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## SHORT COMMUNICATION

# Complete Nucleotide Sequence and Full-Length cDNA Clone of S.A.AR86, a South African Alphavirus Related to Sindbis[1]

DENNIS A. SIMPSON, NANCY L. DAVIS, SEH-CHING LIN,[2] DARCY RUSSELL,[3] and ROBERT E. JOHNSTON[4]

*Department of Microbiology and Immunology, The University of North Carolina, Chapel Hill, North Carolina 27599-7290*

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S.A.AR86 and Girdwood S.A., two South African Sindbis-like arboviruses, are closely related antigenically to the Swedish isolate, Ockelbo82 [Lundström, J. O., Vene, S., Saluzzo, J. F., and Niklasson, B. (1993) *Am. J. Trop. Med. Hyg.* 49(5), 531–537]. Each of these viruses is associated with human disease, and Girdwood S.A. was isolated from a human case. In addition, S.A.AR86 is unique among Sindbis-like viruses in that adult mice remain sensitive to lethal infection with S.A.AR86. The complete genomic sequences of S.A.AR86 and Girdwood S.A. were determined. The S.A.AR86 RNA genome contained 11,663 nucleotides, excluding the 5′ CAP structure and 3′ poly(A) tail. In comparison to the consensus sequence of the prototype Egyptian Sindbis strain AR339, S.A.AR86 differed at 5.57% of the nucleotides, including a 54-nucleotide deletion, two insertions of 6 nucleotides each, and a 3-nucleotide insertion in the 3′ terminal one-third of the S.A.AR86 nsP3 gene. S.A.AR86 is one of only three alphaviruses sequenced to date that does not have an opal termination codon between the nsP3 and the nsP4 genes. These genes are separated by a cysteine codon in the S.A.AR86 genome. The genome of Girdwood S.A. was 11,717 nucleotides in length, excluding the 5′ CAP and 3′ poly(A) tail. Girdwood S.A. contained an opal termination codon between nsP3 and nsP4 and did not have the large 54-nucleotide deletion in nsP3, although Girdwood S.A. did contain the remaining insertions and deletions characteristic of S.A.AR86. S.A.AR86 was more closely related to Girdwood S.A. than to the Egyptian isolate, and the South African isolates as a group were more closely related to the Swedish isolate. Comparison of the S.A.AR86 sequence to that of Ockelbo82, Girdwood S.A., and Sindbis virus AR339 revealed several codons where S.A.AR86 differed from the conserved amino acid encoded by the other three viruses. These changes may be related to the ability of S.A.AR86 to initiate a lethal central nervous system infection in adult mice. To fulfill a prerequisite for testing this hypothesis, a full-length cDNA clone of S.A.AR86 was constructed from which infectious genomic RNA replicas could be derived. The sequence of this clone differed from the S.A.AR86 genomic RNA sequence at four translationally silent positions, and virus derived from the clone reproduced the adult mouse neurovirulence phenotype of its biological progenitor. 

Sindbis virus, the prototype member of the alphavirus genus of the family *Togaviridae,* and viruses closely related to Sindbis are broadly distributed throughout Africa, Europe, Asia, the Indian subcontinent, and Australia, based on serological surveys of humans, domestic animals, and wild birds (*1–6*). Sequence comparisons of Sindbis with a South American alphavirus, Aura, and with the structural gene region of western equine encephalitis virus (WEE) suggest that Sindbis-like viruses also exist in the Western hemisphere (*7, 8*).

The first isolate of Sindbis virus (strain AR339) was recovered from a pool of *Culex* sp. mosquitoes collected in Sindbis, Egypt in 1953 (*9*) and is the most extensively studied representative of this group. Natural isolates associated with human disease include S.A.AR86, Ockelbo82, and Girdwood S.A. These "Sindbis-like" viruses are related to Sindbis strain AR339 but are more closely related to each other based on serological comparisons (*10*). S.A.AR86 was isolated in 1954 from a pool of *Culex* sp. mosquitoes collected near Johannesburg, South Africa during an arbovirus survey (*11*) and was temporally associated with a febrile illness in humans (*2, 12*) similar to that caused by Ockelbo82 (*13*). Ockelbo82 was isolated from *Culiseta* sp. mosquitoes collected in Edsbyn, Sweden in 1982 and was associated serologically with human disease (*14*). Girdwood S.A. was isolated from a human patient in the Johannesburg area of South Africa in 1963 (*15*) and has the distinction of being one of the few Sindbis isolates derived from a human case.

