Ⅱ. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshii, K., Konno, A., Goto, A., Nio, J., Obara, M., Ueki, T., Hayasaka, D., Mizutani, T., Kariwa, H., and Takashima, I.	Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion.	J. Gen. Virol	85	3049-3058	2004
Hayasaka, D., Gritsun, T.S., Yoshii, K., Ueki, T., Goto, A., Mizutani, T., Kariwa, H., Iwasaki, T., Gould, E.A., and Takashima, I.	Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of tick-borne encephalitis virus.	J. Gen. Virol.	85	1007-1018	2004
高島郁夫、早坂大輔、後藤明子、好井健太朗、苅和宏明	日本と極東ロシアのダニ媒介性脳炎ウイルスの系統解析と病原性	ウイルス	55	35-44	2005
Yoshii, K., Hayasaka, D., Goto, A., Kawakami, K., Kariwa, H., and Takashima, I.	Packaging the replicon RNA of the Far-Eastern subtype of tick-borne encephalitis virus into single-round infectious particles: development of a heterologous gene delivery system.	Vaccine	23	3946-3956	2005
Goto, A., Yoshii, K., Obara, M., Ueki, T., Mizutani, T., Kariwa, H., and Takashima, I.	Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. . .	Vaccine	23	3043-52	2005
Obara, M., Yoshii, K., Kawata, T., Hayasaka, D., Goto, A., Mizutani, T., Kariwa, H., and Takashima, I.	Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles.	J. Virol. Methods	134	55-60	2006
Iwasa, M.A., Kariwa, H., Cui, B.Z., Lokugamage, K., Lokugamage, N., Hagiya, T., Mizutani, T., and Takashima, I.	Modes of hantavirus transmission in a population of Clethrionomys rufocanus bedfordiae inferred from mitochondrial and microsatellite DNA analyses.	Arch. Virol.	149	929-941	2004
Lokugamage, K., Kariwa, H., Lokugamage, N., Iwasa, M., Hagiya, T., Araki, K., Tachi, A., Mizutani, T., Yoshimatsu, K., Arikawa, J., Iwasaki, T., and Takashima, I.	Comparison of virulence of various hantaviruses related to hemorrhagic fever with renal syndrome in newborn mouse model.	Jpn. J. Vet. Res	51	143-149	2004
Lokugamage, K., Kariwa, H., Lokugamage, N., Miyamoto,H., Iwasa, M., Hagiya, T., Araki, K., Tachi, A., Mizutani, T., Yoshimatsu, K., Arikawa, J., and Takashima, I.	Genetic and antigenic characterization of the Amur virus associated with hemorrhagic fever with renal syndrome.	Virus Res.	101	127-134	2004
Lokugamage, N., Kariwa, H., Lokugamage, K., Iwasa, M.A., Hagiya, T., Yoshii, K., Tachi, A., Ando, S., Fukushima, H., Tsuchiya, K., Iwasaki, T., Araki, K., Yoshimatsu, K., Arikawa, J., Mizutani, T., Osawa, K., Sato, H., and Takashima, I.	Epizootiological and epidemiological study of hantavirus infection in Japan.	Microbiol. Immunol.	48	843-851	2004
Pattamadilok, S.,Lee,B.H Kumperasart,S., Yoshimatsu, K., Okumura, M., Nakamura, I., Araki, K., Khoprasert,Y., Dangsupa, P., Paniar, P., Jandrig, B., Kruger, D.H., Kiemps, B., Jakel, T., Schmidt, J., Ulrich, R., Kariwa, H., and Arikawa, J.	Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am. .	J. Trop. Med. Hy	75	994-1002	2006
Baek, L.J., Kariwa, H., Lokugamage, K., Yoshimatsu, K., Arikawa, J., Takashima, I., Kang, J.I., Moon, S.S., Chung, S.Y., Kim, E.J., Kang, H.J., Song, K.J., Klein, T.A., Yanagihara, R., and Song J.W.:	Soochong virus: an antigenically and genetically distinct hantavirus isolated from Apodemus peninsulae in Korea.	J. Med. Virol.	78	290-297	2006
Okumura, M., Yoshimatsu., K.., Kumperasart, S., Nakamura, I., Ogina, M., Taruishi, M., Su7ngdee, A., Pattamadilok, S., Ibrahim, I.N., Erlina, S., Agui, T., Yanagihara, R., and Arikawa, J.	Development of Serological Assays for Thottapalayam Virus, an Insectivore-Borne Hantavirus.	Clin. Vac. Immunol.			2007: in press.
Lee, B.H., Yoshimatsu, K., Araki, K., Okumura, M., Nakamura, I., Arikawa, J.	A pseudotype vesicular stomatitis virus containing Hantaan virus envelope glycoproteins G1 and G2 as an alternative to hantavirus vaccine in mice.	Vaccine	24	2928-34	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Maruyama, S., Izumikawa, K., Miyashita, M., Kabeya, H., Mikami, T., Yamanouchi, H., Sasaki,E., Yoshida, H., and Izumikawa.	First isolation of *Bartonella henselae* type I from a cat-scratch disease patient in Japan and its molecular analysis.	Microbiol. Immur	48	103-109	2005
Li, W., Chomel, B., Maruyama, S., Guptil, L, Sander, A., Reoult, D. and Foumier, P.E.	Multi-spacer typing to study the genotypic distribution of *Bartonella henselae* populations.	J. Clin. Microbiol	47	2499-2506	2006
Kabeya, H., Yamasaki, A., Ikariya, M., Negishi, R., Chomel, B. B., and Maruyama, S.	Characterization of Th1 activation by *Bartonella henselae* stimulation in BALB/c mice: Inhibitory activities of interleukin-10 for the production of interferon-γ in spleen cells.	Vet. Microbiol.	119	290-296	2007
Inokuma,,H., Makino, T., Kabeyama, H., Nogami, S., Fujita, H., Asano, M., Inoue, S., and Maruyama, S.	Serological survey of *Ehrlichia* and *Anaplasma* infection of feral raccoons (*Procyon lotor*) in Kanagawa Prefecture, Japan.	Vet Parasitol.			2007 in press
Andoh, M., Nagaoka, H., Yamaguchi, T., Fukushi, H., and Hirai, K..	Comparison of Japanese Isolates of *Coxiella burnetii by* PCR-RFLP and sequence Analysis.	Microbiol. Immur	48	971-975	2004
Hotta, A., Zhang, G.Q., Andoh, M., Yamaguchi, T., Fukushi, H., and Hirai, K.	Use of monoclonal antibodies for analyses of *Coxiella burnetii* major antigens.	J. Vet. Med. Sci.	66	1289-1291	2004
Andoh, M., Nagaoka, H., Yamaguchi, T., Fukushi, H., and Hirai, K..	In vitro susceptibility to tetracycline and fluoroquinolones of Japanese isolates of *Coxiella burnetii.*	Microbiol. Immur	48	661-664	2004
Zhang, G., To, H., Russell, K.E., Hendrix, L.R., Yamaguchi, T., Fukushi, H., Hirai, K., Samuel, J.E.	Identification and characterization of an immunodominant 28-kilodalton *Coxiella burnetii* outer membrane protein specific to isolates asssociated with acute disease.	Infect. Immun.	73	1561-1567	2005
Horisaka, T., Fujita, K., Iwata, T., Nakadai, A Okatani, A.T., Horikita, T., Taniguchi, T., Honda, E., Yokomizo, Y., and Hayashidani, H.	.Sensitive and specific detection of *Yersinia pseudotuberculosis* by loop-mediated isothermal amplification.	J.Clin.Microbiol.	42	5349-5352	2004
Iwata, T., Une, Y., Okatani, A.T., Kaneko, S., Namai, S., Yoshida, S., Horisaka, T., Horikita, T., Nakadai, A., and Hayashidani, H.	*Yersinia enterocolitica* serovar O:8 infection in breeding monkeys in Japan.	Microbiol. Immunol.	49	1-7	2005
Kojitani, E., Horisaka, T., Okatani, A.T., Nomura, Y., Iwata, T.,Hara-Kudo, Y., Kumagai, S., and Hayashidani, H.	Immuno-magnetic separation and agar layer methods for the isolation of Freeze-Injured *Yersinia enterocolitica* O:8 from Water.	J.Vet.Med.Sci.	68	195-199	2006
Kawabuchi, T., Tshuji, M., Sado, A., Matoba, Y., Asakawa, M., and Ishihara, C.	*Babesia microti*-like parasites detected in feral raccoons (Procyon lotor) captured in Hokkaido, Japan.	J. Vet. Med. Sci.	67(8)	825-827	2005
Zamoto, A., Tsuji, M., Wei, Q., Cho, S.H., Shin, E.H., Kim, T.S., Leonova, G.N., Hagiwara, K., Asakawa, M., Kariwa, H., Takashima, I., and Ishihara, C.	Epizootiologic survey for *Babesia microti* among small wild mammals in northeastern Eurasia and a geographic diversity in the beta-tubulin gene sequences.	J. Vet. Med. Sci.	66	785-792	2004
Tshuji, M., Zamoto,A., Kawabuchi, T., Kataoka, T., Nakajima, R., Asakawa, M., and Ishihara, C.	*Babeshia microti*-like parasites detected in Eurasian red squirrels (sciurus vulgaris orientis in Hokkaido, Japan.	J. Vet Med. Sci.	68(7)	643-646	2006
Mitani, H., Talbert, A., and Fukunaga, M	New World relapsing fever *Borrelia* found in Ornithodoros porcinus ticks in central Tanzania.	Microbiol. Immunol.	48	501-505	2004
Tabuchi, N., Tomodo, K., Kawaguchi, H., Iwamoto, H., and Fukunaga., M.	Immunodominant epitope in the C-terminus of a variable major protein in *Borrelia duttonii*, an agent of tick-borne relapsing fever.	Microbiol. Immunol.	50	293-305	2006

Ⅲ. 研究成果の刊行物・印刷

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Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion

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Flaviviruses are assembled to bud into the lumen of the endoplasmic reticulum (ER) and are secreted through the vesicle transport pathway. Virus envelope proteins play important roles in this process. In this study, the effect of mutations in the envelope proteins of tick-borne encephalitis (TBE) virus on secretion of virus-like particles (VLPs), using a recombinant plasmid expression system was analysed. It was found that a single point mutation at position 63 in prM induces a reduction in secretion of VLPs. The mutation in prM did not affect the folding of the envelope proteins, and chaperone-like activity of prM was maintained. As observed by immunofluorescence microscopy, viral envelope proteins with the mutation in prM were scarce in the Golgi complex, and accumulated in the ER. Electron microscopic analysis of cells expressing the mutated prM revealed that many tubular structures were present in the lumen. The insertion of the prM mutation at aa 63 into the viral genome reduced the production of infectious virus particles. This data suggest that prM plays a crucial role in the virus budding process.

