Replication of Poliovirus RNA Containing Two VPg Coding Sequences Leads to a Specific Deletion Event

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Studies of the poliovirus genome-linked protein VPg have shown that this small viral protein is required for replication of virus-specific RNA (Q. Reuer, R. J. Kuhn, and E. Wimmer, *J. Virol.* 64:2967-2975, 1990). To understand the mechanism of RNA replication, we constructed a recombinant poliovirus genome encoding two tandemly arranged VPg coding sequences that were nearly identical in both nucleotide and amino acid sequence. Following transfection of this two-VPg-containing RNA into HeLa cells, we found a specific and selective deletion in the progeny virus genome. Sequence analysis of the recovered viral RNA indicated that the complete nucleotide sequence encoding the second (3C-proximal) VPg coding sequences was removed, restoring the authentic genome sequences in the poliovirus genome. Analysis of viral RNAs following transfection suggested that the deletion event occurred during genome replication. Deletion could have occurred via homologous recombination between two VPg sequences or via intramolecular deletion with loop-out of the template. In vitro translation of the two-VPg-containing transcript RNA indicated aberrant processing of the viral polypeptide. This result suggested that selection of the wild-type genotype in the transfected cells may occur at the level of viral protein synthesis.

Poliovirus VPg, a virus-encoded protein of 22 amino acids (1, 9, 20, 25), is covalently linked to the 5' termini of all newly synthesized viral RNAs recovered from infected cells (35, 37). VPg, a basic protein having a net charge of +4 and a pI of >10 (1), is linked to the polynucleotide chain via a phosphoester bond between the O⁴ hydroxyl group of the tyrosine residue at position 3 of VPg and the 5'-phosphate of the terminal uridine nucleotide in the RNA genome (VPg-Tyr-O⁴-pUAAAA-) (2, 42). This tyrosine residue is conserved in all picornavirus VPgs. The presence of VPg on the RNA is not required for either translation or infectivity, because proteolytic removal of the VPg protein from viral RNA does not affect the infectivity of the RNA (36).

In reactions of poliovirus RNA synthesis with partially purified polypeptides, anti-VPg antibodies have been reported to specifically inhibit initiation of viral RNA synthesis (4, 30). Since the poliovirus-specific RNA polymerase is strictly primer dependent (49), it has been proposed that a uridylylated form of VPg or its precursor 3A-VPg (3AB) serves as a primer for the replication of viral RNA (31, 35, 44, 50). This hypothesis was supported by the observation that in a membranous replication complex, the endogenous VPg can be uridylylated in vitro to VPg-pUpU and subsequently extended to longer RNA molecules (45, 46). Moreover, antibodies raised against VPg do immunoprecipitate VPg as well as a uridylylated form (VPg-pUpU) from poliovirus-infected cells (6). Recently, poliovirus has been synthesized de novo in an extract of uninfected HeLa cells (29), and it was found that anti-VPg antibodies can block virus formation in this reaction by inhibiting RNA replication (27b). Site-directed substitutions of the tyrosine in position 3, the only tyrosine in VPg of poliovirus, produced a lethal phenotype for virus growth. In this mutant, RNA synthesis was found to be abolished (40), an observation suggesting

that the covalent attachment of VPg to plus and minus strands is required for RNA replication.

The genomes of all five picornavirus genera (*Enterovirus*, *Rhinovirus*, *Hepatovirus*, *Cardiovirus*, and *Aphthovirus*) encode VPgs 22 to 25 amino acids in length. However, whereas most of these viruses contain only one copy of the VPg reading frame, the genomes of aphthoviruses (e.g., foot-and-mouth disease virus [FMDV]) contain three distinct VPg reading frames in tandem (10), all of which are important for genome replication (8). The reason for this peculiar complexity of the FMDV genome remains unknown.

In this study, we constructed a poliovirus genome containing two almost identical VPg reading frames in tandem (designated 2VPg), using the cartridge mutagenesis method previously developed (22, 23). The genetic stability of this 2VPg mutant RNA (transcripts of pPVM-2VPg-1) has been tested. Surprisingly, transfection of HeLa cells with the mutant RNA yielded poliovirus in which one VPg sequence was deleted, restoring the original gene order of the viral genome. Biochemical analysis suggested that the 3C-proximal VPg was removed. Our results indicate that poliovirus, unlike FMDV, cannot tolerate two tandem VPg units.

