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Genetic Determinants of Sindbis Virus Neuroinvasiveness

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After peripheral inoculation of mice, Sindbis virus replicates in a variety of tissues, leading to viremia. In some cases, the virus can enter the central nervous system (CNS) and cause lethal encephalitis. The outcome of infection is age and virus strain dependent. Recently, two pairs of Sindbis virus variants differing in neurovirulence and neuroinvasiveness were derived by limited serial passaging in mouse brain. Two early passage isolates (SVA and SVB) were neurotropic but did not cause lethal encephalitis. SVB, but not SVA, was neuroinvasive. A second independent pair of isolates (SVN and SVNI), which had undergone more extensive mouse brain passaging, were highly neurotropic and caused lethal encephalitis. Only SVNI could reach the brain after peripheral inoculation. From these isolates, virion RNAs were obtained and used to construct full-length cDNA clones from which infectious RNA transcripts could be recovered. The strains recovered from these clones were shown to retain the appropriate phenotypes in weanling mice. Construction and analysis of recombinant viruses were used to define the genetic loci determining neuroinvasion. For SVB, neuroinvasiveness was determined by a single residue in the E2 glycoprotein (Gln-55). For SVNI, neuroinvasive loci were identified in both the 5′ noncoding region (position 8) and the E2 glycoprotein (Met-190). Either of these changes on the SVN background was sufficient to confer a neuroinvasive phenotype, although these recombinants were less virulent. To completely mimic the SVNI phenotype, three SVNI-specific substitutions on the SVN background were required: G at position 8, E2 Met-190, and Lys-260, which by itself had no effect on neuroinvasion. These genetically defined strains should be useful for dissecting the molecular mechanisms leading to Sindbis virus invasion of the CNS.

To cause a systemic illness, a virus must first enter the host animal, undergo primary replication at a site near its portal of entry, and then ultimately spread to distant target tissues, such as the central nervous system (CNS). Viral pathogenesis can then be analyzed in terms of a series of stages, and a variety of barriers at different levels can prevent access of the virus to target cells (57). In the case of a neurotropic virus, one of the steps limiting replication in the brain is passage through the blood-brain barrier (BBB) protecting the CNS. To be pathogenic, a neurotropic virus must then be neuroinvasive (defined as the ability for a virus to penetrate the CNS after inoculation and growth at a peripheral site) and also neurovirulent (defined as the capacity of a virus to establish a lethal infection within the CNS) (9).

Alphaviruses, including Sindbis virus, Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEE), and Ross River virus, have been extensively used in murine model systems for studying the pathogenesis of acute viral encephalitis (reviewed in references 10 and 18). This group of mosquito-borne viruses includes several agents (e.g., VEE) which are important causes of acute viral encephalitis in both humans and horses (18, 53). Infectious alphavirus particles (see reference 3 and citations therein) consist of an icosahedral nucleocapsid made up of 240 subunits of basic capsid (C) protein complexed with a single positive-sense ~12-kb genome RNA. This nucleocapsid is surrounded by a host-derived lipid bilayer containing 240 E1E2 glycoprotein heterodimers, which function in virus attachment and entry and are targets for virus-specific neutralizing antibodies (53).

In the case of Sindbis virus, important variables determining susceptibility to virus-induced encephalitis include the age and strain of the animal, the site of inoculation and dose, and the virus strain (reviewed in references 10 and 56). In terms of neurovirulence (measured by intracranial inoculation), infection with natural Sindbis virus isolates such as AR339 and SV leads to rapidly destructive, lethal encephalitis in neonatal mice. In contrast, infection of weanling mice (3 to 4 weeks old) with these strains is usually not lethal (11, 17, 31). Age-dependent neurovirulence seems to correlate not with maturity of the immune system but rather with restriction of viral replication in neurons (8, 23, 25, 55, 56). This decreased replication may involve changes in neuronal receptors (55, 58) or increased resistance to Sindbis virus-induced apoptosis through expression of the *bcl-2* oncogene or related homologs (23–25). Variants which induce lethal encephalitis can be selected by serial passage in the mouse brain (11, 31), and virulence determinants often map to the virion envelope glycoproteins (5, 32, 47, 56, 59). In contrast, some natural Sindbis virus isolates, such as S.A.AR86, are virulent in both neonates and weanling mice when inoculated intracranially (47).

In newborn and suckling mice which lack a developed BBB, peripheral intraperitoneal inoculation with Sindbis virus leads to replication in muscle and other extraneural tissues, resulting in a transient viremia followed by invasion of the CNS (10). Some neurovirulent variants (NSV and SVN) induce fatal encephalitis in suckling mice after intracranial or intraperitoneal inoculation, whereas in weanling mice they induce a fatal encephalitis only after intracranial inoculation (10, 11, 31). Such variants are nonneuroinvasive and unable to invade the CNS of weanling mice (31). This age-dependent resistance to neuroinvasiveness often coincides with the maturation of the BBB (15).

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TABLE 1. Pathogenicity of Sindbis virus strains in weanling (3- to 4-week-old) mice[a]

Strain	Intracranial inoculation		Intraperitoneal inoculation	
	Proliferation in the brain	Death	Invasion into the brain	Death
SVA	+	−	−	−
SVB	+	−	+	−
SVN	+	+	−	−
SVNI	+	+	+	+

[a] Neurovirulent and neuroinvasive phenotypes of SVA, SVB, SVN, and SVNI have been characterized elsewhere (29, 30). Strains which proliferate in the brain after intracranial inoculation and induce death are defined as neurovirulent. Strains which can invade the CNS after peripheral inoculation are defined as neuroinvasive.

Although much has been learned about the genetic determinants affecting Sindbis virus neurovirulence, no study has defined determinants which allow neuroinvasion in mice with a developed BBB. Recently, two pairs of Sindbis virus variants (called SVA/SVB and SVN/SVNI) which differ in neurovirulence and neuroinvasiveness in weanling mice have been isolated (29, 31) (Table 1). Two early-passage isolates (SVA and SVB) were both neurotropic but did not cause lethal encephalitis. After intraperitoneal inoculation, similar levels of peripheral viremia were observed until day 3, when virus could no longer be recovered from the blood (29, 31). SVB, but not SVA, was neuroinvasive and was present in the brain by day 3, reaching peak titers on day 4. A second pair of independent isolates (SVN and SVNI), which had undergone more extensive mouse brain passaging, were both highly neurotropic and caused lethal encephalitis after intracranial inoculation. After peripheral inoculation with either SVN or SVNI, similar levels of viremia were observed, with clearance from the blood by day 3. Only SVNI was neuroinvasive; by day 3, high levels of virus were recovered from the brain, with peak titers reached on day 6, coincident with or shortly before death due to lethal encephalitis. Similar results were obtained in studies using either ICR or Swiss Webster outbred weanling mice. Interestingly, peripheral inoculation with a mixture of SVN and SVNI led to lethal encephalitis, and both strains could be recovered from the brain, suggesting that SVNI might induce a breach in the BBB, allowing SVN to invade the brain (31). In contrast, after intraperitoneal inoculation with a mixture of SVN and SVB, only SVB could be recovered from the brain (31). These observations suggest that different mechanisms are utilized by SVB and SVNI for invasion of the CNS.

In this study, we have defined the genetic determinants responsible for SVB and SVNI neuroinvasiveness. RNA transcripts synthesized by using full-length cDNA clones of SVA, SVB, SVN, and SVNI were infectious, and the resulting viruses retained the biological properties of their parental strains. Construction and analysis of a large number of recombinant viruses allowed mapping of the neuroinvasive loci. For SVB, neuroinvasiveness was conferred by a single amino acid substitution in the E2 glycoprotein. For SVNI, distinct neuroinvasive determinants were identified in both the 5′ noncoding region (5′NCR) and the E2 glycoprotein.

MATERIALS AND METHODS

Cell cultures and viruses. Chicken embryo fibroblasts (CEF) were grown in minimum essential medium containing 4% fetal bovine serum (FBS). BHK-21 cells and Vero cells were grown in minimal essential medium containing 10% FBS. The *Aedes albopictus* C6/36 mosquito cell line was grown at 28°C in Mitsuhashi and Maramoroch insect tissue culture medium supplemented with 10% FBS. For production of virus stocks, only 2% FBS was used in the medium.

