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RNA-RNA Recombination in Sindbis Virus: Roles of the 3′ Conserved Motif, Poly(A) Tail, and Nonviral Sequences of Template RNAs in Polymerase Recognition and Template Switching

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

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

Received 25 October 1996/Accepted 23 December 1996

Sindbis virus (SIN), a mosquito-transmitted animal RNA virus, carries a 11.7-kb positive-sense RNA genome which is capped and polyadenylated. We recently reported that the SIN RNA-dependent RNA polymerase (RdRp) could initiate negative-strand RNA synthesis from a 0.3-kb 3′-coterminal SIN RNA fragment and undergo template switching in vivo (M. Hajjou, K. R. Hill, S. V. Subramaniam, J. Y. Hu, and R. Raju, J. Virol. 70:5153–5164, 1996). To identify and characterize the viral and nonviral sequences which regulate SIN RNA synthesis and recombination, a series of SIN RNAs carrying altered 3′ ends were tested for the ability to produce infectious virus or to support recombination in BHK cells. The major findings of this report are as follows: (i) the 3′-terminal 20-nucleotides (nt) sequence along with the abutting poly(A) tail of the SIN genome fully supports negative-strand synthesis, genome replication, and template switching; (ii) a full-length SIN RNA carrying the 3′-terminal 24 nt but lacking the poly(A) tail is noninfectious; (iii) SIN RNAs which carry 3′ 64 nt or more without the poly(A) tail are infectious and regain their poly(A) tail in vivo; (iv) donor templates lacking the poly(A) tail do not support template switching; (v) full-length SIN RNAs lacking the poly(A) tail but carrying 3′ nonviral extensions, although debilitated to begin with, evolve into rapidly growing poly(A)-carrying mutants; (vi) poly(A) or poly(U) motifs positioned internally within the acceptor templates, in the absence of other promoter elements within the vicinity, do not induce the jumping polymerase to reinitiate at these sites; and (vii) the junction site selection on donor templates occurs independently of the sequences around the acceptor sites. In addition to furthering our understanding of RNA recombination, these studies give interesting clues as to how the alphavirus polymerase interacts with its 3′ promoter elements of genomic RNA and nonreplicative RNAs. This is the first report that an in vitro-synthesized alphavirus RNA lacking a poly(A) tail can initiate infection and produce 3′ polyadenylated viral genome in vivo.

RNA viruses are known to evolve rapidly in nature (1, 14, 19, 24, 26, 32, 60, 61). Emergence of new virus strains with altered virulence and tissue and host specificity is an important consequence of virus evolution. RNA recombination, one of the critical forces in virus evolution, results in genome rearrangements and formation of chimeric RNA genomes (34). Many eucaryotic and procaryotic RNA viruses are demonstrated to undergo recombination (4, 7, 9, 10, 27, 30, 31, 34, 39, 51, 54, 60, 63, 65). Although viral RNA recombination can occur by specific enzymatic cleavages followed by ligation of substrate RNAs, definitive evidence for this mechanism is lacking. Results obtained from several viral systems indicate that template switching of viral RNA-dependent RNA polymerase (RdRp) during replicative RNA synthesis is responsible for RNA recombination (7, 12, 31, 34, 40, 47). The molecular features of substrate RNAs and proteins which regulate polymerase jumping events within or between template RNAs are not fully understood. Some of the RNA determinants implicated in template switching are (i) sequence or structural homology; (ii) cryptic or authentic polymerase recognition motifs; and (iii) potential base pairing between RNA sequences (7, 9, 10a, 11, 34). It is conceivable that the intracellular concentrations of template RNAs, polymerase proteins, and specific host factors also regulate recombination frequency and crossover loci (27).

Sindbis virus (SIN) is one of the best-studied member of the *Alphavirus* genus of the *Togaviridae* family (28, 62). Members of the *Alphavirus* genus are transmitted by mosquitoes to animals and humans, and they cause a variety of human illnesses such as fever, arthritis, and encephalitis (20, 28). The genome of SIN consists of an 11.7-kb positive-sense RNA, which is capped at its 5′ end and polyadenylated at its 3′ end (62). Upon entry into host cells, the incoming genomic RNA of SIN is translated to produce viral RNA polymerase, which in turn binds to the genomic 3′ end to make a negative-sense RNA intermediate. The negative-sense RNA is then utilized as a template to produce full-length genomic RNA and a 4.1-kb 3′-coterminal subgenomic RNA. Conserved motifs located at the termini and at internal locations within the SIN genome regulate RNA synthesis and replication (46, 62). In vitro-synthesized SIN RNAs are extensively used to study regulation of SIN RNA synthesis, development of RNA viral vectors, and pathogenesis of alphavirus infections (6, 16, 18, 20, 22, 35, 36, 49, 51, 53, 62, 67).

The demonstration of a cellular tRNA at the 5′ end of a SIN defective interfering (DI) RNA (42), the indication that western equine encephalitis virus is a recombinant product of SIN and eastern equine encephalitis virus (21, 62a), and the experimental generation of full-length SIN from a DI and a helper RNA (63), taken together, underscore the importance of recombination in alphavirus biology and evolution. We recently

* Corresponding author. Mailing address: Department of Microbiology, Meharry Medical College, School of Medicine, 1005 D. B. Todd Blvd., Nashville, TN 37208. Phone: (615) 327 6687. Fax: (615) 327 6602. E-mail: ramasa25@ccvax.mmc.edu.

TABLE 1. Oligonucleotides used in this study

Name	Sequence	Location[a]	Polarity
Apa1-5	AAGCTGGGGCCCTTAACATTTCAA	3′19	+
Apa1-10	AAGCTGGGGCCCTTAACATTTCAA	3′19	+
AX3-17	AAGCTGGGGCCCACAAAATTTTGTTTTTAACATTGGCTCGAGGACAGA[b]		+
AX3-T	AAGCTGGGGCCCACATTTTTTTTTTTT TTTCATTGGCTCGAGGACAGA		+
AX3-A	AAGCTGGGGCCCACAAAAAAAAAAAAAACATTGGCTCGAGGACAGA		+
AXP-1	AAGCTGGGGCCCACA		+
AXP-2	TCTGTCCTCGAGCCA		−
JC1350	GAAATGTTAAAAACAAAA	3′NTR	−
JC1295-1S	CCTAGAGCTCAAAGTTATGCAGACGCTG	3′NTR	+
JC1295-3	AAAGTTATGCAG	3′NTR	+
JC1200-2	CACTGCGCAGCA	3′NTR	−
JC1000-1	GCTGACTAGCACACGAAG	3′S	+
JC1000-1H	CTGCAGAAGCTTGCTGACTAGCACACGAAG	3′S	+
JC1000-2	GCTTGCAGCATGATGCTGACT	3′S	+
JC3259	AATCAGCAGGGTCATCGC	3′V	−
SP6P	CACATACGATTTAGGTGA	SP6	+
T11350	TAGTCAGCATCATGCTGC	3′S	+
T11820	AGGGGTTCCGCGCACATTTC	3′NV	−
T11600B	GCAGCGTCTGCATAACTT	3′NTR	+
T11200H	CTGCAGAAGCTTATGTAAACCACCAGCTGA	3′S	−
T11900	AGGGAATAAGGGCGACAC	3′NV	−
11750Xho	AAGAATTAATTCCCCTCGAG	3′NV	−
19Xho	CTAGCACTCGAGGAAATGTTAAAAACAAAAT	3′19	−
18TSac	CCTAGAGCTCAGTTTTTTTTTTTTTTTTT	PA	−
12TSac	CCTAGAGCTCAGTTTTTTTTTTTTGAAATG	PA	−
3′29Apa	AAGCTGGGGCCCAATCAACAAAATTTTGTTTTT	3′NTR	+
3′63Apa	AAGCTGGGGCCCCAGCGTCTGCATAACTTTTAT	3′NTR	+
3′CS14	TAGCTAGGGCCCAGTTTTTAACATTTC	3′19	+
3′CS16	TAGCTAGGGCCCATTGTTTTTAACATTT	3′19	+
3′CS20	TAGCTAGGGCCCAATTTTGTTTTTAACATTTC	3′19	+
3′19SAC	CCTAGAGCTCGAAATGTTAAAAACAAAA	3′19	−

[a] The region on the SIN genome to which each oligonucleotide anneals: 3′19, 3′19-nt conserved motif; 3′NTR, internal sequence of the 3′NTR; 3′S, 3′ end of the S-coding region; 3′V, 3′ end of Toto vector (53); 3′NV, 3′ nonviral sequences encoding β-lactamase gene; PA, poly(A) motif.

[b] The 17-nt promoter region is underlined.

discovered that the full-length SIN genome could be generated in vivo by transfecting mammalian cells with two RNA fragments corresponding to the 5′ 7.6 kb and the 3′ 4.1 kb of the SIN genome (51). We also demonstrated that a 0.3-kb 3′-coterminal SIN RNA fragment could recombine with a SIN genomic RNA whose 3′ 0.3 kb was replaced by a 1.8-kb nonviral sequence (22). These results implied that the 0.3-kb 3′-coterminal SIN RNA (donor template) contained all necessary signals for SIN RdRp recognition in vivo to synthesize nascent negative-sense RNAs (NNSR). SIN RdRp apparently made use of these NNSR as primers for elongation on the cotransfected acceptor templates to produce full-length negative-sense RNA by template switching (22). Analysis of viable recombinant viruses generated from these studies (22) revealed the nonhomologous nature of crossovers and suggested a role for cryptic RNA promoter elements in template switching. Here we report studies carried out to unravel the role of promoter elements and adjoining nonviral sequences which regulate polymerase recognition and template switching during negative-strand SIN RNA synthesis.

