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Mutational Analysis of Upstream AUG Codons of Poliovirus RNA

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The 5′ untranslated region of poliovirus type 2 Lansing RNA consists of 744 nucleotides containing seven AUG codons which are followed by in-frame termination codons, thus forming short open reading frames (ORFs). To determine the biological significance of these small ORFs, all of the upstream AUG codons were mutated to UUG. The point mutations were introduced into an infectious poliovirus cDNA clone, and RNA transcribed in vitro from the altered cDNA was transfected into HeLa cells to recover the virus. Mutation of AUG 7 resulted in a virus (called R2-5NC-14) with a small-plaque phenotype, whereas mutation of the other six AUG codons produced virus with a wild-type plaque morphology. To determine whether the small-plaque phenotype of R2-5NC-14 was due to altered translational efficiency of the viral mRNA, we constructed chimeric mRNAs containing the 5′ noncoding region of poliovirus mRNA fused to the chloramphenicol acetyltransferase (CAT) coding sequence. mRNA containing a mutated AUG 7 codon showed decreased translational efficiency in vitro. The results indicate that the upstream ORFs of poliovirus RNA are not essential for viral replication and do not act as barriers to the translation of poliovirus mRNA. AUG 7 and flanking sequences may play a positive acting role in poliovirus RNA translation.

Several distinguishing features of poliovirus polysomal RNA include a 5′ end terminating in pUp (6, 19) and a long 5′ noncoding region (ca. 750 nucleotides) containing seven or eight (depending on the poliovirus serotype) AUG codons. The AUG codons initiate small open reading frames (ORFs), whose polypeptide products have escaped detection both in vivo and in vitro. Three of the AUGs are conserved in position among the poliovirus serotypes (31), but the ORFs specified by these AUGs are not conserved in length or amino acid composition. It is not known whether the upstream AUG codons play a role in poliovirus replication.

Most eucaryotic mRNAs are functionally monocistronic and utilize the 5′-proximal AUG codon as the site of translation initiation. A scanning model proposes that 40S ribosomes (with associated initiation factors) bind at or near the 5′ terminus of an mRNA and scan the 5′ noncoding region until the appropriate initiation codon is reached (11). A small number of mRNAs, however, contain one or more minicistrons preceding the major ORF. It was suggested that in such cases, eucaryotic ribosomes are capable of termination-reinitiation reactions, thus allowing for the expression of downstream ORFs (see, e.g., references 20 and 21). Consistent with this, the polypeptide products encoded by the upstream minicistrons on several bicistronic mRNAs have been detected in vivo (7, 10, 34) and in vitro (4). The presence of upstream minicistrons generally acts to decrease the expression of the downstream major ORF (9, 10, 18, 21, 29), possibly because not all terminating ribosomes become competent for reinitiation. Poliovirus initiates translation, however, by binding of ribosomes to an internal sequence in the mRNA 5′ noncoding region, thus avoiding most of the upstream AUGs (23). Consequently, it is not a priori clear that they play a role in modulating translation from the major downstream ORF.

In this report we examine the possible roles of the upstream AUG codons of poliovirus RNA in translation. To this end, we have used site-directed mutagenesis to change all of the upstream AUG codons to UUG. Our results indicate that none of the upstream ORFs are essential for poliovirus replication. However, a mutant virus containing an altered AUG codon 7 forms small plaques in HeLa cells. When the 5′ untranslated region (UTR) of this mutated viral RNA was linked to the chloramphenicol acetyltransferase (CAT) gene, a decrease in translational efficiency was observed in vitro compared with that of the wild-type RNA. These results suggest a positive role for the adenosine nucleotide of AUG 7 in poliovirus RNA translation.

