tified in March 2001 on the southern island of Mindanao in the Philippines, and two additional cases were identified in July 2001, situated about 800 km to the north on the island of Luzon (Fig. 1). The fourth isolate was from a healthy contact of one of the poliomyelitis patients in Cavite province, close to metropolitan Manila. Sequence comparisons revealed that all four cVDPVs were closely related to each other (≈99% VP1 nucleotide sequence identity), divergent from Sabin 1 (≈97% of VP1 nucleotide sequence identity), and independent of type 1 VDPVs heretofore found elsewhere. Recombinant genomes as all noncapsid sequences downstream of a common crossover site in the 2B region were derived from an as yet unidentified enterovirus. Most of the biological properties of the Philippines cVDPVs were indistinguishable from those of wild-type 1 polioviruses. Thus, the biological and genetic characteristics of the type 1 VDPVs from acute flaccid paralysis cases in the Philippines were similar to those of the cVDPVs reported from Hispaniola, Egypt, and Madagascar.

MATERIALS AND METHODS

Virus isolation and identification. Viruses were isolated from stool specimens on the RD (human rhabdomyosarcoma cells; ATCC CCL-136) and L20B (mouse fibroblast cells expressing the human poliovirus receptor) cell lines by standard methods (64). Polioviruses were identified by microneutralization with type-specific antisera. HEp-2C cells (human cervical carcinoma cells) were also used for preparation of the virus stocks.

Intratypic differentiation of polioviruses. Two different methods, one based on genetic properties (nucleic acid probe hybridization or PCR) and the other based on antigenic properties by enzyme-linked immunosorbent assay (ELISA), were used for intratypic differentiation to distinguish between vaccine and wild polioviruses of the poliovirus isolates (8, 9). Concordance of the intratypic differentiation results by two methods may indicate a vaccine or wild poliovirus. Isolates with discordant intratypic differentiation results (vaccine-like by one method and non-vaccine-like by the other) are further characterized by genomic sequencing.

For the probe hybridization, poliovirus RNA was inactivated by formaldehyde and immobilized on two nylon membranes for probing with digoxigenin-labeled oligonucleotides (14). One probe targeted highly conserved sequences within the 5′ nontranslated region (5′-NTR) of enteroviruses, while the other was an OPV strain-specific probe directed to variable VP1 sequences. Alternatively, viral RNA was amplified by reverse transcription-PCR (RT-PCR) with oligonucleotide primers directed to the same genomic regions targeted by the nucleic acid probes. The results identify a virus isolate as either vaccine-like or wild. The intratypic differentiation-ELISA used serotype-specific polyclonal antisera raised against intact virus particles of the Sabin OPV strains or a reference wild poliovirus strain of each serotype (60). The antiserum was cross-adsorbed against the heterologous strain of the same serotype to remove antibodies directed to shared antigenic sites. Four patterns of reactivity may be observed: vaccine-like, non-vaccine-like, double-reactive, and nonreactive. An isolate with any of the last three reactivity patterns could be either a wild poliovirus or an antigenically drifted OPV-derived poliovirus having a mutation(s) in a capsid surface determinant(s).

Sequencing. Viral RNA was extracted from the virus stocks with the Qiagen viral RNA kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions and used for RT-PCR amplification by standard procedures. The RT-PCR products were purified with the QiaQuick PCR system (Qiagen K.K.). The entire VP1 or full-length genome sequence of the purified PCR products of both strands were determined with oligonucleotide primers with an ABI Prism 310 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

The sequencing primers were designed according to the genome sequence of Sabin 1 or by primer walking. The 5′-end sequence was determined with the 5′-rapid amplification of cDNA ends (RACE) system, version 2.0 (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the CAV-650 (5′-TACTTAGAGTAAACACACTC-3′) primer was used for reverse transcription, and the PV3S-460A (antisense; 5′-GGTTAGGAATTAGCCGCATT C-3′) and Abridged Anchor (sense; provided by the manufacturer) primers were used for PCR amplification. The PV3S-300A primer (antisense; 5′-GGCCCAA GCTACACTCCGGG-3′) was used to determine the 5′-terminal sequence. The 3′-end RT-PCR amplicon was produced with the Access RT-PCR system (Promega K.K., Tokyo, Japan). The PV1S-6530S (sense; 5′-AGTTTGAATGACTC AGTGGC-3′) and 3′-end (antisense; 5′-GACCACGCGTATCGATGTCGACT TTTTTTTTTTTTTTV-3′, where V is a mixture of A, G, and C) primers were used for PCR amplification, and the sequence was determined with the PV1S-7080S primer (sense; 5′-TTGAAACAGTCACATGGGAG-3′). The resultant chromatogram data were analyzed with Sequencher software (Hitachi Software Engineering Co., Ltd., Kanagawa, Japan).

Phylogenetic analysis. The entire VP1 sequences were determined for the initial genetic characterization of poliovirus isolates with discordant intratypic differentiation results. Sequence alignments were performed with the Clustal W software (57), and phylogenetic trees were constructed by the neighbor-joining method with Kimura's two-parameter method (52). The reliability of the tree was estimated with 1,000 bootstrap replicates. The tree was displayed with the program TreeView (49).

Comparative analysis of the genome sequences of VDPVs. The full-length genome sequences of the reference strains of polioviruses, cVDPVs, and species C enteroviruses were obtained from the GenBank database. Pairwise comparisons among nucleotide sequences in each genome region and multiple sequence alignments were analyzed with Genetyx software (Software Development Co., Ltd., Tokyo, Japan). The numbering of nucleotide positions follows that of the original sequence report of Sabin 1 (45).

Neurovirulence test in TgPVR-21 mice. ICR-PVR-Tg21 mice, carrying the human poliovirus receptor gene, were purchased from the Central Laboratory of Experimental Animals (Kanagawa, Japan) and used for neurovirulence tests as described previously (32, 66). Briefly, six mice (three males and three females) were inoculated intracerebrally with 30 μl of each virus dilution of the type 1 cVDPV, attenuated (Sabin 1) and virulent wild (Mahoney) reference strains per mouse. The mice were observed daily for 14 days, and the dose causing paralysis or death in 50% of the mice (PD_{50}) was calculated by the Kärber formula.

Single-step growth kinetics. The growth kinetics of the cVDPV isolates at 39.5°C in HeLa S3 cells (ATCC CCL-2.2) was compared with those of the type 1 reference strains as described previously (66). Virus yields were examined at various times after inoculation (input multiplicity = 10 PFU/cell) in HeLa S3 cell suspensions. Virus titers were determined in duplicate in plaque assays on monolayers of HeLa cells (ATCC CCL-2) at 37°C.

Neutralization titer against monoclonal antibodies. The antigenic properties of the cVDPV isolates were analyzed by a microneutralization assay, with monoclonal antibodies specific to Sabin 1 (8a034 and 8a057) or the Mahoney strain (11m071) (24). Fifty microliters of the monoclonal antibodies was diluted twofold and incubated with an equal volume of the challenge virus (≈100 cell culture infective doses per 50 μl) for 2 h at 36°C. Then, 100 μl of a suspension of HEp-2C cells per well was added, and cytopathic effects were examined for 7 days. The neutralization titers were determined by the Kärber formula.

Nucleotide sequence accession numbers. The VP1 nucleotide sequences of the type 1 wild polioviruses determined in this study have been submitted to GenBank under accession numbers AB180058 to AB180069. The other VP1 sequences were obtained from the GenBank database. The complete genomic sequences of the four cVDPVs from the Philippines have been submitted under accession numbers AB180070 to AB180073.

RESULTS

Epidemiologic and clinical background. Wild poliovirus types 1 and 3 were endemic in the Philippines until 1993 (26, 50), and the Philippines, along with the other countries in the Western Pacific Region, were certified as free of indigenous wild poliovirus in 2000 (63). Continued acute flaccid paralysis and poliovirus surveillance in the Philippines has not detected any wild poliovirus infections after 1993. Polio-free status has thus been sustained in the Philippines.

However, from March to July 2001, three acute flaccid paralysis cases associated with cVDPVs were reported in the Philippines (Table 1). The first patient was an 8-year-old boy from the province of Misamis Oriental in northern Mindanao, an island 800 km south of Manila (Fig. 1). The patient had a history of three doses of OPV, and the onset of paralysis occurred on 15 March 2001. The second patient, a 3-year-old girl from the province of Laguna, close to metropolitan Manila on Luzon Island, had a history of three doses of OPV, with onset of meningitis on 21 July 2001. The third patient was a 14-month-old boy from Cavite province (75 km north of the

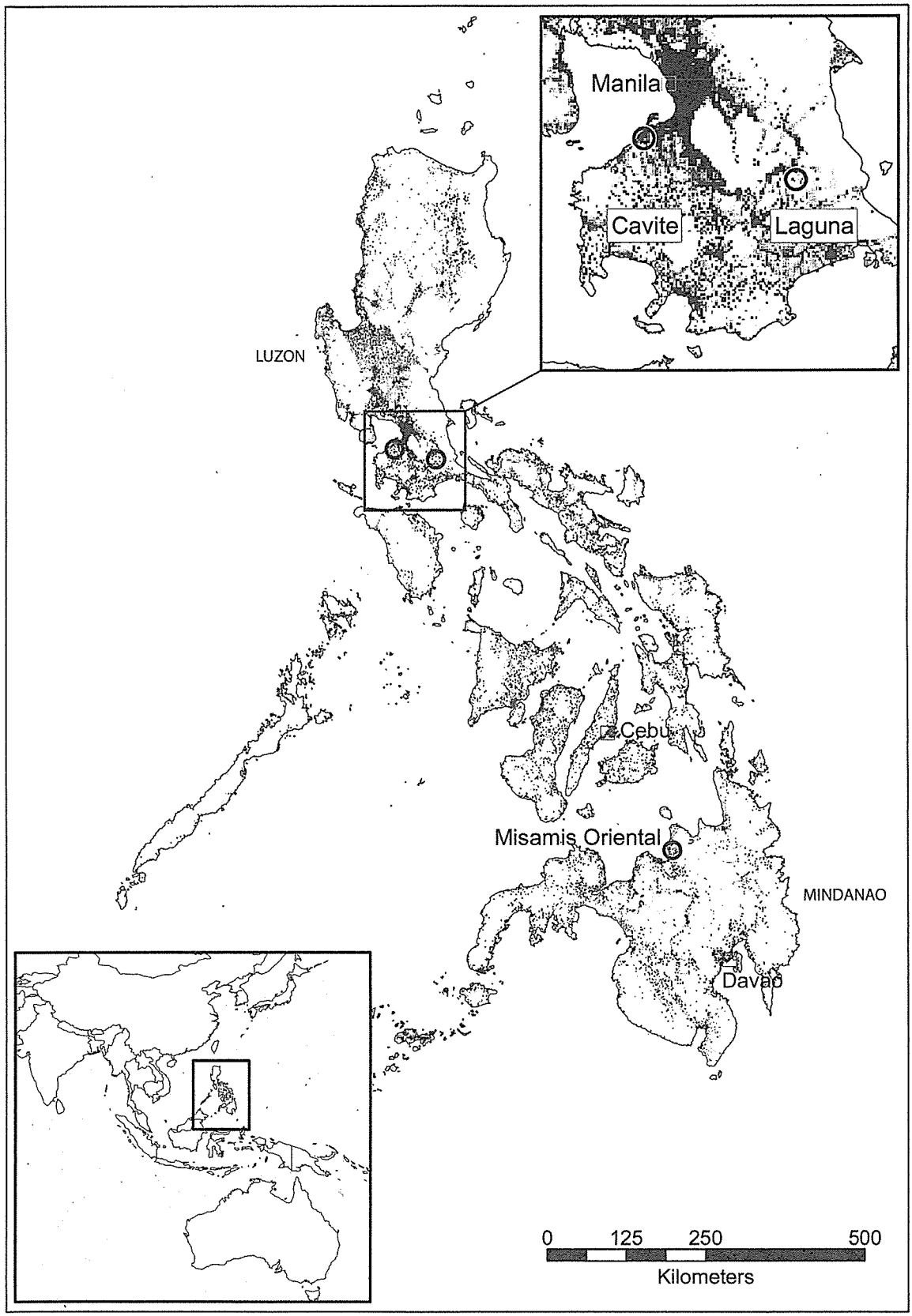

FIG. 1. Distribution of acute flaccid paralysis cases associated with type 1 cVDPVs in the Philippines in 2001. Three acute flaccid paralysis cases, in Misamis Oriental (isolate Mindanao-01-1), in Laguna (isolate Luzon-01-1), and in Cavite (isolate Luzon-01-2), are mapped by open circles. Isolate Luzon-01-2c was from a contact of the Cavite case. Shading indicates approximate population densities.

