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Infectious cDNA Clone of the Epidemic West Nile Virus from New York City

Pei-Yong Shi,[1,2]* Mark Tilgner,[1] Michael K. Lo,[1] Kim A. Kent,[1] and Kristen A. Bernard[1,2]

Wadsworth Center, New York State Department of Health,[1] and Department of Biomedical Sciences, University at Albany, State University of New York,[2] Albany, New York 12201

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We report the first full-length infectious clone of the current epidemic, lineage I, strain of West Nile virus (WNV). The full-length cDNA was constructed from reverse transcription-PCR products of viral RNA from an isolate collected during the year 2000 outbreak in New York City. It was cloned into plasmid pBR322 under the control of a T7 promoter and stably amplified in *Escherichia coli* HB101. RNA transcribed from the full-length cDNA clone was highly infectious upon transfection into BHK-21 cells, resulting in progeny virus with titers of 1×10^9 to 5×10^9 PFU/ml. The cDNA clone was engineered to contain three silent nucleotide changes to create a *Sty*I site (C to A and A to G at nucleotides [nt] 8859 and 8862, respectively) and to knock out an *Eco*RI site (A to G at nt 8880). These genetic markers were retained in the recovered progeny virus. Deletion of the 3′-terminal 199 nt of the cDNA transcript abolished the infectivity of the RNA. The plaque morphology, in vitro growth characteristics in mammalian and insect cells, and virulence in adult mice were indistinguishable for the parental and recombinant viruses. The stable infectious cDNA clone of the epidemic lineage I strain will provide a valuable experimental system to study the pathogenesis and replication of WNV.

West Nile virus (WNV) is found in many regions, including Africa, the Middle East, Europe, Russia, India, Indonesia, and most recently North America (9). Phylogenetic analysis of WNV strains has revealed two distinct lineages (I and II). Lineage I strains are frequently involved in human and equine outbreaks, and lineage II strains are mostly maintained in enzootic cycles (4, 30, 35, 36, 59). Sequence analysis showed that the strain in North America is closely related to other human epidemic strains isolated from Israel, Romania, Russia, and France, all of which belong to lineage I (35, 36). WNV has caused significant human, equine, and avian disease since its appearance in North America in 1999 (2, 28, 36), and the virus has quickly spread from the Northeast to the eastern seaboard and to the Midwest (3). There were 61 human cases (7 deaths) in New York City in 1999 (13); 21 human cases (4 deaths) in New York, New Jersey, and Connecticut in 2000 (42); and 48 human cases (5 deaths) in New York, Florida, New Jersey, Connecticut, Maryland, Massachusetts, Georgia, and Louisiana in 2001 (14).

WNV is a member of the *Flavivirus* genus, a group of arthropod-borne viruses in the family *Flaviviridae*. Besides WNV, many other members of the flaviviruses are important human pathogens, including dengue virus (DEN), yellow fever virus (YF), the tick-borne encephalitis virus complex (TBE), Japanese encephalitis virus (JE), and Murray Valley encephalitis virus (MVE) (9). The flavivirus genome is a single plus-strand RNA of approximately 11 kb in length that encodes 10 viral proteins in a single open reading frame (55). The encoded polyprotein is translated and co- and posttranslationally processed by viral and cellular proteases into three structural proteins (the capsid protein C; the membrane protein M, which is formed by furin-mediated cleavage of prM; and the envelope protein E) and seven nonstructural proteins (the glycoprotein NS1, NS2a, the protease cofactor NS2b, the protease and helicase NS3, NS4a, NS4b, and the polymerase NS5) (15, 39). The 5′ and 3′ untranslated regions (UTRs) of the genomic RNA are approximately 100 and 400 to 700 nucleotides (nt) in length, respectively, and the terminal nucleotides of both the 5′ and the 3′ UTRs can form highly conserved secondary and tertiary structures (7, 8, 55, 60).

The establishment of a reverse genetic system for the WNV strain presently circulating in the United States is a critical step in the study of the epidemic North American strains of WNV. Infectious full-length cDNA clones for a number of flaviviruses have been successfully developed for the study of viral replication and pathogenesis (56). In several cases, assembly of full-length flavivirus clones in a plasmid vector was not straightforward because clones containing large portions of the genome were unstable and deleterious for bacterial hosts. This problem was first circumvented for YF by ligating cDNA fragments in vitro prior to RNA transcription (54). Similar approaches were applied to develop infectious clones for JE (62), DEN type 2 (DEN2) (31), and TBE strain Hypr (40). For other flaviviruses, stable full-length infectious clones were established for DEN4 (34), Kunjin virus (33), TBE strain Neudoerfl (40), MVE (27), and TBE strain Langat (11). Although an infectious clone of the lineage II WNV strain from Nigeria was recently reported (68), no such full-length cDNA clone has been developed for the human epidemic lineage I WNV.

In this report, we describe the construction of a stable full-length cDNA clone of a WNV strain (lineage I) isolated from the epicenter of New York City during the 2000 outbreak (18). RNA transcribed from the cDNA clone was highly infectious upon transfection into cells, as shown by expression of viral proteins, production of progeny virus, and high specific infectivity. Genetic markers engineered into the cDNA clone to

* Corresponding author. Mailing address: Wadsworth Center, New York State Department of Health, Albany, NY 12201. Phone: (518) 473-7487. Fax: (518) 473-1326. E-mail: ship@wadsworth.org.

distinguish recombinant from parental viruses were retained in the recovered virus. The recombinant virus and the parental virus showed similar biological properties in terms of plaque morphology, growth kinetics, and virulence characteristics. The infectious clone of the present epidemic WNV strain in North America will serve as a valuable reverse genetic system to study the molecular mechanisms of WNV pathogenesis and replication.

