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Inefficient Complementation Activity of Poliovirus 2C and 3D Proteins for Rescue of Lethal Mutations

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Poliovirus (PV) 2C protein is a nonstructural polypeptide involved in viral RNA replication, whose biochemical activity(ies) in this process has not been defined. By using site-directed mutagenesis, it was shown previously that disruption of nucleotide-binding motifs present in this protein abolished viral RNA synthesis (C. Mirzayan and E. Wimmer, Virology 189:547–555, 1992; N. L. Teterina, K. M. Kean, E. Gorbalenya, V. I. Agol, and M. Girard, J. Gen. Virol. 73:1977–1986, 1992). We have tested whether PV 2C or 2BC protein provided in *trans* could rescue the replication of these mutated genomes. Rescuing proteins were provided either by cotransfection with helper chimeric PV-coxsackievirus genomes or by expression in cells with a vaccinia virus-T7 RNA polymerase transient-expression system. We report here that replication of mutated RNAs genomes was poorly supported in *trans* both by helper genomes and by expressed 2C or 2BC proteins. Similarly, very inefficient complementation was observed for two mutated genomes with lethal lesions in 3D polymerase coding sequence. Our results indicate that poliovirus RNA replication shows marked preference for proteins contributed in *cis*.

The molecular events in the process of poliovirus (PV) RNA replication are poorly understood. During replication, the genomic plus-strand RNA is used as a template for synthesis of a complementary minus-strand RNA, which in turn serves as template for more plus-strand RNA synthesis (reviewed in references 40 and 43). The viral polypeptide 3D has been identified as the primer- and RNA-dependent RNA polymerase. It is the only protein required for elongation of RNA chains in vitro (17). Although RNA polymerization is thought to be the primary function of 3D, it may perform other functions related to RNA synthesis (12, 33). Toyoda et al. (47) presented evidence suggesting that 3D was involved in uridylylation of VPg, which is proposed to serve as a primer for RNA synthesis (44). In addition, 3CD protein forms a ribonucleoprotein complex, in association with cellular proteins, with the first 100 nucleotides of the genome; complex formation appears to be required for plus-strand but not minus-strand RNA synthesis (1–3). Virtually all of the other viral nonstructural proteins, as well as some intermediates in the polyprotein-processing reaction, also appear to be involved in viral RNA replication. However, despite numerous genetic and biochemical studies designed to define the specific functions performed by the various proteins in PV RNA replication, the roles of most of the viral proteins in this process remain obscure (reviewed in reference 50).

The viral protein 2C (and/or 2BC) appears to have or participate in multiple functions and to play an essential role in viral RNA replication. In infected cells, 2C sequences are associated with virus-induced membranous vesicles at the site of RNA replication (7, 8). Expression of PV 2C or 2BC protein in the absence of other PV proteins induces major structural reorganization of intracellular membranes and formation of vesicles which resemble those found in PV-infected cells (13). In addition, mutations responsible for alteration of the sensitivity of PV RNA replication to guanidine hydrochloride, a specific inhibitor of viral RNA synthesis, map in the 2C coding region (4, 37–39, 46). Guanidine appears to inhibit an initiation step of RNA synthesis but not the elongation reaction, leading to an accumulation of double-stranded RNA (10). A number of different engineered mutations in 2C were shown to inhibit viral RNA replication (6, 27, 28, 32, 36, 45). An insertion mutation after amino acid 254 of 2C produced a mutant virus with a temperature-sensitive phenotype with impaired single- and double-stranded RNA synthesis at the restrictive temperature (27). Surprisingly, a revertant of this mutant had properties indicating an involvement of the 2C protein in virus particle uncoating (28). Some point mutations in the putative amphipathic helix near the N terminus of 2C abolished minus-strand RNA synthesis as well as causing abnormalities in polyprotein processing (36). Finally, the amino acid sequence of 2C contains sequence elements corresponding to the conserved motifs found in typical nucleoside triphosphate (NTP)-binding proteins (20). This NTP-binding pattern consists of the A motif, a Gly-rich loop preceded by a stretch of several hydrophobic residues and followed by a conserved Gly-Lys-Ser/Thr sequence, and the B motif, an Asp residue followed by an Asp or Glu residue and preceded by a stretch of three to five hydrophobic residues (20, 48). ATP/GTP-binding and -hydrolyzing activities have been shown for 2C or fusion protein derivatives expressed from recombinant DNAs in *Escherichia coli* or Sf9 insect cells. (31, 41). Two groups have reported a genetic analysis of the NTP-binding motifs of PV 2C protein (32, 45). Our results showed that alterations of conserved amino acids in the A or B motif of the NTP-binding pattern were lethal for virus viability. No viral RNA replication could be detected in cells transfected with mutated RNA transcripts. These results suggested that NTP-binding and/or -hydrolyzing activity by the 2C protein is essential for viral RNA replication.

Genetic complementation of mutants with deficiencies in

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viral RNA replication is a tool used to define *cis*- versus *trans*-acting functions of viral proteins. The studies addressing this issue are of two types. The first utilized naturally occurring defective interfering particles or engineered genomes with large deletions. A second approach designed to more precisely define the *cis*-dependent loci in PV RNA replication introduced small lesions in the coding regions of different proteins. For 2C mutants, complementation studies support a model involving multiple functions for 2C and/or 2BC. The insertion mutation at position 254 (27) was readily complemented in *trans*, whereas the data on complementation for mutants with guanidine phenotypes are more complicated. Tolskaya et al. (46) reported intra-allelic complementation for viral strains with different guanidine phenotypes upon mixed infection at intermediate guanidine concentration, restrictive for both viral strains. At the same time, asymmetrical complementation under conditions restrictive to only one of the partners, as seen in previous studies (22), was very poor, as if these mutations are *cis* dominant.

In the present study, we address the question whether the mutations within the 2C NTP-binding motifs, which are lethal for viral RNA replication, could be rescued in vivo by wild-type (wt) 2C provided in *trans*. To this end, we used two methods to provide wt 2C. The results reported here demonstrate that the rescue of the replication of these mutated RNAs is very inefficient.

MATERIALS AND METHODS

Cells and viruses. Human HeLa cells were grown as monolayers at 37°C and 5% CO_2 in Earle's minimal essential medium (MEM; Gibco/BRL) supplemented with 8% fetal bovine serum (Hyclone) and antibiotics (100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B per ml; Gibco/BRL). Recombinant vaccinia viruses vPV2C, vPV2C-K135S, vPV2C-D177G, vPV2BC, vPV2BC-K135S, and vPV2BC-D177G were described previously (13). These viruses and vTF7-3 (18) were amplified in suspension HeLa S3 cells as described by Mackett et al. (29).

Bacterial strains and plasmids. *E. coli* DH5α was used as the host for all plasmids. Transcription vector pT7-PV1-52, harboring a full-length cDNA of PV type 1 (Mahoney strain), was originally described by Marc et al. (30). Construction of the plasmids pG1-2C-K135S, pG1-2C-P131N, pG1-2C-D177G, and pG1-2C-D177E was described previously (45). Plasmid pT7-CPV was constructed by replacement of the *Kpn*I-*Nhe*I fragment (nucleotides [nt] 66 to 2470 in PV cDNA) of plasmid pT7-PV1-52 by the equivalent cDNA fragment of plasmid pCP-305 (24) (gift from B. Semler) to yield a chimeric construct of poliovirus cDNA containing coxsackievirus B3 cDNA sequence from nt 66 to 627. To construct plasmid pT7-3D-μ6432, the *Bgl*II-*Eco*RI fragment from plasmid pMV7-2.9-μ6432 (9) was used to replace the equivalent fragment from plasmid pT7-PV1-52. Plasmid pT220-460 (gift from B. Semler) was constructed by Dildine and Semler (16).

