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A factor that specifically binds to the 5′-untranslated region of encephalomyocarditis virus RNA

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A protein factor that specifically binds to the 5′-untranslated region of encephalomyocarditis virus (EMCV) RNA has been found in extracts of ascites carcinoma Krebs-2 cells. This was done using UV-irradiation on extracts supplemented with in vitro synthesized ^{32}P-labelled transcripts followed by analysis of crosslinked proteins by SDS-polyacrylamide gel electrophoresis. The transcripts represented the viral RNA sequence from nt 315 to 1155, its derivatives with internal deletions or truncated forms. This set of transcripts has allowed us to find out that the factor (p58) binds to EMCV RNA within the sequence 315–485.

Translation; Internal initiation; Encephalomyocarditis virus; Protein-RNA interaction

1. INTRODUCTION

Encephalomyocarditis virus RNA as well as RNAs of other picornaviruses are of current interest due to a puzzling mechanism which they use to initiate translation [1–4]. Some RNA properties are not in agreement with Kozak's scanning model of eucaryotic translation initiation [6,7]. It can be explained by suggesting that EMCV RNA is capable of the internal initiation of translation, i.e. independent of the 5′-end entry of the ribosome into the mRNA initiation site. It has recently been proven not only for EMC [5], but also for poliovirus [8]. It is plausible to suggest that there are some special trans-acting cellular factors which trigger the internal initiation.

Using various in vitro synthesized derivatives of the leader region of EMCV RNA, we have shown by the method of UV-crosslinking that ascites carcinoma Krebs-2 cells do contain a protein with an M_r about 58000 which recognizes the 5′-UTR within the sequence 315–485 of EMCV RNA.

2. MATERIALS AND METHODS

The construction of the starting plasmid pTE1 as well as of its truncated and deleted derivatives is described elsewhere [9]. pT7-2-42 containing a DNA copy of AlMV RNA 4 under control of T7-promotor [10] was kindly provided by K. Langereis (Leiden). The plasmids linearized with suitable restriction endonucleases were used in T7 polymerase reaction according to [8] with slight modifications. Specific activity of [α-^{32}P]UTP-labelled (3000 Ci/mmol) transcripts was about 10^5 cpm/μg RNA.

T7 polymerase was prepared from the superproducing strain of *E. coli* (containing pAR 1219) kindly provided by K. Langereis (Leiden) according to [12] with some modifications. The preparation of Krebs-2 cell extracts, microsomal fraction and ribosomal high salt wash was carried out as in [13–15], respectively.

Crosslinking experiments were performed as in [13] with 254 nm light (an incidental dose of 2×10^5 erg/mm^2). Samples (25 μl) with composition, optimized for translation of EMCV RNA in vitro, were incubated for 5 min at 30°C before irradiation [9]. They contained 15 μl of the extracts and 10^5 cpm of a ^{32}P-labelled transcript. After irradiation the samples were treated for 15 min at 37°C with cobra venom ribonuclease (Minor Bio, Estonia) with a final concentration 0.2 μg/μl, and then with a mixture of ribonucleases A (0.4 μg/μl) and T1 (5 U/μl) for 30 min at 37°C with addition of EDTA to a 10 mM concentration. Cross-linked proteins were analyzed by electrophoresis in a gradient PAAG (8–20%) containing SDS [14].

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Abbreviations: EMCV, encephalomyocarditis virus; AlMV, alfalfa mosaic virus; 5′-UTR, 5′-untranslated region; tr, transcript; nt, nucleotide; PAAG, polyacrylamide gel

3. RESULTS

The initial construction, pTE1, contains a part of the 5′-UTR of EMCV genome from 315 to 833 nt and a sequence encoding the 5′-terminal fragment of the polyprotein (up to 1155 nt; fig.1). As we have recently shown, the corresponding transcript has a very high translational activity in vitro and hence, contains all the necessary signals for the initiation process [9]. The same construction through linearization at its corresponding restriction sites, *Hin*dIII and *Bal*I, was used to obtain transcripts truncated from the 3′-end, tr 315–485 and tr 315–837, respectively.

