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# Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of *Tick-borne encephalitis virus*

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A stable full-length infectious cDNA clone of the Oshima strain of *Tick-borne encephalitis virus* (Far-Eastern subtype) was developed by a long high-fidelity RT-PCR and one-step cloning procedure. The infectious clone (O-IC) had four amino acid substitutions and produced smaller plaques when compared with the parent Oshima 5-10 strain. Using site-directed mutagenesis, the substitutions were reverted to restore the parent virus sequence (O-IC-pt). Although genetically identical, parent virus Oshima 5-10 and virus recovered from O-IC-pt demonstrated some biological differences that are possibly explained by the presence of quasispecies with differing virulence characteristics within the original virus population. These observations may have implications for vaccines based on modified infectious clones. It was also demonstrated that the amino acid substitution E-$S_{40}$→P at position 40 in the envelope (E) glycoprotein was responsible for plaque size reduction, reduced infectious virus yields in cell culture and reduced mouse neurovirulence. Additionally, two amino acid substitutions in the non-structural (NS)5 protein (virus RNA-dependent RNA polymerase) NS5-$V_{378}$→A and NS5-$R_{674}$→K also contributed to attenuation of virulence in mice, but did not demonstrate a noticeable biological effect in baby hamster kidney cell culture. Comparative neurovirulence tests revealed how the accumulation of individual mutations (E-$S_{40}$→P, NS5-$V_{378}$→A and NS5-$R_{674}$→K) can result in the attenuation of a virus.

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## INTRODUCTION

*Tick-borne encephalitis virus* (TBEV) is a species of the mammalian tick-borne group within the genus *Flavivirus*, family *Flaviviridae* (Heinz *et al.*, 2000). TBEV is prevalent over a wide area of Europe and Asia (Dumpis *et al.*, 1999; Suss, 2003) and causes a variety of clinical symptoms in humans including subclinical infections, mild or severe fevers and encephalitis with serious sequelae. The virus has a significant impact on public health in these endemic regions (Haglund & Gunther, 2003; Gritsun *et al.*, 2003b) and has been subdivided into Far-Eastern, Siberian and European subtypes (Ecker *et al.*, 1999; Heinz *et al.*, 2000; Hayasaka *et al.*, 2001; Gritsun *et al.*, 2003a). The Far-Eastern subtype, previously known as Russian Spring–Summer Encephalitis (RSSE) virus, causes a severe clinical manifestation and shows a higher case fatality rate (5–20 %) than other Siberian and European strains of TBEV (Shope, 1980; Dumpis *et al.*, 1999; Korenberg & Kovalevskii, 1999).

The flavivirus genome (single, positive-stranded RNA, approximately 11 kb in length) encodes three structural proteins [capsid (C) protein, precursor membrane (prM) protein, and envelope (E) protein] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), within a single long open reading frame (ORF). The 5′ and 3′ termini of the genome have predicted secondary structures (Gritsun *et al.*, 1997; Proutski *et al.*, 1997; Rauscher *et al.*, 1997) and contain important elements for virus replication, translation and packaging of the genome (Chambers *et al.*, 1990; Lindenbach & Rice, 2001).

A number of infectious cDNA clones of flaviviruses have been produced using different strategies to study the different aspects of flavivirus pathogenesis (Rice *et al.*, 1989; Ruggli & Rice, 1999; Sumiyoshi *et al.*, 1992; Kapoor *et al.*, 1995; Mandl *et al.*, 1997; Polo *et al.*, 1997; Gritsun & Gould, 1998; Yamshchikov *et al.*, 2001a, b; Bredenbeek *et al.*, 2003; Zhang *et al.*, 2001; Yun *et al.*, 2003; Puri *et al.*, 2000; Kinney

*et al.*, 1997; Gualano *et al.*, 1998; Sriburi *et al.*, 2001; Lai *et al.*, 1991; Khromykh & Westaway, 1994; Shi *et al.*, 2002; Campbell & Pletnev, 2000; Pletnev, 2001; Hurrelbrink *et al.*, 1999). There are two reports of infectious cDNA clones for TBEV, European subtype strains Neudoerfl and Hypr (Mandl *et al.*, 1997) and Siberian subtype strain Vasilchenko (Vs) (Gritsun & Gould, 1998). The present study reports the construction and characterization of an infectious cDNA clone of the Far-Eastern subtype TBEV Oshima 5-10 strain, which was isolated in Japan in 1995 (Takashima *et al.*, 1997). Genetic and biological differences between the infectious clone and the parent Oshima 5-10 virus were revealed and the parent genotype was recreated from the infectious clone by reversion of all detected amino acid substitutions. Specific mutations within the E glycoprotein and the RNA-dependent RNA-polymerase (NS5) gene were shown to be responsible for modification of plaque phenotype, virus growth in cell culture and virulence for mice.

## METHODS

**Cells and viruses.** Baby hamster kidney (BHK) cells were grown in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co.) supplemented with 8% fetal calf serum (FCS). TBEV strain Oshima 5-10 isolated in Japan in 1995 was used for construction of the infectious cDNA clone (Takashima *et al.*, 1997). The virus was propagated in 1-day-old suckling mice and stored as a 10% mouse brain suspension (MBS).

**Reverse transcription and long-PCR.** Viral RNA extraction and long high-fidelity RT-PCR were described previously (Gritsun & Gould, 1995, 1998). Briefly, viral RNA was extracted from 10% MBS using Catrimox (Iowa Biotechnology Corp.). The RT reaction was carried out with Superscript II reverse transcriptase (Invitrogen) and 14-mer primer (AGCGGGTGTTTTTC). For the long PCR a mixture of thermostable Red Hot DNA polymerase (Advanced Biotechnologies) and Deep Vent DNA polymerase (NEB) was used for a hot start. The PCR programme included 30 cycles of 30 s at 94 °C and 10 min at 72 °C. Primers were designed on the basis of the nucleotide sequence of Oshima 5-10 strain (Hayasaka *et al.*, 2001; Goto *et al.*, 2002). The sense primer Oshi-5'-long included a *Not*I restriction enzyme site, SP6 promoter and 28 nucleotides of 5' untranslated region (UTR) (gtcgac*GCGGCCG*Catttaggtgacactatag*AG*-ATTTTCTTGCACGTGCATGCGTTTGC). The antisense primer Oshi-3'-long has a *Spe*I site and 35 nucleotides of 3'UTR (gcacgt*ACTAGT*-AGCGGGTGTTTTTCCGAGTCACTCATCACCTCCTT). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen).

**Cloning of full-length cDNA clone of TBEV Oshima strain.** Plasmid $pGGVs_{209}$, based on pBR322 (Gritsun & Gould, 1998), was used for the cloning of full-length RT-PCR product amplified from Oshima 5-10 virus (Fig. 1). Plasmid $pGGVs_{209}$ was constructed from the infectious clone for TBEV Siberian strain, Vs, and contains the first 209 nucleotides of the Vs virus genome with the restriction site *Spe*I at position 80 and a *Not*I site upstream of the SP6 promoter. Plasmid $pGGVs_{209}$ and purified long-PCR product were digested with *Not*I and *Spe*I enzymes, ligated with T4 ligase (NEB) and transformed into the low-copy Able-K strain of *E. coli* (Stratagene) by electroporation. Bacterial cells were incubated on TYM agar plates containing 100 μg ampicillin $ml^{-1}$ at 37 °C. Small colonies were chosen and grown in liquid TYM at 28 °C overnight. Plasmid DNAs were extracted and those selected by size (about 15 kb) were verified by restriction analysis to confirm the insertion of virus cDNA.

**Recovery of infectious virus.** Full-length bacterial clones were linearized with *Spe*I and transcribed into the RNA using the SP6-transcription system as described previously (Gritsun & Gould, 1995) or alternatively using mMESSAGE mMACHINE (Ambion). To recover virus, RNA after the SP6-transcription was injected intracerebrally into the young mice. They developed encephalitis within 5–9 days (Gritsun & Gould, 1995, 1998).

For electroporation, the transcription samples were treated with DNase; transcribed RNA after the precipitation with LiCl was dissolved in 10 μl of water. Trypsin-treated BHK cells were washed with cold PBS three times. About 10 μg of transcribed RNA was electroporated to $5 \times 10^6$ BHK cells in 500 μl of cold PBS in 0·4 cm cuvettes using a GenePulser apparatus (Bio-Rad), 1·3 kV, 25 μF and ∞Ω, pulsed two times. After incubation at room temperature for 10 min, cells were kept at 37 °C under 5% $CO_2$.

The identification of the virus in the mouse brain or in cell supernatant medium was carried out by indirect immunofluorescence microsopy using monoclonal antibodies to the E glycoprotein as described in Gritsun & Gould (1995). The full-length clone that produced infectious virus was nominated as O-IC (Oshima virus infectious clone). In the comparative experiments to be described below the virus stocks were derived from the supernatant medium of BHK cells.

**Mutagenesis of the infectious clone.** A range of intermediate plasmids based on pGEM3 and pGEMT vectors (Promega) was constructed to produce reverse-mutations in the infectious clone (pGEM3-HS1, pGEM3-HS2, pGEMT-CprME and pGEMT-NS5, Fig. 1). Plasmid pGEM3-HS2 was constructed to introduce wild-type substitutions (NS5-$T_{794}$, guanine$_{10473}$ and adenine$_{10922}$ in the 3'UTR) to produce a full-length plasmid and virus with genotype $P_{40}A_{378}K_{674}T_{794}$ (Fig. 1).

To substitute $P_{40}$ for $S_{40}$, the parent virus genome fraction between nucleotides 242–2295 was amplified by RT-PCR and cloned (with the introduction of two flanking *Spe*I restriction sites) to construct plasmid pGEMT-CprME. An *Avr*II restriction site (cohesive ends compatible with *Spe*I) was introduced into the plasmid $P_{40}A_{378}K_{674}T_{794}$ (D. Hayasaka and others, unpublished results). This intermediate plasmid and pGEMT-CprME were used to substitute the region 242–2295 between two sets of restriction sites corresponding to *Avr*II or *Spe*I to produce a plasmid and virus with the genotype $S_{40}A_{378}K_{674}T_{794}$ (Fig. 1). Plasmid pGEM-3–HS1 was constructed by subcloning the region between *Hin*dIII and *Spe*I (nucleotides 9197–11100) of the infectious clone O-IC. The $S_{40}A_{378}K_{674}T_{794}$ and plasmid pGEM3-HS1 were used to construct full-length plasmid and corresponding virus with genotype $S_{40}A_{378}K_{674}A_{794}$.

