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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

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We constructed full-length cDNA clones of Sindbis virus that can be transcribed in vitro by SP6 RNA polymerase to produce infectious genome-length transcripts. Viruses produced from in vitro transcripts are identical to Sindbis virus and show strain-specific phenotypes reflecting the source of RNA used for cDNA synthesis. The cDNA clones were used to confirm the mapping of the causal mutation of *ts2* to the capsid protein. A general strategy for mapping Sindbis virus mutations is described and was used to identify two lethal mutations in an original full-length construct which did not produce infectious transcripts. An *Xba*I linker was inserted in the cDNA clone near the transcriptional start of the subgenomic mRNA; the resulting virus retains the *Xba*I recognition sequence, thus providing formal evidence that viruses are derived from in vitro transcripts of cDNA clones. The potential applications of the cDNA clones are discussed.

Sindbis virus is the type species of the *Alphavirus* genus. It is among the least pathogenic of the alphaviruses, a group that includes such important pathogens as Venezuelan, Western, and Eastern equine encephalitis viruses (25). In nature, Sindbis virus is transmitted by mosquitos, and its alternate vertebrate host is usually a bird or a mammal (80). In vitro, Sindbis virus infects a variety of avian, mammalian, reptilian (12), and amphibian cells (40). It also infects many species of mosquitos, a tick (80), and *Drosophila melanogaster* (6). Infection of vertebrate cells in culture is usually characterized by a dramatic cytopathic effect and rapid cell death, whereas growth in mosquito cells often leads to the establishment of chronic or persistent infections.

Since its isolation in 1952 (80), Sindbis virus and the closely related Semliki Forest virus have been widely studied (for a review, see reference 69). The genome of Sindbis virus consists of a single molecule of single-stranded RNA, 11,703 nucleotides (nt) in length (74, 76). The genomic RNA is infectious, is capped at the 5′ terminus and polyadenylated at the 3′ terminus, and serves as mRNA and is therefore by convention of plus polarity. During infection of vertebrate cells, the virus attaches to the cell surface and is endocytosed. Acidification of the endocytic vesicle activates the viral envelope proteins to mediate fusion of the viral envelope with the vesicle wall, thus depositing the genome in the cytoplasm (31). The 5′ two-thirds of the genomic 49S RNA is translated during early infection to produce two polyproteins that are processed by cotranslational or posttranslational cleavage into four nonstructural proteins (called nsP1 through nsP4, numbered in order as they appear in the genome sequence; 74) presumably required for RNA replication. A full-length minus strand complementary to the genomic RNA is first synthesized; this minus strand then serves as a template for the synthesis of new 49S genomic RNA molecules. The three structural proteins are encoded in the 3′ one-third of the genome. They are expressed by transcription of the minus strand at an internal site to produce a 26S subgenomic mRNA that is 4,106 nt long and colinear with the 3′-terminal one-third of the 49S genome. The subgenomic mRNA is capped and polyadenylated. It does not serve as a template for minus-strand synthesis, nor is it packaged into mature virions. Translation of the 26S mRNA produces a polyprotein that is cleaved cotranslationally by a combination of viral and presumably host-encoded proteases to give the capsid protein (C) and the two envelope glycoproteins (E1 and PE2, the precursors of the virion E2). The translation, proteolytic cleavage, glycosylation, fatty acid attachment, and transport of these proteins have been extensively studied both in vivo and in vitro as models for membrane protein biogenesis (for a review, see reference 68). The capsid protein complexes with the 49S genomic RNA to form intracellular icosahedral nucleocapsids, which interact with the cytoplasmic domains of the transmembrane envelope proteins at the cell surface, resulting in the budding of virus from the plasma membrane (23). The virus thus acquires a lipid envelope derived from the host cell. The proteins and RNA in mature virions are exclusively virus encoded.

Genetic analysis of Sindbis virus has been facilitated by the isolation of *ts* mutants defective in RNA replication (RNA^- mutants) and in the production of the structural proteins (RNA^+ mutants). These mutants have been grouped by complementation into three RNA^+ and four RNA^- groups (8–10, 65, 67, 73, 75). Representative mutant-revertant pairs from RNA^+ groups C, D, and E have been analyzed by sequence analysis, and there is excellent correlation between specific sequence changes and phenotypes (2, 27, 44). None of the RNA^- defects has been rigorously assigned to specific nonstructural proteins or RNA sequences.

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Over the past several years, recombinant DNA technology has profoundly advanced the study of RNA viruses. Sequence analysis of cDNA clones of RNA viruses contributed to the identification of potential translation products and, by sequence comparison, of conserved, potentially *cis*-acting sequences implicated in replication and gene expression. However, rigorous tests of these conclusions require the manipulation and expression of functional viral genomes. This has led to the construction of cDNA copies of the bacteriophage Qβ (79) and poliovirus (55, 60, 70) which, after transfection of the appropriate host cells, produce infectious transcripts in vivo. Subsequently, a number of laboratories have succeeded in producing infectious transcripts synthesized in vitro from cDNA clones for several plant viruses (1, 13, 14, 18, 24, 50, 71, 77, 82) and animal viruses (16, 30, 52, 81). Using the same approach, we previously reported the deletion mapping of the *cis*-acting sequences required for the replication and encapsidation of defective-interfering genomes of Sindbis virus (43). We describe here the construction of cDNA clones of Sindbis virus capable of producing infectious transcripts in vitro. We also demonstrate the use of these clones for mapping the causal lesions for mutants with interesting phenotypes and for site-directed mutagenesis to construct novel mutants of Sindbis virus.

MATERIALS AND METHODS

Virus stocks, growth, and purification. Sindbis virus stocks derived from the cDNA clones described below, as well as the HR small plaque strain (HRsp, stock no. 80-5724; 74); temperature-sensitive (*ts*) mutants *ts*2, *ts*6, *ts*11, *ts*18, and *ts*24; and an HR large plaque strain (HR; S. Schlesinger laboratory strain; 8, 9) were grown on monolayers of primary or secondary chicken embryo fibroblasts (CEF) and titers were determined as previously described (73). The *ts* mutants were plaque purified for use in complementation analyses. The virus was purified by polyethylene glycol precipitation from the culture media, followed by successive velocity sedimentation and equilibrium density centrifugations (58). The isolation of intracellular (43, 57, 84) and virion RNA (4, 58, 62) was as described.

General recombinant DNA techniques. Restriction endonucleases and DNA modifying enzymes were purchased from commercial sources and used essentially as recommended by the manufacturer. Plasmids were grown, purified, and analyzed by standard methods with minor modifications (47).

cDNA synthesis, purification, and primer extension. cDNA synthesis and cloning of Sindbis virion RNA from purified virus have been described (62, 74). Double-stranded cDNA restriction fragments used to construct derivatives of Toto5 (see below) were purified by extraction from preparative low-melting-temperature agarose gels (83). For sequence analysis of RNA prepared from Toto1000- and Toto1002-infected CEF cells, a 5′-end-labeled oligonucleotide (complementary to nt 7644 through nt 7662 of the Sindbis virus genome; 74) was annealed to total cytoplasmic RNA and extended with avian myeloblastosis virus reverse transcriptase (15). Discrete extension products corresponding to the 5′ end of 26S RNA were isolated from a preparative sequencing gel and sequenced by the chemical method (48).

