Characterization of the variable region in the 3’ non-translated region of dengue type 1 virus

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The first 84 nt in the 3’ non-translated region (3’ NTR) of dengue type 1 virus (DENV-1) exhibit lower levels of conservation than the other regions; this region is named the variable region (VR). The VR is further divided into two subregions: a 5’-terminal hypervariable region (HVR) and a 3’-terminal semi-variable region (SVR). Recent reports suggested that the VR of DENV-2 is required for efficient virus growth in mammalian cells. To investigate whether this is also true for the VR of DENV-1, deletion or replacement mutations were introduced into the VR by using recombinant DENV-1 cDNA clones. Recombinant viruses with deletion of either or both subregions exhibited reduced growth properties compared with the original virus. Mutants with incompletely reversed or unrelated sequences in the HVR demonstrated growth properties similar to those of the original virus. However, a replacement mutation in the SVR did not cause recovery of growth properties. Furthermore, the amount of viral RNA was decreased in Vero cells infected with the growth-attenuated mutant viruses. Results of reporter translation assays suggest that VR mutations may not affect the translation process of DENV-1. These data indicate that the VR is important for DENV-1 replication and is associated with the accumulation of DENV-1 RNA in mammalian cells, and that the HVR and SVR in the VR may have different roles in DENV-1 replication.

INTRODUCTION

Dengue viruses (DENV-1, 2, 3 and 4) belong to the family Flaviviridae together with other important human pathogens, such as Japanese encephalitis virus, yellow fever virus, tick borne encephalitis virus and West Nile virus. Dengue viruses are the aetiological agents of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The viruses are transmitted to humans by Aedes mosquitoes (Gubler, 1998; Halstead, 1997). Dengue virus infections are a serious cause of morbidity and mortality in most tropical and subtropical areas of the world. Dengue cases are estimated to occur in up to 100 million individuals annually and the case fatality ratio is 1–5% in DHF and DSS patients (Halstead, 1997). DHF and DSS occur more frequently in patients experiencing a secondary dengue virus infection than in those experiencing a primary infection, suggesting that the presence of heterotypic dengue virus antibodies is a risk factor for developing DHF and DSS in secondary infections (Halstead, 1997; Kurane & Ennis, 1997). On the other hand, genotypic differences also appear to be associated with virulence (Rosen, 1977).

Dengue viruses are single-stranded, positive-sense RNA viruses. The genome, approximately 11 kb in length, encodes three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame, and non-translated regions (NTRs) in its 5’ and 3’ terminals (Lindenbach & Rice, 2001).

The flavivirus 3’ NTR, which is about 400–750 nt in length, contains cis-acting sequences that are crucial for virus replication (Lindenbach & Rice, 2001; Markoff, 2004). The 3’ NTR of dengue viruses is divided into two regions based on differences in conservation level: (i) the region immediately after the open reading frame shows low sequence conservation and is, therefore, named the variable region (VR); (ii) the middle and the most 3’-terminal regions are highly conserved among strains and contain several sequence motifs, such as cyclization/conserved sequence (CS1), conserved sequences (CS2 and RCS2), 3’ upstream AUG region (3’ UAR) and four stem–loop structures (Alvarez et al., 2005a; Proustki et al., 1997; Shurtleff et al., 2001). CS1 and the 3’ UAR interact with their complementary sequences in the 5’ region of the viral genome.
and these RNA–RNA interactions are essential for RNA synthesis of mosquito-borne flaviviruses (Alvarez et al., 2005a, b; Hahn et al., 1987; Khromykh et al., 2001; Lo et al., 2003; Men et al., 1996). The stem–loop structure of the 3′ NTR stabilizes the viral RNA genome and also enhances translation initiation, as demonstrated by using a reporter translation assay system (Chiu et al., 2005; Edgil et al., 2003; Holden & Harris, 2004). Other groups also demonstrated, by using reporter replicon systems, that the stem–loop structure is required for RNA replication, but not for viral translation (Alvarez et al., 2005a; Lo et al., 2003; Tilgner & Shi, 2004; Tilgner et al., 2005). Furthermore, well-conserved 3′-terminal dinucleotides (CUOH3′) of the flavivirus genome are indispensable for virus replication (Khromykh et al., 2003). Therefore, the integrity of the structures and motifs of the region is essential for optimal performance of its biological function. In contrast, the VR shows high sequence variability, and nucleotide deletions in this region have been detected in some strains of Japanese encephalitis virus, tick-borne encephalitis virus and yellow fever virus (Bryant et al., 2005; Gritsun et al., 1997; Ma et al., 2003; Nam et al., 2001; Wang et al., 1996; Yang et al., 2004).

