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# Cellular Expression of a Functional Nodavirus RNA Replicon from Vaccinia Virus Vectors

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**RNA replication provides a powerful means for the amplification of RNA, but to date it has been found to occur naturally only among RNA viruses. In an attempt to harness this process for the amplification of heterologous mRNAs, both an RNA replicase and its corresponding RNA templates have been expressed in functional form, using vaccinia virus-bacteriophage T7 RNA polymerase vectors. Plasmids were constructed which contained in 5′-to-3′ order (i) a bacteriophage T7 promoter; (ii) a full-length cDNA encoding either the RNA replicase (RNA 1) or the coat protein (RNA 2) of flock house virus (FHV), (iii) a cDNA sequence that encoded the self-cleaving ribozyme of satellite tobacco ringspot virus, and (iv) a T7 transcriptional terminator. Both in vitro and in vivo, circular plasmids of this structure were transcribed by T7 RNA polymerase to produce RNAs with sizes that closely resembled those of the two authentic FHV genomic RNAs, RNA 1 and RNA 2. In baby hamster kidney cells that expressed authentic FHV RNA replicase, the RNA 2 (coat protein) transcripts were accurately replicated. Moreover, the RNA 1 (replicase) transcripts directed the synthesis of an enzyme that could replicate not only authentic virion-derived FHV RNA but also the plasmid-derived transcripts themselves. Under the latter conditions, replicative amplification of the RNA transcripts ensued and resulted in a high rate of synthesis of the encoded proteins. This successful expression from a DNA vector of the complex biological process of RNA replication will greatly facilitate studies of its mechanism and is a major step towards the goal of harnessing RNA replication for mRNA amplification.**

The process of RNA-dependent RNA synthesis is used for genome replication by all RNA viruses except the retroviruses. The mechanisms of RNA replication differ widely among the positive-stranded, negative-stranded, and double-stranded RNA viruses, but the efficacy of the process is evident from the abundant genome amplifications that occur during the infectious cycles of RNA viruses. It is somewhat surprising, in view of the amplifying power of RNA replication, that the process has been observed naturally only in the context of RNA virus infections. It seems feasible to harness RNA replication to enhance the levels of selected mRNAs expressed from a variety of DNA vectors.

In pursuit of this goal, I undertook a series of experiments designed to express both a functional RNA replicase and its corresponding RNA template from a vaccinia virus (VV) vector system. The choice of which RNA replicase to use for this work was influenced by several considerations: simplicity of subunit structure; activity in a wide range of eukaryotic cells, including cells that could support VV replication; demonstrable activity of the replicase in *trans*, i.e., on an RNA template molecule other than that from which the enzyme itself was translated; and the availability of a biologically active cDNA clone. These considerations led to the choice of the RNA replicase of flock house virus (FHV), which meets each of these criteria (29). FHV is a member of the *Nodaviridae*, a family of small, icosahedral viruses with bipartite, positive-sense RNA genomes (24). Other members of the family include the prototype, Nodamura virus, and the well-studied black beetle virus (BBV) (18). Insects are the natural hosts for FHV, and the virus replicates well in cultured *Drosophila melanogaster* cells, but a productive infectious cycle also results from introducing the viral RNAs into plant cells (30) or into mammalian cells in culture (see below), indicating that FHV RNA replicase can function in a wide range of intracellular environments.

The replicase has not yet been purified to homogeneity, so its subunit composition is unknown (28). However, it is clear that the entire viral contribution to the enzyme is encoded in the larger of the two genomic RNAs (RNA 1 [3,106 nucleotides]), since this molecule can direct its own replication in the absence of the smaller genomic RNA (RNA 2 [1,400 nucleotides], which encodes the viral coat proteins) (6, 7, 12, 15, 19). RNA 1 and its encoded enzyme activities thus constitute an autonomous RNA replicon. Under normal circumstances, however, both RNAs 1 and 2 are templates for the replicase and are copied by a mechanism that involves the synthesis of the corresponding negative-stranded RNAs (28). The viral replication cycle is completed by the copackaging of the two positive strands into progeny virions.

The nucleotide sequence of RNA 1 of BBV reveals two open reading frames: one for a polypeptide of 112.3 kDa (protein A), which spans almost the entire RNA molecule, and one for a polypeptide of 11.6 kDa (protein B), which overlaps the C terminus of protein A in a different reading frame (7, 18). However, protein B is translated not from RNA 1 but from a small subgenomic RNA (RNA 3) which consists of the 3′-terminal 389 nucleotides of RNA 1 and is synthesized during RNA replication (13, 16). Since protein A contains the GDD amino acid sequence motif that is characteristic of RNA polymerases, this protein is probably the catalytic subunit of the RNA replicase. The role, if any, of protein B in RNA replication is unknown, as is the possible involvement of host cell proteins. However, the wide range of cell types in which the RNA replicase is active implies that any necessary cellular components must be highly conserved among insects, plants, and higher animals.

Full-length cDNAs of FHV RNAs 1 and 2 have been inserted into appropriate transcription plasmids and transcribed in vitro to yield FHV RNAs that are infectious for *Drosophila* cells (8). Among the enzymes successfully used for this in vitro transcription is the RNA polymerase of

bacteriophage T7. This enzyme can also be expressed in functional form in higher eukaryotic cells from a VV-T7 RNA polymerase recombinant (14), and therefore this system was chosen for an attempt to express a functional RNA replicon from a DNA vector.

## MATERIALS AND METHODS

**Cells and viruses.** Baby hamster kidney (BHK21) cells were grown at 37°C as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 5% newborn calf serum in an atmosphere containing 5% $CO_2$. Before use, the cells were plated in 35-mm-diameter wells of six-well cluster tissue culture plates and grown overnight to reach confluence at about $10^6$ cells per well. African green monkey kidney (BSC40) cells (17) were grown as monolayer cultures in DMEM containing 5% fetal bovine serum. *D. melanogaster* (Schneider line 1) cells were grown as monolayer cultures in Schneider's medium (GIBCO) containing 15% fetal bovine serum at 28°C in an atmosphere containing 5% $CO_2$.

The recombinant VV that expresses the T7 bacteriophage RNA polymerase (vTF7-3) was kindly provided by Bernard Moss (National Institutes of Health) (14). It was grown and its titer was determined by plaque assay on BSC40 cells. A stock of FHV (previously known as the W17 isolate of BBV) (19, 31) was kindly supplied by Tom Gallagher and Roland Rueckert (University of Wisconsin—Madison). It was grown on *Drosophila* cells and purified as described for BBV by Friesen et al. (11).

**Plasmid origins and construction.** Transcription plasmids were constructed from pGEM4 (Promega Biotec) by the following steps. (i) The *Eco*RI restriction site in the pGEM4 multiple-cloning site was destroyed by cleavage, filling, and religation. (ii) A 140-bp *Bam*HI-*Bgl*II restriction fragment from pET3 (plasmid kindly supplied by William Studier [Brookhaven National Laboratory]) (27) which contained the Tϕ transcription terminator was inserted into the *Bam*HI site of the pGEM4 multiple-cloning site such that the terminator was in the same orientation as the pGEM4 T7 promoter. (iii) A 65-bp *Bam*HI fragment from pJOSP6-self (plasmid kindly provided by J. Bujarski, Northern Illinois University, De Kalb) (9) which encodes the self-cleaving ribozyme of satellite tobacco ringspot virus (sTobRV) RNA was inserted into the unique *Bam*HI site between the T7 promoter and terminator; its orientation was such that transcripts from the T7 promoter would contain the self-cleavage activity. The nucleotide sequence of the sTobRV cDNA fragment in these constructs differed from the published sequence (3) at two positions: it contained T instead of C at position −1 and C instead of T at position +11 relative to the site of self-cleavage between residues −1 and +1. (iv) Full-length FHV cDNAs were inserted as *Pst*I-*Xba*I fragments between the *Pst*I and *Xba*I sites of the multiple-cloning site. Plasmids containing full-length cDNA copies (FHV 1 and 2 cDNAs) of FHV RNAs 1 and 2 were generously provided by Ranjit Dasgupta and Paul Kaesberg (University of Wisconsin—Madison) (8). Both cDNAs had been constructed with unique *Pst*I and *Xba*I cleavage sites at their 5′ and 3′ ends, respectively. The last nucleotide of the *Pst*I cleavage site (G) and the first nucleotide of the *Xba*I cleavage site (T) corresponded to the first and last nucleotides of the FHV sequences, respectively. Step iv in the constructions created plasmids that had 26 extra base pairs between the site of transcriptional initiation and the 5′ ends of the FHV sequences and 43 extra base pairs between the 3′ ends of the FHV sequences and the site of ribozyme-mediated self-cleavage. (v) Thirty-one of the 43 extra base pairs between the 3′ ends of the FHV sequences and the site of ribozyme-mediated self-cleavage were removed by digestion with *Xba*I and *Eco*RI. (The *Eco*RI site was derived from the 65-bp ribozyme fragment; the *Eco*RI site in the pGEM4 multiple-cloning site had been destroyed in step i.) The DNA termini were made blunt by digestion with mung bean nuclease, and the blunt termini were religated. This created plasmids that contained 12 bp (CGATACCCTGTT) between the 3′ ends of the FHV sequences and the site of self-cleavage (see Fig. 1). (vi) Sixteen of the extra 26 bp between the 5′ ends of the FHV sequences and the point of transcriptional initiation by the T7 RNA polymerase were removed by digestion with *Hin*dIII and *Pst*I; the DNA termini were made blunt by digestion with mung bean nuclease, and the blunt termini were religated. This created plasmids that contained 10 DNA base pairs (GGGAGACCCA) between the major site of transcriptional initiation and the 5′ end of the FHV 1 or 2 cDNA insert. (Notice that this sequence differs slightly from that predicted from the sequence of pGEM4.) (vii) In order to further improve the 5′ ends of the FHV cDNA transcripts, the *Pvu*II-*Pst*I DNA fragment of pGEM4 (which contains the T7 promoter) was replaced with a *Sma*I-*Pst*I DNA fragment from pMJ5 (plasmid kindly supplied by Paul Ahlquist [University of Wisconsin—Madison]) (1). This DNA fragment contained the same T7 promoter but had different adjacent restriction enzyme sites, including a *Stu*I cleavage site between nucleotides 2 and 7 from the point of transcriptional initiation. (viii) Twenty-six of the extra 28 bp between the 5′ ends of the FHV sequences and the point of transcriptional initiation were removed by digestion with *Stu*I and *Pst*I of plasmid DNA grown in *dcm*-negative BL21 (DE3) cells. The DNA termini were made blunt by digestion with mung bean nuclease, and the blunt termini were religated. This created plasmids that contained 2 bp (GG) between the major site of transcriptional initiation and the 5′ end of the FHV 1 or 2 cDNA insert (see Fig. 1).

