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RNA–protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA

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ABSTRACT

Various derivatives of the internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV) RNA have been used to analyze by UV-cross-linking its interaction with mRNA binding proteins from ascites carcinoma Krebs-2 cells. A doublet of proteins with M_r 58 and 60 kD bound to two regions of the IRES . One site is centered at nt 420–421 of EMCV RNA whereas the other is located between nt 315–377. Both sites form hairpin structures, the loops of which contain UCUUU motif, conserved among cardio- and aphthoviruses. The interaction of p58 and p60 with IRES is affected by the integrity of the stem-loop structure proximal to the start AUG codon (nts 680–787), although, under similar conditions, cross-linking of these proteins to this region was not detected. Deletions in the main recognition site of p58 strongly reduce the initiation activity of the IRES *in vitro*. However, elimination of p58 (p60) binding by these mutations does not completely abolish the ability of the IRES to direct polypeptide synthesis starting from the authentic AUG codon. The IRES can be assembled *in vitro* from two covalently unlinked transcripts, one containing the target site for p58 and the other encompassing the remaining part of the IRES fused to a reporter gene, resulting in considerable restoration of its activity. Implications of these findings for the mechanism of initiation resulting from internal entry of ribosomes are discussed.

INTRODUCTION

Picornaviral RNAs employ an unusual mechanism to initiate polypeptide synthesis. Its main distinction from that which is used by the bulk of cellular mRNAs is that picornaviral RNAs bind ribosomes directly to internal sites of their 5′-untranslated regions (5′-UTR)[1–3]. Thus, the initiation of translation for these RNAs does not obey one of the principal rules of the scanning mechanism according to which primary binding of ribosomes (40S subunits) to the mRNA must occur at its 5′-end, in close proximity to the cap-structure [4]. Picornaviral RNAs have no cap at the 5′-end and, therefore, do not use cap-dependent initiation factor 4F to trigger the process of translation initiation [5].

The borders of the internal ribosomal entry site (IRES) as well as its essential structural elements have been well determined for a member of the genus *Cardiovirus*, EMCV (the IRES is located between nt 315–834 of the viral RNA) [6,7], and for foot-and-mouth disease virus (FMDV) [8,9], a member of genus aphthoviruses. The borders of the IRES of poliovirus (a member of the enterovirus genus) have been defined less precisely [see 5].

The complex structure of the IRES raises question about components of the translational apparatus which recognize its features. In particular, the existence of special cellular initiation factors specific for IRES elements seemed to be quite probable. Therefore, a search for these factors is being undertaken in several laboratories. The interest was reinforced by the fact that polioviral RNA is faithfully translated in HeLa cell extracts but is not capable of a correct and efficient initiation of translation in rabbit reticulocyte lysates (RRL) [10]. Thus, some of these putative cellular factors may also be tissue-specific.

Recently, we have discovered an mRNA binding protein, p58 in ascites Krebs-2 cells that specifically binds to the 5′-UTR of EMCV RNA, and have carried out a preliminary identification of its recognition sequence [11]. A little later, a similar protein was found in RRL that bound to EMCV RNA [7] and FMDV RNA [12]. In this paper we present data on localization of two binding sites for p58 within the EMCV IRES. The role of this protein in the internal initiation of translation has been estimated by deletion of its binding sites. Additionally we have studied the influence of the integrity of other IRES domains on the interaction of p58 with its target sites. For the first time, it has been demonstrated that the initiation activity of IRES can largely be restored by its reconstitution *in vitro* from two covalently unlinked RNA fragments, one containing the p58 binding sites and the other encompassing the remaining downstream part of the 5′-leader fused to a reporter gene. This is convincing evidence that scanning is not involved in the initiation of EMCV RNA translation.

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MATERIALS AND METHODS

Oligodeoxyribonucleotides were synthesized using methoxy or cyanoethyl phosphoramidite chemistry on a semiautomatic synthesizer of our own construction. Unlabeled nucleoside triphosphates were purchased from Pharmacia and radiolabelled nucleotides were from Tashkent (USSR). Restriction endonucleases and enzymes used in DNA cloning were purchased from Ferment (USSR). Human placental ribonuclease inhibitor (RNAsin) was from Omoutninsk (USSR). RNAse H from *E.coli* was kindly provided by N.V.Chichkova. DNA manipulations were performed according to standard procedures [13].

