The 5′ stem-loop is a conserved sequence element found around the translation initiation site of three collagen mRNAs, α1(I), α2(I), and α1(III). We show here that the 5′ stem-loop of collagen α1(I) mRNA is inhibitory to translation in vitro. The sequence 5′ to the translation initiation codon, as a part of the 5′ stem-loop, is also not efficient in initiating translation under competitive conditions. This suggests that collagen α1(I) mRNA may not be a good substrate for translation. Since the 5′ stem-loop binds protein factors in collagen-producing cells, this binding may regulate its translation in vitro. We studied in vivo translation of collagen α1(I) mRNA after transfecting collagen α1(I) genes with and without the 5′ stem-loop into Mov 13 fibroblasts. The mRNA with the α1(I) 5′ stem-loop was translated into pepsin-resistant collagen, which was secreted into the cellular medium. This mRNA also produced more disulfide-bonded high molecular weight collagen found intracellularly. The mRNA in which the 5′ stem-loop was mutated, but without affecting the coding region of the gene, was translated into pepsin-sensitive collagen and produced only trace amounts of disulfide-bonded collagen. This suggests that the 5′ stem-loop is required for proper folding or stabilization of the collagen triple helix. To our knowledge this is the first example that an RNA element located in the 5′-untranslated region is involved in synthesis of a secreted multisubunit protein. We suggest that 5′ stem-loop, with its cognate binding proteins, targets collagen mRNAs for coordinate translation and couples translation apparatus to the rest of the collagen biosynthetic pathway.

Three fibrillar collagen mRNAs, α1(I), α2(I), and α1(III), are coordinately regulated in fibrotic processes of various organs (1, 2). In the 5′-UTR of these mRNAs there is a stem loop structure (5′ stem-loop) encompassing the translation initiation codon (3). The 5′ stem-loop is located about 75 nt from the cap and has a stability of ΔG = 25–30 kcal/mol. The 5′ stem-loop is well conserved in evolution, differing by only two nucleotides in Xenopus and human collagen mRNAs (4). The sequence flanking the 5′ stem-loop is not conserved. A similar stem-loop structure is also found around the translation start codon of the sea urchin collagen gene (5). Evolutionary conservation of this sequence suggests an important function.

We analyzed previously a regulatory role of the 5′ stem-loop in two experimental systems; quiescent versus activated hepatic stellate cells (HSCs) (6) and fibroblasts cultured in a three-dimensional matrix (7). Activated HSCs are responsible for excessive collagen production in liver fibrosis (8, 9). We found that the 5′ stem-loop prevented expression of the reporter genes in quiescent HSCs, which express low amounts of type I collagen, but allowed for expression in activated HSCs. This inhibitory effect of the 5′ stem-loop was in part mediated by a decreased half-life of the corresponding mRNAs. Reporter genes with the mutated 5′ stem-loop were 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 post-transcriptional mechanism (6).

Second, we studied the role of the 5′ stem-loop on collagen α1(I) mRNA expression in fibroblasts cultured in a three-dimensional matrix where the fibroblasts revert from an activated phenotype to a more quiescent phenotype. This is accompanied by destabilization of endogenous collagen α1(I) mRNA (10). The reporter collagen α1(I) mRNA with the intact 5′ stem-loop was less stable than the identical mRNA with the mutated 5′ stem-loop in the cells grown in the matrix. Thus, the 5′ stem-loop is required for accelerated decay of collagen α1(I) mRNA in cells that down-regulate collagen synthesis (7).

The mechanism by which the 5′ stem-loop targets mRNAs for turnover in HSCs and fibroblasts grown in a three-dimensional matrix is unknown. In quiescent HSCs we could not detect any protein binding to the 5′ stem-loop in vitro. In activated HSCs a cytosolic protein factor(s) of unknown identity binds to the stem-loop and requires a 7mG cap on the RNA for binding (6). An excess of cap analogue completely prevents formation of this complex in vitro. The complex is also found in fibroblasts in polysomal cytoplasmic fraction. Its binding is greatly reduced if the cells are cultured in a three-dimensional matrix (7). One possibility is that the 5′ stem-loop binding activity may increase the steady-state level of collagen mRNAs by diverting them from the degradative pathway (11).