We recently isolated two new DENV-1 strains, which have 17 and 29 nt deletions in the VR, from DF patients who stayed in the Seychelles and Yap Island in Micronesia, respectively (Nukui et al., 2006). Such small nucleotide deletions have also been observed in some strains of DENV-2 and DENV-3 (Leitmeier et al., 1999; Shurtleff et al., 2001). Deletion in the VR of the tick-borne encephalitis virus genome was induced spontaneously during propagation of the virus in cell culture and in mice (Mandl et al., 1998). Mutational analysis of the 3′ NTR in tick-borne encephalitis virus using recombinant cDNA clones indicated that deletion in the VR may not affect virus growth properties in vitro or virulence in mice (Mandl et al., 1998). Analysis using a Kunjin virus replicon also showed that the VR in Kunjin virus is not essential for RNA replication (Khromykh & Westaway, 1997). We also examined the effect of a small deletion (19 nt in length) in the VR on the characteristics of DENV-1 by using recombinant full-length cDNA clones (Tajima et al., 2006). No differences were observed between the parent and the mutant viruses in replication efficiency or plaque size in mammalian or mosquito cells, suggesting that 19 nt of the VR of DENV-1 are dispensable for virus replication and propagation in vitro (Tajima et al., 2006). However, analysis of DENV-4 showed that recombinant viruses with a deletion in the VR exhibited a small-plaque morphology in mosquito C6/36 cells and slightly attenuated replication efficiency in simian LLC-MK2 cells (Men et al., 1996). It was also reported that the VR might play a role in the rate of viral RNA replication of Japanese encephalitis virus (Nam et al., 2001). A recent report showed that the VR of DENV-2 enhances viral replication in BHK cells, whereas it is dispensable in mosquito cells (Alvarez et al., 2005a). These findings indicate that the VR is associated with efficient virus replication and propagation in mammalian cells; however, further analyses are necessary to understand the role of the VR in virus replication and propagation.

In the present study, we tried to define the region in the VR that is critical for efficient virus growth by using recombinant DENV-1 cDNA clones. We reconstituted several DENV-1 mutants that have an incomplete VR in the 3′ NTR and examined the ability of these mutant viruses to replicate and propagate in mammalian and mosquito cells.

**METHODS**

**Cells and viruses.** Vero (NIHSC strain) and C6/36 cells were cultured at 37 and 28 °C, respectively, in 5 % CO2 in Eagle’s minimum essential medium (Sigma) supplemented with 10 % heat-inactivated fetal bovine serum and 100 U penicillin/streptomycin ml−1. Two recombinant DENV-1 viruses, rDENV-1(02-20), which is derived from DENV-1 isolate NIID02-20 (GenBank accession no. AB178040), and rDENV-1m10, were prepared in our laboratory as described previously (Tajima et al., 2006).

