Nonhomologous Recombination between Defective Poliovirus and Coxsackievirus Genomes Suggests a New Model of Genetic Plasticity for Picornaviruses

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ABSTRACT **Most of the circulating vaccine-derived polioviruses (cVDPVs) implicated in poliomyelitis outbreaks in Madagascar have been shown to be recombinants between the type 2 poliovirus (PV) strain of the oral polio vaccine (Sabin 2) and another species C human enterovirus (HEV-C), such as type 17 coxsackie A virus (CA17) in particular. We studied intertypic genetic exchanges between PV and non-PV HEV-C by developing a recombination model, making it possible to rescue defective type 2 PV RNA genomes with a short deletion at the 3′ end by the cotransfection of cells with defective or infectious CA17 RNAs. We isolated over 200 different PV/CA17 recombinants, using murine cells expressing the human PV receptor (PVR) and selecting viruses with PV capsids. We found some homologous (H) recombinants and, mostly, nonhomologous (NH) recombinants presenting duplications of parental sequences preferentially located in the regions encoding proteins 2A, 2B, and 3A. Short duplications appeared to be stable, whereas longer duplications were excised during passaging in cultured cells or after multiplication in PVR-transgenic mice, generating H recombinants with diverse sites of recombination. This suggests that NH recombination events may be a transient, intermediate step in the generation and selection of the fittest H recombinants. In addition to the classical copy-choice mechanism of recombination thought to generate mostly H recombinants, there may also be a modular mechanism of recombination, involving NH recombinant precursors, shaping the genomes of recombinant enteroviruses and other picornaviruses.**

IMPORTANCE **The multiplication of circulating vaccine-derived polioviruses (cVDPVs) in poorly immunized human populations can render these viruses pathogenic, causing poliomyelitis outbreaks. Most cVDPVs are intertypic recombinants between a poliovirus (PV) strain and another human enterovirus, such as type 17 coxsackie A viruses (CA17). For further studies of the genetic exchanges between PV and CA17, we have developed a model of recombination, making it possible to rescue defective PV RNA genomes with a short deletion by cotransfecting cells with the defective PV genome and CA17 genomic RNA. Numerous recombinants were found, including homologous PV/CA17 recombinants, but mostly nonhomologous recombinants presenting duplications of parental sequences preferentially located in particular regions. Long duplications were excised by passages in cultured cells or in mice, generating diverse homologous recombinants. Recombination leading to nonhomologous recombinants, which evolve into homologous recombinants, may therefore be seen as a model of genetic plasticity in enteroviruses and, possibly, in other RNA viruses.**

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The mechanisms underlying the genetic plasticity of picornaviruses, including human enteroviruses (HEVs), such as polioviruses (PVs), are mutation and genetic recombination, followed in all cases by selection (1, 2). Interest in these mechanisms has recently been stimulated by the emergence of circulating vaccine-derived strains of PV (cVDPVs), which have been implicated in poliomyelitis outbreaks (3, 4). These cVDPVs emerged from attenuated vaccine PV strains, through mutation, and, in most cases, recombination with other HEV types (3). Mutations are known to contribute to reversion of the attenuated phenotype, but the contributions of genetic exchanges to the characteristics of HEV recombinants and the mechanisms involved in recombination, particularly when intertypic, remain unclear (1).

HEVs constitute a large genus of viruses that can be classified into four species (HEV-A, -B, -C, and -D) (5). Three species of rhinoviruses have also recently been included in this genus. HEVs are small viruses consisting of a nonsegmented positive-strand RNA genome of about 7,500 nucleotides (nt) surrounded by an

icosahedral capsid composed of four proteins, VP1 to VP4 (6). The viral genome consists of a single large open reading frame (ORF) flanked by two untranslated regions (5′ UTR and 3′ UTR). Both UTRs are highly structured elements involved in the replication of the viral genome. In addition, the 5′ UTR contains an internal ribosome entry site (IRES) driving translation. The ORF is translated into a single polyprotein that is proteolytically processed by viral proteases (2A, 3C, and 3CD) to generate the capsid proteins, which are encoded by the P1 region, and the nonstructural proteins (P2 and P3 regions), including proteases and the RNA-dependent RNA polymerase (3Dpol). Viral replication cycles occur almost entirely in the cytoplasm of infected cells.

PV, a member of the HEV-C species, has a high mutation rate, of the order of 10^{-4} errors per nucleotide per viral genome replication cycle, due to the low fidelity of the 3Dpol and a lack of mismatch repair mechanisms. Furthermore, genetic analyses of several enteroviruses, including PV prototype strains and field and clinical isolates, have shown that these viruses commonly evolve by recombination (1, 2, 7, 8). There are three stable serotypes of PV, defined on the basis of the viral capsid. Natural and engineered intra- and intertypic PV recombinants have been described (9–12). Furthermore, the recombination of PV with other enteroviruses of the HEV-C species can lead to the emergence of new PV strains, in particular cVDPVs (3, 4).

Two principal nonexclusive models have been proposed for the molecular mechanisms underlying the recombination of positive-strand animal RNA viruses: a copy-choice or replicative mechanism, in which RNA template switching occurs during negative-strand synthesis (13), and the joining of RNA fragments, thought to occur without the participation of the viral RNA polymerase by a nonreplicative mechanism (14–16).

Most natural recombinants are homologous recombinants (H recombinants) thought to result from template switching during viral RNA replication. In H recombinants, there is no evidence for the insertion or deletion of sequences on either side of the recombination site when genomes are aligned with the parental sequences. Intertypic H recombinants produced through the replicative mechanism from parental PVs with partial nucleotide identity (85%) have been reported to occur at a frequency 2 orders of magnitude lower than that of intratypic recombinants originating from almost-identical parents (13). The nonreplicative mechanism can generate H or nonhomologous (NH) recombinants (14–16). NH recombinants have deletions or duplications of homologous parental sequences on either side of the recombination site. It has been suggested that in certain cases NH recombinants can occur through a replicative mechanism (17, 18). The nonreplicative mechanism is thought to make a much smaller contribution to the recombination process under natural conditions than the replicative mechanism generating H recombinants. However, the relative importance of these two mechanisms remains to be established (1).

In 1988, the WHO set up a poliomyelitis eradication program based on massive vaccination campaigns with oral polio vaccine (OPV). This vaccine is composed of live attenuated viruses of the three serotypes (Sabin strains 1 to 3), which can multiply to high titers only in the gastrointestinal tract. An inactivated polio vaccine (IPV) has also been developed. After 26 years of massive antipolio immunization programs, poliomyelitis due to wild-type viruses has greatly decreased in frequency worldwide and is considered to be endemic in only three countries (Pakistan, Afghanistan, and Nigeria). Despite this progress, poliomyelitis has not been entirely eradicated, due to low vaccine coverage in some areas of the developing world, including those in which the disease is endemic. Two main issues threaten to undermine the benefits of the eradication program. The first is that low vaccine coverage may lead to the importation of wild PV strains from countries in which PV remains endemic into countries from which wild endemic PVs have disappeared. The second is that suboptimal vaccine coverage may allow the circulation of OPV strains between humans, leading to their genetic drift and the emergence of cVDPVs. These cVDPVs have caused iatrogenic epidemics of paralytic poliomyelitis in several regions of the world (4, 19–21). Most cVDPVs studied to date have similar genomic features: the region encoding the capsid proteins originates from the vaccine strain, but with more than 1% nucleotide substitutions, and some or all of the rest of the genome, particularly the region encoding nonstructural proteins, originates from other HEV-Cs, with certain coxsackie A viruses (CAVs) identified as particularly frequent recombination partners (3).

