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Requirements for the Self-Directed Replication of Flock House Virus RNA 1

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The larger segment (RNA 1) of the bipartite, positive-sense RNA genome of the nodavirus flock house virus encodes the viral RNA-dependent RNA polymerase. Two nonstructural viral proteins are made during the self-directed replication of this RNA: protein A (110 kDa), the translation product of RNA 1 itself, and protein B (11 kDa), the translation product of a subgenomic RNA (RNA 3) that is produced from RNA 1 during replication. To examine the roles of these proteins in RNA replication, specialized T7 transcription plasmids that contained wild-type or mutant copies of flock house virus RNA 1 cDNA were constructed and used in cells infected with the vaccinia virus-T7 RNA polymerase recombinant to make full-length transcripts that directed their own replication. Sequences in the primary transcripts that extended beyond the ends of the authentic RNA 1 sequence inhibited self-directed RNA replication, but plasmids that were constructed to minimize these terminal extensions produced primary transcripts that replicated as abundantly as authentic RNA 1. Truncation or mutation of the open reading frame for protein A eliminated self-directed replication, although the mutant RNA 1 remained a competent template for replication by wild-type protein A supplied in *trans*. These results showed that protein A was essential for RNA replication and that the process was not inseparably coupled to complete translation of the template. In contrast, protein B could be eliminated without inhibiting replication by mutations that disrupted the second of the two overlapping open reading frames on RNA 3. Furthermore, a mutant of RNA 1 in which the first nucleotide of the RNA 3 region was changed from G to U replicated at levels as high as those of the wild type without making either RNA 3 or protein B. However, diminishing replication levels were observed during subsequent replicative passages of RNA from both the mutants that could not make protein B. Roles for this protein that could account for the subtle phenotype of these mutants are discussed.

Flock house virus (FHV) is the best-studied member of the *Nodaviridae*, a family of small spherical riboviruses with bipartite positive-sense RNA genomes (24). The larger segment of the FHV genome (RNA 1; 3,107 nucleotides) replicates autonomously to high levels when transfected alone into cultured *Drosophila* cells (20) or into the cells of a variety of other species (3), indicating that RNA 1 encodes the entire viral contribution to the RNA-dependent RNA polymerase (RNA replicase) that replicates the viral genome. In a natural situation, the RNA replicase is highly template specific and replicates only FHV RNA 1 and the smaller segment of the viral genome (RNA 2; 1,400 nucleotides). RNA 2 encodes a precursor (α) to the viral capsid proteins (β and γ), which assemble with T=3 icosahedral symmetry around the two genomic RNA segments in the mature FHV particle (9, 15, 16, 33). Both RNAs carry cap zero structures at their 5′ ends, and their 3′ ends, which do not have poly(A), are blocked either by an undetermined covalent modification or by the steric hindrance of RNA secondary structure. The apparent simplicity of this RNA replication system, its compatibility with a wide range of intracellular environments, and the great abundance of its RNA and protein products combine to make it attractive both for studies of the basic mechanism of RNA replication and for harnessing to the replication of heterologous RNAs (5).

The nucleotide sequence of FHV RNA 1 (10) reveals a single long open reading frame (orf) of 998 codons whose translation product, protein A, has been detected both in cell-free systems (18) and in transfected cells (20). Since the deduced amino acid sequence of protein A contains a clear polymerase motif (7), including the characteristic sequence Gly-Asp-Asp at amino acid positions 691 to 693, it seems most likely that protein A is the catalytic subunit of the RNA replicase. Since the enzyme has not yet been purified to homogeneity, the involvement of other polypeptide subunits is unknown. During replication of RNA 1, however, a small subgenomic RNA (RNA 3) that is derived from the 3′ end of RNA 1 is also made (21). The first AUG codon in RNA 3 is in the same reading frame as protein A and could therefore initiate synthesis of a 102-residue C-terminal fragment (protein B1). Whether this protein is produced remains to be determined. The second AUG codon in RNA 3 is in the +1 reading frame with respect to protein A, lies in a somewhat more favorable context for initiation (25), and could start the synthesis of a 106-residue protein (B2) whose reading frame overlaps the C terminus of protein A almost entirely. Both in infected cells (16) and in cell-free systems (17), RNA 3 directs the synthesis of one major protein (B) of about 11 kDa, which unpublished studies indicate to be the product of the B2 orf (23). Although the function of the B2 protein is unknown and its deduced amino acid sequence offers no clues, the fact that, like protein A, it is synthesized predominantly early in infection and is not incorporated into mature virions suggests that it probably functions at an early step in the infectious cycle.

The recombinant vaccinia virus (VV) that expresses T7 RNA polymerase (19) was used to achieve intracytoplasmic transcription of full-length cDNA clones (13) of FHV RNAs 1 and 2 (1, 4). Synthesis of transcripts with discrete termini from

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circular DNA templates was accomplished by appropriate positioning of a T7 promoter site at the 5′ end of the FHV cDNA, and by inserting a cDNA that encodes an autolytic ribozyme after it, so that the 3′ end of the transcript was generated by self-cleavage. This approach was successful for the production of both positive-sense (4) and negative-sense (2) transcripts of RNA 2 that could be replicated efficiently, and it has facilitated studies of the *cis*-acting signals required for RNA 2 replication. Moreover, it was observed that an RNA 1 transcript made in this system combined the properties of a functional message with those of a functional template and, like authentic RNA 1, directed its own replication (1). Here, I report the optimization of this plasmid expression system and its use to examine some of the requirements for the autonomous replication of FHV RNA 1.

MATERIALS AND METHODS

Cells and viruses. RNA replication experiments were performed in baby hamster kidney (BHK21) cells grown as monolayers in 35-mm-diameter wells of six-well plates as described before (1). The VV recombinant that expressed T7 RNA polymerase (vTF7-3 [19], generously provided by Bernard Moss, National Institutes of Health) was grown and titers were determined by plaque assay on monolayers of BSC40 cells. FHV was grown and titers were determined by plaque assay on monolayers of *Drosophila* cells (33) from a stock kindly provided by Tom Gallagher and Roland Rueckert (University of Wisconsin—Madison).

