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Identification of Mutations That Occurred on the Genome of Japanese Encephalitis Virus During the Attenuation Process

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Abstract

The total nucleotide sequences of the genomes of two Japanese encephalitis virus (JEV) strains, the attenuated vaccine strain SA_{14}-14-2 and its parental virulent strain SA_{14}, were determined by using the molecular cloning technique. The sequence analysis revealed that both virion RNA molecules were 10,976 nucleotides long with 95 and 585 flanking bases at the 5′ and 3′ untranslated sequences, respectively. A single, long open reading frame spanning 10,296 nucleotides was observed to encode a polyprotein of 3432 amino acid residues. When these sequences were compared with each other, 57 nucleotide substitutions were found to be scattered all over the genome. Of these, 24 resulted in amino acid changes within viral proteins. Structural proteins C and E contain one and eight amino acid changes, respectively. Of the nonstructural proteins, NS1 contains three, NS2a two, NS2b two, NS3 four, NS4a one, NS4b one, and NS5 two amino acid substitutions. The 5′- and 3′-terminal untranslated regions contain one- and two-point mutations, respectively. These data and comparative studies with other JEV strain genomes provide a molecular basis for investigating attenuation mechanisms of JEV.

Introduction

Japanese encephalitis virus (JEV) is a member of the family Flaviviridae, which includes approximately 60 viruses, many of which are of major concern in human health. Flaviviruses are serologically related to each other and share a similar structural feature. The genome of this small envelope virus is a single, positive-stranded RNA of approximately 11 kb that is capped at the 5′ end and unpolyadenylylated at the 3′ end (1). The viral RNA has a single, long open reading frame (ORF) of approximately 10 kb for the synthesis of a viral polyprotein. The polyprotein is subsequently processed to form viral structural and nonstructural proteins (2–7). Studies on the sequences of RNA and of amino acids in viral proteins have provided a precise viral protein map (2,8–10). To date, complete nucleotide sequences of the genomes of two different JEV strains, JaOArS982 (6) and Beijing-1 (7), have been reported, and the gene order is C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5.

Japanese encephalitis (JE) is a widespread viral disease in East, Southeast, and South Asia. JEV that causes JE is transmitted in nature by mosquitoes that preferentially breed in rice fields. The geographic distribution of JE dramatically shifted between 1967 and 1970 (11). In Japan, Korea, and Taiwan, the morbidity rates of this disease have rapidly decreased since 1967. This regional decline has mainly been due to a combination of increased distribution of vaccine and altered agricultural practices. On the other hand, in Vietnam, Thailand, Nepal, and India, epidemic JE has been recognized from 1965 to 1978, and JE still continues to be a significant public health problem in these Asian countries.

A number of cases of JE also occur in most provinces in China (12). Since the revolution in 1949, many studies have been carried out on the epidemiology of JE and the development of the vaccines. Inactivated JE vaccines were prepared from infected primary hamster kidney cell cultures (PHK) (12). Live-attenuated JE vaccines were later prepared for the attainment of higher protection against this disease. The attenuated vaccine strain SA_{14}-14-2 was recognized to be most effective of these (13). This vaccine strain was derived from the virulent virus SA_{14} by multiple passages, mainly through PHK cells (12). In 1987, more than 500,000 children were inoculated with this live vaccine. The results indicated that the attenuated live vaccine was effective in promoting an immune response in recipients, with no side effects. Sometimes, seroconversion rates were more than 90% after administrating only one dose of the attenuated vaccine (13). The live vaccine SA_{14}-14-2 is now being used effectively to prevent JE in China. However, the mechanism that is responsible for the attenuation is totally unknown at present.

As a first effort to give an insight into the molecular basis for the biological differences between the virulent and attenuated JEV strains, we determined the total primary nucleotide sequences of the genomes of the virulent SA_{14} and attenuated SA_{14}-14-2 strains. We report here the complete 10,976-nucleotide sequence

of the JEV genomes and the mutation sites, which were identified by comparing the nucleotide sequences with each other.

Materials and Methods

Cells and viruses

BHK-21 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS) and 0.3% tryptose phosphate broth (TPB). Monolayer-cultured C6/36 were maintained at 27°C in MEM containing heat-inactivated 10% fetal calf serum (FCS) and seven nonessential amino acids at a concentration of 0.2 mM each (14).

JEV live attenuated vaccine strain SA_{14}-14-2 was isolated by Yu Yong-Xin et al. (15) according to the attenuation processes shown in Table 1. The parental virulent virus SA_{14} was obtained from a pool of larvae of *Culex pipiens* mosquitoes by 11 serial passages in mouse brain.

Plaque assays

To measure virus titers, monolayers of BHK-21 cells in 60-mm plastic dishes were washed twice with MEM containing 0.3% TPB, covered with 0.5 ml of a virus solution (a dilution of the virus stock), and kept at room temperature for 20 min. After incubation at 37°C for 20 min, the cells were washed twice with MEM with 0.3% TPB and then covered with the same medium containing 1% agarose and 5% CS. After 7 days of incubation at 37°C, plaques were visualized by staining cells with 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

Table 1. Processes of JEV attenuation

JEV	Attenuation process	Size of plaques[a]
SA_{14} (parent)		Large
$PHKC_{100}$	100 passages of SA_{14} in PHK cells	Large and small
Clone 12-1-7	3 plaque purifications of a small plaque variant of $PHKC_{100}$	Small
Clone 9-7	7 plaque purifications with 1 mouse intraperitoneal passage and 1 mouse subcutaneous passage of clone 12-1-7	Small
Clone 5-3	6 hamster oral passages of clone 9-7, and 2 plaque purifications	Small
Clone 14-2	5 suckling mouse subcutaneous passages followed by 2 plaque purifications	Small

[a] Plaque size was examined using LLC-MK2, chick embryo, or BHK-21 cells.

mide (MTT) (Sigma) in 0.15 M NaCl at 37°C for 1 hr or crystal violet, as previously described (see ref. 19). In some cases, African green monkey kidney cells were also employed to measure virus titers (16).

