

## Characterization of Defective-Interfering RNAs of Rubella Virus Generated during Serial Undiluted Passage

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During serial undiluted passage of rubella virus (RUB) in Vero cells, two species of defective-interfering (DI) RNAs of approximately 7000 and 800 nucleotides (nts) in length were generated (Frey, T. K., and Hemphill, M. L., *Virology* 164, 22–29, 1988). In this study, these DI RNAs were characterized by molecular cloning, hybridization with probes of defined sequence, and primer extension. The 7000-nt DI RNA species were found to be authentic DI RNAs which contain a single 2500- to 2700-nt deletion in the structural protein open reading frame (ORF) region of the genome. The 800-nt RNAs were found to be subgenomic DI RNAs synthesized from the large DI RNA templates. Analysis of the extent of the deletions using a reverse-transcription-PCR protocol revealed that the 3' end of the deletions did not extend beyond the 3' terminal 244 nts of the genome. The 5' end of the deletions did not extend into the nonstructural protein ORF; however, DI RNAs in which the subgenomic start site was deleted were present. Following serial undiluted passage of seven independent stocks of RUB, this was the only pattern of DI RNAs generated. DI RNAs of 2000 to 3000 nt in length were the majority DI RNA species in a persistently infected line of Vero cells, showing that other types of RUB DI RNAs can be generated and selected. However, when supernatant from the persistently infected cells was passaged, the only DI RNAs present after two passages were 7000 nts in length, indicating that this species has a selective advantage over other types of DI RNAs during serial passage. © 1995 Academic Press, Inc.

### INTRODUCTION

Rubella virus (RUB) is a member of the *Togavirus* family and is the sole member of the *Rubivirus* genus (Francki *et al.*, 1991). The RUB genome is a single-stranded, positive-polarity RNA molecule of 9757 nucleotides (nts) in length (reviewed by Frey, 1994). The genome RNA contains two long open reading frames (ORFs). The 5' proximal ORF encodes the nonstructural proteins (NSP-ORF) and the 3' proximal ORF encodes the structural proteins (SP-ORF) in the order NH<sub>2</sub>-C-E2-E1-COOH. Both nonstructural and structural proteins are proteolytically processed from a polyprotein precursor into the individual proteins. Replication of the virus RNA is carried out through a negative-polarity, genome-length intermediate RNA species: initiation at the 3' end of the negative-polarity template leads to production of the genomic RNA while initiation at an internal site on the negative-polarity RNA template leads to production of a subgenomic RNA (SG RNA). The SG RNA is translated to produce the structural proteins. The genome organization and replication strategy of RUB is similar to that of the alphaviruses, the other *Togavirus* genus whose members have been well-characterized (reviewed in Strauss and Strauss, 1986).

RUB generates defective-interfering (DI) RNAs during both serial undiluted passage and persistent infection in cell culture (Norval, 1979; Bohn and van Alstyne, 1981; Terry *et al.*, 1985; Frey and Hemphill, 1988; Abernathy *et al.*, 1990). In a previous report from this lab (Frey and Hemphill, 1988), DI RNAs were detected after four serial passages in Vero cells. Two DI RNA species were generated and maintained through 20 passages: a large species of approximately 7000 nts in length and a smaller species ranging from 700 to 1000 nts in length. Concomitant with the appearance of DI RNAs, a decrease in the amount of standard genome RNA and a drop in virus titer were observed (Frey and Hemphill, 1988), indicating that these DI RNAs interfered with the replication of standard virus. In persistently infected Vero cells, up to seven DI RNAs were detected and the number of these species varied as the persistently infected culture was propagated (Abernathy *et al.*, 1990). The majority of the DI RNAs in persistently infected cells is less than 3000 nts in length (Norval, 1979; Frey and Hemphill, 1988; Abernathy *et al.*, 1990). This paper describes the molecular characterization of DI RNAs generated during serial passage of RUB in Vero cells.

### MATERIALS AND METHODS

#### Cells and virus

Vero cells, obtained from the American Type Culture Collection, were maintained at 35° under 5% CO<sub>2</sub> in Dul-

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becco's minimal essential medium (DMEM) (Gibco-BRL) supplemented with 5% fetal bovine serum, 10% tryptose phosphate, and gentamicin (20  $\mu\text{g}/\text{ml}$ ). Vero cells were routinely subcultured at a 1:10 dilution every 7 days by trypsinization.

The W-Therien strain of RUB was provided by J. Wolinsky and the F-Therien and M33 strains of RUB were provided by J. K. Chantler. Some of these strains were plaque-purified in Vero cells before use. Supernatants from synovial cell explant cultures persistently infected with the F-Therien and M33 strains of RUB were provided by J. K. Chantler. To amplify DI-containing stock from the previous study (Frey and Hemphill, 1988), passage 10 and passage 18 were passed twice in Vero cells and RNA was extracted from cells infected with the second passage (P12 and P20). All other serial passages were done by initially infecting  $1 \times 10^6$  Vero cells with virus stock or supernatant from persistently infected cells. In each passage the supernatant was harvested 48 to 72 hr postinfection and one-fourth of the supernatant was used to infect  $1 \times 10^6$  Vero cells.

#### Intracellular RNA extraction and Northern analysis

Vero cells were infected with standard RUB (plaque-purified W-Therien) at an m.o.i. of 0.1 PFU/cell or serial passage stock for 1 hr at 35°, after which time the inoculum was removed and replaced with growth medium. Intracellular RNA was extracted between 48 and 72 hr postinfection (at the time that cytopathic effects were initially observable) by the method of Sawicki *et al.* (1981). Northern gel analysis of intracellular RNA was as previously described (Hemphill *et al.*, 1988) with the exception that nylon membranes (Micron Separations, Inc.) were used instead of nitrocellulose. Synthesis of and hybridization with  $^{32}\text{P}$ -labeled positive- and negative-polarity RNA probes containing the 3' 587 nts of the RUB genome were as described previously (Hemphill *et al.*, 1988). Northern transfers to be hybridized with radiolabeled oligonucleotide probes were prehybridized in 5X SSC, 1% SDS, 1 mM EDTA, containing 200  $\mu\text{g}/\text{ml}$  of denatured salmon sperm DNA for 2 hr or Rapid Hybridization Buffer (Amersham) for 30 min at 42° followed by addition of the probe. Oligonucleotide probes, synthesized using an ABI Model 381A Synthesizer, were 5' end-labeled with [ $^{32}\text{P}$ ]ATP using polynucleotide kinase as described previously (Frey *et al.*, 1989). Hybridizations in Rapid Hybridization Buffer were done at 42°. For hybridizations in 5X SSC, the hybridization temperature for oligonucleotide probes was calculated using the following formula:  $T_H (\text{in } ^\circ) = 2 \times (A + T) + 4 \times (G + C) - 5$ , where A, T, C, and G correspond to the number of times each nucleotide occurs in the oligonucleotide probe. After hybridization, membranes were washed at the hybridization temperature twice in 2X SSC, 0.1% SDS for 15 min and once in 1X SSC, 1% SDS for 15 min. Washed membranes were wrapped in plastic wrap and exposed to Kodak X-Omat

AR film at -80° between two Cronex Lightning Plus intensifying screens (DuPont).

