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Characterization of Poliovirus Clones Containing Lethal and Nonlethal Mutations in the Genome-Linked Protein VPg

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Viral RNA synthesis was assayed in HeLa cells transfected with nonviable poliovirus RNA mutated in the genome-linked protein VPg-coding region. The transfecting RNA was transcribed in vitro from full-length poliovirus type 1 (Mahoney) cDNA containing a VPg mutagenesis cartridge. Hybridization experiments using ribonucleotide probes specific for the 3′ end of positive- and negative-sense poliovirus RNA indicated that all mutant RNAs encoding a linking tyrosine in position 3 or 4 of VPg were replicated even though no virus was produced. VPg, but no VPg precursor, was found to be linked to the 5′ end of the newly synthesized RNA. Encapsidated mutant RNAs were not found in transfected-cell lysates. After extended maintenance of transfected HeLa cells, a viable revertant of one of the nonviable RNAs was recovered; the revertant lost the lethal lesion in VPg by restoring the wild-type amino acid, but it retained all other nucleotide changes introduced during construction of the mutagenesis cartridge. Mutant RNA encoding phenylalanine or serine rather than tyrosine, the linking amino acid in VPg, was not replicated in transfected cells. A chimeric mutant containing the VPg-coding region of coxsackievirus within the poliovirus genome was viable but displayed impaired multiplication. A poliovirus-coxsackievirus chimera lacking a linking tyrosine in VPg was nonviable and replication-negative. The results indicate that a linkage-competent VPg is necessary for poliovirus RNA synthesis to occur but that a step in poliovirus replication other than initiation of RNA synthesis can be interrupted by lethal mutations in VPg.

Poliovirus consists of a messenger-sense, single-stranded RNA genome approximately 7,440 nucleotides in length packaged in 60 copies each of the capsid proteins VP1, VP2, VP3, and VP4 (14, 32). Poliovirus genomic RNA and mRNA are identical in sequence: they contain a 5′-noncoding region of 742 bases followed by a 6,627-nucleotide open reading frame, a 72-base 3′ untranslated region, and a 3′ polyadenylate tract (14, 30, 45). Both RNA species differ, however, in the structures of their 5′ termini. Whereas viral mRNA terminates in pU, the genomic RNA carries an additional group, the genome-linked viral protein VPg, at the 5′ end (7, 19, 23–25, 27, 28, 44). It has been observed that all newly synthesized viral RNAs are VPg linked (VPg-pU...) (23) but that plus strands destined to become mRNA are unlinked from the 5′-terminal VPg to yield pU..., a process thought to be catalyzed by a cellular enzyme (2, 6).

VPg is a basic, 22-amino-acid oligopeptide that is linked to RNA by a phosphodiester bond between the 5′-terminal uridine residue of the RNA and the O^4-hydroxyl group of tyrosine, the third residue of VPg (1, 31). VPg is not required for infectivity; the RNA is the only component of the virus necessary to initiate a productive infection of a susceptible host cell (44).

VPg is a component of the poliovirus polyprotein (13) and is released from it by two cleavages at glutamine-glycine amino acid pairs (22) (Fig. 1). It is not known at what stages in the replicative cycle these cleavages occur. The cleavage at the C-terminal glutamine of VPg is very rapid and leads, together with upstream proteolytic processing, to the emergence of 3AB (3B is VPg; see reference 32), a polypeptide thought to be a membrane-associated precursor of VPg (34).

The mechanism by which VPg, or a VPg precursor, is linked to RNA has not been identified. Linkage of the protein to positive- and negative-sense RNAs may occur by different mechanisms, since the negative-sense strands contain poly(U) at their 5′ termini, whereas the 5′ termini of positive-sense strands are heteropolymeric.

Despite intensive biochemical and genetic analyses, poliovirus genome replication remains a poorly understood process (17, 36). Replication of poliovirus template RNA in a cell-free system, a means of simplifying the analysis of the role individual proteins and cofactors may play in various stages of RNA replication, has not been achieved. In vitro systems are complicated because genome replication occurs on membranes (9, 21, 26, 37–39). Intracellular poliovirus RNA replication follows the pathway characteristic of other lytic, single-stranded RNA viruses with messenger-sense genomes. However, poliovirus and the other members of the family *Picornaviridae* employ a strategy of gene expression common to only a few plus-strand RNA virus families. Translation of the poliovirus genome yields a single polypeptide that is cleaved by viral proteinases to provide structural and nonstructural polypeptides (10, 22, 41). Among the virus-specific nonstructural proteins is the primer-dependent RNA polymerase, $3D^{pol}$, that is probably involved in the synthesis of negative-sense and positive-sense copies of the genomic RNA (8, 17, 36). Individual steps of RNA synthesis, particularly initiation, are obscure; however, a protein-nucleotidyl moiety containing VPg or a VPg precursor is likely to be generated. Whether this is achieved through uridylylation of polypeptides (37–39) or by cleavage of a polynucleotide chain (8) remains to be seen (discussed in references 17 and 36). Regions of VPg, including the nucleic acid-linking site, are highly conserved among picornaviruses (3) and thus appear to be under strong selective pressure.

