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Genetic Complementation among Poliovirus Mutants Derived from an Infectious cDNA Clone

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We constructed several well-defined mutations in the nonstructural portion of the poliovirus type I (Mahoney strain) genome by making small insertions in an infectious cDNA clone. The derived viral strains carrying the mutations exhibited a variety of distinct plaque phenotypes. Thus, we were able to examine genetic complementation between different pairs of mutants by comparing the yields of progeny virus in mixed and single infections. Two mutants bearing lesions in the 2A and 3A regions of the genome, which are defective in the inhibition of host cell translation and the synthesis of viral RNA, respectively, could be rescued efficiently by genetic complementation; three replication-deficient mutants containing insertions in the 2B, 3D (replicase), and 3′-untranslated regions could not. Both the 2A and 3A mutants could be rescued by each other and by all of the other mutants tested. Because yield enhancement was apparent well before the completion of a single infectious cycle, it is likely that complementation of both mutants involved early diffusion of functional products. These data provide the first unambiguous evidence that the nonstructural portion of the poliovirus genome contains multiple complementation groups. The data also suggest that certain nonstructural functions act only in *cis*.

There is a long history of genetic study of picornaviruses by means of temperature-sensitive mutants obtained by treating infected cells with chemical mutagens (for a review, see reference 11). These mutants have been thought to cover the entire genetic map of each virus. In general, however, it has been difficult to demonstrate significant genetic complementation between pairs of these mutants by comparing yields of progeny virus in mixed and single infections (7, 9, 15). A modest but reproducible enhancement of yield at high temperature has been reported for two such mutants, but only in mixed infections with a particular partner (1, 9). There are several possible explanations for the overall lack of complementation among these mutants. First, it could be that because the picornavirus genome is translated as a single polyprotein, defects which affect the structure of one segment of the polyprotein alter the structure of multiple viral polypeptides (11). A similar explanation is that multiple segments of the polyprotein fold together into a complex before processing, such that substitution of a faulty cleavage product cannot occur. Even if the various cleavage products of the polyprotein fold separately, they might associate rapidly and not freely dissociate. There is substantial evidence that the individual capsid proteins are not released as independent polypeptides after cleavage from the capsid precursor (reviewed in reference 22); hence, it would be predicted that capsid mutants cannot complement each other. Furthermore, it is also possible that some events in the viral life cycle occur in isolated structures or compartments, such that there is not free mixing of products translated from different RNA molecules. An equivalent situation would be obtained if each newly synthesized polyprotein (or segments of each polyprotein) binds immediately to the RNA molecule from which it has been translated and functions in association with that RNA.

Complementation has also been studied in experiments on defective interfering particles and phenotypic mixing. The existence of defective interfering particles containing large deletions in the capsid region of their genomes and requiring a source of wild-type capsid proteins for growth (8) implies that the structural proteins can be provided in *trans*. This finding is supported by repeated demonstrations of free mixing of structural subunits in mixed infections with serologically distinct enteroviruses (12, 16, 29). In these cases it is likely that the entire capsid precursor, rather than individual capsid proteins, was supplied (11). The inability of investigators to find defective interfering particles with deletions outside of the capsid region is consistent with the suggestion raised by the above complementation experiments that the nonstructural functions act in *cis* or at least act effectively in *cis* during the early phase of the replicative cycle (17).

Although these genetic experiments have raised a number of questions about the picornavirus life cycle, the reliability of the results obtained with the temperature-sensitive mutants is unclear. Specific nucleotide sequence changes have never been correlated with specific defects in any of the mutants. It is not at all clear that the phenotype of these mutants can be attributed to a single lesion. In fact, there is evidence that at least some of the mutants contain multiple defects (5, 10, 15). The presence of multiple mutations might help to explain why, even though many studies have shown clearly that capsid proteins can be supplied in *trans*, complementation of a temperature-sensitive mutant which contains structural defects has never been reported.

The construction of an infectious cDNA copy of poliovirus (23) has now made it possible to obtain mutants that can be precisely defined and then used to determine which viral functions can be complemented. Moreover, because it is possible to make different types of specific alterations in the viral sequence and then to examine the phenotypes of the resulting mutants, it should be possible to isolate mutants with a variety of different plaque phenotypes (e.g., temper-

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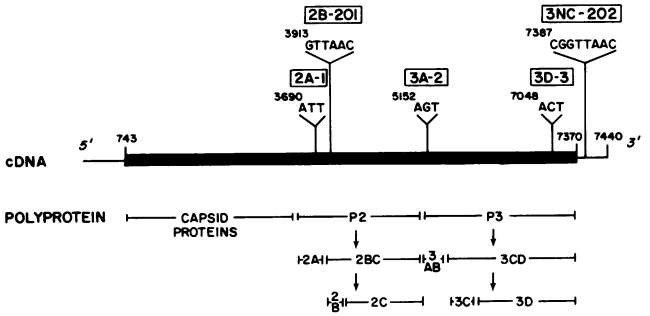

FIG. 1. Sequence of mutants used in this study. Mutants were generated by making small insertions at restriction endonuclease recognition sites of pSV2-polio, a derivative of the mammalian expression vector pSV2 (19) which contains an infectious type I (Mahoney strain) poliovirus cDNA insert. The lesions were mapped by restriction enzyme digestion, and the sequence of a fragment which contains the mutation was determined by the method of Maxam and Gilbert (18). The genomic location of each lesion is shown.

ature sensitive, cold sensitive, host range). Mixed infections can be performed with phenotypically distinct pairs of mutants, and the progeny can be distinguished by plaque assay. We obtained poliovirus mutants which display a variety of phenotypes by making small insertions in the infectious cDNA. Complementation experiments with these mutants provide direct evidence that some, though apparently not all, of the nonstructural functions of the virus can be provided in *trans*.

MATERIALS AND METHODS

DNA procedures. The plasmid from which all mutants were derived, pSV2-polio, was obtained from V. Racaniello. The cloning procedures used to construct the mutants and the transfection procedures used to obtain virus have been described previously (6, 24).

Cells and viruses. CV-1 cells (African green monkey kidney cells) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Hazleton). HeLa cells were grown in suspension in the Joklik modification of minimal essential medium supplemented with 7% horse serum (GIBCO Laboratories).

All of the master virus stocks used (except the 2A-1 stock) were produced by isolating a single plaque from a pSV2-polio transfection and expanding it on HeLa cells by conventional means. The 2A-1 stock was obtained as described in a previous publication (6), in which it was designated HF121.