MATERIALS AND METHODS

Cells, strains of bacteria, and plasmids. Transfections, virus plaque assays, and viral RNA purification were performed with R19 HeLa cell monolayers maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (GIBCO). *Escherichia coli* DH5 was used for selection and propagation of recombinant plasmids. Plasmid pT7-VPg22 carries a full-length poliovirus genome which encodes a VPg protein with a methionine residue at the position 6 (22). The in vitro-transcribed viral RNA from plasmid pT7-VPg22 has the same specific infectivity as does the cDNA clone of poliovirus type 1 carried by pT7PV1-5

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TABLE 1. Summary of oligonucleotides used in the work

Oligonucleotide	Sequence (5' to 3')	Purpose
9g	CCTAACGTGCCACCATTCGGACCGCAAAG	9g, 9h, 9i, and 9j were used for construction of double-VPg mutant pPVM-2VPg-1
9h	TGTACCTTTGCGGTCCGAATGGTGGGCACGTTAGG	
9i	GTACAAGGAGCATACACTGGTTTACCAAACAAGAGG	
9j	CCTCTTGTGTTGGTAAACAGTGTATGCTCCT	
2910	GGGTTGGATAGTTAACATCACCAGCC	Used as primer for PCR and plasmids construction
2912	CCTGCTTGATCTTCGAGCGC	Same as 2910
2933	GCTGTAACAATGTTTCTTTTAGCC	Used as primer for PCR and RNA sequence analysis

(48). Two other type 1 poliovirus cDNA clones, pT7XLD and pT7XL2C (27, 32), were also used in this work.

Construction of pT7-PVM. Restriction enzymes used in cloning DNA were purchased from New England Biolabs. To facilitate mutagenesis in the VPg coding region, we introduced a cleavage site for the restriction enzyme *HpaI* upstream of the VPg gene in clone pT7XLD. To do so, clone pT7XL2C was first digested with restriction enzymes *NheI* (position 2470) and *BglII* (position 5601); the resulting 3.2-kb *NheI-BglII* fragment containing the unique *HpaI* site at position 5242 was then ligated into the *NheI*- and *BglII*-treated vector pT7XLD to give rise to plasmid pT7XLD2C. Finally, the *BglII-EcoRI* fragment (nucleotide positions 5601 and 7527, respectively) of the new clone pT7XLD2C was replaced by the *BglII-EcoRI* fragment derived from clone pT7PV1-5. The genetic order of the resulting cDNA clone, named pT7-PVM, was confirmed by restriction enzyme mapping. The in vitro-transcribed RNA from the cDNA clone pT7-PVM produced plaques faster than did RNA from clone pT7PV1-5 upon transfection of HeLa cell monolayers (18 to 20 h). The pattern of the processed proteins from translation products of pT7-PVM RNA in vivo and in vitro was identical to that of RNA of poliovirus type 1 (Mahoney).

Construction of pPVM-2VPg-1. To insert the second VPg sequence into the poliovirus genome, oligonucleotides 9g, 9h, 9i, and 9j (Table 1) were used for mutagenesis. Plasmid pT7-VPg22 was first digested with *StuI* and *BglII*, resulting in 201-bp and 11-kbp fragments. The 201-bp fragment was blunt-end ligated to the preannealed oligonucleotide pair 9i-9j, whereas the 11-kbp fragment was blunt-end ligated to the oligonucleotide pair 9g-9h. The two new resulting fragments were ligated to give rise to clone pT7-VPg33. The inserted VPg sequence in clone pT7-VPg33, containing two VPg sequences, was confirmed by sequence analysis, using

the dideoxy-chain termination method (43); the exact sequences of VPg1 (3A proximal) and VPg2 (3C proximal) are shown in Fig. 1. As the last step, a 470-bp fragment encompassing the two VPg coding sequences was amplified from clone pT7-VPg33, using the polymerase chain reaction (PCR) (13) with oligonucleotides 2910 and 2912 (Table 1) as primers. Digestion of the 470-bp PCR product with *HpaI-BglII* yielded a 429-bp fragment, which was then inserted into the cDNA clone pT7-PVM at the *HpaI-BglII* sites. The sequence of the VPg gene segment in the new recombinant clone, which was termed pPVM-2VPg-1, was confirmed by sequence analysis. Similarly, a PCR fragment containing the VPg sequence from pT7-VPg22 was generated by using primers 2910 and 2912, and the *HpaI-BglII* fragment of this PCR product was ligated into pT7-PVM at the *HpaI-BglII* sites to give rise to clone pPVM-VPg22. Clone pPVM-VPg22 was used as a positive control throughout this work.

