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Intracellular Modifications Induced by Poliovirus Reduce the Requirement for Structural Motifs in the 5′ Noncoding Region of the Genome Involved in Internal Initiation of Protein Synthesis

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A series of genetic deletions based partly on two RNA secondary structure models (M. A. Skinner, V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond, J. Mol. Biol. 207:379–392, 1989; E. V. Pilipenko, V. M. Blinov, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol, Virology 168:201–209, 1989) was made in the cDNA encoding the 5′ noncoding region (5′ NCR) of the poliovirus genome in order to study the sequences that direct the internal entry of ribosomes. The modified cDNAs were placed between two open reading frames in a single transcriptional unit and used to transfect cells in culture. Internal entry of ribosomes was detected by measuring translation from the second open reading frame in the bicistronic mRNA. When assayed alone, a large proportion of the poliovirus 5′ NCR superstructure including several well-defined stem-loops was required for ribosome entry and efficient translation. However, in cells cotransfected with a complete infectious poliovirus cDNA, the requirement for the stem-loops in this large superstructure was reduced. The results suggest that virus infection modifies the cellular translational machinery, so that shortened forms of the 5′ NCR are sufficient for cap-independent translation, and that the internal entry of ribosomes occurs by two distinct modes during the virus replication cycle.

The genome of poliovirus is a single-stranded, positive-sense, polyadenylylated RNA molecule. It encodes a large polyprotein which is proteolytically processed during the virus replication cycle to produce a series of structural and nonstructural proteins. Unlike cellular mRNAs, the poliovirus genome does not contain a 7-methyl G cap structure but instead has a small virus-encoded protein (VPg) covalently linked to its 5′-terminal nucleotide. This protein is enzymatically removed before translation of the RNA. The 5′ noncoding region (5′ NCR) of the poliovirus genome is unusually large (approximately 750 nucleotides) and includes sequences essential for virus replication and for cap-independent translation. This region has also been shown to be the site of mutations which attenuate virus neurovirulence. It has been suggested that the multiple functions of the 5′ NCR are dependent on its RNA secondary structure as well as its primary structure, and several groups of investigators have used a combination of sequence alignment and biochemical probing methods to derive secondary structure models for the region (17, 25, 26, 29). The models proposed are remarkably similar to each other and contain large complex stem-loops encompassing most of the 5′ NCR. The frequent occurrence of compensatory base changes in homologous stems of the RNAs from related enteroviruses and rhinoviruses indicates that the structures are highly conserved and suggests that they have functional roles in vivo.

The observation that poliovirus genome expression occurs under conditions where the cap-binding protein complex (the p220 component of eukaryotic initiation factor 4F [eIF-4F]) is inactivated (5, 14), together with the absence of a 5′-terminal cap and the presence of multiple AUGs upstream from the known translational start site, suggested that initiation of translation of poliovirus RNA occurs by an alternative mechanism to ribosome scanning. Several groups subsequently showed for poliovirus and other picornaviruses that eukaryotic ribosomes bind internally on the 5′ NCR in order to initiate protein synthesis at the correct AUG (2, 9, 10, 22–24, 31, 32). For poliovirus, the region necessary for internal ribosome binding maps between nucleotides 140 and 631 and has been termed the ribosome landing pad (24). For the cardioviruses and the aphthoviruses, the functionally equivalent region has been termed the internal ribosome entry sequence and comprises a sequence of about 450 nucleotides toward the 3′ end of the 5′ NCR (2, 10) which directs ribosome entry immediately adjacent to the authentic initiation codon (13). The precise location of the sequences which promote internal entry of ribosomes and the role of the extensive secondary structure of the 5′ NCR, however, remain to be determined.

This article identifies the conserved stem-loop structures of the poliovirus 5′ NCR which are required for internal ribosome entry. We show that many of the stem-loops of the 5′ NCR superstructure are essential for initial translation but that after the expression of the poliovirus genome, a greatly reduced RNA structure can provide the same function. The results suggest that when cap-dependent translation is inhibited, viral protein synthesis can occur by using a ribosome landing pad lacking the elements which were otherwise essential.

