JOURNAL OF VIROLOGY, Jan. 1984, p. 116–124
0022-538X/84/010116-09$02.00/0

Vol. 49, No. 1

Early and Late Functions in a Bipartite RNA Virus: Evidence for Translational Control by Competition Between Viral mRNAs

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Received 30 June 1983/Accepted 19 September 1983

It has been shown previously that *Drosophila* cells infected with black beetle virus synthesize an early viral protein, protein A, a putative element of the viral RNA polymerase. Synthesis of protein A declines sharply by 6 h postinfection, whereas synthesis of viral coat protein α continues for at least 14 h. The early shutoff in protein A synthesis occurred despite the presence of equimolar proportions of the mRNAs for proteins A and α, RNAs 1 and 2, respectively. We have now been able to mimic this translational discrimination in a cell-free protein-synthesizing system prepared from infected or uninfected *Drosophila* cells, thus allowing further analysis of the mechanism by which translation of RNA 1 is selectively turned off. The results revealed no evidence for control by virus-encoded proteins or by virus-induced modification of mRNAs by the cell-free system. Rather, with increasing RNA concentration, viral RNA 1 was outcompeted by its genomic partner, RNA 2. This suggests that the early shutoff in intracellular synthesis of protein A is due to decreasing ability of RNA 1 to compete for a rate-controlling translational factor(s) as the concentration of viral RNAs accumulates within the infected cell.

Black beetle virus (BBV), a bipartite RNA virus belonging to the family Nodaviridae (18, 21, 27), multiplies vigorously in cultured *Drosophila* cells (7). Hence, its molecular biology is now accessible for detailed study. As one of the smallest and simplest of the multipartite viruses, it promises to be a useful experimental tool for studying gene interaction at the molecular level.

BBV-infected cells contain three virus-specific messenger-active RNAs (6, 8). Two of these, vRNA 1 (1.1 megadaltons) and vRNA 2 (0.46 megadaltons), are packaged into virions in roughly equimolar proportions (18); the third, RNA 3 (0.15 megadaltons), is not packaged but is regenerated from RNA 1 during each cycle of infection (8). Each of these RNAs directs the synthesis of a single protein in cell-free extracts: protein A (104K) from RNA 1; coat precursor protein α (47K) from RNA 2; and protein B (10K) from RNA 3 (2, 5, 6, 11).

RNAs 1 and 2 are made in roughly equimolar proportions throughout the course of the infection cycle (3, 6), yet their respective proteins A and α are made in widely different proportions. Thus protein A, a putative element of the viral RNA polymerase (5, 12, 23), peaks at about 5 h postinfection and then declines rapidly, whereas synthesis of proteins B and α continues to accelerate. Synthesis of protein B, whose precise function is still unclear, peaks at about 8 h. By the late stages of infection, beyond 14 h, infected cells synthesize mainly coat protein α, which continues to accumulate in large amounts, reaching nearly 20% of the total protein by 48 h (5, 7). The different temporal courses with which proteins A, B, and α are synthesized, together with the finding that RNAs 1 and 3 can replicate independently of RNA 2 (8), support the hypothesis that RNA 1 mediates early functions, through proteins A and B, whereas RNA 2 mediates a late function, through synthesis of coat protein needed for virion assembly.

Earier studies (6) have suggested that the wide variation in the rate of synthesis of proteins A and α, in particular, is due to a modulation in the efficiency with which viral mRNAs are translated and is therefore the result of regulation at the level of translation (for reviews, see references 16, 28). Recent advances in the preparation of cell-free protein-synthesizing extracts from cultured *Drosophila* cells, highly efficient for the translation of homologous mRNA (11, 31), have enabled us to examine the mechanism of this apparent translational control in greater detail. We now provide evidence that the shutoff in synthesis of protein A is due to the limited ability of its mRNA, RNA 1, to compete with other viral messengers for an unidentified, rate-limiting factor(s) in the host translational system as viral RNAs accumulate during the infection cycle. This mechanism allows the virus to shut off synthesis of early protein A while maintaining its ability to synthesize equal proportions of the two messenger-active genomic RNAs needed later for packaging.

MATERIALS AND METHODS

Cell growth and infection. The WR subline of Schneider's line 1 *Drosophila* cells (7, 30) was propagated in roller bottles, using Schneider culture medium (29) supplemented with 15% fetal bovine serum as previously described (5). For infection, log-phase cells (5×10^7 cells per ml) were inoculated with BBV stocks (8) at multiplicities of 5,000 virus particles per cell. Uninfected (control) cultures were inoculated with an equal volume of culture medium lacking virus. After 30 min of gentle agitation, the infected and mock-infected cell suspensions were diluted with fresh culture medium to a final concentration of 5×10^6 to 7.5×10^6 cells per ml. Incubation was continued at 26°C on a gyratory shaker at 60 rpm.

Procedures for extraction of RNA. To isolate total intracellular RNA from *Drosophila* cells, samples (1 ml) of infected and mock-infected cell suspension (7.5×10^6 cells per ml) were withdrawn at the indicated intervals. The cells were collected by centrifugation ($600 \times g$) and washed once with ice-cold culture medium followed once by TNE buffer (0.03

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M Tris, pH 8.1, 0.1 M NaCl, 25 mM EDTA). After resuspension to a concentration of 5×10^6 cells per ml, the cells were lysed by the addition of sodium dodecyl sulfate (SDS) to 1% and immediately extracted twice with phenol and chloroform as described previously (6). The extracted RNA was precipitated overnight with 2 volumes of absolute ethanol at −20°C, collected by centrifugation (12,000 × *g*) for 15 min, redissolved in 50 μl of distilled water, and stored at −70°C.

BBV RNAs 1 and 2 were extracted from purified virus with phenol and chloroform as described elsewhere (5). Ethanol-precipitated virion RNA, consisting of an equimolar mixture of RNAs 1 and 2 (19), was dissolved in water to a final concentration of 6.5 mg/ml and stored at −70°C. The concentration of RNA was determined spectrophotometrically, using an extinction coefficient of 22 mg/ml at 260 nm.