Translation and mRNA decay are coupled processes (12). Therefore, studies on the translation of collagen α1(I) mRNA are required to provide insight into the mechanism of stabilization of this mRNA. All collagen α1(I) mRNA is associated with membrane-bound polysomes and is not found on free
Role of the 5’ Stem-Loop in Translation of Collagen α(I) mRNA

polysomes or in postpolysomal supernatant. It is not known if this association is because of targeting of the mRNA or targeting by the leader peptide after initiation of translation. Collagen are secreted proteins, and their translation is coupled to export of the peptides into the endoplasmic reticulum (13). There is substantial evidence that all three peptides initiate folding into the heterotrimer while still associated with polysomes on the endoplasmic reticulum (14–17). When folding is initiated, the collagen trimer is released in the lumen of the endoplasmic reticulum. In human disease oxygenase imperfection (OI) certain mutations of α(1) chain decrease the rate of assembly of collagen type 1. Unassembled OI α(1) chains are hypermodified on proline and lysine residues and degraded (18, 19). This suggests that modification and assembly processes are in a kinetic equilibrium. It is possible that the 5’ stem-loop binding activity may target collagen mRNAs to translation at such sites.

In this study we investigate the role of the 5’ stem-loop in translation of collagen α(1) mRNA in vitro and in vivo. We found that the 5’ stem-loop inhibits translation in vitro and when more than one mRNA is competing for a limited amount of translational apparatus. In fibroblasts in vivo, the 5’ stem-loop is necessary for efficient folding or synthesis of stable triple helical collagen. To our knowledge this is the first example of an RNA element that affects protein folding.

MATERIALS AND METHODS

 Constructs—Plasmid used for in vitro transcription of PSII mRNA was constructed by cloning of the double-stranded oligonucleotide with the sequence shown in Fig. 1B into HindIII and NarI sites of the pGL3 vector (Promega). Then, the double-stranded oligonucleotide with the sequence of the T7 promoter was cloned into BglII-HindIII sites of the above construct. This plasmid was linearized with HpaI and transcribed in vitro with T7 polymerase and Cap-scribe kit (Roche Molecular Biochemicals) to produce capped PSII mRNA. POLL-START and COLL-OPTSTART constructs were made by cloning double-stranded oligonucleotides with the sequence shown in Fig. 1B into SacI-NarI sites of pGL3 and recloning of the EcoRI-HpaI fragment into EcoRI-Smal sites of the pGEM3 vector (Promega). After linearization with BamHI corresponding capped mRNAs were synthesized with T7 polymerase and Cap-scribe kit (Roche Molecular Biochemicals) to produce capped α(I) mRNA. POLL-START and COLL-OPTSTART constructs were made by cloning double-stranded oligonucleotides with the sequence shown in Fig. 2A into the HindIII-NarI sites of the pGL3 vector and cloning of the T7 promoter as for PSII. Capped mRNAs were made as described for PSII mRNA. The clone producing competitor A mRNA was made by cloning 75 codons of an α(I) cDNA (a kind gift from Dr. M. Breindl) into EcoRI-XhoI sites of the vector pCDNA3 (Stratagene), followed by cloning of an optimal translation start site (Fig. 1B) to allow expression. This plasmid was linearized with NotI, and competitor A mRNA was made as for PSII mRNA. All in vitro produced mRNAs were gel-purified and analyzed by agarose gel electrophoresis. β-globin mRNA and tobacco mosaic virus (TMV) mRNA were prepared from the Roche Molecular Biochemicals in vitro translation kit.

