Roles of Nonstructural Polyproteins and Cleavage Products in Regulating Sindbis Virus RNA Replication and Transcription

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Using vaccinia virus to express Sindbis virus (SIN) nonstructural proteins (nsPs) and template RNAs, we showed previously that synthesis of all three viral RNAs occurred only during expression of either the entire nonstructural coding region or the polyprotein precursors P123 and P34. In this report, the vaccinia virus system was used to express cleavage-defective polyproteins and nsP4 proteins containing various N-terminal extensions to directly examine the roles of the P123 and P34 polyproteins in RNA replication. Replication and subgenomic mRNA transcription occurred during coexpression of P34 and P123 polyproteins in which cleavage was blocked at either or both of the 1/2 and 2/3 sites. For all cleavage-defective P123 polyproteins, however, the ratio of subgenomic to genomic RNA was decreased, suggesting that both the 1/2 and 2/3 cleavages are required for efficient subgenomic RNA transcription. These studies indicate that the uncleaved P123 polyprotein can function as a component of the viral replicase capable of synthesizing both plus- and minus-strand RNAs. In contrast, cleavage-defective P34 was unable to function in RNA replication, even in complementation experiments in which minus-strand RNAs were provided by nsP4. A P34 polyprotein whose cleavage site was not altered could only function in RNA replication in the presence of an active nsP2 protease. Although nsP4, the putative RNA polymerase, was capable of synthesizing only minus-strand RNAs during coexpression with P123, the addition of only 22 upstream residues to nsP4 allowed both replication and transcription of subgenomic RNA to occur. These data show that the conserved domains of both nsP3 and the nsP4 polymerase do not need to be present in a P34 polyprotein to form a functional plus-strand replicase-transcriptase and suggest that the presence of an active nsP2 protease and a cleavable 3/4 site correlates with synthesis of all virus-specific RNA species.

Sindbis virus (SIN) is a plus-strand RNA virus belonging to the *Alphavirus* genus of the family *Togaviridae*. Replication and transcription of SIN RNAs is dependent on the viral nonstructural proteins (nsPs) which are thought to be components of the viral replicase-transcriptase complex responsible for the synthesis of three viral RNAs: the genome-length minus-strand RNA, the plus-strand 49S genomic RNA, and a 26S subgenomic mRNA (45). The nsPs are translated from the genomic RNA as two large polyproteins (P123 and P1234) which are co- and posttranslationally processed by a proteinase activity residing in the C-terminal domain of nsP2 (6, 17) to several intermediate proteins (P12, P23, and P34) with the eventual production of the four individual proteins, nsP1 to nsP4. nsP4, which is thought to be the viral polymerase (2, 13, 23), is produced by read-through of an opal termination codon located between nsP3 and nsP4 and is present in two predominant forms in SIN-infected cells, nsP4 and P34 (43–45). Whether the functional form of the polymerase is nsP4 or P34 or whether both moieties play a role in RNA replication is not known.

Processing of the nonstructural polyproteins appears to be temporally regulated such that the ratio of polyproteins and cleavage products changes over the course of infection (4). The fact that the virus has a complex regulatory scheme for production of its nsPs suggests that the precursor proteins may perform functions distinct from those of the mature cleavage products. In the accompanying report, a vaccinia virus transient expression system was used to determine which viral nsPs and/or polyproteins were necessary for the

formation of active replication complexes (29). Coexpression of the polyproteins P123 and P34 was essential for RNA replication, suggesting that the polyproteins themselves might be actual components of the SIN RNA replication complex. In addition, these studies indicated that nsP4, when expressed independently by inclusion of an extra N-terminal Met, was only capable of synthesizing minus-strand RNAs. Previous work with SIN mutants containing mutations which blocked cleavage at the 1/2 and 2/3 sites suggested that uncleaved P123 could function in replication (41). However, the polyprotein processing and RNA phenotypes of these mutants have not yet been reported. In this paper, the vaccinia virus system has been used to directly examine the ability of cleavage-defective polyproteins to function as components of replication complexes. In addition, nsP4 constructs which contained various amounts of upstream nsP3 sequence were generated to further define the sequences necessary for formation of replication complexes capable of synthesizing both plus- and minus-strand RNAs.

MATERIALS AND METHODS

Viruses and cells. Vaccinia virus (WR strain) and vTF7-3, a vaccinia virus recombinant which expresses the T7 polymerase (10), were propagated and titered on BSC40 cells as previously described (20). The growth conditions for BSC40 cells and CV-1 cells (20), as well as BHK-21 cells (29), were as previously described.

Plasmid constructions. (i) **Cleavage-defective mutants.** Expression constructs were pTM3 derivatives (34) in which various SIN cDNA sequences were positioned downstream of the bacteriophage T7 promoter and the encephalomyo-

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FIG. 1. Vaccinia virus recombinants expressing cleavage-defective polyproteins. The nonstructural coding region of the SIN genome is shown as a box with the four nsPs represented by different fill patterns. Cassettes expressed by vaccinia virus recombinants are shown below. The cleavage sites, either unaltered or mutagenized, are shown with the single-letter amino acid code. The solid diamond indicates the opal termination codon preceding nsP4; replacement of the opal termination codon with a Ser residue is indicated by SER. The nomenclature used for the expressed protein products is indicated to the left. An N prefix refers to a protein in which at least one cleavage site has been eliminated, while a P prefix refers to proteins whose cleavage sites have not been altered. An asterisk is used to indicate cleavage sites which have been blocked. The opal-to-Ser replacement is indicated by (s).

carditis virus internal ribosome entry site (29). Derivatives encoding cleavage-defective SIN polyproteins were constructed by using previously generated substitution mutations (4, 41). Standard cloning methods were used, and constructs encoding cleavage-defective polyproteins or generated by polymerase chain reaction (PCR) were verified by sequence analysis (39). A brief description of the plasmid constructions is given below, and the structures of the encoded polyproteins are shown in Fig. 1.

