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 β-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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特集1 第52回日本ウイルス学会学術集会シンポジウム

1. 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

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北海道と極東ロシアのダニ媒介性脳炎ウイルスの系統解析の結果，北海道株は極東地区において数百年前に出現したと推定された．イルクーツク地区のダニ媒介性脳炎ウイルスはシベリア亜型と同定された．ダニ媒介性脳炎ウイルスのBHK細胞適応変異株はマウスにおける神経侵襲性毒力が低下していた．変異株はエンベロープ蛋白に1ヶ所のアミノ酸置換があり，荷電が陽性に変化する変異であった．変異株はウイルス血症と脾臓でのウイルス価が親株に比べ低下していた．ダニ媒介性脳炎ウイルスの感染性cDNAクローンの作製に成功し，神経毒力の解析を行った．エンベロープ蛋白の1ヶ所とNs5の2ケ所のアミノ酸変異が相乗的に神経毒力の低下に関与していた．

1. はじめに

ダニ媒介性脳炎はフラビウイルスによる人獣共通感染症でマダニ科Ixodidaeに属する各種のマダニにより伝播される．ダニ媒介性脳炎には致死率30%にも及ぶロシア春夏脳炎と比較的軽症で経過する中央ヨーロッパダニ媒介性脳炎が存在する[2, 16]．これまでわが国では長い間ダニ媒介性脳炎の発生報告はなかったが，1993年北海道で本症の患者が発見され，原因ウイルスをイヌ，ノネズミおよびマダニより分離した[17～19]．ロシアでは，毎年10,000人前後の本病の流行が報告されている（**表1**）．我々はこれまで極東ロシアにおいて疫学調査を実施し，ダニ媒介性脳炎ウイルスを分離した．ここではまず日本と極東ロシアのTBEウイルスの系統解析の成績を紹介する．さらに日本のダニ媒介性脳炎ウイルスの病原性について，弱毒変異株および感染性cDNAクローンを用いて解析した成績について紹介する．

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2. 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

北海道のダニ媒介性脳炎ウイルス株の起源を推定するために，極東ハバロフスクにおいてマダニ類を採集してウイルス分離を試み，ウイルスの系統解析を実施した．1998年にハバロフスク地区で採集した550匹のシュルツェマダニから5株のウイルスを分離した[7]．これらの株と北海道分離株のE-タンパク遺伝子の塩基配列を決定し，すでに公表済みのダニ媒介性フラビウイルス各株の塩基配列と比較し，系統樹を作成した（**図1**）[4,7,14]．北海道株（Oshima 5-11，I-1，3-6，5-10，C-1，A-1）はSofjin株とハバロフスク株（KH98-2，98-10，98-5）とともに極東型ウイルスとして同一のクラスターを形成した．次に極東型ウイルス各株の同義置換距離をもとに系統樹を作成した（**図2**）．これらの株の平均同義置換率を計算したところ，2.9×10^{-4}となった．この平均同義置換率と同義置換距離をもとに北海道株とハバロフスク株の分岐の年代を計算したところ，これらの株は約260～430年前に分岐したと推定された．従ってダニ媒介性脳炎ウイルス北海道株は極東地区において数百年前に出現したと推定された[8]．

次に近年，シベリア地区に系統樹解析による極東型とヨーロッパ型に加え，第3の亜型のダニ媒介性脳炎の存在が示唆された．そこでイルクーツク周辺において，マダニを採集しウイルス分離を試みた．新たに6株のダニ媒介性脳炎ウイルスを，シュルツェマダニ（*I.persulcatus*）から分

表1 1992年～2001年のダニ媒介脳炎患者発生状況

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
オーストリア	84	102	178	109	128	99	62	41	60	51
ドイツ	142	118	306	204	109	168	130	82	155	
エストニア	163	166	177	175	177	403	387		272	
ラトビア	287	791	1366	1341	736	884	1037	352	544	303
リトアニア	17	198	284	426	310	646	584		419	
ポーランド	8	241	181	267	259	200				
ロシア	6301	7520	5593	5982	10298	6702	7520	9955	5931	6399
スウエーデン	83	51	116	68	45	76				
フィンランド	14	25	16	23		20	17			
スイス	66	44	97	60	62	123	68			
スロベニア	210	194	492	260	406	269	136			
クロアチア	27	76	87	91	57	25	32			
チェコ	337	629	619	743	571	415	422	490	7191	623
スロバキア	13	50	58	89	93	77				
ハンガリー	206	329	278	240	253	107	84	51		
イタリー		3	2	5	3		6			
フランス	3	5	4	7	1	1				

表2 研究に使用された極東とシベリア地区からのダニ媒介性脳炎ウイルス

Strain	Year of isolation	Geographical origin	Source	Accession no.	
				Envelope	3'-NCR
VL99-m11	1999	Vladiostok	*I. persulcatus*	AB049345	AB049393
KH99-m9	1999	Khabarovsk	*I. persulcatus*	AB049346	–
D1283	1998	Khabarovsk	Human brain	AB049347	–
IR99-1m1	1999	Irkutsk (1)*	*I. persulcatus*	AB049348	AB049397
IR99-1m4	1999	Irkutsk (1)	*I. persulcatus*	AB049349	AB049398
IR99-2m3	1999	Irkutsk (2)	*I. persulcatus*	AB049350	–
IR99-2m7	1999	Irkutsk (2)	*I. persulcatus*	AB049351	AB049399
IR99-2f7	1999	Irkutsk (2)	*I. persulcatus*	AB049352	–
IR99-2f13	1999	Irkutsk (2)	*I. persulcatus*	AB049353	AB049400
Oshima 5-10	1995	Oshima	Dog blood	AB001026	AB049390
Oshima I-1	1996	Oshima	*I. ovatus*	AB022292	AB049391
Oshima A-1	1995	Oshima	*A. speciosus*	AB022293	AB049392
KH98-2	1998	Khabarovsk	*I. persulcatus*	AB022295	AB049394
KH98-5	1998	Khabarovsk	*I. persulcatus*	AB022296	AB049395
KH98-10	1998	Khabarovsk	*I. persulcatus*	AB022297	AB049396
Sofjin-HO	1937	Primorsky	Human brain	AB022703	AB049401

( )*: Virus isolation point number

離し，エンベロープタンパク遺伝子の系統解析を行った(**表2**)．エンベロープタンパク遺伝子の塩基配列に基づく系統樹が**図3**に示されている．系統樹の分岐パターンは，ダニ媒介性脳炎ウイルスの4つのクラスターすなわち，ヨーロッパ亜型，極東亜型，シベリア亜型の3つのダニ媒介性脳炎ウイルスと跳躍病（louping ill）ウイルスに分かれた．ウラジオストックとハバロフスクから新たに分離された株（VL99-m11，KH99-m9とD1283）はOshima株とSofjin株のように，極東亜型と同定されたウイルス株と同一のクラスターを形成した．従ってこれらの分離株はダニ媒介性脳炎ウイルス極東亜型と分離された．しかし，イルクーツク地区からの分離株はVasilchenko株（シベリア亜型のプロトタイプ）とAina株とクラスターを形成した．従ってこれらの株はシベリア亜型のダニ媒介性脳炎ウイルスと同定された．これらのシベリア亜型のウイルス株は極東亜型とヨーロッパ亜型のウイルスと区別されたが，これらシベリア分離株はヨーロッパ亜型よりは極東亜型により近縁であった．

