Assembly of Functional Sindbis Virus RNA Replication Complexes: Requirement for Coexpression of P123 and P34

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A vaccinia virus transient expression system was used to determine which of the Sindbis virus (SIN) proteins and/or polyproteins are necessary for the formation of active replication complexes and, in particular, to analyze the role of nsP4, the putative polymerase, versus P34 in RNA replication. We generated vaccinia virus recombinants in which the cDNA for the entire SIN nonstructural coding region as well as cDNA copies of the individual nonstructural proteins (nsPs) and several intermediate polyproteins were placed downstream of the promoter for T7 RNA polymerase and the encephalomyocarditis virus 5' untranslated region. The proteins expressed by the vaccinia virus recombinants comigrate with authentic proteins synthesized in SIN-infected cells, and the polyproteins appear to be processed to the individual proteins of the correct size. To examine the replication efficiencies of different protein combinations, a vaccinia virus recombinant was designed to express an engineered substrate RNA which could serve as a template for replication and subgenomic mRNA transcription by the SIN nsPs. Expression of the entire SIN nonstructural coding region resulted in the synthesis of high levels of both genomic and subgenomic RNAs derived from the engineered template. No RNA replication could be detected during coexpression of the four individual nsPs, although the proteins were indistinguishable, in terms of electrophoretic mobility, from those synthesized in SIN-infected cells. Coexpression of polyproteins P12, P23, and/or P34 with the individual nsPs also did not result in detectable levels of RNA replication. However, when P123 and P34 were coexpressed, efficient RNA replication and subgenomic mRNA transcription of the substrate RNA was observed. Coexpression of nsP4 with P123 resulted in the synthesis of only minus-strand RNAs. These studies show that expression of both P123 and P34 is necessary for establishment of functional RNA replication and transcription complexes and raise the possibility that the polyproteins themselves may be functional components of these complexes. In addition, these data indicate that an nsP4 moiety expressed independently with an additional N-terminal methionine is capable of functioning in minus-strand but not plus-strand RNA synthesis.

Alphaviruses are small, enveloped plus-strand RNA viruses which replicate in a wide variety of vertebrate and invertebrate cells. Sindbis virus (SIN), the prototype alphavirus, has a single-stranded RNA genome 11,703 nucleotides in length which is capped at the 5' terminus and polyadenylated at the 3' terminus. Upon infection of cells, the genomic RNA initially serves as an mRNA and is translated to produce the viral nonstructural proteins (nsPs) which are necessary for SIN replication (55). RNA replication is initiated by the synthesis of a full-length minus strand complementary to the genomic 49S plus-strand RNA. This minus strand then serves as the preferred template for the synthesis of nascent plus-strand genomic RNA molecules and for transcription of a 26S subgenomic RNA via initiation at an internal site in the minus-strand RNA. Three to 4 h postinfection the synthesis of minus-strand RNA ceases, while the production of plus-strand genomic and subgenomic RNAs continues throughout the infectious cycle (51, 52).

The nsPs involved in SIN RNA replication are translated as two large polyproteins from the 5' two-thirds of the viral genome (55). Translation of the genomic RNA results in a 200-kDa polyprotein, P123, which terminates at an in-frame opal termination codon and encodes nsP1, nsP2, and nsP3. At a low frequency, read-through of the opal termination codon occurs, resulting in the production of a 250-kDa polyprotein, P1234, which encodes nsP1, nsP2, nsP3, and

nsP4. These polyprotein precursors are co- or posttranslationally processed by a protease activity residing in the C-terminal domain of nsP2 (8, 21) to generate several intermediate polyproteins (P12, P23, and P34) and the four individual nsPs (6, 20). Because the synthesis of P1234 is dependent on read-through of an opal termination codon, nsP4 and nsP4-containing polypeptides are underproduced relative to the other nsPs (54). Processing of the SIN nonstructural polyproteins is highly regulated, and during the infectious cycle the predominant species of polyprotein intermediates changes, as the various nsP2 protease-containing polyproteins have different cleavage site preferences (6, 20). The fact that the virus has such a complex regulatory scheme for production of its nsPs suggests that the temporal regulation of protein processing is important for efficient replication.

The possible roles of the nsPs in RNA replication have been analyzed by sequence comparisons and studies on SIN mutants. nsP1 is thought to be involved in minus-strand synthesis (17, 57) and appears to be associated with a methyltransferase activity (39, 40). The carboxy terminus of nsP2 contains a proteolytic activity responsible for cleavage and processing of the viral nonstructural polyproteins (8, 21), while the N-terminal domain of nsP2 contains conserved motifs found in nucleotide-binding and helicase proteins (13). In addition, mutations resulting in the temperature-sensitive synthesis of 26S RNA have mapped to the C-terminal portion of nsP2, suggesting that nsP2 is required for the synthesis of subgenomic RNA (17). Little is known about

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FIG. 1. Vaccinia virus recombinants expressing SIN nsPs and polyproteins. The nonstructural coding region of the SIN genome is shown with the four nsPs represented by different fill patterns. Cassettes expressed by vaccinia virus recombinants are shown below. The solid diamond indicates the opal termination codon preceding nsP4. The nomenclature used for the expressed protein product is indicated to the left. The nsP prefix refers to an individual protein moiety, while a P prefix indicates a polyprotein. As noted in Results, infections in which nsP2-containing polyproteins are expressed will also contain the cleavage products. Observed sizes of the polypeptides during SDS-PAGE are shown to the right.

the function of nsP3. It has been shown that nsP3 is phosphorylated *in vivo* (36), but the role of nsP3 phosphorylation in SIN RNA replication, if any, is not yet known. nsP4 is thought to function as the viral RNA polymerase, as it shares amino acid homology with the RNA-dependent RNA polymerase of poliovirus (26), and mutations which inhibit the elongation activity of the viral replicase have been mapped to nsP4 (3, 16). Data also suggest a role for nsP4 in the regulation of minus-strand synthesis (50). It is not yet known whether the SIN replicase functions as nsP4 or P34 or whether both moieties play a role in RNA replication. One study has shown that an increase in the P34/nsP4 ratio correlates well with the shut-off of minus-strand synthesis. This result led to the hypothesis that nsP4 is the active form of the RNA polymerase for minus-strand synthesis while P34 is responsible for production of plus-strand RNAs (6).

Although genetic studies indicate that the four nsPs, and possibly a host protein, are required for SIN RNA replication, the specific components of alphavirus replication complexes have not been well defined. Studies to identify the components of alphavirus replication complexes have involved the isolation and solubilization of replication complexes capable of elongating preexisting RNA molecules (4, 5, 12, 46). The SIN replication complex has been partially purified from infected cells and has been shown by immunoprecipitation to contain the four individual nsPs and some P34, as well as a 120-kDa host protein (4). Although these isolated complexes can elongate preexisting RNA molecules, there is no direct evidence that they are capable of initiating new polynucleotide chains and, thus, during purification may have lost a polypeptide(s) necessary for the complete RNA replication cycle.

