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Genetics

Construction of a "mutagenesis cartridge" for poliovirus genome-linked viral protein: Isolation and characterization of viable and nonviable mutants

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ABSTRACT **By following a strategy of genetic analysis of poliovirus, we have constructed a synthetic "mutagenesis cartridge" spanning the genome-linked viral protein coding region and flanking cleavage sites in an infectious cDNA clone of the type 1 (Mahoney) genome. The insertion of new restriction sites within the infectious clone has allowed us to replace the wild-type sequences with short complementary pairs of synthetic oligonucleotides containing various mutations. A set of mutations have been made that create methionine codons within the genome-linked viral protein region. The resulting viruses have growth characteristics similar to wild type. Experiments that led to an alteration of the tyrosine residue responsible for the linkage to RNA have resulted in nonviable virus. In one mutant, proteolytic processing assayed *in vitro* appeared unimpaired by the mutation. We suggest that the position of the tyrosine residue is important for genome-linked viral protein function(s).**

Poliovirus, like all members of the picornavirus family, has a messenger-sense, single-stranded RNA genome. The viral RNA is 7441 nucleotides long exclusive of the genetically encoded polyadenylate tract at the 3′ terminus (1). Its sequence is known (2, 3) as are the genetic loci and amino acid sequences of all virus-encoded polypeptides (2). The 5′ terminus of viral RNA is blocked by a genome-linked *v*iral *p*rotein, VPg (4, 5). VPg is a small (22 amino acid), basic protein that is covalently linked to the polynucleotide chain through a phosphodiester bond between the 5′-terminal uridine residue and the O^4 of the tyrosine residue (6, 7). All virus-induced RNAs found in the infected cell, with the exception of mRNA, contain VPg at their 5′ ends (8–10). The release of VPg from mRNA is accomplished by a cellular protein termed the unlinking enzyme that leaves a pU at the 5′ end of the RNA (11). Though the function(s) of VPg is unknown, it has been suggested that it may serve as a primer for the primer-dependent RNA polymerase (8). Indeed, following uridylylation of VPg *in vitro*, the product VPg-pUpU can be elongated into longer RNA polynucleotide chains, a result in support of the primer model for VPg function (12, 13). VPg has also been proposed (ref. 14 and references cited therein) to function as a nuclease, cleaving the hairpin of end-linked double-stranded RNA. It, therefore, seems likely that, despite its small size, VPg may play an integral role in the replication of the virus.

The ability to construct mutations in an infectious cDNA copy of the poliovirus genome and to recover viable viral mutants has been demonstrated (15–19). Such mutants can prove invaluable in deciphering the function of a particular polypeptide or region of the viral genome. The difficulty remains in determining the target site for mutation. Those procedures that are useful for generating a series of mutations in a given DNA sequence, although thorough, usually contain an inherent bias as to the location (insertion/deletion mutants) or to the nature of the nucleotide change (20). However, site-directed oligonucleotide mutagenesis allows one to precisely change nucleotides at will with no bias, though one is limited in the number of changes per oligonucleotide used to generate mismatches. The small size of VPg (22 amino acids), its unusual amino acid composition, and the availability of other picornavirus VPg sequences for homology determinations identified it as an excellent target for extensive site-directed mutagenesis experiments and afforded us an opportunity to examine in detail the amino acid requirements for VPg function(s).

By using infectious cDNA clones (21, 22) and an *in vitro* transcription system to generate highly infectious viral RNA (29), we have constructed a "mutagenesis cartridge" spanning the VPg coding region and flanking proteolytic cleavage sites. The creation of unique restriction sites in the region of the genome coding for VPg coupled with the insertion of synthetic mutant oligonucleotides permits a quick and extensive mutational analysis of the region. This cartridge has been used to investigate the role of the tyrosine residue that links VPg with the RNA. In addition, mutants have been made that create one or more methionine codons within the VPg sequence, allowing for direct labeling of VPg with [^{35}S]methionine. Our results suggest that specific amino acid substitutions in VPg differentially affect the covalent attachment of this protein to RNA and its cleavage from precursor polypeptides.