マウスモデルを用いてイルクーツク分離株（IR99-2f7と-2f13）と極東株（VL99-m11，D1283，KH98-5，Ohima5-10）

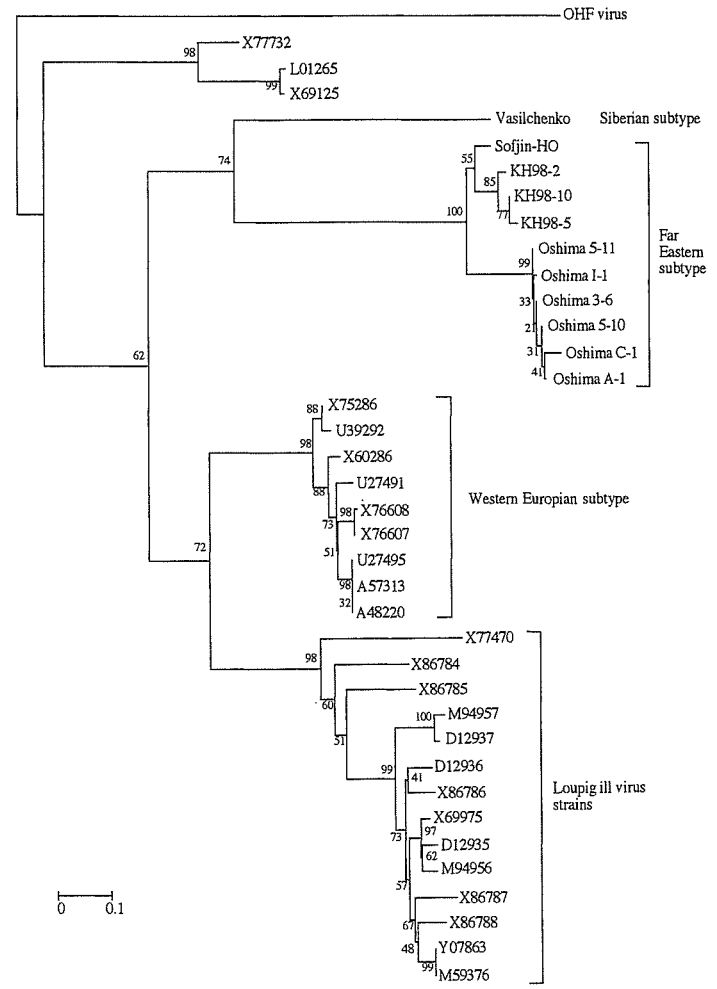

図１　極東亜型，シベリア亜型，西欧亜型ダニ媒介性脳炎ウイルスと跳躍病ウイルスを含めた系統樹

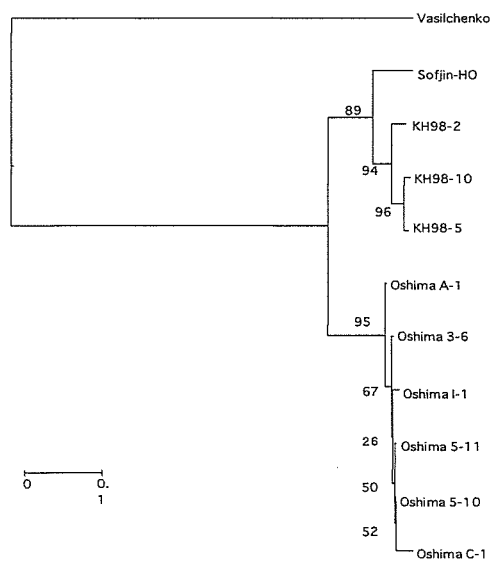

図２　北海道と極東ロシアで分離された極東型ダニ媒介性脳炎ウイルス株の系統樹

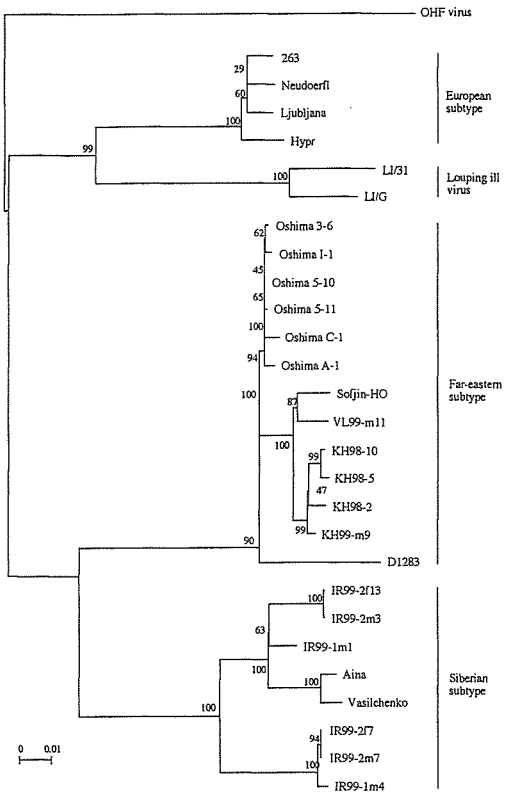

図3 各種ダニ媒介性脳炎ウイルス株とイルクーツク株の系統樹の比較

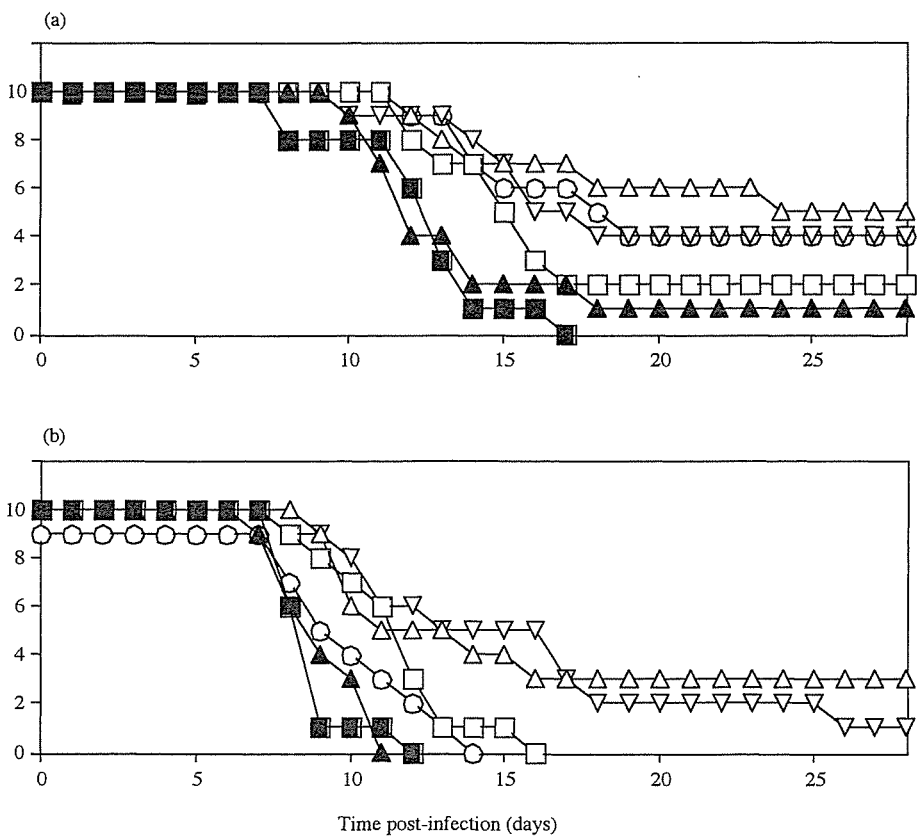

図4 ダニ媒介性脳炎ウイルス株接種後のマウスの生存(a)1000FFU のウイルスを皮下接種, (b)10FFU のウイルスを脳内接種, IR99-2f7(■), IR99-2f13(▲), VL99-m11(□), KH98-5(△), D1283(▽), Oshima 5-10(○)

