Aura Virus Is a New World Representative of Sindbis-like Viruses

TILLMANN RÜMENAPF,† ELLEN G. STRAUSS, and JAMES H. STRAUSS²

Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125

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Aura virus is an alphavirus present in Brazil and Argentina that is serologically related to Sindbis virus (present throughout the Old World) and to Western equine encephalitis (WEE) virus (present in the Americas). We have previously shown that WEE is a recombinant virus whose glycoproteins and part of whose 3' nontranslated region (NTR) are derived from a Sindbis-like virus, but the remainder of whose genome is derived from Eastern equine encephalitis (EEE) virus. We show here that Aura virus is a Sindbis-like virus that shares considerable organizational and sequence identity with Sindbis virus. Certain nucleotide sequence elements present in Aura RNA that are believed to function as promoters are almost identical to their Sindbis counterparts, repeated elements in the 3' nontranslated region are shared with Sindbis virus, and important antigenic epitopes are conserved between the two viruses. Despite their close relationship, the two viruses have diverged significantly, sharing 73% amino acid sequence identity in the nonstructural proteins and 62% identity in the structural proteins. This is about the same as the identities between EEE and Venezuelan equine encephalitis virus whose promoter elements, 3' NTRs, and antigenic epitopes have diverged more radically, such that these two viruses are considered to belong to different subgroups. Importantly, the glycoproteins of WEE are more closely related to those of Sindbis than to those of Aura virus. From this we propose that an ancestral Sindbis-like virus present in the Americas (probably South America) diverged 1000-2000 years ago into a lineage that gave rise to Aura virus and a lineage that gave rise to Sindbis virus and to the Sindbis-like parent of WEE. At some time after this divergence, a Sindbis-like virus belonging to the latter lineage was transferred to the Old World where it gave rise to Sindbis viruses distributed throughout the Old World, and in a separate event a Sindbis-like virus belonging to the same lineage underwent recombination with EEE to give rise to WEE.

INTRODUCTION

Alphaviruses comprise a genus of 26 arthropod-borne viruses (reviewed in Strauss and Strauss, 1994). As a group they have a very wide distribution and many cause serious illness in man. The evolution of this group is being intensively studied as a model for the evolution of RNA viruses (Hahn et al., 1988; Levinson et al., 1989; Shirako et al., 1991; Weaver et al., 1991, 1992a,b, 1993, 1994), a topic made more interesting by the fact that several of the viral replicase proteins are homologous to proteins present in a number of plant viruses, including tobacco mosaic virus (Ahiquisti et al., 1985; Haseloff et al., 1984).

The alphavirus genome is a plus-stranded RNA approximately 12 kb in size (reviewed in Strauss and Strauss, 1994). The genome is translated into nonstructural proteins that form components of the viral replicase. A subgenomic RNA of about 4 kb is also produced in the infected cell and is translated into a capsid protein and two envelope glycoproteins used to assemble progeny virions. All alphaviruses sequenced to date share a minimum of 60% amino acid sequence identity in the nonstructural proteins and 45% identity in the structural proteins, and these viruses form a closely related and coherent taxon of animal viruses. Within the genus, the 26 viruses have been further grouped into seven complexes of more closely related viruses on the basis of serological cross-reactions (Calisher and Karabatsos, 1988). The rate of evolutionary divergence of alphaviruses has been found to be slow in comparison to other virus groups examined to date, perhaps as a result of the requirement for alternation in arthropod hosts and vertebrate hosts (reviewed in Weaver, 1994). Thus, although any two alphaviruses share extensive sequence identity, divergence of ancestral viruses into the current serological groups is estimated to have occurred thousands of years ago.