MATERIALS AND METHODS

Cells and virus. Vero (ATCC CCL-81) cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). BHK-21/WI2 (BHK-21) (64) and *Aedes albopictus* C6/36 (C6/36) (ATCC CRL-1660) cells were grown in Dulbecco's modification of MEM with 10% FBS and 0.1 mM nonessential amino acids. Antibiotics were added to all media at 10 U/ml of penicillin and 10 μg/ml of streptomycin. Cells were maintained in 5% CO_2 at 37°C (Vero and BHK-21) or 28°C (C6/36). The parental WNV strain 3356 was isolated from the kidney of an American crow collected in October 2000 from Staten Island, New York (18). A single viral stock was made from the second passage in Vero cells without plaque purification, stored as aliquots at −80°C, and designated as parental WNV 3356. This stock was used as parental virus in all assays. Plaque assays were performed on Vero cells as described previously (53).

cDNA synthesis and cloning. BHK-21 cells were infected at a multiplicity of infection (MOI) of 0.05 with parental WNV 3356, and virus was harvested from cell culture media at 36 h postinfection. Genomic RNA was extracted from the cell culture media with RNeasy (Qiagen, Valencia, Calif.). cDNA fragments covering the complete genome were synthesized from genomic RNA through ThermoScript reverse transcription (RT)-PCR according to the manufacturer's instructions (Gibco BRL, Rockville, Md.). Plasmid pBR322 was modified by replacement of the *Sph*I-*Eco*RI fragment in the tetracycline resistance gene with a pair of complementary oligonucleotides to create the sequence 5′-*GCATGg*ATCCCGTTGCGCATGCTGATTCGAACCGACTAGT-CTCGAG-TCTAGAATTC-3′ to yield plasmid pBRlinker containing the unique restriction sites *Bam*HI, *Sph*I, *Spe*I, *Xho*I, and *Xba*I (listed in order and underlined). After the modification, the original *Sph*I site of pBR322 (italics) was mutated through a C to G substitution (lowercase italics). The modified pBR322, pBRlinker, was used as the cloning vector throughout the experiments.

Bacterial strain HB101 (Gibco BRL) was used as the host for construction and propagation of cDNA clones. Standard cloning procedures were followed (57), except that constructs with inserts of greater than 3 kb were propagated at room temperature. Electroporation was performed to transfect plasmid into bacteria in 0.2-cm cuvettes, using a GenePulser apparatus (Bio-Rad, Hercules, Calif.) with settings of 2.5 kV, 25 μF, and 200 Ω. The virus-specific sequence of each intermediate cloning product was validated by sequence analysis (Applied Biosystems, Foster City, Calif.) before it was used in a subsequent cloning step. All restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.).

RNA transcription and transfection. Plasmid pFLWNV, containing the full-length cDNA of WNV, was amplified in *Escherichia coli* HB101 and purified through MaxiPrep (Qiagen). For in vitro transcription, 5 μg of pFLWNV was linearized with *Xba*I. Mung bean nuclease (5 U; New England BioLabs) was directly added to the *Xba*I digestion reaction mixture, and the reaction mixture was further incubated at 30°C for 30 min to remove the single-stranded nucleotide overhang generated by the *Xba*I digestion. The linearized plasmids were extracted with phenol-chloroform twice, precipitated with ethanol, and resuspended in 10 μl of RNase-free water at 0.5 μg/μl. The mMESSAGE mMACHINE kit (Ambion, Austin, Tex.) was used to in vitro transcribe RNA in a 20-μl reaction mixture with an additional 2 μl of GTP solution. The reaction mixture was incubated at 37°C for 2 h, followed by the addition of DNase I to remove the DNA template. RNA was precipitated with lithium chloride, washed with 70% ethanol, resuspended in RNase-free water, quantitated by spectrophotometry, and stored at −80°C in aliquots. A mutant RNA transcript with a deletion of the 3′-terminal 199 nt of WNV was generated from pFLWNV linearized with an internal restriction site *Dra*I at nt position 10830. The mutant RNA was synthesized in the same manner as the full-length RNA, as described above. All procedures were performed according to manufacturer protocols.

For transfection, approximately 10 μg of RNA was electroporated to 10^7 BHK-21 cells in 0.8 ml of cold phosphate-buffered saline (PBS), pH 7.5, in 0.4-cm cuvettes with the GenePulser apparatus (Bio-Rad) at settings of 0.85 kV and 25 μF, pulsing three times, with no pulse controller. After a 10-min recovery, cells were mixed with media and incubated in a T-75 flask (5% CO_2 at 37°C) until cytopathic effects (CPE) were observed. Virus was harvested as tissue culture media, clarified by centrifugation at 10,000 × *g*, stored in aliquots at −80°C, and designated as recombinant WNV. Plaque assays were performed on Vero cells as described previously (51).

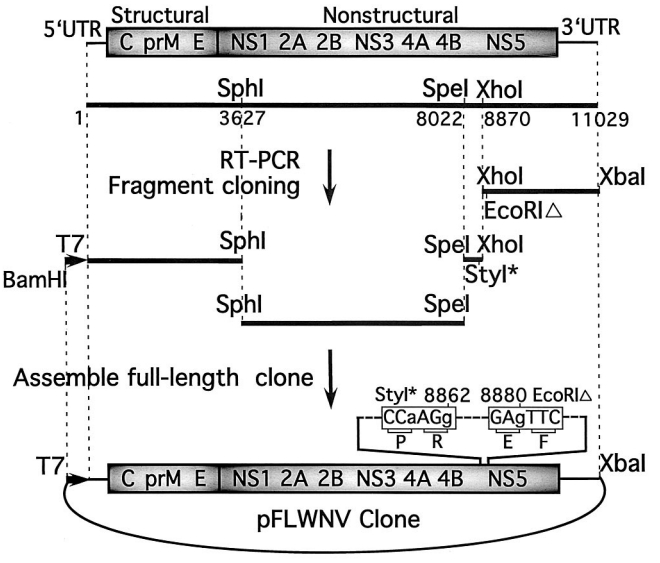

FIG. 1. Construction of the full-length cDNA clone of WNV. Genome organization and unique restriction sites as well as their nucleotide numbers are shown at the top. Four cDNA fragments represented by thick lines were synthesized from genomic RNA through RT-PCR to cover the complete WNV genome. Individual fragments were assembled to form the full-length cDNA clone of WNV (pFLWNV) as described in Materials and Methods. The complete WNV cDNA is positioned under the control of T7 promoter elements for in vitro transcription. Three silent mutations (shown in lowercase) were engineered to create a *Sty*I site (∗) and to knock out an *Eco*RI site (Δ) in the NS5 gene. The numbers are the nucleotide positions based on the sequence from GenBank accession no. AF404756.

Genetic marker analysis of the recombinant and parental virus. Genetic markers of *Sty*I and *Eco*RI were engineered into the cDNA clone (Fig. 1) to distinguish recombinant progeny virus from the corresponding parental virus. Recombinant virus harvested from supernatant on day 3 posttransfection and parental virus were subjected to RNA extraction with RNeasy (Qiagen). A 388-bp fragment including the genetic markers was amplified through RT-PCR from RNA extracted from either recombinant or parental virus with primers 8706V (5′-CATGGCCATGACTGACACTACTC-3′) and 9093C (5′-CTTGGCCTTTCCGAACTCTCCG-3′). The RT-PCR products were digested with *Sty*I or *Eco*RI and analyzed on a 2% agarose gel.

