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Cowpea Mosaic Virus Middle Component RNA Contains a Sequence That Allows Internal Binding of Ribosomes and That Requires Eukaryotic Initiation Factor 4F for Optimal Translation

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Cowpea mosaic virus (CPMV) middle component RNA (M-RNA) encodes two proteins of 105 and 95 kDa, of which translation starts at nucleotide (nt) 161 and nt 512, respectively. In vitro translation of both proteins directed by T7 transcripts of M-RNA was stimulated fourfold by eukaryotic initiation factor 4F (eIF-4F), the cap-binding protein complex. The ratio of the synthesis of both proteins after translation was not influenced by eIF-4F or by any known eIF. Part of the CPMV 5′ sequence was cloned downstream of the 5′ untranslated region of ornithine decarboxylase (ODC); the latter untranslated sequence has a highly stable secondary structure, preventing efficient translation of ODC. Insertion of nt 161 to 512 of CPMV M-RNA upstream of the ODC initiation codon resulted in a marked increase in ODC translation, which indicates that the CPMV sequence contains an internal ribosome-binding site. The insertion conferred stimulation by eIF-4F on ODC translation, showing that eIF-4F is able to stimulate internal initiation.

Cowpea mosaic virus (CPMV) is a type member of the plant comoviruses (for a recent review, see reference 6). The genome consists of two noncapped, VPg-containing, positive-stranded RNAs, designated middle component RNA (M-RNA) and B-RNA (17, 36). Translation of M-RNA in vivo and in vitro initiates at two sites. The AUG at position 161 leads to a protein of 105 kDa, and the translational start at position 512 leads to a 95-kDa protein (20, 25, 36). Both polyproteins are processed by the viral protease, encoded by the B-RNA (38).

The similarity of the CPMV genome to that of the picornaviruses (9) raised the question of whether CPMV M-RNA, like poliovirus, foot-and-mouth disease virus, and encephalomyocarditis virus, has the ability to direct ribosomes to internal sequences rather than to the 5′ end of its RNA. This question was addressed for poliovirus, encephalomyocarditis virus, and foot-and-mouth disease virus by cloning their 5′ untranslated regions (UTRs) between two reporter genes (1, 11, 12, 23). It was shown that translation of the downstream gene was dependent on the presence of the picornaviral 5′ UTR. Translation directed by the 5′ UTR of poliovirus was abolished by insertion of a hairpin structure in the RNA downstream of the ribosomal entry site (23). This strategy was used to investigate whether the CPMV sequence from nucleotides (nt) 161 to 512 forms a ribosomal entry site.

The rat ornithine decarboxylase (ODC) gene has a 303-nt-long 5′ UTR that can be folded into a very stable secondary structure (34). This structure prevents efficient translation of the downstream gene in vivo (10, 18) as well as in vitro (10, 35). The ODC cDNA was placed downstream of the T7 RNA polymerase promoter. The CPMV sequence from nt 161 to 512 was cloned between the ODC 5′ UTR and the ODC initiator ATG. The effect of this insertion on the efficiency of translation of the downstream gene was studied by translation in reticulocyte lysates (21).

Eukaryotic protein synthesis initiation factor 4F is a three-subunit protein complex, consisting of eukaryotic initiation factor (eIF) 4E, the cap-binding protein; eIF-4A, involved in mRNA unwinding (15, 27); and p220, a subunit with unknown function (26, 28, 29). eIF-4F activity is inhibited by cap analogs such as ^{7}mGTP and ^{7}mGpppG (28, 29), and therefore its function is linked to the translation of capped mRNAs (2, 5, 16, 31), implying no function in the translation of noncapped mRNAs such as CPMV, poliovirus, or encephalomyocarditis virus RNA. The effect of eIF-4F on translation of CPMV M-RNA was tested, and surprisingly, translation was stimulated considerably. This ability of eIF-4F to stimulate translation was also transferred to the ODC construct that contained the nt 161 to 512 sequence of CPMV M-RNA.

MATERIALS AND METHODS

Plasmid pTM1G contains a full-length cDNA clone of CPMV M-RNA cloned behind a T7 RNA promoter (7). The plasmid was linearized with *Cla*I and transcribed with or without 0.5 mM ^{7}mGpppG as described before (19, 22). The nucleotide sequence of the transcript is identical to that of viral M-RNA (36) except for one additional G residue at the 5′ end and two nucleotides (UA) at the 3′ end. The yield and integrity of the RNAs were checked by agarose gel electrophoresis.

Translation of transcripts was done in rabbit reticulocyte lysates (21) with 1 μCi of [^{35}S]methionine (1,000 Ci/mmol) per 5 μl of assay mix. Routinely, 75 ng of transcript was used. Incorporation was determined in a 1-μl sample via hot trichloroacetic acid precipitation, and the remaining 4 μl was analyzed via 12.5% polyacrylamide–sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and fluorography.

