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Synthesis of Black Beetle Virus Proteins in Cultured *Drosophila* Cells: Differential Expression of RNAs 1 and 2

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Black beetle virus is an insect virus with a split genome consisting of two single-stranded, messenger-active RNA molecules with molecular weights of 1.0×10^6 (RNA 1) and 0.5×10^6 (RNA 2), respectively. Virions contained two proteins, β with a molecular weight of 43,000 (43K) and γ (5K), and traces of a third protein, α (47K). When translated in cell-free extracts of rabbit reticulocytes, RNA 1 directed the synthesis of protein A (104K), whereas RNA 2 synthesized protein α. The in vitro translation efficiency of the two RNAs was roughly equal. Infection of cultured *Drosophila* cells induced the synthesis of five new proteins: *A*, α, β, γ, and B (10K), detected by autoradiography of polyacrylamide gels after electrophoresis of extracts from [^{35}S]methionine-labeled cultures. All but protein γ could also be detected by staining with Coomassie brilliant blue, indicating vigorous synthesis of viral proteins. Pulse-chase experiments in infected cells revealed the disappearance of protein α and the coordinate appearance of proteins β and γ, supporting an earlier proposal that coat protein of mature virions is made by cleavage of precursor α. Proteins A and B were stable in such pulse-chase experiments. The three classes of virus-induced proteins, represented by A, B, and α, were synthesized in markedly different amounts and with different kinetics. Synthesis of proteins A and B peaked early in infection and then declined, whereas synthesis of coat protein precursor α peaked much later. These results suggest that RNA 1 controls early replication functions via protein A (and also possibly protein B), whereas RNA 2 controls synthesis of coat protein required later for virion assembly.

Black beetle virus (BBV), named after the host insect from which it was first isolated (9), is a member of a newly identified family of viruses whose prototype is Nodamura virus (12, 13, 20). The family name, Nodaviridae, is currently under consideration by the International Committee on Taxonomy of Viruses. These Nodamura-like viruses resemble the picornaviruses in size (30 nm in diameter) and buoyant density (1.33 to 1.34 g/ml) but sediment somewhat more slowly (137S versus 160S). The virion is reported to contain a single major coat protein (molecular weight, 40,000 to 41,000 [40 to 41K]) together with traces of a larger protein (molecular weight, 43 to 44K) thought to be a precursor (4, 14, 15). The viral genome consists of two molecules of single-stranded RNA with molecular weights of about 1.0×10^6 (RNA 1) and 0.5×10^6 (RNA 2), respectively (10). Both RNAs are thought to be packaged in the same virus particle (10, 14). This would make them unique among the other known bipartite viruses (comoviruses, nepoviruses, and tobraviruses), where the two RNAs are packaged in different particles (reviewed in reference 1).

Multipartite riboviruses containing messenger-active RNAs offer unique opportunities for studying the interaction of genes because translation and replication of one RNA can be studied in the absence of the others. Such studies on Nodamura virus have revealed that the two genomic RNAs have independent genetic functions. In cell-free systems, each RNA directed the synthesis of a single polypeptide which was chemically unrelated to the product of the other (15). RNA 1 coded for a 105K-molecular-weight protein (p105), whereas RNA 2 coded for a 43K-molecular-weight protein (p43) which contained all of the tryptic sequences of mature coat protein. In infected cells, both RNAs replicated independently; furthermore, both were required for infectivity (14). Collectively, these findings indicate that RNA 2 represents an independent gene which is not derived from RNA 1.

In vitro cell-free systems have been useful in studying the translational expression of the bipartite riboviruses (6, 15–17), yet little is currently known about the mechanisms used by these viruses to direct their synthesis and assembly in infected cells. This has been largely due

to the lack of convenient cell culture systems favorable for the biochemical analysis of viral multiplication. It was recently found, however, that BBV multiplies unusually well in *Drosophila melanogaster* cell culture (4). The availability of this new system, in addition to the fact that BBV has the smallest genome of the known bipartite riboviruses, has encouraged us to initiate in vivo studies on the life cycle of this virus. We report here that three classes of virus-induced proteins are synthesized in BBV-infected *Drosophila* cells. Furthermore, we provide evidence that each class of protein is under quantitative and temporal control during infection.

MATERIALS AND METHODS

Propagation of *Drosophila* cells. Schneider's *Drosophila* line 1 cells (22) were propagated at 26°C in Schneider culture medium (21) containing 5 mg of bacteriological peptone (Difco Laboratories, Detroit, Mich.) per ml and supplemented with 15% fetal bovine serum. The WR strain of line 1 (4) was adapted to growth in 490-cm^2 roller bottles (Corning Glass Works, Chicago, Ill.; no. 25130) turning at 0.5 rpm. The doubling time of the cells was about 12 h. Thus, bottles seeded with 15×10^6 to 20×10^6 cells in growth medium (100 ml) formed confluent monolayers (about 2×10^9 cells) within 3 to 4 days. Carried longer, the cells detached spontaneously but grew readily if transferred to fresh medium within 6 h. Cells were routinely passaged by flushing confluent monolayers into the spent growth medium with the aid of a pipette and diluting the cells 100-fold into fresh medium.

Stock virus. BBV, passaged twice in larvae of the wax moth *Galleria mellonella* (4), was subsequently passaged three times in *Drosophila* line 1 culture. To this end, WR cells were first inoculated with larva-grown BBV (1 wax moth 50% lethal dose per cell). After 2 days, virus was released by three freeze-thaw cycles, and debris was removed by centrifugation for 20 min at $12,000 \times g$. A volume (1 ml) of the frozen-thawed supernatant, representing 1 to 2% of the total virus yield, was subsequently used to infect an identical culture. After the third such passage, the clarified frozen-thawed supernatant was stored at − 70°C and used as stock virus.

