SHORT COMMUNICATION

and Hypervariable Domains within the C Terminus of nsP3[1]

M. STEVEN OBERSTE,[2] MICHAEL D. PARKER, and JONATHAN F. SMITH

Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Maryland 21702-5011

The complete nucleotide and predicted amino acid sequences of Venezuelan equine encephalitis (VEE) virus subtype IE (isolate 68U201) were determined and compared to those of other antigenic variants within the VEE complex, strains IAB-TrD, IC-P676, ID-3880, IE-MenaII, and II-Fe3-7c. The 68U201 structural proteins were most closely related to their MenaII counterparts (97–100% identity) and more distantly related to VEE strains of other antigenic varieties (83–93% identity). With the exception of nsP3, the 68U201 nonstructural proteins were 94–95% identical to those of TrD, P676, and 3880 (nonstructural gene sequences are not available for MenaII and Fe3-7c). The amino-terminal region of nsP3 (aa 1–329), which is highly conserved among all alphaviruses, was 93–94% identical for all VEE strains. The nsP3 carboxyl region is highly divergent among alphaviruses in general, but well conserved among previously sequenced VEE strains (>90% identity). Surprisingly, the carboxyl region of 68U201 nsP3 (aa 330–563) was only 59–61% identical to that of subtype IAB, IC, and ID viruses, with large insertions and deletions in addition to numerous substitutions. The differences between the 68U201 and other VEE nsP3 carboxyl regions were not randomly distributed, as there were four domains of high similarity within the nonconserved region. To examine this divergence more closely, we sequenced a portion of the MenaII ns3 gene. The 68U201 and MenaII nsP3 nonconserved regions were 85.3% identical and had the same basic domain structure, which was distinct from the IAB, IC, and ID nsP3 proteins, suggesting that the domain structure of nsP3 may be subtype/variety-specific. VEE nsP3 sequence diversity may reflect ecological differences such as adaptation to different mosquito vectors or vertebrate hosts.   © 1996 Academic Press, Inc.

Venezuelan equine encephalitis (VEE) virus, a member of the alphavirus genus of the family Togaviridae, was first isolated in Venezuela in the 1930s from the brain of a horse which had died from encephalitis (*1, 2*). The VEE virus complex is now known to consist of at least six major serological subtypes (I–VI), with additional serologically defined variants within types I and III (*3*). Subtypes IAB and IC have caused numerous equine epizootics in tropical and subtropical regions of the Americas (*4, 5*). Epizootic strains are transmitted to and among equines by mosquitos of several different genera, but the reservoir host is unknown. The remaining viruses (ID-IF and II-VI) appear to be attenuated for equine species and are maintained in geographically restricted enzootic foci in tropical regions of Central and South America, usually closely associated with a preferred vector species (*3*). Small mammals are thought to be the primary reservoir for the enzootic VEE strains, with *Culex* (*Mela-*

noconion subgenus) mosquito species as the principal vector. VEE viruses of subtype IE are found in isolated foci in tropical Central America, from Veracruz state in Mexico to northwestern Panama (*3*). *Culex taeniopus,* a common mosquito species in the subtype IE endemic area, has been shown to readily transmit VEE IE, but it appears to be refractory to infection by epizootic strains (*6*). Epizootic VEE isolates are highly virulent in equines and inbred guinea pigs, whereas most enzootic strains cause only benign infections in these hosts (*7–10*). An exception to the correlation between equine and guinea pig virulence is that subtype ID viruses are fully pathogenic for guinea pigs, despite their lack of virulence for horses. The molecular basis of vector competence and differential virulence remain unknown.