の病原性を比較した（図 4）．神経侵襲性毒力を調べるために，1,000FFU のウイルスをマウスへ皮下接種し，28 日間生存率を記録した（図 4a）．これらのウイルス株は異る神経侵襲性を示した．接種後，生存率は IR99-2f7 の 0%,IR99-2f13 の 10%，VL99-m11 の 20%，D1283 と Oshima5−10 の 40%，KH98-5 の 50% であった．イルクーツク分離株を接種したマウスは極東株接種マウスより 3 ～ 5 日間早く死亡した．これらのウイルス株の神経毒力を調べるために，各ウイルス株の 10FFU を脳内接種して，マウスの生存率を比較した（図 4b）．IR99-2f7，IR99-2f13，VL99-m11，Oshima5-10 株接種マウスはすべて死亡した．しかし IR99-2f7 と IR99-2f13 接種マウスは VL99-m11 または Oshima5-10 接種マウスより約 2 ～ 5 日早く死亡した．D1283 と KH98-5 接種マウスは各々 10%，20% 生存した．これらの成績はイルクーツクに分布するダニ媒介性脳炎ウイルスは極東地区に分布するウイルスと比べ同等かまたはより強い毒力を持つことを示している．

3. 培養細胞に適応したダニ媒介性ウイルス変異株の病原性

ダニ媒介性脳炎ウイルス Oshima5-10 株を BHK 細胞に継代したところ，大きなプラックを形成する培養細胞適応変異株が得られた（図 5a）[5]．この BHK 細胞適応変異株 Oshima Cl-1 株についてマウスにおける病原性と他の生物的性状および遺伝子性状を調べた．Oshima 5-10 株と Oshima Cl-1 株 BHK 細胞での増殖を比較した．Oshima Cl-1 株の培養上清におけるウイルス力価は 9 時間後に増加したが，Oshima5-10 株の力価は 9 時間目まで低く，12 時間後に増加したが Oshima Cl-1 の 1/100 であった（図 5b）．この結果は変異株 Oshima Cl-1 は親株 Oshima5-10 より迅速に増殖することを示す．

ウイルスの神経侵襲性毒力の程度を，マウスの皮下接種の系で調べた．神経毒力の程度を評価するため，マウスへの脳内接種も実施した．各ウイルスを 10,000FFU マウスへ皮下接種したところ，2 株の間に神経侵襲性毒力に有意な違いがあることが明らかになった（図 6a）．Oshima Cl-1 株接種マウスは 80% 生存したのに比べ，Oshima 5-10 株では 30% のマウスが生存した．一方 2 株のウイルスの神経毒力のレベルは 10FFU のウイルスを神経内接種により調べた（図 6b）．両株の間には有意の差は見られなかった．これらの成績から Oshima Cl-1 株の神経侵襲性毒力の程度は Oshima5-10 株より有意に低かったが，神経毒力のレベルは

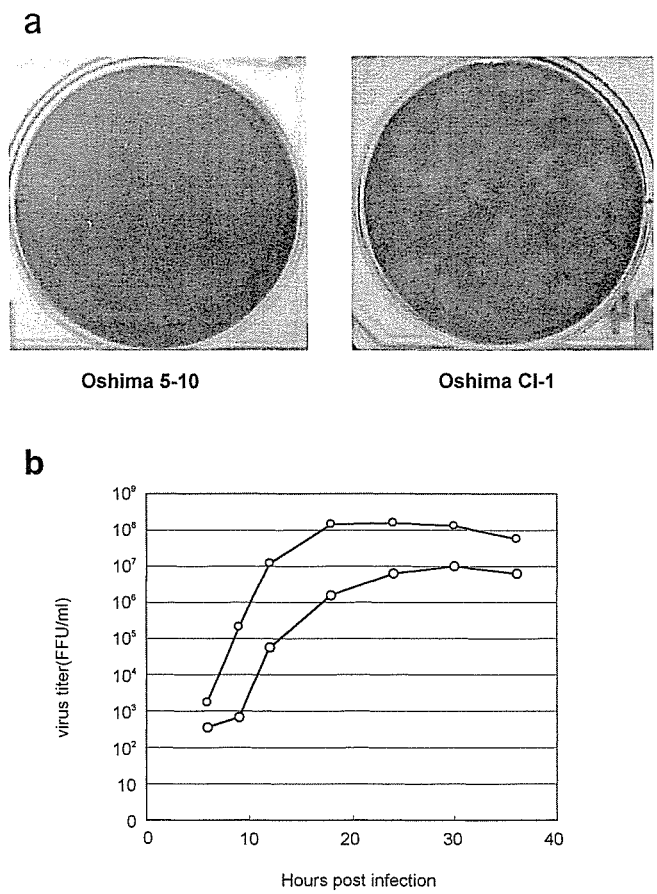

図 5　BHK-21 培養細胞におけるダニ媒介性ウイルスの増殖（a）Oshima5-10 と Oshima Cl-1 感染 120 時間後の BHK-21 細胞上でのプラックの形態（b）BHK-21 細胞でのウイルス増殖曲線，MOI1FFU の Oshima Cl-1（●）または Oshima 5-10（○）を感染させた。

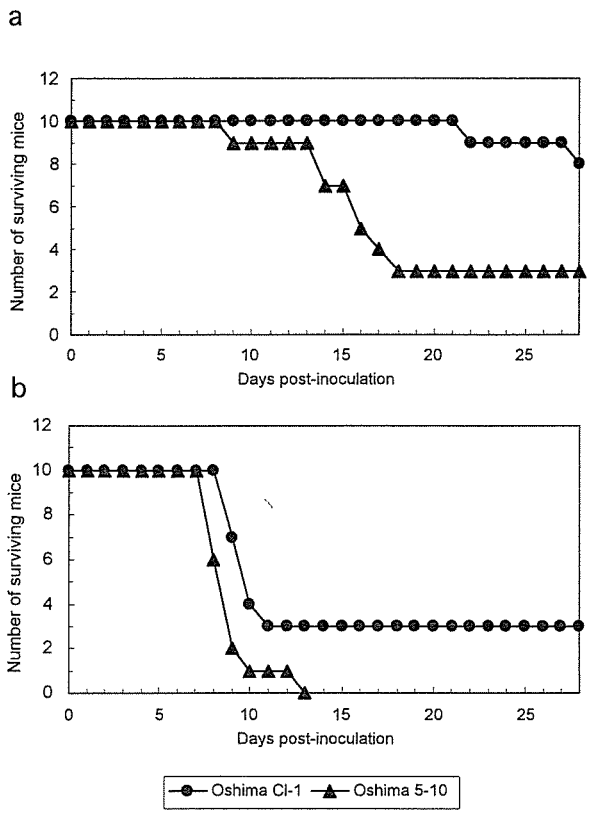

図6 ダニ媒介性脳炎ウイルス接種後のマウスの生存(a)10,000FFU のウイルスを皮下接種(b)10FFU のウイルスを脳内接種, Oshima Cl-1(●), Oshima 5-10(▲)

