JOURNAL OF VIROLOGY, June 1988, p. 2124–2133
0022-538X/88/062124-10$02.00/0

Vol. 62, No. 6

Molecular Analysis of Sindbis Virus Pathogenesis in Neonatal Mice by Using Virus Recombinants Constructed In Vitro†

JOHN M. POLO,[1]* NANCY L. DAVIS,[1] CHARLES M. RICE,[2] HENRY V. HUANG,[2] AND ROBERT E. JOHNSTON[1]

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615,[1] and Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 3 December 1987/Accepted 8 March 1988

Genetic loci affecting Sindbis virus pathogenesis in neonatal mice have been examined by using a full-length cDNA clone of the virus (Toto1101). The full-length cDNA is linked to a bacteriophage SP6 promoter to facilitate the synthesis of infectious RNA transcripts in vitro. Virus derived from Toto1101 showed reduced virulence (attenuation) in neonatal mice. Replacement of the E1 glycoprotein and 6K genes of Toto1101 with cloned E1 and 6K genes derived from a virulent Sindbis virus strain, AR339 (SB), resulted in a new construct, TR2000, that gave rise to virulent virus. Sequence determinations for the entire substituted regions of TR2000, Toto1101, and related virulent and attenuated strains identified three coding differences in E1 between Toto1101 and TR2000. These differences, individually or in combination, may be responsible for the attenuated phenotype. Previous studies in this laboratory identified another attenuating mutation at amino acid position 114 of the E2 glycoprotein (N. L. Davis, F. J. Fuller, W. G. Dougherty, R. A. Olmsted, and R. E. Johnston, Proc. Natl. Acad. Sci. USA 83:6771–6775, 1986). Substitution of Arg-114 in the mutant SB-RL for Ser-114 of SB appears to confer three distinguishing phenotypes: attenuation in neonatal mice, increased sensitivity to specific E2 monoclonal antibodies, and accelerated penetration of BHK cells. Replacement of TR2000 sequences containing the codon for amino acid 114 of E2 with corresponding fragments from cDNA clones of SB or SB-RL produced two strains of Sindbis virus (TR2100 and TR2200) which were isogenic except for the E2 114 codon (Ser and Arg, respectively). The three diagnostic phenotypes cosegregated according to the origin of the codon for amino acid 114 of E2, confirming the dramatic effect of this single amino acid substitution on these three phenotypes.

Understanding viral pathogenesis at the molecular and genetic levels has been an important concern not only because it is an inherently interesting problem but also because it is crucial in the development of safer and more effective vaccines. Much of the progress in this field is attributable to the characterization of virus strains which display altered virulence in animals. Genetic analyses of naturally occurring virulent and attenuated strains and comparisons of virulent and vaccine strains have implicated specific genomic regions as major determinants of the virulent phenotype (20, 23, 31, 36, 48). However, detailed sequence comparisons between such pairs of virulent and attenuated strains often have been frustrated by the presence of numerous coding and noncoding nucleotide differences. Thus, assignment of specific mutations to a particular phenotype is difficult, if not impossible. Another approach has been the isolation of attenuated mutants which are closely related to a parental virulent strain. Such mutants have been found among panels of temperature-sensitive mutants (6, 16) or among variants selected for the ability to escape neutralizing antibody, as in the case of reovirus, rabies virus, and Sindbis virus (15, 25, 28, 47; D. F. Pence and R. E. Johnston, unpublished data). Sequence comparisons of these closely related strains suggest that single amino acids in specific virus protein domains which are exposed to antibody at the virion surface are important determinants of viral pathogenesis in vivo. In many cases, these virulence domains involve proteins that participate in early virus-cell interactions at the plasma membrane, suggesting that changes in the specificity of such interactions in vivo may produce an attenuated phenotype.

Assignment of particular mutations to specific phenotypes of RNA viruses has been assisted greatly by the construction of full-length cDNA clones. Infectious progeny virus is produced when the cDNA is transfected into appropriate host cells (40, 51) or when it is coupled to bacteriophage promoters to serve as templates for the in vitro synthesis of infectious RNA transcripts (see, for example, references 2, 11, 27, and 34). The availability of complete clones allows the construction of strain pairs that are isogenic except for defined base substitutions and has facilitated the study of a number of viral phenotypes, including the pathogenesis phenotype (30, 35, 39). In this report, the power of this approach is again illustrated by the use of a full-length clone of Sindbis virus (42) to identify two loci which modulate the virulence of this virus in neonatal mice.

Sindbis virus is the prototype member of the *Alphavirus* genus, a group of arthropod-borne viruses which includes the agents of eastern, western, and Venezuelan equine encephalitis (3). The virus is composed of a single-stranded RNA genome of positive polarity enclosed in an icosahedral nucleocapsid formed by multiple capsid (C) protein monomers (46). Nucleocapsids are surrounded by a host-derived lipid envelope from which two viral glycoproteins, E1 and E2, protrude to form heterodimeric spikes (43). These surface glycoproteins appear to function in several biological activities of the virus, including attachment and penetration (7), hemagglutination (12), and pathogenesis in vivo (13).

We have previously reported the isolation of an attenuated mutant of Sindbis virus, SB-RL. The parent of SB-RL was our laboratory AR339 strain, SB, which is virulent in neonatal mice but avirulent in adults (4). SB-RL was derived

* Corresponding author.

† Journal series paper 11303 of the North Carolina Agricultural Research Service, Raleigh.

from SB after a limited number of passages in baby hamster kidney (BHK) cells under a stringent selective pressure for rapid growth (5). With respect to SB, SB-RL is characterized by three distinguishing, covariable phenotypes: attenuation in neonatal mice, increased sensitivity to neutralization by two E2-specific monoclonal antibodies, and accelerated penetration into BHK cells (37, 38). SB and SB-RL differ in their glycoprotein genes by only a single nucleotide, which predicts the replacement of a Ser at position 114 of the SB E2 glycoprotein with an Arg in SB-RL (13). Virulent revertants isolated from the brains of moribund, SB-RL-infected mice contain a same-site reversion to Ser at E2 residue 114, as well as reversion of the neutralization sensitivity and penetration phenotypes. These experiments provided strong genetic evidence that the mutation at E2 residue 114 is responsible for the coordinate changes seen in the three phenotypes. However, in the absence of complete sequence data for the entire genomes of these strains, we could not rule out the possibility that a second mutation outside the glycoprotein genes also was involved. To eliminate this possibility, we used a full-length cDNA clone of Sindbis virus, designated Toto1101 (42), to construct additional clones differing only at the E2 114 locus.

In this article, we report (i) the attenuated phenotype of virus derived from Toto1101 in the neonatal mouse system, (ii) the development of a Sindbis virus cDNA clone which produces virulent virus, (iii) the identification of mutations in the E1 glycoprotein of Toto1101 which may be responsible for its attenuated phenotype, and (iv) confirmation of the pleiotropic role of E2 amino acid 114 in attenuation, increased sensitivity to neutralization by E2-specific monoclonal antibodies, and accelerated penetration into BHK cells.

