JOURNAL OF VIROLOGY, Dec. 1989, p. 5216–5227
0022-538X/89/125216-12$02.00/0

Vol. 63, No. 12

A *cis*-Acting Mutation in the Sindbis Virus Junction Region Which Affects Subgenomic RNA Synthesis

ARASH GRAKOUI, ROBIN LEVIS,† RAMASWAMY RAJU, HENRY V. HUANG, AND CHARLES M. RICE*

Department of Molecular Microbiology, Washington University School of Medicine, Box 8093, 660 South Euclid Ave., St. Louis, Missouri 63110-1093

Received 13 June 1989/Accepted 30 August 1989

The synthesis of Sindbis virus minus-strand and genomic and subgenomic RNAs is believed to require specific *cis*-acting sequences or structures in the template RNAs and a combination of virus-specific proteins and host components which act in *trans*. A conserved sequence of about 21 nucleotides in the junction region and encompassing the start site for the subgenomic RNA has been proposed to function as the promoter on the minus-strand template for synthesis of the subgenomic RNA (J.-H. Ou, C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss, Proc. Natl. Acad. Sci. USA 79:5235–5239, 1982). We introduced a three-base insertion in this sequence, which also inserts a single amino acid near the COOH terminus of nsP4, in a cDNA clone of Sindbis virus from which infectious RNA transcripts can be generated. The phenotype of this mutant, called Toto1100CR4.1, was studied after RNA transfection of chicken embryo fibroblasts or BHK cells. The mutation leads to a drastic reduction in the level of the subgenomic RNA but does not alter the start site of the RNA. Probably as a consequence of depressed structural-protein synthesis, very few progeny virions are released and the mutant makes tiny or indistinct plaques even after prolonged incubation. The *cis*-acting effect of this mutation was demonstrated by incorporating either a wild-type or mutant junction region into a defective-interfering RNA and examining the relative synthesis of defective-interfering RNA-derived subgenomic RNA in vivo in the presence of wild-type helper virus. These results show that the junction region is recognized by yet unidentified viral *trans*-acting components for subgenomic RNA synthesis. When the Toto1100CR4.1 mutant was passaged in culture, plaque morphology variants readily arose. A total of 24 independent revertants were isolated, and 16 were characterized in detail. All revertants analyzed showed an increase in the level of subgenomic RNA synthesis. Sequence analysis of the junction region showed that all were pseudorevertants, with only two containing potentially compensating changes in the junction region. An assay was developed to identify revertants with second-site changes in *trans*-acting viral components involved in subgenomic RNA synthesis. At least two such revertants were identified. Mapping of these and other second-site compensating mutations may provide genetic clues as to which virus-specific protein(s) is responsible for interaction with the conserved junction region to promote subgenomic RNA synthesis.

Sindbis virus, the type species of the *Alphavirus* genus (family *Togaviridae*), and the closely related Semliki Forest virus have been widely studied (reviewed in reference 36). Members of this genus are enveloped viruses which mature by budding through host membranes and are transmitted by mosquitos to a wide variety of vertebrate hosts. The genome of Sindbis virus consists of a single molecule of single-stranded RNA, 11,703 nucleotides in length (39). 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. The 5′ two-thirds of the genomic 49S RNA is translated early during infection to produce two polyproteins that are processed by cotranslational and posttranslational cleavage (13) into four nonstructural proteins (called nsP1 through 4, numbered in order as they appear in the genome sequence [39]) which are required for RNA replication. A full-length minus strand complementary to the genomic RNA is first synthesized, and it then serves as a template for the synthesis of new 49S genomic RNA molecules. The minus strand is also transcribed from an internal site to produce a 26S subgenomic mRNA that is 4,106 nucleotides long and colinear with the 3′-terminal one-third of the 49S genome. This subgenomic mRNA is capped, polyadenylated, and encodes the three virion structural proteins (the capsid protein C, and the two envelope glycoproteins E1 and PE2, the precursor of the virion E2). In contrast to the 49S RNA, 26S subgenomic RNA does not serve as a template for minus-strand synthesis nor is it packaged into mature virions.

Synthesis and packaging of Sindbis virus-specific RNAs in vertebrate cells require *trans*-acting components (both virus encoded and host specified) which act on specific viral RNA structures or sequences. The viral proteins and *cis*-acting RNA sequences mediating these regulated steps in the virus life cycle and the molecular mechanisms involved are still largely undefined. Comparison of several different alphavirus sequences (24–26) revealed at least four conserved RNA domains which could act as *cis*-acting RNA sequences or structures. A conserved sequence of 19 nucleotides adjacent to the 3′-terminal poly(A) tail was hypothesized to be important for initiation of minus-strand synthesis (25). Deletion mutagenesis studies, in which a defective-interfering (DI) RNA cDNA clone which could produce biologically active RNA transcripts was used, showed that this sequence was important for replication and/or packaging of DI RNA (17), although direct evidence of its role in initiation of minus-strand synthesis has yet to be obtained. The other three conserved RNA features in the alphavirus genome include a 5′-terminal secondary structure (26), a 51-base conserved sequence near the 5′ end of the genomic RNA

* Corresponding author.
† Present address: National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

(26), and a stretch of 21 bases encompassing the start of the subgenomic RNA in the junction region (24). The junction region is defined as the region of the genome immediately preceding and including the beginning of 26S RNA (24). For DI RNA replication and packaging, the 5'-terminal secondary structure, the 51-base conserved sequence, and the junction region are nonessential (17). Mutagenesis studies aimed at defining the function of these sequences in wild-type Sindbis virus replication have not yet been reported.

The conserved 21-base sequence in the junction region was proposed to function, in the minus strand, as the promoter for subgenomic RNA synthesis (24). This sequence is absent from naturally occurring DI RNA genomes which have been sequenced (16, 23), and such DI genomes do not make DI-derived subgenomic RNAs. Recent experiments have shown that translocation of a segment of the viral genome containing the junction region into a DI genome leads to the production of DI-derived subgenomic RNA, thus allowing us to map the minimal sequences required for subgenomic RNA synthesis (R. Levis, S. Schlesinger, and H. V. Huang, submitted for publication). In this study, by using a full-length cDNA clone of the Sindbis virus genome from which infectious transcripts can be generated by in vitro transcription (29), we report the construction of a three-base insertion mutation in the junction region and the characterization of its effects on subgenomic RNA synthesis in both the Sindbis virus genome and in engineered DI RNA genomes. Furthermore, in the context of the full-length genome, the mutant phenotype is unstable and revertants arise at high frequency. The phenotypes of several independent revertants have been characterized, including two which may contain compensating second-site changes in viral components which allow more efficient recognition of the mutant subgenomic RNA promoter.

MATERIALS AND METHODS

General recombinant DNA materials and methodology. All restriction enzymes, SP6 DNA-dependent RNA polymerase, RNasin, DNase I, T4 DNA ligase, T4 DNA polymerase, and *Escherichia coli* DNA polymerase I and its large fragment (polI-K) were obtained from New England BioLabs Inc., Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, or Promega Biotec. Avian myeloblastosis virus reverse transcriptase was from Life Sciences, Inc., Moloney murine leukemia virus (M-MLV) reverse transcriptase was from Bethesda Research Laboratories, and *Taq* DNA polymerase was from Cetus. All radioactive materials were purchased from either Amersham Corp. or ICN Pharmaceuticals, Inc. Enzymes were used essentially as recommended by the manufacturers. Plasmids were grown, purified, and analyzed by using standard methods, with minor modifications (21).

Plasmid constructions. Plasmids were constructed by using standard methods, and brief descriptions are presented below. The structures of the relevant clones are shown in Fig. 1. Plasmid structures were verified by appropriate restriction digests and sequence analyses. Nucleotide numbers are given relative to the Sindbis virus genomic RNA or DI genomic RNA 5' termini, unless otherwise noted. The genomic and subgenomic RNA sizes do not include that of the poly(A).

Toto1100CR4.1. The nsP4 coding sequence from *Hin*dIII (nucleotide [nt] 6267) to the filled-in *Nco*I site (nt 8038) of Toto1000 (a plasmid capable of yielding infectious RNA transcripts [29]) was subcloned into πAN7 (20), which had been digested with *Hin*dIII and *Hin*cII. The resulting plasmid, πNSP4C (2,638 base pairs), was partially digested with *Sau*96I. Linear molecules were isolated from preparative agarose gels (41), treated with polI-K to fill in the 5' protruding ends, and religated. Mutants were identified and mapped by restriction digestion with *Sau*96I and were verified by sequence analysis. A mutation in which the *Sau*96I site at position 7590 in the Sindbis virus cDNA sequence was filled in was designated CR4.1. The *Hin*dIII (nt 6267) to *Aat*II (nt 7999) fragment from this mutant subclone was then used to replace the corresponding region in Toto1100, giving the plasmid Toto1100CR4.1. Toto1000, Toto1100, and Toto 1101 contain identical Sindbis virus cDNA sequences, with minor differences in the plasmid vector. Plasmids in the Toto1100 series have, downstream from the 3' wild-type tail, a unique *Xho*I site used for production of linearized templates for runoff transcription, which replaces the *Sst*I site in Toto1000. Toto1100 and Toto1101 also contain a deletion in the pBR322-derived sequences between the *Eco*O109 and *Aat*II sites, rendering the *Aat*II site (nt 7999) in the Sindbis virus cDNA sequence unique. Toto1100, in addition, contains a high-copy-number mutation in the plasmid replication origin (3). Virus stocks derived from Toto1000, Toto1100, or Toto1101 are considered to be identical and in this paper are defined as the parental or wild-type virus.