MATERIALS AND METHODS

Cells and Viruses. All virus propagation and plaque assays were performed on HeLa cell monolayers maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum. Wild-type poliovirus used for experiments was a plaque-purified isolate derived following a transfection of COS-1 cells with pEV104 (22). Viral RNA was isolated and sequenced essentially as described by Pincus *et al.* (23). The primer used for sequencing DNA and RNA is complementary to nucleotides (nt) 5449–5463 of the plus-strand viral RNA. The nomenclature of viral mutants follows the suggestion of Bernstein *et al.* (17): name of the laboratory head (W), serotype of poliovirus (1), location of the mutated gene segment (VPg), and number of mutant.

Construction of Synthetic Cartridge. Oligonucleotides were synthesized on a Systec 1450 using phosphoramidite

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Abbreviations: VPg, genome-linked viral protein; nt, nucleotide(s).

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chemistry. The *Bst*EII and *Ava* I restriction sites were inserted into the polio cDNA sequence by using an M13mp10 derivative grown in *Escherichia coli* strain BW313 (24) and oligonucleotide-directed mutagenesis essentially as described by Zoller and Smith (25). The synthetic cartridge was assembled by annealing six complementary oligonucleotides followed by stepwise ligations. The final product was purified by elution from an 8% acrylamide gel and was used to construct pNT15 (Fig. 1). The sequence of the cartridge was confirmed by a modified dideoxy chain-termination procedure (27, 28).

Transcriptions and Transfections. The fragment of DNA containing the desired mutation(s) was inserted into the T7 RNA transcription plasmid pT7PV1-5 (29). Template was prepared and transcription was performed as described (29). *In vitro* translations were carried out as described by Ypma-Wong and Semler (30). Infected virus extracts added to the translations are from a poliovirus-infected HeLa cell S10 fraction (30).

RNA transfections were carried out in T25 flasks (10^6 HeLa cells per flask) and were performed essentially as described by van der Werf *et al.* (29).

RESULTS

Construction of pT7-VPg15. To probe the function(s) of the genome-linked protein, we constructed a synthetic mutagenesis cartridge for the replacement of short sequences of DNA by using new restriction sites inserted into the VPg region of the polio genome. The initial step in the construction of this cartridge was the introduction of two unique restriction sites flanking the VPg coding region. The two sites were chosen such that the primary structure of the polyprotein would be unaltered and that the entire coding sequence for VPg and the flanking cleavage sites would be contained within it (Fig. 2).

The *Bst*EII restriction site at nt 5362 and the *Ava* I restriction site at nt 5440 were created independently by oligonucleotide-directed mutagenesis using an M13mp10-derived vector as shown in Fig. 1. The appropriate mutant oligonucleotide (15 nt long with one mismatch), along with

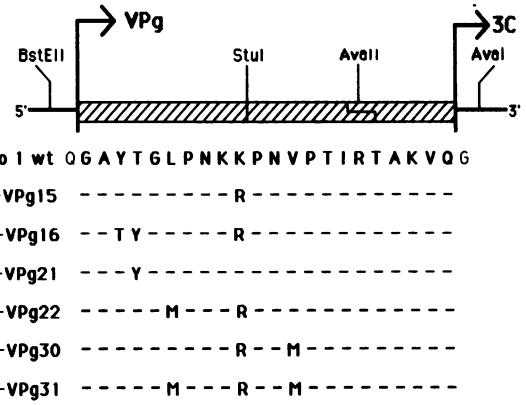

FIG. 2. Schematic of the VPg mutagenesis cartridge and VPg mutants. The limits of the cartridge are defined at the 5′ end by the *Bst*EII site and at the 3′ end by the *Ava* I site. The hatched box represents the primary sequence of VPg, with the one-letter amino acid code for the wild type (WT) (type 1, Mahoney) shown underneath. This sequence differs from the pT7-VPg15 sequence at position 10, in which arginine of pT7-VPg15 replaces the wild-type lysine. This change is present in all mutants generated with the cartridge. The other mutants are shown with their respective alterations replacing the dashed line. The * denotes a mutation that was not made by using the cartridge.

the M13 sequencing primer, was annealed to a template that had been prepared from a Dut^-Ung^- *E. coli* strain (24). The primers were extended with the large fragment of DNA polymerase I (Klenow) and ligated with T4 DNA ligase, followed by digestion with *Hin*dIII to remove the polio cDNA fragment. The polio cDNA fragment was then inserted into the *Hin*dIII restriction site of pNT4 (22). Recombinant clones were screened for the presence of the mutation by digestion with the appropriate restriction enzyme.

