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Mutational Analysis of the Genome-Linked Protein VPg of Poliovirus

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Using a mutagenesis cartridge (R. J. Kuhn, H. Tada, M. F. Ypma-Wong, J. J. Dunn, B. L. Semler, and E. Wimmer, Proc. Natl. Acad. Sci. USA 85:519–523, 1988), we have generated single and multiple amino acid replacement mutants, as well as a single amino acid insertion mutant in the genome-linked protein VPg of poliovirus. Moreover, we constructed three different 5-amino-acid insertion mutants that map close to the C terminus of 3A, a viral polypeptide whose coding sequence is adjacent to VPg. Transfection of HeLa cells with RNA synthesized in vitro was used to test the effect of the mutation on viral proliferation. Mutations were either lethal or nonlethal. A temperature-sensitive phenotype was not observed. The arginine at position 17 of VPg could not be exchanged with any other amino acid without loss of viability, whereas the lysine at position 20, an amino acid conserved among all known polioviruses, coxsackieviruses, and echoviruses, was replaceable with several neutral amino acids and even with glutamic acid. Replacement of poliovirus VPg with echovirus 9 VPg yielded viable virus with impaired growth properties. Our results suggest considerable flexibility in the amino acid sequence of a functional VPg. All insertions in polypeptide 3A proved to be lethal. In vitro translation of mutated viral RNAs gave patterns of proteolytic processing that in some cases was aberrant, even though the mutation was nonlethal.

The development of infectious cDNA clones of poliovirus and other members of the picornavirus family permits a molecular genetic analysis of the viral genome on a level previously unattainable (10, 17, 22, 26, 27, 35). By using such an analysis, it should now be possible to identify the *cis*-acting elements in the viral genome and to precisely define the role of the viral polypeptides in RNA replication. Mutations constructed in both the 5′- and 3′-untranslated regions, along with recombinant viruses of poliovirus and coxsackievirus, indicate that there are *cis*-acting sequences in the untranslated regions which are required for normal growth (8, 13, 28, 32, 36). Such sequences would presumably contain an encapsidation signal that distinguishes viral RNA from mRNA, a signal directing the correct initiation of synthesis of the polyprotein, and polymerase recognition sequences that can differentiate plus and minus RNA strands. For example, a specific segment within the 5′-untranslated region of encephalomyocarditis virus RNA has been implicated in the direct internal binding of ribosomes to viral RNA prior to initiation of polyprotein synthesis (7).

Data implicating some of the proteins involved in genome replication have come from biochemical studies that examined the activities found in both infected cells and purified extracts from infected cells (15). These studies have indicated that there are at least three proteins involved in viral RNA replication: the RNA-dependent RNA polymerase, $3D^{pol}$, the viral proteinase, $3C^{pro}$, and the genome-linked protein, VPg. Moreover, genetic studies and mapping of guanidine-resistant mutants have shown that polypeptide 2C is also involved in RNA synthesis (25). The precise location of these virus-encoded polypeptides and the gene organization of poliovirus is shown in Fig. 1A (11).

The genome-linked protein, VPg, is an oligopeptide 22 amino acids in length that is covalently attached via a tyrosine residue to the 5′ end of all newly synthesized viral RNAs but is absent from viral mRNA (1, 21, 24, 29). It has been previously suggested that VPg may serve as a primer for the poliovirus RNA-dependent RNA polymerase (20). Alternatively, it has been proposed that VPg acts as a nuclease that cleaves the hairpin of end-linked double-stranded RNA and attaches itself to the newly formed 5′ end of the RNA (6). In addition to its role in RNA synthesis, VPg may also play a role in the encapsidation of viral RNA. Only RNA containing VPg at the 5′ end is packaged into virions (20, 21).

In order to delineate the function(s) of VPg in virus replication, we have undertaken a mutational analysis of the genome-linked protein, VPg, of poliovirus by using a cDNA clone from which infectious RNA was synthesized. We have previously constructed a mutagenesis cartridge which spans the VPg region of the genome and contains restriction sites that permit the introduction of short complementary oligonucleotides harboring single or multiple nucleotide substitutions (14). The strategy of mutagenesis by a synthetic cartridge has also been successfully adapted to exchange the neutralization antigenic site I of poliovirus type 1 with that of polioviruses type 3 (18a) and type 2 (18) and to generate viable mutants containing amino acid substitutions in 3C (5). In the study described here, we have used the cartridge spanning the coding region of VPg to study the requirements for some of the positively charged residues in VPg. We have also characterized a chimeric virus (14) in which the coding region for poliovirus VPg was substituted with that of

