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Molecular Aspects of Japanese Encephalitis Virus Persistent Infection in Mammalian Cells

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Abstract: Japanese encephalitis virus (JEV) is the causative agent of a mosquito-borne encephalitis and is transmitted to human via persistently infected mosquito vectors. Although the virus is known to cause only acute infection, there were reports that showed neurological sequelae, latent infection in peripheral mononuclear cells, and recurrence of the disease after acute encephalitis. Innate resistance of certain cell lines, abnormal NS1 expression of the virus, and anti-apoptotic effect of cellular *bcl-2* have been suggested as probable causes of JEV persistence even in the absence of defective interfering (DI) particles. Although possible involvement of DI particles in JEV persistence was suggested, neither has a direct evidence for DI presence nor its molecular characterization been made. Two questions asked in this study are whether the DI virus plays any role in JEV persistent infection if it is associated with and what type of change(s) can be made in persistently infected cells to avoid apoptosis even with the continuous virus replication. DI-free standard stock of JEV was infected in BHK-21, Vero, and SW13 cells and serial high multiplicity passages were performed in order to generate DI particles. Three different-sized DI RNA species which were defective in both structural and nonstructural protein coding genes. Rescued ORFs of all the DI genome maintained in-frame and the presence of replicative intermediate or replicative form RNA of the DI particles confirmed their replication competence. On the other hand, several clones with JEV persistent infection were established from the cells survived acute infections during the passages. Timing of the DI virus generation during the passages seemed coincide to the appearance of persistently infected cells. The DI RNAs were identified in most of persistently infected cells and were observed throughout the cell maintenance. One of the cloned cell line maintained the viral persistence without DI RNA coreplication. The cells with viral persistence released the reduced but continuous infectious JEV particle for up to 9 months and were refractory to homologous virus superinfection but not to heterologous challenges. Unlike the cells with acute infection these cells were devoid of characteristic DNA fragmentation and JEV-induced apoptosis with or without homologous superinfection. Therefore, the DI RNA generated during JEV undiluted serial passage on mammalian cells was shown to be biologically active and it seemed to be responsible, at least in part, for the establishment and maintenance of the JEV persistence in mammalian cells. Viral persistence without DI RNA coreplication, as in one of the cell clones, supports that JEV persistent infection could be maintained with or without the presence of DI particles. In addition, the fact that the cells with JEV persistence were resistant against homologous virus superinfection, but not against heterologous one, suggests that different viruses have their own and independent pathway for cytopathogenesis even if viral cytopathic effect could be converged to an apoptosis after all.

Introduction

JEV is a prototypic member of the family *Flaviviridae.* JEV genome is a linear, single-stranded RNA of approximately 11 kb in length with positive polarity, which has an open reading frame (ORF) more than 10 kb encoding a polyprotein. In the virus-infected cells the polyprotein is processed by cellular and viral proteases into structural proteins, capsid (C), membrane precursor (prM), and envelope (E), and nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (reviewed in Chambers *et al.*,1990). JEV is the causative agent of a mosquito-borne encephalitis in wide area of Asia including India (Umenai *et al.*,1985) and the virus is transmitted to human via persistently infected mosquito vectors. Although the virus is known to cause only acute infection, there were reports that some of the survivors from acute encephalitis showed neurological sequelae (Gourie-Devi, 1984), latent infection in peripheral mononuclear cells, and recurrence of the disease (Sharma et al., 1991).

DI particles are often produced in serial high-multiplicity passages of the standard virus. These particles retain only a portion of the standard viral genome which usually consists essential signals for genome replication and encapsidation and usually lack part or most of viral protein-coding region. Defectiveness of the genome makes DI particles unable to replicate in the absence of standard virus (Huang & Baltimore, 1970). Therefore defective genome must amplifies at the expense of co-replicating standard virus and this type of infection eventually leads reduced cytopathic effect (CPE) due to the interfering activity of DI particles. Thus, it has long been conceived that DI particles may be responsible for the establishment and maintenance of persistent infections.

Persistent infections of flavivirus including yellow fever (Monath *et al.*, 1971), St. Louis encephalitis (Slavin *et al.*, 1943), West Nile (Brinton *et al.*, 1982), and MVE viruses (Poidinger *et al.*, 1991) has been described. Recently, Lancaster *et al.* reported that DI RNA of MVE was produced and molecularly characterized in the persistent infection on Vero cells (1998). Like other flavivirus, persistent infection of JEV in several cultured cell lines as well as in experimental animal has been well documented (Mathur et al. 1986; Schmaljohn and Blair, 1977; Shaha and Gadkari, 1987; Chen et al., 1996, Liao et al., 1998). Innate resistance of certain cell lines (Schmaljohn and Blair, 1979), abnormal NS1 expression of the virus (Chen et al., 1996), and an anti-apoptotic effect of cellular *bcl-2* (Liao et al., 1998) in a cultured system were described as probable causes of JEV persistence even in the absence of any detectable DI virus (Chen et al., 1996). Although possible involvement of DI particles in JEV persistence was suggested (Schmaljohn and Blair, 1979) but neither has the molecular nor the biochemical characterization of the DI particle in conjunction with the viral persistence been made.