The ratio of mass of viral RNAs 1 and 2/unit mass of rRNAs was found to be 0.5 in RNA extracted from infected cells at 12 h postinfection. This ratio was measured from relative fluorescence intensity of the electrophoretically separated acridine orange-stained RNAs (6). The content of *Drosophila* cells is about 1% of the wet weight; hence, the average intracellular concentration of viral RNA is 0.5 × 10 or about 5 mg/ml. Of the total vRNA, about 30% was packaged in virions (measured as described in Fig. 5 of reference 6). Therefore, we estimate the intracellular concentration of mRNA (unpackaged vRNA) to be about (0.7 × 5 =) 3.5 mg/ml at 12 h postinfection. The intracellular concentration of vRNA at 5 h was estimated to be about 0.8 mg/ml from the proportion of radiolabeled uridine incorporation accumulated at 5 h relative to that accumulated in 12 h (6).

Drosophila tRNA, used as a supplement for cell-free protein synthesis (below), was extracted from *Drosophila* line 1 cells with phenol and then purified as described previously (35), using 1 M NaCl and DEAE-cellulose chromatography (DE52; Whatman). Approximately 0.6 mg of tRNA was recovered from 1 g (wet weight) of *Drosophila* cells.

Preparation of infected and uninfected *Drosophila* cell lysates. At the times indicated, infected and mock-infected cells were recovered by low-speed centrifugation and washed three times with ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered *Drosophila* saline (35 mM HEPES, pH 7.6, 100 mM NaCl, 10 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$). The final cell pellet was resuspended in 2 volumes of homogenization buffer (10 mM HEPES, pH 7.6, 1 mM dithiothreitol). After a 10-min incubation on ice, the swollen cells were disrupted with 20 strokes of a tight-fitting Dounce homogenizer constructed to specifications described previously (25). This provided 90% cell breakage. Nuclei and cellular debris were removed by centrifugation at 12,000 × *g* for 15 min (6°C), using the Sorvall SS34 rotor. The cytoplasmic supernatant (S12) was carefully withdrawn, avoiding the top layer of lipid material, frozen in 100- to 200-μl aliquots with an ethanol-dry ice slurry, and stored at −70°C. Each milliliter of extract corresponded to approximately 10^9 cells.

Cell-free protein synthesis in *Drosophila* lysates. Conditions for protein synthesis in *Drosophila* line 1 cell-free extracts were similar to those described by Guarino et al. (11). A standard protein synthesis reaction (30 μl) contained 15 μl of S12 *Drosophila* lysate (prepared as described above) plus the following: 10 mM HEPES (pH 7.6), 20 μM hemin, 27 μg of creatine kinase (Sigma Chemical Co., St. Louis, Mo.) per ml, 10 mM creatine phosphate, 5 mM dithiothreitol, 90 to 100 mM potassium acetate, 0.5 mM magnesium acetate, 90 μg each of 19 unlabeled amino acids per ml, 60 μg of *Drosophila* tRNA per ml, and 1 U of human placental RNase inhibitor (Biotec, Madison, Wis.) per ml. [^{35}S]methionine (1,200 Ci/mmol; New England Nuclear, Boston, Mass.) was added to a final concentration of 1.5 mCi/ml. When translation in infected and uninfected extracts was compared, care was taken to assure that salt concentrations were identical. Protein synthesis was initiated by transferring the reaction mixture from ice to a 26°C water bath. At the indicated intervals, samples (5 μl) were withdrawn, diluted fivefold with solubilizing solution (1% SDS, 0.1% β-mercaptoethanol), and heated for 5 min (100°C). Protein synthesis was monitored by assaying the incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material as previously described (5).

Nuclease treatment of *Drosophila* lysates. Lysates from BBV- and mock-infected line 1 cells were rendered mRNA dependent with micrococcal nuclease as described by Pelham and Jackson (24). A typical digestion mixture contained 92 μl of S12 cell lysate (see above), 2 μl of 1 M HEPES (pH 7.6), 1 μl of 2 M potassium acetate, 1 μl of 0.1 M $CaCl_2$, 1 μl of 4 mM hemin, 1 μl of 5 mg of creatine kinase per ml, and 1 μl of 15,000 U of micrococcal nuclease (Sigma) per ml. After a 10-min incubation at room temperature, nuclease digestion was terminated by the addition of 2 μl of 0.1 M ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid.

A standard mRNA-dependent reaction (30 μl) contained 17 μl of nuclease-treated lysate, the translation components listed above, and 0.8 mCi of [^{35}S]methionine per ml. In vitro protein synthesis (26°C) was initiated by adding exogenous mRNA. When the above cell-free reactions were supplemented with human placental RNase inhibitor (1 U/ml; Biotec), overall protein synthesis increased twofold. Similiarly, the synthesis of protein A relative to that of protein α in supplemented extracts was approximately threefold higher than in nonsupplemented extracts (data not shown). This selective increase in protein A synthesis was attributed to an enhanced preservation of RNA 1, which because of its larger size was more susceptible to nuclease inactivation than RNA 2.

Cell-free protein synthesis in rabbit reticulocyte lysates. The preparation of mRNA-dependent rabbit reticulocyte lysates and conditions necessary for in vitro protein synthesis have been described (32, 33). Addition of *Drosophila* tRNA, potassium acetate, and magnesium acetate to final concentrations of 60 μg/ml, 90 mM, and 0.5 mM, respectively, provided optimum incorporation of [^{35}S]methionine (1.0 mCi/ml) into acid-insoluble material. In vitro protein synthesis was conducted at 30°C for 45 min and then terminated by the addition of solubilizing solution as described above.

Electrophoretic analysis. Samples (25 μl) of solubilized cell-free translation extracts and SDS-disrupted cells (10^6 cells per sample) were prepared for electrophoresis by adding 8.3 μl of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 3.5% Ficoll [Pharmacia Fine Chemicals, Uppsala, Sweden]) and heating for 5 min (100°C). Electrophoresis on SDS-containing polyacrylamide slab gels (0.5 by 17 cm wide and 20 cm long) was conducted according to the method of Laemmli (15) unless otherwise indicated. After electrophoresis, the gels were fixed in a solution containing 10% acetic acid, 25% methanol, and 1% glycerol and then dried under vacuum (Hoefer Scientific Instruments, San Francisco, Calif.). The dried gels were subjected to autoradiography by exposing each to Kodak XAR5 film for 1 to 5 days. Polypeptide bands on a single autoradiogram were quantitated by densitometry, using a

Joyce-Loebl double-beam recording microdensitometer (model MKIIIC) equipped with a linear 0.00 to 1.24 optical density reference wedge. The measured values for the area under each peak were calculated and corrected for possible nonlinearities of the X-ray film, using a computer program written by Mark Pallansch (Ph.D. thesis, University of Wisconsin-Madison, 1982).