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides used in this study are described in Table 1.

Plasmids. Tapa (51) contains the complete coding region for SIN nonstructural and structural (S) proteins positioned downstream of an SP6 promoter (Fig. 1A). In addition to carrying all sequences of the parental Toto 1101 (53), Tapa carries a unique *Apa*I site at the 3′ end of the S-coding region.

TT21f (22) was constructed by digesting Tapa with *Apa*I and *Xho*I, and the 13.4-kb fragment lacking the SIN 3′ nontranslated region (3′NTR) was isolated. A double-stranded oligonucleotide representing 17 nucleotides (nt) of the 19-nt 3′ promoter region of SIN was synthesized by PCR amplification and inserted at the *Apa*I and *Xho*I sites of the 13.4-kb vector fragment. For PCR amplification, oligonucleotide AX3 was used as a template. AXP-1 and AXP-2 were used as 5′ and 3′ primers, respectively. PCR-amplified material was digested with *Apa*I and *Xho*I and directly cloned into the 13.4-kb vector and sequenced by using primer T11750(−) to confirm the identity.

TT21g was made by ligating the 13.4-kb *Apa*I-*Xho*I fragment of Tapa to a 36-nt DNA fragment which carries a poly(T) motif. To obtain the 36-nt poly(T)-containing DNA fragment, the template AX3-T was amplified by PCR with primers AXP-1 and AXP-2 and digested with *Apa*I and *Xho*I.

TT21h carries a poly(A) motif between the *Apa*I and *Xho*I sites of the Tapa plasmid. The poly(A)-containing DNA motif was obtained by PCR amplification of the template AX3-A with primers AXP-1 and AXP-2 and subsequent digestion with *Apa*I and *Xho*I.

S3P (Fig. 1C) was constructed by ligating the 1.5-kb *Apa*I-*Pvu*I DNA fragment carrying the SP6 promoter from JUNCAT (22, 49) to the 1.1-kb *Apa*I-*Pvu*I fragment of Tapa, which contains the complete 3′NTR of SIN, including a poly(A) tail.

To make S3Ps and its derivatives, plasmid S3P was amplified by PCR with primers 19Xho and SP6P to obtain the SIN 3′NTR without the poly(A) tail. The 0.3-kb PCR fragment thus obtained was digested with *Apa*I and *Xho*I and ligated to the 2.3-kb *Apa*I-*Xho*I fragment obtained from S3P.

To make S3Pk and TT21k, the template AX3-17 was PCR amplified with primers AXP-1 and 19Xho, digested with *Apa*I and *Xho*I, and ligated to the 2.3-kb *Apa*I-*Xho*I fragment of S3P to obtain S3Pk. The 2.3-kb *Apa*I-*Sst*I fragment of S3Pk was ligated to the 11.7-kb *Apa*I-*Sst*I fragment of Tapa to obtain TT21k.

To make S3P and TT21 derivatives carrying a truncated 3′NTR of SIN, plasmid S3P was used as a template to PCR amplify portions of the 3′NTR. The negative-sense primer JC3259, which anneals just downstream of the β-lactamase gene of S3P, was used in conjunction with one of the positive-sense 3′NTR-specific primers to amplify S3P. The PCR products were digested with *Sst*I and *Apa*I, and the 2-kb DNA fragments carrying the various mutants of the 3′NTR were ligated with a 0.3-kb *Apa*I-*Sst*I fragment of S3P which carries the SP6 promoter. The 3′NTR-specific positive-sense primers used in PCRs and their motifs (Fig. 1) were as follows: Apa1-5, i; Apa1-10, j; 3′CS14, 1; 3′CS16, m; 3′CS20, n; 3′29Apa, q; and 3′63Apa, r. Plasmid S3Ps was amplified by PCR with the negative-sense primer JC3259 and the following positive-sense primers to obtain the motifs indicated: 3′29Apa, qa; and 3′63Apa, ra. The S3P derivatives carrying each of the mutant 3′ NTRs were sequenced by using primer SP6P to

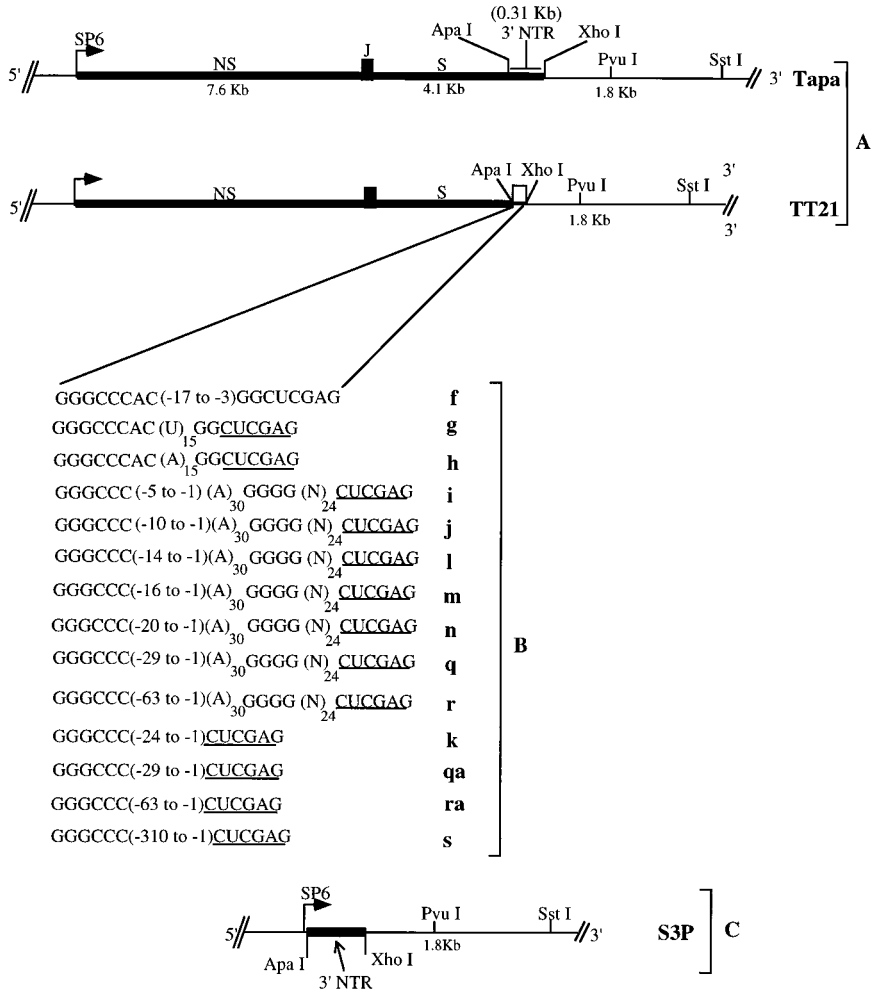

FIG. 1. Structures of plasmid constructs. (A) Tapa, one of the full-length SIN cDNAs carrying the entire SIN sequence; TT21, a derivative of Tapa which lacks the SIN 3′NTR. The sizes of the polymerase-coding region (NS) and structural protein-coding region (S) and the nonviral sequences which can be found at the 3′ end are indicated. The 3′NTR which carries the promoter for negative-strand synthesis is flanked by *Apa*I and *Xho*I sites. These two unique sites in TT21 and S3P were used to introduce the various motifs of the 3′NTR. (B) Motifs f, g, h, i, j, k, l, m, n, q, r, qa, ra, and s, which represent deletion versions of the SIN 3′NTR. The numerals in parentheses refer to the nucleotide positions of the SIN 3′NTR contained within a motif. As originally suggested by Kuhn et al. (33), the −1 position of the 3′NTR correspond to the first viral nucleotide abutting the poly(A) tail at the 3′ end of SIN. The length of the poly(A) tail contained within a motif is indicated by a subscript. The 24-nt nonviral sequence identified as $(N)_{24}$ is AATTCCTCGATAATTAAGCGGCCG. The *Xho*I site at the 3′ end of each motif is underlined. (C) S3P, an RNA expression vector which carries the full-length SIN 3′NTR (22). The full-length 3′NTR includes positions −310 to −1 of the SIN genome (33) and the abutting poly(A) tail. TT21 and S3P derivatives are identified by the name of the parental plasmid followed by a letter corresponding to the motif. For example, plasmid TT21g refers to the TT21 plasmid which carries motif g between the *Apa*I and *Xho*I sites. The *Xho*I or *Sst*I site can be used to linearize the plasmid for in vitro transcription reactions. Each in vitro-transcribed RNAs is identified by the DNA template from which it is derived and the restriction site used to linearize the plasmid. For example, TT12g/Xho refers to an RNA derived from plasmid TT21g, which is linearized with *Xho*I.

confirm the various motifs introduced in S3P. The 2.3-kb *Apa*I-*Sst*I fragment obtained from each of the S3P derivatives was ligated to the 11.4-kb *Apa*I-*Sst*I fragment of Tapa to obtain the various 3′NTR mutants of the SIN genome (TT21 derivatives).