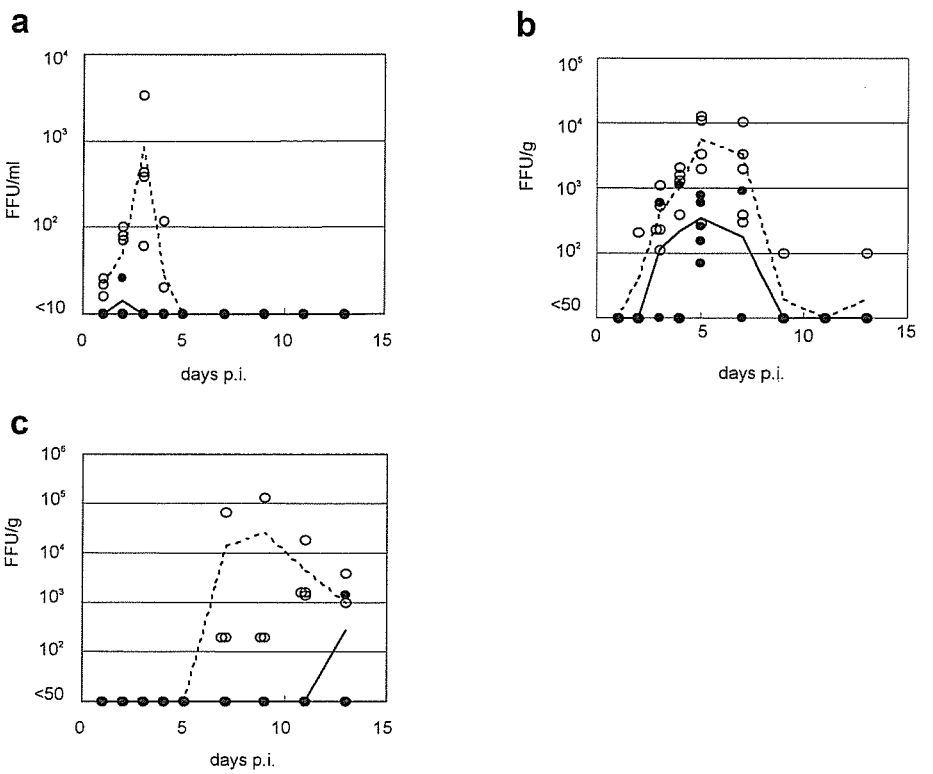

図7 マウスの血液と組織におけるダニ媒介性脳炎ウイルス力価の推移, (a)血液, (b)脾臓, (c)脳, Oshima Cl-1(● — ●), Oshima 5-10(○ ···· ○)

両株で差がなかったことが示された．

Oshima Cl-1の低い神経侵襲性毒力が末梢組織におけるウイルスの増殖または拡散が低下し脳内へのウイルス侵入の低下に原因した可能性がある．この点を調査するために，血液，脳および脾臓におけるウイルス増殖のレベルを両ウイルス株を皮下接種したマウスにおいて比較した（**図7**）．Oshima 5-10接種後，ウイルス血症は接種後1日目に検出され，3日後にピーク（1×10^3FFU/ml）に達し，徐々に減少し5日目には検出できなくなった（**図7a**）．脾臓においてはウイルスは2日目に検出され，5日目に5×10^3FFU/gのピーク値に達した（**図7b**）．脳ではウイルスは7日目に最初に検出され，9日目にピークの2×10^4FFU/gに達した（**図7c**）．他方，Oshima Cl-1接種後，ウイルス血症は2日目に検出され（60FFU/ml），その後減少し，3日目には検出できなかった（**図7a**）．脾臓ではウイルスは3日目に最初に検出され，5日目にピーク（5×10^2FFU/g）に達した．9日目に脾臓ではウイルスは検出できなかった．脳においてはウイルスは11日目まで検出されず（**図7c**），観察期間中，マウスは臨床的な脳炎症状を示さなかった．まとめると，脾臓と血液でのOshima Cl-1のウイルス増殖はOshima 5-10より低く，さらに脳でのウイルス増殖は，Oshima 5-10は7日目に検出されたが，Oshima Cl-1ではほとんど認められなかった．

塩基配列の比較では，Oshima Cl-1はOshima 5-10と比べ3つの塩基と2つのアミノ酸の置換があった．2つのアミノ酸の置換の1つは，NS5タンパクで検出されたが，タンパクの荷電や極性に影響しない変化であった[20]．しかし他の置換は，Eタンパクにあり，Oshima Cl-1のEタンパクの陽性電荷を増加させた．この置換はEタンパクのドメインIIに位置しており[15]，以前の研究でこのドメインの変異は膜融合と赤血球凝集性に影響することが示されていた[3,10]．

特定の培養細胞へのウイルスの適応は，グリコサアミノグルカン（GAGs）へ高い親和性を持つ変異株を選択し，それらの変異株のいくつかは，動物における毒力の低下を来す[1,11,13]．そこで，2株のウイルスのGAGsへの親和性を比較するために，異なるGAGsのウイルス感染性の抑制効果を調べた．Oshima 5-10のBHK-21細胞への感染性はGAGsによりほとんど阻止されなかった（**図8a**）．対照的にOshima Cl-1の感染性は，試験されたすべてのGAGsにより抑制され，その抑制は用量依存的であった（**図8b**）．

これらの成績はウイルスEタンパクのアミノ酸の置換が，マウスモデルでの神経侵襲性の低下を来したことを示している．その変異によりウイルス血症と末梢でのウイルス増殖が低下し，ウイルスが脳内に侵入できなかったと考えられた．このEタンパクの変異により，ウイルスタンパクの