Construction of full-length Sindbis cDNA clones. An original full-length cDNA clone of Sindbis virus HRsp, called Toto1 (Fig. 1), was reconstructed from the four *Hin*dIII subclones of the Sindbis virus genome used for determination of the sequence (74). The *Hin*dIII cDNA fragments were subcloned into Proteus1. Proteus1 consists of the replicon and the beta-lactamase gene of pBR322, from the *Tha*I (position 2522) to *Eco*RI (position 1) sites, with the *Tha*I end fused to the filled-in *Bgl*II end of a *Bgl*II-*Cla*I fragment from SP6 phage containing a SP6 RNA polymerase promoter (E. Butler and P. Little, unpublished data.) The same promoter was subsequently used to construct pSP64 (49). A polylinker sequence (5′-AAGCTTCTAGAGATCTGCAGGTCGAC GGATCCCCGGGAATTCCGCGGAATT-3′) was positioned between the *Cla*I site from SP6 phage and the filled-in *Eco*RI site from pBR322.

The 5′ terminus of Sindbis virus cDNA was fused to the *Sma*I site in the polylinker of Proteus1 (74). The GC tail at the 5′ terminus regenerated the *Sma*I site. The site was converted to a *Cla*I site by cutting with *Sma*I and ligating in *Cla*I linkers (pCATCGATG), and it was used to fuse the Sindbis virus 5′ terminus to the *Cla*I site downstream of the SP6 promoter. This 5′ clone included the *Hin*dIII site at position 125 of Sindbis virus. The 3′ terminus of Sindbis virus cDNA was cloned as a *Hin*dIII (position 6267) to the poly(A)-poly(T) fragment between the *Hin*dIII and *Sma*I sites of Proteus1 (74). The 5′ and 3′ clones were combined by ligating at the respective *Hin*dIII sites. (The *Sst*II site in the polylinker was converted to a *Sst*I site, by using *Sst*I linkers, for subsequent runoff transcription). The two internal *Hin*dIII fragments (nt 125 to 1302 and 1302 to 6267) were then inserted, in the correct order and orientation, in the *Hin*dIII site of the 5′-3′ clone to produce Toto1. Toto1 has 198 nt between the SP6 transcription start and the 5′ nucleotide of the Sindbis virus genome, including 9 G residues, derived during cDNA cloning, immediately 5′ to the Sindbis virus sequences. The 3′-terminal poly(A) tract consists of ca. 35 residues followed by the sequence 5′-G GGAATTCGAGCTC-3′, the last six nucleotides of which comprise the *Sst*I site used for runoff transcription.

Toto2 was derived from Toto1 by inserting a *Cla*I linker (pCATCGATG) into a *Hae*III site in SP6-derived sequences, cutting with *Cla*I, and ligating to the SP6-derived *Cla*I site downstream of the SP6 promoter (see above). This left 48 nt between the SP6 transcription start and the Sindbis virus 5′ nucleotide. Toto3 was derived from Toto2 by deleting all SP6-derived sequences preceding the Sindbis virus sequences. We took advantage of an *Hph*I recognition sequence in the SP6 promoter that directs *Hph*I cutting after the A 1 nt 3′ from the G that corresponds to the major transcriptional initiation site for SP6 RNA polymerase (unpublished observations; 29). We fused the *Hph*I cut site (T4 DNA polymerase treated to remove the 3′ protruding A) to a *Sfa*NI site (filled in by treatment with the large fragment of DNA polymerase I) created by the *Hae*III-*Cla*I linker fusion in Toto2. Toto3 has eight extra G's preceding the Sindbis virus sequences. Toto5, derived from Toto3, had all but one extra G removed. The fragment from Toto3 containing the SP6 promoter and the 5′-terminal 125 nt of Sindbis virus (to a *Hin*dIII site at position 125) was first subcloned into πAN8 (W. C. Hollifield et al., unpublished data). This clone, called πK1, has an unique *Rsa*I site 14 nt downstream from the Sindbis virus 5′ terminus and an unique *Hph*I recognition sequence in the SP6 promoter (see above). A pair of complementary synthetic oligonucleotides, corresponding to the Sindbis virus 5′ terminus through nt 14, was ligated to *Rsa*I- and *Hph*I-digested and T4 DNA polymerase-treated πK1. The resulting clone, πK2, was verified by sequence analysis, and the SP6 promoter-Sindbis virus 5′-terminal sequences were recloned into Toto3 to give Toto5.