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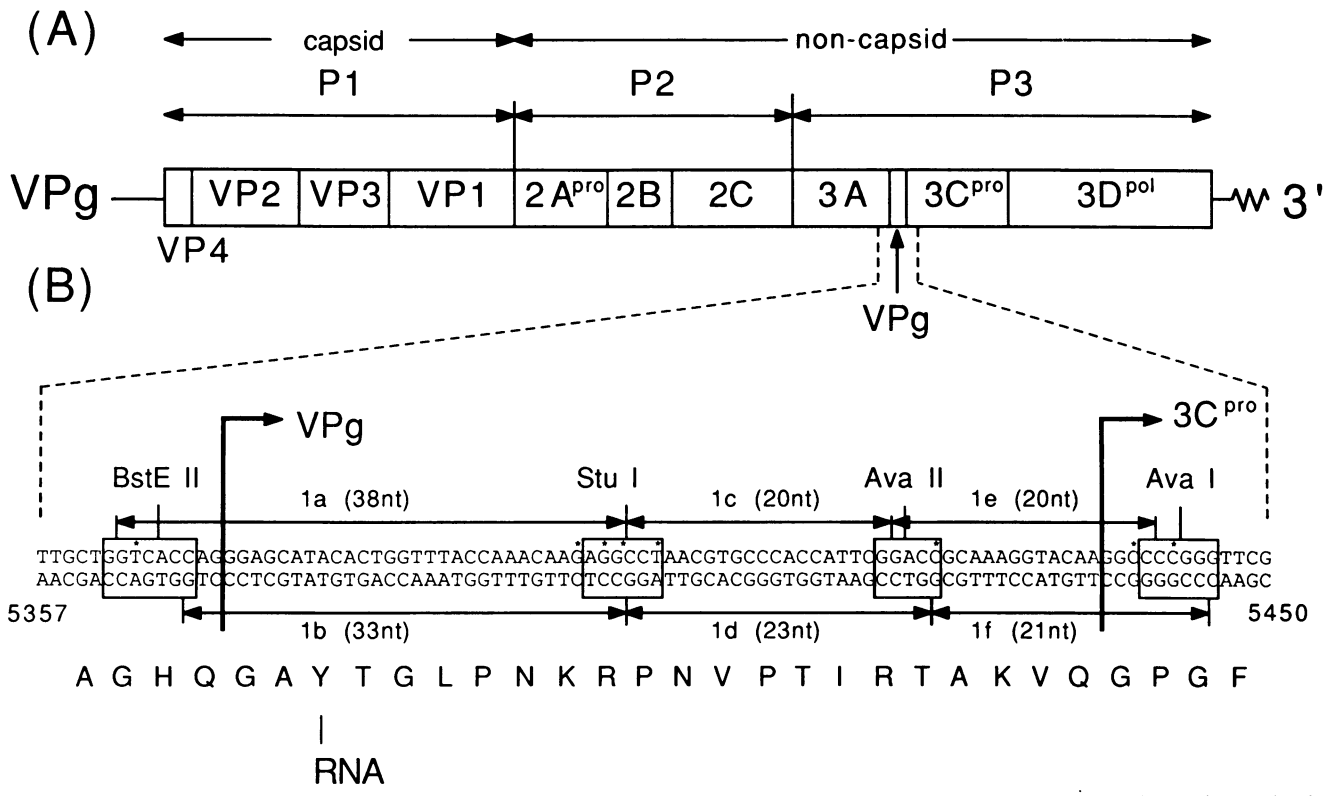


FIG. 1. Location and structure of the mutagenesis cartridge for VPg. (A) The genome of poliovirus, shown schematically, is covalently linked to VPg at the 5′ end. The 3′ end is polyadenylylated. The coding region for the polyprotein is represented by an open box that has been divided into the capsid (P1) and noncapsid regions (P2 and P3). The divisions do not precisely correspond to relative sites of the end products of proteolytic processing. Polypeptides $2A^{pro}$ and $3C^{pro}$ are proteinases, $3D^{pol}$ is the RNA-dependent RNA polymerase. 3AB (3B = VPg) is thought to be a precursor for VPg. 3CD, a precursor for $3C^{pro}$ and $3D^{pol}$, has recently been found to be involved in P1 processing in vitro (see text). The nomenclature proposed by Rueckert and Wimmer (30) has been used throughout this paper. (B) Structure of the VPg mutagenesis cartridge. The nucleotide sequence shown corresponds to the cDNA encoding nucleotides 5357 through 5450 of the poliovirus type 1 (Mahoney) RNA genome. Nucleotide differences between the wild-type sequence and the mutagenesis cartridge are denoted by dots above the appropriate nucleotide. The nucleotides enclosed within a box represent a newly created restriction site. The amino acid sequence is given underneath the nucleotide sequence in the one-letter amino acid code. The 5′ end of the RNA is covalently linked to a tyrosine residue as indicated. The oligonucleotides used to construct the cartridge are symbolized by the line with the double arrow along with their appropriate designation (1a, 1b, 1c, 1d, 1e, and 1f), and their lengths are given in nucleotides (nt) in parentheses.

echovirus 9. The introduction of an additional amino acid in VPg had little effect on virus production, whereas the insertion of five amino acids in the C terminus of 3A, a polypeptide mapping immediately upstream of VPg (33) (Fig. 1A), yielded noninfectious RNA. We have employed an in vitro translation system to study whether proteolytic processing was affected in the mutants constructed. Our results suggest that amino acid substitutions in VPg can exert profound effects not only on the function(s) of VPg in vivo but also on the apparent production of other viral polypeptides in vitro.

MATERIALS AND METHODS

Cells and viruses. All virus propagation and virus plaque assays were performed on HeLa cell monolayers maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The wild-type virus stock used in all experiments was a plaque-purified isolate obtained following transfection of pEV104 (35) into COS-1 cells.

Construction of plasmids. The construction of the synthetic mutagenesis cartridge has previously been described (14). Pairwise complementary oligonucleotides coding for mutations were introduced into the appropriate restriction sites in the mutagenesis cartridge by using the plasmid pNT15. Putative mutants were screened by dideoxy sequencing of the double-stranded plasmid (4, 31). The primer used for sequencing was complementary to nucleotides 5449 through 5463 of the plus-strand viral RNA. A *Bgl*I (nucleotide 5318)-to-*Bgl*II (nucleotide 5601) fragment from a positive clone was used to replace the corresponding fragment from pT7-VPg15.

Plasmid pNT20 was constructed by performing a partial *Ava*II restriction digestion of pNT15, followed by extension of the 5′ overhang with the large fragment of DNA polymerase I (Klenow). Plasmids were screened for the loss of the *Ava*II restriction site within the mutagenesis cartridge. Plasmids pNT37, pNT38, and pNT39 were constructed by linearizing pNT15 at the unique *Bst*EII site (poliovirus nucleotide 5362), filling in the 5′ overhang with Klenow, and ligating the appropriate synthetic DNA linker (10-mer) to the blunt end, a procedure resulting in the insertion of five amino acids. These steps were followed by the replacement of the *Bgl*I-*Bgl*II fragment back into the full-length infectious transcription plasmid pT7-VPg15.