Therefore, we intended to produce DI particles in JEV infection in vitro and characterize them at molecular level. We then questioned whether the DI virus plays any role in JEV persistent infection. We also established JEV persistent infections with the cells survived acute infections. Characteristic features of the cells with viral persistence and the nature of the DI virus co-replication with helper virus were analyzed.

Materials and Methods

Cells and virus. Vero, BHK-21, and SW13 cells were grown and maintained in M199, DMEM, or Eagle's MEM. The media were supplemented with 5% fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine. JEV Nakayama and K94P05 were used in this study and standard virus stocks were obtained from the national collection of Korean National Institute of Health at Seoul, Korea. Strain Nakayama has been used for Japanese encephalitis inactivated vaccine preparation in Korea and strain K94P05 is a Korean isolate and it was obtained from a pool of engorged *Culex triaeniorhynchus* captured at Wando in Korea, 1994. Both virus stocks were obtained as a 10% homogenate of infected suckling mouse brain. The viruses were plaque-cloned twice and propagated in BHK-21 cells at multiplicity of infection (m.o.i.) of 0.1 in order to prepare DI-free standard virus stock.

Virion plaque assay. BHK-21 cells in 35 mm or 60 mm tissue culture plate was incubated with 250 $\mu\ell$ of serially diluted virus inoculum for 90 min. The cells were then overlaid with 1% agarose-containing media. After 3 days incubation at 37℃, cells were fixed with 10% formaldehyde following 0.1% crystal violet staining in order to visualize plaques.

Undiluted serial virus passage. Standard JEV strains Nakayama and K94P05 were passaged serially on fresh cells in 60 mm plate at an initial m.o.i. of 5 plaque forming unit (p.f.u.). Cell culture supernatant was harvested at 72 h postinfection (p.i.), and then undiluted supernatant of 250 $\mu\ell$ (1/20 of the harvest) was used to inoculate freshly prepared Vero cells.

Intracellular RNA extraction and Northern blotting. Cells in 60 mm tissue culture plate was infected with the virus and the cell culture supernatant was removed at 30 h p.i. About 0.5 ml RNA extraction buffer (50 mM sodium acetate [pH 5.0], 0.5% SDS, 1 mM EDTA, protease K [50 μg/ml]) per plate was added and the plate was then kept at room temperature for an hour. An equal amount of Tris-EDTA (pH 8.0) buffer-saturated phenol (pH 6.7) was added to the cell lysate, mixed thoroughly by vortex, and the aqueous phase was harvested by incubating at 56℃ for 5 min to remove protein fraction. After phenol/chloroform extraction, DNase I was treated to remove chromosomal DNA and the RNA fraction was collected by ethanol precipitation. RNA isolate was separated and transferred to a nitrocellulose membrane. A cDNA fragment of JEV Nakayama NS5/3'-untranslated region sequence was utilized as a template to prepare [α-^{32}P] dATP-labeled probe. The primers used in this study were designed mostly based on the JEV JaOArS982 sequence (Sumiyoshi *et al.*, 1987). The membrane blot was prehybridized for 3 h at 65℃ in hybridization buffer (0.25 M Na_2HPO_4 [pH7.2], 7% SDS) and hybridized for overnight under the same condition in the presence of the ^{32}P-labeled cDNA probe. The membrane blot was then washed and air-dried, and autoradiographed to Kodak XAR-5 film at -70℃.

RT-PCR and cloning. In order to localize the exact deletion site of the DI RNA, the intracellular RNA extract from cells infected with a DI particle-containing stock of JEV was subjected to RT-PCR. Two primer pairs used in cDNA synthesis, each specifies a 5'-half and a 3'-half of the JEV genome, were as follows; primers for 5'-half binds nucleotides 1-32 and binds 6433-6462; primers for 3'-half binds 4608-4635 and binds 10932-10963. For reverse transcription, RNA extract was heat-denatured for 2 min at 95℃ and chilled on ice for 5 min in the presence of 100 pM primer. For PCR, 1/5 of cDNA from the RT reaction was

incubated with 30 μℓ of PCR mixture with 50 pM of corresponding primers for 2 min at 95℃ and then following amplification step was carried out for 30 cycles (95℃ for 40 sec, 68℃ for 40 sec, and 72℃ for 8 min).

Confirming the deleted region was located within 5′-half of the virus genome, shorter cDNA fragments for plasmid cloning in order to facilitate sequence determination of the deletion site were produced by a second-round nested PCR with the primers binding to nucleotides 351-376 within C, and either a primer binding to 3696-3720 within NS2A or a primer binding to 4663-4687 within NS3 ORFs. About 1/30 of primary PCR product with 50 pM of each corresponding primers was subjected to 30 cycles of amplification (94℃ for 40 sec, 55℃ for 40 sec, 72℃ for 4 min). Sequence of the nested PCR product was determined directly or after cloning of the product onto a pGEM-T vector plasmid.