Mutant nomenclature. We have adopted a systematic nomenclature for poliovirus mutants. Mutants are designated by the ultimate cleavage product or noncoding region in which their lesion resides (e.g., 2A indicates an alteration in protein P2-2A, and 3NC indicates a mutation in the 3'-untranslated region) and by an arbitrary cardinal number (1, 2, 3, 100, etc.). Hence, 2A-1 is a mutant which contains a mutation in the region of the genome which codes for protein 2A and which has been assigned the number 1. In publications in which mutants from other laboratories are used, we will use the prefix B for our mutants; when mutants of different strains of poliovirus are used we will affix a 1 (for type 1) before the laboratory designation. The full name of 2A-1 will then be 1B-2A-1.

Virus infections and measurement of progeny virus yields. CV-1 infections were performed in 60-mm (diameter) petri dishes as previously described (6), except that after adsorption cells were washed three times to remove excess virus. HeLa cell infections were also performed as previously described (6), but after adsorption cells were collected by centrifugation and washed. The incubation temperature after adsorption was that at which the growth of all mutants was restricted.

At various times after infection, we harvested cells to measure the yield of progeny virus. CV-1 cells were placed on ice, scraped into the medium with a rubber policeman, and then frozen. A portion of each infected HeLa cell culture (100 μl containing 3×10^5 cells) was added to 1 ml of cold minimal essential medium and then frozen. These samples were subjected to three freeze-thaw cycles, and dilutions were prepared so that virus titers could be determined by plaque assay.

The complementation index (CI) was defined as follows. For plaque size mutants (i.e., 2A-1, 2B-201, and 3D-3), CI for mutant A = (titer of mutant A in mixed infection)/(titer of mutant A in single infection), where the titers are measured at the same temperature. For temperature-dependent mutants (i.e., 3A-1 and 3NC-202), CI for mutant A = (titer of mutant A in mixed infection)/(virus yield in single infection at permissive temperature − yield at nonpermissive temperature), where the titer of mutant A in the mixed infection is determined by counting plaques arising at the temperature which is permissive for the growth of that mutant. Because the background of apparent revertants (i.e., variants which grew at all temperatures) often represented a very significant fraction of the total virus obtained in single infections, it was necessary to subtract this value from the denominator to assess the level of complementation accurately.

RESULTS

Description of mutants used in this study. Three of the mutants used in this study, 2A-1 (formerly designated HF121), 3A-2, and 3D-3, were generated by a method which has been described previously (6), in which *Hin*fI sites in the plasmid pSV2-polio were filled in with DNA polymerase to create single-codon insertions. By restriction digest analysis, the mutations were found to reside in regions which encode the 2A, 3A, and Pol (3D) portions of the polyprotein, respectively (see Fig. 1). The other two mutants were constructed by insertion of synthetic DNA linkers into restriction sites. Mutant 2B-201 was generated by linearization of pSV2-polio by partial digestion with the restriction endonuclease *Hin*cII and subsequent ligation of synthetic *Hpa*I linkers containing the sequence GTTAAC. This mutant contains two extra amino acids in the 2B region of the polyprotein (see Fig. 1). The construction of 3NC-202 (formerly designated PTH7387), a mutant which contains a lesion in the 3'-untranslated region, has been described previously in detail (24). Briefly, a *Taq*I site was filled in with DNA polymerase, and *Hpa*I linkers were attached to create a net insertion of eight base pairs (Fig. 1). To show that the insertion, rather than an accidental second alteration in the DNA, was responsible for the phenotype of each mutant, we exchanged a restriction fragment of mutant DNA which contained the insertion with the equivalent wild-type fragment. We then showed that only those clones which contained the insertion gave rise to the mutant phenotype. In addition, we checked the nucleotide sequence of the mutant restriction fragment and showed that the only alteration was the insertion (data not shown).

Each of the mutants used in these experiments has a unique plaque phenotype and distinct physiological proper-

ties (Table 1). Mutant 2A-1 has been described previously in detail (6). This mutant makes minute plaques at all temperatures on monolayers of monkey kidney (CV-1) and HeLa cells. The plaques are so small that they can not be clearly visualized until the day following that on which wild-type and other mutant plaques are ordinarily scored. Mutant 2A-1 lacks the ability to mediate the selective inhibition of host cell translation which normally occurs in the first few hours after infection. An apparent consequence of this defect is the synthesis of only a very small amount of viral protein. Although enough protein is made in CV-1 cells to allow a normal course of RNA synthesis, the production of progeny virus is very low. In HeLa cells, where less viral RNA is made, the production of progeny virus is even lower.

Mutant 2B-201 produces small plaques at all temperatures on both HeLa and CV-1 cells. Although the plaques are considerably smaller than those produced by wild-type virus, if scored at times after wild-type plaques have reached a moderate size, they ordinarily increase only in size and not in number. This mutant produces only about 10% as much RNA as wild-type virus. Viral protein synthesis is reduced to approximately the same extent as viral RNA synthesis, but the cleavage of the polyprotein appears to be normal. In addition, the number of infectious particles produced in a single cycle of infection is reduced about 10-fold (Sarnow and Baltimore, unpublished data).

Mutant 3A-2 contains an insertion in a region of the polyprotein which has been proposed to have a role in the initiation of poliovirus RNA synthesis (4, 26). This mutant is cold sensitive in monkey kidney cells. Nearly wild-type size plaques are produced at 39.5°C, whereas only small plaques, reduced in number at least 100-fold, are made at 32.5°C. At the nonpermissive temperature, RNA synthesis is poor. As expected, very little protein is synthesized, and the yield of particles in a single cycle of infection is at least several hundredfold below the wild-type level (Bernstein and Baltimore, unpublished data).

