Three Poliovirus 2B Mutants Exhibit Noncomplementable Defects in Viral RNA Amplification and Display Dosage-Dependent Dominance over Wild-Type Poliovirus

KYLE L. JOHNSON^{1,2} AND PETER SARNOW^{1,2,3*}

Molecular Biology Program,¹ Department of Biochemistry, Biophysics, and Genetics² and Department of Microbiology and Immunology,³ University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 20 March 1991/Accepted 17 May 1991

Many functions of the poliovirus genome in virally infected cells have been elucidated. However, the role of 2B (and of its precursor polypeptide, 2BC), encoded by the P2 region in the poliovirus genome, remains unknown. We have employed a genetic approach to examine the role of 2B in poliovirus-infected cells. We report here the phenotype of one previously isolated mutant in the 2B coding region, 2B201. In addition, we have constructed one additional mutation in the 2B coding region of an infectious poliovirus cDNA clone. Upon transfection into monkey Vero cells we could recover two 2B mutant polioviruses, 2B204 and 2B205. All three mutants exhibited small-plaque phenotypes on monkey Vero and human HeLa cells and displayed primary defects in viral RNA synthesis. None of the 2B mutants could be complemented by wild-type virus. Instead, the mutants exhibited a dosage-dependent dominance over wild-type poliovirus. Thus, the phenotypes of these 2B mutants implicate 2B and possibly its precursor, 2BC, in viral RNA amplification in poliovirus-infected cells, and the dominance of the 2B mutants suggests a structural role for 2B in viral replication complexes.

Poliovirus, a member of the *Picornaviridae*, has a positive-stranded RNA genome, approximately 7,500 nucleotides in length, with a small viral protein, VPg, covalently attached to its 5' end and a genetically encoded poly(A) tail at its 3' end (see reference 19 for a review). Its single long open reading frame is translated into a 247-kDa polypeptide, which is subsequently processed by three viral proteases, 3C (9), 2A (25), and 3CD (29), into individual viral polypeptides (19) as outlined in Fig. 1. A final cleavage of VP0 to VP2 and VP4 is capsid associated and possibly autoproteolytic (1).

The functions of many of these polypeptides in poliovirus-infected cells have been elucidated by purification and enzymatic assays in vitro and by the study of well-defined poliovirus mutants in vivo. The proteins encoded by the P1 region of the genome, VP1, VP2, VP3, and VP4, are capsid proteins (18). Many functions have been assigned to the nonstructural proteins encoded by the P2 and P3 regions. The P2-2A protease has been shown to be involved in the shutoff of host cell translation (3). P2-2C plays a role in the replication of the viral RNA (15, 17). Proteins encoded by the P3 region (Fig. 1) include P3-3B (VPg), covalently attached to the 5' end of the viral RNA (8, 14); the protease P3-3C that is involved in polypeptide processing (9); and P3-3D, the viral RNA-dependent RNA polymerase (26). However, the functions of the remainder of the viral proteins and their precursors remain unknown. For example, the functions of the P2-2B protein and its precursor, P2-2BC, in the viral life cycle remain an enigma.

Some evidence has suggested that 2B or its precursor, 2BC, is involved in viral RNA synthesis. First, host range variants of two related picornaviruses, human rhinoviruses 2 and 39, have been isolated. These variants are able to replicate in normally nonpermissive mouse L cells and contain several undefined mutations in the 2B and 2BC coding regions, identified by an altered 2B protein mobility

during gel electrophoresis (16, 28). Second, Bienz et al. (4–6) have shown that the 2BC precursor is present in membrane-bound replication complexes in vivo and suggested that 2BC induces the formation of smooth membrane vesicles on whose surfaces the replication complexes are found. Finally, two different poliovirus 2B mutants have been isolated and found to have noncomplementable defects (2, 15).

Despite these indications, there has been no direct evidence for the involvement of 2B in viral RNA synthesis. Here, we demonstrate that three 2B mutant polioviruses exhibit early defects in viral RNA synthesis, cannot be complemented by wild-type poliovirus, and are dominant over wild-type poliovirus in mixed infections. Together, these data suggest a role for 2B or its 2BC precursor in viral RNA amplification in poliovirus-infected primate cells.

MATERIALS AND METHODS

Cells and viruses. Human HeLa and monkey kidney Vero cells were grown in Dulbecco's modified eagle's medium supplemented with 10% calf serum (Irvine Scientific). Wild-type (Mahoney type 1) and 2B mutant polioviruses were isolated from single plaques derived from Vero cells transfected with in vitro-synthesized RNA as previously described (21). High-titer stocks of wild-type and 2B mutant viruses were grown in Vero cells at 37°C. The 3NC202 stock (22) was grown in Vero cells at 32.5°C. Plaque assays were performed as previously described (12). Incubations were performed at 37 and 39.5°C for 42 h and at 32.5°C for 60 h.

