Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolagus 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 cynomolagus monkeys, respectively. Mutations in 3D polymerase (3Dpol) 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, 3Dpol 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
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,
tetraparesis, and ataxia (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 deter-
minants 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
virulence 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 transcrip-
tion 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 EcoRI-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 frag-
ment 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 PvuI-T45 + and 1595R – and then cloned into the above construct following digestion by PstI and Mungl. 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/BamHI – and EV71-1500F + (Table 1) and then cloned into the BamHI 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 SnaBl and MluI, and then cloned into plasmid pHi d40 (a generous gift from Dr E. Wimmer) (Zhao et al., 2000). In this construct, to introduce MluI and SnaBl sites, part of the plas-
mid vector was obtained by PCR using Tbr EXT DNA polymerase (Finnzymes) with primers MluI_vec + and SnaBl_vec –, using plasmid pHi d40 as the template DNA. There were five nucleotide dif-
fferences 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 SnaBl-I-T7
EV71 + and 1595R –, using the viral genome of EV71(BrCr-TR) as the template. The obtained fragment was digested with Mungl and SnaBl and then ligated into the infectious clone. To restore other mutared 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 Mungl and Xmal, with Xmal and Sall or with Sall and SpeI, 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)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
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<td>1595R–</td>
<td>TCCAGCGGGCTGATAGGCACCAC</td>
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<tr>
<td>2784+</td>
<td>CATAACCGCGCTAGGCGAGATG</td>
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<td>71/3393–</td>
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| A2BamHI–    | 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Virus solutions were inoculated at 100 μl (Falcon) containing a Vero-cell monolayer. Tenfold dilutions of 102–106 plaque-forming units (PFUs) were inoculated into 12-well plates (Stripwell Plate; Corning) containing 104 cells 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 CCID50 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. 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 CCID50 measurement.

Temperature sensitivity. The temperature sensitivity of viruses was evaluated by determining the virus titres 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 CCID50 values at 36 and 39°C (ΔCCID50). 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 cynomolagus 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 ketalar and xylazine, 1 ml of each virus solution (containing 107 CCID50 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

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<tr>
<th>Nucleotide position</th>
<th>Site of mutation</th>
<th>Nucleotide change</th>
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<tr>
<td>491</td>
<td>5' NTR</td>
<td>U to C</td>
<td>–</td>
</tr>
<tr>
<td>681</td>
<td>5' NTR</td>
<td>U to C</td>
<td>–</td>
</tr>
<tr>
<td>848</td>
<td>VP4</td>
<td>C to U</td>
<td>–</td>
</tr>
<tr>
<td>1154</td>
<td>VP2</td>
<td>U to C</td>
<td>–</td>
</tr>
<tr>
<td>1707</td>
<td>VP2</td>
<td>G to A</td>
<td>Ala253 to Thr</td>
</tr>
<tr>
<td>2693</td>
<td>VP1</td>
<td>U to C</td>
<td>–</td>
</tr>
<tr>
<td>2784</td>
<td>VP1</td>
<td>U to C</td>
<td>Tyr116 to His</td>
</tr>
<tr>
<td>4034</td>
<td>2B</td>
<td>A to G</td>
<td>–</td>
</tr>
<tr>
<td>4990</td>
<td>2C</td>
<td>C to U</td>
<td>Thr305 to Ile</td>
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</table>
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 *vivo*, 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

### Table 3. Temperature sensitivity of EV71 mutants

<table>
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<th>EV71 mutant</th>
<th>Titre* at:</th>
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<tbody>
<tr>
<td></td>
<td>36 °C</td>
<td>39 °C</td>
</tr>
<tr>
<td>BrCr-TR</td>
<td>4.25</td>
<td>3.0</td>
</tr>
<tr>
<td>ts-TR</td>
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<tr>
<td>ts-ts-TR</td>
<td>2.5</td>
<td>ND (&lt;0.5)</td>
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<tr>
<td>BrCr-ts</td>
<td>3.5</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>ts2784</td>
<td>3.25</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>TR2784</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>S1</td>
<td>4.25</td>
<td>2.25</td>
</tr>
<tr>
<td>S2</td>
<td>4.0</td>
<td>1.75</td>
</tr>
<tr>
<td>S3</td>
<td>3.5</td>
<td>1.25</td>
</tr>
<tr>
<td>3’</td>
<td>4.0</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>S1-3’</td>
<td>4.0</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>3’ (4511, spinal cord)</td>
<td>4.0</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>3’ (4511, brainstem)</td>
<td>4.13</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>3’ (4512, brainstem)</td>
<td>4.0</td>
<td>ND (&lt;0.5)</td>
</tr>
<tr>
<td>S1-3’ (4514, spinal cord)</td>
<td>4.13</td>
<td>ND (&lt;0.5)</td>
</tr>
</tbody>
</table>

*Virus titre represents log<sub>10</sub>(CCID<sub>50</sub>) in 10 μl virus sample. ND, Not detected.

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<sup>pol</sup>-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.
Secondly, mutations corresponding to the major determinants of Sabin 1, 2 and 3, respectively (Fig. 3c). EV71(BrCr) genome, nt 485, 486 and 474 corresponded to typical of the enterovirus IRES (Thompson & Sarnow, 2003).

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 3Dpol 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 Filipenko et al. (1998). (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.

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 3Dpol-coding region and the 3' NTR [EV71(S3) mutant] conferred a strong ts phenotype to EV71(BrCr-TR) (Δ36/39°C, >3-5 log) (Table 3). Therefore, the triple mutations in the 3Dpol-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
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<sup>pol</sup> 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<sup>7</sup> CCID<sub>50</sub> 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

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

<table>
<thead>
<tr>
<th>Virus</th>
<th>Monkey no.</th>
<th>Clinical manifestation*</th>
<th>Virus isolation</th>
<th>Lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tremor</td>
<td>Ataxia</td>
<td>Moribund</td>
</tr>
<tr>
<td>EV71(BrCr-TR)</td>
<td>4507</td>
<td>Day 4</td>
<td>Day 5</td>
<td>Day 6</td>
</tr>
<tr>
<td></td>
<td>4508</td>
<td>Day 4</td>
<td>Day 5</td>
<td>Day 6</td>
</tr>
<tr>
<td>EV71(TR2784)</td>
<td>4509</td>
<td>Day 7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4510</td>
<td>Day 6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EV71(3’)</td>
<td>4511</td>
<td>Day 9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4512</td>
<td>Day 8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EV71(S1-3’)</td>
<td>4513</td>
<td>Day 9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4514</td>
<td>Day 9</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*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 lesions 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, 3Dpol 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 3Dpol protein) affects the oligomerization and uridylylation of viral protein 3BVPg in a temperature-sensitive manner (Paul et al., 2000). Another mutation of nt 7071 (Ile362 of the 3Dpol protein) is required for the ts phenotype of Sabin 1 (Georgescu et al., 1995). The amino acid residue at 362 of the 3Dpol protein is located at interface 1 of 3Dpol (Hansen et al., 1997); together with the mutation of nt 6203, this amino acid residue may also affect the oligomerization of the 3Dpol 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 3Dpol (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 3Dpol-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′)NTR showed a strong ts phenotype, resulting in a similar lesion score, despite 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 3Dpol-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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