JOURNAL OF VIROLOGY, Jan. 1981, p. 500-505
0022-538X/81/010500-06$02.00/0

Vol. 37, No. 1

Translation of Black Beetle Virus RNA and Heterologous Viral RNAs in Cell-Free Lysates Derived from *Drosophila melanogaster*

LINDA A. GUARINO,* DENNIS E. HRUBY, L. ANDREW BALL, AND PAUL KAESBERG

Biophysics Laboratory, Graduate School, and Biochemistry Department, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

A cell-free protein synthesizing system was prepared from cells of *Drosophila melanogaster* line 1 and made mRNA dependent by treatment with micrococcal nuclease. The system was tested with homologous RNA from black beetle virus propagated in *Drosophila* cells, with *Drosophila* heat shock mRNA, and with various heterologous viral mRNA's. Under optimal conditions amino acid incorporation programed with black beetle virus RNAs was 30-fold higher than endogenous incorporation. RNAs 1 and 2 primarily directed the synthesis of proteins with approximate molecular weights of 120,000 and 46,000, respectively. mRNA's, prepared by transcription from vesicular stomatitis virus or vaccinia virus, were translated efficiently and yielded products that comigrated with authentic viral proteins. Brome mosaic virus RNA and encephalomyocarditis virus RNA were translated poorly. The system retained full activity after freezing.

Translation of viral mRNA in cell-free lysates is useful for studying the arrangement and function of viral genes. Although a number of translation systems have been developed that are responsive to the addition of exogenous, heterologous mRNA, it is sometimes desirable to use a system derived from cells in which the virus can replicate. The study of insect viruses has been hampered in this regard by the low activity of translating systems derived from insect cells (6, 9, 10). Preparation of an active system from cultured cells of *Drosophila melanogaster* line 2 has been reported (18). However, this system requires addition of a supernatant extract of rat liver, so it is not strictly homologous for mRNA's of insect origin. Furthermore, the system is not stable to freezing.

Here we describe the preparation and characterization of a freezable cell-free system derived entirely from insect cells, *D. melanogaster* line 1.

To study properties of this system we used mainly RNA from black beetle virus (BBV). This virus, originally isolated from the New Zealand black beetle (*Heteronychus arator*), is a member of a group of small riboviruses with divided genomes (nodaviruses), whose prototype is Nodamura virus (13). Both Nodamura virus and BBV have a major coat protein with a molecular weight of 40,000 and contain two RNAs with approximate molecular weights of 1×10^6 (RNA 1) and 0.5×10^6 (RNA 2). Nodamura virus RNAs are active messengers in wheat embryo and HeLa cell extracts (14). However, no adequate cell cultures are known for Nodamura virus. Recently, BBV has been shown to grow excellently in *D. melanogaster* line 1 cells (7), and this prompted us to develop a protein synthesizing system from these cells and to test it with BBV RNAs.

Drosophila line 1 cells (17), originated by I. Schneider, Walter Reed Army Institute of Research, Washington, D.C., were grown at 26°C in plastic roller bottles (Corning) containing 100 ml of Schneider complete growth medium (16) supplemented with bacteriological culture medium (Difco peptone, 5 mg/ml) and 15% heat-inactivated fetal calf serum (Kansas City Biologicals). For preparation of *Drosophila* lysates, a confluent monolayer (10^9 cells) was dislodged gently by rinsing with a pipette, and 2.5-ml aliquots together with 97.5-ml amounts of fresh growth medium were transferred to each of five roller bottles. Three days later, cells (2.5×10^9 total) were dislodged and harvested by centrifugation at $730 \times g$ for 5 min. The cells were suspended and centrifuged three times in ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (146 mM sodium chloride-35 mM HEPES [pH 7.6]). The final centrifugation was in a graduated conical tube at $730 \times g$ for 10 min in order to measure the packed cell volume. The cells were suspended in 2 volumes of homogenization buffer (1 mM dithiothreitol-10 mM HEPES [pH 7.6]) and allowed to swell on ice for 10 min. The cells were broken with 10 strokes of a Dounce homogenizer; a microscopic examination revealed

greater than 95% cell breakage. The homogenate was centrifuged first at 21,650 × *g* for 10 min, and then the supernatant liquid was transferred to 1.5-ml Eppendorf micro-test tubes and centrifuged at 15,600 × *g* for 2 min. The supernatant was collected, divided into 100-μl aliquots, and frozen at −70°C. Cell lysates prepared in this manner averaged 60 to 120 optical density units (at 260 nm) per ml and 75 to 120 mg of protein per ml.