MATERIALS AND METHODS

Production of plasmids and bicistronic mRNAs. Restriction endonucleases and DNA-modifying enzymes were obtained from Bethesda Research Laboratories and used according to the manufacturer's instructions. Routine cloning procedures, including ligations and transformation of *Escherichia*

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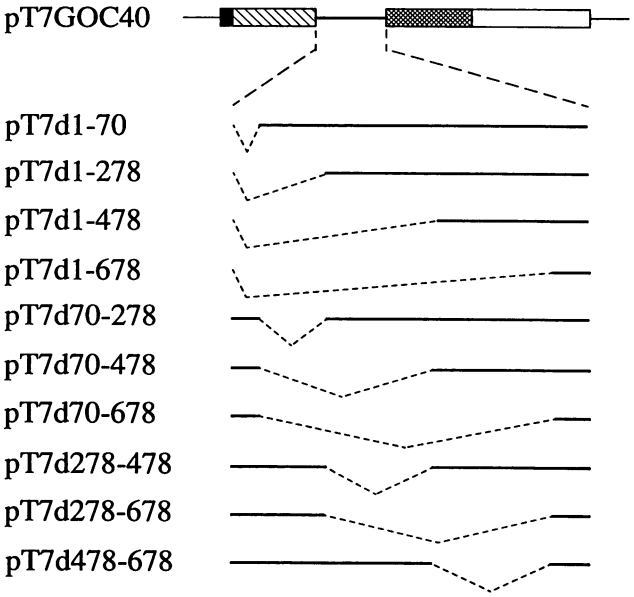


FIG. 1. Plasmids containing deletions in the 5′ NCR of poliovirus based on restriction endonuclease sites. The parent plasmid pT7GOC40 is shown at the top, with the T7 promoter depicted in black, the GUS gene in diagonal hatching, and the CAT gene in cross-hatching. The 5′ NCR of poliovirus type 3 is indicated by the bold line, and the pBR322-based vector sequences are shown as fine lines. The open box represents the simian virus 40 termination and polyadenylylation signals. The deletions created in the 5′ NCR by restriction endonuclease digestion are represented by dashed lines and identified by nucleotide numbers in the plasmid designations.

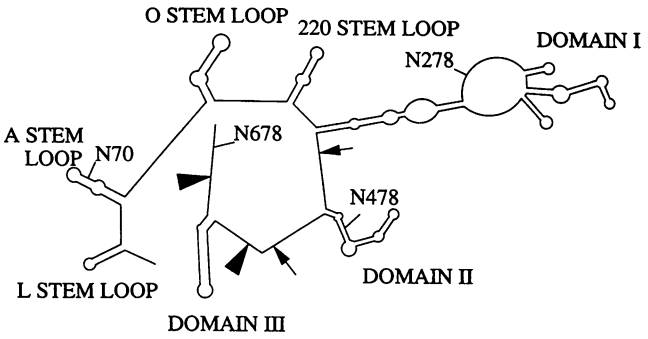


FIG. 2. Secondary structure model of the poliovirus 5′ NCR according to Skinner et al. (29). The nucleotide positions of the restriction endonuclease sites used to produce the plasmids presented in Fig. 1 are indicated as N70, N278, N478, and N678. The arrows and arrowheads indicate the sequences predicted to base pair in the model proposed by Pilipenko et al. (25) in the formation of domains referred to in the text as IIep and IIIep, respectively.

coli, were performed as described previously (18). DNA fragments were separated by agarose gel electrophoresis and purified with Geneclean (Stratagene Inc.). DNA sequences were verified by the dideoxy-chain termination method (27). Oligodeoxynucleotide primers for sequencing and site-directed mutagenesis were synthesized with a Cyclone DNA synthesizer (Biosearch Inc.) and were purified by preparative polyacrylamide gel electrophoresis. Site-specific mutagenesis was carried out as described previously (16) with uracil-enriched templates and specific mutagenic oligonucleotide primers.

The 8.5-kb plasmid pT7GOC40 is shown in Fig. 1. In this plasmid, the chloramphenicol acetyltransferase (CAT)-coding region from the *E. coli* transposable element Tn*9* (derived from pRSVCAT [7]) was fused directly downstream of a cDNA copy of the complete 5′ NCR of poliovirus type 3 P3/Leon/37 and upstream of the simian virus 40 small t intron and polyadenylylation and transcription termination signals. The cDNA encoding the *E. coli* β-glucuronidase gene (GUS) from pRAJ275 (12) was inserted upstream of the poliovirus 5′ NCR and under the control of a bacteriophage T7 promoter so that transcription from this promoter produced a bicistronic mRNA with the structure 5′-GUS-poliovirus 5′ NCR-CAT-3′.

