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Posttranscriptional Regulation of Collagen α1(I) mRNA in Hepatic Stellate Cells

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The hepatic stellate cell (HSC) is the primary cell responsible for the dramatic increase in the synthesis of type I collagen in the cirrhotic liver. Quiescent HSCs contain a low level of collagen α1(I) mRNA, while activated HSCs contain about 60- to 70-fold more of this mRNA. The transcription rate of the collagen α1(I) gene is only two fold higher in activated HSCs than in quiescent HSCs. In assays using actinomycin D or 5,6-dichlorobenzimidazole riboside collagen α1(I) mRNA has estimated half-lives of 1.5 h in quiescent HSCs and 24 h in activated HSCs. Thus, this 16-fold change in mRNA stability is primarily responsible for the increase in collagen α1(I) mRNA steady-state level in activated HSCs. We have identified a novel RNA-protein interaction targeted to the C-rich sequence in the collagen α1(I) mRNA 3′ untranslated region (UTR). This sequence is localized 24 nucleotides 3′ to the stop codon. In transient transfection experiments, mutation of this sequence diminished accumulation of an mRNA transcribed from a collagen α1(I) minigene and in stable transfections decreased the half-life of collagen α1(I) minigene mRNA. Binding to the collagen α1(I) 3′ UTR is present in cytoplasmic extracts of activated but not quiescent HSCs. It contains as a subunit αCP, which is also found in the complex involved in stabilization of α-globin mRNA. The auxiliary factors necessary to promote binding of αCP to the collagen 3′ UTR are distinct from the factors necessary for binding to the α-globin sequence. Since αCP is expressed in both quiescent and activated HSCs, these auxiliary factors are responsible for the differentially expressed RNA-protein interaction at the collagen α1(I) mRNA 3′ UTR.

Cirrhosis is a major medical problem characterized by the excessive production of extracellular matrix proteins in the liver, including type I collagen (36, 41, 42). Hepatic stellate cells (HSCs; also called Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the cirrhotic liver (17, 63). In normal liver, quiescent HSCs store vitamin A (24) and probably regulate hemodynamics (27, 55) but express only trace amounts of type I collagen. Upon a fibrogenic stimulus, HSCs become activated, a process in which they lose vitamin A granules, proliferate, change morphologically into myofibroblasts, and increase their synthesis of extracellular matrix proteins (15, 38). The development of methods for the isolation and culture of HSCs has enabled in vitro studies of these cells (12, 16). Culturing quiescent HSCs on plastic causes activation similar to that seen in liver fibrosis in vivo, including the large accumulation of collagen α1(I) mRNA (16, 19, 28). This provides a simple model system with which to study HSC activation and collagen gene regulation.

The transcriptional regulation of the type I collagen gene α1(I) has been extensively studied (2, 5, 7, 53, 60). In transgenic mice, the human collagen α1(I) gene containing 2 kb of the 5′ flanking and 10 kb of the 3′ flanking sequences is expressed in a proper tissue-specific manner and is responsive to fibrogenic stimulus (8, 68). Promoter analysis of the collagen α (I) gene has identified four sites of protein binding as revealed by DNase I footprinting in vitro (51). Two of these binding sites are for the ubiquitous transcription factors Sp1 and NF1, bindings of which are mutually exclusive (45, 46). A transcriptional enhancer has been reported in the first intron of the α1(I) gene (6, 57); however, studies using transgenic mouse have questioned the biological relevance of this observation (59, 62). Protein factors interacting with the collagen α1(I) gene have also been studied in quiescent and activated HSCs. Footprinting analysis has shown a different pattern of DNase I-resistant sites in the promoter, and DNA binding activity of the Sp1 transcription factor is about twofold higher in activated HSCs than in quiescent cells (52). These results are consistent with a higher transcription rate of the collagen α1(I) gene in activated than in quiescent HSCs. Transforming growth factor β, a cytokine implicated in liver fibrogenesis (10, 32, 44), increases collagen α1(I) mRNA steady-state levels severalfold (26, 49, 65) in fibroblasts and activated HSCs. An increased transcription rate of the gene is, at least in part, responsible for this effect, as demonstrated in nuclear run-on experiments (3).

Posttranscriptional regulation of collagen α1(I) mRNA has been studied almost exclusively in fibroblasts (13, 14, 49, 50), where its stability depended on culturing conditions. In NIH 3T3 fibroblasts, the mRNA has a half-life of between 4 h in semiconfluent cultures and 9 h in confluent cultures (49). In rat fibroblasts, the half-life of collagen α1(I) mRNA was measured to be longer than 16 h (61). Treatment of fibroblasts with transforming growth factor β caused an increase in collagen α1(I) mRNA, which is due in part to stabilization of the mRNA (49, 50).

Stability of most mRNAs is determined by sequences in their 3′ untranslated regions (UTRs) (4, 11, 56). Collagen α1(I) mRNA exists as two species, a 4.7-kb mRNA and a 5.7-kb mRNA, depending which polyadenylation site is used (43). In fibroblasts, a 67-kDa protein binds to the 3′ UTR present in

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both the 4.7- and 5.7-kb collagen α1(I) mRNA species (34, 35). However, its binding site has not been precisely mapped. The binding activity of this protein decreases upon treatment of cells with dexamethasone, which also decreases collagen α1(I) mRNA levels, suggesting a stabilizing role of this protein (34). Recently the same authors have suggested a role of regions other than 3′ UTR in controlling the turnover of collagen α1(I) mRNA, based on experiments with hybrid mRNA composed of the 5′ human growth hormone RNA and 3′ collagen α1(I) RNA (33). There are no studies on the posttranscriptional regulation of the collagen α1(I) gene in HSCs.

In this study, we have measured the steady-state level, the rate of transcription, and the half-life of collagen α1(I) mRNA in quiescent and activated HSCs. The results suggest predominantly posttranscriptional upregulation of collagen α1(I) mRNA in the activation process of HSCs. An RNA binding activity targeted to the C-rich region in the 3′ UTR of this mRNA has been found. This binding activity is present in cytoplasmic extracts of activated but not quiescent HSCs. Mutation of the binding site decreases accumulation of mRNA derived from a collagen minigene in transient transfection experiments. Finally, we show that this binding activity shares at least one subunit, αCP, with the complex which binds to and stabilizes the human α-globin mRNA (64, 67).

MATERIALS AND METHODS

Plasmid constructs. Collagen minigenes contain 220 nucleotides (nt) of the promoter of the mouse collagen α1(I) gene followed by the first six exons ligated to the last four exons within the intronic sequences, truncated at the *Hin*dIII site (277 nt downstream of the stop codon) and thus containing only the first polyadenylation signal.

