Developmental-dependent Replication of Minute Virus of Mice in Differentiated Mouse Testicular Cell Lines

By ESTHER GUETTA, DINA RON† AND JACOV TAL*

Biology Department, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

(Accepted 7 August 1986)

SUMMARY

The replication of the autonomous parvovirus, minute virus of mice (MVM), requires mitotically active cells and depends on certain factors expressed by cells of particular differentiated phenotype. As an approach to the understanding of these helper functions, we studied the interaction of the fibrotropic [MVM(p)] and the lymphotropic [MVM(i)] strains of MVM with two differentiated cell lines from mouse testicular epithelial origins. The relative support given to viral expression by these cell lines varied extensively. Cells from Sertoli origin (TM4) were permissive to MVM(p) but were mostly restrictive to MVM(i). The other cell line, of Leydig cell origin (TM3), was highly restrictive to both viral strains, but the blocks to their growth in these cells were localized at different stages of their growth cycle, suggesting that the replication of MVM in these cells requires tissue-specific helper functions during at least two stages of viral replication.

Autonomously replicating parvoviruses infect a wide spectrum of animal species ranging from mice to humans (Berns, 1984). Their limited genetic capacity, and their inability to induce cellular functions, makes them highly dependent on cellular helper functions for their own replication. Primarily, functions expressed transiently during the S phase of the cell cycle are required for viral DNA replication (Tennant et al., 1969). In addition, the tissue specificity of individual parvovirus strains is determined intracellularly, most likely by the interaction of developmentally regulated host protein(s) with a structural determinant on the viral DNA (Spalholz & Tattersall, 1983). We have recently isolated cell mutants which are deficient in certain tissue-specific helper activities but retain the S phase helper function(s) (Ron & Tal, 1986).

The parvovirus minute virus of mice (MVM) offers an excellent system for studying the structure and function of the viral host range determinant (hrd). There are two, serologically identical strains of MVM with allotropic host range properties. The prototype strain, MVM(p), lytically infects a variety of mouse fibroblasts such as A9 cells, a subline of mouse L-cells. The immunosuppressive strain, MVM(i), infects T-lymphocytes, such as EL4 cells in vitro (Bonnard et al., 1976). MVM(p) and MVM(i) are reciprocally restricted in each other’s permissive cell line (Tattersall & Bratton, 1983). The sequences of the two viruses, now available (Sahli et al., 1985; Astell et al., 1986), reveal 175 differences between them, most of which involve a single base. However, since these changes are scattered extensively along the genome, it is not possible to determine which of them comprise, or participate in, the hrd site.

While mapping of the MVM hrd site (currently underway) is essential to the understanding of the molecular basis for host range selection, it is the interaction with developmentally regulated host components that determines whether an infection will eventually be permissive or restrictive. In this study, we were interested in finding out whether these MVM strains would

† Present address: Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, U.S.A.
Table 1. Growth properties of MVM(p) and MVM(i) in TM3 and TM4 cells*

<table>
<thead>
<tr>
<th>Cell</th>
<th>Infecting virus</th>
<th>Infectious centres (%)†</th>
<th>Infectious virus (p.f.u.)</th>
<th>Binding (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM4</td>
<td>MVM(p)</td>
<td>21</td>
<td>3.8 x 10⁹</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>MVM(i)</td>
<td>6.5</td>
<td>2.8 x 10⁶</td>
<td>28</td>
</tr>
<tr>
<td>TM3</td>
<td>MVM(p)</td>
<td>&lt;0.1</td>
<td>&lt;10²</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>MVM(i)</td>
<td>&lt;0.1</td>
<td>&lt;10²</td>
<td>15</td>
</tr>
</tbody>
</table>

* All cell lines were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 8% foetal bovine serum (Seralab). The preparation of virus stocks was done as described previously (Ron & Tal, 1985). All infections were done at an input multiplicity of 10 p.f.u./cell for 1 h at 37 °C. Infectious virus of both strains was determined by plaque assays in 324K indicator cells (Tattersall & Bratton, 1983).
† Virus-producing cells were determined by infectious centre assay. Following infection, the cells were washed twice with phosphate-buffered saline, then treated with 1% anti-MVM serum for 6 h, trypsinized and seeded over monolayers of 324K indicator cells (5 x 10⁵ cells/60 mm dish). Two h later the cultures were overlaid with 0.6% Difco agar solution in medium containing 5% foetal calf serum and incubated at 37 °C for 5 days. To visualize plaques, the plates were overlaid with an agar-medium solution containing 0.2% neutral red and incubated at 37 °C for 10 h.
‡ Binding assays were performed according to Linser et al. (1977). [3H]Thymidine-labelled viruses were incubated with cells in 1 ml DMEM containing 20 mM-PIPES pH 7.2 and no NaHCO₃. After incubation at 4 °C for 1 h, total radioactive counts were determined by TCA precipitation of a 0.4 ml sample (total virus, A). The rest of the cells were pelleted, and a 0.4 ml sample was withdrawn from the supernatant for radioactive counting (unbound virus, B). The percentage of binding was calculated as (A-B/A) x 100.

