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A Segment of the 5′ Nontranslated Region of Encephalomyocarditis Virus RNA Directs Internal Entry of Ribosomes during In Vitro Translation

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Picornavirus RNAs are uncapped messengers and have unusually long 5′ nontranslated regions (5′NTRs) which contain many noninitiating AUG triplets. The translational efficiency of different picornavirus RNAs varies between different cell-free extracts and even in the same extract, such as micrococcal nuclease-treated rabbit reticulocyte lysates. The effect of the poliovirus 5′NTR on in vitro translation was compared with that of the 5′NTR of encephalomyocarditis virus by the use of synthetic mRNAs, micrococcal nuclease-treated HeLa cell extracts, and rabbit reticulocyte lysates. Artificial mono- and dicistronic mRNAs synthesized with T7 RNA polymerase were used to investigate whether the 5′NTR of encephalomyocarditis virus RNA contains a potential internal ribosomal entry site. The sequence between nucleotides 260 and 484 in the 5′NTR of encephalomyocarditis RNA was found to play a critical role in the efficient translation in both mono- and dicistronic mRNAs. Our data suggest that an internal ribosomal entry site resides in this region.

The *Picornaviridae* comprise one of the largest families of animal viruses whose single-stranded RNA genomes, on the average 7,500 nucleotides (nt) long, have plus-strand polarity. Genomic structure, gene organization, and replication of these viruses are similar (27, 37, 43). The subject of this paper is an in vitro study of translational control mediated by the 5′ nontranslated regions (5′NTRs) of two prominent picornaviruses: poliovirus, which belongs to the genus *Enterovirus*, and encephalomyocarditis virus (EMCV), which belongs to the genus *Cardiovirus*.

Poliovirus and EMCV genomic RNAs, like most eucaryotic mRNAs, are polyadenylated at their 3′ ends. Their 5′ termini, however, are not conventionally capped (2); that is, they lack the 7-methyl guanosine triphosphate group but are covalently linked to a virus-encoded oligopeptide (VPg) (12, 14, 30). Poliovirus-specific polysomal mRNA, isolated from infected cells, is identical to the virion RNA in nucleotide sequence. However, its 5′ terminus is pUp; that is, it lacks VPg (17, 35, 36, 42), and this is probably true for all picornaviruses (16). HeLa cell extracts and rabbit reticulocyte lysates (RRL) contain an enzyme activity that cleaves VPg from the 5′ terminus of picornavirus RNA in vitro (1), and it has been suggested that this activity modifies all newly synthesized viral RNAs destined to be mRNAs in vivo (27). Picornavirus mRNAs encode a single polyprotein which is proteolytically processed to yield mature capsid and noncapsid proteins (26, 34). The initiation sites for translation of all picornavirus polyproteins are preceded by unusually long 5′NTRs ranging from 650 to 1,300 nt (37). Moreover, the 5′NTRs of EMCV and foot-and-mouth disease virus (a picornavirus which belongs to the genus *Aphthovirus*) contain a stretch of poly(C), located approximately 200 to 300 nt downstream of the 5′ terminus, the function of which is obscure. The 5′NTR of EMCV consists of 718 heteropolymeric nt and a poly(C) tract (115 nt long) that is located between nt 149 and 263. Ten AUG codons, scattered throughout the 5′NTR, precede the AUG codon at which synthesis of the EMCV polyprotein initiates (38). The initiation AUG codon of the poliovirus polyprotein is located at nt 743 and is preceded by eight other AUG triplets, scattered throughout the 5′NTR (7).

Internal initiation sequences for translation several thousand bases downstream from the 5′ end occur within the coding frame of poliovirus RNA (9). These initiation sequences are recognized when poliovirus RNA is translated in RRL but not in HeLa cell lysates. The use and role of these sites in vivo, if any, is not clear (9). Moreover, internal initiation of translation in RRL is suppressed by supplementation with a HeLa cell extract (9).

EMCV RNA is an excellent messenger in micrococcal nuclease-treated RRL and yields a pattern of protein products that closely resembles that seen in infected cells (41). The efficiency of translation of EMCV RNA is not changed when the 5′-terminal portion [up to and including the poly(C) tract] of the 5′NTR is removed. In this regard, EMCV RNA is very similar to foot-and-mouth disease virus RNA, in which a cleavage within its poly(C) does not reduce the translational efficiency of the larger fragment (44). In contrast to EMCV RNA, poliovirus RNA is a poor messenger in RRL, and modifications (deletions) of the 5′NTR strongly affect translational efficiency in RRL (33).