JNSH and JNSH.A. JNSH is a modified version of a cDNA clone, KDI25 (17), of a DI RNA genome. Its 3' terminus is derived from Toto1100, and it also contains a fragment encompassing the junction region (see Fig. 1). The 3'-terminal region of KDI25 from the *Nae*I site at base 2245 to the *Eco*RI site at base 2373 (for a description of KDI25, see reference 17) was replaced with the *Mst*I (nt 11546) to *Eco*RI [immediately 3' of the poly(A)] fragment from Toto1100. This clone, called KDI25.3, has a unique *Nae*I site in the DI cDNA at position 1340. The *Ssp*I (nt 7499) to *Hin*cII from TSCAT (the *Hin*cII site is in the polylinker region upstream from the chloramphenicol acetyltransferase [CAT] gene in TSCAT [42]), containing the junction region (from −98 to +117 relative to the subgenomic RNA start), was then inserted into the *Nae*I site of KDI25.3, resulting in JNSH (DI genomic RNA length of 2,671 bases; predicted subgenomic RNA length, 1,280 bases). JNSH.A is identical to JNSH except that the *Ava*II (*Sau*96I) site in the junction region was filled in with polI-K, resulting in the same three-base insertion found in Toto1100CR4.1.

TRCAT and TRCAT.A. TRCAT contains the bacterial CAT gene replacing the coding region for the Sindbis virus structural proteins in Toto1002 ([42] between positions 7612 and 11087). The size of TRCAT genomic RNA is 8,969 nt, and the size of the subgenomic RNA encoding CAT is 1,412 bases. TRCAT.A is identical to TRCAT except that the *Ava*II (*Sau*96I) site in the junction region was filled in with polI-K, creating the same three-base insertion mutation found in Toto1100CR4.1.

DIJCAT and DIJCAT.A. These DI cDNA constructs were derived from KDI25 (17), in which the unique *Hin*dIII site was destroyed by filling in with polI-K. A polylinker containing a *Hin*dIII site was inserted into the *Bal*I site at nt 241 of the DI cDNA. This construct was then fused at this *Hin*dIII site to the *Hin*dIII (nt 6267) site of TSCAT (42). DIJCAT.A is identical to DIJCAT except that the *Ava*II (*Sau*96I) site in the junction region was filled in with polI-K, creating the same three-base insertion mutation found in Toto1100CR4.1.

Cell cultures, virus growth, plaque assay, and revertant isolation. Chicken embryo fibroblasts (CEF) were propa-

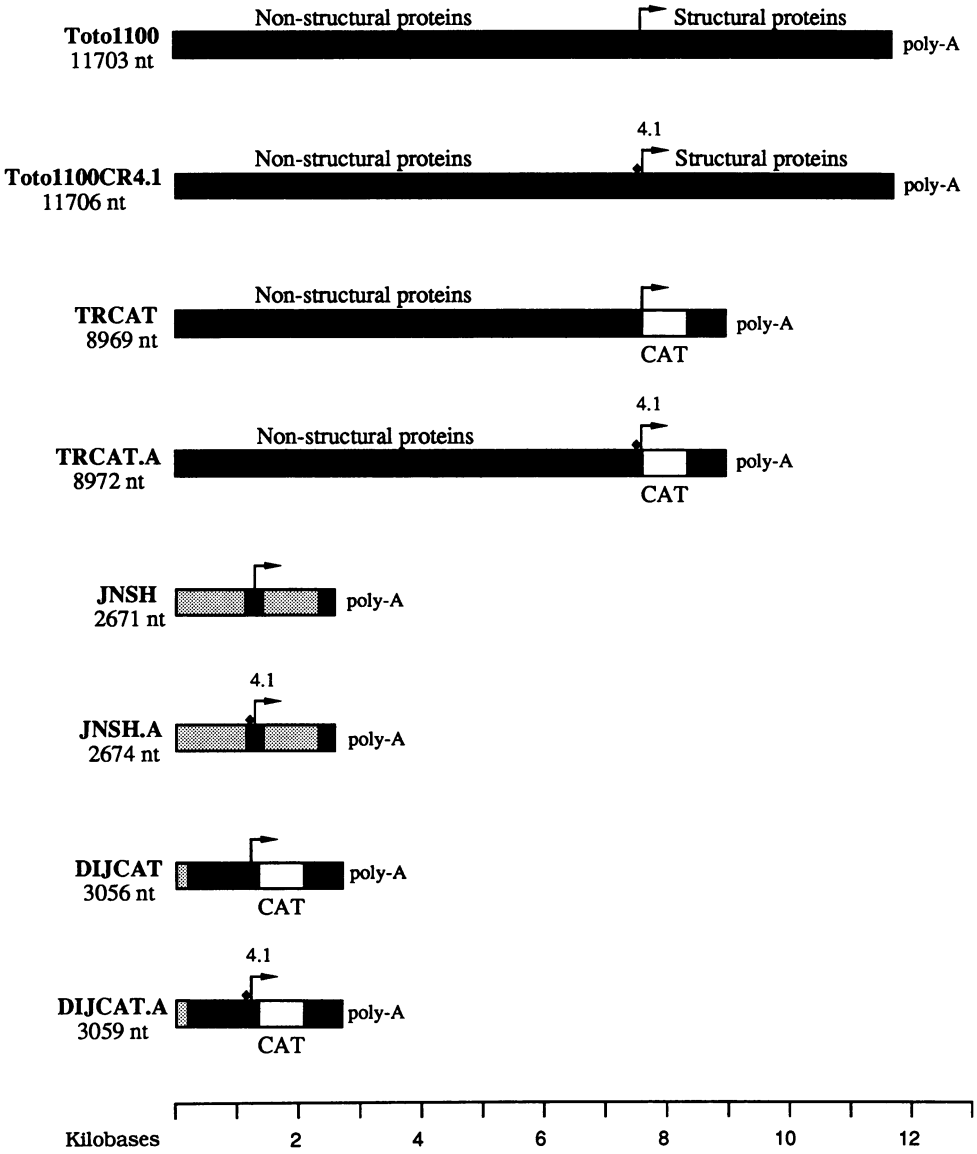


FIG. 1. Structure of clones. Diagrams and sizes of genomic-length RNA transcripts derived by in vitro transcription of plasmid templates used in this study are shown. Details of the construction of the plasmids are given in Materials and Methods. Sindbis virus sequences (solid bar), the CAT gene coding sequences (open bar), and DI RNA sequences (shaded bar) are indicated. Arrows mark the locations in the genomic RNAs corresponding to the start site of subgenomic RNAs. Constructs with the mutant junction (4.1) containing a three-base insertion (solid diamond) are indicated.

gated as previously described (27). BHK-21 cells (from the American Type Culture Collection via Robert E. Johnston, University of North Carolina) were grown in minimal essential medium supplemented with 10% tryptose phosphate broth and 10% bovine calf serum. Virus stocks were derived from either the full-length Sindbis virus cDNA clone Toto1101 or its mutagenized derivatives by in vitro transcription of linearized plasmid DNAs by using SP6 DNA-dependent RNA polymerase, followed by RNA-mediated transfection of CEF cells (29). Virus stocks were titered on CEF monolayers as described previously (37). Revertants were isolated by passaging supernatants from 24 independent Toto1100CR4.1 RNA transfections and were selected on the basis of plaque morphology at 30°C. Since the original mutant produced fuzzy, indistinct plaques under these conditions, revertants which produced clear, discrete plaques at 30°C after 48 h were chosen. Individual plaques were recovered from second-passage supernatants and were purified with an additional cycle of plaque purification on CEF monolayers at 30°C. Stocks were grown at 30°C and harvested at 48 h.

In vitro transcription and capping. 5′-Capped RNA transcripts were synthesized in vitro by SP6 DNA-dependent RNA polymerase by using plasmid template DNAs digested with appropriate restriction endonucleases for production of runoff transcripts (29). 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 by adsorption to DE 81 (Whatman) filter paper (21). Template DNA was removed by digestion

with DNase I, followed by extraction with phenol-chloroform and recovery of the RNA by ethanol precipitation.

