JOURNAL OF VIROLOGY, May 1983, p. 481–489
0022-538X/83/050481-09$02.00/0

Vol. 46, No. 2

Autonomous Replication and Expression of RNA 1 from Black Beetle Virus

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Received 1 December 1982/Accepted 1 February 1983

Black beetle virions contain two RNAs. The smaller one, RNA 2, has previously been shown to be a messenger for viral coat protein. It is shown here, by infecting sensitized *Drosophila* cells with the individually purified RNAs, that the larger one, RNA 1, carries the viral gene(s) required for RNA polymerase functions. RNA 2 was dispensible for synthesis of viral RNA 1 and subgenomic RNA 3 but was essential for synthesis of RNA 2 and virions. Cells infected with RNA 1 alone produced RNA 3 in proportions 10- to 20-fold greater than cells infected with virions. This overproduction of RNA 3 decreased with increasing proportions of RNA 2 in the infecting RNA 1. We conclude that RNA 1 is the previously unidentified progenitor of subgenomic RNA 3, whereas RNA 2 regulates the amount of RNA 3 produced in the infected cell.

Viruses of the family Nodaviridae, of which black beetle virus (BBV) is a member (13), contain two messenger-active virion RNAs, both of which are necessary for formation of infective virions (17). The two RNA species of BBV, RNA 1 (22S) and RNA 2 (15S), are readily separable by centrifugation on sucrose density gradients. In vitro translation of the separated RNAs has shown that protein A (molecular weight, 105,000) is encoded by RNA 1 and that coat protein precursor alpha (molecular weight, 47,000) is translated from RNA 2 (4). A third viral RNA, RNA 3 (9S), is found in BBV-infected *Drosophila* cells. Although the origin of this subgenomic RNA 3 has not been clear, it is known to be the messenger for a 10-kilodalton protein called B (5), which is produced in large amounts in BBV-infected cells.

Synthesis of Nodaviral RNA is resistant to the drug actinomycin D, implying that the virus genome encodes a viral replicase (5, 18). Since RNA 2 was already known to be the messenger for coat protein, RNA 1 was an obvious candidate for a replicase gene. To test this proposal, we set out to determine if RNA 1 could replicate independently of RNA 2 and its gene product.

Earlier studies on a number of bipartite plant viruses, including tobraviruses (11, 22), comoviruses (6, 8), and nepoviruses (21), have demonstrated the ability of one of the RNAs to replicate independently of its partner RNA carrying the gene for coat protein. All of these experiments took advantage of the circumstance that the two RNAs segregate into separate virions (coviruses), some of which could be partitioned into individual components by centrifugation on isopycnic density gradients. We initially attempted to fractionate BBV virions in a similar way but failed, presumably because both RNAs reside in a common particle (12, 18). We were able to surmount this problem by separating the free RNAs on sucrose gradients and by taking advantage of the observation that *Drosophila* cells, like mammalian cells (20), could be sensitized to infection by free viral RNA after treatment with DEAE-dextran, a polycationic polymer.

These infective RNA studies have shown that BBV RNA 1 is indeed able to replicate in the absence of RNA 2, the gene for coat protein, indicating that RNA 1 carries all of the viral genes necessary for synthesis of viral RNA in infected *Drosophila* cells. Our findings also lend additional support to the hypothesis (4, 6) that one function of the split genome is to separate the genes involved in early (replicative) functions from those involved in late (packaging) functions.

MATERIALS AND METHODS

Cells. The WR subline of Schneider line 1 cells (3, 24) was propagated in roller bottles in Schneider culture medium (23) containing 15% fetal bovine serum as described previously (4). The serum was heat inactivated before use by incubation for 30 min at 60°C. The doubling time of the cells at 26°C was about 15 h. Cells were counted in a Neubauer hemacytometer.

Stock virus. BBV was propagated as described pre-

viously (4), except that virus from wax moth larvae (3) was passaged serially three times through *Drosophila* cells at a lower multiplicity (100 particles per cell) to generate stock virus. Purification was as described previously (4), except that virus was released, not by freezing and thawing, but by gently agitating the infected cells for 15 min at 0°C with 0.5% Nonidet P-40. Treatment with this nonionic detergent was about twice as effective as freeze-thaw cycles in releasing progeny virus from infected cell debris. Under these conditions, the virus yield 2 days after infection was about 3 to 5 μg of virus per ml of infected suspension.

Virus to be used as a source of infective RNA was prepared by infecting 10^8 cells in 10 ml of growth medium with 5,000 particles of stock virus per cell. After a 2-day incubation, the yield with this higher infection multiplicity was typically about 200 μg of virus per ml of infected suspension.

This procedure of passaging serially at low multiplicity followed by a single passage at high multiplicity was designed to prevent accumulation of defective interfering particles which otherwise emerge when the virus is passaged serially at the high multiplicities (10,000 particles per cell) used previously (unpublished observations).

