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Nonhomologous RNA-RNA Recombination Events at the 3′ Nontranslated Region of the Sindbis Virus Genome: Hot Spots and Utilization of Nonviral Sequences

MUSTAPHA HAJJOU, KRISTIE R. HILL, S. V. SUBRAMANIAM, JUDY Y. HU, AND RAMASWAMY RAJU*

Department of Microbiology, School of Medicine, Meharry Medical College, Nashville, Tennessee 37208

Received 27 February 1996/Accepted 30 April 1996

The mechanism of RNA-RNA recombination at the 3′ nontranslated region (3′NTR) of the Sindbis virus (SIN) genome was studied by using nonreplicative RNA precursors. The 11.7-kb SIN genome was transcribed in vitro as two nonoverlapping RNA fragments. RNA-1 contained the entire 11.4-kb protein coding sequence of SIN and also carried an additional 1.8-kb nonviral sequence at its 3′ end. RNA-2 carried the remaining 0.26 or 0.3 kb of the SIN genome containing the 3′NTR. Transfection of these two fragments into BHK cells resulted in vivo RNA-RNA recombination and release of infectious SIN recombinants. Eighteen plaque-purified recombinant viruses were sequenced to precisely map the RNA-RNA crossover sites at the 3′NTR. Sixteen of the 18 recombinants were found to be genetically heterogeneous at the 3′NTR. Two major clustered sites within the 3′NTR of RNA-2 were found to be fused to multiple locations on the nonviral sequence of RNA-1, resulting in insertions of 10 to 1,085 nucleotides at the 3′NTR. Sequence analysis of crossover sites suggested only limited homology and heteroduplex-forming capability between substrate RNAs. Analysis of additional 23 recombinant viruses generated by mutagenized donor and acceptor templates supports the occurrence of recombination hot spots on donor templates. Introduction of a 17-nucleotide rudimentary replicase recognition signal in the acceptor template alone did not induce the polymerase to reinitiate at the 17-nucleotide signal. Interestingly, deletion of a 24-nucleotide hot spot locus on the donor template abolished crossover events at one of the two sites and allowed the polymerase to reinitiate at the 17-nucleotide replicase recognition signal inserted at the acceptor template. The possible roles of RNA-protein and RNA-RNA interactions in the differential regulation of apparent pausing, template selection, and reinitiation are discussed.

Viral RNA-RNA recombination, a process amounting to covalent fusion of two or more RNA sequences, is believed to be important in repair and evolution of RNA viral genomes (25, 30, 36, 51, 64, 65, 75). Many plant (2, 9, 10, 15, 36, 46, 56, 70), animal (3, 4, 13, 21, 28, 29, 31, 32, 38, 41, 42, 58), and bacterial (6, 49) RNA viruses are experimentally proven to undergo RNA recombination. Detailed studies carried out in brome mosaic virus (8, 26, 44–46), coronavirus (3, 4, 36, 41, 68, 73), poliovirus (28, 30–32, 36, 58), Sindbis virus (SIN) (42, 55, 69), tombusvirus (70, 71), and turnip crinkle virus (10–12) have set the stage to explore the sequence and structural requirements of RNAs and proteins involved in RNA recombination. It is currently believed that viral RNA recombination is mediated by jumping of viral polymerase from one location to another location on the same or a different RNA. Three major determinants which promote template switching of polymerase were explored in the studies cited above: (i) local RNA-RNA annealing between substrate RNAs; (ii) replicase recognition motifs; and (iii) secondary structures on participating templates. These studies also emphasize the relative importance of the three determinants in individual systems and indicate the diversity of mechanisms which might contribute to template switching.

SIN is a mosquito-transmitted animal RNA virus which belongs to the *Alphavirus* genus of the *Togaviridae* family (62, 66). The SIN genome consists of an 11.7-kb single-stranded positive-sense RNA. The protein coding potential of SIN is expressed from two contiguous open reading frames (ORFs). The first ORF codes for the viral RNA-dependent RNA polymerase complex (nonstructural [NS]) and spans nucleotides (nt) 59 to 7598 of the SIN genome (62, 66). The second ORF, which codes for the viral structural (S) proteins, spans nt 7647 to 11381. The 321-nt nontranslated region located at the 3′ end (3′NTR; nt 11382 to 11703) contains a 19-nt *cis*-acting motif which serves as a promoter for negative-strand RNA synthesis. The availability of well-characterized gene expression modules derived from cDNA copies of the SIN genome facilitated the rapid analysis of *cis*- and *trans*-acting functions encoded by SIN (7, 16, 17, 20, 23, 35, 39, 53, 55, 57, 69, 72).

Although studies of SIN defective interfering particles (42, 61) and sequence analysis of other alphaviruses (21, 64–66, 68a) indicated RNA recombination in alphaviruses, the first experimental evidence in this connection was provided by Weiss and Schlesinger (69). By making use of two separate SIN replicons expressing SIN polymerase or structural proteins, they demonstrated the production of recombinant SIN carrying both coding regions in a single RNA. These studies mapped the RNA-RNA recombination sites, indicated the nonhomologous nature of RNA crossovers, and suggested a copy choice mechanism for recombination between SIN-specific RNAs. Recently, we reported a novel class of recombination events while we were using SIN vectors for studying expression of foreign genes (55). In essence, we demonstrated that two nonreplicative RNA fragments representing the 5′ 7.6-kb and 3′ 4.1-kb regions of SIN could recombine in cul-

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Meharry Medical College, 1005 D. B. Todd Blvd., Nashville, TN 37208. Phone: (615) 327-6687. Fax: (615) 327-6072. Electronic mail address: ramasa25@ccvax.mmc.edu.

TABLE 1. Oligonucleotides used

Name	Sequence	Position[a]	Polarity
JC1350	GAAATGTTAAAAACAAAA	11687–11704	−
JC1295-1	AAAGTTATGCAGACGCTGCGTGGC	11635–11658	+
JC1295-2	ACGCTGCGTGGC	11647–11658	−
JC1295-3	AAAGTTATGCAG	11635–11646	−
JC1200-1	GTTATGTGGCAACACTGCGCAGCA	11544–11567	−
JC1200-2	CACTGCGCAGCA	11556–11567	−
JC1200-3	GTTATGTGGCAA	11544–11555	−
JC1000-1	GCTGACTAGCACACGAAG	11363–11380	+
JC1000-2	GCTTGCAGCATGATGCTGACT	11349–11369	+
T11050	CGTACATTCGCATTCGAG	11051–11068	+
T11150	GCGAACTTTATCGTATCG	11151–11168	+
T11150(+)	CGACTGTCTAGATTATCTAGCGGACGCCAA		+
T11350	TAGTCAGCATCATGCTGC	11353–11570	−
T11550(−)	AGT GAG TCTAGACAACACTGCGCAGCATTA		−
T11750(+)	AAAGGGAATTCCTCGAGGGGGA	11737–11558	+
T11750(−)	TTCAAGAATTAATTCCCCTCG	11752–11772	−
T10650	AACCAGGAGCGTTTGGAG	10651–10668	+
T10300	GGTCTTCGGAGGGGTCTA	10301–10318	+
T13348	TCCGGTAAGCGGCAGGGTCGG	13383–13403	+
AX-3	AAGCTGGGGCCCACAAA<u>ATTTTGTTTTTAACATT</u>GGCTCGAGGACAGA[b]		+
AXP-1	AAGCTGGGGCCCACA		+
AXP-2	TCTGTCCTCGAGCCA		−

[a] Location within SIN Toto 1101 (53, 57).
[b] The 17-nt promoter region is underlined.

tured mammalian cells to produce live virus. We suggested a modified copy choice model involving RNA-RNA and/or RNA-protein interactions to explain recombination events among nonreplicative RNA precursors. In the present work, we provide further evidence for recombination between nonreplicative RNA precursors and demonstrate the ability of SIN polymerase to preferentially terminate RNA synthesis at two selected locations on donor templates and reinitiate on nonviral sequences linked to acceptor templates. In addition to serving as a model system with which to study macromolecular interactions during viral RNA synthesis, studies on SIN recombination are expected to aid in understanding of the evolution of the alphavirus genome (66) and in further development of RNA viral vectors (7, 16, 40b, 53, 61a, 72).