For in vitro transcription to generate the RNA probes used in gel mobility shift experiments, the *Eco*RI-*Hin*dIII fragment of the mouse collagen α1(I) gene from nt −39 to +277 (+1 is the first nucleotide after the stop codon) was subcloned into corresponding sites of the pGEM3zf+ vector (Promega). To generate a shorter probe, the mouse collagen α1(I) gene from nt +23 to +55 was synthesized as a double-stranded oligonucleotide and cloned into *Eco*RI-*Hin*dIII sites of pGEM3zf+. The α-globin template contains nt +29 to +70 from the 3′ UTR of human α-globin gene (64). α-Globin and collagen RNA probes were transcribed from these templates after linearization with an appropriate restriction enzyme. Site-directed mutagenesis was performed by the method of Kunkel (31). Riboprobes used for RNase protection assays were derived from the 375-nt *Pst*I-*Ava*I fragment from rat collagen α1(I) cDNA (clone py1R1; a gift from David Rowe), cloned into *Hinc*II-*Pst*I sites of the pGEM 3zf+ vector and from the pTRI-GAPDH-rat antisense control template (Ambion). To generate an RNA probe to detect expression of the collagen minigenes, exons 5 to 9 of mouse collagen α1(I) cDNA were cloned into *Pst*I-*Hin*dIII sites of pGEM4, and a 348-nt antisense RNA probe was transcribed after linearization with *Nhe*I. Single-stranded DNAs (ssDNAs) used for hybridization in nuclear run-on experiments were made by using helper phage R408 (Promega) after cloning of the rat collagen cDNA *Pst*I-*Ava*I fragment into the pGEM 3zf− vector and the rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA 340-nt *Eco*RI-*Hin*dIII fragment into plasmids pGEM 3zf+ and pGEM 3zf−, respectively.

RNA isolation and analysis. Total cellular RNAs were isolated by the method of Chomczynski and Sacchi (9). RNase protection analysis was performed according to a published procedure (8). Collagen α1(I) and *GAPDH*-specific riboprobes were hybridized with the same RNA sample to account for recovery of the RNA. Reverse transcription-PCR analysis was done with 200 ng of total RNA and a GeneAmp rTth DNA polymerase kit (Perkin-Elmer). For collagen α1(I) mRNA, the primers were TTCCCTGGACCTAAGGGTACT and TTGAGCTCCAGCTTCGCC, derived from two different exons within the region of the gene encoding the triple-helical domain of the protein (positions 1711 and 1824 relative to the start codon in cDNA). These primers give an amplification product of 113 nt from spliced mRNA. For *GAPDH* mRNA, the primers were TGGCCAAGGTCATCCATGAC and GAGTGGCAGTGATGGCA TGG, which give an amplification product of 75 nt. Both sets of primers were used together in a single amplification reaction. All gels were quantified by phosphorimaging.

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 an arabinogalactan gradient as described by Friedman et al. (16). HSCs were collected from the 4.5% arabinogalactan interphase and purity estimated by autofluorescence of the cells by UV microscopy. More than 90% of cells showed the characteristic autofluorescence. These cells were immediately used as quiescent HSCs in all experiments. Cells from the 12% stractan interphase were seeded onto uncoated plastic tissue culture dishes and cultured in Dulbecco's modified medium supplemented with 10% fetal calf serum in a 5% CO_2-humidified atmosphere. After 7 days of culture, the cells were trypsinized and grown for additional 7 days, after which they were harvested and used as activated HSCs.

NIH 3T3 fibroblasts were cultured and transfected according to standard procedures (7). For estimation of mRNA half-lives, HSCs were incubated with 25 μg of actinomycin D per ml or 67 μM 5,6-dichlorobenzimidazole riboside (DRB), and cells were harvested at indicated time points.

Preparation of cell extracts, mobility shift experiments, and UV cross-linking. Quiescent or activated HSCs were resuspended in lysis buffer containing 10 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, 3 mM NaCl, and 0.4% Nonidet P-40 (NP-40). After incubation for 10 min on ice, the cells were centrifuged in an Eppendorf tube for 10 min at 14,000 rpm, and the supernatant was collected as cytoplasmic extract. The protein concentration was measured by the Bradford assay (Bio-Rad), using bovine serum albumin (BSA) as a standard. Extracts were kept at −80°C. Uniformly labeled RNA probes were prepared by standard in vitro transcription protocols using [^{32}P]UTP (3,000 Ci/mmol; ICN) after linearization of plasmids with appropriate restriction enzymes. For mobility shift experiments, 25 μg of extracts was incubated with 40,000 cpm of radiolabeled RNA probes in 15 mM KCl–1 mM dithiothreitol (DTT)–12 mM HEPES (pH 7.8)–0.24 mM EDTA–5 mM $MgCl_2$–0.4% NP-40–200 ng of yeast tRNA per μl in a total volume of 25 μl for 30 min at 30°C. Additionally, 10 μg of yeast tRNA was added to suppress nonspecific binding. Competitor RNAs were added in some experiments as indicated. These RNAs were made by in vitro transcription, and their integrity was assessed by agarose gel electrophoresis. After incubation, 145 U of RNase T_1 (Gibco) was added, and incubation continued for 30 min at 30°C. Finally, 3 μl of heparin (50 mg/ml) was added, and after 10 min of incubation on ice, samples were loaded on a 6% nondenaturing acrylamide gel (acrylamide-to-bisacrylamide ratio, 60:1). The gels were run in 0.3× Tris-borate-EDTA buffer at ambient temperature. For photoaffinity cross-linking experiments, after addition of heparin, the samples were irradiated with 254-nm UV light from a distance of 5 cm for 20 min, using a UVGL-2 lamp (UVP, Inc., San Gabriel, Calif.); 250 U of Benzon nuclease (Sigma) was then added, and RNA was digested for 30 min at 37°C. RNA-protein complexes were resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis. To study the effect of antibodies on RNA-protein binding, 10 μl of ascites fluid containing anti-αCP monoclonal antibody 6B12.A3 was added to the binding reaction, while 10 μl of anti-human smooth muscle actin antibody (DAKO) was added to the control reaction. The total mouse immunoglobulin G (IgG) fraction (2 μg/10 μl) was supplemented with BSA to 5% (as total proteins in ascites fluid) and added as an additional control reaction.

Nuclear run-on experiments. Nuclei were prepared from 1.5×10^7 quiescent or activated HSCs by incubating cells for 10 min on ice in 10 mM Tris-Cl (pH 7.4)–3 mM $CaCl_2$–2 mM $MgCl_2$–1 mM DTT–0.1 mM phenylmethylsulfonyl fluoride–50 U of RNasin (Promega) per ml–0.4% NP-40, followed by Dounce homogenization. Cell lysis was checked microscopically, and samples were mixed with 4 volumes of 0.1 M Tris-Cl (pH 7.4)–10 mM NaCl–3 mM $MgCl_2$ and centrifuged for 10 min at 500 × *g*. The nuclear pellet was resuspended in 200 μl of 50 mM Tris-Cl (pH 8.3)–5 mM $MgCl_2$–0.1 mM EDTA–40% glycerol–1 mM DTT–0.1 mM phenylmethylsulfonyl fluoride–50 U of RNasin per ml, flash frozen in liquid N_2, and stored at −80°C. Nuclear run-on experiments were done by mixing 200 μl of nuclei with 200 μl of solution containing 140 mM KCl, 12.5 mM $MgCl_2$, 6.25 mM DTT, 0.25 mM EDTA, 1 mM each ATP, CTP, and GTP, and 0.5 mCi of [^{32}P]UTP (3,000 Ci/mmol). After a 30-min incubation at 30°C, nuclei were treated with 40 U of RNase-free DNase I (Boehringer) for 15 min at 37°C; 400 μl of a 2% SDS–20 mM Tris-Cl (pH 7.5)–10 mM EDTA solution and 8 μl of proteinase K (20 mg/ml) were added, and incubation was continued for an additional 30 min. Samples were then extracted with phenol-chloroform, ethanol precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of ethanol, redissolved in 200 μl of 10 mM Tris-Cl (pH 7.4)–10 mM NaCl–5 mM $MgCl_2$–1 mM $CaCl_2$, and digested with 200 U of RNase-free DNase I for 60 min at 37°C. RNA was again extracted with phenol-chloroform and ethanol precipitated as described above. Finally, samples were dissolved in hybridization buffer [50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5), 100 mM NaCl, 50 mM sodium phosphate (pH 7), 1 mM EDTA, 5% SDS]. Then 2.5 μg of ssDNA containing reverse complement sequences of the rat collagen α1(I) gene or the *GAPDH* gene (minus strand) and 2.5 μg of ssDNA containing the respective coding sequences (plus strand) were blotted onto a Hybond-N membrane (Amersham). In one control experiment, we had blotted 5 μg of double-stranded plasmid DNA, either without insert or with a collagen α1(I) or *GAPDH* insert (see above). In one experiment, quiescent HSCs produced 5.6×10^6 cpm/1.5×10^7 nuclei, while activated HSCs produced 6.1×10^6 cpm/1.5×10^7 nuclei. Membranes were prehybridized for 2 h at 65°C, after which 5×10^6 cpm of nuclear run-on transcripts from quiescent and activated HSCs was hybridized in 200 μl of hybridization buffer for 3 days. Membranes were washed once for 15 min at room temperature (RT) in 5% SDS–1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and twice for 30 min at 65°C in 0.1% SDS–0.1× SSC. To reduce background, membranes were rinsed once in 100 mM sodium phosphate (pH 7.2) and digested with 10 μg of RNase A per ml and 350 U of RNase T_1 (Gibco) for 30 min at 37°C. Following rinsing in 100 mM sodium phosphate, membranes