To identify negative-sense RNA in RI or RF of DI RNA, a carefully designed RT-PCR was employed. In brief, freshly prepared cells were infected with the DI particle-containing virus stocks harvested at passage 25 in undiluted serial passages. Total intracellular RNA was extracted and the cDNA of the negative-sense viral RNA was synthesized with a primer binding to the nucleotides 39-62 of negative-sense. The cDNA product was then incubated at 95℃ for 5 min to inactivate reverse transcriptase activity and chilled on ice. For PCR, 1/10 of the cDNA product with 50 pM each of two primers targeting nucleotides 39-62 and 5644-5666. In order to ascertain the detection specificity of the RT-PCR for negative-strand DI RNA a second-round PCR was carried out with two nested primers targeting nucleotides 351-376 and 4663-4687 for Nakayama or 4664-4687 for K94P05. PCR product was separated on 1.2% agarose gel electrophoresis and visualized by EtBr staining.

Cloning of persistently infected cells. After survived cells from acute infections were formed a monolayer, they were reinoculated with 10 m.o.i. of DI-free JEV. Survived cell fraction from the repeated virus infection was further incubated until the cell formed a monolayer. Then the cells were trypsinized and resuspended in M199 media, diluted to the range of 10 cells/mℓ, and 100 μℓ of diluted cell suspension per well was seeded into a 96-well microtiter plate. Each well was microscopically observed daily for the formation of cell colonies. Stabilized cells were regularly subcultured at 3 to 4 days interval for 7 months without reinfection.

Indirect immunofluorescence assay. Cells were grown on coverslips with or without virus infection. At 24 h p.i., cells were washed and fixed by immersion in absolute acetone for 30 sec at -20℃ followed by immediate air-dry. After blocking with 5% BSA anti-E antibody was incubated with the fixed cells. Cells were then fluorescein-labeled by incubation with FITC-conjugated anti-mouse IgG and the cells were mounted on slideglass by *p*-phenylenediamine mounting solution. The cells were observed and photographed under a fluorescent microscope at the magnification of ×400.

Superinfection experiment. Homologous superinfection was performed by cross-infection of fresh or persistently infected cells either with the DI-free standard stocks of JEV Nakayama or K94P05 at an m.o.i. of 1. The fresh or persistently infected cells were also subjected to heterologous superinfection with poliovirus 3, echovirus 11, and coxsackievirus B5 at an m.o.i. of 1. Each cell plate was daily examined from day 1 to day 5 after virus infection and the number of viable cells was calculated by trypan-blue dye exclusion.

Results and Discussion

Characteristics of JEV in serial undiluted passages. Naive BHK-21, Vero, and SW13 cells on 60 mm cell culture plate were infected with either DI-free JEV Nakayama or K94P05, at an m.o.i. of 5. The infected cell culture supernatant was harvested at 72 h p.i. and serially passaged up to 20 times on freshly prepared cells without m.o.i. adjustment. The virus titers of the supernatant from each infection during the serial undiluted passages were determined by plaque assay. Infectious virus titers of JEV Nakayama on Vero cells dropped down only after the first or second passage and then a cyclic pattern of virus yields was exhibited (Fig. 1A). The reduced virus titers in the beginning were somewhat recovered at the 6th passage and a fluctuation of the virus titers was continued thereafter. While 8 to 9 $\log_{10}$ p.f.u./ml of Nakayama can be obtained as an average titer in acute infections on Vero cells, the virus titers during the undiluted serial passages recorded at 100- to 1000-fold lower than average. In the case of K94P05, the average virus titer obtainable in Vero cell by acute infections was about 7 to 8 $\log_{10}$ p.f.u./ml, slightly lower than that of Nakayama. Neither was a noticeable reduction nor the fluctuation in viral yields as shown in the undiluted serial passages of Nakayama observable (Fig. 1B). The virus titers at each passage were remained relatively higher than those of Nakayama although they recorded gradually lowered value in the second half of the passages.

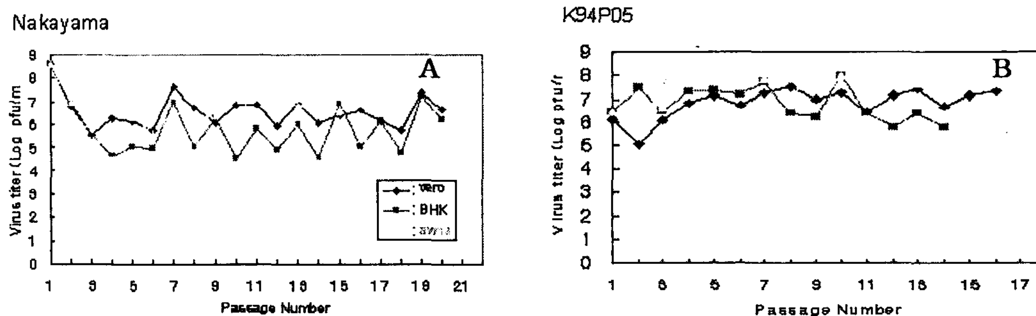

Fig. 1. Titration of serial undiluted passages of JEV strains Nakayama (A) and K94P05 (B) in BHK-21, Vero, and SW13 cells.