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INTRODUCTION

Enveloped viruses acquire their lipid envelopes by budding through the plasma membrane or the membrane of an intracellular organelle, such as the endoplasmic reticulum (ER), the ER to Golgi intermediate compartment or the Golgi complex (Garoff *et al.*, 1998). Flaviviruses are generally thought to bud into the ER of infected cells (Lindenbach & Rice, 2001). Virus particles have been detected by electron microscopy in the lumen of the rough ER, and in the lumen of either the smooth ER or the intermediate compartment (Ishak *et al.*, 1988; Wang *et al.*, 1997). However, the details of the mechanism of the budding process are still almost unknown.

It has been reported that the envelope proteins play an important role in the budding process of many viruses. Expression of the M and E envelope proteins of mouse hepatitis virus, without other viral proteins, led to the secretion of virus-like particles (VLPs), which were morphologically similar to native virions (de Haan *et al.*, 1998; Vennema *et al.*, 1996). The hepatitis B virus (HBV) surface proteins can be secreted as subviral particles, but their morphology is quite different from HBV virions (Patzer *et al.*, 1986; Simon *et al.*, 1988). In the case of flaviviruses, slowly sedimenting haemagglutinin (sHA), which lacks infectivity, is secreted from virus-infected cells (Gritsun *et al.*, 1989; Heinz & Kunz, 1977). sHA has viral envelope proteins but lacks nucleocapsid protein and viral RNA, and its particulate structure is similar to the infectious virion, except for lower density and smaller size. Expression of the prM and E protein of several flaviviruses without other viral proteins results in the secretion of VLPs, which are similar to sHA particles (Allison *et al.*, 1995b; Konishi *et al.*, 1992; Mason *et al.*, 1991).

The flavivirus envelope has two proteins: the major envelope glycoprotein E (molecular mass 52 kDa) and the small membrane protein M (molecular mass 7–8 kDa). Both proteins are synthesized as part of a polyprotein precursor and then co- and post-translationally cleaved into the individual proteins (Lindenbach & Rice, 2001). The M protein is cleaved first into an intermediate precursor called prM, before final processing.

E protein is a well-characterized viral protein in flavivirus. E protein mediates virus entry via receptor-mediated endocytosis and also carries the major antigenic epitopes leading to a protective immune response (Heinz & Mandl, 1993).

The X-ray crystallographic resolution of the structure of the E ectodomain of TBE virus revealed that E protein forms head-to-tail homodimers that lie parallel to the viral envelope (Rey *et al.*, 1995). In low-pH condition, such as in endocytic vesicles, these homodimers dissociate and lead to the irreversible formation of homotrimers (Allison *et al.*, 1995a; Stiasny *et al.*, 2001, 2002).

M is synthesized as precursor protein, prM (molecular mass 25 kDa) in ER, carrying one *N*-linked oligosaccharide. One of the roles of prM protein reported previously is a chaperone-like activity for the folding and maturation of E (Konishi & Mason, 1993; Lorenz *et al.*, 2002). Newly synthesized E and prM proteins associate to form heterodimers that are incorporated into immature virions (Wengler & Wengler, 1989). This heterodimerization leads to the final native conformation of E and protects E from inactivation by acidification in the transport vesicles (Heinz & Allison, 2000). Shortly before release from the cell, the immature particles are converted to the active form by cleavage of the pr-portion from prM by a cellular furin protease in *trans*-Golgi network and prM turns into M (Elshuber *et al.*, 2003; Stadler *et al.*, 1997).

Recent examination of the assembly and maturation of Kunjin virus revealed that the assembly of virions occurs within the lumen of the rough ER (Mackenzie & Westaway, 2001). Furthermore, the structure of immature flavivirus particles containing prM was analysed by cryoelectron microscopy (Zhang *et al.*, 2003). Sixty trimeric spikes were organized icosahedrally on the surface of the particles, in contrast to the smooth surface of mature virions reported previously (Kuhn *et al.*, 2002). In the spike structure, prM covers the fusion peptides of E in a manner similar to the organization of the glycoproteins in alphavirus spikes (Zhang *et al.*, 2002). In this way, various approaches have revealed the morphological assembly and maturation processes of virus particles, but the molecular mechanism of virus budding and secretion remains obscure.

In this study, we constructed plasmids expressing mutant prM and E proteins of TBE virus, and tested the effect of these mutations on the production of VLPs when expressed in mammalian cells. This allowed the identification of a single point mutation in prM that induced a reduction of secretion of VLPs. The mutation in prM did not affect the oxidative folding of the viral envelope proteins nor the chaperone-like activity of prM. The envelope proteins not secreted from the cells due to the prM mutation accumulated in the ER, and the transport of viral envelope proteins to the Golgi complex was also inhibited. By electron microscopy, tubular structures were observed in the lumen of the ER. When the point mutation in prM was introduced into the TBE virus genome, it severely reduced the ability of the mutant viral RNA to produce infectious particles. This data points out the critical role of prM protein in the virus budding process.

METHODS

Cells. Baby hamster kidney (BHK)-21 cells were grown at 37 °C in MEM supplemented with 8 % FCS and 2 mM L-glutamine. 293T cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium, containing 10 % FCS, 2 mM L-glutamine and penicillin–streptomycin (50 U and 50 μg ml^{-1}, respectively).

Antibodies. For detection of TBE virus prM and E proteins, ELISA, immunoprecipitation and immunofluorescence experiments, mouse anti-E mAb 1H4 and 4H8, prepared in our laboratory, were used (Komoro *et al.*, 2000). Rabbit polyclonal anti-prM and anti-E antibodies were prepared by immunization with recombinant prM and E proteins expressed in the pET43 system (Novagen). For the immunofluorescence colocalization studies, anti-calreticulin rabbit polyclonal antiserum (Affinity BioReagents) or anti-giantin rabbit polyclonal antiserum (Covance Research Products) was applied. FITC conjugated anti-mouse IgG antibodies and Texas red conjugated anti-rabbit IgG antibodies (Jackson Immunoresearch) were used as secondary antibodies in the immunofluorescence assays.

Plasmid construction. The production of the recombinant plasmid pCAGprME expressing prM and full-length E, derived from the Oshima 5-10 strain of TBE virus (GenBank accession no. AB062063), was described previously (Yoshii *et al.*, 2003). For the construction of the mutant plasmids, TBE viral RNA was extracted from virus-inoculated suckling mouse brain and RT-PCR was performed as described previously (Takashima *et al.*, 1997). Amplification of mutated DNA coding prM and E gene was carried out twice by error-prone PCR using *Xho*IMEf and *Cla*IrNS1 primers (Table 1), with Ampli*Taq* DNA polymerase (Applied Biosystems), in the presence of a high concentration of Mg^{2+}, at low annealing temperature. To generate the mutant expression plasmid, clone 55, the PCR products were then digested with *Xho*I and *Cla*I, and cloned into the pCAGGS/MCSR plasmid (Niwa *et al.*, 1991).

For the construction of pCAGprMEpr63S, DNA fragments with the site-directed mutation were amplified by PCR using pr63Sf and pr63Sr primers (Table 1). The PCR products were digested by *Xho*I and *Psh*AI and inserted into the pCAGprME wild-type plasmid, also treated with *Xho*I and *Psh*AI. For the pr88E and E276V464P plasmids, DNA fragments amplified using appropriate primers (M160f and M4 for pr88S; 5s and *Cla*IrNS1 for E276V464P, Table 1) were digested by

Table 1. Primers used in this study

Primer	Primer sequence* (5′–3′)
*Xho*IMEf	agcctcgag*atg*GTAGGTTTGCAAAGACG (*Xho*I)
*Cla*IrNS1	ctcatcgat*cta*ATAATTGTCATACCACTCGGATA-CCTCCC (*Cla*I)
pr63Sf†	TAGACCAGGGGGAGGAA*t*CGGTT
pr63Sr†	CG*a*TTCCTCCCCCTGGTCTA
M160f	AGGGGGAGGAACCGGTTGAC (*Age*I)
M-4	CATTGAGGGCTTCCCCTCAG
5s	CGGAGACCTGTCCTTGTTAT

*Viral and nonviral sequences are in upper case and lower case, respectively. Restriction endonuclease sites are underlined and indicated in parentheses. Italics show the start and stop codons.
†Mutations within the viral sequences are depicted in italicized lower case.

restriction enzymes (*AgeI* and *PshAI* for pr88E; *BstZ17I* for E276V464P) and inserted into the pCAGprME wild-type plasmid, as described above.