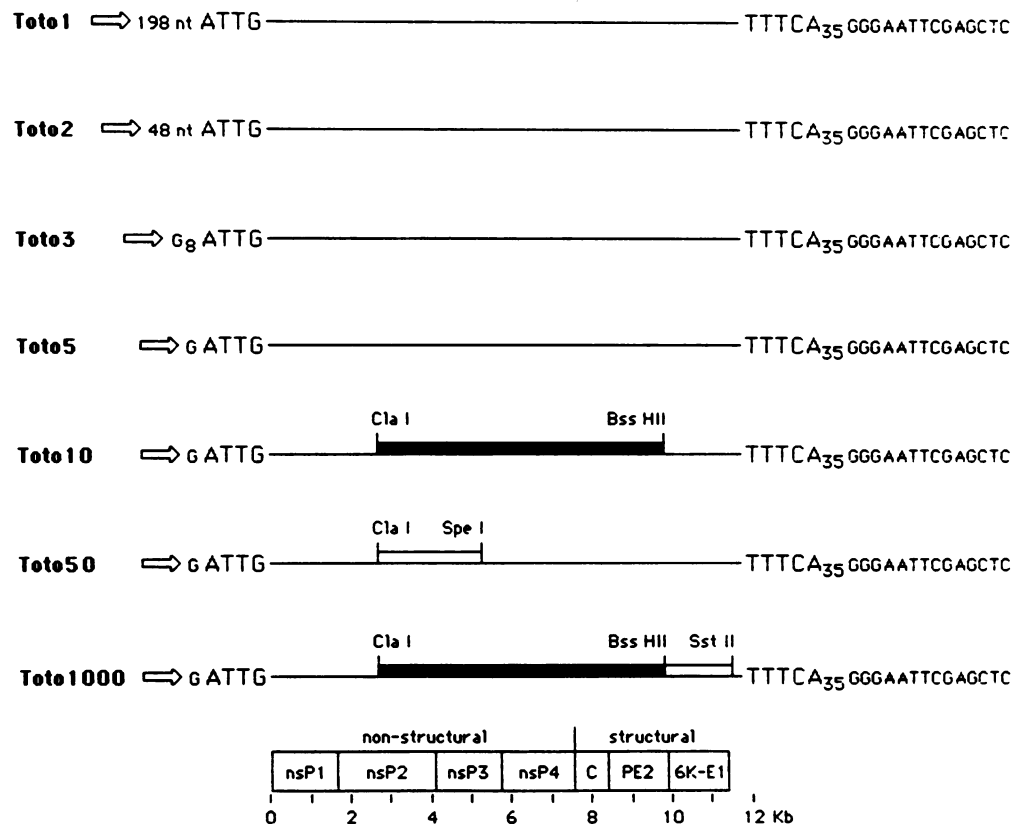

FIG. 1. Structure of full-length cDNA clones of Sindbis virus. The open arrow denotes the SP6 RNA polymerase promoter. Extraneous sequences preceding the Sindbis virus sequences are indicated. The first and last four nucleotides of Sindbis are shown. All clones have a 3' poly(A) tract ca. 35 nt long. The sequence between the poly(A) tract and the *SstI* site used for runoff transcription is shown. All clones consisted of HRsp cDNA except the HR-derived sequences in Toto10 and Toto1000, indicated by filled-in bars extending from *ClaI* 2713 to *BssHII* 9804. Toto1000 contains an additional substitution from *BssHII* 9804 to *SstII* 11484 of HRsp cDNA (open bar). The open bar in Toto50 indicates HRsp-derived sequences, from *ClaI* 2713 to *SpeI* 5262, from an independent HRsp cDNA clone. The coding regions of the viral proteins are outlined at the bottom of the figure (nucleotide positions are indicated in kilobases). The plasmid beta-lactamase gene and replicon are not shown.

A derivative of Toto5 was made by substituting the *ClaI* (nt 2713) to *SpeI* (nt 5262) region of Toto5 with that from an independent subclone (of the *HindIII* [nt 1302] to *HindIII* [nt 6267] region) of cDNA from Sindbis virus HRsp to produce Toto50. Similarly, the *ClaI* to *SpeI* fragment from Sindbis virus HR cDNA was used to replace the corresponding region of Toto5 to give Toto5CS. The same *ClaI* to *SpeI* fragment of Sindbis virus HRsp cDNA was used to replace the corresponding region of Toto3 to give Toto30. The *ClaI* (nt 2713) to *AvrII* (nt 4280) region of Toto5 was replaced with Sindbis virus HR cDNA from Toto1101 (see below) to give Toto5CA. Subclones of Toto5 were made for smaller substitutions between the *ClaI* and *AvrII* sites. Two fragments of Toto5, from *HindIII* (nt 1302) to *BamHI* (nt 4633) and from *BglII* (nt 2288) to *BamHI* (nt 4633), were cloned in πAN7 (46). Various regions of the two subclones were replaced with corresponding regions from the *HindIII* subclone of HRsp cDNA (see above). The *ClaI* (nt 2713) or *BglII* (nt 2288) to *AvrII* (nt 4280) fragment from the resulting clones was then used to replace the corresponding region of Toto5 to produce the clones Toto5CP, Toto5CN, Toto5BN, Toto5NT, Toto5PT, Toto5Bs, and Toto5TA (see Results and Table 2).

The *ClaI* (nt 2713) to *BssHII* (nt 9804) interval of Toto5 was also replaced with the corresponding Sindbis virus HR cDNA, transformants were screened for clones that produced infectious transcripts, and one such clone was called Toto10. The *BssHII* (nt 9804) to *SstII* (nt 11484) interval of Toto10 was further replaced with Sindbis virus HRsp cDNA, and transformants were screened for clones that gave infectious transcripts, one of which was called Toto1000. Toto10 and Toto1000 are therefore hybrids of Sindbis virus HRsp and HR (Fig. 1).

A plasmid clone, called πnsP4C1, consisting of the *AccI* (nt 7492) to *NcoI* (nt 8038) region of Toto1000 cloned in πAN7, was digested with *RsaI* (nt 7611) and ligated in the presence of 43 mM *XbaI* linker (pTCTAGA). After transformation, clones that contain the *XbaI* linker were selected by digestion with *XbaI*, isolation of linear DNA, ligation, and retransformation. A representative clone, πC1R, with a single *XbaI* linker inserted at *RsaI* (nt 7611) was then extended in the 5' direction by cloning in the *HindIII* (nt 6267) to *AccI* (nt 7492) interval from Toto1000. The resulting clone, πnsP4CR, contains unique *HpaI* (nt 6919) and *AatII* (nt 7999) sites, which were used to excise the *XbaI* linker-containing fragment for replacement into Toto1000 to give Toto1002.

λToto1101, a lambda phage clone of a full-length copy of the Sindbis virus genome downstream from the SP6 promoter, was constructed from λgt*WES*-λB (41) by replacing the *SstI*-*XhoI* region (λ positions 25881 to 33498) with the *SstI* to *XhoI* fragment of Toto1101 containing the SP6

promoter and the Sindbis virus genome (Toto1101 is derived from Toto1000 by replacing the 3′ *Sst*I site used for runoff transcription with a *Xho*I site).

Toto *ts*2.1 was constructed by replacing the *Nar*I (nt 7870) to *Mst*II (nt 8892) region of Toto1000 with the corresponding region from a cDNA clone of *ts*2 designated *ts*2A (this clone was produced as described in reference 44 and contains the *ts*2 RNA sequence from the *Hin*dIII site at nt 6267 to the poly(A) tail, and it was generously provided by C. S. Hahn). The presence of the putative *ts*2 mutation (27) in the resulting clone was verified by chemical sequence analysis.

In vitro transcription and capping. RNA transcripts were synthesized in vitro by SP6 RNA polymerase with either supercoiled plasmid templates or plasmid DNAs digested with appropriate restriction endonucleases for production of runoff transcripts. Reactions containing 40 mM Tris chloride (pH 7.6); 6 mM $MgCl_2$; 2 mM spermidine; 1 mM each ATP, CTP, UTP, and GTP; 100 μg of nuclease-free bovine serum albumin per ml; 5 mM dithiothreitol; 500 U of human placental RNase inhibitor per ml; 400 U of SP6 RNA polymerase per ml; and 10 to 100 μg of template DNA per ml were incubated at 38°C for 1 h (11, 36, 49). 5′-capped transcripts were produced by inclusion of 1 mM $m^7G(5')ppp(5')G$ or $m^7G(5')ppp(5')A$ cap analogs in the transcription reaction (34). Trace quantities of ^{3}H-UTP or [α-^{32}P]CTP included in the transcription reactions allowed quantitation and gel analysis of the RNA transcripts. Incorporation was measured either by trichloracetic acid precipitation or by adsorption to DE 81 filter paper (Whatman, Inc., Clifton, N.J.) (47). Template DNAs did not need to be purified by banding on CsCl gradients, and several protocols for rapid plasmid and phage DNA preparation were satisfactory, provided that the template DNA was RNase-free and salt-free (11). Transcripts made from supercoiled plasmid DNA from minipreps were infectious. However, their specific infectivities were variable, typically about 10-fold lower than the transcripts produced by runoff transcription. RNA products which comigrated with full-length runoff transcripts were found when either uncut lambda or supercoiled plasmid templates were used, and such products may result from the falloff of the SP6 polymerase in the poly(A) tract or shortly thereafter. For rapid assay of transcript infectivity or for production of virus stocks, the transcription mix was used directly for transfection (see below). For purified transcripts, the template DNA was removed by digestion with DNase I, followed by extraction with phenol-chloroform and ethanol precipitation.