We studied the cVDPV strains implicated in two epidemics of poliomyelitis occurring in Madagascar in 2002 and 2005 (20, 21). We found that these strains were recombinant PV/CAV that had emerged from an HEV-C viral ecosystem with an unexpectedly high level of diversity, evolving through intra- and intertypic recombination (19, 22, 23). Complex mosaic genomes with sequences originating from different serotypes of OPV strains and containing genomic fragments from different CAV types, including type 13 and 17 CAVs (CA13 and CA17) in particular, were found in Madagascar over a short period of time. These data indicate that a given PV type can evolve rapidly, through several recombination events, when multiplying and circulating in close interaction with other PVs or other HEV-C types.

We previously reported that the 3′ half of the genome of a Madagascan type 2 cVDPV (MAD04) was related to that of a cocirculating CA17 isolate (CA17.67591) (19). We investigated whether this CA17 isolate could recombine with PV, by generating recombinant constructs combining genetic material from the CA17 isolate with genetic material from the Sabin 2 (S2) strain and the type 2 cVDPV (24). We found that *in vitro*-constructed PV/CA17 recombinants were viable. Viruses in which the 3′ portion of the cVDPV genome was replaced with the 3′ half of the CA17 genome had genotypic and phenotypic characteristics similar to those of the cVDPV, including neurovirulence in transgenic mice expressing the poliovirus cellular receptor (PVR) gene. Cocirculation in children and the genetic recombination of HEVs of different types with different biological properties may therefore lead to the generation of pathogenic recombinants. This system thus constitutes an interesting model of viral evolution and emergence. Little detailed information about intertypic recombination processes is currently available.

In this study, we developed an experimental recombination model for studies of the rules and mechanisms governing genetic exchanges between PV and CA17, in which defective PV RNA genomes can be rescued by the cotransfection of cells with defective or infectious CA17 genomes, leading to recombination in the P2 or P3 nonstructural region. The characterization of over 200 PV/CA17 recombinants led to the identification of H recombination sites and a majority of NH recombination sites located in three recombination hot spots. Furthermore, the evolution of NH recombinants into H recombinants in cultured cells and in an

animal model suggested a new model of genetic plasticity for enteroviruses.

RESULTS

Development of an RNA recombination system based on a cotransfection assay. We developed a viral recombination system based on the principle that noninfectious S2 genomic RNA carrying deletions could be rescued by recombination, following the cotransfection of cells with either infectious or noninfectious genomic RNA derived from CA17.67591. We ensured that only recombinant viruses with a capsid from PV were amplified, by cotransfecting mouse L20B cells expressing the human PVR (CD155), but not the putative CA17 receptor, ICAM-1 (24). We checked that L20B cells could produce both infectious CA17 and S2 viruses following transfection with full-length CA17 and S2 RNA genomes, respectively, but that only S2 could infect these cells (data not shown).

The noninfectious S2 and CVA17 RNAs were generated by deleting part of the genomic cDNA under the control of the T7 promoter, in the plasmids pBR-S2 and pBR-CA17, respectively (24, 25) (Fig. 1A). The 3′ end of the 3Dpol coding region and the entire 3′ UTR [from nucleotide 7301 to poly(A)] were deleted from the infectious cDNA of S2 (ΔS2 cDNA), impairing replication. Nucleotides 291 to 480 were deleted from the 5′ UTR of the infectious cDNA of CA17 (ΔCA17 cDNA). This deletion encompassed an essential domain of the IRES, impairing translation. Viral genomic RNA was synthesized *in vitro* from viral cDNA, with the T7 RNA polymerase, and was used to transfect L20B cells, which were subsequently grown on semisolid medium to allow plaque formation.

We investigated the production of recombinant viruses, by incubating cotransfected L20B cells at 37°C and 34°C (Fig. 1B). Consistent with the temperature sensitivity of S2, the parental S2 RNA was more cytopathogenic at 34°C than at 37°C. As expected, the parental CA17 RNA and the ΔS2 and ΔCA17 RNAs produced no plaques on L20B cells at either temperature. When cotransfection was performed at 37°C with the noninfectious ΔS2 RNA and either the infectious CA17 RNA or the noninfectious ΔCA17 RNA, only a few plaques were obtained (Fig. 1B), suggesting that this temperature impaired recombination or the replication of recombinants. When cells were cotransfected at 34°C, numerous large plaques corresponding to infectious recombinant viruses were observed (Fig. 1B). These results indicate that noninfectious S2 genomic RNA carrying deletions can be rescued by recombination, following the cotransfection of cells with either infectious or noninfectious genomic RNA derived from CA17.

We checked that recombination occurred between the RNA molecules used for transfection rather than with residual template DNA, by treating the RNA with DNase after *in vitro* transcription. DNase treatment did not affect multiplication of the positive-control virus, S2, or the generation of recombinants between ΔS2 and CA17 or ΔCA17 RNAs (Fig. 1C). In contrast, RNase treatment abolished the production of S2 and recombinant viruses, confirming that recombination occurred between the RNA molecules used for transfection, or at least that RNA was essential for this process.

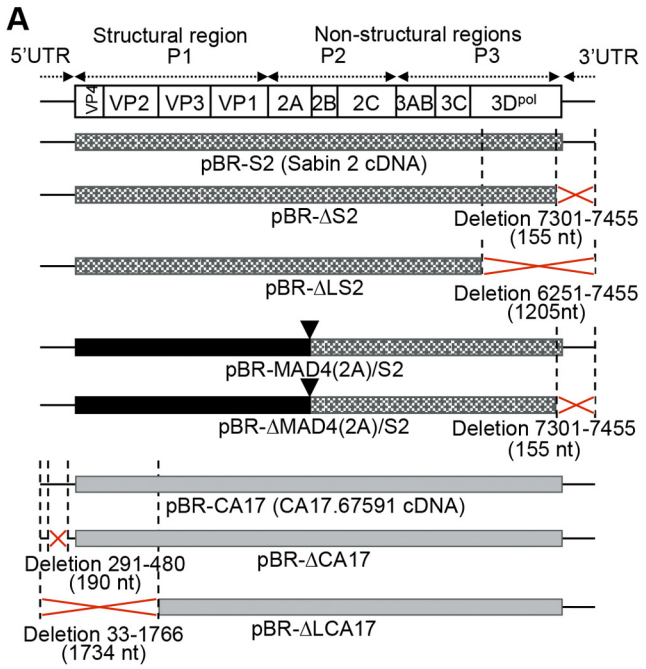
Finally, we checked that the genomes with deletions did not allow residual levels of RNA polymerase activity that might contribute to a replicative mechanism of recombination, by constructing an S2 genome with a larger deletion in the region encoding 3Dpol and the deleted 3′ UTR [from nucleotide 6251 to the 3′ end of the poly(A) tract] (ΔLS2 cDNA) (Fig. 1A). We also deleted almost all of the 5′ UTR and the region encoding the structural proteins VP4 and VP2 [nucleotides 33 to 1766] from the cDNA of the CA17 genome (ΔLCA17 cDNA). We assessed *in vitro* recombination by cotransfecting cells with the ΔLS2 RNA and either the CA17 or the ΔLCA17 RNA (Fig. 1D). Large deletions did not seem to affect the number of recombinant plaques. This suggests that the recombination mechanism operating on these noninfectious genomes with deletions did not require the viral polymerase and might therefore involve a nonreplicative mechanism of RNA recombination, at least for the first step. Recombination events appeared to be rare, based on the number of PFU per μg of genomic RNA used for transfection (Fig. 1E). Similar results were obtained when infectious or noninfectious CA17 RNAs were used.

