JOURNAL OF VIROLOGY, June 1982, p. 986–995
0022-538X/82/060986-10$02.00/0

Vol. 42, No. 3

Black Beetle Virus: Messenger for Protein B Is a Subgenomic Viral RNA

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Received 30 November 1981/Accepted 15 February 1982

Black beetle virus induces the synthesis of three new proteins, protein A (molecular weight, 104,000), protein α (molecular weight, 47,000), and protein B (molecular weight, 10,000), in infected *Drosophila* cells. Two of these proteins, A and α, are known to be encoded by black beetle virus RNAs 1 and 2, respectively, extracted from virions. We found that RNA extracted from infected cells directed the synthesis of all three proteins when it was added to a cell-free protein-synthesizing system. When polysomal RNA was fractionated on a sucrose density gradient, the messengers for proteins A and α cosedimented with viral RNAs 1 (22S) and 2 (15S), respectively. However, the messenger for protein B was a 9S RNA (RNA 3) not found in purified virions. Like the synthesis of viral RNAs 1 and 2, intracellular synthesis of RNA 3 was not affected by the drug actinomycin D at concentrations which blocked synthesis of host cell RNA. This indicated that RNA 3 is a virus-specific subgenomic RNA and, therefore, that protein B is a virus-encoded protein.

Black beetle virus (BBV) is a member of the family Nodaviridae, a recently recognized group of riboviruses with unusually small divided genomes (14). The isometric virions of BBV are about 30 nm in diameter (15) and contain two single-stranded messenger-active RNA molecules (virion RNA 1 [vRNA 1] and vRNA 2), whose molecular weights have been reported (16) to be about 1.0×10^6 and 0.5×10^6, respectively. When translated in cell-free protein-synthesizing systems (5, 6), vRNA 1 directs the synthesis of a 104,000-dalton polypeptide, protein A, which may be involved in viral RNA replication. vRNA 2 directs the synthesis of coat protein α (molecular weight, 47,000). Electrophoretic and pulse-chase studies (5) in cultured *Drosophila* cells infected with BBV have indicated that protein α is a cleavage precursor of virion coat proteins β and γ (molecular weights, 43,000 and 5,000, respectively).

In addition to the proteins described above, infected *Drosophila* cells contain abundant amounts of a fifth protein, protein B, which has a molecular weight of about 10,000 (1, 5). Pulse-chase and kinetic studies have suggested that protein B is not derived from protein A or α (5). Moreover, this protein has not been detected in cell-free extracts programmed with vRNA 1 or vRNA 2 or in uninfected cells. Thus, it has not been clear whether protein B is encoded by one of the two genome vRNAs, by a subgenomic viral RNA, or by the host genome.

In this report we show that in a cell-free protein-synthesizing system, RNA extracted from infected cells directs the synthesis of protein B, as well as the synthesis of proteins A and α. By radiolabeling virus-specific RNAs in BBV-infected cells treated with actinomycin D, we identified the messenger for protein B as a subgenomic virus-encoded RNA (RNA 3). Moreover, the relative messenger activities of the three viral RNAs in infected cells and in cell-free extracts suggest that the synthesis of at least one viral protein, protein A, is regulated at the level of translation.

MATERIALS AND METHODS

Cells. The WR subline of Schneider line 1 cells (4, 25) was propagated at 26°C in roller bottles in Schneider culture medium (24) containing 15% fetal bovine serum, as described previously (5). The serum was heat inactivated before use by incubation for 30 min at 60°C. The doubling time of the cells was about 15 h.

Virus. BBV was prepared as described previously (5), except that stock virus was passaged serially three times through *Drosophila* cells (WR subline) at a lower multiplicity (100 particles per cell). This precaution was taken to minimize accumulation of defective virus when virus stocks were passaged at higher multiplicities (T. Gallagher, personal communication). The development of a reliable plaque assay has been hindered by the minimal cytopathic effects of BBV on *Drosophila* cells. Accordingly, input multiplicities are expressed below in terms of virus particles per cell. Particle concentrations were determined spectropho-

tometrically after purification on sucrose density gradients (5).

Procedure for infection. *Drosophila* cells (5×10^7 cells per ml) in serum-deficient Schneider medium containing 0.5% bovine serum albumin were transferred to a stoppered, siliconized Erlenmeyer flask and inoculated with stock virus at a multiplicity of 10^4 particles per cell. After 30 min of gentle agitation at 26°C on a rotary shaker (60 rpm), the suspension was diluted to a concentration of 7.5×10^6 cells per ml with Schneider medium containing 15% fetal bovine serum, and agitation was continued in the same way.

Radiolabeling of intracellular protein. Infected cell suspensions were radiolabeled with L-[*methyl*-^{3}H]methionine (catalog no. TRK.583; Amersham Corp., Arlington Heights, Ill.) in methionine-deficient growth medium, as described previously (5).

Radiolabeling of intracellular RNAs. Samples (0.75 ml) of infected cell suspension (7.5×10^6 cells per ml) were withdrawn at intervals during infection and incubated in the presence of [5-^{3}H]uridine (90 μCi/ml; catalog no. NET 174; New England Nuclear Corp., Boston, Mass.). Before radiolabeling (30 min), actinomycin D (Dactomycin; Merck & Co., Inc., West Point, Pa.) was added to a final concentration of 5 μg/ml. After labeling, the cells were collected by centrifugation at $800 \times g$ for 5 min, washed with ice-cold growth medium, and suspended in ice-cold TNE buffer (0.03 M Tris, pH 8.1, 0.1 M NaCl, 25 mM EDTA).

