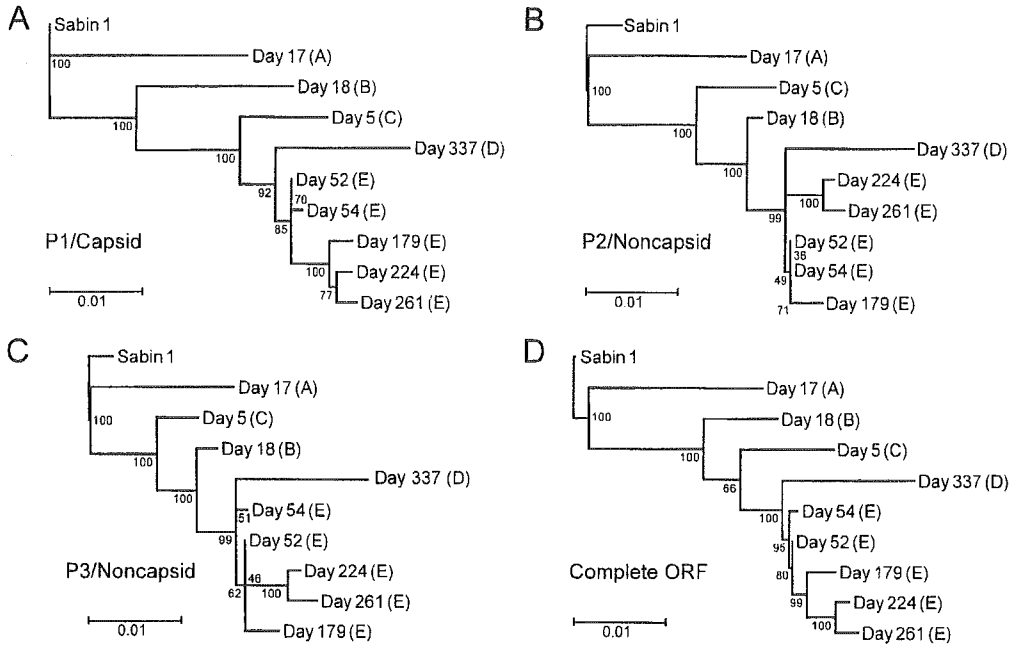

FIG. 2. Maximum-likelihood trees summarizing sequence relationships among the Sabin 1 OPV strain (root of tree) and the nine type 1 iVDPV isolates across different genomic intervals: (A) P1/capsid region (reference interval for evolution rate calculations), (B) P2/noncapsid region, (C) P3/noncapsid region, and (D) complete ORF. Major diverging lineages are labeled A to E.

(34, 73) (data not shown). The Taiwan isolates were identified as iVDPVs in accordance with the World Health Organization classification scheme, where VDPVs are poliovirus clinical isolates having 1% to 15% VP1 nucleotide sequence divergence from the parental OPV strain, and iVDPVs are VDPVs isolated from patients known to have B-cell immunodeficiencies (36).

Subsequent nucleotide sequencing of the complete genomes (7,411 to 7,441 nucleotides) of all nine isolates showed that all sequences were derived from Sabin 1. Most of the genetic differences among isolates were nucleotide substitutions, 82% of which generated synonymous codons. However, three different categories of 5′-UTR deletions were also observed. The day 17 isolate had no 5′-UTR deletions compared to Sabin 1. By contrast, all other VDPV isolates had a deletion of 8 nucleotides at positions 667 to 674, and the day 5 pharyngeal isolate had an additional deletion of 22 nucleotides at positions 695 to 716. The deletions occurred within a highly variable 5′-UTR interval that appears to have little secondary structure (63). Type 1 polioviruses with deletions within this interval have been shown to be viable in cultured cells (24, 44) and in humans (49). Four 5′-UTR nucleotide substitutions (G26A, U344C, U355C, and G480A) were shared by all isolates. All of these substitutions but U344C represent reversions back to the parental Mahoney sequence (59), and all three were consistently found among type 1 cVDPV isolates from Hispaniola (34) and the Philippines (73).

Emergence of multiple iVDPV lineages. Relationships among the complete P1/capsid region sequences of the nine iVDPV isolates were summarized in a tree constructed using the maximum-likelihood algorithm (21) and rooted to the Sabin 1 sequence (Fig. 2A). The tree had a deeply branched topology, with five major branches corresponding to lineages A to E, extending from a single main lineage. The first lineage (A) to branch off from the main lineage is represented by the day 17 isolate. This isolate differed from all others by the absence of any 5′-UTR deletions. Replication of lineage A virus may have ceased soon after onset of paralysis, as no other lineage A isolates were subsequently found. All other isolates contained the 8-nucleotide 5′-UTR deletion, which probably became fixed into the virus population very early in the chronic infection. The P1/capsid sequences of the day 17 (lineage A) and day 18 (lineage B) isolates were quite distinct, differing at 3.9% of nucleotide positions. The extensive divergence of the two isolates was surprising because the viruses originated from stool specimens taken on consecutive days. However, the VP1 sequence of the original day 17 isolate was ambiguous at several positions, indicating that the virus population was a mixture of variants, and the isolate was plaque-purified before complete genomic sequencing. By contrast, the day 18 isolate sequences were unambiguous, so no plaque purification was performed. It is possible that the day 17 specimen also contained lineage B virus that was removed by the plaque-purification step.

The day 18 isolate sequence appears at the tip of a long branch (Fig. 2A), suggesting that the day 18 isolate represents a second lineage that may have terminated soon after onset of paralysis. Two other distinct lineages, C and D, are represented by the day 5 and day 337 isolates, respectively. The 22-nucleotide 5′-UTR deletion apparently occurred after lineage C diverged from the evolutionary pathway leading to lineages D and E. Lineage D is defined by the sequence of the day 337 isolate, from the last poliovirus-positive stool specimen. Lineage E is defined by five closely related isolates from

the day 52, day 54, day 179, day 221, and day 261 specimens. Divergence among isolates of lineage E was limited and probably occurred during the period of sampling.

All sequences of the iVDPV isolates were derived from Sabin 1. Trees of the P2/noncapsid, P3/noncapsid, and complete ORF regions had deeply branched topologies similar to the P1/capsid tree (Fig. 2). However, the four trees were not congruent, as the order of branch nodes and branch lengths differed. For example, the branch nodes of the isolate sequences were ordered day 18 (B) –day 5 (C) –day 337 (D) in the P1/capsid tree, and day 5 (C) –day 18 (B) –day 337 (D) in the P2/noncapsid and P3/noncapsid trees (Fig. 2). Bootstrap support (20, 21) for the order of these nodes was high (>90%) in all trees. Moreover, the branch length of the day 18 isolate from its node was long in the P1/capsid tree and short in the P2/noncapsid and P3/noncapsid trees.

As the window for comparison moved from the P1/capsid region to the P2/noncapsid and P3/noncapsid regions, the branch node of the day 18 isolate shifted away from the root and that of the day 5 isolate shifted toward the root. By contrast, the sequence relationships among most other isolates were similar across the ORF, apart from minor differences in tree topologies that had low bootstrap support. The exception was the day 179 isolate, within lineage E, that was most closely related to the day 224 isolate in the P1/capsid tree, but most closely related to the day 52 isolate in the P2/noncapsid and P3/noncapsid trees. Although some of the differences in tree topologies may be attributable to stochastic variability in the rates of fixation of substitutions across different lineages and genetic intervals, another mechanism probably explains the more pronounced differences.

Recombination across and within iVDPV lineages. Relationships among the aligned complete ORF sequences of the day 5, day 18, and day 52 isolates were initially examined by using the distance plot and bootscan functions of the Simplot program (51), which revealed a possible recombination site between nucleotide positions 2659 and 2678, near the 5′ terminus of the VP1 region (Fig. 3B). Upstream P1/capsid region sequences (nucleotide positions 743 to 2658) of the day 52 isolate more closely matched those of the day 5 isolate (genetic distance 0.006 ± 0.002) than the day 18 isolate (genetic distance 0.035 ± 0.004), whereas the downstream ORF sequences (nucleotide positions 2679 to 7369) of the day 52 isolate more closely matched those of the day 18 isolate (genetic distance 0.007 ± 0.001) than the day 5 isolate (genetic distance 0.019 ± 0.002), a pattern suggestive of recombination.

Because recombination is difficult to detect at low levels of divergence (66), recombination was further investigated using the program DnaSP (71). The sharp discontinuity in the extent of sequence identity, clearly visualized by alignment of polymorphic nucleotide sites among the ORFs of the three isolates (Fig. 3C), was very likely produced by recombination. The likelihood that the observed sequence discontinuities arose by random substitution without recombination is very low ($P < 0.00001$; G test with Williams's correction). Although the summary alignment (Fig. 3B) shows the day 52 isolate genome as a mosaic assembled from upstream sequences similar to those of the day 5 isolate and downstream sequences similar to those of the day 18 isolate, the actual evolutionary relationships among the three isolates is likely to be more complex. For example, runs of sequence differences in the P3/noncapsid regions of the day 18 and day 52 isolates (Fig. 3C) may signal the occurrence of additional recombination events. However, short stretches of recombinant sequences are difficult to distinguish from localized clustering of substitutions (66). More importantly, because the sequences of the parental and progeny viruses directly participating in the recombination events are unknown, and only a small number of representatives of each lineage were available for analysis, the observed mosaic structures of the genomes are explainable by several alternative pathways of recombination.

Similarly, we found that the genome of the day 224 isolate also appeared to be a mosaic, with upstream ORF sequences (nucleotide positions 743 to 3366) more closely related to those of the day 179 isolate and downstream sequences (nucleotide positions 3372 to 7369) more closely related to those of the day 52 isolate (Fig. 3D). Alignment of the polymorphic nucleotide sites (Fig. 3E) revealed discontinuities in the extent of sequence identity that are most likely attributable to recombination ($P = 0.008$ for random substitution without recombination; G test with Williams's correction). The statistical support for recombination among the lineage E isolates was less robust than in the previous example because the number of polymorphic sites distinguishing the ORF sequences of the three isolates was small ($n = 60$).

Estimation of the date of OPV exposure that initiated the chronic iVDPV infection. The topologies of the trees indicated that genetic divergence from Sabin 1 increased with the time of sampling (Fig. 2). An approximately linear relationship ($R^2 = 0.84$) was obtained when the number of synonymous substitutions (K_s) in the P1/capsid region was plotted as a function of the times of collection (zero time: date of last OPV dose) for the nine specimens (Fig. 4). The linear relationship suggests that all lineages were derived from a single OPV dose. The date of the initiating OPV dose, estimated by extrapolation to the time when $K_s = 0$, was 59 days before the fifth and last OPV dose (95% confidence interval: 588 days before to 190 days after the last OPV dose). The rate of P1/capsid evolution estimated from the slope of the regression line was $(2.92 \pm 1.25) \times 10^{-2}$ synonymous substitutions per synonymous site per year, a value very similar to those previously obtained for poliovirus P1/capsid region or VP1 sequences (34, 35, 50, 53). When we performed similar analyses based upon total nucleotide substitutions (K_t) in the P1/capsid region, the intercept was 172 days before the last OPV dose and the evolution rate at all sites was estimated to be $(1.14 \pm 0.45) \times 10^{-2}$ substitutions per site per year (data not shown).

