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Formation of a type I collagen RNA dimer by intermolecular base-pairing of a conserved sequence around the translation initiation site

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ABSTRACT

A symmetrical sequence around the translation initiation site of several collagen genes is highly conserved. Deletions in this sequence increase translational efficiency of an α_2(I) collagen - CAT chimeric gene after DNA transfection of NIH 3T3 fibroblasts (Schmidt, Rossi and de Crombrugghe, submitted). The secondary structure, predicted by the sequence of this segment, was shown to exist in solution in 200 mM NaCl at 37°C. Cell-free translation of the corresponding RNA using a reticulocyte lysate is inhibited 2 to 4-fold by preincubation with a 0.5 M NaCl extract of an NIH 3T3 ribosomal eluate. Cell-free translation of two mutant RNAs, with partial deletions of the conserved sequence, is not inhibited by such preincubation. This inhibition is not due to degradation of the RNA and requires a protein component of the ribosomal eluate, which, however, is not required after the preincubation step. Preincubation of the RNA with the ribosomal eluate from NIH 3T3 fibroblasts causes the reversible formation of an intermolecular dimer in which the conserved symmetrical sequences hybridize to each other. This results in an increase in the degree of secondary structure of the conserved segment around the translation initiation site. We speculate that translational efficiency could be modulated by influencing the equilibrium between monomer and dimer.

INTRODUCTION

Around the translational start site of three different interstitial collagen genes, α_1(I), α_2(I), and α_1(III), a uniquely conserved sequence of about 50 bp is found (1-6). The nucleotide sequence of this conserved element is largely symmetric and contains in its middle a quasi perfectly conserved inverted repeat containing 8-9 bases in each repeat. The two arms of this inverted repeat are separated by five nucleotides whose sequences are less well conserved among the collagen genes. In each gene two AUG codons are found at identical locations within this inverted repeat, the distance between these AUGs being constant. The promoter proximal AUG is followed in frame after a short distance by a non-sense codon. The promoter distal AUG is the one used for translation initiation of the collagen polypeptide. Both the high conservation and the location of this conserved sequence raises the possibility that it might play a role in determining the efficiency of trans-

lation of the type I and III collagen chains. Since a number of studies suggest that the control of type I collagen synthesis has, under certain experimental situations, a translational component (7-12), it is possible that the conserved element might be the target for such control. The view that the conserved element may play a role in determining translational efficiency is supported by experiments using mutants in which portions of the conserved sequence were deleted. These experiments were performed by transfecting an α_2(I) collagen - CAT (chloramphenicol acetyl transferase gene) fusion gene into NIH 3T3 fibroblasts. A deletion in the left part of the inverted repeat causes a 10-fold increase in translation efficiency, a deletion in the right part of the element or of the entire element causes a 30-fold increase in translation, whereas a deletion in the 5' leader upstream of the conserved element has no effect (Schmidt, Rossi, and de Crombrugghe, submitted).

Studies from other laboratories (13-14) have examined the effects of linker sequences inserted in the 5' untranslated leader segments of either thymidine kinase or insulin mRNAs. These linker sequences constitute inverted repeats and presumably result in formation of secondary structures in the RNA which vary in the strength of their ΔG with the length of the linker insertion. Only when the free energy of these secondary structures was at least -30 Kcal was there a significant decrease in the translation of these RNAs. The predicted secondary structure around the translational start sites of the type I and III collagen mRNAs falls below these values since it has a ΔG of ~ only 16 to 20 Kcal. Hence, it is possible that to account for the results with the deletion mutants, additional factors exist which could be present in fibroblasts and which might influence the degree of translation of collagen RNAs.

The aim of the studies presented here is to further examine the mechanism by which this conserved element may influence translation. We first verified that the predicted secondary structure of the conserved element exists in solution. We also tested the possible role of this secondary structure in a reticulocyte cell-free translation system and whether factors present in NIH 3T3 fibroblast affected translation of the wild-type message. Our results indicate that addition of a high salt extract from NIH 3T3 polyribosomes causes a decrease in translation of the wild-type RNA by a reticulocyte lysate. This results in the formation of an intermolecular hybrid in the region of the conserved element which significantly increases the degree of secondary structure around the translation initiation site.

MATERIALS AND METHODS

Preparation of Ribosomal Wash Fluid

Confluent cultures of NIH 3T3 cells were harvested, washed with isotonic buffer, and resuspended in 2 volumes of hypotonic buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM Mg Acetate, 1 mM DDT) and homogenized by 20 strokes in an all-glass Dounce homogenizer. The cell extract was adjusted to a final concentration of 20 mM HEPES, pH 7.4, 120 mM KCl, 5 mM Mg Acetate, 1 mM DTT, and 10% glycerol (buffer B). After centrifugation at 2,000 g for 15 min at 4°C the supernatant was further centrifuged at 12,000 g for 15 min. The resulting supernatant was centrifuged for 2.5 hr at 150,000 g. The supernatant was discarded, and the pelleted polysomes washed several times with buffer B and resuspended in buffer B in less that 1/10 of the original volume of the extract. Four M KCl was added slowly to a final concentration of 0.5 M; the samples were gently stirred with a glass rod and shaken for 30 min at 4°C. The ribosomal wash fluid was collected by centrifuging twice at 150,000 g for 2.5 hr, dialyzed against buffer B and stored at -80°C.

Construction of Plasmids

Plasmid pAZ1010 contains sequences from 2,000 bp upstream of the +1 start of transcription of the mouse α_2(I) collagen gene to 159 bp downstream of this start in a fusion with bacterial CAT gene. The nontranslated α_2(I) collagen RNA segment (138 n) contains two AUG codons preceding the translation initiation AUG for the collagen polypeptide, each followed by only short open reading frames in different frames from the one determined by the collagen initiation codon. In this plasmid, the collagen initiation AUG was placed in the same reading frame as the initiation AUG codon of the bacterial CAT protein. To construct pAZ1010*, the fragment between the SphI site at + 54 and the BamHI site on the 3' side of the CAT gene of pAZ1010 was subcloned in the polylinker region of plasmid pGEM-3 (Promega Biotec) downstream of the SP6 promoter. Plasmids pR7* and pR9* are identical to pAZ1010* except that they contain deletions in portions of the conserved element that are indicated in Fig. 1B. The deletions correspond to those utilized in DNA transfection experiments (Schmidt, Rossi and de Crombrugghe, submitted).

