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Rescue of Sindbis Virus-Specific RNA Replication and Transcription by Using a Vaccinia Virus Recombinant

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A heterologous system expressing functional Sindbis virus nonstructural proteins (nsPs) has several possible uses for studying Sindbis virus-specific RNA replication and transcription in vivo and in vitro. Of the many possible approaches, vaccinia virus offers an attractive transient expression system given that Sindbis virus replication can occur in cells which have been previously infected by vaccinia virus. In this report, a vaccinia virus recombinant (called vSINNS), which contains the cDNA encoding the Sindbis virus nsPs under the control of either the vaccinia virus 7.5K promoter or the bacteriophage T7 promoter, has been constructed and characterized. Upon infection of several cell types with vSINNS, Sindbis virus nsP precursors and processed forms, including nsP1, nsP2, and both phosphorylated and nonphosphorylated forms of nsP3, were synthesized. Proteins containing the putative RNA-dependent RNA polymerase domain (nsP4 and nsP34), which are normally produced in small amounts by readthrough of an opal termination codon, were not detected in vSINNS-infected cells. However, all nsP functions necessary for Sindbis virus-specific RNA synthesis must have been expressed, since both replication and subgenomic mRNA transcription of an engineered Sindbis virus defective interfering RNA in cells infected with vSINNS was observed. Furthermore, vSINNS could be used as a helper virus to amplify, to relatively high titers, a replication-defective Sindbis virus mutant containing an in-frame deletion in the conserved N-terminal domain of nsP3. These data, as well as the observation that normal yields of parental Sindbis virus are produced in cells which have been previously infected with vSINNS, indicate that expression of Sindbis virus nsPs, in the absence of Sindbis virus-specific RNA replication, is not sufficient to block the formation of active RNA replication complexes by superinfecting Sindbis virus.

Sindbis virus (SIN) is an enveloped plus-strand RNA virus belonging to the *Alphavirus* genus of the family *Togaviridae*. The viral RNA genome, whose sedimentation coefficient is 49S, has been completely sequenced and consists of 11,703 nucleotides (nt) (56). The entire replication cycle of the virus occurs in the cytoplasm of host cells. The genomic RNA first functions as the mRNA for the translation of viral nonstructural proteins (nsPs) which are required for viral RNA replication. SIN RNA replication is initiated by the synthesis of a minus-strand RNA complementary to the genomic RNA. This minus-strand RNA is in turn used as a template for the synthesis of two species of plus-strand RNAs: 49S genomic RNA and a 3′-coterminal 26S subgenomic RNA which serves as the mRNA for the translation of viral structural proteins. In vertebrate cells, the production of minus-strand RNA ceases 3 to 4 h after infection, while the synthesis of plus strands (both 49S genomic and 26S subgenomic RNAs) continues throughout virus infection (51).

Four SIN-specific nsPs have been identified and named nsP1 to nsP4 according to the order of their location in the genome (5′ to 3′) (20). The nsPs are encoded in the 5′ two-thirds of the genome by a single open reading frame, except for an interruption between nsP3 and nsP4 by an in-frame opal termination codon (55). As a result, two polyprotein precursors are produced. P123 is the terminated product and contains the sequences of nsP1, nsP2, and nsP3, whereas the readthrough product, P1234, is produced in much smaller quantities and contains the sequences of nsP1, nsP2, nsP3, and nsP4. These polyprotein precursors are cleaved co- or posttranslationally by a proteinase activity residing in the C-terminal domain of nsP2 (9, 21) to generate the four individual nsPs and a reasonably stable nsP34 polyprotein (8, 37, 54). Different polyproteins containing nsP2 are active viral proteinases with different cleavage site preferences (8), and it has been hypothesized that temporal regulation of cleavage at the nsP3-nsP4 site, leading to an increase in the nsP34/nsP4 ratio, may be responsible for shifting the preference towards synthesis of plus-strand RNAs late in infection.

The nsPs are the only virus-encoded proteins required for SIN RNA replication and transcription (59). Mutations in each of the nsPs can result in RNA-negative phenotypes, indicating that they are all required for viral RNA synthesis (17, 18). nsP1 appears to participate in viral methyl and guanylyl transferase activities (41, 52). Mapping of a temperature-sensitive RNA-negative mutant (ts11) also suggests a requirement for nsP1 in minus-strand synthesis (18). As mentioned above, the C-terminal domain of nsP2 carries a proteinase activity responsible for processing viral nsPs (9, 21), and conditional mutations can lead to defects in polyprotein processing and RNA replication (18, 19). The N-terminal domain of nsP2 contains sequence motifs shared by NTP-binding proteins and helicases (13, 14), and mutations in this region can lead to reduced levels of 26S subgenomic RNA (29, 50). nsP3 consists of multiple, electrophoretically distinguishable, phosphorylated forms (38). Although conditional mutations have been found in nsP3 (18, 33), specific effects on viral RNA synthesis have not been reported. nsP4 shares highly homologous amino acid sequences with the RNA-dependent RNA polymerase of poliovirus (28), and a

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single amino acid change in this protein can result in the shutoff of all viral RNA synthesis under nonpermissive conditions (2, 3, 17). Therefore, nsP4-containing products, including nsP4 and nsP34, are potential candidates for polymerases in SIN RNA replication. In addition, nsP4 appears to play a role in the regulation of minus-strand shutoff (49) and interacts with a host component(s) which can have dramatic effects on viral RNA replication (34).

Although the genetic and biochemical studies outlined above have yielded important insights into the involvement of the nsPs in polyprotein processing and RNA replication, the specific functions of the nsP precursors or the cleaved products in SIN RNA synthesis have yet to be determined. To facilitate further in vivo and in vitro studies, we have investigated the use of vaccinia virus-SIN recombinants for the expression of biologically active SIN nsPs. Among several well-developed eukaryotic expression systems, vaccinia virus offers advantages of broad host range, large cloning capacity, and, like SIN, a cytoplasmic replication cycle (40, 42). In addition, in vivo rescue experiments using vaccinia virus recombinants and SIN mutants or SIN-specific defective interfering RNAs (DI RNAs) can be performed, since SIN can replicate in cells which have been previously infected by vaccinia virus (46). In this report, we demonstrate that the SIN nsPs, as expressed via a recombinant vaccinia virus, are correctly processed into mature products which are functional for SIN-specific RNA replication and transcription in vivo.

