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# Genesis of Sindbis Virus by In Vivo Recombination of Nonreplicative RNA Precursors

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**Genetically engineered RNA transcripts coding for various Sindbis virus (SIN) genes were used to study structure and sequence requirements of RNA recombination in BHK cells. Three different groups of RNA transcripts were made: (i) RNAs which retain the ability to replicate and which carry sequences coding for either viral polymerase or viral structural proteins; (ii) RNAs which lack the complete 3′ end of the SIN genome and thus are incapable of replicating; and (iii) RNAs which lack the complete 5′ end of the SIN genome and also are incapable of replicating. BHK cells were transfected with specific combinations of these precursor RNAs, and virus production and RNA synthetic abilities of the released virus were determined. We demonstrate in vivo generation of infectious SIN by fusion of (i) replicative RNAs to nonreplicative RNAs and (ii) two nonreplicative RNA precursors. Both homologous and nonhomologous types of recombinations were observed. In the homologous type of recombination, a 694-nucleotide overlap at the crossover region of the first pair of precursors resulted in the addition of an A residue converting the UAG stop codon of nonstructural protein P4 to a UAA stop codon. In the nonhomologous type of recombination, the crossover sites contained deletion of up to 76 nucleotides from one of the precursors and complete preservation of junction sequence from the other precursor. This is also the first report that a cytoplasmic RNA virus can be generated from biologically nonreplicative RNA precursors. These results have implications for initiation of viral RNA synthesis and recombination between RNA viral genomes in general. We favor template switching as a mechanism for the fusion events described here and suggest inclusion of polymerase scanning of diverse nonreplicative RNAs as an inherent feature of the copy choice model of RNA recombination. Very importantly, the facile nature of RNA recombination occurring between nonreplicative RNA precursors should speed up the production and analysis of targeted mutants of SIN and possibly other RNA viruses.**

Sindbis virus (SIN) is one of the best-studied alphaviruses at the molecular level (54, 59). SIN is transmitted by mosquitoes to animals and humans and is one of the least pathogenic member of the *Alphavirus* genus of the *Togaviridae* family (59). The genome of SIN is composed of an 11.7-kb single-stranded positive-sense RNA (55, 59). The protein-coding potential of the SIN genome is divided mainly into two large open reading frames (ORFs). The first ORF, which encompasses a 7.6-kb 5′ region of the genome, is directly translated into viral polymerase proteins (59). The second ORF, which spans the remaining 3′ region of the genome, is actually translated from a 26S subgenomic RNA, giving rise to viral structural (S) proteins (48, 59). Conserved *cis*-acting sequences located at the 5′ and 3′ ends of the viral RNA are known to be necessary and sufficient to confer replication competency to the viral genome or its derivatives (26, 38, 39, 59). Genetically engineered cDNA copies of the SIN genome and its derivatives have been extensively used to study the *cis*- and *trans*-acting functions of the SIN genome (6, 12, 26, 30, 38, 43, 47, 61). Recently, Weiss and Schlesinger (61) demonstrated the occurrence of RNA recombination between SIN replicons. By making use of replication-competent engineered SIN RNAs, they showed that near-wild-type SIN genomes can be produced from two smaller replicons carrying each of the ORFs (61).

RNA recombination among RNA viruses is a well-studied biological process (11, 16, 17, 27, 29, 57). Animal (3, 4, 10, 14, 16, 24, 29, 40, 65), plant (2, 7, 8, 9, 19, 35, 36, 37, 60, 62), and bacterial (5, 40) viruses are known to undergo RNA recombination. Although RNA recombination is characterized by production of new and chimeric RNA viruses by physical exchange of genetic information, the precise mechanism and forces governing RNA recombination are not completely understood (9, 20, 27, 36, 37, 49, 61). On the basis of sequence homology at the crossover sites, both homologous (20, 24, 25, 32, 36, 40) and nonhomologous (7, 10, 34, 61, 62, 65) types of recombinations were attributed to viral RNA recombinations. Mechanistically, RNA recombinations could be due to breakage and reunion of template RNAs (7, 27) or due to template switching of polymerase during replicative RNA synthesis (23, 24, 27). In the absence of any well-characterized enzyme capable of RNA cleavage, ligation, and editing activities, little attention was given to the breakage and reunion model of RNA recombination (7, 27). On the other hand, the copy choice mechanism, which invokes the template switching of viral polymerase during replicative RNA synthesis, is well accepted, since all known RNA recombinations are known to be associated with replicative RNA synthesis (20, 22, 24, 27, 37, 40, 57, 61).

If the copy choice mechanism is important for RNA recombination, what are the intracellular forces which make the polymerase jump from one template to another? On the basis of generation of defective interfering (DI) and rearranged genomes (41, 53) during high-multiplicity passages of viruses, and on the basis of the association between active replication and RNA recombination (24, 27), we hypothesized that replicative polymerase cycling (RPC) and/or high intracellular concentrations of cognate template RNAs and polymerase proteins may play a crucial role in facilitating template switching.

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We define RPC as a process in which large numbers of polymerase proteins cycle through template RNAs, generating abundant levels of nascent RNAs. It is conceivable that RPC is associated with polymerase crowding on template RNAs, which might lead to frequent slippage and reinitiation at alternate sites or templates. As indicated by others (9, 27, 37), the presence of RNA structures on template RNAs might also influence detachment and reattachment of polymerases. Events such as mixed hybrid formation between limiting negative-sense RNAs and excess positive-sense RNAs (or vice versa) may also result in formation of branched RNAs, leading to expulsion and rerouting of polymerase travel during RNA synthesis. In this study, using engineered SIN RNAs as precursors, we demonstrate that RNA recombination occurs in the SIN system in the absence of RNA replication and hence under low concentrations of cognate precursor RNAs. We postulate multiple modes of template switching by RNA polymerases and suggest that template switching may function as an effective mechanism to generate new replicative RNAs from nonreplicative RNAs in a protein- and RNA-rich milieu.

## MATERIALS AND METHODS

**Plasmids.** Toto 1002, derivative of Toto 1000 (18, 47), contains an *Xba*I site at nucleotide (nt) 7613. Toto 1000 is one of the parental SIN plasmids which contain a functional SIN cDNA. An SP6 promoter located at the 5′ end of the SIN genome drives the synthesis of infectious transcripts. This plasmid contains an *Sst*I site at the 3′ end of the SIN genomic sequence which is used to linearize the plasmid for in vitro transcription. Cells transfected with RNA derived from this plasmid produce large amounts of infectious virus (47).

TRCAT, a well-characterized SIN vector which lacks all structural proteins (63), contains the complete nonstructural (NS) region, subgenomic promoter, and reporter chloramphenicol acetyltransferase (CAT) gene and all *cis*-acting sequences essential for replication. TRCAT contains an *Sst*I site for linearization. Cells transfected with RNA derived from this plasmid express TRCAT genomic RNA, a subgenomic RNA coding for CAT, and high levels of CAT protein. No virus particles are produced from TRCAT RNA-transfected cells.

TRCAT 62 is a replication-competent derivative of TRCAT which lacks all of the 3′ nontranslated region except for the last 62 nt at the 3′ end of TRCAT (18). Like TRCAT, TRCAT 62 expresses CAT protein in transfected cells and lacks the ability to produce any virus particles.

GNTR-Lac, a derivative of TRCAT in which the CAT gene is replaced by a *Bgl*II-*Sac*II fragment of the La Crosse virus S genome (44), lacks approximately 90 nt of 3′ noncoding region of the SIN genome adjacent to and downstream of the S-protein termination codon. In vitro transcripts produced from this plasmid are replication competent, but no virus particles are produced from RNA-transfected cells.

SINrep, one of the latest SIN vectors originally named SINrep 5 (6), lacks all of the S-protein-coding region but contains all *cis*-acting motifs essential for replication and subgenomic RNA synthesis. An *Xho*I site located at the 3′ end of the viral sequence is used to linearize the plasmid. No infectious particles are produced from cells transfected with SINrep RNA.

DI-S is a derivative of DIF20e (43) which contains an SIN DI genome. This plasmid was constructed by cloning the *Xba*I-*Sst*I fragment of Toto 1002 which contains SIN S region into DIF20e at the same sites. This process resulted in deletion of the CAT gene of DIF20e and introduction of the S region downstream of the subgenomic promoter located in DIF20e. RNA transcripts produced from this vector are replication competent and express 26S subgenomic RNA when supplemented with NS proteins in *trans* from another source.