were subjected to autoradiography for 3 days with an intensifying screen and quantitated with a phosphorimager.

Immunohistochemical analysis. Culture-activated HSCs were grown on coverglasses in six-well dishes. Cells were fixed in 4% formalin (in phosphate-buffered saline [PBS]) for 15 min at RT and washed twice with PBS before permeabilization with 0.2% Triton X-100 (in PBS) for 10 min at RT. After two washes with PBS, blocking was performed with 2% goat serum diluted in PBS for 30 min at RT. The slides were incubated for 1 h at RT with either anti-αCP monoclonal antibody 6B12.A3 diluted 1:10 in PBS or anti-smooth muscle actin monoclonal antibody 1A4 (DAKO). Subsequent incubations with biotinylated secondary antibody and avidin-biotinylated enzyme complex was performed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, Calif.) as instructed by the manufacturer. Color was developed by incubating the slides for 5 to 10 min with diaminobezidine, using Fast-DAB tabs (Sigma Chemical Co., St. Louis, Mo.).

Western and Northwestern blots. Cytosolic protein extracts (S100) were prepared as previously described (64). Proteins were separated on an SDS–12.5% polyacrylamide gel and electroblotted onto a nitrocellulose membrane in buffer containing 25 mM Tris, 192 mM glycine, and 15% methanol. For Western analysis, filter blocking and antibody binding were performed as described previously (23). The polyclonal anti-αCP antibody was raised in chickens (Cocalico Biologicals, Inc.) against bacterially expressed and gel purified αCP-1 (to be detailed elsewhere). The antibody was purified from chicken yolks as described previously (40). Primary antibody was used at 1:10,000 dilution followed by secondary antibody (horseradish peroxidase-conjugated goat anti-chicken IgG; Accurate Antibodies) used at 1:7,000 dilution. The antibody complexes were detected by using the Amersham ECL system. For Northwestern analysis, the proteins were renatured in situ by incubating the membrane in renaturing buffer (10 mM Tris-Cl [pH 7.4], 50 mM NaCl, 1 mM EDTA, 1× Denhardt solution, 1 mM DTT) for 2 h at RT. Following renaturation, the membrane was incubated with ^{32}P-labeled poly(C) probe (20,000 cpm/ml) in hybridization buffer (10 mM Tris-Cl [pH 7.4], 50 mM NaCl, 1 mM EDTA, 1× Denhardt solution, 20 mg of tRNA per ml, 5 mg of heparin per ml) for 2 h at RT. The membrane was then washed three times (10 min each) in 10 mM Tris-Cl (pH 7.4)–50 mM NaCl–1 mM EDTA–1× Denhardt solution at RT and exposed to X-ray film with an intensifying screen.

RESULTS

Upregulation of collagen α1(I) mRNA in rat HSCs is mediated by a posttranscriptional mechanism. The steady-state level of collagen α1(I) mRNA was measured by an RNase protection assay in freshly isolated quiescent rat HSCs or activated HSCs cultured on plastic for 14 days. Collagen- and *GAPDH* gene-specific riboprobes were simultaneously hybridized, the latter serving as an internal control for recovery of the RNA. We assumed that there is no change in *GAPDH* mRNA levels between quiescent and activated HSCs, and we normalized expression of collagen α1(I) mRNA to that of *GAPDH* mRNA. In quiescent HSCs, a very low level of collagen α1(I) mRNA is detected (Fig. 1A, lane 5) in this overexposed autoradiogram. The activated HSCs (lane 6) accumulated approximately 70-fold more collagen α1(I) mRNA than did quiescent HSCs. Similar results have been previously reported for quiescent and activated HSCs in vivo and in culture (36, 65).

To assess the contribution of increased transcription of the collagen α1(I) gene in this dramatic upregulation, we performed nuclear run-on experiments with nuclei from freshly isolated HSCs and activated HSCs. Again we compared the rate of transcription of the collagen α1(I) gene to that of the *GAPDH* gene as a control. We hybridized nascent transcripts to ssDNAs immobilized on a membrane to increase the sensitivity of the assay and to distinguish between transcription from the coding and noncoding strands. The minus-strand ssDNA contains sequences complementary to the RNA transcribed from the collagen or *GAPDH* gene, and thus a signal in this slot indicates RNA polymerase II loading on the collagen (or *GAPDH*) gene. The plus-strand ssDNA contains sequences identical to those of the RNA originating from the collagen or *GAPDH* gene and therefore detects transcription in the opposite direction. We hybridized equal amounts (counts per minute) of nascent transcripts of nuclei from quiescent and activated HSCs. We found that activated HSCs have a transcriptional rate of the collagen α1(I) gene that is twofold-higher than that of quiescent HSCs (Fig. 1B). Since the rate of incorporation of radioactivity into nuclei was 15% higher in activated than quiescent HSCs, the activated HSCs had a 2.3-fold-higher transcriptional rate/nucleus of the collagen α1(I) gene. The signal from plus-strand ssDNA is much weaker, suggesting no significant transcription on the noncoding DNA strand or hybridization to plasmid sequences. *GAPDH* gene is also transcribed at a slightly higher rate in activated HSCs than in quiescent HSCs. However, the *GAPDH* gene in activated HSCs also has a signal from plus-strand ssDNA. This probably represents transcription from the noncoding DNA strand of this gene, but the significance of this observation is unknown. We have repeated the nuclear run-on experiment and hybridized the nascent transcripts to double-stranded DNA sequences with the same result. As a negative control, we included an empty plasmid which showed no hybridization (data not shown).