A plasmid that contained the FHV cDNA sequence encompassing the complete open reading frame for protein B was constructed by digestion with *Sfa*NI of the FHV 1-containing plasmid whose structure is shown in Fig. 1. This restriction enzyme cuts the DNA 9 bp to the 3′ side of the site that corresponds to the 5′ end of RNA 3, which is 10 bp before the ATG at the beginning of the open reading frame for protein B. The DNA terminus was made blunt by reaction with the large fragment of *Escherichia coli* DNA polymerase I and ligated to octameric *Hin*dIII linkers. A 585-bp *Hin*dIII-*Kpn*I fragment that encompassed the FHV RNA 3 region, the sTobRV cDNA, and the Tϕ terminator was transferred to pGEM4 between the *Hin*dIII and *Kpn*I sites. This created a plasmid that had 26 bp between the site of transcriptional initiation by T7 RNA polymerase and the ATG codon at the start of the protein B open reading frame and 12 bp between the 3′ end of the FHV sequence and the site of ribozyme-mediated self-cleavage. The final plasmids were purified by banding on cesium chloride-ethidium bromide isopycnic gradients.

**Transcription in vitro.** For the synthesis of $^3$H-labeled RNAs, in vitro transcription was performed for 90 min at 37°C in reaction mixtures containing Tris-HCl ($4 \times 10^{-2}$ M, pH 8.0), $MgCl_2$ ($8 \times 10^{-3}$ M), NaCl ($2.5 \times 10^{-2}$ M), spermidine trihydrochloride ($2 \times 10^{-3}$ M), dithiothreitol ($5 \times 10^{-3}$ M), all four ribonucleoside triphosphates ($2 \times 10^{-3}$ M each), [$^3$H]UTP (50 μCi/ml), circular or linearized template DNA (40 μg/ml), and T7 RNA polymerase (Bethesda Re-

search Laboratories; $5 \times 10^3$ U/ml). For the synthesis of $^{32}$P-labeled RNAs for use as strand-specific hybridization probes, in vitro transcription of linearized DNA was performed for 20 min at 37°C in reaction mixtures containing the same components as the mixtures described above, except that the concentrations of ATP, GTP, and CTP were reduced to $1 \times 10^{-3}$ M and that of UTP was reduced to $7 \times 10^{-5}$ M. [$\alpha$-$^{32}$P]UTP was included at a specific activity of 50 Ci/mmol. In each case, transcription was terminated by digestion of the DNA template for 15 min with RNase-free DNase (Promega Biotec; 1 U/$\mu$g of DNA) followed either by extraction of the [$^3$H]RNA products with phenol-chloroform or by gel filtration of the [$^{32}$P]RNA products on spun columns of Sephadex G-50.

**Purification of FHV RNAs.** FHV RNAs 1 and 2 were extracted from purified virus with phenol and chloroform in the presence of sodium dodecyl sulfate (SDS) at 45°C (15). FHV is extraordinarily stable and was substantially resistant to extraction with phenol-chloroform at room temperature. FHV RNA 1 was purified free of RNA 2 by multiple sequential passages of transfection of *Drosophila* cells using extremely low RNA concentrations. Since RNA 1 is capable of autonomous replication, this procedure resulted in its enrichment and the eventual elimination of RNA 2 (1a). The resulting preparations of RNA 1 were then amplified by replication at higher concentrations in *Drosophila* cells, and the total cytoplasmic RNAs were extracted from the transfected cells, precipitated, and used as a source of (biologically) pure RNA 1. The abundance of FHV RNA 1 in these preparations was approximately the same as that of rRNA (see Fig. 7A). RNA 2 was undetectable even by hybridization (see Fig. 5).

**Infection and transfection of cells.** Monolayers of BHK21 cells were washed once with phosphate-buffered saline containing magnesium chloride ($10^{-3}$ M) and infected with vTF7-3 at a multiplicity of infection of 5 PFU per cell. The virus was allowed to adsorb for 60 min at room temperature before the inoculum was removed and DMEM without serum was applied (1 ml/35-mm-diameter well). The infected cells were incubated for 15 to 30 min at 28°C in an atmosphere of 5% $CO_2$ before a mixture of RNA, plasmid DNA (5 $\mu$g in 10 $\mu$l), and Lipofectin (Bethesda Research Laboratories; 10 $\mu$g in 10 $\mu$l) was added directly to the medium. Incubation at 28°C was continued for the times indicated below for the individual experiments.

**RNA labeling, extraction, and analysis.** For many experiments, it was necessary first to inhibit DNA-dependent RNA synthesis before labeling the products of RNA replication by incorporation of [$^3$H]uridine. Transcription of plasmid DNA by T7 RNA polymerase was found to be unusually resistant to inhibition by actinomycin D, so before labeling, the BHK21 cell monolayers were treated with 10 $\mu$g of actinomycin D per ml for 30 min at 28°C. The medium was then replaced with medium containing 20 $\mu$Ci of [$^3$H]uridine per ml and 10 $\mu$g of actinomycin D per ml (1 ml/35-mm-diameter well), and incubation at 28°C was continued for 1 or 2 h as indicated below for the individual experiments. The cells were lysed in buffer containing Tris-HCl ($10^{-2}$ M, pH 7.4), tetrasodium EDTA ($6.6 \times 10^{-2}$ M), Nonidet P-40 (1%, vol/vol), and sodium deoxycholate (0.4%, wt/vol) at 0°C (200 $\mu$l/35-mm-diameter well), and the cell nuclei were removed by 12 s of centrifugation in a microcentrifuge. Cytoplasmic RNAs were extracted from the supernatants with phenol-chloroform-isoamyl alcohol, reextracted with chloroform-isoamyl alcohol, and precipitated with ethanol. The dried RNA pellets were redissolved in Tris-HCl ($10^{-2}$ M, pH 8.0)–EDTA ($10^{-3}$ M), and samples corresponding to the RNA from about $10^5$ cells were digested with RNase-free DNase. For analysis under denaturing conditions, the RNA samples were treated with formaldehyde at 65°C in the presence of formamide and subjected to electrophoresis on 1% agarose–formaldehyde gels (22). After electrophoresis, either (i) the gels were fixed with 30% methanol–10% acetic acid and subjected to fluorography (2) or (ii) the resolved RNAs were transferred by blotting to GeneScreen Plus (NEN), and the blots were baked in vacuo at 80°C for 2 h and then subjected to hybridization. For analysis under nondenaturing conditions, the RNA samples were subjected to electrophoresis on SDS–5% polyacrylamide gels (21), fixed as described above, and visualized by fluorography (5).

**Hybridization of Northern (RNA) blots.** After being baked, the blots were prehybridized overnight at 68°C in a solution containing 50% formamide, 1.0 M NaCl, 1% SDS, 5× Denhardt's solution, 0.5 mg of yeast RNA per ml, and 0.2 mg of boiled salmon sperm DNA per ml. $^{32}$P-labeled RNA probe ($0.3 \times 10^6$ to $0.8 \times 10^6$ cpm), synthesized by transcription in vitro, was then added directly to the hybridization bag and allowed to hybridize for 18 h at 68°C. The filters were washed successively in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, 2× SSC containing 1% SDS at 65°C, and finally 0.1× SSC at room temperature. The dried filters were exposed to X-ray film at −70°C with intensifying screens.

**Protein labeling, extraction, and analysis.** Before labeling of proteins by incorporation of [$^{35}$S]methionine, the BHK21 cell monolayers were starved for methionine by incubation at 28°C in methionine-free DMEM for 30 min. The medium was then replaced with methionine-free DMEM containing 30 $\mu$Ci of [$^{35}$S]methionine per ml (0.5 ml/35-mm-diameter well), and incubation at 28°C was continued for 1 h. The cells were lysed as described for RNA analysis, the nuclei were removed by 12 s of centrifugation in a microcentrifuge, and samples corresponding to the cytoplasmic proteins from about $4 \times 10^4$ cells were subjected to electrophoresis on SDS–12.5% polyacrylamide gels (21). After electrophoresis, the gels were fixed in 30% methanol–10% acetic acid, dried, and exposed to X-ray film at room temperature.