Transfections. Transcriptions were performed as described previously (40). Transfections were carried out in

25-cm^2 flasks containing confluent monolayers of HeLa cells (10^6 cells per flask). RNA (15 to 30 μg) was diluted in HBSS buffer (5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, 1 g of glucose per liter [pH 7.05]) and then mixed with an equal volume of DEAE-dextran (1 mg/ml in HBSS buffer; Pharmacia Fine Chemicals). The RNA-DEAE-dextran mixture (0.5 ml per flask) was added directly to the cells which had been previously washed twice with DMEM. The flasks were allowed to incubate at room temperature for 30 min after which fresh DMEM supplemented with 10% fetal bovine serum was added and the flasks were placed at 37°C for 2 h. At the end of the incubation, the medium was removed and the cells were washed twice with DMEM and were overlaid with DMEM supplemented with 10% fetal bovine serum. The presence or absence of cytopathic effects were normally observed at 24 to 48 h posttransfection.

Preparation of viral RNA and nucleotide sequencing. Viruses were grown on HeLa cell monolayers (75-cm^2 flask, three flasks per virus) at a multiplicity of infection of 0.01 PFU per cell. Following cell lysis, the flasks were freeze-thawed three times and the cell debris was removed. The supernatant was further clarified by centrifugation for 15 min at 15,000 rpm in a TY30 rotor at 5°C. After the addition of sodium dodecyl sulfate to the supernatant to a final concentration of 0.5%, the virus was pelleted by centrifugation for 4 h at 26,000 rpm in a TY30 rotor at 20°C. The pellet was suspended in 1 ml of 0.02 M sodium phosphate buffer (pH 7.7). Virus was purified by overnight ultracentrifugation in a CsCl density gradient in a SW50.1 rotor at 40,000 rpm. The purified virus was extracted twice with phenol and twice with $CHCl_3$. RNA was then precipitated by the addition of ethyl alcohol. The primer used for sequencing has been described above.

In vitro translation of transcripts derived from mutant cDNA. Translations were carried out at 30°C in a rabbit reticulocyte lysate supplemented with an uninfected HeLa cell extract (42, 43). HeLa cell extract preparation has been described previously (3, 34). Both the uninfected and infected HeLa cell extracts were made mRNA dependent by treatment with micrococcal nuclease (23). The translation mixtures contained 20 μl of rabbit reticulocyte lysate (Promega Biotec) and 3 μl of uninfected HeLa cell extract. The reactions also contained 20 μM of each amino acid (minus methionine), 8 μCi [^{35}S]methionine (Amersham Corp.), 0.2 mg of phosphocreatine kinase per ml, 1 mM ATP, and 0.2 mM GTP. The RNA concentration in the translation reaction was 10 μg/ml. Translations were terminated after 3 h by the addition of cycloheximide and pancreatic RNase to 5 and 10 mg/ml, respectively. Typically, either 3 μl of lysis buffer used for cell extract preparation or 3 μl of infected-cell extract was added to 10 μl of the translation reaction and the incubation was continued at 30°C for 1 h.

All translation reactions were diluted with Laemmli sample buffer and subjected to electrophoresis on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate. After fluorography, the gel was exposed to Kodak XAR-5 film.

RESULTS

Construction and isolation of mutants. The general scheme for constructing the VPg mutations has been previously described (14). Briefly, a mutagenesis cartridge was constructed by inserting two unique restriction sites flanking the coding region of VPg (Fig. 1B). The wild-type sequences between the new restriction sites were replaced by the insertion of three pairs of complementary oligonucleotides. The full-length plasmid containing the mutagenesis cartridge was designated pT7-VPg15, and mutants constructed with the cartridge have been given the prefix pT7-VPg, followed by the mutation number (14). Viral mutants were named according to the nomenclature suggested by Bernstein et al. (2): name of the laboratory head (W), serotype of poliovirus (1), location of the mutated gene segment (VPg), and number of mutant.

Although the insertion of the restriction sites defining the limits of the cartridge was designed not to alter the amino acid sequence, the placement of the internal *Stu*I site resulted in an amino acid change at position 10 in the VPg polypeptide. Since this K10R change (substitution of a lysine at position 10 to an arginine) was conservative and maintained a positive charge at the original wild-type position, the probability was low that it would interfere with VPg function (14). Moreover, both type 2 and type 3 strains of poliovirus have an arginine in place of the lysine at this position (16, 37, 39). Indeed, the pT7-VPg15-derived virus, W1-VPg15, was shown to be identical in its growth pattern to that of the parental virus, an observation suggesting that the alteration did not affect VPg function (14). With this result, mutations could be generated in and around VPg by using the mutagenesis cartridge. A series of mutations involving the tyrosine residue at position 3 and the insertion of methionine residues within VPg have been previously described (14).

RNA transcripts which produced virus usually gave cytopathic effects after 24 to 48 h posttransfection. The supernatants from the lysed cells were diluted and plated onto HeLa cell monolayers. Plaque-purified virus was recovered and used to generate virus stocks. The titers of these virus stocks were determined, and the genomic RNAs of these virus stocks were sequenced. Titration at different temperatures did not reveal any ts phenotypes. For those mutants which failed to produce cytopathic effects following transfection, an attempt was made to recover virus at different temperatures. The supernatant from the transfected HeLa cell monolayers was recovered after 4 to 5 days and was either directly assayed for plaque formation or added to another confluent monolayer of HeLa cells. Incubations were carried out at 32, 37, and 39°C. The cells were monitored for several days for the production of virus.

Mutations directed at the charged amino acids arginine 17 and lysine 20. VPg is a small polypeptide of 22 amino acids, with a net positive charge of 4 (Fig. 1B). Although the known VPgs of picornaviruses vary in their net charges, they usually contain positively charged amino acids in approximately the same positions. One hypothesis to explain this observation is that the positive charges of VPg interact specifically with the negative charges associated with the nucleotides of the viral RNA.

