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Effects of cDNA Hybridization on Translation of Encephalomyocarditis Virus RNA†

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Cell-free translation of the RNA of encephalomyocarditis virus was examined after hybridization of chemically synthesized cDNA fragments to different sites of the 5′ noncoding region of the viral RNA. The following results were obtained. (i) The binding of cDNA fragments to the first 41 nucleotides, to the poly(C) tract (between nucleotides 149 and 263), and to the sequence between nucleotides 309 and 338 did not affect translation of the viral RNA; (ii) the binding of cDNA fragments to the sequence between nucleotides 420 and 449 caused a slight inhibition; and (iii) the binding of fragments to eight different sites between nucleotides 450 and the initiator AUG codon (nucleotide 834) caused high degrees of inhibition. The results suggest that the first part of the 5′ untranslated region, at least to nucleotide 338, may not be required for encephalomyocarditis viral RNA translation; however, the region near nucleotide 450 is important for translation of the viral RNA. The possibility that initiation occurs at an internal site is discussed.

Encephalomyocarditis (EMC) virus is a member of the picornavirus family. Much is known about the protein synthesis pathways of picornaviruses and about the products of the synthesis reactions. However, an important aspect of the viral protein synthesis process, the initiation of translation, is still unclear at present.

For most eucaryotic mRNAs, the ribosome-scanning model has been accepted to explain the translational initiation process (7, 8). In this model, 40S ribosomal subunits first bind to the 5′ end of a messenger and then migrate along the RNA chain until they encounter the first favorable AUG triplet, at which point translation begins. An essential feature of this scanning model is that the 40S subunits recognize and bind only to the 5′ ends of the mRNA molecules before the start of the scanning process. The 5′ cap structure, which is present in most eucaryotic mRNAs, is known to be involved in the ribosome-binding reaction (for a review, see reference 3).

Unlike most eucaryotic mRNAs, picornaviral RNAs do not have a 5′ nucleotide cap structure. Instead, they have a covalently linked protein (VPg) at their 5′ ends (26). This genome-linked protein is not required for translation (18). Therefore, during initiation of picornaviral RNA translation, it is possible that an alternate ribosome recognition and binding mechanism is involved. For example, the ribosome subunits may recognize and initially bind to an internal site of the viral RNA before the scanning process begins.

We have investigated this possibility by using EMC virus as a model. EMC viral RNA is a particularly active messenger in several cell-free protein-synthesizing systems (16, 20, 24). In rabbit reticulocyte lysates, the viral RNA is completely and efficiently translated, and the translation products are accurately processed to form mature viral proteins (16, 20, 21, 24). Recently, the complete nucleotide sequence of EMC virus was determined (14, 25; A. C. Palmenberg and G. M. Duke, manuscript in preparation). The RNA is 7,835 nucleotides long, and the translation initiated AUG codon is located at nucleotide 834 (14). This codon is followed by an open reading frame of 6,876 nucleotides, which encodes the viral polyprotein. The initiator AUG is preceded by 10 other AUG triplets scattered throughout the 5′ noncoding region. The present study was designed to investigate the mechanism by which ribosomes initiate translation at the particular AUG triplet in position 834.

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. After completion of the synthesizing cycles, the oligonucleotide solution from the synthesizer was incubated with 1 ml of concentrated NH_4OH for 4 to 12 h at 50°C to completely remove the blocking groups. The solution was then evaporated to dryness in a rotary evaporator. The dry residue was dissolved in 1 ml of 25 mM triethylammonium bicarbonate (TEAB), pH 7.6, and the solution was evaporated. This TEAB treatment step was repeated twice. The residue was dissolved in 3 ml of TEAB, and the oligonucleotide fragments were isolated by using a Sep-pak cartridge (11), which served to remove prematurely terminated sequences from the complete oligonucleotide chains. The oligonucleotide fraction eluted from the cartridge was dried, and the 5′-protecting groups were removed by dissolving the nucleotides in 1 ml of 80% acetic acid and incubating the solution at room temperature for 25 min. After evaporation, the sample was dissolved in water and dried. The fully unprotected product was further purified by fractionation on a 20% polyacrylamide gel under denaturing conditions. The conditions for electrophoresis and for oligonucleotide isolation from the gel have been described elsewhere (11).