Endogenous mRNA in the *Drosophila* lysates was digested by treatment with micrococcal nuclease (15). A typical nuclease digestion mixture contained 92 μl of lysate, 2 μl of 1 M HEPES (pH 7.6), 1 μl of 2 M potassium acetate, 1 μl of 5-mg/ml creatine kinase (Sigma), 1 μl of 4 mM hemin (in 90% ethylene glycol), 1 μl of 0.1 M calcium chloride, and 1 μl of 15,000-U/ml micrococcal nuclease (Boehringer Mannheim). The mixture was incubated at 23°C for 10 min, and then the nuclease digestion was terminated by the addition of 2 μl of 100 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid neutralized with KOH.

A standard protein synthesis mixture (15 μl) contained 8.3 μl of the nuclease-treated lysate plus the following assay components: 10 mM creatine phosphate; 19 unlabeled amino acids, 100 μM each; 96 μg of *Drosophila* tRNA per ml; 5 mM dithiothreitol; 1.25 mM magnesium acetate; 65 mM potassium acetate; 5 μCi of [^{35}S]-methionine (500 to 750 Ci/mmol, Amersham Corp.); and mRNA as indicated. Incubation was at 23°C for 90 min unless otherwise stated.

BBV was prepared according to Friesen et al. (7) except that suspended cells were infected and virus was harvested by two cycles of precipitation with polyethylene glycol. Typically, 1 mg of purified virus was obtained from 10^8 infected *Drosophila* cells. Virus was dissociated by the addition of an equal volume of 0.2 M Tris (pH 9.0)–0.2 M NaCl–2% sodium dodecyl sulfate, and the RNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was extracted three times with ether, heated at 65°C for 5 min, layered over a 12-ml gradient of 5 to 20% sucrose in 0.01 M Tris (pH 7.6)–0.05 M NaCl–1 mM EDTA–0.2% sodium dodecyl sulfate, and centrifuged in an SW41 rotor at 38,000 rpm and 15°C for 6 h. The peaks of optical density corresponding to 15S (RNA 2) and 22S (RNA 1) were precipitated separately by the addition of 0.15 M sodium acetate and 2 volumes of ethanol and repurified by a second sucrose centrifugation as described above.

Vesicular stomatitis virus mRNA was synthesized in vitro and purified by oligodeoxythymidylate-cellulose chromatography as previously described (4). Vaccinia virus early mRNA species were isolated at 6 h postinfection from mouse L cells infected in the presence of 5 mM hydroxyurea to prevent late gene expression. The RNA was isolated by centrifugation through cesium chloride (8) followed by affinity chromatography on oligodeoxythymidylate-cellulose (5). Brome mosaic virus and encephalomyocarditis virus RNAs were isolated by phenol extraction of purified virus preparations (11, 12). *Drosophila* heat shock mRNA was prepared from *Drosophila* line 1 cells 2 h after a shift from 26 to 37°C. *Drosophila* tRNA was prepared by making a phenol extract of whole cells, followed by 1 M NaCl extraction (3) and chromatography on DEAE-cellulose (DE-52, Whatman) (22).

Variables affecting protein synthesis in the *Drosophila* lysate in response to BBV RNA are plotted in Fig. 1. Maximal protein synthesis was achieved at 23°C (Fig. 1a). At this temperature, protein synthesis was linear for 30 min and continued to increase during the next 30 min. At 28°C, protein synthesis was initially faster than at 23°C, but ceased after 30 min. At 31 and 37°C the system was less active. Endogenous protein synthesis at 28, 31, and 37°C was higher than at 23°C (data not shown). At lower temperatures (18 and 21°C), protein synthetic activity was less than at 23°C (data not shown). The optimum concentration of potassium ions for BBV RNA translation was 65 mM (Fig. 1b). The potassium optimum for endogenous synthesis was slightly higher (85 mM). Maximal translation was observed at a magnesium ion concentration of 1.5 mM (Fig. 1c). In many systems (2), spermidine has a stimulatory effect on protein synthesis at suboptimal magnesium concentrations. In the *Drosophila* lysates, when no magnesium ions were added, spermidine stimulated the incorporation of [^{35}S]methionine. However, no combination of spermidine and added magnesium ions gave activity as great as that induced by added magnesium ions alone. Probably the endogenous level of magnesium ions was too high for observation of a synergystic effect.