RNA transcription. RNA was synthesized from pT7-PV1-52 or its derivatives linearized with *Eco*RI in 20- to 60-μl reaction mixtures with an Ambion Megascript T7 kit as described in the manufacturer's protocol. Briefly, reaction mixtures contained 7.5 mM each of the four NTPs and 2 μCi of [^{3}H]UTP (NEN) in 20 μl of the transcription buffer-enzyme mixture supplied by the manufacturer. A 1-μg portion of linearized DNA template was used in a 20-μl reaction mixture. Incubations were carried out at 37°C for 4 to 6 h. At the end of incubations, 1 μl of RNase-free DNase I (2 U/μl) was added to the reaction, and incubation was continued for an additional 15 min. The yield of RNA was estimated from measurement of the incorporation of [^{3}H]UTP into trichloroacetic acid-insoluble material. Quick Spin columns (Boehringer Mannheim) were used to remove unincorporated nucleotides. RNA transcripts were extracted with phenol and precipitated with ethanol. For the preparation of a ^{32}P-labeled riboprobe complementary to nt 220 to 460 of the PV RNA plasmid, pT220-460 was linearized with *Bam*HI and transcribed with SP6 polymerase (Promega) in the presence of 0.5 mM ATP, GTP, and UTP, 0.05 mM CTP, and 1 μCi of [α-^{32}P]CTP per μl (3,000 Ci/mmol; NEN) for 45 min at 37°C. The transcription mixture was then treated for 10 min at 37°C with RQ 1 DNase (1 U/μg of DNA; Promega). Labeled RNA was purified on a 6% sequencing gel and eluted for 3 to 4 h at 37°C in buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS).

RNA transfection. RNA transfections were performed with DEAE-dextran (molecular weight, 500,000; Sigma Chemical Co), essentially as described previously (45). Briefly, HeLa cell monolayers were prepared by plating 1.2×10^6 to 1.5×10^6 cells in 60-mm dishes and incubating them for 24 h before transfection. RNA was dissolved in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (42) containing 0.5 U of RNasin per μl. For each dish of cells to be transfected, 1 μg of each RNA in 200 μl of HEPES buffer was mixed with 200 μl of DEAE-dextran at 1 mg/ml in HEPES buffer. Samples were incubated on ice for 30 min. Transfection mixtures were added to each dish, previously rinsed twice with medium without serum. Cells were incubated with transfection mixtures for 30 min at room temperature with constant rocking. After incubation, monolayers were overlaid with MEM and incubated at 37°C. After 1 h, the medium was replaced with MEM supplemented with 3% fetal bovine serum.

RNA extraction and RNase protection. Total cytoplasmic RNA was extracted from cells with guanidinium isothiocyanate as described by Chomcynski and Sacchi (14). Cells in culture dishes were washed once with phosphate-buffered saline and lysed directly with 0.4 ml of lysis buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl). Lysed cells were transferred to a 1.5-ml tube, 40 μl of 2 M sodium acetate (pH 4.5) was added, and samples were extracted with phenol. After ethanol precipitation, RNA was dissolved in buffer A (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). RNase protection assays were performed with an RPA II kit (Ambion) as described by the manufacturer. RNA extracted from approximately 5×10^5 cells was used for one RNase protection experiment. Each sample was mixed with 5×10^4 to 9×10^4 cpm of ^{32}P-labelled probe and coprecipitated by ethanol in the presence of 0.5 M ammonium acetate. Coprecipitated RNAs were resuspended in 20 μl of hybridization buffer (Ambion), denatured by heating for 3 min at 90°C, and incubated for 16 h at 42°C. RNase digestion was carried out as described in the manufacturer's protocol. Protected fragments were separated on a 6% sequencing gel. Quantitation of protected RNA was performed by a Molecular Imager System and Phosphor Analyst software (Bio-Rad).

Expression of recombinant 2C and 2BC proteins. Monolayer HeLa cells were coinfected with vTF7-3 and one of the recombinant vaccinia viruses vPV2C, vPV2BC, or mutant forms at a multiplicity of infection of 10 PFU per cell for each virus. Cells were incubated with the inoculum for 40 min, the inoculum was removed, and complete medium containing 3% fetal bovine serum was added. Expression of recombinant proteins at indicated times after infection was analyzed by Western immunoblot essentially as described previously (13). For metabolic labeling at 8 h after infection, the cells were washed once with MEM without methionine and incubated in this medium for 30 min followed by an additional 1 h in MEM without methionine plus [^{35}S]methionine-cysteine (Translabel; ICN) at 30 μCi/ml (final concentration). The cells were washed once in phosphate-buffered saline and lysed in 200 μl of buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40). Cellular DNA was removed by centrifugation at 13,000 × *g* for 2 min at 4°C. The proteins were analyzed on SDS–10% polyacrylamide gels and autoradiographed at room temperature.

RESULTS

We have shown previously that specific point mutations in the NTP-binding motifs of 2C were lethal for RNA replication and virus growth (45). These lethal mutations included all substitutions at Lys-135 or Asp-177, as well as some mutations at Pro-131. As part of a genetic analysis of 2C function, we were interested in determining whether the functions inactivated by mutations in the NTP-binding motifs could be rescued in *trans* by wt 2C or 2BC proteins or whether these activities were required in *cis*. Two different methods to provide wt 2C in *trans* were used in this study (Fig. 1). In both cases, RNA transcripts containing the 2C mutations were synthesized by T7 RNA polymerase in vitro and were introduced into HeLa cells in the presence or absence of wt 2C sequences. The first method utilized a replicating helper virus RNA to provide wt 2C sequences. The helper virus was a PV-coxsackievirus chimera (24), which produced all the PV proteins but allowed us to distinguish the replication of the two genomes by the use of specific hybridization probes. The second method introduced wt 2C or 2BC proteins via recombinant vaccinia virus vectors, in the absence of other PV proteins or RNA, into cells transfected with the mutated RNAs. The various constructs utilized in these studies are shown schematically in Fig. 2, and the mutations in the 2C protein are described in Table 1.

Inefficient coreplication of mutated 2C RNAs in the presence of helper genome. The chimeric virus PCV-305 (24) contains a PV genome in which nt 1 to 627 in the 5′ nontranslated region (5′ NTR) have been replaced with the corresponding region from coxsackievirus B3. In initial experiments, we in-

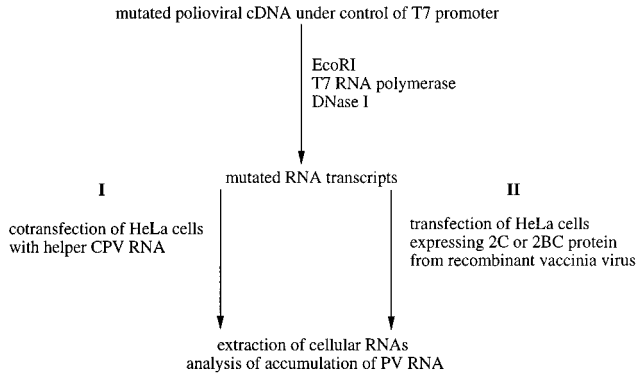

FIG. 1. Two methods used to provide wt 2C and/or 2BC in *trans* with mutated PV RNAs. In method I, CPV chimeric RNA (24) was cotransfected with mutated PV RNA. This helper provides all wild-type PV proteins, while its replication can be distinguished from the replication of mutated PV RNA by the use of probes specific for the 5′ NTR. In method II, expression of PV 2C or 2BC proteins in the absence of other PV proteins or PV RNA was achieved with a recombinant vaccinia virus system.

fected cells with PCV-305 either 1 h before or 1 to 2 h after transfection with RNAs encoding defective 2C proteins. No replication of the transfected mutated genomes was detected by dot blot hybridization. We were concerned, however, that possible differences in the rates and/or compartmentalization of the replication of the two viral RNAs introduced by infection versus transfection might preclude helper function in *trans*. To enable us to introduce both genomes simultaneously by the same procedure, we reconstructed the chimeric 5′ NTR of pCP-305 (plasmid parent of PCV-305) into the full-length cDNA of pT7-PV1-52 (30), which is the parental PV-cDNA plasmid for all mutants in this study. The resulting plasmid, pT7-CPV, is identical to pT7-PV1-52 except for the presence of the coxsackievirus B3 sequence at nt 66 to 627 in the 5′ NTR (Fig. 2). RNA transcripts produced from this plasmid replicated after transfection of HeLa cells at approximately the same rate as did wt transcripts from pT7-PV1-52, and complete cytopathic effects and cell lysis were observed at the same time, about 22 h after transfection (data not shown).

