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cis-Acting Requirements for the Replication of Flock House Virus RNA 2

L. ANDREW BALL* AND YUE LI

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

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To examine the *cis*-acting requirements for RNA replication, a cDNA clone of flock house virus (FHV) RNA 2 was transfected into baby hamster kidney cells and transcribed to yield RNAs that had terminal extensions of different lengths or that lacked internal regions of the molecule. These RNAs were tested for their ability to be replicated by FHV replicase that was provided by cotransfection of purified FHV RNA 1. The results showed that RNA replication was inhibited by terminal extensions, particularly those at the 5′ end of the RNA, despite the fact that these extensions were corrected during RNA replication. A negative-sense transcript with a 12-nucleotide 3′ extension was replicated to produce a positive-sense RNA that had the correct 5′ end, showing that the replicase could select its correct initiation site from within a longer template. A uridine residue at the second position of the positive strand was an important determinant of template activity. RNA molecules with large internal deletions that amounted to almost 50% of the 1,400 nucleotides of RNA 2 replicated as efficiently as full-length molecules, but only if they contained an internal region that lay between nucleotides 538 and 616. Thirty-six spontaneous deletions of RNA 2 that arose during sequential replicative passages all conserved the same internal region of the molecule. These results establish that both terminal and internal regions of FHV RNA 2 play essential roles in making the molecule a competent template for replication.

Flock house virus (FHV) is a member of the *Nodaviridae*, a family of small isometric riboviruses with bipartite positive-sense RNA genomes (16). FHV was first isolated from the New Zealand grass grub, *Costelytra zealandica* (25), but the virus replicates well in wax moth larvae (*Galleria mellonella*) and in *Drosophila melanogaster* cells in culture (14). Moreover, productive infections can also result from introducing the viral RNA directly into plants (26) or cultured mammalian cells (1). The larger segment of the viral genome (RNA 1; 3,107 nucleotides) encodes the viral contribution to the RNA-dependent RNA polymerase which replicates both segments of viral RNA (13). The smaller genome segment (RNA 2; 1,400 nucleotides) encodes a polypeptide precursor (α) which, after virion assembly, undergoes a single proteolytic cleavage to yield the two capsid proteins β and γ (14). In the mature virion, 180 copies of these proteins are arranged with icosahedral symmetry on a T=3 surface lattice (17), surrounding one molecule of each of the viral RNA genome segments (27).

Several features of FHV RNA replication make it an attractive system for studies of the molecular mechanism. Among these features are the abundant activity of the replicase in a broad range of intracellular environments and its natural action in *trans*, i.e., on an RNA template other than that from which the enzyme itself was translated. To this end, we constructed DNA plasmids which directed the intracellular transcription of FHV RNAs that could serve as templates for the FHV RNA replicase (1). These plasmids contained full-length cDNA copies of FHV RNA 1 or 2 (9) inserted between a promoter site for bacteriophage T7 RNA polymerase and a cDNA copy of the self-cleaving ribozyme of satellite tobacco ringspot virus (sTobRV) (6). On transfection into baby hamster kidney (BHK 21) cells which were expressing T7 RNA polymerase from a vaccinia virus (VV) recombinant, such plasmids made FHV RNA transcripts which, after autolytic cleavage, could serve as templates for FHV RNA replicase.

In the work reported here, we used this experimental approach to identify features of FHV RNA 2 that influenced its ability to replicate. The work had two goals: to examine the mechanism of FHV RNA replication and to identify replaceable regions of RNA 2 that could serve as potential sites for the insertion of foreign sequences. The results showed that in addition to strict requirements at the RNA termini, an internal region of the RNA was also important for replication. Moreover, the internal region was strongly conserved in deletions of RNA 2 that arose spontaneously during RNA replication.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK 21) cells, African green monkey kidney (BSC 40) cells, and *D. melanogaster* (Schneider line 1) cells were grown as described before (2). The recombinant VV that expresses the T7 bacteriophage RNA polymerase (vTF7-3 [12]) was kindly provided by Bernard Moss (National Institutes of Health) and was grown and titers of the virus were determined as described before (1). A VVT7 recombinant that had a 1-kb deletion in the gene for VV DNA ligase was constructed by recombining the *Hin*dIII restriction fragment that contained the T7 polymerase gene from vTF7-3 DNA into VV SK20, a DNA ligase deletion mutant of VV that was kindly provided by Shona Kerr and Geoffrey Smith (Oxford University) (18). A stock of FHV, kindly provided by Tom Gallagher and Roland Rueckert (University of Wisconsin—Madison), was grown and purified as described before (1), and the titer was determined by plaque assay on monolayers of *D. melanogaster* cells (27).

Plasmid constructions. Transcription plasmids that contained FHV 2 cDNA all conformed to the general structure

* Corresponding author.

