5% CO_2. Cells were examined for cytopathologic effect for up to 7 days after inoculation.
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5. Total numbers of mosquitoes by species that were collected in 14 towns in Fairfield County, CT, and tested for virus from 6 September through 14 October 1999: *Ae. vexans*, 1688; *Ae. cinereus*, 172; *Ae. trivittatus*, 131; *Ae. taeniorhynchus*, 123; *Ae. sollicitans*, 109; *Ae. cantator*, 63; *Ae. triseriatus*, 28; *Ae. japonicus*, 19; *Ae. canadensis*, 1; *Anopheles punctipennis*, 82; *An. quadrimaculatus*, 4; *An. walkeri*, 2; *Coquillettidia perturbans*, 15; *Culex pipiens*, 744; *Cx. restuans*, 27; *Cx. erraticus*, 4; *Cx. territans*, 1; *Culiseta melanura*, 76; *Cs. morsitans*, 1; *Psorophora ferox*, 4; and *Uranotaenia sapphirina*, 104.
6. M. Z. Ansari, R. E. Shope, S. Malik, *J. Clin. Lab. Anal.* **7**, 230 (1993). Isolates were tested initially in an ELISA against reference antibodies to six viruses, in three families, isolated from mosquitoes in North America. The antibodies were prepared in mice and provided by the World Health Organization Center for Arbovirus Research and Reference, Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine. The antibodies were to Eastern Equine Encephalomyelitis, Highlands J, Cache Valley, LaCrosse, Jamestown Canyon, and St. Louis Encephalitis viruses.
7. Most dead birds were collected by state or town personnel in Connecticut and sent to the Pathobiology Department at the University of Connecticut, Storrs, where they were examined for postmortem and nutritional condition, gross lesions, and microscopic evidence indicative of encephalitis. Brain tissue from birds with presumed encephalitis were frozen at −70°C and then sent to the Connecticut Agricultural Experiment Station, New Haven, for virus testing. Corresponding brain sections were processed for histologic examination. A 10% suspension of each sampled brain tissue was prepared in 1.5 ml of phosphate-buffered saline by triturating with a mortar and pestle (*3*). Two to seven tissue samples from each brain were tested for virus. Alundum was added to facilitate homogenization of tissue. Suspensions were centrifuged at 520*g* for 10 min. The supernatant of each sample was then passed through a 0.22-μm filter before inoculation of a 100-μl sample onto a monolayer of Vero cells. Cells were grown and examined for cytopathologic effect (*3*). Isolates were initially tested against reference antibodies (*6*).
8. Connecticut towns from which dead crows were collected and virus isolated from brain tissues (number of isolates in parentheses): Bridgeport (n = 1), Darien (n = 1), Fairfield (n = 4), Greenwich (n = 3), Hamden (n = 1), Madison (n = 1), Milford (n = 1), New Canaan (n = 1), New Haven (n = 3), North Haven (n = 1), Norwalk (n = 1), Redding (n = 1), Stamford (n = 5), Stratford (n = 1), Weston (n = 1), Westport (n = 1), and Woodbridge (n = 1).
9. The Cooper's hawk was observed alive on the ground on 25 September 1999 and was described as having difficulty standing, spinning in circles, and having seizures. It died 11 hours after being found. Gross pathology of the brain showed extensive hemorrhage.
10. D. L. Swofford, *PAUP: Phylogenetic Analysis Using Parsimony Users Manual* (Illinois Natural History Survey, Champaign, 1993). Data were analyzed by PAUP 4b.1 with maximum parsimony, maximum likelihood, and neighbor-joining analysis. The data set was identical for all analyses. A total of 933 characters was used, including insertions created during (Clustal X) alignment. All characters were unordered and had equal weight; all sites were assumed to evolve at the same rate. Four hundred and forty-six characters were constant, 281 characters were parsimony-uninformative, and 206 characters were parsimony-informative. Gaps were treated as missing. For maximum parsimony analysis, the best tree found = 754; number of trees retained = 1. The branch and bound method of search was used to guarantee finding the shortest tree (or trees). For the bootstrap analysis, 500 replicates were run with the maximum parsimony method. Maximum likelihood analysis settings corresponded to the Felsenstein model. Transition/transversion ratio = 2 (κ = 3.88125); molecular clock was not enforced; trees with approximate likelihoods of 5% or further from the target score were rejected without additional iteration; "MulTrees" option was in effect; topological constraints were not enforced. Score for best tree found by maximum likelihood analysis = 4278.24084; number of trees retained = 1. Trees were run as unrooted. Passage 2 of each virus isolate was grown in Vero cells (*3*) at 37°C. Infected cells were scraped from the bottom of the flask, centrifuged at 4500*g* for 10 min, and the supernatant was discarded. RNA was extracted from the pellet using the Rneasy mini protocol (Qiagen), eluting the column twice with 40 μl of ribonuclease-free water. Two microliters of each eluate were used in a 50-μl reverse transcription–polymerase chain reaction (RT-PCR) with the GeneAmp EZ rTth RNA PCR kit (Perkin-Elmer). Primers WN-233F-GACTGAAGAGGGCAATGTTGAGC and WN-1189R-GCAATAACTGCGGACYTCTGC used in the reaction were designed to specifically amplify WN and Kunjin viruses based on an alignment of six flavivirus isolates listed in GenBank [SLE virus capsid, membrane, envelope: accession M16614; Japanese encephalitis virus polyprotein: accession M73710; Kunjin virus gene for polyprotein: accession D00246; Nigerian WN virus complete genome: accession M12294; Romania WN virus strain R097-50 polyprotein gene, partial, accession AF130362; Romania WN virus strain 96-1030 polyprotein gene, accession AF130363]. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and submitted to the Keck Biotechnology Center at Yale University, New Haven, CT, for sequencing. Sequences were aligned with Clustalx 1.64b [J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **22**, 4673 (1994)].
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22. Cells were fixed at 4°C in a 2.5% (v/v) glutaraldehyde–2% paraformaldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose in 100 mM Na cacodylate buffer (pH 7.4), postfixed in 1% (w/v) OsO_4, dehydrated through an ethanol and acetone series, and embedded in an LX-112–Araldite mixture. Thin sections were poststained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV. Virus particles measured 35 to 40 nm.
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24. We thank J. Correia, J. Shepard, M. Vasil, B. Hamid, C. Scott, and T. Blevins for technical assistance. P. Mazik, P. Mazik, R. Wagner, P. Lucas, T. Capanella, R. Nieves, and S. Nieves helped with the collection of mosquitoes in Greenwich and Stamford. K. Hannon and R. Schaper provided the brain from the Cooper's hawk. Supported in part by Hatch Grant 763 and NIH grant P01-AI-30548.

