Replication of the Parvovirus MVM

I. Dependence of Virus Multiplication and Plaque Formation on Cell Growth

PETER TATTERSALL¹

*Department of Molecular Virology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields,
London WC 2A 3PX, England*

Received for publication 24 July 1972

A plaque assay has been developed for the minute virus of mice. The infectious unit is a single particle. Plaque size is determined by the extent of cell division in the infected monolayer. Infection of quiescent and serum-stimulated cells suggests that virus multiplication depends on host function(s) that are not normally expressed in resting cells.

Members of the parvovirus group of small, isometric deoxyribonucleic acid (DNA) viruses infect a wide variety of animal hosts, in many cases causing abnormal fetal and neonatal development. The viruses can be divided into two subgroups, those dependent upon adenovirus coinfection, and those, described here as autonomous, which do not require helper virus in most cells. The members of both subgroups so far examined contain 1.2×10^6 to 2.0×10^6 daltons of single-stranded DNA (3, 13, 15, 16, 23) and have three structural polypeptides, in the range 55×10^3 to 92×10^3 daltons (8, 9, 17, 18). In each case, the combined molecular weight of the structural proteins equals or exceeds the theoretical coding capacity of the viral genome. If the infectious entity is a single particle containing one DNA molecule with separate cistrons for all the structural polypeptides, then the virus would be unable to code for any nonstructural proteins. In this case, the expression of functions required for autonomous virus multiplication would have to be controlled by host regulatory mechanisms, rather than by viral gene products, unless the coat proteins have dual functions. It has been shown that Kilham's rat virus (RV) and H-1 virus, both autonomous parvoviruses, preferentially infect cells in S phase (6, 20, 21). Such cell cycle dependence would provide a biochemical explanation for the affinity of these viruses for dividing cells which has been observed in pathological studies (13, 22). Similar studies on the growth cycle of the autonomous minute virus of mice (MVM) have been handicapped by the lack of a virus-cell system which provides efficient infection and an assay of viral infectivity. This paper describes a plaque assay for MVM, which requires a dividing cell monolayer

for plaque formation. The dose response of this infectivity assay shows that a single virus particle is infectious. The assay has been used to investigate the multiplication of MVM in resting and serum-stimulated mouse cells.

MATERIALS AND METHODS

Cell cultures. Monolayers of mouse and rat embryo cells, BHK-21 cells (12), and of A-9 cells, a derivative of the mouse L cell line (10), were grown in 80-oz (2.37 liters) roller bottles (7) in D medium (Dulbecco's modification of Eagle minimal medium) supplemented with 10% calf serum. Suspension cultures of A-9 cells were grown in DP medium (calcium-free D medium with 10 X phosphate), supplemented with 5% fetal calf serum. All cultures were grown at 37 C in an atmosphere of 20% CO₂.

Viruses. MVM (CR) was the original isolate described by Crawford (2). MVM (T) was a plaque-purified strain isolated as described below.

Virus propagation and purification. MVM (CR) was grown in mouse embryo cultures as described by Crawford (2). MVM (T) was grown in A-9 cell monolayer cultures. The virus was passaged at low multiplicity by infecting roller bottle cultures with 10 ml of a one-tenth dilution of medium from the earliest passage available. After 1 week, the cells were shaken into the medium and harvested by low-speed centrifugation. The cells were disrupted by three cycles of freezing and thawing, and the virus was extracted with receptor destroying enzyme and 0.25% sodium deoxycholate (2), for 16 hr at 37 C. After removal of cell debris by low-speed centrifugation, the virus was pelleted in an MSE 8 X 50 rotor at 100,000 X g (average) for 3 hr at 4 C. The pellet was resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, and extracted twice with 2 volumes of 1, 1, 2 trichlorotrifluoroethane. Samples (1 ml) of the virus suspension were layered on 3 ml of cesium chloride, density 1.48 g/ml, in 0.2 M Tris buffer, pH 7.5, and sedimented to equilibrium at 74,000 X g (average) for 18 hr at 5 C in Spinco SW39 rotor. Fractions were collected from the bottom of the tube.