Preparation of Synthetic RNAs; Capping and Labeling; Primer Extension

RNAs were generated, after making the plasmids linear with the appropriate restriction enzymes, by transcription using SP6 RNA polymerase (15). RNAs were labeled at the 5' end by sequential treatment with alkaline phosphatase and T4 polynucleotide kinase (Boehringer-Mannheim) using γ^{32}[P]ATP

(New England Nuclear). Capped RNAs were generated by inclusion of m^7GpppG in the transcription reaction as described by Pelletier and Sonenberg (13). All transcription reactions were performed for 2 hrs at 40°C in a total volume of 100 µl using 3 µg of plasmid DNAs as template. The primer-extension experiments shown in Fig. 5 were performed by hybridizing phenol extracted aliquots of the in vitro translation reactions with excess CAT antisense oligonucleotide (5'GCCATTGGGATATATCAACGGTG$^{3'}$) labeled at the 5' end. The conditions for hybridization, reverse transcriptase reaction and electrophoretic analysis of the cDNA products were as previously described (16, 17).

RNase Digestions

5' end-labeled RNAs were digested with RNAse A, T_1, or V_1 (Pharmacia, PL) for 5 min at 37°C, at the concentrations described in the Figure Legends, in the presence of excess tRNA and 200 ng poly U-poly C, 200 µl of a buffer containing 200 mM NaCl, 50 mM Tris-Cl, pH 7.5, 5 mM EDTA. In reactions containing V_1 ribonuclease 10 mM $MgCl_2$ was added. The reactions were stopped by adding 1% SDS and further incubated for 15 min at 37°C with 50 µg/ml of proteinase K, and analyzed by 12% polyacrylamide/7 M urea gel electrophoresis.

Mobility Shift Assays

Between 5 and 10 ng labeled RNA was incubated at 30°C for 20 min with buffer B or ribosomal wash fraction in a 10-15 µl volume. 250 ng of E. coli tRNA were added to the reaction. After the reaction, the samples were treated with 1% SDS, diluted with 10 µg tRNA and 5 volumes of 0.3 M Na Acetate, pH 6.5, extracted with chloroform with two interphase reextractions, ethanol precipitated, resuspended in 10-20 µl of buffer B and 1/5 volume of 50% glycerol, 0.5% xylene cyanol and 0.25% bromophenol blue, and loaded on 4% polyacrylamide nondenaturing gels. Alternatively the samples were directly loaded after the preincubation step on the gel, after adding directly the tRNA and the dyes.

In Vitro Translations

Capped RNAs (generated by SP6 transcription of BamHI linearized plasmids) were translated in vitro in the presence of ^{35}S-methionine after preincubation with buffer B or ribosomal wash fraction. Reactions were linear in the range of time and RNA concentration used. The translation products were monitored by either SDS-PAGE (12% polyacrylamide) followed by fluorography or by TCA precipitations.

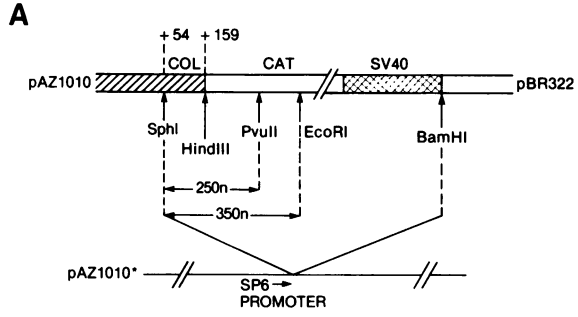
RESULTS

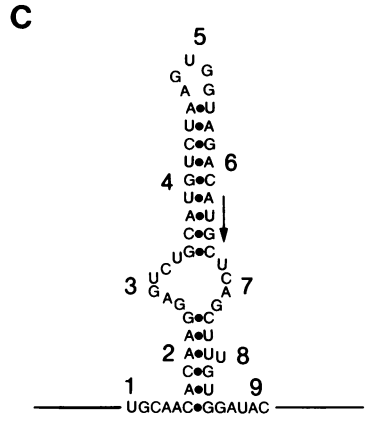
Structure of Wild-Type and Mutant RNAs

Wild-type RNA used in all experiments was derived by SP6 transcription from pAZ1010*, DNA described in Fig. 1A. The collagen portion of this RNA covers the segment from +54 to +159 in the mouse α_2(I) collagen-CAT chimeric gene, the hybrid gene which we previously have used to examine the role of the conserved element in DNA transfection experiments (Schmidt, Rossi and de Crombrugghe, submitted). Since the collagen signal peptide is in the same reading frame as the CAT coding sequence, the fusion polypeptide translated from this RNA includes the N-terminal seven amino acids of the collagen signal peptide, followed by a sequence of three amino acids coded by the HindIII linker, ten amino acids coded by the untranslated segment of the bacterial CAT gene and the rest of the CAT polypeptide. During in vitro translation of this RNA in a reticulocyte lysate system, approximately 70-80 percent of the total synthesized polypeptides initiate at the collagen AUG, producing a polypeptide which is ~2,000 daltons larger than the one initiated at the CAT AUG. The end points of two deletion mutations, pR7* and pR9*, which were used in some experiments, are shown in Fig. 1B. These RNAs are identical to pAZ1010* RNA except that the conserved element has been partially deleted.

Evidence that the Predicted Secondary Structure Around the Translation Initiation Site of the α_2(I) Collagen RNA Exists in Solution

We tested whether the potential stem-loop structure around the translation initiation site of the α_2(I) collagen RNA (Fig. 1C) was formed in solution by the following experiment. An RNA corresponding to a segment between +54 and +412 in the mouse α_2(I) collagen-CAT chimeric gene (see Fig. 1A ~350 n) was transcribed in vitro with the SP6 RNA polymerase using plasmid pAZ1010* digested with EcoRI as template. This RNA was 5' end-labeled with T4 polynucleotide kinase and treated under conditions of partial digestion with both single-strand and double-strand specific RNAses (Fig. 2). Ribonuclease V1, which is specific for double-strand RNA produces cleavages only in those segments which are predicted to form base pairs (segments designated 2, 4, 6 and 8 in the figure). Ribonuclease T1 produces prominent cleavages in three G residues in the loop segment 5 and cleaves to a lesser degree in segment 3. Pancreatic ribonuclease cleaves also in segment 5 and in addition in segments 1, 3, 7, and 9. Much less or no cleavage is produced by either T1 or pancreatic ribonuclease in the segments recognized by V1 ribonuclease. We conclude that a stable stem-loop structure which includes the conserved inverted repeat





B

Conserved Sequence

+54

1 2 3 4 5 6 7 8 9

pAZ1010* GCAUGCCUGCGCCCGCCAGGUGAUACCUCCGCUGGUGACCCAGGGGCUCUGCAACACAAGGA GUCUGCAUGUCUAAGUGGUAGACAUGCUCAGCUUUGUGGAUACGC *caagcuugg*cgagauuuucaggagcuaaggaagcuaaaauggag

pR7* GCAUGCCUGCGCCCGCCAGGUGAUACCUCCGCUGGUGAC- - - - -[GGUACCGGUACCG]- AGACAUGCUCAGCUUUGUGGAUACGC *caagcuugg*cgagauuuucaggagcuaaggaagcuaaaauggag

pR9* GCAUGCCUGCGCCCGCCAGGUGAUACCUCCGCUGGUGACCCAGGGGCUCUGCAACACAAGGAGUCUGCAUGUCUAAGUGGUAG- C*caagcuugg*cgagauuuucaggagcuaaggaagcuaaaauggag

sequence is formed in solution. The same pattern of RNAse T1 digestion as the one shown in Fig. 2 was obtained at 30° either in 200 mM NaCl or 40 mM NaCl.