Bacterial strains and plasmids. *Escherichia coli* HB101, originally obtained from C. Cepko (Harvard Medical School), was used as the host for all plasmids grown. Plasmid pSV2-polio was originally obtained from V. R. Racaniello (Columbia University). Construction of the T7-polio plasmid has been previously described (21). pGEM3 was purchased from Promega.

Enzymes. Enzymes T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, Klenow, and T7 RNA polymerase

* Corresponding author.

FIG. 1. Proteolytic processing map of poliovirus proteins. The location of poliovirus proteins are indicated. Precursor proteins readily detected during infection, as well as the terminal cleavage products, are shown. Arrows indicate the position of the 2B201, 2B204, and 2B205 mutations in 2B and its precursor polypeptides.

were obtained from Promega. Restriction endonucleases were obtained from Promega, Bethesda Research Laboratories, and New England BioLabs.

Construction of linker-insertion mutations. Construction of pSV2-H/H 3916, containing a 6-bp insertion at position 3916 in the 2B coding region, has been described (2). A second 2B mutation was constructed by cleavage of pSV2-H/H 3916 with *HpaI*, which cuts within the inserted base pairs, and insertion of a phosphorylated *SmaI* linker (CCCGGG; Collaborative Research) at position 3919. Plasmid pSV2-H/H/S 3916 bears the 12-bp insertion at position 3916 (Fig. 2). The viral cDNA of pSV2-H/H/S 3916 was cloned into the T7-polio plasmid (21) to allow the synthesis of infectious RNAs in vitro. The resulting plasmid was termed T7-H/H/S 3916.

Isolation of poliovirus 2B mutants. T7-H/H/S 3916 was linearized with *Eco*RI and used as a template for in vitro transcription by T7 RNA polymerase (21). Full-length RNA molecules were transfected into Vero cells by using a DEAE-dextran transfection method (21), overlaid with agar, and incubated at 37°C for 40 to 45 h. Individual plaques were picked, and viral stocks of several plaque isolates were made. For each isolate, viral RNA was purified and the nucleotide sequence of the 2B coding region was determined. In this way two unique 2B mutants, 2B204 and 2B205, were isolated. The plaque phenotypes of these mutants were determined on HeLa and Vero cells.

Reconstruction of mutations into wild-type cDNA. To confirm that the introduced mutations in 2B were solely responsible for the observed viral mutant phenotypes, the mutant sequences were reintroduced into a wild-type poliovirus cDNA by using oligodeoxynucleotide-directed mutagenesis (7) as follows. A 952-bp *AvaII-SphI* fragment from the reconstructed T7-H/H 3916 plasmid (see above) bearing the 2B201 insertion was subcloned into pGEM3. This subclone was cut with either *BstEII*, to remove a 690-bp fragment, or cut with *SspI*, to linearize the plasmid. *BstEII*- and *SspI*-cut DNA molecules were mixed together, and strands were separated in the presence of 1 M KOH and reannealed to yield gapped duplex molecules (7).

Oligodeoxynucleotides 5'-CAGAGTTGTTCTCGGGAAC GACCA-3' and 5'-CAGAGTTGTTCCCGGGAATGACC AATATG-3', bearing the 2B204 and 2B205 insertions, respectively, were annealed to these gapped duplex molecules.

The molecules were then repaired with T4 DNA polymerase and T4 DNA ligase and transformed into *E. coli*; colonies harboring mutagenized plasmids were identified by colony hybridization in which radiolabeled oligodeoxynucleotides were used as probes. Following hybridization, filters were washed with tetramethylammonium chloride (27) and autoradiography was performed. Hybridization-positive colonies were picked, and plasmid DNAs were isolated and screened for the loss of a *Hpa*I site (2B204) or the gain of a *Sma*I site (2B205). The 690-bp *Bsr*EII fragments bearing the 2B204 and 2B205 insertions were isolated, recloned into wild-type T7-polio, and verified by sequencing.

Single-cycle virus yields. Measurement of single-cycle yields for wild-type and 2B mutant polioviruses was performed as previously described (2), except that HeLa cells were infected at a multiplicity of infection (MOI) of 10 and incubated at 37°C for 6 or 8 h. Virus stocks were made, and their titers were determined by plaque assay on HeLa cells at 37°C.

Analysis of viral RNA synthesis. Cells were infected with wild-type and mutant polioviruses at an MOI of 10. Total cytoplasmic RNA was prepared at 2, 4, 6, and 8 h after infection and transferred to nitrocellulose with a slot-blot apparatus (23). Positive-stranded viral RNA was visualized after hybridization to a radiolabeled RNA (poliovirus sequences 670 to 220).

Stability of viral RNA. Measurement of viral RNA stability was performed as previously described (23).