For lack of a plaque assay, virus input during infection was expressed as particles per cell. The latter was calculated from optical density (absorbancy at 260 nm) measurements on virus purified from the above stocks. For example, when 20 ml of stock virus was sedimented on a sucrose density gradient (see below), the 137S virus peak contained an absorbancy of 1.8 at 260 nm. Assuming 100% recovery and an extinction coefficient of 4.15 (10), this virus stock contained 22 μg of virus per ml. When a molecular weight of 5×10^6 for BBV is used (8), this corresponds to about 2.5×10^{12} virus particles per ml of stock virus.

Procedure for infection. WR cells were dislodged from confluent monolayers, washed with Schneider medium containing 0.5% bovine serum albumin, and concentrated to 5×10^7 cells per ml. Routinely, a 5-ml suspension containing 2.5×10^8 cells was inoculated with third-passage virus stock (described above) at a multiplicity of 10^4 virus particles per cell. The cell suspension was agitated gently for 30 min at 26°C to allow for virus attachment. The culture was then diluted to 5×10^6 cells per ml with complete growth medium containing 15% fetal bovine serum, and the infection was allowed to proceed at 26°C. All infections were carried out in stoppered, siliconized Erlenmeyer flasks swirled gently (60 rpm) on a New Brunswick G2 rotary shaker. With constant but gentle swirling, the infected cells remained in suspension. This facilitated quantitative removal of samples during the course of infection.

Radiolabeling of intracellular proteins. At the indicated intervals during infection, samples (1 to 2 ml) of the infected cell suspension were withdrawn, sedimented by centrifugation, and resuspended in methionine-deficient Schneider growth medium containing 2% fetal bovine serum. From 100 to 200 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.; SJ.204) was added per ml of suspension, and incubation was continued at 26°C with gentle swirling. Incorporation of radiolabel was stopped by sedimenting the cells, washing them once with ice-cold Schneider medium, and resuspending them to 4×10^7 cells per ml in distilled water. An equal volume of solubilizing solution (2% sodium dodecyl sulfate [SDS], 1.0 M urea, 0.2% β-mercaptoethanol [β-ME]) was added, and the resulting viscous solution was heated in a boiling water bath for 5 min. SDS-dissociated cell lysates were stored at − 20°C.

Virus purification. BBV was purified as previously described (4). In brief, virus was released from infected cells by three freeze-thaw cycles and pelleted through a 30% sucrose cushion containing 0.05 M sodium phosphate (pH 7.2), 0.1% β-ME, and 0.5% BSA. Centrifugation was at $60,000 \times g$ for 4 h at 6°C. The pellet was resuspended in the above buffer, made 1% with respect to SDS, and sedimented on a 15 to 45% (wt/wt) sucrose gradient containing 0.05 M Tris-acetate, pH 7.2. Centrifugation was at 25,000 rpm ($60,000 \times g$) for 4.5 h at 11°C in a Spinco SW25.1 rotor. The virus-containing fractions, detected by optical density or radioactivity, were pooled and stored at − 70°C.

Purification of BBV RNA. Virion RNA was isolated as described for Nodamura virus (15). Purified virus (1.5 mg/ml in 1% SDS–0.05 M Tris-acetate, pH 7.2) was shaken with 1 volume of phenol for 15 min at room temperature, followed by 0.5 volume of chloroform-isoamyl alcohol (24:1) for 2 min at 45°C. Phases were separated by centrifugation, and then the resulting interface and aqueous phase were reextracted as described above, omitting the 45°C incubation. The final aqueous phase was made 0.2 M with respect to sodium chloride, and RNA was precipitated overnight at − 20°C with the addition of 2 volumes of absolute ethanol. RNA was reprecipitated with ethanol, dissolved in 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS, and sedimented on a 12-ml, 5 to 25% (wt/wt) sucrose gradient in 0.1 M sodium acetate (pH 5.0). Centrifugation was in an SW41 rotor at 26,000 rpm ($80,000 \times g$) for 18 h at 10°C, using [^{3}H]-uridine-labeled BBV RNA markers. The gradient was collected by bottom puncture, and the RNA-containing

fractions were ascertained by assaying for radioactivity. RNAs 1 and 2 were ethanol precipitated and further purified with an additional cycle of sucrose gradient sedimentation. The separated RNAs were precipitated with ethanol, redissolved to 1.2 mg/ml in distilled water, and stored at − 70°C.

Protein synthesis in rabbit reticulocyte lysates. Preparation of rabbit reticulocyte lysates and conditions necessary for protein synthesis have been described by Shih et al. (23, 24). In vitro protein synthesis was conducted at 30°C, using the standard 30-μl reaction mixture which included 58 μg of calf liver tRNA per ml and 1.1 mCi of L-[^{35}S]methionine (Amersham Corp., 1,200 Ci/mmol) per ml. Translation was initiated by the addition of viral RNA to primed reaction mixtures and then stopped by diluting samples (5 μl) in 100 μl of solubilizing solution (1% SDS, 0.5 M urea, 0.1% β-ME) and heating for 5 min at 100°C.

In vitro protein synthesis was monitored by measuring the incorporation of radiolabel into trichloroacetic acid-insoluble material. Paper filter disks (2.3 cm, Whatman 3MM), pretreated with 0.1 ml of 1% SDS–3% Casamino Acids (Difco Laboratories), were spotted with 25-μl samples of SDS-disrupted translation mixtures. The filters were soaked for 30 min at room temperature in 10% trichloroacetic acid–1% SDS–3% Casamino Acids and then heated to 85°C for 20 min in 5% trichloroacetic acid–1.5% Casamino Acids. After the filters were washed with 5% trichloroacetic acid, 95% ethanol, and diethyl ether, respectively, they were dried and assayed for radioactivity.

