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Genomic and Copy-Back 3′ Termini in Sendai Virus Defective Interfering RNA Species

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Direct sequencing of nine Sendai virus defective interfering RNA species revealed two kinds of 3′-terminal sequences. Six RNA species had 3′ termini identical to the virus genome (negative strand), confirming that internal deletions are a frequent cause of Sendai virus defectiveness. The other three RNA species had 3′-terminal sequences identical to that described as the complement of the 5′ terminus of the virus genome (R. A. Lazzarini, J. D. Keene, and M. Schubert, Cell **26**:145–154, 1981), indicating that they are of the copy-back type. Extensive homology between these two types of 3′ sequences evidently accounts for the ability of the copy-back sequence to function as an initiation signal for viral RNA replication. There may not be a selective advantage of one type of terminus over the other, since one defective interfering strain possessed two RNA species, one of which had the genomic 3′ terminus and the other the copy-back type.

Defective interfering (DI) virus particles are of special interest as naturally produced modulators of the severity and duration of infection (6, 8). Since they are deletion mutants, the nucleotide sequences remaining in their genomes are responsible for their biological effects and include signals essential for their replication. The helical nucleocapsid symmetry of the nonsegmented negative-strand RNA viruses permits the survival of defective genomes with widely variant deletions of genetic information. In contrast with this freedom is the marked conservation of terminal nucleotide sequences essential for the initiation of RNA replication: the 5′ terminus of the parental virus genome appears to be always conserved, whereas the 3′ terminus is either conserved or replaced by a close homolog, a complementary copy of the genomic 5′ terminus (14, 18). This homolog has been termed a copy-back sequence, since it is thought to arise by a copy choice mechanism, in which the RNA replicating enzyme erroneously abandons its normal positive strand template and adopts the nascent product strand (including the genomic 5′ terminus) as its template (7, 15).

The number of DI RNA species with conserved genomic 3′ termini described for a rhabdovirus, vesicular stomatitis virus (VSV), is limited to only two published examples (9, 18). In one of these, the sequence derived from the genome terminus is located internally and does not function in the initiation of RNA replication (9). In contrast, recently published oligonucleotide maps of Amesse et al. (1) indicate that genomic 3′ termini are relatively common in DI strains of a paramyxovirus, Sendai virus. RNase T_1-resistant oligonucleotides representing 3′-terminal leader RNA template and nucleoprotein (NP) gene sequences were identified in most of the DI RNA species examined in that work. In this work, we confirmed by direct RNA sequencing that six of nine Sendai virus DI RNAs have genome-type 3′ termini.

MATERIALS AND METHODS

Viral RNA species. The sources of Sendai virus DI strains and procedures for growing them and for isolating their RNA have been reported (1). Sucrose gradient centrifugation (10) was used to provide enriched samples of DI RNA species at the expense of 50S genomic RNA, rRNA, and 4S RNA species (including tRNA) commonly found in egg-grown virions (11, 13).

Sequence analyses. RNA samples were labeled at their 3′ termini by the incorporation of cytidine 3′,5′-[5′-^{32}P]bis(phosphate) (New England Nuclear Corp. or Amersham Corp.), mediated by bacteriophage T4 RNA ligase (P-L Biochemicals) (3). Radioactive RNA species were then denatured with glyoxal (17) and separated by agarose gel electrophoresis (4). After autoradiography of the wet gel, radioactive bands were excised and RNA species were isolated (20) and sequenced by one-dimensional (2) and two-dimensional (16) methods. The 3′-terminal nucleotide of terminally labeled RNA, digested to completion with RNase T_2, was identified by thin-layer chromatography on polyethyleneimine-cellulose (19).

RESULTS

Virus genome 3′ terminus. As a standard of comparison with the DI RNA species, we determined the 3′-terminal sequence of the Sendai

virus 50S negative-strand genome (Fig. 1 and 2). This sequence differed in 8 of the first 25 nucleotides from a preliminary sequence determined by D. Kolakofsky and M. Leppert and quoted by Lazzarini et al. (14). In seven of the eight instances (positions 5, 9, 11, 14, 17, 18, and 24), the quoted sequence has a cytosine where we found a uracil. In the eighth case (position 19) we identified a guanine in place of a tentative adenine.

