The genus *Flavivirus* from the family *Flaviviridae* contains more than 70 viruses, many of which are important human pathogens causing major public health threats worldwide. Dengue virus (DENV)\(^2\) is a mosquito-borne flavivirus responsible for 50–100 million human infections and \(\sim 20,000\) deaths each year (1). Besides DENV, West Nile virus (WNV), Japanese encephalitis virus, yellow fever virus, and tick-borne encephalitis virus also cause significant human diseases (1). No antiviral therapy is currently available for treatment of flavivirus infections. Therefore, development of antiviral therapy is urgently needed for flaviviruses.

The flavivirus genome is a single strand, plus-sense RNA of about 11 kb in length. The genomic RNA contains a 5\(^{\prime}\)-untranslated region (UTR), a single open reading frame, and a 3\(^{\prime}\)-UTR. The single open reading frame encodes a long polyprotein that is processed by viral and host proteases into 10 mature viral proteins (2). Three structural proteins (capsid, pre-membrane, and envelope) are primarily involved in virus particle formation, and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are mainly responsible for viral replication (2). Among these, NS3 and NS5 possess enzymatic activities and have been targeted for antiviral development. NS3 functions as a protease (with NS2B as a cofactor), helicase, 5\(^{\prime}\)-RNA triphosphatase, and nucleoside triphosphatase (3–5). NS5 is the largest and the most conserved viral protein. The N-terminal part of NS5 is a methyltransferase that methylates the N-7 and 2\(^{\prime}\)-O positions of the viral RNA cap structure (6–9); the C-terminal part of NS5 has an RNA-dependent RNA polymerase (RdRp) activity (10, 11). Because the RdRp activity is essential for viral replication, and human host cells are devoid of RdRp, it is an attractive target for antiviral development.

Two approaches have been successfully used to develop RdRp inhibitors in clinical trials for hepatitis C virus (HCV) (12), a virus closely related to flavivirus. The first approach is based on a nucleoside analogue. Nucleoside analogues function as RNA chain terminators to block viral RNA synthesis. The second approach is based on an allosteric inhibitor. Allosteric inhibitors bind to pockets on polymerase to block conformational changes required for enzyme function. Both nucleoside analogues and allosteric inhibitors (targeting one of the four pockets in HCV RdRp) are currently in clinical trials (12). The success of HCV RdRp inhibitors suggests that the same approaches could be used to develop inhibitors of flavivirus RdRp.

Flavivirus RdRp initiates the RNA synthesis via a *de novo* mechanism, which differs from a primer-dependent mechanism used by other viruses, such as poliovirus and SARS-CoV (10, 13–15). Crystal structures of RdRp from DENV-3 and WNV have been reported (16, 17). Like all polymerases, the structure of flavivirus RdRps resembles a right hand with characteristic fingers, palm, and thumb subdomains. Malet et al. (18) recently proposed two cavities (named cavities A and B) that were conserved in the crystal structures of DENV and WNV RdRp. Both cavities are located in the thumb subdomain and could be potentially targeted for development of small molecule inhibitors. However, the biological relevance of the cavities has not been validated.
In this study, we performed a biochemical and genetic analysis of the two cavities conserved among flavivirus RdRp. Mutagenesis analysis was used to analyze the function of the cavities in viral replication. Residues critical for viral replication were subjected to revertant analysis. In addition, the critical residues were further examined for their roles in RdRp activity. Our results demonstrate that cavity B, but not cavity A, is essential for DENV replication. Mutations in cavity B could abrogate viral replication by decreasing the initiation of RdRp activity; alternatively, mutations in cavity B could suppress viral replication by interference with the interaction between the NS5 and NS3 helicase domains. The results have provided functional evidence that cavity B could be a rational target for drug discovery.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Baby hamster kidney cells (BHK-21) and African green monkey kidney cells (Vero) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37 °C. DENV-2 TSV01 strain (GenBank™ accession number AY037116) was first isolated from a patient in Australia in 1993 and then was passaged on African green monkey kidney cells (Vero) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37 °C. DENV-2 TSV01 strain (GenBank™ accession number AY037116) was first isolated from a patient in Australia in 1993 and then was passaged on Vero cells (ATCC) were seeded with a cell density of 1 × 10⁵ per well in a 24-well plate (Nalge Nunc) 2 days in advance. A series of 1:10 dilutions were made by mixing 15 μl of virus sample with 135 μl of RPMI 1640 medium containing 2% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. One hundred microliters of undiluted and 10-fold dilutions of viral supernatant were seeded to individual wells of 24-well plates. The plates were incubated at 30 °C with 5% CO₂ for 1 h with shaking every 15 min, and then the virus inocula were replaced with 0.6 ml of RPMI 1640 medium plus 0.8% methylcellulose (Aquacide II, Calbiochem) and 2% FBS. After 4 days of incubation at 37 °C with 5% CO₂, the cells were fixed with 3.7% formalin and stained with 1% crystal violet. For some mutant viruses, the incubation period was more than 4 days to visualize the plaques.