The ribosome-scanning model has been accepted for most eucaryotic mRNAs to explain the initiation process of translation (21, 22). In this model, a 40S ribosomal subunit binds initially to the 5′ end of the mRNA and migrates along the RNA chain until it encounters the first AUG triplet. If the first AUG codon occurs in the optimal sequence context (ACCAUGG), most, if not all, 40S subunits stop there; that AUG serves as the unique site of initiation. Mechanisms of leaky scanning and reinitiation of 40S ribosomal subunits have been proposed to explain the initiation of translation at AUG codons downstream of the first AUG or the translation of eucaryotic dicistronic messengers (23; reviewed in reference 24). Internal initiation of translation, independent of the

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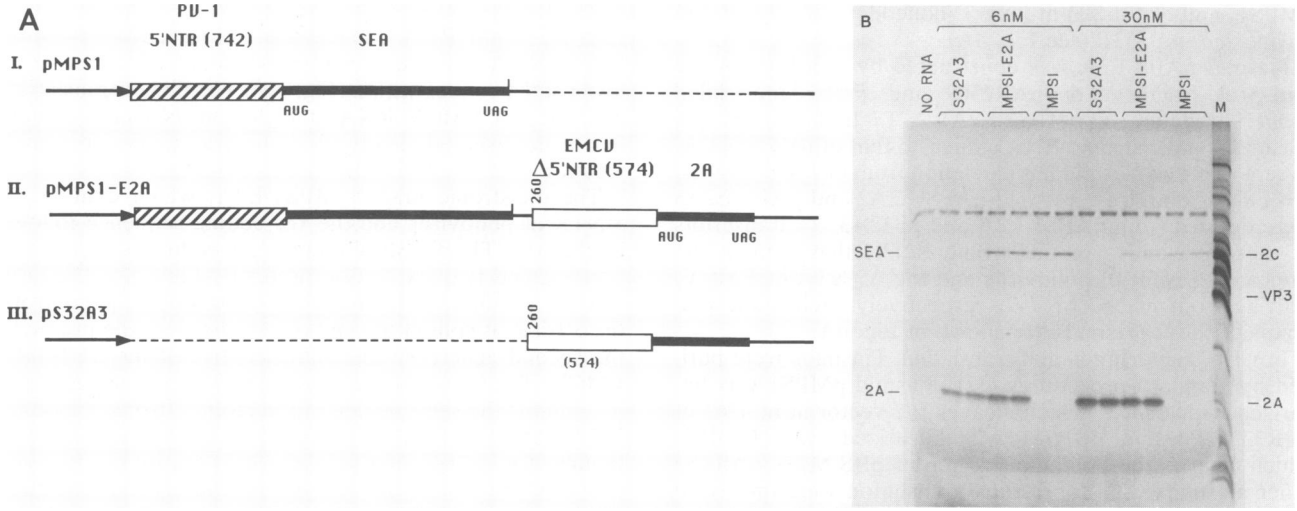

FIG. 1. Effect of RNA concentration on translation of mono- and dicistronic mRNA in RRL. (A) Schematic diagrams of the portion of plasmids that are transcribed to yield mono- or dicistronic mRNAs. Hatched boxes represent the 5'NTR of poliovirus type 1 (Mahoney), and open boxes represent the Δ5'NTR of EMCV. Solid bars represent the *sea* and poliovirus 2A-coding sequences. Arrowheads indicate the location of T7 promoters. Thin lines represent either flanking sequences which follow the *sea* gene in plasmid pS13P or vector sequences. Dotted lines are used to align corresponding sequences. The initiation and the termination codons of indicator genes are indicated as AUG and UAG, respectively. Numbers written vertically indicate the nucleotide number at which the Δ5'NTR of EMCV starts. Full-length 5'NTRs were used for poliovirus mRNAs. Numbers within parentheses indicate the sizes of the 5'NTRs of poliovirus or Δ5'NTRs of EMCV. Most vector sequences are not shown. (B) Effect of RNA concentration on translation of mono- and dicistronic mRNAs. Two different RNA concentrations (6 or 30 nM as indicated above the gel) were used in translation reactions in RRL. Each reaction was duplicated. Samples (2 μl) of translation mixtures were diluted to 14 μl with loading buffer (28) and analyzed on sodium dodecyl sulfate–13.5% polyacrylamide gels. Note that endogenous labeling of a band with a mobility similar to that of 2BC in marker (M) lane occurred throughout. M indicates a lysate of [^{35}S]methionine-labeled poliovirus-infected HeLa cells.

upstream cistron in an mRNA molecule, on the other hand, has never been proven to occur in eucaryotic protein synthesis.

The unusually long 5'NTRs, the absence of a cap structure, and the presence of a long poly(C) tract (in aphthoviruses and cardioviruses) make picornavirus RNA a conspicuously unusual eucaryotic mRNA. We have therefore addressed the question of whether the 5'NTRs of picornavirus RNAs contain potential internal ribosomal entry sites. We have used T7 RNA polymerase to synthesize artificial dicistronic messengers that contain, in the order from 5' to 3', the 5'NTR of poliovirus connected to the coding region of the *sea* oncogene (the first indicator gene) followed by the truncated 5'NTR (Δ5'NTR; from nt 260 to 833) of EMCV connected to the 2A-coding sequence of poliovirus (the second indicator gene). Runoff transcripts of various plasmid constructs and of derivatives with deletions in the 5'NTRs were analyzed for the translational efficiency on translation in RRL or in micrococcal nuclease-treated HeLa extracts. The data obtained can be interpreted to indicate that a specific, internal ribosomal entry site exists within the 5'NTR of EMCV.

MATERIALS AND METHODS

Construction of plasmids. Restriction enzymes and enzymes used in cloning and modifying DNAs were purchased from New England Biolabs, Inc., and Bethesda Research Laboratories, Inc. DNA manipulations were done by standard procedures (32). Plasmid pMPS1 was derived from plasmid pS13P containing viral oncogene *sea* (M. J. Hayman, unpublished data) and from plasmid pMN25 containing the promoter of gene 10 of phage T7 (33). T7 transcripts of pMPS1 contain the full-length 5'NTR and the first 8 nt of the coding sequence of poliovirus RNA, followed by the 1,048-nt *sea*-coding sequence and 95-nt noncoding sequences in pS13P (Fig. 1).