For the construction of the TBE virus infectious cDNA clone containing the Pro→Ser mutation at position 63 in prM, the site-directed mutation was substituted into pGEMT-CprME using the pr63Sf and pr63Sr primers described above. The reconstructed plasmid was then digested with *SpeI* and the fragment containing the mutation was replaced into Oshima IC-pt, as described previously (Hayasaka *et al.*, 2004). The new cDNA clone construct was designated Oshima IC-pr63S.

Transfection. 293T cells, grown to 60–70 % confluence in six-well culture plates, were transfected with 2 μg each plasmid complexed to *Trans*IT-LT1 reagent (PanVera) in Opti-MEM (Invitrogen). At 24 h post-transfection (or as otherwise stated), the cells and supernatant were harvested and used for further experiments.

ELISA. Transfected cells were lysed with 1 % Triton X-100 in 10 mM Tris-buffered saline (TBS) and the supernatants were treated with 1 % Triton X-100. Triton X-100-solubilized samples were added to mAb 1H4-coated wells of 96-well microtitre ELISA plates, previously blocked with 3 % BSA. TBE virus E protein was detected by adding biotinylated MAb 4H8 and HRP-conjugated streptavidin (Sigma). The HRP activity was detected by adding *o*-phenylenediamine dihydrochroride (Sigma) in the presence of 0·03 % H_2O_2.

Immunoprecipitation. 293T cells were transfected with the wild-type or pr63S pCAGprME plasmid as described above. At 24 h post-transfection, the cells were lysed with Triton X-100 in 10 mM TBS, incubated on ice for 20 min and then centrifuged at 16 000 *g* for 20 min. The supernatant, which excluded the nuclear fraction, was precleared on Protein G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. Precleared lysates were combined with protein G-Sepharose beads with MAb 1H4 and precipitated by incubation for 2 h at 4 °C. Immune complexes were pelleted at 10 000 *g* for 10 s and washed four times with 1 % Triton X-100 in 10 mM TBS. Subsequently, the precipitated materials were solubilized by adding Laemmli buffer (Laemmli, 1970) and by heating to 95 °C for 2 min and then analysed by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. Transfected cells were lysed with Laemmli buffer under nonreducing or reducing (in the presence of 2-mercaptoethanol) conditions. Protein samples were electrophoresed through 8 and 15 % polyacrylamide-SDS gels. The protein bands were transferred onto PVDF membranes, then incubated with 1 % gelatin in 25 mM TBS containing 0·01 % Tween 20 (TBST) for 30 min at room temperature. After washing with TBST, the membranes were reacted with polyclonal anti-E or anti-prM rabbit IgG for 1 h, followed by alkaline phosphatase conjugated anti-rabbit IgG (Promega) for 30 min at room temperature. For the detection of glycosylation of envelope proteins, the membranes were treated with biotin conjugated concanavalin A (Honen Corporation) and then with alkaline phosphatase conjugated streptavidin (Sigma). Protein bands were visualized using the AP detection reagent kit (Novagen).

Immunofluorescence assay. 293T cells grown on eight-well chamber slides (Nalge Nunc International) were transfected with the wild-type or pr63S pCAGprME plasmids. At 8 h post-transfection, cells were rinsed with PBS and fixed with 4 % paraformaldehyde for 10 min, then permeabilized with 0·2 % Triton X-100 for 4 min at room temperature. After blocking with 2 % BSA for 30 min, the cells were incubated at room temperature for 1 h with mouse mAb 1H4 and antibodies that recognize marker proteins of various cellular organelles, at dilutions between 1:100 and 1:1000 in antibody-dilution buffer (PBS containing 0·1 % Triton X-100 and 2 mg BSA ml^{-1}). After extensive washing, the cells were incubated at room temperature for 1 h with fluorescence-label conjugated secondary antibodies, diluted 1:200. The cells were washed three times with PBS, followed by mounting of the coverslips on glass slides. Images were viewed and collected with an Olympus IX70 confocal microscope.

Electron microscopy. 293T cells were transfected with the wild-type or pr63S pCAGprME plasmids. At 24 h post-transfection, cells were harvested and centrifuged at 1000 *g* for 5 min. The pellets were fixed with 3 % (v/v) glutaraldehyde in 0·1 M phosphate buffer (pH 7·2) for 3 h and then rinsed three times with 0·1 M phosphate buffer. After post-fixation in a 1 % (w/v) osmium tetraoxide solution for 1·5 h, the pellets were dehydrated through a series of graded ethanols and embedded in Epon 812 via QY1 (Nishin EM). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined under a JEM 1210 transmission electron microscope (JEOL) at an acceleration voltage of 80 kV.

RNA transcription and transfection. Oshima IC-pt or Oshima IC-pr63S were digested with *SpeI* and extracted using a QIAquick gel extraction kit (Qiagen). Infectious RNA was transcribed *in vitro* using mMESSAGE mMACHINE SP6 kits (Ambion) in 20 μl reaction volumes, with an additional 1 μl GTP solution. After the transcription at 37 °C for 2 h, template DNA was removed by DNase I digestion 37 °C for 15 min. RNA was precipitated with lithium chloride, washed with 70 % ethanol, resuspended in RNase-free water, and stored in aliquots at −80 °C.

Approximately 5×10^6 BHK cells in 0·5 ml cold PBS were electroporated with 10 μg RNA in 0·4 cm cuvettes using a GenePulser apparatus (Bio-Rad), pulsing twice at settings of 1·3 kV, 25 μF and maximum resistance. Transfected cells were equally divided into two T-25 flasks. After an overnight recovery, cell debris resulting from electroporation was washed away twice with PBS and fresh medium was added. At various times post-electroporation, aliquots of media were harvested as a source of recovered viruses. Infectious virus titre was assayed by the focus count assay, as described previously (Takashima *et al.*, 1997).

RESULTS

Mutant plasmid which induces a reduction of secretion of VLPs

We previously constructed plasmid pCAGprME, which expresses recombinant TBE virus prM/E proteins (Yoshii *et al.*, 2003). In this system, VLPs are secreted from pCAGprME-transfected cells. In the current study, a mutant plasmid, which induced suppression of VLP secretion in spite of higher levels of intracellular viral proteins, was obtained by error-prone PCR. 293T cells were transfected with pCAGprME wild-type or with the mutant clone, designated clone 55. Although higher levels of E proteins were detected from cell lysates transfected with the mutant clone 55 by ELISA, E proteins in the culture media were drastically reduced (to approximately 1/50 to 1/100), as compared with the media of cells transfected with the wild-type plasmid.

Complete sequencing of the pCAGprME mutant clone 55 allowed identification of 5 nt changes that induce 4 aa changes (Fig. 1). The mutant prM protein had 2 aa changes at positions 63 and 88 in the pr region, which was eventually removed by an intracellular furin protease to produce the

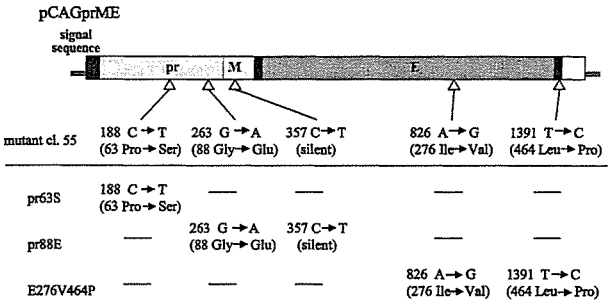

Fig. 1. Schematic representation of the plasmids encoding various parts of TBE virus prM and E proteins. The signal sequence domain and transmembrane regions of prM and E are shown as black boxes. Positions of the mutations in the mutant plasmids used in this study were indicated by open triangles. Numbers of nucleotides and amino acids starting from the amino terminus of prM and E protein according to TBE virus Oshima 5-10 strain genomic sequence (GenBank accession no. AB062063).

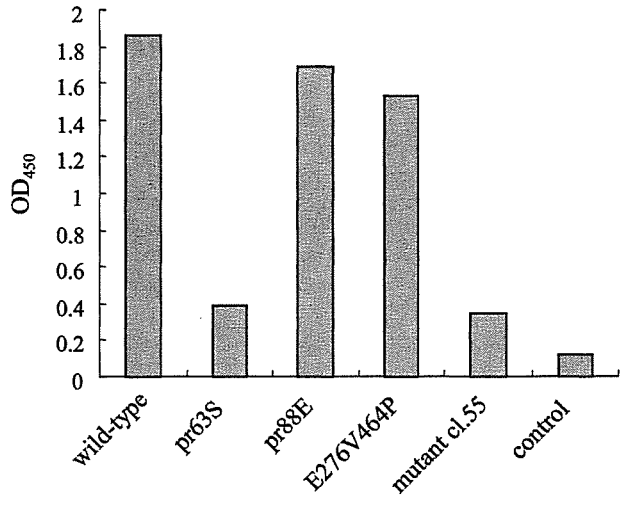

Fig. 2. Identification of the mutation that induces the suppression of VLP secretion. 293T cells were transfected with pCAGprME wild-type, pr63S, pr88E, E276B464P, mutant clone 55 and control plasmid pCAGGS. At 24 h post-transfection, culture media were collected and treated with Triton X-100. E protein in solubilized samples was detected by ELISA using TBE virus E-protein-specific mAb.

mature M protein (Stadler *et al.*, 1997). The mutant E protein had two amino acid changes at positions 276 and 464. The Ile→Val substitution at position 276 is a conservative amino acid change and maps to the domain II of E proteins (Rey *et al.*, 1995). The Leu→Pro substitution at position 464 maps to the first transmembrane region of E protein, which constitutes a membrane anchor (Allison *et al.*, 1999; Mandl *et al.*, 1989; Rice, 1996).

