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Comparative Sequences of Two Type 1 Dengue Virus Strains Possessing Different Growth Characteristics *In Vitro*

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***Abstract*: The complete genome sequences of two dengue-1 virus strains having different growth characteristics (Mochizuki and A88) were compared with other published strains. The sequence analysis indicated several unique amino acid changes throughout the coding region of Mochizuki strain, mostly in envelope (E) protein. A unique amino acid, Ile-69 for Mochizuki strain at E protein resulted in the loss of an Asn-67-linked glycosylation site. A Thr substitution for Ala-114 at C protein and amino acid changes found in E, non-structural NS3, NS4a, and NS5 proteins were unique for A88 strain. These substitutions might be correlated to their different growth characteristics *in vitro*.**

***Key words*: Dengue-1 virus, Growth characteristics, Genome sequence, Envelope protein**

Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have become global public health problems in recent years. There are an estimated 50 million dengue cases annually, including 400,000 cases of DHF with high mortality in Southeast Asian countries (28). DHF cases in Indonesia recently increased rapidly and nearly all provinces of the country were affected (27). In Japan there were 92 confirmed cases of DF during the period of 1985–1995 (29) and 74 cases during the period of 1996–1999 (30), imported from tropical countries, mostly from Southeast Asia.

It is known that the interaction between envelope (E) protein and cell receptor is a critical determinant of infectivity (2, 3) and the loss of an envelope glycosylation site appears to play a role in dengue neurovirulence (11). Among the non-structural proteins, NS3 and NS5 are involved in RNA replication (1, 24) and are membrane-associated proteins, which interact in the perinuclear region and function as the component of putative viral replicase (10, 26).

The mouse-adapted dengue-1 Mochizuki strain (isolated in 1943) (12) and A88 strain (isolated in 1988) (6) were propagated in *Aedes albopictus* C6/36 cells grown at 30 C and African green monkey kidney (Vero) cells grown at 37 C. Virus titers were determined by plaque assay using Vero cells grown in 6-well plates. Viral RNA was extracted from the infected cultured fluids or cells by using Isogen reagent (Nippon Gene, Japan). The reverse transcription (RT) was carried out at 42 C for 60 min. Samples were amplified by 30 cycles of polymerase chain reaction (PCR), 1 min at 94 C, 2 min at 60 C and 3 min at 72 C, respectively. Synthetic oligonucleotide primer pairs were designed based on the published sequence data (22) and were used for RT-PCR and sequencing. Sequencing reactions were performed on the purified PCR product and analyzed using an automated Applied Biosystem PRISM 310 Genetic Analyzer according to the manufacturer's instructions. Homology search and comparison of amino acids were carried out using Genetix Mac ver 9.0 software.

Mochizuki strain grew rapidly in both C6/36 and Vero cell cultures, while A88 strain grew comparatively rapidly only in the cultured C6/36 but slowly in Vero cells (data not shown). Similarly in the previous study (16), the growth of dengue viruses was different in C6/36 and Vero cells at 37 C but not in C6/36 cells at 30 C. The growth rate of Mochizuki strain was considerably higher in Vero cells than that of A88 strain. The growth

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Abbreviations: DEN-1, dengue-1; E, envelope; MOI, multiplicity of infection; NS, non-structural protein; PFU/ml, plaque-forming units per milliliter; RT-PCR, reverse transcription polymerase chain reaction.

titers of Mochizuki strain increased approximately 3 times at the elevated incubation temperature (37 C) in both C6/36 and Vero cells (Table 1). It might be correlated to the high viral RNA production as previously reported for dengue type 2 (20). In contrast, the growth titers of A88 strain slightly decreased at the elevated temperature. Moreover, after freezing at −80 C, the infectivity of A88 strain grown at 37 C significantly decreased compared with that of Mochizuki strain or A88 strain grown at 30 C.

To identify regions of the dengue genome that might contribute to their growth characteristics *in vitro*, the genomic RNA of Mochizuki and A88 strains were sequenced. Comparison of nucleotide and amino acid sequence homologies between Mochizuki and A88 strains were 94.6% and 97.8%, respectively (Table 2). Similarly, the homologies of nucleotide (amino acid) sequences of Nauru, West Pac 74 (22) and Singapore, S275/90 (5) strains compared with Mochizuki strain were 95.2% (97.7%) and 95.0% (97.4%), respectively. Most part of the structural protein of Mochizuki strain had been sequenced previously (31). There was one nucleotide difference (previous nucleotide, G_{374} to A) in prM protein gene resulting in an amino acid change (Val to Met). Several interesting findings were observed in the structural protein genes of Mochizuki and A88 strains. The E protein had lower sequence homology than those of other structural protein sequences, while NS3 and NS5 proteins had high sequence homologies of 94.9% (99.2%) and 95.0% (99.2%), respectively.