Plasmid pS32A3 was derived from pS32A (25) by linker insertion at the 3' end of the poliovirus 2A-coding sequences, which resulted in insertion of two termination codons at the end of 2A. T7 transcripts of this plasmid contain a Δ5'NTR (from nt 260 to 833) and the first 15 nt of the coding sequence of EMCV RNA followed by the last 3 nt of the coding sequence of the poliovirus P1 region and the entire coding sequence of the 2A gene of poliovirus (Fig. 1).

Plasmids pMPS1-E2A, pMPS1-E2A1, pMPS1-E2A2, and pMPS1-E2A3 were derived from pMPS1 and pS32A3. T7 transcripts of these plasmids contain the full-length 5'NTR of poliovirus and the *sea*-coding sequence as in pMPS1 followed by Δ5'NTRs of EMCV starting from nt 260, 485, 563, and 742, respectively. The coding sequence for the second indicator gene is 2A as in pS32A3.

Plasmid pAE2A1, pAE2A2, and pAE2A3 were derived from plasmid pS32A3 and pAR2369 (A. Rosenberg, J. J. Dunn, and F. W. Studier, unpublished data). T7 transcripts of these plasmids are composed of the 2A-coding sequence as in pS32A3 preceded by Δ5'NTRs of EMCV starting from nt 485, 563, and 742, respectively, as in pMPS1-E2A1, pMPS1-E2A2, and pMPS1-E2A3. Schematic diagrams of these plasmids are shown in Fig. 3A.

Plasmid pBPS11 was derived from plasmids pBS-M13+ (Vector Cloning Systems), pMN25, and pS13P. T7 transcripts of this plasmid contain a truncated 5'NTR of poliovirus (from nt 671 to 742), the *sea*-coding sequence as in

pMPS1, and about 800-nt-long 3'-noncoding sequences following *sea* in pS13P (see Fig. 4A).

Plasmid pAPS-E2A was constructed by a ligation of the *Pst*I-*Stu*I fragment of pAR2369 and *Pst*I-Klenow-filled *Bam*HI fragment of pMPS1-E2A. A T7 transcript of this plasmid is a dicistronic RNA similar to that of pMPS1-E2A, except that this plasmid has a deletion from nt 1 to 670 of the 5'NTR of poliovirus. Plasmid pAPS-E2A2 and pAPS-E2A3 were derived from pAPS1-E2A and pS32A3. T7 transcripts of these plasmids are dicistronic RNAs that contain the truncated 5'NTR of poliovirus and the Δ5'NTR of EMCV, starting from nt 567 or 742, respectively. Schematic diagrams of these plasmids are shown in Fig. 4A.

In vitro transcription and translation. Plasmids were purified by standard procedures (32). Plasmid pMPS1 was linearized with *Eco*RI, which cleaves the vector at nt 4361 of pBR322. Plasmid pBPS11 was linearized with *Hin*dIII, which cleaves the polylinker region of pBS-M13+. All the other plasmids used for in vitro translation were linearized with *Dra*I, which cleaves the vector at nt 3943 of pBR322. T7 RNA polymerase was a kind gift of John J. Dunn. Transcription reactions were performed as described previously (48). RNAs were dissolved in H_2O to give final concentrations of 6, 12, or 30 nM in the in vitro translation mixtures after the addition of 1 μl of the RNA stock solutions.

Synthetic RNAs were translated in RRL (Promega Biotech) or in HeLa extracts. The translations in RRL were performed essentially as described by the supplier. The reaction mixtures were incubated at 30°C for 30 min or as otherwise indicated. Incorporation of [^{35}S]methionine was determined by the method of Jackson and Hunt (18).

Preparation of cell-free extracts from HeLa cells and translation in these extracts were performed as described by Lee and Sonenberg (29), except that the final concentration of RNA was 12 nM and the incubation time was 30 min.

In vitro translation products were analyzed in sodium dodecyl sulfate–13.5% polyacrylamide gels using the buffer system of Laemmli (28) modified as described by Nicklin et al. (33). Gels shown in Fig. 2A and 3C were treated with En^{3}Hance (New England Nuclear Corp.). Kodak XAR-5 film was exposed to dried gels for 18 h or as otherwise indicated.

RESULTS

Effect of RNA concentration on the translation of mono- or dicistronic RNAs. Deletion of the 5'NTR of EMCV RNA up to nt 259 [including the poly(C) tract] has been found not to influence the translational efficiency of this messenger. This observation suggested to us that a translation-controlling element may be located between nt 260 and 833. We will refer to the 5'NTR of EMCV spanning nt 260 to 833 as Δ5'NTR. To investigate whether the 5'NTR of EMCV RNA contains a potential internal ribosomal entry site, artificial mono- and dicistronic mRNAs were synthesized by runoff transcription with phage T7 RNA polymerase. The plasmids used as templates for mRNA synthesis are shown in Fig. 1A. Monocistronic messenger MPS1 is a transcript of pMPS1 which consists of the full-length 5'NTR of poliovirus followed by the *sea* indicator gene, which encodes an oncogene product of 38 kilodaltons containing 10 methionine residues (M. J. Hayman, personal communication). MPS1 was translated less efficiently in RRL than was the monocistronic messenger S32A3, which contains a Δ5'NTR of EMCV followed by the coding sequence of the 2A proteinase of poliovirus (17 kilodaltons; six methionine codons) (10, 20). MPS1 RNA showed higher translational efficiency at 6 nM than at 30 nM RNA concentration (Fig. 1B). In contrast, S32A3 RNA showed lower translational efficiency at the lower RNA concentration (Fig. 1B). The higher translational efficiency of EMCV RNA at higher mRNA concentration has been observed also by R. J. Jackson (personal communication).