Plasmid pTMΔAUG1/2, containing a *Sal*I site at position 156 and an *Eco*RI site at position 509 of the M-cDNA (removing the AUGs at 161 and 512), was created by site-directed mutagenesis (36a). The 353-nt *Eco*RI-*Sal*I fragment was inserted into pBS-KS+ (Stratagene), creating

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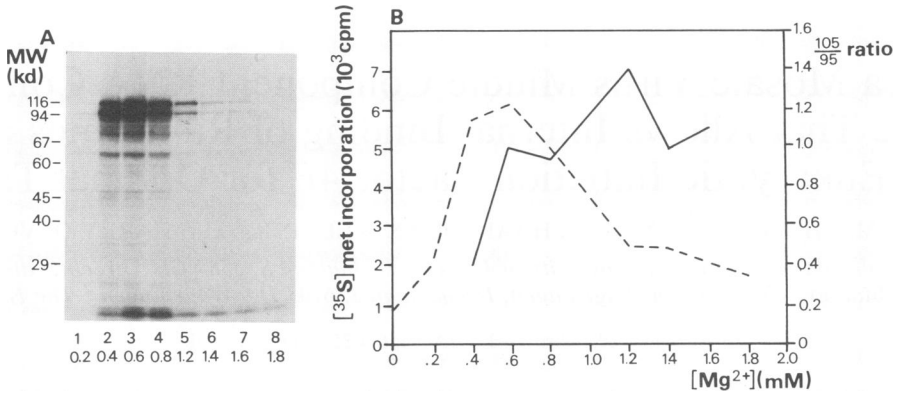

FIG. 1. Mg^{2+} dependence of CPMV M-RNA translation. (A) pTM1 was linearized with *Cla*I and transcribed in the absence of cap analog. Optimal amounts of RNA were assayed in the reticulocyte lysate in the presence of different concentrations of magnesium acetate, as indicated (millimolar). After incubation for 1 h at 30°C, 1.5 μl was assayed for hot trichloroacetic acid-precipitable radioactivity, and the remainder was analyzed on a 10% acrylamide–0.26% bisacrylamide gel. Molecular mass standards are indicated. The 105-kDa protein comigrated with the subunit of β-galactosidase (116 kDa). (B) Several exposures of the fluorogram were scanned, and the ratio of 105-kDa and 95-kDa proteins (——) and total [^{35}S]-methionine incorporation (– – –) were plotted versus the Mg^{2+} concentration during translation.

pBSL2. Plasmid pT7ODC303BH was obtained by cloning the T7 promoter sequence into pODCE10 (containing the cDNA for ornithine decarboxylase [ODC] [33]) directly upstream of the ODC gene. The nucleotides around the initiation codon at position 304 were changed from AGAAC CAACCATGG to GGATCCAACCATGG. pBSL2 was digested with *Hin*cII and *Bam*HI, and a 378-nt fragment (351 nt plus 27 nt of polylinker sequence) was ligated into pT7ODC303BH (35), digested with the same enzymes. This generated pODC-CPMV, containing the T7 promoter, 226 nt of the 5′ UTR of ODC, the 351-nt fragment of CPMV, and 27 nt of polylinker sequence, followed by the ATG initiation codon and the ODC coding sequence.

Plasmid pODC-CPMV was cut with *Bgl*II at position 189 of the CPMV sequence, filled in with Klenow polymerase, and religated, resulting in pODC-CPMV-BglII. All these manipulations were checked by T7 DNA polymerase sequencing (Pharmacia).

Eukaryotic initiation factors of protein synthesis were purified by published procedures (2, 32). The purity of eIF-4F was tested in three types of assay. An eIF-4A-dependent translational assay (32) was not stimulated by eIF-4F, whereas eIF-4F did not contain a (general) stimulatory factor, as encephalomyocarditis virus RNA translation in reticulocyte lysates was not stimulated by extra eIF-4F. Furthermore, cross-linking of eIF-4F to reovirus mRNA did not reveal eIF-2, eIF-3, or eIF-4B contamination, which was also checked by immunoblotting with an amount of factor fivefold higher than that used in translation assays (32a).

The free energies of the 5′ UTRs were calculated as described before (39).

RESULTS

CPMV M-RNA has two functional translation initiation sites. The question was addressed whether both initiation events arise from recognition of the 5′ end of the RNA and scanning towards the initiation codon (13, 14) or by internal initiation, as described for poliovirus (23), encephalomyocarditis virus (11, 12), and foot-and-mouth disease virus (1). To this end, first the Mg^{2+} dependence for translation at both sites was determined with in vitro-transcribed M-RNA (Fig. 1). Figure 1A shows the fluorogram after electrophoretic separation of the proteins synthesized in the reticulocyte lysate from CPMV M-RNA in the presence of different amounts of Mg^{2+}. The two main products are the expected 105- and 95-kDa proteins, synthesized by initiation at nt 161 and 512, respectively (the migration of the 105-kDa protein is similar to that of the subunits of β-galactosidase, with a molecular mass of 116 kDa). Optimal translation was obtained at 0.6 mM Mg^{2+}; the incorporation was drastically inhibited at 0.2 mM and above 1.0 mM Mg^{2+}.