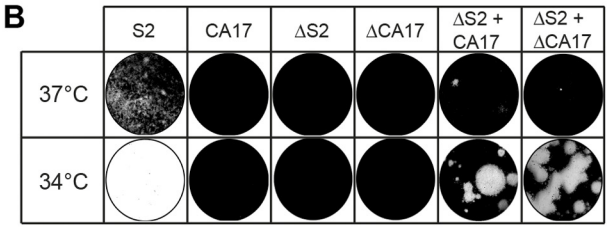
On the basis of these results, we were able to define a model of *in vitro* recombination between S2- and CA17-derived RNAs, probably occurring via a nonreplicative mechanism, at least when cells were cotransfected with two noninfectious RNA partners. We then used noninfectious RNAs with smaller deletions (ΔS2 and ΔCA17 RNAs), allowing recombination in the P2 region and most of the P3 region of the genome.

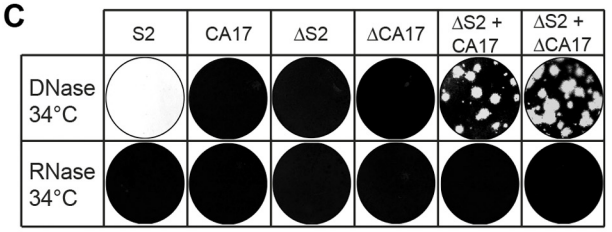
Genomic analysis of recombinant viruses. We investigated the genomic features of recombinant viruses isolated by picking plaques from cotransfected L20B cells. In total, 45 S2CA17 (cotransfection with ΔS2 and CA17 RNAs) and 76 S2CA17Δ (cotransfection with ΔS2 and ΔCA17 RNAs) recombinant viruses were picked, amplified in L20B cells, and analyzed.

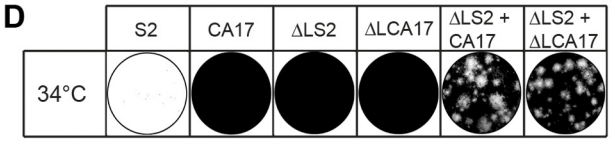
The sequencing of recombinant genomes revealed both H and NH recombination events between parental genomes. A diagram of the different types of recombinants with recombination sites in the 2A coding region is presented in Fig. 2. In H recombinant genomes, there is no evidence of the insertion or deletion of sequences. In contrast, most of the NH recombinant genomes displayed insertions of a variable number of additional codons, at the recombination junction. These inserted sequences created duplications of homologous (but not identical) sequences from the parental genomes, around the recombination site. NH recombinant genomes displaying duplicated sequences without duplication of the cotranslational cleavage sites are referred to as NH-1 recombinants (Fig. 2). NH recombinant genomes with duplicated sequences resulting in a duplication of cotranslational cleavage sites are referred to as NH-2 (two homologous cleavage sites) recombinants. For example, the duplicated homologous sequences of the NH-2 recombinant shown in Fig. 2 correspond to the 3′ end of the VP1 coding region and the 5′ end of the 2A coding region. This results in a duplication of the cotranslational cleavage site between VP1 and 2A. Between the duplicated VP1/2A cleavage sites lies a 2A/VP1 recombinant polypeptide, with residues from S2 and CA17 on either side of the recombination site. If cleavage occurs at both cleavage sites, the recombinant polypeptide is released and unmodified VP1 from S2 and 2A from CA17 are generated. Finally, an example of an NH recombinant with a deletion (NH-Δ recombinant) is shown in Fig. 2. Deletions of one or two codons were sometimes observed at the recombination site. NH recombinant genomes were therefore classified into three types: NH-1, NH-2, and NH-Δ.

Surprisingly, only 33% of S2CA17 and 30% of S2CA17Δ recombinants were the products of H recombination events, the others resulting from NH recombination events. An analysis of









E

Cotransfection temperature	Titer (PFU/μg)				Recombination frequency	
	S2	CA17	ΔS2+ CA17 recombinant	ΔS2+ ΔCA17 recombinant	ΔS2+ CA17	ΔS2+ ΔCA17
34°C	7.00E+04	1.28E+05	1.25E+01	2.27E+01	6.31E-05	1.15E-04

FIG 1 RNA-based cotransfection assay. (A) Genomic structure of the parental viral cDNAs and the viral cDNAs with deletions inserted into plasmids for *in vitro* RNA synthesis. The genetic organization of the PV genome is shown (at the top). The plasmids carrying the full-length cDNA sequences of Sabin 2 and CA17 (pBR-S2 and -CA17, respectively) and those of the cVDPV/Sabin 2 recombinant generated *in vitro* [pBR-MAD4(2A)/S2] have been described elsewhere (24, 25). Red crosses indicate the genomic regions in which the deletions were made. The nucleotide positions and the lengths [numbers of nucleotides deleted are shown in parentheses, including the poly(A) tail of 16 nucleotides] of these deletions are also indicated. Solid triangles indicate *in vitro* recombination sites. (B to E) Cotransfections were performed in L20B

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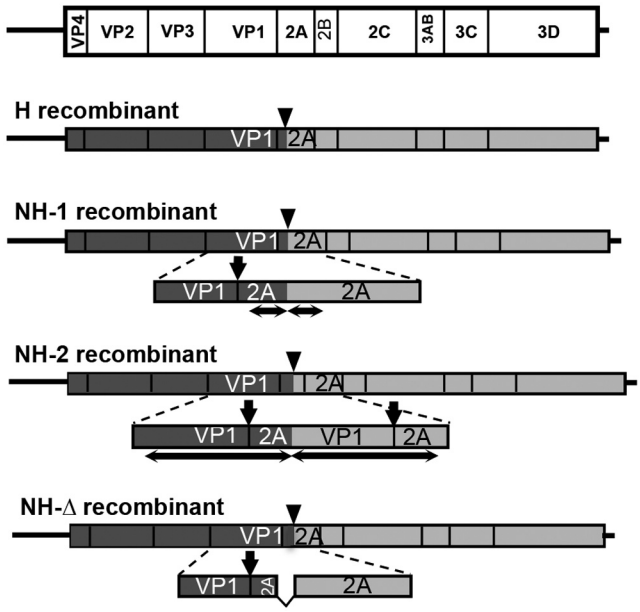

FIG 2 Genomic features of recombinant viruses. The different types of recombinants generated (homologous H and nonhomologous NH-1, NH-2, and NH-Δ) are illustrated. All the recombinants shown in this schematic diagram have a recombination site in the 2A coding region (solid triangle). The recombination site and the homologous duplicated sequences are enlarged for NH-1, NH-2, and NH-Δ recombinants. Vertical arrows indicate the natural VP1/2A cotranslational cleavage site of the viral polyprotein. NH-2 and NH-1 recombinants have duplicated homologous sequences (indicated by horizontal double-headed arrows) flanking the recombination site. NH-2 recombinants also display duplication of the natural VP1/2A cleavage site. NH-Δ recombinants have a short deletion.

the recombination sites showed them to be located in the region encoding the C-terminal part of VP1 and the nonstructural protein P2 and P3 coding regions (Fig. 3A; see also Tables S1 and S2 in the supplemental material). Examples for H and NH recombination sites are shown in Fig. S1A and B, respectively. The proportions of H and NH recombinants and the locations of recombination sites were similar for S2CA17 and S2CA17Δ. We therefore present the data for S2CA17 and S2CA17Δ together.