#### cDNA synthesis and cloning

P12 intracellular RNA was fractionated on a 15 to 30% sucrose gradient in 1X TEN buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 M NaCl] by high-speed centrifugation in a Beckman SW41 rotor at 40,000 rpm for 4 hr at 25°. RNA fractions identified by Northern hybridization to contain DI RNAs were used as a template for oligo-dT-primed cDNA synthesis. First- and second-strand cDNA synthesis was done using a Promega Riboclone cDNA synthesis kit. Double-stranded cDNA was size-selected by spun column chromatography through a 1-ml Sephacryl S-400 (Pharmacia) column. The size-selected cDNA was dCTP-tailed using terminal deoxynucleotidyl transferase as previously described (Frey *et al.*, 1986), annealed with pGEM-3z(f-) (Promega) tailed with dGTP, and transformed into maximum efficiency *Escherichia coli* DH5 $\alpha$  cells (Bethesda Research Laboratories). Colonies containing plasmids with RUB-specific inserts were identified by colony blot hybridization using as a probe the *EcoRI-HindIII* fragment of Robo12 [a plasmid containing a complete cDNA copy of the RUB genome (Wang *et al.*, 1994)] labeled with [ $\alpha^{32}\text{P}$ ]dATP by random priming.

#### PCR amplification and cloning of SP-ORF deletion region

Unfractionated intracellular RNA samples (1  $\mu\text{g}$ ) were used as templates for reverse transcription primed with oligo-dT (200 ng, Promega) or oligonucleotide 51 (5'ACG-TGCATGCCTGCAGT20-3') in 25- $\mu\text{l}$  reaction mixtures containing 0.05 M Tris-HCl (pH 8.3), 0.075 M KCl, 3 mM MgCl<sub>2</sub>, 1 mM dATP, dCTP, dGTP, and dTTP (Pharmacia), 5 mM DTT, 40 units of RNasin ribonuclease inhibitor (Promega), and 200 units of Superscript RNase H-reverse transcriptase (Bethesda Research Laboratories). The reaction was incubated at 45° for 1 hr followed by incubation at 97° for 5 min to inactivate the reverse transcriptase. Ten microliters of the reverse transcription reaction was used as a template for amplification by the polymerase chain reaction (PCR) using one of three sets of oligonucleotide primers: 102 (5'-CCGGTCTAGACT-AGTGCGGGTTTTCGCGC-3', complementary to nts 9708 to 9725 of the RUB genome) and 170 (5'-CCGAATTCG-CGCCAATCTCCACGAC-3', colinear with nts 6360 to 6377 of the RUB genome) (underlined sequences indicate flanking restriction sites incorporated to facilitate cloning); 102 and 173 (5'-CCGGAATTCGACTACAG-CGCGGAGC-3', colinear with nts 6241 to 6258 of the RUB genome); and 58 (5'-ACGTGCATGCCTGCAGTT-3', anchor sequences of oligonucleotide 51 used to prime reverse transcription) and 177 (5'-CCGGAATTCGGTGCT-TTGCCGCCGTT-3', colinear with nts 6120 to 6146 of the RUB genome). The locations of the primer sets with respect to the RUB genome are shown at the top of Fig. 2.

PCR was performed in a 100- or 200- $\mu$ l reaction mixture containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1% Triton X-100, 10% DMSO (Sigma), 80  $\mu$ g/ml bovine serum albumin (New England BioLabs), 0.03%  $\beta$ -mercaptoethanol (Sigma), 200  $\mu$ M dATP, dCTP, dGTP, and dTTP (Pharmacia), 200 ng of each synthetic oligonucleotide primer, and 1.5 units of Hot Tub DNA polymerase (Amersham). The PCR reaction consisted of 30 cycles of 1 min ascending to 94°, 1 min at 94°, 3 min descending to 50°, 1 min at 50°, 2 min ascending to 72°, and 2 min at 72° followed by 1 cycle of 30 min at 72°. PCR products were purified from primers by spun column chromatography through a Sephacryl S-400 column.

PCR products were digested with *Eco*RI and *Xba*I or *Eco*RI and *Pst*I, size-selected by agarose gel electrophoresis or Sephacryl S-400 spun column chromatography, and ligated into appropriately restricted pGEM-3z(f-) (Promega). One-half of the ligation reaction was used to transform competent *E. coli* strain SURE cells (Stratagene). Colonies containing RUB-specific recombinant plasmids were identified by colony blot hybridization using radiolabeled oligonucleotide 86 (5'-TGGTGTGTG-TGCCATAC-3', complementary to nts 9540 to 9524 of the RUB genome) as a probe. Alternatively, following restriction some of the PCR amplification reactions were ligated with a bacteriophage  $\lambda$  vector,  $\lambda$ GEM-2 (Promega). Duplicate plaque lifts were made of plates containing phage plaques. One lift was hybridized to radiolabeled oligonucleotide probe 10 (5'-GGCGAAACAACGGTGGGTGA-3', complementary to nts 6453 to 6434 of the RUB genome) and the other to 86 (5'-TGGTGTGTGTGCCATAC-3', complementary to nts 9540 to 9524 of the RUB genome).

#### Sequence determination

DNA sequencing of double-stranded plasmid templates was performed using the dideoxynucleotide chain termination method of Sanger *et al.* (1977) employing bacteriophage T7 DNA polymerase (Sequenase version 2.0, United States Biochemical) with [ $\alpha$ -<sup>35</sup>S]dATP (1000–1500 Ci/mmol, New England Nuclear) or [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol, New England Nuclear or Amersham), 7-deaza GTP instead of GTP to reduce compression artifacts and synthetic oligonucleotide primers. The plasmid template was denatured prior to primer annealing by alkaline denaturation (Haltiner *et al.*, 1985) or heat denaturation (Andersen *et al.*, 1992). The sequencing reactions were electrophoresed on 8% polyacrylamide-urea sequencing gels run at 2000 V. Sequencing gels were fixed in 10% methanol, 10% glacial acetic acid, dried onto Whatman 3MM filter paper under vacuum, and exposed to Kodak X-Omat AR film at -80°.