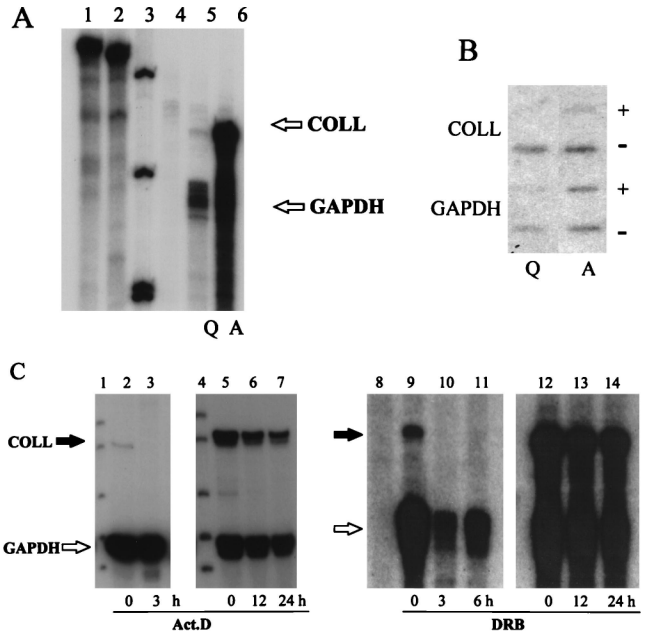

FIG. 1. Collagen α1(I) mRNA expression in HSCs. (A) RNase protection assay with collagen α1(I) (lane 1) and *GAPDH* (lane 2) gene-specific riboprobes and total RNA extracted from quiescent (Q; lane 5) and activated (A; lane 6) HSCs. Protected bands are indicated by arrows. Lane 3, pBR322/*Msp*I size marker; lane 4, tRNA control. (B) Nuclear run-on transcription in nuclei isolated from quiescent (Q) HSCs and (A) activated HSCs. Equal amounts (counts per minute) of nascent RNA were hybridized to ssDNA containing collagen α1(I) or *GAPDH* coding (+) or complementary (−) sequence. (C) mRNA stability assays using either actinomycin D (Act. D; lanes 1 to 7) or DRB (lanes 8 to 14). Reverse transcription-PCR analysis of 200 ng of total RNA extracted from quiescent (lanes 2 and 3) or activated (lanes 5 to 7) HSCs incubated with actinomycin D (25 μg/ml) for the indicated time periods. In lanes 2 to 7, the RNA was simultaneously analyzed with primers specific for collagen α1(I) and *GAPDH* mRNAs. Products of 76 nt (*GAPDH*) and 113 nt (collagen [COLL]) were resolved on a 6% denaturing gel and are indicated by arrows. Lanes 1 and 4 contain a pBR322/*Msp*I size marker. Lanes 8 to 14 represent an RNase protection assay with RNA isolated from quiescent (lanes 9 to 11) or activated (lanes 12 to 14) HSCs treated with 67 μM DRB for the indicated time periods. Arrows indicate protected bands. Lane 8 represents a control reaction with tRNA.

Since the increased transcription rate of the collagen α1(I) gene accounted for only a small increase in the steady-state levels of its mRNA, we decided to investigate the stability of the collagen α1(I) mRNA in quiescent and activated HSCs. We first used actinomycin D to block transcription because previous measurements of the collagen mRNA half-life by

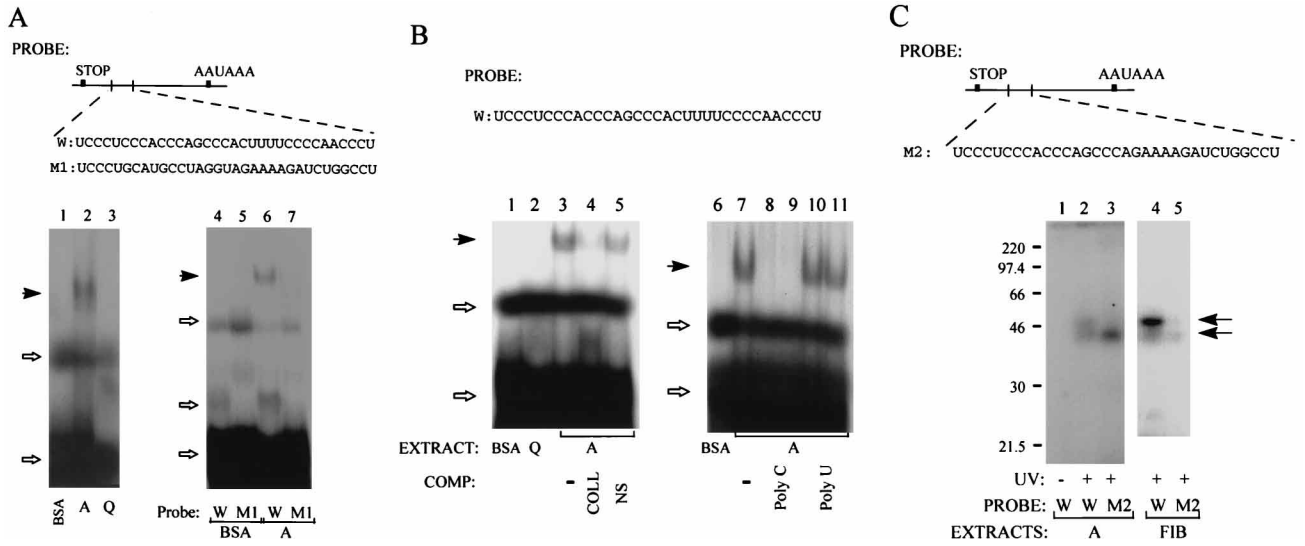

FIG. 2. RNA binding proteins interact with the 3′ UTR of collagen α1(I) mRNA. (A) Gel mobility shift experiment using a 316-nt RNA probe spanning the whole 3′ UTR. The sequences of the wild-type (W) and mutant (M1) RNA probes are shown at the top. In lanes 1 to 3 25 μg of BSA (lane 1) or cytoplasmic extract from activated (A; lane 2) or quiescent (Q; lane 3) HSCs was incubated with 40,000 cpm of the W probe. After digestion with RNase T_1 and addition of heparin, complexes were resolved on a 6% native acrylamide gel. Positions of the RNA-protein complex (full arrow) and free RNA (open arrows) are indicated. In lanes 4 to 7, the W probe (lanes 4 and 6) and M1 probe (lanes 5 and 7) were incubated with BSA (lanes 4 and 5) or activated HSC cytoplasmic extract (lanes 6 and 7) and analyzed by gel mobility shift. (B) Gel mobility shift with the 33-nt C-rich RNA probe. The sequence of the probe is shown at the top. This probe was incubated with 25 μg of BSA (lanes 1 and 6), 25 μg of quiescent HSC cytoplasmic extract (lane 2), or 25 μg of activated HSC cytoplasmic extract (lanes 3 to 5 and 7 to 11). A 100-fold molar excess of specific competitor (collagen [COLL]; lane 4) or nonspecific (NS) competitor (lane 5) was added. Poly(C) and poly(U) had an average size of 200 nt and were added in 100-fold (lanes 8 and 10) and 200-fold (lanes 9 and 11) molar excess. (C) UV cross-linking of proteins to the 3′ UTR of the collagen α1(I) mRNA. The sequence of the W probe is shown in panel A. The mutant probe (M2) has substitutions in a part of the C-rich sequence, as shown at the top. Sixty micrograms of cytoplasmic extract from activated HSCs (A; lanes 1 to 3) or from NIH 3T3 fibroblasts (FIB; lanes 4 and 5) was used in the binding reaction. After UV irradiation and extensive RNase digestion, samples were resolved on an SDS–10% polyacrylamide gel (lanes 2 to 5). Lane 1 is the sample without UV irradiation. The size markers (positions indicated in kilobases) are low-molecular-weight prestained protein size markers from Amersham. The arrows indicate the positions of RNA-protein complexes.