Aura virus is an alphavirus that has been isolated from Aedes serratus in Brazil and northern Argentina (reviewed in Rümenapf et al., 1994). Although the distribution of the virus is wide, the vertebrate host of the virus is unknown and no human illness is known to result from infection by the virus. The virus is serologically related to Sindbis virus and to Western equine encephalitis (WEE) virus, but is distinct from both (Calisher et al., 1988). We previously showed that WEE is a recombinant virus in which most of the genome is derived from Eastern equine encephalitis (EEE) virus and only the two viral glycoprotein genes and part of the 3' nontranslated region (NTR)

† Present address: Federal Research Center for Animal Diseases, P.O. Box 1149, D-72001 Tübingen, Germany.
² To whom reprint requests should be addressed.
are derived from a Sindbis-like virus, and thus the serological relationship of WEE to Sindbis is a function solely of the viral glycoproteins (Hahn et al., 1989). Because Sindbis virus is an Old World alphavirus, whereas WEE and EEE are found only in the Americas, we are investigating South American viruses that belong to the WEE/Sindbis virus serological complex in order to clarify the status of Sindbis-like viruses in the New World.

We previously found that Aura virus, unlike other alphaviruses, packages the subgenomic RNA into virus-like particles (Rümenapf et al., 1994, 1995). The assembly of Aura appears to be less precise than that of other alphaviruses, and the specific infectivity of Aura virions is low. In this report, we present the entire nucleotide sequence of Aura virus and show that it is not a recombinant like WEE or is it an immediate parent of WEE. Rather it is a New World representative of Sindbis-like viruses. The extent of sequence divergence between Aura, WEE, and Sindbis make clear that Aura virus and the Sindbis-like parent of WEE diverged long ago and that the immediate parent of WEE has yet to be identified. These sequence divergences support the hypothesis that the alphaviruses originated in the Americas (Levinson et al., 1990) and suggest that Sindbis virus was transferred to the Old World less than 2000 years ago.

**MATERIALS AND METHODS**

**Cells and virus**

Propagation of Aura virus prototype strain BeAR 10315 has been previously described (Rümenapf et al., 1994).

**Preparation of Aura cDNA libraries**

Preparation of cDNA libraries made to Aura virus RNA was essentially as previously described (Rümenapf et al., 1994). A cDNA library was first made by priming with oligo(dT). This library consisted primarily of clones with about 4-kb inserts complementary to 265 RNA. From the sequence of 2 of these clones, A44 (4.1 kb in length) and A412 (4.2 kb in length), an oligonucleotide called Au1 that was complementary to Aura nt 7639–7654 was synthesized and used to prime cDNA synthesis on Aura RNA; ds cDNA produced that was >7 kb in length was cloned to generate a new library. This new library was screened with a nick-translated XbaI–SspI fragment (nt 55–503) from Sindbis virus cDNA clone Toto57 (a gift from R. J. Kuhn) and 11 clones were obtained, the 2 longest being 7.6 (clone A713) and 7.4 kb (clone A76).

**Sequencing of Aura cDNA clones**

Clones A44 and A412 were sequenced by production of unidirectional nested deletions. The clones were digested with SacI and BanHII followed by treatment with ExoIII and Mung bean nucleases from Stratagene, following the manufacturer’s instructions but using half the re-action volumes. Incubation times between each step of 40 sec at 37° produced deletions that averaged 250 nucleotides. The truncated plasmids were cyclized by ligation and transformed into XL-1Blue cells, and the sizes of the inserts in the resulting clones were analyzed by restriction enzyme digestion. Clones containing inserts with suitable deletions were sequenced using the Sequenase II kit (USB) and [32P]dATP (NEN).

Clone A76 was sequenced by obtaining random subclones of the insert. For this, 50 µg of DNA from clone A76 was digested with NotI to release the 7.6-kb insert, and the insert was isolated by agarose gel electrophoresis. The insert DNA was concatemered with treatment with 20 U of T4 DNA ligase, and the resulting concatenated DNA was fragmented by shearing in a cupshorn sonifier (Branson) at 150 W for 3 x 30 sec at 4°C. The resulting fragments (average size 300 bp) were treated with Escherichia coli DNA polymerase Klenow fragment (0.1 U/µg DNA) and cloned into EcorV-digested pBluescriptII (SK) plasmid. White colonies were picked and tested for the presence of inserts, and 48 suitable clones were sequenced with pBluescript-specific primers (KS and SK), in this way 18 kb of nucleotide sequence was determined (2.3-fold oversampling) which represented 84% of the sequence of A76. Gaps in the sequence were filled by using synthetic oligonucleotides as primers to sequence A76 DNA. We found that the randomly selected fragments were unevenly distributed in the clones sequenced, with some regions well represented and others only poorly represented.