Identification of mutation involved in the reduction of secretion of VLPs

To determine which of the mutations was involved in the reduction of secretion of VLPs, three plasmids were constructed (Fig. 1): pCAGprME pr63S, containing a Pro→Ser mutation at position 63 in prM; pr88E, containing a Gly→Glu mutation at position 88 in prM; and E276V464P, containing Ile→Val and Leu→Pro mutations at positions 276 and 464 in E protein. We then transfected 293T cells with pCAGprME wild-type, mutant clone 55, pr63S, pr88E and E276V464P. At 24 h post-transfection, the culture media were harvested and E protein was detected by ELISA, as described above. Cells transfected with pr88E and E276V464P secreted the same level of E protein as wild-type-transfected cells, but pr63S-transfected cells secreted very low levels of E protein, as did mutant-clone-55-transfected cells (Fig. 2). This indicated that the Pro→Ser mutation at position 63 in prM protein was involved in the reduction of VLP secretion.

To compare the kinetics of protein E secretion from cells transfected with pCAGprME wild-type and pr63S, supernatant and cell lysate samples were collected at 6, 9, 12, 16, 20, 24 and 28 h post-transfection. Levels of protein E in the intracellular and extracellular fractions were detected by ELISA. Regardless of prM mutation, almost equivalent level of protein E was detected by monoclonal antibodies that recognize conformational structures of protein E (Fig. 3a), indicating that prM mutation did not disturb protein E from reaching its final conformational structure. However, protein E secretion was drastically reduced by the prM mutation (Fig. 3b). This indicated that protein E, which was not effectively secreted from mutant-transfected cells, accumulated intracellularly.

Interaction between prM and E proteins

PrM and E proteins form heterodimers, which are involved in the folding and maturation of E protein (Konishi & Mason, 1993; Lorenz *et al.*, 2002). To examine the mechanism of the reduction of secretion of VLPs induced by the Pro→Ser mutation at position 63 in prM protein, we first investigated the interaction between prM and E proteins. To investigate the heterodimer formation of prM and E protein, 293T cells transfected with the wild-type or the pr63S pCAGprME plasmid were lysed with Triton X-100, and the post-nuclear supernatants were immunoprecipitated with anti-E-specific mAb 1H4 (Komoro *et al.*, 2000). Immunoprecipitates were then separated by SDS-PAGE and protein bands were detected by anti-E and anti-prM rabbit IgG, as described in Methods. As shown in Fig. 4(a), prM proteins were detected from immunoprecipitated samples from pr63S-transfected cells as well as from wild-type-transfected cells. The intensities for prM and E bands of pr63S-transfected cells were higher than those of wild-type-transfected cells, as observed in Fig. 3. These data indicated that heterodimerization between prM and E had occurred in spite of prM mutation. The glycosylation of prM and E proteins was examined by concanavalin A, which

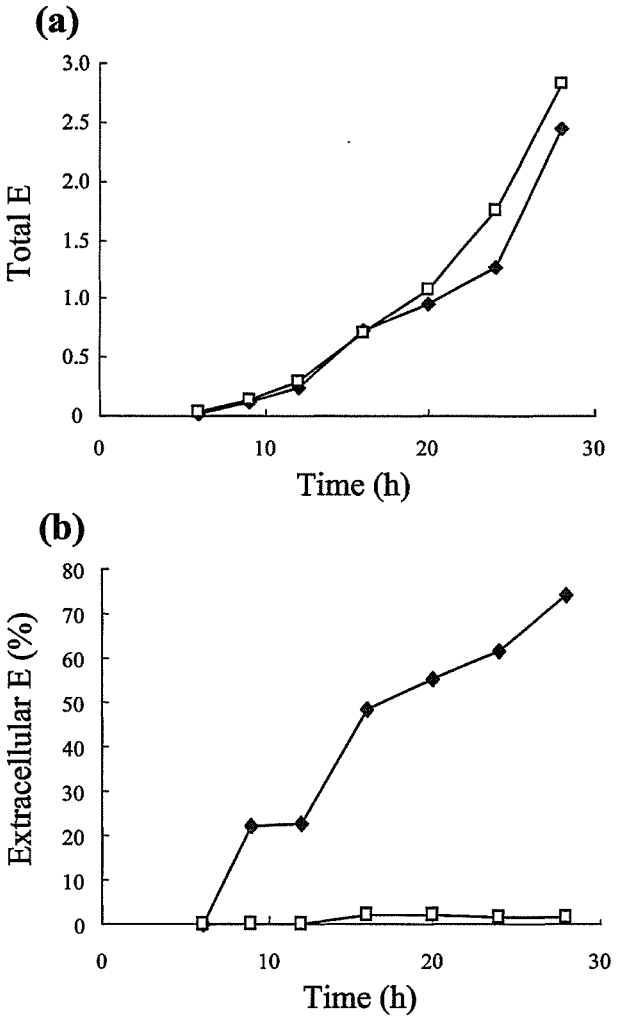

Fig. 3. Time course of protein E expression and secretion. Intracellular and extracellular extracts of 293T cells transfected with pCAGprME wild-type (closed diamond) or pr63S (open square) were collected at 6, 9, 12, 16, 20, 24 or 28 h post-transfection and E protein expression was quantified by ELISA. (a) To calculate total amount of E protein (intracellular and extracellular fractions), E expressed from wild-type-transfected cells at 20 h was set at 1·0. (b) Percentage of total E protein in the extracellular fraction.

binds to *N*-linked glycan. PrM and E proteins were glycosylated well in the immunoprecipitated sample of pr63S-transfected cells, indicating that the two proteins were functionally glycosylated. The effect of Pro→Ser mutation in prM proteins on oxidative folding were further analysed by SDS-PAGE under reducing and nonreducing conditions (Fig. 4b). For prM, a clear difference in the electrophoretic mobility between nonreducing and reducing conditions was visible. This shift was identical for wild-type and mutant samples, indicating that disulfide bond formation occurred normally even in the presence of prM mutation. The corresponding shift in the E protein band was much smaller but was consistently observed regardless of prM mutation. These data showed that the Pro→Ser mutation at position 63 in the prM protein did not affect the interaction and folding of prM and E proteins, regardless of the observed reduction in VLP secretion.

Intracellular localization of recombinant TBE virus envelope proteins

To determine the intracellular distribution of the viral envelope proteins, 293T cells were transfected with pCAGprME wild-type or pr63S plasmids. The cells were fixed, permeabilized and double stained for TBE virus envelope proteins and cellular marker antigens. Anti-calreticulin (Michalak *et al.*, 1992) was used as a marker for ER (Fig. 5b and e), and anti-giantin (Linstedt & Hauri, 1993) was used as a marker for the Golgi complex (Fig. 5h and k). A mouse mAb anti-E (Fig. 5a, d, g and j) was used to stain viral envelope proteins. In wild-type-transfected cells, the distribution of viral envelope protein overlapped almost completely with the ER marker (Fig. 5c) and Golgi marker (Fig. 5i), indicating that viral envelope protein was transported into the Golgi complex. While distribution of viral envelope protein in the ER was observed (Fig. 5f), overlap of viral envelope proteins and Golgi marker was hardly seen in pr63S-transfected cells (Fig. 5l). These data suggest that the mutation at position 63 in prM proteins causes the accumulation of viral envelope proteins in the ER.

Electron microscopy analysis of VLP assembly

As shown in Fig. 6(a), many VLPs were observed in the lumen of the ER in wild-type-transfected cells. However, in pr63S-transfected cells, the spherical VLPs observed in wild-type-transfected cells were hardly seen in the ER lumen; instead, there were many long tubular structures in the ER lumen (Fig. 6b). The tubular particles were 0·1 to 2·0 μm in length and they were not observed in the Golgi complex (data not shown).

Pro→Ser mutation in prM protein affects TBEV infectivity

To investigate the effect of the prM mutation on viral infectivity or on viral budding, the Pro→Ser mutation at position 63 was inserted into an infectious cDNA clone of the TBE virus genome. A full-length TBE viral cDNA containing Pro→Ser mutation was constructed (Oshima IC-pr63S), and BHK cells were transfected with *in vitro*-transcribed TBE viral RNA. Virus production was analysed by focus count assay of the medium harvested from transfected cells at 24 and 48 h post-transfection. As shown in Table 2, the cells transfected with Oshima IC-pr63S secreted fewer infective virus particles than cells transfected with Oshima IC-pt. Total secreted E proteins were also reduced by Pro→Ser mutation and secreted E proteins to f.f.u. ratios were almost same regardless of prM mutation. There was no mutation in recovered virus except for Pro→Ser mutation in prM. These data confirm the