RESULTS

Relative levels of messenger-active viral RNA in BBV-infected *Drosophila* cells. Preliminary experiments have suggested that shutoff in synthesis of protein A late in the infection cycle is not due to a lack of messenger for protein A (6). To confirm that synthesis of protein A was indeed regulated at the level of translation, we have used cell-free synthesis of viral proteins to quantitate the relative in vitro activities of viral mRNAs 1, 2, and 3 present during the course of infection and thereby compare them with the corresponding intracellular rates of synthesis of each viral protein (proteins A, α, and B). To this end, samples (2 ml) of BBV-infected cell suspension were withdrawn every 2 h (from 1 to 14 h after infection) and divided in half. Cells in the first half were pulse-labeled with [^{35}S]methionine for 1 h, whereas the other half was incubated in parallel without radioactivity. At the end of 1 h, cells in both halves were washed and then lysed with SDS. Total intracellular RNA for in vitro translation was extracted from the unlabeled half with phenol. Samples (0.5 μl) of phenolated RNA from equal volumes of infected cell suspension were then translated in mRNA-dependent extracts of rabbit reticulocytes (Fig. 1). Translation was carried out with concentrations of RNA not greater than 20 μg/ml, a concentration well within the range in which protein synthesis was linearly proportional to the amount of RNA added (Fig. 1, inset).

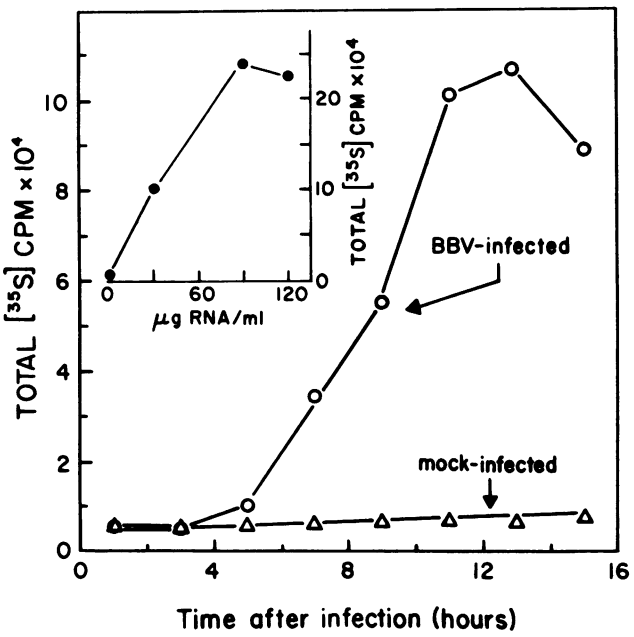

FIG. 1. Relative amounts of messenger-active RNA in BBV-infected and mock-infected *Drosophila* cells. Total intracellular RNA was extracted with phenol from equal volumes (1 ml) of cell suspension withdrawn at the indicated times after infection. Samples (0.5 μl) of purified RNA (from 7.5 × 10^4 cells) were translated in standard 30-μl reaction mixtures prepared from rabbit reticulocytes made mRNA dependent by treatment with micrococcal nuclease as described in the text. The relative amount of protein synthesized in vitro was measured by incorporation of [^{35}S]methionine into acid-insoluble material, using 0.63-μl samples of each reaction. (Inset) Effect of RNA concentration on overall in vitro protein synthesis.

Messenger activity in infected cells increased progressively, reaching a peak at about 10 to 12 h after infection (Fig. 1). This is contrasted with the messenger activity from mock-infected (control) cells carried in parallel. At its highest point (10 to 12 h), the messenger activity in infected cells was 15 to 20 times greater than that in mock-infected cells.

To determine the distribution of functional viral mRNAs (RNAs 1, 2, and 3), the cell-free products of each reaction were examined on a polyacrylamide gel (Fig. 2B). The [^{35}S]methionine-pulse-labeled proteins synthesized by the intact cells from which RNA was extracted were also analyzed on a parallel gel (Fig. 2A). Cell-free messenger activity was already evident within 2 h after infection for proteins A and α and within 6 h for protein B (Fig. 2B). Messenger activity for proteins α and B increased steadily from 4 to 14 h after infection and thus paralleled the increasing rates of synthesis of proteins α and B in intact cells (Fig. 2A). Despite the declining synthesis of protein A in intact cells (lanes e to h), messenger activity for protein A also increased steadily during this period (Fig. 2B, lanes n to p) and in amounts similiar to that of protein α. The heterogeneous collection of fainter bands between proteins A and α (lanes n to p) represented <10% of the total radiolabeled protein present in each lane and probably represents premature termination products of protein A (5). The increasing in vitro messenger activity for protein A indicated that the declining intracellular rate of synthesis of A was not due to a parallel decline in the amount of messenger active RNA 1 available for translation in the infected cell.

Evidence that infected cells contained roughly equal proportions of messenger-active RNAs 1 and 2 was obtained by examining the translation of RNAs 1 and 2 phenol extracted from virions (Fig. 2, lane q). With identical conditions for translation, virion RNA, consisting of an equimolar mixture of RNAs 1 and 2, directed the synthesis of proteins A and α in the same proportions as that directed by total RNA extracted from infected cells (cf. lanes p and q). Thus, the presence of nearly equimolar amounts of messenger-active RNAs 1 and 2, in cells which synthesized a markedly low proportion of protein A relative to protein α, suggested that the efficiency with which RNA 1 was translated in the infected cell was greatly reduced relative to that of RNA 2.

Translational control in cell-free extracts of BBV-infected *Drosophila* cells. To further explore the mechanism by which synthesis of protein A is regulated, we next sought to determine whether the in vivo discrimination against RNA 1 as a messenger could be reproduced in homologous cell-free extracts derived from virus-infected *Drosophila* line 1 cells. To this end, Dounce homogenates of *Drosophila* cells were prepared 5 and 12 h after infection, the periods of maximum and shutoff synthesis of protein A, respectively. The 12,000 × *g* clarified supernatants (S12) were supplemented with components necessary for in vitro protein synthesis, omitting the usually standard addition of micrococcal nuclease to preserve the endogenous viral mRNA present in such extracts. In vitro protein synthesis, initiated by warming the reactions to 26°C, was complete within a 1-h period as judged by the incorporation of [^{35}S]methionine into acid-insoluble material.