In Vitro Translation of Wild-type and Mutant RNAs

Previous DNA transfection experiments have shown that deletions in the conserved sequence around the translation initiation site of the α_2(I) collagen gene cause a 10 to 30-fold increase in the efficiency of translation, whereas a control deletion 5' to the conserved sequence had no effect (Schmidt, Rossi and de Crombrugghe, submitted). We postulated that the disruption of the secondary structure caused by the deletions might, at least in part, be responsible for this increased translational efficiency. To begin to study the mechanisms responsible for this difference in translational efficiency, we first compared wild-type and mutant RNAs by cell-free translation.

RNA was synthesized *in vitro* from each plasmid using SP6 RNA polymerase and translated in a reticulocyte cell-free system. RNA synthesized from pAZ1010* produces approximately as much CAT polypeptide as RNA synthesized from pR7* and pR9* (Fig. 3). In contrast to what was found by DNA transfection of NIH 3T3 fibroblasts, the *in vitro* translational efficiencies of pR7* RNA, pR9* RNA and pAZ1010* RNA are not significantly different. These results suggest that under the conditions of our assays, the secondary structure by itself is not sufficient to inhibit translation in a reticulocyte cell-free system.

Figure 1A: Construction of pAZ1010*. A fragment between the SphI at position +54 in the mouse a_2(I) collagen gene to the BamHI site in pAZ1010 (see Materials and Methods) was subcloned behind the SP6 promoter in the polylinker region of pGEM3 (Promega Biotec).

Figure 1B: Sequence of a segment of the mouse α_2(I) collagen-CAT RNA in pAZ1010* and of two deletion mutants. The first base in the sequence is at +54 in the first exon of the a_2(I) collagen gene. The bases in capital letters correspond to the collagen sequence, the bases in italics to the HindIII linker and the bases in small letters to the CAT sequence. The collagen - CAT fusion protein contains the first seven amino acids of the collagen signal peptide, a sequence of three amino acids encoded by the HindIII linker, ten amino acids specified by the 5' untranslated region of the CAT gene and the rest of the CAT polypeptide. Deleted bases in pR7* and pR9* are indicated by dots. Sequences between brackets corresponds to a KpnI linker used to generate the pR7 deletion. AUG codons are underlined, and a bar is placed over stop codons. The conserved region, which is strongly conserved in several collagen genes, is indicated. Numbers above the sequence correspond to areas defined in Fig. 1C.

Figure 1C: Predicted secondary structure of the RNA in the segment corresponding to the conserved sequence. Arrow indicates AUG codon used to initiate translation of the collagen polypeptides.

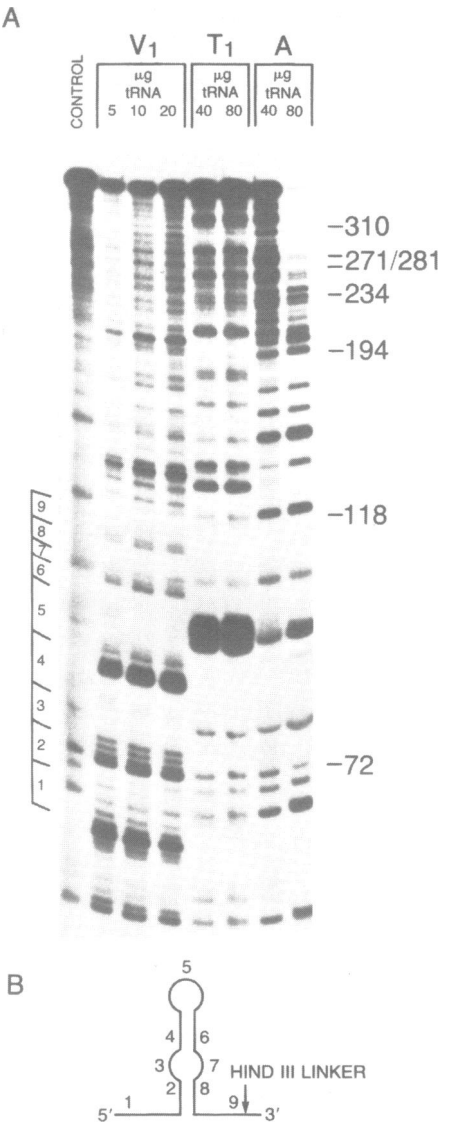


Figure 2: Partial ribonuclease digestion of 5' end-labeled wild-type RNA. A 3350 nucleotide long in vitro transcription product was obtained by SP6 transcription of pAZ1010* linearized by EcoRI. This RNA was labeled at its 5' end with γ^{32}[P]ATP and polynucleotide kinase. (A) Control lane: undigested RNA; RNase V1 digestions were performed with 400 µU of enzyme; RNase T1 digestions with 2 µU; and RNase A digestion with 2 and 20 ng of enzyme. Digestions were performed in presence of the indicated amounts of tRNA. (B) Schematic representation of the predicted secondary structure of the RNA in the area of the conserved sequences. Numbers on the left side of A correspond to the sequences indicated in Fig. 1 and to the segments shown in B.

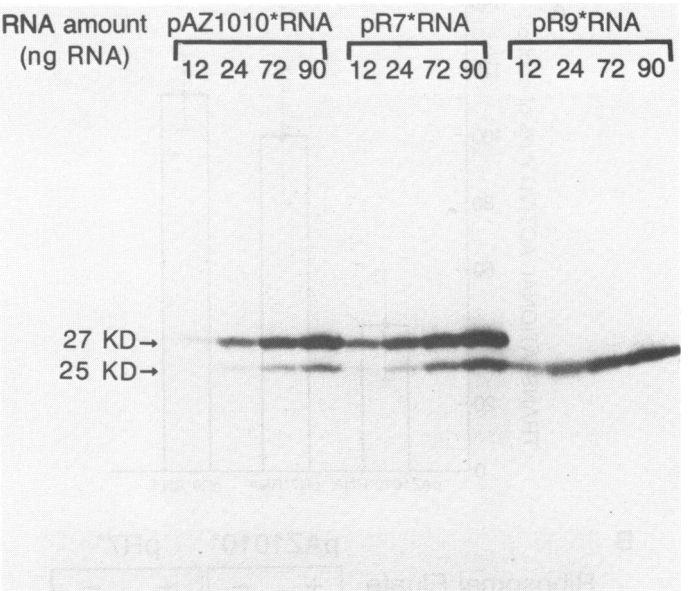


Figure 3: In vitro translation of wild-type and mutant RNAs. Increasing amounts of SP6 generated capped mRNAs were translated in vitro for 1 hr at 30°C using a lysate purchased from New England Nuclear as described in Materials and Methods. Equal amounts of the reactions were analzyed by SDS-PAGE electrophoresis followed by fluorography. The sizes of the ^{35}S labeled proteins are consistent with the size of the CAT polypeptide (25 KD) or of a collagen - CAT fusion polypeptide (27 KD). RNAs were generated in vitro from plasmids pAZ1010*, pR7* and pR9*. RNAs corresponding to pAZ1010* and pR7* generate one major band which represents the collagen-CAT fusion polypeptide and one minor species representing the CAT polypeptide which intitiates at the CAT AUG. RNA transcribed from pR9* generates only one polypeptide which initiates at the CAT AUG.