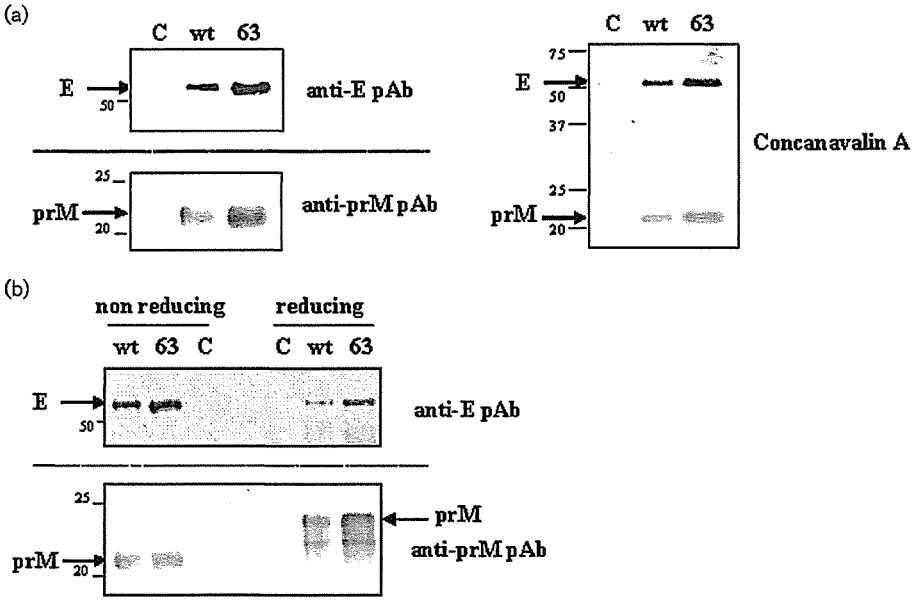

Fig. 4. Interaction between prM and E proteins expressed in transfected 293T cells. (a) Heterodimer formation of prM and E proteins in transfected cells. 293T cells were transfected with pCAGprME wild-type (wt), pr63S (63) or control plasmid pCAGGS (C). At 24 h post-transfection, post-nuclear supernatant was immunoprecipitated with mouse anti-E mAb 1H4, followed by analysis of the proteins by SDS-PAGE (7·5 and 12%, top and bottom, respectively), and transferred to PVDF membranes. Protein bands were detected using anti-E and anti-prM rabbit polyclonal antiserum. Concanavalin A was used for the detection of glycosylated proteins. Positions of the individual proteins are marked, and molecular size is indicated at the side, in kilodaltons. (b) Oxidative folding of prM and E in transfected cells. Post-nuclear supernatant of transfected cells was subjected to SDS-PAGE in nonreducing and reducing conditions and transferred to membrane. Protein bands were detected using anti-E and anti-prM rabbit polyclonal antiserum.

findings of the expression studies concerning the effects of the pr63S mutation on secretion of TBE virus VLPs.

DISCUSSION

In many enveloped viruses, the envelope proteins play important roles in the assembly and budding of virus particles (Garoff *et al.*, 1998). In flaviviruses, it has been shown that virus envelope proteins prM and E are secreted in the form of VLPs when they are expressed recombinantly in mammalian cells without other viral proteins (Allison *et al.*, 1995b; Konishi *et al.*, 1992). Because VLPs have features that are structurally and functionally similar to virus envelope, they have been used as useful tools in the investigation of virus envelope function and the kinetics of viral envelope proteins (Allison *et al.*, 2001; Corver *et al.*, 2000; Lorenz *et al.*, 2003; Op De Beeck *et al.*, 2003). In this study, we constructed a mutant plasmid that reduced VLP secretion in cells expressing recombinant TBE virus prM and E proteins. By analysis of the mutant plasmids, the Pro→Ser mutation at position 63 of the prM protein was found to cause the reduced VLP secretion (Fig. 2). PrM has been reported to serve as a chaperone-like protein in the early steps of virus maturation (Konishi & Mason, 1993; Lorenz *et al.*, 2002). After the cleavage of an N-terminal signal sequence of prM by a host cellular peptidase, prM forms heterodimers with E protein, and E protein attains its native conformation (Stocks & Lobigs, 1995). In addition, it has been thought that prM protects E protein from low-pH rearrangements during transport thorough the acidic compartments of the *trans*-Golgi network by keeping E protein in an inactive structure (Heinz & Allison, 2000). To date, the function of prM in E protein maturation has been studied, but other properties of prM are still unclear. Consequently, this mutation in prM, which reduces VLP secretion, brings a new aspect to the study of prM functions.

The interaction between prM and E proteins is important during the early events of virus particle maturation and secretion. It has been reported that E protein cannot attain full maturity when expressed alone, while prM protein is able to fold independently of other viral components (Lorenz *et al.*, 2002). Furthermore, the secretion of E protein requires cosynthesis with prM, as demonstrated previously in many flaviviruses (Allison *et al.*, 1995b; Konishi & Mason, 1993; Ocazionez Jimenez & Lopes da Fonseca, 2000). Heterodimerization of prM and E leads to the final native conformation of E protein, which is an early process in virus maturation. Therefore, we first examined whether the reduced VLP secretion induced by the position 63 mutation in prM was related to the alteration of interaction between the prM and E proteins. The results of immunoprecipitation

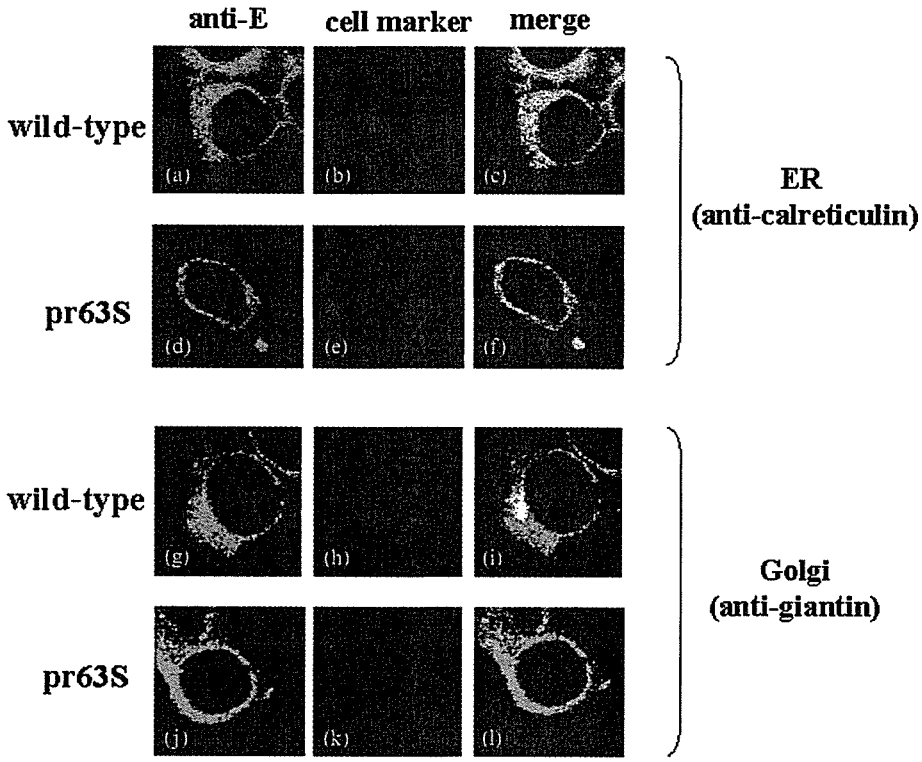

Fig. 5. Intracellular localization of expressed envelope proteins. 293T cells transfected with pCAGprME wild-type (a, b, c, g, h and i) or pr63S (d, e, f, j, k and l) were fixed and subjected to dual staining with TBE virus-specific antibodies and antibodies against marker proteins for cellular organelles as primary antibodies. The samples were then reacted with secondary antibodies conjugated to FITC or Texas red. Shown are cells stained with a mouse anti-E mAb 1H4 (a, d, g and j), immunofluorescent staining of ER with rabbit polyclonal anti-calreticulin (b and e) and immunostaining of the Golgi with rabbit polyclonal anti-giantin antibodies (h and k). Colocalization of viral envelope protein with organelle markers is represented by the yellow regions within each cell in the merged images (c, f, i and l).

with anti-E monoclonal antibodies also indicate that heterodimerization between prM and E, as well as oxidative folding and glycosylation of viral envelope proteins, occurred normally in the presence of the position 63 mutation in prM (Fig. 4). Furthermore, total production level of protein E, which had conformational structures, was not affected by the prM mutation (Fig. 3a), indicating that the position-63 mutation in prM does not detrimentally affect the maturation of protein E. These data suggest that the reduction of VLP secretion induced by the prM mutation was due to a later step of virus particle budding and secretion, not to the prM and E interaction in the early events of virus budding.

It has been reported that flavivirus particles are assembled into the ER lumen (Mackenzie & Westaway, 2001). Thus, the mechanism of flavivirus secretion can be divided into two steps. Virus particle budding in ER membrane, followed by virus transport through the secretory pathway. To identify the influence of the prM position 63 mutation in virus secretion, the intracellular localization of the viral envelope proteins was examined. Envelope proteins expressed with the mutated prM were not transported to the Golgi complex, and accumulated in the ER (Fig. 5). Electron microscopic analysis revealed that many tubular structures, which differed from spherical VLPs in shape, were observed in the ER lumen of cells transfected with a plasmid, with the mutation in prM (Fig. 6). In the Lorenz *et al.* (2003) study, it was reported that similar tubular structures were occasionally seen in cells expressing TBE virus prM and E, and that these structures were not observed in the Golgi complex. The tubular structures observed in cells expressing mutated prM and E in our study may be comparable to those noted in the Lorenz study, and they may not undergo secretion due to their abnormal budding. Another possibility is that the tubular structures are membrane components that detach from the ER lumen due to the damage to membrane structures caused by accumulation of viral envelope proteins. In any case, the position 63 mutation in prM clearly affects the budding process of the virus particle.

