Genetic recombination of poliovirus in a cell-free system

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ABSTRACT Genetic recombination of plus-strand RNA viruses is an important process for promoting genetic variation. By using genetically marked poliovirus RNAs, we have demonstrated that genetic recombination can occur in a cell-free system that generates infective virus from added poliovirus RNA. Recombinant polioviruses were isolated, and the region of crossing over was roughly mapped. Recombinants could be isolated even under conditions where the yield of viruses from one of the parental RNAs was depressed to levels comparable to or less than the yield of recombinant viruses, an observation suggesting that only one of the recombining RNAs needs to be replication-competent. The generation of poliovirus recombinants in a cell-free system offers new possibilities for studying recombination and evolution of RNA viruses.

Rampant genetic variation is a hallmark of RNA viruses. The predominant reason for this phenomenon is the misincorporation of nucleotides during RNA synthesis in the absence of proofreading and editing functions (1). The error rate in RNA synthesis during poliovirus (PV) proliferation is roughly 10^{-4} per base pair per replication cycle (2), a value typical for RNA viruses that thus exist as “quasi-species” (3). Variation of plus-strand RNA viruses is increased by genetic recombination, which is the exchange of genetic elements of different viruses during their replication in the same cell (2, 4). Genetic recombination of an RNA virus (PV) was first described by Hirst in 1962 (5). Subsequent studies determined that the exchange of genetic elements occurs with frequencies matching those of the misincorporation of nucleotides, the former depending on the degree of homology between the parental RNA strands and on the size of the target area between the genetic markers (2). King (6) has estimated that 10–20% of viral genomes may undergo genetic recombination during a single growth cycle. Indeed, evidence has been presented suggesting a remarkably high incidence of genetic recombination between sibling strands in PV-infected cells (7). This phenomenon is considered useful for the RNA virus to eliminate debilitating mutations in the genome or to adapt progeny virus to a new environment. Numerous studies have addressed the mechanisms by which the exchange of genetic elements can occur between two PV genomes. The evidence supports a model of homologous recombination by copy choice, predominantly during minus-strand RNA synthesis (2). Details of the crossover mechanism, particularly the role of viral proteins, remain obscure.

The incubation of PV RNA in cytoplasmic extracts of uninfected HeLa cells leads to viral protein synthesis, RNA replication, and assembly of new virions indistinguishable from the parental virus (8). This cell-free, *de novo* synthesis of PV has nullified the dictum that viruses can replicate only in intact cells. The method allows the manipulation of the replicating

system in the absence of the plasma membrane barrier, a strategy that has been useful to decipher several aspects of PV replication (9–12).

We are interested in exploiting the cell-free synthesis of PV for studying genetic events such as recombination. For this purpose, we have prepared RNAs of two genetically distinct type 1 polioviruses: *wt* PV1 (Mahoney) [PV1(M)*g*^s], whose RNA replication is sensitive to the action of 2 mM guanidine hydrochloride (Gua-HCl), and a guanidine-resistant (*g*^r) variant of a chimeric PV [PV1(RIPO)] whose internal ribosomal entry site (IRES) was exchanged with that of human rhinovirus type 2 (HRV2; 13). PV1(RIPO) is unable to proliferate in neuroblastoma cells (13), a property of host range restriction that was used to select recombinants. Coreplication of PV1(M)*g*^s and PV1(RIPO)*g*^r in a cytoplasmic extract of uninfected HeLa cells yielded genetic recombinants, a result that adds credibility to the cell-free replication system as an authentic medium for the study of PV proliferation.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. HeLa cells (R19) and SK-N-MC cells were grown as described previously (7, 13). Plaque assays were carried out as described previously (14). Plasmid pT7-PVM encodes full-length cDNA of PV RNA downstream of the T7 promoter (15, 16). Plasmid pT7-PV1(RIPO) encodes a chimeric PV in which the IRES of PV has been replaced with that of HRV2 (13).

