Serotype-specific interactions among functional domains of dengue virus 2 nonstructural proteins (NS) 5 and NS3 are crucial for viral RNA replication

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Four serotypes of mosquito-borne dengue virus (DENV), evolved from a common ancestor, are human pathogens of global significance for which there is no vaccine or antiviral drug available. The N-terminal domain of DENV NS5 has guanylyltransferase and methyltransferase (MTase), and the C-terminal region has the polymerase (POL), all of which are important for 5′-capping and RNA replication. The crystal structure of NS5 shows it as a dimer, but the functional evidence for NS5 dimer is lacking. Our studies showed that the substitution of DENV2 NS5 MTase or POL for DENV4 NS5 within DENV2 RNA resulted in a severe attenuation of replication in the transfected BHK-21 cells. A replication-competent species was evolved with the acquired mutations in the DENV2 and DENV4 NS5 MTase or POL domain or in the DENV2 NS3 helicase domain in the DENV2 chimera RNAs by repeated passaging of infected BHK-21 or mosquito cells. The linker region of seven residues in NS5, rich in serotype-specific residues, is important for the recovery of replication fitness in the chimera RNA. Our results, taken together, provide genetic evidence for a serotype-specific interaction between NS3 and NS5 as well as specific interdomain interaction within NS5 required for RNA replication. Genome-wide RNAseq analysis revealed the distribution of adaptive mutations in RNA quasispecies. Those within NS3 and NS5 are located at the surface and/or within the NS5 dimer interface, providing a functional significance to the crystal structure NS5 dimer.

Dengue virus (DENV) is a member of the Flavivirus genus that includes a large number of human pathogens transmitted by mosquitoes, such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), the recently emerging and rapidly spreading Zika virus, or by ticks, such as tick-borne encephalitis virus (TBEV). Four serotypes of dengue viruses (DENV 1–4) cause ~390 million infections annually in >100 countries in tropical and subtropical regions of the world via Aedes mosquitoes (1). DENV exhibit ~70% amino acid identity among serotypes. This antigenic diversity potentiates multiple DENV infections by different serotypes. There is no cross-protection by the immune response from a previous DENV infection. In fact, an immune reaction against a secondary infection by a different serotype could induce severe dengue diseases such as hemorrhagic fever and/or dengue shock syndrome (2, 3).

The viral RNA has single-stranded, positive-sense polarity and codes for one long open reading frame flanked by 5′- and 3′-untranslated regions (UTR). RNA is translated into a polyprotein in the endoplasmic reticulum (ER) of the host and processed by the ER-resident host proteases, as well as the viral serine protease, by co-translational and post-translational mechanisms (4, 5). Three structural proteins, capsid (C), a precursor membrane that is processed into M (prM→M), and envelope (E), form the virion. The virion contains a single copy of a 5′-capped viral RNA that is tightly associated with the very basic capsid protein (4, 6, 7). The nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4A-2K, NS4B, and NS5) are expressed in the infected host and have multiple functions. These include polyprotein processing, viral RNA replication, 5′-capping, the assembly of immature particles that undergo maturation in the trans-Golgi network in conjunction with furin, a cellular serine protease, and finally, the release of the mature virions into the extracellular milieu. The mature virions can then infect other cells and contribute to an increase in viral load. The differences in the pathogenicity and virulence among different subgroups as well as genotypes of the same virus are dictated by the differences in the virus-host interactions.

NS3 and NS5 have multiple enzyme activities that function in viral replication and 5′-capping in the virus-induced ER membranous compartments (8–12). DENV NS3 consists of the N-terminal protease domain, which is activated by interaction with its cofactor, NS2B, to form the active protease and function in polyprotein processing. The C-terminal helicase domain of DENV NS3 (NS3hel) has RNA-stimulated NTPase (13–16), ATP-dependent double-stranded RNA unwinding activity (14–16), 5′-RNA triphosphatase activity, which is required in the first step of 5′-capping (15, 17), and ATP-independent RNA annealing activity (18). The highly conserved NS5 is the largest protein (~100 kDa with 900 aa) and consists of the guanylyl-
NS5 and NS3 interactions in viral replication

transferase and methyltransferase (MTase) domains within the first 270 aa (19–22) and the RNA-dependent RNA polymerase (POL) domain in the C-terminal region of NS5 (264–900 aa). The MTase domain has two activities, guanine N7-methyltransferase and adenosine-2′-O-methyltransferase, which work together in the formation of the type I cap, 7-MeGpppA (2′-OMe) (reviewed in Ref. 23, 24). The C-terminal POL domain of NS5 has RNA-dependent RNA polymerase activity (25–27). In the DENV2-infected cells NS3 and NS5 exist in a complex (28). The crystal structures of full-length DENV2 NS5 (29, 30) and DENV3 NS5 have been solved (31, 32), and they are dimers (32). Thus, the multiple enzyme activities of NS3 and NS5 could participate in both minus- and plus-strand RNA synthesis and 5′-capping. However, the mechanism of how the activities of NS3 and NS5 are coordinated in the ER membrane-bound replication complex is little understood.

In a previous study, we presented evidence that a functional interaction between the MTase and POL domains of DENV2 NS5 is required for viral replication (33). Even though DENV2 and DENV4 NS5 share 74% sequence identity, the replacement of NS5 MTase (1–270 aa) with the corresponding region of DENV4 severely attenuates the viral replication (33). By repeated passages of chimera DENV2 RNA-transfected BHK-21 cells for ~30 days, a replication-competent chimera virus was evolved by an adaptive mutation, K74L, in the DENV4 NS5 MTase domain or D290N in the DENV2 NS3 helicase domain. However, further study was required to dissect the functional domains of NS5 and NS3 that participate in these interactions to achieve replication fitness.

In the current study, we sought a mechanistic insight into the nature of the requirement for serotype-specific interactions between the MTase and POL domains of NS5. We used a novel approach of interserotypic substitutions within DENV2 and DENV4 NS5 as well as NS3 to understand the serotype-specific molecular interactions between NS5 and NS3.

