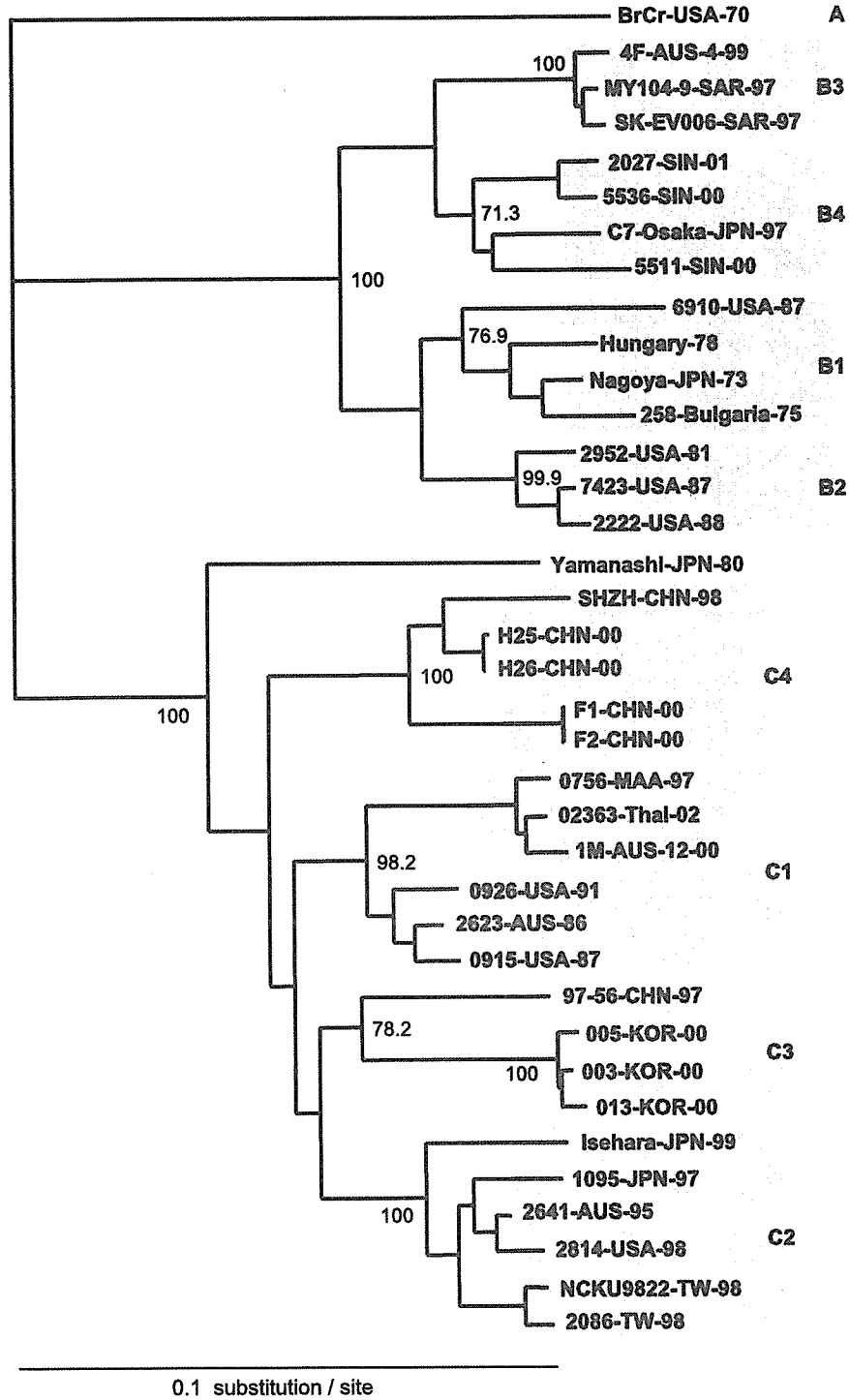

Fig. 1 Phylogenetic analysis of EV71 based on the VP1 sequences. The phylogenetic tree was constructed by the neighbor-joining method and rooted using a prototype BrCr strain as an outgroup. Bootstrap values (%) are indicated at the branch nodes.

molecular epidemiological analyses. In our experimental infection model of cynomolgus monkeys with three different EV71 genogroups, the monkeys developed various neurological manifestations regardless of the infecting EV71 strains.[29] Thus, two major genogroups of EV71 in the WPR have been the major causative agents of HFMD outbreaks in this area, and both are neurovirulent.

Although phylogenetic analyses were performed using different regions of the viral genome, identical clustering of recent EV71 strains was shown regardless of the regions examined. The VP4-based classification was established for the molecular typing of human enteroviruses, and was also used for a more detailed molecular epidemiological analysis. Therefore the VP4 genotyping was mainly used in analyzing

Table 1 Enterovirus 71 isolates sequenced for this study

Isolate	Year	Origin	Clinical outcome	Accession No.
F1-CHN	2000	China (Shanghai)	HFMD	AB115490
F2-CHN	2000	China (Shanghai)	HFMD	AB115491
H25-CHN	2000	China (Shanghai)	HFMD	AB115492
H26-CHN	2000	China (Shanghai)	HFMD	AB115493
97-56-CHN	1997	China (Heilongjiang)	AFP	AB115494
02363	2002	Thailand	HFMD	AB115495

AFP, acute flaccid paralysis; HFMD, hand, foot and mouth disease.

Table 2 Subgenogroup classification based on the VP1 and VP4 regions

VP1	VP4	Representative strain†	Reference
A	A (BrCr)‡	BrCr-USA-70	27
B1	B1 (A-1)	Nagoya-JPN-73	14, 29
B2	B2 (A-2)	7423-USA-87	27
B3	B3 (A-2)	SK-EV006-SAR-97	14, 29
B4	B4 (A-2)	C7-Osaka-JPN-97	14, 29
C1	C1 (B-1)	0195-USA-87	15
C2	C2 (B-1)	1095-JPN-97	29
C3	C3 (B-1)	003-KOR-00	25
C4	C4 (B-2)	SHZH-CHN-98	GenBank

†The oldest EV71 strain, whose VP1 and VP4 sequences were available, was used as representative strains of each subgenogroup. ‡VP4-based genotyping/subgenotyping in our previous reports are shown in parentheses.[14,26,29]

recent and reference EV71 strains in our previous studies.[22,30] The use of the VP4-based system, using a single universal primer pair for RT-PCR and sequencing, is a simple and convenient screening method for the identification of EV71. On the other hand, the VP1-based phylogenetic analysis provides a more strict and reliable genogrouping of EV71 owing to the longer sequencing window and possible correlation of the genogroup with viral antigenicity.[21,25,28] In this study, the VP1-based phylogenetic analysis was conducted using the entire VP1 sequences of recent EV71 strains isolated in the WPR, which were actively accumulated by several other groups, to coordinate different genotyping systems. Moreover, to avoid unnecessary confusion in nomenclature of the genotyping/genogrouping systems of EV71, our previous VP4-based genotyping system (BrCr, A and B) was revised to correspond with those of the VP1-based genogrouping. Although more entire-VP1-sequence data are necessary to completely coordinate the two systems, our revised VP4-based genotyping is preliminarily proposed in Table 2.

In the countries where large HFMD outbreaks have occurred, and in countries that already have established surveillance systems for enterovirus infections, the monitoring of severe enterovirus encephalitis, especially EV71 infections, is currently strict. In the extended areas of the WPR and their surrounding areas, however, enterovirus surveillance systems are not functioning well. Therefore, it is still difficult to accurately survey the epidemics of enterovirus infections throughout the WPR area. In this study, we conducted molecular epidemiological analysis using EV71 isolates from the regions where HFMD epidemics had not been systematically reported, presumably owing to an inadequate enterovirus surveillance system. Consequently, five recent EV71 isolates in Mainland China represented a putative new subgenogroup within genogroup C, supported by a high bootstrap value. Subgenogroup C4 may be an indigenous EV71 lineage in Mainland China. Nevertheless, it would be necessary to study the actual status of EV71 transmission in Mainland China using more EV71 isolates in the future. It is expected that the monitoring of the trend of EV71 transmission will be possible by molecular epidemiological analysis of more EV71 isolates in wider areas. It is important to establish disease and infectious agent surveillance systems that target enterovirus infections in more countries in the WPR.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant for health research from the Regional Office for the Western Pacific, World Health Organization. The authors thank Dr Kunito Yoshiike for critical review of the manuscript. C. Li and N. Onnimala are visiting fellows from the Japan International Cooperation Agency.