MATERIALS AND METHODS

Cells, virus, and virus propagation. Tissue culture cells, both BHK-21 and chicken embryo fibroblasts (CEF), were maintained in Eagle minimal essential medium (MEM) containing 10% (for BHK-21 cells) or 5% (for CEF) (vol/vol) donor calf serum and 10% (vol/vol) tryptose phosphate broth. Sindbis virus strains were propagated and titrated in BHK-21 cells. SB and SB-RL (SB-reduced latent period) are the virulent and attenuated prototype strains of this laboratory. HR-TX was obtained from Dennis Brown (University of Texas), and SS std. is the laboratory strain of Sondra Schlesinger (Washington University, St. Louis, Mo.). All virus stocks used for RNA extraction were tested for the virulence phenotype in newborn mice.

General recombinant DNA methods. DNA manipulations were carried out essentially as described by Maniatis et al. (32). Plasmid DNA was prepared by the boiling method of Holmes and Quigley (22). Colony hybridizations to identify clones containing virus-specific inserts were done essentially as described elsewhere (21) by using alternate probes for the SB and SB-RL libraries. For SB clones, ^{32}P-labeled, first-strand cDNA was synthesized from purified SB genome RNA templates by using calf thymus DNA hexanucleotides as random primers (32). For SB-RL clones, an E2 sequencing primer, E24 (13), was annealed to purified SB-RL genome RNA, serving as the primer for synthesis of a short-copy, ^{32}P-labeled, first-strand cDNA probe. Standard nucleic acid-modifying enzymes used in these procedures were obtained commercially from Promega Biotec, Bethesda Research Laboratories, New England BioLabs, Inc., Boehringer Mannheim Biochemicals, and Amersham Corp.

Molecular cloning of cDNA. Genomic RNA was isolated from purified virus as described by Davis et al. (13). cDNAs, complementary to both SB and SB-RL virion RNAs, were generated by using the technique of Gubler and Hoffman (19). Prior to ligation with vector DNA, SB-derived cDNA was fractionated by Sephacryl S-1000 (Pharmacia) column chromatography to isolate the larger molecules. Blunt-ended cDNA from both strains was ligated into dephosphorylated, *Pvu*II-digested pBR322, transformed into *Escherichia coli* MC1061, and plated onto yeast-tryptone agar containing ampicillin (100 μg/ml). To identify the exact termini of selected, large cDNA inserts, two oligonucleotide primers complementary to sequences on either side of the pBR322 unique *Pvu*II site were synthesized and used in the direct plasmid sequencing procedure of Chen and Seeburg (9). The two 18-mer oligonucleotides, 5′-GACCACGCTGATGAGC TT-3′ and 5′-CACCGTCATCACCGAAAC-3′, which are complementary to bases 2042 to 2059 and 2079 to 2096, respectively, were gifts from W. Dallas (Burroughs Wellcome Co.). Representative cDNA clones of each library are depicted in Fig. 1.

Construction of recombinant viruses. Plasmid pTR2000 was constructed from Toto1101 as shown in Fig. 2 and described below. Numbering of nucleotides follows that of Strauss et al. (49), beginning at the first base of the Sindbis virus insert and continuing consecutively into the vector sequences. Plasmid Toto1101 was partially digested with *Sac*II (four sites present) under conditions optimized to yield a large proportion of single-cut, linear molecules. The linear DNA was purified from agarose gels by electrophoresis onto DE-81 paper (Whatman, Inc.). Elution of DNA from the paper was accomplished by two 60°C incubations for 20 min each in TE buffer (10 mM Tris hydrochloride–0.1 mM EDTA [pH 7.6]) containing 1 M NaCl and 50 mM Arg-HCl. After phenol and ether extractions and ethanol precipitation, the DNA was digested to completion with *Bss*HII (one site). The appropriate Toto1101 vector fragment (minus the 1,680-base-pair [bp] fragment from *Bss*HII [position 9804] to *Sac*II [position 11484]) again was purified from agarose gels. Plasmid pSB4 was digested to completion with both *Bss*HII and *Sac*II. Following agarose gel purification of the 1,680-bp fragment from pSB4, the DNA was ligated into the prepared Toto1101 vector with T4 DNA ligase, resulting in plasmid pTR2000.

Plasmids pTR2100 and pTR2200 were constructed from pTR2000 as shown in Fig. 3. Plasmid pTR2000 was partially digested with *Pst*I (six sites present) under conditions optimized to yield a large proportion of single-cut, linear molecules. Following purification of linear molecules from agarose gels, the DNA was digested to completion with *Stu*I (one site) and the appropriate pTR2000 vector fragment (minus the 548-bp fragment from *Stu*I [position 8571] to *Pst*I [position 9119]) was purified from agarose gels. Plasmids pRL1 and pSB3 were digested to completion with both *Pst*I and *Stu*I. The appropriate 548-bp fragments were purified from agarose gels and ligated into the prepared pTR2000 vector, resulting in plasmids pTR2100 (replacement from pSB3) and pTR2200 (replacement from pRL1). Plasmids isolated from individual colonies were numbered consecutively (e.g., pTR2101, pTR2102).

In vitro transcription and transfection of CEF. For the production of run-off transcripts, all plasmid DNA was linearized with *Xho*I prior to transcription. *Xho*I has a unique site in the plasmid vector (position 11749). After phenol-chloroform and ether extractions, followed by ethanol precipitation, linear template DNA was suspended in sterile H_2O. Transcription reactions were done as described by Rice et al. (42). RNA products were analyzed by agarose