MATERIALS AND METHODS

Enzymes and plasmids. All restriction endonucleases, the large fragment of *Escherichia coli* DNA polymerase I (Klenow), T4 DNA polymerase, T4 DNA ligase, and calf intestinal alkaline phosphatase (CIAP) were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim Biochemicals. Plasmids pGem 2529 (39), pSC11 (5), and pJNTSCAT were kindly provided by M. MacDonald (Department of Anatomy and Neurobiology, Washington University), B. Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases), and B. Weiss and S. Schlesinger (Department of Molecular Microbiology, Washington University), respectively.

pJNTSCAT (23) was constructed by replacing the 1,113-bp *Nae*I-*Sst*I fragment of KDI25.3 (35) with the 1,617-bp *Ssp*I-*Sst*I fragment of TSCAT (59). From *Sst*I-linearized pJNTSCAT, an engineered SIN DI RNA (JNTSCAT) could be generated by in vitro transcription with SP6 RNA polymerase (35).

Viruses and cells. Vaccinia virus (WR strain) was propagated and titered on BSC-40 cells as previously described (22). Virus stocks of SIN and the mutant CR3.4 were produced by transfection of CEF or BHK-21 cell cultures with capped RNA transcripts synthesized by in vitro transcription (47). Parental SIN RNA was derived from the cDNA clone pToto1101 (16). Mutant CR3.4 RNA was derived from the cDNA clone pToto1101:CR3.4, which is isogenic with pToto1101 except for an in-frame deletion of 165 nt (nt 4754 to 4919) in the conserved N-terminal region of nsP3 (27). SIN mutant ts24 was kindly provided by D. Sawicki (Department of Microbiology, Medical College of Ohio). The growth conditions for BSC-40 cells (22), human TK^- 143 cells (5, 45), CV-1 cells (22), BHK-21 cells (26), and CEF cells (38) were described previously.

Construction of the recombinant vaccinia virus (vSINNS) containing SIN nonstructural genes. All DNA manipulations were conducted by using standard methods (48). Plasmid pToto1105, derived from pToto1002 (47) which contains an *Xba*I linker between nt 7612 and 7613, contains an additional *Xba*I linker inserted between nt 14 and 15 (4). Considering the potential use of a recombinant vaccinia virus expressing the T7 DNA-dependent RNA polymerase to enhance the expression of the target genes (12), the *Xba*I-*Xba*I fragment (from SIN sequence nt 15 to 7612 [56]) of Toto1105, containing the SIN nonstructural genes, was subcloned into the unique *Bam*HI site of the vector pGem2529 (39) after the cohesive ends were filled in with the Klenow fragment. The resulting plasmid (pGem-SINNS) had the SIN nonstructural genes flanked by a T7 promoter and terminator. The *Sst*I-*Xba*I fragment, containing the SIN nonstructural genes and the flanking T7 promoter and terminator sequences from pGem-SINNS, was made blunt ended by filling in with the Klenow fragment (*Xba*I site) or by incubation with T4 DNA polymerase (*Sst*I site) and was ligated to *Sma*I-digested pSC11 (5). The resulting plasmid, pSC11-T7-SINNS, contained SIN nonstructural genes under the control of two tandem promoters, the T7 promoter and the vaccinia virus 7.5K early/late promoter. The structure of pSC11-T7-SINNS is shown in Fig. 1.

The corresponding recombinant vaccinia virus, vSINNS, was derived by standard methods by using pSC11-T7-SINNS and homologous recombination with vaccinia virus in CV-1 cells (40, 46). TK^- recombinants were amplified by passage in human TK^- 143 cells in the presence of bromodeoxyuridine (25 μg/ml) (5), followed by plaque assay on BSC-40 cells. Recombinants were identified by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (5), and 100% homogeneous stocks were obtained after three successive cycles of plaque purification.

Protein analysis. Confluent BSC-40 cell monolayers in 35-mm tissue culture dishes were infected with SIN, the recombinant vaccinia virus (vSINNS), or parental vaccinia virus at a multiplicity of infection (MOI) of 1 PFU per cell in 200 μl of phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). After 1 h at 4°C for virus absorption, the infection mixture was removed and 3 ml of Eagle's minimum essential medium-Earle's salts (MEM) containing 3% FCS was added to the cells and the dishes were incubated at 37°C for 10 h. For continuous labeling, the cells were labeled for 3 h in 1 ml of MEM containing 20% of the normal methionine concentration and 20 μCi of [^{35}S]methionine per ml. For pulse-chase labeling, the cells were labeled for 15 min with 40 μCi of [^{35}S]methionine per ml (Translabel; ICN) in MEM lacking methionine and then subjected to different times of chase in regular MEM. After labeling, the cell monolayers were rinsed twice with ice-cold PBS and lysed with 200 μl of 1% sodium dodecyl sulfate (SDS) containing 20 μg of phenylmethylsulfonyl fluoride (PMSF) per ml. SIN-specific proteins in the lysates were immunoprecipitated with antibodies monospecific for SIN nsP1, nsP2, nsP3, or nsP4 (20), and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 6% polyacrylamide) (31). After treatment for fluorography (32), gels were dried and exposed to Kodak XAR-5 film.

The expression of SIN nsPs by vSINNS in CEF and BHK-21 cells was also examined by using the procedure described above. Since vaccinia virus failed to produce plaques on CEF cells under our conditions, its titer on BHK-21 cells, which was about 50-fold lower than on BSC-40 cells, was used for the infection of CEF cells.

RNA transfection. The procedure for the RNA transfection of CEF or BHK-21 cells (16) was adapted from a method for

DNA transfection (11). Transcript RNA (100 ng) was mixed with 200 μl of PBS containing 8 μg of lipofectin (Bethesda Research Laboratories). The transfection mixture was incubated on ice for 10 min and then added to the cell monolayer, which had been rinsed twice with ice-cold PBS. After 10 min at room temperature, the transfection mixture was removed, 3 ml of prewarmed (37°C) MEM containing 3% FCS was added, and incubation was continued at 37°C for the indicated times.

RNA analysis. Confluent BHK-21 or CEF cell monolayers in 35-mm tissue culture dishes were infected with SIN, vSINNS, or wild-type vaccinia virus at an MOI of 1 PFU per cell in 200 μl of PBS containing 1% FCS. Virus absorption was conducted at 4°C for 1 h. The infection mixture was then removed, and 3 ml of MEM was added to each dish. At 3 h postinfection, the monolayers were transfected with 100 ng of JNTSCAT RNA as described above. Following incubation at 37°C for an additional 10 h, cytoplasmic RNAs were isolated, denatured with glyoxal and dimethyl sulfoxide, and analyzed by 1% agarose gel electrophoresis (48).

