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Structural and Functional Analysis of the Ribosome Landing Pad of Poliovirus Type 2: In Vivo Translation Studies

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The naturally uncapped genomic and mRNAs of poliovirus initiate translation by an internal ribosome-binding mechanism. The mRNA 5′ untranslated region (UTR) of poliovirus is approximately 750 nucleotides in length and has seven to eight (depending on the serotype) AUG codons upstream of the initiator AUG. The sequence required for internal ribosome binding has been termed the ribosome landing pad (RLP). To better understand the mechanisms of internal initiation, we have determined the boundaries and critical elements of the RLP of poliovirus type 2 (Lansing strain) in vivo. By using deletion analysis, we demonstrate the existence of a core RLP in the poliovirus mRNA 5′ UTR whose boundaries are between nucleotides 134 and 155 at the 5′ end and nucleotides 556 and 585 at the 3′ end. Sequences flanking the core RLP affect translational activity. The importance of several stem-loop structures in the RLP for internal initiation has been determined. Mutation of the phylogenetically conserved loop sequences in the proximal stem-loop structure of the RLP (stem-loop structure III; nucleotides 127 to 165) abolished internal translation. However, deletion of the second stem-loop in the RLP (stem-loop structure IV; nucleotides 189 to 223) reduced internal translation by only 50%. Internal deletions encompassing nucleotides 240 to 300, 350 to 380, or 450 to 480, predicted to disrupt stem-loop structure V and possibly VI, also abrogated internal initiation. Small point mutations within a short polypyrimidine sequence, highly conserved among all picornaviruses, abolished translation. A conservation of distance between the conserved polypyrimidine tract and a downstream AUG could play an important role in the mechanism of internal initiation.

Poliovirus, a member of the *Picornaviridae* family, is a small icosahedral virus containing a positive-sense single-stranded RNA genome of approximately 7,500 nucleotides in length (42). The genomic RNA contains a covalently attached polypeptide at its 5′ terminus, which is removed in infected cells to generate a pUp-terminated mRNA (12, 13, 27, 32). Thus, poliovirus mRNA, in contrast to all known eukaryotic cellular mRNAs (except organellar), does not possess a 5′ cap structure (m^7GpppN, where N is any nucleotide). Similarly, all picornavirus mRNAs and several other viral mRNAs (e.g., caliciviruses and nepoviruses) do not contain a cap structure (1).

The cap structure plays an important role in translation initiation of cellular mRNAs through the interaction with eIF-4F, the cap-binding protein complex (for reviews, see references 40 and 47). eIF-4F, together with two other initiation factors (eIF-4A and eIF-4B), is thought to be involved in the melting of mRNA 5′ secondary structure to facilitate 40S ribosome binding (47). Inasmuch as poliovirus mRNA is uncapped, it must bypass the cap recognition step for translation initiation and thus translate in a cap-independent manner. This mechanism of translation is consistent with the ability of poliovirus to inactivate eIF-4F by proteolytic degradation (11; for a review, see reference 46). This is an important step in the inhibition of host protein synthesis after poliovirus infection, because poliovirus translation does not require eIF-4F. Poliovirus thus usurps the host protein synthesis machinery for synthesis of its proteins.

How do ribosomes initiate translation on poliovirus mRNA? We have previously reported that ribosomes bind internally to a sequence within the 5′ untranslated region (UTR) (35, 38), and sequences within the poliovirus 5′ UTR have been shown to be important for translation (5, 34). Similar results have been obtained for other members of the *Picornaviridae* family (17, 18, 23). The exact binding sequence is not known, but in vivo and in vitro studies suggest that a large sequence consisting of hundreds of nucleotides (nucleotides 140 to 630) is required for internal initiation of translation (34, 35, 50, 51). The 5′ UTR sequence required for internal initiation has been called the ribosome landing pad (RLP). This conclusion is consistent with genetic analyses of poliovirus mutants, showing that mutations in the 5′ UTR causing a small-plaque phenotype can be suppressed by second-site revertants separated by many nucleotides from the original mutation (22, 50). These data strongly suggest that the RLP assumes a highly ordered tertiary structure that is recognized by a *trans*-acting factor(s) and/or 40S ribosomes. The existence of such a superstructure has not been demonstrated directly, but considerable knowledge of the secondary structure of the poliovirus 5′ UTR has accumulated in recent years (26, 37, 41, 45). The models for the secondary structure of the poliovirus 5′ UTR are based on comparative phylogenetic sequence analysis, chemical modifications, and RNase mapping. According to these models, the 5′ UTR of poliovirus can fold into several stable and significant stem-loop structures (schematically shown in Fig. 3B). These structures are likely to participate in the binding of ribosomes to the RLP. The importance of a pyrimidine stretch in the 5′ UTR of foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus (EMCV) for internal initiation has been reported (19, 23). Here, we tested the importance of several of the stem-loop structures and the

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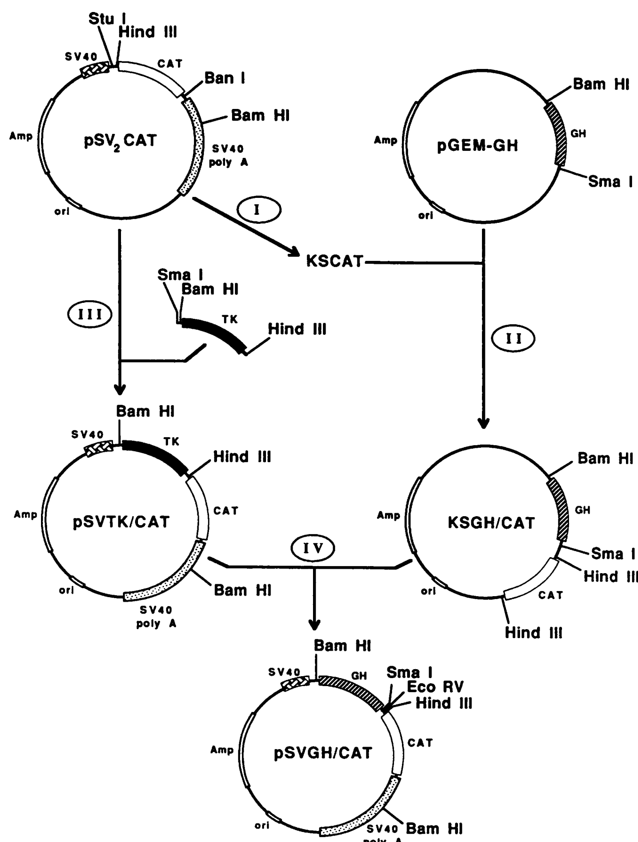