Variants of Sindbis virus used in this work have been previously described (31). Neurovirulent variants SVN (nonneuroinvasive) and SVNI (neuroinvasive) were isolated by serial passage in suckling and weanling mouse brain. Nonneurovirulent variants SVA (nonneuroinvasive) and SVB (neuroinvasive) were isolated from a pool of culicine mosquitoes gathered in Israel (31) by plaque purification. Virus titers were determined by plaque assay using CEF monolayers (50).

Construction of full-length cDNAs and recombinants. Standard recombinant DNA techniques were used to generate cDNA clones (48). For cDNA synthesis, viral RNA was prepared from purified virus (46). Virus was grown in C6/36 cells or Vero cells, concentrated from the culture medium by polyethylene glycol precipitation, and purified by successive sedimentation velocity and equilibrium density centrifugations (41). In preliminary experiments, segments of the viral genomes were amplified by reverse transcription-PCR (RT-PCR) and analyzed by restriction digestion to devise strategies for assembling and analyzing full-length clones (Fig. 1). SP6 transcription plasmids (45) containing full-length SVA, SVB, SVN, and SVNI cDNAs (called pSVA, pSVB, pSVN, and pSVNI) were constructed in a stepwise fashion, using a full-length cDNA recipient clone (pToto1107). pToto1107 is identical to pToto1101 (45) except that silent mutations have been introduced to create *Mlu*I and *Xba*I restriction sites at nucleotide positions 4080 and 5730, respectively. Oligonucleotides were chosen for cDNA synthesis such that convenient restriction enzymes could be used to digest the cDNA and replace the corresponding region of pToto1107. Recombinant full-length clones were identified by restriction analysis and tested for the production of infectious RNA transcripts (44, 45). Clones producing infectious transcripts were used to assemble two independent full-length cDNA clones for each strain. The correct 5′ terminus was created by designing an appropriate synthetic oligonucleotide for second-strand cDNA synthesis based on direct sequencing of SVNI genomic RNA (12).

Recombinant plasmids for mapping neuroinvasive loci were constructed using the following restriction endonuclease sites: *Sst*I (nucleotide [nt] −29, upstream from the SP6 promoter), *Eco*47III (nt 1404), *Cla*I (nt 2712), *Avr*II (nt 4280), *Spe*I (nt 5262), *Aat*II (nt 7995), *Bss*HII (nt 9804), and *Xho*I (nt 11749). Three-piece

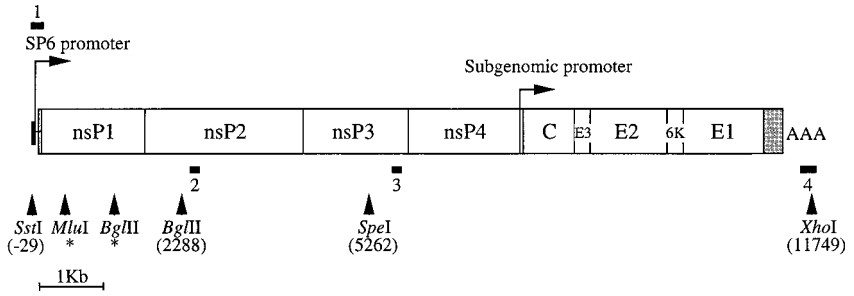

FIG. 1. Structures of Sindbis virus cDNAs. A schematic of the Sindbis virus cDNA is shown with the nonstructural (nsP1 to nsP4) and structural (C, E3, E2, 6K, and E1) protein coding regions indicated. 5′ and 3′ noncoding regions are shaded. As detailed in Materials and Methods, specific primers (primers 1 to 4) for cDNA synthesis (solid bars) and convenient restriction sites (arrows) were used to construct full-length cDNA clones with an upstream SP6 promoter and a unique 3′ *Xho*I site for production of runoff RNA transcripts. Primer 1 (positive sense; 5′-CCCCCGAGCTCATACGATTTAGGTGACACTATAGATTGGCGGCGTAGTACAC ACTA-3′) contains an *Sst*I restriction site and the SP6 promoter followed by the first 22 bases of the SVNI genome (as determined by sequencing of the genomic RNA). Primer 4 contained an *Xho*I restriction site followed by oligo(dT). Primers 2 (5′-CTCAGTCTTAGCACGT-3′) and 3 (5′-ACTCAGAGCTATTGCTTG-3′) were negative sense. For pSVA and pSVB, an additional *Bgl*II site and a unique *Mlu*I restriction site (indicated by asterisks) were also used to assemble the full-length clones.

ligations, using nonunique restriction sites, were used to create constructs for mapping determinants in the E2 gene (*Sna*BI [nt 9221] and *Pst*I [nt 9115]) or the 5′NCR of pSVN/pSVNI (*Ssp*I [nt 502]) or to reintroduce a mutagenized 5′-end fragment (see below) into pSVN/pSVNI (*Nsp*I [nt 490]). To prevent possible contamination with the parental sequences, deleted forms of pSVA, pSVB, pSVN, and pSVNI were used for construction of recombinant full-length clones. For pSVA and pSVB, the sequence between *Stu*I (nt 8569) and *Bss*HII (nt 9804) was deleted or the *Mlu*I site (nt 500) was destroyed by *Mlu*I cleavage, filling in the protruding ends, and religation. For pSVN and pSVNI, recipient plasmids included derivatives with a *Sma*I (nt 767 to 944) or a *Pml*I-*Stu*I (nt 8067 to 8569) deletion. After subcloning of the appropriate regions, recombinant constructs were verified by digestion with appropriate restriction enzymes or, if necessary, by sequence analysis.

In vitro transcription and transfection. 5′-capped RNA transcripts were synthesized by using SP6 polymerase and plasmid DNA templates which had been linearized with *Xho*I (45). Infectivity of RNA was assayed by plaque formation after transfection of confluent monolayers of secondary CEF, using either DEAE-dextran (45) or Lipofectin (GIBCO-BRL) (44). Virus stocks were generated from parental clones or each recombinant, using BHK-21 cells and Lipofectin (44), or Vero cells or C6/36 mosquito cells, using DEAE-dextran (42). vSVA, vSVB, vSVN, and vSVNI are virus stocks derived from the parental full-length cDNA clones.

Animal inoculation and determination of LD_{50}. The phenotypes of virus strains recovered from cDNA and their recombinants were assayed in weanling OF1 (IFFA-CREDO) or ICR (CD1; Charles River) mice after either intraperitoneal or intracerebral injection of 10-fold serial dilutions as described by Lustig et al. (31). For each parental or recombinant plasmid DNA template, two independent transcriptions were used to derive two virus stocks for in vivo assays using groups of seven to eight mice. Fifty percent lethal doses (LD_{50}) were calculated according to the method of Reed and Muench (43). Virus recovered from brains of mice (as a 20% brain homogenate in phosphate-buffered saline) was quantified by plaque assay on Vero cell monolayers.

Site-directed mutagenesis. For site-directed mutagenesis at nucleotide position 45 of the 5′NCR of pSVN and pSVNI, the *Sst*I (nt −29)-*Sma*I (nt 767) fragment of pSVN or pSVNI was cloned into pGEM-3Zf(−) (Promega). Single-stranded uridylated DNA was prepared (28) and used as a template for oligonucleotide-directed mutagenesis (22). Plasmids containing substitution mutations at position 45 were identified by checking for the presence or absence of the *Mun*I restriction site normally present at position 41 of pSVNI but absent in pSVN. The nucleotide sequences of the mutagenized *Sst*I (nt −29)-*Nsp*I (nt 490) fragments were verified before cloning into appropriate full-length clones.

Sequencing. Genomic regions of pSVA, pSVB, pSVN, and pSVNI shown to be responsible for changing the neuroinvasive phenotypes of the recombinant viruses were sequenced with the dideoxy-chain termination method using Sequenase (U.S. Biochemicals). To check for possible reversion of point mutations in some recombinants, RNA extracted from the brains of infected mice by using the RNAzol B method (Tel-Test) was amplified by RT-PCR, and the amplified fragments were sequenced by using the fmol DNA sequencing system (Promega).