In order to investigate the requirement for the charged residues at positions 17 and 20 in the poliovirus VPg, appropriate oligonucleotides, degenerate at position 3 of the codon for either the arginine at position 17 or the lysine at position 20, were synthesized and annealed. Following reconstruction of the altered cDNA sequence back into the full-length clone, an attempt was made to recover virus. Interestingly, all mutations altering the arginine residue at position 17, even an arginine-to-lysine substitution, resulted in a lethal lesion (Fig. 2A). In contrast, all substitutions at the lysine residue at position 20 produced virus. The changes at lysine 20 were as follows: K20R (pT7-VPg27), K20Q (pT7-VPg25), K20L (pT7-VPg23), K20I (pT7-VPg24), and

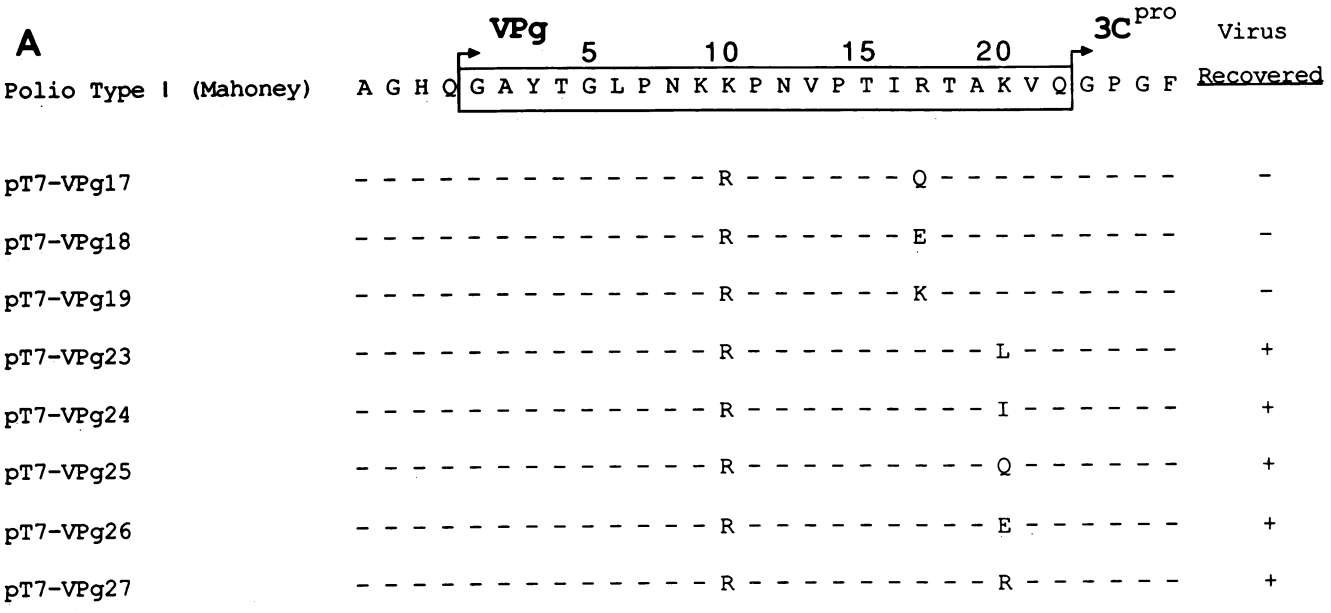
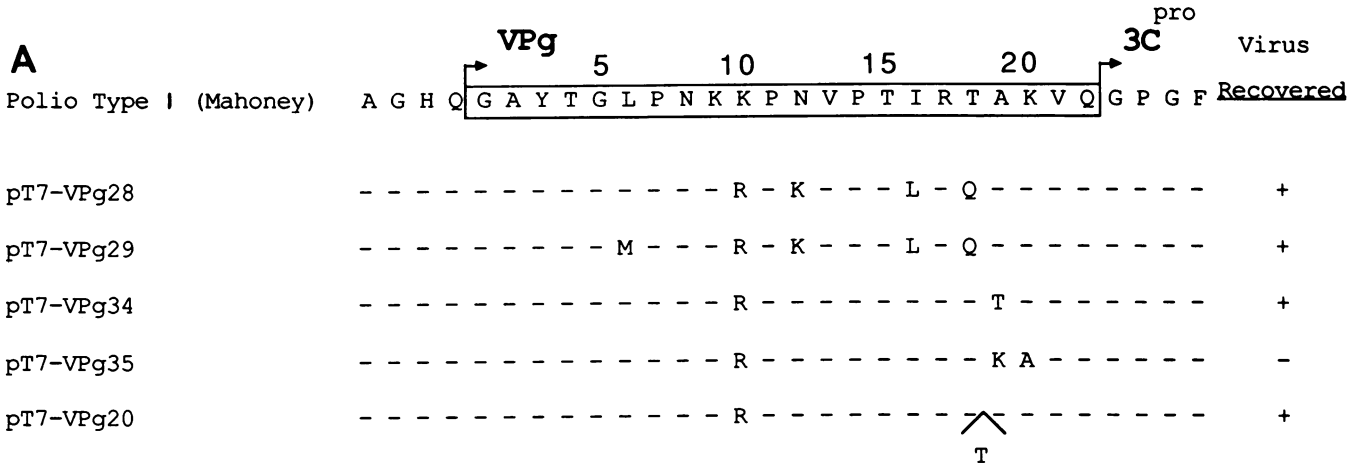


FIG. 2. Mutations introduced at two charged residues in VPg. (A) The amino acid sequence of the wild-type (poliovirus type I) Mahoney VPg is given by the one-letter amino acid code within the boxed region. The names of the mutants are given on the left side, and their appropriate changes are shown replacing the wild-type sequence (- - - - -). Whether infectious virus was recovered is shown to the right. (B) One-step growth curves of the wild-type virus and the five lysine 20 mutant viruses shown in panel A. HeLa cell monolayers were infected at a multiplicity of infection of 25 and incubated at 37°C. At different times postinfection, the cells were removed and virus production was assayed. The results are plotted as the virus titer (log_{10} PFU/ml) against time (hours postinfection [pi]).

