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Template-Dependent Initiation of Sindbis Virus RNA Replication In Vitro

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Recent insights into the early events in Sindbis virus RNA replication suggest a requirement for either the P123 or P23 polyprotein, as well as mature nsP4, the RNA-dependent RNA polymerase, for initiation of minus-strand RNA synthesis. Based on this observation, we have succeeded in reconstituting an in vitro system for template-dependent initiation of SIN RNA replication. Extracts were isolated from cells infected with vaccinia virus recombinants expressing various SIN proteins and assayed by the addition of exogenous template RNAs. Extracts from cells expressing $P123_{C>S}$, a protease-defective P123 polyprotein, and nsP4 synthesized a genome-length minus-sense RNA product. Replicase activity was dependent upon addition of exogenous RNA and was specific for alphavirus plus-strand RNA templates. RNA synthesis was also obtained by coexpression of nsP1, $P23_{C>S}$, and nsP4. However, extracts from cells expressing nsP4 and P123, a cleavage-competent P123 polyprotein, had much less replicase activity. In addition, a P123 polyprotein containing a mutation in the nsP2 protease which increased the efficiency of processing exhibited very little, if any, replicase activity. These results provide further evidence that processing of the polyprotein inactivates the minus-strand initiation complex. Finally, RNA synthesis was detected when soluble nsP4 was added to a membrane fraction containing $P123_{C>S}$, thus providing a functional assay for purification of the nsP4 RNA polymerase.

Sindbis virus (SIN), a plus-strand RNA virus, is the prototype alphavirus (reviewed in reference 37). Upon infection of cells, the genomic RNA serves as an mRNA and is translated to produce the viral nonstructural proteins (nsPs) which are necessary for SIN replication. Viral RNA replication is initiated by the synthesis of a full-length minus-strand RNA complementary to the genomic 49S plus-strand RNA. This minus strand then serves as the preferred template for the synthesis of both 26S subgenomic mRNA and additional genomic RNA. Three to four hours postinfection, the synthesis of minus-strand RNA ceases, while the production of plus-strand genomic and subgenomic RNAs continues throughout the infectious cycle (32, 33).

The nsPs are translated as two large polyproteins (P123 and P1234), which are processed by a papain-like protease activity residing in the C-terminal domain of nsP2 (5, 9), to generate several intermediate polyproteins and the four individual nsPs (4, 8, 34). These polyproteins and cleavage intermediates, as well as the mature nsPs, are thought to function as the viral components of the SIN RNA replication machinery. Evidence suggests that there are distinct complexes responsible for synthesis of plus- and minus-strand RNAs and that during SIN infection, proteolytic processing regulates the composition and template preference of these replication complexes (18, 35).

Efforts to examine the activity of SIN replication-transcription complexes with different nsP compositions have mainly involved in vivo studies of SIN mutants (31, 35) or a vaccinia virus heterologous expression system (16–18). While these studies have been informative, a cell-free assay could be extremely useful for studying initiation events and replicase function. To date, a cell-free system capable of initiating SIN RNA replication upon addition of exogenous template RNA has not been reported. Extracts from SIN-infected cells have been shown to elongate SIN RNAs in vitro; however, direct evidence for de novo initiation was not obtained (1). The inability to obtain efficient initiation with a plus-strand template is perhaps not surprising, given recent in vivo studies that indicate a requirement for either P123 or P23 and mature nsP4 for initiation of minus-strand RNA synthesis (15, 18, 35). These studies also suggest that cleavage at the 1/2 and 2/3 sites switches the template preference of this complex to minus strands, thus promoting synthesis of plus-strand genomic and subgenomic RNAs and inactivating minus-strand RNA initiation (18). Previous attempts to isolate an in vitro system have utilized extracts from SIN-infected cells containing an active nsP2 protease and may have precluded the isolation of complexes containing unprocessed P123 or P23 polyproteins capable of efficient minus-strand initiation. In this paper, using a heterologous system to express the viral components of the SIN replicase, we have been able to isolate a crude in vitro system which can initiate and elongate SIN minus-strand RNA upon addition of SIN-specific plus-strand template RNA. This system has been used to examine the requirement for uncleaved polyproteins in the initiation of minus-strand synthesis.

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MATERIALS AND METHODS

Viruses and cells. vTF7-3, a vaccinia virus recombinant which expresses the T7 polymerase (7), was propagated as previously described (12). The growth conditions for BSC40 cells (12) and BHK-21 cells (16) have been described previously. Recombinant vaccinia viruses were generated by marker rescue on CV-1 cells (22), identified and purified by the *gpt* selection method (6), and partially purified stocks were grown in BSC40 cells (12).

Infection and preparation of the P15 fraction. BHK-21 monolayers in 150-mm-diameter tissue culture dishes were infected at a multiplicity of infection of 10 PFU of each virus per cell in 2.5 ml of phosphate-buffered saline (PBS) containing 1% fetal calf serum. After 1 h at room temperature, the inoculum was removed and the cells were incubated at 37°C in minimal essential medium containing 5% fetal calf serum. At 6 h postinfection, the P15 fraction was obtained by the isolation procedure of Barton et al. (2), with minor modifications. Cells were washed with ice-cold PBS, scraped from the dish in PBS, and collected by centrifugation at 900 × *g* for 5 min at 4°C. Cell pellets were resuspended in 1 ml of hypotonic buffer (10 mM Tris-Cl [pH 7.8], 10 mM NaCl), allowed to swell for 15 min on ice, and disrupted by Dounce homogenization. The nuclei were removed by pelleting at 900 × *g* for 5 min at 4°C, and the postnuclear supernatant was centrifuged at 15,000 × *g* for 20 min at 4°C. The P15 pellet isolated from one 150-mm-diameter dish was resuspended in 120 μl of storage buffer (10 mM Tris-Cl [pH 7.8], 10 mM NaCl, 15% glycerol) and stored in aliquots at −80°C.