Electrophoretic analysis. Procedures for SDS-polyacrylamide gel electrophoresis have been described previously (11). Slab gels (14 by 28 by 0.15 cm) were prepared with 9.8% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 0.1% *N,N,N',N'*-tetramethylethylenediamine, 0.1% SDS (Pierce Chemical Co., Rockford, Ill.; lot 4102-5), and 1.0 M urea in 0.1 M sodium phosphate (pH 7.2). Polymerization was catalyzed by the addition of ammonium persulfate to a final concentration of 0.05%. Samples (50 μl) in 1% SDS–0.5 M urea–0.1% β-ME were prepared for electrophoresis by adding Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and bromophenol blue to 10 and 0.01%, respectively, and heating for 5 min at 100°C. Electrophoresis was conducted at 3 V/cm, while the electrode buffer (0.1% SDS, 0.05 M neutralized 3-mercaptopropionic acid, 0.1 M sodium phosphate, pH 7.2) was recirculated between the upper and lower buffer chambers.

Gel staining and autoradiography. After electrophoresis, each slab gel was soaked for a total of 2 h in two changes (500 ml each) of 25% methanol–10% acetic acid–1% glycerol. Gels were then stained for 3 h in the above solution containing 0.025% Coomassie brilliant blue R250 and destained overnight with several changes of solution alone. The stained gels were then dried under a vacuum and subjected to autoradiography by exposing each to Kodak NS 54T no-screen X-ray film for 1 to 3 days.

Molecular weight determination. Samples of [^{35}S]methionine-labeled virions, cell lysates prepared 5 and 24 h after infection, and cell-free reticulocyte translation mixtures were subjected to electrophoresis on 10% polyacrylamide gels (see above) in lanes adjacent to samples containing a mixture of five unlabeled protein standards: β-galactosidase (Worthington Diagnostics, Freehold, N.J.), phosphorylase *a* and carbonic anhydrase (Sigma Chemical Co., St. Louis, Mo.), and bovine serum albumin and ovalbumin (Schwarz/Mann, Orangeburg, N.Y.). Gels were stained with Coomassie brilliant blue and subjected to autoradiography. Apparent molecular weights were calculated by comparing the electrophoretic mobilities of the radiolabeled viral proteins with those of the stained standard proteins (3).

RESULTS

Effect of input multiplicity on virus-induced protein synthesis. The unusually high yields of BBV produced in *Drosophila* line 1 cells, on the order of 20% of the total cellular protein (4), suggested that intracellular virus-specific proteins ought to be present in quantities sufficiently large to permit detection by merely staining polyacrylamide electropherograms. That this was indeed the case was demonstrated by preliminary experiments in which cells were exposed to serial dilutions of stock virus, lysed 24 h later with hot SDS, and subjected to electrophoresis on SDS-polyacrylamide slab gels. As shown in Fig. 1, virus-specific proteins were readily distinguished from the large background of host *Drosophila* proteins on Coomassie brilliant blue-stained electropherograms.

Three prominent protein bands (α, β, and B) appeared within 24 h after infection. Proteins α and β comigrated with the BBV coat proteins vp44 and vp40, respectively (compare lanes i and j). On the other hand, protein B is a previously undescribed protein not found in purified virions. The two proteins α and B were detected at input multiplicities as low as 10 virus particles per cell (lane d). With virus doses exceeding 300 particles per cell (lane g), the intensities of these two bands remained constant. Even with input multiplicities as high as 100,000 particles per cell, there was no further increase in the relative size and intensity of these two bands (data not shown). Thus, under these conditions, an input multiplicity of at least 300 particles per cell was sufficient for maximum synthesis of virus-specific proteins.

Time course of protein synthesis. Pulse-label experiments were conducted to examine the sequence of viral protein synthesis in BBV-infected cells and to determine the effect of infection on host protein synthesis. To this end, a 50-ml suspension culture was infected as described above at a high multiplicity (10^4 particles per cell) to assure maximal synthesis of viral proteins. Samples (2 ml) of cell suspension were

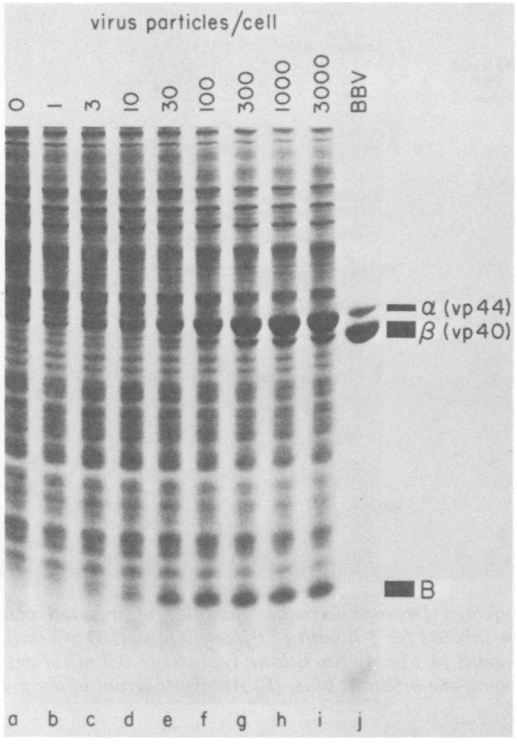

FIG. 1. *Titration of virus stock for ability to induce virus-specific protein synthesis in cultured Drosophila cells. Replicate cultures of Drosophila line 1 cells were exposed to the indicated doses of stock virus (see text) and incubated for 24 h at 26°C. The cells were sedimented, dissolved in hot SDS, and subjected to polyacrylamide gel electrophoresis; proteins were detected by staining the gel with Coomassie brilliant blue. Each lane represents the proteins from 0.2 ml of cell suspension (~10^6 cells). BBV capsid proteins (lane j) are included as markers.*

withdrawn at 6-h intervals and exposed to [^{35}S]methionine for 1 h. Cell lysates were then prepared and analyzed by SDS-polyacrylamide gel electrophoresis. The resulting gel was stained with Coomassie brilliant blue R250 (Fig. 2A) and then subjected to autoradiography (Fig. 2B).