TABLE 1. Mutations in the 2C protein used in this study

Plasmid	Sequence of [a]:		Codon changes
	A-motif	B-motif	
pT7-PV1-52	GSPGTGKS	VVIMDD	
pG1-2C-P131N	GS<u>N</u>GTGKS	VVIMDD	CCC→AAC
pG1-2C-K135S	GSPGTG<u>S</u>S	VVIMDD	AAA→AGC
pG1-2C-D177E	GSPGTGKS	VVIMD<u>E</u>	GAC→GAG
pG1-2C-D177G	GSPGTGKS	VVIMD<u>G</u>	GAC→GGG

[a] Mutations encoded in the plasmids are underlined.

A riboprobe complementary to PV nt 220 to 460 (within the region of coxsackievirus sequence substitution in the helper virus) was used to distinguish the replication of 2C mutant and helper genomes in an RNase protection assay performed on RNA extracted from cells harvested at 10, 15, and 20 h after transfection. This probe hybridized completely with viral RNAs containing the PV 5′ NTR but was imperfectly matched to the corresponding coxsackievirus 5′ NTR sequence. As a result, RNAs containing the PV 5′ NTR protect the entire 240-nt probe sequence (Fig. 3, band indicated PV), whereas chimeric RNAs containing the coxsackievirus 5′ NTR protect only a set of short fragments of the PV probe that result from the presence of a few highly conserved segments of RNA sequence between the two viruses (Fig. 3, bands indicated CPV).

For the coreplication analysis, RNA transcripts coding for the following mutations in the 2C protein NTP-binding motifs were chosen: Pro-131→Asn, Lys-135→Ser (A motif), Asp-177→Glu, and Asp-177→Gly (B motif) (Table 1). These mutations rarely if ever gave rise to revertants (45) and therefore are presumed to be unable to support even low levels of RNA replication by themselves. Cells were transfected with 1 μg of

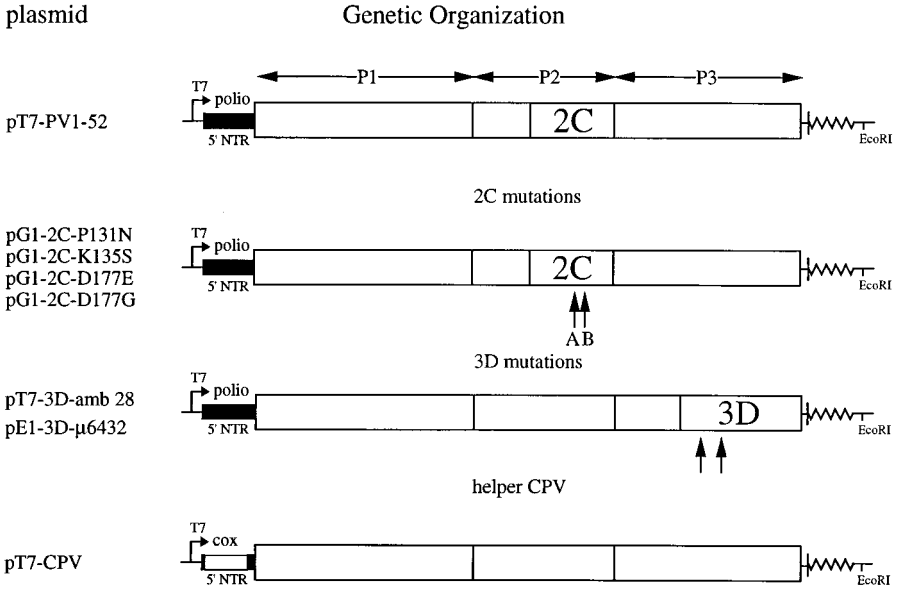

FIG. 2. Schematic representation of PV and various modified genomes used in this study. The promoter for T7 RNA polymerase (T7), the 5′ NTR, coding regions for polyprotein products, the position of 2C and 3D coding sequences, and the 3′ noncoding region and poly(A) tract are indicated. Arrows indicate the positions of mutations in the A or B motif of the NTP-binding pattern in the 2C coding region or of mutation 3D-amb or μ6432 in 3D.

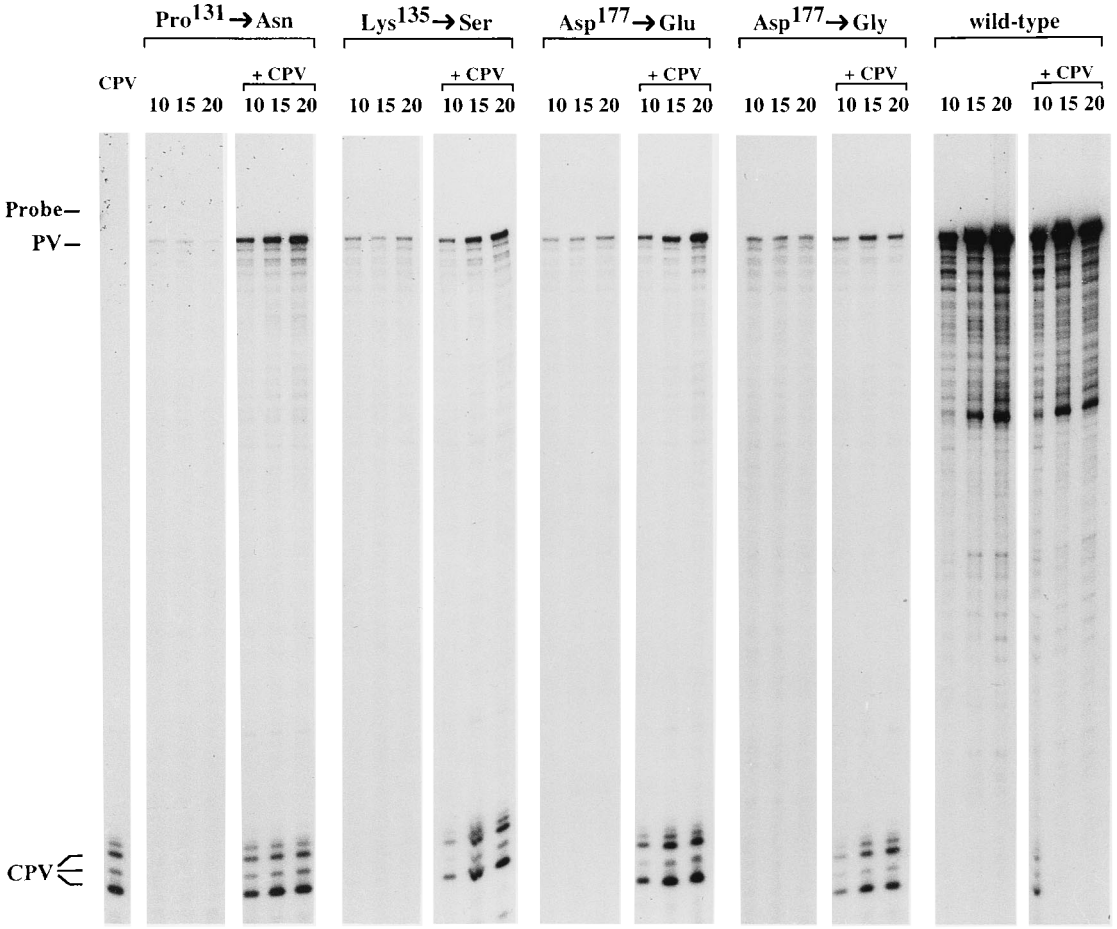

FIG. 3. Accumulation of 2C mutant and CPV helper RNA following cotransfection. HeLa cells were transfected with 1 μg of RNA transcripts coding for PV RNA containing the mutations in 2C protein P131N, K135S, D177E, D177G, or wt PV RNA transcript or cotransfected with these RNA transcripts and helper CPV RNA transcripts. Total cytoplasmic RNAs were extracted at 10, 15, and 20 h after transfection and were subjected to RNase protection with a labeled PV RNA probe complementary to nt 220 to 460 of the positive strand. The 240-nt RNA protected by the PV RNAs and the set of short RNA fragments protected by CPV helper RNA extracted 15 h after transfection are indicated.

mutated RNAs or were cotransfected with the same amount of each mutated and helper CPV transcript. The cells were incubated at 37°C for up to 20 h after transfection, and RNA was extracted and analyzed for the presence of positive-strand viral RNA.