¹ Present address: Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, N.J. 07110.

Plaque assay. A-9 cells from a rapidly dividing suspension culture were suspended at 4×10^6 cells per ml in phosphate-buffered saline (PBS, reference 4) and mixed with an equal volume of virus dilution, also in PBS, for 90 min at 37 C. This suspension was then diluted with D medium, and samples of 10^6 cells were inoculated into 60-mm plastic tissue culture dishes in a final volume of 10 ml. These dishes were incubated for 2 hr at 37 C, by which time all the cells had attached. The medium was then removed, and the cells were overlaid with 5 ml of D medium containing 1.0% agar and 5% fetal calf serum. After 6 days, the monolayers were stained by adding 5 ml of overlay medium without serum, containing 0.006% neutral red and incubated overnight, and the plaques were counted the following morning.

Plaque purification. MVM was cloned by single plaque isolation. Cells and agar were transferred from a well-isolated plaque (whenever possible from a dish containing only one plaque) into 0.2 ml of PBS and frozen and thawed three times. This suspension was either diluted to perform another isolation or used to infect monolayer cultures from which stocks were derived.

Preparation of resting and serum-stimulated cells. Low-serum resting mouse embryo cells were prepared as described by Fried and Pitts (5). Plastic tissue culture dishes (50 mm) containing 2.5×10^6 cells in D medium supplemented with 0.5% calf serum were incubated at 37 C for 4 days. Cells were infected by removing the medium, adding 0.2 ml of MVM suspension in warm PBS, and incubating at 37 C for 90 min. The inoculum was then removed, the monolayers were washed twice with 5 ml of warm PBS, and the original low-serum medium was replaced. Control cells were mock-infected, in a similar fashion, with PBS alone. Synchronous DNA synthesis was induced in these resting cells by exposure to high-serum medium for 12 hr (1, 14). The low-serum medium was removed and replaced with D medium containing 10% calf serum. After incubation at 37 C for 12 hr, this medium was removed, the monolayer was washed with unsupplemented D medium, and the original low-serum medium was replaced. DNA synthesis in these cultures was followed by pulse-labeling cells grown on glass cover slips with ^3H -thymidine ($1 \mu\text{Ci/ml}$, $5 \times 10^{-6} \text{ M}$) for consecutive 12-hr periods. After the pulse the cells were fixed in ice-cold formol saline. Duplicate cover slips were removed and washed, in the cold, once in 10% trichloroacetic acid, twice in 5% trichloroacetic acid, and once in absolute ethanol, and then dried. Each cover slip was placed in 10 ml of toluene containing 2,5 diphenoloxazole per liter and 0.3 g of 1,4-bis (4-methyl, 5-phenoxazole) benzene per liter, and the radioactivity was determined in a liquid scintillation counter. Cell-associated virus was assayed by scraping the cells into the medium, harvesting by centrifugation, and extracting the virus as before. Duplicate dilutions were assayed for infectious virus as described above, and plaques on four dishes per dilution were counted.

RESULTS

A plaque assay was developed for the accurate determination of viral infectivity and the genetic

purification of the virus for subsequent biochemical studies. The assay has been used to investigate the unusual interaction between this small DNA virus and its host cell.

Plaque purification. MVM (CR) was cloned by single-plaque isolation, and the properties of one isolate, MVM (T), were studied in detail. This strain was cloned by five serial isolations, a large plaque being transferred each time. Progeny plaques from this strain exhibited the same size distribution as those from the parental strain, as described below.