The major viral product synthesized during the course of infection was protein α. On stained gels (Fig. 2A), protein α appeared 6 h after infection and reached maximum proportions by 24 h. The major virion protein β did not appear until 18 h after infection but accumulated to nearly the same proportions as did protein α by 48 h. The early appearance of protein α followed later by the appearance of protein β is consistent with the proposal (4) that protein α, also called vp44, represents the precursor of the major coat protein β, also called vp40. Two other proteins, also detected in BBV-infected cells, included the prominent band B (noted above) and a very faint high-molecular-weight band A (Fig. 2A). Both proteins A and B appeared as early as 6 h and were present for as long as 48 h after infection.

Protein A is more apparent in the autoradiogram (Fig. 2B) which illustrates the relative rates of synthesis of the BBV-induced proteins. Proteins A and B were synthesized most rapidly in the 6-h sample. However, protein B was synthesized in greater amounts, and its rate of synthesis declined less rapidly than that of A. The intense labeling of protein α suggested that coat protein is synthesized in amounts greatly exceeding that of proteins A and B. Synthesis of protein α was maximal at 18 h but continued at comparatively high rates for at least 48 h after infection.

Figure 2B also indicates that infection was accompanied by a significant decline in the rate of host protein synthesis occurring as early as 12 h after infection. The constant staining intensity of host proteins throughout infection (compare lanes of Fig. 2A) suggest that cell lysis, which occurs only a low levels late in infection, is not the major cause of this decline. This inhibition of host protein synthesis also implies that the majority of cells were infected.

BBV coat proteins: pulse-chase analysis. Although readily apparent on stained gels (Fig. 2A), the major coat protein β was not detected on autoradiograms of pulse-label experiments (Fig. 2B). This implied that if protein β is derived from protein α, the cleavage must be slow relative to the 1-h labeling periods examined. This hypothesis was tested by pulse-chase experiments conducted during the period of maximum coat protein synthesis. Line 1 cells were pulse-labeled with [^{35}S]methionine 12 h after infection and transferred to complete culture medium containing a 50-fold excess of methionine and 15% fetal bovine serum. The flow of radioactivity was monitored at intervals during the chase by polyacrylamide gel electrophoresis (Fig. 3).

The decrease in protein α and the accompanying appearance of protein β (lanes d to i) indicated that protein α is indeed cleaved to generate protein β in infected cells. These two coat proteins were present in nearly equimolar proportions after the 36-h chase period (lane i). Thus, precursor α has an unusually long half-life (about 1.5 days) compared, for example, with the picornaviral coat precursor with a half-life of only 7 to 10 min (19). A previously undescribed protein, γ, also appeared late in the chase period (lanes d to i). The small size of this protein and its simultaneous appearance with protein β suggested that protein γ is the sister

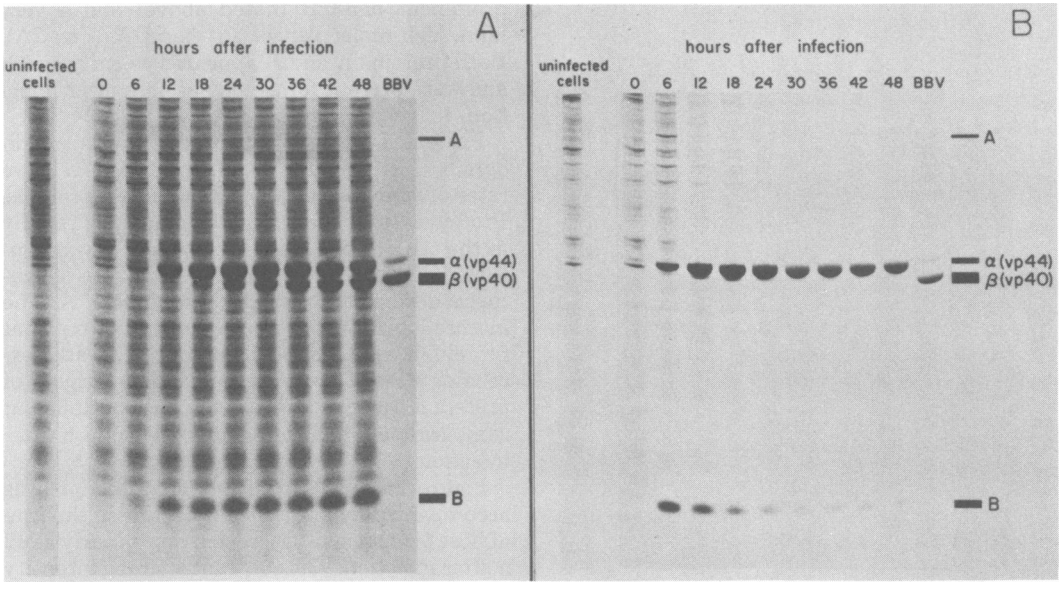

FIG. 2. *Time course of protein synthesis in BBV-infected Drosophila cells. Samples of infected cell suspension were withdrawn at the indicated times, pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/ml), and prepared for electrophoresis as described in the legend to Fig. 1. Each lane represents 0.2 ml of cell suspension. (A) Electropherogram after staining with Coomassie brilliant blue. (B) Autoradiogram of the gel shown in (A).*

fragment generated by cleavage of protein α. Thus, cleavage appears to follow the scheme α → β + γ.