Analysis of the cell-free translation products on SDS-

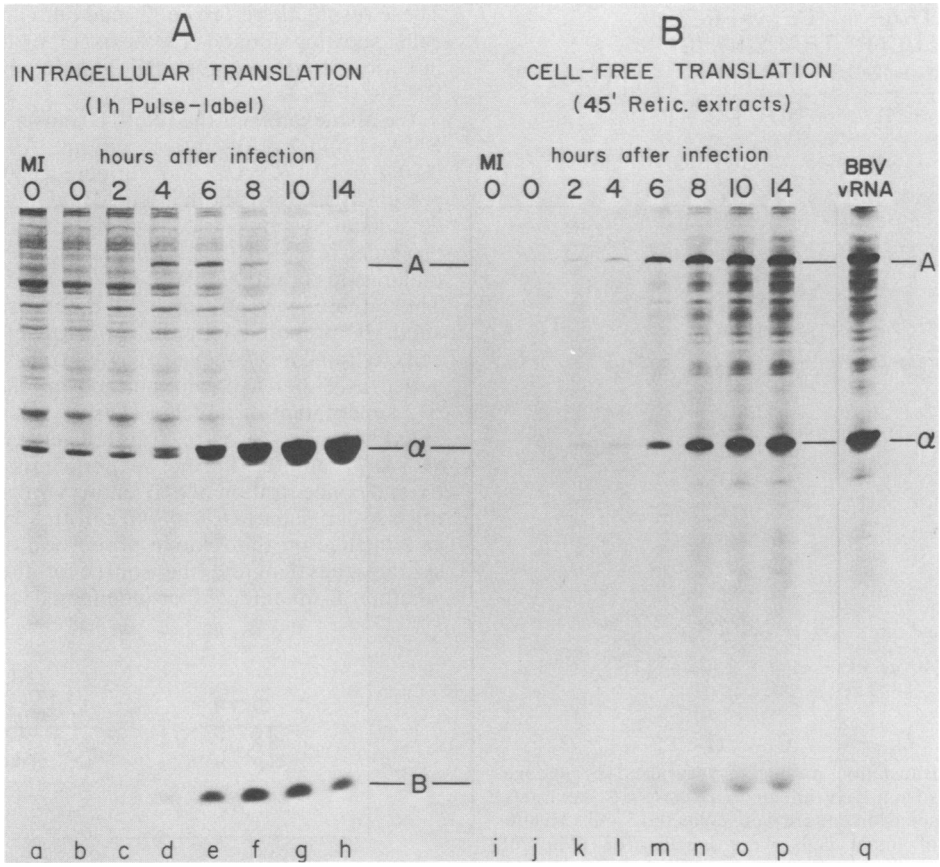

FIG. 2. Intracellular and cell-free messenger activities of RNA from BBV-infected *Drosophila* cells. Samples of infected cell lysates (10^6 cells) and reticulocyte cell-free translation reactions (0.63 μl) prepared as described in the text were subjected to electrophoresis on 10% polyacrylamide slab gels as previously described (5). (A) Autoradiogram (2-day exposure) of proteins synthesized by intact cells labeled at the indicated times with [^{35}S]methionine (100 μCi/ml) for 1 h as previously described (5), using methionine-deficient Schneider growth medium. (B) Autoradiogram (1-day exposure) of [^{35}S]methionine-labeled cell-free translation products synthesized by reticulocyte extracts in response to total intracellular RNA simultaneously extracted from the cells pulse-labeled in (A). Also shown are intracellular proteins synthesized by mock-infected (MI) cells (lane a) and cell-free translation products synthesized in response to (lane i) RNA extracted from mock-infected cells and (lane q) RNA (60 μg/ml) extracted from purified virions (BBV vRNA).

polyacrylamide gels (Fig. 3) revealed that the patterns of protein synthesis by the S12 extracts (lanes c and d) closely resembled the patterns synthesized in infected cells from which the extracts were derived (lanes a and b). Synthesis of protein A in the 12-h extract (lane d), like that of 12-h intact cells (lane b), was dramatically reduced compared with the synthesis of coat protein α and protein B. These results therefore indicated that it was possible to prepare cell-free extracts which would exhibit the same type of discrimination between viral mRNAs (RNAs 1, 2, and 3) as was observed in intact cells.

Translation of exogenous BBV RNA in mRNA-dependent extracts of infected cells. The above experiments suggested that the S12 extracts from infected *Drosophila* cells contained those factors necessary for reproducing translational inhibition of viral RNA in vitro. We initially speculated that this inhibition was due to the accumulation of a virus-encoded protein. Such a regulatory protein might operate by selectively masking the ribosomal binding site of RNA 1 or by modifying the host translational machinery (e.g., ribosomes, initiation factors, or 5′ cap recognition functions) so as to select against RNA 1 as a messenger. BBV protein B, because of its small size (molecular weight, 10,000) and its high concentration in S12 extracts (data not shown), was the most likely candidate for such a negative regulatory protein.

To test the possibility that soluble proteins of viral origin are responsible for the translational discrimination against RNA 1, we next examined the translation of exogenous viral mRNA added to mRNA-dependent extracts prepared from infected and uninfected cells. If soluble regulators were present and active, it was expected that infected extracts would discriminate against RNA 1 in a manner similiar to that of intact cells whereas uninfected extracts would not. *Drosophila* S12 extracts were prepared for mRNA-dependent translation of exogenous RNA by treating with micrococcal nuclease. Nuclease treatment eliminated 98.5% of the endogenous protein-synthesizing activity of infected and uninfected extracts; thus most, if not all, mRNA (viral and host) was susceptible to nuclease degradation.

