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Translational Efficiency of Poliovirus mRNA: Mapping Inhibitory *cis*-Acting Elements within the 5′ Noncoding Region

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Poliovirus mRNA contains a long 5′ noncoding region of about 750 nucleotides (the exact number varies among the three virus serotypes), which contains several AUG codons upstream of the major initiator AUG. Unlike most eucaryotic mRNAs, poliovirus does not contain a m^{7}GpppX (where X is any nucleotide) cap structure at its 5′ end and is translated by a cap-independent mechanism. To study the manner by which poliovirus mRNA is expressed, we examined the translational efficiencies of a series of deletion mutants within the 5′ noncoding region of the mRNA. In this paper we report striking translation system-specific differences in the ability of the altered mRNAs to be translated. The results suggest the existence of an inhibitory *cis*-acting element(s) within the 5′ noncoding region of poliovirus (between nucleotides 70 and 381) which restricts mRNA translation in reticulocyte lysate, wheat germ extract, and *Xenopus* oocytes, but not in HeLa cell extracts. In addition, we show that HeLa cell extracts contain a *trans*-acting factor(s) that overcomes this restriction.

The translation of poliovirus RNA yields a large precursor polypeptide (NCVP00) which is cleaved at specific sites to generate poliovirus proteins (for a review, see reference 22). The manner by which poliovirus RNA is translated is perplexing in several respects. First, poliovirus polysomal mRNA, unlike the vast majority of eucaryotic mRNAs, is not capped at its 5′ end, but terminates in pUp (11, 24). Thus, its translation must occur by a cap-independent mechanism. Second, poliovirus mRNA (Lansing strain) contains a long 5′ noncoding region (745 nucleotides) which harbors seven AUGs shortly followed by in-frame termination codons (other strains of poliovirus contain similar cistrons in the 5′ leader segment), whereas in most eucaryotic mRNAs the 5′-proximal AUG is the initiator AUG (15). The ability of these upstream AUGs to serve as translation initiation codons has not been demonstrated. It is still unclear how the correct initiator AUG of poliovirus is selected.

Translation studies in reticulocyte lysates demonstrated that poliovirion RNA is a weak mRNA compared with encephalomyocarditis virion (another member of the picornavirus family) RNA, which is also uncapped (33). The poor translational efficiency of poliovirion RNA and its consequent inability to compete effectively with other mRNAs for components of the translational machinery were suggested (13) to be reasons why poliovirus, and not encephalomyocarditis virus, evolved a specific mechanism to inactivate eIF-4F (a translation initiation factor required for translation of capped mRNAs [8, 34]). This inactivation is postulated to allow poliovirus mRNA to usurp the cellular translational machinery without the need to compete with cellular mRNAs for components of the translational machinery (for reviews, see references 8 and 34).

In vitro studies of the translational properties of poliovirus by Dorner et al. (7) revealed that in reticulocyte lysates, significant initiation of translation occurred in the P3 region located in the 3′ one-third of the RNA. This apparently aberrant initiation could be suppressed by factors present in HeLa cell extract (7). A similar effect was observed in other studies (3, 30). Thus, reticulocyte lysates might be deficient in a component(s) necessary for 5′-proximal initiation of translation on poliovirus mRNA (3, 7, 30).

To gain a better understanding of the mechanism of translation initiation of poliovirus mRNA, we have analyzed the effects of deletions in the 5′ noncoding region of poliovirus mRNA on its translation in several in vitro systems, as well as in microinjected *Xenopus* oocytes. We have mapped a major domain (between nucleotides 70 and 381) within the mRNA 5′ noncoding region of poliovirus Lansing strain, which restricts its translation in reticulocyte lysate, wheat germ extract, and *Xenopus* oocytes, but not in HeLa cell extracts. This region can act in *cis* when fused to a heterologous mRNA. A model is presented to explain the differences in translational efficiencies observed with poliovirus mRNA in different cell extracts.

MATERIALS AND METHODS

Construction of deletion templates. The construction of the deletion templates and poliovirus-chloramphenicol acetyltransferase (Polio/CAT) fusion constructs has been described in detail (27). The plasmid used to construct the deletion mutants (pP2-5′; see Fig. 1A) was made by inserting a *Hin*dIII-*Sma*I fragment of poliovirus type 2 Lansing cDNA (17), representing bases 1 to 1872 of the viral genome, into pSP64.

In vitro transcriptions. Plasmids were linearized with *Sma*I (for pP2-5′ deletion constructs) or *Bam*HI (for pP2/CAT fusion constructs). Transcriptions were primed with the dinucleotide m^{7}GpppG or GpppG (500 μM) to provide 5′ capped methylated (m^{7}GpppG. . .) or capped unmethylated (GpppG. . .) terminated mRNAs, respectively, and performed as previously described (27).

In vitro translations. Mahoney poliovirion and tobacco mosaic virus (TMV) RNAs were a kind gift of K. A. W. Lee. Brome mosaic virus RNA was purchased from Promega Biotec. Poliovirus mRNAs generated by in vitro transcription were 5′ terminal capped methylated unless otherwise indicated. In vitro translations in micrococcal nuclease-treated rabbit reticulocyte lysates were performed by the method of Pelham and Jackson (26). The translations were performed with 12.5-μl reaction mixtures containing 0.2 μg

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of pP2-5′ deletion mRNAs or 0.5 μg of poliovirion or TMV RNA. Incubations took place for 60 min at 30°C. Following translation, RNase T_1 (200 U) and RNase A (1 μg) in 3 M urea–25 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride (final concentration) were added, and the incubation was continued at 30°C for 1 h. A 4-μl portion of the incubation mixture was withdrawn and processed for electrophoresis. Translations in HeLa cell extracts were as described by Rose et al. (32) with modifications by Lee and Sonenberg (18). Following translations, proteins were analyzed on gradient sodium dodecyl sulfate–10% to 15% polyacrylamide gels (16). Gels were fixed in 40% methanol–7.5% acetic acid, treated with En^{3}Hance (New England Nuclear Corp.), and exposed against X-ray film at −70°C. Translation experiments were repeated twice, with a variation in the results of less than 10%.