Although the two analyses yielded comparable estimates for the time of start of the chronic infection, we generally prefer to base our estimates upon the rate of fixation of synonymous substitutions, which we assume to accumulate at a nearly constant rate by random genetic drift. We also prefer to use the P1/capsid region for our calculations because it is the largest interval within the ORF that can generally be assumed to share a recent common ancestry, as recombination of noncapsid region sequences with heterologous species C enteroviruses frequently occurs in circulating wild polioviruses (10, 49) and cVDPVs (34, 70, 73, 89). However, because sequences in all genomic intervals were derived from a recent common Sabin 1 ancestor, we were also able to estimate the date of the initiat-

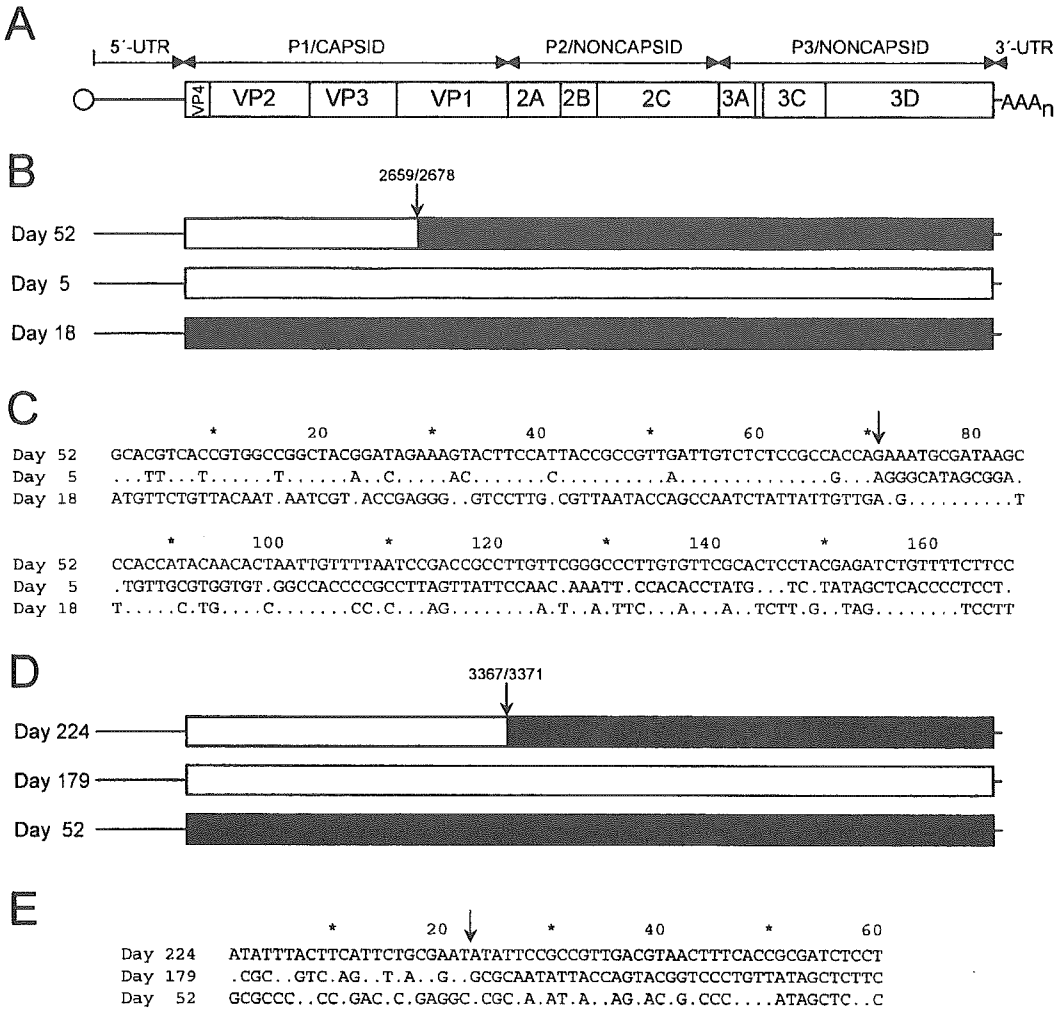

FIG. 3. Recombination across (panels B and C) and within (panels D and E) iVDPV lineages generating mosaic genomes. Panel A: Schematic of the poliovirus genome. The single ORF is represented by a rectangle, flanked by the 5′-UTR and 3′-UTR. Panels B and D: The most recent of the trio of isolates is represented as having the reference mosaic genome, with the sites of sequence discontinuity indicated by arrows. Sequence intervals of the reference mosaic genome most closely matching those of the preceding isolates are shaded accordingly. The 5′-UTR and 3′-UTR sequences were not included in the analyses for recombination and are represented only by lines. Panels C and E: Alignment of ORF nucleotide sites that are polymorphic among each trio of isolates. In each trio, the sequence of the most recent isolate is shown as a reference, with the sequence differences from that reference indicated below for the two preceding isolates. Polymorphic sites are numbered consecutively.

ing dose using the evolutionary rate data for the complete ORF (estimated date: 95 days after the last OPV dose; evolution rate: $[3.70 \pm 0.67] \times 10^{-2}$ synonymous substitutions per synonymous site per year; $R^2 = 0.95$) as well as for VP1 (estimated date: 146 days before the last OPV dose; evolution rate: $[2.41 \pm 1.58] \times 10^{-2}$ synonymous substitutions per synonymous site per year; $R^2 = 0.64$) (data not shown). Recombination across iVDPV lineages would not substantially alter the overall rate estimates provided that all iVDPV lineages evolved at nearly equivalent rates within the same genomic intervals. These evolution rate estimates strongly implicate the fifth and last OPV dose, given 567 days before onset of paralysis, as the OPV exposure event that initiated the chronic iVDPV infection.

Estimated times of divergence of different iVDPV lineages. The topologies of the trees (Fig. 2) suggested that the five major coreplicating iVDPV lineages diverged from each other before or soon after the onset of paralysis. For example, lineage A, whose branch node is near the root in all four trees, apparently diverged from the main lineage very early in the chronic infection. Other lineages diverged subsequently. To estimate the dates of divergence of the major iVDPV lineages, we assumed that all lineages diverged at a single rate of evolution and we used the single rate dated tips model (68) to convert genetic distances into units of time. We further assumed that the chronic infection began with the last OPV dose given 567 days before onset of paralysis. We based our estimates of divergence time upon the sequence relationships within the interval of nucleotides 743 to 2658, the upstream 72% of the P1/capsid region that we assume to have evolved principally by progressive fixation of nucleotide substitutions (Fig. 3B).

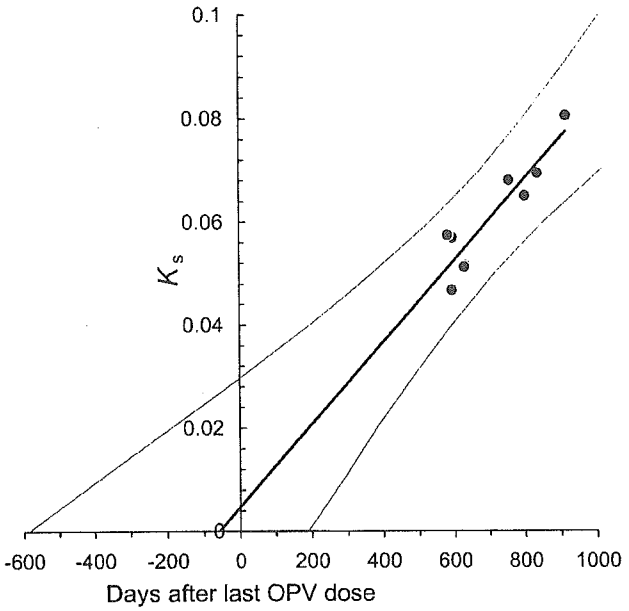

FIG. 4. Estimate of time of the initiating OPV dose from rate of accumulation of synonymous substitutions into the P1/capsid region of the nine type 1 iVDPV isolates. K_s, number of synonymous substitutions at synonymous sites in the P1/capsid region (Sabin 1 sequence set to zero substitutions). The evolution rate was estimated by weighted linear regression (R^2 = 0.84) as described in Materials and Methods. The curves flanking the regression line trace the 95% confidence limits for the time estimates calculated from K_s values.

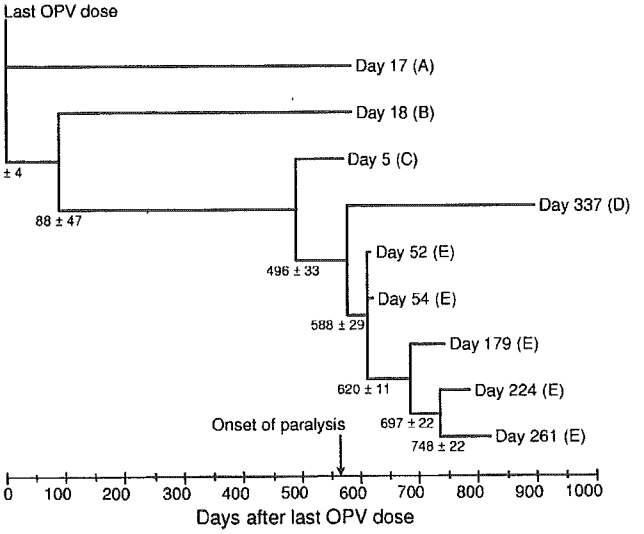

FIG. 5. Estimated times of divergence of major iVDPV lineages based upon the rate of substitution at third-codon positions within the P1/capsid region interval of nucleotides 743 to 2658. Genetic distances and standard errors were scaled under the single rate dated tips model (68) to the time of specimen collection (time zero: date of last OPV dose) as described in Materials and Methods. Note that isolates are identified by the day of specimen collection, not by the day after the last OPV dose.

We obtained a global evolution rate for third codon positions (representing ~91% of all synonymous substitutions) of (3.05 ± 0.34) × 10^{-2} substitutions per site per year for the partial (1916-nucleotide) P1/capsid interval compared. The internal nodes on the phylogenetic tree (Fig. 5) represent point estimates of the times of divergence of lineages A to E during the chronic infection. The topology of the partial P1/capsid tree (Fig. 5) was very similar to that of the complete P1/capsid tree (Fig. 2A) except for differences in the relative branch lengths of lineages B and C. Lineage A was estimated to have diverged from lineages B to E immediately after administration of the last OPV dose. Lineage B diverged from the remaining lineages around day 88, and lineage C around day 496, of the chronic infection. Additional divergences were estimated to have occurred after the onset of paralysis (at day 567 of the chronic infection), with lineages D and E diverging around day 588, and further divergences within lineage E around days 620, 697, and 748 (Fig. 5). Selection of other genomic intervals would have yielded different dates of divergence because of the effects of recombination. However, comparisons across the capsid interval we selected yielded the most deeply branched tree (compare Fig. 2 and 5) and the earliest dates for divergence of lineages A and B. Moreover, most of our phylogenetic comparisons are restricted to P1/capsid region sequences, which encode the defining biological properties of poliovirus (10, 34, 49, 89).

Antigenic divergence of iVDPV lineages. All iVDPV isolates were antigenic variants of the Sabin 1 strain. The day 17 isolate showed non-vaccine-like antigenic properties in the enzyme-linked immunosorbent assay using type-specific cross-absorbed antisera (82), whereas all of the other isolates were nonreactive. The antigenic differences are probably attributable to the numerous amino acid substitutions clustered within or near surface loops forming neutralizing antigenic (NAg) sites 1, 2, and 3a (7, 55, 62) (Fig. 6). Multiple (three to six) amino acid substitutions mapped to the interval forming most of NAg-1 (residues VP1:95 to 106, B–C loop), 1 to 2 substitutions in NAg-2 (at residue VP2:65, E–F loop; and residue VP1:222, G–H loop), and 1 to 4 substitutions within or near NAg-3 (at residues VP3:58 to 60, and VP3:75; loop preceding the B strand). All isolates had amino acid substitutions at site VP3:K060. Substitution at that site is associated with the antigenic reversion (from vaccine-like to non-vaccine-like) of Sabin 1-derived isolates tested with neutralizing monoclonal antibodies (7).