Construction of 5′ NCR deletions. Two sets of 5′ NCR deletions were constructed; the first was based on convenient restriction endonuclease sites, and the second was constructed by site-directed mutagenesis based on the RNA secondary structure models of Skinner et al. and Pilipenko et al. (25, 29) shown in Fig. 2.

The 5′ NCR of poliovirus type 3 (nucleotides [nt] 1 to 745) was subcloned into pUC7, rendering the following restriction sites unique: *Hin*dIII and *Pst*I (both flank the extreme 5′ end of the poliovirus clone), *Kpn*I (nt 70), *Mlu*I (nt 278), *Nco*I (nt 478), *Bam*HI (nt 678), and *Sst*I (nt 751). Deletions were then created by double digestion with the appropriate enzymes, and the resulting termini were rendered blunt ended by treatment with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates. After purification, the DNA was self-ligated, treated with a suitable enzyme to remove undigested molecules, and used to transform competent *E. coli* MC1061. Plasmids of the desired structure were identified, and the modified 5′ NCRs were inserted into pT7GOC40 as *Hin*dIII-*Sst*I fragments. The structures of the resulting plasmids (shown in Fig. 1) were confirmed by direct sequencing of the plasmid double-stranded DNAs.

Deletion mutants based on secondary structure were made by loop-out mutagenesis on a uracil-enriched template (a *Pst*I-*Sst*I fragment cloned into M13mp19) according to the method of Kunkel (16). Each deletant was cloned into pT7GOC40 between GUS and CAT, and its structure was confirmed by direct sequencing of plasmid DNA. A summary of the deletions constructed by this method is shown in Table 1.

Transient expression assays. The expression of GUS and CAT from the plasmids summarized in Fig. 1 and Table 1 was assessed in a transient expression system as described previously (2). Briefly, monolayers of BSC40 cells in 35-mm-diameter dishes were infected with a recombinant vaccinia

TABLE 1. 5′ NCR mutants based on secondary structure

Plasmid	5′ NCR nucleotide changes verified by sequencing
pT7dL^{inv}GOC	nt 10–35 reversed
pT7dLGOC	nt 10–35 deleted
pT7dAGOC	nt 51–79 deleted
pT7dOGOC	nt 125–164 deleted
pT7d220GOC	nt 188–222 deleted
pT7dDIGOC	nt 236–443 deleted
pT7dDIImsGOC	nt 470–538 deleted
pT7dDIIepGOC	nt 451–559 deleted
pT7dDIIImsGOC	nt 585–620 deleted
pT7dDIIIepGOC	nt 564–628 deleted

