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Deduced Consensus Sequence of Sindbis Virus Strain AR339: Mutations Contained in Laboratory Strains Which Affect Cell Culture and In Vivo Phenotypes

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The consensus sequence of the Sindbis virus AR339 isolate, the prototype alphavirus, has been deduced. The results presented here suggest (i) that a substantial proportion of the sequence divergence evident between the consensus sequence and sequences of laboratory strains of AR339 has resulted from selection for efficient growth in cell culture, (ii) that many of these changes affect the virulence of the virus in animal models, and (iii) that such modified genetic backgrounds present in laboratory strains can exert a significant influence on genetic studies of virus pathogenesis and host range. A laboratory strain of Sindbis virus AR339 was sequenced and cloned as a cDNA (pTRSB) from which infectious virus (TRSB) could be derived. The consensus sequence was deduced from the complete sequences of pTRSB and HR_{sp} (E. G. Strauss, C. M. Rice, and J. H. Strauss, Virology 133:92–110, 1984), from partial sequences of the glycoprotein genes of three other AR339 laboratory strains, and by comparison with the sequences of four other alphaviruses closely related to Sindbis virus. The sequence of neither HR_{sp} nor TRSB was representative of the consensus Sindbis virus AR339 sequence. HR_{sp} differed from the consensus sequence by eight coding changes, and TRSB differed by three coding changes. In the 5′ untranslated region, HR_{sp} differed from the consensus sequence at nucleotide (nt) 5. These differences were likely the result of cell culture passage of the original AR339 isolate. At three of the difference loci (one in TRSB and two in HR_{sp}), selection of cell-culture-adaptive mutations was documented with Sindbis virus or other alphaviruses. Selection in cell culture often results in attenuation of virulence in animals. Considering the TRSB and HR_{sp} sequences together, one noncoding difference from the consensus (an A-for-G substitution in the 5′ untranslated region at nt 5) and six coding differences in the glycoprotein genes (at E2 amino acids 1, 3, 70, and 172 and at E1 amino acids 72 and 237) were at loci which, either individually or in combination, significantly affected alphavirus virulence in mice. Although the levels of virulence of isogenic strains containing either nt 5 A or nt 5 G did not differ significantly in neonatal mice, the presence of nt 5 A greatly enhanced the effect of a second attenuating mutation in the E2 gene. These results suggest that minimal differences in the "wild-type" genetic background into which an additional mutation is introduced can have a dramatic effect on apparent virulence and pathogenesis phenotypes. A cDNA clone of the consensus AR339 sequence, a sequence devoid of occult attenuating mutations introduced by cell culture passage, will allow the molecular genetic examination of cell culture and in vivo phenotypes of a virus which may best reflect the sequence of Sindbis virus AR339 at the time of its isolation.

The genetic instability of RNA virus genomes reflects the error rate of viral RNA-dependent RNA polymerases. The high error rate of these enzymes, estimated at 10^{-3} to 10^{-4} substitutions per nucleotide (64), combined with the general absence of proofreading activity, implies that RNA virus preparations represent collections of variants and that the nucleotide sequence which defines an RNA virus is the consensus sequence of the population (15). In nature, sequence variability is limited by the constraints of the natural virus life cycle, which are imposed upon the variant population and which cause only a narrow range of most-fit sequences to be selected from that population. After isolation in the laboratory, however, a new set of environmental constraints is imposed on virus growth. In the case of Sindbis virus, an alphavirus which is maintained in nature by alternating cycles of growth in mosquito vectors and predominantly avian vertebrate hosts, laboratory maintenance has historically involved repeated passage in suckling-mouse brains and/or cell cultures of avian or rodent origin. Under these artificial conditions, it is likely that alternative sequences, other than those favored by the natural history of the virus, will be selected as most fit.

This circumstance presents a distinct challenge for the propagation of live virus vaccine strains and for investigations of RNA virus host range and pathogenesis in that these are precisely the phenotypes which are most likely to be affected by selection under laboratory conditions (25, 39, 46, 49). To minimize selection in culture, positive-sense RNA genomes, like that of Sindbis virus and other alphaviruses, have been converted to a genetically more stable form in cDNA clones (1, 9,

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22, 24, 37, 40). Low-passage virus is recovered after transfection of RNA genomic replicas transcribed in vitro from complete cDNA templates. However, the problem of laboratory selection cannot be resolved simply by enshrining the sequence of a laboratory strain as a cDNA clone.

The objectives of the experiments reported here were to predict the consensus sequence of the prototype Sindbis virus isolate, strain AR339, to identify mutations introduced during laboratory maintenance of this isolate, and to determine the origins and phenotypes of some of these mutations. These results will allow the construction of a complete clone of Sindbis virus AR339 containing the predicted consensus sequence.

Sindbis virus is the type species of the *Alphavirus* genus in the family *Togaviridae* (reviewed in references 50, 59, and 60) and is the prototype of the Sindbis virus-like superfamily. Studies of this virus have played a central and sometimes leading role in the characterization of RNA virus gene expression, genome replication, glycoprotein processing, and pathogenesis. The virus derives its name from Sindbis Village, a community in the Nile Delta where the prototype virus (designated AR339) was isolated in 1952 (61). The isolation was accomplished by inoculation of 3-day-old mice, either intracerebrally (i.c.) or intraperitoneally, with a suspension of triturated mosquitos captured with a light trap. Subsequent isolations were made from other mosquito pools and avian species in the region, and serosurveys suggested infection of a wide variety of domestic livestock, domestic and wild birds, and humans. Since its original identification, Sindbis virus strains have been isolated from South Africa, Europe, Asia, the Indian subcontinent, and Australia (32), and evidence has been obtained for a Sindbis or Sindbis-like virus in the Americas (12, 44). Therefore, the virus has a broad geographic distribution as well as a broad range of natural hosts.

In cell culture, the host range also is broad. The original isolate of AR339 rapidly produced cytopathic effects on chicken embryo fibroblast (CEF) cells (61), and Sindbis virus replication has been observed in primary cells or cell lines from multiple species (60).

The Sindbis virus genome is a single strand of positive-sense RNA composed of 11,703 nucleotides (nt), excluding the 5′ cap structure and the 3′ poly(A) tail (in the HR_{sp} strain [57]). The 5′ two-thirds of the genome contains the genes for the four nonstructural proteins, in the order nsP1 to nsP4, which catalyze the replication and transcription of the viral RNAs. The structural proteins, capsid, and two envelope proteins, E1 and E2, are encoded in the 3′ one-third of the genome and are translated from a 26S subgenomic mRNA transcribed from an internal promoter (41). The genome and 240 copies of the capsid comprise the icosahedral nucleocapsid of the virion. The nucleocapsid is surrounded by a lipoprotein envelope containing E1 and E2. Three E1/E2 heterodimers form each of 80 spikes which protrude from the virion and which are arranged in an icosahedral array reflective of the internal architecture of the nucleocapsid (30).