Signals from cells transfected with mutated RNAs alone were low and somewhat variable, perhaps reflecting some variability in initial transfection efficiencies or sample recovery (Fig. 3). No increase in signal occurred over time, however, confirming that the mutated RNAs failed to replicate in transfected cells. Wild-type RNA alone showed efficient replication levels, as expected (Fig. 3, wild-type lanes). Upon cotransfection of mutated 2C RNA transcripts with the CPV helper RNA, an increased signal due to increased RNA levels occurred with time, reflecting low but significant replication of the mutated RNAs in the presence of helper. The level of coreplication varied among the different mutations, with the Pro-131→Asn mutation being most efficiently complemented and the Asp-177→Gly mutation showing hardly any complementation. Lys-135→Ser and Asp-177→Glu mutations showed intermediate levels of replication in the presence of helper. The different levels of mutated RNA accumulations were not the result of different helper RNA levels, which can be detected in the same experiment (Fig. 3). Thus, the mutated RNA genomes did not act as defective interfering RNAs. In all cases, replication of mutated RNAs was significantly lower than the level of replication of wt RNA, which was relatively unaffected by the presence of the chimeric RNA. Replication of the wt PV RNA appeared to reduce the intensity of the signal from the CPV chimeric RNA (Fig. 3). This apparent inhibition was probably due to competition for limiting amounts of probe by the large amounts of viral RNA present in the wt transfected cells, since no such inhibition was detected when measured by dot blot hybridization with CV-specific oligonucleotide probe. Although wt RNA replication in these samples was readily detected by dot blot hybridization, none of the mutated RNAs were detected (results not shown), and the use of the more sensitive RNase protection assay, as shown in Fig. 3, was required to observe the complementation effect.

Quantitation of these data was performed by phosphor image scanning of the autoradiograms. The relative amounts of PV-specific RNA present at 20 h after transfection, when the maximal signal for mutated RNA was observed, are shown in Fig. 4. Comparison of the relative amount of protected PV-specific RNAs in cells transfected with mutated RNAs alone or cotransfected with helper CPV RNA (Fig. 4A) shows up to 10-fold enhancement of synthesis of mutated RNA in the pres-

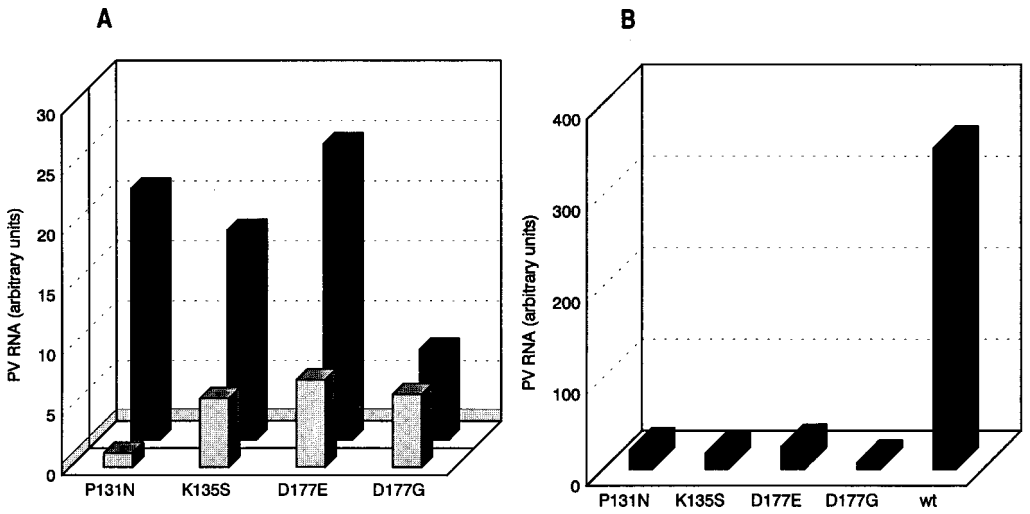

FIG. 4. Relative accumulation of PV-specific RNAs at 20 h after transfection. Autoradiograms of the products of RNase protection were scanned by phosphor imaging. The relative amounts of the PV-specific RNA are shown. (A) Comparison of the relative amounts of protected RNA after transfection with 2C mutant RNAs alone (first row, light bars) or in the presence of helper CPV RNA (second row, dark bars). (B) Comparison of the relative amounts of protected PV-specific RNA after cotransfection with helper CPV RNA for 2C mutant and wt RNA.

ence of helper. However, comparison of the levels of mutated PV-specific RNAs attained during cotransfection with helper with the level of replication of wt RNA under the same conditions (Fig. 4B) shows that the maximal levels of mutated RNAs represent no more than 6% of the level of wt PV RNA. Thus, the rescue of replication of RNAs coding for point mutations in the 2C NTP-binding motifs by coreplication of helper wt 2C sequences occurs but is inefficient.

Inefficient complementation by 2C and 2BC proteins expressed in cells. There was a possibility that the reason for the low level of rescue of the replication of 2C mutated RNAs upon cotransfection with helper was due to low concentrations of this protein in the transfected cells. To address this question, we used a second approach to provide 2C protein in *trans*. 2C protein accumulates in PV-infected cells both as the final cleavage product and in uncleaved precursor forms, mainly as 2BC. Some indirect evidence indicates that the 2BC polypeptide may have some independent function, and the function for which NTP binding is crucial is not known. For this reason, we chose to compare the effect of 2BC, as well as 2C, on the replication of RNAs encoding lethal mutations in 2C protein. Previously, we described construction of recombinant vaccinia viruses vPV2C and vPV2BC, expressing poliovirus 2C or 2BC proteins (13). When HeLa cells were coinfected with one of these recombinant viruses and recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (18), the expression of 2C or 2BC protein was readily detected. At 8 h after infection, more than 80% of cells expressed the protein on the basis of immunofluorescence data. Western blot analysis showed that the level of proteins at this time corresponded to the maximal level in cells infected with PV (at 5.5 h after infection). Although there is no further accumulation of 2C-related proteins in PV-infected cells as the replication cycle is completed, the level of 2C or 2BC proteins continued to increase in cells infected with recombinant vaccinia viruses, so that eventually at least 10-fold more 2C and 2BC proteins was expressed than in the same number of PV-infected cells (13).

To analyze whether the expression of 2C or 2BC proteins would rescue replication of PV RNA coding for mutations in 2C protein, cells were transfected with mutated RNA transcripts 8 h after infection with recombinant vaccinia viruses. Figure 5 shows the expression of protein at this time, analyzed by Western immunoblotting. Moreover, analysis of total cellular proteins labelled at 8 h after infection with recombinant vaccinia viruses showed that 2C and 2BC proteins were among the most abundant proteins synthesized in cells (data not shown).