FIG. 1. Construction of pSVGH/CAT bicistronic vector. Details of the constructions are described in Materials and Methods.

pyrimidine stretch of the poliovirus 5′ UTR for internal initiation.

MATERIALS AND METHODS

Plasmid constructions. The poliovirus 5′ UTR used (pP2-5′) is a Mahoney-Lansing fusion in which Mahoney type 1 sequences constitute the first 68 nucleotides of the 5′ UTR and the remaining sequences are derived from Lansing type 2 (24, 34, 38). The poliovirus 5′ UTR was subcloned into the plasmid pSVGH/CAT (Fig. 1), which is transcribed into a bicistronic mRNA coding for human growth hormone (GH) and chloramphenicol acetyltransferase (CAT) under the control of the early simian virus 40 (SV40) promoter. The poliovirus 5′ UTR was inserted between the coding regions for GH and CAT. To prepare plasmid pSVGH/CAT, pSV2CAT was linearized with *Ban*I, treated with T4 DNA polymerase, and ligated to *Hin*dIII synthetic linkers; after digestion with *Hin*dIII, the CAT coding region was gel purified and ligated into Bluescript KS+ vector (Stratagene) at the *Hin*dIII site to create KSCAT (Fig. 1, step I). The coding region of human GH was removed from pGEM3-GH (a gift from Eric Cohen, Université de Montréal, Quebec, Canada) with *Bam*HI and *Sma*I, gel purified, and ligated into the KSCAT plasmid between the *Bam*HI and *Sma*I sites to generate plasmid KSGH/CAT (Fig. 1, step II). This plasmid was digested with *Bam*HI and *Hin*dIII, and the fragment containing the GH coding region was isolated and inserted into pSVTK/CAT in place of the thymidine kinase (TK) coding region (step IV) (pSVTK/CAT was constructed as described by Pelletier et al. [35] except that the *Hin*dIII site upstream of the CAT sequence was preserved [step III]). The resulting plasmid, pSVGH/CAT, was then linearized with *Eco*RV, the site into which the wild-type and mutant poliovirus sequences were inserted.

The 5′ UTR of poliovirus was removed from pP2-5′ with *Pst*I (which leaves four non-poliovirus nucleotides upstream of the first nucleotide of poliovirus) and *Eco*RV (which cuts 12 nucleotides upstream of the poliovirus initiator AUG). The *Pst*I-*Eco*RV fragment was made blunt by digestion with T4 DNA polymerase and inserted into the *Eco*RV site of pSV2GH/CAT to create pSVGH/Polio-5′-UTR/CAT. The same poliovirus 5′ UTR fragment was also inserted into a Bluescript KS+ vector in the *Eco*RV site (the resulting plasmid is called KSP2RV).

Deletion and mutagenesis of the poliovirus 5′ UTR. To make deletions within the poliovirus 5′ UTR, this region was excised from plasmid pP2-5′ with *Hin*dIII and *Eco*RV and made blunt with T4 DNA polymerase, and synthetic *Eco*RI linkers were attached. The DNA was digested with *Eco*RI, gel purified, and inserted into the KS vector at the *Eco*RI site (the resulting construct is called KSP2RI). The internal deletion construct Δ240-302 and 3′ deletions were generated by linearizing KSP2RI with *Bst*XI and *Bam*HI (for 3′ deletions) or with *Sty*I (Δ240-302), digesting with exonuclease III and then with mung bean nuclease, and religation (Stratagene Bluescript system). The resulting deletions were confirmed by dideoxy sequencing (43) and removed from KS by digestion with *Sac*I and *Pst*I (or *Pst*I and *Apa*LI for the internal deletions), made blunt by T4 DNA polymerase, and inserted into the *Eco*RV site of pSVGH/CAT. The 5′ deletions were generated from KSP2RV by restriction with *Eco*RI, exonuclease III and mung bean nuclease digestion, restriction with *Eco*RV, and gel purification of the fragments. These fragments were inserted into pSV2GH/CAT at the *Eco*RV site.

To create deletion mutants Δ350-380 and Δ450-Δ480, the 5′ UTR was removed from KSP2RV with *Eco*RI and *Hin*dIII

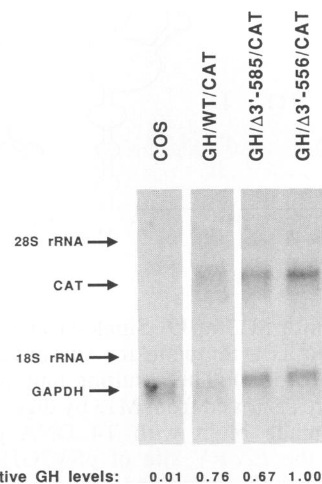

FIG. 2. Northern analysis of bicistronic mRNAs. Total RNA was prepared from COS cells 48 h after transfection with the indicated plasmids. RNA (10 μg) was resolved on a formaldehyde-agarose gel and transferred to nylon membranes as described previously (19a). The blot was first hybridized with a ^{32}P-labeled DNA probe containing a *Hin*dIII-*Bam*HI CAT gene fragment (35) and subsequently with a ^{32}P-labeled DNA probe for GAPDH. rRNA was used for size markers (19a).

