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Mechanism of translational coupling between coat protein and replicase genes of RNA bacteriophage MS2

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ABSTRACT

We have analyzed the molecular mechanism that makes translation of the MS2 replicase cistron dependent on the translation of the upstream coat cistron. Deletion mapping on cloned cDNA of the phage shows that the ribosomal binding site of the replicase cistron is masked by a long distance basepairing to an internal coat cistron region. Removal of the internal coat cistron region leads to uncoupled replicase synthesis. Our results confirm the model as originally proposed by Min Jou et al. (1). Activation of the replicase start is sensitive to the frequency of upstream translation, but never reaches the level of uncoupled replicase synthesis.

INTRODUCTION

Synthesis of the replicase protein encoded by the RNA bacteriophage MS2 is controlled in two ways. First, translation of the replicase gene is inhibited by the MS2 coat protein, which binds to the replicase start region, thus acting as a translational repressor (2,3,4). Second, and this is the subject of this paper, replicase synthesis depends on the translation of the upstream coat protein cistron (5). This translational coupling was deduced from the observation that early amber mutations in the coat protein gene (codon 6) prevent replicase synthesis. On the other hand such translational polarity was not found for late amber mutations (codon 50 and further downstream) (5,6,7). It was therefore proposed that translation of MS2 RNA between codons 6 and 50 of the coat protein sequence activates the ribosomal binding site of the replicase cistron, which lies some 340 nucleotides further downstream (Fig. 1).

Once the relevant sequences of the MS2 RNA molecule became known a molecular explanation for the polarity effect was postulated by Min Jou et al. (1). A region within the coat protein cistron comprising codon 24 to codon 32 (referred to hereafter as the Min Jou-sequence) shows reasonable complementarity to the start region of the replicase gene. Basepairing between the two sequences was proposed to mask the initiation site of the

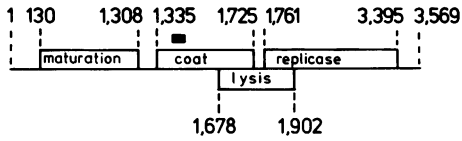


Fig. 1. Genetic map of coliphage MS2. Nucleotide numbers are taken from ref. 15. The Min Jou-sequence (1409-1433) is indicated by a black box.

replicase gene (Fig. 2A). This long range RNA interaction was consistent with the finding that in a partial RNase digest of MS2 RNA the fragments containing the complementary regions comigrated during electrophoresis under non-denaturing conditions (1).

Translation over the Min Jou-sequence should disrupt the basepairing and release the replicase start site. In early coat amber mutants the ribosomes never reach the Min Jou-sequence and no replicase will be synthesized. In agreement with the model, the replicase start site is not accessible to ribosomes in vitro unless the MS2 RNA is denatured or degraded (8,9,10). It may be noted that an alternative RNA secondary structure for the replicase start region exists in the form of a hairpin shown in Fig. 2B (11,12,13). The 59 nucleotide long RNA fragment depicted is protected from RNase T1 digestion in the MS2 RNA-coat protein complex. The hairpin containing the initiation signals of the replicase gene was found to be stabilized by the coat protein.

On the other hand, electronmicroscopy studies on intact MS2 RNA led Jacobson and Spahr (14) to propose an alternative for the Min Jou model. The authors suggest a tertiary RNA structure that brings the start region of the coat- and replicase-genes close together. In this model the preferred binding of ribosomes to the coat cistron physically excludes ribosomal binding to the replicase start. Once the first ribosome moves away from the coat start site, the tertiary structure dissolves and enables subsequent initiations at the replicase start (14).

The availability of cloned MS2 DNA provides a unique opportunity to evaluate both models. If the Spahr model is correct the deletion of the coat protein start site will result in replicase synthesis, but according to the flower model of Fiers et al. (1,15) only removal of the Min Jou-sequence will allow replicase synthesis. Our results confirm the Belgian model. Selected deletions extending into the Min Jou-sequence trigger uncoupled translation of the replicase gene.