This cleavage scheme implied that protein γ might also be a virion protein which until now had not been recognized as such (4, 10). To examine this possibility, virus was radiolabeled to a high specific activity (about 0.3 μCi/μg) by adding [^{35}S]methionine to a culture 12 h after infection and harvesting virus 36 h later. After rapid purification on sucrose density gradients as described above, 137S virions were disrupted with 1% SDS–0.5 M urea–0.1% β-ME and analyzed by electrophoresis. As shown in Fig. 3 (lane j), protein γ is indeed found in virions. It is not yet entirely clear why protein γ has not previously been recognized in virions. In some cases, it was lost by migration past the end of the gel (e.g., Fig. 2). However, since the relative amount of protein γ in different preparations is variable (compare Fig. 3, lane j, and Fig. 8, lane f), it might also be that protein γ is easily lost during purification of virions.

Noncapsid proteins: kinetics of synthesis. The experiment illustrated in Fig. 2B indicated that maximum synthesis of noncapsid proteins A and B occurred during the first 12 h of infection. This early period of synthesis was examined in greater detail by infecting a suspension of *Drosophila* line 1 cells (10^4 particles per cell) and pulse-labeling samples at hourly intervals with [^{35}S]methionine. Figure 4A, which displays only half of the samples analyzed by electorphoresis, shows that synthesis of protein A peaks from 4 to 6 h, whereas that of protein B peaks later, about 8 to 10 h after infection. This is illustrated more fully in Fig. 5, which summarizes the results of densitometric analyses of all 13 samples prepared 0 through 12 h after infection.

Pulse-chase experiments conducted during the period of declining synthesis of proteins A and B (5 to 24 h postinfection) gave no indication that either protein is cleaved to smaller polypeptide products (Fig. 4B). Each protein was stable for at least 36 h after its synthesis. Thus, the observed increase and subsequent decrease in the rates of radiolabel incorporation by these proteins (Fig. 5) are due to differences in the rates of synthesis and not to rapid cleavage or breakdown of newly synthesized protein. The slow decline in intensity of proteins A and B is probably due to low levels of cell lysis occurring late in infection since there is a parallel decrease in intensity of labeled host proteins.

In vitro translation of BBV RNA in cell-free extracts of rabbit reticulocytes. To identify the viral polypeptides encoded by each of the two genomic RNAs of BBV, in vitro translation studies were conducted. RNA was extracted from purified virus, separated by sedimentation on sucrose density gradients (Fig. 6),

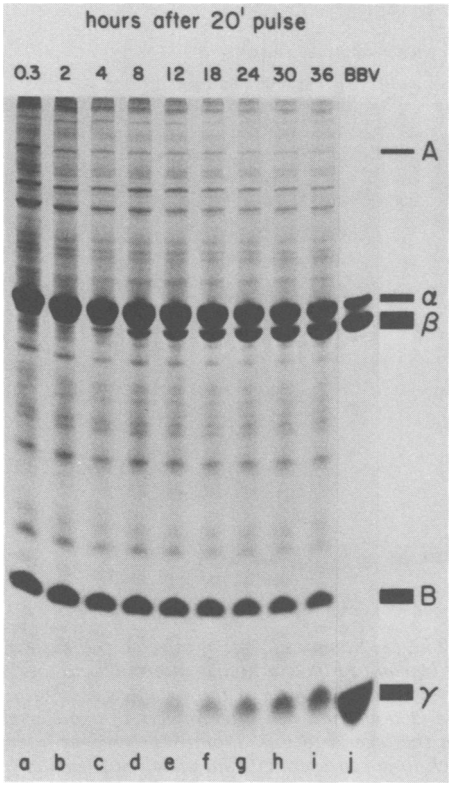

FIG. 3. *Pulse-chase analysis of proteins synthesized in BBV-infected Drosophila cells. Cells infected 12 h previously were pulse-labeled for 20 min with [^{35}S]methionine (200 μCi/ml), washed, and resuspended to their original concentration (5 × 10^6 cells per ml) in complete growth medium containing a 50-fold excess of methionine. Samples (1 ml) were withdrawn at the indicated times during the chase and analyzed by polyacrylamide gel electrophoresis as described in the text. [^{35}S]methionine-labeled virion protein markers (lane j) were prepared as described in the text.*

and translated in nuclease-treated lysates of rabbit reticulocytes as described above.

Both BBV RNAs were unusually active messengers in the reticulocyte system. Maximum amino acid incorporation was obtained when potassium acetate and magnesium acetate were added to concentrations of 80 and 0.5 mM, respectively. In the range of 0 to 75 μg of virion RNA (an unfractionated mixture of RNAs 1 and 2) per ml, incorporation increased linearly and saturated with an RNA concentration of 100 μg/ml or greater. Under optimal conditions, incorporation was 60 to 75 times that of background.

A typical time course of [^{35}S]methionine incorporation in reticulocyte lysates, programed with saturating levels of BBV RNA, is shown in Fig. 7A. Incorporation increased linearly for the first 20 min of incubation and then stopped within 60 min. Purified RNA 1 stimulated about 1.5 times as much incorporation as RNA 2. Incorporation stimulated by the mixed RNAs was about that expected if neither RNA influenced the translation efficiency of the other. Figure 7 also compares the messenger activity of the BBV RNAs with that of encephalomyocarditis virus RNA, another exceptionally active viral message in reticulocyte lysates (23, 24). Synthesis directed by saturating amounts of each RNA (100 μg of BBV RNA per ml; 40 μg of encephalomyocarditis virus RNA per ml) was complete within 60 min, yet incorporation directed by total BBV RNA (4.4 × 10^5 cpm) was 1.6 times greater than that of encephalomyocarditis virus RNA (2.8 × 10^5 cpm).

SDS-polyacrylamide gel analysis of the proteins synthesized in response to the BBV RNAs revealed two major products: proteins A and α (Fig. 7B). Band α appeared within 5 to 10 min after the addition of RNA, whereas band A appeared within 20 min. No evidence for cleavage of either protein was observed when lysates were incubated for as long as 3 h. A heterogeneous collection of minor bands migrating between bands A and α was routinely observed, and we intepret these bands to represent incomplete forms of the larger product A generated by premature termination of nascent polypeptide chains. The absence of a correspondingly large number of minor bands below band α suggests that premature termination occurs less frequently with RNA 2 than with RNA 1.