No substitutions were found in surface residues of NAg-3b (VP2:T72 and VP3:P76) or in parts of NAg-2 (VP2:270) and NAg-3a (VP1:287 to 292) (55, 62) (Fig. 6). Four of the substitutions (VP2:D165N, VP1:K99T, VP1:T106A, and VP1:A222T) restored the amino acid of the parental Mahoney strain, and four other substitutions (VP2:D165G, VP3:A59E, VP3:K60Q, and VP1:N100S) were identical to the monoclonal antibody escape mutations that were originally used to define the NAg sites of Sabin 1 (62, 83). The trypsin cleavage site in NAg-1 (VP1:K99), characteristic of Sabin 1 (22), was consistently eliminated by amino acid substitution (day 5 isolate, VP1:K99M; days 18 to 337 isolates, VP1:K99T) in all but the day 17 isolate. Amino acid substitutions outside of the surface loops were generally conservative and mapped to β-sheet structures.

The NAg sites of the day 17 isolate (lineage A; non-vaccine-

	VP2			VP3			VP1			
	NAg3b	NAg2	NAg2	NAg3a	NAg3a	NAg3b	NAg1	NAg2	NAg3a	
	71	164 .	269.	56 .	70		95 .	221	287 .	
Sabin 1	WTK	DDNQTSPARR	PRL	DLSAKKK	VRLSDKPHTD		SASTKNKDKLFT	SAALGD	KDGTLT	
Day 17 (A)	...	.G.....	...	....S..	.....		.VP.....N...	.....	.....	
Day 18 (B)	...	.N.....	...	...EQ..	.....		.V.ATS...Q.A	.....	.....	
Day 5 (C)	...	.N.....	...	...EQR.	.....Q....		....M.R..Q.A	.....	.....	
Day 337 (D)	...	.N.....	...	...EQ..	.....Q....		...ATS..RQ.A	.T....	.....	
Day 52 (E)	...	.N.....	...	...EQR.	.....Q....		.V.ATS...Q.A	.T....	.....	
Day 54 (E)	...	.N.....	...	...EQR.	.....Q....		.V.ATS...Q.A	.T....	.....	
Day 179 (E)	...	.N.....	...	...EQR.	.....Q....		.V.ATS...Q.A	.T....	.....	
Day 224 (E)	...	.N.....	...	...EQR.	.....Q....		.V.ATS...Q.A	.T....	.....	
Day 261 (E)	...	.N.....	...	...EQR.	.....Q....		.V.ATS...Q.A	.T....	.....	
Mahoney	...	.N.....	...	....T..	.....		P...T.....A	.T....	.....	

FIG. 6. Sequences of amino acid residues within or bounding neutralizing antigenic sites 1 (VP1:95 to 106), 2 (VP2:164 to 173; VP2:269 to 271; VP1:221 to 226), 3a (VP3:56 to 62; VP3:70 to 74; VP1:287 to 292), and 3b (VP2:71 to 73; VP3:75 to 79). Residues defining the type 1 poliovirus neutralizing antigenic sites by mutations conferring resistance to neutralization by monoclonal antibodies (7, 55, 62, 83) are indicated by boldface type. The trypsin cleavage site in NAg-1 (VP1:K99), characteristic of Sabin 1 (22), is underlined.

like antigenicity) were less highly substituted (5 amino acid substitutions) than those of all other iVDPV isolates (9 to 11 amino acid substitutions; nonreactive antigenicities), and only one substitution (VP1:A96V; NAg-1) was shared with any of the other isolates (lineages B and E) (Fig. 6). The non-vaccine-like antigenicity of the day 17 isolate indicates retention of the capacity to bind the anti-wild poliovirus cross-absorbed sera used in the standard enzyme-linked immunosorbent assay intratypic differentiation test (81, 86), a capacity lost by the more highly substituted nonreactive isolates. Lineage A appears to have followed an independent pathway of antigenic divergence from Sabin 1, consistent with its early divergence from the other lineages. The NAg sites of lineages B to E appear to have shared an early common pathway of antigenic divergence from Sabin 1. However, the NAg-1 sequences of the day 5 isolate (lineage C) differed from the lineage B, D, and E isolates at several positions, and may represent another pathway for antigenic evolution within NAg-1. The NAg sites of all five lineage E isolates were identical (Fig. 6), consistent with the view that the antigenic evolution had stabilized in the late stages of the chronic infection.

Neurovirulence of iVDPV isolates in PVR-Tg21 transgenic mice. Sabin 1 is an attenuated derivative of the neurovirulent Mahoney strain (59, 72). Important determinants of the attenuation phenotype in Sabin 1 include nucleotide G480 in the 5′-UTR and amino acid residues VP4:S65 (encoded by U935), VP3:M325 (encoded by A2438), VP1:T106 (encoded by A2795), and VP1:F134 (encoded by U2879) (9, 33). Reversion of the 5′-UTR determinant, G480A, occurred in all iVDPV isolates (Table 1). Reversion of the capsid determinant, VP1:T106A, occurred in eight of the nine isolates, and reversion of another capsid determinant, VP3:M325L, occurred in seven of the nine isolates. Two determinants, VP4:S65 and VP1:F134, did not revert.

Isolates representing each lineage were tested for neurovirulence in PVR-Tg21 transgenic mice expressing the human re-

TABLE 1. Nucleotide and amino acid substitutions at critical determinants of the attenuation and temperature-sensitive phenotypes of Sabin 1 and the corresponding phenotypes of representative Taiwan iVDPV isolates

Virus	Lineage	5′-UTR 480[c]	Viral protein:amino acid position[a,b]						3′-UTR 7441[d]	Log PD_{50}	EOP[e]
			VP4:65[c,d]	VP3:325[c,d]	VP1:88[d]	VP1:106[c]	VP1:134[c]	3D:73[d]			
Sabin 1		G	S	M	A	T	F	H	G	>8.0[f]	0.009
Day 17	A	A	S	M	A	T	F	Y	A	3.7	1.0
Day 18	B	A	S	M	A	A	F	Y	A	3.5	1.0
Day 5	C	A	S	L	A	A	F	Y	A	2.7	1.3
Day 337	D	A	S	L	A	A	F	Y	A	4.8	0.7
Day 52	E	A	S	L	A	A	F	Y	A	5.6	0.8
Day 261	E	A	S	L	A	A	F	Y	G	3.3	1.1
Mahoney		A	A	L	T	A	L	Y	A	2.8	1.0

[a] Amino acid substitutions correspond to the following nucleotide substitutions: VP4:S65A, U935G; VP3:M325L, A2438U; VP1:A88T, G2741A; VP1:T106A, A2795G; VP1:F134L, U2879C; and 3D:H73Y, C6203U.
[b] Amino acid residues at all six critical sites were identical in all five lineage E isolates.
[c] Site of major determinant of attenuation phenotype in Sabin 1.
[d] Site of major determinant of temperature-sensitive phenotype in Sabin 1.
[e] EOP, efficiency of plating, 39.5°C/34.5°C.
[f] The PD_{50} value of the Sabin 1 strain is quoted from previous reports (46, 73).

ceptor for poliovirus (Table 1). All six iVDPV isolates tested were more neurovirulent than Sabin 1, but only the day 5 pharyngeal isolate (lineage C) appeared to be as highly neurovirulent as the reference Mahoney strain. The day 17 (lineage A), day 18 (lineage B), and day 261 (lineage E) isolates were also highly neurovirulent, but the day 337 (lineage D) and day 52 (lineage E) isolates required substantially higher virus titers to induce paralysis or death in the transgenic mice. Surprisingly, the highly neurovirulent day 5 and day 261 isolates had identical alleles at the five critical sites as the less neurovirulent day 52 and day 337 isolates. Moreover, the day 52 isolate appears to be a recombinant between viruses closely related to the day 5 and day 18 isolates, but was markedly less neurovirulent than either (Table 1). These observations suggest that different genetic backgrounds may modify the effects of reversion at critical determinants of the attenuation phenotype, consistent with the composite nature of the determinants of the attenuation phenotype in Sabin 1 (26). As has recently been noted for type 1 cVDPV isolates from the Philippines (73), evolution toward increased neurovirulence is not necessarily irreversible for derivatives of Sabin 1.

Temperature sensitivity of iVDPV isolates. All three Sabin strains have a temperature-sensitive phenotype, producing lower virus yields at supraoptimal temperatures than wild polioviruses (58, 61). Major determinants of the temperature-sensitive phenotype of Sabin 1 include two amino acid residues associated with the attenuation phenotype (VP4:S65 and VP3:M325; see above), and minor determinants include two amino acids not associated with the attenuation phenotype, VP1:A88 (encoded by G2741) and 3D:H73 (encoded by C6203), along with nucleotide G7441 in the 3′-UTR (9, 64). As described above, the determinant of the temperature-sensitive (and attenuation) phenotypes, VP4:S65, did not revert, whereas the determinant VP3:M325 reverted in all but two iVDPV isolates (Table 1).

Another capsid determinant of the temperature-sensitive phenotype, VP1:A88, also did not revert. By contrast, reversions 3D:H73Y and G7441A occurred in all nine isolates, but an A7441G backmutation restored the Sabin 1 allele in the day 261 isolate. Despite the incomplete reversion at all sites conferring the temperature-sensitive phenotype, plaque yields in HeLa cells for the nine iVDPV isolates were similar at 39.5°C and 34.5°C (Table 1). Under our experimental conditions, the iVDPV isolates were like the Mahoney strain and unlike the temperature-sensitive Sabin 1 strain, which had a ~100-fold decrease in plaque counts at the elevated temperature (Table 1). Thus, the type 1 iVDPV isolates from Taiwan resembled the type 1 cVDPV isolates from Hispaniola (34) and the Philippines (73), which had also lost both the attenuation and temperature-sensitive phenotypes.

DISCUSSION

The genetic relationships among the type 1 iVDPV isolates described in this report highlight the dynamics of poliovirus evolution during chronic infection of an immunodeficient person. At least five distinct type 1 iVDPV lineages, derived from a single initiating OPV dose, emerged over the estimated 30 months of the chronic infection. Divergence of separate lineages began at the start of the infection and continued for at least 18 months thereafter. The five main lineages observed represent the minimum that may have emerged during the chronic infection, as some lineages may have terminated before the collection of the first clinical specimen, estimated to have been taken ~19 months after the start of the chronic infection. Moreover, only nine isolates were obtained over 337 days after onset of paralysis, and all but two of the isolates had been plaque purified before sequencing, so that any potentially wider diversity of the viruses in the original clinical specimens may have escaped detection.

Genetic divergence was associated with changes in the antigenic surface of the viruses. Antigenic evolution of the first diverging lineage (lineage A) appeared to be less extensive than, and independent of, the evolution of the other lineages. Antigenic evolution of lineages B to E followed a common early pathway, with an initial burst of substitutions in or near NAg sites 1, 2, and 3a that probably occurred within the first 3 months of the chronic infection. Antigenic evolution of lineages B, D, and E apparently stabilized afterwards, as only four polymorphic amino acid residues were found among the NAg sites of these lineages, and the antigenic surface of all lineage E isolates apparently remained unchanged during the 209 days of replication from day 52 to day 261. By contrast, the NAg-1 sequences of the day 5 isolate (lineage C) differed from the lineage B and E isolates at five sites, possibly signaling the existence of a diverging pathway for antigenic evolution.

Extensive genetic and antigenic diversity may be a characteristic feature of iVDPV isolates from long-term chronic poliovirus excretors. Stool specimens from other immunodeficient patients with prolonged infections have also been shown to contain mixtures of divergent iVDPV variants (29, 35, 53; R. Park, unpublished data). By contrast, the genetic diversity of vaccine-related or wild poliovirus isolates from immunocompetent individuals is typically low, presumably because the duration of each infection is short. cVDPV isolates from outbreaks similarly have low genetic diversity, as the duration of their infections and modes of transmission are probably the same as for wild polioviruses (34, 70, 73, 89). Although some diversification of wild type 3 poliovirus in a single immunocompetent person has been reported, the extent of divergence was far lower than what has been observed with immunodeficient long-term chronic excretors (42).