Plasmid construction, sequencing, and mutagenesis. Two full-length cDNA clones of FHV RNA 1 were generously provided by Ranjit Dasgupta and Paul Kaesberg (University of Wisconsin—Madison) (13). While both clones yielded RNA transcripts that self-replicated with equal efficiency, they differed in that one clone (pKS2) had the 5′ sequence 5′ GTTTTGAA, whereas the other (p1B9) had the 5′ sequence 5′ GTTTTCGAA. Both sequences are found in preparations of FHV RNA 1, although molecules with the latter sequence are more abundant (11). Most of the work reported here used plasmids derived from pKS2.

Conventional methods of DNA manipulation (32) were used for plasmid construction. Transcription plasmids that contained FHV 1 cDNA conformed to the general structure described previously (1) and were all based on pGEM3 or pGEM4 (Promega Biotech). They differed from one another in the number of nucleotides between the site of transcriptional initiation and the first nucleotide of the FHV 1 sequence, designated [N], and in the number of nucleotides between the end of the FHV 1 sequence and the site of ribozyme-mediated autolytic cleavage, designated [M]. Plasmids were designated FHV1[N,M] to indicate these differences (Table 1). Plasmids whose transcripts had extensions of 12 or 43 nucleotides at their 3′ ends contained the ribozyme from satellite tobacco ringspot virus (8); those whose transcripts had no additional nucleotides or lacked 5 nucleotides from the 3′ end contained the ribozyme from the antigenomic strand of hepatitis delta virus (30). Primer extension mapping of the 5′ ends of the downstream cleavage products (4) confirmed that RNA self-cleavage occurred at the positions predicted from the properties of the ribozymes (Table 1). Nucleotide sequences across the 5′ and 3′ junctions of the FHV 1 cDNA inserts were determined by the dideoxynucleotide chain termination method, with denatured plasmid DNA as the template (22), and the critical regions of these junction sequences are shown in Table 1.

Oligonucleotide-directed site-specific mutagenesis was performed with the pALTER system (Promega Biotech). Mutated DNA fragments of minimal convenient size were religated into wild-type clones that had not been subjected to mutagenesis, and the nucleotide sequence of the entire mutated fragment was determined in each case.

Infection and transfection of cells. BHK21 cells were infected with vTF7-3 at a multiplicity of infection of 10 PFU per cell. After virus adsorption at room temperature for 60 min, the inoculum was removed, the cells were washed once with phosphate-buffered saline, and Dulbecco's modified Eagle's medium (1 ml/35-mm-diameter well) was applied. The infected cells were incubated for 15 to 30 min at 28°C in an atmosphere of 5% CO_2 before being transfected with a mixture of DNA (5 μg in 10 μl) and Lipofectin (Gibco-Bethesda Research Laboratories; 10 μg in 10 μl). Incubation at 28°C was continued for 24 h before the cells were metabolically labeled for RNA or protein synthesis. The low incubation temperature was necessary because FHV RNA replicase loses activity at temperatures above 31°C (1).

RNA labeling, extraction, and analysis. The products of RNA replication were specifically labeled by metabolic incorporation of [^{3}H]uridine (20 μCi/ml) in the presence of actinomycin D (10 μg/ml) as described before (1). After 2 h of incorporation at 28°C, the cells were lysed and the RNAs were extracted and precipitated as described earlier (1). The relative levels of self-directed RNA replication were quantitated by scintillation spectrometry of the radioactivity in duplicate samples of acid-precipitable ^{3}H-labeled RNA, which measured the combined levels of RNAs 1 and 3. Under the conditions described, the observed levels of RNA replication were independent of the amount of RNA or plasmid DNA used for transfection. Labeled RNAs were resolved by electrophoresis on 1% agarose-formaldehyde gels (27) and visualized by fluorography (26). For RNA (Northern) blot analysis, the resolved RNAs were transferred to GeneScreen Plus (Dupont-NEN) and probed by hybridization with ^{35}S-labeled RNA 1 of negative sense made by run-off transcription in vitro (1).

Protein labeling, extraction, and analysis. The products of protein synthesis were labeled by metabolic incorporation of [^{35}S]methionine/cysteine (Tran^{35}S label; ICN; 50 μCi/ml) for 2 h in methionine-free Dulbecco's modified Eagle's medium. Radioactively labeled proteins were extracted, analyzed by electrophoresis on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels, and visualized by autoradiography as described before (1).

TABLE 1. Junction sequences of plasmids and self-directed replication of T7 transcripts

Plasmid[a]	5′ Junction[b]	3′ Junction[b]	RNA replication (%)[c]
FHV1[26,43]	↓ CACTATAGGGAGACCCAAGCTTGCATGCCTGCA**GTTTTGA**	**CAGAGGT**CTAGAGGATCCCCGGGTACC GAGCTCGAATTCGATACCCTGTT*ACC	0.0
FHV1[26,12]	As FHV1[26,43]	**CAGAGGT**CGATACCCTGTT*ACC	<1.0
FHV1[10,12]	↓ CACTATAGGGAGACCCA**GTTTTGA**	As FHV1[26,12]	1.0
FHV1[2,12]	↓ CACTATAGG**GTTTTGA**	As FHV1[26,12]	11.3
FHV1[1,12]	↓ CACTATAG**GTTTTGA**	As FHV1[26,12]	54.1
FHV1[0,12]	CACTATA**GTTTTGA**	As FHV1[26,12]	0.0
FHV1[1,12]Δ1	↓ CACTATAG**GTTTGA**	As FHV1[26,12]	45.1
FHV1[1,12]Δ2	↓ CACTATAG**GTTGA**	As FHV1[26,12]	21.1
FHV1[1,0]	As FHV1[1,12]	**CAGAGGT***GGGTCGG	96.9
FHV1[0,0]	As FHV1[0,12]	As FHV1[1,0]	0.0
FHV1[1,−5]	As FHV1[1,12]	**GGGCA***GGGTCGG	0.0

[a] The numbers in square brackets in the plasmid names indicate the numbers of nucleotides in the 5′ and 3′ extensions on the primary transcripts.