TABLE 1. Acute Flaccid paralysis and contact cases associated with type 1 cVDPV in the Philippines

Virus[a]	Area/province	Age (yr)/sex	Source[b]	No. of OPV doses	Date of onset	Date of sampling	Residual paralysis
Mindanao-01-1	Mindanao/Misamis Oriental	8/M	AFP	3	15 March 2001	28 March 2001	No
Luzon-01-1	Luzon/Laguna	3/F	AFP; aseptic meningitis	3	21 July 2001	28 July 2001	No
Luzon-01-2	Luzon/Cavite	1/M[c]	AFP	2	26 July 2001	5 August 2001	Yes
Luzon-01-2c	Luzon/Cavite	3/F	Contact	Not available	Not applicable	23 September 2001	No

[a] Designations indicate geographic origin of specimen–year of isolation–case number and if the isolate was obtained from a contact (c).
[b] AFP, acute flaccid paralysis.
[c] 14-month-old child.

second case), who had a history of two doses of OPV and onset of paralysis on 26 July 2001. Only the third case had residual paralysis after 60 days from the date of onset of symptoms (Table 1). None of the patients had any history of travel outside of their resident areas since their birth, and no direct epidemiological link has been found among the three acute flaccid paralysis cases. A stool sample was collected from a healthy contact of patient 3 in Cavite on 23 September 2001.

To increase population immunity against polio and to interrupt cVDPV circulation, the Department of Health of the Philippines conducted local and nationwide OPV campaigns from December 2001 to March 2002 (50, 62). Consequently, no cVDPV has been identified in the Philippines since October 2001 under the intensified field and laboratory surveillance activities.

Virus isolation and identification. The isolate from the first acute flaccid paralysis case, Mindanao-01-1, was identified as type 1 poliovirus by microneutralization. Probe hybridization and PCR identified the isolate as Sabin 1-like, but it was found to have non-vaccine-like antigenicity by intratypic differentiation-ELISA (Table 2). Nucleotide sequence analysis of the VP1 region revealed that the isolate was 3.1% divergent from the parental Sabin 1 strain, and it was thus classified as a VDPV (Table 2).

In response to the detection of a VDPV in Mindanao, surveillance for acute flaccid paralysis cases was intensified in the affected community and in other parts of the Philippines. Thirty-four poliovirus isolates were identified from 22 acute flaccid paralysis cases between March 2001 and December 2003. All isolates were determined to have vaccine-like genetic properties by nucleic acid probe hybridization or PCR. All but six of the poliovirus isolates tested had vaccine-like antigenic properties by intratypic differentiation-ELISA. Three type 1 isolates (Luzon-01-1 and Luzon-01-2 from acute flaccid paralysis cases and Luzon-01-2c from a contact of an acute flaccid paralysis case in Cavite) were identified from different patients in 2001 and resembled Mindanao-01-1 in having non-vaccine-like antigenic reactivity (Tables 1 and 2). Consequently, the four type 1 isolates with non-vaccine-like antigenic properties were analyzed further. A double reactive result by intratypic differentiation-ELISA occurred with two poliovirus type 1 isolates in 2002 and one type 3 isolate in 2003. However, sequencing subsequently determined that the type 1 isolates had <0.4% nucleotide variation from the VP1 sequence of Sabin 1, with no evidence of recombination in the noncapsid region (data not shown). Based upon the sequencing results, these isolates were identified as Sabin-like.

Nucleotide sequence analysis. The four type 1 isolates with non-Sabin-like antigenic reactivity by intratypic differentiation-ELISA were initially characterized by analysis of their VP1 nucleotide sequences. The four isolates were found to be closely related to Sabin 1, differing by 3.1% to 3.5% of VP1 nucleotides (Table 2), indicating that all were VDPVs. The Philippines cVDPVs were unrelated (<82% VP1 nucleotide identity) to the wild type 1 polioviruses previously endemic in the Philippines (PJ5-92, PJ7-92, and 5380-93) (26, 50), to those in neighboring countries (11, 23, 31, 67), or those circulating until recently in other parts of the world (Fig. 2) (25, 29). When the VP1 nucleotide sequence relationships were summarized on a phylogenetic tree, the Philippines isolates clustered with Sabin 1 and a representative isolate (HAI01-007) from the Hispaniola cVDPV outbreak (Fig. 2).

More detailed phylogenetic analysis of the complete genomic sequence relationships among the type 1 VDPV isolates revealed that the Philippines and Hispaniola cVDPVs followed independent pathways of divergence from Sabin 1 (Fig. 3A). The cVDPVs from both outbreaks were distinct from a sporadic type 1 VDPV isolated in Russia in 1999 (10). The Philippines cVDPV isolates were much more closely related to each other (99.0% to 99.9% complete genomic nucleotide identity) than were the Hispaniola cVDPV isolates (25) (Fig. 3A). When the GenBank database was screened with the BLAST search software, the virus determined to be most closely related to the Philippines cVDPV isolates was Sabin 1 (data not shown).

Recombination. All of the cVDPV isolates from Hispaniola (type 1), Egypt (type 2), and Madagascar (type 2) had highly evolved capsid sequences as well as recombinant noncapsid sequences (25, 51, 66). Although the exact donors of the noncapsid sequences of the cVDPV isolates were not identified, their noncapsid sequences are considered to be derived from some species C enteroviruses (see Discussion).

Sequencing of the full-length genomes of the four Philippines cVDPV isolates revealed that all were recombinants between Sabin 1 and non-Sabin viruses (Table 3). Sequence identities between Sabin 1 and Mindanao-01-1 were >96% in the 5′-NTR, capsid, and 2A regions but <85% in the remain-

TABLE 2. Initial characterization of Philippines type 1 cVDPVs

Virus	Identification by antiserum neutralization[a]	Intratypic differentiation method[b]		Nucleotide diversity from Sabin 1 (%)
		Nucleic acid probe hybridization and/or PCR	ELISA	
Mindanao-01-1	P1	SL	NSL	3.1
Luzon-01-1	P1	SL	NSL	3.4
Luzon-01-2	P1	SL	NSL	3.1
Luzon-01-2c	P1	SL	NSL	3.5

[a] P1, poliovirus type 1.
[b] SL, Sabin-like; NSL, non-Sabin-like.

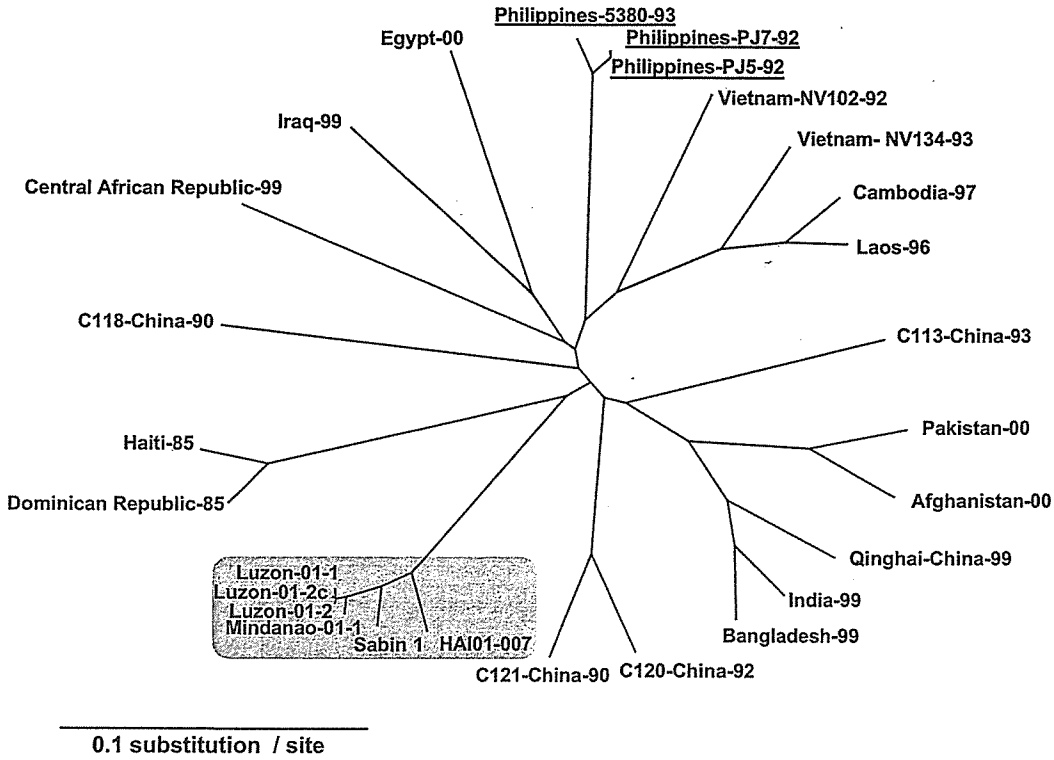

FIG. 2. Phylogenetic analysis of type 1 cVDPVs and the wild type 1 polioviruses based on VP1 nucleotide sequences. The unrooted radial neighbor-joining tree was drawn with the TreeView software. Type 1 cVDPVs (four from the Philippines and one from Haiti, HAI01-007) and Sabin 1 are shaded. Three type 1 wild polioviruses from the Philippines are underlined.

ing noncapsid regions. The other cVDPV isolates showed very similar sequence differences from Sabin 1, and the four shared ≈99% nucleotide sequence identities with each other along their entire genomes, indicative of circulation within the Philippines (Table 3). Furthermore, the four isolates had a common recombination site located in the middle of the 2B region (between nucleotides 3927 and 3949; Fig. 4).

The very high degree of noncapsid sequence identity among the Philippines cVDPV isolates contrasted with the cVDPV isolates from Hispaniola (25) and Egypt (66), in which heterogeneous recombinant noncapsid sequences had been generated by successive recombination events. The noncapsid sequences of the Philippines isolates were generally related to (i) other polioviruses (the three Sabin strains and the Hispaniola isolates with different noncapsid sequences) and (ii) species C enteroviruses (e.g., coxsackievirus types A11, A17, and A20) (Table 3), but no sequence with >95% homology was found in GenBank (data not shown). This result suggests that the recombinant noncapsid sequences might be classified into a major species C enterovirus phylogeny, including all polioviruses and many species C enteroviruses, as recently reported by Brown et al. for the reference species C enterovirus strains (6).

Estimated time of initiating OPV dose. The first cVDPV isolate (Mindanao-01-1, isolated on 28 March 2001) was more closely related to Sabin 1 (96.9% VP1 nucleotide identity) than the last isolate (Luzon-01-2c, isolated on 23 September 2001) (96.5% VP1 nucleotide identity), consistent with a pattern of increasing divergence from Sabin 1 over time (Fig. 3B). Most (≈70%) of the genetic changes in VP1 were substitutions at synonymous sites (data not shown). If one assumes that the rate of VP1 sequence divergence from Sabin 1 was 0.03 substitution per synonymous site per year (17, 25, 27, 35, 37), then we can estimate that the initiating OPV dose was given in early to mid-1999. We further estimate that the four cVDPV isolates were derived from a common ancestral infection that occurred in mid- to late 2000. Such a relationship is evident from the high overall sequence similarity among the four Philippines cVDPV isolates (Fig. 2 and Table 3) and the topology of the tree shown in Fig. 3B.

The Sabin 1-derived genomic sequence of the common ancestral virus is represented by the node joining the sequence of Mindanao-01-1 to the three Luzon isolate sequences (Fig. 3B). The terminal branches extending from that node were shorter than the branch extending back to the Sabin 1 sequence, again suggesting that divergence of the observed cVDPV lineages occurred 1 to 2 years after the initiating OPV dose. The estimated date is based on only four isolates identified over a 6-month period. The earliest isolate was from a March 2001 case which occurred about 2 years after the initiating OPV dose. A more accurate determination could have been made if a greater number of cVDPV isolates had been identified over a longer period.