In an acute infection of both JEV strains on Vero cells, most of the cells are lysed and develop apparent CPE by the virus infection at 72 h p.i. However, the viable Vero cells devoid of the cytolysis in acute infections during the serial passages were began to appear between P5 and P6 and increased in their number gradually thereafter. The number of survived cells in the P10 infection of both Nakayama and K94P05 was estimated to be more than 40% of the initial cell number and it reached up to 70% as serial virus passage was progressed. The cells survived acute infections were indistinguishable in their morphology, but they grew much slower in comparison with that of naive cells.

Another interesting feature observed during the undiluted serial virus passages was the change in virus plaque morphology in terms of its size. Average virus plaque with the standard JEV sizes around 3 to 4 mm in diameter at 72 h p.i. in routine plaque assay on

BHK-21 cells. However, much smaller plaques, ranging from 1.5 to 2.2 mm in diameter, was observable in the titration of P4 to P5 samples and the average plaque size of after P5 virus samples appeared less than 1 mm in diameter (Table 1). Interestingly enough, the emergence of smaller plaques seemed almost coincide with the appearance of survived cells in the acute infection of undiluted serially passaged JEV suggesting that DI generation may predispose the infected cells a certain condition necessary for establishment of viral persistence.

Table 1. Characteristics of plaque morphology mutants of JEV derived from undiluted serial passages.

	Clone I		*Clone II*		*Clone III*	
PJ-Cn	***Size***	***Titer***	***Size***	***Titer***	***Size***	***Titer***
clone1	**None**	**None**	**1.0mm**	**$4.3x10^6$**	**1.0mm**	**$1.7x10^6$**
clone2	**None**	**None**	**1.5mm**	**$5.6x10^6$**	**1.5～2mm**	**$3.5x10^6$**
clone3	**None**	**None**	**2.5mm**	**$4.2x10^6$**	**1.1～2mm**	**$6.0x10^6$**
clone4	**None**	**None**	**1.8～2.5mm**	**$8.4x10^6$**	**2.5mm**	**$3.7x10^6$**
clone5	**None**	**None**	**1.5～2.0mm**	**$2.4x10^6$**	**1.1～1.6mm**	**$4.4x10^6$**
clone6	**None**	**None**	**1.0mm**	**$5.7x10^4$**	**1.0mm**	**$7.3x10^5$**
clone7	**None**	**None**	**1.5～2.0mm**	**$4.4x10^6$**	**1.8～2mm**	**$1.0x10^7$**
clone8	**None**	**None**	**0.9～1.1mm**	**$6.3x10^6$**	**0.9mm**	**$4.3x10^5$**
clone9	**None**	**None**	**1 ～2.1mm**	**$3.6x10^6$**	**1.5～2mm**	**$1.1x10^6$**
clone10	-	-	-	-	-	-
clone11	**None**	**None**	**1.2～1.5mm**	**$4.0x10^6$**	**1.0mm**	**$1.9x10^6$**
clone12	**None**	**None**	**1.0～1.5mm**	**$6.9x10^4$**	**1～1.2mm**	**$3.6x10^6$**

(Small) group I :clone 1, 6, 8, 11, 12; (Medium) " II: clone 2, 3, 5 9
(Large) " III: clone 4, 7; @Wild type plque size ⇒ 3～4 mm (diameter)
@JEV mutants obtained from 15 th serial passaged Vero cells that persistantly infected by the strain Nakayama. Several mutants of plaque morphology released from persistantly infected and stabilzed cells were plaque-cloned three consecutive times.

Defective viral RNA in undiluted serial passages. We analyzed the intracellular viral RNA from Vero cells by Northern hybridization in order to study whether any change in its replication was introduced in correspondence to the drastic CPE reduction and the emergence of smaller plaque-forming viruses during the passages. Naive Vero cells were infected with the Nakayama or K94P05 virus inocula obtained from each passage step and the total intracellular RNA was subjected to Northern hybridization by using ^{32}P-labeled cDNA probe specific to NS5 and 3′-UTR of JEV Nakayama (Fig. 2). Subgenomic size of virus-specific RNA species, estimated about 8.4 kb in length, were observed at rather early passage numbers in addition to the virus genomic RNA (11 kb) and it was detected continuously until the end of the passages (Fig. 2A and 2B). Since flaviviruses do not transcribe subgenomic viral RNA in their replication procedures these subgenomic size viral RNA were believed to be a type of homologous defective RNA. The defective viral RNA species in both JEV strains soon began to accumulate efficiently at the expense of the standard virus RNA replication and the relative quantity of genomic and subgenomic RNA seemed off-balanced on occasion in favor of defective viral RNA (Fig. 2A, lanes p10-p14, p22-24; 2B, lanes p9-p17).

