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A cellular 57 kDa protein binds to two regions of the internal translation initiation site of foot-and-mouth disease virus

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A ribosome-associated 57 kDa protein from rabbit reticulocytes was linked to the internal translation initiation site of foot-and-mouth disease virus by mild UV-irradiation. Binding studies with different RNA fragments revealed that this protein interacts with two distinct sites within the translational control region. One site is located approximately 400 nucleotides upstream from the translational start codon and the second binding site could be confined to 60 nucleotides preceding this codon. Both sequences coincide with hairpin structures at the two opposite ends of a secondary structure model of the internal ribosomal entry site proposed by Pilipenko et al. [(1989) Nucleic Acids Res. 17, 5701–5711].

Translation initiation; Internal initiation; Foot-and-mouth disease virus

1. INTRODUCTION

The translational start site of foot-and-mouth disease virus (FMDV), a member of the family Picornaviridae, is preceded by a long 5′-untranslated region (5′UTR) of 1300 nucleotides containing many unused AUG codons and no cap-structure at the 5′-end [1]. Unlike normal eukaryotic mRNAs, the ribosomes do not scan from the 5′-end of the RNA, but bind most likely directly to an internal entry site, from where they are translocated to the initiator codon [2]. We have shown that the region involved in the regulation of FMDV translation initiation extends 450 nucleotides upstream from the start codon [3] which is in agreement with the findings in other picornaviruses [4–6].

Binding of cellular proteins to multiple sites [7], and interaction of a 52 kDa protein with a specific region of this internal entry site of poliovirus RNA [8] have been reported. Very recently, binding of a 58 kDa protein from mouse ascites cells to the 5′-UTR of encephalomyocarditis virus (EMCV) has been observed upon UV-crosslinking [9].

We demonstrate in this paper that a 57 kDa protein contained in rabbit reticulocyte lysates binds specifically to the internal ribosomal entry site of FMDV. In addition, we determined two binding sites for this protein located at far distant positions within this regulatory region.

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Abbreviations: FMDV, foot-and-mouth disease virus; 5′UTR, 5′-untranslated region

2. MATERIALS AND METHODS

Plasmid pSP449 which contains the complete ribosomal entry site and the first 27 nucleotides of the polyprotein coding region of FMDV is a derivative of plasmid pSP436Δ297–361 [3] and can be transcribed in vitro from an SP6 promoter which is located in front of nucleotide 362 of the FMDV sequence [10]. In plasmid pSP450, which is an analogous construction, initiation of transcription occurs 37 nucleotides further downstream at position 399 of the FMDV sequence. This plasmid is derived from plasmid pSP436Δ348–398 which encodes a mutagenized internal ribosomal entry site with approximately 50% translation efficiency [3]. Plasmid pSP444 contains the FMDV sequence from position 742 to 831 and is derived from plasmid pSP436. This plasmid as well as plasmids pSP436Δ519–559 and pSP436Δ581–672 are also described in [3]. Labeled RNAs were derived from linearized plasmids essentially as described [11] using 10 μM [α-^{32}P]UTP (specific activity 60 Ci/mmol).

Procedures for the preparation of the rabbit reticulocyte lysate and of the ribosomal salt wash (0.5 M KCl) have been described [12,13].

UV-crosslinking of about 10 ng of labeled RNA with 25 μg protein extract (unless otherwise indicated) was performed as described [14] in a total volume of 30 μl in the absence of monovalent cations, but in the presence of 250 μM GTP, 250 μM ATP and 2.5 μg *E. coli* tRNA. Before irradiation for 1 h at 4°C, samples were incubated for 10 min at 25°C. After irradiation the samples were digested with 20 μg of RNase A for 30 min at 37°C and analyzed by SDS-PAGE on 10% gels [15].