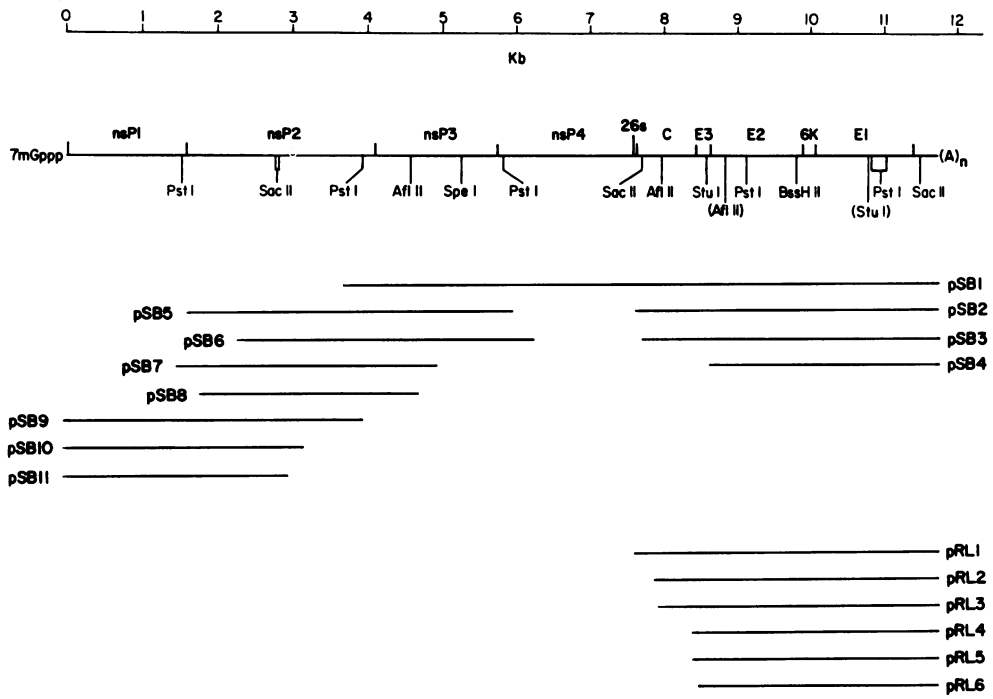

FIG. 1. Representative cDNAs generated from SB and SB-RL genomic RNAs and cloned into pBR322. Plasmids containing virus-specific inserts are designated pSB or pRL (derived from SB or SB-RL, respectively) and are numbered consecutively. The Sindbis virus genome organization is shown, including restriction sites used in the construction of recombinant virus strains. Plasmids pSB1 to pSB4 and pRL1 to pRL6 contain inserts which extend from the 3′ end of the genome and include 9 to 17 nt of the poly(A) tract. Plasmids pSB9, pSB10, and pSB11 contain inserts lacking 25, 14, and 21 nt, respectively, from the 5′ end of the genome. Kb, Kilobases. nsP1, nsP2, nsP3, and nsP4, Nonstructural genes.

gel electrophoresis and visualized by ethidium bromide staining to demonstrate synthesis of genome-length RNA. Approximately half of the product RNA was diluted in phosphate-buffered saline D (no Ca^{2+} or Mg^{2+}) for transfection of CEF monolayers in 24-well tissue culture plates (Costar). Monolayers were washed once with MEM that did not contain serum and then incubated in 1 ml (per well) of MEM containing 50 mM Tris hydrochloride (pH 7.5) and 200 μg of DEAE-dextran (Sigma Chemical Co.) per ml at 37°C for 1 h, followed by removal of the DEAE-dextran. To each well, 100 μl of a diluted transcription mix was added, and the plates were incubated at room temperature for 30 min. At this time, the inoculum was removed and 1 ml of fresh MEM plus 5% donor calf serum was added per well. Control wells were mock transfected with either linearized template DNA or phosphate-buffered saline D alone. Transfected cell monolayers were incubated at 37°C and checked periodically for cytopathic effect. Positive supernatants were harvested (typically 36 to 40 h posttransfection), and virus was passaged one time in BHK cells prior to phenotypic and sequence analyses.

Sequence analysis of recombinant viruses. The complete nucleotide sequence of the substituted region, containing E1 glycoprotein and 6K genes, was determined for both TR2000 and Toto1101 (*Bss*HII [position 9804] to *Sac*II [position 11484]). Also, sequence information was obtained for the entire E2 glycoprotein gene of Toto1101. The sequences of the entire substituted regions of the TR2100 and TR2200 class isolates (*Stu*I [position 8571] to *Pst*I [position 9119]) were verified with both TR2111 and TR2215. In addition, TR2120 and TR2270 were sequenced between nucleotides (nt) 8631 and 9119.

Nucleotide sequence data for recombinant virus strains were obtained by using both the Sanger dideoxynucleotide chain termination method (44) directly on virus genome RNA (1, 54) and the chemical cleavage method of Maxam and Gilbert (33) on plasmid DNA from which virus was derived. Oligonucleotide primers complementary to genomic RNA were used to determine most E1 and E2 glycoprotein gene sequences by dideoxynucleotide sequencing (13). For determination of the sequence in the 3′ untranslated region, a 14-base oligonucleotide, 5′-TTATAGGACTTATG-3′ (identical to bases 11330 to 11343), was synthesized. The 14-mer, designated UT1, was annealed to linearized, heat-denatured plasmid DNA to serve as the primer for dideoxynucleotide sequencing. Finally, the E3, 6K, and remaining E2 gene sequences were determined by the chemical cleavage method on DNA restriction fragments uniquely radiolabeled at one end.

Phenotypic assays of recombinant viruses. Unless otherwise noted in the text, pathogenesis testing of all virus stocks was done by subcutaneous inoculation of 100 PFU (50 μl) into neonatal CD-1 mice (18 to 24 h old, with 8 to 14 mice per sample; Charles River Breeding Laboratories, Inc.). Deaths were recorded for 14 days postinoculation and were used to calculate the percent mortality and mean survival time (4).

Sensitivity to neutralization by monoclonal antibody R6 was measured as previously described by using serial dilutions (1:100 through 1:3,200) of R6 ascites fluid (37). Control wells contained a 1:100 dilution of monoclone 5406 ascites fluid, an antibody raised against a nuclear inclusion protein encoded by tobacco etch virus (D. E. Slade, W. G. Dougherty, and R. E. Johnston, unpublished data). Endpoints were represented as the highest antibody dilution yielding an 80% reduction in the number of plaques compared with that of controls.

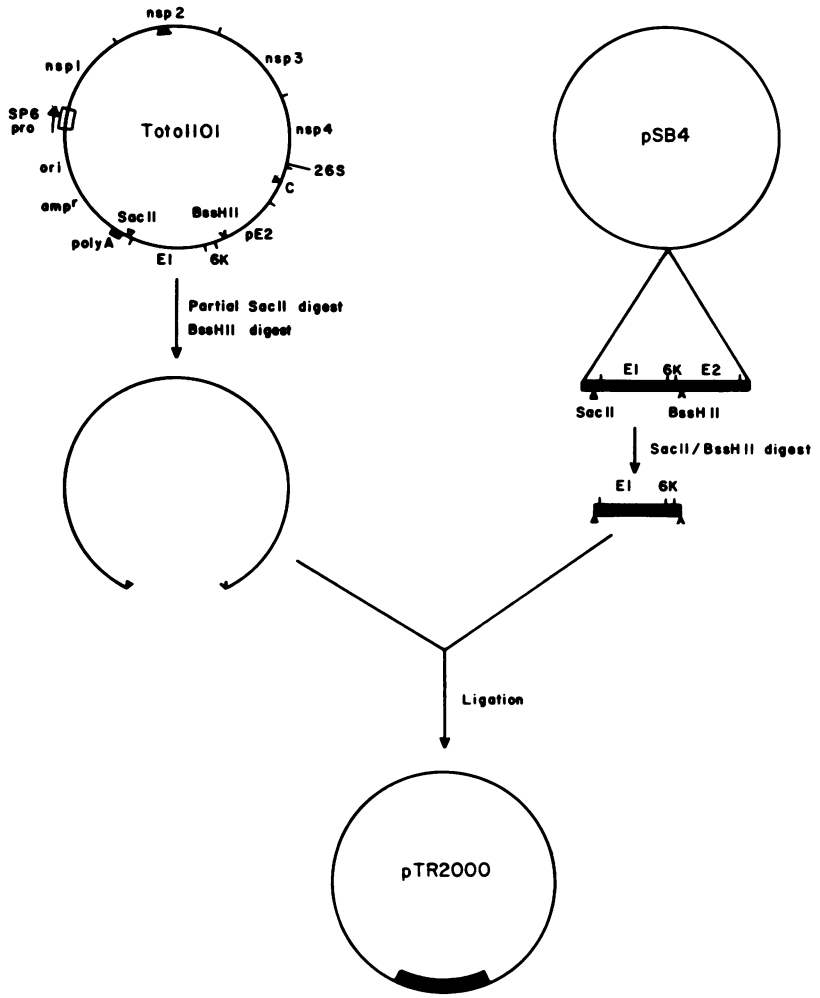

FIG. 2. Construction of plasmid pTR2000 from Toto1101 and pSB4. The full-length Sindbis virus cDNA insert is shown in Toto1101 with the *Bss*HII (V) and *Sac*II (▼) restriction sites indicated. The SB cDNA insert in pSB4 is expanded to show detail. The SP6 promoter sequence is shown as an open box. nsp1, nsp2, nsp3, and nsp4, Nonstructural genes. The construction of pTR2000 is detailed in Materials and Methods.