To examine the polypeptides required for formation of

active replication-transcription complexes, a heterologous system has been used to express SIN nsPs and polyproteins independently of SIN-specific RNA amplification. It was previously shown that functional SIN nsPs could be expressed via a recombinant vaccinia virus (34). On the basis of these results, we have generated vaccinia virus recombinants which express the SIN nsPs, both as the individual proteins and as polyprotein precursors, to study the role of each polypeptide in the various steps of viral RNA replication. By analyzing the ability of different protein combinations to replicate SIN-specific RNAs *in vivo*, the proteins which are required for RNA replication have been further defined.

MATERIALS AND METHODS

Viruses and cells. Vaccinia virus (WR strain) and vTF7-3, a vaccinia virus recombinant which expresses the T7 polymerase (11), were propagated and titered on BSC40 cells as previously described (23). The growth conditions for BSC40 cells and CV-1 cells were as previously described (23). BHK-21 cells were maintained at 37°C in modified Eagle medium (MEM) containing nonessential amino acids and 10% heat-inactivated fetal calf serum (FCS).

Plasmid constructions. Plasmids were constructed by standard methods (49), and a brief description of the constructs is presented below. The structures of the relevant clones are shown in Fig. 1. Unless otherwise stated, pToto1101, a full-length cDNA clone of the HR strain of SIN (47) was used as a source of SIN-specific cDNA for subcloning and for amplification by the polymerase chain reaction (PCR) (48). All subclones obtained from SIN cDNA which were amplified with specific primers were verified by sequence

analysis. The predicted amino and carboxy termini of the SIN nsPs, as assigned by partial amino acid sequence data and by homology among alphaviruses, were utilized to design the nsP-encoding constructs. nsP3 and P123 are present in two forms in infected cells: the predominant form produced by termination at the opal stop codon and a form which is 7 amino acids longer and generated by cleavage of the P34 and P1234 read-through products, respectively. The carboxy terminus of the predominant forms of nsP3 and P123 was used to generate the nsP3- and P123-expressing constructs. Constructs to be used for marker rescue were generated in the plasmid pTM3 (41) which contains, in 5'-to-3' order, the bacteriophage T7 promoter, the encephalomyocarditis virus internal ribosome entry site which facilitates cap-independent ribosome binding, and the T7 termination signal. SIN cDNAs were positioned 3' to the unique *Nco*I site in pTM3 which contains the ATG codon used for translation initiation.

For construction of pTM3/SINnsP1, which contains its own initiation codon, the oligonucleotide primer 5'-TGG TACCATGGAGAAGCCAGTA-3' was used to create an *Nco*I site in which the ATG in this site corresponds to the N-terminal Met of nsP1. pToto1204 (a Toto1101 derivative in which silent substitution mutations were used to create a *Xho*I site at nucleotide [nt] 222 [33]) cDNA was amplified by PCR, and the resultant product was digested with *Nco*I and *Xho*I and ligated into pTM3 which had been digested with *Nco*I and *Xho*I. The remainder of the nsP1 gene with adjacent termination codons was obtained by subcloning a restriction fragment from pnsP1.TFL, a plasmid which contains the nsP1 gene with tandem ochre and amber termination codons at the 3' end of the nsP1 gene (all TFL constructs were generously provided by M. W. LaStarza). Similar methods, using pToto1101 DNA for PCR amplification, were used to position initiation codons immediately preceding the coding sequences of nsP2, nsP3, and nsP4 and tandem termination codons at the 3' ends of the nsP2 and nsP3 genes. The termination codon for the nsP4 cassette was provided by its own naturally occurring stop codon.

Constructs encoding the polyproteins were derived by several subcloning steps. With the exception of plasmids expressing nsP1-containing polyproteins, these constructs begin with an initiating Met codon immediately preceding the coding sequence of the first amino acid of the polyprotein and terminate with tandem termination codons.

Construction of substrate plasmids. Plasmids which would allow the expression of engineered SIN-specific substrate RNAs *in vivo* were generated. Plasmid pTRCAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene replacing the coding region for the SIN structural proteins in pToto1002 (58). An *Ssp*I (nt 7499)-*Bgl*II (nt 9907) fragment from pTRCAT, which contains the SIN subgenomic promoter and the entire CAT gene, was ligated into pKDI25.3 (31), which had been digested with *Nae*I and *Bgl*II, to generate pJNTRCAT. For subsequent cloning purposes the *Sal*I site of pJNTRCAT was removed from the polylinker by digesting with *Xba*I and *Bam*HI, producing blunt ends with Klenow, and recircularization. This plasmid is termed pJNTRCATdXB. The predicted sizes of the genomic and subgenomic RNAs derived by SIN-specific replication and transcription of pJNTRCATdXB transcripts are 2,840 and 1,375 nt, respectively, exclusive of the poly(A).

In order to generate vaccinia virus recombinants expressing the substrate RNA, pJNTRCATdXB was inserted into plasmids pVV5N.27, pVV5.2, and pVV2.T7. Plasmids pVV5N.27 and pVV5.2 contain two different deleted forms

of the vaccinia virus 7.5K early/late promoter flanked by vaccinia virus DNA sequences containing the 5' and 3' ends of the vaccinia virus *tk* gene (18). Plasmid pVV2.T7 contains the bacteriophage T7 promoter and terminator flanked by vaccinia virus DNA sequences containing the 5' and 3' ends of the vaccinia virus *tk* gene. Ligation of pJNTRCATdXB into pVV5N.27, pVV5.2, and pVV2.T7 generated plasmids pRS.7, pRS.2, and pRS.T7, respectively. A fragment from pTM3, which contained the *gpt* gene, was ligated into these plasmids to allow the use of *gpt* selection during the generation of the corresponding vaccinia virus recombinants vRS.7, vRS.2, and vRS.T7.

pJNTSCAT, described previously (34), was used for *in vitro* transcription with SP6 polymerase to generate RNA transcripts for transfection of infected cells.

pGemJICATdXB was used for *in vitro* SP6 transcription of the minus-sense RNA probe and was constructed by inserting the SIN junction region followed by the first 171 nt of the CAT gene of pJNTRCATdXB into the polylinker region of pGem-1 (Promega Biotec). pPROT25CATA+ (24) contains the CAT gene inserted downstream of the SP6 promoter in a pBR322-derived vector, and this plasmid was used for *in vitro* transcription of the plus-sense RNA probe.

Generation of vaccinia virus recombinants. Recombinant vaccinia viruses were generated by marker rescue on CV-1 cells (37) and identified by the *gpt* selection method (9). Virus was plaque purified three times under selective conditions, and stocks were grown and titered on BSC40 cells. Expression of expected SIN-specific proteins by the vaccinia virus recombinants was verified by protein analysis of infected-cell lysates.