We first compared the ability of mRNA-dependent extracts, prepared from infected and mock-infected cells, to translate the endogenous mRNA present in S12 extracts prepared 12 h after infection. Thus, the mRNA translated here was identical to that which served as message in the 12-h "endogenous" reaction above (see Fig. 3, lane d) except that the final concentration of viral mRNA was 7.5-fold

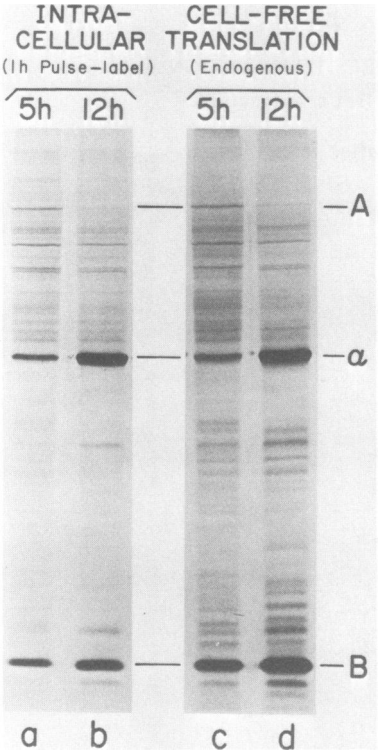

FIG. 3. Cell-free translation products synthesized by endogenous RNA in extracts from BBV-infected *Drosophila* S12 extracts. Cells from a single suspension culture were collected 5 and 12 h after infection. Samples of intact cells were labeled for 1 h with [^{35}S]methionine as described in the legend to Fig. 2. Extracts (S12) from unlabeled cells were simultaneously prepared by Dounce homogenization and programmed for in vitro translation of the endogenous viral mRNA as described in the text. Labeled cell lysates (3×10^5 cells) and cell-free products of 1-h translation reactions (1.5 μl) were subjected to electrophoresis on an 8 to 15% polyacrylamide gradient gel followed by autoradiography (4-day exposure). (Lanes) Intracellular proteins radiolabeled at 5 (lane a) and 12 (lane b) h after infection; cell-free translation products synthesized by endogenous S12 extracts from cells harvested 5 (lane c) and 12 (lane d) h after infection. The concentration of endogenous viral mRNA in the cell-free extracts were approximately 133 (lane c) and 580 (lane d) μg/ml, i.e., one-sixth the intracellular concentrations computed as described in the text.

lower due to removal of background RNA by the nuclease pretreatment. Electrophoretic analysis of the cell-free translation products (Fig. 4, lanes a and c) revealed that all three viral proteins, A, α, and B, were synthesized in abundant amounts by both extracts. Quantitation of the protein bands by densitometry indicated that there was no significant difference in the ability of either extract to translate RNA 1 relative to RNAs 2 and 3. The molar ratios of protein A/protein α synthesized by the 12-h-infected and mock-infected extracts were about the same, 1:6 and 1:4, respectively (assuming that protein A contains twice as much methionine as protein α). Thus, synthesis of protein A relative to protein α was 10- to 15-fold higher than that synthesized by intact cells or S12 extracts containing endogenous viral RNA (see Fig. 3). The abundant messenger activity of RNA 1, detected in S12 supernatants, ruled out the possibility that the low rate of protein A synthesis in endogenous extracts was due to post-transcriptional modification of RNA 1 (i.e., removal of the 5′ cap structure or random nucleolytic degradation). These results therefore confirmed our earlier conclusion that cells showing limited synthesis of protein A (12 h after infection) contained abundant amounts of messenger-active RNA 1 (Fig. 2).

We also examined the relative translational efficiencies of RNAs 1 and 2 purified from virions. An equimolar mixture (150 μg/ml) of RNAs 1 and 2 directed a 60-fold stimulation of protein synthesis in both infected and mock-infected mRNA-dependent systems. Electrophoretic analysis of the in vitro products revealed that both extracts synthesized identical molar ratios of proteins A/α, about 1:6 (Fig. 4, lanes b and d). Thus, there was no apparent difference in the ability of similarly prepared extracts from infected and uninfected cells to translate exogenous RNA 1 (relative to RNA 2) when added as either a phenol-purified mixture or an untreated S12 supernatant of infected cells.

Evidence for in vitro translational competition between vRNAs 1 and 2. Further experiments revealed that the overall concentration of exogenous viral mRNA added to the mRNA-dependent *Drosophila* cell-free systems had a greater influence on the relative proportion of proteins A and α synthesized than did the source of the cell-free system, whether from infected or uninfected cells. For example,

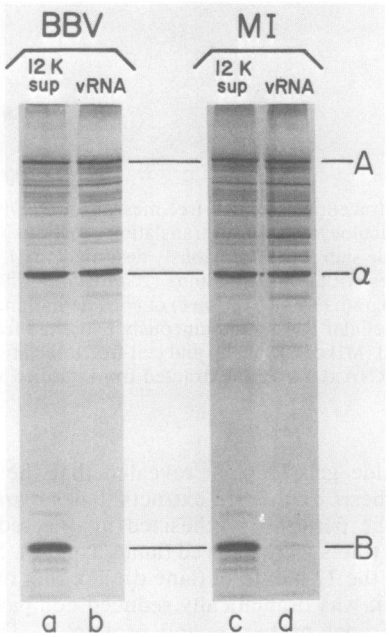

FIG. 4. Cell-free translation products synthesized by mRNA-dependent 12 h BBV-infected (BBV) and mock-infected (MI) *Drosophila* extracts in response to endogenous S12 mRNA and purified vRNA. Samples (4 μl) of S12 supernatant (12K sup), containing endogenous mRNA from 12-h-infected cells and samples (4 μl) of a 1:1 mixture of purified BBV RNAs 1 and 2 (vRNA) phenol extracted from virions were added to nuclease-treated extracts prepared from infected (BBV) or mock-infected *Drosophila* cells. After a 1-h translation period (26°C) in 30-μl reaction mixtures, samples (1.2 μl) were withdrawn and subjected to electrophoresis on 12% polyacrylamide gels followed by autoradiography (5-day exposure). (Lanes) [^{35}S]methionine-labeled cell-free products synthesized by mRNA-dependent 12-h-infected (BBV) extracts in response to (a) 12K supernatant of 12-h-infected cells (77 μg of viral mRNA per ml), 24,000 cpm, or (b) vRNA (150 μg/ml), 30,000 cpm; cell-free products synthesized by mRNA-dependent mock-infected extracts in response to (c) 12K supernatant of 12-h-infected cells (77 μg of viral mRNA per ml), 33,000 cpm, or (d) vRNA (150 μg/ml), 32,000 cpm.