Acid-insoluble radioactivity. Samples (0.1 ml) of radiolabeled cell suspension (2×10^7 cells per ml) in TNE buffer (pH 8.1) were lysed by adding 10% (wt/vol) sodium dodecyl sulfate (SDS) to a final concentration of 1%. Duplicate samples (20 μl) were spotted onto 2.4-cm glass fiber filters (Whatman GF/A). The filters were soaked in 20% trichloroacetic acid for 20 min at 0°C, washed successively with 8% trichloroacetic acid, 95% ethanol, and diethyl ether, and then dried and assayed for radioactivity by liquid scintillation spectroscopy, as described previously (18).

RNA extraction. Cell suspensions (4×10^6 cells per ml) in TNE buffer (pH 8.1) were lysed by adding SDS to 1% and then immediately extracted with phenol as described previously (22). Briefly, cells were blended in a Vortex mixer for 1 min with an equal volume of phenol at 45°C. Chloroform (0.5 volume) was added, and the sample was blended in the Vortex mixer for another 1 min. After phases were separated by centrifugation, the resulting interface and aqueous layer were extracted with phenol and chloroform as described above. The RNA-containing fraction was extracted twice with chloroform alone (1 volume), adjusted to 0.2 M NaCl, and precipitated overnight (−20°C) by adding 2 volumes of absolute ethanol. The RNA was recovered by centrifugation at $12,000 \times g$ for 15 min, dissolved in water, and stored at −70°C.

Electrophoretic analysis of RNA. RNA was subjected to electrophoresis on composite agarose-acrylamide gels by using a modification of the procedure of Floyd et al. (3). Vertical slab gels (17 by 20 cm) containing 2.5% acrylamide, 0.12% *N,N'*-methylenebisacrylamide, 0.5% agarose (Seakem; FMC Corp., Rockland, Maine), 6 M urea, 0.1% SDS, and 0.1% *N,N,N',N'*-tetramethylethylenediamine in Loening buffer (0.036 M Tris, pH 7.8, 0.03 M sodium phosphate, 1 mM EDTA) were polymerized by adding ammonium persulfate to a final concentration of 0.04%. Gels were stored overnight at 6°C before use. No pre-electrophoresis was performed. Unless otherwise stated, the samples were prepared for electrophoresis by diluting [^{3}H]uridine-labeled RNA with an equal volume (15 μl) of Loening buffer containing 10 M urea, 0.05% (wt/vol) xylene cyanol, and 0.05% (wt/vol) bromophenol blue. Electrophoresis was conducted for approximately 6 h at 10 V/cm (6°C) without recirculating the electrophoresis (Loening) buffer.

After electrophoresis, the gels were soaked for 2 h in two changes (250 ml each) of 10% acetic acid–25% methanol–1% glycerol. The gels were then impregnated with fluorographic enhancer (catalog no. NEF-966; New England Nuclear Corp.) and soaked for 1 h in water. After drying under a vacuum, the gels were exposed to Kodak XAR5 film for 1 to 2 days.

Molecular weight determinations. The molecular weights of the BBV-induced RNAs were estimated by using a modification of the glyoxal method of McMaster and Carmichael (2, 17). Stock glyoxal (40%; Aldrich Chemical Co., Milwaukee, Wis.) was deionized with AG-501-X8 mixed-bed ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.). RNA samples were denatured for 1 h at 50°C in 10 mM sodium phosphate (pH 7.2) containing 1 M glyoxal. Before electrophoresis on agarose-acrylamide gels containing 6 M urea (see above), the samples were diluted with an equal volume of Loening buffer containing 10 M urea, 0.05% xylene cyanol, and 0.05% bromophenol blue. After electrophoresis, the gels were stained with acridine orange (30 μg/ml) as described previously (17) and destained in Loening buffer. The relative mobilities of the viral RNAs were compared with those of glyoxal-denatured HeLa cell 28S and 18S rRNAs (molecular weights, 1.75×10^6 and 0.67×10^6, respectively [17]), brome mosaic virus RNAs 1, 2, 3, and 4 (molecular weights, 1.09×10^6, 0.99×10^6, 0.75×10^6 and 0.28×10^6, respectively [11]), and the small nuclear RNAs from HeLa cells (7S, U3, U2, and U1, assuming 350, 216, 188, and 165 bases per RNA, respectively [28]). The size of the HeLa 7S RNA was provided by James Dahlberg (personal communication).

Isolation of polysomes and polysomal RNA. Polysomes from infected and uninfected *Drosophila* cells were prepared essentially as described previously (12, 13). Cells were collected by centrifugation, washed, and suspended to a concentration of 10^8 cells per ml in ice-cold isotonic TNMC buffer (0.03 M Tris, pH 8.1, 0.1 M NaCl, 0.01 M $MgCl_2$, 2 mM $CaCl_2$). After the outer membrane was broken with 0.5% Nonidet P-40 (a nonionic detergent), the nuclei were removed by centrifugation at $12,000 \times g$ for 5 min, and 0.2 ml of the resulting cytoplasmic extract (2×10^7 cells) was layered onto a linear 12-ml 15 to 45% (wt/wt) sucrose gradient in TNMC buffer. Centrifugation was conducted for 90 min at 40,000 rpm ($166,000 \times g$) and 4°C in a Spinco SW41 rotor. Fractions (0.3 ml) were collected from each gradient, top first, using an ISCO density gradient fractionator equipped with a flow cell (path length, 1 cm) and a model UA-5 absorbance monitor. All cell fractionation procedures were conducted at 5°C. RNase inhibitors, which are often used in the isolation of RNA from insect cell cultures (12), were unnecessary under these conditions.