VEE IE-68U201 was isolated in 1968 from the brain of a sentinel hamster near La Avellana, in the Pacific lowlands of southeastern Guatemala (*11*). 68U201 is serologically related to other IE isolates, but as a group, IE viruses cross-react poorly with isolates of other VEE varieties (*12*). We chose this particular IE isolate for our studies because it has been well characterized with respect to pathogenesis (*7, 8*), serological cross-reactivity (*12*), and mosquito transmission (*6*). In addition, 68U201 has infected and caused acute febrile ill-
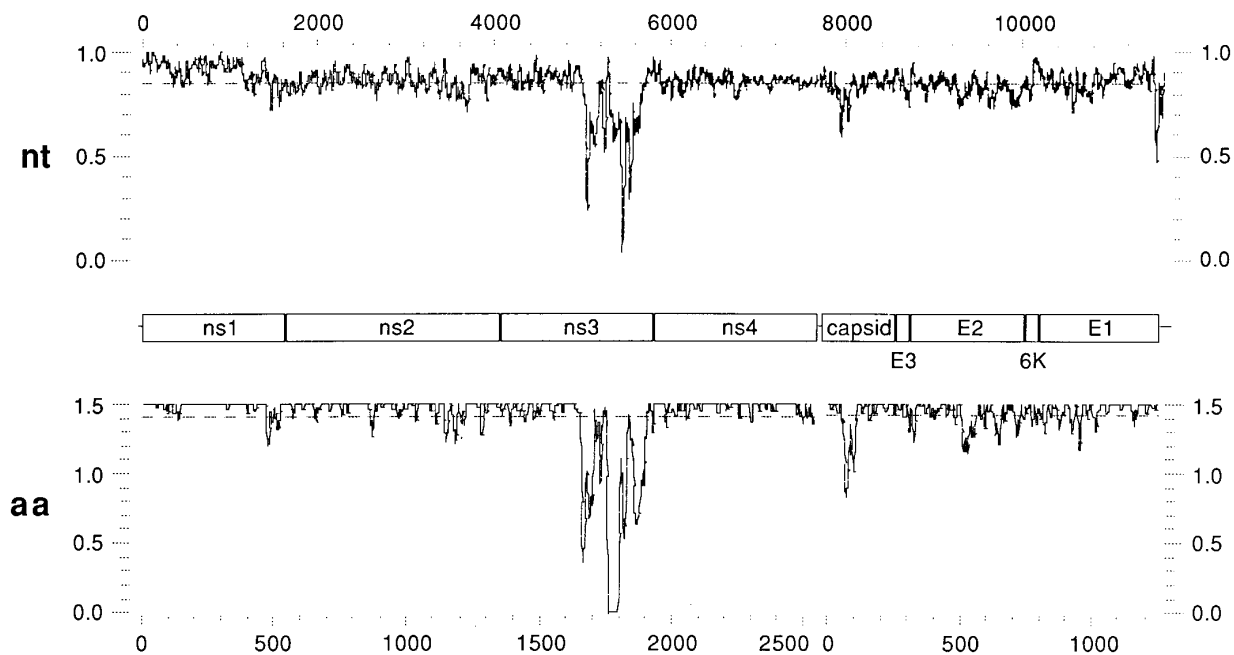
FIG. 1. VEE Nucleotide and amino acid similarity plots. The nonstructural and structural regions were aligned separately with Pileup (Genetics Computer Group, Inc.), by using the complete nucleotide sequences of VEE IAB-TrD, IC-P676, ID-3880, and IE-68U201 and the 26S sequences of IE-Menall and II-Fe3-7c (*13, 15, 16*), all obtained from GenBank. Plotsimilarity (Genetics Computer Group, Inc.) was used to plot sequence variability as a function of residue number. For nucleotide sequences (top), the average match score at each position (match = 1, mismatch = 0) was averaged over a window of 30 residues. The average match score for the entire nucleotide alignment was approximately 0.85 and is plotted as a dashed line. For predicted amino acid sequences (bottom), the average match score at each position (maximum score = 1.5, minimum score = 0) was averaged over a window of 10 residues, using the scoring matrix of Gribskov and Burgess (*36*). The average match scores for the entire nonstructural and structural regions were approximately 1.41 and 1.42, respectively, and are plotted as dashed lines.

ness in lab workers after accidental exposure, despite otherwise adequate responses to previous immunization with a live–attenuated VEE vaccine (TC-83), which was derived from the subtype IAB-TrD strain, demonstrating that the current vaccine does not adequately protect against infection with 68U201 (P. B. Jahrling, personal communication).

Analyses of the complete sequences of VEE strains IAB-TrD, IC-P676, and ID-3880 and of the structural regions of strains IE-Menall and II-Fe3-7c showed that subtypes IAB, IC, ID, and II were closely related to one another, whereas subtype IE was distinct from other subtypes (*13–15*). To help determine the molecular basis for VEE vector competence and differential virulence, to generate reagents for the development of a VEE IE-specific vaccine, and to extend existing sequence analyses to include the nonstructural region of VEE IE, we determined the complete sequence of VEE IE-68U201 (11,464 nt). Sequences derived from random-primed cDNA clones were mapped to the nonstructural region of the genome by alignment to the published sequence of IAB-TrD (*16*) and used to design 68U201-specific primers to facilitate cloning the remainder of the nonstructural region by reverse transcription–PCR (RT–PCR). cDNAs representing the 68U201 structural gene region were synthesized by RT–PCR, using primers based on the published sequence of VEE IE-Menall (*14, 15*) and cloned

prior to sequencing. Additional sequencing templates were generated by RT–PCR and 68U201-specific primers, purified by the Wizard PCR prep kit (Promega Corp., Madison, WI), and sequenced directly. Each nucleotide was determined by sequencing both strands with an automated DNA sequencer (Applied Biosystems Division, Perkin–Elmer Corp., Foster City, CA) and using two or more independent clones or PCR products, with an average of 5.4-fold redundancy. Nucleotide and predicted amino acid sequences were analyzed with programs in the Wisconsin Sequence Analysis Package, version 8.0 (Genetics Computer Group, Inc., Madison, WI).

The alphavirus nonstructural proteins are translated from an mRNA identical to the genomic RNA. The three structural proteins (capsid, E2, and E1) are produced by proteolytic processing of the structural polyprotein (capsid-E3-E2-6K-E1), which is translated from a 26S subgenomic mRNA. The available VEE nonstructural (four strains) and structural (six strains) gene sequences, as well as their predicted polyprotein products, were compared to one another by using Pileup and the similarity scores were plotted with PlotSimilarity (Fig. 1). In both nucleotide and amino acid sequence comparisons, the region of greatest divergence was within nsP3 (Fig. 1 and see below). Overall, the nucleotide sequence of 68U201 was 77–78% identical to the sequences of IAB-TrD, IC-P676, ID-3880, and II-Fe3-7c (26S only) (Table 1).