Plasmid construction and site-specific mutagenesis

Plasmids of the pTE series, pTE1-pTE14, were described in our previous paper [6]. The initial construct, pTE1, contained cDNA corresponding to nucleotides 315–1155 of the EMCV RNA fused via a polylinker to T7 promotor. Plasmid pTE3 was derived from pTE1 by deletion of the Eco RI-Xba I fragment from the polylinker. Other plasmids represented various derivatives of the non-coding part of EMCV from nt 315 to 833.

The procedure to obtain deletions 414–427 and 398–448 in pTE1 was based on the method proposed by Kunkel [14,15]. Two synthetic oligodeoxyribonucleotides were used for this purpose. The first one was 30 nts long and corresponded to nt 401–413 followed by nt 428–444. The other one was 28 nts long and encompassed the sequence from nt 384 to 397 followed by that from nt 449 to 469 of EMCV RNA. Single-stranded phage DNA of negative polarity from pTE1 was annealed to each of the two phosphorylated oligonucleotides in DNA polymerase reaction buffer. The annealing was performed at 100°C for 3 min, then at 65°C for 10 min followed by a slow cooling to room temperature. Primer extension was carried out by T4 DNA polymerase followed by simultaneous addition of Klenow fragment and T4 DNA ligase + ATP. Clones were screened by hybridization with [^{32}P]labelled oligonucleotide probes and the deletions were verified by sequencing. Plasmids pTE15 and pTE16 were thus obtained for the first and second deletions, respectively.

Plasmids pTE17, pTE18 and pTE19 were derived from pTE1, pTE15 and pTE16, respectively, by digestion of the plasmids with BamHI + ApaI followed by nuclease S1 treatment before religation. The cloned sequence for the resulting plasmids thus started at nucleotide 378 of EMCV RNA.

To obtain constructs of different derivatives of the EMCV RNA 5'-UTR fused to a reporter gene, the corresponding pTE plasmids were treated with NcoI + PstI and the large fragments thus obtained were ligated with an NcoI–PstI DNA fragment containing the whole coding sequence of β-glucuronidase (GUS). GUS-containing plasmid, a derivative of pJII1000, was kindly provided by T.M.A. Wilson. Plasmids containing the GUS gene were denoted as pTE-GUS.

Oligodeoxyribonucleotide directed cleavage of IRES with RNAse H

The transcript synthesized *in vitro* from pTE3, that had been linearized at BalI site, was used to generate two IRES fragments by means of oligodeoxyribonucleotide-directed cleavage with RNAse H. For this, the oligonucleotide complementary to nucleotides 424–448 of EMCV RNA was added in a 10-fold molar excess to 3 μg of RNA in RNAse H reaction buffer (20mM Tris-HCl (pH 7,5), 2mM $MgCl_2$, 40mM NaCl, 2mM DTT). The mixture (20 μl) was supplied with RNAsin (20U) and with RNAse H (10 U) and incubated for 2,5–3 h at 4°C. These conditions allowed the oligonucleotide to interact with the RNA only at unpaired nucleotide residues. The extent of cleavage was about 70%.