JNTSCAT-specific genomic and subgenomic RNAs were identified by Northern (RNA) analysis (48). Briefly, the RNA was transferred onto a nitrocellulose membrane after electrophoresis. The membrane was baked at 80°C for 2 h and then prehybridized at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% SDS–100 ng of denatured salmon sperm DNA per ml–50% formamide. After 6 h of prehybridization, a ^{32}P-labeled CAT gene-specific probe (10^7 cpm), labeled by random priming (10) the *Pvu*II-*Nco*I fragment of pJNTSCAT, was added, and the membrane was then incubated at 42°C for an additional 12 h. After hybridization, the membrane was washed three times at room temperature (20 min per wash) with 0.5× SSC–0.1% SDS and was then exposed, with an intensifying screen, to Kodak XAR-5 film.

CAT assay. The method described by Gorman et al. (15) was used with modifications. Virus infection and RNA transfection were conducted as described above. At 10 h posttransfection, cell monolayers were rinsed three times with ice-cold PBS and scraped into 500 μl of PBS. Cells were pelleted for 10 s in a microcentrifuge, resuspended in 200 μl of 0.25 M Tris-HCl (pH 7.5), and broken by three freeze-thaw cycles (on dry ice and at 37°C). Cell debris were pelleted by centrifugation in a microcentrifuge (15 min), and the supernatants were stored at −80°C. CAT enzyme assays, in a final volume of 100 μl, contained 0.25 M Tris-HCl (pH 7.5), 0.1 μCi of [^{14}C]chloramphenicol, 5 mM acetyl coenzyme A, and 10 μl of the cell extract from a single 35-mm culture dish. Reactions were incubated at 37°C for 1 h and stopped by adding 1 ml of ice-cold ethyl acetate. After vortexing and a brief (1-min) centrifugation, the organic layer was transferred to a new tube, dried, and resuspended in 20 μl of ethyl acetate. Samples were spotted on silica gel plates and separated by ascending chromatography developed with chloroform-methanol (95:5). The plates were air dried and exposed to Kodak XAR-5 film. The ^{14}C-labeled substrate and products were localized by autoradiography, excised from the plates, and quantitated by liquid scintillation counting.

Rescue of a replication-defective SIN mutant (CR3.4) in vSINNS-infected cells. All infections in the following experiments were conducted at room temperature in 200 μl of PBS containing 1% FCS. Confluent BHK-21 monolayers were infected with either vSINNS or wild-type vaccinia virus at an MOI of 1 PFU per cell and incubated at 37°C. At 3 h postinfection, the CR3.4 RNA, which was derived from pToto1101:CR3.4 by in vitro transcription with SP6 DNA-dependent RNA polymerase, was introduced into the cells by RNA transfection as described above. The medium was harvested at 24 h posttransfection (called the first passage). Ten percent (100 μl) of the first-passage medium was used to infect fresh cell monolayers which had been infected with either wild-type vaccinia virus or vSINNS 3 h earlier. The medium was harvested at 24 h postinfection (the second passage) and passaged a third time.

To assay samples of the media for infectious SIN particles, infectious vaccinia virus virions (vSINNS or wild-type vaccinia virus) were removed by differential centrifugation and neutralization. Centrifugation for 20 min in a microcentrifuge removed more than 99% of the infectious vaccinia virus virions. Residual infectivity was further reduced by incubation at 37°C for 30 min with a vaccinia virus-specific neutralizing monoclonal antibody (VV-75; kindly provided by Alan Schmaljohn, Virology Division, Department of the Army, U.S. Army Medical Research Institute for Infectious Diseases) at a dilution of 1/100. After these treatments, which did not affect the yield of infectious SIN virions, less than 50 infectious vaccinia virus virions per ml remained when titered on BSC-40 cell monolayers.

Analysis of CR3.4 RNA replication and transcription. Since CR3.4 failed to produce plaques on both CEF and BHK-21 cells, the titers of the amplified CR3.4 mutant in the medium, compared with those of Toto1101, were estimated by RNA dot blot hybridization (25), using a ^{32}P-labeled probe (10) corresponding to the *Nar*I-*Nar*I fragment (nt 10039 to 11311 [56]) of pToto1101 (16). For analysis of CR3.4 RNA synthesis, the third-passage medium (unless otherwise indicated) was used to infect CEF or BHK-21 cell monolayers at an MOI equivalent to 0.1 infectious unit per cell. Parental SIN (Toto1101) was used at an MOI of 10 PFU per cell. At 8 h postinfection, the percentage of infected cells was determined by immunofluorescence (46), using an SIN E1-specific monoclonal antibody (S-33 [53]) and fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) (Boehringer Mannheim Biochemicals). A parallel set of infected monolayers was used to isolate cytoplasmic RNAs for Northern blot analysis with the ^{32}P-labeled probe described above.

RESULTS

Structure of the recombinant vaccinia virus vSINNS. The structure of the recombination plasmid pSC11-T7-SINNS, which was used to construct vSINNS, is schematically shown in Fig. 1. The SIN nonstructural genes were placed downstream of two tandem promoters, the vaccinia virus 7.5K early/late promoter (6) followed by the bacteriophage T7 promoter (12). The first AUG in the mRNA expressed from either promoter is that of nsP1, which initiates the open reading frame encoding the SIN nsPs. This arrangement was presumably maintained in the recombinant vaccinia virus vSINNS but was not examined directly. The SIN sequences in vSINNS did not include *cis* RNA elements in the 5′ and 3′ terminal regions and would therefore not be expected to replicate via SIN-specific RNA replication machinery (30, 36, 43). The term "replication-competent SIN-specific RNA" is used to refer to RNA molecules which carry essential *cis*-acting elements for replication by SIN RNA replication machinery, e.g., SIN genomic RNA and DI RNAs.