The clinical symptoms of human infection with Ockelbo82, S.A.AR86, or Girdwood S.A. are a febrile illness,

[1] The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the Accession Nos. U38304 (Girdwood S.A.) and U38305 (S.A.AR86).

[2] Present address: Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333

[3] Present address: Washington and Lee University, Department of Biology, Parmly Hall, Room 306, Lexington, VA 24450

[4] To whom reprint requests should be addressed. Fax: (919) 962-8103.

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general malaise, macropapular rash, and joint pain which occasionally progresses to a polyarthralgia sometimes lasting from a few months to a few years. The pathogenesis underlying this human disease is not known. Experimental infections with strain AR339 and its laboratory derivatives have explored Sindbis virus virulence and pathogenesis in the mouse model. In newborns of this animal host, inocula approaching a single plaque-forming unit (PFU) administered either subcutaneously (sc) or intracranially (ic) produce a uniformly fatal disease characterized by high levels of viral replication in the brain. However, in mice greater than 2 weeks of age, all natural isolates of Sindbis virus examined to date, with the exception of S.A.AR86, are avirulent even when injected ic at high doses and even though virus replicates in the central nervous system (CNS) to high titers (*16, 17*). S.A.AR86, however, retains neurovirulence for mice regardless of age, inducing uniformly fatal infection after ic inoculation of doses less than that detectable by standard plaque assay in baby hamster kidney (BHK-21) cells (*18*).

Therefore, S.A.AR86 presents an intriguing opportunity to investigate a Sindbis-like virus associated with human disease as well as the mechanism by which S.A.AR86 overcomes the age-related restriction characteristic of other Sindbis and Sindbis-like viruses. In the experiments reported here, the first objective was to determine the complete genomic sequences of S.A.AR86 and Girdwood S.A., and to compare these to each other and closely related Sindbis-like viruses, thus identifying amino acid differences potentially associated with the unique neurovirulence phenotype of S.A.AR86. The second objective was to construct a full-length cDNA clone of the S.A.AR86 genome such that the virus derived from it would reproduce the adult mouse neurovirulence phenotype of the natural isolate. This clone will provide a stable system to examine the molecular genetics of S.A.AR86 pathogenesis.

The sequences of S.A.AR86 and Girdwood S.A. were determined from uncloned RT-PCR fragments amplified from virion RNA (*19*). The sequence of the 5′ 40 nucleotides was determined by directly sequencing the genomic RNA (*20–22*). The S.A.AR86 genome was 11,663 nucleotides in length, excluding the 5′ CAP and 3′ poly(A) tail, 40 nucleotides shorter than the prototype Sindbis strain AR339 (*23*). Compared with the consensus sequence of Sindbis virus AR339 (*24*), S.A.AR86 contained two separate 6-nucleotide insertions, and one 3-nucleotide insertion in the 3′ half of the nsP3 gene, a region not well conserved among alphaviruses. The two 6-nucleotide insertions were found immediately 3′ of nucleotides 5403 and 5450, and the 3-nucleotide insertion was immediately 3′ of nucleotide 5546 compared with the AR339 genome. In addition, S.A.AR86 contained a 54-nucleotide deletion in nsP3 which spanned nucleotides 5256 to 5311 of AR339. As a result of these deletions and insertions, S.A.AR86 nsP3 was 13 amino acids smaller than in AR339, containing an 18-amino-acid deletion and a total of 5 amino acids inserted. The 3′ untranslated region of S.A.AR86 contained, with respect to AR339, two 1-nucleotide deletions at nucleotides 11,513 and 11,602, and one 1-nucleotide insertion following nucleotide 11,664. The total numbers of nucleotides and predicted amino acids comprising the remaining genes of S.A.AR86 were identical to those of AR339.