To recreate the infectious clone O-IC-pt, so that it was genetically identical to parent virus Oshima 5-10, the nucleotides 7365–11 100 of the NS5 gene were amplified from parent Oshima 5-10 virus by RT-PCR and cloned to construct a plasmid pGEMT-NS5 that was subsequently used to substitute the region between nucleotides 7365–11100 (*Aat*II and *Spe*I sites) of the plasmid $S_{40}A_{378}K_{674}T_{794}$. All subcloned plasmids (Fig. 2) were fully sequenced to confirm their authenticity.

**Sequencing of cDNA plasmid and recovered virus.** Recovered viruses were used to infect BHK cells and viral RNA was extracted with Isogen Kit (Nippon Gene). RT-PCR products were amplified using a Thermo Script RT-PCR system (Invitrogen) and sequenced with DNA Sequencing Kit (ABI PRISM) using the fluorescence autosequencer (ABI PRISM 310 Genetic Analyser). To determine the sequences of the 5'- and 3'-ends of the recovered virus, extracted RNAs were de-capped with tobacco acid pyrophosphatase (Wako), ligated with T4 RNA ligase (Takara) and amplified by RT-PCR.

**Immunofluorescent antibody (IFA) focus assay and titration of viruses.** A fluorescent focus assay, developed previously

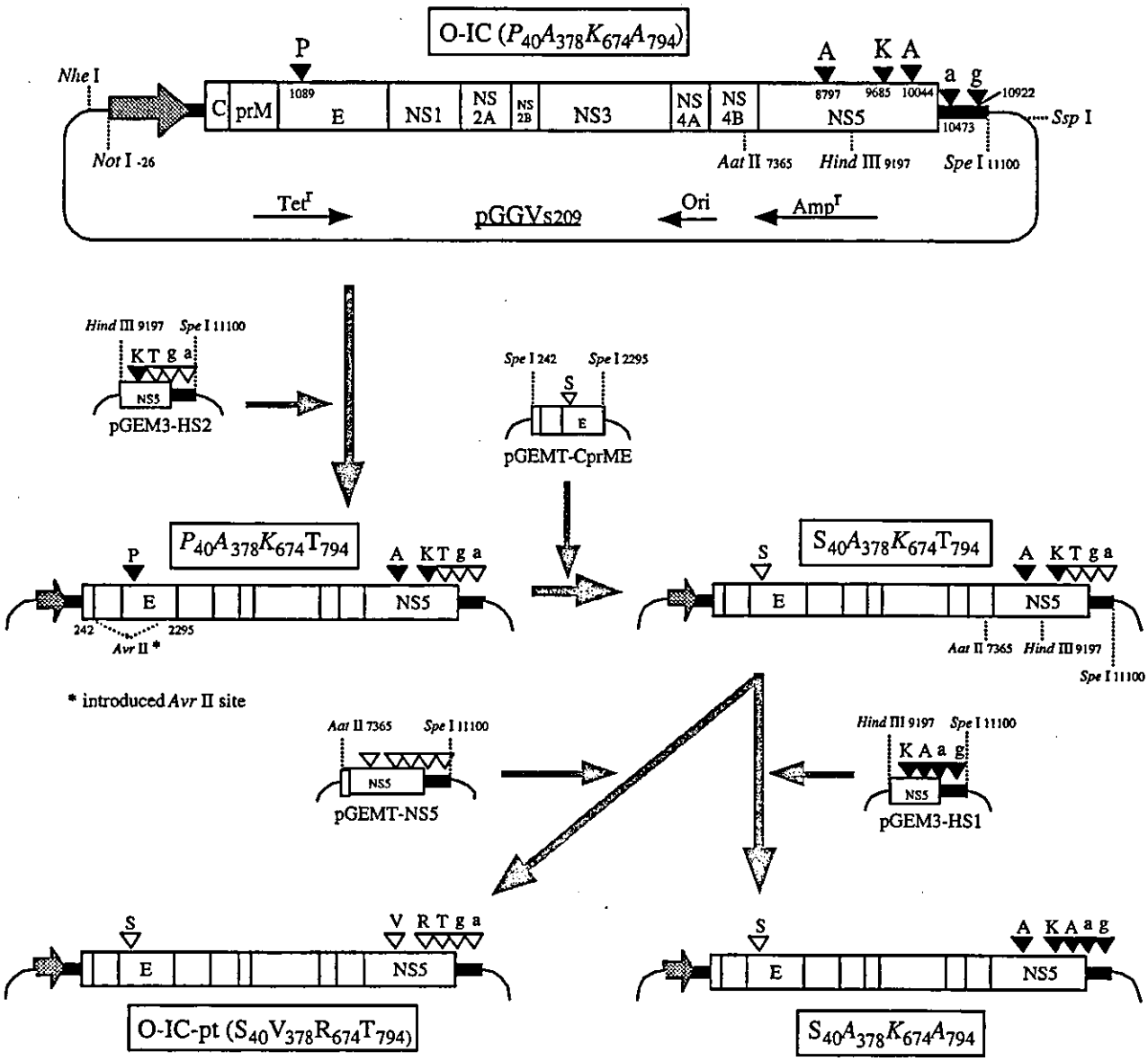


**Fig. 1.** Strategy for the construction of an infectious cDNA clone of TBEV Oshima 5-10 strain. The virus genome (about 11 kb) is depicted as an open box with genes specified through C to NS5. Untranslated regions flanking polyprotein are specified as thick black lines. The primers for amplification of virus RNA in RT (14-mer primer) and long-PCR (Oshi5'-long and Oshi3'-long) are indicated as thin arrows, and the SP6 promoter as a shadowed arrow. Restriction sites *Not*I and *Spe*I were used for cloning the PCR product into the plasmid $pGGVs_{209}$ based on pBR322. The restriction sites used to construct mutant plasmids and viruses are shown above. Authentic features of pBR322 are specified as restriction sites *Nhe*I and *Ssp*I, tetracycline- and ampicillin-resistant genes ($Tet^r$ and $Amp^r$) and origin of replication. The four amino acid substitutions in the polyprotein and two nucleotide changes in the 3'UTR of the infectious clone are indicated by capital letters above the genome with nucleotide positions in numbers next to arrowheads. Subcloned plasmids containing C-prM-E, NS5 and 3'UTR sequences that were amplified by RT-PCR from parent Oshima 5-10 virus RNA and used for the construction of mutants viruses and revertant infectious cDNA clone O-IC-pt. O-IC plasmid (amino acid genotype $P_{40}A_{378}K_{674}A_{794}$) was digested with *Hin*dIII and *Spe*I (between nucleotides 9197–11100) and replaced with a corresponding fragment derived from pGEM3-HS2 to produce plasmid $P_{40}A_{378}K_{674}T_{794}$. The plasmid $S_{40}A_{378}K_{674}T_{794}$ was produced from plasmid $P_{40}A_{378}K_{674}T_{794}$ by two sequential clonings: firstly, an *Avr*II site (cohesive ends compatible with *Spe*I) was introduced into the plasmid $P_{40}A_{378}K_{674}T_{794}$ to create an intermediate plasmid (D. Hayasaka and others, unpublished results) and the fragment between nucleotides 242–2295 was replaced with a corresponding fragment from pGEMT-CprME (digested with *Spe*I enzyme). The sequence between nucleotides 9197–11100 of $S_{40}A_{378}K_{674}T_{794}$ was replaced with an equivalent fragment derived from pGEM3-HS1 to create plasmid $S_{40}A_{378}K_{674}A_{794}$. Plasmid $S_{40}A_{378}K_{674}T_{794}$ was digested at *Aat*II and *Spe*I sites (between nucleotides 7365–11100) and replaced with a fragment of pGEMT-NS5 digested with *Aat*II and *Spe*I to produce plasmid O-IC-pt.

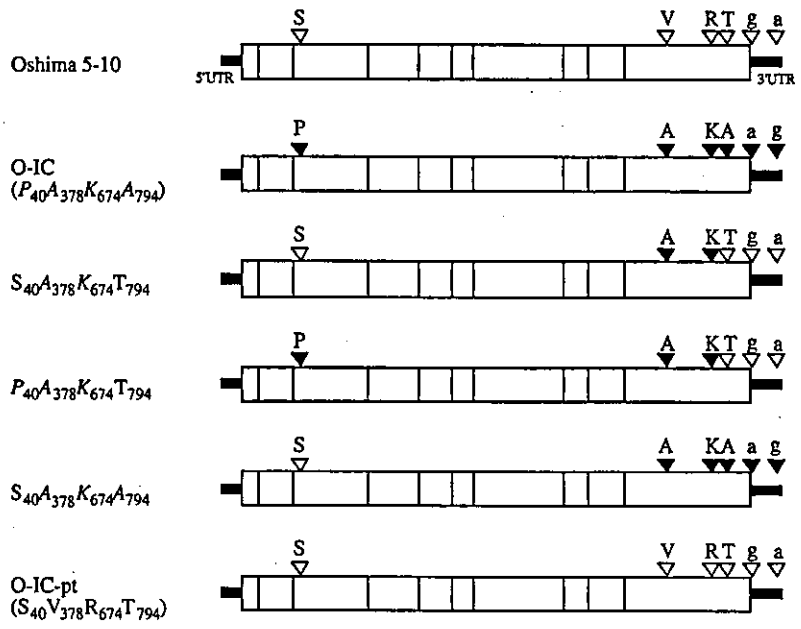


**Fig. 2.** Schematic representation of constructed full-length plasmids and viruses that were recovered from them. The black arrowhead indicates amino acid and nucleotide sequences derived from O-IC. The white arrowhead indicates amino acid and nucleotide sequences of parent Oshima 5-10 virus.

