Flock House Virus: Down-Regulation of Subgenomic RNA3 Synthesis Does Not Involve Coat Protein and Is Targeted to Synthesis of Its Positive Strand

WEIDONG ZHONG AND ROLAND R. RUECKERT*

Institute for Molecular Virology and Department of Biochemistry, University of Wisconsin—Madison, 1525 Linden Drive, Madison, Wisconsin 53706-1596

Received 23 December 1992/Accepted 7 February 1993

Flock house virus is a small insect virus with a bipartite RNA genome consisting of RNA1 and RNA2. RNA3 is a subgenomic element encoded by RNA1, the genomic segment required for viral RNA synthesis (T. M. Gallagher, P. D. Friesen, and R. R. Rueckert, J. Virol. 46:481-489, 1983). Synthesis of RNA3 is strongly inhibited by RNA2, the gene for viral coat protein. Evidence that coat protein is not the regulatory element was obtained by using a defective interfering RNA2 which was messenger inactive. It was also found that RNA2 selectively down-regulated synthesis of positive-strand RNA3 but not of its complementary negative strand. cDNA-generated RNA2 transcripts, carrying four extra nonviral bases at the 3' end, failed to repress synthesis of RNA3 but recovered this activity after a single passage in Drosophila cells in the presence of RNA1, suggesting that down-regulation of RNA3 synthesis is controlled by competition with RNA2 for viral replicase.

Nodaviruses are a family of small insect viruses with a messenger-sense bipartite RNA genome (for a review, see reference 19). Members include at least four serotypes represented by Nodamura virus (35), black beetle virus (13, 23), flock house virus (FHV) (7), and Bolaarva virus (29). The genome of FHV consists of 4,506 bases split between two single-stranded positive-sense RNA segments, RNA1 and RNA2, which are packaged in a single particle (5, 6, 10, 26, 27, 38). Genomic RNA1, 3,106 bases, carries all viral genes required for replication (15). It is the messenger for protein A (112 kDa) (10, 18, 33) and encodes a second protein, protein B (10 kDa), via subgenomic RNA3 (389 bases) which is derived from the 3' end of RNA1 (10, 11, 17). RNA2, 1,400 bases, encodes coat precursor protein α (44 kDa) which is cleaved into a 40-kDa (β) and a 4-kDa (γ) fragment after assembly into provirions (3, 4, 10, 12).

Besides being the messenger for coat protein, nodaviral RNA2 also down-regulates synthesis of subgenomic RNA3 through an unknown mechanism. A preliminary study has shown that Drosophila cells transfected with RNA1 alone produced RNA3 in proportions 10- to 20-fold greater than do cells infected with virions or transfected with both viral RNA1 and RNA2 (15).

We now provide evidence that coat protein, the gene product of RNA2, is not required for down-regulation of RNA3 synthesis. A defective interfering (DI) RNA (DI-634), with large deletions in the open reading frame (ORF) for coat protein (41), retained the ability of RNA2 to down-regulate RNA3 synthesis, and removal of its messenger activity did not impair such function. Additional evidence relevant to the mechanism of the down-regulatory activity is also discussed.

MATERIALS AND METHODS

Propagation of Drosophila cells. The WR strain of Drosophila cells was propagated in roller bottles as previously described (10, 37). Complete growth medium consisted of Schneider's insect medium (36) supplemented with 15% fetal bovine serum. Cells were routinely passaged by flushing confluent monolayers into the spent medium and then diluted 150-fold with fresh medium.

Purification of FHV RNA1 and other viral RNAs. Fifty micrograms of a viral RNA mixture (containing equimolar amounts of RNA1 and RNA2) was isolated from gradient-purified virus particles. The RNA mixture was heated at 65°C for 5 min and was then resolved for about 3 h at 8 V/cm on a 1% low-melting-point (LMP) agarose gel (buffer was 89 mM Tris-Cl-89 mM borate-1 mM EDTA [pH 8.3]). After electrophoresis, the agarose piece containing the RNA1 band was collected, melted at 65°C, and loaded onto another 1% LMP agarose gel for the second round of fractionation. The RNA1 purification step was repeated with a third gel. The final gel piece, with purified RNA1, was melted at 65°C in an equal volume of 0.4 M NaCl-20 mM Tris-Cl (pH 7.0)-2 mM EDTA and then subjected to extraction with phenol-chloroform and ethanol precipitation. Approximately 10 µg of RNA1 was purified from the initial viral RNA mixture (50 µg). There was no detectable RNA2 synthesis assayed by incorporation of [3H]uridine in Drosophila cells transfected with such prepared RNA1. Similarly, FHV RNA2 and DI-634 were also purified through LMP agarose gels.