[b] Nucleotide sequences across the 5′ and 3′ junctions of the FHV 1 cDNA insert are shown, with the FHV-specific nucleotides in boldface letters. T7 promoter sequences are underlined, and the major sites of transcriptional initiation are indicated by vertical arrows. Asterisks indicate the positions that correspond to the sites of ribozyme-mediated cleavage of the RNA transcripts.

[c] RNA replication was measured by [^{3}H]uridine incorporation in the presence of actinomycin D and is expressed relative to that observed in cells transfected with authentic FHV RNA 1 (100%). The values shown are the averages of several independent experiments.

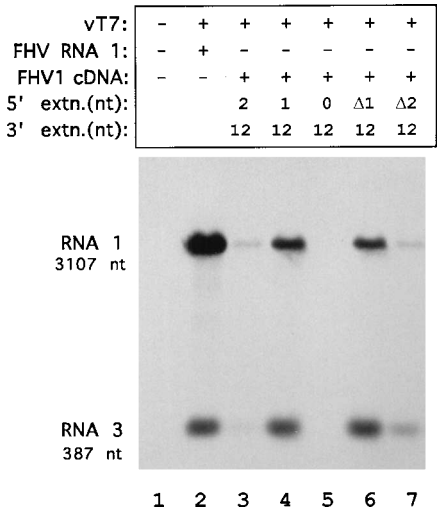


FIG. 1. Products of RNA replication directed by plasmids containing FHV 1 cDNA. BHK21 cells were left uninfected (lane 1) or infected with vTF7-3 (lanes 2 to 7) and transfected with authentic FHV RNA 1 (lane 2) or with the following plasmids (by lane): FHV1[2,12] (lane 3), FHV1[1,12] (lane 4), FHV1[0,12] (lane 5), FHV1[1,12]Δ1 (lane 6), and FHV1[1,12]Δ2 (lane 7). At 24 h post-transfection, the products of RNA replication were labeled by 2 h of 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. extn., extension; nt, nucleotide.

RNA 5′ end mapping by primer extension. The 5′ ends of FHV RNAs 1 and 3 were mapped by extension of oligonucleotide primers that annealed to nucleotides 99 to 80 and 2,819 to 2,801 in the FHV RNA 1 sequence, respectively (10). Primer extension products were labeled by incorporation of [α-^{35}S]dATP and analyzed by electrophoresis on 6% sequencing gels alongside dideoxynucleotide sequence ladders generated by using the same primers and an appropriate DNA template.

RESULTS

Replication of transcripts with terminal extensions. In previous work, colleagues and I demonstrated that transcripts generated by T7 RNA polymerase from the plasmid FHV1[2,12], which have 2 extra 5′ nucleotides and 12 extra 3′ nucleotides beyond the authentic FHV 1 sequence, underwent both translation to yield functional RNA replicase and consequent self-directed RNA replication (1). However, the onset of replication was delayed by several hours and its level was reduced about 10-fold in comparison with that of authentic FHV RNA 1. Since RNA replication was autocatalytic and therefore occurred with initial exponential kinetics (data not shown), the efficiencies of plasmid-directed synthesis and cleavage of the primary transcript were not rate limiting for its subsequent self-directed replication (except in one extreme case described below). Instead, the level of RNA replication reflected the intrinsic message and template properties of the cleaved primary transcript. Since terminal extensions profoundly affect the efficiency of replication of transcripts of the smaller FHV genome segment, RNA 2 (2, 4), we constructed a series of FHV 1 transcription plasmids that were designed to make RNAs with different lengths of terminal extensions. The sequences that specified the 5′ and 3′ ends of the RNA 1 transcripts from these plasmids are shown in Table 1. Plasmids were transfected into BHK cells that expressed T7 RNA polymerase from the VV recombinant vTF7-3, and the levels of self-directed RNA replication of their transcripts were measured by [^{3}H]uridine incorporation in the presence of actinomycin D to inhibit all DNA-dependent RNA synthesis (Table 1). As seen with transcripts of RNA 2, the long terminal extensions on the transcripts from plasmid FHV1[26,34] prevented detectable RNA replication, although synthesis of protein A was unimpaired (see the description below and Fig. 6, lane 4). Also reminiscently of RNA 2, extensions at the 5′ end were observed to be more detrimental to replication than those at the 3′ end. Thus, shortening of the 5′ extension from 10 to 2 to 1 nucleotide increased the level of self-replication from 1 to 11 to 54% of that observed with authentic RNA 1. Removal of the 12-nucleotide 3′ extension (with plasmid FHV1[1,0]) resulted in a further twofold increase in replication, up to a level that was equivalent to that of authentic RNA 1 (Table 1).

The RNA products of self-replication of some of the plasmid-derived transcripts were examined by electrophoresis on agarose-formaldehyde gels and found to be similar, both in size and in the relative abundances of RNAs 1 and 3, from the products of replication of authentic RNA 1 (Fig. 1, lanes 2 to 4). The recovery of cDNA transcripts that could direct self-replication with an efficiency and fidelity comparable to that of authentic RNA 1 made possible the genetic studies reported in this paper.

Plasmids designed to transcribe RNA 1 molecules that had no extra nucleotides at their 5′ ends (FHV1[0,12] and FHV1[0,0]) consistently failed to exhibit detectable RNA replication (Table 1 and Fig. 1, lane 5). The reason for this failure was that the T7 promoter site in this sequence context was inactive, probably because the four U residues at positions 2 to 5 of FHV RNA 1 caused the polymerase to stutter, as observed by Ling et al. (28). Even with purified T7 RNA polymerase in vitro, plasmid FHV1[0,12] failed to yield an RNA transcript that was detectable either by gel electrophoresis (data not shown) or by primer extension mapping of its 5′ end (Fig. 2, lane 5). In contrast, plasmids FHV1[26,12], FHV1[10,12], FHV1[2,12], and FHV1[1,12] (including Δ1 and Δ2) all directed the in vitro synthesis of T7 transcripts whose predominant 5′ ends mapped to the expected sites of transcriptional initiation (Fig. 2, lanes 1 to 4, and 6 and 7). A lack of primary transcription by T7 RNA polymerase similar to that of FHV1[0,0] was seen with plasmids designed to express the negative strand of RNA 2 with no additional 5′ nucleotides (2), although the positive-sense RNA 2 plasmid FHV2[0,0] was transcribed successfully (5).