The emergence of divergent lineages suggests that the iVDPV had established separate sites of replication within the gastrointestinal tract. Poliovirus replicates in the lymphoid tissue of the oropharynx (tonsils), the small intestine (the Peyer's patches in the ileum), and in the mesenteric lymph nodes (8). The isolation of virus from both throat swabs and stool specimens is indicative of prolonged iVDPV replication in both the oropharynx and in the intestinal tract. It is interesting that the day 5 throat isolate (lineage C) was more closely related to the stool isolates of lineages B, D, and E than was the day 17 stool isolate (lineage A). The deep branch structure of the tree suggests that the separate lineages could replicate independently for extended periods of time (for at least 19 months in the case of lineage A virus). However, the detection of recombination across lineages indicates that the tissue compartmentalization of different lineages was incomplete. It is possible that recombination within and across lineages occurs frequently, but it is difficult to distinguish recombinants from

multiple substitution mutants if the parental viruses are very closely related. Such recombinants become even more difficult to recognize if the intervals exchanged are short or if multiple rounds of genetic exchange had occurred.

Although the patient was concurrently infected with E7 for many months, there was no evidence of genetic exchange between the two coinfecting enteroviruses. The lack of genetic exchange may be attributable to barriers to recombination between human enteroviruses of species B (E7) and species C (including polioviruses) (10, 60). Like the iVDPVs, the E7 isolates also showed evidence of extensive evolution and establishment of separate lineages (J.-Y. Yang, unpublished results).

The occurrence of chronic iVDPV infections may permit observation of processes that normally occur during acute poliovirus infections, but are otherwise undetectable because of the short duration of those infections. For example, intertypic recombination among the three Sabin strains is well documented (12, 23, 48) and frequently occurs in immunocompetent individuals fed trivalent OPV (17, 38), but natural intratypic recombination among OPV variants has been more difficult to demonstrate. Recently, Cherkasova et al. presented evidence consistent with intratypic recombination between coevolving lineages of virus derived from Sabin 2 (14). It is likely that intratypic recombination occurs constantly during natural poliovirus infections, as it does under experimental conditions in cell culture (16). Consistent with experimental findings (80), it is also likely that intratypic recombination in humans occurs at much higher frequencies than intertypic recombination. Similarly, it has long been known that polioviruses can colonize different sites in the gastrointestinal tract, as evidenced by the frequent isolation of virus both from stool specimens and throat swabs taken from the same immunocompetent patient. However, most poliovirus infections are cleared before the emergence of multiple variant lineages becomes evident.

Chronic poliovirus infections of immunodeficient persons may differ from acute infections involving person-to-person transmission of polioviruses in other respects. For example, the NAg sites of circulating wild polioviruses and cVDPVs appear to be more stable than those of iVDPVs from chronic excretors. The well-documented antigenic lability of the Sabin OPV strains (55, 58, 82) appears to only partially account for these differences (J. Jorba et al., unpublished results). Fluctuating antibody levels during immunoglobulin therapy may be an important contributing factor for the rapid antigenic evolution of iVDPVs.

The dynamics of genetic and phenotypic variation described here for iVDPVs are analogous to processes that typically occur during chronic infections established by other rapidly evolving viruses, among which human immunodeficiency virus type 1 has been the most extensively studied. Underlying mechanisms for the rapid evolution of human immunodeficiency virus type 1 in chronically infected patients include the emergence of multiple virus lineages, the proliferation of antigenic variants, the localization of different lineages to separate tissue compartments, and recombination across lineages (57, 92).

The two categories of VDPVs present different risks for the World Health Organization strategy to cease OPV use after global eradication of wild polioviruses (4, 19). The emergence and spread of cVDPVs can be prevented by maintenance of high rates of poliovirus vaccine coverage (36). By contrast, the emergence of iVDPVs in immunodeficient patients is rare and sporadic (27, 37) and cannot be prevented by high levels of population immunity to polioviruses. The potential for person-to-person spread of iVDPVs is unknown, because most iVDPV infections detected so far have occurred in well-immunized communities, and secondary infections have rarely been detected. It is possible that the Taiwan iVDPV had spread to close contacts but that all infections had cleared by the time the immunodeficient patient showed signs of paralysis and the case was investigated. It appears likely that iVDPVs can spread to contacts at least as efficiently as virus excreted by many healthy OPV recipients (6, 75). However, it is unknown whether iVDPVs have the same transmissibility as cVDPVs, which appear to be biologically indistinguishable from wild polioviruses (34, 70, 73, 89).

The risks of emergence of long-term chronic iVDPV excretors vary in different settings (4). Many high-income countries in Europe and North America have shifted from OPV to IPV, thereby effectively preventing the emergence of new vaccine-associated paralytic poliomyelitis cases and iVDPV infections (3). The survival rates of patients with B-cell immunodeficiencies are highest in high-income countries because of the availability of supportive immunoglobulin therapy. In such settings, the occurrence of chronic iVDPV infections is rare even among patients with B-cell immunodeficiencies, and most chronically infected patients either spontaneously cease iVDPV excretion or die from complications of their immunodeficiency (37). Nonetheless, some patients in high-income countries appear to have been infected with iVDPVs for up to 10 years or more and either experienced the late onset of paralytic poliomyelitis (5, 35) or remained asymptomatic carriers of iVDPVs (52).

Although most reports of chronic iVDPV excretors have come from high-income countries (5, 11, 29, 35, 37, 52, 53), recent reports of iVDPV infections have come from other middle-income countries such as Argentina (30), Thailand (79), and Peru (85). Middle-income countries with improving levels of sanitation and rising access to medical care may be at increasing (although very low) risk for the occurrence of immunodeficient chronic iVDPV excretors. This risk will continue as long as OPV is used. By contrast, lower-income countries appear to be at the lowest risk for the occurrence of immunodeficient chronic iVDPV excretors because conditions do not favor the survival of persons with B-cell immunodeficiencies (27). It thus appears especially important for middle- and high-income countries that improved methods for detecting iVDPV infections be developed and that effective therapies for clearing chronic poliovirus infections be found.

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A Sabin 3-Derived Poliovirus Recombinant Contained a Sequence Homologous with Indigenous Human Enterovirus Species C in the Viral Polymerase Coding Region†

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Outbreaks of poliomyelitis caused by circulating vaccine-derived polioviruses (cVDPVs) have been reported in areas where indigenous wild polioviruses (PVs) were eliminated by vaccination. Most of these cVDPVs contained unidentified sequences in the nonstructural protein coding region which were considered to be derived from human enterovirus species C (HEV-C) by recombination. In this study, we report isolation of a Sabin 3-derived PV recombinant (Cambodia-02) from an acute flaccid paralysis (AFP) case in Cambodia in 2002. We attempted to identify the putative recombination counterpart of Cambodia-02 by sequence analysis of nonpolio enterovirus isolates from AFP cases in Cambodia from 1999 to 2003. Based on the previously estimated evolution rates of PVs, the recombination event resulting in Cambodia-02 was estimated to have occurred within 6 months after the administration of oral PV vaccine (99.3% nucleotide identity in VP1 region). The 2BC and the $3D^{pol}$ coding regions of Cambodia-02 were grouped into the genetic cluster of indigenous coxsackie A virus type 17 (CAV17) (the highest [87.1%] nucleotide identity) and the cluster of indigenous CAV13-CAV18 (the highest [94.9%] nucleotide identity) by the phylogenic analysis of the HEV-C isolates in 2002, respectively. CAV13-CAV18 and CAV17 were the dominant HEV-C serotypes in 2002 but not in 2001 and in 2003. We found a putative recombination between CAV13-CAV18 and CAV17 in the $3CD^{pro}$ coding region of a CAV17 isolate. These results suggested that a part of the $3D^{pol}$ coding region of PV3(Cambodia-02) was derived from a HEV-C strain genetically related to indigenous CAV13-CAV18 strains in 2002 in Cambodia.

Poliovirus (PV) is a small nonenveloped virus with a single-strand positive genomic RNA of about 7,500 nucleotides (nt) belonging to the family *Picornaviridae*, known as the causative agent of poliomyelitis. Currently, the global eradication program for poliomyelitis is continuing by utilizing both inactivated and live attenuated vaccines (44, 46). The endemicity of indigenous wild PVs was confirmed to be restricted to Afghanistan, Egypt, India, Niger, Nigeria, and Pakistan as of 2004 (http://www.polioeradication.org/progress.asp).

The Sabin strains (Sabin 1, 2, and 3) are attenuated PV strains and have been widely used as live oral PV vaccine (OPV) (44). Following the administration of OPV, the viruses infect the mucosal tissues and are commonly excreted for 3 to 7 weeks from immunocompetent individuals (1, 18) and occasionally for 10 to 22 years from immunodeficient patients (2, 25, 32; reviewed in reference 48). During the replication of the Sabin strains, revertants with increased virulence could emerge and cause vaccine-associated paralytic poliomyelitis in rare cases. The rate of vaccine-associated paralytic poliomyelitis has been estimated as one case per 520,000 doses associated with the first dose of OPV (35). The revertants have been isolated from healthy individuals and also from the environment (21, 52).

Recently, outbreaks of poliomyelitis caused by circulating vaccine-derived PV (cVDPV) have been reported in Egypt, Hispaniola, the Philippines, and Madagascar (6, 8, 10, 24, 51). Sequence analysis of the genomes of cVDPVs showed unidentified sequences in the nonstructural protein coding region. These sequences are considered to be derived from recombination with unidentified nonpolio enterovirus (NPEV) during the circulation of VDPVs for 1 to 10 years (6, 8, 10, 24, 49, 51). However, a highly evolved derivative of Sabin strains without recombination by an unidentified counterpart has been isolated from an acute flaccid paralysis (AFP) case after a long-term circulation (12). Therefore, the biological role of the recombination of cVDPVs with unidentified counterpart remains to be elucidated. At present, increased transmissibility of cVDPVs compared with that of the parental Sabin strains has been proposed as a result of the recombination (3, 16); however, no virological evidence has been provided so far.

Indigenous wild PVs have been eliminated in regions where cVDPVs have been reported (1991 in the Americas [42], 1993 in the Philippines [11], and 1998 in Madagascar [41]) except Egypt. Therefore, the field NPEVs genetically closely related to PV or highly mutated Sabin derivatives are considered the possible counterparts of the recombination. Among NPEVs, coxsackie A viruses (CAVs) belonging to human enterovirus species C (HEV-C) are the suspected origin of the recombination because of the higher similarity of the genomic se-

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TABLE 1. Primers used for sequence analysis of enterovirus isolates

Primer	Sequence[a]	Corresponding site on Sabin 3 genome (nt)
UG16	GTTGGTGGGAACGGTTCACA	5912–5931
EcoRI-3END−	ACTGGAATTCTTTTTTTTTTTTTTTTTTTTTTV	7432–poly(A)tail
UC12	TCAATTAGTCTGGATTTTCCCTG	6485–6507
EVP4	CTACTTTGGGTGTCCGTGTT	544–563
OL68-1	GGTAAYTTCCACCACCANCC	1178–1197
2A2+	TTTKCNGMACCWGGKGAYTGYGGYGG	3683–3708
2C−	GGYTCAATACGGYRTTTGCTCTTGAACTG	4451–4479
292	MIGCIGYIGARACNGG	2604–2619
222	CICCIGGIGGIAYRWACAT	2942–2960

[a] Variable sequence positions in the primers are expressed according to the IUPAC system. Sequences read from the 5′ position at the left end.

quence with that of PV compared with those of other NPEVs belonging to HEV-A, HEV-B, or HEV-D (4, 22). A recent report indicated that HEV-C was frequently isolated in Madagascar (around 50% of NEPV isolates), suggesting the possible involvement of HEV-C in the emergence of the recombinant cVDPVs (41).