Plasmid construction. A full-length DNA copy of FHV RNA2 had been cloned in plasmid pSP64 (Promega) (6). The double-stranded DNA was then recloned into transcription vector pBluescribe M13 (+) (Stratagene) according to the promoter sequence of T3 RNA polymerase. The plasmid was further modified by removing the nonviral bases located between the transcription initiation site of the T3 promoter and downstream viral DNA sequence through oligonucleotide-directed mutagenesis. RNA2 transcripts generated in vitro from the modified plasmid contain 5' ends identical to those in viral RNA2 (data not shown) but four extra nonviral bases at the 3' ends (6). The virion RNA2 or DI-634 sequence was mGpppGUAA..........................AGGU (blocked); the transcript RNA2 or DI-634 sequence was mGpppGUAA..........................AGGUucuag-OH (lower-case letters signify nonviral nucleotides; the 3' terminus of virion RNA2 is inert to the action of the enzyme RNA ligase

* Corresponding author.
and poly(A) polymerase (3), but the blocking agent is not known].

A full-length DNA copy of DI-634 was also cloned in pBluescribe M13 (+) by R. Dasgupta, using the method described previously (1). The DI clone was modified as described above.

Site-directed mutagenesis of the translational initiation codon (AUG) in DI-634. For in vitro mutagenesis, the cDNA clone of DI-634 which carries an M13 origin was used to transform Escherichia coli JM101 competent cells. The transformed cells were then superinfected with M13 helper phage, and particles released from the cells were precipitated with polyethylene glycol. Single-stranded DNA was isolated from the precipitated phages by extraction with phenol-chloroform and ethanol precipitation. The oligodeoxynucleotide (21 bases) used in mutagenesis was synthesized at the University of Wisconsin—Madison Biotechnology Center. The base changes that it introduced (AU-23→UA) are underlined in the following sequences: wild-type, AGU UCC AAA AUC GUU AUA AAC ↔ and mutant, AGU UCC AAA UAG GUU AUA AAC. In vitro mutagenesis was carried out with a mutagenesis kit from Amersham (catalog no. RPN.1523), and the mutations were confirmed by dideoxy sequencing (32) using standard procedures.

In vitro transcription of cloned FHV DNAs. Selected FHV DNA clones were cleaved with the restriction enzyme, XbaI, and the resulting linear DNA templates (0.03 mg/ml) were transcribed with T3 RNA polymerase as described by Konarska et al. (20); 0.5 mM 7-methyguanosine 5′-triphosphate 5′-guanosine [m7G(5′)ppp(5′)G] or the unmethylated analog [G(5′)ppp(5′)G] was included in the reaction mixture to provide capped transcripts. Reactions were terminated by phenol extraction, and RNA transcripts were recovered by either ethanol precipitation (three times) in the presence of 2.5 M ammonium acetate or gel electrophoresis. RNA transcripts prepared by either method were very efficient when used for transfection of Drosophila cells (data not shown).

Preparation of 32P-labeled RNA probes for Northern (RNA) hybridization. [α-32P]CTP-labeled positive- and negative-strand RNA1 in vitro transcripts were used as probes to measure the synthesis of RNA3 in transfected cells. A DNA copy of RNA1 had been independently inserted into transcription plasmids pSP64 and pSP65 (Promega), which were used to generate virus-sense (positive-strand) and virus-complementary (negative-strand) RNA1 transcripts, respectively (33). Positive- and negative-strand RNA1 transcripts were transcribed with SP6 RNA polymerase from XbaI-linearized pSP64 and from PstI-linearized pSP65 clones, respectively. Standard reactions had a total volume of 25 μl and contained 1 mM ATP, 1 mM UTP, 1 mM GTP, 200 μM unlabeled CTP, 20 μCi of [α-32P]CTP (1 Ci = 37 GBq), 10 U of SP6 RNA polymerase, and several other components as described in reference 20. Labeled RNA transcripts were gel purified before use.