The synthetic mutagenesis cartridge was assembled by the ligation of the *Stu* I–*Ava* II oligonucleotide pair to the *Ava* II–*Ava* I oligonucleotide pair. The ligation mixture was electrophoresed on a preparative polyacrylamide gel, and the correct fragment was excised, eluted from the gel, and

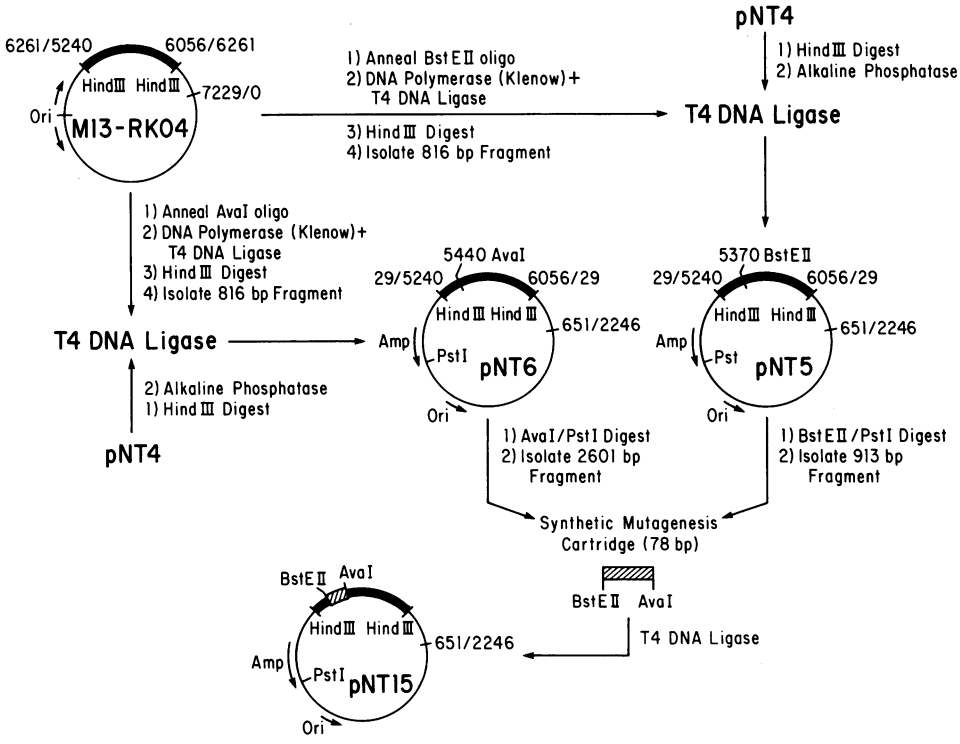

FIG. 1. Strategy for the construction of plasmid pNT15. Polio cDNA sequences are denoted by the solid line. The plasmid pIN-III-C3-7c was used as a convenient donor for the polio sequences required for VPg mutagenesis (26). M13mp10 was linearized by digestion with *Hin*dIII. The polio-specific *Hin*dIII fragment of pIN-III-C3-7c was ligated to the M13 vector generating M13-RK04. The hatched box represents the synthetic mutagenesis cartridge. The origin of plasmid pNT4 is given in ref. 22.

ligated to the *Bst*EII–*Stu* I oligonucleotide pair. The resulting 78-base-pair fragment was again purified by gel electrophoresis. Once assembled, the synthetic cartridge was ligated to DNA fragments from pNT5 and pNT6 to generate pNT15 (Fig. 1). This plasmid contains the functional mutagenesis cartridge: five new restriction sites have been inserted (*Bst*EII, *Stu* I, *Ava* II, *Ava* I, and *Sma* I) and one site has been removed (*Ava* II). With the exception of one amino acid, which will be discussed below, the primary sequence of the wild-type polyprotein has been maintained, although the nucleotide sequence has been altered in eight positions. Dideoxy sequencing was performed directly on the double-strand plasmid DNA to confirm the sequence of the mutagenesis cartridge. Following this step, a *Bgl* I/*Bgl* II digestion was performed, and the resulting 283-base-pair fragment (nt 5318 to nt 5601) was used to replace the corresponding wild-type fragment from pT7PV1-5. The plasmid, designated pT7-VPg15, which was recovered, was sequenced starting from nt 5449 and proceeding through the *Bgl* I site at nt 5318 to confirm its identity. Two independent clones were isolated from each mutant to ensure the mutation created was responsible for the phenotype.