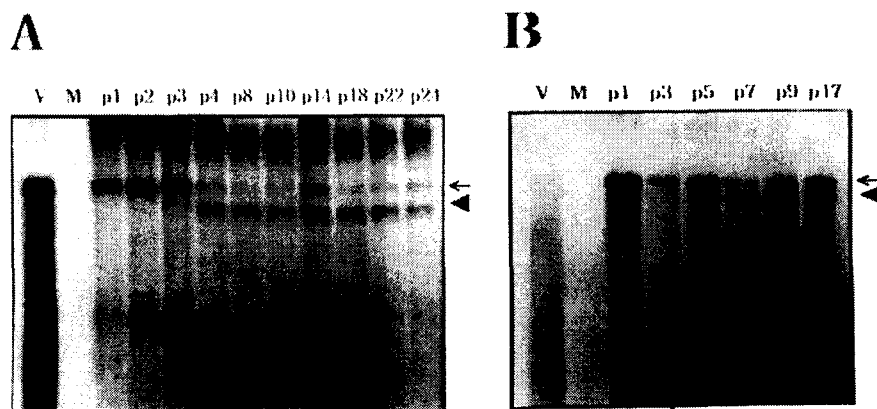

Fig. 2. Northern blotting of intracellular RNA from the cells infected with JEV Nakayama and K94P05. Cells were infected with virus inocula obtained from each passage during undiluted serial passages and intracellular RNA was isolated at 40 h p.i. and subjected to Northern blotting. JEV-specific RNA species in the Vero cells infected with Nakayama (A) or with K94P05 (B) were shown. Arrow and arrow head indicate JEV genomic RNA and putative DI RNA, respectively. V, virion RNA; M, mock infection.

Taken together, these observations lead us to a reasonable assumption that the defective viral RNA species generated in JEV undiluted serial passages may be a genuine homologous DI RNA, so that their efficient accumulation must play a significant role in the reduction of CPE in JEV acute infections by actively interfering the genome replication of helper virus.

Structural analysis of defective viral RNA. In order to localize the exact deletion site of the defective RNA, total RNA extracts of JEV-infected cells were subjected to RT-PCR. P15 sample was chosen for this purpose because the defective RNA accumulation was shown to be efficient among the series of acute infections. The PCR product for 5′ half was separated by nondenaturing agarose gel electrophoresis and a 6.4 kbp DNA fragment was obtained from both the full-length virion RNA and the total RNA extracted from cells infected with P15 sample. In addition, a fast-migrating fragment, estimated about 3.8 kbp, was identified in the PCR with the P15-infected cellular RNA which suggests the deletion happened within 5′ half of the virus genome. After second round nested PCR, the products were then subjected to either direct sequencing after gel-purification or cloned into pGEM-T vector. Direct sequencing result of the PCR products suggested that the deletion expands from prM to the middle of NS1 but the sequence was inconclusive in the vicinity of the deletion. Thus, the precise sequences at the deletion sites from selected clones were determined by automated sequencing. The sequencing results revealed the heterogeneity at the deletion sites as expected from the PCR product direct sequencing and there is a fairly large deletion encompassing the 3′ half of prM, entire E, and the 5′ half of NS1 ORFs (Fig. 3A) in all five clones. The sizes of the deletion were all different ranging 2652 nucleotides for the least and 2753 nucleotides for the most deleted ones. The continuum of translation at the deletion junctions of all the clones were shown to be in-frame despite of the deletion heterogeneity.

When the sequence data from the selected clones of the defective RNA from K94P05 were

compared to the JaOArS982 reference sequence, the deletion of the defective RNA was similar to that of Nakayama, affected the ORFs prM, E, and NS1 (Fig. 3). The positions of the deleted area were, again, different in all clones sequenced but the deletion heterogeneity, ranging 2442 nucleotides for the least and 2754 nucleotides for the most deleted ones, appeared rather broad than that of the defective RNA clones from Nakayama. All the deletions of these clones were also shown to be in-frame despite of their heterogeneity.

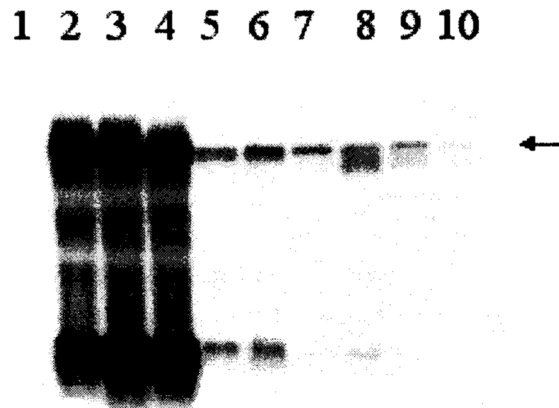

Fig. 3. Nother blot analysis of intracellular RNA from the SW13 cells infected with JEV Nakayama inocula obtained from the undiluted serial passages. Arrow indicates JEV genomic RNA.

Taken the sequence data together, a similar pattern of massive deletion was revealed in the defective RNA species derived from both undiluted serial passages of JEV Nakayama and K94P05. The deletion in all the sequenced clones encompasses the C-terminus of prM, entire E, and N-terminus of NS1 coding regions and this suggests that dispensible viral genes could be limited for being selected positively in a given circumstance. Heterogeneity at the junctions of the deleted sites implies that the defective viral RNA species produced in this study must be a mixture of which the exact site of the deletion may not necessarily be fixed although the possibility that certain type(s) of the defective RNA may exist as a fleeting majority can not be excluded. It is also tempting to assume that the translation continuity of rescued ORFs after deletion may be one of the requirements necessary for efficient replication of defective RNA since the putative ORFs of the defective RNA species in this study are all maintained in-frame.