The distribution of recombination sites seemed to depend on the type of recombinant. NH recombination sites were found exclusively in the three "hot spot" regions (the 2A, 2B, and 3A coding regions), whereas H recombination sites were also found in other regions (the VP1, 2C, 3C, and 3D coding regions) (Fig. 3A). NH-Δ recombinants were rare, with recombination sites found only in the 5′ or 3′ end of the 2A coding region. NH2 recombinants ac-

Figure Legend Continued

cells, in semisolid medium, with 2.5 μg of each RNA. The overlay was added, and cells were incubated at 34°C or 37°C, as indicated, for 4 days before staining. Cells transfected with Sabin 2 RNA (S2) and incubated at 34°C were entirely lysed after 4 days (wells appear white). (B and C) Before cotransfections, genomic RNAs were either left untreated (B) or treated with DNase or RNase for 1 h at 37°C (C) and cells were incubated at 34°C after cotransfection. (D) Cotransfections were performed with RNAs with large deletions. (E) The *in vitro* recombination frequencies after cotransfection with ΔS2 plus CA17 RNAs and ΔS2 plus ΔCA17 RNAs at 34°C are indicated (see Materials and Methods for calculation).

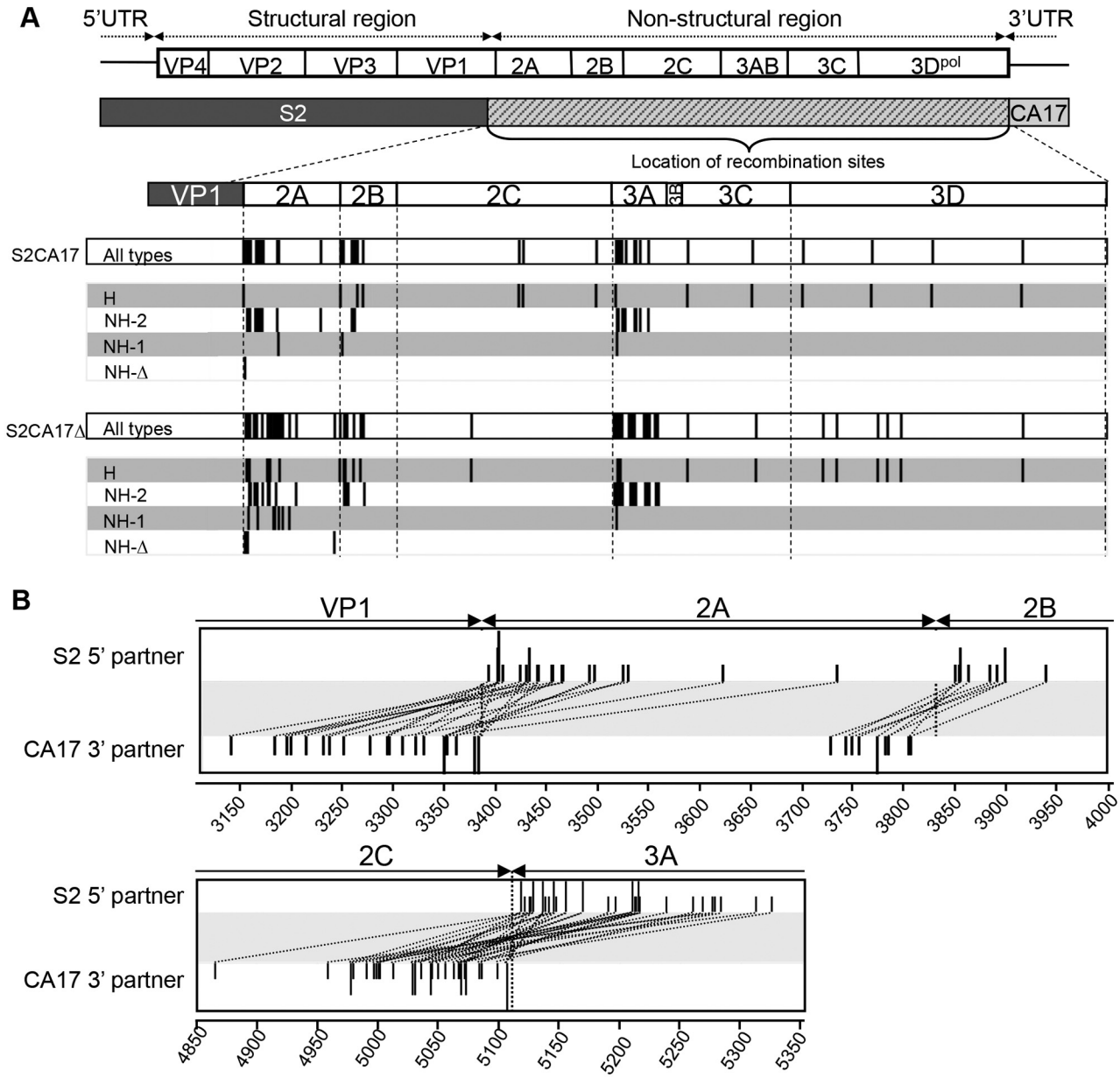

FIG 3 Genomic analysis of recombinant viruses. (A) Distribution of the recombination sites identified in recombinant viruses S2CA17 and S2CA17Δ, isolated at passage P1, following cotransfection with deleted S2 and either CA17 (for S2CA17) or defective ΔCA17 (for S2CA17Δ) RNA. The names of the recombinants are indicated on the left, together with the type of recombination. Black vertical lines indicate recombination sites according to S2 numbering. For NH-2 recombinants, the locations of the recombination sites in the S2 5′ partner are shown. (B) Sites of recombination for NH-2 recombinants, for each RNA partner. Regions with recombination sites are enlarged. The names of the partners are indicated on the left. Vertical lines indicate the sites of recombination for each partner. The dotted lines represent the bond between each partner and a given NH-2 recombinant. Site locations (nucleotide numbering) are indicated at the bottom.

counted for the majority of recombinants in the 2A, 2B, and 3A coding regions (see Fig. S2A in the supplemental material).

In NH-1 recombinants, the recombination sites in the 2B and 3A coding regions were limited to the 5′ ends of these regions, whereas those in the 2A coding region were scattered throughout the 5′ half of this coding region. Within the recombination hot spot regions of NH-2 recombinants, we looked for particular structures or nucleotide motifs in the vicinity of the recombination sites (Fig. 3B). No preferential substrates for recombination were found in either the S2 5′- or the CA17 3′-RNA partners.

The median size of the inserted sequences of NH-2 recombinants (47 codons) was greater than that in NH-1 recombinants (7 codons) (see Fig. S2B in the supplemental material). Similarly, the range of insertion sizes was greater for NH-2 recombinants than for NH-1 recombinants. In addition, insertion size in NH-2 recombinants was more heterogeneous for the 2A and 3A regions than for the 2B coding region. NH-Δ recombinants displayed small deletions, of one or two codons.