Isolation of Poliovirus Mutant W1-VPg15. Following large-scale purification of plasmid pT7-VPg15, the plasmid was prepared for transcription by T7 RNA polymerase by linearization at the unique *Eco*RI site immediately on the 3′ side of the poly(A) tract of the polio cDNA (29). The transcription reaction was performed as described above, and the resulting RNA transcripts were used in transfections of HeLa cell monolayers. Eighteen to twenty-four hours after transfection cytopathic effects were observed. Following total cell lysis, W1-VPg15, the virus recovered, was added to HeLa cell monolayers to generate a stock of virus. To verify the altered genomic sequence of the virus, genomic RNA was purified, and the nucleotide sequence of the VPg region was determined directly by chain-termination sequencing. The sequence of the viral RNA was found to be identical to that of the mutagenesis cartridge.

As was mentioned, the amino acid sequence of the mutagenesis cartridge was not completely identical to the type 1 (Mahoney) sequence. The lysine residue at position 10 of VPg had been replaced by an arginine residue. This alteration was necessitated by the placement of the unique *Stu* I restriction site in the center of the mutagenesis cartridge. The possibility that this amino acid substitution would affect VPg function and virus growth was quite low, because (*i*) this was a conservative change that maintains the charge at the normal position and (*ii*) both the type 2 (Lansing) and the type 3 (Leon) strains of poliovirus have an arginine in place of the lysine found in type 1 (31, 32).

The titer of W1-VPg15 was determined in parallel with wild-type virus at 33.5°, 37.0°, and 39.5°C. At all three temperatures both viruses produced titers that were within 0.5 $\log_{10}$ unit of each other. A one-step growth experiment was carried out to determine the replication efficiency of W1-VPg15 (data not shown). The growth rate for W1-VPg15 was found to be indistinguishable from the wild-type control virus.

VPg Mutants Containing Methionine Residues. The elucidation of the role VPg plays in RNA synthesis has been hampered by the difficulty in detecting free VPg or small nucleotide–protein complexes, the latter being visualized only after incorporation of [α-^{32}P]UTP *in vitro* (12, 13). Though a precursor of VPg, 3AB, can be followed after [^{35}S]methionine labeling, nucleotides have never been found attached to it. To simplify the study of VPg and its function(s), a methionine codon was inserted into the VPg coding region. The amino acid sequence of the VPg from the enterovirus echovirus 9 is highly conserved compared to poliovirus VPg, differing in only one position through the first 11 amino acids (33). This difference occurs at amino acid position 6, where poliovirus has a leucine and echovirus 9 has a methionine. Based on this homology and the conservative nature of the change, a methionine codon was introduced into the synthetic cartridge at position 6 of VPg (Fig. 2). This cartridge was then inserted into the full-length clone generating pT7-VPg22. Following transfection of HeLa cells with RNA described from pT7-VPg22, infectious virus was recovered and designated W1-VPg22. This virus was shown by dideoxy sequencing to contain the methionine codon.

Another mutant containing a methionine codon in the VPg region was fortuitously discovered while sequencing pNT15 clones and contains a methionine in place of a valine at position 13 of VPg (Fig. 2). During either the synthesis of the oligonucleotide or the cloning, an adenosine residue substituted for the wild-type guanosine residue, and an AUG codon was generated. This mutant when placed into the full-length clone also yielded a viable virus with the correct sequence and was designated W1-VPg30. Since this mutant mapped downstream of the *Stu* I restriction site and the methionine codon from pT7-VPg22 mapped upstream of the site, it was possible to combine the two mutations in a single step. Such a recombinant was made, and it too produced infectious virus after transfection. The recovered virus contains two methionine residues in the VPg polypeptide and was designated W1-VPg31 (Fig. 2).