In vitro transcription and RNA transfection. T7 RNA polymerase was prepared by Kevin Harris in our laboratory by expression in *E. coli* (7) and subsequent purification. Transcription of RNA by T7 RNA polymerase was performed as previously described (48) except that a higher final concentration of dithiothreitol (20 mM) was used in the reaction buffer. RNA transfection of HeLa cell (R19) monolayers by using DEAE-dextran was performed as published previously (23). For plaque assays, HeLa cells were first incubated with the RNA-DEAE-dextran mixture at room temperature for 30 min and then overlaid with DMEM-agar containing 5% fetal bovine serum. The transfected cells were further incubated at 37°C until plaques were formed.

Preparation of viral RNA and nucleotide sequencing. HeLa cell monolayers (4×10^6 cells) were infected with virus that resulted from transfection of pPVM-2VPg-1 RNA at a multiplicity of infection of 20. At 7 h postinfection, total cyto-

FIG. 1. Schematic representation of the double-VPg construction pPVM-2VPg-1. Two almost identical, tandemly arranged coding sequences of the genome-linked protein (VPg) were constructed in the cDNA clone pT7-PVM. The open box represents the VPg sequence of the VPg mutant in pT7-VPg22, a derivative of the VPg mutagenesis cartridge in pT7-VPg15 (shaded box). Viruses derived from pT7-VPg15 and pT7-VPg22 were previously characterized; they were indistinguishable from wild-type virus derived from pT7PV1-5 (22). Nucleotides and amino acids which differ from the corresponding wild-type sequences are underlined.

plasmic RNA was extracted as described previously (40). The VPg region of the RNA was sequenced by the dideoxy-chain termination method (43), using [α - 35 S]dATP (Amersham) and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim).

Detection of RNA synthesis by PCR. Total cytoplasmic RNAs were extracted from HeLa cells at various times after the cells were transfected with RNAs derived from plasmid pPVM-2VPg-1. After treatment of the isolated RNA fraction with RQ1 RNase-free DNase, the VPg regions of the virus-specific RNAs were analyzed by cDNA synthesis followed by PCR as described by Molla et al. (29). Primer 2933 or 2910 (Table 1) was used for synthesizing cDNAs derived from plus-strand RNA or minus-strand RNA, respectively. The PCRs were performed by using the cDNAs as templates and oligonucleotides 2933 and 2910 as primers. RQ1 DNase was purchased from Promega, and the *Taq* polymerase used for PCR was purchased from Perkin-Elmer/Cetus.

In vitro translation and immunoprecipitation. In vitro translations of viral RNAs were performed essentially as described by Molla et al. (29). Briefly, viral RNAs transcribed in vitro from pPVM-VPg22 and pPVM-2VPg-1 were mixed with a nuclease-treated cell extract (S10 fraction of the disrupted HeLa cells) plus translation mixture (containing essential ingredients of translation) and 1 mCi of [35 S]methionine (ICN Radiochemicals) per ml. Incubations were performed at 30°C from 2 to 11 h. Samples were analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-13.5% polyacrylamide gels and subsequent autoradiography.

Immunoprecipitation of proteins was performed as follows. Twenty microliters of the translation mixture was preincubated in 1% SDS buffer for 10 min at 65°C. After centrifugation at 10,000 rpm for 10 min, the supernatants were diluted 1:10 with phosphate-buffered saline (PBS) buffer (8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 140 mM NaCl) and then incubated at 4°C overnight with an equal volume of prebound pAS-antibody mixture. (pAS-antibody contains protein A-Sepharose complexed with specific antibody, diluted 1 to 15 in 50% Sephadex G-50. Protein A-Sepharose and Sephadex G-50 were purchased from Pharmacia.) The unbound material was washed off with PBS buffer; the pAS-antibody with bound polypeptides was then boiled for 5 min in Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (24).