Virus growth

The seed viruses prepared by plaque purification of SA_{14} and SA_{14}-14-2 viruses in BHK-21 cells were inoculated to microcarrier-cultured C6/36 cells using Cytodex 1 (Pharmacia), and 3 ls of infected fluid were harvested on each day from the second to fifth day of the culture, as described by Sumiyoshi et al. (6), and the viruses derived from SA_{14} and SA_{14}-14-2 were designated SA(V) and SA(A), respectively, and used in this study.

Mouse neurovirulence test

Each mouse (ddY strain) was inoculated with 30 μl of virus solution intracerebrally. Mice were observed every 12 hr for clinical symptoms and death up to 21 days.

Preparation of virion RNA

The infected fluid (3 ls) was filtered through a paper (Toyo filter paper 24CM2) and centrifuged at 8000 rpm for 20 min at 4°C in a Hitachi RR10A rotor. The supernatant was added with polyethylene glycol 6000 and NaCl to final concentrations of 6% and 0.5 M, respectively, kept at 4°C overnight, and centrifuged at 8000 rpm for 20 min at 4°C. The pellet was suspended in 30 ml STE buffer containing 0.1 M NaCl; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; and centrifuged at 15,000 rpm for 15 min at 4°C in a Beckman type 45Ti rotor. JEV was pelleted from the supernatant by centrifugation at 30,000 rpm for 3 hr at 4°C in a Beckman type 45Ti rotor. The pellet was suspended in a small volume of STE buffer and extracted with $NaDodSO_4$-phenol. Virion RNA was precipitated with ethanol from the aqueous phase.

Molecular cloning and sequencing

Single-stranded cDNA of the JEV genome was synthesized using avian myeloblastosis virus reverse transcriptase and a 16-nucleotide synthetic DNA primer corresponding to the consensus sequence, including the terminal base at the 3' end of JEV genome (6,7). Double-stranded cDNA was then prepared according to the method of Gubler and Hoffman (17) using a cDNA synthesis kit (Amer-

sham). The cDNAs were treated with T4 DNA polymerase to make blunt ends, digested with appropriate restriction enzymes, and then ligated to cloning vector pUC18 or pUC19, which had been treated with the same restriction enzymes as used for digesting cDNAs and/or *Sma*I or *Hin*cII for blunt-end ligations. *Escherichia coli* strain SCS-1 was transformed with the recombinant plasmids, and transformants carrying JEV cDNAs were selected by hybridization using various cDNAs of JaOArS982 (6) and Beijing-1 (7) as probes.

Cloned DNAs were subcloned into the appropriate sites of pUC118 or pUC119, and the nucleotide sequences were determined by the dideoxy termination method (18) using the Sequenase sequencing kit (United States Biochemical Cooperation) with M13 primers or synthetic oligonucleotides corresponding to the appropriate sites of the genome. In some cases, the nucleotide sequences of virion RNAs were directly determined using synthetic oligonucleotides as primers, according to the dideoxy method reported previously (19).

Results

JEV strains and their biological differences

The passage history to obtain JE vaccine strain SA_{14}-14-2 is shown in Table 1. When the parent SA_{14} that showed the large-plaque phenotype was passaged 100 times in PHK cells, small-plaque variants were generated in the virus preparation. After nine plaque purifications in primary-cultured chick embryo (CE) cells and two plaque purifications in PHK cells of a small-plaque variant, an attenuated clone SA_{14}-5-3 was obtained. Five passages of the clone SA_{14}-5-3 in subcutaneous tissue of suckling mice followed by two plaque purifications in PHK cells resulted in the selection of the SA_{14}-14-2 strain. The latter clone was immunogenically superior to the former clone, SA_{14}-5-3 (13). Thus, the attenuated JE vaccine SA_{14}-14-2 is a JEV strain that is adapted to PHK cells, and therefore it grows much more efficiently than the parental virulent SA_{14} in PHK cells (data not shown). However, the SA_{14}-14-2 strain displays smaller plaques in LLC-MK2, CE, and BHK-21 cells than the parent SA_{14} virus (Table 1, Fig. 1).

Both the wild and attenuated strains were plaque purified in BHK cells, and then viruses were grown in insect cells (C6/36) for use in this study. Here, viruses that were derived from SA_{14} and SA_{14}-14-2 viruses are designated SA(V) and SA(A), respectively, as described in Materials and Methods. The size of the plaques of these viruses displayed on BHK-21 monolayer cells were examined (Fig. 1). The result suggests that the plaque-size phenotypes did not change during plaque purification in BHK-21 cells and propagation in C6/36 cells. Furthermore, mouse neurovirulence tests on plaque-purified viruses were performed by intracerebrally injecting the viruses into suckling, 2-week-old and 4-week-old ddY mice to confirm that their neurovirulence phenotypes were also unchanged (Table 2). One PFU of virus SA(V) seemed to be enough for 4-week-old mice to die of