TABLE 1

Comparison of VEE IE⁻ 68U201 Nucleotide and Predicted Amino Acid Sequences with Those of Other Strains of VEE[a]

	IAB-TrD	IC-P676	ID-3880	IE-Menall	II-Fe3-7c
Nucleotides					
Entire genome	77.2	77.5	77.2	NA[b]	NA
Nonstructural	77.1	77.4	76.9	NA	NA
Structural	77.4	77.8	78.0	93.2	77.4
5′ NTR[c]	95.3	95.3	95.3	NA	NA
26S NTR[d]	85.7	85.7	85.7	100.0	80.0
3′ NTR	74.6	77.2	78.1	96.9	76.3
Nonstructural proteins					
nsP1	94.8	94.8	94.8	NA	NA
nsP2	93.8	93.8	93.7	NA	NA
nsP3	82.0	81.4	81.4	88.0[e]	NA
N-terminal[f]	93.3	93.6	93.3	NA	
C-terminal[f]	61.3	59.1	59.4	84.5	
nsP4	94.4	94.4	94.4	NA	NA
Structural proteins					
Capsid	87.3	86.9	86.5	97.1	86.1
E3	86.0	84.2	86.0	98.2	82.5
E2	86.3	87.7	89.2	97.2	84.9
6K	89.3	92.9	91.1	100.0	91.1
E1	92.5	92.3	92.8	99.3	92.5

[a] Sequences were aligned by using the program Gap (Genetics Computer Group, Inc.). All numbers are percentage identical residues using the length of the shorter of the two sequences as the denominator.
[b] NA, sequence not available for this portion of the genome.
[c] NTR, nontranslated region.
[d] Sequences between the ns4 termination codon and the initiation codon of the structural polyprotein. The published sequences of IE-Menall and II-Fe3-7c begin at nt 1 of the 26S RNA (15), which is nt 6 of the nontranslated region.
[e] Only a portion of the Menall ns3 gene was sequenced (see text).
[f] The conserved amino-terminal region (aa 1–329) and nonconserved carboxyl-terminal region, as defined by Strauss et al. (28), were also aligned separately.

The nonstructural and structural coding regions were equally similar, on average, in all cases. The 68U201 26S nt sequence was 93.2% identical to that of Menall, a 1962 IE isolate from northwestern Panama.

The VEE glycoproteins, E1 and E2, contain epitopes which are important as elicitors and targets of the host immune response during natural infection (17–21). The structural proteins of 68U201 and Menall were most closely related to one another, as expected from their serological cross-reactivity (12), exhibiting 97–100% identity in amino acid sequence (Table 1). The predicted E2 protein of 68U201 was 97.2% identical to that of Menall and 86–91% identical to those of subtypes IAB, IC, ID, and II (Table 1), with the region of greatest heterogeneity between residues 179 and 264 (Fig. 2). The epitopes recognized by four anti-IAB-TC-83 neutralizing monoclonal antibodies, two of which also neutralized IE-Menall, have been mapped to this region by using neutralization–escape mutants (19). A linear TrD epitope between E2-240 and E2-264, which elicits a nonneutralizing but protective immune response in mice, was identified by using synthetic peptides (20). The variability of this region makes it difficult to predict whether the amino acid at

a given position would result in the same phenotype (neutralization escape or attenuation) in a different strain. For example, changing E2-209 from glutamate to lysine in a IAB infectious clone results in reduced virulence for mice (22). Strain II-Fe3-7c contains lysine at E2-209 (13); however, its virulence for adult mice is unknown. Presumably, the three-dimensional conformation of VEE glycoproteins determines phenotype, and the conformation may be influenced by a number of different amino acid positions within the protein.