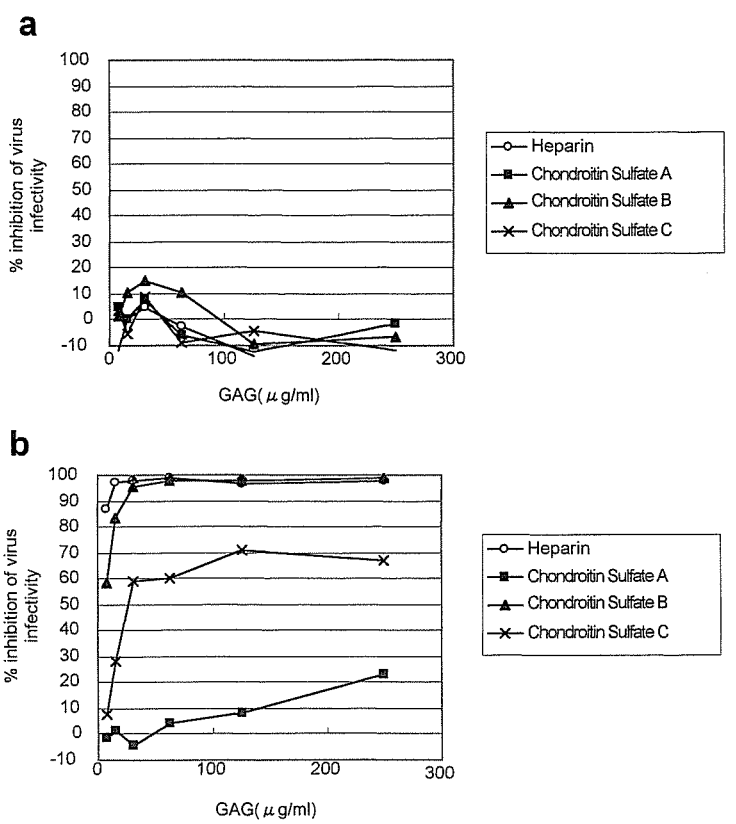

図8　GAGsによるOshima 5-10(a)とOshima Cl-1(b)の感染阻止

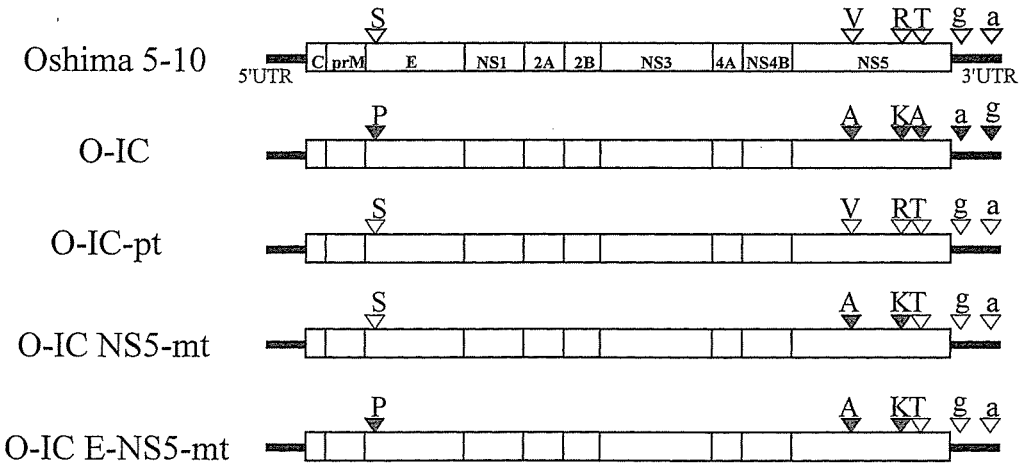

図9 親株と cDNA クローン由来ウイルスのアミノ酸の置換▽は親株のアミノ酸または塩基,▼は O-IC のアミノ酸または塩基

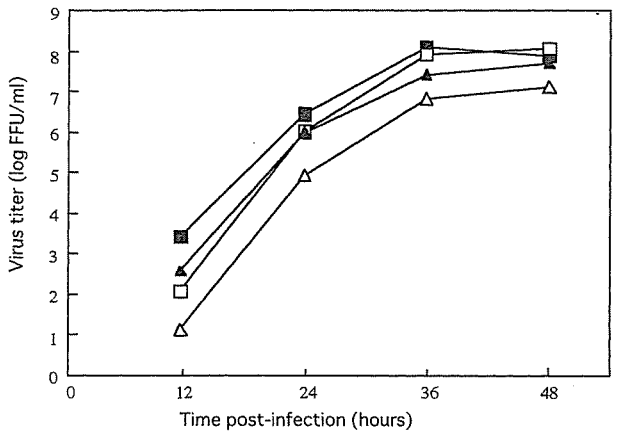

図10 BHK 細胞における親株 Oshima 5-10 と cDNA クローン由来ウイルスの増殖曲線の比較
親株 Oshima 5-10(■), OIC NS5-mt(▲)
OIC-E-NS5-mt(△), OIC-pt(□)

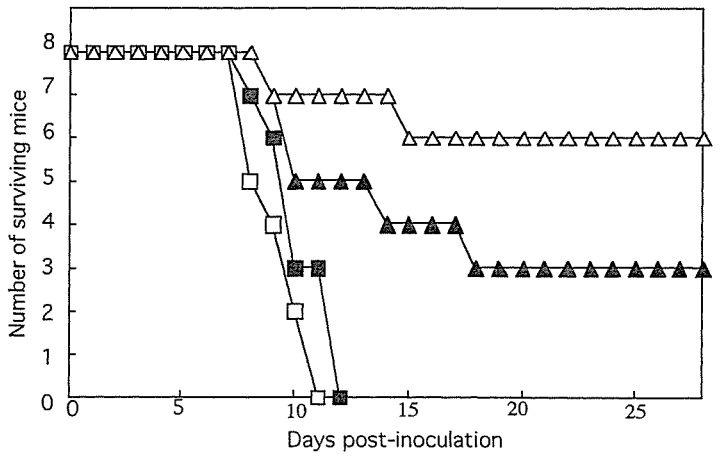

図11 50FFU 脳内接種マウスにおける生存。
親株 Oshima 5-10(■), O-IC NS5-mt(▲)
O-IC-E-NS5-mt(△), O-IC-pt(□)

陽性電荷の程度が増加し，動物体内に一般的に分布するGAGsによりウイルスが捕捉されウイルスの末梢での増殖が低下したためと推定された．

4. 感染性cDNAクローンを用いたダニ媒介性脳炎ウイルスの病原性の解析

ダニ媒介性脳炎ウイルスの感染性cDNAクローンについて，ヨーロッパ亜型のNeudoerfl株とHypr株およびシベリア亜型のVasilchenko株についての報告がある[6,12]．そこで極東亜型のダニ媒介性脳炎ウイルスOshima5-10株について感染性cDNAクローンを作出し，病原性の解析に用いた．

Oshima 5-10株の感染性cDNAクローンの作出は，long high-fiedelity RT-PCRとone-step cloning法により実施した[9]．最初に得られた感染性cDNAクローン0-1Cは構造タンパクに4ヶ所のアミノ酸置換と，3' UTRに2ヶ所の塩基置換を有していた．構造タンパクのアミノ酸置換はE-タンパクに1ヶ所，Ns5タンパクに3ヶ所存在した(**図9**)．このアミノ酸と塩基の置換を親株に部分的修復した種々のクローンおよびすべて親株に修復したO-IC-ptを作成した(**図9**)．

これらのクローンからウイルスを回収し，BHK細胞における増殖を比較した（**図10**）．親株Oshima 5-10と遺伝的に同一の0-IC-ptウイルスの間に感染後12時間で，ウイルス産生量に有意な違いが存在した．これは親株ウイルスストックの集団中にquasi-speciesの存在によるためかも知れない．4株のうちでE-タンパクに置換のある$P_{40}A_{378}K_{674}T_{794}$（O-IC E-NS5-mt）が他の株に比べウイルス産生が低下していた．

次にこれら4株のウイルスのマウスの神経毒力について調べた（**図11**）．50FFUのウイルスをマウスの脳内に接種後，親株Oshima 5-10と親株と遺伝的に同一のO-IC-ptは，接種後に12日以内に100%の致死率を示した．対照的にO-IC-ptウイルスとNs5遺伝子の2ケ所を異にする$S_{40}A_{378}K_{674}T_{994}$ (O-ICNS5-mt)ウイルスは神経毒力が低下しており，18日目に62.5%のマウスを死亡させた．$S_{40}A_{378}K_{674}T_{994}$ウイルスとE-タンパクの1ヶ所のアミノ酸が異なる$P_{40}A_{378}K_{674}T_{994}$（O-ICE-NS5-mt）は，最も低い神経毒力を示し，25%のマウスを死亡させた．これらの成績はEタンパクのS40→Pへの置換とHs5-V_{378}→AとNs5-R_{674}→Kへの置換が相乗的にウイルスの神経毒力の低下に影響していることを示している．

5. おわりに

ダニ媒介性脳炎の患者数はヨーロッパ諸国とロシアを中心に毎年10,000人前後報告されている．ヨーロッパではダニ媒介性脳炎の予防のためワクチン接種を実施している国が多い．毎年400万人以上の日本人旅行者がヨーロッパを訪問している．これらの日本人へヨーロッパにおけるダニ媒介性脳炎の流行情報を正確に提供するとともに，ダニが多数生息する森林や農村地帯に滞在するハイリスクグループの人々にはワクチンの実用化が望まれる．

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Phylogenetic analysis and pathogenicity of tick-borne encephalitis virus from Japan and far-east Russia

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Phylogenetic analysis of tick-borne encephalitis(TBE) virus revaled that Hokkaido strain of TBE virus evolved several hundreds years ago in far-east Russia. TBE virus strains in Irkutsk area were identified as Siberian subtype of TBE virus. BHK-cell adapted mutant of TBE virus showed lower neuro-invasive virulence in mice than parent virus. The mutant carried one amino acid substitution in envelope protein which resulted in increase of positive charge of the protein. The mutant-infected mice showed lower virus titers in bloods and spleens than the parent-infected mice. Infectious c-DNA clone of TBE virus Hokkaido strain was successfully generated and was applied to examine the neurovirulence in mice. One amino acid change in envelope protein and 2 amino acid changes in Ns5 protein showed a synergistic effect on reduced neurovirulence in mice.