Here, we report an isolation and genetic characterization of a Sabin 3-derived PV recombinant (Cambodia-02) from an AFP case in Cambodia in 2002. Based on the previously estimated evolution rates of PVs, it was estimated that Cambodia-02 was isolated within 6 months after the administration of OPV, suggesting that the recombination occurred within 6 months before the isolation. This prompted us to identify the recombination counterpart of Cambodia-02 among the HEV-C isolates in Cambodia. We performed phylogenic analysis and identification of NPEV isolates from AFP cases in Cambodia from 1999 to 2003.

MATERIALS AND METHODS

Cells and viruses. RD cells (derived from human rhabdomyosarcoma), HEp-2c cells (derived from human larynx epidermoid carcinoma) and L20B cells (derived from mouse L tk^- $aprt^-$ fibroblast) were cultured as monolayers in Eagle's minimum essential medium supplemented with 2% fetal calf serum (33, 50). RD, HEp-2c, and L20B cells were used for the virus isolation from fecal samples of AFP cases. Virus stocks were stored at −70°C.

Sequence analysis of the genomes of enterovirus isolates. Viral genomic RNA was isolated from the culture fluid of infected cells by using a High Pure viral RNA purification kit (Roche). DNA fragments used for the DNA sequencing were prepared by reverse transcription-PCR (RT-PCR) using the viral genomic RNA as the template by use of a Titan one-tube RT-PCR system (Roche). PCR products were purified by using a QIAquick PCR purification kit (QIAGEN). DNA sequencing was performed using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems), and then sequences were analyzed by use of an ABI PRIZM 3100 genetic analyzer (Applied Biosystems). The sequences of the 5′ end of the viral genomes were determined by the 5′ rapid amplification of cDNA ends method by using a 5′ rapid amplification of cDNA ends system, version 2.0 (Invitrogen), according to the manufacturer's instructions. The sequence of the 3′ end of the viral genomes was determined from an RT-PCR product obtained with UG16 primer (20) and EcoRI-3END− (Table 1). The percentage of the mutated synonymous sites among all synonymous sites (K_s) was calculated for the VP1 coding region as previously reported (2, 12, 17). Phylogenic trees were constructed by the neighbor-joining method after bootstrapping 1,000 times (14, 45) using PHYLIP software (Joseph Felsenstein 1990, University of Washington). The nucleotide substitutions among the isolates were estimated by the Kimura-2 parameter method (26). The rate of transition-transversion was set at 2.0. Similarity plot analysis of HEV-C isolates was performed by using SimPlot (29).

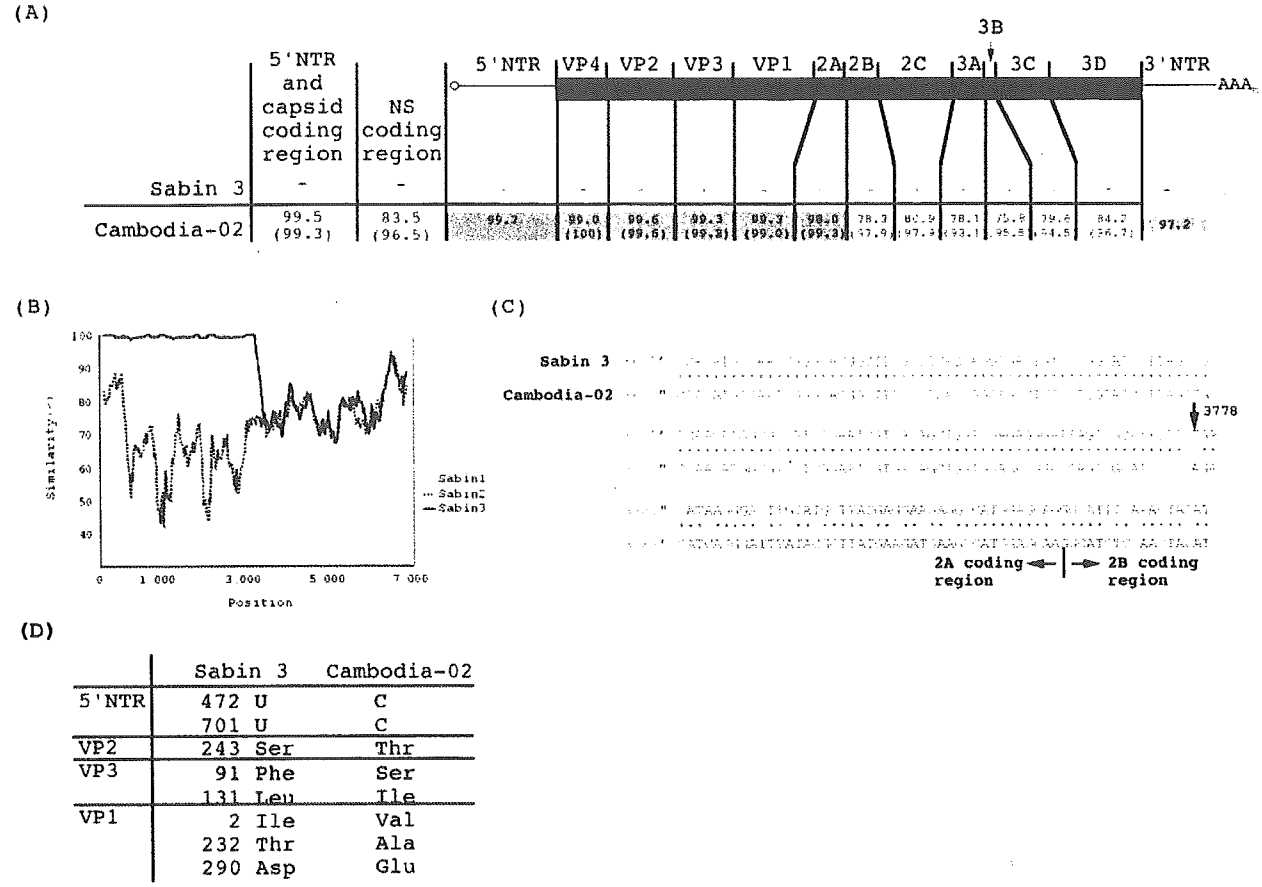
Primers used for the sequence analysis are listed on Table 1. Primers UG16 and UC12 were used for the analysis of a part of the $3D^{pol}$ coding region (20). Primers EVP4 and OL68-1 were used for the analysis of the VP4 coding region (39, 43). Primers 2A2+ and 2C− were designed and used for the analysis of a part of the 2BC coding region. Primers 292 and 222 were used for the initial analysis of the VP1 coding region (37). Genomic sequences used for the phylogenic analysis were as follows: 207 nt of the VP4 coding region (corresponding to nt 743 to 949 of the Sabin 3 genome), 337 nt of the 2BC coding region (corresponding to nt 3854 to 4190 of the Sabin 3 genome), and 352 nt of the $3D^{pol}$ coding region (corresponding to nt 6137 to 6488 of the Sabin 3 genome).

Identification of NPEV isolates. A panel of horse antisera against commonly found NPEVs (RIVM, Bilthoven, The Netherlands), which include echo and coxsackie B viruses, was used for the identification of HEV-B. Antisera against CAVs were purchased from the American Type Culture Collection. A total of 100 50% cell culture infectious doses of enterovirus isolates were incubated with 20 units of antiserum for 2 h in 37°C, and then HEp-2c cell or RD cell suspensions in 10% fetal calf serum–minimum essential medium were added and incubated at 35.5°C (50). Inoculated cells were observed for cytopathic effect until 24 h after the complete appearance of cytopathic effect in the cells inoculated with the isolates in the absence of antiserum.

Accession numbers of the nucleotide sequences. All the nucleotide sequences determined in this study were submitted to the DNA Data Bank of Japan (DDBJ). The GenBank/EMBL/DDBJ accession numbers of each sequence were as follows. The accession numbers of the VP4 coding region, the 2BC coding region, and the $3D^{pol}$ coding region of the NPEV isolates are AB206334 to AB206380, AB206709 to AB206757, and AB205529 to AB205546, respectively (see Fig. 2, 3, and 5 and the supplemental material). The accession numbers of the VP1 coding region of CAM1952, CAM2033, CAM2038, and CAM2083 are AB207264, AB207263, AB207265, and AB207266, respectively (Table 1). The accession numbers of the genomic sequences of Cambodia-02, CAM1900, CAM1972, CAM2069, and CAM2101 are AB205395, AB205397, AB205396, AB205398, and AB205399, respectively.

RESULTS

Isolation of a type 3 PV recombinant from an AFP case. In 2002, type 3 PVs were isolated from three AFP cases in Cambodia. These PV isolates were initially characterized by sequencing of the VP1 coding region, and all the isolates were classified as OPV-like PVs according to the criteria of the World Health Organization (less than 1% nucleotide difference from the parental Sabin 3) (50). However, we found that one of these PV isolates (Cambodia-02) contained an unidentified sequence in the $3D^{pol}$ coding region which was apparently not related to those of the Sabin strains (Fig. 1). Further sequence analysis of the genome of Cambodia-02 showed that the 5′ part of the genome (from nt 1 to 3777), including the 5′ nontranslated region (5′NTR), the structural protein coding region, and a part of the $2A^{pro}$ coding region, was derived from Sabin 3 followed by an unidentified sequence from the $2A^{pro}$ coding region to the 3′ end of the genome (from nt 3778 to the 3′ end) (Fig. 1B and C). The nonstructural protein coding region of Cambodia-02 showed only low similarity with those of Sabin strains (Fig. 1B).



(D)

	Sabin 3	Cambodia-02
5'NTR	472 U	C
	701 U	C
VP2	243 Ser	Thr
VP3	91 Phe	Ser
	131 Leu	Ile
VP1	2 Ile	Val
	232 Thr	Ala
	290 Asp	Glu

FIG. 1. The genomic sequence of Cambodia-02. (A) Alignment of the genome of Cambodia-02 (accession no. AB205395) with that of Sabin 3 (accession no. X00925). The numbers in each region represent the percentages of nucleotide identity with the Sabin 3 genome. The numbers in parentheses represent the percentages of amino acid identity. The genomic regions that showed more than 90% amino acid identity are colored with light gray, and genomic regions that showed more than 96% nucleotide identity are colored with dark gray. The nucleotide identity in the 5′ part of the genome (including the 5′NTR and the structural protein coding region) and in the nonstructural protein (NS) coding region are also shown. (B) Similarity plot analysis of Cambodia-02 and Sabin strains (Sabin 1, accession no. AY184219; Sabin 2, accession no. AY184220) calculated by SimPlot. The nucleotide sequence of the Cambodia-02 genome was used as the reference. A window size of 200 bp with an increment of 20 bp was used. (C) Alignment of the genome of Cambodia-02 with that of Sabin 3 near the putative recombination junction in the $2A^{pro}$ coding region. The part representing unidentified sequence (from nt 3778 to the 3′ end) is colored with light gray. (D) The nucleotide and amino acid differences in the 5′NTR and the capsid proteins of Cambodia-02. Numbers represent the positions of nucleotides in the 5′NTR or of the amino acid residues in each capsid protein.

The nucleotide identity of the 5′ part of the Cambodia-02 genome to Sabin 3 was 99.5%. The K_s value of Cambodia-02 calculated for the VP1 coding region was 1.35×10^{-2} (with a standard error of 0.77×10^{-2}). Using evolution rates of PV observed for immunodeficiency cases (2.85×10^{-2} to 3.28×10^{-2} synonymous substitutions per synonymous site per year) or for transmission of wild PV recombinants (3.45×10^{-2} synonymous substitutions per synonymous site per year) (2, 17, 28), we estimated that Cambodia-02 was isolated within 6 months after the administration of OPV. Cambodia-02 had reversions at the major attenuation determinants of Sabin 3 at nt 472 (U to C) and nt 2034, which resulted in an amino acid change of VP3 Phe91 to Ser (13, 31) (Fig. 1D). The Cambodia-02 genome contained multiple mutations in the structural protein coding region in addition to VP3 Phe91, as previously reported for temperature-resistant revertants of Sabin 3 (15, 31, 34).