22 October 1999; accepted 19 November 1999

Origin of the West Nile Virus Responsible for an Outbreak of Encephalitis in the Northeastern United States

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In late summer 1999, an outbreak of human encephalitis occurred in the northeastern United States that was concurrent with extensive mortality in crows (*Corvus* species) as well as the deaths of several exotic birds at a zoological park in the same area. Complete genome sequencing of a flavivirus isolated from the brain of a dead Chilean flamingo (*Phoenicopterus chilensis*), together with partial sequence analysis of envelope glycoprotein (E-glycoprotein) genes amplified from several other species including mosquitoes and two fatal human cases, revealed that West Nile (WN) virus circulated in natural transmission cycles and was responsible for the human disease. Antigenic mapping with E-glycoprotein–specific monoclonal antibodies and E-glycoprotein phylogenetic analysis confirmed these viruses as WN. This North American WN virus was most closely related to a WN virus isolated from a dead goose in Israel in 1998.

In late August and early September 1999, New York City and surrounding areas experienced an outbreak of human encephalitis consistent with an arboviral etiology. Serological evidence from this outbreak implicated a flavivirus as the etiologic agent. Concur-

rent with this human encephalitis outbreak, a viral encephalitis of unknown etiology was discovered in American crows (*Corvus brachyrhynchos*) and fish crows (*Corvus ossifragus*) dying in the same geographic area. Deaths were also observed among several exotic avian species, including a Chilean flamingo (*Phoenicopterus chilensis*) at the Bronx Zoo. Necropsy samples from these birds were submitted to the National Veterinary Services Laboratories, U.S. Department of Agriculture, and were inoculated into embryonated chicken eggs for virus isolation. Flavivirus-like particles (diameter 40 nm) were observed by electron microscopy in the allantoic fluid 4 days after inoculation. The isolates were forwarded to the Centers for Disease Control and Prevention (CDC) for identification.

The complete nucleotide sequence of one of these viral isolates (WN-NY99, from the dead Chilean flamingo) has now been determined. The viral genomic RNA was amplified and copied into overlapping DNA fragments of ~2 to 3 kb by means of the reverse transcription polymerase chain reaction (RT-PCR) (*1*). Both strands of the purified DNAs were sequenced with the use of primers spaced about 400 bases apart along the entire genome. The complete 11,029-nucleotide genomic sequence of WN-NY99 has been submitted to GenBank (accession number AF196835). The deduced amino acid sequence of the coding region of WN-NY99 (genomic positions 97 to 10,395) is shown in Fig. 1. The WN-NY99 virus genome exhibited standard flavivirus genomic organization, the same overall genomic organization as was described for the WN-Nigeria and Kunjin (KUN) viruses (*2*). A short 5′ noncoding region of 96 nucleotides is followed by an ATG initiation codon at position 97 and a single open reading frame of 10,302 nucleotides coding for three structural proteins—capsid, premembrane (prM), and envelope (E)—and five nonstructural proteins (NS1, NS2a/NS2b, NS3, NS4a/NS4b, and NS5). The coding region of WN-NY99 is followed by a 3′ noncoding region of 631 nucleotides.