Protein analysis. BHK monolayers in 35-mm tissue culture dishes were infected at a multiplicity of infection (MOI) of 5 PFU per cell of each virus in 200 μ l of phosphate-buffered saline (PBS) containing 1% FCS. After 1 h at room temperature, the inoculum was removed and the cells were incubated at 37°C in MEM containing 5% FCS. At the indicated times post infection (p.i.), the medium was removed and the cells were labeled at 37°C in MEM containing 1/40 (2.5%) of the normal methionine concentration, 5% FCS, and 20 μ Ci of [³⁵S]methionine per ml. After labeling, the cells were lysed as previously described (35) and the lysates were immunoprecipitated with antibodies monospecific for SIN nsP1, nsP2, nsP3, or nsP4 (20, 29). The immunoprecipitated products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were treated for fluorography (28), dried, and exposed to pre-flashed X-ray film.

RNA transfection. The procedure for the RNA transfection of BHK-21 cells (15) was adapted from a method for DNA transfection (10). A total of 150 ng of transcript RNA was mixed with 250 μ l of PBS containing 6 μ g of lipofectin. The transfection mixture was incubated on ice for 10 min and then added to the cell monolayer. After 15 min at room temperature, the transfection mixture was removed, 2 ml of prewarmed MEM containing 5% FCS was added, and incubation was continued at 37°C for the indicated times.

RNA isolation and analysis. BHK monolayers in 35-mm tissue culture dishes were infected for 1 h at room temperature with vTF7-3, vRS.7, and various vaccinia virus recombinants expressing SIN proteins, at an MOI of 5 PFU per cell of each virus. The inoculum was removed and replaced with MEM containing 5% FCS, and the cells were incubated at 37°C. At 4 h p.i. the medium was removed and replaced with prewarmed medium containing 1 μ g of dactinomycin per μ l, and the cells were incubated at 37°C (time course analyses

had shown that dactinomycin addition at 4 h p.i. yielded the optimal signal-to-noise ratio in RNase protection assays [data not shown]. At 8 h p.i., cells were washed with ice-cold PBS and then lysed with 1 ml of RNazol B lysis solution (Tel-Test, Inc.). Samples were extracted with chloroform, and the RNA was collected by precipitation with isopropanol. Alternatively, vRS.7 was not included in the coinfection and the substrate RNA was delivered as an RNA transcript which was introduced into cells by lipofection. The same protocol was followed for the remainder of the experiment, except dactinomycin was not included in the media.

Substrate-specific minus-strand RNAs were analyzed by a two-cycle RNase protection assay. The assays were performed essentially as described by Novak and Kirkegaard (42), with minor modifications. For minus-strand analysis, 3.5 µg of total cytoplasmic RNA was suspended in 30 µl of hybridization buffer and incubated at 55°C for 16 h in the presence of 10 ng of in vitro-transcribed pJNTRCATdXB transcript. RNase digestion was for 60 min at room temperature in RNase mixture (500 mM NaCl, 10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 40 µg of RNase A per ml, and 700 U of RNase T₁ per ml). The samples were treated with SDS-proteinase K, phenol extracted, and ethanol precipitated with 5 µg of tRNA. Samples were resuspended in 30 µl of hybridization buffer, and 750 fmol of ³⁵S-labeled RNA probe was added (see below). The samples were denatured at 85°C for 5 min, hybridized overnight at 55°C, and subjected to RNase treatment under the conditions described above. Samples were analyzed on a 5% polyacrylamide-urea sequencing gel; the gel was fixed, transferred to Whatman paper, and dried; and the bands were quantitated with a Betagen Betascope. For plus-strand analysis, 0.5 µg of total cytoplasmic RNA was suspended in 30 µl of hybridization buffer containing 750 fmol of ³⁵S-labeled RNA probe (see below). The samples were denatured at 85°C for 5 min, hybridized overnight at 55°C, and subjected to RNase treatment as described above, except that the RNase mixture did not contain RNase A.

³⁵S- and ³²P-labeled RNA probes were generated by in vitro transcription of plasmid DNA with SP6 polymerase as described previously with minor modifications (38). For minus-strand analysis, a CAT-specific positive-sense RNA probe 289 nt in length was generated by in vitro transcription of plasmid pProt25CAT+ which had been linearized at the *Eco*RI site. After hybridization and RNase digestion, the size of the protected fragment is 251 nt when vRS.7 is used to deliver the substrate RNA. When pJNTSCAT transcript RNA is transfected into cells, the size of the protected fragment is 266 nt (see Results also). For plus-strand analysis, a negative-sense RNA probe 314 nt in length was generated by in vitro transcription of plasmid pGemJICATdXB which had been linearized at the *Ssp*I site. This probe included regions both 5' and 3' to the start site for subgenomic RNA transcription, and after RNase digestion, the sizes of the protected fragments, as determined by hybridization to genomic or subgenomic RNAs, are 283 or 186 nt, respectively.

RESULTS

Construction of vaccinia virus-SIN recombinants. To examine the involvement of the different nsP species in RNA replication, a set of SIN cDNA clones encoding both the individual nsPs as well as the polyprotein intermediates was constructed. Since the nsPs are generated by proteolytic

FIG. 2. Synthesis of SIN nsPs in cells infected with recombinant vaccinia viruses. BHK-21 cells were coinfecting with vTF7-3 and a vaccinia virus recombinant as described in Materials and Methods. The exception includes the lanes labeled 1234 alone, which did not include vTF7-3 in the infection. Cells were labeled with [³⁵S]methionine for 5 h, and SDS-solubilized lysates were prepared and immunoprecipitated with antibodies monospecific for the SIN nsPs (as indicated). Immunoprecipitates were analyzed by SDS-PAGE on 8% gels. The antibodies used for immunoprecipitation are indicated directly above each lane, with the number representing the SIN nsP specificity. The recombinants used for coinfection with vTF7-3 are indicated at the top. Both mock (M) and SIN-infected (SV) cells were infected with vTF7-3 and are included as controls. The positions of the SIN nsPs are indicated on the left, and molecular weight standards are indicated on the right.

cleavage of polyproteins in SIN-infected cells, PCR was used to engineer cassettes encoding individual proteins and polyproteins with flanking initiation and termination codons (see Materials and Methods). nsP cassettes were placed downstream of a promoter for T7 polymerase and the encephalomyocarditis virus internal ribosome entry site such that efficient transcription and cap-independent translation occur in the presence of T7 RNA polymerase, which is supplied by coinfection with vaccinia virus recombinant vTF7-3 (11). These plasmids were used to generate vaccinia virus recombinants, and this set of constructs and the nomenclature used are shown schematically in Fig. 1. Briefly, a P prefix indicates a polyprotein, while nsP refers to a specific protein moiety. A v prefix will refer to the vaccinia virus recombinants used for infection, e.g., v1 refers to the vaccinia virus recombinant expressing the nsP1 protein. In infections in which nsP2-containing polyproteins are expressed, cleavage products will also be present, since nsP2 protease-containing polyproteins can cleave the 1/2, 2/3, and 3/4 sites.