The ratio of initiation at both sites leading to the 105- and 95-kDa proteins (36) was calculated from the data in Fig. 1A and plotted in Fig. 1B. The change in Mg^{2+} concentration had a clear effect on the selection for initiation at nt 161 or 512, the latter being dominant at lower Mg^{2+} concentrations. At higher Mg^{2+} concentrations, when total protein synthesis was already inhibited, synthesis of the longer protein was predominant. A likely explanation for this result is that the local secondary structure at the two initiation sites is different. However, translation at different potassium concentrations affected only total protein synthesis, not the ratio of 105- and 95-kDa proteins synthesized (not shown).

To investigate whether the difference in efficiency of translation can be manipulated with initiation factors of protein synthesis, translation of CPMV-RNA was performed in reticulocyte lysates in the presence of additional amounts of the different known eukaryotic protein synthesis initiation factors. It has been shown for a recombinant mRNA containing the influenza virus 5′ UTR mRNA that eIF-2 influenced the choice of potentially active initiation sites (4). Therefore, it was expected that the addition of eIFs would lead to changes in the 105-kDa/95-kDa protein ratio. However, Fig. 2 shows that the ratio produced after translation was not changed after addition of the initiation factors eIF-2/2B (lanes 9 and 10), eIF-4A (lanes 5 and 6), eIF-4B (lanes 7 and 8), and eIF-4F (lanes 3 and 4) compared with translations in the absence of added eIFs (lanes 1 and 2). The initiation factors eIF-1, eIF-1A, eIF-3, and eIF-5 gave essentially the same results (not shown). However, eIF-4F unexpectedly stimulated translation of the noncapped M-RNA about fourfold (Table 1), whereas all other factors only slightly changed [^{35}S]methionine incorporation. This

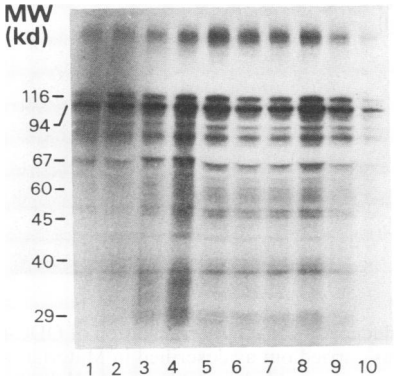

FIG. 2. Initiation factor dependence of CPMV M-RNA translation. Translation of pTM1-derived CPMV RNA in 10-μl assay mixes at 0.6 mM magnesium acetate was done as described in Materials and Methods. Two microliters was used for determination of [^{35}S]methionine incorporation, and the remaining 8 μl was analyzed by SDS-PAGE. Only 2 μl instead of 8 μl was analyzed in the assays containing extra eIF-4F. Lanes 1 and 2 duplicate buffer controls; lane 3, 0.14 μg of eIF-4F; lane 4, 0.28 μg of eIF-4F; lane 5, 0.13 μg of eIF-4A; lane 6, 0.26 μg of eIF-4A; lane 7, 0.1 μg of eIF-4B; lane 8, 0.2 μg of eIF-4B; lane 9, 0.3 μg of eIF-2 plus eIF-2B; lane 10, 0.6 μg of eIF-2 plus eIF-2B. This amount of eIF-2 reduced total incorporation in this assay, but similar results were obtained with smaller amounts. The amount of eIFs tested was sufficient in model assays for maximal translation. Molecular mass standards are indicated.

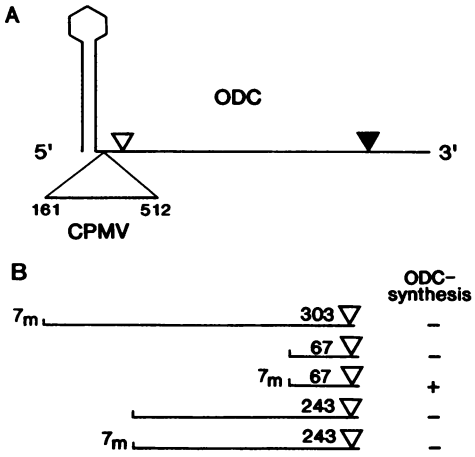

FIG. 3. Schematic representation of ODC-CPMV. (A) ODC, ODC coding sequence; CPMV, sequence nt 161 to 512 of the M-RNA. The structure at the 5′ end schematically indicates the ODC 5′ UTR. Open triangle, initiation site of ODC; solid triangle, termination site. (B) ODC mRNAs used in this study. The numbers indicate the number of nucleotides in the 5′ UTR. Wild-type ODC RNA has a 303-nt 5′ UTR. ^{7}m indicates the presence of a cap structure. The open triangle is the initiation site of translation. The ODC synthesis of the different constructs is indicated (for details, see Fig. 4).

indicates that all the eIFs were present in a reticulocyte lysate in a concentration optimal for CPMV M-RNA translation except for eIF-4F.

