Analysis of intermolecular RNA–RNA recombination by rubella virus

Sandra D. Adams,[1,2] Wen-Pin Tzeng,[1] Min-Hsin Chen,[3] and Teryl K. Frey*

Department of Biology, Georgia State University, Atlanta, GA 30303, USA

Received 1 July 2002; returned to author for revision 19 August 2002; accepted 3 December 2002

Abstract

To investigate whether rubella virus (RUB) undergoes intermolecular RNA–RNA recombination, cells were cotransfected with pairs of in vitro transcripts from genomic cDNA plasmid vectors engineered to contain nonoverlapping deletions: the replicative transcript maintained the 5′-proximal nonstructural (NS) ORF (which contained the replicase, making it RNA replication competent), had a deletion in the 3′-proximal structural protein (SP) ORF, and maintained the 3′ end of the genome, including the putative 3′ *cis*-acting elements (CSE), while the nonreplicative transcript consisted of the 3′ half of the genome including the SP-ORF and 3′ CSE. Cotransfection yielded plaque-forming virus that synthesized the standard genomic and subgenomic RNAs and thus was generated by RNA–RNA recombination. Using transcripts tagged with a 3′-terminal deletion, it was found that recombinants contained the 3′ end derived from the replicative strand, indicating a *cis*-preference for initiation of negative-strand synthesis. In cotransfections in which the replicative transcript lacked the 3′ CSE, recombination occurred, albeit at lower efficiency, indicating that initiation in *trans* from the NS-ORF can occur. The 3′ CSE was sufficient as a nonreplicative transcript, showing that it can serve as a promoter for negative-strand RNA synthesis. While deletion mutagenesis showed that the presence of the junction untranslated region (J-UTR) between the ORFs appeared to be necessary on both transcripts for recombination in this region of the genome, analysis with transcripts tagged with restriction sites showed that the J-UTR was not a hot spot for recombination compared to neighboring regions in both ORFs. Sequence analysis of recombinants revealed that both precise (homologous) and imprecise recombination (aberrant, homologous resulting in duplications) occurred; however, imprecise recombination only involved the J-UTR or the 3′ end of the NS-ORF and the J-UTR (maintaining the NS-ORF), indicating selection pressure against duplications in other regions of the genome.
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Introduction

A number of positive-strand RNA viruses of animals and plants, including members of the picornavirus, togavirus, flavivirus, arterivirus, coronavirus, bromovirus, tombusvirus, and carmovirus families, have been shown to undergo RNA–RNA recombination (reviewed in Lai, 1992; Nagy and Simon, 1997) and RNA–RNA recombination appears to be a strong driving force in RNA virus evolution (Strauss and Strauss, 1998), both within virus genera (eg. Hahn et al.,

1988; Kew et al., 2002) and among virus families (e.g., Zhang et al., 1992). Kirkegaard and Baltimore (1986) provided evidence for a copy-choice or template-switching mechanism for RNA recombination in poliovirus in which the viral RNA-dependent–RNA polymerase (RdRp)-nascent progeny strand complex switches templates during negative-strand transcription of a positive-strand template, leaving one template (the donor strand) and continuing synthesis of the progeny strand on a second template (the acceptor strand). Most subsequent studies have provided evidence consistent with recombination occurring during RNA replication as a result of template switching, during either negative- or positive-strand synthesis, and template switching has been demonstrated in vitro with highly purified viral RdRps (Arnold and Cameron, 1999; Kim and Kao, 2001); however, recombination due to a nonreplicative mechanism has been reported (Gmyl et al., 1999). Both homologous and nonhomologous recombination have been

* Corresponding author. Fax: +1-404-651-3105.

E-mail address: tfrey@gsu.edu (T.K. Frey).

[1] These authors contributed equally to the study.

[2] Present address: Department of Biology, Montclair State University, Upper Montclair, NJ 07043.

[3] Present address: Measles Virus Section, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

documented and high-frequency recombination sites or "hot spots" have been described (Lai, 1992; Nagy and Simon, 1997).

Rubella virus (RUB) is the only member of the *Rubivirus* genus of the family *Togaviridae*. The RUB genomic RNA is a single-stranded, 9762-nt, positive-sense RNA that contains two long open reading frames (ORFs): a 5′-proximal ORF which encodes nonstructural proteins (NSP) that function primarily in viral RNA replication, including the RdRp, and a 3′-proximal ORF which encodes the virion structural proteins (SP), the capsid protein (C), and two envelope glycoproteins, E1 and E2. The genomic RNA serves as a template for synthesis of a complementary minus-strand RNA which is the template for synthesis of both the genomic RNA and the subgenomic (SG) RNA, from which the structural proteins are translated (Frey, 1994). Same-strand, intramolecular recombination by RUB has been reported and studied using genomic cDNA plasmid vectors in which the subgenomic promoter (SGP) for SG RNA synthesis, which lies in the "junction" untranslated region (J-UTR) between the ORFs, has been duplicated for expression of foreign genes (Pugachev et al., 2000; Tzeng and Frey, 2002). Recombination between the two SGPs of these vectors resulted in deletion of the foreign gene and reconstitution of the wild-type (wt) virus genome. Sequence analysis of recombinants showed that both homologous recombination (precise recombination resulting in a wt sequence as defined by Lai, 1992) and aberrant homologous recombination (imprecise recombination between homologous templates resulting in duplications) within the SGP occurred.

RNA recombination has been well studied in the *Alphaviruses,* the other *Togavirus* genus, particularly with Sindbis virus (SIN). Infectious recombinants were initially recovered when cells were cotransfected with different combinations of in vitro transcripts from genomic cDNA plasmid vectors bearing nonoverlapping deletions that rendered them noninfectious (Weiss and Schlesinger, 1991; Raju et al., 1995). Of the pair of transcripts, one was "replicative" since it preserved the NS-ORF plus the 5′ and 3′ ends of the genome (containing *cis*-acting elements, or CSEs) and thus was capable of RNA replication, while the second transcript was "nonreplicative" since it did not contain a complete copy of the NS-ORF and lacked one or both of the CSEs. Homologous, aberrant homologous, and nonhomologous recombination were detected (nonhomologous recombination results from participation of a nonhomologous template resulting in incorporation of unrelated sequence into the recombinant; Lai, 1992). Sequence analysis of the 3′UTR of recombinants suggested that only limited homology existed between the substrate templates at sites of recombination (Hajjou et al., 1996). Additionally, it was found that a short RNA consisting of a conserved motif within the 3′UTR plus a poly(A) tail was sufficient to serve as a donor for recombination with an acceptor transcript in which the 3′UTR was replaced with nonviral sequences (Hill et al., 1997). This result indicated that negative-strand RNA synthesis could initiate on the 3′UTR/poly(A) fragment and thus it was the only sequence necessary for initiation of negative-strand RNA synthesis.

In this study, we investigated RUB intermolecular RNA–RNA recombination between replicating and nonreplicating RNA transcripts containing nonoverlapping deletions. Considering the previous studies on SIN that used recombination to define the minimal signal for negative-strand initiation, we were additionally interested in taking a similar approach to define the minimal RUB 3′CSE's required for negative-strand initiation. The 3′-terminal 305 nts of the genome are retained in RUB DI RNAs generated during undiluted serial passaging, implying that the 3′CSE are located within this region, which includes both the 3′SP-coding sequences and the 60-nt 3′UTR (Frey and Hemphill, 1988; Derdeyn and Frey, 1995). In addition to functioning as a CSE, this region of the RUB genome is also of interest because a stem-loop (SL) structure within it binds an autoantigen, calreticulin (CAL) (Nakhasi et al., 1990; Singh et al., 1994). Mutagenic analysis of the CAL-binding SL, the 3′UTR, and poly(A) tract within the 3′–305-nt region using a RUB genomic cDNA plasmid vector revealed that most of the 3′UTR is critical for replication with the exception of the 3′-five nucleotides and the poly(A) tract (which was rapidly regenerated in vivo) (Chen and Frey, 1999). Additionally, it was found that mutations which destabilized CAL binding to the SL had no effect on replication. However, because most of the 3′-305-nt region encodes E1, extensive mutagenesis of the region could not be done using a genomic cDNA vector. Therefore, the ability to segregate this region as a recombination donor offers the opportunity to test which parts of it are necessary for negative-strand initiation.