It should be noted that because T7 RNA polymerase is expressed in cells during vaccinia virus infection, it was important to eliminate all traces of plasmid DNA from prepared RNA transcripts before transfection. For this reason, the effect of expression of proteins 2C and 2BC on replication of mutated RNA was compared with the replication of the same RNA sample in cells infected with vTF7-3 alone. Control experiments with wild-type RNA transcripts confirmed that vac-

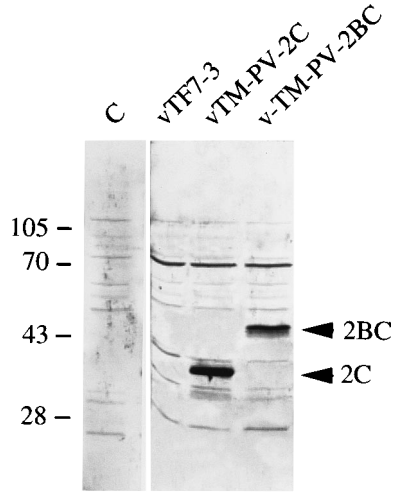

FIG. 5. Immunoblot analysis of proteins in cells infected with recombinant vaccinia viruses expressing PV 2C or 2BC. Extracts of cells infected with the indicated recombinant vaccinia viruses or with no vaccinia virus (lane C) for 8 h were subjected to SDS-polyacrylamide gel electrophoresis before immunoblot analysis with anti-PV 2C serum. Molecular weights of marker proteins are indicated (in thousands) to the left; the positions of PV proteins 2BC and 2C are indicated to the right.

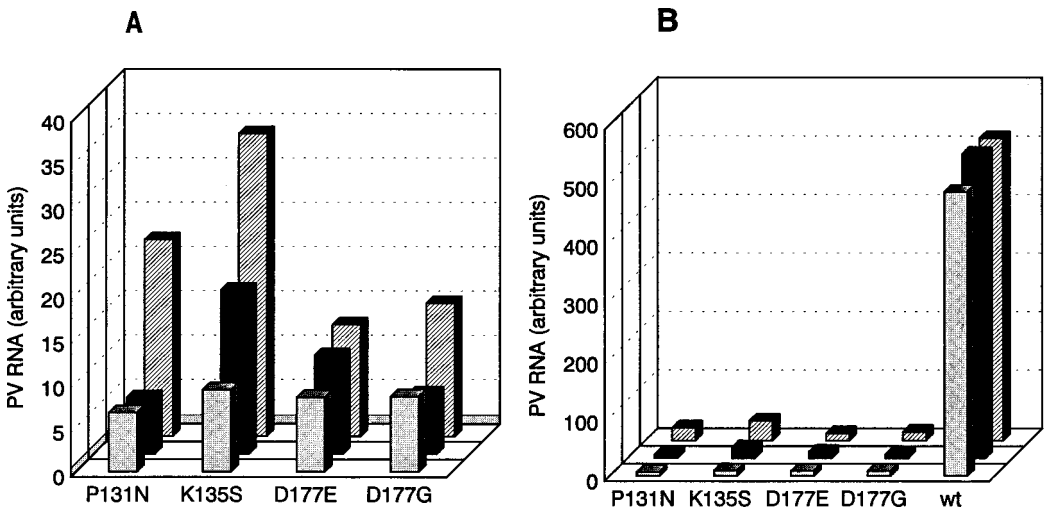

FIG. 6. Relative amounts of PV-specific RNAs in cells infected with recombinant vaccinia viruses. Cells were infected with recombinant vaccinia viruses expressing T7 RNA polymerase (first row [stippled]), PV 2C protein (second row [solid]), or 2BC protein (third row [hatched]). At 8 h after infection, cells were transfected with 2C mutant or wt RNA transcripts. Total RNA was extracted from cells at 15 h after transfection, and the presence of PV-specific RNA was analyzed by RNase protection. The relative intensities of protected fragments were estimated by scanning the autoradiograms of the gel with a phosphor imager. (A) Comparison of the levels of mutated RNAs at 15 h after transfection. (B) Comparison of the levels of mutated and wt RNAs.

cinia virus infection did not interfere with replication of PV RNA after transfection (data not shown). Moreover, we observed no stimulation of viral RNA replication after transfection of vaccinia virus-infected cells. Previously, Pal-Ghosh and Morrow (35) reported increased expression and replication of chimeric PV RNA as well as of PV genomes with lethal mutations in the 2A protein-coding sequence when transfected into vaccinia virus-infected cells. As shown below, no such influence of vaccinia virus infection on replication of genomes bearing mutations in the 2C protein-coding region was observed in these experiments.

Total cytoplasmic RNAs were extracted at 4, 8, 12, and 15 h after transfection and subjected to the RNase protection assay described above, and the gels were scanned to determine the relative amount of protected PV-specific RNAs at 15 h after transfection with each of the mutant RNAs (Fig. 6). Figure 6A shows comparison of the relative amount of each mutant RNA in the cells expressing 2C, 2BC, or T7 RNA polymerase, while the same data are represented in Fig. 6B with respect to the amount of RNA obtained with wt RNA under the same conditions. Consistent with the results obtained with helper CPV RNA, a slight increase in the amount of mutated RNAs present in cells expressing 2C was observed; expression of 2BC protein appeared to enhance replication between two- and sixfold. The stimulation varied for different mutations. However, this enhancement of RNA replication was almost negligible when compared with the level of wt RNA accumulated (Fig. 6B). These results show that the presence of larger amounts of 2C or 2BC protein in the cells did not cause efficient rescue of replication of the RNA coding for the mutation in NTP-binding motifs of 2C protein.

Expression of mutated 2C proteins in cells does not interfere with PV replication. The inefficient replication of PV RNAs encoding altered 2C protein in the presence of protein provided in *trans* could possibly mean that altered forms of 2C are dominant in competing for interaction with RNA and/or other viral and cellular proteins during RNA replication. To address this question, we determined the influence of the expression of some of the altered 2C proteins in recombinant vaccinia viruses on the replication of wt PV. HeLa cells were coinfected with vTF7-3 and one of the recombinant vaccinia viruses vPV2C, vPV2C-K135S, vPV2C-D177G, vPV2BC, vPV2BC-K135S, and vPV2BC-D177G at a multiplicity of infection of 10 PFU per cell for each virus. After incubation for 8 h at 37°C, cells were superinfected with wild-type PV at a multiplicity of infection of 15 PFU per cell. Total cytoplasmic RNA was extracted at 2, 4, 6, and 8 h after PV infection, immobilized on membranes, and hybridized to a PV-specific riboprobe (Fig. 7). In parallel, samples for analysis of 2C-related proteins by Western blot were prepared at 0, 2, 4, 6, and 8 h after PV infection (data not shown). The level of replication of PV RNA in the presence of the altered proteins was compared with the level of replication of viral RNA in cells infected with vTF7-3 virus alone or in cells infected with PV in the absence of vaccinia virus infection. Figure 7 shows that in the presence of mutated 2C (2C-K135S or 2C-D177G) or 2BC (2BC-K135S or 2BC-D177G) proteins, no difference in the amount of RNA synthesis could be detected. Quantitation by phosphor image scanning confirmed this observation (data not shown). Thus, the presence of the altered, inactive forms of 2C protein in the virus-infected cells did not interfere with the replication of wt PV.

