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Nonhomologous RNA Recombination during Negative-Strand Synthesis of Flock House Virus RNA

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During sequential replicative passages of viral RNA from the nodavirus flock house virus, spontaneous deletion of RNA sequences occurred frequently. Families of deleted RNA molecules were derived from both segments of the bipartite viral genome and found to contain single, double, or triple deletions. These deletions were attributed to template switching by the flock house virus RNA replicase, resulting in recombination between distant sequences and excision of the intervening nucleotides. From sequence analysis of the recombination junctions, we concluded that the process of template switching was influenced by both the primary sequence and the secondary structure of the RNA and that it occurred predominantly during synthesis of RNA negative strands.

RNA recombination results in the juxtaposition in a single RNA molecule of sequences that were not adjacent in the parental template(s) from which the RNA was derived. It is thought to occur not by breakage and rejoining but by template switching during RNA replication (16, 18). If the template switch occurs between identical positions on two homologous templates, the result is homologous recombination, whereas if the switch occurs between nonidentical positions or between nonhomologous templates, the result is nonhomologous recombination. Most RNA virus families that have been examined manifest one or the other type of process (3, 4, 19, 21–23).

The mechanistic basis for the distinction between homologous and nonhomologous recombination appears to be that RNA-dependent RNA polymerases differ in their requirements for the minimum length of base pairing that must exist behind the growing point of a nascent RNA strand (18). During the process of polymerase switching from a donor to an acceptor template, this requirement can limit the number of potential sites on the acceptor molecule at which a productive reassociation can take place. At present, there is no experimental system that allows the mechanism of RNA recombination to be examined directly, but much can be inferred from an analysis of the products. Among the picornaviruses for example, in which the process has been studied most closely, the polymerase switches templates during synthesis of the negative strand (13, 17, 19), at sites which, because of their levels of homology and nucleotide compositions, serve to minimize the adverse free energy change involved in the switch (17). The base-paired secondary structure of the template has also been proposed to influence the selection of recombination sites (20, 21, 24, 26).

In the work reported here, we present the sequences of two families of RNA molecules that were derived by spontaneous deletion of regions of flock house virus (FHV) RNAs 1 and 2. FHV is a member of the *Nodaviridae*, a family of small isometric riboviruses with bipartite positive-sense RNA genomes (12). The larger segment of the FHV genome (RNA 1) contains 3,107 nucleotides and encodes the viral RNA replicase (8). The smaller segment (RNA 2) contains 1,400 nucleotides and encodes a precursor to the viral capsid proteins (7). The two RNAs are copackaged into 29-nm-diameter virions (15, 25). With its replicase and capsid proteins encoded by separate molecules, FHV provides an attractive system in which to study RNA recombination, since under appropriate experimental conditions, the behavior of RNA 2 can be observed in the absence of selection pressure for its messenger function. Here we describe an analysis of the nucleotide sequences of the deletion junctions in FHV RNAs 1 and 2 from which we infer that both the primary sequence and the secondary structure of the template influenced the selection of sites for nonhomologous recombination. Moreover, the results indicated that recombination occurred predominantly during negative-strand RNA synthesis.

MATERIALS AND METHODS

Cells and viruses. *Drosophila melanogaster* (Schneider line 1) cells were grown at 28°C as monolayer cultures in Schneider's medium (GIBCO) containing 15% fetal bovine serum. A stock of FHV, originally provided by Tom Gallagher and Roland Rueckert (University of Wisconsin—Madison), was grown, purified, and titered as described before (1).

Transfection and labeling of cells. Monolayers of *Drosophila* cells in 35-mm-diameter wells were washed once with Schneider's medium without serum and then covered with 1 ml of the same medium. A mixture of RNA and 10 μg of Lipofectin (Bethesda Research Laboratories) was mixed with the medium, and the cells were incubated at 28°C for 11.5 h. Actinomycin D was added to a final concentration of 5 μg/ml, and after 30 min of further incubation, replicating RNAs were labeled by incorporation of [^{3}H]uridine (20 μCi/ml) for 2 h at 28°C. Labeled RNAs were purified and analyzed as described before (1).