RNA transfection with lipofectin. For first-cycle analysis, slightly subconfluent monolayers of BHK or secondary CEF cells in 35-mm tissue culture plates (about 10^6 cells) were used for RNA transfection. Transfection mixtures were made by adding 8 μg of lipofectin, a cationic liposome (8; lipofectin was a generous gift from P. L. Felgner and G. M. Ringold, Syntex), to 200 μl of ice-cold phosphate-buffered saline (PBS) (lacking Ca^{2+} and Mg^{2+}) and this was followed by 100 ng of RNA (or in some cases 20 μg of the liposomes and 200 to 250 ng of RNA were used). This ratio was determined empirically, and increasing the amount of the cationic liposomes did not significantly increase transfection efficiency. The transfection mixture was incubated on ice for 10 min prior to use (it is not known if this step is necessary). After washing monolayers twice with PBS, the cells were incubated with 200 μl of the RNA–lipofectin mixture at room temperature for 10 min with occasional rocking. The monolayers were then washed once with PBS and incubated in minimal essential medium containing 2% fetal bovine serum. Incubation times are given in Results, and, unless otherwise indicated, cultures were incubated at 30°C. To examine replication and transcription of DI genomes, DI RNA transcripts and virion RNA were used to cotransfect 60-mm plates of CEF monolayers. A 200-ng portion of the DI RNA was mixed with 250 ng of virion RNA in 160 μl of PBS, and 40 μg of lipofectin was added. This mixture was diluted to 500 μl with PBS and used to transfect the cells as described above.

RNA analysis. Monolayers of BHK or secondary CEF cells were infected with Sindbis virus or revertant stocks at a multiplicity of infection (MOI) of 10 PFU per cell or transfected with RNAs as described above. Virus-specific RNAs were labeled in the presence of minimal essential medium containing 2 μg of dactinomycin and 100 μCi of [^{3}H]uridine per ml for various intervals, as described in the Results. Cytoplasmic RNAs were isolated, denatured with glyoxal and dimethyl sulfoxide, and analyzed by electrophoresis in 1% agarose gels containing 10 mM phosphate buffer (21).

Primer extension analysis. Samples of total cytoplasmic RNA prepared from mock, wild-type virus, mutant, or revertant-infected (or transfected) CEF cells were annealed to 0.5 pmol of a 5′-end-labeled oligonucleotide (complementary to nucleotides 7644 to 7662 of Sindbis virus) and extended by using M-MLV reverse transcriptase. The reaction mix (10 μl) contained 1× polymerase chain reaction (PCR) buffer (10 mM Tris hydrochloride, pH 8.3 [at 20°C], 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 4 mM dithiothreitol, 1.0 mM of each deoxynucleoside triphosphate, 400 U of RNasin per ml, and 2,000 U of M-MLV reverse transcriptase per ml. After incubation at 37°C for 30 min, discrete extension products corresponding to the 5′ end of 26S RNA were resolved by electrophoresis on 5% polyacrylamide sequencing gels (22), visualized by autoradiography, and, in some cases, quantitated by scanning densitometry or excision of the bands and liquid scintillation counting.

CAT assay. At various times after RNA transfection, monolayers were washed three times with PBS, scraped into 0.5 ml of PBS, and transferred to a microcentrifuge tube. After centrifugation, cell pellets were suspended in 0.25 M Tris hydrochloride, pH 8, and stored at −70°C. After thawing and two more freeze-thaw cycles, portions or dilutions of the clarified supernatants were assayed for CAT activity as previously described (9). [^{14}C]chloramphenicol and its acetylated forms were separated by thin-layer chromatography, localized by autoradiography, and quantitated by liquid scintillation counting.

Immunofluorescence. Productively transfected cells were identified and quantitated by immunofluorescence staining of fixed BHK monolayers with a rabbit antiserum specific for Sindbis virus nsP3. BHK cells were seeded in 35-mm tissue culture dishes containing 12-mm cover slips which had been coated with rat tail collagen (2). Monolayers were used for transfection when they were about 40% confluent. Parallel plates were used to determine the number of cells at the time of harvest and for quantitation of CAT activity as described above. For immunofluorescence staining, monolayers were fixed and permeabilized and nonspecific binding sites were blocked as previously described (42). Monolayers were incubated for 30 to 60 min with nsP3 antiserum that was diluted 200-fold with PBS–0.1% bovine serum albumin (BSA). The nsP3 antiserum (13) was preadsorbed with acetone-fixed CEF and BHK cells to reduce nonspecific binding. After washing three times with PBS–BSA, monolayers were incubated for 45 min with fluorescein isothiocyanate-conjugated goat antibody directed against rabbit antibody (1:100 dilution, filtered through a 0.22-μm cellulose acetate membrane), washed three times with PBS–BSA and once with PBS, and mounted in PBS–glycerol (2:1). Stained cells were counted by immunofluorescence microscopy. Fluorescent cells were not seen in mock-transfected monolayers.

PCR amplification and sequencing. To sequence the junction region of Toto1100CR4.1 revertants, total cytoplasmic RNA was isolated from infected (MOI of 10 PFU per cell) or mock-infected CEF cells at 8 h postinfection as previously described (17). Approximately one-tenth of the RNA isolated from a 60-mm tissue culture dish (~3×10^6 cells per plate) was used for reverse transcription. A 0.1-pmol portion of a negative-sense primer corresponding to nts 7690 to 7710 (called PCR2) was annealed to the RNA and extended for 15 min at 37°C with M-MLV reverse transcriptase. The reaction mix (10 μl) contained 1× PCR buffer, 5 mM dithiothreitol, 0.2 mM each deoxynucleoside triphosphate, 400 U of RNasin per ml, and 2,000 U of M-MLV reverse transcriptase per ml. After heating the reaction to 70°C for 10 min, 10 μl of a mixture containing 0.2 mM deoxynucleoside triphosphates, 20 pmol of PCR2 and PCR1 (a positive-sense primer corresponding to nts 7463 to 7483 of Sindbis virus), and 1 U of *Taq* DNA polymerase in 1× PCR buffer was added. The reaction was covered with paraffin oil, and DNA was amplified by 25 consecutive cycles of the PCR (31) with a 1-min denaturation step at 94°C, a 3-min polymerization step at 72°C, and no annealing step. Reactions were terminated by addition of EDTA to 10 mM; 20 μg of tRNA was added as carrier, and the samples were extracted with chloroform, followed by phenol and then chloroform, and the DNA was ethanol precipitated. One-fifth of the DNA from the first round of amplification was further amplified in a 50-μl reaction under the same conditions, except that a 1-min 55°C annealing step was added and only 20 amplification cycles were used. The products were extracted, precipitated and washed with ethanol, and suspended in 10 μl of TE (10 mM Tris hydrochloride, pH 7.5, 0.1 mM EDTA). Since initial attempts to sequence these products by using several modifications of the Sanger method (32) gave unsatisfactory results, we end labeled and sequenced the products by using the chemical method (22). A 1.5-μl portion of the DNAs from the second amplification was digested with *Msp*I (nt 7481) and 3′ end labeled by using polI-K and [α-^{32}P]dCTP. After heating the

samples to 70°C for 10 min to inactivate the polI-K, they were digested with *Hin*fI (nt 7657) and the reaction was terminated by addition of EDTA to 10 mM and formamide to 35%. DNA was denatured by heating to 90°C for 5 min, and the fragments were resolved by electrophoresis on 5% polyacrylamide sequencing gels (30). The 179-nt band corresponding to the junction region was localized by autoradiography, excised, eluted, and sequenced by the chemical method (22, 30).

***trans*-Complementation assay.** Duplicate sets of tertiary CEF cells were infected with Toto1101 or Toto1100CR4.1 revertants at an MOI of 5 PFU per cell. After 1 h at 30°C, the inoculum was removed and the cells were washed three times with PBS. Cells were transfected with 200 ng of capped RNA transcripts derived from either DIJCAT or DIJCAT.A by using 5 μg of lipofectin as described above. After 30 min at 30°C, the monolayers were washed with PBS, minimal essential medium containing 3% fetal bovine serum was added, and incubation was continued for 16 h. The cultures were shifted to 37°C for 2 h, harvested, and assayed for CAT activity as described above. The 37°C incubation was included to increase the sensitivity of the assay, since higher temperatures have been shown to increase the level of CAT produced from preexisting CAT mRNA (42).

RESULTS

Phenotype of virus derived from Toto1100CR4.1. The mutation in Toto1100CR4.1 results in a three-base insertion (GUC) in the conserved sequence of the junction region, between nucleotides −4 and −5 relative to the subgenomic RNA start (Fig. 2). The mutation also inserts an arginyl residue between amino acid residues 608 and 609 of nsP4 (approximately 610 amino acids in length [39]).

