299	堀切勇児(東京大学 薬 製剤), 他	ポリオウイルス受容体の体内分布に基づいたウイルスの組織移行性の機構論的解析	Drug Delivery System(0913-5006)9巻4号 Page263(1994.07) 会議録
300	木村晋亮(石川県保健環境センター), 尾西一, 小坂恵, 他	小児上気道疾患にみられたウイルスの重複分離	石川県保健環境センター年報31号 Page106-111(1994.12) 原著論文
301	木添和博(宮崎県衛生環境研究所), 吉野修司, 山本正悟, 岩城詩子	宮崎県の感染症発生動向調査事業におけるウイルス検出報告(2000年)	宮崎県衛生環境研究所年報(0917-3331)12号 Page61-66(2001.09) 原著論文
302	目黒英典(帝京大学医学部附属市原病院 小児科)	指定伝染病　急性灰白髄炎	治療(0022-5207)77巻10号 Page2675-2678(1995.10) 解説/特集
303	野田雅博(広島県保健環境センター), 徳本静代, 豊田安基江, 他	広島県におけるポリオウイルスの血清疫学的研究	臨床とウイルス(0303-8092)24巻5号 Page409-413(1996.12) 原著論文
304	野本明男(東京大学 医・微生物), 柳谷朗子, 大岡静衣	ウイルス感染の細胞生物学:抗ウイルス剤開発の基盤として　ポリオウイルスの神経病原性発現機構	日本薬学会124年会講演要旨集(0918-9823)1号 Page220(2004.03) 会議録
305	野本明男(東京大学 大学院医学系研究科 微生物学)	【新世紀の感染症学　ゲノム・グローバル時代の感染症アップデート】　感染症の遺伝子学　ウイルスの遺伝子学　プラス1本鎖RNAウイルス ピコルナウイルス科　ポリオウイルス	日本臨床(0047-1852)61巻増刊3 新世紀の感染症学 Page458-462(2003.03) 解説/特集
306	野本明男(東京大学医科学研究所)	ヒト疾患モデルの開発とその感染症への応用 ポリオウイルス病原性と感染モデルマウス	日本細菌学雑誌(0021-4930)54巻1号 Page67(1999.02) 会議録
307	野本明男(東京大学医科学研究所)	【生ウイルスワクチンの動物モデル,ヒトの感染病理】　ポリオウイルス感受性トランスジェニックマウス	臨床とウイルス(0303-8092)26巻1号 Page2-6(1998.03) 解説/特集

308	野本明男(東京大学医科学研究所)	ポリオウイルスの神経特異的病原性発現機構	生化学(0037-1017)69巻11号 Page1324(1997.11)会議録
309	野本明男(東京大学医科学研究所)	【ウイルス】　ポリオ	からだの科学(0453-3038)198号 Page53-56(1998.01) 解説/特集
310	野本明男(東京大学医科学研究所)	【ウイルス最前線】　ポリオウイルス　ポリオウイルスのトロピズム	実験医学(0288-5514)15巻19号 Page2394-2397(1997.12) 解説/特集
311	野本明男(東京大学医科学研究所)	ポリオウイルス感受性トランスジェニックマウス	臨床とウイルス(0303-8092)25巻2号 Pages3(1997.05) 会議録
312	野本明男(東京大学医科学研究所)	ウイルスの性質と遺伝子ベクターとしての利点　ポリオウイルス	治療学(0386-8109)31巻4号 Page459-463(1997.04) 解説
313	野本明男(東京大学医科学研究所)	ウイルスワクチンの新しい展開　ポリオウイルスワクチン	BIO Clinica(0919-8237)11巻9号 Page640-644(1996.08) 解説/特集
314	野本明男(東京大学医科学研究所)	ベールを脱いだ転写後調節の世界　IRESによる翻訳開始の新しい機構　ポリオウイルス病原性から探る	細胞工学(0287-3796)15巻8号 Page1106-1114(1996.08) 解説/特集
315	野本明男(東京大学医科学研究所)	ヒトポリオウイルス受容体遺伝子導入マウス　サルに代わる感染実験動物モデルとして	医学のあゆみ(0039-2359)171巻2号 Page110-111(1994.10) 原著論文
316	野本明男(東京大学医科学研究所)	ポリオウイルス感染性トランスジェニックマウス	Molecular Medicine(0918-6557)臨増マニュアル疾患モデルマウス Page333-337(1994.12) 原著論文