Reverse transcription-PCR. The spontaneous deletion products that arose from FHV RNAs 1 and 2 during multiple replicative passages were recovered by reverse transcription, amplified by polymerase chain reaction (PCR), and molecularly cloned in plasmid pGEM4 (Promega Biotec). Oligonucleotides that were complementary to the 3′ 20 nucleotides of FHV RNA 1 or 2 were used to prime first-strand cDNA synthesis during reverse transcription, and together with oligonucleotides that corresponded to the

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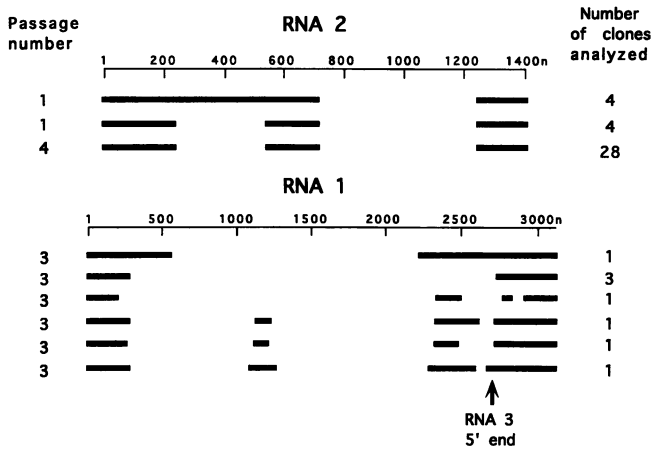

FIG. 1. Schematic representation of the deleted molecules of FHV RNAs 2 and 1 that provided the sequence information shown in Fig. 2 and 3.

5′ 20 nucleotides of these RNAs, they were used for amplification during 35 cycles of PCR. The primers contained unique *Pst*I and *Xba*I cleavage sites, so that after digestion with these enzymes, the PCR products could be ligated between the corresponding sites in the multiple cloning site region of pGEM4. Individual plasmids were sequenced by the dideoxynucleotide chain termination method (11), and sequences that spanned the deletion junctions were verified on both DNA strands.

RESULTS AND DISCUSSION

During the course of our studies of FHV RNA replication, we observed that sequential passage of the viral RNA by repeated transfection into cultured *Drosophila* cells and recovery by extraction with phenol-chloroform resulted in the disappearance of RNA 2 and its replacement by a deleted RNA containing about 630 nucleotides (1). The conditions of sequential passage (high input RNA concentration and recovery by cytoplasmic extraction) were designed to promote the generation, amplification, and recovery of deleted RNA 2 molecules that would be selected for their ability to replicate but not for their mRNA activity or encapsidation efficiency. To examine the structure of the deleted 630-nucleotide RNA and to search for other, less abundant deletions, RNAs from the first and fourth sequential passages were amplified by reverse transcription-PCR, using primers that annealed to the 3′ 20 nucleotides of FHV RNA 1 or 2 positive and negative strands. The amplified cDNAs were molecularly cloned, and the sequences of several different clones were determined (Fig. 1 to 3).

Four unique clones of RNA 2 from the first passage contained single deletions of about 500 nucleotides in the 3′ half of the molecule (Fig. 1). These deletions are identified in

5' Deletions:

#	220 230 240 250 260	500 510 520 530	Number of examples	α ORF
wt	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GAC.UUC.AAC.ACC.GAC.C	.....UCC.GAU.CAG.GUG.UCC.UCA.UUC.AGG.UAC.GCU.U		
1	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GAC.UUC.AAC.ACC.GAC.	(C) AG.GUG.UCC.UCA.UUC.AGG.UAC.GCU.U	1	+
2	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GAC.UU	A.UUC.AGG.UAC.GCU.U	11	+
3	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GAC.U	(U) UC.AGG.UAC.GCU.U	2	-
4	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GAC.	(UUC.A) GG.UAC.GCU.U	4	+
5	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.CCU.GA	(C.) A.UUC.AGG.UAC.GCU.U	1	-
6	U.CUC.AAA.UGU.GCA.UUU.GCA.CCA.C	AC.GCU.U	1	+
7	U.CUC.AAA.	(U) AC.GCU.U	2	+

3' Deletions:

	710 720 730 740 750	1210 1220 1230 1240		
wt	AAC.UUC.UCU.GAG.UCA.UUC.AUC.AAA.GGA.GUG.UUU.UCA.CAG.UC	.....U.GGA.CUG.UCA.GCC.CUU.UUU.GAA.GGA.UUU.GGC.		
8	AAC.UUC.UCU.GAG.UCA.UUC.AUC.AAA.GGA.GU	A.GCC.CUU.UUU.GAA.GGA.UUU.GGC.	2	+
9	AAC.UUC.UCU.GAG.UCA.UUC.AUC.AAA.GGA.GU	C.CUU.UUU.GAA.GGA.UUU.GGC.	1	+
10	AAC.UUC.UCU.GAG.UCA.UUC.AUC.AAA.GGA.GU	CUU.UUU.GAA.GGA.UUU.GGC.	1	-
11	AAC.UUC.UCU.GAG.UCA.UUC.AUC.AAA.G	UU.UUU.GAA.GGA.UUU.GGC.	1	+
12	AAC.UUC.UCU.GAG.UCA.UUC.AUC.A	U.GAA.GGA.UUU.GGC.	1	-
13	AAC.UUC.UCU.GAG.UCA.UUC.AUC.	UU.GAA.GGA.UUU.GGC.	2	-
14	AAC.UUC.UCU.GAG.UCA.UUC.AUC.	GAA.GGA.UUU.GGC.	4	+
15	AAC.UUC.UCU.GAG.UCA.UUC.AUC.	(AA) GGA.UUU.GGC.	2	-
16	AAC.UUC.UCU.GAG.UCA.UUC.AU	A.GGA.UUU.GGC.	1	+
17	AAC.UUC.UCU.GAG.UCA.UUC.AU	UCA.GCC.CUU.UUU.GAA.GGA.UUU.GGC.	1	-
18	AAC.UUC.UCU.GAG.UCA.UUC.A	(U) GAA.GGA.UUU.GGC.	2	-
19	AAC.UUC.UCU.GAG.UCA.UUC.	(A) A.GGA.UUU.GGC.	1	-
20	AAC.UUC.UCU.GAG.UCA.UUC.	GAA.GGA.UUU.GGC.	1	+
21	AAC.UUC.UCU.GAG.UCA.UU	A.GGA.UUU.GGC.	2	+
22	AAC.UUC.UCU.GAG.UCA.	(UU) GGC.	1	-
23	AAC.UUC.UCU.GAG.UC	(A.UU) U.GGC.	2	+
24	AAC.UUC.	GAA.GGA.UUU.GGC.	1	+

FIG. 2. Nucleotide sequences across the 5′ and 3′ junctions found in spontaneous deletions of FHV RNA 2. Nucleotides shown in parentheses could not be assigned unambiguously to one or the other side of the deletion junctions. α ORF, open reading frame for protein α.

