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An RNA Sequence of Hundreds of Nucleotides at the 5′ End of Poliovirus RNA Is Involved in Allowing Viral Protein Synthesis

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Twenty-one mutations were engineered in the 5′ noncoding region of poliovirus type 1 RNA, using an infectious cDNA copy of the viral genome. RNA was made from these constructs and used to transfect HeLa cells. Viable virus was recovered from 12 of these transfection experiments, including six strains with a recognizable phenotype, mapping in four different regions. One mutant of each site was studied in more detail. Mutant 5NC-11, having a 4-base insertion at nucleotide 70, was dramatically deficient in RNA synthesis, suggesting that the far 5′ end of the genome is primarily involved in one or more steps of RNA replication. Mutants 5NC-13, 5NC-114, and 5NC-116, mapping at nucleotides 224, 270, and 392, respectively, showed a similar behavior; they made very little viral protein, they did not inhibit host cell translation, and they synthesized a significant amount of viral RNA, although with some delay compared with wild type. These three mutants were efficiently complemented by all other poliovirus mutants tested, except those with lesions in protein 2A. Our results imply that these three mutants map in a region (region P) primarily involved in viral protein synthesis and that their inability to shut off host cell translation is secondary to a quantitative defect in protein 2A. The exact function of region P is still to be determined, but our data supports the hypothesis of a single functional module allowing viral protein synthesis and extending over several hundred nucleotides.

The genome of poliovirus is a single-stranded molecule of mRNA polarity (positive strand) containing approximately 7,500 nucleotides. Its 5′ end is linked to a small peptide, VPg, and its 3′ end is a stretch of poly(A) 40 to 100 nucleotides long. A total of more than 800 noncoding nucleotides are located at the two ends of poliovirus RNA, 742 at its 5′ end and 65 preceding the poly(A) at its 3′ end (9, 18). An open reading frame initiated by an AUG at position 743 (4) encompasses 6,528 nucleotides and encodes a polyprotein of about 250,000 daltons. Poliovirus mRNA, like that of other picornaviruses, not only has an unusually long untranslated 5′ region, but also is not capped at its 5′ end. It terminates in pUp instead of the "capping" group $m^7G(5')ppp(5')N\ldots$, found on almost all other mRNAs (7, 16).

A highly significant sequence similarity extends through the first 650 nucleotides of the three poliovirus serotypes (26). This marked conservation strongly suggests that the 5′ terminal region serves crucial functions in the virus life cycle. Several potential functions can be imagined for the 5′ noncoding (5NC) region, e.g., initiation of protein synthesis (binding of ribosomes and initiation factors), initiation of synthesis of plus-strand RNA, stabilization of the RNA by secondary structure formation, interaction with the viral capsid proteins during packaging of the RNA, or binding of presumptive regulatory molecules that control the replication and translation of the viral RNA. The 5NC region appears to have a critical role in neurovirulence. Neurovirulent revertants of the Sabin type 3 strain have been isolated from patients with vaccine-associated poliomyelitis; compared with their attenuated parental strain, they were found to have a single base substitution at position 472 of the genome (6). Reinforcing this data, in vitro experiments using recombinants between wild-type and attenuated strains have confirmed that a major determinant of neurovirulence is located in the 5NC region of types 1 (15) and 3 (27) poliovirus.

In an attempt to elucidate the functions of the 5NC region, we have created a number of mutations in this part of poliovirus type 1 (Mahoney) RNA. The mutations were made by mutagenesis of an infectious cDNA clone (19) and by the recovery of altered viral strains, as previously used by us (2, 22) and others (10, 23) to study various aspects of poliovirus molecular biology and genetics. With these mutations, we have been able to delineate at least two functional regions, one primarily involved in the synthesis of the viral RNA, the other in its translation. This last function seems to be accomplished by an extensive sequence of nucleotides, suggesting the role of an RNA superstructure in the initiation of translation. Mutants deficient in the translation function also provide important information on the inhibition of host mRNA translation in poliovirus-infected cells.

MATERIALS AND METHODS

DNA procedures. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass.; DNA polymerase I (Klenow fragment) was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; exonuclease BAL 31 was from International Biotechnologies, Inc., New Haven, Conn.; T7 RNA polymerase and human placenta RNase inhibitor was from Promega Biotec, Madison, Wis.; and avian myeloblastosis virus reverse transcriptase was from Life Sciences Research, Baltimore, Md. All enzymes and compounds were used according to the instructions of the manufacturer.

Mutagenesis was done on a pBR-based subclone containing the first 1,200 nucleotides of poliovirus type 1 (Mahoney) cDNA, downstream of a T7 RNA polymerase promoter (a modification of our original infectious clone) (19). The three protocols used were filling in restriction sites (partial digestion with a restriction enzyme, blunting with Klenow, ligation), deleting restriction site overhangs (partial digestion,

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blunting with T4 DNA polymerase, ligation), and inserting linkers at some restriction sites (partial digestion, blunting with Klenow or T4 DNA polymerase, linker ligation). The *Aat*II site at position 1118 of poliovirus sequence was then used to introduce all the mutated 5′ ends into the full-length clone. Mutations were confirmed by sequencing plasmids by the chemical technique (13), and the derived viral RNA for viable mutants was sequenced by primer extension (21). We are confident that the alterations engineered into clones were responsible for the mutant phenotypes because only the first 1,118 nucleotides were in the altered region and because a number of the mutants were created independently twice with the same phenotypic consequences.

Cells and viruses. HeLa and CV1 cells were grown as previously described (2). HeLa cells on 100-mm-diameter dishes were transfected with 2 to 5 μg of in vitro-synthesized RNA, using the DEAE-dextran procedure (12). All constructs were also tested by DNA transfection (average 10 μg) according to the same protocol. The generation of infectious virus particles was checked by covering the transfected cells with an agar overlay and examining the plates for plaques over 2 to 5 days and also by harvesting cells that had been transfected, treating them by three cycles of freeze and thaw, and using them for a plaque assay on a new monolayer. Stocks from each viable virus were grown according to standard techniques (2).

One-step growth curves. Measurement of virus growth and release was done on 60-mm-diameter HeLa cells dishes, according to standard procedures (2).