**Plaque Assay**—Plaque assay was performed as described previously with minor modifications (24). Briefly, BHK-21 cells (ATCC) were seeded with a cell density of 1 × 10⁵ per well in a 24-well plate (Nalge Nunc) 2 days in advance. A series of 1:10 dilutions were made by mixing 15 μl of virus sample with 135 μl of RPMI 1640 medium containing 2% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. One hundred microliters of undiluted and 10-fold dilutions of viral supernatant were seeded to individual wells of 24-well plates. The plates were incubated at 30 °C with 5% CO₂ for 1 h with shaking every 15 min, and then the virus inocula were replaced with 0.6 ml of RPMI 1640 medium plus 0.8% methylcellulose (Aquacide II, Calbiochem) and 2% FBS. After 4 days of incubation at 37 °C with 5% CO₂, the cells were fixed with 3.7% formalin and stained with 1% crystal violet. For some mutant viruses, the incubation period was more than 4 days to visualize the plaques.

**Selection and Sequencing of Revertant Viruses**—Five mutant viruses defective in viral replication were subjected to revertant analysis by continuously culturing on Vero cells for seven rounds. For each round of culturing, Vero cells (1 × 10⁵ cells) in a T-25 flask were infected with 500 μl of culture supernatant derived from the previous passaging and cultured in DMEM with 2% FBS in 5% CO₂ at 37 °C. The cytopathic effect was monitored under a microscope. The incubation period lasted 5 days unless an apparent cytopathic effect appeared earlier. Viral RNA extracted from culture supernatants of passages 2 and 7 was subjected to RT-PCR using SuperScript III one-step RT-PCR kits (Invitrogen). The entire NS5 region or the complete viral genome was sequenced.
Functional Analysis of Dengue Virus RdRp Cavities