Are mutations in other proteins rescued more efficiently at the level of RNA replication? The low levels of observed complementation for mutations in the 2C coding sequence of PV RNA prompted us to ask whether mutations in other PV proteins, demonstrated to be lethal to RNA replication, could be rescued more efficiently in *trans*. We chose two mutations in 3D polymerase to address this question. One, 3D-μ6432 was previously described by Burns et al. (9). This mutation codes for an Ile insertion at position 149 of 3D polymerase. It was shown that this mutation completely abolished polymerase activity but did not interfere with cleavage at the 3C/3D junction or cleavage of P1 by 3CD (9). We reconstructed this mutation in the full-length transcription plasmid pT7-PV-52. The second 3D mutation was recently described by Novak and Kirkegaard (34). It contains an amber codon at position 28 of 3D polymerase. Novak and Kirkegaard showed that replication of RNA transcripts coding for this mutation can be rescued in the

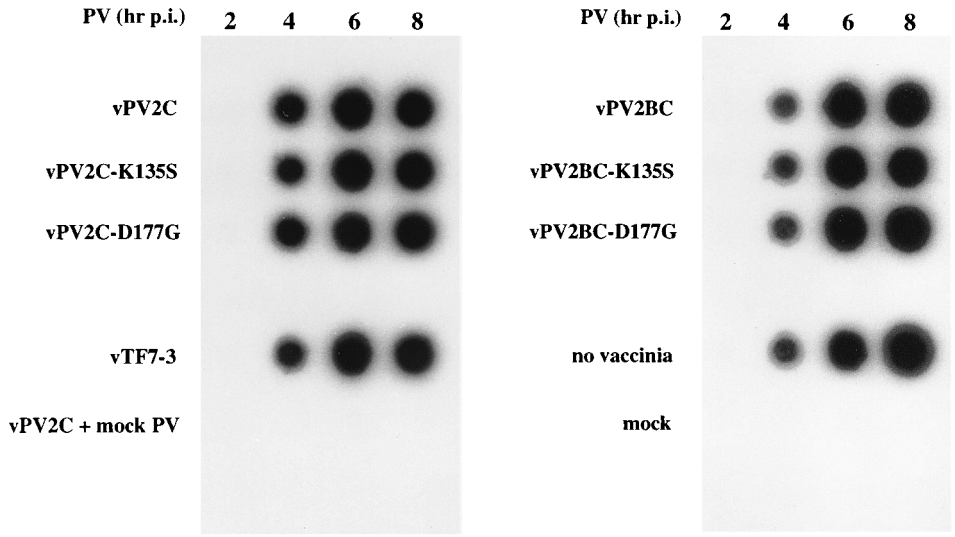

FIG. 7. PV RNA replication in the presence of altered 2C or 2BC protein. HeLa cells were coinfected with recombinant vaccinia viruses vPV2C, vPV2C-K135S, vPV2C-D177G, vPV2BC, vPV2BC-K135S, vPV2BC-D177G, and vTF7-3. At 8 h after infection (hr p.i.), cells were superinfected with PV1 (Mahoney strain). Total cellular RNA was extracted at 2, 4, 6, and 8 h after PV infection, bound to a nylon membrane, and hybridized to a ^{32}P-labeled riboprobe complementary to PV plus-strand RNA.

presence of helper RNA genome. Thus, we utilized this 3D-am28 as a positive control in our experiment.

To determine whether the replication of PV RNAs coding for mutations in 3D protein could be rescued by coreplication of helper RNA, we monitored the accumulation of 3D-am28 or 3D-μ6432 RNAs after cotransfection with helper CPV RNA, as was described above for the 2C mutants. At 10, 15, and 20 h posttransfection, the amount of positive-sense mutated RNA and helper CPV RNA was analyzed by RNase protection (Fig. 8). The production of 3D-specific mutated RNAs in the presence of helper was seen to increase not only 20 h after transfection, as was the case for RNAs encoding defective 2C protein, but also at 15 and even 10 h (compare Fig. 3 and 8). Comparison of the relative quantities of protected fragments, determined by scanning of the gel, is shown in Fig. 9. Although 3D mutant RNA replication was greater than that for the 2C mutants, the level of the mutated RNA in the presence of helper still represented only about 11 to 12% for 3D-μ6432 and less then 10% for 3D-am28.

DISCUSSION

In this study, we performed complementation analysis to determine whether the RNA synthesis defect caused by mutations in 2C protein NTP-binding motifs could be rescued by providing wild-type proteins in *trans*. We have shown that the replication of these mutated RNAs could be rescued in *trans* only inefficiently. This was true whether the defective mutated RNAs were cotransfected with a helper genome or wt PV 2C or 2BC protein were produced in cells by recombinant vaccinia viruses. While the first method provided 2C protein in the form of any potentially functional complexes with other PV-specific proteins, the second led to the presence of larger amounts of protein (2C or 2BC) in the cells. The fact that viral proteins provided by helper CPV RNA were unable to support efficient replication of RNAs encoding defective 2C protein implies that functional 2C protein could not efficiently complement the defect. One possible explanation for the lack of complementation is that the mutations disrupt the structure of the RNA and thereby create *cis*-acting defects. Although this possibility cannot be ruled out as yet, it is not very likely. First, the result was essentially the same for different mutations which have nucleotide changes at different positions. Moreover, different mutations in the same codon, especially in Pro-131, had different impacts on viral RNA replication, and some of them did not abolish this process. Thus, the wt RNA sequence at this particular region is not essential for this process. A more plausible explanation for the inability of the 2C mutant RNA to be replicated in *trans* is that functional replicative proteins could not efficiently interact with RNA.

A previous report by Li and Baltimore (27) described a mutation in 2C protein (mutant 2C-31) that was complemented in *trans*. This mutation placed a 4-amino-acid insertion (REFP) after Ser-254 and produced a temperature-sensitive defect in viral RNA synthesis. A high degree of complementation was estimated for this mutant by plaque assay. The different phenotypes exhibited by mutant 2C-31 and mutants with mutations in NTP-binding motifs might suggest that polypeptide 2C (2BC) participates in different functions required for RNA synthesis, some of which are complementable and some are not. This situation is not unique for PV 2C protein. Efficient complementation was reported for some mutations that mapped in 2A, 3A, and 3D proteins (5, 6, 11, 15), while other mutations in the same proteins were noncomplementable (6, 19, 35). The reason for the differences in complementation susceptibility for these mutations is unknown. It is possible that a given protein with multiple functions disrupted by different mutations is complemented for one function but not for another. Interestingly, a common feature emerges from these data: in all cases when complementation was observed, the mutated genomes retained the capacity for replication, albeit in a defective or conditional-lethal manner, whereas efficient complementation was never reported for nonreplicating mutated genomes. The importance of this difference may be strengthened by data from guanidine-resistant (g^r) and guanidine-dependent (g^d) alleles of the 2C gene. Practically no complementation between guanidine-sensitive (g^s) and g^d or g^r viruses was detected under conditions restrictive for one of the partners (22, 46). In contrast, efficient complementation was