In vitro replication and RNA-dependent RNA polymerase assays. Standard reaction mixtures contained 50 mM Tris-Cl (pH 7.8); 50 mM KCl; 3.5 mM $MgCl_2$; 10 mM dithiothreitol; 10 μg of dactinomycin per ml; 5 mM creatine phosphate; 25 μg of creatine phosphokinase per ml; 1 mM ATP, GTP, and UTP; 0.04 mM CTP; 1.0 mCi of [α-^{32}P]CTP per ml; 800 U of RNasin per ml, 1 μg of template RNA, and 18 μl of the P15 fraction in a total volume of 50 μl. Reaction mixtures were incubated at 30°C for 60 min and terminated by the addition of sodium dodecyl sulfate (SDS) to 2.5% and proteinase K to 100 μg/ml. After extraction with phenol and chloroform, RNA products were ethanol precipitated, denatured with glyoxal, and separated by electrophoresis through a 0.8% agarose gel. Products were visualized by autoradiography of dried gels.

RNase H digestion of RNA products. ^{32}P-labeled reaction products were denatured for 2 min at 95°C in a buffer consisting of 2 mM Tris-Cl (pH 7.5), 0.2 mM EDTA, and 80% formamide. The denatured RNAs were diluted 10-fold and annealed to 150 pmol of each oligonucleotide in a mixture of 20 mM Tris-Cl (pH 7.5), 100 mM KCl, and 2.5 μg of tRNA by slow cooling from 80°C to 30°C. Hybridized oligonucleotides corresponded to the following positions in the SIN genome sequence: 133 (oligonucleotide a: 7567 to 7585; plus sense), 4100 (oligonucleotide b; 1003 to 1019; plus sense), 4777 (671 to 686; minus sense), and 10773 (1157 to 1173; minus sense). An equal volume of digestion buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 20 mM $MgCl_2$, 20 mM dithiothreitol) was added to half of the reaction mixture, and digestion with 0.2 U of RNase H was performed for 20 min at 37°C. The RNA fragments were denatured by adding 4 volumes of deionized formamide (final concentration, 80%) and heating for 2 min at 95°C and then were separated on a 3.5% urea-polyacrylamide gel and visualized by autoradiography.

Template RNAs. pJNTSCATX, which contains a unique 3′ *Xho*I site for production of runoff transcripts, was constructed by replacing the *Bgl*I-*Nsi*I fragment of pJNTSCAT (20) with the corresponding fragment from pToto1101 (30). To generate capped RNA transcripts which served as substrate RNAs, plasmid pJNTSCATX was linearized with *Xho*I and used for in vitro transcription with SP6 polymerase as described previously (23). The resulting RNAs contained a 3′-terminal poly(A) tract of 37 residues followed by the sequence 5′-GGGAATTCCTCGA-3′.

To generate a plus-sense SIN substrate RNA with an authentic 3′ poly(A) tract, an adapter containing sites for the restriction endonucleases *Bsg*I and *Bse*RI was inserted downstream of the poly(A) sequence in pJNTSCATX. The resulting plasmid, designated pJNTSCATX(+), has the sequence 5′-CTCCTC TGCACATGGGCCG-3′ inserted between the *Eco*RI and the *Xho*I sites at positions 2948 and 2954, respectively, of pJNTSCATX [downstream of the 3′ poly(A) tract]. Linearization with *Bsg*I permits synthesis of runoff transcripts terminating with a 34-residue poly(A) tract. By using alternative runoff sites, pJNTSCATX(+) was also used to produce transcripts with truncated or extended 3′-terminal sequences, as indicated in the Results.

To produce a minus-sense substrate RNA for the SIN replicase, the plasmid pJNTSCATX(−) was made by PCR amplification of pJNTSCATX with primers 5′-CAA*CTCGAG*gtaccATTGACGGCGTAGT-3′ (oligonucleotide 265) and 5′-CAC*GAGCTC*TAATACGACTCACTATAGGGTT$_{31}$-3′ (oligonucleotide 305). The resulting PCR product was digested with *Sac*I and *Xho*I (sites shown in italics) and cloned into the corresponding sites of pJNTSCATX to create a T7 promoter (underlined) and the minus-sense strand of pJNTSCATX(+), followed by a *Kpn*I site (lowercase) for production of runoff transcripts with an authentic 3′-terminal G residue (39). Three G residues were inserted between the T7 promoter and the SIN poly(dT) sequence to allow more efficient T7 transcription. The sequence of the region synthesized by PCR was confirmed by sequencing.

Several additional plus-sense capped RNA transcripts or RNA substrates were used to examine template specificity. The predicted sizes and 3′-terminal sequences or structures of these RNAs are summarized in Table 1. Brief descriptions of these RNA substrates follow. pTET/HCV5′T7/FLΔBglII/poly(A) can be used to transcribe an internally deleted (between *Bgl*II sites at 3236 and 8938) hepatitis C virus (HCV) RNA with a 3′-terminal poly(A) tract [called HCVΔ poly(A)]. *Ase*I-digested pTET/HCV5′T7/FLΔBglII/poly(A) was used for in vitro transcription with T7 polymerase. pTET/HCV5′T7/FLΔBglII/poly(U), which is similar to pTET/HCV5′T7/FLΔBglII/poly(A), except that the 3′ nontranslated region (NTR) terminates with a 3′ poly(U) tract, was linearized with *Dra*I and transcribed with T7 RNA polymerase [called HCVΔpoly(U)]. pYF5′3′IV (29), which encodes an internally deleted yellow fever virus RNA, was linearized with *Xho*I and transcribed with SP6 polymerase. pToto1101, a full-length cDNA clone of the SIN genome (30), was linearized with *Xho*I and transcribed with SP6 polymerase. pSP6-SF4, a full-length cDNA clone of Semliki Forest virus (21), was linearized with *Spe*I and transcribed with SP6 RNA polymerase. pVR2, a plasmid encoding a Venezuelan equine encephalitis replicon (3), was linearized with *Not*I and transcribed with T7 RNA polymerase. pRR64, a full-length cDNA clone of Ross River virus (14), was linearized with *Sst*I and transcribed with T7 polymerase. pRobo102, a full-length cDNA clone of rubella virus (38), was digested with *Nsi*I, treated with T4 DNA polymerase to remove a 3′ overhanging end, and transcribed with SP6 polymerase.

In addition, brome mosaic virus (BMV) genome RNAs, RNA 1, RNA 2, and RNA 3 (Promega), were tested as substrates, as well as poly(A) in the absence or presence of oligo(U). Oligo(U)$_{15-30}$ was prepared as previously described (28).