RdRp Assay—Recombinant protein of full-length DENV-4 NS5 was prepared using an E. coli expression system and purified through affinity and gel filtration chromatography. Mutagenesis of the NS5 expression plasmid was performed using QuikChange II XL site-directed mutagenesis kit. The detailed protocols for mutagenesis, protein expression, and purification have been reported previously (25). Two types of RdRp assays were performed, de novo RdRp assay and elongation RdRp assays. The de novo RdRp reaction (25 μl) contained 50 mM HEPES, pH 8.0, 10 mM KCl, 5 mM MgCl2, 2 mM MnCl2, 10 mM DTT, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 2.5 μCi of [α-32P]GTP (10 μCi/μl, 3,000 Ci/mmol; PerkinElmer Life Science), 0.25 μg of NS5, 0.5 μg of RNA template. The RNA template was transcribed in vitro from a PCR product using a T7 MEGAscript kit (Applied Biosystems). The PCR template contained a T7 promoter followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotide 169 to 10,263 (GenBankTM accession number AF038403.1). The de novo RdRp reactions were incubated at 37 °C for 30 min; the mixtures were passed through a MicroSpin G-25 column (GE Healthcare) to remove unincorporated NTPs; and the RNA elute was extracted with phenol/chloroform and precipitated with ethanol. The RNA pellet was dissolved in 10 μl of RNase-free water, mixed with an equal volume of denaturing Gel Loading Buffer II (Applied Biosystems), and then loaded onto a 10% denaturing polyacrylamide gel with 7M urea. A PhosphorImager was used to quantify the 32P-labeled RNA products. The elongation RdRp assay was performed using an RNA template and a 2-fold serially diluted DENV-4 full-length NS3 (0.1–24 μg) or a 0.1–24 μg of RNA generated about 1.2 × 105 pfu/ml on day 4 p.t. (supplemental Fig. 1B). Specific infectivity assay showed that transfection of BHK-21 cells with the in vitro transcribed genome-length RNA exhibited an apparent cytopathic effect on day 5 post-transfection (p.t.). IFA of the transfected cells showed increasing numbers of cells expressing viral E protein from day 1 to 3; more than 80% of the cells were IFA-positive on day 3 p.t. (supplemental Fig. 1B). Plaque assay of the culture fluids showed an increase in viral titer from day 1 to 4, peaking at 1 × 103 to 1 × 104 pfu/ml on day 4 p.t. (supplemental Fig. 1C). Specific infectivity assay showed that transfection of BHK-21 cells with 1 μg of RNA generated about 1.2 × 105 pfu of virus. The results demonstrate that the RNA transcribed from the cDNA clone was highly infectious.

RESULTS

Construction and Characterization of a Full-length cDNA Clone of DENV-2 Strain TSV01—The low copy number plasmid pACYC177 and E. coli HB101 were used to construct the cDNA clone of the DENV-2 TSV01 strain. The selection of pACYC177 as the cloning vector was based on our previous experience in WNV infectious clones (6). The supplemental Fig. 1A shows the overall scheme of the cloning strategy (see details under “Experimental Procedures”). The assembled full-length cDNA clone contained a T7 promoter at the 5′ end for in vitro transcription and a 67-bp hepatitis delta virus ribozyme sequence at the 3′ end for generating the authentic 3′ end viral sequence. The stability of the cDNA clone was tested by six rounds of plasmid extraction and re-formation into E. coli HB101. DNA sequencing of the 6th round plasmid did not show any mutations throughout the cDNA insert (data not shown), demonstrating the stability of the clone.

BH-K-21 cells transfected with the in vitro transcribed genome-length RNA exhibited an apparent cytopathic effect on day 5 post-transfection (p.t.). IFA of the transfected cells showed increasing numbers of cells expressing viral E protein from day 1 to 3; more than 80% of the cells were IFA-positive on day 3 p.t. (supplemental Fig. 1B). Plaque assay of the culture fluids showed an increase in viral titer from day 1 to 4, peaking at 1 × 103 to 1 × 104 pfu/ml on day 4 p.t. (supplemental Fig. 1C). Specific infectivity assay showed that transfection of BHK-21 cells with 1 μg of RNA generated about 1.2 × 105 pfu of virus. The results demonstrate that the RNA transcribed from the cDNA clone was highly infectious.

Structure and Sequence Analyses of Cavities A and B of DENV RdRp—Structure analysis uncovered two cavities conserved in DENV and WNV RdRps (18). The two cavities (A and B) are located on two opposite sides of the thumb subdomain of DENV and WNV RdRp. Fig. 1A shows the two cavities on the crystal structure of DENV-3 RdRp. On DENV-2 RdRp, cavity A is formed by amino acids Lys-756, Glu-760, Ser-763, Asn-777, Cys-780, Ser-785, Thr-806, Glu-807, Asp-808, Met-809, Leu-810, and Tyr-882, among which Lys-756, Thr-806, Glu-807, Asp-808, Met-809, and Leu-810 are completely conserved among various members of flaviviruses (Fig. 1B). Cavity B consists of amino acids Leu-327, Leu-328, Gln-365, Thr-368, Thr-385, Trp-859, Asn-862, Ile-863, and Ala-866, among which Leu-328, Trp-859, and Ile-863 are conserved among flaviviruses (Fig. 1B).