Our results have provided a new insight into the requirement of the linker region of seven amino acids between the MTase and POL domains for the recovery of the replication-competent DENV2 chimera virus. Moreover, the adaptive mutations accumulated within the MTase and POL domains of NS5 as well as in the NS3 helicase (NS3hel) domain. These mutations are located in the NS5 dimer interface and on the NS3 surface. Thus, this study provides functional evidence and significance of the NS5 dimer in viral replication.

Results

DENV2 NS5 chimera containing the DENV4 NS5 POL domain is replication-defective

Initially we sought to analyze the two substitutions of the DENV2 POL domain with the corresponding region of DENV4 POL. DENV4 POL (264–900aa) contains the linker region between the MTase and POL domains in addition to the POL domain sequences of DENV4 NS5 (Fig. 1A). The replication of the DENV2 chimera RNA containing either DENV4 POL (271–900aa) or DENV4 POL (264–900aa) domain was severely attenuated. At day 6 post-transfection, DENV4 POL chimeras did not show any detectable immunofluorescence-positive cells (Fig. 1B), similar to the exchange of DENV2 MTase with that of DENV4 domain (33). It required serial passages of DENV2 chimera RNA-transfected cells for ~30 days for DENV4 POL (264–900aa) or longer for DENV4 POL (271–900aa) (~42 days) to achieve replication fitness (Fig. 1B, bottom panels). When the transfection experiments of the two DENV2 POL chimera RNAs were repeated, DENV4 POL (264–900aa) chimera showed a similar delayed replication. However, the replication of DENV4 POL (271–900aa) chimera RNA was not detected in two subsequent experiments (passaged for >42 days; data not shown). These results suggest that the linker region (residues 264–270 aa) upstream of DENV4 POL (271–900aa) may also be important for the DENV2 chimeric RNA to gain replication fitness.

We then used qRT-PCR to determine the viral copy numbers of DENV2 RNA chimeras containing DENV4 POL domains. Viral RNAs were extracted from the supernatants at different times post-transfection of the DENV2 RNA chimeras. The viral RNA copy numbers were in the following order: WT DENV2 > DENV4 POL (264–900aa) > DENV4 POL (271–900aa) at 14 and 30 days post-transfection (Fig. 1C). DENV4 POL (264–900aa) and DENV4 POL (271–900aa) chimeras grew very slowly and reached near WT DENV2 RNA level at 42 days (Fig. 1C). The DENV4 POL (271–900aa) chimera was the slowest in viral replication until 30 days, although subsequently it gained replication fitness quickly in one of three experiments (Fig. 1C). However, in the other two independent experiments, this chimera never gained replication fitness under similar conditions (data not shown).

The results suggest that the linker region of NS5, 264–270 aa, provides enhanced replication fitness to the DENV2 chimera virus, which contains the DENV4 POL (264–900aa), compared with the DENV4 POL (271–900aa) domain chimera. We thus sought to analyze the contribution of the linker region (264–270 aa) in the interdomain interaction between the MTase and POL domains of NS5. The sequence alignment of the region between residues 264 and 270 in NS5 shows that it is not well-conserved between the DENV2 and DENV4 serotypes (Fig. 2A) and thus may be involved in serotype-specific interactions during viral replication. We constructed three DENV2 chimera RNAs containing NS5 linker regions of DENV4, NS5 (264–270aa), NS5(257–263aa), and NS5 (271–277aa) (Fig. 2B), and analyzed their replication fitness using an immunofluorescence assay (IFA) (Fig. 2C). The transfection of DENV2 chimera RNAs containing DENV4 NS5 substitutions within the 257–277 aa region showed a variety of replication fitness levels, but they all replicated faster than DENV4 POL (264–900aa) and DENV4 POL (271–900aa) chimeras (Fig. 2C versus Fig. 1B). There was a short delay in the replication of DENV2 chimera RNA containing DENV4 NS5 (264–270aa) (Fig. 2C, compare left panels at 2 days). However, the replication progressed quickly, as shown on day 6 post-transfection by IFA, resulting in a cytopathic effect (CPE) on day 14 as shown by staining with DAPI (Fig. 2C). In contrast, the DENV2 RNA chimera containing DENV4 NS5 (257–263aa) or NS5 (271–277aa) linker region did not cause much delay in viral replication compared with the WT DENV2 and showed similar patterns of CPE (Fig. 2C).
The qRT-PCR analysis of the DENV2 chimera RNA containing DENV4 NS5(264–270aa) was consistent with the IFA results (Fig. 2, C and D). The RNA copy number of DENV4 NS5(264–270aa) chimera reached $10^6/\mu l$ at day 14 post-transfection (Fig. 2D). Thus, the linker region of NS5(264–270aa) alone plays a minor role in serotype-specific interactions within NS5, but it plays an important role as an integral part of the DENV4 NS5 POL(264–900aa) domain in the recovery of the DENV2 chimeric virus with enhanced replication fitness.

Adaptive mutations in DENV4 NS5 POL chimeras enhance replication fitness

The substitution of DENV2 POL domain with that of DENV4 POL domain (264–900 or 271–900 aa) was detrimental to viral replication, but it regained viral fitness in ≈30 days. To identify the adaptive mutations in DENV4 POL chimera RNAs, chimera RNAs containing DENV4 POL(264–900aa) and DENV4 POL(271–900aa) were transfected, and viral RNAs were extracted at the end of 30 and 42 days, respectively, from the supernatants. The viral cDNAs were synthesized by the reverse transcriptase reaction as described under “Experimental procedures.” The viral sequences were analyzed using the standard Sanger DNA-sequencing method with the primers listed in Table 1. DENV2 chimera RNAs containing DENV4 POL(264–900aa) and DENV4 POL(271–900aa) showed the common gain-of-function mutation at K76I in the DENV2 MTase domain (Fig. 3, A and C, and Table 2). In addition, the DENV4 POL(271–900aa) chimera acquired an additional mutation, D51N, also in the DENV2 NS5 MTase domain (Fig. 3C and Table 2). In another independent experiment, the DENV4 POL(264–900aa) chimera acquired the E706D mutation in the DENV4 POL domain without the K76I mutation in the DENV2 MTase domain (Fig. 3B; see Fig. 3, D and E, for sequence alignments). The viral RNA was also extracted from...
the supernatants of DENV2 chimera RNA containing DENV4 NS5(264–270 aa) linker-transfected cells at 14 days post-transfection, and sequence analysis showed no acquired mutation (Table 2).