(Takashima *et al.*, 1997), was used to assess virus titres. Virus was adsorbed for 90 min at 37 °C on BHK cells. The inoculum was then replaced with EMEM and 5% carboxymethyl cellulose. After incubation for 36 or 72 h, cells were fixed with methanol and incubated at 37 °C for 1 h with an appropriately diluted ascitic fluid taken from mice hyperimmunized with a tick-borne flavivirus. Following washing with PBS, fluorescein isothiocyanate-conjugated antibody against mouse IgG was added and incubated at 37 °C for 1 h. After washing with PBS, the numbers of fluorescent foci were estimated under a UV microscope. The virus titres were calculated and expressed as focus forming units per ml (f.f.u. $ml^{-1}$).

**Growth curve in cell cultures.** Subconfluent BHK cells were grown as $10^6$ cells per well in 12-well plates and inoculated with each parent or engineered virus at an m.o.i of 0·01 f.f.u. Viruses were diluted with medium containing 4% FCS. After incubation for 90 min, virus inoculum was removed and the cells were washed twice in PBS. Two ml of medium was added to each well and they were then incubated at 37 °C under 5% $CO_2$. The samples were harvested at 12, 24, 36 and 48 h post-inoculation and stored in aliquots at −80 °C prior to titration.

**Virulence in mice.** Viruses were inoculated into 7-week-old male BALB/c mice (SLC) with body weights of about 20 g. Eight mice in each group received 50 f.f.u. of each virus intracerebrally or 1000 f.f.u. of each virus subcutaneously. Morbidity was defined as the appearance of more than 10% weight loss or clinical signs ruffled fur and/or hunched back. Surviving mice were monitored for 28 days post-infection to obtain survival curves and mortality rates.

## RESULTS

### Construction of an infectious clone of TBEV Oshima strain

An infectious clone of Oshima virus was prepared using the one-step cloning procedure (Gritsun & Gould, 1998) as described in Methods. The long high-fidelity RT-PCR product, which was approximately 11 kb, appeared as a single band in agarose gels (data not shown). The cDNA was digested with *NotI* and *SpeI* and cloned into the vector $pGGVs_{209}$ as demonstrated in Fig. 1. Ten out of 88 colonies contained full-length viral cDNA on the basis of restriction enzyme digestion patterns (data not shown). These cDNA clones were transcribed *in vitro* with SP6 RNA polymerase and each of the ten derived RNA preparations was either injected intracerebrally into newborn mice or electroporated into BHK cells. Four out of ten mouse litters developed encephalitis and only one of these four clones also produced virus antigen in the BHK cells as confirmed by IFA tests using TBEV-specific antibody. This clone, designated O-IC, was used for all subsequent experimental work.

To test for stability in bacteria, O-IC plasmids were transformed into *E. coli* strain HB101, grown for various incubation times, (8, 12, 16, 20 and 24 h) and plated on Luria Broth agar containing 100 μg ampicillin $ml^{-1}$. The colony size on the plates was unaltered after five repeated passages and after each subculture, the infectious virus was successfully rescued (data not shown). Sequencing demonstrated that plasmid O-IC and the virus which was recovered from the infectious clone O-IC were identical.

### Properties of the infectious clone

**Formation of fluorescent foci.** Whilst carrying out the focus forming assay, we noticed that the parent Oshima 5-10 virus produced distinct fluorescent foci at 36 h post-infection (Fig. 3). However, O-IC produced only singly infected cells within the same time period and fluorescent foci, of the same size as those produced by the parent virus at 36 h, were visualized only after an incubation period of at least 72 h (Fig. 3).

**Sequencing analysis.** The complete genome sequence of the O-IC plasmid was determined and compared with the parent virus. There were nine nucleotide sequence differences between these viruses and four of the substitutions

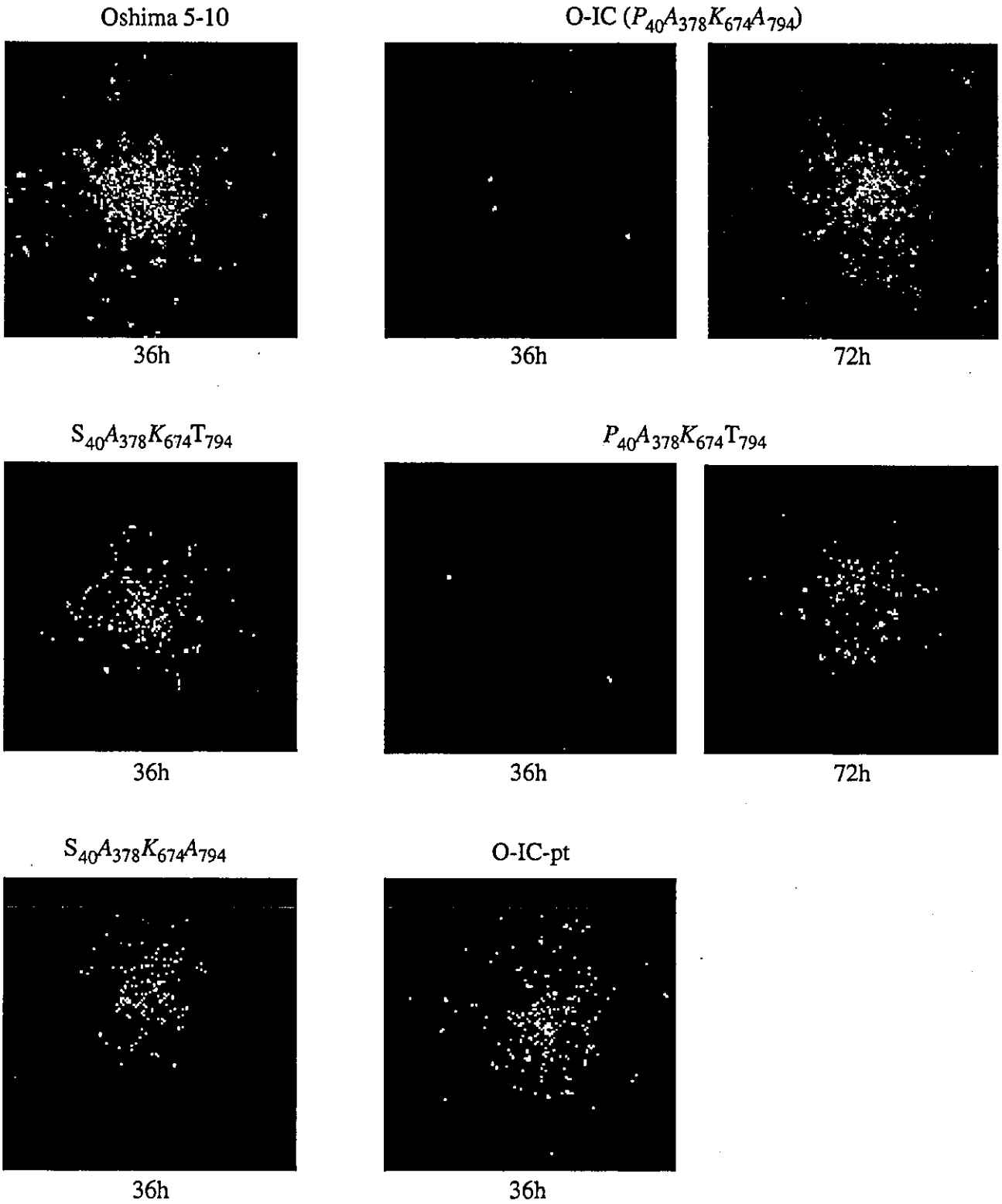


**Fig. 3.** An infectious focus of virus recovered from infectious cDNA clones, 36 and 72 h post-inoculation. Virus antigens were detected in cells fixed with methanol using a 1:100 dilution of mouse ascitic fluid containing high-titre TBEV-reactive antibodies and anti-mouse IgG fluorescein isothiocyanate-conjugated antibody. Viruses are indicated above each photograph. Post-inoculation times are indicated below each photograph.

**Table 1.** Nucleotides and amino acids changes between Oshima 5-10 and O-IC virus

| Nucleotide changes | | | Amino acid changes | | |
|---|---|---|---|---|---|
| Positions | Oshima 5-10 | O-IC | Positions | Oshima 5-10 | O-IC |
| 1089 (ORF) | T | C | $E_{40}$* | Ser | Pro |
| 1286 (ORF) | C | T | | | |
| 1847 (ORF) | A | T | | | |
| 7199 (ORF) | G | A | | | |
| 8797 (ORF) | T | C | $NS5_{378}$ | Val | Ala |
| 9685 (ORF) | G | A | $NS5_{674}$ | Arg | Lys |
| 10044 (ORF) | A | G | $NS5_{794}$ | Thr | Ala |
| 10473 (3'UTR) | G | A | | | |
| 10922 (3'UTR) | A | G | | | |

*The numbers indicate the amino acid position in each protein (E or NS5).

resulted in amino acid changes (Table 1). Comparative protein alignments between all available flavivirus sequences revealed that the infectious clone had a proline in position 40 ($P_{40}$) of the E protein whilst the parent virus Oshima 5-10 and approximately 80 other strains of tick-borne flavivirus, plus *Yellow fever virus* (a mosquito-borne virus) have serine in this position (Fig. 4). Other mosquito-borne and non-vectored flaviviruses have a threonine in this position, and both threonine and serine form a group of polar hydroxyl-containing amino acids. The substitution of threonine or seine for proline could have biological consequences because it changes the hydrophobicity of the E protein in this region.

Three other mutations mapped in the NS5 protein, in regions conserved among tick- and mosquito-borne flaviviruses. The first two substitutions within the NS5 protein $V_{378}\rightarrow A$ and $R_{674}\rightarrow K$ are conserved and should not change the charge or hydrophobicity of the protein. The third substitution $T_{794}\rightarrow A$ is not conserved and also mapped in a highly conserved position in other viruses within the family *Flaviviridae* (Fig. 4).