DNA Manipulation, *in Vitro* Transcription, and Translation. A point mutant changing the 179th amino acid from asparagine to glycine (2 nt changes) of the nonstructural protein 2C that confers resistance to 2 mM of Gua-HCl was PCR-amplified from the cDNA of the original PV*g*^r mutant (17) as an *MluI*–*AvaI* fragment and cloned in pT7-PVM to form pT7-PVM*g*^r. An *XhoI*–*MluI* fragment containing the *g*^r mutation was then released from pT7-PVM*g*^r and used to replace the corresponding region in pT7-PV1(RIPO) to produce pT7-PV1(RIPO)*g*^r. The presence of the *g*^r mutation in all the constructs was confirmed by sequencing.

Plasmid pT7-PV1(RIPO)*g*^r was linearized at the unique restriction site for *FspI*, and 1 μ g was used in transcription reactions by using T7 RNA polymerase as described previously (15). *In vitro* translations were carried out at 30°C in HeLa extracts as described previously (8).

Preparation of RNA. Virion RNAs of PV1(M) and PV1(RIPO)*g*^r were extracted from CsCl purified virus with phenol-chloroform (two cycles), followed by precipitation with ammonium acetate and ethanol. For RNA used in reverse transcriptase (RT)-PCRs, infected SK-N-MC cells (24 hpi) were lysed in 300 μ l Nonidet P-40 lysis buffer (10 mM Tris, pH 7.5/1 mM EDTA/0.5% Nonidet P-40/100 mM NaCl) and extracted twice with phenol-chloroform in the presence of 3 μ l

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Abbreviations: Gua-HCl, guanidine hydrochloride; *g*^r, resistance to Gua-HCl; IRES, internal ribosomal entry site; PV, poliovirus; HRV2, human rhinovirus type 2; pfu, plaque-forming units.

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of 10% SDS. Total nucleic acid was then precipitated with ammonium acetate and ethanol.

Cell-Free Replication. The cell-free replication system was as described by Molla *et al.* (8) with slight modifications. Each 50- μ l reaction was composed of 35.2 μ l of a master mix (see below), 8.8 μ l of virion RNA (1.2 μ g), and 6 μ l of micrococcal nuclease-treated rabbit reticulocyte lysate (RRL; Promega). The 35.2 μ l of master mix contained 19.2 μ l of a S3 HeLa S-10 lysate prepared as described in (8), 6.56 μ l of translation mixture (8 mM ATP/480 μ M GTP/80 mM creatine phosphate/200 μ g/ml creatine phosphokinase/150 mM K-Hepes, pH 7.4/200 μ g/ml calf liver tRNA/100 μ M amino acid mixture without methionine/2 mM spermidine in 10 mM K-Hepes), 545 μ M magnesium acetate, 1.3 mM magnesium chloride, 160 mM potassium acetate, 10 μ M of each amino acid (minus leucine or cysteine; Promega), 0.43 unit/ μ l RNasin (Promega), 284 μ M each of CTP and UTP, and 204 μ M of GTP. The RRL is included in the reaction mixture because, for unknown reasons, it increases the yield of the translation products and virus titer (A.C. and E.W., unpublished results). The HeLa S10 extract was also treated with micrococcal nuclease before its use in the reaction mixture as described (8). When the two parental viral RNAs were included in the reaction mixture, 0.6 μ g of each RNA was added to the reaction. The reaction was carried out at 34°C for 12–15 hr (9).

RESULTS

Genetic Markers Used in the Study. To facilitate the study of cell-free recombination, we have used the following selectable markers: (i) resistance to the drug Gua-HCl (2) and (ii) host range restriction of proliferation in a human neuroblastoma cell line. Wild-type PV RNA synthesis is sensitive to 2 mM Gua-HCl (g^s), but mutants resistant to the drug (g^r) can be selected that carry an amino acid substitution (Asn-179-Gly; resulting from two nucleotide changes) in the coding region for protein 2C (17). The g^r marker has been used previously in numerous studies of PV recombination *in vivo* (2). A PV chimera in which the PV IRES was replaced with that of HRV2 [PV1(RIPO)] was used as host range variant. PV1(RIPO) replicates in HeLa cells with wild-type kinetics; however, it cannot proliferate in human neuroblastoma SK-N-MC cells (13). This chimera was further modified to be g^r . Its genotype differed further from that of wild-type PV1 RNA in that it harbored sequences specifying new restriction sites (*Sac*I, *Stu*I, *Xho*I, see Fig. 1B; ref. 18).