Mutant 3D-3 produce plaques which are somewhat smaller than wild-type plaques at all temperatures on CV-1 cell monolayers. In cells infected by this mutant, viral RNA synthesis reaches normal levels but is somewhat delayed. (At 39.5°C, only about one-third to one-half as much RNA is made, but this result may be due to activation of a nuclease at high temperature at a time after wild-type RNA synthesis is complete [13].) Protein synthesis and particle formation appear to be normal, but because of the delay in RNA synthesis the number of virions produced by the middle of an infectious cycle can be as much as 100-fold below the wild-type level. In some experiments, we used a temperature-sensitive variant of 3D-3 (3D-3*ts*) which arose sponta-

TABLE 1. Phenotypes of mutants used in this study

Mutant designation	Plaque phenotype	Description of phenotype
2A-1	Minute plaque	At all temperatures, mutant plaques clearly visible only 1 day after all other plaques scored
2B-201	Small plaque	At all temperatures, mutant plaques much smaller than wild-type plaques but scored at same time
3A-2	Cold sensitive	At 39.5°C, mutant plaques somewhat smaller than wild-type plaques; at 32.5°C, >100-fold reduction in number of plaques[a]
3D-3	Small plaque[b]	At all temperatures, mutant plaques somewhat smaller than wild-type plaques
3D-3*ts*	Temperature-sensitive variant of 3D-3	At 32.5°C, mutant plaques somewhat smaller than wild-type plaques; at 39.5°C, up to fivefold reduction in number of plaques—mutant plaques much smaller than wild-type plaques but scored at same time
3NC-202	Temperature sensitive	At 32.5°C, mutant plaques somewhat smaller than wild-type plaques; at 39.5°C, >1,000-fold reduction in number of plaques

[a] Observed on monkey kidney cells; cold sensitivity was much less marked on HeLa cells.

[b] Phenotype observed only on monkey kidney cells.

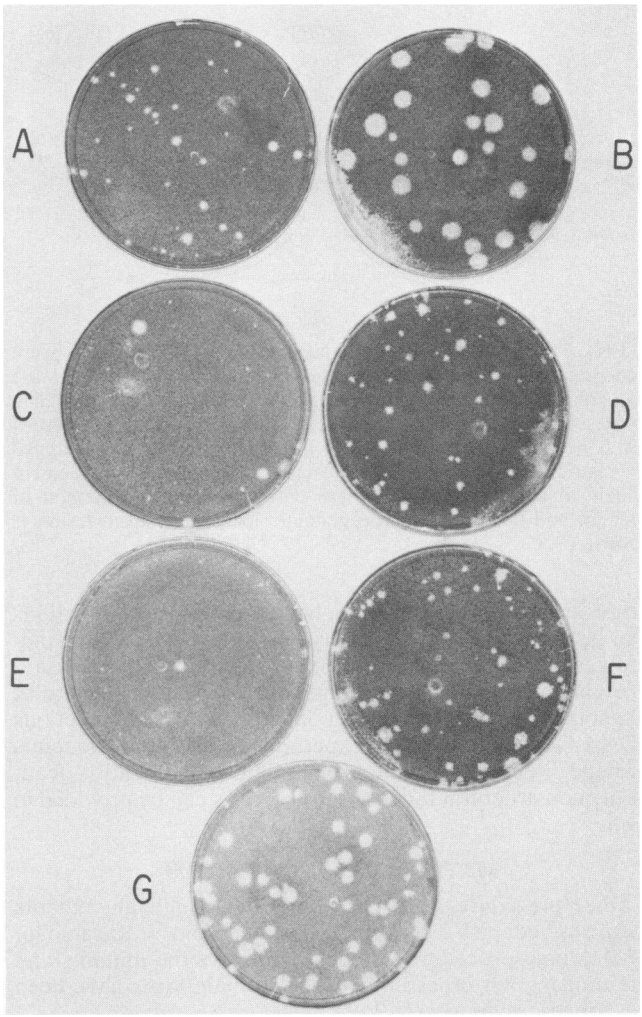

FIG. 2. Distinction of mutants by plaque phenotype. HeLa cells were infected at an MOI of 5 with wild-type virus, 2B-201, 2A-1, or a mixture of the two mutants, each at an MOI of 5, and incubated at 37°C. Titers of virus stocks made from infected cells were determined by plaque assay at the same temperature. Duplicate dishes of HeLa cells were stained approximately 40 h (day 2) or 65 h (day 3) after inoculation with virus. (A) 2B-201, day 2; (B) 2B-201, day 3; (C) 2A-1, day 2; (D) 2A-1, day 3; (E) mixed, day 2; (F) mixed, day 3; (G) wild type, day 2. The mixed stock contained primarily 2A-1 virus, as indicated by the appearance of most of the plaques on day 3 and as confirmed by analysis of the phenotype of the virus contained in the plaques. (The plaques in C were apparent revertants.)

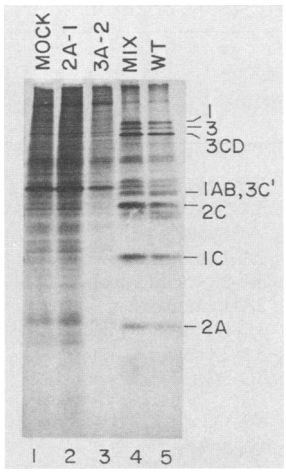

FIG. 3. Pattern of protein synthesis in singly and doubly infected CV-1 cells at 32.5°C. CV-1 cells were infected at an MOI of 10 with a single virus strain or with a mixture of 2A-1 and 3A-2, each at an MOI of 10. At 5 h postinfection, cells were pulse-labeled for 25 min with [^{35}S]methionine. Labeling, preparation of cell extracts, and analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed essentially as described previously (6). Lanes: 1, mock-infected cells; 2, 2A-1-infected cells; 3, 3A-2-infected cells; 4, cells doubly infected with 2A-1 and 3A-1 (MIX); 5, wild-type (WT)-infected cells.

neously during the production of a 3D-3 stock. This variant has the same physiology as its parent, except that at high temperatures a second (apparently unrelated) lesion affects the production of infectious particles and consequently the size and number of plaques.

Mutant 3NC-202 is a highly temperature-sensitive mutant on all cell types; it produces almost no RNA and 10^{-3} to 10^{-4} of the wild-type yield of infectious particles in a single cycle of infection at 39.5°C (24). Most of the plaques generated by this mutant at the nonpermissive temperature are apparent revertants (presumably generated by second-site mutations). Some of the normal cleavages of the polyprotein are not made because of the inability of the virus to replicate and because of the reduction in the level of virally encoded proteases (Sarnow and Baltimore, manuscript in preparation).

The number of wild-type viral particles produced in a single cycle of infection was not significantly reduced when CV-1 cells were coinfected with wild-type virus and 2A-1, 3A-2, or 3D-3, even if eight times more mutant than wild-type virus was added. In addition, a wild-type, and not a mutant, level of viral RNA was produced in HeLa cells infected with equal amounts of wild-type and 2A-1 virus (data not shown). These results indicate that the lesions in these three mutants are not dominant.