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides used in this study are listed in Table 1. The map locations of several of these primers are given in Fig. 1.

Plasmids. Tapa 32 was constructed by digesting plasmid Tapa (23, 53) with *Apa*I and *Xho*I and religating the plasmid after end filling with T4 DNA polymerase in the presence of 1 mM deoxynucleotide triphosphates. This procedure resulted in the deletion of the complete 3′NTR of the SIN genome (Fig. 2a).

TT21f (Fig. 2a) was constructed by digesting Tapa with *Apa*I and *Xho*I, and the 13.4-kb fragment lacking the SIN 3′NTR was isolated. A double-stranded oligonucleotide representing a 17-nt region of the 19-nt 3′ promoter region of SIN was made by PCR amplification and inserted at the *Apa*I and *Xho*I sites of the 13.4-kb vector fragment. For PCR amplification, oligonucleotide AX-3 was used as a template. AXP-1 and AXP-2 were used as 5′ and 3′ primers, respectively. The PCR-amplified material was digested with *Apa*I and *Xho*I, directly cloned into the 13.4-kb vector, and sequenced by using primer T11750(−) to confirm the identity.

S3P (Fig. 2b) was constructed by ligating the 1.5-kb *Apa*I-*Pvu*I DNA fragment carrying the SP6 promoter from JUNCAT (53) to the 1.1-kb *Apa*I-*Pvu*I fragment of Tapa which contains the complete 3′NTR of SIN, including the poly(A) tail.

G26S3-HN (Fig. 2b) was constructed from plasmid G26S-3 (55). Plasmid G26S-3, which carries the complete coding region of SIN S and SIN 3′NTR, was digested with *Nsi*I and *Hin*dIII and religated after end filling.

S3PD1 was made from S3P. Plasmid S3P was PCR amplified with primer T11750(+), which annealed to the 3′ end of the SIN sequence located within S3P, and T11550(−) which annealed to the 5′ boundary of the first pause site and carried an *Xba*I site at its 5′ end (Fig. 2c). The 2.4-kb PCR product thus obtained lacked the 3′ 146 nt of SIN sequence located within S3P and contained an *Xba*I site and *Xho*I site at the termini. A second PCR product representing the 3′ 119 nt of the SIN sequence within S3P was obtained by PCR amplifying S3P with primers T11150(+) and T11750(−). Both PCR products were digested with *Xba*I, ligated in vitro with T4 DNA ligase, and subsequently PCR amplified with primers T11750(−) and T13348(+). The 720-nt PCR product was isolated from agarose gel, purified, and used for in vitro transcription.

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 (53, 55). [α-^{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 were denatured by glyoxal (53, 55) and analyzed on a 1.25% agarose gel. RNA samples smaller than 0.5 kb were analyzed on a 2.8% acrylamide-urea gel as described previously (54).

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 1002 [55, 57]) and recombinant SIN stocks were prepared from BHK cells, and titers were determined on BHK cells or Vero cells, using a standard plaque assay (17, 69). For virus infections, BHK cells grown in 35-mm-diameter petri plates were infected at a multiplicity of infection (MOI) of 0.01 to 5 PFU per cell and incubated at 37°C for desired times.

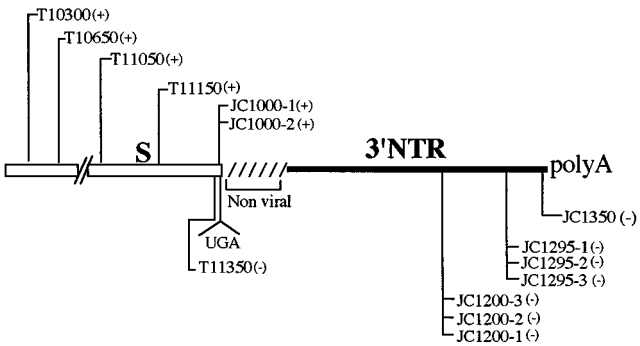

FIG. 1. Map locations of oligonucleotides mapping to the 3′NTR of SIN. The 3′NTR of SIN genome is identified by a boldface line. The SIN S coding region is identified by rectangles. The approximate locations of nonviral sequences that can be found as part of the 3′NTR in viral recombinants are identified by slashes.

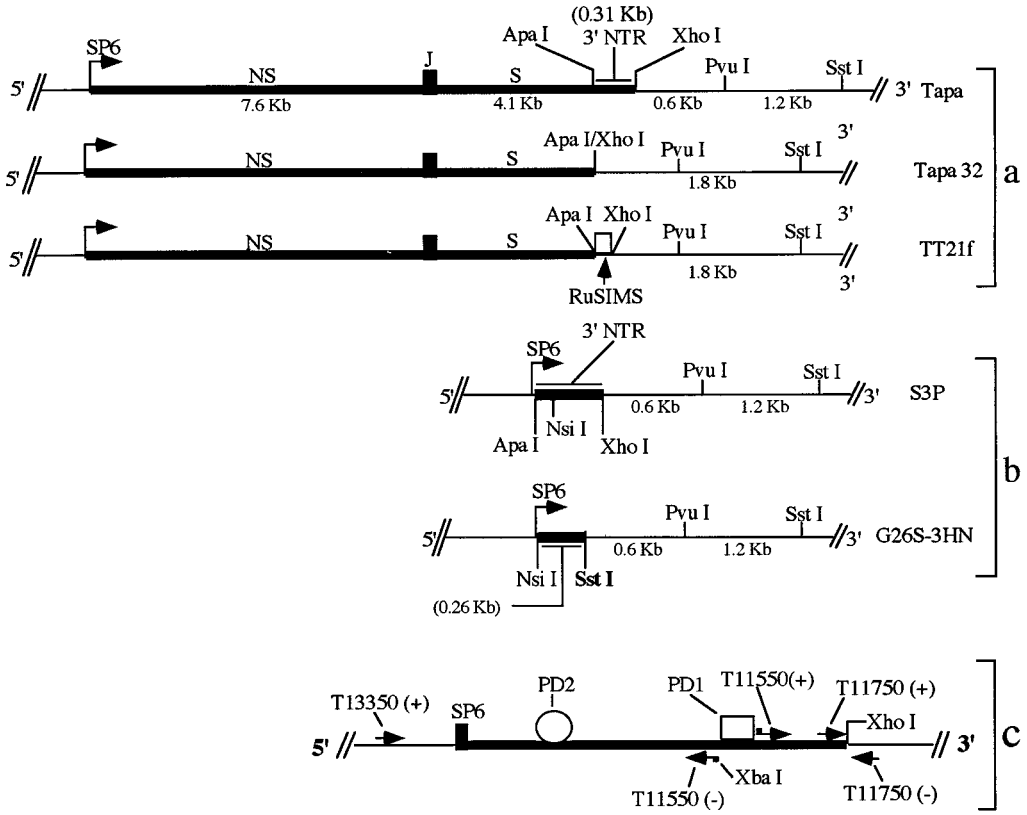

FIG. 2. Structures of plasmids. Tapa, Tapa 32, TT21f, S3P, and G26S-3HN refer to plasmid DNAs which carry various sequences of the SIN genome. (a) Plasmids used for synthesis of acceptor RNA templates; (b) plasmids used for synthesis of donor RNA templates. The sizes of coding, noncoding, and nonviral sequences are indicated. The location of the SP6 promoter is indicated by a right-angled arrow. Virus-specific sequences are identified by thick bars, and the nonviral sequences are identified by thin lines. Restriction sites located downstream of the 3′ ends of viral sequences (*Pvu*I, *Sst*I, and *Xho*I) were used to linearize the plasmid before in vitro transcription. Throughout the text, the RNA transcripts that are derived from these plasmids are identified by the name of the plasmid followed by the name of the enzyme used to linearize the plasmid. NS, genes coding for nonstructural proteins which form the SIN polymerase complex; S, genes coding for SIN structural proteins; J, junction region or intergenic region of SIN where the promoter for 26S subgenomic RNA is located. Apa I/Xho I, the fused location of these two enzyme recognition sequences; RuSIMS, a 17-nt rudimentary promoter homologous to a 19-nt promoter for negative-sense SIN RNA synthesis and virus production (35). (c) Strategy to delete the PD1 sequence from S3P to obtain S3PD1. The relevant restriction sites, primer locations, and the location of PD1 on plasmid S3P are identified. The newly introduced *Xba*I site in oligonucleotide T11550 is indicated. See Materials and Methods for details of the procedure. This mutagenesis procedure resulted in the deletion of a 24-nt segment (5′CCACAUAACCACUAUAUUAACCAU) and the insertion of a 10-nt segment (5′UCUAGCUAGA) at the original PD1 location.

