The regulatory region of phage *fr* replicase cistron. III. Initiation activity of specific *fr* RNA fragments

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ABSTRACT

RNA fragments from phage *fr* covering the complete or part of the replicase cistron initiation region have been used as templates in the formation of a ribosomal initiation complex *in vitro*. The results so obtained together with our earlier findings in a similar approach applied to fragments of the structurally related RNA from phage MS2 have allowed us to pinpoint the boundaries of the replicase cistron initiation region on phage RNA. A structural model of the above initiation region has been provided which shows that besides the minimal initiation region (comprises the Shine-Dalgarno sequence and initiator AUG), the flanking regions are also involved and are responsible for additional interactions with the ribosome. The flanking regions possibly contribute to the stability of specific contact between the ribosome and template realized by the minimal initiation region.

INTRODUCTION

Among the structural determinants of prokaryotic mRNAs involved in the formation of the translation initiation signal, the crucial role is ascribed to the initiator codon and the Shine-Dalgarno purine-rich region preceding it (for reviews see [1,2]). It is also known that the secondary and tertiary structure of mRNA strongly affect its template activity, although the precise mechanisms of this action are still obscure.

A comparison of the primary structure of many mRNAs in the region adjacent to the initiator codon reveals a certain homology in addition to the initiator codon and Shine-Dalgarno sequence [3,20,21]. However, it is still unclear whether this formal homology plays any functional role during initiation. In the case of specific fragments from phage MS2 [4,5] and QB [6] RNA, it has been found that when the initiator codon AUG is

preceded by a stretch of 5'-terminal nucleotides of definite length, the sequence downstream from the AUG codon is not necessary to maintain the full template activity by fragments in initiation.

Using MS2 RNA fragments isolated from the initiation site of the replicase cistron, it was possible to demarcate approximately the 5'-terminal boundary for the localization of the initiation signal [5].

The present study describes template activity data obtained during initiation on specific fragments from phage fr RNA whose structure closely resembles, but is not identical to, that of phage MS2 RNA. These fragments, stretching over the entire initiation site of the replicase cistron or a part of it, are a useful addition to the set of homologous fragments from phage MS2 RNA. Their comparative analysis has led us to formulate a structural model for the initiation site of the replicase cistron (R-region) of RNA phages belonging to the first serological group. It has also helped us to gain a further insight into the general principles of recognition by the ribosome of initiation sites on prokaryotic mRNAs.

The isolation of fr R-region fragments and their structural characterization in comparison with the similar data obtained for the appropriate MS2 R-region sequence has been described previously [7,8].

METHODS

The specific fr R-region fragments were obtained by T₁ RNase digestion (816 units/mg RNA, 30 min at 25°) of repressor complex (initial fr coat protein to fr RNA molar ratio = 9) and purified according to the method developed earlier for the specific RNA fragments from phage R17 and MS2 [4,9].

The binding of f [³H]Met-tRNA to ribosomes was determined in a reaction mixture (100 µl) containing 50 mM tris-HCl, pH 7.4, 50 mM NH₄Cl, 5 mM MgCl₂, 1 mM dithiotreitol, 2 mM GTP, 73.5 pmol ribosomes purified on Sephadex G-200, 2.5 A₂₆₀ tRNA containing 22.3 pmol f [³H]Met-tRNA (5.0 Ci/mmol), purified initiation factors: 0.7 µg IF1, 1.0 µg IF2, 0.9 µg IF3 and the appropriate amounts of template. Following incubation at 37°

for 10 min, the reaction mixture was diluted with 1.5 ml of cold buffer (50 mM tris-HCl, pH 7.4, 50 mM NH_4Cl , 5 mM MgCl_2). The solution was immediately filtered through a 24 mm BA85 nitrocellulose membrane filter, the filters were washed with buffer (4 ml) and dried. Filter-bound radioactivity was measured in a toluene-based scintillation fluid.

To assess the binding efficiency of the second aminoacyl-tRNA, f $^{[3\text{H}]}$ Met-tRNA in the reaction mixture was replaced by unlabeled fMet-tRNA. Purified elongation factors EF-T $_U$ ·T $_S$ (52.7 μg) and 64.5 pmol $^{[3\text{H}]}$ Ser-tRNA (5.5 Ci/mmol) or 34.5 pmol $^{[3\text{H}]}$ Arg-tRNA (3.7 Ci/mmol) or 67.2 pmol $^{[3\text{H}]}$ Pro-tRNA (8.0 Ci/mmol) were also added.