DHBBS (DHBB 5′ SIN) is a plasmid containing SIN DI sequences, a SIN subgenomic promoter, and SIN S-region-coding sequences (6). The RNA transcripts produced from this plasmid are capable of only poor amplification by NS proteins provided in *trans* as a result of mutational alterations in the 5′ end of the vector RNA. In spite of its diminished replication ability, this RNA adequately complements SIN structural protein requirements through synthesis of 26S subgenomic RNA (6).

GNS-2, a plasmid carrying the complete SIN NS region, was made by cloning the NS region from Toto 1002 as an *Sst*I-*Xba*I fragment into plasmid pGem 3 (Promega) at the same sites. The *Xba*I site was used to linearize the plasmid for in vitro transcription. The RNA transcripts produced from this plasmid lacks the complete S region, including the 3′ motifs of the SIN genome. Hence, the GNS-2 transcripts are nonreplicative. However, the GNS-2 transcripts were shown to provide adequate amounts of NS proteins through direct translation in transfected cells (this work).

G26S-2 was made by cloning the *Hpa*I-*Sst*I fragment containing all of the SIN S region of Toto 1002 at the *Hin*cII-*Sst*I location of pGem 3. Positive-sense runoff transcripts were made by linearizing the plasmid at the *Sst*I site. Negative-sense transcripts were made by linearizing at the *Hin*dIII site and using the T7 promoter. The absence of the SIN 5′ motif makes the RNA nonreplicative, although initiation of negative-strand synthesis is indicated by the present work.

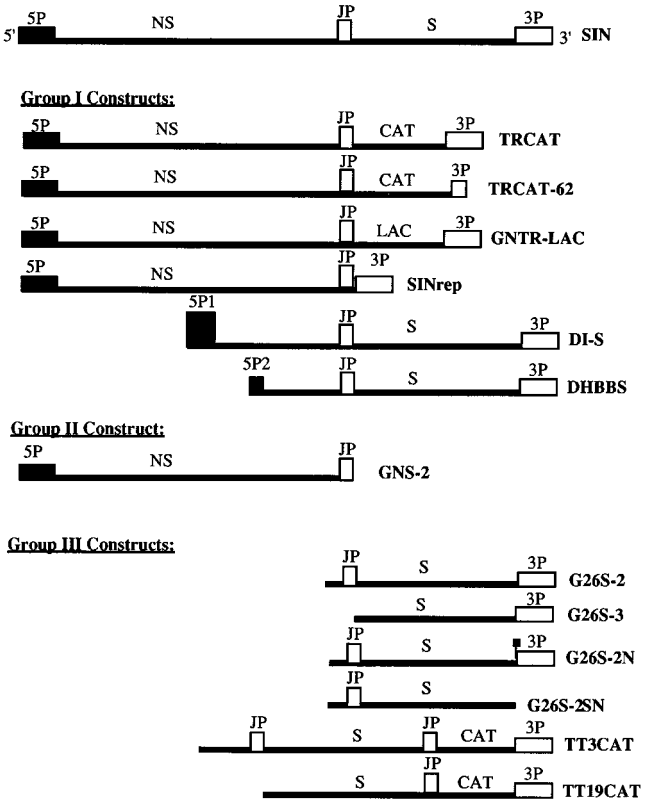


FIG. 1. Structures of engineered SIN RNAs. All RNAs are depicted in plus-sense orientation. Common *cis*-acting sequences are identified uniformly: 5P, 5′ promoter or conserved sequence; 3P, 3′ promoter or conserved sequence; NS, sequences coding for NS proteins which constitute the RNA polymerase complex; S, sequences coding for viral S proteins; JP, junction promoter responsible for 26S subgenomic RNA synthesis; 5P1, 5′ region of SIN DI DNA which contains a tRNA sequence; 5P2, 5′ region of the SIN genome lacking full promoter activity.

G26S-2N was made by digesting plasmid G26S-2 with *Nsi*I and by end filling with T4 DNA polymerase in the presence of 1 mM deoxynucleoside triphosphates. The inactivated *Nsi*I site is indicated as a small stalk in Fig. 1. The inactivated Nsi site served as a reporter in the fusion products.

G26S-2SN was made by deleting the *Nsi*I-*Sst*I fragment of G26S-2 which carries the 3P region (SIN 3′ motif) and religating the plasmid. Runoff transcripts were made by linearizing the plasmid at the *Eco*RI site and using SP6 RNA polymerase. The RNA transcripts produced from this plasmid are nonreplicative since they carry neither 5′ nor 3′ motifs of the SIN genome, but the RNA transcripts code for functional S proteins of SIN.

G26S-3 was made by cloning the S-region-containing *Xba*I-*Sst*I fragment of Toto 1002 at the corresponding sites of pGem 3. Positive-sense transcripts were made by linearizing the plasmid at the *Sst*I site and using SP6 RNA polymerase. Negative-sense transcripts were made by linearizing at the *Xba*I site and using T7 RNA polymerase. Since the RNA transcripts lack the 5′ region of SIN, they are incapable of replication, although initiation of negative-strand synthesis from the 3′ end is indicated by the present work.

TT3CAT carries sequences coding for two subgenomic promoters of SIN. The first subgenomic promoter drives the synthesis of the S region, and the second promoter drives the synthesis of the CAT reporter. The plasmid does not carry any SIN 5′ motifs but does carry functional 3′ motifs. The plasmid was made by cloning the *Xba*I-*Sst*I region carrying the two subgenomic promoters, S region, and CAT region of plasmid TT20c (43) into the same sites of plasmid G26S-2. In vitro positive-sense transcripts are made from this plasmid by linearizing the plasmid at the *Sst*I site. Since the transcripts lack 5′ motifs of SIN, they are nonreplicative, but initiation and elongation of RNA synthesis from the template RNA were indicated by this work.

As described for TT3CAT, TT19CAT was made by cloning the *Xba*I-*Sst*I region of TT20c into G26S-3. In vitro transcripts are made by linearizing the

plasmid at the *Sst*I site and using SP6 RNA polymerase. Other properties of TT19CAT transcripts are identical to those of TT3CAT transcripts.

GNS-2BG was constructed by digesting GNS-2 with *Bgl*II, end filling the linear DNA with T4 DNA polymerase in the presence of 1 mM deoxynucleoside triphosphate, and recircularizing the plasmid. This procedure resulted in introduction of four additional bases in the coding region of the NS polyprotein, leading to translational frameshift and premature termination. RNA transcripts produced from this construct fail to support recombination in vivo.

G3S was constructed by cloning the 2.2-kb region of the SIN S-protein-coding region obtained by *Stu*I digestion of Toto 1002 into the *Hin*cII site of pGem 3. Negative-sense RNA transcripts which hybridize to both the SIN genome and SIN 26S subgenomic RNA are produced from T7 promoter-mediated RNA transcription of G3S.

GNS-1EE carries a 1,050-nt-long SIN NS region flanked by SP6 and T7 promoters. Plasmid GNS-1EE was obtained by cloning the 1050-nt *Eco*RI-*Hpa*I fragment (nt 5870 to 6919 of Toto 1002) in the *Hin*cII and *Eco*RI sites of pGem 3. Linearization of the plasmid with *Hin*dIII and transcription with T7 RNA polymerase gives rise to negative sense RNA transcripts which hybridize to positive-sense RNAs carrying the NS region of SIN.

**In vitro synthesis of RNA transcripts.** Five micrograms of each plasmid was digested with the appropriate restriction enzyme and directly ethanol precipitated. One-third of the DNA was used for in vitro transcription by SP6 RNA polymerase in the presence of a fourfold excess of cap structure as described previously (43, 44). Either [$^3$H]UTP or [$\alpha$-$^{32}$P]GTP was used as a tracer to quantitate the amount of RNA made. After a 1-h incubation, the template DNA was removed by DNase I digestion, and RNA was purified by phenol-chloroform extraction and ethanol precipitation. The amount of RNA made was quantitated by trichloroacetic acid precipitation. Five percent of the RNA samples was denatured by glyoxal (43) and analyzed on a 1.25% agarose gel.

**Cells, viruses, and infection.** BHK-21 cells and Vero cells were maintained in minimal essential medium containing 10% fetal bovine serum. Standard (Toto 1101 or Toto 1002) and recombinant SIN stocks were prepared from BHK cells and titered on BHK cells or Vero cells, using a standard plaque assay (61). For virus infections, BHK cells grown in 35-mm-diameter petri plates were infected with 5 to 10 PFU of virus inoculum per cell diluted in serum-free medium and incubated at 37°C for the desired times.