317	野本明男(東京大学医科学研究所)	ポリオウイルス感受性トランスジェニックマウスの開発 ポリオウイルス研究の歴史と実験動物	実験動物(0007-5124)42巻3号 Page287-290(1993.07) 会議録
318	野本明男(東京大学医科学研究所)	ウイルス感染とレセプター ポリオウイルス感染	ファルマシア(0014-8601)28巻7号 Page731-735(1992.07) 原著論文
319	野本明男(東京大学医科学研究所)	ポリオウイルスの病原性とその制御	実験医学(0288-5514)9巻16号 Page2122-2127(1991.11) 原著論文
320	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ポリオウイルスの組織特異性	ウイルス(0042-6857)42巻1号 Page85-86(1992.06) 会議録
321	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ウイルス疾患の制御 ポリオウイルスの組織特異的病原性とワクチン開発	BIO medica6巻10号 Page959-963(1991.09) 原著論文
322	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	神経系のウイルス感染症 ポリオウイルスの病原性に関する分子生物学的研究の進歩	日本医学会総会23回会誌(0285-6131)1号 Page247(1992.02) 原著論文
323	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ポリオウイルスの医生物学	代謝(0372-1566)27巻臨増 Page43-48(1990.06) 原著論文
324	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ポリオウイルス神経毒性発症の分子機構	阿蘇シンポジウム14回記録 Page43-50(1991.09) 原著論文
325	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ワクチンによる免疫療法 病原ウイルスの感染防御抗原とコード遺伝子 ポリオウイルスの感染防御抗原とコード遺伝子	日本臨床(0047-1852)48巻増刊 臨床免疫 下 Page640-645(1990.08) 原著論文

326	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ポリオウイルスの神経毒性発現	化学と生物(0453-073X)28巻1号 Page40-45(1990.01) 原著論文
327	野本明男(東京都医学研究機構東京都臨床医学総合研究所)	ポリオウイルス感染の分子遺伝学的解析	薬学雑誌(0031-6903)109巻9号 Page622-635(1989.09) 原著論文
328	野本明男,柳谷朗子,大岡静衣：東京大学 医・微生物	【多発性硬化症　revisited】　多発性硬化症の病因・病理　多発性硬化症の動物モデル	日本薬学会124年会講演要旨集,,0918-9823,,1,220,2004.03 会議録
329	矢彦沢裕之(信州大学 第3内科), 井上敦, 高昌星, KimByung S.	構造非依存性抗体エピトープによるタイラー脳脊髄炎ウイルス誘導免疫性脱髄疾患(TMEV-IDD)の抑制	臨床神経学(0009-918X)39巻1号 Page257(1999.01) 会議録
330	矢野一好(東京都立衛生研究所), 吉田靖子, 新開敬行, 他	エンテロウイルス感染症の流行 高度処理下水および河川水からの腸管系ウイルスの分離	臨床とウイルス(0303-8092)21巻1号 Page17-21(1993.03) 原著論文
331	矢野一好(東京都立衛生研究所), 吉田靖子, 藪内清	マガキに添加したポリオウイルスのカチオン性高分子化合物による回収実験	東京都立衛生研究所研究年報(0082-4771)40号 Page26-31(1989.12) 原著論文
332	矢野一好(東京都立衛生研究所), 竹内正博, 真木俊夫	エアロゾルと共に飛散させたウイルスの「セルロース吸着・凝集法」による捕集実験	東京都立衛生研究所研究年報(0082-4771)49号 Page23-28(1999.01) 原著論文
333	矢野拓弥(三重県科学技術振興センター保健環境研究所), 福田美和, 石井淳子, 川田一伸, 杉山明, 櫻井悠郎, 松本正	1998年度の日本脳炎,インフルエンザ,ポリオ流行予測調査の解析	三重県保健環境研究所年報(衛生部門)(1345-4595)1号 Page49-56(2000.03) 原著論文
334	柳原敬(岩手医科大学 細菌), 松本一郎, 川名林治	RD細胞由来のRD-9H8細胞のエンテロウイルス感受性と中和試験への応用	感染症学雑誌(0387-5911)66巻4号 Page484-489(1992.04) 原著論文

335	柳谷朗子(東京大学 大学院医学系研究科 微生物学 講座), 大岡静衣, 野本明男	【神経系ウイルス感染症】 神経細胞が示すポリオウイルス抵抗性	ウイルス(0042-6857)53巻1号 Page41-49(2003.06) 解説/特集
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338	有田峰太郎(東京大学医科学研究所), 他	in vitroの系を用いたポリオウィルス感染初期過程の解析	生化学(0037-1017)68巻7号 Page650(1996.07) 会議録
339	立川真理子(日本大学 薬), 済田清隆, 手塚雅勝, 他	二酸化塩素によるポリオウイルスの不活化作用	衛生化学(0013-273X)39巻6号 Page572-576(1993.12) 原著論文
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341	鈴木葉子(東京女子医科大学附属第二病院 小児科), 他	川崎病患児からのウイルス分離のこころみ(第2報)	Progress in Medicine(0287-3648)18巻2号 Page389(1998.02) 会議録
342	露崎淳(信州大学 第3内科), 井上敦, 高昌星, 池田修一, 京ヶ島守	Theiler脳脊髄炎ウイルスにより誘導される免疫性脱髄疾患(TMEV-IDD)におけるLow-molecular weight heparin(LMWH)の影響について	臨床神経学(0009-918X)39巻12号 Page1313(1999.12)会議録
343	和田義明(東京医科歯科大学神経内科), 他	培養グリア細胞を用いたTheilerウイルス(TMEV)感染に関する検討	臨床神経学(0009-918X)37巻12号 Page1260(1997.12)会議録

