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Unusual ribosome binding properties of mRNA encoding bacteriophage λ repressor

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ABSTRACT

The mRNA encoding repressor cI of phage λ is the only known *E.coli* message which starts directly with the initiation AUG codon. The ability of *in vitro* synthesized cI mRNA fragments (150 or 400 nts) to form ternary initiation complexes has been studied using the toeprint method. In the presence of $tRNA^{Met}_f$, these fragments are capable of forming the ternary complexes at the 5′-terminal AUG codon not only with 30S subunits but also with undissociated 70S ribosomes (70S tight couples). In the latter case, no binding at other positions of cI mRNA can be detected at all. The starting region of cI mRNA has a single stranded conformation and is highly enriched in A-residues. This feature of cI mRNA RBS is suggested to be the main factor which allows cI mRNA to form the initiation complex with the ribosome. Unlike 30S subunits, the binding to 70S tight couples is not affected by any of the initiation factors, although it is as efficient as that to 30S subunits supplemented with the factors. 30S subunits prefer to associate with the internal RBSs of the preformed mRNA molecules, provided that they are not sequestered by the secondary structure. In contrast, 70S tight couples tend to avoid extra sequences upstream of the codon directed to the P site and occupy a position as close as possible to the 5′-end of the message. This has been found to be the case both for $tRNA^{Met}_f$ and for elongator $tRNA^{Glu}_2$. The structural features of mRNA RBSs which influence their different binding for 30S subunits and 70S ribosomes are discussed.

INTRODUCTION

There is general agreement that interaction of prokaryotic mRNAs with ribosomes requires, as a prerequisite, dissociation of the latter into 30S and 50S subunits promoted by initiation factor 3 (IF-3). Subsequently, a small ribosomal subunit binds to the mRNA initiation region (ribosomal binding site, RBS) which encompasses at least 20 nucleotide residues upstream of the initiation codon and at least 15 residues downstream [1–4]. The recognition of mRNA initiation regions is provided by the presence of special signals in their sequences which are situated, as a rule, upstream of the initiation triplet [5–8]. Thus, the 5′-proximal part of the RBS is usually regarded as a site of primary recognition of the message by the ribosome.

The initiation signals may play a dual role. On the one hand, they participate in the selection of RBSs, whereas on the other hand, they increase the affinity of the RBS for the 30S subunit, thereby promoting the process of mRNA accommodation in the mRNA binding site of the ribosome.

Much less is known about the role of the coding region of the RBS in the mRNA-ribosome recognition process. The sequence of its first nucleotides, up to position +15, has been shown to deviate from random [9] and has a bias towards A-residues [10]. The sequence downstream from the start codon for many mRNAs has been found to be complementary to 16S RNA and proposed to be an additional initiation signal for ribosome binding [11,12]. However, this hypothesis has not so far obtained any experimental support.

Taking the above considerations into account, the question arises as to what determines the ability of mRNAs devoid of the 5′-terminal part of the RBS to bind ribosomes. Such mRNAs are known in halophilic microorganisms and mitochondria [13–19]. In *E.coli*, there is only one example of an mRNA starting with the initiation AUG codon; this is the cI mRNA, transcribed from a weak promotor of the λ phage genome (P_{rm}) and encoding λ repressor [20].

Here we have studied the formation of ternary initiation complexes with natural mRNAs by means of the 'toeprint' analysis. This approach [3] is based on the property of the reverse transcription reaction, primed at a distant part of mRNA, to stop at the point where the enzyme reaches the site of contact between the ribosome and the mRNA. The inhibition of reverse transcription of mRNA complexed with the 70S ribosome or 30S subunit occurs strictly at position +16, where +1 is assigned to the first nucleotide of the programmed nucleotide triplet [21]. In the absence of tRNA, reverse transcription is not inhibited. Thus, this method proves to be very suitable for analyzing the mRNA sites recognized by ribosomes and for estimating their potential to form initiation ternary complexes.

Translation initiation regions of two mRNAs, namely the cro and cI mRNAs of the λ phage, have been investigated in this

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paper by the toeprint assay. Both of these mRNAs begin with AUG sequences. The 5'-terminal AUG of the cro mRNA is closely followed by a termination codon and hence may have no functional significance; the actual AUG initiator codon lies further downstream. In both cases, the 5'-terminal AUG codon has been found to determine the ability of these mRNAs to bind undissociated 70S-t ribosomes (70S 'tight couples'). In contrast to 30S subunits, 70S-t ribosomes turned out to be unable to recognize the internal initiation regions of mRNA. Accordingly, we have analyzed the structural features of mRNA regions which determine their difference in binding 30S subunits and 70S ribosomes.

MATERIALS AND METHODS

Ribosomes and initiation factors

30S ribosomal subunits of *Escherichia coli* MRE 600 were isolated as described previously [22]. 70S 'tight couples' were prepared according to Makhno et al [23]. Prior to use, ribosomal subunits were activated by heating in a buffer containing 20 mM Tris/HCl pH 7.4, 20 mM $MgCl_2$, 200 mM NH_4Cl, 2 mM 2-mercaptoethanol for 10–15 min at 40°C, followed by cooling on ice. An equal volume of buffer containing 80 mM K·HEPES pH 7.8, 60 mM KCl, 6 mM 2-mercaptoethanol was added. The subunits were diluted with 80 mM K·HEPES pH 7.8, 10 mM $MgCl_2$, 60 mM KCl, 6 mM 2-mercaptoethanol.

E.coli initiator factors were a kind gift from C.Gualerzi. In all experiments with factors, 30S ribosomal subunits isolated according Makhno et al [23] were used.

tRNA

E.coli $tRNA^{Met}_f$ (Biolar, Latvia), $tRNA^{Phe}$ (Boehringer Mannheim) and $tRNA^{Glu}_2$ (Sigma) were dissolved in the incubation buffer to a concentration of 100 μM.