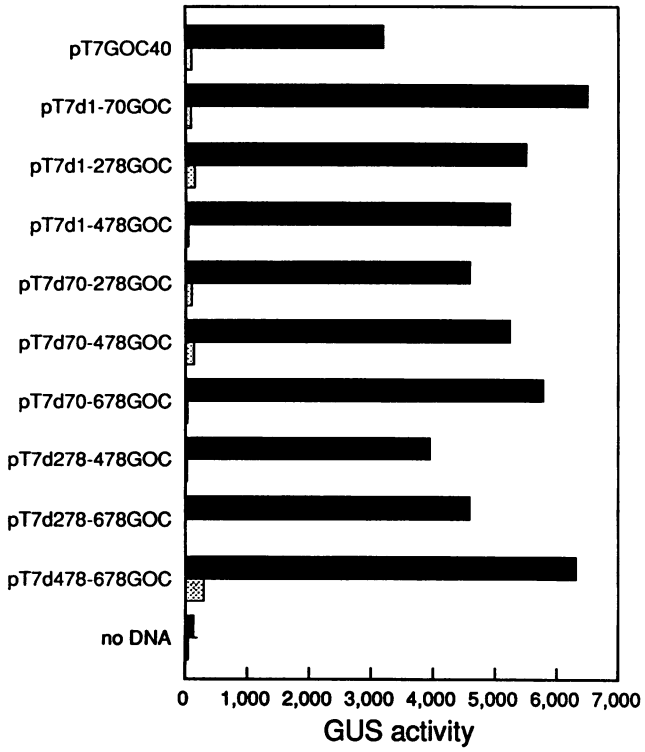


FIG. 3. Expression of GUS from the deletion plasmids shown in Fig. 1 in the presence and absence of expressed poliovirus proteins. Plasmids were introduced into vTF7-3-infected BSC40 cells either alone (■) or with the full-length infectious poliovirus cDNA pT7LEON (▩). Twenty hours after infection, cell extracts were prepared and assayed for GUS activity. The activity is expressed in arbitrary units from a 60-min assay.

virus, vTF7-3, which expresses T7 RNA polymerase (6) and 1 h later were transfected with 5 μg of each plasmid DNA by using lipofectin (Bethesda Research Laboratories). Where required, 5 μg of plasmid pT7LEON containing a full-length infectious poliovirus type 3 cDNA preceded by a T7 promoter (29) was cotransfected with each plasmid. After 20 h, cell extracts were prepared, and enzymic assays were performed on standard aliquots of total cell extract. CAT activity was assessed as described previously (7) except that 0.2 μCi of [^{14}C]chloramphenicol was used in each assay. GUS activity was determined by a fluorimetric assay (11).

RESULTS

The sequence necessary for internal entry of ribosomes has been identified previously as nt 140 to 631 for poliovirus type 1 (24). An initial aim of this study was to define more precisely the primary sequence and/or secondary structures of the 5′ NCR which are essential for this function. We designed an assay system in which the 5′ NCR of poliovirus type 3 could direct translation in a cap-independent manner. Thus, the 5′ NCR derived from plasmid P3/Leon/37 was inserted as an intercistronic spacer between the two reporter genes, the *E. coli* β-glucuronidase gene (*uidA* or GUS) and the CAT gene. These were arranged as a single transcriptional unit driven by a T7 promoter in a plasmid designated pT7GOC40 (Fig. 1). This study employed a transient expression system which used the recombinant vaccinia virus vTF7-3 (6) to express the T7 RNA polymerase in cells transfected with plasmid pT7GOC40 and derivatives thereof. The two translation products from the bicistronic mRNA could then be assayed independently with crude cell extracts (2).

Preliminary experiments on pT7GOC40 transfected alone established that expression occurred from both open reading frames. However, if pT7GOC40 was cotransfected with the full-length infectious poliovirus cDNA pT7LEON, translation of the first cistron (GUS) was abolished by poliovirus-induced host cell shutoff, whereas translation of the second cistron (CAT) was maintained (Fig. 3 and 4). This estab-

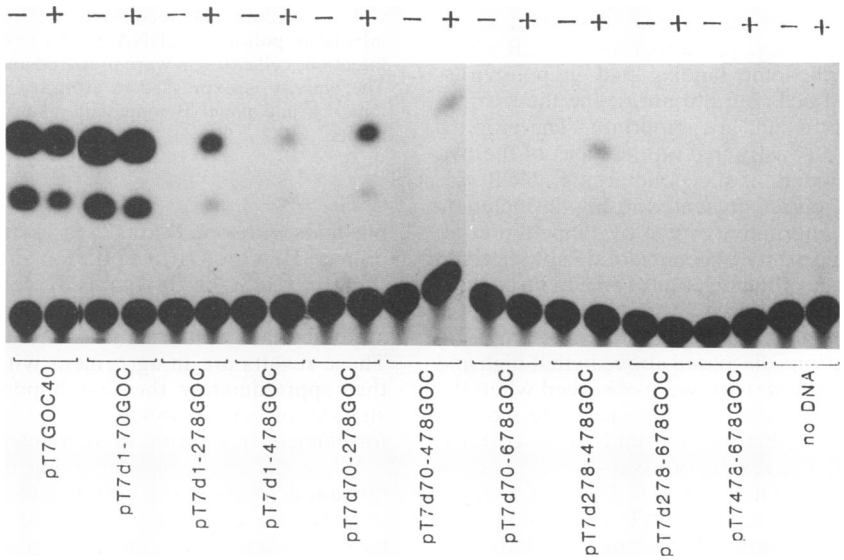


FIG. 4. Expression of CAT from the deletion plasmids shown in Fig. 1 in the presence and absence of expressed poliovirus proteins. Plasmids were introduced into vTF7-3-infected BSC40 cells either alone (−) or with the full-length infectious poliovirus cDNA pT7LEON (+). Twenty hours after infection, cell extracts were prepared and assayed for CAT activity as described in Materials and Methods.

lished that translation of the second cistron occurred by internal initiation on the poliovirus 5′ NCR and that this translation was independent of initiation at the first cistron.