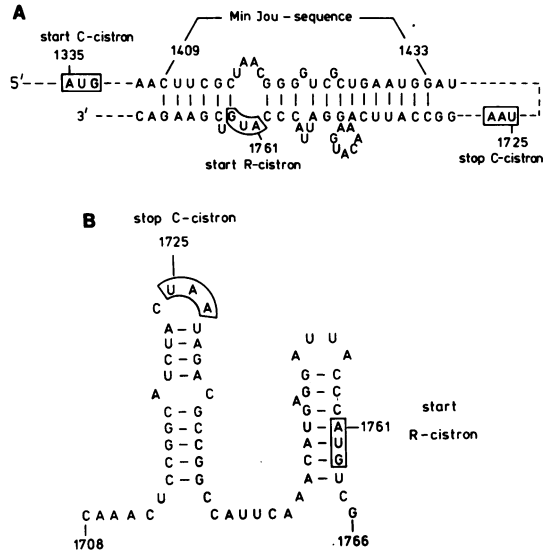


Fig. 2A. Long distance RNA-RNA interaction between the Min Jou-sequence and the ribosomal binding site of the replicase gene (1,15).
2B. Alternative RNA secondary structure for the replicase start region. The 59 nucleotide long RNA fragment depicted is protected against RNase T1 digestion by the coat protein (13). Both hairpins have been demonstrated to exist in the isolated RNA fragment (11,12).

MATERIALS AND METHODS

Strains and plasmids

In all experiments we used E. coli K-12 strain M5219, carrying a defective λ prophage encoding the thermosensitive repressor cIts857 and the transcription antitermination factor N.

MS2 cDNA was cloned by Devos et al. (16) and generously supplied to us by Dr. W. Fiers. All MS2 DNA fragments were cloned as EcoRI-PstI fragments behind the P_L promoter of phage λ in the expression vector pPLa2311 (17). Original EcoRI-sites are present in MS2 DNA at position 103 and 1628, those at 869 and 1305 were introduced by the ligation of EcoRI-linkers to the original PvuII and XbaI sites, respectively (18,19). The PstI-site is located some 250 nucleotides downstream of the actual MS2-sequence.

Plasmid pMS23 contains nearly the complete MS2-information (EcoRI 103-PstI). pMS23Sac$^-$ was obtained by opening the unique SacI site present at position 1490, removing the 3' protruding ends with Klenow fragment of DNA polymerase I and religating. The original sequence is

....CAGAGCTCTG.... and changes to ....CAGCTG.... . The four basepair deletion was checked by sequencing of the SalI-EcoRI fragment (1365-1628) in M13 mp8 (20). Additional confirmation was obtained by the fact that the new sequence forms a PvuII site.

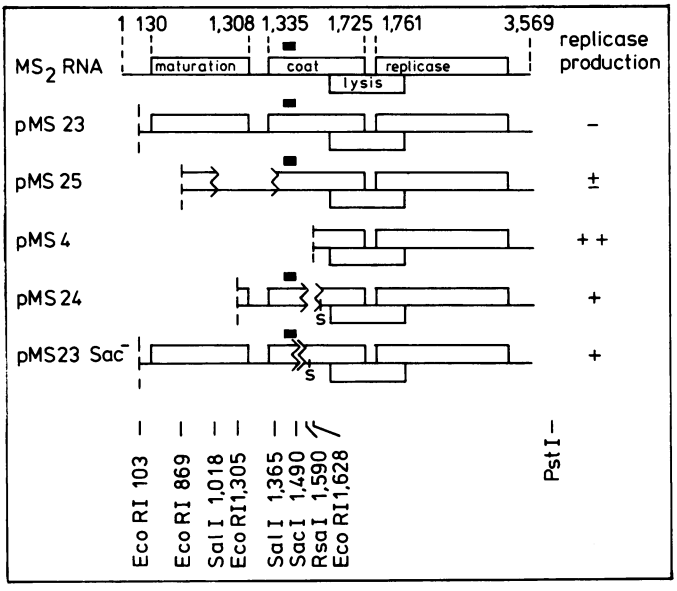
Plasmid pMS4 contains MS2 sequences starting at the EcoRI site 1628 up to the PstI site. pMS24 was made by coligation of two fragments in EcoRI-PstI opened pPLa2311. An EcoRI-RsaI fragment (1305-1590) was isolated and mixed with the EcoRI 1628-PstI fragment of pMS4 , in which the EcoRI site was filled in with the Klenow enzyme. At the fusion of the RsaI and the filled in EcoRI site, the sequence ....GTAATTC.... predicts a stop codon in the coat protein reading frame. The construction was confirmed by sequence-analysis and the presence of a truncated coat protein after induction.