Enzymes

AMV-reverse transcriptase and human placenta RNase inhibitor were obtained from Omutninsk (USSR). Taq-polymerase, terminal nucleotidyl transferase and T4 polynucleotide kinase were purchased from Fermentas (Lithvenia). RNase A was from Sigma, restriction endonucleases *Acc* I and *Taq* I were from Boehringer Mannheim. T7 RNA polymerase was prepared from the superproducing strain of *E.coli* (containing pAR1219) according to Davanloo et al [24].

DNA templates and oligodeoxynucleotides

Oligodeoxynucleotides were synthesized by the phosphoramidate technique on a polymeric support with an Applied Biosystems 380B DNA Synthesizer. Their sequences and annealing sites are shown in Table 1.

The pMM52 plasmid with the cloned *Hind*III-*Bgl*II fragment of λ DNA was described earlier [25]. It contains the intact region of the promotors P_R and P_{RM} and the starting sequences of the cro (22 codons) and cI (130codons) genes.

The pAT7 plasmid pBR derivative containing the *Hind* II fragment of λ DNA with P_{RM} promotor, O_R3 operator and full cI gene was a gift from Dr. A.Zolotuchin (Moscow Univ.)

The *Taq* I fragment encompassing the whole lambda specific region from pMM52 was ligated with M13 mp8 DNA cut by *Acc* I restriction endonuclease. A single-stranded DNA from M13 mp8-52 with P_{RM} and P_{lac} promoters in the opposite orientation was used for the subsequent polymerase chain reaction (PCR). For PCR, the reverse M13 primer (Pr II) and one of the primers (Pr V–Pr VIII) encompassing the start of the cI coding region and the T7 promoter sequence were used.

Isolation of mRNAs from *E.coli* cells

Cro-mini-mRNA was isolated from *E.coli* strain PR13 transformed by the plasmid pMM52 as indicated by Balakin et al [26]. Total mRNA, containing cI mRNA, was isolated from *E.coli* cells XL-1 transformed by plasmid pAT7 according to Jinks-Robertson et al [27]. In some experiments, cI mRNA was partially purified from the total RNA by centrifugation in 5–20% sucrose gradients in a Beckman SW 27 rotor for 17 hrs at 25000 rpm, +4°C.

T7 transcription

With the exception of the 5'-terminal cI mRNA fragments starting with the AUG codon, all other mRNAs were prepared in the T7 polymerase system according to Evstafieva et al [28]. The transcripts beginning with an A residue were prepared in incubation mixtures with the final concentration of GTP reduced to 50 μM, to avoid a non-template initiation of T7 transcription with a G residue. That the transcripts obtained under these conditions did indeed contain the AUG triplet at their 5'-end was checked by their sequencing with addition of terminal nucleotidyl transferase during chase reactions as described in Agranovsky et el [29]. All transcripts were purified by electrophoresis in 4% polyacrylamide gel containing 7 M urea.

Sequencing of RNA

Synthetic oligodeoxynucleotides (Pr I, Pr III and Pr IV) were phosphorylated at their 5'-ends usind T4 polynucleotide kinase and [γ-^{32}P]ATP. Dideoxynucleotide sequencing of RNAs with AMV reverse transcriptase was carried out as described in Barta et al [30], Shelness and Williams [31].

Table 1. Description of oligodeoxynucleotides

Primer	Sequence	Remarks
Pr I	CGAGGTGAATTTCGACCTCT	complementary to mini-cro-mRNA, from 98 to 117
Pr II	AACAGCTATGACCATG	reverse M13 primer.
Pr III	AGCACCACGCCTGACTGC	complementary to c1 mRNA, from 178 to 196
Pr IV	GCAATGCGGCGTTATAAGC	complementary to c1 mRNA, from 132 to 150
Pr V	TAATACGACTCACTATA<u>ATGAGCACAAAAAAGAAACC</u>	T7 promoter fused to the c1 sequence from 1 to 20(underlined)
Pr VI	TAATACGACTCACTATAG<u>ATGAGCACAAAAAAGAAACC</u>	The same as Pr V with additional G between T7 promoter and c1 RNA sequence
Pr VII	TAATACGACTCACTATAGAA<u>ATGAGCACAAAAAAGAAACC</u>	The same as Pr V with additional GAA between T7 promoter and c1 RNA sequence
Pr VIII	TAATACGACTCACTATAGAAAAA<u>ATGAGCACAAAAAAGAAACC</u>	The same as Pr V with additional GAAAAA between T7 promoter and c1 RNA sequence

Reverse transcriptase inhibition analysis

Reverse transcriptase inhibition analysis (toeprinting) was performed as described by Hartz et al [3], with some modifications. The final reaction mixture with a volume of 10 microliters contained 60 nM cro-mini-mRNA or approximately the same concentration of cI mRNA derivatives, 0.4−0.5 μM ribosomes or subunits, 8−10 μM tRNA, 6 units RNasine, 2 units AMV reverse transcriptase in 80 mM K·HEPES pH 7.8, 60 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 0.4 mM of each NTP·Mg^{2+}. After stoping the reaction, RNase A was added to a final concentration of 0.2 mg/ml. The mixture was left at room temperature for 10−20 min, followed by dilution with a buffer containing 0.5 M NH_4Ac, 1 mM EDTA, 0.5% SDS. After extraction with phenol:chloroform (1:1 v/v) and precipitation with ethanol the dried pellets were dissolved in the formamide loading mixture and electrophoresed in an 8% sequencing gel.

RESULTS

Identification of the binding sites on cro mRNA in its complexes with 30S subunits or 70S-t ribosomes in the presence of $tRNA^{Met}_f$.

The initiation AUG codon of the mRNA encoding the Cro protein of bacteriophage λ is located at a position 19 nucleotide residues downstream from its 5′-end (see Fig.1). As is characteristic for the majority of prokaryotic mRNAs, this codon is preceded by the Shine−Dalgarno (SD) sequence. Earlier, we characterized in detail some properties of artificial mini-mRNA molecules containing the 5′-end of the cro gene [21,26]. In particular, with the use of toeprint analysis, it was shown that the 30S subunit in the presence of $tRNA^{Met}_f$ is able to recognize the authentic initiation codon among the several AUG triplets which are found in the cro mRNA initiation region. Only with large concentrations of $tRNA^{Met}_f$, could the attachment of 30S subunits to other AUG triplets be observed [21].