Experiments were also performed on a series of plasmids derived from pT7GOC40 and containing a nested set of 5′ NCR deletion mutations based on convenient restriction endonuclease sites (Fig. 1). When these plasmids were transfected alone, the expression of the first cistron (GUS) was high and comparable for all of the deletions (Fig. 3). The expression of the second cistron (CAT) was relatively unaffected by deletion of the first 70 nt, but deletion of sequences up to nt 278 or beyond abolished CAT expression completely (Fig. 4). This result is in agreement with previously published observations which identified sequences between nt 140 and 631 as containing the internal ribosome binding site (24). When cotransfected with the full-length infectious poliovirus cDNA pT7LEON, GUS activities from all the plasmids were reduced to background levels by virus-induced host cell shutoff, as expected. However, the measurement of expression from the second cistron (CAT) revealed two opposing effects. Plasmids which expressed high levels of CAT activity when assayed alone consistently produced less activity in the presence of pT7LEON. However, some of the plasmids with deletions in the 5′ NCR which were unable to support translation of CAT when transfected alone now produced CAT activity. This was observed with cDNAs carrying deletions from the 5′ terminus up to nt 478, but the activity was lost with deletions which extended to nt 678.

These observations suggest, therefore, that the sequences and/or secondary structures essential for entry of ribosomes on initial poliovirus infection differ from those required after infection is established in the cell.

Since cotransfection with pT7LEON blocked translation of GUS but not of CAT from pT7GOC40 and since expression of CAT required sequences between nt 70 and 678, we can conclude that CAT synthesis was not due to termination and reinitiation events, leaky scanning, or RNA degradation but was the result of internal initiation of protein synthesis from the 5′ NCR.

It has been suggested that secondary structure domains within a picornavirus 5′ NCR are important for the multiple functions of the region including cap-independent translation. The nested set of deletion plasmids described above allowed the detection of a large portion of the 5′ NCR which acts as a functional ribosome landing pad in poliovirus-infected and uninfected cells but did not define the secondary structural elements which are important. Therefore, a series of deletions were constructed on the basis of the two secondary structure models of the poliovirus 5′ NCR (25, 29). The stem-loop structures presented in Fig. 2, including those predicted in the alternative model by Pilipenko et al. (25), were precisely deleted by oligonucleotide site-directed mutagenesis. A summary of the deletions is listed in Table 1, and the expression of GUS activity from these plasmids is shown in Fig. 5. The results obtained were consistent with those for the set of deletions discussed above in that high and comparable levels of GUS activity were observed when the plasmids were transfected alone but activities were reduced to near-background level when the plasmids were cotransfected with pT7LEON. The results of assays for expression of the second cistron (CAT) are shown in Fig. 6. When the plasmids were transfected alone, CAT expression was readily observed from plasmids harboring a deletion or inversion of the L loop (nt 10 to 35) and deletion of the loops termed A (nt 51 to 79), 220 (nt 188 to 222), and domain IIIms (nt 585 to 620). In contrast, CAT activity was not seen in