MATERIALS AND METHODS

Construction of poliovirus mutants. The starting plasmid used for site-directed mutagenesis studies, pP2-5′, has been previously described (22). A *Hin*dIII-*Eco*RI restriction fragment containing nucleotides 1 to 786 of the poliovirus type 2 Lansing 5′ end (14) was inserted into *Hin*dIII-*Eco*RI-digested M13mp19. Point mutations in this fragment were constructed by the two-primer method of Zoller and Smith (35). Mutagenic primers were synthesized on an Applied Biosystems oligonucleotide synthesizer and contained one or two mismatches with the wild-type sequence. The nucleotide sequence of the various mutants was determined by dideoxy sequencing (28). P2(AUG-6)/CAT and P2(AUG-7)/CAT were constructed by reverting the double mutant P2(AUG-6-7). Following mutagenesis, the DNA was linearized with *Eco*RI, filled in with DNA polymerase I (Klenow fragment) and restricted with *Hin*dIII. The poliovirus fragment was directionally subcloned into pSP64 (between the *Hin*dIII and *Hin*cII sites). The resulting constructs were restricted with *Eco*RV (site at nucleotide 730 of the poliovirus type 2 Lansing genome) and *Bam*HI and then ligated to the CAT gene. The CAT coding and 3′ UTR region was isolated from pSV2CAT by restricting with *Hin*dIII, repairing with Klenow fragment, and cleaving with *Bam*HI. The insert was purified from a low-melting-temperature agarose gel (15) and used for ligation. Manipulations involving DNA

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were carried out by established procedures (see reference 17 and references therein).

Isolation of viruses containing mutated AUG codons. The poliovirus 5′ noncoding regions containing mutated AUG codons were built into full-length poliovirus type 2 Lansing cDNA in two steps. First, the *Kpn*I-*Eco*RI restriction fragment encompassing nucleotides 68 to 786 of the poliovirus type 2 Lansing genome (see above) was excised from M13 replicative-form DNAs containing the mutated AUG codon. This DNA fragment was subcloned into *Kpn*I-*Eco*RV-cleaved pJB127, which contains nucleotides 1 to 220 of the poliovirus type 1 Mahoney genome downstream of the bacteriophage T7 promoter (33). These subclones were designated pJB(AUG-*n*) where the number *n* refers to the AUG codon. Next, pJB(AUG-*n*) was cleaved with *Eco*RV and *Pst*I, and the large fragment was gel purified and ligated to a poliovirus type 2 Lansing DNA fragment extending from the *Eco*RV site at nucleotide 730 through the 3′ end of the genome and to the *Pst*I site in pBR322 (14). The resulting constructions, called pT7L(AUG-*n*), consisted of a full-length copy of poliovirus type 2 Lansing cDNA downstream of the T7 promoter and containing the appropriate mutated AUG codon. Note that in this construction, as well as in the original full-length poliovirus type 2 Lansing cDNA (24), the first 68 nucleotides are actually derived form poliovirus type 1 Mahoney; the two viruses differ at four nucleotide positions in this area.

To determine whether infectious virus containing AUG codon mutations could be recovered, pT7L(AUG-*n*) was linearized with *Sac*I (site at the 3′ end of the viral genome) and used as a template for RNA synthesis in vitro by using T7 RNA polymerase as described previously (8). RNA was transfected into HeLa cells with DEAE-dextran as a facilitator (8), and cells were incubated at 37°C until a complete cytopathic effect was observed. Plaque purifications and titrations on HeLa cell monolayers were performed as described previously (14). Nomenclature of mutant viruses is by accepted convention (25). To confirm that viruses contained the desired mutation, viral RNA was prepared and its nucleotide sequence in the area of the AUG codons was determined by the dideoxynucleotide method, with reverse transcriptase and oligonucleotide primers (13).

Analysis of viral replication. Viral protein synthesis, RNA synthesis, and virus production were examined at different times postinfection in triplicate monolayers of 2×10^6 HeLa cells infected at a multiplicity of infection of 10 PFU per cell. Methods used for analysis were essentially as described previously (25). Briefly, virus yields were determined by harvesting cells, freeze-thawing, and quantitating virus by plaque assay. Protein synthesis was examined by pulse-labeling with [^{35}S]methionine for 15 min and fractionating polypeptides by electrophoresis on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel. Viral RNA synthesis was quantitated by hybridization with a slot-blot apparatus. For measurement of RNA levels, the exposed X-ray film was scanned by laser densitometry.

