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An extended RNA/RNA duplex structure within the coding region of mRNA does not block translational elongation

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ABSTRACT

RNA/RNA duplex formation involving the 5'untranslated region of a mRNA can efficiently block translation. Here we investigated the effect on translation of an RNA/RNA duplex between part of the coding region and sequences of the 3'untranslated region of lysozyme mRNA. A cDNA was constructed which contained 2 identical sequences of 150 nucleotides, one of which was an inverted repeat of the other. Cell-free transcription of this cDNA with T7 RNA polymerase resulted in a mRNA with an extended RNA/RNA duplex within the coding region. The presence of the double stranded structure was confirmed by the accessibility of complementary oligonucleotides to this region. mRNA was cleaved by RNaseH, endogenous to the wheat germ lysate, when hybridization of a complementary oligonucleotide occured outside but not within the predicted double stranded structure. When this mRNA was translated in a cell-free wheat germ translation system, the translation product was found to be of the size of full-length prelysozyme and not arrested. We conclude that the extend of a secondary structure within the coding region of a mRNA does not restrict the ability of the ribosome to translate this mRNA efficiently. Our data are consistent with the presence of an activity unwinding RNA/RNA duplexes, which is associated with the translating ribosome.

INTRODUCTION

Hybrid formation of long inverted repeats between the untranslated regions of mRNAs have been shown to efficiently inhibit translation (1). We investigated the possibility of arresting mRNA translation by introducing an inverted repeat within the coding region. The translational arrest of a nascent polypeptide chain at a predetermined site would allow the study of biological functions in which the nascent polypeptide chain is involved. For example, the translocation of secreted proteins across the membrane of the endoplasmic reticulum involves a complex interaction of soluble and membrane-bound components of the translocation machinery with the ribosome and the nascent polypeptide chain (for reviews see 2-3). Since translocation is rapid and in mammalian cells coupled to translation, the nascent polypeptide is expected to only transiently contact these components. For the identification and analysis of components involved at any particular stage of translation, it would therefore be an advantage to "freeze" single steps by arresting the process of translation at defined sites.

In the wheat germ cell-free translation system, the addition of signal recognition particle (SRP), a soluble component required for the translocation of secreted proteins, can

cause elongation arrest (4). This SRP-induced arrest has been used to study the association of nascent preprolactin with the microsomal membrane (5-6). However, the applicability of this approach is limited as the arrest occurs at a limited number of sites only (7).

Several approaches have been tested to arrest elongation of translation. Truncated mRNAs can be generated by cleavage of cloned cDNAs at sites within the coding region prior to transcription in vitro (8). The same effect is achieved when complementary deoxyoligonucleotides or cDNA clones in single stranded vectors are annealed to mRNA followed by digestion with RNaseH (9-11). Cell-free translation of such a truncated mRNA results in a correspondingly shortened polypeptide (8-9,11). However, ribosomes translating these transcripts release the nascent polypeptide chain at the end of the truncated mRNA (9). Consequently, these techniques are of limited applicability where it is desired to arrest ribosome movement at a particular site without dissociating the nascent polypeptide from the translational complex. An alternative approach would be the introduction of extended secondary structures into the coding region.

In this report we test whether extended RNA/RNA duplexes would allow translational arrest without dissociation of the translational complex. We describe the efficient translation of mRNA containing a double stranded segment of 150 base pairs within the 3'end of the coding region. We conclude that even extended secondary structure in the coding region of mRNA has little effect on the translatability of this mRNA in vitro.

MATERIALS

Restriction enzymes, other DNA modifying enzymes and the capping reagent $m^7G(5')ppp(5')G$ were from Boehringer Mannheim. L-[^{35}S]-methionine and ^{32}P-GTP were from Amersham; EN^{3}HANCE from New England Biolabs. The vector pGEM2 and T7 RNA polymerase were obtained from Promega. Deoxyoligonucleotides (oligo no.1: GGG TGT TGA AGT TAC TCT CG; oligo no.2: CAC GCT CGC TGT TAT GTC TG; oligo no.3: CGA CCC ACG CGC TCA TGC CG) were synthesized and purified as described previously (9).