**Construction of recombinant mutant DENV-1 clones and recovery of mutant viruses.** Original recombinant DENV-1 (rDENV-1) clone rDENV-1(02-20)/pMW119 (Tajima et al., 2006) was used for the construction of nucleotide-substitution, deletion and replacement mutant DENV-1 clones. Primers and oligonucleotides were used for constructing mutant nucleotides and were listed in Supplementary Table S1, available in JGV Online. The BamHI–SacI fragment in the N55–3′-terminal region was subcloned into the BamHI–SacI site of pBluescript II SK(+) (Stratagene) (NS5–3′T/pBS). The plasmid was amplified by an inverse PCR method with primer sets D1.10322f and D1.10276r, D1.10358f and D1.10276r, and D1.10358f and D1.10321r, for introduction of the dHVR, dVR and dSVR mutations, respectively. The PCR products were treated with DpnI to digest the plasmid templates, purified, self-ligated and then transformed into Stbl2 (Invitrogen). Plasmid clones with deletions [NS5–3′T(dHVR)/pBS, NS5–3′T(dVR)/pBS and NS5–3′T(dSVR)/pBS] in the 3′ NTR were obtained. To construct replacement mutants, double-stranded oligonucleotide fragments rHVR (rHVRf and rHVRr), rVR (rVRf and rVRr) and rSVR (rSVRf and rSVRr) were ligated into the products of inverse PCR with primer sets D1.10322f and D1.10276r, D1.10358f and D1.10276r, and D1.10358f and D1.10321r, respectively, and plasmid clones with replacement in the variable region of the 3′ NTR [NS5–3′T(rHVR)/pBS, NS5–3′T(rVR)/pBS and NS5–3′T(rSVR)/pBS] were obtained as described above. For introduction of rHVRm1 and rHVRm2 mutations, the plasmid NS5–3′TrHVR/pBS was amplified by the inverse PCR method with primer sets rHVRm1f and rHVRm1r for rHVRm1, and rHVRm2f and rHVRm2r for rHVRm2, and then clones NS5–3′TrHVRm1/pBS and NS5–3′TrHVRm2/pBS were obtained as described above. To complete the mutant rDENV-1 clones, the Sfi–SacI fragment of rDENV-1(02-20)/pMW119 was replaced with the Sfi–SacI fragment of each mutant NS5–3′T/pBS clone. The nucleotide sequence of the viral genome region of the recombinant clones was checked after amplification of the plasmids in Escherichia coli. Recombinant mutant viruses were recovered from these clones as described previously (Tajima et al., 2006).

**Analysis of virus growth and plaque size.** Cells (1 × 106) were plated in a 25 cm2 culture flask and infected with original and mutant DENV-1 at an m.o.i. of 0.0005 p.f.u. per cell for Vero cells and 0.005 p.f.u. per cell for C6/36 cells. Small aliquots of the medium were recovered periodically and the aliquots were titrated by plaque assay on Vero cells grown in six- or 12-well culture plates. To evaluate the...
plaque size, Vero cells were fixed with 3.7% (v/v) formaldehyde solution in PBS for 1 h, then the methylcellulose overlay was removed and the cells were stained with methylene blue solution for 2 h.

**Western blotting.** Aliquots of the culture medium, collected as described above, were subjected to SDS-PAGE on a 10% polyacrylamide gel. The proteins on the gel were transferred to a PVDF membrane filter and the filter was incubated in buffer that contained ascites from DENV-2-infected mice (VR-222; purchased from the ATCC). After being washed, the filter was incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG, and then the products were detected with SuperSignal West Pico chemiluminescent substrate (Pierce).