3. RESULTS

In order to analyze the participation of cellular factors in the internal ribosome entry to the FMDV RNA, the ability of ^{32}P-labeled RNA of the translation control region to react with different samples of a fractionated rabbit reticulocyte lysate was analyzed by UV-crosslinking. RNA containing the complete ribosomal entry site (position 362–831) binds selectively to a 57 kDa protein (p57) under the assay conditions used, as shown in Fig. 1, lane 1. This protein is ribosome-

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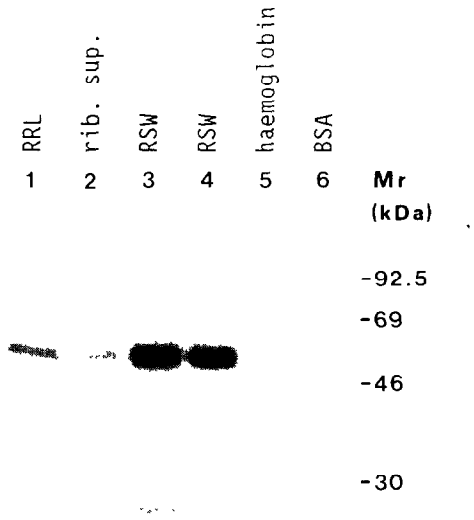

Fig. 1. UV-crosslinking of ^{32}P-labeled RNA from the 5'UTR of FMDV (position 362–831) with: (1) 200 μg protein of a rabbit reticulocyte lysate; (2) 200 μg protein of the post ribosomal supernatant of this lysate; (3) and (4) 25 μg protein each of two independent reticulocyte ribosomal salt wash preparations; (5) 25 μg hemoglobin and (6) 25 μg bovine serum albumin. Samples were analyzed by SDS-PAGE (10% gel) and autoradiography. Molecular masses in kDa are shown on the right.

associated and can be eluted with 0.5 M salt (lanes 3 and 4). As shown in lane 2, not all of p57 fractionates with ribosomes.

Specific labeling of p57 by radioactive RNA from the translational control region was obtained only in the presence of an excess of cold competitor RNA (80 μg/ml of *E. coli* tRNA). No labeled bands were observed using hemoglobin (lane 5) and bovine serum albumin (lane 6) instead of the ribosomal salt wash fraction under the same conditions, or upon addition of proteinase K (not shown), revealing the specificity of this complex and demonstrating in addition that the bands do not derive from unhydrolyzed RNA fragments.

To determine the interaction site(s) of p57 with the RNA within the ribosomal entry site, binding of different subfragments of this sequence (depicted in Fig. 2) to the protein was analyzed. Complex formation can be observed with short RNA fragments starting at position 362 (RNAs 362–434 and 362–527, Fig. 3, lanes 1 and 3), although not as strong as with the complete sequence from position 362 to 831 (lane 5). If transcription initiates 37 nucleotides further downstream at position 399, p57 is not linked to the RNA (RNAs 399–434 and 399–527, lanes 2 and 4). However, when the 5'-deleted RNA contains the complete 3'-sequence of the ribosomal entry site up to the start codon (position 399–831), a protein-RNA complex was formed again (lane 6).

These findings suggest the presence of two binding sites for p57, one between positions 362 and 434 and another in the 3'-part of the translational control region. This view is supported by the fact that several different fragments containing either only the 5'-end (positions 362–668 and 362–742) or only the 3'-end of the region (positions 399–831 and 742–831) bind to p57 with approximately half the strength of the complete sequence (Fig. 4, lanes 3 and 5, or lanes 2 and 7, respec-