Expression of SIN nsPs in vSINNS-infected cells. To examine the ability of vSINNS to express SIN nsPs, vSINNS-infected BSC-40 cells were continuously labeled with

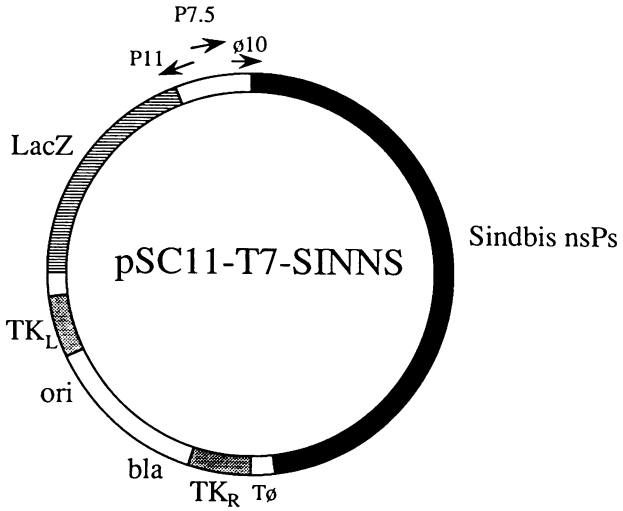

FIG. 1. Structure of pSC11-T7-SINNS. Details of the plasmid construction are described in Materials and Methods. The entire sequence encoding SIN nsPs (black area) is flanked by the bacteriophage T7 promoter (ϕ10) and terminator (Tϕ). Immediately upstream of the T7 promoter is the vaccinia virus 7.5K early/late promoter (P7.5). This plasmid also contains an *E. coli* β-galactosidase gene (LacZ, hatched area) under the control of the vaccinia virus 11K late promoter (P11). The arrows indicate the transcription directions from the corresponding promoters. TK_L and TK_R refer to the left and right regions of the thymidine kinase gene of vaccinia virus (5). Also shown are the replication origin of the plasmid (ori) and the gene conferring ampicillin resistance (bla).

[^{35}S]methionine from 10 to 13 h postinfection. The cell lysates were immunoprecipitated with antibodies monospecific for SIN nsP1 to nsP4, and the protein samples were analyzed by SDS-PAGE (6% polyacrylamide). As shown in Fig. 2A, nsP1, nsP2, and nsP3 were easily detected in vSINNS-infected cells and were indistinguishable, in terms of electrophoretic mobility, from their authentic counterparts synthesized by SIN. nsP4 and nsP34, which were synthesized in small amounts by translational readthrough of the in-frame opal codon preceding nsP4 in SIN-infected cells, were not detected in vSINNS-infected cells. The polyprotein precursor, P123, appeared to be more abundant in vSINNS-infected cells than in SIN-infected cells. Coinfection with vSINNS and a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3 [12]) led to an enhancement in the production of SIN proteins (from 10 to 13 h postinfection) by about threefold (data not shown).

To study the kinetics of protein synthesis, vSINNS-infected cells were pulse-labeled for 1 h with [^{35}S]methionine at different times postinfection. A typical experiment examining the synthesis of SIN nsP2 in vSINNS-infected cells is shown in Fig. 2B. SIN proteins could be detected at 3 to 4 h postinfection, and although maximal synthesis occurred at 10 to 11 h postinfection, synthesis continued even at 24 h postinfection despite dramatic cytopathic effects.

Since most SIN replication experiments have been carried out with CEF or BHK-21 cells and we wished to use these cell types for rescue experiments (see below), the expression of SIN-specific nsPs via vSINNS was also examined with these cell types. As in BSC-40 cells, SIN nsP1, nsP2, and nsP3 were easily detected in both CEF and BHK-21 cells infected with vSINNS, although the levels of the SIN proteins were about 10-fold less than in SIN-infected cells (data not shown).

Processing of SIN nonstructural polyproteins in vSINNS-infected cells. The polyprotein-processing kinetics of SIN nonstructural polyproteins were examined by the pulse-chase analysis of vSINNS-infected cells. At 10 h postinfection, vSINNS-infected BSC-40 cell monolayers were pulse-labeled with [^{35}S]methionine for 15 min and then subjected to different times of chase in the presence of excess unlabeled methionine. Labeled virus-specific proteins in the cell lysates were immunoprecipitated with antibodies monospecific for each of the SIN nsPs, and the immunoprecipitates

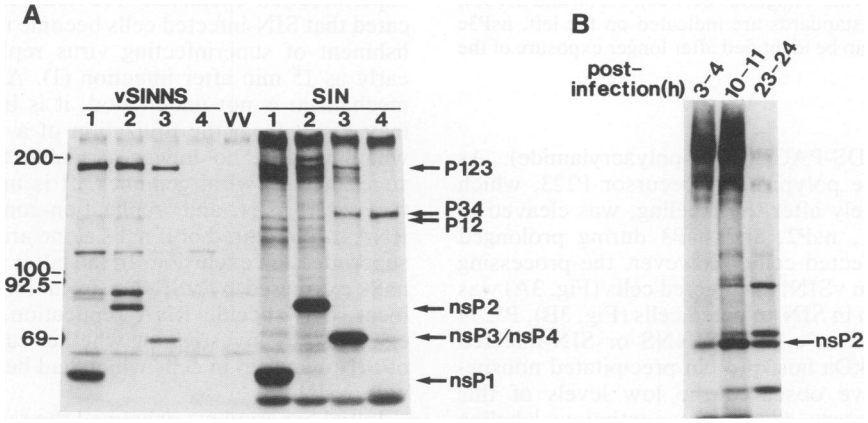

FIG. 2. Synthesis of SIN nsPs in vSINNS-infected cells. (A) BSC-40 cell lysates from either SIN- or vSINNS-infected cell cultures were immunoprecipitated with the antibodies monospecific for SIN nsP1 (lanes 1), nsP2 (lanes 2), nsP3 (lanes 3), or nsP4 (lanes 4). The immunoprecipitates of nsP3 and nsP4 were treated with CIAP prior to SDS-PAGE (38). The sample in lane VV was derived from wild-type vaccinia virus-infected cell cultures and immunoprecipitated with a mixture of all four monospecific antisera. Protein samples were analyzed by SDS-PAGE (6% polyacrylamide). SIN-specific proteins are indicated on the right, and molecular mass standards are indicated on the left. Both nsP3 and nsP34 are observed as a single nonphosphorylated form because of CIAP treatment (38). (B) vSINNS-infected cell cultures were pulse-labeled with [^{35}S]methionine for 1 h at different times postinfection, as indicated. Cell lysates were immunoprecipitated with antiserum to nsP2. Protein samples were analyzed by SDS-PAGE (6% polyacrylamide). The position of nsP2 is indicated. It should be noted that several bands seen in these immunoprecipitation reactions are either host or vaccinia virus-specific but appear to be enhanced when immune complexes, particularly with the nsP2-specific antiserum, are being formed.