**Quantification of DENV-1 RNA.** Primers and oligonucleotides used for detection of viral RNA of DENV-1 are listed in Supplementary Table S1, available in JGV Online. Cells (2.5 × 10^5) were plated in six-well culture plates and infected with the original and mutant rDENV-1 at an m.o.i. of 1 p.f.u. per cell for Vero cells. The cells were recovered at 0 and 27 h after inoculation and total RNA was extracted by using TRIzol reagent (Invitrogen). For detection of viral RNA (mainly positive-sense RNA), cDNA was synthesized by using SuperScript III reverse transcriptase (Invitrogen) and antisense primer D1.151r. The 5’ NTR–core region of the viral genome was amplified by real-time quantitative PCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), a thermal cycler (LightCycler; Roche) and primers D1.27f and D1.151r to quantify the amount of cDNA. For detection of negative-sense viral RNA, positive-sense viral RNA was digested with RNase H to prevent negative-strand viral cDNA synthesis from the 3’ terminus of positive-strand viral RNA as follows: oligodeoxynucleotide m10-D1.10231r was added to the RNA solution to hybridize with positive-sense viral RNA (NS5 region) and treated with RNase H to digest the hybrid region of the positive-strand RNA. RNase H-treated RNA was used for synthesis of cDNA with or without the sense primer D1.ES-T3 and D1.151r. The 5’ NTR–core region of negative-sense viral RNA was quantified as described above with primers D1.ES-T3 and D1.151r. Means ± SD were determined and Student’s t-test was performed for three experiments. After quantitative PCR, the products were subjected to electrophoresis on a 2% agarose gel.

**Reporter translation assay.** The original (NIID02-20) and mutant (dHVR, rHVR, dSVR and rSVR) rDENV-1 clones were used for construction of DENV-1 NTR–reporter plasmids 5D1Rluc3D1-02-20/pMW119, 5D1Rluc3D1-dHVR/pMW119, 5D1Rluc3D1-rHVR/pMW119, 5D1Rluc3D1-dSVR/pMW119 and 5D1Rluc3D1-rSVR/pMW119. Means ± SD were determined and Student’s t-test was performed for three experiments. After quantitative PCR, the products were subjected to electrophoresis on a 2% agarose gel.

### Fig. 1. Schematic representation of the VR of DENV-1 and deletion/substitution regions in mutant recombinant DENV-1 viruses. Mutant viruses used in the present study are shown. Asterisks indicate completely conserved nucleotide positions among all 39 DENV-1 strains for which nucleotide sequences of the 3’ NTR have been determined and dashes indicate gaps in mutant viruses. Lower-case letters in rDENV-1.rHVRm1 and rDENV-1.rHVRm2 indicate nucleotides that are different from those of both the original 02-20 and rHVR viruses.

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pMW119, respectively (Fig. 7a). The Renilla luciferase (Rluc) gene was amplified by PCR from plasmid pRL-SV40 (Promega) with primers Rluc-F1 and Rluc.SflI.r (Supplementary Table S1) and then the PCR products were digested with BsmI and SfiI. The BsmI–SfiI fragment (capsid–NS5 region) of five rDENV-1 clones was replaced in frame by the Rluc gene fragment. Firefly luciferase reporter plasmid FFluc/pBS was constructed by inserting a HindIII–AccI fragment, which contains the firefly luciferase gene of pGL3-Control plasmid (Promega), into the HindIII–AccI site of pBluescript II SK(+) (Stratagene). DENV-1 NTR–reporter plasmids and FFluc/pBS were digested at the 3′ end with SacII and XhoI, respectively, and the linearized DNA was transcribed as described previously (Tajima et al., 2006). DENV-1 NTR–luciferase chimeric RNA (1 μg) and firefly luciferase RNA (0.1 μg) were cotransfected into Vero cells (1 × 10⁵ cells) by using Lipofectamine 2000 (Invitrogen). Eight hours after transfection, cells were harvested and the activities of Renilla and firefly luciferases were measured in lysates by using the Dual Luciferase reporter assay system (Promega) as described previously (Tajima et al., 1998). Means ± SD were determined for three experiments.