Isolation and identification of HEV-C from AFP cases in Cambodia. We analyzed the genome of NPEV isolates from AFP cases around 2002 in Cambodia to identify the putative recombination counterpart of Cambodia-02. In 2002, we isolated NPEVs from 53 AFP cases (one was from a mixed case with PV) among a total of 155 AFP cases (Table 2). For the initial molecular typing of the isolates, we analyzed the VP4 coding region (nt 743 to 949 of the Sabin 3 genome; 207 nt) to classify the isolates into each genomic species (HEV-A, HEV-B, and HEV-C) (23). We found that 21 isolates were grouped into HEV-C by the phylogenic tree analysis of the sequence of the VP4 coding region (data not shown). We identified the serotype of HEV-C isolates by a neutralization assay using type-specific antisera or by sequence analysis of the VP1 coding region. We could not discriminate CAV13 from CAV18 or CAV11 from CAV15 by the sequence analysis or by the neutralization assay, consistent with previous reports (4,

TABLE 2. Virus isolation from AFP cases in Cambodia from 1999 to 2003

Identification	No. of cases				
	1999	2000	2001	2002	2003
AFP	149	230	168	155	128
Virus isolated	49	73	46	55	39
Poliovirus[a]	3	9	4	3	4
Nonenterovirus	6	8	4	3	3
HEV-A	3	6	0	9	3
HEV-B	25	26	24	20	13
HEV-C	12	24	15	21	16

[a] OPV-related isolates.

36). The deduced amino acid sequence of the VP1 protein of CAM2033 and CAM2038 showed a high nucleotide identity with those of CAV17 (94.1%) and CAV11-CAV15 (96.7%), respectively. We could not identify the serotype of CAM2083 from the deduced amino acid sequence of the VP1 protein, which showed low similarity with known enteroviruses. We observed the highest amino acid identity only with CAV24 (DN-19 strain) (74.1%) or with a CAV24 variant (73.1%). Consequently, HEV-C isolates in Cambodia in 2002 consisted of CAV1, CAV11-CAV15, CAV17, CAV13-CAV18, CAV20, CAV24, and an untypable HEV-C strain CAM2083 (Table 3).

Sequence analysis of HEV-C isolates in the 3D^{pol} coding region. We then analyzed the genomic sequence in the 3D^{pol} coding region of the HEV-C isolates. The phylogenic analysis of a part of the 3D^{pol} coding region (corresponding to a region of nt 6137 to 6488 of the Sabin 3 genome; 352 nt) showed that the isolates formed distinct genetic clusters from those of the prototype HEV-C strains, as observed for the sequence analysis of the VP4 coding region (Fig. 2). The phylogenic analysis of the 3D^{pol} coding region failed to show a clear relationship between the serotypes of isolates and their genetic clusters. For CAM1974, CAM2083, and CAM2091, we could not obtain the corresponding DNA fragment by RT-PCR. In the phylogenic analysis, a genetic cluster of indigenous CAV13-CAV18 strains was the closest to Cambodia-02. We found that a CAV13-CAV18 isolate (CAM1900) showed the highest nucleotide identity (94.0%) to Cambodia-02 among the HEV-C isolates. We further analyzed the nonstructural protein coding region of CAM1900 and found that CAM1900 showed a high identity to

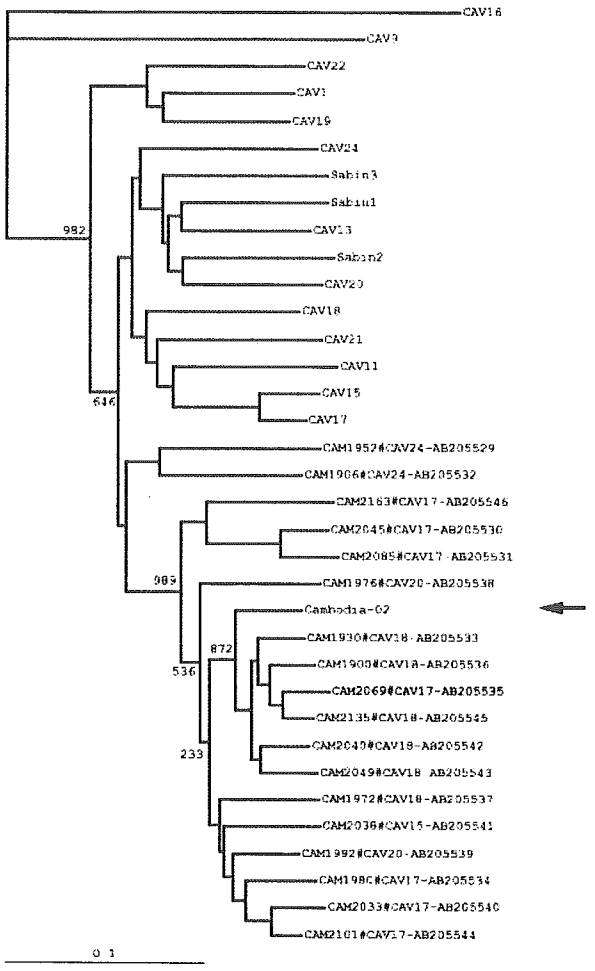


FIG. 2. Phylogenic analysis of the 3D^{pol} coding region. The phylogenic tree was generated from the nucleotide sequences of the 3D^{pol} coding region of Cambodian HEV-C isolates, prototype HEV-C strains, and Sabin strains. The location of Cambodia-02 is indicated by an arrow. A putative HEV-C recombinant (CAM2069) is colored with light gray. The nomenclature of the isolates indicates the names of the isolates, serotypes, and the GenBank/EMBL/DDBJ accession numbers. Bootstrap values are shown at the branch nodes. Bar, 0.1 substitution per site.

TABLE 3. Isolation of HEV-C from AFP cases in Cambodia from 1999 to 2003

HEV-C isolate	No. of isolates				
	1999	2000	2001	2002	2003
CAV1	0	0	0	1	1
CAV11/15	2	3	0	1	3
CAV13/18	2	3	0	7	2
CAV17	1	2	4	7	0
CAV20	2	5	2	2	3
CAV21	0	1	0	0	0
CAV24	2	4	7	2	6
CAM2083	3	6	2	1	1
Total	12	24	15	21	16

Cambodia-02 only in the 3D^{pol} coding region (94.9%) but not in other regions (Fig. 3A and C).

In the phylogenic analysis of the 3D^{pol} coding region, we found that a CAV17 isolate, CAM2069, was located apart from other CAV17 isolates in a genetic cluster of indigenous CAV13-CAV18 strains. The sequence analysis of CAM2069 showed that CAM2069 exhibited a high (95.5 to 97.7%) nucleotide identity to CAM2101 (a CAV17 isolate) for the 2C and the 3AB coding regions. However, the 3CD^{pro} coding region of CAM2069 showed high (93.3 to 95.5%) nucleotide identity to that of CAV13-CAV18, as observed in the phylogenic analysis of the 3D^{pol} coding region (Fig. 2) and in the similarity plot analysis (Fig. 3C). This observation suggested that recombination between CAV13-CAV18 and CAV17 could occur at least in the 3CD^{pro} coding region.

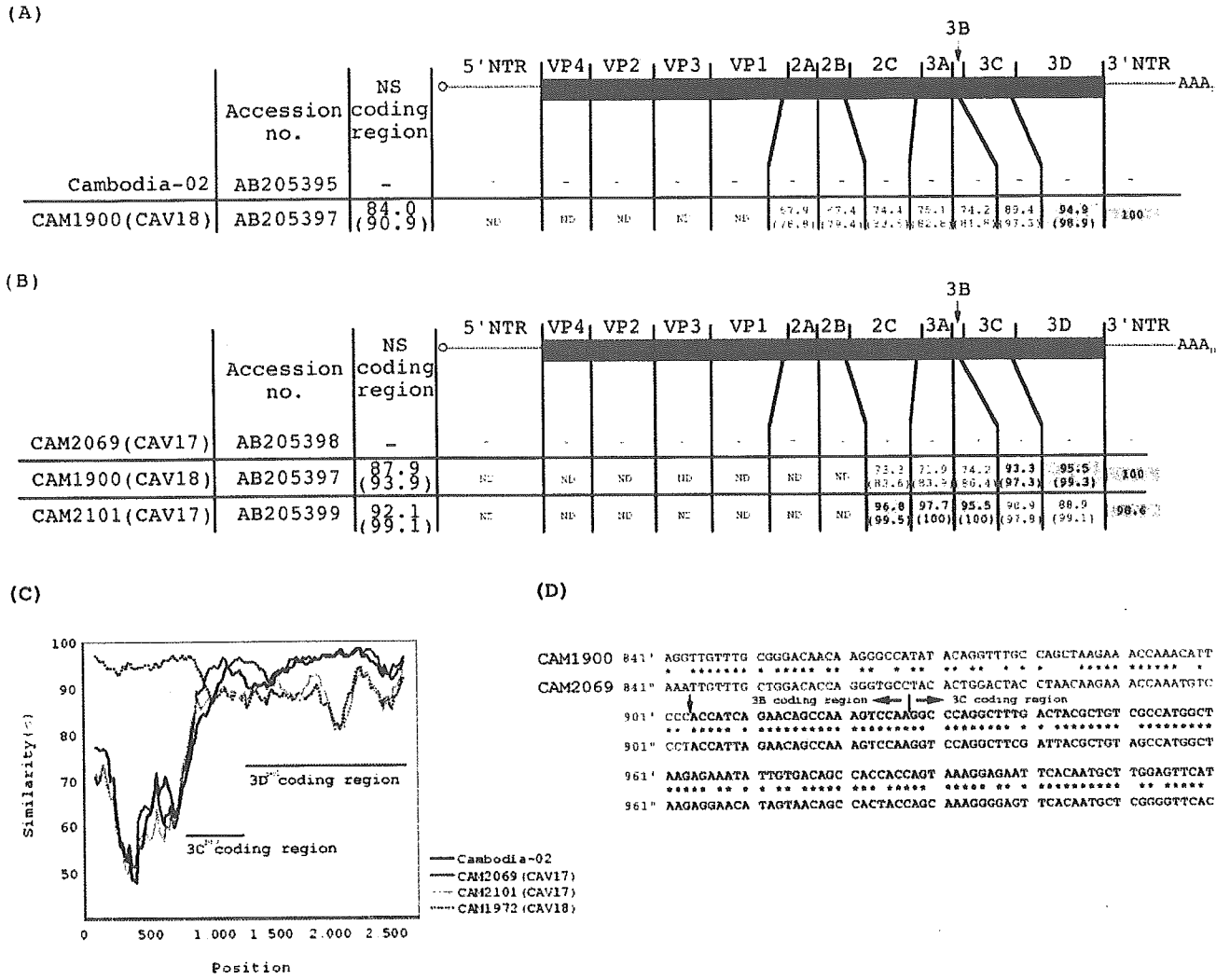


FIG. 3. Alignment of the genomes of Cambodian HEV-C isolates. The numbers in each region represent the percentages of nucleotide identity, and the numbers in parentheses represent the percentages of amino acid identity. The genomic regions that showed more than 90% amino acid identity are colored with light gray, and the genomic regions that showed more than 92% nucleotide identity are colored with dark gray. (A) Alignment of Cambodia-02 with a CAV13-CAV18 isolate (CAM1900). (B) Alignment of a CAV17 isolate (CAM2069) with CAM1900 (CAV13-CAV18) and CAM2101 (CAV17). (C) Multisequence analysis of HEV-C isolates and Cambodia-02 by similarity plot analysis calculated by SimPlot. CAM1900 was used as the reference. A window size of 200 bp with an increment of 20 bp was used. The locations of $3C^{pro}$ and $3D^{pol}$ coding regions are shown in the plot. (D) Alignment of a part of the genome of CAM1900 with that of CAM2069 around the putative recombination junction near the $3C^{pro}$ coding region. The part representing unidentified sequence is colored with gray. NS; nonstructural protein, ND; not determined.