**Transfection of BHK cells with RNA precursors.** Approximately 70 to 90% confluent BHK cells grown in 35-mm-diameter petri plates (Sarstedt) were washed twice with phosphate-buffered saline (PBS) containing no calcium and magnesium and transfected with 200 to 300 μl of transfection mixture. The transfection mixture consisted of 20 μg of Lipofectin (Gibco-BRL) or Transfectace, 50 to 200 ng of each in vitro-transcribed RNA, and PBS (43). The transfection mixture was allowed to incubate in ice for 5 to 10 min, layered onto PBS-washed cells, and continually rocked for 20 to 30 min. At the end of the transfection procedure, the transfection mixture was removed from the cells, carefully washed with medium, and layered with 2 ml of medium containing 10% fetal bovine serum. Using TRCAT as a reporter molecule, we routinely observed transfection of up to 0.5 to 2% of cells per ng of RNA. Electroporation of RNA into BHK cells gave comparable efficiencies. The transfected cells were incubated for 2 to 3 days at 37°C and constantly monitored for development of cytopathic effects. Culture supernatants were recovered from all transfected cells and titered on Vero cells, using a standard plaque assay.

**In vivo labeling and analysis of RNA products.** Five microliters of the culture supernatant (5 to 15 PFU of virus per cell) derived from transfected cells was diluted to 0.2 ml with serum-free medium and allowed to infect BHK cells grown in 35-mm-diameter petri plates (43). At the end of 1 h, 0.6 ml of medium containing 5 μg of dactinomycin per ml was added to the plates. Twenty minutes later, 50 μCi of [$^3$H]uridine was added to each plate, and the infection was continued at 37°C for 6 h. At the end of infection, cells were harvested and cytoplasmic RNA was isolated (43, 44). Approximately 5 μg of RNA was denatured with glyoxal, analyzed on a 1.25% agarose gel, and then fluorographed.

**Reverse transcription-PCR amplification of cytoplasmic RNA and sequencing.** Total cytoplasmic RNA was isolated from infected cells at 6 h postinfection (p.i.) (43), treated with RNase-free DNase (Gibco-BRL), and further purified by proteinase K digestion and phenol extraction. One-fifth of the cytoplasmic RNA was used for reverse transcription and PCR amplification. In brief, the first-strand synthesis involved annealing of 6 pmol of a negative-sense primer (nt 8049 to 8070 of Toto 1000) with 5 μg of cytoplasmic RNA in 0.3 M NaCl and subsequent extension using murine leukemia virus reverse transcriptase. In addition to RNA and primer, the reaction mixture consisted of 50 mm Tris-HCl (pH 8.3), 70 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.35 mM deoxynucleoside triphosphates, and 400 U of murine leukemia virus in a total volume of 30 μl. The reaction mixture was incubated for 1 h at 37°C and subsequently for 20 min at 42°C. At the end of the reaction, an aliquot of the reaction mixture was diluted to 10-fold and used directly for PCR amplification. The PCR mixture consisted of 2 to 5% of the cDNA products, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 100 μg of gelatin per ml, 5 pmol each of negative-sense primer and positive-sense primer (nt 7248 to 7270), 5 U of *Taq* polymerase, and 350 μM deoxynucleoside triphosphates in a volume of 50 μl. After 20 cycles of PCR amplification, the reaction mixture was removed and stored. Ten percent of the PCR products was analyzed on a gel, and the 820-nt band corresponding to the amplified region was isolated from the gel. The isolated DNA fragment was sequenced by Sanger's dideoxy method, using an internal primer (nt 7650 to 7666). Identical results were obtained when gel-purified fused RNA was used for reverse transcription-PCR amplification and sequencing.

**CAT assay.** The CAT assay was performed as described earlier (43, 63). Infected cell monolayers were harvested at indicated times by scraping into 2 ml of PBS. The cells were pelleted at 4,000 rpm in a refrigerated microcentrifuge and suspended in 500 μl of lysis buffer (0.25 M Tris-HCl [pH 8]). After three cycles of freeze-thawing, the cell debris was removed by centrifugation at 15,000 rpm in a refrigerated microcentrifuge, and the cytoplasmic supernatant was recovered. The reaction mixture consisted of 20 to 60 μg of cytoplasmic proteins, 0.1 μCi of [$^{14}$C]chloramphenicol (50 mCi/mmol), 5 μl of 20 mM acetyl coenzyme A, and 90 μl of 0.25 M Tris-HCl (pH 8.0). The contents were incubated for 1 h at 37°C and extracted with ethyl acetate, and the products were analyzed by silica gel-based thin-layer chromatography. The percent conversion of chloramphenicol to acetyl chloramphenicol was determined by cutting each of the radioactive spots and determining the radiactivity by liquid scintillation.

**Northern (RNA) analysis.** Glyoxylated RNA samples were separated on a 1.25% agarose gel for 2 to 3 h. At the end of electrophoretic separation, the gel was soaked in 20 mM NaOH for 10 min and extensively washed with running water. The RNA samples were electrophoretically transferred to a Zetaprobe (Bio-Rad) nylon membrane, using 10 mM Tris–20 mM sodium acetate (pH 7.8) as the transfer buffer for 6 h. Prehybridization and hybridization with RNA probes were done as described earlier (44), using 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 100 μg of RNA per ml, and appropriate RNA probe labeled with [$\alpha$-$^{32}$P]GTP ($2 \times 10^6$ dpm/10 ml).

## RESULTS

**Design of substrate RNAs and assay for recombination.** To study recombination between RNA precursors, three groups (Fig. 1) of RNAs were made by in vitro transcription of plasmid DNAs carrying an SP6 or T7 phage promoter. The group I RNAs contain both the 5′ and 3′ ends of SIN, and hence they are replication competent. Thus, group I RNAs are amplifiable in vivo by viral polymerase. The group II RNA is exemplified by GNS-2. GNS-2 RNA lacks the 3′ one-third of the SIN genome and hence cannot support any RNA synthesis. The majority of group III RNAs contain an authentic 3′ end of the SIN genome, but all of them lack the 5′ two-thirds of the SIN genome. None of individual RNAs in groups I, II, and III can produce live virus when transfected into cells, since they all lack either NS- or S-protein-coding sequences. Since both 5′ and 3′ sequences are essential for replication of SIN-related RNAs, none of the group II and group III RNAs can replicate to produce progeny RNAs.

The experimental strategy involved multiple steps: (i) introduction of one or more of the in vitro made RNA precursors into BHK cells to generate infectious RNA recombinants; (ii) recovery of culture supernatants containing recombinant viruses and titration; and (iii) study of gene expression from recombinant viruses. Although none of the RNAs belonging to group I, II, and III can produce infectious virus when transfected alone, there is a possibility that live virus can be produced when two functionally complementing RNAs are cotransfected. Live virus can be produced either by physical recombination (24, 61) between RNA precursors or by simple complementation between virus proteins (12). To demonstrate recombination between transfected RNA precursors, we resorted to electrophoretic analysis of RNA products (6, 61). The presence of RNA products of ca. 12 kb or more containing both NS and S sequences was used as a confirmatory test for RNA recombination.