In vitro transcriptions and translations. Plasmids were linearized with *Bam*HI, and transcriptions were performed by the method of Pelletier et al. (22). Mock- and poliovirus-infected HeLa cell extracts were prepared at 3 h after infection, essentially as described by Rose et al. (27), with minor modifications (16). For gel analysis of translation products, aliquots from translation mixtures were mixed with SDS sample buffer, boiled for 5 min, and applied on SDS–10 to 15% polyacrylamide gels. Gels were fixed in 40% methanol–7.5% acetic acid, treated with En^{3}Hance (Du Pont, NEN Research Products), and exposed against X-ray film at −70°C. Radioactive bands were quantitated by laser densitometry. Each experiment was repeated at least three times, and the results varied by not more than 20%.

RESULTS

To determine whether the ORFs found within the poliovirus 5′ noncoding region are required for virus replication, we performed site-directed mutagenesis to convert the AUG codons upstream of the poliovirus polyprotein initiation site to UUG codons. None of the introduced mutations resulted in the generation of termination codons in any of the three reading frames. Figure 1 illustrates the positions of the point mutations introduced into the seven upstream AUG codons. To determine the effect of these mutations on virus replication, we constructed full-length cDNA clones of the poliovirus type 2 Lansing viral genome which contained the various mutations. RNA produced from the different templates by T7-directed transcription was subsequently transfected into HeLa cells at 37°C. A cytopathic effect was observed in monolayers transfected with RNAs containing mutations at each of the AUG codons. For further characterization, viruses from these cell supernatants were twice plaque purified. Nucleotide sequence analysis of viral RNA indicated that all seven mutant viruses, called R2-5NC-8 through R2-5NC-14 (Fig. 1), contained the expected base change. All viruses formed wild-type-sized plaques on HeLa cell monolayers and grew to wild-type titers, with the exception of R2-5NC-14, which formed small plaques (Fig. 2A).

To better characterize the mutant virus R2-5NC-14, we measured several parameters of its life cycle. One-step growth curves showed that R2-5NC-14 replicated more slowly than did wild-type poliovirus type 2 Lansing and produced a 10-fold-lower yield of infectious virus (Fig. 2B). Viral RNA synthesis in R2-5NC-14-infected cells was first detected at 4 to 5 h postinfection compared with 2 to 3 h for wild-type virus. Final levels of viral RNA in mutant-infected cells eventually reached ca. 50% of the wild-type level (Fig. 2C). We next analyzed the pattern of viral and cellular proteins synthesized in infected cells. Cells were pulse-labeled with [^{35}S]methionine at various times postinfection, and cytoplasmic extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Synthesis of wild-type poliovirus proteins was first detected at 3 h postinfection, reached maximal levels by 4 h, and declined thereafter. In contrast, viral protein synthesis was absent at 4 h in R2-5NC-14-infected cells, was barely detected by 5 h postinfection, and was maximal by 8 h. Even at this time, the rate of synthesis of R2-5NC-14 proteins was still 10 times lower than that observed in wild-type-infected cells at the time of maximal synthesis (4 h). Furthermore, the kinetics of inhibition of host translation were slower in R2-5NC-14-infected cells. By 2 h postinfection, levels of host cell proteins were clearly lower in wild-type-infected cells. Host translation was maximally inhibited in wild-type-infected cells by 4 h postinfection, at which time there was still residual host protein synthesis in R2-5NC-14-infected cells. The stronger inhibition of protein synthesis compared with RNA synthesis is consistent with the former's being the primary defect in virus replication (32).

The above results indicate that the upstream ORFs are not essential for poliovirus replication in HeLa cells. However, mutations at AUG 7 appeared to lower the efficiency of viral replication. To determine whether this mutation caused a