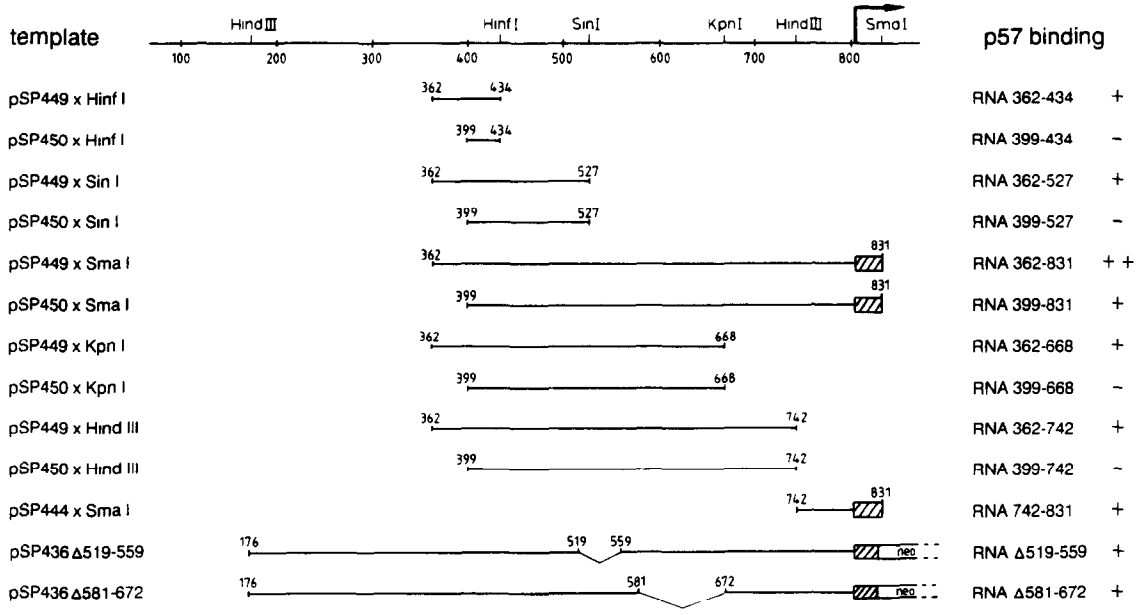

Fig. 2.The top line shows the 5'UTR of FMDV with the translation start site (arrow). Restriction sites for linearization of template DNA are indicated. Numbering of the nucleotides is according to the published FMDV O1K sequence [10]. In the lower part in vitro transcripts of the individual templates (left) are shown. The deleted RNAs Δ519–559 and Δ581–672 are derived from templates linearized downstream from the start codon. On the right, p57 binding of the different RNAs is indicated.

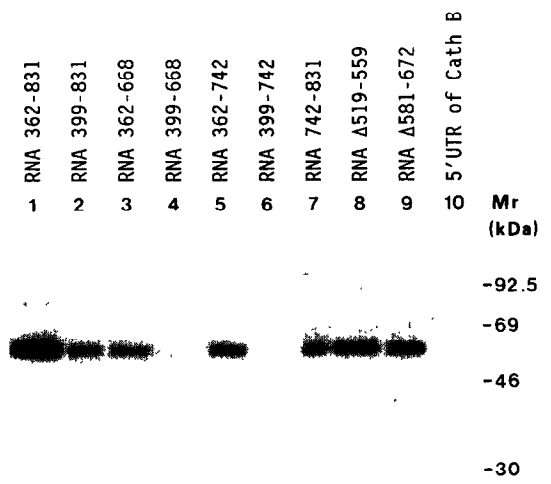

Fig. 3. UV-crosslinking of proteins from the ribosomal salt wash fraction to different transcripts of the FMDV 5'UTR: (1) RNA 362–434; (2) RNA 399–434; (3) RNA 362–527; (4) RNA 399–527; (5) RNA 362–831; (6) RNA 399–831. Molecular masses in kDa are shown on the right.

tively). Binding of p57 to the 3'-region seems not to depend on the presence of the start codon as a fragment from position 742 to 802 thus not containing the AUG codon binds with the same affinity as the fragment from position 742 to 831 (data not shown). In summary, the experiments show that the RNA sequence position 362–399 is necessary and position 362–434 is sufficient for binding of p57 to the first site, and the last 60 nucleotides of the 5'UTR, position 742–802, are necessary and sufficient for binding of p57 to the second site.

The middle part of the ribosomal entry site seems not to be involved in p57 binding. As can be seen from Fig. 4, lanes 8 and 9, deletions between positions 519 and 672 do not interfere with p57 binding. These findings are supported by the results of a complementary experiment: RNA fragments corresponding to the middle region of the internal ribosomal entry site (from positions 399–668 and 399–742) are not crosslinked to p57 (lanes 4 and 6). The 5'UTR of cathepsin B of *Schistosoma mansoni* [16], used as a control for normal mRNA, did also not react with p57 (lane 10).