Translations in *Xenopus* oocytes. Translations in *Xenopus* oocytes were as follows. Ten oocytes were microinjected with [^{3}H]CTP-labeled mRNAs (50 nl per oocyte; 0.2 μg/ml) and incubated at 20°C for 15 h. The oocytes were processed and the CAT activity was determined as detailed by Colman (5). Briefly, oocytes were homogenized in 0.1 M NaCl–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride–20 mM Tris hydrochloride (pH 7.6) and centrifuged in an Eppendorf centrifuge for 5 min, and the supernatant was used to measure CAT activity. Before the assays, portions of homogenates were counted in a scintillation counter to normalize for variations in microinjection and sample preparation.

RESULTS

To study the translational properties of poliovirus mRNA, we used RNA transcribed from a type II (Lansing strain) poliovirus cDNA clone with SP6 RNA polymerase. A truncated cDNA clone (pP2-5′) was used to avoid the analysis of many translation products due to protein processing. This clone extends to nucleotide 1872 in the VP3 region and gives rise to a single 1.8-kilobase RNA species following transcription in the SP6 system and analysis on formaldehyde gels (data not shown). Deletions extending into the poliovirus 5′ end were constructed (Fig. 1A), and translation of the mRNAs was examined in several translation systems: rabbit reticulocyte lysate; extracts from HeLa cells, wheat germ, and mouse L cells; and *Xenopus* oocytes (by microinjection). Because poliovirus genomic and polysomal RNAs are uncapped (9, 11, 19, 24) and translate by a cap-independent mechanism (reviewed in references 8 and 34), we used both 5′ terminal capped unmethylated (GpppG. . .) and capped methylated (m^{7}GpppG. . .) mRNAs for our translation studies. The capped unmethylated mRNAs are functionally analogous to uncapped mRNAs, since it is the methyl group on capped methylated mRNAs which plays a pivotal role in the function of the cap structure (2). We used capped unmethylated mRNAs rather than uncapped mRNAs because the former are considerably more stable (and are as stable as their methylated counterparts) in cell-free translation systems (10).

Translation of pP2-5′ deletion mRNAs in reticulocyte lysates. Translations were initially performed in a reticulocyte lysate to assess the effect of a methylated versus unmethylated cap structure on the expression of pP2-5′ mRNA. *S*-Adenosyl homocysteine was included in the translation incubations to prevent methylation of unmethylated capped transcripts by endogenous methyltransferases (2). Translation products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and are shown in Fig. 2A. Translation of poliovirion RNA was about five times less efficient than that of TMV RNA (compare lane 5 with lane 3; determined by [^{35}S]methionine incorporation, since the methionine contents of both proteins were similar) and was not inhibited by the cap analog m^{7}GDP (compare lane 6 with lane 5), in contrast to the translation of TMV RNA (85% inhibition; compare lane 4 with lane 3). Translation of SP6-derived methylated and unmethylated herpes simplex virus type 1 thymidine kinase mRNA (pX1) was performed as a control to assess the cap dependency of the translation system, since the translation of this mRNA has previously been shown to be cap stimulated (28). Translation of unmethylated thymidine kinase mRNA was not affected by the addition of m^{7}GDP (compare lane 8 with lane 7), whereas the presence of a methylated cap structure stimulated translation of the mRNA about sixfold (compare lane 9 with lane 7). Addition of m^{7}GDP (lane 10) reduced the translation of the mRNA to the level observed with the unmethylated thymidine kinase mRNA (lanes 7 and 8). Thus, as expected, the translation of cap-dependent mRNAs in our reticulocyte lysate is stimulated by the presence of a methylated cap structure. Translation of the pP2-5′ transcripts was very inefficient and cap independent in the reticulocyte lysate (lanes 11 to 14), as was observed with the translation of poliovirion RNA in the reticulocyte lysate (lane 5) (33). Since the protein product is not visible at this exposure, a longer exposure is shown (lanes 19 to 22). Translation of both unmethylated and methylated mRNAs was similar and slightly stimulated (1.5-fold) by the addition of m^{7}GDP (lanes 20 and 22). Such a stimulation has been previously observed with naturally uncapped mRNAs (35) and can also be noticed with poliovirion RNA (compare lane 6 with lane 5). Taken together, these results indicate that poliovirus mRNA translation is indifferent to the nature of the structure present at its 5′ terminus. The protein product synthesized from pP2-5′ mRNA was confirmed to be a truncated poliovirus product containing VP4 since it could be immunoprecipitated by using an anti-VP4 antibody (data not shown). We note that translation of poliovirion RNA (lanes 5 and 6) is much better than that of pP2-5′ mRNA (lanes 11 to 14). A plausible explanation for this difference is that most of the translation products from poliovirion RNA result from internal initiation in the P3 region (compare with reference 7); this region is lacking in pP2-5′ mRNA, which can therefore translate only by 5′-proximal initiation.

We wished to determine whether the low translational efficiency of poliovirus mRNA in reticulocyte lysates was caused by sequences in the 5′ noncoding region. We therefore assayed a series of mutant pP2-5′ mRNAs containing deletions in the 5′ noncoding region and concomitantly assessed the effect of methylated versus unmethylated capped mRNAs on expression. The results are shown in Fig. 2B: Δ5′-33 (lanes 6 and 7), Δ5′-80 (lanes 8 and 9), Δ5′-96 (lanes 10 and 11), and Δ5′-140 (lanes 12 and 13) translated poorly in the reticulocyte lysate, and, like pP2-5′ mRNA (lanes 4 and 5), translated in a non-cap-stimulated fashion (densitometry scanning of these lanes was performed on a longer exposure of Fig. 2). The translation of Δ5′-320, albeit poor, revealed that the methylated mRNA was expressed slightly better (1.5-fold) than its unmethylated counterpart (compare lane 15 with 14). For mutants Δ5′-632 and Δ5′-733, translation of unmethylated mRNAs was remarkably more efficient (ca. 20- to 35-fold) than that of pP2-5′ mRNA (lanes 16 to 19). Translation of methylated Δ5′-632 and Δ5′-733 mRNAs was ca. threefold more efficient than that of their unmethylated counterparts (compare lane 17 with lane 16

