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An Internal 5′-Noncoding Region Required for Translation of Poliovirus RNA In Vitro

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A truncated poliovirus RNA that contains the entire 5′-noncoding region as well as some capsid protein-coding sequences was produced from cloned cDNA inserted into an SP6 transcription vector and subsequently was translated in a mixed rabbit reticulocyte-HeLa cell lysate. Deletions or modifications of regions of the 5′-noncoding sequences had significant effects upon the efficiency of translation. The presence of a 60-nucleotide sequence located at positions 567 to 627 appeared to be essential for active ribosome binding and translation of this uncapped RNA.

The translation of poliovirus mRNA involves an unusual process of ribosome binding and AUG selection that has not been characterized and has some puzzling aspects. Following infection, translation of cellular mRNAs stops, presumably because of the virus-induced inactivation of eIF-4F, a translational initiation factor that binds to the m^7G cap group and thereby identifies the 5′ ends of capped mRNAs for the processes of mRNA unwinding and ribosome binding (6, 34). The translation of poliovirus RNA occurs efficiently in the absence of a functional cap recognition system; this lack of a requirement for a functional cap-binding protein complex is not surprising, since poliovirus mRNA is not capped (8, 12, 23). However, the question is raised as to whether the viral RNA utilizes an alternate, specific, cap-independent mechanism of ribosome binding and translation initiation.

For capped mRNAs, initiation of translation is thought to occur by binding of the ribosome at the 5′ end of the mRNA, followed by migration of the ribosome along the RNA chain and scanning for the first favorable AUG, where translation begins (15). Poliovirus mRNA, in addition to being uncapped, has an extremely long, untranslated 5′ sequence: the initiating AUG is located 743 nucleotides from the 5′ end (4) and is preceded by numerous apparently unused AUG triplets (eight in the case of poliovirus type 1, Mahoney strain) (14, 28). This situation raises the possibility that some internal sequence and/or structure might mediate ribosome binding. Support for this idea has been suggested by study of the translation in vitro of RNA from another picornavirus, encephalomyocarditis virus (33). In that study (33), cell-free translation of encephalomyocarditis virus RNA was examined after hybridization of chemically synthesized cDNA fragments to different sites on the 5′-noncoding region. The results showed that binding of cDNA fragments to RNA between the 5′ end and nucleotide 450 had little or no effect on translation; binding of fragments to the RNA sequence between nucleotide 450 and the initiating AUG (nucleotide 834) caused marked inhibition of translation. The authors (33) concluded that the RNA region near nucleotide 450 is important for translation of encephalomyocarditis virus RNA and that ribosome binding occurs at an internal site rather than at the 5′ end.

The function(s) of the long, untranslated 5′ sequence in poliovirus RNA is unknown. It is thought to have an essential role(s) in virus replication because all picornaviruses contain 5′-noncoding sequences of similar length and regions of similar sequence and/or structure (37; V. M. Rivera, J. D. Welsh, and J. V. Maizel, Virology, in press). In addition, poliovirus cDNA clones containing either deletions or alterations in a variety of sites within the 5′-noncoding region are not infectious or give rise to viruses with altered growth properties (13, 17, 29, 30). Other mutations within the 5′-noncoding region of poliovirus RNAs have been correlated with changes in neurovirulence and translational efficiency (7, 18, 24, 36). This region may be involved in ribosome binding and initiation of translation of plus-strand RNA, in polymerase binding to the 3′ end of minus-strand RNA, and/or perhaps in packaging and assembly of plus-strand RNA in virions.

In the studies described here, we attempted to evaluate the importance for translation of specific regions in the 5′-noncoding sequence. Our approach was to insert poliovirus cDNAs containing deletions or modifications into an SP6 transcription vector and to translate the resulting transcripts in vitro.