TABLE 1. 5′- and 3′-junction sequences of primary transcripts[a]

Plasmid	5′ junction		3′ junction
FHV 2(26, 53)	GGGAGACCCAAGCUUGCAUGCCUGCAGUAAAC	... ...	UAAGGUCUAUAGGCCUCUAGAGGAUCCCCGGG UACCGAGCUCGAAUUCGAUACCCUGUU*
FHV 2(26, 43)	As above	... ...	UAAGGUCUAGAGGAUCCCCGGGUACCGAGCUC GAAUUCGAUACCCUGUU*
FHV 2(26, 25)	As above	... ...	UAAGGUCUAAGGGGAUCCUCGAUACCCUGUU*
FHV 2(26, 12)	As above	... ...	UAAGGUCGAUACCCUGUU*
FHV 2(28, 12)	GGCCUGGAUCCUCUAGAGUCGACCUGCAGUAAAC	... ...	as FHV 2(26, 12)
FHV 2(10, 12)	GGGAGACCCAGUAAAC	... ...	as FHV 2(26, 12)
FHV 2(2, 43)	GGGUAAAC	... ...	as FHV 2(26, 43)
FHV 2(2, 20)	As FHV 2(2, 43)	... ...	UAAGGUCCUGAAUUCGAUACCCUGUU*
FHV 2(2, 12)	As FHV 2(2, 43)	... ...	as FHV 2(26, 12)
FHV 2(1, 12)	GGUAAAC	... ...	as FHV 2(26, 12)
FHV 2(0, 12)	GUAAAC	... ...	as FHV 2(26, 12)
FHV 2(0, 12)U2G	GGAAAC	... ...	as FHV 2(26, 12)
2 VHF(2, 12)	GGACCUUA	... ...	GUUUACCGAUACCCUGUU*

[a] Underlined sequences are the authentic termini of RNA 2; the asterisk indicates the 3′ end generated by autolytic cleavage.

described previously (1) but differed from one another in the number of nucleotides (*N*) between the site of transcriptional initiation and the first nucleotide of the FHV 2 sequence and in the number of nucleotides (*M*) between the end of the FHV 2 sequence and the site of autolytic cleavage. Plasmids that contained the FHV 2 cDNA in the positive orientation with respect to the T7 promoter were designated FHV 2(*N*, *M*) [e.g., FHV 2(0, 12)] to indicate these differences. Those that contained the FHV 2 cDNA in the opposite orientation were designated 2 VHF(*N*, *M*) [e.g., 2 VHF(2, 12)]. Plasmids were purified by banding on cesium chloride-ethidium bromide isopycnic gradients, and the sequences across the 5′ and 3′ junctions of their FHV 2 cDNA inserts were determined by the dideoxy chain termination method with denatured plasmid DNA as template (15). Table 1 shows the sequences at the termini of transcripts derived from the plasmids used in this work. For determination of the 5′-junction sequences and for mapping the 5′ ends of the FHV 2 RNAs by primer extension, an oligonucleotide whose sequence was complementary to nucleotides 99 to 81 in the FHV RNA 2 sequence was used as primer. For determination of the 3′-junction sequences and for mapping the site of autolytic cleavage, an oligonucleotide whose sequence was complementary to nucleotides −92 to −71 relative to the site of transcriptional termination in the T7 terminator was used as primer.

Infection and transfection of cells. These operations were performed as described before (1), except that 2.5 μg of plasmid DNA was used throughout to transfect the cells of a 35-mm monolayer. When required, FHV RNA replicase was provided by cotransfection of authentic FHV RNA 1 that was biologically purified to be free of RNA 2 by multiple replicative passages as described before (2). RNAs were labeled by incorporation of [^{3}H]uridine in the absence or presence of actinomycin D (10 μg/ml) as indicated for the individual experiments. Labeled RNAs were purified from cytoplasmic lysates by extraction with phenol-chloroform and precipitation with ethanol. They were resolved by electrophoresis on 1% agarose–formaldehyde gels (19) and visualized by fluorography (4).

RNA 5′-end mapping by primer extension. The 5′ ends of RNA transcription, cleavage, and replication products were mapped by primer extension with the primers described above at a concentration of 0.28×10^{-6} M; dGTP, dCTP, and dTTP were used at concentrations of 0.5×10^{-3} M; [α-^{35}S]dATP was used at a concentration of 0.5×10^{-4} M (300 μCi/ml); and reverse transcriptase (Moloney murine leukemia virus; Bethesda Research Laboratories) was used at a concentration of 2×10^4 U/ml. Reaction mixtures were incubated at 37°C for 60 min before analysis of the products by electrophoresis on 6% sequencing gels alongside a dideoxy sequence ladder generated by using the same primer and an appropriate DNA template.

Reverse transcription-PCR. The spontaneous deletion products of FHV RNA 2 that arose during multiple replicative passages were analyzed after reverse transcription, amplification by the polymerase chain reaction (PCR), and cloning in pGEM4 (Promega Biotec). An oligonucleotide that was complementary to the 3′-terminal 20 nucleotides of FHV RNA 2 was used to prime first-strand synthesis during reverse transcription. This oligonucleotide and another whose sequence corresponded to the 5′-terminal 20 nucleotides of FHV RNA 2 were used together as primers during 35 cycles of PCR. The primers also contained unique *Xba*I and *Pst*I cleavage sites, respectively, so that after digestion with these enzymes, the PCR products could be ligated into pGEM4 cut with the same two enzymes. Individual plasmids were sequenced as described above (15). Sequences that spanned the deletion junctions were verified on both DNA strands.