Sequence analysis of HEV-C isolates in the 2BC coding region. Next, we analyzed the sequence of another nonstructural protein coding region of HEV-C isolates, because the analysis in the $3D^{pol}$ coding region failed to identify the recombination counterpart. For this purpose, we designed a new primer set for RT-PCR and DNA sequencing, 2A2+ and 2C−, in the $2A^{pro}$ coding region and in a *cis*-acting replication element in the 2C coding region (19, 40), respectively. By using this primer set, we analyzed a sequence of the 2BC coding region (corresponding to nt 3854 to 4190 of the Sabin 3 genome; 337 nt) for all the NPEV isolates (Fig. 4). We then analyzed the sequence of three isolates (CAM1920, CAM1936, and CAM2034) for which we could not analyze the sequence of the VP4 coding region. However, one isolate (CAM1952, a CAV24 strain) failed to give an RT-PCR product. In the phylogenic analysis of the 2BC coding region, we observed a close relationship between the serotypes of isolates and the genetic clusters. In this phylogenic analysis, Cambodia-02 was again located close to the genetic clusters of indigenous HEV-C but not to those of the HEV-C prototypes. CAM2101 (a CAV17 isolate) showed the highest nucleotide identity to Cambodia-02 in the 2BC coding region; however, the identity was not significantly high (86.9%).

HEV-C isolates from AFP cases in Cambodia from 1999 to 2003. To identify the recombination counterpart of Cambodia-02, we further analyzed the NPEV isolates from 1999 to 2003 in Cambodia. HEV-C was a dominant HEV species isolated from AFP cases in this period, suggesting that the prevalence

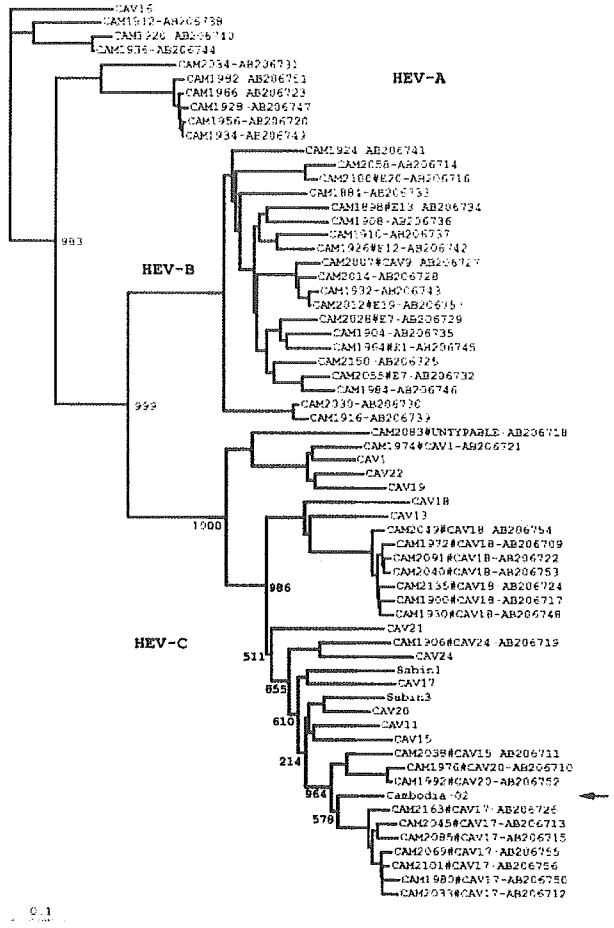


FIG. 4. Phylogenic analysis of the 2BC coding region. The phylogenic tree was generated from the nucleotide sequences in the 2BC coding region of Cambodian NPEV isolates, prototype HEV-C strains, and Sabin strains. The location of Cambodia-02 is indicated by an arrow. The nomenclature of the isolates indicates the names of the isolates, serotypes, and the GenBank/EMBL/DDBJ accession numbers. Bootstrap values are shown at the branch nodes. Bar, 0.1 substitution per site.

of HEV-C was consistently high in Cambodia (Table 2 and 3). The dominant serotypes of HEV-C were different from year to year; in 2002, they were CAV17 and CAV13-CAV18. In the phylogenic analysis of the 2BC coding region, Cambodia-02 was grouped into a cluster of the indigenous CAV15-CAV17-CAV20 isolates. However, the nucleotide identity was not significantly high, and the highest (90.1%) was found with a CAV17 isolate in 2000 (data not shown). Therefore, we could not identify the exact recombination counterpart among the HEV-C isolates examined. However, these results suggested that the recombination counterpart of Cambodia-02 was genetically closely related to the indigenous HEV-C strains in Cambodia.

DISCUSSION

A Sabin 3-derived PV recombinant (Cambodia-02) analyzed in this study was isolated from an AFP case in Cambodia in 2002. Cambodia-02 was classified as OPV-like PV by sequence analysis in the VP1 coding region. In 2002, we isolated three type 3 PVs, including Cambodia-02, from AFP cases in Cambodia, but these PV isolates were genetically unrelated to each other (data not shown). Therefore, this evidence suggested that Cambodia-02 was isolated from a sporadic AFP case and did not result from a circulating strain.

Cambodia-02 was isolated within 6 months after the administration of OPV, as determined on the basis of the previous estimation of the evolution rate of PVs (2, 17). Thus, the unidentified sequence of Cambodia-02 should have retained the original genetic feature of the recombination counterpart. The last indigenous PV case in Cambodia was reported in 1997 (7); therefore, we examined HEV-C strains for the recombination counterpart. We examined HEV-C isolates from AFP cases in Cambodia; however, these isolates represented only a minor population of circulating HEV-C strains that would mostly result in asymptomatic infection. Therefore, through this strategy, we could expect to find some HEV-C strains that were only related to the recombination counterpart. We found that HEV-C was a dominant enterovirus species that could be isolated from the AFP cases in Cambodia (Table 2). The HEV-C isolates consisted of CAV1, CAV11-CAV15, CAV13-CAV18, CAV17, CAV20, CAV21, CAV24, and an untypable serotype represented by CAM2083 (Table 3). HEV-C strains have not been isolated as major NPEVs through the established enterovirus surveillance systems (5, 9) (http://idsc.nih.go.jp/iasr/prompt/circle-g/meningi/menin.html [in Japanese]). However, recently, a high frequency of HEV-C isolation (~50% of the isolates) was reported in Madagascar, where type 2 cVDPVs emerged in 2002 (10, 41). Therefore, HEV-C might be a dominant HEV species among the circulating enteroviruses in tropical areas. However, the prevalence of HEV-C in other tropical areas remained to be further investigated.

We performed a comprehensive sequence analysis of HEV-C isolates in three different genomic regions, including the 2BC coding region and the $3D^{pol}$ coding region (20, 39, 43) (Fig. 2 and 4). We designed a new primer set, 2A2+ and 2C−, for the analysis of the 2BC coding region (Table 1). The 2A2+ primer was designed in the $2A^{pro}$ coding region, and the 2C− primer was designed in a *cis*-acting replication element of the enterovirus (19, 40). This primer set showed a broad spectrum of applicability for HEV-A, HEV-B, and HEV-C isolates, and sequence analysis using this primer set failed for only one isolate (CAM1952 [CAV24]) among 216 NPEV isolates. Recombination junctions of cVDPV have been identified in the 2AB coding region (6, 8, 10, 24). Therefore, with a wide spectrum of applicability for NPEV, this primer set would serve as a useful tool to identify HEV-C strains related to the recombination counterpart of cVDPV.

From the sequence analysis, we found that the nonstructural protein coding regions of Cambodia-02 were grouped into the genetic clusters of the indigenous CAV17 and CAV13-CAV18 strains in Cambodia, distinct from those of the HEV-C prototype strains (Fig. 2 and 4). We isolated CAV17 from 2001 to 2002 and CAV13-CAV18 from 2002 to 2003. Thus, both CAV17 and CAV13-CAV18 were highly prevalent and could be available as the recombination counterpart of Cambodia-02 in 2002. One of the CAV13-CAV18 isolates (CAM1900) showed the highest (94.9%) nucleotide identity to Cambo-

dia-02 in the $3D^{pol}$ coding region (Fig. 4). The nucleotide identities in the $3D^{pol}$ coding region among isolates with the same HEV-C serotype isolated in 2002 were 95 to 96% (Fig. 3), and Cambodia-02 showed a nucleotide identity comparable to indigenous HEV-C isolates in 2002. This suggested that these HEV-C isolates had evolved independently before the putative epidemic in 2002. Interestingly, we found a putative CAV recombinant (CAM2069) in the $3CD^{pro}$ coding region, suggesting frequent interserotypic recombination between CAV17 and CAV13-CAV18 in this region (Fig. 4). Actually, the $3CD^{pro}$ coding region showed high similarities among CAV13-CAV18, CAV11-CAV15, CAV17, and CAV20 (4, 22). This was reminiscent of frequent recombination among HEV-B strains and also among human rhinoviruses where the $3D^{pol}$ coding regions of the same serotype were not monophyletic (27, 30, 38, 47). However, we could not find the exact recombination counterpart among the indigenous HEV-C isolates from AFP cases, although their nonstructural protein coding region was closely related to that of Cambodia-02.

In summary, we isolated a type 3 PV recombinant from an AFP case in Cambodia and suggested that the unidentified sequence of the recombinant was derived from an indigenous HEV-C strain in Cambodia. In addition to the poor population immunity, the high prevalence of HEV-C would be another critical factor for the emergence and/or the evolution of cVDPV. The biological roles of the recombination of cVDPV remain to be further studied.

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Persistence of oral polio vaccine virus after its removal from the immunisation schedule in New Zealand

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See Comment page 351

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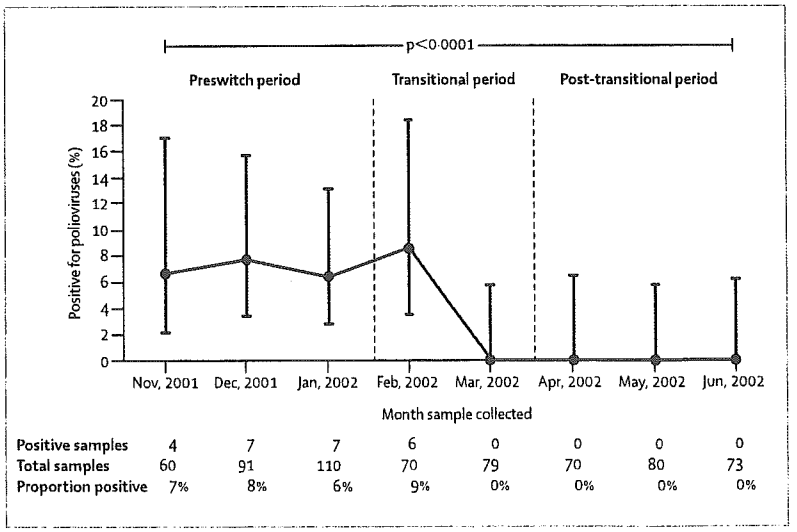
On Feb 1, 2002, inactivated poliomyelitis vaccines replaced live-attenuated oral poliovirus vaccine (OPV) in New Zealand's immunisation schedule, allowing systematic monitoring of OPV virus circulation. Findings of paediatric-inpatient surveillance indicate that 7% of children excreted polioviruses before this switch, but none did so 1 month afterwards. Acute flaccid paralysis surveillance detected no poliovirus during and after the switch, whereas enterovirus surveillance detected poliovirus only once during the switch. Environmental surveillance identified polioviruses in sewage samples until May, 2002, after which they were detected infrequently. Intratypic differentiation and sequencing showed that all polioviruses were Sabin-like. Multiple surveillance methods hence showed that OPV strains did not persist for extended periods after a vaccine switch in a developed country with a temperate climate. Sequence homology with Sabin vaccine parent strains indicated that polioviruses detected more than 4 months after the switch were of recent origin, consistent with importation from OPV-using countries.