Virus penetration of BHK cells, defined as resistance to removal by trypsinization, was assayed as follows. BHK cell monolayers in 60-mm dishes, prechilled at 4°C for 1 h, were infected in the cold with 200 PFU of virus (0.2 ml per dish). Virus was allowed to attach for 1 h at 4°C, a temperature at which virus attaches but does not penetrate. At this time, cells were moved to a 37°C incubator to allow normal infection to proceed. Duplicate plates were removed at 5-, 20-, and 60-min time points and trypsinized for 5 min to remove any virus remaining at the cell surface. Infected cells were isolated after two cycles of differential centrifugation, suspended in 20 ml of MEM, and plated in 96-well tissue culture plates (150 μl per well). The number of infectious centers was calculated by using the Poisson distribution and was compared with a standard plaque assay to calculate the percentage of added virus which had penetrated.

RESULTS

Pathogenesis of virus derived from Toto1101. Subcutaneous inoculation of as little as 1 PFU of virulent SB into neonatal mice (less than 36 h old) consistently produces a fatal paralytic encephalitis, resulting in 100% mortality within 6 days (4, 24, 41). In contrast to SB, our attenuated prototype strain, SB-RL, is characterized by a low mortality rate, typically less than 40% and often 0%, with mean survival times of 10 to 14 days. Virus derived from Toto1101 also was attenuated in neonatal mice. Following subcutaneous inoculation of 100 PFU per animal, mortality was 11% and the mean survival time was 13.5 days. The full-length Toto1101 cDNA (11,703 total nt) is a hybrid containing portions from two Sindbis virus pools: HRsp (nt 1 to 2713 and 9804 to 11703) (50) and the laboratory standard strain of Sondra Schlesinger (SS std.; nt 2714 to 9804). The SS std. virus stock, which contributed 61% of the Toto1101 genome sequence, including those sequences for protein C and most of E2, was virulent, producing 100% mortality, with a mean survival time of 5.1 days. This result suggested that either the sequences derived from HRsp carried an attenuating mutation or during the construction of Toto1101 an attenuating mutation was introduced into the sequence. Since the surface proteins of several viruses have been implicated in the virulence phenotype (13, 15, 16, 28, 45, 47, 53), the glycoprotein genes of Toto1101 were examined for the presence of an attenuating mutation.

Sequence of the Toto1101 E2 glycoprotein gene. The nucleotide sequence of the Toto1101 E2 gene was determined as

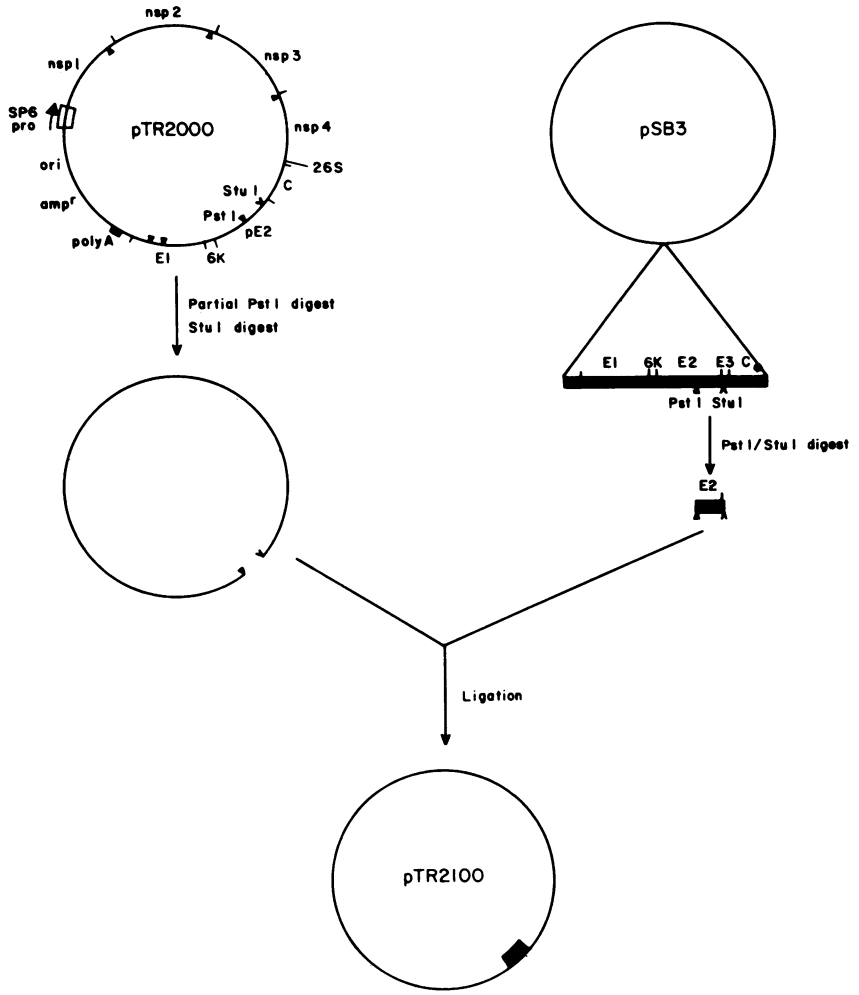

FIG. 3. Construction of plasmid pTR2100 from pTR2000 and pSB3. The full-length Sindbis virus cDNA insert of pTR2000, with E1 and 6K genes from pSB4, is shown with the *Pst*I (▼) and *Stu*I (V) restriction sites indicated. The SB cDNA insert in pSB3 is expanded to show detail. The SP6 promoter sequence is shown as an open box. The construction of pTR2100 is described in Materials and Methods. Plasmid pTR2200 was constructed from pTR2000 by using pRL1 instead of pSB3. Plasmid pRL1 contains an insert spanning approximately the same region of the Sindbis virus genome as pSB3.

described in Materials and Methods and compared with that of virulent Sindbis virus strains. Four coding differences were found between the E2 gene of our virulent laboratory strain, SB, and E2 of Toto1101 (Table 1). Three of the Toto1101 amino acids, those at residues 1, 3, and 70, are identical to amino acids found in the virulent strain HR-TX (10), as well as three additional strains (14) derived from the heat-resistant (HR) strain (8). These amino acid differences appear to be strain-specific changes between SB and those strains derived from the HR strain and appear to be unlikely possibilities for an attenuating mutation. The fourth coding difference, that found at residue 251, was unique to Toto1101 but involved a conservative amino acid substitution (Val for Ala) at a locus which had not been implicated previously in alteration of the pathogenesis phenotype. Therefore, we tentatively concluded that the attenuating mutation in Toto1101 did not reside in the E2 gene, as was subsequently demonstrated by experiments described below.