The stimulation of M-RNA translation by eIF-4F was unexpected, because M-RNA is a naturally noncapped messenger and eIF-4F was reported to stimulate translation of capped mRNAs only, not of noncapped mRNAs such as mengovirus, encephalomyocarditis virus, and poliovirus mRNAs (2, 5, 16, 31). However, Fletcher et al. (8) showed that uncapped southern bean tobacco necrosis virus translation was stimulated by eIF-4F. Furthermore, eIF-4F was active with capped and noncapped RNAs in a model assay, the melting of synthetic, partially double-stranded RNA-RNA or RNA-DNA hybrids (15, 27).

TABLE 1. Dependence of CPMV M-RNA translation on initiation factors[a]

Addition	Amt (μg)	[^{35}S]Methionine incorporation (cpm/2 μl)
Buffer		22,059
eIF-1	0.15	16,819
	0.30	21,977
eIF-2 + eIF-2B	0.5	20,279
	1.0	23,370
eIF-3	3.2	26,369
	6.4	28,988
eIF-4A	0.2	24,960
	0.4	27,247
eIF-4B	0.13	25,755
	0.26	26,835
eIF-4C	0.1	20,936
	0.2	19,053
eIF-4F	0.21	80,809
	0.42	107,746
eIF-5	0.06	18,456
	0.12	16,714

[a] Translation in the absence of mRNA was 2,080 cpm/2 μl.

The length of the 5′ sequence upstream of the initiation site at nt 512, the presence of a nonfunctional AUG at nt 115 (36), the sequence context of the two initiation AUGs (13, 14), and the similarity of the CPMV genome with picornavirus RNAs (9) suggested to us that initiation on CPMV M-RNA may be the result of internal initiation rather than recognition of the 5′ end and leaky scanning to the first and second initiator AUGs. There is no AUG between nt 161 and 512 of the CPMV 5′ sequence (36); in picornaviruses several upstream AUGs exist, which was one of the arguments against 5′-end recognition and scanning in picornavirus 5′ UTRs.

The question whether CPMV M-RNA initiates translation at position 512 according to the scanning model (14) or according to the internal binding model (11, 12, 23) was addressed as follows. The ODC mRNA has a highly structured 5′ UTR that prevents efficient translation of its gene (10, 18, 35). This 5′ UTR was used to block ribosomal entry from the 5′ end of the message in a way similar to that described for the poliovirus internal ribosome entry site (IRES) (23). The CPMV sequence nt 161 to 512 was inserted downstream of the ODC 5′ UTR and just upstream of the ODC initiation codon (Fig. 3A). The resulting ODC-CPMV DNA was transcribed, and capped and noncapped mRNAs were translated in reticulocyte lysates. Two controls were included (Fig. 3B): ODC67 RNA, which is a T7 transcript of

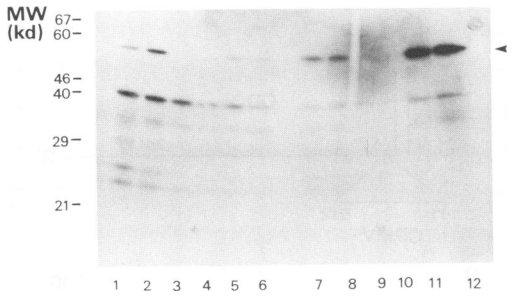

FIG. 4. Translation of capped and noncapped ODC-CPMV RNA. Plasmids were linearized and transcribed under capping and noncapping conditions as described in the text. Odd-numbered lanes, 75 ng of transcript; even-numbered lanes, 150 ng of transcript. Lanes 1 to 6, noncapped transcripts; lanes 7 to 12, capped transcripts. Lanes 1, 2, 7, and 8, ODC-CPMV; lanes 3, 4, 9, and 10, ODC243; lanes 5, 6, 11, and 12, ODC67. The arrow indicates the ODC protein. Molecular mass standards are indicated.

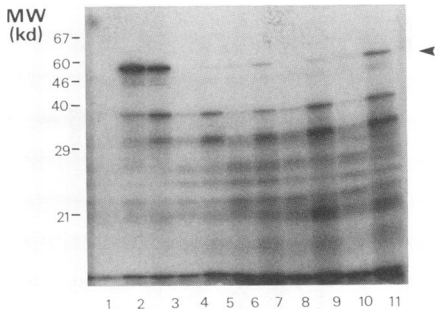

FIG. 6. Influence of eIF-4F on translation of ODC-CPMV RNA. Translation was carried out as described in Materials and Methods. eIF-4F (0.21 μg) was added in odd-numbered lanes. Lane 1, no mRNA added; lanes 2 and 3, ^{7}m-ODC67; lanes 4 and 5, ^{7}m-ODC243; lanes 6 and 7, ^{7}m-ODC-CPMV; lanes 8 and 9, ODC243; lanes 10 and 11, ODC-CPMV. Molecular mass standards are indicated. The arrow indicates ODC protein.

the ODC cDNA with a truncated 67-nt 5′ UTR, that supports cap-dependent ODC translation; and ODC243, which has a 243-nt 5′ UTR. Figure 4 shows that ^{7}m-ODC243 (the capped form of ODC243) gave only low synthesis of full-length ODC (about 55 kDa) compared with that by ^{7}m-ODC67. This observation is described in detail elsewhere in a study on the inhibitory action of the ODC 5′ UTR (35). Like wild-type RNA (303-nt 5′ UTR) (not shown), ODC243 was a very inactive transcript, as expected from the calculated free energy of the 5′ UTRs, −180 and −130 kcal/mol (39). The insertion of nt 161 to 512 from the CPMV sequence into the ODC 5′ UTR (ODC-CPMV) increased ODC synthesis considerably (Fig. 4, lanes 7 and 8), suggesting that this insertion enabled ribosomes to enter downstream of the cap and the ODC 5′ UTR.