A site of potential N-linked glycosylation (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro; ref. 23) at E2-318 was conserved among all VEE strains. All strains except Fe3-7c have NPT at E2-291, but the presence of proline within the recognition sequence suggests that this site is probably not glycosylated. TrD, TC-83, and Fe3-7c have an additional potential glycosylation site at E2-212 (NKT), while 68U201 has a predicted site at E2-406 (NAS) that is absent from all other strains, including IE-Menall. Johnson et al. (24) postulated that both E2-212 and E2-318 of IAB-TrD and IAB-TC-83 are glycosylated in vivo. The E2 glycoproteins of VEE strains TrD, TC-83, and Fe3-7c appear to be larger than those of most other

```
TrD     1   STEELFKEYKLTRPYMARCIRCAVGSCHSPIAIEAVKSDGHDGYVRLQTSSQYGLDSSGNLKGRTMRYDMHGTIKEIPLH  80
P676    1   STEELFKEYKLTRPYMARCIRCAVGSCHSPIAIEAVKSDGHDGYVRLQTSSQYGLDSSGNLKGRTMRYDMHGTIEEIPLH  80
3880    1   STEELFKEYKLTRPYMARCIRCAVGSCHSPIAIEAVKSDGHDGYVRLQTSSQYGLDSSGNLKGRTMRYDMHGTIEEIPLH  80
Menall  1   STEELFKEYKLTRPYMARCIRCAVGSCHSPIAIEAVRSEGHDGYVRLQTSSQYGLDPSGNLKGRTMRYDMHGTIEEIPLH  80
68U201  1   STEELFKEYKLTRPYMARCIRCAVGSCHSPIAIEAVRSEGHDGYVRLQTSSQYGLDPSGNLKGRTMRYDMHGTIEEIPLH  80
Fe3-7c  1   STEELFKEYKLTRPYMARCVRCAVGSCHSPIAIEAVKSDGHDGYVRLQTSSQYGLDPSGNLKSRTMRYNMYGTIEEIPLH  80

TrD     81  QVSLHTSRPCHIVDGHGYFLLARCPAGDSITMEFKKDSVTHSCSVPYEVKFNPVGRELYTHPPEHGVEQACQVYAHDAQN  160
P676    81  QVSLHTSRPCHIVDGHGYFLLARCPAGDSITMEFKKDAVTHSCSVPYEVKFNPVGRELYTHPPEHGAEQACQVYAHDAQN  160
3880    81  QVSLHTSRPCHIVDGHGYFLLARCPAGDSITMEFKKDAVTHSCSVPYEVKFNPVGRELYTHPPEHGAEQACQVYAHDAQN  160
Menall  81  QVSLHTSRPCHIIDGHGYFLLARCPAGDSITMEFKKESVTHSCSVPYEVKFNPVGRELYTHPPEHGAEQPCHVYAHDAQN  160
68U201  81  QVSVHTSRPCHIIDGHGYFLLARCPAGDSITMEFKKESVTHSCSVPYEVKFNPAGRELYTHPPEHGAEQPCHVYAHDAQN  160
Fe3-7c  81  QVSLHTSRPCHIVDGHGYFLLARCPAGDSITMEFKKDSVTHSCSVPYEVKFNPVGRELYTHPPEHGAEQACQVYAHDAQN  160

TrD     161 RGAYVEMHLPGSEVDSSLVSLSGSSVTVTPPVGTSALVECECGGTKISETINKTKQFSQCTKKEQCRAYRLQNDKWVYNS  240
P676    161 RGAYVEMHLPGSEVDSSLISLSGSSVTVTPPVGTSALVKCKCGGTKISETINKAKQFSQCTKKEQCRAYRLQNDKWVYNS  240