To identify the New York virus antigenically, we performed indirect immunofluorescence antibody tests using a panel of well-defined monoclonal antibodies (mAbs) to map various isolates from birds and mosquitoes. The mAb end-point titers with the North American isolates were compared to titers derived with other representatives of the Japanese encephalitis (JE) virus serocomplex of flaviviruses (Table 1). These mAbs, which are specific for the E-glycoprotein, can distinguish WN virus from KUN virus and can also distinguish either of these viruses from other members of the JE virus serocomplex. Viruses were grown in Vero cells, spotted onto 12-well slides, air-dried, and fixed with acetone before staining. All viruses reacted similarly with the broad flavivirus-reactive, positive-control mAb 4G2 (*3, 4*). None of these viruses reacted with the negative-control antibody, which is specific for the E1 glycoprotein of eastern equine encephalitis (EEE), an unrelated alphavirus (*5*). All WN isolates, including those from North America, reacted specifically with the WN virus–specific mAb H5.46, but not with the KUN

cap
MSKKPGGPGKSRAVNMLKRGMPRVLSLIGLKRAMLSLIDGKGPIRFVLALLAFFRFTAIAPTRAVLDRWRGVNKQTAMKHLLSFKKELGTLTSAINRRSS
prM
KQKKRGGKTGIAVMIGLIASVGAVTLSNFQGKVMMTVNATDVTDVITIPTAAGKNLCIVRAMDVGYMCDDTITYECPVLSAGNDPEDIDCWCTKSAVYVR
M
YGRCTKTRHSRRSRRSLTVQTHGESTLANKKGAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFVVLLLLVAPAYSFNCLGMSNRD
E
FLEGVSGATWVDLVLEGDSCVTIMSKDKPTIDVKMMNMEAVNLAEVRSYCYLATVSDLSTKAACPTMGEAHNDKRADPAFVCRQGVVDRGWGNGCGLFGK
GSIDTCAKFACSTKAIGRTILKENIKYEVAIFVHGPTTVESHGNYSTQVGATQAGRFSITPAAPSYTLKLGEYGEVTVDCEPRSGIDTNAYYVMTVGTKT
FLVHREWFMDLNLPWSSAGSTVWRNRETLMEFEEPHATKQSVIALGSQEGALHQALAGAIPVEFSSNTVKLTSGHLKCRVKMEKLQLKGTTYGVCSKAFK
FLGTPADTGHGTVVLELQYTGTDGPCKVPISSVASLNDLTPVGRLVTVNPFVSVATANAKVLIELEPPFGDSYIVVGRGEQQINHHWHKSGSSIGKAFTT
NS1
TLKGAQRLAALGDTAWDFGSVGGVFTSVGKAVHQVFGGAFRSLFGGMSWITQGLLGALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHADTGCAIDIS
RQELRCGSGVFIHNDVEAWMDRYKYYPETPQGLAKIIQKAHKEGVCGLRSVSRLEHQMWEAVKDELNTLLKENGVDLSVVVEKQEGMYKSAPKRLTATTE
KLEIGWKAWGKSILFAPELANNTFVVDGPETKECPTQNRAWNSLEVEDFGFGLTSTRMFLKVRESNTTECDSKIIGTAVKNNLAIHSDLSYWIESRLNDT
WKLERAVLGEVKSCTWPETHTLWGDGILESDLIIPVTLAGPRSNHNRRPGYKTQNQGPWDEGRVEIDFDYCPGTTVTLSESCGHRGPATRTTTESGKLIT
NS2A
DWCCRSCTLPPLRYQTDSGCWYGMEIRPQRHDEKTLVQSQVNAYNADMIDPFQLGLLVVFLATQEVLRKRWTAKISMPAILIALLVLVFGGITYTDVLRY
VILVGAAFAESNSGGDVVHLALMATFKIQPVFMVASFLKARWTNQENILLMLAAVFFQMAYHDARQILLWEIPDVLNSLAVAWMILRAITFTTTSNVVVP
LLALLTPGLRCLNLDVYRILLLMVGIGSLIREKRSAAAKKKGASLLCLALASTGLFNPMILAAGLIACDPNRKRGWPATEVMTAVGLMFAIVGGLAELDI
NS2B
DSMAIPMTIAGLMFAAFVISGKSTDMWIERTADISWESDAEITGSSERVDVRLDDDGNFQLMNDPGAPWKIWMLRMVCLAISAYTPWAILPSVVGFWITL
NS3
QYTKRGGVLWDTPSPKEYKKGDTTTGVYRIMTRGLLGSYQAGAGVMVEGVFHTLWHTTKGAALMSGEGRLDPYWGSVKEDRLCYGGPWKLQHKWNGQDEV
QMIVVEPGKNVKNVQTKPGVFKTPEGEIGAVTLDFPTGTSGSPIVDKNGDVIGLYGNGVIMPNGSYISAIVQGERMDEPIPAGFEPEMLRKKQITVLDLH
PGAGKTRRILPQIIKEAINRRLRTAVLAPTRVVAAEMAEALRGLPIRYQTSAVPREHNGNEIVDVMCHATLTHRLMSPHRVPNYNLFVMDEAHFTDPASI
AARGYISTKVELGEAAAIFMTATPPGTSDPFPESNSPISDLQTEIPDRAWNSGYEWITEYTGKTVWFVPSVKMGNEIALCLQRAGKKVVQLNRKSYETEY
PKCKNDDWDFVITTDISEMGANFKASRVIDSRKSVKPTIITEGEGRVILGEPSAVTAASAAQRRGRIGRNPSQVGDEYCYGGHTNEDDSNFAHWTEARIM
LDNINMPNGLIAQFYQPEREKVYTMDGEYRLRGEERKNFLELLRTADLPVWLAYKVAAAGVSYHDRRWCFDGPRTNTILEDNNEVEVITKLGERKILRPR
NS4A
WIDARVYSDHQALKAFKDFASGKRSQIGLIEVLGKMPEHFMGKTWEALDTMYVVATAEKGGRAHRMALEELPDALQTIALIALLSVMTMGVFFLLMQRKG
NS4B
IGKIGLGGAVLGVATFFCWMAEVPGTKIAGMLLLSLLLMIVLIPEPEKQRSQTDNQLAVFLICVMTLVSAVAANEMGWLDKTKSDISSLFGQRIEVKENF
SMGEFLLDLRPATAWSLYAVTTAVLTPLLKHLITSDYINTSLTSINVQASALFTLARGFPFVDVGVSALLLAAGCWGQVTLTVTVTAATLLFCHYAYMVP
GWQAEAMRSAQRRTAAGIMKNAVVDGIVATDVPELERTTPIMQKKVGQIMLILVSLAAVVVNPSVKTVREAGILITAAAVTLWENGASSVWNATTAIGLC
NS5
HIMRGGWLSCLSITWTLIKNMEKPGLKRGGAKGRTLGEVWKERLNQMTKEEFTRYRKEAIIEVDRSAAKHARKEGNVTGGHPVSRGTAKLRWLVERRFLE
PVGKVIDLGCGRGGWCYYMATQKRVQEVRGYTKGGPGHEEPQLVQSYGWNIVTMKSGVDVFYRPSECCDTLLCDIGESSSSAEVEEHRTIRVLEMVEDWL
HRGPREFCVKVLCPYMPKVIEKMELLQRRYGGGLVRNPLSRNSTHEMYWVSRASGNVVHSVNMTSQVLLGRMEKRTWKGPQYEEDVNLGSGTRAVGKPLL
NSDTSKIKNRIERLRREYSSTWHHDENHPYRTWNYHGSYDVKPTGSASSLVNGVVRLLSKPWDTITNVTTMAMTDTTPFGQQRVFKEKVDTKAPEPPEGV
KYVLNETTNWLWAFLAREKRPRMCSREEFIRKVNSNAALGAMFEEQNQWRSAREAVEDPKFWEMVDEEREAHLRGECHTCIYNMMGKREKKPGEFGKAKG
SRAIWFMWLGARFLEFEALGFLNEDHWLGRKNSGGGVEGLGLQKLGYILREVGTRPGGKIYADDTAGWDTRITRADLENEAKVLELLDGEHRRLARAIIE
LTYRHKVVKVMRPAADGRTVMDVISREDQRGSGQVVTYALNTFTNLAVQLVRMMEGEGVIGPDDVEKLTKGKGPKVRTWLFENGEERLSRMAVSGDDCVV
KPLDDRFATSLHFLNAMSKVRKDIQEWKPSTGWYDWQQVPFCSNHFTELIMKDGRTLVVPCRGQDELVGRARISPGAGWNVRDTACLAKSYAQMWLLLYF
HRRDLRLMANAICSAVPVNWVPTGRTTWSIHAGGEWMTTEDMLEVWNRVWIEENEWMEDKTPVEKWSDVPYSGKREDIWCGSLIGTRARATWAENIQVAI
NQVRAIIGDEKYVDYMSSLKRYEDTTLVEDTVL