Detection of genetic complementation between mutants. In the experiments described here, the principal assay for the rescue of one mutant by another depends on the ability to distinguish plaques of the two mutants. The distinctive plaque phenotypes of the mutants which we constructed enabled us to perform mixed infections of two types. First, we could doubly infect cells with mutants which produce plaques of clearly different sizes. Second, we could infect cells with a mutant which grows well only at one temperature along with another which has a defined plaque size at all temperatures. An example of results obtained from the first type of experiment is shown in Fig. 2. On HeLa cell monolayers, plaques of mutant 2B-201 appeared on day 2 after infection (Fig. 2A; compare with the wild type in 2G) and increased in size (but not number) by day 3 (Fig. 2B). With the exception of variants of the mutant which behaved like wild-type virus (here designated revertants, although they are probably the result of second-site mutations), 2A-1 plaques were not visible on day 2 but became apparent only on day 3 (Fig. 2C and D). Examination of the progeny of a mixed infection with the two mutants showed that the titer of virus yielding visible plaques on day 2 was not greater than that produced in the 2B-201 infection (Fig. 2E; the virus dilution was 4-fold greater than in 2A), but approximately 60-fold more virus yielding plaques on day 3 was produced than in the 2A-1 infection (Fig. 2F; the virus dilution was 40-fold greater than in 2D). We confirmed that most of the plaques produced by mixed stocks which appeared on day 2 were 2B-201 plaques, whereas most that appeared on day 3 were 2A-1 plaques, by showing that when the virus con-

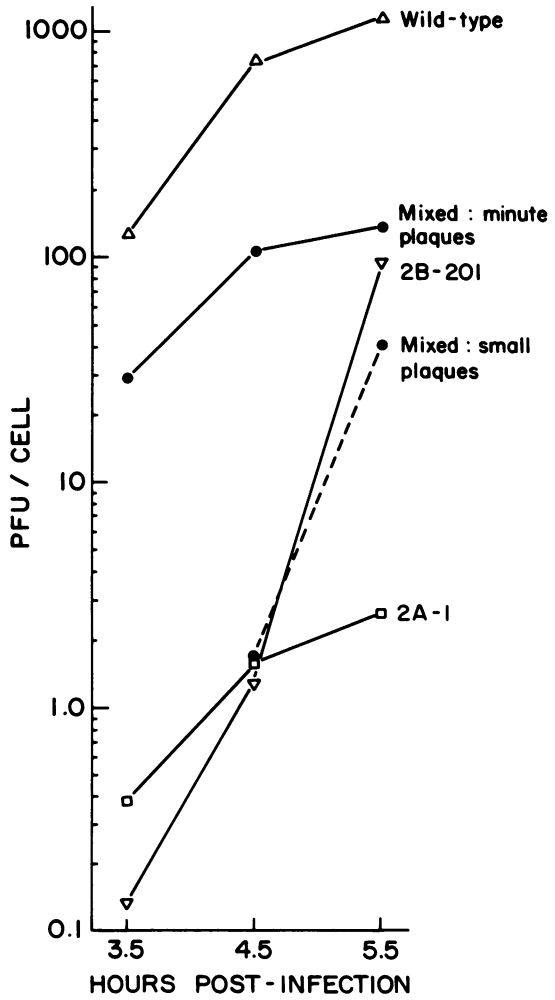

FIG. 4. Complementation test between mutants 2A-1 and 2B-201. Virus titers of stocks made at 3.5, 4.5, and 5.5 h postinfection in the experiment described in the legend to Fig. 2 are shown. We determined the 2B-201 titer in the mixed stock by counting plaques which appeared on day 2 after inoculation and measured the 2A-1 titer by counting only the minute plaques which appeared on day 3. The titer of 2B-201 virus in the 3.5-h mixed stock could not be accurately determined because of the background created by the much greater number of 2A-1 particles.

TABLE 2. Complementation tests between mutants 3A-2 and 2A-1, 3D-3*ts*, or 2B-201[a]

Virus stock	Titer in PFU/cell (CI)[b]				Plaque type
	6 h postinfection		8 h postinfection		
	39.5°C	32.5°C	39.5°C	32.5°C	
Expt 1					
Wild type	35	40	220	260	Wild type
3A-2	0.071	0.012	0.87	0.57	3A-2 + variants (39.5°C); variants (32.5°C) (<wild type)[c]
2A-1	0.34	0.14	3.0	1.5	2A-1 (minute)[d]
3A-2 + 2A-1	3.2 (54)		19 (63)		3A-2 (<wild type)
		9.0 (64)		78 (52)	2A-1 (minute)
3D-3*ts*	1.4	4.8	26	100	3D-3*ts* (small[e] [39.5°C]; <wild type [32.5°C])
3A-2 + 3D-3*ts*	2.4 (41)		23 (77)		3A-2 (<wild type)
		4.5		62	3D-3*ts* (<wild type)
Expt 2					
Wild type	34	29	150[f]	150[f]	Wild type
3A-2	0.090	0.046	0.88[f]	0.60[f]	3A-2 and variants (<wild type)
2A-1	0.29	0.12	1.8[f]	0.82[f]	2A-1 (minute)
3A-2 + 2A-1	3.6 (82)		15 (54)[f]		3A-2 (<wild type)
		7.5 (63)		34 (41)[f]	2A-1 (minute)
2B-201	0.10	0.096	5.8[f]	4.7[f]	2B-201 (small)
3A-2 + 2B-201	0.68 (15)		6.4 (23)[f]		3A-2 (<wild type)
		0.36 (3.8)		5.0 (1.1)[f]	2B-201 (small)

[a] CV-1 cells were infected with wild-type virus or a mutant at an MOI of 5 or with a mixture of two mutants, each at an MOI of 5, and then incubated at 32.5°C. Cells were harvested at various times after infection, and virus stocks were made as described in Materials and Methods.
[b] The CI values (shown in parentheses) were calculated as described in Materials and Methods.
[c] Intermediate-size plaques which were somewhat smaller than those produced by wild-type virus.
[d] Extremely small plaques which were clearly visible only 1 day after all others were scored.
[e] Plaques much smaller than those of wild-type virus but scored at the same time as wild-type plaques.
[f] At 7.5 h postinfection.

tained in the plaques was replated on a fresh monolayer, it had the expected mutant phenotype (data not shown). Moreover, because no more than 2 to 3% of the total virus produced in the mixed infection in this and all other experiments described here was phenotypically wild-type virus (i.e., revertant or recombinant virus), it was clear that recombination between mutants did not significantly contribute to virus yield.