Analysis of viral protein synthesis. In vivo labeling of infected cells was performed as previously reported (23), except that HeLa cells were infected at an MOI of 50 and incubated at 37°C. At various times after infection, cells were pulse-labeled for 20 min in methionine-, cysteine-, and serum-free modified Eagle's medium containing 100 μ Ci of Trans-label ($[^{35}\text{S}]\text{methionine}$ and $[^{35}\text{S}]\text{cysteine}$; New England Nuclear) before harvest. Labeled proteins were analyzed on sodium dodecyl sulfate (SDS)-18% polyacrylamide gels (13). The gels were fixed in a solution of 30% methanol-10% acetic acid and then treated with Fluoro-Hance (Research Products International Corp., Prospect, Ill.), dried, and exposed to X-AR film (Kodak) at room temperature.

In vitro translation of wild-type and mutant viral RNAs. Full-length wild-type and mutant RNA molecules were syn-

FIG. 2. Genotypes of 2B mutant polioviruses. A portion of the wild-type (WT) 2B coding region is shown at top. 2B201 contains a 6-nucleotide insertion (GUUAAC) at position 3916; 2B204 and 2B205 each contain a 12-nucleotide insertion (GUUCUCGGGAAC and GUUCCCGGGAU, respectively) at position 3916. Asterisks denote the difference between actual and predicted 2B204 and 2B205 genotypes.

thesized in vitro, as described above, and precipitated with ethanol. The relative amounts of RNAs produced were determined by Northern (RNA) analysis (23). In vitro translation of equivalent amounts of each RNA species was performed for 3 h at 30°C as previously described (30) by using rabbit reticulocyte lysate (Promega) supplemented with an S10 extract from uninfected HeLa cells (a generous gift from Sandra Dildine and Bert Semler, University of California at Irvine) and 7 µCi of [³⁵S]methionine per reaction. The resulting translation products were analyzed on SDS-12.5% polyacrylamide gels, which were processed as described above.

Complementation and interference assays. Complementation assays were performed as previously described (2, 11). HeLa cells were infected with wild-type or 2B mutant viruses at an MOI of 5 or with a mixture of wild-type and mutant viruses at an MOI of 5 each and then incubated at 37°C. At 6 or 8 h postinfection, cells were harvested and virus stocks were made. These stocks were titrated on Vero cells at 37°C (Vero cells were used because the plaque sizes of wild-type and mutant polioviruses are more homogeneous on Vero than on HeLa cells). Wild-type and 2B mutant plaques were distinguished on the basis of plaque size; wild-type plaques are much larger than 2B mutant plaques, especially on Vero cells (Fig. 3). For each mutant, the complementation index (CI) was calculated; the CI for any

given virus is defined as the titer of that virus in a mixed infection divided by its titer in a single infection (2).

Interference assays by superinfection were performed as follows. HeLa cells were either mock infected or infected in duplicate with mutant viruses at an MOI of 5, 25, or 50 and incubated at 37°C. At 5 h after infection, one plate from each set was superinfected with wild-type virus at an MOI of 5 and the other was "mock superinfected," and the cells were then incubated at 37°C. Cells were harvested 5 h after superinfection, and virus stocks were made. Viral yields were determined by plaque assay on Vero cells, at 37°C for wild-type and 2B mutant viruses or at 32.5 and 39.5°C for 3NC202. Wild-type and 2B mutant plaques were distinguished on the basis of their plaque sizes, and 3NC202 was distinguished from wild type by its ability to form plaques at 32.5 but not at 39.5°C.

RESULTS

Construction and isolation of poliovirus 2B mutants. Two mutations were introduced into the 2B coding region of an infectious poliovirus cDNA clone (Fig. 2). The first mutation consisted of a 6-bp *Hpa*I linker inserted at position 3916. Transfection into mammalian cells of this mutated cDNA yielded mutant 2B201 (2). The second mutation consisted of a 6-bp *Sma*I linker within the initial *Hpa*I insertion, resulting

FIG. 3. Phenotypes of 2B mutant polioviruses. Plaque assays were performed on human HeLa and monkey Vero cells as described in Materials and Methods.

in a net 12-bp insertion (Fig. 2). This mutated viral cDNA was then introduced into a vector containing the promoter for T7 RNA polymerase, and viral RNA was synthesized *in vitro*. Following transfection of the RNA transcripts into Vero cells, several viral plaques were picked and virus stocks were made. For each isolate, viral RNA was purified and the 2B coding region was sequenced.

The sequence analysis of several independent plaque isolates revealed that we had isolated two mutant viruses, 2B204 and 2B205 (Fig. 2). Both 2B204 and 2B205 had insertions with nucleotide sequences that were different from the expected genotype. 2B204 had a single C-to-U change at position 3920 within the inserted *Sma*I linker, resulting in the substitution of a serine for a proline in the predicted 2B amino acid sequence. 2B205 also contained a single C-to-U change, this time at position 3927 in the inserted *Hpa*I linker nucleotide sequence, resulting in the substitution of a methionine for a threonine in the 2B protein. The nucleotide changes observed in the 2B coding regions of 2B204 and 2B205, respectively, were reintroduced into wild-type cDNAs, and mutant viruses were recovered.