**A**

| | 40 |
|---|---|
| Oshi5-10 | CVTITAEGKPSMDVWLDSIY |
| IC10 | .....P..... |
| Za | ..... |
| Vs | ..... |
| Sof | ..... |
| 205 | ..... |
| Hypr | .....A.. |
| Neu | .....A.. |
| LIV | .....A.. |
| LGTV | ...V..D....L..... |
| KFDV | ...L.....V.....D.H |
| POWV | .....I....ED.F |
| TYUV | ....M.D....V.I...E.. |
| SREV | ....V.D.....I...G.. |
| YFV17D | ...VM.PD...L.IS.ETVA |
| YFVAsibi | ...VM.PD...L.IS.ETVA |
| JEVBej | .L..M.ND..TL..RMIN.E |
| JEVSA14142 | .L..M.ND..TL..RMIN.E |
| KUNV | ....MSKD..TI..KMMNME |
| MVEV | .I..M.AD..TL.IRMMN.E |
| SLEV | ...VM.PE..TL.FKVMKME |
| WNV | ....MSKD..TI..KMMNME |
| DENV-1 | ...TM.KD..TL.IE.LKTE |
| DENV-2 | ...TM.KN..TL.FE.IETE |
| DENV-3 | ...TM.KN..TL.IE.QKTE |
| DENV-4 | ...TM.Q...TL.FE.TKTT |
| APOIV | .S.LMV.D..AV.....EVS |
| REV | ....S.QD...V.L...DVI |

**B**

| | 378 |
|---|---|
| Oshi5-10 | VIMRAVNDWI |
| Ic10 | .....A.... |
| Za | ..... |
| Vs | ..... |
| Sof | ..... |
| 205 | ..... |
| Hypr | ..... |
| Neu | ..... |
| LIV | ..T..... |
| KFDV | I..... |
| LGTV | I.....L |
| POWV | .....S..L |
| YFV17D | K..KV..R.L |
| YFVAsibi | K..KV..R.L |
| JEVBej | EVLNETTN.L |
| JEVSA14142 | EVLNETTN.L |
| MVEV | TV.DETTN.L |
| KUNV | YVLNETTN.L |
| WNV | YVLNETTN.L |
| SLEV | Q..DVTT..L |
| DENV-1 | Q..EVTAR.L |
| DENV-2 | KL.KITAE.L |
| DENV-3 | KV.EITAE.L |
| DENV-4 | MV.TTTAN.L |
| APOIV | KV..L.FR.L |
| RBV | IV..K.FT.L |

**C**

| | 674 |
|---|---|
| Oshi5-10 | SGDDCVVRPVDDRFSRALYFLND |
| Ic10 | .....K..... |
| Za | .....I....GK..... |
| Vs | .....I....GK..... |
| Sof | .....G..... |
| 205 | .....G..... |
| Hypr | .....L....GK..... |
| Neu | .....L....GK..... |
| LIV | .....I....GK..... |
| KFDV | .....I....GK..... |
| LGTV | .....K..... |
| POWV | .....K.I....GK..... |
| YFV17D | .....I....GL..SH..A |
| YFVAsibi | .....I....GL..SH..A |
| JEVBej | .....K.L....AT..H...A |
| JEVSA14142 | .....K.L.....T..H...A |
| MVEV | .....K.L.....T..H...A |
| KUNV | .....K.L....ATS.H...A |
| WNV | .....K.L....ATS.H...A |
| SLEV | .....K.I....AT..H...N |
| DENV-1 | .....K.I....AT..TA... |
| DENV-2 | .....K.L....AS..TA... |
| DENV-3 | .....K.I....AN..LA... |
| DENV-4 | .....K.L.E..GTS.L.... |
| APOIV | .....NAL.E..GK..TW..T |
| RBV | .....L.NAC.E..G.S.TW..T |

**D**

| | 794 |
|---|---|
| Oshi5-10 | PVDWVPTGRTTWSIHASGAW |
| Ic10 | .....A..... |
| Za | ..... |
| Vs | ..... |
| Sof | .....A..... |
| 205 | .....C..... |
| Hypr | ..... |
| Neu | .A..... |
| LIV | .A..... |
| KFDV | .I....Q..... |
| LGTV | ..... |
| POWV | ..S...M.....E. |
| YFV17D | .TS...Q.....GK.E. |
| YFVAsibi | .TS...Q.....GK.E. |
| JEVBej | .....S....SK.E. |
| JEVSA14142 | .....S....SK.E. |
| MVEV | .....GK.E. |
| KUNV | ..N.....G.E. |
| WNV | .AN.....K.E. |
| SLEV | ..N.....GK.E. |
| DENV-1 | .....S.....HHQ. |
| DENV-2 | .SH....S.....KHE. |
| DENV-3 | ..H....S.....HHQ. |
| DENV-4 | .TE.F..S.....HHQ. |
| APOIV | .K..F.....V..NHE. |
| RBV | .SN.I.....TKD. |

**Fig. 4.** Fragments of amino acid alignments of E protein (A) and NS5 protein (B, C and D). Viruses are presented according to the recent ICTV scheme (Heinz *et al.*, 2000): flaviviruses of mammalian (ordinary letters) and seabird (ordinary letter, underlined) groups belong to one tick-borne flavivirus group within the genus *Flavivirus*. Mosquito-borne flaviviruses (names in bold letters) and the non-vectored viruses APOIV and RBV (names in bold letters, underlined) are two other ecological groups within the genus. The viruses in capital letters represent virus species. The first eight viruses at the top are strains of TBEV. The designations of viruses with appropriate accession numbers were listed in Heinz *et al.* (2000). Numbers on the top specify mutated amino acids within the E protein (A) and NS5 protein (B, C and D) for O-IC virus.

## A single amino acid substitution within the E protein reduces the size of fluorescent foci

Following the above analysis we carried out mutagenesis on the infectious clone to restore the original sequence of the parent virus and also to identify which mutation or mutations are responsible for reduction in size of fluorescent foci induced by the infectious clone. The strategy for the construction of mutant viruses is described in Methods and is illustrated in Fig. 1. The genotypes of recovered mutant viruses are presented in Fig. 2. The viruses were compared in four different biological tests, viz. relative size of fluorescent foci, growth curve experiments, mouse neurovirulence and neuroinvasiveness.

The parent Oshima 5-10 virus and O-IC-pt virus which were genetically identical and contain serine in position 40 of the E protein (genotype $S_{40}V_{378}R_{674}T_{794}$), produced identical large fluorescent foci at 36 h post-infection (Fig. 3). Viruses $S_{40}A_{378}K_{674}T_{794}$ and $S_{40}A_{378}K_{674}A_{794}$, also with E-$S_{40}$, but different from Oshima 5-10 virus and O-IC-pt virus in the *NS5* gene and the 3′UTR, also formed large foci by 36 h post-infection. Therefore the three mutations in the NS5 protein, in conjunction with mutations in the 3′UTR, do not influence focus development. On the other hand, $P_{40}A_{378}K_{674}T_{794}$ virus with the single substitution E-$S_{40}$→P in comparison with $S_{40}A_{378}K_{674}T_{794}$ virus, formed fluorescent foci only after incubation for at least 72 h. These results indicate that a single amino acid mutation within the E protein ($E_{40}$) was responsible for the focus size reduction described in this report.

## Virus growth curves in cell cultures

Several conclusions arise from the comparative growth characteristics of the mutated viruses (Fig. 5).

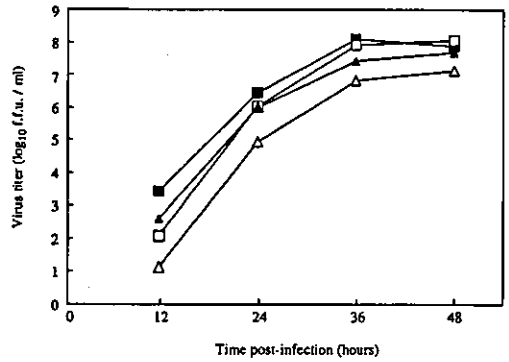


**Fig. 5.** Comparison of the growth curves of recovered viruses in BHK cells which were infected (m.o.i. 0·01) with parent Oshima 5-10 (■), $S_{40}A_{378}K_{674}T_{794}$ (▲), $P_{40}A_{378}K_{674}T_{794}$ (△) or O-IC-pt (□) virus. Supernatant medium was harvested at intervals of 12 h post-infection. Virus infectivity was determined by focus assay. Each point is the mean of three separate experiments. The variation in individual points on the graph did not exceed 0·5$\log_{10}$ f.f.u.

Firstly, there were highly significant differences (orders of magnitude) in the yields of virus at 12 h post-infection between parent virus Oshima 5-10 and genetically identical O-IC-pt virus. This was a reproducible observation that may possibly be explained by the presence of quasi-species in the original parent virus population (see Discussion).

Secondly, although newly synthesized virions began to appear for all viruses between 12–14 h post-infection, the infectious yields at each time point remained higher for $S_{40}A_{378}K_{674}T_{794}$ virus than for $P_{40}A_{378}K_{674}T_{794}$ and since they differ by only one amino acid in position 40 of the E protein (E-$S_{40}$ genotype against E-$P_{40}$ respectively) these results imply that a single substitution in the *E* gene significantly reduces virus reproduction efficiency in cell cultures.

Thirdly, there was no significant difference in the growth curves produced by O-IC-pt ($S_{40}V_{378}R_{674}T_{794}$) and $S_{40}A_{378}K_{674}T_{794}$, despite the fact that they differ by two amino acids (NS5-$V_{378}$→A and NS5-$R_{674}$→K).

Therefore these growth curve experiments demonstrate that only the mutation in the E protein exerts an effect on the phenotype of the virus in cell culture.