As shown in Figure 1, 70S tight couples, which are known to dissociate only at low magnesium concentrations [32], also interact with cro-mini-mRNA, initiation factors being dispensable for this binding. In this case, recognition also occurs at a single AUG triplet, but this triplet occupies the position at the extreme 5′-end of cro mRNA.

On the other hand, 30S subunits derived from this 70S-t ribosome preparations recognize the authentic initiation codon and, therefore, do not differ qualitatively in this respect from other preparations of small ribosomal subunits used in our previous experiments.

Binding of the λ repressor mRNA to 30S subunits or 70S-t ribosomes in the presence of initiator tRNA

The phage λ repressor mRNA transcribed under lysogenic conditions from the P_{RM} promotor uses the 5′-terminal AUG triplet as an authentic initiation codon. In contrast, the 5′-terminal triplet of cro mRNA can only potentially direct the synthesis of a dipeptide.

At the initial stage of this work, the total cellular mRNA was isolated from cells transformed by a plasmid containing the cI gene under control of the P_{RM} promotor with the O_3R operator region. As follows from Figure 2 it turns out that the content of cI mRNA in this preparation is sufficient not just to detect

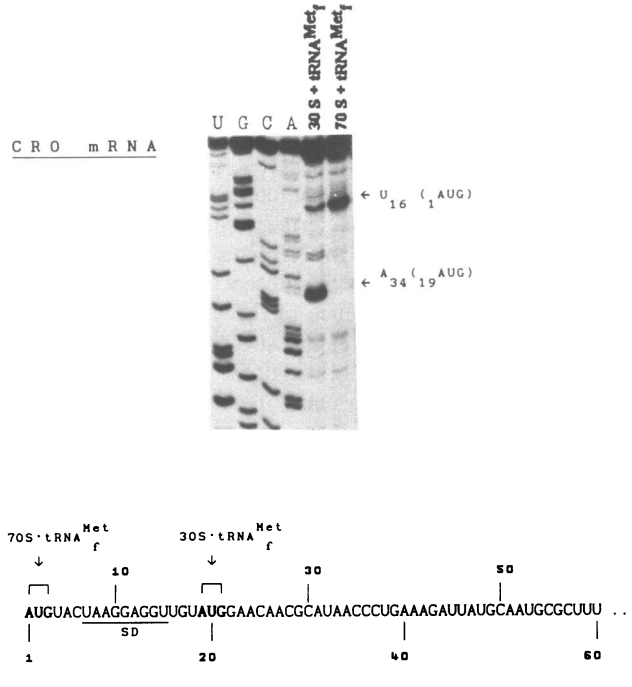

Figure 1. Detection of ternary complexes with cro-mini-mRNA (*in vivo* transcript of pMM 52) with undissociated 70S ribosomes and 30S subunits derived from them. The 5′-terminal part of mini-mRNA is identical to that of phage λ cro mRNA. Arrows with nucleotide positions mark toeprint bands on the autoradiograph. The corresponding codons recognized by $tRNA^{Met}_f$ are indicated in parentheses on the autoradiograph and by brackets in the cro mRNA sequence shown below. The SD sequence is underlined. Lanes U, G, C, and A are sequencing products generated in the presence of dideoxy-5′-triphosphates.

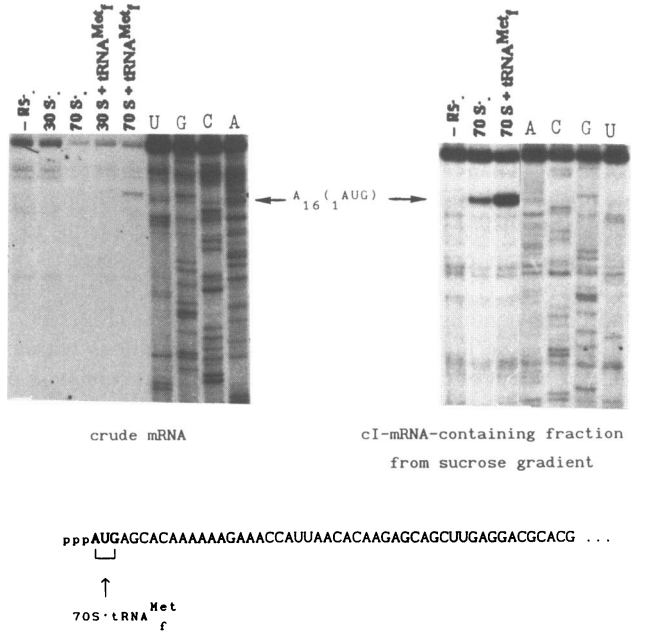

Figure 2. Detection of ternary complexes with cI mRNA using the total RNA from pAT7 transformed cells (left) and the RNA from fractions containing cI mRNA after sucrose gradient separation (right). The oligonucleotide complementary to the region 178−196 (Pr III) of cI mRNA was used for toeprinting.

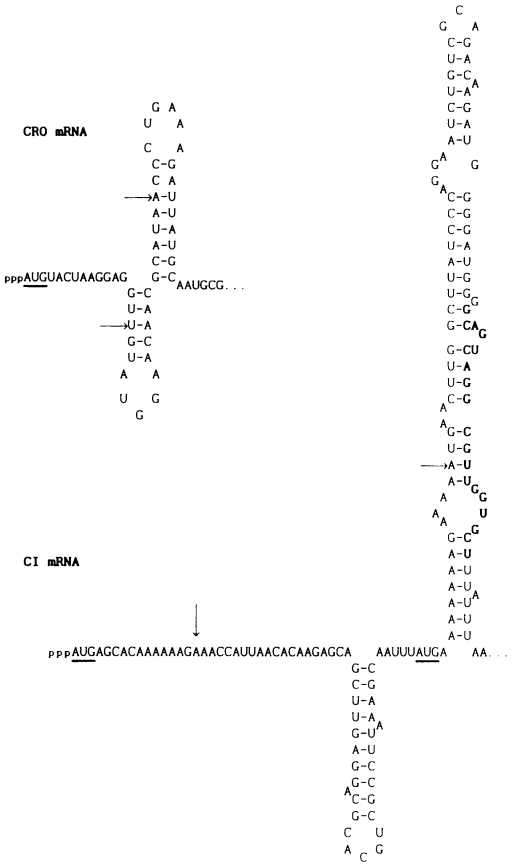

Figure 3. Secondary structures within the leader regions of cro mRNA (experimentally supported, see [26]) and cI mRNA (computer prediction based on the algorithm of Zuker and Stiegler [33]). Arrows show the toeprint positions corresponding to initiation at the underlined AUG triplets. One of two primer binding sites in cI mRNA (Pr IV) is given in bold type.

