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Replication of the genomic RNA of a positive-strand RNA animal virus from negative-sense transcripts

(flock house virus/nodaviruses/vaccinia virus/ribozymes/cis-acting sequences)

L. ANDREW BALL

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

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ABSTRACT **Studies of RNA replication among the positive-strand RNA animal viruses have been hindered by the apparent inability of their RNA-dependent RNA polymerases to initiate replication on the corresponding negative-sense RNAs. However, here I report that in the case of the nodavirus flock house virus (FHV), which has a bipartite positive-sense RNA genome, the viral RNA replicase can replicate a negative-sense transcript of the genome segment that encodes the viral capsid proteins. For this work, the FHV replication cycle was experimentally reconstructed in baby hamster kidney cells that were transfected with specialized transcription plasmids designed to direct the synthesis of RNAs which corresponded closely to the two genome segments of FHV. The RNA replicase encoded by the larger genome segment could utilize either the positive or the negative strand of the smaller segment as a template, and it catalyzed RNA replication to produce similar RNA products in the two situations. Surprisingly, studies of the nucleotide sequences that were required for replication showed that the 3′ end of the negative-strand RNA contained only a minimal cis-acting signal. The success of these experiments will facilitate further studies of the cis- and trans-acting factors involved in the recognition and replication of negative-sense RNA in this system.**

One of the characteristic features of RNA replication among the positive-strand RNA viruses is that positive-strand RNA is synthesized in great excess over the negative strand. The relative levels vary among different viruses and in many cases precise measurements have not been made, but typically the negative-strand RNAs accumulate to only 1–5% of the level of the positive-strand RNAs (1). This implies that the negative-strand RNAs are by far the more efficient templates. Paradoxically, however, most attempts to initiate replication with input negative-strand RNA, either in cell-free replication systems or in cells expressing the appropriate RNA replicase, have been unsuccessful. Only in rare cases has infectivity been recovered from the negative-strand complement of a positive-strand RNA viral genome (2, 3).

The reasons for the general lack of template activity among negative-strand transcripts are unclear, but in some cases they may be due to unexpected nucleotides at the termini of the authentic negative strand (4), while in other cases they may be related to the obligatory cis action of some RNA replicases. The poliovirus enzyme, for example, may be able to initiate replication only on the RNA molecule from which it was translated, a property which would preclude *de novo* interaction between the replicase and the negative strand (5).

I have been studying the mechanism of RNA replication of flock house virus (FHV), a member of the nodaviruses, which comprise a family of small isometric riboviruses with bipartite, positive-sense RNA genomes (6). The larger segment of the FHV genome (RNA 1, 3107 nt) encodes the viral contribution to the RNA replicase (7), whereas the smaller segment (RNA 2, 1400 nt) encodes a capsid protein precursor that undergoes an assembly-dependent cleavage to yield the two virion structural proteins (8). One hundred eighty copies of these capsid proteins copackage the two genome segments into a T = 3 icosahedral virion whose structure has been determined at 3.0-Å resolution (9).

To study RNA replication in this and other systems, I developed specialized transcription plasmids for the intracellular synthesis of RNAs that had defined termini and were therefore competent templates for RNA replication. These plasmids contained a bacteriophage T7 promoter site, a cDNA copy of the RNA to be replicated, a sequence that encoded a cis-acting autolytic ribozyme, and a T7 transcriptional termination signal. In the case of both the negative-strand virus, vesicular stomatitis virus (10), and the positive-strand virus, FHV (11, 12), transfection of such plasmids into cells that were expressing T7 RNA polymerase from a recombinant vaccinia virus (VV; ref. 13) resulted in the synthesis of primary transcripts that self-cleaved to yield RNA molecules with precise termini that were competent templates for RNA replication. These results, and the ability of the FHV replicase to function in trans, which is implicit in the bipartite nature of the viral genome, encouraged me to examine the template activity of negative-sense transcripts of the FHV capsid protein genome segment, RNA 2.