## RESULTS

**Synthesis of discrete cDNA transcripts in vitro and in vivo.** Since the process of RNA replication initiates at the 3′ end of the RNA template, it seemed likely that the structure of this end of the FHV transcripts would be important for their ability to be replicated. Similarly, since the 3′ ends of the negative-stranded RNAs would be generated by replication of the 5′ ends of the positive-stranded transcripts, it seemed likely that the structure of the 3′ end would also be important for replication. Indeed, FHV transcripts made in vitro that have long 3′ or 5′ extensions show greatly reduced or no infectivity (8), and preliminary experiments showed that similar transcripts generated in vivo were not competent templates for replication (data not shown). DNA templates that direct the synthesis of transcripts with precise 5′ termini can usually be constructed by accurate positioning of the promoter site. Furthermore, it is easy to generate transcripts that have perfect or nearly perfect 3′ ends in vitro by using RNA polymerase runoff from template DNA linearized by cleavage at a prepositioned restriction enzyme site. However, attempts to use the same approach to produce RNAs with precise 3′ termini in vivo were unsuccessful. Linearized DNA either was not detectably transcribed in cells infected

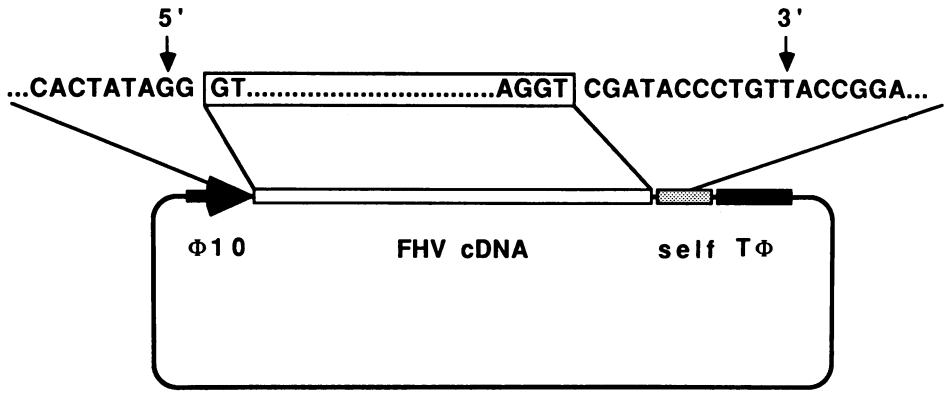


FIG. 1. Diagrammatic representation of the structure of the transcription plasmids used to generate transcripts of FHV 1 and 2 cDNAs. The open boxes encompass the full-length FHV 1 or 2 cDNA sequence, and the two shaded boxes represent a cDNA encoding the sTobRV self-cleaving ribozyme (self) and the bacteriophage T7 transcriptional terminator (Tϕ). ϕ10 represents the T7 ϕ10 promoter sequence. The nucleotides designated 5′ and 3′ correspond in the DNA sequence to the nucleotides predicted for the 5′ and 3′ ends, respectively, of the self-cleaved RNA transcripts synthesized by T7 RNA polymerase.

with a VV-T7 RNA polymerase recombinant or was transcribed only after recircularization within VV-infected cells (data not shown).

A combination of two sequence elements was necessary to achieve a DNA structure which, in circular form, could direct the synthesis of RNA transcripts that had predetermined 3′ ends: (i) a cDNA fragment that encodes a self-cleaving ribozyme derived from sTobRV RNA (4) and (ii) a bacteriophage T7 transcriptional terminator (27). These elements were juxtaposed downstream of full-length cDNA copies of FHV RNA 1 or 2 in transcription plasmids that also contained a bacteriophage T7 promoter (Fig. 1).

The products of in vitro transcription of circular plasmids that contained neither, one, or both of the self-cleaving ribozyme and the T7 terminator were analyzed by electrophoresis on agarose-formaldehyde gels (Fig. 2). DNA linearized by restriction enzyme cleavage at the 3′ end of the FHV 1 cDNA sequence directed the synthesis of a single major runoff transcript (Fig. 2, lane 2) that comigrated with authentic 3.1-kb FHV RNA 1 that was synthesized in cells transfected with FHV RNAs 1 and 2 (Fig. 2, lane 1) or RNA 1 alone (Fig. 2, lane 7). The RNA products directed by circular DNA templates were drastically affected by the presence or absence of the sTobRV ribozyme and T7 terminator. With neither element present, the RNA products were a heterodisperse population that included very large RNA molecules (Fig. 2, lane 3). Inclusion of the ribozyme at the 3′ end of the FHV 1 cDNA instead yielded a single major transcript of about 6.1 kb (Fig. 2, lane 4). This corresponded to the size expected not for a transcript of the FHV 1 cDNA insert but for a transcript of the entire plasmid, and hybridization analysis showed that this RNA contained both insert and vector sequences (data not shown). The 6.1-kb transcript dominated the RNA products presumably because of the highly processive nature of the T7 RNA polymerase reaction (32). Thus, without the ribozyme the RNA products were multimeric concatemers, whereas with the ribozyme the concatemers self-cleaved to yield predominantly plasmid-length transcripts. A small amount of RNA that comigrated with FHV RNA 1 was detectable (Fig. 2, lane 4), with one molecule presumably being produced from the 5′ end of each concatemeric transcript. The lack of RNA larger than 6.1 kb indicated that the ribozyme-mediated cleavage was essentially complete under the conditions of transcription in vitro.

This interpretation was confirmed by the combined effects of the ribozyme and the transcriptional terminator on the pattern of RNA products. Circular DNAs that contained both elements directed the predominant synthesis of RNA that comigrated with 3.1-kb FHV RNA 1 (Fig. 2, lane 5). The

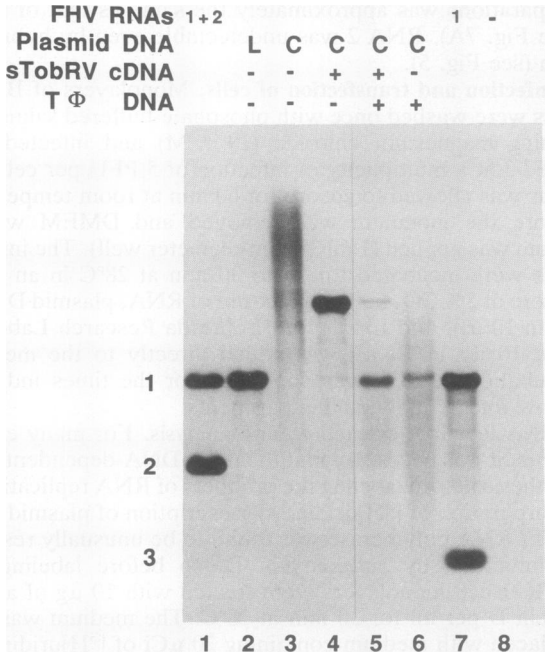


FIG. 2. Analysis of transcripts made in vitro. Plasmids containing FHV 1 cDNA were transcribed by T7 RNA polymerase in vitro, and the $^3$H-labeled products were resolved by electrophoresis on an agarose-formaldehyde gel and visualized by fluorography. Authentic marker RNAs, identified on the left, were labeled during a 2-h incorporation of [$^3$H]uridine in the presence of actinomycin D at 22 h after transfection of BHK21 cells with 100 ng of FHV RNAs 1 and 2 (lane 1) or FHV RNA 1 alone (lane 7) per $10^6$ cells or no RNA (lane 8). RNAs made in vitro were directed by plasmid DNA linearized by cleavage at the 3′ end of the FHV 1 cDNA insert (lane 2) or by circular DNAs containing neither the sTobRV cDNA nor Tϕ (lane 3), the sTobRV cDNA but not Tϕ (lane 4), the sTobRV cDNA and Tϕ (lane 5), or Tϕ but not the sTobRV cDNA (lane 6).

plasmid-length 6.1-kb RNA that was also made by this plasmid can be attributed to incomplete termination of transcription at the T7 terminator. Incomplete termination was also observed in transcription reactions directed by circular DNA that contained only the T7 terminator at the 3′ end of the FHV 1 cDNA (Fig. 2, lane 6) and has been noted by others (27).

A similar set of plasmids that contained full-length FHV 2 instead of FHV 1 cDNA was constructed. Analysis of their products of transcription in vitro confirmed the picture described above: with the ribozyme alone, the RNA products were predominantly of plasmid length (4.4 kb in this case), whereas with both the ribozyme and the T7 terminator, the major RNA product that was directed by circular DNA corresponded to the size of the cDNA insert (1.4 kb in this case) (data not shown). Similarly, a circular plasmid that contained a 380-bp cDNA containing the B protein open reading frame, followed by the sTobRV cDNA and the Tϕ terminator, directed the synthesis in vitro of an RNA that comigrated with authentic FHV RNA 3 (see Fig. 3, lane 8).