Genetic properties of the cVDPV isolates. The complete genomic sequences of the four Philippines cVDPVs were compared with those of Sabin 1 and its neurovirulent parental strain, Mahoney (45). The four cVDPV isolates shared a G-to-A nucleotide substitution at 480 in the domain V of the 5′-NTR, which represents the reversion of a major attenuation determinant of the Sabin 1 OPV strain (Table 4). Two other substitutions at positions 7410 and 7441 of the 3′-NTR distin-

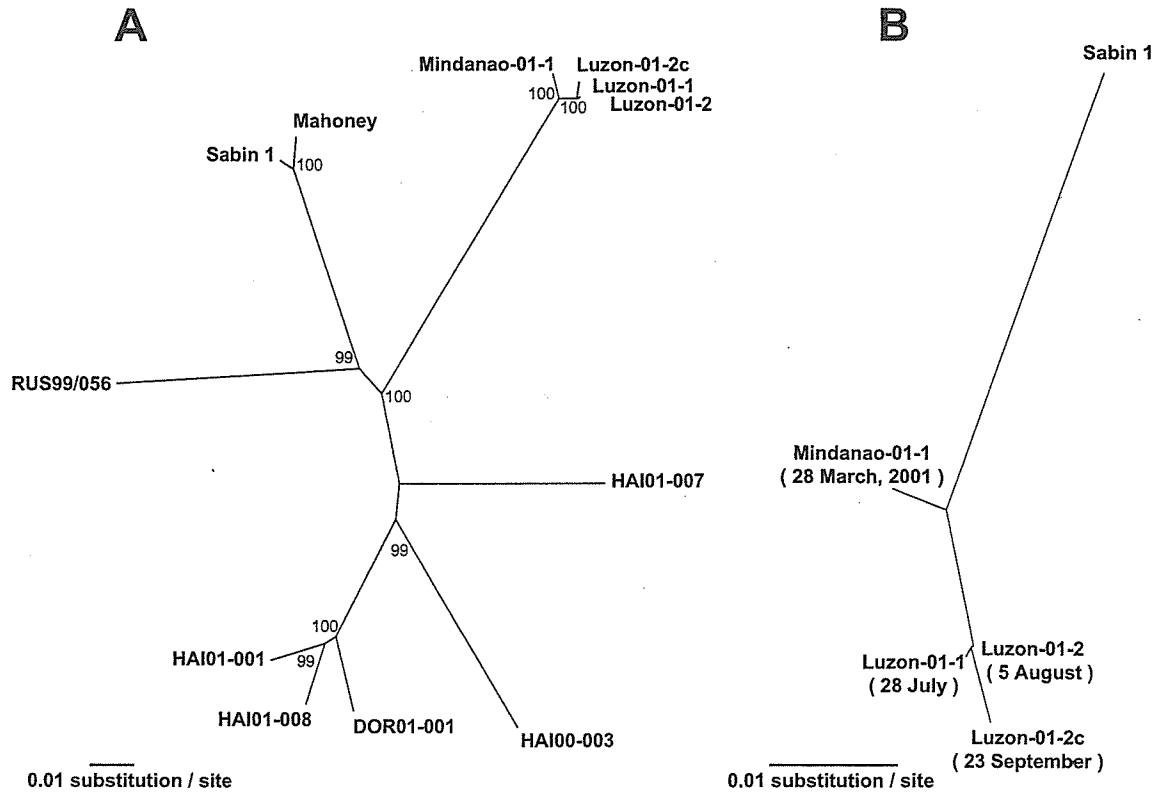

FIG. 3. Phylogenetic analysis of type 1 VDPV isolates. (A) Complete genomic sequence relationships among type 1 VDPV isolates. cVDPV isolates are from the Philippines, Haiti (HAI), and Dominican Republic (DOR) (25). RUS99-056 is a type 1 VDPV from a sporadic acute flaccid paralysis case in Russia (10). Bootstrap values of >80% for each cluster are shown at the branch nodes. (B) Sequence relationships among Philippines type 1 cVDPV isolates based on the nucleotide sequences derived from Sabin 1 (5′-NTR, capsid, and 2A regions, nucleotides 1 to 3927).

guished the cVDPV isolates and Mahoney from Sabin 1. The cVDPV isolates also differed from Sabin 1 at nine amino acid reversions to the parental Mahoney strain (Table 4). Among three amino acid reversions in the capsid proteins, two amino acid changes in VP1 (VP1-99 and VP1-106) were located within neutralization antigenic site 1. Four amino acid differences were mapped in the $3D^{pol}$ region (3D residues 53, 73, 250, and 362). These reversions in $3D^{pol}$ were introduced by recombination and were similar to the amino acid changes found at equivalent positions among the four different recombinant $3D^{pol}$ sequences in the Hispaniola cVDPVs despite the highly diverse nucleotide sequences (Tables 3 and 4) (25). Some of these substitutions, alone or in combination, have been found to contribute to the temperature sensitivity and/or attenua-

TABLE 3. Comparison of the entire genome of Mindanao-01-1 to that of other VDPVs and reference enterovirus strains

Virus	GenBank accession no.	Nucleotide sequence identity to Mindanao-01-1[a] (%)												
		5′-NTR	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D	3′-NTR
Luzon-01-1	AB180071	99.1	99.5	98.9	99.4	98.8	98.4	99.7	99.1	99.6	100	99.6	99.4	100
Luzon-01-2	AB180072	99.1	99.5	98.9	99.4	99.1	98.4	99.7	99.1	100	100	99.6	99.4	100
Luzon-01-2c	AB180073	98.7	99.5	98.5	99.4	98.7	97.8	99.3	99.0	99.6	100	99.5	99.2	100
Sabin 1	V01150	97.7	96.6	98.4	98.2	96.9	97.1	84.2	82.1	75.9	80.3	80.0	84.1	97.2
Sabin 2	X00595	85.2	78.3	72.8	75.5	68.1	82.1	77.7	80.9	75.9	74.2	80.3	83.7	97.2
Sabin 3	K00043	81.7	78.3	71.2	73.6	68.2	80.3	75.6	78.9	73.2	81.8	80.9	84.0	98.6
DOR00-013	AF405690	96.5	94.2	95.8	96.4	95.3	93.3	77.3	79.9	77.4	81.8	79.4	85.4	98.6
DOR00-041c1	AF405682	96.0	92.8	95.5	95.9	95.4	93.1	77.0	79.8	77.4	81.8	79.2	84.5	100
HAI 01-003	AF405669	95.8	96.1	95.8	96.8	94.6	94.6	77.0	78.9	75.5	77.3	79.6	83.7	100
HAI 01-007	AF405666	96.4	94.7	95.0	95.5	94.3	93.7	79.0	80.7	77.0	75.8	79.4	84.2	100
CAV11	AF499635	87.2	75.9	69.0	69.2	64.3	77.4	81.8	78.7	76.6	75.8	79.6	84.9	97.2
CAV17	AF499638	85.8	73.4	69.6	73.0	67.6	80.8	75.9	79.4	77.0	83.3	81.2	85.5	98.6
CAV20	AF499642	88.4	68.6	69.1	71.8	66.9	79.6	79.3	80.5	77.8	77.3	81.6	84.1	98.6

[a] GenBank accession no. AB180070.

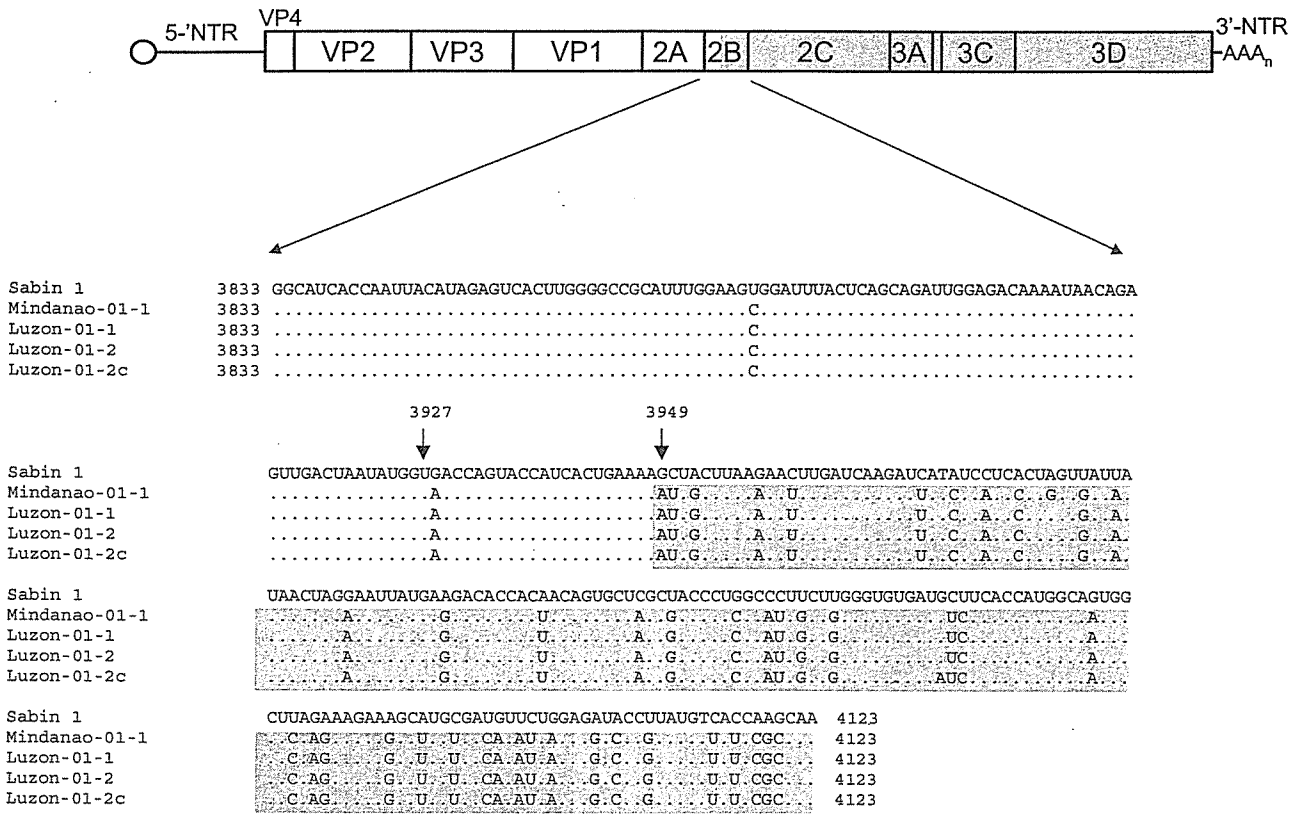

FIG. 4. Putative recombination crossover site in the 2B noncapsid region of the Philippines type 1 cVDPVs. (A) Schematic diagram of the poliovirus genome. (B) Nucleotide sequence alignment. Sequences derived from Sabin 1 are unshaded, while those derived from the unidentified species C enterovirus are shaded.

tion phenotypes of the Sabin 1 strain (5, 12, 19, 20, 40, 48, 59, 61).

Biological properties of the cVDPV isolates. The neurovirulence of the cVDPV isolates in the Philippines was evaluated by intracerebral inoculation of ICR-PVR-Tg21 mice carrying the human poliovirus receptor gene. The Mindanao-01-1 and Luzon-01-2 isolates showed potent neurovirulence (PD_{50} = 2.4 and 2.6 cell culture infective dose per mouse, respectively) that was comparable to that of the virulent Mahoney strain (PD_{50} = 2.6 cell culture infective dose per mouse) (Table 4). By contrast, the Luzon-01-2c strain, which was isolated from a contact of the second case in Luzon (Luzon-01-2), exhibited only moderate attenuation or neurovirulence in ICR-PVR-Tg21 mice (PD_{50} = 4.9 cell culture infective dose per mouse).

The temperature-sensitive phenotype of impaired virus replication at supraoptimal temperatures is a shared characteristic of all three serotypes of the OPV strains. We evaluated the temperature sensitivity of the Philippines type 1 cVDPV isolates by comparing virus yields in single-step growth experiments at 39.5°C in HeLa S3 cells (66). Three cVDPV isolates

TABLE 4. Genetic and phenotypic characterizations of type 1 cVDPV in the Philippines

Virus	Nucleotide and amino acid reversions[a]														Neutralization titer			Neurovirulence[c] (PD_{50})
	5'-NTR at nt:			Capsid			Nonstructural proteins						3'-NTR at nt:					
	26	355	480	VP4-65	VP1-99[b]	VP1-106[b]	2A-134	2B-95	3D-53	3D-73	3D-250	3D-362	7410	7441	8a034	8a057	11m071	
Sabin 1	G	T	G	S	K	T	T	T	N	H	E	I	C	G	102,400	>64,000	<20	>8.0
Mindanao-01-1	A	C	A	A	T	A	S	I	D	Y	K	T	U	A	<20	673	905	2.4
Luzon-01-1	A	C	A	A	T	A	S	I	D	Y	K	T	U	A	<20	800	1,810	ND
Luzon-01-2	A	C	A	A	T	A	S	I	D	Y	K	T	U	A	<20	336	1,076	2.6
Luzon-01-2c	A	C	A	A	T	A	S	I	D	Y	K	T	U	A	ND	ND	ND	4.9
Mahoney	A	C	A	A	T	A	S	I	D	Y	K	T	U	A	<20	450	160	2.6
HAI00-003[d]	A	C	A	A	T	A	A	I	D	Y	K	T	U	A	ND	ND	ND	2.8

[a] Nucleotide and amino acid reversions from the Sabin 1 (Genbank accession no. V01150) to Mahoney (VO1149; the neurovirulent parent of Sabin 1) sequences are indicated. Nucleotide substitutions are given only for the 5'- and 3'-NTRs.

[b] Surface residues forming part of neutralizing antigenic site 1.

[c] ND, not determined.