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Journal of General Virology (2005), **86**, 1391–1401 DOI 10.1099/vir.0.80784-0

Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys

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Enterovirus 71 (EV71) is one of the major causative agents of hand, foot and mouth disease and is sometimes associated with serious neurological disorders. In this study, an attempt was made to identify molecular determinants of EV71 attenuation of neurovirulence in a monkey infection model. An infectious cDNA clone of the virulent strain of EV71 prototype BrCr was constructed; temperature-sensitive (*ts*) mutations of an attenuated strain of EV71 or of poliovirus (PV) Sabin vaccine strains were then introduced into the infectious clone. *In vitro* and *in vivo* phenotypes of the parental and mutant viruses were analysed in cultured cells and in cynomolgus monkeys, respectively. Mutations in 3D polymerase ($3D^{pol}$) and in the 3′ non-translated region (NTR), corresponding to *ts* determinants of Sabin 1, conferred distinct temperature sensitivity to EV71. An EV71 mutant [EV71(S1-3′)] carrying mutations in the 5′ NTR, $3D^{pol}$ and in the 3′ NTR showed attenuated neurovirulence, resulting in limited spread of virus in the central nervous system of monkeys. These results indicate that EV71 and PV1 share common genetic determinants of neurovirulence in monkeys, despite the distinct properties in their original pathogenesis.

Received 25 November 2004
Accepted 3 February 2005

INTRODUCTION

Enterovirus 71 (EV71) belongs to the genus *Enterovirus* of the family *Picornaviridae* and possesses a single-stranded, positive-sense RNA genome of approximately 7500 nt in length (Brown & Pallansch, 1995; Schmidt *et al.*, 1974). Genetically, EV71 is classified as a species A human enterovirus along with some coxsackie A (CA) viruses, such as CA10 and CA16 (Brown & Pallansch, 1995; Pulli *et al.*, 1995). As well as CA10 and CA16, EV71 causes hand, foot and mouth disease (HFMD) and herpangina, which are common and self-limiting diseases that typically occur in children. However, EV71 infection sometimes causes severe neurological diseases, such as brainstem encephalitis and polio-like paralysis (Chumakov *et al.*, 1979; Wang *et al.*, 2003), mainly in infants and young children (McMinn, 2002). A number of fatal encephalitis cases were reported in large-scale HFMD outbreaks in Malaysia in 1997 (Abubakar *et al.*, 1999; Shimizu *et al.*, 1999) and in Taiwan in 1998 and 2000 (Ho *et al.*, 1999; Lin *et al.*, 2003; Lu *et al.*, 2002; Wang *et al.*, 2002). Furthermore, sporadic HFMD cases with severe neurological manifestations have been reported in the Western Pacific region, e.g. in Australia, Singapore, Hong Kong and Japan (Ahmad, 2000; Chan *et al.*, 2000; Fujimoto *et al.*, 2002; Herrero *et al.*, 2003; Komatsu *et al.*, 1999; Lum *et al.*, 1998; McMinn *et al.*, 1999, 2001b). Numerous factors (e.g. virus genotypes or specific mutations, herd protective immunity, individual immunity or association with other infectious agents) could lead HFMD to become a more serious disease. From molecular epidemiological studies of EV71, McMinn *et al.* (2001a) suggested that an amino acid change at position 170 of VP1 (from Ala to Val) is involved in the virulence of EV71. Non-structural proteins of EV71 (2A and 3C proteinases) were responsible for the induction of apoptosis in infected cells *in vitro* (Kuo *et al.*, 2002; Li *et al.*, 2002). However, crucial epidemiological or experimental evidence to identify critical factors of EV71 pathogenesis has yet to be provided (Shimizu *et al.*, 1999).

The occasional association of EV71 infection with serious neurological manifestations suggests that EV71 is highly neurotropic, like poliovirus (PV), which is the causative agent of poliomyelitis. The molecular determinants of the neurovirulence of PV have been studied extensively on the vaccine strains (Sabin 1, 2 and 3) (reviewed by Minor, 1992) in monkeys, as well as in transgenic mice carrying the

The GenBank/EMBL/DDBJ accession numbers for the complete genomic sequences of EV71(BrCr-TR) and EV71(BrCr-ts) are AB204852 and AB204853, respectively.

human PV receptor gene (Horie *et al.*, 1994; Koike *et al.*, 1993; Ren *et al.*, 1990). In contrast, the molecular basis of EV71 neuropathogenicity remains poorly understood, partly due to the lack of appropriate infection models.

Recently, we established an experimental EV71 infection of cynomolgus monkeys by using intravenous inoculation (Nagata *et al.*, 2004). This new experimental system of EV71 consistently induced typical neurological manifestations similar to those observed in human cases, including tremor, ataxia and polio-like paralysis (Nagata *et al.*, 2004). These disorders were caused by encephalomyelitis, involving both the pyramidal and extrapyramidal systems, in monkeys. These neurological manifestations were difficult to assess in current mouse models, where some clinical symptoms, including rash and hind-limb paralysis, were observed and adaptive mutations of EV71 played a critical role in the virulence (Chen *et al.*, 2004; Wang *et al.*, 2004). Not all of the EV71 isolates, irrespective of their clinical backgrounds, could achieve infection in mice (N. Nagata, H. Shimizu & T. Iwasaki, unpublished data). Therefore, we applied a monkey infection model for the evaluation of genetic determinants of EV71 neurovirulence.

In this study, we established an infectious cDNA clone derived from the prototype BrCr strain of EV71 and examined the effect of temperature-sensitive (*ts*) mutations on neurovirulence in a monkey infection model. We analysed a *ts* variant of the BrCr strain [EV71(BrCr-ts)] with an attenuated phenotype (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) to identify the critical mutations for its *ts* phenotype. We examined the effect of *ts* determinants of Sabin strains in the context of the EV71(BrCr) genome on the attenuation.

METHODS

Cells and viruses. Vero cells (derived from African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and were used for virus preparation, titration and measurement of growth kinetics and temperature sensitivity. *ts* and temperature-resistant (*tr*) variants of the prototype BrCr strain (Schmidt *et al.*, 1974), EV71(BrCr-ts) and EV71(BrCr-TR), were isolated previously in cynomolgus monkey kidney (CMK) cells (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983). EV71(BrCr-ts) showed an attenuated phenotype and EV71(BrCr-TR) retained the neurovirulent phenotype of the BrCr strain in cynomolgus monkeys (Hashimoto & Hagiwara, 1983; Nagata *et al.*, 2002, 2004). The variants used in this study were obtained after further plaque purification in Vero cells from the original virus stock. The viral genomes of plaque-purified variants had mutations compared with the parental BrCr strain. The virus stocks were prepared in Vero cells by RNA transfection of the transcripts derived from corresponding infectious clones.

RNA extraction, RT-PCR and sequencing. Viral genomic RNA was extracted from the culture fluid of infected cells by using a High Pure viral RNA purification kit (Roche). RT-PCR was performed by using RevatraAce reverse transcriptase (Toyobo) for reverse transcription and either Advantage 2 polymerase (Clontech) or *Tbr* EXT DNA polymerase (Finnzymes) for PCR. PCR products were purified by using a PCR purification kit (Qiagen). Direct sequence analysis was carried out on the full-length genomic sequences of EV71(BrCr-TR) or EV71(BrCr-ts), using DNA fragments amplified by RT-PCR as the templates of the sequence reaction. The sequence of the 5′ end of the viral genome was determined by using a 5′ RACE (rapid amplification of cDNA ends) system, ver. 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 primers 7200F+ and *Eco*RI-3END− (Table 1). DNA sequencing was performed by using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) and then analysed by an ABI PRISM 310 genetic analyser (Applied Biosystems).

General methods of molecular cloning. Two *Escherichia coli* strains were used for the preparation of plasmids. The TOP10 strain (Invitrogen) was used for direct cloning of PCR products, using a TOPO XL PCR cloning kit (Invitrogen). The XL10gold strain (Stratagene) was used for the preparation of other plasmids. Ligation of DNA fragments was performed by using a Quick Ligation kit (New England Biolabs). Site-directed mutagenesis was performed by using KOD plus DNA polymerase (Toyobo) (Sambrook & Russell, 2001).

