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Plaque Assay for Black Beetle Virus

BERNARD H. SELLING AND ROLAND R. RUECKERT*

Biophysics Laboratory and Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

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A rapidly growing strain of virus was used to develop a reliable plaque assay for Black beetle virus on monolayers of cultured *Drosophila* cells. Cell density of the monolayer was critical for successful plaque formation. The dose-response curve for plaque formation was linear, supporting earlier proposals that both RNA segments of the split genome reside in the same particle. The method greatly facilitates isolation of reassortant and variant strains of virus.

Black beetle virus (BBV) (9, 10), a nonenveloped ribovirus (12) belonging to the *Nodaviridae* (11), contains a split genome (12) composed of two messenger-active RNAs; the larger one, RNA 1 (molecular weight, 1.1×10^6), encodes for viral replication functions (6), whereas the smaller one, RNA 2 (molecular weight, 0.46×10^6), encodes the virion coat protein (molecular weight, 44,000) (14). The naked RNAs are infective, and both are required for infectivity (6). Thus, BBV is a promising model system for studying reassortment between genomic RNA segments and interactions between viral gene products.

One of the last obstacles in developing effective genetic studies with this simple virus has been the lack of an effective plaque assay (5). We now present a solution to this problem and show that the difficulty was due in large part to suppression of plaque formation at high cell density. We also describe the development of a more cytolytic strain of BBV, which facilitated discovery of the conditions required for plaque formation.

Despite its ability to grow vigorously in culture (3), wild-type BBV is not very cytolytic for *Drosophila* cells, possibly because Drosophila is not the natural host for this virus (9, 10). Unfortunately, the only available strain of black beetle cells (1) grows so slowly in culture as to be impractical for routine work. An alternative strategy was to select for a laboratory strain of virus which would grow more rapidly in cultured *Drosophila* cells. This was accomplished by passaging wild-type virus repeatedly through *Drosophila* cells, infecting at low multiplicity (see below) to minimize formation of defective interfering particles (3).

To this end, 5×10^7 *Drosophila* line 1 cells (16) in 10 ml of complete growth medium (CGM) were inoculated with serial 10-fold dilutions of gradient-purified wild-type virus (3). CGM was the insect medium of Schneider (15) supplemented with 15% heat-inactivated fetal calf serum and 5 mg of bacteriological peptone (Difco Laboratories, Detroit, Mich.) per ml. The inoculum virus had previously been cloned by an endpoint dilution procedure (manuscript in preparation). The infected cell suspensions were incubated for 48 h at 26°C. Progeny virus was then purified (5) for each suspension, and the smallest optically detectable (>0.01 optical density U at 260 nm) yield of virus, ranging from 10 to 200 μg, was selected for further passage by the same procedure. The maximum yield obtainable with high multiplicity infections was reproducibly about 1 mg.

During a series of 17 serial passages, progressively smaller amounts of input virus were able to initiate vigorous infections. Beginning with passage nine, partial lysis of the infected suspensions could be seen by 48 h postinfection (multiplicity of 50 virions per cell). This was in marked contrast to wild-type BBV, which produced little cytolysis even at very high multiplicity (e.g., 10^4 virions per cell). By passage 17, substantial lysis was observed at a multiplicity of 1 virion per cell. This cytolytic strain of virus (BBV-W17) was tested for plaque-forming ability, and the results were positive.

For the plaque assay, cells were gently flushed from the walls of a confluent roller bottle with a pipette. After removal of a 0.1-ml portion for counting in a hemacytometer (at least 600 cells), 15 ml of the suspension was centrifuged for 10 min at $280 \times g$ in a plastic conical 15-ml tube (Corning Glass Works, Corning, N.Y.). The sedimented cells were then suspended to 4.2×10^7 cells per ml in CGM. Samples containing 4×10^6 cells in 0.095 ml were pipetted into fresh plastic conical tubes and inoculated with 0.005 ml of virus (diluted in CGM).