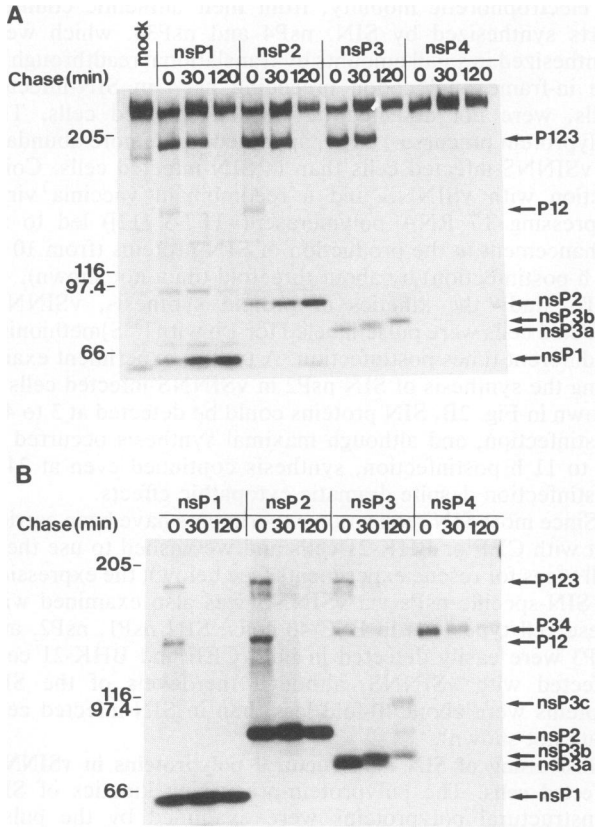

FIG. 3. Processing kinetics of SIN polyproteins in vSINNS-infected cells. BSC-40 cell cultures were infected with either vSINNS (A) or SIN (B), and at 10 h postinfection they were pulse-labeled for 15 min and then subjected to different times of chase, as indicated. Cell lysates were immunoprecipitated with the antibodies monospecific to SIN nsP1, nsP2, nsP3, or nsP4. Lysates from mock-infected cell cultures were immunoprecipitated with a mixture of all four monospecific antisera. Protein samples were analyzed by SDS-PAGE (6% polyacrylamide). SIN-specific proteins are indicated on the right (nsP4 migrates between nsP3a and nsP3b), and the molecular mass standards are indicated on the left. nsP3c but not nsP4 and nsP34 can be identified after longer exposure of the gel shown in panel A.

were analyzed by SDS-PAGE (6% polyacrylamide). As shown in Fig. 3A, the polyprotein precursor P123, which was present immediately after the labeling, was cleaved to produce mature nsP1, nsP2, and nsP3 during prolonged chases in vSINNS-infected cells. However, the processing of these polyproteins in vSINNS-infected cells (Fig. 3A) was apparently slower than in SIN-infected cells (Fig. 3B). P1234 could not be identified in either vSINNS or SIN-infected cells because a ~250-kDa host protein precipitated nonspecifically and may have obscured the low levels of this precursor. As was observed with the continuous-labeling experiments, nsP4 and nsP34 were not detected in vSINNS-infected cells (Fig. 3A; see below), although both products were present in SIN-infected cells (Fig. 3B).

In SIN-infected cells, nsP3 is posttranslationally modified by phosphorylation, resulting in forms which have slower mobilities by SDS-PAGE (38). As in SIN-infected cells (Fig. 3B), nsP3 produced by vSINNS first appeared as a single species with an apparent molecular mass of 76 kDa (nsP3a). This newly synthesized species was gradually converted to a smear of multiple slower-mobility forms ranging from 78 kDa (nsP3b) to 106 kDa (nsP3c) as the times of chase were increased (Fig. 3A). The conversion rate from nsP3a to nsP3c was slower in vSINNS-infected cells (Fig. 3A) than in SIN-infected cells (Fig. 3B). Upon phosphatase treatment, the slower mobility forms were converted to nsP3a (data not shown), confirming that the altered migration of the nsP3 species was due to phosphorylation. As previously reported (38), these results indicate that nsP3 phosphorylation can occur independently of SIN-specific RNA replication.

TABLE 1. SIN growth in vaccinia virus-infected BHK-21 cells[a]

Primary infection or coinfection	Superinfection	SIN titers (PFU/ml)	
		30°C	40°C
SIN	None	1.0×10^9	1.0×10^9
Wild-type VV + SIN	None	1.0×10^9	1.0×10^9
Wild-type VV	SIN	1.0×10^9	9.8×10^8
SIN ts24 + SIN	None	1.4×10^9	9.7×10^8
SIN ts24	None	5.0×10^8	<500
SIN ts24	SIN	1.0×10^9	1.4×10^7
Wild-type VV + SIN ts24[b]	SIN	9.1×10^8	1.3×10^7
vSINNS + SIN	None	1.8×10^9	1.2×10^9
vSINNS	SIN	1.0×10^9	1.0×10^9

[a] Primary infections or coinfections were performed with SIN, wild-type vaccinia virus (VV), ts24, or vSINNS at the same MOI, 10 PFU per cell (as determined on BHK-21 cells). Superinfections, as indicated, were performed at the same MOI, after either 1 h (ts24) or 3 h (wild-type VV and vSINNS). After incubation at 30°C for 10 h, released SIN virions in the medium were harvested and titered on CEF monolayers at the indicated temperatures. The titers shown are the averages of two independent experiments. In infections involving both SIN and ts24, the titers at 40°C reflect only SIN whereas the titers at 30°C represent both SIN and ts24.

[b] In this experiment, monolayers were infected with wild-type VV and incubated at 30°C for 3 h prior to infection with ts24.

Superinfection of SIN in vSINNS-infected cells. A possible complication in the use of vSINNS as a helper virus to supply functional nsPs in *trans* involves the phenomenon of superinfection exclusion. Previous experiments have indicated that SIN-infected cells become refractory to the establishment of superinfecting virus replication complexes as early as 15 min after infection (1). Although the molecular mechanism is not understood, it is believed that this state may result from the utilization of a limited host factor(s), which is then no longer available for the replication of superinfecting viral genomes. It is unknown whether both the viral nsPs and replication-competent virus-specific RNAs are required or if nsPs alone are sufficient to establish superinfection exclusion. In the latter case, we would expect nsPs expressed by vSINNS to inhibit, rather than complement, SIN-specific RNA replication. This possibility was examined by investigating whether superinfection exclusion of SIN occurred in cells which had been previously infected with vSINNS.