Plasmid pSPOV was constructed by inserting the complete poliovirus cDNA sequence into the *Eco*RI site of pSP65 (Promega Biotec). The poliovirus cDNA was obtained from pPV16 (32), kindly provided by Bert Semler, University of California, Irvine. It contains 18 G residues preceding the 5′ terminus of the poliovirus sequence and is flanked by *Eco*RI linkers. An SP6 promoter is contributed by the pSP65 vector. Transcription of pSPOV DNA by SP6 RNA polymerase (Bethesda Research Laboratories, Inc.) produces plus-strand RNA. The plasmid was linearized with *Sal*I prior to transcription, and the resulting transcript was shown to comigrate with virion RNA on agarose gels containing methyl mercury hydroxide (1). In preliminary experiments, a similar plasmid, pTPOV, was constructed by using a T7 promoter to drive transcription of the poliovirus insert. T7 RNA polymerase purified in our laboratory produced more efficient transcription than did commercial enzymes; however, no difference in translational efficiency was observed between SP6- and T7-derived transcripts.

The full-length poliovirus transcript was translated in rabbit reticulocyte lysates. Although somewhat less efficient as a template than virion RNA, the transcripts directed the synthesis of the same polypeptides as did natural viral RNA (data not shown). Translation of poliovirus RNA in rabbit

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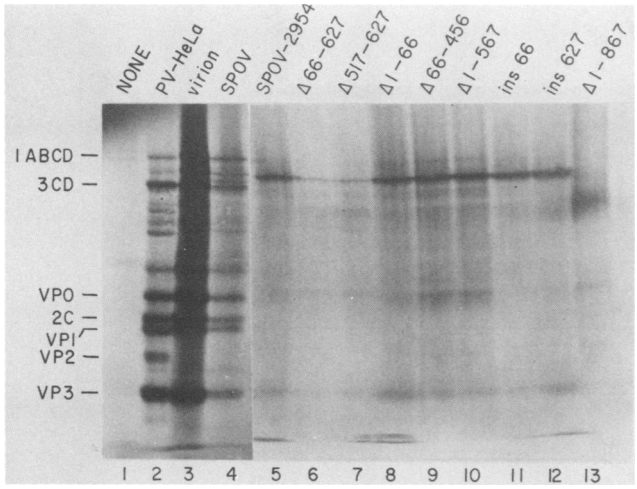

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins synthesized in vitro from poliovirus RNAs modified in the 5'-noncoding region. Derivatives of pSPOV-2954 were linearized with *Sal*I and transcribed (21) with SP6 RNA polymerase. Nucleic acids in the reaction mixture were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the aqueous phase was adjusted to 2.5 M ammonium acetate and precipitated with ethanol. After suspension in water, RNA concentrations were determined by analysis on methyl mercury hydroxide-containing agarose gels, and the RNAs were used to program an in vitro translation reaction (50 μl) with 35 μl of micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) used in accordance with manufacturer instructions and supplemented with 7 μl of HeLa cell S-10 extract. Translation reactions were incubated for 3 h at 30°C, at which time gel sample buffer was added, and a sample (10 μl) was applied to a sodium dodecyl sulfate–10% polyacrylamide gel. After electrophoresis, gels were dried and exposed to film for autoradiography. Lanes: 1, no mRNA; 2, poliovirus-infected HeLa cell cytoplasmic extract, labeled from 2 to 4 h postinfection with [^{35}S]methionine; 3, poliovirus RNA extracted from virions; 4, full-length RNA transcript from pSPOV; 5 through 13, translation products of transcripts from the plasmid (pSPOV-2954) containing the truncated poliovirus DNA sequence (nucleotides 1 to 2954) (lane 5) and those modified by deletion (Δ) or insertion (ins) at the nucleotide positions indicated (lanes 6 through 13). Poliovirus proteins identified from lane 2 are indicated at the left.