Protein analysis. P15 and S15 material from equal numbers of cells was separated by SDS–8% polyacrylamide gel electrophoresis (PAGE), and after electrophoretic transfer, SIN-specific proteins were detected with rabbit antiserum specific for nsP1 (nsP1-2; 1/3,000 dilution), nsP2 (nsP2-2; 1/10,000 dilution), nsP3 (WU136; 1/2,000 dilution), or nsP4 (nsP4-1; 1/5,000 dilution) and standard Western blotting conditions (including successive blocking steps with 3% goat serum and 5% milk).

TABLE 1. Template specificity of the SIN P123$_{C>S}$ + nsP4 replicase in vitro

Template[a]	Length(s) in bases[b]	3′-terminal sequence or structure[c]	Functional template[d]
JNTSCATX	2,957	UUCA$_{37}$gggaauuccucga	+++
JNTSCATX(+)	2,941	UUCA$_{34}$	+++
JNTSCATX(−)	2,943	AUAUCCG	−
Poly(A)	Various	A$_n$	−
Poly(A):oligo(U)	Various	Duplex	−
HCVΔpoly(A)	3,728	GGCCUA$_{26}$uua	−
HCVΔpoly(U)	3,750	UCCCCU$_{37}$	−
YF5′3′IV	4,861	Hairpin + 3 bases	−
BMV	3,234, 2,865, 2,114, 876	tRNA-like	−
Toto1101	11,753	UUCA$_{37}$gggaauuccucga	++
SP6-SF4	11,516	UUCA$_{70}$cuag	+
VR2	7,762	UUCA$_{21}$ggguacgcggcc	+/−
RR64	11,891	UUCA$_{30}$cggaauucg	+
Robo102	9,783	UAGA$_{20}$	−

[a] All template RNAs were tested at a concentration of 20 ng/μl under standard reaction conditions. See Materials and Methods for explanation of the plasmids and nomenclature, linearization sites, and in vitro transcription conditions.

[b] Predicted or known lengths of RNAs tested.

[c] The 3′-terminal sequence or structure of each substrate RNA is indicated. For transcribed RNAs, sequences are predicted assuming complete transcription of the DNA template strand. Additional sequence after poly(A) or poly(U) tracts is shown in lowercase.

[d] Qualititative assessment of RNA transcript activity in the in vitro SIN replicase assay.

RESULTS

Template-dependent initiation of SIN RNA replication in vitro. Vaccinia virus recombinants were generated to express SIN polyproteins and nsPs thought to be essential for the initiation of minus-strand RNA synthesis. The vaccinia virus recombinant v123$_{C>S}$ expresses a P123 polyprotein in which the proteolytic activity residing in nsP2 has been abolished by a Cys-481-to-Ser substitution (36), yet P123$_{C>S}$ still functions efficiently in minus-strand RNA synthesis in vivo (18) in the presence of nsP4. To produce nsP4 in the absence of an active viral protease, vUb-nsP4 (Tyr), a recombinant expressing a ubiquitin-nsP4 fusion protein, was generated. Cellular ubiquitin C-terminal hydrolase should cleave immediately after the C-terminal Gly of ubiquitin (27) and before the first amino acid of nsP4, thus generating an nsP4 with no additional N-terminal residues. It has previously been shown that, when expressed with its authentic N-terminal Tyr residue, nsP4 gen-

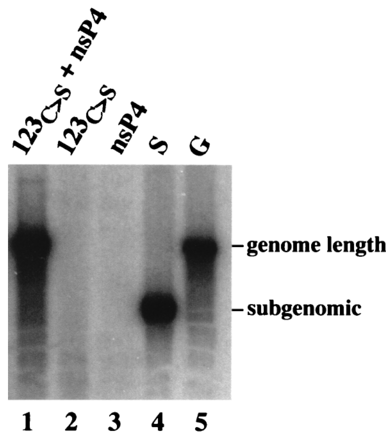

FIG. 1. In vitro synthesis of SIN RNA. P15 fractions were prepared from BHK-21 cells infected with the indicated vaccinia virus-SIN recombinants and vTF7-3 (lanes 1 to 3). Reaction mixtures were incubated with JNTSCATX template RNA at 30°C for 60 min under standard conditions. Denatured products were separated on an agarose gel and visualized by autoradiography. Lanes 4 and 5 are radiolabeled RNA transcript markers corresponding to JNTSCATX subgenomic (S) and genomic (G) RNAs, respectively.

erated from Ub-nsP4 is capable of functioning in the synthesis of both minus- and plus-strand RNAs in vivo (18).

To assay for in vitro polymerase activity, the P15 membrane fraction was isolated from cells coinfected with vTF7-3 and vaccinia virus recombinants expressing $P123_{C>S}$ and Ub-nsP4 (Tyr). JNTSCATX, a SIN-specific plus-sense substrate RNA, was used as an exogenous template. This substrate RNA contains all of the necessary *cis*-acting elements for the synthesis of minus-strand, plus-strand, and subgenomic RNAs, but requires functional SIN nsPs supplied in *trans* for replication and transcription. Addition of JNTSCATX RNA to P15 fractions isolated from cells expressing both $P123_{C>S}$ and nsP4 resulted in the synthesis of a genome-length RNA product (Fig. 1). In the absence of added JNTSCATX RNA or in reaction mixtures containing P15 extracts from cells infected only with vTF7-3, no corresponding product was observed (data not shown; see below). In addition, RNA synthesis was not detected in extracts from cells expressing only $P123_{C>S}$ or nsP4 (Fig. 1), indicating a requirement for both P123 and nsP4. Synthesis of discrete subgenomic RNAs was not detected when P15 extracts containing $P123_{C>S}$ and nsP4 were used. It is possible that the level of RNase in the P15 extract prevents detection of single-stranded subgenomic RNA (see below). Alternatively, this may just reflect the phenotype of the $P123_{C>S}$:nsP4 replicase, since it has been shown in vivo that a complex consisting of uncleaved P123 and nsP4 is very inefficient at transcription of subgenomic mRNA (18).

RNA accumulation. A time course of RNA accumulation in vitro was determined by using extracts containing $P123_{C>S}$ and nsP4. It appears that initiation and elongation by the SIN replicase are rapid, because full-length RNA products are observed within 3 to 5 min after the addition of exogenous template (Fig. 2A and data not shown). Full-length products continued to accumulate for approximately 30 min, reaching a plateau between 30 to 60 min (Fig. 2A and data not shown). This suggests either that the enzyme complex is not very stable or that a component of the reaction mixture, possibly the template RNA, has become limiting. With regard to RNA template stability, observable loss of single-stranded template RNA and intact rRNAs was observed after incubation at 30°C (Fig. 2B).