The dependence of protein synthesis on the concentrations of BBV RNAs 1 and 2 is shown in Fig. 1d. For both RNAs the incorporation of ^{35}S increased linearly with increasing RNA concentration in the range of 0 to 80 μg/ml. Incorporation increased gradually in the range of 80 to 240 μg of mRNA per ml and saturated at 240 to 320 μg/ml. The RNA concentration optimum observed in the *Drosophila* lysate was significantly higher than that observed in wheat embryo or reticulocyte lysates, in which BBV RNA saturated at 60 or 80 μg/ml, respectively. At saturating concentrations the incorporation di-

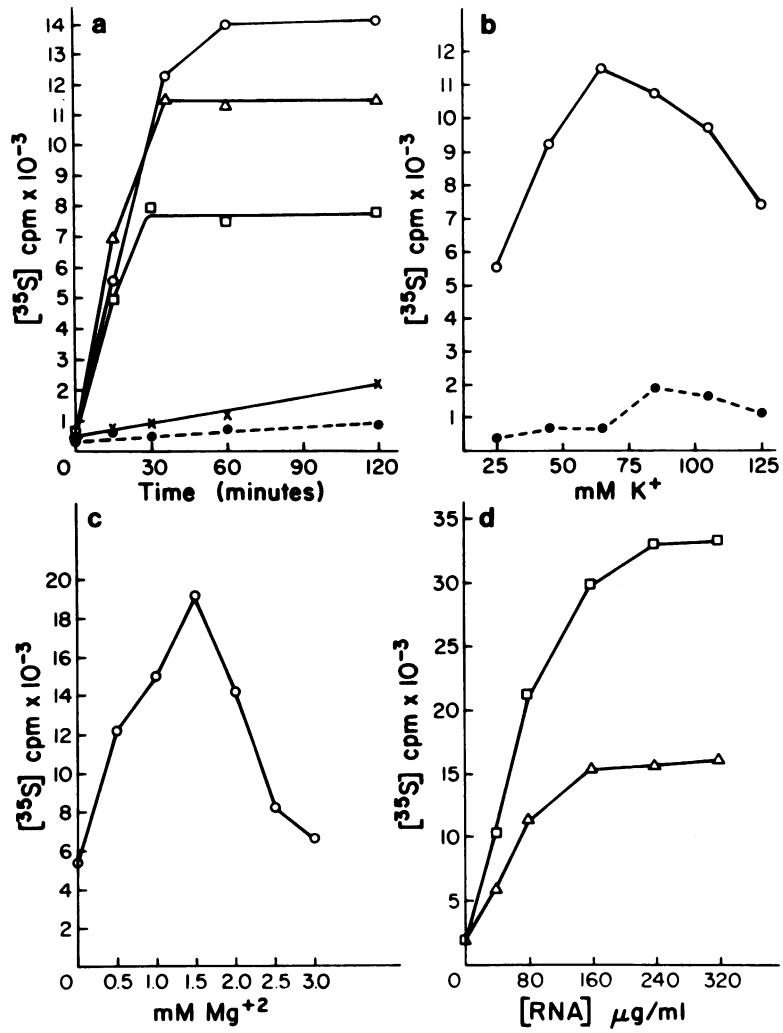

FIG. 1. *(a) Time course of protein synthesis. Protein synthesis was monitored as described in the text except that 30-µl reaction mixtures were made and incubated at 23°C (○, ●), 28°C (△), 31°C (□), and 37°C (×). ○, △, □, and × indicate reactions containing 200 µg of BBV RNA 2 per ml, and ● indicates no added mRNA. At the indicated times 2-µl aliquots were removed and assayed for hot trichloroacetic acid-precipitable radioactivity. (b) Potassium ion optimum of BBV RNA-directed protein synthesis in Drosophila lysates. The reactions were set up as described in the text except that the potassium acetate concentration was varied as indicated. After 2 h at 23°C, 5-µl aliquots were processed for hot trichloroacetic acid-precipitable radioactivity. Symbols: ●, without added mRNA; ○, with 200 µg of BBV RNA 2 per ml. (c) Magnesium ion optimum of BBV RNA-directed protein synthesis in Drosophila lysates. The reactions were as described in the text except that the magnesium ion concentration was varied as indicated. BBV RNAs 1 and 2 were added at 100 µg/ml each. After 90 min at 23°C, 2-µl aliquots were removed and processed. (d) BBV RNA concentration optimum in Drosophila lysates. The reactions were as described in the text. BBV RNA 1 (△) or 2 (□) was added at the concentrations indicated. After 90 min at 23°C, 2-µl aliquots were removed and processed.*

rected by RNA 2 was about twice that of RNA 1.