All hybridizations were done in reaction mixtures (8 μl) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6)–0.1 M NaCl–1 mM EDTA. The RNA concentration was 0.5 mg/ml, and cDNA was added in a fivefold molar excess. The reaction mixtures were heated at 70°C for 10 min followed by incubation at 45°C for

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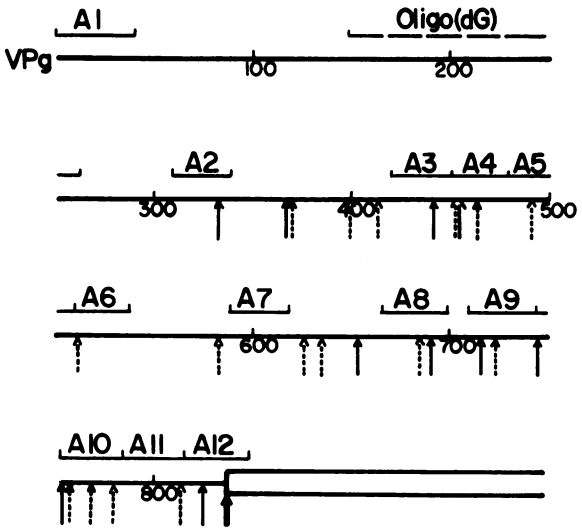

FIG. 1. 5'-Untranslated region of EMC viral RNA. Symbols: —, 5'-noncoding sequence; ➡, location of initiator AUG codon; →, locations of other AUG triplets; ⊸, positions of termination codons; ⌊⌋, cDNA fragments. For the indicated fragments, the lengths and the exact nucleotide locations (given in parentheses) of the sites of hybridization were: A1, 41 (1 to 41); A2, 30 (309 to 338); A3, 30 (420 to 449); A4, 30 (450 to 479); A5, 30 (480, to 509); A6, 30 (510 to 539); A7, 30 (590 to 619); A8, 33 (667 to 699); A9, 34 (710 to 743); A10, 40 (744 to 783); A11, 30 (784 to 813); A12, 41 (814 to 854).

3 h. Nonhybridized control samples were treated identically, except that cDNA was omitted from the reaction mixtures.

The hybridization procedure described above was developed by combining the procedures of Hastie and Held (5) and Liebhaber et al. (10). The first method (5) involved heating mRNA and single-stranded cDNA in an aqueous solution at 65°C for 30 min. The second method (10) involved heating mRNA and double-stranded DNA in a formamide-containing buffer for 10 min at 70°C followed by incubation at 45°C for 3 h. Each of the methods produces complete hybridization.

Translation of the hybridized RNA samples was examined by using the mRNA-dependent rabbit reticulocyte system (17). Reticulocyte lysates were obtained from Green Hectare, Oregon, Wis. The conditions for translation have been described previously (20, 21).

Figure 1 depicts schematically the 5' region of EMC viral RNA and the locations of cDNA hybridization sites. The cDNA fragments, designated A1 through A12, are 30 to 41 nucleotides long, and their positions are indicated.

Translation of cDNA-mRNA hybrids. EMC viral RNA was tested for its ability to direct translation after hybridization to various cDNA fragments. The effects with two of the fragments, A10 and A12, are shown (Fig. 2A). A12 hybridizes to the genomic region encompassing the polyprotein initiation codon, including 18 upstream and 20 downstream nucleotides. A10 hybridizes to a region that is 30 nucleotides 5' to the A12-hybridized region. The data indicate that A10 and A12 caused 93 and 77% inhibition of normal EMC viral polyprotein translation, respectively. The results of a control experiment in which the A12-mRNA complex was dissociated by heating at 97°C before translation are given (Fig. 2B). Heating of the complex eliminated most of the inhibition. We therefore conclude that the decrease in translation observed (Fig. 2A) was the result of hybridization to the A10 and A12 oligonucleotide fragments.

The effects on translation of hybridization of seven other fragments (A4 through A9 and A11) are shown (Fig. 2C, 2D, and 3). The data indicate that fragments A4 through A7 and A11 caused more than 80% inhibition of translation; fragment A8 caused 65% inhibition, and fragment A9 caused 75% inhibition.

Figure 3 depicts amino acid incorporation data for A4- and A3-hybridized RNA. As described above, hybridization of A4 caused a strong inhibition of translation. On the other hand, hybridization of A3, which corresponds to the region immediately 5' to the A4-hybridized region, caused only a low level of inhibition. The translational activity of the A3-hybridized viral RNA was about 70% of that of the control (unhybridized) RNA.