These results demonstrated that circular plasmids with the general structure shown in Fig. 1 could be used to generate RNA transcripts having discrete sizes that corresponded to the sizes of their cDNA inserts. The 5′ ends of the transcripts would be determined by the site of transcriptional initiation of the T7 RNA polymerase, whereas their 3′ ends would be determined by the site of ribozyme-mediated cleavage. The nucleotide sequences of both sites are subject to some constraints. For example, the T7 RNA polymerase has a strong preference for initiation by incorporation of a purine residue and is severely inhibited by A residues near the 3′ end of the template (corresponding to U residues near the 5′ ends of the transcripts) (23). The sTobRV ribozyme has specific sequence requirements for cleavage, some of which involve nucleotides on the 5′ side of the cleavage site (i.e., nucleotides at the extreme 3′ end of the cleaved transcript) (3, 20). Because of these constraints, it has not been possible to construct plasmids that would direct the transcription of FHV RNA molecules with perfect termini. The most successful constructs to date have the junction sequences shown in Fig. 1 and direct the synthesis of FHV RNAs that contain two additional nucleotides at their 5′ ends and 12 additional nucleotides at their 3′ ends. Moreover, the 3′-terminal U residue carries a 2′,3′ cyclic phosphate as a result of the mechanism of cleavage mediated by the sTobRV ribozyme (26).

When circular plasmid DNAs of the general structure shown in Fig. 1 that contain FHV 1, 2, or 3 cDNA were transfected into cells that expressed T7 RNA polymerase from a VV recombinant, they directed the synthesis of transcripts that were similar to those made in vitro and that corresponded closely in size with authentic FHV RNA 1, 2, or 3, respectively (Fig. 3). The levels of transcription in vivo were sufficiently high that the plasmid-derived transcripts were clearly visible among the total spectrum of cytoplasmic RNAs that could be labeled by incorporation of [$^3$H]uridine during a 2-h pulse given 15 h after transfection (Fig. 3, lanes 3 to 5).

**Replication of RNA derived from transcription of FHV 2 cDNA.** I next tested the ability of RNA derived by transcription of plasmids containing FHV 2 cDNA to function as a template for replication by the FHV RNA replicase. As described above, the entire viral contribution to this enzyme activity is encoded in FHV RNA 1, which can replicate autonomously in appropriate cells. Therefore, cells were transfected with purified authentic FHV RNA 1 to provide a

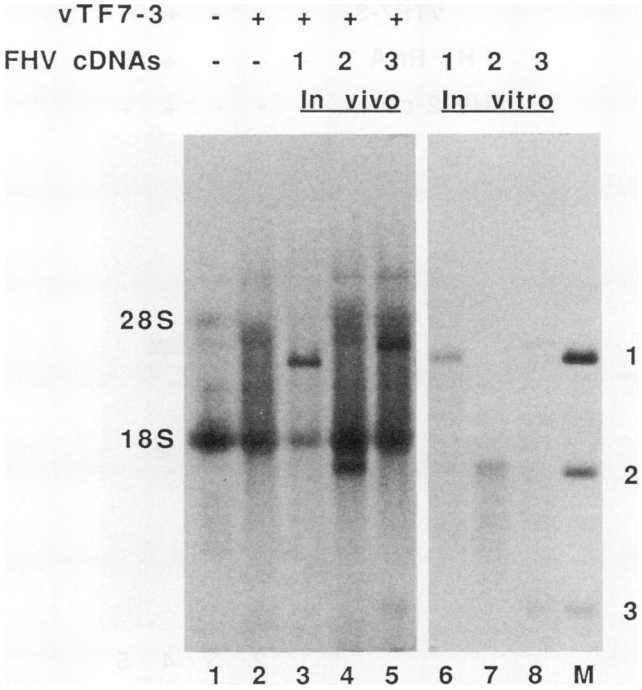


FIG. 3. Comparison of RNAs made by transcription of circular plasmids in vivo (lanes 3 to 5) and in vitro (lanes 6 to 8). BHK21 cells were left uninfected (lane 1) or infected with vTF7-3, a recombinant VV that expresses the DNA-dependent RNA polymerase of bacteriophage T7 (14) (lanes 2 to 5). The cells were then transfected with no DNA (lane 2) or with 5 μg of circular plasmids containing FHV 1 cDNA (lane 3), FHV 2 cDNA (lane 4), or FHV 3 cDNA (lane 5) per $10^6$ cells. After 15 h of incubation at 28°C, the cells were labeled by incorporation of [$^3$H]uridine for 2 h in the absence of actinomycin D, and the cytoplasmic RNAs were extracted, resolved by electrophoresis on agarose-formaldehyde gels, and visualized by fluorography. The same three circular plasmids were transcribed by T7 RNA polymerase in vitro, and their RNA products are shown in lanes 6, 7, and 8, respectively. Authentic FHV RNAs 1, 2, and 3 are shown in lane M and identified on the right. 28S and 18S rRNAs labeled in uninfected cells (lane 1) are identified on the left.

source of replicase activity. Templates for replication were generated by intracellular transcription of plasmids that contained FHV 2 cDNA with T7 RNA polymerase expressed from the VV recombinant. When transfected alone into BHK21 cells at 28°C, FHV RNA 1 replicated efficiently and synthesized both RNA 1 and RNA 3 in a reaction that, like those of other RNA-dependent RNA syntheses, was insensitive to inhibition by actinomycin D (Fig. 4, lane 2). The low temperature was necessary for FHV RNA replicase activity in BHK21 cells. Concurrent infection of the cells with the VV-T7 RNA polymerase recombinant did not greatly affect the pattern or amount of RNA replication (Fig. 4, lane 3). However, when FHV 2 transcripts were supplied by cotransfection of a plasmid that contained FHV 2 cDNA downstream of a T7 promoter, three effects were observed: replication of FHV RNA 2, diminished synthesis of RNA 3, and elimination of a series of labeled RNAs intermediate in size between RNAs 1 and 3 (Fig. 4, lane 4). That the synthesis of RNA 2 was mediated by the FHV RNA replicase in an RNA-dependent reaction, rather than exclusively by the T7 RNA polymerase in a DNA-dependent reaction, was shown by its insensitivity to actinomycin D. Moreover, in the absence of the FHV RNA replicase, no actinomycin

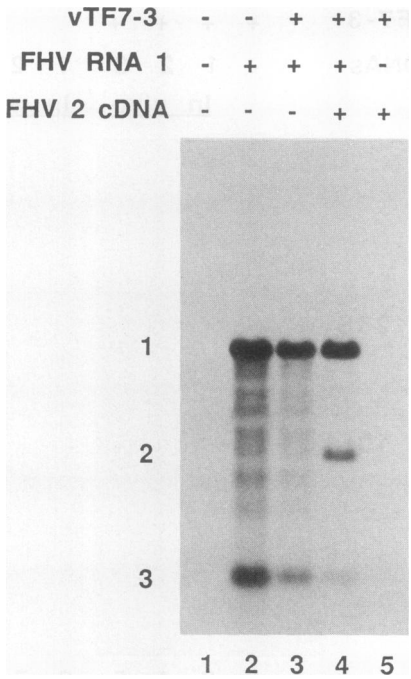


FIG. 4. Replication of authentic FHV RNA 1 and plasmid-derived transcripts of FHV 2 cDNA. BHK21 cells were left uninfected (lanes 1 and 2) or infected with vTF7-3 (lanes 3 to 5). Immediately after infection, the cells were left untransfected (lane 1) or were transfected with FHV RNA 1 (lanes 2 to 4) or with of circular DNA (5 μg/$10^6$ cells) of the structure shown in Fig. 1 that contained FHV 2 cDNA (lanes 4 and 5). After 18 h of incubation at 28°C, the products of RNA replication were labeled by 2 h of incorporation of [$^3$H]uridine in the presence of actinomycin D, extracted, resolved by electrophoresis on agarose-formaldehyde gels, and visualized by fluorography. The migration positions of authentic FHV RNAs 1, 2, and 3 that were run in an adjacent lane are shown on the left.

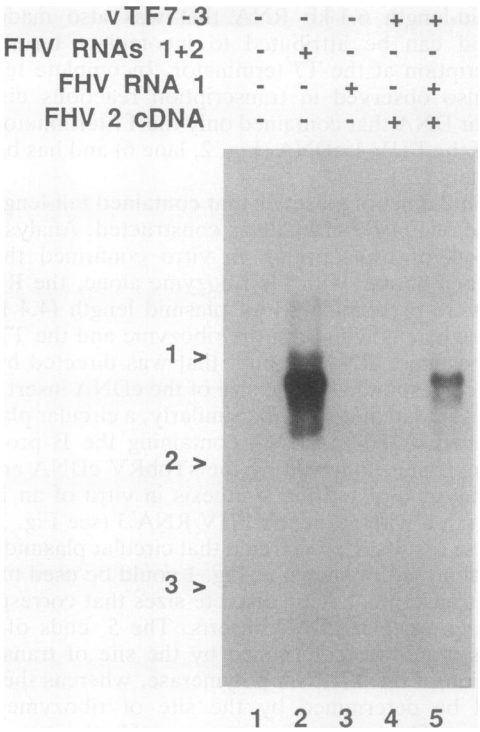


FIG. 5. Analysis of negative-stranded RNAs that accumulated during replication of authentic FHV RNA 2 (lane 2) and plasmid-derived transcripts of FHV 2 cDNA (lane 5). BHK21 cells were left uninfected (lanes 1 to 3) or infected with vTF7-3 (lanes 4 and 5) and then transfected with 180 ng of FHV RNAs 1 and 2 (lane 2) or FHV RNA 1 alone (lanes 3 and 5) per $10^6$ cells or 5 μg of a circular plasmid containing FHV 2 cDNA per $10^6$ cells (lanes 4 and 5). After 23 h (lanes 1 to 3) or 30 h (lanes 4 and 5) of incubation at 28°C, cytoplasmic RNAs were extracted, digested with RNase-free DNase, and resolved by electrophoresis on an agarose-formaldehyde gel. After electrophoresis, the RNAs were transferred by blotting to a charged nylon membrane and hybridized to $^{32}$P-labeled, positive-stranded FHV 2 RNA made by runoff transcription in vitro, and negative-stranded FHV 2 RNAs were detected by autoradiography of the washed membrane. The migration positions of authentic positive-stranded FHV RNAs 1, 2, and 3 are indicated on the left.

