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Transition of the mRNA sequence downstream from the initiation codon into a single-stranded conformation is strongly promoted by binding of the initiator tRNA

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Using an RNA footprinting technique, accessible sites on the mRNA initiation region bound to the ribosome have been determined. Chemical probing experiments have been done both in the presence and absence of the initiator tRNA with dimethyl sulfate, kethoxal and carbodiimide as reagent probes. As an mRNA, a mini-mRNA containing the initiation region of bacteriophage λ gene cro has been used. This region is characterized by a long single-stranded Shine-Dalgarno (SD) sequence followed by two hairpin structures of which the first one comprises in its loop the initiation codon. As compared to a free mRNA, the only nucleotides additionally protected in the binary mRNA-ribosome complex have been those which belong to the S-D sequence and the initiation codon. The protection of other nucleotides has not changed. Addition of the initiator tRNA results in the modification of nucleotides in the stems of the downstream hairpin structures of the initiation region. This reflects their transition into a single-stranded conformation promoted by tRNA. A possible implication of these findings for the decoding process is discussed.

Introduction

The initiation of translation is a multi-step process which starts with the recognition by the ribosome of a special region of mRNA. The sequence of this initiation region can be arbitrarily subdivided into the part upstream from the initiation codon, the initiation codon itself and the sequence downstream from it. Much attention has been recently focused on the structure of the first two of them which are known to represent the primary binding sites for the ribosome (for references see Ref. 1). Much less information is available on the role of the region downstream from the initiation codon both in binding with the ribosome and in the decoding process. The sequence of this part of the initiation region is, nevertheless, non-random [2] and reveals a high preference for A residues [3]. One may infer, therefore, that it tends to be in a single-stranded conformation, and the secondary structure of this region, when present, has to be destroyed by the ribosome at some step of the initiation of translation, thus preparing the message for the decoding process. The aim of our investigations is to gain some information how ribosomes interact with this part of the initiation region.

Here, we have used a mini-mRNA (166 nt) containing the initiation region of bacteriophage λ gene cro where the downstream nucleotides are involved in the secondary structure. Using chemical probing, we have shown that the transition of the downstream sequence bound to the ribosome into a single-stranded conformation is strongly promoted by the initiator tRNA.

Materials and Methods

T4 polynucleotide kinase (EC 2.7.1.78) was from Pharmacia P-L Biochemicals, tRNA$_{Met}^i$, ddNTPs and dNTPs were obtained from Boehringer Mannheim. AMV reverse transcriptase (EC 2.7.7.49) was obtained from Omutninsk (U.S.S.R.).

30 S and 50 S subunits of E. coli MRE 600 were isolated as described previously [4]. Initiation factor IF-3 was prepared as in [5]. Initiation translation complexes were formed in modification buffer (MB-buffer) 80 mM K-Hepes (pH 7.6), 10 mM MgCl$_2$, 60 mM KCl, 6 mM β-mercaptoethanol as described early for toeprinting experiments, only primer, reverse transcript-
ase and dNTPs were omitted from incubation cocktails containing 0.6 μM mRNA, 2–4 μM ribosomal subunits, 20–40 μM tRNA\textsubscript{Met} and 2–4 μM IF-3 [6].

Cro-mini-mRNA was isolated by using the method [7] with some modifications. \textit{E. coli} PR13 (RNase I\textsuperscript{-}, pnp\textsuperscript{-}) transformed with the plasmid pMM 52 were grown to late log phase. Cro-mini-mRNA was separated by electrophoresis in 8% PAG containing 7 M urea and eluted with 0.5 M ammonium acetate, 1 mM EDTA, 0.5% SDS (GES-buffer) and an equal volume of water saturated phenol. Samples were concentrated on a DE-52 column, precipitated with ethanol, dissolved in water and stored in water at −20°C.

The chemical probing of the mRNA structure [8] was done as follows: 1 μl of DMS/ethanol (1:20, v/v) or 1 μl of 0.54% kethoxal (KE) or 10 μl of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate (CMCT) (42 mg/ml) were added to 10 μl of mRNA or initiation complexes in MB-buffer. Samples were incubated at 37°C for 5 min for DMS and 15–20 min for other two chemicals. Next, 1/4 volume of stop-solution (1.5 M sodium acetate (pH 5.0), 1 M β-mercaptoethanol, 0.1 mM EDTA) was added, mixed and then supplemented with 3 vol. of ethanol. Pellets were resuspended in 50 μl of GES-buffer, extracted with phenol/chloroform (1:1, v/v) and precipitated with 3 vol. of ethanol.