RNA transfection. Typically, confluent monolayers of secondary CEF in 35-mm tissue culture plates (about 10^6 cells) were used for transfection. After washing once with Eagle minimal essential medium with Earle salts (MEM) without serum, the cells were incubated with 1.5 ml of MEM containing 50 mM Tris chloride (pH 7.3) (at 25°C) and 200 μg of DEAE dextran per ml (average molecular weight, 500 kilodaltons [kDa]; Sigma Chemical Co., St. Louis, Mo.) for 15 to 60 min at 37°C. This medium was removed and 200 μl of RNA or transcription mix (diluted in phosphate-buffered saline) was added to the cells and incubated at room temperature for 15 to 60 min with occasional rocking. PFU were quantitated by overlaying the monolayers with 2 ml of 1.2% agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) in MEM and 2% fetal calf serum followed by incubation at 37°C for wild-type stocks or at 30 and 40°C for *ts* mutants. Plaques were visualized by staining with neutral red or crystal violet after 24 to 48 h (37 or 40°C incubation) or 48 to 72 h (30°C incubation). For production of virus stocks, the transfection mix was removed and the cells were incubated with 2 ml of MEM containing 2% fetal calf serum for 24 (37°C) or 48 h (30°C).

RNA gel analysis. Monolayers of secondary CEF cells were infected with Sindbis virus stocks at a multiplicity of infection of 20 PFU per cell. Virus-specific RNA was labeled in the presence of MEM containing 1 μg of actinomycin D per ml and 20 μCi of [^{3}H]uridine per ml from 3 to 6 h postinfection. Cytoplasmic RNA was isolated, denatured with glyoxal and dimethylsulfoxide, and analyzed by electrophoresis in 1% agarose gels (47).

Analysis of 5′ and 3′ termini of transcripts and virion RNAs. For 5′-end analysis, in vitro transcripts were labeled by transcription in the presence of [α-^{32}P]ATP. Virion RNAs were labeled in vivo with $^{32}PO_4$. Cells were infected with Sindbis virus HRsp or virus derived from Toto50 and incubated with phosphate-free MEM and 3% chicken serum. At 5 h postinfection, 100 μCi of $^{32}PO_4$ per ml was added, and the infection was allowed to proceed for an additional 9 h. The virus was purified, and the virion RNA was extracted (see above). For 3′-end labeling, [5′-^{32}P]pCp was first synthesized with 3′ CMP and crude [γ-^{32}P]ATP (7,000 Ci/mmol, 200 μCi/μl; ICN Pharmaceuticals Inc., Irvine, Calif.). The reaction contained 3 μl of crude [γ-^{32}P]ATP, 4.5 nmol of 3′ CMP, and 5 U of T4 kinase in a final volume of 5 μl and was incubated for 6 h at 37°C, after which it was judged to be essentially complete by ascending chromatography on polyethyleneimine cellulose (developed in 2M sodium formate, pH 3.5). Approximately 0.5 pmol of either virion RNA or in vitro transcripts was 3′-end labeled with the crude [5′-^{32}P]pCp and T4 RNA ligase (21). The reactions contained 25 mM Tris chloride (pH 8.3), 7 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 15% dimethyl sulfoxide, 70 mCi of [5′-^{32}P]pCp per ml, 1,000 U of T4 RNA ligase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) per ml, and 200 μg of RNA per ml and were incubated at 4°C for 20 h. The end-labeled RNAs were recovered by ethanol precipitation after extraction with phenol and chloroform. The labeled RNAs were further purified by two selections with oligo(dT) cellulose (Pharmacia, Inc., Piscataway, N.J.) by following the directions of the supplier. RNA samples containing 10 μg of carrier tRNA were digested with 1 U of RNase T2 at 50°C for 1 h in 50 mM NH_4OAc (pH 5.3). The digestion products were spotted onto cellulose plates and separated by two-dimensional thin-layer chromatography (34).

Protein analysis. For comparison of structural and nonstructural proteins of parental virus strains and stocks derived from infectious transcripts, secondary CEF cells were infected at a multiplicity of infection of 20 to 50 PFU/cell. At 3 h postinfection, cells were labeled in methionine-free media containing 20 μCi of L-[^{35}S]methionine per ml (ICN translabel) for 1 h. Proteins in *ts*2-infected cells or in cells infected with virus derived from Toto*ts*2.1 were labeled from 7 to 8 h (30°C) or 6 to 7 h (40°C) postinfection. Cell extracts were prepared by washing the monolayers twice with ice-cold phosphate-buffered saline and by lysis of the monolayer with 0.5% sodium dodecyl sulfate containing 40 μg of phenylmethylsulfonyl fluoride per ml. Samples were electrophoresed on 10% discontinuous sodium dodecyl sulfate-polyacrylamide gels (37), treated for fluorography (39), and exposed to X-ray film. Sindbis virus-specific structural and nonstructural protein standards were prepared by immunoprecipitation (63) with monospecific polyclonal rabbit antisera to each of the proteins (63); antisera to nsP1, nsP2, nsP3, and nsP4 were generously provided by W. Reef Hardy, California Institute of Technology, Pasadena.

RESULTS

Infectious in vitro transcripts of Sindbis virus cDNA clones. We constructed full-length cDNA copies of the Sindbis virus RNA genome, positioned downstream from the SP6 RNA polymerase promoter, such that transcripts have either 198 nt (Toto1), 48 nt (Toto2), 8 G's (Toto3, Toto30), or a single G (Toto5, Toto10, Toto50, Toto1000) preceding the Sindbis virus-derived sequences (Fig. 1; Materials and Methods). The clones may be linearized at a *Sst*I site 3' of the poly(A) sequences of Sindbis virus for runoff transcription in vitro.