The 5’ WT-MH-COLL gene was made by cloning the BglII-XhoI fragment of mouse genomic DNA containing 220 nt of the promoter and 115 nt of the 5’ UTR (a kind gift from Dr. M. Breindl) into BglII-XhoI sites of the pGL3 vector and inserting the above construct into the XhoI-BamHI fragment of mouse collagen α(I) cDNA (a kind gift from F. Ramirez). This restores the 5’ stem-loop, which is identical in mouse and human collagen α(I) mRNA and includes the entire coding region and 3’-UTR of human collagen α(I) cDNA. The 5’ MUT-MH-COLL gene was made identically except that a substitution of 18 nt, shown in Fig. 2A, was introduced into the mouse genomic clone but not in the full-size construct.

A riboprobe for analyzing expression from MH-COLL genes was made by cloning the XbaI-KpnI fragment of human cDNA clone into XbaI-KpnI of Bluscript SK vector (Invitrogen). This plasmid was linearized with NotI and transcribed by T7 polymerase in the presence of [35S]UTP as described (20).

In Vitro Translation Reactions—0.08 pmol of gel-purified mRNAs was translated in a 50-μl reaction using nuclease-treated rabbit reticulocyte lysate (Roche Molecular Biochemicals), according to the manufacturer’s instructions. In preliminary experiments 0.08 pmol of mRNA was found to be a nonsaturation concentration of mRNA for a 50-μl reaction. Competitor mRNA was added in 10-fold molar excess (0.8 pmol) to the test mRNA prior to mixing with the lysate. Reactions were incubated for 30 min at room temperature when a 5-μl aliquot was analyzed for luciferase activity. Incubations longer than 30 min did not further increase luciferase activity. For the same test mRNA preparation the reaction was done with and without competitor, and the ratio of luciferase activity was normalized to that of PSII mRNA. All experiments were done with two different mRNA preparations, each done in duplicate.

Transfection of Mov 13 Fibroblasts—Transient transfections were done with the calcium phosphate technique using 10 μg of corresponding MH-COLL plasmids. 24 h after transfection, equal number of cells were split into two dishes and incubation continued for an additional 24 h. The cells were then incubated in 0.2% serum for 24 h and either treated with 4 ng/ml of TGFβ1 (R&D Systems) or left untreated for an additional 24 h. Cells and cellular medium were collected and analyzed by RNase protection assay or Western blot. Stably transfected Mov 13 fibroblasts were developed by transfection of MH-COLL genes and pCDNA3 vector in a ratio of 1:10 and selection with G418 for 3 weeks. G418-resistant cells were pooled and processed as above.

Western Blots—50 μg of cellular proteins were run on 7.5% SDS-PAGE gels under reducing or nonreducing conditions, as indicated. 100 ng of purified rat tail collagen type I (Collaborative Biomedical Products) was included as control. After transfer, the blots were probed with 1:1000 dilution of anti-collagen type I antibody (600-401-103, Rockland) and developed using the ECL system (Amersham Biosciences). Cellular medium was concentrated on Centricron 100 columns (Amicon), and equivalent amounts (corresponding to 4 × 10⁶ cells) were analyzed by Western blot as above. For collagenase digestion, 40 μl of concentrated medium was adjusted to pH 2.5 with acetic acid and digested with 1 μl of 64,000 units/ml of pepsin (Sigma) for 30 min at room temperature. After neutralization, the samples were analyzed by Western blot. For collagenase digestion, 1 μl of 4 units/ml of bacterial collagenase (Roche Molecular Biochemicals) was added to 40 μl of concentrated medium and digested for 30 min at room temperature.

RNase Protection Assay—50 μg of total cell RNA was simultaneously hybridized with collagen-specific riboprobe and glyceraldehyde-3-phosphate dehydrogenase-specific riboprobe (Ambion), as previously described (20). The collagen-specific band has an expected size of 145 nt, and the glyceraldehyde-3-phosphate dehydrogenase-specific band has an expected size of 120 nt.

