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A Combination of Naturally Occurring Mutations in North American West Nile Virus Nonstructural Protein Genes and in the 3′ Untranslated Region Alters Virus Phenotype▽

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We previously reported mutations in North American West Nile viruses (WNVs) with a small-plaque (*sp*), temperature-sensitive (*ts*), and/or mouse-attenuated (*att*) phenotype. Using an infectious clone, site-directed mutations and 3′ untranslated region (3′UTR) exchanges were introduced into the WNV NY99 genome. Characterization of mutants demonstrated that a combination of mutations involving the NS4B protein (E249G) together with either a mutation in the NS5 protein (A804V) or three mutations in the 3′UTR (A10596G, C10774U, A10799G) produced *sp*, *ts*, and/or *att* variants. These results suggested that the discovery of North American WNV-phenotypic variants is rare because of the apparent requirement of concurrent polygenic mutations.

Due to the highly neuroinvasive nature of the strain of West Nile virus (WNV) introduced into North America in 1999, speculation about differences in virulence between North American and Old World strains has arisen (18). Comparative virulence studies have shown that field isolates of WNV from different continents differ in their abilities to cause neuroinvasive disease and mortality in avian and murine models (3, 5, 6, 12, 13, 23). Previous studies also demonstrated that certain WNV strains isolated in Texas in 2003 possess phenotypic differences from typical North American WNV isolates (i.e., changes in plaque morphology, temperature sensitivity [*ts*], in vitro growth kinetics, and attenuation of mouse neuroinvasiveness [*att*]). Furthermore, complete genome sequencing of several phenotypically distinct isolates identified mutations putatively conferring these changes (8).

In this article, we report the characterization of one of these Texas isolates, strain Bird 1153, and demonstrate that a combination of mutations in three regions of the genome is required for the modified phenotype. Compared to the prototypical North American WNV, NY99 (also known as strain 382-99), the Bird 1153 strain exhibits a small-plaque (*sp*) phenotype and is *ts* and *att*. This strain contains only four amino acid substitutions in the WNV polyprotein (in the premembrane protein with the V156I mutation [prM-V156I], the envelope protein with the V159A mutation [E-V159A], nonstructural protein 4B with the E249G mutation [NS4B-E249G], and NS5-A804V) and four nucleotide substitutions in the 3′ untranslated region (3′UTR) (A10596G, C10774U, A10799G, and A10851G), relative to the sequence of the NY99 strain (GenBank accession number AF196835) (8). We have investigated the effects of the Bird 1153-specific mutations by using site-directed mutagenesis and genetic exchanges to incorporate either single point mutations or combinations of mutations into the viral genome of an NY99-derived infectious clone (NY99ic).

A total of nine mutant viruses were made (Table 1) using site-directed mutagenesis of the NY99ic as previously described (4). Following viral rescue from Vero cells, RNA was amplified by reverse transcription PCR and sequenced to confirm the presence of the correct mutation(s) and the absence of unwanted mutations. Initially, amino acid residue 249 of the NS4B protein was mutated from Glu to Gly, and this NS4B-E249G mutant was identified as having a phenotype identical to that of the NY99 strain (i.e., the nonattenuated-virus phenotypes of 3- to 4-week-old female Swiss Webster mice, namely, large plaques [*lp*], a lack of *ts*, and high neuroinvasiveness) (Table 1). Because the NS4B-E249G mutant did not show any of the altered phenotypes found in strain Bird 1153, additional mutant viruses were generated by incorporating combinations of Bird 1153 virus-specific amino acid mutations and 3′UTR nucleotide mutations into the WNV NY99ic (Fig. 1). To incorporate all four Bird 1153-specific 3′UTR ($3'UTR_B$) mutations, a complete 3′UTR exchange was made between the NY99ic and Bird 1153 by engineering a SalI site at nucleotide 10381 at the NS5-3′UTR junction, which resulted in a conservative E901D mutation in NS5. Following the rescue