Initial experiments examined the replication compatibility of SIN and vaccinia virus. SIN was either coinfected with wild-type vaccinia virus or used to superinfect wild-type vaccinia virus-infected BHK-21 cell monolayers. Virus stocks were harvested 10 h after SIN infection and titered on CEF monolayers. No significant differences in SIN yields between cells coinfected with vaccinia virus and SIN and cells infected with SIN alone were observed (Table 1). These results were consistent with a previous report which indicated that SIN could replicate in vaccinia virus-infected cells

(46). Similarly, in coinfection or superinfection experiments using SIN and vSINNS, significant decreases in SIN yields were also not observed (Table 1), indicating that the expression of SIN nsPs by vSINNS was not sufficient to establish superinfection exclusion. Similar results were obtained when CEF cells were used (data not shown). These results suggested that the vSINNS recombinant might be useful for supplying functional nsPs for SIN-specific RNA replication (see below).

Regarding the mechanism of superinfection exclusion, it could be argued that the inability of vSINNS to establish this state might be quantitative rather than qualitative, i.e., insufficient quantities of nsPs might be produced, or vaccinia virus infection per se might abolish superinfection exclusion. Although we cannot exclude the first possibility, the latter explanation was examined by analyzing normal SIN superinfection exclusion in vaccinia virus-infected cells. A SIN temperature-sensitive mutant with low reversion frequency, ts24, was chosen, since it can establish superinfection exclusion of wild-type SIN at 30°C (1) and can be distinguished from the superinfecting wild-type SIN by its inability to form plaques at 40°C. As shown in Table 1, virus yields of superinfecting SIN decreased approximately 100-fold in either the presence or absence of vaccinia virus infection. These results indicated that prior infection by vaccinia virus did not interfere with the establishment of superinfection exclusion by SIN.

Replication and transcription of a SIN-specific DI RNA by SIN nsPs supplied by vSINNS. To directly test the ability of SIN nsPs expressed via vSINNS to form functional replication and transcription complexes, an assay using an engineered SIN DI RNA, called JNTSCAT (Fig. 4A), was employed. JNTSCAT does not encode functional virus-specific proteins, but it contains the *cis*-acting elements necessary for DI RNA replication as well as transcription of a subgenomic RNA. In the presence of functional SIN-specific RNA replication and transcription machinery, JNTSCAT genomic RNA [2,907 nt, excluding the poly(A) tail] is amplified, via complementary minus-strand intermediates, to produce two predominant RNA species: full-length DI genomic RNA and a 3′ coterminal DI subgenomic RNA [1,469 nt, excluding the poly(A) tail] which encodes CAT (Fig. 4A). The assay for CAT activity provides a sensitive, albeit indirect, measure of DI subgenomic mRNA transcription.

As shown in Fig. 4B, after transfection of JNTSCAT RNA transcripts, CAT activity in both SIN- and vSINNS-infected BHK-21 cells, but not in mock or wild-type vaccinia virus-infected cells, was readily detected. The level of CAT activity was about threefold higher in cells infected with SIN than in cells infected with vSINNS. As expected, in the absence of DI RNA transfection, CAT activity was not detected in cells infected with SIN, vSINNS, or wild-type vaccinia virus. Similar results were obtained when the experiments were conducted with CEF cells (data not shown). These data suggested that the SIN nsPs expressed via vSINNS were functional for transcription of the DI subgenomic RNA encoding CAT.

More direct evidence was obtained by Northern blot analysis using a CAT gene-specific hybridization probe. Both genomic and subgenomic JNTSCAT RNAs were observed in SIN- and vSINNS-infected cells but not in mock or wild-type vaccinia virus-infected cells or in the other negative controls (Fig. 4C). Interestingly, the molar ratio of DI genomic RNAs to DI subgenomic RNAs was different for SIN-infected and vSINNS-infected cells. This ratio was

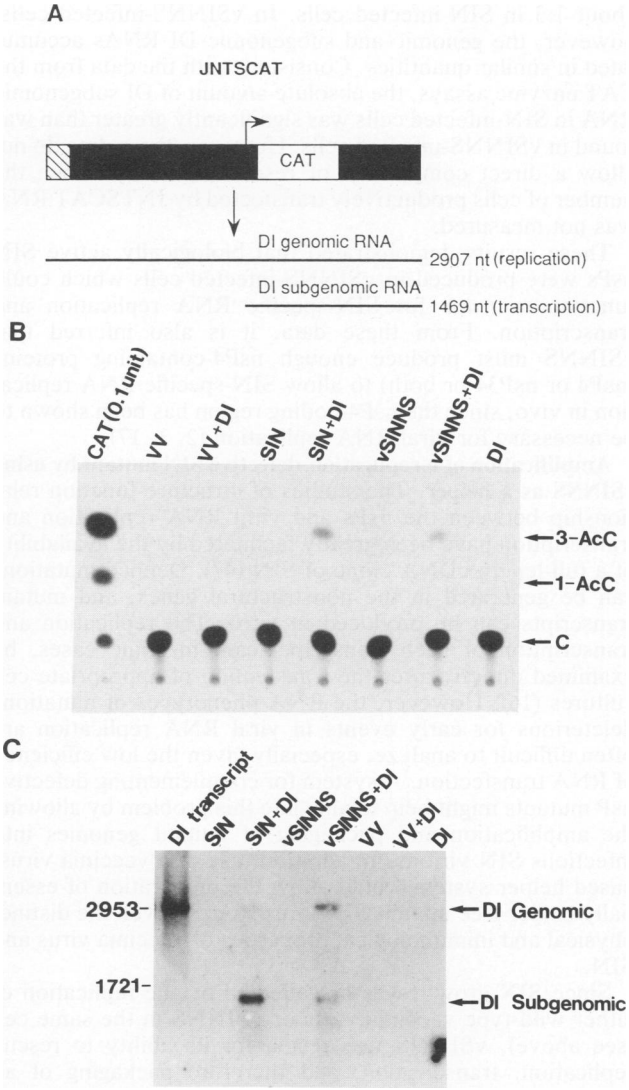

FIG. 4. Rescue of SIN RNA replication and transcription by vSINNS. (A) Schematic of the genomic structure of JNTSCAT, the SIN-specific template used for functional analysis of the SIN nsPs expressed in vSINNS-infected cells. In addition to the *cis*-acting elements essential for replication by SIN-specific RNA replication machinery, JNTSCAT contains the CAT gene (open box) under the control of the SIN subgenomic promoter. The top arrow indicates the transcription direction from the SIN subgenomic promoter. The black and hatched regions indicate sequences derived from SIN and a naturally occurring SIN DI, KDI25 (36), respectively. Plus-strand JNTSCAT RNA species, produced by replication or transcription, are shown below, with the lengths of the RNAs indicated in bases. (B and C) JNTSCAT RNA was transfected into BHK-21 cell cultures which were previously infected with SIN (lane SIN+DI), vSINNS (lane vSINNS+DI), or wild-type vaccinia virus (lane VV+DI) or were mock infected (lane DI). Cell extracts were prepared for either CAT assay (B) or cytoplasmic RNAs isolated for Northern blot analysis (C). Additional negative controls included samples from cells infected with different viruses without DI RNA transfection (lanes SIN, vSINNS, and VV). In panel B, C, 1-AcC, and 3-AcC indicate chloramphenicol, chloramphenicol 1-acetate, and chloramphenicol 3-acetate, respectively. In panel C, the probe used was specific for CAT sequence and therefore would detect both genomic and subgenomic DI RNAs but not the SIN-specific nsP sequence expressed by vSINNS. The DI-specific RNAs are indicated on the right and the positions and sizes (in bases) of RNA markers are indicated on the left.