In vitro transcription

Plasmid DNA (0.3µg) was digested either with EcoRI or SacI. Recessed ends were made blunt using T4 DNA polymerase following the protocol described by Maniatis et al. (12), and the reaction was stopped by heating the reaction mixture at 70°C for 10min. The reaction mixture was extracted sequentially with an equal volume of phenol, phenol: chloroform (1:1) and chloroform, and DNA was precipitated with ethanol and dried under vacuum. The transcription reaction was carried out in a 20µl volume containing 0.1M Hepes, pH 7.4, 0.1M $Mg(OAc)_2$, 0.02M spermidine, 0.1mg/ml bovine serum albumin, 0.01M DTT, 0.5mM each of ATP,CTP,GTP,UTP, 20U RNAsin, 0.25mM m7G(5')ppp(5')G and 25U of T7 polymerase at 40°C for 60min. In studies using labelled mRNA, 1µl of a^{32}P ATP (3,000 Ci/mmole; 10µCi/µl) was included in the transcription reaction. The transcription mixture

was frozen in liquid nitrogen and stored at -80°C. 2µl of transcription mixture was used per analysis.

In vitro translation

Transcripts were translated in wheat germ lysate in the presence of L-^{35}S methionine as described previously (13). Translations were carried out at 25°C for 60min in a total volume of 25µl. In the translation reactions with labelled mRNA, L-^{35}S-methionine was omitted. In some experiments, oligonucleotides complementary to the lysozyme mRNA were included in the in vitro translation mixture at an approximately 50-fold molar excess (9).

Electrophoresis

For the analysis of translation products, cell-free transcription/ translation mixture was dissolved in sample buffer and separated on 22% polyacrylamide gels containing 6M urea as described previously (9). ^{35}S-labelled translation products were visualized by fluorography using EN^{3}HANCE following the instructions of the manufactorers. ^{32}P-labelled transcripts were separated using the same electrophoretic system, but the gel contained only 10% polyacrylamide. The gels were exposed without prior fluorography.

RESULTS

Plasmid construction

In order to obtain a mRNA with a long inverted repeat structure, we isolated a 3'terminal cDNA sequence and ligated it to the wild-type sequence in a tail-to-tail orientation.

Fig.1A illustrates the construction of a lysozyme cDNA clone, in which sequences of the 3'end of the lysozyme coding region were ligated to the 3'end of the lysozyme cDNA in an inverted orientation. The lysozyme cDNA (I) was excised with HindIII from the plasmid pDS5/4 (13) and subcloned into the polylinker (PL) of pGem2, under the control of the T7 promoter (pTlsm). A second fragment (II) including 150 base pairs (bp) of the chicken lysozyme cDNA and 140bp of the 5'end of the chloramphenicol acetyltransferase (cat) gene was excised from pDS5/4 with SacI and PvuII and cloned between the SacI site and the HincII site of the polylinker region of pGem2 (pTlsmC). During this cloning step, the PvuII and HincII-sites were deleted. The fragment was excised with EcoRI and PstI and cloned between the EcoRI and PstI sites of pTlsm. This construct was designated pTlsmL. mRNA derived from this construct contains a 3'terminus which is complementary to nucleotides 381-531 of lysozyme mRNA. The putative secondary structure of the self-complementary mRNA is shown in fig.1B (lsmL folded). It is assumed that the inverted repeat sequences hybridize to each other and form a stem-loop structure. The constructions were verified by restriction analysis (not shown).