The sequence of A76 was confirmed by sequencing one strand of clone A713 in its entirety, using the deletion approach described for clones A44 and A412. The insert in A76 was released by treatment with NotI and cleaved into two fragments with ScaI. Each of the two fragments (3.4 kb representing the 5’ part of the insert and 4.2 kb representing the 3’ part of the insert) was subcloned into a ScaI/NotI-digested pBluescript (SK) plasmid. The two resulting subclones were used to generate nested deletions, using NotI and ApaI digests so as to obtain unidirectional deletions.

To further confirm the sequence resulting from these various clones, an additional cDNA clone (A12/21) was obtained from a fragment prepared by reverse transcriptase-PCR on Aura genomic RNA using as primers Au21 (nt 9983–10002) and Au12 (5063–5068) (PCR for 30 cycles, 94°C for 30 sec, 55°C for 30 sec, 72°C for 150 sec). The resulting 4.9-kb fragment was ligated to EcoRI/NotI adapters and inserted into EcoRI-digested pBluescript (SK) plasmid. Restriction enzyme analysis and partial sequencing of this clone gave results identical to those obtained with the other clones.

**Sequence of the 5’ end of Aura RNA**

The 5’ end of Aura genomic RNA was sequenced directly using a synthetic primer Au23 (nt 301–320) and
reverse transcriptase (Hahn et al., 1989). The sequence was clear but ceased exactly at nt 56, the most downstream nucleotide of the stem-loop structure predicted at the 5' end (see Results). Use of higher temperatures or two other primers (A4, nt 86–106, and A26, nt 58–79) was not successful in extending the sequence. To obtain the 5' end sequence, cDNA clones containing this sequence were prepared as follows. cDNA was made using 

AU23 as primer and 5 U Superscript reverse transcriptase (BRL) at 45°C. The cDNA was purified by ethanol precipitation and the 3' end was extended with 1 U of terminal deoxynucleotidyl transferase in the presence of 0.1 mM dGTP or dATP for 30 min. The tailed cDNA was used as a template for PCR using as primers AU23 and either oligo(dC) or oligo(dT) (12- to 15mers) as appropriate [30 cycles; 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec for oligo(dC); 94°C for 30 sec, 37°C for 30 sec, 72°C for 30 sec for oligo(dT)]. PCR products were flush ended by treatment with T4 DNA polymerase and inserted into an EcoRV-digested pBluescript plasmid. Of the resulting clones examined, two clones from dG-tailed cDNA had the longest inserts and both gave as the 5' end of the Aura genome the sequence 5' AUAGCGGAC. . . 3'.

RESULTS

Sequence of Aura virus RNA

The complete translated nucleotide sequence of Aura virus RNA is presented in Fig. 1. The genome organization of the virus is similar to that of Sindbis virus and the other alphaviruses (Fig. 2). Following the 5' NTR of 77 nucleotides is a reading frame of 7497 nucleotides that encodes the nonstructural proteins. This reading frame is entirely open except for a leaky opal termination codon between nsp3 and nsp4, as is the case for most alphaviruses. The nontranslated nucleotides between the nonstructural and structural domains number 53. The length of the open reading frame encoding the structural proteins is 3732 nucleotides and the length of the 3' NTR is 465 nucleotides, for a total genome length of 11,824 nucleotides.

The nonstructural proteins

The amino acid sequences of the nonstructural proteins of Aura are compared to those of alphaviruses belonging to four serological complexes in Fig. 2. Aura virus is most closely related to Sindbis virus but differs significantly from it. The amino acid sequence identity in the nonstructural proteins between Aura and Sindbis (73%) is the same as that between Venezuelan equine encephalitis (VEE) and EEE, which are estimated to have separated from each other 1400 years ago (Weaver et al., 1992a). Aura shares about 60% sequence identity with VEE, EEE, or Semliki Forest (SF), similar to the sequence identity between VEE and SF.