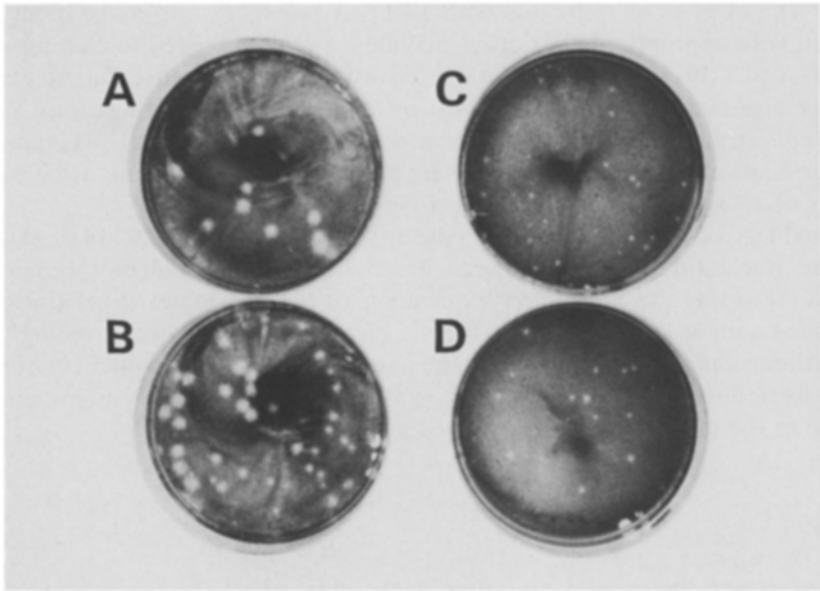

Fig. 1. Plaques displayed on monolayers of BHK-21 cells. The virulent SA_{14} virus and attenuated SA_{14}-14-2 were plaque purified in BHK-21 cells and propagated in C6/36 cells, as described in Materials and Methods. Viruses thus obtained were designated SA(V) and SA(A), respectively, in this study. Plaques of viruses SA_{14} (A), SA(V) (B), SA_{14}-14-2 (C), and SA(A) (D) displayed on BHK-21 cells were stained with crystal violet and are shown in this figure.

Table 2. Mouse neurovirulence tests with intracerebral route

Amount of virus (PFU)	SA(V)	SA(A)			
				Suckling	
	4 week old	4 week old	2 week old	1st exp.[c]	2nd exp.
10^7	0/10[a]	ND[b]	ND	ND	ND
10^6	0/10	10/10	10/10	0/31	0/16
10^5	0/10	ND	10/10	ND	0/8
10^4	0/10	ND	10/10	ND	0/8
10^3	0/10	ND	ND	ND	ND
10^2	0/10	ND	ND	ND	ND
10^1	0/10	ND	ND	ND	ND
10^0	5/10	ND	ND	ND	ND
10^{-1}	10/10	ND	ND	ND	ND

[a] Number of survival mice/number of mice inoculated with virus.
[b] Experiment was not done.
[c] Experiment.

encephalitis, while 2- or 4-week-old mice injected with 10^6 PFU of virus SA(A) did not develop any clinical symptoms, and only suckling mice were sensitive to virus SA(A) (Table 2). Thus, viruses SA(V) and SA(A) appear to have biological properties that are characteristic of viruses SA_{14} and SA_{14}-14-2, respectively.

Cloning and sequencing experiments

Double-stranded cDNAs were prepared from virion RNAs, as described in Materials and Methods, and digested with appropriate restriction enzymes selected by taking the previously determined nucleotide sequences of JEV genomes (6,7) into consideration. DNA fragments thus obtained are joined to cloning vector pUC18 or pUC19 and replicated in competent *E. coli*. Map positions of the JEV-cDNAs were determined by hybridization with specific DNA probes prepared from cDNAs of other JEV genomes (6,7) or by distribution of cleavage sites of restriction enzymes. Thus all cDNA clones selected were physically mapped and the main clones used for sequencing are shown in Fig. 2. Assembly of cDNA clones covers all of the genome except the short stretch at the 5′ terminal region.

Approximately 50 kinds of synthetic DNA primers were synthesized with each 200-nucleotide distance for sequencing cDNAs and virion RNAs. Primer exten-

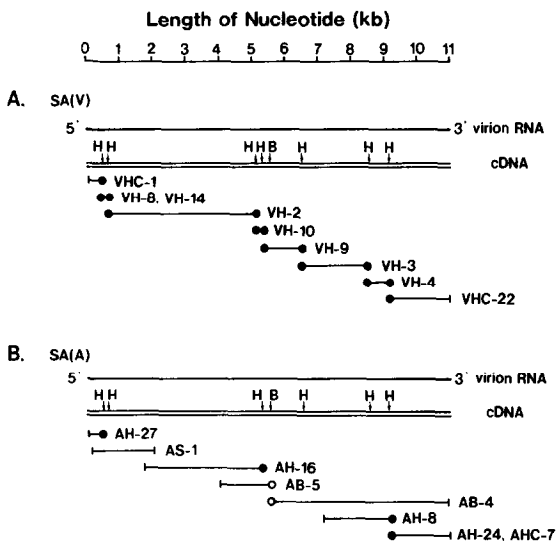

Fig. 2. (A) Restriction cleavage sites on SA(V) and (B) SA(A) cDNAs and the cDNA clones obtained. H and B represent restriction cleavage sites for *Hin*dIII and *Bam*H1, respectively. *Hin*dIII and *Bam*H1 cleavage sites used for molecular cloning are indicated by closed circles and open circles, respectively, at the ends of cDNA clones. Other ends of cDNAs appear to be connected with vector DNA by blunt-end ligations. The length of nucleotides from the corresponding 5′ terminus of the JEV genome is shown in kilobases at the top of the figure.

sion reactions were carried out to determine the nucleotide sequences of cDNAs and RNAs, as described in Materials and Methods. The primer extension method was also employed for sequencing the genome region close to the 5′ terminus, where sequence was not contained in the cloned cDNAs. Nucleotide sequences thus obtained were combined with the aid of reference nucleotide sequences of the genomes of JaOArS982 (6) and the Beijing-1 (7) strains. Thus, the total nucleotide sequences of both the SA(V) and SA(A) genomes were determined, except for two nucleotides at the 5′ terminus, which were difficult to identify by the primer extension method only (6,7), and 16 nucleotides at the 3′ terminus whose sequence was included in the synthetic oligonucleotide primer used for the reverse transcription. The nucleotide sequence of the SA(A) genome and the deduced amino acid sequence are shown in Fig. 3. A single ORF spanning 10,296 nucleotides encoding a polyprotein of 3432 amino acid residues is observed, flanked by 95 bases at the 5′ end and 585 bases at the 3′ end. The genome organization indicated in Fig. 3 follows those of the yellow fever virus (YFV) genome reported by Rice et al. (2) and Chambers et al. (10), and the Kunjin virus genome reported by Speight et al. (8,9).