Measurement of viral RNA synthesis. Monolayer cultures of HeLa cells were infected at a multiplicity of infection (MOI) of 10, and after adsorption at room temperature for 30 min, Dulbecco modified Eagle medium (DME) supplemented with 7% fetal calf serum (Hazleton) was added. At various times after infection, cells were placed on ice, washed once with cold phosphate-buffered saline, and lysed in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–0.1% Nonidet P-40. Nuclei and debris were removed by centrifugation. RNA was isolated by phenol-chloroform extraction and ethanol precipitation. Portions were denatured in 10× SSC (1× is 0.15 M NaCl, 15 mM sodium citrate)–17% formaldehyde and bound to nitrocellulose by aspiration with a dot-blot apparatus. Filters were baked for 2 h at 80°C under vacuum, prehybridized in sealed bags at 37°C for 2 h in 50% formamide–5× SSCPE (1× is 1× SSC, 13 mM KH_2PO_4, 1 mM EDTA)–5× Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–250 μg of herring sperm DNA per ml–500 μg of yeast tRNA per ml; the filters were hybridized overnight at 60°C in the same solution, using [^{32}P]RNA probes generated with SP6 or T7 polymerase as described previously (14). The probes represented nucleotides 670 to 2243 of the poliovirus type 1 Mahoney genome, in either direction. After hybridization, filters were washed in 0.2× SSC three times at 68°C and exposed to X-ray film. Regions in which RNA was bound were then cut out and dissolved in Biofluor (National Diagnostics), and radioactivity was measured with a scintillation counter.

Nucleotide sequencing of RNA. RNA from all viable viruses was prepared as above and was sequenced by the chain-termination technique (21), using reverse transcriptase and synthetic oligonucleotide primers complementary to bases 120 to 143, 298 to 320, 477 to 500, and 758 to 781 of the viral RNA.

Electrophoretic analysis of protein synthesis in infected cells. Infection procedures were as described above. At various times postinfection, cells were placed in methionine-depleted Dulbecco modified Eagle medium containing 25 μCi of [^{35}S]methionine per ml. After 30 min of incubation, cells were put on ice, washed once with cold phosphate-buffered saline, harvested by centrifugation, and lysed in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–0.5% Nonidet P-40. Nuclei were removed by centrifugation, and a portion of the cytoplasmic extract was fractionated by electrophoresis through a 12.5% SDS-polyacrylamide gel. After electrophoresis, gels were treated with Autofluor (National Diagnostics), dried, and exposed to X-ray film at −70°C.

Immunoprecipitation of infected cell extracts. [^{35}S]methionine-labeled cell extracts were immunoprecipitated in IPB (20 mM Tris hydrochloride [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 1 mM EDTA), by using a rabbit polyclonal poliovirus type 1 antivirion antiserum. After incubation for 1 h on ice, the immune complexes were recovered by the addition of a 10% solution of *Staphylococcus aureus*; the complexes were washed three times with IPB, suspended in Laemmli sample buffer (11), boiled, and centrifuged to remove the *S. aureus* cells. The supernatants were analyzed by electrophoresis as described above.

Immunoblot analysis of p220. Cytoplasmic extracts were prepared as described above, and an immunoblot analysis was performed as described by Bonneau and Sonenberg (3), using a rabbit anti-p220 polyclonal antiserum (a gift from I. Edery, McGill University, Montreal, Quebec, Canada).

Complementation experiments. HeLa or CV1 cell infections were performed at 39°C in 60-mm-diameter petri dishes, and yields of progeny virus were measured at various times, as described by Bernstein et al. (1). The complementation index (CI) was defined as follows: CI for mutant A = (titer of mutant A in mixed infection)/(titer of mutant A in single infection), where titers are measured at the permissive temperature. Results for revertants and recombinants, identified by the size of plaques and by the loss of temperature sensitivity, were subtracted from the counts.

RESULTS

Mutagenesis. Not knowing which area of the 5NC region might be critical to a given function, we randomly mutagenized the whole region by insertion or deletion of multiple nucleotides at various convenient sites (see Materials and Methods). Some sites were modified in several ways, sometimes with different results, as will be outlined below. After the mutations were engineered into subclone pL1.4, they were introduced into the full-length clone pXpA, generating constructs pPN-1 to pPN-21. These constructs and their locations are described in Table 1 and Fig. 1. Using T7 RNA polymerase, RNA was made from all the constructs and used to transfect HeLa cells, and the recovery of infectious virus particles was tested by plaque assay on HeLa cells. Three kinds of results were seen (Fig. 1).

First, nine mutations involving seven sites (those in pPN-1, -2, -9, -12, and -16 to -20) were lethal, meaning that no infectious virus particle was recovered from the transfected cells.

Second, five mutations at three different restriction sites (in pPN-5, -6, -7, -10, and -13) did not affect the phenotype of the virus, at least as assessed by plaque assay. Another construct, pPN-21, with a deletion of bases 630 to 723, generated by the sequential use of the *Bal*I restriction enzyme and BAL 31 exonuclease, also gave rise to virus with a wild-type phenotype. Such a result has also been reported with the Sabin type 1 strain, in which viruses

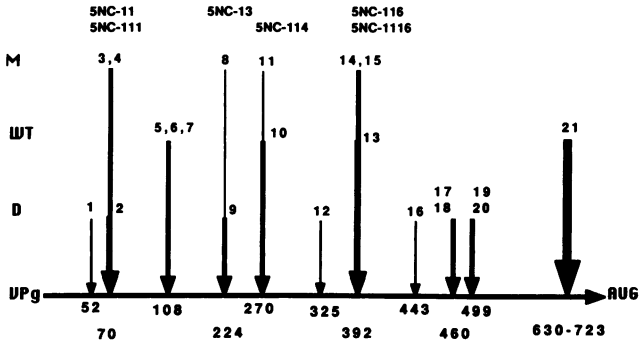

FIG. 1. The result obtained from transfection of HeLa cells with RNA made from the 21 mutated clones is shown. Numbers on top of each arrow correspond to the constructs described in Table 1 (i.e., 1 is pPN-1). Construct pPN-21 has a deletion of the sequence indicated on the bottom line. Short arrows indicate lethal mutations (D), medium-length arrows denote silent mutations (WT), and long arrows point to mutations responsible for a recognizable viable phenotype (M). Names of the mutant viruses are shown at the top of the figure. Numbers on bottom lines indicate the nucleotide positions of the mutations.

lacking the genome region between nucleotides 600 and 726 are fully viable (10).