Seventy-four amino acid substitutions were detected between Mochizuki and A88 strains throughout the coding region, with the majority (19 of 74) in E proteins (Table 3). The predominant amino acid changes in the E protein of Mochizuki strain may be a consequence of dengue virus adaptation during serial passages in mouse brain (9) and in cultured cells as referred to in the previous report (17). Thirty-six unique (conservative change) amino acids were found throughout the coding region of Mochizuki strain and 14 in A88 strain when compared with the published complete sequences (5, 22) and other structural protein sequences (4). Fourteen of 74 amino acid substitutions resulted in a charge difference and 26 resulted in a side chain polarity difference as compared to the structure of the 20 common amino acids (19). Six of the 8 charge differences in amino acids of the structural protein of Mochizuki strain were observed in E protein, mostly in domain II of the virus envelope tertial structure (25), which is very labile and easily destroyed by reduction. Four charge differences

Table 1. Infectivity of the dengue-1 Mochizuki and A88 strains in C6/36 and Vero cells and their infectivity after freezing at −80 C

Virus strains	Freezing	Virus titers (PFU/ml)		
		C6/36 (30 C)	C6/36 (37 C)	Vero (37 C)
MOC	Before[a]	9.0×10^6	3.2×10^7	2.8×10^7
	After[b]	8.2×10^6	2.8×10^7	6.0×10^6
A88	Before	3.2×10^6	2.4×10^6	2.3×10^6
	After	3.1×10^6	2.0×10^5	6.0×10^4

Cell monolayers were infected with virus strains at MOI of 0.02 PFU/cell.

[a] Virus infectivity before freezing at −80 C (7 days post infection).

[b] Virus infectivity after freezing at −80 C.

Table 2. Nucleotide and amino acid homology between the dengue-1 Mochizuki and other strains

	Lengths		Percentage of nucleotide (amino acid) homology		
	Nucleotide	(AA)	A88	Nauru[a]	Singapore[b]
Full length	10,735	(3392)	94.6 (97.8)	95.2 (97.7)	95.0 (97.4)
5'NCR	94		100	100	97.5
C	342	(114)	95.3 (96.5)	96.5 (99.1)	96.8 (97.4)
PrM	273	(91)	96.0 (96.7)	96.3 (95.6)	93.0 (95.6)
M	225	(75)	95.6 (98.7)	96.0 (98.7)	95.1 (97.3)
E	1,485	(495)	93.1 (96.2)	94.1 (96.0)	93.1 (95.8)
NS1	1,056	(352)	95.5 (97.4)	95.5 (97.4)	94.1 (96.9)
NS2a	654	(218)	91.7 (95.9)	93.4 (95.4)	95.7 (98.6)
NS2b	390	(130)	94.4 (96.9)	93.8 (97.7)	93.3 (98.5)
NS3	1,857	(619)	94.9 (99.2)	95.5 (98.7)	95.1 (98.1)
NS4a	450	(150)	92.7 (94.7)	92.0 (96.7)	93.8 (97.3)
NS4b	747	(249)	95.4 (97.6)	95.6 (97.2)	94.9 (97.2)
NS5	2,697	(899)	95.0 (99.2)	95.6 (98.9)	96.1 (98.0)
3'NCR	465		96.6	96.8	97.0

[a] The DEN-1 Nauru (West Pac 74) strain (22).

[b] The DEN-1 Singapore (S275/90) strain (5).

NCR = Noncoding region.

observed at E-37 (Gly-Asp) and E-149 (Tyr-His) of domain I, and at E-228 (Lys-Gln) and E-277 (Lys-Thr) of domain II resulted in a change of secondary structure, while the differences at E-58 (Glu-Lys) of domain II and E-309 (Lys-Glu) of domain III did not change the structure. Amino acid mutations within a potential 'hinge' region between domains I/II (E-58 and E-277) may be involved in fusion of virus with cell membrane. The mutations found at E-305 of A88 or E-309 of Mochizuki strains are predicted to be located within the C terminal of the first putative glucosaminoglycan binding motifs (3). But, these mutations may not change the binding of the virus with a target cell receptor.