#### Primer extension

Primer extension was performed using nonfractionated intracellular RNA samples as templates. Approxi-

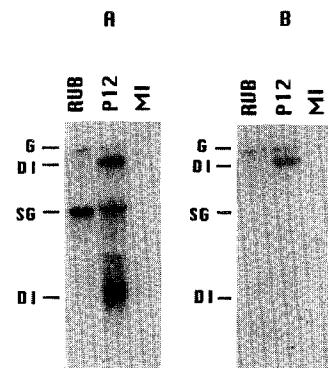


Fig. 1. Northern hybridization of P12 RNA with 3' terminal RNA probes of both positive and negative polarity. Intracellular RNA samples (RUB, standard RUB-infected cell RNA; P12, P12 RNA; MI, mock-infected cell RNA) were denatured in glyoxal and DMSO prior to electrophoresis on a 1% agarose gel. Following electrophoresis, the RNA was transferred to a nylon membrane. The transfer in A was hybridized against a negative-polarity <sup>32</sup>P-labeled RNA probe complementary to the 3' terminal 587 nts of the RUB genome. The transfer in B was hybridized to a positive-polarity <sup>32</sup>P-labeled RNA probe containing the 3' terminal 587 nts of the RUB genome. On the left margins the positions of migration of the genomic (G), subgenomic (SG), and DI RNAs are indicated.

mately 5 ng of <sup>32</sup>P-radiolabeled oligonucleotide 36 (5'-TGGTCTCTTACCCAACT-3', complementary to nts 101 to 117 of the RUB genome) was annealed with 1  $\mu$ g of intracellular RNA in a 15- $\mu$ l reaction mixture containing 10  $\mu$ l of 5 $\times$  Reverse Transcription buffer [0.25 M Tris-HCl (pH 8.3), 0.375 M KCl, 15 mM MgCl<sub>2</sub>] by incubation at 80° for 5 min and slow cooling to 40°. The extension reaction was performed by the addition to the annealed template-primer mixture of 5  $\mu$ l of 100 mM DTT; 2.5  $\mu$ l of 20 mM dATP, dCTP, dGTP, and dTTP; 5  $\mu$ l of actinomycin D (200  $\mu$ g/ml); and 1  $\mu$ l of Superscript RNase H-reverse transcriptase (200 units/ $\mu$ l, Bethesda Research Laboratories). The extension reaction was incubated at 45° for 30 min. Following an incubation at 90° for 5 min, primer extension products were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel.

## RESULTS

### Characterization of RUB DI RNAs generated during serial passage

To amplify DI RNAs for sequence analysis, passage 10 and passage 18 stocks from the previous study (Frey and Hemphill, 1988) were used to infect Vero cells and two subsequent passages were done, expanding the number of cells infected with each passage. RNA was extracted from the cells infected with the second passages of both stocks (P12 and P20). Shown in Fig. 1 is a Northern blot of P12 RNA hybridized with 3' terminal RNA probes of both polarities. Figure 1A shows the presence of two DI RNA populations of 7000 to 7500 and 700 to 850 nts in length. The sizes of the DI RNAs in P12 are similar to those which were previously reported. Figure

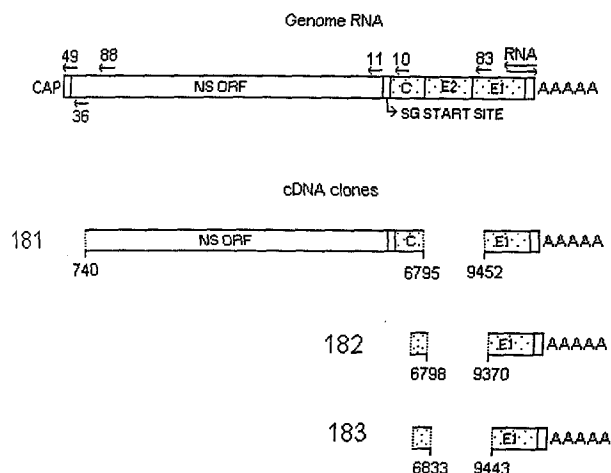


Fig. 2. DI clones isolated from the P12 cDNA library. P12 RNA, fractionated by velocity gradient centrifugation, was used as a template for construction of a cDNA library. From a total of approximately 3000 recombinant colonies recovered, 3 colonies with RUB-specific sequences were identified. A diagram of RUB genome RNA and the sequences present in each of the 3 clones is shown. The numbers of designations of the 3 clones are indicated to the left of each sequence representation. The nucleotide positions of the breakpoints on each side of the deletions are indicated below each clone. The 5' end of clone 181 at nucleotide 740 is indicated. On the genome RNA diagram, the locations of oligonucleotide and RNA probes used in Northern hybridization are indicated by arrows. The direction of the arrow indicates the polarity of the probe.

1B shows the detection of a negative-polarity complement for only the larger DI RNA species.

To clone the smaller DI RNA species, intracellular RNA from P12-infected cells was fractionated by sedimentation through a continuous 15 to 30% sucrose gradient and fractions which contained this RNA species were identified by Northern hybridization. One gradient fraction which contained an abundance of the small DI RNA species and a small amount of the large DI RNA species was used as a template for cDNA synthesis using oligo-dT as a primer. A cDNA library was constructed using a plasmid vector (pGEM-3z) and from the total of approximately 3000 recombinant colonies that were recovered, 3 colonies containing RUB-specific sequences were identified. The sequences of the RUB-specific inserts present in each of these 3 clones were determined (Fig. 2, bottom). All three clones contained a poly-A tail and the exact 3' terminus of the genome, including several hundred nts into the E1 coding region. All three clones contained a single large deletion in the SP-ORF extending from the E1 protein coding region to the capsid protein coding region, with the E2 protein coding region completely deleted. The deletions in the three clones were dissimilar and extended from nts 6795 to 9452 in 181, 6798 to 9370 in 182, and 6833 to 9443 in 183. The size of the deletion in all three clones was approximately 26% of the size of the RUB genome. Upstream from the deletion, two of the clones, 182 and 183, terminated in the capsid protein coding region while the largest clone, 181, extended to nt 740 of

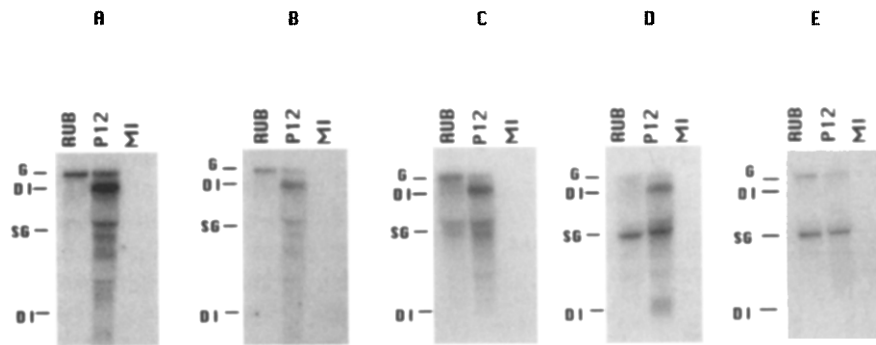
the genome. Except for a large deletion in the SP-ORF, for the extent of the genome which each clone covered, each was a faithful copy of the RUB genome (occasional point mutations did occur in each clone).