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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

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Abstract

The sub-genomic replicon of tick-borne encephalitis (TBE) virus (Far-Eastern subtype) was packaged into infectious particles by providing the viral structural proteins in *trans*. Sequential transfection of TBE replicon RNA and a plasmid that expressed the structural proteins led to the secretion of infectious particles that contained TBE replicon RNA. The secreted particles had single-round infectivity, which was inhibited by TBE virus-neutralizing antibody. The physical structure of the particles was almost identical to that of infectious virions, and the packaged replicon RNA showed no recombination with the mRNAs of the viral structural proteins. Furthermore, heterologous genes were successfully delivered and expressed by packaging TBE replicon RNA with inserted GFP and Neo genes. This replicon packaging system may be a useful tool for the molecular study of the TBE virus genome packaging mechanism, and for the development of vaccine delivery systems.

Keywords: Tick-borne encephalitis virus; Replicon; Virus-like particles

1. Introduction

The genus *Flavivirus* (family *Flaviviridae*) contains important human pathogens, which include the tick-borne encephalitis (TBE) virus, Japanese encephalitis virus, yellow fever virus, Dengue virus, and West Nile virus. Flaviviruses can be divided into three phylogenic and ecological groups: a tick-borne group, a mosquito-borne group, and a vector-unknown group [1–3]. The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e. the core (C), premembrane (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, which is co-translated and cleaved post-translationally [4]. The 5′- and 3′-UTRs have predicted secondary structures that are implicated in viral replication, translation, and packaging of the genomes [5–8].

The TBE virus is a species of mammalian tick-borne flavivirus that is prevalent over wide areas of Europe and Asia [9,10]. It causes severe encephalitis in humans with serious sequela and has a significant impact on public health in these endemic regions [11,12]. The virus has been subdivided into Far-Eastern, Siberian, and European subtypes [13–15]. The Far-Eastern subtype, which was previously known as Russian spring-summer encephalitis (RSSE) virus, causes severe clinical manifestations and has a higher mortality rate (5–20%) than the other TBE virus subtypes [9,16].

The subgenomic replicons of positive-stranded RNA viruses contain genes that are necessary for viral amplification in host cells, but they lack the genes for the viral structural

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proteins. Thus, the replicon RNAs can replicate in cells but cannot be incorporated into virus particles. Therefore, these replicons have been used as a tool for the study of genome replication in various viruses [17–20]. Moreover, little is known about the flavivirus genome packaging mechanism, packaging signal(s), and nucleocapsid formation, mainly because it is difficult to reproduce the packaging of flavivirus genome RNA into nucleocapsids. Therefore, the construction of a packaging system that uses these replicons could be a useful tool for studying the flavivirus genome packaging mechanism.

Another application of these replicon systems is the development of vectors for gene expression and vaccines [20–22]. Replicon-based vectors of positive-strand RNA viruses are becoming more popular for development of antiviral and anticancer vaccines. By using replicon systems, a high level of expression of heterologous genes can be expected, and the inability of replicon RNA to spread from transfected cells gives biological safety for the vaccine application. In several replicon studies of the mosquito-borne flaviviruses, heterologous gene expression following the insertion of genes into the deleted structural protein gene region or 3′-UTR has been reported [23–25]. In a study of the Kunjin virus, the packaging of replicon RNA into particles by providing the structural protein in *trans* was developed and applied for heterologous gene delivery vector and as a vaccine [26–30].

In our previous study, a sub-genomic replicon of the Far-Eastern subtype TBE virus strain Oshima 5–10, which was isolated in Japan [31], was constructed, and heterologous genes were inserted into the 3′-UTR [32]. The heterologous genes were expressed appropriately, and the host cells that were transfected with the replicon RNA showed no cytopathic effect (CPE). In this study, a TBE virus replicon packaging system was developed using the expression of the viral C/prM/E proteins in *trans*. C-prM-E processing was carried out correctly by NS proteins that were produced from the replicon, and replicon RNAs were incorporated into single-round infectious particles without recombination between the replicon RNA and the mRNAs for the structural proteins. Furthermore, heterologous genes were delivered and expressed using this packaging system, whereby, for the first time with a tick-borne flavivirus TBE virus, replicons were inserted with heterologous genes. These data suggest that this newly developed packaging system is a useful tool for the molecular study of the TBE virus genome packaging mechanism, and for the development of gene delivery vectors using the TBE virus replicon.

2. Materials and methods

2.1. Cells and viruses

The baby hamster kidney (BHK)-21 cell line was grown at 37 °C in Eagle's minimum essential medium (MEM) that was supplemented with 8% FCS and L-glutamine. For the selection of cells that expressed the neomycin resistance gene, G418 (1 mg/ml) was added to medium. TBE virus was prepared from the infectious virus recovered from TBEV infectious cDNA O-IC-pt, in which the full-length cDNA of TBE virus Oshima strain (Far-Eastern subtype) was cloned, as described previously [33].

2.2. Antibodies

For the detection of TBE virus E proteins in the ELISA, the mouse monoclonal anti-E antibodies 1H4 and 4H8 [34] were used. Rabbit polyclonal anti-prM, anti-E, and anti-NS3 antibodies were prepared by immunization with recombinant prM, E, and NS3 proteins, respectively, which were expressed in the pET43 system (Novagen, Madison, WI). FITC-conjugated anti-mouse IgG antibodies and anti-rabbit IgG antibodies (Jackson Immunoresearch, West Grove, PA) were used as secondary antibodies in immunofluorescence assay.

2.3. Plasmid construction

The derivation of the recombinant plasmid pCAGprME, which expresses both prM and full-length protein E derived from the Oshima 5–10 strain of TBE virus (GenBank accession no. AB062063), has been described previously (Fig. 1A) [35]. For the construction of pTBECprME, TBE viral RNA was extracted from a virus-inoculated suckling mouse brain, and RT-PCR was performed as described previously [31]. PCR for the amplification of the DNA fragment that contains the gene for protein C and part of the *prM* gene was carried out with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), using the following primers: forward, 5′-AGC*GCTAGC*AGATTTTCTTGCACGTGCAT-3′; and reverse, 5′-GTGTCCCCTTCCTGTGAGAT-3′. The PCR products were digested with *Nhe*I and *Age*I, and inserted into the pCAGprME plasmid, which was predigested with *Nhe*I and *Age*I.

TBE replicon RNA transcripts were prepared form Oshima REPpt, Oshima REP-GFP, and Oshima REP-NEO plasmids, as described previously [32]. For the construction of Oshima REPpt, the NS5 region spanning residues 7881 to 9829 (between the restriction sites for *Afl*II to *Asc*I) in Oshima REP, which has two amino acid mutations in the NS5 gene, was replaced with the parental NS5 gene. The Oshima REP-GFP and Oshima REP-NEO plasmids, in which the GFP and neomycin-resistance genes were inserted into the 3′-UTR, have been described in our previous study [32].