RNAs of the two type 1 parental viruses [PV1(M) g^s and PV1(RIPO) g^r] were compared for their ability to direct translation *in vitro*. The RNAs were isolated from CsCl-purified virus preparations, where the stock of PV1(RIPO) g^r originated from plasmid pT7-PV1(RIPO) g^r transcript-transfected HeLa cells. The yield of translation products and the patterns of polyprotein processing generated by PV1(RIPO) g^r RNA were very similar to that obtained with PV1(M) g^s RNA (Fig. 2A).

In general, PV g^r mutants have impaired growth properties (16). PV1(RIPO) g^r exhibited a small plaque phenotype (Fig. 2C) when compared with wt PV (Fig. 2B). Moreover, the titers obtained from transfections of PV1(RIPO) g^r RNA were lower by one order of magnitude than those obtained with PV1(M) g^s RNA [10^8 plaque-forming units (pfu)/ml and 10^9 pfu/ml, respectively].

HeLa cell-free extracts were then programmed with virion RNAs of either PV1(M) g^s or PV1(RIPO) g^r , yielding *de novo* synthesized virus with titers of approximately 10^6 pfu/ml and 10^2 pfu/ml, respectively, the yields varying with different HeLa cell extracts. The low yield of PV1(RIPO) g^r relative to PV1(M) g^s is most likely related to the g^r mutation. Remarkably, addition of 2 mM Gua-HCl to the cell-free system increased the yield of PV1(RIPO) g^r to 2×10^5 pfu/ml, a phenomenon observed previously (8). Addition of the drug to

FIG. 1. Schematic representation of the genotypes of parental RNA molecules and the resulting recombinant. (A) Genome of Gua-HCl-sensitive wild-type PV1(M) g^s . The sphere at the 5' end represents the genome-linked protein (VPg) that is attached to a clover leaf-like structure. Five stem-loop structures that follow form the IRES for PV (solid line) or HRV2 (broken line). The open box is the PV ORF encoding the polyprotein whose major cleavage products are indicated. The genome is terminated by 72 nontranslated nucleotides plus a poly(A) tail. (B) Genome of PV1(RIPO) g^r , a chimera between PV and HRV2 that, in addition, contains two point mutations at nucleotide 4658/59 (Asn to Gly) leading to resistance (g^r) against 2 mM Gua-HCl. Important restriction sites present only in the cDNA of PV1(RIPO) g^r RNA (18) are shown. (C) The recombinant molecule that may have originated through crossover during minus-strand synthesis of PV1(RIPO) g^r RNA in a region between the *Sac*I and *Stu*I restriction sites.

the cell-free extract and incubation with PV1(M) g^s RNA abolished virus synthesis, as expected (8).

Cell-Free Recombination of PV. Recombinants between PV1(M) g^s and PV1(RIPO) g^r may bypass the restriction of one parent to replicate in the presence of 2 mM Gua-HCl (17) and of the other parent to proliferate in SK-N-MC cells (13). These recombinants could be isolated by incubating progeny virus of mixed cell-free replication on SK-N-MC cells in the presence of the drug. Accordingly, HeLa cell extracts were incubated either with PV1(M) g^s RNA or PV1(RIPO) g^r RNA alone, or with mixtures thereof. After RNase treatment of the incubation mixtures (8), progeny viruses were plated on SK-N-MC cell monolayers in 2 mM Gua-HCl and overlaid with noble agar. As expected, progeny of PV1(M) g^s RNA and PV1(RIPO) g^r RNA alone did not yield any plaques (Table 1, extract A). In contrast, replication of the two RNAs together yielded progeny virus able to replicate under the restrictive conditions (Table 1). Intrinsic to experiments of cell-free virus synthesis is a difference in the efficiency of cellular extracts (8), a variable that, at present, we are unable to control. This is apparent with the virus yields (data not shown) and the subsequent yield of recombinants that were obtained with extracts A and B (Table 1).