Virtually all of the available information on Sindbis virus molecular biology, structure, and pathogenesis has been obtained with a biologically cloned strain of AR339 (33) or the heat-resistant strain (HR) which was derived from it (5). HR was selected by multiple cycles of heat inactivation at 60°C for several minutes, followed by growth of the surviving virus on CEF cultures. HR was the progenitor for many of the temperature-sensitive mutants of Sindbis virus (5, 56). The first complete published sequence of the Sindbis virus genome was the sequence of a small-plaque variant of HR, HR_{sp} (57). HR_{sp} cDNA clones also were major constituents of the first complete cDNA clones of Sindbis virus (40). Therefore, HR_{sp} had undergone considerable passage and biological cloning in cell culture prior to being sequenced and molecularly cloned, suggesting that the HR_{sp} sequence, and that of cDNA clones derived from it, may contain significant changes relative to the sequence of the original AR339 isolate.

This issue is particularly relevant to the examination of alphavirus pathogenesis, as passage in cell culture tends to select for attenuated variants (18, 28), and minimal genomic changes, i.e., single nucleotide changes, can have profound effects on the in vivo phenotype of alphaviruses (6, 8, 20, 26, 34, 36, 51, 62, 63). In the experiments reported here, we have determined the sequence of our laboratory strain of AR339, constructed a cDNA clone of that sequence, and shown that infectious virus derived from the clone, upon inoculation into neonatal mice, reproduced the virulence phenotype of its biological parent. On the basis of comparisons with the sequence of other Sindbis virus strains, the consensus AR339 sequence was predicted. We have demonstrated that at least some of the differences between the predicted consensus AR339 sequence and those of existing laboratory strains arose from passage of the virus in cell culture and that these sequence differences have a significant effect on virulence in mice.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK) cells were obtained from the American Type Culture Collection in passage 52 or 53 and were used between passages 54 and 64. Primary CEF cells were obtained from 9-day-old embryonated eggs. BHK and CEF cells were maintained in Eagle's minimal essential medium with Earle's or Hank's salts, respectively, and with 10% donor calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). BHK medium contained 10% tryptose phosphate broth.

Sindbis virus strain AR339 was isolated from a pool of trapped mosquitos in Sindbis Village, Egypt (61). It was obtained in 1976 from H. R. Bose, University of Texas at Austin, who had obtained the virus previously from E. R. Pfefferkorn, Dartmouth Medical School (33). This virus had been maintained in the Bose laboratory as a low-passage stock on CEF cells and grew poorly on BHK cells. In 200-μl BHK cultures on 96-well plates, cytopathic effect was observed 5 to 6 days postinfection at a multiplicity of infection approaching 1 CEF PFU per well. The virus was cloned biologically by limiting dilution on BHK cells in a 96-well plate, and supernatant was recovered from a well infected at a high dilution and showing cytopathic effects within 48 h. This virus became our laboratory strain of Sindbis virus, designated SB. SB-RL (SB, reduced latent period), a prototype attenuated Sindbis virus strain, was isolated by passage of SB in BHK cells under selection for rapid virus growth (3). HR (Sindbis virus, heat-resistant strain) was derived from AR339 by selection for stability at 60°C (5). Viruses designated with a TR prefix were derived by in vitro transcription of the corresponding complete cDNA clone (which has a pTR prefix) and subsequent introduction of the transcripts into BHK cells (see below).

S.A.AR86 and Girdwood S.A. are South African isolates which were obtained from J. Dalrymple, U.S. Army Medical Research Institute for Infectious Diseases. Ockelbo82 is an isolate from Sweden, the sequence of which was determined by Shirako et al. (53). The sequence of Aura virus, a South American alphavirus, was determined by Rumenapf et al. (44).

General recombinant DNA methods. All DNA manipulations were carried out as described by Sambrook et al. (47). Nucleic acid modifying enzymes were obtained from Promega Biotec, Bethesda Research Laboratories, New England Biolabs, Boehringer Mannheim Biochemicals, and United States Biochemicals. DNA fragments used in cloning were purified from agarose gels with Gene Clean (Bio 101).

Construction of virus clones. The viral cDNA clones consist of a pBR322-based vector, an SP6 bacteriophage promoter, the complete genomic sequence represented as a cDNA, a poly(A) tract, and a unique restriction site for linearization of the plasmid prior to in vitro transcription of infectious genomic RNA replicas. The first alphavirus clone of this type, pToto1101, was constructed by Rice et al. (40) and consists of cDNA sequences derived from several laboratory isolates of Sindbis virus. Construction of pTRSB (a full-length cDNA clone of our laboratory Sindbis virus strain following the SP6 phage promoter) was accomplished by sequential replacement of SB-derived cDNAs into the pToto1101 plasmid background (Fig. 1). Production of the cDNAs used in constructing pTRSB (pSB1 to pSB11) has been described previously (34) as has the construction of pTR2000 (34, 35). Nucleotide numbering follows that of Strauss et al. (57).

The *Stu*I (nt 8571)-to-*Sac*II (nt 11484) fragment of pToto1101 was removed and replaced with the analogous fragment from clone pSB4 to form pTR2000, by using the loss of the pToto1101 *Stu*I site at nt 10770 (a site not present in SB) as

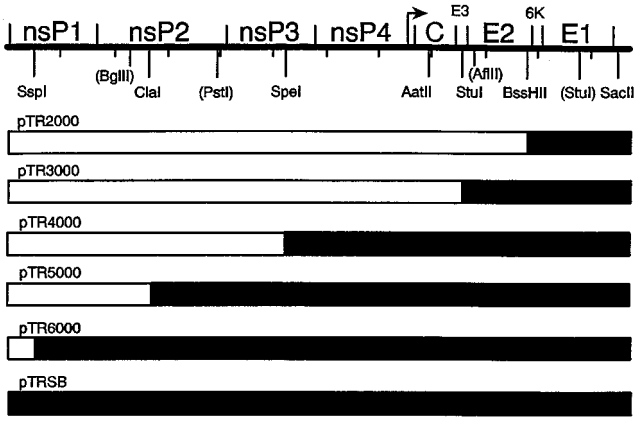

FIG. 1. Chimeric clones derived from pToto1101 and SB cDNAs. Filled bars, sequences derived from SB; open bars, sequences derived from pToto1101. pTRSB was assembled by sequential substitution of cDNAs derived from SB into pToto1101, starting at the 3′ end. The 3′ extent of SB cDNAs in all the constructs is the *Sac*II site. However, the sequence from the *Sac*II site to the end of the genome (nt 11485 to 11703) contains no sequence differences among HR_{sp}, pToto1101, and SB; this region is indicated as SB in origin on the figure. Unique restriction sites in pTRSB are *Cla*I, *Spe*I, *Aat*I, *Stu*I, *Bss*HII, and *Sac*II. There are four *Ssp*I sites in pTRSB, although only the one at nt 504 is shown.

a screen (Fig. 1). The sequences of SB and pToto1101 are identical from nt 11485 to the 3′ end (nt 11703); therefore, Fig. 1 indicates these 3′ sequences to be of SB origin. Construction of pTR3000 was accomplished by the replacement of the *Stu*I (nt 8571)-to-*Bss*HII (nt 9804) fragment of pTR2000 with the analogous fragment from pSB3. The *Afl*II site found at nt 8835 in pToto1101 but absent in SB was used to screen the recombinants. An SB fragment from pSB1, spanning sites *Spe*I (nt 5262) to *Bss*HII, was used to replace the *Spe*I-*Bss*HII fragment from pTR2000 with the *Afl*II site as a screen to form pTR4000. To construct pTR5000, pSB5 was subcloned into pUC119. The *Pst*I site at nt 3953 was ablated by site-directed mutagenesis (23) to change nt 3950 from a U to a C, a translationally silent mutation. The *Cla*I (nt 2713)-to-*Spe*I fragment was removed from the mutagenized subclone and used to replace the analogous fragment of pTR4000 with the ablated *Pst*I site as a screen.