This suggestion was confirmed by the translation of the noncapped forms of the transcripts. The absence of a cap structure on ODC67 caused a drastic decrease in ODC synthesis (Fig. 4, lanes 5, 6, 11, and 12), as expected from a noncapped transcript. The ODC-CPMV construct, however, was not handicapped by the absence of the cap structure (lanes 1 and 2), indicating cap-independent initiation on the CPMV sequence. Remarkably, the synthesis driven by ODC243 (lanes 3 and 4) was now mainly of truncated forms of ODC (about 32 and 40 kDa). This observation will be discussed below.

The cap-independent initiation on internal sequences was investigated further by measuring the effect of the addition of a cap analog, ^{7}mGpppG, to the translation mixtures of capped and noncapped transcripts (Fig. 5). Capped and noncapped transcripts of ODC-CPMV, ODC243, and ODC67 were added in equal amounts to translation assays. Translation of ^{7}m-ODC67 was sensitive to ^{7}mGpppG addition (Fig. 5B, lanes 5 and 6), whereas the synthesis of ODC driven by ^{7}m-ODC-CPMV was not (lanes 1 and 2). The low synthesis of full-length ODC by ^{7}m-ODC243 (Fig. 5B, lanes 3 and 4) was also not cap analog sensitive, an observation discussed later. The same results were found when twice as much transcript was translated (Fig. 5C), indicating that the RNA concentration did not influence the results. Noncapped ODC67 translated poorly (Fig. 5A); longer exposures showed no cap analog inhibition of ODC synthesis (not shown). ODC-CPMV translation was not cap analog sensitive, as expected. The results indicate that irrespective of the presence of a capped 5′ end, ODC-CPMV translation was not cap analog sensitive, indicating 5′-end-independent internal initiation.

Translation of M-RNA was strongly stimulated by eIF-4F (Fig. 2). The question whether the transfer of the IRES of

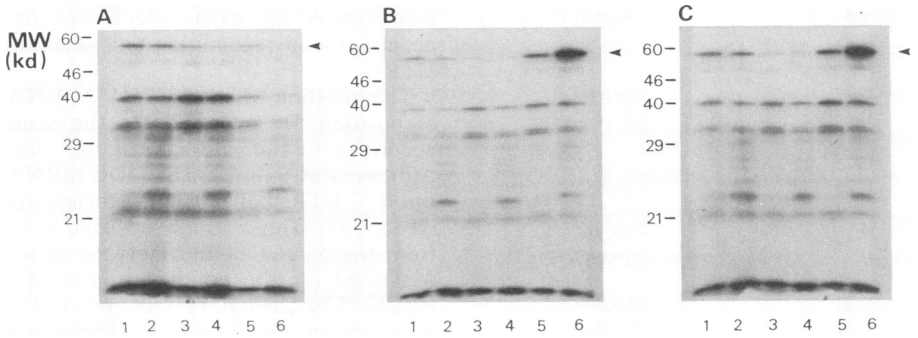

FIG. 5. Influence of cap analog on translation of ODC-CPMV RNA. Capped and noncapped transcripts were prepared as described in Materials and Methods. Either 40 ng of noncapped transcript (A) or 40 ng (B) or 80 ng (C) of capped transcript was translated. Lanes 1 and 2, ODC-CPMV; lanes 3 and 4, ODC243; lanes 5 and 6, ODC67. ^{7}mGpppG was added to a final concentration of 150 μM in all odd-numbered lanes. Molecular mass standards are indicated. The arrow indicates the ODC protein.

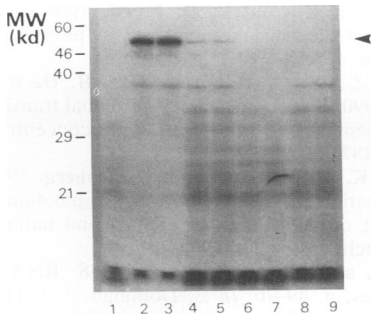

FIG. 7. Influence of a 4-nt insertion on translation of ODC-CPMV RNA. ODC-CPMV-BglII DNA was prepared as described in Materials and Methods. Transcripts (50 ng for the even-numbered lanes, 100 ng for the odd-numbered lanes) were translated as described in the text. Lane 1, no RNA added; lanes 2 and 3, ^{7}mODC67; lanes 4 and 5, ODC-CPMV; lanes 6 and 7, ODC-CPMV-BglII; lanes 8 and 9, ODC243. Molecular mass standards are indicated. The arrow indicates ODC protein.