The requirements of the *Drosophila* lysate for the other components of the system were tested as shown in Table 1. In vitro protein synthesis was strongly dependent upon mRNA and the ATP regenerating system (creatine kinase and creatine phosphate). The activity was reduced by more than 50% when either hemin, *Drosophila* tRNA, or unlabeled amino acids were omitted. The additions of three other compounds known to affect other cell-free protein synthesiz-

TABLE 1. *Stimulation of protein synthesis in Drosophila lysate*[a]

Reaction mixture	^{35}S incorporation (cpm/5 μl)	Activity (% of complete)
Complete	23,504	100
Minus mRNA	849	3.6
Minus hemin	10,289	44.2
Minus creatine kinase	1,835	7.8
Minus creatine phosphate	1,116	4.7
Minus tRNA	10,631	45.2
Minus unlabeled amino acids	10,662	45.3
Plus 1 mM 7-methyl-5'-GMP	10,725	45.6
Plus 5 mM cyclic 3',5'-AMP	31,580	134.3

[a] The complete system was constituted as described in the text with 200 μg each of BBV RNAs 1 and 2 per ml and incubated at 23°C for 90 min. Aliquots (5 μl) were analyzed for hot trichloroacetic acid-precipitable radioactivity.

TABLE 2. *Response of Drosophila lysate to different mRNA's*[a]

RNA	^{35}S incorporation (cpm/5 μl)	Stimulation (fold)
None	1,978	1.0
BBV RNA 1	35,355	17.8
BBV RNA 2	53,832	27.2
BBV RNAs 1 and 2	58,482	29.6
Brome mosaic virus	3,389	1.7
Vesicular stomatitis virus mRNA	27,259	13.8
Encephalomyocarditis virus RNA	4,648	2.4
Vaccinia virus mRNA	11,733	5.9
Drosophila heat shock mRNA	28,039	14.2

[a] Cell-free protein synthesis reactions were performed as described in the text except that [^{35}S]methionine was increased to 10 μCi per assay. The indicated mRNA's were added to 200 μg/ml except for vesicular stomatitis virus mRNA, where the final concentration was 40 μg/ml. After 90 min at 23°C, 5-μl aliquots were processed to determine hot trichloroacetic acid-precipitable radioactivity. The brome mosaic virus and encephalomyocarditis virus RNAs used in this experiment stimulated reticulocyte lysates 50- and 60-fold, respectively.

ing systems were tested. The addition of 7-methyl-5'-GMP, an analog of the cap structure found on many eucaryotic RNAs (19), inhibited translation of the BBV RNA. This is an expected result, as both BBV RNAs are capped (A. Ghosh and L. Guarino, unpublished data), and it suggests that the mechanism of message recognition and binding by the *Drosophila* ribosomes involves the 5' cap. The addition of cyclic 3',5'-AMP resulted in a small stimulation of [^{35}S]-methionine incorporation.

Although the *Drosophila* system was developed primarily to translate BBV RNA in a homologous system, we tested the ability of the system to translate other messages (Table 2). Brome mosaic virus and encephalomyocarditis virus RNAs were translated poorly (1.7- and 2.4-fold stimulation, respectively), whereas messages derived from vesicular stomatitis virus and vaccinia virus directed substantial amino acid incorporation, as did cellular message transcribed in *Drosophila* cells under conditions of heat shock (1). All of the message preparations used in this experiment were also tested in reticulocyte lysates and were found to be quite efficient.

Translation products were analyzed on polyacrylamide gels (Fig. 2). The principal product of translation of BBV RNA 2 (lane c) was a protein with a molecular weight of 46,000 believed from in vivo studies to be the precursor of the single virion coat protein (molecular weight, 40,000). The principal product of translation of BBV RNA 1 (lane b) was a protein with a molecular weight of 120,000. These molecular weights were not significantly different from those reported for Nodamura viral proteins (14). The product with a molecular weight of 46,000 was apparent after 15 min of translation, whereas the product with a molecular weight of 120,000 was visible within 60 min (lanes d through i). Products with identical mobilities were synthesized in cell-free extracts derived from rabbit reticulocytes or from wheat embryo, although in the latter case many additional products were apparent (data not shown).