Replication of defective viral RNA. To confirm whether the defective viral RNA species is indeed an independent replicon and not the truncated by-product of full-length virus genome, a RT-PCR to detect the negative-sense RNA template in replicative intermediate (RI) or replicative form (RF) RNA of the defective RNA was performed (White *et. al*, 1998). For this, naive BHK-21 cells were infected with either Nakayama or K94P05 virus inocula of P25 samples from undiluted serial passages on Vero cells and total intracellular RNA was isolated at 24, 32, and 42 h p.i. Since the PCR product of the negative-sense RNA was shown to accompany with some non-specific bands in the gel (data not shown), a second-round nested PCR was performed for higher specificity and the product was subjected

to the non-denaturing gel electrophoresis (Fig. 4).

The RT-PCR products of negative-sense defective RNA were demonstrated with the predicted size at all three different replication time points (Fig. 4A, lanes 7, 9, and 11). The RT-PCR product of the total RNA extracted at 42 h p.i. appeared much denser than two earlier time points, 24 h and 32 h p.i., and this indicates the replication activity of defective RNA was increasing by time after infection. The amount of RT-PCR products of positive-sense defective RNA, especially at earlier time points, was shown to be much greater than those of negative-sense defective RNA as expected (Fig. 4A, lanes 6, 8, and 10). In the same type of analysis with K94P05, as shown in Fig. 4B, the negative-sense defective RNA was clearly demonstrated and the amount of amplified products was again increasing by time after infection. It was noticeable that the RT-PCR products of both positive- and negative-sense defective RNA formed much less sharp bands than those of Nakayama in the gel electrophoresis and this corresponds the data from structural analysis in that the deletion sites of the K94P05 defective RNA were more heterogenous. Together with the data from Northern blot and structural analyses these results show unequivocally that the defective viral RNA, produced during the undiluted serial passages of JEV, is a biologically active DI RNA of JEV.

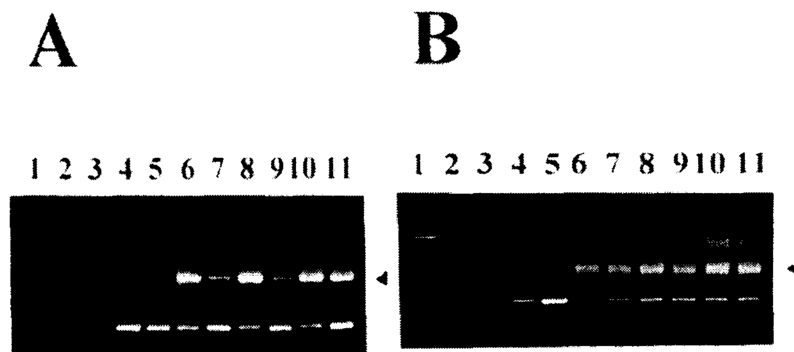

Fig. 4. RT-PCR products of JEV-specific negative-sense RNA from RF or RI forms. Vero cells were infected with P15 JEV Nakayama (A) or JEV K94P05 (B) inocula containing putative DI RNA and intracellular RNA was isolated at 24, 32, and 40 h p.i. Lane 2, mock infection; lane 3, virion RNA; lanes 4, 6, 8, 10, positive-sense RNA for DI-free wildtype JEV and P15 at 24, 32, and 40 h p.i., respectively; lanes5, 7, 9, and 11, negative-sense RNA for those in lanes 4, 6, 8, and 10.

Establishment of JEV persistent infection. JEV persistent infection was established by selecting the cells survived an acute infection. More than 90% of the cells showed severe CPE at 4 days p.i. and the cells were allowed to grow to confluence and reinfected with 10 m.o.i. of the standard JEV to eliminate possibly uninfected cells. Once the survived cells became confluent again, they were routinely subcultured and maintained for up to 6 months without virus reinfection. The cell culture supernatant was collected at each cell passage and subjected to titration. Morphology of these cells was indistinguishable from that of naive cells but they grew about 30% slower. These cells released low titer (10^3-10^4 PFU/ml) of infectious virus particle constantly without apparent CPE until the end of experiment which indicates the cells, or a majority of the cells at least, were infected persistently with JEV. In

comparison with an average plaque size of wildtype JEV (3-5 mm in diameter), that of the virus obtained from persistent infection was measured quite small ranging from 0.1 to 2 mm in diameter at the most (Table 1.).

An indirect immunofluorescence assay using a polyclonal antibody to the viral protein E was performed in order to ascertain the virus replication in persistently infected cells. The 15th subculture of the persistently infected cells was chosen for the assay. Most of the cells in JEV persistent infection revealed the virus E protein antigen (Fig. 5) regardless presence or absence of DI. One of the cloned BHK-21 which had ceased virus release after certain numbers of passage still demonstrated very faint signal but no virus-specific RNA was detectable by Northern blotting or by RT-PCR.