A freshly prepared according to [4] fr coat protein at 15-fold molar excess over template was added in initiation repression experiments.

Formation of the 70S initiation complex was studied by centrifugation of the reaction mixture in a sucrose gradient. The $^{[32\text{P}]}$ labeled fr R-region polynucleotides were used as templates. After incubation for 10 min at 37 $^\circ$, the mixture was cooled and loaded onto 10-40% sucrose gradient in a buffer containing 50 mM tris-HCl, pH 7.4, 50 mM NH_4Cl , 5 mM MgCl_2 . Centrifugation was performed at 4 $^\circ$ for 150 min at 50,000 rpm (SW56 Ti rotor, Spinco L2-65B). Gradient fractions (9 drops each) were collected onto double 24 mm Whatman 3 MM paper disks through a hole in the bottom of a centrifuge tube. Disks were dried and placed in a toluene-based scintillation fluid for radioactivity counts.

Initiation factors and RNases-free ribosomes were isolated by Sephadex G-200 gel filtration of unwashed ribosomes in a 1.5 M NH_4Cl containing buffer as described in [10]; initiation factors were purified according to [11-14].

RESULTS

The structure of specific fr RNA fragments

The individual RNA fragments from the initiation site of phage fr replicase cistron (fr R-region) isolated and characterized structurally [7,8] are depicted in Fig.1. The longest of them - fr R(-53 $\rightarrow$ 35) contains 17 terminal nucleotides of

Fig.1 The structure of polynucleotide fr R(-53→35) and its derivatives. The terminator codon in the coat protein cistron and initiation codon in the replicase cistron are boxed, the putative Shine-Dalgarno sequence is underlined. Other potential initiator triplets are shown by a broken line. Nucleotide substitutions in the corresponding MS2 RNA sequence are presented [19].

the coat protein cistron, 36 intercistronic nucleotides and 35 nucleotides from the start of the replicase cistron. The other fragments are shorter derivatives of fr R(-53→35). Structure differences in the appropriate RNA regions, as compared to phage MS2, are indicated by arrows. The most plausible secondary structure model for fr R(-53→35) calculated according to [15] is presented in Fig.2 together with the appropriate model for MS2 R(-53→6) [4]. The melting curves found experimentally for fr R(-53→35), fr R(-23→35) and MS2 R(-53→6) [8] are in good agreement with the calculated models.

Template activity of fr R-region polynucleotides

Since fr R(-53→35) formally contains four potential initiation codons (Fig.1), we had to find out which of them are actually involved in initiation. To this end, EF-T_U-dependent binding to ribosomes of the second aminoacyl-tRNA was analysed under conditions that rule out elongation of translation. Such an approach is expected to give a definite answer, because the first amino acid codons downstream from each potential initiator codon are different: GUG₋₂₄·CCA(Pro); AUG₋₁₁·AGG(Arg); AUG₃·UCA(Ser). As can be seen from Table 1, the only aminoacyl-tRNA effectively bound to the initiation complex is Ser-tRNA. Equimolar attachment of fMet-tRNA and Ser-tRNA also excludes the involvement of the fourth 3'-terminal

Fig.2 Secondary structure models suggested for fr R(-53→35), (top) and MS2 R(-53→6), (bottom). The free energies ΔG (kcal/mol) of hairpins (a) and (b) were calculated following the method described in [15] and the corresponding experimental values [8] in brackets are indicated.

AUG₃₅ triplet in initiation. This is supported, too, by the inability of the fr R(4→35) polynucleotide isolated by us to form the 70S initiation complex (not shown). Consequently, the only initiating triplet in the fr R(-53→35) appears to be

Table 1. The binding of aminoacyl-tRNA by ribosomes programmed by fr R(-53→35) and the repressor activity of the phage fr coat protein

Template	Bound [³ H]aminoacyl-tRNA, pmol			
	f-Met	Ser	Pro	Arg
fr R(-53→35)	6.19	5.89	0.03	0.06
fr R(-53→35) + coat protein	0.45	0.28	-	-

Fig.3 The binding of $f[{}^3\text{H}]\text{Met-tRNA}$ to ribosomes programmed by fr R-region polynucleotides. 1 - Phage fr RNA; 2 - fr R(-53→35); 3 - fr R(-23→35); 4 - fr R(-23→18). Initiation complex was trapped on nitrocellulose filters, the background radioactivity of $f[{}^3\text{H}]\text{Met-tRNA}$ (0.9 pmol) in the absence of template was subtracted.

the native initiator AUG_3 from the replicase cistron. As had been expected, the translational repressor for this cistron - coat protein of phage fr inhibited initiation (Table 1).