The transcripts synthesized on other plasmids represent either truncated from the 5′-end derivatives of pTE1 or contain deletions in different sites of the 5′-UTR (fig.1). Their translational activity has been recently analyzed by us in Krebs-2 cell extracts [9].

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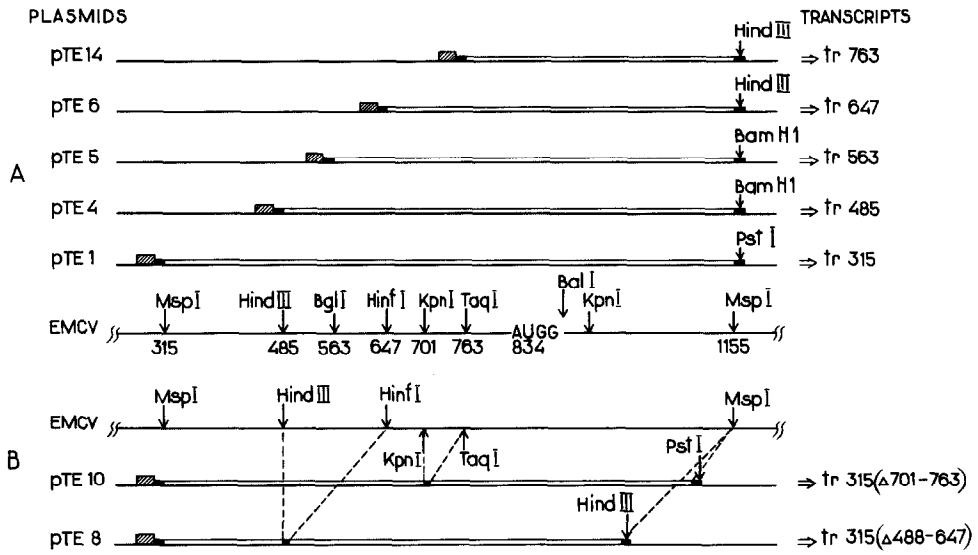

Fig.1. Constructions used to obtain truncated from the 5′-end derivatives of the 5′-UTR of EMCV RNA (A) or its deleted forms (B). T7 promotor is shown by hatched boxes.

Almost all of them proved to be completely inactive. However, in the case of tr 485, some residual activity in the cap-independent initiation still remained. This set of RNAs was supposed to be a convenient tool to look for factors recognizing the 5′-UTR of EMCV RNA.

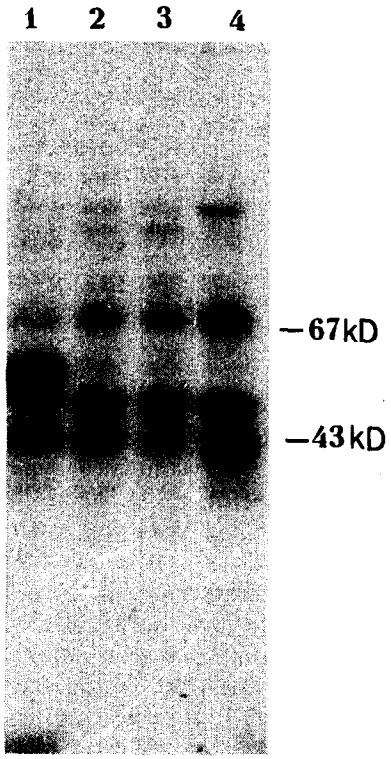

Fig.2. Autoradiographs showing proteins from extracts of ascites carcinoma Krebs-2 cells crosslinked to different derivatives of the 5′-UTR of EMCV RNA. (1) tr 315–837; (2) tr 485; (3) tr 647; (4) anti-tr 315. Arrows indicate positions of marker proteins. Patterns of proteins crosslinked to tr 563 and tr 767 are similar to that for tr 485 and not shown in the figure.

Also, as a control, in vitro synthesized AlMV RNA4 and antisense RNA complementary to tr 315 have been used.

Ascite cell extracts have been supplemented with the ^{32}P-labelled transcripts, UV-irradiated, treated with RNases and then analyzed by electrophoresis in PAAG. As seen in fig.2, most of the radioactive bands are identical for all transcripts mentioned above. Treatment of the samples with proteinase K, proved these bands to be not unhydrolyzed RNA fragments, but UV-crosslinked proteins (data not shown).