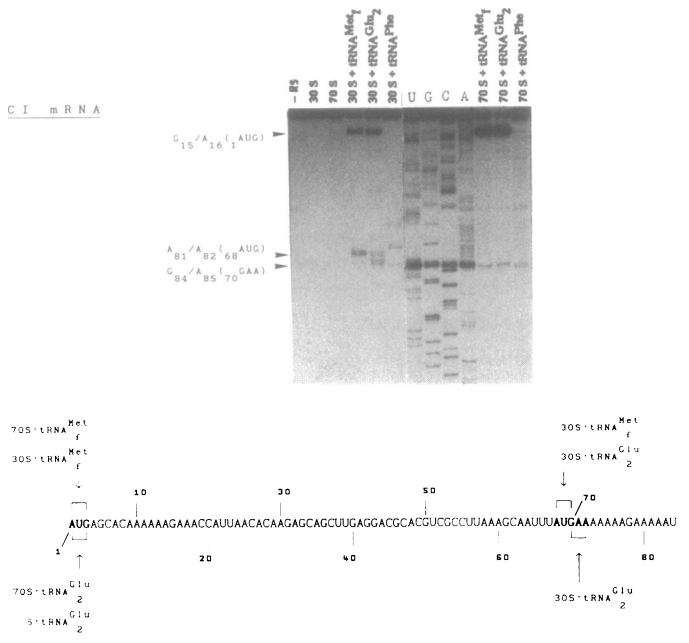

Figure 4. Comparison of ternary complex formation using the 400 nucleotide long 5′-terminal fragment of cI mRNA with 30S and 70S ribosomes directed by the initiator $tRNA^{Met}_f$ and uncharged elongator $tRNA^{Glu}_2$ and $tRNA^{Phe}$. The mRNA fragment was prepared *in vitro* with T7 RNA polymerase. The oligonucleotide complementary to the region 178−196 of cI mRNA (Pr III) was used for toeprinting.

its reverse transcription product using a 5′-labelled oligodeoxyribonucleotide as primer, but also to determine its sequence by the Sanger method.

Moreover, when 70S-t ribosomes and $tRNA^{Met}_f$ were added to this mRNA, annealed with a primer Pr III complementary to position 178−196, a band was observed corresponding to a reverse transcription stop at position +16 with respect to the initiation codon (Fig.2A). In contrast, 30S subunits gave only a band corresponding to the full-length transcript (Fig.2A).

In further experiments, to obtain RNA preparations with an increased content of cI mRNA, we performed a fractionation of the total RNA by centrifugation in a sucrose gradient followed by analysis of each fraction by primer extension. The fractions thus shown to be enriched in cI mRNA occupied the region in the optical density profile between the peak of 16S RNA and that of low-molecular RNAs, with a partial overlapping the latter.

RNA from fractions with the highest content of cI mRNA was analyzed in the translation initiation system by toeprinting. Figure 2 (right) demonstrates the formation of complexes containing cI mRNA, 70S-t ribosome and $tRNA^{Met}_f$. It is of interest that the appearance of the '+16' band was also observed with no addition of $tRNA^{Met}_f$ to the system. There is little doubt that this is due to the presence of endogenous tRNAs in our RNA preparations. A similar phenomenon has been described by Gold and co-workers [3]. However, these authors observed several bands, the most intensive of which corresponded to the initiation complex with $tRNA^{Met}_f$, whereas the others resulted from elongator tRNAs bound to their own codon in the ribosomal P site at positions close to the authentic AUG codon.

It should be noted that when using the same preparations of 70S-t ribosomes, their interaction with cI mRNA occurs much more efficiently than with cro mRNA. Even those preparations of 70S-t ribosomes which do not interact with cro-mini-mRNA at all are able to form the ternary complex 70S*$tRNA^{Met}_f$* mRNA with up to 70% of cI mRNA (data not shown). The reason for kind type of difference is the presence of secondary structure in cro mRNA and the absence of the latter in the 5′-terminal part of cI mRNA. The secondary structures presented in Fig.3 were derived by chemical modification studies [26] for cro-mini-mRNA, and by calculation using the Zuker program [33] for cI mRNA.

Comparison of features of cI mRNA interaction with 30S subunits and 70S-t ribosomes

Total cellular RNA does not appear to be a suitable substrate for studying the properties of the interaction of cI mRNA with 30S ribosomal subunits, because cellular mRNAs with SD-sequences would bind to 30S subunits more efficiently than cI mRNA which lacks such a sequence. The competition between c1 mRNA with other cellular templates appears to account for the absence of toeprint signal in the experiments shown in Figure 2 (left). Accordingly, 5′-terminal fragments of individual cI mRNA were prepared. They were transcribed from cDNA fragments obtained by PCR techniques and contained the T7-promoter and the first 400 or 150 bp of the cI mRNA coding region.