Replication of transcripts with terminal deletions. Deletion of 5 nucleotides from the 3′ end of the RNA 1 transcript (expressed from FHV1[1,−5]) prevented RNA replication (Table 1), suggesting that an intact RNA 3′ end was required, probably for the correct initiation of negative-strand synthesis. However, deletion of one or two of the four U residues near the 5′ end (in the RNAs expressed from FHV1[1,12]Δ1 and FHV1[1,12]Δ2) inhibited RNA replication only partially (Table 1 and Fig. 1, lanes 6 and 7). Interestingly, the synthesis of RNA 3 during replication of these transcripts was inhibited concomitantly with that of RNA 1. In the case of RNA 2, the major *cis*-acting signal at the 5′ end of the positive strand is contained within the first 3 nucleotides, GUA (5a). Since the RNAs transcribed from FHV1[1,12], FHV1[1,12]Δ1, and FHV1[1,12]Δ2 would all have the same sequence for at least 4 nucleotides (GGUU), the observed levels of replication are consistent with a similar situation existing for RNA 1. Notice also that the presence of an additional C residue at position 6 in RNA 1 had no detectable effect on the efficiency of RNA replication (see Materials and Methods).

Mapping the 5′ end of RNA 3. In order to examine the structure and synthesis of RNA 3 in greater detail, its 5′ end was mapped by primer extension. An oligonucleotide primer that was complementary to nucleotides 2819 to 2801 of FHV

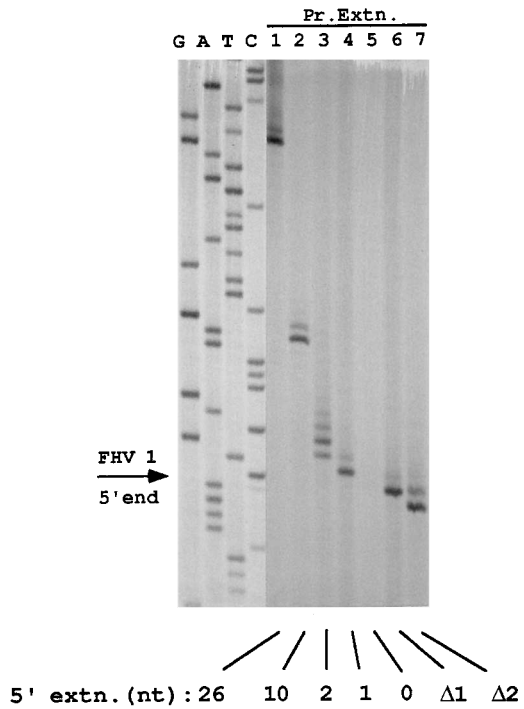


FIG. 2. Primer extension (Pr. Extn.) analysis of transcription products from plasmids. Lanes: 1, FHV1[26,43]; 2, FHV1[10,12]; 3, FHV1[2,12]; 4, FHV1 [1,12]; 5, FHV1[0,12]; 6, FHV1[1,12]Δ1; 7, FHV1[1,12]Δ2. RNAs synthesized in vitro by the action of T7 polymerase on the indicated plasmids were used as templates for extension by reverse transcriptase of an oligonucleotide primer that annealed to nucleotides (nt) 99 to 80 of FHV RNA 1. 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. Lanes G, A, T, and C show a dideoxynucleotide sequencing ladder produced by using the same primer and a plasmid (pKS2) which contained a full-length cDNA clone of FHV RNA 1. The position that corresponds to the 5′ nucleotide of the FHV 1 sequence is indicated to the left of the lanes (arrow).

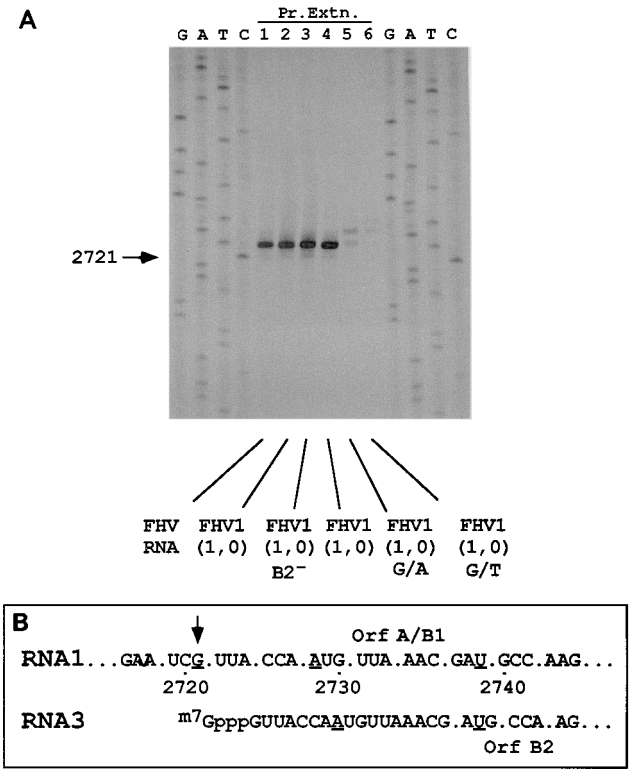


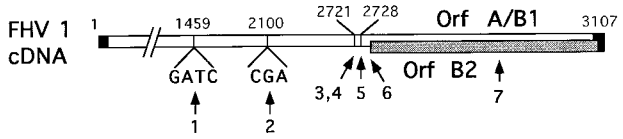
FIG. 3. (A) Primer extension (Pr. Extn.) analysis of the 5′ end of RNA 3 from cells infected with vTF7-3 and transfected with RNAs or plasmids. Lanes: 1, FHV RNAs (1 plus 2); 2 and 4, wild-type plasmid FHV1[1,0]; 3, plasmid FHV1[1,0] with mutations 6 and 7; 5, plasmid FHV1[1,0] with mutation 3; 6, plasmid FHV1[1,0] with mutation 4. Cytoplasmic RNAs were extracted at 24 h posttransfection and used as templates for extension by reverse transcriptase of an oligonucleotide primer that annealed to nucleotides 2819 to 2801 in FHV RNA 1. 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. Lanes G, A, T, and C show a dideoxynucleotide sequencing ladder produced by using the same primer and a plasmid that contained nucleotides 2721 (indicated by the arrow to the left of the lanes) to 3107 of the cDNA clone of RNA 1. (B) Part of the nucleotide sequence of RNA 1 around the start site of RNA 3 (indicated by the vertical arrow). orfs A and B1 are indicated on the upper (RNA 1) sequence, and orf B2 is indicated on the lower (RNA 3) sequence. Some of the nucleotides that were targeted for mutagenesis are underlined: G2721 (mutations 3 and 4); A2728 (mutation 5); and U2739 (mutation 6).