Characterization of the products synthesized in vitro. To characterize the products of the in vitro assay, the $P123_{C>S}$ plus nsP4 ($P123_{C>S}$ + nsP4) reaction products were treated with RNase A, an enzyme specific for single-stranded nucleic acid, and analyzed on either denaturing or nondenaturing RNA gels. Untreated control samples were analyzed in parallel. Similar patterns of labeled RNA were observed for control samples and samples treated with RNase (data not shown). Under these RNase digestion conditions, single-stranded JNTSCATX RNA and rRNA were completely degraded. Furthermore, the predominant RNA species recovered after denaturation of RNase-treated material comigrated with JNTSCATX RNA. These results indicate that the stable full-length or near-full-length reaction products were primarily in the form of double-stranded RNA. Although we did not detect single-stranded genome-length RNA products, it is possible that the level of ribonuclease present in the P15 fraction would have precluded their detection even had they been synthesized.

To determine the polarity of the RNA synthesized in vitro, four oligonucleotides were annealed to the labelled reaction products, and stretches of RNA present in DNA-RNA hybrids were digested with RNase H. Plus- and minus-sense RNAs transcribed in vitro from plasmids pJNTSCATX(+) and pJNTSCATX(−), respectively, were used as controls. All oligonucleotides used were found to promote specific digestion by RNase H only when the complementary strand was present (data not shown). When the reaction product was hybridized with two different oligonucleotides complementary to minus-sense SIN RNA, most of the genome-length RNA was digested to produce minus-strand-specific fragments of the expected sizes (Fig. 3). Plus-sense RNA species were not detected when the product was annealed with two different oligonucleotides complementary to plus-sense SIN RNA (data not shown). These results suggest that the majority of the newly synthesized product was minus-sense SIN RNA. Similar results were obtained by RNase T_1 analysis (data not shown).

Specificity of the SIN replicase. The template specificity of the in vitro replicase was examined with a number of substrate RNAs. Since it is likely that the 3′ end of the template RNA

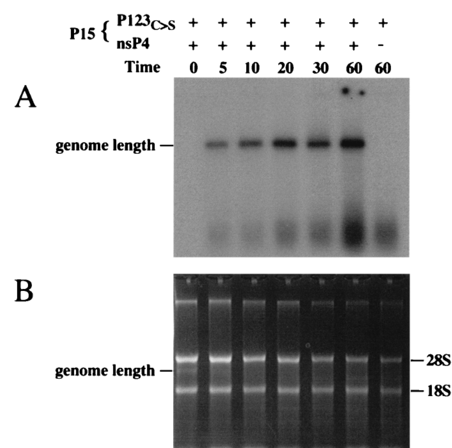

FIG. 2. Time course of RNA accumulation. P15 fractions were prepared from BHK-21 cells infected with vTF7-3 and vaccinia virus recombinants expressing $P123_{C>S}$ and Ub-nsP4 (Tyr) (nsP4) as indicated above each lane. Reactions with JNTSCATX template RNA were incubated at 30°C for the indicated times (in minutes) under standard conditions. Denatured (A) or nondenatured (B) RNAs were separated on agarose gels and visualized by autoradiography (A) or by staining with ethidium bromide (B). In panels A and B, the position of genome-length JNTSCATX RNA is indicated. In panel B, 28S and 18S rRNAs are indicated.

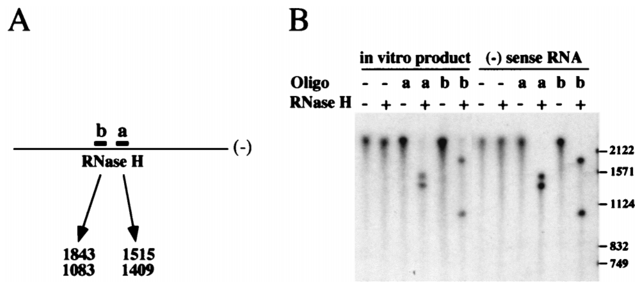

FIG. 3. RNase H analysis of in vitro-synthesized RNA. (A) Diagram indicating where the minus-strand-specific primers anneal to the JNTSCATX template and the lengths (in bases) of the fragments expected after complete digestion with RNase H. (B) P15 fractions were prepared from BHK-21 cells infected with vaccinia virus recombinants expressing $P123_{C>S}$, Ub-nsP4 (Tyr), and vTF7-3. Reaction mixtures were incubated with JNTSCATX template RNA under standard conditions, and the products were denatured, annealed to specific primers, and digested with RNase H (+) or incubated without added enzyme (−). The resulting fragments were separated on a 3.5% polyacrylamide–urea gel and visualized by autoradiography. As a control, a minus-sense transcript from pJNTSCATX(−) was analyzed in parallel. To the right, the positions of various radiolabeled RNA size markers are indicated (bases).

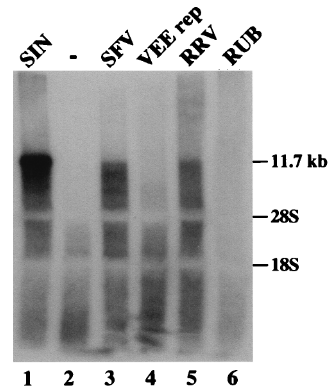

FIG. 4. In vitro replication of full-length SIN RNA and heterologous *Togaviridae* templates by the SIN replicase. In vitro transcripts from the full-length or subgenomic replicon cDNAs were used as templates in P15 extracts containing $P123_{C>S}$ and Ub-nsP4 (Tyr). Reaction mixtures were incubated at 30°C for 60 min under standard conditions. The products were denatured, separated on an agarose gel, and visualized by autoradiography. −, no added template RNA; SFV, Semliki Forest virus; VEE rep, Venezuelan equine encephalitis virus subgenomic RNA replicon; RRV, Ross River virus; RUB, rubella virus. The positions of genome-length SIN RNA (11.7 kb) and 28S and 18S rRNAs are indicated to the right.