344	和田義明(東京医科歯科大学 神経内科), 他	Theilerウイルスの神経毒性に関する検討 Capsid protein VP2の役割について	臨床神経学(0009-918X)36巻12号 Page1474(1996.12)会議録
345	濟田清隆(横浜市衛研), 立川眞理子, 手塚雅勝, 澤村良二	次亜塩素酸のポリオウイルス不活化に対するイソシアヌル酸の影響	衛生化学(0013-273X)44巻6号 Page442-450(1998.12) 原著論文
346	濱田洋文(札幌医科大学 分子医学)	【遺伝子治療の最前線　開発が進む次世代ベクター】　次世代ウイルスベクターの開発と遺伝子治療への応用	細胞工学(0287-3796)20巻9号 Page1216-1221(2001.08) 解説/特集
347	濱野雅子(岡山県環境保健センター), 葛谷光隆, 藤井理津志, 小倉肇, 内川洋之	岡山県のポリオとインフルエンザの感染源調査　平成9年度伝染病流行予測調査	岡山県環境保健センター年報(0914-9309)22号 Page13-15(1998.12) 原著論文
348	濱野雅子(岡山県環境保健センター), 葛谷光隆, 藤井理津志, 他	伝染病流行予測調査(平成7年度)	岡山県環境保健センター年報(0914-9309)20号 Page38-39(1996.12) 原著論文
349	濱野雅子(岡山県保健福祉部), 葛谷光隆, 藤井理津志, 他	伝染病流行予測調査(平成8年度)	岡山県環境保健センター年報(0914-9309)21号 Page37-38(1997.12) 原著論文
350	萬木章(岡山大学 小児科), 小田慈, 清野佳紀	エンテロウイルス群による神経感染症　ポリオウイルス,コクサッキーウイルス,エコーウイルス,エンテロウイルスtype68-72	日本臨床(0047-1852)55巻4号 Page849-854(1997.04) 解説/特集

研究成果の刊行に関する一覧表

Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, Iwasaki T, Miyamura T. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. J Gen Virol 86: 1391-1401, 2005.

Huang QS, Greening G, Baker M, Grimwood K, Hewitt J, Hulston D, Webber L, Fitzsimons A, Garrett N, Graham D, Lennon D, Shimizu H, Miyamura T, Pallansch M. No longterm oral polio vaccine virus persistence after its removal from the immunisation schedule in New Zealand. Lancet (in press).

Kuramitsu M, Kuroiwa C, Yoshida H, Miyoshi M, Okurmura J, Shimizu H, Narantuya L, Bat-Ochir D. Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: prevalence, intrafamilial spread, and risk factors for infection. Epidemiol Infect (in press).

Shimizu H, Thorley B, Paladin FJ, Brussen KA, Stambos V, Yuen L, Utama A, Tano Y, Arita M, Yoshida H, Yoneyama T, Benegas A, Roesel S, Pallansch M, Kew O, Miyamura T. Circulation of type 1 vaccine-derived poliovirus in the Philippines in 2001. J Virol 78: 13512-13521, 2004.

Arita M, Shimizu H, Miyamura T. Characterization of in vitro and in vivo phenotypes of poliovirus type 1 mutants with reduced viral protein synthesis activity. J Gen Virol 85: 1933-1944, 2004.

Nagata N, Iwasaki T, Ami Y, Tano Y, Harashima A, Suzaki Y, Sato Y, Hasegawa H, Sata T, Miyamura T, Shimizu H. Differential localization of neurons susceptible to enterovirus 71 and poliovirus type 1 in the central nervous system of cynomolgus monkeys after intravenous inoculation. J Gen Virol 85: 2981-2989, 2004.

Kew OM, Wright PF, Agol V, Delpeyroux IF, Shimizu H, Nathanson N, Pallansch MA. Circulating vaccine-derived polioviruses: current state of knowledge. Bull World Health Organ 82:16-23, 2004.

Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, Pongsuwanna Y, Miyamura T. Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. Pediatr Int 46: 231-235, 2004.

清水博之，武田直和，宮村達男．ポリオワクチン．小児科診療 67: 1843-1849, 2004.

清水博之，武田直和，宮村達男．不活化ポリオワクチン．綜合臨床 53：1860-1865，2004.

Kuramitsu M, Kuroiwa C, Yoshida H, Miyoshi M, Okurmura J, Shimizu H, Narantuya L, Bat-Ochir D. Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: prevalence, intrafamilial spread, and risk factors for infection. Epidemiol. Infect. 143 (in press)

Hansman GS, Kuramitsu M, Yoshida H, Katayama K, Takeda N, Ushijima H, Surenkhand G, Gantulga D, Kuroiwa C. Viral gastroenteritis in Mongolian infants. Emerging Infect. Dis. 11：181-182，2005.

高尾信一，下薗広行，柏　弘，松原啓太，坂野　堯，池田政憲，岡本尚子，吉田　弘，島津幸枝，福田伸治．本邦において初めて流行が確認された小児の human metapneumovirus 感染症の臨床的，疫学的解析．感染症学雑誌，78：129-137，2004.

Ida-Hosomuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita T, Taya C, Yonekawa H, Koike S. Alpha/beta infeterferon controls tissue tropism and pathogenicity of poliovirus. J Virol 79：4460-4469，2005.

小池　智．ピコルナウイルスベクター．Mebio 21：42-49，2004.

小池　智．ポリオウイルスの標的組織特異性決定機構．ウイルス 54：205-212，200

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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.