Construction of pTR6000 involved the replacement of the *Ssp*I (nt 504)-to-*Cla*I (nt 2713) fragment from pSB10 into pTR5000 as follows. An *Eco*RV fragment of pSB10, derived by digestion at a site in the pBR322 vector upstream of the SB sequences and at SB nt 2750, was cloned into the *Sma*I site of M13mp19. The *Bgl*II site at nt 2288 was ablated by site-directed mutagenesis (nt 2288 A to G), which created a translationally silent restriction site polymorphism with which to screen subsequent recombinants. The mutagenized replicative form was digested with *Ssp*I and *Pvu*II, which removed a fragment from SB nt 504 (*Ssp*I) to the M13 *Pvu*II site 75 nt downstream of the SB sequence (the SB portion of the fragment ending at SB nt 2750). This fragment was cloned into the unique *Ssp*I site in the *amp* gene of pBR322, conserving the upstream *Ssp*I site (nt 504) but ablating the downstream *Pvu*II site. From this clone, the SB sequence (nt 504 to 2750) and the *tet* gene were removed by digestion with *Ssp*I (nt 504) and *Pvu*II (in pBR322 downstream of the SB and *tet* sequences). A second fragment was obtained from pToto1101 by digestion with *Ssp*I. This fragment extended from the *Ssp*I site in the pToto1101 *amp* gene, including the origin of replication and the SP6 promoter, to the *Ssp*I site at nt 504 of the pToto1101 Sindbis virus sequences. The two *Ssp*I fragments were ligated to create a plasmid containing a functional *tet* gene, an origin of replication, an SP6 promoter, Sindbis virus nt 1 to 504 derived from pToto1101, and SB nt 505 to 2750. This recombinant plasmid was digested with *Sac*I (a unique site in the vector immediately upstream of the SP6 promoter) and *Cla*I, and the resulting fragment was used to replace the corresponding fragment of pTR5000, yielding pTR6000. Thus, the Sindbis virus sequences of pTR6000 were composed entirely of SB sequence, except for nt 1 to 504 and 11485 to 11703, which were of pToto1101 origin.

As noted above, the sequences of nt 11485 to 11703 in pToto1101 and SB were identical. In their 5′ terminal 504 nt, there was only one sequence difference: an A at nt 5 in pToto1101 and a G at nt 5 in SB. Site-directed mutagenesis was used to make this change in pTR6000, converting it to pTRSB.

RNA and DNA sequencing. Sequence analyses of RNA and single-stranded DNA were performed by dideoxynucleotide chain termination on RNA or reverse transcription-PCR cycle sequencing methods (United States Biochemical Corp.) applied to uncloned reverse transcription-PCR fragments (2, 6, 14, 48). Double-stranded DNA derived from the pTRSB plasmid was sequenced by the Δ*Taq* cycle sequencing system (United States Biochemical Corp.). Any nucleotide differences from the published Sindbis virus HR_{sp} sequence (57) were confirmed by sequencing SB virion RNA.

In vitro transcription and RNA transfection. Linearization and transcription of full-length cDNA clones, transfection or electroporation of the transcripts, and assay for specific infectivities of the transcripts were performed as described previously (14, 24, 34). RNA transcripts from each of the constructs used in this study had specific infectivities similar to those of TRSB, indicating the absence of adventitious lethal mutations.

Passage of virus in BHK cells. A stock of virus derived by electroporation was diluted in phosphate-buffered saline (PBS) containing 1% calf serum to give an approximate multiplicity of infection of 0.3 PFU per cell in BHK cultures at approximately 80% confluency. Following a 60-min attachment period, additional medium was added and the cultures were incubated at 37°C for 18 h. Additional passages were performed in a similar manner except that virus harvested at each passage was diluted 1:15 for infection of the next group of cultures.

Measurement of penetration phenotype. Penetration was measured as the resistance of virus to neutralization by AR339-specific hyperimmune mouse ascites fluid according to the method described by Pence et al. (31) with some modifications. These modifications included the simultaneous quantitation of attached PFU and additional controls to show that all of the strains were neutralized by greater than 95% by hyperimmune mouse ascites fluid. Percent penetration was calculated by dividing the number of neutralization-resistant PFU present after a 15-min incubation at 37°C by the number of attached PFU and multiplying by 100.

Animal experiments. Unless noted otherwise, virus-containing transfection stocks were assayed for virulence in litters of 12- to 24-h-old CD-1 mice (Charles River Breeding Laboratories, Inc., Raleigh, N.C.). Animals were inoculated either subcutaneously (s.c.) or i.c. (in the right hemisphere) with 100 PFU of virus diluted in 50 μl of PBS–1% calf serum. Animals were observed for a period of 14 days postinoculation, after which percent mortality and average survival times (AST) were calculated for the animals that died. For the 50% lethal dose (LD_{50}) determinations, litters (at least 10 animals per dose) were inoculated either s.c. or i.c. with 10-fold dilutions of virus in PBS–1% calf serum. LD_{50} calculations based on a 14-day observation period were performed by the method of Reed and Muench (38).

RESULTS

The objectives of these experiments were (i) to determine the sequence of SB, our laboratory derivative of Sindbis virus AR339, (ii) to deduce the consensus sequence of AR339, and (iii) to explore possible origins of variation found between the laboratory strains and the consensus sequence. The consensus AR339 sequence was inferred by comparison of the SB sequence with the complete or partial sequences of HR_{sp} (the GenBank sequence [57]), SV1A and NSV (D. Griffin's AR339-derived laboratory strains [26]), and SIN (a laboratory-derived AR339 strain from A. Schmaljohn and J. Dalrymple, U.S. Army Medical Research Institute for Infections Diseases [7, 58]). Each of these viruses was descended from AR339. Where these sequences differed from each other, they also were compared with the amino acid sequences of other viruses closely related to Sindbis virus: Ockelbo82 (53), S.A.AR86 (45, 54; GenBank accession number U38305), Girdwood S.A. (54; GenBank accession number U38304), and the somewhat more distantly related Aura virus (44).