The unique amino acid substitutions for Mochizuki strain were shown at N-terminal of C protein (Asn_3 to Asp) and at the cleavage site of prM/M (Asp_{89} to Gly) with charge differences, while the substitution at E/NS1 (Met_{492} to Thr) was seen with the side chain polarity difference. A single amino acid substitution at M-16 (Thr to Ala) with a side chain polarity difference (nonpolar to

Table 3. Amino acid substitutions between the dengue-1 Mochizuki and A88 strains

Protein	Nucleotide changes (MOC-A88)		Amino acid changes (MOC-A88)		Hydropathy scale[a] (15)	
Core	7	GAC-AAC	3	Asp-Asn	−3.5,	−3.5
	208	GGC-AGC	70	Gly-Ser	−0.4,	−0.8
	340	GCG-ACG	114	Ala-Thr	1.8,	−0.7
PrM	266	GGC-GAC	89	Gly-Asp	−0.4,	−3.5
M	46	GCC-ACC	16	Ala-Thr	1.8,	−0.7
Envelope	110	GGT-GAC	37	Gly-Asp	−0.4,	−3.5
	172	GAA-AAA	58	Glu-Lys	−3.5,	−3.9
	206	ATC-ACC	69	Ile-Thr	4.5,	−0.7
	262	GCG-ACG	88	Ala-Thr	1.8,	−0.7
	349	ACT-GCT	117	Thr-Ala	−0.7,	1.8
	149	TAC-CAC	149	Tyr-His	−1.3,	−3.2
	679	CCC-TCC	227	Pro-Ser	−1.6,	−0.8
	682	AAA-CAA	228	Lys-Gln	−3.9,	−3.5
	830	AAA-ACA	277	Lys-Thr	−3.9,	−0.7
	890	ATG-ACA	297	Met-Thr	1.9,	−0.7
	913	TCA-CCA	305	Ser-Pro	−0.8,	−1.6
	925	AAG-GAG	309	Lys-Glu	−3.9,	−3.5
	1037	ATC-ACC	346	Ile-Thr	4.5,	−0.7
	1253	ATC-ACC	418	Ile-Thr	4.5,	−0.7
	1475	ACG-ATG	492	Thr-Met	−0.7,	1.9
NS1	313	GGG-AGG	105	Gly-Arg	−0.4,	−4.5
	436	GAC-AAC	146	Asp-Asn	−3.5,	−3.5
	782	TCC-TTC	261	Ser-Phe	−0.8,	2.8
NS2a	130	ATA-ACA	44	Ile-Thr	4.5,	−0.7
	442	GCC-ACC	148	Ala-Thr	1.8,	−0.7
	628	GCA-ACA	210	Ala-Thr	1.8,	−0.7
NS2b	208	ACC-GCC	70	Thr-Ala	−0.7,	1.8
NS3	272	ATA-ACG	91	Ile-Thr	4.5,	−0.7
	355	CCT-TCT	119	Pro-Ser	−1.6,	−0.8
	688	GCT-TCT	230	Ala-Ser	1.8,	−0.8
	1438	TCA-CTG	480	Ser-Leu	−0.8,	3.8
NS4a	7	GCA-TCA	3	Ala-Ser	1.8,	−0.8
	268	GCT-TCC	90	Ala-Ser	1.8,	−0.8
	292	TCC-GCC	98	Ser-Ala	−0.8,	1.8
	376	CAA-CTG	126	Gln-Leu	−3.5,	3.8
NS4b	79	ACA-GCA	27	Thr-Ala	−0.7,	1.8
	454	GAT-GTT	152	Asp-Val	−3.5,	4.2
NS5	379	CAC-TAC	127	His-Tyr	−3.2,	−1.3
	404	ACA-ATA	135	Thr-Ile	−0.7,	4.5
	1277	GCA-GAA	426	Ala-Glu	1.8,	−3.5
	1919	GAA-GGA	640	Glu-Gly	−3.5,	−0.4

[a] Scale indicating hydrophilicity (−3.2 to −4.5) or hydrophobicity (1.8 to 4.5). Radical amino acids are underlined.