Antiserum. Antiserum directed against native BBV was collected from rabbits 7 weeks after injection of 0.5 mg of purified BBV emulsified in Freund complete adjuvant (3). Preimmune serum was obtained immediately before injection.

Preparation of infective RNA. Virion RNA was isolated as described previously for Nodamura virus (19). Purified virus (2 mg/ml in 0.05 M Tris-acetate [pH 7.2] containing 1% sodium dodecyl sulfate [SDS]) was shaken vigorously for 1 min at room temperature with an equal volume of phenol which had previously been equilibrated with 0.02 M phosphate (pH 7.2) containing 0.14 M NaCl. Chloroform (0.5 volume) was then added, and shaking was continued for 2 min at 45°C. The phases were separated by centrifugation, and the resulting interface and aqueous phase were reextracted as described above except at room temperature instead of 45°C. Residual phenol was then removed from the aqueous phase by extraction with 1 volume of chloroform.

The final aqueous phase was made 0.2 M with respect to sodium acetate (pH 5.0), and the RNA was precipitated overnight after adding 2 volumes of absolute ethanol. The RNA was recovered by centrifugation at 12,000 × *g* for 20 min at 0°C. The RNA was dissolved in 1 ml of water and stored at −70°C.

Separation of RNAs 1 and 2. The RNA (about 0.75 mg in 0.5 ml of water) was heated for 5 min at 65°C to melt possible aggregates and then chilled quickly to 0°C to minimize reannealing. The preparation was then layered on a 38-ml gradient of 5 to 25% (wt/wt) sucrose in 0.2 M sodium acetate–0.01 M EDTA (pH 5.0). Centrifugation, in a Beckman-Spinco SW27 rotor, was at 25,000 rpm for 20 h at 10°C. After sedimentation, the gradient was passed through an ISCO type 6 optical unit equipped with a model UA-5 absorbance monitor. The separated RNAs 1 and 2 were ethanol precipitated, and each was further purified by two additional cycles of sedimentation on 12-ml gradients of 5 to 25% (wt/wt) sucrose in an SW41 rotor (36,000 rpm for 11 h at 10°C). The heating and cooling regimen was repeated for each cycle of centrifugation. The separated RNAs were precipitated with ethanol and redissolved in water to 1 mg/ml, and samples were stored at −70°C.

Infection with RNA. *Drosophila* cells were sensitized to infection with RNA by a modification of the procedure of Pagano et al. (20). Log-phase cell suspension (5×10^6 cells per ml) was washed three times at ambient temperature by resuspending sedimented cells in isotonic PIPES buffer [100 mM sodium chloride, 10 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 35 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.8]. The cells were then resuspended to 5×10^7/ml in the same buffer containing 0.5% DEAE-dextran (molecular weight, 500,000; Pharmacia, Uppsala, Sweden).

Samples (0.1 ml) of sensitized cell suspension were added immediately to sterile silanized 3-ml vials containing 0 to 5 μg of viral RNA in 2 to 4 μl of distilled water. Infection of sensitized cells with intact virus (0.4 μg, corresponding to about 5×10^{10} particles) was carried out in the same way. Particle concentrations were determined spectrophotometrically, assuming 3×10^{13} particles per unit of absorbance at 260 nm. After 10 min without agitation at ambient temperature, each sample was diluted with 0.5 ml of Schneider medium containing 0.5% bovine serum albumin. The cells were then collected by centrifugation and resuspended to 10^7 cells per ml by adding 0.5 ml of Schneider medium containing 15% fetal bovine serum. The vials were incubated horizontally without agitation at 26°C.

Radiolabeling of intracellular RNA. At intervals during infection, samples (0.2 ml) of cell suspension were removed from each culture and added to 1.5-ml polypropylene Eppendorf micro test tubes containing 3 μg of actinomycin D (Dactinomycin; 0.5 mg/ml; Merck & Co., Inc., West Point, Pa.). After 30 min, [5-^{3}H]uridine (catalog number NET-174; New England Nuclear Corp., Boston Mass.) was added to a final concentration of 200 μCi/ml. After a 2-h labeling period at 26°C, cells were collected by centrifugation at 800 × *g* for 5 min.

Acid-insoluble radioactivity. Radiolabeled cell pellets were suspended in 0.05 ml of 0.05 M Tris-acetate (pH 7.2) (TA buffer) containing 1% SDS. Cells (4×10^7 per ml) were lysed by vigorous blending in a Vortex mixer for 1 min at room temperature. Samples (0.025 ml, corresponding to 10^6 cells) were quickly removed from the resulting homogeneous lysates and spotted onto 2.4-cm GFA glass microfiber filters (Whatman, Inc., Clifton, N.J.). After drying at room temperature, the filters were soaked in 20% trichloroacetic acid for 20 min at 0°C and then washed successively with 8% trichloroacetic acid, 95% ethanol, and diethyl ether. The disks were air dried, and radioactivity was measured by liquid scintillation spectroscopy (14).