Although a substantial proportion of the in vitro transcripts derived from Toto1, Toto2, Toto3, and Toto5 template DNAs appeared to be full-length (e.g., Fig. 2) and contained poly(A) (data not shown), they were not infectious. Since Toto5 has only a single extra G at the 5' terminus, we suspected that our inability to produce infectious transcripts might be due to the presence of one or more lethal mutations in the Sindbis virus cDNA clone. Since Toto5 contains a number of unique restriction sites in the Sindbis virus sequences, we used these sites to replace defined intervals of the Sindbis insert of Toto5 with cDNA derived from an independent HRsp cDNA clone. One of the resulting clones is called Toto50 (Fig. 1). Similarly, Toto10 and Toto1000 contain large regions which have been replaced with cDNA derived from Sindbis virus strain HR. In vitro runoff transcripts of Toto10, Toto50, Toto1000 (Fig. 2), and λToto1101 (see Materials and Methods) produce infective centers when transfected into CEF, suggesting that Toto5 contains one or more lethal mutations in the replaced regions (see below). Table 1 shows that the infective centers must be derived from RNA transcripts of the cDNA clone, since the DNA template is required only for the transcription step and is dispensable after transcription, when infective center formation becomes sensitive to RNase A. Under these conditions, the DNA is not infectious. The cap analog, $m^7G(5')ppp(5')G$, was included during transcription so that the resulting transcripts would contain a 5' cap (34). The

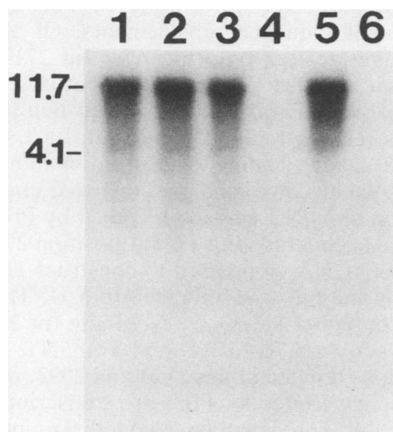

FIG. 2. In vitro transcripts. RNAs isolated from the transcription reactions described in Table 1 were denatured with glyoxal and DMSO and electrophoresed on a 1% agarose gel. Lanes: 1 to 3, transcription products made in the presence of $m^7G(5')ppp(5')A$, $m^7G(5')ppp(5')G$, and no cap, respectively; 4, RNase A digestion after transcription; 5, DNase I digestion after transcription; 6, DNase I digestion before transcription. The markers are indicated and correspond to Sindbis virion RNA (11.7 kb) and a 4.1-kb SP6 premature transcription product (present in minor amounts in lanes 1 to 3 and 5) which comigrates with Sindbis virus 26S RNA (unpublished observation).

TABLE 1. Infectiousness of in vitro transcripts of Toto1000

Conditions[a]	RNA (PFU/μg)[b]
Complete [$m^7G(5')ppp(5')G$]	4.0×10^4
$m^7G(5')ppp(5')A$	1.1×10^4
No cap analog	1.3×10^2
DNase I before transcription	0[c]
DNase I after transcription	1.0×10^4
RNase A after transcription	0[c]

[a] Complete SP6 transcription reaction [including $m^7G(5')ppp(5')G$; see Materials and Methods] except for the modifications listed. Dilutions of the transcription mixes were used directly for transfection.

[b] PFU produced per μg of transcribed RNA. Titrations were done in the range of 0.1 ng to 1 μg of RNA. Values of PFU/μg were extrapolated from titrations in the range of 1 to 10 ng of RNA.

[c] Less than 1 PFU in the entire transcription reaction (the usual yield was 1 to 1.2 μg of transcripts when the template was not pretreated with DNase I).

specific transfectivity of the capped transcripts is about 10-fold less than that of 49S RNA (1×10^5 to 4×10^5 PFU/μg) extracted from Sindbis virions, even when the fraction of apparently full-length transcripts was accounted for. The lower specific infectivity of the in vitro transcripts could be due to nonviral nucleotides at the termini of the transcripts (see below) or the presence of incomplete, premature falloff products from transcription, despite the observation that some of the transcripts appear full length on agarose gels.

Although the in vitro transcripts are expected to initiate with G, we find little difference in the specific transfectivity of transcripts made in the presence of the cap analogs $m^7G(5')ppp(5')G$ and $m^7G(5')ppp(5')A$. The transcripts made in the presence of $m^7G(5')ppp(5')G$ are indeed capped, as demonstrated by analysis of the 5' ends (34) and by the fact that the specific transfectivity of transcripts made in the absence of cap analogs is 100-fold lower. Direct evidence for incorporation of the $m^7G(5')ppp(5')A$ cap analog was not obtained since the Gp spot obscured the $m^7G(5')ppp(5')Ap$ spot in our two-dimensional separations. Additional nucleotides at the 5' end of the transcripts seem to be deleterious, since Toto30 transcripts, identical to Toto50 transcripts except for having 8 extra G-'s at the 5' terminus, were not infectious (<1 infectious center per μg of RNA).

Properties of virus derived from transcripts of the cDNA clones. Virus stocks derived from transfection with in vitro transcripts have efficiencies of plating at 30, 37, and 40°C that are indistinguishable from the parental Sindbis virus strains. Toto50, like its parental Sindbis virus HRsp strain, gave small plaques. Plaque sizes of Toto10 and Toto1000, which are hybrids of Sindbis virus HRsp and HR (Fig. 1), were intermediate between those of Sindbis virus HRsp and HR. Presumably, one or more determinant(s) affecting plaque size maps in the *Cla*I (nt 2713) to *Bss*HII (nt 9804) interval (which encompasses most of nsP2 through to near the end of E2).

RNA of virus derived from infectious transcripts. Both the intracellular, virus-specific RNA species from transfected cells and the virion RNAs of purified virus derived from in vitro transcripts are indistinguishable from that of Sindbis virus by gel electrophoresis after denaturation (results not shown). Transfection efficiencies of virion RNAs of Toto-derived stocks were comparable to 49S virion RNA of the parental Sindbis virus.

We analyzed virion RNAs labeled in vivo with ^{32}P, derived from either HRsp or Toto1000, by two-dimensional chromatography after complete digestion with RNase T2.

The patterns were indistinguishable. However, because of a high background of unidentified nucleosides, neither m^{7}G(5')ppp(5')Ap, m^{7}G(5')ppp(5')Gp, nor pppGp could be unambiguously identified (data not shown). Thus, if transcripts containing an extra G residue are infectious, it is unknown whether this residue is eliminated in subsequent amplification in vivo. The 3'-terminal nucleotides were analyzed in the same manner after enzymatic labeling of the transcripts or virion RNAs with [5'-^{32}P]pCp and RNA ligase, purification on oligo(dT) cellulose, and digestion with RNase T2. The HRsp and Toto1000-derived virion RNAs gave identical patterns, with only A being labeled, whereas the transcripts from Toto1000 contained heterogeneous 3' termini (data not shown). This heterogeneity is presumably due to alternative runoff of the SP6 polymerase at the 3' overhang generated by *Sst*I cleavage (49). It is not known whether the absence of heterogeneous 3'-terminal nucleotides in Toto1000 virion RNAs results from elimination of extra bases during replication or selective replication of transcripts without extra nucleotides [possibly produced by falloff of the SP6 polymerase in the poly(A) tract].