The equilibrium density gradient centrifugation of hemagglutinating activity and infectivity for the cloned virus are shown in Fig. 1. The virus formed two bands of hemagglutinin in cesium chloride, as observed by Crawford (2). Virus from the denser band stained as "full" particles by electron microscopy, whereas the upper band contained predominantly "empty" particles. Infectivity was associated with the denser band and virus from this band had particle-infectivity ratios of 300:1 to 400:1 compared to 5,000:1 to 10,000:1

FIG. 1. MVM (T) grown up from a single plaque was extracted and centrifuged to equilibrium as described in the Materials and Methods. Hemagglutination (HA) titer is expressed as the reciprocal of the virus dilution causing complete agglutination of a 1% guinea-pig red blood cell suspension. Infectivity was measured with the plaque assay described in the text and expressed as plaque-forming units (PFU) per ml.

for density gradient-purified MVM (CR) "full" particles. MVM (T) has the same hemagglutination spectrum, buoyant density, and sedimentation coefficient as the original strain. Antiserum to MVM (CR) had the same titer against MVM (T) and MVM (CR) as measured by hemagglutination inhibition. Both strains grow in primary cultures of mouse and rat origin, mouse A-9 and L strain fibroblasts, and BHK-21 hamster cells. It would appear, therefore, that the plaque-purified virus is physically identical to the virus originally described by Crawford (2).

Characteristics of the plaque assay. At an initial density of 10^6 cells per dish, the cells formed a sparse monolayer which reached confluence by the fourth day. After confluence was attained, little further cell division occurred. Plaques were visible on the fourth day, but dishes were stained routinely on the sixth day, and the plaques were counted the following morning. The plaques formed were of variable size, from 1 to 8 mm in diameter, and did not increase in size or number after the fifth day. Although plaque size varied considerably, it was observed that small-plaque isolates segregated large plaques and vice versa. The distributions of progeny plaque size were unimodal and had essentially the same mean diameter regardless of parental plaque size. This suggested that plaque size was not genetically controlled, in contrast to most other viruses (19).

Observations in this laboratory have indicated that the extent of MVM replication is directly influenced by the extent of cell division in the infected culture (P. J. Tattersall, Ph.D. thesis, Univ. of London, 1971). Consequently, the effect of initial density, and therefore the extent of cell division in the locale of plaque formation, was examined. Figure 2 shows the effect on plaque size, of altering the initial cell density, for a constant virus dose. Plaque size decreases as input cell number increases. When dishes are seeded at confluence, few visible plaque are obtained, and those which appear are extremely small. The addition of fresh overlay medium on the second day does not affect the result, showing that this effect is not due to medium depletion by the higher cell input. This effect suggests that the sensitivity and the reproducibility of plaque assays for MVM and similar viruses will depend upon adhering to standard conditions.

The plot of dose response for the plaque assay (shown in Fig. 3) generates a straight line with a slope of 45° , demonstrating that plaque formation is a one-hit phenomenon. The nonlinearity at virus doses above 40 plaque-forming units (PFU) per dish may be due to coincidence of plaques or interference produced in the monolayer.

Multiplication of MVM in resting and serum-

FIG. 2. Serial doubling dilutions were made of an A-9 cell suspension at 2.56×10^7 cells/ml in PBS. Samples (1 ml) of these cells were added to an equal volume of MVM (T) suspension at approximately 80 PFU/ml. The plaque assay was then continued as described in the text. Initial cell numbers per dish were: (a) 4×10^6 , (b) 8×10^6 , (c) 1.6×10^6 , (d) 3.2×10^6 , (e) 6.4×10^6 .

stimulated mouse cells. The dependence of cytopathic effect on cell growth suggests that cell killing by MVM requires some function expressed only in dividing cells. To establish whether this phenomenon extends to virus replication per se, the kinetics of virus production in resting and serum-stimulated cells were examined. Mouse embryo cells cultivated in low serum exhibit low rates of DNA and ribonucleic acid synthesis and low levels of DNA polymerase activity (5). Such quiescent cells can be induced to incorporate ^3H -thymidine and subsequently divide, in a synchronous fashion, by a short exposure to medium containing high levels of serum (1, 14). The protocol for infection and serum pulsing of such cells is shown in Fig. 4. Incorporation of ^3H -thymidine in these cultures is shown in Fig. 5B and C. Figure 5A shows that infection of these cells with MVM (T) in the absence of a serum pulse leads to a virus yield of 6 PFU per cell by 60 hr, whereas infection followed by a serum pulse increases this yield to over 50 PFU per cell. If infection is de-

layed until 24 hr after the start of the serum pulse, a similar latent period is observed, but the rate of virus production is increased so that over 40 PFU per cell are observed 36 hr after infection.