Extraction of intracellular RNA. The pelleted uridine-labeled cells were resuspended at room temperature in TA buffer containing 1% SDS. The lysate was then diluted 20-fold in TA buffer containing 0.1% SDS and then immediately extracted with phenol as described above. After phenol extraction and ethanol precipitation, intracellular RNA from each sample was dissolved in 0.05 ml of distilled water.

Electrophoretic analysis of RNA. RNA was subjected to electrophoresis on composite agarose-acrylamide gels as described previously (5). In brief, [^{3}H]uridine-

labeled RNA was mixed with an equal volume (0.02 ml) of Loening buffer (0.036 M Tris, 0.03 M sodium phosphate, 1 mM EDTA, pH 7.8) containing 10 M urea and 0.05% xylene cyanol. Electrophoresis without buffer circulation was on vertical slab gels at 6°C for 5 h at 10 V/cm in 2.5% acrylamide and 0.5% agarose containing 6 M urea. After electrophoresis, the gels were soaked for 2 h in 10% acetic acid–25% methanol–1% glycerol and then impregnated for 1 h with fluorographic enhancer (catalog number NEF-966; New England Nuclear Corp.). After soaking for 1 h with water, the gels were dried under vacuum (Hoefer Scientific Instruments, San Francisco, Calif.) and then exposed to Kodak XAR-5 film for 3 to 5 days at −70°C before the autoradioradiogram was developed.

Radiolabeling proteins in heat-shocked cells. At intervals during the viral infection cycle at 26°C, samples (0.2 ml) of cell suspension were withdrawn and centrifuged to sediment the cells. After suspending the cells in methionine-deficient growth medium containing 2% fetal bovine serum and 15 μg of actinomycin D per ml, the temperature was elevated to 37°C for 1 h to reduce synthesis of host proteins. After the temperature was reduced, [^{35}S]methionine (catalog number NEG-009T; New England Nuclear Corp.) was added to a final concentration of 250 μCi/ml, and incorporation was continued for 2 h at 26°C. The cells were then collected by centrifugation, and the pellets were stored frozen at −20°C.

Electrophoretic analysis of proteins. Lysates containing [^{35}S]methionine-labeled proteins were electrophoresed by the procedure of Laemmli (9). In brief, cell pellets (2×10^6 cells) were dissolved in 0.2 ml of solubilizing solution (62.5 mM Tris-hydrochloride [pH 6.8], 2.3% SDS, 5% mercaptoethanol, 3.5% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.]). After boiling in a water bath for 5 min, samples (5×10^5 cells) were electrophoresed on Laemmli slab gels (14 by 15 by 0.2 cm) containing 12% (wt/vol) acrylamide in 13 cm of resolving gel and 5% (wt/vol) acrylamide in the upper 1 cm of stacking gel. Electrophoresis was at 10 V/cm for 5 h at ambient temperature without buffer circulation. After electrophoresis, the gels were soaked overnight in 10% acetic acid–25% methanol–1% glycerol and then dried under vacuum. Autoradiograms were developed after exposing the gels to Kodak XAR-5 film for 3 to 5 days.

RESULTS

Infectivity of viral RNA. BBV is a relatively temperate virus causing little lysis in cultured *Drosophila* cells at low multiplicity. It is therefore difficult to measure its infectivity by plaque assay. This problem was overcome when we discovered that cells pretreated briefly with DEAE-dextran were sufficiently sensitive to infection that it was practical to measure infectivity of RNA by measuring the incorporation of [5-^{3}H]uridine into acid-insoluble material. Incorporation of the radiolabel was made specific for synthesis of viral RNA by treating the cells with the drug actinomycin D. Preliminary experiments established the optimum amount of RNA in the inoculum to be around 1 μg of RNA per culture tube containing 5×10^6 cells. A fivefold increase or decrease in this amount of RNA decreased incorporation about twofold (data not shown). The efficiency of infection by RNA was relatively insensitive to the concentration of DEAE-dextran over a broad range (10 to 3,000 μg/ml), but no RNA infectivity was detectable by the incorporation assay when the polycation was omitted.