reticulocyte lysates results in the production of aberrant polypeptides, apparently because of artifactual initiation reactions that occur in the 3' third of the RNA (5, 27). The addition of a ribosomal salt wash (2) or a cytoplasmic extract (5, 27) from uninfected HeLa cells apparently suppresses the internal initiation and improves the fidelity of translation. In this study, similar results were found for translation of the RNA transcripts; consequently, all translations were performed with a mixed reticulocyte-HeLa cell extract. Lanes 3 and 4 of Fig. 1 show the translation products obtained from translation of virion RNA and pSPOV transcripts, respectively, in the mixed translation system. The products were qualitatively identical to each other and very similar to the viral proteins synthesized in infected HeLa cells (Fig. 1, lane 2). A similar system has been utilized by Ypma-Wong and Semler (38) for translation of T7 transcripts of poliovirus cDNA, with similar results.

Measurements of translational efficiency of poliovirus RNAs are complicated by the requirements for polyprotein processing to obtain recognizable viral proteins. We therefore constructed a plasmid, pSPOV-2954, which produced a truncated RNA transcript that contained the entire 5'-noncoding region, the polyprotein start site, and capsid-coding sequences through nucleotide 2954, which terminates in the middle of the VP1-coding region. The poliovirus DNA sequences in this plasmid, as well as the transcripts derived from it and from other plasmids to be described below, are shown schematically in Fig. 2. Translation of the pSPOV-2954 transcript is predicted to produce a single polypeptide with a molecular weight 84,000, since all known viral protease sequences have been removed from the plasmid. Figure 1, lane 5, shows that a single band of the expected mobility was detected as the predominant translation product of this RNA.

To evaluate the influence of various regions in the 5'-noncoding region on translational efficiency, we constructed a number of deletions or insertions in pSPOV-2954 DNA (Fig. 2). Each of the plasmids was linearized and transcribed, and the resulting transcripts were translated in the mixed reticulocyte-HeLa cell extract. Care was taken in each case to ensure that the translation reaction was saturated with RNA so that apparent differences in translational efficiency were not the result of variable RNA concentrations. Figure 3 shows an RNA saturation curve for SPOV-2954 RNA which was typical of the other transcripts as well. In addition, each transcript was analyzed on agarose gels containing methyl mercury hydroxide and shown to be intact and of the expected size (data not shown).

Analysis of the 5'-terminal nucleotide sequence of poliovirus type 1 has revealed a potential for the formation of a stable stem-loop structure that involves nucleotides 10 to 18 paired with nucleotides 26 to 34 (19). Similar stable secondary structures have been proposed for the 5'-terminal ends of poliovirus types 2 and 3 (3), as well as for other representative cardioviruses, aphthoviruses, rhinoviruses, and enteroviruses for which sequence information has become available (Rivera et al., in press). Deletion of 115 terminal nucleotides from the 5' end of the viral sequence rendered a cDNA clone noninfectious (29), and deletion of a single nucleotide at the base of the hairpin resulted in a temperature-sensitive virus (30). We therefore first examined the effect of removal of the first 66 nucleotides from the poliovirus sequence (Fig. 2, Δ1-66) on the ability of the RNA to be translated in the mixed reticulocyte-HeLa cell extract. Figure 1, lane 8, shows that no significant change in translational efficiency was detected. A similar result was obtained with RNA lacking nucleotides 1 to 13 (data not shown). These constructions also resulted in the loss of the 18 G residues that were present in the transcript of intact pSPOV-2954. These had no detectable effect on translational efficiency.