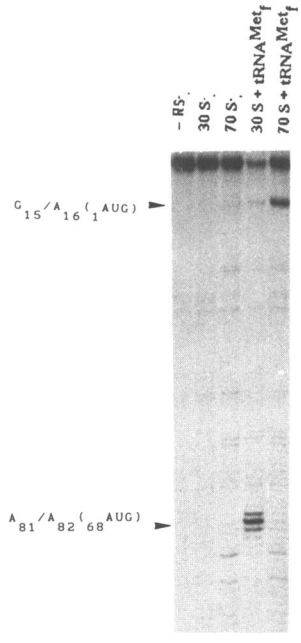

Figure 5. Detection of ternary complexes with the 150 nucleotide long 5′-terminal fragment of cI mRNA with 30S and 70S ribosomes and $tRNA^{Met}_f$. The oligonucleotide complementary to the region 132 – 150 of cI mRNA (Pr IV) was used for toeprinting.

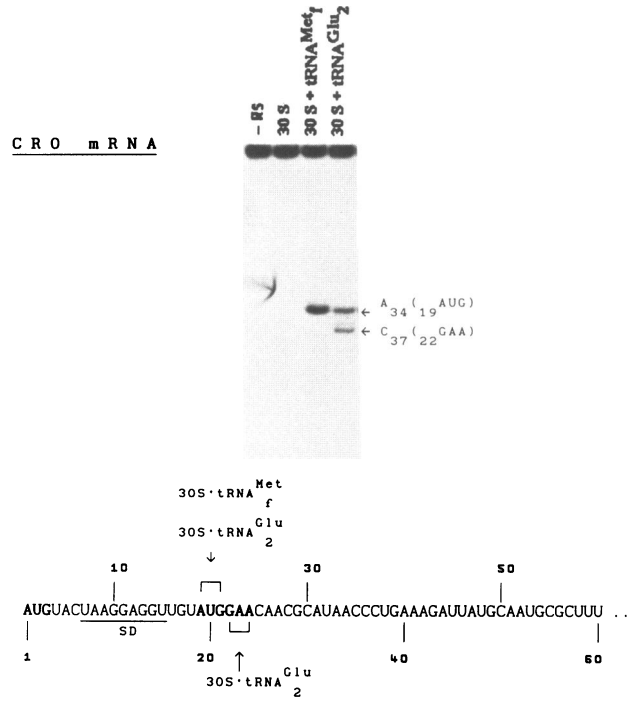

Figure 6. Codon selection properties of $tRNA^{Glu}_2$ in ternary complex formation with cro-mini-mRNA.

Table 2. Influence of initiation factors on ternary complex formation with cI mRNA and fMet-$tRNA^{Met}_f$ (% mRNA in ternary complex*)

	-IFs	IF-2	IF-3	IF-1 IF-2	IF-1 IF-3	IF-2 IF-3	IF-1 IF-2 IF-3
30S	34	61	36	62	42	67	73
70S-t	62	56	58	58	63	58	60

* 1 pmole of each factors was added to 3 pmol of ribosomes in the 'toeprinting' buffer (see Materials and Methods) and the mixture (6μl) was preincubated for 5 min at 37°C. It was then used in reverse transcription inhibition analysis. The amoumts of bound and unbound cI mRNA were quantified by densitometrical scanning of the toeprint autoradiographs.

In the presence of $tRNA^{Met}_f$, the binding of cI mRNA both to 30S subunits and 70S-t ribosomes occurred mainly at the 5′-terminal AUG codon. This is clearly demonstrated by experiments presented in Fig.4. However, under identical conditions, the fraction of mRNA complexed with 30S subunits was much lower than that with 70S-t ribosomes. A quantitative comparison of the data is presented in Table 2.

The toeprinting assay revealed an additional site of 30S binding at the AUG codon starting at position A_{68} (Fig.4). This site becomes predominant for the shorter of the two fragments of c1 mRNA used in this work (see Materials and Methods) or when the 5′-proximal primer Pr IV (see Table 1) was used for the toeprint analysis (Fig.5). This is most probably due to an easier accessibility of the corresponding RBS in the shorter fragment as compared to the larger one, where it seems to be masked by the secondary structure. The computer-assisted modelling of the secondary structure for the 5′-terminal part of cI mRNA supports this suggestion (Fig.3), and also explains why annealing of the primer Pr IV with the large fragment results in unmasking of this RBS.

Earlier, Gold and co-workers [3.34] have shown that not only the initiator tRNA but also elongator tRNAs can form ternary complexes within the mRNA initiation region, provided that their respective codons are located not far from the initiation triplet. We decided to use this property of elongator tRNAs to find out whether the 5′-terminal binding of c1 mRNA to undissociated ribosomes is characteristic of the AUG*$tRNA^{Met}_f$ interaction or it is also the case for some elongator tRNAs.

As elongator tRNAs, we used $tRNA^{Glu}_2$ and $tRNA^{Phe}$. The first of these can be able to recognize the GAG and GAA codons which are present in all our constructions where they either overlap the AUG codon or reside very close to it. The second one served as a control for the codon dependence of tRNA binding since there are no phenylalanine specific triplets in the vicinity of the 5′-end of the cI mRNA.

Unexpectedly, $tRNA^{Glu}_2$ was unable to bind to its cognate codon near the 5′-end of cI mRNA. Instead, $tRNA^{Glu}_2$ directed the 5′-terminal AUG codon to the P site , both with 30S subunits and 70S-t ribosomes as it follows from the experiments presented in Fig.4. In the former case, other less intensive bands were also observed in the autographs. They corresponded to the 30S recognition of the internal initiation site, namely at the AUG codon starting at position A_{68} and at the glutamine specific G-AA codon starting at G_{70}.