Role of Cavity A in Viral Replication—To determine the biological function of cavity A, we selected seven amino acids for mutagenesis analysis. As shown in Fig. 1A (left panel), Lys-756 (red), Gln-760 (green), Cys-780 (blue), and Met-809 (red) were located deep inside the cavity. Thr-806 (magenta), Glu-807 (cyan), and Asp-808 (orange) form the left side wall of the cavity. Among the seven selected residues, five amino acids (Lys-756, Thr-806, Glu-807, Asp-808, and Met-809) are completely conserved among flaviviruses; two residues (Gln-760 and Cys-780) have variations in yellow fever virus and/or tick-borne encephalitis virus RdRp (Fig. 1B). It should be noted that resi-
FIGURE 1. Structure and sequence analyses of cavities A and B from DENV RdRp. A, cavities A and B in DENV-3 RdRp structure. The crystal structure of DENV-3 RdRp (Protein Data Bank entry 2J7U) was used to illustrate cavity A (left panel) and cavity B (right panel). Amino acids constituting the cavities are labeled in yellow or other colors. Residues selected for mutagenesis analysis are colored as follows: for cavity A, Lys-756 (red), Gln-760 (green), Cys-780 (blue), Thr-806 (magenta), Glu-807 (cyan), Asp-808 (orange), and Met-809 (tint); for cavity B, Leu-328 (red), Lys-330 (green), Trp-859 (blue), and Ile-863 (pink). The images of RdRp were produced using PyMOL.

B, amino acid sequence alignment of partial RdRp region from the four serotypes of DENV and other flaviviruses. The RdRp amino acid sequences of DENV-1, DENV-2, DENV-3, DENV-4, WNV, yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV) are derived from GenBank™ accession numbers U88535, AY037116, M93130, AY947539, AF404756, X03700, and AF069066, respectively. Identical amino acids among all RdRps are shaded. The numbering of amino acid sequence is based on DENV-2. The residues involved in the formation of cavity A and B are indicated by • and ▼ above the sequence, respectively. The mutated residues in this study are indicated by * below the sequence.
Functional Analysis of Dengue Virus RdRp Cavities

dues Thr-806, Glu-807, Asp-808, and Met-809 also form the C-terminal part of the priming loop (16).

Each of the selected residues was mutated to Ala in the infectious clone of DENV-2 TSV01. Equal amounts of wild-type (WT) and mutant genome-length RNAs were electroporated into BHK-21 cells. Viral protein synthesis, plaque morphology, and virus production were compared. For viral protein synthesis, IFAs (detecting viral E protein) showed that similar percentages of IFA-positive cells were detected for the WT and mutants T806A, E807A, and D808A RNAs at 72 h p.t.; mutants Q760A, C780A, and M809A showed a slight decrease in the percentage of IFA-positive cells, and mutant K756A remained less than 1% IFA-positive cells at 72 h p.t. (Fig. 2A). For different RNA mutants, the relative IFA-positive cells were similar when the transfected cells were analyzed at 24 and 48 h p.t. (data not shown). No infectious virus was recovered from the K756A RNA-transfected cells, whereas other mutant RNAs yielded similar amounts of infectious viruses with no significant difference in plaque morphology (Fig. 2, A and B). These results suggest that only K756A among the selected mutations in cavity A is essential for viral replication.