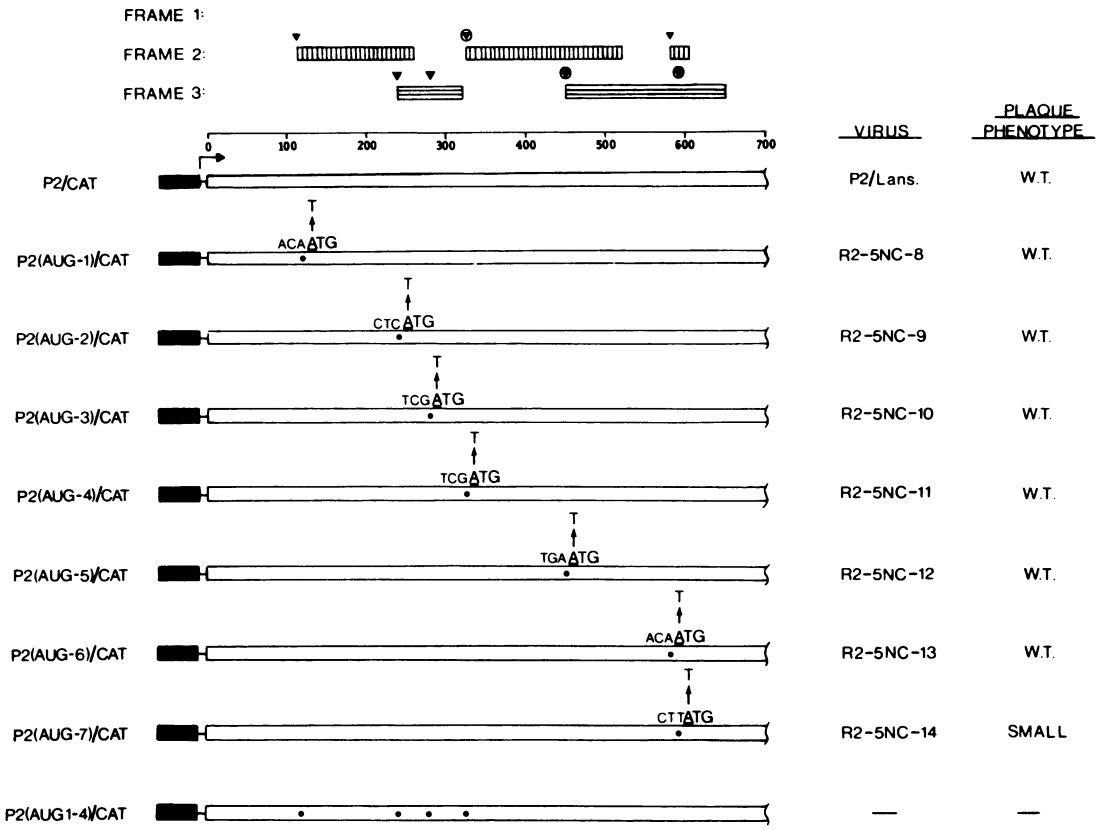

FIG. 1. Point mutations in the upstream AUG codons of the poliovirus 5′ UTR. The open box represents poliovirus type 2 Lansing viral sequences. The potential ORF defined by the AUGs in the 5′ UTR are schematically represented at the top of the diagram. Symbols: ▼, AUG positions; ⓥ, AUGs conserved among the three poliovirus serotypes. The AUG and flanking nucleotide sequences are indicated above the respective mutated codons. The positions of the mutations in the AUG codons are shown (●). The names and phenotypes of the viruses containing the indicated mutations are shown at the right.

reduction in translation efficiency, poliovirus-CAT fusion constructs containing the different mutated AUGs were transcribed by using the SP6 system (22), and the RNA was translated in extracts prepared from mock- and poliovirus-infected HeLa cells. HeLa cells have been traditionally used to study poliovirus-host relationships, and extracts prepared from poliovirus-infected HeLa cells mimic the shutoff of the host-protein synthesis phenomenon observed in vivo (5, 16, 27). For the in vitro experiments, both unmethylated (GpppG. . .) and methylated (m^7GpppG. . .) capped mRNAs were synthesized in the event that alteration of the AUG codons affected the nature of the cap-independent translation of poliovirus mRNA. We used unmethylated capped mRNA rather than uncapped mRNAs, because the former is as stable as capped methylated mRNAs (3), but, similarly to uncapped mRNA, translates inefficiently (2). In all in vitro translations, *S*-adenosyl-L-homocysteine was included to prevent methylation of unmethylated capped mRNAs by endogenous methyltransferases (2).