The observation that $tRNA^{Glu}_2$ is able to direct the AUG codon of mRNA to the ribosomal P site was also found for the authentic initiation codon of cro mRNA. Two types of complex were observed in this case, one with the AUG codon and the other with the adjacent overlapping GAA triplet (Fig.6). Both codons were indeed in the P-site rather than in the A-site, as confirmed by experiments with addition of tetracycline (data not

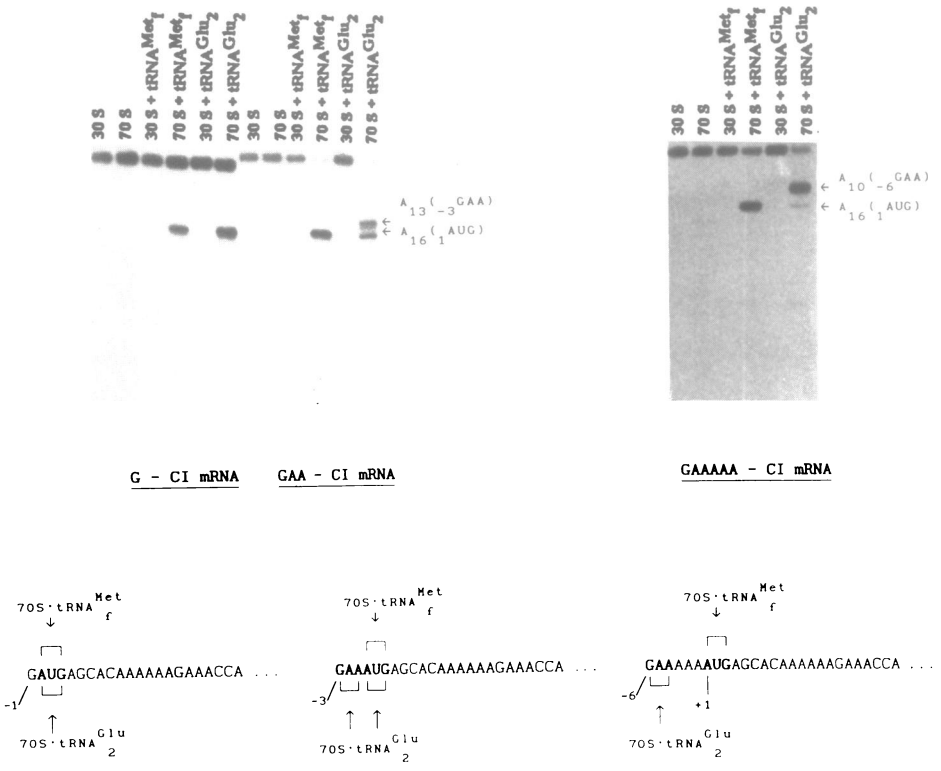

Figure 7. Comparison of the ribosome binding properties of three cI mRNA derivatives in ternary complex formation with two tRNA species. All mRNAs were prepared *in vitro* with T7 RNA polymerase and contained the first 400 nucleotides of phage λ cI mRNA with additional nucleotides at their 5′-ends. The oligonucleotide complementary to the region 132−150 of cI mRNA (Pr IV) was used for toeprinting.

shown) which is known to inhibit tRNA binding to the A-site [35]. In contrast, with cI mRNA, $tRNA^{Glu}_2$ gives only one type of complex, directing the AUG codon to the P site rather than its cognate GAG triplet, which overlaps the AUG. It is known that both GAA and GAG are able to be recognized by $tRNA^{Glu}_2$, although the former is more efficient due to thiolation of the uridine in the tRNA wobble position [36]. As shown in further experiments (see below), $tRNA^{Glu}_2$ can recognize AUG and GAA codons equally efficiently, at least in the absence of competition with the initiator tRNA.

The mechanism of $tRNA^{Glu}_2$ efficient binding to the AUG codon is not clear and deserves special investigations. This mechanism, however, not revealed to these studies. There is no doubt that $tRNA^{Glu}_2$ binds to the ribosomal P site. Therefor, both $tRNA^{Met}_f$ and $tRNA^{Glu}_2$ could be used for the experiments described in the following section.

Ternary complexes formed in the presence of $tRNA^{Phe}$ were observed only with 30S subunits. As seen in Fig.4, they are manifested by a weak band corresponding to the phenylalanine specific codon UUU starting at position U_{65} of cI mRNA.

Ribosome binding properties of cI mRNA derivatives with extra nucleotide sequences at the 5′-end

To analyse the effect of additional nucleotides upstream of the P site directed codon of mRNA, three cI mRNA derivatives containing extra nucleotide sequences at the 5′-end were tested for their binding to 30S subunits and 70S-t ribosomes as indicated in Fig.7. Two of them, GA_2-cI and GA_5-cI had the Glu-specific GAA codon at the 5′-end. The third mRNA contained only a single extra G-residue before the start AUG codon of cI mRNA.

All three mRNAs analogues bound to 30S subunits mainly at their internal regions in the presence of both $tRNA^{Met}_f$ and $tRNA^{Glu}_2$. In the case of 70S-t ribosomes, no difference in the toeprint patterns was found for all three mRNAs in the presence of $tRNA^{Met}_f$. They associated with 70S-t couples at the 5′-proximal AUG codon, in spite of its variable distance from the 5′-end in these mRNAs. However, with $tRNA^{Glu}_2$, the mRNAs bound to 70S-t ribosomes in a different manner. Whereas G-cI mRNA interacted only at the first AUG codon, GA_2-cI mRNA resulted in two complexes, which formed with a similar efficiency. One of these arose from initiation at the 5′-terminal GAA codon and the other resulted from binding at the adjacent AUG triplet. In contrast, GA_5-cI mRNA associated with 70S-t ribosomes in the presence of $tRNA^{Glu}_2$ almost exclusively at the 5′-terminal GAA. Interestingly, for each mRNA, the yield of ternary complex proved to be similar for $tRNA^{Met}_f$ and $tRNA^{Glu}_2$.

Effect of initiation factors on the binding of cI mRNA with ribosomes

Since the process of translation initiation in the cell involves additional protein factors, we considered it necessary to study their influence on the binding of cI mRNA into the ternary initiation complex.