The relative template activity of isolated polynucleotides during the formation of the ribosomal initiation complex was assessed in vitro by measuring incorporation of $f[{}^3\text{H}]\text{Met-tRNA}$ into an initiation complex trapped on nitrocellulose membrane filters (Fig.3) and the $f\text{Met-tRNA}$ stimulated template binding to 70S initiation complex (Fig.4). The two methods give comparable results. The formation of the initiation complex using fr R-region polynucleotides was analysed under conditions optimal for the natural templates (see Methods), the native fr RNA serving as a control. Among the polynucleotides from fr R-region, the best template in initiation was fr R(-53→35) (Fig.3,4a), whose activity was comparable to that of the native phage RNA (in the latter case, initiation mainly proceeds at the coat protein cistron). A fragment of fr R(-53→35), shortened at the 5'-terminal end - fr R(-23→35) exhibited a 2-3-fold decrease in activity, but none-the-less, its template activity was quite appreciable (Fig.3,4b). The shortening of fr R(-23→35) at the 3'-end leads to a gradual decrease in activity reaching zero value under the present experimental conditions. While fr R(-23→18) is still capable of producing an initiation complex (Fig.3,4c), fr R(-23→11) and fr R(-23→5)

show hardly any appreciable activity (Fig.4d).

For the initiation complex to be formed, initiation factors are required, the formation of the 70S complex showing a strong dependence on the presence of fMet-tRNA in the incubation mixture (Fig.4). The same holds true for initiation studies with the native mRNA including phage RNAs which supports the vali-

dity of our methods for initiation complex detection.

The binding of [32 P]-labeled fr R-region polynucleotides to 30 S subunits in the presence or absence of fMet-tRNA observed in sucrose gradients appears to be unspecific because it is not dependent on the initiation factors (not shown). A similar artifactual binding of a template to the 30 S subunit has been indicated earlier in the case of MS2 R-polynucleotides [4] and phage R17 RNA [22].

DISCUSSION

The high degree of homology shown by the primary structure of fr R-region and the appropriate region of phage MS2 RNA (Fig.1) allows a direct comparison of template activity in the fr and MS2 polynucleotide series. This comparison is rather plausible since the region of basic structural determinants covering the AUG codon and the Shine-Dalgarno sequence is characterized by almost identical primary and secondary structures.

As can be seen from Fig.5, among the earlier studied MS2 R-region polynucleotides [5] a dramatic drop in activity in initiation is observed from the highly-active MS2 R(-53→6) and MS2 R(-35/33→6) to the practically inactive MS2 R(-21→6) and MS2 R(-17→6). The left boundary of the template region covered by the ribosome in the initiation complex is located somewhere between A₋₂₁ and C₋₁₇ [16], hence to be efficient the template still requires a nucleotide sequence upstream from this boundary, which in the initiation complex is not protected by the ribosome against RNase attacks. The inactive fragments fr R(-23→5) and fr R(-23→11) are structurally related to the previously mentioned MS2 R(-21→6) and MS2 R(-17→6). They carry, apart from minor differences in the terminal nucleotides, three apparently insignificant nucleotide substitutions in positions -17, -6 and +6 (Fig.1) which do not affect either the Shine-Dalgarno sequence or the nucleotides responsible for the secondary structure of hairpin (b) (Fig.2).

The initiation activity of these minimal polynucleotides appears to be restored by their elongation either toward the 5'-end, cf. MS2 R(-21→6) with MS2 R(-35/33→6) and MS2 R(-53→6), or towards the 3'-end, cf. fr R(-23→5) and

Fig.5 Comparison of the structure of R-region polynucleotides from phage fr and MS2 RNA and their template activity in initiation. The relative activities based on sedimentation analysis data are taken from Fig.4 (fr) and Table 2 [5] (MS2).