In many enveloped viruses, the cytoplasmic domain of the envelope proteins has been assigned an important role in virus assembly. For vesicular stomatitis virus, the cytoplasmic domain is important for incorporation of the

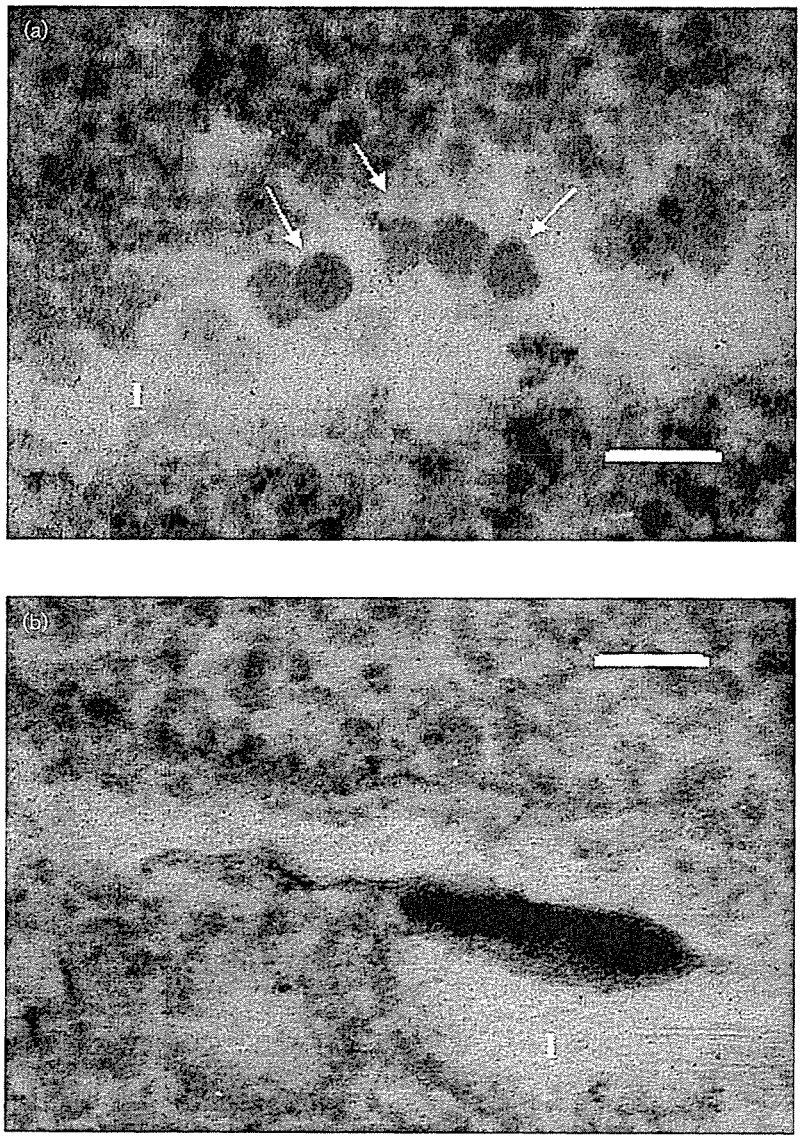

Fig. 6. Electron micrographs of 293T cells transfected with pCAGprME wild-type and pr63S. VLPs (arrow) are seen in the ER lumen of pCAGprME wild-type-transfected cells (a). (b) Shows a long tubular structure (diameter, 120 nm) in the lumen of the ER of pCAGprME pr63S-transfected cells. I, ER lumen. Bars, 50 nm.

glycoprotein (Owens & Rose, 1993; Whitt *et al.*, 1989). For alphaviruses, it has been shown that the cytoplasmic domain of the E2 glycoprotein has a critical role in virus budding (Kail *et al.*, 1991; Owen & Kuhn, 1997; Zhao *et al.*, 1994). Deletion of the cytoplasmic tails of influenza virus haemagglutinin and neuraminidase (NA) leads to irregularly shaped virions, and deletion of the NA cytoplasmic domain reduces the incorporation of NA into virions (Jin *et al.*, 1997; Mitnaul *et al.*, 1996). But unlike these viruses, flavivirus prM and E proteins have cytoplasmic loops consisting of only a few amino acid residues between their two transmembrane segments. Thus, it is

Table 2. Virus titre in media of electroporated BHK-21 cells at 24 and 48 h post-electroporation

Template used for transcription	Virus titre (f.f.u. ml^{-1})		f.f.u./extracellular E*	
	24 h	48 h	24 h	48 h
Oshima IC-pt	$2{\cdot}3 \times 10^5$	$4{\cdot}0 \times 10^6$	1	1·24
Oshima IC-pr63S	$1{\cdot}4 \times 10^4$	$2{\cdot}2 \times 10^5$	0·96	1·29

*Total extracellular E proteins were quantified by ELISA and E proteins to f.f.u. ratios were calculated by setting the ratio of 24 h at 1·0.

thought that the luminal domains, or the two transmembrane domains of prM and E, play more critical roles in the assembly of these viruses.

In a recent study by Zhang *et al.* (2003), the structure of prM-containing immature particles of dengue and yellow fever virus was analysed by cryoelectron microscopy and image reconstruction techniques. The surface of the immature particles was characterized by the presence of 60 fairly prominent projections or spikes, which differed from the smooth surface of mature virus (Kuhn *et al.*, 2002). In the spike structure, prM protein covered the fusion peptides of domain II of the E protein, similar to the case of alphaviruses, where the E2 glycoproteins protect the fusion peptides of the E1 glycoproteins within a trimeric spike (Zhang *et al.*, 2002). Thus, it is suggested that the position 63 mutation in prM may induce conformational changes in the domain exposed on the outer side of the viral envelope, which is important for the virus budding process.

The cellular membranes involved in membrane transport normally form vesicles on the cytoplasmic side, such as clathrin coated vesicles, and COP I and COP II vesicles (Schekman & Orci, 1996). It is possible that prM-E heterodimers, alone or with cellular factors in the ER lumen, assemble laterally and induce the membrane curvature into an isometric lattice, like the assembly of coat proteins in membrane transport vesicles (Keen *et al.*, 1979; Wieland & Harter, 1999). The abnormal budding induced by the prM mutation may be due to a dysfunction in this process, caused by structural changes in prM or by loss of interaction with a cellular component. Alternatively, the prM mutation may be related to the pinching off of particles from the ER membrane, as is the case for dynamin in clathrin-coated vesicles, and it might be possible that VLPs could not be pinched off properly due to the prM mutation (McNiven, 1998).

In summary, by analysis of a prM mutation that induces the reduction of VLP and virus particle secretion, we demonstrated a critical function for prM in the virus budding process. This mutation does not affect the heterodimerization between prM and E, and E proteins can reach the native conformation in spite of the prM mutation, suggesting the preservation of prM's chaperone-like role. Envelope proteins that are not secreted due to the prM mutation accumulate in the ER, indicating the failure of virus particle budding. Molecular approaches focused on the ectodomain of prM protein should enable further investigation of the mechanisms during the virus budding process.

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REFERENCES

Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995a). Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* **69**, 695–700.

Allison, S. L., Stadler, K., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995b). Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. *J Virol* **69**, 5816–5820.

Allison, S. L., Stiasny, K., Stadler, K., Mandl, C. W. & Heinz, F. X. (1999). Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. *J Virol* **73**, 5605–5612.

Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W. & Heinz, F. X. (2001). Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J Virol* **75**, 4268–4275.

Corver, J., Ortiz, A., Allison, S. L., Schalich, J., Heinz, F. X. & Wilschut, J. (2000). Membrane fusion activity of tick-borne encephalitis virus and recombinant subviral particles in a liposomal model system. *Virology* **269**, 37–46.

de Haan, C. A., Kuo, L., Masters, P. S., Vennema, H. & Rottier, P. J. (1998). Coronavirus particle assembly: primary structure requirements of the membrane protein. *J Virol* **72**, 6838–6850.

Elshuber, S., Allison, S. L., Heinz, F. X. & Mandl, C. W. (2003). Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus. *J Gen Virol* **84**, 183–191.

Garoff, H., Hewson, R. & Opstelten, D. J. (1998). Virus maturation by budding. *Microbiol Mol Biol Rev* **62**, 1171–1190.

Gritsun, T. S., Lisak, V. M., Liapustin, V. N., Korolev, M. B. & Lashkevich, V. A. (1989). Slowly-sedimenting hemagglutinin of the tick-borne encephalitis virus. *Vopr Virusol* **34**, 449–454 (in Russian).

Hayasaka, D., Gritsun, T. S., Yoshii, K. & 7 other authors (2004). Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of *Tick-borne encephalitis virus*. *J Gen Virol* **85**, 1007–1018.

Heinz, F. & Kunz, C. (1977). Concentration and purification of tick-borne encephalitis virus grown in suspensions of chick embryo cells. *Acta Virol* **21**, 301–307.

Heinz, F. X. & Mandl, C. W. (1993). The molecular biology of tick-borne encephalitis virus. Review article. *APMIS* **101**, 735–745.

Heinz, F. X. & Allison, S. L. (2000). Structures and mechanisms in flavivirus fusion. *Adv Virus Res* **55**, 231–269.

Ishak, R., Tovey, D. G. & Howard, C. R. (1988). Morphogenesis of yellow fever virus 17D in infected cell cultures. *J Gen Virol* **69**, 325–335.

Jin, H., Leser, G. P., Zhang, J. & Lamb, R. A. (1997). Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. *EMBO J* **16**, 1236–1247.