3880    161 RGAYVEMHLPGSEVDSSLVSLSGSSVTVTPPAGTSALVECECGGTKISETINTAKQFSQCTKKEQCRAYRLQNDKWVYNS  240
Menall  161 RGAYVEMHLPGSEVDSTLLSTSGSSVHVTPPAGQSVLECECGGTKISETINSAKQYSQCSKTAQCRAYRTQNDKWVYNS  240
68U201  161 RGAYVEMHLPGSEVDSTLLSMSGSSVHVTPPAGQSVQVECECGGTKISETINSAKQYSQCSKTSQCRAYRTQNDKWVYNS  240
Fe3-7c  161 RGAYVEMHLPGSEVDSSLVSLSSGLVSVTPPAGTSALVECECSGTTISKTINKTKQFSQCTKKEQCRAYRLQNDKWVYNS  240

TrD     241 DKLPKAAGATLKGKLHVPFLLADGKCTVPLAPEPMITFGFRSVSLKLHPKNPTYLTTRQLADEPHYTHELISEPAVRNFT  320
P676    241 DKLPKAAGATLKGKLHVPFLLADGKCTVPLAPEPMITFGFRSVSLKLHPKNPTYLTTRQLADEPHYTHELISEPAVRNFT  320
3880    241 DKLPKAAGATLKGKLHVPFLLADGKCTVPLAPEPMITFGFRSVSLKLHPKNPTYLTTRQLADEPHYTHELISEPAVRNFS  320
Menall  241 DKLPKAAGETLKGKLHVPFVLTEAKCTVPLAPEPIITFGFRSVSLKLHPKNPTFLTTRQLDGEPAYTHELITNPVVRNFS  320
68U201  241 DKLPKASGETLKGKLHVPFVLTEAKCTVPLAPEPIITFGFRSVSLKLHPKNPTFLTTRQVDGEPAYTHELITHPVVRNFS  320
Fe3-7c  241 DKLPKAAGATLKGKLHVPFLLADGKCTVPLAPEPMITFGFRSVSLKLHPKYPTYLTTRELADEPHYTHELISEPSVRNFS  320

TrD     321 VTEKGWEFVWGNHPPKRFWAQETAPGNPHGLPHEVITHYYHRYPMSTILGLSICAAIATVSVAASTWLFCRSRVACLTPY  400
P676    321 VTEKGWEFVWGNHPPKRFWAQETAPGNPHGLPHEVITHYYHRYPMSTILGLSICAAIVTVSVAASTWLFCKSRVSCLTPY  400
3880    321 VTEKGWEFVWGNHPPKRFWAQETAPGNPHGLPHEVITHYYHRYPMSTILGLSICAAIVTVSVAASTWLFCKSRVSCLTPY  400
Menall  321 VTEKGWEFVWGNHPPQRYWSQETAPGNPHGLPHEVITHYYHRYPMSTILGLSICAAIVTTSIAASVWLFCKSRISCLTPY  400
68U201  321 VTEKGWEFVWGNHPPQRYWSQETAPGNPHGLPHEVITHYYHRYPMSTILGLSICAAIVTTSIAASVWLLWKSRISCLTPY  400
Fe3-7c  321 VTAKGWEFVWGNHPPKRFWAQETAPGNPHGLPHEVIVHYYHRYPMSTITGLSICAAIVAVSIAASTWLLCRSRASCLTPY  400

TrD     401 RLTPNARIPFCLAVLCCARTARA  423
P676    401 RLTPNARMPLCLAVLCCARTARA  423
3880    401 RLTPNARMPLCLAVLCCARTARA  423
Menall  401 RLTPNARMPLCLAVLCCARTARA  423
68U201  401 RLTPNASMPLCLAVLCCARTAKA  423
Fe3-7c  401 RLTPNAKMPLCLAVLCCARSARA  423
```
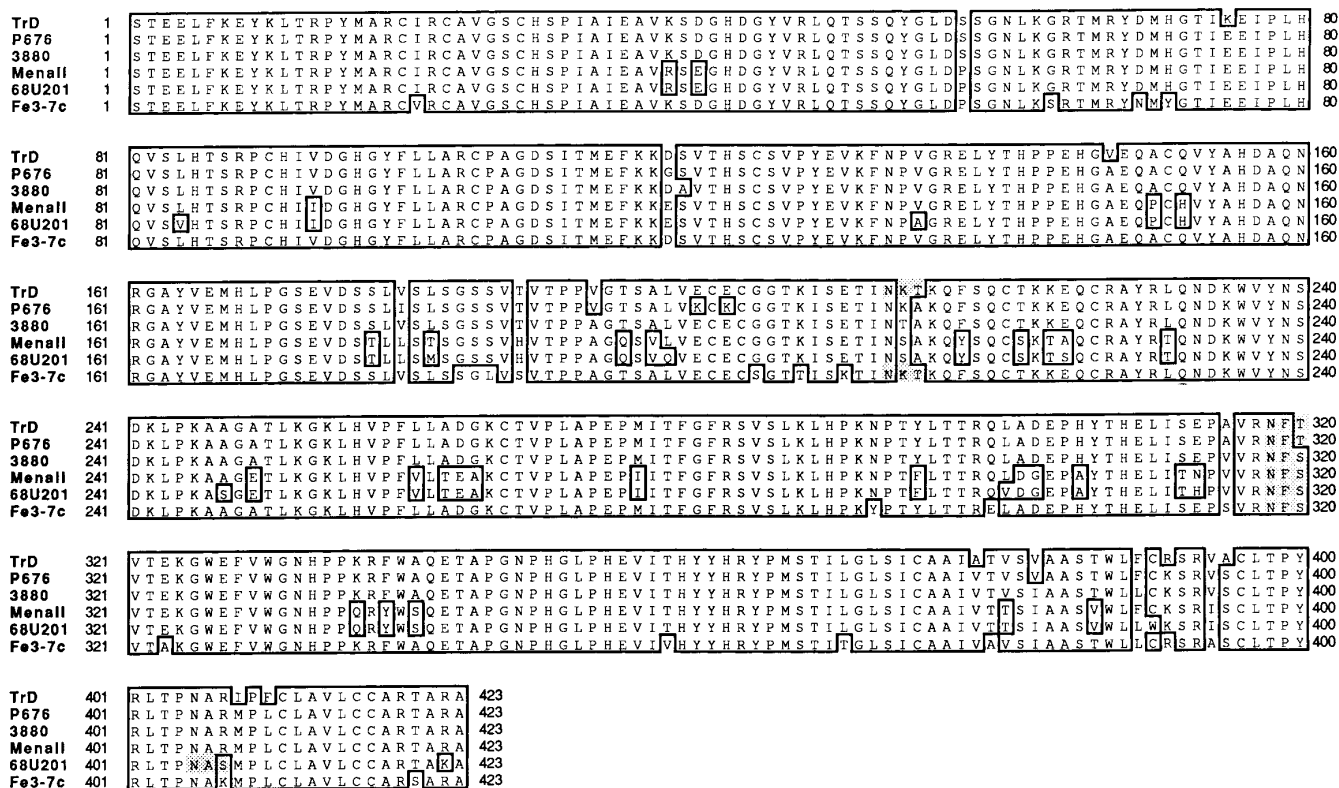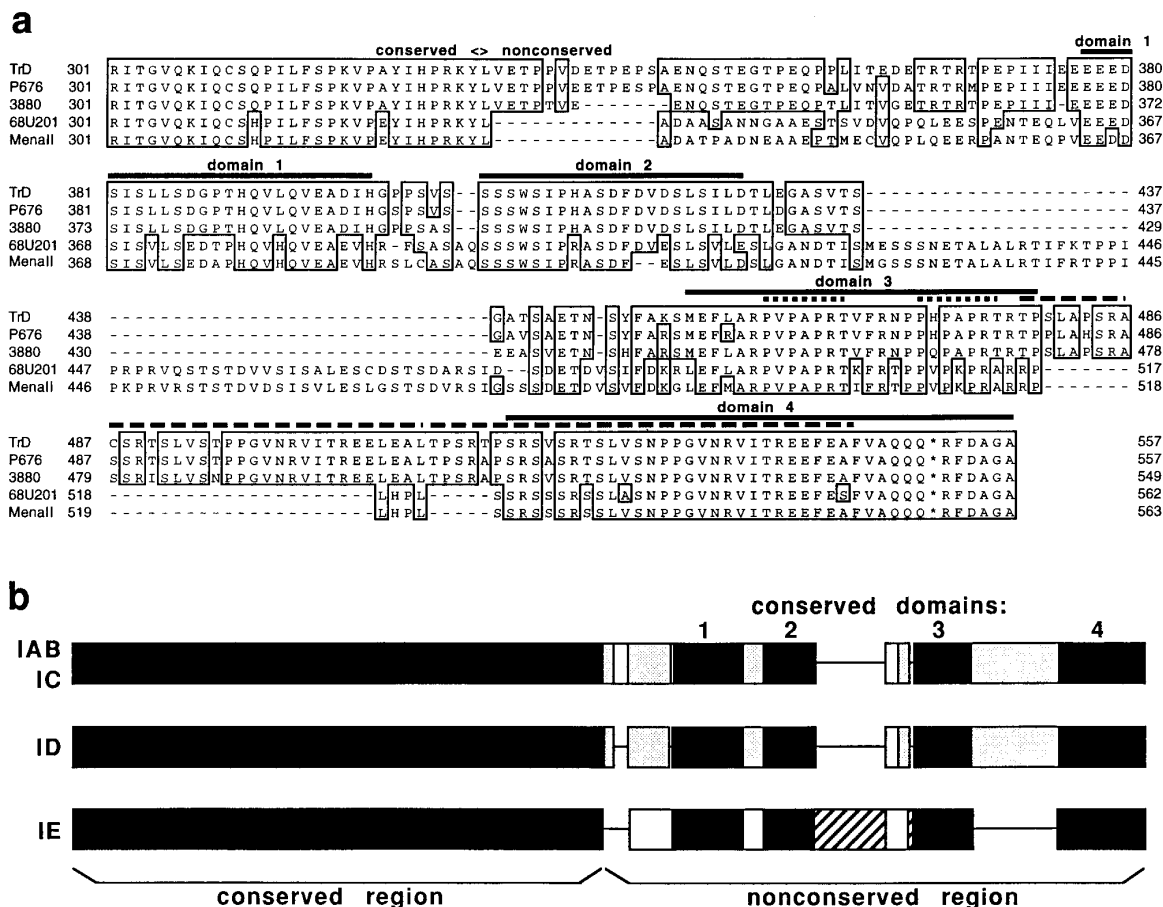
FIG. 2. Alignment of VEE E2 proteins. The predicted VEE E2 protein sequences were aligned using Pileup. Boxed residues are those which were identical in at least four of the six aligned sequences. The residues comprising the predicted sites of N-linked glycosylation are shaded.