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−	ACTGGAATTCTTTTTTTTTTTTTTTTTTTTTTV
EV71-1500F+	GGATTAATCTNCGNACCAACAA
EV71-2800F+	TTCACNTACATGCGCTTTGANGC
EV71-A2−	CCATCGATGGTTTTTTTTTTTTTTTTTTTTTTTTTTGCTATTCTGG
*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

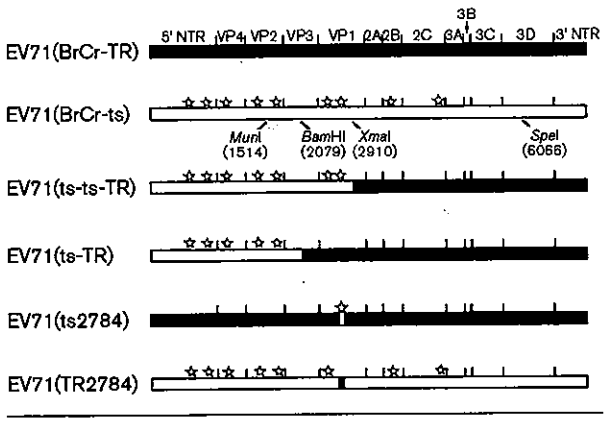

Fig. 1. Schematic diagram of the genomes of EV71 mutants. The sequences derived from the parental EV71(BrCr-TR) genome are represented as closed boxes and the sequences derived from the EV71(BrCr-ts) genome are represented as open boxes. The mutations observed in the EV71(BrCr-ts) genome are shown as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

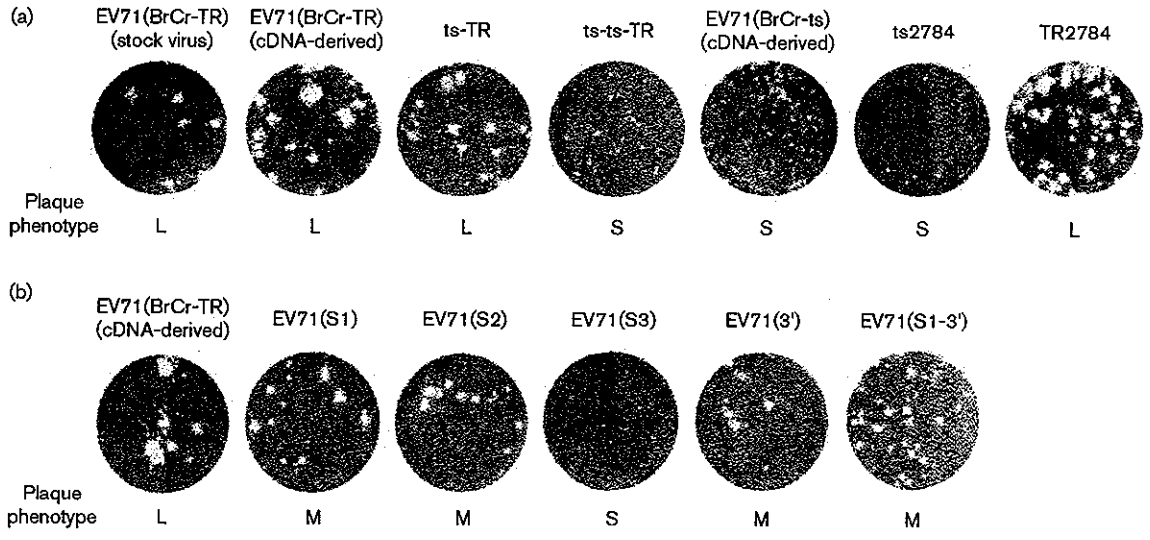

Fig. 2. Plaque phenotype of EV71 mutants. (a) Plaque phenotype of EV71 mutants derived from EV71(BrCr-ts). (b) Plaque phenotype of EV71 mutants carrying mutations of Sabin strains. Mutants EV71(S1), EV71(S2) and EV71(S3) have a mutation of the 5′ NTR of Sabin 1, 2 or 3, respectively. The EV71(3′) mutant 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.