## Virulence for mice

The results of neurovirulence tests for $S_{40}A_{378}K_{674}T_{794}$, $P_{40}A_{378}K_{674}T_{794}$, O-IC-pt and parent virus Oshima 5-10 are shown in Fig. 6. Following intracerebral inoculation, both parent virus Oshima 5-10 and genetically identical O-IC-pt virus produced 100% mortality of mice within 12 days post-inoculation. In contrast, $S_{40}A_{378}K_{674}T_{794}$ virus, that is different from O-IC-pt virus by two amino acid substitutions in the *NS5* gene (NS5-$V_{378}$→A and NS5-$R_{674}$→K) had lower neurovirulence, killing a maximum of 62·5% of mice by day 18 post-inoculation. Virus $P_{40}A_{378}K_{674}T_{794}$, that is different from $S_{40}A_{378}K_{674}T_{794}$ virus by only one amino acid in the E protein, showed the lowest neurovirulence, killing

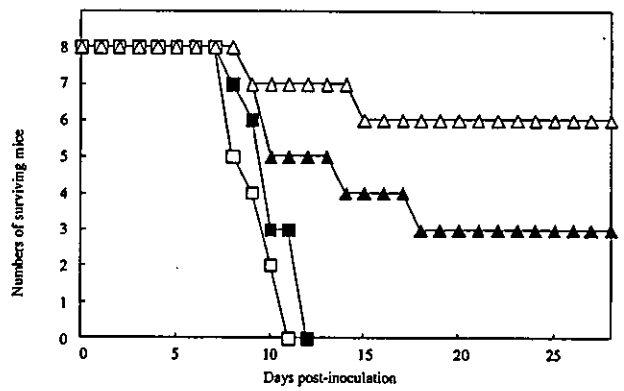


**Fig. 6.** Fate of mice inoculated intracerebrally with each virus. Mice were inoculated with 50 f.f.u. of parent Oshima 5-10 (■), $S_{40}A_{378}K_{674}T_{794}$ (▲), $P_{40}A_{378}K_{674}T_{794}$ (△) or O-IC-pt (□) virus and monitored daily.

**Table 2.** Morbidity and mortality of mice inoculated with 1000 f.f.u. of each virus subcutaneously

| | Morbidity (no. of sick/total) | Mortality (no. of sick/total) |
|---|---|---|
| Oshima 5-10 (parent) | 8/8 | 8/8 |
| O-IC-pt | 7/8 | 0/8 |
| $P_{40}A_{378}K_{674}T_{794}$ | 0/8 | 0/8 |
| $S_{40}A_{378}K_{674}T_{794}$ | 0/8 | 0/8 |

about 25 % of mice. These experiments therefore demonstrate firstly, a biological effect of NS5-$V_{378}$→A and NS5-$R_{674}$→K mutations that was not detectable in cell culture, and secondly it shows the cumulative effect of E-$S_{40}$→P, NS5-$V_{378}$→A and NS5-$R_{674}$→K on attenuation of the virus.

Neuroinvasiveness was also compared for the same viruses, by subcutaneous inoculation of mice with 1000 f.f.u. of each virus. The results of these experiments are presented in Table 2. All mice inoculated with parent Oshima 5-10 virus died between 8 and 12 days post-inoculation. However, all mice inoculated with O-IC-pt virus, that is the virus with an identical genotype, were still alive at 28 days post-inoculation, although seven of the eight mice showed clinical signs such as paralysis or loss of body weight (Table 2). These results may possibly be explained by the presence of virus quasi-species with different pathogenic characteristics in the original virus population (see Discussion).

Comparison of O-IC-pt and $S_{40}A_{378}K_{674}T_{794}$ viruses that differ by two mutations in the NS5 protein, show differences in morbidity but not mortality rate, suggesting that both NS5-$V_{378}$→A and NS5-$R_{674}$→K in addition to neurovirulence also affect virus neuroinvasiveness. The effect of the E-$S_{40}$→$P_{40}$ mutation was not possible to evaluate, since all mice inoculated with $S_{40}A_{378}K_{674}T_{794}$ and $P_{40}A_{378}K_{674}T_{794}$ survived for the entire period of observation and showed no clinical signs.

## DISCUSSION

This is the first report of an infectious clone of a Far-Eastern subtype of TBEV that is commonly referred to as RSSE virus because the disease caused by this virus typically occurs in the spring and summer in the forested regions of Far-Eastern Russia, Asia and Japan (Gritsun *et al.*, 2003b; Ecker *et al.*, 1999; Hayasaka *et al.*, 2001). Infectious clones of two other TBEV subtypes, that is Western European and Siberian, were constructed previously (Gritsun & Gould, 1998; Mandl *et al.*, 1997). Despite their close antigenic and genetic similarity (about 96 % for amino acid sequence), TBEV strains of different subtypes cause diseases with a variety of clinical manifestations and mortality rates. According to statistical reports, Far-Eastern TBEV causes the most severe encephalitis with a higher case fatality than the European or Siberian subtypes (Gritsun *et al.*, 2003a, b; Shope, 1980; Dumpis *et al.*, 1999; Korenberg & Kovalevskii, 1999). The construction of these different but related infectious clones of TBEV therefore provides us with important tools with which to investigate and possibly to resolve the underlying basis of TBEV pathogenicity.

To construct an infectious cDNA clone of Far-Eastern subtype TBEV, we used the Oshima 5-10 strain, which was isolated in 1995 in Japan (Takashima *et al.*, 1997). Although only one case of human TBE has been confirmed in Japan, it is believed that TBEV emerged within the past few centuries and was distributed to a wide area of Hokkaido, the northern island of Japan (Hayasaka *et al.*, 1999; Takeda *et al.*, 1999). Oshima 5-10 virus shares more than 98 % amino acid identity with Sofjin virus, the prototype Far-Eastern TBEV (Goto *et al.*, 2002). In addition, Oshima 5-10 virus shows similar virulence in mice with the viruses recently isolated in Far-Eastern Russia (Chiba *et al.*, 1999; Hayasaka *et al.*, 1999, 2001).

In this study we constructed an infectious clone using the strategy described for the Siberian strain of TBEV, Vs, (Gritsun & Gould, 1995, 1998). Long high-fidelity PCR and one-step cloning procedures were employed to produce bacterial clones containing full-length molecules of TBEV. The bacterial cells were routinely propagated at 28 °C to reduce the mutation rate introduced by the bacteria. We checked 88 clones from bacterial colonies after transformation and ten contained full-length virus sequences. One clone (O-IC) that was infectious for mice and BHK cells was selected for further analysis. A separate non-infectious full-length clone that we sequenced had 15 nucleotide differences from the parent virus and a single nucleotide deletion at position 5120 in the NS3 protein region (data not shown). A similar proportion of 1/12 between infectious and non-infectious cDNA molecules was also detected after cloning the Siberian TBEV strain (Gritsun & Gould, 1998). The fact that bacteria select in favour of non-infectious molecules has been explained by low level expression of regions of flavivirus polyprotein with transmembrane domains that could be toxic for bacteria; therefore clones that accidentally acquired lethal mutations might have a selective advantage (Yamshchikov *et al.*, 2001a). Nevertheless, although only one infectious clone was obtained, and it was stable in *E. coli* strain Able-K and also HB101 at both 28 °C and 37 °C. The original infectious clone O-IC had nine nucleotide substitutions compared with the parent virus Oshima 5-10 (see below), but other full-length infectious clones that were individually modified by reverse mutations to parent virus sequence ($S_{40}A_{378}K_{674}T_{794}$, $P_{40}A_{378}K_{674}T_{794}$, $S_{40}A_{378}K_{674}A_{794}$ and O-IC-pt) were also stable. Therefore, the infectious clone will be highly exploitable for genetic manipulations.

Four amino acid substitutions, one mapping within the E glycoprotein and the other three within the *NS5* gene (RNA-dependent RNA polymerase), were revealed between parent Oshima 5-10 and the original infectious clone O-IC (Table 1). O-IC virus also differed from parent virus in the infectious focus test, producing infectious foci later than those of the parent virus Oshima 5-10 (Fig. 3). We therefore

carried out mutagenesis on the infectious clone to restore the original virus genotype and also to identify which mutations caused the delay in formation and reduction of focus size.

An infectious clone O-IC-pt was reconstructed from O-IC to restore the parent virus genotype. This was achieved using a cassette of intermediate plasmids that have also been used to engineer several viruses with different combinations of mutations (Fig. 1). The viruses were compared in focus assays, growth curves, mouse neuroinvasiveness and neurovirulence. The results of these tests demonstrated that substitution of conserved (among all flaviviruses) hydroxyl-containing amino acids serine or threonine in position 40 of the E protein for the aromatic hydrophobic amino acid proline of the infectious clone was solely responsible for the delay of TBEV focus formation, delayed growth curve characteristics and reduced neurovirulence (Figs 3, 5 and 6). Examination of the crystal structure of the E protein (Rey *et al.*, 1995) shows that amino acid $S_{40}$ is buried within the central domain I, i.e. it is not exposed on the surface of the protein. The effect of E-$S_{40}$→P on virus reproduction might therefore be mediated by conformational changes that could affect virion adsorption, penetration or assembly. Proline is an aromatic amino acid, larger in size then threonine and serine, and it could induce important structural changes to the protein since proline residues are often located at the point of $\beta$-turns. Nevertheless one non-vectored flavivirus – *Apoi virus* – had an alanine in this position (Fig. 4). Therefore it is possible to have a hydrophobic amino acid in this position, although it could change the properties of the protein. The E protein is the most studied protein of the flaviviruses because it is associated with the main biological characteristics of the virus, including virion adsorption, pH-dependent penetration, haemagglutination, induction of virus-neutralizing and protective antibodies, antibody-dependent enhancement and virulence properties (Barrett & Gould, 1986; McMinn, 1997; Heinz & Allison, 2000; Heinz, 2003). Many mutations have been identified that attenuate virus reproduction through different functional regions of the E protein (Lee & Lobigs, 2000; Mandl *et al.*, 2000, 2001; Hurrelbrink & McMinn, 2001; Holzmann *et al.*, 1997; Allison *et al.*, 2001; Monath *et al.*, 2002; Cecilia & Gould, 1991; Gritsun *et al.*, 1995, 2001; Jiang *et al.*, 1993; Rey *et al.*, 1995). At this stage of the investigations it is not possible to define the function of the E protein (adsorption, penetration or virion assembly) that was affected by the E-$S_{40}$→P mutation.

Two nucleotide substitutions were mapped in the 3′UTR. The mutation at position 10473 (position 97 after the stop codon in Oshima 5-10 virus sequence; Table 1) mapped in the hypervariable region of the flaviviruses (Gritsun *et al.*, 1997; Wallner *et al.*, 1995) that was shown not to influence flavivirus infectivity (Mandl *et al.*, 1998). The second mutation, guanine, in genome position 10922 (nucleotide 546 after the stop codon), mapped in a highly conserved region of the 3′UTR (Gritsun *et al.*, 1997; Proutski *et al.*, 1997; Rauscher *et al.*, 1997) where the nucleotide alignment revealed adenine for 25 tick-borne flaviviruses (data not presented). Nevertheless, folding of the 3′UTR of the infectious clone did not predict any change in RNA secondary structure following substitution of adenine for guanine (data not presented).