To generate P123 polyproteins cleavage defective at the 1/2 site (termed N1*23; see Results for a description of the nomenclature) or 2/3 site (termed N12*3), sequences encod-

ing the wild-type cleavage sites in pTM3/SIN123 (29) were replaced with the appropriate regions from pP1-539v/P2-806v (41) or pToto.1234 (4). Two plasmids encoding P123 polyproteins cleavage defective at both the 1/2 and 2/3 cleavage sites were generated. pTM3/SIN1*2*3V was constructed by using pP1-539v/P2-806v and encodes a polyprotein containing Val substitutions at the P2 positions of both the 1/2 and 2/3 cleavage sites. pTM3/SIN1*2*3E was constructed by using pToto.1234 and encodes a polyprotein containing Glu substitutions at the P2 positions of both the 1/2 and 2/3 cleavage sites.

pTM3/SIN1234(s) and pTM3/SIN34(s) were generated by replacing a *Bam*HI fragment of pTM3/SIN1234 and pTM3/SIN34 (29) with the corresponding fragment from pToto1101.SER, a derivative of pToto1101 in which the opal termination codon preceding the nsP4 sequence has been replaced by a Ser codon, but the 3/4 cleavage recognition sequence has not been altered (32). pTM3/SIN123*4(s) and pTM3/SIN3*4(s), which encode polyproteins whose cleavage at the 3/4 site is blocked by a Val substitution at the P1 position (and also contain the opal-to-Ser substitution), were generated by replacing the *Bam*HI fragment of pTM3/SIN1234 and pTM3/SIN34, respectively, with the corresponding fragment from pToto.1234. pToto.1234 and pP1-539v/P2-806v were used to generate plasmid pTM3/SIN1*2*3*4(s), which encodes a polyprotein which is cleavage defective at all three cleavage sites and contains the opal-to-Ser replacement.

Plasmid p'C481>G' encodes a polyprotein containing a Cys-to-Gly substitution at residue 481 of nsP2, which completely abolishes the proteolytic activity of nsP2 in vitro (4, 42). The *Bam*HI-*Eco*RI fragment of p'C481>G' was used to replace the corresponding region in pTM3/SIN123, thereby generating pTM3/SIN123_{C>G}.

PCR amplification of pTM3/SIN1234(s) DNA was used to generate pTM3/SIN123r, which encodes a P123 polyprotein containing 7 additional C-terminal amino acid (aa) residues and is equivalent to the P123 generated by cleavage at the 3/4 site of P1234(s).

(ii) **4n constructs.** Using pTM3/SIN34(s), we constructed several plasmids which encoded nsP4 with various lengths of upstream nsP3 sequence. Digestion of pTM3/SIN34(s) at convenient restriction sites and then fill in and religation were used to generate constructs pTM3/SINnsP4_A, pTM3/SINnsP4_B, and pTM3/SINnsP4_C, which encode proteins containing 316, 251, and 153 aa, respectively, of upstream nsP3 residues.

PCR amplification of pTM3/SIN34(s) DNA was used to generate pTM3/SINnsP4_D, which encodes a protein with only 22 aa of upstream sequence, and pTM3/SINnsP4_{Y>M}, which encodes an nsP4 derivative in which the N-terminal Tyr residue has been substituted by Met.

RNA substrate constructs. vRS.7, a vaccinia virus recombinant which encodes the cDNA for an engineered substrate RNA, has previously been described (29) and was used to express a functional substrate RNA in vivo. pJNTSCAT (21, 31) was used for in vitro transcription by SP6 polymerase to generate RNA transcripts for transfection of infected cells.

Generation of vaccinia virus recombinants. Recombinant vaccinia viruses were rescued with CV-1 cells (33) and identified by the *gpt* selection method (7). Virus was plaque purified three times, and stocks were grown and titered on BSC40 cells. Before growing large-scale virus stocks, expression of appropriate SIN-specific proteins by the vaccinia virus recombinants was verified by protein analysis as described below.

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% fetal calf serum (FCS). After 1 h at room temperature, the inoculum was removed and the cells were incubated at 37°C in modified Eagle medium containing 5% FCS. At the indicated times post infection (p.i.), the medium was removed and the cells were labeled at 37°C in modified Eagle medium 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 (32), and the lysates were immunoprecipitated with antibodies monospecific for SIN nsP1, nsP2, nsP3, or nsP4 (16, 28). The immunoprecipitated products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were treated for fluorography (25), dried, and exposed to X-ray film.

RNA transfection. The procedure for the RNA transfection of BHK-21 cells (12) was adapted from a method for DNA transfection (8) and performed as previously described (29).

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 modified Eagle medium 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 ml, and the cells were incubated at 37°C. 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, transcribed from pJNTSCAT, was introduced into cells by lipofection. The same protocol was followed for the remainder of the experiment, except that dactinomycin was not included in the media.

Substrate-specific RNAs were analyzed by a two-cycle RNase protection assay previously described by Novak and Kirkegaard (35), with minor modifications which are described in detail in the accompanying paper (29). For minus-strand analysis, 3.5 μ g of total cytoplasmic RNA was used for hybridization, while 0.5 μ g of total cytoplasmic RNA was used for plus-strand analysis. Radiolabeling of strand-specific probes and the annealing conditions used are described in the accompanying paper (29). A CAT-specific RNA probe 289 nucleotides (nt) in length was used for minus-strand analysis. After RNase digestion, the size of the protected fragment from hybridization to minus-strand RNA is 251 nt when vRS.7 is used to deliver the substrate RNA. When JNTSCAT RNA is transfected into cells, the size of the protected fragment is 266 nt. For plus-strand analysis, an RNA probe (314 nt) which included regions 5' and 3' to the start site for subgenomic RNA synthesis was used. After RNase digestion, the sizes of the fragments protected by hybridization to genomic and subgenomic RNAs are 283 and 186 nt, respectively.