In view of the possibility that these differences might be strain specific, we sequenced 50S genomic RNA from the R and H strains of Sendai virus that we had obtained from D. Kolakofsky, University of Geneva, Geneva, Switzerland (15). The results were identical to those shown for the Enders strain. D. Kolakofsky has informed us that his recent data on the H strain genomic RNA confirm the sequence in Fig. 2 with two exceptions: he still finds a cytosine at position 5, and he finds a uracil instead of a cytosine at residue 15 (personal communication).

DI RNA termini of the genomic type. We

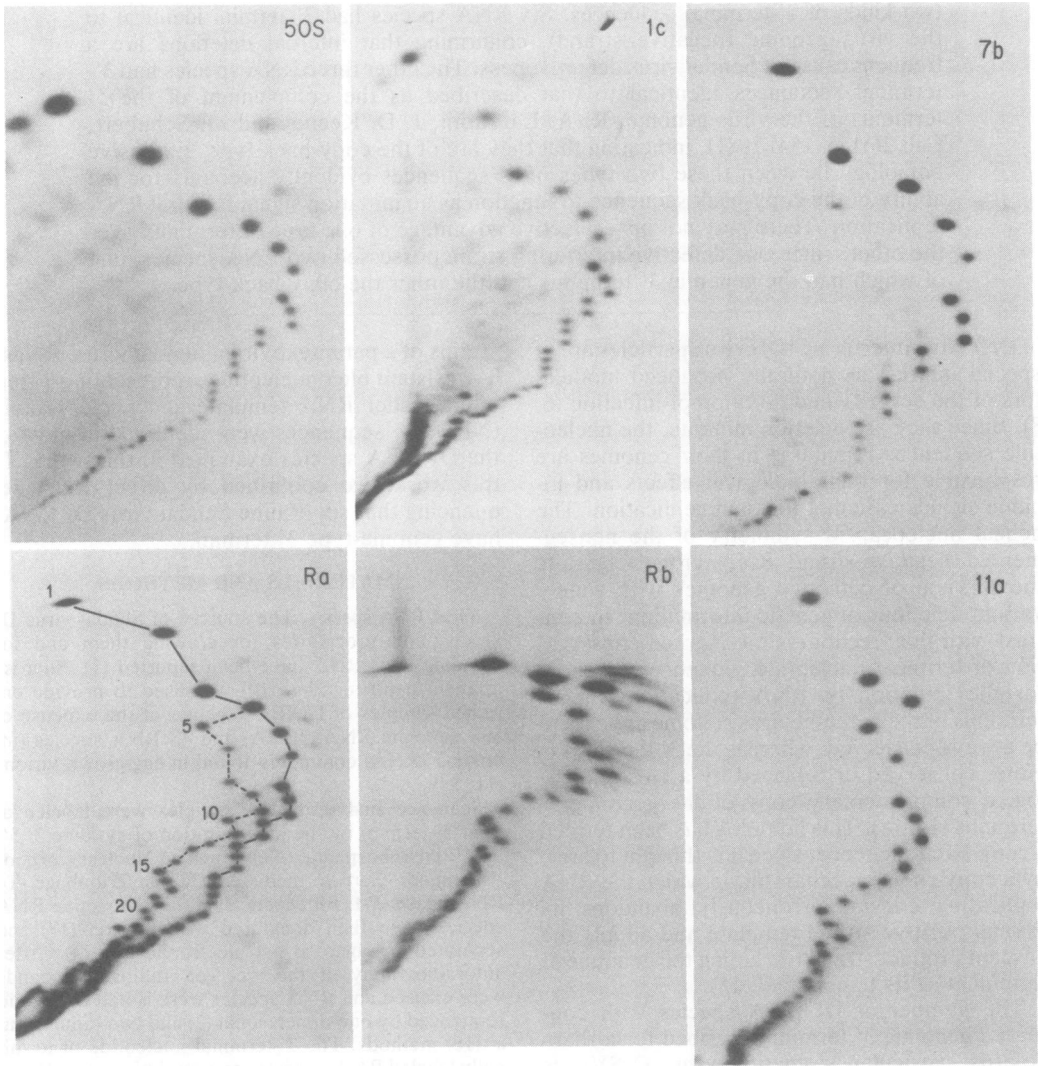

FIG. 1. Two-dimensional sequencing gels of 3'-terminal-labeled Sendai virus DI RNAs. Electrophoretically separated RNA bands were cut from gels like those shown in Fig. 3, hydrolyzed partially at pH 9.5, and subjected to polyacrylamide gel electrophoresis. The origin is at the lower left of each panel; the horizontal dimension buffer was pH 3.5; the vertical dimension buffer was pH 8.3. RNA species are designated in the upper-right corner of each panel. The 50S panel represents Sendai virus genome RNA. The numbers in panel Ra represent the lengths of the oligonucleotides (less the 3'-terminal cytidylate added by in vitro labeling); the relevant sequences are given in Fig. 2. In panels 7b and 11a, the first nucleotide is not shown.

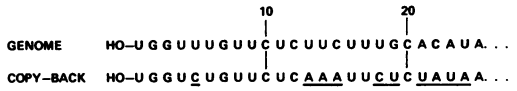

FIG. 2. Sendai virus RNA 3'-terminal nucleotide sequences. Sequences were determined from the data in Fig. 1 and from one-dimensional electrophoresis of 3'-terminal-labeled RNA species digested partially with RNases T_1 and U_2 and *Bacillus cereus* RNase (2). The underlined nucleotides are nonhomologous.