Similarly with the Siberian TBEV virus, Vs, mutations other than those in the E protein of the infectious clone O-IC, appeared to be responsible for the reduction of neurovirulence in mice, although they did not result in any biological consequences for plaque assays or growth cycle characteristics (Gritsun *et al.*, 2001). Two viruses, O-IC-pt and $S_{40}A_{378}K_{674}T_{794}$ were genetically different by two amino acids within the NS5 protein (in positions 378 and 674, Fig. 2 and Table 1) that affected only neurovirulence and neuroinvasive properties of the virus (Fig. 6 and Table 2) with no effect on virus growth characteristics in BHK cell culture (Fig. 5). These two substitutions within the NS5 protein were of a conserved nature ($V_{378}$→A, $R_{674}$→K), but both were located in functionally important domains of NS5 protein, one $V_{378}$→A in the nuclear localization sequence (Forwood *et al.*, 1999) and the other $R_{674}$→K in close proximity to the highly conserved GDD sequence (663–665), which is an RNA-dependent RNA polymerase motif (Rice *et al.*, 1985; Khromykh *et al.*, 1998). Therefore the reduction of neurovirulence due to these two substitutions could be explained by the limitations imposed on protein tertiary structure in highly conserved domains of the NS5 protein. A similar observation for mutations within the NS5 protein and the 3′UTR was previously reported for the infectious clone of the Siberian TBEV Vs virus (Gritsun *et al.*, 2001).

Comparative analyses of three viruses in neurovirulence tests (Fig. 6), namely O-IC-pt (parent genotype), $S_{40}A_{378}K_{674}T_{794}$ (with substitutions $V_{378}$→A and $R_{674}$→K in comparison with parent genotype) and $P_{40}A_{378}K_{674}T_{794}$ (with substitution $S_{378}$→P in comparison with $S_{40}A_{378}K_{674}T_{794}$ virus genotype) have also revealed the cumulative effect of different mutations on attenuating properties of TBEV, as has also been demonstrated for Siberian virus (Gritsun *et al.*, 2001).

Neuroinvasiveness was also compared by subcutaneous inoculation of mice (Table 2). All mice inoculated with parent Oshima 5-10 virus died within 8–12 days post-inoculation. Although mice inoculated with O-IC-pt virus survived until 28 days post-inoculation, some showed clinical signs such as paralysis or loss of body weight (Table 2). Growth curve experiments also demonstrated smaller virus titres at 12 h post-infection for O-IC-pt virus in comparison with parent Oshima 5-10 virus (Fig. 5). In view of these unexpected results, we sequenced the virus recovered from sick mice and confirmed that this O-IC-pt virus had the identical sequence to parent Oshima 5-10 virus. One possible explanation for this result may be that the parent Oshima 5-10 virus consists of a population of quasi-species. Since the parent Oshima 5-10 virus sequence had been determined by direct-sequencing from RT-PCR products (Hayasaka

*et al.*, 1999; Goto *et al.*, 2002), the sequence would be a consensus. We therefore cloned parent Oshima 5-10 virus by plaque assay and noticed several different sizes of plaque (data not shown). We picked a large and a small plaque variant and determined the sequence of the E protein of each. A single but different amino acid substitution was detected in the E protein of each plaque variant. Both viruses showed less neuroinvasiveness than the parent virus (Goto *et al.*, 2003). Whilst we did not identify a plaque variant with the high neuroinvasiveness of the parent virus, these results combined with the concept that quasi-species may show a range of virulence characteristics, provide a rational explanation for the difference in neuroinvasiveness of parent Oshima 5-10 and O-IC-pt virus for mice. It is unlikely that other phenomena such as interferon or defective interfering particles can account for this difference in virulence because both virus stocks were prepared in the same way, that is in BHK cells. Moreover, the infectious clone was passaged only once in BHK cells which would be unlikely to generate a significant level of defective particles.

In summary, we have constructed an infectious clone based on the Far-Eastern subtype of TBEV. The original infectious clone contained four amino acid substitutions that were back-mutated to produce the infectious clone genetically identical to the parent virus. Site-directed mutagenesis on the infectious clone revealed that one amino acid, $P_{40}$, in the E protein and two amino acids, $A_{378}$ and $K_{674}$, in the NS5 protein were responsible for the virus attenuation. We also demonstrated the cumulative effect of point mutations on attenuated characteristics of TBEV.

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# Sub-genomic replicons of *Tick-borne encephalitis virus*

## Brief Report

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**Summary.** We constructed three sub-genomic replicons of *Tick-borne encephalitis virus* (TBEV) (Oshima REP, Oshima REP-GFP and Oshima REP-Neo) by deleting genes coding for structural proteins without or with insertion of green fluorescent protein (GFP) or Neo genes, respectively. BHK cells transfected with Oshima REP expressed the viral non-structural antigens in immunofluorescent and western blot analyses. GFP and viral antigens were co-expressed in the transfected cells with Oshima REP-GFP. G418-resistant cells harboring Oshima REP-Neo consistently expressed the antigens without showing any apparent CPE. These replicons constructed in this study will be useful in studies on the replication, assembly and packaging of TBEV, and to develop vaccines and gene-delivering systems.

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The genus *Flavivirus*, family *Flaviviridae* contains several important pathogens, including *Japanese encephalitis virus*, *Yellow fever virus* (YFV), *Dengue virus* (DENV), *West Nile virus* (WNV) and *Tick-borne encephalitis virus* (TBEV). Phylogenetic analysis revealed that flavivirus is divided into mosquito-borne, tick-borne and no-known-vector virus clades [8, 25]. It is interesting to reveal the background of different pathogenesis such as mosquito-borne, tick-borne and no-known-vector viruses on molecular basis.

The flavivirus genomes (single, positive-stranded RNA, 10–11 kilobases in length) encodes three structural genes coding capsid (C), membrane precursor (prM) and envelope (E) proteins and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) within a single long open reading

frame [5]. The 5′- and 3′-untranslated regions (UTR) have predicted secondary structures [5, 27].

System of sub-genomic replicon is a very useful tool to study viral replication, assembly and packaging, development of vaccine and delivering system of foreign genes. Several sub-genomic replicons of positive-strand RNA viruses have been constructed [1, 2, 3, 11, 16, 27, 29, 31, 33, 38, 40]. With respect to the family *Flaviviridae* sub-genomic replicons were constructed for Kunjin virus [17, 42], WNV [34], DENV [32] and YFV [6]. However, these are that of mosquito-borne viruses and there is no report of these for tick-borne flaviviruses. It is valuable to construct the sub-genomic replicon of tick-borne flaviviruses.

TBEV is prevalent over a wide area on the Eurasian continent and causes severe encephalitis in humans with serious sequelae [7, 10, 12, 35]. Oshima strain of TBEV was isolated from a dog in the endemic area in Hokkaido, Japan and identified as Russian Spring-Summer encephalitis virus known as possessing high neurovirulence [13, 36, 37]. In this study, we constructed the sub-genomic replicons of Oshima strain.

The strategy constructing sub-genomic replicon cDNA is shown in Fig. 1. The first replicon was created from a full-length infectious cDNA clone of Oshima strain (O-IC). Briefly, O-IC was developed by high fidelity long RT-PCR and one-step cloning procedure with a modified pBR322 vector (unpublished data). Infectivity of O-IC was confirmed by production of infectious virus particles in BHK cells following electroporation of the RNA and by pathogenicity in mice after intracerebral inoculation (unpublished data). Recovered O-IC virus showed similar cytopathic effects in cell culture in comparison with that of the parent Oshima strain (unpublished data).

Three parts of Oshima virus genome (nt −26–242, 2295–2554, and 2554–3956, respectively) were amplified by RT-PCR to include SP6 promoter sequence at 5′-terminal and restriction sites (*Not*I$_{-26}$, *Avr*II$_{242,2295}$, *Nsi*I$_{2554}$ or *Eco*RI$_{3956}$). These sequences were cloned into pGEM3 vector (pGEM3-del.CprME). pGEM3-del.CprME had a deletion in the majority of structural proteins sequence of amino acid $C_{38}$–$E_{421}$ and the deletion was combined with *Avr*II. pGEM3-del.CprME was digested with *Not*I$_{-26}$ and *Age*I$_{3946}$ and inserted into O-IC between nt −26 and 3946 (Oshima REP). Recombinant clones containing flavivirus genomes have been known to be unstable during propagation in *E. coli*. Therefore, we confirmed the stability of Oshima REP during propagation in *E. coli* strain HB101 at 37 °C to have no mutation, insertion nor deletion in the recombinants by sequencing (data not shown).

After digestion of replicon cDNA with *Spe*I, *in vitro* transcription by SP6 RNA polymerase was performed using a mMESSAGE mMACHINE (Ambion, Inc., TX U.S.A.). About 10 μg of RNA was electroporated into $1 \times 10^7$ of BHK cells, carried out in 0.2 cm cuvettes using the GenePulser apparatus (Bio-Rad, CA U.S.A.), 140 V and 25 ms, pulsed three times interval time 1 sec. The cells were incubated at 37 °C under 5% $CO_2$ in 8% fetal calf serum (FCS) -Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Japan).