### Evaluation of BHK-21 cell-derived adaptive mutations in DENV2 chimera RNAs for replication fitness in mosquito (C6/36) cells

Thus far, the replication of chimera RNAs and adaptive mutations for DENV4 NS5 MTase were analyzed in mammalian (BHK-21) cells. We then asked whether the adaptive mutations gained in BHK-21 cells were sufficient to support viral replication in mosquito (C6/36) cells, the natural host of DENV. The culture supernatants from the two DENV2 chimera RNAs containing the DENV4 POL(264–900aa) and POL(271–900aa) domains were used to infect C6/36 cells, and their replication fitness was analyzed by qRT-PCR (Fig. 4A). For comparison, the recovered DENV4 MTase(1–270aa) chimera virus containing either the K74I adaptive mutation in the DENV4 MTase domain or the D290N mutation in the DENV2 NS3hel domain (~30-day supernatants of BHK-21 cells (33)) was used to infect C6/36 cells. Both the DENV2 POL and the MTase chimera viruses replicated in a similar manner, although both were still slower than the WT (Fig. 4A). The qRT-PCR results confirmed
that the adaptive mutations gained in BHK-21 cells confer efficient replication fitness in C6/36 cells. To identify changes in the RNA sequence, the viral RNAs were extracted from the supernatants of infected C6/36 cells. The cDNAs were synthesized and sequenced by the standard Sanger DNA-sequencing method (Table 3 and Fig. 4B). In two independent experiments, infection of C6/36 cells with DENV2 chimera virus containing a DENV4 NS5 MTase(1–270aa) substitution and the adaptive mutation, D290N in the DENV2 NS3hel domain, gave rise to an additional mutation, K74E, in the DENV4 MTase(1–270aa) domain. Thus, the Lys74 position in the MTase domain was again targeted for substitution in the C6/36 cells, although a different amino acid (Glu instead of Ile) was chosen (Table 3). When the chimera virus containing the K74I mutation in the DENV4 MTase domain was used to infect C6/36 cells, the virus maintained the K74I mutation but acquired the V226I mutation or a silent mutation at Leu235 in the NS3hel domain in independent experiments. Asp290 and Val226, located in the conserved region of the NS3hel domain (Fig. 4, C and D), suggest that they are involved in functional interaction with the NS5 MTase domain. The appearance of highly selective adaptive mutations within the NS5 MTase and NS3hel domains of DENV2 and DENV4 suggests that there is a cross-talk between these two functional domains.

The DENV2 chimera, containing the NS3hel and the entire NS5 from DENV4, is replication-competent but does not cause CPE

It is clear that specific MTase and POL domain interactions are required for efficient viral replication. In addition, replacement of the entire DENV2 NS5 with DENV4 NS5 did not cause much delay in replication, and no adaptive mutations or CPE appeared (32), suggesting that additional protein-RNA interaction with NS5 is required to complete the viral replication cycle. A likely candidate for this interaction is the NS3hel domain, endowed with RNA-stimulated ATPase, helicase, and 5'-RNA triphosphatase activities, which are essential for RNA synthesis and 5'-RNA capping. Genetic evidence for the interaction between NS3 and NS5 was provided by the appearance of an adaptive mutation in the NS3hel region when the DENV2 MTase domain was replaced with that of DENV4 (33). Thus, we tested whether a serotype-specific interaction between NS3hel and NS5 could render the chimeric RNA as replication-competent as the wild type.

We created the DENV2 chimera RNA containing both the NS3hel domain (170–618 aa) and the entire NS5 from DENV4 (Fig. 5A, DENV4 NS3hel + NS5FL). The NS3hel domain was chosen from position 170–618 aa for substitution of the DENV4 NS3hel domain (Fig. 5, A and B) because this region was shown to have optimal RNA helicase activity in vitro for DENV2 in a previous study (34). Viral replication of DENV2 chimera RNAs containing DENV4 NS3hel + NS5FL, or the full-length DENV4 NS5FL alone was analyzed by IFA and qRT-PCR (Fig. 5, C and D). The DENV2 chimera RNA containing DENV4 NS3hel + NS5FL was slightly delayed compared with the WT DENV2 RNA, but it replicated as efficiently as DENV2 chimera RNA containing DENV4 NS5FL alone (32) (Fig. 5, C and D). Although the replication of WT DENV2 RNA showed CPE within 6 days of post-transfection, the chimera RNAs did not cause CPE even

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**Table 1**

| Primers used for chimera plasmid construction, PCR, and DNA sequencing | Sequencing primers specific for chimeric DENV domains |
|---|---|
| DENV4 POL(264–300aa) | DENV4 7000F-AAG CCT ACC CAT TTC TGT GAT TAG GTT CAG GGT CAG GGA CTT CCT TCC ACT GGC AAA CT-H11032 |
| DENV4 POL(271–300aa) | DENV4 7000R-GAG TCC TGG CCC TAT GAT GGC-3 |
| DENV4 POL(257–270aa) | DENV4 7000F-CTC GGA AGC GGA ACC CGC AGT GTC TCC ACT GAA ACA GAA ATA CCA AAC CTA GAC ATA AT-H11032 |
| DENV4 NS5FL Forward | DENV4 7000R-TAT GTC TAG GTT TGG TAT TTC TGT TTC AGT GGA GAC ACT GCG GGT TCC GCT TCC GAG GT-H11032 |
| DENV4 NS5(264–270aa) Forward | DENV4 5700F-CTC GGA AGC GGA ACC CGC AGT GTC TCC ACT GAA ACA GA-3 |
| DENV4 NS5(257–263aa) Forward | DENV4 5800F-CTC GGA ATT GAA AGT GAG AAA CCA GAC ATG ACA ATC AT-3 |
| DENV4 NS5(270–277aa) Forward | DENV4 8100F-CTC GGA ATT GAA AGT GAG AAA CCA GAC ATG ACA ATC ATT GGG AAA AGA ATA GAA AAA AT-H11032 |
| DENV4 NS3hel | DENV4 8900R-TTT CCC TTC CTG GTG TAG GGC-3 |
| DENV4 NS3NL (DENV4 FL) | DENV4 10001F-GRGG AAC AGA GTG TGG ATA GAA-3 |
| DENV4 NS3hel + NS5FL | DENV4 10100R-CAA ATC CTC TCT TTT CCC TAG-3 |
in 30 days. The recovered virus at day 14 was also subjected to standard Sanger sequence analysis, but there were no adaptive mutations within the DENV4 NS3hel or DENV4 NS5FL domains (data not shown).