Addition of a Ribosomal Eluate Fraction of Fibroblasts Decreases Translation From the Wild-type but Not Mutant RNAs

One possible hypothesis which could account for the discrepancy between the in vivo and in vitro experiments could be that fibroblasts contain factors which might stabilize the secondary structure around the translation initiation site of the α_2(I) collagen RNA and that these factors might be absent, inactivated or neutralized in reticulocyte lysates. We, therefore, preincubated equal amounts of the various RNAs with a 0.5 M salt extract of a ribosomal pellet of NIH 3T3 fibroblasts. This was followed by addition of rabbit reticulocyte lysate and the translation mix. Figure 4A shows that preincubation with ribosomal eluate reduces translation of wild-type RNA but has no effect on pR7* or pR9* RNA. In these experiments protein synthesis was assay-

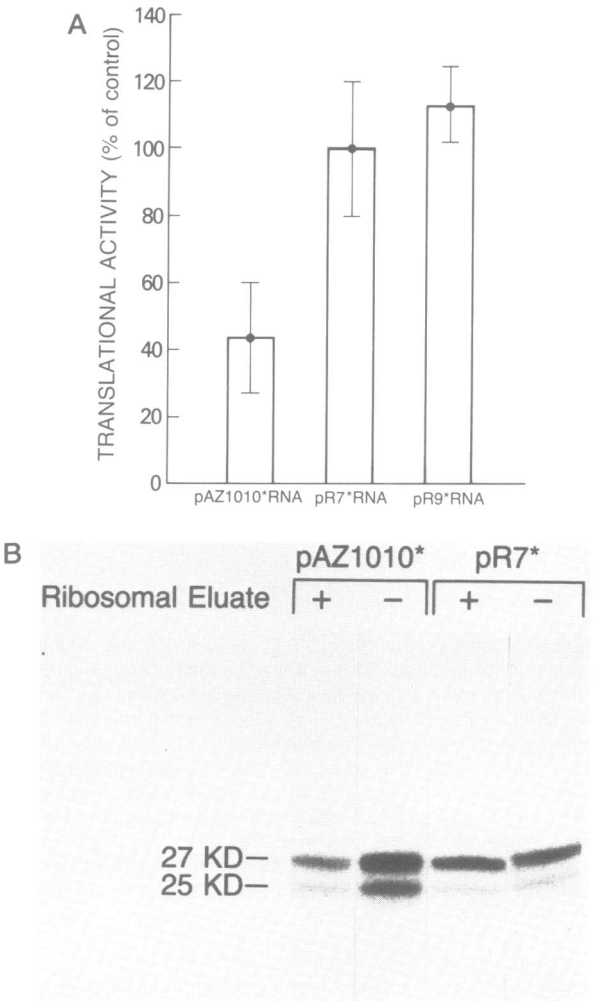
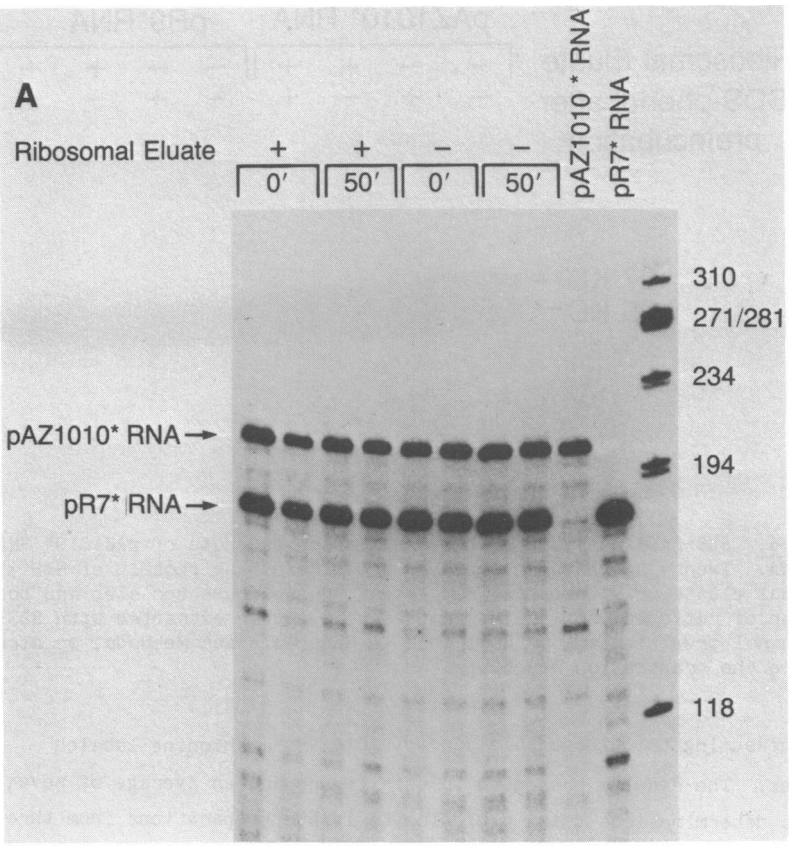


Figure 4: In vitro translation of capped synthetic RNAs after preincubation with the ribosomal wash fluid from NIH 3T3 fibroblasts. (A) Twenty ng RNA were preincubated with 0.5 µg ribosomal eluate in buffer B or with an equal volume of buffer B for 20 min at 30°C and subsequently translated for 1 hr at 30°C using a rabbit reticulocyte lysate in the presence of 35S-methionine. The amount of incorporated 35S-methionine was determined by TCA precipitation. The average ± SD of seven independent duplicate or triplicate determinations are reported. Results are expressed as percent incorporation of reactions preincubated with buffer B only. (B) SDS-PAGE analysis of the translation products of the wild-type RNA (pAZ1010*) and of a mutant RNA (pR7*). Twenty ng RNA were preincubated with 0.5 µg protein of NIH 3T3 ribosomal eluate in buffer B or with buffer B alone under the same conditions as in (A).