D-resistant synthesis of RNA 2 was observed (Fig. 4, lane 5). The inhibition of RNA 3 synthesis was an expected consequence of RNA 2 replication, since it has been shown that authentic RNA 2 of BBV inhibits the synthesis of BBV RNA 3 (15), and similar effects were observed with FHV (compare lanes 1 and 7 of Fig. 2).

The RNAs with sizes intermediate between those of RNAs 1 and 3 (Fig. 4, lanes 2 and 3) were of unknown origin. They were made in an actinomycin D-resistant reaction during replication of RNA 1 alone (see also Fig. 2, lane 7) but not when replicating RNA 2 was present (Fig. 4, lane 4; see also Fig. 2, lane 1). From the size distribution of these RNAs, it seems likely that they were products related to the replication of RNA 1 whose synthesis was suppressed either by authentic RNA 2 or by replicable RNA 2 transcripts. Further work is necessary to verify this interpretation, but the elimination of the intermediate-sized RNAs by replication of the transcripts of FHV 2 cDNA was another indication of the functionality of the plasmid-derived RNA.

Further evidence that the plasmid-derived FHV 2 transcripts were competent templates for replication by authentic FHV RNA replicase was provided by detection of negative-stranded FHV 2-specific RNAs. Cytoplasmic RNAs were resolved by electrophoresis on denaturing agarose-formaldehyde gels, transferred to a charged nylon membrane, and hybridized with a $^{32}$P-labeled positive-sense FHV RNA 2 probe. Negative-sense FHV RNA 2 was readily detected in BHK21 cells that had been transfected with authentic FHV virion RNAs 1 and 2 but was undetectable in cells transfected with FHV RNA 1 alone (Fig. 5, lanes 2 and 3, respectively). Surprisingly, most of the negative-sense RNA 2 migrated significantly more slowly than the 1.4-kb positive-sense RNA 2 and showed an apparent size of about 2.8 kb. It is unclear whether this result was due to incomplete strand separation of a double-stranded replicative intermediate RNA on the agarose-formaldehyde gels or whether the FHV 2 negative-strand sequences were really present in RNA molecules with a predominant size of 2.8 kb. Further examination of the properties of these molecules and the mechanism of FHV RNA replication will be necessary to resolve this point. Nevertheless, negative-stranded RNAs, whatever their size and structure, are clearly diagnostic of RNA replication, and their detection in cells expressing authentic FHV replicase and plasmid-derived transcripts of RNA 2 (Fig. 5, lane 5) confirmed that these transcripts were competent templates for replication.

**Replicase expression from transcripts of FHV 1 cDNA.** The

experiments described above showed that the VV-T7 RNA polymerase expression system could be used for intracellular synthesis of RNA molecules that were competent templates for replication by the FHV RNA replicase translated from authentic FHV RNA 1. I next examined whether the same plasmid expression system could be used to direct the synthesis of enzymatically active replicase. The experiment was designed to detect RNA replicase activity by assaying the replication of authentic viral RNAs provided as templates. BHK21 cells were infected with the VV-T7 RNA polymerase recombinant, transfected with a plasmid of the structure shown in Fig. 1 that contained FHV 1 cDNA, and incubated for 14 h at 28°C. Infected cells that received either no plasmid DNA or authentic FHV RNA 1 were included as negative and positive controls, respectively. In order to provide RNA templates of known replication competence, virion-derived FHV RNAs 1 and 2 were then transfected into the cells in the presence of cycloheximide to prevent any replicase expression from the authentic RNA 1. After a further 12-h incubation, replicating RNAs were labeled by the incorporation of [$^3$H]uridine in the presence of actinomycin D and then analyzed by electrophoresis on agarose-formaldehyde gels.

In cells that received authentic FHV RNA 1, replicase activity was detected by the actinomycin D-resistant synthesis of RNAs 1 and 3 (Fig. 6, lane 4). Moreover, RNA replication continued for at least 12 h in the presence of cycloheximide (Fig. 6, lane 5). When provided with authentic FHV RNA 2, the replicase derived from expression of authentic FHV RNA 1 catalyzed its replication (Fig. 6, lane 6). Conversely, no RNA replication was seen either in untransfected cells or when cells were transfected with authentic RNAs 1 and 2 in the continual presence of cycloheximide (Fig. 6, lanes 1 and 3, respectively). These control experiments showed that it was possible to express stable FHV replicase activity in vivo by transfecting cells with authentic FHV RNA 1 and to detect the enzyme by subsequently providing it with a replication-competent template RNA. Importantly, replicase activity was also detected in cells that expressed plasmid-derived FHV 1 cDNA transcripts. Even in the absence of authentic RNAs as templates, a low level of actinomycin D-resistant synthesis of RNAs 1 and 3 was observed (Fig. 6, lane 7). The provision of authentic RNAs 1 and 2 (in the presence of cycloheximide) resulted in detectable levels of RNA 2 replication (Fig. 6, lane 9). This result showed that functional RNA replicase was expressed from plasmid-derived transcripts of FHV 1 cDNA. No replicase activity was expressed from a plasmid that directed the synthesis of a C-terminally truncated 53.5-kDa fragment of protein A (data not shown).

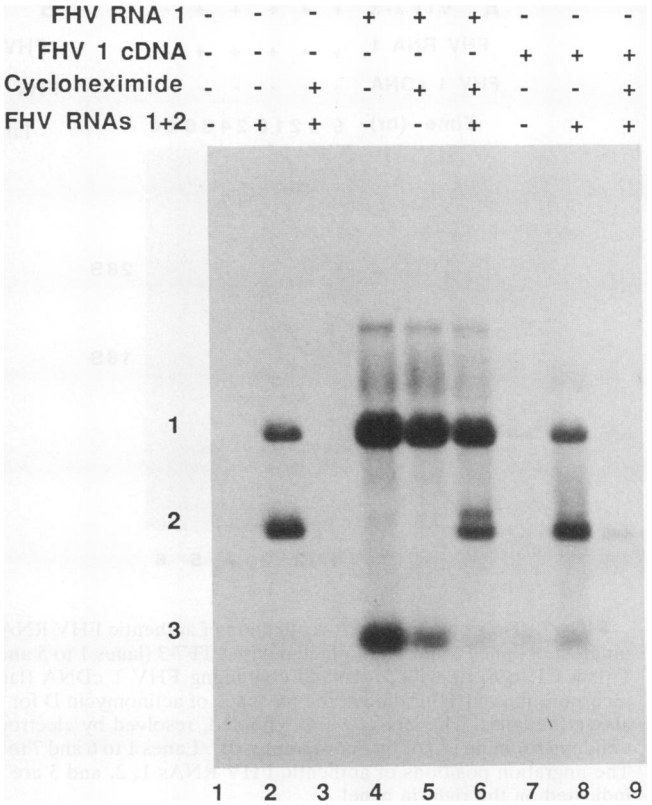


FIG. 6. Detection of RNA replicase activity in cells expressing plasmid-derived transcripts of FHV 1 cDNA. BHK21 cells were infected with vTF7-3 and either left untransfected (lanes 1 to 3) or transfected with FHV RNA 1 (lanes 4 to 6) or with 5 μg of a circular plasmid containing FHV 1 cDNA per $10^6$ cells (lanes 7 to 9). After 14 h of incubation at 28°C, cycloheximide (100 μg/ml) was added to the cells whose RNA products are shown in lanes 3, 5, 6, and 9 and maintained at this concentration throughout the experiment. Thirty minutes later, 100 ng of authentic FHV RNAs 1 and 2 per $10^6$ cells was transfected into the cells whose RNA products are shown in lanes 2, 3, 6, 8, and 9. After a further 12 h of incubation at 28°C, replicating RNAs were labeled by 2 h of incorporation of [$^3$H]uridine in the presence of actinomycin D. Cytoplasmic RNAs were extracted, resolved by electrophoresis on an agarose-formaldehyde gel, and visualized by fluorography. The migration positions of authentic FHV RNAs 1, 2, and 3 are indicated on the left.