Replacement of Toto1101 6K and E1 genes. To test the possibility that the E1 gene of Toto1101 contained the attenuating mutation(s), an alternative approach was undertaken. In poliovirus type 1 (35, 39) and poliovirus type 3 (17, 30) systems, an allele replacement approach was used to identify or confirm sequences involved in attenuation of virulence. As a necessary first step in applying this technique to our study of SB, a cDNA library of SB was generated as described in Materials and Methods. cDNA was synthesized from purified genome RNA templates and cloned into the unique *Pvu*II site of pBR322. Colony hybridizations were used to identify those clones containing virus-specific inserts. The probe was ^{32}P-labeled first-strand cDNA synthesized from purified SB genome RNA templates randomly primed by calf thymus DNA hexanucleotides. Clones containing inserts of up to 8 kilobases in length and including all but the first 14 nucleotides at the 5′ end of the genome were identified in the SB library (Fig. 1). The lack of the 5′-most nucleotides in any of the pSB clones was expected because of the technique used to generate cDNA (19). A similar cDNA library was constructed from SB-RL by using a probe for colony hybridizations which contained primarily sequences complementary to those of E2 and C (Materials and Methods). Clones which included all or most of the structural gene region were identified in the SB-RL library (Fig. 1).

TABLE 1. Coding differences in Sindbis virus E2 glycoprotein genes[a]

Strain	Phenotype	Residue at position:						
		1	3	70	114	172	216	251
Toto1101	Attenuated	Ser	Ile	Lys	Ser	Gly	Glu	Val
HR-TX	Virulent	Ser	Ile	Lys	Ser	Arg	Val	Ala
SB	Virulent	Arg	Thr	Glu	Ser	Gly	Glu	Ala
TR2100[b]	Virulent	Arg	Thr	Glu	Ser	Gly	Glu	Val
TR2200[c]	Attenuated	Arg	Thr	Glu	Arg	Gly	Glu	Val
SB-RL	Attenuated	Arg	Thr	Glu	Arg	Gly	Glu	Ala

[a] Comparison of complete E2 nucleotide sequences (nt 8631 to 9899) for the Sindbis virus strains listed, showing all amino acid coding differences. E3 gene sequences also were obtained for TR2100 and TR2200 class recombinants to include the entire region of substitution (*Stu*I [nt 8571] to *Pst*I [nt 9119]). No additional nucleotide changes were found.

[b] Sequence data were obtained as described in Materials and Methods for two isolates of the TR2100 class, i.e., TR2111 and TR2120.

[c] Sequence data were obtained as described in Materials and Methods for two isolates of the TR2200 class, i.e., TR2215 and TR2270.

TABLE 2. Coding differences in Sindbis virus E1 glycoprotein genes[a]

Strain	Phenotype	Residue at position:		
		72	75	237
HR-TX	Virulent	Ala	Asp	Ser
SS std.	Virulent	Ala	Asp	Ala
Toto1101[b]	Attenuated	Ala	Gly	Ser
TR2000[b]	Virulent	Val	Asp	Ala
SB	Virulent	Val	Asp	Ala

[a] Comparison of complete E1 nucleotide sequences (nt 10065 to 11381) for HR-TX, Toto1101, TR2000, and SB, showing all amino acid coding differences. Partial E1 sequence was determined for SS std. to include codons listed in the table.

[b] The sequences for the entire substituted region (*Bss*HII [nt 9804] to *Sac*II [nt 11484]) were compared for recombinants Toto1101 and TR2000. No additional nucleotide changes were found.

The E1 and 6K genes of Toto1101 were removed by digestion at the unique *Bss*HII site (position 9804) and a *Sac*II site (position 11484) (Fig. 2). This region was subsequently replaced with the corresponding fragment from pSB4, resulting in a new construct designated pTR2000 (plasmid Toto-Raleigh-2000). Individual clones were screened with restriction endonucleases to identify clones of the proper size and arrangement. In addition, the fidelity of the replacement was verified by using the restriction enzyme *Stu*I. *Stu*I cuts once in this region in Toto1101 sequences (at nt 10770) but not in SB-derived sequences. Plasmid pTR2000 was linearized with *Xho*I and transcribed in vitro as described in Materials and Methods. The product RNA was transfected into CEF, and the virus produced was tested in neonatal mice. The virus derived from pTR2000 was virulent, producing 100% mortality, with a mean survival time of 4.3 days. These results indicated that none of the Toto1101 E2 coding differences, including the candidate site at E2 residue 251, were major determinants of the Toto1101 attenuation phenotype. Furthermore, the virulent phenotype of TR2000 suggested that the mutation or mutations responsible for the attenuation of Toto1101 resided within the 6K-E1 replacement.

The sequence of the substituted region was determined for both Toto1101 and TR2000 by using a combination of direct dideoxynucleotide sequencing of virus genome RNA and Maxam-Gilbert sequencing of plasmid DNA from which infectious RNA, and subsequently virus, was derived (Materials and Methods). Table 2 shows the three coding differences found in Toto1101 and TR2000, all in the E1 glycoprotein gene. Again, the sequences of two additional virulent Sindbis virus strains, HR-TX and SS std., were compared. Of the three coding changes found in Toto1101 and TR2000, only the Gly at residue 75 was unique to the attenuated Toto1101 and was not found in any of the virulent strains examined. These data suggest, but do not prove, that substitution of Gly for Asp at E1 residue 75 may be the causal attenuation mutation in Toto1101.

Analysis of the attenuation locus at E2 position 114. Comparisons of the glycoprotein gene sequences of SB, SB-RL, and virulent revertants of SB-RL produced strong genetic evidence that substitution of Arg at E2 position 114 of SB-RL for Ser in SB coordinately altered three phenotypes. These were attenuation in neonatal mice, increased sensitivity to neutralization by two E2-specific monoclonal antibodies, and increased rate of penetration into BHK cells (13, 37). The construction of pTR2000, a full-length clone producing virus lethal for neonatal mice, allowed us to test directly whether the SB-RL E2 mutation was sufficient in itself to produce these three distinguishing phenotypes. Two isogenic strains of virus which differed only at E2 residue 114 were constructed, beginning with the virulent full-length clone, pTR2000. As described in Materials and Methods and shown in Fig. 3, a 548-bp fragment from the *Stu*I site (position 8571) to a *Pst*I site (position 9119) of pTR2000 was replaced with corresponding regions from either the SB or SB-RL cDNA libraries. This substitution included the E2 114 codon. The two classes of recombinant constructs were designated pTR2100 (containing the SB replacement) and pTR2200 (containing the SB-RL replacement) (Fig. 4). A restriction endonuclease, *Afl*II, was used to confirm the replacement. *Afl*II cuts once in this region in Toto1101 and pTR2000 sequences (nt 8835) but not in SB or SB-RL sequences. In addition, two isolates of each type (TR2111 and TR2120, TR2215 and TR2270) were sequenced as described in Materials and Methods. A single nucleotide difference was present at position 8972, a cytidine in the two TR2100 class isolates versus an adenosine in the two TR2200 isolates, representing the single predicted amino acid-coding difference at the codon for E2 residue 114 (Table 1).