TABLE 1. Viral yields in single-cycle infections^a

Virus stock	Titer (PFU/cell) ^b at h (postinfection):	
	6	8
Wild type	600	850
2B201	4.5 (0.8)	85 (10)
2B204	5.0 (0.8)	50 (6)
2B205	3.0 (0.5)	90 (11)

^a HeLa cells were infected with wild-type or 2B mutant polioviruses at an MOI of 10 and then incubated at 37°C. Cells were harvested at 6 or 8 h postinfection, and virus stocks were made as previously described (21). Viral yields were determined by plaque assay on HeLa cells at 37°C.

^b Values in parentheses are 2B mutant titers expressed as percentages of the wild-type yield.

2B201 had been previously reconstructed into a wild-type cDNA clone (2).

The plaque phenotypes of the viruses obtained following reconstruction of the 2B201, 2B204, and 2B205 RNA sequences from the original plaque isolates were indistinguishable from those of the viruses in the original plaques and are displayed in Fig. 3. Mutants 2B201, 2B204, and 2B205 all exhibit small-plaque phenotypes on HeLa and Vero cells at 37°C compared with that of the wild type. The small-plaque phenotype of the 2B mutants was also observed at 32.5 and at 39.5°C (10). To understand the basis for the small-plaque phenotypes of the 2B mutants, we further examined the biological properties of these mutant viruses.

Single-cycle yields of 2B201, 2B204, 2B205, and wild-type polioviruses. First, we determined the amounts of infectious intracellular particles formed for each virus in single-cycle infections. HeLa cells were infected with wild-type or 2B mutant polioviruses and harvested at 6 or 8 h after infection. Virus stocks were made, and viral titers were determined by plaque assay. As shown in Table 1, 2B201, 2B204, and 2B205 infections each produced less than 1% of the wild-type virus yield at 6 h after infection and from 5 to 10% of the wild-type yield at 8 h after infection. Therefore, the low virus yields in mutant-infected cells correlated well with the small-plaque phenotype of the mutant 2B polioviruses.

Analysis of viral RNA synthesis and RNA stability in mutant- and wild-type-infected cells. Next, we examined viral RNA synthesis in mutant-infected cells. Briefly, HeLa cells were infected with wild-type or mutant viruses, total cytoplasmic RNA was isolated at various times after infection, and the amount of positive-stranded RNA was determined (see Materials and Methods). Figure 4 shows that the RNA synthesis of all three 2B mutants is severely impeded compared with that of the wild-type virus during the first 6 h after infection. For example, at 6 h after infection, mutants 2B201, 2B204, and 2B205 synthesized only 15, 19, and 18% as much RNA, respectively, as the wild-type virus; at 8 h after infection, the three mutants synthesized roughly 30 to 50% as much RNA as the wild type. At that time, cells start to lyse and detach from the culture dishes, so analysis at later times is not possible.

To determine whether the decrease in the amount of RNA detected in this assay was due to a difference in the rate of RNA synthesis or was merely a reflection of decreased stability of the mutant RNAs, we analyzed the stability of mutant and wild-type RNAs. HeLa cells were infected with mutant and wild-type polioviruses, and viral RNA synthesis was selectively stopped at 4 h after infection by the addition of 2 mM guanidine hydrochloride to the culture medium. Subsequently, the amounts of positive-strand viral RNAs

FIG. 4. Analysis of wild-type and mutant viral RNA syntheses. The amount of viral RNA was determined as described in Materials and Methods. Shown are the optical densities at 633 nm of the autoradiograph. (A) RNA synthesis in wild-type-, 2B201-, and 2B204-infected cells. (B) RNA synthesis in wild-type- and 2B205-infected cells. Symbols: ●, wild-type virus; ■, 2B201 virus; ▲, 2B204 virus; ◆, 2B205 virus.

were determined at different times after guanidine hydrochloride treatment (23). Figure 5 shows that mutant 2B205 RNA was degraded at a rate similar to that of wild-type RNA, while mutant 2B201 and 2B204 RNAs decayed slightly faster than wild-type RNA. For example, at 2 h after guanidine hydrochloride treatment, 60% of the wild-type RNA remained detectable, compared with 47% of 2B201, 44% of 2B204, and 58% of 2B205. Although the 2B mutant RNAs may be slightly less stable than the wild-type RNA, it is unlikely that these small differences in RNA stability account for the markedly decreased rate of RNA synthesis in mutant-infected cells.