Transfection of BHK cells with RNA precursors. Transfection of BHK cells was carried out essentially as described previously (55). In brief, semiconfluent BHK cells were washed twice with isotonic saline and layered with 0.2 ml of phosphate-buffered saline containing 60 to 300 ng of in vitro-transcribed RNA and 20 μg of Lipofectin and continually rocked for 30 min. At the end of transfection, the transfection mixture was removed, and the cells were replenished with 2 ml of minimal essential medium containing 10% fetal bovine serum and incubated at 37°C. Cells were monitored for cytopathic effect, and culture supernatants were harvested and analyzed at the end of 3 days.

In vivo labeling and analysis of RNA products. BHK cells were infected with various culture supernatants or plaque-purified viruses as described previously (53, 55). 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 to 12 h. At the end of infection, cells were harvested and cytoplasmic RNA was isolated (54, 55). Approximately 5 to 8 μg of RNA was denatured with glyoxal, analyzed on a 1.25% agarose gel, and later fluorographed.

Reverse transcription of cytoplasmic RNA, PCR amplification, and sequencing. Six to 8 μg of cytoplasmic RNA was used for reverse transcription and PCR amplification (55). In brief, the first-strand synthesis involved annealing of 5 to 10 pmol of a negative-sense primer JC1350 or JC1295-1 (Fig. 1) with 5 to 8 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 deoxynucleotide triphosphates, and 400 U of murine leukemia virus reverse transcriptase 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 incubation, an aliquot of the reaction mixture was diluted to 10-fold and used directly for PCR amplification. The PCR reaction mixture consisted of 1 to 5% of the total 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 primers JC1350 or JC1295-1 and JC1000-1 or T11150, 5 U of *Taq* polymerase, and 350 μM deoxynucleotide 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 were analyzed on a gel. Additional primers listed in Table 3 were used for nested PCR analysis. The PCR products were isolated from low-melting-temperature agarose gel and purified by phenol-chloroform extraction and ethanol precipitation. The isolated DNA fragment was sequenced by the dideoxy method of Sanger et al. (59), using Sequenase (U.S. Biochemical). Alternatively, 5% of agarose gel-purified PCR products were subjected to cycle sequencing with a Perkin-Elmer sequencing kit. In some cases, the RNA samples were reverse transcribed, PCR amplified with primers carrying *Hin*dIII or *Sst*I sites, digested with these enzymes, and cloned at the same sites present in pGem3 (Promega).

RESULTS

Design of RNA substrates. To test the general applicability of RNA-RNA recombination between nonreplicative RNA precursors and the scanning model of RNA recombination (55), we chose to study recombination at the 3′NTR of the SIN genome. As depicted in Fig. 2, several parental plasmids were made from which defined RNAs can be transcribed in vitro. Plasmid Tapa 32 codes for all SIN proteins but lacks the 3′NTR, including the promoter for negative-strand RNA syn-

TABLE 2. Production of infectious SIN from transfected precursor RNAs[a]

Template 1	Template 2	Cytopathic effect		Virus titer (PFU/ml)	Plaque size (%)
		36 h	60 h		
Toto 1002	None	+++	++++	8.6×10^7	100
Tapa 32/Sst	S3P/Xho	+	+++	7.5×10^6	20–150
Tapa 32/Sst	G26S-3HN/Sst	+	+++	7.3×10^6	20–150
Tapa 32/Pvu	S3P/Sst	+	+++	6.2×10^6	20–150
Tapa 32/Sst	S3P/Pvu	+	+++	6.8×10^6	20–150
Tapa 32/Sst	G26S-3HN/Pvu	+	+++	8.1×10^6	20–150
Tapa 32/Sst	None	None	None	0	NA
Tapa 32/Pvu	None	None	None	0	NA
S3P/Xho	None	None	None	0	NA
S3P/Sst	None	None	None	0	NA
G26S-3HN/Sst	None	None	None	0	NA
G26S-3HN/Pvu	None	None	None	0	NA

[a] The RNA templates are identified by the names of the plasmids from which they are derived and by the enzymes used to linearize the plasmid (see Fig. 2). BHK cells were transfected with 50 to 80 ng of smaller RNAs and 200 to 300 ng of larger RNAs as described in Materials and Methods and monitored every 4 to 5 h for cytopathic effects. The culture supernatant was harvested after 3 days, and the virus titer was determined on Vero cells. Plaque sizes are estimates in comparison with the plaque size obtained from Toto 1002. +, Low cytopathology; +++, severe cytopathology but still adherent cells; ++++, complete cell death and detachment; NA, not applicable.

thesis. Linearization of Tapa 32 with *Sst*I or *Pvu*I and subsequent transcription gave rise to positive-sense SIN RNAs with truncated SIN 3′ ends but carrying differing lengths of nonviral sequences at their 3′ ends. Plasmid S3P codes for the complete 3′NTR of SIN, and plasmid G26S-3HN is a deletion version of S3P lacking 51 nt from the 5′ region of the SIN 3′NTR. Linearization of plasmid S3P at the *Xho*I site or of plasmid G26S-3HN at the *Sst*I site gave rise to positive-sense RNAs representing the SIN 3′NTR which were devoid of nonviral sequences at their 3′ ends. Linearization of plasmid S3P at the *Pvu*I or *Sst*I site and of plasmid G26S-3HN at the *Pvu*I site gave rise to RNAs with various lengths of nonviral sequences at their 3′ ends. These RNA substrates were designed to address the following questions. Does recombination occur between nonhomologous and nonreplicative RNA substrates at the 3′NTR of the SIN genome? Does the presence of nonviral sequences at the 3′ end of the acceptor template affect production of infectious virus and recombination? Can a short RNA substrate such as the 0.3-kb region of the 3′NTR of SIN serve as a donor template for polymerase, or are additional sequences needed to allow the polymerase to load on the donor template? Is it possible to delete part of 3′NTR sequences and still retain RNA recombination? What exactly are the structures and sequences that can be found at the crossover points of recombinants?

Production and characterization of recombinant viruses from precursor RNAs. To test the effects of 3′ nonviral sequences located on the acceptor templates on recombination, in vitro transcripts were made from Tapa 32 after linearization of the plasmid with *Sst*I or *Pvu*I. Similarly, RNA transcripts were made from plasmids S3P and G26S-3HN after digestion of the plasmids with *Xho*I, *Sst*I, or *Pvu*I. The RNA transcripts were characterized by agarose and acrylamide gel electrophoresis to confirm their size and integrity. A series of BHK cultures were transfected with a combination of donor and acceptor templates as depicted in Table 2. Control cultures received only one of the RNA templates. Cultures were monitored for cytopathic effect every 4 to 5 h. Cultures which were transfected with full-length infectious RNA derived from Toto 1002 (55, 57) produced the earliest cytopathic effect (18 h). Cells which received any of the combinations of donor and acceptor templates showed delayed cytopathic effect, but all of them showed severe cytopathic effect by day 3 of transfection. None of the control cultures which received only one of the nonreplicative RNA substrates showed any cytopathic effect in comparison with mock-transfected cultures. Culture supernatants were recovered from all the cells by the end of day 3, clarified, and stored. The virus titers and plaque sizes were determined on Vero cells. As depicted in Table 2, high levels of virus were produced from cells transfected with combination RNA substrates. In general, we found that recombination between these precursors occurred at even lower concentrations (20 to 100 ng) of input RNA substrates, whereas a previous study (55) of recombination at the NS-S junction found that higher concentrations of RNA templates (100 to 2,000 ng) were needed. This observation suggested that the RNAs under study served as better substrates for recombination. Since all combinations of RNA substrates produced almost similar virus titers, it appears that the presence of nonviral sequences at the 3′ ends of the acceptor templates and the donor templates did not affect the recombination events significantly. Since donor templates such as S3P/Xho (0.26 kb) and S3P/Sst (0.3 kb) served equally well in producing high-titer infectious virus, it appears that substrate RNAs as small as 0.26 kb can be easily recognized by SIN RNA polymerase to initiate RNA synthesis.