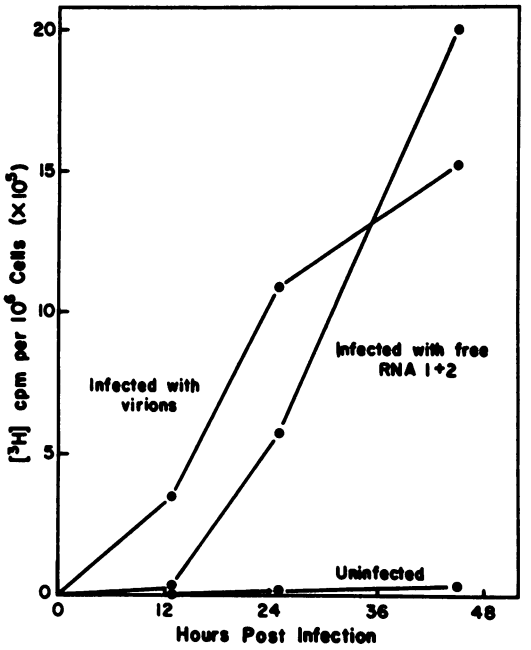

FIG. 1. Time course of viral RNA synthesis in virus-infected, RNA-infected, and uninfected *Drosophila* cells. Cultured *Drosophila* cells (1×10^7 cells in 1.0 ml) were infected as described in the text. At the indicated times after infection, samples (0.2 ml) of these cell suspensions were withdrawn into Eppendorf tubes containing 3 μg of actinomycin D. After 30 min, [5-^{3}H]uridine (50 μCi in 0.05 ml of distilled water) was added. Acid-insoluble radioactivity was determined 2 h later. We attribute the continued strong incorporation, beyond 36 h in RNA-infected cells, to a greater cell mass resulting from continued multiplication of the initially uninfected cell population.

The incorporation curve for RNA-infected cells initially lagged behind the curve observed with virus-infected cells by about 12 h (Fig. 1). During this initial lag period (12 h postinfection), immunofluorescence tests, using antibody specific for viral coat protein, indicated that only 5% of the RNA-infected cells were positive, whereas over 50% were positive in virus-infected cultures. Therefore, we attribute the initial lag to infection of only a small fraction of the cells by RNA. The prolonged upsurge of synthesis, beginning at 12 h in the RNA-infected cells, was evidently due to secondary infection by

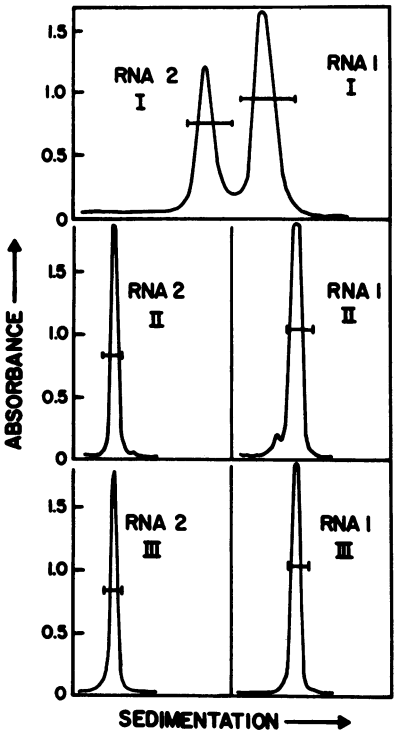

FIG. 2. Separation of BBV RNAs 1 and 2. RNA from virions was ethanol precipitated, dissolved in distilled water, and disaggregated before each sedimentation cycle as described in the text. Gradients were assayed for absorbance by passage through a flow cell. Cycle I was in a Spinco SW27 rotor; cycles II and III were in an SW41 rotor. Horizontal bars (|———|) indicate fractions which were pooled.

newly formed virions because it was completely abolished by adding neutralizing antibody 2 h postinfection (data not shown). Indeed, infection of cultures with RNAs 1 and 2 resulted in the production of particles which cosedimented with authentic BBV on sucrose gradients. Taken together, these data indicate that cells infected with free viral RNA produce infective virus particles.

In summary, these preliminary experiments established that only about 5% of the sensitized cell population was infected even with optimum inoculating concentrations of RNA. Secondary infection, due to newly formed virions, posed a problem for the uridine incorporation assay only if incubtion continued beyond 12 h and could be controlled with neutralizing antiserum.

Stimulation of viral RNA synthesis by isolated RNAs 1 and 2. Having demonstrated that free viral RNA was indeed infective for the *Drosophila* cells, we next examined the infectivity of the individually purified RNAs. Purification was accomplished by three cycles of centrifugation on sucrose density gradients, using a heating and quick cooling step before each cycle to reduce contamination by broken or aggregated RNA molecules (Fig. 2). We estimate, from measurements of peak areas under the absorbance profiles, that the RNAs were enriched at least 10-fold, probably substantially more, by each sedimentation cycle.

Table 1 summarizes the results of a typical experiment carried out with RNA at the second stage of purification (cycle II). RNAs 1 and 2, individually and in combination, were used to infect sensitized *Drosophila* cells, and their ability to stimulate incorporation of radiolabeled uridine into acid-insoluble material was measured. RNA 1 stimulated incorporation ninefold over background (Table 1, column 2) and was at least as active in this regard as a mixture of RNAs 1 and 2, whether reconstituted or unfractionated. RNA 2 exhibited no detectable activity.