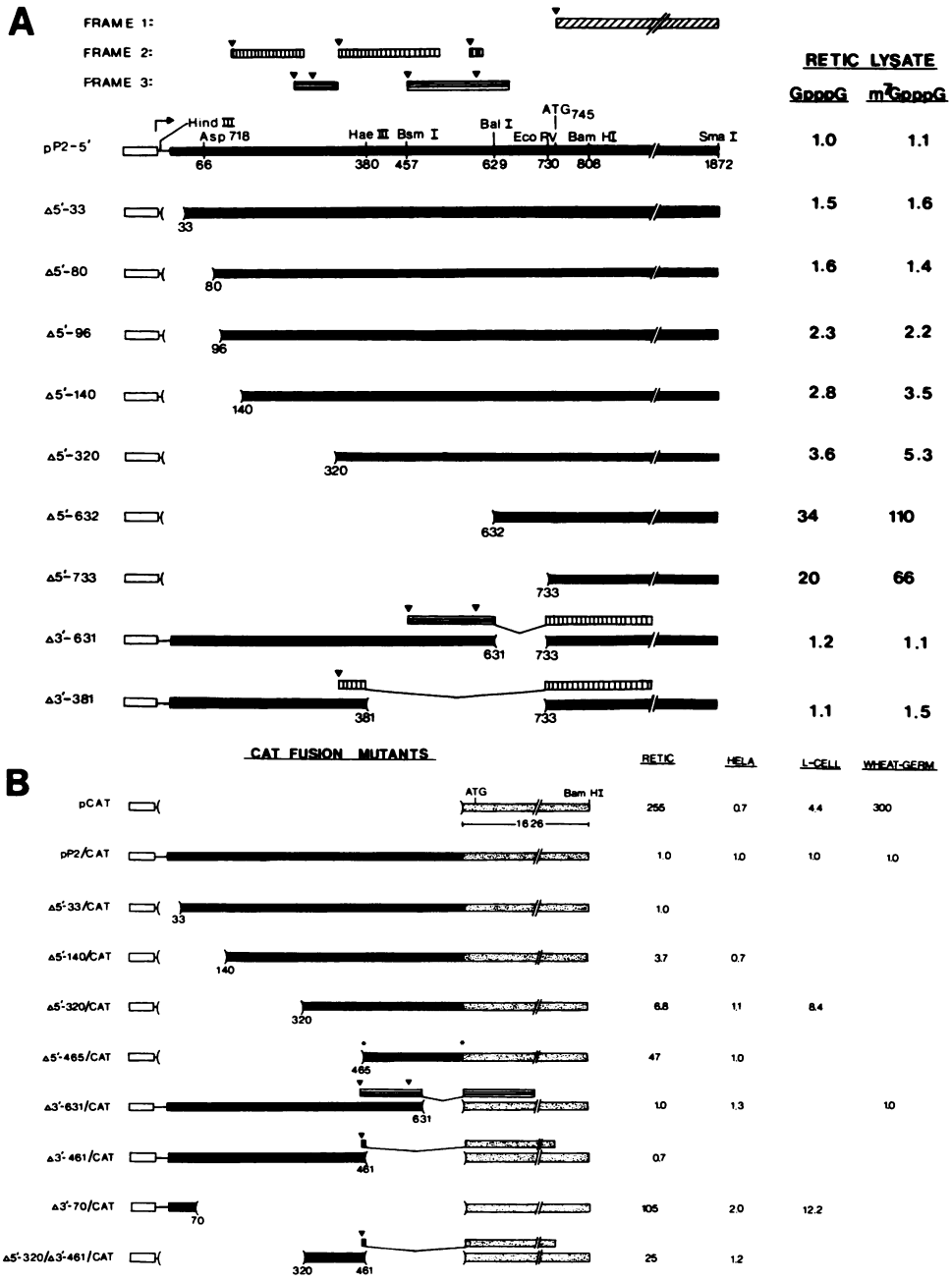

FIG. 1. Maps of poliovirus deletion mutants. (A) Structure of deletion constructs of poliovirus RNA. Potential open reading frames in the 5′ noncoding region are shown at the top of the diagram. ▼, AUG positions. The cleavage site of restriction enzymes used to produce the deletion mutants are shown on the pP2-5′ template. The numbers shown represent distances, in nucleotides, from the first nucleotide of the 5′ end of the viral template. Symbols: ▭, SP6 polymerase promoter; ——, 24 nonviral nucleotides between the SP6 promoter and the first nucleotide of the viral genome; ▬, P2/Lansing viral sequences; deleted sequences are bracketed. RNAs transcribed from these templates contain the initiator ATG (shown at nucleotide 745) of the viral polyprotein encoding an open reading frame for a truncated poliovirus polyprotein consisting of VP4 (69 amino acids), VP2 (271 amino acids), and 37 amino acids of VP3 (predicted total molecular weight, 42,000 [17]). Deletions that cause alterations within upstream open reading frames, resulting in termination within the major poliovirus polyprotein open reading frame, are indicated above the constructs Δ3′-631 and Δ3′-381. The relative translational efficiencies of the deletion constructs obtained in reticulocyte lysates are indicated to the right. Radioactive bands corresponding to poliovirus protein products were quantified by soft-laser densitometry (LKB Instruments, Inc.), and the value obtained for capped unmethylated pP2-5′ mRNA was set as 1.0. (B) Diagram of fusion constructs between poliovirus deletion mutants and CAT coding sequence. Symbols: ▩, CAT gene; ▬, sequences derived from pP2-5′. Alterations in open reading frames caused by deletion mutagenesis are drawn above the appropriate construct. The asterisks above Δ5′-465/CAT denote the positions of synthetic *Hin*dIII linkers. The relative translational efficiencies obtained with the pP2/CAT fusion constructs are indicated to the right. The value obtained for pP2/CAT was set as 1.0.