Translation of pTlsmL results in a full-length product

In order to analyse the translation products of the clones and to obtain size markers for the expected truncated prelysozyme peptide, we linearized the cDNA constructs at unique

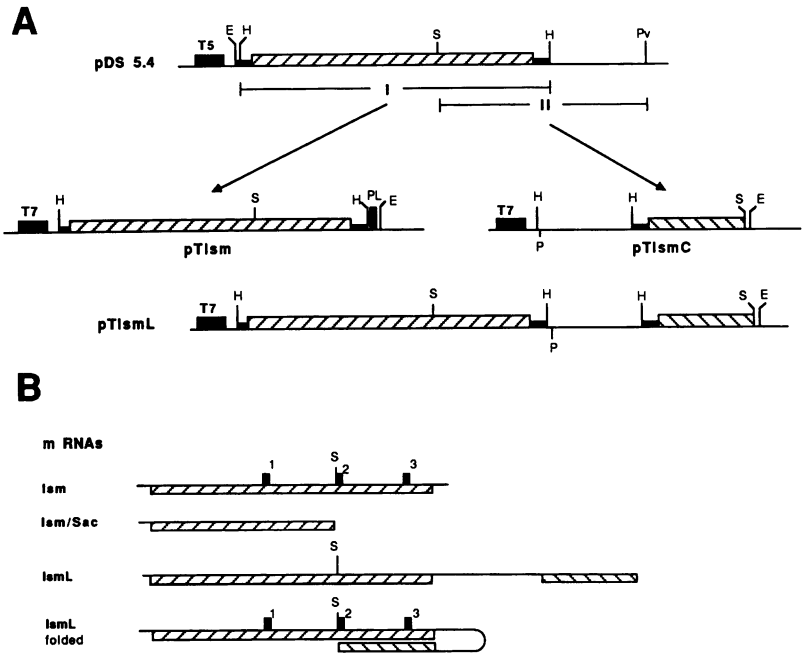

Fig. 1A Construction of pTIsmL
Two DNA fragments (I+II) were excised from plasmid pDS5.4 (13), isolated from agarose gels and subcloned into the polylinker region (PL) of pGem2. Fragment I, representing the lysozyme cDNA, was brought under control of the T7 (T7) promoter (pTIsm). Fragment II, representing the 3'terminus of the lysozyme cDNA and sequences from the vector pDS5, was first cloned between the SacI and HincII site of the PL of pGem2 (pTIsmC). The sequences were excised from pTIsmC with EcoRI and PstI and cloned between the corresponding sites of pTIsm, thus resulting in a tail-to-tail orientation with respect to the fragment I (pTIsmL). The coding region is shown as striped box, the 5'and 3'untranslated regions are indicated as black boxes and vector sequences as lines. The opposite direction of diagonal stripes symbolizes the opposite orientations of the inserts. Restriction sites: E EcoRI; H HindIII; P PstI; Pv PvuII; S SacI.

Fig. 1B RNA Transcripts derived from pTIsm and pTIsmL.
Non coding sequences are represented by lines and coding sequences by striped boxes. The black boxes indicate the sites to which the respective oligonucleotides are complementary. Ism mRNA is derived from construct pTIsm, linearized with EcoRI, Ism/Sac mRNA is derived from pTIsm linearized with SacI and IsmL mRNA is derived from pTIsmL, linearized with EcoRI. IsmL folded shows the putative secondary structure of IsmL mRNA.

restriction sites, transcribed the DNA with T7 RNA polymerase and translated the mRNA in wheat germ lysate.

Digestion of a cDNA at restriction sites located within the coding region leads to truncated mRNA when transcribed in vitro. Consequently, the resulting peptide chain is shortened correspondingly (8). The plasmid pTIsmL was linearized with EcoRI and pTIsm was

