Intracellular RNA Expressed in Black Beetle Virus-infected Drosophila Cells

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SUMMARY

Black beetle virus replicates in Drosophila cells with the synthesis of virus-specific RNA and protein being detected as early as 2 h post-infection and continuing for at least 48 h. Shut-off of host synthesis did not occur in infected cells until 8 to 10 h post-infection. Examination of the RNA from infected cells on denaturing gels or on agarose/acrylamide gels demonstrated the production of four RNA species not found in uninfected cells. Two of these species, being single-stranded, co-migrated with the virion RNA and the other two were the double-stranded replicative stages of the virion RNA. No additional RNA species were found in infected cells, either in the presence or absence of actinomycin D.

Black beetle virus (BBV) is a small RNA virus which contains a bipartite genome. The two species of single-stranded (ss)RNA, RNA 1 (mol. wt. 1.0 \( \times 10^6 \)) and RNA 2 (mol. wt. 0.5 \( \times 10^6 \)), are encapsidated into one type of particle with a diam. of 30 nm and which is composed of a major protein of mol. wt. 40000 (Longworth & Carey, 1976).

Virus RNA 1 and RNA 2 direct the synthesis in vitro of 110000 and 43000 mol. wt. proteins respectively (Crump & Moore, 1981b; Friesen & Rueckert, 1981a; Guarino et al., 1981). The 110000 protein is a putative RNA-dependent RNA polymerase (Guarino & Kaesberg, 1981) and the 43000 protein is the capsid precursor (Crump & Moore, 1981b). The two proteins produced in vitro correspond to those synthesized in BBV-infected Drosophila cells. However, the origin of an additional low molecular weight (8000 to 10000) protein found in infected cells is unclear. This protein, of unknown function, is synthesized early in infection and does not appear to be a cleavage product of either of the other two proteins. Guarino & Ghosh (1981) reported that translation of RNA 2 in Drosophila cell lysate at suboptimal magnesium concentrations produced a protein which co-migrated on SDS–polyacrylamide gels with this protein. However, Friesen & Rueckert (1981b, 1982) identified at least seven virus-specific RNAs from infected cells, and suggested the possible synthesis of a subgenomic messenger (RNA 3) responsible for the production of the third protein.

We report the appearance of RNA in BBV-infected Drosophila cells and conclude that the virus replicative strategy is similar to that of other animal viruses with positive-stranded RNA genomes.

BBV-infected Drosophila cells produce virus polypeptides as early as 2 h post-infection and continue their synthesis for up to 48 h (Crump & Moore, 1981a; Friesen & Rueckert, 1981a). No cell lysis or cytopathic effect except for floating or rounded cells is observed. However, host protein synthesis declines from about 12 h post-infection onwards. It was therefore necessary to study BBV-directed RNA and protein synthesis during the early stages of infection. The rate of synthesis was monitored over a 12 h period by dual labelling cells with \([^{35}\text{S}]\)methionine and \([^{3}\text{H}]\)uridine in the presence and absence of actinomycin D (Act D).

Confluent monolayers of Drosophila cells in 60 mm Nunc Petri dishes were infected with 10 p.f.u./cell. After 1 h adsorption the inoculum was removed and the cells were overlaid with 2 ml maintenance medium. At the times indicated cells were pulsed with 20 \( \mu \text{Ci} \) \([^{35}\text{S}]\)methionine (890 Ci/mmole) and 40 \( \mu \text{Ci} \) \([^{3}\text{H}]\)uridine (25 Ci/mmole) (Amersham International) in 250 \( \mu \text{l} \) methionine-deficient medium after a 15 min starvation period in this medium. Post-pulse cells were collected into 1% Nonidet P40 in phosphate-buffered saline (PBS) and the nuclei were removed by centrifugation (3000 g for 15 min). Products were trichloroacetic acid-precipitated and collected by filtration onto Whatman GF/C glass microfibre paper. Incorporation was
assayed by counting on a Packard Tri-Carb scintillation counter using Triton–toluene–POPOP scintillation fluid.