Sequence analysis of pTRSB. A full-length clone of our laboratory derivative of Sindbis virus AR339 was constructed by sequentially replacing portions of pToto1101 with cDNAs derived from reverse transcription of SB RNA, beginning at the 3′ end of the virus-specific sequences (Fig. 1). The sequence of pTRSB was determined and compared with the genomic sequence of the HR_{sp} Sindbis virus strain (57). Sequence differences between pTRSB and HR_{sp} were confirmed by direct sequencing of uncloned reverse transcription-PCR fragments derived from SB genomic RNA.

Nine of the 10 noncoding changes between HR_{sp} and pTRSB were within the Sindbis virus open reading frames (Table 1). Nucleotide changes at nt 2288 and 3950 were introduced into pTRSB by site-directed mutagenesis to provide restriction site polymorphisms as markers for future cloning experiments. The S.A.AR86 and Ockelbo82 nucleotides matched pTRSB at three sites (nt 353, 7337, and 10469),

TABLE 1. Translationally silent nucleotide differences between the sequence of Sindbis virus HR_{sp} and pTRSB

Nucleotide position[a]	Nucleotide difference (HR_{sp}→pTRSB)
5[b]	A→G[c]
353	C→U[c]
1844	A[c]→U
2288[d]	A→G
3950[d]	U→C
7337[e]	U→C[c]
8345[f]	C→A
9779	U[c]→C
10392	U[c]→C
10469	U→A[c]

[a] Nucleotide numbers follow Strauss et al. (57).
[b] The HR_{sp} nucleotide at this position is specific for the small-plaque isolate (57) and is not changed in other variants of HR. Mutation at this position has a demonstrable effect on virulence in mice.
[c] Nucleotides identical to both the S.A.AR86 and Ockelbo82 sequences at the equivalent position.
[d] Nucleotide changes in pTRSB by site-directed mutagenesis: a mutation at nt 2288 ablates a *Bgl*II site and a mutation at nt 3950 ablates a *Pst*I site.
[e] The change at this position is associated with a *Bam*HI restriction site in the HR_{sp} sequence which is not found in pTRSB.
[f] S.A.AR86 and Ockelbo82 have a G at this position.

matched HR_{sp} at three other positions (nt 1844, 9779, and 10392), and matched neither pTRSB nor HR_{sp} at nt 8345. The remaining noncoding difference was at nt 5 within the 5′ untranslated region (UTR) of the genome. HR_{sp} has an A at this position, while a G was present at nt 5 in pTRSB, S.A.AR86 (54), Girdwood S.A. (54), Ockelbo82 (53), and the HR large-plaque (HR_{lp}) strain (57).

Five nucleotide differences which predicted four amino acid substitutions were found in the nonstructural gene open reading frames (Table 2). In two instances (nt 2992 and 3579), the predicted pTRSB amino acids, nsP2 Leu-438 and nsP2 Glu-634, were in common with the equivalent positions in the S.A.AR86 and Ockelbo82 sequences, suggesting that pTRSB contained the consensus amino acids. A similar comparison at nt 5683 (nsP3 528) suggests that the HR_{sp} residue (Arg) represents the consensus sequence and that the pTRSB residue (Gln) is unique to this strain. Two nucleotide differences were found in nsP1 codon 441 (nt 1380 and 1381). In comparison with the other strains, pTRSB contained the consensus Ile residue common to S.A.AR86, Girdwood S.A., and Aura virus, whereas Ockelbo82 and HR_{sp} contain Ser and Cys, respectively.

Ten coding differences distinguished pTRSB, SV1A, NSV, SIN, and HR_{sp} in the structural gene open reading frame. The E2 His-55 mutation in NSV has been causally associated with enhanced neurovirulence in adult mice after in vivo selection for this property (11, 26). E2 Gln-55 was common to the other AR339-derived viruses and to Girdwood S.A., which are not neurovirulent for adult mice, as well as to S.A.AR86, which is neurovirulent in adult animals. At four codons (E2 3, 23, and 172 and E1 237), the HR_{sp} sequence was unique, with pTRSB, SV1A, NSV, SIN (in E2), S.A.AR86, Girdwood S.A., and

TABLE 2. Nucleotide 5 and amino acid sequence differences among Sindbis virus AR339 isolates compared with each other and with S.A.AR86, Girdwood S.A., Ockelbo82, and Aura virus

Nucleotide(s) (UTR or aa residue)[a]	Nucleotide or amino acid for virus or strain:									
	HR_{sp} (codon)[b]	TRSB (codon)[c]	SV1A[d]	NSV[e]	SIN[f]	S.A.AR86 (aa residue)[g]	Girdwood S.A.[g]	Ockelbo82 (aa residue)[g]	Aura virus (aa residue)[h]	Consensus AR339 sequence
5[i,j] (5′ UTR)	A	G	ND[k]	ND	ND	G	G	G	C	G
1380, 1381 (nsP1 441)	Cys (UGC)	Ile (AUC)	ND	ND	ND	Ile	Ile	Ser	Ile	Ile
2992 (nsP2 438)	Pro (CCC)	Leu (CUC)	ND	ND	ND	Leu	ND	Leu	Leu	Leu
3579 (nsP2 634)	Lys (AAA)	Glu (GAA)	ND	ND	ND	Glu	ND	Glu	His (633)	Glu
5683 (nsP3 528)	Arg (CGA)	Gln (CAA)	ND	ND	ND	Arg (514)	Arg	Arg (530)	ND[l]	Arg
8633[j,m] (E2 1)	Ser (AGC)	Arg (AGA)	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
8638[j,m] (E2 3)	Ile (AUU)	Thr (ACU)	Thr	Thr	Thr	Thr	Thr	Thr	Pro	Thr
8698[i] (E2 23)	Val (GUA)	Glu (GAA)	Glu	Glu	Glu	Glu	Glu	Glu	Glu (24)	Glu
8793[j] (E2 55)	Gln (CAA)	Gln (CAA)	Gln	His	Gln	Gln	Gln	Lys	Arg (56)	Gln
8838[j,m,n] (E2 70)	Lys (AAG)	Glu (GAG)	Lys	Lys	Glu	Glu	Glu	Glu	Gly (71)	Glu[o]
9144[j,m] (E2 172)	Arg (AGA)	Gly (GGA)	Gly	Gly	Gly	Gly	Gly	Gly	Gly (173)	Gly
9255[p] (E2 209)	Gly (GGA)	Gly (GGA)	Arg	Gly	Gly	Gly	Gly	Gly	Gly (210)	Gly
9276[p] (E2 216)	Glu (GAA)	Glu (GAA)	Glu	Glu	Lys	Glu	Glu	Glu	Lys (217)	Glu
10279[j,m] (E1 72)	Ala (GCU)	Val (GUU)	Val	Ala	ND	Ala	ND	Ala	Glu	Ala
10773[j,n] (E1 237)	Ser (UCA)	Ala (GCA)	Ala	Ala	ND	Ala	Ala	Ala	Pro	Ala