Proteins of virus derived from infectious transcripts. The intracellular virus-specific proteins in cells infected with HRsp, HR, Toto10, Toto50, and Toto1000 are compared in Fig. 3. The structural protein patterns are essentially identical, with PE2, E1, and C clearly resolved. These proteins have been shown to be immunoprecipitable with antisera which are monospecific for each of the virion structural proteins (data not shown). Of note is a strain variation affecting the migration of PE2. PE2 of HRsp migrates slightly faster than PE2 from HR, and as shown in Fig. 3, the PE2 of virus derived from Toto10 and Toto1000 comigrates with the HR PE2, whereas the PE2 of virus derived from Toto50 migrates faster and comigrates with the HRsp PE2, reflecting the source of the PE2 cDNA sequences.

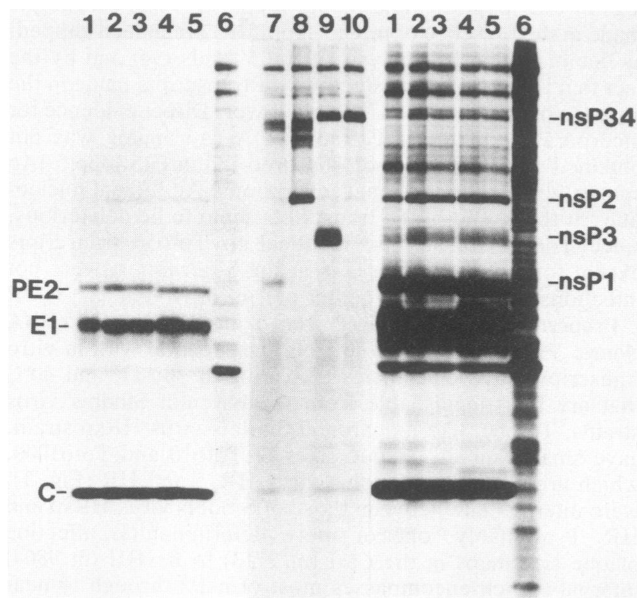

FIG. 3. Structural and nonstructural proteins from parental and in vitro-derived Sindbis strains. CEF monolayers infected with Sindbis virus strains HR, Toto10, Toto1000, HRsp, or Toto50 or mock infected (lanes 1 to 6, respectively) were pulse-labeled with L-[^{35}S]methionine from 3 to 4 h post-infection. Lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel. Immunoprecipitates of a Sindbis virus-infected lysate with antisera specific for nsP1, nsP2, nsP3, and nsP4 were run in lanes 7 to 10, respectively. The positions of structural protein precursors and nonstructural proteins are indicated in the left and right margins, respectively. A longer exposure of lanes 1 to 6, to intensify the bands of the nonstructural proteins, is shown at the right of the figure.

The nonstructural proteins made by virus derived from Toto10, Toto50, and Toto1000 are indistinguishable from those of parental Sindbis virus strains. Although only nsP2, nsP3, and nsP4 can be identified in Fig. 3, the presence of nsP1 (which comigrates with PE2) and nsP4 (present in very small quantities and migrating slightly faster than nsP3), as well as that of nsP2, nsP3, and nsP34, has been verified by immunoprecipitation (G. Li, unpublished data) with monospecific antisera to nsP1, nsP2, nsP3, and nsP4 (W. R. Hardy and J. H. Strauss, manuscript in preparation).

Mapping of Toto5 defect. As mentioned above, the full-length Sindbis virus cDNA clone, Toto5, did not yield infectious RNA transcripts. We suspected that the Sindbis virus sequences in Toto5 contained a lethal mutation(s), perhaps generated during cDNA cloning. This theory was tested by the following strategy, which serves as an example of how an infectious clone can be used to map a specific phenotypic marker (in this case a lethal mutation). Individual segments of Toto5, flanked by unique restriction sites, were replaced with corresponding segments from independent cDNA clones of Sindbis virus HRsp or HR. Of a number of replacement clones tested, infectious in vitro transcripts were obtained when the *Cla*I (nt 2713)-*Spe*I (nt 5262) interval of Toto5 was replaced by that from HRsp cDNA or when the *Cla*I (nt 2713)-*Avr*II (nt 4280) interval was replaced by that from Toto1000 (Table 2). Infectious transcripts were not obtained with replacement of other regions (results not shown). Thus, Toto5 contains one or more lethal mutations in the *Cla*I (nt 2713)-*Avr*II (nt 4280) region. Since this interval does not contain additional unique restriction sites, subclones of it were made, such that some of the sites in the interval are now unique in the subclones. These sites were then used for substituting smaller fragments of HRsp cDNA into Toto5 sequences, after which the *Cla*I-*Avr*II fragment was recloned into Toto5, resulting in Toto5 derivatives containing small sequence replacements. Of a number of such derivatives tested, only the *Cla*I (nt 2713)-*Pvu*II (nt 3103) segment rescued Toto5 (clone Toto5CP; Table 2). Sequence analysis of this region showed that Toto5 has 2 base changes relative to Toto50 and Toto1000, at positions 2824 (a C to T change leading to substitution of nsP2 Ala-382 of Toto50 by Val in Toto5) and 2992 (a T to C change leading to substitution of nsP2 Leu-438 of Toto50 by Pro in Toto5). The sequence of Sindbis virus (74) at position 2992, derived from the same cDNA clone used to construct Toto5, is that of the mutant and must be amended to a U. The two base changes are on either sides of a *Nco*I site (nt 2976), which was used to construct Toto5CN and Toto5NT, which have single mutations at nucleotides 2824 and 2992, respectively. Neither clone gave infectious in vitro transcripts (Table 2). We conclude that Toto5 contains two lethal point mutations.