[d] A representative type 1 cVDPV strain from Hispaniola. Its PD_{50} value is quoted from a previous report (25).

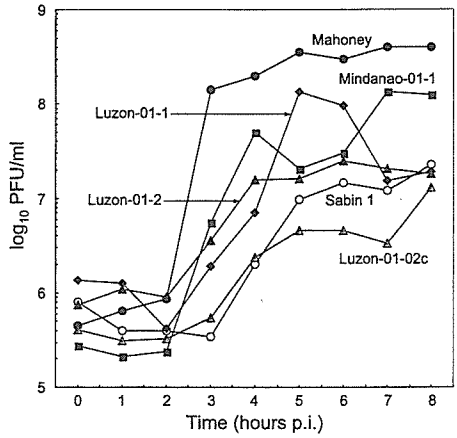

FIG. 5. Single-step growth curves of type 1 Philippines cVDPV isolates, Mahoney, and Sabin 1 strains at 39.5°C in HeLa S3 cells.

(Mindanao-01-1, Luzon-01-1, and Luzon-01-2) exhibited moderate temperature sensitivity with respect to the growth kinetics of temperature-resistant (Mahoney) and temperature-sensitive (Sabin 1) type 1 reference strains, suggesting that they had partially lost the temperature-sensitive phenotype (Fig. 5). The exception was again the last cVDPV isolate, Luzon-01-2c, which had regained the temperature-sensitive phenotype for growth at 39.5°C in HeLa S3 cells (Fig. 5). Both the low neurovirulence in ICR-PVR-Tg21 mice and the temperature sensitivity of Luzon-01-2c were surprising because its nucleotide and amino acid sequences at key sites were identical to those of the other three cVDPV isolates from acute flaccid paralysis cases (Table 4).

The neutralization antigenicities of the Philippines cVDPV isolates were analyzed in neutralization assays with three monoclonal antibodies specific to either Sabin 1 or Mahoney (24). As anticipated from the non-Sabin-like reactivity in the ELISA-intratypic differentiation assay (Table 2), the cVDPV isolates were not neutralized or very weakly neutralized by the Sabin 1-specific monoclonal antibodies (8a304 and 8a057) (Table 4). In a similar manner, the same cVDPVs were efficiently neutralized by a monoclonal antibody specific for the Mahoney strain, 11m071. Amino acid reversions in the capsid proteins (VP1-99 and VP1-106) and/or some additional amino acid substitutions (VP1-66, VP1-90, etc.; data not shown) might be responsible for the altered neutralization antigenicity of the cVDPV isolates. Such amino acid changes have frequently been found in many Sabin 1-derived polioviruses isolated from OPV recipients, vaccine-associated paralytic poliomyelitis patients, and the environment (18, 39, 43).

DISCUSSION

The recent cVDPV outbreak in the Philippines has important implications for the global initiative to eradicate polio. First, it demonstrates that use of OPV with suboptimal coverage rates can lead to the emergence and spread of cVDPVs even in countries where indigenous wild polioviruses have already been eradicated. Second, it again shows that use of OPV at high rates of coverage can prevent further spread of cVDPVs. Third, it reaffirms the importance of maintaining sensitive acute flaccid paralysis and poliovirus surveillance in both polio-free and polio-endemic countries. Finally, it provides additional insights into the conditions permissive for cVDPV emergence and the biological and genetic properties of the emergent viruses.

The cVDPV outbreak in the Philippines differed in key respects from earlier outbreaks reported in Egypt (66) and Haiti in Hispaniola (25) and the subsequent outbreak in Madagascar (51). In the other outbreak countries, OPV coverage rates were particularly low (<50%) in the affected communities and generally low nationwide. Moreover, nearly all of the case patients in the other outbreaks were unimmunized or incompletely immunized children (25, 51, 66). By contrast, nationwide rates of routine coverage with three doses of OPV were reported to have been approximately 80% in the Philippines since the early 1990s (50, 62), and two of the case patients had received three doses of OPV and the third patient had received two doses (Table 1). However, gaps in population immunity probably occurred after 1997, when the mass OPV campaigns in the form of national immunization days were last conducted in the Philippines. Subnational immunization days that covered the urban areas of Manila, Cebu, and Davao (Mindanao) followed in 1998 and 1999 but did not include the three provinces with cVDPV cases (Fig. 1) (62).

It is likely that gaps in OPV coverage developed most rapidly in the slum areas, such as those around metropolitan Manila, and these gaps were aggravated by a temporary shortage of OPV supply in 2000 to 2001. The widening immunity gap, coupled with very high population densities (especially around metropolitan Manila; Fig. 1), poor hygiene or sanitation, and tropical conditions may have established local conditions favoring cVDPV emergence. Once poliovirus circulation starts, three prior OPV doses may not be enough to protect all children from poliomyelitis, particularly in high-risk communities (55). However, overall population immunity appears to have been sufficiently high to restrict cVDPV transmission to a minimally branched chain, in contrast to the pattern of multichain transmission seen in Egypt and Hispaniola (25, 66). The important lesson from the Philippines outbreak is that cVDPVs can emerge even in countries with good rates of OPV coverage nationwide if immunity gaps develop in local areas at highest potential risk for poliovirus circulation.

The detection of the cVDPVs in the Philippines highlights the significant role of poliovirus surveillance in the final stages of global polio eradication. Immediately following the cVDPV outbreak in Hispaniola, intensive screening of cVDPVs was initiated by laboratories within the entire World Health Organization Global Polio Laboratory Network (8, 9). Vaccine-related poliovirus isolates are identified by genetic methods, such as probe hybridization (14), and also characterized for evidence of antigenic divergence from the prototype OPV strains by antigenic tests, such as intratypic differentiation-ELISA (8, 9, 60). The likelihood of antigenic divergence increases with the duration of replication of OPV strains in the human gut (41), and all documented cVDPV isolates have been antigenic variants of the OPV strains (25, 51, 66). Vaccine-related isolates having altered antigenic properties are candidate VDPVs and are characterized further by genomic sequencing. In addition, the World Health Organization is promptly notified of the virologic findings in order to accelerate active surveillance and to prepare for any necessary

supplementary immunization campaigns, as described here for the Philippines (8, 9).

The Philippines cVDPV isolates, as with the other cVDPV isolates described so far (25, 51, 66), have recombinant noncapsid sequences derived from other species C enteroviruses (6). Since OPV contains all three serotypes of the Sabin strains, a recombinant poliovirus among heterogeneous strains readily emerges during virus replication in the gut of vaccinees. Nevertheless, recombination among the vaccine strains is known to occur frequently with serotypes 2 and 3 but rarely with type 1 (4, 7, 13, 16, 21, 22, 38). On the other hand, circulating wild polioviruses with a block of sequence derived from Sabin 1 have been described (34, 35). It appears most likely that the donor of the noncapsid sequences to the Philippines type 1 cVDPV isolates was nonpolio enteroviruses, as the sensitive surveillance scheme for cases of acute flaccid paralysis maintained in the Philippines has not detected any indigenous or imported wild polioviruses since 1993. Although the apparent donor of the recombinant noncapsid sequences of cVDPVs has not been identified, growing evidences indicate frequent recombination between polioviruses and species C nonpolio enteroviruses (6, 22, 25, 28, 30, 34, 35, 51, 66) as well as between serotypes within the same nonpolio enterovirus species (33, 36, 46, 47, 53). Further epidemiological studies of species C nonpolio enteroviruses, especially in tropical areas, are needed to understand the conditions favorable for cVDPV recombination.

Although recombination with other enteroviruses appears to be an indicator of poliovirus circulation (25), the possible role of recombination in the phenotypic reversion of OPV is less clear. Genetic determinants of attenuation and temperature sensitivity in Sabin 1 (but not in Sabin 2 and 3) are mapped in the $3D^{pol}$ noncapsid region (5, 12, 20, 59), so that recombination may be an efficient mechanism to replace these mutations with consensus wild enterovirus sequences. However, the major determinants of attenuation in Sabin 1 map to the 5′-NTR and capsid regions, which were not replaced by recombination in either the Philippines or Hispaniola cVDPVs.

The partially attenuated and temperature-sensitive phenotypes of the most recently identified cVDPV isolate, Luzon-01-2c, from a healthy contact child, were unexpected in view of the close sequence relationship of the contact isolate to the other three Philippines cVDPV isolates that had biological properties similar to those of wild type 1 polioviruses. The temporal and phylogenetic relationships among the Philippines cVDPV isolates suggest that isolate Luzon-01-2c was derived from a more neurovirulent and less temperature sensitive progenitor, raising the possibility that reversion of the attenuated and temperature-sensitive phenotypes of Sabin 1 is not necessarily irreversible during cVDPV evolution. Although the Luzon-01-2c isolate has the same recombinant properties as the other cVDPV isolates, it does differ from the other three isolates at some specific nucleotide and amino acid substitutions (644, 667, and 720 in the 5′-NTR, VP1-224, 2A-101, 2A-129, 2B-75, and 2C-94). Further virologic, epidemiologic, and reverse genetic studies are needed to understand the role of mutation and recombination in poliovirus evolution and cVDPV emergence.

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Characterization of *in vitro* and *in vivo* phenotypes of poliovirus type 1 mutants with reduced viral protein synthesis activity

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Sabin vaccine strains of poliovirus (PV) contain major attenuation determinants in the internal ribosomal entry site (IRES), an area that directs viral protein synthesis. To examine the effect of reduced viral protein synthesis on PV neurovirulence, spacer sequences, consisting of short open reading frames of different lengths, were introduced between the IRES and the initiation codon of viral polyprotein, resulting in PV mutants with reduced viral protein synthesis. These PV mutants had a viral protein synthesis activity 8·8–55 % of that of the parental Mahoney strain as measured in HeLa S3 cells. Only viruses with more than 28 % of the wild-type activity had intact spacer sequences following plaque purification. Mutants with 17 % or 21 % of the wild-type activity were unstable and a mutant with 8·8 % was lethal. The neurovirulence of PV mutants was evaluated in transgenic mice carrying the human PV receptor gene. In this test, mutants with more than 28 % of the wild-type activity remained neurovirulent, while a mutant with 17 % of wild-type activity exhibited a partially attenuated phenotype. This mutant stably replicated in the spinal cord; however, the stability was severely affected during the course of virus infection from the cerebrum to the spinal cord. These results suggest that reduced viral protein synthesis activity as measured in cultured cells (17–55 % of the wild-type activity) is not the main determinant of PV attenuation.

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INTRODUCTION

Poliovirus (PV) is a small non-enveloped virus belonging to the family *Picornaviridae* with a single positive-stranded genomic RNA; it is known as the causative agent of poliomyelitis. The PV genome is about 7500 nucleotides (nt) with a 5′ non-translated region (nt 1–742 in the Mahoney strain) including a cloverleaf structure (Andino *et al.*, 1990) and an internal ribosomal entry site (IRES) (Jang *et al.*, 1988; Pelletier & Sonenberg, 1988). Motor neurons are the target of PV in the central nervous system (CNS). The tropism of PV infection to target cells in the CNS is, in part, attributable to the specific expression of the PV receptor in neurons (Koike *et al.*, 1994; Ren & Racaniello, 1992).

The effect of viral protein synthesis on PV neurovirulence has mainly been studied on live attenuated strains of PV (Sabin 1, 2 and 3) that are widely used as oral PV vaccines (Sabin, 1965). Attenuation determinants of Sabin strains have been defined throughout the virus genome in detail (reviewed by Minor, 1992), including mutations of the 5′ non-translated region, and the coding region of the capsid proteins and polymerase (Bouchard *et al.*, 1995; Cann *et al.*, 1984; Horie *et al.*, 1994; Macadam *et al.*, 1993; Omata *et al.*, 1986). Among these attenuation determinants, a major determinant is mapped on stem–loop V (nt 448–556) of the IRES element in all Sabin strains (nt 480 in Sabin 1, nt 481 in Sabin 2 and nt 472 in Sabin 3) (Cann *et al.*, 1984; Evans *et al.*, 1985; Kawamura *et al.*, 1989; Macadam *et al.*, 1991). The introduction of these attenuation determinants into the wild-type IRES element resulted in a decrease in *in vitro* viral protein synthesis activity (Muzychenko *et al.*, 1991; Svitkin *et al.*, 1985, 1990), possibly due to destabilization of the stem–loop V structure and/or that of the entire IRES element (Macadam *et al.*, 1994; Malnou *et al.*, 2002; Rowe *et al.*, 2001). Furthermore, the introduction of attenuation determinants into the IRES also caused a cell type-specific decrease in IRES activity in a neuroblastoma cell line or cell lysate compared with the parental IRES activity (Gutierrez *et al.*, 1997; Haller *et al.*, 1996). To date, and pioneered by the study of Svitkin *et al.*, Sabin IRES activities have been found to be 12–67 % of the parental or virulent revertant IRES activity (Gutierrez *et al.*, 1997; Haller *et al.*, 1996; Muzychenko *et al.*, 1991; Svitkin *et al.*, 1985, 1990). A study on the neurovirulence of Theiler's murine encephalomyelitis virus (GDVII strain) showed that a putative host factor (neural-specific homologue of

Results of the measurement of protein synthesis activity directed by the IRES mutants in SK-N-MC cells are available in JGV Online.

pyrimidine tract-binding protein) affects both the viral protein synthesis and neurovirulence (Pilipenko *et al.*, 2001).