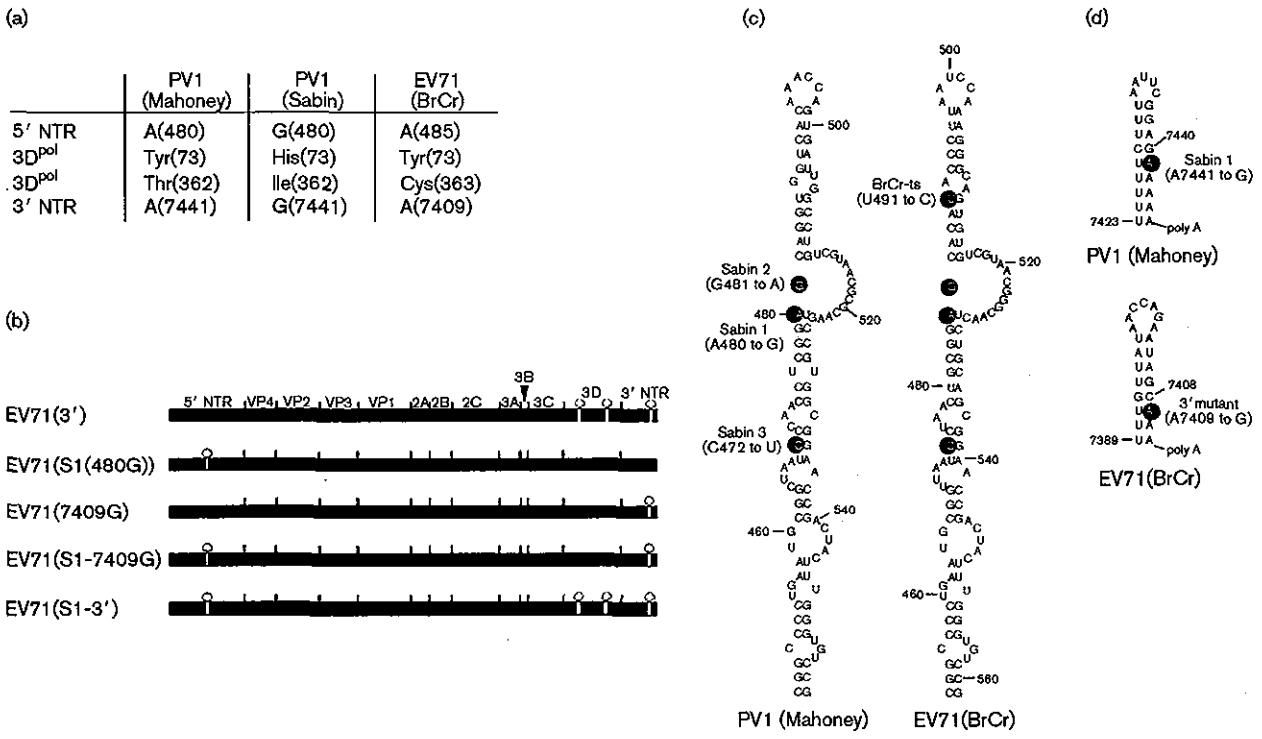

Fig. 3. EV71 mutants containing the corresponding mutations of the *ts* determinants of PV Sabin strains. (a) *ts* determinants of Sabin 1 introduced into the EV71(BrCr-TR) genome. The corresponding sites of the EV71(BrCr-TR) genome were substituted to those of Sabin 1. Numbers in parentheses represent 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

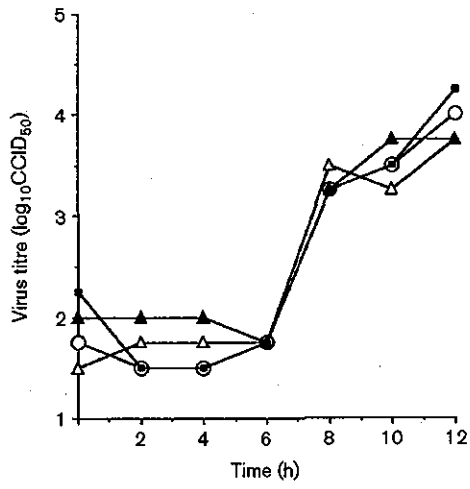

Fig. 4. Growth kinetics of EV71 mutants (BrCr-TR, ○; TR2784, ■; 3′, △; S1-3′, ▲).

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.

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REFERENCES

Abubakar, S., Chee, H. Y., Shafee, N., Chua, K. B. & Lam, S. K. (1999). Molecular detection of enteroviruses from an outbreak of hand, foot and mouth disease in Malaysia in 1997. *Scand J Infect Dis* **31**, 331–335.

Ahmad, K. (2000). Hand, foot, and mouth disease outbreak reported in Singapore. *Lancet* **356**, 1338.

Arita, M., Shimizu, H. & Miyamura, T. (2004). Characterization of *in vitro* and *in vivo* phenotypes of poliovirus type 1 mutants with reduced viral protein synthesis activity. *J Gen Virol* **85**, 1933–1944.

Bouchard, M. J., Lam, D.-H. & Racaniello, V. R. (1995). Determinants of attenuation and temperature sensitivity in the type 1 poliovirus Sabin vaccine. *J Virol* **69**, 4972–4978.

Brown, B. A. & Pallansch, M. A. (1995). Complete nucleotide sequence of enterovirus 71 is distinct from poliovirus. *Virus Res* **39**, 195–205.

Brown, B. A., Oberste, M. S., Alexander, J. P., Jr, Kennett, M. L. & Pallansch, M. A. (1999). Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. *J Virol* **73**, 9969–9975.

Chan, L. G., Parashar, U. D., Lye, M. S. & 9 other authors (2000). Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clin Infect Dis* **31**, 678–683.

Chen, Y.-C., Yu, C.-K., Wang, Y.-F., Liu, C.-C., Su, I.-J. & Lei, H.-Y. (2004). A murine oral enterovirus 71 infection model with central nervous system involvement. *J Gen Virol* **85**, 69–77.

Christodoulou, C., Colbere-Garapin, F., Macadam, A., Taffs, L. F., Marsden, S., Minor, P. & Horaud, F. (1990). Mapping of mutations associated with neurovirulence in monkeys infected with Sabin 1 poliovirus revertants selected at high temperature. *J Virol* **64**, 4922–4929.