In a membranous replication complex, endogenous VPg can be uridylylated in vitro to VPg-pUpU, which can be extended to longer RNA molecules, an observation that was

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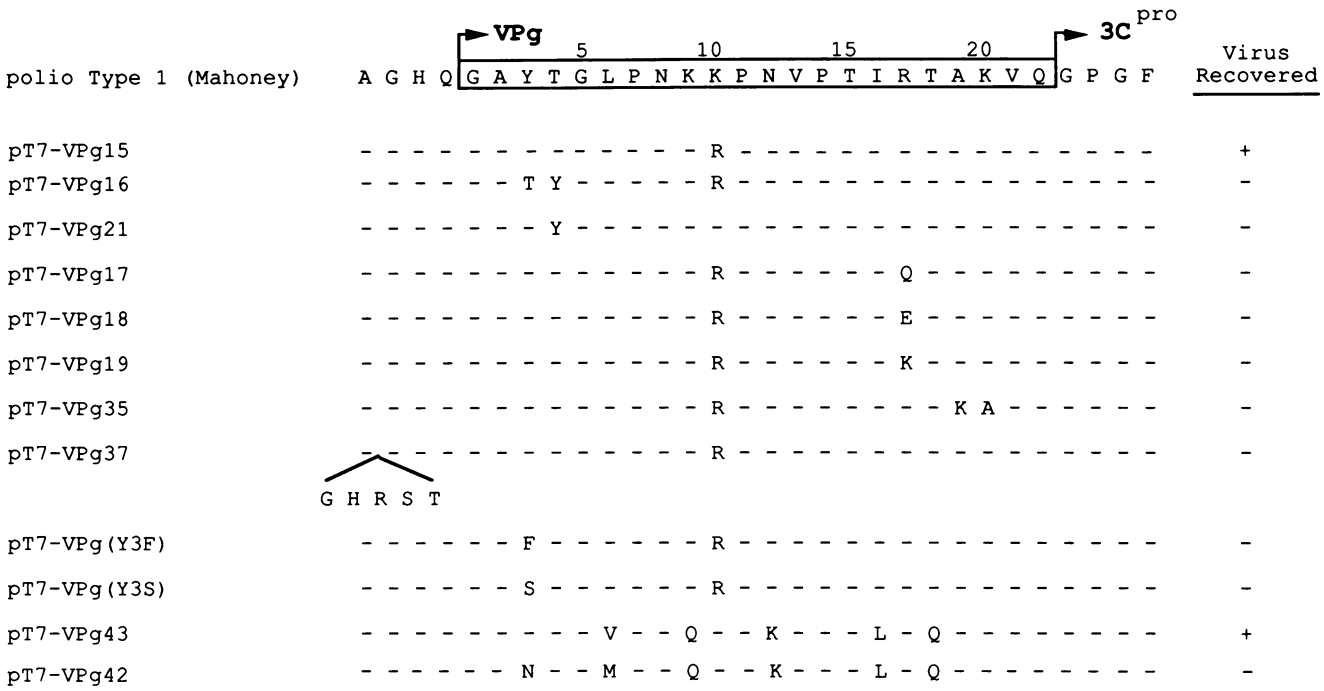


FIG. 1. Schematic representation of poliovirus type 1 (Mahoney) and mutant VPgs. The amino acid sequence of the wild-type VPg is given by the one-letter amino acid code within the boxed region. Mutants are listed below the wild-type sequence, with their amino acid changes replacing the dashed lines. Construct pT7-VPg37 encodes a 5-residue insertion, as indicated. Some of the mutants contain arginine rather than lysine at position 10 of VPg; this conservative substitution was the result of construction of the mutagenesis cartridge. Whether infectious virus was recovered from transfected HeLa cells is shown to the right.

interpreted as suggesting that the uridylylated protein may serve as a primer for RNA-dependent RNA synthesis (23, 37). Priming of genome synthesis with a nucleotidyl polypeptide is not without precedent. For example, nucleotidylated proteins of adenovirus and the DNA bacteriophages ϕ29 and PRD1 serve as a primers in DNA synthesis (12, 44), during which they become linked to the genomic DNAs. Surprisingly, the PRD1 terminal protein shares some amino acid homology with poliovirus VPg (11, 12).

Whether VPg serves as a signal for encapsidation of virion RNA into procapsids or is involved in the maturation cleavage of VP0 to VP4 and VP2 (22) remains to be seen. Whatever the role of VPg in poliovirus replication, the construction of mutants by site-directed mutagenesis seemed a plausible strategy with which to attack the problem. Accordingly, several poliovirus VPg mutants have been generated by Kuhn et al. (15, 16) by using cartridge mutagenesis. Here we report further characterization of these and newly constructed mutants. Although these studies do not allow us as yet to assign a function to VPg, our data suggest that VPg may play a role in RNA synthesis and also in a step of replication that follows RNA synthesis.

MATERIALS AND METHODS

Cells and viruses. Transfections, virus propagations, and assays of viral RNA synthesis and VPg-RNA linkage were performed with R19 HeLa cell monolayers maintained in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum. Poliovirus type 1 (Mahoney strain) stock used in the experiments was a plaque-purified isolate obtained by transfection of COS-1 cells with pT7XL RNA (42) or pEV104 DNA (35). Purification of virions and preparation of viral RNA for sequencing were done as described previously (29).

Construction of plasmids and transfection of cells. The construction of mutant cDNAs by using the VPg mutagenesis cartridge has been described previously (15, 16). Complementary oligonucleotides encoding mutations were inserted into the VPg mutagenesis cartridge contained in the plasmid pNT15. Putative mutants were screened by dideoxy sequencing of plasmid DNA (5, 33). *Bgl*I (nucleotide 5318)-to-*Bgl*II (nucleotide 5601) fragments from mutant clones were used to replace the corresponding fragment in the transcription plasmid pT7-VPg15, which contains a full-length clone of type 1 poliovirus under control of the phage T7 promoter. The sequence of mutants was confirmed by dideoxy sequencing of RNA transcribed from full-length clones (33). Transcription of RNA by T7 RNA polymerase and transfection of cells using DEAE-dextran were done as previously described (16, 42), except that cells were supported in Dulbecco modified Eagle medium containing 5% rather than 10% fetal bovine serum. Mutant RNAs were considered nonviable if no plaque-forming virus was detected by 48 h posttransfection. One-step growth curve experiments with viable mutants were done as previously described (15).