Incubation of PV1(M) g^s or PV1(RIPO) g^r RNAs in separate extracts followed by mixing the *in vitro* mixture and plating it on SK-N-MC cells in the presence of the drug did not yield plaques (Table 1, extract A). Thus, the recombinants observed did not originate by genetic exchanges between preformed, coinfecting viruses in the indicator cells *in vivo*. This was expected because SK-N-MC cells in the presence of Gua-HCl are not permissive to either parental virus. Finally, the unlikely possibility that recombination occurred through RNAs that had survived RNase treatment was eliminated, because coinfection of PV1(M) g^s and PV1(RIPO) g^r RNAs with SK-N-MC cells in the presence of Gua-HCl did not yield plaques (Table 1, extract A).

FIG. 2. Translation patterns and plaque phenotypes obtained from the parental PV genotypes. (A) An SDS/PAGE gel showing the *in vitro* translation products of PV1(M) g^s and PV1(RIPO) g^r viral RNAs at two RNA concentrations (150 and 300 ng). The identity of the cleavage products of the viral polyprotein are indicated on the right. (B–D) Plaque phenotypes on HeLa cells to compare the size of a representative recombinant virus (D) with those produced by wild-type PV1(M) g^s RNA (B) and PV1(RIPO) g^r RNA (C).

Addition of 2 mM Gua-HCl to the cell extract at time 0 of incubation of the mixture of the two parental RNAs was expected to abolish recombination events because PV1(M) g^s RNA would be unable to enter into a replication mode. To our surprise, in two out of six experiments, recombinants could be recovered, albeit at very low yield (Table 1, extract B).

Analysis of the Recombinants. To analyze the plaque phenotype of the recombinants, recombinants were plaque purified and amplified once in SK-N-MC cells in the presence of 2 mM Gua-HCl. Virus from these cells was then used for plaque assays on monolayers of HeLa cells. The plaque size of recombinant viruses was found to be intermediate to that of the parental viruses (Fig. 2, compare D with B and C).

To confirm the presence of the selecting markers in recombinant genomes, and to determine the approximate

region of crossover, we have analyzed 16 independent recombinant plaques for their genotypes. Total RNA of recombinant-infected SK-N-MC cells was isolated and subjected to RT-PCR by using appropriate primers, followed by restriction enzyme and sequencing analyses, where appropriate. The 16 recombinants yielded identical results. In Fig. 3, analyses of two of these recombinants are shown. As control, the RT-PCR products of virion PV1(M) g^s and PV1(RIPO) g^r RNAs were generated. Lack of signals after RT-PCR of RNA extracted from mock-infected cells served as a negative control (Fig. 3A, lane 1). The PV1(M) g^s and recombinant RNAs gave identical products of 830 nt (Fig. 3A, lanes 2, 5, and 6, respectively), whereas the product of PV1(RIPO) g^r RNA was only 699 nt long (lane 3), an observation indicating that the recombinants contained the

Table 1. Titers of recombinants (pfu/ml) in SK-N-MC cells in the presence of 2 mM Gua-HCl

Combination	Experiment no.					
	I	II	III	IV	V	VI
Extract A						
PV1(RIPO) g^r RNA <i>in vitro</i> virus	<20 (0)	<20 (0)	<20 (0)	<20 (0)		
PV1(M) g^s RNA <i>in vitro</i> virus	<20 (0)	<20 (0)	<20 (0)	<20 (0)		
PV1(M) g^s RNA + PV1(RIPO) g^r RNA coreplicated <i>in vitro</i>	60 (3)	120 (6)	80 (4)	60 (3)		
PV1(M) g^s RNA <i>in vitro</i> virus + PV1(RIPO) g^r RNA <i>in vitro</i> virus	<20 (0)	<20 (0)	<20 (0)	<20 (0)		
PV1(M) g^s RNA + PV1(RIPO) g^r RNA	<20 (0)	<20 (0)	<20 (0)	<20 (0)		
Extract B						
PV1(M) g^s RNA + PV1(RIPO) g^r RNA coreplicated <i>in vitro</i>	180 (9)	200 (10)	200 (10)	160 (8)	160 (8)	160 (8)
PV1(M) g^s RNA + PV1(RIPO) g^r RNA coreplicated <i>in vitro</i> with 2 mM Gua-HCl	<20 (0)	20 (1)	<20 (0)	<20 (0)	20 (1)	<20 (0)

Each 50- μ l reaction mixture was spread on a single plate. The total number of plaques obtained (in parentheses) and the approximate titer (pfu/ml) are given for each experiment. The reactions in A and B were carried out using separate HeLa S10 extracts.