Analysis of viral protein synthesis in 2B201-, 2B204-, 2B205-, and wild-type-infected cells. To examine whether the impaired RNA synthesis of the 2B mutants was a consequence of poor translation of the mutant viral genomes, we compared the translation of mutant and wild-type RNAs by analysis of radioactively pulse-labeled nascent viral polypep-

FIG. 5. Stability of wild-type and 2B mutant poliovirus RNAs in infected HeLa cells. Analysis was performed as described by Simoes and Sarnow (23). The amount of RNA detected at the time of addition of 2 mM guanidine-HCl was designated 100%. Symbols: ●, wild-type virus; ■, 2B201 virus; ▲, 2B204 virus; ◆, 2B205 virus.

tides. Figure 6 shows that all three mutants displayed a slight delay in protein synthesis compared with that in the wild type. However, the rate of protein synthesis at 6 h after infection in mutant-infected cells was only slightly impaired compared with that in wild-type-infected cells, and polyprotein processing in mutant-infected cells was not affected. Because RNA synthesis, but not protein synthesis, is severely impaired in mutant-infected cells at 6 h after infection (Fig. 4), it is likely that the early delay in viral protein synthesis is due to the decrease in available mRNAs. Nonetheless, to exclude a primary defect in translation of the mutants more directly, we compared the translation of wild-type and mutant 2B205 RNA molecules *in vitro*.

***In vitro* translation of wild-type and mutant 2B205 RNA molecules.** We chose to examine the translation of 2B205 RNA molecules *in vitro* because 2B205 displayed a greater delay of translation *in vivo* (Fig. 6) than 2B201 and 2B204. Full-length wild-type and 2B205 RNA molecules were synthesized *in vitro* by using T7 RNA polymerase. Various amounts of wild-type and 2B205 RNAs were translated *in vitro* in a rabbit reticulocyte lysate supplemented with a HeLa S10 extract (30), and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Figure 7 shows that equivalent amounts of wild-type and mutant 2B205 RNA were translated with similar efficiency, producing properly processed viral polypeptides. Similar results were obtained when equivalent amounts of 2B201 and 2B204 RNAs were translated in this supplemented extract (10). Therefore, it is likely that the decrease in viral RNA synthesis in mutant 2B-infected cells is due to a primary defect in RNA synthesis rather than in protein synthesis.

FIG. 6. Protein synthesis in wild-type- and 2B mutant-infected HeLa cells. Infected cells were radioactively labeled in 20-min pulses with [35 S]methionine and [35 S]cysteine at 2, 4, 6, and 8 h after infection. Extracts were prepared and analyzed as described in Materials and Methods. An autoradiograph of the SDS-polyacrylamide gel is shown. A labeled extract from mock-infected cells (m) is shown at the left.

2B mutants cannot be complemented by wild-type virus. To gain some insight into how the 2B gene might be involved in viral RNA amplification, we performed a genetic analysis of the 2B mutants. Bernstein et al. (2) examined complementation between different pairs of poliovirus mutants and showed that 2B201 could not be complemented by any of the other mutants tested. We tested whether this lack of complementation was a characteristic of all three 2B mutants or was unique to 2B201. Defects in RNA structure, for example, should be noncomplementable but quite allele specific. HeLa cells were infected with wild-type or mutant viruses either alone at an MOI of 5 each or in a mixed infection with wild-type and mutant viruses at an MOI of 5 each. Progeny virus was harvested at 6 or 8 h postinfection, and titers of wild-type and mutant viruses were determined by plaque assay. Wild-type and mutant plaques were distinguished on the basis of plaque size. If complementation had occurred, one would expect to see a significant increase in the number of mutant (small) plaques produced in a mixed infection over the number produced in a single infection, i.e., a large complementation index (CI) value; in experiments in which one mutant rescued another, CI values of between 50 and 100 were usually observed (2).

As shown in Table 2, at 6 h after infection there was slight complementation of 2B201 and 2B205 but not of 2B204. However, at 8 h postinfection, none of the mutants was complemented by the coinfecting wild-type virus. These data indicate that the defects in 2B201, 2B204, and 2B205 are noncomplementable and suggest that the genetic lesions in 2B act in *cis*. The slight complementation at 6 h after infection may be a result of an early interaction of wild-type virus with the infected host cell that may help the mutants to establish an infectious cycle more rapidly.

2B mutants display a partial dominance over wild-type virus in mixed infections. We noticed a slight but reproducible reduction in wild-type titers during mixed infections, particularly at 6 h postinfection (Table 2). To test whether 2B mutants were dominant over wild-type virus, we determined the titer of wild-type virus after coinfection with the 2B mutants or mutant 3NC202. 3NC202 is a poliovirus mutant bearing an 8-nucleotide insertion in the viral 3' noncoding region. Phenotypically, 3NC202 displays a temperature-sensitive defect in viral RNA synthesis (22). We used 3NC202 as a negative control for dominance over the wild type, since the *cis*-acting defect in 3NC202 should display no dominance over wild-type virus (20). Importantly, the yield of 3NC202 in a single infectious cycle at 37°C is comparable to that of the 2B mutants (Table 3), facilitating the interpretation of the experiments.

Briefly, HeLa cells were infected in duplicate with mutants 2B201, 2B204, 2B205, and 3NC202 at MOIs of 5, 25, and 50 followed by incubation for 5 h at 37°C. After that, the cells were superinfected with wild-type virus at an MOI of 5 and incubated for an additional 5 h. Virus stocks were made, and virus yields were determined by plaque assay.