[a] Nucleotide numbers follow Strauss et al. (57). aa, amino acid.
[b] HR strain isolated by Burge and Pfefferkorn (5) and sequenced by Strauss et al. (57).
[c] Virus derived from pTRSB, a cDNA clone of the Johnston laboratory strain of Sindbis AR339, and sequenced as reported here.
[d] Virus obtained from ATCC by the Griffin laboratory and sequenced by Lustig et al. (26).
[e] Virus derived by alternating passages in brains of neonatal and adult mice by Griffin and Johnson (11) and sequenced by Lustig et al. (26).
[f] Laboratory strain of A. Schmaljohn and J. Dalrymple; sequenced by Davis et al. (7) and Strauss et al. (58).
[g] South African (S.A.AR86 and Girdwood S.A.) and Swedish (Ockelbo82) isolates supplied by J. Dalrymple, U.S. Army Medical Research Institute for Infectious Diseases. Sequence obtained from Russell et al. (45) and Simpson and Johnston (54; GenBank accession number U38305) for S.A.AR86, from Simpson and Johnston (54; GenBank accession number U38304) for Girdwood S.A., and from Shirako et al. (53) for Ockelbo82.
[h] Sequence determined by Rumenapf et al. (44).
[i] The HR_{sp} nucleotide is specific for the small-plaque isolate of the HR strain (57).
[j] A mutation in nucleotides or codons at this position in Sindbis virus or other alphaviruses has a demonstrable effect on virulence in mice (this report and references 8, 13, 26, 35, 62, 63).
[k] ND, not determined.
[l] Divergence at the carboxy end of nsP3 makes a comparison unmeaningful.
[m] A mutation in nucleotides or codons at this position significantly affects early events in alphavirus infection (this report, references 4, 18, 62, and data not shown).
[n] Changes at this position are associated with restriction sites in the HR_{sp} sequence and are not found in pTRSB: *Afl*II (nt 8838) and *Stu*I (nt 10773).
[o] As Lys is rapidly selected during cell culture passage, Glu appears to be the consensus E2 70 amino acid.
[p] Variations at this position are associated with changes in reactivity with neutralizing monoclonal antibodies to the E2b site (7, 27).

TABLE 3. Virulence of Toto1101/TRSB chimeric viruses

Virus	Nucleotides of SB sequence[a]	Mice[b] inoculated s.c.		Mice inoculated i.c.	
		% Mortality[c]	AST ± SD[d]	% Mortality	AST ± SD
Toto1101	NA[e]	30.4 (7/23)	7.3 ± 0.5	61.9 (13/21)	7.2 ± 0.8
TR2000	9804–11703	100 (2/22)	3.4 ± 1.4	100 23/23)	3.8 ± 1.8
TR3000	8571–11703	100 (0/20)	4.5 ± 1.1	100 27/27)	2.4 ± 0.9
TR4000	5262–11703	100 (0/20)	4.0 ± 1.1	100 22/22)	2.2 ± 0.4
TR5000	2713–11703	100 (3/23)	4.0 ± 1.1	100 21/21)	3.0 ± 0.7
TR6000	504–11703	100 (9/89)	3.9 ± 1.2	100 70/70)	3.7 ± 1.5
TRSB	1–11703	100 (1/41)	3.0 ± 0.7	100 84/84)	2.2 ± 1.1

[a] TR2000 and subsequent TR clones contain nt 11485 to 11703 derived from Toto1101. However, there are no differences in this region between the sequence of Toto1101 and the genomic sequence of SB.
[b] Neonatal CD-1 mice, 18 to 24 h old, were inoculated with 100 PFU of virus by s.c. or i.c. routes and were observed for 14 days.
[c] Numbers in parentheses are the number of mice that died during the observation period over the number of mice inoculated.
[d] AST ± standard deviations were calculated for those animals that died.
[e] NA, not applicable.

Ockelbo82 having the consensus residue at these positions. At E2 23, HR_{sp} and HR_{lp} differ, with HR_{lp} and the original HR strain being identical to the other Sindbis virus and Sindbis virus-like strains (57). At E2 70, both Lys and Glu were evident. However, E2 Lys-70 was associated with selection during tissue culture passage (see below), indicating that pTRSB E2 Glu-70 is representative of the consensus sequence. Similar analysis of the two remaining structural protein gene differences suggests that, at these positions, it is the pTRSB sequence which may depart from the consensus sequence. The E1 72 residue was Ala in HR_{sp}, NSV, S.A.AR86, and Ockelbo82, whereas Val was found in pTRSB and SV1A. At the amino-terminal residue of the mature E2 molecule, the pTRSB sequence predicts an Arg residue, while Ser is found in the other available Sindbis virus and Sindbis virus-related sequences and is the consensus amino acid in other alphaviruses sequenced to date.

To summarize the sequence findings, 11 coding differences and a difference at nt 5 in the 5′ UTR were found between the published sequence of the HR_{sp} strain and that of pTRSB. The pTRSB sequence contained the consensus 5′ UTR sequence and the consensus amino acid residues at 8 of the 11 difference sites within the open reading frames (nsP1 441, nsP2 438, nsP2 634, E2 3, E2 23, E2 70, E2 172, and E1 237). At three coding positions, (nsP3 528, E2 1, and E1 72), the pTRSB sequence differed from the consensus sequence. The TRSB sequence also differed from the consensus sequence at five noncoding nucleotides, two of which were introduced purposely. While we cannot rule out an effect from these noncoding changes, none of them were in the 5′ or 3′ UTR sequences or in other known *cis*-acting alphavirus sequences (60).

Virulence of virus derived from pTRSB. Virus (TRSB) was derived from pTRSB and inoculated into mice to confirm that TRSB exhibited the virulence characteristics of its biological progenitor. Neonatal CD-1 mice were inoculated with 100 PFU of TRSB either s.c. or i.c., and percent mortality and AST were calculated. TRSB invariably produced 100% mortality. Average survival times were 3.0 ± 0.7 days (s.c.) or 2.2 ± 1.1 days (i.c.), results comparable to those previously obtained with SB, the laboratory strain of Sindbis virus AR339 from which pTRSB was derived (Table 3).

An attenuating E2 mutation in the TRSB background. A mutation which changed E2 residue 114 from Ser to Arg was identified previously in a biological mutant of SB derived after selection for rapid growth in BHK cells (3, 6). The mutant, SB-RL, was significantly attenuated in neonatal mice. Like the SB parent, virulent revertants of SB-RL, isolated from brains of SB-RL-infected animals, contained Ser at E2 114 (6). An analogous Ser-to-Arg change at E2 114 was observed among mutants (designated S3, S8, and S11) of S.A.AR86 after selection for rapid penetration of BHK cells (45). These results suggested that E2 Arg-114 was a readily selected BHK-cell-adaptive mutation.

