HSCs with a quiescent phenotype, only 2 days after isolation. Our viruses also express GFP from an independent transcription unit, which serves as an indicator of infection efficiency. Equal expression of GFP is associated with comparable expression of the p74WT and p74MUT decoy (Fig. 3). The efficacy of molecular decoys in inhibiting collagen α1(I) gene expression in HSCs was tested during culture activation of these cells. Collagen α1(I) mRNA was decreased by 46% in the presence of p74WT decoy in HSCs (Fig. 4C). Similar results were obtained.
Molecular Decoy Inhibits Collagen α(I) Expression

with NIH 3T3 fibroblasts (Fig. 2). There was no clear correlation between the level of expression of the p74WT decoy and the decrease in steady state level of collagen α(I) mRNA (Fig. 2C). It is possible that even the modest expression of the p74WT decoy is sufficient to titrate the putative 5’ stem-loop binding protein(s). Expression of collagen α2(II) and α1(III) mRNA was also inhibited by the p74WT decoy in HSCs to a similar extent (Fig. 5). The cumulative result was to decrease intracellular procollagen by 70% (Fig. 6). Expression of GAPDH mRNA (Fig. 5), interleukin-6 mRNA (Fig. 5), and αSMA protein (Fig. 6) were unaffected by the p74WT decoy, suggesting a specific effect of the decoy on the three fibrillar collagen mRNAs. Expression of the disulfide-bonded high molecular weight procollagen, a precursor form of fibril formation, was decreased by the p74WT decoy by 70% in rat HSCs (Fig. 6B) and by 50% in human HSCs (Fig. 7B). Collagen secreted into the supernatants of fibroblasts was also inhibited by 50% (Fig. 2D). In addition to inhibiting fibrillar collagen mRNA stability, the p74WT decoy may also inhibit collagen mRNA translation. Such significant effects suggest that decoys based on the p74WT construct may become an effective tool for gene therapy of liver fibrosis.

Although the decoys are based on snRNAs that are nonfunctional in their physiological processes (27, 28), sequestration of Sm proteins by the decoys and competition for transcription factors with other snRNA genes may cause some undesired side effects. As a proof of principle, we have developed fibroblast cell lines that stably express a high level of the 5’ stem-loop decoy (Fig. 2). These cells are morphologically indistinguishable from the control cells with similar growth rates but specifically decrease collagen production. Thus, our study demonstrates that molecular decoys may provide a general method of assessing the functional significance of blocking the interactions of endogenous mRNAs and proteins.

Several gene therapies have been proposed for treatment of liver fibrosis. They are based on enhancing the degradation of extracellular matrix by overexpressing urokinase-type plasminogen activator (34) or matrix metalloproteinase 1 (35) or on stimulation of hepatocyte proliferation by expression of hepatocyte growth factor (36). The molecular decoys described here act by a novel mechanism of preventing excessive production of fibrillar collagens. This effect is significant in HSCs in culture and suggests that molecular decoys may be effective in reducing excessive collagen production in liver fibrosis.

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