IFA. Indirect immunofluorescence assays (IFA) were used to detect viral protein expression in WNV RNA-transfected BHK-21 cells. After electroporation, approximately 10^5 transfected cells were spotted onto 10-mm glass coverslips. Cells on coverslips were analyzed by IFA at various times posttransfection for viral protein synthesis. Cells were fixed in 3.7% paraformaldehyde with PBS, pH 7.5, at room temperature for 30 min followed by incubation in −20°C methanol for 30 min. The fixed cells were washed with PBS, incubated at room temperature for 45 min in WNV immune mouse ascites fluid (1:100 dilution; ATCC, Manassas, Va.), and further reacted with goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate at room temperature for 30 min (1:100 dilution) (KPL, Gaithersburg, Md.). The coverslips were washed with PBS, mounted to a slide using fluorescent mounting medium (KPL), and observed under a fluorescence microscope equipped with a video documentation system (Zeiss, Thornwood, N.Y.).

Specific infectivity assay. Approximately 10 μg of RNA was electroporated to 10^7 BHK-21 cells, as described above. Both transfected and untransfected BHK-21 cells were adjusted to a concentration of 6×10^5 cells per ml. A series of 1:10 dilutions were made by mixing 0.5 ml of transfected cells with 4.5 ml of untransfected cells. One milliliter of cells (6×10^5 cells total) for each dilution

TABLE 1. Oligonucleotides used to construct the full-length cDNA of WNV

Primer[a]	Primer sequence[b]	Amplified fragment[c]
1V[d]	caaaggatcc*taatacgactcactatag*AGTAGTTCGCCTGTGTGAGCTGA (*Bam*HI)	*Bam*HI-*Sph*I
3839C	ATGTTCTCCTGGTTGGTCCA	
3286V	GTAGAGATTGACTTCGATTAC	*Sph*I-*Spe*I
8804C	CGTACTTCACTCCTTCTGGC	
8016V	GCCCCAACTAGTGCAAAGTTATGGATGGAAC	*Spe*I-*Xho*I
8881C	ATTCTTCTCGAGAGCACATcCTtGGACGTTTTTCTCTGGCC (*Xho*I, *Sty*I)	
8865V	GTGCTCTCGAGAGGAgTTCATAAGA (*Xho*I, *Eco*RIΔ[e])	*Xho*I-*Xba*I
11029C	AacaatctagAGATCCTGTGTTCTCGCACCAC (*Xba*I)	

[a] The primers were named after the nucleotide position of viral sequence and polarity. V, viral genomic sense; C, complementary sense. Nucleotide numbering is based on the sequence from GeneBank accession no. AF404756.

[b] Viral and nonviral sequences are in uppercase and lowercase, respectively. Restriction endonuclease sites are underlined and in parenthesis. Silent mutations within the viral sequences are depicted in lowercase.

[c] cDNA fragments used to construct the full-length clone are shown in Fig. 1.

[d] Italicized sequence represents the T7 promoter.

[e] *Eco*RIΔ represents the knockout of an *Eco*RI site in the cDNA clone by a silent A to g substitution.

was seeded per individual well of six-well plates. Triplicate wells were seeded for each cell dilution. The cells were allowed to attach to the plates for 4 to 5 h under 5% CO_2 at 37°C before the first layer of agar was added, as described previously (53). After incubation of the plates for 3 days under 5% CO_2 at 37°C, a second layer of agar containing neutral red was added. Plaques were counted after incubation of the plates for another 12 to 24 h, and the specific infectivity was calculated as the number of PFU per microgram of RNA.

Growth curves. Subconfluent BHK-21 and C6/36 cells in 12-well plates were inoculated with either the parental or recombinant WNV at an MOI of 5 or 0.05 in triplicate wells. Virus stocks were diluted in BA-1 (M199-H [Gibco-BRL], 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g of sodium bicarbonate/liter, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 1 μg of amphotericin B [Fungizone]/ml). Attachment was allowed for 1 h under 5% CO_2 at 37°C or under 5% CO_2 at 28°C for the BHK-21 and C6/36 cells, respectively. The inocula were then removed, the monolayers were washed three times with BA-1, and 2 ml of medium was added to each well. The plates were incubated for up to 6 days under 5% CO_2 at 37°C or under 5% CO_2 at 28°C for the BHK-21 and C6/36 cells, respectively. The medium was sampled immediately after the addition of medium (1-h time point) and at 7.5, 16, 24, 32, 40, 48 and 72 h for BHK-21 and C6/36 cells, as well as at 96 and 124 h for C6/36 cells. The 10-μl samples were stored at −80°C prior to titration as previously described (53). Cells were observed daily for CPE.

Virulence in mice. Mice were housed in an environmentally controlled room under biosafety level 3 conditions and were given food and water ad libitum. Female outbred CD-1 mice (Charles River Laboratories, Wilmington, Mass.) were obtained at 5 weeks of age and were acclimatized for 1 week. All mice were 6 weeks of age at the start of the experiment. Eight mice per group were inoculated with diluent alone or with 10^2 PFU of parental or recombinant virus subcutaneously (s.c.) in the left rear footpad. Diluent was PBS (endotoxin-free) supplemented with 1% FBS. Mice were evaluated clinically and weighed daily for 2 weeks, then monitored daily and weighed thrice weekly for 2 more weeks. Observed clinical signs included ruffled fur, paresis, hindleg paralysis, and tremors. Morbidity was defined as exhibition of greater than 10% weight loss or clinical signs for 2 or more days. Mice were euthanized if they became moribund. Exposure to virus was confirmed in all surviving mice by a positive antibody titer to WNV by enzyme-linked immunosorbent assay on day 28 postinoculation.

Statistical analyses. Microsoft Excel 97 was used for all statistical analyses. A chi-square test was used to compare the morbidity and mortality in mice for the parental and recombinant viruses, and a two-tailed Student's *t* test was used to evaluate the survival time for the two groups.

RESULTS

Construction and sequencing of the full-length WNV cDNA clone. Selection of the appropriate plasmid vectors and host bacterial strains is critical for construction of flavivirus full-length cDNA clones (56). After testing different vectors and bacterial hosts, plasmid pBR322 and *E. coli* HB101 were chosen for cloning throughout the study. A polylinker containing a number of unique restriction sites was engineered into pBR322 to facilitate the cloning procedure (see Materials and Methods). Cloning procedures for bacterial propagation had higher success rates when performed at room temperature than did those performed at 37°C. All constructs with inserts of greater than 3 kb were propagated at room temperature, but later experiments showed that propagation of the clones at room temperature was not necessarily essential once the clones had been constructed.