RESULTS

Deletions in the DENV-1 VR decrease viral replication in Vero cells

To examine the role of the VR in DENV-1 replication in mammalian cells, we first constructed a mutant recombinant DENV-1 clone, rDENV-1.dVR/pMW119, based on the original recombinant clone rDENV-1(02-20)/pMW119 as described previously (Tajima et al., 2006), and obtained mutant viruses using these clones (Fig. 1). Mutant virus rDENV-1.dVR (dVR) had a deletion of the whole VR. To compare plaque morphology induced by the recombinant viruses, Vero cells in six-well plates were inoculated with approximately 50 p.f.u. of the recombinant viruses and cultured (Fig. 2a). Plaques of the dVR mutant were small and faint compared with those of the original virus. We next examined the growth kinetics of the original virus and the dVR mutant in Vero cells (Fig. 2b). The mutant showed slower growth kinetics than the original and m10 mutant viruses. We also checked the amount of viral antigens secreted into the culture medium from Vero cells infected with the original and mutant viruses, and the antigen level was decreased in the dVR mutant (Fig. 2c). These data indicate that the VR is associated with efficient replication of DENV-1 in mammalian cells.

Both the hypervariable and semi-variable regions in the VR are required for efficient replication of DENV-1 in Vero cells

The VR of DENV-1 is divided into two subregions: the 5′-side hypervariable region (HVR; 45 nt in length) and the 3′-side semi-variable region (SVR; 36 nt in length), according to the conservation level among DENV-1 isolates (Fig. 1). To determine the region in the VR responsible for efficient viral growth in Vero cells, we produced two other novel deletion-mutant viruses, rDENV-1.dHVR (dHVR) and rDENV-1.dSVR (dSVR). The mutants dHVR and dSVR have deletions in the HVR and SVR, respectively (Fig. 1). We first compared the plaque morphology induced by the recombinant viruses in Vero cells (Fig. 3a). Both the dHVR and dSVR mutants induced small plaques in Vero cells and the shape of plaques was similar to that of plaques induced by the dVR mutant. Growth kinetics and levels of secreted viral antigens in the culture supernatant fluid of Vero cells infected with the viruses. Cell-culture supernatant fluid was analysed at 4 days post-infection by Western blotting with ascitic fluid of mice immunized with DENV-2. Similar results were obtained in two independent experiments.

![Fig. 2. Growth properties of the DENV-1 VR mutant dVR in Vero cells. (a) Plaque phenotypes of the viruses in Vero cells. (b) Growth curves of the viruses (▲, 02-20; ■, m10; ×, dVR). Cell-culture medium was collected on the days indicated and virus titres were assessed in Vero cells. (c) Detection of virus antigens in the culture supernatant fluid of Vero cells infected with the viruses. Cell-culture supernatant fluid was analysed at 4 days post-infection by Western blotting with ascitic fluid of mice immunized with DENV-2. Similar results were obtained in two independent experiments.](http://vir.sgmjournals.org)
Replacement of the mutations in the HVR of the VR recovers the growth property of the virus

For further analysis, we produced three replacement-mutant viruses, rDENV-1.rHVR (rHVR), rDENV-1.rSVR (rSVR) and rDENV-1.rVR (rVR) (Fig. 3). The mutants rHVR, rSVR and rVR have insertion of completely or incompletely reversed nucleotide sequences of the HVR, SVR and VR into the dHVR, dSVR and dVR viruses, respectively (Fig. 1). Plaques of the rSVR and rVR mutants were smaller than those of the original virus, but similar to those of the dSVR and dVR mutants (Fig. 3a). Growth kinetics and the amounts of secreted viral antigens were also decreased in the rSVR and rVR mutants compared with those of the original virus (Fig. 3b, c). These results suggest that the nucleotide sequence in the SVR may be essential for complete activity of the VR in viral replication in Vero cells. In contrast, the rHVR mutant induced plaques as large as those induced by the original virus (Fig. 3a). Moreover, no apparent difference was observed between the original virus and the rHVR mutant in growth kinetics and the levels of secreted viral antigens (Fig. 3b, c). The results suggest that the growth property of the dHVR mutant is restored by the insertion of reversed nucleotide sequences into the HVR of this mutant. It is possible that the reversed fragment of rHVR forms a secondary RNA structure similar to the authentic HVR. To address this question further, we produced two additional mutant viruses, rHVRm1 and rHVRm2 (Fig. 1). rHVRm1 has 2 nt deletions and 6 nt substitutions and rHVRm2 has 15 nt substitutions in the HVR, compared with the rHVR mutant. In particular, the A+T content of the HVR in the original virus and rHVR mutant (73 mol%) was decreased dramatically in the rHVRm2 mutant (42 mol%). Plaque morphology of the new mutant viruses was compared with that of the original dHVR and rHVR mutant viruses (Fig. 4). Plaque size was similar among the viruses, except for the dHVR mutant. Our data indicate that the HVR of DENV-1 contributes to efficient DENV-1 growth in a nucleotide sequence-independent manner.