Expression of the SIN nsPs by recombinant vaccinia viruses. To examine the expression of SIN-specific proteins by different vaccinia virus recombinants, BHK cells were coinfecting with vTF7-3 and the various recombinants and labeled with [³⁵S]methionine from 3 to 8 h p.i. Cell lysates were immunoprecipitated with antibodies monospecific for SIN nsP1 to nsP4, and the precipitated proteins were analyzed by SDS-PAGE. As shown in Fig. 2, the individual proteins expressed by the vaccinia virus recombinants comigrate with the proteins synthesized in SIN-infected cells. Previously it was shown that nsP3 in SIN-infected cells (apparent mass, 76 kDa) is modified by phosphorylation on Ser and Thr residues to produce a smear of slower-mobility

forms ranging in size from 78 to 106 kDa (36). It appears that nsP3 expressed by itself from v3 is also phosphorylated (Fig. 2), indicating that, as with SIN-infected cells, phosphorylation can occur in this system and that the phosphorylation of nsP3 is not dependent on the presence of any of the other SIN nsPs.

nsP4 is produced in small amounts in SIN-infected cells since its synthesis is dependent on read-through of an opal termination codon (20). In addition, it is thought that nsP4 which is not incorporated into a replication complex is rapidly degraded, since increased synthesis of nsP4 by replacement of the opal termination codon with a serine codon did not result in greatly increased levels of nsP4 (35). In contrast, very high levels of nsP4 can be detected in the vaccinia virus system when nsP4 is expressed as an individual protein (Fig. 2). Because such high levels of nsP4 are detected with v4, it seems likely that the addition of an N-terminal Met residue on nsP4 has greatly increased the stability of this protein (see also Discussion) (7).

The SIN-specific polyproteins expressed by the different vaccinia virus recombinants were next examined (Fig. 2). On the basis of previous *in vitro* studies (6), polyproteins containing the nsP2 domain (P12, P23, P123, and P1234) are expected to be processed to their corresponding cleavage products. P12 and P23 appear to be processed to their respective individual proteins either by *cis* or *trans* cleavages (Fig. 2). In extracts from v34-infected cells, both nsP3 and P34 can be detected, but no nsP4 is detectable. Thus, as with SIN-infected cells, nsP3 is produced by termination at the opal stop codon and P34 is expressed at lower levels by read-through of the termination codon. P123 also appears to be processed efficiently to its corresponding three individual proteins nsP1 to nsP3, while P1234 is processed to the four individual proteins nsP1 to nsP4. As was observed with v3, the nsP3 proteins expressed by v34 and v123, and probably v23 and v1234 (34), appear to be phosphorylated. When produced by cleavage at the 2/3 site (i.e., from P23, P123 or P1234), the level of nsP3 is decreased relative to those of the other nsPs. This is also observed during coinfection with SIN and vaccinia virus and does not affect the efficiency of *trans* complementation of SIN-specific RNA replication and transcription (data not shown).

When driven by the bacteriophage T7 polymerase, the level of protein expression with the recombinant-expressed proteins is approximately fivefold higher than in SIN-infected cells. In the absence of T7 polymerase (Fig. 2, 1234 alone) the level of protein expression is slightly less than observed in SIN-infected cells.

Expression of engineered SIN-specific substrate RNAs to assay replication-transcription efficiencies. To determine which of the nsPs are necessary to form functional replication complexes, the abilities of different protein combinations to replicate and transcribe engineered SIN-specific substrate RNAs were assayed. These engineered substrate RNAs contain all the *cis*-acting elements necessary for the production of both minus- and plus-strand genome-size RNAs as well as a subgenomic RNA. However, the RNAs do not encode functional nsPs and thus can only be replicated by functional SIN replication machinery supplied *in trans*. Replication and transcription of substrate RNAs was analyzed by an RNase protection assay to look at the production and accumulation of both plus- and minus-strand RNAs. In addition, the gene for CAT is located downstream of the subgenomic promoter, allowing transcription of subgenomic RNA to be assayed indirectly by measuring CAT activity.

Two methods for delivery of the substrate RNA into infected cells were analyzed. The first involved transfection of an RNA template derived by *in vitro* transcription of pJNTSCAT. This method allows one to deliver a defined target RNA into cells, but because of low RNA transfection efficiency not all of the cells receive a replication-competent substrate RNA. The second method utilized vaccinia virus recombinants which could express a functional substrate RNA *in vivo*. Recombinants in which the cDNA of the substrate RNA was placed downstream of the bacteriophage T7 promoter (this recombinant is termed vRS.T7) were generated, such that expression occurred during coinfection with vTF7-3, or alternatively, the substrate cDNA was placed downstream of two different deleted forms of the vaccinia virus 7.5K early/late promoter (these recombinants are termed vRS.2 and vRS.7) such that initial transcription by the vaccinia virus DNA-dependent RNA polymerase occurred at levels of approximately 14 and 37% of that of the wild-type 7.5K promoter. On the basis of results from deletion mapping studies, the 5' ends of the early transcripts should be within a few nucleotides of the correct 5' termini (18). This method allows one to deliver the target RNA to the maximum number of cells; however, the vaccinia virus transcripts probably do not end precisely at the 3' end of the substrate RNA, as a transcription terminator is not present.

By using the RNase protection assay, RNA phenotypes were compared in cells infected with vTF7-3 and v1234 and either coinfecting with the various substrate-expressing recombinants or transfected with the RNA transcript from pJNTSCAT. In all four cases, the substrate RNAs were efficiently replicated and transcribed by the SIN nsPs encoded by v1234 (data not shown). In the absence of the nsPs, the level of background transcription observed with vRS.T7 from the T7 promoter was much higher than that observed with the deleted forms of the 7.5K promoter. Slightly higher levels of RNA accumulation were detected in infections involving vRS.7 compared with vRS.2, making vRS.7 the preferred recombinant for these experiments. Multiple repetitions of this experiment indicated that quantitative and reproducible data were generated when the recombinant was used to deliver the substrate RNA. Transfection of substrate JNTSCAT RNA yielded similar results in independent experiments, but actual quantitation of the data showed considerable variance, presumably resulting from differences in RNA transfection efficiency. This would suggest that the recombinant method of target delivery is the one of choice. Unfortunately, when this method is used to analyze minus-strand RNAs, a faint background band, also present in the sample from mock-infected cells, comigrated with the protected band of interest, obscuring low levels of minus-strand accumulation. Because of slight differences in the structure of the substrate RNA produced by the recombinant vRS.7 and the RNA used for transfection (Fig. 3), the protected band of interest generated with the transfected substrate RNA does not comigrate with the background band, allowing detection of very low levels of minus-strand synthesis. Taking these factors into consideration, vRS.7 was used to analyze both plus- and minus-strand RNAs, and in addition, minus-strand RNAs were further analyzed with the transfection protocol. A diagram of the substrate RNAs used for both plus- and minus-strand analyses, along with the expected sizes of protected fragments, is shown in Fig. 3.