Fig. 1. Deduced amino acid sequence of the polyprotein of West Nile virus WN-NY99. The start of each protein is marked by an arrow. Abbreviations for protein names: cap, nucleocapsid; prM, premembrane protein; M, viral membrane protein; E, viral envelope glycoprotein; NS1 to NS5, viral nonstructural proteins. The E-glycoprotein glycosylation motif (NYS) is underlined. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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virus–specific mAb 10A1 (four- to eightfold titer differences) (*6–8*). Similarly, only KUN virus reacted with mAb 10A1, but not with mAb H5.46. No KUN or WN viruses reacted with either the St. Louis encephalitis (SLE) virus–specific mAb 6B5A-2 or the Murray Valley encephalitis (MVE) virus–specific mAb 4B6C-2 (*9, 10*). These results type the North American isolates as WN virus and not as KUN, SLE, or MVE viruses.

WN virus belongs to the family Flaviviridae, genus *Flavivirus*, and is a member of the JE virus serocomplex, which also includes JE, SLE, MVE, and KUN viruses, among others (*11*). Flaviviruses are plus-sense, single-stranded RNA viruses with a genome of ~11,000 nucleotides (*2*). Recently published sequence and phylogenetic data suggest that, within this serocomplex, KUN viruses appear to be a subtype of WN virus rather than a separate viral species (*12*). Although flaviviruses are closely related to each other antigenically and cross-react in serological tests with polyclonal antisera, most have a rather distinctive geographic distribution. Those of the JE serocomplex are maintained in a natural transmission cycle involving mosquito vectors and bird reservoir hosts. Humans and horses are usually incidental hosts.