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TABLE 1. Phenotypic properties of mutant viruses derived from the WNV NY99ic

Virus[a]	Plaque size[b]	Temp sensitivity (log_{10} no. of PFU/ml)			Mouse neuroinvasiveness	
		37.0°C	41.0°C	Δ[c]	i.p. LD_{50} (PFU)	AST ± SD[d]
NY99 (wt virus)	*lp*	8.1	7.6	0.5	0.8	8.0 ± 1.2
NY99ic	*lp*	7.0	7.3	0.3	1.0	7.2 ± 0.6
Bird 1153 (wt virus)	***sp***	7.7	4.8	2.9†	>10,000[e]	NA
NS4B-E249G mutant	*lp*	6.2	6.0	0.2	1.2	7.5 ± 0.4
prM-V156I mutant	*lp*	5.9	6.1	0.2	0.7	8.6 ± 1.4
NS5-A804V mutant	*mp*	6.3	5.5	0.8	5	8.0 ± 0.5
3′UTR_B mutant	*lp*	6.4	5.0	1.4*	0.6	8.8 ± 2.0
prM-V156I + NS4B-E249G mutant	*lp*	7.0	7.2	0.2	1.4	8.8 ± 1.5
prM-V156I + 3′UTR_B mutant	*lp*	6.6	5.1	1.5*	4.2	9.0 ± 1.0
NS4B-E249G + 3′UTR_B mutant	***sp***	6.7	3.9	2.8†	>10,000[e]	NA
NS4B-E249G + NS5-A804V mutant	***sp***	6.2	6.4	0.2	2,000[e]	9.2 ± 1.2
NS5-A804V + 3′UTR_B mutant	*mp*	5.4	4.2	1.2*	>10,000[e]	NA

[a] The wt NY99 and Bird 1153 WNV isolates are indicated. The remaining mutant-virus designations indicate each Bird 1153 virus-specific locus incorporated into the NY99ic viral background.

[b] Plaque sizes were as follows: *sp* were <1.0 mm, *lp* were >3.0 mm, and *mp* were 1 to 3 mm in Vero cells.

[c] Δ indicates the difference between average virus titers at 37.0°C and 41.0°C (triplicate experiments) at 72 h postinfection in Vero cells. † indicates a *P* of <0.001 in a comparison of the viral titer at 41.0°C versus that at 37.0°C, with experiments performed in triplicate. * indicates a *P* of <0.05 in a comparison of the viral titer at 41.0°C versus that at 37.0°C, with experiments performed in triplicate.

[d] AST, average survival time ± standard deviation following intraperitoneal inoculation of virus. NA, not applicable, as no mice died following inoculation.

[e] Attenuated for mouse neuroinvasiveness.

of each mutant, each engineered mutation was confirmed by nucleotide sequencing. The E-V159A mutation has previously been identified as a marker of the dominant North American WNV genotype, isolates of which characteristically display *lp*, non-*ts*, and non-*att* phenotypes, and was, therefore, not considered relevant to the altered phenotypes examined (9). In addition, one of the nucleotide changes in the 3′UTR (A10851G) was present in the NY99ic used in this study and has subsequently been identified in viruses belonging to the dominant genotype as well (9). Because the Bird 1153 isolate did not contain the NS5-E901D mutation and since this mutation, like the E-V159A and 3′UTR (A10851G) mutations, is present in WNVs that have phenotypes identical to that of the NY99 strain, this nearly synonymous mutation was considered to not contribute significantly to the phenotypes of the mutants containing the 3′UTR_B mutations. However, we cannot eliminate the possibility that the E-V159A, 3′UTR (A10851G), and engineered NS5-E901D mutations played some minor role in the phenotypes of the viruses studied here.