FIG. 3. RT-PCR and restriction analyses of representative cell-free recombinants generated in this study. Reverse transcription was carried out by using AMV reverse transcriptase (Boehringer Mannheim) and random nonamers. The cDNAs were amplified with *Taq* DNA polymerase (Boehringer Mannheim) by using specific primers. At the top is a schematic representation of PV1(RIPO)*g^r*, showing the *g^r* mutation and various restriction sites used as neutral markers. The concentration of all agarose gels shown here is 0.8%, except the one on the right in A, which is 2.5% to detect the small RT-PCR fragment released after cleavage with *Sac*I. (A) Analysis in the IRES region. Lanes 4 and 9, DNA molecular weight marker. (B) Analysis to show the presence of a *Stu*I site in the RT-PCR products. Lane 3, DNA molecular weight marker. (C) Analysis of the nonstructural protein region containing the *g^r* mutation. Lane 3, DNA molecular weight marker.

PV1(M)*g^s* IRES. This was supported by digesting the RT-PCR products with *Sac*I. Only the 699-nt-long DNA, originating from PV1(RIPO)*g^r* RNA, yielded two of the expect-

ed-size fragments of 582 and 117 nt (Fig. 3A, compare lane 10 with lanes 7, 8, and 11). Thus, the recombinants also lack the *Sac*I site.

Table 2. Yield of parental and recombinant viruses in the absence of Gua-HCl

100- μ l reactions -Gua-HCl	PV1(M) g^s (SK-N-MC)			PV1(RIPO) g^r (HeLa + Gua-HCl)			Recombinants (SK-N-MC + Gua-HCl)			F
	D	P	T	D	P	T	D	P	T	
I	10^4	18	1.8×10^5	2.5×10^3	166	4.1×10^5	25	2	50	0.8×10^{-4}
II	10^4	21	2.1×10^5	2.5×10^3	140	3.5×10^5	25	3	75	1.4×10^{-4}
III	10^4	19	1.9×10^5	2.5×10^3	144	3.6×10^5	25	2	50	0.9×10^{-4}
IV	10^4	16	1.6×10^5	2.5×10^3	128	3.2×10^5	25	3	75	1.5×10^{-4}
V	10^4	11	1.1×10^5	2.5×10^3	146	3.6×10^5	25	2	50	1.0×10^{-4}
Avg.			1.7×10^5			3.6×10^5			60	1.1×10^{-4}

100- μ l reactions $\begin{cases} \rightarrow 10 \mu\text{l (SK-N-MC)} \\ \rightarrow 40 \mu\text{l (HeLa + Gua-HCl)} \\ \rightarrow 40 \mu\text{l (SK-N-MC + Gua-HCl)} \end{cases}$

D, dilution factor; P, no. of plaques; T, titer in pfu/ml; F, frequency of recombination.

A similar analysis was carried out in the region of viral genomes specifying a *StuI* site that is unique to PV1(RIPO) g^r cDNA (Fig. 1). When cDNAs (731 nt) obtained with appropriate primers were subjected to *StuI* restriction, only the cDNAs of PV1(RIPO) g^r and the recombinants, but not of PV1(M) g^s , yielded two products (423 and 308 nt; Fig. 3B, lanes 2, 4, and 5, respectively). In contrast, PV1(M) g^s cDNA was resistant to the restriction enzyme, as expected (lane 1).

Finally, cDNAs were prepared in the region of the genomes specifying the g^r locus and the *XhoI* site. Sequence analysis of the recombinant cDNA confirmed the presence of the codon for glycine in position 179 of 2C that confers the g^r phenotype (data not shown). Moreover, restriction of the 1510-nt fragment with *XhoI* yielded two fragments for the cDNAs of PV1(RIPO) g^r and the recombinants (Fig. 3C, lanes 2, 4, and 5, respectively), whereas the PV1(M) g^s cDNA was resistant (lane 1). The absence of the *SacI* site and the presence of the *StuI* and *XhoI* sites in the cDNAs of the recombinant genomes indicate that crossover between the two parental strands must have occurred between the *StuI* and *SacI* sites, as indicated in Fig. 1.