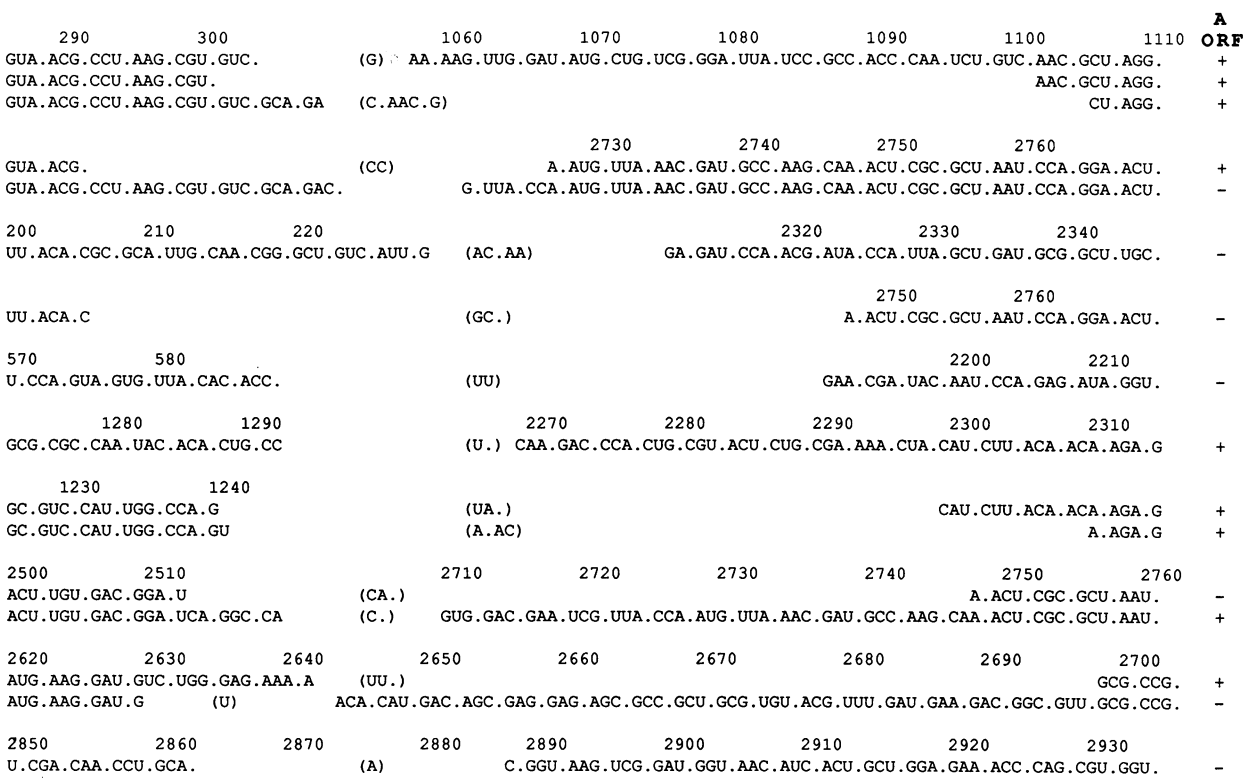

FIG. 3. Nucleotide sequences across the junctions found in spontaneous deletions of FHV RNA 1. Nucleotides shown in parentheses could not be assigned unambiguously to one or the other side of the deletion junctions. A ORF, open reading frame for protein A.

Fig. 2 as numbers 14, 16, 18, and 24. The other four unique clones from passage 1 RNA that were analyzed each contained two deletions: one of about 270 nucleotides in the 5′ half of the molecule in addition to the larger deletion in the 3′ half. These were 5′ deletions 1, 2, and 3 and 3′ deletions 8, 15, 20, and 21 (Fig. 2). Twenty-eight clones of RNA 2 were analyzed from passage 4 RNA, and all contained deletions in both the 5′ and 3′ halves of the molecule (Fig. 1). Although the boundaries of the deletions were similar among these molecules, they were usually not identical for both deletions. From this observation, we could conclude that 22 of the 32 clones with two deletions were derived from 22 unique members of a family of closely similar RNA molecules that contained about 630 nucleotides. Moreover, the fact that so many of the clones were unique implied that the family of RNA molecules probably contained other members whose junction sequences were not determined. However, the possibility of biased amplification during PCR makes it uncertain that the frequency with which each unique clone was isolated reflected the abundance of the corresponding RNA deletion. For this reason, all potential sibling clones were discarded from the data set that was used in the following analysis.

It was striking that all of the RNA 2 clones that were analyzed retained the same internal region, from about nucleotides 530 to 730. An FHV RNA 2 molecule with similar deletions was isolated independently from a culture of *Drosophila* cells that were persistently infected with FHV (5). Sequence analysis showed that this RNA contained the deletion junctions identified as numbers 2 and 18 in Fig. 2 (5). No insertions or rearrangements of sequence were found, although five point mutations were identified among the total 24 kb of RNA 2 sequence that were examined. In every case, the point mutations were far from the deletion junctions. The junction sequences across the 5′ and 3′ deletions are compiled in Fig. 2. Also shown are the number of structurally different molecules in which each deletion junction was observed and whether the deletion preserved (+) or disrupted (−) the open reading frame for protein α, the capsid protein precursor.

When primers specific for RNA 1 were used for reverse transcription-PCR amplification of passage 3 or 4 RNA, cDNA clones of several different sizes between about 570 and 1,500 bp were generated. Eight representative clones were sequenced and found to contain single or triple deletions of the wild-type RNA 1 sequence (Fig. 1). Apart from about 200 to 300 nucleotides at each terminus, no common internal sequence was retained by all of the RNA 1 deletions. The 16 deletion junctions that were identified in the eight different clones (Fig. 3) were more widely distributed along the length of the RNA than were those found in RNA 2, although some clustering of deletion boundaries was evident, near the site of initiation of the subgenomic RNA 3, for example. The possibility of chimeric RNA 1-RNA 2 recombinant molecules was not investigated.