MATERIALS AND METHODS

Cells and Viruses. All expression experiments were performed in baby hamster kidney (BHK21) cells grown as monolayers in the 35-mm diameter wells of 6-well plates as described (11). The recombinant VV that expressed T7 RNA polymerase, vTF7-3 (13), was kindly provided by Bernard Moss (National Institutes of Health). The VV recombinant vF1 (14) was constructed so that VV early transcription (15) would initiate at the first nucleotide of a full-length infectious cDNA clone of FHV RNA 1 (ref. 16; kindly provided by Ranjit Dasgupta and Paul Kaesberg, University of Wisconsin, Madison) (see Table 1). The 3′ end of the FHV1 cDNA was juxtaposed with a sequence that corresponded to the site of RNA self-cleavage mediated by the antigenomic ribozyme of hepatitis delta virus (HDV) (Table 1). This ribozyme was encoded in an 87-nt cDNA derived from plasmid pSA1 (ref. 17; kindly provided by Michael Been, Duke University).

Plasmid Constructions and Sequencing. Conventional procedures (18) were used for the manipulation of DNA fragments and the construction of the plasmids used in this work (see Table 1). The sequence arrangements shown in Table 1 were all assembled within the multiple cloning sites of pGEM-3 or -4 (Promega), and all plasmids conformed to the overall structures shown in Fig. 1 and presented in detail before (10, 11). In plasmid 2VHF (ΔT7,12), the 41 bp up-

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Abbreviations: FHV, flock house virus; HDV, hepatitis delta virus; VV, vaccinia virus.

stream of the T7 transcriptional start site, which contain the T7 promoter, were replaced by an unrelated and nonfunctional sequence of 21 bp. All constructions were verified by determining the nucleotide sequences (19) across the relevant junctions, and the plasmids were purified by banding in cesium chloride/ethidium bromide density gradients.

RNA and Protein Analyses. BHK21 cells were infected at a multiplicity of infection of 10 plaque-forming units per cell, transfected with 5 μg of plasmid DNA per 10^6 cells, incubated at 28°C, and labeled by incorporation of [^{3}H]uridine (20 μCi per ml; 1 Ci = 37 GBq) in the presence of actinomycin D (10 μg/ml). Cytoplasmic RNAs were analyzed by agarose/formaldehyde gel electrophoresis followed by fluorography or Northern blotting as described (11). The intensity of hybridization was quantitated by phosphorimaging. Mapping of RNA 5′ ends by primer extension was performed as described (12). Cytoplasmic proteins were resolved by electrophoresis on SDS/12.5% polyacrylamide gels (20), electroblotted onto nitrocellulose sheets, and probed with a rabbit hyperimmune serum to FHV (kindly supplied by Tom Gallagher and Roland Rueckert, University of Wisconsin, Madison). The immunoreactive proteins were visualized by reaction with a goat anti-rabbit IgG-horseradish peroxidase conjugate (GIBCO/BRL) and the peroxidase substrate 4-chloro-1-naphthol. The experiments presented in Figs. 2–6 were repeated on multiple occasions with good reproducibility.

RESULTS AND DISCUSSION

Transcription Plasmids That Expressed Replicable RNAs. In previous work, I demonstrated the efficacy of cis-acting ribozymes in generating replicable RNAs with defined 3′ ends from intracellular transcripts of circular plasmids that had the general structure shown at the top of Fig. 1 (11, 12). Plasmid names (Table 1) indicate which FHV genome segment they contained (FHV1 or -2), and its orientation with respect to the T7 promoter—e.g., FHV2 (positive) or 2VHF (negative). The numbers in parentheses show how many extraneous nucleotides remained after cleavage of the T7 transcripts at their 5′ and 3′ ends, respectively.

Initially, I used the hammerhead ribozyme from satellite tobacco ringspot virus (21) in constructs that left a minimum of 12 non-FHV nucleotides at the 3′ end of the self-cleaved RNA [as in plasmid 2VHF (16,12), for example]. In contrast, the pseudoknot ribozyme from HDV was readily engineered