In vitro synthesis of RNA transcripts. Five to 10 μg of each plasmid was digested with the appropriate restriction enzyme and precipitated with ethanol. In vitro transcription of template DNA was carried out with 2 to 4 μg of DNA in a total volume of 30 μl essentially as described previously (49–51, 53). All transcription reactions were performed with 1.5 mM cap analog and 1 μCi of [^{3}H]UTP as a radioactive tracer. After 1 h of incubation at 38°C, the template DNA was digested with 1 μg of RNase-free DNase I (Life Technologies), and the 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 with glyoxal (48, 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 (50).

Cells, viruses, and infection. BHK-21 and Vero cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum. BHK cells were used for RNA transfection studies, preparation of virus stocks, and analysis of viral gene expression. Since Vero cells form confluent cultures quickly and consistently, they were used to titrate virus stocks and to isolate individual virus plaques for further studies. Well-separated plaques were recovered and directly suspended in 600 μl of MEM. For virus infections, BHK cells grown in 35-mm-diameter petri plates were infected with 200 μl of the virus suspension at a multiplicity of infection of 0.1 to 2 and incubated at 37°C for the desired times.

Transfection of BHK cells with RNA. Transfection of BHK cells was carried out essentially as described previously (49, 51). Briefly, semiconfluent BHK cells were washed twice with isotonic saline, layered with 0.2 ml of phosphate-buffered saline containing 40 to 800 ng of each of the in vitro-transcribed template RNAs and 25 μg of Lipofectin or Transfectace (Life Technologies), 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 MEM containing 10% fetal bovine serum and incubated at 37°C. Cells were monitored for cytopathic effect (CPE) every 5 to 6 h. The culture supernatant was recovered after 2 to 3 days and stored frozen. To determine the specific infectivities of the RNAs, the transfected cells were layered with agarose and monitored for plaques. Transfection experiments which resulted in no virus production were repeated three times, and the culture supernatant derived from these transfections was passaged twice to confirm the absence of virus production.

In vivo labeling of viral RNAs, isolation and analysis of cytoplasmic RNA. BHK cells were infected with plaque-purified viruses as described previously (22). At 1 to 3 h postinfection, 0.6 ml of MEM containing dactinomycin (5 μg/ml) was added to the plates. Twenty minutes later, 50 μCi of [^{3}H]uridine (NEN) 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 as described previously (50). Approximately 5 to 8 μg of the isolated RNA was denatured with glyoxal, analyzed on a 1.25% agarose gel, and fluorographed as previously described (49, 51).

Reverse transcription of cytoplasmic RNA, PCR amplification, and sequencing. The first-strand synthesis involved annealing of 1 to 4 pmol of appropriate negative-sense primer with 5 to 8 μg of cytoplasmic RNA in 0.3 M NaCl and extension with murine leukemia virus reverse transcriptase (22). 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 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 10-fold and used directly for PCR amplification. The PCR mixture consisted of 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 deoxynucleoside triphosphates in a volume of 50 μl. After 20 cycles of PCR amplification, the reaction mixture was removed and 10% of the PCR products were analyzed on a gel. Since the PCR products corresponded to plaque-purified viruses, a single species of DNA product was obtained almost all times. As previously described (22), several control PCRs and nested PCRs were routinely used to ascertain the authenticity of the PCR products. The size of each PCR product was compared with the agarose gel profile of the corresponding viral RNAs to quickly identify discrepancies. Ten percent of 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 subjected to cycle sequencing using a Perkin-Elmer sequencing kit. Since most of the PCR products carried *Hin*dIII and *Sst*I restriction sites at their termini, they were digested with these enzymes and cloned in plasmid SP72 or pGem3. Plasmid DNA isolated from 1.5-ml cultures was subjected to cycle sequencing to analyze the 3′NTRs of recombinants.

RESULTS

Role of the poly(A) motif in polymerase reinitiation. Previously (22), we made use of a donor template (S3P/Xho [Fig. 1]) which carried the complete 3′NTR of SIN and an acceptor template (TT21f [Fig. 1]) which carried an 1.8-kb nonviral sequence at its 3′ terminus. Since these substrate RNAs lack the ability to replicate individually, as expected, the virus particles released from the transfected cells were found to be recombinants between the donor and acceptor templates (22). Sequence analysis of the individual plaque-purified viruses suggested recombination hot spots on the donor template and the utilization of AU-rich or A-rich RNA motifs as reinitiation sites on the acceptor template. To test if an A-rich RNA motif of an acceptor template induces recombination at that site, cells were transfected with the in vitro-synthesized RNAs TT21h/Sst and S3P/Xho (Fig. 1). As shown in Fig. 1A, the TT21h/Sst RNA carried the entire SIN protein-coding region, a 15-nt internal oligo(A) sequence, and an 1.8-kb nonviral sequence at its 3′ end. On the basis of previous results (22, 51), it was anticipated that the S3P/Xho RNA would serve as a donor template allowing the polymerase to make NNSR. It was also anticipated that the SIN RdRp would utilize these NNSR as primers to reinitiate on the acceptor template TT21h/Sst.

As expected, cells transfected with both RNAs gave rise to high-titer virus particles (Table 2; experiment 36). Control cultures transfected with only one of the template RNAs did not produce any virus (Table 1, experiments 4 and 28). Sixteen individual virus plaques were isolated from the virus stock and were directly used to infect BHK cells. Labeled cytoplasmic RNA was isolated from these cultures and analyzed by denaturing agarose gel electrophoresis. As previously reported (22), all recombinants expressed both genomic RNA and subgenomic RNA of SIN (data not shown). As shown in Table 3, the 3′NTRs of 16 of the recombinants were amplified by PCR, cloned in plasmid SP72, and sequenced to ascertain the crossover junctions. By using JC1000-1H, which annealed to the end of the S-coding region as a primer for sequencing, we determined that 15 of the 16 recombinants had crossover sites well beyond the internal oligo(A) motif (data not shown). This observation indicated that the internal oligo(A) motif itself did not induce the polymerase to reinitiate at this locus. The crossover sites of 11 recombinants were fully characterized (Table 3, assay A). Recombinant ASS17, whose crossover site mapped to the internal oligo(A) motif (−1821), carried six A residues at the crossover site. The crossover sites of other recombinants mapped from −672 to −1650 of the acceptor template. The lengths of these internal oligo(A) motifs in other recombinants varied from 9 to 76 nt, indicating polymerase stuttering at oligo(A) motifs. To test whether reverse transcription and PCR (RT-PCR) amplification of cytoplasmic RNA results in polymerase stuttering, the TT21h/Sst template RNA was subjected to RT-PCR amplification using primers T11750 Xho and T11200H and cloned in SP72. Sequence analysis of 14 of these clones revealed that the length of poly(A) varied from 13 to 29 nt, indicating stuttering during the RT-PCR procedure (data not shown). However, it should be noted that sequencing of plasmid TT21j with *Taq* polymerase consistently gave a 15-nt poly(A) motif. Since the size of poly(A) within the TT21h/Sst-derived viral recombinants varied up to 76 nt, it appears that the viral RdRp undergoes stronger stuttering at the oligo(A) locus than does the SP6 polymerase or reverse transcriptase. It should be noted that the crossover sites on the donor template map to selected internal locations (166 to 168 and 237 to 253) of S3P/Xho (22).

As a control experiment for oligo(A)-induced polymerase reinitiation on acceptor templates, we tested the role of a poly(U) motif by using acceptor template TT21g/Sst (Fig. 1A and B). BHK cells were transfected with TT21g/Sst and S3P/Xho, and the released virus was recovered (Table 2, experiment 35). Sixteen plaque-purified viruses were used to infect BHK cells, and cytoplasmic RNA was isolated and analyzed. As expected, both genomic and subgenomic RNAs were expressed from each of the recombinants (data not shown). The 3′NTRs of 10 of the recombinants were amplified by PCR, cloned in SP72, and sequenced to map the crossover sites. As shown in Table 3, experiment B, only three of the recombinants (TSS4, -5, and -9) reinitiated at the poly(U) motif. The remaining seven recombinants, which had crossover sites within the nonviral sequence of the TT21g template, carried 12 to 17 U residues, indicating limited polymerase stuttering at these residues. Analysis of TT1g/Sst RNA by RT-PCR amplification, cloning in SP72, and sequencing the resulting plasmids indicated that the stuttering at the poly(U) motif varied from 10 to 16 nt. These results indicated that the stuttering on poly(U) motifs by the SIN RdRp is not as strong as on internal oligo(A) motifs, and the poly(U) motif as presented in TT21g/Sst did not significantly influence the RdRp to reinitiate at the poly(U) locus. Donor sites for most of the recombinants mapped to internal locations of the S3P/Xho template (Table 3), suggesting premature termination of RNA synthesis on donor templates or processing of NNSR before recombination.