5′-Capped RNA transcripts were produced by in vitro transcription of *Xho*I-linearized Toto1100CR4.1 plasmid DNA with SP6 DNA-dependent RNA polymerase. CEF transfected with this RNA showed very little if any cytopathic effect even after 72 h at 30°C (with Toto1101 virus, dramatic cytopathic effect is seen after 8 to 12 h at 37°C or 15 h at 30°C). When the ability of the RNA to initiate plaque formation at various temperatures was examined, very small diffuse plaques could be seen when monolayers were incubated for 96 h at 30°C and stained with neutral red. While these plaques were difficult to count, there did not appear to be a significant difference between the specific infectivity of RNA transcripts derived from Toto1100CR4.1 compared with the parental Toto1101. Plaques were apparent but more difficult to visualize at higher temperatures (37 or 40°C). Samples of the media from these primary transfection experiments typically gave very low titers of small diffuse plaques at 30°C (~10^5 PFU/ml, compared with >10^9 PFU/ml for the parental virus) and very indistinct plaques at 40°C which were difficult to quantify. The presence of plaque morphology variants in early passage virus stocks suggested the presence of revertants in the population. These initial results showed that the insertion mutation was deleterious (but not lethal) and suggested that the mutant phenotype was unstable, thus emphasizing the need to study the phenotype as early as possible, i.e., immediately after transfection.

First-cycle analysis. Analysis of the effects of mutations on viral RNA and protein synthesis in transfected cells was hampered by the low transfection efficiency of RNA with DEAE-dextran (29), which at best results in only about 0.1% of the cells being productively transfected. We therefore established RNA transfection conditions by using the cationic liposome, lipofectin (8), which increased the percentage of transfected cells to about 1% for CEF or BHK cells (data not shown; the procedure is given in Materials and Methods).

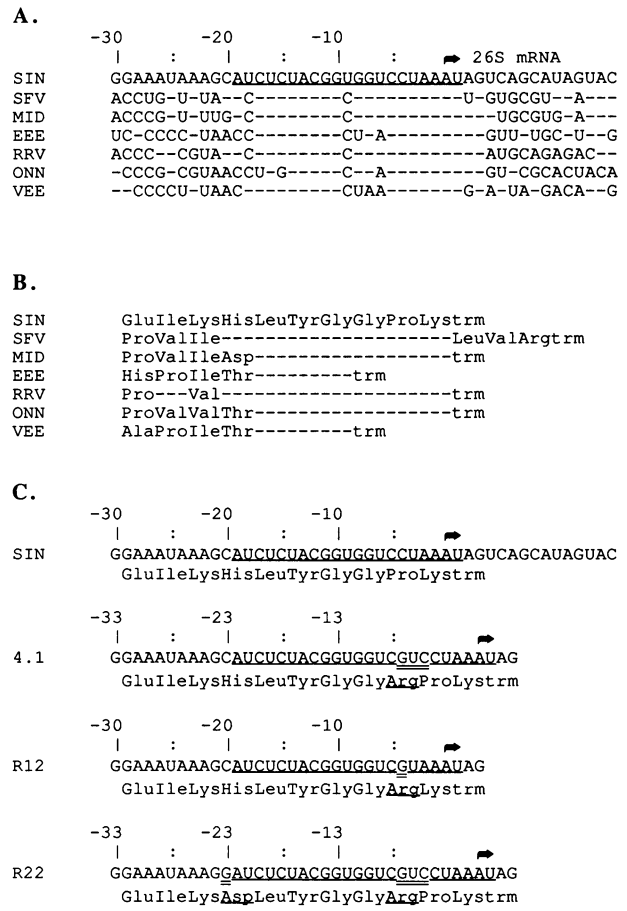


FIG. 2. Alphavirus junction region sequences. (A) Alignment of the genomic sequences in the junction region from several alphaviruses: Sindbis virus (SIN [24]), Semliki Forest virus (SFV [24]), Middelburg virus (MID [24]), Eastern equine encephalitis virus (EEE [7]), Ross River virus (RRV [24]), O'Nyong-nyong virus (ONN [38]), Venezuelan equine encephalitis virus (VEE [15]). The complement of these sequences in the negative-strand template was proposed to function as the promoter for subgenomic RNA synthesis. Sequence identity with respect to the Sindbis virus sequence is indicated by dashes. Nucleotides are numbered relative to the 26S mRNA start (shown by an arrow; see reference 24). (B) Deduced amino acid sequences of nsP4 in this region are compared. (C) Nucleotide (double underline) and amino acid (underline) sequences of the 4.1 mutant and the R12 and R22 revertants that differ from that of the parental virus, Toto1101.

When viral RNA patterns of transfected BHK cells were examined at different temperatures (30, 37, and 40°C), the mutant showed a dramatic reduction in the level of 26S RNA relative to 49S RNA (Fig. 3A). While the 26S RNA species produced by the mutant was barely detectable, the production of small diffuse plaques implied that sufficient levels of structural proteins were produced to allow some virus assembly to occur.

Primer extension analyses were used to verify the existence of the 26S RNA in Toto1100CR4.1-transfected cells and to compare the start site of the subgenomic RNAs produced

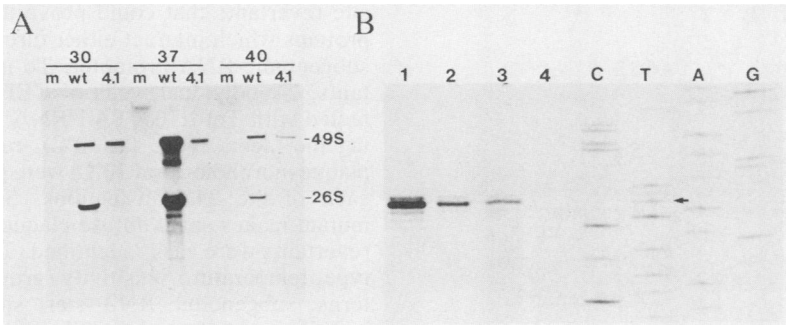


FIG. 3. First-cycle RNA analyses of Toto1100CR4.1. (A) Levels of 49S and 26S RNAs in BHK cells transfected with Toto1101 or Toto1100CR4.1 transcripts. Monolayers of BHK cells were transfected with transcripts from either Toto1101 (wt), Toto1100CR4.1 (4.1), or mock transfected (m), as described in Materials and Methods. After incubation at 30, 37, or 40°C for 2 h, the cells were labeled with [^{3}H]uridine in the presence of 2 μg of dactinomycin per ml. At 8 h posttransfection, cytoplasmic RNAs were isolated, denatured, and separated on a 1% agarose gel. The positions of the genomic 49S RNA and the subgenomic 26S RNA are indicated. The absolute levels of virus-specific RNA synthesis cannot be compared in this experiment due to the dramatic differences in the rate and amount of virus released from the Toto1100CR4.1 mutant versus Toto1101-transfected cells and subsequent infection of additional cells during the course of the experiment. (B) Mapping the initiation site for subgenomic RNA synthesis. Intracellular RNAs were isolated from CEF cells transfected with either Toto1101 or Toto1100CR4.1 or mock transfected as described in Materials and Methods. A 5′-end-labeled negative-sense oligonucleotide hybridizing from nt 47 to nt 65 downstream from the wild-type subgenomic RNA start was extended by using reverse transcriptase. The products were denatured and run on a 5% acrylamide-urea sequencing gel. Extension products from Toto1100CR4.1 and mock-transfected cytoplasmic RNAs were loaded in lanes 3 and 4, respectively. Relative to amounts loaded for the mutant and mock control, 1/25th and 1/100th of the extension products from the Toto1101 intracellular RNA were loaded in lanes 1 and 2, respectively. The four lanes at the right (C, T, A, and G) show extension products from dideoxy sequencing (32) of Toto1101 plasmid DNA with the same primer. The nucleotide complementary to the start site of wild-type Sindbis virus subgenomic RNA is marked in the sequence ladder with an arrow.

from the wild-type versus the mutant junction region. A 5′-end-labeled oligonucleotide complementary to the sequence from 47 to 65 bases downstream from the wild-type subgenomic RNA start was annealed to total cytoplasmic RNA from mutant, wild-type, or mock-infected cells and extended by using reverse transcriptase. Virus-specific extension products which corresponded to the 5′ terminus of 26S RNA were seen for both wild-type and mutant intracellular RNAs (Fig. 3B). Although present in greatly reduced quantities, the sizes and distribution of the extension products of the mutant were identical to those of the wild-type virus. This suggests that the three-base insertion in Toto1100 CR4.1, while dramatically decreasing the level of 26S RNA synthesis, does not alter the start site or the extent of 5′ capping. Since the start site of the wild-type and mutant subgenomic RNAs appeared to be identical by this analysis, the simplest interpretation of these data would argue for a direct effect of the insertion on the efficiency of subgenomic RNA synthesis, rather than an effect on 26S RNA stability. We note, however, that other possible effects, such as the capping efficiency of the 26S RNA affecting its stability, have not been examined.