Marker rescue of the temperature-sensitive mutation of *ts*2. To demonstrate the utility of this approach for mapping *ts* mutations, we replaced the capsid sequences of Toto1000 with cDNA containing the *ts*2 mutation to produce a plasmid called Toto *ts*2.1 (see Materials and Methods). *ts*2 is in RNA$^+$ complementation group C. Sequence analysis of cDNA clones derived from *ts*2 as well as from a temperature-insensitive revertant suggested that the *ts*2 phenotype resulted from a C to U transition at position 701 of the 26S mRNA, such that serine is substituted for proline at residue

TABLE 2. Clones used to map mutations in Toto5[a]

Clone	Toto5 sequence replaced[b]	Rescue[c]
Toto50	*Cla*I 2713-*Spe*I 5262	+
Toto5CS	*Cla*I 2713-*Spe*I 5262	+
Toto5CA	*Cla*I 2713-*Avr*II 4280	+
Toto5CP	*Cla*I 2713-*Pvu*II 3103	+
Toto5CN	*Cla*I 2713-*Nco*I 2976	−
Toto5BN	*Bgl*II 2288-*Nco*I 2976	−
Toto5NT	*Nco*I 2976-*Tth*111I 3912	−
Toto5PT	*Pvu*II 3103-*Tth*111I 3912	−
Toto5Bs	*Bst*XI 3441-*Bst*XI 4175	−
Toto5TA	*Tth*111I 3912-*Avr*II 4280	−

[a] See Materials and Methods for construction of these clones.
[b] Donor sequences were from HRsp cDNA, except that HR cDNA was used for Toto5CS and Toto5CA.
[c] Transcripts of the clone were infectious (+) or noninfectious (−). Base changes in Toto5 relative to Toto50 or Toto1000 are at nt 2824 and nt 2992.

218 of the capsid protein (27). At the nonpermissive temperature (40°C), *ts*2 is defective in cotranslational cleavage of the capsid protein from the nascent structural polyprotein translated from 26S mRNA, resulting in the accumulation of a 130-kDa polyprotein. Monolayers were transfected with dilutions of RNA transcribed from Toto *ts*2.1 and incubated at either 30°C (the permissive temperature) or 40°C (the nonpermissive temperature). Plaques were observed at 30°C but not at 40°C. The virus stock derived from these infectious transcripts was clearly temperature sensitive, having an efficiency of plating at 40°C/30°C of $<2.7 \times 10^{-4}$. We examined the ability of this virus to complement representative *ts* mutants from several other complementation groups. The results in Table 3 show that the virus derived from Toto *ts*2.1 complements all four of the RNA$^-$ complementation groups but does not, as expected, complement *ts*2.

Figure 4 shows the Sindbis virus proteins extracted from cells infected with HRsp, *ts*2, or virus derived from Toto10, Toto1000, or Toto *ts*2.1 and pulse-labeled at the permissive and nonpermissive temperatures. The patterns of both *ts*2 and Toto *ts*2.1 are essentially similar to those of the parental viruses at 30°C, but at 40°C, both viruses produce a prominent species of about 130 kDa characteristic of *ts*2 and with greatly diminished quantities of the cleaved structural proteins. These polypeptides are virus specific, as demonstrated by their absence from patterns of mock-infected monolayers (Fig. 4) and by immunoprecipitation with antisera specific for the structural proteins (data not shown). It is of interest that while both Toto1000 and *ts*2, parents of Toto *ts*2.1, produce large plaques at 30°C, the virus derived from Toto *ts*2.1 has significantly smaller plaques. These different plaque phenotypes of the *ts*2 mutation placed on different genetic backgrounds raise the possibility that during selection or propagation of *ts*2, secondary mutations were selected on the basis of either larger plaque morphology or a selective growth advantage or both.

TABLE 3. Complementation analysis of virus derived from Toto *ts*2.1

Virus	Complementation index[a] with complementation group:					
	A (*ts*24)	B (*ts*11)	F (*ts*6)	G (*ts*18)	C	
					*ts*2	Toto *ts*2.1
*ts*2	3	306	219	27	1	1
Toto *ts*2.1	7	212	77	32	1	1

[a] Complementation index at 40°C (73) defined as yield of the mixed infection divided by the sum of the yields of the individual infections. Numbers are rounded to the nearest integer.

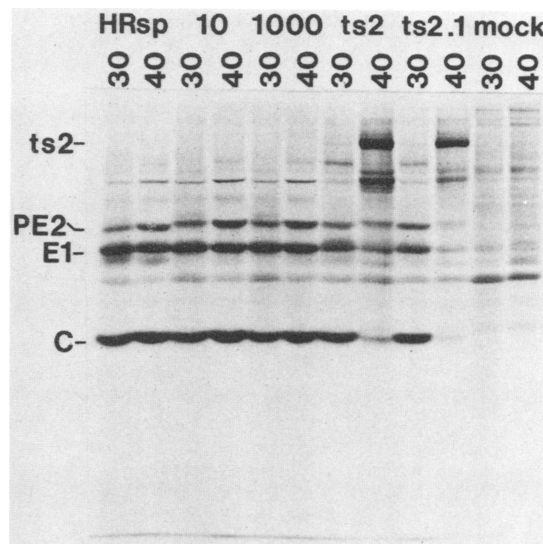

FIG. 4. Proteins made by *ts*2- and Toto*ts*2.1-derived virus. Virus-infected (10, Toto10; 1000, Toto1000; ts2, Sindbis virus *ts*2 mutant; ts2.1, Toto*ts*2.1) or mock-infected (mock) cells were incubated at either 30 or 40°C and pulse-labeled with L-[^{35}S]methionine at the same temperatures from either 7 to 8 h (30°C) or 6 to 7 h (40°C) postinfection. Lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel. The positions of C, PE2, E1, and the protein characteristic of the *ts*2 mutant (ts2; ca. 130 kDa) are indicated in the left margin.

In vitro mutagenesis of Toto1000: formal evidence for infectious in vitro transcripts. We inserted a 6-base-pair TCTAGA *Xba*I linker in the *Rsa*I site 14 nt 3′ of the 26S mRNA transcription start (56, 62). Transcription of this clone, called Toto1002, produced infectious RNA. The virus derived from Toto1002 retains the *Xba*I recognition sequence (Fig. 5), providing formal proof that viruses recovered after transfection of cells are indeed derived from in vitro transcripts. Using the assays described above, we found that the virus is essentially wild type except for a slight underproduction of 26S mRNA relative to 49S RNA.

DISCUSSION

We constructed full-length Sindbis virus HRsp and HRsp-HR hybrid cDNA clones that can be transcribed in vitro to produce infectious transcripts. Cells transfected with the transcripts produce virions that are indistinguishable from Sindbis virus and that retain strain-specific phenotypes reflecting their genetic origin. As has been found for other viruses whose virion RNAs are normally capped (1, 16), capping of in vitro-synthesized Sindbis virus transcripts enhanced their infectivity. Most transcripts synthesized in the presence of m^{7}G(5′)ppp(5′)G are capped and include an extra G residue at the 5′ terminus. In addition, most of the transcripts contain extra 3′ terminal nucleotides. The specific infectivity (PFU/μg of RNA) of the transcripts is about 10% of that of virion RNA. The RNA recovered from Sindbis virions derived from the Toto clones appears to have at least the same 3′ termini as RNA from HR or HRsp virus. Thus, if molecules containing these extra nucleotides are infectious, the extra nucleotides are somehow selectively deleted during virus replication. The eight extra G residues