The bulk of translation of poliovirus mRNA in vivo occurs under restrictive conditions, where host protein synthesis is shut off. This is thought to be due to inactivation of the cap-binding protein complex, eIF-4F (see reference 30 for a review) and a still poorly understood second event (1). Consequently, it was important to assess the effect of the AUG mutations on translation in poliovirus-infected HeLa cell extracts. Translation of poliovirus mRNA proceeds in a cap-independent fashion and requires sequences within its 5′ UTR for its expression in a poliovirus-infected HeLa cell extract (22, 23). CAT mRNA is not translated in these extracts owing to the restriction on cap-dependent mRNA translation (Fig. 4, lanes 2 and 3). However, fusion of the poliovirus 5′ noncoding region to the CAT coding region confers on CAT mRNA the ability to translate in a cap-independent manner (lanes 4 and 5), as was previously shown (22, 23). Mutation of the four 5′-proximal AUGs (lanes 6 and 7) generated an mRNA species which translated with ca. 50% of the efficiency of P2-CAT (lanes 4 and 5), and a mutation of AUG 6 [P2(AUG-6)/CAT; lanes 8 and 9] resulted in an mRNA that translated with efficiency similar to that of P2-CAT mRNA. However, in striking contrast to the latter mutations, mutation of AUG 7 alone [P2(AUG-7)/CAT; lanes 10 and 11] and in combination with AUG 6 [P2(AUG-6-7)/CAT; data not shown] caused an eightfold decrease in translational efficiency as compared with P2/CAT (compare lane 10 with lane 4).

Figure 5 shows the translation of mRNAs containing the mutated AUGs in a mock-infected HeLa extract. This experiment was performed to determine whether the translation of P2(AUG-7)/CAT mRNA could be cap stimulated. We reasoned that if the region encompassing AUG 7 is important for cap-independent translation, translation of P2(AUG-7)/CAT mRNA, in which this region is altered, might proceed by a cap-dependent mechanism. Translation of unmethylated capped CAT mRNA resulted in the synthesis of 16- and 26-kilodalton polypeptides (lane 2). The 26-kilodalton poly-