pulse-chase methods and using actinomycin D produced similar results in fibroblasts (21). Preliminary experiments demonstrated that collagen α1(I) mRNA decays very slowly in activated HSCs; therefore, these cells were incubated for up to 24 h with actinomycin D. During this time period, the cells remained morphologically unchanged. Quiescent HSCs were incubated with actinomycin D for only 3 h. For more sensitive detection of already very low collagen α1(I) mRNA levels in quiescent HSCs, we used a reverse transcription-PCR analysis. Two sets of primers, one specific for collagen α1(I) mRNA and the other specific for *GAPDH* mRNA, were used for this assay. Collagen primers were derived from two exons to discriminate a signal originating from potentially contaminating DNA. Amplification with these primers was not affected by the presence of *GAPDH* primers (data not shown). Both sets of primers were used together in an amplification reaction which was limited to 20 cycles. In quiescent HSCs, the collagen α1(I) mRNA level had decreased to 25% of the starting level at 3 h after the inhibition of transcription (Fig. 1C, lane 3). In activated HSCs, more than 50% of the collagen α1(I) mRNA still remained after 24 h of actinomycin D treatment (Fig. 1C; compare lanes 5 and 7), suggesting a markedly increased stability compared to quiescent HSCs. During this time, *GAPDH* mRNA showed only about 30% decay, indicating that it may be slightly more stable than collagen α1(I) mRNA. To show that actinomycin D had indeed blocked transcription in activated HSCs, we reprobed the same RNA samples for the presence of histone H3 mRNA, which is known to have a half-life of about 3 h in slowly dividing cells (1). As expected, this mRNA could not be detected in cells incubated with actinomycin D for 12 or 24 h (data not shown). We quantitated relative abundances of collagen and *GAPDH* mRNAs in the time zero samples and found that there was 60-fold more collagen α1(I) mRNA in activated than in quiescent HSCs. This result is in a good agreement with data obtained by an RNase protection assay (Fig. 1A and C) and confirms the validity of our reverse transcription-PCR assay. From this experiment, we estimated by interpolation (56) that collagen α1(I) mRNA has half-lives of 1.5 h in quiescent HSCs and 24 h in activated HSCs.

We also measured the half-life of collagen α1(I) mRNA in HSCs by using the inhibitor of transcription DRB and quantitated expression by an RNase protection assay (Fig. 1C, lanes 9 to 14). In quiescent HSCs, collagen α1(I) mRNA was undetectable both 3 and 6 h after addition of DRB (compare lanes 9, 10, and 11). In activated HSCs, there was only a 50% decrease in the level of this mRNA 24 h after addition of the inhibitor. Thus, experiments with two inhibitors, actinomycin D and DRB, and two assays for mRNA levels consistently demonstrate a longer half-life of collagen α1(I) mRNA in activated HSCs than in quiescent HSCs.

RNA binding proteins interact with the collagen α1(I) 3′ UTR and are differentially expressed in HSCs. The stability of most mRNAs is mediated by sequences in their 3′ UTRs and controlled by proteins binding to them (4, 11, 56). Therefore, we decided to examine the 3′ UTR of collagen α1(I) mRNA for protein binding activity. We derived a 316-nt RNA probe spanning the whole 3′ UTR of the mouse 4.7-kb mRNA as well as 39 nt of the coding region (Fig. 2A) and incubated this probe with cytoplasmic extracts made from quiescent and activated HSCs. We detected an RNA-protein complex in extracts from activated but not quiescent HSCs (Fig. 2A, lanes 2 and 3). This RNA has a C-rich region located 22 nt 3′ to the stop codon (Fig. 2A, W). Substitution of this C-rich sequence with 25 nt of an unrelated sequence (M1 in Fig. 2A) greatly

diminishes protein binding to the entire 3′ UTR (compare lanes 6 and 7), suggesting the importance of this sequence for protein interaction. To show that this sequence is not only necessary but also sufficient for the interaction, we derived a short RNA probe spanning only the C-rich sequence (Fig. 2B). Again, in quiescent HSCs no protein binding could be demonstrated (lane 2), while an RNA-protein complex was efficiently formed in extracts from activated HSCs (lane 3). This binding was competed with an excess of specific competitor (lane 4); an excess of nonspecific competitor has almost no effect (lane 5). The binding to the collagen α1(I) mRNA 3′ UTR is also effectively competed with excess poly(C) (lanes 8 and 9) but not with excess poly(U) (lanes 10 and 11).

To provide insight into proteins that are present in the complex which binds collagen α1(I) mRNA, we performed a photoaffinity cross-linking experiment. Using the 316-nt probe (identical to that shown in Fig. 2A) and extracts from activated HSCs (Fig. 2C, lanes 1 to 3) or NIH 3T3 fibroblasts (lanes 4 and 5), two proteins were cross-linked; one protein-RNA complex has a size of about 45 kDa, and the other has a size of about 40 kDa. No cross-linked proteins were detected in extracts from quiescent HSCs. When we used the short probe (identical to that shown in Fig. 2B) in UV cross-linking experiments, the 45-kDa complex, but not the 40-kDa complex, was formed (data not shown). Since U is a photosensitive nucleotide in RNA, proteins are almost always cross-linked to RNA at U residues (22). We reasoned that the C-rich sequence is required for binding of the complex and that the U's found within this sequence are necessary for formation of the cross-link. Therefore, we mutated the 3′ portion of the C-rich region identified in the previous experiment as the protein binding site and analyzed cross-linking to this mutant (M2 [Fig. 2C]). This substitution abolished formation of the 45-kDa complex, while the 40-kDa complex could still be demonstrated (lanes 3 and 5), suggesting that this protein cross-links to another U(s) on this 316-nt RNA.

From the foregoing experiments, we concluded that there is an RNA binding activity that is targeted to the C-rich region in the 3′ UTR of collagen α1(I) mRNA. This activity is absent from cytoplasmic extracts of quiescent HSCs, where the mRNA has a short half-life, and is present in extracts from activated HSCs and fibroblasts, where its half-life is prolonged. Therefore, it may be involved in stabilization of collagen α1(I) mRNA in HSCs. A protein of about 45 kDa seems to be an RNA binding subunit of this activity, as suggested by UV cross-linking experiments.

Expression of collagen minigenes. To provide evidence for a functional significance of this RNA-protein interaction, we used a collagen α1(I) minigene that is suitable for experimental manipulations. Transcription of this gene is driven by 220 nt of the promoter, followed by its first six exons ligated to its four last exons. We truncated the minigene to contain only the first polyadenylation site (Fig. 3A). In the 3′ UTR of this minigene (W), we created the same substitution (M2) which abolished formation of the 45-kDa RNA-protein complex (Fig. 2). W and M2 minigenes with a luciferase reporter gene plasmid (to control for transfection efficiency) were transiently transfected into NIH 3T3 fibroblasts, and accumulation of the mRNA derived from the minigenes and the endogenous collagen α1(I) gene was analyzed by an RNase protection assay. Fibroblasts were used because activated HSCs cannot be efficiently transfected to allow for such analysis. Also, collagen α1(I) mRNA has a long half-life in fibroblasts, which contain the binding activity to the 3′ UTR (Fig. 2). We quantitated the expression of minigene mRNA and corrected for transfection efficiency by analyzing luciferase activity. Figure 3B shows the result of