RESULTS

Plasmids that direct the intracellular synthesis of replicable RNAs. To determine what sequences were necessary for RNA replication, we transfected cells that were expressing both T7 RNA polymerase and FHV RNA replicase with DNA plasmids that could direct the synthesis of replicable FHV RNAs. As described before, runoff transcription from linearized plasmid DNA transfected into VVT7-infected cells was unreliable (1), and the use of a VVT7 recombinant from which the VV DNA ligase gene (18) had been deleted did not improve the results (data not shown). To circumvent this problem, we developed transcription plasmids that expressed a *cis*-acting ribozyme sequence which generated the 3′ end of the RNA by autolytic cleavage (1). Initially, we used the ribozyme from sTobRV (6), which, in our constructs, left a minimum of 12 ribozyme-derived nucleotides at the 3′ ends of the FHV RNAs (Table 1). More recently, we constructed transcription plasmids that contained instead

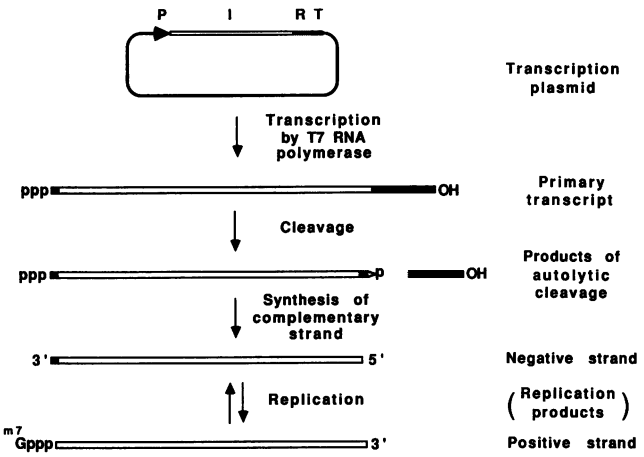

FIG. 1. Schematic representation of RNA synthesis and processing in cells expressing T7 RNA polymerase which were transfected with plasmids that synthesized replicable RNAs. P, T7 promoter; I, FHV 2 cDNA insert; R, sTobRV ribozyme; T, T7 terminator.

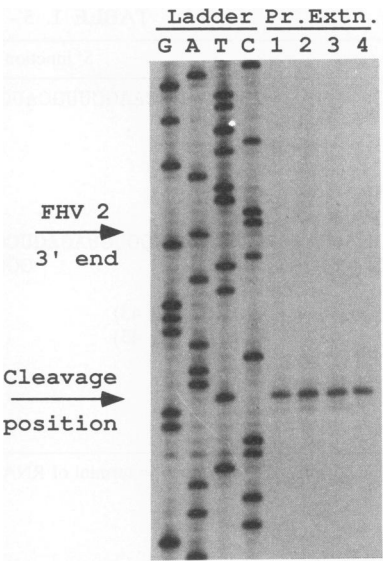

FIG. 2. Primer extension (Pr. Extn.) analysis of the products of ribozyme-mediated autolytic cleavage. BHK 21 cells were infected with VVT7 and transfected with the following plasmids (by lane): 1, FHV 2(2, 43); 2, FHV 2(2, 20); 3 and 4, FHV 2(2, 12). The cells that yielded the lane 4 sample were cotransfected with FHV RNA 1 to provide RNA replicase. Twenty-four hours posttransfection, cytoplasmic RNAs were extracted and used as templates for extension by reverse transcriptase of a primer that annealed to the downstream product of ribozyme-mediated autolytic cleavage (see Materials and Methods). The primer extension products were labeled by incorporation of [α-^{35}S]dATP, resolved by electrophoresis on a 6% sequencing gel, and visualized by autoradiography. The same primer was used to produce a dideoxy sequencing ladder of the plasmid FHV 2(2, 12) and those samples were run in lanes G, A, T, and C. The bottom arrow indicates the site of self-cleavage predicted from the known properties of the sTobRV ribozyme (5), and the top arrow indicates the position of the 3' end of the FHV 2 cDNA sequence.

the ribozyme from the antigenomic strand of hepatitis delta virus (23). RNA expressed from these plasmids self-cleaved to yield a 3' end that corresponded precisely to the 3' end of the inserted cDNA (2a). In the case of transcripts of a cDNA clone of a defective interfering particle of vesicular stomatitis virus, a perfect 3' end was essential for the RNA to be a competent template for vesicular stomatitis virus replication (22). However, transcripts of FHV RNA 2 replicated well despite the presence of 3'-terminal extensions (see below), and all the results presented in this paper were obtained by using plasmids that contained the sTobRV ribozyme.

The series of events expected to occur after transfection of these plasmids into cells is presented in Fig. 1 in order to clarify the assays we have used. The T7 RNA polymerase expressed as a result of infection with the VVT7 recombinant transcribed the transfected plasmids to produce primary transcripts with 5' extensions whose lengths depended on the position of the T7 promoter with respect to the cDNA insert. The 5' ends of the primary transcripts, some of which were capped by the VV guanylyltransferase (11), were mapped by primer extension. The 3' ends of the primary transcripts were generated by transcriptional termination at the Tϕ terminator (24). Next, ribozyme-mediated autolysis generated an upstream RNA product that carried a 2',3' cyclic phosphate at the end of a 3'-terminal extension, whose length depended on the position of the ribozyme with respect to the 3' end of the cDNA. The predicted downstream product of autolytic cleavage was a 170-nucleotide RNA that carried a 5' hydroxyl group. Mapping its 5' end by primer extension was used to define the site of autolysis of the primary transcript. If the transfected cells expressed active FHV replicase and if the cleaved transcript was a competent template for replication, cRNA was made in an RNA-dependent, actinomycin D-resistant reaction. If, in turn, the cRNA was a competent template, full RNA replication ensued. Analysis of the termini of the replication products showed whether the terminal extensions of the primary transcript were corrected during replication.