The results of this experiment are shown in Table 3. At an MOI of 5, all four mutant viruses caused a 10- to 20-fold reduction in the titer of the superinfecting wild-type virus, a result that is probably a consequence of the 5 h of mutant growth prior to superinfection. However, at MOIs of 50 for mutants, 3NC202 still reduced the wild-type titer only 20-fold, while 2B201, 2B204, and 2B205 reduced the wild-type titer by 212-, 133-, and 106-fold, respectively. These results indicate that 2B201, 2B204, and 2B205 can exert partial dominance over wild-type virus in a mixed infection.

FIG. 7. In vitro translation of wild-type and 2B205 RNAs. Samples (50, 10, or 2 ng [lanes 1, 2, and 3, respectively]) of in vitro-synthesized wild-type (WT) and 2B205 RNAs were translated in vitro in a rabbit reticulocyte lysate supplemented with an S10 extract from uninfected HeLa cells. The translation products were analyzed by SDS-PAGE as described in Materials and Methods. The gel was then exposed to X-ray film for 10 days, and the autoradiograph is shown. A translation reaction to which no RNA was added is shown (lane C). Labeled extract from poliovirus-infected HeLa cells (M) is shown at the left.

DISCUSSION

We performed site-directed mutagenesis in the 2B coding region of an infectious poliovirus cDNA; a 6-bp *Sma*I linker was inserted into the unique *Hpa*I site of a viral cDNA bearing a 6-bp *Hpa*I linker at position 3916 (2). Full-length viral RNAs bearing the introduced mutation were synthesized in vitro by T7 RNA polymerase. After transfection into monkey Vero cells, two 2B mutants, 2B204 and 2B205, were isolated. Interestingly, neither of these mutants exhibited the predicted genotype. Instead, each mutant differed from the predicted sequence by a single C-to-U change in the inserted linker: 2B204 contained a C-to-U change at position 3920, and 2B205 carried a U instead of a C residue at position 3927. Both mutations changed the predicted amino acid sequence of the 2B protein.

These mutations were acquired after transfection, because the in vitro-synthesized RNAs contained the predicted nucleotide insertions (10). Most likely, the observed point mutations resulted from errors made by the poliovirus RNA-dependent RNA polymerase, 3D^{pol}. The fact that these mutational events took place only in monkey Vero cells and not in human HeLa cells (10) implies that a Vero cell host factor may have been involved in the process that led to the selection of the mutants.

All three 2B mutant viruses (2B201, 2B204, and 2B205) exhibited small-plaque phenotypes. Further characterization revealed that the mutants produced approximately 10% as

TABLE 2. Complementation tests between wild-type and 2B mutant polioviruses^a

Virus	Titer (PFU/cell) + CI ^b at h (postinfection):	
	6	8
Wild type	112	105
2B201 alone	3.5	12.5
Mixture		
Wild type	75	92.5
2B201	12.5 (3.6)	7.5 (0.6)
2B204 alone	3.8	7.5
Mixture		
Wild type	60	80
2B204	2.5 (0.7)	10 (1.7)
2B205 alone	1.3	32.5
Mixture		
Wild type	60	70
2B205	7.5 (5.8)	15 (0.5)

^a HeLa cells were infected with either wild-type or 2B mutant polioviruses at an MOI of 5 or with a mixture of wild-type and 2B mutant polioviruses at an MOI of 5 each and then incubated at 37°C. Cells were harvested at 6 or 8 h postinfection, and virus stocks were made as previously described (21). Viral yields were determined by plaque assay on Vero cells at 37°C; under these conditions the differences in plaque phenotype between wild-type (large) and mutant (small) plaques were quite clear (Fig. 3).

^b The CI for each mutant (given in parentheses) was calculated as previously described (2) and is defined as the titer of the mutant in mixed infection divided by the titer of the mutant in single infection.

many infectious viral particles as the wild-type virus. Two lines of evidence suggest that the 2B mutants have most likely a primary defect in RNA synthesis and not in protein synthesis. First, the rate of translation at 6 h after infection was only slightly impaired in mutant-infected cells compared with that in wild-type-infected cells. However, there was 10-fold-less viral RNA in mutant-infected cells than in wild-type-infected cells at that time. Therefore, primary defects in translation would have resulted in a decreased amount of viral proteins under those circumstances. Second, in vitro translation of wild-type and 2B mutant RNAs displayed the same rate of translation and polyprotein processing.

The observation that these mutants synthesized very few RNA molecules during the first 6 h of the infectious cycle but then 30 to 50% of the amount of wild-type RNA by 8 h after infection indicated that the mutants have problems early in the infectious cycle. That the defects in RNA synthesis displayed by these mutants are more severe early in infection could result from a variety of factors; for example, it is possible that the 2B function is limiting only early in infection or that the defect in 2B function is at the level of one of its precursors (2BC), which is needed only early in infection.