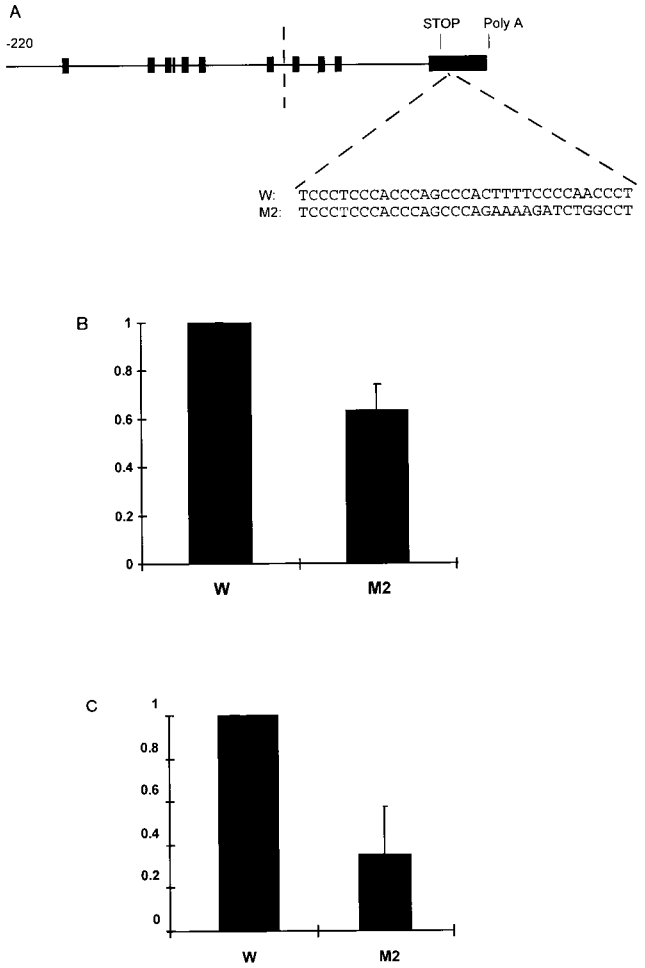

FIG. 3. Expression of collagen α1(I) minigenes. (A) Schematic representation of collagen α1(I) minigenes. Exons are shown as boxes; promoter, introns, and 3′ UTR are represented by lines (not to scale). The point of ligation of the 5′ portion to the 3′ portion of the gene is indicated. The wild-type sequence of the 45-kDa cross-link site (W) and the mutation introduced (M2) are shown. (B) Quantitation of expression of W and M2 collagen α1(I) minigenes. Collagen minigenes were cotransfected with the luciferase gene into NIH 3T3 fibroblasts, and the mRNA level was analyzed by RNase protection assay. In three separate experiments, expression of the minigenes was quantified relative to that of the endogenous collagen α1(I) gene by phosphorimage scanning of the gels and corrected for transfection efficiency by analyzing luciferase activity. Expression of the W minigene was arbitrarily set at 100%. The error bar shows standard deviation. (C) Comparison of the mRNA half-lives of W and M2 collagen α1(I) minigenes. The W and M2 collagen α1(I) minigenes were stably transfected into NIH 3T3 fibroblasts and the stable cell lines were incubated with actinomycin D for 0 or 24 h in two separate experiments. Collagen α1(I) mRNA levels were quantified by RNase protection assays and expressed as the ratio of the mRNA level at 24 h to that at 0 h. This ratio for the W minigene was arbitrarily set at 1.0. The error bar shows standard deviation.

three independent transfections. RNA derived from the M2 minigene accumulated to 60% of the level of RNA transcribed from W minigene. This result is consistent with the model that binding of a protein of approximately 45-kDa to the 3′ UTR of collagen α1(I) mRNA increases the steady-state level of this mRNA. Stable cell lines of NIH 3T3 cells expressing the minigenes produced similar results, with the half-life of the mRNA from the M2 minigene being 35% that of the mRNA from the W minigene (Fig. 3C).

αCP is a subunit of a complex that binds collagen α1(I) mRNA 3′ UTR. αCP is a ubiquitous 39-kDa protein (based on

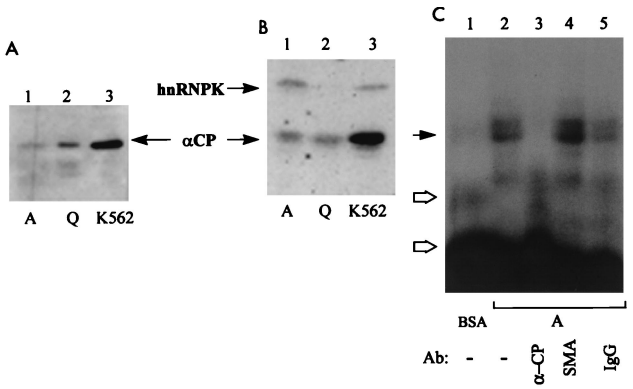

FIG. 4. αCP is a subunit of a protein complex binding the collagen α1(I) mRNA 3′ UTR. (A) Western analysis done with 40 μg of cytoplasmic extracts from quiescent (Q) and activated (A) HSCs and S100 extract from K562 human erythroleukemia cells. The blot was probed with a polyclonal antibody directed against αCP. (B) Northwestern blot with the same extracts, probed with 20,000 cpm of 5′-end-labeled poly(C). Positions of the αCP and hnRNP-K proteins are indicated. (C) Inhibition of collagen α1(I) mRNA 3′ UTR binding activity with the anti-αCP monoclonal antibody (Ab). RNA binding was done by mixing 25 μg of extracts from activated HSCs with 10 μl of 5% BSA (lane 2), 10 μl of ascites fluid containing anti-αCP antibody 6B12.A3 (lane 3), 10 μl of ascites fluid containing anti-smooth muscle actin (SMA) antibody (lane 4), or 2 μg of mouse IgG in 10 μl of 5% BSA (lane 5). Lane 1 is a control reaction without extract. After incubation with the 33-nt probe (Fig. 2B) and RNase T_1 digestion, the complexes were resolved on a 6% native acrylamide gel. Positions of free RNA (open arrows) and RNA-protein complexes (full arrow) are indicated.

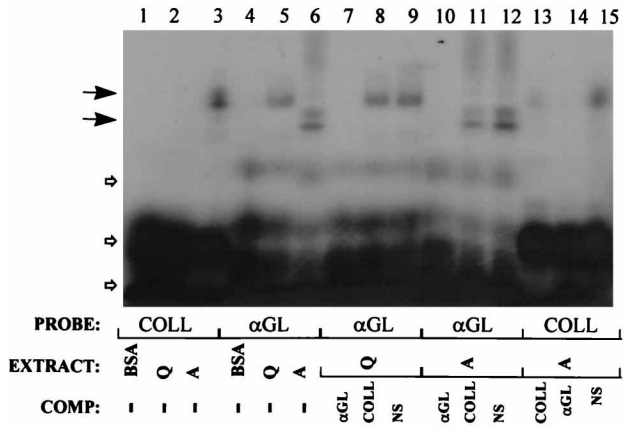

FIG. 5. Cross-competition between collagen α1(I) and α-globin RNA protein binding sequences. A gel mobility experiment was done as for Fig. 2. The RNA probes were the collagen α1(I) (COLL) sequence shown in Fig. 2B and human α-globin (αGL) nt +29 to +70 from the 3′ UTR. The same unlabeled RNAs were used as competitors as indicated. The nonspecific competitor (NS) was a 434-nt RNA derived from the antisense strand of the rat *GAPDH* cDNA. The competitors were added in 100-fold molar excess. The positions of free RNA (open arrows) and RNA-protein complexes (full arrows) are indicated.

the predicted amino acid sequence) which has been cloned by its ability to bind poly(C) nucleic acids (20). Our UV cross-linking and RNA binding experiments (Fig. 2) identified a protein of about 45 kDa that preferentially binds the C-rich sequence in the 3′ UTR of the collagen α1(I) mRNA. Taking into account that cross-links always contain a small portion of RNA, which can affect the apparent molecular weight of the protein, we investigated whether our 45-kDa protein is in fact αCP. First, we determined whether cytoplasmic extracts from HSCs contain αCP by Western and Northwestern analyses. S100 extracts from K562 cells served as a positive control in these experiments. Figure 4A shows that antibodies against αCP detected proteins of corresponding sizes in both quiescent and activated HSCs extracts. The levels were similar or slightly greater in quiescent HSCs.