Primer extensions and dideoxynucleotide sequencing of RNA were performed as described by Shelness and Williams [9]. As a primer, a 20-mer, dCGAGGTAAT-TTCGACCTCT, complementary to positions 98–117 of cro-mini-mRNA [10] was used.

Results

The mini-mRNA used in this study contains the initiation region of bacteriophage \textit{λ} gene \textit{cro} and the \textit{ρ}-independent terminator of transcription of \textit{fd} phage [10]. Its secondary structure is shown in Fig. 1. The predicted folding of the mini-mRNA has been confirmed by enzymatic probing (unpublished) and by chemical modification (this paper). The structure of this mRNA has several important features which, taken together, are not found in mRNAs used by other investigators. It starts by the AUG codon which is not functional under natural conditions of protein synthesis followed by a single-stranded sequence encompassing a very long (9 bp) SD-region. The true initiation codon (nt 19–21) is in the loop of the first short hairpin (nt 14–28) that is probably stabilized by a coaxial stacking interaction with the second longer hairpin encompassing positions 29–50. When using toeprint analysis [6], cro-mini-mRNA reveals a normal stop at position +16 from the natural initiation codon (+1 is the A residue of the initiation codon), in agreement with data of Hartz et al. [11]. This stop is not observed at all in the absence of tRNA, though the long SD-sequence of cro-mini-mRNA appears to provide rather stable binary

![Fig. 1. Cro-mini-mRNA secondary structure, summarizing the results of chemical probing experiments (shown by arrows) and enzymatic digestions (solid lines between nucleotides). SD-sequence and AUG-codon are shown by bold symbols.](image-url)
binary complex is unclear and requires further investigations.

Addition of tRNA to the 30S mRNA complex dramatically changes the pattern of chemical modification of bases situated downstream from the initiation codon. One can observe in this case the modification of U32, U38, U45, U46, U48 by CMCT (Fig. 3, lanes 3–4), G43, G49 and, to some extent, G29 by kethoxal (Fig. 3, lanes 3–4) and C30 and A31 by DMS (Fig. 2, lanes 3–5). One should stress that the protection of the SD-sequence in the binary and tertiary complexes is similar (Fig. 2, lanes 2–5 and Fig. 3, lanes 2–4) and, hence, the amount of mRNA complexed with 30S subunits cannot differ appreciably in these cases. Thus, all these data summarized in Fig. 4 give a strong evidence that the helix 29–35/43–50 unwinds in the course of formation of the 30S-mRNA:tRNA complex.

Unfortunately, we cannot judge with certainty about conformational changes within the initiation hairpin mRNA-ribosome complexes (see below). The only possible interpretation of this disagreement is that reverse transcriptase dislodges the 30S subunit from mRNA in the absence of tRNA.

As seen in Fig. 2, lanes 2–5, addition of 30S subunits to cro-mini-mRNA results in a considerable protection from DMS of SD-bases, particularly, that of A9 and A12. The same is true for the extent of modification by CMCT of U20 (Fig. 3, lanes 2–4), the second base of the initiation codon.

The modification of nucleotides downstream from the initiation codon and upstream from SD-sequence does not change for the 30S mRNA complex as compared to a free mRNA. Thus, only the accessibility of SD-sequence and the AUG codon decreases in the course of formation of the binary 30S mini-mRNA complex. Why the initiation codon is protected in the
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SD sequence

1 5
10 15 20
25
30 35 40
45 50

AUGUCAUAAACG GCCUAGUAAUGCAUAAAGCUGAUAAGCUGAAGACUGAAGCAGAUAAUGCUAAG...

---Ribosome-----++++ ++ + + ++ + + + +

30S--------- - - - +++++ ++ + + + + + + + ++ ++

30S+tRNA Met--------- - - + + +++++ +++++ ++ ++ + ++

Fig. 4. Results of chemical probing of the initiation region of cro-mini-mRNA in a free state, in the binary and tertiary complexes with the 30 S subunit. The SD-sequence and the initiation AUG-codon are underlined. + and - denote accessibility of some nucleotide residues for chemical modification by all three reagents.

14–28, since some of the bases of its stem remain to be protected from chemical modification. From logical consideration, however, the conservation of the secondary structure of this sequence seems unlikely, and the protection of some bases may be a consequence of an interaction with ribosomal components.

Addition of 50 S subunits (Fig. 2, lane 5 and Fig. 3, lane 4) or/and factor IF-3 (Fig. 2, lanes 4–5) to the mixture containing mRNA, 30 S subunits and tRNA does not affect the pattern of chemical modification. This suggests that: (1) the mRNA binding centers at least for the downstream part of the RNA, are located only on the 30 S subunit; (2) these centers are not affected appreciably on formation of the 70 S initiation complex.