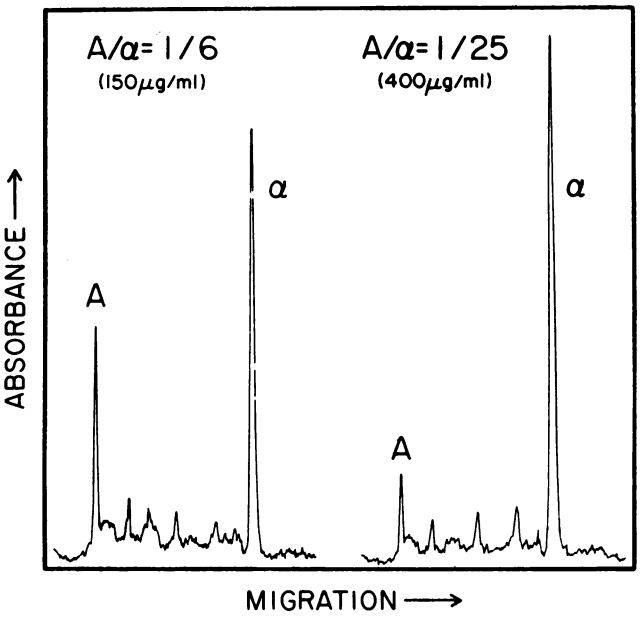

FIG. 5. Densitometer profiles illustrating effect of RNA concentration on the proportion of proteins A and α synthesized by mRNA-dependent extracts from 12-h-infected cells. [^{35}S]methionine-labeled translation products synthesized as described in the legend to Fig. 4, in response to 1:1 mixtures of purified BBV RNAs 1 and 2, were analyzed by electrophoresis on 12% polyacrylamide gels. Autoradiograms were scanned with a Joyce-Loebl microdensitometer equipped with an optical density wedge having a full scale of 1.24 optical density units, a value within the linear exposure range of the film. Translation profiles of BBV virion RNA at 150 and 400 μg/ml, respectively, are shown.

when the concentration of exogenous virion RNA was increased from 150 to 400 μg/ml, *Drosophila* extracts discriminated against RNA 1 and favored RNA 2 (Fig. 5), resulting in a translation pattern which more closely resembled that of cells late in infection.

This discriminatory effect is illustrated in greater detail in Fig. 6. Upon the addition of increasing amounts of a 1:1 mixture of RNAs 1 and 2 to mRNA-dependent S12 extracts of infected *Drosophila* cells, overall protein synthesis increased linearly and then leveled off at concentrations beyond 150 μg/ml (Fig. 6A). At lower (nonsaturating) concentrations of RNA, the synthesized ratio of A/α did not change (Fig. 6C). However, as the concentration of RNA was raised beyond the saturation level, in the range of 200 μg/ml, the ratio of A/α dropped linearly over a fourfold range, from 1:6 to 1:25 (Fig. 6C). This decline occurred even though overall translation remained constant (Fig. 6A). A similiar reduction in the synthesized ratio of A/α was observed when mRNA-dependent S12 extracts of uninfected *Drosophila* cells were used (data not shown).

To determine whether this pattern was unique to the homologous *Drosophila* system, translation was repeated with the heterologous cell-free reticulocyte system (Fig. 6). As the concentration of virion RNA exceeded the saturation level (50 to 60 μg/ml; Fig. 6B), a similiar three- to fourfold decrease in the synthesis of protein A relative to α (Fig. 6D) was observed, indicating that reticulocyte extracts also show preferential translation of RNA 2 at high RNA concentrations. One difference, however, is that at low concentrations of RNA reticulocyte extracts translated RNA 1 more efficiently than *Drosophila* extracts. This difference is discussed below.

Preferential translation of one mRNA over another at high concentrations of RNA is diagnostic of translational competition between mRNAs. For example, studies on the regulation of α- and β-globin synthesis in reticulocyte extracts (4, 14, 22) have suggested that the addition of saturating amounts of mRNA to cell-free translation systems forces different mRNAs to compete for limiting amounts of a rate-controlling factor(s) required for initiation of translation. This competition leads to preferential translation of those mRNAs with the highest affinities for the limiting factor(s) (1, 9, 17). These arguments suggested, therefore, that BBV RNAs 1 and 2 compete in vitro and that because of a lower intrinsic affinity for such translational factors RNA 1 competes less efficiently than its partner, RNA 2.

Restoring the translational efficiency of RNA 1 by diluting endogenous viral mRNA in the cell-free protein-synthesizing system. The above experiments also suggested that competition between viral mRNAs induced by the accumulation of high concentrations of messenger-active RNA might be

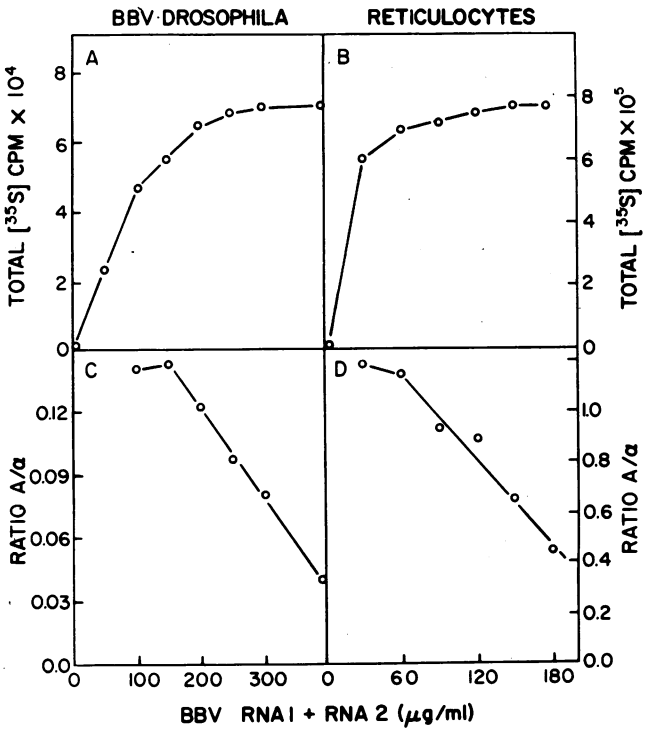

FIG. 6. Change in the in vitro synthetic ratio of proteins A and α as a function of the concentration of exogenous virion RNA. The indicated concentrations of BBV RNA (1:1 mixture of RNAs 1 and 2 phenol extracted from purified virus) were translated for 1 h in mRNA-dependent cell-free extracts. Total protein synthesis was monitored by the incorporation of [^{35}S]methionine into acid-insoluble material by 1.2 μl of 12-h BBV-infected *Drosophila* extract (A) and 0.6 μl of rabbit reticulocyte extract (B), respectively. Cell-free translation products were subjected to polyacrylamide gel electrophoresis and quantified from densitometer tracings of the resculting autoradiograms as shown in Fig. 5. The molar ratio of A/α synthesized in 12-h BBV-infected *Drosophila* extracts (C) and reticulocyte extracts (D) were calculated assuming that protein A contains about twofold more methionine than protein α (see text) and then plotted as a function of the concentration of exogenous RNA. Note scale differences of (C) and (D).

responsible for the reduced translational efficiency of RNA 1 in the infected cell. If this were the case, any reduction in competitive pressure between viral RNAs should result in stimulation of the translation of the "weaker" mRNA, RNA 1, relative to the "stronger," RNA 2 (and possibly RNA 3). We tested this hypothesis by progressively reducing the concentration of endogenous mRNA in extracts of infected cells and measuring the effect upon in vitro synthesis of proteins A, α, and B.