Comparative sequence analysis

The nucleotide sequence of the attenuated strain SA(A) was compared with that of the parent SA(V) by computer analysis, and genetic variations that occurred during attenuation processes were identified. As a result, 57 nucleotide substitutions were found to disperse all over the genome (Table 3). Of these, 24 are amino-acid changes and the rest are silent mutations. As shown in Table 3, many missense mutations are located in viral envelope protein E, although every viral protein, except prM, contains missense mutation(s). Many base substitutions in the genome regions encoding NS2a, NS4b, and a putative viral replicase NS5 occurred in the third letter position of the in-phase codons (Table 3). This resulted in a low frequency of amino acid changes in these coding regions. This result may indicate that conservation of amino acid sequences of these portions of the nonstructural proteins is more important for JEV replication than conservation of those of the viral envelope protein.

Comparative sequence study was also performed on nucleotide sequences of four different JEV strains, JaOArS982, Beijing-1, SA(V), and SA(A). It should

Fig. 3. Nucleotide sequence of the genome of SA(A) and the predicted amino acid sequences of viral polyproteins. The RNA sequence deduced from the corresponding cDNA sequence is shown. The genome organization indicated here follows those of the YFV genome reported by Rice et al. (2) and Chambers et al. (10), and of the Kunjin virus genome by Speight et al. (8,9). Two bases at the 5′ terminus are not identified and follow the previously determined nucleotide sequence of JEV genomes (6,7). The sequence of 16 nucleotides at the 3′ terminus also follows the previous data (6,7), and synthetic oligonucleotide of this sequence was used as a primer for the synthesis of the cDNA, as described in Materials and Methods.