RESULTS

Construction of vaccinia virus-SIN recombinants and expression of SIN cleavage-defective polyproteins. It has previously been shown in vitro that processing at SIN nonstructural polyprotein cleavage sites can be abolished by single amino acid substitutions (4, 41). These substitutions were

used to generate vaccinia virus recombinants expressing cleavage-defective derivatives of P1234, P123, and P34 in order to assess the possible function of these polyproteins in RNA replication complexes (29). Polyproteins expressed by these recombinants and the nomenclature used are shown in Fig. 1. A v prefix will be used to refer to the vaccinia virus recombinant used for infection. To distinguish between expressed polyproteins containing wild-type or altered cleavage sites, an N prefix refers to proteins in which at least one cleavage site has been eliminated, whereas a P prefix refers to a protein whose cleavage sites have not been altered. An asterisk is used to indicate the cleavage site that has been blocked. For instance, v1*23 expresses N1*23, which is the P123 polyprotein in which cleavage at the 1/2 site has been eliminated by mutagenesis. Vaccinia virus recombinants (and their encoded polyproteins) which contain the Ser replacement for the opal codon preceding nsP4 are indicated by (s).

Products expressed by these vaccinia virus recombinants were analyzed by immunoprecipitation of proteins labeled in vivo. BHK cells coinfecting with vTF7-3 and the various recombinants were labeled with [³⁵S]methionine from 3 to 8 h p.i. Cell lysates were immunoprecipitated with antibodies monospecific for SIN nsP1 to nsP4 and analyzed by SDS-PAGE. As expected, cleavage of the polyproteins could not be detected at the sites which were altered, and yet the wild-type cleavage sites were still processed (Fig. 2). For v12*3, blocking cleavage at the 2/3 site did not inhibit cleavage at the 1/2 site, and both nsP1 and N2*3 were produced. Similarly, when cleavage was abolished at the 1/2 site, this did not affect processing at the 2/3 site, as evidenced by the presence of N1*2, although nsP3 could not be detected. It has previously been suggested (29) that nsP3 generated by cleavage at the 2/3 site (i.e., from P23, P123, and P1234) is less stable than the other nsPs in the presence of vaccinia virus. This is also observed during coinfection with SIN and vaccinia virus and does not affect the efficiency of *trans* complementation of SIN RNA replication and transcription (data not shown). Substitution with either Val or Glu at both the 1/2 and 2/3 cleavage sites resulted in the accumulation of N1*2*3. It should be noted that when cleavage was blocked at the 1/2 site, a small protein of approximately 55 kDa is immunoprecipitated specifically with antibodies to nsP1. This protein was also present when P123_{C>G}, a P123 polyprotein in which the nsP2 protease has been inactivated, was used, indicating that it is probably a degradation product or results from premature translation termination (data not shown). A similar nsP1-specific product has also been observed in studies involving *ts17*, a temperature-sensitive mutant of SIN which is defective in protein processing (15).

Compared with the level of P34 observed in lysates from v34- or v1234-infected cells, the opal-to-Ser replacement in both v34(s) and v1234(s) resulted in increased levels of the P34(s) polyprotein. In addition, extracts from cells infected with v1234(s) accumulated slightly higher levels of nsP4 than extracts from v1234-infected cells. A similar increase was not observed in cells infected only with v34(s), since the nsP2 protease was not present to perform cleavage at the 3/4 site. Thus, the protein pattern for extracts from v34(s)-infected cells and v3*4(s)-infected cells was similar. For v123*4(s), which contained a mutation blocking 3/4 cleavage, nsP3 and nsP4 were not detected and there was a subsequent increase in the level of N3*4(s) compared with the level of P34(s) observed with P1234(s). Processing at the 1/2 and 2/3 sites still occurred, resulting in the production of

FIG. 2. Synthesis of cleavage-defective proteins in cells infected with recombinant vaccinia viruses. BHK-21 cells were coinfectd with vTF7-3 and a vaccinia virus recombinant as described in Materials and Methods. Cells were labeled with [³⁵S]methionine from 3 to 8 h p.i., and SDS-solubilized lysates were prepared and immunoprecipitated with antibodies monospecific for the SIN nsPs. Immunoprecipitates were analyzed by SDS-PAGE on 8% gels. The antibodies (Ab) used for immunoprecipitation are indicated directly above each lane, and the recombinant used for coinfection with vTF7-3 is indicated at the top. A lysate from mock-infected cells (mock) which had only been infected with vTF7-3 was included as a control. For immunoprecipitations involving nsP3, see Results. The positions of the SIN nsPs are indicated, as are the molecular mass standards (in kilodaltons).

nsP1 and nsP2. In v1*2*3*4(s) extracts, cleavage could not be detected at any of the cleavage sites; however, the 55-kDa nsP1-specific product described above was present.

Assays for replication-transcription by use of engineered SIN-specific substrate RNAs. To determine whether the cleavage-defective polyproteins could function as components of active replication complexes, their abilities to replicate SIN-specific substrate RNAs were assayed. These engineered substrate RNAs contain all the necessary *cis*-acting elements for the production of both minus- and plus-strand genome-size RNAs as well as a subgenomic RNA encoding chloramphenicol acetyltransferase (CAT) (29). However, the RNAs do not encode functional nsPs and thus can only be replicated by functional SIN nsPs supplied in *trans*. The substrate RNAs were delivered to infected cells either by transfection of an *in vitro*-transcribed RNA or by infection with vRS.7, a vaccinia virus recombinant which encodes the cDNA for an engineered substrate RNA under the control of a deleted form of the vaccinia virus 7.5K early/late promoter (29). When vRS.7 is used, the initial RNA transcripts are generated at a low level by the vaccinia virus DNA-dependent RNA polymerase. Dactinomycin is used to control the level of RNA synthesis by the vaccinia virus polymerase to yield an optimal signal-to-noise ratio. RNase protection assays were used to determine whether different protein combinations could replicate and transcribe the substrate RNAs, and the levels of plus- and minus-strand RNA accumulation were measured with a Betagen Betascope. vRS.7 was used to analyze accumulation of both plus- and minus-strand RNAs, and in addition, minus-strand RNAs were further analyzed by the transfection method of delivery, since a faint background band, also present in mock-infected cells, comigrated with the protected band generated when vRS.7 was used but not with the protected band generated from the transfected transcript. Thus, with the transfection method, even very low levels of minus-strand synthesis can be detected which might otherwise be obscured. Proteins were analyzed in parallel with all RNA analyses to ensure that all proteins were produced and processed as expected (data not shown).