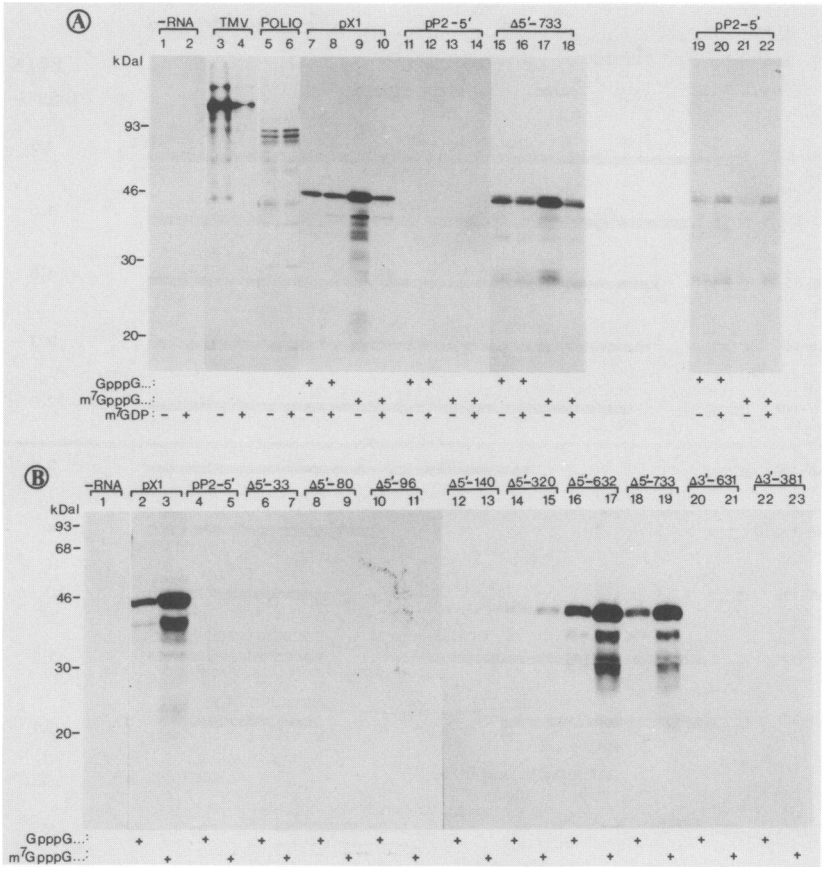

FIG. 2. Translation in rabbit reticulocyte lysates programmed with pP2-5′ poliovirus deletion mRNAs. Values obtained after scanning the autoradiograph relative to the amount of protein synthesized from pP2-5′ mRNA are indicated in Fig. 1A. Kinetic analysis and dose-response curves indicated that the rates of translation were in the linear range. (A) Effect of cap analog on translation in reticulocyte lysates. The following amounts of mRNAs were added: TMV and poliovirion RNA, 0.5 μg; thymidine kinase 0.2 μg; pP2-5′, 0.2 μg; Δ5′-733, 0.2 μg. The protein products in lanes 11 to 14 were difficult to visualize, and the autoradiograph after being exposed five times longer is shown (lanes 19 to 22). m^7GDP (0.6 mM) was added where indicated. Lanes containing unmethylated (GpppG. . .) and methylated (m^7GpppG. . .) capped mRNAs are indicated. (B) Translation of pP2-5′ poliovirus deletion mRNAs. Translations (in 12.5 μl) were performed with 0.25 μg of mRNA. Molecular masses in kilodaltons (kDal) are shown on the left of each panel.

and lane 19 with lane 18). This translation was cap stimulated, since translation of methylated Δ5′-733 mRNA was inhibited by m^7GDP, whereas its unmethylated counterpart was not (Fig. 2A, lanes 15 to 18). The translation of two mRNAs, Δ3′-631 and Δ3′-381, in which distal sequences within the 5′ noncoding region were removed, was very inefficient (Fig. 2B, lanes 20 to 23). The reduced translation obtained with these mRNAs is not due to the presence of a new upstream cistron which now overlaps the major open reading frame of VP4-VP2-VP3 (diagrammed in Fig. 1A). Polypeptides of ca. 17 and ca. 12 kilodaltons are expected from the new cistrons in Δ3′-631 and Δ3′-381, respectively. These proteins, which are expected to contain six (Δ3′-631) and five (Δ3′-381) methionines, were not observed following translation of these mRNAs (Fig. 2B), indicating that the new cistrons are not used. These data indicate that sequence elements upstream of nucleotide 381 form part of a domain which contributes to the reduced translational efficiency of poliovirus mRNA in a reticulocyte system.

Translation of pP2/CAT deletion mRNAs in reticulocyte lysate. To determine whether the inhibitory effect on translation observed with the poliovirus mRNA 5′ noncoding region could be imparted to an mRNA whose translation is very efficient in reticulocyte lysates, we joined the 5′ noncoding region of the deletion mutants to the bacterial CAT coding region (Fig. 1B). Translations of capped unmethylated versus capped methylated mRNAs were performed for all the experiments described below, but only the results with capped methylated mRNAs are presented, since the data obtained with the two types of mRNAs were similar to those obtained in Fig. 2. The results of translating the chimeric deletion mRNAs in a reticulocyte lysate are shown in Fig. 3, and the data are summarized in Fig. 1B. As shown above (Fig. 2), the translation of poliovirion RNA was inefficient (lane 2) as compared with the translation of TMV (Fig. 3, lane 3) and brome mosaic virus (lane 4) RNAs. pCAT mRNA was translated very efficiently (lane 5). Fusion of the 5′ noncoding region of poliovirus mRNA (nucleotides 1 to 733) to the CAT coding sequence drastically reduced CAT protein synthesis (ca. 250-fold reduction; lane 6). A deletion of 33 nucleotides from the 5′ end did not relieve the inhibitory effect of the poliovirus 5′ noncoding sequences (lane 7). However, further deletions gradually led to increases in the translational efficiency of pP2/CAT mRNA. Translation was stimulated 3.7-fold for deletion mutant Δ5′-140/CAT (compare lane 8 with lane 6), 6.8-fold for Δ5′-320/CAT (compare lane 9 with lane 6), and 47-fold for Δ5′-465/CAT (compare lane 10 with lane 6) relative to