RESULTS

Generation of a recombinant poliovirus genome encoding two VPg units. We are interested in understanding the mechanism of RNA replication of picornaviruses. Because of the essential nature of VPg in this process, we introduced two almost identical VPg coding sequences into the poliovirus genome by cartridge mutagenesis (Fig. 1). To investigate possible pathways by which VPg is delivered for RNA linkage, the first VPg coding sequence (3A proximal) contained a methionine residue at position 6, whereas the second VPg coding sequence (3C proximal) contained a leucine at this position. This genetic difference was used as a marker to analyze which VPg would be linked to the viral RNA or, in the case of deletion, which VPg remained in the genome. In the FMDV genome, each of the three different VPgs (10) has been found to be linked to the viral RNA (17). Recent evidence suggests that all three VPgs of FMDV are important for virus viability (8). We originally expected that duplicating the VPg genes in the poliovirus genome would

FIG. 2. Plaque morphology of polioviruses generated by transfection of wild-type RNA derived from pPVM-VPg22 (A and C) and of the mutant RNA derived from pPVM-2VPg-1 (B and D). Transfections of 3 ng of wild-type RNA or 5 μ g of mutant RNA were overlaid with DMEM-agar, and the plaques were visualized after 48 h (A and B) or 68 h (C and D) of incubation at 37°C.

yield a viable virus but that it nevertheless would affect RNA replication.

Deletion of one VPg from a 2VPg poliovirus genome. After transfection of HeLa cells with in vitro-transcribed full-length RNA of pPVM-2VPg-1, containing two VPg units in tandem, viable virus particles were formed. The cytopathic effect (CPE) resulting from transfection with this mutant RNA was apparent after approximately 24 h, whereas CPE from the control RNA of clone pPVM-VPg22 appeared at 18 h posttransfection. When assayed under the DMEM-agar overlay, the monolayer of HeLa cells that had been transfected with the control or mutant RNAs exhibited plaques of different sizes. This difference, however, diminished if the cells were incubated for 68 instead of 48 h. The average size of plaques developed upon transfection with pPVM-2VPg-1 RNA was smaller (Fig. 2B) than that after transfection with pPVM-VPg22 (Fig. 2A) at 48 h posttransfection. In contrast, the plaque sizes were nearly identical after 68 h of incubation (Fig. 2C and D). Quantitative analysis also showed that the RNAs from pPVM-VPg22 and pPVM-2VPg-1 differed in their efficiencies to infect HeLa cells; the RNA prepared from pPVM-VPg22 had a specific infectivity averaging 10^4 PFU/ μ g, whereas the RNA from pPVM-2VPg-1 had a specific infectivity as low as 5 PFU/ μ g. Therefore, a high concentration of mutant RNA (5 to 10 μ g/ 4×10^6 cells) was required to obtain roughly the same number of plaques by transfection under agar overlay as from wild-type RNA.

The reason for the large amount of mutant RNA required for transfection of the HeLa cells is unknown. It is conceivable, although not likely, that the RNA genome of the mutant is less stable as a result of formation of novel cleavage sites for nucleases. Alternatively, a higher number of mutant RNA molecules might be required to finally

generate infectious virus particles, whereas most transfections were aborted. To distinguish between these two possibilities, we added 1 to 5 μ g of tRNA as carrier to 50 μ l of transcription mixtures before or after the *in vitro* transcription with T7 RNA polymerase. The tRNA-protected RNAs were then used to transfect HeLa monolayers. No effect on the transfection was observed with either wild-type or mutant RNA. Although this result is not entirely conclusive, it nevertheless suggests that the low infectivity of mutant RNA might be due to its reduced ability to replicate in transfected cells and not to degradation occurring during the process of transfection.

Virus generated from cells transfected with pPVM-VPg22 and pPVM-2VPg-1 RNAs was used to infect fresh monolayers of HeLa cells. Surprisingly, these virus preparations produced plaques similar in size. Moreover, the titers after secondary infections were about the same (10^9 PFU/ml). We therefore considered the possibility that progeny virus of transfections with pPVM-2VPg-1 RNA contained wild-type gene order -3A-VPg-3C-. Following infection of HeLa cells with pPVM-2VPg-1 progeny virus at a multiplicity of infection of 20, RNA was extracted at 7 h postinfection and the region containing the VPg coding sequence was sequenced by using oligonucleotide 2933 as the primer. Sequence analysis revealed that the genotype of this RNA was exactly the same as that of pPVM-VPg22: the 3C-proximal VPg sequence was deleted in the packaged viral RNA derived from the 2VPg clone pPVM-2VPg-1. We sequenced the VPg coding regions of five independent virus plaque isolates that were obtained following independent transfections with the 2VPg RNA; all isolates had sequences that were identical to that of pPVM-VPg22. The mechanism of the precise deletion of the second VPg is not yet clear (see Discussion), but once it occurred, it must have been followed by a strong selection of the one-VPg-containing RNA in transfected HeLa cells.