Results

RUB has not yet been reported to undergo intermolecular RNA–RNA recombination. Following cotransfection of Vero cells with transcripts from a replicative, defective-interfering RNA construct with a deletion in the SP-ORF (DI-P3) that maintains the NS-ORF and thus is capable of RNA replication (Tzeng et al., 2001) and a nonreplicative (NR) construct containing the 3′ half of the RUB genome (NR-5355-3′; the designation indicates that the construct is nonreplicative and contains the sequences from nt 5355 through the 3′ end of the genome) (Fig. 1A), cytopathic effect (CPE) was observed and plaque-forming virus was recovered from the transfected culture medium. Neither CPE nor recovery of plaque-forming virus occurred following transfection with either transcript by itself, suggesting that recombination between the transcripts had occurred, resulting in generation of virus. This experiment was repeated with transcripts from NR-5355-3′ and several replicative DI constructs with SP-ORF deletions with similar
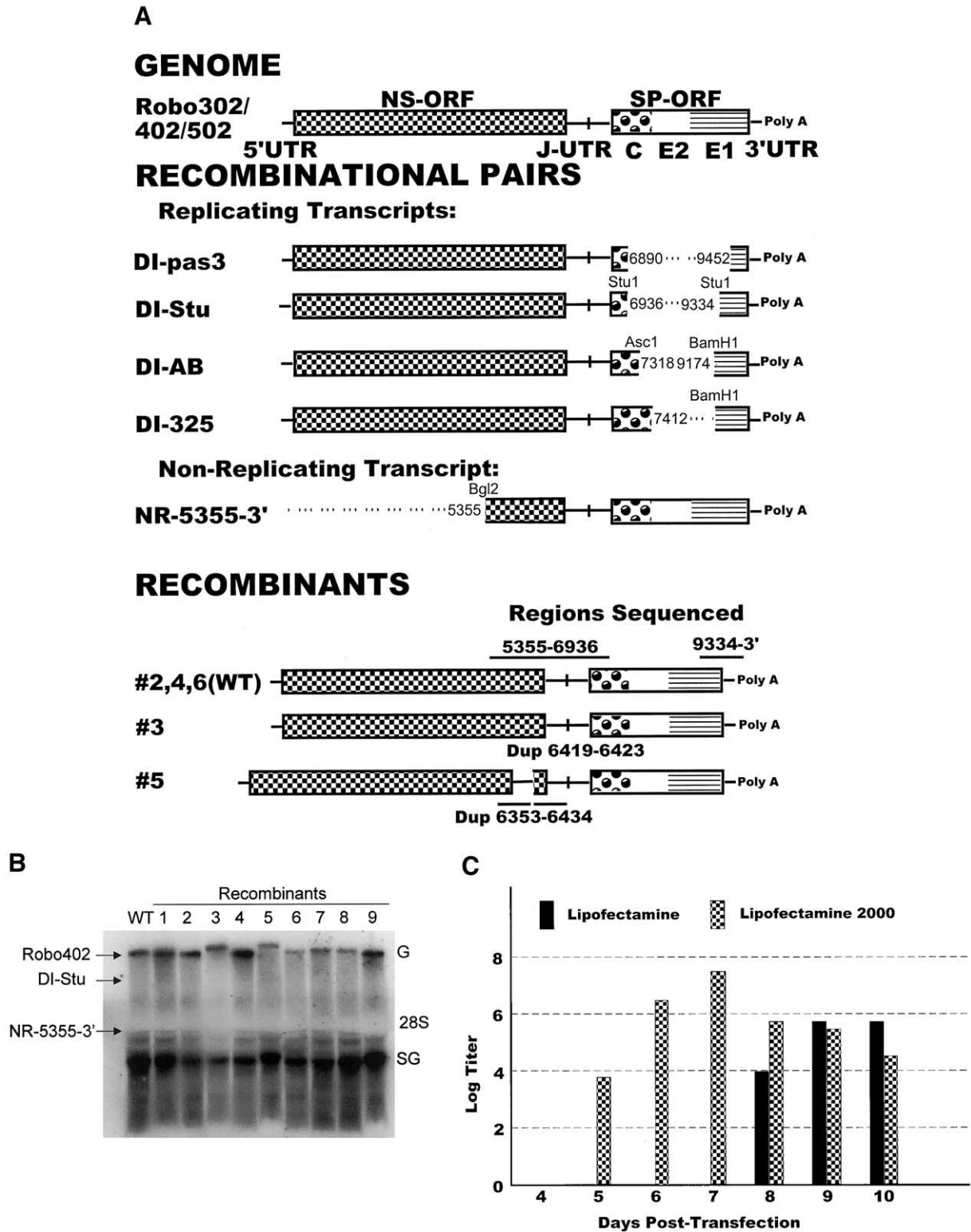
Fig. 1. Initial transcript pairs that yielded recombinants. At the top of (A) is a diagram of the RUB genome (ORFs as boxes, UTRs as lines); the vertical line in the J-UTR represents the SG RNA start site. Recombination was initially observed when the four replicating transcripts shown with deletions in the SP-ORF (the nt number in the RUB genome of the deletion breakpoints along with restriction sites used to generate the deletions are given; deletions are indicated by dotted lines) were individually used in cotransfections with the nonreplicating transcript NR-5355-3′. Virus from nine recombinant plaques generated from DI-Stu X NR-5355-3′ cotransfections were amplified in Vero cells. The amplified stocks were used to infect Vero cells and intracellular RNA was extracted and Northern analysis was used to characterize the viral RNAs present as shown in (B). The positions of migration of Robo402, DI-Stu, and NR-5355-3′ transcripts electrophoresed in the same gel are shown in the left margin, while the genome (G), subgenome (SG), and ^{28}S rRNA (which results in a white space) are indicated in the right margin. The RNA of five of these recombinants (2, 3, 4, 5, and 6) was sequenced across the region of overlap between the parental transcripts; the sequence of three of these recombinants was wild-type, while the other two recombinants had duplications in the 3′ end of the NS-ORF and J-UTR, as shown at the bottom (A). To test the effect of transfection reagent on recovery of recombinant virus, seven plates of Vero cells were cotransfected with DI-Stu and NR-5355-3′ transcripts using either Lipofectamine or Lipofectamine 2000. On days 4–10 posttransfection, the medium from one plate of cells was harvested and the virus present was titered by plaque assay; daily recovery of plaque-forming virus is shown in (C).

results (Fig. 1A). Plates transfected with transcripts from NR-5355-3′ and one of the SP-ORF deletion constructs, DI-Stu, were overlaid with plaque assay agar; plaques were picked and after one round of amplification used to infect Vero cells and the virus-specific RNAs produced were analyzed. As shown in Fig. 1B, virus from all of the plaques produced RNAs corresponding to the genomic and SG RNAs, indicating that RNA–RNA recombination had occurred between the transcripts, resulting in generation of wt virus, rather than replication and copackaging of transcripts as has been observed with SIN (Geigenmuller-Gnirke et al., 1991; Weiss and Schlesinger, 1991).

Two of the recombinants (3 and 5) had genomic RNAs that were larger than the wt genomic RNA and recombinant 5 also produced a SG RNA larger than the wt SG RNA. To determine if duplications or insertions occurred during recombination, after an additional round of amplification, intracellular infected cell RNA was extracted and the regions of overlap between the parental transcripts were sequenced for recombinants 3 and 5 as well as recombinants 2, 4, and 6, which had both wt-sized genome and SG RNAs. No insertions or deletions were detected in the 3′ region of parental overlap with any of these recombinants nor were insertions or deletions detected in the NS-ORF/J-UTR/SP-ORF region of parental overlap in recombinants 2, 4, or 6. Recombinant 3 was found to have a tandem duplication of 6 nts in the J-UTR; it was surprising that such a small duplication would retard the mobility of the genomic RNA to the extent observed in the gel in Fig. 1B. Recombinant 5 was found to have a tandem duplication of nts 6353–6434 encompassing the 3′ end of the NS-ORF and the J-UTR through 2 nts upstream from the SG start site.

In another experiment, the parental regions of overlap in seven recombinant viruses plaque-purified from culture fluid from a plate cotransfected with DI-Stu and NR-5355-33′ transcripts were sequenced with the result that only point mutations, but no deletions or insertions, in the regions of parental overlap were detected.

In subsequent experiments, following cotransfections with pairs of replicative and nonreplicative transcripts, the cells were examined daily for CPE. Following the appearance of putative CPE, the transfected cell-culture fluid was used to infect fresh monolayers of cells, which were then examined for the development of CPE to confirm that recombinant virus had been produced. Cotransfections were repeated at least three times to ensure that the production of virus through recombination between a pair of transcripts was reproducible. In the course of these experiments, both Lipofectamine and Lipofectamine 2000 were used as transfection reagents. In our hands using RUB replicons expressing the GFP reporter gene, Lipofectamine 2000 was roughly seven times more efficient based on the number of cells expressing GFP following transfection of a standard amount of transcripts (5% of the cells fluorescing following Lipofectamine-mediated transfection vs 35% following Lipofectamine 2000 mediated transfection). The different trans-

fection reagents also affected the kinetics of appearance of CPE following cotransfection with replicating and nonreplicating transcripts. In a typical cotransfection experiment with DI-Stu and NR-5355-3′, CPE was first observed 5 days posttransfection when Lipofectamine 2000 was used and 8 days posttransfection when Lipofectamine was used. As shown in Fig. 1C, detection of plaque-forming virus coincided with appearance of CPE and occurred 3 days earlier following Lipofectamine 2000 than Lipofectamine-mediated transfection. As will be shown later, the use of transfection reagent affected recovery of recombinants following cotransfections with specific replicative and nonreplicative transcript pairs; in these cases, recombination was detectable if Lipofectamine 2000 was used, but not if Lipofectamine was used. We interpret this to indicate that transfection efficiency was higher with Lipofectamine 2000 in terms of delivery of transcripts to individual cells as well as to number of cells transfected, resulting in more cells containing both transcripts and the presence of more intracellular transcripts in individual cells on which recombination could occur. Alternatively, the presence of more transcripts delivered to individual cells by Lipofectamine 2000 could have increased the functional half-life during which transcripts were available in the cell for recombination to occur.