Analysis of RNA synthetic abilities of recombinants. We chose to analyze in detail two major populations of viral recombinants produced. The first population corresponded to virus produced from RNA precursors, namely, Tapa 32/Sst and S3P/Xho (TSX cross). The second population corresponded to recombinants produced from Tapa 32/Sst and G26S-3HN/Sst (TGS cross). In both of these RNA crosses, the same acceptor template was used. Although donor templates used in these studies carried identical 3′ viral region and nonviral extensions, the 5′ end of G26S-3HN lacked 52 nt corresponding to the 5′ region of the 3′NTR (Fig. 2b). Since we were interested in studying the regulatory role of the 3′NTR in RNA recombination, we thought that these two populations (TSX and TGS crosses) should serve as a basis for exploring all 3′NTR recombinants. To this end, 16 plaques from TSX cross, 12 plaques from TGS cross, and 8 control plaques of Toto1002 were isolated from Vero cells and suspended in 2 ml of culture medium. Duplicate BHK cultures were infected with each of the suspended viruses. The first set of cultures was used for labeling intracellular RNA. The second set was used to pre-

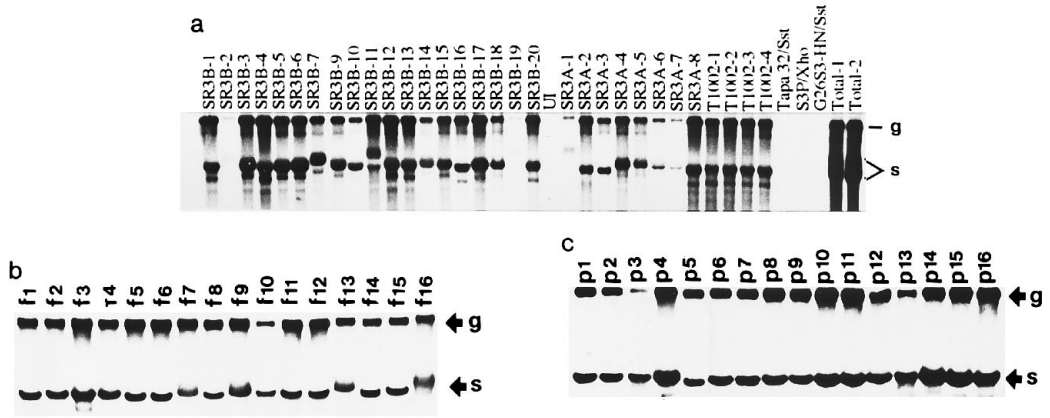

FIG. 3. Viral RNA synthesis in infected cells. BHK cultures were infected with the indicated viruses at an MOI of 0.01 to 0.1, and virus-specific RNAs were labeled with [^{3}H]uridine for 12 h. Approximately 5 to 8 μg of cytoplasmic RNA isolated from infected cells was denatured with glyoxal, analyzed on a 1.25% agarose gel, and fluorographed. (a) RNA synthesis from viruses produced from TGS and TSX crosses. The individual plaqued viruses, SR3B-1 to SR3B1-12 and SR3A1 to SR3A-4, were derived from culture supernatants of cells transfected with Tapa 32-Sst and S3P-Xho RNAs (TSX cross). Total-1, total virus obtained from TSX cross. Viruses SR3B-12 to SR3B-20 and SR3A-5 to SR3A-8 were derived from culture supernatants of cells transfected with Tapa 32-Sst and G26S-3HN-Sst (TGS cross). Total-2, total virus obtained from TGS cross. Viruses T1002-1 to T1002-4 represent four independent control plaques derived from Toto 1002 RNA-transfected cells. Tapa 32/Sst, S3P/Xho, G26S-3HN/Sst, culture supernatants obtained from cells transfected with only one of the RNAs. UI, uninfected; g, genomic RNA of recombinants; s, subgenomic RNA. (b) RNA synthesis from viruses produced by TTSX crosses. BHK cells were infected with 1 to 2 PFU of the indicated plaque-purified recombinant viruses per cell and labeled for 5 h. (c) RNA synthesis from viruses produced by TTSP crosses. BHK cells were infected with the indicated plaque-purified recombinant viruses and labeled for 6 h.

pare a virus stock. Analysis of cytoplasmic RNA from virus-infected cells (Fig. 3a) demonstrated the presence of both 49S genomic RNA and 26S subgenomic RNAs characteristic of SIN infection. Interestingly, the mobility of the 26S subgenomic RNA was found to be altered in many recombinant virus-infected cells, suggesting possible deletions and/or insertions. No virus-specific RNAs were demonstrable in cells infected with the medium derived from cells transfected with only one of the substrate RNAs (Fig. 3a, lanes Tapa 32/Sst, S3P/Xho, and G26S-3HN/Sst). All control virus-infected cells showed identical RNA patterns of both 49S and 26S RNAs (Fig. 3a, lanes T1002-1, T1002-2, T1002-3, and T1002-4). Cells infected with unplaqued total virus population (Fig. 3a, lanes Total-1 and Total-2) gave broad subgenomic RNA bands. As shown in Tables 3 and 4, each of the plaque-purified viruses grew well in mammalian cells and yielded high-titer virus stocks. As evident from Tables 3 and 4, no correlations could be made between plaque size and virus titer. These results indicated the ability of nonreplicative RNA precursors to undergo recombination at the 3′NTR of SIN and to produce viable recombinant SIN with altered gene expression.

Mapping of the 3′NTRs of recombinants. A detailed study was undertaken to analyze the 3′NTRs of recombinants from TSX and TGS crosses. In brief, cytoplasmic RNAs depicted in Fig. 3a were reverse transcribed by using primer JC1295-1, and the resulting cDNA was PCR amplified by making use of primers JC 1295-1 and JC1000-1. To confirm the authenticity of the PCR products and to rule out nonspecific and PCR artifacts, a series of additional primers (Fig. 1) were used in the initial reverse transcription as well as in the nested PCR analysis of the first round of PCR products. Figure 4a shows a representative picture of PCR products obtained from some of the RNA samples under discussion. All of the RNA samples corresponding to individual plaque-purified viruses yielded a single species of amplification product. Almost all of them showed altered mobility compared with the PCR product (0.3

TABLE 3. Characterization of recombinants produced from Tapa 32/Sst and S3P/Xho RNAs (TSX cross)[a]

Recombinant	Plaque identification	Plaque size (%)	Virus titer (PFU/ml)	3′ deletions (bases [position])	3′ insertions (bases)	Total length of 3′NTR (bases)	Common COS bases
a	SR3A-2	75	5.3×10^7	169 (144–313)	10	152	AU
b	SR3A-3	50	8.5×10^6	178 (135–313)	0	134	A
c	SR3A-4	75	6.2×10^7	54 (259–313)	366	623	AU
d	SR3B-1	100	9.1×10^6	56 (257–313)	247	504	None
e	SR31-4	50	2.6×10^7	178 (135–313)	541	673	AUA
f	SR3B-6	75	1.9×10^7	177 (136–313)	458	594	None
g	SR3B-7	25	6.5×10^6	56 (257–313)	776	1,031	AA
h	SR3B-10	75	3.2×10^7	56 (257–313)	241	498	G
i	SR3B-11	25	2.9×10^6	181 (132–313)	1,085	1,213	UUAA
j	SR3B-12	150	4.6×10^6	0	248	561	None

[a] The virus stock obtained from TSX cross (Table 2) was plaqued on Vero cells, and well-separated representative plaques were isolated and suspended in 2 ml of minimal essential medium. Each virus suspension thus obtained was used to infect duplicate BHK cultures at an MOI of 0.01 to 0.1. The first set of cultures was labeled with uridine and harvested at 12 h postinfection. Supernatants from the second set of cultures were recovered at 24 h postinfection, and titers were determined on Vero cells. The cytoplasmic RNA isolated from the first set of cultures was reverse transcribed, PCR amplified, and sequenced, and crossover sites were mapped. The magnitudes of deletions and insertions as well as common bases located at the crossover site (COS) were determined from sequence data (Fig. 5 and 6a). Plaque sizes are estimates with respect to that formed by Toto 1002.