examined a total of nine RNA species from five DI strains (Table 1). The electrophoretic separation of seven of the species after ^{32}P labeling of their 3' termini is shown in Fig. 3. Proportions of individual species in strains 7 and R differed from those shown previously (1) due to variations in their recovery from sucrose gradients. RNA species 1c, not shown in Fig. 3, is the smaller of the two originally described as natural passengers in the Enders strain of Sendai virus (1, 10). It had a 3' terminus identical to that of the virus genome (Fig. 1 and 2). This was true also for RNA species 1a, the higher-molecular-weight companion of 1c (data not shown). By oligonucleotide mapping, RNA 1a had 3'-proximal genetic information from the leader RNA template and a portion of the NP gene, whereas 1c possessed leader template sequences but no NP sequence (1).

Strain 7 from our collection contained three DI RNA species (Fig. 3). One of these, 7b, possessed leader RNA template sequences as determined by oligonucleotide mapping (1). As shown in Fig. 1, 7b has the genomic 3' terminus. This was also the case for 7a and 7c, which had not been examined by oligonucleotide mapping (data not shown).

Copy-back termini. A different sequence was found at the 3' ends of RNA species Rb and Ha,

TABLE 1. Properties of Sendai virus DI RNAs[a]

RNA species	Mol wt ($\times 10^6$)	3' Terminus	
		Genomic	Copy-back
1a	1.44	+	
1c	0.91	+	
7a	1.24	+	
7b	0.70	+	
7c	0.55	+	
11a	0.40		+
Ha	0.49		+
Ra	1.13	+	
Rb	0.92		+

[a] Molecular weights were estimated from the electrophoretic mobilities of the RNA species compared with rRNA species under denaturing conditions (1). The molecular weight of the 50S Sendai virus genome is 4.8×10^6 (12).

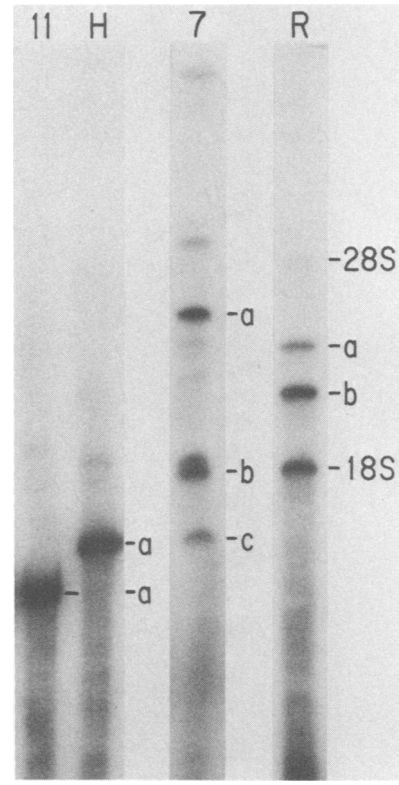

FIG. 3. Electrophoresis of 3'-terminal-labeled Sendai virus DI RNA species. After in vitro labeling with cytidine 3',5'-[5'-^{32}P]bis(phosphate), RNA species were denatured with glyoxal and separated by electrophoresis in acid-urea-agarose gels. Above each lane is the designation of the DI virus strain. Lowercase letters represent DI RNA species; the numbers 18 and 28 designate rRNAs that are present in virus particles.