The dicistronic mRNA MPS1-E2A, which contains the 5'NTR of poliovirus and the *sea*-coding sequence followed by the Δ5'NTR of EMCV and the 2A-coding sequence (Fig. 1A), yielded two translation products (Fig. 1B). The larger was the product of the first gene (*sea*) under the control of the 5'NTR of poliovirus, and the smaller was the product of the second gene (2A) under the control of the Δ5'NTR of EMCV. The translational efficiency of the second gene (2A) was almost the same as that of the monocistronic messenger S32A3 at both high and low RNA concentrations, even though it was preceded by the poorly translated first gene (*sea*). At the higher RNA concentration, the translational efficiency of the first gene decreased similar to that of monocistronic RNA MPS1, whereas the translational efficiency of the second gene increased similar to that of monocistronic RNA S32A3. These results indicate that the translation of the second gene may be independent of translation of the first gene.

Kinetics of translation of mono- or dicistronic RNAs. Time course experiments were performed to analyze further the translational efficiency of the second gene in vitro. MPS1, MPS1-E2A, and S32A3 RNAs at 30 nM were used for translation in RRL. Detectable amounts of the 2A protein under the control of the Δ5'NTR of EMCV were observed within 4 min of incubation regardless of whether the gene occurred in the context of mono- or dicistronic RNAs (Fig. 2A, lanes II and III at 4 min). However, the *sea* gene product under the control of the 5'NTR of poliovirus was not detected until after 9 min of translation of either mono- or dicistronic RNAs (Fig. 2A, lanes I and II at 9 min). Although the ratio of the 2A product translated from mono- and dicistronic RNAs varied slightly at very early times in different sets of experiments, the times at which *sea* appeared relative to 2A was consistently delayed by several minutes.

The translational efficiency of the mono- and dicistronic mRNAs was normalized by dividing the measured radioactivity of each band by that of the protein product obtained after 20 min of incubation. Normalized translational efficiencies at each time point are shown in Fig. 2B. Fifty percent of maximal translation of 2A was reached after about 8 min of incubation from both mono- and dicistronic RNAs. By contrast, this stage was reached after about 11 min of incubation for *sea*. The longer period of translation necessary to observe *sea* may be due to the bigger size of *sea* relative to 2A. In any case, yields of 2A were much higher than those of *sea*. This is immediately apparent if one considers the intensity of the bands in Fig. 2A and the content of methionine residues in the polypeptide products (10 in *sea* and 6 in 2A). These observations strongly suggest that the translation of the second gene is independent from that of the first gene in the dicistronic mRNA MPS1-E2A.

Determination of a region critical for highly efficient translation of EMCV RNAs. Serial deletions were made in the Δ5'NTR of mono- or dicistronic RNAs (Fig. 3A) to identify a structural region in the Δ5'NTR of EMCV that might be responsible for the high translational efficiency and/or for the independence of translation of the second gene. Monocistronic RNA AE2A1, which contains a deletion up to nt 484