**Figure 3. Acquired mutations in NS5 gene in the viruses recovered after electroporations with DENV4 POL chimera RNAs.** A, in one experiment, DENV4 POL(264–900aa) chimera RNA-transfected cells gained a K76I mutation in the DENV2 MTase domain and a Glu642 silent mutation (GAA to GAG) in the DENV4 POL domain. B, in another experiment, DENV4 POL(264–900aa) chimera RNA acquired an E706D mutation in the DENV4 POL domain. C, in only one experiment, the DENV4 POL(271–900aa) chimera RNA gained two amino acid alterations, D51N and K76I, in the DENV2 MTase domain. In two additional experiments, this chimera RNA failed to gain replication fitness. Amino acid sequence alignments of NS5 MTase (70–85 aa) (D) and NS5 POL (698–714 aa) (E) domains of four serotypes of DENV are shown.

**Analysis of DENV2 quasispecies in chimera RNA populations generated during replication**

Thus far, the acquired mutations in DENV2 chimera viruses subsequent to serial passages were analyzed first by the standard Sanger DNA-sequencing method using PCR (33) (Tables 2 and 3 and Figs. 3 and 4 in this study). However, this method can only detect dominant mutations but not sequence diversity present as RNA quasispecies in the virus populations. The diversity of RNA virus populations has been shown to help viruses to evolve and acquire gain of function in replication (35–37) (see a review in Ref. 38). To further examine and identify whether RNA quasispecies are involved during the evolution of WT and chimeric RNA to gain replication fitness, we
analyzed the diversity of the virus populations in both BHK-21 and C6/36 cells by next-generation sequencing (NGS). DENV2 WT RNA was transfected into BHK-21 cells. At day 6 posttransfection there was an abundant loss of cells due to CPE. However, the serial passages of WT DENV2 RNA-transfected BHK-21 cells were made possible by the addition of naive BHK-21 cells along with fresh medium every 4 days. C6/36 cells were infected with the 6-day supernatant from the DENV2 RNA-transfected BHK-21 cells and were serially passaged every 4 days. The infected C6/36 cells did not exhibit CPE. The viral sequence diversity between DENV2 WT cultured in BHK cells and C6/36 cells for 30 days was compared first. RNA quasispecies diversity in the chimera RNA. Mutations within these domains (Table 5) were dramatically different from the WT DENV2 passaged in BHK-21 cells. Both WT DENV2 and DENV2 chimera RNA-transfected BHK-21 cells contained adaptive mutation in the NS1 gene (29.4 and 21.7%, respectively) (Tables 4 and 5). However, in contrast to the WT DENV2 RNA, which showed low-frequency mutations in the nonstructural protein genes, NS2A to NS5 (Table 4), chimera viruses acquired mutations as quasispecies in NS2A (19.3%), NS3 (7.1%), NS4A (~7%), and NS5 (3.0–99%) genes. In particular, the DENV2 chimera virus containing DENV4 MTase or DENV4 POL had multiple alterations within these domains (Table 5). For the DENV4 MTase chimera, the most predominant mutation (~99%) was K74I in the MTase domain in the NGS analysis, confirming the results determined previously by the conventional Sanger DNA-sequencing method (33). For the DENV2 chimera containing DENV4 POL (264–900aa), the predominant mutations (~20%) were found in E protein, NS5, and 3'-UTR. In particular, NS5 contained K76R (41%) and P137S (53%) in the DENV2 MTase domain and E650K (53%) and P693L (30%) in the DENV4 POL domain. In contrast, the DENV4 NS3hel + NS5FL chimera, which was able to replicate with better efficiency than the DENV4 MTase or POL chimera, had predominant mutations in the E protein (Q400H), NS5 POL (a silent mutation at Ala383), and 3'-UTR (position nt 10414, A→T) (Table 5). However, two common mutations were observed in the chimera viruses containing DENV4 POL and DENV4 NS3hel + NS5FL, one at the NS4A gene (T135P) and the other in the 3'-UTR (A→T at nt 10414) (Table 5). These results indicate that either the replacement of DENV2 MTase or POL domain, or the replacement of the region encoding both NS3hel and NS5FL, with the corresponding region of DENV4, generated RNA quasispecies diversity in the chimera RNA. Mutations were distributed throughout the genome, including 3'-UTR, during evolution to achieve replication fitness (Table 5).

**DENV2 chimera virus, with acquired mutations from growth in BHK-21 and C6/36 cells, exhibits CPE and forms infectious particles**

Our results thus far indicated that gain-of-replication fitness by DENV2 chimera virus containing the DENV4 MTase domain, even after acquiring the K74I mutation from BHK-21 cells, was not sufficient to cause CPE or form plaques. Therefore, we analyzed whether the DENV2 chimera viruses that gained additional mutations in mosquito C6/36 cells, namely, DENV4 MTase-K74I + DENV2 NS3hel-V226I, NS3hel-L235* (asterisk indicates silent mutation), or DENV4 MTase-K74E + DENV2 NS3hel-D290N (see Table 3), could cause CPE and form plaques, which are hallmarks of productive infection producing progeny virus particles. As a control, we used a DENV2

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**Table 2**

Amino acid alterations gained within NS5 and NS3 genes of chimera viruses in BHK-21 cells

| Chimera type                  | No. of repeats | No. of cases | NS5 MTase | NS5 POL | NS3hel |
|-------------------------------|----------------|--------------|-----------|---------|---------|
| DENV4 NS5 MTase              | 3              | 2            | K74I      | ND      | ND      |
| DENV4 NS5 MTase              | 1              | 1            | ND        | ND      | D290N   |
| DENV4 POL(264–900aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 POL(271–900aa)         | 3              | 1            | K76I 'Silent' | ND | ND |
| DENV4 NS3(264–270aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 NS5(257–263aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 NS5(271–277aa)         | 2              | 2            | ND        | ND      | ND      |

a Independent transfections were performed.
b ND, not detected.
c Teramoto et al. (33).
d Klema et al. (32).
e Silent mutation at Glu642 (GAA to GAG) was observed.
f Two experiments were not successful in producing IFA-positive cells even beyond 42 days.