The VR does not affect the replication of DENV-1 in mosquito C6/36 cells

To evaluate the effect of the mutations in the VR on viral growth in mosquito cells, growth kinetics were compared in Aedes mosquito C6/36 cells (Fig. 5). In contrast to the results with Vero cells, no significant differences were detected among these viruses. The results suggest that the VR is dispensable for DENV-1 replication in Aedes mosquito cells.

Fig. 3. Growth properties of DENV-1 VR mutant viruses (dVR, dHVR, dSVR, rHVR, rSVR and rVR) in Vero cells. (a) Plaque phenotypes of the viruses in Vero cells. (b) Growth curves of the viruses (○, 02-20; ■, dHVR; ×, dSVR; ▲, rHVR; *, rSVR; ●, rVR). (c) Detection of virus antigens in culture supernatant fluid of Vero cells infected with the viruses. Cell-culture supernatant fluid was analysed at 4 days post-infection. Similar results were obtained in two independent experiments.
Growth-attenuated VR mutants of DENV-1 had lower levels of viral RNA in Vero cells

Although our results and a previous report by Alvarez et al. (2005a) have shown that the VR of DENV-1 and 2 contributes to efficient viral replication, the mechanism by which VR mutations cause a decrease in viral replication has not yet been determined. To examine whether the growth-attenuation mutations in the VR influence the accumulation of viral RNA in infected cells, the levels of viral RNA, mainly positive-sense RNA, were compared among Vero cells inoculated with the original and mutant viruses by using a conventional real-time RT-PCR method (Fig. 6a). The amounts of viral RNA were reduced in cells inoculated with the dHVR, dSVR and rSVR mutants. However, the RNA level in rHVR-infected cells was similar to that in cells infected with the original virus. We also compared the levels of negative-sense viral RNA among the inoculated cells (Fig. 6b). The amounts of negative-sense RNA were reduced in cells inoculated with dHVR, dSVR and rSVR mutants, but not in the cells inoculated with rHVR. In our method for detecting negative-sense viral RNA, viral cDNA was amplified only when the sense RT primer T3-5N was present in the reverse-transcription process (Fig. 6c), suggesting that our method overcame the problem of mispriming on excess positive-sense viral RNA. These results indicate that both the HVR and SVR in the VR are involved in the regulation of viral RNA level in Vero cells.

Growth attenuation in VR mutants is not associated with the translation efficiency of DENV-1 in Vero cells

To examine the effect of VR mutations on the translation of DENV-1, we used a reporter translation assay system. In vitro-transcribed DENV-1 NTR–luciferase chimeric RNA with the original (5D1Luc3D1-02-20), dHVR (5D1Luc3D1-dHVR), rHVR (5D1Luc3D1-rHVR), dSVR (5D1Luc3D1-dSVR) and rSVR (5D1Luc3D1-rSVR) 3’ NTR, as described in Fig. 7(a), was transfected into Vero cells and luciferase activity in cell lysates was measured (Fig. 7b). No significant differences were observed among these reporter RNAs, indicating that the VR mutations may not affect the translation process of DENV-1 and, therefore, that growth attenuation of the dHVR, dSVR and rSVR mutants may not be attributed to a decrease in the translation efficiency of viral RNA.