Influence of the 3′ nonviral sequences of acceptor templates on recombination. All of the recombination studies on the 3′NTR of the SIN genome were carried out by using acceptor templates whose 3′ regions carried a 1.8-kb nonviral sequence (Fig. 1). Since these acceptor templates served as excellent substrates for recombination, the presence of lengthy nonviral sequences may have facilitated the landing and movement of the RdRp complex on the acceptor template. To understand

TABLE 2. Production of infectious viruses from in vitro-made RNAs[a]

Expt	Template 1	Template 2	CPE				Virus titer (PFU/ml)
			16 h	28 h	44 h	72 h	
1	TT21g/Xho	None	None	None	None	None	0
2	TT21g/Sst	None	None	None	None	None	0
3	TT21h/Xho	None	None	None	None	None	0
4	TT21h/Sst	None	None	None	None	None	0
5	TT21f/Xho	None	None	None	None	None	0
6	TT21i/Xho	None	None	None	None	None	0
7	TT21j/Xho	None	None	None	None	None	0
8	TT21j/Sst	None	None	None	None	None	0
9	TT21k/Xho	None	None	None	None	None	0
10	TT21k/Sst	None	None	None	None	None	0
11	TT21l/Xho	None	None	None	+	++	1.4×10^5
12	TT21l/Sst	None	None	None	None	None	0
13	TT21m/Xho	None	None	None	+	++	0.9×10^6
14	TT21m/Sst	None	None	None	None	None	0
15	TT21n/Xho	None	+	++	+++	++++	1.5×10^8
16	TT21n/Sst	None	++	+++	++++	++++	1.5×10^8
17	TT21q/Xho	None	++	+++	++++	++++	4.3×10^7
18	TT21q/Sst	None	++	+++	++++	++++	6.1×10^7
19	TT21r/Xho	None	++	+++	++++	++++	1.8×10^8
20	TT21r/Sst	None	++	+++	++++	++++	1.1×10^8
21	TT21s/Xho	None	+	++	+++	++++	1.8×10^8
22	TT21s/Sst	None	None	None	None	+	1.7×10^4
23	TT21qa/Xho	None	None	None	None	+	4.2×10^4
24	TT21qa/Sst	None	None	None	None	None	0
25	TT21ra/Xho	None	None	+	++	+++	1.9×10^6
26	TT21ra/Sst	None	None	None	None	None	0
27	Tapa/Sst	None	++	+++	++++	++++	2.0×10^8
28	None	S3P/Xho	None	None	None	None	0
29	None	S3P/Sst	None	None	None	None	0
30	None	S3Pn/Xho	None	None	None	None	0
31	None	S3Pn/Sst	None	None	None	None	0
32	None	S3Pr/Xho	None	None	None	None	0
33	None	S3Ps/Xho	None	None	None	None	0
34	None	S3Ps/Sst	None	None	None	None	0
35	TT21g/Sst	S3P/Xho	+	++	+++	++++	2.0×10^7
36	TT21h/Sst	S3P/Xho	+	++	+++	++++	1.4×10^7
37	TT21f/Xho	S3P/Xho	+	++	+++	++++	1.6×10^7
38	TT21g/Xho	S3P/Xho	+	++	+++	++++	2.8×10^6
39	TT21h/Xho	S3P/Xho	+	++	+++	++++	2.1×10^7
40	TT21j/Sst	S3Pn/Xho	None	+	++	+++	3.2×10^6
41	TT21j/Sst	S3Pn/Sst	None	None	++	+++	2.9×10^6
42	TT21j/Sst	S3Pra/Xho	None	None	None	None	0
43	TT21j/Sst	S3Ps/Xho	None	None	None	None	0
44	TT21j/Sst	S3P/Xho	+	++	+++	++++	3.2×10^7

[a] BHK cells were transfected with 200 to 800 ng of genome-length RNAs (template 1) and/or 40 to 250 ng of template 2 RNAs as indicated. After transfection, cells were overlayed with MEM containing 10% fetal bovine serum, and the appearance of CPE was monitored every 4 to 6 h. Culture supernatants were recovered after 72 h, and titers were determined on Vero cells. Although the amount of virus released from the transfected cells varied depending on the template RNA used, in general plaque-purified viruses from all the stocks grew well and gave comparable titers at the end of 24 h.

how recombination events are affected by the absence of lengthy nonviral sequences, we made use of the acceptor templates TT21f/Xho, TT21g/Xho, and TT21h/Xho. As shown in Fig. 1, the 3′ terminus of each of these templates carried the 17-nt rudimentary replicase motif f, the poly(U) motif g, or the internal oligo(A) motif h, followed by a 6-nt *Xho*I site. BHK cells were transfected with either of these acceptor templates and the donor template S3P/Xho. Early CPE and high-titer virus stock were obtained from cells transfected with the donor and acceptor templates (Table 2, experiments 37 to 39). The individual template RNAs themselves did not produce any virus (Table 2, experiments 1, 3, and 5). This result indicated that even a short stretch of nontranslated 3′ terminus, as found in these acceptor templates, was capable of supporting a high level of recombination with a donor template such as S3P/Xho.

To analyze the crossover sites, 16 viral plaques from each of the three recombinant crosses were used to infect BHK cells, and cytoplasmic RNA was isolated. As shown in the Fig. 2A, genomic and subgenomic RNAs characteristic of SIN infection were expressed by all recombinants. The 3′ regions of the recombinants were amplified by RT-PCR and sequenced to map the crossover junctions. As shown in Table 4, assay A, recombinants derived from the TT21f/Xho × S3P/Xho cross were lacking 17 to 29 nt from the 3′ terminus of the TT21f/Xho RNA. Only one recombinant (SX11) carried the bulk of the 3′ extension located within the TT21f/Xho template. Interestingly, the donor sites for most of these recombinants were mapped to 132 to 144 or 250 to 259, which strikingly agrees with the previously reported hot spot loci (22). Since these two donor sites were frequently used in the S3P/Xho RNA even

TABLE 3. Roles of poly(A) and poly(U) motifs of acceptor templates in reinitiation[a]

Assay recombinants	Plaque	Acceptor site/donor site		Poly(A) length (nt)
		Junction sequence	Map location	
A (TT21h/Sst × S3P/Xho derived)	ASS17	AAAAAA/AAUGCUGCG	−1821/−166	6
	ASS3	UCUAAAU/AUAAUGCU	−1651/−168	36
	ASS4	UCUAAAU/AUAAUGCU	−1651/−168	19
	ASS14	AAGAUUUA/UAAUGCU	−1651/−167	27
	ASS8	UUAUACAU/UAAUGCU	−1637/−167	47
	ASS15	UUAUACAU/UAAUGCU	−1637/−167	20
	ASS16	UUAUACAU/UAAUGCU	−1637/−167	20
	ASS11	UUCUCGU/AUCAGGCU	−1315/−253	31
	ASS12	UUCUCGU/AUCAGGCU	−1315/−253	30
	ASS10	UAAAUUU/AGAUCCCC	−672/−237	13
	ASS18	UAAAUUU/AGAUCCCC	−672/−237	14
B (TT21g/Sst × S3P/Xho derived)	TSS4	GCCCUUUUU/AUAUUAACC	−1816/−136	
	TSS5	GCCCUUUUU/AUAUUAACC	−1816/−136	
	TSS9	GGGCCCAC/AAACUGAUGU	−1810/−291	
	TSS1	GAGGGGAAU/AUAACCACU	−1791/−144	
	TSS2	AUGAGACAA/ACAUAACCA	−1621/−146	
	TSS6	GUAUGAGUA/AAUAUAGCA	−1613/−215	
	TSS8	GUAUUCAAC/AACUGAUGU	−1562/−270	
	TSS7	UGAAAAAGG/AAAUGAUCC	−1535/−309	
	TSS3	UGAAAGUAA/AUAGCAACA	−1485/−212	
	TSS10	GUUUUCCA/ACUUCCGAGG	−1378/−281	

[a] Cytoplasmic RNAs isolated from the indicated plaques were reverse transcribed with primer 3′19SAC, which annealed to the 3′ end of the recombinant, and PCR amplified with JC1295-1S and JC1000-1H. The single species of PCR product thus obtained was digested with *Sst*I and *Hin*dIII, cloned in plasmid SP72, and sequenced by using primers JC1000-1, JC1295-1, JC1395, and SP6P to map the crossover sites. As previously described (22), the map positions of the crossover sites are based on the numbering of the 3′ terminus of the acceptor templates and the donor templates as −1. However, it should be noted that 3′, the poly(A) tail of the donor template is excluded in the numbering of the donor template (33). Thus, the poly(A) and poly(U) motifs are positioned just upstream of −1806 of TT21 vector.

when the acceptor template did not carry long nonviral sequences at the 3′ end, the primary sequence of the nonviral region located at the 3′ terminus of the acceptor template appears to have little effect on the process of polymerase detachment from the donor template.