Recombination in the junction region

The standard transcript pair in a recombination assay shown in Fig. 1 overlapped in two regions of the genome: the region containing the 3′ end of the NS-ORF through the J-UTR and the 5′ end of the SP-ORF (1535–1959 nts, depending on the replicative transcript used); and the region at the 3′ end of the genome (310–591 nts). To produce virus, minimally one recombination in the former region needs to occur. To investigate the requirements for recombination in this region, a series of cotransfections was performed with transcripts containing deletions as shown in Fig. 2 and Table 1. When the overlap was minimized less than 100 nts in the SP-ORF (DI-AB or DI-325 cotransfected with NR-7318-3′; a region of overlap of 8 and 95 nts, respectively), no recombination was detected. When the SP-ORF was replaced with the GFP reporter gene in the replicative transcript (RUBrep/GFP), recombination was still detected when NR-5355-3′ was used as the nonreplicative transcript. A series of constructs that progressively deleted the 5′ end of the nonreplicative NR-5355-3′ transcripts was used in cotransfections with RUBre/GFP. Transcripts which retained part of the NS-ORF (NR-6263-3′ and NR-6313-3′) yielded recombinants, while transcripts deleted into the J-UTR (NR-6408-3′ and NR-6436-3′) did not. The region of overlap in these latter two cotransfections was reduced to 108 and 76 nts, respectively; however, when the same series of transcripts as used in cotransfections with DI-Stu, the same observations were made even though region of overlap with NR-6408-3′ and NR 6436-3′ was increased to 553 and 525 nts, respectively. When Lipo-
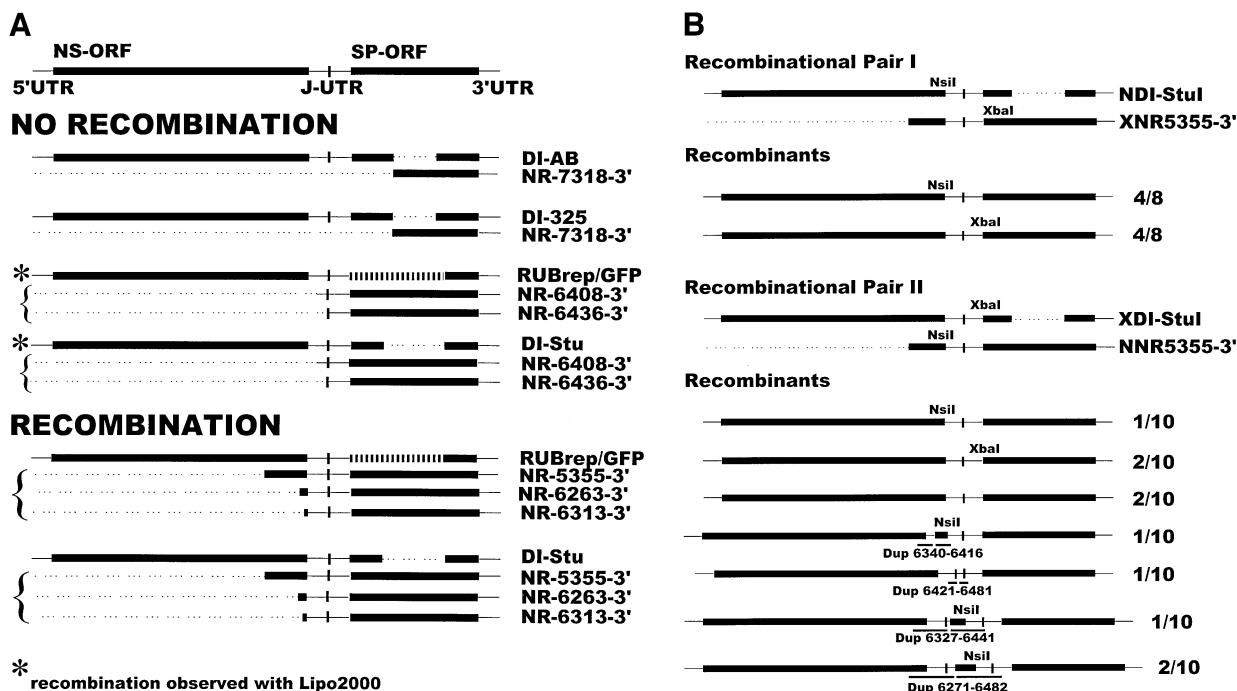
Fig. 2. Recombination in the J-UTR. Genomic diagrams of pairs of replicating and nonreplicating transcripts are shown in (A) that either failed to yield recombinants (NO RECOMBINATION) or yielded recombinants (RECOMBINATION) when Lipofectamine was used as the transfection reagent. Brackets indicate nonreplicating transcripts used individually in cotransfections with a common replicating transcript with a similar result. In some cases, transcript pairs that failed to yield recombinants following Lipofectaine-mediated transfection did yield recombinants when Lipofectamine 2000 was used, as indicated by an asterisk. To determine whether the J-UTR was a recombinational hot spot, DI-Stu and NR-5355-3′ transcripts generated from constructs that had been genetically tagged with restriction sites (*Nsi*I at the 5′ end of the J-UTR or *Xba*I at the 3′ end of the J-UTR) were used in cotransfections (Lipofectamine 2000) and virus recovered from recombinant plaques was sequenced across the region of overlap in the middle of the genome. As shown in (B), recombinants were recovered that had no exchange of the parental markers, exchange of parental markers, and duplications of the 3′ NS-ORF and J-UTR.

fectamine 2000 was used as the transfection reagent, recombinants were recovered when NR-6408-3′ and NR-6436-3′ were cotransfected with RUBrep/GFP or DI-Stu, indicating that although recombination occurred, it was inefficient. These observations suggested that the J-UTR was a potential hot spot for recombination relative to the neighboring sequences in the NS- and SP-ORFs or that its presence in the donor strand might be necessary for recombination to occur in this region of the genome. To determine whether or to what extent the J-UTR could be deleted while preserving the ability of recombination to occur, an extensive series of constructs that introduced deletions at the 5′ end of the nonreplicative transcript through the junction UTR was made and these transcripts were cotransfected with DI-Stu transcripts using Lipofectamine 2000 reagent. As shown in Table 1, the minimal nonreplicative transcript that yielded recombinants was NR-6479-3′, the 5′ end of which still contains the thirty-three 3′ nt of the J-UTR (although the overlap with DI-Stu was 482 nts). In cotransfections between DI-Stu and nonreplicative constructs with deletions extending beyond nt 6436, the appearance of CPE was delayed (Table 1).

To resolve whether the J-UTR was a hot spot for recombination, transcripts were used that were genetically tagged with restriction sites at either end of the J-UTR: *Nsi*I was

introduced at the 5′ end of the J-UTR to produce NDI-Stu and NNR-5355-3′, while *Xba*I was introduced at the 3′ end of the J-UTR to produce XDI-Stu and XNR-5355-3′; Robo502 virus with either of these restriction sites produces titers equivalent to Robo502 virus; Robo502 and XRobo502 virus produce similar plaques, and NRobo502 virus produces smaller plaques. As shown in Fig. 2B, when NDI-Stu and XNR-5355-3′ transcripts were combined in a cotransfection (Lipofectamine 2000) followed by overlaying the transfection plate with plaque assay agar, isolation, and analysis of individual plaques, only recombinants with the parental J-UTRs were recovered. This indicated that recombination had occurred either upstream (recombinants with *Xba*I site) or downstream (recombinants with *Nsi*I site) from, but not within, the J-UTR. When the reciprocal recombination assay was done, XDI-Stu/NNR-5355-3′, of 10 recombinants analyzed, 3 had parental J-UTRs (two with *Xba*I and one with *Nsi*I); 2 had a recombinant J-UTR (neither site), while 5 had tandem duplications: 1 within the J-UTR and 4 including both the 3′ end of the NS-ORF and the 5′ end of the J-UTR. In each of these latter four recombinants, the upstream duplication lacked the *Nsi*I site, while the downstream duplication contained it, indicating that in all four cases transcription had proceeded into the NS-ORF on the nonreplicative transcript (NNR-5355-3′) and then

Table 1
Recombination between transcripts with sequential deletions across the junction UTR

Replicating Transcript[a]	Nonreplicating Transcript[a]	Lipo[a,b]	Lipo2000[a,b]
DI-AB (Δ7318-9174)	NR-7318-3′	−	−
DI-325 (Δ7412-9174)	NR-7318-3′	−	−
RUBrep/GFP (Δ6512-9934)	NR-5355-3′	+	+
	NR-6263-3′	+	+
	NR-6313-3′	+	+
	NR-6408-3′	−	+
	NR-6436-3′	−	+
DI-Stu (Δ6936-9334)	NR-5355-3′	+	+
	NR-6263-3′	+	+
	NR-6313-3′	+	+
	NR-6408-3′	−	+
	NR-6436-3′	−	+
	NR-6462-3′	ND	+*
	NR-6467-3′	ND	+*
	NR-6473-3′	ND	+*
	NR-6479-3′	ND	+*
	NR-6486-3′	ND	−
	NR-6622-3′	ND	−
	NR-6651-3′	ND	−
	NR-6751-3′	ND	−
	NR-6851-3′	ND	−
	NR-6951-3′	ND	−

[a] Vero cells were co-transfected with the indicated replicating and non-replicating transcripts; each replicating transcript contained a deletion in the SP-ORF (extent indicated) while each non-replicating transcript contained the 3′ end of the genome starting at the indicated nt (the J-UTR covers nts 6388–6512). Transfection was done either using Lipofectamine (Lipo) or Lipofectamine 2000 (Lipo2000) as the transfection reagent. +: recombination observed; −: recombination not observed; ND: not done.