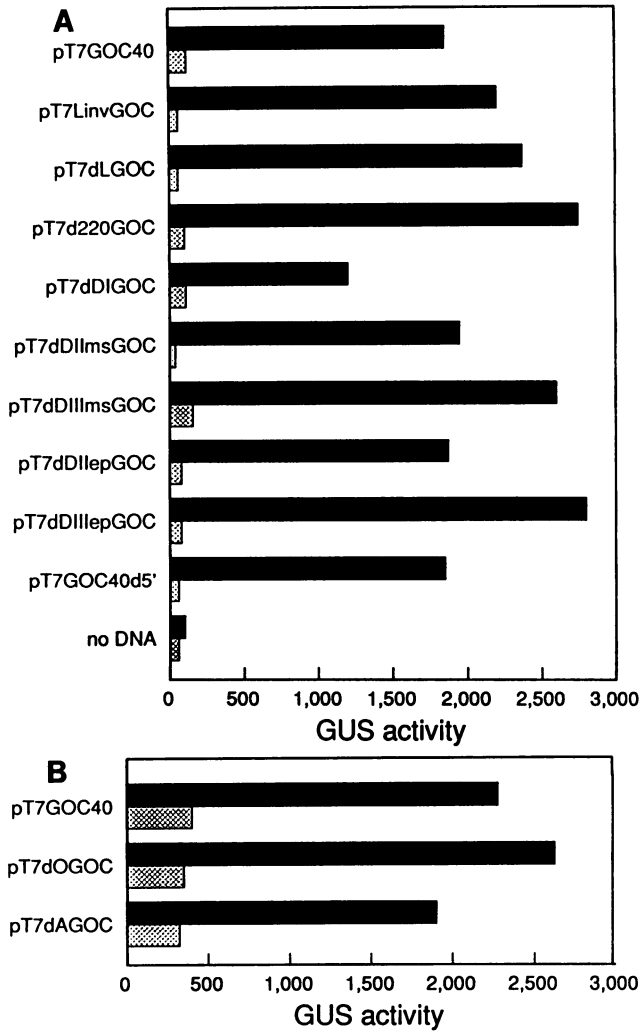
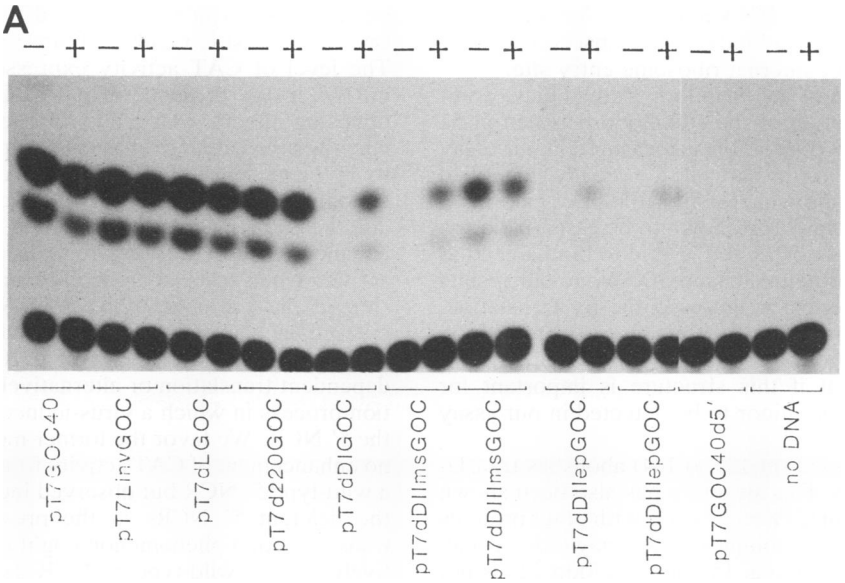


FIG. 5. Expression of GUS from the 5′ NCR deletions created by site-directed mutagenesis (Table 1) in the presence and absence of expressed poliovirus proteins. Plasmids were introduced into vTF7-3-infected BSC40 cells either alone (■) or with the full-length infectious poliovirus cDNA pT7LEON (▩). Twenty hours after infection, cell extracts were prepared and assayed for GUS activity. The activity is expressed in arbitrary units from a 60-min assay. Panel A and panel B represent results from independent experiments.

plasmids with loop O (nt 125 to 164), domain I (nt 236 to 443), domain IIep (nt 451 to 559), domain IIms (nt 470 to 538), or domain IIIep (nt 564 to 628) deleted. This experiment, therefore, identifies the secondary structure domains which are essential for internal ribosome entry in uninfected cells. These results are in agreement with previous observations that approximately the first hundred nucleotides and the stem-loops from nt 188 to 222 and 585 to 620 are dispensable for efficient translation in uninfected cells (4, 8).