Rescue of one mutant by another, as measured by enhancement of virus yield, was strongly correlated with physiological differences between singly and doubly infected cells. Under conditions in which the mutants described here grew poorly, induction of the dramatic morphological changes in adherent cells which normally precede cell death (the cytopathic effect) was greatly delayed. When either one or both mutants in a mixed infection were rescued, the cytopathic effect proceeded at a rate which was similar to that seen after infection with wild-type virus. Rescue of mutants also correlated with changes in the pattern of macromolecular biosynthesis. An example is shown in Fig. 3, which illustrates protein synthesis in infected CV-1 cells at the onset of the linear phase of RNA replication. Because mutant 2A-1 failed to inhibit host cell translation, the pattern of proteins produced 5 h postinfection at 32.5°C was very similar to that seen in mock-infected cells (Fig. 3, lanes 1 and 2). Mutant 3A-2, on the other hand, produced very little viral protein because it synthesized RNA poorly at this temperature; nevertheless sufficient protein was made to effect inhibition of host cell translation (Fig. 3, lane 3). When cells were infected with the two mutants together, however, the pattern of protein synthesis was very similar to that observed in wild-type-infected cells (Fig. 3, compare lanes 4 and 5). Host cell translation was inhibited, and a high level of virus-specific protein was produced. It should be emphasized that although these physiological changes correlated with virus rescue, they gave no indication as to which mutant had been rescued.

Rescue of mutant 2A-1 by other cDNA-derived mutants. By taking advantage of the observation that 2A-1 plaques appear unusually late after infection and that the virus grows at all temperatures, whereas some other mutants do not, we found that the titer of mutant 2A-1 increased dramatically when either HeLa or CV-1 cells were coinfected with any other cDNA-derived mutant. Figure 4 shows the complete data of the experiment from which the results displayed in Fig. 2 were taken. HeLa cells were infected with wild-type virus, 2A-1, 2B-201, or a mixture of the two mutants. The titer of 2A-1 stocks made at different times after infection was several hundredfold below that of wild-type virus. In a mixed infection with 2B-201, however, an increase in titer of 50- to 100-fold was apparent both at midcycle (3.5 h postinfection), when significant accumulation of progeny virions is first observed (3), and toward the end of the infectious cycle (4.5 and 5.5 h postinfection). In addition, the level of apparent revertants of 2A-1 was substantially reduced in stocks made from the mixed infection (data not shown). The five- to sevenfold-lower titer of 2A-1 in the mixed infection compared with that of wild-type virus was probably in part an experimental artifact. The slightly lower particle/PFU ratio often found for 2A-1 stocks plated on HeLa cells (data not shown) suggested that plaque assays on this cell line gave an underestimate of the virus titer because of the relative inability of the mutant to form visible plaques.

Mutant 2A-1 could also be complemented by the cold-sensitive mutant 3A-2 (Table 2, experiment 1). CV-1 cells were infected with wild-type virus, 2A-1, 3A-2, or an equal mixture of the two mutants and then incubated at 32.5°C. Virus stocks were made both at midcycle (6 h postinfection)

TABLE 3. Complementation tests among mutants 2A-1, 3D-3, and 3NC-202[a]

Virus stock	Titer in PFU/cell (CI)[b]				Plaque type
	3.25 h postinfection		4.75 h postinfection		
	39.5°C	32.5°C	39.5°C	32.5°C	
Expt 1					
Wild type	60	63	300	330	Wild type
3NC-202	<0.0003	0.006	0.0075	0.098	Variants (39.5°C) (mixed)[c]; 3NC-202 + variants (32.5°C) (<wild type)[d]
2A-1	0.13		3.5		2A-1 (minute)[e]
3NC-202 + 2A-1	9.5 (73)		74 (21)		2A-1 (minute)
Expt 2					
Wild type	74[f]				Wild type
3NC-202	0.0028[f]	0.020[f]			Variants (39.5°C) (mixed); 3NC-202 + variants (32.5°C) (<wild type)
2A-1	0.054[f]				2A-1 (minute)
3NC-202 + 2A-1	8.8 (160)[f]	–[g]			2A-1 (minute)
3D-3	2.4[f]				3D-3 (<wild type)
3D-3 (day 3)[h]	2.3[f]				3D-3 (<wild type)
3NC-202 + 3D-3	5.8 (2.4)[f]	7.5[f]			3D-3 (<wild type)
2A-1 + 3D-3	5.0 (2.1)[f]				3D-3 (<wild type)
	8.3 (150)[f]				2A-1 (minute)

[a] CV-1 cells were infected with wild-type virus or a mutant at an MOI of 5 or with a mixture of mutants, each at an MOI of 5, and then incubated at 39.5°C. Cells were harvested at various times after infection, and virus stocks were made as described in Materials and Methods.

[b] The CI values (shown in parentheses) were calculated as described in Materials and Methods.

[c] Plaques of mixed size, ranging from very small to wild-type size.

[d] Intermediate-size plaques which were somewhat smaller than those produced by wild-type virus.

[e] Extremely small plaques which were clearly visible only 1 day after all others were scored.

[f] At 3 h postinfection.

[g] –, 3NC-202 titer could not be calculated. The mutant titer must have been small compared with the background of recombinant and revertant plaques because there was no significant increase in the number of relatively large plaques which appeared at 32.5°C.

[h] Plaques counted 1 day later than usual, at the same time that minute plaques were scored.

and late after infection (8 h). The 2A-1 titer in the 2A-1 and mixed stocks was scored by allowing plaques to develop for 4 days at 32.5°C. In the mixed stock, virtually no plaques appeared before 4 days because 3A-2 produces very few plaques at 32.5°C and evidently also because very few apparent revertants of either mutant arose during the mixed infection. Calculation of the CI revealed that at both time points the titer of 2A-1 was increased 50- to 70-fold in the mixed infection, such that the number of infectious particles was only a fewfold below that produced in a wild-type infection. Titration of the 2A-1 stock at 39.5°C, however, revealed a twofold cold sensitivity of the mutant (Table 2). This observation suggested that the amount of 2A-1 virus produced in the mixed infection may have been even closer to the wild-type level than was evident by measurement of the titer at low temperature. (A second experiment which yielded very similar results is shown in Table 2, experiment 2.)