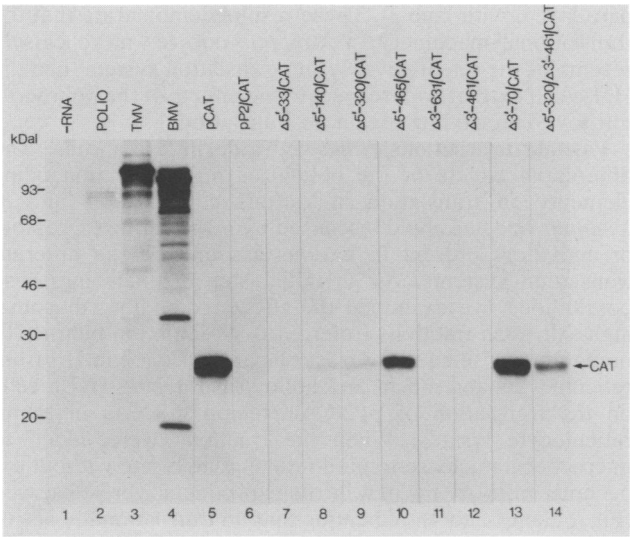

FIG. 3. Translation in rabbit reticulocyte lysates of pP2/CAT deletion mRNAs. Translations were performed as described in Materials and Methods. Values obtained by scanning of the autoradiograph relative to the amount of protein synthesized from pP2/CAT mRNA (set as 1.0) are shown in Fig. 1B. The amounts of mRNA used was as follows: poliovirion, 0.25 μg; TMV, 0.25 μg; brome mosaic virus (BMV), 0.25 μg; pP2/CAT, 0.25 μg. Molecular masses in kilodaltons (kDal) are shown on the left.

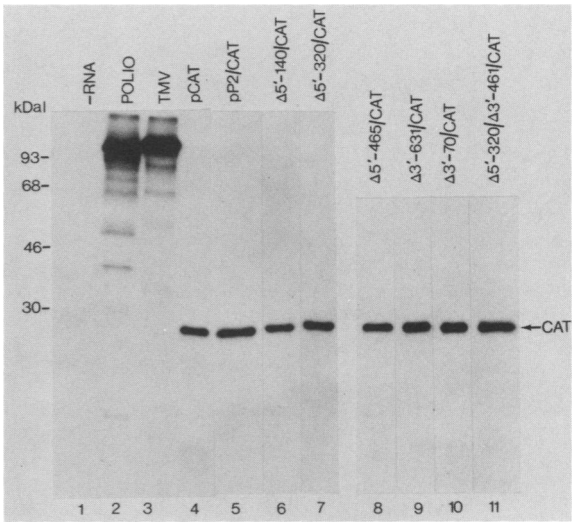

FIG. 4. Translation of pP2/CAT deletion mRNAs in mock-infected HeLa cell extracts. Translations were performed, as described in Materials and Methods, with 0.4 μg of capped methylated pP2/CAT deletion mRNAs and 0.6 μg of poliovirion and TMV RNAs, and the products were resolved on gels and processed as described in Materials and Methods. Molecular masses in kilodaltons (kDal) are shown on the left.

pP2/CAT. The inhibition obtained with Δ5'-465/CAT was only 5-fold, compared with the 250-fold inhibition obtained with the full-length poliovirus 5' noncoding region. Translation of both Δ3'-631/CAT and Δ3'-461/CAT mRNAs was as inefficient as translation of mRNA containing the full-length 5' noncoding sequence (lanes 11 and 12), consistent with the previous conclusion that the major inhibitory sequence is upstream of nucleotide 465. When a larger deletion mutant (Δ3'-70) was tested, translation was vastly increased and approached the efficiency exhibited by pCAT mRNA (compare lane 13 with lane 5). These results are consistent with those of Fig. 2 and map the translational inhibitory element to sequences between nucleotides 70 and 461. A fragment containing nucleotides 320 to 461 was fused to the CAT coding sequence and tested for its ability to translate in a reticulocyte lysate. Translation of this mRNA was inhibited 10-fold more than that of CAT mRNA (compare lane 14 with lane 5). This inhibition, although significant, cannot account for the large decrease in translation (250-fold) caused by the full-length 5' noncoding region of poliovirus mRNA when fused to CAT mRNA. Therefore, sequences upstream of nucleotide 320 are required for the inhibition. Since deletion of sequences downstream of nucleotide 381 did not mitigate the inhibition (Fig. 2), we conclude that a domain exists between nucleotides 70 and 381 which prevents efficient translation of poliovirus mRNA in a reticulocyte lysate.

Translation of pP2/CAT deletion mRNAs in extracts from HeLa cells. Translations in HeLa cell extracts were performed with capped methylated deletion mRNAs of pP2-5' and pP2/CAT. The results obtained were similar with both sets of constructs, and only those for pP2/CAT mRNAs are shown (Fig. 4; summarized in Fig. 1B). Translation of poliovirion RNA (Fig. 4, lane 2) was as efficient as that of TMV RNA (lane 3) in HeLa extracts. The efficient translation of poliovirion RNA in HeLa cell extracts is in contrast to its inefficient translation in reticulocyte lysate (Fig. 2 and 3). Juxtaposition of the poliovirus 5' noncoding region in front of the CAT mRNA slightly increased translation compared with that of pCAT mRNA (1.5-fold; compare lane 5 with lane 4), in sharp contrast to the inhibitory effect observed in the reticulocyte lysate (Fig. 3). Stepwise deletions originating from the 5' end of the mRNA had no significant effect on the translational efficiency of the mRNAs (lanes 6 to 8). Similarly, removal of 102 nucleotides from the 3' end of the noncoding region (nucleotides 631 to 733) had no significant effect on translation (1.3-fold higher than that of pP2/CAT mRNA; lane 9). When most of the 5' noncoding region was removed by a 3' deletion up to nucleotide 70, translation was again stimulated twofold over that of pP2/CAT mRNA (compare lane 10 with lane 4). Fusion of the fragment containing nucleotides 320 to 461 to CAT (Δ5'-320/Δ3'-461/CAT) did not affect CAT translation (lane 11), in contrast to the inhibition obtained in the rabbit reticulocyte lysate (Fig. 3). These results demonstrate that the *cis*-inhibitory elements present in the 5' noncoding region of poliovirus mRNA do not function in HeLa cell extracts and are consistent with the finding that poliovirion RNA translates efficiently in HeLa cell extracts (Fig. 4, lane 2).