Placement of the E2 Arg-114 mutation into Sindbis virus clone pTR2000, pTR3000, pTR4000, or pTR5000 by site-directed mutagenesis demonstrated that E2 Arg-114 was indeed an attenuating mutation on the basis of comparison of E2 Arg-114-containing viruses with viruses derived from otherwise isogenic clones, pTR2000 to pTR5000 (35, 36, 51). The attenuating phenotype of E2 Arg-114 was retained when this mutation was placed into the TRSB background (TRSBr114) (Table 4). However, the degree of attenuation of TRSBr114, as reflected in mortality and AST values, was not as great as the

TABLE 4. Virulence of TRSB containing mutations at nt 5 and E2 114

Virus	Nucleotide at nt 5	Amino acid at E2 114	Mice[a] inoculated s.c.		Mice inoculated i.c.	
			% Mortality[b]	AST ± SD[c]	% Mortality	AST ± SD
TRSB	G	Ser	100 (41/41)	3.0 ± 0.7	100 (84/84)	2.2 ± 1.1
TRSBr114	G	Arg	72.9 (43/59)	7.4 ± 2.7	76.1 (67/88)	6.1 ± 2.4
TR6000	A	Ser	100 (89/89)	3.9 ± 1.2	100 (70/70)	3.7 ± 1.5
TR6500	A	Arg	22.1 (15/68)	8.2 ± 1.5	34.5 (20/58)	9.1 ± 3.1

[a] Neonatal CD-1 mice, 18 to 24 h old, were inoculated with 100 PFU of virus by s.c. or i.c. routes and were observed for 14 days.
[b] Numbers in parentheses are the number of mice that died during the observation period over the number of mice inoculated.
[c] AST ± standard deviations were calculated for those animals that died. AST for TRSB differed significantly from AST of TRSBr114 and TR6500 by the Student *t* test ($P < 0.001$) for both s.c. and i.c. inoculated groups. The differences between TR6500 and TR6000 AST, for both s.c. and i.c. inoculated mice, were also significantly different ($P < 0.001$). The differences between TRSB and TR6000, in both s.c. and i.c. infected mice, were not distinguishable ($P < 0.2$).

TABLE 5. LD_{50} for TRSB containing mutations at nt 5 and E2 114

Virus dilution	% Mortality of infected mice[a] at virus dilution:								PFU[b] at virus dilution 10^{-7}	LD_{50} (PFU)[c]
	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Inoculations										
Subcutaneous										
TRSB[d]	ND	ND	ND	ND	100	100	100	20	0.3	1.1
TR6000	ND	ND	ND	ND	100	100	100	10	0.1	0.7
TRSBr114	60	70	100	90	90	70	70	30	2.2	122
TR6500	0	30	20	20	27	20	20	10	2.7	$>1.9 \times 10^7$
Intracerebral										
TRSB	ND	ND	ND	ND	100	100	100	67	0.3	<0.3
TR6000	ND	ND	ND	ND	100	100	100	50	0.6	0.6
TRSBr114	100	100	100	100	90	83	55	50	2.2	16.4
TR6500	80	10	10	10	20	20	0	20	2.7	3.6×10^6

[a] Groups of 10 to 13 neonatal CD-1 mice, 18 to 24 h old, were inoculated with dilutions of virus stocks by s.c. or i.c. routes and were observed for 14 days.
[b] The PFU delivered to animals from the highest dilutions were determined by plaque assay of the inocula.
[c] LD_{50}s were determined by the method of Reed and Muench (38).
[d] The base at nt 5 and the amino acid at E2 114 are given in Table 4.

attenuation when this same mutation was placed in the chimeric clones shown in Fig. 1 (35, 36, 51; see below).

Attenuation specified by nt 5 alone and in combination with E2 Arg-114. One of the differences between the pTRSB sequence and those of HR_{sp} and the chimeric clones pTR2000 to pTR6000 was at nt 5. TRSB and the consensus Sindbis virus sequence have a G residue, whereas HR_{sp} and the chimeric clones have an A. A previous report showed that variable virulence effects resulted from multiple nucleotide deletions in the 5′ UTR (21). Comparison of TRSB and viruses derived from the chimeric clones pTR2000 to pTR5000 (Table 3), as well as comparison of TRSB and TR6000, which differed only at nt 5, suggested that A at this position was not significantly attenuating as measured by mortality, AST (Table 4), or LD_{50} (Table 5). However, the degree of attenuation observed when the nt 5 A mutation was combined with E2 Arg-114 (TR6500) (Tables 4 and 5) was much greater than that seen with either mutation alone (TR6000 and TRSBr114). Therefore, the nt 5 A mutation inherent in the Toto1101 and HR_{sp} genetic backgrounds can act synergistically with attenuating mutations elsewhere in the genome, although the nt 5 A mutation alone produced no obvious effect. This suggests that studies of virulence, when performed in the background of Toto1101, HR_{sp} clones, or the chimeric pTR2000 to pTR6000 clones, are likely to be influenced significantly by the presence of the nt 5 A mutation.

Adaptive mutations for growth in BHK cells. One potential source of the differences among the sequences of the laboratory strains is the different cell culture passage histories of these viruses. This possibility is suggested by the propensity of many viruses to become attenuated upon cell culture passage (25, 46), and previous work showing that selection for efficient growth (3) or efficient penetration (18, 29) of alphaviruses on BHK cells coselects at a high frequency for attenuating mutations in the E2 gene. The experiments reported in this section focus on E2 residue 70, at which position mutation affected both growth in BHK cells and virulence in neonatal mice.

Among mutants of S.A.AR86 selected for rapid penetration of BHK cells (45) was one (designated S14) which harbored a G-to-A mutation at nt 8838, resulting in a Lys substitution for Glu (the Sindbis virus consensus residue) at E2 70. This change was identical to that which distinguishes the HR_{sp}, SV1A, and NSV E2 Lys-70 codons from that of the consensus sequence (Table 2). S.A.AR86 and S14 were inoculated i.c. into groups of 10 (S.A.AR86) or 12 (S14) 6-week-old female CD-1 mice at a dose of 100 PFU. The S.A.AR86-inoculated animals suffered 100% mortality, with an AST of 6.6 ± 1.3 days. In contrast, mortality was reduced to 42% among S14-inoculated mice, with an AST of 7.4 ± 2.3 days. This result suggested that significant attenuation was associated with E2 Lys-70.

The E2 Lys-70 mutation was recreated in the TR3000 background by site-directed mutagenesis to yield pTR3070, and the substituted E2 gene from pTR3070 was placed into the TR4000 and TR5000 backgrounds to construct pTR4070 and pTR5070, respectively. The E2 Lys-70 mutation significantly reduced virulence in each of these genetic backgrounds, demonstrating its attenuating effect in otherwise isogenic clones (Table 6). Furthermore, comparison of the AST for TR3070 and TR5070 suggested that the attenuating effect of the E2 Lys-70 mutation was modulated by differences in the two genetic backgrounds contained within the nsP2 and nsP3 genes.