Role of the P123 polyprotein in RNA replication. To determine whether the P123 polyprotein itself can function in RNA replication, RNA analyses were performed on coinfections involving v34(s) and recombinants expressing various cleavage-defective P123 polyproteins. v34(s) was used in these experiments rather than v34, because during coinfection with v123, higher levels of RNA accumulated with v34(s) than with v34, allowing easier detection of inefficient RNA replication (see Fig. 4). As shown in Fig. 3 and in Table 1, minus-strand RNAs accumulate to high levels during coinfection with v34(s) and either v1*2*3 or v123*3, which express polyproteins cleavage defective at the 1/2 and 2/3 cleavage sites, respectively. Coexpression of P34(s) with N1*2*3 polyproteins, in which both the 1/2 and 2/3 cleavage sites were blocked, also resulted in the synthesis of minus-strand RNAs (Fig. 3). The same results were obtained for both forms of N1*2*3 examined, one containing Val (N1*2*3V) and another containing Glu (N1*2*3E) substitutions at both cleavage sites. The level of minus-strand accumulation with N1*2*3V plus P34(s) was comparable to that observed with P123 plus P34(s), indicating that in this system, N1*2*3V could function as efficiently as P123 and its cleavage products. The level of minus-strand accumulation with N1*2*3E plus P34(s) was approximately half the level observed with P123 plus P34(s), suggesting that the Glu substitutions may have deleterious effects on the ability of the N1*2*3E polyprotein to function in RNA replication.

Plus-strand RNA analysis was performed on the same set of samples to determine whether processing of the P123 polyprotein was necessary to allow synthesis of either plus-strand genomic or subgenomic RNAs. From these results (Fig. 3 and Table 1), it appears that all cleavage-defective forms of P123 were able to function in the synthesis of plus-strand genomic RNA. Although the level of genomic RNA accumulation in most coinfections was comparable to that observed with v123 plus v34(s), slightly higher levels were observed during coexpression of the proteins N12*3 and P34(s) compared with the wild-type P123 polyprotein and P34(s) (Table 1). Similar to what was observed during minus-strand analysis, the level of genomic RNA accumula-

FIG. 3. Replication and transcription of an engineered substrate RNA by cleavage-defective 123 polyproteins. 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. Panels A and B show negative- and positive-strand analyses, respectively. Seven times as much total cytoplasmic RNA was used for minus-strand analyses than was used for positive-strand analysis (see Materials and Methods), and hence, the signals cannot be directly compared. The major protected RNA fragment for the minus-strand RNA is 251 nt. Major protected fragments for the genomic and subgenomic RNAs are 283 and 186 nt, respectively. As controls, the indicated amounts of negative (–) and positive (+)-sense RNA transcripts were subjected to the RNase protection assay. Protected fragments for controls are 266 nt for minus-strand RNA and 283 nt for plus-strand RNA. Protected fragments for controls are 266 nt for minus-strand RNA and 283 and 193 nt for plus-strand genomic and subgenomic RNA, respectively. Probe, undigested probe.

tion with N1*2*3E plus P34(s) was approximately half that observed with P123 plus P34(s). The cleavage-defective P123 polyproteins could also function in subgenomic mRNA transcription. However, when either the 1/2 or 2/3 cleavage site was altered, the molar ratio of subgenomic RNA to genomic RNA was approximately 1:1, which is significantly lower than the approximately 5:1 ratio observed in the v123 plus v34(s) infection. Nevertheless, these data indicate that the P123 polyprotein can function as a component of viral replicases synthesizing both minus-strand and plus-strand genomic and subgenomic RNA.

Role of the P34 polyprotein in RNA replication. The ability of the P34 polyprotein to function in RNA replication was examined with both P34 and P1234 cleavage-defective polyproteins. The ability of the P1234(s) and P34(s) polyproteins to function in RNA replication was first analyzed. The levels of both minus- and plus-strand RNAs produced during infection with v1234(s) or v123 plus v34(s) were higher than those obtained during infection with v1234 and v123 plus

TABLE 1. Accumulation of viral RNAs during expression of cleavage-defective 123 polyproteins

Sample	Relative RNA accumulation ^a			Sub/Gen molar ratio ^b
	Minus strand	Genomic	Subgenomic	
123 + 34(s)	1.0	1.0	1.0	4.7, 4.5
1*23 + 34(s)	0.9, 0.9	0.8, 0.7	0.2, 0.3	0.9, 1.0
12*3 + 34(s)	1.4, 1.5	3.4, 3.1	1.2, 1.1	1.0, 1.2
1*2*3V + 34(s)	1.3, 1.4	1.1, 1.1	0.2, 0.2	0.9, 0.9
1*2*3E + 34(s)	0.4, 0.3	0.5, 0.5	0.1, 0.2	0.7, 1.0

^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 as previously described (29). The accumulation of each sample was normalized with respect to the value obtained for v123 plus v34(s) and is expressed relative to the value for v123 plus v34(s), which is set at 1.0. The values given represent two independent experiments.

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

v34, respectively (Fig. 4 and Table 2), indicating that the opal-to-Ser replacement did not decrease the ability of the P34 polyprotein to function in replication and thus, P1234(s) and P34(s) could serve as controls for examining 3/4 cleavage-defective polyproteins. RNA analyses indicated that when cleavage at the 3/4 site was blocked in either the N123*4(s) or N3*4(s) polyprotein, neither plus- nor minus-strand RNAs accumulated (Fig. 4), although all of the proteins were produced as expected (data not shown). Rescue of this negative phenotype was not observed during coinfection with v4 but could be obtained in the v123 plus v3*4(s) infection by coinfection with v34 (a recombinant expressing the wild-type P34 polyprotein which can be cleaved), although the levels of RNA accumulation were very low (Fig. 4). The simplest interpretation of these results suggests that in the absence of P34 cleavage, RNA replication cannot occur. However, one cannot rule out the possibility that the lack of RNA replication was due to an effect of the Val substitution at the 3/4 site on the function of P34 itself rather than a result of P34 not being cleaved. To address this possibility, studies were performed by coinfection with v34(s) and v123_{C>G}, a recombinant expressing a P123 polyprotein in which the proteolytic activity residing in nsP2 has been abolished by a Cys-481-to-Gly substitution (42). For this experiment, the 3/4 cleavage site has not been altered but cleavage of P34 is not expected to occur in the absence of an active protease (5, 46). The P123 polyprotein also will not be cleaved, but the absence of P123 processing should not prevent RNA synthesis since it was already shown that the P123 polyprotein is capable of functioning in RNA replication. RNA analyses indicated that neither plus- nor minus-strand RNA synthesis could be detected in the v34(s) plus v123_{C>G} coinfection (Table 3).