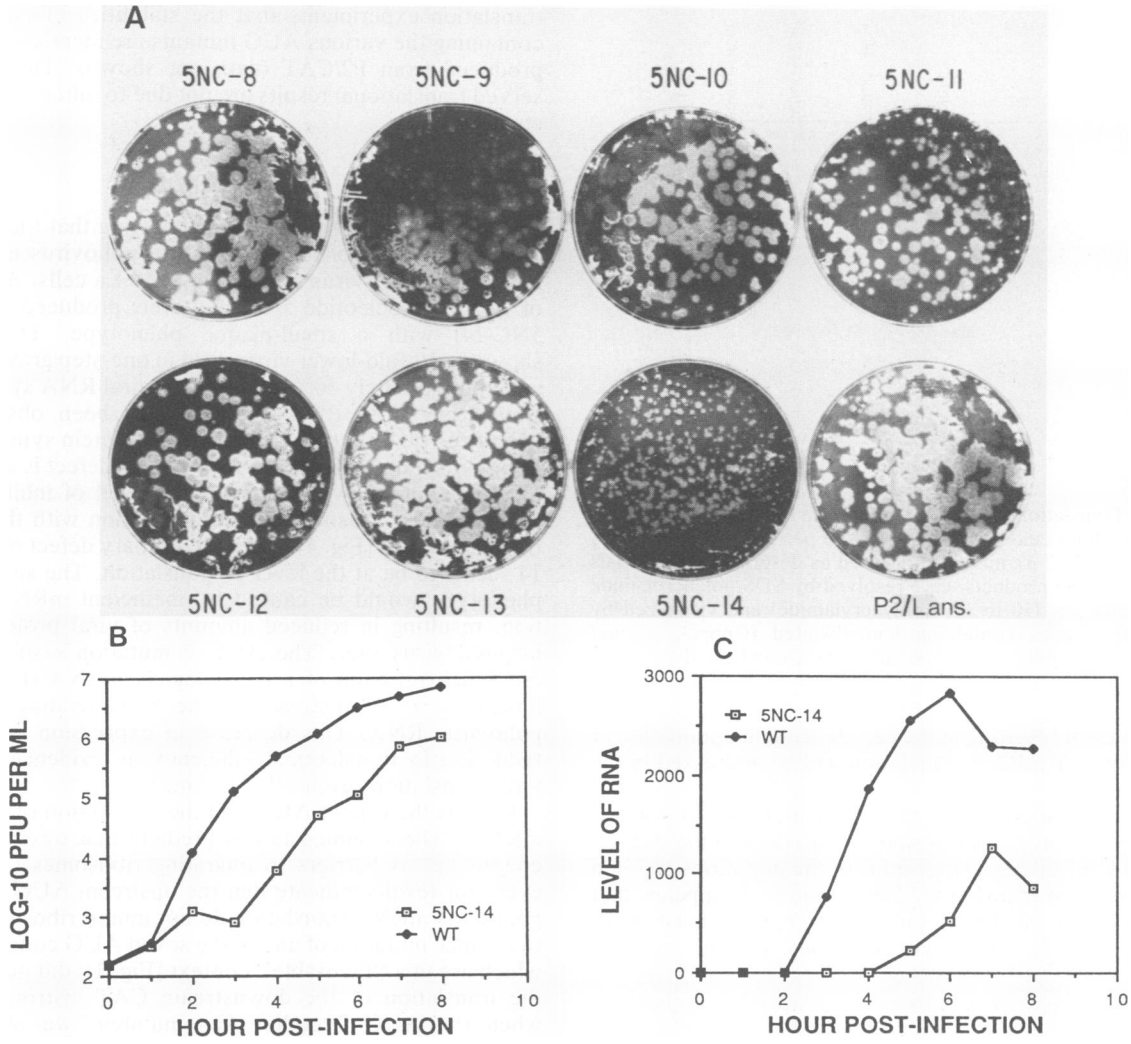

FIG. 2. (A) Plaque phenotype of AUG mutants in HeLa cells. (B) One-step growth curve of R2-5NC-14. (C) Viral RNA synthesis in virus-infected cells. Units on the ordinate refer to the area under the peaks of densitometry tracing. WT, Wild type.

peptide is the CAT protein, whereas the 16-kilodalton polypeptide is related to CAT, since it can be immunoprecipitated with a monoclonal antibody directed against CAT (data not shown), and probably results from readthrough of the first CAT AUG codon with subsequent initiation at a downstream in-frame AUG codon. This phenomenon has been previously observed (22). The presence of a methylated cap structure on CAT mRNA resulted in a ninefold increase in translational efficiency as compared with cap unmethylated mRNA (compare lane 3 with lane 2). Fusion of the poliovirus 5′ noncoding region to the CAT coding sequence generated a chimeric mRNA, which translated in a cap-independent fashion (compare lane 5 with lane 4), as previously described (22). The translation of P2(AUG-1-4)/CAT (lanes 6 and 7) and P2(AUG-6)/CAT (lanes 8 and 9) mRNAs was only marginally (ca. 30%) cap stimulated. However, capped unmethylated P2(AUG-7)/CAT mRNA, in which AUG 7 is mutated, translated with lower efficiency than the wild type did (compare lane 10 with lane 4), and, strikingly, the presence of a methylated cap structure significantly enhanced translation of this mRNA (threefold; compare lane 11 with lane 10). This result indicates that the adenosine nucleotide in AUG 7 is important for cap-independent translation.

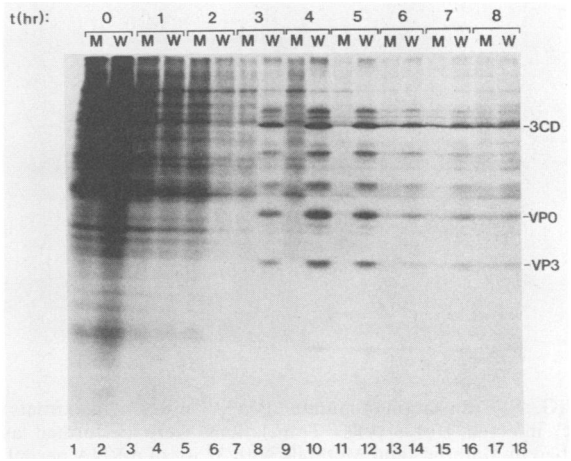

FIG. 3. Protein synthesis in virus-infected cells. HeLa cells were infected with wild type (W) or P2(AUG-7)/CAT mutant (M) virus, and protein synthesis was examined by pulse-labeling as described in Materials and Methods.