Chumakov, M., Voroshilova, M., Shindarov, L. & 16 other authors (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Arch Virol* **60**, 329–340.

Evans, D. M., Dunn, G., Minor, P. D., Schild, G. C., Cann, A. J., Stanway, G., Almond, J. W., Currey, K. & Maizel, J. V., Jr (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature* **314**, 548–550.

Fujimoto, T., Chikahira, M., Yoshida, S., Ebira, H., Hasegawa, A., Totsuka, A. & Nishio, O. (2002). Outbreak of central nervous system disease associated with hand, foot, and mouth disease in Japan during the summer of 2000: detection and molecular epidemiology of enterovirus 71. *Microbiol Immunol* **46**, 621–627.

Georgescu, M. M., Tardy-Panit, M., Guillot, S., Crainic, R. & Delpeyroux, F. (1995). Mapping of mutations contributing to the temperature sensitivity of the Sabin 1 vaccine strain of poliovirus. *J Virol* **69**, 5278–5286.

Hagiwara, A., Yoneyama, T. & Hashimoto, I. (1983). Isolation of a temperature-sensitive strain of enterovirus 71 with reduced neurovirulence for monkeys. *J Gen Virol* **64**, 499–502.

Hansen, J. L., Long, A. M. & Schultz, S. C. (1997). Structure of the RNA-dependent RNA polymerase of poliovirus. *Structure* **5**, 1109–1122.

Hashimoto, I. & Hagiwara, A. (1983). Comparative studies on the neurovirulence of temperature-sensitive and temperature-resistant viruses of enterovirus 71 in monkeys. *Acta Neuropathol* **60**, 266–270.

Hashimoto, I., Hagiwara, A. & Kodama, H. (1978). Neurovirulence in cynomolgus monkeys of enterovirus 71 isolated from a patient with hand, foot and mouth disease. *Arch Virol* **56**, 257–261.

Herrero, L. J., Lee, C. S. M., Hurrelbrink, R. J., Chua, B. H., Chua, K. B. & McMinn, P. C. (2003). Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997–2000. *Arch Virol* **148**, 1369–1385.

Ho, M., Chen, E.-R., Hsu, K.-H., Twu, S.-J., Chen, K.-T., Tsai, S.-F., Wang, J.-R. & Shih, S.-R. (1999). An epidemic of enterovirus 71 infection in Taiwan. *N Engl J Med* **341**, 929–935.

Hogle, J. M., Chow, M. & Filman, D. J. (1985). Three-dimensional structure of poliovirus at 2·9 Å resolution. *Science* **229**, 1358–1365.

Horie, H., Koike, S., Kurata, T. & 11 other authors (1994). Transgenic mice carrying the human poliovirus receptor: new animal model for study of poliovirus neurovirulence. *J Virol* **68**, 681–688.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmakol* **162**, 480–483 (in German).

Kauder, S. E. & Racaniello, V. R. (2004). Poliovirus tropism and attenuation are determined after internal ribosome entry. *J Clin Invest* **113**, 1743–1753.

Kawamura, N., Kohara, M., Abe, S., Komatsu, T., Tago, K., Arita, M. & Nomoto, A. (1989). Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. *J Virol* **63**, 1302–1309.

Koike, S., Horie, H., Sato, Y., Ise, I., Taya, C., Nomura, T., Yoshioka, I., Yonekawa, H. & Nomoto, A. (1993). Poliovirus-sensitive transgenic mice as a new animal model. *Dev Biol Stand* **78**, 101–107.

Komatsu, H., Shimizu, Y., Takeuchi, Y., Ishiko, H. & Takada, H. (1999). Outbreak of severe neurologic involvement associated with enterovirus 71 infection. *Pediatr Neurol* **20**, 17–23.

Kuo, R.-L., Kung, S.-H., Hsu, Y.-Y. & Liu, W.-T. (2002). Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death. *J Gen Virol* **83**, 1367–1376.

Li, M.-L., Hsu, T.-A., Chen, T.-C., Chang, S.-C., Lee, J.-C., Chen, C.-C., Stollar, V. & Shih, S.-R. (2002). The 3C protease activity of enterovirus 71 induces human neural cell apoptosis. *Virology* **293**, 386–395.

Lin, T.-Y., Twu, S.-J., Ho, M.-S., Chang, L.-Y. & Lee, C.-Y. (2003). Enterovirus 71 outbreaks, Taiwan: occurrence and recognition. *Emerg Infect Dis* **9**, 291–293.

Lu, H.-H., Alexander, L. & Wimmer, E. (1995). Construction and genetic analysis of dicistronic polioviruses containing open reading frames for epitopes of human immunodeficiency virus type 1 gp120. *J Virol* **69**, 4797–4806.

Lu, C.-Y., Lee, C.-Y., Kao, C.-L. & 8 other authors (2002). Incidence and case-fatality rates resulting from the 1998 enterovirus 71 outbreak in Taiwan. *J Med Virol* **67**, 217–223.

Lum, L. C. S., Wong, K. T., Lam, S. K. & 7 other authors (1998). Fatal enterovirus 71 encephalomyelitis. *J Pediatr* **133**, 795–798.