The infected suspensions were gently rocked for 60 min at room temperature to allow the virus to attach to cells. The suspensions were then diluted with 4.9 ml of NPA buffer, suspended with a vortex mixer, and poured into 60-mm tissue culture dishes (Corning). NPA buffer was 0.1 M NaCl–0.025 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.75)–0.1% bovine serum albumin. Cells were allowed to attach to the dishes for 1 h at room temperature. The buffer was then replaced with 3 ml of an overlay consisting of 1.8% Seaplaque agarose (FMC Corp., Rockland, Maine) in CGM at 37°C. After the overlay had gelled (about 10 min), an additional 2 ml of CGM was added, and the dishes were placed in a humidified incubator at 26°C. After 52 h, the overlays were removed by a flick of the wrist, and the monolayers were stained for 10 min with 0.1% crystal violet in 20% ethanol; excess stain was removed with a water rinse. An occasional overlay was difficult to flick out of its dish. This problem was solved by pipetting about 1 ml of Formalin into the dish and removing the overlay with forceps a few minutes later.

Early attempts to obtain plaques with BBV-W17 yielded erratic results until it was recognized that plaque formation depended critically upon the seeding density used to form the cell lawn. Plaque size was maximimal when 2×10^6 to 4×10^6 cells were seeded into dishes (Fig. 1, panels B and C, respectively). With 1×10^6 cells, contrast was unacceptably low, whereas with 10×10^6 cells plaques were so small as to be barely visible (panel F). A density of 4×10^6 cells gave optimum contrast (panel C); under these conditions, indica-

* Corresponding author.

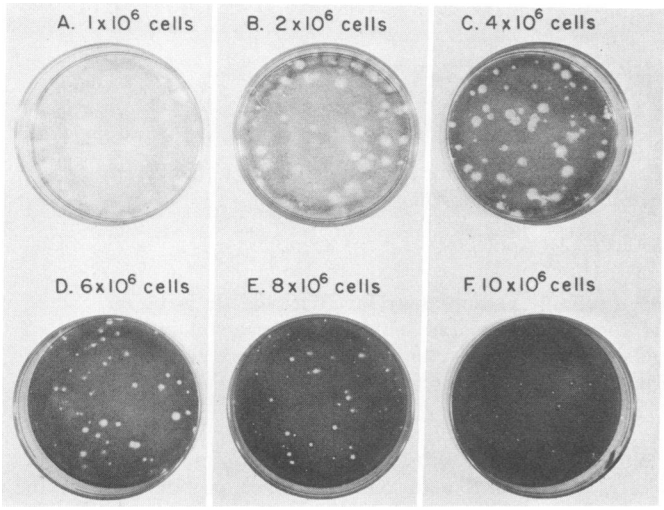

FIG. 1. Effect of cell density on size of BBV plaques. The indicated numbers of *Drosophila* cells were inoculated with 5 μl of BBV-W17. Attachment was carried out at a cell concentration of 4 × 10^7 cells per ml for 1 h at room temperature. The suspensions were then diluted to 5 ml with NPA, vortexed, and poured into 60-mm tissue culture dishes. Plaques were developed after 52 h at 26°C.

tor cells were about 70% confluent at the time plaques were scored. Antiserum against wild-type BBV (5) completely eliminated plaques, whereas a control antiserum against cricket paralysis virus (gift from P. D. Scotti) (17) had no effect (data not shown).

The plaque assay revealed a linear relationship between PFU and the number of particles (Fig. 2), supporting the contention that infection can be initiated by a single virion, i.e., that both RNA 1 and RNA 2 are contained in the same particle. This supports a conclusion drawn previously from the observation that RNAs 1 and 2 became cross-linked when Nodamura virus (the prototype nodavirus) was irradiated with UV light (13).

Plaques ranged from 0.5 to 4 mm in diameter. This variation in plaque size was not primarily due to genetic variation between virions because progeny from a single (large) plaque exhibited the same behavior. This variation may be related to asynchrony in the penetration or uncoating process required to initiate an infection. Another possibility, which remains to be explored, is that virus replication begins only after the cell has reached an appropriate stage in its growth cycle, as is the case for parvoviruses (19) and retroviruses (7, 8). The latter hypothesis might also account for the strong effect of cell density on plaque formation, since growth conditions are known to affect progression through the cell cycle.