We repeated the experiment with RNA of poliovirus genomic construct pPVM-2VPg-2, which was identical to pPVM-2VPg-1 except that the order of the two VPgs was reversed. In pPVM-2VPg-2, the 3A-proximal VPg had the sequence of pT7-VPg15 whereas the 3C-proximal VPg contained the marker methionine of pT7-VPg22 in position 6. Transfection of the RNA derived from pPVM-2VPg-2 yielded progeny virus with a phenotype similar to that of pPVM-2VPg-1 (data not shown). Sequence analysis of the recovered viral RNA (purified from total progeny viruses and four additional independent isolates) revealed that it contained only one VPg unit and the genotype was identical to that of pT7-VPg15, that is, with leucine at position 6.

Replication of pPVM-2VPg-1 RNA in HeLa cells. It was of interest to determine the point in the replication cycle at which the specific deletion of the 3C-proximal VPg had occurred. To answer this question, HeLa cells were transfected with RNAs derived from pPVM-2VPg-1. Total intracellular RNA was then extracted at 0, 2, 5, 8, 14, 18, and 22 h posttransfection and subjected to reverse transcription, using either oligonucleotide 2933 or oligonucleotide 2910 as a primer specific for plus- or minus-strand reverse transcription, respectively. The reverse transcripts were then amplified by PCR. Figure 3a shows the amplified PCR product derived from the plus-strand viral RNA of pPVM-2VPg-1; Fig. 3b shows that of minus strands. For marker fragments, we PCR amplified plasmid DNA of pPVM-2VPg-1 and pPVM-VPg22 with the same oligonucleotides as primers, a reaction yielding DNAs of 327 and 261 bp, respectively (Fig. 3a and b, lanes 8 and 9, respectively). Deletion of one VPg unit, as indicated by the shortening of the PCR product

FIG. 3. Detection of replication products generated from transfections with pPVM-2VPg-1 RNA. (a) Cytoplasmic RNAs were isolated at different times posttransfection, the plus-strand RNAs were reverse transcribed and PCR amplified, and the products were subjected to agarose gel electrophoresis as described in Materials and Methods. Lanes: 1 to 7, RNAs isolated at 0, 2, 5, 8, 14, 18, and 22 h posttransfection; 8, plasmid DNA of pPVM-2VPg-1 as the PCR template; 9, plasmid DNA of pPVM-VPg22 as the template. The arrows indicate the specific PCR products from the 2VPg genome (upper) and from the 1VPg genome (lower). (b) The same analysis as in panel a except that reverse transcription was specific for minus strands.

derived from the plus-strand RNA, was detectable at 2 h posttransfection (Fig. 3a, lane 2). At 22 h posttransfection, when CPE of the transfected cells just started, most of the plus-strand RNA retained only one VPg in the genome (Fig. 3a, lane 7). A different result was obtained for minus-strand RNA synthesis. Throughout the cycle, minus strands with two complementary VPg sequences were the predominant form detectable by reverse transcription-PCR, whereas the deleted form appeared only 14 h posttransfection and remained a minor species (Fig. 3b, lanes 5 to 7).

Reverse transcription-PCR analysis of the RNA genome resulting from transfection of cells with pPVM-2VPg-2 RNA showed results similar to those obtained with pPVM-2VPg-1 (data not shown).

In vitro translation of pPVM-2VPg-1 RNA and proteolytic processing. In our search to identify the steps in viral replication responsible for the strong selection for the deleted (wild-type) genome, we analyzed protein synthesis and proteolytic processing directed by mutant 2VPg RNA. For this purpose, we made use of the HeLa cell-free system developed by Molla et al. (29) which faithfully translated poliovirus RNA *in vitro* with concomitant proteolytic processing nearly indistinguishable from that observed *in vivo*. Translation of the RNAs derived from pPVM-2VPg-1 and pPVM-VPg22 were carried out for 2, 5, 8, and 11 h at 30°C (Fig. 4). As expected, the proteins translated from the pPVM-VPg22 RNA showed patterns resembling that of the poliovirus-infected cell extract (lanes 2, 4, 6, and 8) with the exceptions of 3D^{pol} and 3C^{pro}, which were produced in very low amounts.