When the plasmids containing the precise stem-loop or domain deletions were cotransfected with pT7LEON, GUS activity was essentially abolished in each case, and again two opposing effects on the expression of CAT were observed. The level of CAT activity from pT7GOC40, pT7L^{inv}GOC, pT7dLGOC, pT7d220GOC, and pT7dDIIIms GOC was decreased, whereas CAT activity was now ob-



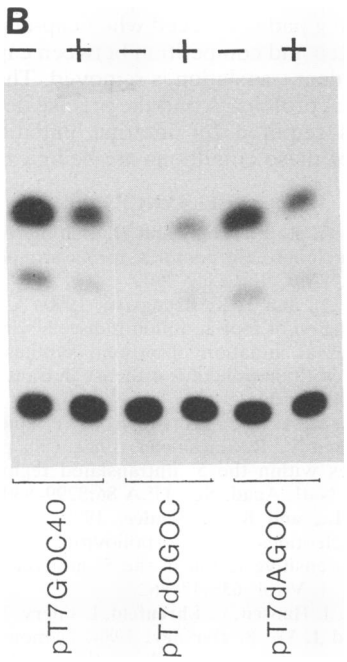


FIG. 6. Expression of CAT from the 5′ NCR deletions created by site-directed mutagenesis (Table 1) in the presence and absence of expressed poliovirus proteins. Plasmids were introduced into vTF7-3-infected BSC40 cells either alone (−) or with the full-length infectious poliovirus cDNA pT7LEON (+). Twenty hours after infection, cell extracts were prepared and assayed for CAT activity as described in Materials and Methods. CAT assays were performed on the cell extracts used for the GUS assays whose results are shown in Fig. 5. Panel A and panel B represent results from independent experiments.

served from plasmids pT7dDIGOC, pT7dDIIepGOC, pT7dDIImsGOC, and pT7dDIIIepGOC, which were inactive in the absence of pT7LEON. Thus, in contrast to the situation in which the plasmids were transfected alone, none of the secondary structural domains presented in Fig. 2 were essential for ribosome entry in cells cotransfected with pT7LEON and expressing poliovirus proteins. The results imply that poliovirus infection modifies the translational apparatus of the cell so that ribosomes can be readily recruited for internal initiation and can enter via a smaller region of the landing pad than is necessary early in infection of the cell.

DISCUSSION

The initiation of translation of poliovirus RNA occurs by a mechanism different from that of cellular mRNAs because of the fact that poliovirus RNA is uncapped. Early in the replication cycle, poliovirus RNA must compete for translation with capped cellular mRNAs in the presence of fully functional ribosomes. Later, after the production of poliovirus protein 2A and the resulting inactivation of the cap-binding complex (the p220 component of eIF-4F), cap-dependent translation is inhibited, resulting in the inhibition of host cell protein synthesis, and translation occurs by a cap-independent mechanism. The precise sequences and/or RNA secondary structures required for efficient translation of poliovirus RNA under these two sets of conditions have not been characterized hitherto.

The observations presented in this article help to define more accurately the sequences of the poliovirus 5′ NCR required for internal entry of ribosomes. The analysis of a series of 5′ NCR deletion mutants, each included as the intercistronic spacer in a bicistronic mRNA, indicates that in uninfected cells sequences included in the secondary structural domains O (nt 125 to 164), I (nt 236 to 443), IIep (nt 451 to 559), IIms (nt 470 to 538), and IIIep (nt 564 to 628) are essential for this function but that sequences which reside in domains L (nt 10 to 35), A (nt 51 to 79), 220 (nt 188 to 222), and IIIms (nt 585 to 620) are not required. The conclusion concerning the last two domains is strengthened by the observation that their deletion in full-length infectious cDNAs does not destroy infectivity (unpublished results).

There have been several reports describing the binding of cellular factors to stem-loop structures in picornavirus 5′ NCRs, and it is likely that some of these interactions are involved in ribosome entry. For example, Jang and Wimmer (10) have demonstrated that a cellular protein, p57, binds to a conserved secondary structure motif (stem-loop E) of

encephalomyocarditis virus RNA in conjunction with an as yet unidentified factor which binds to the conserved pyrimidine-rich segment of the internal ribosome entry site.