RNA 1 was annealed to RNA extracted from cells that were replicating authentic RNA 1. The primer was extended by reaction with reverse transcriptase in the presence of [α-^{35}S] dATP, and the labeled products were resolved by electrophoresis on a 6% polyacrylamide sequencing gel (Fig. 3, lane 1). For comparison, adjacent lanes contained a dideoxynucleotide sequencing ladder generated with the same primer on a plasmid containing nucleotides 2721 to 3107 of the FHV 1 sequence downstream of a T7 promoter site (Fig. 3, lanes G, A, T, and C).

A single major product of primer extension corresponding to a 5′ end at nucleotide 2720 of RNA 1 was observed. Since, under these reaction conditions, reverse transcriptase incorporated a cytidylate residue in response to the guanylate residue of the cap (5a, 14), this result indicated that the uncapped 5′ terminus of authentic RNA 3 corresponded to nucleotide 2721 of RNA 1, i.e., 5′ GUUA (Fig. 3B). The same 5′ end was seen with RNA 3 that resulted from the self-replication of transcripts of FHV1[1,0] (Fig. 3, lanes 2 and 4). These results disagree with those of Guarino et al. (21) who, from direct sequence determination of RNA 3 of the closely related black beetle virus, concluded that the 5′ end corresponded to a position 2 nucleotides further towards the 5′ end of RNA 1, i.e., 5′ UCGUUA. It is unlikely that this reflects a difference between FHV and black beetle virus, since the RNA 1 sequences of the two viruses are 99% identical overall and 100% identical for the 3′ 569 nucleotides, which encompass the entire RNA 3 region (10, 12). More probably, the discrepancy is due to the difficulty of determining the 5′ sequence of an end-labeled RNA by the enzymatic and chemical cleavage methods (21). The Wisconsin group agrees that by primer extension, the 5′ end of FHV RNA 3 maps to nucleotide 2721 of the RNA 1 sequence (31). These results show that the three FHV RNAs share the initial dinucleotide GU and thus have the following 5′ sequences: ^{m7}GpppGUUU (RNA 1), ^{m7}Gppp GUAA (RNA 2), and ^{m7}GpppGUUA (RNA 3).

Another copy of the GUUA sequence occurs 9 nucleotides downstream of the RNA 3 start site (Fig. 3B), and initiation there would yield an RNA that contained only the B2 orf instead of both the B1 and B2 orfs. However, no evidence for an RNA species with this truncated 5′ end was found by the primer extension experiments reported here, even when the natural start site for RNA 3 was inactivated by mutation (see the description below; Fig. 3, lane 6).

Mutation of FHV 1 cDNA. To examine the roles in RNA replication of the protein products of orfs A, B1, and B2 and of RNA 3 itself, seven mutations were introduced separately into



Mutation	orf A	Effect on: orf B1	orf B2
1. 1459(GATC)1460	473(frameshift) 475(Stop)	None	None
2. 2100(CGA)2101	686(Arg)687	None	None
3. G2721A	Ser894Ser	None	None
4. G2721T	Ser894Ser	None	None
5. A2728C	Met897Leu	Met1Leu	None
6. T2739C	Asp900Asp	Asp4Asp	Met1Thr
7. C2910A	Ile957Ile	Ile61Ile	Ser58Stop

FIG. 4. Schematic representation of FHV 1 cDNA showing the positions (nucleotide numbers above the diagram) at which the seven different mutations (numbered arrows) were introduced. The open bar represents orfs A and B1, and the shaded bar represents orf B2. At the bottom of the figure, the seven mutations are specified and their effects on the three orfs, A, B1, and B2, are shown.

FHV 1 cDNA (Fig. 4) and examined for their effects on RNA replication (Fig. 5), the initiation of RNA 3 synthesis (Fig. 3), and the synthesis of proteins A and B (Fig. 6).

(i) Mutations 1 and 2. Mutations 1 and 2 disrupted protein A, either by introducing a frame shift after amino acid residue 473 which led to termination at residue 475 (mutation 1) or by inserting an arginine residue between amino acids 686 and 687 which lie in the heart of the polymerase motif (mutation 2). Transcripts of mutant 1 plasmid directed synthesis of the expected 54 kDa N-terminal fragment of protein A, but no full-length A or B proteins were observed (Fig. 6, lane 7), nor was any self-directed RNA replication noted (data not shown). Transcripts of mutant 2 plasmid directed the synthesis of full-length protein A, but the inserted arginine residue evidently rendered it catalytically inactive, because no self-directed RNA replication or synthesis of protein B was observed (data not shown). However, RNAs from both mutants 1 and 2 were

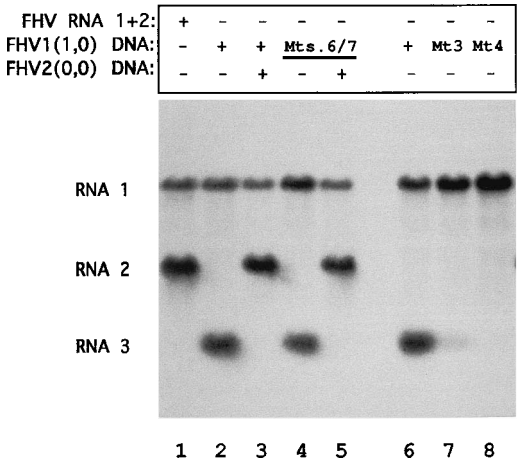


FIG. 5. Products of RNA replication directed by plasmids. Lanes: 2 and 6, FHV1[1,0]; 3, as lane 2 plus FHV2[0,0]; 4, FHV1[1,0] with mutations (mts) 6 and 7; 5, as lane 4 plus FHV2[0,0]; 7, FHV1[1,0] with mutation 3; 8, FHV1[1,0] with mutation 4. Lane 1 shows the RNA products of replication of authentic FHV RNAs 1 and 2. RNAs were labeled in the presence of actinomycin D and analyzed as described for Fig. 1.