In vitro translation of the individual BBV RNAs demonstrated that protein A is the product of RNA 1, whereas protein α is the product of RNA 2 (Fig. 8). The major product directed by RNA 1 (lane d) had an apparent molecular weight of 104K (Table 1) and an electrophoretic mobility identical to that of protein A found in infected cells (lane b). The profile of protein directed by RNA 2 (lane e) revealed one major product, which had an electrophoretic mobility identical to that of coat protein α (molecular weight, 47K; Table 1) found in infected cells (lane b). In addition to protein α, bands with molecular weights exceeding the theoretical coding capacity of RNA 2 were routinely observed. The origin of these bands is uncertain but might conceivably be due to contaminating fragments of RNA 1. Under these conditions, synthesis of virus-induced protein B (molecular weight, 10K; Table 1) was not detected in lysates programed with RNA 1 or 2 or mixtures thereof.

The combined electrophoretic pattern of RNA 1 (lane d) and RNA 2 (lane e) is essentially identical to the pattern produced by the translation of total virion RNA (lane c). Thus, trans-

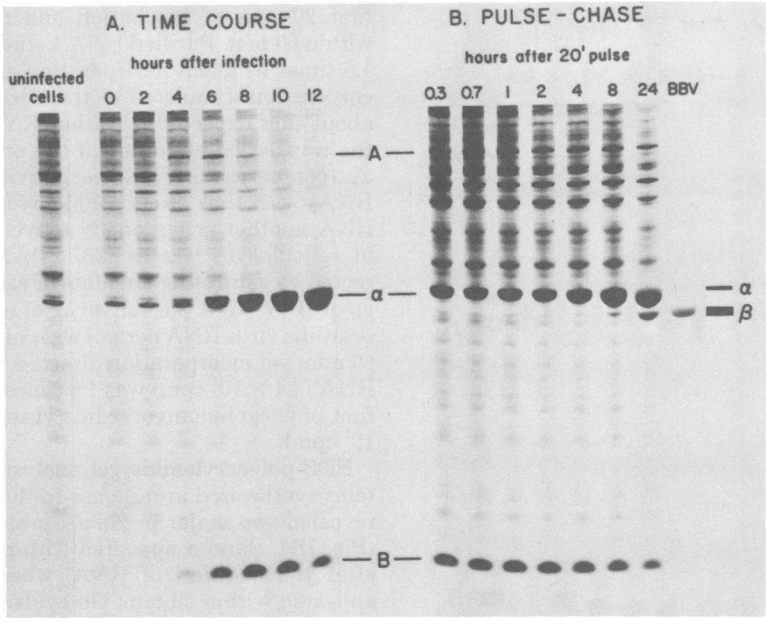

FIG. 4. *Kinetics of BBV-induced protein synthesis (0 to 12 h). (A) Samples (2 ml) of infected cell suspension were pulse-labeled with [^{35}S]methionine as described in the legend to Fig. 2 at hourly intervals and analyzed by polyacrylamide gel electrophoresis. The autoradiogram shows only half of the samples analyzed. (B) Infected cells were pulsed for 20 min with [^{35}S]methionine (200 μCi/ml) 5 h after infection, transferred to growth medium with unlabeled methionine as described in the legend to Fig. 3, and analyzed at intervals thereafter by gel electrophoresis and autoradiography. Each lane represents 0.2 ml of cell suspension (~10^6 cells).*

lation of a mixture of RNAs 1 and 2 is not significantly different from that of each individual RNA. This suggests that under these conditions, the translation product of one RNA does not significantly influence the translation or processing of the other. Translation of equal amounts of RNAs 1 and 2 resulted in nearly equal synthesis of both products A and α (Fig. 8, lane c). This is contrasted with the greatly disproportionate synthesis of proteins A and α observed in infected cells (Fig. 2 and 4).

DISCUSSION

Model for the translation and processing of BBV protein. Our current picture of the synthesis and assembly of BBV protein is shown in Fig. 9. When translated in cell-free extracts of rabbit reticulocytes, each of the two RNAs directed the synthesis of a single polypeptide, protein A (104K) from RNA 1 and protein α (47K) from RNA 2. Thus, the in vitro translation pattern of BBV RNAs closely resembles that of Nodamura virus whose proteins p105 (homologous to protein A) and p43 (homologous to protein α) have previously been shown to share no common sequences detectable by tryptic analysis (15). Oligonucleotide fingerprints of RNase T_1 digests similarly fail to reveal any evidence of extensive homologies between BBV RNAs 1 and 2 (A. Ghosh and L. Guarino, personal communication). This supports the conclusion that RNAs 1 and 2 are independent genes; i.e., RNA 2 is not a subgenomic derivative of RNA 1 (14, 15).

Our studies of virus-induced protein synthesis in BBV-infected cells have added three new features to the above picture. First, the primary translation product of RNA 2, coat protein α, is cleaved in the cell; second, another protein, B, not synthesized in cell-free extracts, was produced in large amounts in the cell; and third, the synthesis of all three major products, A, B, and α, were under quantitative and temporal control not observed in the cell-free system.

Processing and assembly of virions. In vivo pulse-chase experiments (Fig. 3) revealed that protein α disappeared coordinately with the appearance of coat proteins β (molecular weight, 43K) and γ (molecular weight, 5K). This supports the hypothesis that protein α is the in vivo precursor of coat protein. The similar masses of the α (molecular weight, 47K) and β + γ chains (molecular weight, 48K) are also consistent with this picture; the small discrepancy in masses lies

well within the experimental uncertainty of the empirical electrophoretic method used to obtain the molecular weight values (Table 1).