Deletion or inversion of the stem-loop formed between nt 10 and 35 (L) and deletion of the stem-loop between nt 51 and 79 (A) do not affect translation as measured in our assay system. These loops form part of a recently identified cloverleaf structure comprising the first 100 nt of picornavirus mRNAs (1) which has been shown to be essential in the plus strand for RNA synthesis and is able to bind a cellular protein and the viral proteins 3C and 3D. While our results indicate that this cloverleaf is not essential for translation, there is a recent report that an insertion into the L loop produces a mutant deficient in translation in vivo (28). We therefore conclude that if this structure is important for translation, its role is too minor to be detected in our assay system.

Deletion of stem-loop O (nt 125 to 164) abolishes translation in uninfected cells. This structure has also been shown to bind a cellular protein (3) which, along with other proteins (including eIF-2α), forms a complex which may represent an early step in ribosome binding. In contrast, loop 220 is not required for translation, and we can therefore conclude that the 50-kDa membrane-associated protein reported by Najita and Sarnow (20) to bind to this region is unlikely to be involved in ribosome entry. It is noteworthy that viable mutants which lack sequences which include this structure have been isolated (reference 4 and unpublished results).

Deletion of the two largest structures, domains I and II, results in total loss of translation in uninfected cells, indicating that these domains are essential for ribosome entry in primary translation. Domain II contains the major attenuating mutation of neurovirulence found in the three poliovirus serotypes (nt 480, 481, and 472 in types 1, 2, and 3 respectively) which has been reported to affect translation of the RNA. Extensive mutagenesis in this region has confirmed that the disruption of a conserved stem-loop at the base of domain IIms causes the attenuation phenotype (unpublished results). It is of interest that neither of these domains has yet been shown to bind cellular factors.

Deletion of domain IIIms (which forms part of domain IIIep) did not prevent translation in our system, although the level was considerably reduced. This is consistent with the observation that mutagenesis of a conserved AUG in this region results in reduced translational efficiency (21). This domain has also been shown to bind a cellular factor, p52 (19), which may be equivalent to the "initiation correction factor" identified by Svitkin et al. (30). Removal of the complete domain (i.e., domain IIIep [nt 564 to 628]), on the other hand, abolished translation completely, suggesting that the highly conserved pyrimidine-rich region between nt 564 and 585 is required. This is consistent with the results of Kuge and Nomoto (15), who showed that a deletion between nt 564 and 726 (poliovirus type 1 numbering) gave rise to an attenuated virus and further deletions extending upstream were lethal.

The analysis of deletion mutants in cells cotransfected with a complete poliovirus cDNA unexpectedly revealed that under conditions in which virus proteins were being synthesized, considerably reduced 5′ NCR structures retained the ability to direct internal initiation of protein synthesis. Deletion of the individual domains indicated that none of the major stem-loop structures in the models proposed by Skinner et al. (29) and Pilipenko et al. (25), including domains IIep (nt 451 to 559) and IIIep (nt 564 to 628), were essential under these conditions. However, the deletions up to nt 678 indicated that some sequences between this position and nt 70 are required for this activity. The level of CAT activity expressed from the bicistronic mRNA in the presence of pT7LEON is the result of two opposing effects. Plasmids which expressed CAT activity when assayed alone consistently expressed a reduced activity in the presence of pT7LEON. We attribute this reduction to competition between the 5′ NCR of the bicistronic mRNA and the poliovirus genome. Despite this effect, we observed enhanced CAT expression from plasmids which produced no activity when assayed alone. The activation of CAT expression in the presence of pT7LEON may represent an increased availability of cellular factors resulting from the poliovirus-induced cleavage of p220 and inhibition of cap-dependent translation or alternatively may be a transactivation process in which a virus-induced protein interacts with the 5′ NCR. We favor the former model, since we observed no enhancement of CAT activity from pT7GOC40 containing a wild-type 5′ NCR but observed increased expression from the deletant 5′ NCRs in the presence of pT7LEON. A transactivation phenomenon ought to work at least as effectively on the wild-type 5′ NCR as on the deleted forms. Hence, we propose that the requirement for a complete ribosome landing pad is reduced when cap-dependent translation is inhibited and competition between cap-independent and cap-dependent translation is removed. The involvement of the poliovirus protein 2A and the precise determination of the sequences required for internal initiation of protein synthesis under these conditions are being explored.

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