Quantitative examination of the effects of the Toto1100 CR4.1 mutation. We measured the relative levels of subgenomic RNA synthesis by using two packaging-defective but replication-competent Sindbis virus genomes: TRCAT (42) and TRCAT.A (Fig. 1). In TRCAT, the Sindbis virus structural genes are replaced by the CAT gene, such that a subgenomic RNA encoding CAT is produced by the Sindbis virus replication-transcription machinery (42). TRCAT.A is identical to TRCAT except for the three-base insertion corresponding to the Toto1100CR4.1 mutation in the junction region. Since these genomes lack the structural protein genes, no virus will be released from transfected cells and the levels of genomic and subgenomic RNAs in transfected cells can be more easily compared. Furthermore, translation of the subgenomic RNA leads to production of enzymatically active CAT, which can then be assayed to provide a more sensitive albeit indirect estimate of the relative subgenomic RNA levels. When the level of CAT produced per cell by these constructs was compared at different times posttransfection, TRCAT.A produced between 200- to 500-fold less CAT per cell than TRCAT did (Table 1). Examination of the dactinomycin-resistant RNA patterns from BHK cells transfected with these constructs, incubated at 30°C, showed that while the three-base insertion drastically reduces subgenomic RNA synthesis, relatively little difference was seen in the level of genome-length RNAs (data not shown). This implies that the mutation does not lead to any obvious defects in genomic RNA synthesis at this temperature.

Effects of the three-base insertion on subgenomic RNA production in *trans*. Since the Toto1100CR4.1 mutation also affects the structure of nsP4 (Fig. 2C) by insertion of an arginyl residue two residues from the C terminus of the

TABLE 1. Measurement of CAT activity in cells transfected with TRCAT or TRCAT.A[a]

Time (h p.t.)[b]	CAT expression[c]		CAT trimers per cell[d]		Ratio of trimers per cell
	TRCAT	TRCAT.A	TRCAT	TRCAT.A	TRCAT/TRCAT.A
15	6×10^{-5}	4×10^{-8}	4×10^{5}	8×10^{2}	5×10^{2}
20	7×10^{-5}	1×10^{-7}	5×10^{5}	3×10^{3}	2×10^{2}
23	1×10^{-4}	1×10^{-7}	1×10^{6}	3×10^{3}	3×10^{2}

[a] Duplicate plates of BHK monolayers were transfected with TRCAT or TRCAT.A transcripts or mock transfected for each time point, as described in Materials and Methods. After incubation at 30°C for the indicated times, one monolayer was harvested and assayed for CAT. The duplicate monolayer was fixed and stained with antisera specific for Sindbis virus nsP3 to determine the number of productively transfected cells.

[b] Monolayers were harvested for CAT assay or fixed for immunofluorescence at the indicated times posttransfection (p.t.).

[c] Units of CAT per monolayer, as determined in reference 9.

[d] Total number of enzymatically active CAT trimers in the extract divided by the total number of positively staining immunofluorescent cells.

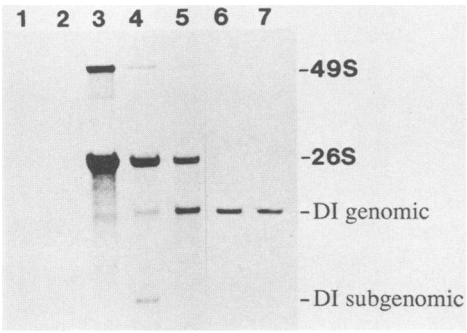


FIG. 4. Effect of the insertion mutation on subgenomic RNA synthesis in *trans*. CEF monolayers were transfected with RNA from JNSH (lane 1), JNSH.A (lane 2), 49S virion RNA (lane 3), cotransfected with virion RNA and JNSH (lane 4), or virion RNA and JNSH.A (lane 5). RNAs were labeled with [^{3}H]uridine in the presence of 2 μg of dactinomycin per ml from 1 to 15.5 h posttransfection. Total cytoplasmic RNAs were isolated, denatured, and separated on a 1% agarose gel. Transcripts from JNSH and JNSH.A used for transfection were run in lanes 6 and 7, respectively. The positions of the genomic 49S RNA (11703 bases), the subgenomic 26S RNA (4106 bases), the DI genomic RNA (DI genomic, 2671 bases), and the DI subgenomic RNA (DI subgenomic, 1280 bases) are indicated at the right. The identity and structure of the DI subgenomic RNA was verified by S1 analysis of in vivo-labeled intracellular RNAs (Levis, Ph.D. thesis).

protein, the phenotypes of Toto1100CR4.1 and TRCAT.A may result from deleterious effects of the insertion mutation on the subgenomic RNA promoter, an effect of the inserted amino acid on the function of nsP4, or both. The predicted carboxyl termini of alphavirus nsP4s are rather variable (Fig. 2). The nsP4 coding region of Semliki Forest virus contains three additional carboxyl-terminal residues, whereas Venezuelan and Eastern equine encephalitis viruses contain a stop codon three residues upstream from the position homologous to the Toto1100CR4.1 insertion mutation. Although these observations suggest that this region of nsP4 may not be functionally significant, and, therefore, that the Toto1100CR4.1 insertion mutation acts primarily in *cis*, a direct test is clearly desirable.

In studies described in detail elsewhere (Levis et al., submitted), insertion of a fragment containing the junction region into a DI RNA results in the synthesis of a DI-derived subgenomic RNA in the presence of a helper virus. We constructed DI RNAs which contained either the wild-type (JNSH) or the mutant (JNSH.A) junction region (Fig. 1) and tested their ability to support subgenomic RNA synthesis when wild-type nonstructural proteins were supplied in *trans* by helper virus. The respective DI RNAs and wild-type virion RNA were used to cotransfect CEF cells, and the virus-specific RNAs were examined after labeling with [^{3}H]uridine in the presence of dactinomycin. The DI genome containing the junction region with the three-base insertion (JNSH.A) produced little, if any, subgenomic RNA compared with the DI with the wild-type junction region (JNSH, Fig. 4). These results clearly showed that the mutation was *cis* acting. Thus, the depressed synthesis of 26S RNA by Toto1100CR4.1 is primarily due to the deleterious effect of the three-base insertion on the efficiency of the subgenomic RNA promoter.

Isolation of Toto1100CR4.1 revertants. While plaque morphology variants of the Toto1100CR4.1 mutant made the initial characterization of the mutant phenotype difficult, the variants might allow us to isolate and study potential second-site revertants that could prove useful for identifying viral proteins which interact either directly or indirectly with the subgenomic RNA promoter. To isolate independent revertants, 24 individual wells of CEF monolayers were transfected with Toto1100CR4.1 RNA transcripts. After passaging the media twice (at 30°C), revertants showing altered plaque morphology (at 30°C) were plaque purified twice from each of the 24 transfections. Since the Toto1100CR4.1 mutant makes small diffuse plaques under these conditions, revertants were easily identified. The data on plaque phenotype, temperature sensitivity, growth properties, RNA patterns, subgenomic RNA start site, and sequence of the junction region for many of these revertants (called Toto1100 CR4.1R1 through 24 and abbreviated here as R1 through R24) are summarized in Table 2 and described below.

All of the revertants produced plaques at 30°C, clearly distinguishable from Toto1100CR4.1, ranging from small or medium plaques to large plaques (revertant R12) indistinguishable from Toto1101. With the exception of R12, most revertants either did not plaque at 40°C or produced small diffuse plaques, indicating some temperature sensitivity in plaque formation. The RNA patterns and the growth properties of the revertants that gave reasonably high titers were examined in detail. Differential growth curves for R1, R3, R9, R11, R12, R17, R22, and R23 compared with Toto1101 virus at 30°C are shown in Fig. 5. With the exception of R12, all of these revertants showed delayed virus production relative to Toto1101 but eventually produced high (10^7 to 10^9 PFU/ml) virus yields. Examination of virus-specific RNA patterns revealed that R12 made nearly wild-type levels of 26S RNA, compared with 49S RNA. The other revertants produced greatly elevated levels of 26S RNA relative to Toto1100CR4.1 (see Fig. 3A) but were still depressed for subgenomic RNA synthesis relative to Toto1101 (Fig. 6A). In all cases examined thus far (Fig. 6B), the 26S RNA start site was unchanged, except that R9 shows a weak additional product approximately four bases longer than the wild-type 26S RNA extension product.

To examine the junction region of the revertants for possible local changes that might compensate for the original three-base insertion, the sequence of this region was determined for Toto1101, Toto1100CR4.1, and most of the revertants by chemical sequencing of PCR-amplified cDNA (see Methods). The revertant R12, which made nearly wild-type levels of 26S RNA, contains a three-base deletion in the junction region, with the net result being a single base change at the −4 position (C to G) which also restores the wild-type nsP4 carboxyl-terminal sequence (Fig. 2C). Revertant R22 retained the original three-base insertion but has a C to G change at the −23 position, resulting in the substitution of Asp for His at residue 604 of nsP4 (Fig. 2C). This residue is present in the homologous position in Middelburg virus (Fig. 2), and one possibility is that the inserted Arg residue in Toto1100CR4.1, if deleterious, is compensated by the acidic Asp substitution. The remaining revertants were identical to Toto1100CR4.1 in the region sequenced (from nt 7555 to nt 7655 of the genomic RNA sequence, or from −43 to +58 relative to the subgenomic RNA start). These results demonstrate that all of the revertants thus far characterized are second-site revertants and that many involve changes outside of the junction region.