Mapping the sites of self-cleavage by primer extension. The sites of self-cleavage of the primary transcripts from plasmids FHV2(2, 43), FHV 2(2, 20), and FHV 2(2, 12) (Table 1) were mapped by analysis of the products of primer extension on the downstream products of autolysis by using a primer that annealed to a sequence in the T7 terminator (see Materials and Methods). Cytoplasmic RNAs were extracted from VVT7-infected, transfected cells, were digested with RNase-free DNase to remove any residual plasmid DNA, and were used as templates for primer extension. The results showed that cleavage occurred at the same position with respect to the ribozyme in RNA from all three plasmids (Fig. 2), and this position corresponded to the known site of autolysis by the sTobRV ribozyme (5). Moreover, the efficiency of cleavage, which can be estimated from the intensity of the primer extension products, was approximately the same in each case. Ongoing replication of the upstream product of autolysis (see below) did not affect cleavage of the primary transcript (Fig. 2, lane 4).

Relative abundances of transcription and replication products. The relative abundances of the products of transcription and replication were compared for four plasmids that expressed FHV RNA 2 transcripts with different 5' ends. Transcription products were labeled by incorporation of [^{3}H]uridine in the absence of actinomycin D for a 2.5-h period starting 24 h posttransfection. Replication products were labeled for a similar period, but in the presence of 10 μg of actinomycin D per ml added 30 min before labeling and in cells that had been cotransfected with authentic FHV RNA 1 to provide a source of RNA replicase activity. Throughout

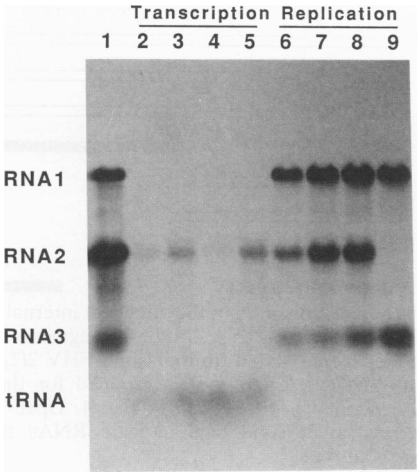

FIG. 3. Products of transcription and replication directed by the following plasmids (by lane): 2 and 6, FHV 2(2, 12); 3 and 7, FHV 2(1, 12); 4 and 8, FHV 2(0, 12); 5 and 9, FHV 2(0, 12)U2G. BHK 21 cells were infected with VVT7 and transfected with the plasmids described. The cells that yielded the samples in lanes 6 through 9 were cotransfected with FHV RNA 1 to provide RNA replicase. Twenty-four hours posttransfection, cells were labeled for 2.5 h with [^{3}H]uridine (20 μCi/ml) in either the absence (lanes 2 through 5) or the presence (lanes 1 and 6 through 9) of 10 μg of actinomycin D per ml. After labeling, cytoplasmic RNAs were extracted, resolved by electrophoresis on a 1% agarose-formaldehyde gel, and visualized by fluorography. Lane 1 shows authentic FHV RNAs labeled 12 h after transfection of cells with RNAs 1 and 2.

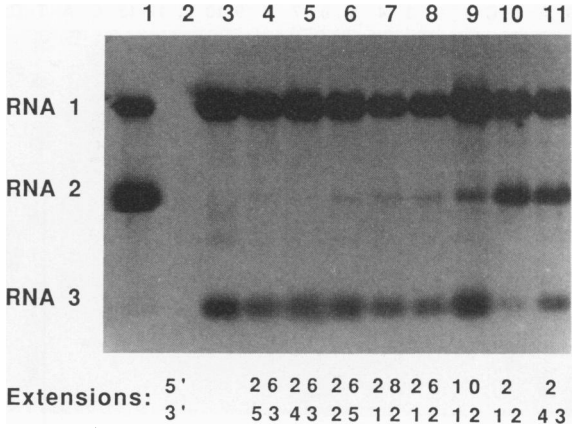

FIG. 4. Products of replication directed by the following plasmids (by lane): 1 and 3, none; 4, FHV 2(26, 53); 5, FHV 2(26, 43); 6, FHV 2(26, 25); 7, FHV 2(28, 12); 8, FHV 2(26, 12); 9, FHV 2(10, 12); 2 and 10, FHV 2(2, 12); 11, FHV 2(2, 43). BHK 21 cells were infected with VVT7, transfected with the plasmids described, and cotransfected with authentic FHV RNAs 1 and 2 (lane 1) or RNA 1 alone (lanes 3 through 11). Twenty-four hours posttransfection, the products of RNA replication were labeled by a 3.5-h incorporation of [^{3}H]uridine in the presence of actinomycin D. Labeled cytoplasmic RNAs were resolved by electrophoresis on a 1% agarose–formaldehyde gel and visualized by fluorography.