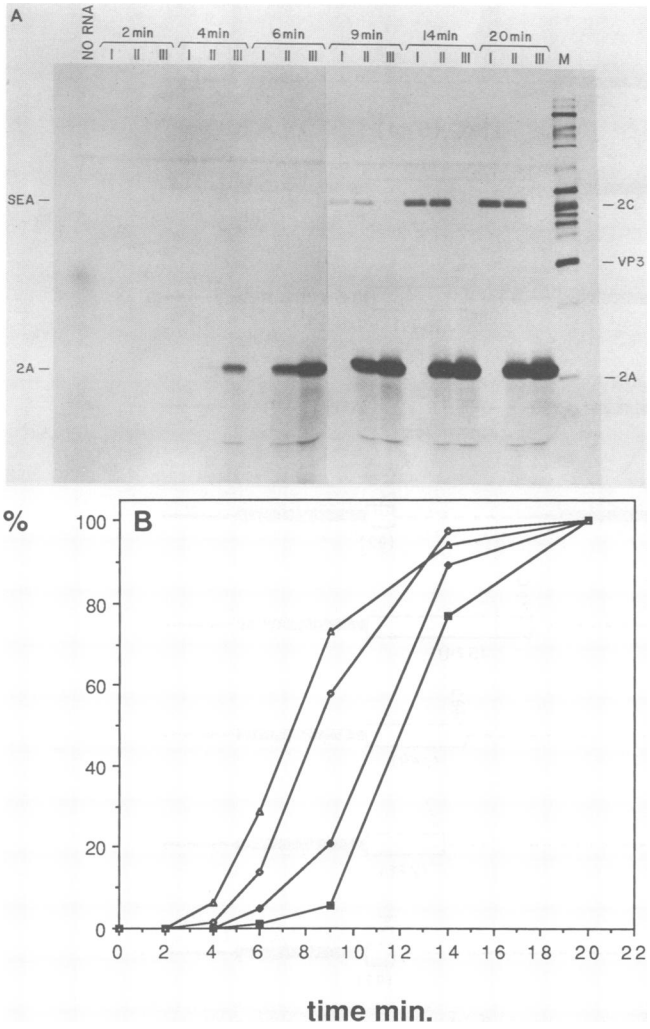

FIG. 2. Time course of mono- and dicistronic mRNA translation. (A) Kinetics of mono- and dicistronic mRNA translation in RRL. Reaction mixtures initially contained 30 nM RNA in a total volume of 60 μl. Samples (7 μl) were withdrawn from reaction mixtures at the indicated times and mixed with 15 μl of cold water; 22 μl of 2× loading buffer was added to each mixture. Samples (15 μl) were analyzed on sodium dodecyl sulfate–13.5% polyacrylamide gels which were then treated with En^{3}Hance. Two films were combined as one figure. Lanes I, II, and III indicate the translation products of RNAs MPS1, MPS1-E2A, and S32A3, respectively (see Fig. 1A for details). M indicates the same marker proteins as described in Fig. 1B. (B) Relative translational efficiency as a function of time. The radioactivity (counts per minute) of each protein in Fig. 2A was measured with the Ambis Beta Scanning System (Automated Microbiology Systems, Inc.), and the radioactivity of a specific protein is shown as a percentage of the radioactivity of the corresponding protein after 20 min of translation. Symbols: ■ and ◆, *sea* product synthesized by RNAs MPS1 and MPS1-E2A, respectively; ◇ and △, 2A product synthesized by RNAs MPS1-E2A and S32A3, respectively.

in the 5′NTR of EMCV (Fig. 3B), did not produce detectable amounts of 2A in RRL under conditions in which the undeleted RNA S32A3 produced large amounts of 2A (lanes AE2A1 and S32A3, respectively). However, RNAs resulting from further deletions up to nt 562 or 741 in the 5′NTR of EMCV were translated to yield 2A, albeit at a reduced level (Fig. 3B, lanes AE2A2 and AE2A3). Such partial reactivation of translation after extensive deletion may be due to translational readthrough from the 5′ termini of the RNAs. The second gene was not translated at all from derivatives of MPS1-E2A in which a similar set of deletions in the 5′NTR of EMCV had been engineered (Fig. 3A and B, lanes MPS1-E2A1, MPS1-E2A2, and MPS1-E2A3). These results suggest that a region critical for the high efficiency of the translation of EMCV RNA, as well as for the independence of the translation of a second gene in dicistronic RNA, is located between nt 260 and 484 of the 5′NTR of EMCV. The precise structure in EMCV 5′NTR responsible for these effects has not yet been determined.

Translation in HeLa cell extracts. We then investigated whether translation of these synthetic mRNAs in a cell-free extract of HeLa cells showed the same effect as in RRL. In HeLa extracts, the gene (*sea*) linked to the poliovirus 5′NTR showed higher translational efficiency than the gene (2A) linked to the EMCV Δ5′NTR (Fig. 3C, lanes MPS1 and S32A3). Monocistronic RNA deleted up to nt 484 of the 5′NTR of EMCV did not direct translation of any 2A product (Fig. 3A, lane AE2A1). Moreover, in contrast to the result in RRL, resumption of translation of the 2A gene in HeLa extracts did not occur after reduction in the size of the 5′NTR of EMCV in monocistronic messengers (Fig. 3C, lanes AE2A2 and AE2A3). These results indicate that the 5′NTRs of EMCV with deletions up to nt 741 lack element(s) required for efficient translation in HeLa extracts, although some of these deletion derivatives (AE2A2 and AE2A3) directed reduced levels of synthesis in RRL (Fig. 3B). The dicistronic RNA MPS1-E2A produced two translation products, and the translational efficiency of each product was almost the same as that from the corresponding monocistronic RNAs (Fig. 3C, compare lane MPS1-E2A with lanes MPS1 and S32A3). Further deletions in the 5′NTR of EMCV abolished translation of the second gene (Fig. 3C, lanes MPS1-E2A1, MPS1-E2A2, and MPS1-E2A3). Note that the deletion up to nt 484 of the 5′NTR of EMCV abolished the translation of 2A in HeLa extracts for both mono- and dicistronic messengers. This may indicate that the same internal structural element is responsible for the efficient functioning of the 5′NTR of EMCV in both mono- and dicistronic mRNAs, and that the contribution of the 5′ terminus per se for the translational efficiency is small.

Effect of an efficient first translation unit on the translation of the second cistron. Although the translational efficiency of the second cistron in dicistronic RNA was similar to that of the corresponding gene in monocistronic RNA, we observed consistently that the presence of a preceding cistron slightly stimulated the translation of the following cistron (Fig. 1B, lane 6 nM MPS1-E2A; Fig. 3C, lane MPS1-E2A). This effect could be explained if the 40S ribosomal subunit can reinitiate translation after its engagement in translation of the first cistron without dissociation from mRNA as postulated by Liu et al. (31). Another possible explanation for this phenomenon is that the translational machinery might enter the next round of translation by binding to a putative internal entry site in the 5′NTR of the second gene. Translation of the first gene might enhance the translation of the second gene by increasing the local concentration of components involved in translation near the putative internal entry site.