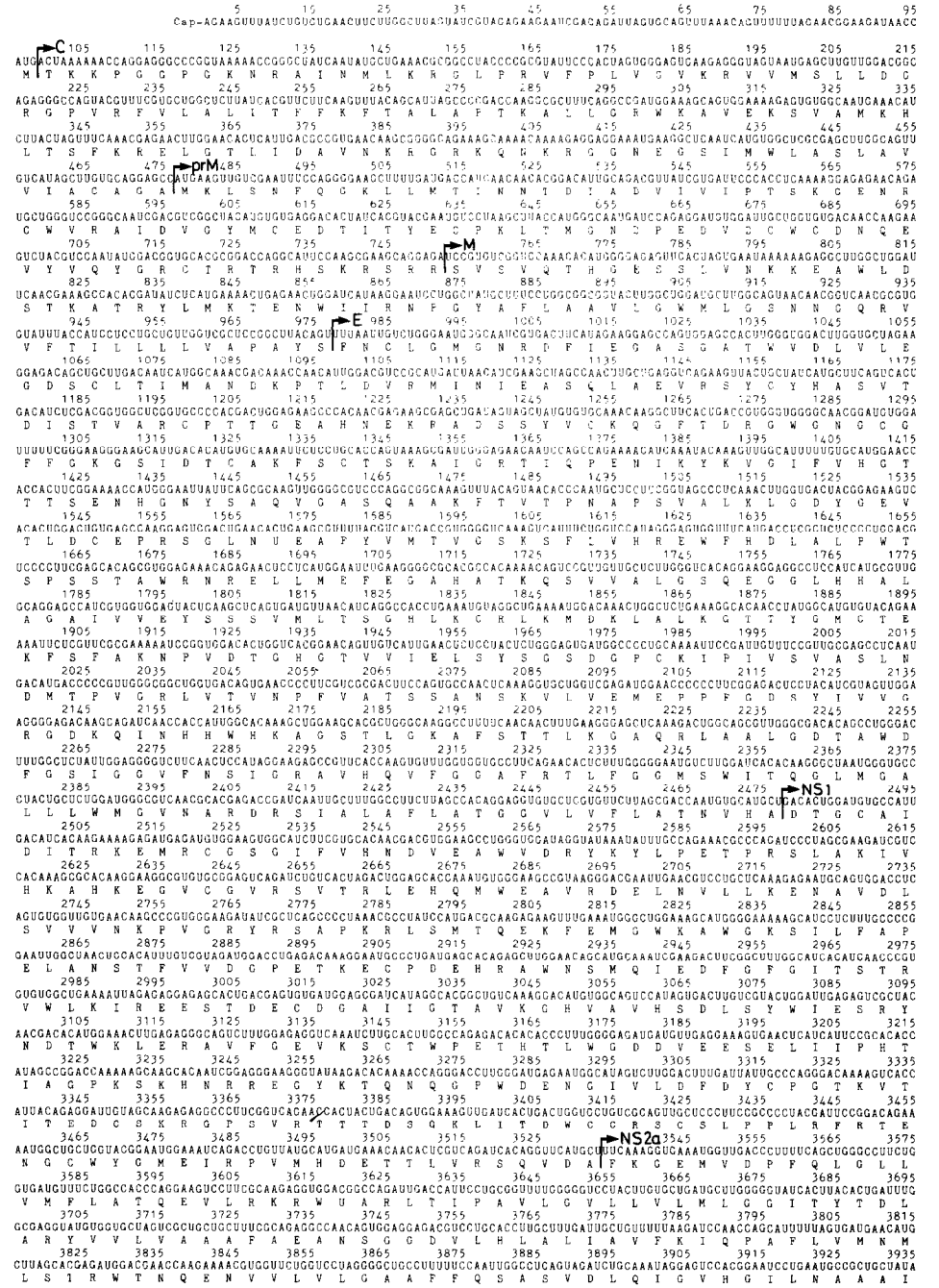
Cap-AGAAGUUUAUCUGUCUGAACUUCUUGGCUUAGUAUCGUACAGAAGAAUCGACACAUUAGUGCAGUUUAAACAGUUUUUAACAACGGAAGAUAACC
C
AUGACUAAAAAACCAGGAGGGCCCGGUAAAAACCGGGCGAUCAAUAUGCUGAAACGCGCCGUACCCCGCGUAUUCCCACUAGUGGGAGUGAAGAGGGUAGUAAUGAGCUUGUUGGACGGC
M T K K P G G P G K N R A I N M L K R G L P R V F P L V G V K R V V M S L L D G
R G P V R F V L A L I T F F K F T A L A P T K A L L G R W K A V E K S V A M K H
CUUACUAGUUUCAAACGAGAACUUGGAACACUCAUUGACGCCGUGAACAAGCGGGGCAGAAAGCAAACAAAAGAGGACGAAAUGAAGGCUCAAUCAUGUGGCUCGCGAGCUUCGCAGUU
L T S F K R E L G T L I D A V N K R G R K Q N K R G G N E G S I M W L A S L A V
prM
VIACAGA MKLSNFQGKLLMTINNTDIADVIVIPTSKGENR
M
VYVQYGRCIRTRHSKRSRSVSVQTHGESSLVNKKEAWLD
E
VFTILLLVAPAYSFNCLGMGNRDFIEGASGATWVDLVLE
GDSCLTIMANDKPTLDVRMINIEASQLAEVRSYCYHASVT
DISTVARCPTTGEAHNEKRADSSYVCKQGFTDRGWGNGCG
FFGKGSIDTCAKFSCTSKAIGRTIQPENIKYKVGIFVHGT
TTSENHGNYSAQVGASQAAKFTVTPNAPSVALKLGDYGEV
TLDCEPRSGLNTEAPYVMTVGSKSFLVHREWFHDLALPWT
SPSSTAWRNRELLMEFEGAHATKQSVVALGSQEGGLHHAL
AGAIVVEYSSSVMLTSGHLKCRLKMDKLALKGTTYGMCTE
KPSFAKNPVDTGHGTVVIELSYSGSDGPCKIPIVSVASLN
DMTPVGRLVTVNPFVATSSANSKVLVEMEPPFGDSYIVVG
RGDKQINHHWHKAGSTLGKAFSTTLKGAQRLAALGDTAWD
FGSIGGVFNSIGRAVHQVFGGAFRTLFGGMSWITQGLMGA
ELANSTFVVDGPETKECPDEHRAWNSMQIEDFGFGITSTR
VWLKIREESTDECDGAIIGTAVKGHVAVHSDLSYWIESRY
NDTWKLERAVFGEVKSCTWPETHTLWGDDVEESELIIPHT
IAGPKSKHNRREGYKTQNQGPWDENGIVLDFDYCPGTKVT
ITEDCSKRGPSVRTTTDSGKLITDWCCRSCSLPPLRFRTE
NS2a
NGCWYGMEIRPVVMHDETTLVRSQVDAFKGEMVDPFQLGLL
VMFLATQEVLRKRWTARLTIPAVLGVLLVLMLGGITYTDL
ARYVVLVAAAFAEANSGGDVLHLALIAVFKIQPAFLVMNM
LS1RWTNQENVVLVLGAAFFQSASVDLQIGVHGILNAAAI

Fig. 3.