[b] With transcript pairs that produced recombinants, CPE was usually observed 8–12 days post-transfection when Lipofectamine was used and 5–8 days post-transfection when Lipofectamine 2000 was used. Co-transfections in which appearance was delayed (9–10 days post-transfection with Lipofectamine 2000) are marked with an *.

switched to the replicating template (XDI-*Stu*I) within the J-UTR (such an event would have produced recombinant 5 in Fig. 1). Thus of the 18 recombinants tested, the J-UTR was not involved in 11 recombination events, was the site of recombination in 3, and participated in recombination with the NS-ORF in 4. Therefore, while the J-UTR could be involved in recombination, it was not the only site at which recombination occurred. The asymmetry of the results in this experiment (i.e., the recombination within the J-UTR when the genetic tags were in one orientation, but not the other) could have been due to selection against recombinants with both restriction sites that would have resulted from a recombination event in the J-UTR in the NDI-Stu/XNR-5355-3′ cotransfection. However, a Robo502 construct engineered to contain both sites yielded virus with a wild-type phenotype.

Recombination at the 3′ end of the genome

When DI-Stu transcripts were cotransfected with non-replicative transcripts from NR-5355-3′ constructs in which the 3′UTR had either been deleted (NR-5355-Δ3′UTR) or replaced with the 3′UTR from SIN (NR-5355-SIN3′UTR), recombinants were recovered when both Lipofectamine and Lipofectamine 2000 were used as the transfection reagent (Fig. 3A). Recombination between replicative constructs

that lacked the 3′CSE and nonreplicative constructs that contained the 3′CSE was next explored. It has not been demonstrated that a construct containing the 5′CSE and NS-ORF but lacking the 3′CSE can undergo RNA replication; however, these constructs were considered replicative in the context of these experiments because they contained the NS-ORF and thus could theoretically produce the viral replicase. Unexpectedly, when the 3′UTR was only present on the nonreplicative transcript, recombination was not detected when Lipofectamine was used as the transfection reagent. This included cotransfections with transcripts with minimal overlap [R-5′-7318/NR-7318-3′; R-5′-7318 denotes a replicative (R) transcript containing sequences from the 5′ end of the genome through nt 7318], with transcripts with overlap in the 3′NS-ORF/J-UTR/5′SP-ORF region (R-5′-7318/NR-5355-3′), and with replicative transcripts lacking only the 3′UTR (R-Δ3′UTR; replicative transcript lacking the 3′UTR) or with the 3′UTR replaced with the SIN 3′UTR (R-SIN3′UTR) or nonviral vector sequences (R-3′NVS) and the standard NR-5355-3′ nonreplicative transcript. When Lipofectamine 2000 was used as the transfection reagent, recombinants were recovered from these cotransfections, with the exception of the R-5′-7318/NR-7318-3′ cotransfection of transcript with minimal overlap. Additionally, using Lipofectamine 2000, recombinants were recovered from cotransfections with the R-Δ3′UTR,

A

NS-ORF   SP-ORF

5'UTR   J-UTR   3'UTR

NO RECOMBINATION

R-5'-7318
NR-7318-3'

R-5'-7318
NR-5355-3'

ΔUTR R-Δ3'UTR
3'SIN R-SIN3'UTR
NVS R-3'NVS
NR-5355-3'
NR-3'CSE

ΔUTR R-Δ3'UTR
3'SIN R-SIN3'UTR
NVS R-SIN3'NVS
NR-3'UTR

RECOMBINATION

DI-Stu
ΔUTR NR-5355-Δ3'UTR
DI-Stu
3'SIN NR-5355-SIN3'UTR

* recombination observed with Lipo2000

B

NS-ORF/3'CSE in *cis*;
Higher Efficiency (Lipofectamine)

i)

ii)

NS-ORF/3'CSE in *trans*;
Lower Efficiency (Lipo2000)

iii)

iv)

C

RECOMBINATIONAL PAIR

Δ5 DI-Stu-3'Δ5
NR-5355-3'

RECOMBINANTS

Δ5 7/7

0/7

RECOMBINATIONAL PAIR

DI-Stu-3'
Δ5 NR-5355-3'Δ5
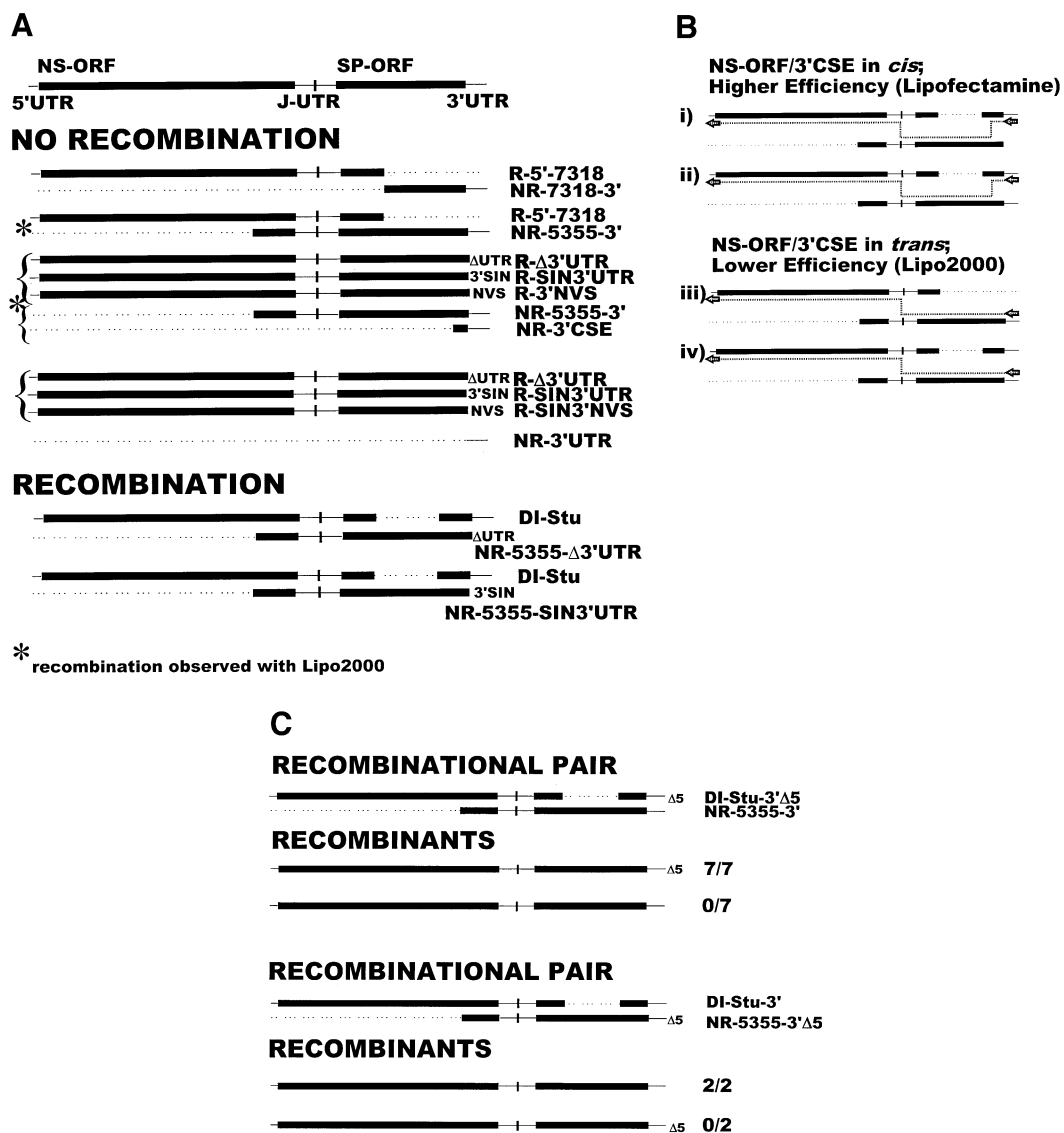
RECOMBINANTS

2/2

Δ5 0/2

Fig. 3. Recombination at the 3' end of the genome. Genomic diagrams of pairs of replicating and nonreplicating transcripts are shown in (A) that either failed to yield recombinants (NO RECOMBINATION) or yielded recombinants (RECOMBINATION) when Lipofectamine was used as the transfection reagent. Brackets indicate both replicating and nonreplicating transcripts used individually in cotransfections with a common replicating transcript(s) with a similar result. In some cases, transcript pairs that failed to yield recombinants following Lipofectaine-mediated transfection did yield recombinants when Lipofectamine 2000 was used, as indicated by an asterisk. In (B) is shown a model based on observations reported in (A) and previous reports that initiation of negative-strand synthesis is preferential for the NS-ORF and 3'CSE in *cis*. When one of the transcripts has the NS-ORF and 3'CSE in *cis* (i), negative-strand initiation and subsequent recombination is efficient to the extent that recombination is observed when Lipofectamine is used as the transfection reagent. When the NS-ORF and 3'CSE are in *trans* (iv), negative-strand initiation and recombination are less efficient and recombination is only observed when Lipofectamine 2000 is employed. This model predicts that when the NS-ORF and 3'CSE are available either in *cis* or in *trans* (ii and iii), initiation of minus-strand synthesis will occur preferentially on the 3'CSE in *cis* with the NS-ORF. To determine from which transcript the 3' end of the recombinant was derived in such a situation, DI-Stu and NR-5355-3' transcripts generated from constructs with a wt 3' end or a construct with a deletion of 5 nt from the 3' end of the genome [these constructs maintained the poly(A) tract] were used in cotransfections (Lipofectamine 2000) and virus recovered from recombinant plaques was sequenced across the region of overlap at the 3' end of the genome. As shown in (C), recombinants uniformly had the 3' end derived from the replicating parental transcript (DI-Stu).