pMS25 (EcoRI 869-PstI) was obtained by deleting the unique SalI fragment between sites 1018-1365. Plasmids pMS25Δ1380, Δ1419, Δ1420 and Δ1432 all derive from pMS25, in which BAL 31-induced DNA digestion was initiated at the unique and fused SalI site 1066/1365. After ligation and transformation the obtained clones were tested by restriction analysis. Four selected clones were sequenced as follows. The EcoRI fragment (869-1628) was end-labelled and subsequently recut with RsaI (1590), the isolated EcoRI-RsaI fragment (869-1590) was subjected to the sequencing-protocol according to Maxam and Gilbert (21). In pMS25Δ1432 the EcoRI site at position 869 was removed by the BAL 31-digestion. To sequence this clone, the unique EcoRI site at position 1628 was end-labelled. After cutting with XhoI, the XhoI-EcoRI fragment comprising the P_L promoter was isolated over a polyacrylamide gel and sequenced. The XhoI site lies upstream the P_L promoter in the Kan^R-gene of the vector. Deletions were as follows; 979-1379 for pMS25Δ1380, 1004-1418 for pMS25Δ1419, and 1004-1419 for pMS25Δ1420. In pMS25Δ1432 the deletion starts at position 103 of the P_L leader (17) and proceeds up to MS2 position 1431.

Protein analysis

Clones were induced at 42°C for the time indicated. Cells of a 1 ml culture were collected by centrifugation and analyzed by SDS-PAGE according to Laemmli (22). Replicase is a membrane bound protein (23). Boiling of the pelleted cells in Laemmli buffer does not solubilize the protein. Instead it was essential to first treat the bacteria with lysozyme-EDTA followed by several freeze-thaw cycles, before boiling the sample in

Table I MS2 cDNA present in the various subclones used in this study. In each construction the P_L promoter is positioned just upstream the MS2 sequences depicted. The manipulations in pMS24 and pMS23Sac$^-$ create a stopcodon (indicated by S) in the coat protein reading frame. Replicase synthesis as directed by the constructs is indicated in the right hand column. See text for further details. The Min Jou-sequence is indicated by the black box.



Laemmli buffer. All samples for electrophoresis contained the same amount of cells as based on their optical density at 650 nm.

Labelling of cultures with radioactive aminoacids was as described (24).

RESULTS

Expression of MS2 DNA subclones was carried out in the vector pPLa2311, where transcription of the inserted DNA fragment is controlled by the thermo inducible promoter P_L from phage λ (17). At 28°C the promoter is silent due to the presence of the repressor gene cIts857 in the host chromosome. At 42°C the repressor is unstable and transcription is turned on.

Let us compare pMS25 and pMS4 for their potential to synthesize the replicase protein. pMS25 contains the MS2 DNA region 869-3569 from which an internal SalI fragment (1018-1365) is deleted (Table I). The deletion includes the start of the coat protein cistron but leaves the Min Jou-sequence (1409-1433) intact. In pMS4 only the MS2 DNA region 1628-3569