B

TRANSLATIONAL ACTIVITY (cpm)

	− Ribosomal Eluate	+ Ribosomal Eluate
pAZ1010* RNA	60190	16900
	58260	14150
pR7* RNA	64810	72800
	84200	83200

Figure 5: Primer extension analysis of 4 ng of wild-type (pAZ1010*) and mutant (pR7*) RNAs before (0 min) and after 50 min translation with a reticulocyte lysate. Samples were preincubated for 20 min at 30°C with 0.5µg protein NIH 3T3 ribosomal eluate (+) or with buffer B (-). Translation reactions contained 35S-methionine. Each duplicate translation reaction using wild-type RNA was mixed with the corresponding reaction using mutant RNA before nucleic acid extraction and primer extension. In the two lanes on the right, the cDNA primer extension products of 4 ng wild-type or mutant RNAs are shown. (B) An aliquot of each reaction was removed at the end of the 50 min translation and precipitated with TCA to measure amounts of 35S-methionine incorporated. The amount of 35S-methionine incorporated at 0' time was subtracted.

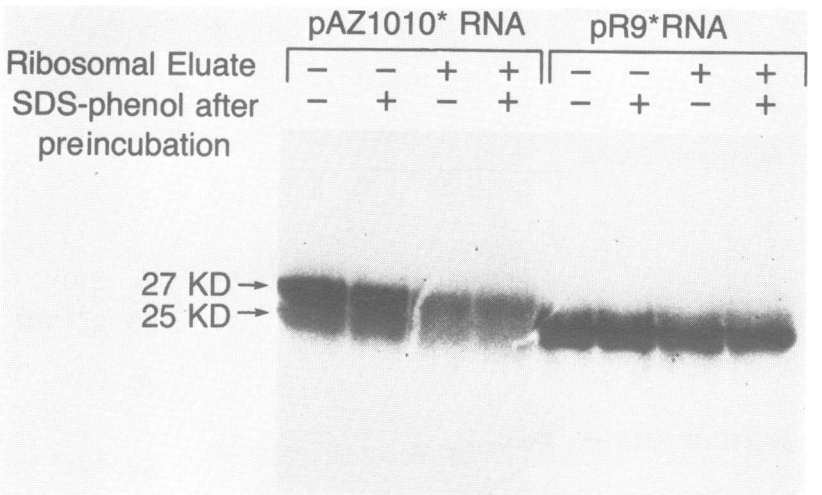


Figure 6: SDS-PAGE analysis of the translation products of pAZ1010* RNA and pR9* RNA. Twenty ng RNA were preincubated with 0.5 µg protein of NIH 3T3 ribosomal eluate or with buffer B. After the preincubation step and before addition of reticulocyte lysate, samples were either extracted with SDS-phenol and ethanol precipitated, as described in Materials and Methods, or directly added to the translation reactions.

ed by measuring trichloroacetic precipitable ^{35}S-methionine labeled peptides. The results presented in this figure are an average of seven independent determinations using reticulocyte lysate preparations from three different manufacturers. The degree of inhibition with wild-type RNA varied between 2 and 4-fold in different experiments. A similar inhibition of pAZ1010* RNA translation was also observed when the translation products were analyzed by electrophoretic fractionation (Fig. 4B).

One possible explanation for these results would be that the ribosomal eluate would contain a double-strand specific RNase activity which would digest preferentially the wild-type RNA. This RNase could be analogous to the dsRNA and ATP dependent RNAse which is stimulated by the products of the interferon-induced oligoA-synthetase (18). This hypothesis was tested by comparing the integrity of the wild-type RNA and mutant pR7* RNA before and after *in vitro* translation, following preincubation with and without ribosomal eluate. The primer extension method was used to compare the levels and size of the RNAs using an oligonucleotide primer complementary to a segment located 3' to the conserved element. Since the wild-type RNA is larger than the deletion mutant RNA, each reaction containing wild-type RNA was mixed at the

end of the experiment with the corresponding reaction containing pR7 RNA to control for possible loss during the subsequent procedures needed to complete the primer extension experiment. After phenol extraction, the SP6-generated RNAs were hybridized to an excess of the primer which was labeled at its 5' end. After reverse transcription, the cDNA products were analyzed on a sequencing gel (Fig. 5A). There was no change in either the size or the amounts of the wild-type and the mutant RNAs, whether or not these RNAs had been preincubated with the ribosomal wash fraction. Aliquots of these translation reactions were precipitated by TCA and monitored for ^{35}S-methionine incorporation. In this experiment, the wild-type RNA showed a 4-fold inhibition by the ribosomal eluate, whereas no decrease was seen with pR7* RNA (Fig. 5B).

Another possible explanation for the selective inhibition of translation of the wild-type RNA would be that in the absence of ribosomal eluate, translation factors (including unwinding activities) are in excess in the reticulocyte lysate. The RNAs present in the ribosomal eluate could titrate those factors producing limiting amounts of unwinding factors. This might reduce translation of the wild-type RNAs but not of the mutant RNA containing deletions in the inverted repeat. We, therefore, extracted the ribosomal eluate with SDS-phenol before the preincubation, removing the protein component but presumably retaining the nucleic acids. When the RNAs were preincubated with this deproteinized fraction, no selective inhibition of translation was observed (data not shown).

We conclude from these experiments that preincubation of the wild-type RNA with a ribosomal wash fraction, decreases subsequent translation. This phenomenon is not observed with two mutant RNAs in which part of the conserved sequences have been deleted. The inhibition is not due to a nuclease which would specifically degrade double-stranded RNA. The inhibition requires a protein component in the ribosomal eluate.

Protein Component of the Ribosomal Eluate Is Not Required After the Preincubation

Several other possible hypotheses could be considered to explain the selective inhibition by the ribosomal eluate: 1) binding of a factor to the conserved sequences in the wild-type RNA which would stabilize the secondary structure and inhibit translation; 2) activation by the RNA containing the inverted repeat of a mechanism which modifies the translation machinery (such as the ATP and dsRNA-dependent, interferon-induced protein kinase which inhibits translation by phosphorylation of initiation factor E1F2) (19, 20); 3)