The third type of result was obtained from six mutations, affecting four sites, all of which generated infectious viruses with a phenotype easily distinguishable from that of the wild type.

(i) Mutants 5NC-11 and 5NC-111. Deleting bases 67 to 70 by eliminating the free overhang of a *Kpn*I site was found to be lethal (pPN-2); however, duplicating the same four bases by filling in the overhang left by its isoschizomer, *Ban*I (pPN-3), generated mutant 5NC-11. The further addition of an *Eco*RI linker at this filled-in site (pPN-4) created mutant 5NC-111. Both mutants generate minute plaques compared with those of wild-type virus and are temperature sensitive (the titer at 32°C is 200-fold greater than the titer at 39°C).

(ii) Mutant 5NC-13. The insertion of 4 bases at position 224, taking advantage of a *Bam*HI site (pPN-8), gave rise to mutant 5NC-13. 5NC-13 is temperature sensitive (titer at 32°C is 100-fold greater than the titer at 39°C); moreover, the sizes of the plaques it generates at both temperatures are smaller than those of wild-type virus.

(iii) Mutant 5NC-114. The *Hin*fI site at position 267 could be filled in without effect on the phenotype of the virus (pPN-10); the further insertion of an 8-base linker (pPN-11) generated mutant 5NC-114. 5NC-114 is slightly temperature sensitive (5- to 10-fold) and has a small-plaque phenotype.

(iv) Mutants 5NC-116 and -1116. The insertion of 4 bases at position 392 by filling in a *Nco*I site (pPN-13) did not significantly affect the size of viral plaques; when the same site was further modified by the introduction of a linker (pPN-14) and when this linker was moreover blunted (pPN-15), mutants 5NC-116 and 5NC-1116, respectively, were obtained. Both mutants show a phenotype that resembles the one of 5NC-114 in temperature sensitivity and plaque size.

Assuming that slightly different modifications of the same site would alter the same function, 5NC-111 and 5NC-1116 were not further characterized. Being only slightly temperature sensitive, 5NC-114 and 5NC-116 were studied at 37°C.

One-step growth curves. To characterize the defects in the four phenotypically mutant viruses, we examined some basic parameters of the poliovirus life cycle. One-step growth curves (Fig. 2) show that under nonpermissive conditions, all of these mutants replicate more slowly than the wild-type parental strain and produce a final progeny yield that is lower.

Viral RNA synthesis. As a first step toward defining why their replication was inefficient, we assessed the ability of the 5NC mutants to synthesize RNA. This is usually done by measuring the incorporation of [^{3}H]uridine into viral RNA in the presence of actinomycin D, a specific inhibitor of cellular transcription. However, we found that the replication of all of our 5NC mutants was sensitive to actinomycin D, in contrast to wild-type virus (data not shown). The reason for this sensitivity to actinomycin D is unknown but has been reported in other mutants that map in the 5NC region (20) or elsewhere (M. Girard, personal communication). We therefore analyzed viral RNA synthesis by dot blot, as described

TABLE 1. Mutagenesis

Clone	Wild-type sequence	Mutant sequence	Phenotype[a]
pPN-1	AGT-52-ACT	AGT-52-CCCCGGGACT	D
pPN-2	G-66-GTACC	G-66-....C	D
pPN-3	GGTAC-70-C	GGTAC-70-GTACC	M (5NC-11)
pPN-4	GGTAC-70-C	GGTAC-70-GGAATTCCGTACC	M (5NC-111)
pPN-5	CTTA-108-G	CTTA-108-TTAG	WT
pPN-6	CTTA-108-G	CTTA-108-GGAATTCCTTAG	WT
pPN-7	CTTA-108-G	CTTA-108-GGAATTAATTCCTTAG	WT
pPN-8	GGATC-224-C	GGATC-224-GATCC	M (5NC-13)
pPN-9	GGATC-224-C	GGATC-224-GGAATTCCGATCC	D
pPN-10	GAAT-270-C	GAAT-270-AATC	WT
pPN-11	GAAT-270-C	GAAT-270-GGAATTCCAATC	M (5NC-114)
pPN-12	GAGT-325-C	GAGT-325-AGTC	D
pPN-13	CCATG-392-G	CCATG-392-CATGG	WT
pPN-14	CCATG-392-G	CCATG-392-GGAATTCCCATGG	M (5NC-116)
pPN-15	CCATG-392-G	CCATG-392-GGAATTAATTCCCATGG	M (5NC-1116)
pPN-16	GAAT-443-C	GAAT-443-GGAATTCCAATC	D
pPN-17	GAATG-460-CGGC	GAATG-460-..GC	D
pPN-18	GAATG-460-CGGC	GAAT-460-..GGATTCCGC	D
pPN-19	CAG-499-TGATTG	CAG-499-...TTG	D
pPN-20	CAG-499-TGATTG	CAG-499-...GGAATTCCTTG	D

[a] D, Dead (lethal mutation); WT, wild type (silent mutation); M, mutant.