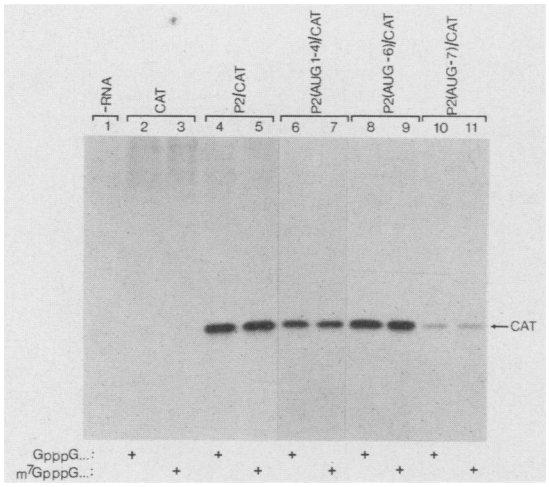

FIG. 4. Translation of P2/CAT mRNAs in extracts from poliovirus-infected HeLa cells. Translations were performed at an mRNA concentration of 32 μg/ml and processed as described in Materials and Methods. The products were resolved by SDS-polyacrylamide gel electrophoresis (10 to 15% polyacrylamide) and visualized by fluorography. Lanes containing unmethylated (GpppG. . .) and methylated (m^7GpppG. . .) capped mRNAs are indicated.

The mechanism resulting in the cap-dependent stimulation of P2(AUG-7)/CAT mRNA translation will be addressed in the Discussion.

These results suggest that the upstream ORFs of poliovirus mRNA do not act as translational barriers to the expression of the CAT cistron, since none of the mutations resulted in an increase in translation. Furthermore, it appears that AUG 7 plays a positive regulatory role in translation of poliovirus mRNA. We have confirmed for the in vitro translation experiments that the stabilities of the mRNAs containing the various AUG mutants are identical to mRNA produced from P2/CAT (data not shown). Thus, the observed translational results are not due to differential mRNA stability.

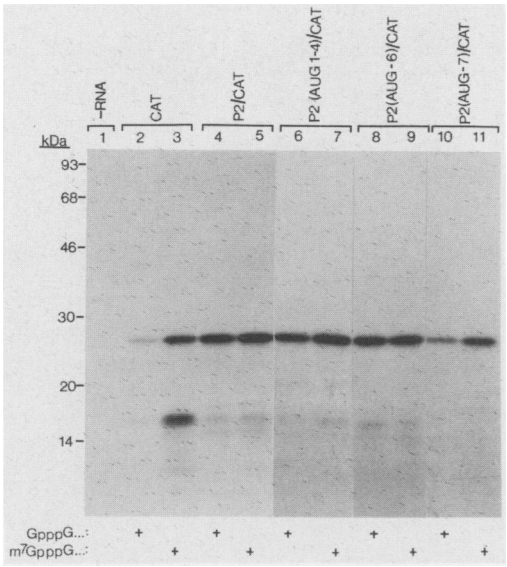

FIG. 5. Translation of mutated P2/CAT mRNAs in extracts from mock-infected HeLa cells. Translations were performed as described in Materials and Methods with 32 μg of mRNA per ml, and the products were resolved by SDS-polyacrylamide gel electrophoresis (10 to 15% polyacrylamide) and visualized by fluorography. Lanes containing unmethylated (GpppG. . .) and methylated (m^7GpppG. . .) capped mRNAs are indicated. kDa, Kilodaltons.

DISCUSSION

In this report, we present data showing that the upstream AUGs in the 5′ noncoding region of poliovirus mRNA are not essential for virus replication in HeLa cells. A mutation of AUG 7 (nucleotide 588), however, produced virus (R2-5NC-14) with a small-plaque phenotype. This mutant showed a 10-fold-lower virus yield in one-step growth curves and approximately 50% reduction in viral RNA synthesis. A similar effect on RNA synthesis has been observed for poliovirus type 1 mutants defective in protein synthesis (32). Poliovirus mutants in which the primary defect is at the level of replication show a much greater level of inhibition (25, 32). For these reasons and in conjunction with the in vitro translation data (Fig. 4 and 5), the primary defect of R2-5NC-14 seems to be at the level of translation. The small-plaque phenotype would be caused by inefficient mRNA translation, resulting in reduced amounts of viral proteins and a lowered virus titer. The AUG 7 mutation resulted in decreased expression of a heterologous mRNA (CAT) when linked in *cis* downstream of the 5′ noncoding region of poliovirus RNA. This decrease in expression is due to a reduction in translational efficiency as evidenced from in vitro translation results (Fig. 4 and 5).