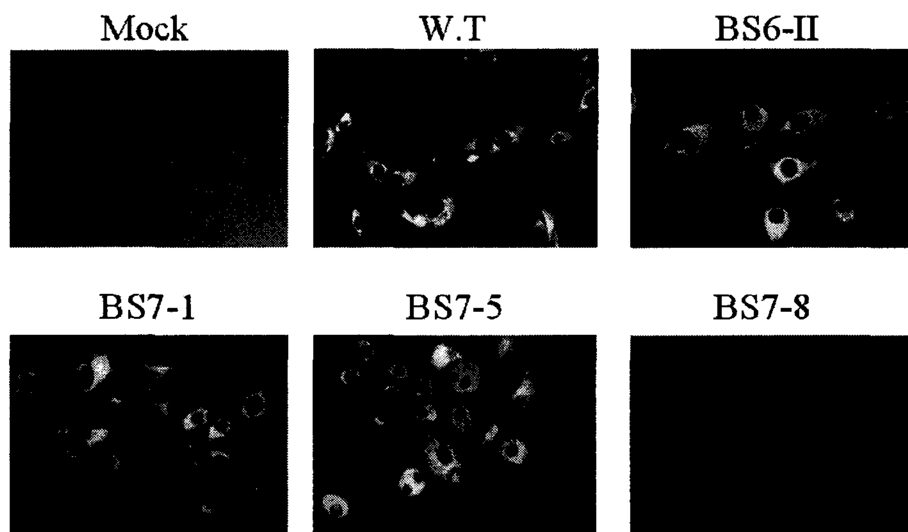

Fig. 5. Indirect immunofluorescence assay of BHK-21 cell clones persistently infected with JEV K94P05. Mouse polyclonal anti-E antibody was used for JEV-specific antigen detection.

Since an 'immunity' of the cells in viral persistence against homologous superinfection has often been regarded as one of the important characteristics, the cells in JEV persistence were subjected to superinfection experiment. Wildtype JEV for homologous superinfection, and poliovirus, coxsackievirus, and echovirus for heterologous superinfection were employed based on the permissiveness of Vero cells to these viruses. Almost all of the persistently infected cells was shown to be viable at this superinfection with either Nakayama or K94P05 throughout the study (Fig. 6). On the other hand, the heterologous superinfection with poliovirus, coxsackievirus, and echovirus, began to reveal severe CPE at 24 h p.i. in persistently infected cells and less than 5% of the cells barely maintained its viability at 5 days p.i. (Fig. 6). These results clearly demonstrate that cells with viral persistence were specifically resistant to the homologous superinfection and the phenomenon appeared not to be restricted to the given strain of JEV used in establishing viral persistence.

Based on the reports that apoptosis is the early and major cause of CPE in JEV infection (Chen *et. al,* 1997, 1998) DNA fragmentation, Northern blot for *bcl-2,* and FACS analyses were performed in order to study wether theses persistently infected cells are refractory to

the virus-induced apoptosis. At transciptional level, bcl-2 expression appeared not to be affected by virus infection in both naive and persistently infected clones (Fig. 7) being consistent with a previous observation. Chromosomal DNA was isolated after 32 h p.i. of wildtype JEV superinfection and subjected to agarose gel electrophoresis. All the persistently infected cell clones but one, revealed very limited DNA fragmentation in comparison to the naive cells with the virus infection (Fig. 8). In FACS analysis of the same cells a majority of the persistently infected cells was at G0/G1 stage and virus-induced apoptotic cell death was not apparent while most of the naive cells with JEV infection was at M/G2 stage.

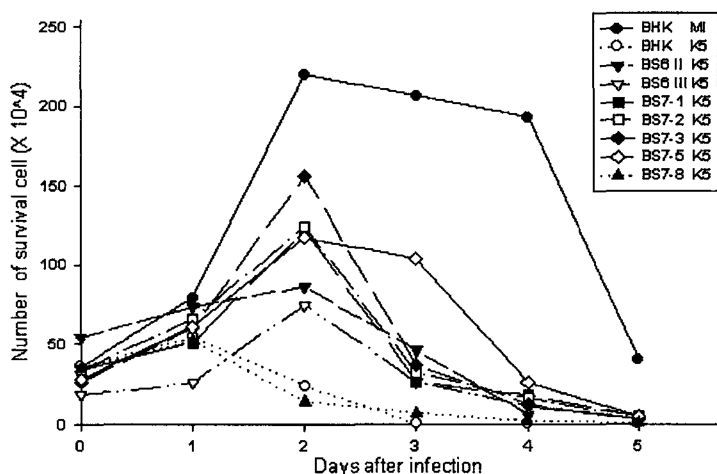

Fig. 6. Superinfection experiment for BHK-21 cell clones persistently infected with JEV K94P05. Number of viable cells after virus challenge was estimated by Trypan-blue dye exclusion ability. MI, mock infection; K5, wildtype JEV K94P05.