2.4. Cell transfection

Cells that were grown to 60–70% confluence in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) were transfected with 2 μg of pCAGprME or pTBECprME that was complexed with the *Trans*IT-LT1 reagent (Pan-Vera Corp., Madison, WI) in Opti-MEM (Invitrogen) without

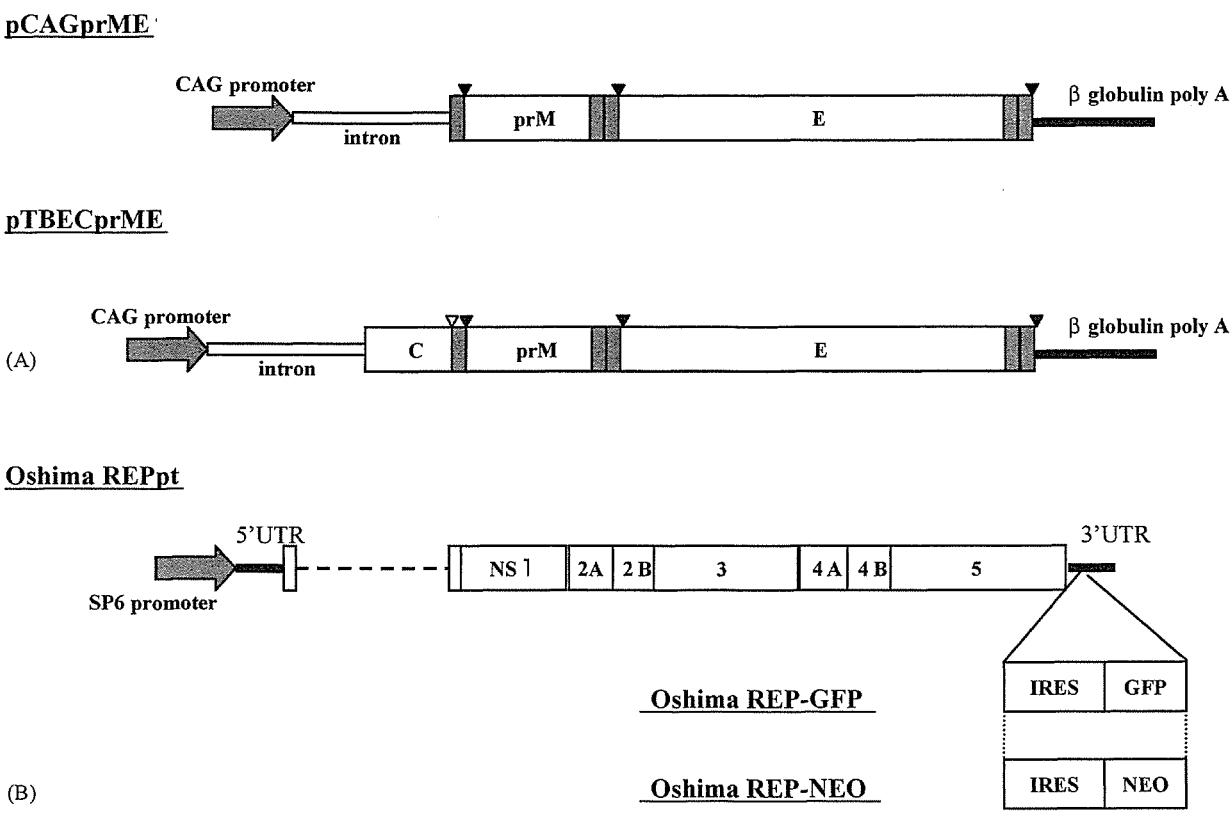

Fig. 1. Schematic representation of the plasmids used in this study. (A) Mammalian expression vector that encodes the TBE virus structural proteins. The TBE virus prM/E and C/prM/E regions were cloned into the pCAGGS vector [52] and designated pCAGprME and pTBECprME, respectively. The transmembrane domain and signal sequence are indicated by a gray box. The intracellular protease cleavage sites are indicated by black triangles, and the NS2B-3 protease site is indicated by an open triangles. (B) Constructs of TBE replicon. TBE replicon regions with or without the IRES GFP/NEO gene in the 3′-UTR were inserted under the control of the SP6 promoter, as described previously for the Oshima REP plasmid [32].

serum or antibiotics. Twenty-four hours post-transfection (unless stated otherwise), the cells and supernatants were harvested and used for further experiments.

2.5. SDS-PAGE and Western blotting

Transfected cells were lysed with Laemmli buffer [36] and electrophoresed in 8% and 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels under non-reducing condition. The protein bands on the gels after SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with 1% Gelatin in 25 mM TBS that contained 0.01% Tween-20 (TBST), for 30 min at room temperature. After washing with TBST, the membranes were reacted with the anti-E and anti-prM rabbit IgG for 1 h, and then with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (Promega, Madison, WI) for 30 min at room temperature. The protein bands were visualized with the AP Detection Kit (Novagen).

2.6. Preparation of encapsidated particles

Oshima REPpt was digested with *Spe*I and extracted using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The mMESSAGE mMACHINE SP6 Kit (Ambion Inc., Austin, TX) was used to in vitro-transcribe infectious RNA in a 20-μl reaction mixture that contained an additional 1 μl of GTP solution. After transcription at 37 °C for 2 h, the DNA template was removed by DNase I digestion at 37 °C for 15 min. The RNA was precipitated using lithium chloride, washed with 70% ethanol, resuspended in RNase-free water, quantitated by spectrophotometry, and stored at −80 °C in aliquots.

Approximately 5×10^6 BHK cells in 0.5 ml cold PBS were electroporated with 10 μg of RNA in 0.4-cm cuvettes using the GenePulser apparatus (Bio-Rad Laboratories, Hercules, CA), with two separate pulses, without the pulse controller at settings of 1.3 kV, 25 μF, and at maximum resistance. The transfected cells were aliquoted into 24-well culture plates. After 24 h, the cells were transfected with 2 μg of pTBECprME per well. At 36 h post-transfection, the supernatant was harvested and cleared by low-speed centrifugation at $1000 \times g$ for 10 min. The particles in the cleared supernatant were precipitated with 10% polyethylene glycol (PEG; MW = 8000) and 1.9% NaCl for 2 h at 4 °C, and pelleted at $10{,}000 \times g$ for 20 min. The pellets were resuspended in PBS that was supplemented with RNase A (20 μg/ml),

left to dissolve overnight at 4 °C, and then used in further experiments.

2.7. Infectivity assays

In order to determine the titers of the encapsidated particles, BHK cells were grown on 16-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) and infected with serially diluted encapsidated particle solutions for 1.5 h at 37 °C. In the particle neutralization test, 1000 infectious units of encapsidated particles were incubated with at a 1:100 dilution of the anti-TBE E protein monoclonal antibody 1H4, which has neutralizing activity against the TBE virus, prior to infection of BHK cells. The culture supernatant was replaced with fresh medium and incubated for 24 h at 37 °C. The cells were rinsed with phosphate-buffered saline (PBS), subjected to fixation with 4% paraformaldehyde for 10 min, and 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 anti-NS3 rabbit IgG antibodies, at a dilution of 1:100, in antibody-dilution buffer (PBS that contained 0.1% Triton X-100 and 2 mg/ml BSA). After extensive washing with PBS, the cells were incubated at room temperature in the dark for 1 h with fluorescence-labeled secondary antibodies, which were diluted 1:200. The cells were washed three times with PBS, followed by mounting of the coverslips on glass slides. The images were viewed and recorded using confocal microscopy.

The infectious titer of the packaged particles was calculated using the following formula: titer (infectious unit [IU] per ml) $= N \times (SW/SIA) \times 1/V \times 10^n$, where N is the average number of anti-NS3 positive cells in the image area, as calculated from 12 image areas in different regions of the coverslips; SW is the surface of the well in a 16-well plate (70 mm^2); SIA is the surface of the image area (3.6 mm^2); V is the total volume of the diluted particle solutions; and 10^n is the dilution factor.

2.8. Particle analysis

Recombinant subviral particles (RSPs) were prepared from pCAGprME-transfected cells, and encapsidated particles were precipitated with PEG (as described above), and then subjected to equilibrium density centrifugation at 35,000 rpm in a 10–50% sucrose gradient at 4 °C for 14 h (P45AT rotor; Hitachi Koki Co., Ltd., Tokyo, Japan). Fractions of 0.5 ml were collected from the tops of the tubes. Aliquots of the fractions were treated with 1% Triton X-100, and added to MAb 1H4-coated wells of 96-well microtiter ELISA plates, which were blocked with 3% BSA. TBE virus-specific antigen in the fractions was detected using the biotinylated MAb 4H8 and horseradish peroxidase (HRP)-conjugated streptavidin (Sigma Chemical Co., St. Louis, MO). HRP activity was detected by adding 100 μl of *O*-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03% H_2O_2.