The three viruses with the methionine substitutions in VPg were examined to determine whether these mutations imparted any deficiencies on the resultant viruses. Plaque titrations at 33.5°, 37.0°, and 39.5°C showed the normal variation as did wild type (data not shown). The one-step growth curves for these viruses indicated that they grow as well as the original mutant W1-VPg15 and the wild-type virus (data not shown). To determine whether the processing of viral proteins in the mutant viruses has been altered, infected cells were pulse-labeled with [^{35}S]methionine 3 hr after infection. The pattern of viral proteins produced in each mutant is similar to that produced from the wild-type control (Fig. 3*A*). There appears to be less-effective inhibition of host-cell protein synthesis in some of the lanes, the significance of which is uncertain. When a higher multiplicity of infection was used, host-cell shut-off was normal. The immunoprecipitations (Fig. 3*B*) demonstrate that, in each mutant virus, the altered VPg polypeptide and its precursors can be recognized by the appropriate antisera. Although not included in Fig. 3*B*, W1-VPg31 also shows the same pattern of immunoprecipitated products present in the other mutants. Note that VPg can be seen in those cases where it contains a methionine residue. This data confirms that free VPg exists in the infected cells (35).

VPg of echovirus 9 differs only in 4 amino acids from poliovirus VPg (Met-6, Lys-12, Leu-16, and Gln-18; ref. 33). We have changed the corresponding amino acids in W1-VPg15 to those of echovirus 9 VPg and obtained a mutant virus (W1-VPg29) that was viable at 32°C and at 37°C and yielded plaques indistinguishable in size from those obtained with W1-VPg15. The results of this experiment demonstrate that an allele replacement in the region of VPg between two human enteroviruses is compatible with viral replication.

Mutagenesis of the Tyrosine Residue. The tyrosine residue at position 3 in VPg is responsible for the linkage of VPg to the RNA. A comparison of the amino acid sequence of 27 picornaviral VPgs indicates an invariance for the tyrosine residue at position 3. To examine more closely the role of the tyrosine residue in VPg, two mutations were constructed. The first mutation, created by oligonucleotide site-directed mutagenesis in M13 prior to the construction of the synthetic mutagenesis cartridge, inserts a tyrosine reside in place of the threonine residue at position 4 (pT7-VPg21). This generates a VPg molecule that contains two tyrosines adjacent to