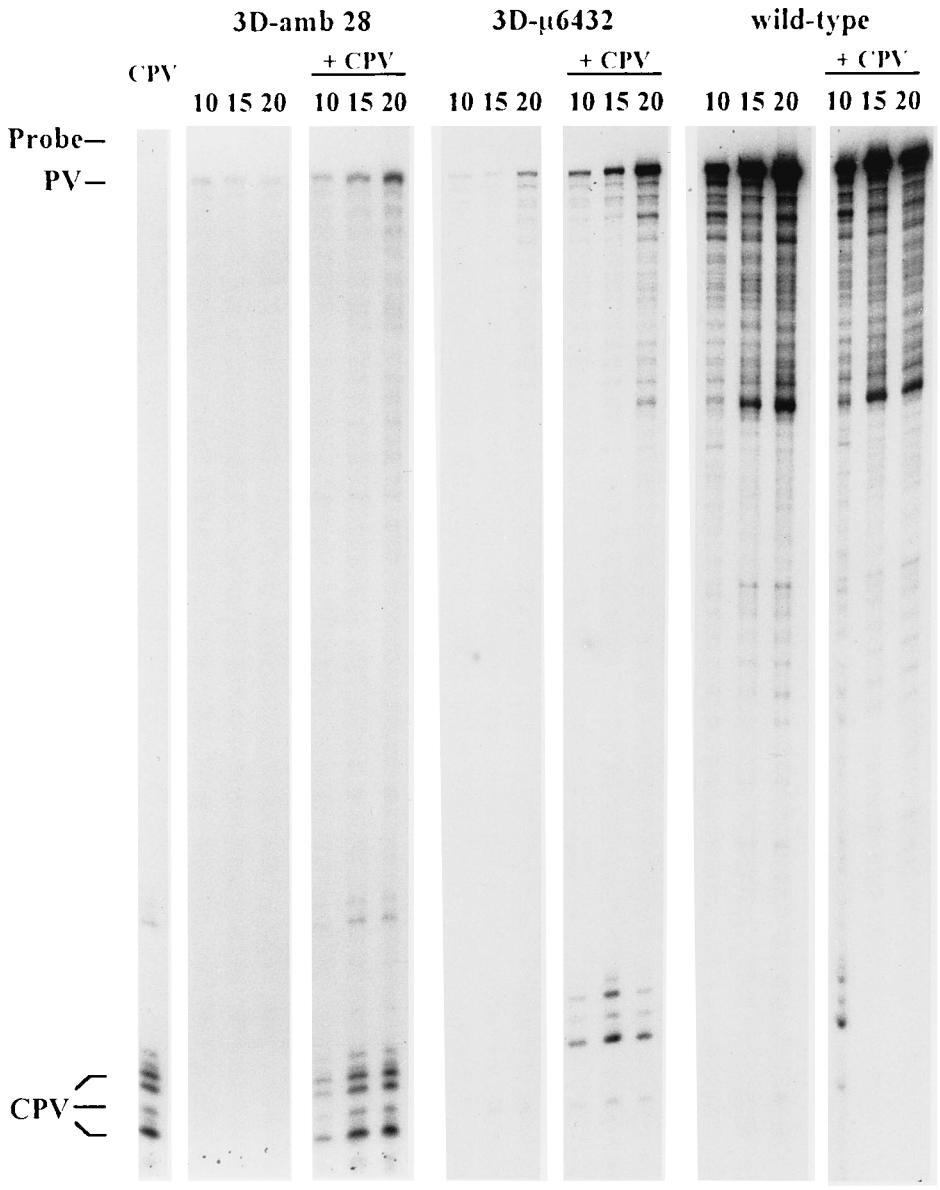

FIG. 8. Accumulation of 3D mutant RNAs following cotransfection with CPV helper RNA. HeLa cells were transfected with 1 μg of RNA transcripts coding for the mutations in 3D protein 3D-am28 or 3D-μ6432 or with wt PV RNA transcript or cotransfected with these RNAs and helper CPV RNA transcript by the DEAE-dextran method. Total cytoplasmic RNAs were extracted at 10, 15, and 20 h after transfection and were subjected to RNase protection with a labeled RNA probe complementary to nt 220 to 460 of the PV positive strand. The 240-nt RNA protected by the PV RNAs and the set of short RNA fragments protected by CPV helper are indicated.

observed under intermediate conditions, partially restrictive for both partners. These data were interpreted as indicating possible oligomerization of 2C protein (46). In this study, we analyzed two lethal mutations in the 3D gene for comparisons of the efficiency of complementation and observed similar low levels of replication to those observed for the replication-negative mutated genomes encoding defective 2C protein in the presence of helper virus.

The first data demonstrating that nonreplicating genomes could not be rescued in *trans* were obtained in studies utilizing naturally occurring defective interfering particles or engineered genomes with large deletions. It was shown that all replicating genomes containing deletions in the capsid coding region preserved the translation reading frame through the deletion junctions (15, 21, 25, 26), whereas no genomes containing out-of-frame deletions were able to replicate, even in the presence of helper, which provided the missing PV proteins in *trans*. When the deletion was extended into the 2A coding region, replication occurred, albeit slowly, in the presence of helper, but further extension of the deletion into the 2B coding region abolished the replication capacity. These data led to the hypothesis that PV RNA replication may depend on translation of the genome in *cis*, although other possible effects of these large deletions on RNA structure or of *trans*-dominant effects of the truncated proteins were not excluded. Recently, Novak and Kirkegaard (34) used a new approach to test the requirement for translation of the viral RNA in *cis*. They compared the replication of amber nonsense mutations in the PV

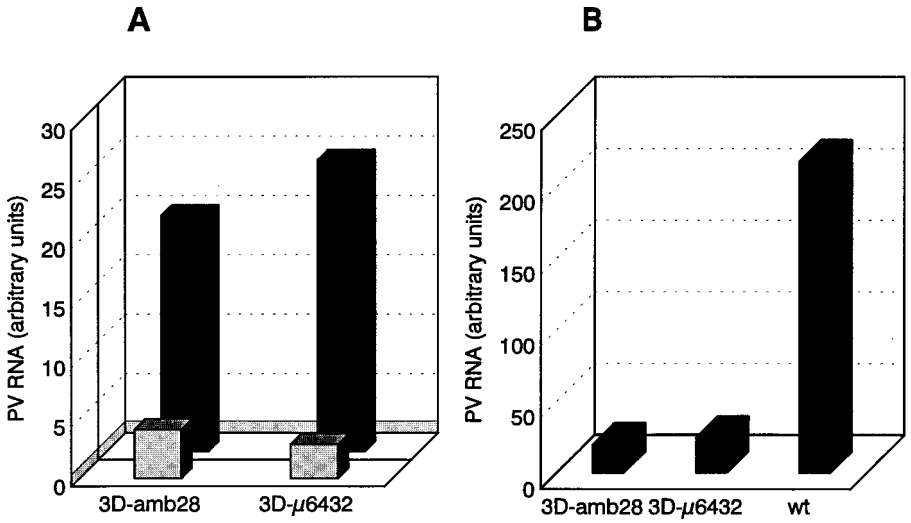

FIG. 9. Relative accumulation of PV-specific RNAs at 20 h after transfection. Autoradiograms of the products of RNase protection were scanned by phosphor imaging. The relative amounts of the PV-specific RNA are shown. (A) Comparison of the relative amounts of protected RNA after transfection with 3D mutant RNAs alone (first row, light bars) or in the presence of helper CPV RNA (second row, dark bars). (B) Comparison of the relative amount of protected PV-specific RNAs after cotransfection with helper CPV RNA for 3D mutant and wt RNA.

genome in nonpermissive cells in the presence of helper genome and in a permissive cell line carrying an amber suppressor. A genome that contained an amber mutation in the 2A coding sequence replicated in amber-suppressing cells, ruling out the possibility that the mutation introduced defects into RNA structure that rendered it unable to replicate. This mutated genome, however, was unable to replicate in the presence of a helper genome which provided all nonstructural proteins. Thus, at least some nonstructural proteins could not be provided in *trans* to support viral RNA replication.

An explanation for the lack of complementability of nonreplicating genomes has not been formulated. One possibility is that nonfunctional proteins are dominant for replication. Such dominance has been reported for 2B protein mutants (23). However, we were unable to detect even competition by altered 2C or 2BC proteins expressed by recombinant vaccinia virus, which might argue against a dominant negative effect. A second possibility is that wild-type proteins produced from the helper viral RNA are not able to physically contact and interact with the mutated template. Since PV RNA replication is a membrane-associated process, replicating RNA templates and the proteins synthesized from them may be sequestered in their membrane environment, so that the probability of interaction of nonreplicating RNA with proteins synthesized from the replicating template is low. A third consideration is that newly synthesized proteins, essential for RNA replication, preferentially interact with the RNA genome from which they have been made, resulting in the channeled formation of a replication initiation complex in *cis*. Precedence for such *cis*-preferential replication was demonstrated previously for turnip yellow mosaic virus RNA (49).

We attempted to overcome some of these potential effects by overexpression of functional 2C or 2BC protein in the absence of replicating helper RNA genome. Regardless of a high level of expression of these proteins, no efficient complementation was detected. Previously, we showed that expression of 2C and 2BC proteins in cells induced membrane rearrangements which resemble those found in PV-infected cells (13). 2C and 2BC proteins were associated with these membrane structures. It remains unclear if membrane-associated proteins are accessible for interaction with RNA or other PV proteins.