Thus, the intertypic recombination model generated H recombinants together with a large number of NH recombinants with

TABLE 1 Stability of the insertions in NH-2 recombinants, following passages in cells

Parental virus name	Type	Recombination site	Insertion length, nt	Preservation of insertion				
				Duplicate	Status at passage no.[a]: P8	P13	P18	Recombination site
S2CA17Δ cl 9.2	NH-2	S2-3401 (2A)	8	A	Yes	Yes	Yes	Parental[b]
		CA17-3396 (VP1)		B	Yes	Yes	Yes	Parental
S2CA17 cl 1.1	NH-2	S2-3454 (2A)	86	A	No			S2-3407 (2A)[c]
		CA17-3215 (VP1)		B	Yes	Yes	Yes	Parental
S2CA17Δ cl 11.1	NH-2	S2-3622 (2A)	101	A	Yes	No		S2-3458 (2A)
		CA17-3338 (VP1)		B	No			S2-3501 (2A)
S2CA17 cl 2.1	NH-1	S2-3840 (2B)	3	A	Yes	Yes	Yes	Parental
		CA17-3850 (2B)		B	Yes	Yes	Yes	Parental
S2CA17Δ cl 8.1	NH-2	S2-5125 (3A)	87	A	No			S2-5003 (2C)
		CA17-4883 (2C)		B	No			S2-5060 (2C)
S2CA17 cl 4.4	NH-2	S2-5209 (3A)	70	A	Yes	Yes	M[d]	Mixture[d]
		CA17-5018 (2C)		B	Yes	Yes	M	Mixture
S2CA17Δ cl 12.2	NH-2	S2-5214 (3A)	49	A	Yes	Yes	Yes	Parental
		CA17-5086 (2C)		B	Yes	Yes	Yes	Parental

[a] Number of passages in cells. Preservation or loss of insertion (duplicated homologous sequences) is indicated, following passages.
[b] Recombination site unchanged, identical to that in the original virus.
[c] Homologous recombination site: nucleotide position in the Sabin 2 genome is indicated.
[d] Indicates a mixture of NH and H recombinants.

insertions of duplicated homologous sequences preferentially located in certain genomic domains and often extending to several dozens of residues.

Phenotypic characteristics and evolution of NH recombinant viruses in cells. One NH-1 and six NH-2 recombinants (the most abundant type of recombinant) with recombination sites in the 2A, 2B, and 3A coding regions were selected for further analysis of their characteristics and evolution *in vitro* (Table 1). We first assessed whether the presence of duplications in NH recombinant genomes affected their capacity for growth. The replication kinetics of the selected S2CA17 and S2CA17Δ viruses were compared with those of the parental viruses (S2 and CA17) in HEp-2c cells at a low multiplicity of infection (MOI) (0.1 50% tissue culture infective dose [$TCID_{50}$] per cell). All the selected recombinants had replication kinetics similar to that of S2. Representative growth curves of viruses displaying recombination in one of the three hot spot regions are presented in Fig. S3 in the supplemental material. The duplicated sequences had little or no effect on replication efficiency under these conditions.

We then evaluated the stability of the duplicated sequences in NH S2CA17 recombinant genomes after serial passages of viruses in L20B cells infected at a low MOI (0.1 $TCID_{50}$ per cell). The selected NH-1 and NH-2 recombinants were passaged in duplicate (passage series A and B, respectively, for each recombinant). We checked for the presence of duplicated regions in the recombinant genomes, at passages 8, 13, and 18, by reverse transcription-PCR (RT-PCR) and sequencing (Table 1). The NH-2 recombinant genomes (S2CA17Δ cl 9.2 and S2CA17Δ cl 12.2) with the smallest insertions (8 and 49 codons, respectively) were stable until passage 18. In contrast, the three NH-2 recombinant genomes (S2CA17 cl 1.1, S2CA17Δ cl 8.1, and S2CA17Δ cl 11.1) with the largest insertions (86, 87, and 101 codons, respectively) had mostly lost their inserted sequences by passage 8, in at least one of the two passage series (Table 1). At passage 13, only recombinant S2CA17 cl 1.1 from series B retained the insertion. The locations of the homologous recombination sites created following the deletion of insertions in the S2CA17Δ cl 8.1 virus differed between the passage series A and B, indicating that two different genomic rearrangement events occurred in these two passage series. The same was true for the S2CA17Δ cl 11.1 recombinant.

The passaging of the NH-2 recombinant S2CA17 cl 4.4 with an insertion of intermediate size (70 codons) led to a mixture of H and NH recombinants at passage 18 in both passage series (Table 1). The genomes of the viruses in the mixture were analyzed in both series. RT-PCR products overlapping recombination sites were inserted into bacterial plasmids for sequencing (190 bacterial clones analyzed). The original 70-codon insert (Fig. 4A) was found in 166 clones, whereas diverse H recombinants with different recombination sites were identified in 20 clones (Fig. 4B). A few clones displayed short insertions and/or deletions and frameshifts, suggesting the presence of nonfunctional genomes. We followed the evolution of the viral population further, by continuing the passaging process until passage 35 (Fig. 4C). We analyzed 43 bacterial clones containing RT-PCR products. The initial duplication (70-codon insert) was retained in 11 clones but lost in the other 32, leading to the generation of homologous recombination sites. Most of the homologous recombination sites were located at sites similar to those previously identified at passage 18, but several new sites were also identified.

Thus, the duplicated sequences present in NH S2CA17 recombinant genomes may remain stable after passages in L20B cells, particularly if they are no more than 49 codons long. In contrast, the recombinant genomes with the largest insertions seemed to evolve more rapidly toward H recombinants, through the deletion of duplicated residues, generating a panel of different recombination sites.

Evolution of NH recombinant viruses in mice. We also evaluated the stability of NH recombinants *in vivo*, following the inoculation of transgenic homozygous PVR-Tg21 mice, which constitutively express the human PVR CD155 (26). The inoculation of these mice with pathogenic PVs via the parenteral or intranasal (i.n.) (mucosal) route leads to the development of symptoms similar to those observed in humans (paresis, acute flaccid paralysis,