αCP alone can bind poly(C) homopolymers but not degenerate C-rich sequences such as the α-globin sequence (29, 64). Results of Northwestern analysis using an ssDNA poly(C) oligonucleotide as a probe were similar to those of the αCP Western blot analysis (Fig. 4AB), suggesting that αCP is present in extracts of quiescent and activated HSCs. The [^{32}P]poly(dC) probe also reacts in the Northwestern analysis with hnRNP-K, which is sometimes seen in cytoplasmic extract preparations as a result of nuclear contamination. Therefore, we performed immunostaining of activated HSCs to investigate subcellular localization of αCP in HSCs. αCP appears uniformly distributed in activated HSCs, being present in the cytoplasm as well as in the nucleus (data not shown). To establish whether αCP is actually present as a component of binding activity to the collagen α1(I) 3′ UTR, we performed a binding experiment in which we added either an αCP-specific antiserum or nonspecific antiserum to the extracts of activated HSCs and compared their abilities to bind the collagen probe by gel retardation analysis. The αCP-specific antibody (Fig. 4C, lane 3) abolished formation of the RNA-protein complex whereas nonspecific antibodies (lanes 4 and 5) had no effect, suggesting that αCP is required for formation of this RNA binding activity. Since activated but not quiescent HSCs can bind to the collagen α1(I) 3′ UTR, although they both contain αCP, it seems that αCP is necessary but not sufficient to promote protein binding to the collagen α1(I) 3′ UTR. Alternatively, αCP may be modified in activated HSCs.

α-Globin and collagen α1(I) 3′ UTR binding activities. Collagen α1(I) mRNA has a half-life of 24 h in activated HSCs (Fig. 1), and a 45-kDa protein (as estimated by UV cross-linking [Fig. 2]), probably as a part of a multiprotein complex, interacts with the C-rich sequence in its 3′ UTR. This interaction may be responsible for stabilization of the mRNA. Human α-globin mRNA has half-life of days in reticulocytes (67), and the 3′ UTR of this mRNA has a C-rich sequence, which is not identical to that of collagen α1(I) mRNA (64). A ubiquitous poly(C) binding protein of 39 kDa was identified as a component of this binding activity and shown to be identical to αCP (29). We investigated the relationship between the RNA binding proteins that bind to these C-rich sequences in the collagen α1(I) and α-globin mRNAs 3′ UTRs. We performed RNA binding and competition experiments with collagen α1(I) and α-globin sequences (Fig. 5). In agreement with our previous data (Fig. 2), there is no binding activity to the collagen α1(I) C-rich sequence in quiescent HSCs (lane 2), while it is readily observable in activated HSCs (lane 3). To our surprise, in quiescent HSCs we could detect a complex formed on the α-globin C-rich sequence (lane 5). In activated HSCs, there is a binding activity to the α-globin sequence as well, although it has higher electrophoretic mobility and is resolved as two bands (lane 6). It also has a different electrophoretic mobility than the complex formed on the collagen probe (compare lanes 3 and 6). These data suggest that the complexes binding collagen α1(I) and α-globin C-rich sequences are composed of different subunits. Although quiescent HSCs contain αCP, the accessory component(s) necessary for binding the collagen α1(I) mRNA is lacking, but the accessory components necessary for binding to the α-globin mRNA are present.

Next we performed cross-competition studies where we competed binding to the collagen α1(I) sequence with the α-globin sequence and vice versa in cytoplasmic extracts from

quiescent and activated HSCs (Fig. 5, lanes 7 to 15). In quiescent HSCs, interaction with the α-globin probe was not competed with a 100-fold molar excess of the collagen α1(I) sequence (lane 8) or with excess nonspecific sequence (lane 9). It was effectively competed with the same molar excess of specific competitor (lane 7). Therefore, the collagen sequence neither binds proteins nor competes for binding to the α-globin sequence in extracts from quiescent HSCs. In activated HSCs, the collagen α1(I) sequence partially inhibited binding to the globin probe (compare lanes 11 and 12, which contains the nonspecific competitor), although not as well as the α-globin sequence itself (lane 10). Thus, in activated HSCs, there is some degree of cross-competition between α-globin- and collagen C-rich sequences, implying that they share at least one component required for binding. Since activated HSCs contain all accessory components to promote formation of an αCP-containing complex on the collagen sequence, it is likely that sequestration of αCP in these complexes by an excess of the collagen sequence inhibited binding to the α-globin probe. The opposite also seems to be true in that the α-globin sequence efficiently competes binding to the collagen probe (lane 14), again somewhat better than the collagen sequence itself (lane 13). Presence of a nonspecific RNA sequence has no influence on binding (lane 15).

DISCUSSION

We have investigated mechanisms controlling the increase of collagen α1(I) mRNA during activation of HSCs and have demonstrated three main observations: (i) the regulation of collagen α1(I) mRNA is predominantly posttranscriptional, (ii) this regulation is correlated with a differentially expressed RNA binding activity, and (iii) this binding activity contains as a subunit αCP, which is also involved in stabilization of α-globin mRNA (67). There is 60- to 70-fold increase in collagen α1(I) mRNA steady-state level in activated HSCs compared to quiescent HSCs (Fig. 1A). Similar increases were observed by other investigators for activated HSCs in culture (25, 37, 65) and in vivo (36, 66). In our study, activated HSCs transcribe the collagen α1(I) gene at a rate twofold higher than that for quiescent HSCs (Fig. 1B), which is insufficient to account for a 60- to 70-fold increase in the mRNA accumulation.

Injection of CCl_4 into experimental animals results in approximately a 30-fold increase in collagen α1(I) mRNA in total liver RNAs (unpublished observation and reference 8). There is about a twofold increase in collagen α1(I) gene transcription in total liver nuclei from CCl_4-treated rats compared to normal liver nuclei (47, 48). Taking into account that HSCs are the major liver cells capable of upregulating collagen α1(I) mRNA following a fibrogenic stimulus (36), these results are in excellent agreement with what we have found in this study for culture-activated HSCs. On the other hand, expression of a reporter gene driven by the collagen α1(I) minimal promoter was increased about 40-fold in activated compared to quiescent HSCs (25). The different results from the reporter gene assay and the nuclear run-on assays might reflect aberrant expression of the multicopy reporter gene, which was missing collagen α1(I) gene regulatory elements (references 3 and 54 and unpublished observations). Alternatively, the nuclear run-on assays could overestimate synthesis of the full-length mRNA in quiescent HSCs, such as could be produced by a block of transcriptional elongation (30).