To determine more precisely the relationships between the WN-NY99 virus and other related virus strains, we performed a phylogenetic analysis on an informative region of the E-glycoprotein gene (genome positions 1402 to 1656) (*12, 13*). Aligned nucleic acid sequence data from 33 WN viruses, seven KUN viruses, and one JE virus were analyzed with the use of algorithms for parsimony (PAUP), distance (MEGA; Fig. 2), and maximum likelihood (fastDNAml) (*14–17*). The phylogenetic trees generated by these analyses had the same overall topology as that previously observed, insofar as all WN and KUN viruses are separated into two major lineages (*12, 13*). Viruses in lineage 1 are primarily of West African, Middle Eastern, Eastern European, and Australian origin. Lineage 2 consists exclusively of viruses from the African continent that have apparently not been involved in human or equine outbreaks, but rather are maintained in enzootic cycles.

Within lineage 1, the KUN viruses and the Indian WN viruses both appear as monophyletic sister clades to the European and African WN viruses. The WN-NY99 virus is found within lineage 1 and is most closely related to WN viruses that have recently been isolated from North Africa, Romania, Kenya, Italy, and the Middle East. Of particular note is the close relationship between the WN-NY99 virus and a WN virus isolated from the brain of a dead goose in Israel in 1998. Phylogenetic analysis of a portion of the gene encoding the NS5 protein and of the 3′ noncoding region (830 bases) of 12 WN and KUN viruses also generates trees with nearly identical topology, with WN-NY99 demonstrating the closest relationship with lineage 1 WN viruses (*18*). Flavivirus sequences amplified from brain specimens from fatal hu-

Table 1. Antigenic characterization of North American WN viruses. Ig, immunoglobulin; nd, not done.

mAb*	Virus specificity	Ig	Virus†								Vero‡
			KUN	Rom WN	Egypt WN	NYC WN	India WN	SLE	MVE	EEE	
H5.46	WN	IgM	20	160	160	80	80	≤10	≤10	nd	≤10
10A1	KUN	IgG2b	80	≤10	≤10	≤10	≤10	≤10	≤10	nd	≤10
2B2	WN/KUN	IgG2a	80	80	160	160	160	≤10	≤10	nd	≤10
4B6C-2	MVE	IgG2a	≤10	≤10	≤10	≤10	≤10	≤10	≥320	nd	≤10
6B5A-2	SLE	IgG2a	≤10	≤10	≤10	≤10	≤10	≥320	≤10	nd	≤10
4G2	Flavivirus	IgG2a	≥320	≥320	≥320	≥320	≥320	≥320	≥320	nd	≤10
1B1C-4	EEE-E1	IgG2b	≤10	≤10	≤10	≤10	≤10	≤10	≤10	320	≤10

*The mAbs H5.46, 4B6C-2, 6B5A-2, 4G2, and 1B1C-4 were mouse ascitic fluids; mAbs 10A1 and 2B2 were cell culture supernatants; mAb 1B1C-4, used as a negative control antibody, is specific for the E1 glycoprotein of the alphavirus EEE. †Virus strains used: KUN, Kunjin MRM-16; Rom WN, Romania mosquito isolate 97-50; Egypt WN, Eg101; NYC WN, New York City mosquito isolate NY-99-6922; India WN, 2266; SLE, MSI-7; MVE, original; EEE, strain NJ/60. ‡Uninfected cell control.