Figure 1 shows the overall scheme of the cloning strategy. The primers used to generate individual fragments are listed in Table 1. The full-length cDNA clone was constructed in four steps. First, a fragment from *Spe*I to *Xho*I was amplified by primers 8016V and 8881C and cloned into pBRlinker at their respective sites (Fig. 1), yielding plasmid pSpe-Xho. In order to distinguish the recombinant virus from the parental virus, a *Sty*I site (underlined) was designed in primer 8881C containing silent mutations of nucleotide C to A and A to G at positions 8859 and 8862 (underlined lowercase), respectively. Second, a fragment covering *Xho*I to *Xba*I was amplified by primers 8865V and 11029C and inserted into plasmid pSpe-Xho to generate clone pSpe-Xba (Fig. 1). Primer 8865V contained an A to G substitution at nt 8880 (underlined lowercase) to knock out an *Eco*RI site within the sequence of the parental virus (*Eco*RIΔ in Fig. 1 and Table 1). Third, a fragment covering *Sph*I to *Spe*I was amplified by primers 3286V to 8804C and cloned into pBRlinker at their respective sites, resulting in plasmid pSph-Spe. The fragment from *Sph*I to *Spe*I was then subcloned into pSpe-Xba to yield pSph-Xba (Fig. 1). Finally, a cDNA fragment from *Bam*HI to *Sph*I was amplified by primer 1V and 3839C (Table 1) and cloned into plasmid pSph-Xba at the sites of *Bam*HI and *Sph*I. Primer 1V contained the T7 promoter sequence (italicized lowercase) following the *Bam*HI cloning site (underlined lowercase in Table 1). The resulting plasmid, pFLWNV, contained the complete WNV cDNA under the T7 promoter for in vitro transcription. For RNA synthesis, the full-length cDNA plasmid was linearized by *Xba*I and the overhang nucleotides resulting from *Xba*I digestion were removed by mung bean nuclease. RNA that was in vitro transcribed from this DNA template had an authentic 3′ end of the WNV genomic RNA and a 5′ nonviral G nucleotide derived from the T7 promoter. Analysis of the RNA transcript on a formaldehyde-denaturing agarose gel showed a single band

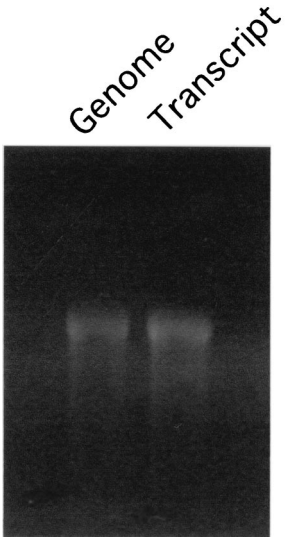

FIG. 2. Transcription of WNV RNA. Formaldehyde-denaturing 1.0% agarose electrophoresis of RNA transcript together with genomic RNA purified from WNV.

with a mobility identical to that of genomic RNA extracted from WNV (Fig. 2).

Sequence analysis of the full-length cDNA clone showed 11 nucleotide changes compared with the parental virus sequence (35) (Table 2). All nucleotide changes were silent mutations except a T to C transition at nt 7826, which resulted in a conservative change from a valine to an alanine residue. Three nucleotide changes were intentionally designed, two to create a *Sty*I site (C to A and A to G at nt 8859 and 8862, respectively) and a third to knock out an *Eco*RI site (A to G at nt 8880). These restriction sites were used as genetic markers to distinguish the recombinant virus from the parental virus (see below). Other mutations in the cDNA clone may derive from the quasispecies of the original virus stock because the parental virus was not plaque purified. It is also possible that some of the mutations occurred during the cloning procedures.

RNA transcript from WNV cDNA clone was highly infectious. Capped RNA transcript was synthesized from the *Xba*I-linearized full-length cDNA plasmid by using T7 RNA polymerase and an optimized 4/3 ratio of methylated cap analogue to GTP. Approximately 30 to 40 μg of RNA was generated from 2 μg of DNA template in a 20-μl reaction mixture. Increasing the ratio of cap analogue to GTP substantially reduced the RNA yield. We routinely electroporated 10 μg of RNA transcript to 10^7 BHK-21 cells as described in Materials and Methods. The transfected cells were incubated and observed for CPE. Apparent CPE were observed in cells on day 3 posttransfection. The cell culture medium was harvested, and virus titer was determined on Vero cells by plaque assays. High viral titers (1×10^9 to 5×10^9 PFU/ml) were consistently obtained.

IFA were used to detect viral protein expression in BHK-21 cells transfected with WNV RNA transcript (Fig. 3). No IFA staining was observed in cells 12 h posttransfection. At 24 h posttransfection, fluorescence was detected in the majority of cells. The staining intensity varied among the IFA-positive cell population. The fluorescent signal increased, and all cells were IFA-positive at 36 h posttransfection. To eliminate the possibility that the positive IFA was merely derived from translation of the transfected RNA rather than from RNA replication in cells, we transfected cells with a mutant RNA containing an expected lethal deletion of the 3′-terminal 199 nt of the genomic RNA. The 3′ deletion RNA was synthesized from the cDNA plasmid digested with a WNV-unique *Dra*I site (nt position 10830). No positive IFA staining was detected in cells at any time points posttransfection (data not shown). These results indicated that the positive IFA signals were initially derived from the replication of RNA in transfected cells and that progeny virus was subsequently generated and spread to neighboring cells through new rounds of infection.

The specific infectivity of RNA transcribed from the full-length cDNA was determined in order to evaluate the efficiency of the system. The specific infectivity of RNA was estimated to be 5×10^4 to 1×10^5 PFU/μg of RNA. Similar specific infectivity was obtained for a mutant RNA containing an extra four nucleotides (5′-CUAG-3′) at the 3′ end. The mutant RNA was synthesized from the *Xba*I-linearized DNA template without mung bean nuclease treatment. Genomic RNA purified from virus showed a specific infectivity of 5×10^5 to 1×10^6 PFU/μg, approximately 10-fold higher than that of transcript RNA. Since uncapped RNA exhibits specific infectivity 10^2- to 10^3-fold lower than that of the capped transcript (40, 54), the discrepancy of infectivity between viral and transcript RNA is most likely due to incomplete capping of the in vitro-transcribed RNA population or to sequence differences, as outlined in Table 2.

The stability of the clone was tested by propagating the *E.coli* HB101 hosting the full-length cDNA plasmid for six continuous passages. Restriction enzyme analysis of the plasmid purified from each of these passages showed digestion patterns identical to that of the original pFLWNV (data not shown). BHK-21 cells transfected with RNA transcripts synthesized from the DNA of passage six showed specific infectivity and CPE indistinguishable from those of passage one cells, indicating that the full-length clone was stable.