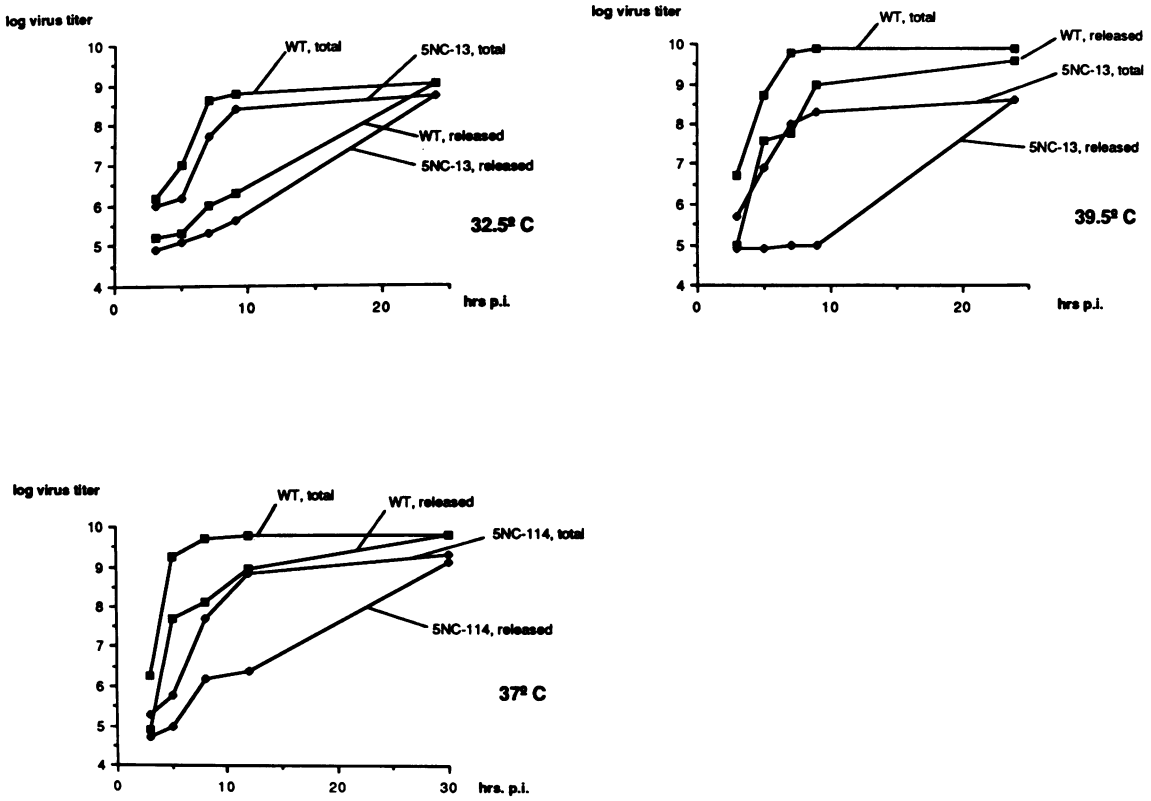

FIG. 2. One-step growth curve of 5NC mutants. Infection was done on HeLa cell monolayers as described in Materials and Methods. Virus released into the medium and total virus production was determined at each time point. (Top) 5NC-13 at 32.5°C (left) and 39.5°C (right); (bottom) 5NC-114 at 37°C. Similar patterns were obtained from 5NC-11 and 5NC-116 (data not shown).

in Materials and Methods (Fig. 3; only the result for positive-strand RNA is shown, because the ratio of positive- to negative-strand RNA was indistinguishable from that of wild type with all mutants). 5NC-11 differed strikingly from 5NC-13, 5NC-114, and 5NC-116 with regard to viral RNA synthesis; at both 32 and 39°C, 5NC-11 made less than 1% of the wild-type amount of RNA (Fig. 3A). Further work is under way to define which step of RNA synthesis is affected in this mutant; therefore, 5NC-11 will not be further described here.

5NC-13, 5NC-114, and 5NC-116 behaved grossly similarly regarding RNA synthesis, they made 30 to 60% of the wild-type level of RNA, both plus and minus strand (Fig. 3B and C). There was, however, a delay in RNA synthesis by all of the mutants; for instance, at 37°C, wild-type RNA synthesis peaked at 4 h postinfection, whereas 5NC-114 and 5NC-116 reached their maximal amounts of RNA synthesis at 6 h postinfection. Although the three mutants are somewhat defective in RNA synthesis, this is not their primary defect. A comparison of progeny virion yield and RNA synthesis makes this point (Fig. 3D). 5NC-114 (and the others similarly) made, at 6 h postinfection, about 50% of the normal yield of RNA, with a normal ratio of single- to double-stranded RNA (data not shown), but produced only 0.3% of the normal yield of progeny.

Protein synthesis in infected cells. To examine what might be the primary defect in these mutants, we analyzed the pattern of viral and cellular proteins synthesized in infected cells. For this analysis, cells were pulse labeled with [^{35}S]methionine at various times after infection and cytoplasmic extracts were fractionated by electrophoresis through SDS-polyacrylamide gels. In striking contrast to wild-type virus, 5NC-13 (at 39°C), 5NC-114, and 5NC-116 did not shut off host cell translation, even late after infection (Fig. 4A and B). This failure to induce shutoff could be partly relieved by increasing the MOI, as shown with 5NC-114 (Fig. 4C). To resolve the virus-specific proteins that were made, we immunoprecipitated the extracts with antiviral antibodies that reacted with the capsid proteins (Fig. 5). In all three mutants, virus-specific protein synthesis was markedly decreased compared with that of wild type, detectable levels appearing only several hours after infection. The processing of the virion proteins appeared, however, to be normal, and no excess of precursor accumulated at any point.

Fate of p220. Shutoff of host cell translation correlates with the cleavage of one of the components of the cap-binding complex, eucaryotic initiation factor 4F, also called p220 (2, 5; for a review, see reference 24). To examine whether our mutants failed to induce this cleavage, we performed an immunoblot analysis of infected cell cytoplasmic extracts, using a polyclonal anti-p220 antibody (Fig. 6). In wild-type virus-infected cells, the cleavage of p220 was almost completed at 3 h postinfection (at 37 or 39°C). In mutant-infected cells, only partial cleavage was seen at 6 h postinfection; at its nonpermissive condition (39°C), 5NC-13 did not induce any cleavage of p220. As already seen with host cell translational shutoff, an MOI effect was observed; when cells were infected with increasing MOIs of 5NC-114, a better, although still delayed, rate of cleavage of p220 was observed.

Genetic analysis. As demonstrated above, all three mutants we have examined here behaved similarly in the