Within the nonstructural proteins, the level of sequence identity between Aura and Sindbis is not uniform. The identity in nsp1 is 78%, in nsp2 is 69%, in the conserved N-terminal domain of nsp3 is 68%, and in nsp4, the highly conserved RNA polymerase of alphaviruses, is 80%.

Alphavirus nsp3s contain two domains, a conserved N-terminal domain and a nonconserved C-terminal domain (Strauss et al., 1988). The latter is not conserved as to sequence or length among different alphaviruses, and only in very closely related strains of an alphavirus is sequence identity present. In the case of Aura virus and Sindbis virus, there is no detectable sequence identity in this domain of nsp3, although these domains are similar in size in the two viruses (219 residues in Aura and 231 residues in Sindbis). This lack of sequence conservation is particularly striking in view of the fact that the repeated sequence elements in the 3' NTR, which are not conserved between different alphaviruses although they are conserved among strains of the same virus, are conserved between Aura and Sindbis, as described below. The function of the nonconserved C-terminal domain of nsp3 is unknown, but the lack of conservation suggests that the precise sequence of this domain is irrelevant to its function. Mutagenesis studies in Sindbis (LaStarza et al., 1994a,b) and in VEE (Davis et al., 1993) have shown that many changes in this region, including insertions, substitutions, and large deletions, are tolerated. Deletion of most of this domain in Sindbis resulted in a viable virus whose RNA synthesis rate was depressed early after infection (LaStarza et al., 1994a), suggesting that this domain functions in RNA synthesis but is not absolutely required for RNA replication. This domain is rich in serine residues and is thought to bear most or all of the phosphate residues present in nsp3 (LaStarza et al., 1994a; Li et al., 1990).

The structural proteins

The structural proteins of Aura are also compared to those of alphaviruses belonging to four serological complexes in Fig. 2. The structural proteins of Aura virus are most closely related to those of Sindbis virus, with sequence identities of 77% in the capsid protein, 56% in glycoprotein E2, and 61% in glycoprotein E1, for an overall average identity of 62%. The sequence identity between Aura and Sindbis is slightly greater than that between VEE and EEE, which share 58% identity in the structural protein region.

Also shown in the figure are comparisons of Aura structural proteins with those of WEE. The Aura glycoproteins are distinct from those of WEE and, in fact, are less closely related to WEE than are the glycoproteins of Sindbis to those of WEE. Thus, Aura could not have served as the immediate parent of WEE. Furthermore, the fact that Sindbis is more closely related to WEE than to Aura suggests that a Sindbis-like ancestor of Aura and
FIG. 1. Complete translated nucleotide sequence of Aura RNA. The beginning of each protein encoded in the genome is indicated. Nucleotides are numbered from the 5' end of the genome and amino acids are numbered from the start of each protein.
Sindbis, present in the Americas, separated into the Aura lineage and the Sindbis (and WEE) lineage before the transfer of a Sindbis-like virus to the Old World. These sequence comparisons also make clear that Aura is not a recombinant virus like WEE but a Sindbis-like virus.

The serological relationships between Aura, Sindbis, and WEE are the result of sequence relationships in the glycoproteins. An important set of antigenic epitopes responsible for virus neutralization has been shown to be largely or totally contained in the E2 domain between residues 175 and 220 (Strauss et al., 1991; Weng and Strauss, 1991). This domain is compared for Sindbis, Aura, and WEE in Fig. 3; also shown are the sequences...
of alphaviruses belonging to three other serological complexes. The three viruses belonging to the Sindbis complex have a carbohydrate chain attached to residue 197 (Aura numbering) which is present in a different position in other alphaviruses. They also share extensive sequence identity in the region; particularly striking is the sequence from 194–216, which is highly conserved in the viruses of the Sindbis complex but different in the viruses belonging to other serological complexes. The fact that all three cysteines in this domain, as well as one glycine and one valine, are invariant in all alphavirus sequences suggests that all alphavirus E2s have the same structure in this region but that different epitopes are exposed on the surface of the virus.