The following constructions (Fig. 4A) were made to address this question. The indicator gene *sea* was placed under the translational control of a poliovirus 5′NTR deleted up to nt 670, which has previously been shown to direct efficient translation of an indicator gene in RRL (33). BPS11 RNA, containing a truncated 5′NTR of poliovirus, stimulated effi-

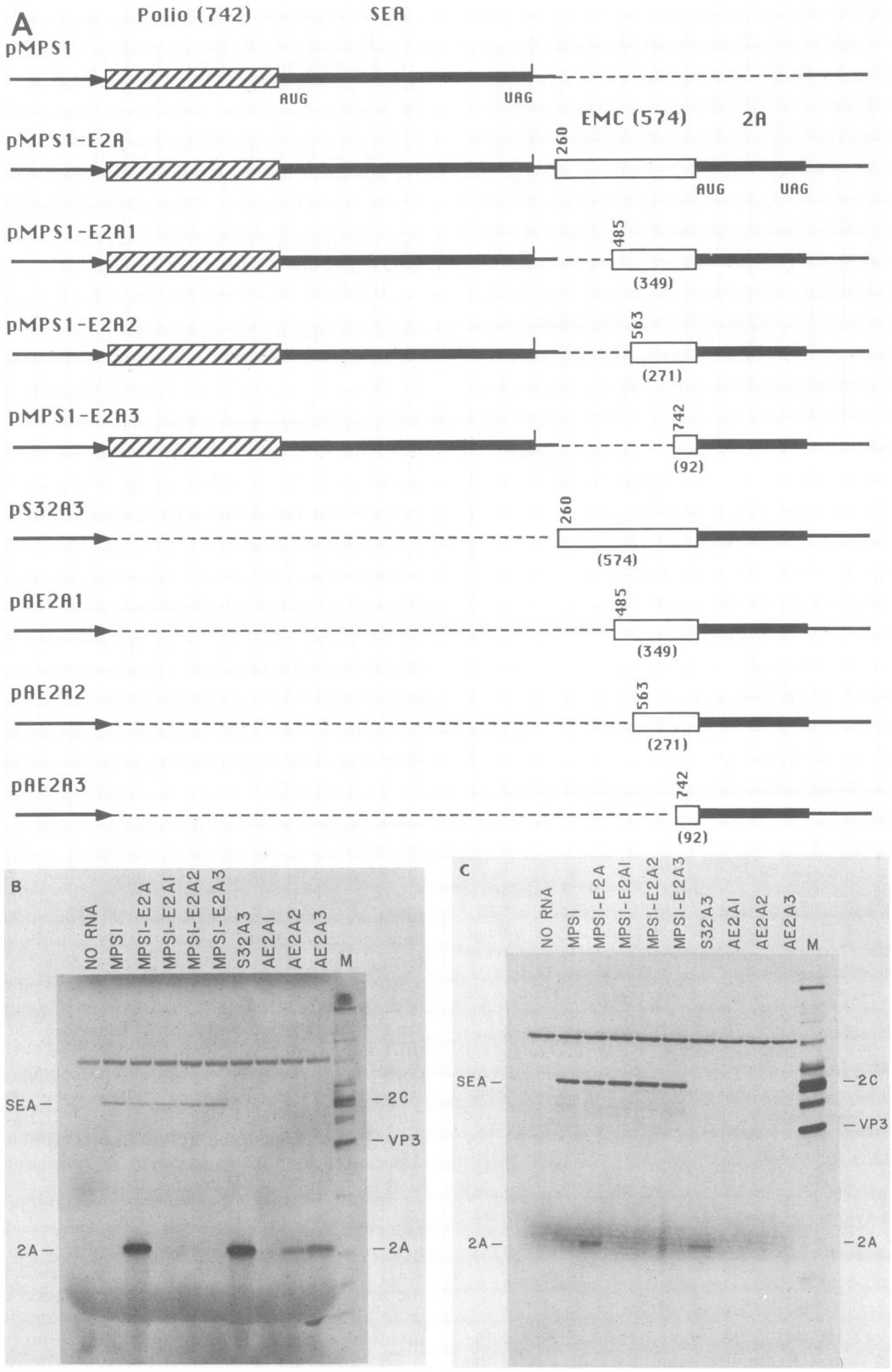

FIG. 3. Determination of the region in the 5'NTR of EMCV responsible for high translational efficiency and/or independent translation of the second gene. (A) Schematic diagrams of the portions of plasmids that are transcribed to yield synthetic mRNA. Symbols and letters are as described in the legend to Fig. 1A. Most of the vector sequences are not shown. (B) Effect of deletions in the 5'NTR of EMCV on mono- and dicistronic RNA translation in RRL. A concentration of 30 nM RNA was used in the translation reactions. Samples (3 μl) of translation mixtures were diluted to 7 μl with water, mixed with 7 μl of 2× loading buffer, and then analyzed on sodium dodecyl sulfate–13.5% polyacrylamide gel. M indicates the marker proteins described in the legend to Fig. 1B. (C) Effect of deletions in the 5'NTR of EMCV on mono- and dicistronic RNA translation in HeLa extracts. A concentration of 12 nM RNA was used in the translation reactions. Samples (3 μl) were analyzed as in B, except that the gel was treated with En^{3}Hance after electrophoresis. M indicates the same marker proteins as described in the legend to Fig. 1B.