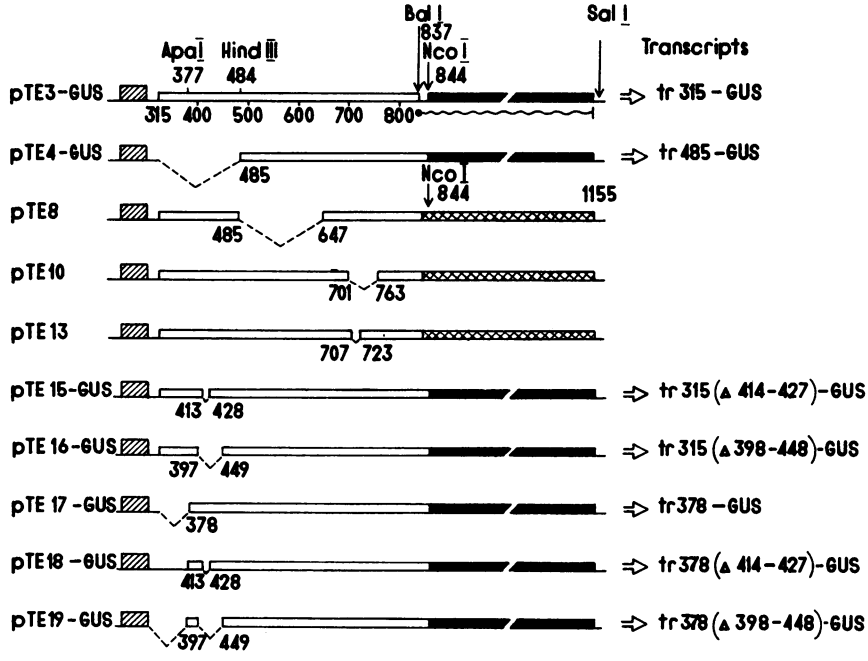

Figure 1. Schematic representation of plasmid constructions used in the work. Hatched, crossed and filled rectangles denote T7 promotor, the N-terminal part of EMCV polyprotein coding sequence and the GUS-coding sequence, respectively. Only those sites of restriction endonucleases are shown which were used to linearize plasmids for a subsequent preparation of transcripts.

In vitro transcription and translation

T7 polymerase directed transcription was done as indicated in [6] using enzyme prepared in our laboratory. The transcripts to be used in translation and cross-linking experiments were purified by the $CaCl_2$ precipitation method [16] followed by two ethanol precipitations and washing in 70% ethanol. This procedure proved to be especially useful for elimination of unreacted nucleoside triphosphates and some impurities when isolating transcripts from the PAA gel.

Translation of the transcripts was performed in extracts of ascites carcinoma Krebs-2 cells as described earlier [6]. Products of *in vitro* translation were analyzed on gradient SDS-PAA gels (8–20%). The gels were fixed in 10% ethanol–7,5% acetic acid, dried and exposed to X-ray film. A set of different exposures was analyzed using a Video Densitometer (Biomed Instruments).

Reconstitution of IRES from two covalently unlinked fragments

Plasmids pTE3 and pTE4 (or pTE4-GUS) linearized at HindIII and PstI restriction sites, respectively, were transcribed *in vitro* resulting in the formation of tr 315–484 and tr 485 (or tr 485-GUS). tr 315–484 was purified from PAA gel. The annealing of tr 315–484 with tr 485 (or tr 485-GUS) at a molar ratio of 10:1 was done in HB buffer (50 mM Tris-HCl, 540 mM KCl, 1mM EDTA) by heating samples at 85–90°C for 3–4 min followed by a cooling to 0°C. Translation of the annealed samples was done in Krebs-2 extracts after adjustment of ionic conditions to those optimal for EMCV RNA translation (170 mM K^+).

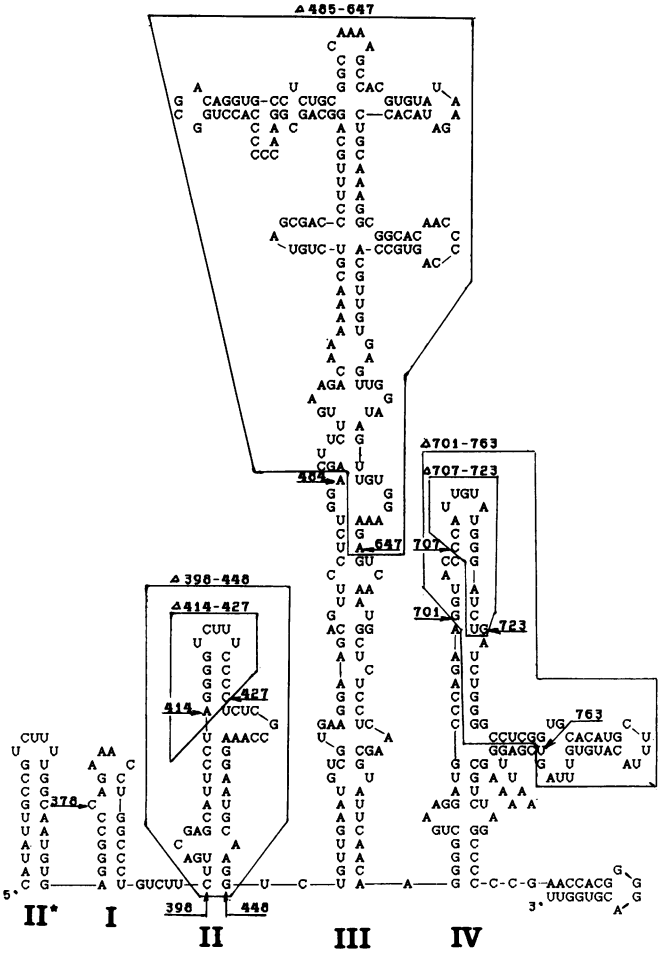

Figure 2. The secondary structure of EMCV IRES as proposed by Pilipenko et al. [17]. The deletions used in some plasmid constructions (see Fig.1) are boxed. With the exception of domain II*, numbering of the domains is according to [17].