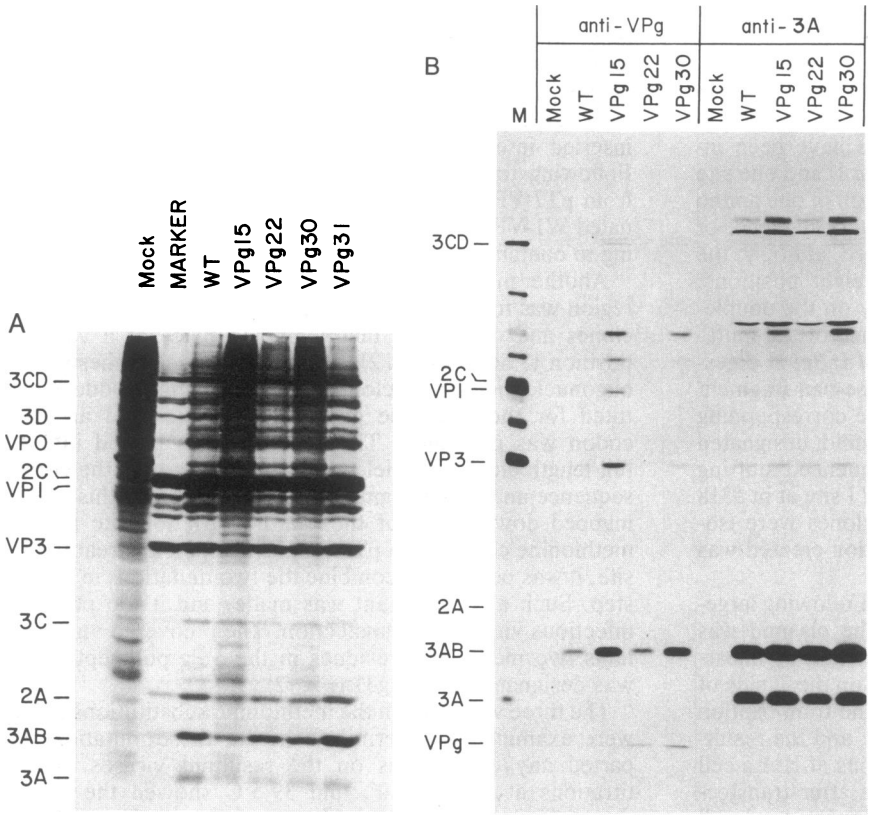

FIG. 3. (*A*) [^{35}S]Methionine labeling of HeLa cells infected with wild-type poliovirus and the VPg mutants at multiplicity of infection of 25. Labeling was performed from 3 to 5 hr after infection, at which time lysates were prepared and electrophoresed on a 12.5% NaDodSO$_4$/polyacrylamide gel. (*B*) Lysates were prepared as described in *A* except cells were infected at a multiplicity of infection of 100. Lysates were subjected to immunoprecipitation with anti-VPg (N10+C7) sera (12, 34) and anti-3A serum and electrophoresed. The rabbit anti-3A serum was induced with a carrier-linked synthetic decapeptide corresponding to the N-terminal sequence of polypeptide 3A (R.J.K., T. Takegami, and E.W., unpublished results) and specifically recognizes 3A and its precursors. Like anti-VPg sera, anti-3A nonspecifically precipitates capsid proteins. WT, wild type; M, marker.

each other and preserves the original position of the tyrosine residue. Because it is not a derivative of pNT15, it contains only the threonine → tyrosine change at position 4 and not the lysine → arginine change at position 10. The second mutation removes the tyrosine from the conserved third position and inverts it with respect to the threonine at position 4, generating a molecule having a tyrosine at position 4 and a threonine at position 3 (pT7-VPg16).

Both of these mutants described above failed to produce virus under the RNA transfection assay conditions. Cells had been transfected with the mutant RNAs at 33.5°, 37.0°, or 39.5°C, either under agar or under liquid medium, however, infectious virus was never recovered. To determine whether this result was due to an intrinsic defect in the functioning of VPg or whether it might be the result of a conformational change affecting the cleavage site and altering or preventing the cleavage by proteinase 3C^{pro}, RNA was transcribed by T7 polymerase *in vitro*. This was followed by *in vitro* translation of the RNA under conditions that allow optimal proteolytic processing (30). The results of this experiment (Fig. 4) show that the proteinase 3C^{pro} is capable of processing the glutamine/glycine cleavage sites defining VPg for pT7-VPg16 but not for pT7-VPg21. The translation products of wild-type viral RNA, pT7PV1-5, pT7-VPg15, and pT7-VPg16 RNA are completely identical with or without the addition of an infected viral extract. Therefore, the data suggest that the lesion introduced by modification of the tyrosine residue in pT7-VPg16 acts directly on the function of VPg and does not inhibit proteolytic processing.

The situation for pT7-VPg21 appears to be more complex. Processing of the polyprotein is reduced in this mutant as evidenced by a decrease in the appearance of the capsid polypeptides VP0 and VP1. More dramatic is the decrease in the P3 polypeptides, even after immunoprecipitation (data not shown), which suggests the block to virus production may lie in the inability of the proteinase to process the glutamine/glycine cleavage site separating protein 3A and VPg. However, it is not known whether the uncleaved precursor of VPg can be uridylylated *in vivo*. An additional protein band appears that migrates faster than band 3CD (≈70 kD) and is not present in the other lanes. This polypeptide can be immunoprecipitated by anti-2A, -2C, -3A, and -VPg (but not by anti-3C) antibodies and may be polypeptide P2-3AB (36). The addition of an infected virus extract containing proteinase activity to the translation mix of pT7-VPg21 results in further processing of the capsid proteins to levels that are seen in the wild-type control, whereas the level of 3CD is unaffected by the addition of exogenous viral proteins.

DISCUSSION

We have followed a strategy of genetic analysis of poliovirus by constructing a mutagenesis cartridge spanning the region of the poliovirus genome encoding VPg and its flanking cleavage sites. This cartridge was designed to be easily accessible to manipulation and to contain sufficient restriction sites such that oligonucleotide synthesis would be kept to a minimum. This approach has allowed us to perform a genetic analysis of this polypeptide with the objective of determining its function(s) and exploring how the primary and secondary structures of VPg carry them out. The data presented here indicate that mutations can be introduced into VPg with either little or no effect on the resulting virus or that they can result in a complete inactivation of the virus. It is assumed that intermediates, that is, conditional-lethal mutants mapping in VPg, will also be isolated.