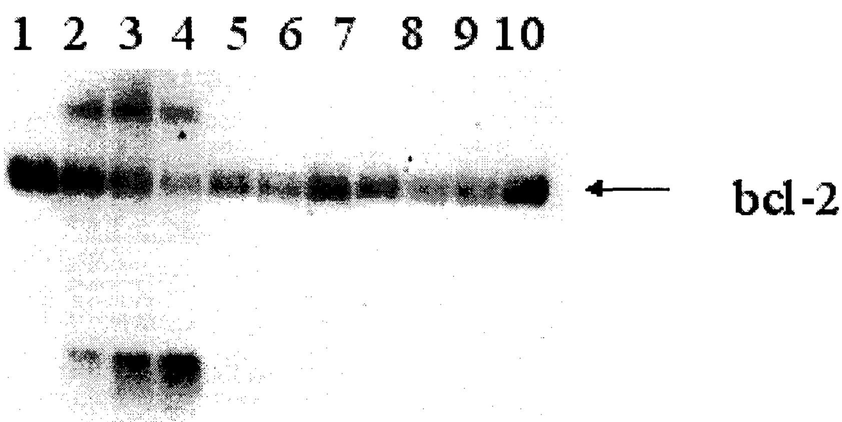

Fig. 7. Northern blot analysis of bcl-2 expression in BHK-21 cell clones persistently infected with K94P05. Lanes 1, mock infection; 2-4, wildtype JEV K94P05 at 24, 32, and 40 h p.i.; 5-9, BHK-21 persistently infected cell clones; 10, a cured BHK-21 clone, BS7-8, after persistent infection by JEV K94P05.

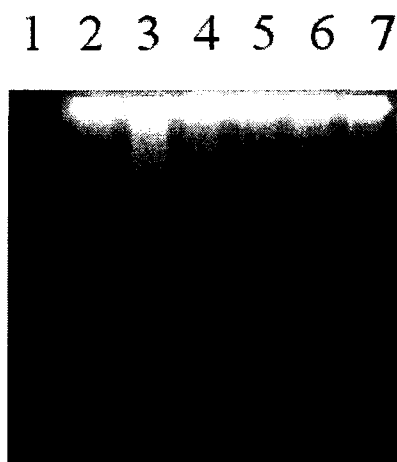

Fig. 8. DNA fragmentation profile in JEV-infected cells. Agarose gel electrophoresis of DNA extracted from naive or persistently infected BHK-21 cell clones. Lanes 2-3, naive BHK-21 wthout or with wildtype JEV infection (m.o.i of 5). Lanes 4-7, persistently infected BHK-21 cell clones challenged with K94P05 of JEV (m.o.i of 5). Low-molecular-weight DNA was isolated from the infected cells at 32h p.i. Lane 1, 100-bp DNA ladder.

DI RNA in JEV persistent infection. To question if the DI RNA were involved in the state of this viral persistence, a series of cell culture supernatant obtained during the maintenance of the persistently infected Vero cells was inoculated on to freshly prepared Vero cell plates and the intracellular RNA was subjected to Northern blotting using JEV-specific cDNA probe (Fig. 9). The JEV genomic RNA showed a continuous replication throughout the cell maintenance (Fig. 9, lanes pl-p60) although the genome synthesizing activity seemed unstable time to time. A significant amount of DI RNA was clearly demonstrated from the point of the persistently infected cell establishment (Fig. 9, lane pl) to the end of the cell maintenance (Fig. 9, lanes p15-p60), lasted for more than 6 months. The DI RNA, as shown in lanes p15, p25, and p60, frequently exhibited its enrichment at the expense of standard virus genome after certain period of cell maintenance. However, adventitious dominance of standard virus genome replication over DI RNA was also observed during the cell maintenance (Fig. 9, lane p55) which, in turn, suggests that there is some type of competition for replication between the genomic RNA and the DI RNA probably due to their continuous coexistence in the same space.

In conclusion, a defective RNA was generated and detected in the undiluted serial passages of JEV on Vero cells. The defective RNA contained a large deletion, about 2.7 kb, encompassing prM, E, and NS1 genes of JEV. Sequence analysis revealed the sequence heterogeneity of the defective RNA at the deletion sites and the level of heterogeneity was more significant in the defective RNA derived from one strain than the other. A continuum of ORF in most of the defective RNA species in this study was maintained in-frame after the deletion The presence of negative-sense RNA corresponding the size of the defective RNA strongly supports that the defective RNA is also biologically active and thus leads us to a reasonable assumption that the defective RNA should be the DI RNA of JEV. The DI RNA

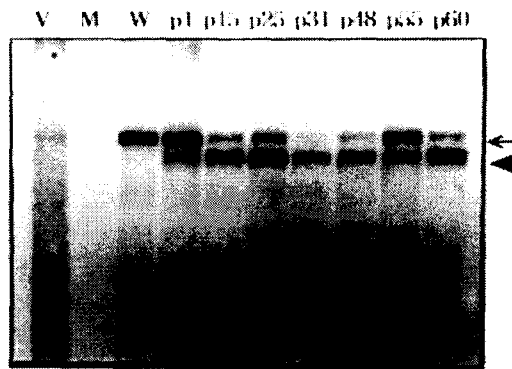

Fig. 9. Northern blot analysis for JEV-specific RNA in a persistently infected Vero cell clone. Cell culture supernatant harvested during cell maintenance which is persistently infected with JEV Nakayama and inoculated naive Vero cells. Intracellular RNA was isolated at 40 h p.i. V, virion RNA; M, mock infection; W, wildtype JEV Nakayama.

seemed competes for its replication to the genomic RNA of parental virus and may play an important role in establishment and maintenance of JEV persistence in Vero cells.

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