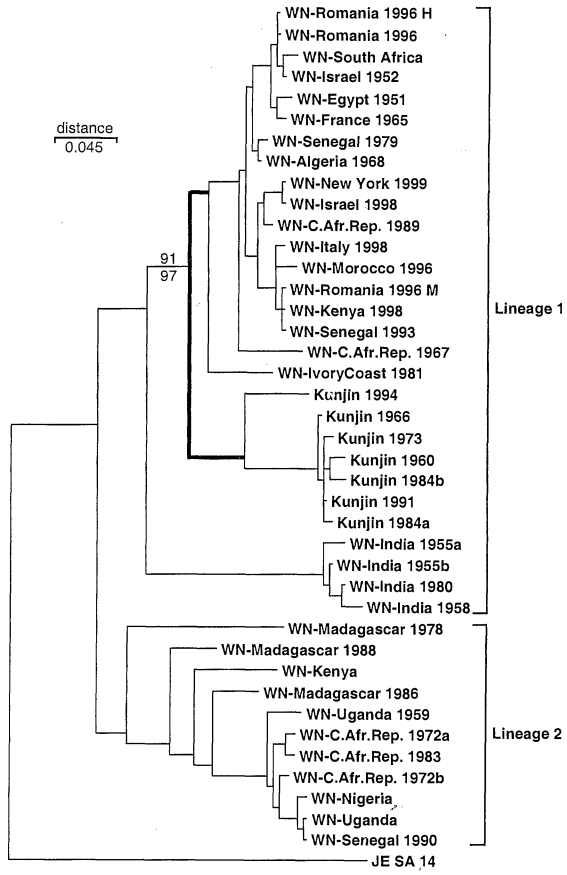

Fig. 2. Phylogenetic tree based on E-glycoprotein nucleic acid sequence data (255 base pairs). The tree was constructed with the program MEGA by neighbor-joining with Kimura two-parameter distance (scale bar). Bootstrap confidence level (500 replicates) and a confidence probability value based on the standard error test (*22, 23*) were calculated using MEGA and are included on the tree (top and bottom values, respectively), illustrating support for the division between the lineage 1 WN virus group (not including the India isolates) and the KUN virus group. The best estimated length of the segment (bold line) separating these groups, in units of expected nucleotide substitutions per site, is 0.06928 and is statistically significantly positive ($P < 0.01$) by the likelihood ratio test (fastDNAml maximum likelihood program). An approximate 95% confidence interval for the true length of this segment is 0.03347, 0.10737. The isolate history of strains used in this tree and the alignment used for analysis are available upon request from the authors. GenBank accession numbers for the sequences included in the tree are as follows: WN-Romania 1996 H, AF130363; WN-Romania 1996, AF205879; WN–South Africa, AF205880; WN-Israel 1952, AF205881; WN-Egypt 1951, AF001568; WN-France 1965, AF001560; WN-Senegal 1979, AF001569; WN-Algeria 1968, AF001567; WN–New York 1999, AF196835; WN-Israel 1998, AF205882; WN-C.Afr.Rep. 1989, AF001558; WN-Italy 1998, AF205883; WN-Morocco 1996, AF205884; WN-Romania 1996 M, AF130362; WN-Kenya 1998, AF146082; WN-Senegal 1993, AF001570; WN-C.Afr.Rep. 1967, AF001566; WN–Ivory Coast 1981, AF001561; Kunjin 1994, AF196495; Kunjin 1966, AF196509; Kunjin 1973, AF196515; Kunjin 1960, D00246; Kunjin 1984b, AF196498; Kunjin 1991, AF196491; Kunjin 1984a, AF196519; WN-India 1955a, AF205885; WN-India 1955b, AF196525; WN-India 1980, AF196526; WN-India 1958, AF196524; WN-Madagascar 1978, AF001559; WN-Madagascar 1988, AF001574; WN-Kenya, AF001571; WN-Madagascar 1986, AF001564; WN-Uganda 1959, AF001562; WN-C.Afr.Rep. 1972a, AF001563; WN-C.Afr.Rep. 1983, AF001557; WN-C.Afr.Rep. 1972b, AF001565; WN-Nigeria, M12294; WN-Uganda, AF001573; WN-Senegal 1990, AF001556; JE SA 14, U04522.

Table 2. Percent identity among West Nile virus strains over a 1278-nucleotide base region of prM and E proteins.

	WN virus strain*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	WN-NY99		99.8	99.9	99.8	99.9	99.9	99.8	99.9	99.8	99.8	96.9	95.1	87.1	75.8
2	WN-Crn-CT99			99.9	99.8	99.9	99.9	99.8	99.9	99.8	99.8	96.9	95.2	87.2	75.8
3	WN-Crow-NJ99				99.8	100.0	100.0	99.9	100.0	99.8	99.9	97.0	95.3	87.2	75.9
4	WN-Crow-NY99					99.8	99.8	99.8	99.8	99.7	99.8	96.8	95.1	87.0	76.0
5	WN-C.pipiens-NY99						100.0	99.9	100.0	99.8	99.9	97.0	95.3	87.2	75.9
6	WN-Eq.-NY99							99.9	100.0	99.8	99.9	97.0	95.3	87.2	75.9
7	WN-HB709-NY99								99.9	99.8	99.8	96.9	95.2	87.2	76.0
8	WN-HB743-NY99									99.8	99.9	97.0	95.3	87.2	75.9
9	WN-USAMRIID99										99.9	97.2	95.3	87.3	76.0
10	WN-Israel 1998											97.1	95.4	87.4	76.1
11	WN-Rom.-mosq.96												94.7	87.2	76.4
12	WN-Rom.-hum.96													87.5	76.9
13	Kunjin-MRM-16														76.3
14	WN-Nigeria														

*Sources of WN virus strains are as follows: WN-NY99, virus isolate from Bronx Zoo flamingo 382-99 (entire genome sequenced); WN-Crn-CT99, NVSL isolate from a sandhill crane, Fairfield County, Connecticut; WN-Crow-NJ99, American crow, Hunterdon County, New Jersey; WN-Crow-NY99, American crow, Nassau County, New York; WN-C.pipiens-NY99, *Culex pipiens* pool, Nassau County, New York; WN-Eq.-NY99, NVSL isolate from a horse, Suffolk County, New York; WN-HB709-NY99, human brain tissue 61709, Queens County, New York; WN-HB743-NY99, human brain tissue 61743, Queens County, New York; WN-USAMRIID99, USAMRIID virus isolate from a crow found in the Bronx Zoo; WN-Israel 1998, Pasteur Institute virus isolate from goose, Israel, 1998; WN-Rom.-mosq.96, virus isolate from *Culex pipiens* pool, Romania, 1996 (GenBank accession number AF130362); WN-Rom.hum.96, virus isolate from human cerebrospinal fluid, Romania, 1996 (GenBank accession number AF130363); Kunjin-MRM-16, Kunjin virus (GenBank accession number D00246); WN-Nigeria, West Nile virus (GenBank accession number M12294). Unless otherwise noted, sequencing was performed on original noncultured tissue specimens.

man cases occurring in the New York City outbreak have been analyzed by comparing genomic sequences from the nonstructural protein genes to single strains of KUN and WN viruses (*19*). Briese *et al.* concluded that the agent responsible for the New York City area outbreak was most closely related to KUN virus, and accordingly called the outbreak virus Kunjin/West Nile–like. The phylogenetic tree in Fig. 2 compares 40 WN and KUN viruses and is more representative of the phylogeny among WN viruses.