Construction of the infectious cDNA clone of EV71(BrCr-TR). A DNA fragment containing 6 kb of the 3′ region of the viral genome was amplified by RT-PCR using Advantage 2 polymerase (Clontech) from the viral genome of EV71(BrCr-TR) with primers EV71-1500F+ and EV71-A2− (Table 1). The resultant cDNA fragment was cloned into plasmid pCR-XL-TOPO by using a TOPO XL PCR cloning kit (Invitrogen). Next, the 5′ end sequence of EV71(BrCr-TR) was amplified by RT-PCR with primers *Pvu*T7-45+ and 1595R− and then cloned into the above construct following digestion by *Pvu*I and *Mun*I. However, the RNA transcript derived from the resultant full-length cDNA of EV71(BrCr-TR) did not produce any viable viruses after RNA transfection into Vero cells (data not shown). Therefore, to remove possible lethal mutation(s) in the construct, the 3′ part of the cDNA fragment of EV71(BrCr-TR) was obtained by RT-PCR using *Tbr* EXT DNA polymerase (Finnzymes) with primers A2*Bam*HI− and EV71-1500F+ (Table 1) and then cloned into the *Bam*HI site of the above construct. Transfection of the RNA transcript derived from this cDNA clone produced viable viruses in Vero cells. This infectious clone of EV71(BrCr-TR) was digested with *Sna*BI and *Mlu*I, and then cloned into plasmid P/H d40 (a generous gift from Dr E. Wimmer) (Zhao *et al.*, 2000). In this construct, to introduce *Mlu*I and *Sna*BI sites, part of the plasmid vector was obtained by PCR using *Tbr* EXT DNA polymerase (Finnzymes) with primers *Mlu*I-vec+ and *Sna*BI-vec−, using plasmid P/H d40 as the template DNA. There were five nucleotide differences between the sequence of the EV71(BrCr-TR) genome and that of the resultant infectious clone. To restore the sequence of the clone to the consensus sequence of the EV71(BrCr-TR) genome, the 5′ fragment was amplified again by RT-PCR with primers *Sna*BI-T7-EV71+ and 1595R−, using the viral genome of EV71(BrCr-TR) as the template. The obtained fragment was digested with *Mun*I and *Sna*BI and then ligated into the infectious clone. To restore other mutated sites, DNA fragments obtained with primers EV71-1500F+ and 71/3393−, with primers EV71-2800F+ and tr-6300R− or with primers E2 CF2+ and tr-6300R− were digested with *Mun*I and *Xma*I, with *Xma*I and *Sal*I or with *Sal*I and *Spe*I, respectively, and then ligated sequentially into the infectious clone. Finally, the resultant infectious clone was sequenced and confirmed to have the consensus sequence of the EV71(BrCr-TR) genome. This infectious clone of EV71(BrCr-TR) was designated pEV71(BrCr-TR).

Construction of *ts* mutants. We constructed *ts* mutants of EV71 by introducing the mutations of a *ts* variant of the BrCr strain [EV71(BrCr-ts)] (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) (Fig. 1). For the construction of a cDNA clone of EV71(ts-TR), a cDNA sequence was amplified from the EV71(BrCr-ts) genome by

Table 1. Primers used for the construction of the infectious clone of EV71(BrCr-TR)

Primer name	Sequence (5′–3′)
1595R−	TCCAGCGGGCTGATAGGCACCACC
2784+	CATAACCGGTCATGCGCAGATG
2784−	CATCTGCGCATGACCGGTTATG
3564−	CTCACTAGCTTCTACAAACACCAAACTAGG
6153C+	GAACCTGATGAGCACGTGACACAGGC
6153C−	GCCTGTGTCACGTGCTCATCAGGTTC
7023AT+	GATAAATCACCTATTTTCAATGAGGTTAC
7023AT−	GTAACCTCATTGAAAATAGGTGATTTATC
7200F+	AACACTCAAGATCACGTGCGCTCCC
71/3393−	GGCGGTTRACCACYCTDAAGTTGCCCAC
7409G−	AAAAACGCGTTTTTTTTTTTTTTTTTTTTTTTTTTCGCTATTCTGGTTATAAC
A2*Bam*HI−	AAAAGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTGCTATTCTGG
E2 CF2+	GAGCAAACACCGTATTGAACCTGTATG
*Eco*RI-3END−	ACTGGAATTCTTTTTTTTTTTTTTTTTTTTTTTTV
EV71-1500F+	GGATTAATCTNCGNACCAACAA
EV71-2800F+	TTCACNTACATGCGCTTTGANGC
EV71-A2−	CCATCGATGGTTTTTTTTTTTTTTTTTTTTTTTTTGCTATTCTGG
*Mlu*I-vec+	TCAAACGCGTTTGAAGACGAAAGGGCCTCG
*Pvu*T7-45+	AAATTCGATCGAAATTAATACGACTCACTATAGGTTAAAACAGCCTGTGGGTTGCA-CCCACTCACAGGGCCCACTGGGC
S472+	GAATGCGGTTAATTCTAACTGCGGAGCAC
S472−	GTGCTCCGCAGTTAGAATTAACCGCATTC
S480+	CCTAACTGCGGGGCACATACCCT
S480−	AGGGTATGTGCCCCGCAGTTAGG
S481+	CCTAACTGCGGAACACATACCCTTAATC
S481−	GATTAAGGGTATGTGTTCCGCAGTTAGG
*Sna*BI-vec−	AAATTACGTAATTTCGATAAGCCAGTCGAG
*Sna*BI-T7-EV71+	TTAATACGTATTAATACGACTCACTATAGGTTAAAACAGCCTGTGGGTTGCACC
TR2784+	GACATAACCGGTTATGCGCAGATGCGC
TR2784−	GCGCATCTGCGCATAACCGGTTATGTC
TR5000F+	ATAGCGTCGACACCGTGGTATCGG
tr-6300R−	AGGATGTGTCTTTTCTTGATGCCC

RT-PCR with primers *Sna*BI-T7-EV71+ and 1595R− and then cloned into pEV71(BrCr-TR) after partial digestion with *Sna*BI and *Bam*HI. This cDNA clone was designated pts-TR. For the construction of a cDNA clone of EV71(ts-ts-TR), a DNA fragment was obtained from the EV71(BrCr-ts) viral genome by RT-PCR with primers EV71-1500F+ and 3564− and then cloned into pts-TR after partial digestion with *Xma*I and *Bam*HI. This cDNA clone of mutant ts-ts-TR was designated pts-ts-TR. For the construction of a cDNA clone of EV71(BrCr-ts), a DNA fragment was obtained from the EV71(BrCr-ts) viral genome by RT-PCR with primers EV71-1500F+ and A2*Bam*HI− and then cloned into pts-ts-TR after digestion with *Xma*I and *Spe*I. This cDNA clone of mutant EV71(BrCr-ts) was designated pEV71(BrCr-ts). cDNA clones of EV71(ts2784) and EV71(TR2784) were prepared by site-directed mutagenesis by PCR with primers 2784+ and 2784− or with primers TR2784+ and TR2784−, using pEV71(BrCr-TR) or pEV71(BrCr-ts) as the template.

Next, we constructed cDNA clones of other *ts* mutants by introducing the corresponding mutations of the attenuation and *ts* determinants of PV Sabin strains (Fig. 3). For the construction of a cDNA clone of EV71(3′), site-directed mutagenesis was performed by PCR with primers 6153C+ and 6153C−, using pEV71(BrCr-TR) as the template. The clone obtained was subjected to site-directed mutagenesis with primers 7023AT+ and 7023AT−. Next, a DNA fragment was obtained by PCR with primers TR5000F+ and 7409G−, using the obtained clone as the template, and then cloned into pEV71(BrCr-TR) following digestion with *Spe*I and *Mlu*I. This cDNA clone of EV71(3′) was designated pEV71(3′). A cDNA clone of EV71(S1-3′) was obtained by site-directed mutagenesis by PCR with primers S480+ and S480−, using pEV71(3′) as the template. cDNA clones of EV71(S1), EV71(S2) and EV71(S3) were obtained by site-directed mutagenesis with primer set S480+ and S480−, with S481+ and S481− or with S472+ and S472−, respectively, using pEV71(BrCr-TR) as the template.