Synthesis of radiolabeled polynucleotide probes. Radioactively labeled probes specific for positive- and negative-sense poliovirus RNA were prepared by transcription from restriction endonuclease-digested plasmids containing poliovirus cDNA downstream from a T7 promoter. Transcription mixtures contained 1 mCi of [α-^{32}P]ATP (specific activity, 650 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per ml. Digestion of 10 μg of pT7PV1-2 with *Pvu*II and subsequent transcription produced a 386-base polyribonucleotide com-

plementary to the 3′ end of positive-sense poliovirus RNA. *Kpn*I digestion of 10 μg of pT7XL, a plasmid derived from pT7PV1-5 that contains a full-length poliovirus cDNA, allowed transcription of a 70-base polyribonucleotide complementary to the 3′ end of negative-sense poliovirus RNA. Labeled probes were purified by passage through 2 ml of Sephadex G-50 (particle size, 20 to 80 μm) columns.

RNA blotting and hybridization. HeLa cell monolayers in 35-mm dishes were transfected with 200 ng of RNA or were mock transfected. As a positive control, some monolayers were transfected with RNA transcribed from plasmid pT7XL or pT7-VPg15. RNA transcribed from pT7-VPg15 encodes arginine rather than lysine at position 10 of VPg, but the multiplication of virus derived from VPg15 RNA is similar to that of wild-type virus, as indicated by one-step growth experiments. RNA lacking a complete RNA polymerase-encoding region was transcribed from *Pvu*II-digested pT7PV1-5 and served as an additional negative control. At various times posttransfection, the growth medium was aspirated and the monolayers were rinsed twice with cold phosphate-buffered saline. Cells were lysed in 200 μl of lysis buffer (10 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.2% [vol/vol] Nonidet P-40). The lysates were clarified by centrifugation at 5,000 × *g* for 5 min, and the RNA was denatured by the addition of 120 μl 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) (20) and 80 μl of 37% (wt/vol) formaldehyde followed by incubation at 60°C for 15 min. RNA was spotted on Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.) with a Micro-Sample filtration manifold (Schleicher & Schuell, Inc., Keene, N.H.) (40). Prehybridization of membranes was done in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA) (20), 10× Denhardt reagent (1× Denhardt reagent is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) (20), 0.75% sodium dodecyl sulfate (SDS), and 50 μg of herring sperm DNA per ml for 2 h at 42°C. The prehybridization solution was then replaced with hybridization solution (6× SSPE, 0.75% SDS, 50% [vol/vol] formamide, 50 μg of herring sperm DNA per ml) containing 5×10^7 cpm of radiolabeled probe. Hybridization was done at 59°C for 16 h. After two 15-min washes in 6× SSPE–0.3% SDS at room temperature and two 15-min washes in 1× SSPE–0.75% SDS at 37°C, the degree of hybridization was measured by exposure of the membranes to XAR-2 film (Eastman Kodak Co., Rochester, N.Y.).

Labeling of VPg-pUp. HeLa cell monolayers in 10-cm dishes were transfected with 2 μg of RNA or were mock transfected and incubated at 37°C. Nine hours posttransfection, the medium was aspirated and replaced with medium containing 2.5 μg of dactinomycin per ml. Fifteen minutes later, $^{32}P_i$ was added to a concentration of 2 mCi/ml. At 20 h posttransfection, the medium was aspirated, the monolayers were rinsed once with cold phosphate-buffered saline, and the cells were lifted from the plates with trypsin. Cells were washed once with cold phosphate-buffered saline and lysed in 200 μl of lysis buffer. Clarified lysates were extracted once with phenol, ethanol precipitated, and treated with 0.5 mg of RNase A per ml and 0.2 mg of RNase T_1 per ml for 30 min at 37°C. Immunoprecipitations were done with antiserum specific for VPg or 3A as previously described (39). However, protein A-Sepharose was used in place of *Staphylococcus aureus* cells. A VPg marker was prepared by labeling a 100-ml suspension culture (4.5×10^5 cells per ml) with 5 μCi of [^{14}C]lysine per ml 45 min after infection with PV1. At 6 h postinfection, the ^{14}C-labeled cells were harvested, and poliovirions were gradient purified as previously described (29). The virions were denatured and treated with RNase A and RNase T_1 as described above, and ^{14}C-labeled VPg-pUp was immunoprecipitated with VPg-specific antiserum. Immunoprecipitated material was denatured for 5 min at 65°C in loading buffer and electrophoresed in 13.5% bisacrylamide-cross-linked polyacrylamide gels (18). Gels were fixed in 12% (vol/vol) acetic acid–40% (vol/vol) methanol, dried, and exposed to Kodak X-Omat AR film.

Fractionation of viral RNA and poliovirions. R19 HeLa cells in 6-cm dishes were transfected with 2 μg of RNA transcribed from pT7-VPg16, -17, -21, and -35. Cells were labeled with $^{32}P_i$ at a concentration of 125 μCi/ml as described above. Clarified lysates were layered onto 30-ml 15 to 30% sucrose gradients containing a top layer of 1% deoxycholate, 1% Brij 58, and 10% sucrose in reticulocyte standard buffer plus Mg^{2+} (25). The gradients were centrifuged in an SW28 rotor at 53,000 × *g* for 16.7 h when migration of RNA was assayed and at 89,000 × *g* for 2.5 h when migration of poliovirions was assayed. Gradients were fractionated, and trichloroacetic acid-precipitable counts were measured. CsCl gradient-purified poliovirions, phenol-chloroform-extracted virion RNA, and HeLa cell rRNA were used as markers in the sucrose gradients.

RESULTS

Accumulation of poliovirus RNAs in transfected HeLa cells. To determine the effect of mutations in VPg on RNA replication, HeLa cells were transfected with full-length transcripts, and at various times after transfection total intracellular RNAs were recovered, denatured with formaldehyde, and immobilized on membranes. The immobilized RNAs were separately identified by hybridization with ^{32}P-labeled polyribonucleotide probes as described in Materials and Methods. The probes were specific for the detection of negative- and positive-sense RNA and allowed analysis of the kinetics of synthesis of the two species of viral RNA.