Still to be clarified are the virus or host origin of the protease responsible for cleavage of protein α, the stage in viral morphogenesis at which cleavage takes place, and the role of protein γ whose identity and association with virions have only now been recognized.

Another point which merits further examination is the proportion of RNAs 1 and 2 found in purified virions. A ratio of 0.4 to 0.6 found for RNA 1/RNA 2 (e.g., Fig. 6) is inconsistent with the current model (Fig. 9) in which each virus

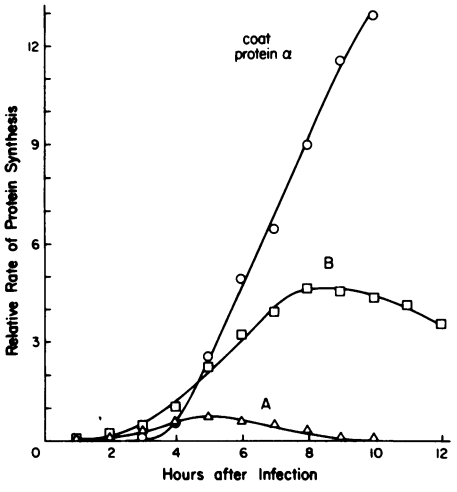

FIG. 5. *Relative rates of synthesis of the major BBV-induced polypeptides in Drosophila line 1 cells. Autoradiograms like that in Fig. 4 were quantified by densitometry. To measure protein synthesis, the area under each peak was normalized to sample size and plotted in arbitrary units. The results were not corrected for nonlinearities in the relationships between radioactivity and film density. This error causes the highest values to appear too low.*

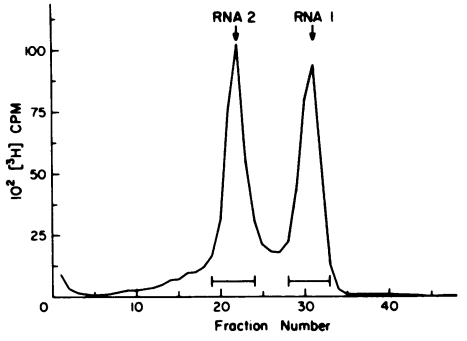

FIG. 6. *Sedimentation profile of [^{3}H]uridine-labeled RNA of BBV propagated in Drosophila cell culture. Purified BBV (radiolabeled with [^{3}H]uridine from 24 to 48 h after infection) was extracted, precipitated with ethanol, and centrifuged on a sucrose density gradient as described in the text. RNA in 0.1 M sodium acetate (pH 5.0) was heated for 5 min (65°C) and quickly cooled to 0°C before centrifugation. Sedimentation is from left to right. Before translation in cell-free extracts, each RNA was further purified by precipitating the indicated fractions (——) with ethanol and resedimenting as described above.*

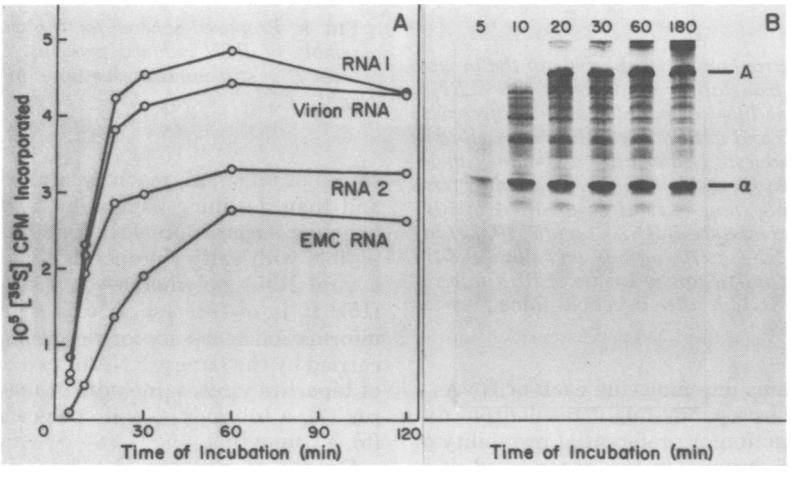

FIG. 7. *Time course of protein synthesis in cell-free extracts of rabbit reticulocytes programed with BBV RNA. Translation was initiated by the addition of RNA to nuclease-treated reticulocyte lysates and stopped at the indicated times with hot SDS as described in the text. (A) Incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material in lysates programed with total virion RNA, RNA 1, RNA 2, (100 μg/ml each), and encephalomyocarditis virus RNA (40 μg/ml). (B) Electrophoretic analysis of a reticulocyte lysate programed with total virion RNA of BBV.*