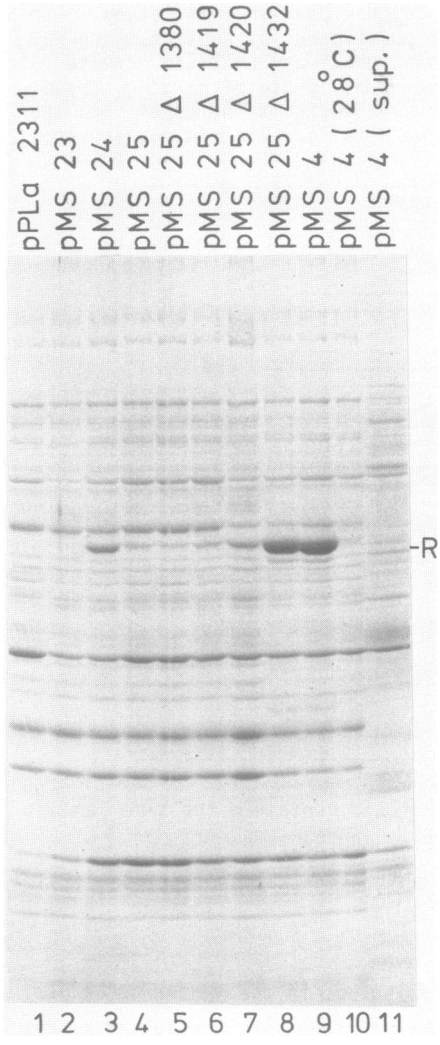


Fig. 3. In vivo replicase (R) synthesis as directed by the various DNA-constructs. After induction of the host cells at 42°C for 30 min., cells were collected, treated with lysozym and fractionated by centrifugation (see materials and methods). The replicase protein segregated with the insoluble membrane fraction, which was solubilized in sample buffer and analyzed on a 12,5 % SDS-PAGE gel. All lanes contain the equivalent of 3×10^8 cells. Lane 1 shows the protein pattern of the host M5219 with the expression vector pPLa2311. In lane 2 pMS23 was analyzed. Although this construction carries the complete MS2-information (see Table 1), no replicase was synthesized due to the presence of the coat protein, acting as a translational repressor. A non-induced sample (28°C) of pMS4 is present in lane 10. Lane 11 shows the soluble cell fraction of pMS4; no replicase protein can be detected.

is present, consequently the basepairing proposed by Min Jou cannot take place. If the Min Jou model is correct, pMS4 should synthesize much more replicase than pMS25. In the Jacobson and Spahr model, however, no large differences are expected since in both clones the coat protein start site is absent and access to the replicase start should therefore be free. As shown in Fig. 3, pMS4 (lane 9) makes a large amount of replicase protein, whereas pMS25 (lane 4) hardly produces any. This supports the long range RNA-RNA interaction proposed by Fiers and coworkers. As a control we show

the uninduced protein pattern for pMS4 in lane 10.

Starting from the two extremes, the non-producer pMS25 and the replicase overproducer pMS4, we determined which MS2 RNA region between 1365 and 1628 is actually responsible for the repression of replicase synthesis. pMS25 was subjected to limited BAL 31 digestion starting at the unique fused SalI site (1018/1365). Four subclones were selected for further study after determining the deletion endpoints by DNA sequencing . In pMS25 Δ 1380 (where the 3'deletion end point is at nucleotide 1379) the Min Jou-sequence (1409-1433) is still present and as shown in Fig. 3 lane 5 this clone still makes very little replicase. In pMS25Δ1419 and pMS25Δ1420 about half of the Min Jou-sequence is deleted and indeed replicase synthesis increases with respect to pMS25 (lanes 6 and 7). Finally, in pMS25 Δ 1432 virtually the complete Min Jou-sequence is removed and the same large amount of replicase as in pMS4 is made (lane 8). In fact replicase yields have been measured for several additional clones with MS2 DNA cut-off points downstream of position 1432. No further increase of replicase yield above that of pMS25Δ1432 and pMS4 was found. In some of the clones presented here the amount of replicase was quantitated by labeling the cultures with ^{14}C-aminoacids. The results are presented in the inset in Fig. 4 and demonstrate that removal of the Min Jou-sequence leads to a nine fold increase in replicase synthesis. Our findings thus fully support the model put forward by the group of Dr. Fiers. A summary of the results is given in Fig. 4.

It is assumed that on intact phage RNA the replicase start is exposed temporarily by the movement of ribosomes over the Min Jou area. The availability of cloned MS2 DNA allowed us to answer two more questions concerning this problem. First, how effective is ribosomal movement in exposing the replicase start as compared to the situation where the Min Jou-sequence is absent? Secondly, how does the frequency of ribosomal passage over the Min Jou area affect replicase synthesis? To answer these questions we needed clones that allow translational starts at the coat cistron, but do not yield the mature coat protein, which would repress replicase synthesis. Accordingly, we manipulated the coat gene sequence to obtain a premature translational stop. In one of the clones, pMS24, an RsaI-EcoRI restriction fragment (1590-1628) was removed creating a nonsense codon at the new junction. In the other clone, pMS23 Sac$^-$, the removal of 4 nucleotides at the SacI site (1490) leads to out of phase termination at position 1561. In Fig. 3, lane 3 we show the amount of replicase present