***trans*-Complementation assay to identify second-site revertants in *trans*-acting viral components.** Most of the second-site revertants described above retained the mutant junction region and must therefore contain compensating changes elsewhere in the genome. To screen for revertants which

TABLE 2. Characterization of Toto1100CR4.1 revertants

Strain[a]	Incubation temp				Junction sequence[b]	Ratio of CAT activity (DIJCAT/DIJCAT.A)[c]	% Conversion relative to Toto1101 on wild-type junction[d]
	30°C		40°C				
	Plaque morphology[e]	Titer[f]	Plaque morphology[e]	Titer[f]			
Toto1101	L	2×10^8	M	2×10^8	wt	4.1, 5.5, 6.0	100, 100, 100
Toto1100CR4.1	SD	ND	vSD	ND	4.1	—	—
R1	S	2×10^7	SD	(4×10^6)	4.1	ND, 8.3, 7.9	ND, 49, 14
R2	S	2×10^7	SD	(2×10^6)	4.1	—	—
R3	M	5×10^8	N, CPE	ND	4.1	—	—
R4	SM	3×10^7	N, CPE	ND	4.1	—	—
R5	S	2×10^6	N, CPE	ND	4.1	—	—
R6	S	4×10^7	N, CPE	ND	4.1	—	—
R7	vS	2×10^5	N	ND	ND	—	—
R8	M	4×10^7	SD	(2×10^7)	4.1	—	—
R9	SM	2×10^8	SD	(2×10^7)	4.1	—	—
R10	SM	5×10^7	vSD	(3×10^6)	4.1	—	—
R11	S + M[g]	1×10^8	vSD	(1×10^7)	4.1	1.2, 2.4, 1.1	19, 32, 11
R12	L	6×10^8	M	3×10^7	+	ND, 1.4, 1.1	ND, 9, 17
R13	S	3×10^4	SD	(3×10^3)	ND	—	—
R14	M	2×10^4	N	ND	4.1	—	—
R15	M	5×10^6	SD	(2×10^6)	4.1	ND, 2.7, 2.7	ND, 28, 24
R16	M	4×10^4	N	ND	4.1	—	—
R17	ML	4×10^7	N, CPE	ND	4.1	ND, 2.5, 2.0	ND, 18, 12
R18	S	5×10^1	N	ND	4.1	—	—
R19	SM	1×10^4	SD	(2×10^3)	4.1	—	—
R20	SM	2×10^4	N	ND	4.1	—	—
R21	M	2×10^5	SD	(2×10^4)	4.1	—	—
R22	M	3×10^7	N, CPE	ND	+	—	—
R23	M	2×10^6	N, CPE	ND	4.1	ND, 4.5, 5.2	ND, 15, 41
R24	SM	8×10^5	N	ND	4.1	—	—

[a] Virus stocks of Toto1101 and Toto1100CR4.1 were derived from RNA transfection supernatants. Revertant stocks were derived from independent transfections and plaque purified twice as described in Materials and Methods.

[b] Nucleotide sequences from nt 7555 to 7655, as determined from sequencing PCR products and as described in the text. No revertants were found with sequences in this region identical to wild-type Sindbis virus (wt). Except for R12 and R22, all revertants sequenced were identical to Toto1100CR4.1 (4.1) in the junction region. Two sequences were not determined. +, Sequences determined for the junction regions of R12 and R22 are shown in Fig. 2.

[c] The ability of the revertant to use the 4.1 junction region incorporated into a DI RNA better than the wild-type parent was assayed as described in the text and Fig. 7. This number gives the ratio of enzymatically active CAT produced in CEF monolayers transfected with DIJCAT RNA divided by the level produced in cells transfected with DIJCAT.A RNA in the presence of either the wild-type virus or the revertants indicated as helper viruses. The results of two or three independent repetitions of the experiments are presented. Although transfection efficiency is quite variable between experiments, this ratio is quite consistent. ND, Not determined. —, Level of CAT activity detected in *trans*-complementation assay was too low for reliable comparisons or the titer of the revertant was too low for a high multiplicity infection.

[d] Percentage of the chloramphenicol aceylated normalized to the amount converted by using Toto1101 as the helper virus for DIJCAT. The levels of CAT produced were normalized to that of the wild-type virus in each experiment to correct for the variable transfection efficiencies between experiments. Although the levels of CAT produced by the revertants are lower than the those found with Toto1101, these values are still 10- to 100-fold greater than the background levels of CAT activity seen in the absence of helper virus. ND, Not determined. —, As defined in footnote *c*.

[e] Plaque morphology is given relative to Toto1101: S, small; M, medium; L, large; SM, ML, intermediate sizes; SD, small and diffuse; vSD, very small and diffuse plaques; N, no discrete plaques; CPE, cytopathic effect apparent at lower dilution, although discrete plaques could not be counted.

[f] Titer is given in PFU/ml. Those numbers enclosed in parentheses are tentative due to difficulties in counting the small diffuse plaques present at 40°C. ND, The absence of plaques or the inability to accurately count small diffuse plaques did not allow titers for these samples to be determined.

[g] A heterogeneous mixture of small and medium plaques was noted for R11.

might contain compensating changes in *trans*-acting viral proteins important for subgenomic RNA transcription, an assay was developed on the basis of the activity of the junction region when translocated to DI RNAs (Fig. 7). DI clones were constructed with the CAT gene downstream from a wild-type (DIJCAT) or mutant junction (DIJCAT.A) region (Fig. 1). These constructs do not encode functional nonstructural proteins and are inactive in the absence of helper virus. In the presence of Toto1101 helper virus, DIJCAT RNAs replicate, transcribe a subgenomic RNA, and express high levels of CAT enzyme. In the case of DIJCAT.A, much less CAT enzyme is made. Presumably, this is due to lower levels of subgenomic RNA production, as was found for JNSH.A. The Toto1100CR4.1 revertants were then tested as helpers, screening for those that show enhanced recognition of the mutant junction region. CEF cells were infected with either Toto1101 or the Toto1100 CR4.1 revertants and were then transfected with capped transcripts from either DIJCAT or DIJCAT.A. Cells were harvested after ~20 h, and the level of CAT enzyme activity was determined (Table 2). The ratio of CAT activity (DIJCAT/DIJCAT.A; shown in Table 2, second to last column) was used as a measure of the use of the mutant junction region relative to the wild-type junction region by each virus. Toto1101 appeared to use the mutant junction region 4- to 6-fold less efficiently than the wild-type junction region. Revertants R11 and R12 gave similar CAT expression with wild-type and mutant junction regions, suggesting that they contain changes in viral proteins which facilitate use of the mutant junction region relative to the wild-type junction region in *trans*. Revertants R15 and R17 seem to recognize the mutant junction region somewhat less efficiently than R11 and R12, while revertants R1 and R23, like Toto1101, appear to recognize the mutant junction region poorly by this

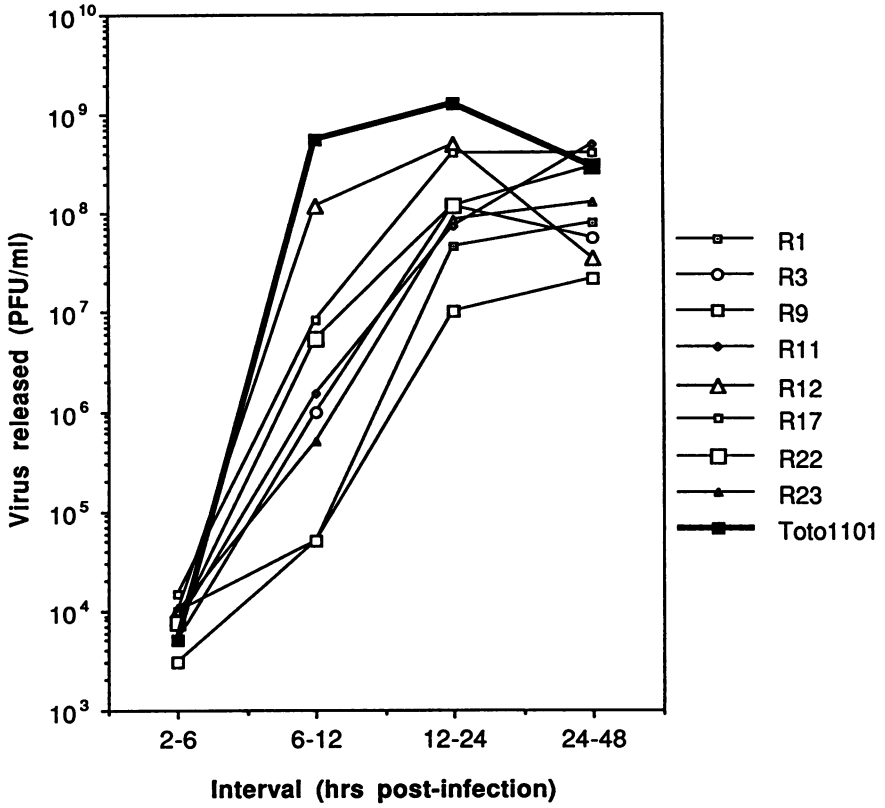


FIG. 5. Differential growth curves of selected Toto1100CR4.1 revertants. CEF monolayers were infected with Toto1101 or Toto1100CR4.1 revertants R1, R3, R9, R11, R12, R17, R22, and R23 at an MOI of 10 PFU per cell. The amount of virus released during incubation at 30°C, for the time intervals shown, was determined by plaque assay on CEF monolayers at 30°C.

assay. The ratios obtained in independent experiments were consistent, although there was variation in the absolute levels of CAT produced (Table 2, last column).