UV-cross-linking of transcripts

UV-cross-linking experiments were carried out as described earlier [11] using either an S_{30} cell extract or the 25–40% ammonium sulfate fraction of ribosome salt wash (RSW) of ascites carcinoma Krebs-2 cells. After irradiation samples were treated with nucleases and analyzed on a gradient 11–18% SDS-PAA gel.

RESULTS

Interaction of mRNA binding proteins with the IRES as revealed by UV-cross-linking

The plasmids used in this work are depicted in Fig.1. The secondary structure of the IRES of EMCV RNA from nt 347 to 808 as proposed in [17] is presented in Fig.2.

Cleavage of tr 315–837 (obtained from pTE3 linearized with BalI) with RNase H in the presence of oligodeoxynucleotide complementary to nt 420–449 at 4°C (see Materials and Methods) resulted in the production of two RNA fragments which were separated in 4% PAA gel containing 7M urea (Fig.3). As shown by sequencing of the large fragment, the main cut occurs within nts 445–448. Among the proteins cross-linked to the small fragment (Fig.4), one can see protein p58 that has been previously shown to be specific for EMCV RNA [11]. As p58 also cross-links to tr 378–484 (Fig.5 A, lane 2), the stem-loop structure centered at positions 420–421 (domain II in Fig.2) was suggested to contain a binding site for this protein.

Closer analysis of the autoradiographs reveals a doublet of protein bands binding to all transcripts containing domain II analyzed in S30 Krebs-2 cell extracts. The stronger band corresponds to p58, the weaker to a protein with molecular weight about 60 kD. Deletions in domain II of tr 378 (see below) always resulted in the disappearance of both bands from autoradiographs.

Figure 3. Oligonucleotide directed RNAse H cleavage of IRES into two fragments. tr 315–837 was treated with RNAse H (see Materials and Methods) in the presence (1) and absence (2) of oligodeoxynucleotide complementary to positions 424–448 of EMCV RNA. The reaction products were separated by 7M urea-4% PAA gel electrophoresis. I, S and L denote the initial untreated RNA and small and large fragments of IRES, respectively.

One more band corresponding to the protein of approximately 56kD is clearly detectable only when RSW is used for cross-linking (see Fig.4).

p58 and p60 appear to have another binding site, upstream from the main one (Fig.5A, lane 3).

However, P58 was cross-linked to both [^{32}P-UTP] and [^{32}P-ATP]-labelled transcripts containing nt 315−373 of the 5′-UTR, whereas p60 was cross-linked only to [^{32}P-ATP]-labelled transcripts (Fig.5B, compare lanes 1,2 with 4 and 5). The reason for this difference (which was apparent even after prolonged exposure of autoradiographs) is not yet known.

Tr 315−373 binds at least one more protein with Mr 54 kD (Fig.5). p54 was previously shown to cross-link specifically to EMCV 5′-UTR as (lower) one of the quadruplet bands referred to as p58 in [11].

In addition, other cross-linked proteins, including p52, are seen in the autoradiographs. They have been previously shown to be non-specific for EMCV RNA [11].

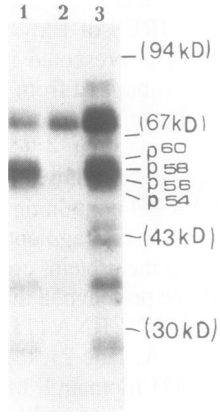

Figure 4. Proteins from 25−40% ammonium sulfate fraction of ribosomal high salt wash cross-linked to L and S fragments of IRES (see Fig.3). 1—tr 315−837; 2—L fragment; 3—S fragment. The same amount of radioactivity is used in experiments 2 and 3. The molecular weight markers are indicated in parenthesis.