strains, based on their mobility on SDS–polyacrylamide gels (25, 26). Kinney et al. (13) and Sneider et al. (15) hypothesized that the differences in gel mobility may be due to differential glycosylation. For example, the Menall E2 protein (one glycosylation site) had a relative mobility of about 54 kDa, whereas those of TrD, TC-83, and Fe3-7c (two glycosylation sites) had mobilities of approximately 57–58 kDa (25, 26). The mobility of 68U201 E2, about 58 kDa (25), suggests that E2-406 may be glycosylated in 68U201, as the predicted molecular weight of the protein component of the glycoprotein is approximately the same for all strains (data not shown). Thus, Menall and 68U201 may differ in their pattern of glycosylation, yet remain antigenically similar.

The functions of the alphavirus nsP1, nsP2, and nsP4 proteins in viral replication and transcription have been partially elucidated through biochemical studies with purified Sindbis and Semliki Forest virus proteins (27). nsP3 is phosphorylated on serine and threonine residues and is thought to be involved in viral RNA synthesis, but its precise role in replication remains largely unknown. nsP1, nsP2, and nsP4 are relatively conserved among all alphaviruses (27) and the VEE nsP1, nsP2, and nsP4 proteins are all highly conserved among VEE strains IAB, IC, ID, and IE (93.7–99.6% identity, with no gaps), with IAB-C-D most closely related (Table 1 and ref. 13). The

amino-terminal portion of nsP3 (VEE IAB aa 1–329), which is highly conserved among all alphaviruses (28), was 93–94% identical for all VEE strains (Table 1). The nsP3 carboxyl region is highly divergent among alphaviruses in general (28), but >90% identical among the previously sequenced VEE strains IAB, IC, and ID (13). Surprisingly, the carboxyl region of 68U201 nsP3 (aa 330–563) was only 59–61% identical to that of TrD, P676, and 3880, with large insertions and deletions in addition to numerous substitutions (Table 1 and Fig. 3a). To determine whether the structure of the nsP3 nonconserved domain of 68U201 was peculiar to this isolate only or was a general feature of IE strains, we sequenced a portion of the ns3 and ns4 genes of VEE IE-Menall, corresponding to nucleotides 4928 to 5785 (858 nt; 68U201 numbering). This sequence encodes 31 amino acids of the nsP3 conserved region, the entire nonconserved region, and 23 amino acids at the amino terminus of nsP4. The Menall nucleotide sequence (861 nt) was 87.8% identical to that of 68U201 (data not shown) and the predicted amino acid sequences were 88.0% identical (Table 1 and Fig. 3a). Within the nsP3 nonconserved region, the Menall and 68U201 nucleotide and amino acid sequences were 86.8 and 84.5% identical, respectively.