Genetically manipulated PV IRES mutants and their revertant viruses have been used to analyse the role of PV IRES, mainly focusing on stem–loops II and V, and the oligopyrimidine/cryptic AUG motif (Haller *et al.*, 1996; Iizuka *et al.*, 1989; Shiroki *et al.*, 1997; Slobodskaya *et al.*, 1996). The stem–loop V structure was found to be involved in a neuronal cell-specific IRES activity and in virus release from cells (Haller *et al.*, 1996; Stewart & Semler, 1999), and the importance of the cryptic AUG (nt 586 to 588 in the Mahoney strain) in neurovirulence has been reported (Iizuka *et al.*, 1989; Slobodskaya *et al.*, 1996). The stem–loop II structure has a role in PV host-range phenotype by modulating viral protein synthesis (Shiroki *et al.*, 1997). A *cis*-element for replication (Borman *et al.*, 1994; Shiroki *et al.*, 1993, 1995) and an encapsidation signal (Johansen & Morrow, 2000) have been suggested to exist in the IRES element. These observations suggest that a small structural disturbance in the 5′ non-translated region of the PV genome could have pleiotropic effects on the virus life cycle.

In this study, we examined the significance of viral protein synthesis on type 1 PV neurovirulence. We constructed PV mutants with reduced viral protein synthesis activity in HeLa S3 cells, and examined their neurovirulence in transgenic mice expressing the human PV receptor (hPVR).

METHODS

Cells and viruses. HEp-2c and HeLa S3 cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum (FCS). Viruses were prepared in HEp-2c cells incubated at 35 °C by RNA transfection of transcripts synthesized from corresponding infectious clones by the DEAE–dextran method, until all the cells showed a cytopathic effect (CPE) (Lu *et al.*, 1995). Virus stocks were stored at −70 °C.

General methods of molecular cloning. *Escherichia coli* strain XL10gold (Stratagene) was used for plasmid transformation and propagation. DNA fragments were ligated using a Quick Ligation kit (NEB). Site-directed mutagenesis (SDM) was performed by PCR following a standard procedure using cloned *Pfu* DNA polymerase (Stratagene) or KOD plus DNA polymerase (TOYOBO) (Sambrook & Russell, 2001). The Titan one-tube RT-PCR system (Roche) was used for RT-PCR. PCR products were purified using a QIAquick PCR Purification kit (QIAGEN). Samples for sequencing were prepared using a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (ABI) and analysed using the ABI PRIZM 310 Genetic Analyser (ABI). Primers used for DNA construction are listed in Table 1.

Construction of dicistronic replicons. Firefly luciferase (Fluc) cDNA was obtained from plasmid $(Gtx133\text{-}141)_{10}(SI)_9b/RPh$ by PCR amplification using primers Fluc-H(+) and Fluc-SacI-T(−) (Chappell *et al.*, 2000). The DNA fragments encoding the PV IRES site were obtained by PCR amplification using primers PV IRES-SalI-H(+) and PV IRES-Fluc-T(−) with pT7PV1M (a generous gift from Dr E. Wimmer), an infectious clone of type 1 Mahoney strain, as the template. Next, these DNA fragments were fused together and reamplified by PCR with primers PV IRES-SalI-H(+) and Fluc-SacI-T(−). The resulting DNA fragment was digested with *Sal*I and *Sac*I, and then cloned into the HRPF-luc vector (Zhao & Wimmer, 2001). Next, the *Renilla* luciferase (Rluc) DNA fragment was obtained by PCR amplification using primers EcoRI-SmaI-Rluc-H(+) and Rluc-SalI(−) with plasmid $(Gtx133\text{-}141)_{10}(SI)_9b/RPh$ as the template. The DNA fragment was digested with *Eco*RI and *Sal*I, and then cloned into the above construct. The resulting dicistronic vector was named (−)IRES-PV dc. *Eco*RI and *Xma*I sites were used for the construction of IRES mutants (Fig. 1A). Next, the spacer sequence was obtained from the pEGFP-C1 vector (Clontech) using EcoRI-EGFP-H(+) and SmaI-EGFP-T(−), digested with *Eco*RI and *Xma*I, and then cloned into (−)IRES-PV dc. The 232 nt spacer sequence or 139 nt spacer sequence contained part of the coding region for enhanced green fluorescence protein (EGFP) (nt 99–327 or nt 99–234, respectively) followed by three nucleotides (AAG) just upstream of the initiation codon of Rluc. The PV IRES sequence obtained by PCR amplification using PV IRES-EcoRI-H(+) and PV IRES-SacI-SmaI-T(−) primers was digested with *Eco*RI and *Mfe*I, and then cloned into the above construct. The resulting dicistronic vector was named PV-232(+6+14aa)-PV dc and had two short open reading frames (sORFs) on the spacer sequence, which were out-of-frame compared to the original EGFP coding. Based on this construct, sORFs were introduced on the spacer sequence by the introduction of an initiation codon (AUG) and a termination codon by SDM. PV-232(−)-PV dc, PV-232(+6aa)-PV dc and PV-232(+6+4aa)-PV dc were obtained by SDM from PV-232(+6+14aa)-PV dc. To construct PV-232(+6aa)-PV dc, successive SDM steps were performed with primer set EGFP3-H(+) and EGFP3-T(−), followed by a second round of SDM using primer set EGFP6-1-H(+) and EGFP6-1-T(−) and the plasmid obtained from the first round of SDM. PV-232(−)-PV dc was obtained from PV-232(+6aa)-PV dc by SDM using EGFP6-2-H(+) and EGFP6-2-T(−) primers. PV-232(+6+4aa)-PV dc was obtained from PV-EGFP(+6aa)-PV dc by SDM using EGFP7-H(+) and EGFP7-T(−) primers. PV-232(+14aa)-PV dc was obtained by successive SDM from PV-232(+6aa)-PV dc using the primer sets AUG(1+)+ and AUG(1+)−, 10AA+ and 10AA−, as well as 14AA+ and 14AA− for each round of SDM. DNA fragments used for the construction of PV-139(−)-PV dc, PV-139(+6aa)-PV dc, PV-139(+14aa)-PV dc and PV-139(+25aa)-PV dc were obtained by PCR amplification using primers PV IRES-EcoRI-H(+) and space107− with PV-232(−)-PV dc, PV-232(+6aa)-PV dc, PV-232(+14aa)-PV dc and PV-139(+25aa) mc as the templates, respectively. These fragments were digested with *Eco*RI and *Xma*I, and then cloned into PV-232(−)-PV dc. For the construction of Mahoney-PV dc and Sabin 1-PV dc, IRES sequences were obtained by PCR or RT-PCR amplification using primers PV110(+) and PV-SmaI(−) with pT7PV1M or the Sabin 1 virus genome as the template, respectively. The DNA fragments were digested with *Eco*RI and *Xma*I, and then cloned into (−)IRES-PV dc.

Construction of monocistronic replicons. Plasmid PVM/Luc, which encodes a monocistronic Fluc PV replicon (Li *et al.*, 2001), was a generous gift from Dr E. Wimmer. Monocistronic replicons for IRES mutants were constructed as outlined below. The Fluc-coding region was obtained by PCR amplification using primers Fluc(+) and Fluc(−) with the PV-232(−)-PV dc as the DNA template. The DNA fragment obtained was digested with *Xma*I and *Sac*I, and then cloned into PV-232(−)-PV dc. The resultant monocistronic replicon was named PV-232(−) mc. For the construction of PV-232(+6aa) mc, PV-232(+6+4aa) mc and PV-232(+14aa) mc, DNA fragments were obtained using primers PV110(+) and RIPO669(−) with PV-232(+6aa)-PV dc, PV-232(+6+4aa)-PV dc and PV-232(+14aa)-PV dc as the templates, respectively. These PCR products were digested with *Eco*RI and *Xma*I, and then cloned into PV-232(−) mc. PV-139(−) mc, PV-139(+6aa) mc and PV-139(+14aa) mc were constructed as described above for

Table 1. Primers used in this study

Name	Sequence of primer (5′→3′)
10AA+	CAAGCTGGCCCTGGAGTTGATCTGCACCAC
10AA−	GTGGTGCAGATCAACTCCAGGGCCAGCTTG
14AA+	CTGGAGTTGGTCTGCACCATAGGCAAGCTG
14AA−	CAGCTTGCCTATGGTGCAGACCAACTCCAG
25+	GTTGGTCTGCACCATACGCAAGCTGCCCG
25−	CGGGCAGCTTGCGTATGGTGCAGACCAAC
AUG(1+)+	CGGCAAGCTGGCCCTGAAGTTC
AUG(1+)−	GAACTTCAGGGCCAGCTTGCCG
EcoRI-EGFP-H(+)	GCCCGAATTCGGGCGAGGGCGATGCCACCTAC
EcoRI-SmaI-Rluc-H(+)	GCATGAATTCATAATGGGAGCTCCCGGGACTTCGAAAGTTTATGATCC
EGFP(+)	CCCGAATTCGGGCGAGGGCG
EGFP3-H(+)	CCCCGACCACTTGAAGCAGCAC
EGFP3-T(−)	GTGCTGCTTCAAGTGGTCGGGG
EGFP6-1-H(+)	CAAGTCCGCCTTGCCCGAAGG
EGFP6-1-T(−)	CCTTCGGGCAAGGCGGACTTG
EGFP6-2-H(+)	GGCGAGGGCGTTGCCACCTAC
EGFP6-2-T(−)	GTAGGTGGCAACGCCCTCGCC
EGFP7-H(+)	GAAGGCTAGGACCATGTGCGCACCATAGTCTTCAAGG
EGFP7-T(−)	CCTTGAAGACTATGGTGCGCACATGGTCCTAGCCTTC
Fluc(+)	CTTCCCCGGGGAAGACGCCAAAAACATAA
Fluc(−)	ATTGGAGCTCCAATTTGGACTTTC
Fluc-H(+)	ATGGAAGACGCCAAAAACATAAAG
Fluc-SacI-T(−)	AATTGGAGCTCCAATTTGGACTTTCCGCCCTTCTTGGCCT
MahSacI+	GTATCATAATGGGAGCTCAGGTTTCATCAC
MahSacI−	GTGATGAAACCTGAGCTCCCATTATGATAC
PV110(+)	GCGTGAATTCACGCACAAAACCAAGTTC
PV740I(−)	ATGGGAGCTCCCATTATGATACAATTGTC
PV83+	ATTGCGGTACCCTTGTACGCCTG
PV IRES-EcoRI-H(+)	GCGTGAATTCACGCACAAAACCAAGTTCAA
PV IRES-Fluc-T(−)	CTTTATGTTTTTGGCGTCTTCCATTATGATACAATTGTCTGATTG
PV IRES-SacI-SmaI-T(−)	AGCTCCCGGGAGCTCCCATTATGATACAATTGTCTGATTG
PV IRES-SalI-H(+)	GCGTGTCGACACGCACAAAACCAAGTTCAATAGA
PV-SmaI(−)	TAACCCCGGGGTTAAAAGTCATTATGATACAATTG
RIPO669(−)	GTAGAACCACCATACGCTCTATTTG
Rluc-SalI-T(−)	ACGCGTCGACTTATTGTTCATTTTTGAGAACTCGCTCAACGAAC
SacI(−)	ATGGGAGCTCCCATCTTGGTCTTGTAGTTG
SacI-PV120+	ACTGAGCTCAGTAACTTAGACGCACAAAAC
SmaI(−)	TAACCCCGGGGTTAAAAGTCATCTTGG
SmaI-EGFP-T(−)	TAACCCCGGGGTTAAAAGTCATCTTGGTCTTGTAGTTGCCGTCGTCCTT-GAAGAAGATGGTGCGCTCCTGGACCTAGCCTTC
spa107-s	ATGGGAGCTCCCATCTTGTGGTCGGGGTAG
space107−	TAACCCCGGGGTTAAAAGTCATCTTGTGGTCGGGGTAGCGG

monocistronic replicons with a 232 nt spacer, but using space107− primer instead of RIPO669(−) primer for the PCR amplification. PV-139(+25aa) mc was obtained by SDM using a primer set 25+ and 25− with PV-232(+14aa) mc as the template.