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**NS5 and NS3 interactions in viral replication**

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**Table 2**

Amino acid alterations gained within NS5 and NS3 genes of chimera viruses in BHK-21 cells

| Chimera type                  | No. of repeats | No. of cases | NS5 MTase | NS5 POL | NS3hel |
|-------------------------------|----------------|--------------|-----------|---------|---------|
| DENV4 NS5 MTase              | 3              | 2            | K74I      | ND      | ND      |
| DENV4 NS5 MTase              | 1              | 1            | ND        | ND      | D290N   |
| DENV4 POL(264–900aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 POL(271–900aa)         | 3              | 1            | K76I 'Silent' | ND | ND |
| DENV4 NS3(264–270aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 NS5(257–263aa)         | 2              | 2            | ND        | ND      | ND      |
| DENV4 NS5(271–277aa)         | 2              | 2            | ND        | ND      | ND      |

a Independent transfections were performed.
b ND, not detected.
c Teramoto et al. (33).
d Klema et al. (32).
e Silent mutation at Glu642 (GAA to GAG) was observed.
f Two experiments were not successful in producing IFA-positive cells even beyond 42 days.
chimera RNA containing DENV4 MTase-K74I mutation, which could replicate in BHK-21 cells although significantly more slowly than WT DENV2 (33)). We showed that the DENV2 chimera virus containing DENV4 MTase/K74I and DENV2 NS3hel-V226I mutations gained the ability to replicate (Fig. 6A, row 3), caused CPE, and formed plaques (Fig. 6, B and D). The qRT-PCR analysis showed that the viral RNA copy number is comparable with that of WT DENV2 RNA (Fig. 6C). Other chimera viruses acquired replication fitness, as shown by IFA and qRT-PCR, but they were still defective when compared with WT in causing CPE and forming plaques (Fig. 6A, rows 4 and 5, and D).

**Discussion**

In this study, we have shown that genetic interactions among NS3hel, NS5 MTase, and NS5 POL are required for viral replication, using a DENV2 chimera RNA containing the corresponding regions from DENV4. The DENV2 chimeras RNAs gain a variety of mutations as quasispecies (Table 5), which are likely to contribute toward overcoming the replication block...
when a functional domain from a heterologous serotype is substituted. Despite gaining replication fitness, the DENV2 chimera viruses did not show any CPE or ability to form plaques. We have shown that the RNA quasispecies generated in BHK-21 cells are not sufficient to form infectious particles and that additional passaging in the mosquito C6/36 cells is important for gaining this ability. The patterns of RNA quasispecies generated by WT DENV2 RNA during replication in BHK-21 and C6/36 cells, as well as the DENV2 chimera RNA with MTase or POL domain substitutions during replication in BHK-21 cells, are different (Tables 4 and 5). This study underscores the importance of versatile adaptation of RNA viruses to different host environments to promote their life cycle. Interestingly, the compensatory mutations in the MTase and POL domains occur in the serotype-specific residues for DENV2 and DENV4 NS5 (Table 5 and Fig. 7), presumably to restore the interdomain interactions. We mapped the locations of the compensatory mutations (Asp51, Lys76, Pro137, Glu650, Glu226), and p < 0.01 was confirmed by one-way ANOVA with Bonferroni post-test.

### Table 3

Amino acid alterations gained after infection of mosquito (C6/36) cells with chimera viruses recovered from BHK-21 cells

| Chimera virus                  | No. of repeats | No. of cases | C6/36-acquired mutations in DENV2 chimera RNA containing DENV4 NS5 MTase(1–270aa) | DENV2 NS5 POL(271–900aa) | NS3hel |
|-------------------------------|----------------|-------------|---------------------------------------------------------------------------------|--------------------------|--------|
| DENV4 MTase-K74I              | 2              | 1           | K74I                                                                             | ND                       | V226I  |
| DENV4 MTase- DENV2NS3 D290N   | 2              | 2           | K74I                                                                             | K74I                     | D290N  |

*Infection of C6/36 cells and analysis were performed twice using the same supernatant collected ~30 days post-transfection by electroporation in BHK-21 cells (33).

*ND, not detected.

*Silent mutation at Leu235 (CTA to CTG) was observed.

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**Figure 5.** DENV2 chimera containing DENV4 NS3hel + DENV4 NS5FL analyzed for replication fitness. **A,** the region replaced with DENV4 NS3hel + NS5FL is depicted. **B,** amino acid sequences in the boundary between NS3 protease and helicase domains in DENV2 and DENV4 are compared. **C,** replication of DENV2 chimera RNA containing NS3hel + NS5FL substitution was analyzed by immunofluorescence staining using anti-NS1 antibody. **D,** extracted WT DENV2, DENV2 chimera RNAs containing DENV4 NS5, or DENV4 NS3hel + DENV4 NS5FL RNA from the supernatants of BHK-21 cells was measured as to viral RNA copy number. The viral RNA copy numbers represent mean values ± S.E. (n = 2), and p < 0.01 was confirmed by one-way ANOVA with Bonferroni post-test. NT, not tested by qRT-PCR.
NS5 and NS3 interactions in viral replication

Table 4
RNA quasispecies generated in BHK-21 cells transfected with DENV2 WT RNA or C6/36 cells infected with supernatant from 6-day culture of BHK-21-transfected cells

This experiment is performed in detail under “Experimental procedures.” The original amino acid sequence, position, altered sequence, and frequency (%) are shown in parentheses. At 3′-UTR, the original RNA sequence, altered sequence, and frequency (%) are shown. The cutoff for inclusion in the Table is 3% abundance. ND, not detected. The numbers outside the parentheses indicate the nucleotide positions that result in amino acid changes indicated within the parentheses.