The mutant viruses that were designed to have methionine within the amino acid sequence of VPg allowed us to label VPg with [^{35}S]methionine. This facilitates the study of the metabolism of VPg *in vivo* and *in vitro*. Moreover, we have exchanged VPg of poliovirus with that of echovirus 9 with little effect on virus growth. Thus, a number of residues in poliovirus VPg appear to have spacer function and are replaceable.

The mutation in plasmid pT7-VPg16 that was directed at altering the position of the linkage amino acid and the

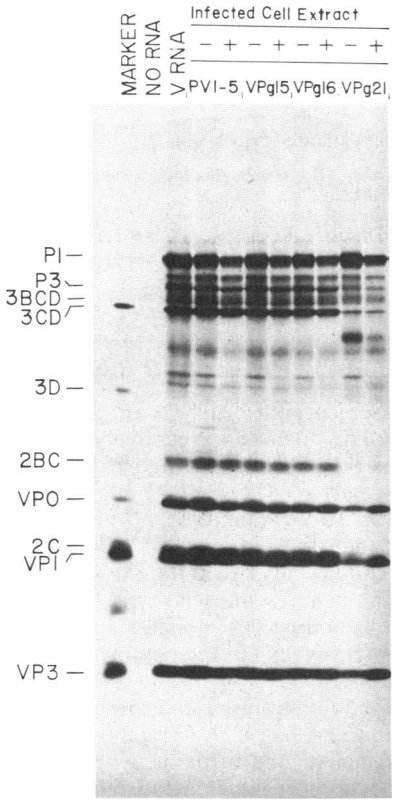

FIG. 4. *In vitro* translation of T7-transcribed RNA from pT7PV1-5, pT7-VPg15, pT7-VPg16, and pT7-VPg21. Translations were performed as described (30). Following translations an infected viral extract, competent for proteolytic processing, was added to the mixtures as indicated and incubated at 30°C for 60 min. The products were then electrophoresed on a 10% $NaDodSO_4$/polyacrylamide gel and visualized by fluorography. VRNA, viral RNA. Absence (−) or presence (+) of infected cell extract is indicated.

mutation in plasmid pT7-VPg21 that altered the target for the linking enzyme failed to produce infectious virus. In the latter case, where a tyrosine was substituted for a threonine directly adjacent to the linkage tyrosine, the protein processing pattern, as assayed by *in vitro* translation of the T7-transcribed RNA, shows a dramatic alteration in the P3 region of the polyprotein. This aberrant processing may itself be the primary block to virus replication since the 3A/3B-glutamine/glycine cleavage site no longer appears to be a substrate for the viral proteinase or the block may be coupled to an inability to link RNA to this mutated polypeptide. Since processing of the capsid precursors can proceed normally after the addition of an infected extract, whereas certain P3 precursors are unaffected by the addition, it is likely the insertion of the tyrosine residue disturbs the folding of the polyprotein on its carboxyl-terminal side. Such a misfolding may account for the reduced level of processing and the inhibition of exogenously added proteinase to cleave the P3 region polypeptides.

The mutation affecting the position of the tyrosine, pT7-VPg16, can be interpreted more precisely. Since the processing of the polyprotein is unaffected by the amino acid alteration, the defect lies in VPg function. Every picornavirus VPg sequenced to date has the tyrosine residue at position 3 conserved whereas those amino acids surrounding the tyrosine residue are quite variable. We suggest that the position of the tyrosine may be more important for VPg function than the immediately surrounding amino acid context.