K20E (pT7-VPg26). The K20E change is particularly noteworthy, because it involves substitution of the lysine by glutamic acid. An analysis of the growth pattern of the mutant isolates is shown in Fig. 2B. Three of the viruses, pT7-VPg24 (K20I), pT7-VPg25 (K20Q), and pT7-VPg26 (K20E), showed a deviation from the kinetics of virus production seen with the wild-type virus, the difference being greatest at 3.5 h postinfection in all cases. The slopes of the lines between 2.5 and 3.5 h suggest that there was an impairment to virus growth during this period. After 3.5 h, the slopes of the lines either exceeded the wild-type rate or were approximately equal, an observation suggesting that the block in growth has been removed or overcome. To examine the protein processing pattern of these mutants, infected cells were labeled with [^{35}S]methionine for 2 h, starting at 3.5 h postinfection. The cells were harvested, and the labeled proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The protein patterns produced by all of the mutants were identical to those observed for the wild-type virus, except for polypeptide 3AB. Protein 3AB is a putative precursor to VPg and in some cases exhibited aberrant migration in some of the mutants (data not shown). This is not surprising, since the mutations map to 3B (VPg). (Note that free VPg can usually not be detected in cell lysates by the methods used here [33]). The greatest defect in growth is seen in pT7-VPg24, which is a lysine-to-isoleucine substitution.

Substitution of poliovirus VPg with the corresponding echovirus 9 VPg. As we reported previously (14), replacement of the poliovirus VPg with the VPg of echovirus 9 yielded a viable virus following transfection. The construction of this mutant required a two-step assembly. The first step was the replacement of a leucine residue at position 6 with a methionine residue, which is found in the echovirus 9 sequence (41). This was accomplished by the appropriate change in the 1a and 1b oligonucleotides (Fig. 1B). The corresponding virus, designated W1-VPg22, grows normally (14). The second step of the construction involved changing the remaining



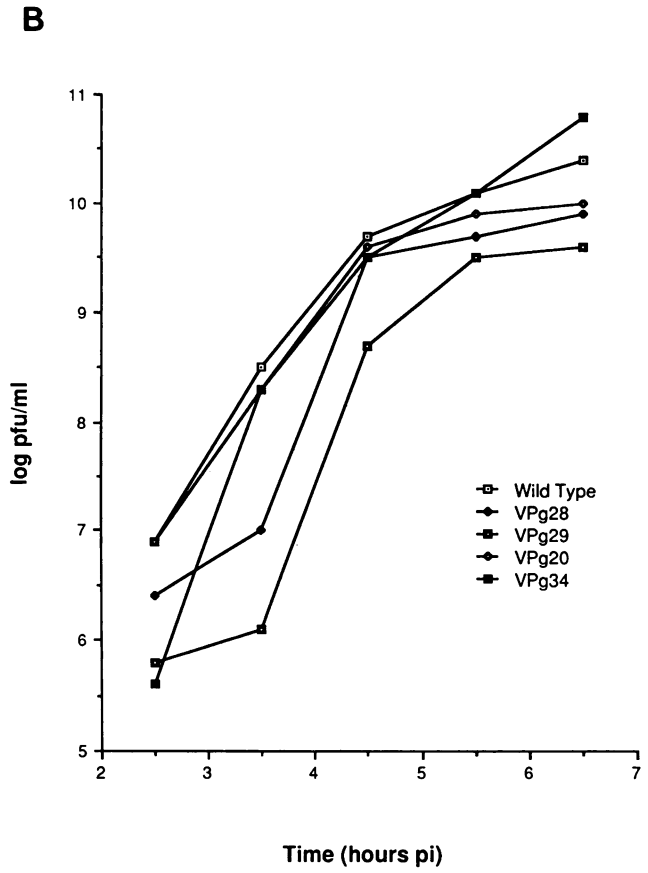


FIG. 3. Diagram depicting additional mutations introduced into VPg. (A) This diagram is similar to that in Fig. 2A. In addition to amino acid substitutions, pT7-VPg20 contains an insertion of a threonine between amino acids 18 and 19 of VPg. (B) One-step growth curves of the wild-type virus and the mutant viruses described in panel A.

three amino acids which were located in the C-terminal half of VPg. Two complementary oligonucleotides (1c plus 1e and 1d plus 1f [Fig. 1B]) were synthesized accordingly and cloned into pNT15 to subsequently generate pT7-VPg28 (Fig. 3A). Following transfection, virus W1-VPg28 was recovered, a positive result on which basis we predicted that complete allele replacement of poliovirus VPg with the echovirus 9 VPg may yield virus.

The construction of a poliovirus-echovirus 9 chimeric virus was completed by joining the N-terminal half of VPg from pT7-VPg22 to the C-terminal half of pT7-VPg28 at the unique *Stu*I site to generate pT7-VPg29 (Fig. 1B and 3A). After linearization of the plasmid DNA, RNA was transcribed and transfected onto HeLa cell monolayers. Virus was recovered, and its RNA sequence was confirmed. The chimeric virus was designated W1-VPg29.

To analyze the replication of the two viruses, W1-VPg28 and W1-VPg29, a one-step growth experiment was performed (Fig. 3B). As was seen with some lysine 20 mutants, there was a lag in growth at 3.5 h postinfection. The production of the hybrid virus W1-VPg29 at 3.5 h postinfection was approximately 100-fold below that of wild-type virus. W1-VPg28 replicated at a rate between that of wild-type and W1-VPg29 viruses. Since W1-VPg22 replicates at a normal rate (14), the juxtaposition of a methionine at position 6 appears to compound the phenotype of reduced growth rate seen in W1-VPg28. The basis for this result is not known. Labeling of the viral polypeptides, as described above, was performed, and the processing pattern for both viruses was similar to that for the wild type (data not shown).