A mixture of the two RNAs stimulated RNA synthesis only 5 to 6% as much as did virus (Table 1, column 3). Since we concluded above that only 5% of the sensitized cells were infected by RNA alone, this indicates that RNA synthesis is at least as vigorous in cells infected with RNA as in a normal infection with virions. Assuming RNA 1 alone also infects 5% of the sensitized cells, it induces a similarly vigorous synthesis of RNA.

Electrophoretic analysis of RNA-infected cells. To further identify the RNAs, phenolated extracts of the samples radiolabeled as described in Table 1 were prepared and subjected to gel electrophoresis (Fig. 3). In agreement with earli-

TABLE 1. Stimulation of viral RNA synthesis by isolated RNAs 1 and 2

Inoculum	Counts[a]	Stimulation of RNA synthesis (%)
None	3,100	0
RNA 1	27,300	7.3
RNA 2	2,800	0
Reconstituted RNAs 1 + 2	18,300	4.6
Virion RNAs 1 + 2	23,200	6.1
Virions	334,000	100

[a] *Drosophila* cell suspension, inoculated with the indicated agents, was incubated for 12 h at 26°C. Actinomycin D (20 μg/ml) was then added to each culture. After 30 min, [5-^{3}H]uridine was added, to 200 μCi/ml. After a 2-h labeling period, cells were sedimented and dissolved in SDS, and samples representing 10^6 cells were spotted onto glass microfiber filters and washed with trichloroacetic acid before being counted in the scintillation spectrometer. The remaining samples were phenolated for electrophoretic analysis of the RNA. Final inoculum RNA 1 concentrations were identical in cultures infected with RNA 1 alone, reconstituted RNAs 1 + 2, and virion RNAs 1 + 2.

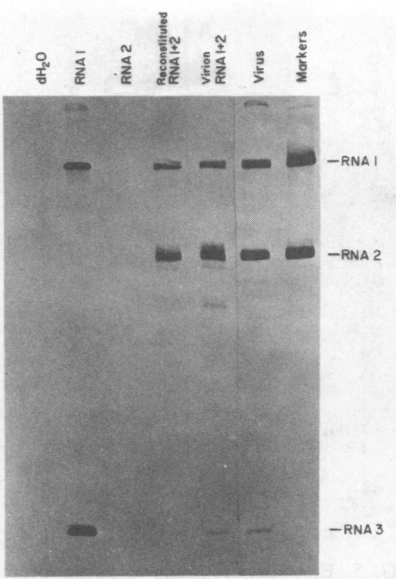

FIG. 3. Electrophoretic analysis of the actinomycin D-resistant RNAs produced in cells infected with isolated RNAs 1 and 2. Total cellular RNA from each of the six samples described in Table 1 was precipitated with ethanol and analyzed by gel electrophoresis. Each lane contained the RNA from 5×10^5 cells, except for lane 6 (infected with virions), on which only one-tenth as much extract was applied. Marker RNAs 1 and 2 (right lane) were extracted from purified virions.

er reports (5), virus-infected cells (Fig. 3, lane 6) contained large amounts of RNAs 1 and 2 together with much smaller amounts of RNA 3. Similar patterns were obtained from cells infected with mixtures of RNAs 1 and 2. There was no evidence of any labeled RNA in mock-infected control cells (Fig. 3, lane 1) or in cells infected with RNA 2 alone. By contrast, cells infected with RNA 1 alone contained RNA 1 in about the same amounts as cells infected with both RNAs. They also contained RNA 3 in a proportion much larger than normally observed in an infection with both RNAs. This result indicated that RNA 1 is capable of independent replication; it also suggested that RNA 1 is the parent of subgenomic RNA 3.

Suppression of RNA 3 synthesis in the presence of RNA 2. The abnormally large proportion of RNA 3 in cells infected with RNA 1 alone suggested that RNA 2 might exert a suppressing effect upon its synthesis. This hypothesis was reinforced by examination of products made in cells inoculated with RNA 1, cycles I, II, and III, taken from the sucrose gradients described in the legend to Fig. 2 above. The proportion of RNA 3 synthesized in RNA 1-infected cells decreased progressively with increasing proportions of RNA 2 in the inoculum (Fig. 4). Cells normally infected with virions made a full complement of RNA 2 but very little RNA 3 (Fig. 4, inset). There was a progressive decrease in the fraction