FIG. 3. MVM (T) from the dense band in a cesium chloride equilibrium gradient (3 to 400 particles/PFU) was used at various dilutions to infect A-9 cells for plaque assays as described in the text. At least 150 plaques were counted for each point, and the error bars indicate the sample standard deviation for each virus dose.

FIG. 4. Mouse embryo cultures were maintained in low-serum (LS) medium for 4 days and then subjected to three different regimes of serum pulsing and infection (at a multiplicity of 4 PFU per cell). These cultures were assayed at 12-hr intervals for infectious virus, and parallel infected and mock-infected cultures were pulsed for 12-hr periods with ^3H -thymidine. Culture a was infected and maintained in LS medium; culture b was infected, exposed to high-serum (HS) medium from 0 to 12 hr, and then maintained in LS medium; culture c was exposed to HS medium from 0 to 12 hr, and then maintained in LS medium, and infected at 24 hr. Horizontal arrows show the times for which the cultures were maintained in the two media. Vertical arrows indicate the times of infection.

FIG. 5. Figure 5A shows the kinetics of cell associated virus production in cultures a (●), b (○), and c (△) (see legend to Fig. 4). Fig. 5B and C show the incorporation of ^3H -thymidine in infected (—) and mock-infected (---) cultures under regimes a and b, respectively. Essentially no difference was detected in ^3H -thymidine incorporation between cultures under regimes b and c. Results are plotted as the mean of counts per minute (cpm) for duplicate cover slips.

DISCUSSION

MVM has been shown to replicate without a helper virus in dividing mouse fibroblast monolayers, giving rise to plaques. The infectious unit is a single particle, presumably containing one molecule of single-stranded DNA (3). The dependence on dividing cells may be explained by the requirement for some host function expressed in these cells. If the period of ^3H -thymidine in-

corporation in serum-stimulated cells is equated with the S phase of the normal cell cycle (1), it would appear that a host function is expressed during late S or G2 phase which directly affects the rate of synthesis of progeny virus.

A small amount of incorporation due to manipulation of the cultures was detected in the absence of a serum pulse (Fig. 5B). This imperfection in the experimental system may be responsible for the small amount of virus produced in the resting cells, since the yields of virus in cultures a and b (Fig. 5A) correlate well with the total DNA synthesis observed in these cultures (Fig. 5B and C). It is clear that MVM is not capable of inducing host DNA synthesis in this system, in contrast to polyoma virus (5). Thus it is possible that MVM is absolutely dependent upon, but incapable of inducing, some host activity which is not expressed in cells arrested in G1 phase.

Infection of rapidly dividing cells with MVM does not markedly alter their rate of DNA synthesis, although after 14 hr a considerable proportion of the intracellular DNA (over 25% by 20 hr) is viral intermediate and progeny DNA (P. J. Tattersall, Ph.D. Thesis, Univ. of London, 1971; Tattersall, *Fed. Proc.* 31:913, 1972; Tattersall and Crawford, *manuscript in preparation*). Taken with the results presented here, this suggests that the replication of the viral DNA requires activation of the host replication system, which the virus is then able to utilize preferentially. This would render a major process in virus multiplication dependent upon the temporal regulation of the host cell.

MVM shares, with all other parvoviruses examined, the unusual property that the information required to code, separately, for its structural polypeptides would saturate the theoretical capacity of its genome (Tattersall and Shatkin, *unpublished results*; D. C. Ward and G. N. Godson, *personal communication*). Thus, it may be severely restricted in coding potential for non-structural proteins, explaining its extreme dependence on the expression of host functions. However, it is possible that one or more of the structural proteins are derived from one polypeptide by cleavage or condensation. Alternatively, the virus may incorporate one or more host proteins into the virion. These possibilities are currently under investigation.

ACKNOWLEDGMENTS

I thank Chris Syrett for electron microscopy. I also thank L. V. Crawford and A. M. C. Fried for useful discussion during the

course of this work and R. R. Bürk, J. Cairns, and A. J. Shatkin for critically reading the manuscript.