Insertion of a threonine between positions 18 and 19 of VPg. The position of the *Ava*II site in the mutagenesis cartridge (Fig. 1B) can be utilized by filling in the 3-base-pair overhang produced by *Ava*II digestion to generate a single in-frame amino acid insertion in the VPg sequence (Fig. 1A and 3A). Insertion of one amino acid (a threonine) in this position was done prior to the mutations of arginine 17 and lysine 20 to investigate whether the spacing between the charged residues of positions 17 and 20 might be critical to VPg function. Following transfection of RNA derived from pT7-VPg20, virus was recovered (W1-VPg20) that had growth properties nearly identical to those of wild-type virus (Fig. 3B). In addition, the processing pattern of viral polypeptides in vivo was identical with that of wild-type virus (data not shown).

Mutations affecting proteolytic processing. The determinants which govern the cleavage sites used by the poliovirus polypeptide $3C^{pro}$ (proteinase) are not well understood. Although Q-G is always the amino acid pair that is cleaved, not every Q-G pair is subject to cleavage and it is assumed that additional primary sequence signals or higher-order structure or both play a role. Nicklin et al. (19) analyzed the amino acids surrounding the Q-G sites in poliovirus polypro-

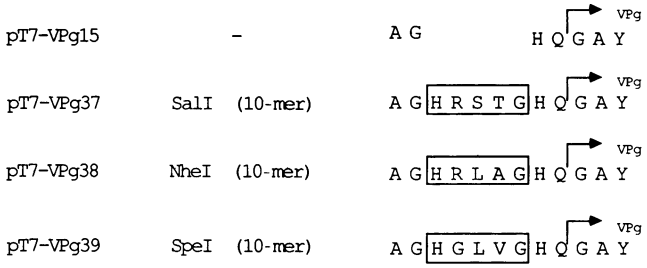


FIG. 4. Diagram describing the linker-insertion mutants created at the *Bst*EII site. The sequence of the C-terminal four amino acids of polypeptide 3A and three amino acids of VPg are shown. The Q-G cleavage site for 3A-VPg is indicated by the arrow. The inserted amino acids for the appropriate linker are given within the box.

tein and concluded that no definitive sequence or structure could be shown associated with those sites that are cleaved. It was observed, however, that Q-G signals recognized by the proteinase 3C preferentially contain alanine, as well as proline, valine, and threonine in the P4 position (position 4 preceding the scissile bond). In contrast, Q-G pairs with lysine, tyrosine, or serine in P4 appear not to be cleaved. Analysis of Q-G cleavage sites of other picornavirus polyproteins also suggests that there is some constraint placed upon the amino acid in the P4 position, since a neutral aliphatic amino acid is typically present (for a review, see reference 12).

Two mutations were made which altered the P4 position (an alanine at residue 19) in VPg (Fig. 3A). In plasmid pT7-VPg34, the alanine was replaced by a threonine, and in pT7-VPg35, the alanine-lysine (at positions 19 through 20) were inverted such that the lysine now occurs in the P4 position. It should be noted that while pT7-VPg35 has a 2-amino-acid substitution, position 20 appears to be tolerant to substantial diversity in the amino acid side chain present at that position, as demonstrated by mutants W1-VPg23-27. Upon transfection of transcripts, virus was recovered from pT7-VPg34 but not from pT7-VPg35. W1-VPg34 has growth properties similar to those for wild-type virus (Fig. 3B). Experiments are currently being carried out to determine if a single substitution (Ala → Lys) at position 19 (P4) is sufficient to block VPg-3C Q-G cleavage.

Linker-insertion mutants in 3A. By using the *Bst*EII restriction site in the 3A polypeptide, a series of synthetic linker oligonucleotides were inserted which resulted in the displacement of VPg from a strongly hydrophobic sequence found in 3A, thus moving the VPg sequence away from a putative membrane anchor. As was previously reported, polypeptide 3AB resides in the smooth membranes of virus-infected HeLa cells (33). To investigate whether there is a positioning effect, subclone pNT15 was linearized at the unique *Bst*EII restriction site, the overhangs were filled in with the large fragment of DNA polymerase I (Klenow), and an appropriate linker (10-mer) was inserted by ligation. Three different mutants were constructed in this way, each having a 5-amino-acid insertion close to the C terminus of 3A (Fig. 4). Note that two of the three linker insertions retain an alanine or a threonine in the P4 position of the Q-G site between 3A and VPg. Following reconstruction of the full-length clones containing these mutations, RNAs containing these linker insertions were transcribed and transfected onto cells. None of the mutants produced viable virus.

In vitro translation of mutant RNAs. To determine whether those mutants which failed to produce virus might be defective in their ability to process the viral polypeptides, in vitro translations of synthetic RNAs (transcripts) were performed. Translations were done under conditions found to be optimal for proteolytic processing (42). A poliovirus-infected HeLa S10 extract, containing the virus-specific proteinases, was added to a portion of the translation mixture to investigate whether a mutant polyprotein can be cleaved by exogenous, virus-encoded proteinase(s).

The protein patterns observed after translation of mutant RNAs in vitro can be divided into two simple categories, normal and abnormal, although this phenotype does not necessarily covary with the ability of the mutant RNAs to produce viable virus (Fig. 5A through D). Examples are shown in Fig. 5A for transcripts derived from pT7-VPg23, -VPg24, and -VPg25 (viable) and transcripts of pT7-VPg17 and -VPg18 (nonviable), all of which give normal protein patterns. A similar situation exists for transcripts derived from pT7-VPg26 (viable; Fig. 5B, lane 4), pT7-VPg34 (viable; Fig. 5D, lane 4) and pT7-VPg37 (nonviable; Fig. 5B, lane 12). In the case of the nonviable constructs, pT7-VPg17 (R17Q) and pT7-VPg18 (R17E), one could cautiously conclude that the lesion must affect VPg function per se, rather than alter processing. That is, the positively charged amino acid in position 17 of VPg has a specific VPg-related function. Replacement of this arginine by lysine is also lethal (pT7-VPg19; Fig. 2A). Thus, the arginine in position 17 of VPg, not just a positive charge, may be uniquely required for VPg function. On the other hand, the pattern of proteolytic processing produced by VPg19 RNA (R17K, nonviable) is clearly abnormal (Fig. 5A, lane 8). In principle, this abnormality in protein synthesis could be cited as the reason for nonviability of the pT7-VPg19 genotype were it not for the nearly identical abnormal processing pattern produced by pT7-VPg20 RNA (Fig. 5A, lane 10), a genotype which is infectious (Fig. 3B).