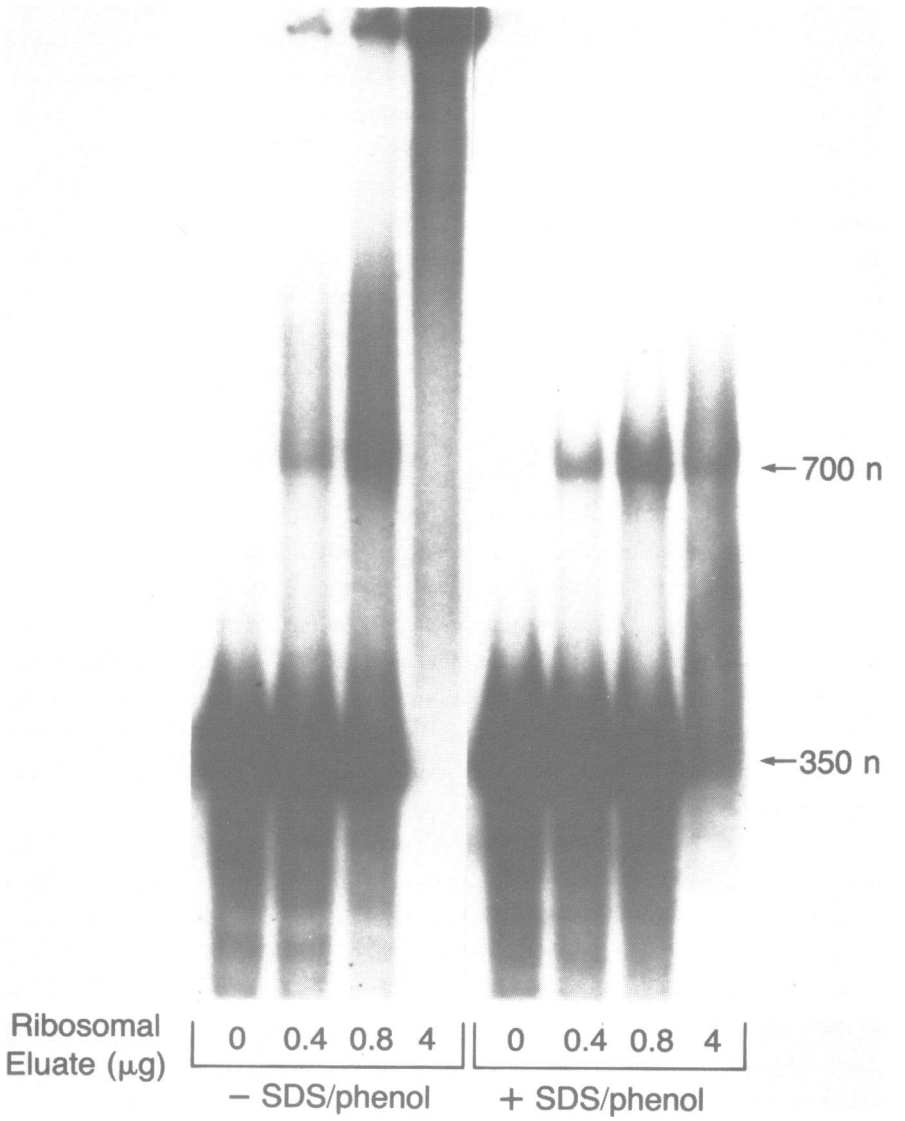


Figure 7: A) Electrophoretic analysis of reactions containing wild-type RNA and ribosomal eluate. A 350 n RNA was obtained by SP6 transcription of pAZ1010* that was made linear with EcoRI. The 350n 5' end-labeled RNA was incubated with buffer B or with increasing amount of NIH 3T3 ribosomal eluate for 20 min at 30°C. The samples were loaded on a nondenaturing 4% acrylamidebisacrylamide gel immediately after the preincubation. B) Same as A, but the samples were extracted with SDS-phenol, ethanol precipitated and resuspended, as described in Materials and Methods, before the electrophoretic analysis.

modification of the mRNA.

To test some of these possibilities, we extracted the reactions with SDS-phenol at the end of the preincubation but before the translation reaction. Figure 6 shows that translation of pAZ1010* RNA is still selectively inhibited after such treatment. This observation favors the third hypothesis, i.e., that a modification may occur in the RNA during the preincubation, which depends both on the presence of the conserved element in the RNA and on a factor from the ribosomal wash.

Evidence That Preincubation of Wild-type RNA With the Ribosomal Eluate Produces a Dimeric RNA

To test whether a factor of the ribosomal eluate would bind to the RNA, we examined the RNA at the end of the preincubation by polyacrylamide gel electrophoresis under the non-denaturing conditions used for gel retardation assays. Figure 7 (left panel) shows such an experiment and indicates the appearance of a slower migrating RNA band when the RNA is preincubated with the ribosomal eluate. The intensity of the slower migrating RNA band increases with higher concentrations of ribosomal eluate. However, at the higher concentrations, the RNA is mostly retarded at or near the origin (Fig. 7, left panel, lane marked 4). This mobility shift is not due to the binding of a protein, since it is still observed after SDS-phenol treatment of the samples at the end of the preincubation (Fig. 7, right panel). Under these conditions, the amounts of the slower migrating band is again dependent on the ribosomal eluate concentration. We noted that at the higher concentrations of ribosomal eluate (Fig. 7, right panel, lane marked 4) a significant portion of the RNA remains at the interphase during phenol extraction presumably in a SDS resistant ribonucleoprotein complex. At these higher concentrations, it appears that between 20 to 50 per cent of the RNA recovered in the aqueous phase after phenol extraction is retarded. The amount of RNA which was retained at the interphase was also dependent on the preparation of ribosomal eluate. No modification is induced by the ribosomal eluate in the mobility of mutant pR9 RNA (data not shown). These results suggest that the slower migrating band is not a protein-RNA complex.

The size of the slower migrating band suggested the possibility that the RNA had become a dimer. Evidence for this interpretation comes from the following experiment. RNAs of two different sizes (350 n and 250 n) were generated by cleavage of the DNA template at two different restriction sites before transcription with the SP6 RNA polymerase. In this experiment we used capped RNAs. The RNAs were not labeled at their 5' end as in Fig. 7 but were