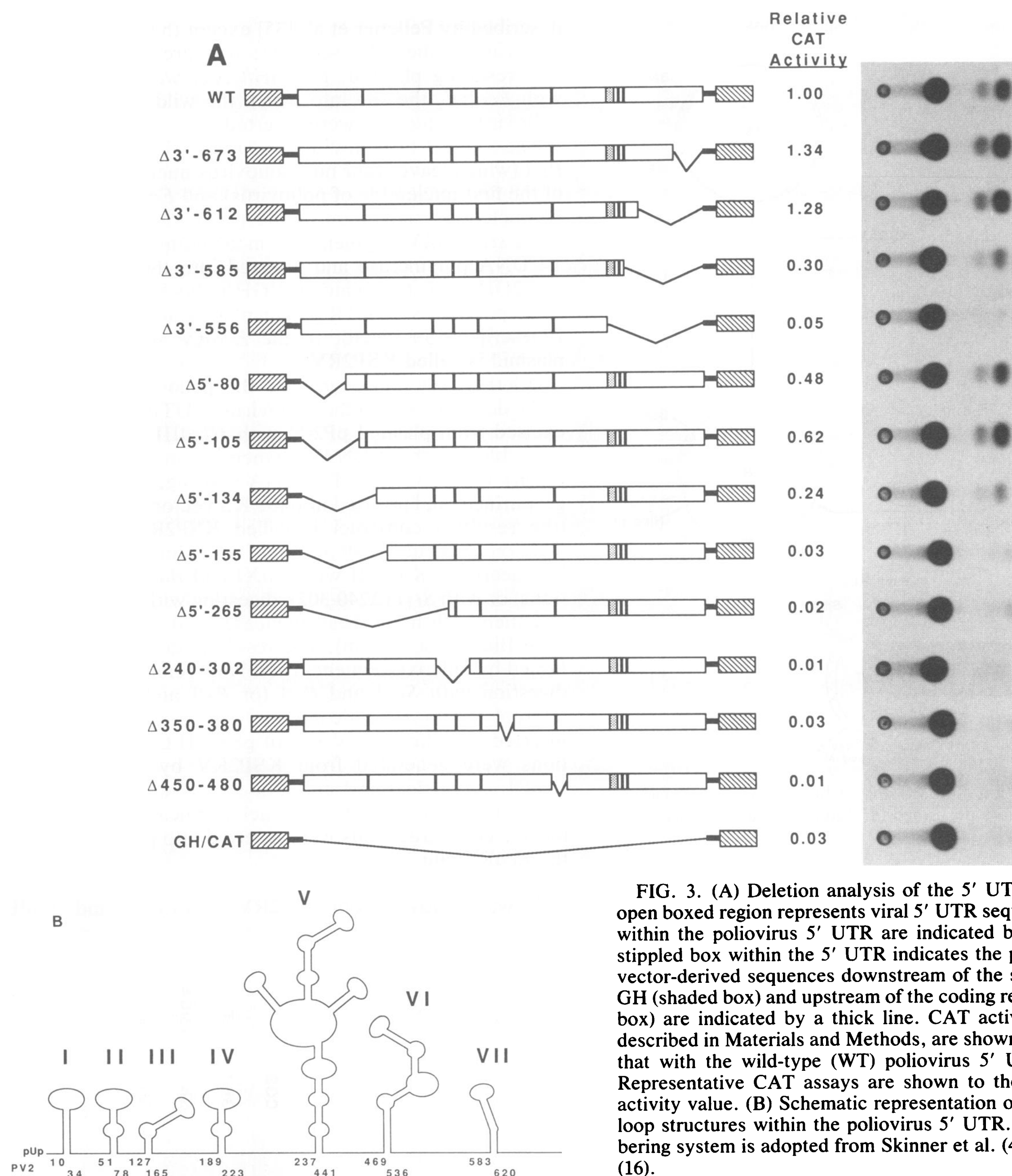

FIG. 3. (A) Deletion analysis of the 5′ UTR of poliovirus. The open boxed region represents viral 5′ UTR sequences. AUG codons within the poliovirus 5′ UTR are indicated by vertical lines. The stippled box within the 5′ UTR indicates the pyrimidine tract. The vector-derived sequences downstream of the stop codon of human GH (shaded box) and upstream of the coding region of CAT (shaded box) are indicated by a thick line. CAT activities, determined as described in Materials and Methods, are shown as values relative to that with the wild-type (WT) poliovirus 5′ UTR (value of 1.00). Representative CAT assays are shown to the right of each CAT activity value. (B) Schematic representation of the predicted stem-loop structures within the poliovirus 5′ UTR. The stem-loop numbering system is adopted from Skinner et al. (45) and Jackson et al. (16).

and inserted into M13mp19. Single-stranded DNA was isolated and used as a template for site-directed mutagenesis (Amersham protocol). The resulting mutants, Δ350-380 and Δ450-480, were removed from M13 by digestion with *Hin*dIII and *Eco*RI, made blunt with T4 DNA polymerase, and inserted into the *Eco*RV site of pSV2GH/CAT. For stem structure III and IV mutations, the 5′ UTR was removed from KSP2RV with *Bam*HI and *Hin*dIII and inserted into M13mp19. Single-stranded DNA from this construct was used to create the 5′-proximal mutants (S1, L1, and R2) and mutants M1 to M4. Mutant fragments were removed from M13 by *Bam*HI and *Sty*I digestion, except for M1 to M4 that were released with *Kpn*I and *Eco*RV, and inserted into KSP2RV. These mutants were released from the KS plasmids with *Sma*I and *Eco*RV, or *Kpn*I and *Eco*RV for M1 to M4, and inserted into pSVGH/CAT at the same sites. Mutant M5 was as described previously (33). Mutants M6 and M7 were kindly given by K. Meerovitch. All mutant inserts were sequenced in their entirety to ensure the absence of second-site mutations.