In situ plaque hybridization. To measure the number of SVN PFU in the brains of coinoculated animals, in situ plaque hybridization was used to discriminate between SVN and SVNI sequences at the 5′ and E2 neuroinvasive loci. Probes were either of two synthetic oligonucleotides complementary to nt 1 to 15 (5′NCR) or 9192 to 9206 (E2 gene) of SVN which contained single-base mismatches with the SVNI RNA sequence at positions 8 and 9199, respectively. In situ plaque hybridization and washing were conducted as described previously (27) except that the hybridization temperature was 36°C instead of 42°C. Under these conditions, a strong signal was detected only for SVN.

Cell-free translation. 5′-capped RNA transcripts were synthesized and quantified by incorporation of [^{3}H]UTP as previously described (45). Serial dilutions were translated in vitro, using nuclease-treated rabbit reticulocyte lysates (Promega) as specified by the manufacturer. Ten-microliter translation reaction mixtures contained 12 μCi of [^{35}S]methionine (Amersham) and were incubated for 1 h at 30°C. Proteins were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

Localization of the SVB neuroinvasive determinant. As detailed in the introduction, SVA and SVB are neurotropic but nonneurovirulent in weanling mice; SVB is neuroinvasive, whereas SVA is nonneuroinvasive (Table 1). To identify the neuroinvasive determinants of SVB, we constructed full-length infectious cDNA clones for these strains (Fig. 1 and Materials and Methods). The existence or absence of convenient restriction endonuclease recognition sites was determined by analysis of segments amplified by RT-PCR and used to devise a strategy for assembling full-length clones (called pSVA and pSVB) (Fig. 1). The neuroinvasive phenotypes of the virus stocks generated from these clones (vSVA and vSVB) were assessed by measuring the levels of virus in the brain after intraperitoneal inoculation and were indistinguishable from those of the parental strains (Fig. 2).

Recombinant viruses for mapping were constructed by exchanging genomic fragments of pSVA and pSVB (Fig. 2). Since determinants involved in Sindbis virus pathogenesis often map to the glycoprotein genes (reviewed in reference 10), our initial set of recombinants focused on the structural region, in particular E2 (Fig. 2). These results indicate that the neuroinvasive determinant of vSVB is located in the 3′ half of the genome. The *Aat*II-*Bss*HII fragment (nt 7995 to 9804), encompassing most of the E2 coding region of pSVB, was able to confer a neuroinvasive phenotype on recovered virus when exchanged with the corresponding fragment in pSVA (recombinant 21 [R21]). Consistent with this result, the corresponding fragment of pSVA when placed in the SVB background yielded virus with a nonneuroinvasive phenotype (R24). Sequence analysis of these two regions of pSVA and pSVB revealed only two differences in the E2 gene: a silent change at nucleotide position 186 (T, SVA; C, SVB) and a single amino acid difference at E2 residue 55 (Arg, SVA; Gln, SVB). These results suggest that a Gln at E2 position 55 is responsible for SVB neuroinvasiveness.

Localization of SVNI neuroinvasive determinants. In contrast to the SVA/SVB pair, SVN and SVNI are both neurovirulent in weanling mice. After intraperitoneal inoculation, SVN and SVNI replicate to similar titers in the periphery, but only SVNI can efficiently invade the CNS and cause lethal encephalitis (31) (Table 1). To localize the genetic determinant(s) of SVNI neuroinvasiveness, full-length clones (called pSVN and pSVNI) were constructed (see Fig. 1 and Materials and Methods), and neuroinvasiveness was measured by determining the PFU/LD_{50} after intraperitoneal injection (PFU/$IPLD_{50}$). As summarized in Fig. 3, the neuroinvasive and neurovirulence phenotypes of virus stocks generated from these clones (vSVN and vSVNI) were indistinguishable from those of the parental isolates (Fig. 3). Recombinant viruses were constructed by exchanging genomic fragments of pSVN and pSVNI. Analysis of the first set of chimeras (R7 to R12) indicated that a region of 1805 bases of SVNI, encompassing most of the E2-coding region, was able to confer a neuroinvasive phenotype when introduced into SVN background (R9) (although the PFU/LD_{50} titer was increased by 1 order of magnitude). In the SVNI background, the corresponding region of SVN increased the PFU/LD_{50} titer by nearly 2 orders of magnitude but did not abolish neuroinvasion (R12). These results provided evidence for a second neuroinvasive determinant in the 5′ portion of the SVNI genome. Construction and analysis of an additional set of recombinants (R13 to R18) demonstrated that this neuroinvasive determinant mapped to the 5′ 1406 bases of SVNI (R15). However, both the 5′ and E2 SVNI neuroinvasive determinants (R31) were required to produce a virus with neuroinvasive properties indistinguishable from those of SVNI. Reciprocal recombinants in the SVNI background corroborated these results: the 5′ region from SVN dramatically reduced neuroinvasiveness when combined with the SVN E2 determinant (compare R12 with R18 [Fig. 3]).

Genetic differences in the mapped regions were determined by sequence analysis of the SVN and SVNI cDNA clones. Identical results were obtained for two independent cDNA clones of each strain. Three differences were observed in the 5′ region: two in the 5′NCR at nt 8 (U, SVN; G, SVNI) and 45 (C, SVN; U, SVNI) and one in nsP1 at residue 425 (Cys, SVN; Arg, SVNI). Three differences were also found in the E2 gene

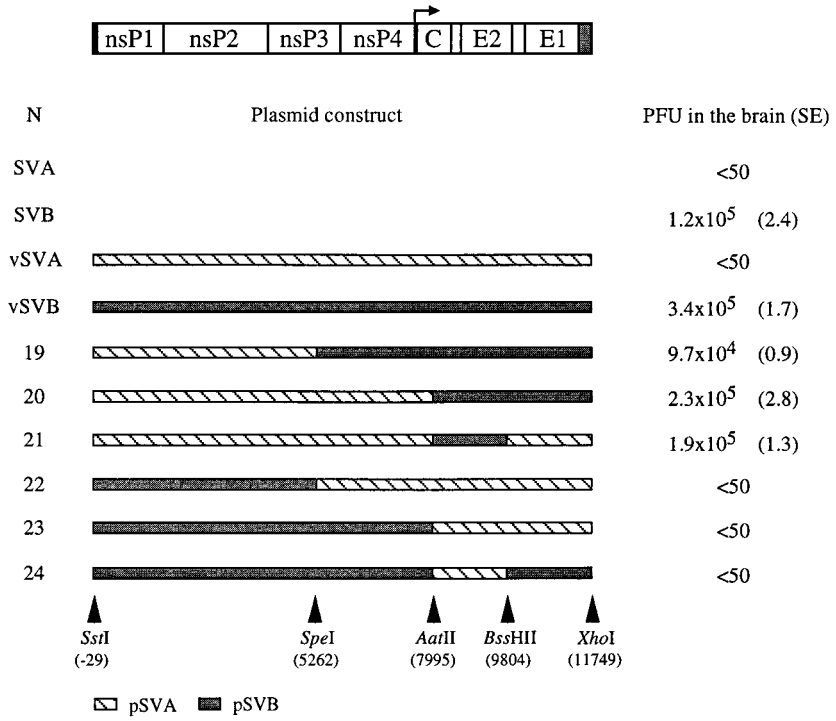

FIG. 2. Localization of the vSVB neuroinvasive determinant. A diagram of the Sindbis virus genome is shown at the top, and the restriction sites used to generate the recombinants are indicated at the bottom. The names (N) of viruses analyzed, either parental strains (SVA and SVB), virus recovered from cDNA (vSVA and vSVB), or various vSVA/vSVB recombinants (numbered) are given at the left. cDNA constructs used for recovery of these viruses are diagrammed to show the sequences derived from pSVA (hatched box) and pSVB (shaded box). Since SVA and SVB are nonneurovirulent, groups of seven to eight weanling mice (3.5 to 4 weeks old) were inoculated intraperitoneally with 1,000 PFU of virus, and neuroinvasiveness was measured by titrating virus recovered from the brain at day 5. Mean titers per brain (± standard errors [SE]) are given. At this time, peripheral viremia is absent and virus replication in the brain is maximal (31).

at residues 121 (Val, SVN; Ala, SVNI), 190 (Lys, SVN; Met, SVNI), and 260 (Glu, SVN; Lys, SVNI).