Vesicular stomatitis virus mRNA directed the synthesis of polypeptides which comigrated with the viral polypeptides N, NS, and M (lane k). No products were detected that correspond in molecular weight to the viral glycoprotein (G) or its unglycosylated precursor. Vesicular stomatitis virus has recently been shown to grow in *Drosophila* cells, although, interestingly, a defect in the synthesis or maturation of the viral G protein was observed (23).

Brome mosaic virus RNA (unfractionated mixture of RNAs 1, 2, 3, and 4) directed synthesis of a small amount of a single protein having the mobility of brome mosaic virus coat protein (Fig. 2, lane j). Brome mosaic virus RNA is a very efficient message in most cell-free systems. Attempts to improve the translation of brome mosaic virus in *Drosophila* lysates by optimizing the magnesium, potassium, and mRNA concentrations were not successful. No products stimulated by encephalomyocarditis virus RNA were

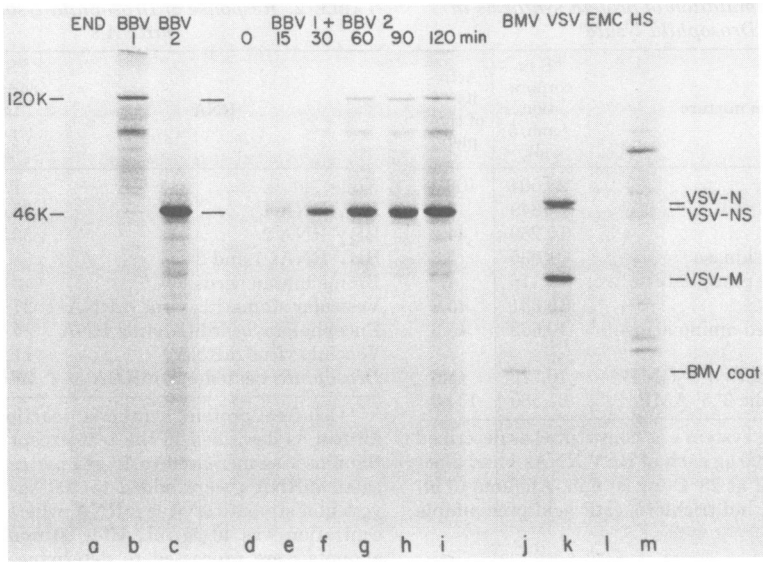

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel analysis of cell-free translation products in Drosophila lysates. Lanes: a, endogenous synthesis; b, products synthesized in response to 200 μg of BBV RNA 1 per ml; c, products synthesized in response to 200 μg of BBV RNA 2 per ml; d through i, time course of proteins synthesized in response to 200 μg of BBV RNAs 1 and 2 per ml; j through m, products synthesized in response to brome mosaic virus (BMV), vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMC), and Drosophila heat shock message, as described in the footnote to Table 2. The gel was run according to Studier (21). The molecular weights of the viral proteins were calculated by co-electrophoresing an unlabeled mixture of known molecular weights: myosin, 2 × 10⁶; β-D-galactosidase, 1.16 × 10⁵; phosphorylase B, 9.4 × 10⁴; carbonate dehydratase, 2.9 × 10⁴; tomato bushy stunt coat protein, 4 × 10⁴; BMV coat protein, 2 × 10⁴; tobacco mosaic virus coat protein, 1.75 × 10⁴, myoglobin, 1 × 10⁴.*

observed (lane l). This RNA is actively translated in many cell-free systems (20), but is poorly translated in wheat embryo lysates. Recently, it has been shown that addition of a ribosomal wash fraction from rabbit reticulocytes greatly enhances the translation of encephalomyocarditis virus RNA in wheat embryo lysates (5). It is possible that in the *Drosophila* lysate, as in the wheat embryo lysate, some factor(s) necessary for translation of encephalomyocarditis RNA is absent. The spectrum of products synthesized in response to *Drosophila* heat shock mRNA (lane m) was similar to that of proteins synthesized in *Drosophila* cells after heat shock. Although we did not make explicit tests, we judged from the relative lack of products shorter than the expected proteins that the system was reasonably free of nucleases. We attribute this to the special care we took in avoiding breakage of nuclei during its preparation.

This *Drosophila* system should be useful for analyzing the structure and expression of genes homologous to these cells. Various translating systems described in the literature differ greatly in efficiency of protein synthesis in response to heterologous messengers and even in ability to initiate translation in some cases. We judge that the *Drosophila* system may be useful in defining the variables that influence the correct and efficient translation of heterologous messengers.