this work, a standard incubation temperature of 28°C was used because the activity of the FHV replicase was inhibited at higher temperatures (2). Cytoplasmic RNAs were extracted, resolved by electrophoresis on an agarose-formaldehyde gel, and visualized by fluorography. From three of the four plasmids, transcripts that comigrated with authentic FHV RNA 2 were synthesized at rates that compared with that of tRNA, the major cytoplasmic product of cellular transcription under these labeling conditions (Fig. 3, lanes 2, 3, and 5). On the other hand, plasmid FHV 2(0, 12), in which the T7 polymerase initiation site coincided with the first nucleotide of the FHV 2 sequence, was transcribed much less efficiently, and its product (which also comigrated with authentic RNA 2) could be detected only after a longer exposure than that shown in Fig. 3 (lane 4). The negative influence of uridylate residues that lie downstream of the initiation site for T7 RNA polymerase has been documented (21) and can be clearly seen by comparing the levels of transcription of FHV 2(0, 12) and FHV 2(0, 12)U2G (Fig. 3, lanes 4 and 5, respectively).

The efficiency of transcription differed markedly from that of RNA replication. Transcripts with 5′ extensions of two or one G residues replicated to about 10 times their transcription level (Fig. 3, lanes 6 and 7), whereas those from FHV 2(0, 12) with no additional 5′ nucleotides replicated to about 100 times their transcription level (Fig. 3, lane 8). The relative intensities of the RNA 2 bands in Fig. 3, lane 1 (authentic RNA) and lane 8 [FHV 2(0, 12)-derived RNA], show that the cDNA transcript replicated with an efficiency comparable to that of authentic RNA 2. Although changing the second nucleotide of the transcript from U to G increased its efficiency of transcription, it prevented replication (Fig. 3, lane 9). Because all three authentic FHV RNAs have 5′-terminal sequences (after the cap) of GU. . ., the U residue at position 2 may be an important determinant of RNA synthesis (7, 8; data for RNA 3 from our unpublished results). The intense bands of FHV RNAs 1 and 3 in lanes 6 through 9 of Fig. 3 resulted from the self-replication of FHV RNA 1 that was cotransfected to provide a source of RNA replicase in these samples.

Influence of terminal extensions on RNA replication. The influence of terminal extensions on the ability of an RNA transcript to be replicated was examined further by comparing eight plasmids that directed the synthesis of different FHV 2 transcripts. FHV 2(26, 53) and FHV 2(26, 43) made RNAs that failed to replicate detectably (Fig. 4, lanes 4 and 5), but further shortening of the extension on either end yielded replicable RNAs (Fig. 4, lanes 6 and 11). The 5′ extension was more inhibitory than the 3′ extension (compare lanes 9 through 11 in Fig. 4) and its length, rather than its sequence, appeared to be the significant factor (compare lanes 7 and 8 in Fig. 4).

5′-end mapping by primer extension. The 5′ ends of RNA 2 transcripts expressed from different plasmids were mapped by primer extension and compared with the 5′ ends of their positive-strand replication products and authentic FHV RNA 2. RNAs were extracted from VVT7-infected, plasmid-transfected cells after a 24-h incubation in the absence or presence of RNA replicase that was supplied, as before, by cotransfection with authentic FHV RNA 1. A primer that annealed to nucleotides 99 to 81 of RNA 2 was extended to the 5′ end of the RNA by reverse transcription, and the products were analyzed by electrophoresis on a 6% sequencing gel. A dideoxynucleotide sequencing ladder of FHV 2(2, 12) DNA with the same primer was run alongside the extension products for comparison.

FHV RNA 2 extracted from purified virions yielded a single primer extension product that was one residue longer than expected from the known 5′ sequence: GUAAA. . . (Fig. 5, lane 13). This was due to reverse transcriptase incorporating a single deoxynucleotide residue in response

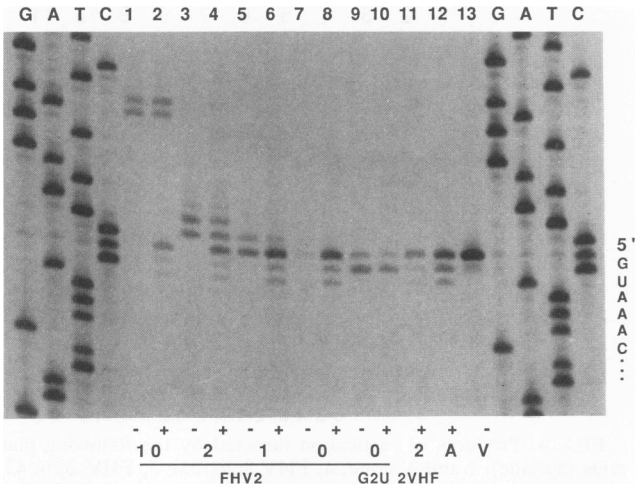

FIG. 5. Primer extension analysis of the products of transcription and replication directed by the following plasmids (by lane): 1 and 2, FHV 2(10, 12); 3 and 4, FHV 2(2, 12); 5 and 6, FHV 2(1, 12); 7 and 8, FHV 2(0, 12); 9 and 10, FHV 2(0, 12)U2G; 11, 2 VHF(2, 12); 12, RNA extracted from cells transfected with FHV RNAs 1 and 2 was used as the template for primer extension; 13, RNA extracted from purified FHV virions was used as the template for primer extension. BHK 21 cells were infected with VVT7 and transfected with the indicated plasmids in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, 10, and 11) of purified authentic FHV RNA 1. Twenty-four hours after transfection, cytoplasmic RNAs were extracted and used as templates for extension by reverse transcriptase of a primer that annealed to nucleotides 99 to 81 of FHV RNA 2. The primer extension products were labeled by incorporation of [α-^{35}S]dATP, resolved by electrophoresis on a 6% sequencing gel, and visualized by autoradiography. The same primer was used to produce a dideoxy sequencing ladder of plasmid FHV 2(2, 12), and those samples were run in lanes G, A, T, and C. The sequence in this ladder that corresponds to the 5′ end of the FHV 2 cDNA is indicated to the right of the figure.