These negative results might be explained if the Cys-to-Gly nsP2 substitution in P123_{C>G} not only blocks proteinase activity but also renders this polyprotein incompetent for RNA replication and this, rather than the absence of P34 cleavage, accounts for the lack of RNA synthesis in the v34(s) plus v123_{C>G} coinfection. To determine whether the P123_{C>G} polyprotein could function as a component of a replication complex, RNA analysis was performed on a coinfection involving v123_{C>G} and v4. We have previously shown that nsP4, whose expression does not require cleavage since it is expressed as the individual protein, is capable

FIG. 4. Replication and transcription of an engineered substrate RNA by cleavage-defective 34 polyproteins. Experimental procedures and the figure labels are as described in the legend to Fig. 3 and in Materials and Methods.

of synthesizing minus-strand RNAs when coexpressed with a wild-type P123 polyprotein (29). RNA analysis on the v123_{C>G} plus v4 coinfection indicated that minus-strand RNAs were produced (Table 3), albeit at very low levels which could not be accurately quantitated.

This suggests that P123_{C>G} can function in RNA replication, at least in the synthesis of minus-strand RNAs. Hence, the fact that RNA synthesis was not detected in the v123_{C>G} plus v34(s) coinfection further supports the idea that cleavage of P34 is necessary to form a functional replicase molecule. Alternatively, it has been hypothesized that nsP4 functions as the minus-strand replicase and P34 functions in the synthesis of plus-strand RNAs (4). If that is the case, then one might expect that synthesis of both plus- and minus-strand RNAs would be observed only when v4 is included in the v123_{C>G} plus v34(s) coinfection, as nsP4 would be necessary to generate minus-strand templates for the subsequent synthesis of plus-strand RNAs by P34. RNA analysis on the coinfection involving v123_{C>G} plus v34(s) plus v4 indicated that minus-strand RNAs were being synthesized. Surprisingly, neither plus-strand genomic nor subgenomic RNA synthesis could be detected in this infection. Thus, even in the presence of minus-strand RNAs, P34 was not able to synthesize plus-strand RNAs. To ensure that P123_{C>G} could function in the synthesis of plus-strand RNAs, a complementation experiment involving P123_{C>G}, P34(s), and an active SIN protease (P23) supplied in *trans*

TABLE 2. Accumulation of viral RNAs during expression of cleavage-defective 34 polyproteins

Sample	Relative RNA accumulation ^a			Sub/Gen molar ratio ^b
	Minus strand	Genomic	Subgenomic	
1234	1.0	1.0	1.0	4.7, 4.5
1234(s)	2.3, 2.1	1.8, 1.7	1.6, 1.5	4.5, 4.5
123*4(s)	—	—	—	
123*4(s) + 4	—	—	—	
123*4(s) + 34	—	—	—	
1*2*3*4(s)	—	—	—	
123 + 34	1.7, 1.6	1.4, 1.2	1.5, 1.4	4.6, 4.6
123 + 34(s)	2.7, 2.6	2.3, 2.5	2.6, 2.6	4.8, 4.5
123 + 3*4(s)	—	—	—	
123 + 3*4(s) + 4	—	—	—	
123 + 3*4(s) + 34	0.1, 0.1	0.3, 0.3	0.3, 0.3	4.0, 4.0
123 + 4	— ^c	—	—	

^a See footnote a to Table 1. The accumulation of each sample was normalized with respect to the value obtained for v1234 and is expressed relative to the value for v1234, which is set at 1.0. —, the sample was less than 10% of the wild-type value, and a protected fragment was not detected with long autoradiographic exposures.

^b See footnote b to Table 1.

^c Although minus-strand synthesis cannot be detected above background when the recombinant is used to deliver the substrate RNA, it is clear from the results with the transfected template RNA that minus strands are synthesized with the 123 + 4 combination (29) (Fig. 6).

TABLE 3. Accumulation of viral RNAs during expression of an inactive SIN protease

Sample	Detection of RNA ^a		
	Minus strand	Genomic	Subgenomic
123 _{C>G} + 4	+	—	—
123 _{C>G} + 4 _{Y>M}	+	—	—
123 _{C>G} + 4 _C	—	—	—
123 _{C>G} + 34(s)	—	—	—
123 _{C>G} + 34(s) + 4	+	—	—
123 _{C>G} + 34(s) + 4 _{Y>M}	+	—	—
123 _{C>G} + 34(s) + 4 _C	—	—	—
123 _{C>G} + 34(s) + 23	ND	+	+
123 _{C>G} + 4 _C + 23	ND	+	+

^a Cytoplasmic RNAs were isolated from cells infected with vTF7-3 and various vaccinia virus recombinants and either infected with vRS.7 or transfected with an RNA transcript of JNTSCAT. RNAs were subjected to an RNase protection assay, and the samples were separated on a 5% polyacrylamide-urea gel. As previously discussed (29), a yes or no answer can be obtained from the transfected substrate experiments, but it is difficult to get reproducible quantitation because of variability in RNA transfection efficiencies. In addition, the levels of RNA synthesis in the presence of 123_{C>G} were too low to be accurately quantitated, hence, the data are presented in a plus-minus table. A plus indicates a protected fragment of the correct size was detected. A minus indicates that no protected fragment corresponding to the correct size could be detected. These data are compiled from several different experiments, and the experiments have been repeated at least two times. ND, not determined.

was performed. Proteolytic cleavage at the 3/4 site, as well as the 1/2 and 2/3 sites, could be detected when P23 was coexpressed with P123_{C>G} and P34(s), although cleavage was very inefficient (data not shown). Plus-strand RNA analysis on the v123_{C>G} plus v34(s) plus v23 coinfection indicated that inefficient synthesis of plus-strand RNAs was occurring, and presumably, minus-strand RNA synthesis was also (Table 3). Therefore, the synthesis of all three viral-specific RNAs appears to correlate with cleavage of the 3/4 site.