Recovered WNV derived from the cDNA clone retained ge-

TABLE 2. Summary of sequence differences between the infectious cDNA clone and parental WNV strain 3356

Nucleotide no.[a]	Strain 3356 genome	cDNA clone	Amino acid change	Location
1285	T	C	Silent	E
3840	T	C	Silent	NS2A
7015	C	T	Silent	NS4B
7826	T	C	V → A	NS5
8067	G	A	Silent	NS5
8859[b]	C	A	Silent	NS5
8862[b]	A	G	Silent	NS5
8880[c]	A	G	Silent	NS5
9123	C	T	Silent	NS5
10613	C	T	Silent	3′UTR
10783	C	T	Silent	3′UTR

[a] Nucleotide position and sequence are based on WNV strain 3356 (GenBank accession no. AF404756).

[b] Mutations were designed to generate an endonuclease *Sty*I site as a marker for recombinant virus (Fig. 4).

[c] This mutation was designed to knock out the endonuclease *Eco*RI site as a marker for recombinant virus (Fig. 4).

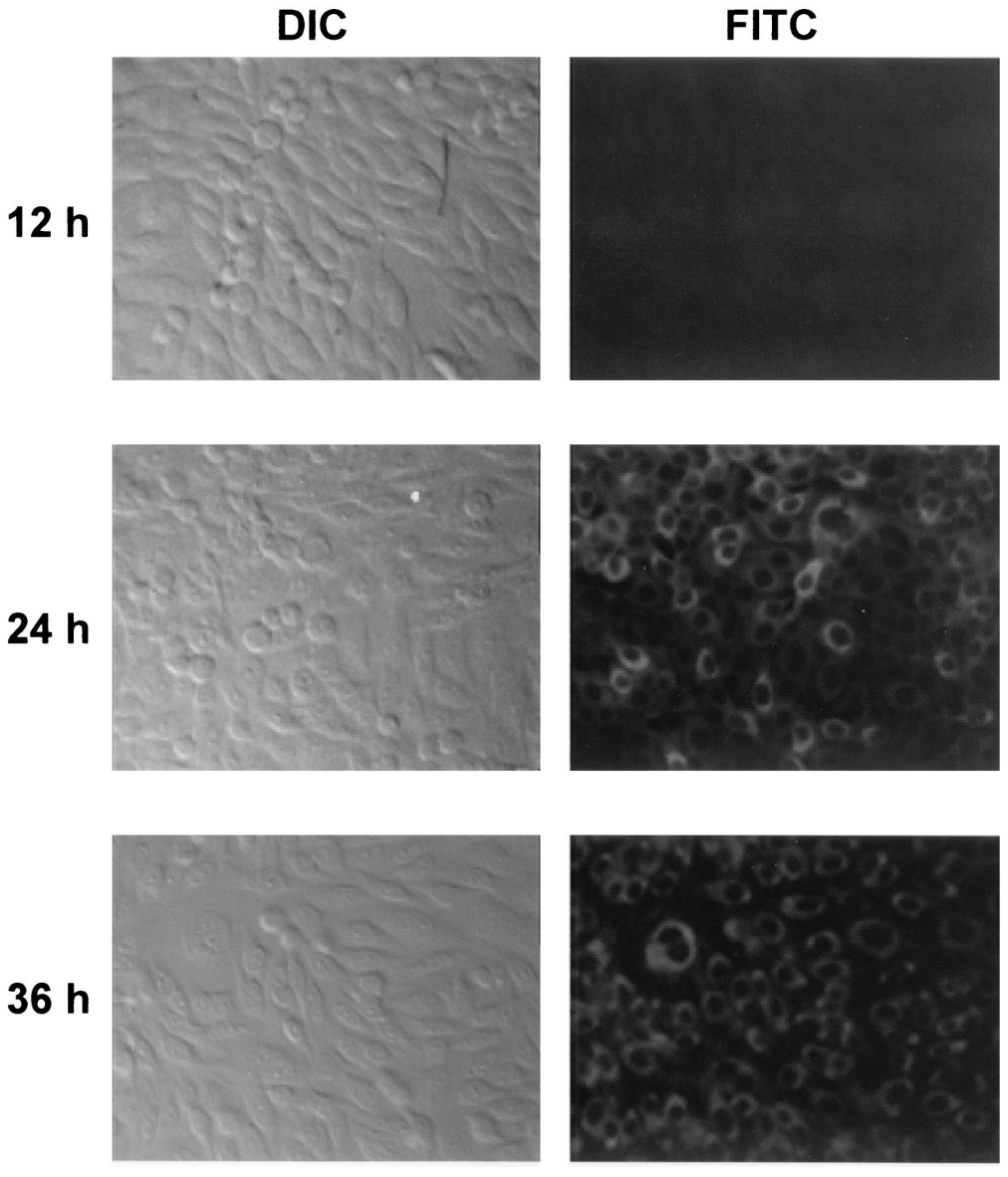

FIG. 3. IFA of viral protein expression in cells transfected with full-length WNV RNA transcript. BHK-21 cells transfected with full-length WNV RNA transcript were analyzed by IFA at the indicated times posttransfection. Photomicrographs were taken at magnifications of ×400. The left and right panels represent the same field of view for each time point. The left panels were visualized with differential interference contrast (DIC), and the right panels were visualized with a fluorescein isothiocyanate (FITC) filter set. For the IFA, WNV immune mouse ascites fluid and goat anti-mouse immunoglobulin G antibody conjugated with FITC were used as primary and secondary antibodies, respectively.

netic markers. To exclude the possibility that the virus recovered from the transfected cells was due to contamination by the parental virus, we engineered genetic markers during the construction of the cDNA clones (Fig. 1). A *Sty*I site was created and an *Eco*RI site was knocked out in the NS5 gene of the recombinant virus. A 388-bp fragment spanning the genetic markers from nt 8706 to 9093 was amplified through RT-PCR from RNA extracted from either parental or recombinant virus. Digestion of the RT-PCR products with *Sty*I and *Eco*RI revealed different cleavage patterns, depending on the origin of the RNA (Fig. 4). As expected, PCR products amplified from parental virus were cleaved by *Eco*RI to generate fragments of 173 bp and 215 bp (lane 4 in Fig. 4B), but were not digested by *Sty*I (Fig. 4B, lane 3). In contrast, PCR products derived from the recombinant virus were not cleaved by *Eco*RI (Fig. 4B, lane 8) but were digested by *Sty*I to generate fragments of 152 and 236 bp (Fig. 4B, lane 7). As a negative control, cells were transfected with RNA containing a deletion of the 3′-terminal 199 nt of the WNV genome (Fig. 4B, 3′ dltn). These cells did not yield any RT-PCR product (lane 9). These results clearly show that virus recovered from the transfected cells was derived from the infectious full-length RNA transcript, not from contaminating parental virus.