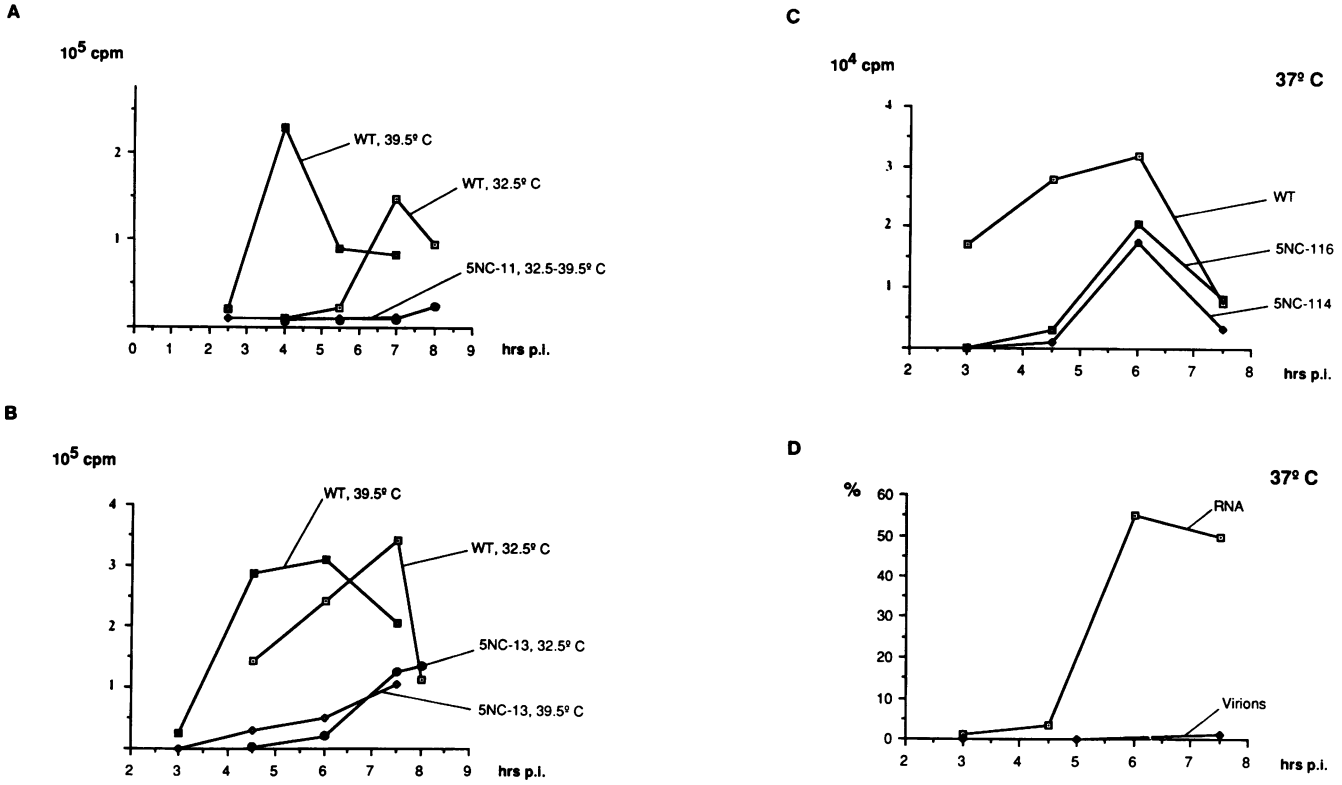

FIG. 3. RNA synthesis in virus-infected cells. Analysis was done by dot-blot as described in Materials and Methods. (A) 5NC-11 at 32.5 and 39.5°C. (B) 5NC-13 at 32.5 and 39.5°C. (C) 5NC-114 and 5NC-116 at 37°C. (D) Comparison of progeny yield (as determined by plaque assay) and RNA synthesis for 5NC-114 with that of wild type.

infected cell, showing poor viral protein synthesis, absence of host cell translational shutoff, and significant but delayed viral RNA synthesis. We could not, however, conclude from the experiments described so far what was the primary defect created by the mutations. Genetic complementation is a useful tool for identifying the effects of mutations in viruses. In such an approach, cells are infected with one virus alone or with two viruses together, and the yields of the single and mixed infections are compared. A CI is calculated from the ratio of these yields. If the growth of one of the partners is enhanced in mixed infection, it is then known to be defective in a function that can be provided in *trans*. The existence of defective interfering particles in poliovirus (8) demonstrates that the capsid proteins can be provided in *trans*. It was also shown recently that two nonstructural proteins, 2A and 3A, involved in translational shutoff and RNA synthesis, respectively, carry out a *trans* rather than a *cis* activity; other nonstructural functions (proteins 2B and 3D, 3NC region) seemingly act mainly in *cis* (1).

On the basis of these precedents, we analyzed the complementation behavior of our mutants by testing their ability to complement and be complemented by other well-defined mutants. The ones we used were (Fig. 7) 1C-41, which maps in the capsid region; 3D-56, which maps in the replicase gene; 3NC-202, which maps in the 3NC region; and 2A-1 and R2-A2-2, which map in the region encoding for the 2A protein. The latter mutants, 2A-1 and R2-2A-2 (a gift from V. R. Racaniello), both exhibit an inability to induce host cell-specific translational shutoff, make a normal amount of RNA, and produce in HeLa cells an early global translational shutoff, inhibiting both cellular and viral protein synthesis (2; V. R. Racaniello, personal communication); cleavage of p220 is also not induced in HeLa cells infected with these mutants. To some extent, therefore, they produce phenocopies of 5NC-13, 5NC-114, and 5NC-116; a major difference is that the 5NC mutants do not induce a global shutoff.

The results of the complementation experiments are best considered in three groups.

First, 5NC-13, 5NC-114, and 5NC-116 did not complement each other (Table 2, lines 1 to 3). There was no enhancement of the total progeny yield in mixedly infected cells compared with that of singly infected cells. Thus, the mutants fall in a single complementation group. When cells were infected at 39°C with 5NC-13, a strongly temperature-sensitive mutant, and 5NC-114, which does not exhibit the same degree of temperature sensitivity, the growth of 5NC-13 was enhanced but only to a level that was still a few fold lower than the growth of 5NC-114. The growth of this latter mutant was itself not enhanced. This suggested that these mutants are defective to different degrees in the same function, and that this function can be provided in *trans*; the function made by the less-defective mutant was available for use by both.

This analysis of one-way intragenic complementation was confirmed by the second type of result. The 5NC mutants were efficiently complemented by mutants 1C-41, 3D-56, and 3NC-202 (Table 2, lines 5 to 10), confirming the *trans*-acting nature of their defect. The three mutants that complemented the 5NC mutants grew very poorly if at all at 39°C, but they dramatically increased the progeny yield of the 5NC mutants in mixed infections. Therefore, they provided to the 5NC mutants a function that the 5NC mutants could not perform.