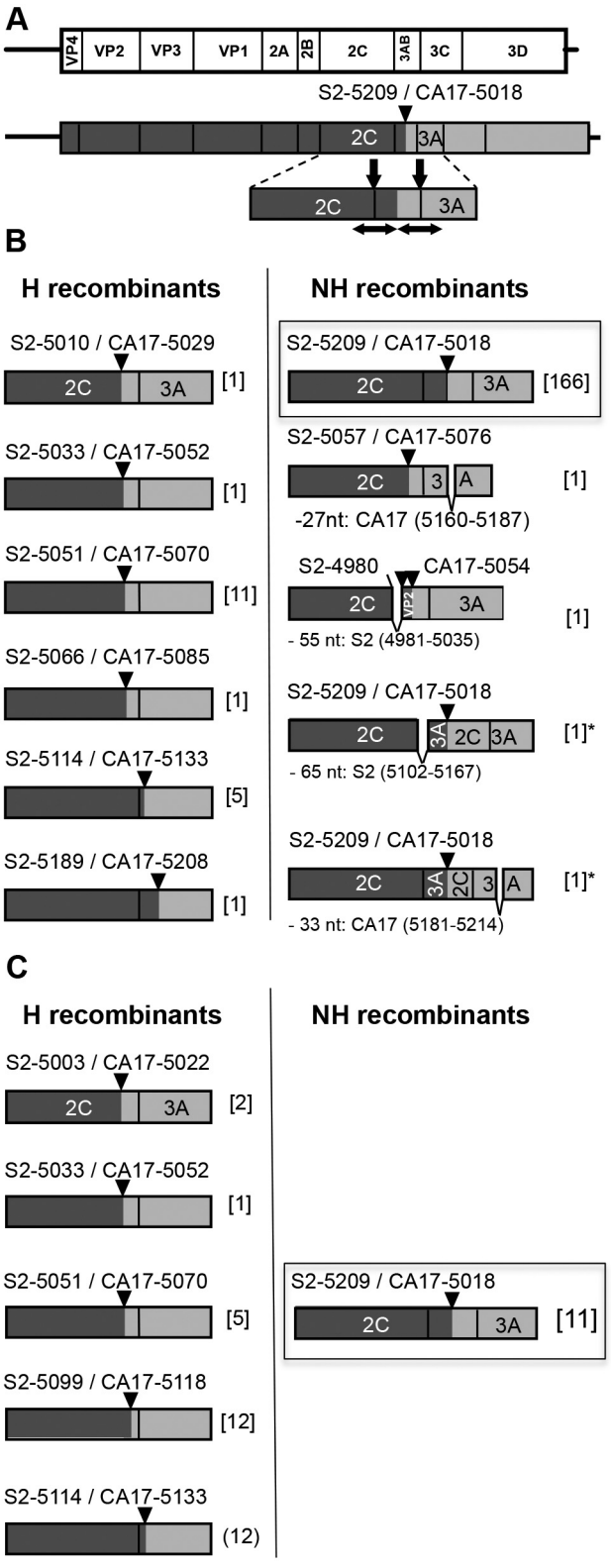

FIG 4 Evolution of the S2CA17 cl 4.4 viral genome following passaging in cells. (A) Schematic diagram of the genomic structure at passage P3 of the NH-2 recombinant S2CA17 cl 4.4 with a recombination site (solid triangle) in the 3A coding region and an insertion of 70 codons. Dark and light gray shadings indicate sequences from the Sabin 2 and CA17 genomes, respectively. The recombination site (according to Sabin 2 and CA17 numbering, respectively) and the homologous duplicated sequences (indicated by horizontal double-headed arrows) are enlarged, and vertical arrows indicate the 2C/3A cleavage sites. (B and C) At passages P18 and P35, the genomic region harboring the recombination site was amplified by RT-PCR. The amplification product, which was heterogeneous in size (two major bands), was inserted into a plasmid vector, which was then introduced into *Escherichia coli*. Inserts present in several bacterial plasmids were sequenced. The structures of the recombined regions at passages 18(B) and 35(C) are shown. The 2C and 3A coding regions and recombination site numbering are represented as in panel A. The original structure of the NH-2 parental recombinant S2CA17 cl 4.4 is framed. The lengths of deletions and nucleotides deleted in NH-Δ recombinants are shown. The fortuitous insertion of a VP2 nucleotide fragment into the recombination site of an NH-1 recombinant (2C S2-4980/VP2 S2-1125-1195/2C CA17-5054) is shown. The number of products cloned for each type of recombinant is indicated in brackets. Asterisks indicate genomic rearrangements disrupting the original open reading frame, suggesting the presence of noninfectious viral genomes.

(Continued)

and death). As expected, all S2CA17 and S2CA17Δ recombinants were attenuated, consistent with the presence of attenuation determinants in the part of the genome derived from S2 (27, 28). However, Jegouic et al. (24) previously showed that an S2 recombinant [MAD4(2A)/S2] in which the 5′ half of the genome had been replaced with that of an S2-derived cVDPV (cVDPV MAD04) was pathogenic in PVR-Tg21 mice, confirming that particular mutations in the 5′ UTR and P1 genomic regions are essential for pathogenicity.

We therefore constructed a noninfectious ΔMAD4(2A)/S2 genome from the MAD4(2A)/S2 recombinant (25), by deleting the 3′ end of the 3D coding region and the 3′ UTR [from nucleotide 7301 to the poly(A) tail] (Fig. 1A). We then generated a new set of recombinant viruses (MADCA17 and MADCA17Δ), by rescuing the noninfectious ΔMAD4(2A)/S2 RNA through the cotransfection of L20B cells with the infectious CA17 or the defective ΔCA17 RNA. We isolated 62 MADCA17 and 72 MADCA17Δ recombinants. As for the S2/CA17 partner pair, the proportion of H recombinants was low (26% and 11%, respectively) (see Table S2 in the supplemental material). Hot spot regions of recombination were identified, at locations similar to those observed in S2CA17 and S2CA17Δ recombinants (see Fig. S4).

Two NH-1 and two NH-2 recombinants with recombination sites in the 2A and 2B coding regions were selected and used to inoculate mice (Table 2). Most of the recombinant viruses were found to be neurovirulent following the i.n. or intraperitoneal (i.p.) inoculation of mice. However, the NH2 recombinant MADCA17Δ cl 1, with a 91-codon insertion in the 2B coding region, induced no clinical signs of disease during the 21 days following i.n. inoculation but was neurovirulent following i.p. and intracerebral (i.c.) inoculation (Table 2).

We investigated the stability of the insertion *in vivo*, by extracting viruses from the spinal cords of paralyzed or dead mice after i.n. or i.p. inoculation with each of the MADCA17Δ recombinants. Surprisingly, all the recombinant genomes were found to have conserved the duplicated sequence (Table 2). Viruses were also extracted from the spinal cords of the five mice that died or were paralyzed following i.c. inoculation with the MADCA17Δ cl 1 recombinant (Table 2). The insertion was present in the genomes of only two of the five progeny viruses. Partial sequencing of the other three viruses recovered led to the identification of three different homologous recombination sites (at nucleotide positions 3728, 3830, and 3845 according to the numbering for

TABLE 2 Stability of the insertions in NH recombinants, following the inoculation of mice

Inoculation route	Virus	Type	Recombination site	Insertion length, nt	Proportion of sick mice[a]	Stability[b]
Intranasal	MADCA17Δ cl 19	NH-2	S2-3407 (2A) CV-A17-3252 (VP1)	58	3/5	Yes
	MADCA17Δ cl 28	NH-1	S2-3426 (2A) CV-A17-3430 (2A)	5	4/5	Yes
	MADCA17Δ cl 1	NH-2	S2-3889 (2B) CV-A17-3635 (2A)	91	0/5	NA[c]
	MADCA17Δ cl a37	NH-1	S2-3849 (2B) CV-A17-3856 (2B)	4	4/5	Yes
Intraperitoneal	MADCA17Δ cl 19	NH-2	S2-3407 (2A) CV-A17-3252 (VP1)	58	4/6	Yes
	MADCA17Δ cl 28	NH-1	S2-3426 (2A) CV-A17-3430 (2A)	5	3/6	Yes
	MADCA17Δ cl 1	NH-2	S2-3889 (2B) CV-A17-3635 (2A)	91	3/6	Yes
	MADCA17Δ cl a37	NH-1	S2-3849 (2B) CV-A17-3856 (2B)	4	3/6	Yes
Intracerebral	MADCA17Δ cl 1	NH-2	S2-3889 (2B) CV-A17-3635 (2A)	91	5/6	No[d]

[a] Number of paralyzed or dead mice/number of animals inoculated. Regarding the inoculated doses and the inoculation routes (Materials and Methods), the nonpathogenic S2 and the neurovirulent S2/4568 or MAD04 virus paralyzed or killed 0% and >75% of inoculated mice, respectively.
[b] Stability of the insertions in viruses isolated from the medulla of paralyzed or dead mice.
[c] NA, not applicable.
[d] Excision of the insertion leading to homologous recombinants in the medulla of 3 of 5 paralyzed or dead mice.

S2), consistent with different mechanisms for deletion of the duplicated inserted homologous sequences after inoculation with the MADCA17Δ cl 1 recombinant. The genome of MADCA17Δ cl 1 was also unstable during *in vitro* passages (data not shown).