Within its boundaries, clone 181 contained a faithful copy of the NSP-ORF and SG start site. Since the template for this clone was presumably the large DI RNA species, it was likely that this species contained a complete copy of the nonstructural ORF and the SG start site. The large deletion in the SP-ORF would account for the reduction in the size of the large DI RNA species as compared to the genome. Thus, the large DI RNA species could be capable of self-replication and the small DI RNA could be a SG RNA synthesized from the large DI RNA template (accounting for the lack of a negative-polarity equivalent). These possibilities were investigated by Northern hybridization to oligonucleotide probes representing different regions of the RUB genome (locations of probes with respect to the genome RNA are shown in the top of Fig. 2).

Northern blots of P12 RNA were first hybridized with radiolabeled oligonucleotide probes which were complementary to nts 1 to 45 (oligonucleotide 49, Fig. 3A) 100 to 117 (oligonucleotide 36, Fig. 3B), and 230 to 253 (oligonucleotide 88, data not shown) of the RUB genome. All three of these 5' terminal oligonucleotide probes hybridized to the large DI RNA species but not to the small DI RNA species, indicating that the small DI RNA does not contain 5' terminal sequences. Next, hybridization to Northern blots of P12 RNA was done with two radiolabeled oligonucleotide probes which are complementary to sequences immediately upstream (nt 6410 to 6430, oligonucleotide 11, Fig. 3C) and immediately downstream (nt 6430 to 6450, oligonucleotide 10, Fig. 3D) from the SG start site (nt 6430). The large DI RNA species hybridized to both probes while the small DI RNA hybridized to only the downstream probe, indicating that the small DI RNA contains sequences downstream but not upstream from the SG start site. Finally, to determine whether the majority of DI RNAs present in P12 RNA contained a deletion in the SP-ORF, an oligonucleotide probe which is complementary to a sequence located within the E1 protein coding region (nts 8472 to 8488, oligonucleotide 83, Fig. 3E) that was deleted in all three cDNA clones was hybridized to P12 RNA. Neither of the P12 DI RNA species hybridized to this probe, indicating that a deletion in the SP-ORF was present in both the large and small populations of DI RNAs. In total, these data from oligonucleotide probe hybridization analysis are completely consistent with the hypothesis that the large DI RNA species contain a single large deletion in the SP-ORF and that the small DI RNA species are SG RNAs synthesized from the large DI RNA templates.

#### Analysis of the 5' terminus of DI RNA generated during serial passage

As shown in Figure 3A, oligonucleotide probes complementary to the exact 5' terminal sequences of the



**Fig. 3.** Northern hybridization of P12 RNA with oligonucleotide probes. Intracellular RNA samples (RUB, standard RUB-infected cell RNA; P12, P12 RNA; and MI, mock-infected cell RNA) were denatured in glyoxal and DMSO prior to electrophoresis on a 1% agarose gel. Following electrophoresis, the RNA was transferred to a nylon membrane. The transfer in A was hybridized against  $^{32}\text{P}$ -labeled oligonucleotide 49 (complementary to nts 1 to 45 of the RUB genome), the transfer in B was hybridized to oligonucleotide 36 (complementary to nts 100 to 117 of the RUB genome), the transfer in C was hybridized against oligonucleotide probe 11 (complementary to nts 6410 to 6430 of the RUB genome), the transfer in D was hybridized against oligonucleotide 10 (complementary to nts 6430 to 6450 of the RUB genome), the transfer in E was hybridized against oligonucleotide 83 (complementary to nts 8472 to 8488 of the RUB genome). The locations of these probes are shown on the genome diagram in Fig. 2. In A, B, C, and D, background hybridization is observed resulting in the presence of two artifactual bands which migrate near the SG RNA. The upper band is created by the presence of the 28S ribosomal RNA and lower bands are created by random breakdown of rubella virus-specific intracellular RNAs. The actual SG RNA migrates between the two artifactual bands. On the left margins the positions of migration of the genomic (G), subgenomic (SG), and DI RNAs are indicated.

RUB genome hybridized to the large DI RNAs. To confirm that these DI RNAs contained the authentic 5' end of the genome, primer extension was performed on intracellular RNA from standard RUB-infected cells and P12 RNA. Approximately 1  $\mu\text{g}$  of each RNA was used as a template. Figure 4 shows the results of primer extension using radiolabeled oligonucleotide primer 36 which is complementary to sequences located 100 nts from the 5' end of the genome. Two primer extension products were produced from both RUB-infected cell RNA and P12 RNA. The lower of these is caused by termination at a stable secondary structure formed by 5' terminal sequences while the upper product is due to termination at the 5' end of the genome (Dominguez *et al.*, 1990). Even though P12 RNA contained some genomic RNA, which can serve as a template for primer extension, the 7000-nt DI RNA was clearly the most abundant template (refer to Fig. 1). Theoretically, DI RNAs which served as a template for primer extension (the majority of 7000 nt DI RNAs hybridized to primer 36, Fig. 3B) and contained 5' termini that differed from the rubella virus genome would produce a primer extension product which migrated differently from that produced from the genome RNA. Only one primer extension product representing the 5' termini of the RNA template was produced from both RUB-infected cell RNA and P12 RNA, indicating that the majority of the large DI RNAs contain a 5' terminus similar to the standard genome.

#### DI RNAs produced by passage of independent RUB stocks

To determine whether the DI RNA species present in P12 RNA were representative of DI RNAs routinely generated during undiluted serial passage of RUB in Vero cells, serial passages in Vero cells of seven other RUB

stocks were made and the DI RNAs produced were analyzed by Northern hybridization to the 3' terminal negative-polarity RNA probe. These stocks were plaque-purified W-Therien [WTh(pp); this is a plaque-purified independent stock from that used to initiate the passage series that includes P12 and P20], F-Therien received from another lab and passaged either before (FTh) or after plaque-purification [FTh(pp)] in this lab; M33; and supernatants from three persistently infected cultures: Vero cells persistently infected with W-Therien [piV(Th); subculture 183, Day 1281 postinitiation (Abernathy *et al.*, 1990)] and human synovial explants persistently infected with F-Therien [piS(Th); Day 40 postinitiation] and M33 [piS(M33); Day 45 postinitiation]. The DI RNA species present in P20 RNA were also analyzed. As shown in Figs. 5A and 5C, after 5 to 10 serial passages of each of these stocks, except for the synovial explant persistent infection supernatants (Fig. 5C, lanes 4 and 5), a DI RNA species similar in size to the large P12 DI RNA was generated. In most of these passages, a short DI RNA species was also generated, although the size and relative amount varied. Hybridization of Northern blots of P20, piV(P5), WThP10, and FThP10 RNA with the 3' terminal positive-polarity RNA probe (Fig. 5B) and the oligonucleotide probes used earlier (data not shown) confirmed that similar to the P12 DI RNAs, only the large DI RNAs present in each of these passage series contained 5' terminal sequences and produced a complementary negative-polarity species (Fig. 5B), that the small DI RNAs lacked sequences upstream from and contained sequences downstream from the SG start site, and that both DI RNA populations contained a deletion in the SP-ORF.