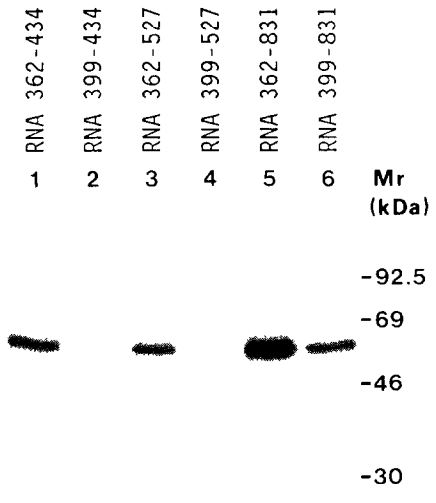

Fig. 4. UV-crosslinking of proteins from the ribosomal salt wash fraction to further transcripts of the FMDV 5'UTR: (1) RNA 362–831; (2) RNA 399–831; (3) RNA 362–668; (4) RNA 399–668; (5) RNA 362–742; (6) RNA 399–742; (7) RNA 742–831; (8) RNA Δ519–559; (9) RNA Δ581–672; (10) normal eukaryotic mRNA (cathepsin B of *Schistosoma mansoni*). The transcript was derived from the *Hin*cII linearized plasmid pSPCB1 [16]. Molecular masses in kDa are shown on the right.

4. DISCUSSION

The experiments described here demonstrate that a ribosome-associated 57 kDa protein from rabbit reticulocytes (p57) interacts with two specific regions of the 5'UTR of FMDV which are part of a sequence required for the internal translation initiation of this virus [3]. Binding of the protein seems not to depend on the complete sequence of this region which may fold in a complicated secondary/tertiary structure as suggested [17].

We cannot fully exclude that p57 consists of two components with very similar molecular weight which bind individually to different RNA domains. In fact, on some short exposures of the autoradiographs, p57 appears as a double band. However, the same doublet of protein bands was observed in complexes with both, RNA fragments from the 5'-end as well as from the 3'-end of the internal ribosome binding site, pointing to two variant forms of a single factor rather than to two distinct proteins with different binding specificities.

p57 binds in approximately equal amounts to the two binding sites. Deletion of the 5'-binding site diminishes binding of the factor approximately two-fold and leads to a proportionally reduced translation efficiency [3]. This would hint to a translational enhancing function of p57. However, deletion of the 3'-site which reduces binding of p57 to a similar degree, abolishes translation almost completeley [3] pointing to a different function of this protein-RNA complex.

Most probably, p57 represents only one of several cellular factors implicated in the specific ribosome-RNA interaction, since binding of proteins to multiple sites of the 5'UTR of poliovirus has been observed [7]. The identity of p57 is not clear. From the molecular weight, its presence in the ammonium sulphate 25–40% saturation fraction (N. Luz, unpubl.) and its low concentration in the cell it seems improbable that it represents one of the main translational initiation factors or a subunit thereof.

A 58 kDa protein from ascites cells has recently been identified which binds specifically to the 5'UTR of EMCV [9]. The binding site of this protein was limited to a sequence of 350 to 520 nucleotides in front of the start codon and could thus correspond to the 5'-binding site of p57 in FMDV. In contrast to our results, however, no binding to a second site near the start codon has been observed. The ascites factor may differ also in other characteristics from p57 since no EMCV RNA binding activity could be observed in reticulocyte lysates by these authors.

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NOTE ADDED IN PROOF

Jang and Wimmer [18] detected at the same time that a 57 kDa protein of rabbit reticulocytes binds to the 5'UTR of encephalomyocarditis virus. Probably this protein is identical to p57, since it binds to the same 5'-site in the RNA secondary structure model [17]. Furthermore the authors could show that the stem-loop structure of this 5'-binding site is essential for p57 binding.

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