Kail, M., Hollinshead, M., Ansorge, W., Pepperkok, R., Frank, R., Griffiths, G. & Vaux, D. (1991). The cytoplasmic domain of alphavirus E2 glycoprotein contains a short linear recognition signal required for viral budding. *EMBO J* **10**, 2343–2351.

Keen, J. H., Willingham, M. C. & Pastan, I. H. (1979). Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell* **16**, 303–312.

Komoro, K., Hayasaka, D., Mizutani, T., Kariwa, H. & Takashima, I. (2000). Characterization of monoclonal antibodies against Hokkaido strain tick-borne encephalitis virus. *Microbiol Immunol* **44**, 533–536.

Konishi, E. & Mason, P. W. (1993). Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J Virol* **67**, 1672–1675.

Konishi, E., Pincus, S., Paoletti, E., Shope, R. E., Burrage, T. & Mason, P. W. (1992). Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* **188**, 714–720.

Kuhn, R. J., Zhang, W., Rossmann, M. G. & 9 other authors (2002). Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108**, 717–725.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lindenbach, B. D. & Rice, C. M. (2001). *Flaviviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 991–1041. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Linstedt, A. D. & Hauri, H. P. (1993). Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol Biol Cell* **4**, 679–693.

Lorenz, I. C., Allison, S. L., Heinz, F. X. & Helenius, A. (2002). Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J Virol* **76**, 5480–5491.

Lorenz, I. C., Kartenbeck, J., Mezzacasa, A., Allison, S. L., Heinz, F. X. & Helenius, A. (2003). Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. *J Virol* **77**, 4370–4382.

Mackenzie, J. M. & Westaway, E. G. (2001). Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J Virol* **75**, 10787–10799.

Mandl, C. W., Guirakhoo, F., Holzmann, H., Heinz, F. X. & Kunz, C. (1989). Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. *J Virol* **63**, 564–571.

Mason, P. W., Pincus, S., Fournier, M. J., Mason, T. L., Shope, R. E. & Paoletti, E. (1991). Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* **180**, 294–305.

McNiven, M. A. (1998). Dynamin: a molecular motor with pinchase action. *Cell* **94**, 151–154.

Michalak, M., Milner, R. E., Burns, K. & Opas, M. (1992). Calreticulin. *Biochem J* **285**, 681–692.

Mitnaul, L. J., Castrucci, M. R., Murti, K. G. & Kawaoka, Y. (1996). The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *J Virol* **70**, 873–879.

Niwa, H., Yamamura, K. & Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.

Ocazionez Jimenez, R. & Lopes da Fonseca, B. A. (2000). Recombinant plasmid expressing a truncated dengue-2 virus E protein without co-expression of prM protein induces partial protection in mice. *Vaccine* **19**, 648–654.

Op De Beeck, A., Molenkamp, R., Caron, M., Ben Younes, A., Bredenbeek, P. & Dubuisson, J. (2003). Role of the transmembrane domains of prM and E proteins in the formation of yellow fever virus envelope. *J Virol* **77**, 813–820.

Owen, K. E. & Kuhn, R. J. (1997). Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. *Virology* **230**, 187–196.

Owens, R. J. & Rose, J. K. (1993). Cytoplasmic domain requirement for incorporation of a foreign envelope protein into vesicular stomatitis virus. *J Virol* **67**, 360–365.

Patzer, E. J., Nakamura, G. R., Simonsen, C. C., Levinson, A. D. & Brands, R. (1986). Intracellular assembly and packaging of hepatitis B surface antigen particles occur in the endoplasmic reticulum. *J Virol* **58**, 884–892.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature* **375**, 291–298.

Rice, C. M. (1996). *Flaviviridae*: the viruses and their replication. In *Fields Virology*, 3rd edn, pp. 931–959. Edited by B. N. Fields, D. N. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus. Philadelphia: Lippincott-Raven.

Schekman, R. & Orci, L. (1996). Coat proteins and vesicle budding. *Science* **271**, 1526–1533.

Simon, K., Lingappa, V. R. & Ganem, D. (1988). Secreted hepatitis B surface antigen polypeptides are derived from a transmembrane precursor. *J Cell Biol* **107**, 2163–2168.

Stadler, K., Allison, S. L., Schalich, J. & Heinz, F. X. (1997). Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol* **71**, 8475–8481.

Stiasny, K., Allison, S. L., Mandl, C. W. & Heinz, F. X. (2001). Role of metastability and acidic pH in membrane fusion by tick-borne encephalitis virus. *J Virol* **75**, 7392–7398.

Stiasny, K., Allison, S. L., Schalich, J. & Heinz, F. X. (2002). Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J Virol* **76**, 3784–3790.

Stocks, C. E. & Lobigs, M. (1995). Posttranslational signal peptidase cleavage at the flavivirus C-prM junction in vitro. *J Virol* **69**, 8123–8126.

Takashima, I., Morita, K., Chiba, M. & 8 other authors (1997). A case of tick-borne encephalitis in Japan and isolation of the virus. *J Clin Microbiol* **35**, 1943–1947.

Vennema, H., Godeke, G. J., Rossen, J. W., Voorhout, W. F., Horzinek, M. C., Opstelten, D. J. & Rottier, P. J. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. *EMBO J* **15**, 2020–2028.

Wang, J. J., Liao, C. L., Chiou, Y. W., Chiou, C. T., Huang, Y. L. & Chen, L. K. (1997). Ultrastructure and localization of E proteins in cultured neuron cells infected with Japanese encephalitis virus. *Virology* **238**, 30–39.

Wengler, G. & Wengler, G. (1989). Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J Virol* **63**, 2521–2526.

Whitt, M. A., Chong, L. & Rose, J. K. (1989). Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. *J Virol* **63**, 3569–3578.

Wieland, F. & Harter, C. (1999). Mechanisms of vesicle formation: insights from the COP system. *Curr Opin Cell Biol* **11**, 440–446.

Yoshii, K., Hayasaka, D., Goto, A. & 8 other authors (2003). Enzyme-linked immunosorbent assay using recombinant antigens expressed in mammalian cells for serodiagnosis of tick-borne encephalitis. *J Virol Methods* **108**, 171–179.

Zhang, W., Mukhopadhyay, S., Pletnev, S. V., Baker, T. S., Kuhn, R. J. & Rossmann, M. G. (2002). Placement of the structural proteins in Sindbis virus. *J Virol* **76**, 11645–11658.

Zhang, Y., Corver, J., Chipman, P. R. & 7 other authors (2003). Structures of immature flavivirus particles. *EMBO J* **22**, 2604–2613.

Zhao, H., Lindqvist, B., Garoff, H., von Bonsdorff, C. H. & Liljestrom, P. (1994). A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. *EMBO J* **13**, 4204–4211.

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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 5×10^6 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 *Hind*III 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

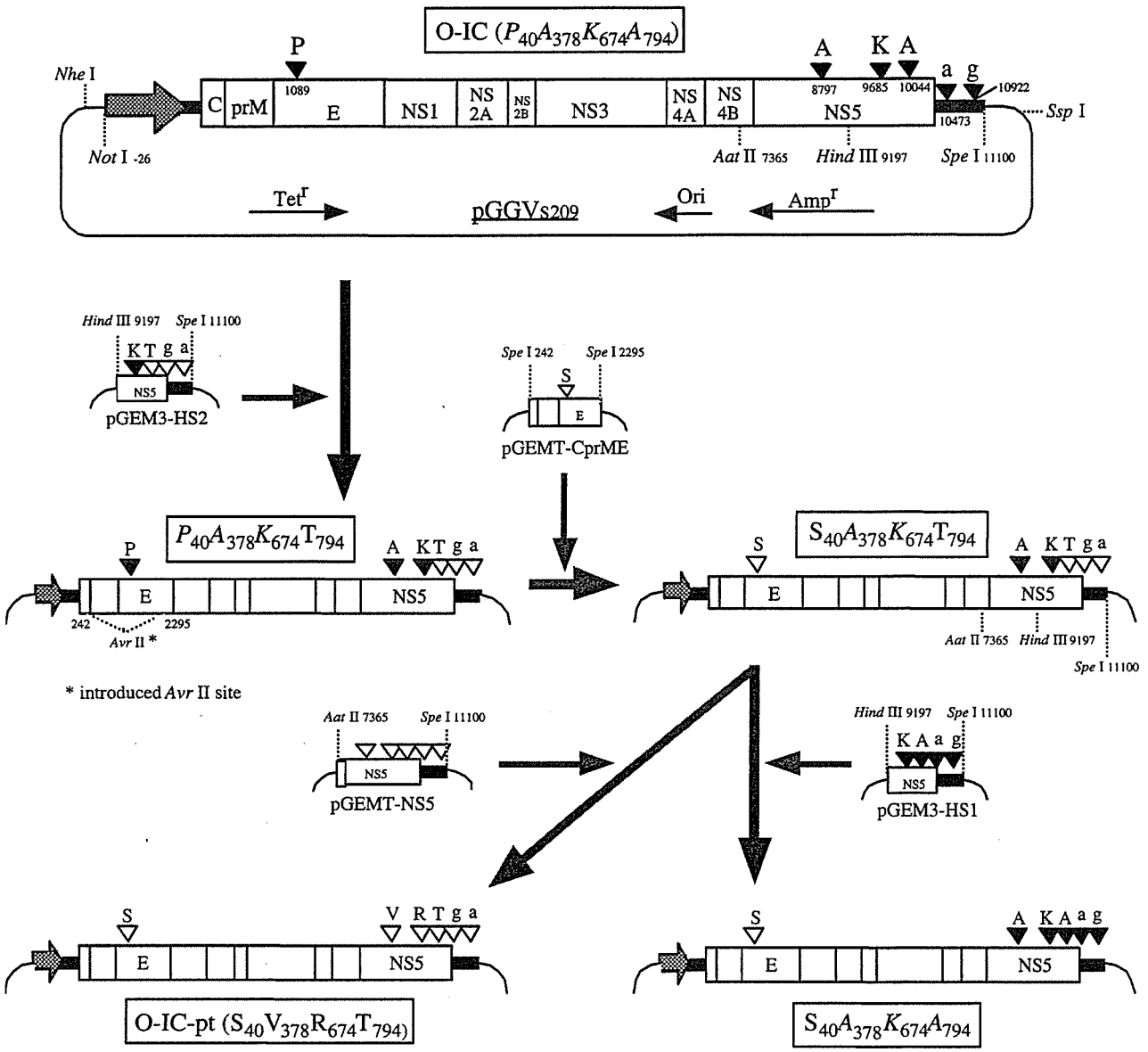

Fig. 1. Strategy for the construction of an infectious cDNA clone of TBEV Oshima 5-10 strain. The virus genome (about 11 kb) is depicted as an open box with genes specified through C to NS5. Untranslated regions flanking polyprotein are specified as thick black lines. The primers for amplification of 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 *Hind*III 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.