E2 Met-190 is a major determinant of SVNI neuroinvasiveness. To identify the specific residue(s) in the SVNI E2 determinant responsible for neuroinvasiveness, point mutations were introduced into either the SVN background or a recombinant background consisting of SVNI with the 5′ determinant from SVN. Figure 4a demonstrates that Met-190 in the SVN background was sufficient to confer neuroinvasiveness (R39). The reciprocal substitution of Lys-190 in the SVNI E2 determinant (R38) reduced neuroinvasiveness to the same level as R18. Substitutions at E2 residues 121 or 260 did not alter neuroinvasiveness. However, some unexpected effects of E2 residue 260 were noted. When Met-190 and Glu-260 were present in the same construct (R35 and R39), paralysis instead of death was observed. This was also the case after intracerebral injection, suggesting that this combination of E2 residues alters neurovirulence but not neuroinvasiveness. The phenotype of R40, with both Met-190 and Lys-260 on the SVN background, was indistinguishable from that of R9, which contained all three SVNI E2 substitutions. Similarly, substitution of SVN residues Lys-190 and Glu-260 on the SVNI background (with the SVN 5′ determinant) abrogated neuroinvasiveness, and this recombinant (R41) was phenotypically indistinguishable from R18 and SVN (Fig. 4). Thus, Met-190 is the major SVNI neuroinvasive determinant in the E2 region. Interestingly, different combinations of residues at positions 190 and 260 altered neurovirulence (paralysis versus death).

Given the observation that E2 Met-190 was sufficient to promote neuroinvasion on the SVN background, our previous results identifying a second determinant in the 5′ region might be explained by the emergence of variant with Met-190 during replication in mice. To address this possibility, total mouse brain RNA was isolated from two or three animals infected with R12 or R15, respectively, after peripheral viremia had disappeared. The E2 region was amplified by RT-PCR and sequenced. Samples from animals infected with R9 or SVNI served as controls. Serial dilutions of the cDNAs used for PCR demonstrated that at least 10^3 molecules were amplified for sequence analysis. In each case, only the amino acid present in the original construct was detected at E2 position 190 (Lys for R12 and R15; Met for R9 and SVNI) (data not shown).

The 5′ neuroinvasive determinant of SVNI maps to position 8 in the 5′NCR. The 5′ neuroinvasive determinant was identified by construction and analysis of additional recombinant viruses. Initially, two recombinants were constructed to separate the nsP1 change from those in the 5′NCR (Fig. 4b). R32 was obtained by introducing the 5′NCR of SVNI into the SVN background; R33 contained the SVN 5′NCR in the SVNI background with the SVN E2 determinant (to abrogate neuroinvasiveness mediated by the E2 determinant). As shown in Fig. 4b, R32 and R15 had identical neuroinvasive phenotypes, whereas R33 and R18 were both nonneuroinvasive. These results map the 5′ SVNI neuroinvasive determinant to the 5′NCR and suggest that the substitution in nsP1 does not play a role in neuroinvasion. Since SVN and SVNI differed at two positions in the 5′NCR, single substitutions were introduced at position 8 or 45 in either the SVN background or the SVNI background with the SVN E2 determinant (R12). In the SVN background, G at position 8 (R43) produced a neuroinvasive virus, whereas T at position 45 (R42) did not alter the SVN phenotype. The reciprocal constructs, used for examining the

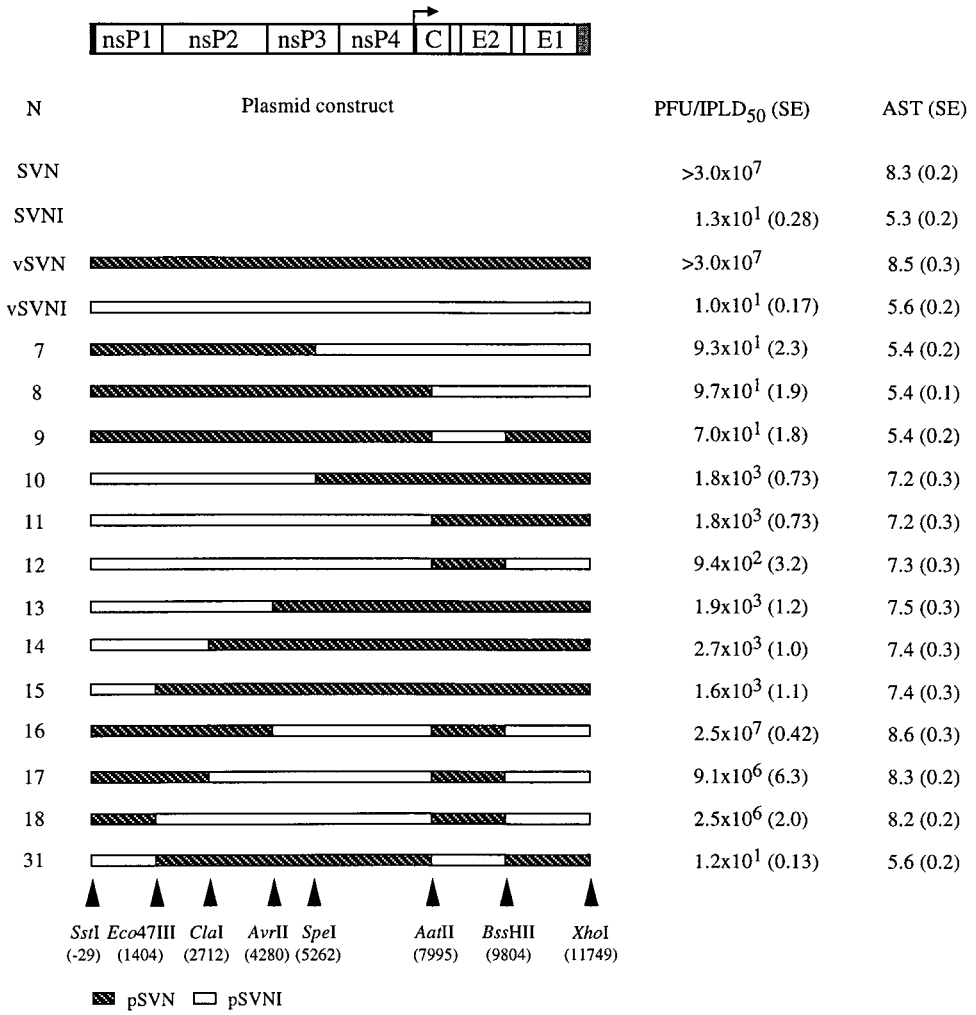

FIG. 3. Mapping of the vSVNI neuroinvasive loci. The format and labels are as for Fig. 2. Viral titers, given as PFU/IPLD$_{50}$ (± standard errors [SE]), were determined after intraperitoneal injection of weanling mice. Mice were injected with 0.1 ml of serial 10-fold dilutions of the indicated virus stocks, whose titers ranged from 2×10^9 to 4×10^9 PFU/ml. The AST (in days) and standard errors are given. After intracranial inoculation, all parental viruses and recombinants induced death, and their LD$_{50}$ titers by this route were similar (data not shown).

effect of these changes on the R12 background, produced complementary results. Substitution of C at position 45 (R44) had no effect on neuroinvasion, whereas substitution of T for G at position 8 abolished neuroinvasion (R45). Thus, the 5′ neuroinvasive determinant of SVNI maps to the G residue at position 8 of the 5′NCR.

The G at position 8 of the 5′NCR and the E2 Met-190 were combined on the SVN background (R46) and compared to SVNI. The PFU/LD$_{50}$ for R46 was about 1 order of magnitude higher than that for SVNI (Fig. 4c). Mice infected, with R46 either died or were paralyzed after intraperitoneal inoculation, whereas 100% mortality was observed by the intracranial route. Inclusion of E2 Lys-260 (R47) was necessary to produce a recombinant with a PFU/LD$_{50}$ titer and average survival time (AST) indistinguishable from those of SVNI. This suggests that E2 Lys-260 can modulate neuroinvasiveness in the context of the principal 5′NCR (G at position 8) and E2 (Met-190) determinants.