retain their reciprocal host specificity when they interacted with differentiated cell lines of epithelial origin as well. These cell lines, TM3 and TM4, were obtained from mouse testicular Leydig and Sertoli cells, respectively, of 10- to 13-day-old BALB/c mice, and they maintain many of their differentiated functions (Mather, 1980; Mather et al., 1982). Their growth properties in culture (i.e. doubling time, saturation density and plating efficiency) are very similar, thus eliminating possible bias in the results due to differences in cell cycle. They are, however, extensively different in their support of virus replication. As shown in Table 1, TM4 cells were about 1000-fold better producers of MVM(p) than of MVM(i). The initial percentage of cells involved in MVM(i) virus replication was nevertheless quite high, about 30% that of MVM(p)-producing cells, as measured by infectious centre assay. However, unlike infection with MVM(p), MVM(i) infection caused only transient and limited c.p.e., after which the culture continued to grow at a rate comparable to that of uninfected cells.

In comparison to TM4 cells, TM3 cells were highly restrictive to both viruses. Infection of TM3 cells with either virus did not alter the normal growth rate of these cells, there was no visible c.p.e. and only background levels of infectious centres (Table 1) or fluorescent nuclei (data not shown) were detected. Binding assays demonstrated that the two virus strains adsorb to both cell lines with comparable efficiencies, indicating that the interference with virus replication occurs later in the infection cycle.

Measurements of intracellular viral DNA synthesis, utilizing the dispersed cell assay, correlated well with infectious virus production. Fig. 1 shows that in TM3 cells neither MVM(p) nor MVM(i) DNAs were amplified. TM4 cells were also poor in supporting MVM(i) DNA synthesis, but MVM(p) DNA synthesis in these cells was very efficient. Intracellular viral DNA measurements of infected A9 and EL4 cells, also included in Fig. 1, confirm the allotropic nature of MVM(p) and MVM(i) in these cells.

The apparent non-permissiveness of TM3 cells to the two virus strains prompted us to follow the intracellular fate of the infecting viral DNA. TM3 cultures were infected with MVM(p) or MVM(i) and at various times after the infection were subjected to dispersed cell assay as well as to size analysis of viral DNA in Hirt extracts. Both assays showed accumulation of MVM(p) DNA at day 1. The levels of hybridization were 10- to 15-fold lower than those obtained in homologous infections [i.e. MVM(p) in A9 cells and MVM(i) in EL4 cells; Fig. 1], and were followed by a gradual decrease which reached the limits of detection by 6 days post-infection (Fig. 2). Size analysis showed that the MVM(p) DNA corresponded to monomer and dimer
Fig. 1. Kinetics of viral DNA amplification in cells infected with MVM(p) or MVM(i). Monolayer cultures were infected with virus at input multiplicities of 10 p.f.u./cell. At each time point shown, the dispersed cell assay (Lavi & Etkin, 1981) was performed. Briefly, samples of $8 \times 10^6$ cells were trapped on nitrocellulose filters, denatured, neutralized, baked (80 °C, 4 to 8 h) and hybridized to $^{32}$P-labelled plasmid pPT206 containing 95% of the MVM genome (Merchlinsky et al., 1983). The filters were then washed, dried and counted in a beta scintillation counter. ---, MVM(p); -- -, MVM(i). (a) A9 cells; (b) EL4 cells; (c) TM3 cells; (d) TM4 cells.

Fig. 2. Fate of MVM(p) and MVM(i) DNAs in TM3 cells. Monolayer cultures were infected at 10 p.f.u./cell. At the times shown, $8 \times 10^6$ cells were subjected to dispersed cell assay. ●, MVM(p); ○, MVM(i). Inset: at the times shown (days post-infection), $5 \times 10^6$ MVM(p)-infected cells were extracted by the Hirt method (Hirt, 1967) and the viral DNA was analysed by electrophoresis on 1% agarose gels followed by Southern blotting and hybridization. ss, Position of the single-stranded viral DNA; RF$_1$ and RF$_2$, monomer and dimer RF DNA.
Fig. 3. Co-infection of TM3 cells with MVM(p) and MVM(i). TM3 cells were co-infected with 3 × 10^4 c.p.m. of 32P-labelled MVM(i) and increasing amounts of unlabelled MVM(p). Forty-eight h post-infection Hirt supernatants were prepared and the viral DNA was electrophoresed on 1% agarose gel. (a) Viral DNA visualized by ethidium bromide staining. Lanes 1, 2 and 3 show infections with 5, 1 and 0 p.f.u. respectively, of MVM(p). Lane 4 shows A9 cells infected with 32P-labelled MVM(p). (b) Autoradiography of the dried gel.

replicative form (RF) DNA (Fig. 2, inset). The RF DNA bands seen on days 1 and 3 represent amplified RF DNA. The sensitivity of the assay was not sufficient to detect input single-stranded viral DNA at day 0 (4 h post-infection) but, judging from the intensity of the RF DNA, synthesis and accumulation of single-stranded progeny DNA should have been visible in this assay. Clearly, however, single-stranded progeny DNA was not synthesized and the RF DNA was diluted out, following an initial amplification stage, as a result of cell proliferation. It is concluded, therefore, that in TM3 cells MVM(p) replication is blocked after RF DNA synthesis and amplification and before progeny DNA and viral protein synthesis take place. In contrast, MVM(i) DNA synthesis in TM3 cells was not detected, indicating that the block to MVM(i) replication in these cells is an early one, prior to RF DNA synthesis.