To this end, a sample of S12 extract (200 μl) of 12-h-infected cells was divided into two equal parts; one part was digested with micrococcal nuclease to remove endogenous mRNA. Both parts were then supplemented identically with components necessary for in vitro protein synthesis, and translation reactions (30 μl) were prepared by mixing untreated extract with increasing proportions of nuclease-treated extract from infected cells (dilution series A). This procedure progressively diluted the amount of endogenous mRNA in each reaction yet maintained a constant translational environment (i.e., each reaction contained identical concentrations of host ribosomes, tRNAs, initiation factors, and viral proteins). As a control, a second series of dilutions was prepared by mixing infected S12 extract with nuclease-treated extract from mock-infected cells (dilution series B). Samples were withdrawn at intervals after initiating translation and assayed for acid-insoluble radioactivity (Fig. 7). Synthesis was complete in all reactions within 1 h (Fig. 7, inset).

The dilution of endogenous viral mRNA in each reaction was accompanied by a corresponding drop in incorporation of [^{35}S]methionine (Fig. 7). Incorporation by the mRNA-containing extract decreased linearly when diluted with nuclease-treated extract from infected cells (series A) but decreased more slowly when diluted with nuclease-treated extract from mock-infected cells (series B).

Analysis of the cell-free products on SDS-gels (Fig. 8) indicated that as the mRNA-containing infected extract was diluted with nuclease-treated infected extract (series A, Fig. 7A), synthesis of proteins α and B declined steadily (lanes b to f) in amounts proportional to the drop in concentration of their corresponding mRNAs. However, despite a parallel drop in the concentration of RNA 1, synthesis of protein A increased. At the 5- and 10-fold dilutions, the proportions of A to α synthesized were 10 times higher than that synthesized by the "undiluted" extract (cf. lanes d to e with lane a). Since a constant translational environment was maintained in all reactions, the selective stimulation in translation of RNA 1 was not due to the dilution of virus-specific regulatory proteins but rather to the declining concentrations of mRNA or, in other words, to the reduction in competitive pressure between mRNAs.

In vitro synthesis of proteins A, α, and B behaved similarly when mRNA-containing infected extract was diluted with nuclease-treated extract from mock-infected cells (series B, Fig. 8B). At the 5- to 10-fold dilutions (lanes l to m), the synthesis of protein A increased about 15-fold relative to proteins α and B. The similiar efficiencies with which dilution with nuclease-treated extracts from virus-infected (series A) and mock-infected (series B) cells restored the translation of RNA 1 (cf. Fig. 8A and B) suggested, moreover, that competition was for limiting amounts of *Drosophila* host factors required for translation.

DISCUSSION

In this report we have shown that the shutoff in synthesis of protein A, which begins about 6 h after infection, was not due to a decline in the intracellular supply of its messenger RNA 1, which remained functionally intact as a messenger and accumulated in amounts comparable to that of RNA 2. Thus, we confirm our earlier conclusion (6) that synthesis of protein A is regulated at the level of translation. Our studies with cell-free protein-synthesizing extracts from infected and uninfected *Drosophila* cells revealed no evidence for involvement of viral protein in this regulation. Furthermore, the ease with which RNA 1 messenger activity was restored by dilution (Fig. 8) argues against the idea that translational inhibition is due to the sequestering of RNA 1 as, for example, in a ribonucleoprotein particle or subcellular vesicle.

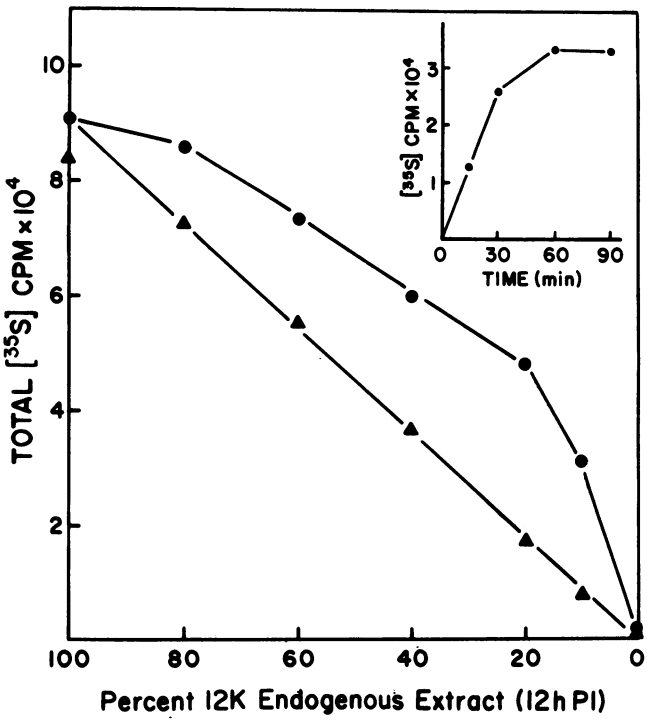

FIG. 7. Relative amounts of protein synthesized in extracts of BBV-infected cells diluted with mRNA-deficient extracts. S12 extracts from 12-h-infected cells were mixed with increasing proportions of micrococcal nuclease-treated S12 extracts of 12-h-infected cells (dilution series A, ▲) or 12-h-mock-infected cells (dilution series B, ●), as indicated, to give a final reaction volume of 30 μl. After a 1-h translation period (26°C), in vitro protein synthesis was measured in each reaction by assaying samples (1.2 μl) for the incorporation of [^{35}S]methionine into acid-insoluble material. Reactions with 0% endogenous extract (far right) illustrate background protein synthesis after nuclease treatment. (Inset) Time course of incorporation of [^{35}S]methionine in a cell-free reaction containing 40% 12-h-infected endogenous extract and 60% nuclease-treated 12-h-infected extract.