Liposome-mediated transfection of Drosophila cells. Confluent monolayers of Drosophila cells (2 × 106 cells per well in a 24-well tissue culture plate [Fisher Scientific]) were washed three times with serum-free Schneider culture medium (36). RNA mixture (5 μl), containing 40 ng of viral RNA1 and 20 ng of RNA2 or 10 ng of DI-634, was mixed with an equal volume of Lipofectin (Bethesda Research Laboratories) and incubated at room temperature for 15 min (8). The prepared RNA-Lipofectin mixture was then combined with 150 μl of serum-free Schneider culture medium and loaded onto the Drosophila cell monolayers. The cells were incubated at 26°C for 1 h, after which 1 ml of Schneider culture medium supplemented with 15% fetal bovine serum was added. The RNA-treated cells were propagated at 26°C before labeling steps.

Radio labeling and extraction of intracellular RNA. Viral RNA synthesis in infected Drosophila cells was detected by incorporation of [3H]uridine in the presence of actinomycin D. At about 12 h postinfection, the cells were treated for 30 min with actinomycin D (12 μg/ml; Sigma Chemical Co.), which inhibits synthesis of cellular RNA but not of viral RNA; viral RNA was then labeled during a 2-h incorporation of [5,6-3H]uridine (100 μCi/ml; New England Nuclear Corp.). To extract total intracellular RNA, the cells were washed once with ice-cold TNE buffer (0.03 M Tris-Cl [pH 8.1]–0.1 M NaCl–25 mM EDTA) and lysed with 0.4 ml of TNE–0.1% sodium dodecyl sulfate (SDS) solution. The cell lysate was immediately extracted twice with phenol and chloroform (14), and RNA was precipitated with 2 volumes of absolute ethanol. The RNA pellet was washed with 70% ethanol, redissolved in 50 μl of distilled water, and stored at −70°C.

Analysis of 3H-labeled RNA samples. Aliquots of [3H]-labeled RNA samples were resolved on a 1.4% agarose gel (containing 0.5 μg of ethidium bromide per ml) for about 3 h at 8 V/cm. After electrophoresis, the gel was photographed, treated with En3Hance (New England Nuclear), and fluorographed overnight at −70°C with an intensifying screen.

Cell-free protein synthesis in rabbit reticulocyte lysate. The rabbit reticulocyte translation in vitro system was purchased from Promega (catalog no. L4250). For each translation assay, the template RNA was preheated at 67°C for 10 min, chilled immediately on ice, and then included, at a final concentration of 10 μg/ml, in reaction mixture containing 35 μl of nuclelease-treated reticulocyte lysate, 40 μl of RNasin, 1 mM amino acid mixture (minus methionine), and 40 μCi of [35S]methionine in a total volume of 50 μl. Reactions were performed at 30°C for 60 min, and the translation products were analyzed on an SDS–15% polyacrylamide gel followed by autoradiography.

Electrophoretic analysis of 35S-labeled proteins. Electrophoresis was performed on discontinuous SDS-polyacrylamide gels (21). Protein samples were combined with 2× sample buffer (25 mM Tris-Cl [pH 6.8], 10% glycerol, 5% SDS, 6 mM 2-mercaptoethanol, 0.02% bromophenol blue) and heated for 5 min at 100°C. Slab gels (100 by 80 by 0.75 mm) were prepared with 15% acrylamide in the resolving gel and 5% acrylamide in the stacking gel. Electrophoresis was performed at 100 V for approximately 3 h, and the gels were dried under vacuum and subjected to autoradiography.