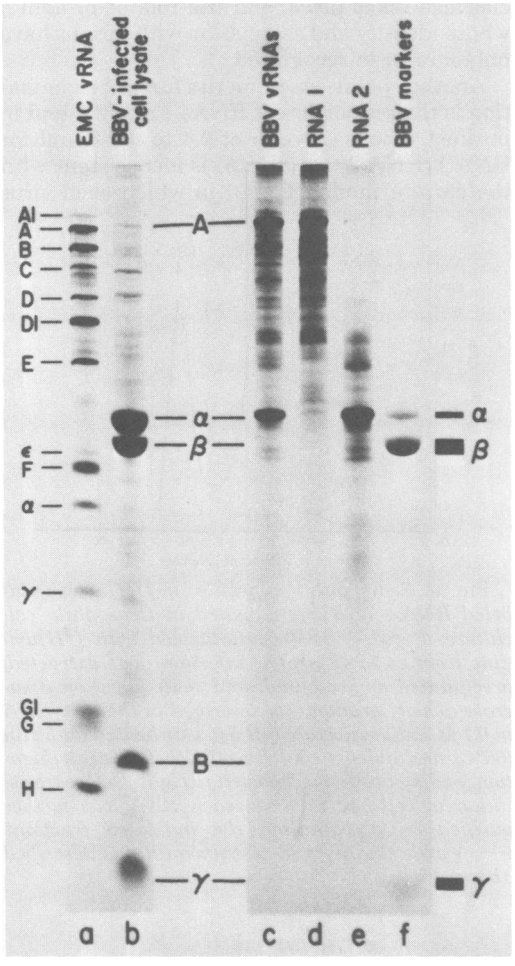

FIG. 8. *Electrophoretic comparison of the in vitro and in vivo translation products of BBV RNA. [^{35}S]methionine-labeled samples were subjected to electrophoresis and autoradiography as described in the text. Reticulocyte lysates after a 60-min translation period with (lane a) encephalomyocarditis virus RNA (40 μg/ml), (lane c) total virion RNA of BBV (unfractionated mixture of RNAs 1 and 2; 100 μg/ml), (lane d) BBV RNA 1 (100 μg/ml), and (lane e) BBV RNA 2 (100 μg/ml). (Lane b) Lysate of BBV-infected Drosophila cells 48 h after infection; (lane f) virion protein markers.*

particle contains one molecule each of RNAs 1 and 2. This discrepancy might result from differential extraction or preferential instability of RNA 1 from virions. On the other hand, it is worth recalling that the model of two RNA species in every particle has not yet been unequivocally established.

Role of protein A. Another new result from these studies on infected cells is the finding that protein A is synthesized mainly during the early

TABLE 1. *Apparent molecular weights of viral polypeptides synthesized in BBV-infected Drosophila cells and cell-free extracts of rabbit reticulocytes programmed with BBV RNAs 1 and 2*

Protein band	Apparent mol wt[a]
A	104,000
α	47,000
β	43,000
B	10,000
γ	5,000

[a] Calculated from the mobility of each protein band on SDS-polyacrylamide gels relative to that of five protein standards; β-galactosidase (116,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), ovalbumin (46,000), and carbonic anhydrase (29,000). A straight line was fitted by regression analysis to the plot of log molecular weight against mobility (3).

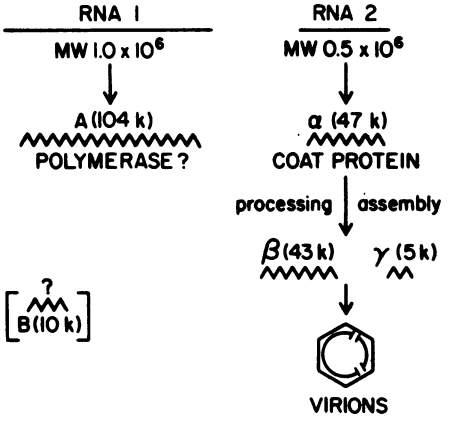

FIG. 9. *Proposed scheme for the translation and assembly of BBV-induced proteins. The origin of protein B is still unclear (see text). MW, Molecular weight.*

stages of infection, reaching a maximum at 5 h and then declining to low levels (Fig. 5). This behavior suggests an early function and is compatible with earlier proposals that protein A is a viral RNA polymerase or a subunit thereof (15). It is of interest in this regard that the information necessary for viral replication is also carried by the largest RNA of two other families of bipartite viruses, including tobacco rattle virus (7), a tobravirus, and cowpea mosaic virus (5), a comovirus.

Origin of protein B. The origin of virus-induced protein B is not clear (Fig. 9). It was not produced in cell-free extracts programed with BBV RNAs 1 and 2. Furthermore, pulse-chase experiments gave no indication that it was derived by cleavage of viral protein A or α (Fig. 4). It is conceivable that protein B represents a host

product whose synthesis is induced upon infection. However, we favor the idea that B is a virus-coded protein. The absence of protein B in cell-free extracts programed with RNA from virions might then be explained by the need for a regulatory factor which is inactive or absent in cell-free extracts or by the existence of a subgenomic RNA found in infected cells and not in virions. Synthesis of virus-specific proteins from subgenomic RNAs has been reported for a number of viruses (6, 18, 25). Indeed, preliminary experiments reveal that BBV-infected cells, treated with actinomycin D to suppress synthesis of host RNAs, contain, in addition to viral RNAs 1 and 2, minor amounts of several other RNAs (unpublished data). This RNA from infected cells contained a message which directed the synthesis of protein B when added to reticulocyte extracts. Moreover, this message which was not found in uninfected control cultures sedimented more slowly than did RNA 2 on a sucrose density gradient. These observations suggest that the B-messenger is a virus-coded subgenomic RNA; however, proof will require isolation of the message and determination of its sequence relationship to RNAs 1 and 2.

Regulation of viral protein synthesis. The independent kinetics and markedly different rates at which each of the three classes of virus-induced protein, A, B, and α, are synthesized (Fig. 5) indicate that the synthesis of each product is independently regulated in BBV-infected cells. This regulation is not, however, apparent in the cell-free reticulocyte system. Quantitation of the relative proportions of each viral RNA during infection will be valuable in determining whether the different rates of synthesis are controlled by the amount of viral messenger available or by the efficiency with which viral messenger is translated.

A final question raised by this study concerns the possible function of a segmented genome in the regulation of viral synthesis. It has recently been reported (5) for cowpea mosaic virus, another bipartite virus, that the smaller middle-component RNA codes for late functions (capsid proteins), whereas the larger bottom-component RNA codes for early functions (possibly an RNA replicase). This has led to the proposal (5) that each RNA codes exclusively for either early or late proteins. If so, then the purpose of segregating genes might be to facilitate independent regulation of early and late functions. Our findings with BBV appear to follow this same pattern; i.e., the larger RNA 1 codes for an early protein, A, whereas the smaller RNA 2 codes for a late protein, capsid precursor α. Because of its apparent simplicity, BBV may provide a good model for further investigation of this proposal.

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