The global eradication of wildtype poliomyelitis by mass immunisation campaigns with live-attenuated oral poliovirus vaccine (OPV) is imminent, despite outbreaks of this disease in Nigeria in 2004–05 and persisting small reservoirs in Africa and Asia (http://www.polioeradication.org). A priority is to develop strategies of when and how to stop OPV immunisation once poliomyelitis is eradicated. However, whether vaccine virus transmission is sustained after withdrawal of OPV from immunisation schedules remains unknown. This question is important, since persistent circulation of OPV viruses increases the risk of reversion to fully neurovirulent vaccine-derived poliovirus strains in unvaccinated populations.[1]

After OPV vaccination, poliovirus is excreted by healthy children for 2–3 months and its persistence in populations is limited.[1] Reports[2] from several developing countries though indicate that circulating neurovirulent vaccine-derived poliovirus strains can be sustained for extended periods and cause poliomyelitis when population immunity is low. Since 1961, New Zealand has maintained OPV coverage of about 85%. However, after two instances of vaccine-associated paralytic poliomyelitis, inactivated poliovirus vaccine (IPV) replaced OPV in the infant immunisation schedule on Feb 1, 2002. This change provided an opportunity to monitor the persistence of OPV strains excreted by the last cohorts of children immunised with OPV. We did systematic population-based surveillance for OPV virus circulation and evolution before, during, and after the OPV/IPV switch with combined paediatric-inpatient, acute flaccid paralysis, enterovirus laboratory, and environmental surveillance systems. Based on Cuba's experience of annual mass immunisation campaigns, we postulated that polioviruses would be isolated during the preswitch period and then decline over a 2-month transitional period, after which no further polioviruses would be isolated.[3]



	Nov, 2001	Dec, 2001	Jan, 2002	Feb, 2002	Mar, 2002	Apr, 2002	May, 2002	Jun, 2002
Positive samples	4	7	7	6	0	0	0	0
Total samples	60	91	110	70	79	70	80	73
Proportion positive	7%	8%	6%	9%	0%	0%	0%	0%

Figure 1: Poliovirus prevalence (95% CI) between November, 2001, and June, 2002 by paediatric inpatient surveillance

The Wellington Ethics Committee approved this study on behalf of Auckland and Waikato Ethics Committees. All patients or their parents or guardians provided written consent. We surveyed paediatric inpatients for 8 months (3 months before, 2 months during, and 3 months after the OPV/IPV switch) at three hospitals in Auckland, Hamilton, and Wellington. To detect a decline in OPV strain prevalence from 4·5% (previous national enterovirus surveillance data) to 0·5% (80% power, 95% significance, two-tailed test of difference in proportions), we approached every month 35 children (younger than age 15 years) consecutively admitted to each of the three hospitals with expectation of 80% participation.

Of 861 patients recruited, 633 (74%) provided stool samples for testing. The results of paediatric-inpatient surveillance indicate that vaccine viruses disappeared quickly after the switch (figure 1). During the preswitch and transition periods, we isolated polioviruses from 18

of 261 (7%, 95% CI 4·1–10·7) and six of 149 children (4%, 1·7–8·2), respectively. We isolated the last poliovirus from a stool sample collected 1 month after the OPV/IPV switch. We did not detect any polioviruses in 223 stool samples collected during the post-transitional period. The Cochram-Armitage test shows a significant trend ($p<0·0001$) in prevalence across the three periods. We recorded the demographic features of the 24 poliovirus-positive and 609 poliovirus-negative children (webappendix 1). All polioviruses were isolated from vaccinees who had OPV within 10 weeks of being admitted to hospital and who were younger than age 6 months. Poliovirus isolation rates were similar across all socioeconomic groups. 24 inpatients yielded 30 Sabin-like polioviruses (seven type 1, 12 type 2, 11 type 3).

Since 1997 there has been continued monitoring of acute flaccid paralysis in children younger than age 15 years.[4] Between January, 2001, and September, 2003, we analysed stool samples from 22 of 33 reported cases of acute flaccid paralysis. Only one child, aged 2 months and with spinal muscular atrophy, had Sabin-like polioviruses type 1 and type 2 isolated from each of two stool samples collected on Feb 28 and March 1, 2001. She had received her first dose of OPV 11 days previously.

Enterovirus surveillance used the national laboratory network, which investigates mainly inpatients with febrile illnesses not associated with acute flaccid paralysis (about 1200 stool samples annually).[5] We analysed data for 33 months (13 months before, 2 months during, and 18 months after the switch) and noted that polioviruses disappeared rapidly after the OPV/IPV switch (webappendix 2). Before the switch, we identified 38 poliovirus-positive children. In the transition period, we isolated Sabin-like polioviruses type 1 and type 2 from a 2-month-old boy without acute flaccid paralysis, whose stool sample was collected 5 days after the OPV/IPV switch. 19 months later, we identified a Sabin-like poliovirus type 2 in a 10-month-old girl with conjunctivitis. Sequencing in the VP1 region showed 99·9% homology to the parental Sabin strain. Almost all poliovirus-positive cases from enterovirus surveillance were aged 6 weeks to 5 months. 40 children without acute flaccid paralysis yielded 48 Sabin-like polioviruses (19 type 1, 18 type 2, 11 type 3).

For environmental surveillance, we collected weekly sewage samples over 18 months (3 months before, 2 months during, and 13 months after the switch) from three sewage treatment plants in Auckland, Hamilton, and Porirua (a satellite city of Wellington) where the surveillance hospitals were located. The catchment populations were 900 000, 100 000, and 65 000, respectively. Before the OPV/IPV switch, the poliovirus isolation rate was 94%. This proportion decreased after the switch, but not as rapidly as with other surveillance methods (figure 2). The decline was maintained in the post-transitional period (April, 2002, to April, 2003), such that after May, 2002, polioviruses were only detected once every 3 months.

We isolated 71 Sabin-like polioviruses as a result of environmental surveillance (nine type 1, 36 type 2, 26 type 3). Sequencing of environmental polioviruses during the post-transitional period confirmed these as Sabin-like with more than 99% homology with parental Sabin strains (webappendix 3). In particular, the five Sabin-like polioviruses identified by environmental

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See for webappendix 1

See for webappendix 2 and webappendix 3

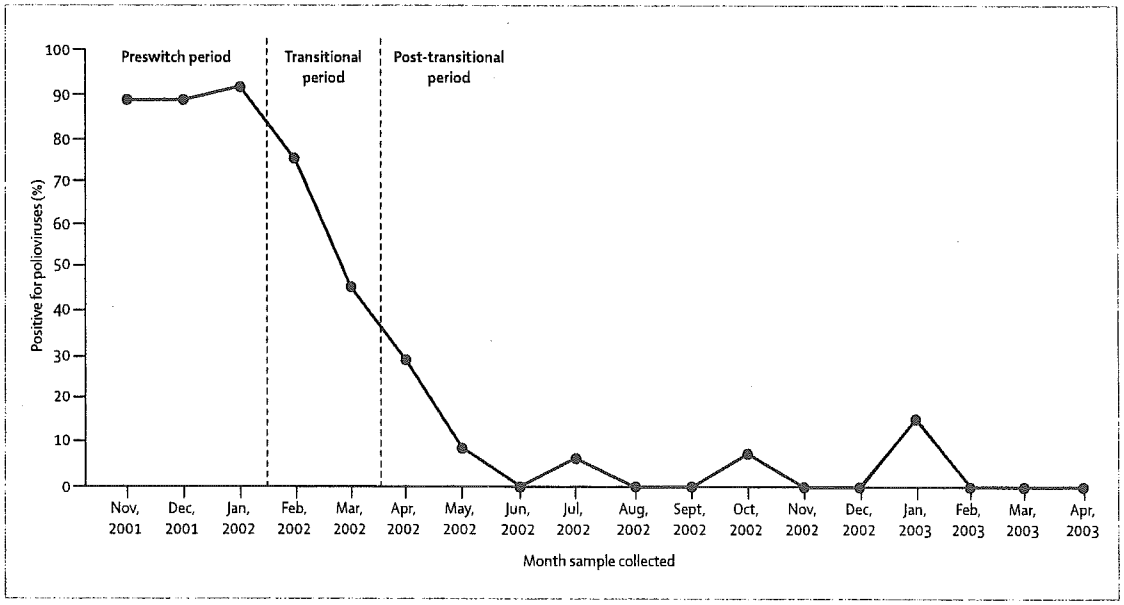

Figure 2: **Poliovirus prevalence between November, 2001, and April, 2003, by environmental surveillance**

surveillance 6, 9, and 12 months after the OPV/IPV switch had 99·7–100% homology with parental strains.

We noted limited circulation of OPV strains in New Zealand after the switch to IPV. First, with one exception, all polioviruses isolated from enterovirus surveillance during 2001–02 were from children aged 6 weeks to 5 months who should have received at least one dose of OPV. Second, OPV viruses detected by paediatric-inpatient surveillance were found only in vaccinees. Third, intratypic differentiation and sequence data for polioviruses obtained from paediatric-inpatient, acute flaccid paralysis, and enterovirus surveillance confirmed that all polioviruses were Sabin-like.

Since polioviruses evolve at a constant rate of 1% nucleotide substitutions per year,[6] environmental isolates 6–12-months post-switch with 99·7–100% sequence homology to parental Sabin strains infer that these viruses were derived from OPV administered 1–3-months previously. Rather than being from either the last cohorts of OPV immunised children or immune-deficient long-term excretors,[7] these viruses are more likely to have originated in recently vaccinated children or their close contacts from an OPV-using country. This finding shows that New Zealand remains vulnerable to vaccine or wildtype virus importation.

Every surveillance method revealed a different rate of OPV virus decline. Acute flaccid paralysis surveillance examines as few as 1 in 100 000 children younger than 15 years for poliovirus excretion. Its sensitivity for detecting sporadic vaccine-derived poliovirus is limited since only 0·1–0·5% of non-immune children infected with virulent strains will manifest paralytic poliomyelitis. Every year, enterovirus surveillance examines stool samples from roughly one in 3000 (1200 of 3 737 277) New Zealanders suspected of enteroviral infections. Paediatric-inpatient surveillance attempted to measure poliovirus excretion in a moderately representative population by sampling one in 648 (633 of 410 181) children living in three cities. Environmental surveillance obtained composite samples from sewage systems that serve 28% of the population. The higher and more prolonged poliovirus detection rates in sewage indicate the increased sensitivity of this method of surveillance over that of paediatric-inpatient surveillance in the same urban areas.

OPV strains do not persist for long after an OPV/IPV switch in a developed country with a temperate climate. Our study should be repeated in tropical, developing countries, however, where transmission of OPV viruses is likely to be more intense. The findings of such studies are vital to formulate polio immunisation policies in the postcertification era. Simultaneous global cessation of OPV after a mass immunisation campaign to maximise population immunity and minimise vaccine-derived poliovirus circulation could be adopted if there is minimum risk of sustained vaccine-derived poliovirus circulation.[2] Meanwhile, the continued risk of poliovirus importation means that New Zealand should maintain high IPV coverage. Finally, multiple surveillance methods, particularly environmental surveys, provide increased sensitivity for detection of poliovirus circulation, which will be essential in the posteradication era.

Contributors

Q S Huang, G Greening, M G Baker, and K Grimwood designed the study, supervised the virological, clinical, and environmental components, interpreted the data and their analysis, and wrote the report. J Hewitt, D Hulston, L van Duin, and A Fitzsimons established and did the laboratory tests. N Garrett did the study size calculations, contributed to the design, and undertook statistical analyses. K Grimwood, D Graham, and D Lennon established paediatric-inpatient surveillance. H Shimizu and T Miyamura undertook the sequence analysis and interpretation. M A Pallansch assisted in study design and interpretation.

Conflict of interest statement

We declare that we have no conflict of interest.

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