Measurement of the collagen α1(I) mRNA half-life in HSCs has not been done previously. The data presented in Fig. 1C suggest that an important posttranscriptional mechanism regulating collagen α1(I) mRNA involves stabilization of the mRNA. We have estimated by interpolation (56) that the half-lives of collagen α1(I) mRNA are 1.5 h in quiescent HSCs and 24 h in activated HSCs (Fig. 1C). Although we did not precisely measure the half-life, this is a dramatic change in stability of this mRNA. With only a moderately increased transcription rate of the gene, this increase in stability is primarily responsible for the observed increase in steady-state levels of collagen α1(I) mRNA in HSCs. Experiments with fibroblasts have shown that collagen α1(I) mRNA can be regulated at the level of mRNA stability, and in these cells the stability can be modulated by culturing conditions and hormonal treatment (14, 15, 33, 48, 49). The half-life of collagen α1(I) mRNA in NIH 3T3 and human skin fibroblasts, cultured under conditions similar to those used for HSCs in our study, was estimated to be 9 h (21, 49), while it is more than 16 h in rat fibroblasts (reference 61 and unpublished results) and even longer in activated HSCs.

The synthetic pathway of the collagen α1(I) mRNA in HSCs is unknown. Collagen α1(I) mRNA may be associated with polysomes and translated throughout its functional life, so that it would synthesize abundant protein even if relatively inefficiently translated. Alternatively, it may be sequestered within a cytoplasmic compartment, with only a fraction targeted for translation. The conserved stem-loop structure overlapping the translation initiation codon in collagen α1(I) mRNA, also found in α2(I) and α1(III) mRNAs (69), may be involved in regulation of these processes. This stem-loop structure can inhibit in vivo and in vitro translation of a chimeric mRNA (unpublished observation).

Theoretically, in quiescent HSCs, collagen α1(I) mRNA may be actively degraded, may be unstable by a default mechanism and stabilized during activation of HSCs, or may be regulated by a combination of the two effects. In activated HSCs, this mRNA undergoes a dramatic stabilization (Fig. 1C). Stability of most mRNAs is regulated by sequences in their 3′ UTRs and determined by proteins interacting with these sequences (4, 11, 56). An RNA binding activity to the 3′ UTR of collagen α1(I) mRNA has been previously identified in fibroblasts, but its binding site was not mapped (34). Here we show that in HSCs, an RNA binding activity is targeted to the C-rich sequence located 23 nt 3′ to the stop codon (Fig. 2) and that its binding correlates with accumulation of collagen α1(I) mRNA in HSCs. At the similar position in the 3′ UTR of the α-globin gene there is a sequence consisting of three C-rich regions, which combines with cytosolic proteins to form a sequence-specific complex, the α complex. Mutations within these C-rich regions block complex formation in vitro (64). The α complex is composed of three protein subunits (29, 64). The smallest component is αCP, which belongs to the KH domain family of RNA binding proteins and by itself is capable of binding poly(C) and poly(dC) single-stranded nucleic acids (20, 39, 64). However, the αCP cannot bind the α-globin sequence on its own; this interaction appears to be dependent on the presence of accessory proteins. Mutation of the stop codon in the α-globin gene into a missense codon is a naturally occurring mutation that is associated with low levels of α-globin mRNA and severe anemia (thalassemia Constant Spring) (67). It has been proposed that ribosomes translating into the 3′ UTR of Constant Spring α-globin mRNA are capable of displacing the α complex, causing destabilization of the α-globin mRNA. In transient transfection experiments, mutation of the binding site for the α complex diminished accumulation of α-globin mRNA to 30% of the wild-type level (67).

We performed a similar type of experiment in which mutation of the collagen α1(I) mRNA C-rich sequence decreased accumulation of mRNA derived from collagen minigenes to 60% of the wild-type level (Fig. 3). We also stably transfected

NIH 3T3 cells with W and M2 collagen minigenes and measured the half-lives of endogenous collagen α1(I) mRNA and minigene RNA with actinomycin D. Full-size collagen α1(I) mRNA had a half-life of about 6 h, in accordance with previously published results (49), while minigene mRNA had a half-life of longer than 24 h. However, the M2 minigene mRNA had a 35% lower half-life than the W minigene mRNA (Fig. 3C). The less pronounced effect of the mutation on collagen minigene mRNA may reflect one of several possible underlying mechanisms. Collagen minigene mRNA does not contain all of the sequences present in full-size α1(I) mRNA, and some long-range interactions important for proper folding of this molecule may be missing (18, 58). In fact, the minigene mRNA is more stable than the endogenous mRNA and therefore might blunt the effect of the destabilizing mutation. In the mutation tested, we substituted 12 nt and abolished cross-linking to a 45-kDa protein; however, cross-linking to a 40-kDa protein persisted (Fig. 2C). Even changing the entire C-rich sequence does not completely abolish protein binding to the 3′ UTR (Fig. 2A, lane 7). It is possible that this yet uncharacterized interaction also contributes to the stability of collagen α1(I) mRNA. Therefore, results with minigene mRNAs cannot be quantitatively extrapolated to naturally occurring collagen α1(I) mRNA. Furthermore, additional regions of the collagen α1(I) mRNA, such as the 5′ UTR or coding region, may also contribute to its increased stability in activated HSCs.

Our findings, nevertheless, suggest that the RNA-protein interaction at the C-rich sequence in the 3′ UTR may be required for high-level accumulation of collagen α1(I) mRNA. We believe that it stabilizes this mRNA because it involves an RNA binding activity that shares at least one subunit with the α complex. Based on inhibition of binding by the anti-αCP antibody (Fig. 4C), competition of binding by poly(C) (Fig. 2B), and UV cross-linking (Fig. 2C), this subunit seems to be αCP.

The collagen α1(I) C-rich sequence is shorter and different from that of the α-globin sequence. However, in activated HSCs, the α-globin sequence efficiently competes for binding to the collagen sequence and the collagen sequence competes, although slightly less effectively, for binding to the α-globin sequence (Fig. 5), again suggesting interaction with a common factor(s). The difference in the effectiveness of competition may reflect slightly higher affinity of protein binding to the α-globin sequence. αCP is a ubiquitous protein and is found in extracts from quiescent HSCs (Fig. 4). We tested many extracts from quiescent HSCs but could not detect binding to the collagen sequence, while binding to the α-globin sequence was readily demonstrable. In the quiescent HSC extracts, the collagen sequence could not compete for binding to the α-globin sequence. Since αCP alone cannot bind to degenerate C-rich sequences, accessory components required for binding to the collagen sequence are lacking in these extracts but those necessary for binding to the globin sequence are present. Thus, accessory factors mediating binding of αCP to the collagen α1(I) and α-globin 3′ UTRs are clearly different. This conclusion is also suggested by the different electrophoretic mobilities of complexes formed on collagen and globin probes in activated HSCs extracts (Fig. 5; compare lanes 3 and 6). It is possible that αCP has the ability to form an array of heteromers by association with various auxiliary factors. In HSCs, these auxiliary factors are differentially expressed and probably regulatory for collagen α1(I) mRNA accumulation. Alternatively, it is possible that αCP is modified in activated HSCs such that it binds the collagen 3′ UTR without participation of auxiliary factors. If this is true, then such a modification is subtle since we have not seen a difference in electrophoretic mobility of αCP in extracts of quiescent and activated HSCs (Fig. 4).

It is almost certain that more long-lived mRNAs that are stabilized by αCP-containing complexes will be discovered. Identification and characterization of auxiliary factors involved in formation of the complex that binds collagen α1(I) mRNA 3′ UTR in HSCs is an important goal. This analysis will provide insight into the critical regulatory mechanism of collagen α1(I) gene expression in HSCs and may lead to development of drugs for treatment of liver fibrosis.

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