Determinants facilitating coinvasion of SVN. Previous studies demonstrated that cointraperitoneal inoculation of SVN and SVNI increased the efficiency of CNS invasion by SVN (31). In the absence of SVNI, SVN appears in the brain in only 0 to 15% of infected animals. In contrast, coinoculation with SVNI increases the efficiency of SVN neuroinvasion to 80 to 100%. Selected recombinants generated in the mapping study described above were used to determine the SVNI determinant responsible for this coinvasion phenomenon. After coinoculation and disappearance of peripheral viremia (which occurs by day 3), mouse brains were harvested on day 5 and titered for infectious virus by plaque assay. In situ plaque hybridization (27) and appropriate washing conditions were used to selectively detect and quantify vSVN in the brains of mice which had been infected with a mixtures of vSVN and vSVNI or various SVNI recombinants (see Materials and Methods).

Table 2 summarizes the data for coinoculation of vSVN with vSVNI, R31, R9, or R15. The detection of vSVN in the brains of 87.5% of the mice inoculated intraperitoneally with a mixture of vSVN and vSVNI derived from cDNA confirms the results previously obtained for the parental strains (31). Coinoculation of vSVN with R31, which contains both neuroinvasive determinants in the SVN background, produced similar results. R9 or R15, containing either the E2 or the 5′ neuroinvasive determinant in the SVN background, respectively, fa-

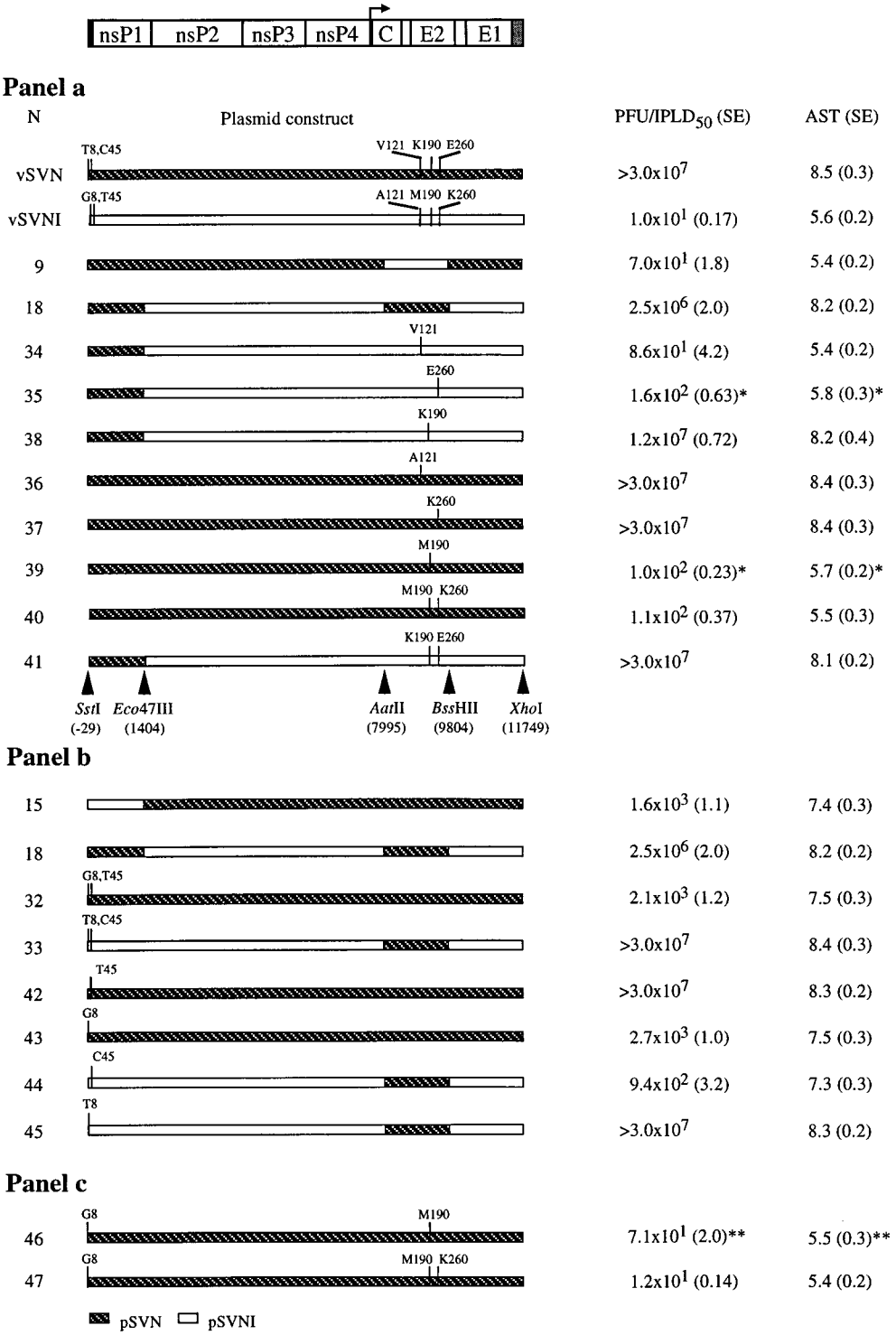

FIG. 4. Identification of specific mutations involved in SVNI neuroinvasiveness. Recombinants generated to map the E2 (a) and 5′ end (b) or synergistic effects of combined (c) SVNI determinants. For the SVN and SVNI parents, nucleotide (positions 8 and 45 in the 5′NCR) or amino acid (E2 positions 121, 190, and 260) sequence differences in the neuroinvasive loci are indicated. For the recombinants, the indicated nucleotide or amino acid residues are those normally present in the other strain. PFU/IPLD$_{50}$ titers and AST values (± standard errors [SE]) were determined as described in Materials and Methods and the legend to Fig. 3. Infection of mice with R35 and R39 induced paralysis instead of death, and the values given represent PFU yielding 50% paralysis rather than death (*). Infection of mice with R46 induced either death or paralysis (**). In these latter cases, AST values represent the average times before paralysis or death was observed. After intracranial inoculation, parental viruses and recombinants had similar PFU/LD$_{50}$ titers (data not shown). R35 and R39 also induced paralysis by this route.

TABLE 2. Neuroinvasion by vSVN after cointraperitoneal injection with vSVNI or selected recombinants

Virus strain[a]	n[b]	Total PFU/brain[c]	% of mice positive for vSVN in the brain[d]	vSVN PFU/brain[d]
Expt 1				
vSVNI + vSVN	8	$(1.5 \pm 2.8) \times 10^7$	87.5	$(1.4 \pm 2.5) \times 10^7$
R31 + vSVN	8	$(4.8 \pm 2.5) \times 10^6$	75	$(3.0 \pm 2.8) \times 10^6$
R9 + vSVN	8	$(1.9 \pm 1.6) \times 10^7$	62.5	$(5.1 \pm 4.3) \times 10^6$
R15 + vSVN	5	$(6.0 \pm 5.9) \times 10^6$	60[e]	$(1.3 \pm 1.0) \times 10^6$
	3	$<10^2$		
vSVN	1	6.5×10^5	12.5	6.5×10^5
	7	$<10^2$		
vSVNI	8	$(1.4 \pm 1.3) \times 10^7$		
vSVN (intracranial)	8	$(2.6 \pm 3.3) \times 10^7$	100	$(2.6 \pm 3.3) \times 10^7$
Expt 2				
vSVNI + vSVN	10	$(8.7 \pm 9.2) \times 10^6$	70	$(5.5 \pm 3.0) \times 10^6$
R43 + vSVN	6	$(2.2 \pm 1.7) \times 10^7$	50[e]	$(7.2 \pm 1.8) \times 10^6$
	4	$<10^2$		
vSVN	1	3.0×10^6	10	3.0×10^6
	9	$<10^2$		
vSVN (intracranial)	10	$1.7 \pm 1.2 \times 10^7$	100	$(1.7 \pm 1.2) \times 10^7$

[a] 10^4 PFU of each strain was injected intraperitoneally.
[b] n, number of mice per group. In some groups (R15 + vSVN and vSVN alone), neuroinvasion varied between individual animals.
[c] Mouse brains were harvested on day 5 postinfection, used to prepare a 20% homogenate in phosphate-buffered saline, and titered by plaque assay on CEF.
[d] The presence of vSVN in the brain as detected by in situ plaque hybridization. A 5′-^{32}P-labeled oligonucleotide probe that hybridizes with nt 9192 to 9206 (E2 gene) of the vSVN RNA sequence was used to detect vSVN when mixed with vSVNI, R31, or R9. An oligonucleotide probe hybridizing to nt 1 to 15 (5′NCR) of vSVN RNA was used to detect vSVN when mixed with R15. The threshold for vSVN detection in a mixed population was 2 to 3 orders of magnitude lower than the total number of PFU in the brains. For calculating the percentages of mice with vSVN detected in the brain, a mouse was considered negative if no vSVN plaque was detected at this threshold.
[e] Calculated by taking only the mice for which virus was detected in the brain as the total number.