Translation patterns of lysine-20 mutant RNAs were generally normal (pT7-VPg23, -VPg24, and -VPg25 [Fig. 5A, lanes 12, 14, and 16, respectively] and pT7-VPg26 [Fig. 5B, lane 4]), as would be expected since these mutations yielded viable virus. An exception is the processing pattern generated by pT7-VPg27 (K20R) which is abnormal (Fig. 5B, lane 6), although this RNA is infectious (Fig. 2B). Thus, as with VPg20, an abnormal processing pattern in vitro is not necessarily an indicator of nonviability.

The insertions at the C terminus of 3A also had unexpected consequences for in vitro translation. RNA-directed protein synthesis of mutant pT7-VPg37 yielded a polyprotein that appeared to undergo correct proteolytic processing (Fig. 5B, lane 12), an observation suggesting that the lesion in 3A may interfere with the function(s) of 3A or 3AB. The pattern produced by RNAs of pT7-VPg38 and VPg39, on the other hand, was highly abnormal (Fig. 5C, lanes 1 and 3), although the P4 position in these cases contains permissive amino acids (alanine and valine). It is likely that the insertions in mutant RNAs pT7-VPg38 and -39 resulted in extensive misfolding of the polypeptide chain within the P2-P3 domains, since 3CD was not seen even after addition of an infected-cell extract (as a source of exogenous proteinase), whereas processing of the P1 region to the capsid polypeptides VP0, VP1, and VP3 occurred (Fig. 5C, lanes 2 and 4).

The alteration from alanine to lysine of the amino acid in P4 of the Q-G site between VPg-3C (pT7-VPg35) also led to a pattern in which 3CD was absent, as expected. In this case,

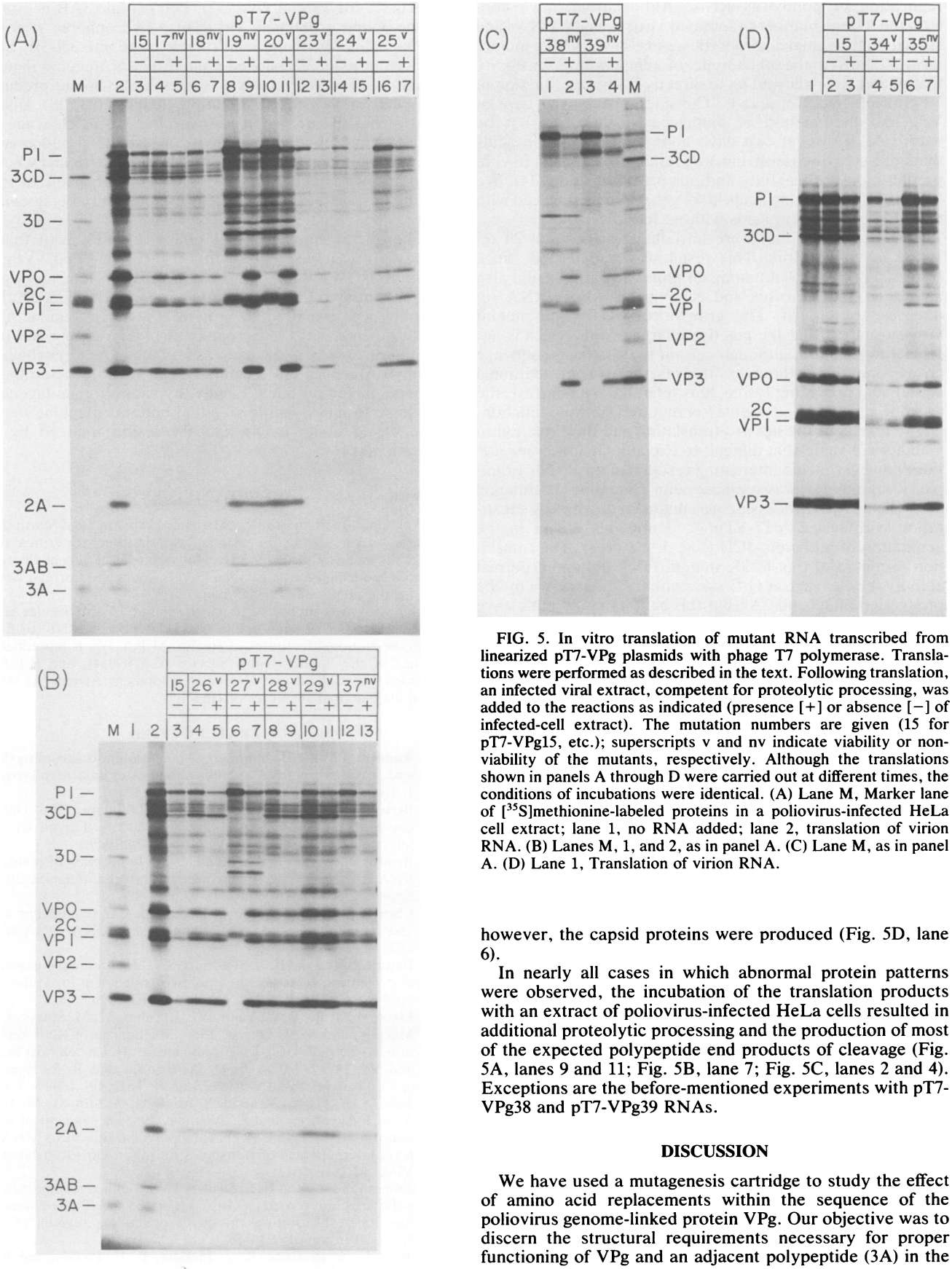


FIG. 5. In vitro translation of mutant RNA transcribed from linearized pT7-VPg plasmids with phage T7 polymerase. Translations were performed as described in the text. Following translation, an infected viral extract, competent for proteolytic processing, was added to the reactions as indicated (presence [+] or absence [−] of infected-cell extract). The mutation numbers are given (15 for pT7-VPg15, etc.); superscripts v and nv indicate viability or nonviability of the mutants, respectively. Although the translations shown in panels A through D were carried out at different times, the conditions of incubations were identical. (A) Lane M, Marker lane of [^{35}S]methionine-labeled proteins in a poliovirus-infected HeLa cell extract; lane 1, no RNA added; lane 2, translation of virion RNA. (B) Lanes M, 1, and 2, as in panel A. (C) Lane M, as in panel A. (D) Lane 1, Translation of virion RNA.

however, the capsid proteins were produced (Fig. 5D, lane 6).