A notable feature of the deduced amino acid sequence of S.A.AR86 was the cysteine codon in place of an opal termination codon between nsP3 and nsP4. S.A.AR86 is the only Sindbis virus isolate, and one of just three alphavirus isolates sequenced to date, which do not contain an opal termination codon between nsP3 and nsP4 (*25, 26*).

The genome of Girdwood S.A. was 11,717 nucleotides long excluding the 5′ CAP and 3′ poly(A) tail. The extra nucleotides relative to AR339 were in the nonconserved half of nsP3, which contained insertions totalling 15 nucleotides, and in the 3′ untranslated region which contained two 1-nucleotide deletions and a 1-nucleotide insertion with respect to the consensus Sindbis AR339 genome. The insertions found in the nsP3 gene of Girdwood S.A. were identical in position and content to those found in S.A.AR86, although Girdwood S.A. did not have the large nsP3 deletion characteristic of S.A.AR86. The remaining portions of the genome contained the same number of nucleotides and predicted amino acids as Sindbis AR339. Overall, Girdwood S.A. was 94.5% identical to the consensus Sindbis AR339 sequence, differing at 655 nucleotides not including the insertions and deletions. These nucleotide differences resulted in 88 predicted amino acid changes or a difference of 2.3%. A plurality of amino acid differences were concentrated in the nsP3 gene, which contained 32 of the amino acid changes, 25 of which were in the nonconserved 3′ half. We were unable to resolve the Girdwood S.A. nucleotides at positions 1, 3, and 11,717. Because the primer used during the RT-PCR amplification of the 3′ end of the genome assumed a cytosine in the 3′ terminal position (5′-CTAACGGCTCGAGTCTAGAT$_{25}$G-3′), we were unable to determine with certainty the identity of this nucleotide. However, in all alphaviruses sequenced to date there is a cytosine in this position. This, combined with the fact that no difficulty was encountered in obtaining an RT-PCR product for this region with an oligo(dT) primer ending with a 3′ G, suggested that Girdwood S.A. also contained a cytosine at this position. The ambiguity at nucleotide positions 1 and 3 resulted from strong stops encountered during the RNA sequencing.

Table 1 examines the relationship of S.A.AR86 and Girdwood S.A. to each other and to other Sindbis-related viruses. This was accomplished by aligning the nucleotide and deduced amino acid sequences of Ockelbo82,

TABLE 1

Differences between Sindbis AR339, OCKELBO82, and Girdwood S.A. Viruses with Respect to S.A.AR86 Virus

| | Nucleotides[a] | | | Amino acids[a] | | |
|---|---|---|---|---|---|---|
| | AR339 | OCK82 | GIRD | AR339 | OCK82 | GIRD |
| Regions | Number (%) | | | Number (%) | | |
| 5′ untranslated | 0 (0.0) | 0 (0.0) | 1 (1.7) | — | — | — |
| nsP1 | 76 (4.7) | 37 (2.3) | 15 (0.9) | 9 (1.7) | 6 (1.1) | 2 (0.4) |
| nsP2 | 137 (5.7) | 86 (3.6) | 45 (1.9) | 15 (1.9) | 8 (1.0) | 12 (1.5) |
| nsP3 | | | | | | |
| Conserved[b] | 51 (5.7) | 35 (3.9 | 13 (1.6) | 6 (2.0) | 1 (0.3) | 1 (0.4) |
| Nonconserved[c] | 116 (6.6) | 83 (4.4) | 70 (2.2) | 45 (9.7) | 34 (7.0) | 27 (3.7) |
| nsP4 | 111 (6.1) | 68 (3.7) | 19 (1.1) | 8 (1.3) | 2 (0.3) | 4 (0.6) |
| 26s junction | 1 (2.1) | 0 (0.0) | 1 (2.1) | — | — | — |
| Capsid | 36 (4.5) | 26 (3.3) | 7 (0.9) | 1 (0.4) | 3 (1.1) | 0 (0.0) |
| E3 | 17 (8.9) | 5 (2.6) | 4 (2.1) | 1 (1.6) | 0 (0.0) | 0 (0.0) |
| E2 | 71 (5.6) | 43 (3.4) | 18 (1.4) | 12 (2.6) | 6 (1.4) | 2 (0.5) |
| 6K | 10 (6.1) | 9 (5.4) | 4 (2.4) | 2 (3.6) | 2 (3.6) | 1 (1.8) |
| E1 | 49 (3.7) | 31 (2.3) | 16 (1.2) | 7 (1.6) | 6 (1.4) | 2 (0.9) |
| 3′ untranslated | 14 (4.5) | 8 (2.5) | 1 (0.3) | — | — | — |
| Totals | 689 (5.5) | 431 (3.3) | 214 (1.4) | 106 (2.3) | 68 (1.4) | 51 (0.9) |