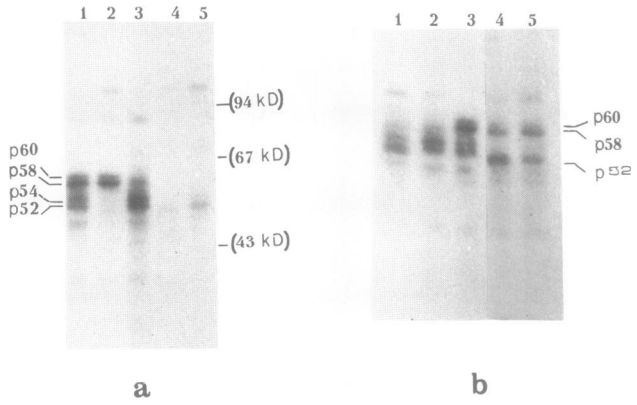

Figure 5. UV-cross-linking of proteins from extracts of ascites carcinoma Krebs-2 cells to different derivatives of EMCV RNA 5′-UTR.(A) 1—tr 315−484; 2—tr 378−484; 3—tr 315−373; 4—tr 378−484 (Δ414−427); 5—tr 378−484 (Δ398−448). (B) 1—tr 315−484 (Δ398−448); 2,5—tr 315−484 (Δ414−427); 3,4—tr 315−484. Lanes 1−3 represent proteins cross-linked to [^{32}P]U-labelled and lanes 4,5—to [^{32}P]A-labelled RNA templates. The molecular weight markers are indicated in parenthesis.

Structural elements of domain II that are required for binding of p58 and p60

Neither p58 nor p60 were cross-linked to tr 378−484 (Δ414−427), so the UCUUU loop with its stem appears to be needed for their binding (Fig.5A, lane 4). To rule out the possibility that p58 interacted with the part of the stem of domain II that does not include U residues (and which could therefore not be detected by cross-linking to [^{32}P-UTP]-labelled transcripts we also analyzed the cross-linking of cellular proteins to [^{32}P-ATP] and [^{32}P-GTP] labelled transcripts. Under these conditions cross-linking of p58 to tr 378−484 (Δ414−427) was not detected (data not shown). Longer [^{32}P-ATP]-labelled transcripts, such as tr 315−484 (Δ414−427) still bind p58 (Fig.5B, lane 5), most probably within the domain immediately upstream of domain I (hereafter referred to as domain II*).

Effect of other domains of EMCV IRES on the interaction of p58 with its target site(s)

The EMCV IRES has a complex and compact structure and it is thus possible that the integrity of IRES domains other than domains II and II* may affect the interaction of p58 with its binding sites. To test this, the [^{32}P] labelled transcripts containing deletions in different parts of the IRES were prepared. Their abilities to cross-link p58 were compared under strictly identical conditions using identical molar amounts of the transcripts added to ascites cells extracts. The data on cross-linking of these derivatives of the IRES to p58 are presented in Fig.6.

It is apparent that tr 315−848 (Δ701−763) containing a large deletion in domain IV binds p58 more than two times less efficiently than intact tr 315−848. tr 315−848 (Δ707−723) with a smaller deletion in the same domain occupies an intermediate position (Fig.6A). At the same time, tr 315−848 (Δ485−647) lacking a 'cross' characteristic of cardio- and aphthoviral IRES (domain III) binds p58 as efficiently as intact tr 315−848 (Fig.6B, lanes 1 and 2). The amount of the protein cross-linked to tr 315−484 is also lower than that for the control RNA (Fig.6B, lane 3). As expected, unlabeled transcripts with deletions in domain IV inhibited the cross-linking of p58 to the whole IRES less efficiently than those with an intact domain IV (data not shown). These results proved to be highly reproducible. They suggest that the interaction of p58 with its target site(s) is affected by the stem-loop structures proximal to the start AUG codon of EMCV RNA.

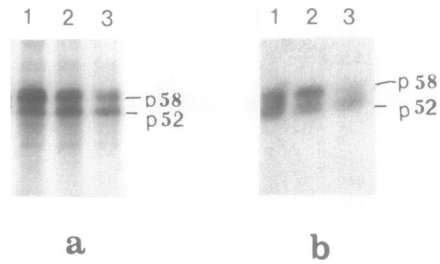

Figure 6. Effect of deletions in IRES domains other than domains II and II* on the UV-cross-linking of p58. (A) 1—tr 315−848; 2—tr 315−848 (Δ707−723); 3—tr 315−848 (Δ701−763). (B) 1—tr 315−848; 2—tr 315−848 (Δ485−647); 3—tr 315−484. The transcripts were added to the samples for irradiation in the same molar amounts calculated on the base of their U-content.