This research was supported by Public Health Service research grants AI 1466, AI 15342, and AI 16356 and salary awards AI 00378, AI 21942, AI 05894, and T32 CA09075 from the National Institutes of Health and by grant 7800002 from the Competitive Grants Program of the Science and Education Administration of the U.S. Department of Agriculture.

We are indebted to Roland Rueckert and Paul Friesen for discussions and especially for informing us about their work with BBV and its hosts.

LITERATURE CITED

1. **Ashburner, M., and J. J. Bonner.** 1979. The induction of gene activity in *Drosophila* by heat shock. Cell **17**: 241–254.
2. **Atkins, J. F., J. B. Lewis, C. W. Anderson, and R. F. Gesteland.** 1975. Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. J. Biol. Chem. **250**:5688–5695.
3. **Aviv, H., I. Boime, and P. Leder.** 1971. Protein synthesis directed by encephalomyocarditis virus RNA: properties of a transfer RNA-dependent system. Proc. Natl. Acad. Sci. U.S.A. **68**:2303–2307.
4. **Ball, L. A., and C. N. White.** 1978. Coupled transcription and translation in mammalian and avian cell-free systems. Virology **84**:479–495.
5. **Boone, R. F., and B. Moss.** 1978. Sequence complexity and relative abundance of vaccinia virus mRNA's synthesized in vivo and in vitro. J. Virol. **26**:554–569.

6. **Fox, A. S., J. Kan, S. H. Kang, and B. Wallis.** 1965. Protein synthesis in cell-free preparations from *Drosophila melanogaster*. J. Biol. Chem. **240**:2059–2065.
7. **Friesen, P., P. Scotti, J. Longworth, and R. R. Rueckert.** 1980. Black beetle virus: propagation in *Drosophila* line 1 cells and an infection-resistant subline carrying endogenous black beetle virus-related particles. J. Virol. **35**:741–747.
8. **Glisin, V., R. Crkuenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
9. **Goldstein, E. S., and L. A. Snyder.** 1972. A cell-free system for protein synthesis from newly fertilized eggs of *Drosophila melanogaster*. Biochim. Biophys. Acta **281**:130–139.
10. **Ilan, J., and F. Lipmann.** 1966. A cell-free protein synthesis system from pupae of *Tenebrio molitor*. Acta Biochim. Pol. **13**:353–359.
11. **Hruby, D. E., and W. K. Roberts.** 1976. Encephalomyocarditis virus RNA: variations in polyadenylic acid content and biological activity. J. Virol. **19**:325–330.
12. **Lane, L. C., and P. Kaesberg.** 1971. Multiple genetic components in brome mosaic virus. Nature (London) New Biol. **232**:40–43.
13. **Longworth, J. F., and G. P. Carey.** 1976. A small RNA virus with a divided genome from *Heteronychus arator* (F.) [Coleoptera:Scarabaeidae]. J. Gen. Virol. **33**:31–40.
14. **Newman, J. F. E., T. Matthews, D. R. Omilianowski, T. Salerno, P. Kaesberg, and R. Rueckert.** 1978. In vitro translation of the two RNAs of Nodamura virus, a novel mammalian virus with a divided genome. J. Virol. **25**:78–85.
15. **Pelham, H. R. B., and R. D. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
16. **Schneider, I.** 1964. Differentiation of larval *Drosophila* eye-antennal discs *in vitro*. J. Exp. Zool. **156**:91–104.
17. **Schneider, I.** 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. J. Embryol. Exp. Morph. **27**:353–365.
18. **Scott, M. D., R. V. Storti, M. L. Pardue, and A. Rich.** 1979. Cell-free protein synthesis in lysates of *Drosophila melanogaster* cells. Biochemistry **18**:1588–1594.
19. **Shatkin, A. J.** 1976. Capping of eucaryotic mRNAs. Cell **9**:645–653.
20. **Shih, D. S., C. T. Shih, C. Zimmern, R. R. Rueckert, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30**:472–480.
21. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins in slab gels. J. Mol. Biol. **79**:237–248.
22. **Waters, L. C., and G. D. Novelli.** 1971. Analytical reversed-phase chromatography of *Escherichia coli* amino acid-tRNA. Methods Enzymol. **20**:39–44.
23. **Wyers, F., C. Richard-Molard, D. Blondel, and S. Dezelee.** 1980. Vesicular stomatitis virus growth in *Drosophila melanogaster* cells: G protein deficiency. J. Virol. **33**:411–422.