DISCUSSION

In this study, we evaluated the functional significance of the VR by using a recombinant DENV-1 production system that was established previously in our laboratory (Tajima et al., 2006). Our data indicate that the VR is associated with efficient replication of DENV-1 in Vero cells. Alvarez
et al. (2005a) also reported that the VR of DENV-2 was involved in efficient viral growth in BHK21 cells. However, the sizes of the deletions in our recombinant DENV-1 clones were smaller (36 nt in dSVR, 45 nt in dHVR and 81 nt in dVR) than that of DENV-2 (155 nt) (Alvarez et al., 2005a), suggesting that partial deletion of the VR is sufficient for disrupting the function of the VR in mammalian cells. Moreover, our data showed that replacement mutations in the VR (rVR and rSVR) induced a reduction in growth rate, suggesting that insertion of a reporter or selective marker gene into the VR may affect the growth properties of dengue viruses in mammalian cells.

The VR of DENV-1 consists of two subregions, HVR and SVR. Our data demonstrate that both of these subregions are required for efficient viral growth of DENV-1 in Vero cells. However, HVR and SVR have different roles in DENV-1 replication: mutant DENV-1 viruses with reversed (rSVR) or unrelated (rVR) sequences at the SVR exhibited reduced growth properties, whereas the growth of mutant viruses with reversed (rHVR) or unrelated (rHVRm1, rHVRm2) sequences at the HVR was equivalent to that of the original virus. These results suggest that the nucleotide sequence in the SVR and the number of nucleotides in the HVR might be important for viral replication in DENV-1. It is probable that the SVR may form a critical secondary...
structure. In fact, the nucleotide sequence of the region resembles a hairpin-forming motif (RYF) in the VR of yellow fever virus (Bryant et al., 2005). It is also possible that the region interacts with a mammalian cellular factor that is important for efficient growth of DENV in mammalian cells. This hypothetical structure and interaction might be abolished by the replacement mutations. In contrast, the nucleotide sequence and A+T-rich status of the HVR are not crucial for viral replication. However, viral growth in Vero cells is reduced by a deletion of the whole (45 nt) HVR, although a smaller deletion (19 nt) does not affect viral replication (Tajima et al., 2006). Thus, a 26 nt sequence in the HVR may be sufficient for full growth activity of DENV-1 in mammalian cells. It is possible that the region forms a loop structure and helps other regions to function in viral replication in mammalian cells (Zhou et al., 2006).

None of the mutations that we introduced into the VR of recombinant viruses in this study resulted in alterations of the growth nature of DENV-1 in mosquito C6/36 cells, and these results are in accordance with previous findings (Alvarez et al., 2005a). However, an in vivo infection experiment using Aedes mosquitoes may be needed to confirm whether the VR of dengue viruses is dispensable for viral replication in mosquitoes.

Our data show that growth-attenuation mutations in the VR decrease the amount of viral RNA in Vero cells, indicating that the VR is associated with synthesis or retention of viral RNA in Vero cells. However, VR mutations did not affect DENV-1 translation when we analysed the VR by using NTR–reporter chimeric RNA. Other groups have also examined the role of the 3′ NTR in viral transcription and translation by using reporting replicons and have suggested that the 3′ NTR is important for transcription, but not for translation (Alvarez et al., 2005a; Lo et al., 2003). These findings and our results raise the possibility that the VR of dengue viruses is involved in viral RNA synthesis in mammalian cells.

In conclusion, we have demonstrated that the VR is important for efficient replication of DENV-1 in mammalian cells and that the HVR and SVR in the VR have different roles in DENV-1 replication. However, it is also apparent that the VR is not essential for viral replication in mammalian cells in vitro. Thus, investigation of the effect of mutation in the VR on replication and virulence of dengue viruses in vivo is needed for understanding the real role of the VR in nature.

ACKNOWLEDGEMENTS

This work was partly supported by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Japan Health Science Foundation (H18-sinkou-ippan-9), a Grant-in-Aid for young scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (16790276), and the Global Environment Research Fund from the Ministry of Environment, Japan (S-4).

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