SIN nsPs and/or polyproteins necessary for the production of different viral RNA species. Using these RNase protection assays, we analyzed which of the SIN proteins and/or polyproteins were capable of forming active replication

FIG. 3. Diagram of SIN-specific substrate RNAs used for functional analysis of the SIN nsP combinations. In addition to the *cis*-acting elements necessary for replication by SIN nsPs, the substrate transcripts contain the CAT gene (open box) under the control of the SIN subgenomic promoter (indicated by an arrow). The shaded regions indicate sequences derived from SIN. Both plus- and minus-strand RNA species, produced by SIN-specific replication and transcription, are shown below as solid lines, with the lengths of the RNAs (excluding homopolymer tails) indicated in bases. Major protected RNA fragments for the plus- and minus-strand RNase protection analyses (using the probes described in Materials and Methods) are indicated below as solid and hatched boxes, respectively, and the lengths of the protected RNA fragments are indicated below. Two different substrate RNAs are shown. (A) Diagram of the substrate RNA transcribed by vaccinia virus recombinant vRS.7. The 3' terminus of this substrate has not been defined and is probably heterogeneous. (B) Diagram of the substrate RNA produced by SP6 transcription of the *Xho*I-linearized pJNTSCAT template. This RNA contains 15 additional nucleotides 5' to the CAT coding sequence (shown as a black bar) which have been deleted in the substrate RNA shown in panel A. This difference results in a protected fragment of 266 nt during minus-strand analysis.

complexes. Cells were coinfecting with vTF7-3, vRS.7 (the recombinant expressing the substrate RNA), and various combinations of vaccinia virus recombinants expressing the different SIN nsPs. At 8 h p.i. cytoplasmic RNAs were harvested and subjected to RNase protection assays with probes specific for either the plus- or the minus-sense RNAs. Minus-strand analyses were also performed by the transfection procedure. SIN-specific proteins were analyzed in parallel to confirm that all proteins were being produced and processed as expected (data not shown), and all of the experiments were repeated at least three times. Quantitation of the protected fragments was performed on experiments using vRS.7, and these data are shown in Table 1.

Analysis of minus strands produced by infection with v1234 shows the accumulation of a minus strand-specific protected fragment which migrates at the expected size (Fig. 4) and comigrates with the band produced during an infection involving SIN in the presence of vaccinia virus (data not shown). The level of minus-strand RNA accumulation was

readily detected above the background band in mock-infected cells (Fig. 4). Because it is not known what the functional form of the SIN replicase is, infections involving nsP4, P34, and both moieties were included in all sets of experiments to see if this resulted in any differences in the RNA phenotypes. As can be seen in Fig. 4 and Table 1, the inclusion of v34 in the coinfection with v1234 slightly inhibited the level of minus-strand accumulation, while the inclusion of v4 in the coinfection with v1234 significantly diminished the level of minus-strand accumulation. Possible explanations for this will be discussed later. Plus-strand analysis indicated that the v1234 infection allowed production of plus-strand genomic and subgenomic RNAs, since specific protected fragments which migrate at the expected sizes were observed (Fig. 4). These bands were not detected in mock-infected cells. The molar ratio of subgenomic RNA to genomic RNA was approximately 4.6:1, which is somewhat higher than the 3:1 ratio usually observed in SIN-infected cells. As was observed during minus-strand synthesis, the inclusion of v4 in the coinfection also decreased the overall level of plus-strand RNA synthesis, suggesting the inhibition by nsP4 was not specific to the synthesis of a particular RNA species. These results indicate that when the entire SIN nonstructural coding region is expressed in this system, functional replication complexes can be formed.

Since the nsPs are normally processed to four individual proteins during SIN infection, we next asked whether the four individual nsPs alone were sufficient to support RNA replication. When the individual nonstructural proteins nsP1, nsP2, nsP3, and nsP4 were coexpressed, no minus-strand RNA species could be detected, even when P34 was present (Fig. 4). Since the results with P1234 indicated that nsP4 could act as an inhibitor of RNA replication, a coinfection in which the nsP4 domain was supplied only by P34 was also analyzed. Still, no RNA synthesis could be detected. As expected, plus-strand protection analyses of RNAs from these infections indicated that neither genomic RNA replication nor subgenomic mRNA transcription was occurring. The lack of detectable RNA replication was not a consequence of the high MOI used, because of the number of recombinants necessary for infection, since control experiments using v1234 and wild-type vaccinia virus indicated that the MOI did not affect the level of RNA replication (data not shown). Thus, even though the nsPs were indistinguishable, in terms of electrophoretic mobility, from those synthesized in SIN-infected cells, coexpressed individual nsPs were not capable of forming active replication complexes.

Although alternative explanations are possible, these results suggest that the precursors to the nsPs are necessary for RNA replication. To examine this possibility, the RNA phenotypes produced in the presence of various polyproteins were examined (Fig. 4). In coinfections involving v12 and various combinations of v3, v4, and v34, neither plus- nor minus-strand RNA synthesis could be detected. Likewise, no RNA synthesis could be detected in similar coinfections involving v23. Protein analysis of these infections indicated that all proteins were produced and processed similarly to what is observed in SIN-infected cells (data not shown), indicating the lack of replication was not a result of aberrant protein processing. It appears then that the presence of the P12 and P23 polyproteins is not sufficient to allow the formation of active replication complexes.

In contrast, minus-strand RNAs were produced very efficiently in the coinfection involving v123 and v34 (Fig. 4). The level of minus-strand accumulation in the v123 plus v34 infection appears to be only slightly less than that observed

FIG. 4. Replication and transcription of an engineered substrate RNA by vaccinia virus-expressed nsPs. Monolayers of BHK-21 cells were infected with vTF7-3, vRS.7, and various vaccinia virus recombinants at an MOI of 5 PFU per cell for each virus. Cytoplasmic RNAs were isolated, and the levels of specific RNAs were determined by an RNase protection assay. The recombinants used for coinfection with vTF7-3 and vRS.7 are shown above each lane. For lanes marked mock, only vTF7-3 and vRS.7 were present in the infection. Panels A and B show minus- and positive-strand analyses, respectively. The major protected RNA fragment for the minus-strand RNA is 251 nt. Major protected fragments for genomic and subgenomic RNAs are 283 and 186 nt, respectively. As controls, the indicated amounts of negative (-) or positive (+)-sense RNA transcripts were subjected to the RNase protection assay. It should be noted that the structures of some of these control transcripts contain minor differences compared with those generated from vRS.7. Hence, some of the protected fragments differ in size. The size of the major protected fragment from the negative-sense control transcript is 266 nt. Protected fragments for plus-strand genomic and subgenomic control RNAs are 283 and 193 nt, respectively.

with v1234. As was seen with v1234, the inclusion of v4 in the v123 plus v34 infection significantly diminished the level of minus-strand accumulation. Coinfection with v123 and v34 also resulted in the efficient accumulation of plus-strand genomic and subgenomic RNAs (Fig. 4). The molar ratio of subgenomic RNA to genomic RNA was approximately 4.5:1, which is similar to that observed with v1234. Again, the inclusion of v4 in the coinfection resulted in an overall decrease in the synthesis of plus-strand RNAs. These data demonstrate that during coinfection with v123 and v34, both plus- and minus-strand RNAs can be synthesized with an efficiency comparable to that observed for v1234 (Table 1).