The polysome-containing fractions were pooled, made 25 mM in EDTA and 0.1% in SDS, and extracted

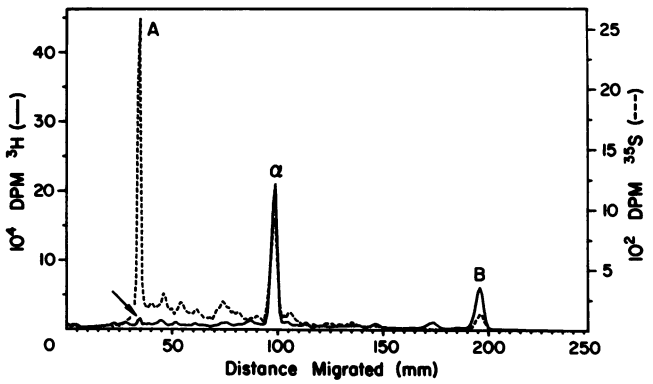

FIG. 1. Electrophoretic profiles of in vitro products synthesized from intracellular BBV RNA and the proteins synthesized in infected *Drosophila* cells. A sample (3×10^5 dpm) of [^{35}S]methionine-labeled products (dashed line) from a reticulocyte extract programmed with RNA from cells 9 h after infection was mixed with a lysate (4×10^6 dpm) of cells radiolabeled with [^{3}H]methionine from 4 to 14 h after infection (solid line). The protein lysates were subjected to electrophoresis on a single polyacrylamide gel as described previously (18). The gel was fractionated into 1-mm segments and assayed for radioactivity.

twice with phenol as described above. The RNA was precipitated twice with ethanol and dissolved in 0.1 M sodium acetate (pH 5.0). Samples (0.2 ml) containing the RNA from 10^7 cells were heated to 65°C for 5 min, quickly cooled on ice, and layered onto a 12-ml 5 to 25% (wt/wt) sucrose gradient in 0.1 M sodium acetate (pH 5.0). Centrifugation was conducted for 18 h at 27,000 rpm (89,000 × *g*) and 10°C in the SW41 rotor. Gradients were fractionated, top first, and the RNA-containing fractions were precipitated with ethanol and stored at −70°C.

Protein synthesis in cell-free extracts of rabbit reticulocytes. The preparation of rabbit reticulocyte lysates and the conditions necessary for protein synthesis have been described previously (26, 27). Lysates were made mRNA dependent by the method of Pelham and Jackson (23). Optimum in vitro translation of BBV RNA required the addition of calf liver tRNA, magnesium acetate, and potassium acetate to concentrations of 58 μg/ml, 0.25 mM, and 80 mM, respectively (5). Typically, a translation mixture (10 μl) consisted of 9 μl of primed lysate containing 1.1 mCi of L-[^{35}S]methionine (1,200 Ci/mmol; catalog no. SJ.204 Amersham Corp.) per ml and 1 μl of purified RNA in water. In vitro protein synthesis was conducted at 30°C and terminated by the addition of solubilizing solution (1% SDS, 0.5 M urea, 0.1% β-mercaptoethanol), followed by heating for 5 min at 100°C. In vitro translation products were analyzed by electrophoresis on 10% polyacrylamide gels and were detected by autoradiography as described previously (5).

RESULTS

Cell-free synthesis of protein B. Previous studies have shown that protein B is not synthesized when vRNAs 1 and 2 extracted from BBV virions are translated in vitro (5). To test the possibility that RNA from infected cells might contain a unique messenger for protein B, we extracted RNA from infected cells 9 h after infection, the period of maximum intracellular synthesis of protein B (5). This intracellular RNA was then translated in cell-free extracts of rabbit reticulocytes. The resulting [^{35}S]methionine-labeled translation products were mixed with differentially radiolabeled protein from BBV-infected cells and subjected to electrophoresis on a polyacrylamide gel (Fig. 1).

The electrophoretic profile of the in vitro translation products (Fig. 1, dashed line) revealed three peaks, which comigrated with proteins A, α, and B from infected cells (Fig. 1, solid line). Thus, the messengers for all three proteins, including the messenger for protein B, were active in the cell-free system. RNA from uninfected cells failed to synthesize any of these three proteins in the cell-free system (data not shown).

The relative in vitro translation activities of the three viral proteins (Fig. 1, dotted line) differed from the translation activities in the intact cells from which the RNA was extracted (Fig. 1, solid line). In particular, the large amount of protein A synthesized in the cell-free system relative to proteins α and B contrasted with the small proportion of protein A typically synthesized in infected cells (Fig. 1, arrow). This marked difference between the cell-free and intracellular messenger activities for protein A (approximately 25-fold relative to protein α) is discussed below. The absence of coat proteins β and γ in the same profile was due to the short labeling period used (4 to 14 h after infection) relative to the long half-life (about 36 h) of coat precursor α (5).