It was possible that the low degree of inhibition observed with A3 was due to weak or incomplete hybridization with viral RNA. However, this possibility was ruled out by primer extension experiments. The electrophoresis patterns

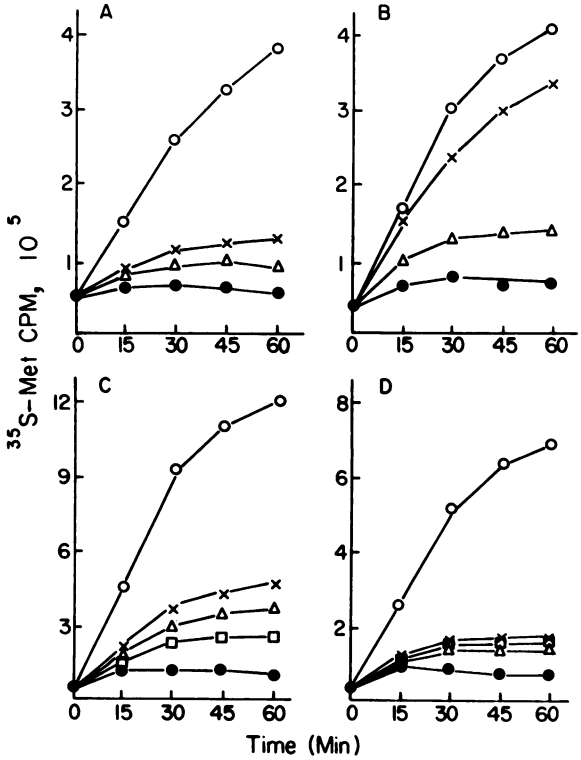

FIG. 2. Inhibitory cDNA fragments. The conditions for hybridization and for translation were as described in the text. The control RNA samples were heated and incubated in the same manner as the hybridized RNA samples, except that cDNA fragments were omitted. (A) Effect of hybridization of A10 and A12. Symbols: ●, minus RNA control; △, A10; ×, A12; ○, minus cDNA control. (B) Effect of dehybridization of the annealed complexes of A12 and EMC viral RNA. The hybridization solution containing the annealed complexes was divided into two aliquots. One was translated directly, and the other was heated at 97°C for 3 min, quickly frozen in a dry ice-ethanol bath, thawed, and translated. Symbols: ●, minus RNA control; △, A12-mRNA complexes; ×, dehybridized mixture; ○, minus cDNA control. (C) Effects of A8, A9, and A11. Symbols: ●, minus RNA control; □, A11; △, A9; ×, A8; ○, minus cDNA control. (D) Effects of A5 to A7. Symbols: ●, minus RNA control; □, A5; △, A6; ×, A7; ○, minus cDNA control.

of primer extension products synthesized from RNA templates primed with A3 (nucleotides 420 to 449) or with A11 (nucleotides 784 to 813) are shown (Fig. 4). The formation of only one major product was seen in each case. The chain length was approximately 350 nucleotides for the major A3-extension product and 750 nucleotides for the major A11 extension product. Densitometer tracings (not shown) of the gel profiles confirmed that equal molar amounts of product DNA were synthesized in reactions with the two primers (A3 and A11), indicating that the extent of hybridization of A3 to RNA was similar to that of A11. Therefore, although the two cDNAs differed significantly in the ability to inhibit EMC viral polyprotein translation, these properties were not caused by differences in hybridization efficiencies.

The hybridization of A1, which corresponds to the first 41 nucleotides, caused no detectable inhibition of translation of the viral polyprotein (Fig. 5A). The same result was also obtained when the hybridization reaction was done by heating the solution to a much higher temperature (97°C) before the annealing step (Fig. 5B). Similarly, the hybridization of oligo(dG) or of A2, which corresponds to the sequence from nucleotides 309 to 338, had no effect on translation (Fig. 5C and D).

The translation data presented above (Fig. 2, 3, and 5) demonstrated a sequential transition of the effect of cDNA hybridization from strongly inhibitory to partially inhibitory to noninhibitory. The area of viral RNA that is critical for efficient translation seemed to center around nucleotide 450, which is the 5′-most nucleotide within a region, to which cDNA fragment A4 was hybridized, leading to strong inhibition.

In addition to the hybridization-translation studies described above, we examined translation of EMC viral RNA after the deletion of specific segments of the 5′ noncoding region by RNase H digestion of cDNA-mRNA hybrids. We found that deletion of the 5′ sequence to approximately nucleotide 338 caused no discernible effect on translation, but deletion of the sequence extending beyond nucleotide 450 diminished the translation activity (data not shown).

Analysis of translation products. The protein products synthesized by various hybridized RNA samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 6, lanes a, b, and c, represents 45-min translation reactions directed by A2-, oligo(dG)-, and A1-hybridized RNA, respectively. Also shown are the products from intact virion RNA (Fig. 6, lane h). The protein bands (Fig. 6, in lanes a, b, c, and h) were indistinguishable in position, although there was some variation in band intensity. This variation could be attributed to the fact that the translation of the different RNA samples was done at different times. Variations in the conditions of lysate preparation and in the components of the protein synthesis reaction mixture could certainly have caused differences in the rates of translation and processing, which would directly affect the product pattern.