*Note.* All nucleotide positions and gene boundaries are numbered according to that used for the Sindbis AR339, $HR_{SP}$ variant (Genebank Accession No. J02363; (*23*)) unless otherwise noted. These comparisons utilized the deduced Sindbis AR339 sequence (*24*).

[a] Differences include insertions and deletions.

[b] Conserved region nucleotides 4100 to 5000 (aa1 to aa300).

[c] Nonconserved region nucleotides 5001 to 5729 (aa301 to aa542, S.A.AR86 numbering).

AR339, and Girdwood S.A. to those of S.A.AR86 and then calculating the percentage identity for each gene using the programs contained within the Wisconsin GCG package (*27*). The analysis suggests that S.A.AR86 is most similar to the other South African isolate, Girdwood S.A., and that the South African isolates are more similar to the Swedish Ockelbo82 isolate than to the Egyptian Sindbis AR339 isolate. These results also suggest that it is unlikely that S.A.AR86 is a recombinant virus like western equine encephalitis virus (*7*).

Since Girdwood S.A., Ockelbo82, and S.A.AR86 were so closely related by sequence, we confirmed that only S.A.AR86 displayed the adult mouse neurovirulence phenotype. Groups of four female CD-1 mice (3–6 weeks of age) were inoculated ic with $10^3$ PFU of S.A.AR86, Girdwood S.A., or Ockelbo82. Neither Girdwood S.A. nor Ockelbo82 infection produced any clinical signs of infection. In contrast, infection with S.A.AR86 produced neurological signs within 4 to 5 days and ultimately killed 100% of the mice as previously demonstrated (*18*).

Table 2 lists those amino acids of S.A.AR86 which might explain its neurovirulence phenotype in adult mice. We scored a position as potentially related to the S.A.AR86 adult neurovirulence phenotype if the S.A.AR86 amino acid differed from that which otherwise was absolutely conserved at that position in the other viruses.

As a first step in investigating the unique adult mouse neurovirulence phenotype of S.A.AR86, a full-length cDNA clone of the S.A.AR86 genome was constructed. The sources of cDNA included conventional cDNA clones (*28*) as well as uncloned RT-PCR fragments derived from the S.A.AR86 genome. As illustrated in Fig. 1, these were substituted, starting at the 3′ end, into

TABLE 2

S.A.AR86 Amino Acids Potentially Related to the Adult Neurovirulence Phenotype

| Position[a] | | S.A.AR86 amino acid | Conserved amino acid |
|---|---|---|---|
| nsP1 | 583 | Thr | Ile |
| nsP2 | 256 | Arg | Ala |
| | 648 | Ile | Val |
| | 651 | Lys | Glu |
| nsP3 | 344 | Gly | Glu |
| | 386[b] | Tyr | Ser |
| | 441 | Asp | Gly |
| | 445 | Ile | Met |
| | 537 | Cys | Opal |
| E2 | 243 | Ser | Leu |
| 6K | 30 | Val | Ile |
| E1 | 112 | Val | Ala |
| | 169 | Leu | Ser |

[a] S.A.AR86 numbering.

[b] Last amino acid before 18-amino-acid deletion found in S.A.AR86.