CPMV M-RNA to ODC RNA also transferred eIF-4F dependence was investigated (Fig. 6). Capped ODC67-directed translation was not stimulated by exogenous eIF-4F (lanes 2 and 3), indicating that a sufficient amount of eIF-4F was present in the lysate to allow efficient cap-dependent translation. However, noncapped ODC-CPMV transcripts were better translated with extra eIF-4F (lanes 10 and 11). Apparently, the target for eIF-4F stimulation in the ODC-CPMV RNA resides in the nt 161 to 512 CPMV sequence. Translation of ODC243, whether capped or not, was slightly stimulated by eIF-4F (lanes 4, 5, 8, and 9), although the efficiency of translation remained well below that of ODC-CPMV. Translation of capped ODC-CPMV was also stimulated by eIF-4F (lanes 6 and 7).

Finally, confirmation of the specific stimulation of ODC translation by CPMV sequences was obtained by translating transcripts from DNA in which the *Bgl*II site at position 189 (of the CPMV-RNA) was filled in and the DNA was recircularized (ODC-CPMV-BglII). As shown in Fig. 7, ODC synthesis was prevented by the 4-nt insertion (lanes 6 and 7) compared with ODC-CPMV (lanes 4 and 5). Apparently, the mutation either prevented recognition of the IRES by ribosomes or initiation factors or prevented recognition of the initiator AUG.

A similar mutation was made in CPMV M-RNA (37). After translation, no 105-kDa protein was made, due to the 4-nt insertion, but the AUG at position 512 was still functional as an initiation signal (37). Apparently, the combination of the presence of the ODC 5′ UTR and the *Bgl*II mutation prevented recognition of the CPMV IRES, while in the M-RNA transcript the *Bgl*II mutation was not sufficient to prevent initiation.

Finally, the integrity of the transcripts after translation was checked to exclude the possibility that RNase action had created free 5′ ends on ODC-CDMV. The annihilation of internal initiation by the *Bgl*II mutation already made it highly unlikely that 5′-end-dependent initiation had occurred. Figure 8 shows that ODC-CPMV and ODC-CPMV-BglII were degraded in the lysate with essentially the same kinetics, excluding the possibility that ODC-CPMV translation was induced by a higher RNase sensitivity. ODC243 translated poorly, and the transcript had an even slightly higher stability (Fig. 8).

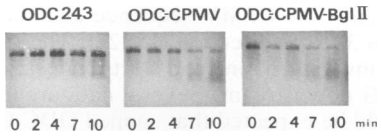

FIG. 8. Analysis of integrity of transcripts during translation. ODC243, ODC-CPMV, and ODC-CPMV-BglII were transcribed in the presence of [^{35}S]UTP. A 25-μl translation assay was initiated with approximately 80,000 cpm (about 100 ng) of transcript. At the times indicated, a 3-μl aliquot was transferred to a phenol-chloroform-H_2O (12.5:12.5:22) mixture, and after centrifugation, 15 μl was loaded onto a 1% agarose gel. After electrophoresis, the RNA was transferred to Hybond. The autoradiogram is shown.

DISCUSSION

This article describes the use of a very stable 5′ secondary structure to prevent cap-dependent entry of ribosomes into the ODC coding region. In this way, the increase in protein synthesis brought about by the insertion of a CPMV sequence indicates the insertion of an IRES (1, 11, 12, 23) in a very inefficient message. In the literature, an upstream gene has been used that prevented translation of the second gene (1, 11, 12, 23) due to the monocistronic character of eukaryotic genes. In our experimental set-up, no ribosomes were translating the RNA, as indicated by the lack of synthesis of ODC243, unless an IRES was introduced and translation was started.

The initiation of translation of CPMV M-RNA on both AUGs showed a different sensitivity to the Mg^{2+} concentration. The synthesis of the longer protein was more resistant to higher Mg^{2+} concentrations. This result suggested either that the secondary structure around each initiation site was different or that initiation occurred by different mechanisms. The initiation on nt 512 occurs by internal binding of the translational machinery, as shown in Fig. 4 to 6. No conclusion can be drawn for the initiation on nt 161, although we speculate that internal binding also governs initiation at this site. If the modified scanning model (14) applies for the AUG at nt 161, differences in initiation factor requirements would be expected. This proved not to be the case (Fig. 2). The results indicate that the nt 161 to 512 sequence of CPMV M-RNA constitutes an internal ribosome-binding site. Whether this site is able to direct the ribosome to both initiation codons or whether two IRES regions are present in the RNA has to be determined.