plays a role in minus-strand initiation, substrates with various 3′ termini were tested as templates. The 3′-terminal sequence or structure of each substrate and the results are summarized in Table 1. Transcripts generated from pJNTSCATX(+), which are SIN-specific plus-sense RNAs that terminate with an authentic poly(A) tract, functioned as a template for the SIN replicase. In contrast, RNA synthesis was not observed when a minus-sense transcript from pJNTSCATX(−) was used as a template. This may indicate that the SIN replicase cannot initiate replication on a minus-sense RNA, or it may just reflect the phenotype of the $P123_{C>S}$:nsP4 replicase, since it has been shown in vivo that this complex is very inefficient at plus-strand synthesis (18). Because the SIN genome contains a 3′ poly(A) tract, poly(A), in the presence or absence of oligo(U), was tested in the in vitro reaction. If poly(A) was active as a template, reaction products should be heterogeneous, producing a smear of species when analyzed by gel electrophoresis. No such smear was observed with these templates, although interpretation of this experiment is difficult, since various levels of smaller labeled products are often seen in the in vitro reactions (independent of SIN-specific replicase components). HCV RNA transcripts (3.7 kb) terminating with either poly(A) or poly(U) were also unable to direct the synthesis of genome-length RNA products. To examine whether the SIN replicase could utilize 3′ secondary structures to initiate RNA synthesis, a transcript containing a predicted 3′-terminal hairpin was generated from a deleted form of the yellow fever virus genome (YF5′3′IV). When tested in the in vitro reaction, RNA synthesis was not detected with this template. Likewise, synthesis of discrete products was not observed with BMV RNAs 1, 2, and 3, which contain 3′ tRNA-like structures.

Besides the JNTSCATX RNA substrate, we also examined the activity of the $P123_{C>S}$ + nsP4 P15 replicase on longer RNAs, including full-length SIN RNA and heterologous RNAs of other members of the *Alphavirus* genus. Addition of full-length SIN RNA yielded efficient synthesis of an 11.7-kb RNA product (Fig. 4, lane 1). For Semliki Forest virus and Ross River virus, some full-length RNA products were also observed, but most appeared to be incompletely transcribed or partially degraded (Fig. 4, lanes 2 and 4). Neither Venezuelan equine encephalitis virus replicon RNA lacking the structural region (~7 kb; Fig. 4, lane 4) nor rubella virus RNA (Fig. 4, lane 6), which lacks the conserved 3′-terminal RNA element thought to be important in initiation of alphavirus minus-strand RNA synthesis (37), was efficiently utilized by this SIN replicase preparation.

Authentic SIN genome RNA terminates with poly(A). To examine the importance of the SIN 3′ end for template activity in the in vitro assay, pJNTSCATX(+) was linearized with different restriction enzymes, and these templates were used to produce RNAs with different 3′ termini (Fig. 5A). RNA synthesized from *Bsg*I-linearized pJNTSCATX(+) should terminate with poly(A) and was efficiently utilized by the in vitro SIN replicase (Fig. 5B, lane 3). No effect, and perhaps even enhanced activity, was observed when a short (32-base) sequence was added 3′ to the poly(A) (Fig. 5B, lane 4). Longer 3′ (184- or 859-base) extensions of nonviral sequences past the

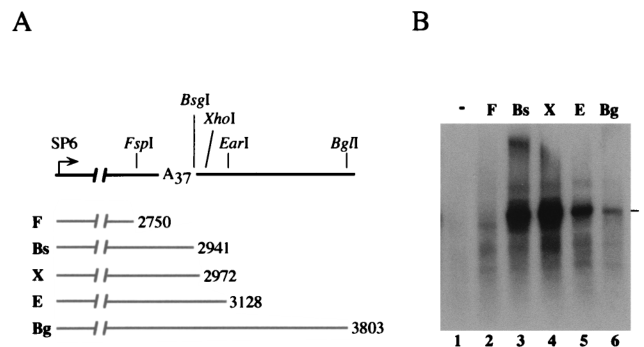

FIG. 5. In vitro replication of SIN RNA templates with different 3′ termini. (A) Diagram representing the JNTSCATX(+) cDNA with the positions of the various runoff sites indicated. Shown below are the predicted RNA substrates generated after transcription. Letters to the left are abbreviations of the restriction enzymes used to linearize pJNTSCATX(+); F, *Fsp*I; Bs, *Bsg*I; X, *Xho*I; E, *Ear*I; Bg, *Bgl*I. The numbers to the right indicate the transcript length (in bases). (B) As diagrammed in panel A, plus-sense SIN RNAs with different 3′ termini were tested in the in vitro replication assay. After pJNTSCATX(+) was linearized with various restriction enzymes, 3′-protruding ends were removed with Klenow enzyme, and RNA templates were synthesized with SP6 polymerase. In vitro replication assays were performed with P15 extracts containing $P123_{C>S}$ and Ub-nsP4 (Tyr) under standard conditions, and the products were analyzed by gel electrophoresis. The size of genome-length RNA produced from pJNTSCATX(+) linearized with *Bsg*I is indicated by the bar to the right.