polar) resulted in a change of hydrophilicity for Mochizuki M protein. The same charge change at prM-89 from negative to neutral (Asp to Gly) was seen at E-37. The Asn-linked glycosylation sites were conserved between the two strains at prM-69, E-153 and NS1-130, while unique amino acid substitution at E-69 (Thr to Ile) of domain II resulted in the loss of Asn-linked glycosylation site at E-67 for Mochizuki strain. This substitution leads to a higher pH threshold conformational change (8). A consequence of substitution at E-69 may be the basic amino acid (Lysin) substitution found at E-228 and E-277 of domain II and E-309 of domain III. However, the low pH-induced fusion is necessary for virus entry into both mosquito and vertebrate cells and is likely to be mediated by the E glycoprotein (23).

The charge difference at E-277 (Thr to Lys) and the side chain polarity differences at E-346 and 418 (Thr to Ile) resulted in the increasing number of T-cell epitopes (Rothbard/Taylor pattern) for Mochizuki strain, while a unique amino acid substitution for Mochizuki at E-492 (Met to Thr) resulted in the loss of T-cell epitopes. The substitutions found at E-346 and E-418 of Mochizuki strain are predicted to be located within the target cell binding motifs (2). These substitutions may have increased the binding of the virus with a target cell receptor.

The amino acid charge differences in the non-structural proteins were seen at NS1, NS4b, and NS5. A charge difference at NS1-105 (Arg to Gly) resulted in a change of hydrophilicity, while a charge difference at NS1-146 (Asn to Asp) did not change the hydrophilicity profile. The unique amino acid substitution for Mochizuki at NS3-230 (Ser to Ala) was seen in nucleotide triphosphate binding site (7) with hydrophilicity change. A charge difference at NS5-127 from neutral to positive (Tyr to His) was unique for Mochizuki (His). The substitution was observed in the methyltransferase binding site (14). The charge differences at NS5-426 (Glu to Ala) and NS5-640 (Glu to Gly) were unique for Mochizuki and A88, respectively. Substitution in the latter was observed in RNA polymerase site (13). The charge differences found in C, prM, E, NS1, NS4b and NS5 proteins of Mochizuki strain and in E, NS3, NS4a, and NS5 of A88 strain might be correlated to their growth characteristics in cell cultures.

The present findings on radical amino acid changes between DEN-1 Mochizuki and A88 strains are similar in part to those found in DEN-2 by Leitmeyer and others (18). The amino acid charge differences were seen in the prM, E, NS4b and NS5 genes, while Mangada and Igarashi (21) found changes mostly in the NS1 and NS2a genes. They also found radical amino acid changes mostly in the non-structural region.

Concerning difference in viral replication, the replication of mice passaged virus was significantly higher than that of unpassaged virus (17). Also, the E glycoprotein of the passaged virus mutant migrated faster than that of the parental virus in the electrophoresis observation (1, 11). In a previous study, Mochizuki and A88 strains grew comparatively rapidly in BHK cell cultures (6). However, in the present study, A88 strain grew slowly in mammalian (Vero) cells and its growth rate and yields were considerably lower than those of Mochizuki strain. In addition, A88 strain was relatively heat labile (Table 1). These results seem to be related to the several amino acid substitutions in the structural proteins, especially E protein (Table 3). The substitutions found at E protein of Mochizuki strain, especially the loss of its glycosylation site at E-67 might be correlated to the loss of its human pathogenicity due to serial mouse brain passages as reported previously (9). The change at E-297 of A88 strain was predicted to be located between the domains I/III (aa 298 to 394) interface (25). It may influence its growth rate and yields by affecting the low-pH conformational change as previously reported (25). The unique substitution for A88 at the cleavage site of C/prM junction (Ala_{114} to Thr) changed its hydrophobicity profile (Table 3) that was conserved among all four dengue virus serotypes. The amino acid substitutions found in the non-structural proteins, NS3-119, NS4a-90, NS4a-126, NS5-135 and NS5-640 of A88 strain could lead to the changes of the viral growth in Vero cells. It is known that the dengue virus mutants grew more slowly than the parent strain and induced smaller plaques in Vero cells (8).

Our comparative analyses of amino acid substitutions between dengue-1 strains suggest the potential mechanisms determining the replication property and pathogenicity of viruses. To identify the determinant site for biological activities, several studies using infectious DNA and animal experiments are needed and are beeing carried out in our laboratory.

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