Two of the mutant viruses had an *sp* phenotype (the NS4B-E249G + 3′UTR_B and NS4B-E249G + NS5-A804V mutants), and two mutants had an intermediate plaque size (*mp*) (the NS5-A804V and NS5-A804V + 3′UTR_B mutants), while all others displayed a typical *lp* morphology. All mutants containing the 3′UTR from Bird 1153 displayed statistically significant reductions in plaque titer in Vero cells at 41.0°C versus that at 37.0°C at 72 h postinfection. Only one mutant, the NS4B-E249G + 3′UTR_B mutant, displayed a statistically significant reduction in plaque titer ($\Delta = 2.8$ log_{10} PFU/ml; $P < 0.001$ by Student's *t* test) comparable to that of strain Bird 1153 at the higher temperature. The other three mutant viruses (the 3′UTR_B, prM-V156I + 3′UTR_B, and NS5A804V + 3′UTR_B mutants) containing the Bird 1153-specific 3′UTR replicated to a titer that was 1.2 to 1.5 log_{10} PFU/ml lower at 41°C, displaying *ts* phenotypes at a level of significance (*P*) of <0.05 (Table 1). Interestingly, when NY99ic plasmids containing NS4B-E249G + NS5-A804V + 3′UTR_B were generated for transfection, several attempts at viral rescue resulted in either no infectious virus or reversion to the wild-type (wt) NY99 sequence at either the NS4B-249 or NS5-804 locus. The inability to rescue virus without reversion to the wt NY99 sequence likely reflects the inability of mutants containing the full spectrum of deleterious mutations to replicate following transfection. The rapid selection of virus containing reversion to the wt sequence at the NS4B or NS5 locus strongly reinforces the deleterious effects of the NS4B-E249G and NS5-A804V mutations on WNV fitness and virulence in the presence of the 3′UTR_B.

To measure the in vitro multiplication kinetics of the mutant viruses, Vero cells were infected in triplicate at a multiplicity of infection of 0.1 for growth curve analysis. The NY99ic virus and the NS4B-E249G, NS5-A804V, and 3′UTR_B infectious-clone mutants reached high mean peak titers of 8.2 to 8.6 log_{10} PFU/ml. However, Bird 1153 virus and the NS4B-E249G + 3′UTR_B, NS4B-E249G + NS5-A804V, and NS5-A804V + 3′UTR_B mutants attained lower mean peak titers of 7.0 to 7.4 log_{10} PFU/ml, suggesting that these mutants replicated less efficiently in Vero cells (Fig. 2).

Previous studies of Texas 2003 field isolates revealed that several *sp* and/or *ts* isolates were attenuated for mouse neuroinvasiveness by as much as 10,000-fold when intraperitoneal (i.p.) 50% lethal doses (LD_{50}s) were compared to those of typical field isolates (8). In the present study, we determined the i.p. LD_{50} of each WNV mutant in a mouse model to characterize the neuroinvasive properties of each virus. Both *sp* mutant viruses (the NS4B-E249G + 3′UTR_B and NS4B-E249G + NS5-A804V mutants) were attenuated for mouse neuroinvasiveness (i.p. LD_{50} of >10,000 PFU and 2,000 PFU, respectively). Additionally, the NS5-A804V + 3′UTR_B *mp* mutant had an attenuated phenotype, with an i.p. LD_{50} of >10,000 PFU. All other mutant viruses produced i.p. LD_{50} values similar to those of wt NY99 and the NY99ic viruses (Table 1). Interestingly, incorporation of each point mutation alone, or even the exchange of the entire 3′UTR from Bird