Cell culture and transfection. COS cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected by DEAE-dextran with 10 μg of test plasmid as described previously (3).

CAT and GH assays. At 48 to 56 h posttransfection, a fraction of the cell medium was removed, centrifuged to remove cells, and assayed for GH activity by a commercially available radioimmunoassay kit (Nichols Institute Diagnos-

tics of San Juan Capistrano). Fresh complete medium was used for serial dilutions of the samples to ensure that assays were performed in the linear range. Assays were performed in duplicates. Cells were harvested, and extracts were analyzed for CAT activity (3). The acetylated and unacetylated forms of [^{14}C]chloramphenicol were cut out of the thin-layer chromatography plates and quantitated in a scintillation counter. CAT activities were normalized to the amount of protein used (determined by Bio-Rad assay) and the level of GH activity. Values represent averages from at least three different experiments. CAT activity varied by less than 30% among the different experiments.

RESULTS

Boundaries of RLP. To determine the boundaries of the poliovirus 5′ UTR RLP, we constructed a series of 5′ and 3′ nested deletions. The mutated sequences were inserted into the bicistronic expression vector pSV2GH/CAT between the two cistrons (Fig. 1). Upon transfection into cells, the RNA transcribed from these constructs is translated into the GH protein, which is encoded by the first open reading frame (ORF), presumably in a 5′-end-dependent manner. Because all of the constructs used are identical at the 5′ end, the level of GH activity should accurately reflect the amount of RNA, assuming that mutations in the poliovirus sequence inserted downstream do not have differential effects on the synthesis of GH protein. This was confirmed by Northern (RNA blot) analysis with several of the constructs; the bicistronic mRNA levels always correlated with GH protein levels, which did not vary more than twofold (with a few exceptions in experiments not included in this article) for the different bicistronic constructs.

Figure 2 shows an example of a Northern analysis for the wild-type construct (GH/WT/CAT) and two 3′ deletion mutants and the corresponding levels of GH protein. As an internal control, we probed for glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) RNA levels. Also, the sizes of the different mRNAs were as expected, and only one major mRNA species was produced from each construct (Fig. 1 and data not shown). Therefore, GH activity was used to normalize for variations in transfection efficiencies. Thus, the level of CAT expression reflects the internal translational activity of the RLP.

The effects of deletions originating from the 3′ end of the 5′ UTR on CAT expression are shown in Fig. 3A. A deletion extending upstream to nucleotide 612 had a slight stimulatory effect on CAT synthesis (~30%). This deletion removed 133 nucleotides that are not conserved among poliovirus serotypes (49). Previous results showed that this sequence is not required for virus growth (22) or cap-independent translation in vitro (34, 35). A further deletion to nucleotide 585 reduced translation about threefold (Fig. 3A, Δ3′-585). The region deleted between nucleotides 585 and 612 includes stem-loop structure VII (Fig. 3B) and the seventh upstream AUG in the 5′ UTR (AUG-7). It has previously been demonstrated that mutation of the A nucleotide in AUG-7 reduced the in vitro translational efficiency of a downstream CAT reporter gene by approximately three- to eightfold, depending on the translation system used (33). Consistent with this, virus containing a mutated AUG-7 exhibited a small-plaque phenotype (33). We will show below that AUG-7 is responsible for the translation-stimulatory activity of this region. A deletion extending to nucleotide 556 abolished translation, indicating that a critical region for internal translation was deleted (Δ3′-556). This region includes a phylogenetically conserved pyrimidine tract (see Fig. 5). Further analysis of this region is detailed below. Thus, the 3′ border of an RLP core element lies between nucleotides 556 and 585.

Deletions of 80 or 105 nucleotides from the 5′ end of the poliovirus UTR resulted in about a twofold reduction in CAT expression (Fig. 3A, Δ5′-80 and Δ5′-105). The deletion of 80 nucleotides removes the two 5′-proximal stem-loop structures (Fig. 3B). A further deletion to nucleotide 134, which removes part of the base of stem-loop structure III (Fig. 3A and B), resulted in a fourfold reduction in translation. Removal of all of stem-loop structure III by deletion to nucleotide 155 abrogated translation. Thus, the 5′ border of the RLP core element lies between nucleotides 134 and 155.

The above analysis of the borders of the 5′ UTR RLP presents direct evidence that the RLP is composed of hundreds of nucleotides, in accord with earlier in vitro data (34, 35) and in vivo genetic evidence (50). However, there is evidence from in vivo studies that sequences which are within the boundaries of the RLP are dispensable for virus growth (9) (see below). We therefore made several internal deletions within the core element of the RLP to determine whether sequences within the RLP are important for translation (Fig. 3). These deletions removed between 30 and 60 nucleotides, encompassing sequences that are parts of the branched stem-loop structure V, the spacer region between stem-loops V and VI, and part of stem-loop VI (Fig. 3B). All of these deletions (Δ240-302, Δ350-380, and Δ450-480) caused complete abolition of CAT expression (Fig. 3A). These results suggest that stem-loop V and possibly VI are critical for internal initiation.

Sequences encompassing stem-loop structure III but not IV are essential for internal initiation of translation. Next, we made mutations and deletions in the 5′ UTR region encompassing stem-loop structures III and IV. Stem-loop structure III (nucleotides 127 to 165) of the poliovirus 5′ UTR is conserved among all enteroviruses. While the primary sequence within the stem of this structure varies phylogenetically, the secondary structure is highly conserved. The sequences within the loop of this structure vary in all positions except 145 to 147, which contain a highly conserved CCA sequence (data not shown). The conservation of this structure and the fact that deletions from the 5′ end of the 5′ UTR extending within this structure destroy translation implicate this region as being important for internal initiation. To address the importance of stem-loop structure III, we mutated part of the stem to destabilize the helix structure. When six proximal base-pairing nucleotides of the stem were altered, internal initiation was abolished (Fig. 4, construct S1). We also examined the significance of the conserved sequence CCA in the loop. An alteration of the two cytidines to guanosines eliminated internal initiation (Fig. 4, construct L1).