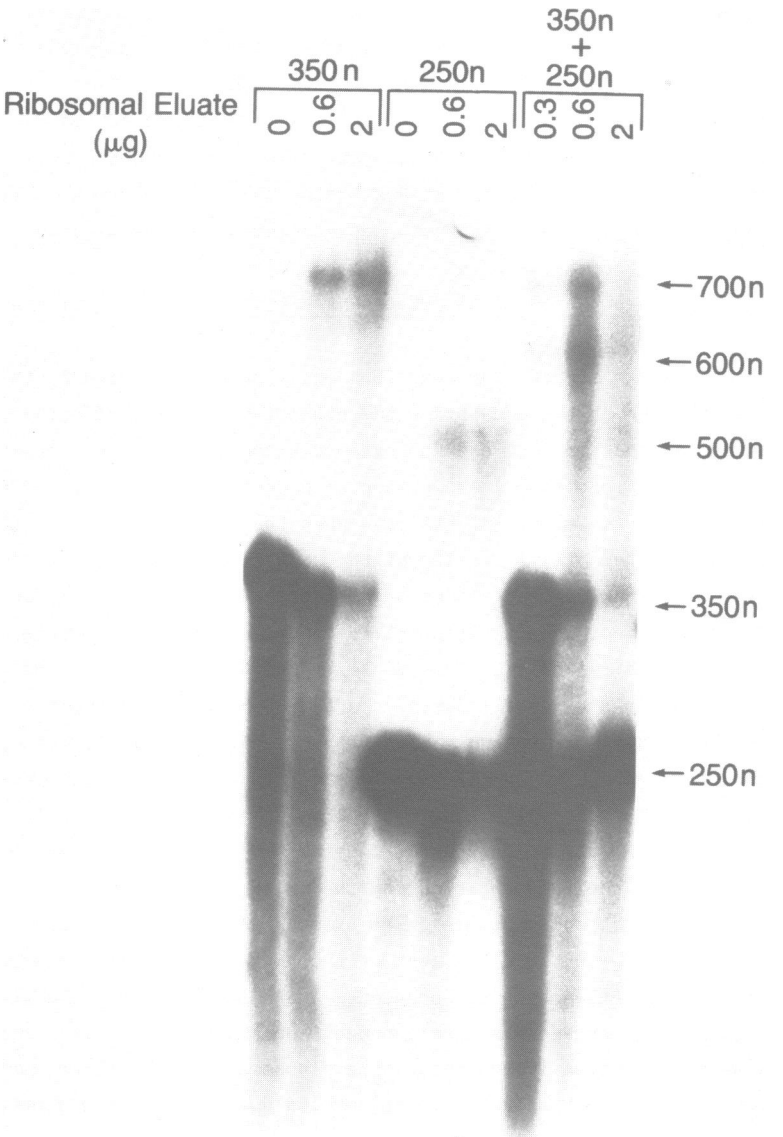


Figure 8: Electrophoretic analysis of reactions containing α^{32}[P]GTP uniformly labeled and capped wild-type RNA incubated with ribosomal eluate. Both a 350 n transcript obtained by cleaving pAZ1010* DNA with EcoRI and a 250 n transcript obtained by cleaving pAZ1010* DNA with PvuII were used. The three lanes on the right represent results obtained with an equimolar mixture of the two RNAs (350 n + 250 n). RNAs were incubated for 20 min at 30°C with the indicated amounts of NIH 3T3 ribosomal eluate, and then treated by SDS-phenol. RNAs were fractionated on a 4% polyacrylamide gel.

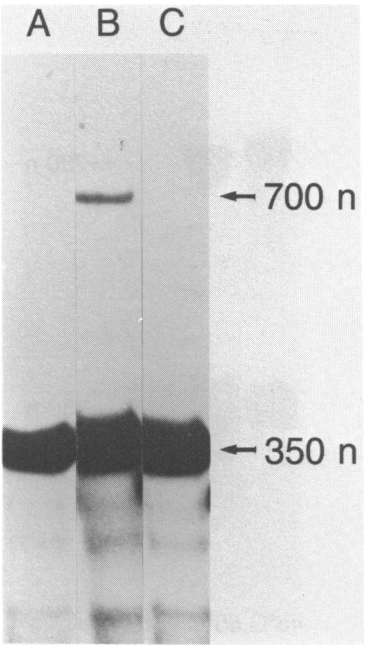


Figure 9: Electrophoretic analysis of a 5' end labeled wild-type RNA (transcribed from pAZ1010* DNA cleaved with EcoRI). Samples were heated for 5 min at 75°C, followed by slow cooling to room temperature. A) 5 ng labeled wild-type RNA (350 n); B) 5 ng labeled wild-type RNA mixed with 200 ng of the same unlabeled RNA; C) 5 ng of labeled wild-type RNA mixed with 200 ng of unlabeled pR7* RNA (transcribed from pR7* DNA cleaved with EcoRI).

uniformly labeled. This accounts for their higher rate of degradation. With the 350 n RNA, the slower migrating species has an approximate size of 700 n, whereas with the 250 n RNA, the retarded band has an approximate size of 500 n (Fig. 8). Mixing the two RNAs results in a slower migrating band of intermediate size (600 n). Furthermore, comparing the experiments in Figs. 7 and 8, we can conclude that the slower migrating species is observed whether or not the 5' end is capped.

The experiment presented in Fig. 8 strongly suggests that the slower migrating band produced after preincubation with ribosomal eluate, is due to the formation of an RNA-RNA dimer. The free energy resulting from intermolecular base-pairing of the conserved inverted repeat sequence between two pAZ1010* mRNA molecules has a theoretical ΔG of -39.8 Kcal, more than twice the ΔG resulting from intramolecular base-pairing of this RNA, which has a value of only -16.9 Kcal. Construction of pAZ1010* and pR9* included the

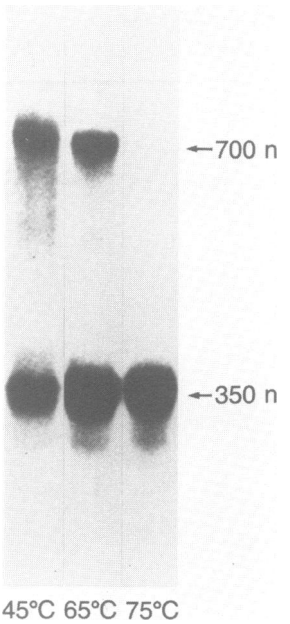


Figure 10: Electrophoretic analysis of wild-type RNA after incubation with 1.0 µg protein of NIH 3T3 ribosomal eluate and SDS-phenol treatment. Incubations were for 20 min at 30°C. Before the electrophoretic analysis, the samples were heated at 45°C, 65°C or 75°C, quickly cooled to 45°C and immediately loaded on a 4% polyacrylamide gel.

placement of a HindIII linker between the collagen and CAT sequences. It should be noted that the free energy of intermolecular base-pairing of the inverted repeat sequence in the HindIII linker has a value of -29.4 Kcal. In pAZ1010* RNA the intermolecular base-pairing of the conserved inverted repeat with a ΔG = -39.8 Kcal would be favored. However, as noted earlier, no ribosomal eluate dependent dimer formation was observed after incubation of pR9* RNA.

The dimer can also be obtained in the absence of ribosomal eluate if the RNA is first heat denatured followed by slow renaturation (Fig. 9). In this experiment the small amounts of labeled RNA are not sufficient to produce a dimer signal unless high concentrations of cold RNA containing an intact conserved inverted repeat are added. When excess cold mutant RNA is added, no dimerization of pAZ1010* RNA occurs. Furthermore, the dimer induced in the wild-type RNA by the ribosomal wash can be converted to the monomeric form by heat denaturation at 75°C (Fig. 10). Interestingly, heat denaturation of the

Table 1. Partial Reversibility of Ribosomal Eluate Effect

		25°C	75°C	RELATIVE INCREASE IN IN TRANSLATION AFTER HEAT DENATURATION
EXP. 1	Ribosomal Eluate			
	--	248	240	0.97
	1 µg	75	115	1.54
EXP. 2				
	--	281	272	0.96
	1µg	95	170	1.79
	2 µg	70	121	1.72

10 ng RNA, transcribed from pAZ1010*, was preincubated without or with ribosomal eluate from NIH 3T3 cells, then extracted with phenol-SDS and either kept at room temperature or heated at 75°C for 5 min. After quick cooling, the RNA was translated in a reticulocyte lysate. The data are expressed as TCA precipitable ^{35}S-methionine cpm x 10^{-3}.

wild-type pAZ1010* RNA, after preincubation with the ribosomal wash followed by SDS-phenol treatment, increases its translatability, whereas it does not affect the translatability of the same RNA preincubated with the control buffer (Table 1). The incomplete reversibility could be due to RNA losses at the phenol-water interphase during extraction of the RNA after preincubation with the ribosomal eluate, an interpretation consistent with results presented in Figs. 7 and 8. Thus, the inhibition of translation is at least partially reverted after heat denaturation, in parallel to the heat induced reversion to the monomeric form.