**Self-replication of transcripts of FHV 1 cDNA.** Since this expression system was able to direct the synthesis of both a replicable RNA template and an active RNA replicase, I next examined whether these two properties could be combined in a single transcript which could function, like authentic FHV RNA 1, as an autonomous RNA replicon. BHK21 cells were infected with the VV-T7 RNA polymerase recombinant and then transfected either with authentic FHV RNA 1 or with a plasmid of the structure shown in Fig. 1 that contained FHV 1 cDNA. After various intervals of incubation at 28°C, the products of RNA replicase activity were labeled by incorporation of [$^3$H]uridine in the presence of actinomycin D, resolved by electrophoresis on agarose-formaldehyde gels, and visualized either by staining with ethidium bromide (Fig. 7A) or by fluorography (Fig. 7B). In cells transfected with authentic FHV RNA 1, RNA replication was first detected by the synthesis of RNAs 1 and 3 after 12 h and continued at a constant high rate until at least 30 h posttransfection (Fig. 7B, lanes 1 to 5). By this time, FHV RNAs 1 and 3 were clearly visible as ethidium bromide-stained bands and RNA 1 had accumulated to a level comparable to the levels of the 28S and 18S rRNAs (Fig. 7A, lane 5). In cells expressing plasmid-derived transcripts of FHV 1 cDNA, RNA replication was first detected only after 24 h of incubation, but it increased substantially in rate by 30 h of incubation (Fig. 7B, lanes 11 and 12). Both RNA 1 and RNA 3 were synthesized in a reaction that was insensitive to inhibition by actinomycin D, a characteristic of RNA-dependent RNA synthesis. The synthesis of RNA 3 was particularly significant, since this RNA is thought to be made only during RNA replication by internal initiation on the negative strand of RNA 1. Indeed, Northern blot analysis of these samples showed that the time of first appearance of negative-stranded RNA 1 coincided with the onset of detectable RNA

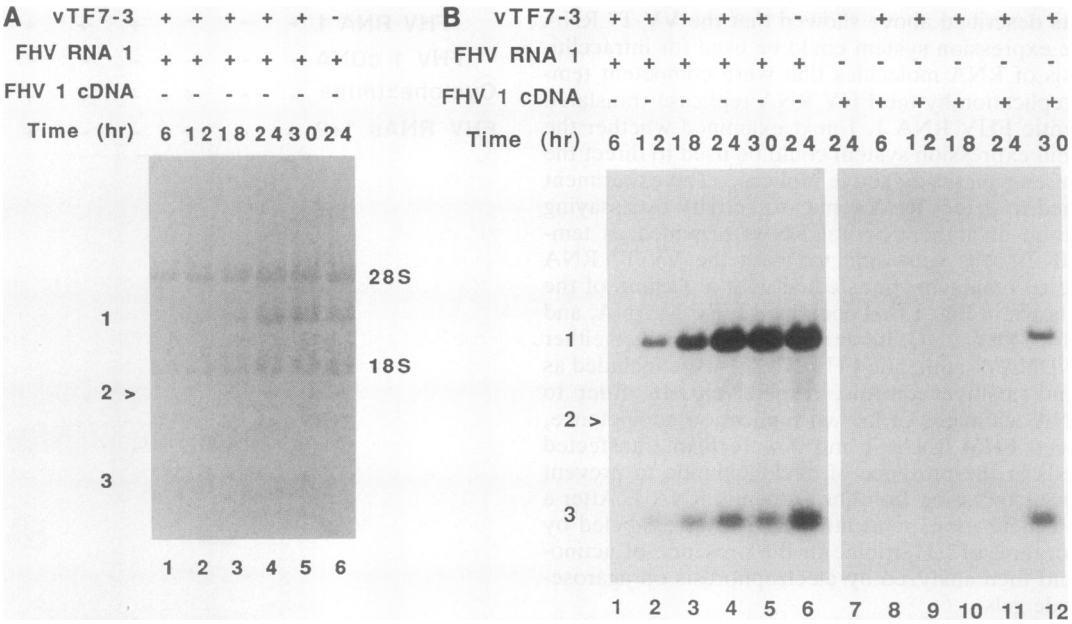


FIG. 7. Time courses of self-replication of authentic FHV RNA 1 and plasmid-derived transcripts of FHV 1 cDNA. BHK21 cells were left uninfected (lanes 6 and 7) or infected with vTF7-3 (lanes 1 to 5 and 8 to 12). The cells were then transfected either with authentic FHV RNA 1 (lanes 1 to 6) or with a plasmid containing FHV 1 cDNA (lanes 7 to 12) and incubated at 28°C. Cytoplasmic RNAs were labeled by incorporation of [$^3$H]uridine in the presence of actinomycin D for 1-h periods before being harvested at the times after transfection indicated above the lanes. The RNAs were extracted, resolved by electrophoresis on an agarose-formaldehyde gel, and visualized by staining with ethidium bromide (A) or by fluorography (B). Lanes 1 to 6 and 7 to 12 in panel B were exposed to X-ray film for 24 h and 14 days, respectively. The migration positions of authentic FHV RNAs 1, 2, and 3 are indicated on the left in both panels, and those of 28S and 18S rRNAs are indicated on the right in panel A.

replication (data not shown). No replication products were detected by labeling or blotting in cells expressing a C-terminally truncated 53.5-kDa fragment of protein A (data not shown). These results showed that plasmid-derived transcripts of FHV 1 cDNA were capable of autonomous replication in BHK21 cells, although the level of replication was lower and its onset was slower than observed with authentic FHV RNA 1. Notice the difference in exposure times of the fluorographs of lanes 1 to 6 and 7 to 12 in Fig. 7B (see legend thereto).

These conclusions were extended by analyzing the RNA replication products by electrophoresis on a 5% polyacrylamide gel under conditions that were not denaturing for double-stranded nucleic acids. Under these conditions, double-stranded RNAs corresponding to authentic FHV RNAs 1, 2, and 3 could be resolved as hypersharp bands (Fig. 8, lanes 1 and 2) that were insensitive to digestion by low levels of RNase (Fig. 8, lane 4). These double-stranded RNAs amounted to only a small percentage of the total labeled FHV RNA. This gel system also resolved single-stranded RNAs 2 (Fig. 8, lane 1) and 3 (Fig. 8, lane 2), but single-stranded RNA 1 remained in the 4.5% polyacrylamide stacking gel and is not shown in Fig. 8. These single-stranded RNA species were sensitive to digestion by low levels of RNase. RNA that was labeled in cells that expressed plasmid-derived transcripts of FHV 1 cDNA contained RNA species that comigrated exactly with double-stranded RNAs 1 and 3 and single-stranded RNA 3 (Fig. 8, lane 3). This result confirmed that the plasmid-derived transcripts of FHV 1 cDNA were capable of autonomous replication and showed that they produced both single- and double-stranded RNA 3 species in the process.

**Protein synthesis during replication of transcripts of FHV 1 cDNA.** The proteins synthesized during replication of plasmid-derived transcripts of FHV 1 cDNA were analyzed by incorporation of [$^{35}$S]methionine, electrophoresis on SDS-polyacrylamide gels, and autoradiography. Superimposed on the background pattern of late VV-specific proteins (Fig. 9A, lane 1), two proteins that comigrated with FHV proteins A and B were readily detectable (Fig. 9A, lane 2). As in cells transfected with authentic FHV RNA 1 (Fig. 9A, lane 3), protein B was by far the more prominent product and exceeded even the major late proteins of VV in its intensity of labeling. Since the methionine content of protein B is not unusual (2.8%), the relative intensities of labeling of the polypeptides shown in Fig. 9A give an approximate measure of the relative rates of synthesis of the different polypeptides. After 24 h of replication of plasmid-derived FHV 1 cDNA transcripts, the rate of synthesis of protein B exceeded that of all other proteins in infected cells. Protein A was synthesized at a lower rate than protein B, but nevertheless its rate of synthesis exceeded that observed in cells transfected with authentic FHV RNA 1.

The mRNA for protein B is RNA 3, which, as described above, is exclusively a product of the RNA replicase. This was demonstrated directly by examining the proteins expressed from transcripts of FHV 1 cDNA at 37°C, a temperature at which the T7 RNA polymerase was active but the FHV RNA replicase was inactive. Under these conditions, plasmid-derived transcripts of FHV 1 cDNA still directed the synthesis of protein A, but the synthesis of protein B was undetectable (Fig. 9B, lane 3). On the other hand, a plasmid containing a cDNA that encompassed only the protein B open reading frame, which was transcribed by T7 RNA polymerase to yield mRNA for protein B directly (Fig. 3, lanes 5 and 8), made similar levels of protein B at 37 and 28°C