Translation of pP2/CAT deletion mRNAs in other translation systems. The results presented so far point to striking translational differences among the various 5' deletion mutants in reticulocyte lysates and HeLa cell extracts. Consequently, it was of interest to determine whether these differences occur because the reticulocyte lysate is peculiar with respect to poliovirus RNA translation. We used two additional in vitro translation systems: extracts from mouse L cells (a fibroblastic tumor cell line) and plant wheat germ. The translation results obtained with these systems are summarized in Fig. 1B. Translation of pCAT in L-cell extracts was reduced only 4.4-fold when the 5' noncoding region of poliovirus mRNA was joined to the CAT coding region. Somewhat higher (12-fold) translational efficiency than that of pP2/CAT mRNA was obtained with the Δ3'-70 deletion construct mRNA. Deletion of the 5'-proximal 320

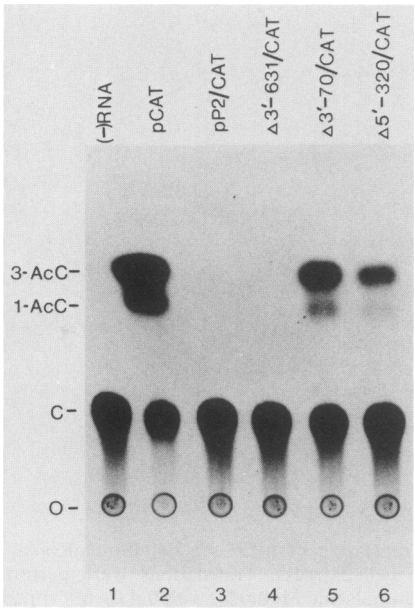

FIG. 5. Translation of pP2/CAT deletion mRNAs in *Xenopus* oocytes. Kinetic analysis and concentration curves indicated that the observed CAT activity for all constructs was in the linear response range. The positions of chloramphenicol (C) and acetylated forms of chloramphenicol (AcC) are indicated. O, Origin.

nucleotides of poliovirus mRNA 5′ noncoding region (Δ5′-320) resulted in increased translational efficiency (8.4-fold higher than that of pCAT). Thus, the behavior of the 5′ noncoding region of poliovirus mRNA in L-cell extracts resembles the situation in HeLa cell extracts more closely, since it exerts a relatively weak (as compared with the reticulocyte lysate) *cis*-inhibitory effect on CAT translation in L-cell extracts.

A strikingly different pattern from that observed in L-cell extracts emerged when translation was conducted in wheat germ extract (results summarized in Fig. 1B). Fusion of the poliovirus mRNA 5′ noncoding region to the CAT mRNA caused a dramatic decrease in translation relative to pCAT (a ca. 300-fold difference). Similarly, the deletion mutant Δ3′-631/CAT translated inefficiently. Thus, the results from the wheat germ translation system indicate that this system resembles the reticulocyte lysate with respect to poliovirus translation and is considerably different from HeLa and L-cell extracts.

In addition to the in vitro studies, we wished to assess the translational properties of the mutant mRNAs in vivo. To this end, we used the *Xenopus* oocyte system, in which translation of pCAT mRNA was determined by analysis of CAT activity after injection of mRNA. Extracts from oocytes not injected with mRNA showed no CAT activity (Fig. 5, lane 1), whereas those injected with pCAT mRNA exhibited CAT activity (77% of chloramphenicol converted to the acetylated form; lane 2). Fusion of the poliovirus mRNA 5′ noncoding region to the CAT coding sequence completely abolished CAT activity (lanes 3 and 4). A 3′ deletion extending to nucleotide 70 (Δ3′-70/CAT) resulted in appreciable CAT activity relative to that obtained with pCAT mRNA (twofold less than that of pCAT mRNA; compare lane 5 with lane 2), whereas injection of mRNA with a 5′ deletion extending to position 320 resulted in some CAT activity (16-fold less than that of pCAT mRNA; compare lane 6 with lane 2). These results demonstrate that the translational machinery in *Xenopus* oocytes more closely resembles the reticulocyte lysate translation system than the HeLa cell extract with respect to the effects of the poliovirus mRNA 5′ noncoding region on translation.

Possible translational *trans*-activator in HeLa cells. The differential effects of the poliovirus mRNA 5′ noncoding elements on translation in various cell extracts and in *Xenopus* oocytes could be caused by *trans*-acting repressors or activators present in various amounts in the different translation systems. As an initial step in analyzing these possibilities, we examined the effects of adding ribosomal high-salt wash fractions (referred to as initiation factor [IF] preparations, since these fractions are enriched in IF) from reticulocytes and mock- and poliovirus-infected HeLa cells on the translation of pP2/CAT fusion mRNAs in rabbit reticulocyte lysates. When the fractions were added to micrococcal nuclease-treated reticulocyte lysate without exogenous mRNA, no new protein products were observed (Fig. 6, lanes 2 to 4), indicating that no translationally active RNA copurified with the IF. The addition of reticulocyte IF had no effect on the translation of pCAT mRNA (compare lane 6 with lane 5), whereas addition of IFs from mock- and poliovirus-infected HeLa cells caused a considerable decrease in pCAT mRNA translation (ca. 10-fold; compare lanes 7 and 8 with lane 5). The reasons for inhibition of pCAT mRNA translation by IFs from mock-infected HeLa cells are not clear, although a similar effect has been previously observed (4). Translation of pP2/CAT mRNA was very inefficient (lane 9), as shown in Fig. 3, and IFs from the reticulocyte lysate did not stimulate translation of this mRNA (Fig. 6, lane 10). However, addition of IFs either from mock-infected (lane 11) or from poliovirus-infected (lane 12) HeLa cells stimulated translation of pP2/CAT mRNA to the level observed when HeLa IFs were added to pCAT mRNA translation (lane 7). We also tested the deletion mutant Δ3′-70/CAT and found that it translated with similar characteristics to pCAT mRNA: expression of this mRNA was not stimulated by IF from reticulocytes (compare lane 14 with lane 13), but was inhibited by IF from mock-infected HeLa extracts (lane 15) and further inhibited by IF from poliovirus-infected HeLa extracts (lane 16). These results suggest that HeLa cells contain a *trans*-acting factor that facilitates efficient translation of poliovirus mRNA.