The nucleotide sequences at some of the deletion junctions in both RNAs 1 and 2 were such that it was impossible to define the recombination site precisely. For deletion junction 4 in Fig. 2, for example, which was found in four different RNA molecules, recombination could have occurred at any of the five internucleotide bonds in the sequence UUCA and the resulting molecules would have had the same sequence. Similarly, for deletion junction 27 (Fig. 3), recombination could have occurred at any one of six

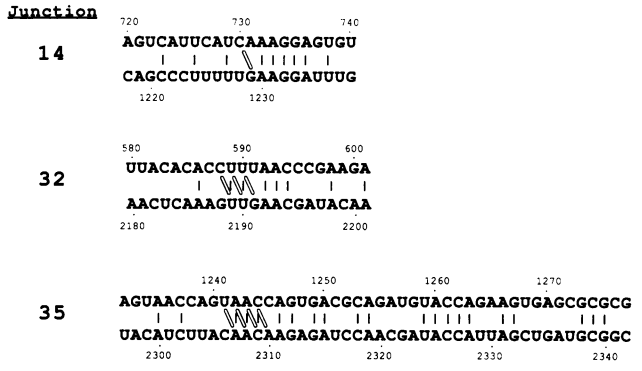

FIG. 4. Alignment of sequences from which deletions 14, 32, and 35 were derived (numbered as in Fig. 2 and 3). Vertical lines indicate identical nucleotides in the donor and acceptor templates at or near the sites of recombination, which are indicated by the diagonal bars.

internucleotide bonds. This was a reflection of the fact that at 24 of the 40 junctions analyzed, a region of identical sequence from one to five nucleotides long was found at either side of the junction, and in the deleted RNAs, the corresponding bases could not be assigned unambiguously to one or the other side of the deletion junction. Such ambiguous nucleotides are shown in parentheses in Fig. 2 and 3. Of these 24 junctions, the 11 with single ambiguous nucleotides provide little evidence at first sight of a role for primary sequence in determining recombination junctions, because 11 of 40 (27.5%) is very close to the 25% frequency of junctions that would be predicted to show single ambiguous nucleotides if recombination occurred without reference to primary nucleotide sequence. Furthermore, in 16 of the 40 cases, there were no ambiguous nucleotides at the junctions, which showed that the FHV polymerase could extend a nascent RNA despite mispairing of the 3'-terminal nucleotide. On the other hand, the frequencies of junctions with two to five ambiguous nucleotides were between 2 and 26 times higher than expected on the basis of a random model of template switching. These results provided evidence that recombination may occasionally be favored by base pairing between the template and nascent strands.

To examine further the role of primary sequence in guiding the recombination process, we aligned and compared the nucleotide sequences surrounding the boundaries of each of the deletions. The most frequent 3' deletion junction of RNA 2 (number 14 in Fig. 2) was particularly revealing, in that each boundary of the unambiguous recombination site lay next to the same sequence of five nucleotides, AAGGA (Fig. 4). On a random basis, this situation would be expected to occur once in every 1,024 junctions; in fact, it occurred at 4 of the 26 (total) 3' junctions analyzed, strongly implicating the sequence in somehow guiding the recombination process. Figure 4 also shows two other junctions (numbers 32 and 35 in Fig. 3) that had a higher than random level of sequence identity at or near the site of recombination. All 26 (total) 3' junctions of RNA 2 deletions that were analyzed are aligned in Figure 5B, with those that may have been guided by the sequence AAGGA shown by solid bars. Figure 5A shows a compilation of the 22 (total) 5' junctions of RNA 2 deletions that were analyzed. The solid bars indicate those that may have been influenced by the sequence UUCA that was identified above as being a major site of recombination (junction 4 in Fig. 2).

It is striking that the recombination site, where it could be identified unambiguously, lay on the 5' side of the region of sequence identity that was postulated to guide the reassociation step of the template switch. This is particularly clear for the junctions shown in Fig. 4 but can also be seen from