DISCUSSION

Alphavirus subgenomic RNA promoter. We have shown that the conserved sequence in the Sindbis virus junction region is indeed a *cis*-acting element involved in subgenomic RNA transcription. The three-base insertion in the putative subgenomic RNA promoter of Toto1100CR4.1 depresses subgenomic RNA synthesis, leading to extremely low virus yields and indistinct plaques. DI RNAs containing either the wild-type or the mutant junction region were used to demonstrate that the mutation led to depressed levels of subgenomic RNA in *cis* in the presence of wild-type nsP4. Thus at

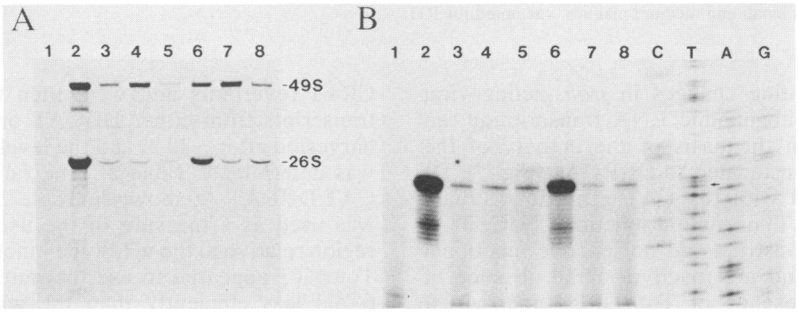
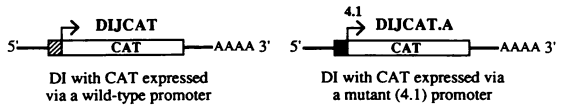


FIG. 6. Virus-specific RNA synthesis and subgenomic RNA start site of Toto1100CR4.1 revertants. (A) Patterns of virus-specific RNAs. CEF cells were either mock infected (lane 1), infected with Toto1101 (lane 2), or infected with Toto1100CR4.1 revertants R1, R3, R9, R12, R17, and R22 (lanes 3 through 8, respectively) at an MOI of 10 PFU per cell. Cultures were incubated at 30°C, and RNAs were labeled with [^{3}H]uridine in the presence of 2 μg of dactinomycin per ml from 4 to 8 h posttransfection. Total cytoplasmic RNAs were isolated, denatured, and separated on a 1% agarose gel. (B) Primer extension mapping of the subgenomic RNA initiation site for these RNA samples. Primer extensions were done as described in Materials and Methods and the legend to Fig. 3. The RNA samples used for primer extension, corresponding to lanes 1 through 8, are as described for panel A. In the four lanes at the right (C, T, A, and G), Toto1101 plasmid DNA was sequenced by using the same primer by the dideoxy method. The nucleotide complementary to the start site of wild-type subgenomic RNA is marked in the sequence ladder with an arrow.

A. Screening for second-site revertants in trans-acting viral proteins



B. Predicted results of trans-complementation assay

Helper Virus	Level of CAT produced DIJCAT	DIJCAT.A
Wild-type (Toto1101)	high	low
Toto1100CR4.1 mutant	?	low
Second-site revertants		
Class I changes in junction region	high	low
Class II increased packaging efficiency	high	low
Class III changes suppressing nsP4 defect	high	low
Class IV changes in trans-acting viral proteins	?	low-high

FIG. 7. Screening for second-site revertants in *trans*-acting viral proteins. (A) A schematic of the rationale used for design of an assay to screen for revertants with potential changes in *trans*-acting viral proteins involved in subgenomic RNA synthesis. In the presence of a helper virus, the CAT activity produced was determined for DI RNAs which expressed a CAT subgenomic RNA either under the control of the wild-type promoter (DIJCAT) or the promoter containing the three-base insertion mutation (DIJCAT.A). A discussion of some of the possible classes of pseudorevertants (panel B) which could compensate for the Toto1100CR4.1 insertion mutation is presented in the text. The results of this assay for several of the second-site revertants are presented in Table 2. The behavior of the original Toto1100CR4.1 mutant in this assay is unknown, since high-titered stocks of this virus, free of revertants, could not be obtained. If the insertion mutation does not affect the activity of nsP4, Toto1100CR4.1 should use the mutant and wild-type junction regions like the wild-type helper virus.

least part of the mutant phenotype is due to a direct *cis*-acting effect of the insertion mutation on promoter efficiency.

The extent of 5′ and 3′ sequences actually necessary for a functional Sindbis virus subgenomic promoter element in the context of a DI RNA have been mapped, and they include the conserved junction sequence (Levis et al., submitted). The three-base insertion mutation described here disrupts the conserved sequence originally proposed to be the subgenomic RNA promoter element ([24] Fig. 2A). The mechanism by which the insertion mutation leads to depressed subgenomic RNA synthesis is unknown. One likely possibility is that the insertion of three bases alters the interaction of the promoter element with one or more *trans*-acting components (either viral or host) required for subgenomic RNA transcription. Since Toto1100CR4.1 subgenomic RNA transcripts apparently initiate at the same position as normal 26S RNA, the insertion of these three bases near the middle of the conserved sequence neither abolished transcription completely nor affected the subgenomic RNA start.

***trans*-Acting transcription factors.** Little is known about the identity of the factor(s) involved in initiation of subgenomic RNA synthesis. Minimally, promoter recognition and RNA polymerase activities are necessary. In addition, the 26S RNA is capped and methylated. Some clues have begun to emerge from mapping the causal lesions in Sindbis virus *ts* mutants defective in RNA replication (RNA^- mutants), which were previously grouped by genetic complementation into four groups (4–6, 14, 33–35, 37; reviewed in reference 40). These data indicate that nsP1 may play a role in negative-strand synthesis (group B [12, 34]) and methyl transferase activity (22a), that lesions in nsP2 affect the ratio of genomic to subgenomic RNA and polyprotein processing (groups A and G [12, 33]), and that lesions in nsP4 can exhibit generalized defects in RNA synthesis (group F [1, 11]) and shutoff of negative-strand synthesis (35; D. L. Sawicki, D. B. Barkhimer, S. G. Sawicki, C. M. Rice, and S. Schlesinger, submitted for publication). Thus at least one possibility is that nsP2 and nsP4 play major roles in initiation of subgenomic RNA synthesis. Indeed, genetic data exist which can be interpreted as evidence for a complex of these two proteins (11).

In this study, we have begun to characterize second-site revertants which may help to identify virus-specific proteins that participate in subgenomic RNA synthesis. There are at least four formal classes of second-site revertants which might compensate for the Toto1100CR4.1 mutation (Fig. 7). One class could involve *cis*-acting secondary changes in the Toto1100CR4.1 junction region that restore or partially restore promoter function. A second class might involve changes in the structural proteins or RNA sequences involved in encapsidation, increasing the efficiency of that process even in the presence of low concentrations of the structural proteins. A third class could involve reversions which compensate for a possible effect of the insertion mutation on nsP4 function. A fourth class involves changes in the protein(s) which recognize the promoter, increasing the efficiency of transcription initiation at the mutant junction region. Since large numbers of revertants are easily obtained, rapid methods for screening and classifying are clearly of value for identifying representatives in each of these classes.

Our current emphasis is on the fourth class of revertants, and a rapid assay was developed and used to identify at least two second-site revertants, R11 and R12, with potential compensating changes in virus-encoded *trans*-acting factors that allow better recognition of the Toto1100CR4.1 mutant junction region. Revertant R12 has a three-base deletion in the junction region, changing the wild-type nsP4 carboxyl-terminal sequence and resulting in a single base substitution at the −4 position of the putative promoter (Fig. 2C). This revertant makes nearly normal levels of subgenomic RNA. In addition to recognizing its own promoter, this revertant also recognizes the wild-type promoter and the Toto1100 CR4.1 mutant promoter, as shown by the *trans*-complementation assay. It may also contain additional changes elsewhere in the genome. The other revertant, R11, retains the Toto1100CR4.1 mutation and recognizes both the mutant and the wild-type promoter. It, too, is likely to have changes in one or more *trans*-acting viral transcription factors.