The S.A.AR86-derived isolate S14, in which the E2 Lys-70 mutation was first encountered, was originally selected for rapid penetration of BHK cells. To demonstrate that this phenotype resulted from the E2 Lys-70 genotype, the penetration phenotypes of TR3070, TR4070, and TR5070 were compared with those of their respective isogenic progenitor clones and to that of TR5500, which contains the E2 Arg-114 mutation on the TR5000 background (Fig. 2). The percentage of attached virions which penetrated was defined functionally as the per-

TABLE 6. Virulence of E2 Lys-70 mutants in infected mice[a]

Virus	Amino acid at E2 70	% Mortality[b]	AST ± SD[c]
TR3000	Glu	100 (11/11)	3.5 ± 0.9
TR4000	Glu	100 (13/13)	3.1 ± 0.6
TR5000	Glu	100 (10/10)	4.4 ± 1.1
TR3070	Lys	28.2 (11/39)	10.4 ± 2.7
TR4070	Lys	34.8 (8/23)	7.9 ± 1.9
TR5070	Lys	39.3 (22/56)	6.9 ± 2.0

[a] Neonatal CD-1 mice, 18 to 24 h old, were inoculated s.c. with 100 PFU of virus and were observed for 14 days.
[b] Numbers in parentheses are the number of mice that died during the observation period over the number of mice inoculated.
[c] AST ± standard deviations were calculated for those animals that died. Differences in AST between otherwise isogenic Glu- and Lys-containing viruses were significant by the Student *t* test (TR3000/TR3070 and TR4000/TR4070, $P < 0.001$; TR5000/TR5070, $P < 0.05$). The increased AST of TR3070 compared with that of TR5070 was significant ($P < 0.1$).

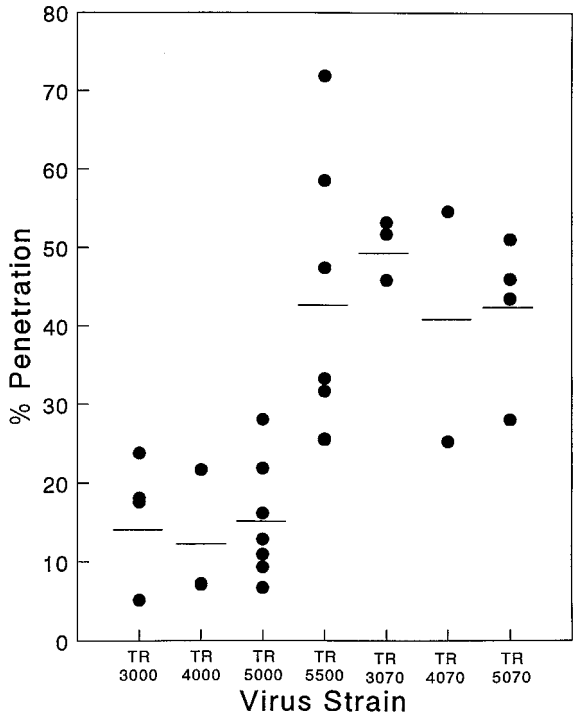

FIG. 2. Penetration of cloned Sindbis virus containing E2 Lys-70. Virus was derived from chimeric pToto1101-SB clones containing either E2 Glu-70 (TR3000, TR4000, and TR5000) or E2 Lys-70 (TR3070, TR4070, and TR5070) but which were otherwise isogenic. TR5500, containing E2 Arg-114 on the TR5000 background, was included as a rapidly penetrating control. Fifteen minutes after the infection of BHK cells with a known number of PFU, high-titered neutralizing antibody was added to block further penetration, as described in Materials and Methods. The percentage of virus initiating infectious centers under these conditions reflects the rate of penetration. Each filled circle represents an independent determination for each of the viruses (TR5000 and TR5500, $n = 7$; TR3000, $n = 5$; TR4000 and TR3070, $n = 3$; TR4070, $n = 2$; TR5070, $n = 4$). The E2 Lys-70 viruses and TR5500 differed significantly from TR5000 by the Student t test ($P < 0.02$).

centage of infectious centers which formed after neutralization of residual extracellular virus by polyclonal anti-Sindbis virus antibody added 15 min after infection. Ten to 15% of TR3000, TR4000, and TR5000 penetrated in 15 min, compared with 40 to 50% penetration for TR5500, TR3070, TR4070, and TR5070. All of the more rapidly penetrating viruses were significantly faster than TR5000 (as determined by the Student t test, $P < 0.02$). These results suggest that the E2 Lys-70 mutation in HR_{sp} could have resulted from passage in BHK cells, during which a more rapidly penetrating variant was selected.

The following experiment, utilizing a virus designated TRSB-E2S1, reproduced the selection of E2 70 during passage in BHK cells. TRSB-E2S1 is isogenic with TRSB except for the replacement of E2 Arg-1 with Ser, the Sindbis virus consensus amino acid at this position (13). TRSB-E2S1 thus represents the first modification of pTRSB toward the construction of a consensus Sindbis virus AR339 clone. Previously published results with this virus show that its specific infectivity for BHK cells is reduced by 2 orders of magnitude relative to that of TRSB (13), suggesting that passage of this virus on BHK cells would exert a strong selective pressure for mutations favoring growth in these cells. To determine whether the E2 Lys-70 mutation could be selected readily by cell culture passage, TRSB-E2S1 was subjected to five successive blind passages in BHK cells. Six isolates of the predominant large-plaque phenotype were derived from the fifth passage, grown to high titer by an additional pass in BHK cells, and gradient purified. The genomes contained in these preparations were sequenced across the first 140 codons of the E2 gene. All six contained an E2 Lys-70 mutation but were otherwise identical to the sequence of the starting virus, TRSB-E2S1, in the region sequenced. It is unlikely that these isolates were contaminants, as this genotype was not present previously in any Sindbis virus strain in our laboratory. RNA genomes, derived from bulk virus populations present at passage levels 1, 2, 3, and 5, were sequenced across the E2 70 locus to determine the rapidity with which the E2 Lys-70 mutation arose and became predominant. The passage 1 population was heterogeneous at the first position of the E2 70 codon, indicating that both E2 Glu-70 (GAG) and E2 Lys-70 (AAG) were represented in the passage 1 virus population. By the second passage, the majority E2 70 codon was AAG.

DISCUSSION

Genetic dissection of viral pathogenesis has progressed from comparison of disparate virus isolates to characterizing the effects of specific virus genes and presently to examining cloned viruses differing from each other by single-amino-acid substitutions (10, 16, 17, 19, 26, 34, 42, 43, 55). Perhaps the most remarkable general finding from such studies is that specific single-amino-acid changes, or indeed substitutions of critical single nucleotides in noncoding regions, can dramatically alter the virulence and pathogenesis of viral diseases. Moreover, when such mutations are combined, the phenotype often is not predictable from the phenotypes conferred by the individual mutations. This implies that the virulence or pathogenetic phenotype of a given mutation may vary considerably depending on the genetic background in which it arises.