Northern blot analysis of positive- and negative-strand RNA3 accumulation. Equal numbers of infected Drosophila cells were withdrawn at indicated intervals postinfection, and total cellular RNA was extracted. Duplicate samples (from 10⁶ cells each) were dissolved in the sample buffer containing 50% formamide–2.2 M formaldehyde and incubated at 85 to 90°C for 15 min. The heated RNA samples were chilled quickly on ice and fractionated for about 5 h at 5 5 V/cm on two 1% denaturing agarose gels containing 2.2 M formaldehyde (the running buffer was 20 mM morpholinepropanesulfonic acid [MOPS; pH 7.0]–8 mM sodium acetate–1 mM EDTA [pH 8.0]) (31). After electrophoresis, RNA was transferred from the gels to Zeta-Probe blotting filters (Bio-Rad Laboratories) and hybridized separately with 32P-labeled positive- and negative-sense RNA1 transcripts, which detected negative- and positive-strand RNA3, respectively. The hybridization was done for about 20 h at 65°C in blotting buffer containing 50% formamide, 0.8 M NaCl, 0.5%
FIG. 1. Down-regulation by RNA2 of subgenomic RNA3 synthesis. Duplicate monolayers of *Drosophila* cells (2 × 10⁵ cells per monolayer) in complete growth medium were infected with FHV virions (lanes 1 and 4) (see reference 10 for the infection procedure), transfected with virion RNA1 and RNA2 (40 ng of RNA1; 20 ng of RNA2) (lanes 2 and 5), or 40 ng of RNA1 alone (lanes 3 and 6). At 12 h postinfection, viral RNA was labeled during a 2-h incorporation of [³H]uridine (100 μCi/ml) in the presence of actinomycin D (12 μg/ml), which inhibits synthesis of cellular RNA but not of viral RNA. Total RNA was then extracted from the cells, and samples from 4 × 10⁶ cells were separated on a 1.4% agarose gel and detected by ethidium bromide staining (lanes 1 to 3) and fluorography (lanes 4 to 6). 18S and 28S rRNAs are indicated on the left.

SDS, 10x Denhardt's solution, and 0.25 mg of denatured salmon sperm DNA per ml. The filters were washed twice with 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS and twice with 0.1x SSC-0.1% SDS for 10 min each time at room temperature and then once at 65°C with 0.1x SSC-0.1% SDS. The filters were dried at room temperature and measured by autoradiography.

RESULTS

Down-regulation of RNA3 synthesis by RNA2. The virions of FHV contain equimolar amounts of RNA1 and RNA2 (5, 10, 26); a third RNA species, RNA3, which is a subgenomic element encoded by RNA1, is also synthesized in FHV-infected *Drosophila* cells but is not packaged into virions (10, 11, 17). An earlier report has shown that synthesis of RNA3 is substantially inhibited when RNA2, which encodes viral coat protein, is present in the cells (15). Figure 1 shows an ethidium bromide-stained electropherogram of RNA extracted from *Drosophila* cells infected with virions (lane 1), RNA1 and RNA2 from virions (lane 2), or RNA1 alone (lane 3). RNA from cells infected with virions, or cotransfected with RNA1 and RNA2, revealed both RNA1 and RNA2 bands along with large amounts of 18S and 28S RNA. Cells transfected with RNA1 alone, however, revealed a new band, RNA3, which was not observed in the other two lanes (the low recovery of 28S rRNA in the case of lane 3 was occasionally observed with the extraction procedure used and was not consistently seen).

Identity of the viral RNAs was confirmed by radiolabeling with [³H]uridine (Fig. 1, lanes 4 to 6) from 12 to 14 h postinfection in the presence of actinomycin D, which inhibits synthesis of host RNA but not of viral RNA. The minor band below RNA1 in lanes 4 and 5 corresponds to double-stranded RNA2.

DI-634 mimics the ability of RNA2 to down-regulate synthesis of RNA3. DI-634 is a DI RNA derived from RNA2 (41). It contains 634 bases with two large deletions (bases 250 to 516 and 729 to 1227) in RNA2, contains a small ORF consisting of two segments from coat protein (shown in black) and a 13-amino-acid (AA) out-of-frame carboxyl terminus (hatched) which predicts a 159-residue (molecular weight, 17,000) protein product. (B) Duplicate monolayers of *Drosophila* cells (2 × 10⁵ cells per monolayer) were transfected with 40 ng of RNA1 alone (lanes 3 and 6), 40 ng of RNA1 plus 20 ng of RNA2 (lanes 2 and 5), or 40 ng of RNA1 plus 10 ng of DI-634 (lanes 1 and 4). Viral RNA was labeled during a 2-h incorporation of [³H]uridine starting at 12 h postinfection. Total RNA was extracted from the infected cells, and samples from 4 × 10⁶ cells were separated on a 1.4% agarose gel and detected by ethidium bromide staining (lanes 1 to 3) and fluorography (lanes 4 to 6). The weaker bands migrating between RNA1 and DI-634 (lanes 1 and 4) are a double-stranded DI form.