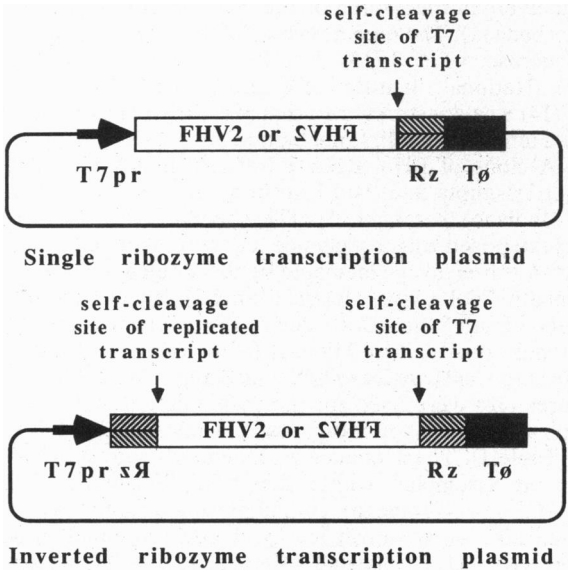

FIG. 1. Schematic representations of the transcription plasmids. T7pr, bacteriophage T7 promoter site; Rz, ribozyme from satellite tobacco ringspot virus or HDV; Tφ, T7 transcriptional terminator.

Table 1. Junction sequences of virus and plasmids

Virus or plasmid	5′ junction	3′ junction
vF1 (0,0)	..AAATATATTCTAATCTGCA↓GTTTTCG...	GAGGT*GGGTCGG..
FHV1 (1,0)	....CACTATAG↓GTTTTCG...	As vF1 (0,0)
FHV2 (1,0)	....CACTATAG↓GTAAACA...	AAGGT*GGGTCGG..
FHV2 (0,0)	.....CACTATA↓GTAAACA...	As FHV 2 (1,0)
FHV2 (δ0,0δ)	....CCGACCC*GTAAACA...	As FHV 2 (1,0)
2VHF (16,55)	.TAG↓GCCTGGATCCTCTAGACCTTAG...	GTTTACTGCAGGTCGAC TCTAGAGGATCCCCGGG TACCGAGCTCGAATTCG ATACCCTGTT*ACCGG..
2VHF (16,12)	As 2VHF (16,55)...	GTTTACCGATACCC TGTT*ACCGGA...
2VHF (2,55)	...CACTATAG↓GACCTTAG...	As 2VHF (16,55)
2VHF (2,12)	As 2VHF (2,55)...	As 2VHF (16,12)
2VHF (0,12)	.....CACTATA(↓)ACCTTAG...	As 2VHF (16,12)
2VHF (2,0)	As 2VHF (2,55)...	GTTTAC*GGGTCGG..
2VHF (1,0)	....CACTATAG↓ACCTTAG...	As 2VHF (2,0)
2VHF (0,0)	As 2VHF (0,12)...	As 2VHF (2,0)
2VHF (δ0,0δ)	....CCGACCC*ACCTTAG...	As 2VHF (2,0)
2VHF (ΔT7,12)	.ACCCGGGGATCCTCTAGACCTTAG...	As 2VHF (16,12)

FHV sequences are shown in boldface type. Promoter sequences are underlined and the vertical arrows indicate the major sites of transcriptional initiation. Asterisks indicate the positions of ribozyme-mediated RNA cleavage.

to yield cleavage products that retained no extraneous 3′ nucleotides [as in plasmids FHV2 (1,0) and 2VHF (2,0), for example], which increased the efficiency of replication of FHV RNAs 1 and 2 to levels that were comparable to those of authentic RNAs purified from virions. The self-replication of transcripts of FHV1 (1,0) is shown in Fig. 2, lane 11, and the self-replication of RNAs 1 and 2 in the complete plasmid-derived reconstructed system is shown in Fig. 2, lane 12. The replication of authentic RNAs 1 and 2 is shown in Fig. 2, lane 13, for comparison. All the labeled RNAs shown in Figs. 2 and 3 were products of the FHV RNA replicase, since DNA-dependent transcription was suppressed by the presence of actinomycin D during the labeling period. The smallest band (RNA 3) is a subgenomic RNA that is produced during replication and consists of the 3′-terminal 387 nucleotides of FHV RNA 1. The observation that RNA 3 synthesis is suppressed by the replication of RNA 2, which is apparent in lanes 12 and 13 of Fig. 2, has been well documented (7, 22).