Unexpectedly, IF-3 turned out to be dispensable for the cI mRNA binding to 30S subunits. According to Gold and co-workers [34,37], initiator factor IF-3 promotes the association of natural mRNAs with the ribosome in the presence of $tRNA^{Met}_f$ and aminoacylation of the latter is not essential for its action. Accordingly, we studied this point in more detail and

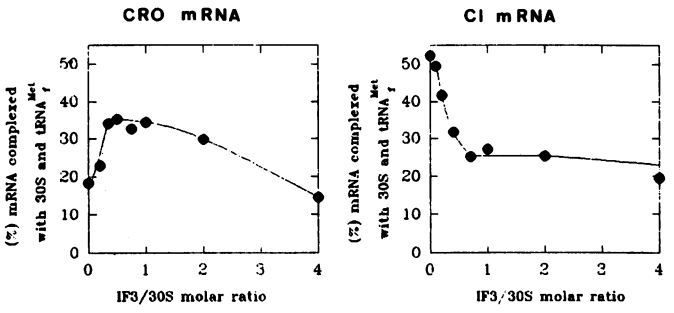

Figure 8. Effect of IF-3 on ternary complex formation with cro-mini-mRNA (left panel) and cI mRNA (right panel) in the presence of $tRNA^{Met}_f$. The amount of mRNA in the ternary complexes was quantified by densitometrical scanning of the toeprint autoradiographs.

compared the effects of IF-3 on cro and cI mRNAs. Unlike cI mRNA, the organization of the initiation region of cro mRNA resembles much more that of the majority of prokaryotic messages.

As seen from Fig.8 where the yield of ternary complex is represented as a function of IF-3 concentration, maximal formation of the complex with cro mRNA is observed at the ratio of 0,3−0,5 mole of IF-3 per mole of 30S subunits. In contrast, the efficiency of ternary complex formation with c1 mRNA decreases after addition of only minimal amounts of IF-3. However, in the combinations with other initiation factors, this effect of IF-3 is not seen. It may be totally compensated by IF-2, whose binding to 30S subunits is promoted by IF-3 [6].

As seen in Table I, the process of ternary complex formation with the participation of cI mRNA and fMet-$tRNA^{Met}_f$ was stimulated by initiation factors only in the case of 30S subunits. Instead of uncharged $tRNA^{Met}_f$, we used here its aminoacylated and formylated form in order to allow the binding of initiator tRNA to IF-2.

However, the same combination of initiation factors added at the same concentrations had no effect on the association of cI mRNA with 70S-t ribosomes in the presence of fMet-$tRNA^{Met}_f$. The amount of complexed mRNA was about 60%, either in the presence or absence of the factors. In the case of 30S subunits, the same level of binding could only be achieved by addition of the initiation factors. Otherwise, only half as much mRNA was found in the ternary complex. As follows from the data presented in Table I, the stimulatory effect can be almost totally attributed to IF-2.

DISCUSSION

The starting point of our investigations was the unexpected finding that the 5′-terminal fragment of cro mRNA was able to bind to ribosomal 70S tight couples in the presence of $tRNA^{Met}_f$, with the binding occurring exclusively at the 5′-terminal AUG rather than at the authentic initiation codon at position +19. In contrast, 30S subunits associated preferably with the cro initiation triplet. This prompted us to investigate the ribosome binding properties of cI repressor mRNA of phage l, for which the 5′-terminal AUG is known to be the authentic initiation codon. We found that this mRNA does indeed bind very well to 70S tight couples at its first AUG triplet, and that initiation factors are dispensable for the interaction. Although we see no reason that would prevent 70S* cI mRNA*fMet-$tRNA^{Met}_f$ complexes from being recruited into further steps of the translational process, we realize that a toeprinting signal should not necessarily mean formation of a productive ternary complex. Special experiments should be designed to demonstrate the ability of λ repressor to be synthesized in the absence of IF-3 both *in vitro* and *in vivo*. At present, on the base of only ribosome binding properties of the c1 mRNA, it is somewhat prematurely to question the old dogma of molecular biology that dissociation of 70S ribosomes into subunits is necessary for initiation complex formation with all natural mRNAs.

30S subunits are also capable of binding cI mRNA at the first AUG codon, provided that the other AUG triplets are sequestered by secondary structure or have a non-optimal nucleotide context. Otherwise, 30S subunits prefer to associate with internal AUG codons as exemplified by their association with the AUG_{68-70} of cI mRNA. This AUG, however, is sequestered by secondary structure in the case of cI mRNA fragments longer than 150 nucleotide residues.

Unlike 70S-t ribosomes, the binding of cI mRNA to 30S subunits is strongly stimulated by IF-2 (when using the aminoacylated form of $tRNA^{Met}_f$) and, probably, by IF-1. However, even in the presence of optimal factor concentrations, the amount of binding never exceeded that found for undissociated 70S ribosomes with no factors at all. IF-3 is dispensable for c1 mRNA binding to 30S subunits, and its addition even inhibited the ternary complex formation. This contrasts the IF-3 requirement for binding of other natural messages [34]. Whether this difference is connected with the absence of the 5′-leader region in cI mRNA RBS or wether some special features of the initiation region are involved remains to be established.

What are the structural features of the cI mRNA initiation region which account for its unusual properties in ribosome binding? A comparison of sequences downstream from the 5′-terminal AUG codon in cro and cI mRNAs allows one common property to be discerned; both mRNAs have a single-stranded conformation. However, cI mRNA binds to 70S ribosomes more strongly than cro mRNA. We speculate that this may be accounted for by a longer single stranded stretch of nucleotides. Moreover, the starting part of cI mRNA coding sequence is highly enriched in A residues and in this respect reveals a striking similarity to the consensus for this region of mRNA RBSs as proposed by Dreyfus [10]. This organization of the c1 mRNA RBS appears to be very important for the formation of stable ternary complexes not only with initiator tRNA but also with some elongator tRNAs. It should be mentioned that similar features of the region downstream from the initiation codon could account for the rather efficient binding of 30S subunits to AUG_{68-70} of cI mRNA which is not preceded by any known initiation signal. The corresponding region turns out to be blocked by the secondary structure in the 400-nucleotide long cI mRNA fragment, whereas it is open in the shorter fragment.