The 5’ NTR

Alphavirus 5’ NTRs are known to have important regulatory signals for replication and translation of the viral RNA. The 5’ NTRs of Sindbis virus and Aura virus are compared in Fig. 4. Mutational studies of Sindbis showed that virus replication is sensitive to changes in the first 44 nucleotides of the 5’ NTR, which can be folded into a stem–loop structure as shown, but that the bases between this stem–loop structure and the start codon for nsP1 could be deleted without apparent effect upon virus replication (Nies ters and Strauss, 1990a). This stem–loop structure is postulated to serve as a promoter at the 3’ end of the minus strand for initiation of plus-strand RNA on the minus-strand template. There is only modest sequence identity between Aura and Sindbis RNAs in the 5’ NTRs but the stem–loop structure is highly conserved. Several conserved features in the stem–loop structures are noteworthy. The first stem begins in each case with two or three AU pairs and then becomes very GC rich. This stem contains an unpaired A residue in both viruses. The second stem, shown projecting to the left in the figure, is AU rich, in contrast to the first stem. A third short stem terminates in an A-rich loop (shown pointing upwards in the figure) that contains the sequence AAUYAA.

The sequence downstream of the stem–loop structure, which as described above can be deleted in Sindbis virus without apparent effect, exhibits similarities in the two viruses. The sequence ACUACCAUCA present in Sindbis is also present in the Aura sequence but in three clusters separated by nucleotides not found in the Sindbis sequence.

Repeated sequences in the 3’ NTR

The 3’ NTRs of alphavirus RNAs contain two domains. The highly conserved 3’-terminal 19-nucleotide se-
3’ NTRs of other alphaviruses illustrating the location of repeated elements and the fact that these elements differ in sequence, location, length, and number of repeats among different viruses. The fact that Sindbis and Aura share these repeated elements is all the more informative because two Old World viruses, SF and Ross River (RR) viruses, which are even more closely related than are Sindbis virus and Aura virus (Figs. 2 and 3), have entirely unrelated repeated elements, as illustrated schematically in Fig. 6 and whose sequences are shown in Figs. 5B and 5C. O’Nyong-nyong (ONN) is also a member of the SF complex of alphaviruses and its repeated elements are again different (Fig. 6).

It is of some interest that EEE contains repeated sequence elements that are related to parts of the Sindbis and Aura repeats (Figs. 5A and 6). There are four copies of a repeated element which is related to the 3’-terminal part of the Sindbis repeated elements. A portion of the 5’ part of the Aura/Sindbis sequence element is also present in EEE in a single copy, so that one copy of an element that is very similar to the Sindbis repeat is present in EEE. We can speculate that the presence of this element in EEE made possible the recombination between EEE and a Sindbis-like virus to give rise to a virus (EEE) able to persist in nature.

Apart from the repeated elements and the 3’-terminal 46 nucleotides of Aura, the sequences in the 3’ NTRs of Aura, WEE, and Sindbis appear unrelated except for a short sequence just downstream of the last repeat elements of Aura and WEE (U_{20}GUCACA . . . UAUCA-YAYUA). The lack of sequence conservation outside of the repeated elements suggests that the repeated elements must perform an important function in viral replication.

**Conserved sequence elements present in Aura**

Three sequence elements whose nucleotide sequences are highly conserved in all alphaviruses have been described (reviewed in Strauss and Strauss, 1994). The sequences of the three elements in Aura are shown in Fig. 7 and compared with the Sindbis elements.

The 51-nt element shown in Fig. 7A begins at residue 173 of the Aura genome; the sequence of the Aura element differs at only 2 nucleotides (boxed in Fig. 7A) from the Sindbis sequence; these differences are found in the loop regions of two stem–loop structures that can be formed. This sequence element is present in all alphaviruses; there is a maximum of 6 nucleotide differences between any two alphaviruses examined to date, with most of the changes occurring in the first loop, in the sequence between the two stems, or in the first nucleotide of the second loop, consistent with the differences seen between Aura and Sindbis. Mutagenesis studies have shown that most single nucleotide substitutions introduced into the element have drastic effects upon virus replication (because the sequence is coding, only
Fig. 5. Sequences of repeated elements in alphavirus 3' NTRs. (A) The three aligned repeats in Aura compared with the corresponding elements in Sindbis (three copies), WEE (two copies), and EEE (one copy of the 5' part of the element and four copies of the 3' part of the element). The element appears to consist of two domains separated by nucleotides that are less well conserved, only one of which is repeated in EEE. (B) The sequence of the second of two copies of a repeated element in SF. (C) This first of four repeats in Ross River (RR) virus. The underlined nucleotides are invariant in all four copies of the RR repeat and in all three copies of the related element in Getah virus. The RR repeat appears to consist of two core elements that are very highly conserved and separated by a few nucleotides exhibiting some variation, analogous to the case for the repeats shown in A. Downstream of these two core elements in RR is a set of A stretches that are conserved in all cases the numbering begins with the first nucleotide of the 3' NTR (i.e., with the first nucleotide of the termination codon that ends the structural protein ORF), and the symbols on the right are used to illustrate the corresponding repeat in Fig. 6. Two domains of the sequences in A are indicated separately, as shown. Sequences are from Ou et al. (1982), Hahn et al. (1986), and Faragher et al. (1986).