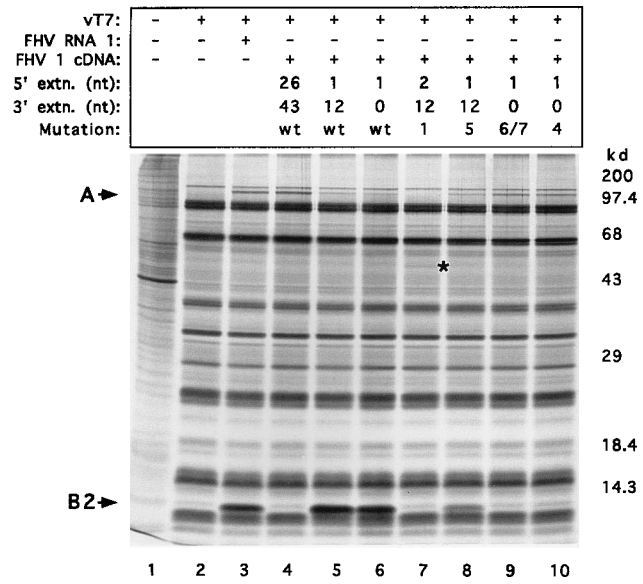


FIG. 6. Proteins produced in cells infected with vTF7-3 and transfected with RNA or plasmid. Lanes: 3, FHV RNA 1; 4, FHV1[26,43]; 5, FHV1[1,12]; 6, FHV1[1,0]; 7, FHV1[2,12] with mutation 1; 8, FHV1[1,12] with mutation 5; 9, FHV1[1,0] with mutations 6 and 7; 10, FHV1[1,0] with mutation 4. Lane 1 shows the proteins from uninfected, untransfected cells, and lane 2 shows those from vTF7-3 infected, untransfected cells. At 24 h posttransfection, BHK21 cells were labeled for 1 h by metabolic incorporation of [^{35}S]methionine/cysteine before the cytoplasmic proteins were harvested, subjected to electrophoresis on an SDS–12.5% polyacrylamide gel, and visualized by autoradiography. The migration positions of the FHV A and B proteins are shown to the left of the lanes (arrows), and those of molecular markers are shown to the right. *, 54-kDa N-terminal fragment of protein A. extn., extension; nt, nucleotide; wt, wild type.

competent templates for RNA replication and the synthesis of RNA 3 when wild-type protein A was provided from another plasmid (see Fig. 5 of reference 5 for mutant 2). These results established that functional protein A was essential for RNA replication.

(ii) Mutations 3 and 4. Point mutations 3 and 4 changed nucleotide G2721 in the FHV sequence to A and T, respectively. As shown in Fig. 3, this nucleotide corresponded to the 5′ terminus of RNA 3, and it was mutated to test the effect on subgenomic RNA synthesis. Neither mutation affected orf A, since each substituted one serine codon for another at amino acid 894. However, the effects of these mutations on RNA replication were striking: transcripts from both mutant plasmids directed the self-replication of RNA 1 at levels at least as high as that of the wild-type transcript (Fig. 5, lane 6), but the synthesis of RNA 3 was greatly reduced by mutation 3 (G2721A), and essentially eliminated by mutation 4 (G2721T) (Fig. 5, lanes 7 and 8). Northern blot analysis of the replication products of these mutants confirmed that RNA 1 accumulated to levels similar to those of the wild-type and that RNA 3 from mutant 4 (G2721T) was undetectable (data not shown).

This profound perturbation of subgenomic RNA synthesis was further examined by primer extension mapping of the 5′ end of RNA 3. In cells that were replicating RNA from mutant 3, primer extension confirmed the inhibition of authentic subgenomic RNA synthesis and revealed the presence of minor RNA species with aberrant 5′ ends 1 or 2 nucleotides longer than that of authentic RNA 3 (Fig. 3, lane 5). Trace amounts of primer extension products corresponding to these aberrant RNA 5′ ends were also detectable with mutant 4 (Fig. 3, lane 6). These results indicated that the G residue at position 2721, which represents the 5′ nucleotide of RNA 3, was essential for

normal synthesis of the subgenomic RNA during replication and that changes at this site may have caused the viral RNA polymerase to attempt subgenomic RNA synthesis from a position 1 or 2 nucleotides 5′ to the authentic start site. Moreover, these results showed that the synthesis of normal levels of RNA 3 was not required for full replication of RNA 1. In accordance with the lack of RNA 3, the synthesis of protein B was not detected in cells that were replicating RNA from mutant 4 (Fig. 6, lane 10). However, the residual levels of RNA 3 made by mutant 3 were sufficient to produce almost normal levels of protein B (data not shown).

(iii) Mutation 5. Point mutation 5 changed the first AUG codon in RNA 3 to CUG, thereby introducing the conservative substitution Met897Leu into orf A, and eliminating the potential initiation codon for protein B1. Transcripts from this mutant plasmid were fully active in self-directed RNA replication (data not shown) and synthesized substantial levels of proteins A and B (Fig. 6, lane 8). These results showed that the abundant 11 kDa B protein was not the product of the B1 orf and that the B1 protein (if it is a product of wild-type RNA) was not essential for RNA replication under these conditions.