These results indicate that the presence of inserted duplicated residues does not prevent most NH recombinants from being neurovirulent in mice. Insertions appeared to be generally stable *in vivo* under these experimental conditions. Nevertheless, in certain cases they may impair pathogenicity and be deleted following multiplication in animals, thereby generating diverse recombination sites, as observed following passaging in cultured cells.

DISCUSSION

We developed a model of recombination, in which defective PV genomes could be rescued by the cotransfection of mouse L20B cells with CA17 genomic RNAs, for investigation of the molecular mechanisms involved in intertypic genetic recombination between HEVs and the subsequent evolution of recombination products. Similar results were obtained for defective and infectious CA17 genomes: some H and numerous NH PV/CA17 recombinants were found, revealing the presence of recombination hot spots in the regions of the genome encoding nonstructural proteins.

Nonreplicative and replicative mechanisms of genomic recombination and rearrangement. We showed that rescue could occur when both genomic CA17 and PV RNA partners had large deletions at their 5′ and 3′ ends, resulting in defective translation and polymerase activities, respectively. The mechanism operating in this case, therefore, probably involved a nonreplicative viral RNA recombination event (a cleavage-joining or joining mechanism) as a first step, as previously described for recombination between defective PV genomic RNAs in cells (15, 29). Furthermore, both we and Gmyl et al. observed the frequent occurrence of NH recombinants (15, 29), contrasting with the homologous features of enterovirus recombinants predominantly observed in classical replicative recombination models (13, 30). Unlike regions with a high frequency of recombination sites supposed to be generated by the template switch mechanism, the recombination hot spots identified in this study did not map preferentially to regions of high nucleotide sequence identity between partners (13). Moreover, inverted segments of the 3′ and 5′ partners likely to form heteroduplexes and to favor template switching as previously described (31–33) were not particularly prevalent in the recombination hot spot. Similar results, describing the *in vivo* recombination of nonreplicative RNA precursors to generate homologous and nonhomologous recombination products, have also been reported for other plus-strand RNA viruses, including alphavirus, pestivirus, and, recently, hepacivirus recombinants (14, 16, 34).

Whatever the initial mechanism of recombination in our model, the rescue of defective genomic RNAs led to the generation of replication-competent genomes. Classical viral replication could then occur in the transfected cells, resulting in replicative recombination, genomic rearrangements such as deletions, and viral selection processes (1). A comparative analysis of the PV/CA17 recombinants obtained following the rescue of defective PV RNA genomes with infectious or defective CA17 RNA genomes showed no significant difference in terms of the number of recombinants, the ratio of H to NH recombinants, or the location of recombination sites. This was somewhat surprising, because infectious CA17 RNA genomes are known to initiate replication after the transfection of L20B cells. Classical replicative recombination processes may be favored by the switch of the CA17 polymerase from the CA17 genome to the PV RNA fragment (13). Although unlikely, we cannot rule out the possibility that the rescue of defective poliovirus RNAs by infectious and noninfectious CA17 RNAs resulted from different initial recombination processes, with the observed similarities in the patterns of recombi-

nants resulting from similar postrecombination genomic remodeling and selection mechanisms. We also cannot exclude that some of the NH recombinants isolated here were the product of the imprecise switch of a viral polymerase during the synthesis of a replication-competent genome. Indeed, it has been shown that NH recombination can sometimes happen via a replicative mechanism (17, 18).

In nature, such NH recombinants would likely be selected against in the competition with wild-type viruses. In our model of recombination, there is no competition between recombinants and parental genomes, giving the recombinants enough time to emerge and replicate. However, *in vitro*-made recombinants may correspond in nature to transient, rare recombinants.

NH recombinants and the duplication of polyprotein cleavage sites. In PV/CA17 recombinants, we found recombination sites scattered throughout the genomic region encoding nonstructural proteins, but most of the NH recombination sites were located in three recombination hot spots corresponding to the coding sequences of viral proteins 2A, 2B, and 3A of PV. Most of these NH recombination sites were flanked by duplicated sequences from the two parental strains, including a polyprotein cleavage site (VP1/2A, 2A/2B, and 2C/3A, respectively). These cleavage sites are among the first to be cleaved in the processing cascade of the PV type 1 polyprotein (35). The presence of duplicated sites flanking a potentially cleavable recombinant viral protein sequence may be seen as an advantage, because this cleavage can release the chimeric inserted nonfunctional peptide (e.g., a 2A-VP1 peptide in the case of a duplicated VP1/2A cleavage site), leaving the flanking viral parental proteins from PV and CA17 unmodified. This suggests that these parental proteins may be more frequently selected in an unmodified than in a recombinant form in recombinants. Indeed, before this first description of these naturally occurring recombinant PVs, a vector was developed for the expression of foreign antigenic peptides via the insertion of exogenous sequences encoding the antigen and artificial proteolytic sites into the PV open reading frame (36). Insertions at the N-terminal end of the PV polyprotein and between proteins VP1 and 2A lead to viable viruses, and this approach has been successfully used to induce immune responses to non-PV antigens in animals (37).

Evolution of NH into H recombinants. It has been reported that pestivirus strains (bovine viral diarrhea viruses) can evolve by NH RNA recombination, leading to deletions, insertions of cellular RNA sequences, and the duplication of viral RNA sequences (38, 39). The passaging of two of these pestivirus NH recombinants in cell cultures was found to lead to the generation of H recombinants, through the deletion of duplicated sequences by homologous recombination. The conservation of duplicated sequences during evolution could also potentially account for the presence of duplicated (but not necessarily related) 2A and Vpg viral sequences in the Ljungan and foot-and-mouth disease picornaviruses, respectively (40, 41).

The homologous nature of most of the enterovirus recombinants found in nature raises questions about the large number of NH recombinants with duplicated sequences observed in our model. However, studies of the evolution of these duplicated sequences showed that some were frequently deleted following passaging in cells or animals, resulting in H recombinants. The four NH recombinants with the largest inserts (>70 codons) were found to be unstable, evolving rapidly into H recombinants following passaging in cells or animals. However, recombinants with smaller duplicated sequences appeared to be remarkably stable under similar conditions. However, they would probably evolve into H recombinants following additional passages or in the context of more stringent selection. The location of the insertions may also play a role in their stability, because it may affect functions of the modified protein and/or the processing of the polyprotein, thereby affecting viral fitness.

The isolation of viable NH recombinants with insertions of nucleotides derived from other regions of the PV genome into the 3′ UTR has been reported before (29). These recombinants isolated following the cotransfection of cells with noninfectious PV genomic fragments were shown to be genetically unstable, being converted into canonical H recombinants after only a few passages.

We found that deletions of duplicated sequences in NH recombinants gave rise to different homologous recombination sites in the duplicated region, thereby generating a diversity of recombinants. This mechanism may cope with the amino acid differences between parental homologous viral proteins, allowing a fine-tuning of the functions of the intertypic recombinant proteins generated by the deletion of duplicated sequences. Intertypic NH recombinants with duplicated sequences behave like precursors, giving rise to different H recombinants that are subjected to selection on the basis of their fitness. This implies that differences in nucleotide and amino acid sequences between the recombination partners may affect the rapidity and/or efficiency of the genomic rearrangement and selection process leading to H recombinants. In agreement with this hypothesis, when defective ΔS2 RNAs were rescued following the cotransfection of L20B cells with the S2 luciferase RNA replicon Luc-PVS2 (42), in which the P1 coding region is replaced with the luciferase gene and from which the IRES sequences have been deleted, as in ΔCA17, only 43% of the S2S2 rescued recombinants were found to be NH recombinants, versus 70% and 89% for S2CA17Δ and MAD4CA17Δ recombinants, respectively. In these experiments, cotransfection efficiencies and NH recombination sites were similar for all partner pairs (our unpublished data). Thus, sequence differences between parental strains may play a role in the generation and/or selection of H and NH recombinants.