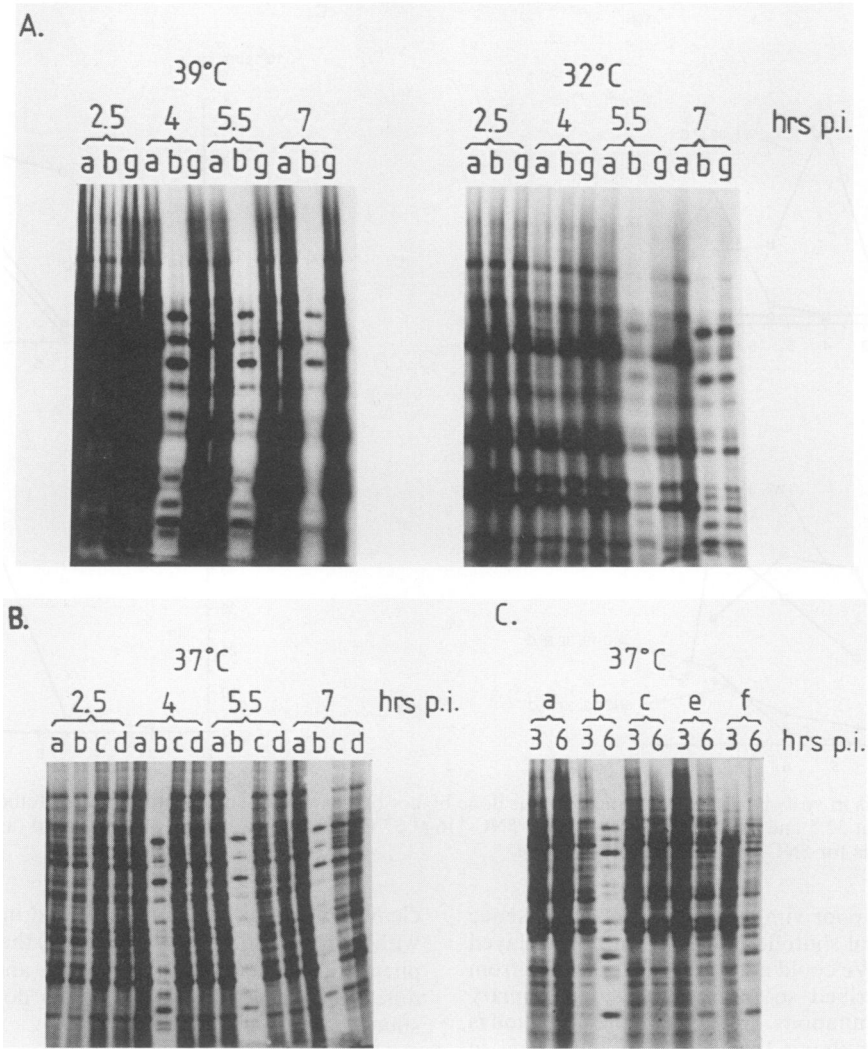

FIG. 4. Protein synthesis in virus-infected cells. Cells were pulsed at different times after infection with [^{35}S]methionine, and extracts were fractionated by electrophoresis through a 12.5% polyacrylamide gel which was then autoradiographed. Initial MOI was 10 PFU per cell unless specified. (A) 5NC-13 at 39 and 32°C. (B) 5NC-114 and 5NC-116. (C) Effect of increasing MOIs of 5NC-114. Lanes: a, Mock-infected cells; b, wild-type virus; c, 5NC-114; d, 5NC-116; e, 5NC-114 (MOI, 50 PFU per cell); f, 5NC-114 (MOI, 100 PFU per cell); g, 5NC-13.

As a control, 3NC-202 and 3D-56 did not detectably complement each other (line 4).

Third and most importantly, mutants mapping in the 2A region did not efficiently complement and were not efficiently complemented by the 5NC mutants (Table 2, lines 12, 13, 15, and 16), even though the 2A function is an easily complementable one (lines 11 and 14) (1). It appears that mutations in the 5NC and the 2A region have deleterious consequences for the same function. Again, there is some unidirectional enhancement of 5NC-13 by 2A-1, presumably by a mechanism similar to the one described earlier (see also Discussion).

These results were confirmed by analyzing [^{35}S]methionine-labeled cytoplasmic extracts obtained from singly and mixedly infected cells (Fig. 8). Compared with cells infected with a single virus, cells coinfected with 3NC-202 and one of the 5NC mutants showed an enhancement of host cell translational inhibition and of virus-specific protein synthesis (Fig. 8A, compare lanes 3, 6, 8, and 10 with lanes 7, 9, and 11; best seen by looking at protein 3CD, shown by arrow). On the other hand, in HeLa cells co-infected with

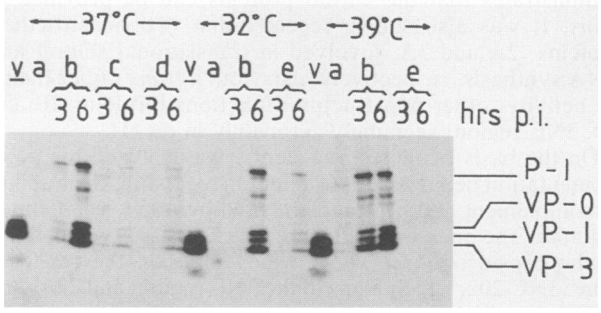

FIG. 5. Analysis of virus-specific protein synthesis. Radiolabeled cytoplasmic extracts were immunoprecipitated with a polyclonal antivirion antibody and analyzed by electrophoresis through a 12.5% polyacrylamide gel. Lanes: v, [^{35}S]methionine-radiolabeled virion; a, mock-infected cells; b, wild-type virus; c, 5NC-114; d, 5NC-116; e, 5NC-13. The designation of the corresponding protein is indicated on the right.

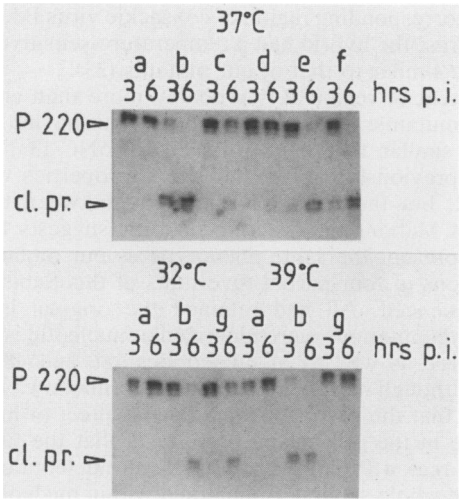

FIG. 6. Fate of p220 in mock-, wild type-, and mutant-infected cells. HeLa cells were infected at an MOI of 10 unless otherwise specified. Cytoplasmic extracts harvested at 3 or 6 h postinfection were electrophoresed through a 6.5% polyacrylamide gel, and the resolved proteins were transferred to nitrocellulose. The filters were probed with a polyclonal anti-p220 antiserum. Lanes: a, mock-infected cells; b, wild-type virus; c, 5NC-114; d, 5NC-116; e, 5NC-114 (MOI, 50 PFU per cell); f, 5NC-114 (MOI, 100 PFU per cell); g, 5NC-13. cl. pr., p220 cleavage products.