R-SIN3'UTR, or R-3'NVS replicative transcripts and a nonreplicative transcript consisting of the 3'CSE, but not a nonreplicative transcript consisting of the 3'UTR.

It was previously shown that RUB replication is preferential, if not exclusive, for templates that contain both the 5'UTR and the NS-ORF in *cis* with the 3'CSE (Liang and

Gillam, 2000; Tzeng et al., 2001). Therefore, we reasoned that replication initiated preferentially at the 3' end of templates which contained the NS-ORF and 3'CSE in *cis* and that recombination to produce virus resulted if replication subsequently switched to the nonreplicating transcript and then back to the replicating transcript (Fig. 3Bi). Much less

efficiently, recombination resulted from initiation at the 3′ end of the nonreplicating template followed by template switching to the replicating transcript, such that it was only observed when Lipofectamine 2000 was used as the transfection reagent (Fig. 3Biii). To test this hypothesis, a genetic tag consisting of a deletion of the 3′ five nts preceding the poly(A) tract (Chen and Frey, 1999) was introduced into one of the transcripts used in a DI-Stu/NR-5355-3′ cotransfection using Lipofectamine 2000 reagent. As shown in Fig. 3C, when DI-Stu transcripts with the Δ5 tag were cotransfected with NR-5355-3′ transcripts, all of the recombinants had the Δ5 tag. When the reciprocal cotransfection (DI-Stu/NR-5355-3′Δ5) was done, none of the recombinants had the Δ5 tag. This demonstrated that when both constructs contained the 3′CSE (Fig. 3Bii and 3Biv), initiation of replication occurred on the transcript with the 3′ end in *cis* with the NS-ORF, confirming our hypothesis.

Discussion

This study provides the first evidence that RUB can undergo intermolecular RNA–RNA recombination. We previously showed that intramolecular recombination could occur, specifically between the SGPs of double subgenomic expression vectors resulting in deletion of the expressed foreign gene (Pugachev et al., 2000; Tzeng and Frey, 2002). To detect intermolecular recombination, cotransfection with transcripts containing nonoverlapping deletions was employed. One of these transcripts contained the 5′ end of the genome, including the 5′CSE through the NS-ORF and J-UTR, a deletion in the SP-ORF, and the 3′CSE, and therefore was capable of RNA replication. The other transcript, a nonreplicative transcript, contained the 3′ half of the genome beginning from the 3′ end of the NS-ORF. Plaque-forming virus was recovered that synthesized the standard genomic and subgenomic RNAs, demonstrating that RNA–RNA recombination between the transcripts had occurred to generate the standard viral genome rather than replication and copackaging of the transcripts as has been reported with alphaviruses (Geigenmuller-Gnirke et al., 1991; Weiss and Schlesinger, 1991). To account for incorporation of sequences from both transcripts into the recombinant virus, it is presumed that recombination occurs due to template switching by the viral RdRp during negative-strand RNA synthesis (Kirkegaard and Baltimore, 1986).

The initial replicative transcripts contained the 3′CSE in *cis* with the 5′CSE and NS-ORF; the nonreplicative transcripts contained the 3′CSE as well. Transfections with replicative transcripts that lacked the 3′CSE failed to yield recombinants when the lower efficiency transfection reagent Lipofectamine was employed, but were successful when the higher efficiency transfection reagent Lipofectamine 2000 was used. A requirement for the presence of the 3′CSE in *cis* with the NS-ORF for RNA replication was recently demonstrated in two studies (Liang and Gillam, 2001; Tzeng et al., 2001) and we therefore reasoned that negative-strand RNA synthesis initiated preferentially on the replicative transcript with the 3′CSE in *cis* with the NS-ORF followed by copy choice transfer of the replication complex to the nonreplicative transcript, resulting in recombination. At a lower efficiency, only detectable when Lipofectamine 2000 was employed as the transfection reagent, initiation occurred on transcripts with the 3′CSE in *trans* from the NS-ORF. Transcripts genetically tagged with a deletion at the exact 3′ end of the genome were used to prove that in the standard cotransfection in which both the replicative and the nonreplicative transcripts contained the 3′CSE; the 3′CSE in the recombinant was derived from the replicative transcript, even when Lipofectamine 2000 was used, as predicted by our hypothesis. Interestingly, this means that the recombinants recovered from the standard cotransfection were predominantly, if not exclusively, double recombinants, as shown in Fig. 2Bii. The recovery of recombinants from cotransfected transcripts with the NS-ORF and the 3′CSE in *trans* is the first demonstration that RUB negative-strand RNA synthesis can be initiated in *trans*.

Studies on recombination using SIN virus showed that a transcript consisting of the 3′ twenty-nucleotides plus the poly(A) tail was sufficient as a nonreplicative transcript when the replicative transcript was the genome with the 3′UTR replaced with nonviral-plasmid vector sequences, indicating that negative-strand RNA synthesis can initiate on this small 3′-terminal sequence (Hajjou et al., 1996; Hill et al., 1997). We were interested in taking a similar approach to define the minimal RUB 3′ *cis*-acting elements required for negative-strand initiation. The 3′-terminal 305 nts of the genome are retained in RUB DI RNAs generated during undiluted serial passage, implying that the complete 3′CSE is located within this region, which includes both 3′ end of the SP-ORF and the 60 nt 3′UTR (Frey and Hemphill, 1988; Derdeyn and Frey, 1995). Previous mutagenic analysis using both the genomic and the replicon infectious cDNA plasmid vectors had shown that the whole region was necessary for optimal replication but that only the 3′UTR with the exception of the 3′-five nucleotides and the poly(A) tract (which were rapidly regenerated in vivo) is essential for replication (Chen, 1998; Chen and Frey, 1999), a region that does not include the calreticulin-binding stem-loop. However, because most of the 3′-305-nucleotide region encodes E1, extensive mutagenesis of the region could not be done using an infectious cDNA genomic vector. Therefore, the ability to segregate this region into a recombination donor offers the opportunity to test which parts of it are necessary for negative-strand initiation. Following the design of the SIN experiments, we cotransfected replicative transcripts consisting of the genome with the 3′UTR either deleted or replaced with heterologous sequences such as the SIN 3′UTR or nonviral-plasmid vector sequences and nonreplicative transcripts consisting of the 3′CSE or 3′UTR.

Because of the *cis* preference for initiation of negative-strand synthesis, recombination did not occur following transfection with Lipofectamine, but with Lipofectamine 2000, recombinants were recovered when the 3′CSE but not the 3′UTR was used as the nonreplicative transcript. Hence, the 3′CSE is capable of initiating negative-strand synthesis. This result may indicate that the 3′UTR cannot serve as a promoter for negative-strand synthesis; however, there was no sequence overlap between the 3′UTR and these replicative transcripts and, as demonstrated with recombinational templates with minimal overlap in the SP-ORF, some overlap appears to be necessary for recombination to occur. Additionally, although Lipofectamine 2000 allowed for recovery of recombinants from R-Δ3′UTR/-, R-3′SINUTR/-, and R-3′NVS/3′CSE cotransfections, appearance of CPE was delayed to the extent that individual plaques could not be recognized due to senescence of the monolayer and when bulk transfection plate culture fluid was harvested and amplified, the recombinant virus was found to have the wt 3′ sequence (data not shown). In contrast, in similar cotransfections, the SIN 3′UTR was found to be able to accommodate extensive rearrangements, including the presence of nonviral sequences. The apparent inability of the RUB 3′UTR to tolerate rearrangements would also serve to limit recombination between replicative transcripts with heterologous 3′UTRs and nonreplicative transcripts consisting of short 3′-terminal fragments. Thus, this experimental model is not applicable to RUB.