2.9. RT-PCR

RNA was extracted from the encapsidated particles, which were purified by centrifugation in sucrose gradients, using the Isogen Kit (Nippon Gene, Toyama, Japan). The reverse transcription (RT) reaction was carried out with Superscript II reverse transcriptase (Invitrogen) and the primer HO_2 (5′-AGCGGGTGTTTTCCGAGTC-3′). PCR was carried out as described previously [31] using the following primers: for amplification of the region between the 5′-UTR and NS1, (forward) 5′-AGATTTTCTTGCACGTGCAT-3′ and (reverse) 5′-GTATGCATAATTGTCATACC-3′; and for amplification of the partial prM gene and E gene, (forward) 5′-TCTGCAAAGACGTGGCAAAA-3′ and (reverse) 5′-CATTGAGGGCTTCCCCTCAG-3′.

3. Results

3.1. Expression of the TBE virus structural proteins by the pTBECprME plasmid

For the expression of the TBE virus structural proteins that were used to package the TBE replicon RNA, the TBE C-prM-E region was cloned into the pCAGGS expression vector, thereby generating pTBECprME (Fig. 1A). To examine the expression of the viral structural proteins and cleavage by the signal peptidase, BHK cells were transfected with pTBECprME, and viral structural protein expression was detected by specific immune sera in Western blots (Fig. 2). In the pTBECprME-transfected cells, E proteins with the same molecular size as those expressed in cells that were transfected with pCAGprME, which encodes the TBE prM-E gene, were detected. On the other hand, a band larger than that in prM was detected by the TBE prM-specific antibodies, while the prM band was detected in pCAGprME-transfected cells. This band corresponds to the C-prM polyprotein, which is not cleaved by the ER signal peptidase. It has been reported previously that the C-prM junction is first cleaved by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER luminal side [37–39]. Therefore, in this case, the C-prM polyprotein was not cleaved due to the absence of the NS3 protease.

It is known that the expression of the flavivirus envelope proteins prM and E leads to the secretion of recombinant subviral particles (RSPs). E protein that was derived from secreted RSPs was detected in the supernatants of pCAGprME-transfected cell cultures, as described previously [35]. However, E protein was not detected in the culture supernatants of cells that were transfected with pTBECprME. This was due to a failure to digest the C-prM junctions. Thus, the viral envelope proteins were unable to assemble into RSPs.

These data suggest that the TBE C-prM-E proteins are expressed in an appropriate manner following pTBECprME transfection. These cassettes were used in further studies to

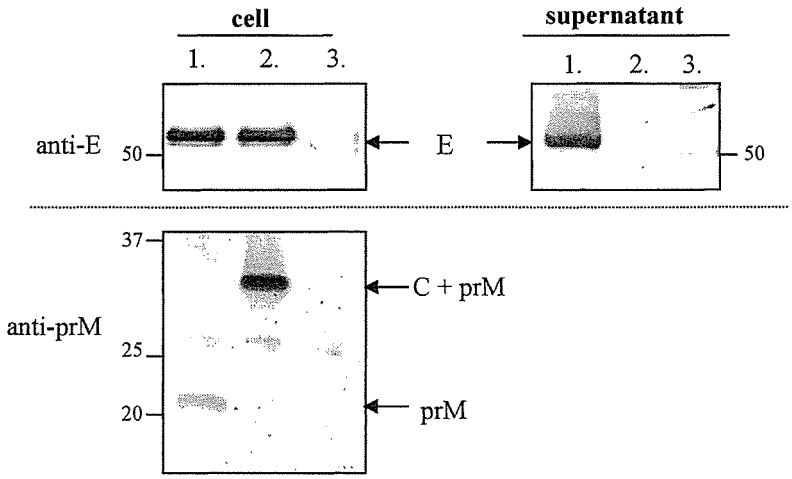

Fig. 2. Expression of TBE virus structural proteins. BHK cells were transfected with pCAGprME (lane 1), pTBECprME (lane 2), or the control pCAGGS plasmid (lane 3). At 24 h post-transfection, the cells and PEG-precipitated supernatant samples were separated by 12% SDS-PAGE under non-reducing condition and subjected to Western blotting. The viral proteins were visualized using anti-E (upper panel) and anti-prM (lower panel) rabbit polyclonal antibodies. The positions of the individual proteins are marked, and the molecular size (in kDa) is indicated at the side.

construct a system for packaging the TBE replicon RNA into infectious particles.

3.2. Packaging of the TBE replicon RNA into single-round infectious particles by trans-complementation

For the RNA packaging experiment, TBE virus replicon RNA was prepared from the Oshima REPpt plasmid, which was constructed from the Oshima REP plasmid with two mutations in NS5 region, as described previously [32]. It was necessary to use a TBE replicon with high transfection efficiency for packaging in doubly transfected cells (i.e., cells transfected with the TBE replicon and pTBECprME plasmid that expresses the TBE structural proteins). This replicon construct showed high efficiency of transfection into BHK21 cells, with 90–100% of the cells being positive in the IFA tests (data not shown). Consequently, the Oshima REPpt replicon was used in the subsequent packaging studies.

In preliminary experiments, transfection of pTBECprME, which expresses the TBE structural proteins, prior to Oshima REPpt replicon electroporation gave no secretion of infectious particles. This result was in accordance with the finding of the Kunjin virus study, which showed that the replicon had to accumulate before the structural proteins were expressed [30]. Therefore, in subsequent experiments, the BHK21 cells were first electroporated with TBE replicon Oshima REPpt, and then 12 h or more later, transfected with pTBECprME, thus ensuring the secretion of infectious particles.

The culture supernatants from the BHK21 cells that were transfected with pTBECprME and replicon Oshima REPpt were cleared by low-speed centrifugation and precipitated with PEG 8000. The precipitated pellets were resuspended in PBS that contained RNase A, and used for further study. In the BHK21 cells that were infected with PEG-precipitated supernatant from pTBECprME- and replicon-transfected cells, the production of TBE NS3 proteins was detected by the immunofluorescence assay (Fig. 3A), in contrast to the lack of viral protein production observed in cells that were infected with the supernatant of replicon-transfected cells (Fig. 3B). A second passage, in which the supernatants of the infected BHK21 cell cultures were transferred to fresh BHK21 cells, resulted in infection in the case of the wild-type control of RNA transfection from TBE virus infectious cDNA O-IC (authentic virus particles; Fig. 3F) but not in the case of transfection with pTBECprME and replicon Oshima REPpt (Fig. 3D). These data demonstrate that the particles (virus-like particles; VLPs) secreted by pTBECprME- and TBE replicon Oshima REPpt-transfected cells have only single-round infectivity potential.

To optimize the conditions for efficient packaging of replicon RNA into VLPs, various time-points between the transfections (Fig. 4A), and between the second transfection and harvesting the VLPs (Fig. 4B), were examined. First, the optimal time period between replicon RNA electroporation and pTBECprME transfection was determined. BHK21 cells were electroporated with Oshima REPpt RNA, and seeded into cell culture dishes at 40–50% confluency for the second plasmid transfection. After incubation for 12, 18, 24, or 30 h, the cells were transfected with pTBECprME, and the culture supernatant was recovered from each dish 48 h after the second transfection. The recovered supernatants were subjected to PEG precipitation and used for the titration of VLPs by IFA with anti-NS3 antibodies. As shown in Fig. 4A, the highest titer of VLPs was obtained from cells that were transfected with the 24-h interval (6.2×10^6 IU/ml). This time delay for optimal transfection was also observed in the Kunjin virus study [30]. This increase in VLP titer with time between transfections indicates that viral RNA and/or viral non-structural proteins are required for the efficient packaging of replicon RNA by *trans*-complementation of structural proteins. Second, the optimal time for VLP harvesting after