Sequence analysis of the recombinants derived from the TT21g/Xho × S3P/Xho cross (Table 4, assay B) indicated that four to five U residues from the poly(U) stretch of the acceptor template were retained during recombination. The donor sites on eight recombinants mapped to the first hot spot locus (−134 or −138) of the donor template, substantiating our conclusion that the hot spot locus on the donor template is not influenced by nonviral sequences on the acceptor template. Sequence analysis of recombinants generated from the TT21h/Xho × S3P/Xho cross (Table 4, assay C) indicated that none of the recombinants carried the poly(A) motif located at the 3′ terminus of the acceptor template. It is likely that the length of the poly(A) stretch was longer than 15 nt because of polymerase stuttering. In spite of the possible presence of a variable length of poly(A), no reinitiations occurred within the poly(A) motif. Recombinants AS5 and AS10 carried a sequence from the SIN S region as the donor site, indicating that RdRp jumped from the donor template to an internal sequence within the acceptor template and made a second jump to the end of the acceptor template to generate these recombinants. Similar crossover events were reported by us previously (22). It should be noted that the donor sites for nine recombinants generated by TT21h/Xho × S3P/Xho cross mapped to the −139 position (hot spot locus 1) of the S3P template.

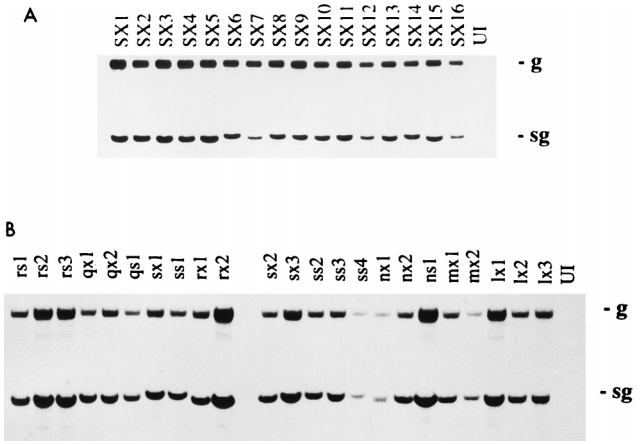

FIG. 2. Gene expression from plaque-purified viruses. BHK cells were infected with 200 μl of the virus suspension obtained from individual plaques at a multiplicity of infection of 0.1 to 2 and labeled with [^{3}H]uridine for 12 h from 1 h postinfection. Cytoplasmic RNA was isolated from each of the infected cultures; 6 to 8 μg of RNA was denatured with glyoxal, analyzed on a 1.25% agarose gel, and fluorographed. The name of the viral plaque used in each experiment is given above each lane. UI, uninfected. (A) Recombinant viruses derived from the TT21f/Xho × S3P/Xho cross; (B) the representative viruses derived from 3′NTR mutants of TT21 RNA. g and sg, genomic and subgenomic RNAs.

Mapping of the core RNA motif of the SIN genome responsible for negative-strand synthesis and genome replication. Results reported above suggested that neither an internal oligo(A) nor an oligo(U) motif serves as a cryptic promoter to allow SIN RdRp to reinitiate on acceptor templates. At this juncture, it became clear to us that the elements of a minimal promoter needed to be defined before we could design a meaningful cryptic promoter. Previous studies reported by Levis et al. (35) and Kuhn et al. (33) suggested that the core promoter responsible for DI RNA replication may differ from the core promoter for SIN genomic RNA replication. As shown in Fig. 1B, we introduced a series of RNA motifs (i, j, k, l, m, n, q, r, s, qa, and ra) in plasmids TT21 and S3P to obtain their corresponding derivatives, to further define the promoter for negative-strand RNA synthesis. These constructs were linearized

TABLE 4. Roles of the nonviral extensions of acceptor templates in the polymerase reinitiation events[a]

Assay (recombinants)	Plaque	Acceptor site/donor site	
		Junction sequence	Map location
A (TT21f/Xho × S3P/Xho derived	SX6	AAGAUGACG/UGCAUCAG	−29/−255
	SX8	AAGAUGACG/UGCAUCAGG	−29/−255
	SX9	AAGAUGACG/UGCAUCAGG	−29/−255
	SX10	AAGAUGACG/UGCAUCAGG	−29/−255
	SX11	CAUUGGCUC/AAUGCAUGC	−3/−257
	SX1	CCACAAA/AUAACCACUAUA	−22/−144
	SX2	CCACAAA/AUAACCACUAUA	−22/−144
	SX3	CCACAAAAU/UAUAUUAA C	−17/−136
	SX4	CCACAAAA/AUUAACCAUUU	−23/−134
	SX5	CCACAAAA/AUUAACCAUUU	−23/−134
	SX7	CCACAAAA/AUUAACCAUUU	−23/−134
	SX13	CCACAAAAU/UAACCAUUUA	−23/−132
	SX15	AAGAUGACGG/UAGCGGAC	−28/−119
B (TT21g/Xho × S3P/Xho derived)	TS9	CACUUUU/AACUCGAUGU	−25/−292
	TS10	CACUUUU/AACUCGAUGU	−28/−292
	TS4	UUUUUUU/UCAGGCUGG	−23/−251
	TS14	UUUUUU/UCAGGCUGGU	−23/−251
	TS1	CACAUUUUU/ACUAUAUU	−25/−138
	TS2	CACAUUUUU/ACUAUAUU	−25/−138
	TS3	CACAUUUUU/ACUAUAUU	−25/−138
	TS5	CACAUUUUU/ACUAUAUU	−25/−138
	TS8	CACAUUUUU/ACUAUAUU	−25/−138
	TS11	CACAUUUUU/ACUAUAUU	−25/−138
	TS6	CACUUU/UAUUAACCAUU	−27/−135
	TS12	CACUUU/UAUUAACCAUU	−27/−135
C (TT21h/Xho × S3P/Xho derived)	AS1	GGGCCCA/CACUAUAUU	−27/−139
	AS2	GGGCCCA/CACUAUAUU	−27/−139
	AS7	GGGCCCA/CACUAUAUU	−27/−139
	AS11	GGGCCCA/CACUAUAUU	−27/−139
	AS12	GGGCCCA/CACUAUAUU	−27/−139
	AS13	GGGCCCA/CACUAUAUU	−27/−139
	AS14	GGGCCCA/CACUAUAUU	−27/−139
	AS16	GGGCCCA/CACUAUAUU	−27/−139
	AS4	UGACGGG/CCAGCUUUU	−31/−297
	AS6	UGACGGG/CCAGCUUUU	−31/−297
	AS5	GGGCCCA/AGAAGACAA	−27/11178
	AS10	GGGCCCA/AGAAGACAA	−27/11178

[a] Cytoplasmic RNAs (Fig. 2) isolated from the indicated plaques were reverse transcribed with primer 3′19Sac, which annealed to the 3′ end of the recombinant, and PCR amplified with 3′19Sac and JC1000-1H. The single species of PCR product thus obtained was digested with *Sst*I and *Hin*dIII, cloned in plasmid SP72, and sequenced by using primers JC1000-2 and SP6P to map the crossover sites. The recombinants are arranged in groups on the basis of their crossover sites.

with either *Xho*I or *Sst*I, transcribed in vitro, analyzed by denaturing agarose gel electrophoresis, and quantitated. BHK cells were transfected with each of these RNAs individually, culture supernatants were harvested, and the virus titer was determined (Table 2, experiments 6 to 26). Cells transfected with TT21i or TT21j RNA, which carried 5 or 10 nt, respectively, of the 3′ conserved motif of the SIN genome plus the poly(A) tail, failed to produce any virus (Table 2, experiments 6 to 8). Similarly, the TT21k RNA, which carried the 3′-terminal 24 nt of the SIN genome without the poly(A) tail, also failed to produce any virus (Table 2, experiments 9 and 10), indicating that the 24-nt motif alone was not sufficient to support negative-strand synthesis or genome replication. The TT21l/Xho RNA, which carried the poly(A) tail and the 3′ 14 nt of the conserved motif, produced a delayed CPE and lower virus titer (Table 2, experiment 11). The TT21l/Sst RNA, which carried the 1.8-kb nonviral sequence downstream of the poly(A) sequence, failed to produce any virus (Table 2, experiment 12). Similar results were obtained for TT21m/Xho and TT21m/Sst RNAs, which carried a poly(A) tail and the 3′ 16 nt of the conserved motif (Table 2, experiments 13 and 14). The specific infectivities of TT21l/Xho and TT21m/Xho RNAs were approximately 12-fold lower than that of the Tapa RNA, but they reproducibly generated infectious virus in BHK cells. The delayed CPE and lower specific infectivities of these RNAs may be due to the production of viral variants. Viral RNA was isolated (Fig. 2B) from individual plaque-infected cells, amplified by PCR, and sequenced. As shown in Fig. 3A and B complete preservation of the original 3′ motif was observed for all of the RNA samples. The inhibitory effects of nonviral sequences on the replication of TT21l/Sst and TT21m/Sst RNAs indicate alterations in the secondary structure of the 3′ promoter regions of these RNAs, leading to inhibition of negative-strand RNA synthesis or genome replication or both.