Dot blot autoradiograms are shown in Fig. 2A. RNAs of mock-transfected cells yielded barely detectable dots. As another negative control, RNAs of cells transfected with transcripts from *Pvu*II-digested pT7XL, in which a portion of $3D^{pol}$ and all of the 3′-nontranslated region are deleted, yielded dots of an intensity similar to that of the dots of mock-transfected cells, an observation confirming that the remaining genetic elements were unable to direct synthesis of viral RNA and that the amount of transfecting RNA by itself was too low to yield a signal under the conditions applied. On the other hand, all mutant RNAs, except VPg42, can direct viral RNA replication. VPg15 is a variant without phenotype, which was expected since the K10R change yields a VPg that occurs in type 2 and type 3 polioviruses (15, 16), whereas none of the mutant RNAs except VPg43 yielded viable virus in HeLa cells. It was apparent from the hybridization signal intensities that in transfections with nonviable mutants there was a lag period before RNA replication commenced, and overall less RNA was synthesized in comparison with PV1 (Mahoney) or VPg15.

Of particular interest to us were mutations of tyrosine 3, the amino acid that links VPg to the viral genome. Among these was VPg16, which carries an inversion of amino acids 3 and 4 (TY instead YT). Apparently, this mutation allows RNA replication, albeit at an impaired level (Fig. 2A). Absence of a tyrosine residue, as in mutant VPg42, abolished RNA synthesis (Fig. 2A). Since VPg42 had additional amino acid replacements, we constructed mutants in which

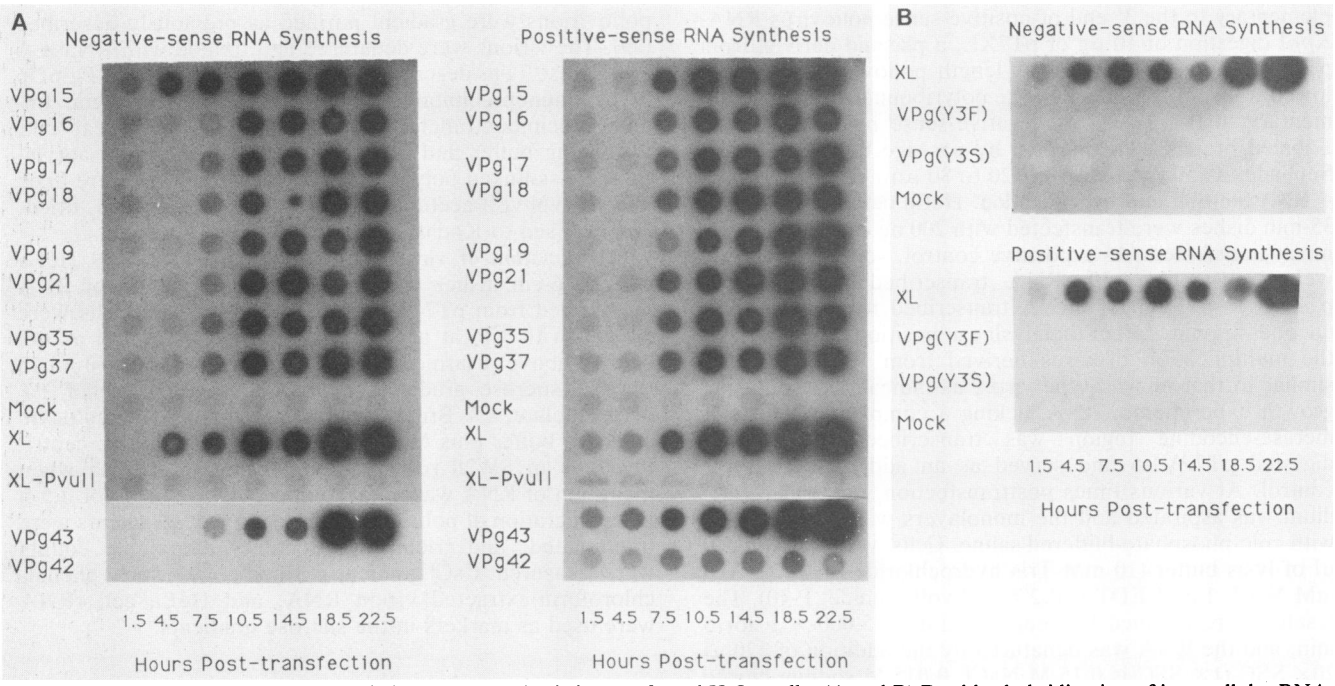


FIG. 2. Quantitative comparison of viral RNA synthesis in transfected HeLa cells. (A and B) Dot blot hybridization of intracellular RNA harvested at various times posttransfection. RNA samples were denatured, spotted on nylon membranes, and probed with equal levels of ^{32}P-labeled polyribonucleotides specific for the 3′ end of negative- and positive-sense poliovirus RNA. The initial number of cells in lysates of all samples was 10^6.

only the third residue of VPg was changed to phenylalanine [VPg(Y3F)] or serine [VPg(Y3S)]. Neither VPg(Y3S)- nor VPg(Y3F)-transfected cells displayed viral RNA synthesis (Fig. 2B), an observation proving the pivotal role of the amino acid in position 3 of VPg.

Is the viral RNA linked to VPg? To determine whether viral RNAs synthesized in transfected HeLa cells were linked at their 5′ termini to VPg, it was necessary to prepare poliovirus RNA labeled to high specific activity with ^{32}P. After phenol-chloroform extraction to remove proteins not covalently bound to the RNA (including intracellular free VPg-pUpU; see below), the labeled material was digested with a mixture of RNases T_1 and A, two enzymes that degrade the RNA to mono- and oligonucleotides with 3′ phosphates. If VPg was linked to newly synthesized RNA in transfected cells, it could be identified as [^{32}P]VPg-pUp after RNase treatment (since the 5′ end of VPg-linked RNA is VPg-pUpU...) through immunoprecipitation and polyacrylamide gel electrophoresis. Immunoprecipitations were carried out with antisera specific for VPg or 3A (39). Immunoprecipitation with antiserum specific for 3A did not yield a detectable labeled product (Fig. 3A). RNAs linked to a VPg precursor were not lost during phenol extraction of cell lysates (e.g., 3AB-RNA entering the phenol phase), as unextracted material examined in parallel with extracted samples also failed to yield ^{32}P-labeled material recognized by anti-3A (data not shown). All the nonviable VPg mutants yielded material immunoprecipitated by anti-VPg that migrated to the same position as [^{14}C]-lysine-labeled marker VPg-pUp (Fig. 3B). The immunoprecipitated VPg-pUp is not derived from free VPg-pUpU in the transfected cells, as extraction with phenol-chloroform removed all VPg-pUpU from the lysates, a phenomenon described previously by Ambros and Baltimore (1, 2). Accordingly, immunoprecipitations with anti-VPg sera after phenol-chloroform extraction but before nuclease digestion did not yield VPg-pUpU in our experiments (data not shown).