RNA transfection. RNA transcripts were obtained by using a RiboMAX large-scale RNA production system–T7 kit (Promega) with *Mlu*I-linearized DNA as the template. The *in vitro*-synthesized RNA transcripts were transfected onto the monolayer of Vero cells in six-well plates (Falcon) by the DEAE–dextran method and incubated at 35 °C in 2 ml 5% FCS/DMEM per well (Lu *et al.*, 1995). Cytopathic effects (CPE) on Vero cells were observed at 24 h post-transfection (p.t.). The cells were harvested when all of the cells exhibited CPE (4–7 days p.t.) and stored at −70 °C. The titres of recovered EV71 mutants were 10^5–10^6 50% cell culture infectious dose ($CCID_{50}$) ml^{-1}.

Virus titration. Virus titre was determined by measuring $CCID_{50}$ by the microtitration assay in Vero cells, as described previously

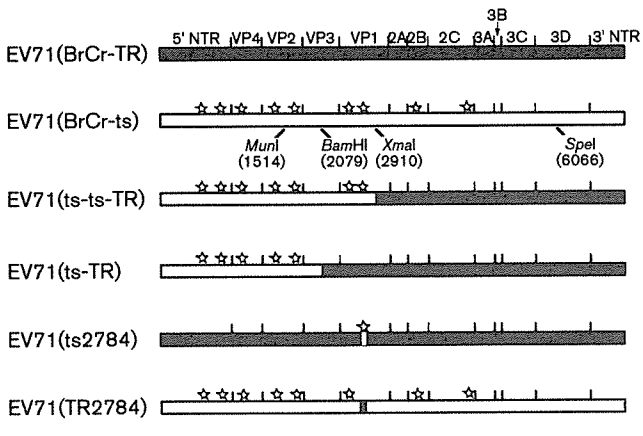

Fig. 1. Schematic diagram of the genomes of EV71 mutants. The sequences derived from the parental EV71(BrCr-TR) genome are represented as closed boxes and the sequences derived from the EV71(BrCr-ts) genome are represented as open boxes. The mutations observed in the EV71(BrCr-ts) genome are shown as open stars on each mutant genome. Restriction-enzyme sites are shown on the genome of EV71(BrCr-ts).

(Nagata *et al.*, 2002). Briefly, inoculated Vero cells were cultured at 36 °C for 10 days and observed for CPE. The value of $CCID_{50}$ was calculated according to the Behrens–Kärber method (Kärber, 1931).

Growth kinetics of EV71 mutants. Vero-cell monolayers were cultured in 96-well plates (Stripwell Plate; Corning) containing $2{\cdot}4 \times 10^4$ cells per well. EV71 mutants were inoculated into the cells at an m.o.i. of 1·0 ($2{\cdot}4 \times 10^4$ $CCID_{50}$ virus per well) and then incubated for 1 h at 36 °C. Inoculated cells were washed three times with 5% FCS/DMEM and then 100 µl 5% FCS/DMEM was added per well. The cells were incubated at 36 °C and harvested at the times indicated, from 2 to 12 h post-infection. The titre of virus was determined by $CCID_{50}$ measurement.

Plaque assay. The plaque assay was performed in 12-well plates (Falcon) containing a Vero-cell monolayer. Tenfold dilutions of virus solution were inoculated at 100 µl per well and incubated for 30 min at 36 °C. Then, 1 ml 2% FCS/modified Eagle's medium (MEM) containing 0·5% agarose ME (Iwai Kagaku) was added per well and incubated at 36 °C. After incubation for 4 days, 0·5% agarose ME in 2% FCS/MEM was overlaid on the first layer of agarose gel and further incubated for 3 days at 36 °C. The cells were fixed in formaldehyde and then stained with 0·5% crystal violet.

Temperature sensitivity. The temperature sensitivity of viruses was evaluated by determining the virus titre in Vero cells at 36 °C, which we used for the isolation of EV71 from clinical samples, and at a supraoptimal temperature, 39 °C. Temperature sensitivity was expressed as logarithmic difference of the $CCID_{50}$ values at 36 and 39 °C ($\Delta CCID_{50}$). We defined temperature sensitivity from 2·0 to 2·75 logarithmic difference as a slight *ts* phenotype, and those with more than 2·75 logarithmic difference as a strong *ts* phenotype.

Monkey neurovirulence test. Eight 17–21-year-old female cynomolgus monkeys were used for the determination of neurovirulence of EV71 mutants. All animal procedures were approved by the Committee for Biosafety and Animal Handling and the Committee for Ethical Regulation of the National Institute of Infectious Diseases, Japan. Animal care, breeding, virus inoculation and observation were performed in accordance with the guidelines of the committees.

Under light anaesthesia with ketaral and xylazine, 1 ml of each virus solution (containing 10^7 $CCID_{50}$ virus) was inoculated intravenously into the right tibial vein. Neurological manifestations of monkeys were checked daily for 10 days and autopsy was performed on day 10 post-inoculation (p.i.) after anaesthesia. Moribund monkeys before 10 days p.i. were sacrificed under deep anaesthesia. At autopsy, various parts of the central nervous system (CNS) were sampled for histopathological and virological analyses. The method of scoring the histological changes of the CNS (lesion score) was described previously (Nagata *et al.*, 2002). For virus isolation, a portion of excised tissues was stored at −80 °C. After freezing and thawing, 10% (w/v) tissue homogenates in MEM containing 2% FBS were centrifuged at 10 000 *g* for 10 min to remove cell debris. Supernatants were subjected to virus isolation in Vero cells. The cells were checked for CPE for 1 week and then blind passage was conducted for CPE-negative samples after freezing and thawing of the first-round passage. If CPE was not observed in the first- or second-round cultures, the result of virus isolation was recorded as negative.

RESULTS

Identification of the *ts* determinant of EV71(BrCr-ts)

To map the critical *ts* mutation of a *ts* variant of EV71 [EV71(BrCr-ts)], the entire genome sequence was determined and compared with that of a *tr* variant [EV71(BrCr-TR)]. The EV71(BrCr-ts) genome had nine nucleotide changes compared with that of EV71(BrCr-TR) and three of them were non-synonymous (Table 2). The three amino acid changes were located in capsid proteins VP2 and VP1 and in non-structural protein 2C (Table 2). We introduced these mutations into the infectious clone of EV71(BrCr-TR) to generate EV71 mutants, as described in Methods (Fig. 1). Viable viruses were recovered from all six clones, although their virus titres were different (Table 3).

To identify the *ts* determinant of EV71(BrCr-ts), temperature sensitivity was analysed for the parental and mutant

Table 2. Mutations of the EV71(BrCr-ts) genome

Mutations are from the BrCr-TR to the BrCr-ts genome.

Nucleotide position	Site of mutation	Nucleotide change	Amino acid change
491	5′ NTR	U to C	–
681	5′ NTR	U to C	–
848	VP4	C to U	–
1154	VP2	U to C	–
1707	VP2	G to A	Ala253 to Thr
2693	VP1	U to C	–
2784	VP1	U to C	Tyr116 to His
4034	2B	A to G	–
4990	2C	C to U	Thr305 to Ile

Table 3. Temperature sensitivity of EV71 mutants

EV71 mutant	Titre* at:		Δ36/39 °C
	36 °C	39 °C	
BrCr-TR	4·25	3·0	1·25
ts-TR	3·75	1·75	2·0
ts-ts-TR	2·5	ND (<0·5)	>2·0
BrCr-ts	3·5	ND (<0·5)	>3·0
ts2784	3·25	ND (<0·5)	>2·75
TR2784	5·0	3·0	2·0
S1	4·25	2·25	2·0
S2	4·0	1·75	2·25
S3	3·5	1·25	2·25
3′	4·0	ND (<0·5)	>3·5
S1-3′	4·0	ND (<0·5)	>3·5
3′ (4511, spinal cord)	4·0	ND (<0·5)	>3·5
3′ (4511, brainstem)	4·13	ND (<0·5)	>3·6
3′ (4512, brainstem)	4·0	ND (<0·5)	>3·5
S1-3′ (4514, spinal cord)	4·13	ND (<0·5)	>3·6

*Virus titre represents $\log_{10}(CCID_{50})$ in 10 μl virus sample.
ND, Not detected.

viruses by measuring the virus titre at 36 and 39 °C. Although the virus titres of EV71(ts2784) and EV71(ts-ts-TR) were much lower than that of the cDNA-derived EV71(BrCr-TR), even at 36 °C, the two mutants did not grow at 39 °C as well as EV71(BrCr-ts) (Table 3).