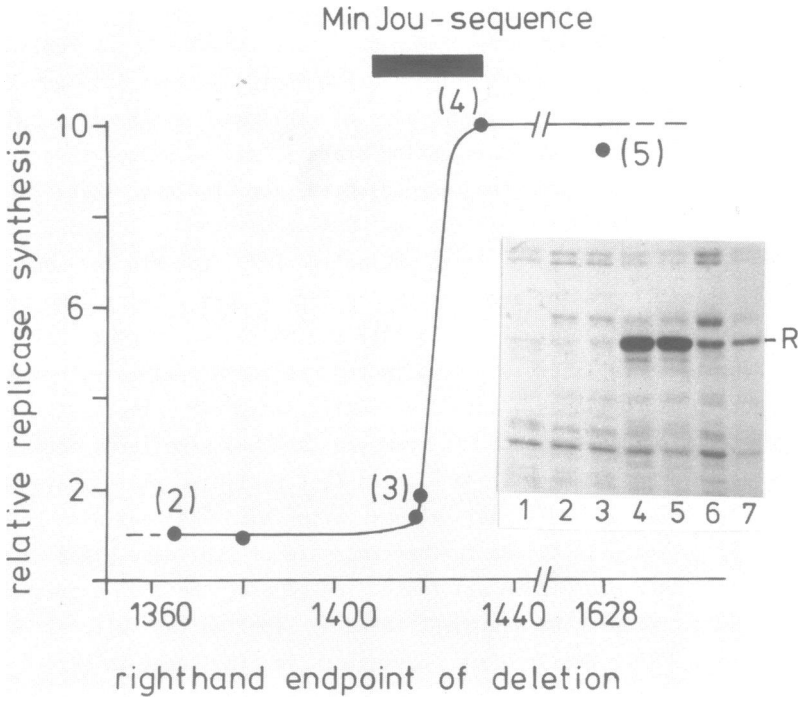


Fig. 4. The effect of deletions in MS2 DNA on replicase synthesis. The amount of replicase protein (R) produced was determined from the radioactive protein gel shown in the inset. The relative amount of the replicase protein was calculated by determining the ratio of counts in the replicase band to the counts in the rest of the lane. Induction and [^{14}C]-aminoacid labelling was for 20 min. at 42^{o}C. Lane 1 shows a non-induced sample (28^{o}C) for control. The insoluble fraction of the cells, containing the replicase, was applied to the gel (see materials and methods). Lane 2: pMS25; lane 3: pMS25Δ1419; lane 4: pMS25Δ1432; lane 5: pMS4; lane 6: pMS24; lane 7: pMS23Sac^{-}. Replicase production in pMS25 is arbitrarily defined as 1.

in pMS24. Evidently much less is made than in pMS4 where the Min Jou-sequence is absent. It appears therefore that ribosomal movement over the Min Jou-sequence does not expose the replicase start to its full potential. This seems plausible since the duplex structure may reform after every ribosome passage. If this were true, increased coat cistron translation would lead to elevated replicase levels. For this purpose pMS23Sac^{-} was constructed. Although we cannot accurately measure the relative yields of the coat protein fragments made in pMS24 and pMS23Sac^{-}, due to their apparent instability and small size, we know from our previous

work that the corresponding parent plasmids synthesize coat protein at different rates due to their differing 5' cut-off points (24). Consistent with these data, the rate of replicase synthesis in pMS23Sac$^-$ is about 2 times that of pMS24 (Fig. 4, lanes 7 and 6). Thus the frequency of translation across the Min Jou-sequence affects the rate of replicase synthesis. Further experiments are needed to determine if the two parameters are proportional.

DISCUSSION

In the present study we have further analyzed the molecular mechanism that makes replicase synthesis dependent on coat cistron translation. We have found that deletions in the sequence between 1419 and 1432 abolish the coupling. This result is exactly what is predicted by the model of Min Jou et al. (1). The alternative explanation for the translational polarity of coat protein amber mutations is based on the close proximity in space of the coat protein and replicase ribosomal binding sites (14). It was proposed that occupancy of one site (the stronger coat cistron) precludes ribosome binding to the other. Our finding that deleting the start of the coat cistron (pMS25 to pMS25Δ1419) barely influences replicase synthesis is at odds with this model. In their recent electron microscopy study, Jacobson et al. do not detect any long range RNA-RNA interaction involving the start region of the replicase cistron. Their results offer no explanation for the polarity phenomenon (25).