(iv) Mutations 6 and 7. Point mutations 6 and 7 disrupted the B2 orf by eliminating the initiation codon and introducing a stop codon, respectively. Mutation 6 changed the AUG codon at the start of the B2 orf to ACGThr, whereas mutation 7 changed the codon UCA for Ser-58 in the B2 orf to UAAStop. Both mutations were silent in the protein A reading frame (Fig. 4). The two mutations were introduced together into the same FHV 1 plasmid to securely block the potential for synthesis of protein B2. Transcripts from the double-mutant plasmid directed both the self-replication of RNA 1 and the synthesis of RNA 3 at levels similar to those of transcripts of the wild-type plasmid (Fig. 5; compare lanes 2 and 4). Northern blot analysis confirmed that RNAs 1 and 3 accumulated to the same levels as in the wild-type situation (data not shown), and primer extension mapping showed that the 5′ end of RNA 3 was fully capped (Fig. 3, lane 3). Moreover, when a plasmid that expressed a positive-sense transcript of FHV RNA 2 (plasmid FHV2[0,0]) was supplied by cotransfection, the RNA replicase made in cells that received the B2$^-$ double mutant was fully active in *trans*, as judged by levels of RNA 2 replication that were similar to those of wild-type replicase (Fig. 5; compare lanes 1, 3, and 5). Protein A was produced during replication of the B2$^-$ double mutant, but protein B was undetectable (Fig. 6, lane 9). This result confirmed that the abundant 11-kDa protein that accumulated during replication of wild-type FHV RNA 1 was indeed the product of the B2 orf but showed, unexpectedly, that the B2 protein was not essential for RNA replication under these conditions.

Recovery of infectious FHV. Although FHV RNA could replicate to high levels when introduced by transfection into BHK cells, FHV itself was unable to infect such cells (3), so any infectious virus produced as a result of the coexpression of FHV 1 and FHV 2 plasmids was unable to spread through the BHK cell culture. However, it was possible to recover infectious FHV from plasmids expressed in BHK cells by amplifying and growing plaques of the progeny virus on monolayers of *Drosophila* cells, which are susceptible to infection by FHV but not by VV (data not shown). In contrast, attempts to recover infectious FHV that was carrying both mutations 6 and 7, which doubly blocked the B2 orf (Fig. 4), were unsuccessful. Infectious virus was recovered from BHK cells that coexpressed FHV2[0,0] and an FHV 1 plasmid carrying mutation 6 alone, but when its phenotype was examined, the rescued virus was found to have recovered the ability to produce protein B, presumably by reversion of mutation 6. These results strongly suggest that although it is dispensable for RNA replication per se, the B2 protein is beneficial for some other aspect of the complete infectious cycle of FHV.

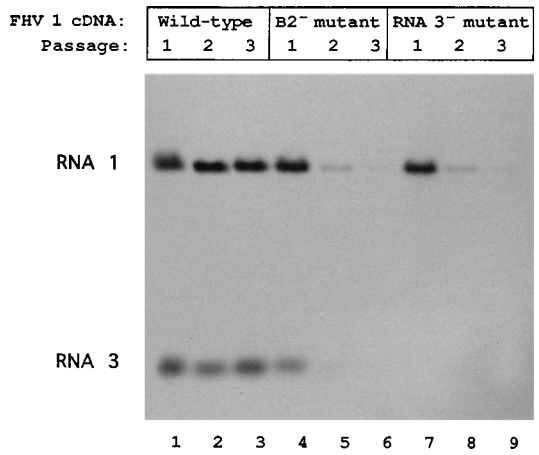


FIG. 7. Serial replicative passages of RNAs originally transcribed from plasmids. Lanes: 1 to 3, FHV1[1,0]; 4 to 6, FHV1[1,0] with mutations 6 and 7; 7 to 9, FHV1[1,0] with mutation 4. BHK21 cells were infected with vTF7-3 and transfected with wild-type FHV1[1,0] or with plasmids having the indicated mutations. At 24 h posttransfection, the RNA products of replication were labeled in the presence of actinomycin D as described for Fig. 1 and were then harvested and used for transfection of fresh, uninfected BHK21 cells. Twenty-four hours after the second transfection, the replication products were again labeled, harvested, and transfected into fresh uninfected cells. For passages 2 and 3, 4% of the total RNA from the previous passage was used for transfection. The labeled RNAs were analyzed as described for Fig. 1.

Repeated passage of mutant RNAs. The observation that the B2 protein was dispensable for RNA replication, at least in VV-infected BHK cells that were supporting the continued transcription of RNA 1, was unexpected and prompted us to examine more closely the replication of the mutants that could not produce protein B2 or RNA 3. Cytoplasmic RNAs were extracted from cells expressing either the wild-type plasmid FHV1[1,0] or similar plasmids that contained both mutations 6 and 7 (B2$^-$) or mutation 4 (RNA 3$^-$). RNA samples derived from equivalent numbers of first-passage cells were transfected into fresh, uninfected BHK cells, and their replication products were labeled by [^{3}H]uridine incorporation in the presence of actinomycin D. At the end of the 2-h labeling period, cytoplasmic RNAs were again extracted and amounts derived from equivalent numbers of second-passage cells were transfected into fresh BHK cells for a third replicative passage. The labeled products of RNA replication in each of these three passages were analyzed by electrophoresis on agarose-formaldehyde gels (Fig. 7). Whereas the wild-type RNA maintained its level of self-directed replication during these repeated passages (Fig. 7, lanes 1 to 3), replication of both the B2$^-$ and RNA 3$^-$ mutants diminished progressively (Fig. 7, lanes 4 to 6 and 7 to 9). For both mutants, the level of replication in passages two and three was about 10% of that seen in the preceding passage and it could not be increased by using more RNA for transfection. A similar progressive decrease in replication occurred when the repeated passages were performed in VV-infected cells (data not shown). These results showed that the mutants had significantly impaired replication when the replicative cycle was initiated by transfection of mutant RNA, but this phenotype was obscured when mutant RNA was generated intracellularly by transcription from a plasmid.

DISCUSSION

FHV RNA 1 combines the properties of a message for an RNA replicase subunit with those of a template for replication by the same enzyme, and therefore specifically directs its own replication in the cytoplasm of appropriate cells. The process is very efficient, since during 24 h of replication, authentic RNA 1 reaches an abundance that is roughly equivalent to that of the rRNAs. To reconstruct this autonomous RNA replication system from cDNA clones, the VV-T7 polymerase recombinant vTF7-3 (19) was used to direct cytoplasmic synthesis of primary transcripts from plasmids containing FHV 1 cDNA. These transcripts were designed to undergo ribozyme-mediated autolysis to generate competent templates for self-directed RNA replication. As observed for both the positive (4) and negative (2) strands of FHV RNA 2 expressed in a similar manner, minimization of the terminal extensions at both ends of the primary transcript (within the constraints of T7 promoter function) was critically important in generating RNA molecules which could replicate as well as authentic RNA 1. For example, transcripts of FHV1[26,43] directed the synthesis of abundant protein A (Fig. 6, lane 4) which was functional (5) but they were nevertheless incapable of replication (Table 1). In contrast, transcripts of FHV1[1,0] self-replicated to about the same level as authentic RNA 1 (Table 1 and Fig. 5, lane 2). This result made possible the genetic studies described in this paper.