TABLE 4. Characterization of recombinants produced from Tapa 32/Sst and G26S-3HN/Sst (TGS cross)[a]

Recombinant	Plaque identification	Plaque size (%)	Virus titer (10^7 PFU/ml)	3′ deletions (bases [position])	3′ insertions (bases)	Total length of 3′NTR (bases)	Common COS bases
k	SR3A-5	75	5.2	117 (144–261)	502	644	AU
l	SR3A-6	75	4.9	11 (250–261)	298	546	CA
m	SR3A-7	25	6.9	6 (255–261)	166	420	A
n	SR3B-14	50	6.6	128 (133–261)	299	430	AU
o	SR3B-15	50	6.6	5 (256–261)	341	596	C
p	SR3B-16	50	6.9	117 (144–261)	156	299	A
q	SR3B-18	50	7.1	11 (250–261)	298	546	CA
r	SR3B-20	200	6.8	5 (256–261)	81	336	A

[a] The analysis was carried out as described in the footnote to Table 3. COS, crossover site.

kb) corresponding to wild-type SIN, Toto 1002. However, the PCRs corresponding to unplaqued virus mixtures always gave a smear indicating the presence of heterogeneous population of viruses with respect to the 3′NTR of SIN (Fig. 4b). As depicted in Fig. 4b, all of the eight plaques corresponding to Toto 1002 yielded a 0.3-kb PCR product, substantiating the specificity of the PCR amplification process. In addition, PCR analysis of a control region representing nt 10650 to 11350 of the SIN S coding domain of plaqued and unplaqued viruses, as expected, yielded a 700-nt band (data not shown), further supporting the fidelity of the PCR analysis even when mixed virus populations were used as the inoculum.

The PCR products corresponding to each of the plaqued viruses were purified from an agarose gel and subjected to dideoxy sequencing (59). In the first round, each of the PCR products was sequenced twice, using primers JC1295-1 and JC1000-1, respectively. On the basis of these sequence data, an additional round was performed with primer JC1200-1, which annealed to an internal sequence within the 3′NTR (Fig. 1). These experiments allowed us to precisely characterize the junction region between the UAG codon of the S coding sequence and the 3′NTR, the RNA-RNA crossover sites, and the 3′ sequences on the recombinants following the crossover site. In addition to the sequence data, we made use of the unique restriction enzyme sites located in the donor templates (S3P/Xho and G26S-3HN/Sst) and the 3′ nonviral sequences (bacterial β-lactamase gene) to construct a map of all recombinants (Fig. 5 and 6a) and to compare the sizes of the PCR fragments with the anticipated lengths of the 3′ regions of the recombinants. From these comparative analyses, we concluded that no multiple crossovers occurred within the two pairs of donor-acceptor systems studied in detail.

Crossover sites on donor and acceptor templates. Figure 5 gives the nucleotide sequences of donor and acceptor templates and the precise locations of crossover sites corresponding to all of the recombinants. Figure 6a gives a map of all crossover events and tracks the predicted route of polymerase travel during the template switching event. The two RNA motifs designated PD1 and PD2 appear to function as recombination hot spots. The first hot spot locus corresponds to nt −132 to −144, and the second site corresponds to nt −250 to −259. These two hot spot loci were found to be associated with similar secondary structures (data not shown). As discussed in our previous work (55), the viral polymerase probably initiated RNA synthesis at the 3′ terminus of the donor template (S3P or G26S-3HN), jumped onto the acceptor template (Tapa 32), and completed synthesis of full-length negative strand. It is logical to conceive that these hot spot loci function as polymerase detachment sites during RNA synthesis. Since the same two sites were used in both donor templates, it appears that the presence of additional sequences in S3P RNA does not influence the possible polymerase stalling at these two sites. No striking nucleotide homology between the two hot spot loci was apparent.

Contrary to the two-cluster nature of 3′ junction sites found on the donor templates, the reinitiation sites were found scattered over a range of 600 nt from the termination codon for

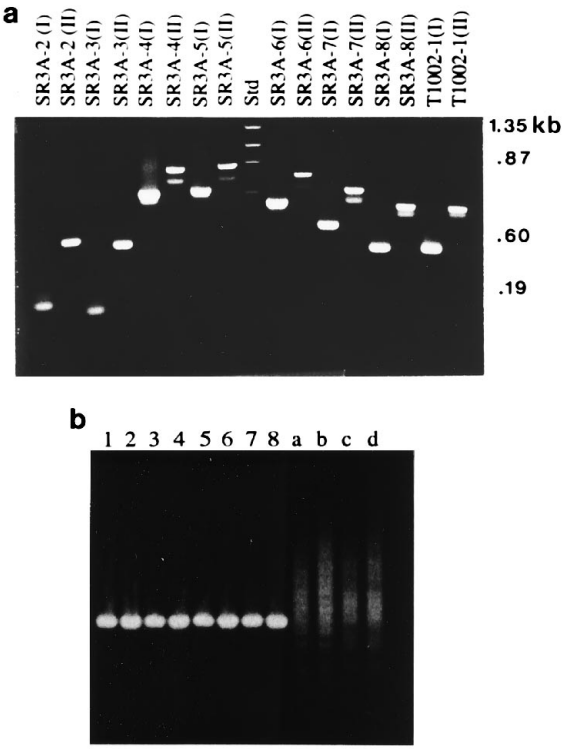

FIG. 4. PCR amplification of the 3′NTRs of viral RNAs. Five percent of a 100-μl aliquots of PCR products was analyzed on a 1.5% agarose gel. The samples used are indicated at the top. Cytoplasmic RNAs isolated from various virus (see below)-infected BHK cells were reverse transcribed with JC1295-1. One percent of the cDNA products obtained through reverse transcription of various cytoplasmic RNAs was amplified with either the first (I, JC1295-1 and JC1000-1) or second (II, JC1295-1 and T11150) set of primers. (a) Representative PCR reaction products corresponding to TGS and TSX crosses; (b) PCR reaction products corresponding to some controls. Lanes 1 to 8 correspond to eight different plaque-purified Toto 1002 virus-infected cells. Lanes a and b correspond to virus mixtures obtained from TSX cross, infected at MOIs of 0.01 and 1, respectively. Lanes c and d correspond to virus mixtures obtained from TGS cross infected at MOIs of 0.01 and 1, respectively. The PCR amplification was carried out with the first set of primers.

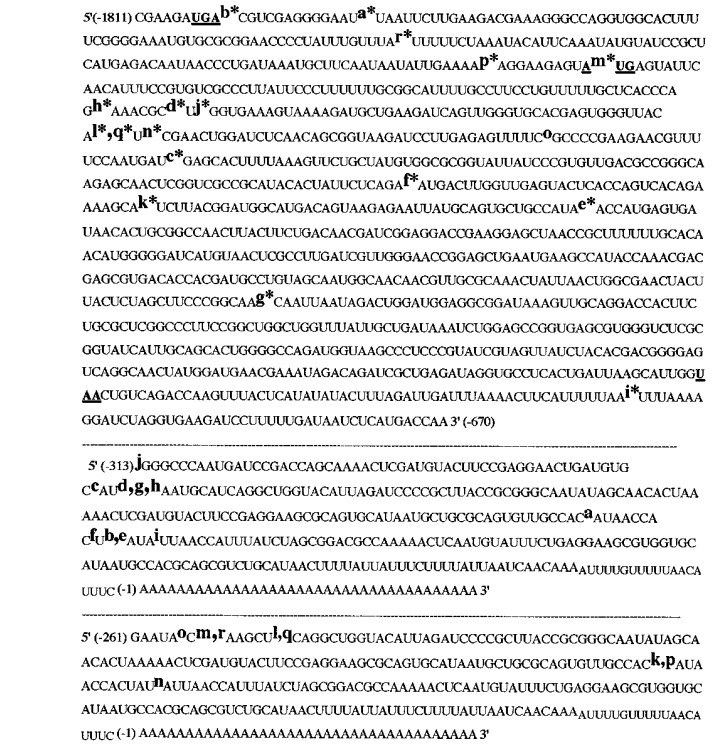

FIG. 5. Sequences of RNA substrates and the precise locations of crossover sites. The complete sequences of both the donor templates and the relevant portion of the 3′ nonviral region of the acceptor template are depicted. As suggested by Kuhn et al. (35), the numbering of donor templates starts from the −1 position of the 19-nt conserved region. Similarly, the extreme 3′ end of the acceptor template is numbered −1. The precise contributions of donor and acceptor RNAs toward the formation of individual recombinants are identified as follows. The boldface superscript at the 5′ side of a nucleotide position of donor sequences indicates the left boundary of the RNA sequence derived from the donor templates. Thus, RNA sequences downstream of the superscript are derived from the donor template. The same superscript with an asterisk at the 3′ side of a specific nucleotide position in Tapa 32/Sst represents the 3′ boundary of the sequences derived from the acceptor template. Thus, sequences upstream of a superscript with an asterisk represent the 5′ region of the recombinant RNA. The UGA stop codon of the SIN S coding region is indicated at nt −1805 of Tapa 32/Sst, and the AUG and UAA codons of the β-lactamase gene are underlined. Top, acceptor template, Tapa 32/Sst; middle, donor template, S3P/Xho; bottom, donor template, G26S-3HN/Sst.