Once the critical importance of seeding density for indicator cells was recognized, it was also possible to obtain plaques with the slower-growing wild-type virus by allowing plaques to develop for a longer time. Figure 3 compares the relative plaque sizes of wild-type BBV (panel B) and BBV-W17 (panel A) after a 72-h development period. Flock house virus (2, 18), a serological relative of BBV, could also be plaqued by the same method (panel C).

Now that BBV can be plaque purified, many experiments with reassortant viruses are feasible. We have already found that infectious reassortant virions can be made by infecting *Drosophila* cells with RNA 1 from BBV and RNA 2 from

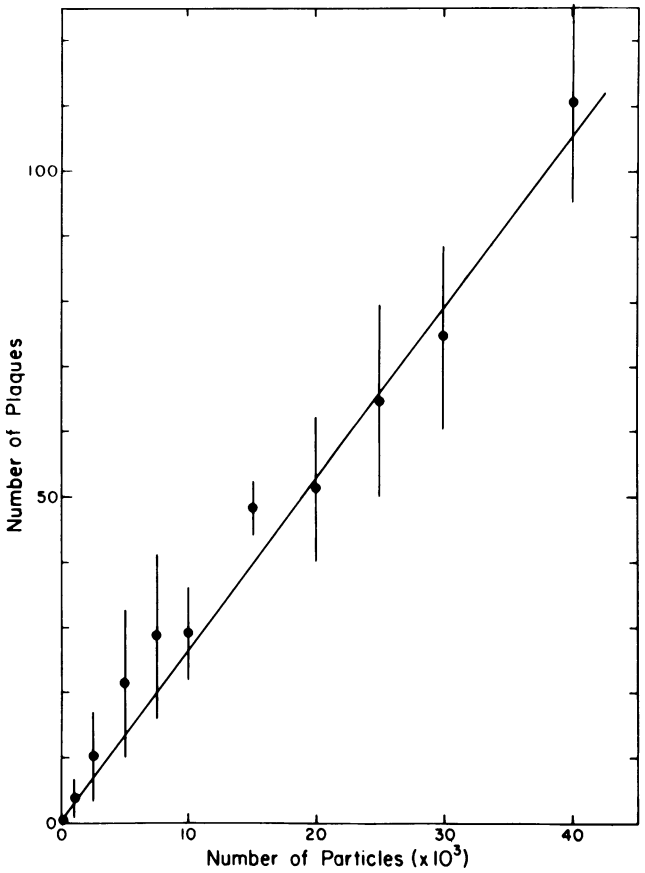

FIG. 2. Dose-response curve for BBV-W17 on *Drosophila* monolayers. The particle-to-PFU ratio, calculated from the slope, was about 400. Particle concentration was calculated from optical density measurements of purified preparations, using an $E_{260}^{0.1\%}$ of 4.15 (10) and a particle mass of 8 megadaltons (6a). Particle-to-PFU ratios could be lowered severalfold by allowing longer attachment periods (e.g., 5 h), but such long attachment periods were unnecessary for most applications. Error bars represent 2 standard deviations about the mean plaque count from triplicate plates.

flock house virus, and vice versa (data not shown). Infectious reassortants can also be made by mixedly infecting cells with individual RNAs isolated from a relatively noncytolytic (wild-type) strain of BBV and the adapted strain of BBV described in this paper. In future experiments, we

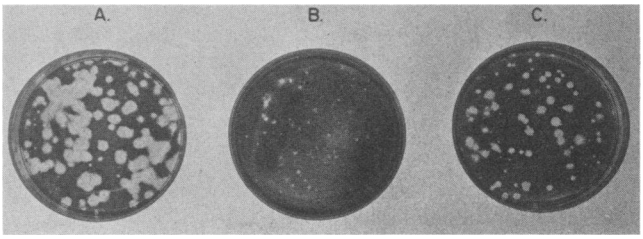

FIG. 3. Comparison of plaques from (A) BBV-W17, (B) wild-type BBV, and (C) flock house virus. Plaques were developed for 72 h at a cell seeding density of 4 × 10^6 cells per 60-mm dish.

anticipate using reassortants to pinpoint which of the two genomic RNAs carries the locus for many traits, such as plaque phenotype and drug and temperature sensitivity.

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