**Strong SIN replicons expressing NS proteins fuse with weak replicons expressing S proteins.** Almost all of the RNA recombination studies reported in the literature made use of live virus or RNA substrates which carried both the 5′ and 3′ ends of viral sequences. Since the copy choice mechanism is widely accepted by RNA virologists, and since the copy choice mechanism predicts a requirement for replication competency of substrate RNAs, perhaps it was logical that all of the studies

TABLE 1. Cytopathic effects and release of virus particles from transfected cells[a]

| Expt no. | Transfected RNA-1 | Transfected RNA-2 | Cytopathic effect | | Virus titer (PFU/ml) | Plaque size (%) |
|---|---|---|---|---|---|---|
| | | | 24 h | 48 h | | |
| 1 | TRCAT | None | None | None | 0 | NA |
| 2 | TRCAT 62 | None | None | None | 0 | NA |
| 3 | GNTR-Lac | None | None | None | 0 | NA |
| 4 | SINrep | None | None | None | 0 | NA |
| 5 | DI-S | None | None | None | 0 | NA |
| 6 | DHBBS | None | None | None | 0 | NA |
| 7 | GNS-2 | None | None | None | 0 | NA |
| 8 | G26S-2 | None | None | None | 0 | NA |
| 9 | G26S-3 | None | None | None | 0 | NA |
| 10 | G26S-2N | None | None | None | 0 | NA |
| 11 | G26S-2SN | None | None | None | 0 | NA |
| 12 | TT3CAT | None | None | None | 0 | NA |
| 13 | TT19CAT | None | None | None | 0 | NA |
| 14 | Toto 1002 | None | ++++ | ++++ | $3.1 \times 10^8$ | 100 |
| 15 | TRCAT | DI-S | + | ++++ | $4.1 \times 10^7$ | 40–80 |
| 16 | TRCAT 62 | DI-S | + | ++++ | $2.2 \times 10^7$ | 20–60 |
| 17 | TRCAT | DHBBS | ++ | ++++ | $2.1 \times 10^7$ | 40–60 |
| 18 | TRCAT 62 | DHBBS | ++ | ++++ | $5.6 \times 10^7$ | 20–60 |
| 19 | SINrep | DHBBS | ++ | ++++ | $6.5 \times 10^6$ | 60–100 |
| 20 | TRCAT | G26S-2 | + | ++++ | $8.3 \times 10^6$ | 20–80 |
| 21 | TRCAT | G26S-2 ($-^b$) | None | None | 0 | NA |
| 22 | TRCAT | G26S-2SN | None | None | 0 | NA |
| 23 | TRCAT 62 | G26S-2 | ++ | ++++ | $6.0 \times 10^6$ | 40–60 |
| 24 | TRCAT 62 | G26S-2N | ++ | ++++ | $2.8 \times 10^6$ | 40–60 |
| 25 | TRCAT 62 | G26S-3 | + | +++ | $9.1 \times 10^5$ | 40–60 |
| 26 | GNTR-Lac | G26S-2N | ++ | ++++ | $8.5 \times 10^6$ | 60–100 |
| 27 | SINrep | G26S-2 | ++ | ++++ | $2.1 \times 10^7$ | 40–60 |
| 28 | GNS-2 | DHBBS | ++ | +++ | $5.0 \times 10^6$ | 60–100 |
| 29 | GNS-2 | G26S-2 | ++ | ++++ | $9.3 \times 10^6$ | 60–80 |
| 30 | GNS-2 | G26S-3 | + | +++ | $5.4 \times 10^5$ | 20–60 |
| 31 | GNS-2 | G26S-2 (−) | None | None | 0 | NA |
| 32 | GNS-2 | G26S-3 (−) | None | None | 0 | NA |
| 33 | GNS-2 | G26S-2SN | None | None | 0 | NA |
| 34 | GNS-2 | TT3CAT | ++ | ++++ | $7.2 \times 10^6$ | 20–60 |
| 35 | GNS-2 | TT19CAT | + | +++ | $6.0 \times 10^5$ | 20–60 |

[a] Structures of individual RNAs are described in Fig. 1. Cells were periodically monitored to determine necrotic cytopathology. No significant cytopathology was observed in cells which were transfected with any one of the nonreplicative RNAs or RNAs which do not code for structural proteins in comparison with untransfected cells. +, low cytopathology; ++, moderate cytopathology; +++, severe cytopathology but still adherent; ++++, complete cell death and detachment. The plaque size is an approximate estimate with respect to that formed by Toto 1002. NA, not applicable.

[b] −, negative strand.

made use of replicative viral derivatives. In fact, the first reported study of the recombination between SIN RNAs made use of highly replicative RNAs (61). Initially, we tested the ability of strong SIN replicons to recombine with weak replicons. The strong replicons TRCAT, TRCAT 62, and SINrep code for polymerase proteins. The weak replicon DHBBS codes for structural proteins (Fig. 1). When DHBBS was used in conjunction with either one of the strong replicons in transfection experiments, infectious viruses were produced (Table 1, experiments 17 to 19). Cells infected with each of the recombinant viruses expressed two genomic and two subgenomic RNAs (Fig. 2a). The faster-moving genomic RNA and subgenomic RNAs are derived from the corresponding strong replicons (compare Fig. 2a and 3b). The slowly moving genomic RNA which hybridizes to both NS- and S-specific probes of SIN (data not shown) is the recombinant RNA product. The slowly moving subgenomic RNA corresponds to 26S subgenomic RNA expressed from the recombinant RNA. These results are reminiscent of the original studies by Weiss and Schlesinger (61). As demonstrated by Bredenbeek et al. (6), the parental DHBBS replicon was not packaged from transfected cells. Unlike earlier work reported by Bredenbeek et al. (6), DHBBS clearly recombined with all of the replicons used in these studies. These results indicated that recombination does occur when weakly replicating RNA substrates are used as substrates. Interestingly, only very low levels of fusion products were demonstrable in cells transfected with precursor RNAs, by [$^3$H]uridine labeling of intracellular RNAs, or by Northern analysis of cytoplasmic RNAs using riboprobes (42).

**Strong replicons expressing NS proteins fuse with a nonreplicating RNA.** The weak replicon DHBBS has a defect in the 5′ *cis*-acting sequence (6). In spite of this defect, DHBBS was shown to serve as a substrate for RNA recombination. To further test the role of the 5′ *cis*-acting sequence in recombination, cells were transfected with one of the strong replicons such as TRCAT, TRCAT 62, SINrep, or GNTR-Lac and one of the nonreplicative RNAs (G26S-2 or G26S-3). These nonreplicative RNAs lack the 5′ end of the SIN genome but carry genes coding for SIN S proteins (Fig. 1). As shown in Table 1 (experiments 20 to 27), infectious viruses were clearly released from cells which were transfected with two complementing RNAs. Infection of cells with these viruses resulted in the intracellular accumulation of both 49S- and 26S-like RNAs characteristic of SIN infection (Fig. 2a and b). These results indicated that 5′ end of at least one of the substrate RNAs was dispensable for RNA fusion. G26S-2N RNA, which had an