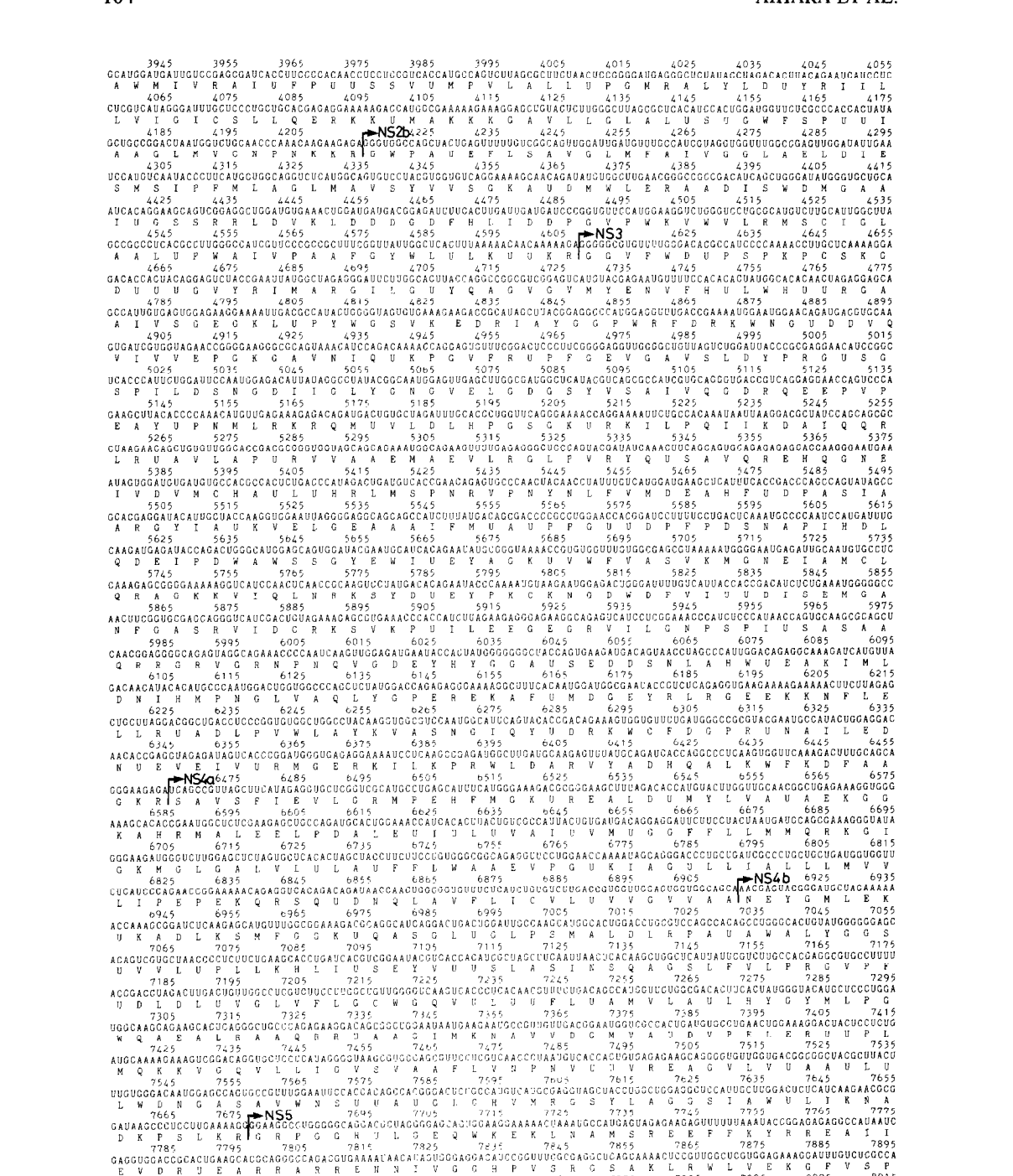

Fig. 3 (continued).

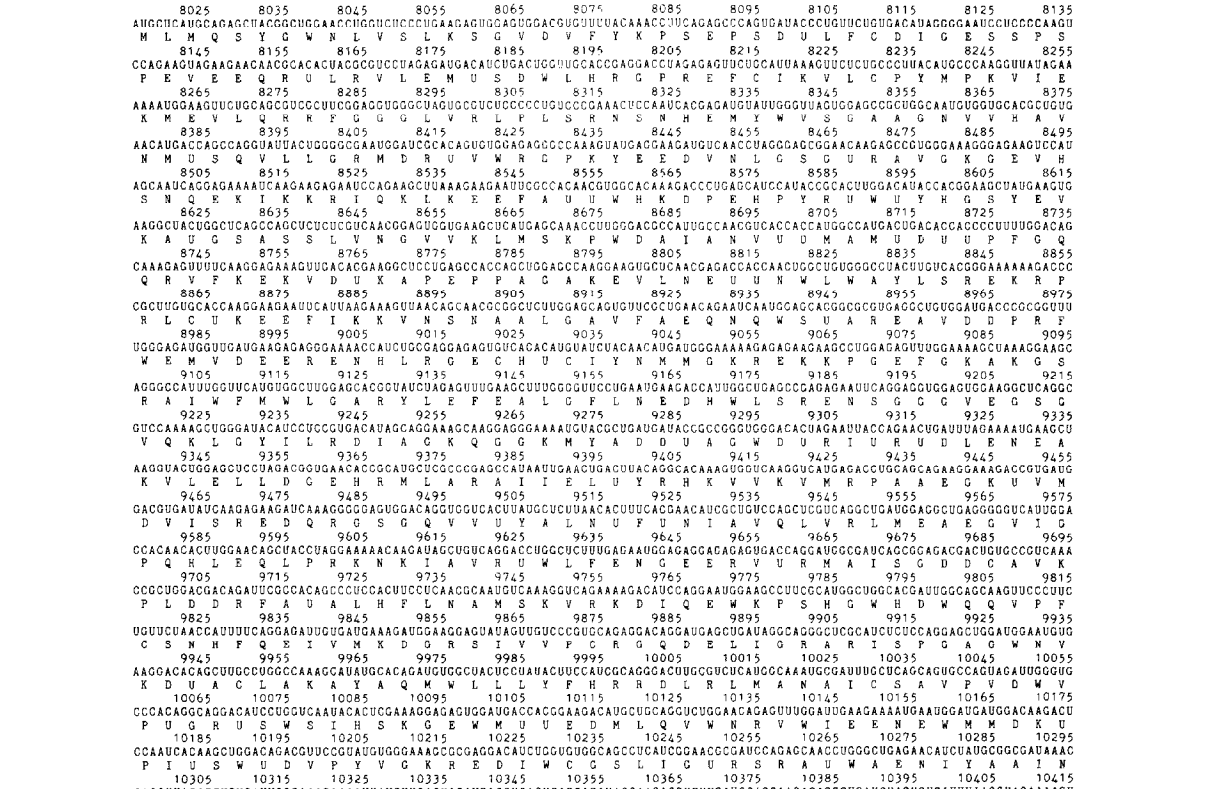

Fig. 3 (continued).

be noted that there are several wrong nucleotide sequences in a published genome sequence of Beijing-1 strain (7) because of the misreading of sequence ladders. The corrected nucleotide sequence of the Beijing-1 genome has already been sent to GenBank. As to the nucleotide sequence of the noncoding regions, highly homologous sequences are observed in both termini of the genome (Fig. 3 and refs. 6,7). Conservation of these nucleotide sequences must be very important for JEV replication, although the biological functions of these parts of the genome have not yet been elucidated. Differences in the primary structure of the coding