RESULTS

Translation of Reporter mRNAs with Collagen 5’-UTR Sequences—Fig. 1A shows the sequence of the 5’ stem-loop of mouse collagen α(I) mRNA. To the right is shown the consensus 5’-stem-loop sequence, which can be derived from α(I), α(2), and α(I)III mRNAs of evolutionary distant organisms (ranging from fish to humans). Since the sequence around the collagen α(I) mRNA start codon, as a part of the 5’ stem-loop, does not match the consensus sequence derived by Kozak (22, 23), one set of mRNAs was constructed to investigate how the sequence of collagen α(I) mRNA immediately 5’ to the start codon affects translation. Therefore, we constructed a reporter mRNA containing only the last 25 nt of the mouse collagen α(I) 5’ stem-loop linked in-frame with a luciferase mRNA (COLL START, Fig. 1B). In this construct the 5’ stem-loop cannot form because its 5’-region was deleted, but it contains the collagen α(I) start codon in its natural sequence context. Control mRNAs had a 5’-UTR of 35–36 nt without any structural elements or short upstream open reading frames (uORF) (PSII and COLL-OPSTART, Fig. 1B). The COLL-OPSTART differs from the COLL-START mRNA by 9 nt preceding the start codon, which were optimized to conform to the Kozak rules, while in COLL-START they were from mouse collagen α(I) mRNA. PSII mRNA had a 5’-UTR derived from the pGL3 vector, which is optimized for efficient translation and was used as control. 3’ to the start codon all constructs had the rest of the sequence of the 5’ stem-loop (underlined in Fig. 1B), followed by the luciferase ORF. The mRNAs were made in...
Role of the 5′ Stem-Loop in Translation of Collagen α1(I) mRNA

A conserved stem-loop structure (5′ stem-loop) found around translation initiation codon of fibrillar collagen mRNAs. The sequence of the mouse collagen α1(I) 5′-UTR is shown to the left and a consensus sequence found in α1(I), α2(I), and α1(III) mRNAs of various organisms is shown to the right. Translation initiation codon is in bold. B, test mRNAs used to assess the functionality of the translation initiation site of collagen α1(I) mRNA. Sequence of the 5′-UTR of test mRNAs is shown. This sequence is followed in frame by the luciferase consensus sequence found in H9251 and H9252 organisms is shown to the right. C, mRNAs shown in A were transcribed in vitro with a 7mG cap, and their integrity was analyzed by agarose gel electrophoresis (Fig. 1C). These mRNAs did not contain a poly(A) tail, because of the small effect that the poly(A) tail has on translation in vitro (21). These test mRNAs were translated in rabbit reticulocyte lysate with or without of 10-fold molar excess of a competitor mRNA. The competitor mRNA had the optimal start site followed by an ORF of 75 amino acids (competitor A). The sequence of the 5′-UTR of competitor A is shown in Fig. 1B (COMP A). Without competitor A all three test mRNAs yielded similar amounts of the luciferase protein. However, in the presence of a 10-fold amount of competitor A the efficiency of translation was reduced 5-fold for PSII mRNA, 19-fold for COLL-START mRNA, and 8.4-fold for COLL-OPT-START mRNA. In Fig. 1D this result is shown normalized to the inhibition of PSII mRNA. Because the highest inhibition was observed when the collagen α1(I) sequence preceded the start codon (COLL-START), we concluded that this sequence is suboptimal in promoting translation initiation when competing with another mRNA for the translation machinery.

Another set of reporter mRNAs was designed to address the role of the 5′ stem-loop in translation. 5′ WT-SL reporter contained 63 nt of the mouse collagen α1(I) 5′-UTR with the 5′ stem-loop in-frame with LUC, while the 5′ MUT-SL reporter had substitutions in the 5′ stem-loop to abolish its formation (Fig. 2A). Translation of these reporters was compared in vitro to the PSII mRNA (described above) without competitor mRNA or under competitive conditions. The integrity of the mRNAs is shown in Fig. 2B. Without competitor, the 5′ WT-SL mRNA was translated about 3-fold less efficiently than PSII (arbitrarily set as 1) and 5′ SL-MUT mRNAs. The latter two were translated with comparative efficiency (Fig. 2C). This was not due to preferential degradation of the 5′-SL mRNA in the lysate, because extraction of the RNAs from the lysate after a 1-h incubation and retranslation in fresh lysate yielded the same result (not shown).