Introduction of a single nucleotide substitution at nt 2784 (U to C) into the EV71(BrCr-TR) genome resulted in impaired virus growth at 39 °C (Δ36/39 °C, >2·75 log) (Table 3). In contrast, a reciprocal substitution (C to U) at the same position of the EV71(BrCr-ts) genome resulted in only a slight *ts* phenotype [mutant EV71(TR2784); Δ36/39 °C, 2·0 log]. The plaque sizes of EV71(BrCr-TR) (cDNA-derived), EV71(ts-TR) and EV71(TR2784) were similar to that of the parental strain (Fig. 2a). On the other hand, EV71(ts-ts-TR) and EV71(ts2784) viruses showed smaller plaques, as well as EV71(BrCr-ts). These results indicate that a single nucleotide substitution at nt 2784 (VP1-Tyr116) is mainly responsible for both the *ts* and small-plaque phenotypes of EV71(BrCr-ts).

Construction of EV71 mutants carrying the *ts* determinants of PV Sabin strains

To generate *ts* mutants that could show growth comparable with that of the parental strain *in vitro*, we examined the effect of the *ts* determinants of PV Sabin strains. As illustrated in Fig. 3, we constructed a series of EV71(BrCr) mutants carrying corresponding nucleotide substitutions.

Firstly, we focused on the mutations in the 5′ non-translated region (NTR) within domain V of the genomes of Sabin strains (at nt 480, 481 and 472 for Sabin 1, 2 and 3, respectively; Fig. 3c), which act as a *ts* determinant and also as the major determinant of attenuation. The 5′ NTR of the EV71(BrCr) genome had a type I internal ribosome entry

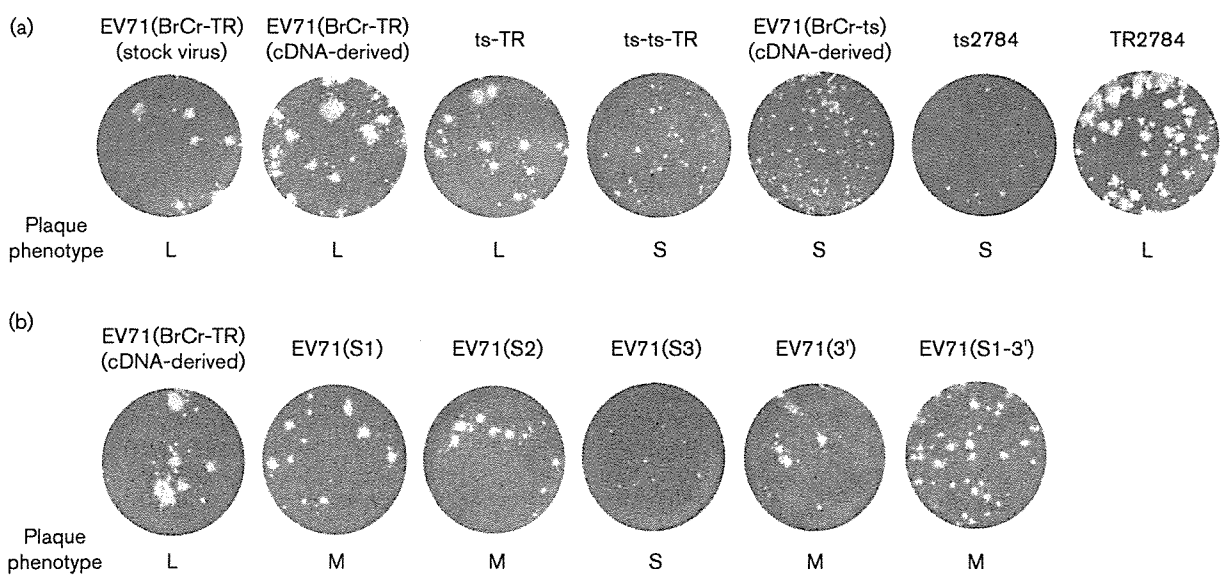

Fig. 2. Plaque phenotype of EV71 mutants. (a) Plaque phenotype of EV71 mutants derived from EV71(BrCr-ts). (b) Plaque phenotype of EV71 mutants carrying mutations of Sabin strains. Mutants EV71(S1), EV71(S2) and EV71(S3) have a mutation of the 5′ NTR of Sabin 1, 2 or 3, respectively. The EV71(3′) mutant has mutations in the $3D^{pol}$-coding region and 3′ NTR. The EV71(S1-3′) mutant contains all the mutations of the EV71(S1) and of EV71(3′) mutants. The assay was performed on Vero-cell monolayers incubated at 36 °C. L, Large-plaque phenotype; M, medium-plaque phenotype; S, small-plaque phenotype.

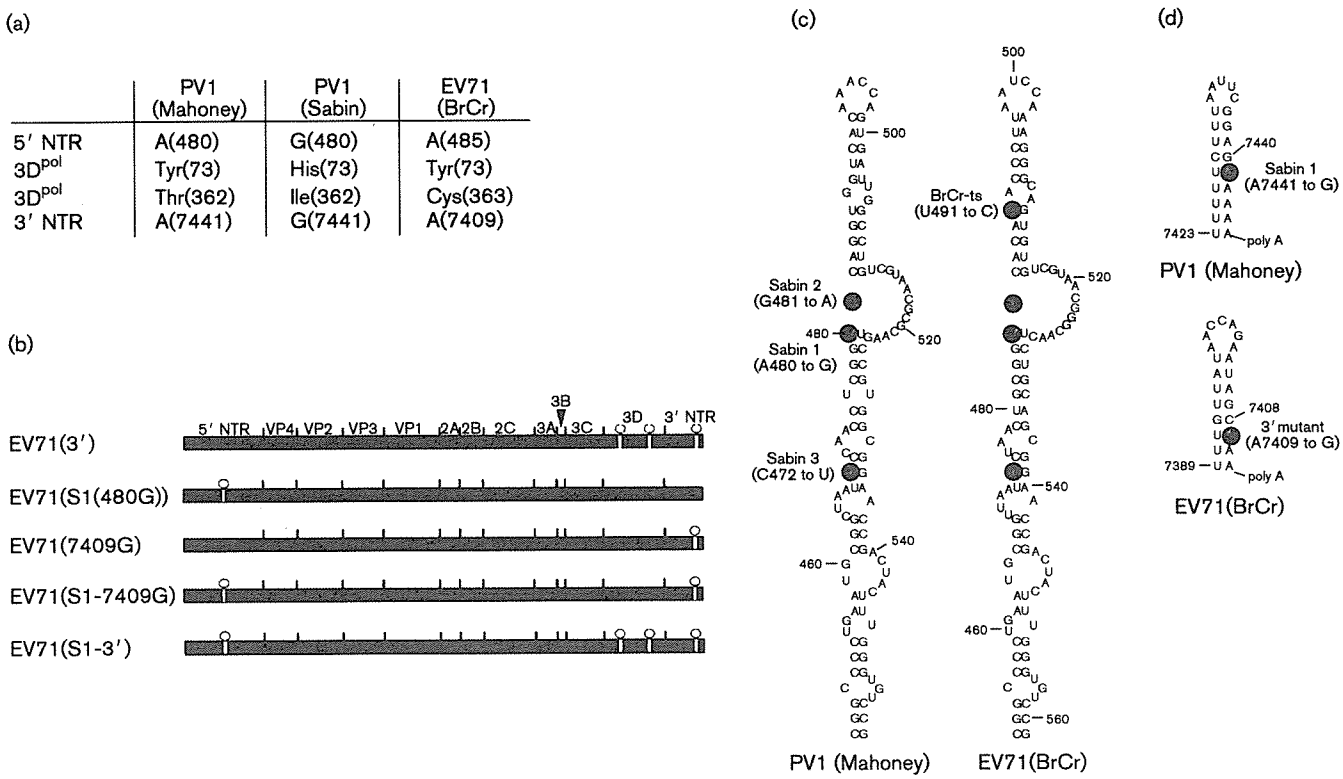

Fig. 3. EV71 mutants containing the corresponding mutations of the *ts* determinants of PV Sabin strains. (a) *ts* determinants of Sabin 1 introduced into the EV71(BrCr-TR) genome. The corresponding sites of the EV71(BrCr-TR) genome were substituted to those of Sabin 1. Numbers in parentheses represent the nucleotide position of the mutations on the genomes (5′ NTR and 3′ NTR) or the position of amino acids on the $3D^{pol}$ protein. (b) Schematic diagram of the genomes of EV71 mutants. Sequences derived from the parental EV71(BrCr-TR) genome are represented as closed boxes and mutations derived from the Sabin 1 genome are represented as open boxes with open circles. EV71 mutants carrying mutations of Sabin 2 or 3 in the 5′ NTR [mutants EV71(S2) and EV71(S3), respectively] are not shown. (c) RNA secondary-structure model of domain V in the IRES of PV1(Mahoney) and EV71(BrCr-TR), proposed by Pilipenko *et al.* (1989). (d) RNA structural models of the 3′ NTR of PV1(Mahoney) and that of EV71(BrCr-TR), obtained by the MFOLD 3.1 program (http://www.bioinfo.rpi.edu/applications/mfold/). The position of a *ts* determinant of Sabin 1 (G7441) and the corresponding site of the EV71(BrCr-TR) genome (A7409) are shown in closed circles.

site (IRES) activity, like that of the PV genome, and its structure model revealed an RNA secondary structure that was typical of the enterovirus IRES (Thompson & Sarnow, 2003). In a predicted secondary-structure model of domain V of the EV71(BrCr) genome, nt 485, 486 and 474 corresponded to the mutations of Sabin 1, 2 and 3, respectively (Fig. 3c).