It was of interest that serial passage of supernatant from persistently infected Vero cells [piV(P5)] gave rise to DI RNAs similar to P12 since when the DI RNAs pres-

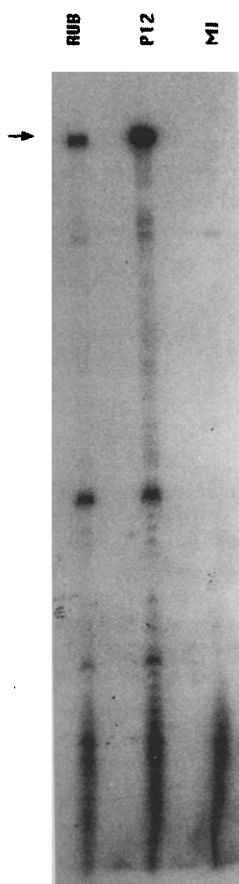


Fig. 4. Analysis of the 5' termini present in P12 RNA by primer extension. Primer extension was performed on unfractionated intracellular RNA (RUB, standard RUB-infected RNA; P12, P12 RNA; MI, mock-infected cells RNA) by reverse transcription using primer 36, which is complementary to nts 100 to 117 of the RUB genome. Primer extension products were analyzed by electrophoresis on an 8% polyacrylamide gel. The position of migration of the primer extension band corresponding to the 5' end of the genome is indicated on the left margin. The other band is a strong stop caused by a stem-loop structure formed by 5' terminal sequences (Dominguez *et al.*, 1990).

ent in that persistent infection were characterized it was found that although a large DI RNA species was present at certain passages, the majority DI RNA species was less than 3000 nts in length (Abernathy *et al.*, 1990). Subculture (sc) 133 of the persistently infected cell line was rescued from liquid nitrogen and after seven subcultures (sc140) supernatant fluid was harvested and passaged twice in Vero cells. RNA was extracted from the persistently infected cell line and cells infected with the first and second passage. As shown in Fig. 6, the primary RNA species present in persistently infected cells was a species of 2000 to 3000 nts in length, although a large DI RNA and two shorter DI RNAs were present. Hybridization with the 3' terminal positive-polarity RNA probe and oligonucleotide 49 showed that the 2000- to 3000-nt DI RNAs contained 5' and 3' terminal sequences and synthesized a negative-polarity complement (data not shown). With exception of one of the short DI RNA species these species were present following one serial

passage, although the relative amount of the 2000- to 3000-nt DI RNA species was greatly reduced. By the second passage, only the large and short DI RNA species were present.

#### Analysis of SP-ORF deletion in DI RNAs generated during serial passage

The 3 cDNA clones obtained from P12 RNA contained nonidentical deletions, indicating that the DI RNA population was heterogeneous with respect to the deletions which they contained. The extent of the deletions in the P12 DI RNA population was analyzed by a protocol in which intracellular RNA was a template for reverse transcription and PCR amplification (RT-PCR) using primer pairs which flank the deleted region. The strategy is diagrammed in the top of Fig. 7. Amplification products were cloned into pGEM-3z and 18 clones were isolated and sequenced. The extent of the deletion in each clone is given in Table 1. The deletions were heterogeneous, ranging in size from 2504 to 3047 nts. All of the deletions were different although some deletions shared one breakpoint. Unexpectedly, 17 of the 18 RT-PCR clones had deletions which included the SG start site [the only P12 RT-PCR clone (120) which retained the SG start site was the result of a mispriming event during PCR amplification in which the 5' primer annealed downstream from the SG start site and thus selected for an amplification product that retained sequences downstream from the SG start site]. This RT-PCR technique was used to obtain information about the deletions present in DI RNAs from two other passages, P20 and piV(P5). Of the 6 clones generated from P20 RNA, all 6 retained the SG start site. Of 3 RT-PCR clones generated from piV(P5) RNA, all 3 contained a deletion of the SG start site.

That the preponderance of RT-PCR clones from P12 RNA lacked the SG start site was unexpected since it was in contrast to the results from the cDNA library. To confirm that this was not due to selective cloning of RT-PCR products lacking the SG start site into the plasmid vector used (i.e., that sequences around the SG start site are not stable in this vector), the original P12 and P20 RT-PCR reactions were ligated into the  $\lambda$  bacteriophage vector,  $\lambda$ GEM2, and recombinant plaques were screened by plaque hybridization to determine the proportion of P12 and P20 recombinants which retained the SG start site. Plaques were transferred to nylon membranes in duplicate and hybridized against two oligonucleotide probes, 86 which is complementary to sequences near the 3' end of the RUB genome and 10 which is complementary to sequences immediately downstream from the SG start site. All plaques containing RUB-specific inserts will hybridize to oligo 86 while only those containing the SG start site will hybridize to oligo 10. Ninety-seven of 100 (97%) of the P12 plaques with RUB-specific inserts and 12 of 47 (25%) of the P20 plaques contained inserts