Discussion

The results presented in this study suggest that an mRNA containing an extended SD-sequence can form a rather stable preinitiation binary complex with the 30 S subunit (without unwinding of the secondary structure of the mRNA initiation region downstream from the AUG initiation codon). Addition of tRNA strongly promotes transition of the nucleotide sequence downstream from the AUG codon into a single-stranded form. This is not necessary to mean that the ribosome in the presence of tRNA actively melts the secondary structure of mRNA. One can also accept the point of view of De Smit and Van Duin [12] that the ribosome fix an unfolded conformation of mRNA that preexists in the population of mRNA molecules. If so, the tRNA in the presence of mRNA actively melts the secondary structure of mRNA. One can also accept the point of view of De Smit and Van Duin [12] that the ribosome fix an unfolded conformation of mRNA that preexists in the population of mRNA molecules. If so, the tRNA just displaces the equilibrium between different conformations of mRNA towards the unfolded state of the polynucleotide chain. However, in contrast to the conception of these authors, in our case, the selection of an extended conformation of the downstream sequence should occur rather when mRNA is already bound to the ribosome by means of its SD-sequence than directly from solution.

That the downstream sequence of the initiation region exists in a single-stranded form in the tertiary complex may be inferred from data of Gold and coworkers [11] and our own results on toeprinting of different mRNAs [6]. In all these cases the only invariant stop at position +16 (starting from the AUG initiation triplet) has been found. However, in our opinion, these data also suggest that at least distal nucleotides of the downstream sequence are fixed in the ribosome and the tRNA promotes this fixation. Indeed, in the absence of tRNA the toeprint signal is either absent or very weak. Therefore, the single-stranded conformation of the downstream part of the initiation region, if it preexists, must be energetically more advantageous per se and not only because it is not able to occlude SD-sequence or the initiation triplet. That would make an additional contribution to the stability of the mRNA-ribosome complex and it is indeed reflected in its consensus sequence [3]. In this connection, it is pertinent to mention one of our findings (manuscript in preparation) where we have succeeded to bind mini-mRNA to undissociated 70 S ribosomes (tight couples) in the presence of tRNA. We have found only one toeprint stop in this case, namely, that originating from the 5'-terminal AUG triplet of mini-mRNA. As seen from Fig. 1, this AUG triplet is followed by a single-stranded downstream sequence.

It is difficult to say now whether only tRNA Met or also other tRNAs promote unwinding of mRNA. Besides, it is not clear what plays the major role in this process: codon-anticodon interaction which may destroy first the hairpin 14–28, then the coaxial stacking of two helices and, finally, the stem of the hairpin 29–50, or some conformational transitions in the P-site of the 30 S subunit or both. The role of all initiation factors in this transition is also to be elucidated. These investigations are now in progress. However, it is tempting to speculate here on the point of a mutual influence of tRNA–mRNA (or tRNA–ribosome) and mRNA–ribosome interactions.

Indeed, if tRNAs affect the conformation of the downstream sequence and its fixation by the ribosome it is logical to suggest an influence in the opposite direction. From this point of view, it is easier to understand some cases described in literature when the secondary structure just downstream from the decoding site affects the decoding process. One can recall, for example, frame-shifting in the translation of Rous
sarcoma virus mRNA where slip-page of tRNAs is dependent on the formation of a hairpin stem-loop structure that is immediately downstream [13] or frameshifting with the genomic RNA of the coronavirus IBV which is dependent on the downstream pseudoknot [14]. Also, a very stable hairpin is present immediately downstream from the jump "take-off" site for the mRNA of phage T4 gene 60 where the ribosome hops over a 50 nucleotide interruption in the coding sequence [15]. In all these cases, the downstream secondary structure is not the only factor that accounts for frameshifting or hopping. However, they may provide some support to the proposed mutual influence of the decoding site of the ribosome and the mRNA structure downstream from it.

As seen from the scheme in Fig. 1, all the bases starting from position 29 are sensitive to modification in the tertiary complex and, hence, are unlikely to be involved in the interaction of the downstream sequence with the ribosome. One can speculate that this interaction is provided by its ribose-phosphate backbone. In this connection, it is pertinent to note data of Murakawa and Nierlich [16] who have found protection of the downstream sequence of the lac Z ribosome binding site mainly from the modification by 1,10-phenanthroline-copper ion oxidative complex. The modification by this reagent is supposed to involve a backbone sugars of the polynucleotides [17]. Nevertheless, many more different initiation regions should be probed before drawing final conclusions.

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