Macadam, A. J., Arnold, C., Howlett, J. & 9 other authors (1989). Reversion of the attenuated and temperature-sensitive phenotypes of the Sabin type 3 strain of poliovirus in vaccinees. *Virology* **172**, 408–414.

Macadam, A. J., Pollard, S. R., Ferguson, G., Dunn, G., Skuce, R., Almond, J. W. & Minor, P. D. (1991). The 5′ noncoding region of the type 2 poliovirus vaccine strain contains determinants of attenuation and temperature sensitivity. *Virology* **181**, 451–458.

Malnou, C. E., Werner, A., Borman, A. M., Westhof, E. & Kean, K. M. (2003). Effects of vaccine strain mutations in domain V of the internal ribosome entry segment compared in the wild type poliovirus type 1 context. *J Biol Chem* **279**, 10261–10269.

McMinn, P. C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol Rev* **26**, 91–107.

McMinn, P., Stratov, I. & Dowse, G. (1999). Enterovirus 71 outbreak in Western Australia associated with acute flaccid paralysis. Preliminary report. *Commun Dis Intell* **23**, 199.

McMinn, P., Lindsay, K., Perera, D., Chan, H. M., Chan, K. P. & Cardosa, M. J. (2001a). Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. *J Virol* **75**, 7732–7738.

McMinn, P., Stratov, I., Nagarajan, L. & Davis, S. (2001b). Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. *Clin Infect Dis* **32**, 236–242.

Minor, P. D. (1992). The molecular biology of poliovaccines. *J Gen Virol* **73**, 3065–3077.

Minor, P. D., Dunn, G., Evans, D. M. A. & 8 other authors (1989). The temperature sensitivity of the Sabin type 3 vaccine strain of poliovirus: molecular and structural effects of a mutation in the capsid protein VP3. *J Gen Virol* **70**, 1117–1123.

Muzychenko, A. R., Lipskaya, G. Yu., Maslova, S. V., Svitkin, Y. V., Pilipenko, E. V., Nottay, B. K., Kew, O. M. & Agol, V. I. (1991). Coupled mutations in the 5′-untranslated region of the Sabin poliovirus strains during in vivo passages: structural and functional implications. *Virus Res* **21**, 111–122.

Nagata, N., Shimizu, H., Ami, Y. & 7 other authors (2002). Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. *J Med Virol* **67**, 207–216.

Nagata, N., Iwasaki, T., Ami, Y. & 8 other authors (2004). Differential localization of neurons susceptible to enterovirus 71 and poliovirus type 1 in the central nervous system of cynomolgus monkeys after intravenous inoculation. *J Gen Virol* **85**, 2981–2989.

Omata, T., Kohara, M., Kuge, S. & 8 other authors (1986). Genetic analysis of the attenuation phenotype of poliovirus type 1. *J Virol* **58**, 348–358.

Paul, A. V., Mugavero, J., Yin, J., Hobson, S., Schultz, S., van Boom, J. H. & Wimmer, E. (2000). Studies on the attenuation phenotype of polio vaccines: poliovirus RNA polymerase derived from Sabin type 1 sequence is temperature sensitive in the uridylylation of VPg. *Virology* **272**, 72–84.

Pilipenko, E. V., Blinov, V. M., Romanova, L. I., Sinyakov, A. N., Maslova, S. V. & Agol, V. I. (1989). Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. *Virology* **168**, 201–209.

Pulli, T., Koskimies, P. & Hyypiä, T. (1995). Molecular comparison of coxsackie A virus serotypes. *Virology* **212**, 30–38.

Ren, R., Costantini, F., Gorgacz, E. J., Lee, J. J. & Racaniello, V. R. (1990). Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. *Cell* **63**, 353–362.

Rowe, A., Ferguson, G. L., Minor, P. D. & Macadam, A. J. (2000). Coding changes in the poliovirus protease 2A compensate for 5′NCR domain V disruptions in a cell-specific manner. *Virology* **269**, 284–293.

Sambrook, J. & Russell, D. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn, pp. 13.19–13.25. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmidt, N. J., Lennette, E. H. & Ho, H. H. (1974). An apparently new enterovirus isolated from patients with disease of the central nervous system. *J Infect Dis* **129**, 304–309.

Shimizu, H., Utama, A., Yoshii, K. & 13 other authors (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Jpn J Infect Dis* **52**, 12–15.

Svitkin, Y. V., Maslova, S. V. & Agol, V. I. (1985). The genomes of attenuated and virulent poliovirus strains differ in their *in vitro* translation efficiencies. *Virology* **147**, 243–252.

Svitkin, Y. V., Pestova, T. V., Maslova, S. V. & Agol, V. I. (1988). Point mutations modify the response of poliovirus RNA to a translation initiation factor: a comparison of neurovirulent and attenuated strains. *Virology* **166**, 394–404.

Svitkin, Y. V., Cammack, N., Minor, P. D. & Almond, J. W. (1990). Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation C472→U. *Virology* **175**, 103–109.

Tardy-Panit, M., Blondel, B., Martin, A., Tekaia, F., Horaud, F. & Delpeyroux, F. (1993). A mutation in the RNA polymerase of poliovirus type 1 contributes to attenuation in mice. *J Virol* **67**, 4630–4638.