Tryptic analysis of protein B. To confirm the identity of the protein B synthesized in response to RNA extracted from infected cells, [^{35}S]methionine-labeled in vitro protein B was eluted from an SDS-polyacrylamide gel and mixed with a

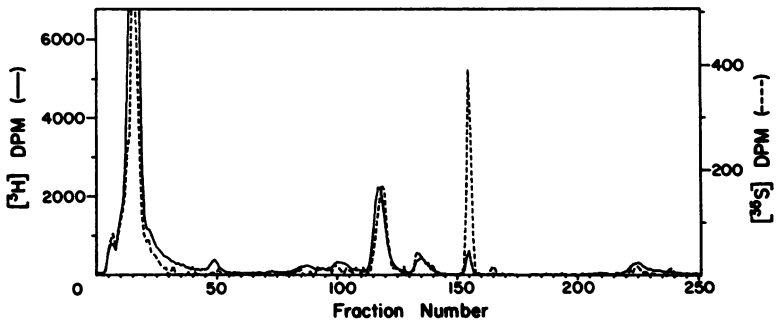

FIG. 2. Tryptic fingerprints of [^{3}H]methionine-labeled protein B isolated from infected cells (solid line) and [^{35}S]methionine-labeled protein B-like product synthesized in cell-free reticulocyte extracts (dashed line). Isolation of the electrophoretically separated proteins, tryptic digestion, and chromatography on a cation-exchange column were performed as described previously (10).

similarly isolated sample of protein B from infected cells labeled with [^{3}H]methionine. The differentially labeled proteins were then digested with trypsin, and the peptide products were separated by chromatography on a cation-exchange column (Fig. 2).

The tryptic fingerprint of protein B made in the cell-free system was similar but not identical to the fingerprint of authentic protein B from infected cells. The in vitro product exhibited a larger peak at fraction 155 and a minor shift in the peak at fraction 118. A possible explanation for these differences is that protein B undergoes post-translational processing and that processing in the cell-free system differs in some way from processing in cells. An alternative possibility is that the in vitro product was contaminated by traces of radiolabeled rabbit reticulocyte globin which comigrated with protein B in our electrophoretic system (see below).

Time course of viral RNA synthesis in BBV-infected *Drosophila* cells. Although it has been suggested that protein B is a virus-encoded protein (5), the possibility that protein B is synthesized by a silent host gene which is switched on after infection with BBV has not been ruled out. To distinguish between these two possibilities, we tried to determine whether synthesis of the mRNA for protein B could be detected in cells treated with actinomycin D, a drug which blocks cellular RNA synthesis but not nodaviral RNA synthesis (20; P. Scotti, personal communication). To this end, infected cells were first pulse-labeled with [^{3}H]uridine in the presence and absence of actinomycin D.

As Fig. 3A shows, the relative rates of RNA synthesis in BBV-infected cells treated with actinomycin increased linearly for the first 24 h and reached a maximum by 36 h. Conversely, the drug reduced synthesis in mock-infected

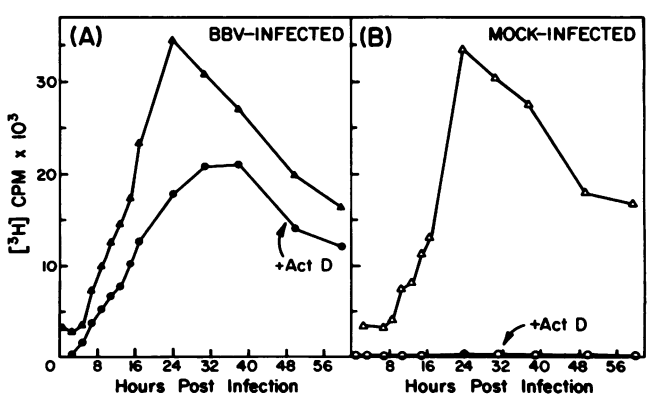

FIG. 3. Time course of the rates of viral RNA synthesis in BBV-infected *Drosophila* cells. At different times duplicate 0.75-ml samples were withdrawn from suspensions (7.5×10^6 cells per ml) of BBV- and mock-infected cells. To one of the duplicate samples, 5 μg of actinomycin D (Act D) per ml of suspension was added, and 30 min later [5-^{3}H]uridine (90 μCi/ml) was added to both samples. After 1 h, the radiolabeled cells were collected, washed, and lysed with SDS. The incorporation of radiolabel into acid-insoluble material was determined on duplicate samples as described in the text.