Coinfection with v4 and v123 also resulted in the accumulation of minus-strand RNAs, albeit at low levels compared with those for v1234 or v123 plus v34. Minus-strand synthesis could not be detected above background when the recombinant was used to deliver the substrate RNA (Fig. 4A, lane 123-4); however, it is clear from results with the transfected template RNA that minus strands are synthesized with this combination (Fig. 5, lane 123-4), demonstrating that nsP4 can function in the synthesis of minus-strand

RNAs. A particularly intriguing result was that neither plus-strand genomic nor subgenomic RNA synthesis was detected during coinfection with v4 and v123. This result was observed with either the vaccinia recombinant (vRS.7) or the transfection method (JNTSCAT) to deliver the substrate RNA. Thus, it appears that nsP4 can only synthesize RNA when coexpressed with P123, and even then nsP4 is capable of synthesizing only minus-strand RNAs. In contrast, P34 also requires coexpression with P123 to form an active replicase complex, but P34 and P123 can function in the synthesis of both plus- and minus-strand RNAs.

DISCUSSION

In this report, a heterologous expression system was developed to reconstruct SIN-specific replication complexes *in vivo*. Vaccinia virus recombinants which express the SIN nsPs, both individually and as polypeptide precursors, were generated to examine which viral proteins are necessary to form replicase complexes capable of replicating and transcribing SIN-specific RNAs. This system provides a means

TABLE 1. Accumulation of viral RNAs in vaccinia virus-infected BHK-21 cells

Sample	Relative RNA accumulation ^a			Sub/Gen molar ratio ^b
	Minus strand	Genomic	Subgenomic	
1234	1.0	1.0	1.0	4.6, 4.5
1234 + 4	0.4, 0.3	0.4, 0.4	0.3, 0.3	4.2, 4.2
1234 + 34	0.7, 0.7	0.9, 0.8	0.8, 0.7	4.5, 4.5
1 + 2 + 3 + 4	—	—	—	
1 + 2 + 3 + 34	—	—	—	
1 + 2 + 3 + 4 + 34	—	—	—	
12 + 3 + 4	—	—	—	
12 + 34	—	—	—	
12 + 34 + 4	—	—	—	
1 + 23 + 4	—	—	—	
1 + 23 + 34	—	—	—	
1 + 23 + 34 + 4	—	—	—	
123 + 34	0.9, 1.0	0.9, 1.0	0.8, 0.9	4.5, 4.5
123 + 34 + 4	0.6, 0.7	0.5, 0.5	0.2, 0.3	4.0, 4.1
123 + 4	—	—	—	

^a Cytoplasmic RNAs were isolated from cells infected with vTF7-3, vRS.7, and various vaccinia virus recombinants (the v prefix has been omitted), and the RNAs were subjected to an RNase protection assay. Samples were separated on a 5% polyacrylamide-urea gel, and the levels of RNA were quantitated with a Betagen Betascope. The accumulation of each sample was normalized with respect to the value obtained for v1234 and is expressed relative to that of wild-type v1234, with the value for v1234 being 1.0. The values given represent the results from two independent experiments. —, the sample was less than 10% of the wild-type value, and a protected fragment was not detected with long autoradiographic exposures.

^b The Sub/Gen column indicates the molar ratio of subgenomic RNA molecules to genomic RNA molecules.

of producing viral proteins in the absence of viral RNA replication, permitting the roles of these proteins in each step of replication to be studied. Similar approaches have been used to study the replication of a number of viruses (2, 14, 25, 27), the most comprehensive studies being those on vesicular stomatitis virus, in which RNA replication as well as particle assembly and budding were observed in cells expressing the five individual vesicular stomatitis virus proteins and an RNA substrate (43–45).

In our studies, we generated a recombinant vaccinia virus which could provide the intracellular synthesis of engineered substrate RNAs that could serve as templates for replication by the SIN replication machinery. The ability to utilize a virus for delivery of the template RNA provides a powerful means of delivering the substrate RNA to cells with maximum efficiency and consistency. Transcription of the template RNA by the vaccinia virus DNA-dependent RNA polymerase yielded transcripts which probably did not end precisely at the 3' end of the substrate RNA. Apparently, though, the SIN nsPs were able to utilize these extended templates, since the RNAs were replicated efficiently. This supports previous results which showed that the presence of extra 3'-terminal sequence on a SIN defective interfering RNA did not prevent replication of the transfected defective interfering RNA (32). Although transcripts generated by the vaccinia virus polymerase could be replicated, other strategies which might produce RNAs with termini more closely resembling the normal SIN 5' and 3' ends may improve the ability of the template RNA to be replicated (2).

These results demonstrate that as expressed, the four

FIG. 5. Minus-strand analysis with a transfected substrate RNA. RNA derived by transcription of pJNTSCAT was used to transfect monolayers of BHK-21 cells which had previously been infected with vTF7-3 and various vaccinia virus recombinants. Cytoplasmic RNAs were isolated, and the level of minus-strand RNA was determined by the RNase protection assay described in Materials and Methods. The recombinants used for coinfection with vTF7-3 are shown above each lane. 123 (no transcript), cells were coinfecting with vTF7-3 and v123, but no RNA substrate was included in the transfection mix. As controls, the indicated amounts of negative (–) and positive (+) sense RNA transcripts were subjected to the RNase protection assay. Only a subset of the samples analyzed is shown. The major protected RNA fragment for the minus-strand RNA is 266 nt (Fig. 3B). A slightly smaller background band of unknown origin is also present.

individual nsPs are not capable of forming functional replication complexes, even in the presence of P34. What effect the engineered initiation and termination codons may have on the activity of the proteins is not known. Other than with P1234, synthesis of both minus- and plus-strand RNAs was detected only during coexpression of the polyproteins P123 and P34. This may indicate that the nsPs have to be synthesized as polyproteins, either to attain their correct conformation or to favor their interaction for complex formation. Alternatively, the polyproteins themselves may have a role in RNA replication as actual components of the replication complex. In support of this alternative, a previous study has shown that mutants of SIN which are defective in cleavage at the 1/2 and 2/3 sites are viable (53). Although these mutants grow very poorly, the fact that viable virus could be obtained suggests that the P123 polyprotein can function in SIN replication.