The differences between the subtype IE and other VEE nsP3 nonconserved regions were not randomly distrib-

FIG. 3. Alignment and domain structure of VEE nsP3 sequences. (a) The predicted VEE nsP3 protein sequences, beginning at residue 301, were aligned using Pileup. Boxed residues are those which were identical in at least three of the five aligned sequences. Sequence gaps are indicated by a hyphen. The boundary between the N-terminal (well conserved among all alphaviruses) and C-terminal (nonconserved) regions, as defined by Strauss *et al.* (*28*), is indicated. Repeat regions are indicated by dashed bars above the alignment: short-dashed bars, short repeat units; long-dashed bars, long repeat units. Solid bars above the alignment indicate relatively conserved domains within the C-terminal region. (b) Diagram summarizing the domain structure of predicted VEE nsP3 proteins, derived from the alignment in (a). Filled boxes, conserved regions (≥80% identity among all VEE subtypes); shaded boxes, regions of ≥80% identity among subtypes IAB-C-D only; open boxes, regions <80% identical among subtypes IAB-C-D or IAB-C-E; striped boxes, regions unique to subtype IE; thin lines, deletions relative to other subtypes. Not to scale.

uted, as there were four domains of high similarity within the nonconserved region (Fig. 3). The first of these was highly acidic, with 7 of 25 residues either aspartate or glutamate and no lysine or arginine residues. The second domain of similarity was rich in serine (8 of 24 residues). The third conserved domain contained an imperfect, 7-residue repeat, PVPAPRT-PXPAPRT, separated by the sequence XFRXP. In 68U201 and Menall, the second repeat unit was PVPKPRA. The 40 residues at the carboxyl terminus of all VEE nsP3 proteins were identical at 36 positions (including the opal stop). This final conserved domain contained most of a 34-residue motif which was repeated imperfectly between the third and fourth conserved domains in the TrD, P676, and 3880 strains but was absent from 68U201 and Menall. A TrD infectious clone, which contained only one copy of the 34-residue motif, was fully viable and phenotypically indistinguishable from an isogenic virus which contained both copies (*29*). The second and third conserved domains were sep-

arated by a 46-amino-acid serine- and threonine-rich sequence unique to the two IE strains (Fig. 3a). This unique sequence was flanked by imperfect repeats and contained an additional copy of the 7-residue imperfect repeat, suggesting that it may have arisen by genetic duplication. The 68U201 and Menall nsP3 nonconserved regions had the same basic domain structure, which was distinct from that of the IAB, IC, and ID proteins, suggesting that the domain structure may be subtype/variety-specific (Fig. 3b).

The Sindbis virus nsP3 nonconserved region tolerates linker insertion mutations and large deletions (*30*), whereas certain mutations in the conserved region render the virus temperature-sensitive for minus-strand RNA synthesis in mammalian cells (*31, 32*). Despite the relatively minor defect in RNA synthesis in mammalian cells, replication of mutants with large deletions in the nonconserved region was greatly impaired in mosquito cells (*30*). Based on these observations, LaStarza *et al.* (*30*)

proposed that the nonconserved region may be more important for viral replication in the mosquito vector, with a relatively minor role in replication in a mammalian host. One model to explain nsP3 sequence heterogeneity among alphaviruses, and now among divergent VEE strains, is that the relatively conserved regions interact with other viral proteins in the replication complex and that the divergent regions interact with as yet unidentified host-specific factors. For example, the four conserved domains may interact with other viral proteins, or with host proteins that are relatively conserved among species, whereas the hypervariable domains may interact with proteins/cellular structures that are unique to a specific insect vector or mammalian host to which it has adapted in nature. The availability of highly divergent nsP3 sequences within the VEE complex will make it possible to address the question of nsP3 function in mosquito and mammalian host systems by using chimeric viruses derived from infectious clones.

If the nsP3 nonconserved region is somehow involved in viral replication in the mosquito host, one might expect the nsP3 sequence to be somewhat specific for the different mosquito vector species. Vector competence studies demonstrated that there is a mesenteronal barrier to infection of *C. taeniopus,* the natural vector of VEE IE, with VEE strains of other varieties (*6, 33*). Oral infection of these mosquitos with IAB viruses required high doses, and even when midgut infection was achieved by high-dose feedings, there appeared to be a barrier to dissemination of virus to other tissues (*33, 34*). The block appeared to be nonreciprocal, as *C. cedecei,* a species closely related to *C. taeniopus,* was infected by and efficiently transmitted subtypes IAB, II (sympatric virus), and IE (*35*). Nonetheless, *C. taeniopus* transmitted the sympatric IE virus efficiently and subtypes IAB, IC, ID, II, III, and IV were transmitted at only low frequency (*33*). IAB infection of *C. taeniopus* may be blocked at multiple levels; e.g., virus receptor/cell attachment and replication in certain cell types, and each of these barriers may be controlled by a different viral protein. Chimeric viruses would make it possible to address in detail these questions of vector competence and differential virulence in animals by allowing, for example, substitution of TrD structural genes into an infectious clone containing 68U201 nonstructural genes or substitution of the 68U201 ns3 gene into a IAB infectious clone. The differential vector competence of *C. taeniopus* was recently confirmed (M. Turell, personal communication), providing an experimental system in which to test the phenotypes of such chimeric viruses.

Expanded sequencing studies are underway to measure the extent of VEE nsP3 sequence diversity and to determine whether these differences correlate with serological subtype (or set of closely related subtypes) or with different transmission cycles. The construction of a full-length 68U201 cDNA clone from which infectious RNA can be transcribed is also in progress. Such a clone will enable the construction of chimeric viruses for vector competence and guinea pig virulence studies, as described above, and will also serve as the basis for the development of live–attenuated and nonreplicating IE-specific vaccine candidates.