Removal of the majority of the 5'-noncoding sequences (Fig. 2, Δ66-627), however, almost totally abolished translation (Fig. 1, lane 6). This deletion eliminated all of the AUG codons that precede the functional one. The failure of this RNA to be translated was not due to accelerated instability in the lysate, suggesting that some internal sequence(s) and/or structure(s) in SPOV-2954 RNA (and therefore in viral RNA) was important for directing translation at the AUG at position 743. Restoration of the sequences in the 3'-terminal half of this deletion produced Δ66-456 (Fig. 2) and restored the translatability of the RNA (Fig. 1, lane 9). On the other hand, a transcript with only nucleotides 517 to 627 deleted (Fig. 2) failed to be translated (Fig. 1, lane 7). Finally, deletion of all sequences upstream of nucleotide 567 produced a transcript (Fig. 2, Δ1-567) that was translated with normal efficiency (Fig. 1, lane 10); thus, the 60-nucleotide

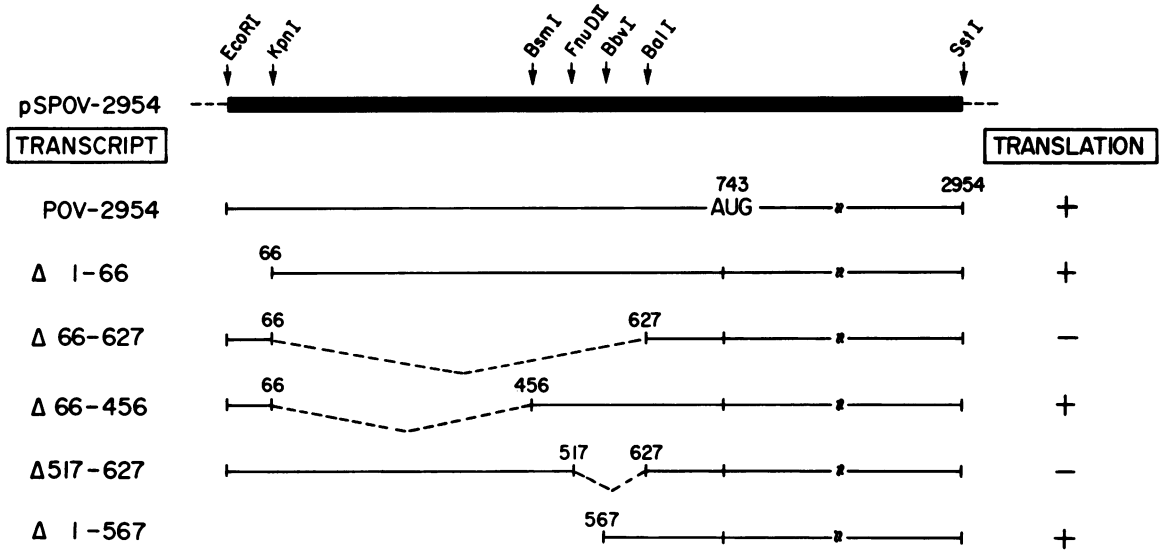

FIG. 2. Deletions and insertions in the 5′-noncoding region of poliovirus RNAs. pSPOV-2954 is a truncated transcription plasmid obtained by digesting pSPOV (see the text) DNA with *Sna*BI (cuts the poliovirus sequence at nucleotide 2954, in the middle of the VP1-coding sequence) and *Sma*I (cuts pSP65 in a multiple cloning site, just beyond the 3′ end of the poliovirus sequence). The digestion products were subjected to electrophoresis in an agarose gel, and the ~6-kilobase vector plus the 5′ end of poliovirus cDNA purified and ligated prior to transformation into *Escherichia coli* C600. The SP6 RNA polymerase transcription product from this plasmid is shown schematically as POV-2954 and contains the entire 5′-noncoding sequence, the initiating AUG at position 743, and the coding sequence for capsid proteins up to nucleotide 2954. Each of the other transcripts shown was transcribed from DNA which contained deletions or was modified by digestion of pSPOV-2954 with the indicated restriction endonuclease, polishing of the ends with the Klenow fragment of DNA polymerase (if necessary), addition of linkers (if necessary), and ligation of fragments to generate plasmids which, when prepared for use as transcription templates, generated the transcripts shown schematically.

region between nucleotides 567 and 627 is able to restore translatability to an RNA which is otherwise unable to be translated (e.g., Δ66-627).