Four independent clones of the pTR2100 class (pTR2111, pTR2120, pTR2136, and pTR2147) and seven clones of the pTR2200 class (pTR2215, pTR2218, pTR2270, pTR2282, pTR2284, pTR2289, and pTR2290) were transcribed in vitro and transfected into CEF. The virus produced by each clone was tested for virulence in neonatal mice (Table 3). All isolates of the TR2100 class (Ser at residue 114) were virulent, producing 100% mortality, with mean survival times of 4.2 to 5.3 days, while all isolates of the TR2200 class (Arg at residue 114) were attenuated, producing mortality rates of 10 to 37.5%, with mean survival times of 12.3 to 13.9 days. The pathogenesis phenotypes of these recombinant viruses accurately reflected the phenotypes of the virulent (SB) and attenuated (SB-RL) prototype strains (37) and demonstrated the importance of E2 residue 114 in virulence in neonatal mice.

The effects of the Arg substitution at E2 residue 114 on the neutralization sensitivity and penetration phenotypes also were examined. Neutralization assays were performed with monoclonal antibody R6 as described in Materials and Methods. The four isolates which had been sequenced were tested. The TR2200 isolates (Arg at residue 114) were significantly more sensitive to neutralization by R6 than were the TR2100 isolates (containing Ser). Both TR2111 and

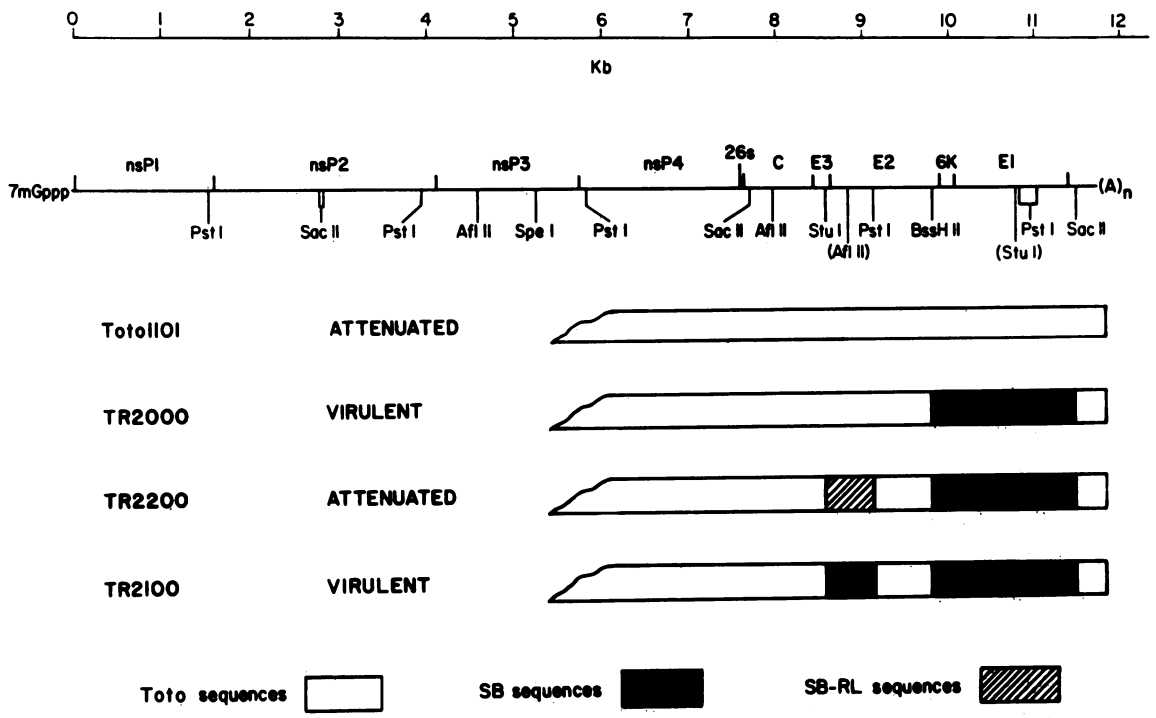

FIG. 4. Genome organization and derivation of recombinant strains of Sindbis virus. The structural gene region of recombinants derived from Toto1101 and cDNA clones of SB and SB-RL are shown with the corresponding recognition sites for restriction endonucleases used in their construction. Unique Toto1101 restriction sites, used in screening constructions, are shown in parentheses.

TR2120 had 80% plaque reduction neutralization endpoints of less than 1:100, as did the SB control (Table 3). The two TR2200 isolates, TR2215 and TR2270, had endpoints of greater than 1:3,200. Penetration assays were performed on the same four recombinant viruses. The two isolates of the TR2200 class and SB-RL consistently showed higher percentages of penetration at all three time points compared with those of SB or the isolates of the TR2100 class (Table 3). These data confirmed that in addition to attenuation in neonatal mice, increased neutralization sensitivity and accelerated penetration resulted from a single nucleotide change which alters E2 residue 114 from Ser to Arg.

TABLE 3. Phenotypic comparisons among in vitro recombinant strains

Strain	Neonatal mouse pathogenesis[a]		80% Neutralization endpoint[b]	% Penetration into BHK cells at[c]:		
	% Mortality	Mean survival (days)		5 min	20 min	60 min
SB	100	3.6	<1:100	6	23	57
TR2111	100	4.3	<1:100	2	18	62
TR2120	100	4.2	<1:100	3	10	65
SB-RL	25	12.3	1:1600	15	44	93
TR2215	37	12.3	>1:3200	10	48	100
TR2270	25	12.9	>1:3200	11	36	100

[a] Pathogenesis is reported as the percent mortality and mean survival time (days) after subcutaneous inoculation (8 to 14 mice per strain) as described in Materials and Methods. The mean survival time was determined by using an observation period of 14 days. Pathogenesis data were obtained for four isolates of the TR2100 class (mortality, 100%; mean survival time for the group, 4.7 ± 0.5 days) and seven isolates of the TR2200 class (mortality, 22.7 ± 10.4%; mean survival time for the group, 13.4 ± 0.6 days).

[b] Neutralization endpoint, versus monoclonal antibody R6, was determined as the highest serial dilution of antibody resulting in an 80% plaque reduction compared with that of controls.

[c] Virus penetration into BHK cells is expressed as a percentage of the original inoculum and calculated as described in Materials and Methods.

DISCUSSION

Previous reports by our group and others have implicated the surface glycoproteins of several alphaviruses as important virulence determinants. In addition to our results with the Sindbis virus-neonatal mouse system, which have identified at least three loci in the E2 glycoprotein that influence virulence (13, 25; D. F. Pence and R. E. Johnston, unpublished results), J. H Strauss and D. E. Griffin (personal communication) have described mutations at E2 residue 55 and one or more E1 residues (at positions 72 and 313), a combination of which appears to be required for the acquisition of Sindbis virus virulence for adult mice. In addition, a naturally occurring isolate of Ross River virus, avirulent in 1-week-old mice, contains an in-frame deletion of amino acids 55 to 61 in the E2 glycoprotein (52). Finally, the attenuation phenotype of a temperature-sensitive mutant (*ts*126) of Venezuelan equine encephalitis virus has been linked to an alteration in the E1 glycoprotein (16).