We also investigated recombination in the other region of the genome that overlapped between the standard replicative–nonreplicative transcript pair, i.e., the 3′NS-ORF/J-UTR/5′SP-ORF. Deletion analysis indicated that the presence of NS-ORF or the SP-ORF in both transcripts was not necessary for recombination; however, the presence of the J-UTR in both transcripts was necessary for efficient recombination (i.e., Lipofectamine-mediated transfection) and the 3′ end of the J-UTR was essential. This was not due to minimal overlapping sequence requirements since, despite the deletions into the J-UTR, an overlap of 482 nts in the SP-ORF was maintained between the transcripts. Thus, we hypothesized that the J-UTR was a recombinational hot spot. To test this hypothesis, the standard cotransfection was done with transcripts genetically marked on either end of the J-UTR with a restriction site, *Nsi*I at the 5′ end or *Xba*I at the 3′ end. When *Xba*I-marked replicative and *Nsi*I-marked nonreplicative transcripts were used, recombinants that retained the single site as well as recombinants that had neither site were recovered, indicating that recombination both outside the J-UTR (resulting in recombinants with a single site) and within the J-UTR (resulting in recombinants with neither site) had occurred. Interestingly, the largest class of the recombinants had duplications either within the J-UTR or between the 3′NS-ORF and the J-UTR; the latter recombinants resulted from participation of both regions in the recombination event. Thus, recombination occurred in

all regions of the overlap between the transcripts. When *Nsi*I-marked replicative and *Xba*I-marked nonreplicative transcripts were used, half of the recombinants had only the *Nsi*I site and the other half had the *Xba*I site; to recover the former recombinant, the recombination event was in the SP-ORF downstream from the J-UTR, while to recover the latter recombinant, the recombination was in the NS-ORF upstream from the J-UTR. A recombination in the J-UTR would have yielded a recombinant with both restriction sites and we initially thought that this type of recombinant was selected against, since recombination in the J-UTR clearly occurred based on results with the reciprocal transcripts. However, a Robo502 construct containing both restriction sites had a wild-type phenotype and the reason for the asymmetry of recombinant recovery is therefore not clear. Nevertheless, these results show that recombination can readily occur outside of the J-UTR. Thus, based on this experiment, the J-UTR does not appear to be a high-avidity recombinational hot spot compared to neighboring sequences in the NS-ORF and SP-ORF. An alternative hypothesis is that a sequence within the J-UTR is necessary in the donor template or on both templates for recombination to occur in this region, whether or not the J-UTR itself is involved in the recombination event. For example, secondary structure in this sequence could interrupt transcription or this sequence could bind a factor that attracts the RdRp complex, facilitating strand transfer. In a plant virus system, it was found that secondary structures that facilitated template switching and recombination were also promoters for RNA replication and bound the viral RdRp (Nagy et al., 1999). In this regard, in the predicted secondary structure of the J-UTR, there is a stable stem-and-loop structure ($\Delta G = -21.24$ kcal/mol) formed by the 3′ end of the J-UTR (data not shown).

In the 3′ region of the genome, only precise recombinants were recovered, while in the J-UTR region, both precise recombinants and duplications of varying lengths were detected. These events have been defined as homologous and aberrant homologous recombination, respectively (Lai, 1992). In a study on intramolecular recombination between SGPs in a double subgenomic vector, both precise recombinants and recombinants with duplications in the J-UTR were recovered with the majority being precise recombinants (Tzeng and Frey, 2002). In this study, which covered a larger region of the genome, the duplications involved only the J-UTR or a combination of the 3′NS-ORF and the J-UTR and thus in all cases the integrity of both the NS-ORF and the SP-ORF as well as the 3′UTR was preserved. A similar assortment of recombinants in the J-UTR was recovered when pairs of transcripts corresponding to the transcripts used in this study were used in a study on SIN recombination (Weiss and Schlessinger, 1991). Therefore, there was apparent selection pressure on maintenance of both ORFs and the 3′UTR. In previous studies, we have found that while the 3′UTR is intolerant of mutagenesis, the

J-UTR will accommodate extensive manipulation (Chen and Frey, 1999; Tzeng and Frey, 2002). Presumably, both precise and imprecise recombinational events occur throughout the genome, but in most of the genome only precise recombinants are viable. It is also possible that duplications caused by imprecise recombination are rapidly removed by intramolecular recombination, as was the case with our double subgenomic vectors (Tzeng and Frey, 2002). This could explain the discrepancy observed in Northern gels of recombinant viruses in which the RNA species were larger than the standard RNAs, but the duplications detected by sequencing after an additional passage were found to be <100 nts (Fig. 1A and B). An evolution toward the wild-type sequence was also observed with passage of SIN recombinants with duplications in the J-UTR region (Weiss and Schlesinger, 1991). Alternatively, duplications could have been present in other regions of the genome that did not overlap between the cotransfected transcripts and were not sequenced in the recombinants.

Finally, aberrant homologous recombination events also allow determination of recombination sites, which are thought to be associated with secondary structure or regions of complementarity (Lai, 1992; Nagy and Simon, 1997). However, the only tendency revealed by analysis of the exchange sites in aberrant recombinants recovered in this study was that the donor site was always in a single-stranded or loop region of the predicted secondary structure. There was no evidence of complementarity between the donor and acceptor site.

Materials and methods

Recombinant DNA procedures

Standard recombinant techniques were used throughout this investigation with minor modifications (Sambrook et al., 1989). Restriction enzymes were purchased from Promega (Madison, WI), New England Biolabs (Beverly, MA), or Roche (Indianapolis, IN) and were used with protocols and buffers supplied by the manufacturers. Restriction fragments were purified from agarose gels using GeneClean II (Bio101, Carlsbad, CA) or Qiagen Gel Extraction (Qiagen, Valencia, CA) kits. Standard PCR reactions consisted of 20 ng of linearized plasmid template, 400 ng of each primer (primers used for PCR are listed in Table 1), dNTPs (2.5 mM each), and 5 units of Takara *Ex* Taq DNA polymerase (PanVera, Madison, WI) in $1\times$ *Ex* Taq buffer [20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, and 50% glycerol], 0.05% β-mercaptoethanol, and 10% dimethyl sulfoxide (DMSO) in a total volume of 50 μl. After 30 cycles of a reaction protocol of 20 s at 95°C for denaturing, 30 s at 50°C for annealing, and 2 min at 72°C for extension followed by one cycle of 10 min at 72°C, the reaction products were analyzed by agarose gel electrophoresis and then pu-

rified using Qiaquick (Qiagen) or Wizard PCR Preps (Promega, Valencia, CA) kits. The purified PCR products were restricted with appropriate enzymes prior to ligation. Ligation reactions containing approximately equimolar concentrations of DNA fragments and 10 units/μl of T4 DNA ligase (New England Biolabs) in a total volume of 20 μl of $1\times$ reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 10 mM dithiothreitol, 1mM ATP, 25 μg/ml bovine serum albumin) supplied by the manufacturer were incubated at 14°C overnight or at room temperature for 1 h. To replace a restriction fragment in a vector with the corresponding restriction fragment from another vector or a PCR product with a mutation, the vector was digested with the appropriate enzymes followed by agarose gel electrophoresis. The larger fragment containing the plasmid backbone was isolated and then ligated with the corresponding restriction fragment isolated from another vector or produced by PCR amplification.

Vectors and plasmid constructs

Escherichia coli strains MC1061 and JM109 were used as bacterial hosts. All plasmid constructs were checked by restriction digestion and sequencing to confirm the correct ligation of fragments and/or verify the presence of designed mutations. Automated sequencing was performed using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and appropriate primers. The sequencing reactions were purified on Centrisep columns (Princeton Separations, Adelphia, NJ) and then resolved using an ABI 373 sequencer (Perkin–Elmer Corp, Foster City, CA).

The following RUB cDNA vectors have been described previously: Robo302/402/502 (infectious cDNA clones using pCL1921, pBR322, and pUC18 as the backbone vector, respectively; Pugachev et al., 1997, 2000; Tzeng et al., 2001; Tzeng and Frey 2002); NRobo402 (Robo402 derivative with a unique *Nsi*I site immediately downstream from the NS-ORF at the beginning of the junction UTR; Tzeng and Frey, 2002); Robo302-3′Δ5 [Robo302 derivative with a deletion of the 3′-terminal 5 nt preceding the poly (A) tract; referred to as Robo302–340 in Chen and Frey, 1999]; dsRobo402/GFP (a Robo402 derivative with a second subgenomic promoter driving synthesis of a second subgenomic RNA from which GFP is expressed; Pugachev et al., 2000); Robo DI-pas3, DI-Stu, DI-AB, and DI-325 (DI cDNA vectors with deletions in the SP-ORF; Tzeng et al., 2001); and RUBrep/GFP (replicon expressing green fluorescent protein in place of the SP-ORF; Tzeng et al., 2001). XRobo502, a Robo502 derivative with a unique *Xba*I site immediately preceding the SP-ORF, was constructed by first amplifying a PCR product from Robo502 template with primers 1032 (*Nsi*I and *Xba*I sites followed by the 5′-fourteen nts of the SP-ORF, nts 6512–6526 of the RUB genome) and 157 (complementary to nts 6716–6733). Following restriction with *Nsi*I and *Not*I, this product was used

to replace the *Nsi*I-*Not*I fragment of pGEM474, a construct containing nt 6398 through the *Eco*RI linearization site of Robo402 cloned between the *Nsi*I and *Eco*RI sites of the MCS of pGEM-11Zf(−) (see below). The *Xba*I-*Eco*RI fragment from this construct was then used to replace the *Xba*I-*Eco*RI fragment of RUBrep/GFP.