The phenotypes of recombinant and parental WNV were indistinguishable. There was no difference in plaque size or morphology on Vero cells between the recombinant and pa-

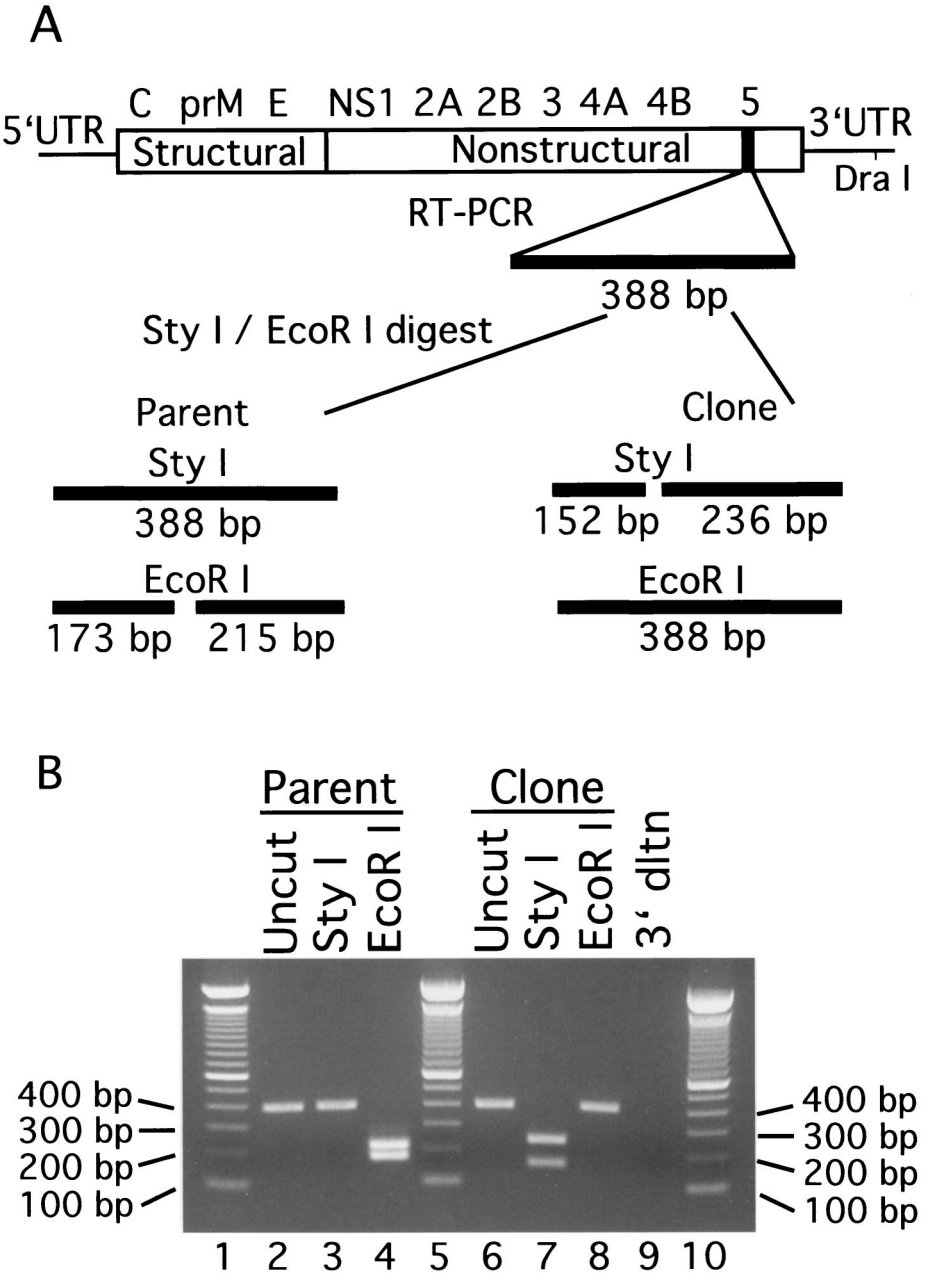

FIG. 4. Recombinant WNV retains the genetic markers engineered during cDNA construction. A *Sty*I site was created and an *Eco*RI site was knocked out in the NS5 gene of the recombinant virus to serve as genetic markers to distinguish recombinant virus from parental virus. A 388-bp fragment (from nt 8706 to 9093) spanning the *Sty*I or *Eco*RI site was amplified through RT-PCR from RNA extracted from either recombinant virus or parental virus. The RT-PCR fragments were subjected to *Sty*I and *Eco*RI digestion. The 388-bp fragment derived from recombinant virus should be cleaved by *Sty*I but not by *Eco*RI; the RT-PCR fragment amplified from parental viral RNA should be digested by *Eco*RI but not *Sty*I. (A) Schematic drawing of genetic marker analysis. The expected sizes of the digestion products are indicated. (B) Agarose gel analysis of genetic markers. Expected digestion pattern as depicted in panel A was observed. As a negative control, no RT-PCR products were detected from the extracted supernatant collected from cells 5 days after transfection with a mutant RNA containing a deletion of the 3′-terminal 199 nt of the genome (lane 9, 3′ dltn). A 100-bp ladder was loaded on lanes 1, 5, and 10 as a standard.

rental viruses (Fig. 5). One-step growth curves at a high MOI of 5 were similar for both recombinant and parental viruses on BHK-21 and C6/36 cells, and their growth characteristics at a low MOI of 0.05 were also equivalent on both cell types (Fig. 6). Furthermore, no quantitative or qualitative differences in CPE were observed between the viruses at each MOI. These data suggest that the parental and recombinant viruses are indistinguishable in replication and spread in both mammalian and insect cells.