from D. Kolakofsky's Sendai virus DI strains, and RNA species 11a, from one of our DI strains (1), as shown in Fig. 1, 2, and 4. This sequence was identical to that reported as the copy-back type in a review by Lazzarini et al. (14). Direct sequencing of the 5' end of the Sendai virus genome confirmed that the copy-back sequence was its complement (G. G. Re, K. C. Gupta, and D. W. Kingsbury, unpublished data). In the cases of RNA species Rb and Ha, our sequence data confirmed the ascription of copy-back sequences to their 3' ends by Leppert et al. (15) on the basis of hybridization analyses of terminally labeled RNA. However, in the oligonucleotide mapping of 10 Sendai virus DI RNA species reported by Amesse et al. (1), Rb was the only one that did not possess an oligonucleotide assignable to 3'-proximal sequences; all 14 of the oligonucleotides it yielded were apparently derived from the L gene. RNA species 11a and Ha both possessed oligonucleotide 10, from the NP

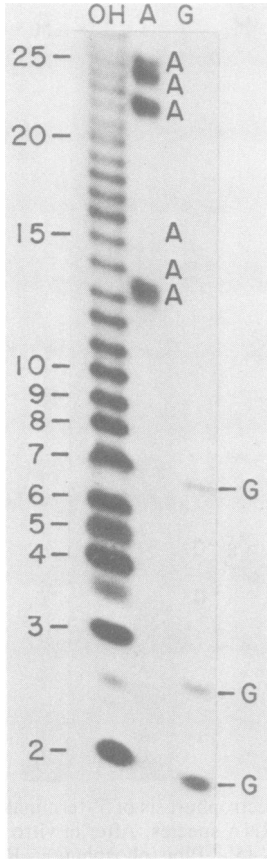

FIG. 4. Adenine and guanine residues in the copy-back 3′ terminus of Sendai virus DI RNA species Rb. Terminally labeled Rb RNA was isolated by electrophoresis, partially hydrolyzed with base-specific nucleases, and subjected to electrophoresis in a 20% polyacrylamide gel containing 7 M urea. Lane designations: OH, alkaline hydrolysis; A, U_2 RNase hydrolysis; G, T_1 RNase hydrolysis.

gene, in addition to L gene-specific oligonucleotides; 11a also had oligonucleotide 7, thought to represent leader RNA template. Thus, Rb may be the only one of these 10 DI RNA species that is a simple copy-back DI RNA, containing only a copy-back 3′ terminus and a 5′-terminal fragment of the L gene, as defined by the models of Huang (7) and Leppert et al. (15).

Special case of strain R. The companion of RNA species Rb, the higher-molecular-weight species Ra (1, 15), had exhibited four oligonucleotides from the NP gene, the maximum representation of this 3′-proximal gene in any of the RNA species that we examined (1). As shown in Fig. 1, two RNA sequence trails were obtained from Ra. One of these trails corresponded to the genomic 3′ terminus and the other to the copy-back 3′ terminus. Since the two RNA species possessing these termini had the same molecular weight and since full-length plus-strand antigenomes of standard virus and DI RNA genomes are incorporated into Sendai virus particles (10, 13), it appeared that the genomic 3′ terminus in Ra belongs to the negative strand of the DI RNA and that the copy-back sequence is the 3′ terminus of its complementary plus strand. A similar situation is visible in the two-dimensional sequencing map of RNA species 7b (Fig. 1). Here, the trail of the copy-back sequence is less intense than in the case of Ra, indicating that 7b plus strands are relatively less abundant. (In contrast, only one sequence trail is expected or observed in DI RNAs possessing the copy-back sequence, irrespective of the proportions of plus and minus strands, since the 3′ end of a plus strand copied normally from the 5′ terminus of the viral genome is identical to the copy-back sequence.) Since RNA species Ra and 7b were selected by size, we considered it unlikely that the copy-back sequence seen in each case represented the negative strand of a distinct DI RNA species, but we have not formally ruled out that possibility.