To investigate these relationships further, we derived additional sequence data from the structural gene region of the genome (positions 549 to 1826 in the genes encoding the prM and E proteins) from selected isolates obtained during the 1999 epidemic and compared them to other WN strains within lineage 1 for which sequence data from this region were available. Table 2 displays the percent identity among these viruses. The high degree of sequence similarity (>99.8%) among the various strains circulating throughout New York City and surrounding counties and states indicates that a single WN strain was introduced and circulated during the U.S. WN virus outbreak. The identical genomic sequences identified from human brain specimens also confirm the association of this WN-NY99 virus with human disease. The small number of nucleotide substitutions observed among the strains analyzed is indicative of viral microevolution occurring during the outbreak.

A high degree of similarity between all of the U.S. WN viruses and the WN virus isolated in Israel in 1998 (>99.8%) was observed. Within these 1278 nucleotides (genome positions 549 to 1826), only two nucleotide differences occurred between WN-NY99 and WN-Israel 1998. Although this high degree of homology was unexpected, it could not have resulted from cross-contamination of U.S. viruses with the Israeli virus; the sequencing of the WN-Israel 1998 virus was performed independently at the Pasteur Institute, whereas the isolation and sequencing of New York isolates was carried out independently at CDC. For comparison, analysis of this same region of WN-NY99 with another virus within the same lineage (Romania 1996, mosquito isolate) revealed 37 nucleotide differences (96.9% identity). The cumulative data support the hypothesis that the epidemic and epizootic observed in the late summer of 1999 in the northeastern United States (primarily New York, New Jersey, and Connecticut) are attributable to a WN virus that has been circulating in the Mediterranean region since 1998. It is noteworthy that the WN-Israel 1998 virus was associated with increased pathogenicity for birds, a property also observed in the U.S. outbreak and previously observed only experimentally (*20*). The absence of reported human cases during this Israeli epizootic may be due to background human immunity to the WN virus in Israel.

The northeastern U.S. outbreak is the first documented incidence of the WN virus in the Western Hemisphere. This virus has a widespread distribution in Africa, West Asia, and the Middle East, occasionally causing epidemics in Europe that are thought to be initiated by viruses introduced by migrant birds (*21*). The current epidemic of WN virus in New York City is unprecedented and underscores the ease with which pathogens can move among the population centers of the world. It is not yet known how the virus was introduced, nor how long it has been in the United States. The extent of its geographic distribution remains a mystery, as does the long-term impact it may have on human and animal health. The WN virus could have entered the Western Hemisphere through a number of mechanisms, including travel by infected humans, importation of illegal birds or other domestic pets, or unintentional introduction of virus-infected ticks or mosquitoes. Additional surveillance as well as field and laboratory studies are in progress to help address these questions. Because it cannot be predicted whether the WN virus will reappear in the year 2000 transmission season, all components of the public health system must be prepared with rapid surveillance and clinical detection systems in place.

References and Notes

1. The following RT-PCR and sequencing protocol was used. RNA was extracted from chicken embryo allantoic fluid with the QIAamp Viral RNA kit (Qiagen); 140 μl of starting material was extracted, according to the manufacturer's protocol, and the RNA was resuspended in a final volume of 100 μl of ribonuclease-free water. DNA templates for sequencing were then generated as follows: The entire RNA genome of WN-NY99 (flamingo 382-99) was converted/copied into six overlapping double-stranded DNA fragments in multiple RT-PCR reactions using the WN-specific primer pairs 109/1442c, 1248/2737c, 2414/5237c, 5119/7990c, 7336/9794c, and 9661/10,489c (where c = complementary). The 3′ end of WN-NY99 virus was amplified by addition of a polyadenylate [poly(A)] tail onto the genome with poly(A) polymerase, followed by a RT-PCR reaction with the WN-specific primer 10,141 in combination with an oligo(dT) anchor primer. The 5′ end of WN-NY99 was amplified with the 5′ RACE System kit (Life Technologies, Gaithersburg, MD) and WN-specific primers 619c and 348c. Sequencing of additional WN isolates in the prM-E region (see text) was accomplished with the use of the primer pairs in the corresponding region of the genome, as described above. All WN-specific RT-PCR and sequencing primers were designed with the use of OLIGO (Molecular Biology Insights Inc., Cascade, CO) and the published sequences of WN (GenBank accession numbers M12294 and M10103) and Kunjin (GenBank accession number D00246). RT-PCR reactions were performed with the TITAN One Tube RT-PCR kit (Boehringer Mannheim) following the manufacturer's protocol. The resulting DNA fragments were purified by electrophoresis on 1% agarose gels; the DNA bands

were excised, then isolated using the QIAquick gel extraction kit (Qiagen). Both strands of the purified DNAs were sequenced with the use of the Taq DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer/Applied Biosystems) and a total of 51 WN-specific primers spaced about 400 bases apart on the genome (primer sequences are available upon request). Cycle sequencing was performed by combining ~400 ng of gel-purified DNA (0.2 pmol) with 30 pmol of WN-specific primer and following the manufacturer's protocol.