The virulence phenotypes of the parental and recombinant viruses were compared in adult mice by inoculating 10^2 PFU s.c. The morbidity, mortality, and average survival times are reported in Table 3. The mortality after s.c. inoculation of 10^2 PFU of parental and recombinant viruses was 62 and 50%,

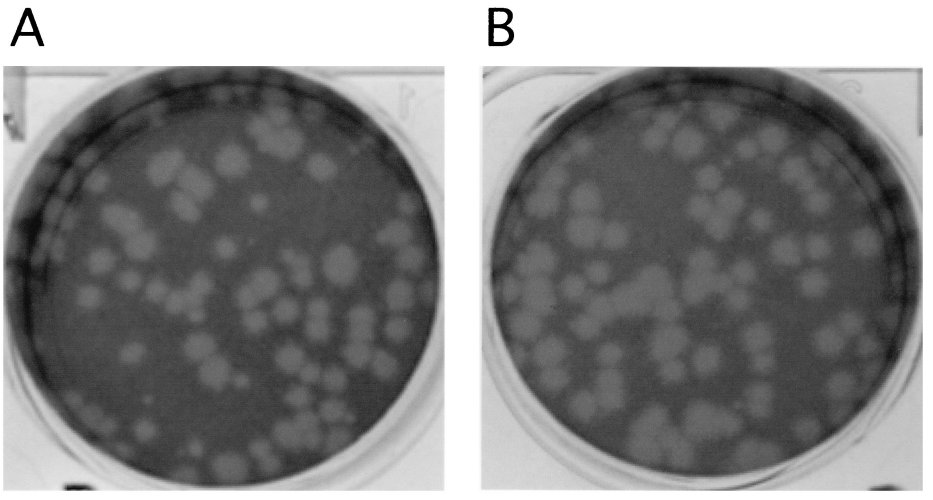

FIG. 5. Plaque morphology of parental and clone-derived WNV on Vero cells. Vero cells in six-well plates were infected with 100 PFU of parental WNV 3356 (A) or 100 PFU of WNV derived from pFLWNV (B). Plaques were visualized 3 days postinoculation by staining for 24 h with neutral red.

respectively. There were no observable differences in the severity or quality of the clinical signs, and the survival curves were very similar. Furthermore, there were no statistical differences in the morbidity ($P = 0.25$), mortality ($P = 0.61$), or average survival times ($P = 0.58$). Thus, the virulence phenotypes were indistinguishable for the parental and recombinant viruses.

DISCUSSION

We report the construction of the first full-length cDNA clone of the human epidemic strain of WNV (lineage I). RNA transcripts transcribed from the cDNA clone were highly infectious upon transfection into BHK-21 cells. The identification of genetic markers engineered into the clone confirmed that the progeny virus was derived from the cDNA clone and thus was not a contaminant. The infectivity of the cDNA-derived RNA was further supported by the finding that a mutant RNA with an expected lethal deletion of the 3′-terminal 199 nt of the genome was not infectious. The recombinant virus showed biological properties indistinguishable from those of the parental virus, including plaque morphology, growth kinetics, and virulence characteristics. These results indicate that an efficient reverse genetic system has been established for lineage I WNV.

A common difficulty in assembling full-length clones of flaviviruses is that plasmids containing long flavivirus-specific inserts are unstable during propagation in bacteria. A number of approaches have been developed to assemble full-length cDNA clones of flaviviruses. (i) Full-length cDNA clone can be assembled through in vitro ligation of cDNA fragments. This approach avoids cloning and propagating full-length clones in bacteria and has been successfully applied to generate infectious RNA of YF (54), JE (62), DEN2 (31), and TBE strain Hypr (40). (ii) Genome-length cDNA containing an upstream promoter for transcription can be directly synthesized by a one-step RT-PCR without any cloning. This rapid method was successfully used to generate infectious RNA of TBE (19). However, this approach has the limitation that the uncloned PCR-derived cDNA will produce a heterogeneous RNA population, derived from mutations during RT-PCR or from quasispecies of the original virus stock. (iii) Full-length cDNA clone can be assembled in yeast cells through homologous recombination. This method was successfully used to assemble a full-length clone of DEN2 (49). (iv) Full-length cDNA can be cloned under the control of a eukaryotic promoter, and introns are introduced into the problematic regions of the cDNA to avoid mutations during their propagation in bacteria. This approach requires transfection of eukaryotic cells with plasmid cDNA rather than RNA. An infectious JE clone was recently developed, using this approach, in which genomic RNA was made in situ by nuclear transcription and intron splicing in transfected eukaryotic cells (67). (v) Stable full-length cDNA clone can be constructed using low- or medium-copy-number vectors and selective bacterial hosts. This approach has been applied to a number of flaviviruses, including DEN4 (34), Kunjin virus (33), TBE strain Neudoerfl (40), MVE (27), TBE strain Langat (38), lineage II WNV (68), and lineage I WNV (this report).

TABLE 3. Morbidity and mortality of parental and recombinant WNV in adult mice[a]

Inoculum	Morbidity (no. sick/total)	Mortality (no. dead/total)	Avg survival time (days [SD])
Diluent	0/8	0/8	NA
Parental WNV	7/8	5/8	9.4 (1.95)
Recombinant WNV	5/8	4/8	10.2 (2.50)

[a] Mice were inoculated with 10^2 PFU s.c. in the left rear footpad. SD, standard deviation; NA, not applicable.

During the construction of our WNV clone, we found that the most unstable region of the genome was within its 5′ quarter and that cDNA from this region should be assembled last in order to obtain full-length clones. These results are consistent with previous reports that cDNAs of structural regions are more likely to be unstable during cloning (67, 68). We also found that bacterial propagation at room temperature