Inverted Ribozyme Transcription Plasmids. The first indication that negative-sense transcripts might be competent templates for RNA replication in this system came from plasmid constructs that were designed to use ribozyme-mediated RNA cleavage to generate both ends of a replicable RNA. A second copy of the HDV ribozyme was inserted in inverted orientation between the T7 promoter and the 5′ end of the FHV2 cDNA to give plasmid FHV2 (δ0,0δ), whose overall structure is shown at the bottom of Fig. 1. The promoter-proximal copy of the ribozyme was intended to cleave the replicated RNA to yield a perfect 3′ end on the complementary strand.

The ability of FHV2 (δ0,0δ) to yield replicable RNA was tested in cells that were infected with vTF7-3 to provide the T7 RNA polymerase necessary to make the primary transcripts and cotransfected with FHV RNA 1 [purified by multiple passages (11)] to provide the RNA replicase. [^{3}H]-Uridine incorporation in the presence of actinomycin D showed that the inverted ribozyme plasmid (Fig. 2, lane 6)

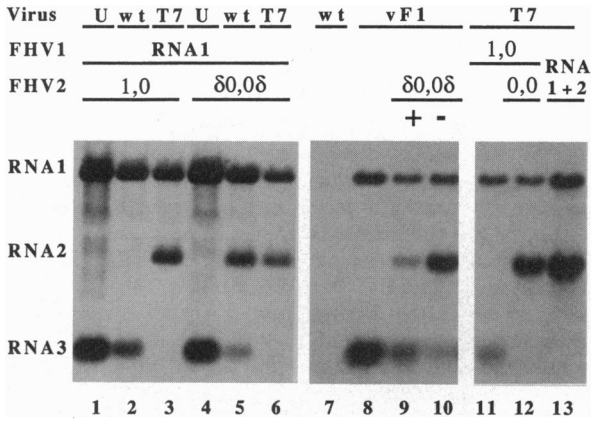

FIG. 2. Replication of FHV RNA 2 from single and inverted ribozyme transcription plasmids. BHK21 cells were left uninfected (U) or were infected with wild-type VV (wt), vTF7-3 (T7), or vF1 as shown on the top line above the lanes and cotransfected with various plasmids as shown on the next two lines. The cells were labeled 24 h after transfection for 2 h with [^{3}H]uridine in the presence of actinomycin D. The products of RNA replication were resolved by electrophoresis on agarose/formaldehyde gels and visualized by fluorography. Lanes 4–6 and 9: FHV2 (δ0,0δ); lane 10: 2VHF (δ0,0δ). The sample in lane 13 was from vTF7-3 infected cells that were transfected with 100 ng of FHV RNAs 1 and 2. The positions of RNAs 1, 2, and 3 are noted at the left.

directed the synthesis of RNA that could replicate about as well as that from the single ribozyme plasmid FHV2 (1,0) (Fig. 2, lane 3). As expected, the ability of plasmid FHV2 (1,0) to make replicable RNA depended on T7 RNA polymerase expressed from the VV recombinant, since no RNA 2 replication was observed in uninfected cells or in cells infected with wild-type VV (Fig. 2, lanes 1 and 2, respectively). Unexpectedly, however, plasmid FHV2 (δ0,0δ) gave replicable RNA even in wild-type VV-infected cells (Fig. 2, lane 5). I attribute this result to the presence, somewhere within the plasmid, of a cryptic promoter site that was recognized by the promiscuous VV DNA-dependent RNA polymerase. Wherever the primary transcript initiated, its cleavage, its replication, and the subsequent cleavage of its complement would yield an RNA 2 molecule with no extraneous nucleotides at either end.

Since the location of the putative cryptic promoter site for VV RNA polymerase was unknown, the polarity of the primary transcript from FHV2 (δ0,0δ) was also unknown. I therefore compared the behavior of this plasmid with that of plasmid 2VHF (δ0,0δ) in which the orientation of the FHV2 cDNA was reversed (Table 1). Since T7 RNA polymerase was not required to generate a primary transcript from these inverted ribozyme plasmids, they were transfected into cells that were infected instead with vF1 (Table 1), a VV recombinant that expressed FHV replicase. This recombinant virus used the 7.5k early promoter of VV (15) to drive the synthesis of a self-cleaving RNA 1 transcript that acted both as message and template for the RNA replicase. Cells infected with this recombinant virus supported RNA 1 replication and the synthesis of RNA 3 at levels comparable with those achieved by authentic RNA 1 (Fig. 2, compare lanes 2 and 8). Both inverted ribozyme plasmids directed the synthesis of replicable RNA 2 in vF1-infected cells, irrespective of the orientation of the RNA 2 sequence (Fig. 2, lanes 9 and 10). Interpretation of the different levels of RNA replication seen with these two plasmids was complicated by the fact that the structure and polarity of their primary transcripts were unknown, but the demonstration that they both made replicable RNA 2 suggested that both polarities of primary transcript might be recognizable by FHV replicase.