Assays for encapsidation of RNA. We next analyzed the nature of the viral RNAs in transfected cells and whether virus particles could be detected in gradients. For this purpose, cellular lysates of HeLa and COS-1 cells transfected with nonviable mutant RNAs were harvested at 20 h posttransfection and analyzed on CsCl gradients. None of the mutants yielded virions or empty capsids (data not shown). Total RNAs of ^{32}P-labeled cells transfected with VPg16, -17, -21, and -35 were fractionated on 15 to 30% sucrose gradients. Peaks of radioactivity corresponding to free viral RNA were observed (Fig. 4A and B). However, no virions were detected in cells transfected with the replication-positive, nonviable RNAs (Fig. 4C).

HeLa cells transfected with replication-positive, virus-negative RNA were labeled with [^{35}S]methionine from 8 to 20 h posttransfection in an attempt to analyze the proteolytic processing of virus-specific proteins translated from the nonviable RNAs. Whereas poliovirus proteins could be detected by immunoprecipitation from extracts of cells transfected with wild-type poliovirus RNA, no viral proteins were observed when nonviable mutant RNAs were used (data not shown). This observation suggests that viral protein synthesis may be severely impaired in mutant RNA-transfected cells.

Four to six days after transfection with VPg16 and VPg18 transcripts, a small percentage of COS-1 cells in transfected monolayers began to display cytopathic effect characteristic of cells transfected with wild-type RNA, although no plaque-forming particles could be recovered (data not shown). The reason for the cytopathogenic effect is not known.

Recovery of a viable revertant. HeLa and COS-1 cells transfected with the nonviable VPg mutant RNAs were maintained for extended intervals to encourage production