TABLE 2. Complementation experiments

Line no.	Virus pair[a]	CI at 3 h PI[b]	CI at 6 h PI
1	5NC-13 + 5NC-114	1 (total) 1 (5NC-114) 15 (5NC-13)	1 (total) 1 (5NC-114) 6 (5NC-13)
2	5NC-114 + 5NC-116	1.2 (total	1 (total)
3	5NC-13 + 5NC-116	1 (total)	1.5 (total)
4	3NC-202 + 3D-56	1.3	1
5	**5NC-13** + 3NC-202	120	140
6	**5NC-114** + 3NC-202	150	43
7	**5NC-13** + 3D-56	180	50
8	**5NC-114** + 3D-56	40	20
9	**5NC-13** + 1C-41	90	ND[c]
10	**5NC-114** + 1C-41	40	ND
11	3NC-202 + **2A-1**[d]	50	ND
12	5NC-13 + 2A-1[d]	1.2 (total) 20 (5NC-13)	ND
13	5NC-114 + 2A-1[d]	2 (total)	ND
14	3D-56 + **R2-2A-2**	40	ND
15	5NC-13 + R2-2A-2	2 (total)	ND
16	5NC-114 + R2-2A-2	4 (total)	ND

[a] The mutant complemented is in bold type.
[b] CI was calculated as described in Materials and Methods. PI, Postinfection.
[c] ND, Not determined.
[d] Done on CV1 cells.

2A-1 and one of the 5NC mutants, viral protein synthesis was not enhanced, specific shutoff was not observed, and instead a global inhibition of translation, both viral and cellular, took place as in cells infected with 2A-1 alone (Fig. 8B, compare lane 3 with lanes 7, 8, and 9). This last point makes a strong argument that the 5NC mutants are defective in providing the 2A function; if it were not the case, then they should have been able to prevent an event thought to be a consequence of the absence of a functional 2A (24; see Discussion).

DISCUSSION

We used a cDNA copy of poliovirus type 1 to generate 21 mutations in the 5NC region of the genome. RNA was made from each of these clones, transfected into HeLa cells, and scored for the ability to generate infectious virus particles. Some mutations, distributed evenly over much of the 5NC region, were lethal, meaning that no infectious virus was recovered from transfected cells. Much of the 5NC region appears therefore necessary to viral growth. Several mutations were silent, including a deletion extending from nucleotide 630 to 723. Thus some regions can be altered without significantly inhibiting viral growth, and one region is dispensable for in vitro growth. Six mutations, targeting four different sites, generated viruses with a recognizable phenotype; one mutant from each of these four sites was further studied. Mutant 5NC-11, which has a 4-base insertion at nucleotide 70, was primarily deficient in RNA synthesis; work is in progress to define more precisely which step of RNA synthesis is impaired. A mutant with a deletion of nucleotide 10 was reported previously (20); it also showed a major impairment of RNA synthesis, suggesting that the far 5' end of the genome is likely to be primarily involved in one or more steps of RNA replication.

The three other mutants studied in some detail, 5NC-13, 5NC-114, and 5NC-116, mapping at positions 224, 270, and 392, respectively, showed similar behavior; they made very little viral protein, they did not inhibit host cell translation, and they synthesized a significant amount of viral RNA, although with some delay compared with the wild type. This defect in RNA synthesis was not reflected in any change in the type of RNA made and was probably due to their deficiency in protein synthesis.

To further analyze the nature of the three mutants defective in protein synthesis, we examined their complementation by other poliovirus mutants. Although pairwise infections among the 5NC mutants gave no overall yield enhancement, they were efficiently complemented by all tested mutants, except those with lesions in protein 2A. A direct interpretation would be that the three 5NC mutants lack a single function that can be complemented in *trans* by all cistrons other than 2A. We will call the sequence within which these mutants fall region P, to denote its role in

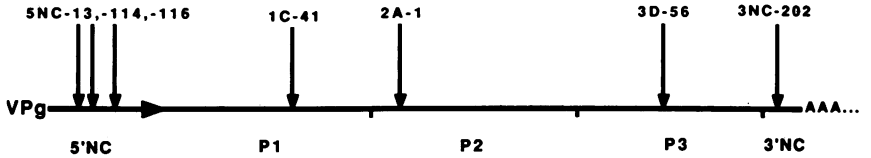

FIG. 7. Mutants used in complementation experiments. The approximate genomic location of the mutants described in the text is shown. R2-2A-2 contains a mutation mapping close to the one in 2A-1.

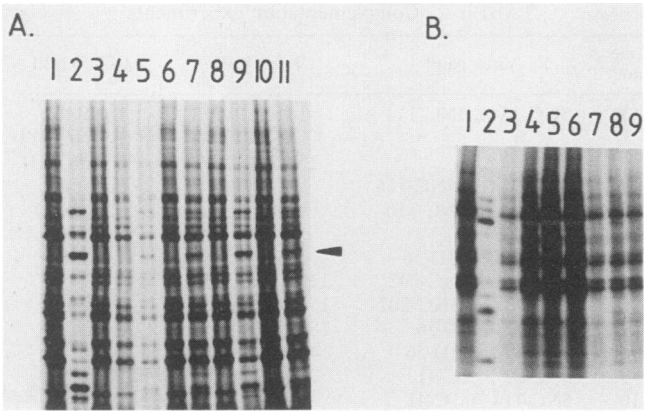

FIG. 8. Complementation experiments. [^{35}S]methionine-labeled cytoplasmic extracts were prepared at 6 h postinfection from singly and mixedly infected cells and analyzed as described in the text. The mutants used are those shown in Fig. 7. (A) Complementation of 5NC mutants by a 3NC mutant. Lanes: 1, mock-infected cells; 2, wild type; 3, 3NC-202; 4, 2A-1; 5, 3NC-202 + 2A-1; 6, 5NC-13; 7, 5NC-13 + 3NC-202; 8, 5NC-114; 9, 5NC-114 + 3NC-202; 10, 5NC-116; 11, 5NC-116 + 3NC-202. The arrow shows protein 3CD. (B) Absence of complementation of 5NC mutants by a 2A mutant. Lanes: 1, mock-infected cells; 2, wild type; 3, 2A-1; 4, 5NC-13; 5, 5NC-114; 6, 5NC-116; 7, 5NC-13 + 2A-1; 8, 5NC-114 + 2A-1; 9, 5NC-116 + 2A-1.