Complementation analysis was performed to determine whether these defects could be rescued by providing wild-type 2B in *trans*. In two previous studies, Bernstein et al. (2) and Li and Baltimore (15) showed that two 2B mutants, 2B201 and 2B-11, exhibited noncomplementable defects. Our results further support that 2B mutants cannot be complemented by wild-type poliovirus. Noncomplementation of 2B mutants was not allele specific. This supports the ideas that none of the alleles have defective RNA structures and that it is the 2B protein which cannot be complemented. One possible explanation for this lack of complementation is that the 2B protein may be effectively sequestered through interactions with the RNA from which it was translated, with

TABLE 3. Interference between wild-type and mutant polioviruses in mixed infections^a

Virus and MOI ^b (PFU/cell)	Titer (PFU/cell) ^c and phenotype	Inhibition index ^d
Wild type, 5	106 (l)	
2B201		
5	93 (s)	
25	111 (s)	
50	114 (s)	
Wild type + 2B201		
5	9 (l)	12
	79 (s)	
25	1 (l)	106
	104 (s)	
50	0.5 (l)	212
	117 (s)	
2B204		
5	75 (s)	
25	115 (s)	
50	154 (s)	
Wild type + 2B204		
5	5 (l)	21
	88 (s)	
25	0.8 (l)	133
	138 (s)	
50	0.8 (l)	133
	103 (s)	
2B205		
5	110 (s)	
25	110 (s)	
50	109 (s)	
Wild type + 2B205		
5	9 (l)	12
	75 (s)	
25	2 (l)	53
	91 (s)	
50	1 (l)	106
	133 (s)	
3NC202		
5	57 (3NC202)	
25	82 (3NC202)	
50	119 (3NC202)	
Wild type + 3NC202		
5	9 (wild type)	12
	94 (3NC202)	
25	8 (wild type)	13
	70 (3NC202)	
50	5 (wild type)	21
	106 (3NC202)	

^a HeLa cells were either mock infected or infected in duplicate with mutant viruses at MOIs of 5, 25, or 50 and incubated at 37°C. At 5 h postinfection, one plate from each set was superinfected with wild-type virus at an MOI of 5 and the other was mock superinfected; the cells were then incubated at 37°C. Cells were harvested 5 h after superinfection, and virus stocks were made as previously described (21). Thus, cells infected with mutant viruses either alone or in combination with wild-type virus were incubated for a total of 10 h, whereas cells infected with wild-type virus alone were incubated for only 5 h. Viral yields were determined by plaque assay on Vero cells, at 37°C for wild-type and 2B mutant viruses or at 32.5 and 39.5°C for 3NC202.

^b MOIs shown are for 2B mutant and 3NC202 viruses. MOIs of wild-type viruses were always 5 PFU per cell.

^c Wild-type and 2B mutant viruses were distinguished on the basis of plaque size; wild-type plaques were large (l), whereas 2B mutant plaques (scored at the same time as wild-type plaques) were small (s). Wild-type and 3NC202 viruses were distinguished on the basis of their growth phenotypes at 32.5°C (wild type and 3NC202) or 39.5°C (wild type only).

^d The inhibition index is defined as the fold by which wild-type virus titer is reduced in mixed infection with mutant viruses.

other viral proteins, or perhaps with membranes. The finding by Bienz et al. (4–6) that the 2B precursor, 2BC, is found in the membrane-bound replication complexes which are the site of RNA synthesis in vivo lends support to such a hypothesis.

Are 2B mutants *cis* dominant or can the 2B proteins exhibit their inhibitory effect in *trans*? We found that the 2B mutants displayed partial dominance in a dosage-dependent manner over wild-type virus. The fact that this dominance was apparent at a time when very little viral RNA was being synthesized again suggests that the mutant protein, rather than the mutant RNA, exerted this dominant effect. Partial dominance of defined poliovirus mutants over wild-type virus has been observed among particular mutant alleles of VP1 (11). A partial dominance of capsid mutants is expected, since it is known that they function as a complex, i.e., the poliovirion. In the case of partial dominance of 2B mutants, a likely explanation for such dominance is that the mutant 2B protein interacts with a limiting factor, for example, a protein or a membrane attachment site, which is also used by the wild-type protein. Perhaps the mutant 2B protein could have an interaction with its target that is of the same or of higher affinity than the wild-type protein with the same target, and thereby, mutant 2B protein could effectively out compete the wild-type 2B. An example could be the interaction of 2BC with 3AB (24) in membrane-associated replication complexes (5). It would then be the subsequent activities, whatever they are, of 2B that are defective in the mutants.

It is possible that the 2B sequences serve only to modify 2C function. However, at this point, it is impossible to separate the functions of 2B and its 2BC precursor. While it is clear from analysis of these poliovirus 2B mutants that 2B (or 2BC) does play a role in viral RNA synthesis in vivo, its exact role remains unknown. Further analysis will be required to determine the exact mechanism by which 2B, 2BC, or both participate in poliovirus RNA synthesis.