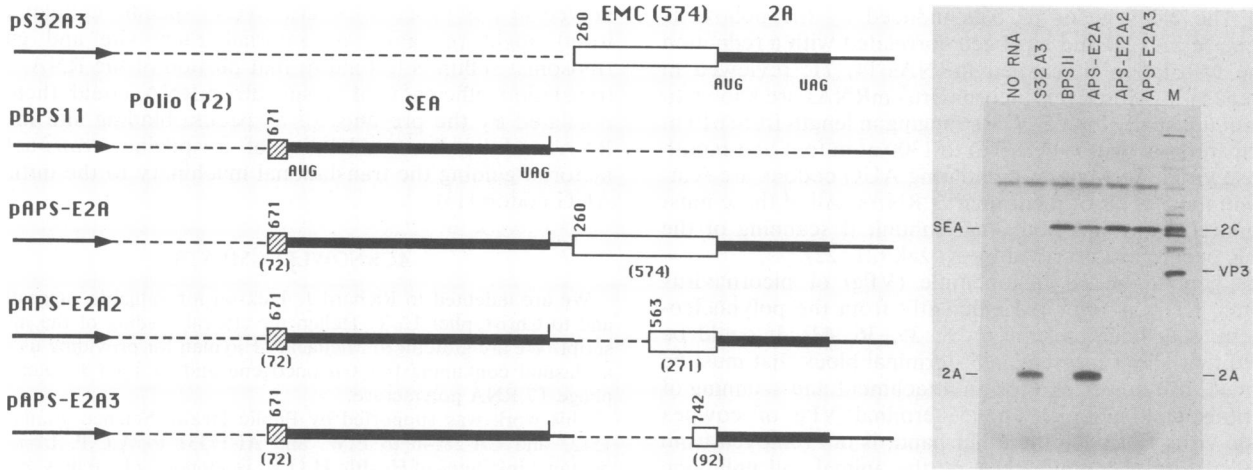

FIG. 4. Effect of a highly efficient first gene on second gene translation. (A) Schematic diagrams of the portions of plasmids that are transcribed to yield synthetic mRNAs. Symbols and letters are as described in Fig. 1A. Note that the first gene (*sea*) is under the control of the truncated 5'NTR of poliovirus. Most of the vector sequences are not shown. (B) Effect of the first gene under the control of the truncated 5'NTR of poliovirus on translation of the second gene in RRL. Analysis of 3-μl samples was done as described in the legend to Fig. 3B, except that the X-ray film was exposed for 6 h. M indicates the marker proteins described in the legend to Fig. 1B.

cient translation and yielded amounts of product comparable to those derived from a messenger containing the Δ5'NTR of EMCV (Fig. 4B, lanes S32A3 and BPS11). The translational efficiency of the second gene in the dicistronic RNA APS1-E2A was indeed enhanced in the presence of a highly efficient first cistron. Slightly reduced translation of the first gene was observed, which may be due to competition for components involved in translation. However, in dicistronic RNAs, which contain deletions in the EMCV 5'NTR up to nt 562 or 742, no detectable amount of 2A was observed, even though this second cistron was preceded by a very efficient first cistron (Fig. 4B, lanes APS-E2A2 and APS-E2A3). These results support our notion that reentry, rather than resumption of scanning, is necessary for the translation of a second gene in RRL.

RNA stability. The stability of dicistronic mRNA MPS1-E2A in RRL was tested by the use of ^{32}P-labeled RNA. No specific cleavages that could separate the dicistronic RNA into two monocistronic RNAs were observed (data not shown).

DISCUSSION

Artificial mono- or dicistronic messengers have been synthesized by using the in vitro T7 RNA transcription system, and the translational efficiency was analyzed in RRL and in HeLa cell-free extracts. The translational efficiency of the second cistron was not reduced in the presence of a poorly translated preceding cistron as long as the second cistron remained under the control of the 5'NTR of EMCV truncated up to nt 259. We observed, however, that further deletions in the 5'NTR of EMCV up to or beyond nt 484 reduced the translation of the second gene to undetectable levels. These observations and time course experiments suggest that the translation of the second gene is independent of translation of the first gene. We interpret these results to indicate that an internal ribosomal entry site is present in the 5'NTR of EMCV and that it resides between nt 260 and 484 of this structural element of the viral genome.