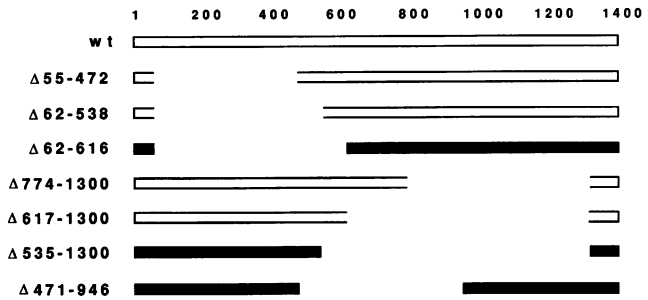

FIG. 6. Schematic diagram of the effects of internal deletions (Δ) on the replication ability of RNA 2. Internal segments of the FHV 2 cDNA sequence were deleted from plasmid FHV 2(2, 12), and the corresponding deleted RNAs were examined for their ability to replicate as described in the legend to Fig. 4. Open bars indicate RNAs that replicated; solid bars indicate RNAs that failed to replicate. wt, wild type.

to the cap at the 5′ end of the RNA template (10). FHV RNA 2 from which the cap had been removed by incubation with tobacco acid pyrophosphatase now directed the synthesis of a primer extension product that was one residue shorter and corresponded exactly to the initiating residue of RNA 2 (data not shown). RNA from cells that were replicating authentic RNA 2 gave three primer extension products that differed in length by 1 nucleotide (Fig. 5, lane 12). The largest corresponded to full-length capped RNA; the next, which was less intense, may have been derived from uncapped RNA; and the smallest and least intense apparently corresponded to RNA that was incomplete by 1 nucleotide. These three primer extension products were characteristic of actively replicating RNA 2.

Primer extension on plasmid-derived transcripts usually yielded two products that differed in length by 1 nucleotide (Fig. 5, lanes 1, 3, 5, and 9). The smaller of each pair of products corresponded to RNAs initiated by recognition of the first C residue in the template strand of the T7 promoter site: 3′-. . .GTGATATCC. . .-5′. By comparison with the products of primer extensions performed on RNA made in vitro, the larger products from in vivo RNA probably corresponded to mixtures derived from uncapped RNAs that initiated at the T residue preceding the major site of initiation and capped versions of the major starts. However, transcripts from plasmid FHV 2(0, 12) directed synthesis of only the larger of the usual pair of products (Fig. 5, lane 7). It is not clear whether this was due to unusually efficient capping of the RNA transcribed from this plasmid or to suppression of transcriptional initiation at the C residue by its unfavorable context. In either case, the small amount of primer extension product from this construct confirmed its low transcriptional activity shown in Fig. 3, lane 4.

When FHV replicase was provided by cotransfection of authentic RNA 1, RNA replication occurred and three additional 5′ ends were detected (Fig. 5, lanes 2, 4, 6, and 8). The primer extension products from replicated transcripts were indistinguishable from those derived from authentic replicating RNA 2 (compare lanes 8 and 12 of Fig. 5), indicating that correction of the 5′-terminal extensions occurred during RNA replication. The absence of primer extension products with lengths intermediate between those of transcripts and those of replication products suggested that correction occurred as a single-step process rather than by terminal trimming. This is particularly clear from lanes 1 and 2 of Fig. 5. Apparently, the FHV replicase accurately selected its correct initiation site for positive-strand synthesis from within a longer negative-strand template, because only the authentic positive-strand 5′ end(s) could be detected among the replication products of a negative-sense transcript that had a 3′-terminal extension of 12 nucleotides (Fig. 5, lane 11).

The relative intensities of the primer extension products from RNA derived from different plasmids confirmed the conclusions concerning the replication efficiency of different transcripts that were drawn from the results presented in Fig. 3. The high efficiency of replication of transcripts of FHV 2(0, 12) was particularly clear (Fig. 5, lanes 7 and 8) and contrasted with the absence of replication of transcripts of FHV 2(0, 12)U2G (Fig. 5, lanes 9 and 10).