Role of Cavity B in Viral Replication—Four residues were selected for mutagenesis analysis of cavity B. As depicted in Fig. 1A (right panel), Leu-328 (red) and Ile-863 (pink) are located at the bottom of the cavity; Trp-859 (blue) is at the side of cavity wall; and Lys-330 (green) is on the surface of the cavity. The four residues were individually mutated to Ala in the genome-length RNA of DENV-2 TSV01. Unlike cavity A mutants, genome-length RNAs containing the mutations in cavity B showed a significant decrease in viral protein synthesis in the transfected BHK-21 cells. At 72 h p.t., ~5% of the cells transfected with I863A RNA expressed viral protein; less than 1% of IFA-positive cells were detected for L328A and K330A; no IFA-positive cells was detected in cells transfected with W859A RNA (Fig. 2C). In agreement with the IFA results, only the I863A RNA-transfected cells yielded viruses (Fig. 2D) with tiny plaques (Fig. 2C); no plaque was detected from the L328A, K330A, and W859A RNA-transfected cells, up to 120 h p.t. (Fig. 2, C and D). Overall, the results indicate that cavity B plays a critical role in viral replication.

Effects of Mutations on RdRp Activity—To determine whether the cavities play a direct role in RdRp activity, we prepared a panel of full-length DENV-4 NS5 proteins and analyzed the mutational effects on polymerase activity. The reason to choose DENV-4 NS5 was that this protein was more stable than DENV-2 NS5 (data not shown). All five replication-defective NS5 mutations (L328A, K330A, K756A, W859A, and I863A) were engineered into recombinant full-length NS5 proteins. The proteins were analyzed in two types of RdRp assays. First, a de novo RdRp assay was performed using a viral subgenomic RNA as template. As shown in Fig. 3A, mutants L328A, W859A, and I863A exhibited only 15, 10, and 7% of the WT activity, respectively, whereas mutants K330A and K756A retained 80 and 82% of the WT activity, respectively.

Because the de novo RdRp assay includes steps of initiation and elongation, we next analyzed the mutant NS5 in an elongation RdRp assay (26). In contrast to the de novo synthesis results, all mutants retained greater than 70% of the WT RNA elongation activity (Fig. 3B). Fig. 3C summarizes the effects of mutations on de novo and elongation RdRp activities. The comparable elongation activities between the WT and mutant NS5 suggest the following: (i) the engineered mutations do not change the global conformation of the NS5 protein; and (ii) the observed defect in de novo RNA synthesis for mutants L328A, W859A, and I863A is not due to variations in protein preparations. These results allowed us to conclude that L328A, W859A, and I863A reduce viral replication on or before de novo initiation, whereas K330A and K756A decrease RNA replication through a nonenzymatic mechanism.

Revertant Analysis of Cavity A—Among the mutated amino acids from cavities A and B, five of them resulted in replication-defective viruses (L328A, K330A, K756A, W859A, and I863A; Fig. 2). Only K756A belongs to cavity A; L328A, K330A, W859A, and I863A belong to cavity B (Fig. 1A). We performed revertant analysis by continuouspassaging of culture supernatants on Vero cells for seven rounds (P1 to P7). The culture fluids from passages 2 (P2) and 7 (P7) were subjected to plaque assay and sequencing analysis. For cavity A, both the P2 and P7 supernatants of the K756A mutant produced plaque morphology similar to the WT; sequencing of the viruses revealed a true reversion to the WT sequence (data not shown). Together with the result that the K756A RNA-transfected cells (P0) did not yield any infectious virus (Fig. 2, A and B), we conclude that residue Lys-756 in cavity A is essential for viral replication.

Revertant Analysis of Cavity B—For cavity B, both P2 and P7 culture fluids derived from the L328A, K330A, and I863A RNAs produced plaques. Sequencing of the recovered viruses showed various reversion events. For L328A mutant, the P2 virus had a mixed population of Val (GTA) and Leu (CTA), whereas the P7 virus became a single population of Leu (CTA) (Fig. 4A). These results suggest that the L328A mutant underwent a stepwise reversion of Ala (GCA) → Val (GTA) → Leu (CTA) (mutant nucleotides are underlined), each step with a single nucleotide change.