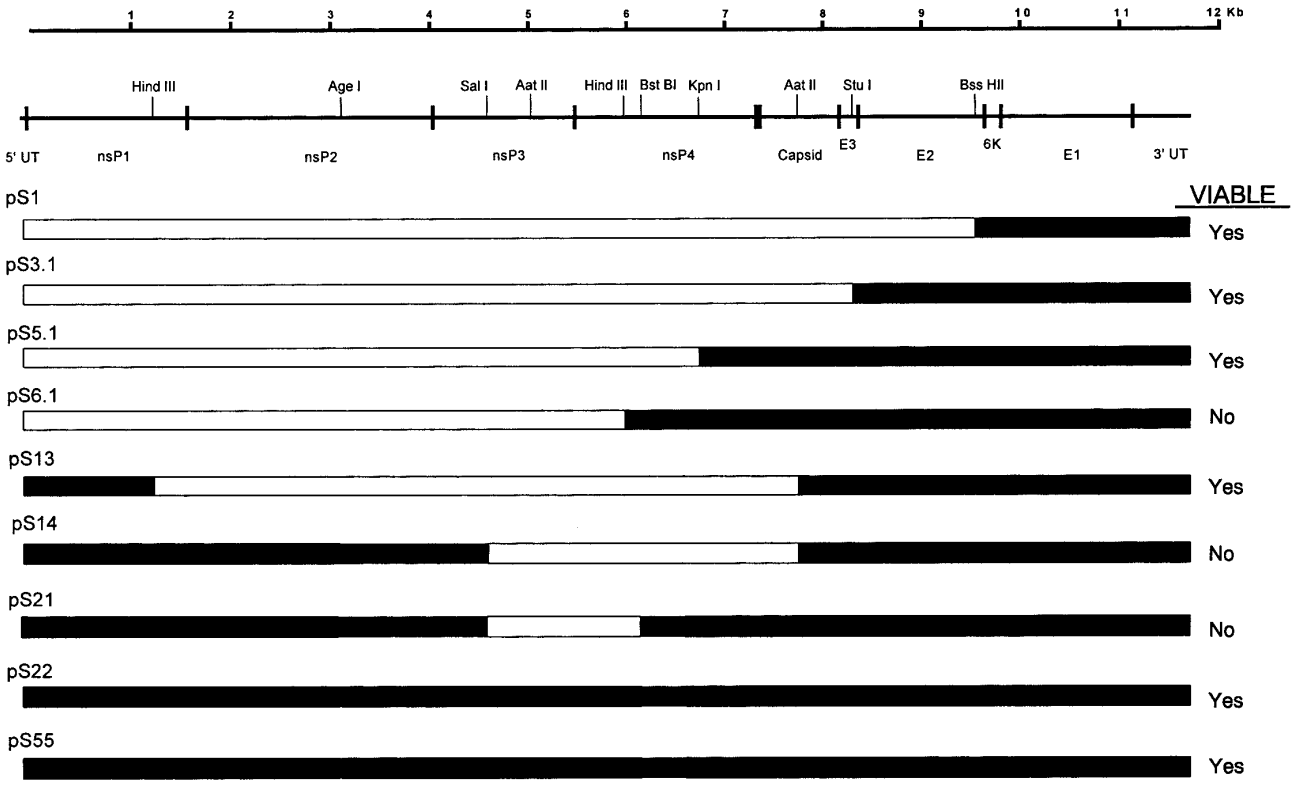


FIG. 1. Construction of full-length S.A.AR86 cDNA clones and chimeric AR339/S.A.AR86 clones. Clones were constructed by substituting S.A.AR86 specific cDNA's (■) for the analogous regions in pTR5000 (□). Substitutions proceeded in both 3′ and 5′ directions. Viability was measured as specific infectivity of RNA transcripts derived from the clones. Virus derived from a clone was considered viable if the PFU/cpm of $^{35}$S-UTP-labeled transcripts was greater than 10% of the PFU/cpm of $^{35}$S-UTP-labeled transcripts derived from pTR5000 (*25*).