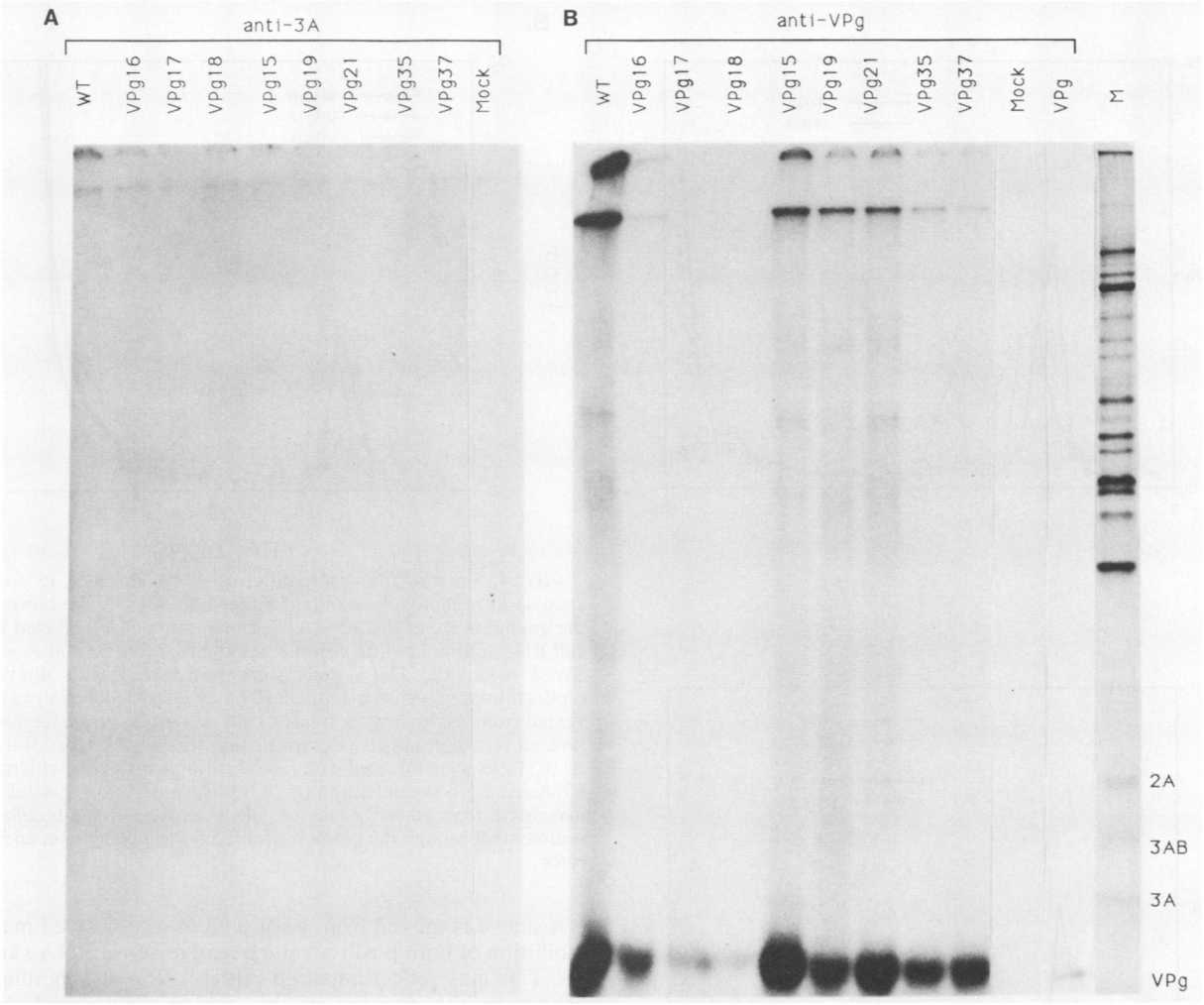


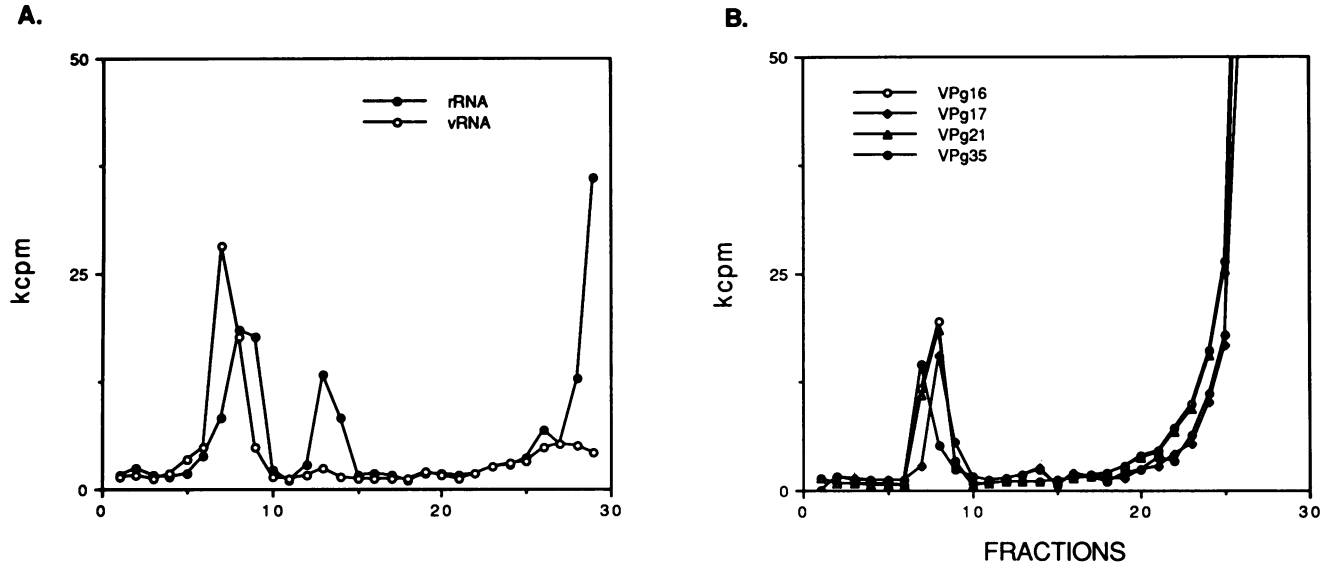
FIG. 3. Immunoprecipitation of poliovirus-specific proteins by VPg- and 3A-specific antiserum. Lysates were prepared from transfected cells labeled with $^{32}P_i$. The lysates were phenol extracted and treated with RNase A and RNase T_1. Immunoprecipitations were done with antiserum specific for 3A (A) or VPg (B), and immunoprecipitated material was electrophoresed in a 13.5% SDS–polyacrylamide gel. [^{14}C]lysine-labeled VPg and [^{35}S]methionine-labeled poliovirus-infected HeLa cell extract are used as markers. The positions of 2A, 3AB, 3A, and VPg are indicated to the right.

of viable revertants. We have recovered infectious virus 72 h posttransfection from HeLa cells transfected with VPg17 RNA (Fig. 5). VPg17 was generated by introducing a G-to-A substitution in the position 17 codon of the VPg-coding region, a mutation resulting in an arginine-to-glutamine substitution. The RNA sequence of the viable revertant retained the nucleotide changes used to introduce restriction sites into the mutagenesis cartridge but possessed the wild-type codon at position 17 and thus has the genotype of VPg15. Indeed, the plaque size of the revertant was similar to that of VPg15 and XL virus (data not shown).

Substitution of coxsackievirus VPg for poliovirus VPg. The replacement of poliovirus VPg with that of coxsackie B virus yielded VPg43, a viable chimeric virus. The VPg of this virus contained amino acids of CBV1, CBV3, and CBV4, a fortuitous result of construction of the mutagenesis cartridge (possibly due to mixtures in the oligonucleotides used) and/or molecular cloning. Specifically, the valine residue at position 6 corresponds to the VPg of coxsackievirus B3, while the lysine residue at position 12 is found in the VPg of coxsackieviruses B1 and B4. Cells transfected with chimeric transcripts displayed cytopathic effect at 40 h posttransfection and were completely lysed by 60 h posttransfection. VPg43 had a small-plaque phenotype (data not shown) and replicated less efficiently than PV1 in one-step growth curve experiments (Fig. 6). Production of VPg43 virus was 100 to 1,000 times lower than that of PV1 during the replication cycle. As mentioned above, VPg42, a mutant with amino acid replacements similar to those of VPg43 but with an asparagine in position 3, was nonviable.

DISCUSSION

A poliovirus cDNA containing a mutagenesis cartridge spanning the VPg-encoding region permitted rapid generation of site-specific mutations by insertion of complementary oligonucleotides at unique restriction sites. Previous mutational analyses of VPg function have identified amino acids in the protein that can be replaced without loss of virus production by transfected cells. However, generation of a large number of nonviable mutants demonstrated that certain amino acids must be conserved if viability is to be