TABLE 3. Neuroinvasion by vSVN after cointraperitoneal injection with R39[a]

Virus strain(s)	Intracranial inoculation (100 PFU)				Intraperitoneal inoculation (1,000 PFU)			
	n[b]	% Mortality	% Paralysis	PFU/brain[c]	n	% Mortality	% Paralysis	PFU/brain
vSVN	7	100		$(6.2 \pm 0.8) \times 10^5$	10	10		5.4×10^5
R39	7		100	$(4.9 \pm 0.3) \times 10^2$	8		100	$(3.7 \pm 0.5) \times 10^2$
R39 + vSVN	8	100		$(5.4 \pm 0.6) \times 10^5$	9	55.5		$(6.7 \pm 0.9) \times 10^5$[d]
							44.5	$(6.1 \pm 0.4) \times 10^2$

[a] Since R39 does not induce death but induces paralysis, mortality after intraperitoneal injection of vSVN together with R39 is taken as an indicator of vSVN neuroinvasion.
[b] n, number of mice used per group.
[c] Virus content of mouse brain harvested on day 6 postinfection.
[d] Virus isolated from the brain behaved like vSVN: generally no clinical signs following intraperitoneal injection (100 PFU) and 100% mortality after intracranial inoculation (100 PFU).

cilitated vSVN neuroinvasion, although somewhat less efficiently than vSVNI. Similar results were obtained with R43 (SVN with G at position 8) or R39 (SVN with Met at E2 position 190), indicating that the mutations determining SVNI neuroinvasiveness were also responsible for facilitating SVN neuroinvasion in coinoculation experiments (Tables 2 and 3).

Cell-free translation of RNAs differing at position 8 in the 5′NCR. Among many possibilities, the nucleotide at position 8 of the 5′NCR might modulate virus replication by affecting genome RNA translation. To examine this, we compared the efficiency of protein synthesis in a cell-free translation system programmed with full-length 5′-capped RNAs transcribed from pSVN, pSVNI, and R43 (which differs from pSVN only at position 8; G instead of T) templates. Serial dilutions of each mRNA were translated to examine relative translation efficiency under nonsaturating conditions (Fig. 5). The yield of translation products was proportional to the amount of input RNA, and the total amounts of protein synthesized at each RNA concentration were similar for all three constructs. Thus,

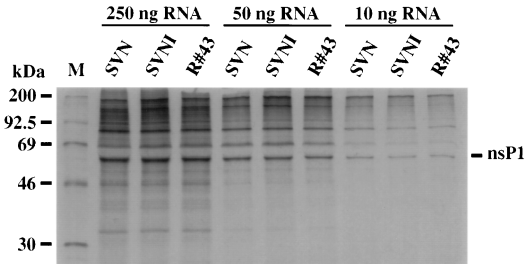

FIG. 5. Cell-free translation of RNAs with G versus U at position 8. 5′-capped RNA transcripts from pSVN (U at position 8), pSVNI (G at position 8; U at position 45) or R43 (G at position 8) were translated in lysates of rabbit reticulocytes. ^{35}S-labeled proteins obtained from translation of 250, 50, or 10 ng of each transcript RNA were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and visualized by autoradiography. The sizes of ^{14}C-labeled protein molecular weight markers (M) are indicated at the left. The position of nsP1, determined by immunoprecipitation with nsP1-specific antiserum (data not shown), is indicated at the right.

TABLE 4. Sequences of the neuroinvasive determinants[a]

Construct	5′NCR			nsP1		E2												
	5	8	45	425	441	3	23	55	70	116	120	121	150	190	208	260	385	390
HRsp	a	g	t	R	C	I	V	Q	K	V	S	A	I	K	T	K	A	C
AR339anc	g	g	t	R	I	T	E	Q	E	V	S	A	I	K	T	K	A	C
NSV	ND	ND	ND	ND	ND	T	E	H	E	V	S	A	I	K	T	K	A	C
pSVA	g	g	t	ND	ND	T	E	***R***	A	A	A	A	V	K	K	K	V	C
pSVB	g	g	t	ND	ND	T	E	Q	A	A	A	A	V	K	K	K	V	C
pSVN	g	***t***	***c***	***C***	I	T	E	H	K	V	S	***V***	I	K	T	***E***	A	R
pSVNI	g	g	t	R	I	T	E	H	K	V	S	A	I	***M***	T	K	A	R

[a] Compilation of nucleotide (5′NCR) or amino acid (nsP1 and E2) residues where differences were noted between the SVA/SVB or SVN/SVNI sequences and those of AR339-derived laboratory strain HRsp (51). Also shown are residues present at these positions for the deduced AR339 ancestral sequence (AR339anc [34]) and NSV (32). NSV is a neuroadapted Sindbis virus variant isolated after six intracranial passages of the AR339 prototype strain (11). Residues shown in boldface italics are those which are different in each noninvasive-invasive pair. ND, not determined. pSVA and pSVB sequences were determined in the region containing the mapped neuroinvasive determinant (nt 7995 to 9809). Relative to the published HRsp sequence (51), the following nucleotide substitutions were identified: 8015 (T); 8024 (T); 8034 (T); 8063 (A); 8144 (T); 8177 (G); 8201 (T); 8246 (G); 8294 (C); 8345 (A); 8372 (C); 8378 (T); 8414 (A); 8462 (C); 8555 (A); 8638 (C); 8663 (A); 8666 (T); 8672 (T); 8693 (C); 8698 (A); 8708 (T); SVA only, 8794 (G, E2 Arg-55); 8795 (G); SVA only, 8816 (T); 8837 (C); 8838 (G); 8839 (C); 8977 (C); 8978 (T); 8988 (G); 9077 (G); 9078 (G); 9144 (G); 9155 (C); 9227 (C); 9253 (A); 9254 (A); 9260 (T); 9362 (T); 9371 (T); 9383 (T); 9398 (C); 9413 (A); 9446 (T); 9575 (A); 9584 (T); 9713 (C); 9734 (T); 9740 (T); and 9784 (T). pSVN and pSVNI sequences were determined in the regions containing the 5′ (nt 1 to 1406) and structural region (nt 7995 to 9809) neuroinvasive determinants. Changes in the 5′NCR are indicated. In the nsP1 coding region, nucleotide changes relative to the HRsp sequence (51) were as follows: 353 (T); 749 (A); SVN only, 1332 (T, nsP1 Cys-425); 1380 (A); and 1381 (T). Changes in the structural region determinant were as follows: 8345 (A); 8638 (C); 8698 (A); 8795 (T); SVN only, 8992 (T, E2 Val-121); 9144 (G); SVNI only, 9199 (T, E2 Met-190); SVN only, 9418 (G, E2 Glu-260); and 9798 (C).

at least in this system, genome RNA translation efficiency was not affected by G versus U at position 8 in the 5′NCR.