The formation of an RNA dimer, due to intermolecular self-hybridization of the conserved sequence (Fig. 11) should result in stabilization of the secondary structure around the translational start site. This may cause the initiation AUG codon to become less accessible (21). To test this hypothesis, we performed an experiment in which 5' end-labeled RNA was treated by reticulocyte lysate alone or in combination with fibroblast ribosomal eluate and assayed for sensitivity to T1 ribonuclease (data not shown). One major difference was that the segment around the AUG initiation codon, which was destabilized by the reticulocyte lysate, became protected if the sample was preincubated with ribosomal eluate. Addition of the reticulocyte lysate alone

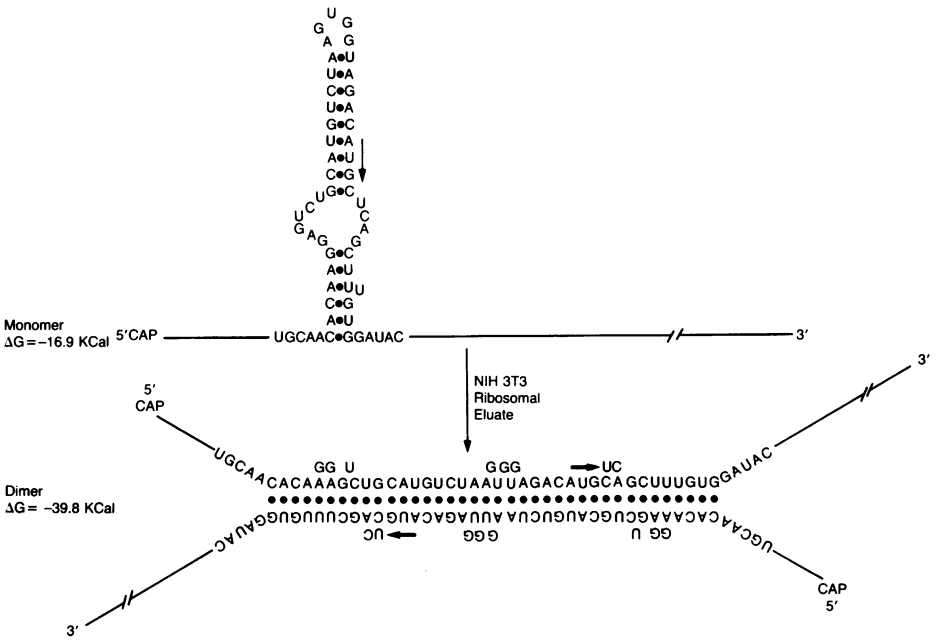


Figure 11: Schematic representation of the dimerization of the mRNA mediated by preincubation with a ribosomal eluate from NIH 3T3 fibroblasts. Short arrows indicate the position of the collagen translation initiation site.

increases the accessibility of segments 2 and 3, and that of segment 6 which contains the translation initiation AUG. A similar destabilization of α and β globin RNA-cDNA duplexes due to the presence of reticulocyte lysate has been previously reported (22). Addition of both the ribosomal eluate and the reticulocyte lysate results in a loss of the accessibility in areas 2, 3 and 6 (not shown). Hence, preincubation with fibroblast ribosomal eluate leads to an increase in the stability of the secondary structure of the conserved element.

DISCUSSION

The results described in this paper suggest the following conclusions: (1) The predicted secondary structure of the conserved element around the translation initiation site of the α_2(I) collagen gene exists in solution. (2) Although deletions in this conserved element increase translational efficiency of a collagen - CAT chimeric RNA after DNA transfection of fibroblasts, no significant differences are detectable by translation of the corresponding RNAs using a reticulocyte lysate cell-free system. (3) However,

preincubation with a salt extract of fibroblasts polyribosomes selectively decreases subsequent translation from the wild-type RNA but not from mutant RNAs. (4) A protein component present in the ribosomal eluate is needed only during the preincubation not during the translation part of the experiment. (5) No preferential degradation of the wild-type RNA occurs during the reaction. (6) During preincubation at least part of the wild-type RNA forms a dimer. We do not know whether the dimeric form is entirely stable during the electrophoretic run. In some experiments, as much as 50% of the RNA was found as a dimer in the gel retardation assay. Formation of the dimer appears to be dependent on the integrity of the conserved element and is reversible. Since the free energy of dimer formation is -39.8 Kcal compared to -16.9 Kcal for the formation of the hairpin structure in the monomer, the RNA dimer is a more stable structure than the monomer and might inhibit translation to a greater extent.

The following arguments suggest that the conserved inverted repeat sequence is the RNA segment involved in the ribosomal eluate dependent dimer formation. (1) No ribosomal eluate dependent dimer formation is observed in mutant RNAs in which the conserved element has been in part deleted. (2) In the 350 n fragment used in the studies of dimer formation, the intermolecular hybrid formed by base-pairing of the inverted repeat sequence has a higher free energy than any other intermolecular hybrid between sequences in this fragment. (3) Formation of the dimer can also be detected by heat denaturation followed by renaturation of the end-labeled wild-type RNA if excess amounts of this unlabeled RNA is present. However, no dimer is formed if the same experiment is performed using labeled wild-type RNA and excess mutant RNAs.

We have not yet studied whether the formation of the dimer is an enzyme catalyzed reaction. We have noted that ATP or GTP are not required but that the reaction is inhibited by addition of EDTA (data not shown). We also do not know yet whether any specific RNA sequences within the conserved element of the collagen gene are needed for dimer formation.

We have no evidence yet for the existence of an RNA dimer *in vivo*. We also do not know whether the formation of a dimeric RNA accounts for the differences in translational efficiency between wild-type and mutant RNAs observed in intact fibroblasts after DNA transfection.

The formation of an RNA dimer by intermolecular self antisense hybridization might be an interesting model to study how the stability of a segment containing an inverted repeat can be increased or decreased. Since the

initiation AUG of the collagen RNAs is present within the conserved inverted repeat element, it is tempting to speculate that modulation of the equilibrium between monomeric and dimeric forms could influence the rate of translation initiation of these RNAs.

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