| RNA locus | BHK-21 cells | C6/36 cells |
|-----------|--------------|-------------|
| 5′-UTR    | ND           | ND          |
| prM       | ND           | ND          |
| (M: 712–936) | 718 (A94S, 3.9%) | ND |
| E         | 1104 (Leu26, 3.4%) | 1382 (H149P 8.8%) |
|           | 1177 (S81R, 28.9%) | 1455 (Ala1162, silent 4.5%) |
|           | 1483 (M183V, 4.6%) | 1493 (S186F, 9.0%) |
|           | 1622 (S229L, 3.9%) | 1782 (H282Q, 7.9%) |
|           | 1656 (Phe240, silent 6.3%) | 1787 (K284R, 5.0%) |
|           | 2162 (I454T, 9.3%) | 2229 (Ser233G, 29.4%) |
| NS1       | 3118 (S233G, 29.4%) | 2964 (Ser181, silent 3.7%) |
| NS2A      | ND           | 3607 (I44V 4.9%) |
| NS2B      | 4221 (Gly36, silent 5.5%) | ND |
| NS3pro    | ND           | ND          |
| NS3hel    | ND           | ND          |
| NS4A      | 6778 (T135P, 7.7%) | ND |
| NS4B      | 7561 (T246P, 4.0%) | ND |
| NS5MT     | ND           | ND          |
| NS5POL    | 8555 (T329K, 4.0%) | ND |
| 3′-UTR    | ND           | 10293 (C to T 4.7%) |
|           |              | 10384 (C to T 4.0%) |
|           |              | 10387 (T to A 4.4%) |
|           |              | 10416 (C to T 3.4%) |
|           |              | 10418 (G to A 3.9%) |
|           |              | 10422 (G to A 5.0%) |

Pro693, and Glu706) accumulated in the viral RNA encoding chimeric NS5 containing DENV2 MTase/DENV4 POL domain on the monomeric and dimeric structures of DENV3 NS5 (32) (Fig. 7; Tables 2 and 5). Since the crystal structure of DENV2 NS5 is not known, the corresponding residues of DENV2 NS5 are shown on the DENV3 NS5 structure in Fig. 7. The six residues align to one side of the NS5 molecule in the NS5 monomer. Except for Asp61 and Pro693, the MTase and POL residues are located on the opposite end of the NS5 molecule, and thus are unlikely to be involved in direct interaction. However, these residues are located near the monomer–monomer interface in the NS5 dimer. Although the oligomeric state of NS5 in the functional replication complex is not known, cellular interaction and pulldown assays indeed demonstrate that NS5 interacts with itself (42, 43), suggesting that intermolecular interactions between NS5 monomers, i.e. dimer formation, may be important for viral replication.

In a previous study (44) using West Nile virus full-length RNA, the conserved Asp146 residue within the Lys61-Asp146-Lys182-Glu218 motif, which is essential for both 2′-O MTase and N-7 MTase activities, was mutated to Leu, Pro, Arg, or Ser. Only the D146S mutant RNA replicant produced a viable virus with compensatory mutations in the MTase (K61Q) and POL (W751R) domains as well as in the 5′-terminal stem-loop of the genomic RNA (a G35U substitution or U38 insertion). Thus, compensatory mutations would account for both restoring enzymatic activity and necessary protein-protein or protein-RNA interactions during genome replication. In the DENV2 chimera RNA containing the DENV4 NS5 MTase domain studied here, the highly conserved Lys61-Asp146-Lys182-Glu218 motif or any other active site residues in the MTase or POL domains were not perturbed, as they are conserved in the MTase or POL domains of both serotypes. Thus, the lack of replication of the chimera RNA and subsequent compensatory mutations would suggest perturbation of essential protein–protein or protein-RNA interaction sites rather than enzymatic defects. Consistent with this conclusion, analyses of the MTase and POL enzyme activities of the DENV2 chimera NS5FL, containing the DENV4 NS5 MTase domain were also similar to that of WT DENV2 NS5FL (33).

The linker region of NS5, consisting of residues 264–270 (DENV2 numbering) in DENV4 NS5POL(264–900aa), is also involved in viral replication and recovery of replication-competent virus (Fig. 2). Mutation studies of the NS5 linker, guided by the crystal structure, suggest that the flexibility of the linker and the identity of residues are important for viral replication (31, 45). Our data show that the linker residues are also important for serotype-specific interactions in NS5. The linker indeed contains four serotype-specific residues, Asn264, Ile265, Gly266, and Ile267, in the DENV2 sequence, which are Ser, Val, Ser, and Thr, respectively, in DENV4 (32). It has been shown previously that residues 263–271 (DENV3 numbering) fused to the N terminus of the DENV3 POL domain contribute to the stability and de novo initiation of nascent RNA synthesis by the polymerase. The DENV3 POL(265–900aa) domain had enhanced polymerase activity compared with DENV3 POL(272–900aa) (46). Thus, interaction between the linker and the POL domain from the same DENV serotype may contribute to robust viral replication and the recovery of the DENV2 chimera virus with replication fitness.

Our results indicated that although DENV2 chimera RNA containing DENV4 MTase produced K74I in the MTase and D290N, V226I, and T317S in the DENV2 NS3hel domains, DENV2 chimera RNAs containing DENV4 NS5FL and DENV4 NS5FL + DENV4 NS3hel were able to be replicated without much delay or any adaptive mutations (Ref. 32 and this study). These results also suggest that specific interaction between NS3hel and NS5 may not involve serotype-specific residues. The NS3hel residues mapped near the RNA-binding site on the protein surface in the crystal structure (Fig. 7), suggesting their functional interaction with the NS5 MTase domain.