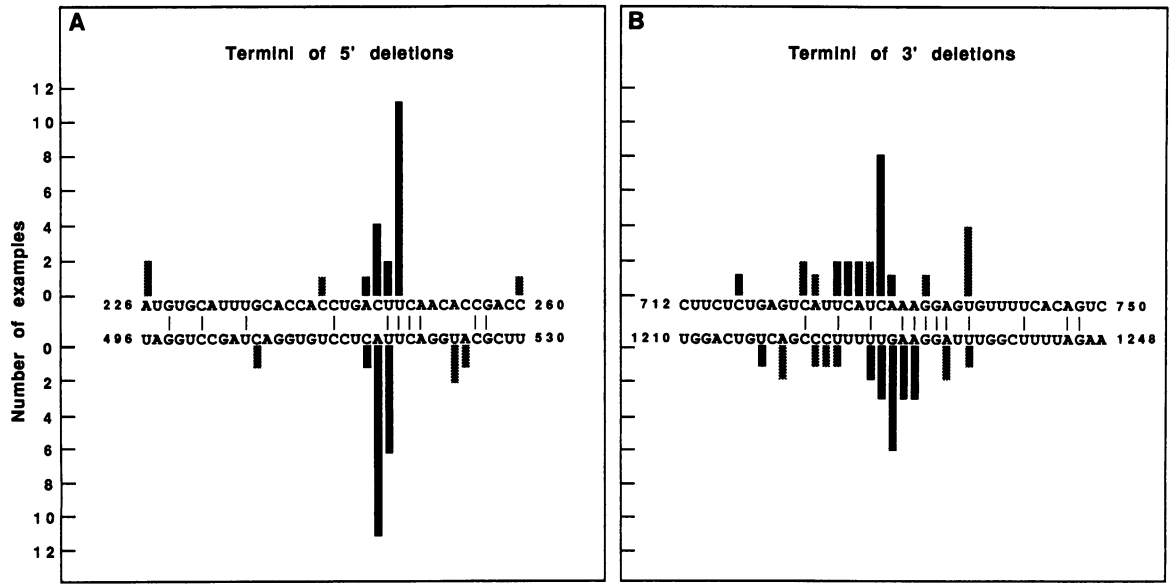

FIG. 5. Alignment of the termini of the 5' deletions (A) and the 3' deletions (B) of RNA 2. The terminal nucleotide at the 5' boundary of each deletion is indicated by the vertical bars above the corresponding sequence, and that from the 3' boundary is indicated below. Bar height corresponds to the number of different molecules in which each deletion junction was observed. Ambiguous nucleotides (see Fig. 2) were arbitrarily placed at the 3' boundaries. Black and hatched grey bars, respectively, indicate junctions where recombination may and may not have been influenced by the putative guide sequences (UUCA for 5' junctions; AAGGA for 3' junctions). The criterion for this distinction was the presence of a single copy of the putative guide sequence in the recombinant molecule.

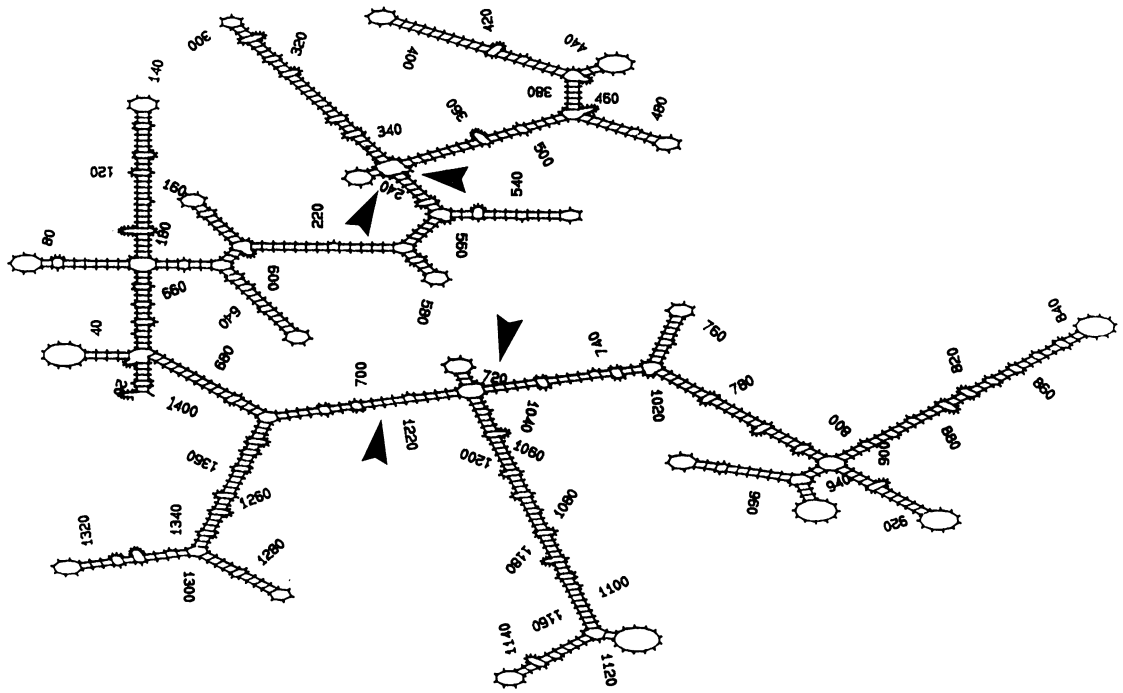

FIG. 6. Predicted secondary structure of the positive strand of FHV RNA 2. The structures were generated by using a modified version of the FOLD program and portrayed by using SQUIGGLES (10). The upper pair of arrowheads point to nucleotides 249 (left) and 517 (right), the approximate boundaries of the 5′ deletions. The lower pair of arrowheads point to nucleotides 1222 (left) and 726 (right), the approximate boundaries of the 3′ deletions.