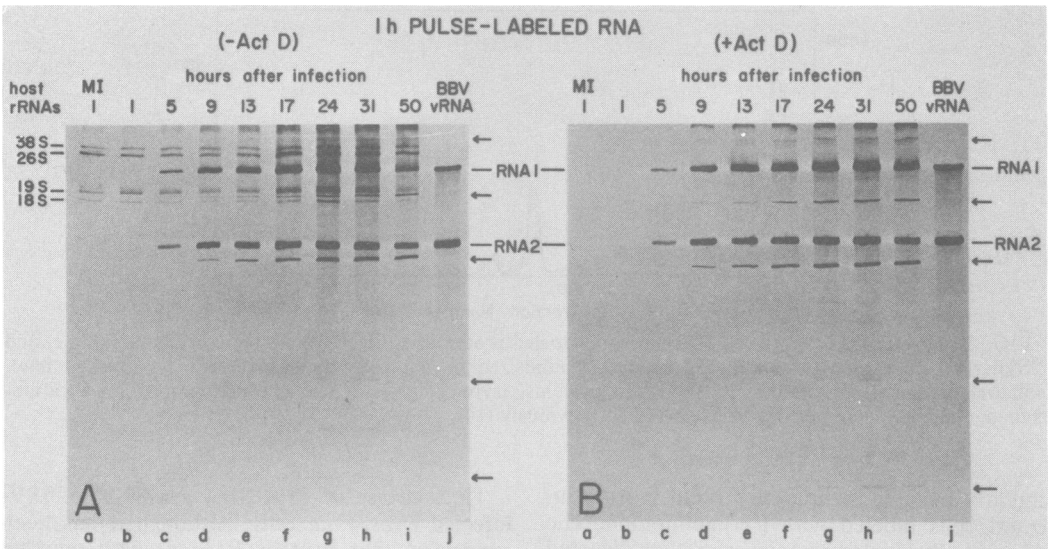

FIG. 4. Time course of BBV-induced RNA synthesis: electrophoretic analysis. Samples (0.75 ml) of an infected cell suspension were withdrawn at different times and pulse-labeled for 1 h as described in the legend to Fig. 3. The cells were lysed and extracted twice with phenol. The ethanol-precipitated RNA from 10^5 cells (30 μl) was subjected to electrophoresis on composite agarose-acrylamide gels containing 6 M urea. (A) Fluorogram of RNA from infected cells. (B) Fluorogram of RNA from infected cells labeled in the presence of actinomycin D (Act D). [^{3}H]uridine-labeled BBV vRNAs 1 and 2 extracted from virions were included as markers (lane j). RNA from mock-infected (MI) cells was also included (lane a).

cells to background levels (Fig. 3B). Thus, viral RNA synthesis, unlike host RNA synthesis, was resistant to actinomycin.

Figure 3A also shows that more than one-half of the total RNA synthesis was virus specific (actinomycin resistant) within 8 h after infection. Nonetheless, the overall profile of RNA synthesis in infected cells (no actinomycin) was not significantly different from that in uninfected cells (Fig. 3A and B). Both profiles showed a 15-fold increase in the rate of synthesis from 0 to 24 h, followed by a decline thereafter. The parallel rate profiles of RNA synthesis in infected and uninfected cells suggest that the observed decline in RNA synthesis (Fig. 3A) was not due to infection by virus but rather to metabolic changes associated with transferring cells to new culture medium.

Electrophoretic analysis of virus-induced RNA. To examine further the nature of the RNA made in the presence and absence of actinomycin, samples of the radiolabeled cells described above were extracted with phenol. The ethanol-precipitated RNA was then subjected to electrophoresis on denaturing (6 M urea) gels (Fig. 4).

The dominant species of RNA synthesized in the absence of actinomycin (Fig. 4A) comigrated with BBV vRNAs 1 and 2 extracted from virions (Fig. 4A, lane j). Also apparent were several host cell RNAs, including the 18S and 19S RNAs found in mature *Drosophila* ribosomes, as well as their 26S and 38S precursors (9). Host RNA continued to be synthesized for at least 50 h after infection, the last time point examined. In infected cells treated with actinomycin, RNAs 1 and 2 were already apparent 5 h after infection (Fig. 4B, lane c). Both of these RNAs were synthesized at high rates as late as 50 h after infection. Moreover, at all time points analyzed, RNAs 1 and 2 were synthesized in nearly equimolar amounts, as determined by densitometric analyses of bands 1 and 2 (Fig. 4B). There was no evidence for the synthesis of specific RNAs in uninfected cells treated with actinomycin (Fig. 4B, lane a).

The electrophoretic patterns described above also revealed several less prominent RNA bands (Fig. 4, arrows) not detected in purified virions. A viral origin is indicated by the resistance of the synthesis of these RNAs to actinomycin (Fig. 4B). The presence of RNase inhibitors (12) during purification did not alter the pattern of intracellular RNA illustrated in Fig. 4 (data not shown). The presence of these supplemental virus-specific RNAs suggested the possibility that a viral RNA other than BBV RNA 1 or 2 might direct the synthesis of protein B in infected cells. Therefore, we set out to determine whether the messenger for protein B could be detected in polysomes from infected cells.

Polysomes from infected and uninfected *Drosophila* cells. Since peak synthesis of protein B