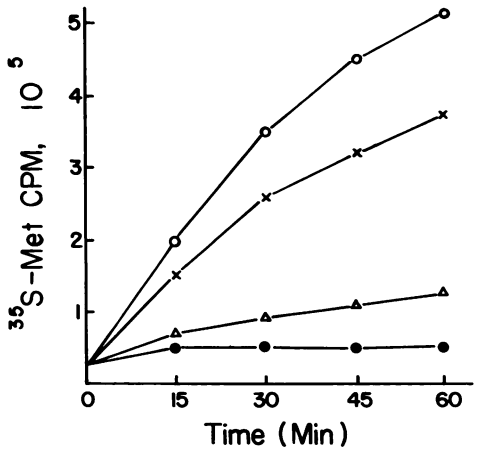

FIG. 3. Activities of viral RNA hybridized to A3 or A4. Symbols: ○, minus cDNA control; ×, A3; △, A4; ●, minus RNA control.

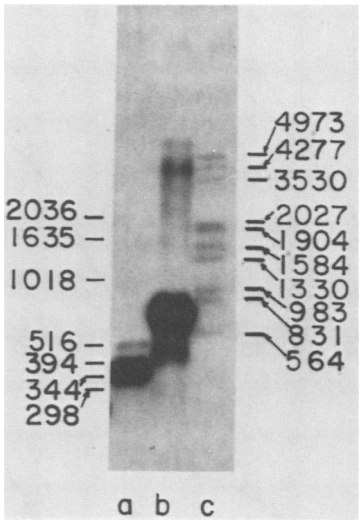

FIG. 4. Gel analysis of the primer extension products from A3- and A11-hybridized primers. A3 and A11 were hybridized separately to the viral RNA. Each hybridized sample (8 μg of RNA) was precipitated with ethanol and dried under vacuum. The residue was dissolved in 15 μl of water and added to a reverse transcription reaction mixture. The transcription reaction was done by the method of Ahlquist et al. (1), based on the original procedures of Land et al. (9) and Kacian and Myers (6). The cDNA products were analyzed on 2% alkaline agarose gels by the procedure of McDonnell et al. (13). Lanes: a, products from A3-hybridized template; b, products from A11-hybridized template; c, radioactively labeled standards. The positions of stained molecular weight markers are shown at the left of lane a and at the right of lane c.

The products from RNA hybridized to A8, A10 plus A12, A10, and A12 are shown (Fig. 6, lanes d through g, respectively). In agreement with the amino acid incorporation data (Fig. 2 and 3), much less of the viral protein was produced in these samples when compared with the control sample.

Our data demonstrated that binding of cDNA fragments to the beginning portion of EMC viral RNA (5′ to nucleotide 338) had no detectable effect on polyprotein translation, but binding of cDNA fragments to sequences 3′ to nucleotide 450 severely inhibited translation. Furthermore, we found that removal of the first 338 nucleotides by RNase H digestion had no discernible effect on subsequent translation activity of the remaining RNA fragment, while removal of nucleotide sequences extending beyond nucleotide 450 virtually abolished the messenger activity.

Our results are in complete agreement with experimental data from other studies. The L fragment generated by RNase H digestion of oligo(dG)-hybridized foot-and-mouth disease virus RNA serves as very active mRNA in vitro and produces the same products as full-length virion RNA (5). Deletion and recombination experiments with cloned