Evidence that coat protein is not required for down-regulation of RNA3 synthesis. Despite the large deletions in RNA2, DI-634 contains a small ORF consisting of two disjointed segments of coat protein corresponding to residues 1 to 76 and 166 to 235 (shown in black), respectively, and a 13-amino-acid out-of-frame region (hatched) at the C terminus (Fig. 2A); such an ORF predicts a protein product containing 159 amino acid residues (molecular weight, 17,000). To inactivate the ORF and thereafter the messenger activity of DI-634, the translational start codon (AUG) at position 23...
was converted to a UAG codon via a cDNA clone of DI-634. The resulting construct was named DI-uag.

As previously reported, cDNA-generated FHV RNA transcripts, with a few extra nonviral bases at 5' and 3' termini, were about 10% as infectious as authentic RNA from virions (6). The cDNA clones used in this study synthesized in vitro transcripts containing 5' ends identical to those in authentic virion RNA2 but with four extra nonviral bases at the 3' ends (see Materials and Methods). To remove those extra bases, the transcripts of DI-uag were transfected into Drosophila cells together with RNA1, which encodes the replicase needed for viral RNA replication; in vivo-propagated DI-uag molecules were recovered from the cells at about 16 h postinfection. The resulting DI-uag RNA was electrophoretically purified by using LMP agarose and tested for messenger activity and ability to inhibit RNA3 synthesis.

FHV RNA2 encodes viral coat precursor protein α (molecular weight, 44,000) containing 407 amino acid residues (4); it was an active message in cell extracts of rabbit reticulocyte (Fig. 3, lane 2). As seen in lane 3, DI-634 was also messenger active but produced a smaller protein (pDI) consistent with the mass of 17 kDa predicted by its nucleotide sequence (Fig. 2A). However, DI-uag, a modified form of DI-634, completely obliterated any evidence of messenger activity (lane 4), despite overexposure of the autoradiogram to enhance detection sensitivity.

These experiments showed that DI-uag had no detectable messenger activity in cell extracts. Similar studies on intact Drosophila cells transfected with DI-uag also failed to reveal evidence of any protein product (data not shown).

As shown in Fig. 4, DI-uag inhibited RNA3 synthesis (lanes 1 and 4) as thoroughly as did DI-634 (lanes 2 and 5) when cotransfected into Drosophila cells with RNA1. Thus, it appears very unlikely that viral coat protein is involved in the ability of RNA2 to down-regulate synthesis of RNA3.

RNA2 down-regulates synthesis of positive-strand RNA3 but not of its complementary negative strand. The existence of nodaviral negative-strand RNA3 has been documented by isolating a double-stranded form of RNA3 from virus-infected Drosophila cells and sequencing its two strands (17). To examine whether synthesis of the negative-strand RNA3 of FHV is regulated differently than that of its positive strand, duplicate suspensions of Drosophila cells were transfected with RNA1 alone and with equivalent amounts of RNA1 and RNA2. Aliquots of the infected cells were withdrawn at indicated intervals (4, 5, 6, and 7 h) postinfection, and total RNA was extracted. The RNA samples were electrophoretically resolved under denaturing conditions and analyzed by Northern blot hybridization using probes specific for positive- and negative-strand RNA3 (Fig. 5). As shown in Fig. 5A, synthesis of positive-strand RNA3 in cells...
cotransfected with both RNA1 and RNA2 (lanes 5 to 8) was substantially lower than in those transfected with RNA1 alone (lanes 1 to 4). Quantitative analysis further indicated that synthesis of positive-strand RNA3 was about 10-fold lower at each time point in the presence of RNA2 (data not shown). However, as shown in Fig. 5B, RNA2 did not decrease the production of negative-strand RNA3 (compare lanes 5 to 8 with lanes 1 to 4). Note that the gel in Fig. 5B was exposed 40-fold longer than that in Fig. 5A to accommodate the much smaller amounts of negative-strand RNA3. Additional control tests showed that there was no detectable cross-reaction between the riboprobes used under these hybridization conditions (see the legend to Fig. 5). These results indicate that RNA2 selectively inhibits synthesis of positive-strand RNA3 but not of its complementary negative strand.