We also assessed the ability of the temperature-sensitive mutant 3NC-202 to complement 2A-1 in experiments performed at 39.5°C. CV-1 cells were infected with wild-type virus, 2A-1, 3NC-202, or an equal mixture of the two mutants (Table 3, experiment 1). Virus stocks were made at midcycle (3.25 h postinfection) and late after infection (4.75 h). At 39.5°C the mixed stock yielded primarily 2A-1 plaques which could be visualized on day 3. A small number of plaques appeared on day 2, but these were all of wild-type size and contained virus which had a wild-type phenotype; the same number of such plaques arose if the plaque assay was performed at 32.5°C. These plaques were apparently generated by either revertant or recombinant viruses. At the early time point, coinfection with both 2A-1 and 3NC-202 enhanced the titer of 2A-1 progeny about 70-fold. At 4.75 h, the increase was about 20-fold, but the reduction in enhancement was a consequence of the difference in growth kinetics in the 2A-1 and mixed infections. Whereas production of progeny tapered off between the two time points in the mixed infection (as in the wild-type infection), the increase in new virus in cells infected with 2A-1 alone was more marked. It was clear, however, that in a mixed infection 3NC-202 could increase the burst size of 2A-1 by the end of a cycle of infection to a level only a fewfold lower than that of wild-type virus.

In another experiment performed at 39.5°C, it was possible to demonstrate that 3D-3 also complemented 2A-1. Because the linear phase of RNA synthesis and the concomitant assembly of most of the progeny virions are delayed in 3D-3-infected cells, there is a short period during which 3D-3-infected cells contain 30- to 100-fold fewer infectious particles than wild-type cells. To perform complementation experiments with 3D-3, it was necessary to harvest cells during this period. Infected CV-1 cells were harvested at 3 h (Table 3, experiment 2). Both 2A-1 and 3D-3 grew at this temperature but, by the time 2A-1 plaques were observable, 3D-3 plaques were relatively large and could be easily distinguished. The titer of minute (2A-1) plaques in the mixed stock was over 100-fold greater than that found in the 2A-1 stock. 3D-3 rescued 2A-1 to approximately the same extent as 3NC-202 did in the same experiment. In both mixed stocks, the 2A-1 titer was eight- to ninefold lower than the wild-type titer but, given the potential differences in growth curves, this number might have been reduced if a later time point could have been taken.

Rescue of mutant 3A-2 by other cDNA-derived mutants. The distinctive cold sensitivity of 3A-2 enabled us to demonstrate its rescue by genetic complementation. In the experiments in which it was possible to test the ability of 3A-2 to complement 2A-1 by determining the titers of mixed

TABLE 4. Complementation test between mutants 3NC-202 and 2B-201[a]

Virus stock	Titer in PFU/cell (CI)[b]						Plaque type
	3 h postinfection		4 h postinfection		5 h postinfection		
	39.5°C	32.5°C	39.5°C	32.5°C	39.5°C	32.5°C	
Wild type	57	53	230	240	410	340	Wild type
3NC-202	$<10^{-4}$	0.0053	0.0046	0.013	0.0070	0.022	Variants (39.5°C) (mixed)[c]; 3NC-202 + variants (32.5°C) (<wild-type)[d]
2B-201	0.017	0.013	2.7	2.4	28	26	2B-201 (small)[e]
3NC-202 + 2B-201	–[f]	–[f]	5.8 (2.1)	7.8	49 (1.8)	41	2B-201 (small)

[a] HeLa cells were infected with wild-type virus or a mutant at an MOI of 5 or with a mixture of the two mutants, each at an MOI of 5, and then incubated at 39.5°C. Cells were harvested at 3, 4, and 5 h after infection, and virus stocks were prepared as described in Materials and Methods.
[b] The CI values (shown in parentheses) were calculated as described in Materials and Methods.
[c] Plaques of mixed size, ranging from very small to wild-type size.
[d] Intermediate-size plaques which were somewhat smaller than those produced by wild-type virus.
[e] Plaques much smaller than those of wild-type virus but scored at the same time as wild-type plaques.
[f] –, The titers of both mutants were relatively small compared with the background of revertants and recombinants.

stocks generated at low temperature at 32.5°C, we were also able to determine whether 2A-1 could complement 3A-2 by determining the titers of the same stocks at 39.5°C (Table 2, experiment 1). At low temperature, 3A-2 grew poorly and the virus population in the 3A-2 stocks was composed largely of apparent revertants. Calculation of the CI indicated a greater than 50-fold increase in the 3A-2 titer in mixed stocks prepared at both 6 and 8 h postinfection. The relatively large plaques which appeared at high temperature did not appear at the lower temperature and contained cold-sensitive virus; hence, it could be concluded that they were produced by 3A-2 particles and not by revertants or recombinants. Even after rescue by genetic complementation, however, the titer of 3A-2 was 10-fold lower than that of wild-type virus and a fewfold lower than that of the mutant it had rescued (2A-1). (A second experiment which yielded very similar results is shown in Table 2, experiment 2.)

It was of interest to determine whether 3A-2 could also be complemented by other mutants. In principle, analogous experiments can be performed with the other small-plaque mutants described above. Attempts to assess the ability of 3D-3 to rescue 3A-2 by using only the methods outlined here, however, were hampered by a combination of two difficulties. First, the plaques produced by 3A-2 and 3D-3 at 39.5°C were not distinguishable by inspection. Second, the titers of mixed stocks at 39.5°C were only a fewfold higher than those of 3D-3 stocks, even if the cells were harvested relatively early after infection (data not shown). Such a result did not exclude a substantial rescue of 3A-2. To circumvent the problem of distinguishing plaques produced by the two mutants, we performed complementation experiments with a temperature-sensitive variant of 3D-3 (3D-3*ts*). Stocks of 3D-3*ts* yielded fewer plaques at 39.5°C than at 32.5°C, and the relatively tiny plaques which were produced could be distinguished from those made by 3A-2 (though, unlike 2A-1 plaques, 3D-3*ts* plaques were visible when 3A-2 plaques were scored). We assessed the ability of 3D-3*ts* to rescue 3A-2 in the same experiments in which we examined complementation between 2A-1 and 3A-2 (Table 2, experiment 1). At both midcycle and late after infection, the 3A-2 titer was very similar to that obtained from virus stocks made from cells coinfected with 2A-1 and 3A-2. Thus, it appeared that 3D-3*ts* rescued 3A-2 as effectively as 2A-1 did.

We also tested the ability of 2B-201 to complement 3A-2 (Table 2, experiment 2). The problems described in the preceding experiment did not occur because the titer of 2B-201 through most of the infectious cycle was much lower than that of 3D-3 and because the plaques produced by 2B-201 on CV-1 monolayers at 39.5°C, like those of 3D-3*ts*, were much smaller than 3A-2 plaques. Infected cells were harvested at 6 and 7.5 h postinfection; the latter time was chosen because it was the latest at which there was a substantial (30- to 50-fold) difference in titer between 2B-201 and wild-type virus. For comparison, rescue of 3A-2 by 2A-1 was assessed in parallel. Rescue of 3A-2 by 2B-201 was apparent at both time points; the titer of 3A-2, however, was clearly severalfold less than that obtained in the mixed infection with 2A-1. Hence, it appeared that 3A-2 was rescued less effectively by 2B-201 than by the other mutants described here.