RNA synthesis increased in infected cells in the first 2 h of infection; this continued, but became less pronounced as cellular RNA synthesis fell. The rate, however, did not fall as low as that in infected cells to which 5 μg/ml Act D had been added, suggesting that infection by BBV does not totally inhibit cellular RNA synthesis. Virus protein synthesis rose sharply compared with uninfected cells in the first 3 h of infection. The decrease in protein synthesis in infected cells after 3 h infection suggests viral inhibition of cell protein synthesis as shown by Friesen & Rueckert (1981a). The incorporation of [35S]methionine in untreated infected cells fell close to that of infected cells treated with Act D, demonstrating almost total suppression of host protein synthesis.

RNA synthesis in BBV-infected Drosophila cells, propagated as monolayers (Crump & Moore, 1981a), was examined by electrophoresis on denaturing (methylmercuric hydroxide-containing) and non-denaturing (agarose/acrylamide) gels.

Confluent monolayers of BBV-infected (100 p.f.u./ml) and mock-infected cells were labelled with 2 μCi/ml [2-14C]uridine (53 mCi/mmol) (Amersham International) for 1 h at times indicated in the text. When Act D (5 μg/ml) was required it was added 2 h before pulse labelling. After labelling, the cell sheet was washed with 5 ml PBS and the cells scraped off into a further 5 ml PBS. The cells were pelleted at 1000 g for 5 min, washed twice in PBS, resuspended in sterile TNE buffer (10 mM-Tris pH 7-4, 100 mM-NaCl, 1 mM-EDTA), and intracellular RNA was extracted as described by Reavy & Moore (1981) after removal of nuclei by lysing with Nonidet P40 and centrifugation.

RNA was analysed by electrophoresis on agarose/acrylamide non-denaturing gels (Loening, 1967; Studier, 1973) and methylmercuric hydroxide-denaturing gels (Bailey & Davidson, 1976; Watson et al., 1979). Non-denaturing gels contained 0·5% agarose (Low EEO, Sigma), 1·5% acrylamide, 0·1% N,N'-methylenebisacrylamide (Bethesda Research Laboratories), 0·1% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0·05% ammonium persulphate in Loening's buffer (35 mM-Tris pH 7·7, 30 mM-NaH2PO4, 1 mM-EDTA). Denaturing gels contained 1·5% agarose and 5 mM-methylmercuric hydroxide in borate buffer [50 mM-boric acid, 5 mM-borax (disodium tetraborate), 10 mM-Na2SO4, 1 mM-EDTA adjusted to pH 8·2 with NaOH]. RNA was denatured in 25 mM-methylmercuric hydroxide in borate buffer for 15 min at room temperature. Before loading, the samples were adjusted to 1% Ficoll, 0·001% bromophenol blue and electrophoresis was carried out at 35 V (= 10 mA) for 16 to 18 h using 1 x borate buffer. Upon completion gels were soaked in 0·5 M-ammonium acetate plus 10 μg/ml ethidium bromide. Gels were either photographed under u.v. light or dried for autoradiography.

Fig. 1 shows the intracellular RNA synthesized at 8 h post-infection. Ethidium bromide staining detected at least four additional RNA species specific to infected cells (Fig. 1a, lanes 3 and 4). The two lower species (vRNA 1 and vRNA 2) co-migrated with RNAs 1 and 2 from purified virus. Two larger virus-induced species were observed (ds 1 and ds 2), which were partially resistant to RNase T1 digestion (data not shown). On close examination each of these consisted of two closely migrating species.

The virus-induced RNA was examined after methylmercuric hydroxide denaturation (Fig. 1b). Under these conditions only two additional species (vRNA 1 and vRNA 2) were observed in infected cells. These corresponded to the virion RNAs. Thus, the larger species observed under non-denaturing conditions are of a double-stranded nature and are candidate replicative forms and replicative intermediates for the virion RNAs.