Another important component of the cI mRNA RBS is the 5′-terminal AUG triplet. This codon seems to have a higher intrinsic affinity for the P site of the ribosome as compared to other codons. Otherwise it would be difficult to understand why AUG forces $tRNA^{Glu}_2$ to bind to it at the P site, even when there is a nearby Glu-specific triplet. This contention is supported by the experiments with cro mRNA. Indeed, the binding of $tRNA^{Glu}_2$ to the authentic start site of cro mRNA has been found to be distributed between the initiator AUG codon and the next

cognate GAA triplet. The same is true for the derivative of cI mRNA starting with the sequence GAAAUGAG, but on the other hand no binding of $tRNA^{Glu}_2$ to the weaker GAG codon overlapping the AUG has been observed at all. However, other elongator tRNAs tested (see [34]) do not form ternary complexes with the AUG codon. This leads us to suggest that, for some unknown reasons, $tRNA^{Glu}_2$ is able to form stable complexes with the AUG, although its anticodon cannot form any canonical Watson–Crick pairs in this case. The role of the nucleotide context of AUG in such an interaction is not yet clear.

At all events, whether some elongator tRNAs can fulfill the role of initiator tRNA when interacting with 70S ribosomes in a factor independent way or whether, under natural conditions, they would be always outcompeted by $tRNA^{Met}_f$, no confusion in selection of the correct cI mRNA reading frame has been observed in the presence of all tRNAs in the toeprint assay.

As mentioned above, the 30S subunit prefers to associate with internal initiation regions of mRNA, if they are not sequestered by the secondary structure. This seems to be the case even when there are no apparent initiation signals (e.g. an SD-sequence) upstream of the initiation triplet, as exemplified by the initiation at AUG_{68-70}. Thus, the 5′-part of the RBS does indeed contribute to the stability of mRNA binding to the 30S subunits. In contrast, 70S ribosomes tend to avoid extra sequences upstream of the codon directed to the P site, if these sequences include the same codon or other nucleotide triplets 'acceptable' by a tRNA used in the formation of the ternary initiation complex. Indeed, whereas $tRNA^{Met}_f$ entirely disregards the extra G, GAA or even GAAAAA sequences at the 5′-end of cI mRNA, $tRNA^{Glu}_2$ binds efficiently to the AUG in the AUGAG and GAUGAG context, binds partially in the case of GAAAUGAG and does not bind at all for the cI mRNA derivative starting with G-AAAAAAUGAG. In the latter case, the binding is totally directed to the 5′-terminal Glu-specific GAA codon. We cannot exclude, however, that this '5′-end proximity rule' could be modulated by a specific nucleotide context of the initiation triplet, such as that characteristic for translation initiation in eukaryotic systems.

In our opinion, the strange behavior of 70S-t ribosomes in selection of mRNA initiation regions may be accounted for by an inability for rapid accommodation of the 5′-untranslated leader of the mRNA. This process should also include some unwinding of its secondary structure. According to our model of mRNA topography in the 70S ribosome, the sequence upstream of the initiation codon is situated in a space between the small and large ribosomal subunits [38], and therefore the corresponding part of the 30S mRNA binding site may not be easily accessible for the template. Thus, for a fast primary binding of mRNA, the 70S-t ribosome is forced to use only the initiation codon and the sequence downstream from it, and this is followed by a slower accommodation of the 5′-part of the RBS.

In support of these considerations, it should be noted that the 30S subunit in 70S ribosomes appears to be unable to use the 3′-end of its 16S RNA for 'fishing out' the majority of mRNA initiation regions, since the anti-SD sequence in 70S tight couples is not available for interaction with the SD-sequences of mRNA (Skripkin et al., unpublished observations). Finally, the small ribosomal subunit in association with the large one may be incapable of unwinding the secondary structure of mRNA RBSs, due to the absence of associated initiation factors.

There is general agreement that the mRNA binding site of the ribosome is entirely confined within its small subunit. Thus, the 30S subunit in 70S-t ribosomes may be regarded as a special form of the 30S ribosomal subunit lacking an anti-SD region, whose ability to accommodate mRNA is greatly reduced. It may be not by chance that the behavior of this form of the 30S subunit is reminiscent of that found for the eukaryotic 40S ribosome. As a rule, the latter cannot associate with internal regions of mRNA. At limited concentrations of special accommodating initiation factors, the 40S subunit is able to bind only to single stranded 5′-terminal parts of mRNA, the binding taking place as close as possible to the 5′-end of the message. Moreover, even in the presence of normal amounts of the initiation factors, the binding of the 40S subunit to most cellular mRNAs also occurs near their 5′-ends, since these factors unwind and help to accommodate only the extreme 5′-terminal regions of eukaryotic mRNAs [39,40]. In this connection, our data on the ribosome binding properties of the small ribosomal subunit in its different states may prove to be more relevant to those systems where the translational initiation takes place on preformed mRNA molecules. Like many other prokaryotic mRNAs, translation of cI mRNA is most probably coupled with its transcription (see [41]).

What bearing might our results have on the expression of the λ repressor in the cell? Although 70S tight couples are thought to be a natural and rather abundant form of the ribosome in the prokaryotic cell [42], we have at present no data as to whether they are directly used to initiate translation of some mRNAs *in vivo*. Therefore, we can only speculate on this point. It is conceivable that the ability of cI mRNA to bind directly to 70S ribosomes in the absence of initiation factors gives it an advantage over most of the other 'standard' cellular mRNAs under unfavorable conditions of cell growth. Indeed, under these conditions, the amount of 30S subunits that are supplied with the whole set of initiation factors may be limited. In this case, cI mRNA could avoid competition with cellular mRNAs by initiating λ repressor synthesis directly on 70S ribosomes, thereby maintaining an optimal concentration of the repressor for the lysogenic state of the cell.

Whether some 5′-leaderless mRNAs found in other organisms possess the ribosome binding properties similar to those for the c1 mRNA remains to be established. It is however noteworthy that classical ternary initiation complex formation with authentic mRNAs and small subunits of animal mitochondrial [17–19] or halophilic ribosomes [16] has not been demostrated. It is not difficult to find an analogy between the lysogenic state of λ infected *E.coli* cell and induction of bacteriorhodopsin synthesis under unfavorable conditions of *Halobacterium halobium* growth [43].

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