ACKNOWLEDGMENTS

We thank Karla Kirkegaard and Sandra Jacobson for critical reading of the manuscript and Karla Kirkegaard for suggesting the interference assay by superinfection. We are grateful to Sandra Dildine and Bert Semler for generously providing the HeLa-S10 extracts.

This study was supported by Public Health Service grants AI-25105 and AG-07347 from the National Institutes of Health. This work was supported in part by a grant from the Lucille P. Markey Charitable Trust.

REFERENCES

1. Arnold, E., M. Luo, G. Vriend, M. G. Rossmann, A. C. Palmenberg, G. D. Parks, M. J. Nicklin, and E. Wimmer. 1987. Implications of the picornavirus capsid structure for polyprotein processing. *Proc. Natl. Acad. Sci. USA* 84:21–25.
2. Bernstein, H. D., P. Sarnow, and D. Baltimore. 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. *J. Virol.* 60:1040–1049.
3. Bernstein, H. D., N. Sonenberg, and D. Baltimore. 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. *Mol. Cell. Biol.* 5:2913–2923.
4. Bienz, K., D. Egger, and L. Pasamontes. 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. *Virology* 160:220–226.
5. Bienz, K., D. Egger, Y. Rasser, and W. Bossart. 1983. Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures. *Virology* 131:39–48.

6. Bienz, K., D. Egger, M. Troxler, and L. Pasamontes. 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. *J. Virol.* **64**:1156-1163.
7. Dalbadie-McFarland, G., L. W. Cohen, A. D. Riggs, C. Morin, K. Itakura, and J. H. Richards. 1982. Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. *Proc. Natl. Acad. Sci. USA* **79**:6409-6413.
8. Flanagan, J. B., R. F. Pettersson, V. Ambros, M. J. Hewlett, and D. Baltimore. 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5'-terminus of virion and replicative intermediate RNAs of poliovirus. *Proc. Natl. Acad. Sci. USA* **74**:961-965.
9. Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer. 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage of glutamine-glycine pairs. *Proc. Natl. Acad. Sci. USA* **79**:3973-3977.
10. Johnson, K. L., and P. Sarnow. Unpublished data.
11. Kirkegaard, K. 1990. Mutations in VP1 of poliovirus specifically affect both encapsidation and release of viral RNA. *J. Virol.* **64**:195-206.
12. Kirkegaard, K., and D. Baltimore. 1986. The mechanism of RNA recombination in poliovirus. *Cell* **47**:433-443.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
14. Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer. 1977. A protein covalently linked to poliovirus genome RNA. *Proc. Natl. Acad. Sci. USA* **74**:59-63.
15. Li, J.-P., and D. Baltimore. 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. *J. Virol.* **62**:4016-4021.
16. Lomax, N. B., and F. H. Yin. 1989. Evidence for the role of the P2 protein of human rhinovirus in its host range change. *J. Virol.* **63**:2396-2399.
17. Pincus, S. E., and E. Wimmer. 1986. Production of guanidine-resistant and -dependent poliovirus mutants from cloned cDNA: mutations in polypeptide 2C are directly responsible for altered guanidine sensitivity. *J. Virol.* **60**:793-796.
18. Putnak, J. R., and B. A. Phillips. 1981. Picornaviral structure and assembly. *Microbiol. Rev.* **45**:287-315.
19. Rueckert, R. R. 1990. Picornaviridae and their replication, p. 507-548. *In* B. N. Fields and D. M. Knipe (ed.), *Virology*. Raven Press, New York.
20. Sarnow, P. Unpublished data.
21. Sarnow, P. 1989. Role of 3'-end sequences in infectivity of poliovirus transcripts made in vitro. *J. Virol.* **63**:467-470.
22. Sarnow, P., H. D. Bernstein, and D. Baltimore. 1986. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. *Proc. Natl. Acad. Sci. USA* **83**:571-575.
23. Simoes, E. A. F., and P. Sarnow. 1991. An RNA hairpin at the extreme 5' end of the poliovirus RNA genome modulates viral translation in human cells. *J. Virol.* **65**:913-921.
24. Takegami, T., B. L. Semler, C. W. Anderson, and E. Wimmer. 1983. Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. *Virology* **128**:33-47.
25. Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer. 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. *Cell* **45**:761-770.
26. Van Dyke, T. A., and J. B. Flanagan. 1980. Identification of poliovirus polypeptide p63 as a soluble RNA-dependent RNA polymerase. *J. Virol.* **35**:732-740.
27. Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn. 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. *Proc. Natl. Acad. Sci. USA* **82**:1585-1588.
28. Yin, F. H., and N. B. Lomax. 1983. Host range mutants of human rhinovirus in which nonstructural proteins are altered. *J. Virol.* **48**:410-418.
29. Ypma-Wong, M. F., P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semler. 1988. Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. *Virology* **166**:265-270.
30. Ypma-Wong, M. F., and B. L. Semler. 1987. *In vitro* molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. *Nucleic Acids Res.* **15**:2069-2088.