In the collection of clones used in this study four levels of replicase can be distinguished, three of which also occur during (mutant) phage infection. In pMS23 repression of replicase is virtually complete due to the presence of the coat protein (Fig. 3, lane 2). This situation mimics that of a wild type phage infection where only very small amounts of replicase are allowed to accumulate. The next level is that of pMS25 where repression only occurs through the Min Jou complementarity. This can be compared to infections with early coat ambers (sus3); control is still tight but a low level is reached (Fig. 3, lane 4). It is known that phage RNA replication in the sus3 mutant is retarded but not absent. The third level is obtained in pMS24 and pMS23Sac$^-$. The Min Jou-sequence is translated allowing a further increased rate of replicase synthesis. This situation reflects that occurring in infections with late coat ambers, where relative overproduction of the replicase takes place due to the absence of an active repressor. Finally the highest level is reached in pMS4 and pMS25Δ1432 where the Min Jou-sequence is absent. This situation

has no analogy with phage infection as far as we know. Nevertheless, the results obtained with pMS4 tell us that the replicase start is used in a suboptimal way in vivo. In this respect it may be noted that replicase is a very toxic product to the bacterial cell . Induction of pMS4 for a few minutes is survived by only 0,1% of the cells (results not shown, see also reference 26). Such a detrimental effect on bacterial growth was also reported for infection of a late coat amber in a Su^--host (27); a situation that like pMS24 and pMS23Sac^- will give rise to a high level of replicase.

We have measured the 4 replicase levels discussed by labelling cells with ^{14}C-aminoacids and have determined the following ratio ; pMS23:pMS25:pMS24:pMS4 = ~ 0:1:2:9.

Translational polarity was first discovered in the RNA phages, but has now also been described for the trp, the gal and some ribosomal protein operons (28-32). The molecular mechanisms underlying these couplings have not yet been described, although, the close proximity of stop and start codons of some coupled genes has suggested a reinitiation event involving a single ribosome. However, the phage MS2 system shows that the coupling mechanism between genes is not necessarily related to the site of upstream translation termination. Recently translational coupling between the T7 genes 13 and 14 was studied. There is a similarity with the MS2 phage system described here in the sense that progressing deletions from the 5' side of the upstream gene 13 result in uncoupled translation of gene 14 (33).

A further feature of the RNA phage is that replicase as well as lysis protein synthesis are under control of coat cistron translation. As we have seen replicase coupling involves the Min Jou-sequence. Translation across the Min Jou-sequence is however not sufficient to activate the lysis gene start . For this to occur ribosomes must travel much further on the messenger. The "coupling point" has not been identified in detail yet, but is most likely located beyond position 1628 since coat ambers in this position fail to lyse the host cell.

It appears then that the intact MS2 RNA molecule initially has only one ribosome entry site, that ultimately services three cistrons. (Expression of the A protein is assumed to take place only on nascent chains (44)). The reason for having such a single control point is probably to facilitate the conversion of phage RNA from messenger RNA to template for replication by having the incoming ribosome and the replicase protein compete for the

same binding site as suggested by Kolakofsky and Weissmann (34). A single ribosome entry site, that is also being recognized by a repressor molecule, also seems the solution to obtain control over the polycistronic ribosomal protein transcripts (32).

The existence of long range RNA-RNA interactions has been accepted for tRNA, tRNA-like structures at the 3' termini of some plant viral RNA's, ribosomal RNA, and for splicing eukaryotic mRNA (35-38). Such interactions have not been expected to play a role in regulating expression in prokaryotic mRNA. A few examples have been described however. The translation of the T7 genes 1.1 and 1.2 is controlled by RNase III. A region complementary to the ribosome-binding site of the upstream gene 1.1 is present just downstream of gene 1.2 as part of a local hairpin structure. Once this region is released from the hairpin by RNase III cleavage it can block translational starts at gene 1.1 and indirectly the expression of the translationally coupled gene 1.2 (39).

Repression by long range RNA-RNA complementarity is also supposed to play a role in the expression of the trp leader peptide gene (40). A region between the leader peptide gene and the trpE gene shows reasonable complementarity to the ribosome binding site of the leader peptide gene. Deletion of this sequence results in a 10 fold increase in leader peptide synthesis. Structural studies on trp mRNA were consistent with the proposed interaction (41).

Finally, translational control by complementary RNA molecules can block initiation regions by intermolecular basepairing (42,43).

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