Secondly, mutations corresponding to the major *ts* determinants of Sabin 1 in the 3D polymerase ($3D^{pol}$)-coding region and 3′ NTR were introduced into the infectious clone of EV71(BrCr-TR). The locations of the triple mutations in the EV71(BrCr-TR) genome (Tyr-73 and Cys-363 in $3D^{pol}$ and 7409A in the 3′ NTR) were predicted from the amino acid or nucleotide sequence alignment between EV71(BrCr-TR) and Sabin 1 (Fig. 3a).

As shown in Fig. 2(b), all mutants except the EV71(S3) mutant formed medium-sized plaques, which were smaller than those of the parental strain, but larger than those of the EV71(S3) mutant. The EV71(S3) mutant showed small-sized plaques, similar to those of the BrCr-ts variant. A PV1 mutant carrying a mutation of the 5′ NTR of the Sabin 3 genome showed a significant reduction in virus growth (Malnou *et al.*, 2003).

Next, we examined the temperature sensitivity of EV71 mutants by measuring the virus titre in Vero cells at 36 or 39 °C (Table 3). The results indicated that mutations in the 5′ NTR [EV71(S1) mutant] were involved in a slight *ts* phenotype (Δ36/39 °C, 2·0 log) and that triple mutations in the $3D^{pol}$-coding region and the 3′ NTR [EV71(3′) mutant] conferred a strong *ts* phenotype to EV71(BrCr-TR) (Δ36/39 °C, >3·5 log) (Table 3). Therefore, the triple mutations in the $3D^{pol}$-coding region and 3′ NTR could serve as the strong *ts* determinants in the genetic context of EV71(BrCr-TR), as well as in the Sabin 1 genome.

In vitro growth kinetics of three EV71 mutants that were

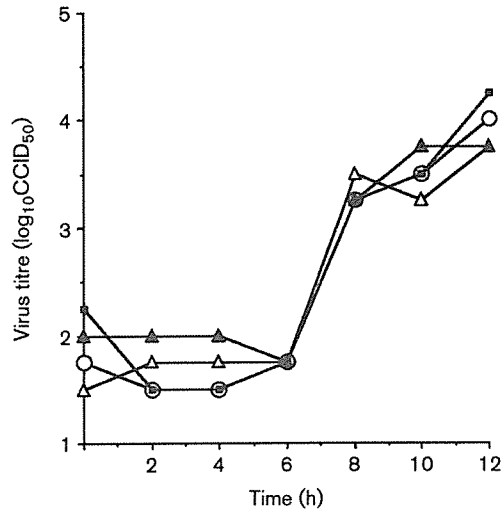

Fig. 4. Growth kinetics of EV71 mutants (BrCr-TR, ○; TR2784, ■; 3′, △; S1-3′, ▲).

used for the neurovirulence test in monkeys (see below) were measured in Vero cells at 36 °C (Fig. 4). All of the mutants showed growth kinetics similar to those of the parental EV71(BrCr-TR) strain.

The above results indicated that the introduction of *ts* mutations of the Sabin 1 genome into the 5′ NTR, $3D^{pol}$ coding region and 3′ NTR of the EV71(BrCr-TR) genome effectively generated *ts* mutants that retained *in vitro* growth kinetics comparable with those of the parental strain.

Neurovirulence of EV71 mutants in cynomolgus monkeys

We determined the neurovirulence of cDNA-derived EV71 mutants in cynomolgus monkeys by intravenous inoculation. Two monkeys inoculated with 10^7 $CCID_{50}$ of the cDNA-derived EV71(BrCr-TR) clone became moribund within 6 days p.i., similar to those inoculated with the parental EV71(BrCr-TR) strain (Nagata *et al.*, 2004). The cDNA-derived EV71(BrCr-TR) induced characteristic neurological manifestations, such as tremor and ataxia, from days 4 and 5 p.i., respectively (Table 4). In contrast, monkeys inoculated with the same dose of cDNA-derived mutants [EV71(TR2784), EV71(3′) and EV71(S1-3′)] showed mild neurological manifestations and histological changes. None of the six monkeys that were inoculated with EV71 mutants became moribund or showed ataxia within 10 days p.i. Moreover, mutant viruses in the CNS were detected in the spinal cord and in the brainstem, whilst the parental strain showed disseminated distribution (Nagata *et al.*, 2002). The total lesion scores of mutants were decreased (Table 4). For the infection of EV71(S1-3′), no viable virus was recovered from the CNS of an inoculated monkey on day 10 p.i. (monkey 4513) and the virus was only isolated from the spinal cord in another inoculated monkey (4514). Thus, the infection of EV71(S1-3′) resulted in the most limited clinical manifestations and in restricted distribution of the virus in the CNS.

To examine the selection pressure in the CNS of monkeys against the temperature sensitivity of EV71, we examined the *ts* phenotype of EV71(3′) and EV71(S1-3′) viruses recovered from the CNS of inoculated monkeys. All of the recovered viruses retained a strong *ts* phenotype, similar to that of the original EV71(3′) and EV71(S1-3′) viruses (Table 3). This suggests that the temperature sensitivity of EV71 is not the critical factor to achieve infection in the CNS of monkeys.

DISCUSSION

The BrCr strain was isolated from an aseptic meningitis patient as the prototype strain of EV71 (Schmidt *et al.*, 1974). Its entire genome sequence is far from that of PV (Brown & Pallansch, 1995). Epidemiological analyses of

Table 4. Summary of the clinical manifestations of monkeys and virus isolation

Virus	Monkey no.	Clinical manifestation*			Virus isolation				Lesion score
		Tremor	Ataxia	Moribund	Spinal cord	Brainstem	Cerebellum	Cerebrum	
EV71(BrCr-TR)	4507	Day 4	Day 5	Day 6	+	+	+	+	2·29
	4508	Day 4	Day 5	Day 6	+	+	+	+	2·51
EV71(TR2784)	4509	Day 7	–	–	+	+	–	–	0·78
	4510	Day 6	–	–	+	–	–	+	0·72
EV71(3′)	4511	Day 9	–	–	+	+	–	–	1·07
	4512	Day 8	–	–	–	+	–	–	0·59
EV71(S1-3′)	4513	Day 9	–	–	–	–	–	–	0·0
	4514	Day 9	–	–	+	–	–	–	1·09

*Time post-inoculation when the monkey started to show the clinical manifestation is indicated. Monkeys were sacrificed at day 6 (for 4507 and 4508) or day 10 (for 4509, 4510, 4511, 4512, 4513 and 4514) post-inoculation.

EV71 revealed that the BrCr strain is not related closely to two major genogroups of EV71 (B and C); thus, it is the sole member of genogroup A (Brown *et al.*, 1999). Previous studies showed that cynomolgus monkeys inoculated with the BrCr strain exhibited typical neurological manifestations and histopathological legions after intraspinal or subcutaneous inoculation (Hashimoto & Hagiwara, 1983; Hashimoto *et al.*, 1978). Furthermore, a cell culture-selected *ts* variant of the BrCr strain [EV71(BrCr-ts)] had been generated (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) and was one of the initial candidates of attenuated EV71 strains. Therefore, we first examined this laboratory variant of the BrCr strain to generate attenuated EV71 strains.