protein synthesis. The lack of complementation by 2A mutants highlights the role of region P because 2A is a function easily complemented by all mutants other than the 5NC mutants (1). Knowing that region P is involved in allowing translation of the viral RNA, it would appear that the 5NC mutants make so little of protein 2A that it becomes the limiting factor in the efficiency of viral infection. Thus, all mutants that can provide any 2A, that is, all mutants but those in 2A, can complement 5NC mutants. The extra 2A in co-infected cells inhibits host cell protein synthesis, a process that 5NC mutants are unable to carry out because they make too little 2A by themselves.

We interpret the effect of providing 2A to region P mutants as follows. The cellular ribosomes, no longer attracted to cell mRNA when 2A function is provided, can better translate poliovirus RNA with region P lesions, and thus the mutants grow much more effectively. Because the region P mutants make significant yields of RNA without complementation, their particle yield is presumed to be a direct function of the amount of capsid protein they make, and thus any increase of viral protein synthesis causes a yield enhancement and is scored as complementation. Only 2A mutants will not produce the effect. Even they complement a bit, presumably because they make totally normal RNA yields and thus provide a large pool of translatable RNA, although without inhibition of host cell mRNA translation, this viral RNA has a very limited translational potential.

The mutants in region P are not null mutants, they grow and therefore exhibit partial function. The dead mutants in region P, as represented in Fig. 2, we believe are true null mutants, and we suggest that region P is absolutely required for significant translation to occur.

Unitary function of region P and its role in neurovirulence. The three mutants we found in region P clearly participate in the same function; their physiology is almost indistinguishable, and they do not complement each other. They span nucleotides 224 to 392. Consistent with this interpretation is the result of substituting nucleotides 220 to 670 of poliovirus with the corresponding region of coxsackie virus B3, another picornavirus; the hybrid had a temperature-sensitive phenotype quite similar to that of our mutants (23).

The extent of region P is probably more than is evident from our mutants. A mutant of Sabin 1 virus having a 4-base insertion similar to the one present in 5NC-13 has been reported previously (10); its biological properties were not described, but the degree of similarity between the 5NC regions of Mahoney and Sabin 1 strains suggests that it is likely to present the same phenotype as our mutants. The 5NC regions of four partial revertants of this Sabin mutant were sequenced. All had retained the original insertion; second-site mutations were identified at nucleotides 186 and 525 in three as well as at nucleotides 186 and 480 in the fourth. Although no mix-and-match experiment was done to ascertain that the reversion was not the effect of mutations elsewhere in the genome, this suggests that the functional extent of region P may be at least from nucleotides 186 to 525. If the whole sequence contained within nucleotides 186 to 525 belongs to the same functional unit, it will include nucleotides that have been found to be major determinants of the attenuation of type 3 (27) and, to a lesser extent, of type 1 (15) neurovirulence. This would suggest that attenuation in vaccine strains might come from a specific inability of motor neurons, as opposed to intestinal cells, to translate the viral genome. It has actually been shown that vaccine strains translate more poorly than their wild-type parents, at least in some in vitro systems (25). If such a model is true, the 5NC region of poliovirus could be used as a target to engineer live-virus vaccines with full immunogenic properties but better stability than the ones currently in use.

Function of region P. Our data does not directly suggest a function for region P beyond the clear demonstration of some role in viral protein synthesis. Using an in vitro approach, Pelletier et al. (17) have obtained data also consistent with an important role for this region in protein synthesis. It is easiest to imagine a *cis*-acting role, for instance providing what is usually a cap-dependent entry site for ribosomes on the mRNA. Although all of our data is consistent with such a *cis*-acting function, we also cannot rule out a *trans*-acting process like ribosome modification. The lack of complementation by 2A mutant argues against a *trans*-active function but is not conclusive.

Viral protein needed for RNA synthesis. The data here emphasize that very little viral protein is needed for viral RNA synthesis to occur. The region P mutants, making very little viral protein (perhaps 2% of normal), still make about 50% of the normal yield of RNA, albeit somewhat more slowly than wild-type virus. Our previous analysis of mutant 2A-1 suggested that viral RNA synthesis requires little viral protein (2), but the data here are even more compelling. This conclusion is reasonable in that in virions each RNA molecule is coated by 60 copies of the capsid proteins and therefore RNA synthesis could require only 1/60 of the amount of protein needed for maximal particle yield. Thus, in adopting a genetic strategy using only one open reading frame for translation of viral RNA, poliovirus never found the need for the inevitable huge excess of RNA synthetic proteins made to satisfy the need for capsid proteins.

Viral protein needed for host cell translational inhibition. The results of our experiments show that more viral protein is needed to inhibit host cell translation than to synthesize viral RNA; the 5NC mutants do not induce this inhibition because they fail to produce enough protein 2A. This defect in 2A is also underscored by the inability of the 5NC mutants to prevent the early global inhibition of translation induced

by 2A mutants in HeLa cells. There are suggestions that this global inhibition is due to the phosphorylation of eucaryotic initiation factor 2, a phenomenon observed with other viruses; the 2A protein might directly or indirectly prevent it (24). Probably due to a delayed accumulation of viral RNA, the 5NC mutants do not induce this global shutoff; however, in mixed infection, when this global shutoff is induced by a 2A mutant, they are unable to prevent it.

5NC nucleotides in cellular mRNAs. The existence of a long 5' untranslated element in poliovirus mRNA raises the issue of whether cellular mRNAs might not have such 5' structural modules. They might also use them in initiating protein synthesis. In favor of such a suggestion is the presence and interspecies conservation of long 5' ends on certain cellular mRNAs. If, however, such structural modules served in the place of 5' caps as initiators of protein synthesis, then there should be a class of cellular mRNAs that continues to be translated in poliovirus-infected cells. Such a class has not been detected, although one mRNA is less sensitive than others (that for grp78; P. Sarnow and D. Baltimore, unpublished data) and rare mRNAs might not have been evident.

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