However, apart from the proteins common to all RNAs an additional broad band with M_r in the range of 50000–60000 is revealed for EMCV tr 315–837.

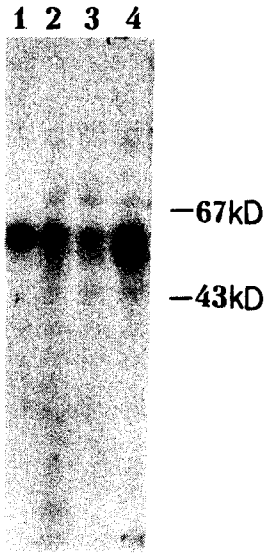

Fig.3. Proteins from extracts of ascites cells UV-crosslinked to: (1) tr 315–837; (2) tr 315–485; (3) tr 315(Δ701–763); (4) tr 315–701(Δ488–647).

This band is absent in the case of antisense RNA, trs 485, 563, 647 and 763 and hardly seen for AlMV RNA4 (fig.2A). The EMCV RNAs with deleted nt 701–763 and 488–647 retain the ability to bind this protein(s) (fig.3). This raised the possibility that the recognition site of the factor(s) is located somewhere between positions 315 and 485 of EMCV RNA. Indeed, the experiments with tr 315–485 (fig.3, lane 2) have confirmed this suggestion.

At a lower exposure of the gels, the broad band of the crosslinked products specific for the EMCV 5′-UTR is often revealed as a quadruplet. Preliminary experiments with the use of mobility-shift electrophoresis [18] seem to indicate that it is accounted for by incomplete digestion of oligonucleotides crosslinked to a single protein with M_r about 58000 (hereafter referred to as p58) rather than by a set of different protein factors (our unpublished observations). The extracts with lower ionic strength reveal an additional crosslinked protein with a similar M_r for all the transcripts including antisense-tr 315 (see some examples in fig.4). Its relation to p58 is unclear. At any rate, under all conditions the intensity of the respective band for transcripts encompassing nt 315–485 of EMCV RNA is much stronger than for all other RNAs.

p58 is unlikely to be ATP- or GTP-dependent protein since the presence or absence of these nucleotides in extracts does not affect its crosslinking to the 5′-UTR of EMCV RNA (data not shown).

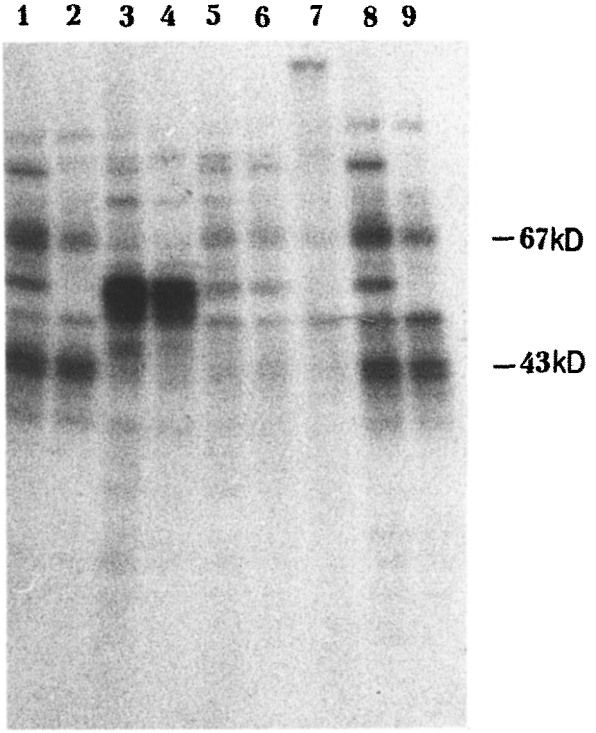

Fig.4. Proteins crosslinked to different derivatives of the 5′-UTR of EMCV RNA at 50 mM K (lanes 1, 3, 5, 6 and 8) and 170 mM K^+ (lanes 2, 4, 7 and 9). (1,2) tr 563–763; (3,4) tr 315(Δ488–647); (5) tr 647; (6,7) tr 563; (8,9) anti-tr 315.