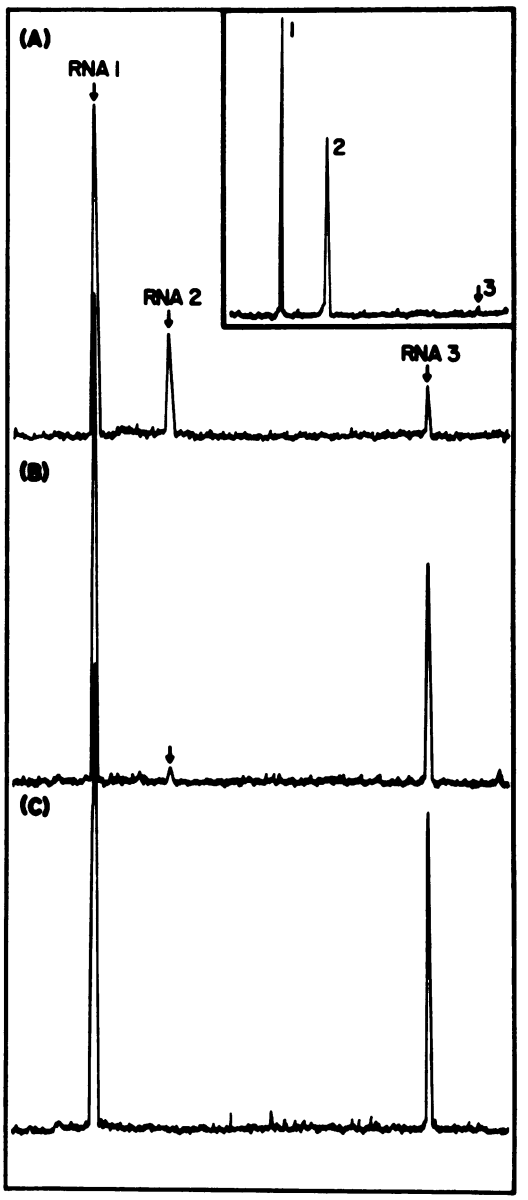

FIG. 4. Enhancement of viral RNA 3 synthesis in cells infected with RNA 1 of increasing purity. A dextran-sensitized cell suspension, prepared as described in the text, was divided into three parts which were inoculated with the preparations of purified RNA 1 described in the legend to Fig. 2. (A) Fraction I; (B) fraction II; (C) fraction III. Labeling with [5-^{3}H]uridine was for 2 h, beginning 15 h postinfection. RNA from 5×10^5 cells was then subjected to electrophoresis. Fluorograms were scanned with a Joyce-Loebl microdensitometer. Inset, Viral RNA profile from cells infected with intact virions.

of RNA 2 made in cells inoculated with increasingly pure preparations of RNA 1, and this decrease in synthesis of RNA 2 was accompanied by an increase in the production of RNA 3 until, with the most highly purified RNA (Fig. 4C), containing probably less than one RNA 2 molecule for every 1,000 molecules of RNA 1, there was no detectable synthesis of RNA 2 at all. Moreover, a parallel sample of the latter culture, radiolabeled at 38 to 40 h postinfection (not shown), still revealed no evidence of RNA 2, indicating that cycle III of RNA 1 was effectively free of RNA 2. The rate of incorporation at 38 to 40 h was similar to that at 12 to 14 h, indicating that infective virions were not produced in RNA 1-infected cultures. These observations strongly reinforce the conclusion that RNA 3 is derived from RNA 1. They also support the idea that RNA 2 is an independent genetic entity and not a subgenomic element of RNA 1.

Proteins synthesized in cells infected with isolated RNA 1. Since authentic RNA 3 is a messenger for protein B(5), we sought to determine if RNA 1-infected cells do indeed make protein B. Cells were radiolabeled from 12 to 14 h after infection with [^{35}S]methionine and then lysed and electrophoresed on polyacrylamide gels (Fig. 5A). To reduce the large background, due to synthesis of host proteins in a culture containing 95% uninfected cells, the cultures were heat shocked by raising the temperature to 37°C for 1 h, before radiolabeling at the normal incubation temperature (26°C) in the presence of the drug actinomycin D. The heat shock blocks synthesis of normal *Drosophila* cell proteins (1), whereas actinomycin D prevents transcription of messengers for a number of proteins which are otherwise normally induced (10).

Synthesis of viral RNA (Fig. 5B, lane d) and protein (Fig. 5A, lane d) exhibited the expected patterns in virus-infected cells. The small amount of protein A relative to proteins B and alpha is a normal feature of the virus infection cycle (4). Unshocked control cultures carried in parallel exhibited similar virus patterns (not shown), except that protein A was obscured by the heavy background of newly synthesized host proteins. These results indicate that synthesis of BBV proteins is resistant to the effects of heat shock, similar to the synthesis of a number of other RNA virus proteins (15, 25).

Viral proteins A, B, and alpha were also detected in cells infected with virion RNAs 1 and 2 (Fig. 5A, lane b) and in about the ratios seen in virus-infected cells (Fig. 5A, lane d). However, the virus protein pattern was considerably weaker because of the low fraction of cells infected. The low fraction of cells infected by RNA is also evident from the weak band