P. T. held an I.C.R.F. Bursarship.

LITERATURE CITED

1. Bürk, R. R. 1970. One step growth cycle for BHK 21/13 hamster fibroblasts. *Exp. Cell Res.* 63:309-316.
2. Crawford, L. V. 1966. A minute virus of mice. *Virology* 29:605-612.
3. Crawford, L. V., E. A. C. Follett, M. G. Burdon, and D. J. McGeoch. 1969. The DNA of a minute virus of mice. *J. Gen. Virol.* 4:37-46.
4. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis virus. *J. Exp. Med.* 99:167-182.
5. Fried, M., and J. D. Pitts. 1968. Replication of polyoma virus DNA. 1. A resting cell system for biochemical studies on polyoma virus. *Virology* 34:761-770.
6. Hampton, E. G. 1970. H-1 virus growth in synchronized rat embryo cells. *Can. J. Microbiol.* 16:266-268.
7. House, W., and P. Wildy. 1965. Large scale production of tissue cells and virus. *Lab. Pract.* 14:594-595.
8. Johnson, F. B., H. L. Ozer, and M. D. Hoggan. 1971. Structural proteins of adenovirus-associated virus type 3. *J. Virol.* 8:860-863.
9. Kongsvik, J. R., and H. W. Toolan. 1972. Capsid components of the parvovirus H-1. *Proc. Soc. Exp. Biol. Med.* 139:1202-1205.
10. Littlefield, J. W. 1964. Three degrees of guanylic acid-inosinic acid pyrophosphorylase deficiency in mouse fibroblasts. *Nature (London)* 203:1142-1144.
11. McGeoch, D. J., L. V. Crawford, and E. A. C. Follett. 1970. The DNAs of three parvoviruses. *J. Gen. Virol.* 6:33-40.
12. Macpherson, I. A., and M. G. P. Stoker. 1962. Polyoma transformation of hamster cell clones. An investigation of genetic factors affecting cell competence. *Virology* 16:147-151.
13. Margolis, G., and L. Kilham. 1966. Rat virus, an agent with an affinity for the dividing cell, p. 361. In *Slow, latent and temperate virus infections*. N.I.N.D.B. Monograph no. 2.
14. Pitts, J. D., and M. Fried. 1969. The binding of DNA polymerase to polyoma DNA. *J. Gen. Virol.* 4:189-201.
15. Robinson, D. M., and F. M. Hetrick. 1969. Single-stranded DNA from the Kilham rat virus. *J. Gen. Virol.* 4:269-283.
16. Rose, J. A., K. I. Berns, M. D. Hoggan, and F. J. Koczot. 1969. Evidence for a single-stranded adenovirus-associated virus genome. Formation of a DNA density hybrid on release of viral DNA. *Proc. Nat. Acad. Sci. U.S.A.* 64:863-869.
17. Rose, J. A., J. V. Maizel, Jr., J. K. Inman, and A. J. Shatkin. 1971. Structural proteins of adenovirus-associated viruses. *J. Virol.* 8:766-770.
18. Salzman, L. A., and White, W. L. 1970. Structural proteins of Kilham rat virus. *Biochem. Biophys. Res. Commun.* 41:1551-1556.
19. Takemoto, K. K. 1966. Plaque mutants of animal viruses. *Progr. Med. Virol.* 8:314-348.
20. Tennant, R. W., K. R. Layman, and R. E. Hand, Jr. 1969. Effect of cell physiological state on infection by rat virus. *J. Virol.* 4:872-878.
21. Tennant, R. W., and R. E. Hand, Jr. 1970. Requirement of cellular synthesis for Kilham rat virus replication. *Virology* 42:1054-1063.
22. Toolan, H. W. 1968. The picornaviruses: H, RV and AAV. *Int. Rev. Exp. Pathol.* 6:135-180.
23. Usategui-Gomez, M., H. W. Toolan, N. Ledinko, F. Al-Lami, and M. S. Hopkins. 1969. Single-stranded DNA from the parvovirus H-1. *Virology* 39:617-621.