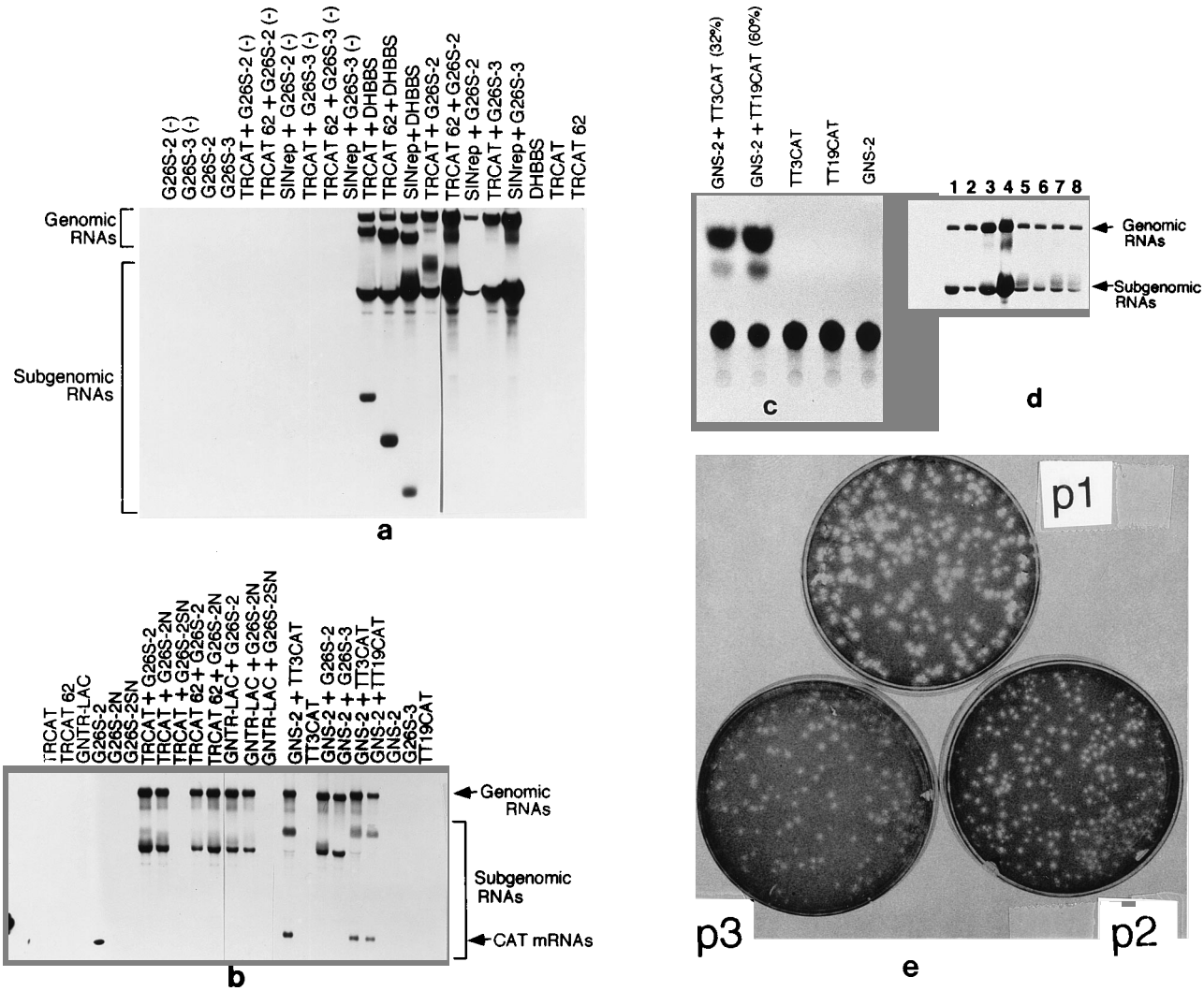


FIG. 2. (a) Recombination between replicative and nonreplicative RNAs. BHK cells were transfected with template RNAs, and culture supernatant containing released virus was recovered. Fresh BHK cells were infected with 5 to 10 PFU of recovered virus per cell and labeled with uridine, and 5 to 7 μg of RNA was separated on a 1.25% gel and fluorographed. The names of RNA substrates used to produce primary virus stocks are indicated above the lanes. Some lanes carry two genomic RNAs and two subgenomic RNAs. In these cases, the slowly moving genomic RNA corresponds to the fusion product. The faster-migrating genomic RNA corresponds to the strong replicon used in transfection. Likewise, the slowly moving subgenomic RNA in each lane corresponds to subgenomic RNA derived from fused genomic RNA. The faster-moving second subgenomic mRNAs are derived from substrate RNAs such as TRCAT, TRCAT 62, and SINrep. (−), negative strand. (b) Recombination between replicative and nonreplicative RNAs. RNA analysis was done as described for panel a. The term genomic RNA corresponds to the fusion product. The subgenomic RNAs are all expressed from fusion products. In addition to 26S subgenomic RNA, two other subgenomic RNA are found. The fastest-moving mRNA labeled CAT mRNA is derived from the second subgenomic promoter found in the TT3CAT and TT19CAT precursors. The slowest-moving subgenomic mRNA is a dicistronic mRNA which contains both the S region of SIN and the CAT region (41), but this dicistronic mRNA is translated to give only S proteins. (c) Expression of CAT protein from recombinant viruses. BHK cells were infected at 20 PFU per cell with recombinant viruses generated from template RNAs indicated above the lanes. After 6 h of infection at 37°C, cells were harvested and cytoplasmic extracts were prepared. Ten (lane 2) or 50 (lanes 1, 3, 4, and 5) μg of cytoplasmic proteins was used to assay CAT activity. The percentage conversion of choramphenicol to acetyl chloramphenicol is indicated in parentheses. (d) Intracellular RNA products expressed from plaque-purified viruses. Four plaques each were isolated from duplicate sets of plates p2 and p3 (see below) and were suspended in minimal essential medium containing 2% serum. One-tenth of the diluted plaque virus was used to infect fresh BHK cells, and intracellular viral RNA was labeled with uridine and analyzed as described for panel a. Lanes 1 to 4 correspond to RNA samples corresponding to the four viral plaques from plate p3; lanes 5 to 7 correspond to RNA samples of the four viral plaques from plate p2. (e) Size and morphology of plaques formed by recombinants. Culture supernatants were serially diluted and used to infect Vero cells in culture, overlaid with agarose, and incubated at 37°C for 2 to 3 days. Plaques became visible after 36 h. After fixation with 3% paraformaldehyde, the cells were stained with crystal violet and photographed. p1, plaque formation by Toto 1002 (size, 100%); p2, plaque formation by recombinant viruses generated by fusion of GNS-2 and G26S-2 (size, 60 to 80%); p3, plaque formation by recombinant viruses generated by fusion of GNS-2 and G26S-3 (size, 20 to 60%).

inactivated *Nsi* site (nt 11452 of SIN) as a mutational marker in the 3′ end, was demonstrable in the fused RNA product (data not shown). Additionally, G26S-2SN RNA, which lacked both the 5′ and 3′ motifs of SIN, although encoding S-protein sequences, failed to support RNA recombination (Table 1, experiment 22; Fig. 2b). Minus-sense RNAs corresponding to G26S-2 and G26S-3 also failed to serve as templates for RNA recombination (Fig. 2a). It is interesting that none of the replicative RNAs (TRCAT, TRCAT 62, and SINrep) used in the original transfection experiment was found to be expressed in the recombinant virus-infected cells. The original transfected cells were expected to release particles containing (i) recombinant RNA capable of expressing 49S and 26S RNAs and (ii) the parental replicon (TRCAT, TRCAT 62, or SINrep) which

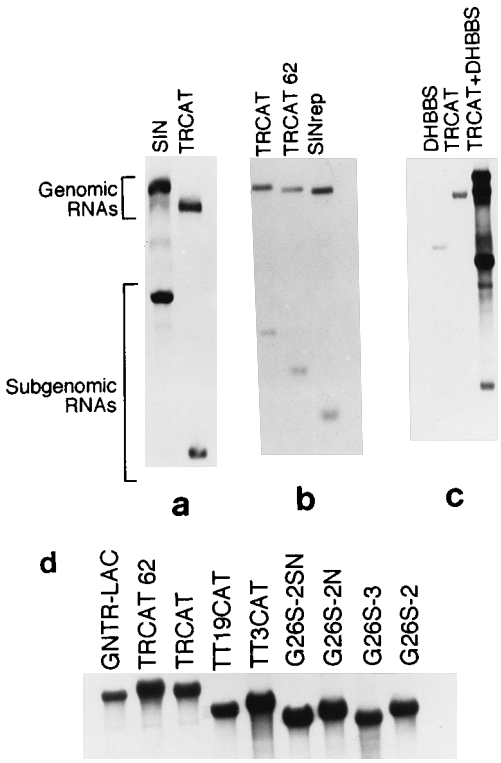


FIG. 3. (a and b) In vivo-made RNAs in transfected BHK cells. BHK cells were transfected with in vitro-transcribed, capped RNAs, and virus-specific RNAs were labeled with uridine in the presence of dactinomycin for 6 to 8 h. Six micrograms of cytoplasmic RNAs was denatured by glyoxal and analyzed on a 1.25% agarose gel. The name of the RNA template used in transfection is indicated above each lane. Note that each template RNA makes a longer genomic RNA and a shorter subgenomic RNA. The gel corresponding to panel a was run for a longer time than the one in panel b. (c) Comparison of in vitro-made RNAs and their in vivo products upon transfection. Lanes corresponding to DHBBS and TRCAT represent in vitro-made RNAs loaded on the gel as a marker. Lane TRCAT+DHBBS represents uridine-labeled in vivo RNA products made in cells which were infected with the virus released from cells transfected with TRCAT and DHBBS RNAs. BHK cells were infected with 5 PFU of virus inoculum per cell and labeled for 4 h. Five micrograms of cytoplasmic RNA was analyzed as in panel a. The gel was run for a shorter duration than in panels a and b. Note the presence of a slowly moving band ahead of TRCAT which is the fusion product between DHBBS and TRCAT and is positive for NS and S regions of SIN. Also note the abundant 26S subgenomic RNA (center band) made from the recombinant product. (d) Some of the in vitro transcripts used as substrates. Five percent of the [$\alpha$-$^{32}$P]GTP-labeled transcripts were treated with DNase I to remove the DNA template, purified, denatured with glyoxal, and separated for 1 h on a 1.25% agarose gel. Descriptions of corresponding plasmids are given in Materials and Methods. All of the transcripts except G26S-2SN were runoff transcripts at an *Sst*I restriction site of each plasmid. The G26S-2SN plasmid was linearized at the *Eco*RI site. All plasmids were transcribed with SP6 RNA polymerase.

failed to undergo recombination. If these two kinds of genomes were found in the released particles, then infection of fresh cells with this population should have led to the production of 49S RNA, 26S RNA, the parental replicon, and its subgenomic RNA. Since only 49S and 26S RNAs are found in the recombinant virus-infected cells (Fig. 2a and b), it appears that either the parental replicon is not packaged and released from the transfected cell or there is a differential gene expression in recombinant virus-infected cells.