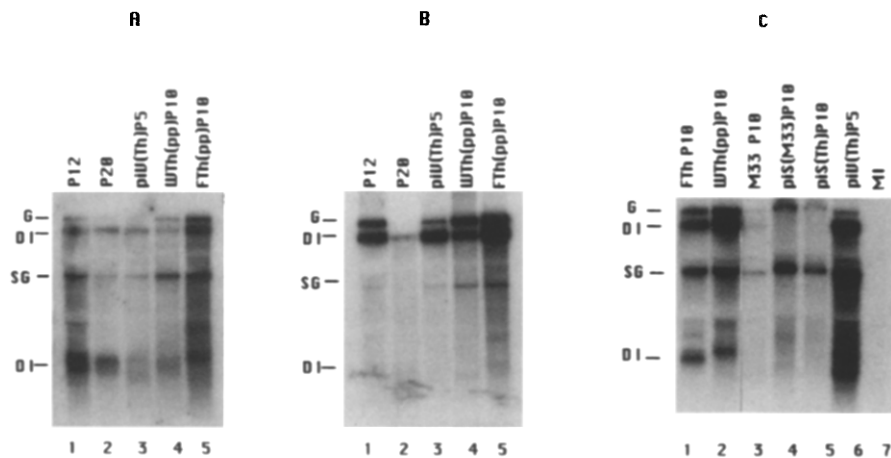


FIG. 5. Northern hybridization of RNA from independent serial passages with 3' terminal RNA probes of both positive and negative polarity. Intracellular RNA samples were denatured in glyoxal and DMSO prior to electrophoresis on a 1% agarose gel. Following electrophoresis, the RNA was transferred to a nylon membrane. The transfers in A and C were hybridized against the  $^{32}\text{P}$ -labeled RNA probe complementary to the 3' terminal 587 nts of the RUB genome. The transfer in B was hybridized against the positive-polarity  $^{32}\text{P}$ -labeled RNA probe containing the 3' terminal 587 nts of the RUB genome. The negative-polarity RNA species migrating near the position of migration of the SG RNA are presumed to be the result of inherent RNase digestion of double-stranded replicative intermediate RNAs present in infected cells and have been observed previously (Hemphill *et al.*, 1988). The RNA samples analyzed in A and B are as follows: P12 (lane 1), P20 (lane 2), piV(Th)P5 (lane 3), WTh(pp)P10 (lane 4), FTh(pp)P10 (lane 5). The RNA samples analyzed in C are as follows: FThP10 (lane 1), WTh(pp)P10 (lane 2), M33 P10 (lane 3), piS(M33)P10 (lane 4), piS(Th)P10 (lane 5), piV(Th)P5 (lane 6), mock-infected cells (lane 7). On the left margin the positions of migration of the genomic (G), subgenomic (SG), and DI RNAs are indicated.

with deletions of the SG start site. These results were similar to those obtained from the analysis of P12 RT-PCR plasmid clones and thus the characterization of infectious RYMV cDNA clones products which contained the SG start site was not due to the instability of the RT-PCR products containing the SG start site in plasmid vectors.

The locations of the 5' and 3' breakpoints of the deletions in all of these clones are shown in the bottom of Fig. 7. The 5'-most breakpoint, which was observed in 2 independent clones, occurred at nt 6383 and preserved

the UAA termination codon of the NSP-ORF. Thus, the deletion extended to, but never into, the ORF. Seven of the breakpoints which clustered within a 300-nt region 20 nts upstream from the SG start site. This sequence is the end of a 20-nt region which shares homology with the alphavirus SG promoter (Frey, 1994). Of the 10 clones which retained the SG start site, all retained at least 200 nts downstream from the start site, and 9 retained over 360 nts downstream. The 3' breakpoints of the deletions were distributed over a 300-nt region and all of the clones retained at least 244 nts from the 3' terminus of the genome. Two regions of clustering of 3' breakpoints were observed. The first cluster occurred between nts 9228 and 9234 (UUCAAGA) and contained the breakpoints of 6 clones. The second cluster occurred between nts 9443 and 9455 (CCAAGUCAG-CUGC) and contained the breakpoints of 11 clones. Of the 10 RT-PCR clones which retained the SG start site and extended into the SP-ORF, 6 maintained the reading frame of the SP-ORF across the deletion. No consensus sequence or sequence homology was identified on either side of any single deletion which could account for its generation. Analysis of computer-generated secondary structures of both sides of the deletion did not reveal any significant secondary structures which could be responsible for generation of the deletions (data not shown).

## DISCUSSION

In this study, it was found that of the two species of novel virus-specific RNAs generated by serial undiluted passage of RUB in Vero cells, the larger species was an

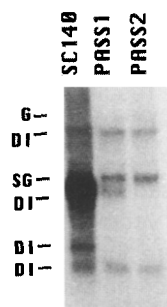


FIG. 6. Analysis of DI RNAs present in persistent infection. Supernatant from a persistently infected Vero cell line at subculture 140 was passaged twice in Vero cells. Intracellular RNA was extracted from the persistently infected cell line and after each passage and analyzed by Northern hybridization for the presence of DI RNAs. Intracellular RNA samples (sc140, subculture 140 of the persistently infected cell line; pass1, first passage of sc140 supernatant; pass2, second passage of sc140 supernatant) were denatured in glyoxal and DMSO prior to electrophoresis on a 1% agarose gel. Following electrophoresis, the RNA was transferred to a nylon membrane. The transfer was hybridized against an oligonucleotide probe complementary to nts 9524 to 9540 of the RUB genome. On the left margin the positions of migration of genomic (G), subgenomic (SG), and DI RNAs are indicated.