Fig. 6. Schematic illustration of the repeated elements in alphavirus 3' NTRs. The downward pointing arrow indicates the beginning of the 3' NTR and the solid box the 19- to 23-nucleotide conserved sequence element at the 3' end of the RNA just before the poly(A) tract. The different repeated elements are indicated by different symbols, which are the same as in Fig. 5. Note that two of the elements in RR and two in ONN overlap. A scale in nucleotides (Nts) is shown below.
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FIG. 7. Conserved nucleotide sequence elements in Aura RNA. The sequences of three conserved sequence elements are shown. The 5'-nucleotide element (A) can be folded into two stem-loop structures as shown. Numbering is from the 5' end of the genome. The subgenomic promoter (B) surrounds the beginning of the subgenomic RNA (rightward pointing arrow); numbering is from the 5' end of the genome. (C) The sequence of the conserved 19-nt element at the 3' end of the RNA, just before the poly(A) tract. In all cases the sequences are identical to the corresponding sequences in Sindbis virus except for the boxed nucleotides.

A limited set of substitutions that did not lead to a coding change could be examined (Niesters and Strauss, 1990b), and although the exact function of this element is not known, it is clear that the precise sequence of this element is required for efficient viral replication. A similar element is also found in rubella virus, although a stem-loop structure cannot be formed in this case (reviewed in Frey, 1994).

A 24-nt element positioned around the start site of the subgenomic RNA has been shown to function as the promoter for synthesis of this RNA (Levis et al., 1990). The Aura promoter differs at only 2 nucleotides from the Sindbis promoter (Fig. 7B), and both differences are at the ends of the promoter element. There are up to 7 nucleotide differences between the promoters of any two alphaviruses examined to date, and these differences have been found to result in differences in the ability of the Sindbis replicase to utilize different alphavirus promoters for subgenomic RNA synthesis (Hertz and Huang, 1992). A related promoter is found in rubella virus (Frey, 1994).

The 19-23 nucleotides at the 3' end of the alphavirus genome constitute another conserved sequence element. This element is 19 nucleotides in Aura and differs at only 1 nucleotide from the Sindbis element (Fig. 7C). This element is believed to function as a promoter for the initiation of a minus-strand copy of the plus-strand genome. Mutagenesis studies have shown that the precise sequence of this element is important for RNA replication (Kuhn et al., 1990).

The sequence immediately upstream of the 19-nt 3'-terminal element is AU rich but not otherwise conserved among alphaviruses (Ou et al., 1982). The sequence from position 20 to 60 in the Aura RNA (beginning with the 3' terminus) is conserved between Aura and Sindbis (alignment requires the insertion of three A residues in the Sindbis sequence and there are four additional mismatches) (Rümenapf et al., 1994), after which the Aura and Sindbis sequences appear to be unrelated except for the repeat elements described above.