Next we compared translational efficiency of the 5′ WT-SL mRNA and 5′ MUT-SL mRNA to PSII mRNA in the presence of 10-fold molar excess of competitor mRNAs. Three competitor mRNAs were added in 10-fold molar excess to the reaction, competitor A (described above), β-globin mRNA, and TMV. With competitor A 5′ WT-SL reporter mRNA was translated about 30-fold less efficiently, when compared with PSII mRNA. 5′ MUT-SL mRNA was translated only 5.5-fold less efficiently relative to PSII mRNA (Fig. 2D). When β-globin mRNA was used as competitor the respective ratios were 4.6- and 3.6-fold (Fig. 2E). When viral RNA was used as a competitor the translation of 5′ WT-SL and PSII mRNAs was not affected, while translation of the 5′ MUT-SL mRNA was increased 2-fold (Fig. 2F). Based on the results in Fig. 2, we concluded that the 5′ stem-loop is inhibitory for translation in vitro in the absence of a competitor mRNA, while various competitor mRNAs have either a strong inhibitory effect or show only a small effect. We analyzed if reticulocyte lysates contain the 5′ stem-loop binding activity by performing gel mobility shift analysis using capped 5′ stem-loop RNA or inverted 5′ stem-loop RNA as probes, as described (6). We could not detect specific binding to the 5′ stem-loop (data not shown).

Expression of Collagen α1(I) Reporter Genes in Mov 13 Fibroblasts—Mov 13 fibroblasts were derived from mice in which insertion of a retrovirus into the first intron of collagen α1(I) gene had inactivated the transcription of this gene (24). Mov 13 fibroblasts...
fibroblasts transcribe the \(\alpha_1(I)\) gene and thus provide a unique opportunity to study translation of collagen type I mRNAs and assembly of the collagen trimer when the \(\alpha_1(I)\) mRNA is encoded by various transgenes. Fig. 3 shows characterization of Mov 13 fibroblasts. No collagen \(\alpha_1(I)\) polypeptides can be detected by Western blot among cellular proteins of Mov 13 fibroblasts (Fig. 3A, lane 1). For comparison, cellular proteins of NIH 3T3 fibroblasts and purified collagen from rat tail were analyzed in lanes 3 and 2, respectively. Pro-\(\alpha_1(I)\) (about 175 kDa) and \(\alpha_1(I)\) (about 120 kDa) peptides were seen in the NIH 3T3 sample. The antibody used did not detect the \(\alpha_2(I)\) chain. The \(\alpha_1(I)\) monomer and higher molecular weight cross-links of type I collagen are seen in the rat tail sample, which served as markers.

To assess the role of the 5’ stem-loop in collagen type I synthesis in vitro we constructed two genes. One gene contained 220 nt of the promoter of the mouse collagen \(\alpha_1(I)\) gene followed by the mouse collagen 5’-UTR including the 5’ stem-loop, ligated to the full-size human collagen \(\alpha_1(I)\) cDNA (5’ WT-MH-COLL, Fig. 3B). The gene has an open reading frame encoding a full-size human collagen pro-\(\alpha_1(I)\) polypeptide. The other gene is identical, except it has an 18-nt mutation within the 5’ stem-loop, which destroys its formation (5’ MUT-MH-COLL). This mutation does not affect the coding region of the gene, it encodes for the identical polypeptide as the 5’ WT-MH-COLL gene. The genes were transiently transfected into Mov 13 fibroblasts and mRNA analyzed by RNase protection assay (Fig. 3C). Untransfected Mov 13 fibroblasts show no expression of collagen \(\alpha_1(I)\) mRNA. Transfected cells show high level of mRNAs transcribed from both transgenes, enabling us to study how collagen mRNAs with the 5’ stem-loop and without it are translated in vivo.