about 1:3 in SIN-infected cells. In vSINNS-infected cells, however, the genomic and subgenomic DI RNAs accumulated in similar quantities. Consistent with the data from the CAT enzyme assays, the absolute amount of DI subgenomic RNA in SIN-infected cells was significantly greater than was found in vSINNS-infected cells. However, these data do not allow a direct comparison of rescue efficiency, since the number of cells productively transfected by JNTSCAT RNA was not measured.

These results demonstrated that biologically active SIN nsPs were produced in vSINNS-infected cells which could function in *trans* for SIN-specific RNA replication and transcription. From these data, it is also inferred that vSINNS must produce enough nsP4-containing proteins (nsP4 or nsP34 or both) to allow SIN-specific RNA replication in vivo, since the nsP4-coding region has been shown to be necessary for viral RNA replication (2, 3, 17).

Amplification of a replication-defective SIN mutant by using vSINNS as a helper. The studies of structure-function relationship between the nsPs and viral RNA replication and transcription have been greatly facilitated by the availability of a full-length cDNA clone of SIN (47). Defined mutations can be generated in the nonstructural genes, and mutant transcripts can be produced in vitro. The replication and transcription of such transcripts can, in some cases, be examined directly after the transfection of appropriate cell cultures (16). However, the RNA phenotypes of mutations deleterious for early events in viral RNA replication are often difficult to analyze, especially given the low efficiency of RNA transfection. A system for complementing defective nsP mutants might help to alleviate this problem by allowing the amplification and packaging of mutant genomes into infectious SIN virions. In addition, use of a vaccinia virus-based helper system would allow the preparation of essentially helper-free mutant SIN virus stocks, given the distinct physical and immunological properties of vaccinia virus and SIN.

Since SIN growth was not affected by the replication of either wild-type vaccinia virus or vSINNS in the same cell (see above), vSINNS was tested for its ability to rescue replication, transcription, and therefore packaging of an apparently lethal nsP3 deletion mutant genome, called CR3.4. CR3.4 contains an in-frame deletion of 165 nt (from nt 4754 to 4919 of SIN), corresponding to 55 amino acids in the conserved N-terminal domain of nsP3. No replication, as evidenced by plaque formation or viral RNA synthesis, could be detected upon transfection of CEF or BHK-21 cell cultures with CR3.4 RNA transcripts. In three independent attempts, plaque-forming revertants were not observed, making CR3.4 a good candidate for testing vSINNS as a helper system for supplying functional nsP3.

CR3.4 transcripts were used to transfect wild-type vaccinia virus- or vSINNS-infected BHK-21 cell cultures, and the medium was harvested at 24 h postinfection. The medium containing the putative amplified and packaged CR3.4 was then passaged twice more in vaccinia virus- or vSINNS-infected cells. By RNA blot hybridization, the supernatant from the third passage in the presence of vSINNS contained $\sim 1 \times 10^6$ infectious units per ml as compared with a parental SIN stock generated in BHK cells (assuming an equal particle/infectious unit ratio). Continued passaging did not result in an increase in CR3.4 titer. Fewer than 10^4 infectious units per ml (which was the limit of detection) were present in the supernatants from the first two passages in vSINNS-infected cells or passages in wild-type vaccinia virus-infected cells.

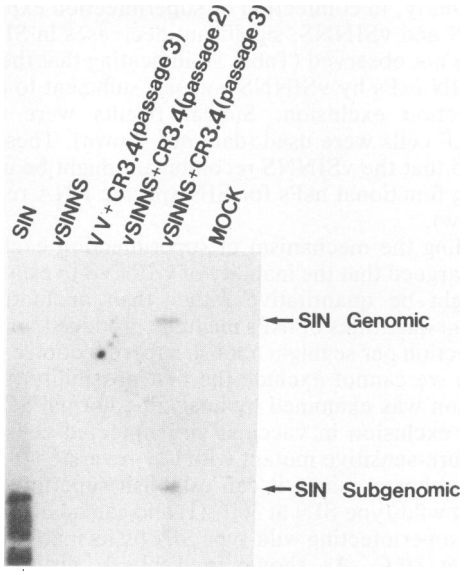

FIG. 5. RNA phenotype of a replication-defective deletion mutant (CR3.4) rescued by vSINNS. As described in the text, samples of medium from different passages (as indicated) were depleted of vaccinia virus and used to infect BHK-21 cells. At 8 h postinfection, cytoplasmic RNAs were isolated and SIN-specific RNAs were identified by Northern blot analysis using a ^{32}P-labeled probe specific for the SIN structural region. CR3.4 amplified by passaging in the presence of vSINNS (lanes vSINNS+CR3.4), CR3.4 passaged in wild-type vaccinia virus-infected cells (lane VV+CR3.4), vSINNS passaged by itself (lane vSINNS), and BHK-21 cells infected with SIN at an MOI of 10 PFU per cell (lane SIN) are shown. In a parallel set of infections, the number of productively infected cells was estimated by immunofluorescence. The amount of RNA loaded from SIN-infected cells corresponds to approximately 1/10 that loaded for cells infected with CR3.4 (passage 3 in the presence of vSINNS). The 49S genomic and 26S subgenomic RNAs of SIN are indicated.

To examine whether SIN RNA synthesis could be detected upon infection of BHK-21 cells with the passaged CR3.4 stock in the absence of helper virus, supernatants were depleted of infectious vaccinia virions by centrifugation and neutralization (see Materials and Methods) and used to infect fresh BHK-21 cell cultures. Cytoplasmic RNAs were isolated and examined by Northern blot analysis using a SIN structural region-specific probe. As shown in Fig. 5, both genomic 49S and subgenomic 26S SIN-specific RNAs were detected after infection with the third passage supernatant. SIN-specific RNA species were not observed after infection with supernatants from the first two passages, nor were they observed when supernatants from passages in mock or wild-type vaccinia virus-infected cells were used.