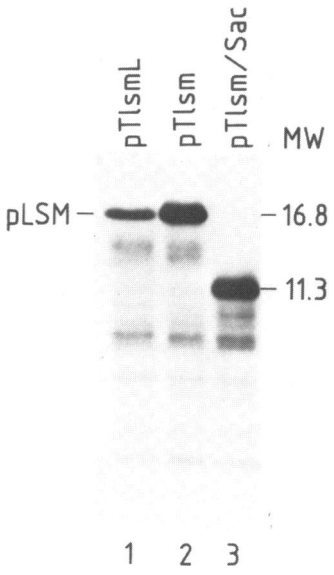

Fig. 2 In vitro translation of lysozyme cDNA constructs.
Transcripts were obtained from pTlsm, linearized with either EcoRI or SacI and from pTlsmL, linearized with EcoRI. The transcripts were translated in wheat germ lysate in the presence of ^{35}S-methionine. The translation products were separated on polyacrylamide gels (22% acrylamide, 6M urea). The gels were autoradiographed following fluorography. Lane 1: Translation product from pTlsmL, linearized with EcoRI; lane 2: translation product from pTlsm, linearized with EcoRI, lane 3: translation product from pTlsm, linearized with SacI. The molecular weight (MW) is shown in kDa.

linearized either with SacI or with EcoRI prior to transcription (fig. 1B). The transcription products were translated and the respective translation products were analysed by SDS-PAGE followed by fluorography (fig.2, lanes1-3). The main translation product of pTlsm mRNA was a protein of the size of prelysozyme with the expected MW of 16.8kDa (fig.2, lane2). Translation of mRNA transcribed from pTlsm, linearized with SacI, resulted in a truncated polypeptide of MW 11kDa (fig.2, lane3). This corresponds in size to a peptide which would be generated as a result of translational arrest caused by the secondary structure in lsmL mRNA. A peptide of identical size was produced from mRNA transcribed from pTlsmL, linearized with SacI prior to transcription (not shown). When mRNA derived from pTlsmL, linearized with EcoRI, was translated, the major translation product was of the size of full-length prelysozyme (fig.2, lane1).

The synthesis of full-length prelysozyme from pTlsmL mRNA was unexpected and showed that an inverted repeat of 150 nucleotides within a mRNA molecule does not arrest translation. There are two obvious explanations for the synthesis of full-length prelysozyme from mRNA lsmL:

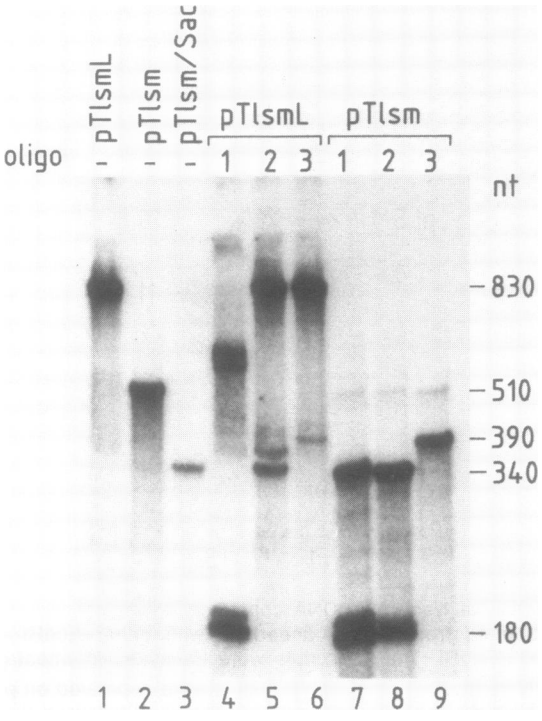

Fig.3 Stability of mRNAs in wheat germ lysate in the presence of complementary oligonucleotides. Labelled transcripts were synthesized from constructs pTlsmL, linearized with EcoRI (lanes1, 4-6) and from pTlsm, linearized either with EcoRI (lanes2, 7-9) or with SacI (lane3) as described in materials and methods. The transcripts were incubated under translation conditions in wheat germ lysate for 60min either in the absence (lanes1-3) or in the presence (lanes 4-9) of complementary oligonucleotides and analysed on 10% polyacrylamide gels, containing 6M urea. Lanes 4&7: transcripts after annealing to oligonucleotide no.1; lanes 5&8: transcripts after annealing to oligonucleotide no.2; lanes 6&9: transcripts after annealing to oligonucleotide no.3. The approximate fragment sizes (nt) are indicated on the right-hand side.