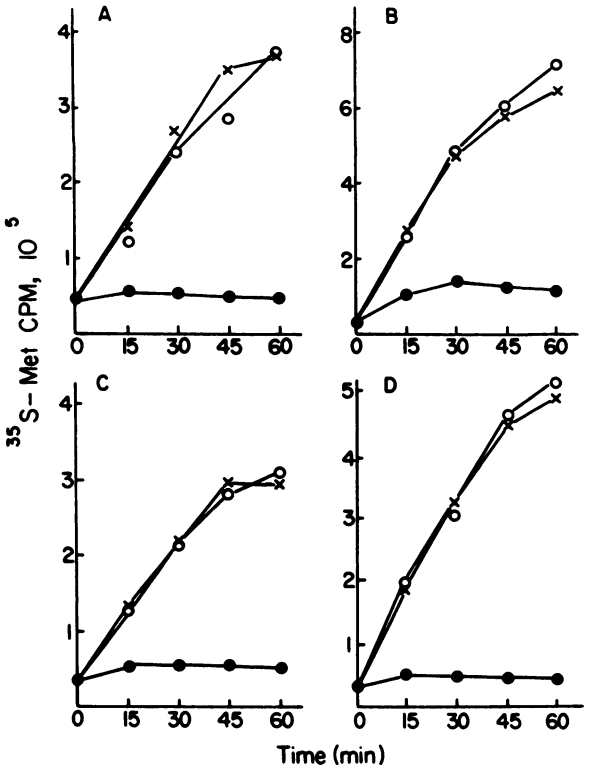

FIG. 5. Noninhibitory cDNA fragments. (A) Effect of A1. Hybridization was done under standard conditions. (B) Effect of A1. The hybridization reaction mixture was heated at 97°C instead of at the normal 70°C. (C) Effect of oligo(dG). The oligonucleotide was added at a fivefold molar excess of the entire poly(C) region (115 nucleotides). (D) effect of A2. Symbols: ●, minus RNA control; ×, hybridized samples; ○, minus cDNA control.

cDNAs have shown that the translational properties of transcribed EMC viral mRNAs reside exclusively in the 5′ noncoding portion of the viral genome, specifically in the segment extending 3′ from (approximately) nucleotide 460 to the AUG codon that begins the polyprotein. The 5′-most 460 nucleotides, the intact polyprotein reading frame, and the 3′ noncoding regions are not necessary for efficient mRNA translation (15; Palmenberg et al., in preparation).

Thus it is reasonable to conclude that the initial sequence (about 400 bases) of the EMC viral RNA is not necessary for translation; however, the region near nucleotide 450 is important. On the basis of this conclusion, we propose that for the initiation of EMC viral RNA translation the 40S ribosomal subunits may first bind at a site near or including nucleotide 450 and that from this site the subunits migrate toward the initiator AUG site and begin protein synthesis.

If our internal initiation hypothesis is correct, an obvious question is which factors are responsible for ribosome recognition and binding? The Shine-Dalgarno sequence is thought to act as a ribosome-binding site in procaryotic mRNAs (22, 23). This conserved, purine-rich sequence is complementary to the 3′ end of bacterial 16S rRNA. Since the 3′ ends of eucaryotic 18S rRNAs also contain a conserved sequence (2, 4) which is very similar to the 3′ sequence of the 16S rRNA, it is reasonable to suggest that the 3′ end of 18S rRNA may be involved in the initiation of translation of some eucaryotic mRNAs. In fact, the hypothetical involvement of 18S rRNA in translation initiation has already been proposed (2, 4, 19). For picornavirus RNAs, the possibility of mRNA-rRNA interaction is particularly relevant because of the lack of the 5′ cap structure of the viral RNA. Examination of the EMC viral RNA sequence reveals a partial complementarity (GAAGCUUCUUGAAG [underscores indicate a potential base pairing]) existing between EMC viral nucleotides 483 to 496 and the 3′ end of rabbit reticulocyte 18S rRNA (2, 4). This limited complementarity may not be sufficient for effective ribosome binding. However, the possibility of other sequence complementation between the 5′ noncoding regions of EMC virus and different parts of the 18S rRNA cannot be ruled out. In this regard, it is interesting to note that a recent study showed that poliovirus RNA can hybridize (under stringent conditions) to a specific region of the 18S rRNA as well as to several regions of 28S rRNA of higher eucaryotes (12).

The studies presented here provide new insight into the mechanisms of translation of EMC viral RNA and suggest avenues for future experimentation. In light of the high translational efficiency of this particular mRNA, we hope that clarification of the translational mechanism may serve as a model for other experimental systems.

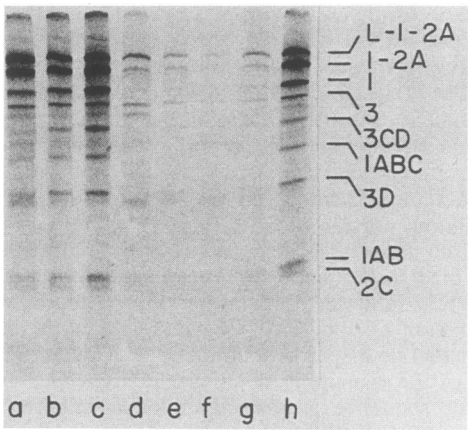

FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of translation products from hybridized RNA samples. All samples were withdrawn after 45 min of translation. Lanes: a through g, products synthesized from viral RNA hybridized to A2, oligo(dG), A1, A8, A10 plus A12, A10, and A12, respectively; h, products from a control RNA sample. The viral proteins are indicated at the right of lane h.

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