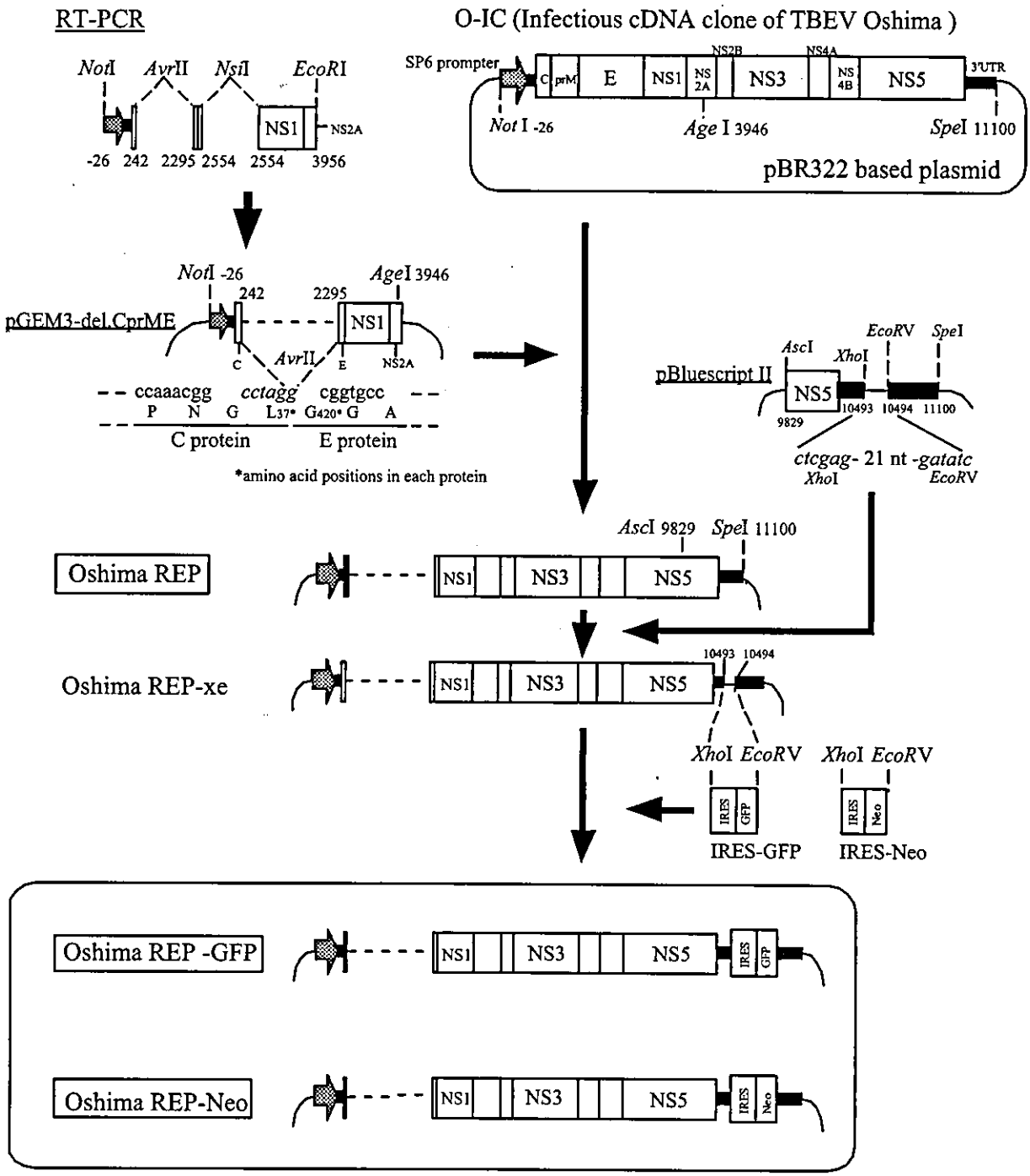



**Fig. 1.** Strategy of construction of sub-genomic replicons

The expression of virus proteins in transfected BHK cells was analyzed using an indirect immunofluorescent analysis (IF). Electropolated cells were cultured on 8 wells of glass slides for 24, 48 and 96 hours. Cells were fixed in 10%

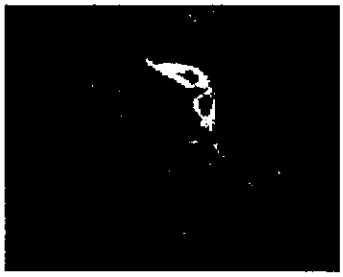
formaldehyde in PBS for 10 min and were treated in 0.5% triton-X100 in PBS for 5 min. After blocking 1 h at 37 °C with 20% Block Ace (Dainippon Pharmaceutical CO., LTD, Japan) in PBS, 1:100 diluted anti-Langat virus mouse poly-clonal ascites was applied on each well and incubated at 37 °C for 1 h. This ascites was obtained from hyperimmunized mice with live *Langat virus* [36], which was approximately 85% homology in the coding gene sequence with that of Oshima strain in amino acids [4, 15, 30]. After wash in 0.02% Tween 20 in PBS, 1:500 diluted Alexa Fluor 532 goat anti-mouse IgG (H+L) (Molecular Probe, Inc., OR, USA) was reacted as second antibody. Cells were examined using a Spectral Con-focal Scanning System, TCS SP2 (Leica, Microsystems, Germany).

The TBEV antigens were detected in cells transfected with Oshima REP at 48 h after electroporation by IF (Fig. 2A, anti-Langat). The antigens localized mainly in the cytoplasm. These antigen positive-cells showed a similar morphology and viability in comparison with normal (virus antigen negative and mock) cells (Fig. 2A, DIC and 2C, BHK DIC). Any apparent cytopathic effects (CPE) was not observed in the TBEV antigen-positive cells (Fig. 2A). The antigens were also detected at 96 h post-transfection without any CPE (data not shown). Only a few amounts of virus antigens were detected at 24 h post-transfection (data not shown). These results indicated that replicon RNA expressed virus proteins until 48 h post-transfection and lasted at least 96 h post-transfection.

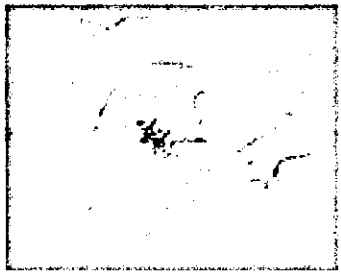
A number of heterologous genes have been inserted flavivirus sub-genomic replicons [17, 34, 41]. Previous studies showed that insertion of heterologous genes into 3′ UTR in the minus-sense orientation did not express any heterologous proteins, while replicon RNAs were replicatable [17, 34]. The sizes of 3′ UTR sequences of TBEV strains are various and several strains have a poly-A tail in 3′ UTR [14, 39]. Oshima strain has 3′ UTR sequence of approximately 720 bases in length and *AAACAAAAA* sequence similar to poly (A) signal is present in the variable region [14]. We inserted green fluorescent protein (GFP) into 3′ UTR of Oshima REP following the *AAACAAAAA* sequence in sense orientation. As shown in Fig. 1, two fragments (9829–10493 and 10494–11100) of RT-PCR products were sub-cloned into pBluescript II plasmid with *Asc*I$_{9829}$, *Xho*I$_{10493}$, *Eco*RV$_{10494}$ and *Spe*I$_{11100}$ sites. Then the sequence between *Asc*I$_{9829}$ and *Spe*I$_{11100}$ was replaced into the same region of Oshima REP (Oshima REP-xe). As the result, Oshima REP-xe contained thirty-three extra *Xho*I$_{10493}$ and *Eco*RV$_{10494}$ sites including 21 nucleotides derived from pBluescript II. Oshima REP-xe also showed the expressions of replicon proteins (data not shown). IRES-GFP sequence was amplified from pIRES2-EGFP (Clontech, CA, U.S.A.) and introduced into Oshima REP-xe in the plus-sense orientation into 3′ UTR with *Xho*I and *Eco*RV sites (Oshima REP-GFP).

**Fig. 2.** Confocal laser microscopic and differential interference contrast (DIC) analyses of BHK cells transfected with TBEV subgenomic replicons, Oshima REP, at 48 h after electroporation (**A**), Oshima REP-GFP at 96 h after electroporation (**B**) and Oshima REP-Neo for days 30, 4 passages (**C**)

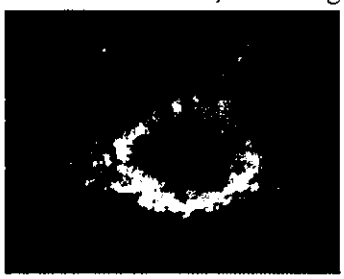
**A.** Oshima REP, anti-Langat



Oshima REP, DIC



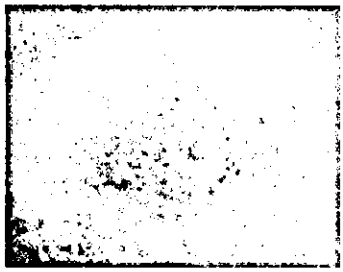
**B.** Oshima REP-GFP, anti-Langat



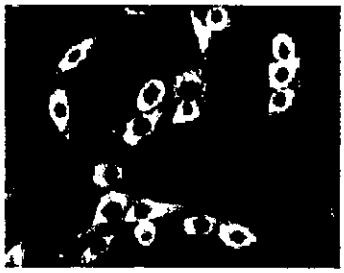
Oshima REP-GFP, EGFP

Oshima REP-GFP, merge

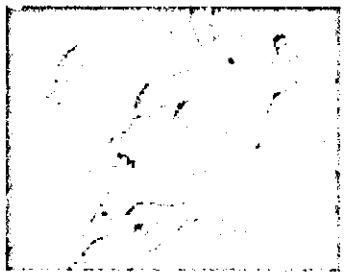
Oshima REP-GFP, DIC



**C.** Oshima REP-Neo, anti-Langat

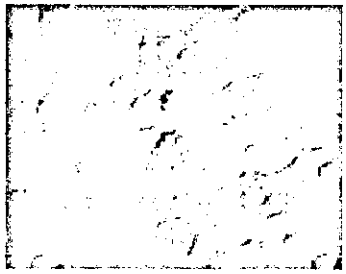


Oshima REP-Neo, DIC



BHK, anti-Langat

BHK, DIC



Expressions of GFP and virus antigens in BHK cells transfected with Oshima REP-GFP RNA were determined at 96 h post-transfection (Fig. 2B, anti-Langat and EGFP). These proteins were co-expressed in the same cells (Fig. 2B, merge). Virus antigens localized in cytoplasm (Fig. 2B, anti-Langat). In contrast, GFP was observed both in cytoplasm and nucleus (Fig. 2B, EGFP). Morphology and viability of antigen positive cells were not distinguished from normal BHK cells and any CPE was not seen (Fig. 2B, DIC). Replicon antigen and GFP positive cells were seen at 48 h post-transfection, whereas the expression was very few at 24 h post-transfection (data not shown). These results indicate that Oshima REP-GFP can express foreign gene of GFP.