To generate attenuated strains of EV71, we focused on temperature sensitivity as an *in vitro* marker. In general, temperature sensitivity of PV vaccine strains serves as an *in vitro* phenotypic marker of attenuation. However, the extent of temperature sensitivity does not necessarily correlate with the extent of attenuation of PV (Bouchard *et al.*, 1995; Christodoulou *et al.*, 1990; Georgescu *et al.*, 1995; Macadam *et al.*, 1989, 1991; Minor, 1992; Omata *et al.*, 1986). Moreover, the *ts* revertant could retain its attenuated phenotype, suggesting that there is no direct link between the arbitrary *ts* phenotype and attenuation (Rowe *et al.*, 2000). Thus, among the *ts* determinants, only some could serve as the attenuation determinants. We examined the genome of EV71(BrCr-ts) to identify the *ts* determinant that could be an initial candidate of the attenuation mutation. We identified a mutation at nt 2784 (VP1-Tyr116 to His) as the *ts* determinant of EV71(BrCr-ts). However, this mutation also conferred a small-plaque phenotype (Fig. 2a). Alignment of the amino acid sequences of EV71(BrCr-TR) and PV1(Mahoney) in the VP1 region suggests that the corresponding amino acids of PV1(Mahoney) would be Thr115, which is located near an interface of protomers between the adjoining VP1, via Gln233 of VP3 (Hogle *et al.*, 1985). Tyr116 of the BrCr-ts strain is located in a region of VP1 that is highly conserved among different EV71 strains (data not shown). One of the attenuation determinants of Sabin 3 is located in the capsid protein (Phe91 of VP3) near an interface between protomers (Minor *et al.*, 1989; Westrop *et al.*, 1989) and affects the virus-assembly process in a temperature-dependent manner (Minor *et al.*, 1989). Therefore, we could not obtain *ts* mutants with a growth activity comparable with that of the parental strain by utilizing the *ts* determinant of EV71(BrCr-ts).

We examined the effect of the *ts* determinants of Sabin strains on the temperature sensitivity of EV71. We focused on *ts* determinants of Sabin 1 that are located in structurally and functionally conserved regions among enteroviruses, i.e. the 5′ NTR, $3D^{Pol}$ and 3′ NTR (Kawamura *et al.*, 1989; Omata *et al.*, 1986). Between PV and EV71, the predicted secondary structures of 5′ NTR and 3′ NTR are highly conserved (Fig. 3). Mutations in the 5′ NTR of Sabin strains cause a reduction in the IRES activity (Muzychenko *et al.*, 1991; Svitkin *et al.*, 1985, 1988, 1990) and act as a *ts* determinant, as well as the major attenuation determinant (Bouchard *et al.*, 1995; Christodoulou *et al.*, 1990; Evans *et al.*, 1985; Georgescu *et al.*, 1995; Macadam *et al.*, 1989, 1991; Minor, 1992; Omata *et al.*, 1986). The attenuation determinant in the 5′ NTR of Sabin 3 leads to translation defects in neuronal and non-neuronal organs *in vivo* (Kauder & Racaniello, 2004). However, in PV1 infection, the reduced level of translation was not the main determinant of attenuation (Arita *et al.*, 2004). Therefore, the mechanism of the attenuation effect of the mutations in the 5′ NTR of Sabin strains remained to be further elucidated.

The mutation of nt 6203 (His73 of the $3D^{Pol}$ protein) affects the oligomerization and uridylylation of viral protein $3B^{VPg}$ in a temperature-sensitive manner (Paul *et al.*, 2000). Another mutation of nt 7071(Ile362 of the $3D^{Pol}$ protein) is required for the *ts* phenotype of Sabin 1 (Georgescu *et al.*, 1995). The amino acid residue at 362 of the $3D^{Pol}$ protein is located at interface I of $3D^{Pol}$ (Hansen *et al.*, 1997); together with the mutation of nt 6203, this amino acid residue may also affect the oligomerization of the $3D^{Pol}$ protein. The mutation in the 3′ NTR, which is located in a stem–loop structure (Fig. 3d), has been suggested to have an effect on the *ts* phenotype, along with other mutations of $3D^{Pol}$ (Georgescu *et al.*, 1995) or the mutation in the 5′ NTR (Christodoulou *et al.*, 1990), by an unknown mechanism. EV71 mutants with mutations in the $3D^{Pol}$-coding region and 3′ NTR showed a strong *ts* phenotype, in contrast to a slight *ts* phenotype that was caused by mutations in the 5′ NTR (Table 3). These results indicate that EV71 and PV1 share common genetic determinants of temperature sensitivity, based at least in part on the conserved replication machinery.

We examined the neurovirulence of EV71 mutants by intravenous inoculation into cynomolgus monkeys. The inoculation route is a critical factor for neurological disorders of EV71 infection in the monkey model. After intraspinal inoculation of the BrCr strain, monkeys showed flaccid paralysis, but not tremor or ataxia, due to intraspinal spread of the virus (Nagata *et al.*, 2004). Therefore, we applied an intravenous inoculation model to avoid direct involvement of the inoculated virus in the CNS, which would not occur in the natural course of EV71 infection.

All three EV71 mutants caused mild neurological symptoms in monkeys after intravenous inoculation, but did not cause lethal neurological disorders that were observed in infection with EV71(BrCr-TR) (Table 4). Interestingly, mutant EV71(TR2784) had only a minor *ts* phenotype, but showed a slightly attenuated phenotype. Infections caused by EV71(TR2784) or EV71(3′), which showed a strong *ts* phenotype, resulted in a similar lesion score, despite their different temperature sensitivity. Therefore, temperature sensitivity could not serve as an absolute indicator of the attenuated neurovirulence of EV71. The distribution of EV71 mutants in the CNS was also restricted, compared

with that of the parental strain. Among the mutants examined, EV71(S1-3′) resulted in the most limited clinical manifestations and in a restricted distribution of the virus in the CNS. A cumulative effect of the mutations in the 5′ NTR and $3D^{pol}$-coding region on the attenuation of PV has been reported (Tardy-Panit *et al.*, 1993). However, infection by EV71(S1-3′) was still associated with minor neurological symptoms in inoculated monkeys and virus was isolated from the spinal cord. Thus, mutation of Sabin 1 in the 5′ NTR could not completely suppress infection by EV71 in the spinal cord of monkeys inoculated by the intravenous route. This observation might suggest that the spinal cord serves as a preferred site of infection for both PV and EV71 (Arita *et al.*, 2004). However, because of the different mechanisms of pathogenesis, the effect of mutations of Sabin 1 on the infection of EV71 remain to be further elucidated.

EV71 antigen was detected in the early phase of infection, followed by clinical manifestations, in monkeys (Nagata *et al.*, 2002). It was possible that EV71 mutants replicated in the CNS in a disseminated manner, as well as the parental virulent strain, in the early phase of infection. Therefore, the tissue specificity of EV71 mutants in the CNS remains to be further studied.

In conclusion, we have generated a cDNA-derived virulent strain of EV71 that maintained the *in vitro* and *in vivo* phenotypes of a neurovirulent strain, EV71(BrCr-TR). Based on this infectious cDNA clone of EV71, we identified several molecular determinants conferring *ts* and attenuated phenotypes to EV71. The cDNA-derived virulent and attenuated strains of EV71 should serve as a valuable tool for the elucidation of EV71-induced severe neurological disorders and development of a vaccine strain.

ACKNOWLEDGEMENTS

We are grateful to Ms A. Harashima, Ms Y. Sato and Ms J. Wada for their excellent assistance. This work was supported by grants-in-aid from the Japan Society for Promotion of Science and from the Ministry of Health, Labour and Welfare, and by a grant for health research from the Regional Office for the Western Pacific, World Health Organization.