DISCUSSION

The Sindbis-like viruses are worldwide in distribution. Viruses called Sindbis have been isolated from Europe, Africa, Asia, the Philippines and Australia, and a related virus, Whataroa virus, has been isolated from New Zealand (Niklasson, 1988; Olson and Trent, 1985; Rentier-Delrue and Young, 1980; Shirako, 1994; Shirako et al., 1991; Strauss and Strauss, 1994). Our current results extend the range of the Sindbis-like viruses to South America. Although the sequence identity between Sindbis and Aura viruses is not particularly high, being less than the sequence identity between SF and RR and about the same as the identity between EEE and VEE, the conserved structures and sequence elements characteristic of alphaviruses are almost identical between Sindbis and Aura. Important antigenic epitopes on the glycoproteins are conserved between the two viruses, and Aura and Sindbis share repeated sequence elements in the 3' NTR. Thus the promoters in the viral RNAs used for RNA replication and packaging have been conserved, and the sharing of antigenic epitopes results in extensive serological cross-reaction despite fairly extensive sequence divergence. The conservation in sequence and number of the repeated elements in the 3' NTRs of the two viruses is remarkable in view of the fact that the repeated elements in RR and SF appear to be unrelated (Fig. 6) even though these viruses are more closely related than are Aura and Sindbis (Fig. 2), and the number of repeats is not conserved in different strains of RR (Faraghor and Dalgarno, 1986). The repeated sequence elements in the 5' NTR of Sindbis appear, in fact, to be a hallmark of the Sindbis-like viruses. There are precisely three copies in all of the strains of Sindbis virus and its relatives sequenced to date (Ou et al., 1982; Shirako et al., 1991) and these repeated elements are different from those found in all other alphaviruses examined, with the exception of EEE, which shares part of the element with Sindbis (Fig. 6). Why this sequence element should have been conserved in all of the Sindbis-like viruses when it is otherwise so variable in different alphaviruses is a mystery but may be related in some way to
the ecology of the viruses. The function of these repeated elements is not known but it may be to bind cellular proteins and to regulate viral RNA translation. Deletion of the three repeated elements from Sindbis virus results in a virus that grows moderately well in chicken cells, that is, its growth rate is within an order of magnitude of the growth rate of the wild-type virus, but which grows very poorly in mosquito cells, being delayed by 4 hr in its growth and yielding virus at a rate that is 2 to 3 orders of magnitude less than that of the parental virus (Kuhn et al., 1990). Because of the difference in the effect of such deletions upon the replication of virus in different cell lines, we propose that they function to bind cellular proteins. Because the repeated elements are not found in defective-interfering RNAs which replicate vigorously but which are not translated, we have suggested that the function of these repeated elements is to regulate RNA translation (reviewed in Strauss and Strauss, 1994). If so, regulation of translation may be more critical in mosquito cells than in vertebrate cells.

The sequencing data make clear that Aura virus is Sindbis-like and not a recombinant like WEE. We have previously suggested that alphaviruses originated in the New World and were transferred twice to the Old World, once to found the Sindbis group of viruses and once to found the Semliki Forest group of alphaviruses (Lavinson et al., 1990). Our findings that Aura virus is a Sindbis-like virus found in the New World is consistent with this hypothesis. Weaver et al. (1992a) estimated that VEE and EEE diverged about 1400 years ago, based upon the extent of nucleotide sequence divergence between these two viruses and the measured rates of (recent) nucleotide sequence divergence in EEE (1.4 × 10^{-4} substitutions/nucleotide/year) (Weaver et al., 1994) and VEE (5 × 10^{-4} substitutions/nucleotide/year) (Weaver et al., 1992a) populations in the Americas. The fact that Sindbis and Aura viruses have diverged to about the same extent as have VEE and EEE suggests that Sindbis and Aura diverged about the same time as VEE and EEE and thus, given the uncertainties in such calculations, that Sindbis and Aura viruses diverged 1000–2000 years ago. Because Sindbis is more closely related to WEE than it is to Aura, it appears, if our hypothesis is correct, that the ancestor to Sindbis virus existed in the Americas for some period after the Sindbis and Aura lineages diverged and, thus, that the transfer of Sindbis to the Old World occurred more recently than the Sindbis–Aura divergence. However, the fact that Sindbis is very widely distributed in the Old World and that it has diverged into a number of distinct lineages (Shirako et al., 1991; Rentier-Delrue and Young, 1980) also suggests that the transfer took place shortly after the divergence of the Sindbis lineage from the Sindbis-like ancestor of WEE.

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