The work presented in this report has expanded our understanding of the molecular basis of alphavirus pathogenesis by using the recently developed full-length cDNA clone of Sindbis virus, Toto1101 (42), in conjunction with cDNA clones of our virulent (SB) and attenuated (SB-RL) prototype strains. These experiments have defined independent functional domains on each of the two glycoproteins which are important in neonatal mouse virulence. The attenuating mutation in Toto1101 was localized to the E1-6K gene region by replacement of this region with the equivalent sequences of SB. The resulting TR2000 recombinant was virulent in

neonatal mice and differed from Toto1101 at three amino acid positions. Of these differences, only E1 Gly-75 was unique to Toto1101 and was not found in any of the virulent strains sequenced. While this finding suggests that the previously undescribed mutation at E1 residue 75 (Asp to Gly) of Toto1101 may be responsible for the attenuated phenotype of virus derived from the clone, it does not rule out the possibility that changes at E1 residues 72 and 237, either alone or in combination with E1 Gly-75, contribute to the attenuated phenotype of Toto1101. Confirmation of the role of E1 Gly-75 in attenuation of virulence in neonatal mice will be obtained by using site-directed mutagenesis to construct virus strains which differ only at E1 residue 75.

It is interesting to note that one of the candidate E1 attenuation mutations lies at the amino-terminal end of a region which is highly conserved among all alphaviruses for which sequences have been published (E1 residues 75 to 108) (29). This sequence contains a highly hydrophobic domain which has been postulated to provide the fusogenic properties of alphaviruses observed after treatment at low pH (18).

In addition to studies of the E1 glycoprotein, the biological effects of alterations at E2 residue 114 have been defined further. By engineering two virus strains which differ only at this position, we have shown definitively that the substitution of Arg for Ser at E2 residue 114 is sufficient in itself to cause attenuation in neonatal mice, increased sensitivity to neutralization by monoclonal antibody R6, and accelerated penetration of BHK cells. E2 residue 114 is contained within a stretch of 18 hydrophobic and uncharged residues. It is likely that this region is not exposed to the aqueous environment surrounding the glycoprotein spike but is buried by the conformational folding of E2 or by the interaction of E2 with E1 in the virion structure. The mechanism whereby mutation at this locus could affect pathogenesis, neutralization sensitivity, and penetration phenotypes is not yet known. However, the substitution of an Arg for a Ser at an internal E2 residue would alter hydrophobic, electrostatic, and hydrogen bonding patterns and would require the accommodation of an amino acid with a much larger side chain either within E2 itself or within the E2-E1 complex. Such internal structural alterations may be sufficient to modulate the activity of external virion domains which interact directly with antibodies or which interact with cell surface structures governing penetration or tissue tropism.

Given the central role of the E2 locus at position 114 in influencing attenuation, penetration, and neutralization sensitivity of SB, one can consider either that E2 residue 114 is a major determinant of a multifunctional domain or that independent but overlapping domains controlling these phenotypes are all affected by this critical amino acid residue. We have taken advantage of this possible relationship by using accelerated penetration into BHK cells as a selective pressure for the generation of mutants attenuated in animals. With SB, such a selection resulted in the isolation of SB-FP, a rapidly penetrating, attenuated mutant (38) which acquired the Arg-for-Ser substitution at E2 residue 114 (13). With another alphavirus, Venezuelan equine encephalitis virus, eight rapidly penetrating mutants have been isolated, and seven of these were avirulent in animal models (26). Such mutants will be extremely useful in mapping attenuating mutations in alphaviruses. Ultimately, detailed knowledge of loci which control virulence, combined with the availability of full-length cDNA clones of these viruses, will allow the construction of genetically stable, live-virus vaccines having multiple, well-defined attenuating mutations. We are currently testing the feasibility of this approach in the Sindbis virus model system by using the E1 and E2 mutations described here.

The effect of genetic background is a key consideration in evaluating the phenotypic expression of specific point mutations in various genetic contexts. In the recombinants described here, introduction of an Arg into TR2000 at E2 residue 114 produced the same constellation of phenotypes which were observed with SB-RL, even though the genetic backgrounds of the two viruses were not identical. A priori, this need not have been the case. It is anticipated that additional loci exist, either in E2 or other genes, at which nucleotide differences can modulate or suppress the effect of the amino acid at E2 114. If this type of second-site mutation had been present in TR2000, the effect of the E2 114 Arg substitution would have gone undetected. We are attempting to identify such loci by isolating second-site revertants of SB-RL and other mutants containing Arg at E2 residue 114.

In summary, we have shown that determinants in each of the Sindbis virus glycoproteins can independently affect viral pathogenesis in neonatal mice. The amino acid residue at position 114 in the E2 glycoprotein plays a key role in multiple biological functions. In glycoprotein E1, residues 72, 75, or 237, either alone or in combination, influence Sindbis virus virulence in neonatal mice. It is likely that additional domains which affect Sindbis virus pathogenesis exist in the glycoprotein genes as well as in other regions of the genome and that alternative selection procedures will lead to their identification.

ACKNOWLEDGMENTS

We thank David Pence for technical assistance, Fred Fuller and Mark Conkling for help in the initial phases of cDNA cloning, James Carrington and William Dougherty for advice on Maxam-Gilbert sequencing techniques and DNA fragment isolation, and Walt Dallas for the gift of pBR322 sequencing primers. We also thank James Strauss and Diane Griffin for sharing data with us before publication.

This work was supported by the North Carolina Agricultural Research Service and Public Health Service grant A122186 from the National Institutes of Health. C.M.R. is a Pew scholar in the biomedical sciences and is supported by a grant from the Pew Memorial Trust and grant A124134 from the National Institutes of Health. H.V.H. is supported by a Monsanto-Washington University biomedical research contract.

LITERATURE CITED

1. **Ahlquist, P., R. Dasgupta, and P. Kaesberg.** 1981. Near identity of 3′ RNA secondary structure in bromoviruses and cucumber mosaic virus. Cell **23**:183–189.
2. **Ahlquist, P., R. French, M. Janda, and S. Loesch-Fries.** 1984. Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc. Natl. Acad. Sci. USA **81**:7066–7070.
3. **Andrews, C. H., and H. G. Pareira.** 1972. Viruses of vertebrates, 3rd ed. Bailliere Tindall Publishers, London.
4. **Baric, R. S., D. B. Moore, and R. E. Johnston.** 1980. In vitro selection of an attenuated variant of Sindbis virus. ICN-UCLA Symp. Mol. Cell. Biol. **18**:685–694.
5. **Baric, R. S., D. W. Trent, and R. E. Johnston.** 1981. A Sindbis virus variant with a cell-determined latent period. Virology **110**: 237–242.
6. **Barrett, P. N., B. J. Sheahan, and G. J. Atkins.** 1980. Isolation and preliminary characterization of Semliki Forest virus mutants with altered virulence. J. Gen. Virol. **49**:141–147.
7. **Bose, H. R., and B. P. Sagik.** 1970. The virus envelope in cell attachment. J. Gen. Virol. **9**:159–161.
8. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30**:204–213.
9. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a