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

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Lancet 2005; 366: 394–96
Published online May 3, 2005
DOI: 10.1016/S0140-6736(05)66386-6

See Comment page 351

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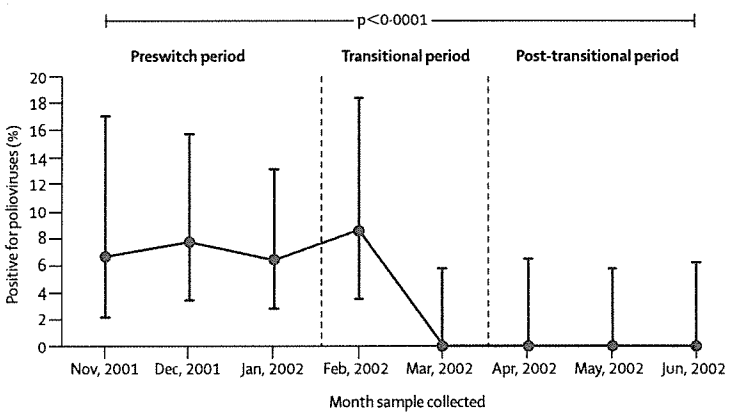
On Feb 1, 2002, inactivated poliomyelitis vaccines replaced live-attenuated oral poliovirus vaccine (OPV) in New Zealand's immunisation schedule, allowing systematic monitoring of OPV virus circulation. Findings of paediatric-inpatient surveillance indicate that 7% of children excreted polioviruses before 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]

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



	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**

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 Lancet Online for webappendix 1

See Lancet Online for webappendix 2 and webappendix 3

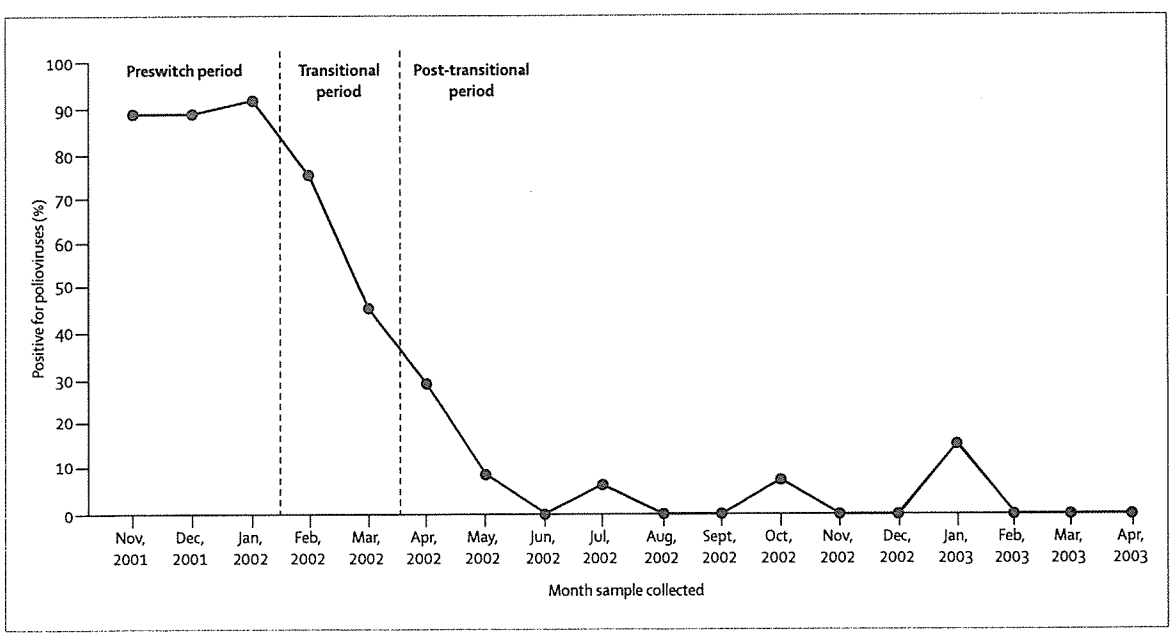

Figure 2: Poliovirus prevalence between November, 2001, and April, 2003, by environmental surveillance

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.

Acknowledgments

We thank David Wood, David Featherstone, and Walter Dowdle for their helpful advice; Elizabeth Sneyd and Ruth Pirie for help with data analysis; Andi Utama for sequence analysis; the paediatric nurses at Middlemore, Waikato, and Wellington Hospitals for stool and questionnaire collection; staff at the Porirua, Hamilton, and Mangere sewage treatment plants for sewage collection; and staff at the Institute of Environmental Science and Research who provided logistic, administrative, and technical support.

The study was supported by a grant (B3/181/124) from the Department of Vaccines and Biologicals, WHO. Surveillance of enterovirus and acute flaccid paralysis received financial support from the New Zealand Ministry of Health who permitted publication of the relevant data. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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JOURNAL OF VIROLOGY, Oct. 2005, p. 12650–12657 Vol. 79, No. 20
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.20.12650–12657.2005
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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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Received 14 April 2005/Accepted 20 July 2005

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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† Supplemental material for this article may be found at http://jvi.asm.org.

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).

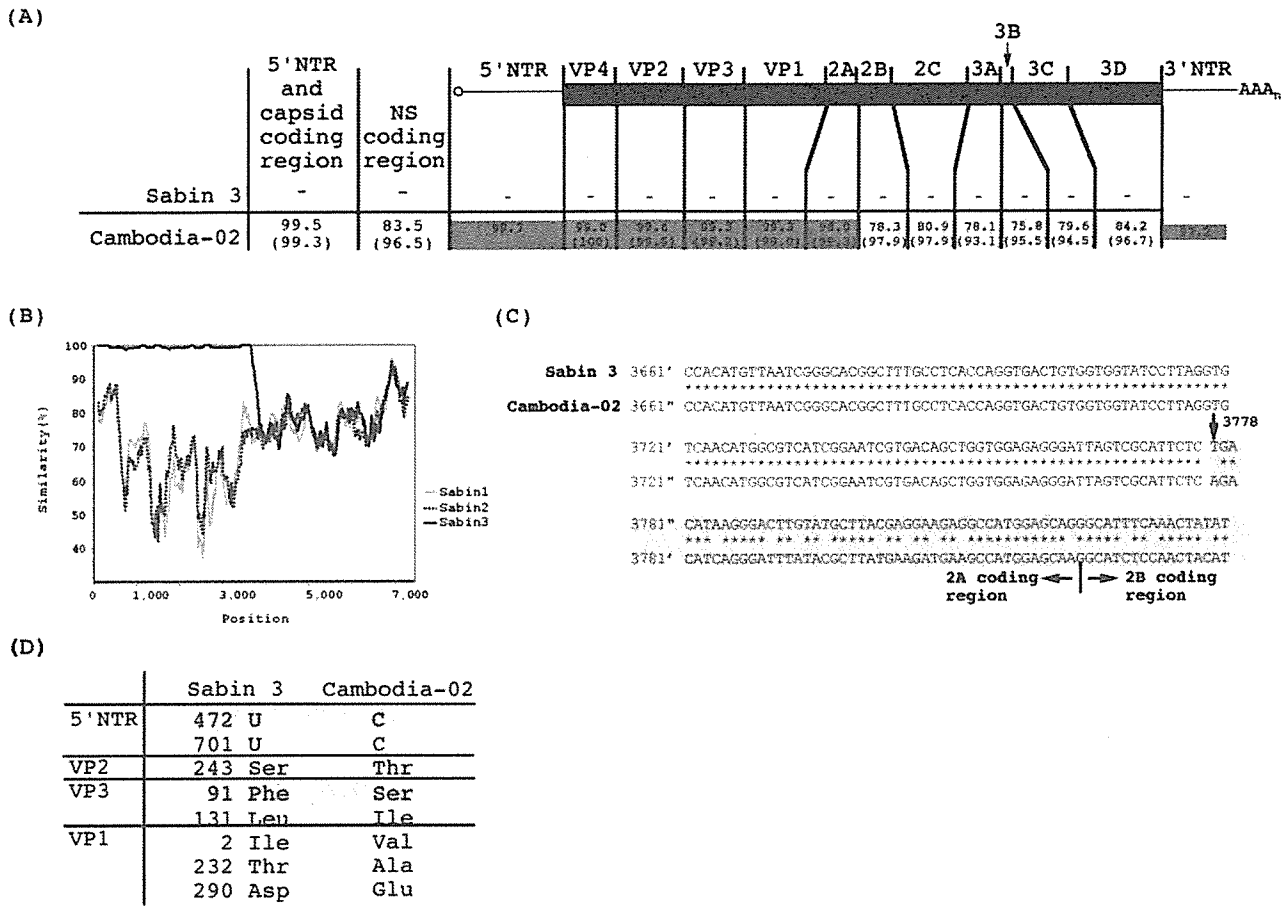
Primers used for the sequence analysis are listed on Table 1. Primers UG16 and UC12 were used for the analysis of a part of the 3D^{pol} coding region (20). Primers EVP4 and OL68-1 were used for the analysis of the VP4 coding region (39, 43). Primers 2A2+ and 2C− were designed and used for the analysis 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).



	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,