Evidence that other cDNA-derived mutants cannot be complemented. It was also possible to determine whether 2B-201, 3D-3, and 3NC-202 could be complemented by exploiting their unique plaque phenotypes. We found that 2B-201 could not be rescued by any other mutant. HeLa cells infected with both 2B-201 and 2A-1 and then incubated at 37°C did not yield a higher 2B-201 titer than did cells infected with 2B-201 alone (Fig. 4). Less than a twofold enhancement of 2B-201 titer was observed when HeLa cells were infected with 2B-201 and 3NC-202 and incubated at 39.5°C (Table 4). In this experiment, we determined 2B-201 titers by performing plaque assays at 39.5°C. The small increase in 2B-201 titer may simply have been a consequence of more rapid viral growth resulting from the use of a total multiplicity of infection (MOI) of 10 in the double infections (5 for each mutant) compared with 5 in the single infections. Virus stocks were also made from CV-1 cells infected with 2B-201 and 3A-2 or with 2B-201 alone and then incubated at 32.5°C (Table 2, experiment 2). Despite an apparent fewfold enhancement of the 2B-201 titer in the mixed stock at 6 h postinfection, little enhancement was observed by 7.5 h.

3D-3 was also not complemented to a significant extent by other mutants. To determine whether the lag in the development of 3D-3 titer could be eliminated in a mixed infection, it was necessary to harvest cells relatively early after infection. In one experiment, 3D-3 titers in virus stocks prepared from CV-1 cells infected with 2A-1 and 3D-3, 3NC-202 and 3D-3, or 3D-3 alone and incubated at 39.5°C for 3 h were compared (Table 3, experiment 2). The 3D-3 titers were determined by performing plaque assays at 39.5°C. The twofold enhancement of the 3D-3 titer observed in both mixed infections was not regarded as significant and again may have merely been an MOI effect. In other experiments, CV-1 cells were infected with both 3D-3 and the cold-sensitive mutant 3A-2 and incubated at 32.5°C for 6 h. When

plaque assays were performed at 32.5°C, there was no significant difference in virus titer between the 3D-3 and mixed stocks (data not shown).

We also tested the ability of the plaque size mutants to rescue 3NC-202. In these experiments, 3NC-202 titers were determined by plaque assay at 32.5°C. Stocks made from CV-1 cells infected with both 3NC-202 and 2A-1 contained a small amount of virus which yielded relatively large plaques at low temperature, but the same number of plaques also formed at high temperature (data not shown). These plaques were produced primarily by variants which were presumably either revertants or recombinants. Although relatively low, the level of such variants was always higher than the level of virus in 3NC-202 stocks. For example, at 4.75 h postinfection (Table 3, experiment 1), variants amounted to approximately 2 PFU per cell, a level which exceeded the titer of virus in the 3NC-202 stock by 20-fold. As a consequence, this methodology would not have been sensitive to a very low level of 3NC-202 rescue. It is clear, however, that substantial rescue of the mutant did not occur. It was more difficult to test complementation of 3NC-202 by 3D-3 because the 3D-3 titer in mixed stocks was more than 2 orders of magnitude greater than the titer of 3NC-202 in single-infection stocks (Table 3, experiment 2). Anything less than a several-hundredfold rescue of 3NC-202 would not have been detected by plaque assay. A slightly higher titer was observed in the mixed stock at 32.5°C than at 39.5°C. Twenty-five plaques produced at low temperature were picked, however, and none had a temperature-sensitive phenotype. Finally, mixed infections were performed in HeLa cells to test the ability of 2B-201 to rescue 3NC-202 (Table 4). The titer of virus obtained from the mixed stocks was very similar at both high and low temperatures, and plaques almost exclusively of the 2B-201 phenotype were observed. Because of the large difference in titer of the two mutants, however, only dramatic rescue of 3NC-202 could have been observed.

DISCUSSION

We studied the rescue of five well-defined mutants of poliovirus type I primarily by comparing virus yields in single and mixed infections. In contrast to many results obtained with unmapped mutants, we obtained strong evidence that defects in at least two of the nonstructural functions of the virus can be complemented to a very significant extent. This finding implies that the nonstructural portion of the polyprotein contains multiple complementation groups. The unambiguous rescue of mutants which contain lesions in regions encoding proteins 2A and 3A implies that the protein which mediates inhibition of host cell translation and one of the components involved in RNA synthesis can be provided in *trans*. The rescue of poliovirus mutants which we observed is distinct from previous reports of mutant rescue in two important respects. First, both 2A and 3A mutants were rescued in mixed infections with all other mutants which contain lesions in other parts of the genome and which are not *trans* dominant, rather than in just one particular mixed infection. Second, the titer of both mutants was enhanced in mixed infections at a time when significant accumulation of progeny virus was first detected, not just at the end of a full cycle of growth. These observations help to support the notion that the rescue we observed involved true genetic complementation, that is, compensation for defects by the diffusion of functional gene products, and was not due to a nonspecific effect. Our studies are also distinct from previous work in that we corroborated the virus yield data with independent evidence of complementation. We correlated enhancement of virus titer with increases in the synthesis of virus-specific macromolecules and in the progression of the cytopathic effect. We also found that mutant rescue correlated with a reduction in the proportion of apparent revertants in virus stocks prepared from mixed as compared with single infections. This observation suggests that complementation reduces the selective pressure which favors variants that grow better than the parental strain. Moreover, these studies describe the first example of two-way rescue of poliovirus mutants.

In addition to demonstrating complementation of two nonstructural mutants, we also found that two mutants which are defective in RNA synthesis could not be complemented to a significant extent by other mutants. Although a low level of complementation cannot be completely excluded, the titer of a mutant which contains an insertion in the 3′-untranslated region of the RNA was also not greatly enhanced in mixed infections with other mutants.

Comparison of the rescue of 2A-1 and 3A-2. The titers of both 2A-1 and 3A-2, which contain single amino acid insertions in the 2A and 3A regions of the polyprotein, respectively, could be increased greater than 50-fold in mixed infections with other mutants. In both cases, the levels of complementation observed both at midcycle and late after infection were similar. Presumably, either diffusion of the functional product was continuous or production of a small amount of functional protein early after infection was sufficient to effect rescue. There were two respects, however, in which the complementation patterns of these two mutants differed. First, the rescue of 2A-1 was more complete than that of 3A-2. That is, the titer of 2A-1 which resulted from a mixed infection was only a fewfold lower than that of wild-type virus, whereas the titer of 3A-2 was approximately 10-fold lower. Second, although complementation of 2A-1 always brought the yield of that mutant to the same level relative to wild-type virus, the degree of rescue of 3A-2 was more variable.