Mutations that truncated orf A or that inserted an Arg residue near the conserved Gly-Asp-Asp motif that is characteristic of RNA polymerases (7), eliminated self-directed replication. However, the mutant RNAs remained competent templates for replication when functional protein A was provided from another source (5). This result established that protein A is essential for RNA replication, probably because it is the catalytic subunit of the RNA replicase. Moreover, the ability to selectively eliminate either the mRNA or template functions of RNA 1 transcripts (from mutant plasmid 1 and FHV1[26,43], respectively, for example) showed that in this system, unlike that of poliovirus (29, 34), replication of an RNA molecule is not inseparably coupled to its translation. This will allow the mRNA and template functions of RNA 1 to be examined independently of one another.

The synthesis of RNA 3 was affected by terminal extensions or deletions in the RNA 1 transcript concomitantly with the replication of RNA 1 itself (Fig. 1). This result was unexpected, since it showed that RNA 3 synthesis, which is thought to occur by initiation at an internal promoter site on a partial or full-length negative strand of RNA 1 (24), was influenced by the 5′ terminal nucleotide sequence of RNA 1, 2.7 kb away from the site of internal initiation. The effect was on the ability of RNA 1 to produce a competent template for RNA 3 synthesis rather than on its translation to produce replicase, since even nonreplicating RNA 1 transcripts made in the VV-T7 expression system yielded enough enzyme to support abundant RNA synthesis. The result suggests that the synthesis of RNA 3 is tightly coupled to the replication of RNA 1 as a consequence of having similar template requirements. Moreover, it implies that a full-length negative strand of RNA 1 must be made before the synthesis of RNA 3 can occur, and thus before protein B1 or protein B2 becomes available.

The 5′ end of RNA 3 was mapped by primer extension to nucleotide 2721 of RNA 1, indicating that RNA 3, which is thought to be 3′ coterminal with RNA 1, contained 387 rather than 389 nucleotides as was concluded previously (21). This result was satisfying because it meant not only that RNA 3 started with a purine rather than a pyrimidine residue but also that it shared a common 5′ dinucleotide (GU) with RNAs 1 and 2. The marked inhibition of RNA 3 synthesis by mutations 3 and 4 (G2721A/T) was entirely consistent with this being the site of initiation of the subgenomic RNA. Other essential features of the internal promoter site remain to be examined.

Although mutation 4 (G2721T) eliminated the synthesis of RNA 3, the replication of RNA 1 was either slightly enhanced (during replication initiated by plasmid transfections [Fig. 5, lane 8]) or partially reduced (in subsequent RNA transfections [Fig. 7, lanes 8 and 9]). This showed that neither RNA 3 nor its translation products were absolutely required for RNA replication, a conclusion that was confirmed by the effects of mutations 5, 6, and 7, which disrupted the B1 and B2 orfs. Indeed, since RNA 3 and hence the B protein(s) require RNA replication for their own synthesis and are not packaged into virions, it is difficult to imagine how they could be essential for RNA replication, at least at the early stages. Nevertheless, the replicative abilities of RNA carrying mutation 4 (RNA 3^-) or mutations 6 and 7 (protein $B2^-$) were substantially different when assayed during the first passage, which was initiated by transfection of plasmids, or in subsequent passages which were initiated by transfection of RNA. A similar but more marked detrimental effect of mutation 7 on RNA replication was observed by Harper and Rueckert (23), who used transfection of mutant RNA 1 transcripts made in vitro to initiate FHV RNA replication in cultured *Drosophila* cells.

The subtlety of the phenotype of these mutants excluded straightforward conclusions concerning the role of the B2 protein in RNA replication. However, among the few hypotheses that are consistent with all the available data is the possibility that the B2 protein is involved in regulating the partitioning of positive-sense RNA between the conflicting demands of translation and replication. FHV RNAs 1 and 2, like the genomic RNAs of all positive-strand RNA viruses, are both translated and replicated, with the balance between these two processes changing during the viral life cycle. It seems likely that viral mechanisms that exert a dynamic control over the distribution of genomic RNA molecules between translation and replication have evolved, and bacteriophage Qβ provides a well-studied example of one way that such a mechanism can operate (6). For the RNA 3^- and $B2^-$ mutants described above, a major difference between the first passage, which obscured the effects of the mutations, and the subsequent passages, which evidenced a clear phenotype, was that during the first passage there were two continuous sources of functional RNA 1: plasmid-directed transcription and self-directed RNA replication. In subsequent passages, and in the experiments of Harper and Rueckert (23), self-replication was the sole source of RNA 1. It is possible that the availability of fully functional RNA 1 from continued transcription circumvented the need for precise partitioning of the RNA between translation and replication and thereby obscured the mutant phenotype. In contrast, the absence of protein B2 in subsequent passages may have resulted in an unbalanced distribution of RNA between these two processes such that one of them was starved for template. Thus, this hypothesis suggests that the B2 protein optimizes RNA replication by modulating the competition for RNA between ribosomes and the replicase.

An alternative hypothesis is that the B2 protein increases the fidelity of replication, such that in its absence multiple rounds of RNA synthesis and translation lead to error catastrophe. Although there is no evidence of increased length heterogeneity in the 5′-terminal 80 nucleotides of RNA 3 made by the $B2^-$ polymerase (Fig. 3, lane 3), this assay would detect only insertions and deletions and would not detect misincorporation of nucleotides. Comparison of the error rates of the wild-

type and B2$^-$ polymerases will require the development of a more sensitive assay. Both of these proposals can be addressed experimentally by using the system developed in this laboratory, and further work will be necessary to examine their validity for the function of the B2 protein.

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