DISCUSSION

Alphavirus neuroinvasion. There are multiple potential pathways for virus entry into the CNS: ascending infection through peripheral nerves, invasion via the olfactory tract, and hematogenous entry across the BBB (57). It has been suggested that some alphaviruses, after replication in extraneural tissues and generation of viremia, invade the CNS by crossing the BBB. Virus antigen has been found in suckling mouse brain endothelium after infection with SIN (16) and in brain endothelial cells of adult mice infected with SFV (49), suggesting that the alphaviruses may invade the CNS by direct infection of brain capillary endothelial cells. Moreover, SFV replicates well in an in vitro mouse brain endothelial cell model (6, 49). Alternatively, a passive mechanism for SFV neuroinvasion across the capillary wall has also been suggested, since virions are rarely seen within mouse brain endothelium but could be found in the basal lamina (40). Transendothelial transport may also occur via diapedesis of infected monocytes, leukocytes, and lymphocytes (4, 57), but no experimental evidence supporting this mechanism exists for alphaviruses. Recently, it has been shown that neuroinvasion of VEE can occur via the olfactory neuroepithelium and a novel route by invasion of the tooth pulp and subsequent spread of virus to the CNS via the trigeminal nerve (1). Soluble factors that increase vascular permeability and transient BBB breakdown may also facilitate alphavirus invasion of the CNS (30) (see below).

From this brief overview, it is obvious that multiple mechanisms are likely to be involved in alphavirus neuroinvasion. Using genetically well defined Sindbis virus strains in a murine pathogenesis model, we hope to eventually understand some of the molecular mechanisms by which specific changes in the viral genome act at the cellular and organismal level to facilitate neuroinvasion. In this study, we focused on two novel pairs of Sindbis virus isolates which differ in their ability to invade the CNS of weanling mice. As described in the introduction and below, these pairs also differ in neurovirulence, neuroinvasion mechanism, and genetic background. For each pair, construction of functional cDNA clones and analysis of recombinant viruses have allowed us to define specific genetic determinants responsible for neuroinvasiveness (Table 4). Our results point to an important role for E2 in neuroinvasion which can be modulated by changes elsewhere in the genome, such as the 5′NCR.

The SVA/SVB pair. The neurotropic but nonneurovirulent SVA/SVB pair was derived from a pool of culicine mosquitoes collected in Israel in 1983 and biologically cloned after limited passaging in suckling mice (29, 31). A single amino acid residue in the E2 glycoprotein, Gln-55, was found to be responsible for SVB neuroinvasion. SVA contains an Arg residue at this position but is nonneuroinvasive although it replicates to similar titers in the periphery or in the brain (after intracranial inoculation). Partial sequence analysis suggests that SVA and SVB differ from the AR339 Sindbis virus prototype strain, which was isolated in Egypt 30 years earlier (54), by about 2.5% at the nucleotide level (Table 4 and data not shown). Like SVB, most laboratory strains derived from AR339, as well as the S.A.AR86 and Girdwood S.A. isolates, contain Gln at E2 position 55 (see reference 34 and citations therein). More distantly related Sindbis-like viruses, Ockelbo and Aura, contain Lys or Arg, respectively, at the homologous E2 position (34). Most of these strains, with the exception of S.A.AR86, are nonvirulent in adult mice after intracranial inoculation; their neuroinvasive properties in weanling mice have not been reported.

In the AR339 background, E2 position 55 has been shown to affect neurovirulence (32, 56). A His (instead of Gln) residue appears to be selected upon mouse brain passaging and increases neurovirulence in adult mice after intracranial inoculation (11, 32, 56) (Table 4, NSV strain). Experiments suggest that this increased neurovirulence correlates with the ability of the virus to overcome protective host factors, such as *bcl-2*, and trigger apoptosis (26, 59). Interestingly, His was also found at position 55 for the mouse brain-passaged SVN/SVNI pair (Table 4), although its role in neurovirulence has not been studied. The mechanism by which SVB enters the CNS remains to be elucidated, but it is tempting to speculate that Gln-55 facilitates SVB neuroinvasion by altering tissue tropism, either at the level of receptor binding or during later stages in the entry process. Several lines of evidence suggest that E2 is involved in receptor binding (55, 58, 61) and penetration (5) and thus a likely candidate for modulating tissue tropism. Also consistent

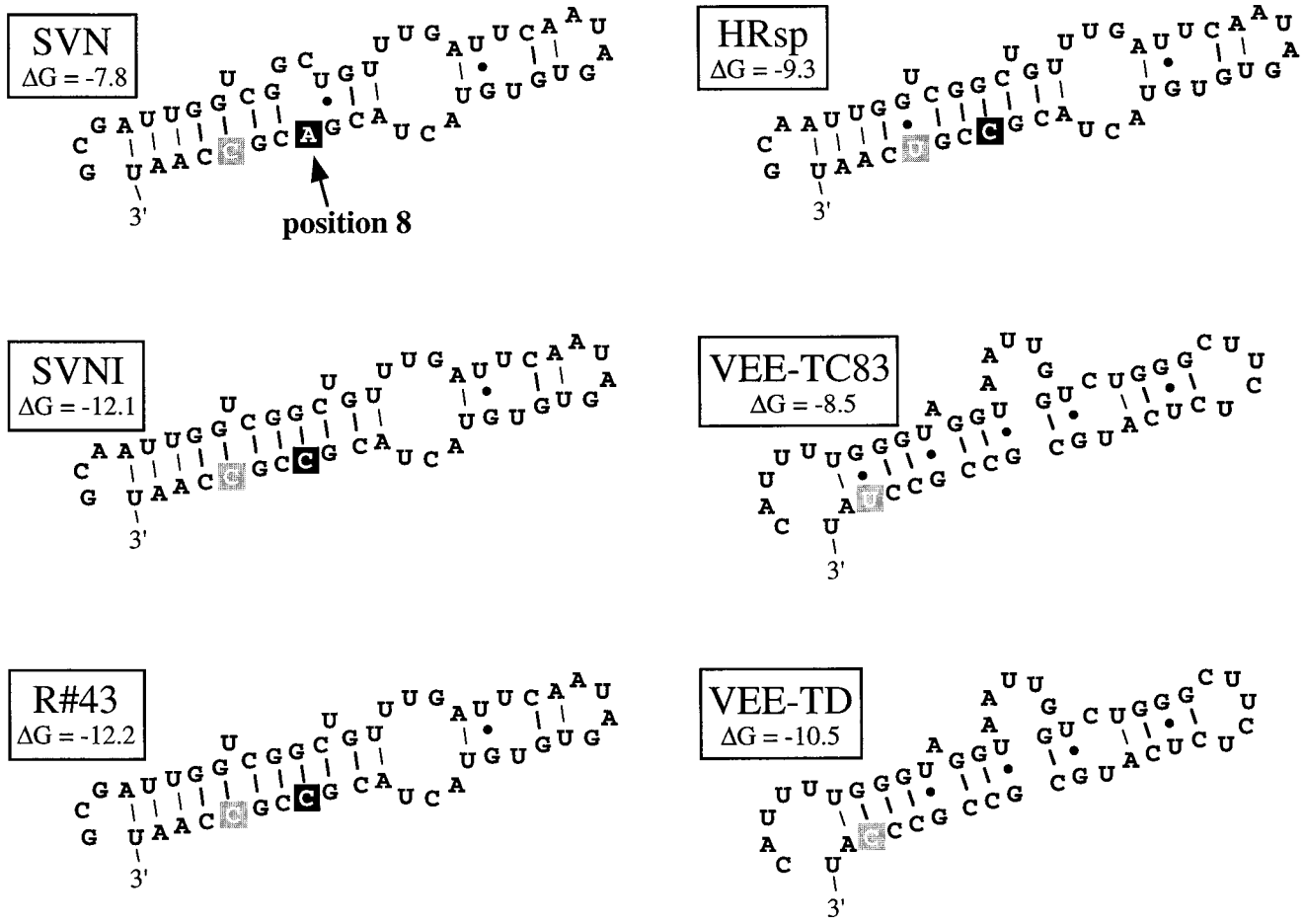

FIG. 6. Effects of position 8 substitutions on secondary structures predictions for the 3′ end of minus-strand RNA. Optimized secondary structures for the 3′ 45 nt of SVN, SVNI, R43, HRsp (51), VEE TC83 (19, 20), and VEE TRD (19, 20) minus-strand RNAs were predicted by using the energy minimization program of Zuker and coworkers (14, 60).

with the involvement of this E2 subregion in receptor binding is the observation that an insertion mutation close to position 55 (between residues 69 and 74) reduces Sindbis virus binding to BHK-21 cells and CEF (7).