Construction of infectious clones of PV mutants with reduced viral protein synthesis activity. A *Sac*I site was introduced into pT7PV1M by SDM using primers MahSacI+ and MahSacI−. The resultant construct was named pMah-SacI. For the construction of infectious clones of the 232(−), 232(+6aa), 232(+6+4aa) and 232(+14aa) viruses, a DNA fragment encoding the IRES and a spacer sequence was obtained by PCR amplification using primers PV110(+) and SacI(−), digested with *Age*I and *Sac*I, and then cloned into pMah-SacI. For the construction of infectious clones of the 139(−), 139(+6aa), 139(+6+4aa), 139(+14aa) viruses, a DNA fragment encoding the IRES and spacer sequence was obtained by PCR amplification using primers PV110(+) and spa107-s, digested with *Age*I and *Sac*I, and then cloned into pMah-SacI. The parental Mahoney strain used in this study was prepared from RNA transcripts synthesized from pMah-SacI.

RNA transfection. RNA transcripts were obtained using a RiboMAX Large-Scale RNA Production System T7 kit (Promega) with *Dra*I-linearized DNA as the template. RNA transcripts were

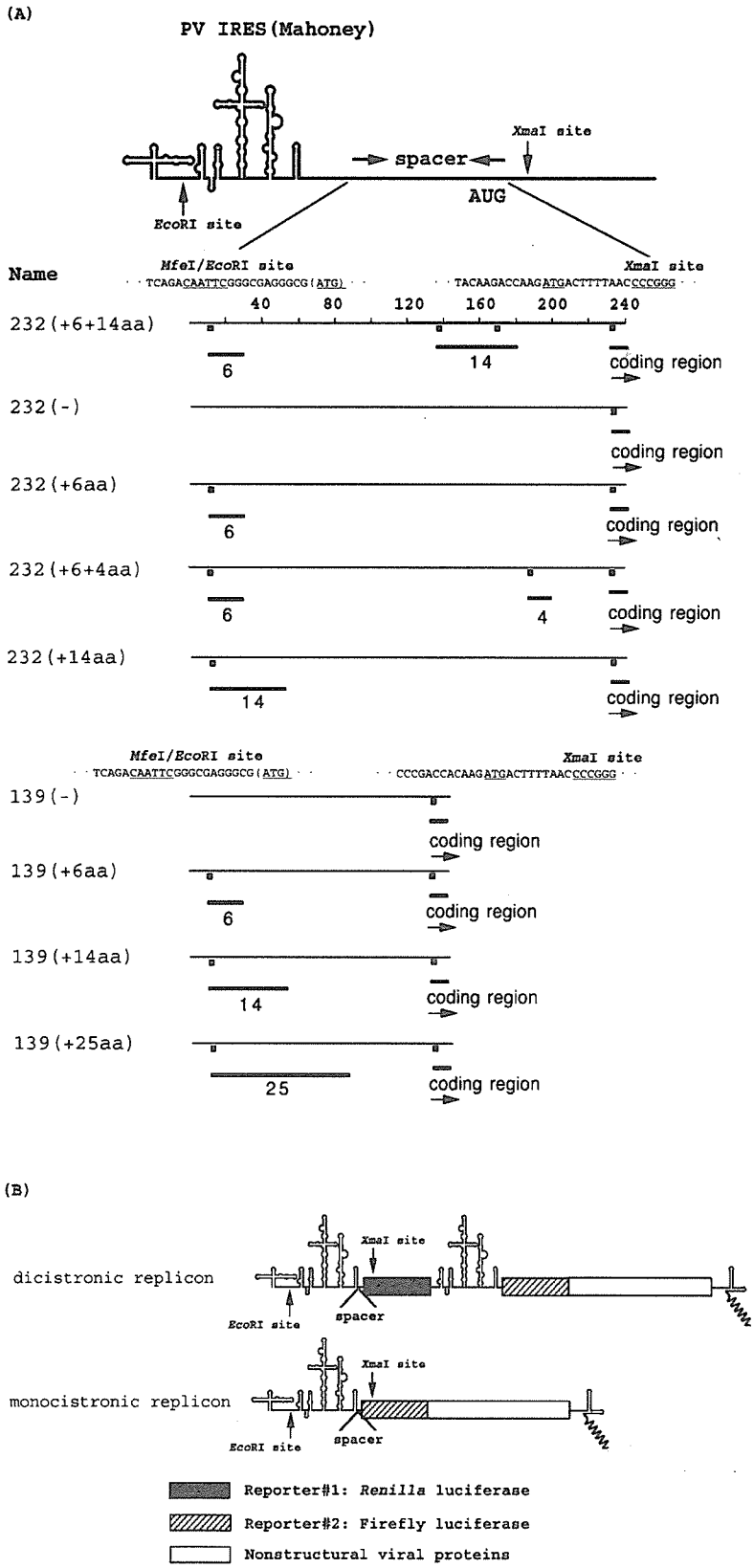

Fig. 1. Schematic view of spacers and luciferase replicons. (A) Schematics of spacer sequences with sORFs and restriction enzyme sites used for construction are shown. Sequences flanking the spacers are shown with initiation codons and restriction sites. Spacers of 232 nt or 139 nt were used with or without sORFs, and the number depicted above the spacer sequence represents the nucleotide number on the spacer sequence and in the coding region. Bars in the spacer represent sORFs and the number below each bar represents the number of amino acid residues they encode. Small closed boxes represent AUG codons on the spacer sequences. (B) Schematics of luciferase replicons.

transfected to a monolayer of HEp-2c or HeLa S3 cells by the DEAE–dextran method (Lu *et al.*, 1995). Confluent (100 %) HEp-2c cells in six-well plates (Falcon) incubated at 35 °C in 2 ml of 5 % FCS/DMEM per well were used for virus preparation. Confluent HeLa S3 cells in 96-well plates (Falcon) incubated at 37 °C in 150 µl of 5 % FCS/DMEM per well were used for the luciferase assay.

Luciferase assay. After RNA transfection to HeLa S3 cells, the cells were harvested at the times indicated by adding 20 μl of passive lysis buffer per well (Promega). Luciferase activity was measured with a Dual Luciferase kit (Promega) using a TR717 Microplate luminometer (ABI) according to the manufacturer's instructions.

Virus titration. Plaque assay was performed in six-well plates (Falcon) containing HEp-2c cell monolayers. Virus solution (100 μl) was inoculated into each well and incubated for 30 min at 36 °C, then 2 ml of 5 % FCS/DMEM containing 0·5 % agarose ME (Iwai Chemicals Company Ltd, Tokyo, Japan) was added and the plates were further incubated for 24 h at 36 °C. The same medium with agarose ME and 0·005 % neutral red was overlaid on the first layer of agarose gel, and the plates were further incubated for 4 days at 36 °C. For the 50 % cell culture infective dose ($CCID_{50}$) measurements, virus solution was diluted with 5 % FCS/DMEM, inoculated into HEp-2c cell monolayers on 96-well plates (Falcon) and then incubated at 36 °C for 1 week. $CCID_{50}$ was calculated according to the Behrens–Kärber method (Karber, 1931).

Neurovirulence test in hPVR-expressing transgenic mice. hPVR-expressing transgenic mice, ICR TgPVR21 (TgPVR21) (Central Laboratory of Experimental Animals, Kanagawa, Japan) (Abe *et al.*, 1995), 4–5 weeks old, were used for the measurement of 50 % paralytic doses (PD_{50}) of mutant viruses and virus growth in the spinal cord. For the measurement of PD_{50}, 10-fold serial dilutions of virus solution were made from the stock virus solution of each mutant, and then 30 μl of the virus solution was intracerebrally inoculated. Six TgPVR21 mice (three males and three females) were used for the injection with each dose. The inoculated TgPVR21 mice were observed for paralysis, weakness and death up to 14 days. Mice that showed paralysis were counted for the calculation of PD_{50} values. For the intraspinal inoculation, 5 μl of virus solution was inoculated into the spinal cord of TgPVR21 mice.

Isolation of viral genomic RNA from TgPVR21 mice. The viral genomic RNA was directly extracted from the spinal cords of inoculated TgPVR21 mice that showed paralysis or death. A part of the spinal cord (1–1·5 cm in length) was recovered from the mice, and then subjected to homogenization in 200 μl of 5 % FCS/DMEM. The homogenate was centrifuged at 10 000 *g* for 5 min at 4 °C, and then a 50 μl aliquot of the supernatant was used for the isolation of the viral genomic RNA using a High-pure-viral RNA purification kit (Roche). Isolated virus genomic RNA was used as the RNA template in the RT-PCR for sequence analysis.

***In vivo* replication of 139(+25aa) mutant.** The stock solution of 139(+25aa) mutant was prepared by RNA transfection in HEp-2c cells incubated for 14 h at 37 °C in 1 ml of 5 % FCS/DMEM per well. After freezing and thawing, the collected supernatant was centrifuged at 10 000 *g* for 5 min at 4 °C, then stored at −70 °C before use in the measurement of PD_{50} and for the intraspinal inoculation. The virus was recovered from a section of the spinal cord (1–1·5 cm) of TgPVR21 mice at the indicated days post-inoculation. Titration of 139(+25aa) mutant was performed by $CCID_{50}$ measurement and the titre was determined from the CPE appearance, and also from the results of RT-PCR analysis of the cell suspension in each well after freezing and thawing, using primers PV110(+) and RIPO669(−) to detect the virus genome.

Computer-based secondary structure prediction of 139 nt spacer sequence. The secondary structure model of a spacer sequence was obtained using the MFOLD 3.1 program and selected constraints on base pairing as indicated. The program is run on a server at The Bioinformatics Center at Rensselaer and Wadsworth (http://www.bioinfo.rpi.edu/applications/mfold/).

RESULTS

Construction of PV IRES mutants with reduced translation activity

First, we constructed PV IRES mutants with reduced protein synthesis activity but without direct modification of the IRES core structure. For this purpose, we introduced a series of spacer sequences between the intact PV IRES element and the initiation codon using the *Mfe*I site (at nt 736) with or without sORFs of different lengths (Fig. 1A). We used a spacer sequence from the EGFP gene as an arbitrary sequence, with or without the introduction of sORFs encoding six, 14 or 25 amino acid residues for constructs containing spacers 232(+6aa) and 139(+6aa), or spacers 232(+14aa) and 139(+14aa), or spacer 139(+25aa), respectively. For spacers 232(+6+14aa) and 232(+6+4aa), two sORFs were introduced.

Protein synthesis directed by the PV IRES with sORFs was quantified using dicistronic PV replicons (Fig. 1B). Dicistronic replicons express *Renilla* luciferase as the first cistron directed by the PV IRES with sORFs, and firefly luciferase as the second cistron directed by the wild-type PV IRES that acts as an internal control of protein synthesis. For the evaluation of the protein synthesis activity of sORF mutants, luciferase activities were measured 2 h post-transfection of corresponding RNA transcripts in HeLa S3 cells, i.e. before starting the replication process of the replicons. The introduction of spacer sequences without sORFs did not affect the *Renilla* luciferase activity relative to the firefly luciferase activity, consistent with previous observations (Table 2) (Hellen *et al.*, 1994; Kuge *et al.*, 1989; Poyry *et al.*, 2001). The introduction of sORFs into these spacer sequences resulted in moderate to marked changes in the relative *Renilla* luciferase activity. For the 139 nt spacer, the introduction of sORFs encoding six, 14 or 25 amino acid residues resulted in protein synthesis activity of 45 %, 28 % or 17 % of the wild-type activity, respectively (Table 2). We also measured the Sabin 1 IRES activity and observed slightly less IRES activity (88 %) than that observed for the parental Mahoney IRES. These results showed that the regulation of viral protein synthesis was achieved by introducing spacer sequences with sORFs between the IRES element and the initiation codon.

Effect of viral protein synthesis activity reduction on the time-course of luciferase activity of PV luciferase replicons

We then analysed the effect of reduced viral protein synthesis activity on an *in vitro* phenotype of PV monocistronic luciferase replicons. Spacer sequences with or without sORFs were introduced between the PV IRES and the initiation codon of firefly luciferase (Fig. 1). The luciferase activity was measured as an indication of replication (Herold & Andino, 2000). As a result, the phenotypes determined by the luciferase activity were classified into four groups. The 139 nt spacer without a sORF [139(−)]

Table 2. Protein synthesis directed by mutants and the wild-type PV IRES

Sample	Rluc*	Fluc*	Rluc/Fluc*	Relative translation activity (%)†
Mock-treated	83	32	–	–
232(+6+14aa)	290	416	0·54 (0·00)‡	8·8
232(−)	3270	528	6·4 (0·50)	105
232(+6aa)	1640	479	3·4 (0·70)	55
232(+6+4aa)	548	385	1·3 (0·30)	21
Mahoney IRES	2440	407	6·1 (0·60)	100
Sabin 1 IRES	1880	365	5·4 (0·70)	88
Mock-treated	85	46	–	–
139(−)	4630	697	7·0 (1·1)	107
139(+6aa)	1200	425	2·9 (0·10)	45
139(+14aa)	1050	580	1·8 (0·0)	28
139(+25aa)	525	438	1·1 (0·20)	17
232(+14aa)	958	471	2·1 (0·20)	32
Mahoney IRES	1790	307	6·5 (1·1)	100

*Rluc, Fluc and Rluc/Fluc indicate *Renilla* luciferase activity, firefly luciferase activity and the ratio between them. Luciferase activity was measured 2 h after transfection of HeLa S3 cells by RNA transcripts of luciferase replicons.