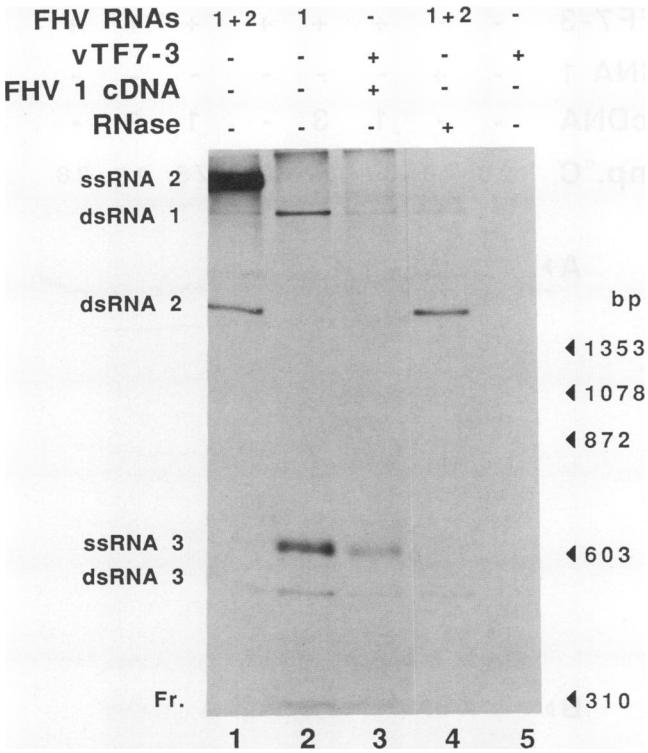


FIG. 8. Analysis of the products of RNA replication by gel electrophoresis under nondenaturing conditions. BHK21 cells were left uninfected (lanes 1, 2, and 4) or infected with vTF7-3 (lanes 3 and 5). The cells were then transfected with either authentic FHV RNAs 1 and 2 (lanes 1 and 4), authentic FHV RNA 1 (lane 2), or a plasmid containing FHV 1 cDNA (lane 3). After 26 h of incubation at 28°C, cytoplasmic RNAs were labeled by 2 h of incorporation of [$^3$H]uridine in the presence of actinomycin D, extracted, resolved by electrophoresis under nondenaturing conditions on an SDS–5% polyacrylamide gel, and visualized by fluorography. The samples in lanes 1, 2, and 4 correspond to the RNA from $2 \times 10^4$ cells; those in lanes 3 and 5 correspond to the RNA from $1 \times 10^5$ cells. Before electrophoresis, the sample in lane 4 was digested with 40 ng of RNase A for 5 min at room temperature. The single-stranded (ss) and double-stranded (ds) RNA species that were resolved under these conditions are identified on the left; Fr. indicates the position of the electrophoresis front. The migration positions of marker fragments of ds DNA and their sizes are indicated on the right. Notice that ds and ss RNAs migrate very differently under these conditions and that the ds DNA fragments are valid size markers only for the ds RNAs.

(Fig. 9B, lanes 4 and 7, respectively). These results show that 24 h after transfection of cells with a plasmid containing FHV 1 cDNA, the most rapidly synthesized protein in the cell was the translation product of an mRNA that was produced exclusively by RNA replication.

## DISCUSSION

The experiments presented in this article describe the cellular expression from circular DNA plasmids of a transcript that could act both as an mRNA for a functional eukaryotic RNA-dependent RNA polymerase and as a template for replication by the same enzyme. Together, these activities constitute the essential components of an autonomous RNA replicon. Upon expression of the replicon in BHK21 cells, one of the RNA products directed protein synthesis at a rate which exceeded that of all other proteins in the infected, transfected cells.

Generation of replicable FHV RNAs by intracellular transcription of plasmids that contained cDNAs depended on the development of a method for the synthesis, from circular DNA templates, of transcripts having defined ends that contained few extraneous nucleotides. This was necessary for two reasons: (i) the FHV RNA replicase has limited tolerance for additional nucleotides at the termini of its templates, and (ii) it was not possible to find conditions for the reproducible synthesis in vivo of runoff transcripts from linearized plasmid DNA. Combination of a self-cleaving ribozyme located directly at the 3′ end of the cDNA with a downstream transcriptional terminator provided replicable transcripts. The transcriptional terminator was necessary to interrupt the highly processive T7 RNA polymerase reaction, but when used alone it yielded transcripts that were predicted to contain 117 terminator-encoded nucleotides at their 3′ ends. These transcripts were not competent templates for RNA replication. Combination of the terminator with a cDNA fragment encoding the sTobRV self-cleaving ribozyme was suggested by the work of Dzianott and Bujarski (10) and resulted in the efficient synthesis of transcripts that were predicted to contain only 12 extraneous 3′ nucleotides. These transcripts were replicable. This method of in vivo generation of RNAs that have predetermined and functional 3′ ends should be generally applicable, since it depends only on the nucleotide sequence of the primary transcript. To test this prediction, VV recombinants in which the VV DNA-dependent RNA polymerase will be used to synthesize replicable RNAs are currently being constructed. Furthermore, the known sequence requirements for activity of the sTobRV ribozyme (3) suggest that it should be possible to construct DNA that directs the synthesis of transcripts with only five or six extra 3′ nucleotides, and these constructions are also in progress. Ultimately, reengineering of the ribozyme itself should allow the construction of DNA that directs FHV RNA transcripts that have only a single nucleotide residue beyond their natural 3′ ends.

Although further improvements at the RNA termini may improve template activity, the results presented above show that plasmid-derived FHV 2 cDNA transcripts with 2 extra 5′ nucleotides and 12 extra 3′ nucleotides were ultimately replicated with an efficiency that was comparable to that of authentic FHV RNA 1 (Fig. 4, lane 4). Authentic FHV RNA 2 replicated with higher efficiency, as was evident from the Northern blot shown in Fig. 5, but it appears that the terminal extensions of the plasmid-derived FHV 2 cDNA transcripts did not greatly impair their ability to initiate replication. It remains to be determined whether the termini of the RNA were corrected during replication, either by nuclease activity or by selection of the correct initiation sites by the RNA replicase, and whether the 3′ ends of the replicating RNAs acquired the chemical and enzymatic nonreactivity that is characteristic of authentic nodavirus RNAs (18). An examination of these questions and of the effects on replication of additional nucleotides at each end of the transcripts is in progress, and the results will be reported elsewhere.

The apparent size of about 2.8 kb for the negative-stranded FHV RNA 2 that was made during replication of either authentic RNA 2 or transcripts of FHV 2 cDNA was a surprise (Fig. 5). The most likely explanation is that despite treatment with formaldehyde and formamide, the RNAs were not completely denatured and migrated as partial

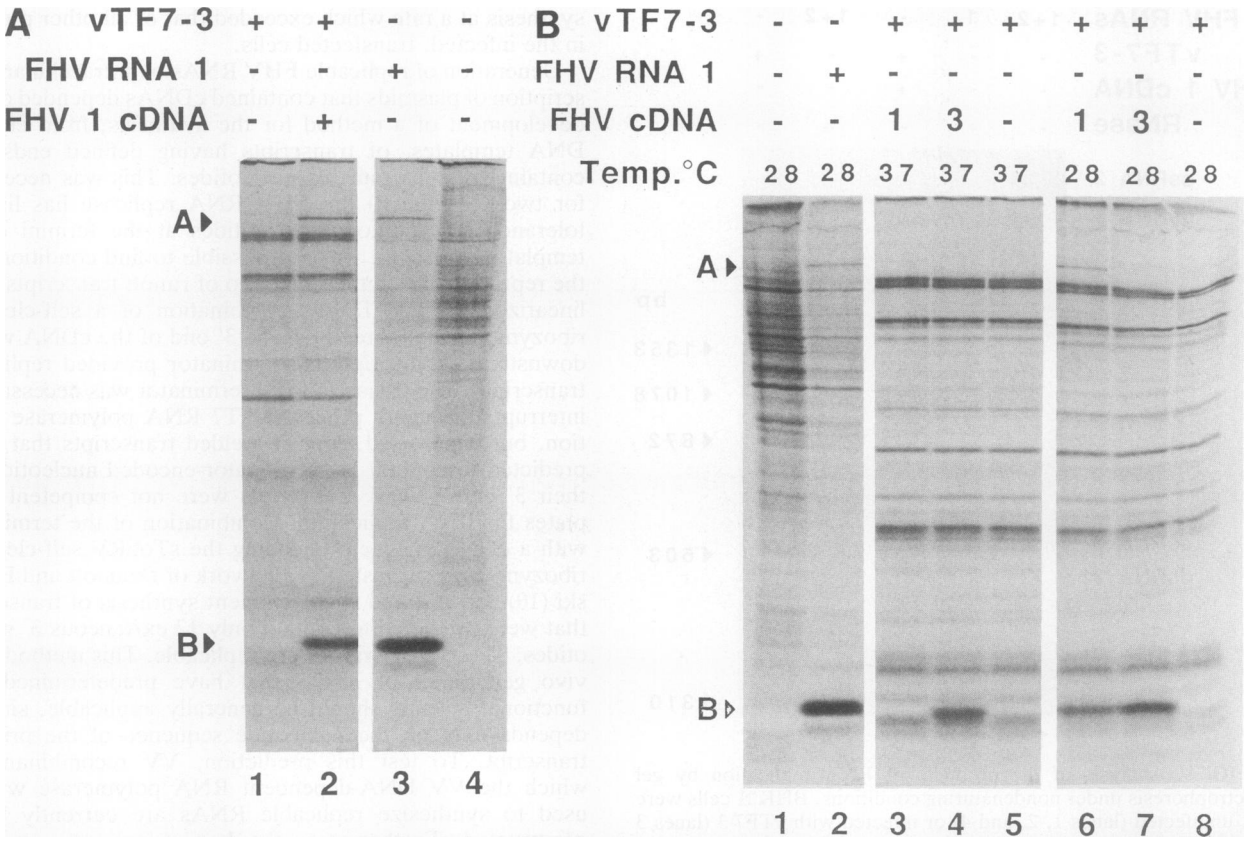


FIG. 9. (A) Protein synthesis during replication of authentic FHV RNA 1 and of plasmid-derived transcripts of FHV 1 cDNA. BHK21 cells were infected with vTF7-3 (lanes 1 and 2) or left uninfected (lanes 3 and 4) and then transfected either with a circular plasmid containing FHV 1 cDNA (lane 2) or with authentic FHV RNA 1 (lane 3). After 23 h of incubation at 28°C, proteins were labeled by 1 h of incorporation of [$^{35}$S]methionine. Cytoplasmic proteins were resolved by electrophoresis on an SDS–12.5% polyacrylamide gel and visualized by autoradiography. (B) Protein synthesis directed by plasmid-derived transcripts of FHV 1 and 3 cDNAs at 28 and 37°C. BHK21 cells were left uninfected (lanes 1 and 2) or infected with vTF7-3 (lanes 3 to 8). They were then transfected with authentic FHV RNA 1 (lane 2) or with circular plasmids containing either FHV 1 cDNA (lanes 3 and 6) or FHV 3 cDNA (lanes 4 and 7) or left untransfected (lanes 5 and 8). After 23 h of incubation at 28°C (lanes 1, 2, and 6 to 8) or 37°C (lanes 3 to 5), proteins were labeled by 1 h of incorporation of [$^{35}$S]methionine. Cytoplasmic proteins were resolved by electrophoresis on an SDS–12.5% polyacrylamide gel and visualized by autoradiography. In both panels, arrowheads A and B indicate proteins A and B, respectively.