In nearly all cases in which abnormal protein patterns were observed, the incubation of the translation products with an extract of poliovirus-infected HeLa cells resulted in additional proteolytic processing and the production of most of the expected polypeptide end products of cleavage (Fig. 5A, lanes 9 and 11; Fig. 5B, lane 7; Fig. 5C, lanes 2 and 4). Exceptions are the before-mentioned experiments with pT7-VPg38 and pT7-VPg39 RNAs.

DISCUSSION

We have used a mutagenesis cartridge to study the effect of amino acid replacements within the sequence of the poliovirus genome-linked protein VPg. Our objective was to discern the structural requirements necessary for proper functioning of VPg and an adjacent polypeptide (3A) in the

replication of poliovirus RNA. Although we have constructed a large number of mutated virus-specific RNAs and studied their biological properties, none of the viable mutant viruses showed the phenotype of temperature sensitivity that would have allowed us to directly probe the function of VPg in infected HeLa cells. Considering the small size of VPg and the method of mutagenesis, this may not be surprising. However, we have now identified amino acids that appear to be essential in VPg function, and thus, in viral proliferation in this study and our previous study (14). We have also found amino acids in VPg that can be replaced with many different amino acids without loss of viability.

All the changes that were introduced at position 20 resulted in viable virus. This result was unexpected, since lysine 20 is conserved among all poliovirus strains and also among coxsackieviruses and echoviruses whose RNA sequences are known. The growth curves for the mutant viruses suggest that the positive charge of this lysine is not crucial to function, although certain replacements influence growth kinetics. Moreover, the insertion of an additional amino acid preceding lysine 20 is tolerated. In contrast, the arginine residue at position 17 is required for VPg function.

The results of the in vitro translation and their relation to viability are somewhat difficult to explain. On closer inspection, however, some interesting results emerged. For example, if arginine 17 was replaced with glutamine or glutamic acid, the in vitro processing was unaffected, whereas substitution with lysine (pT7-VPg19) identified a defect in the generation of authentic 3CD (Fig. 5A, lane 8). The translation products of this R17K mutant RNA exhibited normal activity for cleavage at Q-G sites within P2 (as shown by the production of 2C and 2A), but this activity could not cleave P1 at its two Q-G sites to produce VP0, VP1, and VP3. It may be concluded, therefore, that the activity cleaving P2 differs from that cleaving P1, an observation made previously (42). Interestingly, two other mutants which produced the same aberrant pattern as pT7-VPg19 in the in vitro translation system (pT7-VPg20 and pT7-VPg27) were viable. When the protein patterns of HeLa cells infected with the corresponding mutant viruses (W1-VPg20 and W1-VPg27) were analyzed, normal processing was observed at 37 and 32°C (data not shown). In addition, pulse-chase experiments failed to show any hint of a processing defect (data not shown). Exactly why a processing defect would appear in vitro but not in vivo is not clear. Most likely, the phenomenon is due to differences in local concentrations of the activity (or activities) that must cleave in *trans*. Because in vitro translation yields relatively small amounts of protein, the effect of an altered substrate or enzyme interaction may be accentuated. Recent data obtained in several laboratories (9, 41a, 42; M. J. H. Nicklin, K. S. Harris, H. Toyoda, P. Palai, and E. Wimmer, submitted for publication) have shown that 3CD, and not 3C alone, is necessary for the complete processing of the capsid precursor P1 in vitro. 3CD was missing among the translation products (Fig. 5A, lanes 8 and 10). We propose that the absence of 3CD from translation products is the reason that no trace of VP0, VP1, and VP3 can be seen. Addition of an infected HeLa cell extract containing 3C and 3CD, on the other hand, results in the cleavage of P1 in these reaction mixtures (Fig. 5A, lanes 9 and 11). Interestingly, pT7-VPg35, which is unable to cleave the VPg-3C Q-G site and therefore does not produce 3CD, does efficiently cleave the capsid precursor P1. We suggest this activity is carried out by polypeptide 3BCD or polypeptide P3 or both.

It has been speculated that 3AB (3B = VPg) is the precursor for VPg in vivo (33). Polypeptide 3AB is a membrane-bound protein by virtue of a hydrophobic region of amino acids upstream from the cleavage site 3A-3B. Since the synthesis of VPg-linked poliovirus RNA occurs in membrane-associated complexes, a membrane-bound precursor may deliver VPg to the initiation complex (33, 38). Disruption of the spacer region between the 3A-3B cleavage site and the hydrophobic region by insertion of amino acids resulted in noninfectious RNA. Experiments to test whether or not any of the noninfectious RNAs are capable of replicating in transfected HeLa cells are currently being carried out.

The allele replacement of poliovirus VPg with that of echovirus 9 yielded viable virus (14) (Fig. 3A, pT7-VPg29). This virus (W1-VPg29) showed normal protein processing patterns in vivo (data not shown) and in vitro (Fig. 5B, lane 10) but was impaired in growth (Fig. 3B). Similar allele replacements with VPg from coxsackievirus or even from rhinoviruses may allow us to assess the extent by which the poliovirus replication machinery can tolerate heterologous genome-linked proteins. Finally, by saturation mutagenesis, we hope to find conditional-lethal mutants mapping to VPg that would allow us to find the lesion induced by the mutation(s).

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