In addition to the deletions described above, we also constructed plasmids that contained linker insertions at position 66 or 627. These plasmids were transcribed, and the resulting RNAs were translated with normal efficiency (Fig. 1, lanes 11 and 12). Deletion of the entire noncoding region and the initiating AUG abolished all translation (Fig. 1, lane 13).

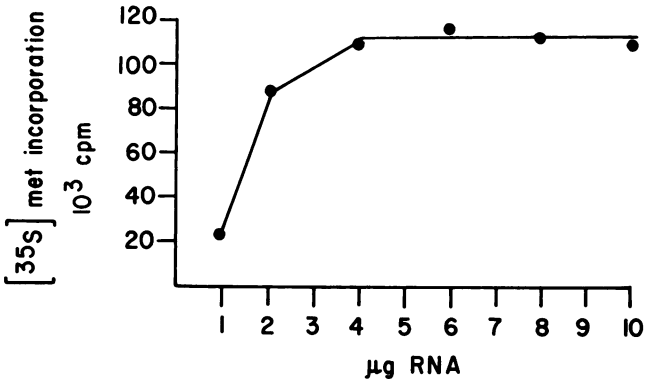

FIG. 3. Effect of RNA transcript concentration on in vitro translation. pSPOV-2954 was linearized with *Sal*I and transcribed with SP6 RNA polymerase, and the nucleic acids were purified as described in the legend to Fig. 1. A sample was analyzed on a 0.7% agarose gel containing methyl mercury hydroxide (1) and stained with ethidium bromide. The RNA concentration was estimated by comparing the stained band with RNA standards of known concentrations. The indicated amounts of RNA were used to program protein synthesis in a 50-μl reaction mixture containing a mixed reticulocyte-HeLa cell extract and [^{35}S] (methionine) [^{35}S] met) for 3 h at 30°C, and samples (2.5 μl) were analyzed for trichloroacetic acid-precipitable radioactivity. Additional samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to confirm the synthesis of the 84-kilodalton polypeptide (data not shown).

Pelletier et al. (26) recently used a similar approach to identify sequence elements within the 5′-noncoding region of poliovirus mRNA that functions in *cis* to mediate cap-independent translation. They localized a region between nucleotides 320 and 631 as a major determinant of the required element. The 60-nucleotide sequence defined in this study falls within the region localized by these investigators and thus more narrowly focuses the *cis*-acting element. Curiously, an RNA transcript lacking almost all of the 5′-noncoding sequence (Δ5′-632) was seen in the study by Pelletier et al. to be translated in a HeLa cell extract approximately twice as efficiently as an RNA transcript with the intact poliovirus 5′-noncoding sequence. We have observed a similar enhanced translation activity of Δ66-627 in unsupplemented reticulocyte lysates but not in HeLa cell extracts or in the mixed reticulocyte-HeLa cell extract used here (unpublished observations). Nicklin et al. (22) and B. Semler and R. Jackson (personal communication) have also reported increased translation of poliovirus RNAs with large deletions in the 5′-noncoding region in unsupplemented reticulocyte lysates. However, as shown here, these investigators also saw a loss of almost all the translation activity of these RNAs in reactions supplemented with HeLa cell extract.

The finding of a specific region of 5′-noncoding sequence that affects the efficiency of translation sheds some light on at least one of the possible functions of this long leader sequence. Thus far, analyses of mutations isolated or constructed in the 5′ end have involved determining the effects

of the modified sequences on the overall infectivity or growth rate of the virus (13, 17, 29, 30). The results of such studies clearly indicate that the 5′ end does contain important regulatory signals, but it has not been possible to pinpoint which step in the viral replication cycle is affected. By measurement of the translational efficiencies of modified RNA transcripts in vitro, signals governing the translation process can be determined independent of those affecting other steps in viral replication.