Replication of Negative-Strand RNA 2. To examine this possibility directly, I constructed several single ribozyme plasmids that contained FHV2 cDNA in the negative orientation with respect to the T7 promoter (Table 1). The ability of these plasmids to direct the synthesis of replicable negative-strand RNA 2 was examined in cells that were infected with vTF7-3 to provide T7 RNA polymerase and cotransfected with plasmid FHV1 (1,0) to provide the RNA replicase. Three of the plasmids made negative-sense primary transcripts that replicated: 2VHF (2,12) (Fig. 3, lane 5); 2VHF (2,0) (Fig. 3, lanes 7 and 10); and 2VHF (1,0) (data not shown). The remote possibility that the replicable transcripts made from these plasmids were in fact positive-sense RNAs initiating at some cryptic promoter on the other DNA strand can be discounted because terminal extensions that are longer than a few nucleotides completely prevent the replication of positive-sense RNA 2 transcripts (12). Moreover, removal of the T7 promoter site [in plasmid 2VHF (ΔT7,12); Table 1] abrogated the synthesis of replicable RNA 2 (Fig. 3, lane 11) and thus confirmed that the replicating primary transcripts were, indeed, negative sense. However, I cannot exclude the possibility that the first positive-sense RNA was made by an unidentified, nonspecific, host cell RNA-dependent RNA polymerase, rather than by the FHV enzyme.

Plasmids 2VHF (0,12) and 2VHF (0,0) failed to direct the synthesis of replicable negative-sense RNA 2 (Fig. 3, lanes 6 and 8). However, examination of these two plasmids as templates for T7 RNA polymerase showed that even *in vitro* they were transcribed very poorly (data not shown), presumably because the site of transcriptional initiation had an unfavorable sequence for T7 polymerase (23). Because both of the inverted ribozyme plasmids, neither of which left extraneous terminal nucleotides, directed the transcription of replicable RNAs, it seems likely that the lack of RNA replication in cells transfected with plasmids 2VHF (0,12) and 2VHF (0,0) was due to the absence of primary transcription. These results reinforced the conclusion that the 2VHF primary transcripts that initiated RNA replication were negative sense. Moreover, the observation that the products of negative-strand RNA replication (such as those shown in Fig. 3, lane 10) continued to replicate on subsequent passages (data not shown) established that the negative-sense primary transcripts initiated complete, reiterative RNA 2 replication.

Extraneous terminal nucleotides (beyond those required for primary transcription) were detrimental to replication, particularly nucleotides at the 5′ end of the negative-sense primary transcript. At least 12 (but not 55) extra nucleotides

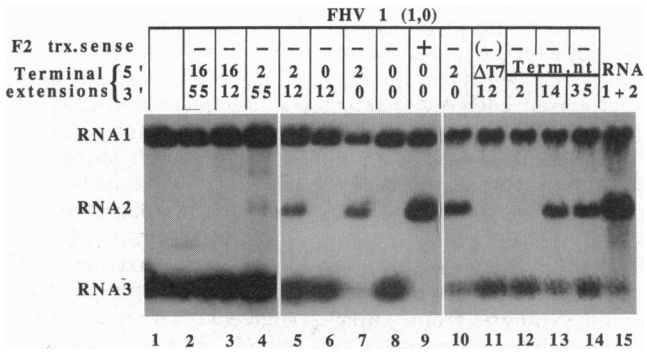

FIG. 3. Replication of FHV RNA 2 from negative-sense primary transcripts. BHK21 cells were infected with vTF7-3 and cotransfected with plasmid FHV1 (1,0) (lanes 1–14) and plasmids that expressed FHV RNA 2 transcripts having the polarities and terminal extensions listed above lanes 2–14. The products of RNA replication were analyzed as described in the legend for Fig. 2. The sample in lane 15 was from vTF7-3-infected cells that were transfected with 100 ng of FHV RNAs 1 and 2. trx, Transcript.

could be tolerated at the 3′ end (Fig. 3, lanes 2, 4 and 5), but 16 extra nucleotides at the 5′ end prevented replication altogether (Fig. 3, lane 3).