Thompson, S. R. & Sarnow, P. (2003). Enterovirus 71 contains a type I IRES element that functions when eukaryotic initiation factor eIF4G is cleaved. *Virology* **315**, 259–266.

Wang, J.-R., Tuan, Y.-C., Tsai, H.-P., Yan, J.-J., Liu, C.-C. & Su, I. J. (2002). Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. *J Clin Microbiol* **40**, 10–15.

Wang, S.-M., Lei, H.-Y., Huang, K.-J., Wu, J.-M., Wang, J.-R., Yu, C.-K., Su, I.-J. & Liu, C.-C. (2003). Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. *J Infect Dis* **188**, 564–570.

Wang, Y.-F., Chou, C.-T., Lei, H.-Y. & 8 other authors (2004). A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. *J Virol* **78**, 7916–7924.

Westrop, G. D., Wareham, K. A., Evans, D. M. A. & 8 other authors (1989). Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. *J Virol* **63**, 1338–1344.

Zhao, W. D., Lahser, F. C. & Wimmer, E. (2000). Genetic analysis of a poliovirus/hepatitis C virus (HCV) chimera: interaction between the poliovirus cloverleaf and a sequence in the HCV 5′ nontranslated region results in a replication phenotype. *J Virol* **74**, 6223–6226.

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Circulation of Type 1 Vaccine-Derived Poliovirus in the Philippines in 2001

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In 2001, highly evolved type 1 circulating vaccine-derived poliovirus (cVDPV) was isolated from three acute flaccid paralysis patients and one contact from three separate communities in the Philippines. Complete genomic sequencing of these four cVDPV isolates revealed that the capsid region was derived from the Sabin 1 vaccine strain but most of the noncapsid region was derived from an unidentified enterovirus unrelated to the oral poliovirus vaccine (OPV) strains. The sequences of the cVDPV isolates were closely related to each other, and the isolates had a common recombination site. Most of the genetic and biological properties of the cVDPV isolates were indistinguishable from those of wild polioviruses. However, the most recently identified cVDPV isolate from a healthy contact retained the temperature sensitivity and partial attenuation phenotypes. The sequence relationships among the isolates and Sabin 1 suggested that cVDPV originated from an OPV dose given in 1998 to 1999 and that cVDPV circulated along a narrow chain of transmission. Type 1 cVDPV was last detected in the Philippines in September 2001, and population immunity to polio was raised by extensive OPV campaigns in late 2001 and early 2002.

Immunization with the oral poliovirus vaccine (OPV) is the cornerstone of the World Health Organization's program for the global eradication of poliomyelitis (15, 44, 55, 56, 65). The attenuated OPV strains of the three poliovirus serotypes (Sabin 1, 2, and 3) replicate in the gut of OPV recipients and can efficiently induce type-specific humoral and mucosal immunity (55), mimicking natural infection. However, replication of OPV in humans is frequently accompanied by genetic change of the vaccine virus, including reversion of key attenuating mutations (5, 42), introduction of other mutations throughout the genome, and *intertypic recombination* among OPV strains (7, 16). The phenotypic reversion of the OPV strains to neurovirulence is the underlying mechanism for the rare cases of vaccine-associated paralytic poliomyelitis among OPV recipients or their close contacts (41, 54, 55). Cases of vaccine-associated paralytic poliomyelitis in immunocompetent persons are generally associated with poliovirus types 2 and 3 and very rarely with type 1 (54). The large majority of OPV isolates from healthy individuals, the environment, or patients with vaccine-associated paralytic poliomyelitis are closely related to the original OPV strain (Sabin-like), diverging by <1.0% of nucleotide sequences encoding the major capsid protein VP1 (8, 9, 39). The low nucleotide sequence diversities from the respective OPV strains are consistent with the short duration of most poliovirus infections (1) and the usually restricted spread of OPV virus (3).

It is now apparent that in areas with widening gaps in population immunity to poliovirus, especially where wild poliovirus circulation has ceased, viruses derived from OPV may circulate within a population, cause cases of paralytic poliomyelitis, and accumulate further mutations. OPV-derived polioviruses with 1 to 15% sequence divergence from the Sabin strains are now defined as vaccine-derived polioviruses (VDPVs) (8, 9), with the extent of divergence roughly proportional to the duration of viral replication (2, 27, 37) or circulation (25, 30, 66) since administration of the initiating OPV dose. In 2000 to 2001, a poliomyelitis outbreak associated with circulating VDPVs (cVDPVs) occurred on the island of Hispaniola (which is divided into the Dominican Republic and Haiti), underscoring the risk of using OPV at low rates of coverage in polio-free areas (15, 25, 28, 30, 44). Furthermore, retrospective genetic studies identified the endemic circulation of a type 2 cVDPV in Egypt from about 1983 to 1993 (66). More recently, intensified acute flaccid paralysis and laboratory surveillance led to the identification of cVDPV outbreaks in the Philippines in 2001 (type 1) (58, 62) and Madagascar in 2001 to 2002 (type 2) (51).

In this report, we describe the circulation of the type 1 cVDPV in the Philippines in 2001 and the genetic and biological properties of the four cVDPV isolates. Three of the isolates were from children with acute flaccid paralysis, a common clinical manifestation of poliomyelitis. The first case was iden-

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