SIN S polyprotein. Since the assay used in these studies was tailored to detect only viable SIN recombinants, as expected, all of the reinitiation sites were mapped to the nonviral sequence tagged onto the acceptor template, Tapa 32 (Fig. 6a). Examination of all reinitiation sites including a 15-nt flanking sequence revealed 30 to 60% homology between reinitiation sites (data not shown). Similarly, heteroduplex analysis of donor and acceptor templates also did not indicate any strong local RNA-RNA annealing processes (data not shown). Hence, it is not clear if sequence homology or local complementarity between substrate RNAs could have played a major role in these types of nonhomologous recombination events.

Roles of the SIN 3′ motif and hot spot loci in recombination. Since local RNA-RNA annealing did not seem to have played a major role in the reinitiation events reported above, we thought that RNA-protein interactions might have played a more dominant role in the selection of reinitiation sites. Since the initial recombination events between the template RNAs should have occurred during negative strand synthesis, it was possible that the jumping polymerase recognized an RNA mo-

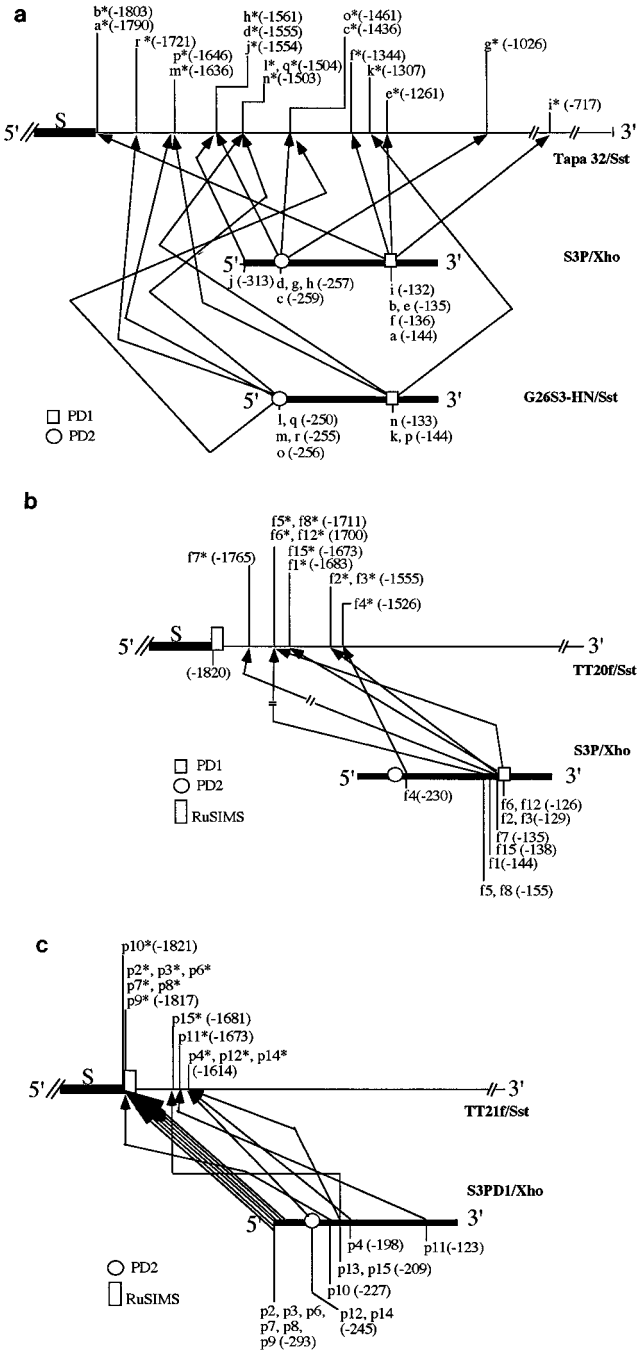

FIG. 6. Maps of crossover events between acceptor and donor templates. The donor and acceptor templates (positive sense) are numbered from their respective 3′ ends. Virus-specific sequences are identified as solid bars, and nonviral sequences are identified as thin lines. Each crossover event is identified by an arrow starting at a specific location of a donor template and ending at a specific location of an acceptor template. The crossover points are denoted with the same letters and asterisks as in Fig. 5. The exact distance of each crossover point from the 3′ terminus of the corresponding template is indicated in parentheses. Thus, each recombinant contains all sequences downstream of the start point of the arrow on the donor template and all sequences upstream of the end of the arrow (reinitiation site) on the acceptor template. Interruptions within a connecting arrow indicate multiple crossovers. The large arrowhead with multiple lines indicates the presence of six recombinants with the identical crossover point. PD1 and PD2, putative pause detachment sites; RuSIMS, a 17-nt rudimentary signal for initiation of minus-strand synthesis. (a) Crossovers between Tapa 32/Sst and S3P/Xho or G26S-3HN/Sst; (b) crossover events between TT21f/Sst and S3P/Xho; (c) crossover events between TT21f/Sst and S3PD1/Xho.

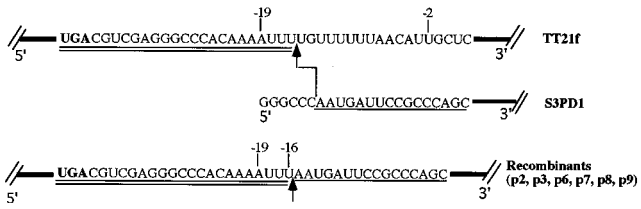

FIG. 7. Structures of recombinants, p2, p3, p6, p7, p8, and p9. The sequence derived from the donor template, S3PD1, is identified by a single underline. The sequence derived from the acceptor template, TT21f/Sst, is identified by a double underline. The sequence corresponding to −2 to −19 represent the 17-nt rudimentary signal for initiation of negative-strand synthesis (RuSIMS) or the replicase recognition signal. The recombinants carry four nucleotides (−16 to −19) of the RuSIMS at the crossover site.

tif resembling the promoter for initiation of negative-strand SIN RNA synthesis. To test this possibility, plasmid TT21f (Fig. 2a) was constructed. Plasmid TT21f carried a 17-nt motif which was identical to the 5′ 17 nt of the 19-nt SIN promoter for negative-sense RNA synthesis (35, 39). Plasmid TT21f was linearized at the *Sst*I site to produce an acceptor RNA containing the same nonviral sequences that were found in Tapa 32/Sst. BHK cells were transfected with TT21f/Sst and S3P/Xho RNAs (TTSX cross), and a recombinant virus stock was produced. Cells transfected with either one of the RNAs did not produce any virus. Sixteen individual plaque isolated viruses derived from TTSX cross were tested for their RNA synthetic abilities. As shown in Fig. 3b (lanes f1 to f16), SIN-specific 49S and 26S RNA species were expressed in all infected cells. Cytoplasmic RNAs isolated from 10 infected cells were PCR amplified and sequenced, and a crossover map was constructed. As shown in Fig. 6b, none of the 10 recombinants analyzed mapped to the 17-nt recognition motif located within the acceptor template. Interestingly, 9 of the 10 recombinants demonstrated pausing in the vicinity of the PD1 site on the donor template, indicating that the 17-nt insertion within the acceptor template did not significantly influence the possible polymerase pausing on the donor templates. More interestingly, the reinitiation sites within the acceptor template were found scattered within the proximal 0.3-kb region of nonviral sequences located just downstream of the termination codon for the viral S polyprotein (Fig. 7b).