The TT21n, TT21q, and TT21r RNAs, which carried 20, 29, and 63 nt, respectively, of the 3′ terminus in addition to a poly(A) tail, produced high-titer virus and showed the earliest CPE (Table 2, experiments 15 to 20). The presence of nonviral sequences downstream of the poly(A) sequence in these RNAs did not inhibit virus production, indicating that proper folding of the 3′ terminus or interaction with sequences elsewhere in the genome (62) is not affected by the presence of nonviral sequences in these RNAs. Sequence analysis of viral RNAs isolated from cells infected with the individual viruses indi-

A. TT21l/Xho : GGGCCCAGUUUUUAACAUUUC$(A)_{30}$ $(N)_{29}$
plaque, lx1: GGGCCCAGUUUUUAACAUUUC$(A)_{78}$
plaque, lx2: GGGCCCAGUUUUUAACAUUUC$(A)_{18}$
plaque, lx3: GGGCCCAGUUUUUAACAUUUC$(A)_{63}$

B. TT21m/Xho: GGGCCCAUUGUUUUUAACAUUUC$(A)_{30}(N)_{29}$
plaue, mx1: GGGCCCAUUGUUUUUAACAUUUC$(A)_{18}$
plaque, mx2: GGGCCCAUUGUUUUUAACAUUUC$(A)_{17}$
plaque, mx3: GGGCCCAUUGUUUUUAACAUUUC$(A)_{17}$

C. TT21n/Xho: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{30}(N)_{29}$
plaque, nx1: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{82}$
plaque, nx2: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{18}$
plaque, nx3: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{25}$
plaque, nx4: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{27}$

D. TT21n/Sst: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{30}(N)_{1.8kb}$
plaque, ns1: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{18}$
plaque, ns2: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{18}$
plaque, ns3: GGGCCCAAUUUUGUUUUUAACAUUUC$(A)_{31}$

E. TT21q/Xho: GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{30}(N)_{29}$
plaque, qx1: GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{18}$
plaque, qx2 GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{18}$
plaque, qx3 GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{22}$

F. TT21q/Sst: GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{30}(N)_{1.8kb}$
plaque, qs1: GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{19}$
plaque, qs2: GGGCCCAAUCAACAAAAUUUUGUUUUAACAUUUC$(A)_{18}$

G. TT21r/Xho: GGGGCCC(-63 to -1)$(A)_{30}(N)_{29}$CUCGA
plaque, rx1: GGGGCCC(-63 to -1)$(A)_{18}$
plaque, rx2: GGGGCCC(-63 to -1)$(A)_{18}$

H. TT21ra/Xho: GGGGCCC(-63 to -1)$(A)_{31}$
plaque, rax1: GGGGCCC(-63 to -1)$(A)_{18}$
plaque, rax2: GGGGCCC(-63 to -1)$(A)_{18}$
plaque, rax3: GGGGCCC(-63 to -1)$(A)_{18}$
plaque, rax4: GGGGCCC(-63 to -1)$(A)_{26}$

I. TT21s/Xho: GGGCCC (-310 to -1)CUCGA
plaque, sx1: (-66 to -1)$(A)_{18}$
plaque, sx2: (-66 to -1)$(A)_{19}$
plaque, sx3: (-66 to -1)$(A)_{24}$
plaque, sx4: (-66 to -1)$(A)_{19}$
plaque, sx5: (-66 to -1)$(A)_{52}$
plaque, sx6: (-66 to -1)$(A)_{31}$
plaque, sx7: (-66 to -1)$(A)_{18}$
plaque, sx8: (-66 to -1)$(A)_{18}$
plaque, sx9: (-66 to -1)$(A)_{18}$

J. TT21s/Sst: GGGCCC (-310 to -1)CUCGAG$(N)_{1.8Kb}$
plaque, ss1: (-66 to -1)CUC$(A)_{27}$
plaque, ss2: (-66 to -1)CUC$(A)_{18}$
plaque, ss3: (-66 to -1)CUC$(A)_{20}$
plaque, ss4: (-66 to -1)CUC$(A)_{53}$
plaque, ss5: (-66 to -1)CUC$(A)_{19}$
plaque, ss6: (-66 to -1)CUC$(A)_{19}$
plaque, ss7: (-66 to -1)CUC$(A)_{18}$
plaque, ss8: (-66 to -1)CUC$(A)_{27}$
plaque, ss9: (-66 to -1)CUC$(A)_{19}$
plaque, ss10: (-66 to -1)CUC$(A)_{18}$
plaque, ss11: (-66 to -1)CUC$(A)_{19}$
plaque, ss12: (-66 to -1)CUC$(A)_{18}$

FIG. 3. Sequences of the 3′NTRs of the SIN produced from TT21 derivatives. The cytoplasmic RNA corresponding to each plaque-purified virus was reverse transcribed with the negative-sense primer 18TSac and T11200H. The single of species of PCR product thus obtained was cloned in SP72 and sequenced by using primer JC1000-2 or SP6P. The parental RNA used to generate the virus, its sequence, and the sequences of 3′NTRs of the representative viruses analyzed are given. Since the negative-sense primer, 18TSac, annealed to the poly(A) tail of the viral genome, only those viruses which carry the poly(A) tail would be cloned by this procedure. The sequencing of the viral RNA belonging to group I was carried out with the T11600B primer.

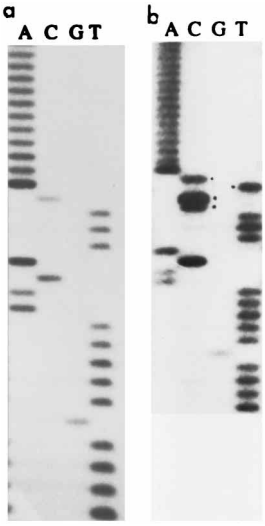

FIG. 4. Sequences of the 3′ termini of TT21s/Xho (A)- and TT21s/Sst (B)-derived SIN RNAs. The sequences correspond to the plaques sx1 and ss1 (first-passage stocks from BHK cells) identified in Fig. 3I and J. The insertion of CUC motif at the terminus is identified with dots.

cated the preservation of their original 3′NTRs (Fig. 3C to G). Interestingly, TT21s/Xho and TT21s/Sst RNAs, which carried the complete 3′ NTR without the poly(A) tail, also produced infectious virus, albeit to different extents (Table 2, experiments 21 and 22). The specific infectivities of these RNAs were 15- and 90-fold lower, respectively, than that of Tapa. Although the sizes of the plaques formed by TT21s/Xho were comparable to that of Tapa/Xho, only minute plaques were formed by TT21s/Sst-derived virus (data not shown). Sequence analysis of viral RNAs expressed from TT21s/Xho and TT21s/Sst demonstrated the presence of a poly(A) tail (Fig. 3I and J). Although the TT21s/Xho RNA (Fig. 1A and B) contained an additional 5 nt downstream of the 3′ 19-nt conserved motif, the progeny viruses effectively eliminated these extra nucleotides, in addition to adding the poly(A) tail. Sequence analysis of the first-passage viral RNA derived from TT21s/Sst revealed the presence of a poly(A) tail and a trinucleotide, CUC, derived from the *Xho*I sequence (Fig. 3J and 4). Multiple passage of TT21s/Sst-derived viruses in BHK cells resulted in the evolution of viruses with larger plaque size and wild-type 3′ terminus (data not shown). Similarly, the TT21ra/Xho and TT21qa/Xho RNAs, which lack the poly(A) tail, also produced infectious virus (Table 2, experiments 23 and 25). The specific infectivities of these RNAs were 20- and 100-fold lower, respectively, than that of Tapa. Plaque-purified viruses derived from TT21ra/Xho expressed both genomic and subgenomic RNAs and retained their original 3′NTR motifs in addition to adding a poly(A) tail (Fig. 3H). It is not known if poly(A) tails are present on the in vivo products of TT21qa/Xho. Interestingly, TT21qa/Sst and TT21ra/Sst RNAs, which carried the 1.8-kb nonviral sequence, failed to produce any virus even upon multiple passaging (Table 2, experiments 24 and 26). Taken together, these results indicate that the TT21 RNAs carrying the motifs s, qa, and ra can be utilized by SIN RdRp for negative-strand synthesis. The lack of a poly(A) tail could have resulted in decreased stability and translatability of these RNAs, which might explain their low infectivities.