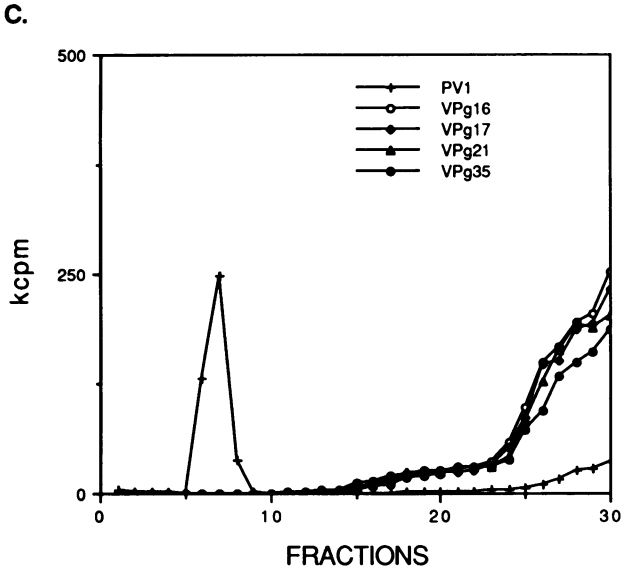


FIG. 4. Fractionation of poliovirus RNA through 15 to 30% sucrose in reticulocyte standard buffer plus Mg^{2+}. The bottoms of the gradients are at the left. (A) Sedimentation of ^{32}P-labeled HeLa cell RNA and poliovirus virion RNA for 16.7 h at 53,000 × *g* in an SW28 rotor. (B) HeLa cells were transfected with nonviable, replication-positive VPg mutant RNA. ^{32}P-labeled RNA was harvested from dactinomycin treated cells at 18 h posttransfection and layered on the gradients. Centrifugation was as described for panel A. (C) Cells were infected with poliovirus or transfected with mutant RNA. At 7.5 h postinfection or 20 h posttransfection, lysates were harvested from the ^{32}P-labeled, dactinomycin-treated cells and sedimented through the gradients for 2.5 h at 89,000 × *g* in an SW28 rotor.

maintained. Nonviable VPg mutants, none of which displayed a temperature-sensitive phenotype, were used in this study to identify the step(s) in poliovirus replication that are impaired or halted by changes of these amino acids.

To assay viral RNA synthesis in cells transfected with RNAs encoding mutations in VPg, intracellular RNA was harvested at various times posttransfection and probed with poliovirus-specific polyribonucleotides. All nonviable RNAs that encoded tyrosine in position 3 or 4 of VPg were replicated in transfected HeLa cells. With most mutants, selective inhibition against positive- or negative-sense RNA synthesis was not apparent, although we repeatedly observed a lag phase before RNA synthesis in cells transfected with nonviable, RNA-positive mutants, and this lag appeared to be more pronounced for negative-sense RNA. Some of the mutants overcame the initial delay and approached the level of RNA synthesis displayed by cells transfected with wild-type transcripts. VPg16, in which the tyrosine was moved from position 3 to 4, displayed marked inhibition of both positive- and negative-sense RNA synthesis. This may reflect impaired uridylylation of VPg due to a change in the position of the linking amino acid and/or defective priming of viral RNA synthesis. Overall, the poliovirus RNA replication system displayed a remarkable degree of tolerance for variation in VPg sequence, as long as a tyrosine was available for linkage of the protein to RNA. The high rate of RNA synthesis shown by the mutants cannot be explained by reversion of the RNA sequences to the wild type, as assays of cell medium collected during the hybridization experiments did not detect plaque-forming virus (data not shown). However, in separate experiments, a viable revertant has been recovered from HeLa cells transfected with VPg17 RNA after long-term incubation of the plates. The revertant RNA retained the nucleotide changes resulting from formation of unique restriction sites in the VPg mutagenesis cartridge and encoded VPg molecules with the primary structure of VPg15. We are currently searching for revertants of other nonviable mutants. The low rate of reversion exhibited by the nonviable, replication-positive mutants has yet to be explained. Thus far, all attempts to find second-site revertants have failed.

Labeling experiments revealed that VPg was linked to the 5′ ends of the replication-positive, nonviable RNAs. The presumed precursor of VPg, 3AB, was not found at the 5′ end of the nonviable RNAs synthesized in transfected cells. If 3AB is involved in replication at all, cleavage of this polypeptide may occur concomitant with, or before, uridylylation of VPg.