Replication of transcripts of cDNA deletions. Elimination of either terminus of the FHV 2 cDNA yielded transcripts that failed to replicate (data not shown). However, large internal regions of the cDNA could be deleted without a marked effect on RNA replication. The results are summarized in Fig. 6. RNA molecules lacking nucleotides 55 through 472 or 62 through 538 replicated well, but those lacking nucleotides 62 through 616 were severely inhibited. Similarly, RNAs lacking nucleotides 774 through 1300 or 617 through 1300 replicated as efficiently as full-length RNA 2, whereas a molecule lacking nucleotides 535 through 1300 failed to replicate. Maintenance or disruption of the open reading frame for protein α did not affect the replication ability of the

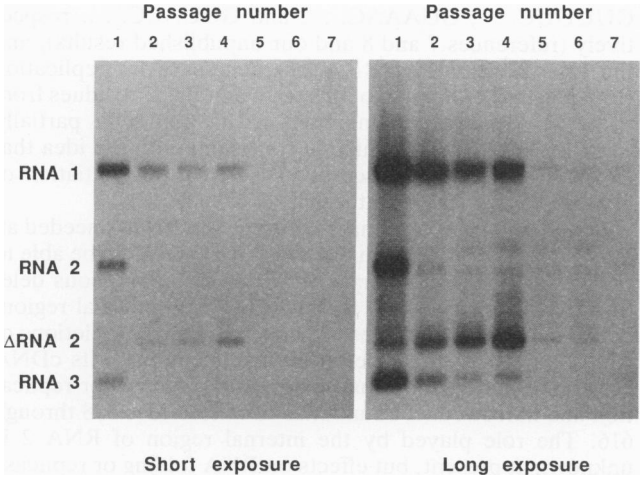

FIG. 7. Spontaneous deletion of FHV RNA 2 during multiple replicative passages. In passage 1, *D. melanogaster* cells were transfected with virion-derived FHV RNAs at a high RNA concentration, incubated for 12 h, labeled by a 2-h incorporation of [^{3}H]uridine in the presence of actinomycin D, and used as the source of cytoplasmic RNAs for transfection into fresh *D. melanogaster* cells (passage 2) as described in the text. Seven such sequential passages were performed before the labeled RNAs from each passage were resolved by electrophoresis on a 1% agarose–formaldehyde gel and visualized by fluorography. Two fluorographic exposures of the same gel are shown, that on the right being 10 times as long as that on the left. ΔRNA 2, RNA 2 with internal deletions.

deleted RNA 2 molecules. These results identified an internal region, comprising nucleotides 538 through 616 of RNA 2, that was important for replication. In accord with this, a molecule lacking nucleotides 471 through 946 failed to replicate. From the results shown in Fig. 6, it appears that the required terminal sequences, which probably include signals involved in the initiation and termination of positive- and negative-strand synthesis, are contained within the first 54 and last 100 nucleotides of the positive strand, but these signals remain to be mapped more precisely.

Spontaneous deletions of RNA 2. To examine further the requirement for an internal region for replication of RNA 2, we subjected FHV RNA to sequential replicative passages in cultured *D. melanogaster* cells under conditions of high RNA concentration. Initially, RNA from purified virions was transfected into *D. melanogaster* cells at a concentration that was equivalent to a multiplicity of infection of 10^5 virus particles per cell. After a 14-h incubation at 28°C, cytoplasmic RNA was extracted with phenol-chloroform and 10% of the yield was transfected into fresh cells for a second round of replication. These conditions of transfection at high input RNA concentration and RNA recovery by cytoplasmic extraction were designed to promote the generation, amplification, and recovery of replication-competent deleted RNA 2 molecules. Seven sequential passages were performed, the RNA from each being labeled by incorporation of [^{3}H]uridine in the presence of 5 μg of actinomycin D per ml for 2 h immediately before harvest. The RNAs were resolved by electrophoresis on an agarose-formaldehyde gel and were visualized by fluorography (Fig. 7). After the first passage, RNA 2 essentially disappeared and was replaced by an RNA of about 630 nucleotides which accumulated up to the fourth passage. Thereafter, total RNA replication decreased sharply, although a long exposure of the gel showed that the overall pattern of RNA products in passages 5 and 6 resembled that in passages 2 through 4 (Fig. 7, right panel). By passage 7, a pattern of RNAs resembling authentic FHV RNAs 1, 2, and 3 was reestablished. A repeat of these sequential replicative passages under the same conditions gave closely similar results, including the appearance of the 630-nucleotide RNA in passages 1 through 4 and the abrupt decrease in RNA replication in passage 5 (data not shown).

Northern (RNA) blot analysis of the RNAs from these sequential passages showed that the 630-nucleotide RNA was related to FHV RNA 2 (data not shown), so RNAs from passages 1 and 4 were used as templates for amplification by reverse transcription-PCR with primers specific for the termini of RNA 2. The products were cloned and sequenced to elucidate the structures of the RNA 2-related molecules that arose during the sequential passages.

Four of the eight unique clones analyzed from passage 1 RNA contained single deletions of about 500 nucleotides in the 3′ half of the RNA 2 molecule (Fig. 8). The other four contained double deletions: one of about 270 nucleotides in the 5′ half of the molecule in addition to the larger deletion in the 3′ half. Twenty-eight clones from RNA of passage 4 of the first or second series of sequential passages were analyzed, and all contained both the 5′ and the 3′ deletions (Fig. 8). Although the boundaries of the deletions were similar among these molecules, they were seldom identical for both deletions, indicating that most of the double-deletion clones