Translational control by viral mRNA competition. Our data are most consistent with a model in which the regulation of viral protein A synthesis results from competition between viral mRNAs for rate-controlling factors in translation. We propose, therefore, that RNAs 1 and 2, which are made in roughly equal proportions throughout infection, are initially translated with similar efficiencies during the early stages of infection (Fig. 2, lanes c and d) because the intracellular concentration of viral mRNA is relatively low. As infection proceeds, synthesis of messenger-active viral RNA increases to very high levels, exceeding the amount of host

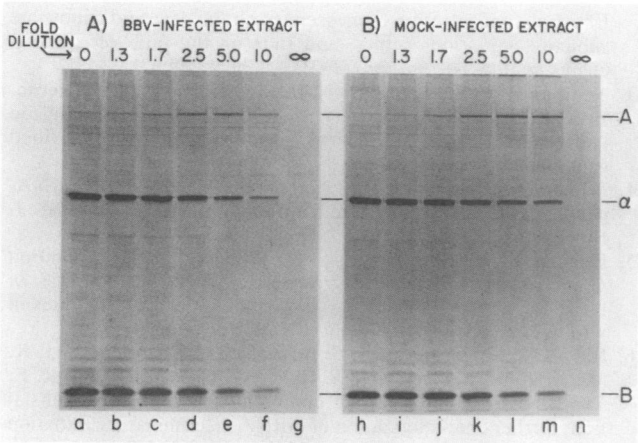

FIG. 8. Effect of diluting BBV-infected extract on the cell-free synthesis of viral protein A, α, and B. Cell-free translation in S12 extracts of 12-h-infected cells mixed with increasing proportions of micrococcal nuclease-treated S12 extract of 12-h-infected (A) or mock-infected (B) cells was conducted as described in the legend to Fig. 7. [^{35}S]methionine-labeled cell-free products from 1-h translation reactions (1.2 μl per sample) were subjected to electrophoresis on 12% polyacrylamide gels followed by autoradiography (24-h exposure). Lanes g and n illustrate proteins synthesized in 12-h-infected and mock-infected extracts, respectively, after nuclease digestion.

messenger by a factor of 15 to 20 (Fig. 1). At these high intracellular concentrations of viral messenger, which exceed 500 μg/ml by 5 h postinfection, the host translation system becomes saturated and forces viral mRNAs to compete for limiting amounts of a factor(s) required for protein synthesis. Since competition favors those mRNAs with the highest affinity for such a factor(s) (1, 9, 17), translation of RNA 2 is preferred to that of RNA 1 (Fig. 6). As a result, the rate of synthesis of protein A declines, whereas that of coat protein α continues to increase (Fig. 2, lanes e to h).

In summary, we have shown that RNA 2 outcompetes RNA 1 in a cell-free translation system which appears to mimic translation in intact *Drosophila* cells. The in vitro concentration of viral RNA required to halve the translation ratio of proteins A/α is high, on the order of 300 μg/ml (Fig. 6B). It is worth recalling, therefore, that BBV is an unusually vigorous virus, accumulating in amounts up to 20% of the total cell mass (7), and that the intracellular concentration of viral mRNA is already on the order of 800 μg/ml (see legend to Fig. 3) by 5 h, the time at which the rate of protein A synthesis begins to decline markedly (see Fig. 5 of reference 5). Thus, these experiments provide strong support for the idea that RNA 2 regulates translation of RNA 1 by competing for some limiting component in the translational machinery of the host cell.

Nevertheless, it is already clear that RNA 2 is not the only element capable of constraining translation of RNA 1 because cells transfected with highly purified RNA 1 alone, and containing little if any RNA 2, continue to make only small amounts of protein A (8). In this situation, however, where RNA 2 is absent, RNA 3 reaches abnormally high concentrations, 20- to 100-fold greater than in the presence of RNA 2 (see Fig. 4 in reference 8). Thus, it may be that the virus has two mechanisms for early shutoff of protein A synthesis, one involving RNA 2 and the other involving RNA 3. However, it has not yet been possible to measure the competitive effectiveness of RNA 3 by translation in cell-free *Drosophila* extracts because of the scarcity of RNA 3. Unlike RNAs 1 and 2, RNA 3 is not packaged into virions; hence it is difficult to purify in amounts required for competition studies in cell-free extracts. Further examination of the role of RNA 3 as a competitive regulator awaits development of a more effective procedure for its purification.

Role of the bipartite genome in regulation of protein synthesis. Since BBV RNA replicase and coat protein genes are encoded by separate genomic RNAs (5, 8), competition of viral RNAs at the level of translation provides an efficient means for the independent regulation of early and late viral functions. By selectively regulating the translational efficiency of RNAs 1 and 2, it is possible to accommodate differential synthesis of those proteins which are evidently needed to accomplish both early RNA replication functions (protein A or B or both) and late assembly functions (coat precursor α). Such regulation occurs while the virus maintains the ability to synthesize equal amounts of its two messenger-active genomic RNAs, required later for packaging into virions. Studies on the cell-free translation of the divided-genome plant viruses (13, 32, 36) have also provided evidence that mRNA competition is involved in the regulation of viral protein synthesis and suggests that this type of translational control may be a general feature in the replication strategy of RNA viruses which contain segmented, messenger-sense genomes.

Identity of the rate-controlling factor(s) in BBV translational competition. Our results suggest that competition between BBV mRNAs is for a component(s) of the host translational system. Indeed, recent studies (4, 10, 14, 20, 26) have indicated that mRNA competition in cell-free translation systems may be relieved by the addition of cellular proteins involved in the initiation of protein synthesis, including eIF-2, eIF-4A, and cap-binding protein also known as eIF-4F. The unusually high efficiency with which reticulocyte extracts translate RNA 1 at low RNA concentrations compared with *Drosophila* extracts (Fig. 2 and 6) also suggests that reticulocytes are relatively rich in factors which limit translation of RNA 1 in *Drosophila* extracts. Purification of such reticulocyte factors may therefore prove useful in further elucidation of the nature of translational competition between BBV RNAs.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA-08662 from the National Cancer Institute. P.D.F. was supported in part by Public Health Service training grant 5-T32-GM07215 from the National Institute of General Medical Sciences.

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