The many shorter products synthesized on noncapped ODC243 point to aberrant initiation. Our explanation is that the 5′ stem-loop structure masks the initiation site, forcing the translational machinery to other AUGs to start translation, as described for poliovirus RNA (30). A capped 5′ end would guide the ribosome to the 5′ end, which in the case of ODC243 is on the upstream side of the stable stem-loop structure. Therefore, after translation of ODC243, truncated products were made, especially with the non-capped RNA (Fig. 4). This aberrant form of initiation translation can also be regarded as internal binding. However, the results with ODC243 show that this type of scanning for internal AUGs is very inefficient. Apparently, some picornaviruses and now also a comovirus (and a potyvirus [3]) have found a way to more efficiently guide the ribosome along internal sequences to an internal AUG. The trigger for internal binding is probably the recognition of a defined (internal) sequence by an initiation factor. The eIF-4 initiation factors are the most likely candidates. It could be hypothesized that the eIF-4

factors recognize the CPMV sequence and start unwinding upstream in a 3′ to 5′ direction (15, 27). The ribosome then binds to the unwound 5′ end and starts translation at the first initiator AUG downstream. Several data argue against this point. It would be expected that capped ODC-CPMV would show higher efficiency of translation in this model, which proved not to be the case (Fig. 4). Second, it has been shown that an IRES induces translation only of the downstream gene, not of the upstream gene (1, 11, 12, 23).

Furthermore, the ODC 5′ UTR inhibits translation of the downstream gene extremely potently, and this inhibition can only be overcome by considerable deletion of 5′ UTR regions (35).

Lastly, the *Bgl*II mutation (Fig. 7) can hardly be regarded as an obstacle for a hypothetical 3′ to 5′ movement and 5′-end-dependent initiation of the ribosome.

The need for eIF-4F in supporting internal initiation on ODC-CPMV RNA (Fig. 6) was surprising. The factor does not have a general stimulatory effect; for example, encephalomyocarditis virus translation was not stimulated by eIF-4F (12; results not shown). Compared with capped 5′-end constructs, the CPMV IRES apparently has a low affinity for eIF-4F, and higher amounts of this factor are needed for optimal translation.

The eIF-4F stimulation of CPMV M-RNA and ODC-CPMV translation (Fig. 2 and 6) and the cap analog sensitivity (Fig. 5, not shown for M-RNA) are two unlinked phenomena, suggesting that part of the eIF-4F function is not dependent on the action of eIF-4E. This suggestion is strengthened by the observation that translation of non-capped reovirus mRNA was also stimulated by eIF-4F (24). The same suggestion was made by Fletcher et al., who measured the eIF-4F dependence of uncapped STNV translation (8). One can speculate that it is the unwinding activity of eIF-4F that is needed for initiation on CPMV M-RNA.

It can be argued that the CPMV sequence insertion into the stable ODC 5′ UTR destroys the inhibitory secondary structure, allowing initiation to occur. The standard free energy of the 243-nt ODC 5′ UTR was calculated to be −130 kcal/mol (39). This very stable structure is formed with almost only GC pairs, without many bulges or internal loops (34). The nt 161 to 512 sequence of CPMV M-RNA is very AU-rich (36) and therefore cannot interfere with the ODC structure.

The ODC-CPMV *Bgl*II mutant showed that a 4-nt insertion prevented efficient translation (Fig. 7), pointing to the importance of intactness of the CPMV stretch for efficient translation. Furthermore, the *Bgl*II-induced decrease in initiation is not compatible with the suggestion that internal initiation on ODC-CPMV RNA is induced by RNase-mediated generation of free 5′ ends.

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REFERENCES

1. **Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64**:5389–5395.
2. **Buckley, B., and E. Ehrenfeld.** 1987. The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. J. Biol. Chem. **262**:13599–13606.
3. **Carrington, J. C., and D. D. Freed.** 1990. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. **64**:1590–1597.
4. **Dasso, M. C., S. C. Milburn, J. W. B. Hershey, and R. J. Jackson.** 1990. Selection of the 5′-proximal translation initiation site is influenced by mRNA and eIF-2 concentrations. Eur. J. Biochem. **187**:361–371.
5. **Edery, I., K. A. W. Lee, and N. Sonenberg.** 1984. Functional characterization of eukaryotic mRNA cap binding protein complex: effect on translation of capped and naturally uncapped RNAs. Biochemistry **23**:2456–2462.
6. **Eggen, R., and A. Van Kammen.** 1988. RNA replication in comoviruses, p. 49–70. *In* E. Domingo, J. J. Holland, and P. Ahlquist, (ed.), RNA genetics, vol. 1. CRC Press, Boca Raton, Fla.
7. **Eggen, R., J. Verver, J. Wellink, A. De Jong, R. Goldbach, and A. van Kammen.** 1989. Improvements of the infectivity of in vitro transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. Virology **173**:447–455.
8. **Fletcher, L., S. D. Corbin, K. S. Browning, and J. M. Ravel.** 1990. The absence of a m^7G cap on β-globin and alfalfa mosaic virus RNA 4 increases the amounts of initiation factor 4F required for translation. J. Biol. Chem. **265**:19582–19587.
9. **Franssen, H., J. Leunissen, R. Goldbach, G. Lomonossoff, and D. Zimmern.** 1984. Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. EMBO J. **3**:855–861.
10. **Grens, A., and P. J. Scheffler.** 1990. The 5′- and 3′-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. J. Biol. Chem. **265**:11810–11816.
11. **Jang, S. K., H.-G. Kraüsslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
12. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63**:1651–1660.
13. **Kozak, M.** 1988. A profusion of controls. J. Cell Biol. **107**:1–7.
14. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
15. **Lawson, T. G., K. A. Lee, M. M. Maimone, R. D. Abramson, T. E. Dever, W. C. Merrick, and N. Sonenberg.** 1989. Dissociation of double-stranded polynucleotide helical structures by eukaryotic initiation factors, as revealed by a novel assay. Biochemistry **28**:4729–4734.
16. **Lee, K. A. W., and N. Sonenberg.** 1982. Inactivation of cap-binding proteins accompanies the shut-off of host protein synthesis by poliovirus. Proc. Natl. Acad. Sci. USA **79**:3447–3451.
17. **Lomonossoff, G. P., and M. Shanks.** 1983. The nucleotide sequence of cowpea mosaic virus B RNA. EMBO J. **2**:2253–2258.
18. **Manzella, J. M., and P. J. Blackshear.** 1990. Regulation of rat ornithine decarboxylase mRNA translation by its 5′-untranslated region. J. Biol. Chem. **265**:11817–11822.
19. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
20. **Pelham, H. R. B.** 1979. Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. Virology **96**:463–477.
21. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
22. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8**:1103–1112.
23. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived