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1. Yogo, Y. & Wimmer, E. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1877–1882.
2. Kitamura, N., Semler, B. L., Rothberg, P. G., Larsen, G. R., Adler, C. J., Dorner, A. J., Emini, E. A., Hanecak, R., Lee, J. J., van der Werf, S., Anderson, C. W. & Wimmer, E. (1981) *Nature (London)* **291,** 547–553.
3. Racaniello, V. R. & Baltimore, D. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4887–4891.
4. Lee, Y. F., Nomoto, A., Detgen, B. M. & Wimmer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 59–63.
5. Flanegan, J. B., Pettersson, R. F., Ambros, V., Hewlett, M. J. & Baltimore, D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 961–965.
6. Rothberg, P. G., Harris, T. J. R., Nomoto, A. & Wimmer, E. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4868–4872.
7. Ambros, V. & Baltimore, D. (1978) *J. Biol. Chem.* **253,** 5263–5266.
8. Nomoto, A., Detjen, B., Pozzatti, R. & Wimmer, E. (1977) *Nature (London)* **268,** 208–213.
9. Pettersson, R. F., Flanegan, J. B., Rose, J. K. & Baltimore, D. (1977) *Nature (London)* **268,** 270–272.
10. Nomoto, A., Kitamura, N., Golini, F. & Wimmer, E. (1977) *Proc. Natl. Acad Sci. USA* **74,** 5345–5349.
11. Ambros, V., Pettersson, R. F. & Baltimore, D. (1978) *Cell* **15,** 1439–1446.
12. Takegami, T., Kuhn, R. J., Anderson, C. W. & Wimmer, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7447–7451.
13. Takeda, N., Kuhn, R. J., Yang, C.-F., Takegami, T. & Wimmer, E. (1986) *J. Virol.* **60,** 43–53.
14. Flanegan, J. B., Young, D. C., Tobin, G. J., Stokes, M. M., Murphy, C. D. & Oberste, S. M. (1987) in *Positive Strand RNA Viruses*, eds. Brinton M. A. & Rueckert, R. R. (Liss, New York), pp. 273–284.
15. Bernstein, H. D., Sonenberg, N. & Baltimore, D. (1985) *Mol. Cell. Biol.* **5,** 2913–2923.
16. Sarnow, P., Bernstein, H. D. & Baltimore, D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 571–575.
17. Bernstein, H. D., Sarnow, P. & Baltimore, E. (1986) *J. Virol.* **60,** 1040–1049.
18. Racaniello, V. R. & Meriam, C. (1986) *Virology* **155,** 498–507.
19. Dewalt, P. G. & Semler, B. L. (1987) *J. Virol.* **61,** 2162–2170.
20. Shortle, D., DiMaio, D. & Nathans, D. (1981) *Annu. Rev. Genet.* **15,** 265–294.
21. Racaniello, V. R. & Baltimore, D. (1981) *Science* **214,** 916–919.
22. Semler, B. L., Dorner, A. J. & Wimmer, E. (1984) *Nucleic Acids Res.* **12,** 5123–5140.
23. Pincus, S. E., Diamond, D. C., Emini, E. A. & Wimmer, E. (1986) *J. Virol.* **57,** 638–646.
24. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
25. Zoller, M. J. & Smith, M. (1984) *DNA* **3,** 479–488.
26. Hanecak, R., Semler, B. L., Ariga, H., Anderson, C. W. & Wimmer, E. (1984) *Cell* **37,** 1063–1073.
27. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
28. Chen, E. & Seeburg, P. H. (1985) *DNA* **4,** 165–170.
29. van der Werf, S., Bradley, J., Wimmer, E., Studier, F. W. & Dunn, J. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2330–2334.
30. Ypma-Wong, M. F. & Semler, B. L. (1987) *Nucleic Acids Res.* **15,** 2069–2088.
31. LaMonica, N., Meriam, C. & Racaniello, V. (1986) *J. Virol.* **57,** 515–525.
32. Stanway, G., Cann, A. J., Hauptmann, R., Hughes, P., Clarke, L. D., Mountford, R. C., Minor, P. D., Schild, G. & Almond, J. W. (1983) *Nucleic Acids Res.* **11,** 5629–5643.
33. Werner, G., Rosenwirth, B., Bauer, E., Scifert, J., Werner, F. & Besemer, J. (1986) *J. Virol.* **57,** 1084–1093.
34. Semler, B. L., Anderson, C. W., Hanecak, R., Dorner, L. F. & Wimmer, E. (1982) *Cell* **28,** 405–412.
35. Crawford, N. M. & Baltimore, D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7452–7455.
36. Takegami, T., Semler, B. L., Anderson, C. W. & Wimmer, E. (1983) *Virology* **128,** 33–47.