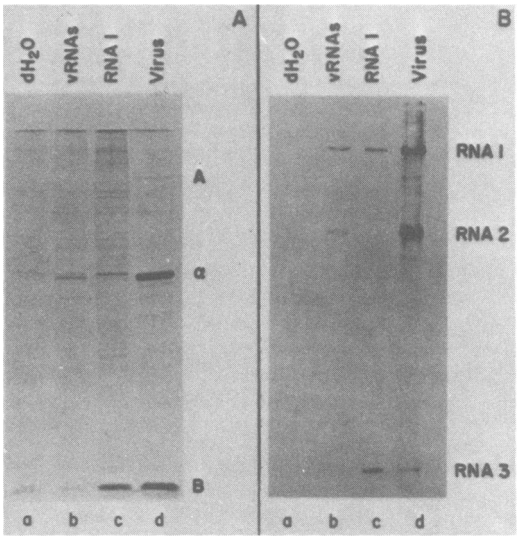

FIG. 5. Electropherograms showing proteins (A) and RNAs (B) synthesized in heat-shocked *Drosophila* cells after mock infection (a) or infection with virion RNAs 1 plus 2 (vRNAs) (b), RNA 1 alone (c), or virions (d). Each lane represents material from 5×10^5 cells. A cell suspension was divided into four equal parts and infected as indicated. After 11 h at 26°C, the temperature was raised to 37°C, and actinomycin D was added to a final concentration of 15 μg/ml. After 1 h, all samples were returned to 26°C. Each sample was divided into two 0.2-ml aliquots. [^{35}S]methionine (50 μCi) was added to one; [^{3}H]uridine (50 μCi) was added to the other. After a 2-h labeling period, the cells were sedimented. The ^{35}S-labeled samples were lysed and subjected to electrophoresis as described in the text. RNA was extracted from the corresponding uridine-labeled samples, electrophoresed, and autoradiographed as described in the text.

intensity of RNA 1 (Fig. 5B, lanes b and c). Thus, the limited, virtually undetectable, synthesis of protein A (Fig. 5A, lanes b and c) is not unusual because it is also synthesized in low proportion in virus-infected cells (Fig. 5A, lane d).

Cultures infected with RNA 1 alone (Fig. 5B, lane c) synthesized more RNA 3 than virus-infected cultures (lane d), even though RNA 1 infected only one-tenth as many cells as did virus (compare the RNA 1 bands in lanes c and d). The same RNA 1-infected cells made correspondingly large proportions of protein B (Fig. 5A, lane c). This viral protein pattern demonstrates that most, if not all, of the RNA 3 generated in RNA 1-infected cells represents authentic messenger for protein B. However, because of the small amount of protein A produced in these cells, we cannot draw any definitive conclusions as to whether the RNA 1 band is predominantly mRNA 1 or its complementary strand.

Careful inspection of Fig. 5A, lane c, also shows that RNA-1 infected cells contain no detectable amounts of protein alpha (Fig. 5A) as expected from the absence of RNA 2 in the same cultures (Fig. 5B). The protein migrating near alpha (Fig. 5A, lane c) is a host protein, probably actin (see lane a).

DISCUSSION

Our current picture of the genome strategy of BBV is illustrated in Fig. 6. We have shown here that viral RNAs 1 and 2 are both required for formation of infective virions. Newman and Brown (17) drew a similar conclusion from RNA infectivity studies on Nodamura virus, the prototype member of the Nodavirus family. In addition, our finding that RNA 2 is dispensible for replication of RNAs 1 and 3 helps clarify the functional relationships of genomic RNAs 1 and 2.

Functions of RNA 1. The results presented here prove that RNA 1 carries the viral gene(s) required for synthesis of the viral RNA polymerase in *Drosophila* cells. They also identify RNA 1 as the previously unknown progenitor of RNA 3. Thus, RNA 3 must now also be considered a potential contributor to viral RNA replication. However, it is still not clear whether one or both gene products are involved in viral RNA synthesis. Protein A has already been identified as a potential candidate (4, 19), and this is supported by enrichment of the protein in partially purified preparations of the enzyme (7). Definitive evidence on this point awaits further purification of the enzyme. Protein B is made in large amounts during the early stages of infection, suggesting that it plays an early function, but little else is yet known about its function.

Functions of RNA 2. Our results also reveal a second role for RNA 2, in addition to its previously recognized function as messenger for coat protein. It also exerts a regulatory function on production of subgenomic RNA 3 in infected cells. The mechanism by which RNA 2 restrains synthesis of RNA 3 without restraining synthesis of RNA 1 is not currently clear but may be accessible to further analysis by studying the effects of exogenously added RNA 2 or its translation product, protein alpha, to an in vitro RNA-synthesizing system (7) prepared from *Drosophila* cells infected with RNA 1 alone. Finally, its absence in cells infected with highly purified RNA 1 is evidence that RNA 2, unlike RNA 3, is not a subgenomic derivative of RNA 1 but is a necessary template for synthesis of new RNA 2 by the virus-induced polymerase.