**Synthesis of Collagen Polypeptides from 5’ WT-MH-COLL and 5’ MUT-MH-COLL Genes**—First, we measured the intracellular activity of the \(\alpha_1(I)\) gene in Mov 13 fibroblasts transduced by the luciferase reporter described above. The results are shown in Fig. 3D and 3E. The luciferase activity produced by PSII mRNA was arbitrarily set as 1. The result shown is from two independent experiments each performed in duplicate. The error bar is S.D. is shown. D, in vitro translation of the mRNAs shown in B under competitive conditions with competitor A. The experiment was performed as in Fig. 1. The result shown is from two independent experiments each performed in duplicate. The error bar is S.D. is shown. E, same experiment as in D, but the competitor was \(\beta\)-globin mRNA. F, same experiment as in D, but the competitor was tobacco mosaic virus mRNA (TMV).
cellular steady-state levels of collagen polypeptides by Western blot after transient transfections of 5′ WT-MH-COLL and 5′ MUT-MH-COLL genes into Mov 13 cells (Fig. 4A). We also treated the cells with 4 ng/ml of active TGFβ1, to assess how this profibrogenic cytokine (25, 26) would affect the expression. The blot was done under nonreducing conditions to assess the synthesis of disulfide-bonded collagen species. The major collagen detected was the pro-α1(I) chain, and both genes synthesized a similar level of the peptide. Its steady-state level was unaffected by TGFβ. However, the 5′ WT-MH-COLL gene yielded some of the disulfide-linked higher molecular weight collagen species (HMW COLL), while the 5′ MUT-MH-COLL gene did not (Fig. 4, compare lanes 1 and 2 to lanes 3 and 4). We could not distinguish, with our antibody, whether these species were homo or hetero multimers of type I collagen, although these collagen moieties comigrated with the collagen species found in rat tail type I collagen (lane 5). The result suggested that the 5′ stem-loop, although an RNA element, is involved in more efficient formation of disulfide-bonded collagen monomers, suggesting better registration of collagen chains. This prompted us to investigate if the collagen synthesized by 5′...
WT-MH-COLL mRNA is more efficiently secreted into the cellular medium.

We collected the cell medium and analyzed the equivalent amounts by Western blot under reducing conditions. Very little of pro-α(1) polypeptide (175 kDa) was secreted out of non-stimulated cells and in the same amount for 5′ WT-MH-COLL and 5′ MUT-MH-COLL genes (Fig. 4B, lanes 3 and 5). Only tracing amounts were processed to mature α(1) chain of about 120 kDa. With TGFβ stimulation a much higher amount of pro-α(1) chain was found in the cellular medium, but both genes produced similar amount of the protein (lanes 3 and 5).

Since there was no change in mRNA level with TGFβ stimulation (not shown), we concluded that TGFβ-stimulated translation or secretion of type I collagen independent of the 5′ stem-loop. Alternatively, TGFβ may have decreased extracellular degradation of collagen. In stably transfected Mov 13 fibroblasts TGFβ also stimulated extracellular accumulation of collagen, but no difference between the 5′ WT-MH-COLL and 5′ MUT-MH-COLL genes was seen (Fig. 4C).