Replication of negative-strand transcripts opened the way for identification of the cis-acting signals involved in recognition of RNA 2 negative strands by the replicase. Experiments that addressed the same question for the positive strand showed that, whereas 50–60 nucleotides from the 3′ end of the positive strand of RNA 2 were necessary for fully efficient replication, fewer than 6 nucleotides were needed from the 5′ end (L.A.B. and X. Wu, unpublished data). Since the initiation of positive-strand synthesis, which is by far the major RNA 2 synthetic event, occurs on the complement of the 5′ end, it was surprising to find such a minimal sequence requirement at that position. Moreover, the result implied that only a few nucleotides from the 3′ end of the negative strand would be required for fully efficient replication. To test this prediction directly, three deletions of 2VHF (2,0) were constructed which synthesized negative-sense primary transcripts that lacked nucleotides 36–80, 15–80, or 3–80 numbered from their 3′ ends. Transcripts from the first two deletion plasmids replicated as efficiently as full-length, negative-sense RNA 2 (Fig. 3, lanes 14, 13, and 10, respectively), whereas transcripts that retained only 2 of the 3′-terminal 80 nucleotides failed to replicate in this assay (Fig. 3, lane 12). These results confirmed the observations made with positive-strand transcripts: a minimal number of nucleotides (between 3 and 14) present at the 3′ end of the negative strand (or at the 5′ end of the positive strand) were sufficient for fully efficient replication of RNA 2.

RNA 5′ End Mapping by Primer Extension. To characterize the products of plasmid transcription and RNA replication in more detail, their 5′ ends were examined by analysis of the extension products of oligonucleotide primers that annealed to the positive and negative strands of RNA 2. The replication products of authentic RNA 2 yielded a characteristic set of primer-extension products, which have been described before (12) and correspond to capped and uncapped positive-sense RNA (Fig. 4, lane 1). Transcripts from plasmid FHV2 (0,0) in the absence of RNA replication yielded similar primer-extension products (Fig. 4, lane 3). However, no products of primer extension on the authentic RNA 2 negative strand were detected even after an autoradiographic exposure that was 10 times longer than that shown in Fig. 4, lane 1. It is likely that the excess positive-sense RNA interfered with primer binding to the negative strands. In contrast, negative-sense transcripts directed by plasmid 2VHF (2,0) in the absence of RNA replication yielded the predicted primer-extension product, 2 nt longer than the product expected from the authentic negative strand (Fig. 4, lane 4). As seen during the replication of authentic RNA, only positive-sense RNA was detectable by primer extension after the replication of both positive- and negative-sense primary transcripts (Fig. 4, lanes 5 and 6, respectively). Moreover, the 5′ ends of the positive-sense replication products were authentic and were the same whatever the polarity of the primary transcripts. The same result was seen with plasmid 2VHF (2, 12) (data not shown), suggesting that the FHV replicase can select the correct initiation site for positive-strand synthesis from within a somewhat larger negative-strand template (see figure 5 of ref. 12). The disappearance (Fig. 4, lane 6) of the primer-extension product that corresponded to the negative-sense transcript was consistent with the suggestion that the excess positive-sense RNA produced by replication interfered with primer binding to the negative RNA produced by transcription or replication. These results confirmed that the negative-sense transcript of 2VHF (2,0) was competent to replicate and showed that, as during the replication of the positive-sense transcript of FHV2 (0,0) and