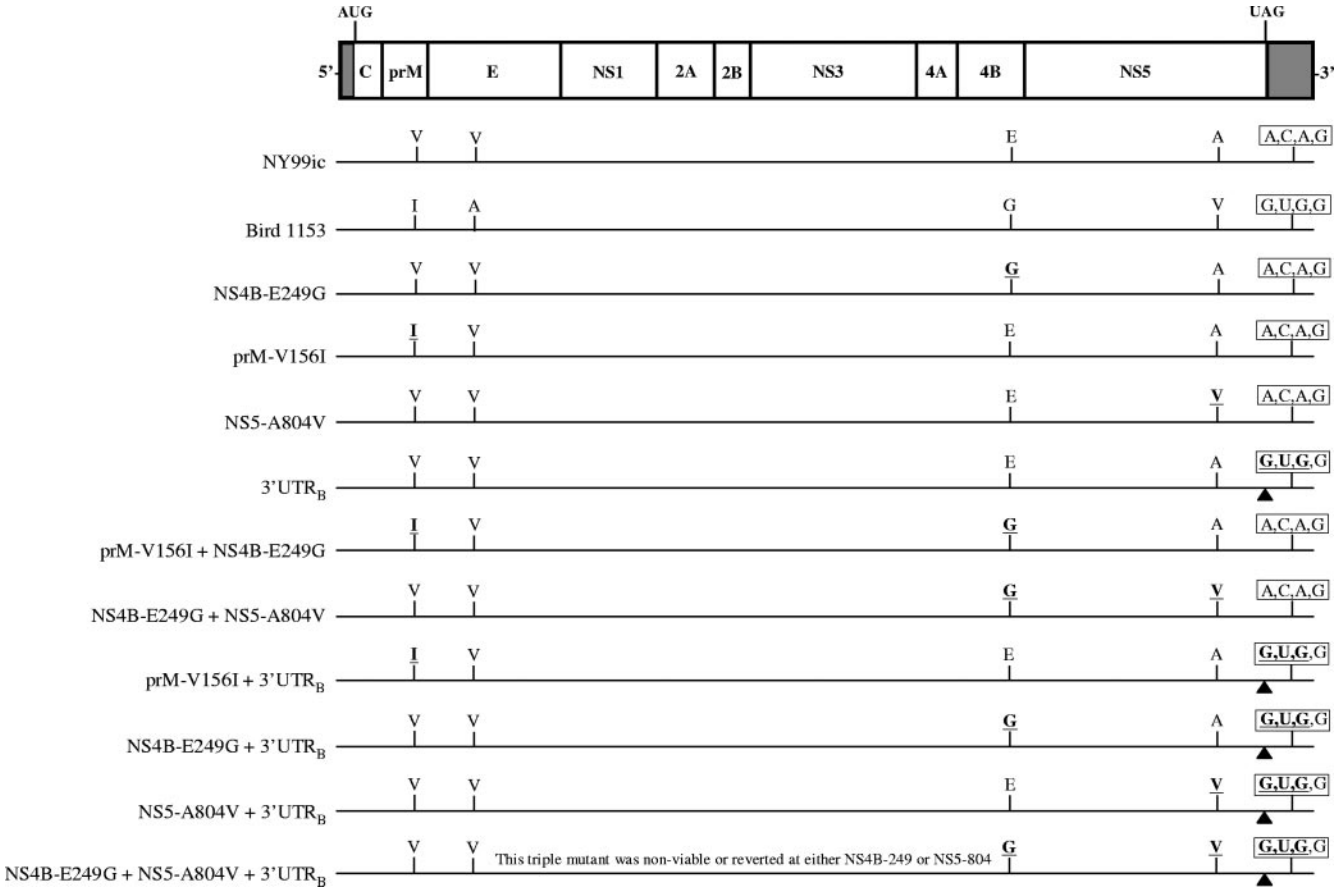

FIG. 1. Infectious-clone-derived WNV mutants generated for this study. The organization of the flaviviral genome is shown at the top. 5′, 5′UTR (left shaded box); C, capsid structural protein; 3′, 3′UTR (right shaded box). NS1is followed by NS2A, -2B, -3, -4A, -4B, and -5. The AUG initiation codon and UAG termination codon for the translated polyprotein are indicated. Line drawings representing the genomes of the viruses tested in this study are shown below the genomic map. The Bird 1153 virus isolate contains four amino acid substitutions in the WNV polyprotein (prM-V156I, E-V159A, NS4B-E249G, and NS5-A804V) and four nucleotide substitutions in the 3′UTR (A10596G, C10774U, A10799G, and A10851G), relative to the published NY99 strain sequence (GenBank accession number AF196835). All of these viruses, including the NY99ic virus, contained nucleotide 10851-G. Each Bird 1153 virus-specific locus cloned into the NY99ic background is shown in bold underlined letters for each engineered mutant. 3′UTR$_B$ indicates the entire 3′UTR$_B$. The solid triangles indicate the relative position of a conservative NS5-E901D mutation that was engineered to incorporate a SalI site that permitted exchange of the 3′UTR$_B$.

1153, into the NY99ic virus, produced mutants that exhibited neuroinvasive phenotypes. The infectious-clone NS4B-E249G + 3UTR$_B$ and NS4B-E249G + NS5-A804V mutants both possessed *sp* phenotypes and reduced multiplication kinetics in vitro, as well as being attenuated in a mouse model. However, not all mutants containing NS4B-E249G were *ts*, suggesting that the NS4B mutation was critical for the *sp* phenotype but not the *ts* phenotype and that the *sp* phenotype was associated with less-efficient replication in Vero cells (Fig. 2). Similarly, the 3′UTR$_B$ was necessary for the *ts* phenotype. Only mutants containing the 3′UTR$_B$, singly or in combination with the mutation in prM, NS4B, or NS5, showed a significant *ts* attribute. This study demonstrated that a point mutation at residue 249 of the NS4B protein from a Glu to Gly, in combination with a mutation in the NS5 protein at residue 804 (Ala to Val) or with three mutations in the viral 3′UTR (A10596G, C10774U, and A10799G), produces variants with *sp*, *ts*, and/or *att* phenotypes.

The NS4B protein of WNV is of unknown function, and inferences concerning the influence of the E249G mutation remain speculative. Predictive structural models of this protein suggest that residue 249 is located in the carboxy-terminal portion of the protein in a region within the tail on the cytoplasmic side of the endoplasmic reticulum (20). Previous studies of WNV and other closely related flaviviruses have shown this protein to be important to viral replication and pathogenesis and indicate that the NS4B protein of various flaviviruses is able to inhibit the interferon-signaling cascade by blocking STAT-1 phosphorylation (24, 11, 17). Recent studies have also identified the NS4B-E249G mutation in WNV replicons that have established persistent infections in different cell lines (20; S. Rossi and P. Mason, personal communication), and there is also recent evidence that the NS4B-E249G mutation may affect replication in a host-dependent manner by attenuating RNA synthesis but not by affecting the NS4B-mediated inhibition of the STAT-1-mediated interferon pathway (20). The NS5 gene of WNV encodes a single protein believed to possess both an N-terminal region with methyltransferase activity and a C-terminal region with RNA-dependent RNA polymerase activity (1). The NS5-A804V mutation is located in the C-terminal region of the protein but is found outside any of the