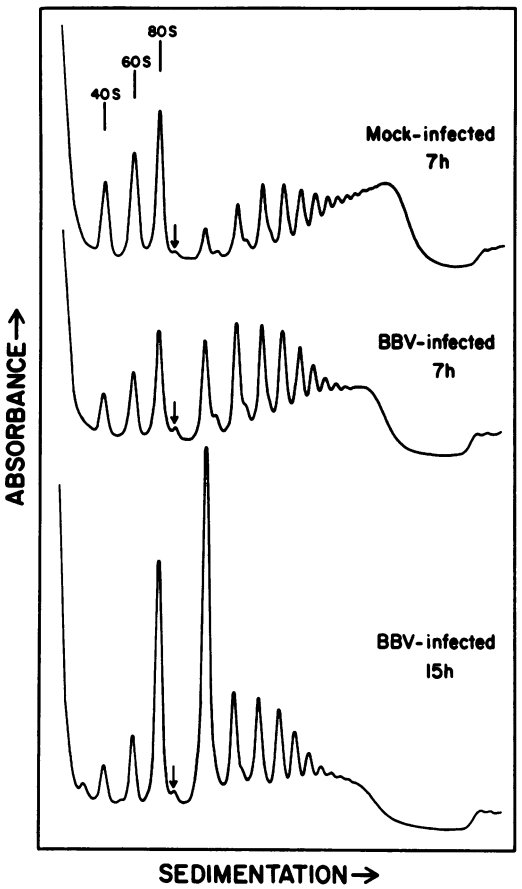

FIG. 5. Sedimentation of polysomes from BBV- and mock-infected *Drosophila* cells. Polysomes prepared 7 and 15 h after infection by using 0.5% Nonidet P-40 were sedimented on 15 to 45% (wt/wt) sucrose density gradients (10^7 cells per gradient) as described in the text. Gradients were fractionated with an ISCO gradient fractionator equipped with an absorbance (254 nm) monitor. Intact 80S ribosomes and subunits (40S and 60S) are indicated.

occurs between 7 and 12 h after infection (5), we expected that the protein B messenger would be most abundant during this interval. Therefore, polysomes were isolated from radioactive uridine-labeled cells at 7, 10, and 15 h. Figure 5 shows the sedimentation profiles of these polysomes isolated at 7 and 15 h.

The largest polysomes from mock-infected cells contained about 15 to 20 ribosomes per message (Fig. 5). The profile of this preparation also revealed, in addition to 80S ribosomes and their 40S and 60S subunits, a small but distinct peak sedimenting just ahead of each polysome peak (Fig. 5, arrow). This peak was also observed in the profile of polysomes from infected cells and may have represented the so-called "halfmers" (mRNA initiation complexes carrying 40S subunits) previously observed in cells of the yeast *Saccharomyces cerevisiae* (8).

Infection led to a progressive decline in the average number of ribosomes per message (Fig. 5). This was especially apparent after 15 h and is consistent with a previous report of a reduction in host cell protein synthesis after infection (5). The 15-h profile also shows a dramatic increase in the disome peak. However, reconstruction experiments have shown that virions (137S) cosediment with the disome peak (data not shown). Thus, the large increase in the size of the disome peak was probably due to the accumulation of newly assembled virions, which began to appear by 12 h after infection (T. Gallagher, personal communication).

Identification of the mRNA for protein B. RNA was extracted from [^{3}H]uridine-labeled polysomes harvested 10 h after infection and was sedimented on a sucrose density gradient. The resulting gradient fractions were subjected to electrophoresis on 6 M urea gels, and the radiolabeled, actinomycin-resistant RNA was detected by fluorography (Fig. 6A). The viral mRNA was resolved into four size classes which sedimented at 22S (fractions 20 through 22), 15S (fractions 14 through 16), 11S (fraction 10), and 9S (fractions 7 through 9). The 9S RNA, in particular, was concentrated in polysomes; this is illustrated by comparing the electrophoretograms of equal amounts of RNA extracted from intact cells (Fig. 6A, lane a) and from polysomes of infected cells (lane b).

The RNA recovered by ethanol precipitation from each gradient fraction was also translated in the cell-free reticulocyte system. The resulting in vitro products were subjected to electrophoresis on SDS-polyacrylamide gels and were detected by autoradiography (Fig. 6B). Figures 6A and B show that the mRNAs that sedimented in the positions of vRNA 1 (22S) and vRNA 2 (15S) directed the synthesis of proteins A and α, respectively. However, protein B was synthesized by the RNA that sedimented at 9S (fractions 7 through 9). Neither RNA 1 nor RNA 2 made any detectable amount of protein B. The prominent 11S RNA displayed no detectable messenger activity in the cell-free system.

We concluded from these in vitro translation experiments that protein B is synthesized from the actinomycin-resistant 9S RNA (designated RNA 3). We attribute the fainter bands that comigrated with protein B and were present in all fractions (Fig. 6B) to rabbit reticulocyte globin. Reticulocytes contain massive amounts of globin mRNA, so it is not surprising that traces of this message survive the nuclease treatment routinely used to remove endogenous mRNA from the cell-free system (23). Similarly,