Table 3. Comparison of genome sequences between SA(V) and SA(A) strains

Substitutions					Substitutions				
Nucleotide position	Base		Amino acid		Nucleotide position	Base		Amino acid	
	SA(A)	SA(V)	SA(A)	SA(V)		SA(A)	SA(V)	SA(A)	SA(V)
39	A	T*[b]			4782 (1)	G	A	Val	Met*
292 (2)[a]	C	T	Ser	Leu*	4825 (2)	A	G	Lys	Arg
1061 (3)	C	T			4921 (2)	G	C	Gly	Ala*
1217 (3)	C	T			4922 (3)	C	T		
1296 (1)	T	C	Phe	Leu*	5634 (1)	T	A	Trp	Arg*
1389 (1)	A	G	Lys	Glu*	6008 (3)	T	C		
1503 (1)	G	A	Val	Ile*	6425 (3)	G	A		
1506 (1)	G	A	Ala	Thr*	6634 (2)	C	T	Thr	Ile*
1769 (3)	T	G	His	Gln*	6728 (3)	A	G		
1813 (2)	T	A	Met	Lys*	6944 (3)	G	A		
1921 (2)	T	C	Val	Ala*	7121 (3)	T	C		
2293 (2)	G	A	Arg	Lys*	7193 (3)	T	C		
2441 (3)	A	G			7227 (1)	G	A	Val	Ile*
2691 (1)	A	C			7655 (3)	G	T		
2843 (3)	C	T			7736 (3)	T	C		
3351 (1)	A	G	Ser	Gly*	8099 (3)	T	C		
3493 (2)	T	G	Met	Arg*	8276 (3)	T	C		
3528 (1)	C	G	His	Asp*	8394 (1)	T	C		
3539 (3)	A	T	Lys	Asn*	8832 (1)	T	C	Tyr	His*
3599 (3)	A	G			8882 (3)	T	A		
3652 (2)	T	C	Val	Ala*	8891 (3)	T	C		
3677 (3)	T	C			9688 (2)	C	T	Ala	Val*
3776 (3)	T	C			9695 (3)	A	G		
3801 (1)	T	C			9818 (3)	T	C		
3929 (3)	T	C			10046 (3)	A	G		
4106 (3)	G	A			10139 (3)	T	C		
4403 (3)	T	G	Asp	Glu*	10217 (3)	C	T		
4408 (2)	G	A	Gly	Asp*	10428	C	T*		
					10784	T	C		

[a] Numbers in parentheses represent positions of in-phase codons.

[b] Nucleotides in the untranslated sequences and amino acids in the translated sequence that are common in the virulent JaOArS982, Beijing-1, and SA(V) strains are indicated by asterisks.

Table 4. Genetic variation in the translated region among JEV strains

JEV	versus	JEV	Nucleotide substitutions (%)	Amino acid substitutions (%)	Missense mutations (%)
SA(A)		Beijing-1	2.75 (283)[a]	1.37 (47)[b]	16.6[c]
		JaOArS982	1.94 (200)	1.08 (37)	18.5
		SA(V)	0.52 (54)	0.07 (24)	44.4
SA(V)		Beijing-1	2.34 (241)	0.82 (28)	11.6
		JaOArS982	1.46 (150)	0.17 (18)	12.0
Beijing-1		JaOArS982	2.87 (296)	0.79 (27)	9.1

[a] Number of nucleotide substitutions observed is indicated in parenthesis.
[b] Number of amino acid substitutions observed is indicated in parenthesis.
[c] Ratio of missense mutations to the total nucleotide substitutions.

sequences among these four JEV strains were determined with the aid of a computer (Table 4). The data suggest that three virulent JEV strains that were independently isolated are genetically related to each other. The ratios of missense mutations to the total nucleotide substitutions between any two JEV strains were calculated and are shown in Table 4. Interestingly, the highest rate (44.4%) of missense mutations among those between any two strains of genomes was observed when the SA(V) and SA(A) genomes were compared with each other, although the genetic variation between these two strains was the smallest. This observation may reflect mutations occurring in the attenuation processes shown in Table 1, during which the wild JEV strain is adapted to PHK cells. If this is the case, there must be a certain selection pressure on JEV replication in PHK cells, leading to adaptation mutations, some of which may contribute to the attenuation phenotype of JEV.

Amino acids in viral proteins of the virulent JaOArS982 (6) and Beijing-1 (7) strains that correspond to amino acid differences between SA(V) and SA(A) were examined. Common amino acids among the three virulent strains JaOArS982, Beijing-1, and SA(V) are indicated by asterisks in Table 3. It is of interest that all the amino acids, except for one in NS3, are common in these virulent strains. Furthermore, out of three point mutations in the untranslated regions, two nucleotides of the SA(V) genome, indicated by asterisks in Table 3, are common in the genomes of other virulent strains. Thus many mutations may be determinants of the attenuation phenotype.

Discussion

Recently, Eckels et al. (20) proposed a vaccine lot of strain SA_{14}-14-2 that was adapted to an alternative cell substrate, primary canine kidney (PCK) cell cultures. Quality control of the attenuated vaccine may be easier in PCK cell cultures as compared with PHK cell cultures (20). Although the live, attenuated JE strain

appears to be fairly stable in their attenuation phenotype during the replication in PHK and PCK cells, the molecular genetic background of the attenuation phenotype is totally unknown at present. Elucidation of the genetic background may be essential to use the live attenuated vaccine for humans worldwide, since a similar study has already been conducted with live attenuated poliovirus vaccines (16,21,22) and the results have been used effectively to test the genetic stability of the vaccine (S. Abe, N. Iizuka, K. Tago, and A. Nomoto, manuscript in preparation) and to construct new vaccine candidates (21,23,24).