Generation of the SIN genome from a replicative RNA such as TRCAT and a nonreplicative RNA such as G26S-2 suggested that the polymerase proteins produced from TRCAT initiated RNA synthesis at the 3′ terminus of G26S-2 and jumped onto the TRCAT template to make the SIN genome. This reasoning is based on the copy choice mechanism of RNA recombination (23, 24). The RNA substrates which function as templates to initiate RNA synthesis and subsequently donate the polymerase complex are known as donor templates. Thus, RNAs such as DHBBS, G26S-2, and G26S-3 can be considered donor templates (20). The RNA substrates such as TRCAT, TRCAT 62, and SINrep which accept the jumping polymerase complex are known as acceptor templates. As depicted in Fig. 4, the donor templates contribute to the 5′ end of the new transcript and the acceptor templates contribute to the 3′ end of the new transcript.

**A nonreplicating RNA expressing NS proteins fuses with a weak replicon expressing S proteins.** Recombination events described above made use of amplifiable acceptor templates with a potential to express high levels of viral polymerase. To test if low levels of polymerase proteins could still catalyze RNA recombination, we made use of GNS-2 RNA (Fig. 1). GNS-2 RNA lacks the complete 4.1-kb 3′ region of the SIN genome, and hence it cannot replicate. When cells were transfected with GNS-2 RNA and DHBBS RNA, infectious virus was produced from transfected cells (Table 1, experiment 28). Cells infected with the recombinant virus produced both 49S and 26S RNAs, but as expected, no RNA corresponding to DHBBS was demonstrable (data not shown). These results indicated that a low amount of viral polymerase protein translated from GNS-2 RNA was sufficient to initiate RNA synthesis at the 3′ end of DHBBS RNA and could effectively switch to GNS-2 RNA to make recombinant RNAs. Studies carried out with the brome mosaic virus system indicate that terminal 3′ truncations of brome mosaic virus RNA-2 (45) and RNA-3 (36) could be repaired by other cotransfecting wild-type RNAs. These results confirm the existence of a pathway among plant and animal alpha-like viruses to repair terminal deletions of viral genomes.

**Two nonreplicating RNA precursors recombine.** Results described above suggested that a minimally functional donor template (e.g., G26S-2) must possess at least the 3′ motif of SIN, and the minimally functional acceptor template (e.g.,

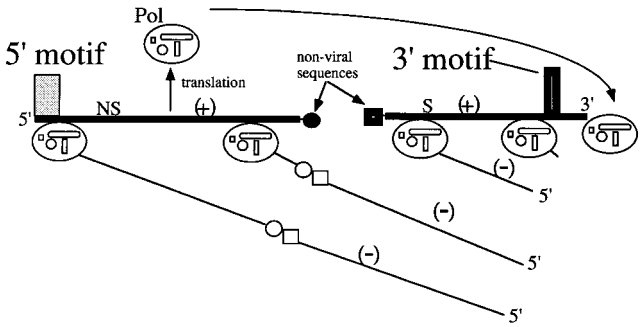


FIG. 4. Model for nonhomologous recombination of nonreplicative RNA precursors (GNS-2 and G26S-3). In this model, polymerase protein synthesized from one of the templates scans and identifies cognate 3′ *cis*-acting sequences to initiate RNA synthesis, independently of the precise 5′ and 3′ sequences of the priming template. The polymerase complex along with its nascent transcript jumps onto a new template some time during transcription. The site of polymerase jumping may be dictated by base pairing between the donor template and the acceptor template at or near the crossover site. Alternatively, the acceptor template may be selectively recruited from a pool of available substrates by base pairing between 3′ *cis*-acting sequences (or an alternate motif) of the donor template and the 5′ *cis*-acting sequences (or an alternate motif) of the receiver template. Once the acceptor template is recruited to the vicinity of the transcription complex, the polymerase may switch to the receiver template independently of any local base-pairing requirement, by specific RNA-protein interactions. Alternatively, the polymerase complex may slide through several RNA templates until a cognate RNA motif or template is chosen for reinitiation.

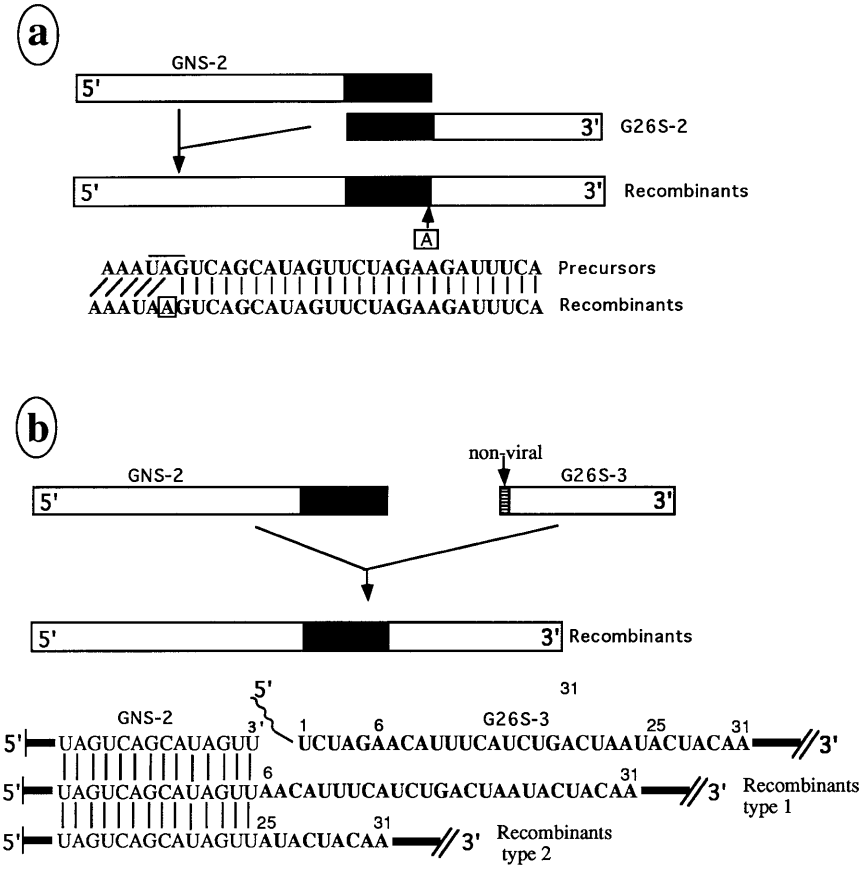


FIG. 5. Characterization of crossover sites of recombinants. All of the eight cytoplasmic RNA samples used for Fig. 2d were reverse transcribed with murine leukemia virus reverse transcriptase and PCR amplified as described in Materials and Methods. The PCR products were purified with low-melting-temperature agarose and sequenced by using a junction primer. (a) Recombination between GNS-2 and G26S-2 RNAs. The 694-nt overlap region is depicted as solid bars. The open bars represent nonhomologous sequences. The location of the single A insertion in the recombinant is indicated. RNA samples corresponding to all four plaques contained the same insertion. (b) Recombination between GNS-2 and G26S-3. These two template RNAs did not possess any discernible homology at the crossover site. The G26S-3 precursor (donor template) contained 51 nt of nonviral sequences derived from pGem 3 at the 5′ end, which is denoted as a wavy line. All four recombinant products corresponding to the four plaque-purified viruses contained the precise 3′ end of GNS-2 used for recombination. None of the four recombinants contained the 51-nt nonviral sequences carried by the G26S-3 precursor. In addition, the type 1 recombinants lost the first 5 nt of the viral noncoding sequence. Thus, type 1 recombinants lack 56 nt of G26S-3 from its 5′ end. Type 2 recombinants, in addition to lacking 51-nt nonviral sequence, also lack the first 25 nt of the viral noncoding sequence. Thus, type 2 recombinant RNAs lack 76 nt from the 5′ end of G26S-3 RNAs.