General approach and importance of first-cycle analysis. The experiments described here are encouraging for the use of molecular genetics to help understand the mechanism of alphavirus replication and transcription. The initial step in this approach is the creation of a deleterious mutation in the region of interest, characterization of the mutant phenotype

(see below), and the isolation of a set of preferably early-passage second-site revertants. The compensating changes in the second-site revertants may be mapped by using essentially the same strategy previously outlined (10–12, 19, 22a, 29). The mapping and characterization of such second-site revertants should be extremely valuable in elucidating functional interactions between the nonstructural proteins and in understanding the interactions of the nonstructural proteins with *cis*-acting regulatory sequences.

Since care was taken to isolate early-passage revertants in our experiments, we expect relatively few changes in the revertants relative to the Toto1100CR4.1 mutant. Also, once the compensating changes in the revertants are cloned, more thorough studies of the phenotypes of the revertants on the Toto1100CR4.1 and wild-type (Toto1101) backgrounds can be undertaken. Such studies will help to identify those viral proteins involved in recognizing the junction region for transcription of the subgenomic RNA.

An important observation emerging from the work reported here and elsewhere (10, 18) is the remarkable plasticity of the alphavirus genome, as well as the facile generation of revertants. Deleterious mutations which allow RNA replication, even at low levels, coupled with the high mutation rates characteristic of RNA viruses will strongly select for revertants. As shown by sequence analysis of pseudorevertants of Toto1100CR4.1, changes involving either substitutions or deletions arise even in early-passage stocks. While the genome flexibility and high mutation frequency are useful in allowing revertants to be easily isolated, our results with Toto1100CR4.1 underscore the need to study the mutant phenotypes as early as possible, ideally without generating virus stocks. In the case of Toto1100CR4.1, if we had transfected cells, harvested the media, and produced a stock after plaque purification, we would have been studying a pseudorevertant (which upon sequencing would probably contain the mutant junction sequence). Precautions that we have adopted include routine examination of the specific infectivity of mutant RNA transcripts and the characterization of the phenotype of a mutant immediately after primary transfection, and these then serve as a standard for comparison with the phenotype of any subsequent virus stocks generated by passaging. With these precautions, there is less risk of reporting a mutant phenotype that is actually that of a revertant.

ACKNOWLEDGMENTS

We thank our colleagues for helpful suggestions on the manuscript and S. Schlesinger for help with the DI constructs.

This work was supported by Public Health Service grants AI24134 and AI26763 from the National Institutes of Health, a grant from the Pew Memorial Trust, and the Monsanto/Washington University Biomedical Research Contract. C.M.R. is a Pew Scholar in the Biomedical Sciences.

LITERATURE CITED

1. **Barton, D. J., S. G. Sawicki, and D. L. Sawicki.** 1988. Demonstration in vitro of temperature-sensitive elongation of RNA in Sindbis virus mutant *ts*6. J. Virol. **62**:3597–3602.
2. **Bornstein, M. B.** 1958. Reconstituted rat-tail collagen used as substrate for tissue cultures on coverslips in maximow slides and roller tubes. Lab. Invest. **7**:134–137.
3. **Boros, I., G. Pósfa, and P. Venetianer.** 1984. High-copy number derivatives of the plasmid cloning vector pBR322. Gene **30**: 257–260.
4. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30**:204–213.
5. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Complementation between temperature-sensitive mutants of Sindbis virus. Virology **30**:214–223.
6. **Burge, B. W., and E. R. Pfefferkorn.** 1968. Functional defects of temperature-sensitive mutants of Sindbis virus. J. Mol. Biol. **35**:193–205.
7. **Chang, G. J., and D. W. Trent.** 1987. Nucleotide sequence of the genome region encoding the 26S mRNA of Eastern equine encephalomyelitis virus and the deduced amino acid sequence of the viral structural proteins. J. Gen. Virol. **68**:2129–2142.
8. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84**:7413–7417.
9. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
10. **Hahn, C. S., C. M. Rice, E. G. Strauss, E. M. Lenches, and J. H. Strauss.** 1989. Sindbis virus *ts*103 has a mutation in glycoprotein E2 that leads to defective assembly of virions. J. Virol. **63**: 3459–3465.
11. **Hahn, Y. S., A. Grakoui, C. M. Rice, E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA$^-$ temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. J. Virol. **63**:1194–1202.
12. **Hahn, Y. S., E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA$^-$ temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J.Virol. **63**:3142–3150.
13. **Hardy, W. R., and J. H. Strauss.** 1988. Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo by using monospecific antibodies. J. Virol. **62**:998–1007.
14. **Keränen, S., and L. Kääriäinen.** 1979. Functional defects of RNA-negative temperature-sensitive mutants of Sindbis and Semliki Forest viruses. J. Virol. **32**:19–29.
15. **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.
16. **Lehtovaara, P., H. Söderlund, S. Keränen, R. F. Pettersson, and L. Kääriäinen.** 1982. 18S defective-interfering RNA of Semliki Forest virus contains a triplicated linear repeat. Proc. Natl. Acad. Sci. USA **78**:5353–5357.
17. **Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger.** 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell **44**:137–145.
18. **Li, G., and C. M. Rice.** 1989. Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. J. Virol. **63**:1326–1337.
19. **Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss.** 1988. Molecular basis of Sindbis virus neurovirulence in mice. J. Virol. **62**:2329–2336.
20. **Lutz, C. T., W. C. Hollifield, B. Seed, J. M. Davie, and H. V. Huang.** 1987. Syrinx 2A: an improved lambda phage vector designed for screening DNA libraries by recombination in vivo. Proc. Natl. Acad. Sci. USA **84**:4379–4383.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.

22a. **Mi, S., R. Durbin, H. V. Huang, C. M. Rice, and V. Stollar.** 1989. Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. Virology **170**:385–391.

23. **Monroe, S., and S. Schlesinger.** 1984. Common and distinct

regions of defective-interfering RNAs of Sindbis virus. J. Virol. **49**:865–872.

24. **Ou, J.-H., C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss.** 1982. Sequence studies of several alphavirus genomic RNA's in the region containing the start of the subgenomic RNA. Proc. Natl. Acad. Sci. USA **79**:5235–5239.
25. **Ou, J.-H., E. G. Strauss, and J. H. Strauss.** 1981. Comparative studies of the 3′ terminal sequences of several alphavirus RNAs. Virology **109**:281–289.
26. **Ou, J.-H., E. G. Strauss, and J. H. Strauss.** 1983. The 5′ terminal sequences of the genomic RNAs of several alphaviruses. J. Mol. Biol. **168**:1–15.
27. **Pierce, J. S., E. G. Strauss, and J. H. Strauss.** 1974. Effect of ionic strength on the binding of Sindbis virus to chick cells. J. Virol. **13**:1030–1036.
28. **Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston.** 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. J. Virol. **62**:2124–2133.
29. **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.
30. **Rice, C. M., and J. H. Strauss.** 1981. Synthesis, cleavage, and sequence analysis of DNA complementary to the 26S messenger RNA of Sindbis virus. J. Mol. Biol. **150**:315–340.
31. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Sawicki, D. L., and S. G. Sawicki.** 1985. Functional analysis of the A complementation group mutants of Sindbis HR virus. Virology **144**:20–34.
34. **Sawicki, D. L., S. G. Sawicki, S. Keränen, and L. Kääriäinen.** 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. J. Virol. **39**:348–358.
35. **Sawicki, S. G., D. L. Sawicki, L. Kääriäinen, and S. Keränen.** 1981. A Sindbis virus mutant temperature-sensitive in the regulation of minus-strand RNA synthesis. Virology **115**:161–172.
36. **Schlesinger, S., and M. J. Schlesinger (ed.).** 1986. The Togaviridae and Flaviviridae. Plenum Publishing Corp., New York.
37. **Strauss, E. G., E. M. Lenches, and J. H. Strauss.** 1976. Mutants of Sindbis virus I. Isolation and partial characterization of 89 new temperature-sensitive mutants. Virology **74**:154–168.
38. **Strauss, E. G., R. Levinson, C. M. Rice, J. Dalrymple, and J. H. Strauss.** 1988. Nonstructural proteins nsP3 and nsP4 of Ross River and O'Nyong-nyong viruses: sequence and comparison with those of other alphaviruses. Virology **164**:265–274.
39. **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.
40. **Strauss, E. G., and J. H. Strauss.** 1980. Mutants of alphaviruses: genetics and physiology, p. 393–426. *In* R. W. Schlesinger (ed.), The Togaviruses. Academic Press, Inc., New York.
41. **Weislander, L.** 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. **98**:305–309.
42. **Xiong, C., R. Levis, P. Shen, S. Schlesinger, C. Rice, and H. V. Huang.** 1989. Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science **243**:1188–1191.