It is generally assumed that nsP4 is the viral polymerase, as it contains the Gly-Asp-Asp motif conserved among several RNA-dependent RNA polymerases (22, 26), and a mutation in this protein can result in the shutoff of all viral RNA synthesis under nonpermissive conditions (3, 16). Whether the active form of the RNA polymerase is nsP4 itself or the P34 polyprotein or whether both moieties play a role in replication is unknown. We observed that when nsP4 was coexpressed with P123 only minus-strand RNAs were synthesized and that the level of minus-strand accumulation was very low. One possible explanation for the low levels of minus-strand accumulation is that the vaccinia virus-expressed form of nsP4 cannot function efficiently because of misfolding or the presence of the extra N-terminal Met. Alternatively, since nsP4 can only synthesize minus-strand RNAs, the input plus-strand RNA templates do not get amplified, and it is possible that these templates are limiting for minus-strand synthesis, particularly if nsP4 does not release the plus-strand template once it has completed synthesis of a minus-strand RNA. A third possibility is that expression of high levels of nsP4 may be detrimental to RNA replication. In SIN-infected cells the level of nsP4 is regu-

lated at translational and posttranslational levels. It is translated in small amounts relative to the other nsPs because its synthesis depends on read-through of an opal termination codon (20), and its production also appears to be temporally regulated (6). In addition, although nsP4 appears to be stable early during SIN infection, it has been suggested that nsP4 which is not incorporated into a replication complex is rapidly degraded (19, 35) and that degradation may be mediated by the N-end pathway of ubiquitin-dependent proteolysis (7). According to the N-end rule, an N-terminal Met is a stabilizing residue which decreases the rate of protein degradation (1). As expressed in the vaccinia virus system, nsP4 has an additional N-terminal Met residue which may increase the stability of this protein. The combination of a high level of nsP4 expression by vaccinia virus and the overexpression of nsP4 via its increased stability may have an inhibitory effect on RNA replication since, presumably, the balance of the viral proteins is important for efficient replication.

It is particularly interesting that nsP4 cannot function in the synthesis of plus-strand RNAs. This finding may support the hypothesis that both nsP4 and P34 function as viral polymerases. On the basis of *in vitro* studies, it has been postulated that processing of the viral polyproteins in SIN-infected cells is temporally regulated by a population of nsP2 protease-containing polyproteins which have different cleavage site preferences; it is this temporal regulation which accounts for the observation that nsP4 accumulates early during SIN infection, whereas later in infection P34 accumulates. Because the timing of the shift in the P34/nsP4 ratio correlates well with the shutoff of minus-strand synthesis, de Groot et al. have postulated that nsP4 is the active form of the RNA polymerase for minus-strand synthesis while P34 is responsible for production of plus-strand RNAs (6). The fact that both plus- and minus-strand RNAs can be synthesized during coexpression of P123 plus P34 while only minus-strand RNAs are synthesized with P123 plus nsP4 could be interpreted in support of this hypothesis. Alternatively, in SIN-infected cells nsP4 may function as the viral replicase capable of synthesizing both plus- and minus-strand RNAs, but by expressing nsP4 alone, rather than by processing of a polyprotein, the structure of nsP4 may be altered such that it can only function as a minus-strand replicase. The latter possibility is supported by experiments presented in the accompanying paper which show that nsP4, expressed with only limited upstream sequences, is capable of functioning in both minus- and plus-strand RNA synthesis (30).

It was also observed that when v4 was included in either the v1234 or the v123 plus v34 infection, the level of both plus- and minus-strand RNA accumulation was reduced. If the activity of the vaccinia virus-expressed nsP4 is decreased because of misfolding or a substantial fraction of the protein is inactive, this might explain why the inclusion of v4 in infections results in decreased overall levels of RNA synthesis. In this scenario, defective nsP4 might be functioning as a competitive inhibitor by interacting with factors necessary for RNA replication (such as RNA templates, initiation factors, or other viral or host proteins) which are present in limited quantities. Alternatively, the vaccinia virus-expressed nsP4 may specifically inhibit minus-strand synthesis, which in turn could affect the accumulation of plus-strand RNAs if the level of plus-strand synthesis is determined by the number of minus-strand templates.

In addition to their role(s) in RNA replication, expression of the various polyproteins has given some insight into the *in vivo* proteolytic activities of several of the polyproteins.

When expressed alone, P123, P12, and P23 are processed to their respective individual proteins, indicating the nsP2 protease can function *in vivo* in the form of these polyproteins. By using the vaccinia virus constructs it is also clear that nsP2 and P12 are capable of cleaving the 3/4 site, albeit inefficiently (data not shown). These results are in contrast to those previously observed *in vitro* showing that cleavage at the 3/4 site could not be detected unless nsP3 was present in the protease (6). Although it is not known whether P12 or the cleaved product nsP2 is functioning as the protease, in either case cleavage at the 3/4 site can occur *in vivo* in the absence of nsP3 sequence in the protease, although cleavage is much more efficient when nsP3 is present in the protease. In addition, protein analysis on extracts from cells infected with v34 indicated that the cleaved nsP4 product could not be detected in the absence of proteins containing the nsP2 protease. These *in vivo* data confirm previous *in vitro* results showing that SIN nsP4 does not function as an autoprotease (7) but contrast with studies reported for Semliki Forest virus nsP4 (56).

Together with previous genetic studies (3, 16) and sequence comparisons (22, 26), the findings reported here indicate that nsP4 does not need to be expressed as a P34 polyprotein to function as a polymerase. What is required to generate a replicase capable of efficient genomic RNA replication and subgenomic mRNA transcription remains to be determined. In addition, it appears that expression of P123 and P34 is essential for RNA replication and suggests that these polyproteins may be necessary precursors, or actual components, of the replication complex. Further studies examining the ability of cleavage-defective polyproteins to function in RNA replication are described in the accompanying paper (30).