A deletion of nucleotides 221 to 224 in the 5′ UTR of an infectious cDNA copy of the poliovirus genome of poliovirus type 1, Mahoney, produced a temperature-sensitive (*ts*) virus with a small-plaque phenotype (9). Selection for non-*ts* revertants yielded a virus, R2, with a deletion of 45 proximal nucleotides (nucleotides 184 to 228). It was concluded that this sequence is dispensable for virus growth in HeLa cells (9). This sequence constitutes the entire stem-loop structure IV within the RLP sequence. It was therefore important to determine whether this structure plays a role in internal translation. Deletion of nucleotides 180 to 223, which encompass stem-loop structure IV in poliovirus type 2, resulted in only a twofold reduction in CAT expression (Fig.

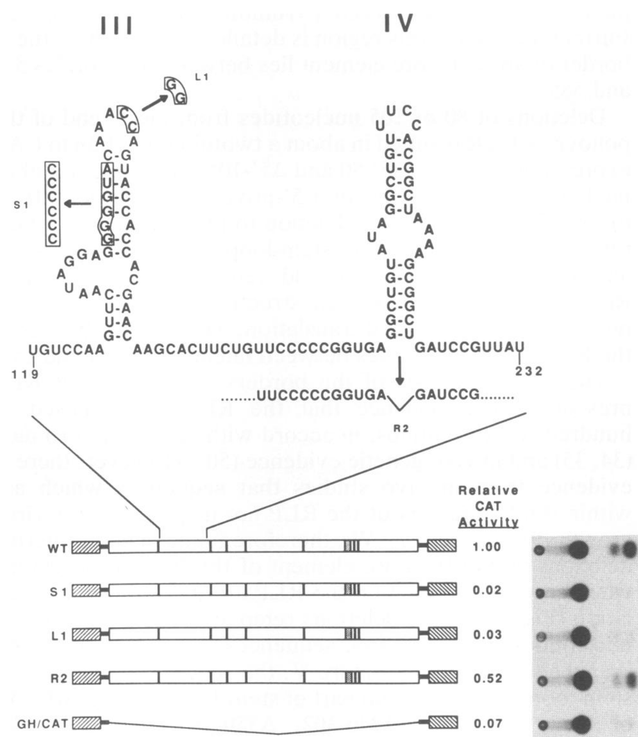

FIG. 4. Mutational and deletion analysis of the predicted secondary structures III and IV near the 5′ end of the poliovirus RLP. The stem of stem-loop structure III is mutated in construct S1. Two highly conserved nucleotides (CC) within the loop of stem-loop structure III are mutated in construct L1. Stem-loop structure IV is completely deleted in construct R2. The boxed regions and relative CAT activities are as described in the legend to Fig. 3. Representative CAT assays are shown to the right of the CAT activity values.

4). We conclude that stem-loop structure IV is not needed for internal initiation.

The polypyrimidine region upstream of AUG-7 is essential for internal initiation of translation. The 29-nucleotide sequence between 556 and 585 contains a polypyrimidine region that is highly conserved in the *Picornaviridae* family (Fig. 5). To determine the importance of this region for poliovirus translation, we examined the effects of various point mutations within the polypyrimidine sequence on internal initiation (Fig. 6). Changing the three proximal uridines in M2 (Fig. 6, nucleotides 560 to 562) or the two cytidines in M1 (Fig. 6, nucleotides 563 and 564) to guanosines resulted in a complete loss of CAT expression (Fig. 6). Mutations of the first distal uridine (nucleotide 565) in M3 had no effect on translation, while mutation of the four distal uridines in M4 reduced internal initiation by only 28%. This suggests that the five proximal nucleotides 560-UUUCC-564 are essential for internal initiation. A comparison of the available picornavirus 5′ UTR polypyrimidine sequences shows a strong conservation of the proximal cytidine and the adjacent upstream three uridines (Fig. 5), and our preliminary data showed that the second cytidine is dispensable for translation (data not shown). It is therefore likely that the important sequence is 560-UUUC-563. These findings are consistent with earlier results showing that 3′ deletions in the 5′ UTR of an infectious RNA that extend to the two

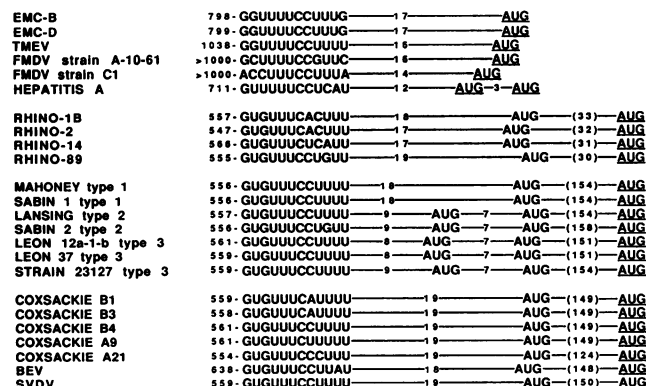

FIG. 5. Alignment of the polypyrimidine region of the 5′ UTR of picornaviruses. The polypyrimidine region upstream of the initiator AUG (underlined) is shown. The distance in nucleotides between the pyrimidine tract and the downstream AUGs is to scale and is indicated. The distance in nucleotides to the initiator AUG is not to scale and is indicated in parentheses. Several serotypes of the cardiovirus, aphtovirus, and the hepatitis A virus genera are grouped together in the upper block of sequences. TMEV, Theiler's murine encephalomyelitis virus. The second block are rhinoviruses. The third block are several serotypes and strains of poliovirus. The bottom block are enteroviruses. BEV, bovine enterovirus; SVDV, swine vesicular disease virus. The source of the sequences was the EMBL and GenBank data bases. The two potential AUG initiators for hepatitis A virus are underlined.

cytidines yield viable virus (albeit exhibiting a small-plaque phenotype), while further deletions destroy virus viability (15, 22).