It is apparent that the open reading frame of the 2VPg RNA was intact, as the capsid precursor P1 is efficiently cleaved to VP0, VP1, and VP3, a cleavage catalyzed by 3CD^{pro} (16, 51); 3CD^{pro} maps to the C terminus of the polyprotein. Proteinase 2A is also produced at nearly wild-type levels, an observation indicating that its N-terminal *cis* cleavage and also its C-terminal *trans* cleavage (the latter being catalyzed by a polypeptide containing 3C^{pro} activity)

FIG. 4. In vitro kinetics of protein synthesis and processing directed by wild-type or mutant RNA. RNAs, transcribed in vitro, were translated in an HeLa cell extract as described in Materials and Methods. Incubations were performed from 2 to 11 h as indicated. Lanes: 2, 4, 6, 8, 11, and 13, reactions with pPVM-VPg22 RNAs; 3, 5, 7, 9, 12, and 14, reactions with pPVM-2VPg-1 RNAs; 1, cytoplasmic extract of [35 S]methionine-labeled poliovirus type 1 (Mahoney)-infected HeLa cells as the marker; 10, no RNA; 11 to 14, polypeptides immunoprecipitated with anti-2C serum (14).

were normal. In contrast, proteolytic processing of the polyprotein consisting of 2BC-3ABCD (3B = VPg) followed an unusual pathway. The appearance of a prominent, new band of 50 kDa, designated 2C-3AB, was easily recognizable (Fig. 4, lanes 3, 5, 7, and 9). At the same time, generation of 2C and 3A was very slow (compare lane 4 with lane 5). Immunoprecipitation with anti-2C antibodies (Fig. 4, lanes 11 to 14) and anti-3A antibodies (data not shown) confirmed the identity of the 50-kDa band. Other unusual intermediates (P2-3AB and 3BCD) were also seen with the 2VPg RNA (lanes 3, 5, 7, 9, 12, and 14). We conclude that the cleavages between 2C-3A and 3A-3B were severely impaired in the polyprotein containing two tandemly arranged VPg sequences. Cleavage between the two VPg sequences, however, appeared to have occurred rapidly.

It should be noted that translation and proteolytic processing of pPVM-2VPg-2 RNA showed results identical to those obtained with pPVM-2VPg-1 RNA (data not shown).

DISCUSSION

Cartridge mutagenesis of segments of the poliovirus genome has proven to be a versatile method in analyses of genome replication (22, 23, 40) or of the biological properties of the poliovirus capsid (33, 34). This method has allowed us to construct a poliovirus genome with two tandemly arranged VPg sequences and to test the replication of such a mutant within HeLa cells. This experiment was prompted by the report from Forss et al. (10) that FMDV, another picornavirus belonging to the genus *Aphthovirus*, encodes three VPg units in tandem, all of which are used in a nearly

random fashion (17). Even though poliovirus has no apparent need for more than one VPg, we expected that the 2VPg mutant would replicate, albeit with a phenotype that, in turn, might shed light on the function of VPg. The results reported here showed that poliovirus containing two VPg units in tandem was unable to proliferate sufficiently so that we were able to isolate virus stocks for biochemical analysis. In contrast, FMDV cannot proliferate with just one VPg coding sequence, whereas, surprisingly, FMDV mutants containing four VPg sequences in tandem have been reported to be viable (8).