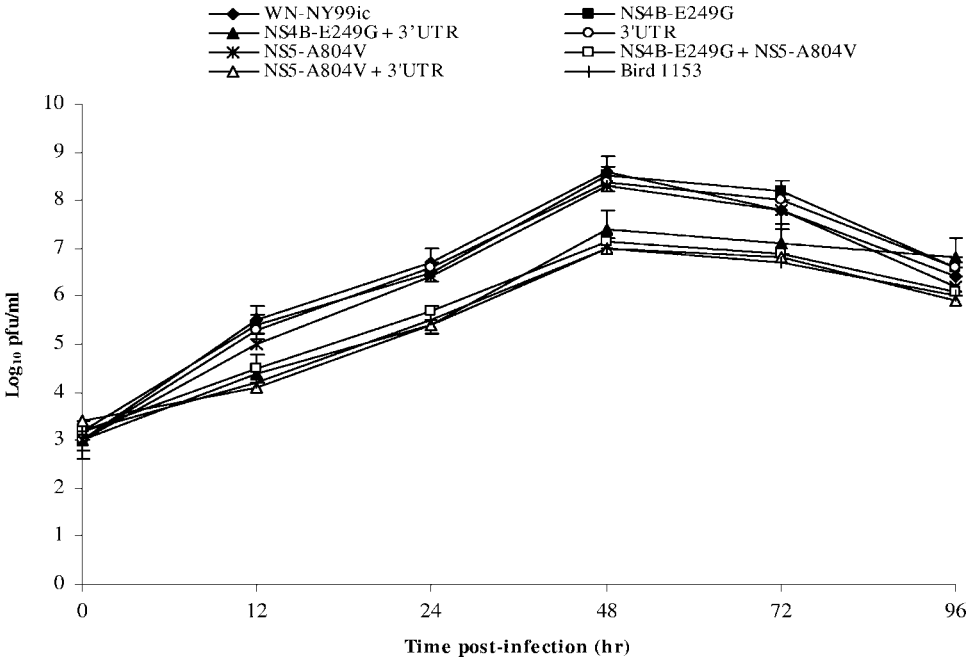

FIG. 2. Viral growth curves of WNV mutants in comparison to virus derived from the NY99ic. Each time point represents the average titer ± standard deviation, as determined by plaque assay of three replicates. The multiplicity of infection was 0.1 in Vero cells.

conserved motifs previously described as important to RNA-dependent RNA polymerase activity. The *mp* phenotype of the NS5-A804V mutant (Table 1) and its slightly lower replication profile in Vero cells than that of the NY99ic virus (Fig. 2) may indicate a replicative effect of this NS5 mutation.

To evaluate the effects of the 3′UTR$_B$ mutations, the predicted RNA secondary structure of a portion of the WNV 3′UTR containing the 3′UTR mutations was performed using Mfold (version 3.1) (26). Interestingly, the C10774U and A10799G mutations were found to be located in or near the 5′ stem-loop structure (dumbbell 1 [DB1]) of the 3′UTR (Fig. 3). The mapping of these mutations to 3′UTR DB1 of WNV suggests their involvement in maintaining the RNA secondary structure of DB1, which in previous studies has been shown to be important for maintaining the function of the 3′UTR (22). Studies have shown that deletions and mutations of conserved nucleotides in any one of the three stem-loop structures predicted in the WNV 3′UTR reduced the replication efficiency of the mutant viruses produced (10, 14). In studies of dengue-4 and tick-borne encephalitis viruses, 3′UTR mutants have been