Contribution of the upstream part of the IRES to its activity in initiation of translation

The influence of deletions in domains III and IV on the initiation of translation was studied in our previous paper [6]. In this work, the contribution of the upstream part of the IRES to its activity in the internal initiation of translation has been analyzed. For this, various derivatives of IRES were fused to the coding region of the GUS gene (β-glucuronidase).

The efficiency of translation of tr 315-GUS was consistently 1.5−2 times greater than that of tr 378-GUS (Fig.7A). In line with these observations is the finding that tr 315−484 supplied *in trans* inhibited the translation of tr 315-GUS more strongly than tr 378−484 (Fig.7B). This may be accounted for by the higher ability of the former to interact with mRNA binding proteins located in the upstream part of IRES.

Partial deletion of domain II in tr 315 (Δ414−427)-GUS totally eliminates the binding of p58 and strongly reduces the activity of the IRES. In this instance, it amounts to only 10−20% of the initial activity (Fig.8, lanes 8,9,10). However, it remains appreciably higher than for the transcripts lack domain II completely (2−4% of the control, Fig.8, lanes 1,2,3). Moreover, some enhancing effect of sequence 315−377 noted above is lost upon the complete deletion of domain II. Indeed, a residual activity of tr 315 (Δ398−448)-GUS appears to be even lower than that of tr 378 (Δ398−448)-GUS (data not shown).

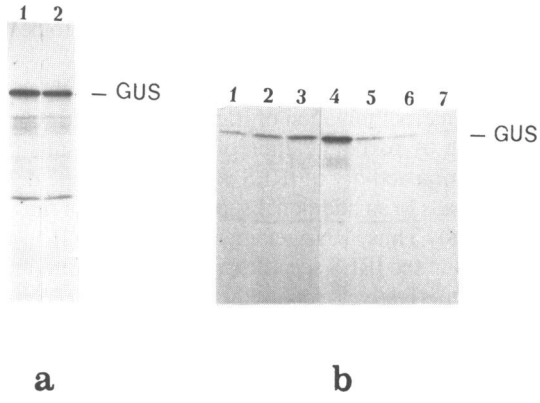

Figure 7. (A) Comparative analysis of translation of tr 315-GUS (1) and tr 378-GUS (2) in ascites carcinoma Krebs-2 cells extracts. The translational efficiencies were compared for 0,5 μg of each transcript per 25 μl of sample volume. (B) Inhibition of translation of tr 315-GUS by tr 315−484 and tr 378−484. The amount of tr 315-GUS was maintained constant in all assays (1 μg/25 μl). 1,2,3—tr 378−484 added in a 60-, 30- and 15-fold molar excess; 5,6,7—tr 315−484 added in a 10-, 20- and 40-fold excess; 4—tr 315-GUS with no addition of competitor RNA.

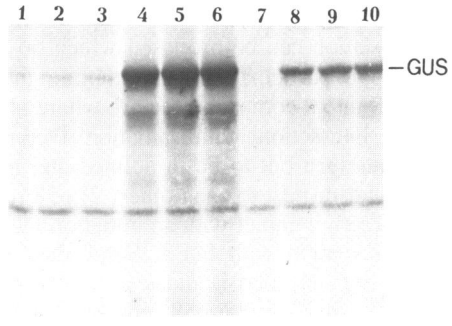

Figure 8. Translation efficiencies of transcripts containing various derivatives of EMCV IRES fused to the GUS gene coding sequence. lanes 1−3—tr 315-GUS (Δ398−448) , lanes 4−6—tr 315-GUS; lanes 8−10—tr 315-GUS (Δ414−427); lane 7—translation without supplying extracts with any exogenic messenger; the samples (25 μl) contained 0,25 μg (lanes 1,4,8), 0,5 μg (lanes 2,5,9) and 1 μg (lanes 3,6,10) of each of the transcripts.