The SVN/SVNI pair. The SVN/SVNI pair of neurovirulent isolates was obtained after prolonged passaging in mouse brain. Limited sequence analysis suggests that SVN and SVNI are remarkably similar to the prototype AR339 strain (34), differing by only ~0.3% at the nucleotide level (Table 4 and data not shown). SVNI neuroinvasiveness mapped to two different loci. As for SVB, one SVNI determinant mapped to the E2 glycoprotein but involved a different residue, Met-190. Lys is found at this position in SVN as well as in AR339-derived strains (Table 4). A second neuroinvasive determinant was found in the 5′NCR at position 8, which is G in SVNI and U in SVN. Either of these SVNI determinants on the SVN background increased neuroinvasiveness and virulence, as measured after intraperitoneal inoculation. However, both neuroinvasive determinants, as well as a Lys (instead of Glu) residue at E2 position 260, were necessary to produce a virus with neuroinvasive and virulence properties indistinguishable from those of the SVNI parent.

As for SVB, the SVNI E2 determinant (Met-190) might modulate neuroinvasiveness by affecting tissue tropism at the level of receptor binding and/or entry. Residue 190 is located in a region of E2 (from residues 170 to 220) which contains several neutralization epitopes (52). This region has also been implicated in virus-receptor interactions (55, 61; see reference 53 for a review). In particular, anti-idiotypic antibodies against monoclonal antibody (MAb) 49 (a neutralizing MAb which maps to this subregion) define a potential Sindbis virus receptor on CEF. Interestingly, the Met substitution for Lys-190 (Table 4) was isolated earlier as an AR339 mutant which escaped neutralization by a different MAb directed against this E2 subregion (52). Alternatively, the SVNI E2 determinant may affect host cell biology and facilitate neuroinvasion in other ways. For example, specific residues in E2 can influence the ability of some Sindbis virus strains to trigger apoptosis in certain cell types (26, 59). A similar effect of SVNI on endothelial cells at the BBB could lead to invasion of the CNS.

The second SVNI determinant, at position 8 of the 5′NCR, is located in a predicted hairpin structure of approximately 44 bases (36). A stable secondary RNA structure is also predicted for the complementary sequence at the 3′ end of the minus-strand RNA (Fig. 6). The roles of this region in genome mRNA translation or, in the complementary minus strand, in the initiation of plus-strand RNA synthesis are not well defined. Deletion mutations which disrupt the predicted hairpin structure are deleterious or lethal for virus replication (35) and lead to attenuated neurovirulence in suckling mice (21). In

particular, deletion of the G at position 8 (which is also G in HRsp [Table 4]) yielded a mutant with a temperature-sensitive phenotype (35). Many of the 5′NCR deletion mutations have different effects on replication in chicken versus mosquito cells, hinting that cellular factors might associate with these proposed hairpin structures in the plus- and/or minus-strand RNAs (see reference 53 for review). In this regard, multiple high-affinity binding sites for the La autoantigen have been identified in this region of the minus-strand RNA (37, 39). These interactions are not altered, however, by deleting the C at position 8 (38) but are strengthened by deleting residue 5 (38), a mutation which is lethal for virus replication (35). While the details remain to be worked out, it seems clear that sequence changes in this region can have dramatic host cell-dependent effects on virus replication and pathogenesis. Hence, it is tempting to speculate that G at position 8 of the 5′NCR affects SVNI replication in a cell type which then facilitates neuroinvasion in this weanling mouse model. It remains to be determined whether this change acts at the level of translation, RNA replication, or both. The similarity in translational efficiencies of SVN, SVNI, and R43 (SVN with G at position 8) RNAs observed in rabbit reticulocyte lysates (Fig. 5) should be interpreted with caution since it may or may not hold true in cells relevant to SVNI neuroinvasion. In this regard, changes in the poliovirus 5′NCR which attenuate neurovirulence have been linked to a translational defect which is specific to neuronal cells (13, 33).

In terms of a possible effect on RNA replication, we note that the SVN substitution at position 8 destabilizes the RNA structure predicted for the 3′-terminal 45 bases of the minus-strand RNA. This could modify binding of cellular factors, such as La, and thereby affect the efficiency of plus-strand initiation. It is striking that position 3 in the VEE genome has also been mapped as an important virulence determinant, distinguishing the TC83 vaccine strain from the virulent Trinidad donkey (TRD) parent (19). As in the case of SVNI, the locus in the TRD 5′NCR acts synergistically with a determinant in the E2 glycoprotein (E2 Thr-120). The attenuated TC83 strain with TRD E2 Thr-120 was essentially avirulent for 6-week-old ICR mice by the intraperitoneal route. When the TRD G residue at 5′NCR position 3 was included on this background, an apparently neuroinvasive virulent recombinant similar to parental TRD was produced. Similar to the SVN/SVNI RNA secondary structures, the TC83 substitution destabilizes the predicted structure for the 3′-terminal 45 bases of TC83 minus-strand RNA relative to that of TRD (Fig. 6). Further studies are required to directly examine the structure of these RNA elements, to define their cognate binding factors, and to understand how these interactions modulate viral replication and neuroinvasion.

Synergistic effects of multiple determinants and genetic background. A common theme linking our study with earlier work on alphaviruses and other RNA viruses is that multiple determinants and genetic background can dramatically influence the outcome of viral pathogenesis. As mentioned earlier for VEE, combining the TRD 5′NCR and E2 determinants markedly increased neuroinvasive character (19). In the case of SVNI, determinants in either the 5′NCR or E2 increased neuroinvasiveness (as measured by PFU/IPLD_{50}) by 4 to 5 orders of magnitude. Combination of these determinants showed a weak synergistic effect (Fig. 4). In a recent study examining Sindbis virus neurovirulence in neonatal mice, a dramatic synergistic effect involving position 5 of the 5′NCR and E2 residue 114 was uncovered (34). Two AR339-derived strains, TRSB and TR6000, differ at 5′NCR position 5: TRSB contains a G at this position (typical of most AR339-derived strains, as well as SVN/SVNI and SVA/SVB [Table 4]), whereas an A residue is present at position 5 of TR6000 (derived from HRsp). Both TRSB and TR6000 are highly virulent for neonatal mice (<1 PFU/LD_{50} after intracerebral injection). When Arg was substituted for E2 Ser-114 on these genetic backgrounds (Arg-114 was previously shown to attenuate neurovirulence [5, 42]), the resulting viruses were significantly attenuated but to vastly different degrees: about 50-fold for TRSB and $>10^6$-fold for TR6000 (34). Given that minor differences at the sequence level can have dramatic consequences on neuroinvasion and/or neurovirulence, it is perhaps not surprising that it is difficult to predict the impact of specific changes on a different viral genetic background. For example, the 5′NCRs of SVA and SVB are identical to SVNI, yet SVA is nonneuroinvasive. Conversely, Gln at E2 position 55 determines neuroinvasiveness for the SVA/SVB pair, yet SVN and SVNI do not differ at E2 position 55. Perhaps common themes will eventually emerge for these different strains and pathogenesis models, but a much deeper understanding of key virus-host interactions at the molecular level will be required.

Defining different molecular mechanism(s) involved in Sindbis virus neuroinvasion. For studying underlying mechanisms involved in Sindbis virus neuroinvasion, SVB and SVNI are of particular interest since they both enter the CNS of mice with a developed BBB. Previous work suggests that SVB and SVNI use different neuroinvasive mechanisms (29, 31). Peripheral coinoculation of SVB and SVN does not enhance the ability of SVN to penetrate the CNS. In contrast, coinoculation of SVN and SVNI facilitates entry of SVN. As shown here, either SVNI neuroinvasive determinant can facilitate entry of SVN, although the E2 determinant seems to be more effective (Table 2). Of many possible coinvasion mechanisms, SVNI could induce a physical breach in the BBB by direct infection and damage of endothelial cells or indirectly, by inducing the production of a host factor mediating BBB breakdown (2, 30). In any case, we now have a set of recombinant viruses with limited and defined genetic changes which exhibit dramatic differences in neuroinvasiveness. These strains are currently being used in pathogenesis studies to further define the routes and molecular mechanisms involved in Sindbis virus neuroinvasion.

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