Mutations in AUG-7 reduce but do not eliminate internal initiation. We have shown in in vitro experiments that mutation of the adenosine of AUG-7 or the other nucleotides of the AUG triplet reduced translation between three- and eightfold, depending on the translation system used, and reduced virus growth in HeLa cells (29, 33). We wanted to measure the effect of mutations in all of the other nucleotides of AUG-7 on translation in vivo. A mutation of A-588

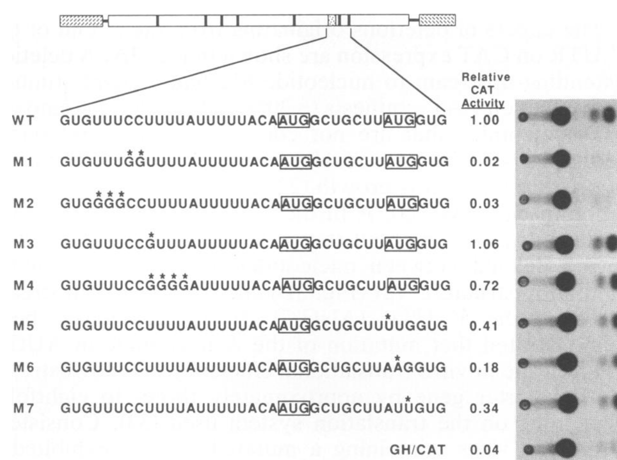

FIG. 6. Mutational analysis of the 5′ UTR of poliovirus. Mutations of the polypyrimidine region and AUG-7 in the poliovirus 5′ UTR are indicated by an asterisk (*) over the altered nucleotide. Upstream AUG codons 6 and 7 are boxed. For a description of the wild-type construct, see Fig. 3.

reduced CAT expression by 2.5-fold (M5, Fig. 6). Mutation of U-589 into an A nucleotide reduced CAT expression by more than fivefold (M6), whereas mutation of G-590 into a U nucleotide reduced CAT expression by threefold (M7). It is not clear how significant is the differential reduction in translation by the individual mutations in the AUG triplet. We have not seen such differences in in vitro translation assays (29). However, if significant, it argues that AUG-7 does not function as an initiator of translation, in accord with earlier suggestions (33).

DISCUSSION

The results reported here demonstrate that the RLP in the poliovirus 5′ UTR consists of at most approximately 400 nucleotides. Here we have shown that the RLP contains a core region spanning nucleotides 134 to 585 that is essential for internal initiation on poliovirus RNA. Not all of the sequences within the core RLP are critical for translation (Fig. 4). The core RLP is flanked by sequences that enhance internal ribosome binding to the poliovirus 5′ UTR.

The 5′ boundary of the core RLP is between nucleotides 134 and 155 and the 3′ boundary is between nucleotides 556 and 585 (Fig. 3A). A deletion extending to nucleotide 80 or 105 reduced translation by 50% only, and further deletion to position 134 reduced CAT expression by approximately fourfold (Fig. 3A). It is likely that the deletion of eight nucleotides at the base of the stem of stem-loop structure III is responsible for most of this decrease. However, since we consistently found a small effect (approximately twofold) on translation by deleting sequences upstream of nucleotide 80, it is possible that 5′-proximal sequences modulate translational efficiency to some extent. A deletion which removes most of stem-loop structure III was deleterious for translation. Therefore, this stem-loop structure might play a critical role in internal initiation. Our results are in general agreement with the demarcation of the RLP by in vitro studies with the authentic poliovirus coding sequence, although deletion of sequences between nucleotides 140 and 320 reduced but did not abrogate translation by fivefold in poliovirus-infected cell extracts (34). In the latter report, it was also shown that when the poliovirus 5′ UTR was linked to the CAT ORF, the 5′-proximal 320 nucleotides were almost dispensable for translation in poliovirus-infected HeLa cell extracts. Thus, in this respect, translation from a bicistronic mRNA in vivo in the current report required more 5′ upstream sequences than the previous translation results in vitro. One possible explanation for the discrepancy is that we have previously used monocistronic mRNAs, whereas here we used bicistronic mRNAs. In support of this explanation, we have noted that the sequence between nucleotides 140 and 320 is also absolutely required for internal initiation on a bicistronic mRNA in vitro (32a).

The fourth stem-loop structure in the 5′ UTR, encompassing nucleotides 189 to 223, is not a critical component of the RLP, since its removal had only a marginal effect on CAT expression (ca. twofold reduction). These results are in accord with earlier studies showing that deletion of this stem-loop had only a small effect on virus growth (9). In addition, stem-loop structure IV is completely missing in bovine enteroviruses (10). It thus appears that the second stem-loop structure in the RLP is not essential for ribosome internal binding. Stem-loop structures V and possibly VI are also important for translation, as deletions which affect their stability abolished translation (Fig. 3).

Our results are at variance with a recent report by Simoes and Sarnow (44), who showed that insertion of extra nucleotides into stem-loop I (nucleotides 10 to 34) dramatically reduced translation of a downstream luciferase reporter gene following RNA transfer into tissue culture cells. It is possible that the insertion into stem-loop I engendered a general conformational change that affected the RLP structure. It has been shown, however, that the structure of stem-loop II is important for RNA replication (2).