For the K330A mutant, transfection of this mutant RNA into BHK-21 cells did not yield any infectious virus (Fig. 2, C and D). However, the culture fluids from P2 and P7 yielded infectious viruses with plaques smaller than the WT virus (Fig. 4B). The sequencing result showed that both the P2 and P7 viruses retained the engineered mutation (Fig. 4B); complete genome-length sequencing of the P2 virus revealed a second site nucleotide mutation G→A at position 10,087, which resulted in a Gly→Arg amino acid change at position 840 of NS5. To demonstrate whether the G840R substitution rescued the replication defect of K330A RNA, we prepared two mutants genome-length RNAs containing the G840R alone or the K330A/G840R double mutations. Transfection of BHK-21 cells with equal amounts of RNA showed that the G840R RNA generated fewer IFA-positive cells than the WT RNA did, but the double mutant K330A/G840R RNA produced more IFA-positive cells than the G840R RNA did (Fig. 4C). At 72 h p.t., equivalent amounts of IFA-positive cells were observed between the WT and the K330A/G840R double mutant RNA-transfected cells. The plaque size of G840R virus was smaller than K330A/G840R viruses, and the plaques of both viruses are fainter and slightly smaller than that of the WT virus (Fig. 4C). These results dem-
FIGURE 2. Mutagenesis of cavities A and B using an infectious cDNA clone of DENV-2. A and C, analysis of viral E protein synthesis and plaque morphology for cavity A and B mutant viruses, respectively. BHK-21 cells were transfected with WT and mutant genome-length RNAs (10 μg), and analyzed for viral E protein expression by IFA at 72 h post-transfection (upper panel). Plaque morphologies of WT and mutant viruses were shown (lower panel). N.D., not detectable. B and D, production of cavity A and B mutant viruses after transfection, respectively. Viruses from culture supernatants were collected every 24 h. Viral titers were determined by plaque assay on BHK-21 cells. Error bars indicate the standard deviations from two independent experiments; dashed line, limit of sensitivity of the plaque assay.
onstrate that the G840R adaptation is responsible for the restoration of the replication of mutant K330A (see further mechanistic analysis below).

For the I863A mutant, the P0 culture fluids showed tiny plaques (Fig. 2C). The P2 virus showed plaques of different sizes, and the P7 virus showed only large plaques (Fig. 5A). In agreement with the plaque morphology, sequencing analysis showed that the P2 virus had a mixed population of Val (GTC) and Ala (GCC), whereas the P7 virus became a single population of Val (GTC) (Fig. 5A). These results suggest that the I863A mutant underwent a reversion of Ala (GCC) → Val (GTC) through a single nucleotide change (mutant nucleotides are underlined). Engineering the I863V substitution into genome-length RNA restored viral replication. After transfection into BHK-21 cells, the I863V RNA and WT RNA generated equivalent amounts of IFA-positive cells from 24 to 72 h p.t. (compare Figs. 5B with 4C). The I863V RNA generated an infectious virus with plaque morphology similar to that of the WT virus (Fig. 5B). In addition, comparable viral titers were detected for WT and I863V mutant virus at various time points post-transfection; sequencing of the recovered virus showed that the engineered I863V mutation was retained without any other mutations (data not shown). Next, we compared the RdRp activity of recombinant NS5 containing the I863A or I863V mutation. In the *de novo* synthesis assay, the I863V and I863A NS5 exhibited 100 and 5% of the WT RdRp activity, respectively (Fig. 5C). In the elongation assay, the I863V and I863A NS5 exhibited 101 and 73% of the WT RdRp activity, respectively (Fig. 5D). These results strongly indicate that the reversion of I863A → I863V improves viral replication through enhancement of RdRp activity, especially at the step of initiation of RNA synthesis.