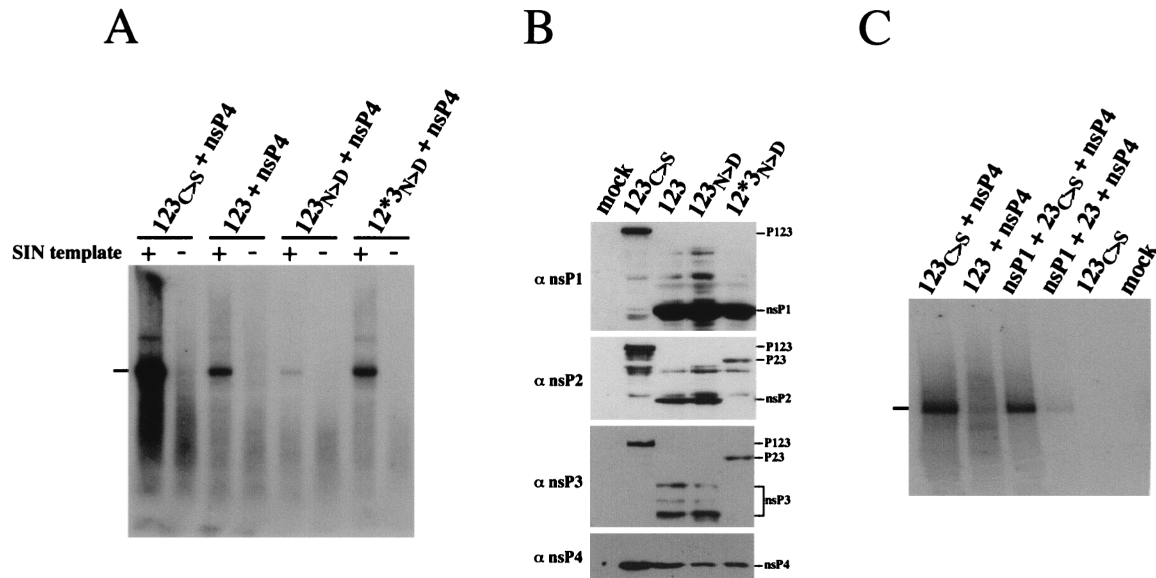

FIG. 6. Comparison of the in vitro activities of replication complexes containing cleaved and uncleaved SIN nonstructural components. (A) P15 fractions were prepared from BHK-21 cells infected with vTF7-3, vUb-nsP4 (Tyr), and vaccinia virus-SIN recombinants expressing the indicated SIN polyproteins. In vitro assays were performed with SIN-specific JNTSCATX RNA substrate (+) or a heterologous HCVΔpoly(A) RNA substrate (−) under standard conditions. Denatured products were separated on an agarose gel and visualized by autoradiography. (B) SIN-specific proteins in the P15 fraction were separated on an SDS–8% polyacrylamide gel and detected by Western blotting with antisera (α) specific for nsP1, nsP2, nsP3, or nsP4. A lysate from mock-infected cells (mock) which had only been infected with vTF7-3 was included as a control. The positions of the SIN polyproteins and nsPs are indicated. Note that SIN nsP3 migrates as multiple species because of differential phosphorylation (19). (C) Experimental procedures were as described for panel A. In panels A and C, the bar on the left indicates the position of genome-length JNTSCATX RNA.

poly(A) reduced RNA synthesis significantly (Fig. 5B, lanes 5 and 6). As expected from previous work (11, 13), transcripts terminating in the SIN 3′ noncoding region upstream of the 3′-terminal 19-base conserved sequence and poly(A) tract were no longer substrates for the replicase (Fig. 5B, lane 2). Since the mobilities of the predominant products appear to be identical and correspond in size to the RNA produced from the *Bsg*I-linearized template (Fig. 5B, lanes 3 to 6), it is likely that initiation of viral RNA synthesis occurred at or near the poly(A) in all templates. For the RNA substrates with detectably longer 3′ extensions, this suggests that minus-strand initiation can occur at an internal site on the template. Alternatively, plus-strand templates resulting from premature termination in the poly(A) during SP6 transcription or from partial degradation in the in vitro assay may be preferentially utilized for minus-strand initiation.

Test of the regulatory model by using the in vitro assay. As described previously (18, 35, 37), our current model of SIN RNA replication is that uncleaved P123 or the P23 cleavage intermediate is required for the initiation of minus-strand synthesis. Processing at the 1/2 and 2/3 sites causes a conformational change, resulting in replication complexes inefficient at initiating minus-strand synthesis. To examine the requirement for polyproteins in minus-strand synthesis, extracts from cells infected with vaccinia virus recombinants expressing nsP4 and various P123 derivatives were tested for their ability to synthesize RNA in vitro. One extract contained P123, a P123 polyprotein that has an active nsP2 protease and is capable of being processed. A second extract contained $P123_{N>D}$, a P123 polyprotein which contains an Asn-614-to-Asp substitution in nsP2 that enhances the efficiency of in vitro proteolytic processing such that uncleaved P123 cannot be detected (36). A third extract contained $P12*3_{N>D}$, a polyprotein that contains the nsP2 hyperprocessing mutation but that cannot be processed at the 2/3 cleavage site because of a single amino acid substitution that blocks cleavage at this site (the asterisk indicates a cleavage site that cannot be processed). As shown in Fig. 6A, extracts containing nsP4 and uncleaved $P123_{C>S}$ exhibited efficient replicase activity upon addition of exogenous template, whereas RNA synthesis was greatly decreased in extracts containing cleavage-competent P123 and nsP4. RNA synthesis was barely detectable in extracts from cells infected with recombinants expressing nsP4 and $P123_{N>D}$, the hyperprocessing mutant. However, in extracts containing $P12*3_{N>D}$, in which the hyperprocessing mutation was present in a polyprotein that could not be cleaved at the 2/3 site, replicase activity was restored. This suggests that the inefficient RNA synthesis with $P123_{N>D}$ was not a result of the Asn-614-to-Asp substitution, but rather resulted from the lack of any uncleaved P123 or P23 polyprotein.

The SIN-specific proteins in the P15 extracts were examined by Western blot analysis with antibodies specific for nsP1, nsP2, nsP3, and nsP4. As expected for the $P123_{C>S}$ + nsP4 replicase preparation, high levels of the $P123_{C>S}$ polyprotein were present, and processing at the 1/2 and 2/3 sites was not observed (Fig. 6B). During expression of P123 and $P123_{N>D}$, cleavage products nsP1, nsP2, and nsP3 were produced, but polyproteins P123, P12, and P23 were not detected. For $P12*3_{N>D}$, efficient processing at the 1/2 site resulted in the production of P2*3 and nsP1, but the P12*3 polyprotein could not be detected.

Recent studies have also shown that a complex consisting of nsP1, $P23_{C>S}$, and nsP4 can function in RNA replication in vivo (15). This complex efficiently synthesizes minus-strand and plus-strand genomic RNAs, but is inefficient at transcription of subgenomic mRNA. To examine whether this complex could function in the in vitro assay, the vaccinia virus recombinant vUb-$P23_{C>S}$ was utilized. This recombinant expresses a P23 polyprotein that contains no additional N-terminal residues and is not processed because of an inactive nsP2 protease (15).