Finally, the genetic interaction between NS5 and 3′-UTR was observed also in the DENV2 chimera RNA containing either DENV4 NS5 POL(264–900aa) or DENV4 NS3hel + NS5FL (Table 5). Both chimera RNAs had the same A→T mutation at 10414 nt. Because the RNA structure of 3′-UTR is recognized by the NS5 complex prior to minus-strand RNA synthesis, serotypic differences in the DENV4 POL domain may have required that mutation in the DENV2 3′-UTR. In conclusion, our study reveals that specific interactions among NS3 helicase, NS5 MTase, NS5 POL, and 3′-UTR play an essential role in viral replication (reviewed in Ref. 12).
Table 5

Analysis of RNA quasispecies in DENV2/DENV4 chimera viruses in BHK-21 cells

The original amino acid sequence, position, altered sequence, and frequency (%) are shown in parentheses. At 5′- and 3′-UTR, the original RNA sequence, altered sequence, and frequency (%) are shown. The cutoff for inclusion in the Table is >3% abundance. ND, not detected. The numbers outside the parentheses indicate the nucleotide positions that result in amino acid changes indicated within the parentheses.

| RNA locus   | DENV4 MTase(1–270aa) | DENV4 POL(264 – 900aa) | DENV4 NS3hel(170 – 618aa) + NS5FL |
|-------------|----------------------|------------------------|----------------------------------|
| 5′-UTR      | ND                   | ND                     | 54 (C to T, 6.1%)                |
| C           | ND                   | ND                     | 167 (S24L, 3.2%)                 |
| prM (N: 712–936) | 540 (Cys44, silent 26.7%) | 842 (I135T, 15.2%) | 868 (I144L, 7.0%)                 |
| E           | 1015 (H277, 3.8%)    | 1142 (T69I, 23.3%)     | 1345 (Y137H, 3.1%)                |
| NS1         | ND                   | 3977 (V167A, 19.3%)    | 1660 (N242D, 3.4%)                |
| NS2A        | ND                   | ND                     | 2136 (Q400H, 84.9%)               |
| NS2B        | ND                   | ND                     |                                  |
| NS3pro      | ND                   | ND                     |                                  |
| NS3hel      | 5470, 5477 (T317S, 7.1%) | 5296 (Leu296, silent 3.8%) | 6061 (E514L, 3.1%)                 |
| NS4A        | 6951 (Ala190, silent 3.6%) | 6778 (T135P, 7.1%)     | 6778 (T135P, 6.7%)                |
| NS4B        | ND                   | ND                     | 7762 (I65L, 5.9%)                 |
| NS5MT       | 7790 (K74L, 99.2%)   | 7718 (T50K, 3.9%)      | 8718 (Ala190, silent 87.1%)       |
| NS5POL      | 8265 (Val212, silent 3.7%) | 7720 (D51N, 8.8%)     | 9136 (D523H, 6.8%)                |
| 3′-UTR      | ND                   | ND                     | 9408 (Thr113, silent 4.5%)        |

**Experimental procedures**

**Construction of cDNAs encoding DENV2 chimera RNAs**

The full-length DENV2 cDNA clone (New Guinea C strain, pRS424-FLDENV2) was a gift from Dr. Barry Falgout, U.S. Food and Drug Administration. The construction of this clone in the yeast/Escherichia coli shuttle vector, pRS424, has been described (47). The sequence of this clone differs from the one deposited in GenBankTM (M29095.1) (48) at 16 nucleotide positions (described in Table 2 of Ref. 47). For ease of mutagenesis of the DENV2 cDNA cloned into pRS424 using homologous recombination in yeast (Polo et al. (47)), we used Dr. Falgout’s DENV2 cDNA for all of our studies. The construction of cDNAs encoding the DENV2 chimera containing the DENV4 MTase domain alone (33) or the DENV4 NS5FL (32) in the yeast/E. coli shuttle vector have been described previously. In this study, DENV2 chimera containing the DENV4 POL domains (264–900 and 271–900 aa) and the DENV4 NS5 linker regions containing amino acid residues 264–270, 257–263, and 271–277 were created in a similar manner. The replacement region was amplified by PCR using the sequence-specific primer pair (Table 1) and pRS424-DENV4 cDNA (a gift from Dr. Barry Falgout) as the template. The amplified DNA fragment in each case was mixed with AatII-digested pRS424-FLDENV2 cDNA encoding full-length DENV2 RNA. A yeast recombination method was used to create chimeric virus cDNAs having indicated portions of DENV4 NS5 in their DENV2 backbone. The DENV4 NS3hel + NS5FL cDNA was created with the PCR product of DENV4 NS3hel (primer pair, Table 1 and the template pRS424-DENV4 cDNA) and the XhoI-digested DENV2 chimera cDNA containing DENV4 NS5FL (32). The chimera cDNA clones were sequenced for verification. All chimera plasmids were linearized using the BglI enzyme at the 3′-end of the viral cDNA sequence, and these were used as the template for in vitro transcription catalyzed by SP6 RNA polymerase (Epicenter Biotechnologies) in the presence of the 7-MeGpppG cap analog.

**Electroporation, mammalian and mosquito cell culture, and immunofluorescence assay**

RNAs (~3 µg) were transfected by electroporation (Amara Nucleofector II system, Amara Biosystems, Cologne, Germany) into BHK-21 (American Type Culture Collection, Manassas, VA) cells (~1 × 10⁶ cells resuspended in 100 µl of Ingenio solution (Mirus Bio, Madison, WI). The pulsed cells were transferred into Eagle’s minimum essential medium supplemented with 5% fetal bovine serum and 1× streptomycin/penicillin. Cells were cultured by splitting them on day 2 from a T-25 to a T-75 flask. This procedure was repeated using one-third of the trypsinized cells with fresh medium for growth in T-75 flask every 4 days. For the immunofluorescence assay, at the end of the indicated time periods RNA-transfected cells were seeded into a slide (LabTek) and fixed by acetone. Cells were incubated with a 1:200 dilution of 7E11, a monoclonal antibody against DENV2 NS1. FITC-labeled goat anti-mouse immunoglobulin G conjugate (Kirkegaard & Perry Laboratories) was used as a secondary antibody at a 1:100 dilution. Immunofluorescence photomicrographs (~200 magnification) were acquired using an Olympus IX-71 inverted Epifluorescence microscope coupled to the Olympus automated photographic system.

**Quantitative real-time PCR with viral RNA extracted from cell-culture supernatants**

Viral RNA was extracted from supernatants of BHK cells that were electroporated with either WT or chimeric RNAs using a ZR viral RNA kit (Zymo Research). The cDNAs were synthesized from the extracted RNAs using M-MuLV reverse transcriptase (New England Biolabs) at 42 °C incubation for 2 h.
qPCR, performed in duplicate, included a no-template control and a standardized DENV 2 cDNA fragment. Each sample was mixed with iTaq™ Universal SYBR Green Supermix (Bio-Rad) and 8 pmol of primers targeting the DENV2 NS4A region (7000F and 7200R, shown in Table 1) in 20 μl of reaction volume. qPCR was performed with two-step reactions: 40 cycles of 95 °C for 10 s and 60 °C for 30 s after initial denaturation at 95 °C for 3 min using iQ5 Optical System software, version 2.1 (Bio-Rad). The average Ct values were converted to viral copy numbers in comparison with the standard DENV2 DNA.