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REFERENCES

1. Bachmair, A., D. Finley, and A. Varshavsky. 1986. *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234**:179-186.
2. Ball, L. A. 1992. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. *J. Virol.* **66**:2335-2345.
3. Barton, D. J., S. G. Sawicki, and D. L. Sawicki. 1988. Demonstration *in vitro* of temperature-sensitive elongation of RNA in Sindbis virus mutant *ts6*. *J. Virol.* **62**:3597-3602.
4. Barton, D. J., S. G. Sawicki, and D. L. Sawicki. 1991. Solubilization and immunoprecipitation of alphavirus replication complexes. *J. Virol.* **65**:1496-1506.
5. Clewley, J. P., and S. I. T. Kennedy. 1976. Purification and polypeptide composition of Semliki Forest virus RNA polymerase. *J. Gen. Virol.* **32**:395-411.
6. de Groot, R. J., W. R. Hardy, Y. Shirako, and J. H. Strauss. 1990. Cleavage-site preferences of Sindbis virus polyproteins containing the nonstructural proteinase: evidence for temporal regulation of polyprotein processing *in vivo*. *EMBO J.* **9**:2631-2638.
7. de Groot, R. J., T. Rümenapf, R. J. Kuhn, E. G. Strauss, and J. H. Strauss. 1991. Sindbis virus RNA polymerase is degraded by the ubiquitin proteolytic pathway. *Proc. Natl. Acad. Sci. USA* **88**:8967-8971.
8. Ding, M., and M. J. Schlesinger. 1989. Evidence that Sindbis virus nsP2 is an autoprotease which processes the virus non-

- structural polyprotein. *Virology* 171:280–284.
9. Falkner, F., and B. Moss. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J. Virol.* 62:1849–1854.
 10. 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.
 11. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83:8122–8126.
 12. Gornatowski, P. J., L. Kääriäinen, S. Keränen, M. Ranki, and D. L. Sawicki. 1980. Semliki Forest virus replication complex capable of synthesizing 42S and 26S nascent RNA chains. *J. Gen. Virol.* 49:61–69.
 13. Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov. 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* 17:4713–4730.
 14. Gotoh, H., T. Shioda, Y. Sakai, K. Mizumoto, and H. Shibuta. 1989. Rescue of Sendai virus from viral ribonucleoprotein-transfected cells by infection with recombinant vaccinia viruses carrying Sendai virus L and P/C genes. *Virology* 171:434–443.
 15. Grakoui, A., R. Levis, R. Raju, H. V. Huang, and C. M. Rice. 1989. A *cis*-acting mutation in the Sindbis virus junction region which affects subgenomic RNA synthesis. *J. Virol.* 63:5216–5227.
 16. 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.
 17. 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.
 18. Hahn, Y. S., J. H. Strauss, and C. M. Rice. Unpublished data.
 19. Hardy, W. R., Y. S. Hahn, R. J. de Groot, E. G. Strauss, and J. H. Strauss. 1990. Synthesis and processing of the nonstructural polyproteins of several temperature-sensitive mutants of Sindbis virus. *Virology* 177:199–208.
 20. Hardy, W. R., and J. H. Strauss. 1988. Processing of the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo using monospecific antibodies. *J. Virol.* 62:998–1007.
 21. Hardy, W. R., and J. H. Strauss. 1989. Processing the nonstructural proteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in *cis* and *trans*. *J. Virol.* 63:4653–4664.
 22. Haseloff, J., P. Goelet, D. Zimmermann, P. Ahlquist, R. Dasgupta, and P. Kaesberg. 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. *Proc. Natl. Acad. Sci. USA* 81:4358–4362.
 23. Hruby, D. E., L. A. Guarino, and J. R. Kates. 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. *J. Virol.* 29:705–715.
 24. Huang, H. Unpublished data.
 25. Huang, T., P. Palese, and M. Krystal. 1990. Determination of influenza virus proteins required for genome replication. *J. Virol.* 64:5669–5673.
 26. Kamer, G., and P. Argos. 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal, and bacterial viruses. *Nucleic Acids Res.* 12:7269–7282.
 27. Karacostas, V., K. Nagashima, M. A. Gonda, and B. Moss. 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA* 86:8964–8967.
 28. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
 29. Lemm, J. A., M. E. Jones, G. Li, and M. W. LaStarza. Unpublished data.
 30. Lemm, J. A., and C. M. Rice. 1993. Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. *J. Virol.* 67:1916–1926.
 31. Levis, R., S. Schlesinger, and H. V. Huang. 1990. Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* 64:1726–1733.
 32. 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.
 33. Li, G. Unpublished data.
 34. Li, G., B. Pragai, and C. M. Rice. 1991. Rescue of Sindbis virus-specific RNA replication and transcription by using a vaccinia virus recombinant. *J. Virol.* 65:6714–6723.
 35. 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.
 36. Li, G., M. W. L. Starza, W. R. Hardy, J. H. Strauss, and C. M. Rice. 1990. Phosphorylation of Sindbis virus nsP3 *in vivo* and *in vitro*. *Virology* 179:416–427.
 37. Mackett, M., and G. L. Smith. 1986. Vaccinia virus expression vectors. *J. Gen. Virol.* 67:2067–2082.
 38. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.
 39. 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.
 40. Mi, S., and V. Stollar. 1991. Expression of Sindbis virus nsP1 and methyltransferase activity in *Escherichia coli*. *Virology* 184:423–427.
 41. Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst. 1990. New mammalian expression vectors. *Nature (London)* 348:91–92.
 42. Novak, J. E., and K. Kirkegaard. 1991. Improved method for detecting poliovirus negative strands used to demonstrate specificity of positive-strand encapsidation and the ratio of positive to negative strands in infected cells. *J. Virol.* 65:3384–3387.
 43. Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz. 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* 69:1011–1020.
 44. Pattnaik, A. K., and G. W. Wertz. 1990. Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. *J. Virol.* 64:2948–2957.
 45. Pattnaik, A. K., and G. W. Wertz. 1991. Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles. *Proc. Natl. Acad. Sci. USA* 88:1379–1383.
 46. Ranki, M., and L. Kääriäinen. 1979. Solubilized RNA replication complex from Semliki Forest virus-infected cells. *Virology* 98:298–307.
 47. 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.
 48. 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.
 49. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 50. Sawicki, D. L., D. B. Barkhimer, S. G. Sawicki, C. M. Rice, and S. Schlesinger. 1990. Temperature sensitive shut-off of alphavirus minus strand RNA synthesis maps to a nonstructural protein, nsP4. *Virology* 174:43–52.
 51. Sawicki, D. L., S. G. Sawicki, S. Keränen, and L. Kääriäinen. 1981. Specific Sindbis virus coded functions for minus strand

- RNA synthesis. *J. Virol.* **39**:348–358.
52. **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.
53. **Shirako, Y., and J. H. Strauss.** 1990. Cleavage between nsP1 and nsP2 initiates the processing pathway of Sindbis virus nonstructural polyprotein P123. *Virology* **177**:54–64.
54. **Strauss, E. G., C. M. Rice, and J. H. Strauss.** 1983. Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. *Proc. Natl. Acad. Sci. USA* **80**:5271–5275.
55. **Strauss, E. G., and J. H. Strauss.** 1986. Structure and replication of the alphavirus genome, p. 35–90. *In* S. Schlesinger and M. J. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
56. **Takkinen, K., J. Peränen, S. Keränen, H. Söderlund, and L. Kääriäinen.** 1990. The Semliki Forest virus-specific nonstructural protein nsP4 is an autoprotease. *Eur. J. Biochem.* **189**:33–38.
57. **Wang, Y., S. G. Sawicki, and D. L. Sawicki.** 1991. Sindbis Virus nsP1 functions in negative-strand RNA synthesis. *J. Virol.* **65**:985–988.
58. **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.