GNS-2) must possess the 5′ motif of SIN. The other terminal motif of SIN may not be needed in the donor and acceptor templates to support recombination. These predictions were suggested by results of experiments in which one of the nonreplicative templates was used in conjunction with a replication-competent template. To test if two nonreplicating minimally functional templates could support recombination, BHK cells were transfected with G26S-2 and GNS-2 RNAs. As depicted in fig. 1 and 5a, these two substrate RNAs contain a 694-overlap region at their meeting ends. As shown in Table 1 (experiment 29), infectious virus was released from cells transfected with this pair of nonreplicative RNAs. Both 49S and 26S RNAs characteristic of SIN were also demonstrable in the recombinant virus-infected cells (Fig. 2b). Sequence analysis of four independent recombinants revealed insertion of a single A residue converting the UAG stop codon of NS protein P4 to a UAA stop codon. These results indicated that two nonreplicative minimally functional RNA substrates could undergo homologous recombination to evolve into an infectious virus.

Although we were surprised to observe recombination between two nonreplicative RNA substrates, we thought that the presence of an overlap region between the two substrate RNAs could have facilitated the recombination. As found for the poliovirus system (24), the 3′ growing end of negative-sense nascent RNA made from the donor template (G26S-2) could have effectively base paired with the right end of the accetor template (GNS-2), facilitating homologous recombination. To determine whether homology at the crossover sites of template RNAs was required to support recombination, we made use of GNS-2 and G26S-3 substrate RNAs. GNS-2 and G26S-3 RNAs do not share any homology at their meeting ends (Fig. 5b). In fact, G26S-3 RNA contains a 51-nt nonviral sequence at its 5′ end which is derived from the pGem 3 vector. Except for this nonviral sequence, the GNS-2 and G26S-3 RNAs correspond to the two fragments of a nicked SIN RNA genome. As shown in Table 1 (experiment 30), transfection of cells with these RNAs led to production of infectious virus. Cells infected with the recombinant virus produced both 49S and 26S RNAs of SIN (Fig. 2b). Both 49S and 26S RNAs continue to accumulate during the course of infection and, as expected, contain viral NS and S coding sequences (Fig. 6). These results clearly demonstrate the apparent ability of two fragments of nicked SIN genome to recombine in vivo and to produce infectious virus. Sequence analysis of four plaqued recombinants

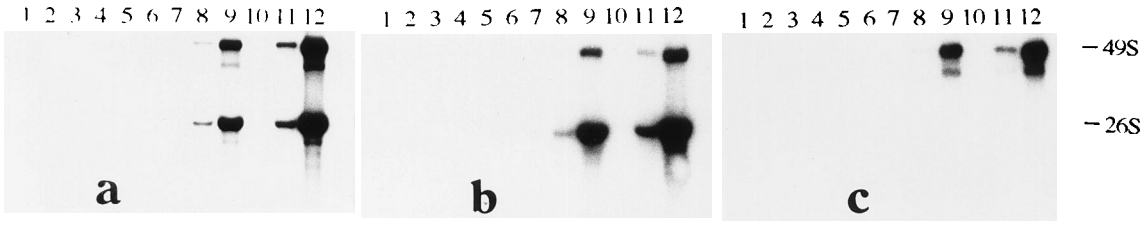


FIG. 6. Controls for RNA-mediated recombination. Ten percent of various control culture supernatants (see below) was used to infect BHK cells, and the intracellular virus-specific RNAs were labeled with uridine as described in Materials and Methods. Cytoplasmic RNAs were isolated from all BHK cells, and triplicate RNA samples (5 μg of each) were separated on a 1.25% gel. The first part of the gel was fluorographed (a). The second and third sets were blotted and probed as described below (b and c). The culture supernatants used in this experiment as virus inocula were derived from BHK cells transfected with RNA or DNA. In brief, BHK cells were transfected with 150 ng of appropriate RNA or 2 to 5 μg of DNA (see below) and incubated at 37°C for 52 h, and the culture supernatants were recovered. Thus, each lane depicts the ability of a previously transfected RNA or DNA to undergo recombination and release of infectious virus. The biological nature and names of the substrates used for the initial transfection experiments are as follows: lanes 1, GNS-2 RNA precursor treated with DNase I and purified by phenol extraction and ethanol precipitation; lanes 2, G26S-3 RNA transcript treated with DNase I and purified by phenol extraction and ethanol precipitation; lanes 3, GNS-2 and G26S-3 RNA transcripts treated with DNase-free RNase and subsequently purified by proteinase K digestion, phenol extraction, and ethanol precipitation; lanes 4, linearized GNS-2 and G26S-3 DNA templates mock transcribed in the presence of only ATP and GTP (omitting UTP and CTP) and purified by phenol extraction and ethanol precipitation (this sample did not receive DNase or RNase treatment, and linearized DNA templates were demonstrable in this preparation); lanes 6, RNA samples derived from GNS-2BG and G26S-3 templates, treated with neither RNase nor DNase, used for transfection; lanes 7 to 9, GNS-2 and G26S-3 RNAs which were treated with RNase-free DNase I and purified and cells treated with 0.5 μg of dactinomycin per ml for 20 min prior to transfection and after transfection until virus harvest; lanes 10 to 12, RNA transcripts derived from plasmid Toto 1002, treated with RNase-free DNase I. The cells were labeled from 1 h to 8 h p.i. for samples corresponding to lanes 1 to 6. The labeling times for the remaining lanes are as follows: lanes 7 and 10, 1 to 2 h p.i.; lanes 8 and 11, 1 to 4 h p.i.; lanes 9 and 12, 1 to 8 h p.i. (a) Fluorography of separated RNAs; (b) Northern analysis of samples 1 to 12, using negative-sense RNA probe G3-S RNA, which detects both the SIN genome and 26S RNA; (c) Northern analysis of samples 1 to 12, using negative-sense RNA probe GNS-1EE, which detects the SIN genome and a minor NS-protein-coding RNA which is often found in all SIN-infected cells. The biological significance of the second NS-protein-coding RNA is not known.

indicated deletion of all nonviral and some noncoding viral sequences of the donor template at the crossover site (Fig. 5b). No nucleotide insertions were detectable within the 92-nt nucleotide vicinity of the crossover site. Interestingly, the precise 3′ end of the acceptor template, GNS-2, is preserved in all of the recombinants (Fig. 5b). It is to be noted that these recombination experiments were designed to monitor recombination at noncoding sequences at the intergenic region of the SIN genome, which is not known to regulate viral replication. Hence, it is less likely that the virus population released from RNA-transfected cells represents a skewed population of recombinants. This point is also supported by the fact that SIN vectors (6, 12, 43, 61) carrying many foreign sequences at this region efficiently undergo replication.

The in vivo fusion of nonreplicative RNA substrates such as GNS-2 and G26S-3, which carry no known homology, indicated to us that other RNAs which carry SIN 3′ motifs might recombine with GNS-2 RNA. To test this proposal, we made use of RNA substrates which code for the reporter, CAT (43). As depicted in Fig. 1, TT3CAT and TT19CAT contain CAT-coding sequences in addition to SIN S-protein-coding sequences and an authentic SIN 3′ motif. Transfection of cells with one of these precursor RNAs along with GNS-2 RNA gave rise to virus particles (Table 1, experiments 34 and 35). Cells infected with the recombinant virus produced a genomic RNA which was slightly longer than the 49S RNA of SIN (Fig. 2b). Since the donor template contained two subgenomic RNA promoters (43), the recombinant genomic RNA expressed two subgenomic RNAs (Fig. 2b). High levels of CAT activity (Fig. 2c) were also demonstrable in the virus-infected cells, indicating that the second subgenomic mRNA which codes for the CAT protein was functional. These results clearly indicate that the polymerase protein can initiate RNA synthesis on any RNA template containing a SIN 3′ motif and then jump onto a suitable acceptor template and make replicative RNAs.