Reconstitution of IRES from two covalently unlinked fragments

The initiation activity of the whole IRES can be restored by its *in vitro* reconstitution from two covalently unlinked RNA fragments, one containing the target sites for p58, and the other encompassing the remaining part of IRES fused to a reporter gene. For this, tr 485-GUS and tr 315−484 were annealed and an aliquot of the incubation mixture was tested for *in vitro* translation in ascites cell-free extracts. As a control, tr 485-GUS that had been subjected to the same treatment was analyzed in parallel. As seen in Fig.9A, the annealing resulted in a considerable increase in the IRES activity. Judging by incorporation of [^{35}S] methionine, in this particular experiment, it amounted to 20−25% of that for the intact tr 315-GUS. For some unknown reasons, the extent of restoration of the initiation activity varied from one assay to another. The best result was obtained when using tr 485−1155 annealed with tr 315−484 (Fig.9B). tr 485−1155 contains a short coding sequence for the N-terminal part of EMCV polyprotein and lacks the termination codon. The translation of transcripts of this series was described in our previous paper [6]. It should be stressed that addition of the fragments one after another to the cell-free system did not result in an increase of the 5′-UTR activity.

DISCUSSION

As revealed by UV-cross-linking, the EMCV IRES functions in association with cellular mRNA binding proteins. One of these proteins, p58, has earlier been shown to be specific for the 5′-UTR of EMCV RNA [11]. In fact, p58 is always associated with a second protein of M_r 60 kD, but since the relationship between them is not yet known, we shall, for simplicity, refer to the protein doublet as p58.

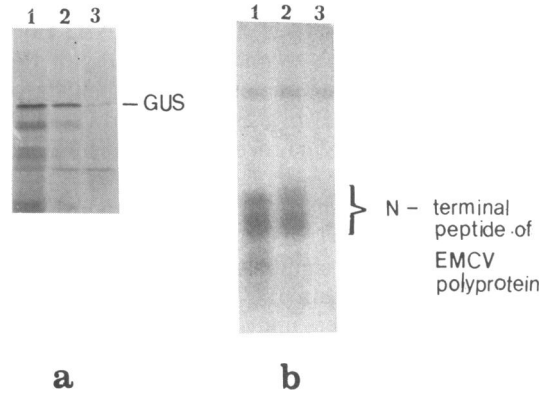

Figure 9. Translation of transcripts with the IRES reconstituted from two covalently unlinked fragments. (A) 1—tr 315-GUS; 2—tr 315−484 + tr 485-GUS; 3—tr 485-GUS; (B) 1—tr 315−1155; 2—tr 315−484 + tr 485−1155; 3—tr 485−1155. For annealing procedure see Materials and Methods. Before adding to the cell extract, the intact control transcripts were subjected to the same treatments as the experimental ones.

Jang and Wimmer [7] and Luz and Beck [12] have identified a protein with M_r 57 kD in rabbit reticulocytes lysates (RRL) that is specific for the 5'-UTRs of EMCV RNA and FMDV RNA, respectively. The suggestion [7] that p57 is similar or identical to p58 from ascites cells has been confirmed in this paper.

The content of p58 in ascites cells is much higher than in reticulocytes (our unpublished observations, T.V.Pestova, personal communication). The same is true for polioviral RNA specific p52 [18] and, as we suspect, for some other specific mRNA binding proteins. That is why a strong signal of cross-linked p58 can be detected even when using the whole extract from ascites cells rather than RSW proteins. The binding of p58 to EMCV RNA is strong and can be easily detected on increasing salt concentration up to 0,3 M of K^+ (unpublished). Under similar conditions, RRL results in a very weak band unless RSW proteins are used in cross-linking experiments. Nevertheless, this low concentration of p58 in RRL appears to be sufficient to provide the effective translation of EMCV RNA in this cell-free system, due to the high affinity binding of this protein to EMCV IRES. However, it may be too low for weaker p58 target sites in other mRNAs. Thus, RRL does not seem to be a quite adequate system to study translation of picornaviral RNAs. It may lead to many conflicting results on the role of different elements of these RNAs in the internal translation initiation [for review see 5].

The main binding site for p58 has been localized within domain II (stem-loop E) of EMCV IRES in agreement with [7]. In addition, we have found a second binding site for this protein, most probably within domain II*, which has the same UCUUU loop of both cardio- and aphthoviruses as that occurs in domain II. The second binding site may have an auxiliary role since the corresponding sequence (315−377) produces some enhancing effect on translation initiation. The effect is lost on deletion of domain II.