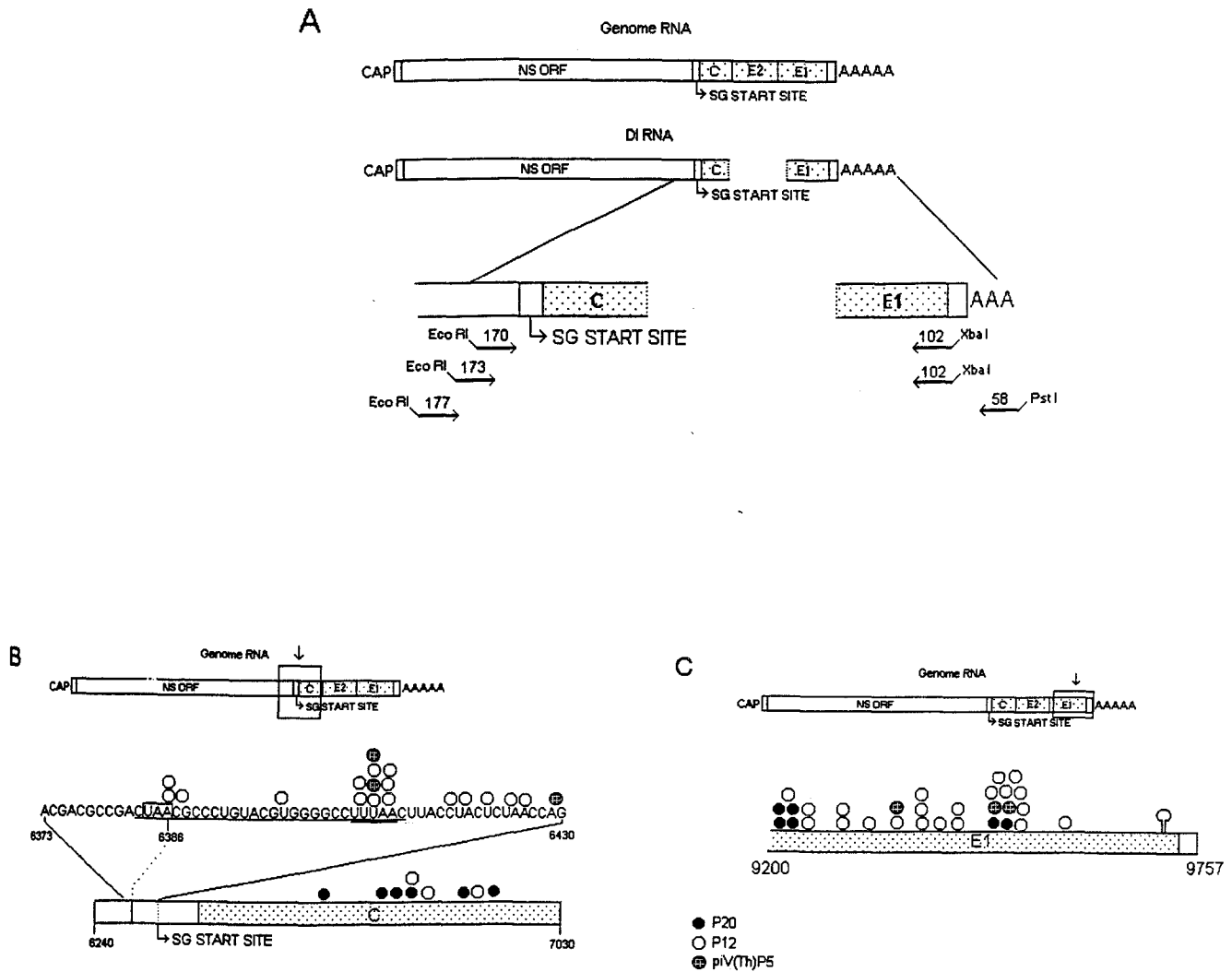


Fig. 7. Strategy of RT-PCR amplification of the SP-ORF and locations of breakpoints of deletions. (A) Diagrams of the RUB genome RNA and the large DI RNA, the relative locations of the three PCR primer sets used in amplification. Primer numbers and restriction enzyme sites included in each primer to facilitate cloning are indicated. (B) the locations of the 5' breakpoints of the deletions in each clone plotted along the RUB nucleotide sequence. Open circles are clones derived from P12 RNA, black circles are clones derived from P20 RNA, and hatched circles are clones derived from piV(P5) RNA. The boxed region indicates the enlarged area. Nucleotide numbers correspond to the RUB genome. The boxed termination codon UAA signals the end of the NSP-ORF. The 20-nt region which shares homology with the alphavirus subgenomic promoter region is underlined. The UUUAA sequence in which nine clones have breakpoints is underlined twice. (C) The locations of the 3' breakpoints of the deletion in each clone which were plotted along the RUB nucleotide sequence. Nucleotide numbers correspond to the RUB genome. A predicted stem-loop structure shown to bind host cell proteins is indicated near the end of the E1 coding region.

authentic DI RNA that contained a single deletion in the SP-ORF and the smaller species was a SG RNA synthesized from the large DI RNA. Evidence supportive of the conclusion that the smaller species was a SG RNA was as follows. First, no negative-polarity complement of the small DI RNA was detected, indicating that it was not replicated directly through an intermediate negative-polarity species. A negative-polarity complement of the small DI RNA species was detected previously by Frey and Hemphill (1988) but was most likely a result of the method of intracellular RNA extraction that was used, which also resulted in the detection of a negative-polarity complement for the SG RNA. These results were most likely due to nonspecific RNase digestion of single-

stranded regions of double-stranded full-length replicative intermediates which contain both positive- and negative-polarity RNAs. Second, cloning of DI RNAs from P12 intracellular RNA resulted in recovery of one clone, 181, that contained the majority of the NSP-ORF and SG start site. The DI RNA from which this clone was derived was thus theoretically capable of synthesizing (or serving as a template for the synthesis of) a SG RNA. Third, both the large and small DI RNA species hybridized to a probe complementary to the 5' end of the SG RNA. However, only the large DI RNA species hybridized to a probe complementary to sequences immediately upstream, indicating that the small DI RNA species does not contain sequences upstream from the SG start site. Finally, the



TABLE 1  
SUMMARY OF THE SP-ORF DELETIONS PRESENT IN RT-PCR  
AND cDNA CLONES

Clone	Source <sup>a</sup>	Junction <sup>b</sup>	Deletion size <sup>c</sup>	SG start site <sup>d</sup>
120	P12	7019-9513	2504	+ORF
124	P12	6407-9443	3036	-
126	P12	6418-9405	2987	-
128	P12	6409-9449	3040	-
U-1	P12	6410-9328	2918	-
U-2	P12	6408-9355	2947	-
U-3	P12	6408-9234	2826	-
U-4	P12	6386-9370	2985	-
L-1	P12	6408-9455	3047	-
L-2	P12	6425-9336	2911	-
L-3	P12	6399-9234	2835	-
G-2	P12	6408-9455	3047	-
S-4	P12	6409-9420	3011	-
S-5	P12	6419-9422	3003	-
S-7	P12	6426-9425	3007	-
177-1	P12	6386-9447	3061	-
177-2	P12	6387-9329	2942	-
177-3	P12	6422-9359	2937	-
181*	P12	6795-9452	2658	+
182*	P12	6798-9370	2572	+ORF
183*	P12	6833-9443	2610	+
20U1	P20	6830-9234	2404	+ORF
20U2	P20	6788-9231	2443	+ORF
20U5	P20	6792-9229	2437	+ORF
20L6	P20	6795-9452	2657	+
20L7	P20	6630-9232	2602	+ORF
20L10	P20	6834-9446	2602	+
54	piV(Th)P5	6427-9447	3020	-
177-5	piV(Th)P5	6405-9454	3049	-
177-7	piV(Th)P5	6408-9360	2952	-

<sup>a</sup> Intracellular RNA source from which clone was derived.

<sup>b</sup> RUB nts on either side of the deletion.

<sup>c</sup> In nts.

<sup>d</sup> (+) Indicates the presence of the SG start site, (-) indicates the absence of the SG start site, and (ORF) indicates maintenance of the translation frame of the SP-ORF across the deletion.

\* Indicates cDNA clones.

small DI RNAs lacked 5' terminal sequences as shown by Northern hybridization. Additional evidence that the small DI RNA population represents SG DI RNAs was that it was shown by Frey and Hemphill (1988) that the large DI RNAs, but not the small DI RNA, were encapsidated.