Fig.6 Structural organization of the replicase cistron initiation site in phages fr and MS2. The minimal initiation site comprising the AUG codon and Shine-Dalgarno sequence (SD) are boxed, the regions involved in additional interaction with the ribosome are shown with a thick solid line, the rest of the template being depicted with a fine solid line.

fr R(-23→11) with fr R(-23→18) and fr R(-23→35), however in the latter case this is not fully achieved (Fig.5).

Virtually identical template activity of MS2 R(-53→3) and MS2 R(-53→50) [5] has led us to conclude that initiation determinants are absent from the template region downstream from the initiator codon. However, the absence of 5'-terminal sequences required for initiation, as noted earlier, may be compensated for, although only partially, by elongation of the template polynucleotide towards the 3'-end.

Consequently, the template region involved in the formation of the initiation complex at the replicase cistron is not limited by the ribosomal binding site only, but exceeds it in length and is characterized by considerable functional heterogeneity. The proper initiator region containing the complete set of structural determinants responsible for the entire translational initiation signal may be subdivided into functionally different regions (Fig.6). Firstly, this is the minimal initiation site with its main determinants, such as the initiation codon and the Shine-Dalgarno sequence. It is located downstream from the left boundary of the ribosomal binding site around A₋₂₁ and stretches to the initiator codon including G₃. The minimal site is apparently responsible for initiation specificity, but does not necessarily provide for the full stability of the initiation complex. In this particular case, for the stable initiation complex to be formed, an additional interaction of the ribosome with the template is required involving the adjacent re-

gions on both sides of the minimal region. The crucial role here is apparently played by the adjacent 5'-terminal region whose left boundary lies about A₃₅ and which is capable of restoring the activity of the minimal initiation site up to 70% of the upper value (cf. MS2 R(-21→6) with MS2 R(-35/33→6) and MS2 R(-53→6)). The 3'-terminal region, involved in additional interaction, only partially makes up for the loss of this 5'-terminal sequence since the activity of fr R(-23→35) constitutes only 40% of that of R(-53→35) (see Fig.5). The righthand boundary of the additional interaction region is probably located near G₁₈ corresponding to the righthand boundary of the ribosomal binding site [16], as the elongation of fr R(-23→18) toward the 3'-end resulting in fr R(-23→35) only slightly enhances template activity (Fig.5).

It can be assumed that the observed additional interaction only serves to enhance the binding of ribosome to the template determined by the main initiation determinants, this process being characterized by low specificity. This is also supported by the fact that the initiation process is unresponsive even to major structural changes in the 5'-terminal region involved in additional interaction. This region in the fr and MS2 R-region polynucleotides shows a considerable variation both in the primary structure and stability of the secondary structure of the hairpin (a) (see Fig.1,2). Moreover, in MS2 R(-35/33→6), in contrast to MS2 R(-53→6) this region is not included in a secondary structure element, however, the two polynucleotides retain comparable activity.

The additional interaction considered here may have a different effect when extended to other cases. For instance, the phage R17 RNA fragment covered by the ribosome during initiation at the A-protein cistron, unlike the corresponding regions of the replicase and coat protein cistron, was active in ribosome binding [17]. In this case, the Shine-Dalgarno sequence of the A-protein cistron exhibits a more pronounced complementarity to the 3'-terminal end of the 16S ribosomal RNA than in other cases. Such a relatively strong interaction itself seems to be sufficient for the formation of a stable initiation complex.

It is reasonable to suggest that the proposed structural

model of the initiation site for the replicase cistron of RNA phages belonging to the first serological group, such as MS2, R17, fr, etc. (Fig.6) cannot be directly extrapolated to other cases without experimental verification or some form of modification. The model presented here is only the first attempt to localize the translational initiation site using a particular template. We have not considered the role of individual determinants of initiation signals, or such important factors as the secondary and tertiary structure of initiation site. It should be emphasized, however, that in the P_{RM} -transcript of λ phage, the translational initiation codon for the cI gene occupies the ultimate site at the 5'-end of the template. Despite the absence of nucleotide sequence to the left of the AUG codon, the template is nevertheless able to initiate and synthesize λ -repressor in vivo, although with low efficiency [18].

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