Sequence requirements for a replicase capable of RNA replication and subgenomic mRNA transcription. The preceding experiments suggest that P34 does not function in RNA replication and would imply that nsP4 may function as the viral replicase which synthesizes both plus- and minus-strand RNAs. We have previously shown that during coinfection with v123 and v4 only minus-strand RNAs are synthesized (29). In this case, the inability of nsP4 to function as a plus-strand replicase may be a result of the extra N-terminal Met residue or may be due to the fact that nsP4 must be expressed as a polyprotein to obtain a conformation which allows it to function in the synthesis of plus-strand RNAs. To investigate the minimal upstream sequences which are necessary to produce an nsP4 moiety capable of synthesizing both plus- and minus-strand RNAs, pTM3/SIN34(s) was used to generate a series of constructs which expressed nsP4 with various lengths of upstream nsP3 sequence (Fig. 5). The number of upstream residues ranged from 549 aa, for the entire P34(s) polyprotein, to 22 aa (P4_D). In addition, we made a construct in which the expressed nsP4 product should contain a Met residue substituted for the N-terminal Tyr residue rather than an additional Met residue, as was present on the previously characterized nsP4 product which was only active for minus-strand synthesis (29). It has been previously shown that when this Tyr-to-Met substitution is present in the SIN genome, efficient RNA

FIG. 5. Vaccinia virus recombinants expressing nsP4 proteins with N-terminal extensions. The P34(s) polyprotein is shown with the two nsPs represented by different fill patterns. Replacement of the opal termination codon by a Ser residue is indicated by SER. Cassettes expressed by vaccinia virus recombinants are shown below. The nomenclature used for the expressed protein products is indicated to the left. The number of residues derived from upstream sequence is shown at the right. The nsP prefix refers to an individual protein moiety, while a P prefix indicates a polyprotein.

replication and virus production occur (30). The ability of the extended forms of nsP4 to replicate and transcribe a substrate RNA during coexpression with P123 was tested by DNA transfection and measurement of the level of CAT activity to assess subgenomic mRNA accumulation (29). P4_A to P4_D were all capable of efficient replication and subgenomic RNA transcription, as evidenced by production of CAT, whereas transfection with nsP4_{Y>M} did not result in detectable levels of CAT (data not shown).

A subset of the constructs was used to generate vaccinia virus recombinants and the RNA phenotypes analyzed during coinfection with v123. All nsP4 moieties were capable of synthesizing minus-strand RNAs during coexpression with P123 (Fig. 6). In fact, with P4_C, which has only 153 upstream residues, higher levels of minus-strand RNA accumulated than was observed with P34(s), the entire P34 polyprotein. During coexpression of P123 and P4_D, which has only 22 upstream residues, the level of minus-strand RNA accumulation was only slightly less than that observed with P123 and P34(s), while nsP4_{Y>M} produced levels of minus-strand RNA comparable to that observed with the nsP4 expressed with an additional N-terminal Met (29) (Fig. 6A). Plus-strand analyses indicated that P4_C was also capable of synthesizing both plus-strand genomic and subgenomic RNAs more efficiently than P34(s) (Fig. 6). In addition, P4_D replicated and transcribed plus-strand RNAs as efficiently as the entire P34 polyprotein when coexpressed with P123. As was observed with the nsP4 expressed with the additional Met, plus-strand RNAs were not generated with the nsP4_{Y>M} protein. The RNA phenotypes produced during coexpression of these proteins with N1*2*3V were also analyzed. Similar RNA patterns were observed in that P4_C and P4_D efficiently synthesized both plus- and minus-strand RNAs, while nsP4_{Y>M} only synthesized minus-strand RNAs (Fig. 7). These results indicate that the addition of only 22 upstream residues to nsP4 allowed the formation of a replicase which was capable of efficient genomic RNA replication and subgenomic mRNA transcription.

To determine if this upstream sequence must be expressed as part of nsP4 in order for replication to occur, studies were performed with nsP4 plus P123r, the minor species of P123 normally produced in SIN-infected cells by 3/4 cleavage of the polyprotein P1234. P123r contains seven additional res-

FIG. 6. Replication and transcription of an engineered substrate RNA by various N terminally extended forms of nsP4. Monolayers of BHK-21 cells were infected with vTF7-3 and various vaccinia virus recombinants at an MOI of 5 PFU per cell for each virus, and either transfected with a substrate RNA transcribed from pJNTSCAT (A) or coinfecting with vRS.7 (B). Cytoplasmic RNAs were isolated and used for an RNase protection assay. The recombinants used for coinfection with vTF7-3 are shown above each lane. Panels A and B show negative- and positive-strand analyses, respectively. Two gel exposures are shown in panel A to show the range of RNA accumulation with different combinations. The major protected RNA fragment for the minus-strand RNA is 266 nt. Major protected fragments for the genomic and subgenomic RNAs are 283 and 186 nt, respectively. As controls, the indicated amounts of negative (-) and positive (+)-sense RNA transcripts were subjected to the RNase protection assay. (C) RNase protection results are summarized as follows: a plus indicates that a protected fragment of the expected size was observed, and a minus indicates that a protected fragment corresponding to the correct size was not detected. Relative levels of RNA are designated by the number of pluses. This format is used since, as discussed in the accompanying paper (29), the substrate RNA was delivered by transfection and the levels of accumulated RNA cannot be accurately quantitated. The experiments have been repeated at least two times.

idues at the C terminus of P123, which are immediately upstream of nsP4, and thus contains all 22 residues shown to be important for replication. Although P123r functioned efficiently in the synthesis of both plus- and minus-strand RNAs during coexpression with P4_D, coexpression of P123r with nsP4 did not allow plus-strand RNA synthesis to occur (data not shown), suggesting that these 22 residues must be

FIG. 7. Replication and transcription of an engineered substrate RNA by cleavage-defective P123 polyproteins and various forms of nsP4. Experimental procedures and the figure labels are as described in the legend to Fig. 6 and in Materials and Methods.

expressed as part of nsP4 to allow formation of a replicase which can function in the synthesis of plus-strand RNAs.