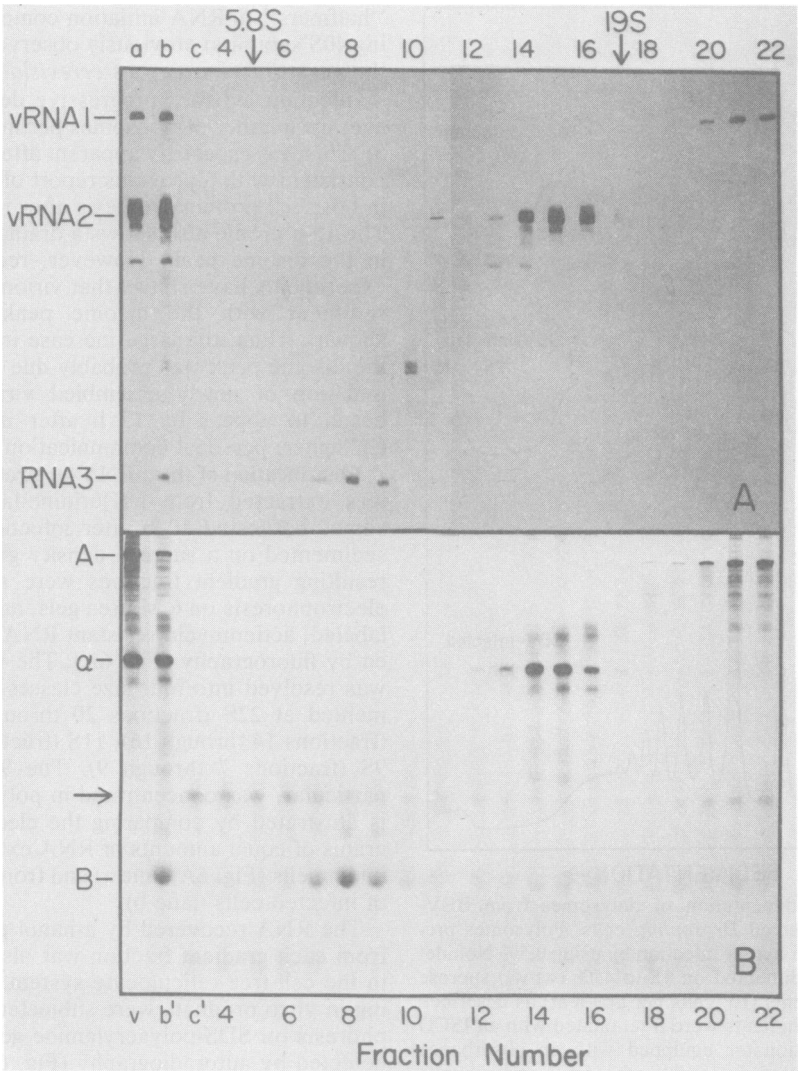

FIG. 6. Identification of intracellular BBV mRNAs. Polysomes from a cell suspension (10^7 cells) treated with actinomycin D and radiolabeled with [^{3}H]uridine (100 μCi/ml) beginning 7 h after infection were harvested 10 h after infection. RNA was extracted with phenol and sedimented on a 12-ml 5 to 25% (wt/wt) sucrose density gradient. The RNA from each gradient fraction (0.3 ml) was precipitated and dissolved in water (5 μl). Samples (1 μl) were either subjected to electrophoresis on 6 M agarose–acrylamide gels or translated in cell-free extracts of rabbit reticulocytes. (A) Fluorogram of actinomycin-resistant RNA from gradient fractions 4 through 22. Lane a, RNA extracted from intact cells radiolabeled as described above; lane b, RNA extracted from polysomes from intact cells radiolabeled as described above; lane c, no RNA. (B) Autoradiogram of an SDS-polyacrylamide gel showing in vitro translation products synthesized from gradient fractions 4 through 22. Lane v, in vitro products from vRNAs 1 and 2 from virions; lane b′, products from total polysome RNA; lane c′, no RNA. The arrow indicates an unidentified reticulocyte protein (molecular weight, 23,000) synthesized in all fractions (see text). The sedimentation positions of *Drosophila* 19S and 5.8S rRNAs are indicated.

an unidentified reticulocyte protein with an apparent molecular weight of 23,000 (Fig. 6B, arrow) was also synthesized in the absence of added RNA (Fig. 6B, lane c′).

Molecular weights of BBV RNAs. The molecular weight of BBV RNA 3 was determined by denaturation with glyoxal, followed by electrophoresis on polyacrylamide gels (Table 1). This method removes native secondary and tertiary structures of nucleic acids such that electrophoretic mobility is reduced to a simple function of molecular weight (17). Our estimate of the molecular weight of BBV RNA 3 (0.15×10^6, or about 450 bases) is sufficiently large to accom-

TABLE 1. Apparent molecular weights of the three major intracellular BBV RNAs

RNA	Apparent mol wt[a]
vRNA 1	1.12×10^6
vRNA 2	0.46×10^6
RNA 3	0.15×10^6

[a] Apparent molecular weights were determined by electrophoresis of denatured RNAs, using appropriate RNA standards, as described in the text. vRNAs 1 and 2 were measured on a 2.5% acrylamide gel; RNA 3 was measured on 4 and 6% gels.

modate the 300 or so bases required to encode a protein with a molecular weight of 10,000 (protein B). The molecular weights for BBV vRNAs 1 and 2 which we determined (Table 1) are in good agreement with the values reported previously for BBV RNA 1 (1.0×10^6) and RNA 2 (0.5×10^6) (16) and Nodamura virus RNA 1 (1.15×10^6) and RNA 2 (0.46×10^6) (20).

DISCUSSION

Model for the expression of the BBV genome: synthesis of a subgenomic RNA. Our current scheme for the synthesis and assembly of BBV proteins is illustrated in Fig. 7. In this report we have shown that virus-infected cells contain 22S and 15S mRNAs indistinguishable in electrophoretic mobility (Fig. 4) and in in vitro translation activity (Fig. 6) from vRNAs 1 and 2, respectively, extracted from virions. We have also shown that infected cells contain a 9S RNA (RNA 3), not found in virions, which cosediments in sucrose gradients with the messenger for protein B. All three RNAs were synthesized in the presence of actinomycin D at concentrations of the drug which completely blocked RNA synthesis in uninfected cells (Fig. 3).

That RNA 3 is a true subgenomic RNA and not merely the product of nuclease action on RNA 1 or 2 is suggested by the observation that in vitro translation of RNA 3 in the presence of a cap analog inhibits the formation of protein B (L. Guarino, personal communication). Such behavior is characteristic of RNAs which are capped at the 5′ end.