To investigate BBV-directed RNA synthesis, infected and non-infected cells were pulsed with [14C]uridine for 1 h. Act D was added 4 h pre-pulse when needed. Virus-specific RNA was induced in large amounts in infected cells as early as 2 h post-infection (Fig. 2a, lane 1) with relatively little inhibition of the host synthesis (compare lanes 1 and 3, Fig. 2a), whereas adding Act D to the cell cultures almost totally inhibited host RNA synthesis in both infected (Fig. 2a, lane 2) and uninfected (lane 4) cells. Later in infection, however, host cell RNA synthesis decreased and virus RNA synthesis increased compared with the controls (Fig. 2b, c).

All the intracellular virus-specific RNA was actively synthesized throughout infection and
the ratios, calculated from densitometer scans of the gels, of the species appeared to be constant. Relative proportions were ascertained from peak areas by cutting and weighing; the molar ratio was calculated by the weight of each peak divided by the total weight of the peaks multiplied by the molecular weight of the RNA species. The ratio of vRNAs did not change significantly during the 38 h infection period and remained about five times greater than the replicative species. vRNA 1 and vRNA 2 were not synthesized in equimolar amounts, but in a molar ratio of approximately 0.7. None of the RNA species was under any differential synthetic control.

In *Drosophila* cells, BBV RNA and proteins are synthesized continuously between 2 and 48 h post-infection. The virus disrupts host macromolecular synthesis only late in infection. The replication of BBV RNA appears similar to that of picornaviruses (Perez-Bercoff, 1979). The large double-stranded (ds) species detected early in infected cells, and which are denatured by methylmercuric hydroxide, are likely candidates for the replicative forms or replicative intermediates. Because of its complex secondary and tertiary structure the replicative intermediate (RI) of picornaviruses migrates very slowly (if at all) when electrophoresed in loose polyacrylamide/agarose gels (Perez-Bercoff, 1979). However, the RNAs of the Nodaviruses are less than one-third the size of picornavirus RNA, and the RIs are therefore probably less complex and possess fewer nascent strands. Their migration into the agarose/acrylamide gel could account for the appearance of the two pairs of closely migrating bands in the high molecular weight region.

Synthesis of RNA appears to continue even though mature virus particles are produced and apparently released from the cell (Crump & Moore, 1981c). All the RNA species are synthesized continuously throughout infection and do not appear to be under any temporal control within
Fig. 2. (a) Autoradiogram of $^{14}$C-labelled RNA synthesized in BBV-infected Drosophila cells. Cells were pulsed for 1 h with 2 μCi $[^{14}$C]uridine at 1 h post-infection (lanes 1 to 4), 5 h post-infection (lanes 5 to 8) and 8 h post-infection (lanes 9 to 12). RNA was extracted and analysed on an agarose/acrylamide gel. Lanes 1, 5 and 9, infected cells; lanes 2, 6 and 10, infected cells plus Act D; lanes 3, 7 and 11, control cells; lanes 4, 8 and 12, control cells plus Act D. (b, c) Autoradiogram of RNA synthesized at late times after infection of Drosophila cells by BBV. Infected cells in the presence and absence of Act D were pulsed with $[^{14}$C]uridine and the purified RNA analysed on (b) non-denaturing and (c) denaturing gels. Lane 1, infected cells plus Act D 12 h post-infection; lane 2, infected cells 12 h post-infection; lane 3, control cells 12 h post-infection; lane 4, infected cells plus Act D 16 h post-infection; lane 5, infected cells 16 h post-infection; lane 6, infected cells plus Act D 20 h post-infection; lane 7, infected cells 20 h post-infection; lane 8, infected cells plus Act D 30 h post-infection; lane 9, infected cells 30 h post-infection; lane 10, infected cells plus Act D 38 h post-infection; lane 11, infected cells 38 h post-infection; lane 12, control cells 38 h post-infection.
the infected cell. This suggests that protein synthesis in infected cells (Friesen & Rueckert, 1981a) is regulated at the level of translation.

Recently Friesen & Rueckert (1982) detected several less prominent RNA species in BBV-infected cells. They were unable to eliminate the possibilities of the presence of another virus or unidentified nucleolytic cleavages. We were unable to detect any such minor RNA species on either of the gel systems employed in this study.

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REFERENCES


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