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REFERENCES

1. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′- end of viral RNA. EMBO J. **12:**3587–3598.
2. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell **63:**369–380.
3. **Andino, R., G. E. Rieckhof, D. Trono, and D. Baltimore.** 1990. Substitutions in the protease ($3C^{pro}$) gene of poliovirus can suppress a mutation in the 5′ noncoding region. J. Virol. **64:**607–612.
4. **Baltera, R. F., Jr., and D. R. Tershak.** 1989. Guanidine-resistant mutants of poliovirus have distinct mutations in peptide 2C. J. Virol. **63:**4441–4444.
5. **Bernstein, H. D., and D. Baltimore.** 1988. Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. J. Virol. **62:**2922–2928.
6. **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.
7. **Bienz, K., D. Egger, T. Pfister, and M. Troxler.** 1992. Structural and functional characterization of the poliovirus replication complex. J. Virol. **66:** 2740–2747.
8. **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of P2 genomic region. J. Virol. **64:**1156–1163.
9. **Burns, C. C., M. A. Lawson, B. L. Semler, and E. Ehrenfeld.** 1989. Effects of mutations in poliovirus $3D^{pol}$ on polymerase activity and on polyprotein cleavage. J. Virol. **63:**4866–4874.
10. **Caliguiri, L. A., and I. Tamm.** 1973. Guanidine and 2-(α-hydroxybenzyl)-benzimidazole (HBB): selective inhibitors of picornavirus multiplication, p. 257–293. *In* W. A. Carter (ed.), Selective inhibitors of viral functions. CRC Press Inc., Cleveland.
11. **Charini, W. R., C. C. Burns, E. Ehrenfeld, and B. L. Semler.** 1991. *trans* rescue of a mutant poliovirus RNA polymerase function. J. Virol. **65:**2655–2665.
12. **Cho, M. W., O. C. Richards, T. M. Dmitrieva, V. Agol, and E. Ehrenfeld.** 1993. RNA duplex unwinding activity of poliovirus RNA-dependent RNA polymerase $3D^{pol}$. J. Virol. **67:**3010–3018.
13. **Cho, M. W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology **202:**129–145.
14. **Chomcynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation

by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
15. **Collis, P. S., B. J. O'Donnell, D. J. Barton, J. A. Rogers, and J. B. Flanegan.** 1992. Replication of poliovirus RNA and subgenomic RNA transcripts in transfected cells. J. Virol. **66:**6480–6488.
16. **Dildine, S. L., and B. L. Semler.** 1992. Conservation of RNA-protein interactions among picornaviruses. J. Virol. **66:**4364–4376.
17. **Flanegan, J. B., and T. A. Van Dyke.** 1979. Isolation of a soluble and template-dependent poliovirus RNA polymerase that copies virion RNA in vitro. J. Virol. **32:**155–161.
18. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
19. **Giachetti, C., S.-S. Hwang, and B. L. Semler.** 1992. *cis*-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. J. Virol. **66:**6045–6057.
20. **Gorbalenya, A. E., and E. Koonin.** 1989. Viral proteins containing the purine NTP-binding sequence pattern. Nucleic Acids Res. **17:**8413–8440.
21. **Hagino-Yamagishi, K., and A. Nomoto.** 1990. In vitro construction of poliovirus defective-interfering particles. J. Virol. **63:**5386–5392.
22. **Ikegami, N., H. J. Eggers, and I. Tamm.** 1964. Rescue of drug-requiring and drug-inhibited enteroviruses. Proc. Natl. Acad. Sci. USA **52:**1419–1426.
23. **Johnson, K. L., and P. Sarnow.** 1991. Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus. J. Virol. **65:**4341–4349.
24. **Johnson, V. H., and B. L. Semler.** 1988. Defined recombinants of poliovirus and coxsackievirus: sequence-specific deletions and functional substitutions in the 5′ noncoding regions of viral RNAs. Virology **162:**47–57.
25. **Kaplan, G., and V. R. Racaniello.** 1988. Construction and characterization of poliovirus subgenomic replicons. J. Virol. **62:**1678–1696.
26. **Kuge, S., I. Saito, and A. Nomoto.** 1986. Primary structure of poliovirus defective-interfering particle genomes and possible generation mechanisms of the particles. J. Mol. Biol. **192:**473–487.
27. **Li, J.-P., and D. Baltimore.** 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. J. Virol. **62:**4016–4021.
28. **Li, J.-P., and D. Baltimore.** 1990. An intragenic revertant of a poliovirus 2C mutant has an uncoating defect. J. Virol. **64:**1102–1107.
29. **Mackett, M., G. Y. Smith, and B. Moss.** 1985. The construction and characterisation of vaccinia virus recombinants expressing foreign genes, p. 191–211. *In* D. Rickwood and B. D. Hames (ed.), DNA cloning, vol. 2. IRL Press, Washington, D.C.
30. **Marc, D., G. Drugeon, A.-L. Haenni, M. Girard, and S. van der Werf.** 1989. Role of myristoylation of poliovirus capsid protein VP4 as determined by site-directed mutagenesis of its N-terminal sequence. EMBO J. **8:**2661–2668.
31. **Mirzayan, C., and E. Wimmer.** 1994. Biochemical studies on poliovirus polypeptide 2C: evidence for ATPase activity. Virology **199:**176–187.
32. **Mirzayan, C., and E. Wimmer.** 1992. Genetic analysis of an NTP-binding motif in poliovirus polypeptide 2C. Virology **189:**547–555.
33. **Neufeld, K. L., J. M. Galarza, O. C. Richards, D. Summers, and E. Ehrenfeld.** 1994. Identification of terminal adenylyl transferase activity of the poliovirus polymerase $3D^{pol}$. J. Virol. **68:**5811–5818.
34. **Novak, J. E., and K. Kirkegaard.** 1994. Coupling between genome translation and replication in RNA virus. Genes Dev. **8:**1726–1737.
35. **Pal-Ghosh, R., and C. Morrow.** 1993. A poliovirus minireplicon containing an inactive 2A proteinase is expressed in vaccinia virus-infected cells. J. Virol. **67:**4621–4629.
36. **Paul, A. V., A. Molla, and E. Wimmer.** 1994. Studies of a putative amphipathic helix in the N-terminus of poliovirus protein 2C. Virology **199:**188–199.
37. **Pincus, S. E., D. C. Diamond, E. A. Emini, and E. Wimmer.** 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. **57:**638–646.
38. **Pincus, S. E., H. Rohl, and E. Wimmer.** 1987. Guanidine-dependent mutants of poliovirus: identification of three classes with different growth requirements. Virology **157:**83–88.
39. **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.
40. **Richards, O. C., and E. Ehrenfeld.** 1990. Poliovirus RNA replication. Curr. Top. Microbiol. Immunol. **161:**89–119.
41. **Rodríguez, P. L., and L. Carrasco.** 1993. Poliovirus protein 2C has ATPase and GTPase activities. J. Biol. Chem. **268:**8105–8110.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Semler, B. L., R. J. Kuhn, and E. Wimmer.** 1988. Replication of the poliovirus genome, p. 23–48. *In* J. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics, vol. 1. CRC Press, Inc., Boca Raton, Fla.
44. **Takeda, N., R. J. Kuhn, C.-F. Yang, T. Takegami, and E. Wimmer.** 1986. Initiation of poliovirus plus-strand synthesis in a membrane complex of infected HeLa cells. J. Virol. **60:**43–53.
45. **Teterina, N. L., K. M. Kean, E. Gorbalenya, V. I. Agol, and M. Girard.** 1992. Analysis of the functional significance of amino acid residues in the putative NTP-binding pattern of the poliovirus 2C protein. J. Gen. Virol. **73:**1977–1986.
46. **Tolskaya, E. A., L. I. Romanova, M. S. Kolesnikova, A. P. Gmyl, A. E. Gorbalenya, and V. I. Agol.** 1994. Genetic studies on the poliovirus 2C protein, an NTPase—a plausible mechanism of guanidine effect on the 2C function and evidence for the importance of 2C oligomerization. J. Mol. Biol. **236:**1310–1323.
47. **Toyoda, H., C.-F. Yang, N. Takeda, A. Nomoto, and E. Wimmer.** 1987. Analysis of RNA synthesis of type 1 poliovirus by using an in vitro molecular genetic approach. J. Virol. **61:**2816–2822.
48. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP-synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
49. **Weiland, J. J., and T. W. Dreher.** 1993. Cis-preferential replication of the turnip yellow mosaic virus RNA genome. Proc. Natl. Acad. Sci. USA **90:**6095–6099.
50. **Wimmer, E., C. U. T. Hellen, and X. M. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.