Although expression of replicon proteins or GFP were detectable by IF (Fig. 2), the percentage of positive cells was less than 5%. We examined the expressions of NS proteins in Oshima REP and Oshima REP-GFP by western blot. However, NS proteins were not detected in cells transfected with both replicons at 60 h after transfection (data not shown), probably due to a small number of cells expressing NS proteins. Sub-genomic replicon does not produce any infectious particles due to deletion in the structural proteins and cell divisions are not always associated to harbor replicon RNA. Therefore, cells harboring the replicons tend to be overgrown by normal cells during cell culture.

To establish a cell line harboring sub-genomic replicon of TBEV persistently, we constructed Oshima REP-Neo containing neomycin phosphotransferase (Neo gene) in 3′ UTR as the same way of Oshima REP-GFP. The sequence of Neo gene with IRES sequence was amplified from pIRESneo3 (BD Biosciences Clontech, CA, USA) and inserted into Oshima REP-xe (Fig. 1). To select G418-sulfate resistant-cells, cells transfected with Oshima REP-Neo RNA were incubated with 8% FCS-EMEM for 2 h after electroporation and then add G418 sulfate (Geneticine; Invitrogen Corp., Calsbad, CA U.S.A.) up to 1 mg/ml. A number of G418-resistant cells were grown, while none of normal BHK cells were alive under the presence of G418 (data not shown). G418-resistant cells were passaged with 1:5 split every 5–7 days. As shown in Fig. 2C (anti-Langat), all cells were expressing virus antigens in cytoplasm for days 30 (4 passages). Cell morphology and viability of the G418-resistant cells were similar to normal BHK cells (Fig. 2C, DIC). Importantly, none of these cells exhibit any apparent CPE. Replicon antigens were detected for at least day 50 (data not shown).

Expression of NS proteins in Oshima REP-Neo harboring cells was determined by western blot analysis by the method described previously [9]. Briefly, Oshima REP-Neo harboring cells, parent Oshima virus infected cells and normal BHK cells were harvested and lysed with sodium dodecyl sulfate (SDS) buffer without 2-mercaptoethanol. After boiling, each sample was applied in SDS-PAGE. Anti-Langat virus poly-clonal ascites and anti-E protein of TBEV rabbit serum were used to detect virus proteins (unpublished results). NS3 protein (69 kDa) was detected in Oshima REP-Neo and Oshima virus lanes by anti-Langat virus antibody (Fig. 3A), while no band was detectable in untransfected BHK cells. E protein (53 kDa) was not detectable in Oshima REP, while a large amount of E protein was present in Oshima virus by anti-E antibody. Presumably, other NS

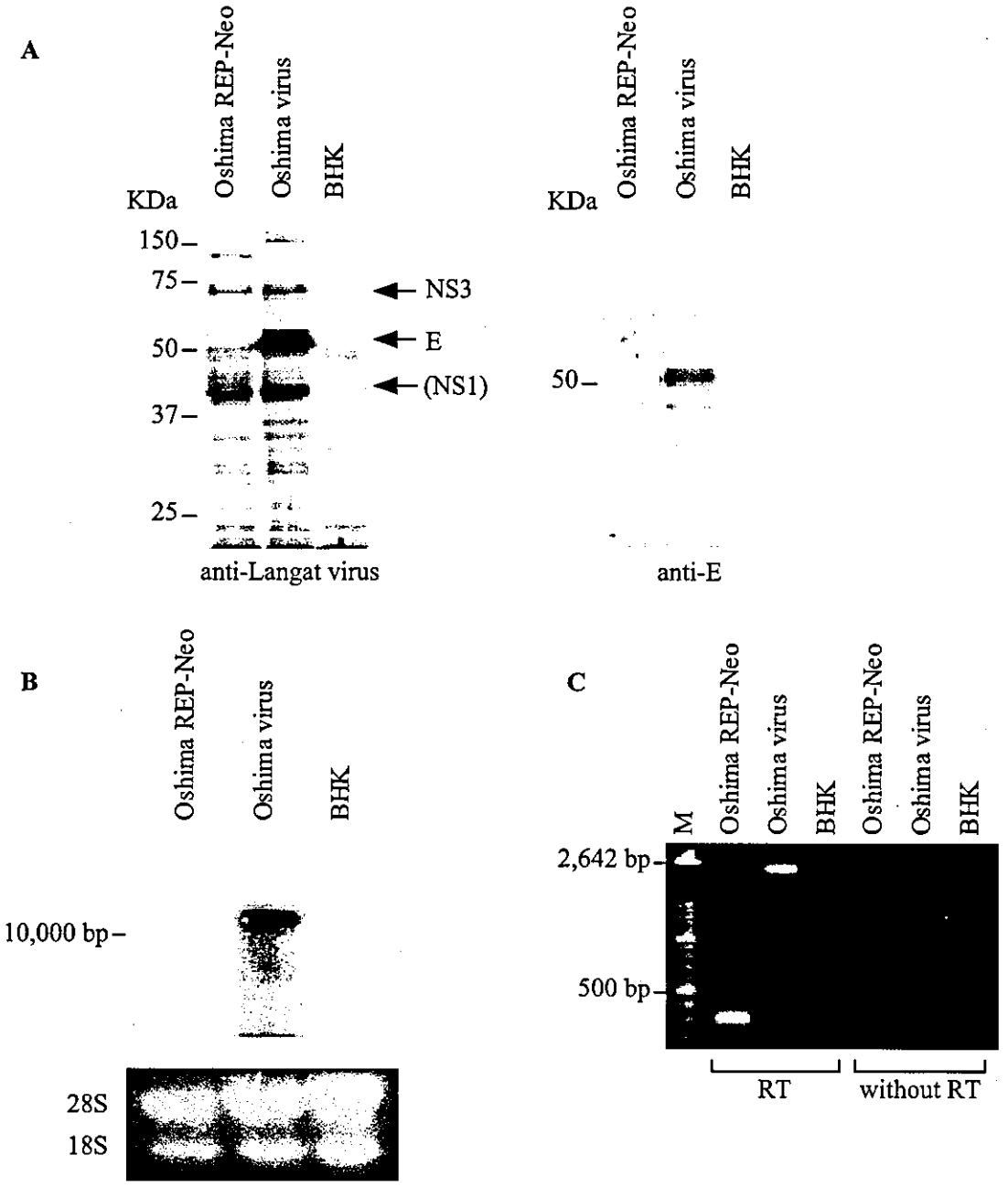



**Fig. 3.** **A** Detection of NS proteins by western-blot analysis. Oshima REP-Neo; Oshima REP-Neo harboring cells (5 passages), Oshima virus; cells infected with parent Oshima virus (60 h post-infection at multiplicity of infection: 0.01), BHK; untransfected with replicon RNA or uninfected with Oshima virus. **B** Detection of virus RNA by northern blot analysis. Samples were from same cells in **A**. Ribosomal RNAs (28S and 18S) were also shown. **C** Results of RT-PCR. RNAs were extracted from same cells of **A** and **B**. PCR was performed using cDNA with or without RT. *M* is DNA Molecular Weight Marker XIV (Roche Diagnostics K.K., Tokyo Japan)

proteins, e. g. NS4B (27 kDa) and NS2A (25 kDa), were also detected in Oshima REP and Oshima virus by anti-Langat virus antibody. However, it was difficult to determine which bands were those NS proteins, because several non-specific bands were also seen in untransfected BHK cells. A broad band between 37 kDa and 50 kDa in replicon harboring cells and virus infected cells was likely to be undenatured NS1 (49 kDa).

To chase RNA replication in transfectant, northern blot analysis was carried out as described previously [9]. 10 μg of RNA was applied in each lane. A partial sequence of NS5-3′ UTR (nt 9829–10493) was used as a probe. Unexpectedly, RNA was not detectable in Oshima REP-Neo cells by the northern blot (Fig. 3B, Oshima REP-Neo), whereas a large amount of RNA was detected in positive control of cells infected with the parent Oshima virus (Fig. 3, Oshima virus). Presumably, RNA was replicating at a low level in cells harboring Oshima REP-Neo, although efficient expressions of NS proteins were detectable by IF and western blot.

Therefore, we confirmed the existence of Oshima REP-Neo RNA in this transfectants by RT-PCR (Fig. 3C). RT-PCR was carried out with a Thermo Script RT-PCR system (Invitrogen Corp., Calsbad, CA, U.S.A.). Briefly, 0.1 μg of RNA was used in 10 μl RT reactions. RT primer was identical to the complementary sequence of last 20 nucleotides in the 3′ UTR (nt 11081–11100). PCR was performed at a 30 cycle PCR consisting of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 68 °C. Sense and anti-sense primers were nt 1–20 and 2382–2363, respectively. A single band, 462 bp in size, was amplified in the cells harboring Oshima REP-Neo. This size is compatible with a deletion of structural protein sequence between nt 243 and 2294. In contrast, 2,382 bp band, including the sequence of sturctural genes, was amplified in the infected cells with Oshima strain. No amplification was observed in normal BHK cells and the transfected and infected cells without RT (Fig. 3C, BHK with RT, without RT).

These results indicated that selected G418-resistant cells were consistently expressing replicon antigen (NS proteins) without any apparent CPE, although replicon RNA is replicating at low level.

Predicted secondary structure exhibited the base-paring interactions between the 5′ genomic region and 3′ UTR of flavivirus, and destruction of the base-parings abolished viral replication [23]. Oshima REP had first 37 amino acid sequences of C protein containing proposed the cyclization sequence at nt 114–124 and 163–173 [23]. Oshima REP had also a C-terminal partial of E protein including signal sequence of NS1 protein. These cyclization and signal sequences were required for replication of sub-genomic replicons of flavivirus [17]. The resultant deletion of structural protein was more than 88% of C-prM-E protein. This indicates that main part of structural protein is not indispensable for replication of TBEV as like as mosquito-borne flavivirus as shown previously [17, 34].

Previous report showed that plus-sense replicon RNA to minus-sense was approximately 100:1 [34]. However, quantification of plus- and minus-sense replicon RNA was not possible in this study, because any plus-sense replicon RNA was not detectable in the northern blot analysis (Fig. 3B). Thus, we performed