The standard replicating vector contained a deletion between nts 6936 and 9334 of the genome (two *Stu*I sites) within the SP-ORF. Constructs with this deletion were produced by digesting a genomic cDNA vector (NRobo502, XRobo502, Robo302-3′Δ5) with *Stu*I, isolating the large fragment containing the vector backbone and 5′ and 3′ ends of the Robo cDNA sequences, and religating. Robo302-Δ3′UTR, a Robo302 derivative which lacks the 3′UTR but retains the poly(A) tract, was described previously (as Robo302–315 in Chen and Frey, 1999). To create a replicating vector that contained nonviral sequences at the 3′ end of the transcripts in place of the 3′UTR, PCR was done using Robo302Δ3′UTR template, upstream primer 52b (nts 9174–9187 including a unique *Bam*HI site at nt 9174) and downstream primer 315-1 (*Eco*RI site followed by sequences complementary to nt 9687–9707, the 3′ end of the SP-ORF). The amplified fragment was restricted with *Bam*HI and *Eco*RI and used to replace the corresponding fragment in Robo302. This vector, Robo302-3′NVS, could be linearized with *Ssp*I or *Spe*I to produce transcripts with 3′-terminal nonviral sequences of 357 and 685 nts, respectively, in place of the 3′UTR and poly(A) tract. Robo502-SIN3′UTR, a Robo502 derivative which contains the SIN 3′UTR in place of the RUB 3′UTR, was constructed by PCR using pTE5′ZJ/CAT as a template and upstream primer 694 (nts 11,302–11,318 of the SIN genome, including an *Mfe*I site) and downstream primer 695 (*Eco*RI site followed by a T_{20} tract and sequences complementary to the 3′-twenty nts of the SIN genome). The amplified fragment was restricted with *Mfe*I and *Eco*RI and inserted at the *Eco*RI site in NR-3′NVS. Finally, Robo302 5′-7318, which terminates at nt 7318, was generated by digesting Robo302 with *Asc*I and *Eco*RI [the linearization site following the poly (A) tract], treating the digested fragments with Klenow (New England Biolabs) to produce blunt ends, isolation of the larger DNA fragment containing the vector, and cDNA sequences from the 5′ end through the *Asc*I site by agarose gel electrophoresis, and self-ligating this fragment.

The standard nonreplicating vector, NR-5355-3′, contained the 3′ end of the Robo cDNA from nt 5355 through the 3′ *Eco*RI linearization site following the poly(A) tract in a pGEM plasmid vector under control of the SP6 RNA polymerase promoter. NR-5355-3′ was constructed by restriction of Robo402 with *Bgl*II and *Eco*RI, and the *Bgl*II and *Eco*RI fragment was used to ligate with *Bam*HI-*Eco*RI-restricted pGEM3Z (Promega). NR-5355-3′Δ5 was constructed by replacing the *Bam*HI-*Eco*RI fragment in NR-5355-3′ with the amplified PCR product from Robo302 template with primers 52 (nts 9163–9180, including a *Bam*HI site at nt 9174) and 340 [*Eco*RI followed by oligo

(dT) and sequences complementary to nts 9738–9757]. NR-5355-3′ΔUTR, which lacks the 3′UTR (nts 9703–9762) but contains a poly(A) tract, was constructed by PCR using Robo302 template, upstream primer 52, and downstream primer 315 (*Eco*RI site followed by an T_{20} tract and sequence complementary to nts 9687–9706 of the genome). The amplification product was restricted with *Bam*HI and *Eco*RI and used to replace the corresponding fragment in NR-5355-3′. To create a nonreplicative vector that contained the SIN-3′UTR in place of the RUB 3′UTR, the *Bgl*II-*Eco*RI fragment of Robo502-SIN3′UTR was introduced into pGEM11Zf(−). NR-7318-3′, which contains the 3′ end of the Robo cDNA sequences from nt 7318 (an *Asc*I site) through the 3′ *Eco*RI linearization site, was constructed by annealing T4 polynucleotide kinase (New England Biolabs) phosphorylated oligos 518 and 519 to produce an SP6 RNA polymerase promoter flanked by 5′ *Hin*dIII and 3′ *Asc*I overhangs and using this linker to replace the *Hin*dIII-*Asc*I fragment in Robo302. NNR-5355-3′ and XNR-5355-3′ were constructed by restricting NRobo502 and XRobo502, respectively, with *Bgl*II and *Eco*RI and cloning the *Bgl*II-*Eco*RI fragment into *Bam*HI-*Eco*RI-restricted pGEM-11Zf(−). NR-3′CSE, a construct containing the 3′CSE and poly(A) tract, constructed by PCR amplification using primers 565 (*Not*I site followed by nts 9457–9471) and 105 [*Eco*RI site followed by oligo (dT)] and Robo402 template, restricting the amplification product with *Not*I and *Eco*RI and cloning it into *Not*I-*Eco*RI restricted pGEM-11Zf(−). A nonreplicative construct containing the 3′UTR and poly(A) tract, NR-3′UTR, was constructed by PCR amplification using primer 498 (*Hin*dIII site followed by a T7 RNA polymerase promoter and nts 9706–9720 of the RUB genome) and primer 105 (*Eco*RI site followed by T_{20}), restriction of the amplified product with *Hin*dIII and *Eco*RI, and ligation into *Hin*dIII-*Eco*RI restricted pUC18.

NR-6263-3′, NR-6313-3′, pGEM-474, NR-6408-3′, NR-6436-3′, and NR-6462-3′ were constructed by digestion of dsRobo402/GFP, dsRobo402/GFP/B438, dsRobo402/GFP/B474, dsRobo402/GFP/B440, dsRobo402/GFP/B441, and dsRobo402/GFP/B442, respectively (Tzeng and Frey, 2002), with *Nsi*I and *Eco*RI and cloning the *Nsi*I-*Eco*RI fragment into *Nsi*I-*Eco*RI-restricted pGEM-11Zf(−). A series of constructs containing sequential deletions of the SGP was made by PCR using Robo402 template, a mutagenic upstream primer consisting of an *Nsi*I site followed by 15 nts of the RUB genome downstream from the deletion site, and downstream primer 157 (complementary to nts 6716–6733, downstream from a *Not*I site at nt 6622). The amplified fragment was restricted with *Nsi*I and *Not*I and used to replace the corresponding fragment in pGEM474. The constructs (the number corresponds to the nt of the genome at which the RUB sequences begin) and upstream mutagenic primers used in their construction were as follows: Robo3′6467, primer 993; Robo3′6473, primer 995; Robo3′6479, primer 994; Robo3′6486, primer 963; Robo3′6651, primer 964; Robo3′6751, primer 965;

Robo3′6851, primer 966; and Robo3′6951, primer 967. Additionally, Robo3′6622 was constructed by digesting pGEM474 with *Not*I and *Eco*RI and cloning the *Not*I-*Eco*RI fragment into pGEM-11Zf(−).

In vitro transcription, transfection, and infection

All plasmids used for in vitro transcription were purified by cesium chloride isopycnic gradient centrifugation (Sambrook et al., 1989). For each construct, one plasmid clone with the desired manipulation or mutation confirmed by sequencing was used for in vitro transcription and subsequent transfection. Plasmids were linearized with *Eco*RI prior to transcription (with the exception of Robo302-3′NVS, which was linearized with *Ssp*I or *Spe*I). One microgram of linearized template plasmid was used in each transcription reaction. Transcription reactions with SP6 or T7 RNA polymerase in the presence of cap analog were done as previously described (Pugachev et al., 1997; Chen and Frey, 1999) followed by digestion with RNase-free DNase (1 U/μl) (Promega) for 30 min at 37°C and phenol–chloroform extraction. The resulting transcripts were analyzed by agarose gel electrophoresis and relative concentrations were approximated by estimating the intensity of the RNA bands after ethidium bromide staining. Aliquots of reaction mixtures were used directly for transfection.

Transfection was performed on ~80–95% confluent Vero cells grown at 35°C in 35-mm^2-diameter plates in Dulbecco's minimal essential medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (5%FBS-DMEM) and gentamycin (10 μg/ml). The cells were washed twice with phosphate-buffered saline (PBS) and once with Opti-MEM I (Gibco-BRL) and transfected with a mixture that contained 200 μl Opti-MEM I (Gibco-BRL), approximately equal amounts of each RNA transcript (4–7 μl of each transcript), and 5 μl of Lipofectamine reagent or Lipofectamine 2000 (Gibco-BRL). After mixing, the transfection mixture was incubated for 45 min at room temperature followed by addition of 0.8 ml Opti-MEM I to the washed cells. For the lipofectamine-mediated transfection, the cells were incubated for 3 to 5 h at 35°C and then 1 ml of 5% FBS-DMEM was added to the cells. The transfection mixture was removed after overnight incubation and replaced with 3 ml of 2% FBS-DMEM and the cells were monitored daily for development of CPE. For Lipofectamine 2000 mediated transfection, ~95% confluent cells were used and, following addition of the transfection mixture, cells were incubated for 3–5 h before the transfection mixture was removed and replaced with 2% FBS-DMEM and monitored daily for development of CPE. After the detection of CPE, transfected culture supernatants were harvested and 250 μl was used to infect fresh Vero cells which were then incubated and monitored for appearance of CPE to confirm the presence of virus.