Mutations that prevent VPg uridylylation (and thus link-

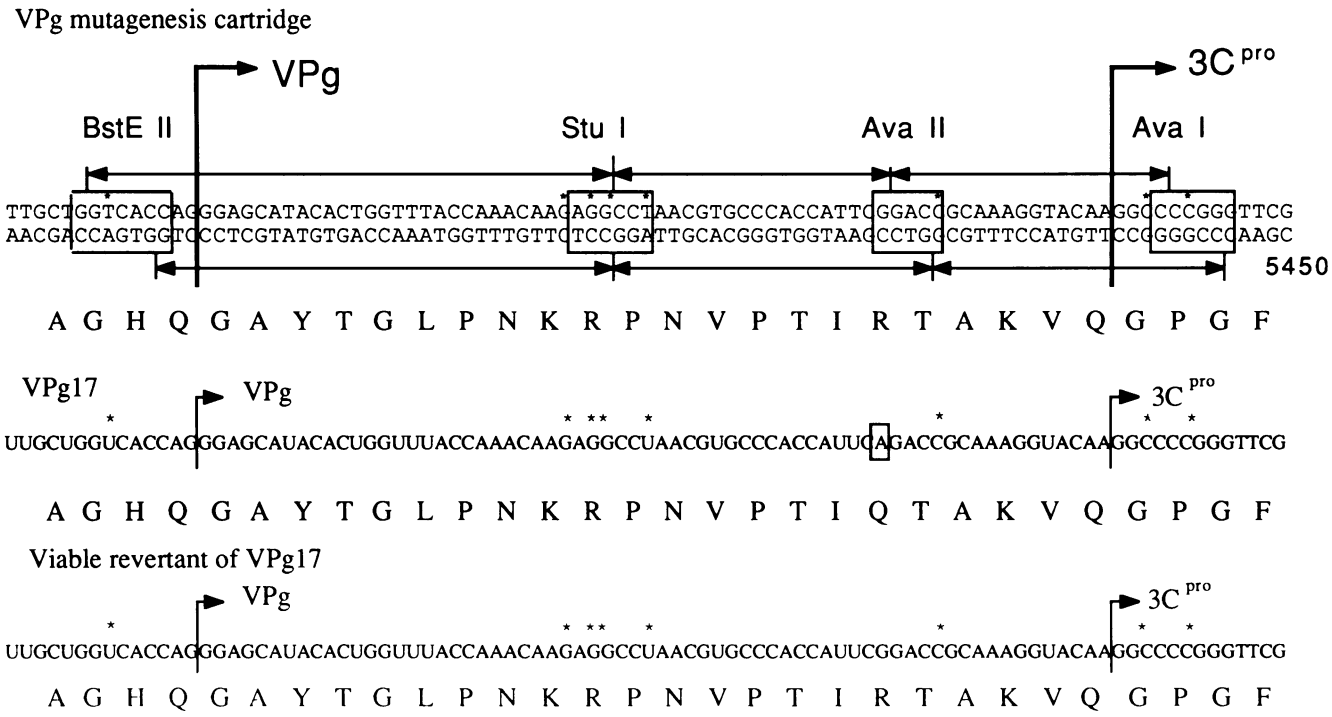


FIG. 5. Structure of the VPg mutagenesis cartridge and the nucleotide sequence of nonviable VPg17 RNA and the viable VPg17-specific revertant. Nucleotide sequences shown correspond to nucleotides 5357 through 5450 of the poliovirus type 1 (Mahoney) genome. Nucleotide substitutions resulting from introduction of restriction sites into the mutagenesis cartridge are denoted by asterisks above the nucleotides. The G-to-A nucleotide substitution used to generate VPg17 is boxed in the VPg17 sequence. The amino acid sequence of the corresponding region of the PV1 polyprotein is given below each nucleotide sequence in the one-letter amino acid code.

age of VPg to RNA) abolish synthesis of viral RNA in transfected cells, and this was found to be true for the three amino acid replacements Y3F, Y3S, and Y3N. Mutant VPg(Y3S) was particularly interesting to us because serine is

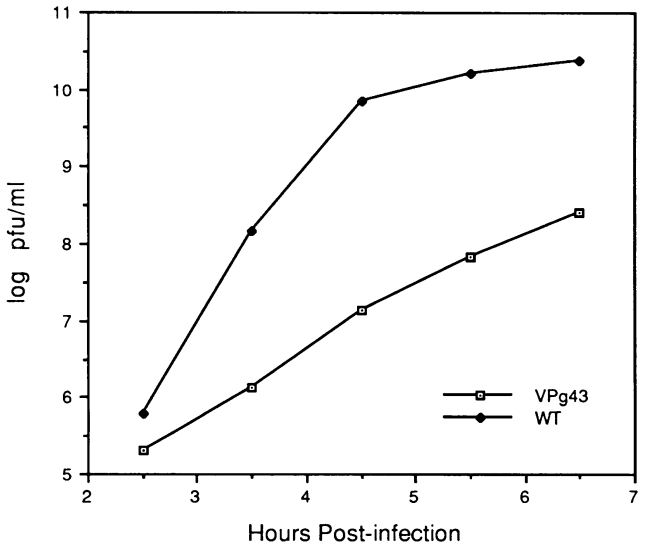


FIG. 6. One-step growth curve of poliovirus type 1 (Mahoney) and the VPg43 mutant virus. HeLa cell monolayers were infected at a multiplicity of infection of 25 and incubated at 37°C. At various times postinfection, cells were harvested and virus production was measured by plaque assay. Data are plotted as the virus titer ($\log_{10}$ PFU per milliliter) versus hours postinfection.

the linking amino acid in VPg of cowpea mosaic virus RNA (46) and also in the terminal protein of adenovirus DNA (4). However, the poliovirus replication system does not appear to allow substitution of tyrosine with serine. VPg(T3F) was undoubtedly nonviable and RNA replication defective because of the absence of a phenolic hydroxyl group with which a phosphodiester could be formed with the 5'-terminal uridylic acid. An essential role for tyrosine is likely for all picornavirus VPgs, since in all cases the tyrosine in position 3 is conserved (3). Together with the analyses of the 5' termini of viral RNA isolated after transfection, these results support the hypothesis that a uridylylation-competent VPg is essential in poliovirus RNA synthesis.

A function of VPg other than involvement in RNA synthesis may be defective in the mutants that were RNA replication positive but nonviable. Genomic masking experiments are under way to examine the possible role of VPg in the encapsidation of viral RNA (43). The hypothesis to be tested is whether VPg can serve as a specific signal for packaging of RNA into capsids. We must entertain, however, a more trivial explanation, which is that defective virus-specific protein synthesis in mutant-transfected cells may result in levels of capsid proteins inadequate for production of virions. Indeed, [^{35}S]methionine-labeled viral proteins could not be detected in immunoprecipitates of cells transfected with the RNA replication-positive, nonviable mutants.

Mutants VPg37 and VPg35 are the most difficult to comprehend. VPg37 contains an insertion in polypeptide 3A, and its VPg is identical to that of VPg15, which replicates with wild-type kinetics. Since transfection with VPg37 produced VPg15-like, VPg-linked RNA, why was this RNA not encap-

sidated? The insertion in 3A may result in defective interaction of 3AB with the membranous replication complex; the perturbation of 3AB function could be responsible for an impairment in encapsidation. In VPg35, the cleavage site AXXQ*G at the C terminus of VPg was changed to KXXQ*G, which is considered the least favorable of all cleavage sequences (22). This mutant produced VPg-linked RNA; however, as VPg35 produced no cleavage between 3B and 3CD in vitro (16), processing may take place at an impaired level in vivo.

Replacement of poliovirus VPg with that of coxsackievirus yielded a viable chimeric virus that did not replicate as efficiently as wild-type poliovirus. This is probably not due to a host cell restriction of virus multiplication, as multiplication of coxsackievirus strains is supported by R19 HeLa cells. Rather, the defect in multiplication may be due to an impairment of interaction between PV1 proteins and the heterologous VPg.

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