Several pieces of evidence suggested that the large DI RNAs conserve the entire NSP-ORF and contain a single deletion in the structural ORF. The largest clone recovered from P12 RNA, clone 181, extended from the poly-A tail to nt 740 of the genome and contained a faithful copy of the nonstructural ORF from nt 740 to the end of the ORF. Although the 5' end of the genome and NSP-ORF was not present in any of the cDNA clones generated, Northern hybridization with probes complementary to sequences upstream from nt 740 demonstrated that the large DI RNAs contained the 5' end of

the genome and the 5' end of the NSP-ORF. A primer extension experiment indicated that the large DI RNA contained the authentic 5' end of the genome RNA. The only deletion observed in clone 181 was located within the SP-ORF and the shorter clones recovered from P12 RNA (182 and 183) also contained a large deletion of the same region of the SP-ORF that was deleted in clone 181. Northern hybridization with a probe within the deleted region showed that both the large and small DI RNA population uniformly contained this deletion. The sizes of DI RNAs that contained the complete NSP-ORF and deletions of the structural ORF present in clones 181, 182, and 183 would be 7099, 7147, and 7185 nts, respectively, consistent with the size of the large DI RNAs. Analysis of the extent of the SP-ORF deletion by RT-PCR revealed that the 5' most deletion site preserved the termination codon of the NSP-ORF and no deletions were detected that extended into the NSP-ORF.

RT-PCR analysis of the extent of the SP-ORF deletions in the DI RNA population showed that the deletions were completely heterogeneous and ranged in size from 2504 to 3047 bases. The 5' most breakpoint was at the exact 3' end of the NSP-ORF while at least 244 nts of 3' terminal sequence were maintained in all of the clones. This includes the predicted stem-loop structure located 58 nts from the 3' terminus that has been shown by Nakhasi *et al.* (1990) to interact with host cell proteins. Analysis of the breakpoints of the deletions present in these clones did not reveal any consensus sequence or significant secondary structure that could be responsible for their generation. The breakpoints were distributed over regions of several hundred nts in the capsid protein and the E1 glycoprotein coding regions. However, clusters of breakpoints were observed on either side of the deletion.

An unexpected result of the RT-PCR analysis was that in the majority of the P12 clones (17 of 18) the SG start site was deleted. The abundance of SG DI RNA detected in P12 RNA by Northern hybridization suggested that the large DI RNAs commonly retained the SG start site. Additionally, oligonucleotide probes complementary to sequences immediately upstream and downstream from the SG start site hybridized to the large DI RNA in P12 RNA. This result was also in contrast with the clones generated by cDNA synthesis, in all 3 of which the 5' breakpoint of the deletion was downstream from the SG start. This result was not due to the inability of template containing the SG start site to be amplified by PCR since the majority of the P20 RT-PCR clones contained the SG start site. It was also not due to instability of sequences in the vicinity of the SG start site in the plasmid vector pGEM-3z since when P12 RT-PCR products were cloned into a bacteriophage  $\lambda$  vector, 97% of the RUB-specific recombinant plaques contained deletions of the SG start site. The preponderance of P12 RT-PCR clones that lack the SG start site possibly was due to selective amplifica-

tion of smaller templates during PCR (i.e., those that lacked the SG start site).

In contrast to RT-PCR clones from P12 RNA, most of the RT-PCR clones from P20 RNA retained the SG start site. Thus, DI RNAs that both retain and delete the SG start site were maintained in the DI RNA population during serial passaging and the relative abundance of each varied. This is in distinct contrast to alphavirus DI RNAs which have been characterized to date, all of which delete the SG promoter and start site (Schlesinger, 1988). It is thought that deletion of the SG promoter provides a selective advantage for replication of the DI RNA over standard genomes because alphavirus DI RNA negative-polarity templates are uniformly available for replication and not used for SG RNA synthesis. It is not clear why RUB DI RNAs that retain the SG start site and produce a SG message are not only retained in the DI RNA population but are also the majority species at certain passages. In addition to maintenance of the SG start site, the overall structure of RUB DI RNAs also contrasts with the structure of alphavirus DI RNAs, which usually contain a complex array of multiple internal deletions, duplications, rearrangements, and occasionally cell-derived sequences (Schlesinger, 1988). Unlike RUB DI RNAs, alphavirus DI RNAs have usually deleted most of the genomic sequences and are thus relatively small [often a fraction (i.e. one-fourth) of the total genome size].

Interestingly, all of the clones in which the SG start site is retained also contained 200 to 360 nts downstream from the start site. The presence of functional fusion ORFs created by the junction of noncontiguous regions of mouse hepatitis virus (MHV) gene 1 in two MHV DI RNAs was reported by Makino *et al.* (1988, 1990). Novel proteins translated from the fusion ORFs of both DI RNAs were detected in infected cells. Thus, translation of fusion ORFs may be a characteristic of MHV DI RNAs. Supporting evidence that MHV DI RNA fusion ORFs are translated was provided by de Groot *et al.* (1992) who constructed a DI cDNA clone derived from a MHV DI RNA which contained a fusion ORF and then generated nonsense and frameshift mutants that did not preserve the fusion ORF. It was found that translation of the ORF was required for efficient amplification of the MHV DI RNA transcripts. Of the 10 P12 and P20 clones which retain the SG start site, only 6 maintained the reading frame of the SP-ORF. Thus translation of a fusion protein does not appear to be necessary for amplification of RUB DI RNAs.

Similar species of RUB DI RNAs were generated in five of seven independent serial passages and no other patterns of DI RNA generation were observed. In two other reports on RUB DI RNA generation during serial passage, the species detected were also 7000 to 9000 nts in length (Bohn and van Alstyne, 1981; Terry *et al.*, 1985). As observed in analysis of DI RNAs present in persistent infection, other types of RUB DI RNAs can be generated. The 2000–3000 nt DI RNA that was the major-

ity intracellular species contained both 5' and 3' terminal sequences. Thus, the prevalence of the large DI RNA species during serial passage is probably due to its having a selective advantage over other DI RNA species. One possibility is efficiency of encapsidation, a feature more critical for maintenance during serial passage than persistent infection. Both the long DI RNA and the 2000- to 3000-nt species from persistent infection were encapsidated as evidenced by their ability to be passaged, but the long DI RNA outcompeted the 2000- to 3000-nt species within two serial passages. However, the RUB encapsidation sequences have not been defined and the disappearance of the 2000- to 3000-nt species within two passages could have been due to factors other than encapsidation. Another possible selective advantage of the large DI RNA is that DI RNAs which contain the entire NSP-ORF can theoretically synthesize their own nonstructural proteins and thus self-replicate. Considering that the m.o.i. of standard virus in serially passaged stock is 0.1 to 0.01 PFU/cell (Frey and Hemphill, 1988) and that the eclipse phase during RUB infection approaches 12 hr (Hemphill *et al.*, 1988), a DI RNA which can self-replicate in a cell while waiting for infection of the cell by a standard helper virus would have a selective advantage over DI RNAs which do not contain the NSP-ORF and thus cannot self-replicate.

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