Protein analysis indicated that some cleavage at the 3/4 site was occurring in the v123 plus v4_C coinfection, although cleavage was very inefficient. It was not clear whether cleavage at the 3/4 site was occurring with P4_D, because of the small difference in migration between the cleaved and uncleaved products. To determine whether cleavage of the extended forms of nsP4 was necessary for RNA replication to occur, complementation experiments similar to those performed with P34(s) were performed with P4_C. P4_C was used rather than P4_D so that cleavage at the 3/4 site could be clearly monitored by a change in the proteins' migration during SDS-PAGE. During coexpression of P4_C with P123_{C>G}, neither plus- nor minus-strand RNA synthesis could be detected (Table 3), although all proteins were produced as expected (data not shown). However, when v23 was included in the v123_{C>G} plus v4_C infection, inefficient cleavage could be detected at the 3/4 site, and synthesis of plus-strand RNAs occurred, albeit inefficiently (Table 3).

DISCUSSION

Using cleavage-defective proteins, we have demonstrated that the P123 polyprotein can function as a component of viral replication complexes capable of synthesizing plus- and minus-strand RNAs. Thus, it appears that complete RNA replication can occur in the absence of detectable levels of P12, P23, and the individual proteins nsP1, nsP2, and nsP3, although low levels of polyprotein processing cannot be excluded. Because the origin of the ~55-kDa product which immunoprecipitates specifically with antibody to nsP1 is unknown, the possibility that this is a functional product also cannot be ruled out. These results support previous studies showing that mutants of SIN with these same mutations blocking cleavage at the 1/2 and 2/3 sites could replicate, although minute plaques and very low virus titers were obtained (41). This mutant phenotype could, at least in part, be a consequence of depressed subgenomic mRNA accumulation, as we observed when cleavage-defective P123 polyproteins were assayed in the vaccinia virus expression system (see below). Whether the P123 polyprotein actually functions as a component of the viral replicase in SIN-infected cells is unknown. Recent studies have shown that an Asn-to-Asp substitution at residue 614 in the nsP2 protease domain is apparently lethal for virus replication. It is intriguing that this mutation increased the efficiency of processing *in vitro* such that uncleaved P123 could not be detected (42). The replication complexes isolated by Barton et al. (3) did not contain detectable P123; however, it is possible that proteolytic cleavage occurred during purification, thereby eliminating detection of P123. In addition, these complexes were isolated late in infection, whereas the P123 polyprotein may perform functions early in infection distinct from those of its cleavage products, which may also be components of active replication complexes.

The fact that P123 can function as part of the viral replicase presents a scenario similar to that observed with tobacco mosaic virus, a plant virus. Translation of the tobacco mosaic virus genomic RNA results in a 126-kDa polyprotein and a 183-kDa polyprotein read-through product which are necessary for the replication of tobacco mosaic virus RNAs (11, 22). It is generally believed that these large proteins are not proteolytically processed but rather function directly in viral RNA replication (19). The coding sequences of these proteins show a high degree of amino acid similarity with the SIN nsPs nsP1, nsP2, and nsP4, suggesting that they may perform analogous functions in RNA replication (1, 18). An important difference, however, is that the putative polymerase domain is present as part of the polyprotein in the plant system, whereas the SIN nsP4 domain cannot function as part of the P1234 polyprotein. In two other plant viruses, alfalfa mosaic virus and brome mosaic virus, two domains which exhibit a high degree of amino acid similarity with SIN nsP1 and nsP2 occur on single polypeptides which are not processed to the individual proteins (9, 24, 38). Thus, while the functional form of the SIN P123 polyprotein may be similar to that of several closely related plant viruses, processing of P123 (and P1234) during SIN replication may provide proteins which confer properties to SIN that are distinct from those of the plant viruses.

Many RNA viruses produce their proteins as precursors which are subsequently cleaved into mature protein products. Proteolytic processing often occurs via alternative pathways, providing a means for the virus to temporally regulate the production of various protein intermediates. Rather than just being precursors, these intermediate pro-

teins may perform functions in viral replication which are different from those of the individual proteins. Polyproteins may function early during viral replication, yet the subsequent processing of these polyproteins could be necessary to generate products which are also required for viral replication. Since we have shown that the SIN P123 polyprotein can function in the synthesis of all three substrate RNAs, the fact that this polyprotein is proteolytically processed during SIN infection suggests that the cleavage products may be necessary for some aspect of viral replication. One possible role of the cleaved products is in the temporal regulation of RNA synthesis. For instance, although the cleavage-defective P123 polyproteins were all capable of efficient RNA replication when coexpressed with P34(s), a decrease in the level of subgenomic mRNA transcription was observed. Although the cleavage site substitutions themselves might affect subgenomic RNA synthesis, the simplest interpretation of these data is that cleavage at both the 1/2 and 2/3 sites is necessary for efficient subgenomic mRNA synthesis. It is interesting to note that several temperature-sensitive mutants of SIN, which at the nonpermissive temperature are defective in the synthesis of subgenomic RNA, are also defective in proteolytic processing such that they accumulate P123 and P1234 at the nonpermissive temperature (14). The defects responsible for accumulation of the polyproteins, as well as the decreased subgenomic RNA synthesis, have been mapped to single mutations in nsP2, a protein which is thought to be involved in subgenomic RNA synthesis and which also contains the active site of the papain-like viral protease (6, 14, 17, 40). Whether the lack of P123 processing results in decreased subgenomic RNA synthesis or whether this is merely a by-product of the mutations in nsP2 which affect both its processing activity and its ability to function in subgenomic RNA synthesis is unknown. It may be that there are two mechanisms by which mutations in nsP2 affect subgenomic RNA synthesis: direct effects on the function of nsP2 in subgenomic synthesis, and effects on proteolytic processing such that cleavage products involved in regulating subgenomic RNA synthesis are not produced.