Function of the pyrimidine stretch in internal binding of ribosomes. The absolute requirement of the proximal portion of the pyrimidine stretch sequence (560-UUUC-563) for internal initiation is of particular interest because this sequence is conserved in all picornaviruses (Fig. 5). This conservation is striking in light of the lack of other conserved sequences in the rest of the 5′ UTR of picornavirus mRNAs. It is likely, therefore, that this sequence plays a critical role that is common for all picornaviruses. Another striking finding is the presence of a conserved AUG triplet ca. 15 to 20 nucleotides downstream from the pyrimidine stretch. In some genera, such as aphthoviruses and cardioviruses, this AUG is the translation initiation codon, but in enteroviruses and rhinoviruses it is not. In the latter genera, the initiator AUG is the next AUG, located approximately 30 nucleotides downstream in rhinoviruses and approximately 150 nucleotides downstream in enteroviruses (Fig. 5). However, the upstream AUG strikingly affects the translational efficiency of poliovirus (tested for Lansing type 2 [33]). It is conceivable that the homologous AUG in other enteroviruses and rhinoviruses will have a similar effect. This result demonstrates that, although the poliovirus equivalent of the cardiovirus and aphthovirus initiator AUG is not functional as an initiation codon, it plays a role in ribosome binding.

The importance of the conserved distance between the pyrimidine stretch and AUG-7 is further supported by our finding that a deletion of 13 nucleotides between these elements had a significant inhibitory effect on translation (31a). The nonequivalence in the function of the conserved AUG downstream from the pyrimidine stretch among the different picornavirus genera might have important implications for the mechanism by which the ribosome selects the initiator AUG. For EMCV, the ribosome binds directly to the initiator AUG, which is located ~20 nucleotides from the conserved pyrimidine stretch (20). This binding is also dependent on sequences upstream from the pyrimidine stretch. It is very likely that for poliovirus (and other entero- or rhinoviruses), initial ribosome binding occurs in a manner similar to that in EMCV and FMDV, possibly binding at the pyrimidine stretch; but instead of initiating translation at the conservatively spaced AUG, it translocates by an unknown mechanism to the more downstream initiator AUG (16). This model is supported by the findings that insertion of secondary structure or AUG codons downstream of AUG-7 reduces translation in vitro (35) or virus growth (21).

How does the polypyrimidine tract function in translation initiation? It was suggested that base complementarity between the pyrimidine stretch of FMDV RNA and a purine-rich sequence at the 3′ end of 18S rRNA promote the interaction between the picornavirus mRNA and the small ribosomal subunit in a manner analogous to that of the prokaryotic Shine-Dalgarno sequence (4, 23). A similar model could be proposed for the interaction of part of the poliovirus pyrimidine stretch (UUCCUUU) with the 3′ end of 18S rRNA. However, the purine-rich region at the 3′ end of 18S rRNA is engaged in stable secondary structure (7, 39), and this helical structure is highly conserved among prokaryotes and eukaryotes. Although it is plausible that this helical

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       (469-536)                                      (586-616)

                                                           U
A                                                   600-A.   U
                                                         C   G
                                                         U   A
       |   |                                             A   U
       |   |                                             A   U
       A   U                                             C   G
   480-G   U                                             A   U
       G   C                                             G   U
       C   G                                             U   A-
       A   U-530                                         G   U
       C   G   UAC                                      -G   C
      AC   G  C   U                                      U   A
     A      C  A U                                       A   U
      UC   G   G U                                 580   U   A
       C   G   C G    .    |    .    |    .    |         U   A
5'-AAU   A--AC GGUGUCCGUGUUUCCUUUUAUUUUUACAAUGGCUGC       AGC-3'
                   ***  *******
            3'-U GGCA  AAGGAAA  G--G C-5'(660)
               U A  G U|      U G  G U
          1690-U A   U 1200   U A  G   G
               C G-1210       C G  G C
               C G            A U  C A
               C G           A   A C G
               G C          A      U  U-----Central Domain
               U A           G   U |        (660--1088)
               | |            U G  |
               | |            C G  U G-1089
       3' Major Domain        G U  G C
          (1207--1692)        A U  G C
                             A   G | |
                              A C  (1089--1160)
                                |
                              1180
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B

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Poliovirus type 2 (Lansing)                 (556) 5'-CGU-guUUCCUUUu-3' (568)
                                                     ***   *******

    Eukaryotes
1.  Homo sapiens (18s)                     (1206) 3'-GCAguuAAGGAAAu-5' (1193)
2.  Oryctolagus caniculus                  (1197) 3'-GCAguuAAGGAAAu-5' (1189)
           (Rabbit, 18s)
3.  Rattus norvegicus (18s)                       3'-GCAguuAAGGAAAu-5'
4.  Rat (18s)                                     3'-GCAguuAAGGAAAu-5'
5.  Mus musculus (18s)                            3'-GCAguuAAGGAAAu-5'
6.  Xenopus laevis (18s)                          3'-GCAguuAAGGAAAu-5'
7.  Artemia salina (18s)                          3'-GCAguuAAGGAAAu-5'
8.  Saccharomyces cerevisiae (Yeast, 18s)         3'-GCAguuAAGGAAAu-5'
9.  Dictyostelium discoideum (17s)                3'-GCAguuAAGGAAAu-5'
10. Tetrahymena thermophila (17s)                 3'-GCAguuAAGGAAAu-5'
11. Oryza Sativa (Rice, 17s)                      3'-GCAguuAAGGAAAu-5'
12. Zea mays (Maize, 17s)                         3'-GCAguuAAGGAAAu-5'
13. Glycine max (18s)                             3'-GCAguuAAGGAAAu-5'