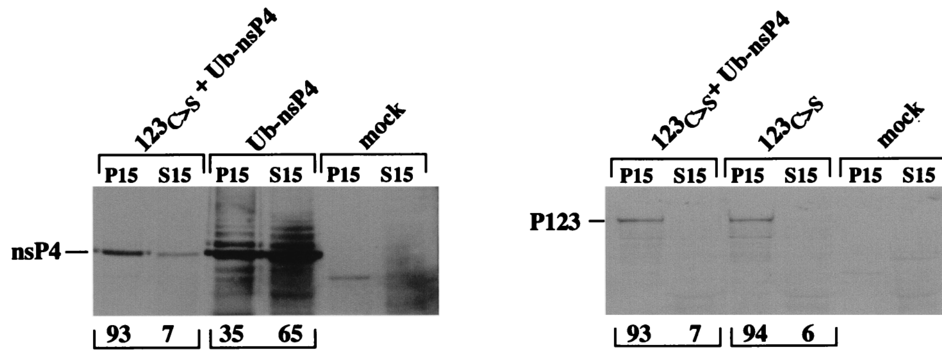

FIG. 7. Distribution of SIN nsPs in P15 and S15 fractions. P15 and S15 fractions were prepared from BHK-21 cells infected with the indicated vaccinia virus-SIN recombinants and vTF7-3. Material from equal numbers of cells was separated by SDS-PAGE on 8% polyacrylamide gels, and SIN-specific proteins were detected by Western blotting and probing with antiserum specific for nsP2 or nsP4. A lysate from cells which had only been infected with vTF7-3 (mock) was included as a control. The levels of SIN-specific protein were quantified with a Betagen Betascope, and the percentage of a particular SIN nsP present in the P15 and S15 fractions is indicated at the bottom.

Upon addition of template RNA, P15 extracts containing nsP1, $P23_{C>S}$, and nsP4 synthesized genome-length RNA products at levels somewhat lower than that observed with $P123_{C>S}$ and nsP4, and synthesis of subgenomic RNA was not detected (Fig. 6C). In contrast, very little, if any, RNA synthesis was observed with extracts containing nsP1, nsP4, and a cleavage-competent P23 (Fig. 6C). Western blot analysis was performed with these extracts with antibodies monospecific for nsP1 and nsP2. As expected, the $P123_{C>S}$ + nsP4 and the nsP1 + $P23_{C>S}$ + nsP4 extracts contained uncleaved P123 and P23, respectively. Extracts from cells infected with vaccinia virus recombinants expressing P123 + nsP4 or nsP1 + P23 + nsP4 contained predominantly nsP1 and nsP2, with very little uncleaved P123 or P23 precursor present (data not shown). These results are consistent with previous in vivo observations suggesting that initiation of minus-strand RNA synthesis requires a complex containing uncleaved P123 or P23 (15, 18).

Soluble polymerase activity. Although alphavirus replication complexes are membrane associated, a solubilized complex capable of elongating SIN RNAs has been isolated from SIN-infected cells (2). However, we were unable to solubilize the in vitro minus-strand initiation complex by using deoxycholate or Triton X-100 (data not shown). As another approach to obtaining a soluble replicase, we examined whether the SIN-specific proteins in the S15 fraction, rather than the P15 fraction, could function in RNA replication. Upon addition of exogenous template, polymerase activity could not be detected in S15 fractions from cells infected with vaccinia virus recombinants expressing $P123_{C>S}$ and nsP4, even when mixed with a P15 fraction from mock-infected cells (data not shown). Protein analysis of these fractions revealed that when coexpressed, only low levels of $P123_{C>S}$ and nsP4 were present in the S15 fraction (Fig. 7), which could account for the lack of replicase activity. Additional mixing experiments were performed to determine whether SIN proteins in the S15 fraction could complement a P15 fraction lacking one of the replicase components essential for initiation of RNA synthesis. Addition of S15 extract containing $P123_{C>S}$ to a P15 fraction from cells expressing nsP4 resulted in no detectable RNA synthesis (data not shown); however, very little $P123_{C>S}$ was present in the S15 fraction (Fig. 7). In contrast, high levels of nsP4 are found in the S15 fraction when nsP4 is expressed in the absence of other SIN nsPs (Fig. 7). Addition of S15 extract containing nsP4 to a P15 fraction from cells expressing $P123_{C>S}$ resulted in a functional replicase that synthesized genome-length RNA (Fig. 8). This result shows that an active minus-strand initiation complex can be formed by adding soluble polymerase to the $P123_{C>S}$ P15 fraction and provides an assay for purification of the SIN nsP4 polymerase.

DISCUSSION

In this paper, we report the first isolation of an alphavirus replicase capable of initiating RNA synthesis in vitro. Replicase activity was dependent on the addition of exogenous template, required both P123 and nsP4 (or nsP1, P23, and nsP4), and was specific for SIN plus-strand RNA templates. Elements key to the development of this template-dependent assay were (i) the utilization of a heterologous system for expression of functional SIN polyproteins and nsPs and (ii) the observation that uncleaved polyprotein P123 or P23 and mature nsP4 were necessary for efficient minus-strand initiation. Based on previous studies with a vaccinia virus transient expression system, along with other in vivo studies (31, 35), a model for the composition of SIN replicase complexes and the temporal regulation of SIN minus- and plus-strand RNAs has been pro-

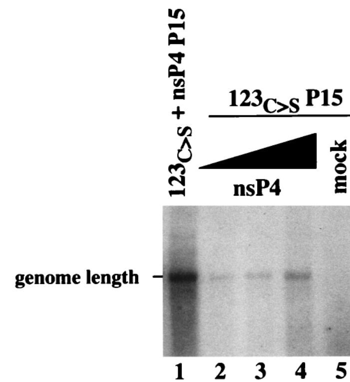

FIG. 8. Extracts containing soluble nsP4 allow initiation of SIN RNA replication in vitro. P15 fractions were prepared from BHK-21 cells infected with vaccinia virus-SIN recombinant $v123_{C>S}$ and vTF7-3 (lanes 2 to 5). The S15 fraction was prepared from cells coinfected with vTF7-3 and vUb-nsP4 (Tyr) or from cells infected with vTF7-3 alone (mock). In vitro reaction mixtures containing the P15 fraction and increasing amounts of nsP4-containing (5, 10, and 18 μl in lanes 2, 3, and 4, respectively) or mock (18 μl in lane 5) S15 fraction were incubated under standard conditions with JNTSCATX template RNA, and the denatured products were separated on an agarose gel. A P15 fraction from cells coinfected with vTF7-3, $v123_{C>S}$, and vUb-nsP4 (Tyr) was assayed in parallel as a positive control (lane 1).