Structural requirement of a minimally functional donor template for recombination. The results presented above indicated that an RNA motif consisting of a 3′ 20-nt sequence plus the poly(A) tail or a larger portion of the 3′NTR of the SIN genome without a poly(A) tail could effectively serve as a signal for negative-strand synthesis and genome replication. Next, we tested if the same core motif could function as a polymerase recognition signal in a donor template. Since preliminary studies with TT21i/Sst and TT21j/Sst as acceptor templates indicated that TT21j/Sst carried a cryptic promoter (sequence j) for reinitiation (see below), we made use of this RNA as an acceptor template for further studies. BHK cells were transfected with the acceptor template TT21j/Sst and one of the following donor templates: S3P/Xho, S3Ps/Xho, S3Pn/Xho, or S3Pn/Sst. As shown in Table 2 (experiments 41 to 44), S3P/Xho, S3Pn/Xho, and S3Pn/SSt effectively functioned as donor

TABLE 5. Role of cryptic promoter j in reinitiation[a]

Assay (recombinants)	Plaque	Acceptor site/donor site		Poly(A) length (nt)
		Junction site	Map location	
A (TT21j/Sst × S3P/Xho derived)	IS1	ND	Nonviral	40
	IS2	ND	Nonviral	21
	IS3	ND	Nonviral	38
	IS5	GGGCCC/UGCGCAGUG	Apa /−161	0
	IS6	AAAAAA/UGCUGCGCA	Poly(A)/−164	14
	IS8	AAAAAA/UGCUGCGCA	Poly(A)/−164	13
	IS10	AAAAAA/UUUCUUUU	Poly(A)/−40	14
	IS12	AAAAAAU/UAACCACU	Poly(A)/−144	32
	IS13	ND	Nonviral	27
	IS14	AAAAAA/UUAACCAC	Poly(A)/−144	39
	IS15	UUCAAA/ACAUUUAU	3′C/−128	3
	IS16	CAAAAA/CUCAAUGUA	3′C/−104	5
	IS18	AAAAAA/UUAACCACU	Poly(A)/−143	28
	IS19	AAAAAA/UUAACCAC	Poly(A)/−143	31
	IS20	ND	Nonviral	52
B (TT21j/Sst × S3Pn/Xho derived)	JNX1	AAAAAA/AAUUUU	Poly(A)/−20	52
	JNX2	AAAAAA/AAUUUU	Poly(A)/−20	49
	JNX3	AAAAAA/AAUUUU	Poly(A)/−20	49
	JNX8	AAAAAA/AAUUUU	Poly(A)/−20	49
	JNX9	AAAAAA/AAUUUU	Poly(A)/−20	49
	JNX4	CUCGAA/AAUUUU	Nonviral/−20	ND
	JNX6	AUACAA/AAUUUU	Nonviral/−20	ND
	JNX5	AAAAAA/AAUUUU	Poly(A)/−20	25
	JNX7	AAAAAA/AAUUUU	Poly(A)/−20	22
C (TT21j/Sst × S3Pn/Sst derived)	JNS1	AAAAAA/GGGGCCC	Poly(A)/−27	13
	JNS2	AAAAAA/GGGGCCC	Poly(A)/−27	20
	JNS4	AAAAAA/(UAC)GGG	Poly(A)/−27	34
	JNS5	AAAAAA/GGGGCCC	Poly(A)/−27	15
	JNS3	AAAAAA/GGGCCC	Poly(A)/−26	17
	JNS6	AAAAAA/GGGCCC	Poly(A)/−26	13
	JNS8	AAAAAA/GGGCCC	Poly(A)/−26	16
	JNS7	AAAAAA/GGGGCCC	Poly(A)/−27	19
	JNS9	AAAAAA/GGGGCCC	Poly(A)/−27	5
	JNS10	AAAAAA/GGGGCCC	Poly(A)/−27	26

[a] Crossover sites were characterized as described in the footnote to Table 4. The crossover sites were mapped by sequencing the clones with primer JC1000-2 (for assay A) and 3′19SAC (for assays B and C). Poly(A), poly(A) motif of the cryptic promoter j; 3′C, the 10-nt 3′ conserved sequence included in motif j; nonviral, nonviral sequences located at the 3′ end of TT21j/Sst. ND, not determined.

templates, and large quantities of infectious virus were recovered. Cells transfected with acceptor or donor templates alone did not produce any virus (Table 2, experiments 28 to 34). The S3Ps/Xho RNA consistently failed to serve as a donor template (Table 2, experiment 43), although the motifs within the context of a full-length genome (Table 2, experiment 21) efficiently functioned as a replication signal. These results demonstrated that a donor template carrying the 20-nt sequence of the 3′NTR plus the poly(A) tail (S3Pn/Xho) is sufficient to serve as a promoter for negative-strand synthesis from a donor template. It is not known if S3Pl/Xho and S3Pm/Xho will also function as donor templates.

The viruses recovered from these crosses were plaqued, and BHK cells were infected with the individual plaque suspensions. The viral RNA isolated from the infected cells was subjected to RT-PCR analysis of the 3′ region of the recombinants and sequenced to determine the precise crossover sites. As shown in Table 5, assay A, the reinitiation sites of 10 of the 16 plaques viruses derived from TT21j/Sst × S3P/Xho mapped to the vicinity of cryptic promoter locus j of TT21j/Sst. The crossover sites of other recombinants mapped to nonviral sequences located at the 3′ end of TT21j/Sst. The length of poly(A) in all recombinants varied from 3 to 52 nt. Donor sites for the all recombinants whose junction sites were determined mapped to internal locations of the donor template (−40 to −164). Since the poly(A) motif of TT21h/Sst failed to induce reinitiation at the poly(A) locus (Table 3, assay A), these results appear to indicate that an RNA motif consisting of the 10-nt sequence of the 3′ conserved motif in conjunction with a poly(A) tail functions as a cryptic promoter for polymerase reinitiation. Further studies making use of TT21l and TT21m as acceptor templates are needed to substantiate these results.

As shown in Fig. 1, S3Pn/Xho carries the terminal 20-nt sequence of the 3′NTR, a poly(A) tail, and a 33-nt nonviral sequence at its 3′ end. S3Pn/Sst carries, in addition to the sequences in S3Pn/Xho, a 1.8-kb 3′ nonviral extension. As the initiation of negative-strand RNA synthesis is not affected by the presence of nonviral sequences (22, 51), both donor templates were expected to produce significant amounts of NNSR. We made use of these two donor templates to test the role of 3′ nonviral extensions on junction site selection during recombination. As shown in Table 5, assay B, the reinitiation sites for seven of the nine recombinants derived from TT21j/Sst × S3Pn/Xho mapped to the poly(A) motif of TT21j/Sst. The length of the poly(A) motif located at the crossover site ranged from 22 to 52 nt. The structure of the 3′NTR region of each of the resulting recombinants is as follows: 5′ ApaI-(−10 to −1 of the 3′NTR)-poly(A)-(−20 to −1 of the 3′NTR)-poly(A) 3′. Thus, the 3′NTRs of these recombinants carried two poly(A) motifs flanked by different lengths of the 3′ conserved motif. This observation indicated that recombinants with two closely spaced poly(A) motifs interrupted by the 3′ 20-nt conserved

motif are viable and infectious. The donor sites of all recombinants mapped to the −20 position of the conserved motif. Although the donor template S3Pn/Xho carried the motif GGGGCCC at its 5′ end, none of the recombinants carried this sequence. Analysis of the 10 plaque-purified viruses derived from the TT21j × S3Pn/Sst cross revealed that all of them made use of the poly(A) motif as their reinitiation site (Table 5, assay C). The length of poly(A) motif at the crossover sites of these recombinants ranged from 5 to 34 nt. Importantly, all recombinants derived from TT21j/Sst × S3Pn/Sst carried the GGGGCCC motif from the donor template, suggesting that the presence of nonviral sequences at the 3′ end of the donor template induced the polymerase to completely transcribe the donor template before template switching. Since the S3Pn/Sst template was completely transcribed and utilized during template switching, processing of the nascent negative strands does not appear to account for the absence of the GGGGCCC motif at the crossover site of recombinants generated by TT21j/Sst × S3Pn/Xho.

DISCUSSION

We carried out a detailed study to understand how SIN RdRp recognizes the 3′ end of the SIN genome to initiate synthesis of negative-sense RNAs and undergoes template switching and reinitiation on acceptor templates. We found that RT-PCR analysis of plaque-purified viruses was more reliable than a similar analysis done on a mixture of recombinants found in the RNA-transfected cell (23). In addition, the availability of plaque-purified recombinants allowed us to do further studies on gene expression and stability upon multiple passages. Analysis of representative viruses for sequence alterations at the crossover sites during three successive passages failed to reveal any sequence alterations (23). To further restrict possible rearrangements within a recombinant virus, the total virus population was plaqued only once. A variety of recombination events involving both translatable and nontranslatable regions of SIN can be envisaged to occur in cells that are transfected with acceptor and donor RNAs. Only those recombinants which are able to replicate and assemble into virus particles can be expected to be released into the culture medium. In spite of these caveats, much information can still be gathered from analyses of packaged recombinants (22, 51).

The sequences and structures that regulate negative-strand RNA synthesis from positive-stranded RNA viruses appear to be complex (2, 5, 15, 17, 25, 29, 37, 44, 56, 58). Using DI RNAs of SIN as templates, Levis et al. (35) defined the importance of the 3′ 19-nt conserved sequence abutting the poly(A) tail in negative-strand RNA synthesis and DI RNA replication. Kuhn et al. (33) reported that part of the 19-nt conserved sequence was dispensable for full-length SIN genome replication when the rest of the 3′NTR was kept intact, suggesting a compensatory role played by sequences within the 3′NTR. Kuhn et al. (33) also reported that the 3′-terminal 26-nt sequence along with the adjoining poly(A) tail of the 3′NTR was sufficient to allow negative-strand RNA synthesis and genome replication of the SIN in chick cells. Our present results on the deletion analysis of the 3′ terminus of the SIN genome indicate that the 3′-terminal 20-nt sequence of the SIN genome along with the poly(A) tail is sufficient to allow negative-strand RNA synthesis and genome replication in BHK cells. As documented by Kuhn et al. (33), the precise promoter elements needed for 3′-end recognition appear to vary between different host cells. Although infectious SIN readily arose from genomic RNAs carrying only 14 nt from the 3′NTR plus a poly(A) tail, it is not known if any sequence alterations occurred within the genes coding for viral RdRp.