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25 October 1999; accepted 18 November 1999

Binding of Transcription Termination Protein Nun to Nascent RNA and Template DNA

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The amino-terminal arginine-rich motif of coliphage HK022 Nun binds phage λ nascent transcript, whereas the carboxyl-terminal domain interacts with RNA polymerase (RNAP) and blocks transcription elongation. RNA binding is inhibited by zinc (Zn^{2+}) and stimulated by *Escherichia coli* NusA. To study these interactions, the Nun carboxyl terminus was extended by a cysteine residue conjugated to a photochemical cross-linker. The carboxyl terminus contacted NusA and made Zn^{2+}-dependent intramolecular contacts. When Nun was added to a paused transcription elongation complex, it cross-linked to the DNA template. Nun may arrest transcription by anchoring RNAP to DNA.

Transcription termination in bacteria may occur when elongating RNAP encounters a template sequence characterized by a region of dyad symmetry followed by a stretch of poly(dT). Alternatively, termination may require a factor that is not a component of the elongation complex, such as the *E. coli* Rho protein (*1*). Factor-dependent termination is generally not highly template- or site-specific. The Nun protein of phage HK022, in contrast, blocks transcription elongation uniquely on phage λ templates (*2*). Nun binds to boxB RNA (BOXB) of the nascent transcript of the λ pL and pR operons, blocks translocation of RNAP in vitro (*3*), and terminates transcription in vivo (*4*). Nun is a 109–amino acid protein that carries the arginine-rich motif (ARM) RNA binding motif found in several RNA binding proteins (Fig. 1A). The ARM motif is located in the Nun NH_2-terminus. The COOH-terminus of Nun inhibits RNA binding, particularly in the presence of Zn^{2+} (*5*). The *E. coli* transcription factor NusA binds the COOH-terminus of Nun and stimulates RNA binding (*5*). Deletion of the COOH-terminus eliminates the association between Nun and RNAP in solution and prevents transcription arrest or termination (*5*).

Within the COOH-terminal region, a tryptophan residue at position 108 is also necessary for termination and arrest, as determined by λ plaque-forming assays (*6*) (Fig. 1B). Mutant Nun protein Trp^{108}→Ala (W108A) promotes template switching, the movement of RNAP from the end of one template to a second without release of nascent transcript (*7*, *8*). We postulated that Trp^{108} intercalates into DNA template and brakes RNAP translocation. This model is supported by the finding that wild-type (WT) Nun cannot arrest transcription on single-stranded template (*8*).

Here we describe photochemical cross-linking and directed DNA cleavage assays that identify contacts made by the Nun COOH-terminus. Nun, which lacks cysteine residues, was modified by the addition of cysteine to the COOH-terminus, by polymerase chain reaction (PCR)–directed mutagenesis (Fig. 1A). This modified Nun protein, Nun Cys^{110}, was active in terminating transcription in a λ *pL-nutL-lacZ* fusion assay in which the pL promoter of λ was fused upstream of the gene for β-galactosidase. Tester strains carrying either WT or mutant Nun formed white (Lac^-) colonies on MacConkey-Lactose indicator plates, indicating that Nun was terminating transcription and preventing expression of β-galactosidase (*9*).

We used the modified protein to conjugate a photoreactive cross-linker, ^{125}I-*N*-[(2-pyridyldithio) ethyl]-4-azidosalicylamide (AET), to the added cysteine residue via a disulfide bond (*10*). By using this cross-linker, radiolabeled ^{125}I can be transferred to the target of the cross-link by treatment with dithiothreitol (DTT). The labeled ^{125}I-AET-Nun conjugate was irradiated with ultraviolet (UV) light in the presence or absence of Zn^{2+} and NusA (*11*). Zn^{2+} promoted formation of intramolecular Nun cross-links (Fig. 2). Thus, the ^{125}I-labeled Nun protein migrated with the same mobility on an SDS–polyacrylamide gel before and after DTT treatment. NusA and ^{125}I-AET-Nun formed an ^{125}I-labeled complex after UV irradiation. The ^{125}I-label was transferred to NusA after DTT treatment (Fig. 2).

This intramolecular cross-linking supports the model that the Nun COOH-terminus inhibits RNA binding in the presence of Zn^{2+} by occluding the NH_2-terminal RNA binding

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[2] **Nucleotide Sequence of Yellow Fever Virus: Implications for Flavivirus Gene Expression and Evolution**

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Science, New Series, Vol. 229, No. 4715. (Aug. 23, 1985), pp. 726-733.

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