(1) Despite the extensive inverted homology, the expected RNA/RNA duplex is either not formed or is unstable under the assay conditions. (2) The duplex structure is formed, but it is unwound by the migrating ribosome complex.

We used complementary oligonucleotides as a tool to test the integrity of the predicted stem-loop structure and to distinguish between the two possible explanations.

RNA/RNA duplex prevents hybridization of complementary oligonucleotides

It has been shown that hybridization of oligonucleotides complementary to mRNA coding sequences leads to shortened polypeptides, if translation is carried out in wheat germ lysate (9). This is due to truncation of the mRNA by endogenous RNaseH activity in the lysate (10-

11). We utilized this endogenous activity to test the existence of the RNA/RNA duplex structure of lsmL mRNA under cell-free translation conditions.

Different oligonucleotides complementary to the lysozyme coding region (fig.1B) were used: oligonucleotide no.1 corresponded to a sequence preceding the putative duplex, oligonucleotide no.2 to a sequence at the start of duplex and oligonucleotide no.3 in the middle of the duplex. A tight duplex structure would inhibit access of oligonucleotides to that region and consequently, no oligonucleotide-mediated cleavage by RNaseH should occur. Conversely, cleavage of the mRNA would indicate that the double stranded structure is not formed or that it is unstable.

mRNAs were radioactively labelled in the transcription reaction and translated in wheat germ lysate in the absence (fig.3, lanes 1-3) or presence (fig.3, lanes 4-9) of complementary oligonucleotides. pTlsm cDNA, linearized with either EcoRI or SacI was transcribed into a product of 510nt and 340nt respectively, and pTlsmL cDNA was transcribed to a product of 830nt. In the presence of oligo no.1, pTlsmL mRNA was cleaved once to yield a product of 180nt (containing the 5'terminus of the mRNA) and a larger one containing the 3'end of the mRNA (fig. 3, lane 4). Oligos nos.2&3 did not lead to a significant cleavage of pTlsmL mRNA, leaving the full-length transcript the main product (fig.3, lanes 5-6). The minor cleavage products could represent a small percentage of mRNA molecules not folded in the expected structure. Alternatively, they could be the result of the translating ribosome transiently destabilizing the double strand and thus allowing access of a complementary oligonucleotide. In contrast, pTlsm mRNA was cleaved almost to completion in the presence of each of the 3 oligonucleotides (fig. 3, lanes 7-9).

The molar concentration of oligonucleotides and the endogenous RNaseH activity were sufficient to completely cleave lsm mRNA at all three sites and also lsmL mRNA, when an oligonucleotide complementary to a region upstream of the putative stem-loop was used. Consequently, the failure of oligonucleotides nos.2&3 to efficiently mediate cleavage of lsmL mRNA is consistent with the presence of the double stranded structure, which prevents hybridization of the oligonucleotide and subsequent RNaseH cleavage.

DISCUSSION

In this report we tested whether an artificially introduced RNA/RNA duplex within the coding region of a mRNA would block translation. We constructed a lysozyme cDNA (pTlsmL) which after transcription resulted in a mRNA that contained an inverted repeat sequence of 150 nucleotides. These sequences form an RNA/RNA duplex close to the 3'terminus of the coding region. The free energy (ΔG) of this structure calculated according to Zuker and Stiegler (14) is -345 kcal x mole-1. The linearized cDNA was transcribed in vitro and the transcript was translated in a wheat germ cell-free lysate. The major translation product was that of the full-length prelysozyme. The presence and tightness of the predicted duplex

structure was confirmed using oligonucleotides complementary to a region within the expected double strand. While wild-type mRNA was efficiently cleaved by endogenous RNaseH after annealing of the oligonucleotides, no significant cleavage was observed when the same oligonucleotides were annealed to the mutant mRNA prior to translation. Our results demonstrate clearly that despite the formation of a double stranded structure, the translating ribosomes have the ability to break the RNA/RNA hybrid and to complete translation. Similarly it has been found that mRNA/cDNA heteroduplexes within the coding region do not arrest translation of the mRNA (15-16). Since neither the formation of mRNA/cDNA hybrids nor the introduction of intramolecular RNA/RNA duplexes arrest elongation, we conclude that approaches others than the introduction of secondary structure have to be sought to obtain site-specific arrest of peptide synthesis.