cDNA-generated RNA2 transcripts lack the ability to repress RNA3 synthesis. As noted above, the transcripts of RNA2 contain four extra nonviral bases at the 3′ ends. The inhibitory effects on RNA3 synthesis between transcript RNA2 and authentic RNA2 from virions were compared (Fig. 6). Unlike virion RNA2 (lanes 4 and 9), which strongly inhibited synthesis of RNA3, the RNA2 transcripts were defective in such function (lanes 3 and 8) when cotransfected into Drosophila cells with virion RNA1. Similar results were also observed for the transcripts of DI-634 (lanes 1 and 6). These results seem to suggest that intact 3′ end of RNA2 is required for efficient down-regulation of RNA3 synthesis. As seen earlier, DI-uag transcripts, after a single passage in transfected Drosophila cells, recovered the ability to down-regulate RNA3 synthesis (Fig. 4, lanes 1 and 4); a similar result was also obtained with use of cellularly passed RNA2 transcripts (Fig. 7, lane 1).

**DISCUSSION**

We have shown here that the down-regulatory activity of RNA2 is selectively targeted to synthesis of the positive strand of subgenomic RNA3 but not to synthesis of its negative strand. We have also provided evidence, based on modifications of the ORF for coat protein, that the down-regulatory activity of RNA2 is not mediated through viral coat protein. It is not clear why transcripts from cDNA of RNA2, carrying four extra nonviral bases at the 3′ ends, lacked the ability of authentic RNA2 to down-regulate RNA3 synthesis in transfected Drosophila cells. However, recovery of the down-regulatory activity after a single passage of RNA2 transcripts in Drosophila cells is likely due to end repair, i.e., removal of the extra nonviral bases during replication of the transcripts in transfected cells. Direct sequencing to test for end repair has been hampered because the 3′ end of RNA2, including cellularly passed transcripts, is blocked by an unidentified agent (3, 6).

Further characterization of the down-regulatory mechanism now requires a better understanding of the mechanism by which RNA3 is replicated. Three types of models have been proposed for generation of subgenomic RNAs. One is initiation of transcription at an internal promoter site on the negative strand of genomic RNA, a mechanism used by the alphaviruses (22, 28, 30, 40) and by the plant virus bromovirus mosaic virus (9, 24, 25). A second mechanism is leader-primed transcription, in which a segment of RNA, transcribed from one end of the negative strand of genomic RNA, dissociates from its template and then rejoins the template at various downstream transcription initiation sites to serve as a primer for transcription of subgenomic RNAs. This mechanism had been proposed for the synthesis of coronavirus subgenomic mRNAs (2) but requires reconciliation with the observations that subgenomic replicative intermediates and subgenomic negative-stranded RNAs were detected in virus-infected cells (34, 39). A third mechanism is premature termination during negative-strand synthesis, fol-
cells is time dependent, correlating with the accumulation of RNA2 as it replicates (11, 15). As shown in Fig. 6, the four extra nonviral bases at the 3' ends of positive-strand RNA2 transcripts reduced the efficiency of these transcripts as a template for viral replicase (lanes 1 and 3). The reduced affinity of RNA2 transcripts for viral replicase would free more replicase molecules to act upon negative-strand RNA3 template and would thus favor the synthesis of positive-strand RNA3. Once the 3' ends of RNA2 transcripts were repaired, they would again compete efficiently with negative-strand RNA3 and thereby recover the down-regulatory activity on positive-strand RNA3 synthesis.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI23742 and AI22813. We thank Ranjit Dasgupta for providing the original FHV RNA2 and DI-634 cDNA clones and purified DI RNA and for access to his sequence for FHV RNA1.

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