The progeny virus that was recovered after transfections with 2VPg RNA contained only one VPg, regardless of the order in which two genetically distinct VPgs were arranged. The retained VPg had always kept the amino acid at position 6 from the 3A-proximal VPg. It should be noted that the VPgs, which differed only in position 6 (leucine or methionine), are functionally fully competent (22). Therefore, a preference of the deletion based on the single amino acid difference would not have been expected and, indeed, was not observed. The mechanism by which the 3C-proximal VPg was deleted in numerous different analyses is unknown. One possibility is homologous recombination whereby crossover occurred between the identical RNA sequences of 48 nucleotides downstream from the codons for Met and Leu. Alternatively, loss of the 3C-proximal VPg could have occurred by a mechanism of intramolecular deletion during either minus- or plus-strand RNA synthesis. The mechanism of deletions in poliovirus replication is poorly understood and may be divided into homologous deletions (of genetic units in viral RNA with homologous sequences) or illegitimate deletions, as in the formation of defective interfering particles (21). In the former, the nascent RNA strand would stop elongation, partially dissociate from the template (14), and resume elongation at a downstream homologous sequence of the same template sequence. A hallmark of genetic recombination is its precision; that is, deletion or insertions are not observed (15, 19, 41). Whether that is true for homologous deletions remains to be seen. We have not observed any deletions or insertions in the retained VPg sequences which may be the result of selection. The frequency of recombination in poliovirus replication has been estimated to be 10^{-3} to 10^{-4} and is therefore surprisingly high (15). The difference in specific infectivity between wild-type RNA (10^4 PFU/ μ g) and 2VPg RNA (5 PFU/ μ g) observed by us is of the same order of magnitude. This finding could be interpreted to suggest recombination. However, differentiation between recombination and intramolecular deletion requires cotransfection with RNAs that are genetically marked both upstream and downstream of the VPg region. These experiments are in progress.

Analysis of replicating viral RNA from transfected HeLa cells suggests that synthesis of the deleted (wild-type) plus strands surpasses that of the 2VPg RNA by 8 h posttransfection (Fig. 3a). Although quantitative PCR (15) was not used in these studies, it nevertheless is apparent that the largest signal for 1VPg plus-strand RNA synthesis was observed by 22 h, coincident with CPE of the HeLa cells detectable at 22 h posttransfection. The analysis of minus strands, however, yielded a different result. Surprisingly, the signal of 2VPg minus-strand RNA was strong throughout the incubation of the transfected cells, whereas the signal for 1VPg minus-strand RNA was detectable only 14 h posttransfection and remained very weak.

The strong signal of 2VPg minus strands is in contrast to an unexpected weaker signal of 2VPg plus strands. One

possible explanation is that the 2VPg plus strands are degraded, which seems unlikely. Another intriguing possibility is that the products of proteolytic processing which contain aberrant products abrogate the down regulation of initiation of 2VPg minus strands that is normally seen in infections, an effect seen for 1VPg minus-strand synthesis. Apparently, this defect cannot be complemented in *trans*. Whether this reflects the possible *cis* action of certain gene products in poliovirus RNA replication (18) remains to be seen.

In vitro translation and polypeptide processing of wild-type 1VPg and mutant 2VPg RNAs allowed us to conclude that the mutant polyprotein, although synthesized in seemingly normal quantity, was not properly processed, particularly in the 2BC-P3 region. This region contains polypeptide domains essential for genome replication: 3D^{pol}, the RNA polymerase (49); 3AB, a precursor for VPg (11, 44) and a cofactor for 3D^{pol} (37a); and 2C, an ATPase (27a) that has been implicated in RNA replication by mutational analysis (26, 27, 38, 39, 47). Moreover, 2BC has been suggested to be essential for vesicle formation occurring during poliovirus replication (5). Finally, 3CD^{pro} appears to be involved in viral RNA replication via its propensity to form, together with an unknown host cell factor, a complex with the 5' end of viral plus strands (3). The aberrant processing pathway of the 2BC-P3 region of the viral polyprotein observed in 2VPg RNA translation may, therefore, be deficient in supplying the required polypeptides in sufficient quantities for specific steps of RNA replication. We propose that this is the reason for the strong selective pressure in vivo favoring the deleted genotype. An alternative mechanism of selection that is based on restricted encapsidation of the 2VPg genome due to the 0.89% increase of its size may be envisioned. This is highly unlikely, however, in view of the recent construction of viable dicistronic poliovirus (28) containing a genome that is larger by 8% than that of the wild-type virus RNA. Selection at the level of encapsidation is also unlikely because the deletion event is apparent relatively early in replication, and the cleavage between the two tandemly arranged VPgs is very fast, at least in vitro.

Aberrant processing of poliovirus mutant polyproteins, particularly those with alterations in the 2BC-P3 domain, has also been observed by Giachetti et al. (11) and by us (37b). It is likely that the 2BC-P3 region is processed predominantly if not exclusively by an intramolecular mechanism (in *cis*). It appears that the insertion of the second VPg into the P3 region interferes with the normal cascade of *cis* cleavages of 2BC-P3. The result is an imbalance of the normal processing products that may lead to the observed severe inhibition of plus-strand RNA synthesis. Removal of the VPg insert by recombination then provides the strong selection for the recombinant with wild-type gene order.

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