Intertypic recombination leading to NH recombinants, which subsequently evolve into H recombinants, may therefore be seen as a model of genetic plasticity for enteroviruses and, possibly, for other positive-sense RNA viruses.

A modular evolution process. Some intertypic enterovirus recombinants with recombination sites in the genomic region encoding capsid proteins VP1 to VP4 (region P1) have already been described (3, 43). However, in most cases, recombination in the P1 coding region appears to be restricted, probably due to structural and functional constraints during the selection process (4, 19, 23, 44, 45). The recombination hot spots and the frequent duplication of cleavage sites in NH recombinants, leaving unmodified the parental viral proteins flanking the duplicated sequences, as observed here, suggest that intertypic recombination is also restricted in genomic regions encoding the 2A, 2BC, and 3ABCD viral proteins, respectively. These three functional genomic regions may be considered to be recombination modules like the P1 coding region. Indeed, we cannot exclude the possibility that the modular aspects of the genetic exchanges observed between PV and CA17 are dependent on the parental strains concerned. However, mostly NH recombinants and similar preferential recombi-

nation sites were identified when PV [ΔMAD4(2A)/S2] and CA11 genomic RNAs were used (our unpublished data). Evidence indicating that recombination is highly restricted in certain functional genomic regions, identifying these region segments as putative recombination modules, has been provided by an analysis of recombination between complete genome sequences from a large number of picornaviruses (2, 45). A theory of modular evolution based on genetic exchanges of interchangeable functional modules has been proposed for temperate DNA bacteriophages (46). This theory may also be applicable to certain aspects of the genetic plasticity and evolution of enteroviruses and, possibly, other RNA viruses.

In conclusion, we have developed a recombination model in which a noninfectious deleted PV genomic RNA can be rescued by the cotransfection of cultured cells and subsequent recombination with coxsackievirus RNAs. The application of this model led to the isolation of more than 200 H and NH PV/CA17 recombinants. The presence of hot spot regions for recombination and the different mechanisms underlying the evolution of NH recombinants into H recombinants suggest that a modular mechanism of recombination, possibly involving NH recombinant precursors, may function alongside the classical copy-choice mechanism of recombination, shaping the mosaic RNA genomes of intertypic recombinant enteroviruses.

MATERIALS AND METHODS

Construction of full-length and deleted cDNA-derived RNAs. Deletions in viral cDNA as schematized in Fig. 1A were performed using full-length S2 cDNA, MAD4(2A)/S2 cDNA, and CA17.67591 cDNA cloned in a modified pBR-322 vector as previously described (24, 25). Details of construction are given in Text S1 in the supplemental material. The T7 RNA^{pol} promoter upstream from the cloned viral cDNA was used to transcribe infectious RNA and RNA with deletions from plasmids, and DNA templates were eliminated by treatment with RNase-free DNase. RNA was then purified, quantified, and used for cotransfection experiments.

Cotransfection assays in semisolid medium. L20B cells were transfected with a mixture of genomic RNAs and maintained in semisolid medium, to allow viral plaque formation. Six-well plastic plates were seeded with 4×10^6 L20B cells per well, in Dulbecco modified Eagle medium (DMEM) supplemented with 3% fetal calf serum (FCS). They were then incubated at 37.0°C, under an atmosphere containing 5% CO_2, for about 24 h. Cell monolayers were washed twice with DMEM without FCS; transfected with 2.5 μg of each RNA per well, in the presence of Lipofectamine 2000; and incubated at 37.0°C, under an atmosphere containing 5% CO_2, for 30 min. We added 2 ml of DMEM without FCS to each well, and the plates were incubated for a further hour at 37.0°C, under an atmosphere containing 5% CO_2. The inoculum was then removed and replaced with 3 ml of a mixture containing 1 volume of 2.4% Avicel (FMC Biopolymer) in water and 1 volume of 2× DMEM supplemented with 4% FCS. Plates were incubated at 34.0°C or 37.0°C, under an atmosphere containing 5% CO_2, for 4 days. Avicel-containing medium was then removed, and the cells were washed twice with phosphate-buffered saline (PBS) (without $CaCl_2$ or $MgCl_2$) and stained. For the isolation of recombinant viruses, L20B cell monolayers were cotransfected and maintained in semisolid medium, as described above, except that the Avicel solution was replaced with agarose (see Text S1 in the supplemental material). Plaques were then picked and amplified on L20B cells. Some recombinant viruses were plaque purified again, to prevent the production of mixed viral stocks. Analysis of recombinant genomes was performed using RT-PCR and sequencing (see Text S1).

Determination of recombination frequency. Recombination frequency was evaluated by dividing the RNA infectivity titer of recombinants (PFU per μg of RNA used for transfection) by the sum of the titers of the parental viruses, as previously described (13). The titers of the parental S2 and CA17 RNAs were determined by transfecting L20B cells and HEp-2c cells, respectively, in a semisolid medium. The yield of the recombinant viruses with an S2 capsid was determined by cotransfecting L20B cells in semisolid medium.

Genetic stability of recombinant viruses *in vitro*. The viral stocks obtained at passage P3 were used for 15 serial passages. These passages were performed by infecting L20B cell monolayers at a low MOI, 0.1 $TCID_{50}$ per cell, in DMEM supplemented with 3% FCS, and incubating them at 37.0°C, under an atmosphere containing 5% CO_2. At passages P8, P13, and P18, we analyzed the genomic features of the recombinant viruses by amplifying the cDNA fragments overlapping recombination sites (RT-PCR) and sequencing. When mixtures of recombinant genomes were found, RT-PCR products were inserted into the pCR II TOPO plasmid vector for the transformation of TOP10 competent cells and positive clones were analyzed by sequencing.

Genetic stability of recombinant viruses *in vivo*. Genetic stability was assessed in homozygous PVR-Tg21 mice (generously provided by A. Nomoto) (26). A given dose of virus (10^8 $TCID_{50}$/ml) was used for the i.c. (30 μl per mouse), i.p. (500 μl per mouse), and i.n. (10 μl per mouse) inoculation of groups of five or six PVR-Tg21 mice. Mice were examined daily for 21 days after inoculation, for paralysis and death. The spinal cord of paralyzed or dead mice was removed for analysis of the genome of recombinant viruses by sequencing. Under similar conditions, the nonpathogenic virus S2 and the neurovirulent S2/4568 or MAD04 paralyze or kill 0% and >75% of inoculated mice, respectively (19, 24, 25). All animal model studies reported here were approved by and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at the Pasteur Institute and complied with French laws and regulations. This study is registered as no. 08188 (Experimental infection of mice with poliovirus). Before i.n. and i.c. inoculations, mice were anesthetized by the i.p. injection of 0.25 μg xylazine (Rompun; Bayer) and 2.5 μg ketamine (Imalgene; Merial) in a total volume of 100 μl PBS.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01119-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.4 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

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F.D. conceived and designed the experiments. B.H., S.J., and M.-L.J. performed the experiments. B.H., S.J., M.-L.J., B.B., and F.D. analyzed the data and wrote the paper.

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