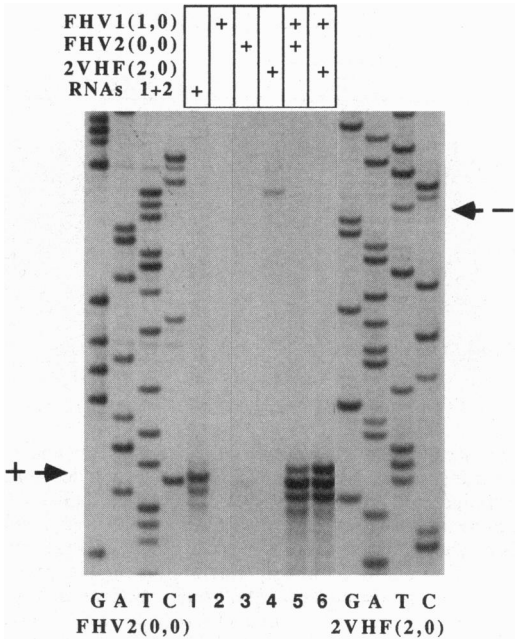

FIG. 4. Analysis of the products of primer extension directed by transcription and replication products of positive- and negative-sense FHV RNA 2. BHK21 cells were infected with vTF7-3 and transfected with FHV RNAs 1 and 2 (lane 1) or with the plasmids shown above lanes 2–6. Cytoplasmic RNAs isolated 24 h after transfection were used as templates for extension of mixed primers that annealed to the positive and negative strands of RNA 2. Dideoxynucleotide sequencing ladders of positive- and negative-sense plasmids generated by using the same two primers are on the left and right of lanes 1–6, respectively, and show the positions that correspond to the 5′ nucleotides of the positive and negative strands of RNA 2. Lanes 3–6 have been overexposed to reveal the faint bands that correspond to the primary transcripts in lanes 3 and 4.

authentic RNA 2, positive-sense RNA was produced in great abundance over its complement.

Northern Blot Hybridization of the Products of Transcription and Replication. Northern blot hybridization yielded more precise quantitation of the relative amounts of positive- and negative-sense RNAs that accumulated under the different conditions of transcription and replication. Cells transfected with authentic FHV RNAs 1 and 2 contained, in addition to the major 1.4-kb species of positive-sense RNA 2, two minor species: one migrated with the apparent size of dimeric RNA 2 (2.8 kb) and the other, which varied in intensity among different experiments, comigrated with 18S ribosomal RNA (Fig. 5, lane 1). RNA species with the same three mobilities hybridized to a positive-strand RNA 2 probe (Fig. 5, lane 7), although their relative abundances were very different, and together these three species constituted only about 1% of the abundance of the 1.4-kb positive-sense RNA 2. (Note that the blot of lanes 7–12 of Fig. 5 was exposed 50 times longer than the upper blot of lanes 1–6.) The major species of negative-sense RNA 2 had an apparent size of 2.8 kb, as described (11). A minor RNA species of 1.4 kb was also usually present, but the band that comigrated with 18S ribosomal RNA varied greatly in intensity among different experiments (Fig. 5, lane 7). This variability and the reported direct interactions between some viral RNAs and ribosomal RNAs (24) suggest that the 18S band was artifactual. The 2.8-kb band, on the other hand, had the mobility and hybridization properties expected for positive- and negative-sense dimers of RNA 2, or perhaps for double-stranded RNA 2 that had resisted denaturation.

In the absence of RNA replication, plasmids FHV2 (0,0) and 2VHF (2,0) directed the synthesis of low levels of

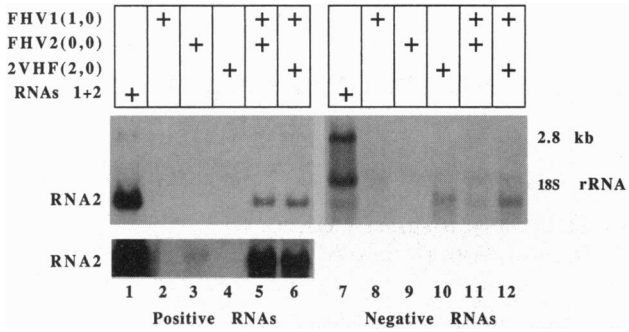

FIG. 5. Northern blot analysis of the products of transcription and replication of positive- and negative-sense FHV RNA 2. BHK21 cells were infected with vTF7-3 and transfected as shown above each lane. Cytoplasmic RNA isolated 24 h after transfection was resolved by agarose/formaldehyde gel electrophoresis, blotted to a charged nylon membrane, and probed with ^{35}S-labeled negative-sense (lanes 1–6) or positive-sense (lanes 7–12) FHV RNA 2 of equal specific activity. The autoradiograph shown in the upper left panel was exposed 1/50 of the time of the other two panels.