    Archaebacteria
14. Halobacterium cutirubrum (16s)                3'-GCGguuAAGGAAAu-5'
15. Halobacterium halobium (16s)                  3'-GCGguuAAGGAAAu-5'
16. Halobacterium volcanii (16s)                  3'-GCGguuAAGGAAAu-5'
17. Halococcus morrhuae (16s)                     3'-GCGguuAAGGAAAu-5'
18. Methanospirillum hungatei (16s)               3'-GCGguuAAGGAAAu-5'
19. Methanococcus vannielii (16s)                 3'-GCGguuAAGGAAAu-5'
20. Methanobacterium formicicum (16s)             3'-GCGguuAAGGAAAu-5'
21. Sulfolobus solfataricus (16s)                 3'-GCGguuAAGGAAAu-5'
22. Thermoproteus tenax (16s)                     3'-GCGguuAAGGAAAu-5'

    Eubacteria
23. Escherichia coli (16s)                  (925) 3'-GCAguuAAGuAAAc-5' (912)
24. Agrobacterium tumefaciens (16s)               3'-GCAguuAAGGAAAc-5'
25. Desulfovibrio desulfuricans (16s)             3'-GCAguuAAGGAAAc-5'
26. Myxococcus xanthus (16s)                      3'-GCAguuAAGGAAAc-5'
27. Pseudomonas testosteroni (16s)                3'-GCAguuAAGGAAAc-5'
28. Proteus vulgaris (16s)                        3'-GCAguuAAGuAAAc-5'
29. Bacillus subtills (16s)                       3'-GCAguuAAGGAAAc-5'
30. Heliobacterium chlorum (16s)                  3'-GCAguuAAGGAAAc-5'
31. Mycoplasma capricolum (16s)                   3'-GCAguuAAGGAAAc-5'
32. Mycoplasma sp. (16s)                          3'-GCAguuAAGGAAAc-5'
33. Bacteroides fragilis (16s)                    3'-GCAguuAAGGAAAc-5'
34. Flavobacterium heparinum (16s)                3'-GCAguuAAGGAAAc-5'
35. Anacystis nidulans (16s)                      3'-GCAguuAAGGAAAc-5'

    Chloroplasts
36. Euglena gracilis (16s)                        3'-GCAguuAAGGAAAc-5'
37. Chlamydomonas reinhardii (16s)                3'-GCAguuAAGGAAAc-5'
38. Zea mays (16s)                                3'-GCAguuAAGGAAAc-5'
39. Nicotiana tabacum (16s)                       3'-GCAguuAAGGAAAc-5'

    Mitochondria
40. Zea mays (18s)                                3'-GCAguuAAGGAAAc-5'
41. Triticum aestivum (18s)                       3'-GCAguuAAGGAAAc-5'
42. Oenothera sp. (18s)                           3'-GCAguuAAGGAAAc-5'
43. Homo sapiens (12s)                            3'-GCGguccAGGAAAc-5'
44. Mus musculus (12s)                            3'-GCGguucAGGAAAc-5'
45. Rattus norvegicus (12s)                       3'-GCGguucAGGAAAc-5'
46. Xenopus laevis (12s)                          3'-GCGguucAGGAAAc-5'
47. Aspergillus nidulans (15s)                    3'-cCAgauuAcuAAAG-5'
48. Saccharomyces cerevisiae (15s)                3'-GCAgauAAcaAAAc-5'

                                                     ***   *******
Poliovirus type 2 (Lansing)                 (556) 5'-CGU-guUUCCUUUu-3' (568)
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FIG. 7. (A) Possible base-pairing model between poliovirus type 2 (Lansing, referred to as PV2L) and human 18S rRNA. The model shows the two stem-loop structures which are highly stable and significant in the RNA folding of polioviruses (25, 26). The RNA secondary structure of the human 18S rRNA encompassing nucleotides 660 to 1692 is based on phylogenetic comparison analysis (7, 14). The complementarity occurs between 5′-CGUguUUCCUUU-3′ (556 to 567) of PV2L and 3′-GCAguuAAGGAAA-5′ (1194 to 1206) of human 18S rRNA. In the structure model of rabbit 18S rRNA, four of five A bases in the sequence 3′-AAGGAAA-5′ are strongly reactive toward single-strand-specific chemical probes (diethyl pyrocarbonate or dimethyl sulfate [39]). One A and one out of two G bases are weakly reactive toward single-strand-specific chemical probes (39). The asterisks between two sequences denote base-pairing between the poliovirus sequence and human 18S rRNA. (B) Sequence alignment of the putative base-pairing region in 18S rRNAs and 16S rRNAs (14).

structure is melted to enable the interaction with the mRNA, we have identified another candidate sequence, 3′-AAAG GAA-5′, spanning nucleotides 1194 to 1200 in human 18S rRNA that can base-pair with the pyrimidine stretch (Fig. 7A). This sequence is evolutionarily conserved (Fig. 7B), and chemical and RNase probing results suggest that this region is not base-paired (39). Another region complementary to the pyrimidine stretch exists between nucleotides 1822 and 1837. This region is not base-paired and is highly conserved throughout evolution (39). We believe, however, that this region is less likely to be of functional importance because it does not pair with nucleotides 562 and 563 (UC), which are important for internal initiation. Base complementarity between the pyrimidine stretch and 28S rRNA has also been suggested (15). If this is functionally significant, then a process involving 60S joining to the initiation complex is also mediated by the pyrimidine stretch of the picornavirus mRNA 5′ UTR.

Ribosome binding to the RLP most probably requires the participation of *trans*-acting cellular and possibly viral factors. Cellular proteins that interact with different regions of the 5′ UTRs of poliovirus and other picornavirus 5′ UTRs have been identified (6, 8, 19, 30, 31) but their mechanism of action in promoting ribosome binding is not understood. Clearly, a complex RNA structure and *trans*-acting factors, in addition to the canonical set of initiation factors, facilitate the efficient internal initiation of translation on poliovirus and other picornavirus mRNAs.

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