pTR5000 (*24*), a full-length Sindbis clone from which infectious genomic replicas could be derived by transcription with SP6 polymerase *in vitro.* The viability of the chimeric clones was tested by comparing the specific infectivities (*19, 24*) of the transcripts from each clone with that of transcripts derived from pTR5000. Transcripts from chimeric clones extending from the 3′ end of the genome into the 3′ half of the nsP4 gene (clones pS1, pS3.1, and pS5.1) were infectious, whereas replacement of additional nsP4 sequence to the *Hin*dIII site with S.A.AR86 sequences rendered the resulting transcripts noninfectious (clone pS6.1). The loss of viability was not due to defective sequence in the *Hin*dIII to *Kpn*I fragment, because the same sequence is a constituent of viable full-length S.A.AR86 clones pS22, pS24, and pS55. Rather this suggested that not all of the pTR5000 and S.A.AR86 nonstructural proteins may be compatible with each other and/or that individual chimeric nonstructural proteins may not be functional. However, viable transcripts were produced from clone pS13, which contained S.A.AR86 gene sequences from the 5′ end of the genome into the 3′ half of the nsP1 gene and from the *Aat*II site in capsid to the 3′ end of the genome. Noninfectious transcripts were derived from pS14 and pS21, the latter having pTR5000 sequences only from the *Sal*I site in nsP3 to the *Bst*BI site in nsP4. The remaining pTR5000 sequences were replaced with S.A.AR86 sequences by replacing the *Age*I (nsP2) to *Bst*BI (nsP4) region of clone pS21 with a corresponding S.A.AR86 cDNA clone. Transcripts derived from this clone (pS22) were infectious, although the resulting virus was temperature-sensitive, exhibiting a three to four log reduction in titer at 37° as compared to the titer of virus grown at 29° (data not shown). We repeated the replacement of the *Age*I to *Bst*BI region using a different primary S.A.AR86 cDNA clone to construct clone pS24, which corrected the temperature-sensitive defect.

Unlike the S.A.AR86 biological isolate, virus derived from pS24 failed to display a neurovirulent phenotype upon ic inoculation of adult mice. Therefore, the nucleotide sequence of pS24 was determined by direct sequencing of the clone itself or by sequencing subclones used during its construction. Comparison of the pS24 sequence to the genomic RNA sequence of S.A.AR86 revealed several coding and silent nucleotide differences. The coding differences were repaired by replacing the mutated regions with analogous uncloned RT-PCR fragments generated from S.A.AR86 virion RNA. The end result was pS55, a clone from which infectious transcripts could be produced and which contained four nucleotide changes (G for A at nt 215;