positive- and negative-sense RNA 2 transcripts, respectively (Fig. 5, lanes 3 and 10). When RNA replication occurred, as a consequence of the cotransfection of plasmid FHV1 (1,0) to provide the RNA replicase, substantial levels of positive-sense RNA 2 accumulated by replication of either the positive-sense primary transcripts (Fig. 5, lane 5) or the negative-sense primary transcripts (Fig. 5, lane 6). Quantitation of all the positive- and negative-sense products made under these two conditions showed that the sense of the primary transcript had little effect on the relative abundances of the complementary products of replication. The ratio of total positive- to negative-sense species of RNA 2 after 24 h of replication of authentic RNA (Fig. 5, lanes 1 and 7) was about 100:1. Similarly, a positive-sense primary transcript (Fig. 5, lanes 5 and 11) gave a ratio of about 150:1, and a negative-sense primary transcript (Fig. 5, lanes 6 and 12) gave a ratio of about 50:1. In all three situations, positive-sense RNA was by far the major product of replication, indicating that the mechanism by which strand asymmetry was achieved was largely able to overcome the perturbation of encountering a negative-sense primary transcript.

Analysis of Capsid Protein Synthesis. Since the Northern blot showed that abundant positive-sense RNA 2 was made by replication of the negative-sense primary transcript from plasmid 2VHF (2,0), I examined the proteins that accumulated under these conditions for the presence of the FHV capsid protein precursor α. Cytoplasmic proteins were harvested from cells that had been transfected in the same six ways that were used for the RNA analyses shown in Figs. 4 and 5. The proteins were resolved by electrophoresis on an SDS/polyacrylamide gel and FHV capsid protein was detected by immunoblotting (Fig. 6, lanes 2–7). A sample of proteins from 1 μg of purified FHV served as a marker (Fig. 6, lane 1).

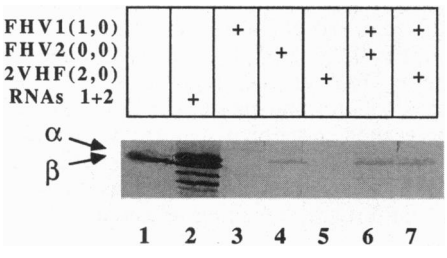

FIG. 6. Western blot of FHV capsid protein synthesis. BHK21 cells were infected with vTF7-3 and transfected as shown above each lane. Total cytoplasmic proteins were resolved by SDS/PAGE 24 h after transfection, electroblotted onto nitrocellulose, and probed with an antiserum to FHV. Lane 1 shows the uncleaved (α) and cleaved (β) capsid proteins from 1 μg of purified FHV.

In the absence of RNA replication, the capsid protein precursor α accumulated in cells that transcribed positive-sense RNA 2 from plasmid FHV2 (0,0) (Fig. 6, lane 4), but not in those that transcribed negative-sense RNA 2 from plasmid 2VHF (2,0) (Fig. 6, lane 5). However, when RNA replicase was provided by cotransfection of plasmid FHV1 (1,0), functional capsid protein mRNA was made by replication of primary transcripts of either sense, as shown by the accumulation of protein α (Fig. 6, lanes 6 and 7). These results confirmed that the negative-sense transcript from plasmid 2VHF (2,0) was competent as a template for the replicative synthesis of functional mRNA.

In summary, these results show that, unlike most other RNA replicases from positive-strand RNA viruses that have been examined, the FHV enzyme can recognize and replicate a negative-strand RNA template. It is not clear whether this ability is due to an unusual, intrinsic property of the FHV enzyme, perhaps related to its need to function in trans during replication of the two viral genome segments, or whether it is a consequence of the technical approach used in these experiments. The VV/T7 plasmid transcription system allows prolonged synthesis of RNAs in the cytoplasm and thereby offers an extended opportunity for the replication of molecules that may have suboptimal template activity or may need to encounter the replicase at a particular concentration or time during replication. Whatever the explanation for the success of these experiments, it will now be possible to examine further the requirements for recognition of the RNA 2 negative strand as well as the mechanism of its replication.

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