Only the 5′ WT-MH-COLL Gene Expresses Properly Folded Collagen—Next, we probed the structure of secreted collagen from Mov 13 fibroblasts by digestion of cellular medium with pepsin and collagenase. We used the medium of cells treated with TGFβ1, because it contained the higher amount of collagen that facilitated the analysis. The medium was subjected to digestion with pepsin and collagenase as described under “Material and Methods” and analyzed by Western blot. In undigested medium under reducing conditions, the predominant collagen species was the pro-α(1) chain (Fig. 5A, lane 1). When the medium of cells expressing the 5′ WT-MH-COLL gene was digested with pepsin the molecular mass of this chain was reduced to about 120 kDa (lane 2). This suggests cleavage of the globular domains, but folding of the core domain into pepsin-resistant triple helix. When the medium was digested with bacterial collagenase, no collagen peptides could be detected, suggesting the specificity of the bands (lane 3). Thus, mRNA with the 5′ stem-loop directs synthesis of triple helical collagen, which accumulated in the cell medium. When the medium of 5′ MUT-MH-COLL-expressing cells was digested with pepsin no pepsin-resistant fragment was obtained (lane 5), although the amount of collagen secreted was comparable to that of the 5′ WT-MN-COLL gene (lane 4). Thus, mRNA without the 5′ stem-loop directs synthesis of structurally aberrant collagen that could not resist limited pepsin digestion. Digestion with collagenase served as the specificity control (lane 6). The result obtained with the medium from stably transfected Mov 13 cells is shown in Fig. 5B. Again, the 5′ WT-MH-COLL gene yielded secreted triple helical collagen (lane 2), while the 5′ MUT-MH-COLL gene produced a pepsin-sensitive collagen (lane 5). We concluded from these experiments that the 5′ stem-loop is necessary for productive collagen synthesis and, although it was mutated without affecting the coding region of the mRNA, has a profound effect on collagen protein folding or stability.

Discussion

Unique features of the three fibrillar collagen mRNAs, α1(I) mRNA, α2(I) mRNA, and α1(III) mRNA, is the 5′ stem-loop structure that encompasses the start codon (3). The 5′ stem-loop has an important role in regulating α1(I) mRNA stability (3, 6, 7) and is conserved in collagen mRNAs of evolutionary distant species (4, 5). Since the start codon is part of this stem-loop, the sequence constraints required to maintain the 5′ stem-loop dictate the sequence around translation initiation. Therefore, the start codon in collagen mRNAs is not in the sequence context necessary for optimal translation initiation (22, 23). We have shown here that it is not efficiently recognized in vitro if more than one mRNA species are competing for the translation machinery. When the start codon was optimized we could increase translation 4-fold under competitive conditions (Fig. 1C). The 5′ stem-loop structure has a stability of 25–30 kcal/mol. This is insufficient to block scanning ribosomes to reach the start codon, because stem-loop structures of about 70 kcal/mol are needed (27, 28). Nevertheless, reporter mRNA with the 5′ stem-loop (5′ WT-SL) was translated 3-fold less efficiently, even in the absence of competitor, than similar mRNA in which the stem-loop was mutated (5′ MUT-SL, Fig. 2C). It seems that the structure of the 5′ stem-loop together with its suboptimal translation start site is responsible for this effect.

Various competitor mRNAs inhibit in vitro translation of a reporter mRNA with collagen α1(I) 5′-stem-loop (5′ WT-SL) to a different degree. Competitor A inhibited translation of 5′ WT-SL mRNA 30-fold relative to a control mRNA (PSII) and 6-fold relative to the identical mRNA without the stem-loop (5′ MUT-SL) (Fig. 2D). β-globin mRNA had a smaller effect. Viral mRNA showed no inhibition on 5′ WT-SL mRNA, while 5′ MUT-SL mRNA is translated better. The reason for this is unclear, but TMV mRNA with its Omega sequence may titrate an inhibitor of translation (29). The result with competitors corroborates the finding that the 5′ stem-loop may compromise translation of collagen α1(I) mRNA, which may be a weak substrate for translation in the absence of its RNA-binding proteins. Since the 5′ stem-loop binds protein factors in collagen-producing cells, it is possible that binding of these factors regulates translation of collagen α1(I) mRNA. Cloning of these proteins and their addition to the in vitro translation reaction will address this question.