DISCUSSION

We have mapped an element(s) in the 5′ noncoding region of poliovirus mRNA that exhibit a strong *cis*-inhibitory effect on translation in reticulocyte lysate, wheat germ extract, and *Xenopus* oocytes. This element(s), as deduced from translation of a series of deletion mRNAs in a reticulocyte lysate, appears to reside between nucleotides 70 and 381 (Fig. 2 and 3). A fragment containing nucleotides 320 to 461 inhibited translation 10-fold when fused to CAT mRNA (Fig. 3). This inhibition is 25-fold lower than that caused by the full-length 5′ noncoding region. However, deletion of nucleotides 1 to 320 resulted in a 35-fold-higher inhibition of CAT translation (Fig. 3). A further deletion extending to nucleotide 465 (Δ5′-465/CAT; Fig. 3) inhibited CAT expression only fivefold. These results suggest that there may be two or more inhibitory elements between nucleotides 70 and 381 which operate synergistically and which have been bisected by some of the 5′ deletions reported here. Alternatively, it is possible that nucleotides 70 to 381 contain one *cis*-inhibitory

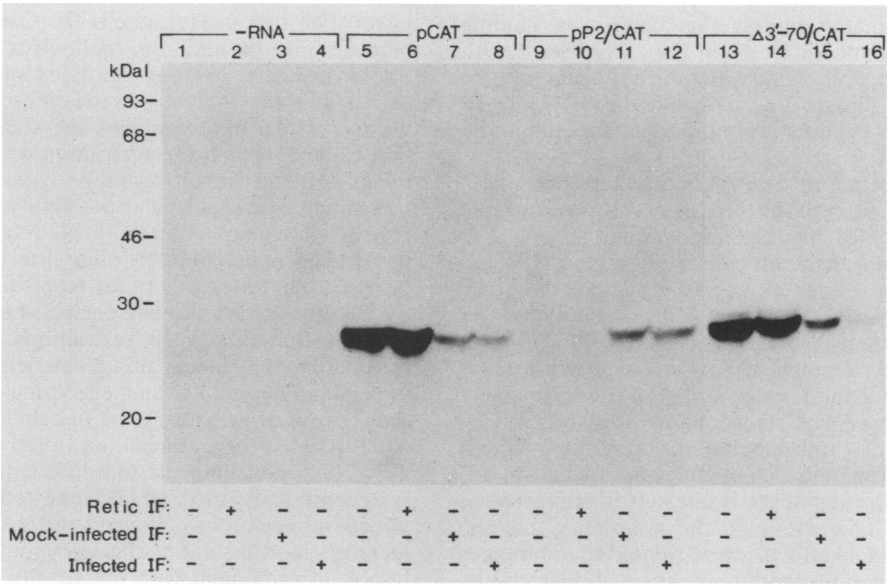

FIG. 6. Effect of IF addition on translation of pP2/CAT mRNAs in a reticulocyte lysate. Reaction mixtures (13 μl) were programmed with 0.2 μg of pCAT, pP2/CAT, or Δ3'-70/CAT mRNA. Reaction mixtures contained 3 μl of IF (ca. 31 mg/ml) from reticulocytes or mock- or poliovirus-infected HeLa cells where indicated. After incubation at 30°C for 60 min, samples of 4 μl were processed for gel electrophoresis. The gels were processed for autoradiography as described in Materials and Methods. Molecular masses in kilodaltons (kDal) are shown on the left.

element. These possibilities are also supported by the oocyte microinjection data (Fig. 5), which show that deletion of the 5'-proximal 320 nucleotides partially relieved the translational inhibitory effect exerted by the 5' noncoding region. Additional work is necessary to distinguish between these two possibilities.

It is unlikely that the translational *cis*-inhibitory effects of the poliovirus mRNA 5' noncoding region are caused by upstream AUGs, which would act to impede the 5'-to-3' movement of ribosomes toward the initiator AUG_{745} and thus reduce translational initiation (15). If this were the case, then removal of upstream AUGs would be expected to stimulate translation of poliovirus mRNA. We tested this hypothesis by systematically mutating the 5' upstream AUGs to UUGs and did not find such an effect in the reticulocyte lysate (J. Pelletier, V. R. Racaniello, and N. Sonenberg, unpublished observations). A similar case has been reported by Katz et al. (14), in which deletion of an upstream AUG had no effect on the translation of avian retrovirus mRNA. At present, a simple inverse relationship between the existence of upstream AUGs in the 5' noncoding region of poliovirus mRNA and its translational efficiency does not exist.

The restrictions imposed by the 5' noncoding region of poliovirus mRNA on translation in a reticulocyte lysate might be relevant to the unusual pattern of proteins synthesized in the reticulocyte lysate as described by Brown and Ehrenfeld (3) and Dorner et al. (7). Dorner et al. showed that in a reticulocyte lysate a significant proportion of translation initiation originates in the P3 region located in the 3' one-third of the RNA (7). Evidence from electron microscopy studies showing ribosome binding to the mRNA P3 region support this conclusion (20). It is possible that in reticulocyte lysates, in which initiation of translation at the 5' end of poliovirus mRNA is weak, an internal initiation site near the P3 region becomes available for ribosome binding. Under conditions when 5' end initiation is efficient (in HeLa extracts), one might not expect to see initiation at the P3 region owing to competition. In agreement with these observations, Phillips and Emmert (30) presented evidence that reticulocyte lysates are limiting in a factor required for utilization of the 5' major initiator AUG. Supplementation of a reticulocyte lysate with HeLa cytoplasmic extract resulted in a qualitative improvement in the selection of the correct initiation site (3, 7, 30). Brown and Ehrenfeld also reported a stimulatory effect of a ribosomal salt wash from HeLa cells on translation of poliovirus RNA in a reticulocyte lysate (3). However, a similar stimulation was also observed when a ribosomal salt wash from reticulocytes was added (3). The activity we described here is specific for HeLa cells, since addition of a ribosomal high-salt wash from the reticulocyte had no effect on translation of pP2/CAT mRNA (Fig. 6, lane 10), whereas HeLa fractions from mock- and poliovirus-infected cells had a significant stimulatory effect (Fig. 6, lanes 11 and 12). Thus, the activity in HeLa cells described here is apparently different from that described by Brown and Ehrenfeld (3).