The process of translation can be divided into initiation, elongation and termination. Base pairing between sequences of the untranslated regions of zein mRNA from maize can occur efficiently and it inhibits initiation of translation completely (1). It is well established that secondary structure within the 5′ untranslated region of mRNAs influences the translatability of mRNAs by interfering with the process of initiation. These observations have been made by using in vitro translation systems (1,17) and cells transfected with cDNA constructs (17-18). It is the current view that 40S ribosomal subunits when moving along the 5′untranslated region, have only a limited ability to disrupt secondary structure (18) and it has been suggested that the effect of secondary structure on migration of 40S subunits determines the correct initiation of translation at the most 5′positioned AUG codon (19). Although the duplex formation in the case of lsmL mRNA is energetically far more favorable, it does not interfere with the process of elongation.

It has been known for some time that eukaryotic mRNAs have extensive secondary structures (20-21) and obviously, these mRNAs are translated in vivo. However, translation is a non-uniform event in bacteria (22-24) and in eukaryotic cells (25). Two different mechanisms have been proposed to explain translational pauses; firstly, extensive secondary structure could cause a slowing down of the translating ribosome (22). Alternatively, the limited availability of certain charged tRNAs required for frequently used codons could result in discontinuous translation (25-26). Indeed, we always observed radiolabelled bands shorter than the full length product, although they were of low abundance. These bands were translation products of the lysozyme mRNA, as no bands were detected when mRNA was omitted from the translation reaction (not shown). However, we did not detect any translational pause at the site of the double stranded structure. Since the pattern of the shortened peptides obtained from translation of lsm mRNA and lsmL mRNA is identical, we conclude that RNA/RNA hybrid structures are not the main cause of discontinous translation, although we cannot exclude that extensively folded mRNAs could have a general inhibitory effect on translation, which is not site specific.

Our data are consistent with the presence of an activity associated with the translating 80S ribosome, which unwinds double stranded mRNA. Recently, the presence of an unwinding activity in the rabbit reticulocyte cell-free translation system, which destabilizes mRNA/cDNA hybrids has been suggested (15-16). This mRNA/cDNA hybrid destabilizing activity was only associated with ribosomes engaged in translation since hybrids involving the untranslated regions were not disrupted. In our opinion, the introduction of intramolecular RNA/RNA hybrids provides a more stringent test for the ability to unwind a duplex structure because formation of such a structure should occur at a higher rate and more efficiently than the formation of a heteroduplex. It remains to be seen whether the activity inferred from our experiments is the same as the one which unwinds mRNA/cDNA duplexes.

In fertilized Xenopus eggs an RNA/RNA unwinding activity is present which unwinds hybrids between mRNA and anti-sense RNA (27-28). This activity is independent of the process of translation and it is developmentally regulated; high levels of it are found in eggs and during early embryogenesis, whereas in oocytes and in late blastula stages this activity is diminished. Both of these characteristics distinguish it from the unwinding activity observed in our experiments.

Activities, which specifically destabilize duplex formation within the coding regions of mRNAs have now been described in a mammalian and in a plant cell-free system. This would argue for a universal presence of such an activity in eukaryotic cells. The association of the destabilizing activity solely with the 80S translating ribosome and not with 40S ribosomal subunit would explain, why secondary structures within the 5'untranslated region, although energetically less favorable than the secondary structure of lsmL mRNA, have a more profound effect.

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