A consideration of the functions impaired by the two lesions suggests an explanation for these differences in complementation pattern. Experiments with guanidine, an inhibitor of viral replication, have shown that at an MOI of 10 or more, the small amount of protein produced by input RNA is sufficient to mediate effective inhibition of host cell translation (20). In addition, mutants such as 3A-2 and 3NC-202, which synthesize RNA in a highly temperature-dependent manner, are capable of inhibiting host cell translation at the nonpermissive temperature in the absence of significant viral protein synthesis (Fig. 3, lane 3; reference 24). Hence, the observation that effective rescue of 2A-1 is relatively independent of the amount of protein or RNA produced by the other mutant in mixed infections is consistent with the notion that only small amounts of the translational inhibitor are required. This hypothesis is reinforced by a report that a 3NC-202 cDNA-bearing cell line partially complements the mutant under conditions in which the cells produce too little viral protein to be easily detected by other means (24; Sarnow and Baltimore, unpublished data). Furthermore, effective rescue of 2A-1 by mutants such as 3NC-202 implies that the function which mediates inhibition of host cell translation probably does not work by selectively promoting virus-specific translation, since such a function would almost certainly be required in higher amounts than the rescuing mutant can provide.

By contrast to the mutation in 2A-1, the insertion in 3A-2

disrupts a function involved in replication which may be required in substantial amounts. This notion appears particularly attractive given that the lesion maps to a segment of the polyprotein which is contained in precursors of VPg (4, 26), the short protein which is found at the 5′ end of every poliovirus RNA molecule, and therefore may affect a function associated with VPg. The extent of 3A-2 rescue in mixed infections correlated with the amount of protein ordinarily synthesized by the other mutant. The best complementation was obtained with 3D-3*ts*, which normally produces wild-type levels of protein, and 2A-1, which can synthesize high levels of viral protein once host-specific translation is inhibited. The least effective rescue was obtained with 2B-201, a mutant that produces only a small fraction as much RNA and protein as does wild-type virus. These data are consistent with the notion that rescue of 3A-2 is at least in part dependent on the level of virus-specific products synthesized by the complementary mutant. What is more difficult to explain is the observation that rescue of 3A-2 was never as complete as rescue of 2A-1. It has been shown that poliovirus can synthesize a normal amount of RNA even if virus-specific protein synthesis is greatly depressed (6). Hence, in mixed infections involving 3A-2 and, say, 3D-3*ts*, the synthesis of the complementary gene product probably far exceeded the theoretical amount required for full rescue of the mutant. A possible explanation for the incomplete rescue of 3A-2 is that the required gene product might not be uniformly distributed, presumably because of a compartmentalization problem or because of hindrance of free diffusion (see also below).

Lack of rescue of three nonstructural mutants. We were unable to demonstrate significant rescue of three mutants which contain replication defects. Although partial rescue of one of these mutants, 3NC-202, is not totally excluded by the data, it seems unlikely that a mutant which has a lesion in a noncoding region of the genome could be rescued in *trans*. One possible explanation for our inability to demonstrate complementation of the 2B and 3D mutants is that the insertions do not alter protein function but rather disrupt the structure of the RNA and thereby create *cis*-acting defects. This explanation is not likely, however, given that at least at a gross level comparative sequence analysis does not yield evidence of important RNA structures within the coding region. The RNA sequence is clearly conserved among different strains of poliovirus (28) and different picornaviruses (2, 14, 27) at the 5′ and 3′ ends of the genome, but only the amino acid sequence appears to be conserved in the coding region. The explanation that the mutations appear *cis* acting because they affect cleavage of the polyprotein is also unlikely because no gross alteration of the cleavage pattern was observed in either 2B-201- or 3D-3-infected cells. A far more likely explanation is that the defective functions in 2B-201 and 3D-3 simply cannot be supplied in *trans*. If this explanation holds true, and if the insertions we introduced do not alter functions which reside in distant parts of the polyprotein, then the overall picture of complementation presented here suggests that the *trans*-acting functions of the virus do not reside in adjacent segments of the polyprotein but rather are interspersed among *cis*-acting functions. Such a suggestion is not incompatible with the notion that the nonstructural portion of the polyprotein must fold as a single unit for some segments to be functional. But it does imply that removal of internal segments of the polyprotein can occur without disruption of the other nonstructural functions. Of course, the lack of complementation of 2B-201 and 3D-3 may not be related to polyprotein folding at all but rather may be due to some phenomenon which results in the effective compartmentalization of the defective proteins.

One hypothesis to explain the *cis*-acting behavior of these mutations is that the RNA replicase is a complex of proteins which uses the specific plus-strand molecule from which it was translated as a template to synthesize a minus strand. If proteins 2B and 3D are part of this complex, it would be understandable why lesions that destroy their function are not complementable even by mutant 2A-1, which should be able to make the functional forms of all proteins downstream from 2A (6). Given evidence that guanidine sensitivity is poorly complemented (K. Kirkegaard and D. Baltimore, unpublished data), protein 2C, to which this genetic marker maps (21, 25), might also be a *cis*-active part of the complex. Even protein 3A might be part of the complex initially, but the complementation of 3A-2 by mutants containing lesions in 2B and 3D argues that it must be reversibly bound, whereas the other elements are effectively irreversibly bound to the complex and the RNA.

Nature of the poliovirus genome. One implication of these experiments is that the noncapsid region of the poliovirus genome cannot be considered to consist of a single genetic unit whereby all functions reside in a single posttranslationally processed polypeptide. Our results show that at least two of the segments of the polyprotein act as independent genetic units. Perhaps part of the reason that the poliovirus genome consists of a single polyprotein is that certain segments must be produced as part of a larger precursor or need to fold together into a complex to function properly. But clearly this is not the only reason, because some segments can function independently (although their folding may be directed by other parts of the polyprotein). Presumably, the virus has adopted a polyprotein genome in part because of the efficiency of gene expression which such a strategy affords.

This study establishes the minimum number of "cistrons" in the nonstructural region at three. Many more well-defined mutants will have to be constructed before a complete complementation map of the virus can be derived. Such a map may provide clues as to how the virus replicates its genome and performs other functions and may also help to explain why the virus uses a unique genetic strategy.

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