the compiled results shown in Fig. 5. It is difficult to see how the regions of sequence identity could have influenced recombination during positive-strand RNA synthesis, since they lie downstream of the recombination site, but this feature of the junctions can be readily understood if recombination occurred during synthesis of the negative strand, since in this case the complement of the region of sequence identity would have been incorporated into the nascent strand immediately before the replicase dissociated from the template, and it would thus have been in a position to guide the reassociation process. At first sight, it might seem surprising that recombination should occur predominantly during negative-strand synthesis, since positive-strand RNA synthesis is by far the major process during FHV RNA replication. The likely explanation is that during template switching, the reassociation step is rate limiting for recombination, and as a second-order reaction, its rate will be strongly influenced by the concentration of acceptor template molecules, i.e., positive RNA strands during negative-strand synthesis (13). For both RNAs 1 and 2, positive strands were about 100-fold more abundant than negative strands during FHV RNA replication (unpublished results).

Thus, both for homologous recombination in picornaviruses and for nonhomologous recombination in FHV, the sequence similarities between the donor and acceptor templates lay on the 3′ side of the recombination site, indicating that recombination occurred during negative-strand synthesis. Although the lengths of the regions that showed the sequence similarity were much shorter for FHV than for poliovirus, the similarities were more noticeable because of the lack of overall sequence homology. The site at which the replicase dissociated from the template during generation of the 3′ deletions of RNA 2 was unusually rich in U residues, which might have increased the probability of dissociation by destabilizing the template-nascent strand complex. However, the equivalent site for the 5′ deletions showed no unusual nucleotide composition, so this was not a general feature of polymerase switch sites in this system.

The influence of primary sequence on recombination described above was insufficient to account for the highly nonrandom distribution of deletions in RNA 2. Two other factors could be identified. One was that the region between the two deletions (approximately nucleotides 530 to 730) contained a sequence that was required in *cis* for RNA 2 replication (1, 2), and RNAs that retained this region were inevitably selected during replicative passages. The other influential factor was the base-paired secondary structure of the RNA 2 template.

A secondary structure model has been proposed for RNA 2 of the related nodavirus black beetle virus on the basis of the optimization of Watson-Crick base pairing (6). However, although the sequence of black beetle virus RNA 2 is about 80% identical to that of FHV RNA 2, the predicted secondary structures of the two RNAs differ substantially (15). We therefore generated a secondary structure model for FHV RNA 2, using a version of the FOLD program of the University of Wisconsin Genetics Computer Group software package (10), modified to accommodate an RNA of 1,400 nucleotides (Fig. 6). In such large RNA structures, predictions of short-range interactions, such as those that form hairpin loops, can be considered fairly reliable, particularly if they recur when different sections of the molecule are folded separately and if they persist over a range of energy parameters. Predictions of long-range interactions, on the other hand, are much less reliable because their calculated stabilities differ insignificantly from those of very different