For the W859A mutant, no infectious virus was recovered (Fig. 2C and D), even after seven rounds of passaging (data not shown), indicating that Trp-859 is essential for viral replication. To examine whether amino acids other than Trp at position 859 could support viral replication, we substituted Trp-859 with Phe, Lys, Asp, His, or Leu in the context of genome-length RNA. Equal amounts of WT and mutant RNAs were transfected into BHK-21 cells. Only the cells transfected with W859F RNA showed a few number of IFA-positive cells (less than 1%) at 72 h p.t. (Fig. 6). None of the Trp-859 variant RNAs produced any infectious viruses, as judged by plaque assays (data not shown). These results demonstrate that Trp-859 in cavity B is essential for viral replication, even an aromatic amino acid substitution (W859F) could not sufficiently support viral replication.
K330A Mutation Affects Viral Replication by Decreasing NS3/NS5 Interaction—K330A mutation abolished viral replication (Fig. 2, C and D) but did not affect RdRp activity (Fig. 3C), suggesting that the mutation affects viral replication through a nonenzymatic mechanism. Revertant analysis showed that a second site mutation (G840R) in the RdRp domain could rescue the replication defect of K330A mutation (Fig. 4C). These results prompted us to test the hypothesis that K330A mutation affects replication through decreasing the interaction NS5 with other replication component(s). Because the NS3/NS5 interaction has been reported for DENV and other flaviviruses (28, 29), we validated the biological relevance of the two cavities that could be used for development of allosteric inhibitors (18). Using both genetic and biochemical approaches, we probed the biological relevance of the two cavities. Fig. 8A summarizes the results about all critical residues identified in this study. For cavity A, we found that among the seven mutated amino acids, only K756A mutation was lethal for DENV replication; the other six mutated residues did not significantly affect viral replication (Fig. 2, A and B). It is worth noting that four of the mutated residues (Thr-806, Glu-807, Asp-808, and Met-809) reside in the C-terminal region of the priming loop (colored in yellow, Fig. 8B). For the K756A mutation, only true reversion could restore viral replication. In terms of mode-of-action, we found that the K756A mutation affect neither de novo RNA synthesis nor RNA elongation activity (Fig. 3). The latter result indicates that the K756A mutation reduces viral replication through a nonenzymatic mechanism and possibly by affecting interaction with other replicate component(s). However, structure analysis showed that residue Lys-756 is located deep inside cavity A; the residue is behind the

K330A Mutation Affects Viral Replication by Decreasing NS3/NS5 Interaction—K330A mutation abolished viral replication (Fig. 2, C and D) but did not affect RdRp activity (Fig. 3C), suggesting that the mutation affects viral replication through a nonenzymatic mechanism. Revertant analysis showed that a second site mutation (G840R) in the RdRp domain could rescue the replication defect of K330A mutation (Fig. 4C). These results prompted us to test the hypothesis that K330A mutation affects replication through decreasing the interaction NS5 with other replication component(s). Because the NS3/NS5 interaction has been reported for DENV and other flaviviruses (28, 29), we validated the biological relevance of the two cavities that could be used for development of allosteric inhibitors (18). Using both genetic and biochemical approaches, we probed the biological relevance of the two cavities. Fig. 8A summarizes the results about all critical residues identified in this study. For cavity A, we found that among the seven mutated amino acids, only K756A mutation was lethal for DENV replication; the other six mutated residues did not significantly affect viral replication (Fig. 2, A and B). It is worth noting that four of the mutated residues (Thr-806, Glu-807, Asp-808, and Met-809) reside in the C-terminal region of the priming loop (colored in yellow, Fig. 8B). For the K756A mutation, only true reversion could restore viral replication. In terms of mode-of-action, we found that the K756A mutation affect neither de novo RNA synthesis nor RNA elongation activity (Fig. 3). The latter result indicates that the K756A mutation reduces viral replication through a nonenzymatic mechanism and possibly by affecting interaction with other replicate component(s). However, structure analysis showed that residue Lys-756 is located deep inside cavity A; the residue is behind the
C terminus of the priming loop (Fig. 8B). It is conceivable that during RNA synthesis, a local conformational change exposes residue Lys-756 to the protein surface, allowing Lys-756 to interact with other replicase component(s). Nevertheless, our results argue that cavity A is not an ideal target for designing small molecular inhibitors.