Thus, Sendai virus DI strain R contains two DI genomes with different 3′ termini, whereas 3′ termini exclusively of the genomic type were present in two other RNA strains possessing multiple RNA species (Table 1).

DISCUSSION

These results directly confirm the thesis that Sendai virus DI RNA species frequently retain the genomic 3′ terminus and do not substitute a copy-back sequence for it (1). This contrasts with VSV, whose DI RNAs are mainly of the copy-back type (18). The basis for this difference may reside in the replication machinery of each virus or in the ways the DI species have been derived (1).

Because the copy-back sequence is identical to the legitimate 3′ end of the positive-strand template that specifies negative-strand replicas, it is an effective functional substitute for the genomic 3′-terminal sequence. There is much homology between the two sequences, but considerably less than that between the analogous sequences of VSV (14). As shown in Fig. 2, both sequences are rich in uracil. The first 4 nucleotides are identical, and there is only one nucleotide difference in the first 12. However, nine differences appear in the next 13 nucleotides. Despite the differences between these sequences, each of them has been fully conserved among the DI RNA species that possess it. It seems paradoxical that no mutations have accumulated in either sequence among the DI RNAs, since the viral RNA replicating machinery is flexible enough to recognize both.

The preferential synthesis of minus strands during viral RNA replication (13) suggests that the copy-back 3′ terminus is a more efficient signal for chain initiation than the genomic 3′ terminus. However, this idea appears to be contradicted by the peaceful coexistence of the two RNA species in Sendai virus DI strain R. If the copy-back 3′ end seen in RNA species Rb is more efficient in initiating RNA replication than the genomic end in Ra, how has Ra been able to hold its own? Perhaps repeated high-multiplicity passages of strain R, putting more selective pressure on Ra, are needed to test this point fully.

The presence of Ra in strain R and the existence of the copy-back terminus in RNA 11a, derived from the Enders strain, show that DI RNA species with both kinds of 3′ termini can be generated from both Sendai virus strains. Indeed, oligonucleotide mapping of the Enders and R strains (1) and the identities of the 3′-terminal sequences of the Enders, R, and H strains demonstrated in the present work indicate that these three strains of Sendai virus are closely related.

Smaller DI RNA species tend to have 3′ termini of the copy-back type. This is seen in 11a and Ha, which occupy the lower end of the size spectrum, representing about 9 and 11% of the molecular weight of the genome, respectively. In addition, Rb, possessing the copy-back sequence, is the smaller of the two RNA species in strain R. But this generalization cannot be taken very far, since RNA species 7b, 7c, and 1c, all smaller than Rb, have the genomic 3′ terminus. If the model described by Huang (7) and Leppert et al. (15) of copy-back DI RNA generation is correct, we might expect copy-back DI RNAs to be smaller, since a copy-back DI RNA can arise in a second step of evolution from a DI RNA species that retains both genome termini, as discussed by Amesse et al. (1). However, the situation may be more complex for RNAs 11a and Ha, since both appeared to possess, as determined by oligonucleotide mapping, 3′-proximal genome sequences that should be eliminated in copy-back DI RNA generation (1, 7, 15). Perhaps these 3′-proximal sequences are located immediately adjacent to the copy-back 3′-terminal sequence as in the case of VSV DI RNA species LT_2 (9). Further sequence information extending inward from the 3′ termini of these and other Sendai virus DI RNAs is needed to explain more fully how they were generated.

Lastly, we note that there is considerable homology between the 3′ termini of the Sendai virus and VSV genomes, with nine identities (mainly uracil residues) in the first 20 nucleotides (positions 1, 2, 4, 5, 9, 11, 13, 14, and 18) (14). This may represent conservation of sequence information essential to the proper initiation of RNA synthesis, which was present in the common ancestor of the paramyxoviruses and the rhabdoviruses. Another striking example of conservation of regulatory sequences between these virus groups involves the termini of Sendai virus and VSV genes (5). For Sendai virus, the common gene-terminating sequence is 3′-AUUC$(U)_5$-5′, whereas for VSV it is 3′-AUAC$(U)_7$-5′.

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