How does SIN RdRp get access to the 3′ promoter elements in a genomic RNA? Some plus-stranded RNA viruses appear to deliver polymerase proteins to the 3′ end of the genome by coupling genome translation and replication (38, 45). Although it is well established that SIN RdRp supports replication of DI RNA templates (35, 42, 57), the pathway of polymerase delivery to and initiation of RNA synthesis from the 3′ promoter may differ between full-length SIN genome and nontranslatable RNAs such as DI genomes and S3P/Xho. In this context, the role of the 3′ poly(A) tail of SIN genome is of interest. Kuhn et al. (33) reported that infectious SIN could be produced from in vitro-synthesized SIN RNA containing a 7-nt insertion at the junction between the 3′ conserved motif and poly(A) tail, indicating that limited displacement of the poly(A) tail from the conserved motif was tolerated by the polymerase. Studies using the poliovirus system appear to indicate the requirement a of poly(A) tail for infectivity (55, 59, 66). We found that large quantities of infectious SIN were produced from TT21s/Xho and TT21ra/Xho, which carry no poly(A) tail. The resulting virus regained the poly(A) tail at the correct 3′ terminus of the SIN genome. If indeed the poly(A) tail is required for polymerase binding and initiation of negative-strand synthesis, then we have to envisage in vivo polyadenylation of these transfected RNAs. Since TT21s/Xho and TT21ra/Xho carried 1 to 5 additional nonviral nt of the *Xho*I sequence downstream of the −1 position of the viral 3′ end, it is not clear how these nonviral sequences are removed in vivo, so that polyadenylation could occur at the precise 3′ end. It is possible that nonspecific cellular 3′ exoribonucleases removed the nonviral sequences and facilitated subsequent polyadenylation. Alternatively, the transfected TT21s/Xho RNA may not have been polyadenylated in vivo, but the polymerase could have recognized the 3′ promoter located close to the 3′ end of the RNA and made a correct negative-sense RNA with a poly(U) tail. Another possibility is that negative strands without poly(U) were made from templates lacking a poly(A) tail, but during positive-strand synthesis from these negative strands, polyadenylation could have occurred. The fact that TT21s/Sst RNA, which carried a long nonviral sequence at its 3′ end, produced polyadenylated SIN RNA with a 3-nt insertion in vivo suggests that transfected RNAs such as TT21s/Sst are not polyadenylated per se, but partial recognition of the masked promoter by SIN RdRp could have led to the production of negative-sense transcripts.

Although the presence of the 3′ 20-nt sequence plus a poly(A) tail confers infectivity on TT21n/Xho, TT21k/Xho carrying the 3′ 24 nt but lacking the poly(A) tail fails to produce infectious virus. Therefore, it appears that TT21ra/Xho and TT21s/Xho RNAs, which carry the 3′ 63 and 310 nt, respectively, of the 3′NTR of the SIN genome, were recognized in vivo, whereas an RNA carrying only the 3′ 24 nt of the 3′NTR (TT21k/Xho) was not. These results suggest the occurrence of specific polyadenylation signals within the terminal 63 nt of the SIN 3′NTR. Alternatively, initiation of negative-sense RNAs may require the presence of either a poly(A) tail with a minimal 3′ promoter or an extended 3′NTR as in TT21s/Xho or TT21ra/Xho in the absence of a poly(A) tail. Although it is believed that the poly(A) tail confers stability to cytoplasmic RNAs (3), it is difficult to imagine how nonpolyadenylated RNAs such as TT21s/Xho and TT21ra/Xho could possess greater stability and translatability than TT21k/Xho in vivo, which could explain their retention of infectivity.

The pathway of utilization of RNAs such as S3P/Xho, S3Pn/Xho, and S3Pn/Sst, which carry only the 3′NTR or its compo-

nents, as donor templates during SIN recombination is also unclear. Even though the 3′ ends of TT21s/Xho and S3Ps/Xho RNAs carry identical sequences, only the 3′ promoter in TT21s/Xho was recognized by SIN RdRp. The ability of TT21s/Xho RNA to serve as an mRNA to produce SIN RdRp could have conferred easy access to the polymerase, thereby obviating the need for poly(A) as a polymerase capture signal. How the absence of poly(A) tail in a donor template such as S3Ps/Xho affects polymerase recognition and template switching remains to be determined.

The nonviral sequences located downstream of the poly(A) tails of many full-length infectious RNAs of SIN (Tapa/Sst, TT21n/Sst, TT21q/Sst, and TT21r/Sst) did not alter infectivity. Similarly, S3P/Sst and G26S-3HN/Sst also functioned efficiently as donor templates in recombination studies (22). These results indicate that SIN RdRp is able to efficiently scan both full-length and short nonreplicative RNAs, to identify promoter elements which regulate negative-strand RNA synthesis, even when the 3′ promoters are flanked by nonviral sequences. Previously (22, 51) we reported that the precise 5′ and 3′ sequences of donor templates did not affect polymerase loading and promoter selection for negative-strand synthesis. These results also suggest the ability of RdRp to efficiently destabilize deleterious secondary structures that are formed by flanking sequences around the native negative-sense promoter. It appears that only selected negative-sense promoters are endowed with the ability to shield themselves from adverse interactions of neighboring sequences. For example, TT21l/Xho, TT21m/Xho, TT21ra/Xho, and TT21s/Xho RNAs, which carry some form of deletions in the 3′NTR of the SIN genome, are infectious, albeit to different levels, and produce infectious particles only when they carry a short or no nonviral sequence at their 3′ ends. When these RNAs carried a 1.8-kb nonviral sequence at their 3′ ends, all RNAs except TT21s/Sst lost infectivity. The TT21s/Sst RNA was found to be 200- to 500-fold less infectious than TT21s/Xho. Thus, these suboptimal promoters, although functional, fail to refold and regain their promoter function when confronted with lengthy nonviral sequences at their 3′ ends. We propose that the native RNA promoters of alphaviruses that regulate negative-sense RNA synthesis, in addition to serving diverse functions such as polymerase capture and accurate initiation of RNA synthesis, regulate their refolding, perhaps in concert with RdRp, to regain biological activity when subjected to adverse RNA-RNA or RNA-protein interactions.

We continue to be amazed by the ease with which recombination occurs at the 3′NTR of the SIN genome. The donor template S3P/Xho recombined with almost every acceptor template tested, leading to the release of large quantities of infectious virus within 24 h after transfection. Although limited sequence homology and similar RNA secondary structures are demonstrable at a majority of the reinitiation sites, their significance is unclear (data not shown). For example, base pairing between the poly(U) motif of the nascent transcript associated with the jumping polymerase and the poly(A) motif in the TT21h/Sst RNA was expected; similarly, the poly(U) motif within TT21g/Sst was expected to base pair with the poly(A) tail of the donor template S3P/Xho. Since these RNA-RNA interactions did not appear to have induced the polymerase to reinitiate at the respective motifs on the acceptor template, the polymerase-RNA interactions may play a more dominant role in selection of reinitiation sites (68). The fact that replacement of the poly(A) motif with the cryptic promoter j on the acceptor template (Fig. 1B) was able to significantly induce the polymerase to reinitiate at the j locus was suggestive of polymerase recognition of this cryptic promoter. When S3Pn/Xho or S3Pn/Sst, which carry only the 20-nt sequence from the 3′NTR plus the poly(A) tail, was used as a donor template in conjunction with TT21j/Sst, most of the recombinants mapped to the j motif of the acceptor template. Thus, recognition of cryptic promoter-like elements by the polymerase appears to be dependent on the length and sequence complexity of the transcript carried by the polymerase complex. Although nonhomologous recombination events on locations with no discernable base pairing ability are well known to occur during retroviral reverse transcription (13, 18a, 21a), it is not known if cryptic promoter-like sequences are involved in these events.

The 5′ termini of donor templates were reported to function as hot spots for recombination in the tombusvirus system (64, 65). But in the SIN system, a few selected internal locations on the donor template S3P/Xho were consistently utilized as crossover sites. These hot spots of recombination on the donor template are not influenced by the length and complexity of the nonviral sequences on the acceptor template. In addition, when the 1.8-kb nonviral sequence of the acceptor templates was replaced by a short stretch of poly(A), poly(U), or an AU-rich motif (f), the efficiency of recombination and selection of donor sites were not significantly altered. This finding is suggestive of a self-regulated premature termination of negative-strand synthesis from the donor template, probably by the structure of the donor template. It is not known if possible interactions of the donor template with 5′ regions of acceptor templates regulate the transcriptional pausing on the donor template. Although junction site selection can be influenced by donor-acceptor interaction at or near the crossover site (7, 43, 54), it does not seem to apply to recombination events observed here. Studies on the role of homology and base-pairing abilities between donor and acceptor templates on junction site selection should address this point.

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