FIGURE 7. Compensatory mutation G840R rescued the replication defect of K330A through restoration of the NS3/NS5 interaction. A and B, SPR measurements of the interaction between the wild-type and mutants of NS5 and the full-length NS3 and its helicase domain, respectively. 3-Fold serially diluted DENV-4 full-length NS3 (0.1–24 μM) or its helicase domain (1–244 μM) were injected across immobilized DENV-4 NS5 (and its mutants) in duplicate (only one of the duplicates is shown in figure for clarity) for 1 min and allowed to dissociate in running buffer for another 2.5 min. The aligned and double-referenced sensorgrams were globally fit to a simple 1:1 interaction model (thin red lines). To verify the accuracy of our rate parameters, we also fit the same data to a bivalent model (BIAEvaluation version 4.1, Biacore, GE Healthcare) and the results tallied with the 1:1 model. Hence, we chose to present the 1:1 model fit to avoid complication and to provide a better comparison between the full-length NS3 data and the NS3 helicase domain data. C, summary of the resultant affinities for interaction between full-length DENV-4 NS3 and its helicase domain with wild-type NS5, its single mutant (K330A and G840R), and its double mutant (K330A/G840R). The resultant affinity for interaction is reflected by $K_D$ (equilibrium dissociation constants), which was calculated using the ratio of $k_d/k_a$ from the SPR assay. RU, response units.
In contrast to cavity A, all four mutated residues in cavity B significantly reduced viral replication (Fig. 2, C and D). None of the mutations decreased RNA elongation activity by >27%; however, three mutations (L328A, W859A, and I863A) reduced de novo RNA synthesis by ≥85% (Fig. 3). Correlated with the de novo RNA synthesis results, revertant analysis showed the following: (i) only true reversion could restore the replication of L328A RNA (Fig. 4A); (ii) no revertant virus could be selected for W859A RNA; (iii) only amino acid Val, similar to the wild-type Ile-863, was selected to restore the replication of I863A RNA (Fig. 5). These results indicate that L328A, W859A, and I863A mutations reduced viral replication by decreasing the initiation of RNA synthesis. Although these residues are away from the active site and RNA tunnel (Fig. 8C), they may participate in the formation of the RNA template-RdRp complex. In support of this notion, the 1 loop on the surface of thumb subdomain of HCV RdRp was previously shown to regulate the conformations needed for de novo initiation and for elongation of RNA synthesis; mutations that affect the 1 loop conformation selectively reduced de novo initiation (35).

Unlike the three residues in cavity B discussed above, mutation K330A abolished viral replication but retained 80 and 81% of the wild-type activities of de novo and elongation RNA synthesis, respectively (Fig. 4A). These results indicate that the K330A mutation abrogated RNA replication through a nonenzymatic mechanism. In support of this hypothesis, our SPR results showed that the K330A mutation reduced the NS3/NS5 interaction. Furthermore, revertant analysis showed that a second site mutation G840R in the RdRp domain could restore the replication of the K330A mutant. In agreement with the genetic data, our SPR results showed that, although the compensatory mutation G840R alone is also defective in NS3/NS5 interaction, the double mutant K330A/G840R restored the NS3/NS5 interaction. On the crystal structure, Gly-840 (blue in Fig. 8C) is 24.1 Å away from Lys-330 but is located on the surface of the thumb subdomain. The switch from the wild-type Lys-330 to the revertant K330A/G840R maintained one positively charged residue, indicating that the positive charge on the surface of NS5 is important for the NS3/NS5 interaction. In support of our results, Johansson et al. (30) previously suggested that a region between residues 320 and 368 of DENV NS5 interacts with NS3 helicase domain.

In summary, our results have demonstrated that cavity B is essential for viral replication and could be targeted for development of the allosteric inhibitor of flavivirus NS5 polymerase.
Pharmacological blockage of cavity B could potentially lead to suppression of initiation of viral RNA synthesis and/or inhibition of NS3/NS5 interaction. The SPR assay described in this study is amendable for high throughput screening to identify inhibitors of NS3/NS5 interaction.

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