To authenticate the generation of functional and infectious SIN from precursor RNAs, GNS-2 and G26S-2 (or G26S-3), we applied several criteria: (i) production and accumulation of RNA of ca. 12 kb which contains both NS and S genes of SIN (Fig. 6); (ii) synthesis and continued accumulation subgenomic RNA characteristic of SIN during the course of infection (Fig. 6); (iii) protein profile and immunoreactivity pattern of recombinant virus-infected cells (42); (iv) characteristic plaque formation by recombinant viruses (Fig. 2e); and (v) sequence analysis of the putative crossover sites demonstrating nucleotide alterations (Fig. 5). We also critically analyzed alternate possibilities which might explain the generation of new viruses (Fig. 6). The RNA precursors used in our experiments were all derived from plasmid DNAs. The plasmid DNAs were all digested with DNase I, and the RNAs were purified by phenol-chloroform extraction before transfection. It is possible that residual plasmid DNAs present in the sample underwent recombination in vivo to generate recombinant plasmids which could have led to the synthesis of full-length SIN RNA. We ruled out this possibility by two experiments. First, RNase-treated RNA preparations failed to produce any virus (Fig. 6). Second, transfection of cells with mock-transcribed plasmids failed to produce any virus or SIN-specific RNAs. Third, the production of recombinant virus was insensitive to dactinomycin, which also indicated the absence of any DNA-dependent RNA synthetic process contributing to the observed recombination. These studies substantiate the central role of precursor RNAs in generating recombinant SIN virus. Deletion mutants in the NS coding region of RNA precursors resulted in loss of virus production, indicating the importance of exogenously introduced NS coding sequence in the generation of SIN virus (Fig. 6) (42).

## DISCUSSION

Recombination among alphaviruses was originally suggested by Hahn et al. (14). The first experimental evidence for recombination in alphaviruses was provided by Weiss and Schlesinger (61). Intrigued by a recombination event between a severely debilitated SIN DI RNA (DHBBS) and a SIN vector (TRCAT), we initiated a systematic study of the sequence requirements of SIN RNAs to support recombination. Our present results have defined the minimal sequence requirements of SIN RNA substrates to participate in RNA recombination. For the first time, we demonstrated that two RNAs

lacking the ability to replicate individually could undergo recombination in vivo to form a replicative RNA. We suggest that the presence of 3′ SIN motif in a donor template and 5′ SIN motif in an acceptor template may be necessary and sufficient to make them undergo recombination in the presence of polymerase proteins. We also demonstrated that minimal amounts of polymerase proteins that can be translated from a transfected mRNA such as GNS-2 RNA were sufficient to catalyze RNA recombination between nonreplicative RNA substrates.

As discussed by Lai (27), RNA cleavage coupled with ligation of precursor RNAs and template switching of polymerase complex are two of the possible mechanisms which can explain the present observations. Our attempts to demonstrate the inheritance of transfected precursor RNAs in the fused recombinant RNAs were unsuccessful. We were also unsuccessful in demonstrating in vivo ligation of radioactively labeled RNA substrates which lack the ability to express SIN polymerase. In addition, cells transfected with fragments of the SIN genome split at the polymerase-coding region failed to produce any virus (42). These results suggest no role for the host cell in mediating functional RNA fusion in the absence of viral polymerase. The absence of nucleotide specificity at the fusion site of recombinants poses further constraints in invoking an RNA cleavage-ligation model. Despite these arguments, it is possible that an RNA ligase and editing activity with a broad nucleotide specificity can play a role in RNA fusion, perhaps in concert with viral polymerase.

We favor a modified form of the copy choice model (Fig. 4). Unlike the earlier proposed models (22–24), this modified model accommodates nonreplicative RNAs as templates. On the basis of the fusion event occurring between the nonreplicative precursors, GNS-2 and G26S-2 (or G26S-3), we propose that SIN RNA-dependent RNA polymerase intrinsically possesses the ability to scan a variety of intracellular RNAs irrespective of their 3′ and 5′ sequence motifs. This hypothesis is supported by the fact that G26S-2 and G26S-3 precursors which lack authentic 5′ motifs of the SIN genome effectively function as templates. Hence, the polymerase should have initiated RNA synthesis at the 3′ end of the donor template (G26S-2 or G26S-3) without any cooperative role from the 5′ region of the donor template. The fact that the 5′ end of the donor template contained 51 nt of nonviral sequences also reinforces the point that authentic SIN 5′ motifs do not play a role in the initiation of RNA synthesis from the priming template. Thus, it is expected that the SIN 26S subgenomic RNA (30, 43, 48) which carries no 5′ SIN motifs should function as a template for initiation of negative-strand RNA synthesis. Since there are no reports indicating the presence of negative-sense copies of SIN 26S RNA in infected cells, it is possible that all negative-sense RNAs made from 26S subgenomic RNA were effectively utilized for 49S negative-strand synthesis. Other possibilities such as alternate modes of template switching by polymerase and nonavailability of 26S RNA for negative-strand synthesis in natural SIN infection cannot be ruled out at this time.

The absolute requirement of a 3′ SIN motif in the priming template is demonstrated by G26S-2NS mutant RNA, which failed to produce any virus or recombinant RNA (Table 1; Fig. 3b), but the precise locations of 3′ motifs within the priming templates were shown to be not important (42) to facilitate RNA recombination. When the priming templates contained several hundred nucleotides of nonviral sequences at both the 3′ and 5′ sequences, these templates were still effectively used for RNA recombination. In fact, the original work on infectious transcripts of SIN (44) showed that in vitro transcripts containing hundreds of nonviral sequences beyond the precise 3′ end are infectious. These results indicate that the polymerase can load on any RNA irrespective of its precise 5′ and 3′ motifs and search for cognate 3′ motifs. Once cognate 3′ motifs are found during scanning, the polymerase appears to initiate RNA synthesis and continue elongation. Since none of the negative strands served as templates for fusion, the model proposed in Fig. 4 depicts template switching during negative-strand synthesis. As suggested by Jarvis and Kirkegaard (20), additional experiments may be needed to demonstrate the strand preference during template switching. Alternatively, the recognition of negative-strand template by polymerase may be a complex process which could obscure our present experimental strategies.

The presence of 694 nt of sequence homology at the meeting point of GNS-2 and G26S-2 RNAs probably explains the observed homologous type of recombination with a single nucleotide addition which converted the UAG stop codon to UAA. Since the homology region extended up to 685 nt into the polymerase-coding sequence, it is not known if any other nucleotide alterations occurred within this region. The loss of all nonviral sequences and some noncoding viral sequences (from G26S-3) at the crossover site between GNS-2 and G26S-3 indicates a nonhomologous type of recombination. Analysis of secondary structures (9, 27) and heteroduplex-forming capabilities (7, 37) at the crossover site of these RNAs showed very limited base-pairing abilities. Hence, we hypothesize (i) the involvement of unidentified internal or other distal sequences of GNS-2 and G26S-3 precursors in aiding RNA-RNA fusion and (ii) RNA-protein recognition which favors template switching. As recently suggested for the coronavirus system (56, 63a), RNA-protein interactions may be critical in this kind of nonhomologous recombination event. Thus, it is possible the template RNAs are not brought close together at all, but the polymerase complex actually travels and screens cytoplasmic RNAs in search of a foster template to continue its RNA synthesis. The sequence and structural elements which are recognized by the traveling polymerase for attachment and reelongation of RNA synthesis remains to be characterized.

Elegant studies reported by many earlier workers strongly suggest a copy choice mechanism of RNA recombination (20, 22, 24, 27, 37, 40, 57). Since the substrate RNAs GNS-2, G26S-2, and G26S-3 RNAs did not have the ability to be amplified individually, it is logical to conclude that RNA fusion took place in the presence of a minimal amount of precursor RNAs in vivo. This observation seriously questions the notion of replicative polymerase cycling and aberrant behavior of overcrowded cytoplasmic polymerase as the cause of template switching described here. If indeed the generation of DI particles is due to polymerase jumping, and if polymerases can jump even under relaxed conditions such as described in this work, then what is unique about high-multiplicity infections which is conducive to DI genome generation? It appears that alternate modes of polymerase jumping exist in vivo which might be influenced by other unknown factors and events associated with active viral replication. Recent work by Jarvis and Kirkegaard (20) implicates intracellular RNA concentration as a key factor in augmenting the frequency of recombination. Since the assay described by these workers appears to be very powerful, it will be highly informative to do a quantitative study on the diverse kind of viral recombinants made at low- and high-multiplicity infections which will illuminate the different modes of polymerase jumping.

Results described in this report demonstrate fusion events in a noncoding region flanked by coding sequences. We have already demonstrated several fusion events between nonrepli-

cative RNA precursors at the 3′ noncoding region of the SIN genome (42). It is possible that other noncoding sequences in the SIN genome can also be exploited to study RNA fusion events. We anticipate that at least the members of the SIN superfamily (1, 11, 13, 15, 59) and possibly many more positive-strand RNA viruses of plant and animal origin will be found to exhibit similar recombination events.

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