The above results indicate that in TM3 cells the replication of MVM(p) and MVM(i) are restricted at two different stages of their replication cycles. To corroborate this, TM3 cells were co-infected with 32P-labelled MVM(i) and with increasing multiplicities of unlabelled MVM(p). Viral DNA was extracted 24 h later and subjected to electrophoresis in agarose. Ethidium bromide staining showed multiplicity-dependent accumulation of viral RF DNA (Fig. 3a), but autoradiography of the same gels revealed that all the radioactive label was in the single-stranded DNA band (Fig. 3b). Thus, TM3 cells which allow some replication of MVM(p), restrict MVM(i) replication prior to the conversion of its DNA to a double-stranded form. The co-infection with MVM(p) DNA did not provide a helper activity in trans to the arrested MVM(i) DNA.
Table 2. Uncoating of MVM(p) and MVM(i) in TM3 cells*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Total number of plaques†</th>
<th>Infectious virus (%)‡</th>
<th>Uncoating (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVM(p)</td>
<td>7</td>
<td>111</td>
<td>6.4</td>
</tr>
<tr>
<td>MVM(i)</td>
<td>1</td>
<td>23</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Cells were infected at 10 p.f.u./cell for 1 h at 37 °C, after which the virus inoculum was removed and replaced by serum-containing medium. Six h post-infection the cells were washed with phosphate-buffered saline, suspended in TE buffer (50 mM-Tris·HCl, 5 mM-EDTA, pH 8.7) and subjected to three cycles of freezing and thawing. Cell debris was removed by low-speed centrifugation and the clear supernatant was assayed for the presence of infectious virus on 324K indicator cells. In the reconstruction experiments, cells and virus were mixed and the cells were immediately subjected to lysis and assayed for infectious virus. Each extract was assayed at several dilutions in phosphate-buffered saline.

† Total number of plaques was determined in a 10⁻⁴ dilution of an extract from 2 × 10⁶ cells.
‡ Values are (p.f.u. infection/p.f.u.; reconstruction) × 100.

To find out if MVM(i) was uncoated, TM3 cells were infected with MVM(p) or MVM(i) at 10 p.f.u./cell and 6 h after the infection were harvested and lysed. As a control, cells were mixed with virus at p.f.u./cell ratios identical to those used in the infection experiments, and immediately lysed. Infectious virus recovered by this procedure was quantified by plaque assaying the cell lysates on 324K indicator cells. Table 2 shows that, in the control experiments, most of the input infectivity was recovered. In contrast, only 4% and 6% of the infectivities of MVM(i) and of MVM(p), respectively, were recovered, suggesting that over 90% of both viruses were uncoated in TM3 cells. It should be noted, however, that this assay does not distinguish between uncoating and other processes which may lead to the inactivation of intracellular virus.

The blocks to the replication of MVM(p) and MVM(i) in TM3 cells indicate that these stages in the virus replication cycle may require host-coded helper activities. The block to MVM(p) replication, which is subsequent to viral RF DNA amplification and before viral proteins are made, is reminiscent of the restriction to MVM(i) replication in A9 cells, and may be identical to it (Tattersall & Bratton, 1983; Ron & Tal, 1985, 1986). The block of MVM(i) replication early in the infection suggests that another host helper activity may be required at or soon after the uncoating stage. This block resembles deficiency in S phase helper function(s) because infection of permissive, but mitotically quiescent cell cultures with MVM(p) results in similar persistence of viral single-stranded DNA in the culture (unpublished observations). Since, however, these cells do provide S phase helper activity [to MVM(p)], another helper activity, which is not related to the S phase helper function, must be provided. It is possible that this helper activity is a cellular function that can interact with MVM(p) but not with MVM(i), suggesting that it is of a developmental nature.

We thank Dr Jennie P. Mather for the TM3 and TM4 cell lines and Dr David Phillips for valuable discussions. This work was supported by a grant from the Chief Scientist, Israel Ministry of Health. J.T. is a fellow in Cancer Research, supported by the David Laser Memorial Fund of the Israel Cancer Research Fund.

REFERENCES

ASTELL, C. R., GARDINER, E. G. & TATTERSALL, P. (1986). DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. Journal of Virology 57, 656-669.


Short communication


(Received 24 March 1986)