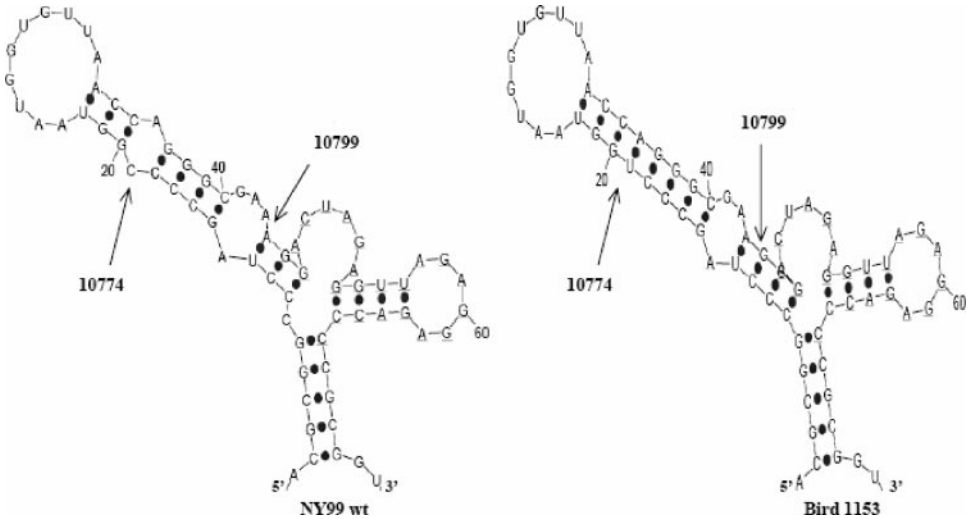

FIG. 3. Predicted RNA secondary structure of a portion of the nucleotide sequence of the Bird 1153 virus 3′UTR compared to that of the NY99 virus 3′UTR. The portion shown comprises the 5′ dumbbell structure (DB1) of the WNV 3′UTR, which contains RCS2. Predicted secondary structures for each sequence were prepared using Mfold, version 3.1, with default folding parameters (26). Folding was predicted at 37°C. Predicted structures at suboptimum energies were similar to those predicted at optimum energies. Underlined nucleotides comprise RCS2.

highly attenuated, with impaired growth characteristics (15, 19). It has been proposed that mutations/deletions in the dumbbell structures lead to a structural rearrangement of the dumbbell directly or to pseudoknots located at the tips of the predicted dumbbells, which may disrupt the ability of the stem-loop to stabilize and compartmentalize the replication complex during viral replication or to form binding sites for viral or host cellular proteins important for replication or RNA synthesis (7, 10, 14). Interestingly, the A10799G mutation is located adjacent to a region of 5′ DB1 that is highly conserved in the *Flavivirus* genus, known as repeated conserved sequence 2 (RCS2) (Fig. 3). Although the function of this region remains unknown, it has been suggested that the RCS2 region is involved in viral replication (16). Based on the Mfold-predicted RNA secondary-structure comparison of the Bird 1153 3′UTR to that of wt NY99, this mutation appears to slightly alter the size and relative position of the RCS2 secondary structure, as well as the stem and bulge immediately adjacent to the 5′ end of the RCS2, which may affect functional aspects of this motif during viral replication. The mutation of a C to U at nucleotide 10774 is also of interest because it appears to produce a straightening of the DB1 stem around this nucleotide and its base-pairing partner (Fig. 3). Thus, this mutation may also influence proper base pairing and folding patterns of the DB1 stem-loop structure and consequently affect functional aspects of this region in viral replication. The mutation at nucleotide 10596 is less likely to influence the structure and functionality of the 3′UTR because it is found outside any known conserved elements within the WNV 3′UTR and does not appear to influence any changes in predicted RNA secondary structure (data not shown). Regardless, the *ts* phenotype of isolates containing these three mutations may be the result of alterations to the 3′UTR secondary structure rendering the DB1 structure and/or the RCS2 motif more sensitive to high-temperature conditions. Under these conditions, it is possible that the function of this stem-loop region is impaired, resulting in reduced replication.

While the mutations in the 3′UTR may influence *ts*, the 3′UTR mutations alone did not attenuate the infectious-clone-derived virus, suggesting that these mutations alone do not alter viral replication under the physiological conditions of the mouse model. None of the mutant viruses generated in this study had significantly reduced growth kinetics in Vero cell culture or attenuation of neuroinvasiveness unless at least two mutations were present, suggesting that these mutations act in concert to significantly alter the biological properties of the virus due to additive effects of the mutations on the functionality of viral genes, proteins, and/or the 3′UTR. Thus, we demonstrated that combinations of mutations in two or more genes and/or in the 3′UTR were necessary to modify several phenotypic markers of the virulent North American NY99 strain of WNV to attain the *sp*, *ts*, and *att* phenotypic characteristics of strain Bird 1153. Although other molecular determinants of attenuation of WNV clearly exist (2, 4, 13, 21, 24, 25), based on genomic sequencing of multiple North American isolates, such combinations of mutations may be relatively rare events and may explain why few examples of North American WNV isolates with modified phenotypes have been reported.

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