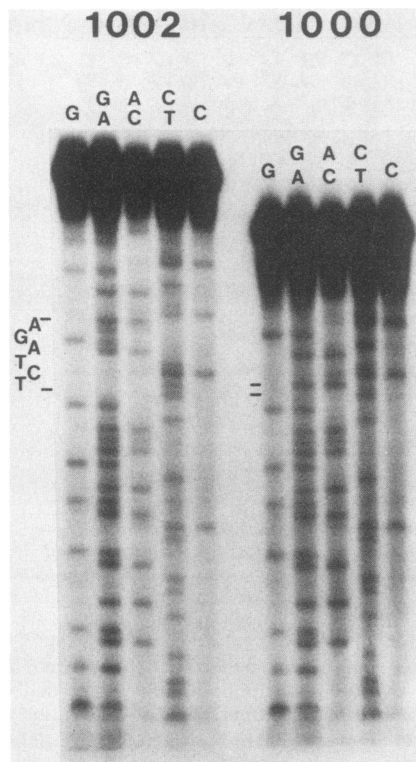

FIG. 5. Sequence of 26S mRNA 5′ untranslated region of Toto1000 and Toto1002. Discrete primer extension products of intracellular RNA from either Toto1000- (1000) or Toto1002- (1002) infected cells corresponding to the 5′ end of 26S RNA were isolated and sequenced (see Materials and Methods). Dashes in the Toto1000 ladder indicate the *Rsa*I site used for insertion of the *Xba*I linker in Toto1002. The linker sequence in the Toto1002 ladder is indicated, i.e., 3′-AGATCT-5′.

at the 5′ terminus of Toto30 led to inactive transcripts. Thus, Sindbis virus seems to be more sensitive to extraneous 5′-terminal sequences than are certain other RNA viruses. In the case of poliovirus cDNA clones transcribed with T7 RNA polymerase, the in vitro transcripts showed a 50-fold increase in specific infectivity (to 5% of that of virion RNA; 81) when 58 extra nucleotides at the 5′ terminus (leaving 2) and 619 extra nucleotides at the 3′ terminus (leaving 7) were removed. In vitro transcripts of cDNA clones from another picornavirus, human rhinovirus 14, are infectious even though they contain 21 additional 5′-terminal nucleotides (52). For black beetle virus, RNA 2 transcripts containing 20 extra 5′ nucleotides are infectious, but in this case, their removal does not increase specific infectivity (16). It is possible that engineering Sindbis virus in vitro transcripts with proper 5′ and 3′ termini may yield specific infectivities closer to those of virion RNA.

Mapping of mutant phenotypes. The cDNA clones of Sindbis virus can be used to define precisely the sequence changes responsible for the phenotype of any Sindbis virus variant by the strategy of exchanging segments of the wild-type clone with cDNA of the variant and determining the phenotype of the resulting clones (33, 38, 54, 59). Complex phenotypes due to multiple sequence changes (e.g., *ts*24; 65, 66) may be identified as such and dissected into single changes whose individual contributions can be determined. The mapping of the two lethal mutations of Toto5 illustrates this approach. The strategy is feasible whenever two strains of virus are closely related enough to share usable restriction sites for exchanging segments. Although Sindbis virus HRsp was derived from HR and subsequently propagated separately, and although HRsp and HR are known to differ at a number of nucleotide positions, we have not found any differences in the restriction maps of their cDNAs.

The infectious Sindbis virus cDNA clones may be used to map a number of interesting phenotypes of Sindbis virus. These phenotypes include mutants which affect virion morphogenesis (72), host range (35), virulence in neonatal mice (17, 53), enhanced neurovirulence in adult mice (26), cytopathogenicity (84), actinomycin D resistance (3), immunodominant epitopes on the envelope glycoproteins, and the ability to grow in low concentrations of methionine in mosquito cells (20). We have also begun to map temperature-sensitive mutations in each of the four RNA$^-$ complementation groups.

An important advantage of this approach is that any mutation that is mapped is also preserved as a DNA clone, with a correspondingly much lower mutation rate (32). The mutation is also placed upon a known genetic background. This placement makes possible the study of subtle effects of the mutation and of the interaction between a mutation and a particular genetic background and allows the facile construction of viruses with multiply defined mutations. The smaller plaque size of virus derived from Toto *ts*2.1 when compared with the *ts*2 or the Toto1000 parents provide initial evidence that the genetic background can be important.

Study of structure-function relationships via site-directed mutagenesis. The cDNA clones may be mutagenized by any of a number of methods to generate transcripts with novel mutations, i.e., lethal, viable or conditional, which can be studied both in vivo and in vitro (5, 7, 19, 28, 45, 51, 64). The characterization of these mutants, in conjunction with the traditionally derived mutants, will further our understanding of the molecular biology of Sindbis virus. In addition to Toto1002 described here, we have constructed a set of single- and multiple-codon insertion mutations of the nonstructural protein genes and are characterizing the replication of the corresponding mutant viruses. The structural proteins of Sindbis virus are better understood, and current experiments include the targeted mutagenesis of the capsid protein to define the amino acid residues responsible for its autoprotease activity (27; C. Hahn and J. H. Strauss, personal communication).

Thus far, essentially all of the revertants of Sindbis virus *ts* mutants derived by chemical mutagenesis (single-base substitutions) are true revertants resulting in restoration of the wild-type sequence. The insertion-deletion or multiple-base-change mutations that now can be introduced into the Sindbis virus genome should be less easily revertable, thus facilitating the selection of revertants with compensating mutations elsewhere in the genome. Mapping and characterization of such second-site revertants should be extremely valuable in elucidating functional interactions between the nonstructural proteins and of the interactions of the nonstructural proteins with *cis*-acting regulatory sequences. We have obtained revertants from a number of codon-insertion mutations in the nonstructural region and are screening them for true second-site revertants. Similar approaches may be taken to study viral maturation to help define the interactions of PE2 with E1, of E2 with E1, and of capsid protein with the cytoplasmic extensions of the envelope proteins or with 49S RNA.

Other applications. The cDNA clones described here consist of functional cDNA copies of the nonstructural and structural protein genes of Sindbis virus. These sequence modules may be inserted into existing gene expression vectors to express large quantities of the corresponding proteins for in vivo and in vitro studies, as well as to provide *trans* complementation for the amplification and study of lethal mutations. A vaccinia virus recombinant that expresses the Sindbis virus structural proteins has been described previously (61). The structural proteins have also been expressed in yeast cells (85).

Finally, given the rapid and high level of expression of its structural proteins, Sindbis virus can be used as a self-replicating gene expression vector. We have shown that Sindbis virus defective-interfering genomes can be used to express the bacterial chloramphenicol acetyltransferase (*cat*) gene (42). We show elsewhere that Sindbis virus, like bromegrass mosaic virus (22) and tobacco mosaic virus (78), can be used to express efficiently the *cat* gene (C. Xiong et al., manuscript in preparation).

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