Two binding sites for p58 (p57) have also been found for FMDV RNA [12]. One of them is similar in structure and position to domain II of EMCV IRES; the other occurs in the vicinity of the initiation codon. No appreciable cross-linking to the latter site has been revealed for EMCV RNA [11, this paper, Fig.4], though we cannot totally exclude its existence in EMCV IRES.

On the face of it, apart from the conserved CUUU motif, there is no apparent similarity between the proposed binding sites for p58, especially between domain II of EMCV IRES and the proximal to the start codon site of FMDV RNA. However, a careful examination of the secondary structures of IRES [17] allows one to discern some common features within the proposed p58 binding regions. These are a single-stranded U-rich sequence adjoined to a rather long stem broken approximately in the middle by a short defect.

In spite of a general similarity of the FMDV and EMCV IRES secondary structures [17], they differ in some details and, especially, in functional significance of their elements. First, FMDV IRES lacks domain II* and the proposed secondary structure of the element immediately upstream from the polypyrimidine tract differs from that in EMCV RNA [17]. Second, deletions of domain II or IV from EMCV IRES produce much more deleterious effect on translation than in the case of FMDV RNA, whereas the central parts or pyrimidine-rich sequences proximal to the start codons are equally crucial in these two RNAs [6,7,9, this paper].

Other determinants of EMCV RNA important for the internal initiation reside, therefore, downstream of domain II. Indeed, deletions downstream from position 485 result in complete inactivation of EMCV IRES. Of them, one can mention deletions in domains III, IV [6] or in the pyrimidine-rich sequence proximal to the initiating AUG codon [7]. The role of the first one is especially intriguing since no function for this region of IRES has ever been suggested.

Thus, if the position of p58 within IRES is essential for the internal initiation, it may be provided in a different way for the two RNAs. The influence of the domain IV integrity on the interaction of p58 with its main target site found in this work may be a key to the problem. We speculate that the interaction of p58 and/or of its main binding site with domain IV may bring the protein to the proximity to the start codon of EMCV RNA, thus providing position of p58 in space equivalent to the one found in FMDV RNA. Such an interaction may partially account for our successful reconstitution of the IRES activity from two covalently unlinked RNA fragments, one containing the p58 binding sites and the other encompassing the remaining downstream part of IRES. In addition, this experiment gives one more strong evidence that there is no ribosomal scanning along EMCV IRES, at least up to position 484 (see also [19]).

Alternatively, p58 may form a multimeric structure that is able to establish several contacts within IRES. The corresponding sites may differ in their affinity to p58 or in their ability to be detected by UV-cross-linking.

We do not think that p58 is a key component which entirely underlies the internal initiation of translation. Indeed, elimination of p58 binding by partial deletion of domain II results in a high residual initiation activity of IRES whereas complete deletion of this domain leads to an additional substantial drop of the initiation activity (Fig.8). Thus, RNA elements of domain II itself seem to be involved in the IRES specific organization. More important, both deleted derivatives retain the ability of IRES to direct ribosomes preferentially to the authentic start AUG codon at positions 834−836 since even tr 485 which possesses some residual activity [6,18, this paper] has been shown to reveal high preference to the authentic AUG start codon [19]. Therefore, p58 is considered to play an accessory role, probably by enhancing the rate of initiation complex formation.

This is a very important difference between our results and those carried out by Jang et al [7] who did not detect any of the residual translation activity of the constructs with the eliminated p58 binding site, including the transcript starting from nt. 485 [2]. One possible explanation for this discrepancy is that we use the Krebs-2 translation system which is a natural one for EMCV reproduction.

In conclusion, two possible roles for p58 in the internal initiation of translation can be proposed which do not exclude each other: 1) p58 participates in assembling the stem-loop elements of IRES into a compact structure where all RNA sites needed for the interaction with initiation factors and 40S ribosomal subunits turn out to be in defined and more or less fixed positions with respect to each other; 2) p58 (or one of its subunits) may directly interact with an initiation factor, thereby stabilizing its association with IRES. Some data suggest that initiation factor 2 can be a good candidate as a partner of p58 [20].

It is attractive to suppose that p58 represents one of the first examples of cellular positive trans-factors involved in mammalian translation initiation. p58 has been shown to interact with mRNAs with high selectivity [6] and, hence, may turn out to be that tool by means of which one can identify cellular mRNAs employing the mechanism of internal initiation. Given their cap-independence, such messages may represent a special class of mRNAs involved in essential cellular processes.

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