The results presented here strongly suggest (i) that a substantial proportion of the sequence divergence evident among laboratory strains of Sindbis virus has resulted from selection for efficient growth in cell culture, (ii) that many of these changes, now considered part of a particular genetic background, can affect pathogenesis in and of themselves, and (iii) that such modified genetic backgrounds can exert a significant influence on genetic studies of virus pathogenesis and host range. These effects have become apparent only because one can now control laboratory-induced variation by stabilizing a sequence as a cDNA clone. It is likely therefore, that similar levels of divergence exist among laboratory strains of other RNA viruses and that such divergence of genetic background will have similar large effects on pathogenesis and host-range phenotypes in these systems.

This circumstance is illustrated well by the effects on mouse virulence of individual changes at Sindbis virus nt 5 and E2 residue 114 and by the interplay between these two loci in double mutants. The substitution of Arg for Ser at E2 114 in the TRSB background (in TRSBr114) increased the LD_{50}, significantly reduced mortality, and doubled the survival time for neonatal mice inoculated either s.c. or i.c. with 100 PFU of the virus. In contrast, the A-for-G substitution at nt 5 had no significant effect on mortality, survival, or LD_{50} in the TRSB background. However, when the E2 Arg-114 substitution was placed in a genetic background containing nt 5 A, the degree of attenuation was greatly enhanced, with the s.c. and i.c. LD_{50} being increased from 122 and 16 PFU to $>1.9 \times 10^7$ and 3.6×10^6 PFU, respectively. Therefore, even in virus derived from molecular clones which were isogenic, with the exception of the E2 114 amino acid, the apparent effect of E2 Arg-114 was highly dependent on the genetic background of the clone into which it was placed. This was the case even though the viru-

lence phenotypes of the two genetic backgrounds, one with nt 5 G and the other with nt 5 A, were indistinguishable from each other. In this context, small sequence differences among biological laboratory strains of a virus considered to be the wild-type genetic background, or in homologous cDNA clones derived from them, may have a profound influence on the interpretation of genetic studies of pathogenesis.

The origin of sequence diversity among Sindbis virus laboratory strains may be traced to the selective pressures of growth in cell culture, especially with respect to the glycoprotein genes. Even HR strains propagated in different laboratories differ from the published HR_{sp} amino acid sequence in two to five codons in the E2 gene (7). Here we present strong evidence that two such changes occurred at E2 70 and E2 1. At the E2 70 locus, a Lys-for-Glu substitution was present in HR_{sp} and in strains specifically selected for rapid penetration of BHK cells (S.A.AR86 mutant S14). Blind passage of a Sindbis virus strain which grows inefficiently on BHK resulted in selection of the same mutation. Therefore, it is likely that laboratory strains harboring E2 Lys-70 acquired this mutation during cell culture passage. Consistent with other mutations which increase efficiency of growth in cell culture (e.g., E2 Arg-114), E2 Lys-70 exerted an attenuating effect on virulence in neonatal mice.

The amino acid at E2 position 1 of TRSB (Arg) differed from that of the Sindbis virus consensus amino acid (Ser), suggesting that the Arg substitution also may have arisen during growth in cell culture. SB, the biological progenitor of pTRSB, was derived from a low-passage CEF stock of Sindbis virus AR339, and the biological cloning of SB from this stock included a selection for efficient production of cytopathic effects on BHK cells (see Materials and Methods). The uncloned parental AR339 stock contained E2 Ser-1 (unpublished results). Comparisons between TRSB (E2 Arg-1) and TRSB-E2S1 (an isogenic virus containing E2 Ser-1) demonstrate that the E2 Arg-1 substitution increased the specific infectivity of the virus for BHK cells by approximately 2 orders of magnitude while decreasing the specific infectivity for neonatal mice by a similar degree (13). Therefore, two conclusions may be drawn from these results. First, like E2 Lys-70, E2 Arg-1 represents a laboratory adaptation which has an attenuating effect in vivo. Second, changing E2 Arg-1 to E2 Ser-1, the first step in making the consensus clone, caused a dramatic increase in virulence for neonatal mice and likely altered pathogenesis. Animals infected with the E2 Ser-1 virus die with an AST of 2.3 days (13), far too quickly for the development of a classical encephalitis, the usual cause of death ascribed to Sindbis virus infections in the neonatal mouse model.

Other differences between the glycoprotein gene sequences of the laboratory strains and that of the Sindbis virus consensus sequence also affect cell culture and/or in vivo phenotypes. The possibility that substitutions at Sindbis virus E2 codon 3 (as in HR_{sp} E2 Ile-3) influence its pathogenesis phenotype is suggested by avirulent mutants of Venezuelan equine encephalitis virus which contain tandem mutations at E2 residues 3 and 4 (8, 18). These conferred both cell-culture-accelerated penetration and in vivo attenuation phenotypes. Similarly, cell culture passage of Venezuelan equine encephalitis virus during the selection of the vaccine strain TC-83 resulted in a mutation at nt 3 of the 5′ UTR (20). This mutation is associated with attenuation of the vaccine, suggesting that it may be analogous to the nt 5 mutation in Sindbis virus.

HR_{sp} residues E2 Arg-172, E1 Ala-72, and E1 Ser-237 all demonstrate an attenuating effect in mice when placed individually into otherwise isogenic clones (26, 35, 62). In addition, variation at E2 172 and E1 72 has been associated with changes in attachment (62) and fusion phenotype (4), respectively. In total, the HR_{sp} sequence differed from the consensus sequence at nt 5 and in 8 codons. Not surprisingly, it is considerably less virulent than a biological isolate closer in sequence to the consensus sequence (52).

Given the potential magnitude of virulence and pathogenetic differences associated with minimal differences in genetic background, what is the appropriate genetic background in which to examine mutations affecting pathogenesis of Sindbis virus? One argument is that the inoculation of vertebrates with nearly homogeneous virus stocks derived from cDNA clones misrepresents a natural inoculum, which would consist of a population of variants with a predominant genotype present in a persistently infected mosquito. This suggests interesting experiments in which molecularly cloned virulent and attenuated viruses are utilized to examine the molecular dynamics of quasispecies generation in mosquitoes and the subsequent genetic selection upon their transmission to vertebrates. A second point of view is that a cloned genetic background containing a complement of marginally attenuating mutations provides a more sensitive detection system for identification of other mutations affecting virulence. This suggestion is substantiated by the dramatic effect of the A-for-G substitution at nt 5 on the virulence phenotype of other substitutions in the E2 gene. However, comparative pathology studies utilizing such strains will be complicated by the presence of the background mutations. A third, more attractive possibility lies with the consensus sequence of the Sindbis virus AR339 isolate. The consensus sequence represents the closest approximation of the sequence of the original AR339 isolate, and currently is the most relevant genetic background for the study of Sindbis virus virulence and pathogenesis. A cDNA clone of the consensus AR339 sequence, a sequence devoid of occult attenuating mutations introduced by cell culture passage, offers the exciting opportunity to examine cell culture and in vivo phenotypes of a virus which is as close as possible to a native Sindbis virus isolate.

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