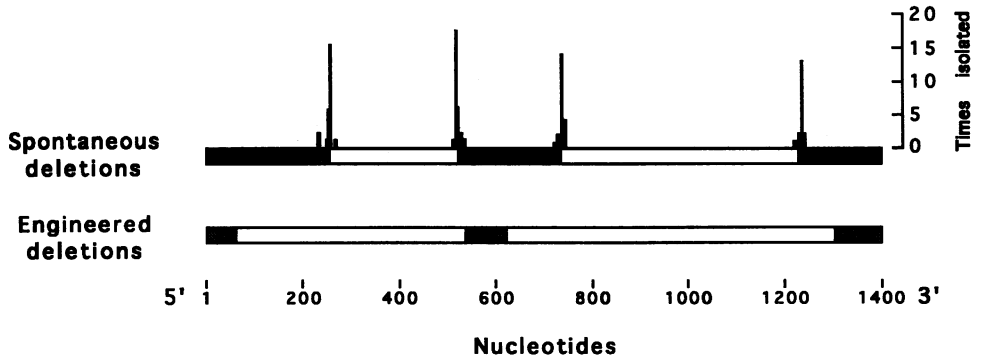

FIG. 8. Schematic representation of the boundaries of spontaneous deletions that were found in RNA 2 after sequential replicative passages. The boundaries of the essential internal region that was identified from the cDNA deletions shown in Fig. 6 are indicated on the lower bar for comparison. Nucleotides that can be deleted are shown as open regions.

were derived from unique RNA molecules of about 630 nucleotides in length. We infer that these deletions arose from separate spontaneous molecular events in which the RNA replicase probably jumped along the template. Analysis of the junction sequences revealed aspects of the mechanism of this process which are discussed elsewhere (20).

The most striking aspect of these results was that each of the spontaneously deleted molecules retained an internal region of RNA 2 between about nucleotides 525 and 725, a region that included the part of the molecule shown by the engineered deletions to be important for replication. Moreover, Zhong et al. (28) recently reported the independent isolation and characterization of an FHV RNA 2 double deletion that retained nucleotides 517 through 728.

DISCUSSION

The experimental approach that we developed to examine RNA replication, which is presented schematically in Fig. 1, works for both positive- and negative-strand RNA viruses (1, 22). It has some distinct advantages over the more conventional approach of synthesizing RNA in vitro by runoff transcription and transfecting it into cells (9). Plasmid DNAs are more readily available in abundant amounts and are considerably more stable than their RNA transcripts. In VVT7-infected BHK 21 cells incubated at 28°C, transfected plasmids are transcribed continuously for at least 72 h, which provides not only a large window for experimental analysis but also an extended opportunity for replication of RNA templates with borderline activity (for example, see Fig. 4, lanes 6 through 8).

Analysis of the 5′ ends of replicating RNA 2 positive strands that were derived from different transcripts showed that 5′ extensions of the original transcripts were corrected during replication, apparently by the replicase selecting its correct initiation site for positive-strand synthesis despite a 3′ extension on the negative strand (Fig. 5). Correction of 5′ extensions was also seen by Dasmahapatra et al. (9) in RNA from infectious FHV that was recovered from cDNA transcripts. In view of this, it was surprising that different primary transcripts showed such marked and persistent differences in template activity, because correction of the termini would have generated identical RNAs (Fig. 4). The explanation appeared to be that transcripts with extensions at the 5′ end could inhibit replication of authentic RNA in *trans*. Cells transfected with plasmid FHV 2(26, 12), for example, contained transcription products that were much more abundant than the replication products and which inhibited their replication (data not shown). Conversely, plasmid FHV 2(0, 12) transcribed poorly but produced RNA that was perfect or almost perfect at its 5′ end, and in this case, replication of RNA 2 far exceeded its transcription (Fig. 3 and 5).

The 3′-terminal extensions of the positive-strand transcripts inhibited replication less than the 5′ extensions (Fig. 4), which was consistent with the replicase being able to select its correct initiation site from within a longer RNA. While we have not proved that these 3′ extensions were corrected during replication, this is strongly suggested by the demonstration that an RNA 2 negative strand with a 12-nucleotide 3′ extension directed synthesis of a correctly initiated positive strand (Fig. 5, lane 11). However, these results imply that the inhibitory effect of 5′ extensions must be exerted at a step other than the initiation of replication of the complementary strand.

The 5′-terminal sequences of FHV RNAs 1, 2, and 3 are GUUUUC. . ., GUAAAC. . ., and GUUACC. . ., respectively (references 7 and 8 and our unpublished results), and the U at position 2 of RNA 2 is necessary for replication (Fig. 3, lane 9). Deletion of one or two of the U residues from the RNA 1 sequence inhibited replication only partially (unpublished results), which is consistent with the idea that an initial GU. . . sequence plays a role in the initiation of positive-strand RNA synthesis.

In addition to its terminal requirements, RNA 2 needed an internal region between nucleotides 538 and 616 to be able to replicate efficiently. Every one of the 36 spontaneous deletions that were analyzed retained the same internal region, between about nucleotides 525 and 725 (Fig. 8). Deletions of RNA 2 that were engineered by manipulation of its cDNA confirmed that this internal region was required for replication and narrowed its boundaries to nucleotides 538 through 616. The role played by the internal region of RNA 2 is unknown at present, but effects on RNA folding or replicase binding can be imagined, among other possibilities. Moreover, Dasmahapatra et al. (8) noticed that this region of RNA 2 contains a sequence of 10 nucleotides (555 to 564) that are complementary to a region of the negative strand of RNA 1 just upstream of the start site for RNA 3. Further experiments will be necessary to determine whether this 10-nucleotide region is the critical sequence element and whether it is responsible for RNA 1-RNA 2 interaction.

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