It remains to be determined whether RNA 3 is derived from virion RNA 1 or RNA 2. That RNA 1 is the parent of RNA 3 is suggested by the preliminary finding that *Drosophila* cells transfected with purified vRNA 1 synthesize RNA 1 and RNA 3 but not RNA 2 (T. Gallagher, manuscript in preparation). A primary sequence analysis currently in progress in the laboratory of Paul Kaesberg should answer this question definitively. Knowledge of the sequence relationships between RNA 3 and the virion RNAs 1 and 2 will also guide the development of systems for clarifying the mechanisms by which subgenomic RNA 3 is generated.

BBV RNA replication. Pulse-label experiments (Fig. 4) indicated that BBV RNAs 1 and 2 are the two most prominent RNAs synthesized in infected cells. Unexplained, however, is the presence of several minor RNAs other than RNA 3. The role of these minor RNAs during infection is currently under investigation. At the present time, we cannot entirely rule out the possibility that these actinomycin-resistant RNAs are the products of specific nucleolytic cleavage or of another related virus (e.g., helper virus).

Our estimate that more than one-half of the RNA synthesized in infected cells is virus specific (actinomycin-resistant synthesis) (Fig. 3) is consistent with the large yields of virus (up to 20% of the cell mass) produced by this *Drosophila* cell line (4). The abundant synthesis of viral RNA in the presence of actinomycin (Fig. 3 and 4) implies that BBV induces synthesis of a virus-specific RNA polymerase. Indeed, such an activity has already been partially purified and characterized (7), and BBV protein A (molecular weight, 104,000) is a likely candidate for this viral polymerase or a subunit thereof (5, 21).

None of the three viral RNAs from polysomes nor vRNAs 1 and 2 extracted from virions bound to oligodeoxythymidylic acid cellulose under conditions where polyadenylic acid-containing polioviral RNA bound quantitatively (data not shown). The vRNA from Nodamura virus also

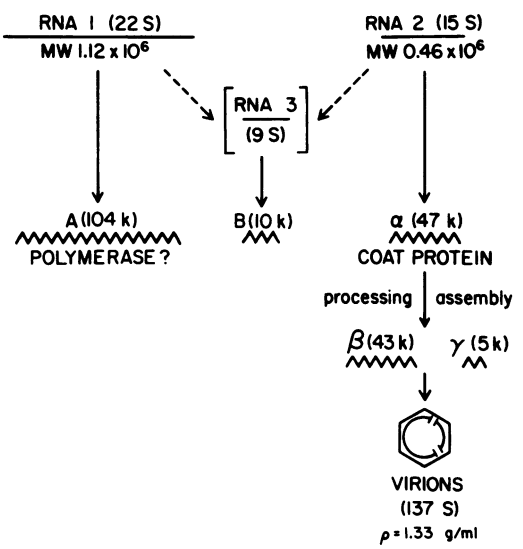

FIG. 7. Current model for the expression of BBV in cultured *Drosophila* cells. RNA 3 is a subgenomic viral message for protein B. The parentage of RNA 3 (vRNA 1 or vRNA 2) remains to be determined. MW, Molecular weight; k, kilodaltons.

lacks such affinity (19). These observations suggest that nodaviral RNAs, whether from virions or polysomes, lack the polyadenylic acid tract found in most mRNAs.

Intracellular synthesis of protein A is regulated at the level of translation. Previous studies on BBV-infected *Drosophila* cells have shown that proteins A, B, and α are synthesized in markedly different amounts and with different kinetics (5). In particular, the synthesis of protein A peaks very early in the infection cycle. This protein is made in amounts much smaller than coat protein α. However, it is clear from the vigorous in vitro synthesis of protein A by RNA extracted from cells at this time (Fig. 1) that the low rate of intracellular synthesis cannot be attributed to a lack of messenger for protein A. Moreover, RNA extracted from infected cells directed nearly equimolar synthesis of proteins A and α in the cell-free system. This proportion is identical to that synthesized in response to RNA extracted from virions and thus suggests that the ratio of mRNAs 1 and 2 found in infected cells is about the same as the equimolar ratio found in virions.

The large difference between the in vitro and in vivo messenger activities for protein A cannot be attributed entirely to preferential translation of RNA 1 by reticulocyte ribosomes. At saturating levels of message, the two virion RNAs are translated in the reticulocyte system with the same relative frequencies (5). There does appear to be a slight discrimination against RNA 1 in a cell-free system made from *Drosophila* cells (6), but it is not yet clear whether this represents true regulatory discrimination or merely greater sensitivity of the larger RNA 1 molecule to nuclease activity.

In conclusion, our findings suggest that infected cells contain an abundance of messenger for protein A even at times when cells synthesize very little protein A. Thus, synthesis of protein A in infected cells appears to be regulated at the level of translation. Such a regulatory mechanism could account for the sudden turnoff of protein A synthesis early in infection (5). The search for a factor or factors with the capacity to modulate the translation frequency of RNA 1 is currently under way.

ACKNOWLEDGMENTS

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

We thank Dan Omilianowski for chromatography of tryptic digests. We are indebted to James Dahlberg for the gift of small nuclear RNAs from HeLa cells and Ranjit Dasgupta for the gift of brome mosaic virus RNA. We also thank Paul Kaesberg and Linda Guarino for discussions and especially for information concerning their work with BBV RNA 3.

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