To further understand the role of hot spot loci on donor templates, a 24-nt region corresponding to the PD1 locus in S3P RNA was deleted to obtain S3PD1 as described in Materials and Methods (Fig. 2c). In vitro transcripts were made from S3PD1 and TT21f, and a recombinant virus stock was produced by transfecting these two RNAs (TTSP cross) into BHK cells. Sixteen individual viral plaques were tested for the ability to produce 49S and 26S SIN RNAs (Fig. 3c, lanes p1 to p16) and PCR amplified to characterize the crossover sites. The 3′NTRs of 13 of the recombinants were completely characterized, and a map of crossover events was constructed. As shown in Fig. 6c, 6 of the 13 recombinants carried identical crossover sites. These six recombinants (p2, p3, p6, p7, p8, and p9) carried the entire donor sequence except for the terminal 6 nt at the 5′ end of the donor and part of the 17-nt SIN promoter (Fig. 7). Recombinants p12 and p14 made use of the second pause site, PD2. The remaining five recombinants made use of alternate locations within the donor template as crossover points (Fig. 6c). These results support the notion that specific sequences within the donor template can function as hot spot loci and removal of such sequences allows frequent readthrough of templates.

Common bases and nontemplated nucleotides at crossover sites. Comparison of nucleotide sequences surrounding the crossover sites of recombinants derived from TGS and TSX crosses (Fig. 5; Tables 3 and 4) indicated that one to four common bases can be found at the junction sites located in donor and acceptor templates. Similar results were obtained for TTSX and TTSP crosses as well (data not shown). Almost all of the recombinants contained only one set of these common bases, suggesting that they were templated only from one of the substrates. This observation is suggestive of a minimal base pairing between the 3′ end of nascent negative-sense RNA and the common bases at the reinitiation site on the acceptor template.

Illegitimate recombinations in RNA genomes have been known to introduce nontemplated bases at crossover sites and are thought to strongly indicate polymerase-mediated recombination (33) rather than RNA cleavage-ligation-mediated recombinations. In the present study, 4 of 41 recombinants carried nontemplated bases at the crossover site. Recombinant SR3B-10 (h) carried a pentamer (5′GAGGC3′) at the crossover site and reinitiated RNA synthesis at a site which contains a G as the common base. Recombinant SR3B-12 (j) carried a single A residue as a nontemplated base at the crossover site, but the reinitiation site did not possess any common base. Recombinant SR3A-5 (k) carried an octamer (5′AAAGAAG T3′) at the crossover site, and a single A residue was found as a common base at the reinitiation site. Finally, recombinant f7 carried a nontemplated octamer (ACGGCCGC) at its crossover site. Although numerous mammalian sequences were found to encode these oligomers during a homology search analysis, the exact donor for these oligomers is not known. RNA recombination events resulting in transduction of cellular sequences have been documented in many situations (13, 29, 33, 34, 42, 43), pointing to the role of viral polymerase in recombination processes.

Of the 41 recombinants whose 3′NTRs were completely characterized, 2 showed multiple crossover events within the viral genome. The 3′NTR of recombinant f5 contained two RNA motifs of the SIN genome, arranged in 5′-to-3′ orientation as found in the SIN genome. The first motif (Fig. 8, M1) corresponded to nt 6376 to 6352, and the second motif (Fig. 8, M2) corresponded to nt 9852 to 9831 on the Toto 1101 SIN clone. Although the crossover junctions in f5 did not contain any nontemplated sequences, one to three common bases could be found at the junctions of both donor and acceptor templates. As depicted in Fig. 8, each of these two noncontiguous sequence motifs, apparently transduced by the jumping polymerase, was arranged in the 5′-to-3′ orientation as found

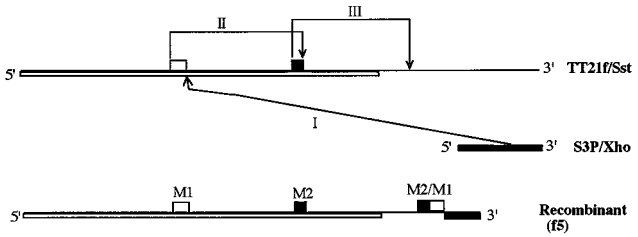

FIG. 8. Possible polymerase jumping events leading to the formation of recombinant f5. The donor template S3P/Xho is identified by as a solid bar. The virus-specific sequence of the acceptor template, TT21f/Sst, is identified as a long empty bar, and the nonviral sequence located at the 3′ region is identified as a thin line. The two internal motifs transduced from the coding region of acceptor templates are identified as M1 and M2. M2/M1 indicates the location and order of the transduced motifs within the 3′NTR of recombinant f5. The possible pathway for polymerase travel during the generation of this recombinant is indicated by lines with arrows.

in the SIN genome but in the reverse order. The 3′NTR of recombinant f7 carried one internal RNA motif from the SIN genome (nt 4906 to 4840) and a nontemplated RNA motif (ACGGCCGC) at the first crossover site. These observations suggest that the jumping viral polymerase can transduce internal sequences of the SIN genome and fuse them to the 3′NTR to generate novel recombinants.

Deletions and insertions within the 3′NTR. Tables 3 and 4 summarize the organization of the 3′NTRs of plaque-purified viruses whose 3′NTRs were mapped and sequenced. Sixteen of the 18 recombinants derived from TGS and TSX crosses were genetically heterogeneous with respect to their 3′NTRs. Only one of them was represented twice in the population. This finding indicated the randomness of recombination events and suggested the existence of a low selection pressure on the replication and packaging of recombinant RNAs. It is also clear from Tables 3 and 4 that majority of the recombinants lack some of the sequences from the donor template. The deletions ranged from 5 to 181 nt. All of the recombinants except recombinant SR3A-3 (b) acquired nonviral sequences from the 3′ end of the acceptor template. The addition of nonviral sequences to the 3′NTR ranged from 10 to 1,085 nt. Fourteen of the recombinants had nonviral insertions of less than 550 nts, suggesting a constraint in the recombination event or packaging of large viral RNAs. One recombinant (SR3B-7 [g]) had a 776-nt nonviral insert. Recombinant SR3B-11 (i) carried an insert of 1,085 nt which encompassed the complete coding sequence for the β-lactamase gene from Tapa 32/Sst RNA. The total length of the resultant 3′NTR for each of the recombinants (Tables 3 and 4) ranged from 134 to 1,213 nt. Results obtained from the analysis of the 23 recombinants generated by TTSX cross (Fig. 6b) and TTSP cross (Fig. 6c) confirmed the occurrence of deletions and insertions within the 3′NTRs of the recombinants. Taken together, these results demonstrate that a multitude of viable deletions and insertions can be introduced at the 3′NTR of SIN by transfection of cells with nonreplicative RNA precursors. These results also indicate that the 3′NTR of SIN can be engineered to expression foreign sequences by in vivo recombination of nonreplicative RNA precursors.

DISCUSSION

Although conclusive studies to eliminate a major role for the cleavage-ligation model for viral RNA recombination are yet to be done, the existing evidence strongly favors template switching of viral polymerase as a major mechanism for viral RNA recombination. Kirkegaard and Baltimore (32) pointed out that at least one of the participating RNA genomes did not need to replicate to function as a substrate for RNA recombination. Studies carried out in plant viral systems (9, 26, 44, 56) confirmed this observation and substantiated the fact that dislodged polymerases along with nascent negative-strand can load on positive-sense RNA templates devoid of authentic 3′ ends and reinitiate RNA synthesis, leading to formation of viable recombinants. By making use of terminally truncated versions of a genomic RNA and a defective interfering RNA derived from separate members of the tombusvirus family, White and Morris (70, 71) demonstrated the in vivo generation of a functional hybrid plant viral genomes. Our recent demonstration (55) that the two halves of the SIN genome can recombine in vivo to produce live virus, the present studies on recombination at the 3′NTR of SIN, and the results of White and Morris (70, 71) confirm the ability of nonreplicative RNA templates to undergo recombination in vivo both in animal and plant cells to produce infectious viruses. Since none of the transfected negative-sense RNAs served as substrates for RNA recombination (55), and since all of the transfected nonreplicative RNAs are of positive polarity, it is logical to conclude that at least the initial recombination should have occurred during negative-strand synthesis. The central role played by the transfected RNAs in generating viral recombinants was also demonstrated by the inability of RNase-treated RNA samples to produce any infectious virus (55). The importance of exogenously supplied viral polymerase in mediating the intracellular recombination event was also verified by transfecting an RNA substrate which carried an interrupted polymerase coding region (55).