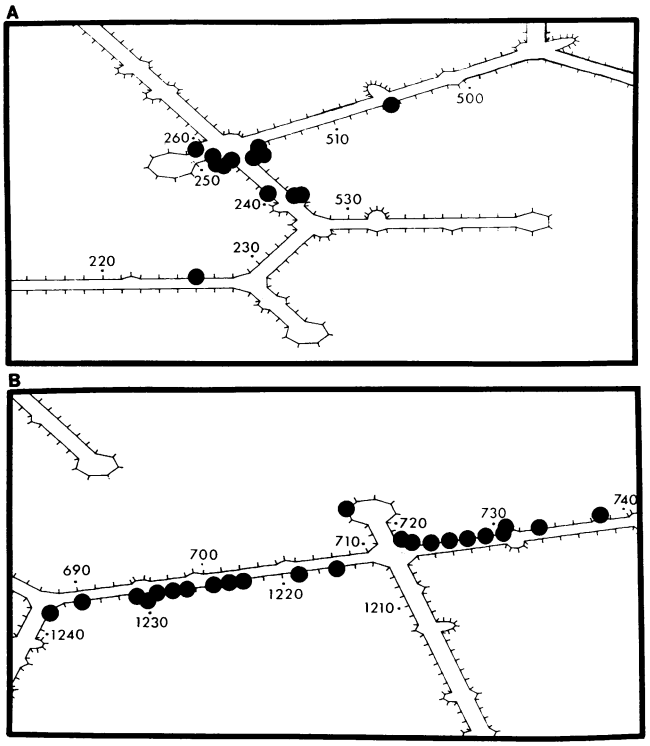

FIG. 7. Predicted secondary structure of the positive strand of FHV RNA 2 in the region of the 5′ deletion (A) and the 3′ deletion (B) junctions. The larger dots indicate the nucleotides at the termini of the individual deletions.

structures. According to these considerations, the predicted structure of the 5′ half of RNA 2 was fairly reliable, and indeed, it included several of the hairpin loops predicted for black beetle virus RNA 2 (6). The proposed structure of the 3′ half of the RNA was less reliable, since the predictions for this region were very sensitive to small changes in folding parameters.

Striking results were obtained by projecting the boundaries of the deletions in the 5′ and 3′ halves of RNA 2 onto the corresponding folded structures (Fig. 6 and 7). It appeared that the most frequent 5′ deletion of RNA 2 (number 2 in Fig. 2) resulted from the replicase dissociating from the positive strand at nucleotide 517, just before the double hairpin loop structure that includes nucleotides 261 to 515, and reassociating at nucleotide 249, which was immediately adjacent to nucleotide 517 in the folded structure (Fig. 7A). The boundaries of the other 5′ deletions were also near one another in the predicted secondary structure, as were the boundaries of the 3′ deletions (Fig. 7B), although the distance between the latter boundaries may reflect a shortcoming of the model for this region of the RNA. Notice that it is the secondary structure of the positive strand that is presented in Fig. 6 and 7 and which appears to have influenced recombination. The boundaries of both deletions were widely separated on the structure predicted for the negative strand (not shown). This result provides an independent line of evidence that recombination occurred predominantly during negative-strand synthesis, since during this process, secondary structure in the positive-strand template could be expected to influence the positions of replicase dissociation and reassociation.

One aspect of the results was unexpected: the fact that the open reading frame for protein α was maintained across 86% of the 5′ deletions and across 58% of the 3′ deletions in RNA 2 (Fig. 2). Our experimental approach was designed not to exert selection pressure for translation of RNA 2, so we expected that two out of three of the spontaneous deletions of this RNA would disrupt the reading frame; the results were very different (Fig. 2). Translation of RNA 2 was not necessary for its replication, since engineered deletions of FHV 2 cDNA yielded transcripts that could be replicated whether or not the reading frame was maintained (1) and inhibition of protein synthesis by cycloheximide did not inhibit RNA 2 replication (data not shown). The simplest explanation for this unexpected finding is that untranslated RNAs are less stable and were therefore underrepresented in the pool of replicating molecules. However, the results presented above showed that recombination occurred predominantly during negative-strand RNA synthesis, when the template can also be used (in the other direction) for translation. We cannot exclude the possibility that the observed tendency of spontaneous deletions to maintain the open reading frame was somehow a consequence of the conflicting processes of translation and replication. Similar selection pressure for the maintenance of an open reading frame across a deletion junction has also been observed in defective interfering RNAs of mouse hepatitis virus (9) and clover yellow mosaic virus (27). In these cases also, the influence of translation on RNA stability was considered a likely explanation. Interestingly, spontaneous deletions of influenza virus RNAs, which are negative stranded, showed no evidence of selection pressure for open reading frame maintenance (14).

In summary, the results of our analysis of the junction sequences of spontaneous deletions of FHV RNAs 1 and 2 were fully consistent with the following picture of RNA recombination in this system. Recombination occurred by template switching during synthesis of negative-strand RNA by FHV replicase. Base pairing between the nascent RNA and the acceptor template at or near the point of RNA elongation was not necessary for a successful template switch, because the FHV replicase could evidently elongate a nascent strand despite mispairing of its terminal nucleotide. However, short regions of such base pairing were found more frequently than expected on a random basis, indicating that the reassociation step of a template switch event was sometimes facilitated and guided by annealing of the nascent strand to the acceptor template. Finally, the base-paired secondary structure of the template appeared to influence the positions at which the most frequent template switches occurred, in that the major sites of replicase dissociation and reassociation were held close together in the predicted secondary structure of the template.

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