Where indicated, transfections were done using 60-mm^2 plates and after a 3–4 h incubation with the transfection mixtures, the cells were overlaid with plaque assay agar [MEM containing 1% FBS, 0.1% DEAE dextran, 0.1% penicillin/streptomycin, and 0.4% agar (Oxoid agar No. 1; Ogdensburg, NY)]. Single plaques were located by microscopy and excised, and virus was eluted in 500 μl DMEM at 4°C overnight, followed by one round of amplification in Vero cells. Additionally, where indicated, culture fluid in transfected cells maintained in liquid medium was passaged after appearance of CPE, as described, to fresh cells which were then overlaid with agar after a 1-h adsorption.

Analysis of virus-specific RNA produced by recombinant viruses

Total cellular RNA was extracted from monolayers of infected Vero cells 4–8 days postinfection (corresponding to appearance of CPE) with RNA Wiz (Ambion, Inc., Austin, TX) following the manufacturer's protocol. RNA extracted from a 35-mm^2 plate of Vero cells was dissolved in 20 to 50 μl of diethylpyrocarbonate-treated water and stored at −70°C. Northern analysis was done as previously described (Tzeng et al., 2001) using NR-6622-3′ that was ^{32}P-labeled by nick-translation. To sequence the regions of the genome in recombinant viruses that overlapped in the transcripts used in cotransfection, RT-PCR followed by automated sequencing of the amplification product was employed; the primer pairs used for RT-PCR and sequencing are listed in Table 2. The negative-sense primer (50 or 100 ng) was annealed at 55°C with 5 μl of the extracted RNA template in Superscript Reverse Transcriptase buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$] (Gibco–BRL) in enzyme-free water for 2 min followed by addition of 100 mM DTT, 20 mM of each deoxynucleotide triphosphate (dNTPs), 1 μl RNase inhibitor (RNasin, 40 U/μl), and 1 μl SuperscriptII Reverse Transcriptase (200 U/μl) in a total volume of 20 μl. The reaction mixture was incubated for 60 min at 45°C and 1 μl RNaseH (0.5–2 U/μl) was then added to the reaction mixture and incubation was continued for 30 min at 37°C, at the end of which time 5 μl was used for PCR amplification using the paired positivex and negative-sense primers listed in Table 2. Cycle sequencing of the amplified PCR product was done as described above.

Table 2
Oligonucleotides used in this study

Number (polarity)[a]	Sequence (5′-3′)	Properties[b]
Vector Construction[c]		
1032 (+)	GCATATGCATCTAGAATGGCTTCTACTACC	*NsiI*/*XbaI* followed by 6512–6526
157 (−)	CGCGGATCCTACTACCAGTCCCTGCGCTGGCC	*BamHI* followed by *c*6716–6733
52b (+)	CGGGATCCACGCCCAC	9174–9187 including *BamHI*
315-1 (−)	CGTGAATTCCCACTAGCGCGGCGCTATAG	*EcoRI* followed by *c*9687–9707
694 (+)	ACACCAATTGTTTTCGGCGGCGCCTCG	11302–11318 (SIN) including *MfeI*
695 (−)	CGGAATTCT$_{20}$GAAATGTTAAAAACAAAATTT	*EcoRI* followed by oligo-dT and *c*11687–11703 (SIN)
326 (+)	GAGATCTTCGCCGGCATGT	5354–5372 including *BglII*
105 (−)	ACGTGAATTCT$_{20}$	*EcoRI* followed by oligo-dT
52 (+)	AATGCCCGAGTGGATCCA	9163–9180 including *BamHI*
340 (−)	CGTGAATTCT$_{20}$CAGCAACAGGTGCGGGGATC	*EcoRI* followed by oligo-dT and *c*9738–9757
52 (+)	AATGCCCGAGTGGATCCA	9163–9180 including *BamHI*
315 (−)	CGTGAATTCT$_{20}$CCACTAGCGCGGCGCTATAG	*EcoRI* followed by oligo-dT and *c*9687–9706
518 (−)	AGCTTATTTAGGGG	*HindIII* 5′ *overhang* followed by SP6 promoter
519 (+)	CGCGCCCCTAAATA	*AscI* 5′ *overhang* followed by *c*SP6 promoter
565 (+)	GCATGCGGCCGCTGCGGGGGCGAGAG	*NotI* followed by 9457–9471
105 (−)	ACGTGAATTCT$_{20}$	*EcoRI* followed by oligo-dT
498 (+)	TCGAAGCTT*AATACGACTCACTATA*GGCCCCCGCGCGAA	*HindIII*, *T7 promoter* followed by 9706–9720
105 (−)	ACGTGAATTCT$_{20}$	*EcoRI* followed by oligo-dT
963 (+)	CCCCCATGCATATTCGGGAGAGCCCC	*NsiI* followed by 6486–6499
964 (+)	CCCCATGCATCCTCCGGAGATGACTCCG	*NsiI* followed by 6651–6671
965 (+)	CCCCATGCATCCCGGAGGAGCGGCAAGAA	*NsiI* followed by 6751–6771
966 (+)	CCCCATGCATGGGGGCTCTGCCCCGCG	*NsiI* followed by 6851–6867
967 (+)	CCCCATGCATAGGCACCCACCGAGGCCTGC	*NsiI* followed by 6951–6970
993 (+)	CCCCATGCATTGGGTACCCAACTTTTG	*NsiI* followed by 6467–6483
994 (+)	CCCCATGCATTTTTGCCATTCGGGAG	*NsiI* followed by 6479–6494
995 (+)	CCCCATGCATCCCAACTTTTGCCATTC	*NsiI* followed by 6473–6489
RT-PCR 3′ end of genome[d]		
F1 (−)	CGCGAATTC(T)$_{20}$CTATACAGCAACAGGTGC	*EcoRI* followed by oligo-dT and *c*9745–9762
52 (+)	AATGCCCGAGTGGATCCA	9163–9180 including *BamHI*
F1 (−)	CGCGAATTC(T)$_{20}$CTATACAGCAACAGGTGC	*EcoRI* followed by oligo-dT and *c*9745–9762
52b (+)	GGGATCCACGCCCAC	9174–9182 including *BamHI*
105 (−)	ACGTGAATTCT$_{20}$	*EcoRI* followed by oligo-dT
52 (+)	AATGCCCGAGTGGATCCA	9163–9180 including *BamHI* (+)
105 (−)	ACGTGAATTCT$_{20}$	*EcoRI* followed by oligo-dT
52b (+)	GGGATCCACGCCCAC	9174–9182 including *BamHI*
216 (−)	CCGGTCTAGACTATACAGCAACAGGTGCGGCAATCT	*XbaI* followed by *c*9737–9762
52 (+)	AATGCCCGAGTGGATCCA	9163–9180 including *BamHI*
3′NS-ORF/J-UTR/5′SP-ORF		
325 (−)	CGGGATCCCCGGCGCGCGCGGTG	*BamHI* followed by *c*7399–7413
173 (+)	CCGGAATTCCGACTACAGCGCGGAGC	*EcoRI* followed by 6244–6260
197 (−)	CACGAAGCTTGTGCCTCGAGGGCCTTC	*HindIII* followed by *c*6550–6566
177 (+)	CCGGAATTCGGTGCTTTGCCGCCGTT	*EcoRI* followed by 6124–6140
550 (−)	CGCAGGCCTCGGTGGGTG	*c*6954–6971 including *StuI*
362 (+)	GCCCTTTTCGCCAGATCC	5196–5213
1033 (−)	GTACTCTAGATTCGGGCACCCTGGGGCTC	*XbaI* followed by *c*6494–6512
715 (+)	GCATGCGGCCGCTTTTCGCCAGATCCC	*NotI* followed by 5200–5214
159 (−)	CGCGGATCCTACTAAAAGACCGCGCCTTCGCC	*BamHI* followed by *c*6995–7014
177 (+)	CCGGAATTCGGTGCTTTGCCGCCGTT	*HindIII* followed by 6124–6140

[a] Restriction sites (underlined), RNA polymerase promoters (italics) and colinear or complementary (*c*) sequences of the RUB (no designation) or SIN genome included in the oligonucleotide primer are given. (+) polarity primers are colinear with the genomic RNA sequence while (−) polarity primers are complementary.

[b] The oligonucleotide primer pairs used to generate individual vector constructs, in order as described in the Methods section, are paired by brackets.

[c] Oligo primers 963–967 (+) and 993–995 (+) were each used with oligo 157 (−) in vector construction.

[d] The bracketed oligonucleotide primer pairs were used for RT-PCR to amplify regions of the genome of recombinant viruses that overlapped in the parental transcripts, the 3′ end of the genome and the regions containing the 3′ end of the NS-ORF, the J-UTR, and the 5′ endof the SP-ORF (see Fig. 1). The amplification products were then used for sequencing.

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