Studies of majority of the viral systems made use of full-length viral genomes or infectious transcripts to study recombination in vivo. Since our present studies made use of nonreplicative RNA fragments, no interference or competition from wild-type helper virus was anticipated. It is prudent to expect recombinants resulting in lethal mutations within protein coding sequences of the acceptor template to be selectively eliminated. Similarly, it is possible for recombinants with replicative advantages to be selectively amplified and hence overrepresented in the virus population. However, the data obtained from TGS, TSX, and TTSX crosses indicated that most of the recombinants were represented only once, suggesting a minimal selection pressure during virus production. Since the present studies involved recombination at the 3′NTR of the SIN genome, and since the 3′NTR of the SIN genome was found to accommodate several foreign sequences (7, 20, 53), it was also logical to expect minimal selection pressure to operate during virus production.

The finding that the majority of the recombinants had a 3′NTR in the range of 150 to 600 nt strikingly agrees with the natural length variance of 3′NTRs of various alphaviruses (35, 66). It is possible that the size of the 3′NTR affects replication and packaging efficiencies of the viral genome. It is also possible that the structures and sequences within the 3′NTR in turn regulate the capacity of this region to accommodate foreign sequences. Kuhn et al. (35) reported extensive mutagenesis studies on the 3′NTR of the SIN genome and demonstrated the remarkable plasticity of this region. Our present studies confirm their conclusion and indicate additional features of the plasticity such as the ability to accommodate foreign sequences of several hundred nucleotides.

The donor and acceptor templates used in this study did not possess any significant homology between them. Examination of all crossover sites also failed to reveal any appreciable base-pairing ability. Perfect heteroduplexes longer than 20 bp between donor and acceptor templates were proposed to be required to support recombination in brome mosaic virus (44). Since such sequences and structures were not found in our study, these recombination events may be driven by RNA-protein interactions as recently described for the coronavirus system (68, 73). However, the transcription and/or recombination events leading to the synthesis of coronaviral mRNAs are known to be site specific. It is also possible that negative homology search data may not completely rule out a role for RNA-RNA interaction for efficient polymerase switching. At the least, the occurrence of one to four common bases at the crossover locations of many recombinants suggests limited base pairing between nascent transcripts and acceptor templates. Analysis of recombinants generated by TTSX cross (Fig. 6b) failed to suggest a strong role for the SIN promoter-like 17-nt motif in recombination. Since the same 17-nt sequence was also found at the 3′ terminus of the donor template (S3P/Xho), a homologous type of recombination could have taken place. Since none of the recombinants generated by

TTSX cross (Fig. 6b) mapped to the 17-nt motif located in the TT21f template, it was possible that the 17-nt motif was not accessible to the jumping polymerase as well as for any base-pairing event with donor or nascent RNA. However, the results obtained from TTSP cross (Fig. 6c) indicated that removal of a putative pause site sequence, PD1, from the donor template resulted in the generation of at least 50% of recombinants which map to the 17-nt motif in the TT21f RNA. It is also possible that these six recombinants with identical crossover sites are actually the same virus possessing high replicative advantage. These results appear to suggest a role for donor template or donor-derived RNA transcript in modifying the structure of the acceptor template, resulting in unmasking of cognate reinitiation sites embedded within acceptor templates. Further studies making use of acceptor templates carrying the 17-nt motif at alternate locations within the 3′ nonviral sequences are needed to explore the role of the 17-nt motif, its context, and the influence of the donor template. It is interesting that studies of the brome mosaic virus system indicated that homology of as little as 15 nt between donor and acceptor templates could support homologous recombination (45).

Specific polymerase recognition signals and structures were proposed to play an important role in the recombination events between turnip crinkle virus and its satellite RNAs (10–12). The apparent occurrence of two hot spot loci within the donor templates S3P and G26S3-HN suggests a sequence and/or structure associated with these two sites. Although little sequence homology could be demonstrated between the two hot spot loci PD1 and PD2, similar secondary structures could be formed by folding a 80-nt region surrounding these two sites. These two hot spot loci might be considered putative pause sites for the viral polymerase. Interestingly, the S3PD1 donor template, which lacks the PD1 site, formed an alternate kind of stem-loop structure (data not shown). Although it is possible that specific secondary structures formed at the putative pause detachment sites facilitate dislodging of polymerase from the donor template, further studies are needed to confirm this hypothesis. Results with the tombusvirus system suggested that strong secondary structure in the donor template facilitated dissociation of the replicase from its template (71). Sequence comparison and homology search analysis of all of the reinitiation sites described in the present work also suggested the involvement of AU-rich sequences in recombination events. AU-rich sequences have been shown to facilitate the generation of imprecise homologous recombinants in brome mosaic virus (46). Since the 3′NTR of SIN, the SIN 3′ promoter, and numerous locations within the 3′ nonviral sequences of acceptor template are rich in AU-rich sequences, the role of AU-rich sequences in SIN recombination remains to be defined. It is also to be noted that the leader-body fusion events during coronavirus transcription take place at AU-rich intergenic sequences (68, 73).

Previously (55) we used an acceptor template (GNS-2) devoid of any nonviral sequences at its 3′ end and showed that the polymerase was able to precisely utilize all 3′ sequences of the template. Our present results seem to suggest that the presence of nonviral sequences located at the 3′ region of the acceptor template (Tapa 32) actually facilitates RNA-RNA recombination. Although it is not completely clear how a dislodged polymerase complex chooses an acceptor template among a pool of available RNAs within the cytoplasm, it is logical to assume that RNA-RNA and/or RNA-protein interactions play a role in this process. The apparent polymerase jumping events observed in recombinant f5 (Fig. 8) suggest some clues in this regard. During the formation of recombinant f5, the polymerase appears to have first jumped from the donor template to a site closer to 5′ region of acceptor template. After copying a 25-nt region of the acceptor template, the polymerase made a second jump to a location 3′ to the first site on the acceptor template. At the second site, a 21-nt region of the acceptor template was copied, and the polymerase appears to have made a third jump to a location 3′ to the second site. The third site happened to fall within the nonviral sequences located at the 3′ end of the acceptor template. From the third reinitiation site, the polymerase appears to have traveled all the way to the 5′ end of the acceptor template and made a functional recombinant. The successive relocations of polymerase from the 5′ to the 3′ region of the acceptor template can also mean movement of template in the 5′ direction relative to the polymerase. These observations seem to suggest that the polymerase can load on internal locations within the acceptor template and apparently scan the acceptor template in both 5′ and 3′ directions. It is also possible that the two internal motifs found within recombinant f5 were derived from two different acceptor templates. It is to be noted that the majority of the recombinants analyzed carry only one crossover junction. Even if multiple polymerase jumps are a prerequisite for all recombination events, they do not seem to result in multiple reinitiations (crossover events). It is also possible that most of the recombinants which have undergone multiple crossover events in the SIN system that we used are not viable because of the deleterious rearrangements introduced within protein coding and other regulatory sequences.

The fact that most of the recombinants carried only sequences which were associated with donor or acceptor templates seems to suggest that the viral polymerase exhibits specificity in selecting template RNAs during its travel within the cytoplasm. Many RNA viruses, including SIN, are suggested to possess limited base-pairing abilities between the 5′ and 3′ ends of their genomes (35, 47, 48, 66). Thus, the 3′ end of the donor template could have base paired with the 5′ end of the acceptor template, albeit to a limited extent, facilitating the association of donor and acceptor templates. It is also possible that the polymerase plays a primary role in selecting the template by recognizing a terminal or internal motif within the acceptor template RNA. Once an appropriate template is selected, the precise site of reinitiation may be facilitated by bidirectional polymerase movement and accompanying RNA-RNA and/or RNA-protein interactions. Although some of these concepts are speculative, they are experimentally testable.

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