The number of RNA strands engaged in translation in cell-free systems as a proportion of total RNA strands is usually small. It could be argued that degradation of the synthetic mRNAs in the cell-free systems produced a small population of fragmented RNA molecules that, having escaped the constraint of the large dicistronic environment, produce the effects generated in this report. This is very unlikely for the following reasons. First, up to 50% of EMCV RNA, added into cell-free extracts of MOPC cells, was incorporated into polysomes at limiting concentrations of RNA (15). Second, deletion of the 5'NTR up to nt 670 of poliovirus yields highly efficient mRNAs where the Δ5'NTRs of poliovirus are connected to indicator genes (Fig. 4B; also described in reference 33). Activation of the 5'NTR of poliovirus RNA by degradation, however, was not seen during incubation of either mono- or dicistronic RNAs (Fig. 1B). Third, deletion of segments of the Δ5'NTR of EMCV in dicistronic mRNA molecules leads to the total loss of translation of the second cistron (Fig. 3B). This phenomenon is difficult to explain unless the region between nt 260 and 485 in the 5'NTR of EMCV promotes specific degradation of the dicistronic RNA. This is highly unlikely in view of the data obtained with monocistronic mRNAs containing the derivatives of the EMCV Δ5'NTR (Fig. 3B). Moreover, we have not observed specific fragmentation of dicistronic mRNA in RRL. Finally, the increased translational activity of some downstream cistrons when they are preceded by efficient upstream cistrons (Fig. 4B) cannot easily be explained if only cleaved RNA molecules are active in translation.

The independence of translation of the second gene from translation of the first gene reported here is in contrast to the reduced translational efficiency of a second gene observed with polycistronic mRNAs constructed by Peabody and Berg (39), Peabody et al. (40), and Kaufman et al. (19). This discrepancy is probably due to the difference in the 5'NTRs that control expression of the second genes. The cDNAs of naturally capped messengers were used by these authors to construct their polycistronic mRNAs, whereas we used the 5'NTR of uncapped picornaviral RNAs. Picornavirus mRNAs differ in several respects from mammalian cellular mRNAs. For example, translation of the uncapped poliovirus mRNA appears not to be influenced by cleavage of p220, a host protein associated with the cellular cap-binding com-

plex. The cleavage of p220 is induced by the poliovirus proteinase 2A (25) and has been correlated with a reduction in the translation of capped mRNAs (4, 11; reviewed in reference 47). Moreover, picornavirus mRNAs are known to contain unusually long 5'NTRs ranging in length from 610 nt in human rhinovirus type 2 (46) to 1300 nt in foot-and-mouth disease virus (5). Many noninitiating AUG codons are scattered in the 5'NTR of picornavirus RNAs. All of these must be ignored by a 40S ribosomal subunit if scanning of the 5'NTR occurs as described by Kozak (21, 22).

The genome-linked oligopeptide (VPg) of picornavirus genomic RNA is removed efficiently from the polynucleotides by a cellular enzyme (1, 8, 35, 36, 42). It could be argued that VPg represents a 5'-terminal block that must be removed to free a 5' end for an attachment and scanning of the ribosomal subunit. The 5'-terminal VPg of cowpea mosaic virus RNA, on the other hand, is not removed from the plant virus genomic RNA by the animal cell unlinking enzyme, yet cowpea mosaic virus RNA is efficiently translated in RRL (6). Also, an artificial addition of the cap structure at the 5' end of the poliovirus RNA did not influence the translational efficiency in RRL (J. Bradley, S. van der Werf, M. Girard, and E. Wimmer, unpublished data). Moreover, a deletion of the 5'NTR up to nt 220 of poliovirus and up to nt 259 of EMCV did not influence the translational efficiency of these RNAs in RRL (unpublished data). Finally, indirect evidence for the presence of an internal ribosomal entry site in the 5'NTR of EMCV was also presented by D. S. Shih et al. (45). These authors identified a critical region for efficient translation centered around nt 450 by using synthetic oligodeoxyribonucleotides complementary to different portions of the 5'NTR of EMCV RNA. This result is compatible with our results in that a deletion up to nt 484 of the 5' NTR of EMCV diminished the translation of EMCV messenger in both mono- and dicistronic RNAs.

Translation of cellular mRNAs under normal circumstances in vivo or in vitro is likely to occur predominantly by binding of components of the initiation complex to the 5'-terminal cap of the polyribonucleotides. The possibility cannot be excluded, however, that eucaryotic ribosomal subunits can enter an internal site in cellular mRNA without prior binding to a 5' end. Such a mechanism may occur in cells in which p220, a component of the cap-binding complex, has been cleaved (3), although the translation of cellular mRNAs under these circumstances is inefficient. mRNAs of eucaryotic cells are usually monocistronic. It follows that internal initiation is unnecessary for protein synthesis directed by cellular mRNAs. Indeed, internal binding of the 40S ribosomal subunit to cellular mRNAs is generally undesirable because it would lead to aberrant translation products, and it may be suppressed by the elaborate and efficient process of cap binding. It can be envisioned, on the other hand, that many virus systems have evolved such that translation of their mRNAs occur in a cap-independent manner, and hence that scanning of 40S ribosomal subunits from the 5' end to the initiator AUG codon does not occur. The uncapped mRNAs of picornaviruses may be such viral entities that function in protein synthesis without involvement of a 5' end, that is, by internal binding of an initiation complex. mRNAs of certain eucaryotic viruses may thus contain a linear sequence signal or higher order structure(s) that are efficiently recognized by translational factors and/or ribosomal subunits and that can be used as an internal binding site to initiate protein synthesis. Conventional eucaryotic initiation factors might be involved in this process. Alternatively, specific cellular factor(s) might recognize an internal entry site and guide ribosomal subunits to bind to that portion of the RNA. The translation efficiency of a specific mRNA could then be regulated by the presence of a specific binding site in the RNA and by the availability of a specific, *trans*-acting factor(s) guiding the translational machinery to the initiator AUG codon (13).

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