REFERENCES

1. Beck, C. E., and Wyckoff, R. W. G., *Science* 88, 530 (1938).
2. Kubes, V., and Rios, F. A., *Science* 90, 20–21 (1939).
3. Walton, T. E., and Grayson, M. A., In "The Arboviruses: Epidemiology and Ecology" (T. P. Monath, Ed.), Vol. IV, pp. 203–231. CRC Press, Boca Raton, FL, 1988.
4. Johnson, K. M., and Martin, D. H., *Adv. Vet. Sci. Comp. Med.* 18, 79–116 (1974).
5. Groot, H., In "Venezuelan Encephalitis," pp. 7–16. Pan American Health Organization, Publ. 243, Washington, DC, 1972.
6. Scherer, W. F., Cupp, E. W., Dziem, G. M., Brenner, R. J., and Ordonez, J. V., *Am. J. Trop. Med. Hyg.* 31, 1030–1037 (1982).
7. Jahrling, P. B., Heisey, G. B., and Hesse, R. A., *Infect. Immun.* 17, 356–360 (1977).
8. Scherer, W. F., and Chin, J., *Am. J. Trop. Med. Hyg.* 26, 307–312 (1977).
9. Walton, T. E., Alvarez, O., Jr., Buckwalter, R. M., and Johnson, K. M., *J. Infect. Dis.* 128, 271–282 (1973).
10. Dietz, W. H., Jr., Alvarez, O., Jr., Martin, D. H., Walton, T. E., Ackerman, L. J., and Johnson, K. M., *J. Infect. Dis.* 137, 227–237 (1978).
11. Scherer, W. F., Dickerman, R. W., and Ordonez, J. V., *Am. J. Trop. Med. Hyg.* 19, 703–711 (1970).
12. Scherer, W. F., and Pancake, B. A., *J. Clin. Microbiol.* 6, 578–585 (1977).
13. Kinney, R. M., Tsuchiya, K. R., Sneider, J. M., and Trent, D. W., *Virology* 191, 569–580 (1992).
14. Weaver, S. C., Bellew, L. A., and Rico-Hesse, R., *Virology* 191, 282–290 (1992).
15. Sneider, J. M., Kinney, R. M., Tsuchiya, K. R., and Trent, D. W., *J. Gen. Virol.* 74, 519–523 (1993).
16. Kinney, R. M., Johnson, B. J. B., Welch, J. B., Tsuchiya, K. R., and Trent, D. W., *Virology* 170, 19–30 (1989).
17. Mathews, J. H., and Roehrig, J. T., *J. Immunol.* 129, 2763–2767 (1982).
18. Roehrig, J. T., and Mathews, J. H., *Virology* 142, 347–356 (1985).
19. Johnson, B. J. B., Brubaker, J. R., Roehrig, J. T., and Trent, D. W., *Virology* 177, 676–683 (1990).
20. Johnson, A. J., Hunt, A. R., and Roehrig, J. T., *Virology* 185, 840–842 (1991).
21. Sviatchenko, V. A., Pereboev, A. V., Agapov, E. V., Razumov, I. A., Sabirov, A. N., Mizenko, G. A., Samukov, V. V., and Loktev, V. B., *Vopr. Virusol.* 38, 162–167 (1993).
22. Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J. B., Smith, J. F., and Johnston, R. E., *Virology* 183, 20–31 (1991).
23. Bause, E., *Biochem. J.* 209, 331–336 (1983).

24. Johnson, B. J. B., Kinney, R. M., Kost, C. L., and Trent, D. W., *J. Gen. Virol.* **67**, 1951–1960 (1986).

25. France, J. K., Wyrick, B. C., and Trent, D. W., *J. Gen. Virol.* **44**, 725–740 (1979).

26. Kinney, R. M., Trent, D. W., and France, J. K., *J. Gen. Virol.* **64**, 135–147 (1983).

27. Strauss, J. H., and Strauss, E. G., *Microbiol. Rev.* **58**, 491–562 (1994).

28. Strauss, E. G., Levinson, R., Rice, C. M., Dalrymple, J., and Strauss, J. H., *Virology* **164**, 265–274 (1988).

29. Davis, N. L., Willis, L. V., Smith, J. F., and Johnston, R. E., *Virology* **171**, 189–204 (1989).

30. LaStarza, M. W., Lemm, J. A., and Rice, C. M., *J. Virol.* **68**, 5781–5791 (1994).

31. LaStarza, M. W., Grakoui, A., and Rice, C. M., *Virology* **202**, 224–232 (1994).

32. Wang, Y.-F., Sawicki, S. G., and Sawicki, D. L., *J. Virol.* **68**, 6466–6475 (1994).

33. Scherer, W. F., Weaver, S. C., Taylor, C. A., Cupp, E. W., Dickerman, R. W., and Rubino, H. H., *Am. J. Trop. Med. Hyg.* **36**, 194–197 (1987).

34. Weaver, S. C., Scherer, W. F., Cupp, E. W., and Castello, D. A., *Am. J. Trop. Med. Hyg.* **33**, 953–960 (1984).

35. Weaver, S. C., Scherer, W. F., Taylor, C. A., Castello, D. A., and Cupp, E. W., *Am. J. Trop. Med. Hyg.* **35**, 619–623 (1986).

36. Gribskov, M., and Burgess, R. B., *Nucleic Acids Res.* **14**, 6745–6763 (1986).