hybrids that contained both positive- and negative-stranded RNA molecules. Analysis of replicating FHV RNAs by gel electrophoresis under nondenaturing conditions shows the presence of double-stranded RNAs 1, 2, and 3 (Fig. 8). However, the structures of these molecular species and their roles in RNA replication are unclear. Taken at face value, the results shown in Fig. 5 would suggest that the double-stranded FHV RNA 2 molecules were, in fact, covalently linked hairpin structures containing 1.4 kbp. Intermediates of this structure have been proposed for poliovirus RNA replication by Tobin and coworkers (33), but the proposal remains controversial (25). For nonpolyadenylated RNAs like those of FHV, it is particularly difficult to imagine a mechanism of replication that involves covalently linked positive and negative RNAs yet preserves all the terminal sequence information. Further examination of the negative-stranded FHV RNA sequences under a variety of conditions will be necessary to resolve this question.

Plasmid-derived transcripts of FHV 1 cDNA expressed RNA replicase that was active both on authentic FHV RNAs (Fig. 6) and on the transcripts themselves (Fig. 7 and 8). The latter result constitutes the first demonstration of the cellular expression of an autonomous RNA replicon from a DNA vector system, and the work opens the way for a detailed analysis of the mechanism of FHV RNA replication.

Furthermore, it is now possible to determine which *cis*-acting nucleotide sequences in RNAs 1 and 2 are necessary for replication as well as which sequences in RNA 1 are necessary for the synthesis of RNA 3. This information may make it possible to impart template activity to other, heterologous RNA molecules and thus achieve the goal of using RNA replication to amplify the levels of selected mRNAs expressed from DNA-based vectors. The potential of such a system is illustrated by the fact that in cells expressing transcripts of FHV 1 cDNA, the rate of synthesis of protein B, whose mRNA was produced exclusively by the RNA replicase, exceeded those of all other proteins by 24 h after transfection (Fig. 9A). Moreover, this occurred against the background of VV-mediated shutoff of host cell protein synthesis and at a time when many of the VV structural proteins were themselves being made at substantial rates. Thus, it appears that RNA replication is an effective way to produce abundant functional mRNA. The same approach may be applicable to other DNA-based vector systems.

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## REFERENCES

1. **Allison, R. F., M. Janda, and P. Ahlquist.** 1988. Infectious in vitro transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. J. Virol. **62**:3581–3588.
1a. **Ball, L. A., J. M. Amann, and B. K. Garrett.** 1992. Replication of nodamura virus after transfection of viral RNA into mammalian cells in culture. J. Virol. **66**:2326–2334.
2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
3. **Bruening, G.** 1989. Compilation of self-cleaving sequences from plant virus satellite RNAs and other sources. Methods Enzymol. **180**:546–558.
4. **Buzayan, J. M., W. L. Gerlach, and G. Bruening.** 1986. Satellite tobacco ringspot virus RNA: a subset of the RNA sequence is sufficient for autolytic processing. Proc. Natl. Acad. Sci. USA **83**:8859–8862.
5. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132–135.
6. **Dasgupta, R., A. Ghosh, B. Dasmahapatra, L. A. Guarino, and P. Kaesberg.** 1984. Primary and secondary structure of black beetle virus RNA 2, the genomic messenger for BBV coat protein precursor. Nucleic Acids Res. **12**:7215–7223.
7. **Dasmahapatra, B., R. Dasgupta, A. Ghosh, and P. Kaesberg.** 1985. Structure of the black beetle virus genome and its functional implications. J. Mol. Biol. **182**:183–189.
8. **Dasmahapatra, B., R. Dasgupta, K. Saunders, B. Selling, T. Gallagher, and P. Kaesberg.** 1986. Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. Proc. Natl. Acad. Sci. USA **83**:63–66.
9. **Dzianott, A. M., and J. J. Bujarski.** 1988. An in vitro transcription vector which generates nearly correctly ended RNAs by self-cleavage of longer transcripts. Nucleic Acids Res. **16**:10940.
10. **Dzianott, A. M., and J. J. Bujarski.** 1989. Derivation of an infectious viral RNA by autolytic cleavage of in vitro transcribed viral cDNAs. Proc. Natl. Acad. Sci. USA **86**:4823–4827.
11. **Friesen, P., P. Scotti, J. Longworth, and R. Rueckert.** 1980. Black beetle virus: propagation in *Drosophila* line 1 cells and an infection-resistant subline carrying endogenous black beetle virus-related particles. J. Virol. **35**:741–747.
12. **Friesen, P. D., and R. R. Rueckert.** 1981. Synthesis of black beetle virus proteins in cultured *Drosophila* cells: differential expression of RNAs 1 and 2. J. Virol. **37**:876–886.
13. **Friesen, P. D., and R. R. Rueckert.** 1982. Black beetle virus: messenger for protein B is a subgenomic viral RNA. J. Virol. **42**:986–995.
14. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
15. **Gallagher, T. M., P. D. Friesen, and R. R. Rueckert.** 1983. Autonomous replication and expression of RNA 1 from black beetle virus. J. Virol. **46**:481–489.
16. **Guarino, L. A., A. Ghosh, B. Dasmahapatra, R. Dasgupta, and P. Kaesberg.** 1984. Sequence of black beetle virus subgenomic RNA and its location in the viral genome. Virology **139**:199–203.
17. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. J. Virol. **29**:705–715.
18. **Kaesberg, P.** 1987. Organization of bipartite insect virus genomes: the genome of black beetle virus, p. 207–218. *In* D. J. Rowlands, M. A. Mayo, and B. W. J. Mahy (ed.), The molecular biology of the positive strand RNA viruses. Academic Press, London.
19. **Kaesberg, P., R. Dasgupta, J. Y. Sgro, J. P. Wery, B. H. Selling, M. V. Hosur, and J. E. Johnson.** 1990. Structural homology among four nodaviruses as deduced by sequencing and X-ray crystallography. J. Mol. Biol. **214**:423–435.
20. **Koizumi, M., S. Iwai, and E. Ohtsuka.** 1988. Construction of a series of several self-cleaving RNA duplexes using synthetic 21-mers. FEBS Lett. **228**:228–230.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
23. **Ling, M. L., S. S. Risman, J. F. Klement, N. McGraw, and W. T. McAllister.** 1989. Abortive initiation of bacteriophage T3 and T7 RNA polymerases under conditions of limiting substrate. Nucleic Acids Res. **17**:1605–1618.
24. **Moore, N. F., B. Reavy, and L. A. King.** 1985. General characteristics, gene organization and expression of small RNA viruses of insects. J. Gen. Virol. **66**:647–659.
25. **Paul, A. V., C.-F. Yang, S.-K. Jang, R. J. Kuhn, H. Tada, M. Nicklin, H.-G. Krausslich, C.-K. Lee, and E. Wimmer.** 1987. Molecular events leading to poliovirus genome replication. Cold Spring Harbor Symp. Quant. Biol. **52**:343–352.
26. **Prody, G. A., J. T. Bakos, J. M. Buzayan, I. R. Schneider, and G. Bruening.** 1986. Autolytic processing of dimeric plant virus satellite RNA. Science **231**:1577–1580.
27. **Rosenberg, A. H., B. N. Lade, D. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
28. **Saunders, K., and P. Kaesberg.** 1985. Template-dependent RNA polymerase from black beetle virus-infected *Drosophila melanogaster* cells. Virology **147**:373–381.
29. **Scotti, P. D., S. Dearing, and D. W. Mossop.** 1983. Flock house virus: a nodavirus isolated from *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae). Arch. Virol. **75**:181–189.
30. **Selling, B. H., R. F. Allison, and P. Kaesberg.** 1990. Genomic RNA of an insect virus directs synthesis of infectious virions in plants. Proc. Natl. Acad. Sci. USA **87**:434–438.
31. **Selling, B. H., and R. R. Rueckert.** 1984. Plaque assay for black beetle virus. J. Virol. **51**:251–253.
32. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
33. **Tobin, G. J., D. C. Young, and J. B. Flanegan.** 1989. Self-catalyzed linkage of poliovirus terminal protein VPg to poliovirus RNA. Cell **59**:511–519.