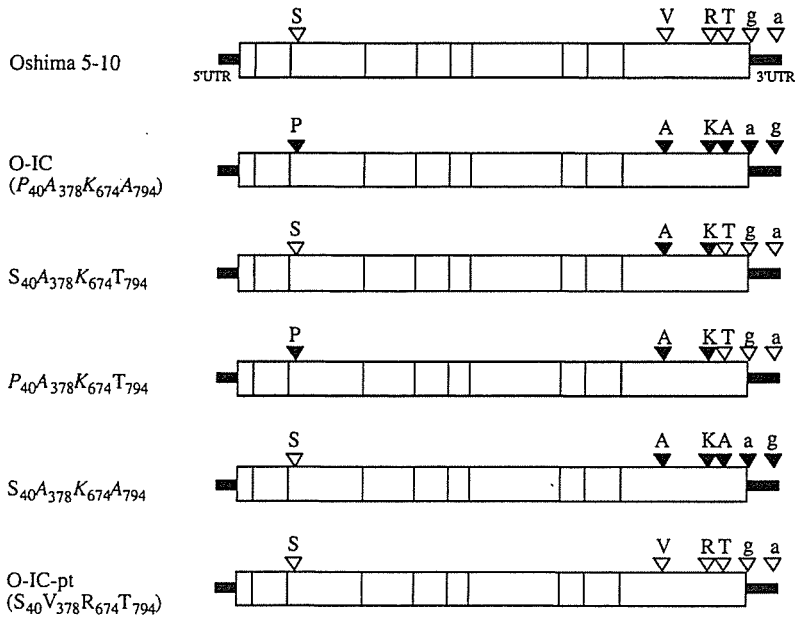

Fig. 2. Schematic representation of constructed full-length plasmids and viruses that were recovered from them. The black arrowhead indicates amino acid and nucleotide sequences derived from O-IC. The white arrowhead indicates amino acid and nucleotide sequences of parent Oshima 5-10 virus.

(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

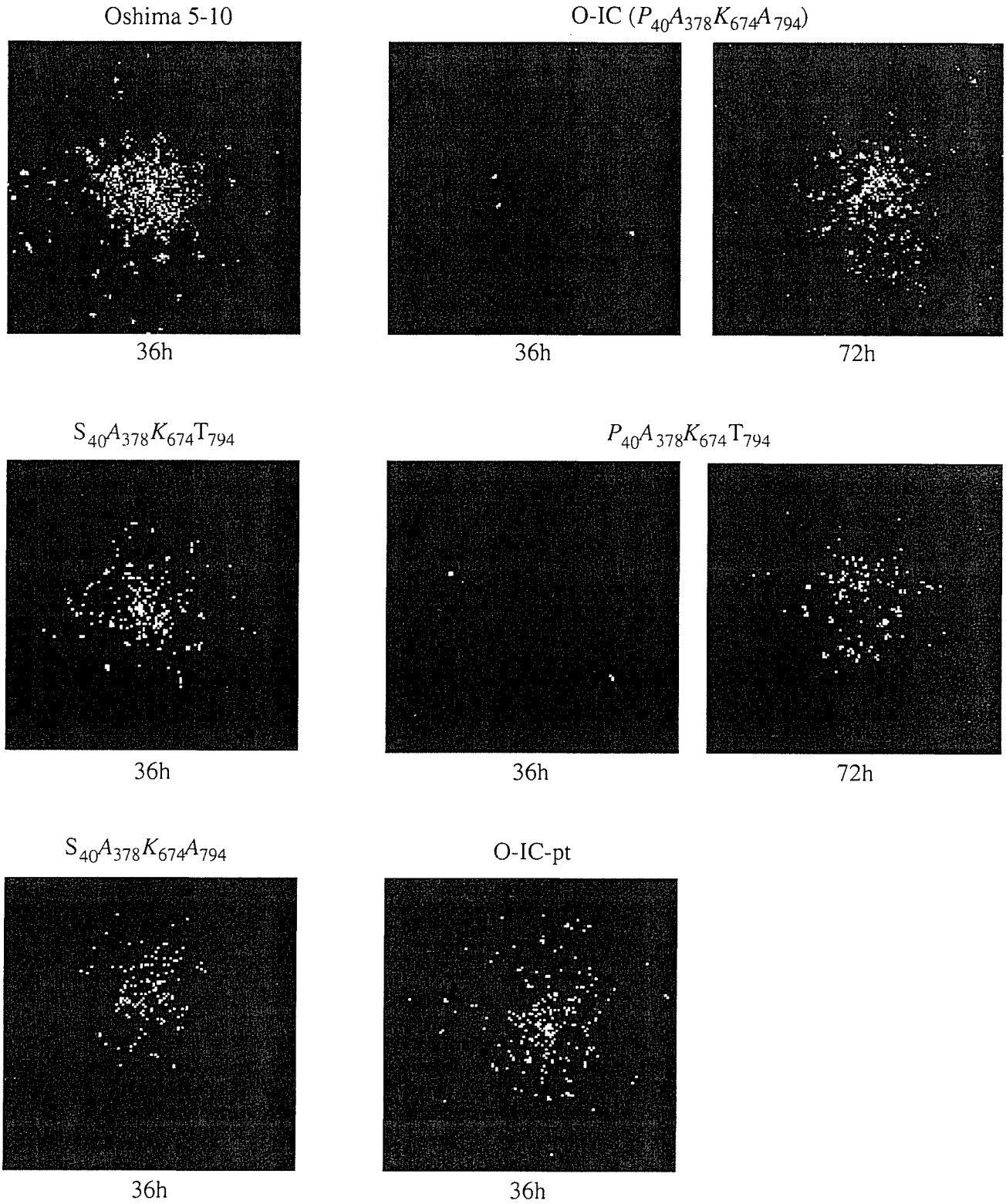

Fig. 3. An infectious focus of virus recovered from infectious cDNA clones, 36 and 72 h post-inoculation. Virus antigens were detected in cells fixed with methanol using a 1 : 100 dilution of mouse ascitic fluid containing high-titre TBEV-reactive antibodies and anti-mouse IgG fluorescein isothiocyanate-conjugated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

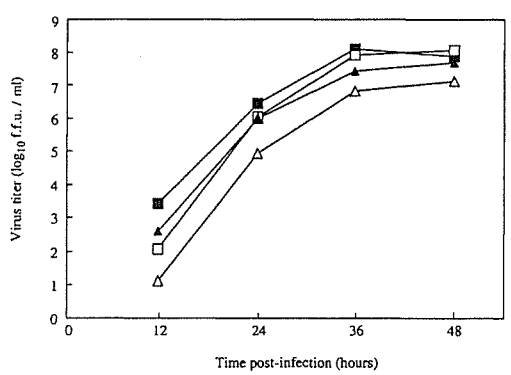

Fig. 5. Comparison of the growth curves of recovered viruses in BHK cells which were infected (m.o.i. 0·01) with parent Oshima 5-10 (■), $S_{40}A_{378}K_{674}T_{794}$ (▲), $P_{40}A_{378}K_{674}T_{794}$ (△) or O-IC-pt (□) virus. Supernatant medium was harvested at intervals of 12 h post-infection. Virus 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 0·5log_{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

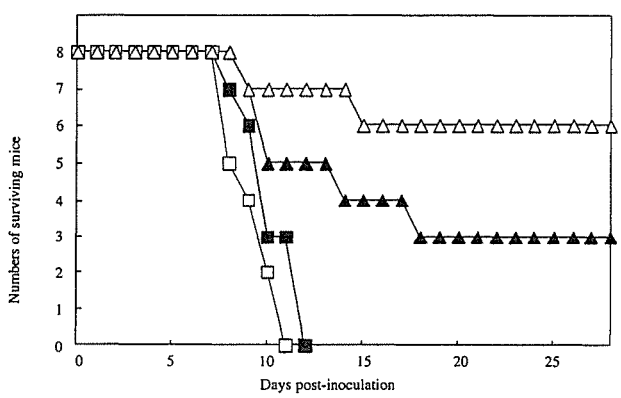

Fig. 6. Fate of mice inoculated intracerebrally with each virus. Mice were inoculated with 50 f.f.u. of parent Oshima 5-10 (■), $S_{40}A_{378}K_{674}T_{794}$ (▲), $P_{40}A_{378}K_{674}T_{794}$ (△) or O-IC-pt (□) virus and monitored daily.