Alternatively, processing of the viral polyproteins may have evolved to generate specific cleavage products involved in aspects of viral replication which affect the host cell. Infection of vertebrate cells with alphaviruses, as with many viruses, sometimes results in the inhibition of cellular DNA synthesis and transcription (for a review, see reference 47). Although it has been difficult to define the components responsible for the observed inhibition, an obvious candidate for this function in alphavirus-infected cells would be nsP2, since a fraction of nsP2 localizes to the host cell nucleus during infection with either SIN (26) or Semliki Forest virus (36). What function nsP2 performs in the nucleus is unknown, but it has been postulated that nsP2 may be involved in such processes as shutting down host cell transcription or DNA replication (36).

These studies have also yielded information regarding the functional form of the SIN RNA polymerase. Although genetic studies (2, 13) and the presence of the conserved Gly-Asp-Asp sequence found in known and putative viral RNA-dependent RNA polymerases (23) have implicated nsP4 as the viral polymerase, it was recently hypothesized that nsP4 is involved in the synthesis of minus-strand RNAs while P34, rather than just serving as a precursor for nsP4, functions in the synthesis of plus-strand RNAs (4). This model was recently modified to suggest that nsP4 might also be capable of plus-strand synthesis (46). In this report, we observed that a cleavage-defective P34 polyprotein was

unable to function in RNA synthesis during coexpression with P123. Since the effect of the altered cleavage site on P34 function is unknown, a definitive interpretation of these results is not possible. However, the P34 polyprotein which does not have an altered cleavage site was not able to function in the synthesis of virus-specific RNAs in the absence of an active viral protease. This phenotype was observed even in the presence of minus-strand RNAs provided by nsP4 and would suggest that synthesis of all three viral RNA species correlates with the presence of an active nsP2 protease. The ability to synthesize RNA was probably not the result of P123 cleavage since it was shown that the cleavage-defective P123 polyprotein functioned efficiently in RNA replication. Hence, taken together, these results suggest that either cleavage at the 3/4 site is necessary to form a functional replicase and/or that the Cys residue in the proposed catalytic site of the viral protease is important for an interaction with nsP4 which is necessary for RNA replication. It should be mentioned, however, that although plus-strand RNA synthesis could be detected in the v34(s) plus v123_{C>G} plus v23 coinfection, cleavage at the 3/4 site was inefficient and the level of RNA accumulation was very low. It is possible that the Cys-to-Gly substitution renders the P123_{C>G} polyprotein defective for RNA replication. In this regard, it would be interesting to generate additional nsP2 protease-defective P123 polyproteins, with different substitutions at this site or at other residues important for catalytic function, and study their abilities to form functional replication complexes. Alternatively, the inefficient cleavage at the 3/4 site may not produce sufficient quantities of nsP4 for efficient replication, although this does not seem likely since efficient RNA replication can occur in the presence of very low levels of nsP4 in SIN-infected cells (16, 27, 32).

If cleavage at the 3/4 site is necessary to form an active replicase complex, this would imply that nsP4 can function in the synthesis of both plus- and minus-strand RNAs. However, we have previously shown that during coexpression with P123, nsP4 was only able to synthesize minus-strand RNAs (29). Comparisons involving P34(s) and a series of nsP4 proteins with various N-terminal extensions were used to determine the upstream region necessary to generate a functional replicase molecule. Interestingly, the addition of only 22 upstream residues to nsP4 allowed assembly of replicases functional for synthesis of plus- and minus-strand RNAs. Although it is clear that this region, in addition to an active protease, is necessary for replication, the function of this upstream sequence is currently not known. Studies with P123r indicate that these 22 residues cannot be supplied as part of the minor P123 species normally produced in SIN-infected cells by cleavage at the 3/4 site of P1234, but rather must be expressed as part of nsP4 to allow formation of a replicase which can function in the synthesis of plus-strand RNAs. It is possible that this upstream sequence is necessary for interaction with other proteins (either host or viral) in the replication complex and, thus, may allow the integration of nsP4 into a replication complex capable of functioning in replication and subgenomic mRNA transcription. Alternatively, these residues may affect the ability of nsP4 to interact with factors required specifically for plus-strand synthesis, such as a host or viral protein which modulates template switching, or the putative promoter sequences or structures on minus-strand RNAs which direct synthesis of plus-strand RNA molecules. These residues could also be necessary to allow correct folding of nsP4 into its active conformation or to allow interaction of nsP4 with nsP2, or nsP2-containing polyproteins, and subsequent cleavage at

the 3/4 site which may precede attainment of a conformation active for the synthesis of plus-strand RNAs. A situation similar to the latter model has been reported for poliovirus in which cleavage of 3CD is necessary to form an active replicase (37).

Studies with the cleavage-defective P123 polyproteins have also given some insight into the *in vivo* processing and proteolytic activities of several of the polyproteins. It appears that when cleavage at the 2/3 site is abolished, processing at the 1/2 site is not affected. This is in agreement with the results of previous *in vitro* studies (41). Likewise, when cleavage is abolished at the 1/2 site, cleavage still occurs at the 2/3 site. This result is in contrast to *in vitro* studies which showed that abolishing cleavage at the 1/2 site prevented processing at the 2/3 site (41) and that polyproteins containing nsP1 were unable to cleave the 2/3 site (4). The reason for these observed differences is unclear, although it may result from differences in active protease concentrations in cell-free translations versus *in vivo* transient expression assays. Studies involving P34(s) indicate that a slightly higher proportion of the P34(s) polyprotein is cleaved during coexpression with N1*2*3, which does not get processed, than with P123. This is consistent with *in vitro* studies which suggest that uncleaved P123 can efficiently cleave the 3/4 site (4).

In summary, these studies have shown that a polyprotein can function as a component of active alphavirus replication complexes. In addition, these data provide evidence that a region within 22 residues upstream of nsP4 is important for viral replication and indicate that the conserved domain of nsP3 and the nsP4 polymerase do not need to be present in a P34 polyprotein to form a functional replicase. Although this might suggest that nsP4 is the viral polymerase, biochemical approaches which allow a direct examination of active viral replicase-transcriptase complexes will ultimately be required. The constructs generated in these studies should provide useful tools for further research on the SIN nsPs using *in vitro* enzymatic studies and biochemical approaches as well as *in vivo* studies to analyze what effect the overexpression of specific proteins has on regulated processes during SIN infection.

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