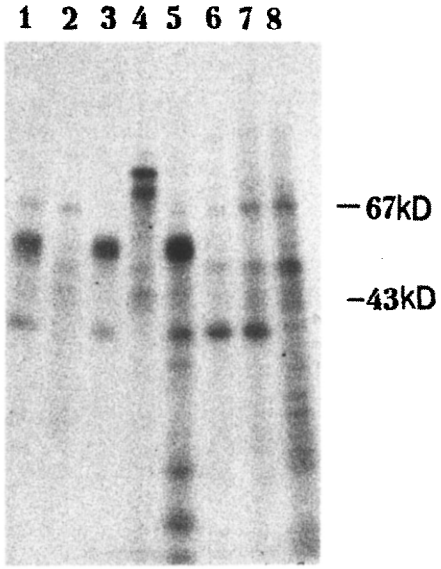

Fig.5. Proteins from a ribosomal high salt wash crosslinked to deleted derivatives of tr 315. (1,3 and 6) 25–40% ammonium sulfate fraction of the ribosomal high salt wash; (2,4 and 7) 40–70% fraction; (5,8) unfractionated factors. (1,2) tr 315(Δ701–763); (3,4 and 5) tr 315(Δ488–647); (6,7 and 8) AlMV RNA-4. The samples (25 μl) contained 2.5 A_{280} units of 25–40% fraction or 1.8 A_{280} units of 40–70% fraction or both.

Subcellular distribution of p58 has not been determined in this work. Nevertheless it is clear that p58 is present in a ribosomal high salt wash, particularly in ammonium sulphate 25–40% saturation fraction (fig.5).

It is of interest that on removal of some proteins, including p58 from unfractionated ribosome-bound factors, new proteins can be efficiently crosslinked to some derivatives of the 5′-UTR of EMCV RNA (fig.5, lanes 3–5). This suggests a competition between some cellular factors for the same sequences in the leader of EMCV RNA.

4. DISCUSSION

The results obtained strongly suggest that ascites carcinoma Krebs-2 cells contain a protein factor (p58) which specifically binds to the 5′-UTR of EMCV RNA. The recognition sequence locates between nt 315 and 485 of the viral genome. It is important to note that the factor crosslinks equally well to tr 315–485 alone or when it is a part of a longer sequence (tr 315 or tr 315–837). In the latter case, the 3′-terminal part of the 315–485 sequence which is thought to be functionally important for translation [19] must be incorporated, according to Pilipenko et al. [20], into a long helix of the 5′-UTR (domain III). Then it appears that the disruption of the lower part of the helix does not abolish the recognition site. Further experiments will clarify whether the p58 recognition site is located in the 5′-part, or in the 3′-part of 315–485 fragment.

Rabbit reticulocyte extracts either do not contain p58 or its concentration in these cells is much lower than in ascite cells. Furthermore, on fractionation of the crude KCl wash from ascites cells, p58 does not co-elute with any of the known translational factors (our unpublished data). These observations argue against the possibility that p58 represents one of the main initiation factors common to all mammalian cells.

At present, we have no evidence that p58 is indeed a cellular trans-factor that confers the cap-independent initiation of translation of EMCV RNA. Moreover, a high efficiency of translation of EMCV RNA in rabbit reticulocyte extract [21] seems to argue against this possibility. However, it is not unlikely that either small amounts of p58 in reticulocytes are sufficient for EMCV RNA translation initiation or that another protein(s) is responsible for similar triggering function in these extracts.

Apparently, p58 and its corresponding recognition site cannot determine entirely the internal initiation within the 5′-UTR of EMCV RNA. Indeed, even a small deletion in the 5′-UTR, far downstream from nt 485 (Δ707–723), completely abolishes the activity of the leader of EMCV RNA in the initiation of translation [9].

Recently, N. Sonenberg et al. [22] have found a protein factor p52 specific for poliovirus RNA which appears to be involved in the internal initiation of translation in HeLa cells. The relation of p52 to p58 is unclear. The role of p58 in expression of both EMCV RNA and probably some cellular RNAs will be the aim of further experiments.

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