To rule out the possibility that the observed viral RNA synthesis might be due to an RNA recombination event between vSINNS transcripts and CR3.4 that restored nsP3, the polymerase chain reaction, using primers hybridizing within the nsP3-coding region, and DNA sequencing techniques were used to analyze virus-specific RNAs produced in BHK-21 cells infected with the third-passage supernatant. The results confirmed that the rescued RNA genome still carried the CR3.4 nsP3 deletion after three passages with the vSINNS helper (data not shown). However, the possibility that changes which allowed more efficient RNA replication by rescued CR3.4 had occurred elsewhere in the genome

cannot be excluded on the basis of these data. In this regard, we have observed differences in the ratio of 49S genomic RNAs to 26S subgenomic RNAs in independent CR3.4 rescue experiments. In the experiment shown in Fig. 5, the ratio was approximately 1:1, which was different from the 1:3 ratio found for SIN-infected cells at the same time postinfection. In an independent experiment, however, a 49S/26S ratio similar to that of SIN was observed (data not shown). Using immunofluorescence to determine the proportion of CR3.4-infected cells to SIN-infected cells (data not shown), we estimate that the level of 49S genomic RNA in CR3.4-infected cells is at least 10-fold lower than in SIN-infected cells. These data indicate that the CR3.4 deletion results in impaired SIN RNA synthesis, a result consistent with a previous report localizing an RNA-negative conditional mutation to nsP3 (18).

DISCUSSION

In this report, we constructed and characterized a vaccinia virus-SIN recombinant, vSINNS, which contained the entire SIN nsP coding region but lacked *cis*-acting sequences essential for SIN-specific RNA replication (30, 36, 43). The SIN nsPs, as synthesized via vSINNS, appear to be produced by authentic processing of the polyprotein precursors and to undergo posttranslational modifications similar to those observed for SIN-infected cells. Previous infection of cells with vSINNS did not lead to a state in which replication of superinfecting SIN was blocked or partially restricted. Hence, we were able to show that the SIN nsPs expressed by vSINNS were biologically active as assessed by their ability to catalyze the synthesis of both genomic and subgenomic SIN-specific RNAs in *trans*. These results indicate that establishment of superinfection exclusion by SIN may require not only the synthesis of functional nsPs but also replication-competent SIN-specific RNAs.

It was noticed that the processing of SIN nonstructural polyproteins, in particular P123, is significantly slower in vSINNS-infected cells than in SIN-infected cells and that the readthrough products, nsP4 and nsP34, could not be detected in vSINNS-infected cells. Since the level of nsP production by vSINNS was lower than the level observed with SIN-infected cells, the kinetics of SIN polyprotein processing in vSINNS-infected cells might be analogous to an early stage in SIN infection. According to the current model (8), P123 can cleave the nsP3-nsP4 site efficiently in *trans*, leading to low levels of nsP34 relative to nsP4 early in infection. Previous experiments, examining the stability of nsP4 in vivo (8, 20, 37) and in vitro (cited in reference 57), have led to the hypothesis that nsP4, which is not actively involved in RNA replication, is subject to rapid turnover. This scenario could then explain the inability to detect nsP4 in vSINNS-infected cells, given the absence of active SIN-specific RNA replication. Alternatively, these observations might also be explained if vaccinia virus infection altered processing and stability of the SIN nsPs or affected readthrough efficiency. However, this explanation is considered unlikely, since nonstructural polyprotein processing and the levels of nsP4 and nsP34 are normal in cells coinfected with SIN and vaccinia virus (data not shown).

We demonstrated that vSINNS can successfully rescue the RNA replication of a DI RNA (JNTSCAT) and a SIN mutant (CR3.4) which contains a defined deletion in nsP3. Replication and transcription of JNTSCAT by vSINNS was nearly as efficient as rescue by SIN; DI RNA synthesis was detectable directly after RNA transfection. Hence, it should be possible to use the vaccinia virus system to express mutant SIN nsPs and assay the effect of such mutations on DI RNA replication and transcription. Similar approaches have recently been employed for studying vesicular stomatitis virus (44) and influenza virus (24) replication. Another important use of vSINNS might be as a helper system to supply wild-type SIN nsPs in *trans* for complementing SIN mutants with lethal lesions in the nsPs. For the CR3.4 mutant, replication could not be detected directly after RNA transfection of vSINNS-infected cells (data not shown), and at least three passages in vSINNS-infected cells were necessary before sufficient titers were obtained to allow analysis of the mutant RNA phenotype in the absence of vSINNS. Similar experiments examining the use of vSINNS for rescuing lethal in-frame deletion mutations in the other nsPs or mutants in several RNA-negative complementation groups (ts6, group F; ts11, group B; ts24, group A) have not been successful (data not shown). Although discouraging, these results are consistent with previous observations which indicate that functional complementation between conditional lethal mutants of RNA viruses, including SIN, poliovirus, and bacteriophage f2, often tends to be inefficient (3, 7, 58). It is possible that translation of altered or inactive nsPs from the mutant RNAs interferes with the utilization of their functional counterparts expressed in *trans* by vSINNS, thus leading to the more efficient rescue of DI RNA replication as opposed to replication of CR3.4 or the other nsP mutants.

The expression of functional SIN nsPs via vSINNS opens many possibilities for further investigation of these proteins. vSINNS provides a convenient, heterologous system for the characterization of SIN nsPs that is free of replication-competent SIN-specific RNAs and structural proteins. It is therefore tempting to use extracts from vSINNS-infected cells as a source of SIN nsPs for the possible construction of an in vitro system which replicates exogenous SIN-specific RNAs. SIN nsPs prepared from vSINNS-infected cells may also be useful for in vitro biochemical analyses of RNA binding or enzymatic activities presumably associated with the nsPs, such as the nsP1-associated methyl and guanylyl transferases (41, 52), the nsP2-associated proteinase (9, 21) and RNA helicase (14), and the nsP4-associated RNA-dependent RNA polymerase (2, 17, 28). Additional vaccinia virus recombinants which express individual nsPs or different cleavage-defective nonstructural polyproteins (8) should allow a direct examination of the roles of these nsP species in the regulated synthesis of SIN-specific plus- and minus-strand RNAs.

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