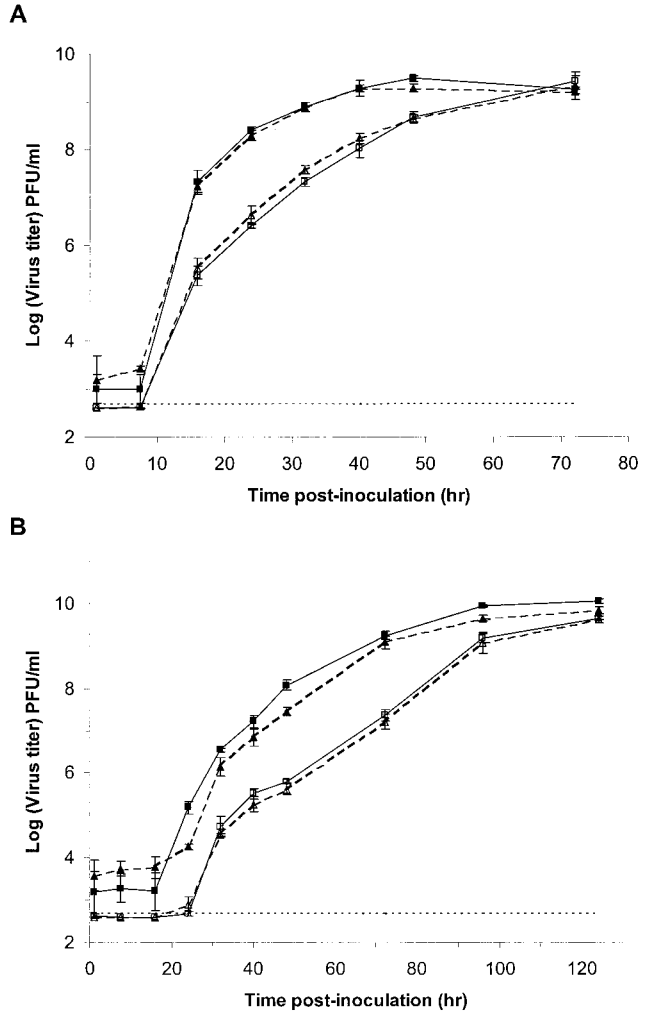

FIG. 6. Comparison of the growth kinetics of recombinant and parental WNV. The growth of recombinant and parental viruses was compared at high and low MOIs on BHK-21 or *Aedes albopictus* C6/36 cells. (A) Growth in BHK-21 cells. (B) Growth in C6/36 cells. Viruses were inoculated at an MOI of 5.0 (filled symbols) or an MOI of 0.05 (open symbols) in triplicate in 12-well plates. Recombinant virus is designated by squares along a solid line. Parental virus is designated by triangles along a dashed line. Error bars represent ± standard deviation of triplicate wells. Dotted line indicates the limit of detection (500 PFU/ml).

rather than at 37°C yielded a higher success rate of cloning intact inserts. A recent report showed that cloning at room temperature was essential to the construction of infectious full-length DEN2 cDNA using high-copy-number plasmid vectors (61).

The 3′ UTR of flaviviruses is believed to function as a promoter for initiation of minus-strand RNA synthesis. The 3′-terminal nucleotides of flavivirus genomic RNA were thermodynamically predicted and experimentally demonstrated to form distinct secondary structures, including a short stem-loop (SL) adjacent to a long SL (8, 50, 52, 60). This secondary structure is conserved among divergent flaviviruses, although only short stretches of sequence are conserved. Structural analysis of WNV 3′ RNA reveals that the loop of the short SL interacts with the lower portion of the neighboring long SL to form a pseudoknot structure (60). Three host proteins bind specifically to the WNV 3′ SL RNA (5), and one of these cellular proteins is the translation elongation factor, eF1-α (6). The NS5 (RNA-dependent RNA polymerase) and NS3 (protease and helicase) of JE were shown to bind specifically to the 3′ SL RNA (17). The cyclization sequences in the 5′- and 3′-terminal regions of the genome were recently demonstrated to be essential for flavivirus replication, both in vivo in Kunjin virus (32) and in vitro in DEN (1, 69). Furthermore, the 3′ SL of WNV RNA was reported to suppress translation of mRNA (38). All of the above reports strongly suggest that the 3′-terminal region of the flavivirus genome plays an essential role in viral replication and possibly regulates translation. Our results show that deletion of the 3′-terminal 199 nt of the genome abolishes the infectivity of WNV RNA. These results agree well with previous reports that deletions in the 3′ UTR of DEN4 (45) and many chimeric 3′ UTRs between DEN2 and WNV (70) are lethal for viral replication. Since the 3′-terminal 199-nt deletion in the mutant RNA included the 3′ cyclization sequence (from nt 10925 to 10932) and the downstream 3′-terminal two-SL structure (from nt 10935 to 11029), it is important to dissect their individual roles during viral replication through systematic mutagenesis analysis.

Many different genetic determinants of virulence have been identified for the flaviviruses (43). For WNV, the studies have been limited. Chambers and coworkers (16) found that neuroinvasion correlates with a mutation in the E gene and determinants outside the E gene. For the related viruses of the JE serocomplex, determinants of neuroinvasion and neurovirulence are in the E gene (12, 21, 22, 37, 44, 47, 65) and the NS1 gene (20). For other flaviviruses, many of the putative virulence determinants are in the E gene (23–26, 29, 48, 58), but mutations in the NS1 gene (10, 46, 51), NS5 gene (66), and the 5′ and 3′ UTR (10, 41) are also associated with virulence. Site-directed mutagenesis of the cDNA clone in this report will allow identification of molecular determinants of virulence for the epidemic strain of WNV.

Lineage I WNV strains have been mostly isolated from epidemic outbreaks and epzootics in birds and equines and have a worldwide distribution. In contrast, lineage II strains have been incidentally isolated from humans with mild febrile disease or without symptoms and are restrictedly found in sub-Saharan Africa and Madagascar (4, 35, 36, 59). Based on sequence analysis of the complete genomes, nucleotide identity between the two lineages is approximately 75% (35). Limited information is known about the pathogenic differences between lineages I and II and among strains within each lineage. Similar growth kinetics were observed for a lineage II Nigerian strain (68) and our lineage I New York strain for both mosquito and mammalian cells. Replacement of the 3′-terminal 1,438 nt of the Nigerian strain (lineage II) with the equivalent sequence (including the complete 3′ UTR and sequence encoding the carboxy-terminal 287 amino acids of NS5) from the prototype WNV Eg101 strain (lineage I) yielded a chimeric virus that showed growth kinetics similar to those of the wild-type Nigerian strain (68). Others have shown differences in neuroinvasiveness in mice between viruses from lineage I and II (D. W. C. Beasley, L. Li, M. T. Suderman, and A. D. Barrett, International Conference on the West Nile Virus, New York Academy of Science Poster Section **1:**5, 2001). Lineage I

strains can be further divided into three clades: clade 1a includes viruses from Africa, Europe, Russia, Middle East, and United States; clade 1b includes Kunjin virus from Australia; and clade 1c includes viruses from India (35). Within clade 1a, all U.S. isolates (including the New York crow strain 3356 used in this study) have a nucleotide identity of 99.8%, 99.7% with an Israeli 1998 strain, and 95.2 to 96.4% with strains from Europe, Russia, and Egypt. Kunjin virus in clade 1b exhibits a nucleotide identity of 86.6 to 87.2% with strains in clade 1a (35, 36). During the recent WNV outbreaks, bird mortality was observed in the United States and Israel but not in Europe; therefore, it was speculated that genetic variability within lineage I strains could affect pathogenicity (35, 36, 63). Lanciotti et al. (35) recently showed that six amino acid changes are consistent with the geographic origin of these viruses and might confer the pathogenic difference among these lineage I strains. It will be very interesting to use the infectious clone described here to experimentally test these observations.

Many factors could contribute to the fact that lineage I WNV strains are frequently involved in human outbreaks, while lineage II viruses are mostly maintained in enzootic cycles (4, 36). In addition to possible differences in virulence, differences in vector competence and transmission cycles as well as host immunity may contribute to the difference in disease pattern between the two lineages. The infectious cDNA clones of WNV will serve as a valuable tool to address many of these questions.

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