**Sanger DNA sequencing of viral RNAs**

The synthesized viral cDNA from collected supernatants was amplified by PCR using NEBNext High Fidelity 2× polymerase (New England Biolabs) and the viral sequence-specific primers (Ref. 33 and Table 1) to make overlapping fragments (each ~800 bp) that spanned the entire genome. PCR products were purified by agarose gel (Zymo Research), and the nucleotide sequence of each fragment was determined by GENEWIZ Inc.

**Next-generation sequencing (RNAseq)**

Supernatants (~10 ml) from BHK-21 or C6/36 cells cultured in T-75 flasks were used for viral RNA extraction. Briefly, the debris in the supernatants was removed by centrifugation at 1500 rpm for 5 min, and a 2.5 ml of 40% PEG 8000 was added to 10-ml supernatants. The mixed solutions were stirred at 4 °C for 2 h and refrigerated further for 3 h without stirring. After centrifugation at 11,000 rpm for 45 min at 4 °C, each pellet was collected with ~0.5 ml of the remaining solution and mixed with 1.5 ml of TRIzol-LS (Ambion). The pellet and the solution were dispersed and well-mixed by pipetting. Chloroform (0.4 ml) was added and mixed further. The solution was centrifuged
at 12,000 × g for 15 min, and the upper layer was collected and precipitated by the addition of ethanol. The pellet was resuspended in nuclease-free water, and the final RNA concentrations were prepared in a range between 25 and 100 ng/μl.

cDNA library preparation and next-generation sequencing procedures were performed by ABM Inc. (Richmond, Canada). Briefly, a quality control check was performed with the Agilent 2100 Bioanalyzer. All samples had rRNA peaks. To remove the rRNA, a WT DENV2 RNA sample (1.1 μg) from cultured C6/36 cells was subjected to the Illumina RiboZero rRNA depletion kit (for mosquito cells). The other samples (330–800 ng) from WT DENV2 RNA from cultured BHK-21 cells and DENV2 chimeras containing the DENV4 POL (264–900 aa), DENV4 MTase, and DENV4 NS3 hel domains were subjected to NEBNext rRNA depletion (for mammalian cells). All samples were input into the Illumina TruSeq stranded mRNA library prep kit, which consists of RNA fragmentation followed by first- and second-strand cDNA synthesis, adenylation of the 3’-ends, adapter ligation, and DNA fragment enrichment via PCR (10 cycle). The libraries' quality control check was performed using an Agilent 2100 Bioanalyzer. All samples had rRNA peaks. To remove the rRNA, a WT DENV2 RNA sample (1.1 μg) from cultured C6/36 cells was subjected to the Illumina RiboZero rRNA depletion kit (for mosquito cells). The other samples (330–800 ng) from WT DENV2 RNA from cultured BHK-21 cells and DENV2 chimeras containing the DENV4 POL (264–900 aa), DENV4 MTase, and DENV4 NS3 hel domains were subjected to NEBNext rRNA depletion (for mammalian cells). All samples were input into the Illumina TruSeq stranded mRNA library prep kit, which consists of RNA fragmentation followed by first- and second-strand cDNA synthesis, adenylation of the 3’-ends, adapter ligation, and DNA fragment enrichment via PCR (10 cycle). The libraries’ quality control check was performed using an Agilent 2100 Bioanalyzer showed a range of library sizes, with the curve being mostly between 200 and 400 base pairs long. The libraries were pooled together with equal amounts based on the Agilent quantification. Real-time qPCR was used to quantify the pooled libraries for optimal clustering during sequencing. All samples were sequenced on a single Illumina NextSeq500 run, generating paired-end 75-base-pair readings. The .bcl files generated by the sequencer were converted to Fastq data immediately after the runs using the software bcl2fastq v2.17.1.14. Alignment analyses of DENV2 WT and chimeric genomes were performed with Geneious 9.1 software (Biomatters), which differentiates statistically significant variants from total SNPs identified within reads. Briefly, paired-end sequences were made from Fastq data R1 and R2. Sequenced fragments (contigs) were trimmed with the quality scores limit set to 0.05. The trimmed contigs were aligned to the reference (input viral cDNA) sequence, and the SNPs were analyzed. Nucleotide alterations at more than 3% frequency are shown.

**Analysis of CPE and infectivity of DENV2 chimera viruses with gain-of-replication fitness**

Supernatants collected from BHK cells after electroporation of chimera RNAs were used for infection of C6/36 cells. Chimeric viruses (5 × 10⁷ viral RNA copies, ~100 μl of the supernatant measured by qRT-PCR) were added to 5 × 10⁵ C6/36 cells. Cells were expanded to a T-75 flask and passaged by splitting every 5–6 days by mixing one-third of the infected cells in a fresh growth medium (Eagle’s minimum essential medium supplemented with 5% FBS, 1× streptomycin/penicillin, 1× non-essential amino acids mixture (Gibco), and 25 mM HEPES). Supernatants were collected when the cells were split and used later to infect BHK-21 cells as well as Vero cells. Plaque assays were performed using Vero cells. Serially diluted supernatants were mixed with 5 × 10⁵ cells/well in paired wells of 6-well cell culture plates for 2 h. Cells overlaid with 0.5% SeaPlaque agarose (Lonza)-containing growth medium were incubated for 9 days at 37 °C and stained with neutral red.
**NS5 and NS3 interactions in viral replication**

**Author contributions**—T. T., A. B., and R. P. conceived and designed the experiments. T. T. and A. B. performed the experiments. T. T., A. B., K. H. C., and R. P. analyzed the data. T. T., A. B., and K. H. C. contributed reagents/materials/analysis tools. T. T., K. H. C., and R. P. wrote the paper.

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NS5 and NS3 interactions in viral replication