Additionally, RT-PCR analysis of the RNA from plaques of recombinant viruses obtained when the recombination reactions were carried out in the presence of Gua-HCl confirmed that these viruses were true recombinants (data not shown).

Yield of Parental and Recombinant Viruses. To calculate the frequency of recombination, the reaction mixture was scaled up to 100 μ l and, after incubation, divided into three portions as shown in Table 2. The results of five independent reactions show an unexpected increase in the titer of the PV1(RIPO) g^r parent when its RNA was coincubated with that

of PV1(M) g^s RNA. We believe this stimulation in the replication of PV1(RIPO) g^r is a result of complementation by the PV1(M) g^s parent.

In six 50- μ l recombination reactions carried out in the presence of Gua-HCl (Table 3, I–VI) only one yielded recombinants (I). It was of interest to determine whether recombination in the presence of Gua-HCl is a result of complementation of PV1(M) g^s RNA by PV1(RIPO) g^r RNA, an event that would generate two replicating parental strains in spite of the presence of Gua-HCl. In eight reactions, all attempts failed to recover PV1(M) g^s after coincubation of this parent's RNA with PV1(RIPO) g^r RNA in the presence of the drug (Table 3, reactions I–VI), even if the entire reaction mixture of 50 μ l was plated on SK-N-MC cells (Table 3, VII and VIII). At present, we are unable to explain the lack of complementation by the PV1(RIPO) g^r parent.

For the reactions in which no Gua-HCl was added (Table 2), by using the titers obtained for both the parents and the recombinants in five reactions, an apparent average recombination frequency of 10^{-4} was calculated. Assuming that a reciprocal crossover event would yield an equal number of recombinants, the frequency of recombination may be 2×10^{-4} . The recombination frequencies in reactions carried out in the presence of Gua-HCl are difficult to assess. At a total yield of 8×10^4 pfu/ml of the PV1(RIPO) g^r parent in six reactions (Table 3), crossover to the genomic RNA of the other parent to yield 29 pfu/ml of recombinants can be estimated to have occurred at a frequency of approximately 3.6×10^{-4} . This frequency of recombination is comparable to that obtained for reactions carried out in the absence of Gua-HCl. The assay that we have employed to detect PV1(M) g^s in the mixed reactions

Table 3. Yield of parental and recombinant viruses in the presence of Gua-HCl

50- μ l reactions +Gua-HCl	PV1(M) g^s (SK-N-MC)			PV1(RIPO) g^r (HeLa + Gua-HCl)			Recombinants (SK-N-MC + Gua-HCl)			F
	D	P	T	D	P	T	D	P	T	
I	2.0×10^2	0	<200	2.0×10^3	10	2.0×10^4	29	1	29	1.5×10^{-3}
II	2.0×10^2	0	<200	2.0×10^3	8	1.6×10^4	29	0	<29	$<1.5 \times 10^{-3}$
III	2.0×10^2	0	<200	2.0×10^3	5	1.0×10^4	29	0	<29	$<1.5 \times 10^{-3}$
IV	2.0×10^2	0	<200	2.0×10^3	7	1.4×10^4	29	0	<29	$<1.5 \times 10^{-3}$
V	2.0×10^2	0	<200	2.0×10^3	6	1.2×10^4	29	0	<29	$<1.5 \times 10^{-3}$
VI	2.0×10^2	0	<200	2.0×10^3	4	0.8×10^4	29	0	<29	$<1.5 \times 10^{-3}$
VII	20	0	<20							
VIII	20	0	<20							
Avg.			<8			1.3×10^4			≤ 5	$\leq 3.6 \times 10^{-4}$

50- μ l reactions $\begin{cases} \rightarrow 5 \mu\text{l (SK-N-MC)} \\ \rightarrow 5 \mu\text{l (HeLa + Gua-HCl)} \\ \rightarrow 35 \mu\text{l (SK-N-MC + Gua-HCl)} \end{cases}$

D, dilution factor; P, no. of plaques; T, titer in pfu/ml; F, frequency of recombination.

in the presence of Gua-HCl does not allow to rule out the occurrence of very low levels of replication. Nevertheless, it appears that input RNA inhibited in replication can participate in recombination.