Previous reports suggested that N- and C-terminal peptides of type I collagen inhibit translation of collagen α1(I) mRNA (30, 31). We did not see any inhibition when these recombinant peptides were added to the in vitro translation reaction (data not shown). Also, the α1(I), α2(I), and α1(III) collagen mRNAs contain two short uORF preceding the start codon. We did not see any change in translation of our reporter mRNAs in vitro when these uORF were abolished (data not shown). Although it is known that uORFs can regulate translation in yeast (32, 33), there are only a few examples of their role in translational regulation in higher organisms (34–36).
Based on electron microscopy data, assembly of the collagen type I heterotrimer occurs on the membrane of the endoplasmic reticulum, while the individual chains are still associated with polysomes or shortly after their release (14, 17). Lysozyme, one of the key enzymes in collagen modifications, is also associated with the membrane of the endoplasmic reticulum (37). Membrane association may couple folding starting from the C terminus of collagen chains, to concomitant modifications of the selected lysine residues. For collagen type I, this implies that α1(I) and α2(I) chains may be synthesized by ribosomes positioned in close proximity on the endoplasmic reticulum membrane. Such coordinated translation would greatly increase local concentration of the chains. We hypothesized that the sequence elements that modulate loading of ribosomes on collagen α1(I) mRNA may be involved in targeting for such coordinated translation. Therefore, we mutated the 5’ stem-loop and analyzed production of collagen trimers in vivo from the hybrid mouse-human collagen genes (5’ WT-MH-COLL and 5’ MUT-MH-COLL) (Fig. 5). The human collagen α1(I) gene could rescue the phenotype of Mov 13 mice, proving that human collagen α1(I) polypeptide is functional in mouse (38, 39). The 5’ WT-MH-COLL gene produced triple helical collagen in Mov 13 fibroblasts; however, the 5’ MUT-MH-COLL gene produced collagen, which was sensitive to digestion with pepsin (Fig. 5). The structurally aberrant collagen was produced although the 5’ MUT-MH-COLL gene had the identical coding region. This pepsin-sensitive collagen may represent individual α1(I) chains, which were not efficiently folded into triple helix and were secreted as monomers, or alternatively, the monomers were not properly modified and an unstable triple helix was secreted. The 5’ MUT-MH-COLL chains had identical electrophoretic mobility to the 5’ WT-MH-COLL chains, excluding a major difference in post-translational modification, although subtle differences may remain undetected. If the chains were not efficiently folded, the 5’ stem-loop may be required to increase their local concentration, which would facilitate the chain registration. The result shown in Fig. 4 where the 5’ WT-MH-COLL gene produced more disulfide-linked high molecular weight collagen suggests that this may be the case. If the chains were not properly modified, the 5’ stem-loop may target collagen mRNAs for translation to discrete regions of the endoplasmic reticulum where there is optimal concentration of collagen-specific modifying enzymes and molecular chaperones (40). Although we do not have direct evidence for this, we think that the 5’ stem-loop also couples the translational machinery to the rest of the collagen biosynthetic pathway, because collagen biosynthesis requires coordinate action of translational apparatus, modifying enzymes, and molecular chaperones (41). In patients with OI, where folding of collagen type I chains is impaired, the mutant chains are hypermodified and subjected to degradation (19, 42). Interestingly, one patient with OI type I was described who had a mutation in the 5’ stem-loop in the absence of any other mutation of the collagen α1(I) gene (43).

There are many examples that mRNAs are targeted for translation at discrete subcellular sites to produce proteins with the concentration gradient within the cell. Most targeting signals are located in the 3’-UTR of these mRNAs (44). To our knowledge this is the first example that a RNA element located in the 5’-UTR is involved in synthesis of a secreted multisubunit protein. This study demonstrates that the conserved collagen 5’ stem-loop has specific functions. In the absence of RNA-binding proteins, the 5’ stem-loop renders the collagen mRNAs inefficient for translation and therefore susceptible to regulation, such as by TGFβ. In collagen-producing cells, the 5’ stem-loop has a novel function of directing the post-translational modification of collagen to produce mature triple helices. The 5’ stem-loop also certainly acts through its cognate RNA-binding proteins. Cloning of these protein factors will help us elucidate the complex biosynthesis of type I collagen.