†Mahoney IRES activity was taken to represent 100 % translation activity.

‡Numbers in parentheses represent the standard deviation obtained from three independent experiments.

did not affect the kinetics of luciferase activity, although the 232 nt spacer [232(−)] slightly reduced it at the maximum point (group I phenotype) (Fig. 2). The introduction of sORFs encoding six and 14 amino acid residues resulted in a moderate reduction in the maximum luciferase activity (10-fold reduction in the maximum activity compared with the wild-type level), with a slight delay in the peak luciferase activity observed at 12 h post-transfection [group II phenotype, 232(+6aa), 232(+14aa), 139(+6aa) and 139(+14aa) spacers]. The introduction of a 25 amino acid-encoding sORF or the tandem insertion of two sORFs (encoding six and four amino acid residues) markedly affected both the maximum luciferase activity and its kinetics, with an approximately 100-fold reduction compared with the wild-type level and the peak activity at 14 h post-transfection [group III phenotype, 139(+25aa) and 232(+6+4aa) spacers, Fig. 2]. The insertion of two sORFs (encoding six and 14 amino acid residues) further reduced the maximum luciferase activity, but an increase in luciferase activity was still detectable [group IV phenotype, 232(+6+14aa) mutant, Fig. 2C]. These results showed that the kinetics and the level of luciferase activity of luciferase replicons could be classified into four groups in a sORF-length-dependent manner, presumably due to reduction of viral protein synthesis.

In vitro phenotypes of PV mutants with reduced viral protein synthesis activity

To analyse the phenotype of spacer-containing mutant viruses, we attempted to recover PV mutants with the spacers using an infectious clone of the type 1 PV (Mahoney) (Fig. 3). After RNA transfection of transcripts from corresponding infectious clones to HEp-2c cells, viruses were obtained for mutants 232(−), 232(+6aa), 232(+14aa), 232(+6+4aa), 139(−), 139(+6aa), 139(+14aa) and 139(+25aa), but not for mutant 232(+6+14aa) (Fig. 3 and Table 3). The plaque phenotypes of mutant viruses showed some heterogeneity as observed previously (Burns *et al.*, 1992). Mutants 232(−) and 139(−) formed medium-sized plaques that were smaller than those formed by the parental Mahoney strain. Mutants 232(+6aa), 232(+14aa), 139(+6aa) and 139(+14aa) formed small plaques. Mutants 232(+6+4aa) and 139(+25aa) formed large plaques. After plaque purification, IRES mutants 232(−), 232(+6aa), 232(+14aa), 139(−), 139(+6aa) and 139(+14aa) retained intact spacer sequences, but mutants 232(+6+4aa) and 139(+25aa) showed a deletion in, or mutation of, the spacer sequence which disrupted the sORF (data not shown). Thus, the latter two mutants showed unstable virus production in contrast to that observed for the other six mutants. PV mutants exhibiting more than 28 % of the wild-type protein synthesis activity were capable of supporting stable virus production; however, mutants with less than 21 % of the wild-type activity showed unstable virus production or a lethal phenotype *in vitro*.

In vivo phenotypes of PV mutants with reduced viral protein synthesis activity

Next, we examined the neurovirulence of mutant viruses that showed stable virus production *in vitro*, by the intracerebral inoculation of transgenic mice (TgPVR21) (Koike

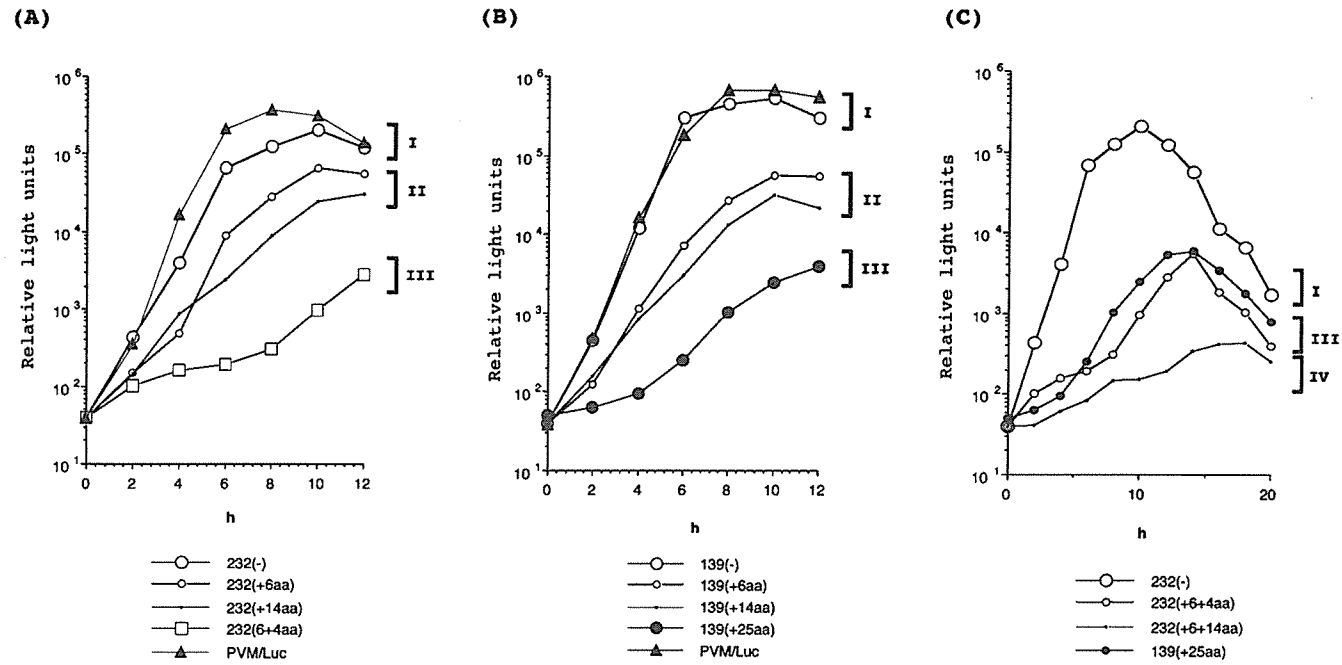

Fig. 2. Time-course of luciferase activity of monocistronic luciferase replicons with mutant IRES. Relative light units of firefly luciferase activity used as a reporter were measured for (A) mutants with the 232 nt spacer, (B) mutants with the 139 nt spacers and (C) mutants with delayed kinetics. Based on the kinetics of the increase and the peak of luciferase activity, mutants were classified into four groups (group I, II, III, IV). The group numbers of each mutant are indicated on the right side of each graph.

et al., 1991). The neurovirulence of 139 nt spacer mutants [139(−), 139(+6aa) and 139(+14aa) mutants] and the parental Mahoney strain were compared. Results showed that all of the spacer mutants examined retained high neurovirulence ($PD_{50}=3{\cdot}2$ to $3{\cdot}6$ $\log_{10}CCID_{50}$) but were slightly attenuated compared with that of the parental strain ($PD_{50}=2{\cdot}6$ $\log_{10}CCID_{50}$). To confirm that the observed neurovirulence was not due to the emergence of revertant viruses, we determined the stability of the introduced spacer sequence or mutation in mutants 139(−), 139(+6aa) and 139(+14aa) during *in vivo* replication. After intracerebral inoculation of each virus mutant to TgPVR21 mice, the spinal cords were collected from those which had exhibited paralysis and then the virus particles were recovered from the tissue (see Methods). Introduced spacers and sORFs were retained in the virus genomes of all three mutants for 5–14 days post-inoculation (data not shown). These results showed that the virulence of PV mutants only slightly decreased as the translational activity decreased.

Characterization of mutant 139(+25aa)

To further define the viral protein synthesis activity required for an attenuated phenotype of PV, we examined the *in vivo* replication phenotype of mutant 139(+25aa) which showed unstable virus production *in vitro*. We prepared 139(+25aa) virus by collecting particles after a single round of replication to reduce the possibility of emergence of revertant viruses (see Methods). To determine the *in vivo* stability of the mutant, the replication phenotype in the spinal cord was determined by intraspinal inoculation of TgPVR21 mice. After inoculation, the mice showed signs of paralysis or died within 7 days post-inoculation (Table 4). For all nine inoculated mice, the virus genome recovered from the spinal cords retained an intact spacer sequence, suggesting that mutant 139(+25aa) was stable during replication in the spinal cords of the TgPVR21 mice.

Next, we determined the neurovirulence of mutant 139(+25aa) following intracerebral inoculation of TgPVR21 mice. The virus showed a partially attenuated phenotype ($PD_{50}=5{\cdot}1$ $\log_{10}CCID_{50}$) (Table 3). The 139(+25aa) viruses recovered from the spinal cords were mostly revertants (six out of nine mice) with a disrupted initiation AUG codon of the sORF or with deletion of the spacer sequence (Fig. 4). This instability is in marked contrast with that observed for the intraspinal inoculation. This suggests that low viral protein synthesis activity was a limiting factor in the pathway of PV infection from the cerebrum to the spinal cord in the TgPVR21 mouse. These results suggest that PV mutants with reduced viral protein synthesis activity observed in cultured cells (17–55 % of the wild-type activity) were not attenuated completely in these mice, although their translational activity in the cultured cells is lower than that of Sabin 1 IRES. This indicates that

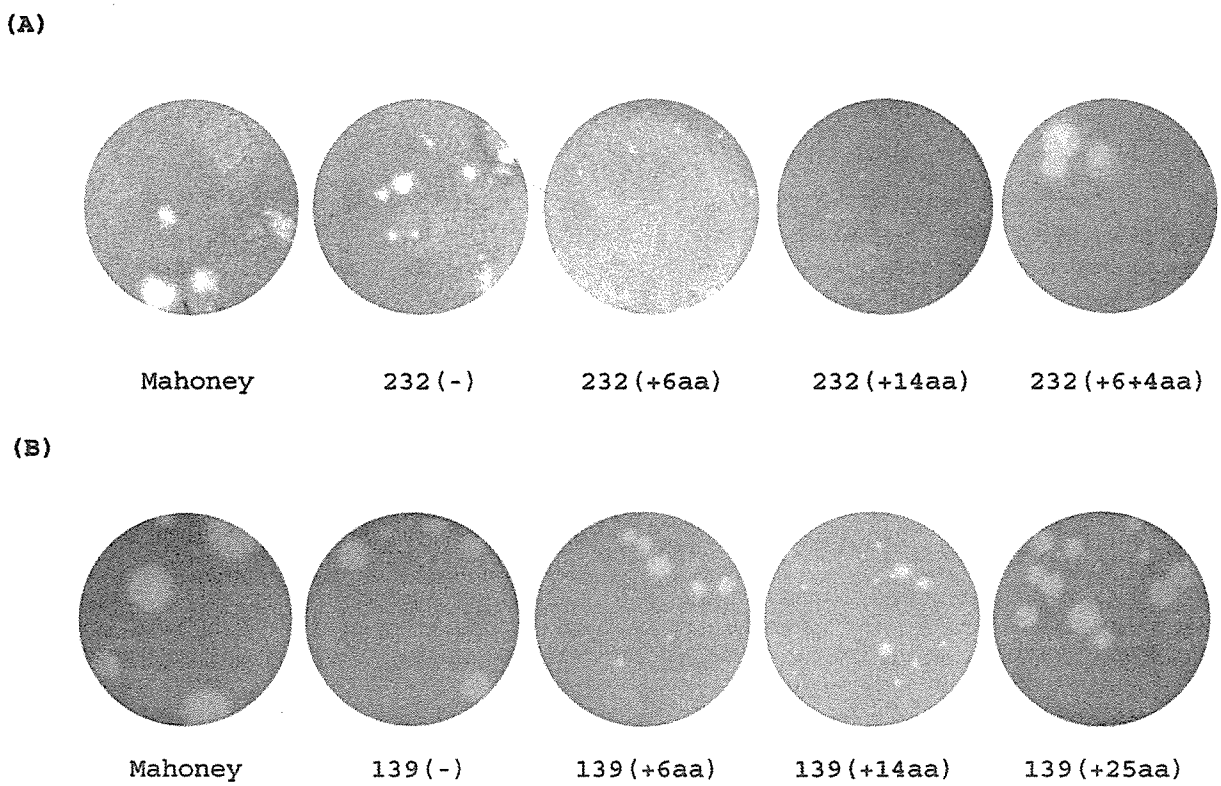

Fig. 3. Plaque phenotype of PV mutants. HEp-2c cells inoculated with virus solution were incubated for 1 day at 36 °C after the addition of 5 % FCS/DMEM containing 0·5 % agarose ME. The same medium with agarose ME and 0·005 % neutral red was overlaid and the plates were further incubated for 4 days at 36 °C for staining. (A) Plaque phenotype of PV mutants with a 232 nt spacer. (B) Plaque phenotype of PV mutants with a 139 nt spacer.