posed (18, 35). This model predicts that uncleaved P123 or the P23 cleavage intermediate and nsP4 are required for initiation of minus-strand synthesis. This complex is also capable of plus-strand synthesis, although initiation of genomic and subgenomic synthesis is inefficient. Processing at the 1/2 and 2/3 sites converts the complex into a replicase containing nsP1, nsP2, nsP3, and nsP4, causing a conformational change that switches the template preference of this complex from plus to minus strands, leading to more efficient plus-strand synthesis. Cleavage at the 1/2 and 2/3 sites inactivates the ability of the complex to function efficiently in minus-strand initiation and results in the shutoff of minus-strand synthesis. These recent insights into the early events in SIN RNA replication could explain the difficulty that we, and others, have had in developing an in vitro assay for initiation of SIN RNA synthesis, since these attempts have most likely used extracts from SIN-infected cells which contain an active protease. The vaccinia virus system provided a means of expressing the required polyprotein intermediates, by using mutations which inactivate the SIN nsP2 protease, while still allowing expression of the nsP4 polymerase. In addition, this system allows one to express the replicase components in the absence of a replication-competent SIN RNA, eliminating the need to remove any endogenous RNA which might prevent the replicase from accepting an exogenous template.

A number of RNA template-dependent polymerase preparations have been isolated from cells infected with eukaryotic positive-strand RNA viruses; however, very few systems have been able to carry out complete RNA replication (10, 40). The reaction product in the SIN in vitro system consisted primarily of genome-length minus-strand RNA. Even when the template RNA was introduced in the presence of Lipofectin (Gibco-BRL), which has been shown to allow plus-strand RNA synthesis to occur for flock house virus (41), there was no significant increase in the amount of minus-strand RNA produced, nor was there a stimulation of plus-strand synthesis (data not shown). The inability of the $P123_{C>S}$:nsP4 replicase to catalyze complete replication of RNA could result because the preparation lacks a cofactor necessary for plus-strand synthesis. It has recently been demonstrated that the La protein in mosquito cells and chicken cells binds to the 3′ end of SIN minus-strand RNA with high affinity (24, 25), and it has been postulated that this protein may be involved in the initiation of plus-strand RNA synthesis. Alternatively, inefficient plus-strand RNA synthesis in vitro would mimic the in vivo phenotype of this replicase (18) and may simply reflect the nature of a replication complex consisting of uncleaved P123 and nsP4. Processing of this complex in vitro may be necessary to convert it to a plus-strand replicase which is active enough to allow detection of plus-strand genomic and subgenomic RNA synthesis. It will be of interest to examine the polarity of RNA products synthesized with the nsP1 + P23 + nsP4 replicase, since this complex can synthesize plus-strand RNA in vivo (15).

The SIN replicase exhibited specificity in that it did not utilize a number of RNA templates tested as substrates. The highest efficiency was observed for the SIN genome RNA, but the SIN replicase could also utilize Semliki Forest virus and Ross River virus RNAs. This is likely due to the conserved sequence elements that are found at the 5′ and 3′ ends of all alphavirus genomes that are believed to function as *cis* elements for initiation of RNA synthesis. It has been postulated that the 19-base element adjacent to the poly(A) tail and sequences in the 5′ NTR or nsP coding region, in particular the 51-nucleotide element near the 5′ end, may play a role in minus-strand initiation (37). Analyses of artificial 5′ or 3′ NTR chimeras between SIN and Ross River virus have demonstrated that Ross River virus 3′ NTR elements can be readily utilized by the SIN replicase in vivo, but that the 5′ NTR is less interchangeable (14). The ability of the SIN replicase to utilize, albeit less efficiently, heterologous alphavirus templates in vitro is consistent with these in vivo data. In addition, the deletion which eliminated the 19-base element and poly(A) abolished the ability of the SIN template RNA to function in the in vitro assay, which also fits with in vivo genetic analyses of the 3′ NTR (11, 13).

To test the requirement for P123 or P23 in minus-strand initiation, we compared the abilities of P15 fractions containing nsP4 and either uncleaved polyproteins or cleavage-competent proteins to function in RNA synthesis. RNA synthesis was observed in extracts from cells infected with recombinants expressing a cleavage-competent polyprotein, although at very low levels. It is possible that this activity results from uncleaved polypeptide present in these extracts in amounts that are below the level of detection. Alternatively, the cleavage products may be able to form a replicase that functions inefficiently in minus-strand RNA synthesis under these conditions. The level of RNA synthesis was even lower, however, in the $P123_{N>D}$ extract than in extracts in which P123 was expressed. The Asn-614-to-Asp mutation could be suppressed by inclusion of a mutation blocking processing at the 2/3 site ($P12{*}3_{N>D}$), suggesting the reduced activity observed for $P123_{N>D}$ was due to the hyperprocessing phenotype conferred by this mutation, rather than some other effect of the substitution on replicase efficiency. Unprocessed P23 ($P23_{C>S}$) was also shown to function more efficiently than cleavage-competent P23 as a minus-strand replicase component. Consistent with the model based on in vivo data, these in vitro results suggest that an uncleaved polypeptide is required for efficient initiation of SIN RNA synthesis and that processing at the 2/3 site is the critical cleavage inactivating the minus-strand initiation complex.

Although the minus-strand initiation complex could not be solubilized by detergent treatment, soluble nsP4, which could form an active minus-strand initiation-elongation complex when added to a P15 extract containing $P123_{C>S}$, was obtained from the S15 fraction. This provides a useful assay for purification and characterization of the nsP4 RNA-dependent RNA polymerase. The inability to obtain a functional replicase with other P15-S15 combinations may be due to the low concentration of nsPs in certain fractions or may reflect a requirement for membrane association of specific nsPs. A possible candidate for this might be nsP1, since it has recently been shown to be membrane associated (26). Alternatively, given the complex regulatory scheme SIN uses for production of its nsPs, the timing of the appearance of the various nsPs may be crucial for promoting specific interactions necessary for active complex formation.

The template-dependent initiation-elongation assay presented in this paper provides a system with which to study the mechanisms involved in the initiation of SIN RNA replication and a means of analyzing the roles of the virus- and host-encoded subunits in RNA replication and transcription. In particular, this system should be useful for examining the processes involved in conversion of a SIN minus-strand replicase to one which efficiently synthesizes plus-strand RNAs and allow the identification of the components and RNA-protein interactions involved in minus-strand initiation.

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