DISCUSSION

Genetic recombination has been demonstrated to occur frequently in mixed infections of closely related species of a large number of plus-strand RNA viruses (2, 4, 19). In these cases, recombination is considered an important viral strategy to eliminate detrimental mutations or to ensure greater genetic diversity. Indeed, it recently has been shown that a deleterious insertion in a quasi-infectious PV genome is rapidly eliminated by genetic recombination, the crossover occurring predominantly between sibling viral strands (7). Genetic recombination of more distantly related viral RNA genomes leading to viable viruses may be very rare. However, analyses of the genotype of western equine encephalitis virus strongly suggested that this human pathogen emerged by recombination of eastern equine encephalitis virus and a Sindbis-like virus (20).

It has been suggested that crossover can occur at any point of the PV genome (21, 22). Agol and his colleagues, however, have argued that crossover regions are nonrandomly distributed and that they involve secondary structures present in the parental genomes (23, 24). King (19) has analyzed numerous crossover sequences on the basis of which he proposed that specific sequence motifs favor strand-switching. Clearly, these questions remain to be solved just as much as the remarkable observation that crossover is precise; no deletions or insertions have been found, even if the exchange of strands occurred in a noncoding region (2, 22). Also unknown is the mechanism by which the replication complex pauses to allow invasion of a nonresident template strand.

The experiments described here show unambiguously that recombination between two replicating PV genomes can also occur in the cell-free system developed by Molla *et al.* (8). The data are compatible with a crossover between the RNA strands between the *StuI* and *SacI* sites of the parental molecules, possibly during minus-strand synthesis of the PV1(RIPO)*g^r* parent (Fig. 1C), a mechanism for which Kirkegaard and Baltimore (25) provided evidence with experiments of PV recombination *in vivo*. The frequency of recombination scored in the experiments here (10^{-4}) is lower than those reported for intracellular PV recombination ($>10^{-3}$; ref. 2), which may be related to deficiencies of the cell-free system.

The remarkable increase of virus yield in cell-free replication of PV1(RIPO)*g^r* RNA in the presence of 2 mM Gua-HCl, an effect observed previously (8), remains unexplained. Genetic loci of guanidine resistance and guanidine dependence map to the multifunctional PV polypeptide 2C (2, 17, 26), but the mechanism of guanidine action on PV proliferation is not understood (2). Most surprising also was the observation that the cell-free synthesis of PV1(RIPO)*g^r* increased by three orders of magnitude when its RNA was coreplicated with the RNA of PV1(M)*g^s*. We consider it likely that this effect is because of complementation in the cell-free system. However, no reciprocal complementation by the PV1(RIPO)*g^r* parent was observed when RNA of this parent was coreplicated with that of PV1(M)*g^s* in the presence of Gua-HCl in the reaction mixture. Whether the unidirectional complementation is because of the *g^r* mutation in the 2C protein remains to be determined. Finally, the observation that recombination between PV1(M)*g^s* and PV1(RIPO)*g^r* could be scored in the presence of 2 mM Gua-HCl, albeit at low incidence, suggests that template strands not engaged in RNA synthesis may

participate in recombination. A similar result was reported previously with PV1(M)*g^s*-infected HeLa cells that were superinfected with a *g^r* variant under addition of Gua-HCl (25). Thus, the cell-free system described here may allow the analysis of recombination events with RNA templates incapable of replication even in the absence of the drug. Indeed, our results may explain the evolution of a PV strain that had acquired a segment of ribosomal RNA (27).

While our experiments were in progress, Tang *et al.* (28) also reported poliovirus genetic recombination in the cell-free system described by Molla *et al.* (8). Recombination in the study by Tang *et al.* (28) was detected by quantitative RT-PCR of RNA from the replication reactions, a strategy that did not allow the selection of recombinant viruses.

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