from poliovirus RNA. Nature (London) **334**:320–325.
24. **Ray, B. K., T. G. Brendler, S. Adya, S. Daniels-McQueen, J. K. Miller, J. W. B. Hershey, J. A. Grifo, W. C. Merrick, and R. E. Thach.** 1983. Role of mRNA competition in regulating translation: further characterization of mRNA discriminatory initiation factors. Proc. Natl. Ac. Sci. USA **80**:663–667.
25. **Rezelman, G., A. van Kammen, and J. Wellink.** 1989. Expression of cowpea mosaic virus M RNA in cowpea protoplasts. J. Gen. Virol. **70**:3043–3050.
26. **Rhoads, R. E.** 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. Trends Biol. Sci. **13**:52–56.
27. **Rozen, F., I. Edery, K. Meerovitch, T. E. Dever, W. C. Merrick, and N. Sonenberg.** 1990. Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol. Cell. Biol. **10**:1134–1144.
28. **Sonenberg, N.** 1987. Regulation of translation by poliovirus. Adv. Virus Res. **33**:175–204.
29. **Sonenberg, N.** 1989. Poliovirus translation: a paradigm for a novel initiation mechanism. BioEssays **11**:128–132.
30. **Svitkin, Y. V., T. V. Pestova, S. V. Maslova, and V. I. Agol.** 1988. Point mutations modify the response of poliovirus RNA to a translation initiation factor: a comparison of neurovirulent and attenuated strains. Virology **166**:394–404.
31. **Tahara, S. M., M. A. Morgan, and A. J. Shatkin.** 1981. Two forms of purified m^7G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. J. Biol. Chem. **256**:7691–7694.
32. **Thomas, A., H. Goumans, H. Amesz, R. Benne, and H. O. Voorma.** 1979. A comparison of the initiation factors of eukaryotic protein synthesis from ribosomes and from the postribosomal supernatant. Eur. J. Biochem. **98**:329–337.
32a. **van Heugten, H. A. A., M. A. M. Kasperaitis, A. A. M. Thomas, and H. O. Voorma.** Evidence that eukaryotic initiation factor (eIF) 2 is a cap-binding protein that stimulates cap recognition by eIF-4B and eIF-4F. J. Biol. Chem., in press.
33. **Van Kranen, H. J., L. Van de Zande, C. F. Van Kreijl, A. Bisschop, and B. Wieringa.** 1987. Cloning and nucleotide sequence of rat ornithine decarboxylase cDNA. Gene **60**:145–155.
34. **Van Steeg, H., C. T. M. Van Oostrom, H. M. Hodemaekers, and C. F. Van Kreyl.** 1990. Cloning and functional analysis of the rat ornithine decarboxylase-encoding gene. Gene **93**:249–256.
35. **Van Steeg, H., C. T. M. van Oostrom, H. M. Hodemaekers, L. Peters, and A. A. M. Thomas.** 1991. The translation in vitro of rat ornithine decarboxylase mRNA is blocked by its 5′ untranslated region in a polyamine-independent way. Biochem. J. **274**:521–526.
36. **Van Wezenbeek, P., J. Verver, J. Harmsen, P. Vos, and A. Van Kammen.** 1983. Primary structure and gene organization of the middle-component RNA of cowpea mosaic virus. EMBO J. **2**:941–946.
36a. **Verver, J., and J. Wellink.** Unpublished data.
37. **Vos, P., J. Verver, P. Van Wezenbeek, A. Van Kammen, and R. Goldbach.** 1984. Study of the genetic organisation of a plant viral RNA genome by in vitro expression of a full-length DNA copy. EMBO J. **3**:3049–3053.
38. **Vos, P., J. Verver, M. Jaegle, J. Wellink, A. van Kammen, and R. Goldbach.** 1988. Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. Nucleic Acids Res. **16**:1967–1985.
39. **Zuker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamic and auxilliary information. Nucleic Acids Res. **9**:133–148.