The sequences implicated in this study as being important for translation in vitro (nucleotides 567 to 627) lie within the region that is highly conserved (nucleotides 509 to 639 [17]) among all poliovirus strains and serotypes. This region includes most of a U-rich sequence which is also observed in this region of the genomes of rhinoviruses and coxsackieviruses and includes a 7-base consensus sequence (positions 583 to 590) that is perfectly conserved in picornavirus genomes (see references cited in reference 17). Deletion analysis of an infectious cDNA clone has suggested that the region between nucleotides 564 and 599 carries genetic information required to maintain the efficiency of some step in viral replication (17). This is consistent with the findings reported here, which implicate this region as mediating translational efficiency.

An even more striking characteristic of this region emerged when a comparative sequence analysis approach was used to predict stable secondary structural features of the 5′-noncoding regions of the enterovirus and rhinovirus subgroups of picornaviruses (Rivera et al., in press). Among the numerous stem-loop foldings predicted by this computer analysis, one extending from positions 584 to 615 and including a perfectly matched stem of 14 base pairs was noticeably conserved in all these viruses. Thus, this stem-loop may maintain a position of structural importance with respect to the AUG and/or the ribosome which could be important for ribosome binding. There was a consistent absence of AUG triplets between this structure and the initiating AUG.

Although the scanning model for translational initiation has continued to gain experimental support, many examples of internal initiation on eucaryotic mRNAs have accumulated in recent years. In some cases, terminators of upstream reading frames precede the internal initiation site, and the mechanism of initiation appears to be in fact a reinitiation event (16, 25). In other cases, however, it appears as though the ribosome is able to bind at the internal site directly. An example of the latter case was described by Hassin et al. (10), who showed that an SP6 transcript containing the adenovirus DNA polymerase mRNA sequence directed the translation of a 62-kilodalton protein from an internal AUG in the middle of the mRNA in addition to the 120-kilodalton protein that resulted from translation of the complete open reading frame from the AUG at the 5′ end of the mRNA. Hybrid-arrested translation experiments suggested that the ribosome did not scan along the mRNA for the internal AUG, since the ribosome would have had to move through a very extensive region of the DNA-RNA hybrid. In fact, the internal site competed very effectively with the 5′ end of the molecule, although capping of the RNA transcript resulted in increased utilization of the 5′ end, with a corresponding decrease in the formation of the internally initiated polypeptide. Other examples of apparent de novo internal initiation have also been described (e.g., references 10 and 11 and references cited therein). In the case of poliovirus RNA, in which the 5′ end is not capped and very extensive secondary structure occupies the proximal sequences, the competition between 5′-end binding and scanning and internal initiation may be strongly shifted in favor of internal initiation.

The ability of an mRNA to initiate translation in the absence of a cap group and a functional cap recognition system is dependent upon structural and/or sequence characteristics that have not been defined. One of the functions of the cap-binding protein complex, eIF-4F, is to promote or mediate an ATP-dependent RNA unwinding activity (31) which appears to be significantly inhibited by a 5′-proximal secondary structure (20). Sonenberg et al. (35) have reported that capped mRNAs with reduced secondary structures have a reduced requirement for the cap-binding protein complex and that such mRNAs can initiate translation in an extract derived from poliovirus-infected cells, in which the cap-binding protein complex is inactive. It is possible that the cap-independent translation demonstrated in picornavirus mRNAs could be affected by internal structures that are required to maintain the absence of a secondary structure elsewhere in the RNA. Alternatively, it is possible that specific sequences are required for cap-independent ribosome binding; e.g., pyrimidine-rich sequences such as the one at poliovirus RNA positions 564 to 577 have been suggested to serve as recognition sites for the conserved purine-rich region found near the 3′ end of all eucaryotic 18S rRNAs (9). At this time, we cannot distinguish whether the decrease in translational efficiency of RNAs lacking nucleotides 567 to 627 results from a change in primary nucleotide sequence or an alteration of the secondary structure or both. Further analysis of the region defined in this study as being important for efficient translation of poliovirus RNA will be required to understand the biochemical function being provided.

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