Both attenuated SA_{14}-14-2 and virulent virus SA_{14} strains are closely related to each other genetically, with only 57 nucleotide differences in the entire 10,976-nucleotide long genome. Furthermore, several biological markers that may correlate with the virulence of the JEV have been well investigated. These markers include mouse neurovirulence and plaque size in LLC-MK2, CE, and BHK-21 cells, which can easily be tested in the laboratory. Thus these two JEV strains provide an excellent experimental model to investigate the molecular mechanisms of JEV attenuation.

There must be many kinds of mutations that lead to viral attenuation. In the case of all three poliovirus vaccine strains, strong determinants of the attenuation phenotype were discovered to reside in the 5′ untranslated region, and it is therefore possible that the attenuation phenotype may be due to lowered efficiency in a certain step of the viral replication in the central nervous system (16,21,22,24). On the other hand, Lustig et al. (25) reported that mutation(s) reducing the neurovirulence of Sindbis virus exists in the genome region encoding the surface glycoproteins. Therefore, a decrease in the affinity of the virus to cellular receptors may be involved in the attenuation of Sindbis virus. In this study, we identified one and two point mutations in the 5′ and 3′ untranslated sequences, respectively. Furthermore, eight amino acid replacements were identified in the E protein of JEV that corresponded to the surface glycoprotein of Sindbis virus. For the identification of mutations that influence the attenuation phenotype, it is essential to construct chimera viruses between the two strains. Infectious cDNA clones of both strains are currently under construction. This kind of study should also elucidate the relationship between mutations for the adaptation to PHK cells and those for attenuation in mouse neurovirulence.

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References

1. Westaway E.G., Brinton M.A., Gaidamovich S.Ya., Horzinek M.C., Igarashi A., Kaariairnen L., Lvov D.K., Porterfield J.S., Russel P.K. and Trent D.W., Intervirology *24*, 183–192, 1985.
2. Rice C.M., Lenches E.M., Eddy S.R., Shin S.J., Sheets R.L. and Strauss J.H., Science *229*, 726–733, 1985.
3. Castle E., Leidner U., Nowak T., Wengler G. and Wengler G., Virology *149*, 10–26, 1986.
4. Dalgarno L., Trent D.W., Strauss J.H. and Rice C.M., J Mol Biol *187*, 309–323, 1986.
5. Hahn Y.S., Galler R., Hunkapiller T., Dalrymple J.M., Strauss J.H. and Strauss E.G., Virology *162*, 167–180, 1988.
6. Sumiyoshi H., Mori C., Fuke I., Morita K., Kuhara S., Kondou J., Kikuchi Y., Nagamatsu H. and Igarashi A., Virology *161*, 497–510, 1987.
7. Hashimoto H., Nomoto A., Watanabe K., Mori T., Takezawa T., Aizawa C., Takegami T. and Hiramatsu K., Virus Genes *1*, 305–317, 1988.
8. Speight G., Coia G., Parker M.D. and Westaway E.G., J Gen Virol *69*, 23–34, 1988.
9. Speight G. and Westaway E.G., Virology *171*, 299–301, 1989.
10. Chambers T.J., McCourt D.W. and Rice C.M., Virology *169*, 100–109, 1989.
11. Burke D.S., in the Committee on Issues and Priorities for New Vaccine Development (ed.) *New Vaccine Development, Establishing Priorities, Vol. II. Diseases of Importance in Developing Countries*. National Academy Press, Washington DC, 1986, pp. 223–240.
12. Huang C.H., in Lauffer M.A., Bang F.B., Maramorosch K. and Smith K.M. (eds.) *Advances In Virus Research, Vol. 27*, Academic Press, New York, 1982, pp. 71–101.
13. Ao J., Yu Y.X., Tang Y.S., Cui B.C., Jia D.J. and Li H.M., Chin J Microbiol Immunol *3*, 245–248, 1983.
14. Igarashi A., J Gen Virol *40*, 531–544, 1978.
15. Yu Y.X., Wu P.F., Ao J., Liu L.H. and Li H.M., Chin J Microbiol Immunol *1*, 77–83, 1981.
16. Kawamura N., Kohara M., Abe S., Komatsu T., Tago K., Arita M. and Nomoto A., J Virol *63*, 1302–1309, 1989.
17. Gubler U. and Hoffman B.J., Gene *25*, 263–269, 1983.
18. Sanger F., Nicklen S. and Coulson A.R., Proc Natl Acad Sci USA *74*, 5463–5467, 1977.
19. Kuge S. and Nomoto A., J Virol *61*, 1478–1487, 1987.
20. Eckels K.H., Yu Y.X., Dubois D.R., Marchette N.J., Trent D.W. and Johnson A.J., Vaccine *6*, 513–518, 1988.
21. Kohara M., Abe S., Komatsu T., Tago K., Arita M. and Nomoto A., J Virol *62*, 2828–2835, 1988.
22. Evans D.M.A., Dunn G., Minor P.D., Schild G.C., Cann, A.J., Stanway G., Almond J.W., Currey K. and Maizel J.V. Jr., Nature (London) *314*, 548–550, 1985.
23. Nomoto A., Iizuka N., Kohara M. and Arita M., Vaccine *6*, 134–137, 1988.
24. Iizuka N., Kohara M., Hagino-Yamagishi K., Abe S., Komatsu T., Tago K., Arita M. and Nomoto A., J Virol *63*, 5354–5363, 1989.
25. Lustig S., Jackson A.C., Hahn C.S., Griffin D.E., Strauss E.G. and Strauss J.H., J Virol *62*, 2329–2336, 1988.