Table 3. Summary of *in vitro* phenotype and neurovirulence of PV mutants

Sample name	Protein synthesis (%)	Virus titre*	Replication kinetics	Plaque phenotype†	PD_{50}
232(−)	105	6·25	+++	M	ND
232(+6aa)	56	6·00	+++	S	ND
232(+14aa)	32	6·00	+++	S	ND
232(+6+4aa)	21	6·25(PI)	++	L(PI)	ND
232(+6+14aa)	8·8	(Lethal)	+	−	ND
139(−)	108	7·38	++++	M	3·2
139(+6aa)	45	6·50	+++	S	3·7
139(+14aa)	28	6·25	+++	S	3·6
139(+25aa)	17	5·50(PI)	++	L(PI)	ND
139(+25aa)§	17	5·00	++	−	5·1
Mahoney	100	7·50	++++	L	2·6
Sabin 1	88	7·50	ND	L	>8·3‡

ND, Not determined.

*Virus titres represent $\log_{10}CCID_{50}$ in 10 µl. The titre of 139(+25aa) virus was measured by counting wells that showed CPE caused by revertant virus or by counting RT-PCR-positive wells to detect virus genome in the $CCID_{50}$ measurement.

†L, Large; M, medium; S, small; PI, pseudoinfection.

‡The data for the Sabin 1 strain were obtained from our previous report (Yoneyama *et al.*, 2001).

§Virus solution was collected 14 h post-transfection of the corresponding RNA transcript.

Table 4. Replication of 139(+25aa) mutant after intraspinal or intracerebral inoculation

Inoculation route	Mouse number	Inoculated virus titre*	Recovered virus titre*	Post-inoculation (day)	Condition	Spacer sequence†
Intraspinal	1	4·7	6·2	3	Paralysed	I
	2	4·7	6·2	3	Dead	I
	3	4·7	6·7	3	Dead	I
	4	3·7	5·9	3	Dead	I
	5	3·7	6·2	3	Paralysed	I
	6	1·7	5·2	5	Paralysed	I
	7	1·7	4·0	7	Paralysed	I
	8	1·7	5·5	6	Dead	I
	9	1·7	4·7	7	Dead	I
Intracerebral	1	5·5	ND	5	Dead	I
	2	5·5	ND	6	Dead	R
	3	5·5	ND	6	Dead	I
	4	5·5	ND	6	Dead	D
	5	5·5	ND	7	Dead	R
	6	5·5	ND	7	Dead	I
	7	4·5	ND	7	Dead	R
	8	5·5	ND	7	Dead	D
	9	5·5	ND	9	Dead	R

ND, Not determined.

*Virus titres represent $\log_{10}CCID_{50}$. TgPVR21 mice were intraspinally inoculated with 5 μl of virus solution, or intracerebrally inoculated with 30 μl of virus solution. Animals were observed for up to 14 days after inoculation for signs of paralysis or death. The titre of 139(+25aa) virus was measured by counting wells that showed CPE caused by revertant virus or by counting RT-PCR-positive wells to detect virus genome in the $CCID_{50}$ measurement.

†I, Intact; R, reversion; D, deletion.

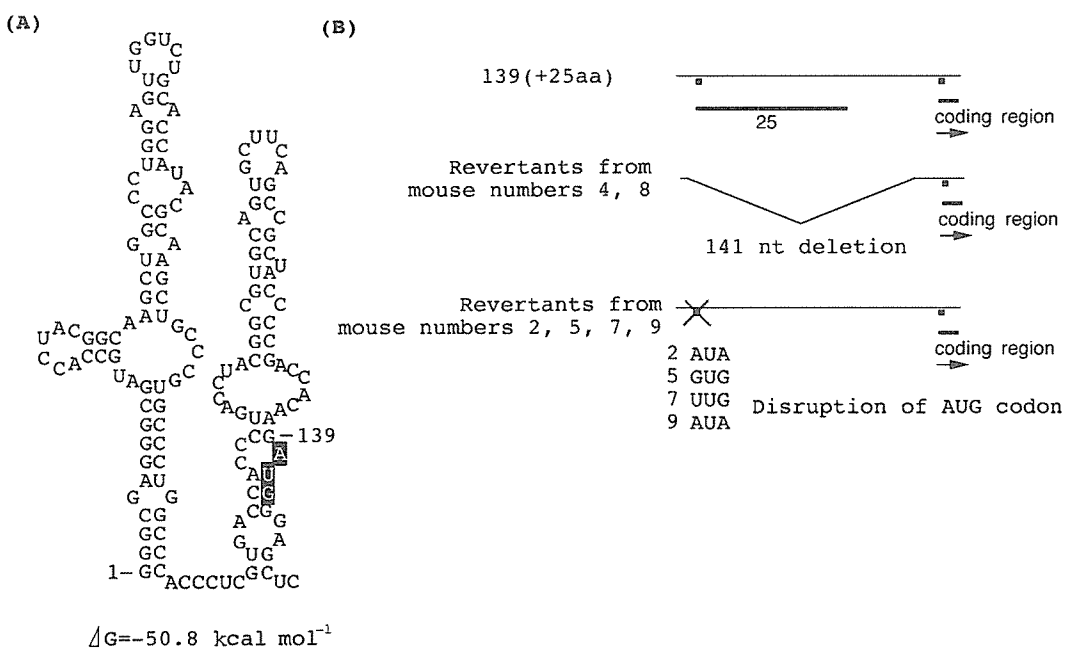

Fig. 4. RNA structural model of spacer sequence of 139(+25aa) mutant. (A) A model of secondary structure of the spacer sequence of the 139(+25aa) mutant estimated by MFOLD 3.1. (B) Schematics of spacer sequences of revertant viruses isolated from mice inoculated intracerebrally with the 139(+25aa) mutant (Table 3).

the translational activity observed in cell culture does not correlate with the neurovirulence of PV mutants.

DISCUSSION

To determine the effect of viral protein synthesis on PV infection, we constructed a series of PV mutants showing reduced viral protein synthesis activity in HeLa S3 cells. We used the dicistronic PV replicon for the measurement of viral protein synthesis. We applied the reinitiation mechanism of ribosomes (Kozak, 1987), by introducing a sORF in a spacer sequence between the IRES element and the initiation codon to construct PV mutants (Fig. 1). The enterovirus genome has an endogenous spacer sequence well conserved in length (approx. 150 nt) without a putative primary or secondary RNA structure between the core IRES structure (from the 5′ end of the genome to the last cryptic AUG) and the initiation codon, although the biological role of this spacer remains to be elucidated (Hellen *et al.*, 1994; Kuge *et al.*, 1989; Kuge & Nomoto, 1987). Recognition of an initiation codon downstream of the PV IRES by ribosomes has been demonstrated to utilize both scanning and shunting mechanisms through this endogenous spacer sequence (Hellen *et al.*, 1994; Kuge *et al.*, 1989; Poyry *et al.*, 2001). The efficiency of reinitiation is influenced by several factors, such as the length of the upstream ORF (Kozak, 1987; Luukkonen *et al.*, 1995), the distance between the upstream ORF and the initiation codon (Kozak, 1987) and the presence of translation initiation factors (Garcia-Barrio *et al.*, 1995; reviewed by Kozak, 1999). We used part of a sequence derived from the EGFP gene as the spacer to introduce a sORF between the IRES element and the intiation codon, i.e. following the endogenous spacer sequence of the PV IRES (Fig. 1, 4). This spacer sequence has a secondary structure consisting of two stem–loop structures with a total free energy of $-50{\cdot}8$ kcal mol^{-1} ($-212{\cdot}5$ kJ mol^{-1}) (Fig. 4). In a previous report, a single stem–loop structure with a free energy of -50 or -60 kcal mol^{-1} markedly inhibited translation mediated by the scanning mechanism, but a stem–loop structure with -30 kcal mol^{-1} did not (Kozak, 1986). The introduction of spacer sequences without sORFs had a minor effect, if any, on protein synthesis (Fig. 1, Table 3), possibly because of loose secondary structures that could not affect the ribosome scanning or shunting on the PV IRES (Hellen *et al.*, 1994; Kuge *et al.*, 1989; Poyry *et al.*, 2001). However, the introduction of the spacer sequence affected the plaque phenotype of mutant viruses (Fig. 3), possibly because of the uncoating process and/or the encapsidation process via an unknown mechanism. The introduction of sORFs severely affected protein synthesis in a manner similar to that of the leaky scanning model (Fig. 1, Table 2). Consequently, we obtained PV IRES mutants that showed reductions in viral protein synthesis activity to between 8·8 % and 55 % of that of the parental Mahoney strain (Fig. 1, Table 2).

We recovered PV mutants with reduced protein synthesis activity (Fig. 3). There was a good correlation among this activity, monocistronic replicon phenotype and the stability of mutant viruses (Fig. 2, Tables 2 and 3). This decreased activity resulted in phenotypes showing delayed kinetics and levels of replication and in instability or lethality of virus production. We found that protein synthesis activity from 17 to 21 % of the wild-type level was on the boundary between stable virus production and the lethal phenotype *in vitro*.

We analysed the *in vivo* phenotype of PV mutants to evaluate their neurovirulence using a transgenic mouse model (TgPVR21) (Abe *et al.*, 1995). The Sabin 1 IRES activity has been determined to have a range of around 32–67 % of the IRES activity of the parental strain (Haller *et al.*, 1996; Muzychenko *et al.*, 1991; Svitkin *et al.*, 1985, 1990). Therefore, it was of interest to evaluate the neurovirulence of the type 1 PV mutants with reduced viral protein synthesis. Among those which showed stable virus production *in vitro*, mutant 139(+14aa) showed the lowest protein synthesis activity (28 % of the wild-type value) (Fig. 3). Despite this low activity, the mutant was highly neurovirulent in TgPVR21 mice ($PD_{50}=3{\cdot}6$ $\log_{10}CCID_{50}$), only slightly attenuated compared with the parental strain ($PD_{50}=2{\cdot}6$ $\log_{10}CCID_{50}$). To further define the viral protein synthesis activity required for the attenuation of type 1 PV, we examined mutant 139(+25aa), which showed the lowest viral protein synthesis activity with unstable virus production, and observed partial attenuation of its neurovirulence ($PD_{50}=5{\cdot}1$ $\log_{10}CCID_{50}$). The apparent attenuation of 139(+25aa) was supported in part by the probability of emergence of revertants caused by the low viral protein synthesis activity as described below (Fig. 4, Table 4). A high ratio of emergent revertants might result in an overestimation of the neurovirulence of this mutant. These results showed that the effect of reduced viral protein synthesis activity in the range examined on the attenuation of PV was limited, hence other steps in the infection process may play major roles in the attenuation. The mutants examined in this study make a contrasting example to the RIPO mutant, which is a PV mutant with rhinovirus IRES that replicated well in HeLa cells but was not neurovirulent in PV receptor-expressing transgenic mice (Gromeier *et al.*, 1996).

Following intracerebral inoculation of mice with mutant 139(+25aa), revertants with a disrupted sORF emerged rapidly (Fig. 4, Table 4). The revertants had either deletion or substitution mutations that disrupted the sORF (Fig. 4). This suggests that it is not the instability of the spacer sequence but the low protein synthesis activity that causes the emergence of revertants. Therefore, PV infection was limited to the stage of viral protein synthesis in some specific sites in the CNS other than the spinal cord. In contrast to the intracerebral inoculation results, mutant 139(+25aa) exhibited partially stable replication in the spinal cord when inoculated intraspinally into the TgPVR21 mice (Table 4). This observation suggests that

motor neurons in the spinal cord might provide a 'rich' environment for PV replication that may possibly alleviate constraints on viral protein synthesis during PV infection. This suggests that different parts of the CNS provide different limitations on the replication of PV at the step of viral protein synthesis.

In summary, we found that the PV mutant with 17 % of the protein synthesis activity of the parental Mahoney strain was attenuated to only a minor extent. Therefore, the viral protein synthesis activity observed in cultured cells (17–55 % of the wild-type activity) did not determine strong attenuation of PV. This confirmed that reduced *in vitro* translational activity did not cause the attenuation of PV. The nature of *in vivo* PV infection remains to be further elucidated, but the IRES mutants constructed in this study would serve as useful tools for exploring the link *in vivo* between viral protein synthesis and pathogenesis in PV infection.

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