RNA 3 as a possible regulator of protein A translation. The inhibitory effect of RNA 2 on the synthesis of subgenomic RNA 3 is the second regulatory system to be observed in BBV-infected cells. The first is the shutdown of protein A synthesis which occurs early, at about 5 h, in the infection cycle, and it has been shown that this early shutdown is not due to lack of competent RNA 1 messenger in the infected cell (5). Thus, it seems likely that some accumulating viral component may be involved. In support of this hypothesis, we have found that RNA 2 competes more effectively than RNA 1 for the host factor(s) limiting translation of the two RNAs in a cell-free translation system prepared from *Drosophila* cells (manuscript in preparation).

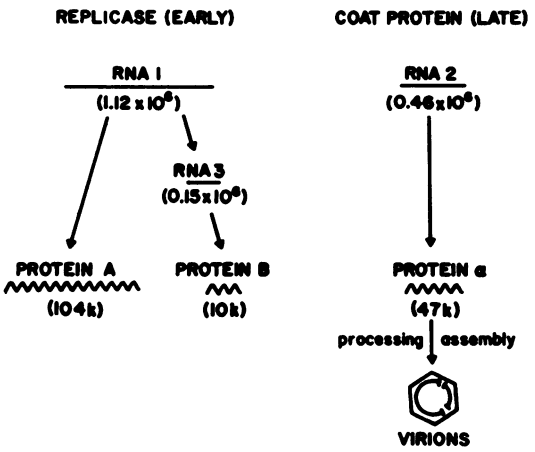

FIG. 6. Model illustrating functions of BBV RNAs 1 and 2 and origin of subgenomic RNA 3. RNA 1, with the possible participation of its subgenomic RNA 3, contains all the viral genes required for synthesis of viral RNA. RNA 2 makes coat proteins required for formation of virions. The roles of proteins A and B in viral RNA synthesis have not yet been defined. k, $\times 10^3$.

This competition, which would be expected to favor translation of RNA 2 as viral messengers accumulate in the cell, cannot, however, be solely responsible for the low translational efficiency of RNA 1. RNA 1-infected cells, as well as virus-infected cells, synthesize less protein A relative to protein B than would be expected from the relative proportions of their respective RNAs (Fig. 5, compare A and B, lanes c and d). This observation appears to exclude RNA 2 or its coat protein product as the only translational inhibitor of RNA 1. One possible explanation is that RNA 1 also competes with RNA 3. The effect may be exaggerated in the absence of RNA 2, which permits RNA 3 to accumulate in abnormally high proportions. Thus, one function of RNA 3 or its gene product, protein B, may be to regulate the translational efficiency of RNA 1.

Immunity of viral messengers to heat shock. Resistance of BBV to inhibition by heat shock

has proved a most useful property in studying the pattern of viral protein synthesis in situations in which the background is high owing to synthesis of host proteins. BBV is at least the third type of RNA virus whose translation is unaffected by heat shock. All three viruses differ widely in structure. One resistant virus is an unclassified virus with double-stranded RNA (25); a second, cricket paralysis virus (15), is a picornavirus which presumably carries polyadenylic acid at the 3′ end and VPg, a covalently bound protein, at the other end. BBV RNA, on the other hand, appears to be capped at the 5′ end (L. A. Guarino and P. Kaesberg, personal communication) and appears to carry no polyadenylic acid at the 3′ end (4). These findings suggest that comparative studies with these and other RNA viruses might be a fruitful approach in defining the structural features endowing RNAs with resistance or sensitivity to heat-shock inhibition.

Genetic studies on other Nodaviruses. The finding that *Drosophila* cells can be infected with individually purified Nodaviral RNAs raises the question of whether RNA 1 can be propagated indefinitely in the absence of RNA 2. It also prepares the way for studies on hybrid viruses prepared by mixing the large RNA of one Nodavirus with the small RNA of another. Such crossings offer an alternative approach to analysis of regulatory functions and determinants of host range. Nodamura virus, which is reported to be serologically distinct from BBV (16) and which also has a distinctly different host range (2, 12), is a suitable candidate for such crosses.

We have found that Nodamura viral RNA infects *Drosophila* to produce three RNAs with electrophoretic mobilities similar, but not identical, to those of BBV. Intact Nodamura virions, however, did not induce viral RNA synthesis under the same conditions, suggesting that resistance to infection is caused by lack of exposed Nodamura viral receptors on the surface of the host cells.

Preliminary attempts to make hybrid virus by crossing BBV RNA 1 with Nodamura viral RNA 2 resulted in synthesis of BBV RNAs 1 and 3 but not of Nodamura viral RNA 2, suggesting that the BBV RNA polymerase was unable to recognize the heterologous RNA 2 as a template. Clearly, studies on the compatibility of heterologous RNAs should be a useful adjunct to serology, host range, and structural studies on the nucleic acids in defining evolutionary relationships between other Nodaviruses.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA-08662 from the National Cancer Institute. T.M.G. and P.D.F. were supported by Public Health Service Training Grant 5-T32-M07215 from the National Institute of General Medical Sciences.

We thank K. C. Medappa for valuable discussions on the use of DEAE-dextran for infecting cells with RNA.

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