What is the role of AUG 7 in the expression of poliovirus mRNA? The scanning model predicts that upstream AUG codons act as barriers to migrating ribosomes (11). However, our results indicate that the upstream AUG codons in poliovirus mRNA translation do not inhibit ribosome migration, since mutation of any of the seven AUG codons (two of which are in a "favorable" context [Fig. 1]) did not increase the translation of the downstream CAT cistron. Instead, when the AUG 7 codon was mutated, we observed a decrease in CAT expression, a result contrary to that predicted by the scanning model. These data are, however, consistent with the evidence that initiation of translation on poliovirus mRNA occurs via internal binding of ribosomes on the mRNA 5′ noncoding region (23). In this scenario, ribosomes bind internally on the mRNA, bypassing most of the upstream AUG codons, but require an important signal encompassing AUG 7 for efficient translation (see below). This is also supported by the finding that mutation in AUG 7 causes decreased internal initiation (J. Pelletier and N. Sonenberg, unpublished observations). The facilitative effect of a 5′ cap structure on the translation of P2(AUG-7)/CAT (Fig. 5) in extracts from mock-infected cells is consistent with this. It is likely that in P2(AUG-7)/CAT, where internal initiation is inefficient, 5′-end-mediated initiation can outcompete internal initiation and thus render the mRNA cap stimulated in vitro.

According to the termination-reinitiation model of translation, ribosomes can reinitiate at a downstream ORF upon termination of translation of an upstream ORF (7, 9, 10, 18, 20, 21, 29, 34). It is unlikely, however, that mutagenesis of AUG 7 affects reinitiation at the CAT AUG, since AUG 7 is an internal methionine in the ORF initiated by AUG 5 (see the layout of the upstream ORFs in Fig. 1). In addition, if scanning ribosomes were somehow to miss AUG 5 (which is in a weak context sequence), they would then encounter AUG 6, which is in a favorable context sequence and in a

different reading frame. Translation of this ORF would shunt ribosomes past AUG 7 (Fig. 1). It is therefore unlikely that AUG 7 is important in the context of an AUG initiator codon or even an internal AUG codon, because mutation of AUG 5, which is in frame with AUG 7, showed a wild-type phenotype in vivo.

It is more likely that disruption of the adenosine nucleotide of AUG 7 alters a motif normally important for translation initiation. The importance of this motif for translational efficiency is consistent with a report that deletion of the region containing AUG 7 resulted in a poliovirus mutant displaying a small-plaque phenotype (12) and that an octamer sequence encompassing AUG 7 is perfectly conserved among picornaviruses (12). Significantly, AUG 7 lies within a 14-nucleotide region which participates in forming a stable stem-loop structure perfectly conserved among the poliovirus serotypes, rhinoviruses, and enteroviruses (26). It seems that we may have fortuitously mutated a region important for translation initiation of poliovirus mRNA. It is important to determine the contribution of other nucleotides in this highly conserved octamer region for poliovirus translation. The importance of this region for translation is consistent with our previously reported data that the cap-independent region maps to nucleotides 320 to 630 (22) and that different loci around positions 200 and 500 within the poliovirus 5′ UTR might interact with each other (12). Hence, the octamer region within which AUG 7 is found could conceivably be important for internal binding of ribosomes.

Recently, scanning linker insertion mutagenesis failed to show a direct correlation between the disruption of upstream ORFs and virus viability, consistent with the data presented here (32). In addition, our mutation falls within the "P region" defined by these authors, a region shown to be important for viral protein synthesis.

Although these experiments indicate that the upstream ORFs have no role in poliovirus replication in HeLa cells, it is possible that such ORFs have a role in pathogenesis. Alternatively, the sequences of any of the AUG codons may serve functions specific for production of disease, as does, for example, nucleotide 472 (13). Since poliovirus type 2 Lansing can cause poliomyelitis in mice, this hypothesis is currently being tested by determining the neurovirulence of these viruses in mice.

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