fast and simple method for sequencing plasmid DNA. DNA Lab. Methods **4**:165–170.
10. **Condreay, L. D., and D. T. Brown.** 1986. Exclusion of superinfecting homologous virus by Sindbis virus-infected *Aedes albopictus* (mosquito) cells. J. Virol. **58**:81–86.
11. **Cress, D. E., M. C. Kiefer, and R. A. Owens.** 1983. Construction of infectious potato spindle tuber viroid cDNA clones. Nucleic Acids Res. **11**:6821–6835.
12. **Dalrymple, J. M., S. Schlesinger, and P. K. Russell.** 1976. Antigenic characterization of two Sindbis envelope glycoproteins separated by isoelectric focusing. Virology **69**:93–103.
13. **Davis, N. L., F. J. Fuller, W. G. Dougherty, R. A. Olmsted, and R. E. Johnston.** 1986. A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. Proc. Natl. Acad. Sci. USA **83**:6771–6775.
14. **Davis, N. L., D. F. Pence, W. J. Meyer, A. L. Schmaljohn, and R. E. Johnston.** 1987. Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. Virology **161**:101–108.
15. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80**:70–74.
16. **Emini, E. A., and M. E. Wiebe.** 1981. An attenuated mutant of Venezuelan equine encephalitis virus: biochemical alterations and their genetic association with attenuation. Virology **110**: 185–196.
17. **Evans, D. M. A., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, J. W. Almond, K. Currey, and J. V. Maizel, Jr.** 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature (London) **314**:548–550.
18. **Garoff, H., A. M. Frischauf, K. Simons, H. Lehrach, and H. Delius.** 1980. Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. Nature (London) **288**: 236–241.
19. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene **25**:263–269.
20. **Hahn, C. S., J. M. Dalrymple, J. H. Strauss, and C. M. Rice.** 1987. Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. Proc. Natl. Acad. Sci. USA **84**:2019–2023.
21. **Hanahan, D., and M. Meselson.** 1980. Plasmid screening at high colony density. Gene **10**:63–67.
22. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
23. **Johnson, B. J. B., R. M. Kinney, C. L. Kost, and D. W. Trent.** 1986. Molecular determinants of alphavirus neurovirulence: nucleotide and deduced protein sequence changes during attenuation of Venezuelan equine encephalitis virus. J. Gen. Virol. **67**:1951–1960.
24. **Johnson, R. T., H. F. McFarland, and S. E. Levy.** 1972. Age-dependent resistance to viral encephalitis: studies of infections due to Sindbis virus in mice. J. Infect. Dis. **125**:257–262.
25. **Johnston, R. E., N. L. Davis, D. F. Pence, S. Gidwitz, and F. J. Fuller.** 1987. Nucleic acid sequence analysis of Sindbis pathogenesis and penetration mutants, p. 467–476. *In* M. A. Brinton and R. L. Rueckert (ed.), Positive strand RNA viruses. Alan R. Liss, Inc., New York.
26. **Johnston, R. E., and J. F. Smith.** 1988. Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. Virology **162**:437–443.
27. **Kaplan, G., J. Lubinski, A. Dasgupta, and V. R. Racaniello.** 1985. *In vitro* synthesis of infectious poliovirus RNA. Proc. Natl. Acad. Sci. USA **82**:8424–8428.
28. **Kaye, K. M., D. R. Spriggs, R. Bassel-Duby, B. N. Fields, and K. L. Tyler.** 1986. Genetic basis for altered pathogenesis of an immune-selected antigenic variant of reovirus type 3 (Dearing). J. Virol. **59**:90–97.
29. **Kinney, R. M., B. J. B. Johnson, V. L. Brown, and D. W. Trent.** 1986. Nucleotide sequence of the 26S mRNA of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and deduced sequence of the encoded structural proteins. Virology **152**:400–413.
30. **La Monica, N., J. W. Almond, and V. R. Racaniello.** 1987. A mouse model for poliovirus neurovirulence identifies mutations that attenuate the virus for humans. J. Virol. **61**:2917–2920.
31. **La Monica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
34. **Mizutani, S., and R. J. Colonno.** 1985. In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. J. Virol. **56**:628–632.
35. **Nomoto, A., M. Kohara, S. Kuge, N. Kawamura, M. Arita, T. Komatsu, S. Abe, B. L. Semler, E. Wimmer, and H. Itoh.** 1987. Study on virulence of poliovirus type 1 using in vitro modified viruses, p. 437–452. *In* M. A. Brinton and R. L. Rueckert (ed.), Positive strand RNA viruses. Alan R. Liss, Inc., New York.
36. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. USA **79**:5793–5797.
37. **Olmsted, R. A., R. S. Baric, B. A. Sawyer, and R. E. Johnston.** 1984. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. Science **225**: 424–427.
38. **Olmsted, R. A., W. J. Meyer, and R. E. Johnston.** 1986. Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. Virology **148**: 245–254.
39. **Omata, T., M. Kohara, S. Kuge, T. Komatsu, S. Abe, B. L. Semler, A. Kameda, H. Itoh, M. Arita, E. Wimmer, and A. Nomoto.** 1986. Genetic analysis of the attenuation phenotype of poliovirus type 1. J. Virol. **58**:348–358.
40. **Rancaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
41. **Reinarz, A. B. G., M. G. Broome, and B. P. Sagik.** 1971. Age-dependent resistance of mice to Sindbis virus infection: viral replication as a function of host age. Infect. Immun. **3**:268–273.
42. **Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang.** 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. **61**:3809–3819.
43. **Rice, C. M., and J. H. Strauss.** 1982. Association of Sindbis virion glycoproteins and their precursors. J. Mol. Biol. **154**:325–348.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
45. **Schlesinger, J. J., M. W. Brandriss, and T. P. Monath.** 1983. Monoclonal antibodies distinguish between wild and vaccine strains of yellow fever virus by neutralization, hemagglutination inhibition, and immune precipitation of the virus envelope protein. Virology **125**:8–17.
46. **Schlesinger, R. W. (ed.).** 1980. The togaviruses: biology, structure, replication. Academic Press, Inc., New York.
47. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53**:926–934.
48. **Stanway, G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond.** 1984. Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin

vaccine derivative P3/Leon $12a_1b$. Proc. Natl. Acad. Sci. USA **81**:1539–1543.

49. **Strauss, E. G., C. M. Rice, and J. H. Strauss.** 1984. Complete nucleotide sequence of the genomic RNA of Sindbis virus. Virology **133**:92–110.
50. **Strauss, E. G., and J. H. Strauss.** 1980. Mutants of alphaviruses: genetics and physiology, p. 393–426. *In* R. W. Schlesinger (ed.), The togaviruses: biology, structure, replication. Academic Press, Inc., New York.
51. **Taniguchi, T., M. Palmieri, and C. Weissmann.** 1978. Qβ DNA containing hybrid plasmids giving rise to Qβ phage formation in the bacterial host. Nature (London) **274**:223–228.
52. **Vrati, S., S. G. Faragher, R. C. Weir, and L. Dalgarno.** 1986. Ross River virus mutant with a deletion in the E2 gene: properties of the virion, virus-specific macromolecule synthesis, and attenuation of virulence for mice. Virology **151**:222–232.
53. **Webster, R. G., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50**:665–666.
54. **Zimmern, D., and P. Kaesberg.** 1978. 3′-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **75**:4257–4261.