What might be the nature of the translational restrictive elements? We believe that it is likely to be secondary structure, since such elements have been shown to inhibit translation (28). It is of interest that computer-aided modeling of the secondary structure of poliovirus mRNA revealed extensive secondary structure in the 5' noncoding region that is conserved in the three poliovirus serotypes (J. Maizel, personal communication). It is possible that the secondary structure interdicts the translation of poliovirus mRNA in reticulocyte lysates, wheat germ extracts, and *Xenopus* oocytes. We have recently shown (27) that the poliovirus 5' noncoding region has a sequence element necessary for cap-independent translation. Our data suggest that reticulocyte lysates are limiting in a factor(s) necessary for cap-independent translation. This would explain the low translational efficiency of pP2/CAT in reticulocyte lysates. In this scenerio, ribosomes would be inhibited from scanning the poliovirus leader region by secondary structure between nucleotides 70 and 380. In HeLa extracts, however, ribo-

somes would presumably bypass this barrier by binding internally to the 5′ noncoding region (29). Consistent with this idea, we have recently shown both in vivo and in vitro that ribosomes can initiate internally on the 5′ leader of poliovirus mRNA (J. Pelletier and N. Sonenberg, submitted for publication).

The possible existence of *trans*-acting factors that affect the translation of poliovirus mRNA in a cell type-specific manner is novel and of interest in several respects. The differential effects on translation exhibited by the poliovirus mRNA 5′ noncoding sequences can be explained either by the existence of negative regulatory *trans*-acting factors in reticulocytes, wheat germ extracts, and *Xenopus* oocytes or, alternatively, by the presence of positive regulatory *trans*-acting factors in HeLa and L cells. Our data (Fig. 6) support the second possibility, since ribosomal high-salt wash fractions from HeLa cells (both mock and poliovirus infected) relieve the *cis*-inhibition imposed by the poliovirus mRNA 5′ noncoding region in reticulocyte lysates. It is important to emphasize that we have excluded the possibility that the cell-specific translational effects are attributed to differences in the in vitro translation conditions, since similar results were obtained under different salt concentrations and temperatures (J. Pelletier, unpublished observations).

What is the nature of the translational *trans*-activator? One possibility is that an initiation factor that is required in larger amounts for the translation of poliovirus mRNA is more abundant in HeLa and L cells than in the specialized cells we used here. In fact, such a property was attributed to eIF-4A by Daniels-McQueen et al. (6), who concluded that higher levels of eIF-4A are required for poliovirus mRNA than for encephalomyocarditis virus mRNA translation. This is an interesting possibility, because eIF-4A was suggested to possess RNA-unwinding activity (31), and the restriction of poliovirus mRNA translation could be attributed to a high degree of secondary structure. There are several reasons, however, why we do not believe that the low levels of translation of poliovirus mRNA in reticulocyte lysates, wheat germ extracts, or *Xenopus* oocytes can be attributed to limiting levels of eIF-4A per se. The addition of purified eIF-4A had no effect on the translation of pP2/CAT mRNA in reticulocyte lysate (Pelletier, unpublished observations). Addition of IF preparations from reticulocytes (which are a very rich source of eIF-4A) could not relieve the *cis*-inhibition imposed by the poliovirus 5′ noncoding region on pCAT mRNA translation, whereas IF preparations from HeLa cells partially relieved this inhibition (Fig. 6). Furthermore, it was shown by Blair et al. (1) that eIF-4A had no stimulatory effect on the translation of poliovirus mRNA in an ascites-reconstituted translation system, whereas the translation of encephalomyocarditis virus mRNA was stimulated. Because of these reservations, an alternative possibility is that HeLa cells contain a factor that facilitates cap-independent and internal binding of ribosomes to the 5′ noncoding region of poliovirus mRNA and that this factor is limiting in reticulocyte lysates, wheat germ extracts, and *Xenopus* oocytes. As mentioned above, translational initiation in these extracts probably occurs by a 5′-end-mediated event and is restricted by a domain found between nucleotides 70 and 381 (presumably in the form of secondary structure).

The possibility that poliovirus mRNA translates efficiently only in certain cell types is consistent with recent results showing that translation of poliovirus RNA in U-937 (a hystiocytic cell line that contains an equal number of poliovirus receptors to that in HeLa cells [23]) is considerably lower than that in HeLa cells (L. Carrasco, personal communication). In addition, poliovirus infection of several established human blood cell lines showed that viral replication differed depending on the differentiation stage and cell lineage (25). In those studies, the stage in the viral replication cycle at which the restriction occurred was not determined, but our results would suggest that a block at the translational level is a clear possibility. Thus, the translation restriction phenomenon may be related to the restricted tissue tropism exhibited by poliovirus (12, 25). Although it is believed that poliovirus tissue tropism is determined largely by the distribution of cell-specific virus receptors (12), it is possible that additional restrictions are imposed by the translational machinery of a given cell. Our study raises the interesting possibility that *cis*-acting sequences similar to those of poliovirus also exist in cellular mRNAs and determine tissue specific translation. Such elements could play a key role in development and differentiation.

Recently, Nicklin et al. (21) reported that deletion of the 5′ noncoding region of poliovirus mRNA resulted in a marked increase in translation efficiency in a rabbit reticulocyte lysate, in agreement with our results. Together, these data point to the existence of a *cis*-inhibitory element(s) within the 5′ noncoding region of poliovirus RNA. This element(s) restricts mRNA translation in reticulocyte lysates, *Xenopus* oocytes, and wheat germ extracts, but not in HeLa cell extracts.

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