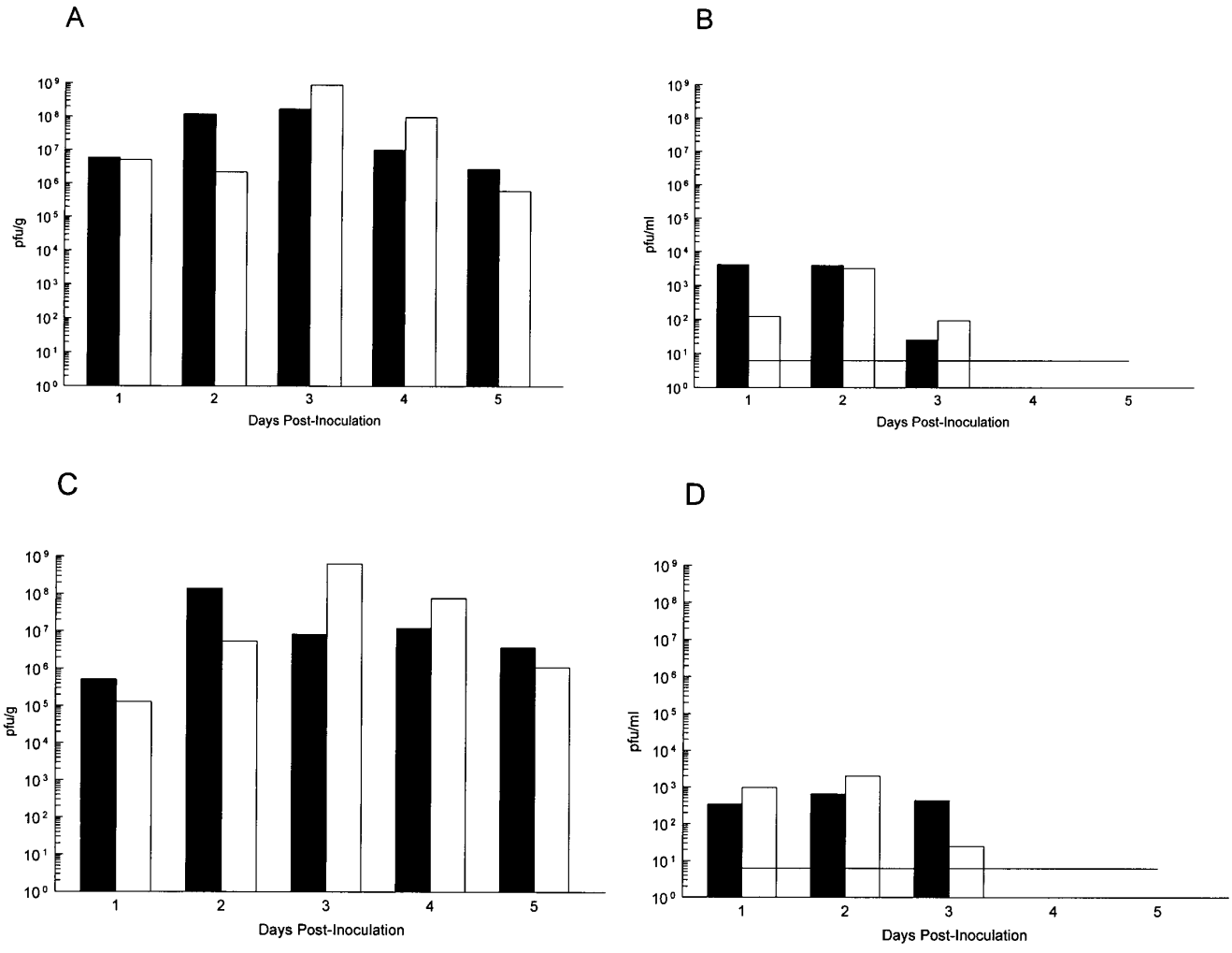


FIG. 2. Virus growth of S.A.AR86 and S55 *in vivo.* S.A.AR86 and S55 titers in the brain and serum of 25-day-old female CD-1 mice inoculated ic with $10^3$ PFU of virus were compared. Each bar represents a single animal at each time point. The line in panels B and D represents the minimum level of detection at each time point. (A) S.A.AR86 brain titers; (B) S.A.AR86 serum titers; (C) S55 brain titers; (D) S55 serum titers.

G for C at nt 3863; G for A at nt 5984; and C for T at nt 9113) but no amino acid differences with respect to the S.A.AR86 genomic RNA.

To determine if virus derived from pS55 (S55) reproduced the defining adult neurovirulence characteristic of S.A.AR86, the virulence phenotype and replication of S55 were compared to S.A.AR86 in adult (25-day-old) female CD-1 mice. Groups of four animals were inoculated ic with $10^3$ PFU of either S55 or S.A.AR86 and were monitored for mortality and signs of infection. Both viruses produced 100% mortality, and the average survival times for each group of animals were indistinguishable (6.5 $\pm$ 1.0 days and 6.0 $\pm$ 0.0 days for S55 and S.A.AR86, respectively). To determine if S55 and S.A.AR86 replicated to equivalent titers *in vivo,* the titers of S55 and S.A.AR86 found in the brain and serum of the animals at 1 through 5 days post-ic-inoculation with $10^3$ PFU was determined (Fig. 2). The S55 and S.A.AR86 profiles of virus growth in the CNS and periphery were very similar. From these data we conclude that the silent changes found in virus derived from clone pS55 had little or no effect on its growth or virulence, and that this molecularly cloned virus accurately represents the biological isolate, S.A.AR86.

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